Escherichia coli ATCC 8739 Biosensor for Preservative Efficacy Testing

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In memory of late father, Mr Alex Choong, wished you were here to witness this.

Philippians 4:13 - I can do all things through Christ who strengthens me.

Abstract

The preservative challenge test is a regulatory requirement specified in various pharmacopoeias to determine the efficacy of preservatives. However, such testing is a labour-intensive repetitive task and often requires days before results can be generated. Microbial biosensors have the potential to provide a rapid and automated alternative to the traditional viable counting currently in use. However, the selection of appropriate promoters is essential. The bioluminescent reporter strains used in the current study comprise the *Photorhabdus luminescence lux CDABE* reporter genes under the control of five individual constitutive *Escherichia coli* promoters: outer lipoprotein (*lpp*); twin arginine translocase (*tatA*)*;* lysine decarboxylase (*ldc*)*;* lysyl t-RNA (*lysS*); and ribosomal protein (*spc*). The promoter plus *lux CDABE* constructs were cloned, ligated into the plasmid vector pBR322 and transformed into *E. coli* ATCC 8739. The bioluminescence intensity in the decreasing order of constitutive promoter was *lpp* > *spc*> *tatA*> *ldc* > *lysS*. The five biosensor strains tested successfully in PET assays and demonstrated accuracy with a minimum detection limit of 10^3 CFU/ml, a detection range of 6 orders magnitude, and yielded equivalent results to methods currently recommended by the pharmacopoeias. The bioluminescent biosensors were used to monitor the efficacy of preservatives; sorbic acid at concentrations of 0.031% to 0.2% at pH 5.0, and benzalkonium chloride at concentrations of 0.0062% to 0.00039% alone and in combination with 0.03% EDTA. The 99.9% percentage of bioluminescence reduction of *tatA-lux, ldc-lux, lysS-lux,* and *spc-lux* was statistically equivalent to the 3 $\log_{10}$ CFU/ml reduction as required by the Pharmacopeias'. Strong significant correlations between bioluminescence and the methods recommended by the pharmacopoeias were obtained when the biosensor strains were challenged with preservatives, for all except *lpp-lux E. coli*. The bioluminescence expressed by the *lpp-lux* biosensor was significantly lower during long-term stationary phase than it was for any of the other biosensors and was also significantly lower than for any of the other biosensors in the presence of preservatives. Since the plasmid copy number and viable counts for *lpp-lux* did not

change under these conditions, it suggests that perhaps *lpp-lux* was down regulated under stress conditions. There were no statistically significant differences between the results of the bioluminescence assays and the results of the viable count and ATP chemiluminescence assay. Virtual foot printing (using Regulon DB database) demonstrated that two *crp* binding sites overlapping the -10 regions are located on the negative strand of the *lysS* promoter sequences and that one *crp* binding site is located in *lpp*. The biosensor strains *ldc-lux* exhibited levels of bioluminescence per cell significantly lower than *spc* in the presence of preservatives whilst there was a significant increase in bioluminescence per cell by *tatA-lux* under alkaline conditions (pH 8.9) during long-term stationary phase. Amongst the five biosensor strains tested in the current work, it was determined that the *spc-lux* strain would be the most attractive candidate for further work, since the bioluminescence expressed per cell was significantly greater, by 10-1000 times, than that expressed by the other four promoters when challenged with the preservatives tested with excellent significant correlations between bioluminescence expression and viable counts in the PET assays with the various preservatives in this study (R^2: 8.79-1.00). The bioluminescent biosensor strains showed no statistical differences from the control strains (wildtype *E.coli* ATCC 8739 and *E.coli* carrying a promoterless [pBR322.*lux*] for adneylate energy charge (AEC), plasmid copy number (PCN) bioluminescence or viable counts over 28 days. The emission of bioluminescence by the four bioreporter strains across 28 days is reflected by the stability of PCN with correlations of 0.78-0.90, except for *lpp-lux* with R^2: 0.59. The following promoter elements were found likely to assist greater expression of bioluminescence: an A+T level of approximately 50% between the -40 and -60 regions (the UP element); a G+C level of approximately 50% within the -10 and +1 regions; the extended -10 region and -10 region of consensus sequence RpoD ($\sigma^{70/D}$).

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List of Tables

Table Number	Titles	Page
1.1	A comparison of the advantages and disadvantages imposed on the various conventional and alternative methods discussed	38
1.2	Functions of *E.coli* σ factors	59
1.3	Criteria for evaluating preservative effectiveness of topical formulations defied by the European Pharmacopeia	67
1.4	Criteria for evaluating preservative effectiveness of parental and ophthalmic formulations defined by the European Pharmacopeia	68
1.5	Criteria for evaluating preservative effectiveness of oral formulations defined by the European Pharmacopeia	68
2.1	Forward and Reverse primers used for amplification of Constitutive promoters	80
2.2	The average recoveries (%) obtained for the bioluminescence and conventional plate count method in high and low bacterial suspensions	90
2.3	Determination of Precision and equivalence of the Bioluminescence Method and other methods analyzed within a range of bacterial suspension	93
2.4	The minimum limit of detection (MDL) (CFU/ml) and Range comparatively to the different measurements	100
2.5	A compilation of sequences in the promoter regions of the five constitutive promoters analyzed against the consensus of RpoD $\boldsymbol{\sigma^{70/D}}$ *E.coli* consensus	104
3.1	Primer sequences of Bla and Dxs	137
3.2	Volume of reagents required and PCR conditions for *bla* and *dxs* amplification	138
3.3	Volume of reagents required and conditions for qPCR analysis	139
4.1	Concentrations of undissociated and dissociated form of sorbic acid in solution and cytoplasm calculated according to Henderson-Hasselbalch equation	191
4.2	Example of a mathematical calculation based on the Henderson-Hasselbalch equation as a representative for all sorbic acid concentrations	192

4.3	Application of whole-cell bioluminescence as a rapid microbiological screening of antimicrobial preservative and evaluation of European and British Pharmacopeia defined sorbic acid effectiveness	207
5.1	The percentage of chemicals used, without the addition of BAK as a preservative acting ingredient	235
5.2	Amounts of BAK required for respective end concentrations	236
5.3	Application of whole-cell bioluminescence as a rapid microbiological screening of antimicrobial preservative and evaluation of European Pharmacopeia defined effectiveness of benzalkonium chloride	258
5.4	Application of whole-cell bioluminescence as a rapid microbiological screening of antimicrobial preservative and evaluation of European Pharmacopeia defined effectiveness of benzalkonium chloride with 0.03% EDTA	269

List of Figures

Figure Number	Title	Page
1.1	The schematic diagram above shows the layout of a biosensor	39
1.2	Reporter protein signal transcribed by the promoter by the target molecule	48
1.3	Arrangement of *lux CDABE* open reading frames in a single polycistronic operon	50
1.4	The fatty acid reduction pathway catalysed by luxD, luxE, and luxC gene products	52
1.5	RNA polymerase interactions at the promoter and the initial steps to transcription initiation	58
1.6	Structural Characteristic of *E.coli* σ^{70}	60
1.7	The inducible and constitutive promoters functional behaviour	62
2.1	Summary the various steps employed to validate the biosensor constructs	76
2.2	An illustrated image of the construct in *E.coli* ATCC 8739	81
2.3	The Celcis Advance Luminometer	83
2.4 (a)	Relationship between log RLUand log CFU between all construct strains	94
2.4 (b)	Relationship between log ATP and log CFU between all constructs and control strains	94
2.4 (c)	Relationship between log RFU and log CFU between all construct and strains	95
2.4 (d)	Relationship between log TVC and log CFU between all constructs and strains	95
2.5 (a)	Relationship between log RFU and log RLU between all constructs and strains	97
2.5 (b)	Relationship between log ATP and log RLU between all constructs and strains	97
2.5 (c)	Relationship between log TVC and log RLU between all constructs and strains	98

2.6	The comparisons of the sampling time and analysis (minutes), Incubation time (Hours), and preparation time (Minutes) per sample for the five methods	102
2.7	Constitutive promoter strength determined at high cell density	103
3.1	Stages of bacterial growth	123
3.2	The principle of SYBR green based assay in each PCR step	127
3.3	Summarises the various studies in this chapter in characterizing the five constructs	132
3.4	A flow chart describing the methodology steps involved in plasmid copy number quantification	134
3.5 (a)	Bioluminescence expression by the five bioluminescent *E. coli* ATCC 8739 reporter strains (*lpp-lux*, *tatA-lux*, *ldc-lux*, *lysS-lux*, and *spc-lux*) over 48 hours of incubation	146
3.5 (b)	Population kinetics of the five bioluminescent *E. coli* ATCC 8739 reporter strains (*lpp-lux*, *tatA-lux*, *ldc-lux*, *lysS-lux*, and *spc-lux*) over 48 hours of incubation	148
3.5 (c)	The bioluminescence to viable count ratio (RLU:CFU) of the five *E. coli* ATCC 8739 reporter strains (*lpp-lux*, *tatA-lux*, *ldc-lux*, *lysS-lux* and *spc-lux*) over 48 hours of incubation	150
3.6 (a)	Bioluminescence expression of reporter strains over extended incubation periods (1, 2, 7, 14,21and 28 d of incubation	153
3.6 (b)	Population kinetics of constructs and control strains over extended incubation periods (1, 2, 7, 14,21and 28 d of incubation	153
3.6 (c)	The bioluminescence to viable count ratio (RLU:CFU) of the five *E. coli* ATCC 8739 reporter strains over incubation periods	154
3.7	The average pH of the growth medium in the cultures at 0, 1, 2, 7, 14, 21 and 28 days of incubation during long-term culture of the *E. coli* strains	154
3.8 (a-f)	Fluorescence imaging of *E.coli* cells	156
3.9 (a &b)	qPCR curves for serial dilutions of gDNA and pDNA versus cycle number (C_T) for *dxs* and *bla*)	159
3.10(a)	Standard curves of C_T value versus $\log_{10}$ number of gene copies for both the *dxs* and *bla* amplification from *lpp-lux Esherichia coli* 8737	160

3.10 (b)	Standard curves of C_T value versus $\log_{10}$ number of gene copies for both the *dxs* and *bla* amplification from *tatA-lux Esherichia coli* 8739	160
3.10 (c)	Standard curves of C_T value versus $\log_{10}$ number of gene copies for both the *dxs* and *bla* amplification from *ldc-lux Esherichia coli* 8739	161
3.10 (d)	Standard curves of C_T value versus $\log_{10}$ number of gene copies for both the *dxs* and *bla* amplification from *lysS-lux Esherichia coli* 8739.	161
3.10 (e)	Standard curves of C_T value versus $\log_{10}$ number of gene copies for both the *dxs* and *bla* amplification from *spc-lux Esherichia coli* 8739	162
3.11 (a)	Melting curve for serial dilutions of the gDNA extracted from *E. coli* 8739 *ldc-lux* after 24 hours of incubation.	163
3.11 (b)	Melting curves for serial dilutions of the pDNA extracted from *E. coli* 8739 *ldc-lux* after 24 hours of incubation	163
3.12	Plasmid copy numbers (PCN) of the five bioluminescent constructs within the *E. coli* strains over a 28 day incubation period	164
3.13	Standard curve of ATP concentration versus Chemiluminescence for the ATP-Chemiluminescence assay using luciferin-luciferase	165
3.14	The intracellular ATP Concentration of the *E. coli* reporter and control strains over an extended incubation period of 28 days	166
3.15	The Adenylate Energy Charge (AEC) of the *E. coli* reporter and control strains over an extended incubation period of 28 days	167
4.1	Chemical Structure of Sorbic acid	182
4.2	A schematic diagram which shows the stress response of a cell challenged with weak acids	184
4.3 (a&b)	Determination of Minimum Inhibition Concentration (MIC) of sorbic acid through the measurements of absorbance and bioluminescence readings after 24 hour incubation	193
4.4 (a,b, c)	Bioluminescence, population counts, and bioluminescence per cell without preservative over 28 days	196
4.5	Bioluminescence levels (RLU/ml) expressed by the five constructs when challenged with sorbic acid	203

4.6	Viable Counts (CFU/ml) of the five constructs when challenged with sorbic acid	204
4.7	Bioluminescence per cell (RLU:CFU) of the five constructs when challenged with sorbic acid	205
4.8	ATP-Chemiluminescence levels (RLU/ml) of the five constructs when challenged with sorbic acid	206
5.1	The chemical structure of Benzalkonium Chloride (BAK)	231
5.2	The illustration above show progressive effect of BAK	233
5.3 (a&b)	Determination of Minimum Inhibition Concentration (MIC) of benzalkonium chloride (BAK) through the measurements of absorbance and bioluminescence readings after 24 hour incubation	241
5.4 (a&b)	Determination of Minimum Inhibition Concentration (MIC) of benzalkonium chloride (BAK) + 0.03% EDTA by measurements of absorbance and bioluminescence readings after 24 hour incubation	243
5.5 (a &b)	Determination of Minimum Inhibition Concentration (MIC) of Ethylenediaminetetraaceticacid (EDTA) by measurements of absorbance and bioluminescence readings after 24 hour incubation	246
5.6	Bioluminescence, population counts, and bioluminescence per cell without preservative over 28 days	248
5.7	Bioluminescence levels (RLU/ml) expressed by the five constructs challenged in benzalkonium chloride (BAK)	255
5.8	Population levels (CFU/ml) by the five challenged in benzalkonium chloride (BAK)	256
5.9	Bioluminescence per cell (RLU:CFU) of the five challenged in benzalkonium chloride	257
5.10	ATP- Chemiuminescence levels (RLU/ml) of the five constructs challenged in benzalkonium chloride (BAK)	258
5.11	Bioluminescence levels (RLU/ml) expressed by the five constructs challenged benzalkonium chloride (BAK) with 0.03% EDTA added	266
5.12	Population levels (CFU/ml) expressed by the five constructs challenged in benzalkonium chloride (BAK) with 0.03% EDTA added	267

5.13	Bioluminescence per cell (RLU:CFU) of the five challenged in benzalkonium chloride (BAK) with 0.03% EDTA added	268
5.14	ATP Bioluminescence levels (RLU/ml) of the five constructs challenged in benzalkonium chloride (BAK) with 0.03% EDTA added	269

List of Equations

Equation Number	Title	Page
2.1	Bioluminescence Determination	82
2.2	Colony Forming units Determination	83
2.3	Fluorescence Determination	85
2.4	Microscopy Counts	86
2.5	Standard Error of Means	86
3.1	DNA Purity Formula	135
3.2	Formulation of Gene Copy Numbers	140
3.3	Adenylate Energy Charge Formula	142
4.1	Henderson-Hasselbalch Equation	189

List of Abbreviations

E.coli : *Escherichia coli*

MFC: Microbial fuel cell

PET: Preservative Efficacy Test

lpp: Outer lipoprotein membrane gene

tatA: Twin Arginine Translocase gene

ldc: Lysine decarboxylase gene

lysS: Lysyl- Transfer Ribonucleic acid (tRNA) gene

spc: Ribosomal Protein gene

lux: Represents Lux CDABE operon

RLU: Relative Light Units

CFU: Colony Forming Units

RLU:CFU: Bioluminescence per cell

RFU: Relative Fluorescence unit

TVC: Total Viable Count

A U: Absorbance unit

R^2: Correlation coefficient

SEM: Standard Error Mean

E.P: European Pharmacopeia

B.P: British Pharmacopeia

pK_a: log acid dissociation

SA: Sorbic Acid

MIC: Minimum Inhibition Concentration

BAK: Benzalkonium Chloride

EDTA: Ehylenediaminetetraacetic acid

DNA: DeoxyNucleic Acid

dNTP: DeoxyNucleotideTriPhosphate

TSA: Tryptone Soy Agar

qPCR: Quantitative Polymerase Chain Reaction

PCR: Polymerase Chain Reaction

VNBC: Viable but non culturable

ATCC: American Type Culture Collection

ATP: Adenosine Triphosphate

AEC: Adenylate Energy Charge

SEM: Standard Error of the Means

PCN: Plasmid Copy Number

C_T: Cycle threshold

Tm: Melting point

SD: Standard Deviation

OD: Optical Density

A: Adenine

G: Guanine

T: Thymine

C: Cytosine

ml: Mililiter

µl: Microliter

mg: Miligram

g: Gram

fg: Femto gram

bp: base pairs

ATCC: American Type Culture Collection

Table of Contents

Dedication.....1

Abstract..... iii

Acknowledgements..... 1-5

List of Tables 6

List of Figures 7

List of Equations 10

List of Abbreviations 11

1 : General Introduction 24

1.1 : Conventional and Rapid Methods for Detection and Enumeration of bacteria 24

1.1.1 : Conventional Methods 25

1.1.2 : Rapid/Alternative Enumeration Methods 30

1.1.2.1 : Biochemical Methods 30

1.2 : Introduction to Biosensors 39

1.2.1 : Whole-cell Microbial Biosensors 40

1.2.2 : Advantages of using Microorganisms as Bio-sensing Elements 41

1.2.3 : Types of Whole-cell Microbial Biosensors 42

1.3 : Gene Expression and Promoter Functions 57

1.4 : Biological Functions of Promoters 62

1.4.1 : Lysyl-tRNA Synthetase (lysS).....63

1.4.2 : Ribosomal Protein (spc).....63

1.4.3 : Outer membrane lipoprotein (lpp) 64

1.4.4 : Twin-Arginine Translocase (tatA) 64

1.4.5 : Lysine Decarboxylase (ldcc) 64

1.5 : Preservative Efficacy Testing 65

1.5.1 : Preservative Efficacy Testing in Accordance with European and British Pharmacopeia Regulations 65

1.6 : Rationale of Study 70

1.7 : Aim 71

2 : The Validation of the Five Bioluminescent reporter strains *E. coli* Constructs- as a Rapid Real Time Microbiological Testing Method. 73

2.1 : Introduction 73

2.1.1 : Validation Criterias' set by the Pharmacopoeia Regulations 74

2.2 : Rational of Chapter 2 75

2.3 : Objectives 77

2.4 : Materials and Methods 78

2.4.1 : Construction of Bioluminescent Reporter Strains *E. coli* 78

2.4.2 : Media and Solutions 81

2.4.3 : Bioluminescence Determination 82

2.4.4 : Viable Counts Determination 83

2.4.5 : ATP-Chemiluminescence Determination 84

2.4.6 : Determination of Fluorescence readings of Viable Cells Using LIVE/DEAD stains . 85

2.4.7 : Epifluorescence Microscopy Counts 85

2.4.8 : Statistical Analyses 86

2.4.9 : Validation 87

2.4.10 : Bioinformatics Analysis of the Promoter Regions 89

2.5 : Results 90

2.5.1 : Recovery Determination of Bioluminescence and Plate Counting 90

2.5.2 : Determination Of The Precision And Equivalence Of The Whole-Cell Bioluminescence Method And Compared To The Conventional Plate Counting Method 92

2.5.3 : Trends and relation to conventional plating method 96

2.5.4 : Trends and relation to Bioluminescence Method 98

2.5.5 : Minimum Limit Of Detection And Working Range Of Methods Employed 100

2.5.6 : Comparison of Time Required to Undertake Each Method 102

2.5.7 : Analysis of Promoter Strength of the Five Constructs 103

2.6 : Discussion 107

2.6.1 : Validation of the Five Bioluminescent Constructs as a Rapid Microbiological Method In Comparison With Traditional Plate Counting 107

2.6.2 : Evaluation of the Promoter Strength, And Its Relationship with Bioluminescence Expression 114

2.7 : Conclusions 121

3 Comparisons of Bioluminescence, Growth, Adenylate Energy Charge (AEC), and Plasmid Copy Number over Extended Incubation period 123

3.1 : Introduction 123

3.1.1 : Bacterial Growth and Survival Phases 123

3.1.2 : Plasmid Copy Number 125

3.1.3 : ATP as a Prime Energy Source for Bacteria 129

3.2 : Rational of Chapter 3 131

3.3 : Objectives 132

3.4 : Materials and Methods 133

3.4.1 : Construction of Growth Curves 133

3.4.2 : Fluorescence Imaging 133

3.4.3 : Plasmid Copy Number (PCN) Determination 134

3.4.4 : Adenosine Measurements 141

3.4.6 : Statistical Analysis 142

3.5 : Results 143

3.5.1 : Bioluminescence and viable count Kinetics in the Growth and Survival Curve 143

3.5.2 : Morphological and Physiological Changes to the Bacterial Cell Over Extended period of 28 days 155

3.5.3 : Plasmid Copy Numbers (PCN) Determination 157

3.5.4 : ATP standard 165

3.6 Discussions 169

3.6.1 : Physiology, Morphology, Metabolic Status, And Plasmid Copy Number Of The Five *E. Coli* Biosensor Strains And The Control *E. Coli* Strains 169

3.6.2 : Relationship Between Extended Stationary phase and Bioluminescence 176

3.7 : Conclusions 180

4 Whole-Cell Bioluminescence Evaluation Using Sorbic Acid 182

4.1 : Sorbic Acid as a Preservative 182

4.1.1 : Mode of Action 183

4.2 : Rationale of Chapter 4 185

4.3 : Objectives 186

4.4 : Materials and Methods 187

4.4.1 : Preparation of Bacterial Initial Inoculum and Preservative Solutions 187

4.4.2 : Preservative Efficacy Testing (PET) 188

4.4.3 : Determination of the Minimum Inhibitory Concentration (MIC) of Sorbic acid 188

4.4.4 : Calculation of the Intracellular Undissociated Acid and Dissociated Acid Anion Concentration Present in the Experiments 189

4.4.5 : Statistical Analysis 189

4.5 : Results 190

4.5.1 : Concentrations of the undissociated and dissociated forms of sorbic acid in solution and in the cytoplasm, calculated according to Henderson-Hasselbalch equation 190

4.5.2 : Minimum Inhibitory Concentration (MIC) of Sorbic Acid 193

4.5.3 : Bioluminescence and Viable Counts of the Preservative-Free Negative Controls ... 196

4.5.4 : Effect of Sorbic Acid on Bioluminescence, Viable Counts and ATP-Chemiluminescence for the Five Biosensor Strains and the Control Strains of *E. coli* ATCC 8739 in a Preservative Efficacy Test 199

4.6 : Discussions 212

4.6.1 : Minimum Inhibitory Concentration (MIC) of Sorbic Acid 212

4.6.2 : Calculation of Undissociated and Dissociated levels of Sorbic Acid by Henderson-Hasselbalch Equation....214

4.6.3 : Effect of Sorbic Acid on Bioluminescence, Viable Counts and ATP-Chemiluminescence of Biosensor and Control *E. coli* ATCC 8739 Strains in Preservative Efficacy Test (PET) 215

4.6.4 : The Overall Comparisons between the Constitutive Promoters Expression Bioluminescence to the ATP-Chemiluminescence and Viable Counts Methods....221

4.6.5 Bioluminescence Expression in Relation to the Promoters Employed....224

4.6.6 : Selected Promoters under Acidic Conditions at pH 5.0 226

4.7 : Conclusions....228

5 Evaluation of Whole-Cell Bioluminescence Using Benzalkonium Chloride (BAK)....230

5.1 : Benzalkonium Chloride as a Preservative 230

5.1.1 : Mode of Action....231

5.2 : Rationale of Chapter 5 234

5.3 : Objectives 235

5.4 : Materials and Methods....236

5.4.1 : Preparation of Bacterial Initial Inoculum and Preservative Solutions....236

5.4.2 : Preparation of Preservative Solutions....236

5.4.3 : Preservative Efficacy Test 238

5.4.4 : Determination of the Minimum Inhibitory Concentration (MIC) ofBenzalkonium Chloride (BAK), BAK +0.03% EDTA, and EDTA 238

5.4.5 : Statistical Analysis....239

5.5 : Results....240
5.5.1 : Minimum Inhibitory Concentration (MIC) Benzalkonium Chloride (BAK)....240
5.5.2 : Minimum Inhibitory Concentration (MIC) of Benzalkonium Chloride (BAK) when Supplemented with 0.03% EDTA....243
5.5.3 : Minimum Inhibitory Concentration (MIC) of EDTA....245
5.5.4 : Bioluminescence and Viable Counts of the Preservative-Free Negative Controls ...247
5.5.5 : Effect of Benzalkonium Chloride (BAK) on Bioluminescence, Viable Counts and ATP-Chemiluminescence for the Five Biosensor Strains and also the Control Strains of *E. coli* ATCC 8739 in a Preservative Efficacy Test....251
5.5.6 : Effect of Benzalkonium Chloride (BAK) with the addition of 0.03% EDTA on Bioluminescence, Viable Counts and ATP-Chemiluminescence for the Five Biosensor Strains and the Control Strains of *E. coli* ATCC 8739 in a Preservative Efficacy Test....262
5.6 : Discussions....273
5.6.1 : The Effect of Benzalkonium Chloride (BAK) on Biosensor and Control Strains....273
5.6.2 : The Effect of 0.03 % EDTA and EDTA on the Biosensor and Control Strains upon Bioluminescence, Viable counts and ATP-Chemiluminescence....278
5.6.3 : Overall Comparison of the Bioluminescent Biosensors with the Viable Counts and ATP-Chemiluminescence Methods in Preservative Efficacy Testing....283
5.7 : Conclusions....285
Chapter 6....286
6 : General Discussion and Conclusion....287
6.1 : Whole-cell Bioluminescence as a Rapid Method....287

6.2 : Selection of the Best overall Constitutive Promoter 290

6.3 : Future work 295

6.4 : Conclusion 297

References 298

Appendix Chapter 2 341

Appendix Chapter 3 354

Appendix Chapter 4 380

Appendix Chapter 5 397

Chapter 1

1 : General Introduction

1.1 : Conventional and Rapid Methods for Detection and Enumeration of bacteria

Microbiological testing is a critical tool in process monitoring control, quality control, surveillance, and in providing inputs to risk assessment (Hoorfar, 2011). It is a key process in the pharmaceutical, biotechnology, cosmetic, food and beverages industries (Meder et al., 2012). The importance of microbiological testing is to ensure the level of microorganisms encountered does not exceed a point where it becomes hazardous to health or the environment (Hoorfar, 2011). Conventional methods are the gold standard for the determination of microbiological quality as they are reliable, easy to carry out, demands no expensive infrastructure and are rather cheap in consumables. Nevertheless, these methods are time consuming procedures both in operation, data collection and labour intensive (Jasson et al., 2010). Moreover, they depend on the ability of microorganisms to yield visible colonies after an incubation period of typically 3 days, but can go up to 14 days (European & United States Pharmacopeia), and demands large volumes usage of liquid and solid media (Jasson et al., 2010; Meder et al., 2012). There are various conventional methods employed, such as the conventional pour/spread plate method, culture turbidity and most probable number method (MPN) (Discussed in Section 1.1.1). These standardised classical culture methods are still in use by many labs, especially by regulatory agencies because they are regarded as harmonised methods and these are considered as important aspects in international trade and compliance testing. However, interest has risen in the development of more rapid methods during the last decades. The term ‘rapid methods’ is defined by Feng (1996) and Fung (1994) as ‘methods that significantly shorten the analysis time compared to conventional detection procedures’. Many rapid methods have been developed and marketed in recent years (Discussed in Section 1.1.2).

1.1.1 : Conventional Methods

1.1.1.1 : Wet and Dry Cell Weight

This method is widely used for the estimation of bacterial biomass as well as for basic calibration of other methods. Wet cell weight is determined after washing and centrifugation or filtration of cells. The wet weight method has high variability and is inaccurate. The dry weight of bacterial cells is obtained by either freeze drying or heating in an oven at 105 °C until no further weight change occurs (Hobson et al., 1996). The determination of bacterial biomass is achieved by converting bacterial volume into organic carbon. The conversion factor involved is calculated from values of buoyant density, the dry weight/wet weight ratio, and carbon weight/dry weight of bacterial cells. By using a conversion factor of 0.22 g of Ccm^{-3}, the carbon content in bacterial biomass is estimated (Bratbak & Dundas, 1984). This method is simple, but it is laborious, inaccurate and unable to distinguish viable from dead cells. Furthermore, errors are introduced during the process in decomposition of biological materials (Hobson et al., 1996). Both are also susceptible to operator error in dilution or evaluation. At a minimum, neither method provides real-time information for process control.

1.1.1.2 : Turbidity

Turbidity is another widely used method for the estimation of cell density in suspension. The turbidity of a suspension can be determined by using a spectrophotometer to measure the loss of light from a beam due to scattering and absorption (Singh et al., 1994). The measurement of turbidity is affected by the osmotic potential across cell membranes, as this leads to changes in cell surface area/volume ratio and refractive index resulting in changes to the turbidity of cell suspension (Hobson et al., 1996). Turbidity measurements can usually be made without destroying or significantly disturbing the sample and results are easy to interpret. With that, turbidity

measurements are widely employed to monitor the growth rate of microbial cultures (Madigan & Martinko, 2006). However, turbidity measurements do not distinguish between viable and dead cells, therefore this method cannot be used to measure death kinetics of microbes.

1.1.1.3 : Viable Counts

Viable cell counting methods are widely used to estimate viable microbial populations. Evaluation of viable cell count is based upon the ability of the cells to grow and multiply in a liquid nutrient culture medium, on an agar gelled nutrient culture medium or on the surface of a membrane filter laid onto a nutrient agar medium. The plate count and membrane filter methods are examples of these techniques. These methods are highly recommended as they have been validated as one of the compendia methods (British & European Pharmacopeia). However, these methods are laborious, require high operating skills, and a long incubation period (24 – 72 hour) (Hobson et al., 1996). Roszak and Colwell (1987) demonstrated the plate counting procedure as an underestimation method for enumeration of viable cells, where counts were lower as compared to direct epifluorescence microscopy counts.

1.1.1.4 : Plate Counting Methods

There are a few plate counting methods such as the pour plate, agar droplet, spread plate, and surface drop. The common feature between these methods is that the bacterial suspensions are diluted (normally tenfold) in diluents such as ringers solution to ensure that the number of bacterial colonies are within a countable range (*i.e.* 30 to 300 colonies) on agar petri dishes.

In the pour plate method, 1 ml of an appropriate dilution is dispensed into a petri dish, where molten nutrient agar is then poured, tempered to 50°C, and mixed carefully. Once the mixture solidifies, it is

incubated at an optimum temperature for the expected bacterial species (Thatcher & Clark, 1968). The agar droplet method is an improved version of the pour plate method. The dilutions of the bacterial suspensions are prepared directly in molten nutrient agar, instead of liquid diluents (Sharpe & Kilsby, 1971). The colonies are formed in the solidified droplets (0.1 ml) during incubation. The advantage of this method is that the preparation of agar can be done on the day to avoid potential contamination.

The spread plate method involves the use of solidified pre-poured nutrient agar plates. Volumes (normally 0.1 ml) of the appropriate dilutions are spread evenly over the surface using a sterile L-shaped glass rod. The spread plate method offers improved aeration for bacterial growth and minimal desegregation of colony formation (Pepper & Gerba, 2009). Furthermore, heat sensitive cells are not killed by the molten agar, which may occur in the pour plate and agar droplet methods when the temperature is too high. Secondly, the colonies developed on the surface of the agar and they can be easily observed and sub-cultured if needed.

Meanwhile, the surface drop method is a modified method of the spread plate known as the Miles and Misra method. Calibrated pipettes dispensing 0.02 ml are used to dispense five separate droplets of diluted sample on solidified agar (Miles & Misra, 1938).

1.1.1.5 : Membrane Filter Method

The membrane filter method is based upon the use of a highly porous pore-size cellulose acetate membrane filter that hinders the passage of bacterial cells, which in turn allows the small number of cells that may be present in large volumes of water to be concentrated (Hobson et al., 1996). Bacteria that are retained by the filter are cultured by applying the filter to the surface of a solidified nutrient agar plate, followed by incubation at an optimum temperature after which the number of colonies formed are counted. The most common pore size for membrane filters used is 0.45µm (Hobson et al.,

1996). The main advantage of the membrane filtration method over the plate count method is that large sample volumes containing low and dispersed cell populations can be processed (Jones, 1979). However, this technique can be very laborious and time consuming.

1.1.1.6 : Most Probable Number (MPN)

Most probable number (MPN) is a method used to estimate the concentration of viable microorganisms in a sample by growth in replicate volumes of liquid broth and is normally performed using ten-fold dilutions. The inoculated samples are incubated and the number of tubes showing growth (turbidity) at each dilution is used to estimate the cell count by referring to a probability table (Meynell & Meynell, 1970). This method offers real advantages as an enumeration tool (Sutton, 2010) for the detection of microorganisms with low numbers and that grow poorly on agar plates. Unlike other direct quantitative procedures, MPN only measures live and active microbes. However, the drawbacks of MPN method are its poor selectiveness of growth for some microorganisms that have different nutritional, physiological requirements and incubation conditions used. The MPN method is not as accurate and precise as the plate count method (Hobson et al., 1996).

1.1.1.7 : Modified and Automated Conventional Methods

Various improvements have been made to the laborious methods mentioned, in an attempt to increase their convenience and ease-of-use. Automation in enumeration methods can be very useful to reduce the time needed for various activities such as the preparation of media, serial dilutions, counting colonies.

The spiral plating method is an example of an automated system that can be used to obtain a viable cell count (Spiral Biotech). A stylus is used to spread a sample of liquid on the surface of a pre-poured agar plate in an Archimedean spiral. This gives a concentration gradient starting from the centre and decreasing as the spiral progresses outward on the rotating plate (Fung, 2002). The agar plate is then incubated (24 – 72 hours) for the colonies to develop. The colonies that grow can be counted manually or electronically using a laser counter. The time required for plating is only several seconds as compared to minutes when conventional methods are used (Fung, 2002).

In addition, chromogenic or fluorogenic substrates are also used in selective media for detection, enumeration, and identification of bacteria, where these procedures can be performed directly on an isolation plate (Manafi, 1996). These compounds yield brightly coloured or fluorogenic products when reacting with specific bacterial enzymes or bacterial metabolites for identification of bacteria species (Boer & Beumer, 1999).The incorporation of such fluorogenic or chromogenic enzyme substrates into a selective medium can eliminate the need for subculture and further biochemical tests to establish the identity of certain microorganisms (Manafi et al., 1991, Manafi, 1996).

The SimPlate system (Biocontrol) automates the dispensing of samples into 84 wells (Fung, 2002). Once the sample is dispensed, rehydrated liquid nutrient medium is added into the wells. The mixture is distributed evenly into the wells by swirling the SimPlate. After incubation, the plate is placed under UV light. Wells which demonstrate fluorescence are counted and the number is converted into MPN by using standard MPN tables (Fung, 2002). TEMPO (Bio-Mérieux), and Colilert (IDEXX Laboratories) are examples of automated MPN principle of enumeration method (Jasson et al., 2010).

1.1.2 : Rapid/Alternative Enumeration Methods

Rapid methods in this context can be interpreted as a shorter time for microbial detection and/or the capacity for handling large throughput of samples for the convenience of routine testing (Jasson et al., 2010). The main methods discussed are: biochemicals capture, and specialized equipment methods employed in rapid testing.

1.1.2.1 : Biochemical Methods

Biochemical methods are based on the measurement of biochemical properties or interactions of microorganisms. Key methods adopted in industries and research labs include ATP-chemiluminescence, direct epifluorescence filter technique (DEFT), enzyme linked immunosorbent assay (ELISA), and fluorescence antibody technique.

1.1.2.2 : ATP-Chemiluminescence

ATP-chemiluminescence is used to determine the approximate number of living organisms present in a sample by determining intracellular ATP present. The viable cell count is estimated by determining the usage of a conversion factor to quantitate the amount of ATP present in the sample, via the firefly luciferase assay (Sharma & Malik, 2012). ATP-chemiluminescence is dependent upon the viability of the cells in the sample, as ATP degrades rapidly after cell death (Deininger & Lee, 2001). ATP-chemiluminescence is one of the most widely used rapid detection methods in microbiology (Sakakibara et al., 2003). The rapid response time makes this system very suitable for on-line monitoring in hazard analysis critical control point (HACCP) manufacturing protocols (Sharma & Malik, 2012).The ATP-chemiluminescence method reduces the test time to approximately one third of that required for traditional methods (PDA, 2000). The Commercial MicroStar system developed

by Millipore Corp (Benford, Mass., USA) combines the ATP-chemiluminescence technique with trapping bacteria on a specialized membrane filter (Milliflex).

The ATP-chemiluminescence assay is an excellent and sensitive method requiring only a few minutes (Fung, 2002). However, the extraction process for intracellular ATP is regarded as a laborious method when conventional extraction methods are applied. The ATP-chemiluminescence method is prone to contamination during the extraction of intracellular ATP. Non-microbial sources of ATP in foodstuffs and bodily fluids could be measured indirectly. Desquamated human skin cells will also be present in most environments. Beggs et al. (2008) estimated that an adult sheds some 10^8 cells in a 24 hour period. Furthermore, each cell is associated with about 100 bacteria.

Hence, the ATP-chemiluminescence method does not differentiate intracellular microbial ATP from other forms of ATP. Other limitations include sensitivity to pH (Boer & Beumer, 1999), temperature (Boer & Beumer, 1999) and the presence of quenchers (Wen et al., 2000) influences light emission.

1.1.2.3 : Direct Epifluorescence Filter Technique (DEFT)

The direct epifluorescence filter technique (DEFT) is a direct method that is used for enumeration of microorganisms, and is based upon the binding properties of a fluorochrome such as acridine orange or DAPI. This technique utilizes the principle of membrane filtration where the samples are pre-treated with detergents and proteolytic enzymes, and then stained with a fluorescent compound, followed by visualisation under fluorescence microscopy (Hobson et al., 1996).

DAPI and acridine orange are able to permeable to cells, and they interact with DNA and RNA by intercalation or electrostatic attractions is visible under fluorescence microscopy. However, nonspecific binding of the fluorescent dyes such as DAPI and acridine orange in heterogeneous

matrix limits the benefits of this technique (Klauth et al., 2004). Recently, the application of novel nucleic acid dyes has shown a lot of potential in the replacement of DAPI and acridine orange. In comparison, there is a better signal to background ratio due to an increase in fluorescence, specifically during DNA binding (Klauth et al., 2004). The new generation of nucleic acid dyes includes: SYBR Green; SYTOX; PICO green; and SYTO dyes (Klauth et al., 2004). Investigation of the viable but non culturable (VBNC) phenomenon using fluorescent redox dyes is on the increase (Besnard et al., 2000).

The redox dye 5-cyano-2, 3-ditolyl tetrazolium chloride (CTC) has been used as an artificial electron acceptor that directly competes with molecular oxygen. The reducing power generated by the electron transport system converts the CTC into insoluble, fluorescent, CTC-formazon crystals (*i.e.* clearly visible with UV optics and epi-illumination) which accumulate in metabolically active bacteria (Besnard et al., 2000).

Thus, the staining procedure is simple and when coupled with direct viable counts is able to demonstrate greater sensitive compared to plate counts (Rowan, 2004). However, this technique requires skilled personnel to operate the microscope.

1.1.2.4 : Enzyme-Linked Immunosorbent Assay (ELISA)

Enzyme-linked immunosorbent assay (ELISA) is a diagnostic tool that uses antibodies and colour change to identify a substance, usually an antigen, in a sample (Gracias & McKillip, 2004). Although enzyme-linked immunosorbent assays (ELISA) are widely used in clinical and food analysis, it was not until recently that these methods were applied to pharmaceutical quality control. They are many ways to perform antigen-antibody reactions, but the most popular format in recent years is the 'Sandwiched' ELISA test (Fung, 2002). This process involves a primary antibody which is specific

to go the target molecule sample. A secondary antibody is added, which forms a 'sandwich' of an antigen between two antibody molecules. When the assay is developed a colorimetric change is observed upon addition of substrate. Alternatively, the secondary antibody may be conjugated to a fluorophore and be observed by fluorescence excitation. Based on the principle of antibody-antigen interaction, this test allows for easy visualization of results and can be completed without the additional concern of radioactive materials use.

ELISA has been successful for detection of whole cell antigens of pathogenic bacteria (Gracias & McKillip, 2004). The ELISA method is reported to be sensitive - with a minimum detection limit of 10^3- 10^4 CFU/ml (Cox et al., 1987; Hobson et al., 1996). Many diagnostic companies such as BioControl, Organon, Tecra, and Molecular Circuitry have marketed ELISA test kits. The ELISA test kits are examples of automated systems, which can perform the entire ELISA procedure automatically and can complete an assay in minutes (Fung, 2002). However, the drawbacks of all of these methods are the requirement for skilled personnel and expensive equipment, which can render routine testing uneconomical.

1.1.2.5 : Fluorescent Antibody Technique

The fluorescent-antibody technique can be used to enumerate specific groups of microorganisms *in situ*. The principle of this method is based on fluorescent probes designed to bind to specific target sites on or in cells. These fluorescent probes are usually antibodies which fluoresce when stimulated by an energy source (*e.g.* laser). The emission of fluorescence occurs extremely rapidly after the absorption of excitation light, upon conjugation of fluorescent antibody. This requires a short incubation time between probes and microorganisms. However, this method is costly and loss of fluorescence signal occurs over time. This method is widely used for bacterial enumeration in microbial ecology studies, and pathogen monitoring (Hobson et al., 1996).

1.1.2.6 : Capture Methods

The capturing methods in this section involve molecular techniques and magnetic, super-paramagnetic particles and magnetization of microbial cells.

1.1.2.7 : Molecular Techniques

A range of molecular targets have been utilised in microbiological assays. Nucleic acid sequence-based methods target specific nucleic acid sequences of bacteria (Noble & Weisberg, 2005). Experiments utilising the polymerase chain reaction (PCR) have been used to detect DNA sequences and identify as well as enumerate bacterial species (Keer & Birch, 2003). The PCR-based methods for detection of foodborne microbial pathogens are recognized and standardised by International Organization for Standardization (ISO) guidelines (ISO, 2010). Other molecular techniques include reverse transcriptase polymerase chain reaction (RT-PCR), quantitative PCR (qPCR), nucleic acid sequence based amplification (NASBA), and microarrays. The applications of DNA microarrays (Ye et al., 2001) and peptide nucleic acid (PNA) probes (Stender et al., 2002) have been employed to increase the speed and specificity of detection of bacterial species present in a sample (Stender et al., 2002; Tomas et al 2009; Ye et al., 2001).

The International Standardization Organization (ISO) recently published standards which address the PCR based methodology for the detection of food-borne pathogens quality assurance of food-borne pathogens in food and dairy products. Monnet et al. (2006) have demonstrated the application of qPCR in quantifying *Corynebacterium casei,* which is present on the surface of cheese after ripening. In addition, the quantification of *Penicillium roqueforti* and *Penicillium camemberti* with qPCR was

used as a biomass indicator to monitor changes in fungal population growth during cheese ripening (Le Drean et al., 2010).

However, the limit of quantification is approximately 10^3-10^4 CFU/ml (Jasson et al., 2010). This limit is achievable by other rapid methods and is insufficient for hygiene indicators under GMP conditions, as they require a minimum limit of 10 CFU/ml to a maximum of 10^4 CFU/ml (Jasson et al., 2010).

Nevertheless, the use of mRNA as a marker of viability is preferred as compared to DNA, for mRNA is a highly labile molecule with a very short half-life (seconds) and therefore should be more closely correlated with the viability of the status than DNA-based methods (Keer & Birch, 2003). Molecular recognition approaches have the potential to be for being more rapid, more sensitive and adaptable to a wider class of bacteria and pathogens. However, molecular approaches require careful optimisation of the experimental design which could be time consuming, in addition requires expensive reagents.

1.1.2.8 : Magnetic, Super-Paramagnetic Method

Many magnetic or magnetisable carriers are super paramagnetic, where they only exhibit magnetic properties in the presence of an external magnetic field. Since the particles are not magnetic in themselves, they are not attracted to each other and therefore they can be easily suspended into a homogeneous mixture in the absence of any external magnetic field (Lea et al., 1988). However, they can be easily removed from suspension by application of a magnetic field. This approach uses the paramagnetic beads as binding platforms or linkers between analytes and labels, which also allows for easy separation of target analytes from a mixed sample by simply using a magnet (Maalouf et al., 2008). Impedance spectrometry has been used to monitor *E. coli* O157:H7 binding to antibodies conjugated to paramagnetic beads and captured on nanoporous membranes (Chan et al., 2012). This

method has demonstrated ultra-low sensitivity levels as low as 10 CFU/ml for *E.coli* O157: H7 (Chan et al., 2012). However, these methods are not commonly used in routine testing, despite the level of sensitivity that can be achieved.

1.1.2.9 : Other Specialized Equipment for Bacterial Detection

Since technology has advanced tremendously, a wide range of specialised equipment can now be used to detect bacteria. Examples of novel alternative rapid methods that can be used to detect and enumerate bacterial populations include: solid-phase flow cytometry (Marie et al., 1999); confocal scanning laser microscopy (CLSM) (Auty et al., 2001), and scanning electron microscopy (SEM) (Sanders et al., 2012). Amongst microscopy methods, a solid-phase cytometry method for conducting total direct counts of bacteria was found less biased, and it also performed significantly better, than the other microscopy methods tested (Lisle et al., 2004).

Recent attempts to monitor cellular metabolism in bacterial cultures have employed nuclear magnetic resonance (NMR) spectroscopy, which relies on measuring phosphates in bacterial cultures as an indicator of cellular metabolism (Gupta et al., 2012). Short wavelength near infrared spectroscopy (Sonnleiter et al., 1992) and Fourier transform infrared spectroscopy (FTIR) (Davis & Mauer, 2010) have also been used to measure the metabolic state of microorganisms. In contrast, traditional plate count methods are unable to distinguish the metabolic state of microorganisms.

Table 1.1 shows the many methods, both conventional and alternative, that have been reported and discussed to detect, enumerate, and screen microorganisms and compares the advantages and disadvantages of the conventional and alternative methods presented. The disadvantages include: requirement for either a long incubation time; highly skilled personnel; expensive reagents or equipment; and also possibly, limited suitability use in routine testing. These features could

potentially make a product more difficult to market to consumers. A method which circumvents the undesirable traits of current rapid methods would be advantageous in rapid microbiological testing. Hence, the application of reporter genes in a whole-cell microbial biosensor could potentially serve as a novel real-time rapid microbiological method. Whole-cell biosensors incorporating reporter genes offer faster responses than traditional plate count methods and can be used to monitor process critical control points in real time whilst being cost effective.

Table 1.1: A comparison of the advantages and disadvantages of the various conventional and alternative microbiological testing methods

Method	Advantage	Disadvantage
Wet and Dry cell weight	Cost effective	Laborious, inaccurate, does not distinguish live and dead cells
Turbidity	Cost effective, Easy interpretation of results	Does not distinguish live and dead cells
Viable counts	Considered as a 'gold standard' and recognized by international consensus	Long incubation time, laborious
Membrane filter method	Favorable for large sample volumes. Able to recover dispersed cell populations	Laborious and time consuming
Most probable Number method	Able to recover low numbers of cells. Only measures live and active microbes	Laborious, dependent upon special requirements
ATP- bioluminescence	Sensitive and rapid	Background bioluminescence. Special conditions required for testing (e.g. pH, temperature, chemical). Prone to contamination
Direct Epifluorescence Filter technique	Sensitive in recovering small populations of microbes	Prone to nonspecific binding Requires trained personnel
ELISA	Easy interpretation of results	Expensive materials and equipment
Fluorescent Antibody Technique	High sensitivity and specificity in microbial ecology studies	Expensive reagents and the need for antibody synthesis
Molecular Techniques	Specific and adaptable to a wider range of bacteria	Requires optimisation of the experimental parameters
Magnetic, super paramagnetic method	Sensitive	Not applicable for routine testing

1.2 : Introduction to Biosensors

The last two decades have witnessed remarkable progress in the development of biosensors and their application in areas such as environmental protection, biotechnology, medical diagnostics, drug screening, food safety and bioterrorism detection and protection. The word 'biosensor' has been defined in different ways in journals and text books. 'Biosensor' is defined by the International Union of Pure and Applied Chemistry (IUPAC) as "a compact analytical device incorporating a biological or biologically-derived sensing element either integrated within, or intimately associated with, a physiocochemical transducer".

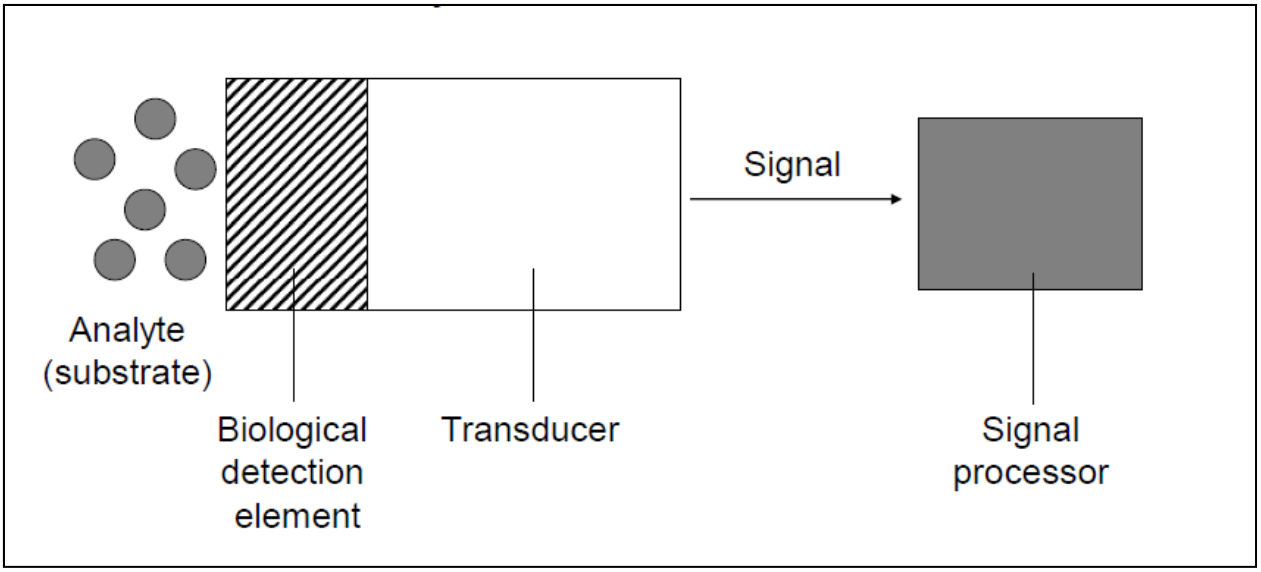


Figure 1.1: Schematic diagram showing the layout of a biosensor. It consists of 3 parts; the biological detection element, a transducer and a signal processor.

A biosensor consists of a biological sensing element, a transducer and a signal processor (Figure 1.1). The biological sensing element acts as the sensing device which contains biological molecules such as whole cells, enzymes, paramagnetic nanoparticles particles attached to whole cells (Maalouf et al., 2007), peptides, antibodies and enzymes (D'Souza, 2001).The biological sensing element is linked to a transducer which converts the signal from the sensor into a quantifiable signal (Figure

1.1). These signals are then amplified so that they can be processed and analysed (Mulchandani & Rogers, 1998).

The variety of different types of transducers give rise to different categories of biosensors such as potentiometric, amperometric, conductimetric, voltammetric, microbial fuel cell, optical and magnetic biosensors (Jianrong et al., 2004) (Discussed in Section 1.3). The application of biosensors has the capability to shorten the time between sampling and results, and potentially to save a proportion of the cost involved. The use of biosensors allows both miniaturization and automation. It also allows sample volumes in the range of microliter or less (Zhang et al., 2009). Commercial biosensors are rapid, reliable, compact and user-friendly instruments. Examples of commercially available biosensors include glucose biosensors (MiniMed Paradigm® by Medtronic), pregnancy test (Clearblue®) and a recently developed HIV biosensor (OraQuick® In-Home HIV Test by OraSure).

1.2.1 : Whole-cell Microbial Biosensors

Microbial whole cells microbial have been used successfully in biosensors (De Souza, 2001; Lei et al., 2006; Su et al., 2011). Many studies have attempted to construct biosensors that incorporate microbial whole-cells within microfabricated devices, and significant progress has been made towards this goal over the past decade (Shiku et al., 2008). Microbes can be used in either a viable or a non-viable form, with viable cells becoming considerably more significant in the production and development of biosensors (Burlage & Kuo, 1994; Riedel et al., 1993; Arikawa et al., 1998; Simonian et al., 1998).

Viable microbes have the ability to metabolise organic substrates, either anaerobically or aerobically, resulting in end products such as ammonia, carbon dioxide, and organic acids. These end products

can then be monitored as a proxy for the presence and concentration of the substrate or the metabolic activity of the cell. Viable whole-cell biosensors monitor the respiratory and metabolic functions of the cell, with the target analyte being either a substrate that enhances activity or an inhibitor that reduces it (D'Souza, 2001). Non-viable cells can also be an economical source of intracellular enzymes. These can be incorporated into simple biosensor applications which do not require metabolic respiratory activity or cofactor regeneration (Mulchandani & Rogers, 1998; Svitel et al., 1998; D'Souza, 1999, 2001). Alternatively, classical analytical methods such as Affinity chromatography (AC), Ion exchange chromatography (IEC), Gas Chromatographic (GC) and High Pressure Liquid Chromatography (HPLC) methods are used to determine the concentration, and possibly identity of analyte/(s).

The classical analytical methods require extraction and separation of the target analyte to be performed and, depending on the number of analytes tested, this process can take hours or even days for completion (Spier et al., 2012). Whole-cell biosensor methods permit the detection of specific target analytes by making specified genetic alterations to the cells, as well as enabling analysis to be completed in minutes.

1.2.2 : Advantages of using Microorganisms as Bio-sensing Elements

Microbes offer a number of advantages as biological sensing elements. These advantages include:

I. Microbes are ubiquitous in the environment (Lei et al., 2006). Culturing of many microbial species is quick and inexpensive.

II. Microbes consist of numerous enzymes and cofactors/coenzymes with the ability to respond to a wide range of chemicals, which means that they can potentially sense many different analytes (Su et al., 2011).

III. Microbes are amenable to genetic modification through mutation or recombinant DNA technology (Liu et al., 2005). This allows the construction of novel cells capable of sensing the substrate of interest (Su et al., 2011).

IV. Microbes have a broad operating range for both pH and temperature, and can adapt their metabolism in response to the relative scarcity or excess of a particular resource (Salis et al., 2008).

V. Microbes are able to respond to a wide range of environmental changes, which makes them suitable for ecotoxicity testing and environmental monitoring (Bentley et al., 2001).

VI. Microbes will accept the introduction of reporter genes in extrachromosomal DNA (plasmid) or chromosomal DNA, which they can replicate without losing physiological function.

1.2.3 : Types of Whole-cell Microbial Biosensors

Whole-cell microbial biosensors can be classified on the basis of the transducers used to process the signal from the sensing element. This signal can be further interpreted by an electronic device. There are two classifications of microbial biosensors; electrochemical microbial biosensors and optical microbial biosensors.

1.2.3.1 : Amperometric Electrochemical Whole-cell Microbial Biosensors

The basic principle of an amperometric whole-cell microbial biosensor is that it operates at a fixed electrical potential with respect to a reference electrode and output potential is correlated with the concentration of the target analyte. In an amperometric biosensor, the output current is generated by the oxidation or reduction of either cations or anions on the surface of the electrode (Lei et al., 2006). Amperometric biosensors are the most common form of electrochemical whole-cell microbial

biosensors. Amperometric whole-cell microbial biosensor have been extensively exploited for environmental applications (D'Souza, 2001), food and fermentation field analysis (D' Souza, 2001).

An example of a commonly used amperometric microbial biosensor is one that was developed to determine the biochemical oxygen demand (BOD) of a water sample; a value related to the concentration of biodegradable organic pollutants in aqueous solutions (Liu et al., 2000).The traditional method for the determination of BOD measures the microorganisms' oxygen consumption/respiration over a period of 5 days (Chan et al., 1999) and is reported as BOD_5. The time taken to process samples by conventional methods can be in the order of hours whilst the response time of an amperiometric biosensorto pollutants is of the order of minutes; for example a response time of 3-5 minutes was achieved by entrapping *Pseudomonas syringae* in a highly porous micro-cellular polymer conjugated with an oxygen electrode (Kara et al., 2009).

Phenol and phenolic compounds are known to be very toxic, so there is an urgent need for innovative analytical tools or devices to facilitate the detection of these compounds. A *fabA* promoter fused to a reporter geneen coded β-galactosidase in an *E.coli* strain has been demonstrated to detect phenol between 1.6 and 16 ppm in 20 minutes (Neufeld et al., 2006). Other toxic chemicals such as pesticides can also potentially be found in wastewaters, and so hence the use of biosensors is increasing compared to chemical analysis in wasterwater testing. Evans et al. (1998) described the incorporation of activated sludge on the electrode for an amperometric biosensor to determine rapidly the toxicity of wastewaters.

Amperometric microbial biosensors have also found uses in the food industry. Milk stored for prolonged periods becomes rancid. Rancidity in milk products is caused by the liberation of short-chain fatty acids (C_4-C_{12}) from milk lipids by endogenous or microbial enzymatic activity.

Arthrobacter nicotianae has been shown to possess enzymes of the β-oxidation of fatty acid pathway with a high specificity towards short-chain fatty acids. These cells have been immobilized onto an oxygen electrode with calcium-alginate gel and can be used to analyse the concentrations of short-chain free fatty acids in milk samples. This sensor does not require any form of pre-treatment and its response time is only 3 minutes (Schmidt et al., 1996).

In addition, thiamine and tyrosine play important roles in maintaining a healthy nervous, cardiovascular functioning of the body and so are necessary supplements in food products. Despite their necessity for good health, it can also be a risk-factor of hypertension when consumed in large doses. Hence, levels of thiamine and tyrosine are monitored and determined in clinical analysis, food processing, pharmaceutical and biotechnological processes. Consequently, genetically modified *Saccharomyces cerevisiae* have been developed and coupled with an amperometric oxygen electrode for the detection of thiamine (Vitamin B1) and tyrosine (Akyilmaz et al., 2006; Di Paolantonio & Rechnitz, 1983) and have been shown to be more sensitive than classical analytical methods such as chromatography and spectrometry.

1.2.3.2 : Potentiometric Electrochemical Whole Cell Microbial Biosensors

Potentiometric microbial whole-cell biosensors have made use of a variety of microbial species to detect specific targets. Potentiometric microbial biosensors are dependent upon a change in electrical potential resulting from ion accumulation or depletion. The conventional potentiometric microbial whole-cell biosensors consist of an ion-selective electrode (*e.g.* pH, ammonium, chloride) coated with an immobilized microbe layer. A microbe consuming an analyte generates a change in potential resulting from ion accumulation or depletion. Potentiometric transducers then measure the difference between a working electrode and a reference electrode, with the difference being correlated to the

concentration of an analyte (Mulchandani & Rogers, 1998; Simonian et al., 1998). However, this method requires a very stable reference electrode, which can be a challenge.

Organophosphorous compounds widely used as pesticides, insecticides and chemical warfare agents have created public concern because of their widespread use and toxicity. Potentiometric microbial whole-cell biosensors based on modification of a glass pH electrode with genetically modified *E. coli* that expresses intracellular organophosphorus hydrolase were able to detect up to 3 µM organophosphate (Brim et al., 2000; Gaberlein et al., 2000; Mulchandani et al., 1999; Simonian et al., 1998). The principle of detection is based upon the release of protons during hydrolysis of organophosphorus, and the concentration of protons released is directly correlated with the concentration of organophosphorus present in the sample. Similar potentiometric whole-cell microbial biosensors have been developed to monitor penicillin concentrations of up to 30 mM. To produce these biosensors the recombinant *E.coli* transformed with a plasmid encoding β-lactamase and penicillinase synthesis were incorporated into membranes comprising a mixture of gluten and acetylcellulose overlaid onto pH electrodes (Galindo et al., 1990 & Chan et al., 1999). Other target substrates that have been successfully analysed by biosensors include tryptophan, urea and trichloroethylene (Lei et al., 2006).

While pH electrodes are the most widely used ion selective electrodes for microbial biosensors, other ion selective electrodes have also been utilized. For example, an ammonium ion selective electrode was coupled with urease-yielding *Bacillus* sp. isolated from soil to develop a disposable microbial biosensor for monitoring the presence of urea in milk (Verma & Singh, 2003). Similarly, a chloride (Cl^-) ion selective electrode was modified with the trichloroethylene (TCE) degrading bacterium *Pseudomonas aeruginosa* JI104 for TCE monitoring in batch and continuous modes in wastewaters (Han et al., 2002).

1.2.3.3 : Microbial Fuel-Cell Electrochemical Whole- cell Microbial Biosensors

Microbial fuel cell (MFC) technology harnesses energy stored in waste streams and organic-rich environments, providing electrons to an electrode by microorganisms (Bullen et al., 2006; Logan et al., 2006; Loveley, 2006). These microorganisms have the ability to donate electrons to an electrode under anoxic conditions to support oxidation of electron donors such as lactate, glucose, acetate and a number of mixed wastes (Rabaey & Verstraete, 2005). Currently, microbial fuel cells (MFC) have been used as biosensors for *in situ* analysis and monitoring of microbial respiration, as changes in the rate of respiration relate to the reduction in the concentration of a contaminant (Du et al., 2007; Li et al., 2011).

Microbial fuel-cell electrochemical whole-cell biosensors can be applied to the monitoring of groundwater contaminant plumes undergoing natural attenuation or bioremediation, since this requires routine monitoring of various contaminant, biogeochemical, and water quality analyte. Furthermore, this provides information regarding contaminant flux and the processes that are central to contaminant remediation (Ling et al., 2003). Characterization of microbial respiration rates and delivery of reducing equivalents to affected areas would also improve the understanding of natural attenuation and bioremediation efforts. Therefore, the development of innovative sensor technologies could potentially reduce the costs of site monitoring, whilst meeting the informational needs of regulators and site managers.

This is a platform in development of MFC-systems into a sensing technology for *in situ* water quality monitoring applications. MFC biosensor system for the detection of acetate has been developed (Tront et al., 2008). *Geobacter sulfurreducens* was used as an external electron acceptor for a system operated with an influent solution containing acetate at various concentrations and monitored for the

change of current generated indicated the change in acetate concentration. An excellent positive correlation between the acetate concentration and the electrical current (R^2= 0.92) was reported (Tront et al., 2008).

1.2.3.4 : Optical Whole-cell Microbial Biosensors

Optical detection is a technique commonly used in microbial biosensors. Optical detection is usually based on the measurement of luminescent, fluorescent, colorimetric, or other optical signals produced by the interaction of microorganisms with the analytes, correlating the observed optical signal with the concentration of target compounds. Genetically modified microorganisms are widely used as optical whole-cell microbial biosensors.

Bio-reporter genes are the primary driver for optical microbial whole-cell biosensors. The signals produced may be detected by luminescent, fluorescent or colorimetric. Reporter gene technology involves controlling the expression of genes by defined *cis*-regulatory sequences (response elements), which correlates with changes in gene regulation and thus expression in host microbial cells (Figure 1.2).The principle behind this technology is relatively straightforward since changes in the abundance of the corresponding reporter protein(s) are indicative of the transcriptional activity of the promoter. The bioreporter genes discussed within these sections are: green fluorescence protein (*gfp*), firefly luciferase (*luc*), beta-galactosidase (*β-gal*), and bacterial luminescence (*lux*).

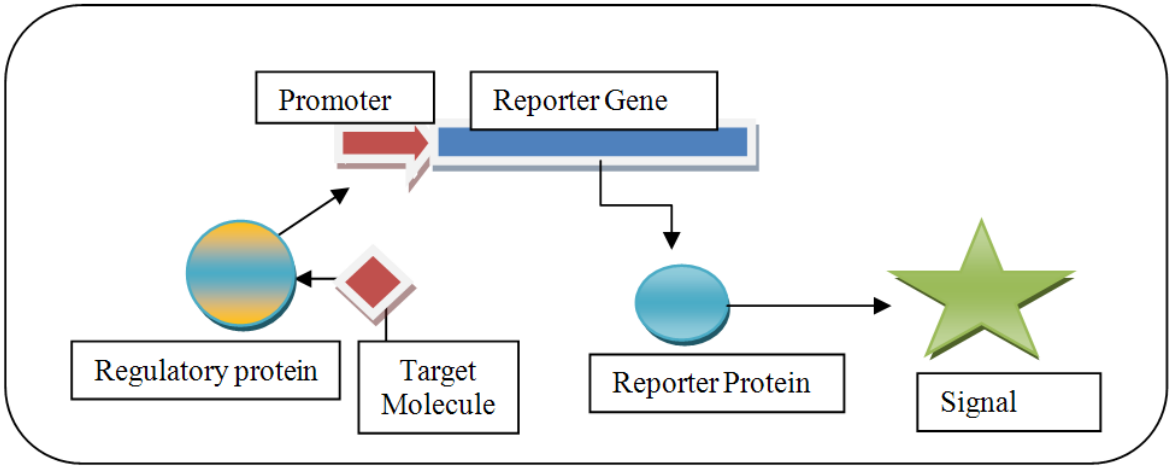


Figure 1.2: Schematic diagram of how a target molecule interacts with a reporter and generates a quantifiable signal.

1.2.3.4.1 : Fluorescent Whole-cell Microbial Biosensors

Green fluorescent protein (GFP) is one of the many bio-reporter genes commonly used in microbial whole-cell biosensors. GFP is a 27-kD protein from the jellyfish, *Aequorea Victoria,* which exhibits bright green fluorescence when folded correctly as polypeptide chain. Residues; Ser65, Tyr66, and Gly67 form an extended and rigidly encapsulated conjugated π system that results in bright green fluorescence (Jackson et al., 2006). The emission of fluorescence requires no addition of any substrate or cofactor, hence it can be used in many species for live cell detection (Jackson et al., 2006). GFP is very stable under a variety of conditions; however this stability limits its application in transcriptional induction studies since the fluorescence may decline at a much slower rate than transcription (Li et al., 1998). Recent efforts have sought to decrease the half-life of GFP, so as to increase its suitability for real time analysis (Matthysse et al., 2006). In biosensor systems, expression of *gfp* is influenced by changes in the transcriptional activity of the promoter which correlated with the concentration of the target analyte. For example, detection of zinc has been achieved developed by the transformation of *Bacillus megaterium* with enhanced green fluorescent protein (*egfp*) under the transcriptional control of the promoter for the *smt* operon (Date et al., 2010). The detection range for this biosensor was from 10^{-6} to 10^{-4}M of the analyte. Monitoring the levels of

uranium in water is vital due to the toxicity, especially in the marine environments. This was undertaken by using a GFP reporter attached to an inducible *urcA* promoter in a *Caulobacter crescentus* biosensor strain which would then exhibit fluorescence in the presence of uranium (Hillson et al., 2007).

1.2.3.4.2 : Colourimetric Whole-cell Microbial Biosensor

Colourimetric whole-cell microbial biosensors utilise the generation of a coloured compound as a signal which can be measured and correlated with the concentration of analyte present in a sample. The coloured product can be distinguished by the naked eye or by spectrophotometry readings. This method gives an easy-to-interpret response, a high turnover rate, is stable, and is easy to quantify; however it may require the addition of a chromogenic substrate.

One example of a colourmetric system is based upon the 464 kDa β-galactosidase enzyme extracted from *E.coli* (Naylor, 1999). The activity of the enzyme can be demonstrated by using the chromogen ortho-nitrophenyl-β-D-galactopyranoside (ONPG) which is an artificial substrate for the enzyme. ONPG is colourless, while its product, orthonitrophenol (ONP) is yellow (λ_{max}= 420 nm). Therefore, enzyme activity can be measured by the abundance of yellow colour using a spectrophotometer. In addition, the artificial substrate X-gal can be used to β-galactosidase activity.The substrate X- gal also forms an intense blue when it is cleaved by β-galactosidase (Naylor, 1999). X-gal is commonly used to monitor β- galactosidase expression in bacterial colonies on agar whilst ONPG is employed for quantifications in solution. Ramanathan et al. (1997) successfully demonstrated the detection of arsenite by monitoring the activity of β- galactosidase, whilst Shin (2012) showed the expression of β- galactosidase in the presence of phenolic compounds.

The discovery of novel colorimetric compounds broadens the scope for colorimetric applications. The detection and quantification of arsenite is performed by the construction of the promoter region of the *ars* operon and the arsinite resistance gene (*ArsR*) cloned from *E.coli*, were conjugated to the *crtA* gene from photosynthetic bacterium, *Rhodovulum sulfidophilum* (Fujimoto et al., 2006). The *crtA* gene is responsible for carotenoid synthesis, and so the biosensor strains will change colour from yellow to red in the presence of arsinite (Fujimoto et al., 2006). The limit of detection for this biosensor was reported to be 5 µg/L arsenite (Fujimoto et al., 2006).

1.2.3.4.3 : Bioluminescent Whole- cell Microbial Biosensor

A bioluminescent microbial whole-cell biosensor is associated with the emission of light by living microorganisms. The bioluminescence expression acts as a reporter for gene expression, a feature which marks the widest application in molecular genetics (Nordeen, 1988; Schauer, 1988). The *lux* genes that control luminescence are arranged in a single polycistronic operon, *lux CDABE* (Meighen, 1991).

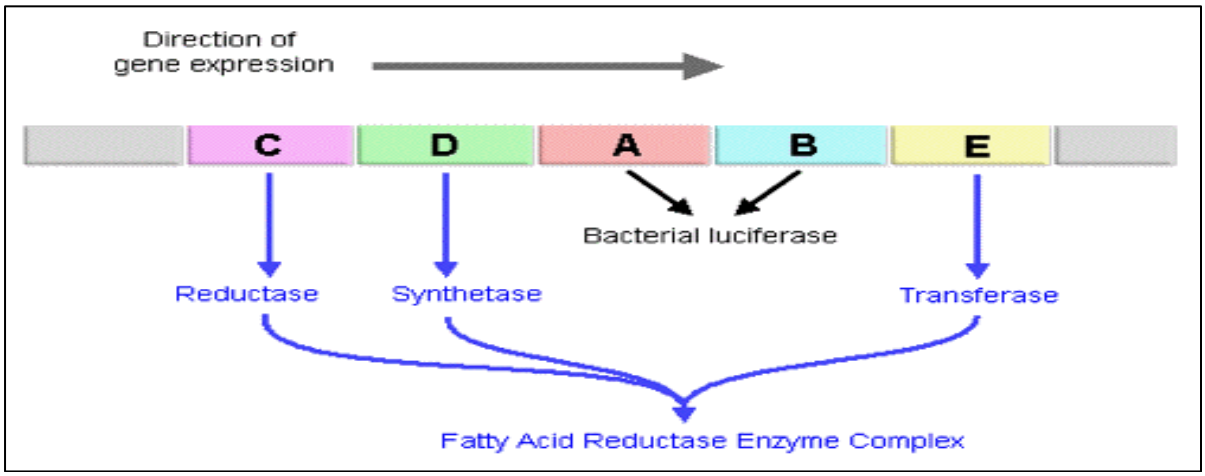


Figure 1.3: Arrangement of *lux CDABE* open reading frames and a single polycistronic operon (Adapted from Lin and Meighen, 2009).

Bacterial luciferase is a heterodimeric enzyme (αβ) of 78k-Da containing two non-identical subunits, α and β, located in a polycistronic operon as shown in Figure 1.3. *luxA* and *luxB*, the genes encoding

α and β subunits, possess 30% sequence identity (Meighen, 1994). Bacterial luciferase oxidizes a long chain aldehyde (RCHO) in the presence of reduced flavin mononucleotide ($FMNH_2$) to produce a long chain fatty acid and blue-green light at 490nm (Meighen, 1991). The $FMNH_2$ is readily provided from the electron transport chain in all bacteria. Although the biosynthesis of riboflavin and FMN is carried out in multiple steps by enzymes that are not encoded by the *lux* gene system, these enzymes are generally present in bacteria as riboflavin and FMN syntheses are required for bacterial growth.

This reaction is highly specific for $FMNH_2$ and any modifications to the flavin ring or removal of the phosphate group decreases its activity significantly (Meighen, 1991). The reduced flavin, $FMNH_2$, binds to the enzyme, and reacts with oxygen (O_2) to form a peroxyflavin. This complex interacts with aldehyde to form a highly stable intermediate which decays slowly. This results in the emission of light along with the oxidation of the substrates. The luciferase enzymes undergoes a single catalytic cycle as the rate of chemical oxidation of $FMNH_2$ is higher than the turnover rate of luciferase in the bioluminescence reaction, resulting in a decay of luminescence with time in a first-order process that reflects the turnover number of the enzyme under the assay conditions (Meighen, 1991). The production of the reduced substrates $FMNH_2$ requires an electron transport system which is driven by the central reactions of carbon and energy metabolism. Any perturbation of these processes or of electron transport would have an effect on bioluminescence intensity. Consequently, monitoring of changes in bioluminescence intensity provides a direct assessment of the perturbant effect on microbial metabolism (Scheerer et al., 2006). Furthermore recent studies have been demonstrated that the bioluminescence emission of a reporter gene under stress conditions correlated to gene expression in host strain using real-time PCR (Burton et al., 2010; Kim & Gu, 2006) and cDNA microarray techniques (Kim & Gu, 2006). Bacterial bioluminescence expression is real-time where monitoring of bioluminescence occurs with time frame of measurements of 3 to 5 minutes.

Additional advantages include very low background noise, high sensitivity and a wide dynamic range which enables very weak to very strong rranscriptional activity to be measured (Sunya et al., 2012).

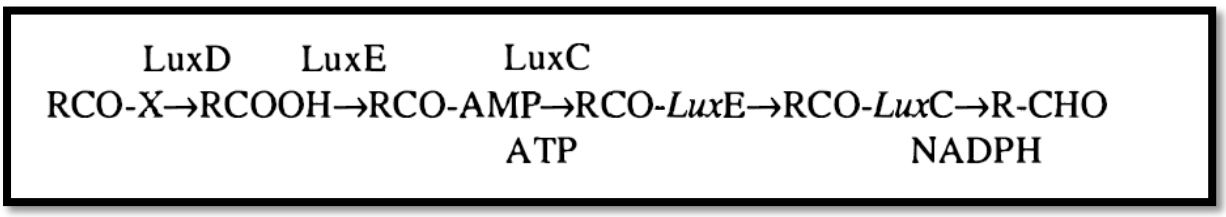


Figure 1.4: The fatty acid reduction pathway catalysed by luxD, luxE, luxC gene products (Meighen, 1994).

The synthesis of aldehydes for the bioluminescence reaction is catalyzed by a multienzyme fatty acid reductase complex containing three proteins; a reductase (lux C), a transferase (lux D*)* and a synthetase (lux E) (Figure 1.5). *lux CDE* encodes the fatty acid reductase complex involved in synthesis of the aldehyde substrate (Figure 1.4). Lux D, the first thioesterase, cleaves tetradecanoyl-Acyl-carrier protein (ACP). The fatty acid product is activated by luxE with ATP to form fatty acyl-AMP. In the presence of Lux C, the acyl group is transferred from acyl-AMP to LuxE, consequently LuxC finally reduces the acyl-AMP to fatty aldehyde (Meighen, 1991). These fatty acid reductase genes make the *lux*-operon 'self-sufficient' with no additional reagents required.

On the contrary, a recent study by Yagur-Kroll and Belkin (2011) demonstrated an improved version of bioluminescent bioreporter is done by splitting *luxCDABE* genes of *Photorhabdus luminescens*, into two smaller functional units: *luxAB* encodes luciferase enzyme and *luxCDE* encodes enzymatic complex responsible for synthesis of aldehyde proved to be superior to the native *luxCDABE* configuration. This suggests that there is an improved efficiency in the transcription and translation of two subunits rather than a large gene. The best combination was of an inducible *luxAB* and a constitutive *luxCDE* due to aldehybe being a limiting factor in the bioluminescence system (Yagur-Kroll &Belkin, 2011). However, mathematical modelling studies byWelham and Stekel (2009)

contradict the findings of Yagur-Kroll and Belkin (2011) indicating optimal performances by constitutive *luxAB* and inducible *luxCDE*. The studies of upgrading bioluminescence expressions by splitting lux cassette is novel in contrast to traditional attempts to improve whole-cell sensors' output by reducing background emission level, promoter strength and manipulation of host cells (membrane permeability) (Yagur-Kroll & Belkin, 2011). However, until recently, only a few research dealing with the splitting of *lux* genes have been studied (Welham & Stekel, 2009; Yagur-Kroll & Belkin, 2011).

Today there are eleven bioluminescent bacterial species classified into four genera. Three genus are of marine origin: *Vibrio, Photobacterium* and *Alteromonas.* The fourth, more recently discovered one is of terrestrial origin: *Photorhabdus* (ex *Xenorhabdus*) (Meighen & Szittner, 1992). Amongst the species known *V. fischeri*, *V. harveyi*, and *Photorhabdus luminescens* appear to have usable thermal stability to temperatures of 30, 37 and 42°C, respectively, with *P. luminescens* having a half-life of 3 h at 45°C (Meighen, 1991). Furthermore, the entire *luxCDABE* operon for *V. harveyi* and *P. luminescens* emits strong bioluminescence signals expressed in *E. coli* at 37°C (Meighen & Szittner, 1990). A comparison between *V.harveyi* and *P.luminscence* indicates a total loss of luciferase activity in *V.harveyi*, whilst *P. luminescens* retained 100% activity at 37°C (Meighen & Szittner, 1990). This indicates that the *P. luminescenes lux* operon is the most suitable from the three genera for further study and incorporation into whole-cell biosensor constructs.

Very effective bioluminescent microbial biosensors have been developed for environmental toxicity monitoring (Liu et al., 2011). Of the several possible bio-reporter systems (Kohler et al., 2000; Magrisso et al., 2008), the use of bacterial bioluminescence genes (*luxCDABE*) is most favoured due to the high sensitivity conferred by enzymatic photon generation, the capacity for continuous online monitoring (Marincs, 2000), and independence from an exogeneous substrate supply. There is no prerequisite for any special genotypes in the recipient strain in order to obtain expression, while the

response of bioluminescence is obtained in real time without cell disruption (Steward, 1990). More importantly, the emission of bioluminescence is proportional to the rate of transcription of *lux*, hence this meets the requirements for a whole-cell biosensors and also gene expression studies. Furthermore, whole-cell bioluminescence not only correlates to growth but also reflects on the metabolic status of the cell (Ellison et al., 1994b; Hill., 1993; Marincs, 2000; Stewart, 1990, 1993 1997; Stewart & Williams, 1992, 1993; Stewart et al., 1991, 1993, 1996, 1997). This implies that any stress responses that directly or indirectly affect the production of intracellular $FMNH_2$ can be monitored in *lux*-recombinant bacteria in the form of light output per cell (Stewart, 1990). This attribute acts as an indication of global changes in intracellular biochemistry reflecting detrimental changes in bioluminescence (Stewart, 1990). This feature is regarded as important in monitoring the real-time recovery of microbial cells from sub-lethal injury, which is of immense importance to the enumeration of microorganisms in food and environment (Stewart, 1990). Previous studies also demonstrated that the recovery of *S. typhimurium* cells from freeze injury can be observed in real-time using bioluminescence (Ellison et al., 1991a). However, gene expression using *lux* gene fusions under anaerobic conditions are limited by the need of oxygen and flavin mononucleotide (Meighen, 1991).Contrary to this, Phillip-Jones (2000) demonstrated successful construction of *lux AB* to aerotolerant anaerobe *Clostridium perfringens* in monitoring expression of *cpa* (virulence gene). Today, the application of bioluminescence genes has extended to the medical field. Recent studies on the application of bioluminescence as a rapid screening method for acute myeloid leukaemia (AML) patients before undergoing chemotherapy presented in the study by Alloush et al., 2010.

The current most widely used bioluminescent microbial toxicity testing system is marketed as Microtox™ and is based upon the wild-type bioluminescent bacteria, *Vibrio fischeri*. In the last 18 years, the Microtox® system has been used by several agencies to assess the impact of chemicals in the environment. Toxic agents that affect metabolism or compromise bacterial viability cause a

reduction in light output. The results are expressed as effective concentration values at which there is a 50% decrease in light emission (EC_{50}) (Sinclair et al., 1999). However, the use of *V.fisheri* in toxicity testing has also some limitations: due to the marine origin of bacteria, which requires high salinity (2% NaCl) and bioluminescence expression is unstable above 30^{o} degrees (Close et al., 2010). In addition, Gellert et al. (2000) demonstrated that *V.fisheri* growth was weakened in the presence of nutrient broth. Due to the disadvantages, luciferase genes, *lux*CDABE genes from *Photorhabdus luminescens* has been often a better alternative used for recombinant luminescent bioreporters and bioluminescence expression is stable up to 40^{o} degrees (Meighen, 1991).

Over the past decade, a new contaminant has found its way into water supplies around the world. Oestrogen comes from multiple sources, both natural and synthetic. This has raised concerns about possible health effects when consuming drinking water from contaminated sources. Most water treatment plants have not implemented any processes to remove oestrogen (Dery, 2009). Therefore it is likely that routine measurement of oestrogen in water supplies is likely to be implemented in the relatively near future. A bioluminescent whole-cell biosensor that is able to detect oestrogen has been constructed in *S. cerevisiae* (Hollis et al., 2000). Both reporter genes *lux* and *lacZ* have been successfully constructed into biosensors for screening, chemical sensing, and real time monitoring of oestrogen compounds and endocrine disrupting chemicals in the environment (Sanseverino et al., 2005).

A wide range of bacteria has been transformed with bioluminescent reporter constructs in which the *lux* operon has been placed under the control of an inducible promoter to create analyte-specific bioluminescent biosensors. These biosensors have demonstrated great value in determining the presence and concentration of specific pollutants. For example, *Pseudomonas fluorescence* HK44 carrying the *nah-lux* reporter plasmid, which is capable of degrading both salicylate and naphthalene,

has been developed as a bioassay to assess the bioavailability of naphthalene and salicylate in contaminated soils (Burlage et al., 1990; King et al., 1990). Similarly, a biosensor in which a fusion of the Tn21 mercury resistance promoter (*mer*) with *lux* operon was constructed to detect inorganic Hg (II) in natural water in the 0.1 to 200 ppb range (Selifonova et al., 1993). Moreover, bioluminescent microbial biosensors to detect chlorophenols (Sinclair et al., 1999), nitrate (Preston et al., 2000), cadmium and lead (Tauriainen et al., 1998) have been developed and tested. A new generation of bioluminescent microbial biosensor of genetically modified spores of *Bacillus subtilis* is able to induce bioluminescence upon germination. This enables screening of many preservatives to be carried out rapidly and in real-time mode and also further evaluate inhibitory actions of the preservatives (Ciarciagline et al., 2000). Bioluminescence offers benefits over other reporter systems, where it has been shown to yield a faster and more sensitive measure and related compounds in samples when compared to fluorescence (Liu et al., 2011).

In addition, firefly (*luc*) genes can also be utilized in luminescent reporters for measures of chemibioluminescence. The Firefly luciferase (LUC) is a 62 kDa protein from the firefly *Photinus pyralis* (DeWet et al., 1985). Firefly luciferase catalyses the oxidative decarboxylation of luciferin to oxyluciferin in the presence of ATP, magnesium ions, and oxygen producing an emission of photons at 562nm wavelength (Lippincott-Schwartz & Patterson, 2003). The advantage of this reaction is applicable to broad range of species (including mammalian cells). However, this reaction involves the addition of reagents, and oxygen and magnesium ions are also required (Naylor, 1999).

One of the greatest limitations of whole-cell biosensors is the availability of strong constitutive promoters or ones that respond only to the relevant stimuli (induced promoters) (Sorensen et al., 2006). To circumvent this obstacle, more knowledge on gene regulatory networks in bacteria is needed. Linking transcriptomic studies and metatranscriptome analysis in microbial cells could

provide an immense source of new regulatory elements in the future (Cases & De Lorenzo, 2005). An alternative approach is to synthesize ‘super promoters’ based on consensus sequences obtained from comparative genomic studies of different promoters in known regulatory networks (Dreier et al., 2002).

Therefore, the aim of the study described in this thesis is to investigate the application, sensitivity, stability of *lux* as a reporter system in *Escherichia coli* ATCC 8739 when controlled by 5 different constitutive promoters.

1.3 : Gene Expression and Promoter Functions

Gene expression is a process by which information from a gene is used in the synthesis of a functional gene product. Regulation of gene expression gives the cell control over both its structure and its functions. Gene expression in bacteria is controlled by the action of the multi subunit RNA polymerase, which catalyses DNA template-dependent RNA synthesis (Lee et al., 2012) illustrated in Figure 1.5.

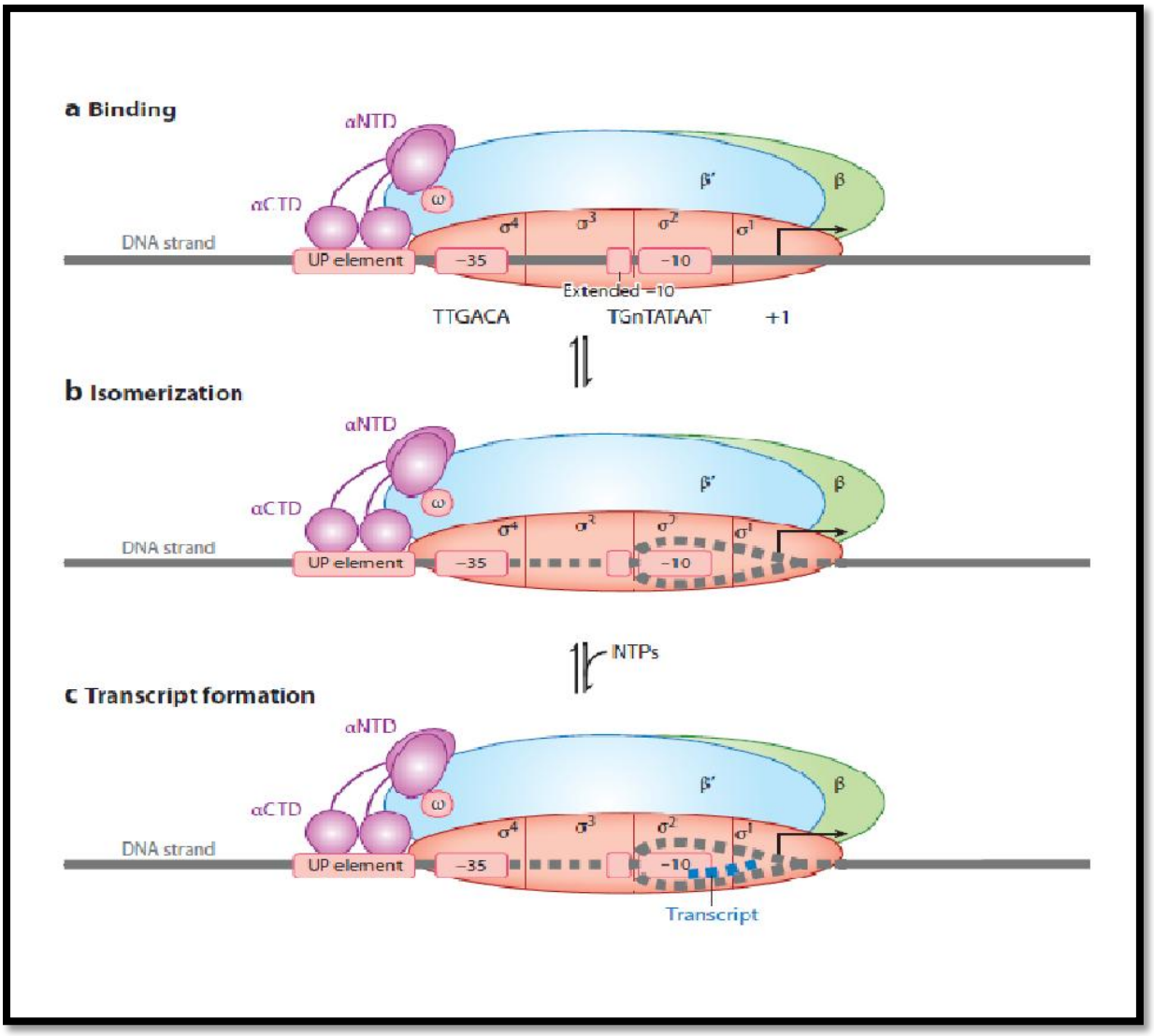


Figure 1.5: Schematic representation of RNA polymerase interactions at the promoter and the initial steps to transcription initiation.
(a): The process of transcription begins when RNA polymerase recognizes and binds to DNA elements within a promoter sequence.
(b): Isomeriation to the open complex is signified by unwinding of the duplex DNA around the transcription start site.
(c): Formation of transcript, with addition of nucleoside triphosphate (NTP) Adapted from Browning & Busby, 2004.

In bacteria, RNA polymerase is composed of a core of multiple subunits ($\beta\beta'\alpha_1\alpha_2\omega$) that is tightly associated with a sigma (σ) factor (Browning & Busby, 2004) (Figure 1.5). The core contains the active site for polymerase activity, and is thus capable of synthesizing mRNA whilst the associated sigma factor controls when and where transcription is initiated. Transcription initiation requires the

interaction of RNA polymerase with promoter DNA and the formation of an open complex, in which the duplex DNA a round the transcript start-point is unwound (De Haseth et al., 1998). Synthesis of the DNA template-directed RNA chain then begins with the formation of the first phosphodiester bond between the initiating and adjacent nucleoside triphosphates (Figure 1.5 c). After this initiation phase, RNA polymerase is moved into the elongation complex, which is responsible for RNA-chain extension (Figure 1.5). The main step in initiation is promoter recognition by RNA polymerase and the different DNA sequence elements that are responsible for this have been studied intensively (Gross et al., 1998; Busby & Ebright, 1994). Four different sequence elements have been identified. The two principal elements are the -10 hexamer and the -35 hexamer, which are located 10 and 35 base pairs (bp) upstream from the transcript start site respectively.

Table 1.2: Functions of *E.coli* σ factors

Organism	σ factor	Gene	Function
Escherichia coli	$\sigma^{70}(\sigma^{D})$	rpoD	Housekeeping genes
	$\sigma^{32}(\sigma^{H})$	rpoH	Heat shock
	$\sigma^{24}(\sigma^{E})$	rpoE	Periplasmic Stress
	$\sigma^{28}(\sigma^{H})$	fliA	Flagellar-based motility
	$\sigma^{38}(\sigma^{S})$	rpoS	Stationary/starvation phase
	$\sigma^{54}(\sigma^{N})$	rpoN	Nitrogen-regulated gene
	σ^{fecI}	fecI	Ferric citrate uptake (ECF)

The text in blue indicates the second family of sigma factors

The highest intracellular concentration of the σ factors belongs to the σ^{70} family, reflecting their relationship to the principal σ factor of Escherichia coli, σ^{70} regulating housekeeping genes (Table 1.2 in black). A second family of σ factors, the σ^{54} family, comprises proteins that are functionally similar to, but structurally distinct from, σ^{70} of *E. coli*. For the purposes of this thesis, we will limit

our discussion to the σ^{70} family.σ^{70} can be divided into four major regions (regions 1, 2, 3, and 4) (Browning & Busby, 2004). Prokaryotes have multiple sigma factors; a primary σ factor that is needed for the expression of housekeeping genes during exponential growth and alternate σ factors that are used under certain conditions of growth or stress (Gruber & Gross, 2003; Paget & Helmann, 2003). As the metabolic state of the cell changes, other sigma factors can dominate and control transcription (Table 1.2).

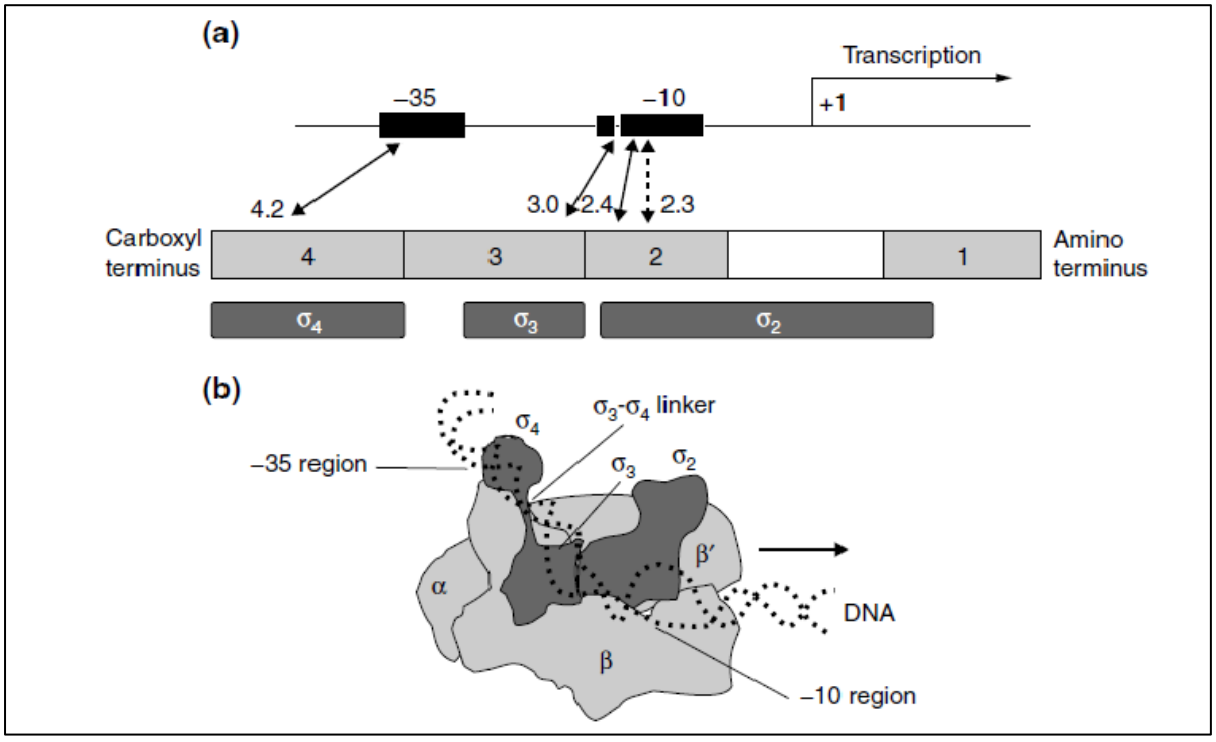


Figure 1.6: Schematic diagram of the structural Characteristic of the *E.coli* σ^{70}.
(a) The promoter DNA sequence has been divided into four distinct regions that are recognized by σ factors.
(b) A model based on crystallographic analysis for the interaction of RNA polymerase holoenzyme (containing β, β', 2α, ώ subunit in addition to the σ factor) with promoter DNA and holoenzyme-model DNA complexes. Adapted from Young et al. 2002

Promoter region -10 elements are recognized by region 2 of the RNA polymerase σ subunit (region2.3 and 2.4) (Figure 1.6). The subregion 2.3 is thought to interact primarily with single stranded DNA in the open complex (dashed arrow in Figure 1.6). The promoter region -35 element is recognized by region 4 (subregion 4.2) of the RNA polymerase σ subunit. The two other important

promoter elements are the extended –10 element and the UP element (-40 to -60). The extended –10 element is a 3 to 4 bp motif located immediately upstream of the –10 hexamer, which is recognized by region 3 of the RNA polymerase σ subunit (Murakami et al., 2002a; Bown et al., 1994; Sanderson et al., 2003). Whilst, UP element is a ~20 bp sequence located upstream of the promoter region –35 hexamer, which is recognized by the C-terminal domains of the RNA polymerase α subunits (Ross et al., 2001).The discovery that the σ3-σ4 linker is located in the RNA exit channel of the RNA polymerase suggests a mechanism of promoter clearance that involves the nascent RNA displacing the linker, in turn weakening the interaction between the core RNA polymerase and the σ4 domain and ultimately the rest of the σ factor (Murakami et al., 2002a; Vassylyev et al., 2002).

Promoters can also be categorized by their strength; with strong promoters generating high levels of transcription whilst weak promoters generate low levels of transcription. In general, strong promoters have sequences that are similar to the consensus sequence (Pribnow, 1975). However, studies conducted by Hook-Barnard & Hinton (2007) indicate that the direct and indirect interactions of σ factor (s) with bases within the consensus elements (i.e. UP elements, -35 region, and -10 region) contribute to the strength of promoters.

There are two groups of promoters commonly employed in whole-cells bio-sensing; these are constitutive and inducible promoters. Biosensor systems based upon these two types of promoters which have different benefits and limitations in bio-sensing (Gu et al., 2004). The use of constitutive expression relies upon a promoter that is highly expressed under normal conditions, where the expression levels of the bio-reporter signal will change directly with the addition of chemicals of interest (Figure 1.7a). Constitutive systems provide an overall picture of the metabolic state of individual cells. On the other hand, inducible promoters respond to specific analytes of interest and the bio-reporter signals increase when the target analyte is detected (Gu et al., 2004) (Figure 1.7b).

Compared to the constitutive systems, inducible systems provides a more sophisticated and specific detection of a certain compound. However, the target analyte is restricted to the specific promoter gene.

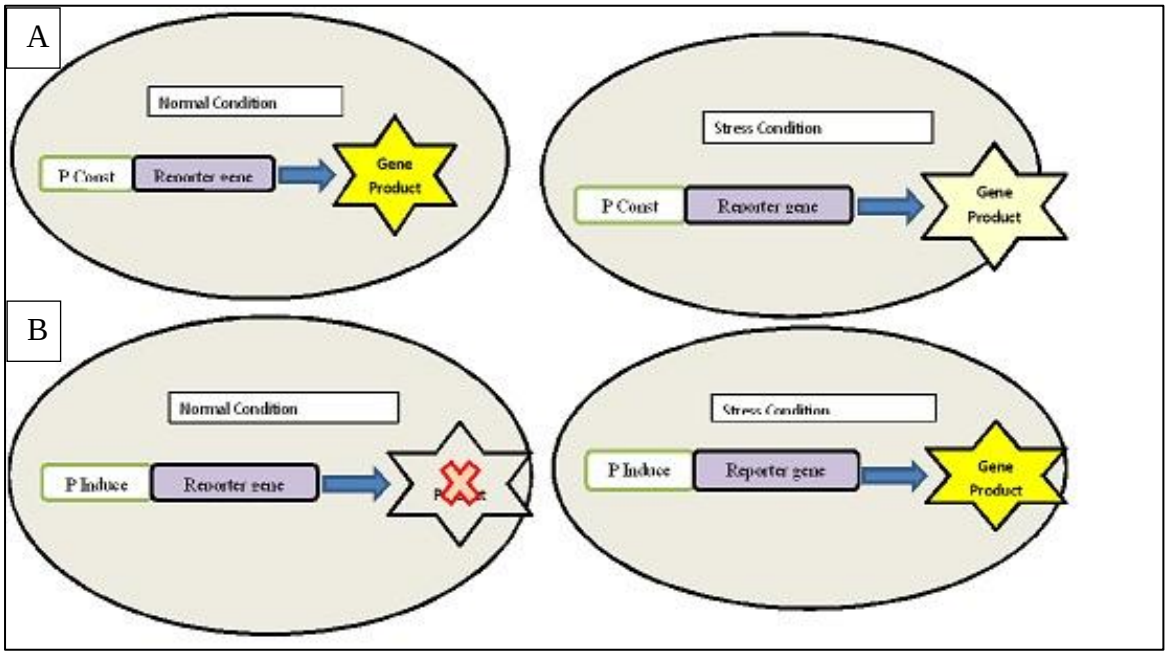


Figure 1.7: The functional behaviour of inducible and constitutive promoters.
A: The reporter gene fused to a constitutive promoter (P_{const}) has high expression levels under normal conditions, whilst reporter protein activity decreases when exposed to stress.
B: is an inducible biosensor fused to an inducible promoter (P_{Induce}) responding to a stress agent inducing a stress response. The reporter protein is expressed at high levels under stress conditions, but is low or nil under normal conditions.

1.4 : Biological Functions of Promoters

There are many constitutive promoters that are used for gene expression studies and in biosensors. Amongst the many constitutive promoters, five constitutive promoters were selected for the study described in this thesis. These promoters were lysyl tRNA synthetase (*lysS)*, ribosomal protein (s*pc)*, outer membrane lipoprotein (*lpp*), twin arginine translocase (*tatA*), and lysine decarboxylase (l*dc*). The biological functions are discussed below.

1.4.1 : Lysyl-tRNA Synthetase (lysS)

The role of aminoacyl-tRNA synthetases plays a key role in protein synthesis by catalysing the covalent attachment of amino acids to the 3' end of tRNAs (Chan & Bingham, 1991). Lysyl-tRNAsynthetase catalyses the formation of lysyl-transfer RNA, and transfers lysine in to polypeptides (Freist & Gauss, 1995). Lysine is important for proteins since it is one of only two proteinogenic amino acids carrying an alkaline functional group (Freist & Gauss, 1995). In *E.coli* there are two lysyl-tRNAsynthetases encoded by *lysS* (VanBogelen et al., 1983) and *lysU* (Emmerich et al., 1987). The *lysS* gene is expressed constitutively (Hirshfield et al., 1984) whilst lysU can be induced under selective conditions (*i.e.* in the presence of L-leucine, L-alanine, or glycyl-L-leucine, D-fructose and also at elevated temperature (Hirshfield et al., 1984). The *lysS* promoter was chosen due to its constitutive expression under all growth conditions because of its essential role in protein synthesis.

1.4.2 : Ribosomal Protein (spc)

The genes encoding the 52 ribosomal proteins (r-proteins) of *Escherichia coli* are organized into approximately 19 operons scattered throughout the chromosome (Cerreti et al., 1983). One of these, the *spc* operon, contains the genes for eleven ribosomal proteins including *secY* (Zengel & Lindahl, 1994); L14; L24; L5; S14; S8; L6; L18; S5; L30 and L15 (*rp1N, rp1X, rp1E, rpsN, rpsH, rp1F, rp1R, rpsE, rpmD,* and *rp1O*) (Cerreti et al., 1983).The *spc* promoter has a long sequence of 2 fold symmetry centred within the Pribnow box (Leonard et al.,1978). The *spc* promoter was chosen due to its important role in *E.coli,* where the synthesis of ribosomal proteins is subjected to tight control and regulates ribosome biosynthesis (Leonard et al., 1978). The crucial role of *spc* in metabolism, and its high levels of expression, indicates that it would make an effective constitutive promoter for a bioluminescent reporter strain that could be used to monitor toxicity in samples.

1.4.3 : Outer membrane lipoprotein (lpp)

The *E.coli* outer membrane contains approximately 7.5 x 10^5 molecules of lipoprotein, which is the most abundant protein found in *E.coli* (Inouye & Inouye, 1985). The strong expression of the gene *lpp* from its promoter contributes to the abundance of the lipoprotein in *E. coli* (Nakamura & Inouye, 1982), as does the stability of the lpp mRNA (Hirashima & Inouye, 1972) and the efficient translation of the *lpp* mRNA (Nakamura et al., 1980). The *lpp* gene is expressed constitutively and has an A-T rich promoter region (-45 bp), which suggests it would be an excellent candidate for a biosensor (Nakamura & Inouye, 1982).

1.4.4 : Twin-Arginine Translocase (tatA)

The twin-arginine translocation (t*atABCE*) operon encodes an export pathway which is found in the cytoplasmic membranes of most bacteria (Berks et al., 2003). This functions as an export pathway of pre-folded proteins and translocates redox enzymes into the *E.coli* periplasm (Santini et et al., 1998). The *tatA* gene are expressed constitutively, indicating a requirement for the Tat export machinery under all growth conditions. Hence, this suggests it could make a suitable promoter for a whole-cell biosensor for monitoring viability.

1.4.5 : Lysine Decarboxylase (ldcc)

There are two lysine decarboxylases, one of which is an inducible decarboxylase (CadA) and the other is a constitutive decarboxylase (LdcC) (Lemonnier & Lana, 1998). The function of constitutive-expressed ldcc is to catalyse the synthesis of putrescine and spermidine, which are polyamines that are needed for ribosomal functions and growth (Tabor & Tabor, 1985).

Stewart (1990) noted that the speedy response of bioluminescent bacteria to the action of antimicrobial substances brings close to reality the prospect of on-line microbial assays for those agents. Thus the purpose of the work described in this thesis was to examine the effectiveness of the five bioluminescent biosensor constructs towards monitoring the levels of preservatives

1.5 : Preservative Efficacy Testing

Antimicrobial preservatives are substances added to multi-use products to protect the products from microbial contamination and spoilage. Microbes can be introduced inadvertently during manufacturing or during the repeated use of a product. The test for Antimicrobial efficacy is used to demonstrate the effectiveness of any added antimicrobial preservative(s). This test, also known as the *preservative efficacy test (PET),* is performed to determine if the chosen preservative is appropriate for a product formulation. Testing is performed according to compendial requirements of the European Pharmacopoeia Section 5.1.3 to evaluate the antimicrobial effectiveness of a product. The effectiveness of the preservative is evaluated by comparing the reduction in viable counts of microorganisms from their initial level, and is tested at various stipulated time intervals over a period of 28 days storage at room temperature.

1.5.1 : Preservative Efficacy Testing in Accordance with European and British Pharmacopeia Regulations

Over the last 20 years or so, a number of alternatives to traditional colony counts methods have been developed to enumerate viable microbial cells in foods, pharmaceuticals and cosmetics (Connolly et al., 1994). Antimicrobial preservatives are used to prevent or inhibit the growth of microorganisms that could present a risk of infection to patients and consumers, or degradation of medicinal and other products (Beveridge, 1999). There are many factors that affect the efficacy of a preservative

which include the concentration, pH, temperature and chemical composition of the product, the physiological condition of the contaminant microorganisms, the presence or absence of interfering substances, and the possible chemical interactions of a preservative with containers and closures. These aspects must be considered when choosing a suitable antimicrobial agent as a preservative, as it is an integral part of a pharmaceutical formulation's early development (Russell, 2003). However, it is difficult to predict accurately the ultimate effectiveness of a preservative in any pharmaceutical formulation. Therefore, it is necessary to assess the preservative efficacy in order to provide assurance that the product is adequately protected.

The pharmacopoeia published by different authorities, *e.g.* European Pharmacopeia (EP), British Pharmacopoeia (BP), Japanese Pharmacopeia (JP) and United States Pharmacopoeia (USP), each describe methods that are intended to assess the efficacy of an antimicrobial preservative. The methods vary only slightly from one pharmacopeia to another and they all share basic similarities with the same aim.

The organisms specified for testing purposes in the current BP (British Pharmacopoeia), USP (United States Pharmacopoeia) and EP (European Pharmacopoeia) are standard species and strains. The test microorganisms that are to be used are *Pseudomonas aeruginosa* ATCC 9027 (EP, USP, JP), *Staphylococcus aureus* ATCC 6538 (EP, USP, JP), *Candida albicans* ATCC 10231(EP, USP, JP), *Aspergillus niger* ATCC 16404 (EP, USP, JP), *Zygosaccharomyces rouxii* (EP, JP), and *Escherichia coli* ATCC 8739 (EP (oral preparations), USP, JP).

The accepted evaluation is dependent on the type of preparations (*i.e.* Parenteral and ophthalmic, topical, and oral) as shown in in Tables 1.3, 1.4 and 1.5. There are two categories of criteria for acceptance described, these are the target (EP-A) and acceptable (EP-B) level criteria. Efficacy results at the EP-B level are acceptable if there are good reasons for EP-A levels not being fulfilled.

Table 1.3: The criteria for evaluating preservative effectiveness in topical formulations as defined by the European Pharmacopeia

		Log_{10}reduction			
		2d	7d	14d	28d
Bacteria	A	2	3	-	NI
	B	-	-	3	NI
Fungi	A	-	-	2	NI
	B	-	-	1	NI

NI = No Increase

Table 1.4: The criteria for evaluating preservative effectiveness in parenteral and ophthalmic formulations as defined by the European Pharmacopeia

		Log_{10}reduction				
		6 h	24h	7d	14d	28d
Bacteria	A	2	3	-	-	NI
	B	-	1	1	3	NI
Fungi	A	-	-	2	-	NI
	B	-	-	-	1	NI

NI = No Increase

Table 1.5: The criteria for evaluating preservative effectiveness in oral formulations as defined by the European Pharmacopeia

		Log_{10}reduction	
		14d	28d
Bacteria	A	3	NI
Fungi	A	1	NI

NI = No Increase

The criteria for evaluating preservative effectiveness for topical formulations indicates that the target for reduction (A) of bacterial viable counts is 2 log_{10} CFU/ml from the initial count at 2 days and 3 log_{10} CFU/ml at 7 days with no increase permitted at 28 days, whist the reduction in fungal viable counts must be 3 log_{10} CFU/ml from the initial count at 14 days with no increase permitted at 28 days.

For parenteral and ophthalmic preparations, the target reduction (A) for bacterial viable counts is 2 log_{10} CFU/ml from the initial count at 6 hours and 3 log_{10} CFU/ml at 24 hours with no increase in counts after 28 days whilst the fungal viable counts must be reduced by2 log_{10} CFU/ml from the initial count at 14 days with no increase at 28 days. In contrast, the criteria for oral formulations do not provide target and acceptable criteria. For bacteria, oral products require a 3 log_{10} CFU/ml

reduction in viable counts from the initial count at 14 days and no increase at 28 days, whilst for fungi a 1 $\log_{10}$ CFU/ml reduction and no increase is permitted at 28 days.

Since plate count methods require several days of incubation to yield visible colonies, then viable count methods can be time-consuming (Chollet et al., 2008). Many companies these days manufacture products under tight time constraints, and delays in laboratory testing can significantly increase the cost of production. Moreover, traditional microbiological techniques require skilled personnel for sample handling and interpretation of results (Morris, 1998). Consequently, bioluminescent reporter strains could prove to be an effective alternative to viable counts for monitoring changes in microbial population density, and thus find potential applications in antimicrobial efficacy testing (Marincs, 2000).

Bioluminescent reporters have the potential to provide continuous real-time results, which means that formulations failing the PET acceptance criteria can be identified early on in the process – therefore potentially saving a lot of time. Bioluminescent reporters can also be used as a rapid screening tool for the selection and development of novel formulations, antimicrobial products and preservatives (Naseby, 2006). A constitutive promoter fused to the *lux* bio-reporter transformed in to biosensor microorganisms could serve as a rapid system for preservative efficacy screening. High levels of bioluminescence would indicate the presence of a large number of viable microbial cells and *vice versa* under normal conditions. In the presence of preservatives, a reduction in bioluminescence would indicate a reduction in the number of viable cells. Consequently, the research described in this thesis set out to assess the various features of five novel *E. coli* biosensor strains in monitoring PET constructed at the University of Hertfordshire in testing a selection of preservatives with unique modes of antimicrobial action. This work will be described further in chapters 4 and 5.

1.6 : Rationale of Study

The rationale behind this study was to investigate and assess *lux* bio-reporter constructs controlled by five different constitutive promoters transformed into *Escherichia coli* ATCC 8739 for the application as a rapid microbiological method in preservative efficacy test (PET). Stewart (1989) stated that the speedy response of bioluminescent signals encoded by *lux* operon, therefore brings the prospect of on-line microbial assays closer to reality. Hence, the hypothesis of this study is that the ability of the whole-cell bioluminescence method to replace the traditional plate counting method. Therefore, successful evaluation of whole-cell bioluminescent method would make a great contribution as a real-time monitoring system in PET.

The promoters play an important role in regulating the bioluminescence gene expression within the *lux* operon. Thus, the promoters' sequences were analysed, in relation to the level of bioluminescence expression and consensus sequences of sigma factors. Sorensen et al. (2006) remarked that one of the greatest limitations of whole-cell biosensors is the strength of the promoter. Thus, a comparison of the sequence of the five selected promoters with the consensus sequence could indicate whether promoter strength was affected by similarity, or otherwise.

In this study, five Escherichia coli ATCC 8739 transformed with five different constructs were validated as novel rapid microbiological method according to the regulations recommended by the British Pharmacopeia Vol 5 SC IV L & European Pharmacopeia 5.1.6, Section 31, for preservative efficacy applications. The criteria of accuracy, precision, reliability, linearity, limit and range of detection were assessed. The five *E.coli* constructs and two control strains were evaluated and compared to four other current microbiological testing methods: Plate counting; ATP chemi-luminescence; Fluorescence Spectrometry; and Epifluorescence Microscopy (Chapter 2). The promoter sequences within the -60 to +1 regions of the constructs were analyzed, and compared to

the consensus sequences of different σ-factors. Measurements of whole-cell bioluminescence, adenylate energy charge (AEC) and viable counts were evaluated throughout the 28 days of incubation to further characterize the constructs. The plasmid copy number (PCN) was determined by quantitative polymerase chain reaction (qPCR), to assess the number of plasmid copies/cell throughout the incubation period This was performed to ensure that any changes in bioluminescence expression levels over the extended incubation periods were not a result of changes to the plasmid copy number per cell (Chapter 3).

The constructs were further challenged with two different preservatives in accordance to the British Pharmacopeia Appendix XVI C and European Pharmacopeia Section 5.1.3 regulations to assess the value of a bioluminescent biosensor as an alternative testing method to conventional microbiological approaches (Chapter 4 and 5).

1.7 : Aim

1) The aim of this project was to assess five genetically modified *E.coli* constructs as novel rapid real time reporters for their application in preservative efficacy studies (PET) studies in replacement of conventional plate counting method.

Chapter 2

2 : The Validation of the Five Bioluminescent reporter strains *E. coli* Constructs- as a Rapid Real Time Microbiological Testing Method.

2.1 : Introduction

Microbial analysis of food, cosmetic, and pharmaceutical products are an integral part of the management of microbial safety. Both control authorities and individual business operators monitor microbial levels in order to detect emerging microbial risks. Microbial analysis is also a valuable tool for compliance testing to define microbiological criteria or to assess the performance of management strategies based upon Hazard Analysis Critical Control Points (HACCP). Standardized methods (*e.g*. ISO and Pharmacopoeia methods) are acknowledged to be the gold-standard analytical methods for assessment of microbial load (Jasson et al., 2010). These standardization methods involve classical culturing methods using broth and media to grow, isolate or enumerate bacterial cells. These methods have been developed historically based upon expertise and decided upon by international consensus (Jasson et al., 2010). These classical culture methods are still used by many labs, and especially by regulatory agencies, because they are harmonized methods. However, a serious drawback is that they are laborious, demand large volumes of liquid, solid media and reagents, and involve time consuming procedures-both in operation and data collection. The term 'rapid method' can be defined as any method or system that reduces the time taken to obtain a microbiological result (Feng, 1996; Fung, 1994). In addition, rapid methods may also be automated to improve efficiency in handling multiple/large samples.

Because of the many rapid microbiological methods available, it is important to develop a comprehensive and holistic approach for the validation process to ensure that a given rapid microbiological testing method is suitable for its intended use. Rigorous scientific validation is a

critical part of obtaining regulatory approval for a rapid microbiological testing method (Riley, 2004). A new method should be at least equivalent to the existing methods. Since the existing methods are predominantly the microbial growth based compendia methods, a comparison should be made between the new and the compendia methods. In order to determine validity between the old and new methods, this is discussed in the following text (British & European Pharmacopeia).

2.1.1 : Validation Criterias' set by the Pharmacopoeia Regulations

The essential criteria for validation of a method are its accuracy, precision, specificity, limit of detection, range, linearity, and robustness (British & European Pharmacopoeia; PDA, 2000).

Accuracy is defined as the closeness between the results of a new test method with the compendia methods (British & European Pharmacopoeia; PDA, 2000). Since the expected results are usually based on the existing method, a test of accuracy is essentially a comparison between the old and new methods. The accepted criterion is that the new method should give equivalent or better results than the current method. A standard deviation of ± 0.3 is accepted for a new microbiological method (British & European Pharmacopoeia; PDA, 2000). The accepted criterion is at least 70% recovery (British & European Pharmacopoeia; PDA, 2000).

Precision is defined as the degree of agreement among individual test results when the procedure is applied repeatedly to multiple samplings or suspensions across the range of test. Precision can be a measure of either the degree of reproducibility or the repeatability of the new microbiological method under optimum conditions. The recommended statistical method of comparing the precision of the two methods is the application of the F value. If the calculated ratio for that degree of freedom exceeds the value in the F distribution table, a significant difference exists between the precision of the two methods (British & European Pharmacopoeia; PDA, 2000).

Limit of detection is the lowest number of microorganism in a sample that can be detected or counted. The limit of detection is particularly important for qualitative tests.

The working range of assay is defined as the interval between the upper and lower levels of microorganisms that have can be determined with precision, accuracy, and linearity with the new microbiological method.

The linearity of a microbiological test method is its ability to furnish results which are proportional to the concentration of microorganisms present in the sample, within a given working range. The criterion for acceptable criterion of correlation of coefficient is 0.9 or better.

The robustness/ ruggedness of an assay is defined as its ability to tolerate slight deviations in test parameters and still provide accurate results.

An ideal rapid method would enable a 'real time' measurement of microbial content in a sample. Furthermore, rapid tests are very useful tools in hazard analysis and critical control points (HACCP) programmes and in conditions where microbial analysis is required, instead of relying on laborious methods that take hours or days to yield results.

2.2 : Rational of Chapter 2

This chapter focuses on the validation process for a whole-cell bioluminescence method, to be used as an alternative rapid method against the compendia method (*i.e.* conventional plate counting). Validation was performed following the guidelines of the Parenteral Drug Association (PDA) Technical Report No 33 'Evaluation, Validation, and Implementations of New Microbiological Testing Methods', as described by the British Pharmacopoeia Volume 5 SC IV L and European

Pharmacopoeia Chapter 5.1.6. The validation criteria examined were accuracy, precision, limit of detection, linearity, range and equivalence. Three other current methods: ATP-bioluminescence; fluorescence spectrometry; and epifluorescence microscopy were performed for comparison with the whole-cell bioluminescence method. The bioluminescence expression levels exhibited by the five bioluminescent reporter strains *E. coli* strains were evaluated. The promoter regions of the five promoters were identified and compared with the consensus sequences of RpoD ($\sigma^{70/D}$). The aim of this was to determine whether the promoter strength of the five constructs was affected by similarity, or otherwise, to the consensus sequence.

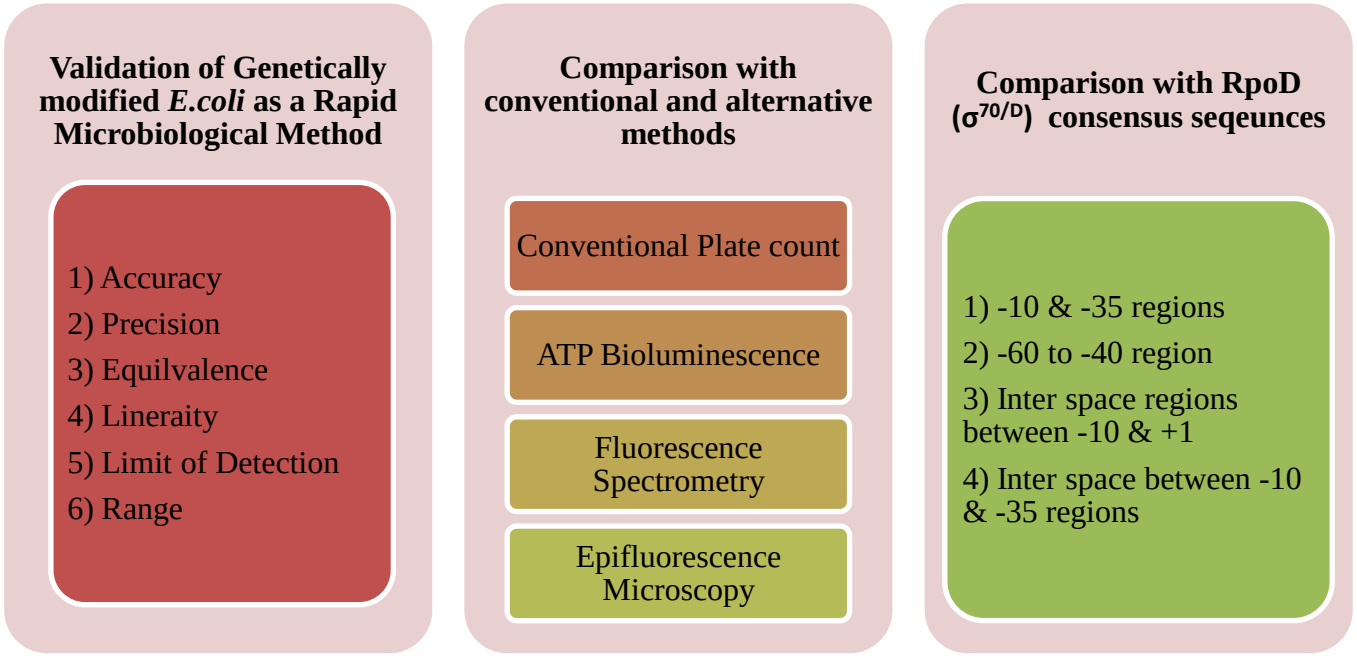


Figure 2.1: Summarises the various steps employed in chapter 2

2.3 : Objectives

1) To validate the five constructs for their accuracy, precision, equivalence, linearity, range and limit of detection in accordance to the European and British Pharmacopoeia as a rapid microbiology method for future preservative efficacy test
2) To compare and contrast the whole-cell bioluminescence method with the plate count method, ATP-bioluminescence, fluorescence spectrometry, and epifluorescence microscopy counts.
3) To identify the promoter regions, and to compare them with the RpoD ($\sigma^{70/D}$) consensus regions of -10 and -35, the spacer lengths between -10 and -35, AT (%) of -60 to -40 regions, and GC (%) between the -10 and +1 regions are analysed.

2.4 : Materials and Methods

2.4.1 : Construction of Bioluminescent Reporter Strains *E. coli*

The *E. coli* ATCC 8739 constitutive promoters used in this study are shown in Table 2.1. The plasmid pSB417, containing a 5.8kbp *lux* cassette, was obtained from Dr. Michael Winson (Winson et al., 1998). The *lux* cassette was flanked at either end by an *Eco*R1 and *Bam*H1 restriction site. The *lux* cassette was also excised by double restriction; using a total volume of 20 µl that consisted of 1 x Multicore buffer (Promega), 1.5 µg pSB417, 2 µg BSA, 10 U *EcoR1* (Invitrogen) and 10 U *BamH1* (Promega). Reactions were carried out at 37°C for 4 hours, and restriction enzymes were inactivated at 80° C for 10 minutes. Success of the reaction was determined by 1.0 % (w/v) agarose gel electrophoresis, and the 5.8 kbp fragment was purified using the QIAquick Gel Extraction Kit (Qiagen) as outlined in the manufacturer's protocol.

Plasmid pBR322 was obtained from Promega and restricted with restriction enzymes *Eco*R1 and *Bam*H1 using the procedure described above for pSB417. Plasmid pBR322/*lux* was constructed by ligation of the purified 5.8 kbp *lux* cassette from pSB417 to the restricted pBR322. After the addition of 0.2 U of T4 DNA ligase (Invitrogen) and 1 x ligase buffer (Invitrogen) to give a final reaction volume of 20 µl, the pBR322/*lux* mix was heated to 65° C and cooled rapidly to 4° C for 16 h.

Constitutive promoters were isolated using the primers shown in Table 2.1. The forward primers had an artificial *Eco*R1 restriction site designed into the 5'end. An 'in frame' artificial stop codon and a *Sna*B1 restriction site were designed into the 5'end of each reverse primer. The promoter regions were amplified by PCR with addition of the following reagents: 200 µM of each deoxynucleoside

triphosphate (dNTP); 0.5 µM of each reverse and forward primers; 2.5 mM $MgCl_2$; 1 x PCR buffer; 1 U platinum Taq DNA polymerase (Invitrogen), and 5 µl of genomic template. The thermal cycling conditions for the PCR reactions were initial denaturation at 94°C for 2 minutes, followed by 35 cycles of denaturation at 94°C for 30s, annealing at 60°C for 30s and extension at 72°C for 30s. A final extension at 72°C for 10 minutes was then performed. The PCR products were analysed by 1.0% (w/v) agarose gel electrophoresis and stained with ethidium bromide.

Plasmid p322/lux (0.9ug) was digested in a 50 ul reaction consisting of 25 U of EcoR1 (Invitrogen) and SnaB1 (New England Biolabs), 1 x Multicore buffer (Promega) and 5 ug BSA (New England Biolabs). Approximately 0.5 ug of each of the purified promoter regions described above were also digested in a total reaction volume of 50 ul consisting of 25 U of EcoR1 and SnaB1, 1 x Multicore buffer and 5ug BSA. Each digestion reaction was performed at 37^{o}C for 4 h followed by inactivation of the enzymes at 80^{o}C for 10 min. The pBR322/*lux* construct was restricted and each of the individual promoters was then ligated with it using DNA ligase. The plasmid pBR322/*lux* constructs were then transformed by electroporation into electrocompetent *E. coli* ATCC 8739 cells. The transformation was performed using a Gene-Pulser system (Bio-Rad) under the following conditions: 25µF; 2.5 kV; and 200 ohms. Figure 2.2 illustrates the construct.

To perform DNA sequencing of the constructs, the plasmid was extracted from the transformed *E. coli* ATCC 8739 using the QIAprep Spin Miniprep kit (Qiagen). The forward primer sequence 5'-ACAAATAGGGGTTCCGCGCA-3' and the reverse primer sequence 5'-TCACGAATGTATGTCCTGCGTC-3' were used for the DNA sequencing analysis of the construct promoter regions with PCR conditions of; initial denaturation at 94°C for 2 min followed by 35 cycles

of denaturation at 94°C for 30 s, annealing at 50°C for 30 s and extension at 72°C for 1 min. A final extension at 72°C for 10 min was then performed. Sequencing was performed in accordance with the protocol of GATC biotech sequencing. The *E. coli* ATCC 8739 biosensor strains were constructed by Dr Daniel Hoeffl in 2007.

Table 2.1: Forward and reverse primers used for amplification of the constitutive promoters

Promoter region	Forward Primer (5'-3')	Reverse Primer (5'-3')
P_{lysS} (Lysyl tRNA synthetase)	CTTCAAGAATTCATGTCG ATACCGCCCTCTGG[1]	CTGATTACGTAG**TCA**TTC CGTGAGGTCCTGAATGGGA[2]
P_{spc} (Ribosomal Protein operon)	CTTCAAGAATTCTTGAAA TCCGCGGAATGCCGT	CTGATTACGTAG**TCA**GTT GTCGGCGACGTTCAGCA
P_{lpp} (Outer Membrane Lipoprotein)	CTTCAAGAATTCTGTATA TCGAAGCGCCCTGATG	CTGATTACGTAG**TCA**TGC CAGCAGAGTAGAACCCAGGA
P_{tatA} (Twin-arginine Translocase)	CTTCAAGAATTCACATTC TTGTTGGTCAGCCGA	CTGATTACGTAG**TCA**ATC GGAACCGATGGAGCCGAGC
P_{ldc} (Lysine Decarboxylase)	CTTCAAGAATTCTGATCG ACTCCATCATCCCGGA	CTGATTACGTAG**TCA**CG ACTCCAGTTCTTTGATGGGCTC

[1] Underlined sequences indicate either *EcoR1* sites (for the forward primers) or *SnaB1* sites (for the reverse primers).
[2] Bold sequences indicate an artificial 'in frame' stop codon

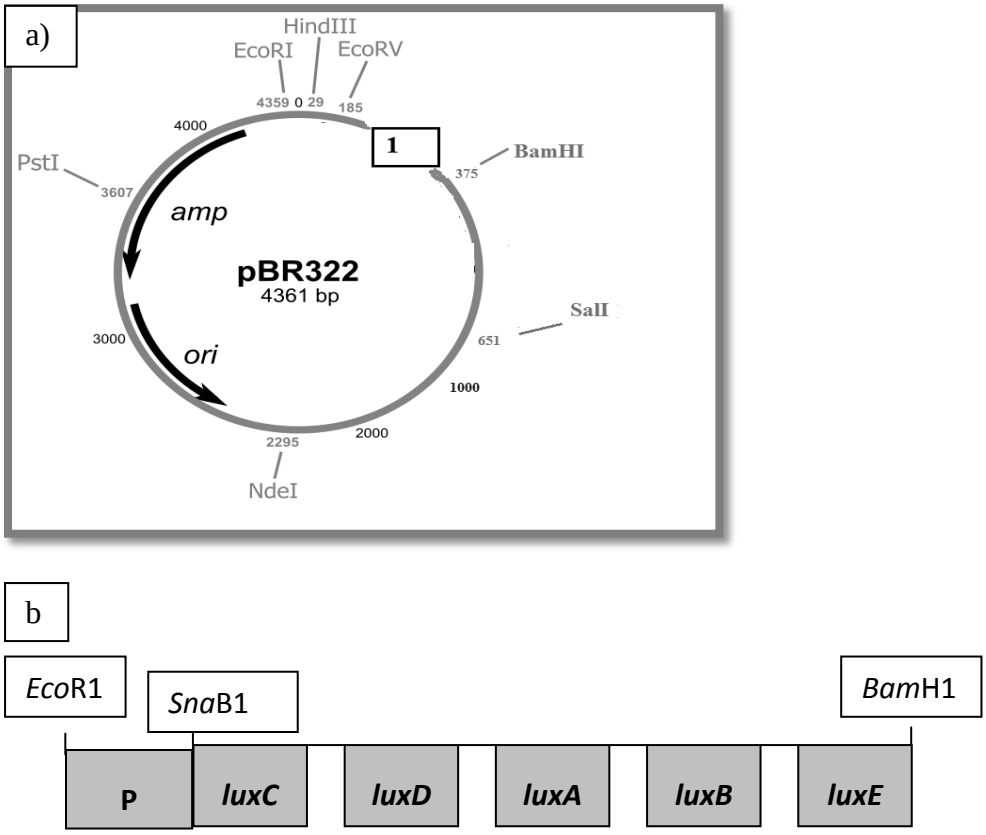


Figure 2.2: a) A diagram of the modified pBR322 vector into which the bioluminescent reporter cassette was inserted. b) A schematic diagram of the bioluminescent reporter construct denoted as [1]. P denotes the promoter, and can represent either *lpp*, *tatA*, *ldc*, *lysS* or *spc*. The promoter and *lux* operon fusion was ligated into position 1 of the plasmid in Figure 2.2a.

2.4.2 : Media and Solutions

The transformed *E. coli* strains were maintained on tryptone soy agar (TSA) (Oxoid) with the addition of 100 µg/ml ampicillin. A single colony of each culture was inoculated into 10 ml tryptone soy broth (TSB) and grown overnight aerobically at 32^{o}C to produce a starter culture for the experiments (as specified by the E.P & B.P). For all experiments, a 50µl volume of the overnight starter culture of each strain was inoculated into 50 ml of TSB supplemented with 100 µg/ml ampicillin, except for the untransformed *E. coli* ATCC 8739 parental strain. The bioluminescent reporter strains *E. coli* strains: *E. coli* [pBR322. *lpp-lux*] (which will be referred to as *lpp-lux E. coli* through the remainder of the text); *E.*

coli [pBR322. *tatA-lux*] (*tatA-lux E. coli*); *E. coli* [pBR322. *ldc-lux*] (*ldc-lux E. coli*)*; E. coli* [pBR322. *lysS-lux*] (*lysS-lux E. coli*)*; E. coli* [pBR322. *spc-lux*]) (*spc-lux E. coli*); and also the control *E. coli* ATCC 8739 [pBR322.*lux*] and *E. coli* ATCC 8739 strains were incubated aerobically on an orbital shaker (Thermo Scientific) at 100 rpm, 32 ^{o}C for 24 hours. Cultures were diluted tenfold in buffered peptone water (10^{-1} to 10^{-8}) (BPW) (Oxoid) in readiness for the experiments described in section 2.4.3, 2.4.4, 2.4.5, 2.4.6, and 2.4.7. This was carried out in five replicates.

2.4.3 : Bioluminescence Determination

Relative light units (RLU) were measured using a Celsis Advance Luminometer. The Celsis Advance Luminometer is designed to run bioluminescence assays using an automated sampling system.

Volumes (1.0 ml) of each dilution, prepared in section 2.4.2 above, were transferred to culture tubes (Fischer Brand disposable borosilicate glass culture tubes 12 x 75 mm) and the bioluminescence readings measured with the Celsis Advance Luminometer. This experiment was repeated 5 times. A blank control containing buffered peptone water was used. The true bioluminescence readings were calculated by subtracting the blank value from the found value and then division of the result by the dilution factor as in equation 2.1.

$$\frac{Bioluminescecne\ readings\left(\frac{RLU}{ml}\right) - Blank\ Reading}{Dilution\ Factor} \quad \text{..... Equation 2.1}$$

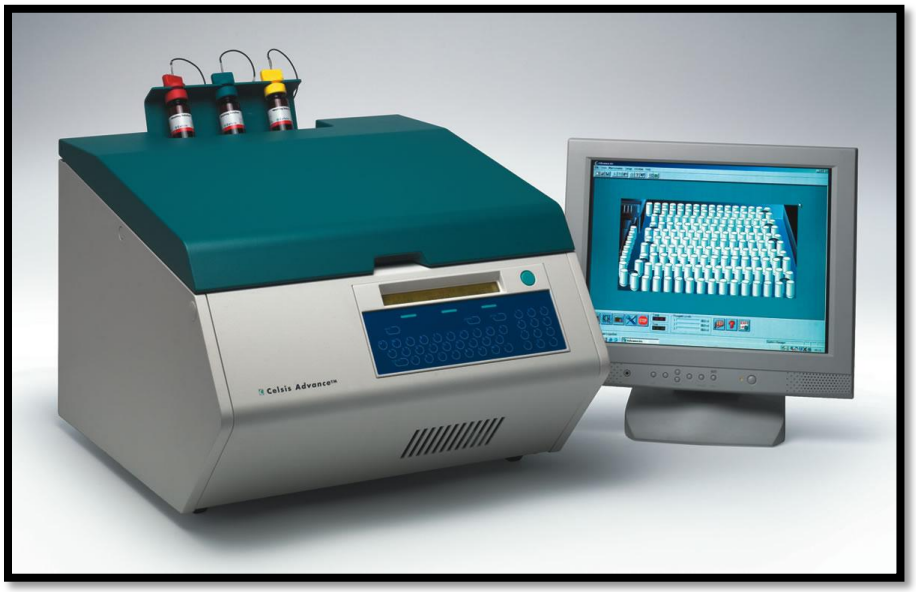


Figure 2.3: The Celsis Advance Luminometer (http://www.celsis.com/rapid/celsis-systems/advance-system/advance-luminometer)

2.4.4 : Viable Counts Determination

The viable counts of the *E. coli* strains were determined by spread-plating 100µl of the 10^{-7} and 10^{-8} diluted culture, prepared as described in section 2.4.1 above, onto TSA agar containing 100 µg/ml ampicillin. Colonies were counted after incubation at 32 $^{\circ}$C for 72 hours. CFU/ml counts was calculated based on the average of five replicates divided by the dilution factor and multiplied by the inverse of the volume used (0.1 ml) as in equation 2.2.

$$\frac{Number\ of\ Colony\ forming\ units}{Dilution\ Factor}\ x\ Volume \quad \text{..... Equation 2.2}$$

2.4.5 : ATP-Chemiluminescence Determination

2.4.5.1 : Preparation of samples

Volumes (1.0ml) of the bacterial suspension, prepared as described in section 2.4.1 above, were centrifuged at 8000 x*g* for 4 minutes (Accu Spin Micro, Fisher Scientific). Supernatant of culture were discarded and cell pellet was washed by resuspending and centrifugation with 1.0ml of cold sterile deionized water to get rid of any broth, three times repetitively. Pellets were resuspended and vortexed with 1.0ml of dilution buffer (Roche). Subsequently, a 500µl volume of cell lysis reagent (Roche) was added to the resuspended pellets, and the mixture incubated for 5 minutes at 20 ± 2.5°C. After mixing, the samples were centrifuged for 2 minutes at 8000 x*g*. Finally, 500µl of the supernatant was transferred to a sterile culture tube and kept on ice until measurement was performed.

The ATP Bioluminescence Assay Kit HS II (Roche) was used for the ATP chemiluminescence determination. Lyophilised ATP (Roche) was diluted (1:10) in dilution buffer, and concentrations ranging from 10.8 fg/ml to 108 µg/ml were prepared – these were expressed as -log mg/ml. Controls for background luminescence (Dilution Buffer) were run using Celsis Advanced luminomter, and these readings were subtracted from the sample readings. 30.0ml of dilution buffer was added to the lyophilized luciferin-luciferase enzyme mix. The Celsis Advanced luminometer was programmed to dispense 200µl of luciferin-luciferase reagent (Roche) into each experimental sample. The luminometer injector was washed with 70% ethanol and rinsed twice with sterile distilled water before use. All ATP preparations were carried out in a BIOMAT^2 Class Microbiological Safety Cabinet to maintain sterility. Gloves were worn at all times to avoid any exogenous ATP contamination of the reagents. Reagents and samples were kept on ice at all times to avoid loss of enzymic activity. The luciferase enzyme was mixed by inversion and not by vortexing, as required by the manufacturer's protocol.

2.4.6 : Determination of Fluorescence readings of Viable Cells Using LIVE/DEAD stains

A 1.0 ml volume of each dilution (10^0- 10^{-6}), prepared as described in section 2.4.1 above, was centrifuged (13,000 x*g*) for 2 minutes (Accu Spin, Fisher Scientific) and resuspended with 1.0 ml of 0.85% NaCl. A 1ml volume of the cell suspension was stained with 1 µl of fluorochrome dyes (0.5 µl of each) SYTO-9 and propidium iodide (PI) (BacLight Live/Dead kit, Molecular Probes). The mixture was incubated for 15 minutes in the dark at 20 ^oC, before 200 µl of each sample was pipetted into separated wells of a 96-well black/white microtiter plate. The fluorescence of the samples was measured using a Promega GloMax detection system with blue filters (excitation at 490 nm, emission at 510-570 nm) and green filters (excitation at 525 nm, emission at 510-570 nm). A control with sterile deionized water was used to determine the background fluorescence. True fluorescence readings were calculated by the deduction of the control readings from the sample readings and division of the result by the dilution factor and multiplication by the volume used (0.2 ml) as in equation 2.3. This was repeated 5 times for each sample.

$$\frac{Fluorescence\ Readings - Blank}{Dilution\ factor}\ x\ Volume \quad \text{..... Equation 2.3}$$

2.4.7 : Epifluorescence Microscopy Counts

Samples of the 10^{-1} - 10^{-3} bacterial suspensions, prepared as described in section 2.4.1 above, and mixed with the BacLight Live/Dead dyes were enumerated using an Improved Neubauer haemocytometer and a fluorescence microscope (Nikon EFD-3) with UV lamp (Nikon Hg lamp), under the following conditions: excitation at 410-490nm, emission at 505-520nm; and 1000x magnification . The bright

green viable cells were counted within a total of 20 (0.005cm x 0.005 cm) with a relevant dilution which yielded 5 to 20 bacteria per small square squares (volume of each: 0.25 x 10^{-6} cm^3. The calculation of total viable cells were done by taking the total of 30 squares divided by the proportion of chamber counted and multiplied by the volume of the chamber (4.0 x 10^{-6} cm^3). The final total viable cell count (TVC) was obtained by dividing the count by the dilution factor as in equation 2.4.

$$\frac{Total\ number\ of\ Squares}{Proportion\ of\ Chamber\ x\ Volume\ of Chamber} \text{ x } \frac{1}{\text{Dilution Factor}} \quad \text{..... Equation 2.4}$$

2.4.8 : Statistical Analyses

Initially, a Shapiro-Wilk normality test was carried out for all data obtained in sections 2.4.1 to 2.4.7 whereby the alpha value tested was α=0.05. The null hypothesis was that the population tested was normally distributed. The P value obtained was less than 0.05 indicating that the null hypothesis should be rejected. Thus the raw count data were found to be not normally distributed. Consequently, all readings were log-transformed to ensure that they conformed to a normal distribution before performing further statistical analyses. Two way ANOVA; Post Hoc Tukey analysis and Pearson's Correlation analysis (α= 0.05) of the data were performed using IBM SPSS statistics Version 20. Standard means of errors (SEM) were calculated by standard deviations (S.D) of replicates divide square root of replicates (n).

$$\frac{\sigma}{\sqrt{n}} \quad \text{..... Equation 2.5}$$

2.4.9 : Validation

The novel bioluminescent method was validated and compared with the four current microbial analytical methods to determine the reliability of the whole-cell bioluminescent method for microbial analysis: the standard plate counting method; ATP chemiluminescence; fluorescence spectrometry; and epifluorescence microscopy. The following parameters were evaluated for the validation: accuracy; precision; equivalence; limit of detection; linearity; and range (B.P & E.P).

1. The accuracy of the whole-cell bioluminescence assay and conventional plate counting method were determined by preparing a suspension of the microorganism at the upper and lower end of the working range for the assay. The whole-cell bioluminescence and colony forming unit readings were obtained upon the testing of 10^8 CFU/ml as the upper limit and a 10^4 CFU/ml as the lower limit. The readings obtained were regarded as the actual result (100%). A further dilution in BPW of (*i.e.* 75%, 50%, 25%, and 10%) made from the upper and lower range, were regarded as the expected result. The expected results were divided by the dilution factor (*i.e.* 75%, 50%, 25%, and 10%). The recovery results for both range were obtained by the ratio of expected to the actual reading multiplied by hundred per cent. The average recoveries of the upper and lower limit were deduced.

2. The range of precision was determined at suspension of whole-cell bioluminescence (RLU/ml) to plate counts at 10^8, 10^7. 10^6, 10^5 and 10^4 CFU/ml. The SEM was calculated of whole-cell bioluminescence readings and plate counts; a relative standard deviation in the 15% to 30% range is acceptable according to the Pharmacopoeia standards. Statistical analysis was performed using the F-test to obtain the F value for precision comparisons between the whole-cell

bioluminescence and conventional plate counting method. The F value obtained was then compared to the expected F value in the F-distribution table.

3. The equivalence of the novel bioluminescent reporter to the standard test methods was tested at the range of suspensions (10^8, 10^7. 10^6, 10^5, 10^4 and 10^3 CFU/ml) as above in the precision section. The results of the whole-cell bioluminescence assay and the conventional plate count method were compared by two-way ANOVA (α= 0.05).

4. The minimum limit of detection (MDL) of the ATP-chemiluminescence, fluorescence spectrometry and epifluorescence microscopy method was determined by producing serial dilutions of the bacterial suspensions graph and plotted against whole-cell bioluminescence readings. The lowest level of whole-cell bioluminescence was deduced and incorporated to the y=mx+c equations in the whole-cell bioluminescence vs CFU counts graph to obtain the MDL in CFU counts.

5. The linearity was determined by calculating the correlation coefficient of whole-cell bioluminescence, ATP chemiluminescence, fluorescence spectrometry and epifluorescence microscopy readings and CFU counts; linearity of whole-cell bioluminescence and ATP-chemiluminescence, fluorescence spectrometry and epifluorescence microscopy were obtained by Pearson`s correlation.

6. The range was determined from the lowest limit of detection (CFU/ml) to the highest limit of detection (CFU/ml) for whole-cell bioluminescence, ATP chemiluminescence, fluorescence spectrometry and epifluorescence microscopy methods.

The time taken per sample analysis (minutes), preparation time per sample (minutes) and incubation time (hours) were recorded throughout this study. The average time to perform the procedure was calculated from the times taken to perform each method 10 times. The time taken per sample analysis included the time taken: to prepare the dilutions; by the equipment processing the samples; and incubation times for the ATP-chemiluminescence, fluorescence spectrometry and epifluorescence microscopy methods. The preparation time also included the time needed to prepare the universal bottles, pipette tips, eppendorf tubes, diluents, and agar for autoclaving.

2.4.10 : Bioinformatics Analysis of the Promoter Regions

The GATC sequencing results obtained were analysed using the BPROM Softberry programme. The Softberry programme allows tabulation of sequences specifically on the bacterial -10 and -35 promoter regions (http://www.softberry.com). Other promoter regions were also tabulated concurrently (the -40 & -60 regions, the inter-regions between -10 and +1, and the inter-regions between -10 and -35).

2.5 : Results

This results section contains experimental results for the validation process, and bioinformatics tabulation of promoter sequences

Sections **2.5.1, 2.5.2, 2.5.3, 2.5.4, 2.5.5, and 2.5.6** contain data for the validation of the bioluminescent reporter strains constructs, whilst section **2.5.7** relates the promoter strength to the consensus sequences of RpoD ($\sigma^{70/D}$)**.**

2.5.1 : Recovery Determination of Bioluminescence and Plate Counting

Bioluminescence recoveries (%) were higher than plate count recoveries at high bacterial suspensions (Table 2.2) for *tatA-lux* and *lysS-lux* (125.61% and 135.19%), whilst *lpp-lux*, *ldc-lux*, and *spc-lux* (87.18%, 86.17%, and 87.18%) showed recoveries similar to the conventional method at high bacterial suspension (87.71 – 99.60%). The range of recoveries for the whole-cell bioluminescence at high concentration was 86.71-135.19 %, compared to 82.1-133.19% for the conventional plate counting method.

At low bacterial suspensions, the plate count method resulted in a greater percentage of recovery (102.21% - 139.17%) compared to the whole-cell bioluminescence method (78.17 – 112.68%). The overall recoveries percentages of the bioluminescence method were 78.71 – 135.19% which was similar to the plate count method (82.10-139.17%).

No significant difference was found in the recoveries of the whole-cell bioluminescent and conventional plate count method for both concentrations.

Table 2.2: The average recoveries (%) obtained for the whole-cell bioluminescence and conventional plate count methods in high (10^8 CFU/ml) and low (10^4 CFU/ml) bacterial concentration

Bioluminescent reporter strains *E. coli* Strain	High bacterial concentration		Low bacterial concentration	
	Average Bioluminescence Recovery (%)[#]	Average Conventional Plate count Recovery (%)[#]	Average Bioluminescence Recovery (%)[#]	Average Conventional Plate count Recovery (%)[#]
Lpp-lux	87.18(±0.125) [ns]	99.6(±0.075) [ns]	83.76(±0.075) [ns]	137.01(±0.14) [ns]
TatA-lux	125.61(±0.125) [ns]	98.81(±0.01) [ns]	112.68(±0.01) [ns]	139.17(±0.15) [ns]
Ldc-lux	86.71(±0.12) [ns]	87.65(±0.02) [ns]	79.38(±0.01) [ns]	128.81(±0.12) [ns]
LysS-lux	135.19(±0.15) [ns]	82.10(±0.02) [ns]	78.71(±0.08) [ns]	114.39(±0.08) [ns]
Spc-lux	87.18(±0.125) [ns]	87.71(±0.08) [ns]	81.70(±0.08) [ns]	125.56(±0.01) [ns]
E. coli [pBR322-*lux*]	n/a	133.19(±0.03)	n/a	129.86(±0.05)
Wildtype *E. coli* ATCC 8739	n/a	101.28(±0.04)	n/a	102.21(±0.03)

[#] The average bioluminescent/ plate count recoveries of high (10^8CFU/ml) / low (10^4CFU/ml)/ bacterial suspensions at 75%, 50%, 25%, and 10%. (n=5, ± S.E.M)

[ns] denotes no significant difference between the whole-cell bioluminescence and conventional plate count methods

2.5.2 : Determination Of The Precision And Equivalence Of The Whole-Cell Bioluminescence Method And Compared To The Conventional Plate Counting Method

To determine the precision of the whole-cell bioluminescence method in comparison with the plate counting method, the F-test was applied and the results of this analysis are shown in Table 2.3 The critical value obtained from F distribution, $F_{4,18;0.05}$=2.93. The calculated F values obtained were, in increasing order t*atA* (F= 1.715), *lpp* (F=1.717), *ldc* (F=1.849), s*pc* (F=1.884), and *lysS* (F=2.656). The most precise construct is *tatA* followed by *lpp, ldc, spc,* and *lysS*. The F values obtained by the bioluminescent reporter strains strains were lower than the critical value (F=2.93) from the F distribution signifying no significant difference between the two methods

Table 2.3: The Precision analysis of the Whole-Cell Bioluminescence Method Compared to the Plate Count Method over a Range of Concentrations.

Bioluminescent reporter strains *E. coli* Strain	Dilution Factor	**Serial dilution of Bacterial Suspension**		**Statistical Analysis**
		Bioluminescence (Average $\log_{10}$ RLU/ml) [#]	Plate count (Average $\log_{10}$ CFU/ml) [#]	F-Value[+] (P-value)[++]
lpp-lux	10^{0}	7.36 (±0.04)	8.5(±0.05)	1.717, (p= 0.18)
	10^{-1}	7.32(±0.01)	7.5(±0.05)	
	10^{-2}	6.54(±0.05	6.5(±0.05)	
	10^{-3}	5.65(±0.01)	5.5(±0.05)	
	10^{-4}	4.69(±0.02)	4.5(±0.05)	
	10^{-5}	3.72(±0.02)	3.5(±0.05)	
tatA-lux	10^{0}	6.37(±0.02)	8.53(±0.08)	1.715, (p=0.15)
	10^{-1}	6.40(±0.05)	7.53(±0.08)	
	10^{-2}	5.50(±0.05)	6.53(±0.08)	
	10^{-3}	4.60(±0.03)	5.53(±0.08)	
	10^{-4}	3.61(±0.008)	4.53(±0.08)	
	10^{-5}	2.71(±0.02)	3.53(±0.08)	
ldc-lux	10^{0}	5.99(±0.03)	8.58(±0.08)	1.849, (p=0.12)
	10^{-1}	6.12(±0.02)	7.58(±0.08)	
	10^{-2}	5.18(±0.06)	6.58(±0.08)	
	10^{-3}	4.24(±0.01)	5.58(±0.08)	
	10^{-4}	3.32(±0.01)	4.58(±0.08)	
	10^{-5}	2.42(±0.012)	3.58(±0.08)	
lysS-lux	10^{0}	5.87(±0.03)	8.65(±0.08)	2.656, (p=0.09)
	10^{-1}	5.95(±0.04)	7.65(±0.05)	
	10^{-2}	5.02(±0.03)	6.65(±0.05)	
	10^{-3}	4.12(±0.03)	5.65(±0.05)	
	10^{-4}	3.27(±0.10)	4.65(±0.05)	
	10^{-5}	2.33(±0.12)	3.65(±0.05)	
spc-lux	10^{0}	6.57(±0.04)	8.64(±0.09)	1.884, (p=0.1)
	10^{-1}	6.71(±0.08)	7.64(±0.09)	
	10^{-2}	5.86(±0.05)	6.64(±0.09)	
	10^{-3}	4.92(±0.05)	5.64(±0.09)	
	10^{-4}	4.02(±0.07)	4.64(±0.09)	
	10^{-5}	3.06(±0.03)	3.64(±0.09)	

[#] The average bioluminescent and plate count in a tenfold dilution series (n=5 ± S.E.M))

+The degrees of freedom analysed were $F_{4,18};_{0.05}=2.93$, no significant difference

++ P value obtained from ANOVA at $\alpha=0.05$

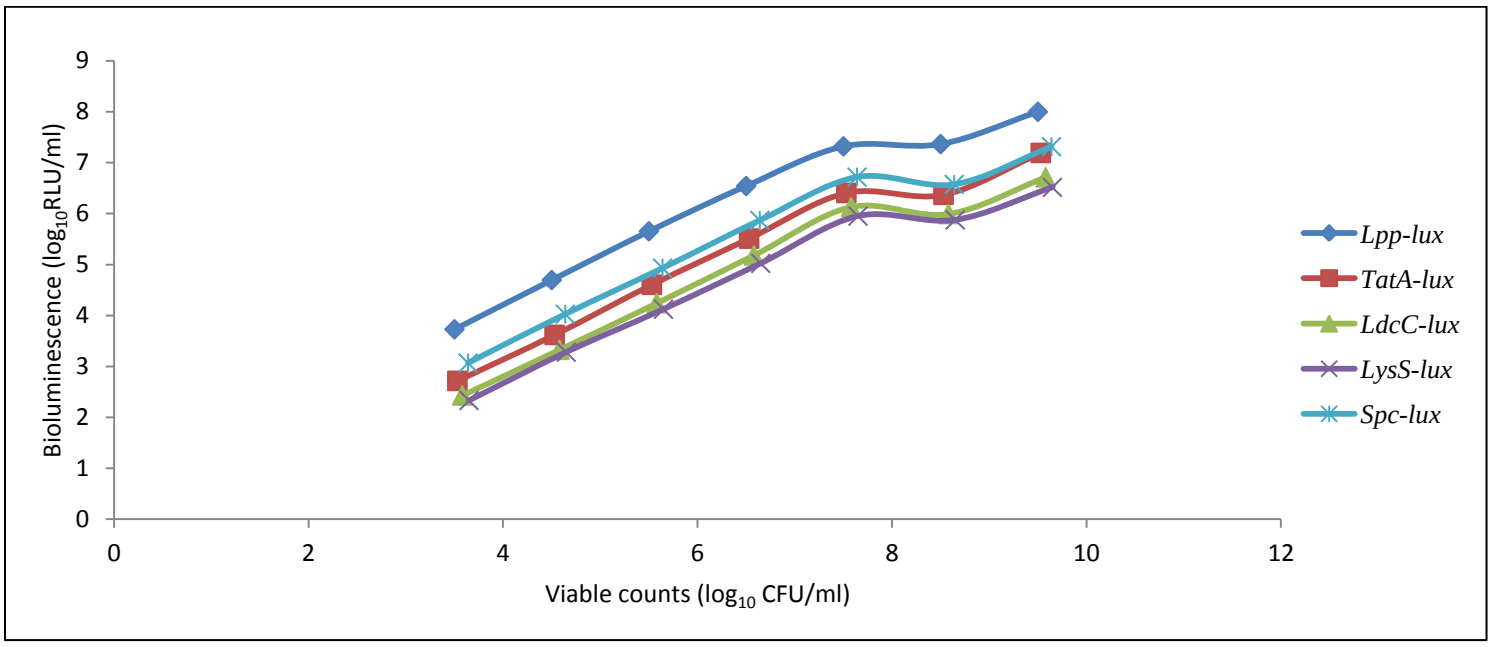


Figure 2.4 (a): The relationship between bioluminescence and viable counts for each of the biosensor strains. The equations for each of the lines are as follows: *lpp* y=0.8857x+1.042; *tatA* y=0.794x+1.064; *ldc* y=0.9271x+0.9455; *lysS* y=0.9254x+1.0143; *spc* y=0.8987x+0.8448. The correlation coefficient for each was *lpp* R^2 = 0.976; *tatA* R^2 = 0.988, *ldc* R^2 = 0.968, *lysS* R^2 = 0.963and *spc* R^2 = 0.971.

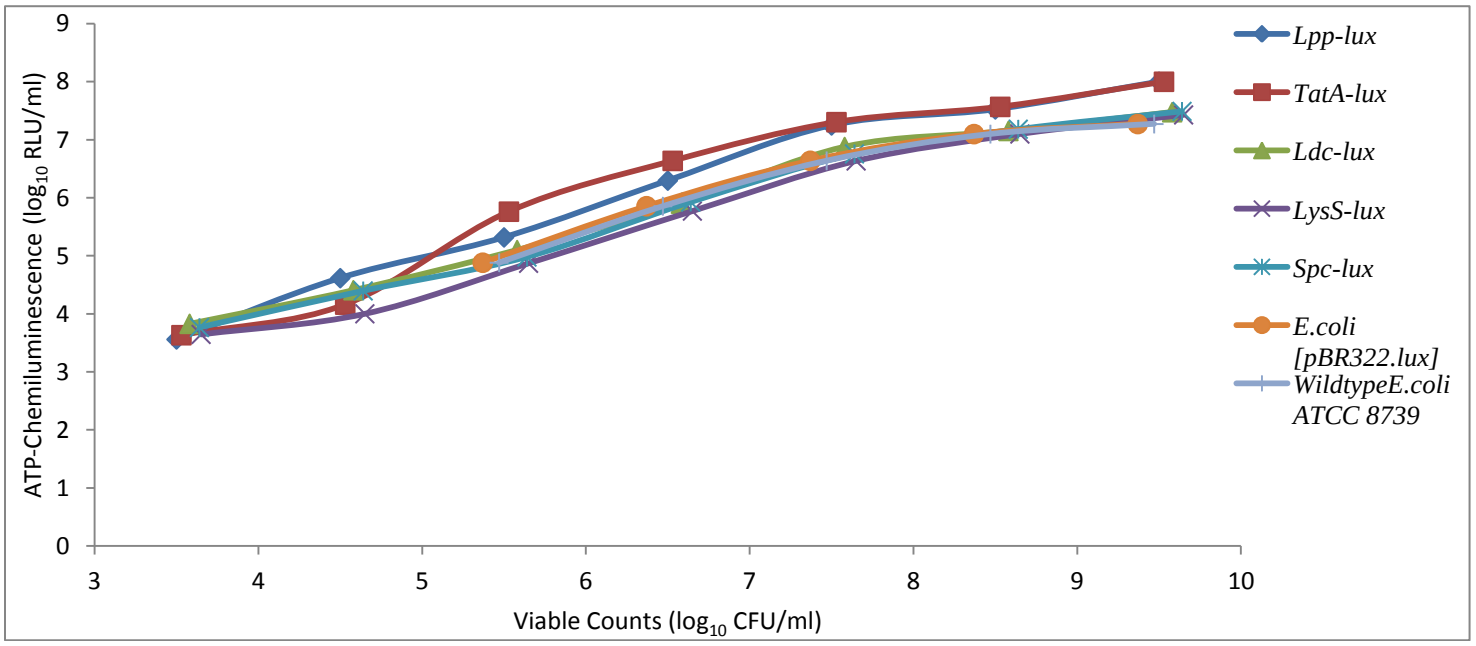


Figure 2.4 (b): The relationship between ATP-chemiluminescence and viable counts for each of the biosensor strains. The correlation coefficient for each was *lpp* R^2 = 0.965; *tatA* R^2 = 0.954; *ldc* R^2 = 0.917; *lysS* R^2 = 0.939; *spc* R^2 = 0.881; *E. coli* [pBR-322.*lux*] R^2 = 0.848; and *E. coli* ATCC 8739 R^2 = 0.848.

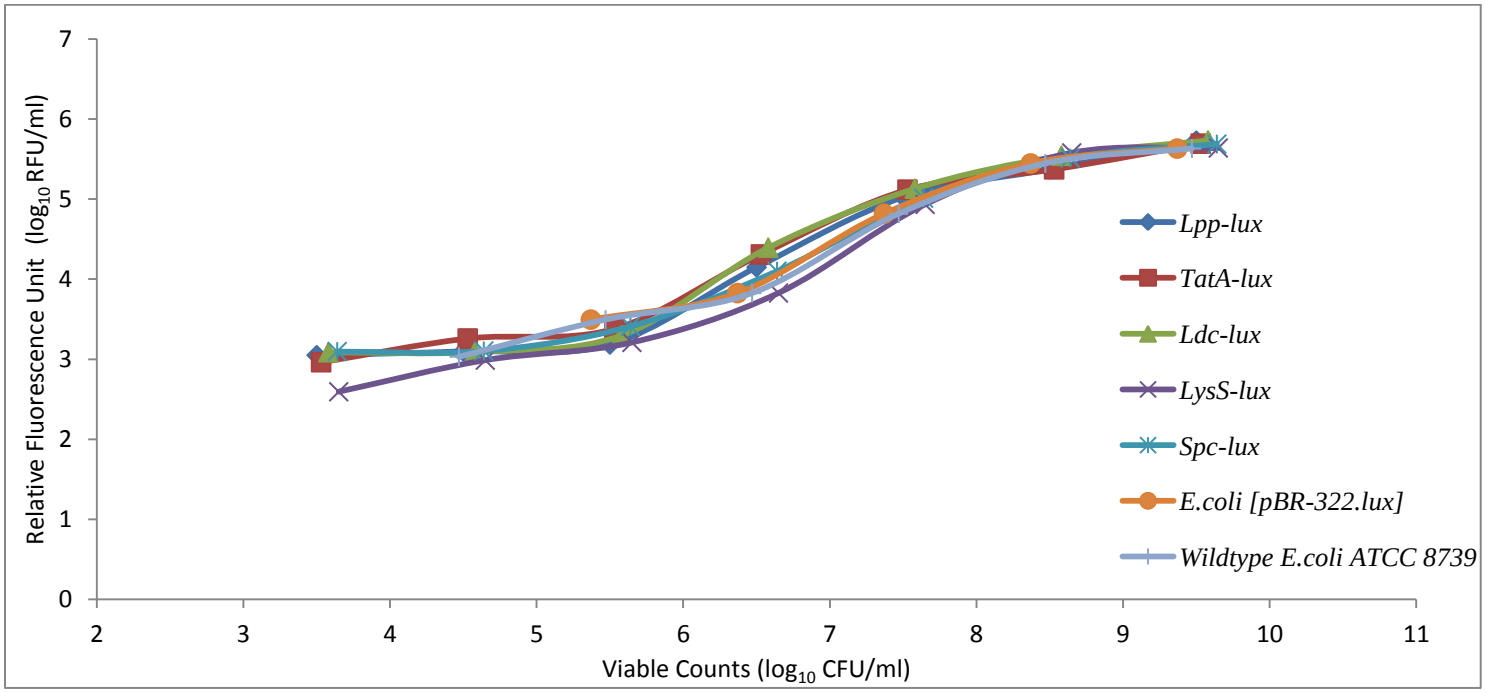


Figure 2.4 (c): The relationship between fluorescence values and viable counts. The correlation coefficient for each was *lpp* r^2 = 0.936; *tatA* R^2 = 0.914; *ldc* R^2 = 0.856; *lysS* R^2 = 0.766; *spc* R^2 = 0.851; *E. coli* [pBR-322.*lux*] R^2 = 0.864; and,*E. coli*ATCC 8739 R^2 = 0.864.

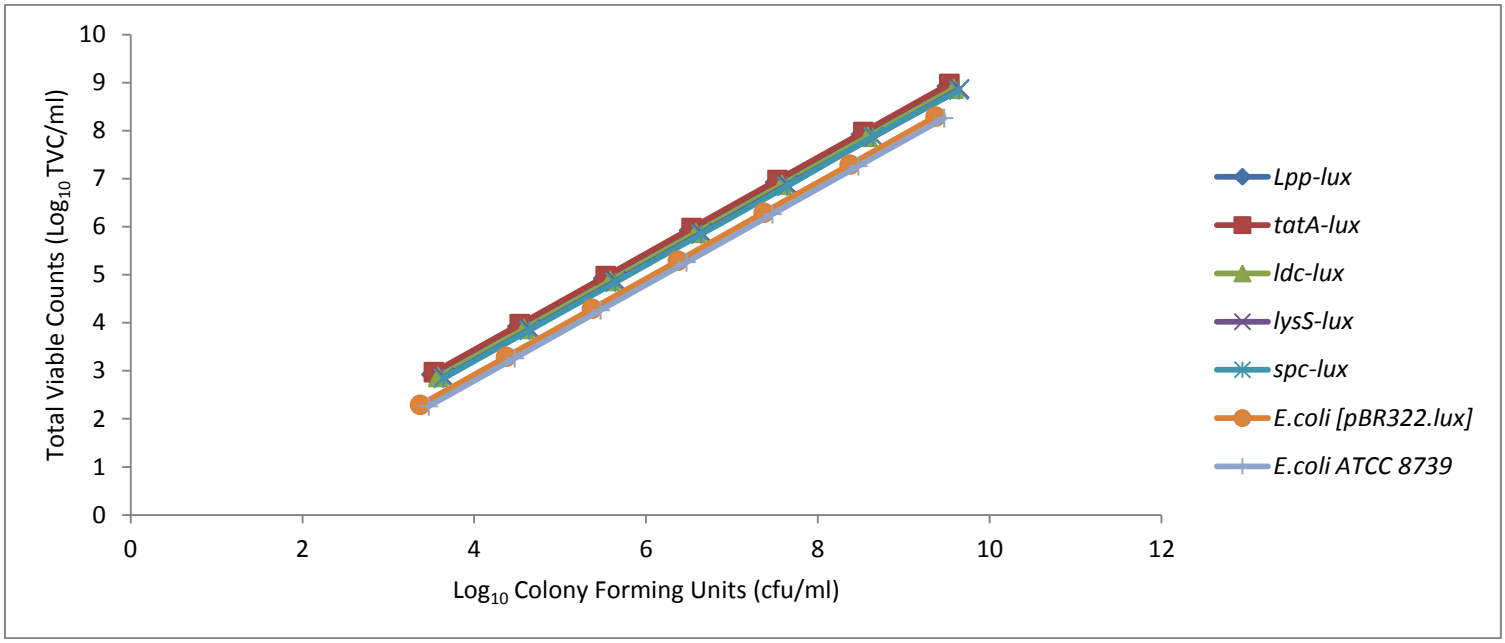


Figure 2.4 (d): Relationship between total viable count (TVC) from microscopic counts using LIVE/DEAD stains and viable counts (plate counting method) of all constructs and strains. The correlation coefficients (r^2 *Lpp*=1.00, *TatA*=1.00, *Ldc*=1.00 *LysS*= 1.00 and *Spc*= 1.00, *E. coli* [pBR-322.*lux*]=1.00, *E. coli* ATCC 8739=1.00).

2.5.3 : Trends and relation to conventional plating method

Figures 2.4 (a, b, c, and d) show the relationships of CFU against four microbial quantification methods. (*i.e*. Whole-cell Bioluminescence, ATP-chemiluminescence, Fluorescence spectrometry, and epifluorescence microscopy). Bioluminescence readings was lower at high populations (8.5 ± 0.05 $\log_{10}$ CFU/ml), compared to bioluminescence readings at 7.5 ± 0.05 $\log_{10}$ CFU/ml. Never the less, strong correlations were obtained for bioluminescence readings of five bioluminescent reporter strains constructs (R^2= 0.963-0.988) against the compendia plate counting method. The ATP-bioluminescence method displayed strong correlations in relation to conventional plate count method (|R^2= 0.881-0.965). At low concentration of CFU/ml, the ATP bioluminescence levels were concentrated at about 10^4 RLU/ml, for the bioluminescent reporter strains constructs and control strains. The relation of fluorescence spectrometry method and conventional plate count method follows a sigmoid curve where the reading plateaus at mid CFU concentration (10^6 CFU/ml) with significant correlation readings of R^2= 0.776-0.936 ($P \leq 0.05$, Pearson Correlation). The epifluorescence method resulted in correlation coefficient of R^2= 1.00 throughout all constructs and control strains.

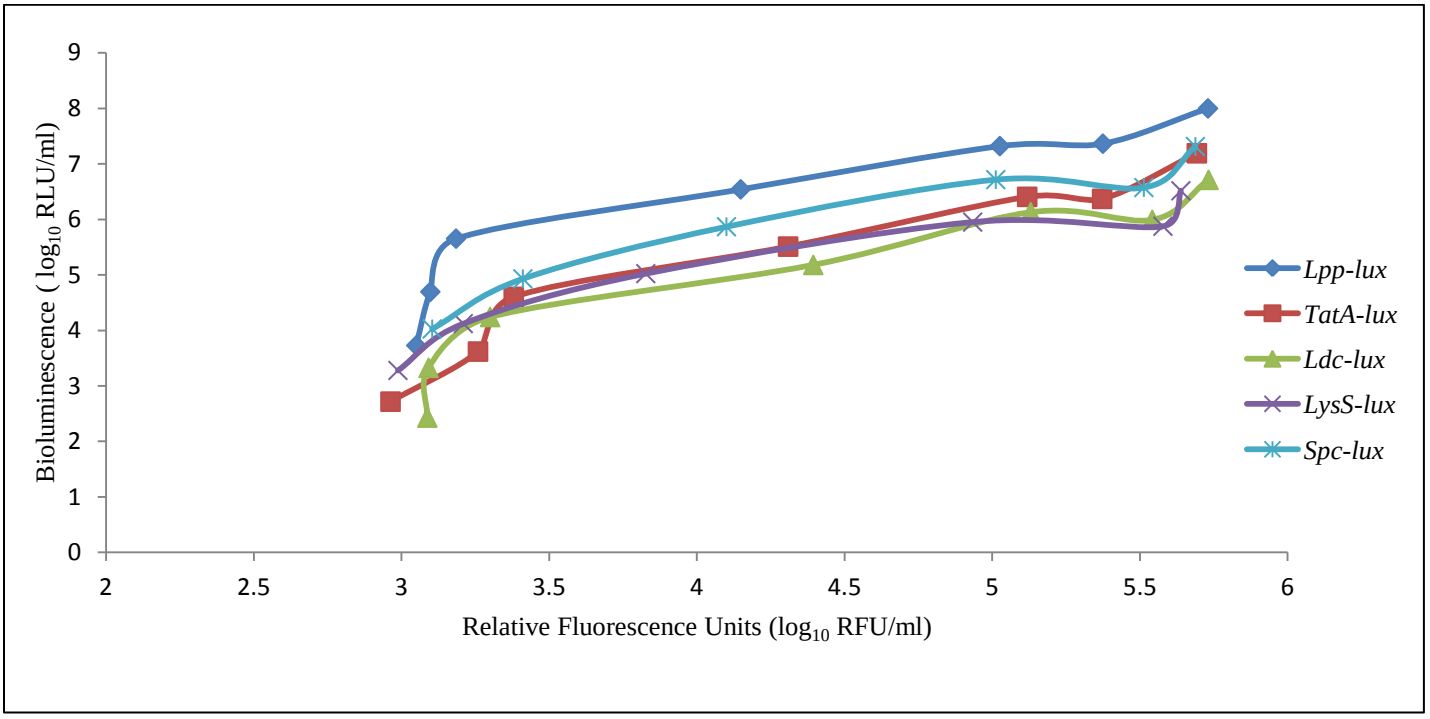


Figure 2.5 (a): The relationshiop between bioluminescence and fluorescence values for each of the biosensor strains. The correlation coefficient for each was (*lpp* R^2 = 0.978; *tatA* R^2 = 0.948; *ldc* R^2 = 0.900; *lysS* R^2 = 0.827; and *spc* R^2 = 0.971.

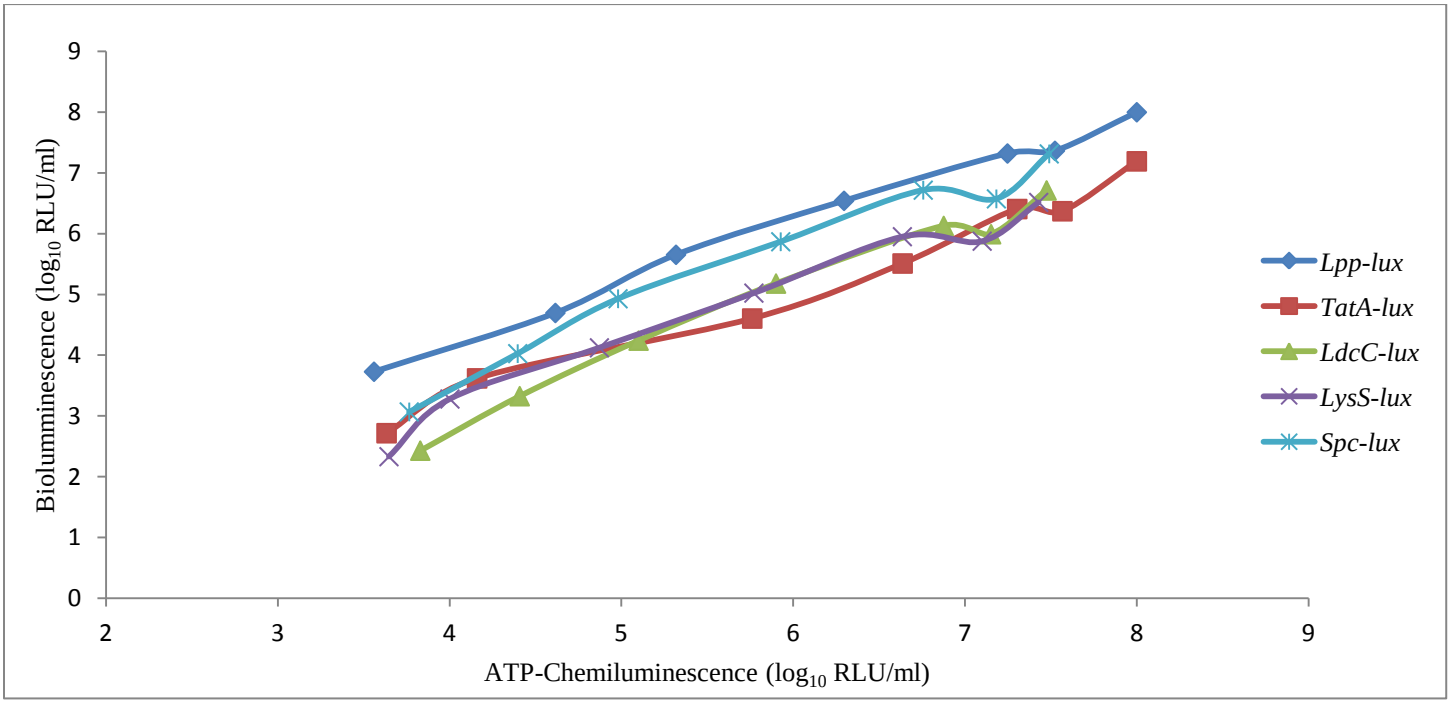


Figure 2.5 (b): The relationship between bioluminescence and ATP-chemiluminescence for each of the biosensor strains. The correlation coefficient for each was *lpp* R^2 = 0.993; *tatA* R^2 = 0.976; *ldc* R^2 = 0.957; *lysS* R^2 = 0.955; and *spc* R^2 = 0.907.

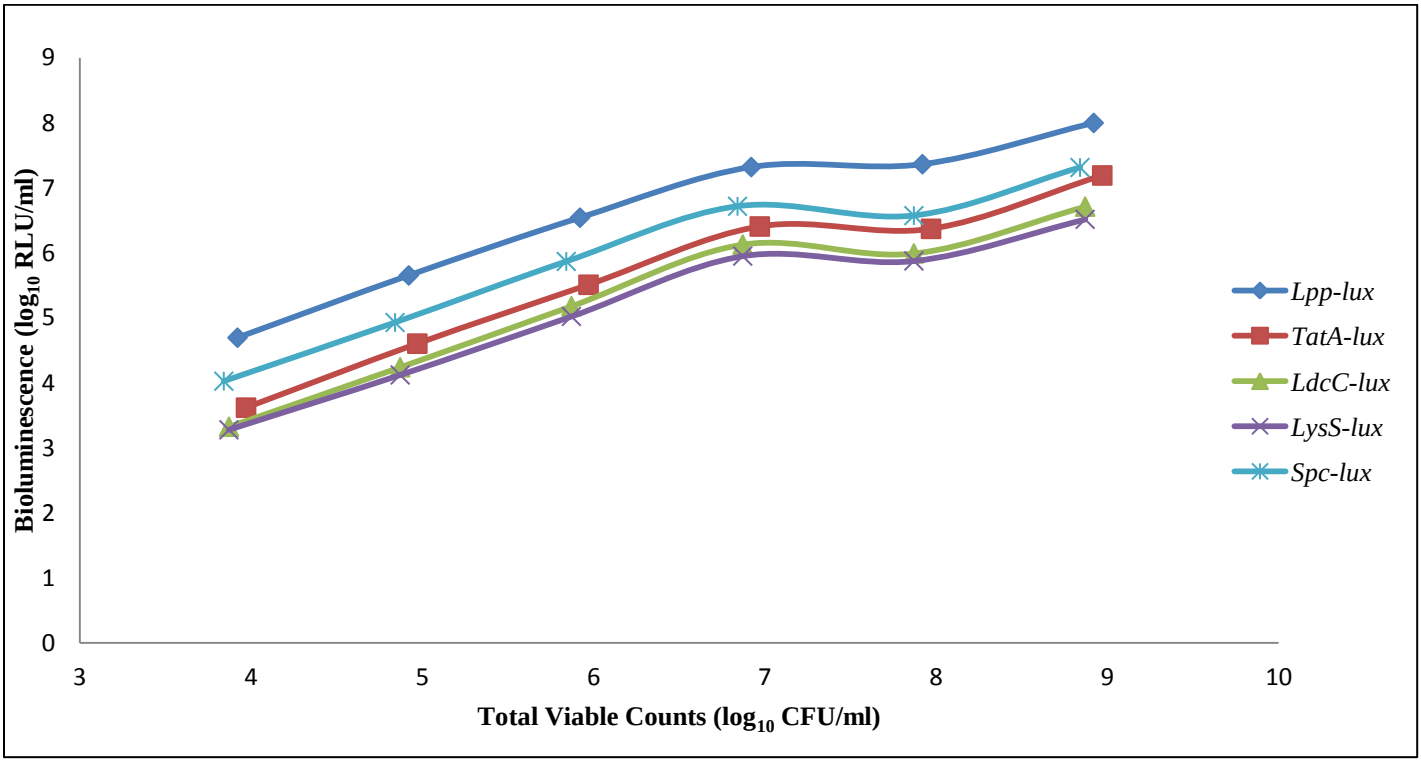


Figure 2.5 (c): The relationship between bioluminescence and viable counts from microscopic counts using LIVE/DEAD stains for each of the biosensor strains. The correlation coefficient for each was (*lpp* R^2 =0.976; *tatA* R^2 =0.988; *ldc* R^2 =0.968; *lysS* R^2 = 0.963; and *spc* R^2 = 0.971).

2.5.4 : Trends and relation to Bioluminescence Method

There was an increasing trend of ATP-bioluminescence, fluorescence units, and total viable counts observed in all the methods, with increasing bioluminescence (RLU/ml) (Figures 2.5 (a, b, c, and d)). Bioluminescence readings increased proportionally with relative fluorescence measured. At low concentration of RLU/ml, the fluorescence readings were concentrated at approximately 10^3 RLU/ml, similarly in Figure 2.4 (c). There was a proportional increase in ATP-bioluminescence and bioluminescence for all five bioluminescent reporter strains constructs *E. coli*. Strong significant correlations of bioluminescence were obtained between these methods ATP bioluminescence (R^2= 0.907-0.993) (Pearson Correlation), fluorescence spectrometry (R^2= 0.827-0.978) (Pearson Correlation)., epifluroscence microscopy (R^2= 0.963-0.988) (Pearson Correlation). The range of

correlations values obtained between the fluorescence method and the whole-cell bioluminescence method was higher in comparison to the ATP-bioluminescence and epifluorescence methods.

2.5.5 : Minimum Limit Of Detection And Working Range Of Methods Employed

The minimum limit of detection for each method employed was determined from whole-cell bioluminescence relations from Figures 2.5 (a, b, c, and d), were converted to CFU/ml using y= mx + c equations in Figure 2.4 (a) for ATP-bioluminescence, fluorescence spectrometry, and epifluorescence microscopy methods.

Table 2.4: The minimum detection limit of (MDL) and range of the methods employed

Bioluminescent reporter strains *E. coli* Strain	**Bioluminescence Method**		**ATP-Bioluminescence Method**		**Fluorescence spectrometry Method**		**EpiFluorescence Microscopy**	
	MDL[#]	Range	MDL	Range	MDL	Range	MDL	Range
lpp-lux	3.16×10^3	6 orders of magnitude	5.22×10^2	8-7 orders of magnitude	3.70×10^4	5-6 orders of magnitude	4.44×10^3	6-7orders of magnitude
tatA-lux	3.80×10^3		1.25×10^2		3.76×10^3		4.42×10^2	
ldc-lux	3.80×10^3		5.10×10^1		3.19×10^3		3.39×10^2	
lysS-lux	4.46×10^3		4.40×10^1		1.41×10^3		5.08×10^2	
spc-lux	3.36×10^3		3.88×10^2		5.75×10^4		4.95×10^3	

The MDL was determined from Figures 2.5 (a, b, c, & d) and converted to CFU/ml using regression equations in Figure 2.4 (a) to permit expression in terms of the viable count (CFU/ml)

The minimum detection limit (MDL) for the whole-cell bioluminescence method ranged from 3.16 - 4.46 x 10^3 CFU/ml, depending upon the biosensor strain, and each displayed a 6 orders of magnitude working range. The MDL for each biosensor strain was the lowest when the

ATP-chemiluminescence method was employed, ranging from 4.40 x 10^1 – 5.22 x 10^2 CFU/ml, and displayed a 7 – 8 orders of magnitude working range. The MDL for each biosensor strain when the Epifluorescence method was employed ranged from 3.39 x 10^2 - 4.95 x 10^3 CFU/ml, and displayed a 6-7 orders of magnitude working range. The highest MDL measured in this study was observed when fluorescence spectrometry was employed, ranging from 1.41 x 10^3 - 5.75 x 10^4 CFU/ml and displaying a 5-6 orders of magnitude working range.

2.5.6 : Comparison of Time Required to Undertake Each Method

The time required to prepare the samples for the ATP-chemiluminescence, fluorescence spectrometry, epifluorescence microscopy, and whole-cell bioluminescence method was 15 minutes, whilst the plate method required 30 minutes of preparation time. The plate count method was the only one that required an incubation time of 72 hours, whilst the other four microbial enumeration methods did not require any overnight incubation time. Amongst the analytical methods employed, the whole-cell bioluminescence and fluorescence spectrometry methods required the least time for sample analysis, at only 15 minutes, whilst the epifluorescence microscopy method required the longest at an hour (Figure 2.6). On the other hand, the ATP-chemiluminescence method required 30 minutes for sample analysis and the plate count method required 35 minutes per sample.

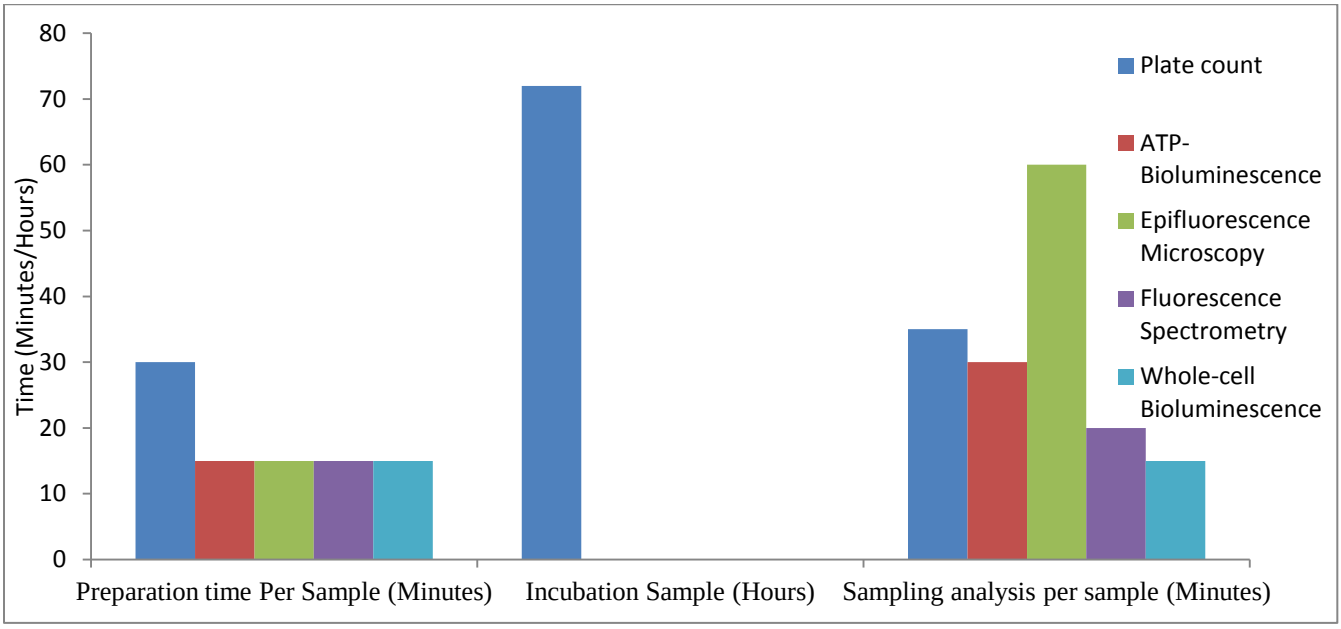


Figure 2.6: The preparation time (minutes), incubation time (hours), and sample analysis time (minutes) per sample for each of the analytical methods employed.
SEM for preparation time did not exceed 3 min for each method
SEM for sampling analysis time did not exceed 1 min for each method

2.5.7 : Analysis of Promoter Strength of the Five Constructs

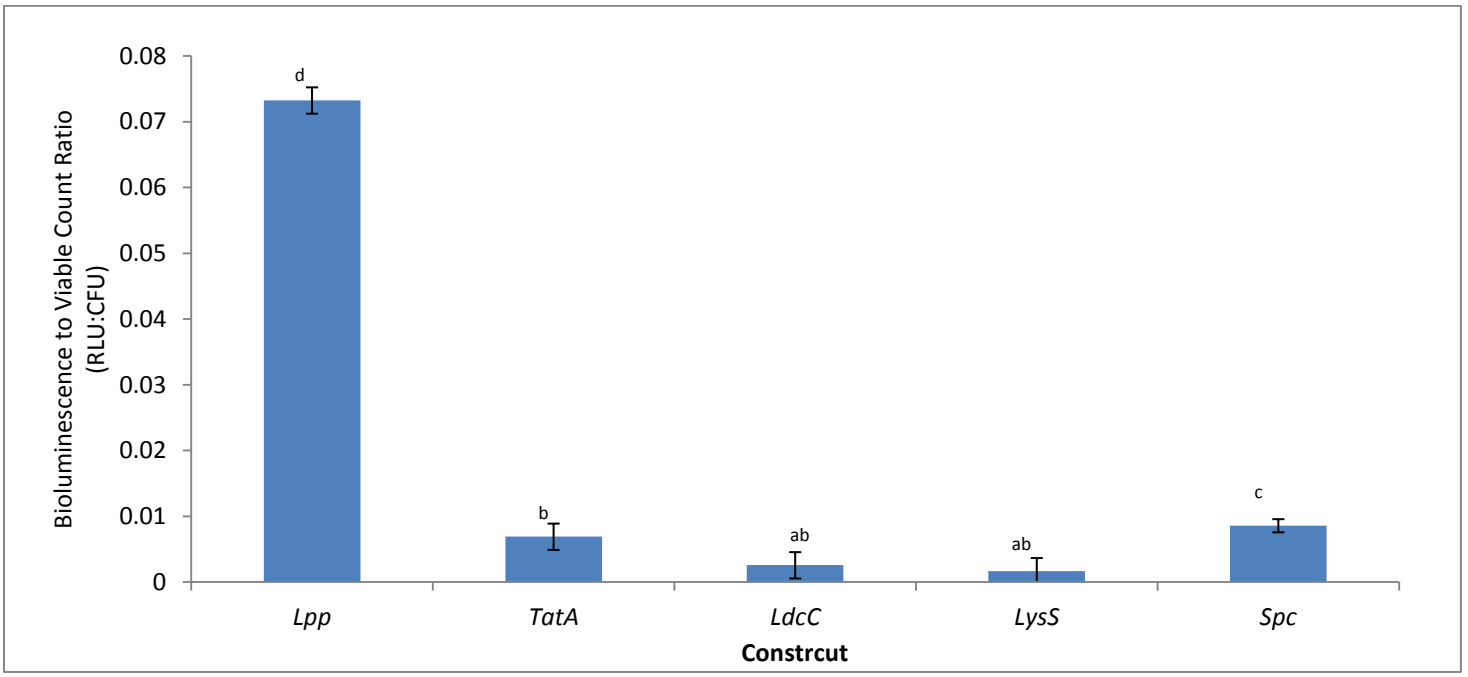


Figure 2.7: Constitutive promoter strength measured as the ratio (RLU:CFU) for each biosensor construct, determined at a high bacterial cell density (10^8 CFU/ml)
[a,b,c,d] Letters within a column indicate significant differences at $P \leq 0.05$, n=5, with error bars representing SEM

At 24 hours of incubation the bioluminescence expression per cell (RLU:CFU) for the *lpp-lux* (0.0732) was statistically significantly greater than for each of the other constructs ($P \leq 0.001$, Tukey *Post Hoc*) followed by *spc-lux* (0.0086) construct expressed significantly ($P \leq 0.001$, Tukey *Post Hoc*) greater bioluminescence per cell than the *tatA-lux* (0.0069) construct ($P \leq 0.01$, Tukey *Post Hoc*). *ldc-lux* (0.0025) and *lysS-lux* (0.00169) were statistically significantly lower ($P \leq 0.001$, Tukey *Post Hoc*) to *lpp-lux*, *tatA-lux*, and *spc-lux* at 24 hours and both expressed the lowest bioluminescence per cell.

Table 2.5: A comparison of sequences within the promoter regions of the promoters used in the bioluminescent reporter strains with the *E. coli* RpoD ($\sigma^{70/D}$) consensus sequence

Promoter	**Extended $^{-15}$TGn^{-13} element**	**-10 region sequence**	**Number of spacers (nt) between the -35 and -10 regions**	**-35 region sequence**	**A+T (%) in UP element**	**G+C (%)between -10 and +1 regions**
Outer Membrane Lipoprotein (*lpp*)	TGT	AATACT	15	TTCTCA	65	34
Twin Arginine Translocase (*tatA*)	ACG	TATAAT	19	TTCATC	40	38
Lysine Decarboxylase (*ldc*)	GGC	TATGAT	17	TTTTTA	50	44
Lysyl-tRNASynthetase (*lysS*)	GAA	GAAAAT	13	TTTATG	50	52
Ribosomal Protein (*spc*)	TGT	TATAAT	18	TTTCTA	45	50
***E. coli* σ^{70} consensus sequence**	**TGn**	**TATAAT**	**17**	**TTGACA**		

*** (Pribnow, 1975)**

Positions -15,-14,-13, (extended element). Positions -12,-11,-10,-9,-8,-7 reads from left to right for -10 regions. Positions -35,-34,-33,-32,-31,-30 (left to right)

Indicates differences from the σ^{70} *E. coli* consensus sequence

Indicates differences from the consensus in spacer length (nt), between the -35 regions and -10 regions

The *lpp* promoter strength was greatest, followed by *spc*, *tatA*, *ldc* and *lysS*, in terms of the bioluminescence per cell (RLU:CFU) (Figure 2.7). The bioluminescence expressed by *lpp-lux* was 43-fold greater than that expressed by any of the other constructs (*lysS-lux*, *ldc-lux*, *tatA-lux*, and *spc-lux*). The sequences of the five constitutive promoters employed were compared to the *E. coli* global consensus sequence (Table 2.5).

The regions analysed were the extended -10 elements, the -10 region, the number of spacers between the -35 and -10, the -35 region, and the UP elements (-40 to -60 regions). *Lpp* and *Spc* display consensus sequences at positions -15 and -14 which is T and G.

The RpoD ($\sigma^{70/D}$) -10 region consensus sequence is TATAAT. Both *spc* and *tatA* exhibited the same sequence as the consensus sequence in the -10 region, whilst *lpp* and *lysS* have the T at position -12 of the consensus sequence with a nucleotide replacement of an A and a G, respectively. In contrast, position -10 of *lysS* has an A instead of a T, whilst *ldc* has a nucleotide replacement of a G instead of the consensus T at the -9 position. *Lpp* has a nucleotide replacement of a C instead of the consensus T at the -8 position.

The spacer length between the -35 and -10 regions is smallest for *lysS* compared to the other four promoters. The *ldc* promoter has the same number of spacers between the -35 and -10 regions as the consensus sequence.

At position -33, in the -35 region, there is a C substituted for a G in the *lpp* and *tatA* promoters whilst for *ldc*, *lysS*, and *spc* a T is replaced. The consensus of the -35 region is TTGACA. At position -30, there are nucleotide replacement of a C in *tatA* and G in *lysS* instead to the consensus A whilst at position -31, constructs *tatA-lux, ldc-lux, lysS-lux* and *spc-lux* showed a T instead of the C consensus. At position -32, there is a substitution of a T for into the *lpp* and *ldc* promoter sequences, whilst a C is substituted for *spc*. At position -33, all constructs showed a nucleotide difference to the consensus G whilst nucleotide sequence at positions -34 and -35 agreed with consensus for all 5 constructs.

The percentages of A+T in the UP elements and G+C in the regions between -10 and +1 were within the ranges of 45-65% and 34-52% respectively. The rank order for A+T percentage of the UP elements was as follows *lpp>ldc=lysS>spc>tatA*, whilst the rank order for G+C percentage between the -10 and +1 regions was as follows *lysS>spc>ldc>tatA>lpp*.

2.6 : Discussion

2.6.1 : Validation of the Five Bioluminescent Constructs as a Rapid Microbiological Method In Comparison With Traditional Plate Counting

The five bioluminescent reporter *E. coli* strains demonstrated an accuracy level which is expressed as the percentage recovery of bioluminescence exhibited by the constructs in the whole-cell bioluminescence assay. The accuracy of the whole-cell bioluminescence method is the closeness of the actual test results obtained by the current compendial method which is the conventional plate counts (PDA, 2000)

The average bioluminescence recovery was between 78.71 and 135.19 %, whilst the average recovery for the conventional plate count methods were between 82.10 and 139.17% with no significant difference (Table 2.2) for both high and low concentrations of cells. This demonstrates that the bioluminescence method has similar accuracy to the plate counting method. In addition, recovery values obtained from the bioluminescence method were higher as compared to recovery values obtained from ATP-chemiluminescence method as shown in Kramer et al (2008) especially at low concentration. This suggests that the whole-cell bioluminescence method has higher accuracy as compared to ATP-chemiluminescence method.

Numerous studies of whole-cell bioluminescent measurement have demonstrated prospects of replacing the conventional plate count method (Ellison et al., 1994a & b; Hill et al., 1993; Marincs, 2000; Stewart, 1990, 1993, 1997; Stewart & Williams, 1992,; Stewart et al., 1991, 1993, 1996, 1997). However, to the author`s best knowledge, previous studies have not demonstrated the evaluation of accuracy of the whole-cell bioluminescent method as a rapid microbiological testing method (PDA, 2000).

The F values suggest that there was a greater variance between the whole-cell bioluminescence method and the conventional plate counting method for *lysS-lux*, with F=2.656, than for *ldc-lux* (F= 1.849), *spc-lux* (F= 1.884), *tatA-lux* (F= 1.715), and *lpp-lux* (F=1.717), as shown in Table 2.2. However, the calculated ratios did not exceed the critical F value obtained in the F distribution, which is 2.93. Therefore, according to the technical report No 33 of evaluation, validation, and implementation of new microbiological testing methods, there is no significance difference ($P \leq 0.05$, ANOVA) between the precision of the bioluminescence method and the conventional plate counting method.

The whole-cell bioluminescence recorded when the viable counts were 8.5 $\log_{10}$ CFU/ml was not 10 fold higher than the whole-cell bioluminescence recorded when the viable counts were 7.5 $\log_{10}$ CFU/ml. This could be explained by scattering of the light by the high cell density of the bacterial culture resulting in less light per cell being detected by the luminometer. Konstantinov et al. (1993) demonstrated that bioluminescent cultures not only emit light but also absorb and scatter and the light extinction effect, known as the "inner filter effect," is significant in high-density cultures. This phenomenon is observed in the whole-cell bioluminescence method at 8.5 $\log_{10}$ CFU/ml which is well above the working concentration required by the Pharmacopeial regulations. Nevertheless, at cell densities below 8.5 $\log_{10}$ CFU/ml the bioluminescence method showed great linearity supported with excellent correlation with the conventional plate count method (Figure 2.4 (a)).

The correlation coefficients for the whole-cell bioluminescence from the five bioluminescent reporter strains compared with the conventional plate counts were significantly higher compared to the correlation coefficients for the ATP-chemiluminescence versus plate counts values of (R^2 = 0.968-0.988 and R^2 = 0.881-0.965, respectively; Figures 2.4 (a) and (b), respectively). The underlining relationship between the bioluminescence and viability involves the oxidation of reduced

$FMNH_2$ and an aldehyde molecule by molecular oxygen (Meighen, 1991). The production of $FMHN_2$ is dependent on the electron transport thus only live cells are able to produce bioluminescence (Stewart, 1997). The relationship between whole-cell bioluminescence readings versus plate counts is significantly higher compared to the correlation coefficients of fluorescence spectrometry readings versus plate counts for the biosensor strains (R^2 = 0.968-0.988 and R^2= 0.776-0.936, respectively; Figures 2.4 (a) and (c), respectively. This demonstrates that the whole-cell bioluminescence method showed greater correlation with the plate counts than ATP-chemiluminescence or fluorescence spectrometry. However, the epifluorescence microscopy method demonstrated the greatest correlation with the conventional plate count method (R^2 = 1.00; Figure 2.4 (d).

Roszak and Colwell (1987) demonstrated that plate counts would the viable count of a bacterial population, whilst direct viable counts determined by microscopy would overestimate the viable count. However, in the study described in this thesis, direct microscopic viable counts yielded very similar viable counts to the plate count method, with correlation coefficients of R^2= 1.00 for each of the constructs. This could be due to the enumeration of *E. coli* cells at early stationary phase, when all the cells could have been viable and culturable in comparison to the natural water sample in Roszak and Colwell (1987). Therefore the comparison of the direct microscopic counts between the whole-cell bioluminescent method lead to high correlation of R^2= 0.963-0.976 (Figure 2.5 (c).

The correlations between ATP-chemiluminescence and whole-cell bioluminescence for the five constructs were significantly excellent at R^2= 0.907-0.993 (Figure 2.5 (b)). The ATP-chemiluminescence method has been validated by Kramer et al. (2008) and has been recognized by the Pharmacopoiea as a rapid microbiological testing method for the preservative efficacy test (PET). The ATP-chemiluminescence method has been widely applied in the food industry for bacterial

enumeration (Griffiths, 1993; Siragusa et al., 1996; Sharma & Malik, 2012). There are many easy-to-use kits, with pre dispensed reagents, which can be used with relatively low cost hand-held luminometers for rapid determination of ATP levels and so to estimate the microbial load (Sharma & Malik, 2012). The ATP-chemiluminescence method is no doubt very rapid however there is the potential of extracellular ATP affecting the readings, creating false positive (Dostalek & Branyik, 2005). Furthermore, the ATP-chemiluminescence method can potentially suffer 'quenching' due to the presence of cations and anions which can cause significant decreases or even increases in the light emission (Wen et al., 2001; Dostalek & Branyik, 2005).

The use of SYTO 9 and propidium iodide (PI) stains has grown tremendously. SYTO 9 stains all bacteria in a population, even those with damaged membranes (Armstrong & He, 2001). In contrast, PI only penetrates bacteria with damaged membranes. It quenches the green fluorescence of SYTO 9 and replaces it with its own red fluorescence (Armstrong & He, 2001). Microscopically, the green and red cells can be distinguished easily (Armstrong & He, 2001) whilst the fluorescence intensity can be measured using a fluorescence spectrometer. The correlation coefficients between the whole-cell bioluminescence and the fluorescence spectrometry for the five biosensor strains were poorer than for any of the other assay methods, since they gave the largest range (R^2= 0.827-0.978; Figure 2.5 (a) with no significant difference). This could have resulted from inconsistent diffusion of the dye across the cell membranes. Previous studies have shown that the outer membrane in stationary phase cells of *E. coli* can act as a barrier to SYTO 9 (Berney et al., 2007). This phenomenon seems to be specific to gram negative cells and so may be related to the presence of an outer membrane (Berney et al., 2007). In addition, SYTO 9 binds non-specifically to any DNA-containing contaminants for example lysed cells (Klauth et al., 2004). Previous studies have found it difficult to distinguish microbial cells from other features of a similar shape and size, and this could have implications for the use of epifluorescence with pharmaceutical products (Nunan et al., 2001). The

SYTO 9 stain is widely used in epifluorescence microscopy for the enumeration of bacteria (Joux & Lebaron, 2000; Klauth et al., 2004). However this technique requires extensive training and a high technical skills proficiency, especially in the loading and handling of the haemocytometer as operator errors can introduce non-random distribution of cells across its surface and it is very fragile. Moreover, fixing the cells on haemocytometer can results in cell shrinkage and so can induce biased light-scattering measurements which can lead to a decrease in fluorescence emissions for SYTO dyes (Del Giorgio et al., 1997).

Despite the use of the same fluorochromes (SYTO 9 and propidium iodide (PI)) in both the epifluorescence microscopy and the fluorescence spectrometry, the minimum detection limit (MDL) for epifluorescence microscopy in the current study was 10^2-10^3 CFU/ml, which was a log unit lower than for the fluorescence spectrometry method at 10^3-10^4 CFU/ml. This could be due to limits to the sensitivity of the spectrometry equipment, whereas the haemocytometer has the potential to manage samples with low viable counts. The limit of detection for epifluorescence microscopy determined in the current study agrees with that determined by Pettifer (1983). On the other hand, epifluorescence microscopy studies conducted by Lemarchand et al. (2001) using microbial cells labelled with SYBR green II found the detection limit to be considerably higher than the current study at 10^4 cells/ml.

The MDL for the whole-cell bioluminescence method is 3.16-4.46 x 10^3 CFU/ml (Table 2.3) with working range of 5-6 orders of magnitude. The MDL obtained were in line with previous work carried out by Rattray et al., 1990. The genetically engineered *E.coli* DH1 [pUCD607.*luxCDABE*], *E.coli* MM294 [pBTK5.*luxRICDABE*], *E.coli* MM294 [pEJ205.*luxABE*], *E.coli* MM294 [pEMR1.*luxABE*], deduced a limit of detection between 5.0 x 10^1 – 1.211 x 10^3 CFU/ml in liquid culture. The variation that was obtained was due to the fact that the bioluminescence measurements carried out in Rattray et al. (1990) was during exponential phase and the different vector and portions

of the *lux* cassette ligated. In contrary to the bioluminescence measurements at stationary phase carried out in this study yet, the MDL falls within the range of 10^3 CFU/ml.

In contrast, the minimum detection limit (MDL) for the ATP-chemiluminescence method was the lowest of all those tested at 4.40 x 10^1 – 5.22 x 10^2 CFU/ml, and it also the greatest working range (7-8 orders of magnitude) (Table 2.4). This agrees with the minimum detection limit for ATP-chemiluminescence as determined by Kramer et al. (2008). However, the MDL obtained by the whole-cell bioluminescent method is equipped for the preservative efficacy testing (PET) which requires that at least a 3 $\log_{10}$ reduction in the viable counts from an initial viable count of 6 $\log_{10}$ CFU/ml occurs, then both of these methods would have a sufficient working range and minimum detection limit to be used in PET.

Amongst the five analytical methods assessed in the current study, the epifluorescence method required the longest time for sample analysis, at an hour (Figure 2.6). The time taken to view and count each sample is strenuous, and does not allow the rapid screening of multiple samples which could be classified as a disadvantage for a rapid method. The fluorescence spectrometry method required 20 minutes to complete the analysis of each sample, which was 5 minutes more than the whole-cell bioluminescence method. The extra time required for fluorescence spectrometry is due to the incubation process of the stains in the dark, before the data were obtained. The ATP-bioluminescence method required five minutes less the plate counting method. The plate counting method involved serial dilution, followed by plating on solid agar and the counting of colonies after an extensive incubation period. On the other hand, although the ATP-bioluminescence method also involved a serial dilution, along with the additional time required for the extraction of intracellular ATP and the time taken to measure the chemiluminescence from the luciferin-luciferase reaction it did not require an extensive incubation period. The use of a kit minimized the time needed for the

extraction process, but conventional extraction , when kits are not available or affordable, involve the use of boiling buffers or ice cold acid which can be very time consuming.

However, the ATP bioluminescence assay kit HS II and the BacLight LIVE/DEAD kits are both costly, whereas the whole-cell bioluminescence method has minimal costs (Appendix 2). Moreover, the ATP extraction process requires a sterile environment to prevent contamination with exogenous ATP-rich cells, which can be difficult to maintain. On the other hand, despite the minimal cost for agar and diluent buffers, the plate count method required a 72 hour incubation time plus 30 minutes for sample preparation which makes it much slower in yielding results and this can have an impact on the rate at which product can be cleared for market by a pharmaceutical manufacturer. Conventional plate counts require 15 minutes more preparation time than the whole-cell bioluminescence method because of the time taken to prepare the agar plates. Consequently, the whole-cell bioluminescence method needed only a short time for sample preparation and analysis, with no incubation time required, as well as requiring only low-cost materials (Appendix 2).

2.6.2 : Evaluation of the Promoter Strength, And Its Relationship with Bioluminescence Expression

Amongst the five constitutive promoters tested for their suitability as a whole-cell bioluminescent method, *lpp-lux* exhibited the highest bioluminescence output per colony forming unit whilst *lysS-lux* exhibited the lowest, after 24 hours of incubation (Figure 2.7). To permit comparison between the bioluminescent biosensor constructs, the light per CFU was converted to a percentage value, with the light output from *lpp-lux* normalised to 100% as it gave the highest light output. Consequently, *spc-lux* expressed 11.7% of the light output of *lpp-lux*, followed by *tatA-lux* with 9.45%, *ldc-lux* with 3.52%, and *lysS-lux* with 2.3% gave the lowest light output. Therefore the rank order for the promoter strength of each of the constructs under standard culture conditions was as follows; *lpp> spc>tatA>ldc>lysS*.

The outer membrane lipoprotein (*lpp*) promoter controls the expression of the enzymes which produce outer membrane lipoprotein, and thus is directly responsible for the levels of lipoprotein present. Since lipoprotein is the most abundant protein in *E. coli*, with approximately 7.5 x 10^5 molecules per cell (Inouye & Inouye, 1985), this could explain why the intensity of bioluminescence per CFU from the *lpp-lux* strain was significantly the greatest. The *spc* operon encodes the ribosomal protein which is regulated at the translational level (Merianos et al., 2004), demonstrating constitutive characteristic of 11.7% bioluminescence per cell whilst the *tatA* promoter controls the protein transport pathway in cytoplasmic membranes (Jack et al., 2001) and *ldc* promoter controls the decarboxylation of lysine, a basic amino acid (Lemonnier & Lane, 1998) with 9.45% and 3.52% bioluminescence per cell .The *lysS* promoter controls the expression of aminoacyl- tRNA synthetase in protein synthesis expressing bioluminescence per cell at 2.3%. The five promoters chosen imply the constitutive levels at varying levels as a whole-cell bioluminescent biosensor. Since after 24 hours of incubation, the culture had

reached early stationary phase it may be that a large amount of outer membrane lipoprotein is required at this stage and so greater levels of expression occur to provide this. In comparison it may be that the expression of ribosomal protein, protein transportation and synthesis, and decarboxylation of basic amino acid does not need to be so strong at this stage of growth. Nakamura and Inouye (1982) demonstrated a 30 times stronger expression in *lpp* promoter than *lac* promoter when inserted upstream of β- galactosidase gene.

The strength of promoter activation is dependent upon the binding affinity of the RNA polymerase to the promoter regions (Ishihama, 1988; Jensen & Hammer, 1997) it was decided to perform a bioinformatics analysis of the promoter regions to understand promoter strength (Table 2.4).

Gene expression in bacteria is due to the action of RNA polymerase that catalyses DNA template-dependent RNA synthesis. In order to recognize promoters, RNA polymerase must associate with a sigma (σ) factor, which is directly responsible for binding of the RNA polymerase complex to the promoter sequence elements (Lee et al., 2012). Formation of a 'close complex' occurs when the sigma (σ) factor is in contact with subregions of 2.4 and 4.2 to interact with the -10 and -35 promoter DNA elements. The sigma (σ) factor orchestrates the transcription process. The sigma subunit of bacterial RNA polymerase is strictly required for promoter recognition. The primary (housekeeping) sigma factor of *Escherichia coli*, sigma RpoD ($\sigma^{70/D}$), is responsible for most gene expression (Gregory et al., 2005).

One of the regions recognized by RpoD ($\sigma^{70/D}$), is the $^{-15}$TGn^{-13} motif, also known as the extended -10 sequence (Keilty & Rosenberg, 1987). Optimal expression activity requires the presence of both the -15 T and -14 G (Keilty & Rosenberg, 1987). Extended regions of *spc* and *lpp* promoters have both of the

nucleotides suggesting the potential for strong promoter activity with RpoD ($\sigma^{70/D}$). The amino terminal part of region 3 of RpoD ($\sigma^{70/D}$) makes contact with the conserved $^{-15}$TGn^{-13} in the extended -10 element is in contact with the amino terminal part of region 3 of RpoD ($\sigma^{70/D}$) and so mediates recognition of the -10 region and melting of the DNA (Paget & Helmann, 2003). This potential for strong promoter activity is reflected in the greater emission of light at early stationary phase by the *spc-lux* and *lpp-lux* constructs in comparison with the others. Barne et al. (1997) and Campbell et al., (2002) reported that promoters which conformed to the consensus sequence of $^{-15}$TGn^{-13} may not require a -35 region or have -35 regions that deviate significantly from the consensus sequence.

Mutagenesis studies carried out by Inouye and Inouye (1985) demonstrated an increase in expression activity when the -10 region of a promoter was the same as the consensus sequence of TATAAT. Both *tatA* and *spc* have a -10 sequence that conforms to the consensus sequence (Table 2.4). However the highest expression levels of light were seen with the *lpp-lux* construct. The -10 region interacts intimately with subregions 2.3 and 2.4 of sigma factor which corresponds to the α-helix in region 2 of RpoD ($\sigma^{70/D}$) (Paget & Helmann, 2003). The -12 position of the *lysS* promoter is the only encoding a G. Several studies have shown that suppression in subregion 2.4 of the σ^{70} can occur when there is a G at the -12 position, this indicates possible suppression of the promoter activity in this region (Waldburger et al., 1990; Siegele et al., 1989). In addition, Dombroski, (1997) demonstrated that within the -10 region, only mutations at positions -12, -11, and -10 were deleterious for binding by σ^{70}. Since *lysS* had the highest number of differences in the -12, -11, and -10 positions (Table 2.5), this could explain the low levels of light expression per cell from this construct compared to the other four (Figure 2.7). Weakened recognition of the promoter by the RNA polymerase and σ subunit, due to a reduction in their

ability to unwind the DNA strand and generate an open complex for initiation of transcription could offer the explanation for this observation (Browning & Busby, 2004).

At positions -11 and -7 of the -10 region, there were no nucleotide differences between the constitutive promoter sequences and the consensus sequence. The -11A and -7T positions interact with RNA polymerase (Liu et al., 2011). The -11A and -7T are flipped out of the base stack, with their bases buried in pockets on the protein surface (Liu et al., 2011). This recognition of the bases at -11 and -7 are specific and conserved (Liu et al., 2011). The five constructs did not show any difference to the consensus at positions -11 and -7 therefore there is relative strength in bioluminescence expression for all constructs, despite the various strengths.

The -35 element of the promoter forms a helix-turn-helix motif when in contact with the carboxy-terminal part of region 4 (subregion 4.2) of RpoD ($\sigma^{70/D}$). The consensus sequence for the -35 region is TTGACA, and both *lpp* and *tatA* differ from the consensus by encoding a C instead of a G at position -33. Interestingly, other studies have shown that transcription occurs more frequently when the non-consensus A or C bases are found at the -33 position than the more highly conserved G (Keener & Nomura, 1993). This could be one of the factors influencing the high expressions of *lpp*.

The conserved regions between -40 and -60 are known as the UP region. Studies have revealed that an A+T-rich sequence of the UP element was enough to significantly enhance transcription from some promoters (Chan & Busby, 1989). The percentage of A+T in the UP region of *lpp* was the highest at 65%, followed by *ldc* and *lysS* with 50%, *spc* with 45%, and *tatA* with 40%. These regions are bound specifically by the C-terminal domain of the α-subunits of the polymerase complex (Gaal et al., 1996;

Ross et al., 1993). This region is in contact through minor grooves of the promoter DNA at position -42 and interacts directly with region 4 of the σ subunit bound to the -35 element (Naryshkin et al., 2000; Ross et al., 2001). Moran et al. (1981) demonstrated that the AT rich sequences in the UP region are known to play a role in promoter strength.

The *lpp* promoter has the highest percentage of A+T within the UP elements. This suggests that the high expression of *lpp* could be influenced greater expression levels compared with the other four promoters. Recent structural studies from Feklistov and Darst (2011) show that interactions between the σ factor and the -10 element take place only after promoter melting. This coincides with the initial encounter of the polymerase with the promoter, involving the promoter UP element and -35 elements, along with the downstream elements. This suggests that there is a strong relation between the -35 element and UP element for gene expression levels, and has implications for the choice of promoter for an efficient whole-cell biosensor, where high levels of expression may be desirable.

The levels of G+C in the region between -10 and +1 influences the kinetic stabilization of the open complexes at the promoter. Consequently, since *lpp* had lower G+C levels in this region, the stability of the open complexes may have been lower and allowed for quicker formation of the first phosphodiester bond when transcribing the *lux* gene (De Haseth et al., 1998). The *ldc*, *tatA*, *spc* and *lysS* promoters all have a higher G+C percentage than *lpp*, which suggests that the high levels of bioluminescence expression by *lpp* in the early stationary phase could partly have been due to the lower energy needed for the formation of phosphodiester bonds.

The number of spacers between the -35 and -10 regions is important for promoter recognition by RNA polymerase (Hawley & McClure, 1980). This distance is set, in part, by the interaction of RpoD ($\sigma^{D/70}$) in domain 4 with the core structure called the ββ' flap at domain 4, which functions to contact at the –35 region (Kuznedelov et al. 2002; Murakami et al. 2002b; Vassylyev et al. 2002). Amongst the promoters, *lpp*, *tatA,* and *spc* have 18 and 16 base pairs of spacers between the -35 and -10 regions, whilst *ldc* has the consensus length of 17 nucleotides and *lysS* has lowest length of 13 nucleotides. Promoters with a spacer length close to 17 have been shown to have a stronger activity *in vitro* as well as *in vivo*, compared with those that have shorter or longer spacers (Ayers et al., 1989*;* Mulligan et al.,1985; Stefano et al.,1982). However, Harley and Reynolds, (1987) demonstrated that inter region spacing of 16 and 18 bp should not affect the promoter activity compared to the consensus sequence which is 17bp suggesting that the promoter activity of *lpp, tatA,* and *spc* were not affected. In contrast, the number of spacers between the -35 and -10 region was the least for *lysS* suggesting that this could been important factor in the lower levels of bioluminescence expressions from this promoter.

It is clear that the activity of the promoter is not reliant on any single particular conserved region, but the sum of the interactions between the RNA polymerase with all the conserved regions. In this study, the presence of a sequence similar to the consensus sequence in the extended region, along with high A+T% in the UP regions and low GC levels within the -10 and +1 region in *lpp* could together have resulted in high expression levels from this promoter. In contrast, the small number of spacers, only 13 nucleotides between the -35 and -10 regions could have resulted in the low levels of bioluminescence expression by *lysS-lux*. Nevertheless, bioluminescence expression by all five of the biosensor constructs demonstrated a good correlation with all of the methods tested (*i.e.* conventional plate counting, ATP-chemiluminescence, and direct epifluoresence microscopy).

The decay of bacterial luminescence with time is a first-order process and reflects the turnover number of the luciferase enzyme (Meighen, 1991) with a half time between 1s and 20 s at room temperature (Meighen, 1993). This attribute of bacteria luciferase is real time whereby the production light is dependent on the metabolic activity and physiology state of the bacteria (Meighen, 1993). Furthermore, the bacterial bioluminescence is known as *real-time* and non-invasive bio-reporter (Carmi et al., 1987). Therefore this real-time system could allow continuous monitoring of bacterial suspension and growing bacterial cultures, without the need for any external substances to be added which are prerequisites for a number of other rapid microbiological testing methods (Marincs, 2000).

Consequently, this is a platform for the introduction of *lux* genes into other Pharmacopoiea bacterial and fungi strains (Naseby, 2006). The use of whole-cell bioluminescent strains could find significant applications in both minimum inhibitory concentration (MIC) and preservative efficacy testing (PET). The whole-cell bioluminescent constructs could serve approve to be good alternatives to laborious and time-consuming conventional methods.

2.7 : Conclusions

1) The bioluminescent reporter strains *E. coli* demonstrated a level of accuracy, precision, linearity, and equivalence to the conventional plate count method, and proved to have a minimum detection limit of 10^3 CFU/ml with a 6 order of magnitude working range, (European Pharmacopoiea 5.1.6) to suggest they were suitable as a rapid microbiological testing method.

2) Amongst the five constructs tested, the rank order for bioluminescence expression was as follows: *lpp-lux E. coli> spc-lux E. coli > tatA-lux E. coli > ldc-lux E. coli >lysS-lux E. coli*. The promoter regions play an important role in the expression of bioluminescence by these constructs. In this study, the similarity of the -10 and the extended -10 region nucleotide sequences with the consensus promoter sequence, along with a high AT% within the UP elements, may influence bioluminescence expression to a positive extent at early stationary phase.

3) The whole-cell bioluminescence method required a shorter time for preparation and sample analysis, with no incubation time required, compared with the other methods tested. In addition, the low cost of the materials needed gives it an additional advantage over the ATP-chemiluminescence, fluorescence spectrometry, and epifluorescence methods.

Chapter 3

3 Comparisons of Bioluminescence, Growth, Adenylate Energy Charge (AEC), and Plasmid Copy Number over Extended Incubation period

3.1 : Introduction

3.1.1 : Bacterial Growth and Survival Phases

Bacterial growth is defined as an increase in the cell number of a population, which occurs through cell growth and division. In this chapter, the cell viability (measured as CFU/ml) of *Escherichia coli* growing in optimal laboratory conditions (rich medium, 32°C and agitation) revealed a characteristic growth pattern comprised of five phases which agrees with the work done by Zambrano and Kolter (1996); Finkel (2006). This is in contrast to the standard textbook description of the bacterial life cycle which comprises of just three phases.

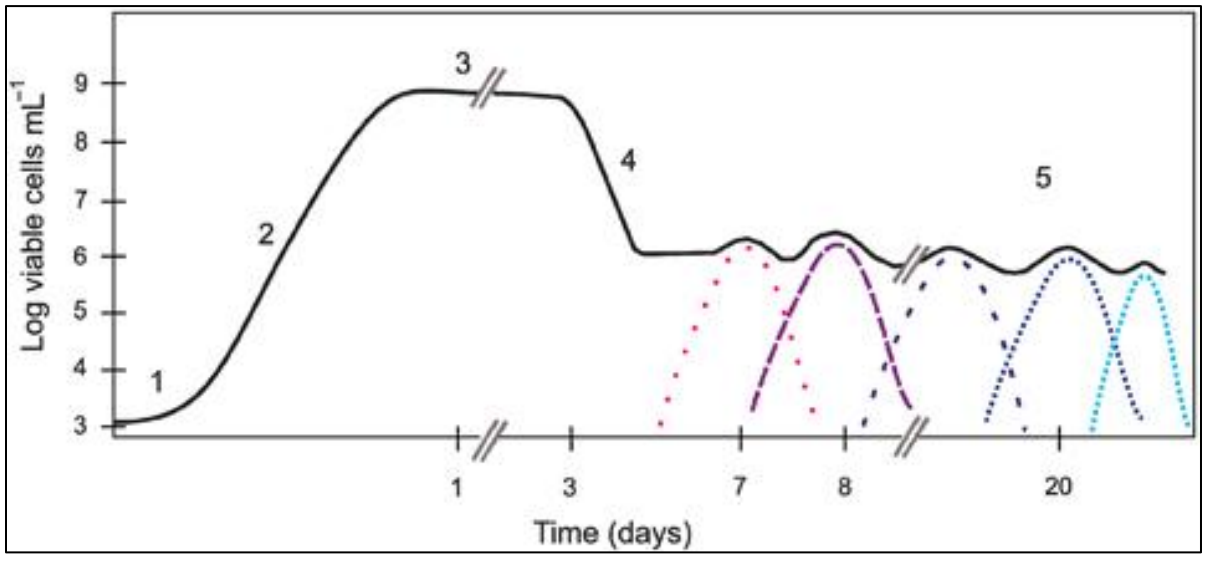


Figure 3.1: Stages of bacterial growth. Phase 1= lag; 2= exponential; 3= stationary; 4=death phase; 5= long term stationary phase (the red, magenta, purple, blue and cyan dotted lines) (Finkel, 2006)

Phase 1 is the lag phase. During lag phase, cells undergo intracellular changes in an effort to adjust to a new environment, and little or no cell reproduction takes place (Yates & Smotzer, 2007). This phase is characterized by metabolic reprogramming of the cell that enables it to thrive in the current environment, and the length of the lag phase is determined by several factors such as the bacterial species, the shifting environmental parameters, and also the incubation time of inoculum before (Pin & Baranya, 2008). Once cells have adapted to a new environment, they start to grow and divide exponentially; a state known as exponential phase (Phase 2). Cells divide asexually by binary fission at a constant rate during exponential phase. The growth rate of a bacterial population is known as the doubling time per hour. This rate varies depending on the environmental conditions with, for example, a slower rate in nutrient-poor media and faster in nutrient-rich media. The primary sigma factor RpoD ($\sigma^{70/D}$) is essential for general transcription in exponential phase, and can also be replaced by alternative sigma factors to coordinate gene expression under particular conditions. This includes diverse functions, such as stress responses, and morphological development (Paget & Helmann, 2003).

In exponential phase, bacterial growth exhausts the nutrient resources available, and eventually the population enters stationary phase (phase 3) when no further increase in cell number is observed. In Gram negative bacteria, the metabolic status of bacterial cells changes over time, during the transition from exponential to stationary phase. Starvation triggers accumulation of the alternative sigma factor RpoS ($\sigma^{S/38}$) which controls 10% of *E. coli* genes, these genes prepare the cell for survival in stress settings (Lacour & Landini, 2004). Nutrient depletion in stationary phase leads to the accumulation of waste products, whilst a lack of nutrients then leads on into the death phase (phase 4) and the number of viable cells (CFU/ml) decreases. *Escherichia coli* cells will tend to enter death phase after around 3 days of uninterrupted incubation (Finkel, 2006). In this phase, 90%-99% of the cell population can die, which

can release nutrients back into the nutrient depleted medium, and this can be used subsequently by the remaining cells. The number of viable cells in older bacterial populations can remain constant for months or even years, and this is termed as long-term stationary phase (phase 5) (Finkel, 2006). The balance between dying and growing cells provides a dynamic equilibrium where the final output is a stable number of viable cells in the population.

3.1.2 : Plasmid Copy Number

The plasmid copy number (PCN) is defined as the number of copies of a plasmid present per copy of the chromosome in a bacterial cell (Gerhardt et al., 1994). Chromosome replication is tightly coordinated with the cell cycle such that all origins are initiated synchronously at the same cell mass per cell cycle whilst plasmid replication is independent (Nordstrom & Dasgupta, 2006). However, plasmid replication can mimic growth curve or otherwise. Control of the plasmid copy-number within cells is achieved by either limiting the supply of initiation factors (RNA or protein) or by inactivating the initiator through dimerization or regulation of iteron complexes (Nordstrom, 1990).

The plasmid pBR322 was selected as the vector for the *lux* operon bio-reporter. The widespread use of pBR322 in recombinant studies has revealed that it has three important features as an effective cloning vector. These are that the plasmid is: stably-maintained within cells; offers reliable selection options for recombinants; and is versatile in accommodating recombinants genes (Balbas et al., 1986). Chiang and Bremer (1988) have also noted that there was no indication of the accumulation multimers which is known to interfere with the stability of pBR322. Plasmid multimers are able to confuse the intracellular control circuits which can lead to a decrease in the number of plasmids within the cell. Plasmid

multimers out-replicate monomeric plasmids and accumulate within the cell, resulting in populations with a significantly-increased rate of plasmid loss (Summers, 1998).

The plasmid copy number is important for recombinant studies, as the level of gene expression within the host cell correlates with it. Consequently, a number of methods to determine plasmid copy number have been devised. Most of the methods available to determine the plasmid copy number are known to be laborious, resulting in low reproducibility and a narrow dynamic range (Schmidt et al., 1996). The methods available include caesium chloride-ethidium bromide (CsCl-EtBr) centrifugation (Weisblum et al., 1979), gel electrophoresis (Projan et al., 1983), and high-performance liquid chromatography (Coppella et al., 1987). Quantitative real-time PCR has demonstrated excellent sensitivity to plasmid copy number and so it was used in this study to quantify the number of intracellular pBR322 plasmid copies. This method is well established and widely used for detecting the copy number of genes, and has been established to determine the plasmid copy number (Lee et al., 2006a; Lee et al., 2006b). Quantitative PCR (qPCR) extends the usefulness of PCR technology by permitting reliable determination of the starting DNA or RNA template concentration. It offers simultaneous detection and quantification of DNA or RNA in one reaction. The most significant advantage over conventional approaches is its superiority in terms of accuracy, sensitivity, and in the elimination of post-PCR steps.

The most common qPCR protocols are either the Taqman probe or the SYBR green I fluorophore. The Taqman probe is based on the specific hybridization of a double dye oligonucleotide probe target to a DNA or RNA molecule. Whilst SYBR green real time PCR assay is based on binding of the fluorescent dye SYBR green I to the minor groove of the double stranded PCR product (Hernandez et al., 2003; Howell et al., 1999; Ririe et al., 1997; Wittwer et al., 1997).

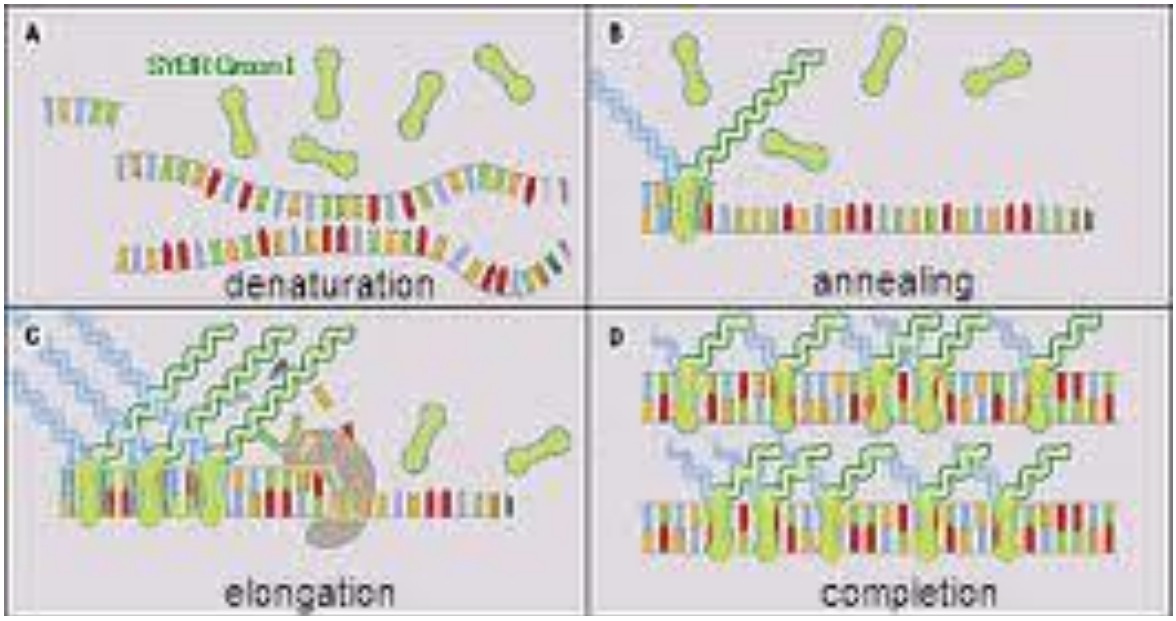


Figure 3.2: The principle of the SYBR green-based qPCR assay: step A is the denaturation stage when the dsDNA is melted; step B is the annealing stage where the primers anneal to the ssDNA; step C is the elongation stage when dNTPs are added to the nascent strand; and step D is the completion stage, when the SYBR green binds to the dsDNA and will emit a fluorescent signal

.
SYBR green I exhibits a baseline fluorescence intensity when irradiated with the excitation wavelength when unbound in solution, but it will emit a much stronger fluorescence signal after it has bound to dsDNA (Figure 3.2). In qPCR, the fluorescence is measured at the end of the elongation step of each PCR cycle to measure DNA amplification. The advantage of using this method for qPCR is that it is relatively cheap, since it can be used with any pair of primers for any target DNA. However, since the dye will bind to any dsDNA present and so yield fluorescence, the specificity of this assay will be greatly reduced by amplification of non-specific products and also primer-dimers. However, a melting curve analysis can be used to distinguish a specific PCR product from non-specific contaminants (Wittwer et al., 1997), and thus to identify and eliminate contamination. It was decided to use the SYBR green assay to investigate plasmid copy number in the work described in this thesis.

There are two quantification strategies that are applied to analyse quantitative PCR, which are absolute and relative quantification of the PCR product (Pfaffl, 2012). Absolute quantification relates the PCR signal to plasmid copy number by means of a calibration curve using known concentrations of the gene of interest (Pfaffl, 2012). Absolute quantification is dependent on the reliability and validity of the standard curve. In this study, absolute quantification of PCN was chosen as the generation of a standard curve can allow highly specific, sensitive and reproducible quantification data (Pfaffl, 2012). Bustin and Nolan (2004) noted that this approach is more accurate but often more labour intensive due to the construction of standard curves for every time point evaluated. Relative quantification instead compares changes in the expression levels of two or more gene targets. In relative quantification, the difference in expression levels between the target gene and the reference gene gives a measure of gene expression. Hence, two general mathematical models can be used for relative quantifications, one of which is based on the ‘delta delta ct model’ (Livak & Schmittgen, 2001) and the other on the ‘efficiently corrected

model' (Pfaffl, 2001). To ensure the integrity of the scientific literature, promote consistency between laboratories, and to increase experimental transparency when performing qPCR the Minimum Information of Quantitative Real-time PCR Experiments (MIQE) Guidelines were developed (Bustin et al., 2009). The important parameters for qPCR quantification include: qPCR efficiency; linear dynamic range; limit of detection; and precision (Bustin et al., 2009).

3.1.3 : ATP as a Prime Energy Source for Bacteria

Adenosine triphosphate (ATP) is the principle energy carrier for all living organisms. It is in this form that cells expend energy on all processes that require it, including cellular energetics, intracellular signalling, metabolic regulation, motility and various homeostasis functions.

The catalyses the synthesis of ATP involes adenosine diphosphate (ADP) and inorganic phosphate which are driven by a flux of protons across the membrane down the proton gradient known as proton motive force (pmf), generated by electron transfer. The flux creates an electrochemical potential from the potential difference across the cytoplasmic membranes catalyzed by ATP synthase. This reaction is fully reversible, so ATP hydrolysis generates a proton gradient by a reversal of this flux (Boyer, 1988).

$$ADP + Pi \overrightarrow{\leftarrow} ATP$$

The importance of the adenine nucleotides as major regulatory factors in controlling metabolic processes as a result of their influence on key regulatory enzymes has been well established. Comparison of the effects exerted on individual enzymes *in vivo* with regulation of metabolism by the energy state of the cell is facilitated by use of the adenylate energy charge (AEC) (Ball & Atkinson, 1975). The adenylate energy charge is defined as [(ATP) + 1/2(ADP)]/ [(ATP) + (ADP) + (AMP)], is a linear measure of the amount of metabolic energy stored in the adenine nucleotide pool and represents the relative saturation of the

adenylate pool expressed as a ratio (Atkinson & Walton, 1967; Ball & Atkinson, 1975). The methods that are most frequently employed for adenine nucleotide determinations involve enzymatic conversions of ADP and AMP to equivalent levels of ATP followed by a quantitative analysis of ATP via the firefly chemibioluminescence reaction.

To the best of this author's knowledge previous studies have not demonstrated the AEC levels of genetically modified bioluminescent bioreporter strains (thereby transformation of plasmids carrying bioreporter genes *lux* to host cell) and wildtype microorganism. The *lux* operon is regarded as an energy expanding system as the primary reaction of fatty acid reductase requires ATP (Meighen, 1993). In addition, Galluzzi and Karp (2007) and Koga et al. (2005) investigated bioluminescence expressions from plasmid-based *lux* fusion and have concluded that intracellular redox pool affects bioluminescence expressions. Therefore, this could lead to the hypothesis that the genetically modified bioluminescent *E.coli* constructs could possibly utilize higher energy levels possibly resulting in lower AEC values than the wildtype organism. Thereby, in this chapter, the analysis of AEC of the genetically modified bioluminescent constructs and control strains ([pBR322.*lux*]. *E.coli* and *E.coli* ATCC 8739) throughout the growth cycle is carried out in this chapter to bridge the gap of knowledge.

3.2 : Rational of Chapter 3

In this chapter, a detailed investigation of the physiology and behaviour of the five *E. coli* constructs and also the control strains was undertaken throughout the course of a full growth cycle. This was achieved by measuring whole-cell bioluminescence (RLU/ml), viable counts (CFU/ml), the adenylate energy charge (AEC) and the plasmid copy number (PCN) over 28 days of incubation. In addition, the morphology of the genetically modified bioluminescent bioreporter strains and the control strains was determined by fluorescence microscopy over the 28 days of incubation. Furthermore a comparison of the promoter sequences for the bioreporters with the consensus sequences for RpoS ($\sigma^{38/S}$), RpoE ($\sigma^{24/E}$), and RpoF ($\sigma^{28/F}$) was performed to understand the activity of the promoter sequences for the five strains in the different growth phases.

The adenylate energy charge (AEC) was determined over the 28 days of incubation by using the luciferin-firefly luciferase assay for ATP combined with enzymatic conversion of AMP to ADP to ATP. The aim of this was to relate the levels of bioluminescence expressed by the bioreporter strains to the AEC during the phases different of growth. To examine the stability of the plasmid copy number for the five constructs, and to permit this to be related to bioluminescence expression by the bioreporter strains, over the 28 days of incubation the plasmid copy number was quantified by using quantitative real-time polymerase chain reaction (qPCR).

The relationship between the different elements of the research described in this chapter is summarised in figure 3.3

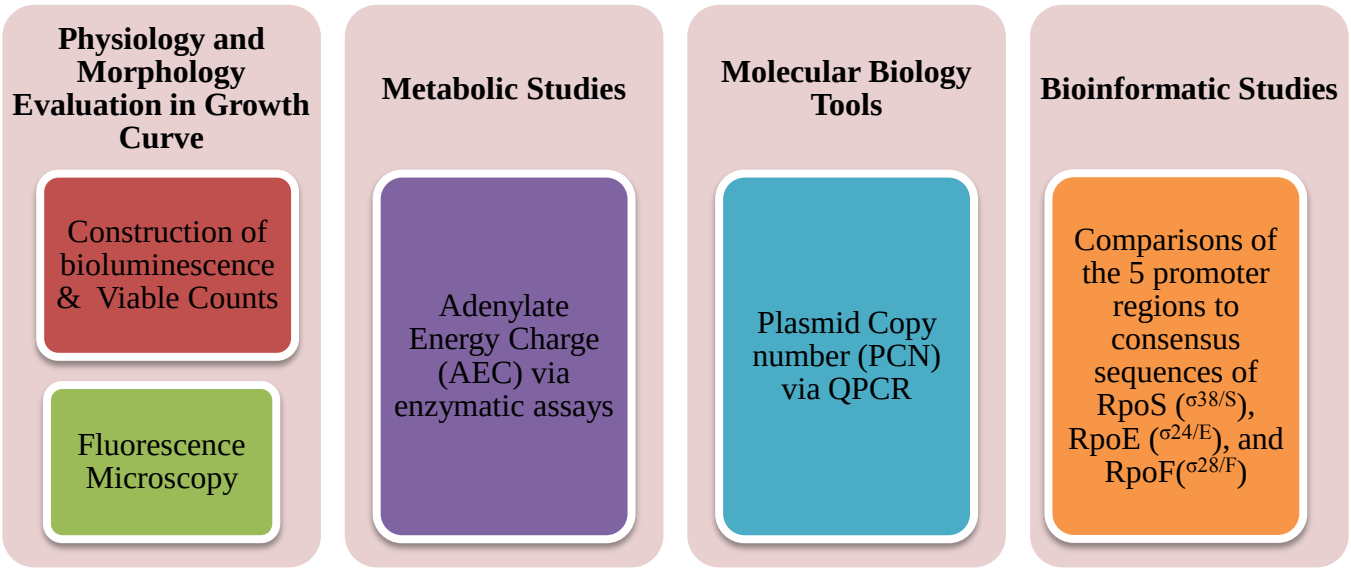


Figure 3.3: Summarises the various studies in this chapter in characterising the five constructs

3.3 : Objectives

1) To perform bioluminescence and viable count growth curve analyses for both the five bioluminescent bioreporter strains and also the control *E. coli* strains, over 28 days of incubation.
2) To determine, using qPCR, the plasmid copy number for the five bioreporter strains over 28 days of incubation.
3) To measure the adenylate energy charge (AEC) of the five bioreporter strains and also the control *E. coli* strains over 28 days of incubation.

3.4 : Materials and Methods

3.4.1 : Construction of Growth Curves

Inoculum preparation and inoculation of the experimental cultures was carried out as described in Section 2.4.1. Whole-cell bioluminescence readings and the viable counts for the five bioluminescent bioreporter strains and the control strains during 28 days of incubation were measured as described in sections 2.4.3 and 2.4.4. The measurements were taken every hour for the first 5 hours of incubation, and then subsequently every half an hour until the 20^{th} hour of incubation. From the 20^{th} hour of incubation, measurements were taken every hour until the 30^{th} hour, followed by every 2 hours until the 40^{th} hour, and then every 4 hours till the 48^{th} hour of incubation. However, the viable counts (CFU/ml) for the control strains were determined every hour until the 24^{th} hour, followed by every 4 hours until the 48^{th} hour of incubation. This was followed by the long term incubation periods at 7 days (168 hours), 14 days (336 hours), 21 days (504 hours), and 28 days (672 hours). The ratio of RLU divided CFU were denoted as bioluminescence per cell (RLU:CFU) across all time points for all five bioreporter *E.coli* strains. The average pH of the growth medium for each of the *E. coli* strains was measured using a calibrated pH meter (Toledo Mettler) upon inoculation (0 hour), and thereafter at 6 h, 24 h, 168 h (7 days), 336 h (14 days), 504 h (21 days) and 672 h (28 days) of incubation.

3.4.2 : Fluorescence Imaging

Samples of the cultures (1.0 ml) taken at 0, 6, 24, 168, 336, 504 and 672 hours of incubation were centrifuged (13,000 x*g*) for 2 minutes (Accu Spin, Fisher Scientific), and resuspended with 0.85% NaCl. The suspensions were stained according to the methods described in section 2.4.6, and viewed

microscopically as described in section 2.4.7. The images were observed at a total magnification of 1000X using a fluorescence microscope (excitation at 450-490nm, emission at 505-520nm, Nikon EFD-3) with a mercury lamp (Nikon), whilst images were taken using a GX-CAM scientific imaging camera and pictures were edited with GX capture software.

3.4.3 : Plasmid Copy Number (PCN) Determination

A brief flow chart of the procedure that was followed to determine the PCN of the five constructs is shown below (Figure 3.4) and the procedure is described in greater detail in the following sections;

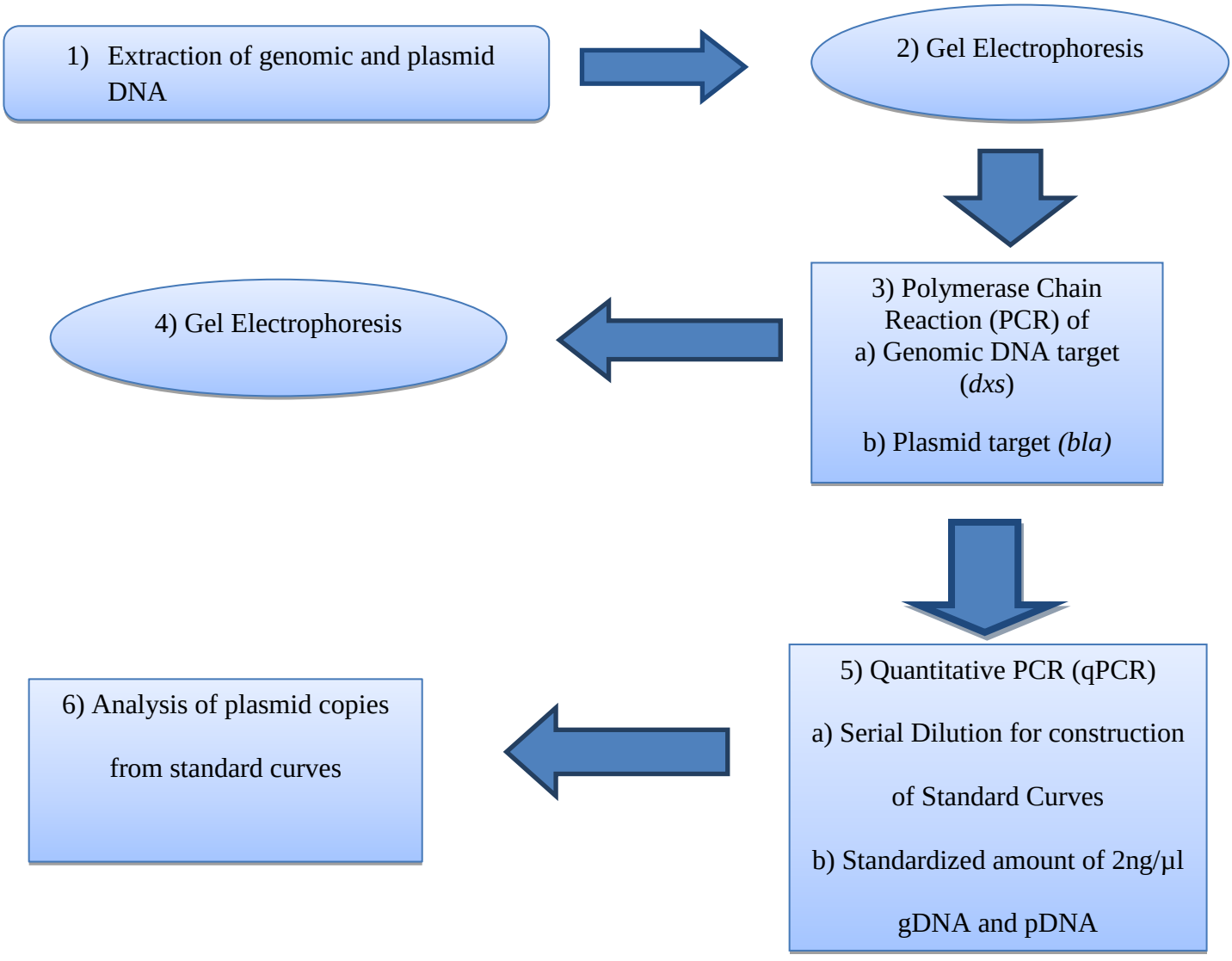


Figure 3.4: A flow chart describing the methodology steps involved in plasmid copy number quantification

3.4.3.1 : Extraction of Genomic DNA (gDNA) and Plasmid DNA (pDNA)

Genomic DNA (noted as gDNA) and plasmid DNA (noted as pDNA) were extracted from the cultures immediately after inoculation (0 hours), and then again at 6, 24, 168, 336, 504 and 672 hours of incubation. The Gen Elute bacterial genomic DNA kit (Sigma-Aldrich) and the Gen Elute HP Plasmid Miniprep kit (Sigma-Aldrich) were used to extract the genomic and plasmid DNA from the cells following the manufacturer's protocol. The concentration of extracted DNA was measured using biophotometer (Eppendorf). The DNA samples were diluted and transferred into quartz cuvettes, and then the absorbance was measured using the biophotometer at wavelengths (λ) of 260nm and 280 nm. The quality and yield of the DNA extract was determined by the OD ratio (260/280). A high ratio (1.8-2.0) indicates a relatively pure sample, with little protein contamination. The DNA concentration was estimated, after the non-nucleic acid absorbance (measured at 320 nm) had been deducted, using equation 3.1 below;

DNA Purity (A_{260}/A_{280}) = $\frac{(A260\ reading - A320\ reading)}{(A280\ reading - A320\ reading}$ ……. Equation 3.1

3.4.3.2 : Gel Electrophoresis

Genomic and plasmid DNA samples and also the standard DNA molecular weight markers (1 kbp and 100 bp, Promega) were prepared by adding gel loading dye (10% Ficoll 400, 20% sucrose, 10mM EDTA and 0.25% bromophenol blue) to the DNA at a ratio of 1:5 respectively and loaded onto the gels as carefully as possible. The 1 kbp DNA markers were used when gDNA and pDNA was loaded, whilst for PCR products, a 100 bp ladder was used. Genomic DNA and plasmid DNA samples were loaded on a 0.8% agarose gel (Invitrogen) in 5µl aliquots. The gel was placed in a gel

tank containing 250ml of TBE buffer (0.09M Tris/HCl, 0.09M boric acid, and 2.5mM EDTA, pH 8.2).

The gels were run at 110V for 60 minutes for genomic/plasmid DNA whilst 45 minutes for PCR products. The gels were subsequently stained with ethidium bromide solution (5 mg/mL) for 15 minutes and then de-stained with sterile distilled water for 5 minutes before visualizing under UV irradiation using a UV-transilluminator (Ingenius Syngene Bioimaging) to ensure that extracted DNA had not been degraded.

3.4.3.3 : Design of primer sets for Polymerase Chain Reaction (PCR) and Quantitative Polymerase Chain Reaction (qPCR)

Two primer sets specific to the beta-galactosidase gene and to D-deoxyxylulose-5-phosphate synthetase (dxs) used were as cited in previous studies in Lee et al., 2006; Rozen & Skaletsky, 2000 denoted as *bla* and *dxs* primers were purchased from Invitrogen. The *bla* is present in single copy in pBR322 (Watson, 1998) whilst the *dxs* is present in single copy in the *E. coli* chromosome (Hahn et al., 2001). Consequently, quantification of the *bla* and *dxs* content of the samples indicates the corresponding amounts of pBR322 plasmid, and *E. coli* chromosomal, DNA present in the samples. Prior to the obtaining the optimal concentrations of magnesium chloride and annealing temperature, magnesium chloride concentrations of 3.5mM, 3mM, 2mM, 1.5mM and 1mM were optimized by using a temperature gradient from 50 ºC to 60ºC for optimal temperature.

Table 3.1: Primer sequences for *bla* and *dxs*

Gene	Accession no.	Primers (5'->3')[n]	Length (nt)	Product size (bp)	T_m (^{0}C)	GC %
bla	J10749	F: CTACGATACGGGAGGGCTTA	20	81	54	55
		R: ATAAATCTGGAGCCGGTGAG	20		52	50
dxs	AF035440	F: CGAGAAACTGGCGATCCTTA	20	113	52	50
		R: CTTCATCAAGCGGTTTCACA	20		50	45

[n] F and R denote the forward and reverse primers, respectively

Upon resuspension of the freeze dried primers in 100µl TE buffer pH 8. 5 µl of each of the primer suspensions was run on a 1.0% agarose gel at 100 V for 60 min, followed by staining with 5 mg/ml ethidium bromide and UV illumination, to check for the presence of the primers before the commencement of PCR.

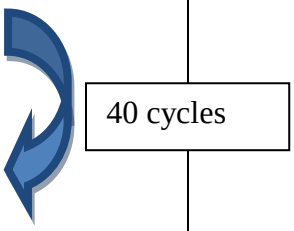
3.4.3.4 : Polymerase Chain Reaction (PCR)

The extracted genomic and plasmid DNA samples were used as the template for PCR. Initial working solutions of 25 µM of primers were prepared. Both the stock solutions and the working solutions of the primers were stored at -20^{o}C until required. Reaction buffer, magnesium chloride ($MgCl_2$), deoxynucleotides (DNTPs), and *taq* polymerase were all purchased from Promega Ltd. The following reagents as stated in Table 3.2 were added give a total volume of 25 µL. The reaction tubes were placed in the Gradient PCR thermocycler (Eppendorf Mastercycler Gradient). The reaction conditions and the volumes used for PCR are listed in table 3.2. The PCR products were

analysed by gel electrophoresis using a 2.0% agarose gel following the procedure described in section 3.7.1.

Table 3.2: Volume of reagents required and PCR conditions for *bla* and *dxs* amplification after optimization

Reagent	Volume (µL)	Steps	Temperature (^{0}C)	Duration (minutes)
10x Reaction buffer	2.5	Initial Denaturation	95.0	5
50mM $MgCl_2$	1.5	Denaturation	95.0	0.5
10mM dNTP	0.5	Annealing	58.0	0.5
25µM Forward primer	1	Extension	72.0	1.0
25 µM Reverse primer	1	Final extension	72.0	5.0
5U/ µM Taq polymerase	0.2	Hold	4.0	Until electrophoresis
DNA template	5			
Sterile water	13.3	n/a		
Total volume	25	n/a		



3.4.3.5 : Real Time Quantitative PCR (qPCR) using SYBR Green I Dye

Real time qPCR amplification was performed using a Chromo4 detector attached to a DNA engine (Bio-Rad) and analysis was done using MJ DNA engine software (Version 3.1; Bio-Rad). Low profile 0.2 ml PCR tube strips with optical flat caps (Bio-Rad), were used to contain the qPCR reactions. DNA extracted as described in Section 3.6.3.1 was used as the template. Platinum SYBR Green qPCR SuperMix-UDG kit purchased from Invitrogen. The volumes and conditions used for each qPCR reaction are given in table 3.3.

Table 3.3: Volume of reagents required and conditions for qPCR analysis

Reagent	Volume (µl)	Steps	Temperature (^{o}C)	Duration (minutes)
Platinum SYBR Green qPCR SuperMix-UDG	25.0	Initial Denaturation	95.0	10
Forward primer (25µM)	1.0	Denaturation	95.0	0.17
Reverse primer (25µM)	1.0	Annealing	58.0	0.17
Magnesium Cloride (50mM)	1.5	Extension	72.0	0.17
DNA Template (normalized to 2ng/µl)	x			
Sterile distilled water	11.5-x	n/a		
Total volume	40.0	n/a		

40 cycles

After amplification, a melting curve analysis with a temperature gradient of 0.1^{0}C/s from 70^{0}C to 95.0^{0}C was performed to confirm that only the products of specific interest had been amplified. Finally, the samples were cooled down to 40^{0}C for 30 s. Negative controls, where no DNA was added, were included in each run. The concentration of the DNA template (gDNA and pDNA) was standardised to 2 ng/µl for all samples. The analyses were carried out in triplicate.

3.4.3.6 : Construction of Standard Curves of Genomic DNA and Plasmid DNA

Samples of gDNA and pDNA taken at each time point of 0, 4, 6, 24, 168, 336, 504 and 672 hours were 10-fold serially diluted (1:10, 1:100 and 1:1000) 10-fold serially diluted whereby 2µl of serially diluted DNA was added to the master mix as indicated on table 3.3 to prepare a standard curve of C_T values against copy numbers. The preparation of the standard curves complied with the MIQE requirements for a linear dynamic range of least 3 orders of magnitude, and C_T values $\geq$ 40 were disregarded as these imply low efficiency of qPCR reaction and can indicate a false positive (Bustin et al., 2009). The efficiency (E) of qPCR reaction is calculated as= $10^{(-1/slope)}$-1 (multiplied to 100%). The range and linearity of standard curve were determined by using the correlation coefficient (R^2) between the C_T values and copy numbers. The minimum limit of detection (MDL) was ensured that ct values (Y-axis) are within 95% of log copies axis (x-axis) to allow accurate quantification of plasmid copy numbers whilst precision of qPCR reaction was interpreted as the calculated SEM.

The initial copy numbers for the Deoxy-Xylulose-5-Phosphate gene (*dxs*) and ampicillin resistance gene (*bla*) were calculated using the following formula:

$$\frac{(6.02\ x\ 10E23\frac{copy}{mol} x\ DNA\ amount(g)}{(660\ g/mol/bp\ x\ Genomic\ or\ plasmid\ size\ (bp)}$$ .....Equation 3.2

(Argyropoulos & Savva, 1997)

Escherichia coli 8739 has a genome of 4746218 bp (NCBI Reference sequence NC_010468), whilst pBR322 with the *lux* operon insert is 10184 bp. The region promoter size varies according to the individual biosensor construct. The sizes of the promoter regions were as follows: *lpp* = 373bp; *tatA* = 300bp; *ldc* = 300bp; *lysS* = 273 bp; and *spc* = 310bp. The *bla* and *dxs* are single copy genes found

in pBR 322 and *E. coli* genomic DNA, respectively. Consequently, the ratio of *bla* to *dxs* is equal to the plasmid copy number of pBR322. Absolute quantification was performed using the standard curves constructed. The ratio of *bla* to *dxs* in the plasmid DNA and genomic DNA were determined from the corresponding standard curves using the C_T values.

3.4.4 : Adenosine Measurements

3.4.4.1 : Adenosine Triphosphate Chemiluminescence Assay

Cell contents were extracted in triplicates described in section 2.4.5.1 for ATP, ADP, and AMP measurements. The ATP chemiluminescence assay was performed as described in Section 2.4.5.1.

3.4.4.2 : ADP Measurement

The ADP in the cell extract was first converted to ATP by the addition of 200 µl of a solution mixture of 25 mM HEPES (Sigma-Aldrich), 10 mM $MgSO_4$ (Fisher Scientific) and 10 mM KCl (Fisher Scientific) adjusted to pH 7.0, 0.4mg/ml of phosphoenolpyruvate (PEP) and 10 µl of pyruvate kinase in ammonium sulphate (32 mg/ml) (Sigma-Aldrich)) to 500µl of cell extract. This was then incubated at 30°C for 30 minutes and the total amount of ATP in the samples was then measured as described in Section 2.4.5.1. This value for total ATP content is the product of the ATP + ADP content of the sample. The luminescence readings of ADP present was finally calculated by deducting the ATP content, determined in section 3.6.4.1, from the total ATP+ADP content. This was repeated three times.

3.4.4.3 : AMP Measurement

The AMP and ADP in the cell extract were converted to ATP by the addition of 200 µl of mixture solution of 25mM of HEPES, Sigma-Aldrich, 10mM $MgSO_4$ (Fisher Scientific), 10mM KCl (Fischer Scientific) adjusted to pH 7.0, 0.4 mg/ml of phosphoenolpyruvate (PEP), 10 µl of pyruvate kinase in ammonium sulphate (Sigma-Aldrich) and 100µl of adenylate kinase (28mg/ml) (Sigma-Aldrich)) to

500µl of cell extract. This was then incubated for 30 minutes at 30°C and the total amount of ATP present in the samples was then measured as described in Section 2.4.5.1. This value for total ATP content is the product of the ATP+ADP+AMP content of the sample. The bioluminescence readings of AMP present was finally calculated by deducting the ATP and ADP bioluminescence readings, determined in section 3.6.4.2, from the total ATP+ADP+AMP content. This was repeated three times.

3.4.4.4 : Calculation of Adenylate Energy Charge (AEC)

The adenylate energy charge (AEC) was calculated from the mean ATP, ADP and AMP content of the cells, as determined in sections 3.6.4.1-3, using the formula given in equation 3.3.

$$AEC = \frac{ATP + 0.5\,(ADP)}{(ATP+ADP+AMP)}$$ .....Equation 3.3

(Atkinson, 1968)

3.4.5 : Statistical Analysis

Statistical Analysis was carried out as described in Section 2.4.8. Pearson correlation analysis was performed to compare plasmid copy number (PCN) with bioluminescence per cell (RLU:CFU). Standard error of means (SEM) is obtained by SPSS program.

3.5 : Results

This results section contains four experimental parts.

Section **3.7.1** reports the bioluminescence and population kinetics. Section **3.7.2** reports the morphology of the *E. coli* strains over the 28 days of incubation. Section **3.7.3** reports the plasmid copy numbers present in each of the bioreporter strains. Section **3.7.4** reports the adenylate energy charge (AEC) within the five bioreporter strains and also the control strains over the 28 days of incubation.

3.5.1 : Bioluminescence and viable count Kinetics in the Growth and Survival Curve

3.5.1.1 : Bioluminescence Kinetics in the Growth and Survival Curve

Figure 3.5 (a) shows the bioluminescence kinetics across 48 hours. The initial bioluminescence (RLU/ml) expressed by each of the 5 bioluminescent bioreporter strains was on average between 4.16 ± 0.03 $\log_{10}$ RLU/ml at 0 hour. The strains carrying the *lpp-lux* and *spc-lux* constructs yielded the highest initial bioluminescence, measured at 4.3 ± 0.02 $\log_{10}$ RLU/ml. The initial bioluminescence expressed by the other bioreporter strains, in increasing order, was as follows: *tatA-lux*, 4 ± 0.03 $\log_{10}$ RLU/ml; *ldc-lux*, 4.03 ± 0.04 $\log_{10}$ RLU/ml; *lysS-lux*, 4.2 ± 0.01 $\log_{10}$ RLU/ml; *spc-lux* and *lpp-lux*, 4.3 ± 0.04 $\log_{10}$ RLU/ml. Each of the 5 bioreporter strains exhibited a decrease in bioluminescence expression, of an average of 0.4 ± 0.07 $\log_{10}$ RLU/ml, with no significant difference between the strains, and remained at that level in the first three hours of incubation. The bioluminescence expressed by each of the bioreporter strains began to increase from the 4th hour up to the 6th hour of incubation, at a rate approximately 2.3 ± 0.13 $\log_{10}$ RLU per hour. A Tukey Post hoc analysis revealed there was a significant increase ($P \leq 0.05$, ANOVA) for bioreporter strains; *lpp-lux*, *tatA-lux*, *ldc*-lux *lysS-lux* and *spc-lux* between the 5th and 6th hour of incubation from 0h

inoculation time with a significant ($P \leq 0.05$, ANOVA) increase of 2.21 ± 0.05 $\log_{10}$ RLU/ml, whilst *tatA-lux* increased significantly ($P \leq 0.05$, Tukey *Post Hoc*) by 1 ± 0.04 $\log_{10}$ RLU/ml, *spc-lux* significantly by 0.71 ± 0.07 $\log_{10}$ RLU/ml, *ldc-lux* by 0.655 ± 0.1 $\log_{10}$ RLU/ml and *lysS-lux* by 0.586 ± 0.01 $\log_{10}$ RLU/ml. At the 5.5th hour of incubation, the bioluminescence peaked the highest with *spc-lux* (8.70 ± 0.04 $\log_{10}$ RLU/ml), followed by *lpp-lux* (8.64 ± 0.05 $\log_{10}$ RLU/ml), *tatA-lux* (8.19 ± 0.04 $\log_{10}$ RLU/ml), *ldc-lux* (7.65 ± 0.06 $\log_{10}$ RLU/ml), *lysS-lux* (7.56 ± .03 $\log_{10}$ RLU/ml)

In total, the bioluminescence expressed by the bioreporter strain carrying the *lpp-lux* construct increased significantly ($P \leq 0.05$, Tukey *Post Hoc*) by 4.31 ± 0.1 $\log_{10}$ RLU/ml over the first 6 h of incubation. The bioluminescence expressed by the *spc-lux* increased significantly ($P = 0.00$, Tukey Post Hoc) by 4.4 ± 0.15 $\log_{10}$ RLU/ml over the first 6 h of incubation whilst that expressed by the *tatA-lux* strain increased significantly ($P = 0.00$, Tukey *Post Hoc*) by 4.19 ± 0.1 $\log_{10}$ RLU/ml, that expressed significantly ($P = 0.00$, Tukey *Post Hoc*) by the *ldc-lux* strain increased by 3.62 ± 0.2 $\log_{10}$ RLU/ml, and that expressed significantly ($P = 0.00$, Tukey *Post Hoc*) by the *lysS-lux* strain increased by 3.36 ± 0.23 $\log_{10}$ RLU/ml (Figure 3.3a). Overall, Tukey *Post Hoc* analysis revealed that there was no significant difference between the high bioluminescence readings of *spc-lux* and *lpp-lux* ($P = 0.900$, Tukey *Post Hoc* analysis) whilst a significant difference between *tatA-lux, ldc-lux* and *lysS-lux* ($P = 0.00, 0.00, 0.00$, Tukey *Post Hoc* analysis

Approaching stationary phase, the level of bioluminescence expressed by each of the bioluminescent bioreporter strains remained relatively stable from the 7.5th hour to the 40th hour for all five constructs with no significant difference. During the stationary phase, the level of bioluminescence expressed by the bioreporter carrying the *lpp-lux* was the highest up to 35th hour of incubation, after which the bioluminescence decreased to levels similar to the other 4 constructs. After the 40th hour of incubation, there was a decrease significantly ($P \geq 0.05$, ANOVA) in the level of bioluminescence expressed of around 1-2 $\log_{10}$ RLU/ml. The level of bioluminescence expressed by the control strains

E. coli ATCC 8739 [pBR322.*lux*] and the negative control, *E. coli* ATCC 8739, remained unchanged at 0 $\log_{10}$ RLU/ml. The overall standard error of the means (SEM) of the bioluminescence kinetics is 0.065 (Figure 3.3 (a)).

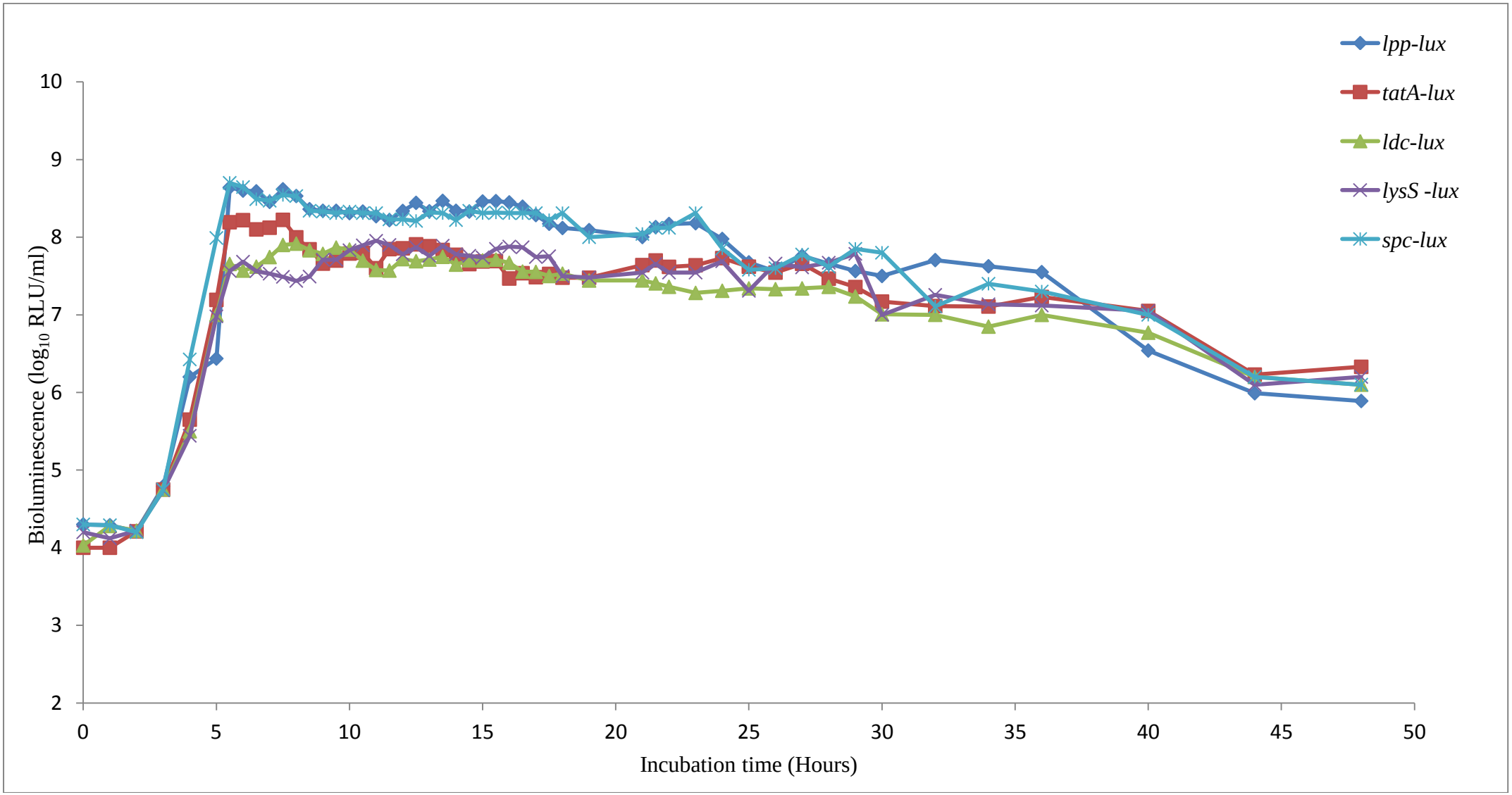


Figure 3.5 (a): Bioluminescence expression by the five bioluminescent *E. coli* ATCC 8739 bioreporter strains (*lpp-lux*, *tatA-lux*, *ldc-lux*, *lysS-lux*, and *spc-lux*) over 48 hours of incubation n=3. # The overall SEM of each is 0.065 at 95% confidence interval.

3.5.1.2 : Viable Counts Kinetics in the Growth and Survival Curve

The initial viable counts for the five bioreporter strains were approximately 5.88 ± 0.07 $\log_{10}$ CFU/ml whilst the initial viable counts for the control strains were approximately 5.53 ± 0.06 $\log_{10}$ CFU/ml with no significant difference between the biosensor and control strains (Figure 3.5 (b)). Each of the strains demonstrated a lag during the first 2 hours of incubation, and there was a slight decrease in the viable counts between 0.25 ± 0.03 $\log_{10}$ CFU/ml with no significant difference, during that period. After 4 h of incubation, the cultures entered exponential phase, and continued to multiply at a rate of approximately 1.5 ± 0.07 $\log_{10}$ CFU/ml every hour until the 7.5th hour. At the 7.5th hour, the viable counts of the bioluminescent bioreporter strains were between 9.8 ± 0.1 $\log_{10}$ CFU/ml whilst 9.66 ± 0.1 $\log_{10}$ CFU/ml those of the *E.coli* strains were significantly increased ($P \leq$ 0.05, ANOVA) from the initial incubation time for all *E.coli* strains. From the 10th hour until the 40th hour of incubation, the viable counts remained statistically unchanged. From the 40th to 48th hour of incubation, there was a decreased significantly ($P \leq 0.05$, ANOVA) of 1.5 ± 0.1 $\log_{10}$ CFU/ml in the viable counts with no significant difference between the *E.coli* strains. The SEM for the population kinetics in Figure 3.3 (b) is 0.083. The significant correlation coefficients between the RLU and the CFU throughout the 48 hours of incubation were: *lpp-lux* R^2 = 0.82; *tatA-lux* R^2 = 0.85; *ldc-lux* R^2 = 0.89; *lysS-lux* R^2 = 0.92; and *spc-lux* R^2 = 0.81, whilst the F value obtained overall for the growth curves across 48 hours was $F_{(228, 21, 0.05)} = 3.88$.

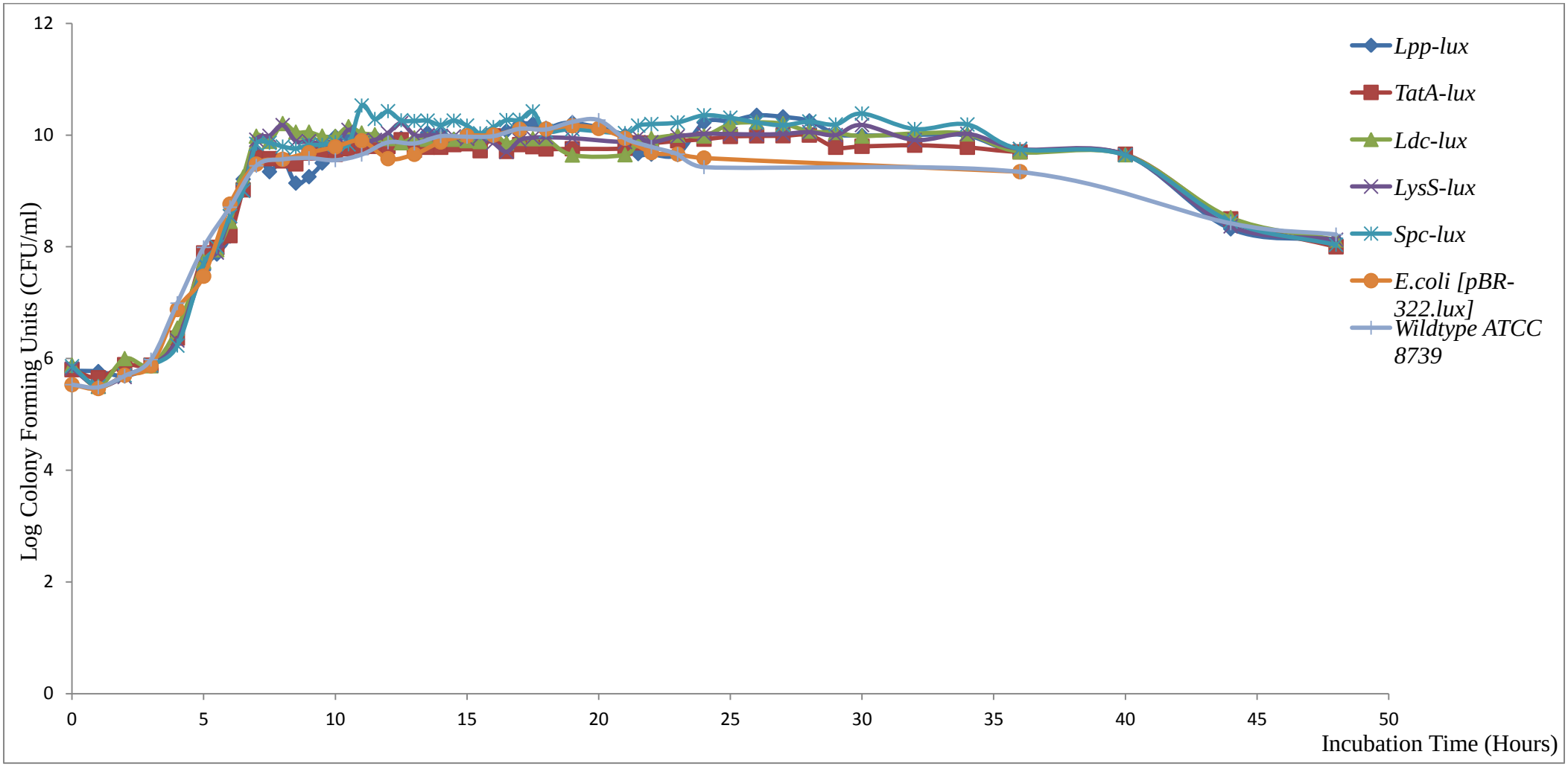


Figure 3.5 (b): Viable counts of the five bioluminescent *E. coli* ATCC 8739 bioreporter strains (*lpp-lux*, *tatA-lux*, *ldc-lux*, *lysS-lux*, and *spc-lux*) and controls (*E.coli* [pBR322.*lux*] and wildtype ATCC 8739 over 48 hours of incubation n=3. # The overall SEM is exceed 0.083 at 95% confidence interval. RLU vs CFU correlation coefficients: *lpp-lux* R^2 = 0.82; *tatA-lux* R^2 = 0.85; *ldc-lux* R^2 = 0.89; *lysS-lux* R^2 = 0.92; and *spc-lux* R^2 = 0.81; F test of (228, 21, 0.05)= 3.88

3.5.1.3 :Ratio of Bioluminescence to Viable Counts (RLU: CFU) in the Growth and Survival Curve

The ratio of bioluminescence to viable counts (RLU: CFU), gives an indication of the level of bioluminescence expressed per cell (Figure 3.5 (c). The RLU: CFU ratio was relatively low for all of the bioreporter strains carrying bioluminescent constructs on average 0.055 ± 0.13 during the first 3 hours of incubation. In contrast, the RLU: CFU ratio reached a peak between at the 5.5^{th} hour of incubation. The bioreporter strain carrying the *lpp-lux* construct demonstrated the highest peak ratio of RLU: CFU of 5.884, and this was followed by the *spc-lux* strain with an RLU: CFU peak of 5.01, then *tatA-lux* at 1.59, and *ldc-lux* at 0.51, whilst the lowest RLU: CFU ratio was exhibited by *lysS-lux* at 0.46. A Tukey post hoc statistical analysis revealed that there is a statistical significance ($P \leq 0.05$, Tukey Post Hoc) increase of RLU:CFU for *lpp-lux*, and *spc-lux* between the 5^{th} and 6^{th} hour of incubation. From the 6.5 hour to 10^{th} hour of incubation, the RLU:CFU ratios decreased significantly ($P \leq 0.05$, ANOVA). Finally, between the 10^{th} and the 48^{th} hour of incubation, the RLU: CFU ratio for each of the five strains was low averaging at 0.008 ± 0.3 with no statistical differences between the *E.coli* strains in figure 3.3 (c)).

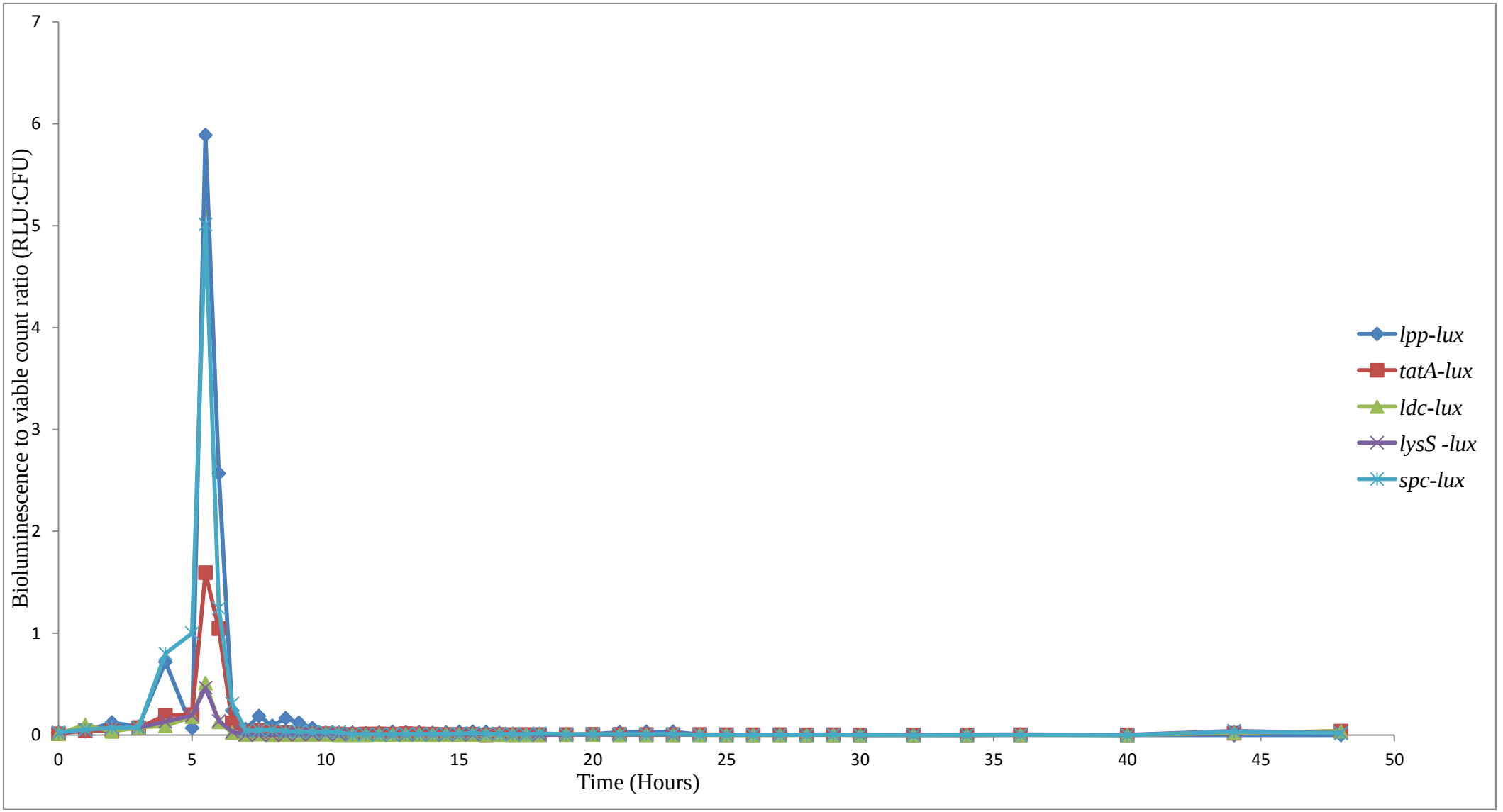


Figure 3.5 (c): The bioluminescence to viable count ratio (RLU:CFU) of the five *E. coli* ATCC 8739 bioreporter strains (*lpp-lux*, *tatA-lux*, *ldc-lux*, *lysS-lux* and *spc-lux*) over 48 hours of incubation n=3. # The overall SEM is 0.19 at 95% confidence interval.

3.5.1.4 : Bioluminescence, Viable Counts And Bioluminescence Per Cell (RLU: CFU) For The Bioreporter Strains Over 28 Days Of Incubation

The bioluminescence and viable counts were determined at 1, 2, 7, 14, 21, and 28 days of incubation (denoted as hours for graphical purposes) (Figures 3.6 a & b). Bioluminescence and population levels at 24 hours were on average 7.71± 0.03 $\log_{10}$ RLU/ml and 10.10± 0.02 $\log_{10}$ CFU/ml exhibited by the five constructs. Bioluminescence and population counts decreased at the 48th hour significantly, whilst sustaining levels of bioluminescence in terms of bioluminescence per cell (RLU:CFU) and populations across the significantly from the 48th hour to 168th hour.

The bioluminescence readings in the 168th hour decreased significantly to between 4.99 and 6.22 $\log_{10}$ RLU/ml; with *Spc-lux* retaining the highest bioluminescence at 6.22 ± 0.01 $\log_{10}$ RLU/ml, followed by *tatA-lux* (5.66 ± 0.013 $\log_{10}$ RLU/ml), *lysS-lux* (5.51 ± 0.02 $\log_{10}$ RLU/ml), *ldc-lux* (5.45 log10 ± 0.03 RLU/ml), and *lpp-lux* (4.99 ± 0.009 $\log_{10}$ RLU/ml) from the 48th hour. Whilst, viable counts decreased to an average 7.9 ± 0.008 $\log_{10}$ CFU/ml at 168 hours for all five constructs.

After the 336th hour of incubation, the bioluminescence levels decreases significant (P = 0.00, Tukey *Post Hoc*), in increasing order; *Lpp-lux* construct declined to 2.42 ± 0.001 $\log_{10}$ RLU/ml, *lyss* 3.42 $\log_{10}$ ± 0.02 RLU/ml, *tatA-lux* 4.10 ± 0.03 $\log_{10}$ RLU/ml, followed by *ldc-lux* to 4.89 ± 0.009 $\log_{10}$ RLU/ml, and *spc-lux* to 5.17 ± 0.001 $\log_{10}$ RLU/ml. Viable counts for all strains declined to an average of 0.2 ± 0.001 $\log_{10}$ CFU/ml measured at 336th hour with significantly ($P \geq 0.05$, Tukey Post Hoc).

At the 504th hour, bioluminescence levels of the four out of the five constructs increased from 336th hour by *tatA-lux* significantly ($P \leq 0.05$, Tukey Post Hoc) as compared to *ldc-lux lysS-lux, spc-lux* and *lpp-lux. Lpp-lux* continued to decrease significantly sharply from the 504th hour to 2 $\log_{10}$

RLU/ml at the 672th hour. Simultaneously, viable counts further decreased from 336th to the 672th hours with no significant differences. Bioluminescence per cell (RLU:CFU) expressed by *tatA-lux* were significantly higher at the 504th hour compared to *ldc-lux, lpp-lux, spc-lux* and *lysS-lux*.

Overall, a Tukey post doc test revealed the RLU:CFU exhibited by *lpp-lux* showed a significantly lower bioluminescent (P = 0.0, Tukey *Post Hoc*) compared to all the other four bioluminescent bioreporter stains in the extended phase of growth whilst no significant difference between *tatA-lux, ldc-lux, lysS-lux* and *spc-lux*. Statistically significant correlation coefficients between RLU vs CFU; *Lpp-lux*= 0.62; *TatA-lux*= 0.75; *Ldc-lux*= 0.74; *LysS-lux*= 0.95 and *Spc-lux* = 0.96 over extended period in Figure 3.6 (a & b).

The SEM for bioluminescence readings over the extended periods was 0.005, whilst the viable counts SEM was 0.014 (Figures 3.6 (a & b)).

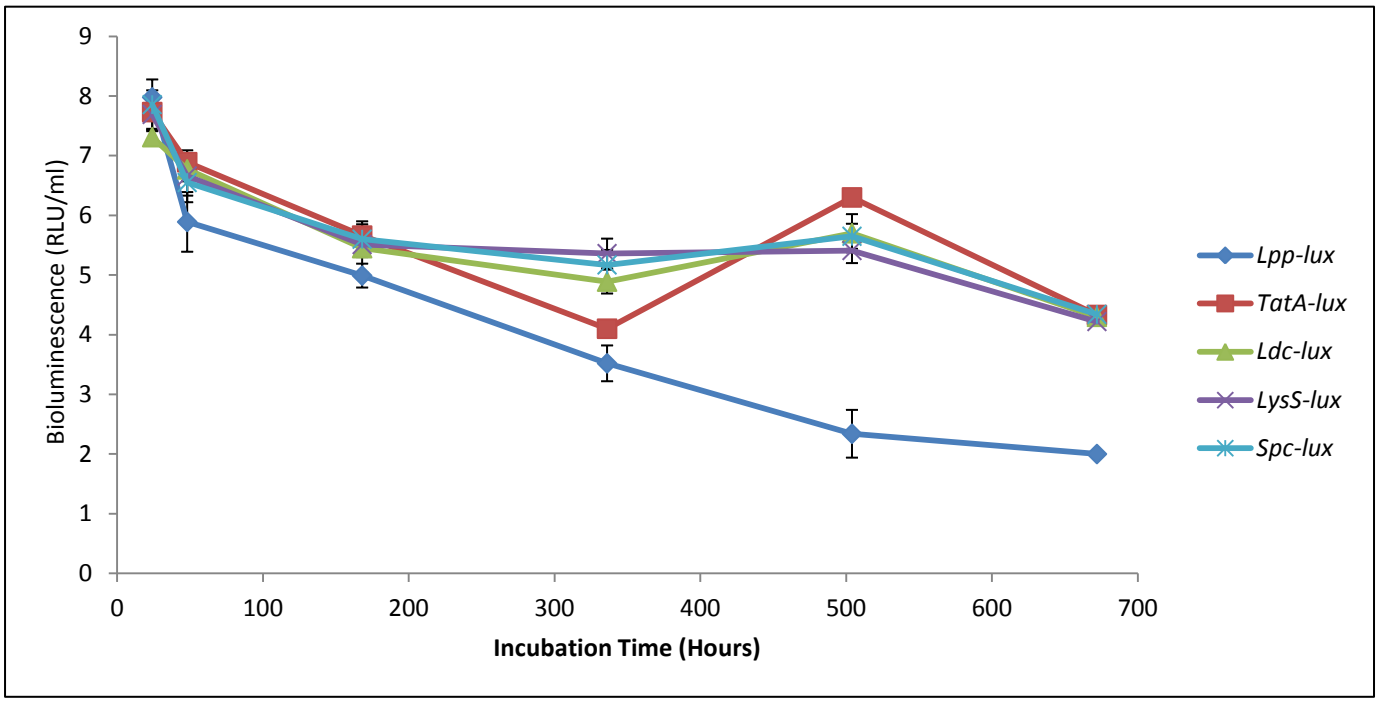


Figure 3.6 (a): Bioluminescence expression of bioreporter strains over extended incubation periods (1, 2, 7, 14,21and 28 d of incubation. n=3 #The overall SEM is 0.005 at 95% confidence interval

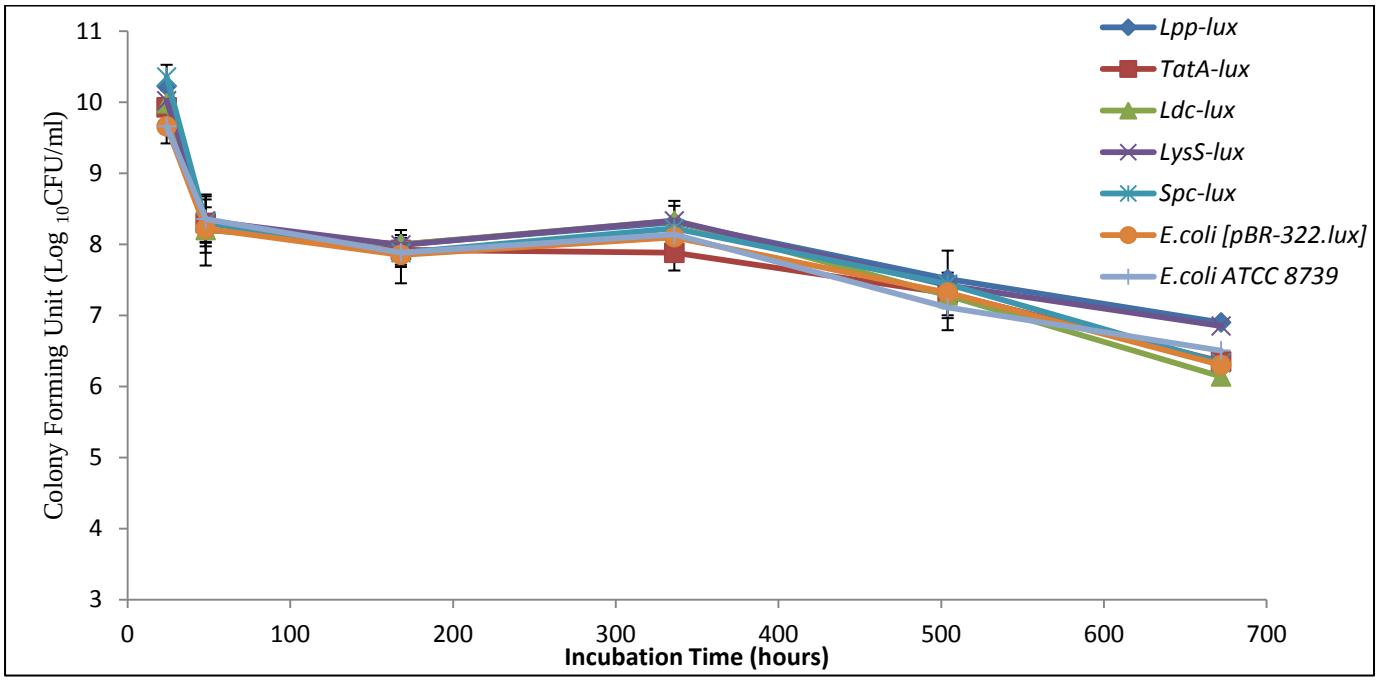


Figure 3.6 (b): Viable Counts of constructs and control strains over extended incubation periods (1, 2, 7, 14,21and 28 d of incubation. #The overall SEM of each biosensor does not exceed 0.014 at 95% confidence interval. n=3

Correlation coefficients RLU vs CFU; *lpp-lux*= 0.62; *tatA-lux*= 0.75; *ldc-lux*= 0.74; *lysS-lux*= 0.95 and *spc-lux* = 0.96 over extended period

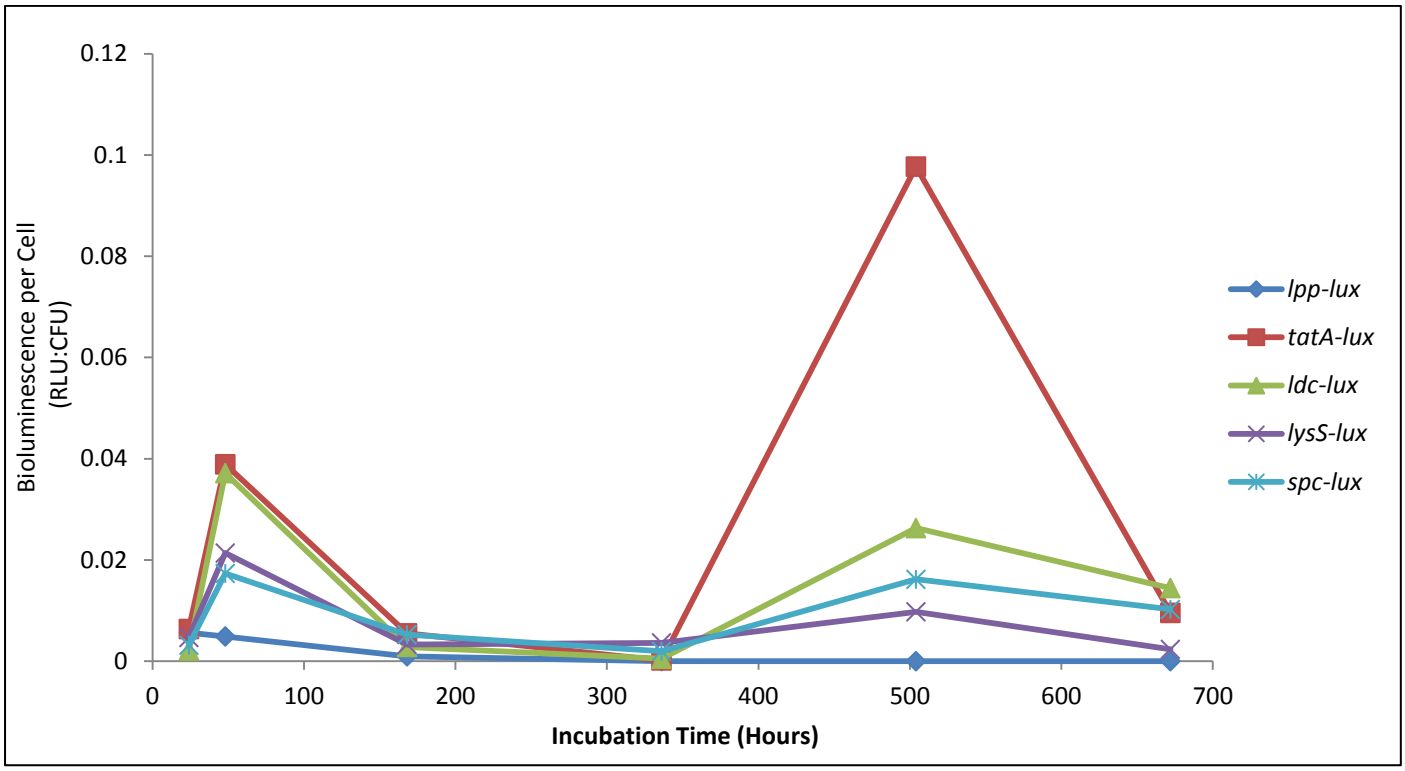


Figure 3.6 (c): The bioluminescence to viable count ratio (RLU:CFU) of the five *E. coli* ATCC 8739 bioreporter strains over incubation periods (1, 2, 7, 14,21 and 28 d of incubation (*lpp-lux*, *tatA-lux*, *ldc-lux*, *lysS-lux* and *spc-lux*) over 28 days of incubation. n=3
The SEM for RLU:CFU is 0.01 at 95% confidence Interval

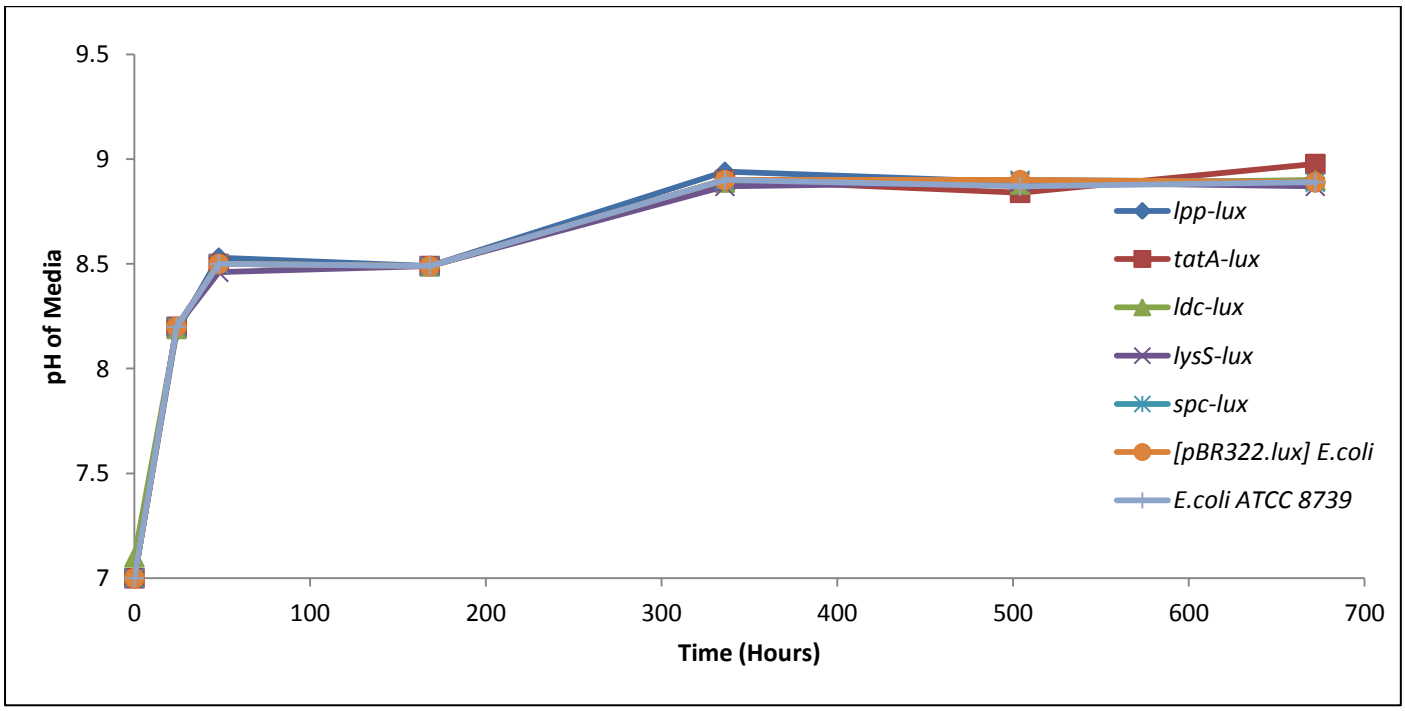


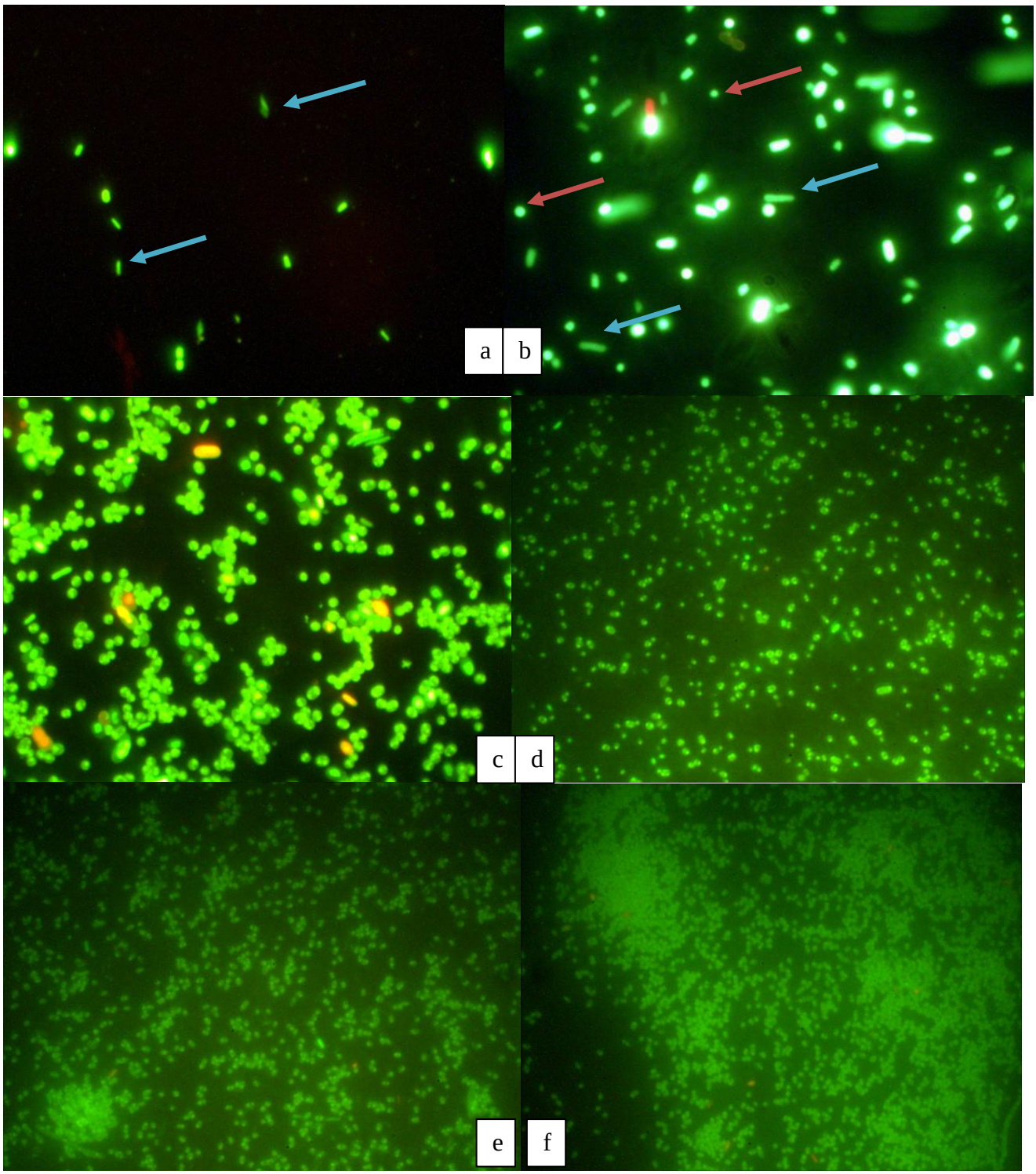
Figure 3.7: The average pH of the growth medium in the cultures at 0, 1, 2, 7, 14, 21 and 28 days of incubation during long-term culture of the *E. coli* strains. n=3
The SEM for pH in the media did not exceed 0.003 for each *E.coli* strain

3.5.1.5 : Culture Medium pH During Long-Term Incubation of 28 Days

The pH of the initial growth medium was pH 7, and this increased to pH 8.2 ± 0.001 after 24 hours of incubation with the bacterial cultures of all seven strains (Figure 3.7). The pH was between 8.53-8.49 ± 0.003 at 48 to 7 days (168 hours) of incubation, and continued increasing until pH 8.9 ± 0.001 at 28 days (672 hours) of incubation. The pH of the culture medium did not differ significantly between the *E. coli* strains at any of the time points.

3.5.2 : Morphological and Physiological Changes to the Bacterial Cell Over Extended period of 28 days

The *E. coli* bioreporter strains and also the control strains when examined microscopically immediately after inoculation were rod shaped (indicated by the blue arrows in figure 3.8 (a)). After 24 hours of incubation there was a mixture of cell morphologies, with almost 60% of cells demonstrating a spherical morphology (indicated by the red arrows in figure 3.8 (b) and approximately 40% of cells remaining rod-shaped (indicated by the blue arrows in figure 3.8 (b)). In contrast, all of the *E. coli* cells in incubated for 7, 14, 21 and 28 days demonstrated a spherical morphology (figures 3.8 (c), (d), (e), & (f) showed by the red arrows). The images here are representative of all of the *E. coli* strains grown.



Figures 3.8 (a) – (f): Fluorescence micrographs showing examples of *E. coli* cells, stained with SYTO-9 dye and captured at x1000 magnification. (a) the *lpp-lux* culture immediately after inoculation; (b) the *ldc-lux* at culture after 1 day of incubation; (c) the *lpp-lux* culture after 7 days; (d) the *E. coli* [pBr-322.*lux*] culture after 14 days; (e) the *lysS-lux* culture after 21 days; (f) the *lysS-lux* culture after 28 days.

Blue arrow indicates rod shaped *E. coli*
Red arrow indicates spherical *E. coli*

3.5.3 : Plasmid Copy Numbers (PCN) Determination

Figures 3.9 (a) & (b) are representative of the graphical output from the MJ DNA software showing fluorescence intensity against cycle number in a serial dilution of gDNA and pDNA. Figure 3.9 (a) shows an example of the cycle number (C_T value) versus fluorescence for the *dxs* target, whilst figure 3.7(b) shows an example of the cycle number (C_T value) versus fluorescence for the *bla* target.

The C_T values for the *dxs* and *bla* amplicons from each of the bioluminescent bioreporter strains were plotted against $\log_{10}$ gene copy number, and there was excellent correlation between C_T value and copy number for each (figure 3.10 (a), (b), (c), (d) & (e)) for all construct *E. coli* strains up to 24 hours of incubation with ≥ 4 orders of magnitude as determined by the MIQE guidelines. The linear equations for the calibration curves at 0 hour, 4 hour, 6 hour, 7 days, 14 days, 21 days and 28 days of incubation are reported in appendix III.

The slope for the relationship between C_T value and copy number (both for *dxs* and *bla*) were -2.5 to -3, which correspond to qPCR efficiencies of 110% to 150% for 0 hours and 4 hours of incubation, respectively (Appendix 3). Whilst slopes of between -3.6 and -2.8 were obtained at between 6 hours and 28 days of incubation, correspond to qPCR efficiencies of between 90% to 127% (Figures 3.10 (a), (b), (c), (d) & (e), and Appendix 3). The standard curves were within the dynamic range of 5 or 6 $\log_{10}$ magnitude order and PCN were tabulated within the 95% of log copies axis (x-axis) vs C_T values (Y-axis) and this was recommended by the MIQE guidelines (Bustin et al., 2009) across all time points. This demonstrates precision and reliability of quantification data. However, the qPCR efficiencies at 0 and 4 hours were between 110% to 150% for all constructs, which was higher than efficiencies obtained at 6 hours, 24 hours, 168 hours, 336 hours, 504 hours and 672 hours of all five constructs. This could be due to the lower DNA purity (1.5-1.69) (Appendix 3) extracted for

construction of standard curve at 0 and 4 hours. The correlation coefficients (R^2) for ct value versus copy number (both for *dxs* and *bla*) were between 0.87-0.99 from 0 hours to 4 hours incubation whilst the R^2 values obtained from 6 hours to 28 days of incubation were between 0.9-0.99. The SEM of the qPCR reaction obtained at all time points was between 0.005 and 0.05.

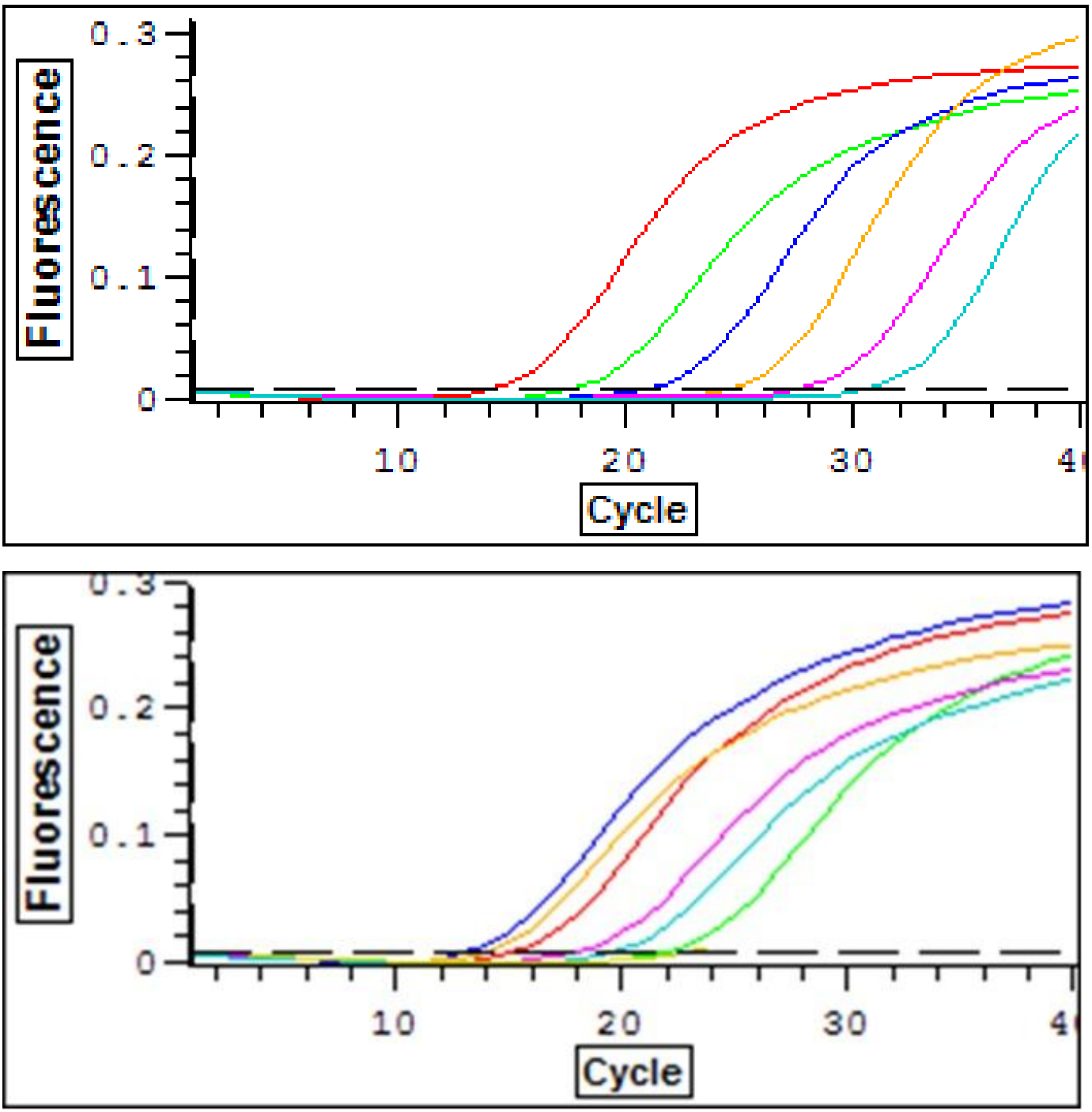


Figure 3.9 (a) & (b): qPCR curves for serial dilutions of gDNA and pDNA versus cycle number (C_T) for *dxs* and *bla*).

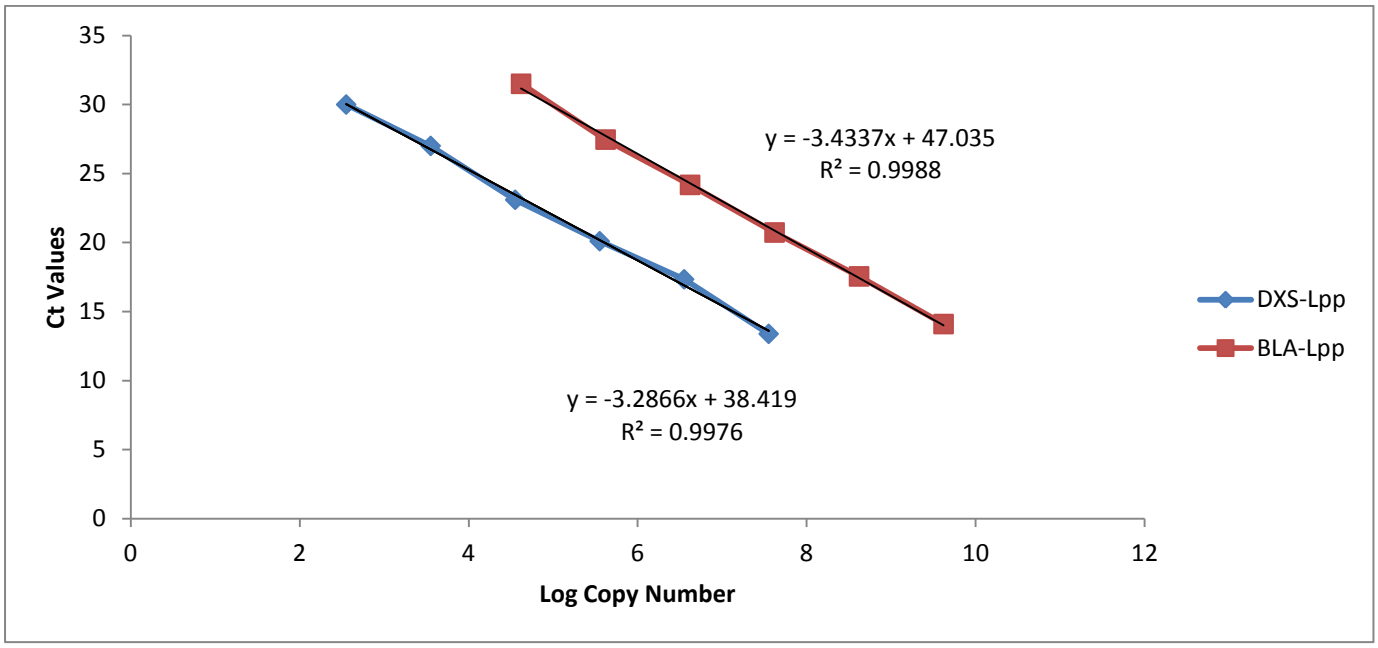


Figure 3.10 (a): Standard curves of C_T value versus $\log_{10}$ number of gene copies for both the *dxs* and *bla* amplification from *lpp-lux Esherichia coli* 8739. The C_T values showed an excellent correlation with the gene copy number for both the *dxs* and the *bla* genes n=3 (R^2>0.99 with qPCR efficiencies 92.38%, 94.31% respectively).

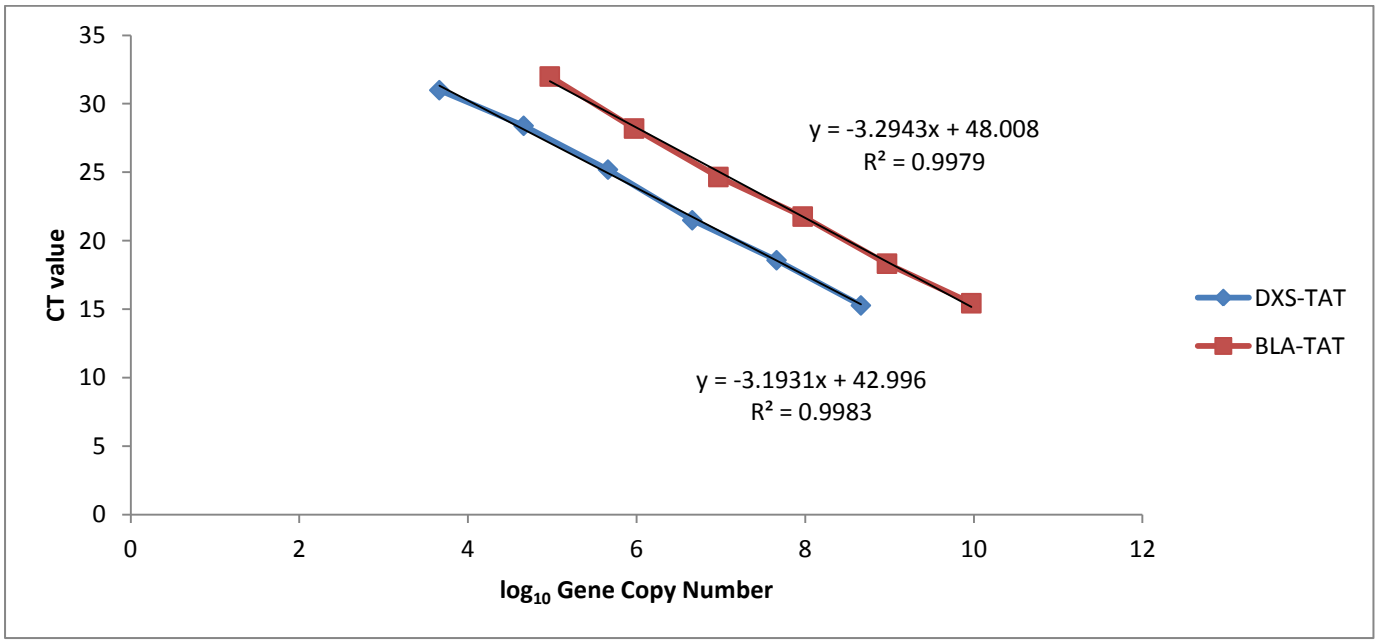


Figure 3.10 (b); Standard curves of C_T value versus $\log_{10}$ number of gene copies for both the *dxs* and *bla* amplification from *tatA-lux Esherichia coli* 8739. The C_T values showed an excellent correlation with the gene copy number for both the *dxs* and the *bla* genes n=3 (R^2>0.99 with efficiencies 101.16%, 105.67% respectively).

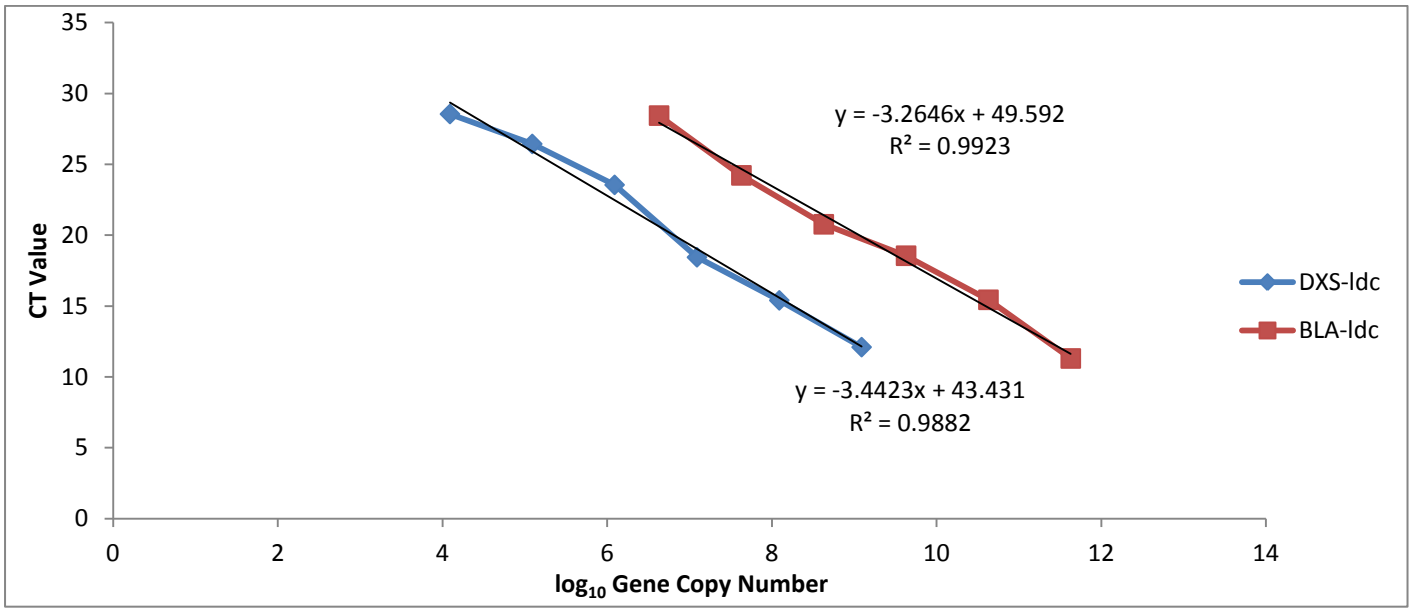


Figure 3.10 (c); Standard curves of C_T value versus $\log_{10}$ number of gene copies for both the *dxs* and *bla* amplification from *ldc-lux Esherichia coli* 8739. The C_T values showed an excellent correlation with the gene copy number for both the *dxs* and the *bla* genes n=3 (R^2>0.99 with efficiencies 97.53%, 101.26% respectively).

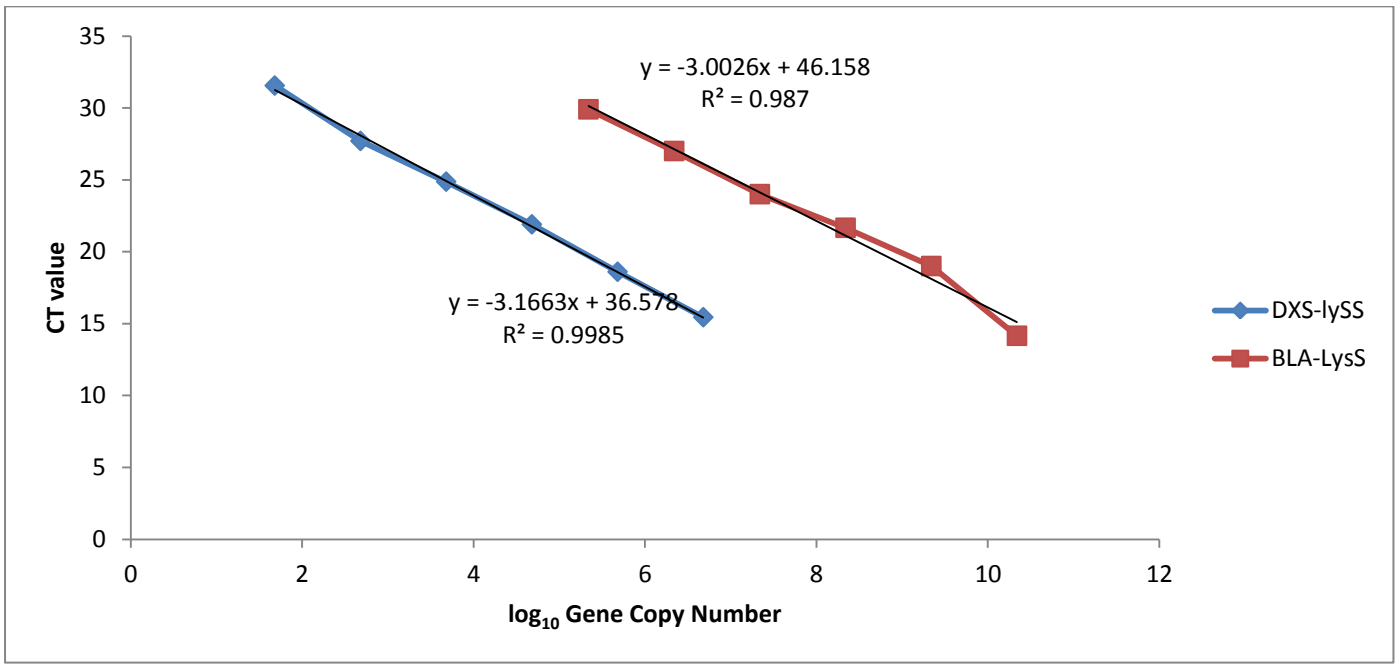


Figure 3.10 (d); Standard curves of C_T value versus $\log_{10}$ number of gene copies for both the *dxs* and *bla* amplification from *lysS-lux Esherichia coli* 8739. The C_T values showed an excellent correlation with the gene copy number for both the *dxs* and the *bla* genes n=3 (R^2> 0.99 with efficiencies 104.07%, 115.46% respectively).

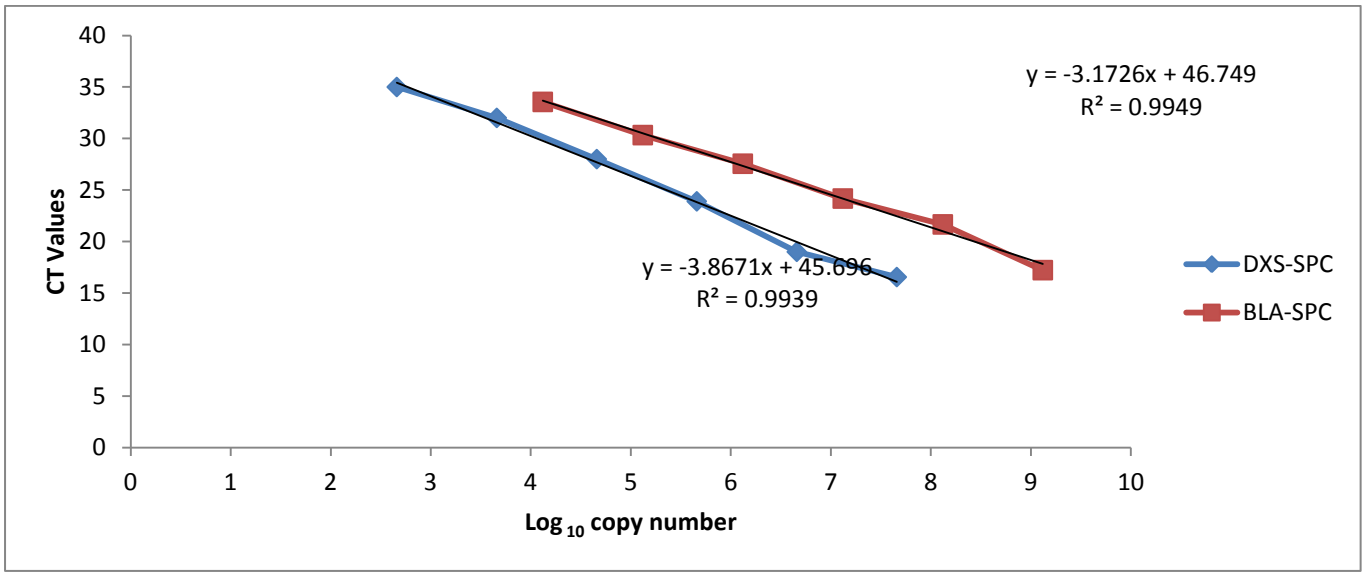


Figure 3.10 (e): Standard curves of C_T value versus $\log_{10}$ number of gene copies for both the *dxs* and *bla* amplification from *spc-lux Esherichia coli* 8739. The C_T values showed an excellent correlation with the gene copy number for both the *dxs* and the *bla* genes, n=3 (R^2> 0.99 with efficiencies 81.38%, 114.51% respectively).

The standard curve equations at other time points (*i.e.* 0, 4, 6, 168, 336, 504, and 672 hours can be referred in appendix 3

3.5.3.1 : Melting Curve and Fluorescence Intensity Curves

The melting curve generated by the qPCR instrument (Bio Rad) yields two items of information: these are the relative intensity of fluorescence (red arrow in figures 3.11 (a) & (b)); and fluorescence derivative (-dI/dT) (blue arrow in figures 3.7(a) & (b)).

The melting temperature for the *dxs* amplicon was 83.6 ± 0.05°C (Figure 3.11 (a)). Alongside figure 3.11 (a) is a photograph of a 2.0% agarose gel in which the reaction products were run: lane 1 shows the molecular weight markers (100bp ladder); lane 2 shows the *dxs* PCR product (113 bp). The melting temperature for the *bla* amplicon was 84.2 ± 0.05°C (figure 3.7 (b)). Alongside Figure 3.11 (b) is a photograph of a 2.0% agarose gel in which the reaction products were run: lane 1 shows the molecular weight markers (100bp ladder); lane 2 shows the *bla* PCR product (81 bp).

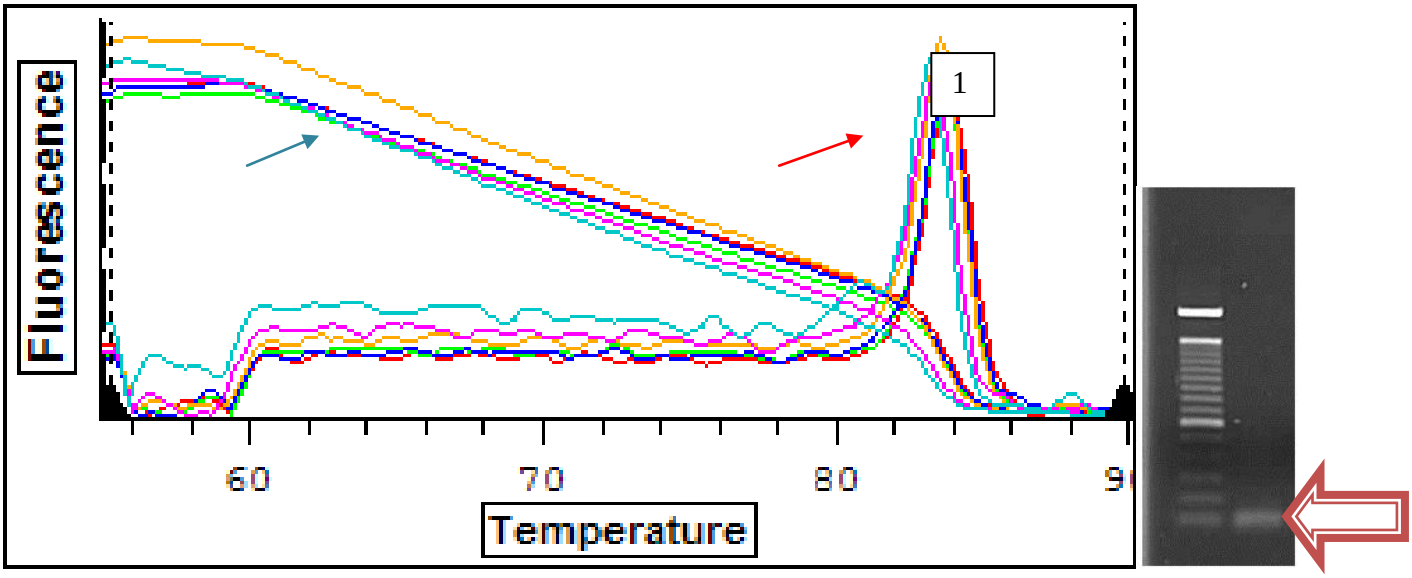


Figure 3.11 (a); Melting curve for serial dilutions of the gDNA extracted from *E. coli* 8739 *ldc-lux* after 24 hours of incubation. Peak (1) is due to melting of the *dxs* amplicons (at 83.6 ± 0.05°C). The picture alongside shows the *dxs* PCR product run on a 2.0% agarose gel along with a 100 bp ladder. The *dxs* product size is 114 bp

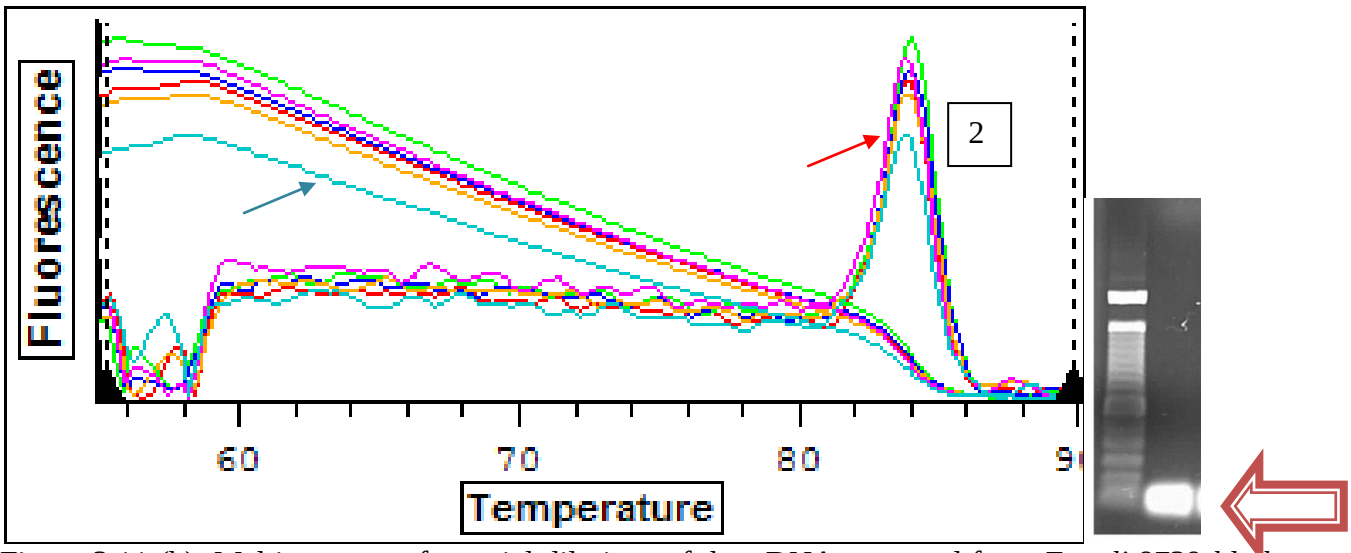


Figure 3.11 (b); Melting curves for serial dilutions of the pDNA extracted from *E. coli* 8739 *ldc-lux* after 24 hours of incubation. Peak (2) is due to melting of the *bla* amplicons at (84.2± 0.05°C). The picture alongside shows the *bla* PCR product run on a 2.0% agarose gel along with a 100 bp ladder. The *bla* product size is 81 bp.

3.5.3.2 : Plasmid Copy Number (PCN) for the Bioluminescent Constructs Within the *E. coli* Bioreporter Strains over 28 days of Incubation

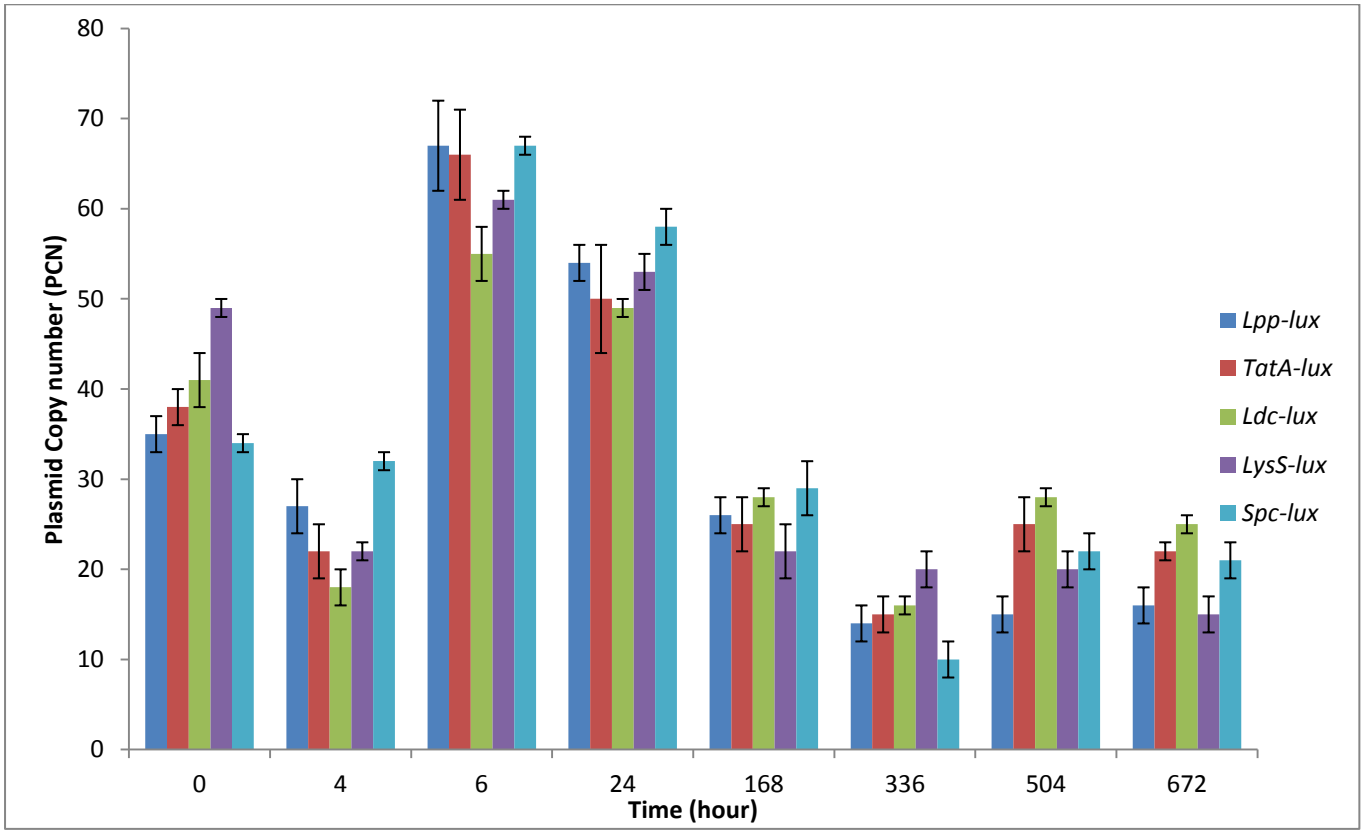


Figure 3.12: Plasmid copy numbers (PCN) of the five bioluminescent constructs within the *E. coli* strains over a 28 day incubation period, n=3 ± SEM.

Figure 3.12 shows the plasmid copy number (PCN) for the bioluminescent constructs in *E. coli* at each time point during 28 days of incubation. Immediately after inoculation (0 hour), the PCN for the *lysS-lux* construct was 49 copies per cell. The PCN at 0 h for *ldc-lux* was 41; for *tatA-lux* it was 38; for *lpp-lux* it was 35; and for *spc-lux* it was 34 with no significant difference amongst the constructs. The PCN in all of the bioreporter strains was statistically significantly lower at the 4th hour of incubation, in all of the strains, at approximately 18 to 32 copies/cell ($P \leq 0.05$, Tukey Post Hoc) After 6 hours of incubation, the PCN had recovered significantly to between 55 and 67 copies/cell. Tukey post hoc test revealed that there was statistically significant higher ($P \leq 0.05$, Tukey Post Hoc than PCN at 0, 4, 168, 336, 504, 672 hours. After 24 hours of incubation the PCN was between 49

and 58 copies/cell. A Tukey post-hoc test revealed that at 24 hour, there was statistically significantly higher ($P \leq 0.05$, Tukey Post Hoc) than 168, 336, 504 and 672 hours compared to 0, 4, and 6 hours.

The highest PCN at 24 h was demonstrated by *spc-lux* with 58, followed by *lpp-lux* with a PCN of 54, *lysS-lux* with a PCN of 53, *tatA-lux* with a PCN of 50, and last of all *ldc-lux* with a PCN 49. The PCN decreased significantly after 24 hours for all constructs and remained between 15-29 copies from 1 day to 28 days of incubation ($P \leq 0.05$, Tukey *Post Hoc*)

The coefficient of correlation between the RLU:CFU and the PCN values for each bioreporter strain was as follows: *lpp-lux* $R^2 = 0.59$; *tatA-lux* $R^2 = 0.90$; *ldc-lux* $R^2 = 0.84$; *lysS-lux* $R^2 = 0.78$; and *spc-lux* $R^2 = 0.80$.

3.5.4 : ATP standard

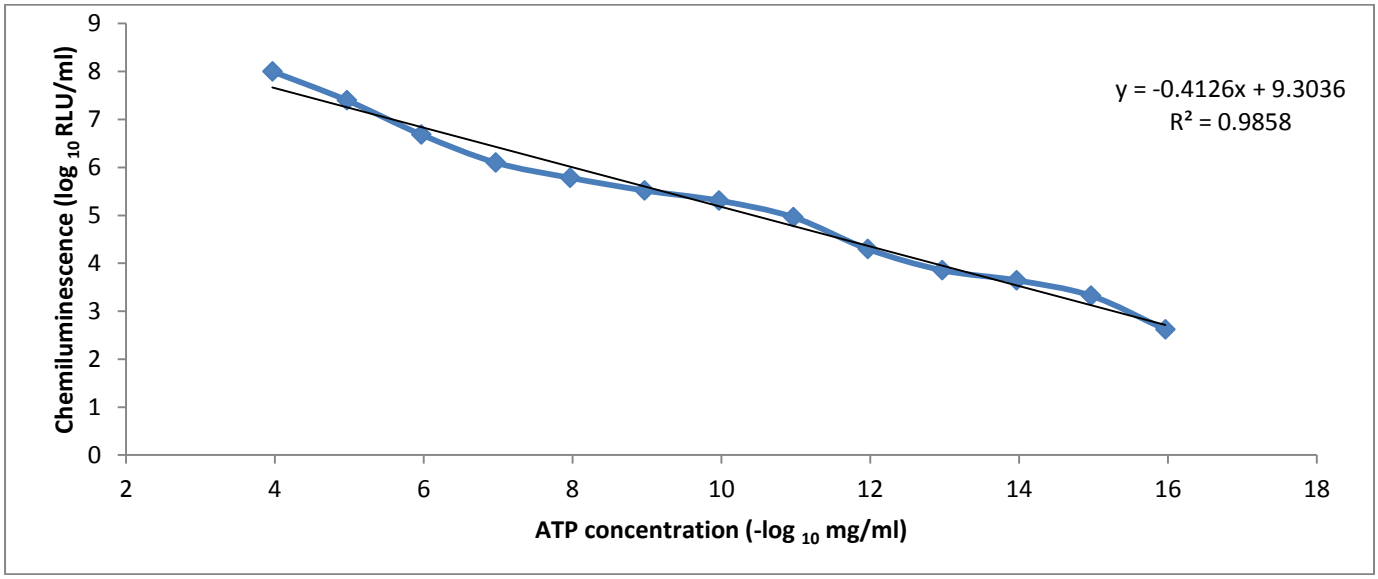


Figure 3.13: Standard curve of ATP concentration versus Chemiluminescence for the ATP-Chemiluminescence assay using luciferin-luciferase

The concentration of ATP in the standard solutions ranged from 10.8 fg/ml to 108µg/ml, which yields chemiluminescence of 3-8 $\log_{10}$ RLU/ml in the ATP-chemiluminescence assay. The chemiluminescence showed excellent correlation with the ATP concentration, over a range of 6 orders of magnitude (R^2: 0.9858).

3.5.4.1 : Intracellular ATP Content of the *E. coli* Bioreporter Strains Over 28 Days of Incubation

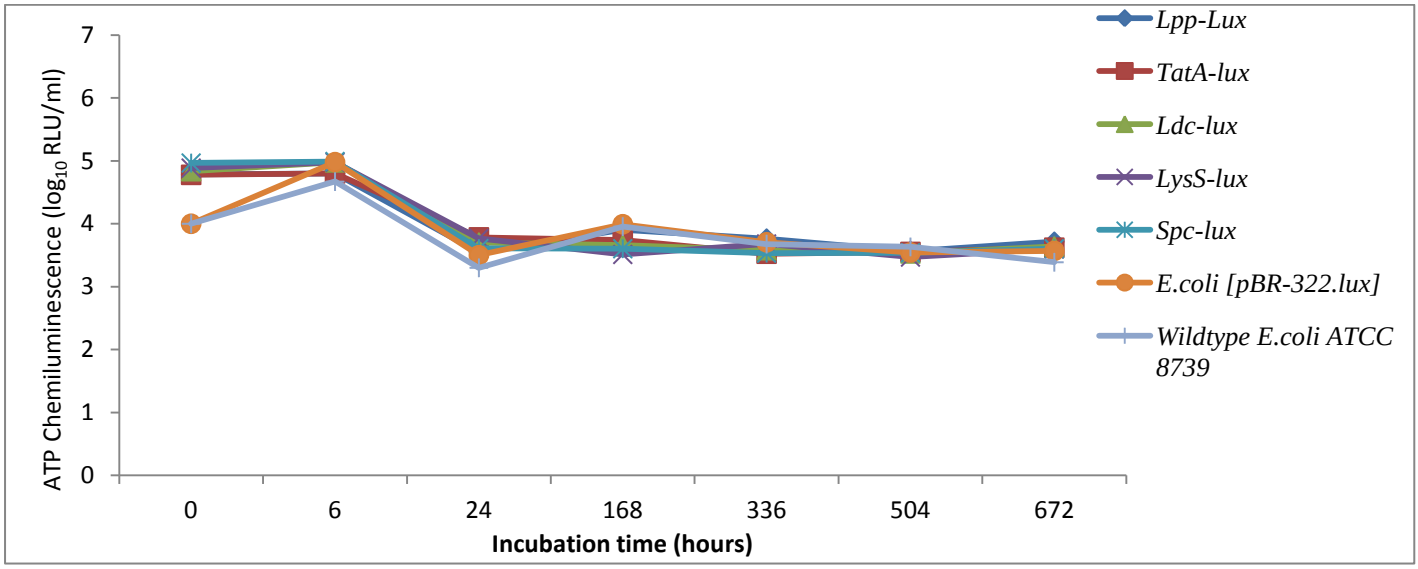


Figure 3.14: The intracellular ATP Concentration of the *E. coli* bioreporter and control strains over an extended incubation period of 28 days.# The overall SEM is 0.006 at 95% confidence interval, n=3

The amount of ATP present in each cell is expressed as $\log_{10}$ RLU/ml output from the ATP-bioluminescence assay. The range of initial ATP-chemiluminescence levels were between 4-4.9 $\log_{10}$ RLU/ml for the five constructs and the control strains. The amount of ATP present in the cells, as demonstrated by ATP-bioluminescence assay, peaked at the 6th hour of incubation for all strains between on 4.67-4.99 $\log_{10}$ RLU/ml with no statistical differences at this point. The amount of ATP present in all strains decreased significantly ($P \geq 0.05$, ANOVA) between 6 to 24 hours of incubation, as demonstrated by a 1.0-1.3 $\log_{10}$ RLU decrease in ATP-chemiluminescence; by all *E. coli* strains. From 168th to 672th hour of incubation, the levels of ATP chemiluminescence were on

average 3.6 ± 0.05 $\log_{10}$ RLU/ml by the five construct and control strains with no statistical differences.

The chemiluminescence readings for ADP and AMP and the levels of ATP, ADP and AMP readings were converted mg/ml from the standard curve obtained in figure 3.13. Due to the huge amount of figures obtained, the readings are located in the appendix 3.

3.5.4.2 : Adenylate Electron Charge (AEC) of the *E. coli* Bioreporter Strains Over 28 Days of Incubation

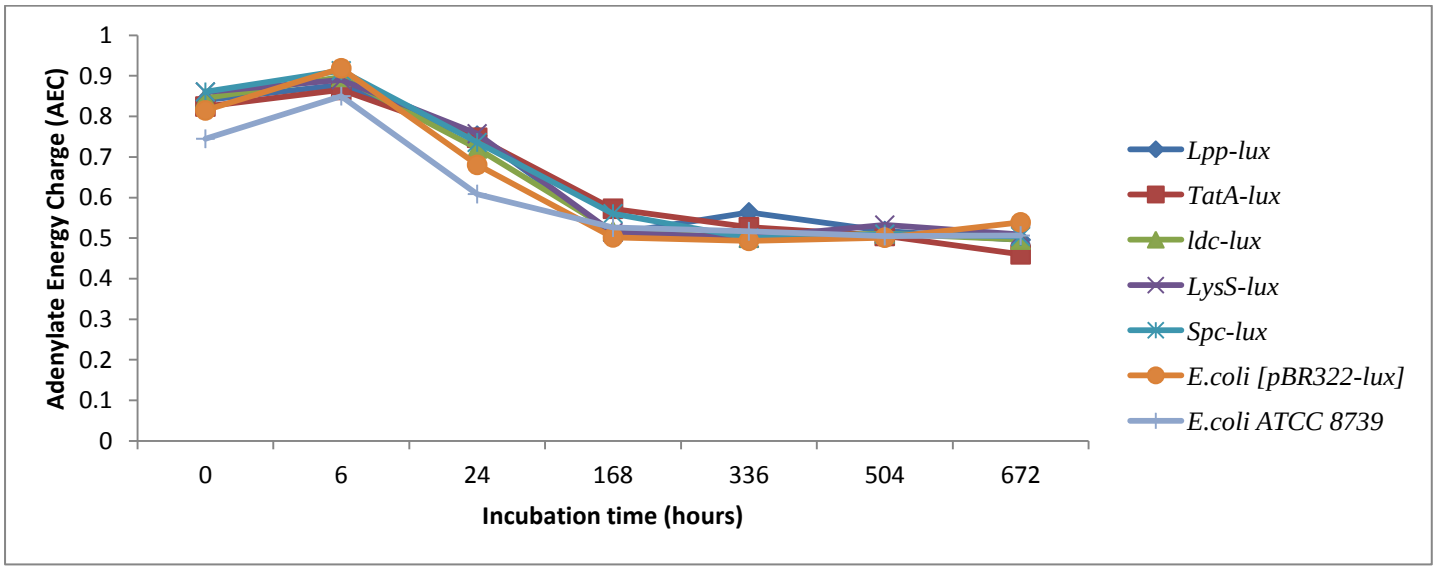


Figure 3.15: The Adenylate Energy Charge (AEC) of the *E. coli* bioreporter and control strains over an extended incubation period of 28 days. # The overall SEM is 0.003 at 95% confidence interval, n=3

The AEC peaked after 6 hours of incubation and, overall, followed the same trend as that shown by the ATP levels (Figure 3.10 versus Figure 3.11). Immediately after inoculation, the AEC was between 0.74 and 0.86 for each the five bioreporter and control strains, with no significant difference between the *E.coli* strains. At the 6th hour of incubation, the AEC values were between 0.85 and 0.91 for all of the *E. coli* strains with no significant difference. The AEC decrease significantly ($P \leq 0.05$,

Tukey Post Hoc) from 6th hour to 24th hour, the AEC values were between 0.60 and 0.75 for all of the *E. coli* strains. From the 7th to 28th day of incubation, the AEC of all of the *E. coli* strains fell steeply by between 0.5 and 0.57. A Tukey post-hoc test revealed no significant difference in the AEC levels from 24 hours to 168 hours.

3.6 Discussions

3.6.1 : Physiology, Morphology, Metabolic Status, And Plasmid Copy Number Of The Five *E. Coli* Biosensor Strains And The Control *E. Coli* Strains

The bioluminescence emission patterns resulting from *lux* expression by five constructs in the bioreporter strains followed the classical bacterial growth curve of a lag, an exponential, a stationary and a death phase. The lag phase is the period during which the microbial population adapts to its environment when it is changed suddenly, leading to a delay in the commencement of growth (Swinnen et al., 2004). The results obtained in this chapter reveals the lag phase of the bioluminescence, viable counts and bioluminescence per cell profiles lasted for the first 4 h of incubation (Figure 3.5 (a, b & c)) with no significant differences. During this adaptation phase the *E. coli* cells undergo intracellular changes to adjust to the nutrients in the new medium in order to initiate exponential growth, and at the same time little cell reproduction occurs (Buchman & Cygnarowics, 1990; Yates & Smotzer, 2007). Consequently, the limited growth and bioluminescence expression during the first 4 h of incubation in the experiments described in this thesis fits well with expectations. The reduction in bioluminescence expressions is also the result from the requirement of reduced cofactors during the adaptation process, during the lag phase in accordance with transient changes of internal metabolite concentrations (*e.g.* ATP; NADP; NADH; intracellular glucose, phosphoenolpyruvate; glucose-6-phosphate) (Buchholz et al., 2002; Hoque et al., 2005). During the lag phase, the housekeeping sigma subunit RpoD ($\sigma^{70/D}$) expression has been shown to produce a 2-fold increase during lag phase, beginning at about 20 minutes, and continuing into the exponential phase of growth (Rolfe et al., 2012). RNA synthesis during the lag phase is a prerequisite for the production of proteins required to equip the bacteria for exponential growth. It has been reported that the intracellular concentration of the core RNA polymerase (RNAP) remains constant during the

exponential and stationary phase, at around 2,000 complexes per chromosome equivalent in *E. coli* (Ishihama, 2000), with the activity of the RNAP being modulated by competition between the different sigma factors (Grigorova et al., 2006).

In this phase, the plasmid copies demonstrated lower copies during the first 4 hour of incubation, the plasmid copy number decreased to 18 - 32 copies at 4 hours decreased significantly ($P \geq 0.05$, Tukey *Post Hoc*) compared with 34 - 49 copies per cell initially (Figure 3.12). Previous studies by Lee et al (2006a) demonstrated lower PCN during lag phase due to the adjustment to the changes in the environment with minimal cell growth and DNA replication. This also coincides with previous studies carried out by Chao-Lin and Bremer (1986), and Klumpp (2011) where plasmid copy number of pBR322 is proportional to the doubling time of growth. During lag phase, the growth rate decreases as shown in this study and hence there is higher competition of RNA polymerase for plasmid and genomic replication (Klumpp, 2011) resulting in lower PCN, CFU and RLU as demonstrated in Figures 3.5 (a & b). Additional tabulation of the growth rate was carried out to demonstrate the relation between growth rate and PCN (Appendix 3).

The relation of bioluminescence expression and growth requires energy, the amount of metabolically available energy is stored in the adenylate system denote as adenylate energy charge (AEC) (Chapman et al., 1971). There was no statistical difference between the AEC levels between the five bioluminescent bioreporter strains and the control *E.coli* strains throughout the 28 days (Figure 3.15). Although initially, it was a concern that the biochemical pathway involving the oxidation of reduced $FMNH_2$ and aldehyde by molecular oxygen controlled by constitutive expressions would increase levels of kinase activity resulting in higher metabolic turnovers by the constructs with lower AEC values. Therefore, it is clear that the same metabolic load is exerted throughout 28 days and this demonstrated the suitability of the bioluminescent measurement exhibited by the bioreporter *E.coli*

ATCC 8739 strains when challenged in PET instead of the conventional plate counting method. In addition, previous report cited that luminometer (Multi-Lite, Biotrance Ltd) was capable of detecting 30 fg ATP, moreover, the celsis luminometer was able to obtain a lower detection of 10.8 fg ATP. Nevertheless, the amount of ATP per *E.coli* cell measured by the Celsis luminometer agreed with the measurements made by Multi-Lite which is approximately 3 fg per colony forming unit (Kyriakides & Patel, 1994).

The bioluminescence reached mid-exponential phase 30 minutes earlier than the viable counts at 5-6 hours (Figures 3.5 (a) & (b)). This resulted in large peaks in bioluminescence per cell (Figure 3.5 (c)) around mid-exponential phase. Previous study have demonstrated increased bioluminescence expressions resulting from the sudden change of metabolic activity from a non-limited glucose growth to a glucose limited condition led to higher concentration of $FMNH_2$ and consequently an strong and sharp signal of bioluminescence (Sunya et al., 2012). Bioluminescence levels per CFU (RLU:CFU) were significantly higher for *lpp-lux* and *spc-lux* than *ldc-lux*, *lysS-lux* and *tatA-lux* (Figure 3.5 (c)). The increased of constitutive expression for *lpp* and *spc* compared to the other three promoters were due to the expression levels contributed by the binding affinity of promoter regions with sigma factors (Jensen & Hammer, 1998) and also the possibility of the native promoter functions which this will be discussed in detail in the next section. On the contrary, recent study from Sunya et al. (2012) showed that the strong bioluminescence expressions is not dependent on the strains or on the type of promoter-*lux* fusions but is rather dependent on the metabolic activity of the cells and the biochemistry of bioluminescence. Hence, this study therefore presents the relation of bioluminescence expressions with combined effect of various constitutive promoters, metabolic activity and biochemistry of bioluminescence.

The increased bioluminescence and bioluminescence per cell is related to the significant increase of PCN from 0 hour to the 6th hour of incubation resulted in plasmid copies per cell of 55 to 67 copies/cell. The significant increase of PCN coupled with CFU at the 5th to 6th hour lead to a greater proportions of bioluminescence per cell expressed and possibly because a large proportion of the rod cells were elongated prior to septation and cell division at this point in time (Figures 3.8 (b)). Therefore, bioluminescence output per cell (RLU:CFU) is expected to be greater between the 5th and 6th hour. Previous studies by Amin-Hanjani et al. (1993) reported similar phenomenon of high copy number contributed to the higher luminescence per cell. This was supported by relation of correlations values (R^2: 0.59-0.9) between bioluminescence per cell levels and PCN for the five constructs except *lpp-lux* which is discussed in the later test. Similar analysis in studies conducted by Rattray et al. (1990) demonstrated bioluminescence per cell exhibited by *E.coli* DH1 [pUCD607.*lux CDABE*], *E.coli* MM294 [pBTK5.l*ux RICDABE*] at exponential phase was 0.13 (RLU:CFU). According to Rattray et al. (1990), the variation levels of bioluminescence between plasmids (*i.e.*pUCD607 and pBTK5) were reflected by the differences in plasmid copy number and constitutive promoter expressing bioluminescence. Hence, in this study, the bioluminescence per cell (RLU:CFU) was higher (0.467 - 5.88) in the exponential phase than in the stationary phase (0.001-0.0009) for each of the biosensor strains. This suggests that the five promoter chosen have higher constitutive activity in expressing high bioluminescence levels and stable plasmid copy number.

Proceeding on to exponential phase, there were approximately 55-67 copies of pBR322 in the five *E. coli* bioreporter strains, which corresponds well with previous observations that *E. coli* harbours between 30 and 70 copies of pBR322 (Atlung et al., 1999; Lee et al., 2006a). pBR322 replication is regulated by the ratio of its self-encoded promoters, RNA I and RNA II, where RNA II is known as an initiation preprimer. The high copy number of pBR322 during exponential phase would suggest that high levels of RNA II, which initiates replication efficiently, are present in the cell, and at the

same time it suggests and absence of the RNA I/RNAII duplex, to inhibit plasmid replication (Atlung et al., 1999).

Exponential bacterial growth and replication involves multiple rounds of DNA synthesis, coupled with transcription and translation, to synthesize the necessary macromolecules. In the exponential stage, there is a high demand for energy, hence the AEC for all of the *E. coli* strains in exponential phase was between 0.9 and 0.93, which compares well and agrees with previous studies that demonstrated that growth was possible with an AEC of 0.8 or above in *E.coli* (Chapman et al., 1971). The increase in the energy status of the cell, as reflected in a rise in the AEC is due to the increased amount of phosphoenolpyruvate that is converted to pyruvate and oxaloacetate for use in the biosynthetic pathways and for energy production through glycolysis, the tricarboxylic acid cycle (TCA), electron transport and oxidative phosphorlyation (Liao & Atkinson, 1971). Furthermore, bioluminescence emission, CFU counts and PCN are at their highest during exponential phase, when replication of the chromosome and the plasmid are also at their maximum.

The plateau in the bioluminescence and viable counts from 7.5 to 40 hours of incubation, is typical of the stationary phase. During this phase, no increase was observed in either the bioluminescence or the viable counts. The depletion of nutrients and the accumulation of bacterial waste products (eg ammonium cation) resulted in an increase of the pH of the growth from 7 to 8.5 by 48 hours of incubation. Clifton (1937) reported the pH of *E.coli* cultures have rapidly shifted to alkaline values reporting at pH 8.4 or higher in peptone medium. At high pH *E. coli* requires a greater import of protons to counteract the alkaline stress on cytoplasmic pH (Maurer et al., 2005) which could lead to death phase.

The death phase occurred from the 40th hour to 48th hour of incubation during which there was a significant decline of 1-2 $\log_{10}$ CFU/ml. During the death phase, dead cells are lysed and release nutrients in to the depleted growth medium that can then be exploited by the surviving population of *E. coli* (Navarro Llorens et al., 2010). As mentioned in chapter 1, the light emitting reaction involves an intracellular, luciferase catalysed by oxidation of reduced $FMNH_2$, molecular oxygen and aldehyde. Since reduced $FMNH_2$ production depends upon fractional electron transport, only live cells are able of producing light. With strong correlations obtained during the growth curve, this confidently demonstrated the relationship between cellular viability and light that endows bioluminescence with the 'reporting' power on the viability status. This attribute of *invivo* bioluminescence as a bioreporter of viability has been demonstrated in many past studies (Hill et al., 1993; Ellison et al., 1994a & b; Marincs, 2000; Steward, 1990, 1993; Stewart & Williams, 1992,; Stewart et al., 1991, 1993, 1996, 1997).

During long term stationary phase, from 7 to 28 days of incubation, the AEC values were between 0.50 and 0.58, with no significant difference between the *E. coli* bioreporter and control strains. This agrees with the work carried out by Chapman et al. (1971) which showed that cell viability could be maintained, but that growth was not possible, at AECs of between 0.5 and 0.8. The significantly lowered AEC from 24 hours to the extended incubation time of 7 to 28 days suggest that there will be less energy available for transcription process of the *lux* cassette to produce bioluminescence (Figure 3.15). This coincides with the significant decrease of PCN from 24 hours to 7, 14, 21 and 28 days in long term stationary phase. Therefore, the combined effect of less energy and lowered PCN values in the extended time frame resulted in the decreased bioluminescence.

The bioluminescence and viable counts were lower between 7 and 14 days of incubation, which may result from the specific targeting of the reduced metabolic activity upon maintenance of cell viability

by, for example repairing the macromolecular damage accumulated in stationary phase (Rolfe et al., 2012). Between the 14th and 21st days of incubation, there was an increase significantly in bioluminescence levels from both the *tatA-lux E. coli* and the *ldc-lux E. coli*. The greater increase of bioluminescence expression with resulted in the higher RLU:CFU for *tatA-lux E. coli* at the between the 14th and 21st day. Previous studies from Maurer et al (2005) demonstrated a significant increase in *tatA* expression at pH 8.7 whilst the pH recorded in this study was pH 8.89 between day 14 and 21 (Figure 3.7). This suggests the potential of *tatA* constitutive promoter to remain high bioluminescence intensity in high alkaline conditions.

The fluorescence microscopy images suggest that during long term stationary phase (from 7 to 28 days) the *E. coli* cells undergo both physical and morphological changes, becoming smaller and more spherical (Figure 3.8 (a-f). Loewen and Hengge-Aronis (1994) showed that this move to a coccoid morphology was a result of induction of the *bolA* gene. The cytoplasm becomes condensed, while the volume of the periplasm increases. The composition of the cell membrane is altered to produce a less fluid membrane and the nucleoid becomes condensed by replacement of some DNA-binding proteins with other species of DNA binding protein, which results in a multi-resistant state, when the cells become more thermotolerant and more resistant to oxidative, acid, and osmotic stresses than when they are in exponential phase (Loewen & Hengge-Aronis, 1994). There is also an increase in the amount of DnaA protein, which is required for initiation of chromosome replication in the late stationary phase (Talukder et al., 1999). Under these conditions, *E. coli* cells with a growth advantage in the stationary phase (GASP) phenotype may grow within the culture to either coexist with the parental majority or displace the parent (Zambrano et al., 1993).

This attribute is important to ensure the bioluminescence readings reflect the viability across the PET time frame. Furthermore, the plasmid copy numbers measured for the five constructs

demonstrated stability across the PET time frame. This chapter covers the extensive study of the bioluminescent bioreporter strains where there are no statistical differences between the bioluminescence, viable counts, AEC, and PCN across 28 days which thereby demonstrated legitimate of the PET application in the proceeding chapters.

3.6.2 : Relationship Between Extended Stationary phase and Bioluminescence

Along growth and survival phase, the *E.coli* strains spend a considerably long amount of time in stationary phase where there is no significant increase in viable counts. In stationary/starvation phase, the expression of genes of both the RpoS (encoding $\sigma^{38/S}$) and the RpoD (encoding $\sigma^{70/D}$) regulons are important, as both σ factors compete for the core polymerase since they both regulate expression of genes required for survival in stationary/starvation phase (Finkel, 2006). However, the levels of active RpoS (encoding $\sigma^{38/S}$) are extremely low during exponential growth (Jishage et al., 1996), and increases by up a 3 to 6 fold increase in RpoS mRNA levels occurs during the transition from the exponential to stationary phase (Yamashino et al., 1995) and RpoS regulates more than 30 genes (Hengge-Aronis, 1996; Nystrom, 1994). Consequently, a different set of recognition sequences is required for genes to be expressed in stationary phase than is required in exponential phase. Furthermore, anti-sigma factors have been identified, such as the anti-RpoD ($\sigma^{70/D}$) factor, and so it is possible that the activity of RpoD ($\sigma^{70/D}$) is controlled to permit RpoS ($\sigma^{38/S}$) to up-regulate transcription of the genes that it regulates more effectively (Hughes & Mathee, 1998).

RpoS ($\sigma^{S/38}$) regulates expression in stationary/starvation phase by recognition of particular promoter regions. The -10 region consensus sequence is identical for both RpoS ($\sigma^{S/38}$) and RpoD ($\sigma^{D/70}$) except that in the -8 position the adenine (A) of RpoD ($\sigma^{D/70}$) is replaced with a cytosine (C) in RpoS ($\sigma^{S/38}$). At the-8 position, *tatA*, *ldc*, *lysS* and *spc* carried an adenine (A), whilst *lpp* carried a cytosine (C) which is the consensus of RpoS ($\sigma^{S/38}$). This did not significantly affect the bioluminescence

levels of *tatA-lux, ldc-lux, lysS-lux,* and *spc-lux* throughout stationary phase except for *lpp-lux.* RpoS ($\sigma^{S/38}$) has a preference for either a T or G nucleotide at position -14, and since *spc* and *lpp* have a G this suggests they would have an increased affinity for σ^{S} during starvation/stationary phase (Becker & Hengge-Aronis, 2001). However, bioluminescence expression by *spc-lux* was not significantly increased during the long term stationary phase. Never the less, bioluminescence expression from the *lpp-lux E. coli* decreased significantly more rapidly than it did from the *lysS-lux, ldc-lux, tatA-lux,* and *spc-lux* under long-term incubation in comparison to the bioluminescence expression exhibited by *lpp-lux* in early stationary phase.

Becker and Hengge-Aronis (2001) reported that the difference in the extended regions of RpoD ($\sigma^{D/70}$) to RpoS ($\sigma^{S/38}$) is the preference of G at the -13 position (Table 2.4) to a C at position -13 of RpoS ($\sigma^{S/38}$). This relates to accommodation of gene expression at different phase of growth for RpoD ($\sigma^{D/70}$)-controlled promoters during exponential phase whilst expression of genes regulated by RpoS ($\sigma^{S/38}$) is acquired under conditions of low or no growth (in stress conditions). RpoS ($\sigma^{S/38}$) and RpoD ($\sigma^{D/70}$) diverged relatively recently in evolutionary terms, and are still relatively similar, but different selective pressures have been acting upon RpoS ($\sigma^{S/38}$) and RpoD ($\sigma^{D/70}$) mediated gene expressions (Becker & Hengge-Aronis, 2001). This selective pressure of sigma factors of RpoS ($\sigma^{S/38}$) and RpoD ($\sigma^{D/70}$) during the growth phase (*i.e.* lag, exponential, stationary phases) could be one of the factors which could influence the expression of bioluminescence, more significantly during starvation/stress periods.

In relation to the bioluminescence per cell expressed over 28 days, bioluminescence per cell exhibited by *lpp-lux E. coli* was significantly much lower than from the other four constructs over the extended incubation period. The significant ($P \leq 0.05$, Tukey *Post Hoc*) reduction in the *lux* expression for *lpp* may suggests down-regulation of this promoter during long-term stationary phase, which has not previously been reported in the literature. Previous studies have demonstrated strong

induction of starvation lipoprotein, *slp*, in response to entry to stationary phase (Alexander & St John, 1994). It has been demonstrated that the *slp* promoter resembles the consensus sequence for σ^{70} (-35 region: ATGAAA, -10 region: TATTAT) (Alexander & St John, 1994). In addition, the *slp* promoter contains a region that has interrupted dyad symmetry between -36 and +5. The region modulates the expression of *slp* during periods of growth and starvation (Alexander &St John, 1994). The role of *slp* is to maintain the structural integrity of the cell surface layers and help stabilize the outer membrane of *E. coli* during carbon starvation and the stationary phase (Alexander & St John, 1994).

Amongst the other four bioreporter strains, *tatA-lux* strains exhibited significantly higher bioluminescence per cell relatively (Figure 3.6 (c)) compared to *ldc-lux*, *spc-lux* and *lysS-lux* in the extended phase in alkaline pH. The biological function of *tatA* is in exporting pre-folded proteins from the cytoplasmic membrane to periplasm (Santini et al., 1998). Therefore, this relates the levels of bioluminescence expressions to the biological functions of *tatA*, where in starvation/stationary phase, this suggest that there would be an increased need to export proteins for viability maintenance. The biological function of *ldc* is to synthesize polyamines which are needed for ribosomal functions and growth (Tabor & Tabor, 1985). In addition, cells respond to starvation by reduced biosynthesis in ribosomal proteins and DNA replication (Llorens et al., 2010), hence expressions of *spc and lysS* would be expected to reduce in extended incubation period. Neusser et al., (2010) denoted that mRNA levels of lysyl- t-RNA synthetase enzyme (*lysS*) decreased during stationary phase which would explain lowest RLU:CFU after *lpp-lux* in the extended phase. Regulation of an alternative lipoprotein, *slp* was known to be regulated under long term stationary phase (Alexander & St John, 1994) which explains the significant decreased in bioluminescence levels. Meanwhile, high amount mRNA levels of *spc* operon were expressed during exponential phase (Wei et al., 2001) which agrees with the results obtain in exponential phase. Therefore there is an indispensable relation between the biological function of the promoter and sigma factor

competitions which relates to expression levels bioluminescence at different growth and survival phases.

In summary, the *E. coli* bioluminescent biosensor strains have demonstrated similar physiology to control strains, in terms of the growth and survival curves, and adenosine energy charge. The relative stability of the plasmid copy number per cell over the 28 days of incubation contradicts the suggestion made by Turdean (2011) which mentioned that one of the disadvantages of whole-cell biosensors is a lack of stability due to the loss of plasmids from the bioreporter cells. However, the PCN results in this study have shown otherwise, indicating no significant decrease of PCN after 7 days to 28 days of incubation. However, studies from Bechor et al. (2002) showed decrease bioluminescence intensity measured from plasmid based *lux* strain compared to chromosomal integration strain. Therefore, despite the use of plasmid strain in this study, low bioluminescence background were also measured under glucose limited condition for biosensor and control strains; promoter-less *lux E.coli* ATCC 8739 strain, in contrary to *rpoS* controlled promoter in Notley and Ferenci, (1996).

Furthermore, Turdean (2011) also stated that the experimental conditions such as medium pH, incubation time, and buffer and reagent composition could have affect bioluminescence expression, and thus biosensor performance. However, the excellent stability of the bioluminescence exhibited by the biosensor strains over 28 days of incubation under changing conditions, such as increasing pH, again contradicts Turdean (2011). Hence, it would seem that these bioluminescent constructs with the chosen constitutive promoters have the potential to succeed in whole-cell microbial biosensors.

3.7 : Conclusions

1) The growth curves indicate that the four biosensor constructs are indeed expressed constitutively with strong correlations (R^2: 0.74-0.92) values were obtained between RLU and CFU across 28 days for all constructs. However, the expression of bioluminescence by the *lpp-lux E. coli* decreased more rapidly than it did in the other four strains.
2) There is no statistical differences between the bioluminescence, viable counts, AEC, and PCN across 28 days which thereby demonstrated legitimate of the PET application in the proceeding chapters.
3) There was no significant difference in the total AEC in either the five bioreporter strains or the *E. coli* control strains over 28 days demonstrating equal metabolic load.
4) There is an indispensable relation between the biological function of the promoter and sigma factor competitions which relates to expression levels bioluminescence at different growth and survival phases.

Chapter 4

4 Whole-Cell Bioluminescence Evaluation Using Sorbic Acid

4.1 : Sorbic Acid as a Preservative

Sorbic acid (2,4-hexadienoic acid) is a straight chain unsaturated fatty acid with a molecular weight of 112.13 g/mol and the formula: CH3- CH = CH - CH = CH – COOH (Figure 4.1) (Sofos & Busta,1981).

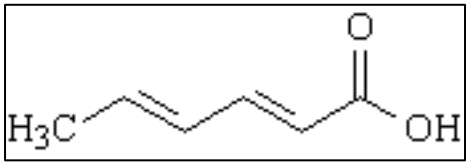


Figure 4.1: Chemical Structure of Sorbic acid

Sorbic acid is commercially produced in powder or granule form; it has a characteristic acrid odour and acid taste (Sofos & Busta, 1981). The carboxyl (COOH) group in sorbic acid is very reactive and can form salts with calcium, sodium and potassium (Sofos & Busta, 1981). A.W. Van Hoffman first isolated sorbic acid from berries of the mountain ash tree in 1859 (Sofos & Busta, 1981) and the antimicrobial properties of sorbic acid were first recognized in the 1940's. Exposure to sorbate results in an extension of the lag phase, regardless of the growth rate of the bacterial culture (Chung & Lee, 1982, Greer, 1982; Larocco & Martin, 1981; Tsay & Chou, 1989; Zamora & Zaritzky, 1987). The effectiveness of sorbate salts against bacteria, yeasts and molds gave rise to its extensive use in foods, cosmetic products, and ophthalmic products (Sofos & Busta, 1981). The addition of small amounts of sorbic acid to food also does not alter the taste, flavour and nutrient content of the food.

4.1.1 : Mode of Action

The primary mode of action of sorbic acid is a result of the partial dissociation of a weak acid. Weak acids exist in varying mixtures of dissociated and undissociated molecules in aqueous solutions. The undissociated form of a weak acid can readily permeate the plasma membrane of microbial cells and is therefore able to diffuse freely into the cytoplasm (Booth & Kroll, 1986) (Figure 4.2). This diffusion of undissociated acid reaches an equilibrium when the internal and external concentrations become equal. Most neutralophilic microbes maintain a pH gradient across their cytoplasmic membranes, with the internal pH higher than the external. Consequently, the acid molecules will tend to undergo dissociation once they have diffused into the cell until the pK_a is achieved. This means that further undissociated acid molecules will diffuse into the cell in order to achieve equilibrium, so fuelling further increases in the dissociated acid anion and proton concentrations. The accumulated high levels of charged weak acid in the cytoplasm results in a decrease in internal pH (pH_i). This drives the proton translocation activity of the H^+-ATPase, in order to expel hydrogen ions and maintain pH_i homeostasis (Bracey et al., 1998; Cole & Keenan, 1987; Eraso & Gancedo, 1987; Salmond et al., 1984). The maintenance of pH_i homeostasis can be energetically expensive (Eraso & Gancedo, 1987; Serrano, 1980, 1984), and can result in consumption of 40%-60% of the total intracellular ATP by the membrane H^+-ATPase (Serrano, 1991). Therefore, the maintenance of pH_i homeostasis in the presence of weak acids preservatives may deplete intracellular ATP levels significantly (Cole & Keenan, 1987). Such depletion of the ATP would result in growth restriction and an indirect result of growth inhibition by weak acids may be to cause membrane disruption (Bracey et al., 1998; Freese et al., 1973; Stratford & Anslow, 1998), leading to a disturbance in essential cell functions such as ATP synthesis, active transport of nutrients, cytoplasmic regulation, inhibition of essential metabolic enzymes (Krebs et al., 1983), cell growth cycle arrest (Booth et al.,1989; Cole et al., 1987; Krebs et al., 1983), and the accumulation of toxic anions (Eklund, 1983).

Sorbic acid was chosen to challenge the bioluminescent *E. coli* bioreporter strains, as it requires an energy-dependent response to counteract the detrimental effect of sorbic acid (Plumridge et al., 2004). Sorbic acid is considered to be very effective against *E. coli* and it has been ranked as the third most effective, after disodium sulfite and benzoic acid, against *E. coli* O157:H7 amongst the weak acid antimicrobials *E. coli* (Lu et al., 2011). However, sorbic acid is less toxic than benzoic acid against humans and animals (Turantas et al., 1999), and so is preferred as a preservative. The multiple effects of sorbate upon the microbial cell such as the depletion of cellular energy therefore present a particular challenge to the biosensor system because of its potential impact upon the expression of bioluminescence.

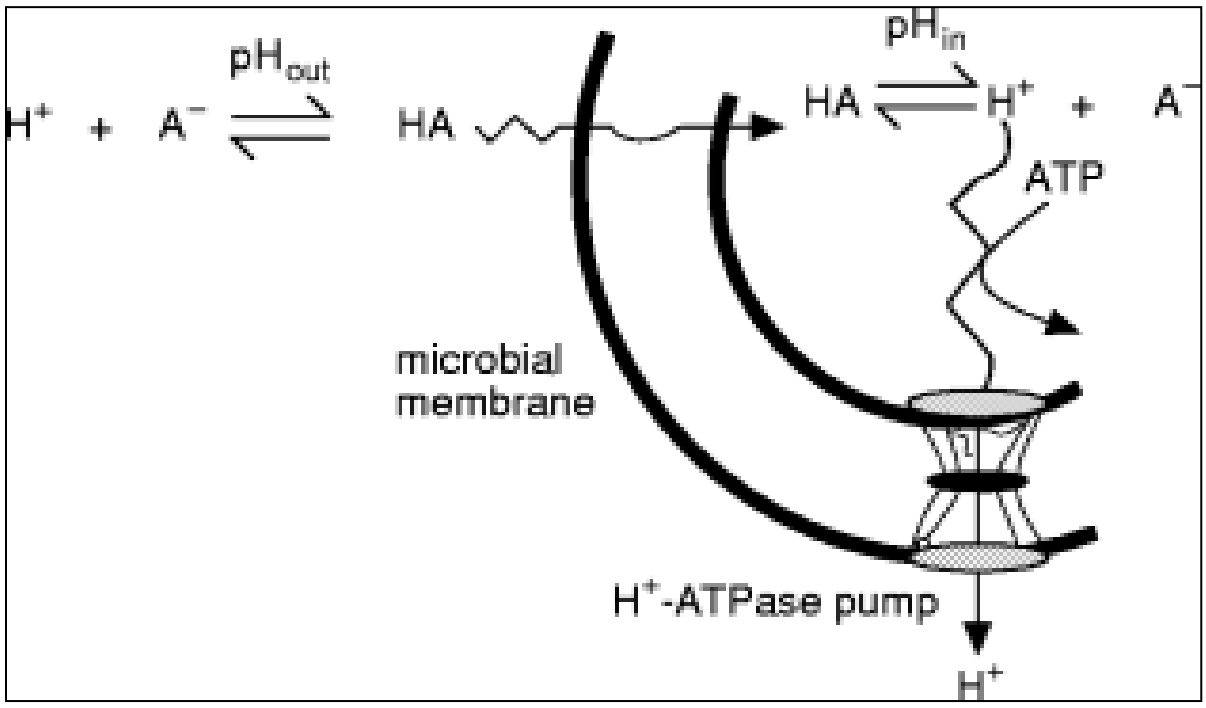
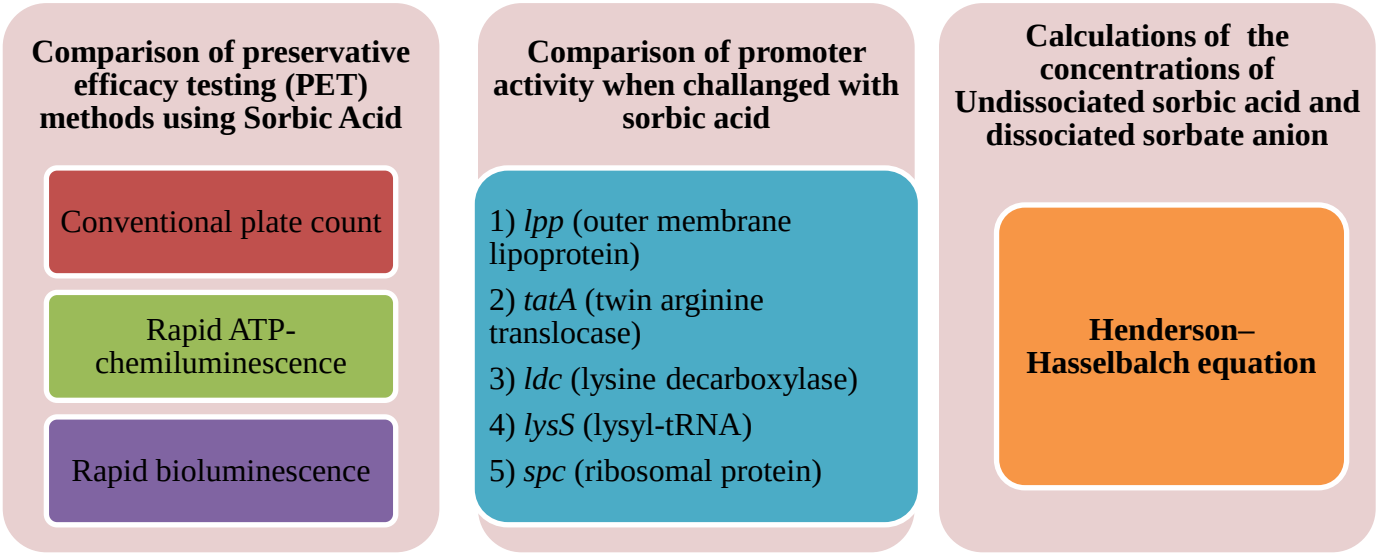


Figure 4.2: A schematic diagram of the mode of action of sorbic acid. Uncharged molecules (HA) diffuse through the plasma membrane and can dissociate to protons (H^+) and anions (A^-) in the cytoplasm. The charged protons are expelled by the membrane H^+ATPase, leaving the acid anions within the cytoplasm (Piper et al., 1998).

4.2 : Rationale of Chapter 4

This chapter describes an investigation into the application of whole-cell biosensors as a rapid microbiological method in preservative efficacy studies. The inocula were standardised in accordance with the requirements set out by the British & European Pharmacopoeias, as were the reductions in viable counts when exposed to the preservatives tested. The whole-cell bioluminescence method was employed to screen a range of concentrations of 0.2% to 0.0031% at pH 5.0. The whole-cell bioluminescence method was performed concurrently with two other methods that are described by the pharmacopoeias: the conventional plate count method; and the ATP chemiluminescence method. The aim of the work described in this chapter was to determine the accuracy and reproducibility of the whole-cell bioluminescence method in comparison with the currently accepted methods.

The chapter that follows covers the following comparisons;



4.3 : Objectives

1) To compare the response of the 5 bioluminescent bioreporter strains after exposure to sorbic acid at 0.2% to 0.031% at pH 5.0
2) To compare the response of the bioluminescent reporter strains to sorbic acid, at 0.2% to 0.031% at pH 5.0, with the existing methods prescribed by the British and European pharmacopoeias.
3) To rationalize and compare the five promoters, expressing bioluminescence in sorbic acid efficacy test at pH 5.

4.4 : Materials and Methods

4.4.1 : Preparation of Bacterial Initial Inoculum and Preservative Solutions

1.0 g of sorbic acid (Sigma-Aldrich) was weighed and dissolved in 150ml of deionized water. The pH of the solution was adjusted to pH 5.0 by adding 0.1M NaOH. This solution was then topped up with deionized water to 250.0 ml in the volumetric flask to produce a 0.4% stock solution. 60ml of the stock 0.4% (w/v) sorbic acid solution was then sterilized by filtering through a 0.2 µm Millipore Minisart syringe filter. Twofold serial dilutions of the 0.4 % (v/v) sorbic acid stock solution were made using sterile deionized water to obtain the following range of concentrations: 0.2% (v/v); 0.1% (v/v); 0.05% (v/v); 0,025% (v/v); 0.0125% (v/v); 0.062% (v/v); and 0.0031% (v/v).

The five bioluminescent *E. coli* bioreporter strains and control strains (*E.coli* [pBR322.*lux*] and *E.coli* ATCC 8739 were grown overnight as described in Section 2.2 to provide an initial inoculum. 10ml of the overnight culture was centrifuged at 20,000 *g* (Sorvall, RC 5B with SS-34 rotor) for 20 minutes, at 4° C. The supernatant was removed and the cell pellet was washed by resuspending and centrifugation with 10ml of sterile deionized water three times. The resulting cell pellet was finally resuspended with 10ml of sterile deionized water. Ten-fold serial dilutions of the washed bacterial suspension were prepared using sterile buffer peptone water (Sigma), to obtain an initial cell density of ~ 10^8 CFU/ml. This was determined by pre-constructed standard curves from chapter 2. A 30µl aliquot of the prepared 10^8 CFU/ml initial inoculum was inoculated into 2970µl of the sorbic acid dilutions (0.4%-0.0031%) prepared as described above along with a negative control of sterile double distilled water (ddH_20). This resulted in an initial population of ~ 10^6 CFU/ml (USP, E.P & B.P). Ten-fold serial dilutions were prepared with samples of each of the bacterial suspensions exposed to preservative and these were spread onto TSA plates, which had been supplemented with

100 µg/ml ampicillin for those plates inoculated with transformed *E. coli* strains, to confirm the initial counts.

4.4.2 : Preservative Efficacy Testing (PET)

The sorbic acid dilutions, inoculated with bacterial suspensions as described above, were incubated at 20ºC ± 2.5 ºC for a period of 28 days. The viable counts (as described in Section 2.4.4), bioluminescence (as described Section 2.4.3) and ATP chemiluminescence (as described in Section 2.4.5) were evaluated at 0 hours, 1 day, 2 days, 7 days, 14 days, 21 days, and 28 days after inoculation as prescribed by the European Pharmacopoeia –A criteria (for topical, ophthalmic and parenteral preparations. Both the British and European Pharmacopoeia require a minimum reduction in viable counts of 2 $\log_{10}$ CFU/ml after 2 days of exposure to a preservative, and a minimum reduction in viable counts of 3 $\log_{10}$ CFU/ml after 7 days of exposure for topical preparations. Whilst, a minimum reduction in viable counts of a 2 $\log_{10}$ CFU/ml after 6 hours of exposure and a minimum reduction in viable counts of 3 $\log_{10}$ CFU/ml after 24 hours of exposure for parenteral and ophthalmic preparations. Lastly a minimum reduction in viable counts of 3 $\log_{10}$ CFU/ml after 14 days of exposure is required for oral preparations. The experiments were performed in triplicate. The time taken to achieve a viable count of $\leq$ 3 $\log_{10}$ CFU/ml, a bioluminescence of $\leq$ 3 $\log_{10}$ RLU/ml and an ATP-chemiluminescence of $\leq$ 3 $\log_{10}$ RLU/ml was recorded for each of the five bioluminescent biosensor strains and control strains.

4.4.3 : Determination of the Minimum Inhibitory Concentration (MIC) of Sorbic acid

A stock solution of sorbic acid was prepared as described in section 4.5.1. Twofold serial dilutions were prepared using TSB to yield working concentrations of 0.2%, 0.1%, 0.05%, 0,025%, 0.0125%, 0.062%, and 0.0031%. Initial inocula of the *E. coli* biosensor and control strains were prepared as

described in section 4.5.1, inoculated into the sorbic acid solutions and incubated at 32 °C. The initial optical density of the bacterial suspensions was determined at 620nm (Cecil CE1011 1000 series), as was the initial bioluminescence of the suspensions before the preservatives were added. The optical density and bioluminescence were again determined after 24 hours' exposure to the sorbic acid dilutions. The MIC was defined as the lowest concentration of sorbic acid that demonstrated no growth of the bacterial culture. Plate counting was carried out before addition of preservatives and at the MIC concentration to confirm a 99.9% reduction.

4.4.4 : Calculation of the Intracellular Undissociated Acid and Dissociated Acid Anion Concentration Present in the Experiments

The pKa of sorbic acid is 4.76 (Cowles, 1941; Cerruti et al., 1990; Freese et al., 1973; Hoffamn et al., 1944; Sofos & Busta, 1981; Rahn & Conn, 1944; Pethybridge et al.,1983), the pH of cytoplasm is 6.8 (Salmond et al., 1984) and the pH of the sorbic acid solution was measured at 5.0. The concentrations of undissociated sorbic acid molecules and also dissociated sorbate anions of were calculated at both pH 5.0 and pH 6.8 using the Henderson-Hasselbalch equation (equation 4.1).

$$\frac{pKa + \log[A-]}{[HA]} \quad \text{.....Equation 4.1}$$

The sorbic acid concentrations calculated were as indicated in section 4.4.1.

4.4.5 : Statistical Analysis

Statistical analysis was performed as described in section 2.4.8. The bioluminescence, viable counts and ATP chemiluminescence were compared by Pearson correlation analysis.

4.5 : Results

The results section contains two experimental parts;

Section **4.6.1** presents the undissociated acid and dissociated acid anion concentrations calculated using the Henderson-Hasselbalch equation. Section **4.6.2** presents the MIC of sorbic acid determined by bioluminescence and turbidity methods, whilst sections **4.6.3, 4.6.4,** and **4.6.5** evaluate various features of sorbic acid (SA) in preservative efficacy testing (PET).

4.5.1 : Concentrations of the undissociated and dissociated forms of sorbic acid in solution and in the cytoplasm, calculated according to Henderson-Hasselbalch equation

Table 4.10 shows the ratio of the un-dissociated and the dissociated form (anion) to the total concentration of sorbic acid at pH 5.0, as calculated according to the Henderson-Hasselbalch equation for sorbic acid concentrations of 35.65mM, 17.84mM, 8.92mM, 4.46mM, 2.23mM, 1.12mM, 0.56mM and 0.28mM (*i.e.* 0.2%, 0.1%, 0.05%, 0.0025%, 0.00125%, 0.00062% and 0.0031%). At a pH of 5.0, the ratio of undissociated to total sorbic acid was 0.37, whilst the ratio of dissociated anion to total sorbic acid was 0.63.

The ratio of the undissociated and the dissociated form (anion) to the total concentration of sorbic acid at the cytoplasmic pH of 6.8 were also calculated. At a pH of 6.8, the ratio of undissociated to total sorbic acid was 0.01, whilst the ratio of dissociated anion to total sorbic acid was 0.99.

Table 4.10: Concentrations of the undissociated and dissociated forms of sorbic acid in solution and in the cytoplasm, calculated according to the Henderson-Hasselbalch equation

Concentration of sorbic acid	Concentration of undissociated acid in solution [HA]		Concentration of discociated acid in solution [A^-]		Concentration of dissociated acid in cytoplasm		Concentration of remaining undissociated acid in cyctoplasm	
(mM)*	(mM)	(Ratio)[1]	(mM)	(Ratio)[2]	(mM)	(Ratio)[3]	(mM)	(Ratio)[4]
17.84	6.51	0.37	11.33	0.63	6.45	0.99	0.06	0.01
8.92	3.25	0.37	5.67	0.63	3.23	0.99	0.03	0.01
4.46	1.62	0.37	2.84	0.63	1.62	0.99	0.015	0.01
2.23	0.81	0.37	1.42	0.63	0.81	0.99	0.008	0.01
1.12	0.40	0.37	0.71	0.63	0.41	0.99	0.004	0.01
0.56	0.20	0.37	0.36	0.63	0.21	0.99	0.002	0.01
0.28	0.10	0.37	0.18	0.63	0.11	0.99	0.001	0.01

*The concentrations of 0.2%, 0.1%, 0.05%, 0.025%, 0.0125%, 0.0062%, and 0.0031% were converted to mM

[1] ratio of [undissociated]/[total sorbic acid (SA)] at pH 5.0

[2] ratio of [dissociated]/[total SA] at pH 5.0

[3] ratio of [undissociated]/[total SA at pH 6.80

[4] ratio of [dissociated]/[total SA at pH 6.8

Table 4.2: Example mathematical calculation for the ratio of undissociated to dissociated sorbic acid

Concentration of undissociated acid [HA] in the medium

$pH = pK_a + \log [A-] / [HA]$
$5.00 = 4.76 + \log [A-] / [HA]$
$1.74 = [A-] / [HA]$ (Ratio of HA to A^- is 1/2.74 or 37%)
Therefore, [HA] = (1/2.74) (Concentration of sorbic acid)
[HA] = (1/2.74) x (35.67mM)
[HA] = 13.01mM

Concentration of dissociated acid anion[A^-] in the medium

$pH = pK_a + \log [A-] / [HA]$
$5.00 = 4.76 + \log [A-] / [HA]$
$1.74 = [A^-] / [HA]$ (Ratio of A^- to HA is 1.74 /2.74 or 63%)
Therefore, $[A^-]$ = (1.74 /2.74) (Concentration of sorbic acid)
$[A^-]$ = (1.74 /2.74) x (35.67mM)
$[A^-]$ = 22.65 mM

Concentration of dissociated acid anion and protons $[A^-] + [H^+]$ in cytoplasm

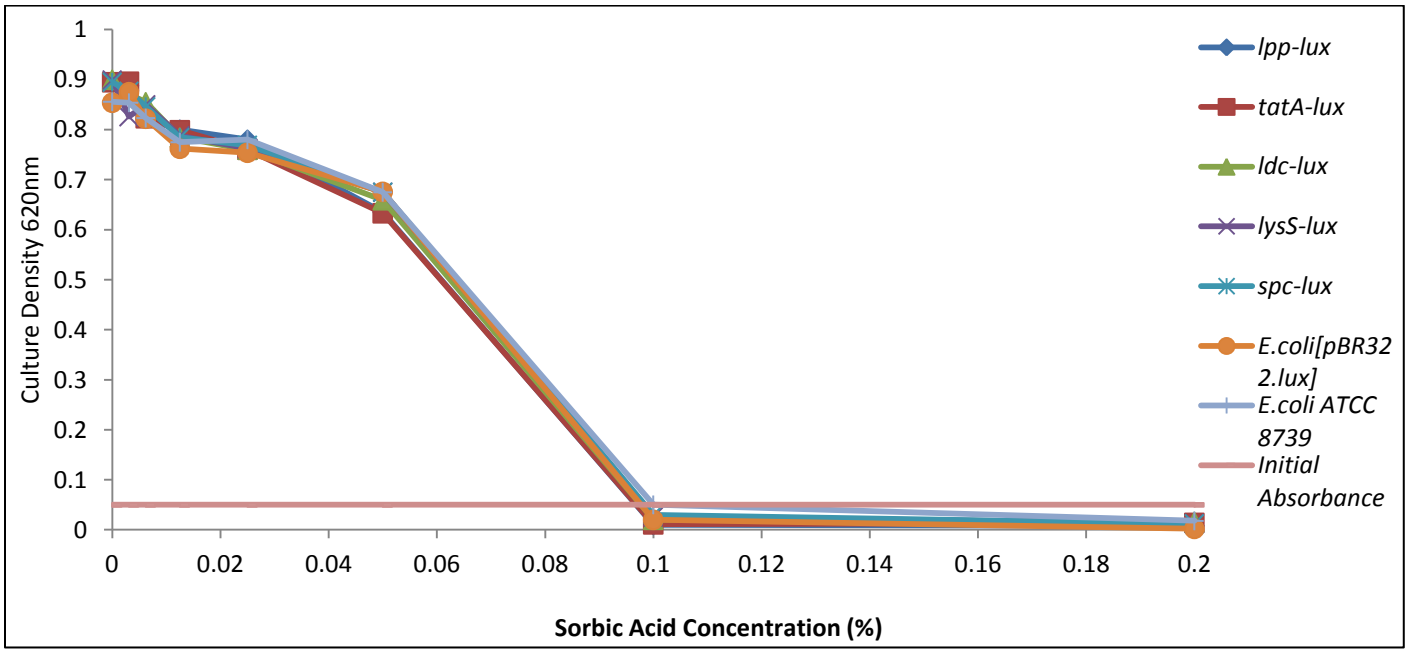
$pH = pK_a + \log [A-] / [HA]$
$6.80 = 4.76 + \log [A-] / [HA]$
$109.64 = [A-] / [HA]$ (Ratio of $[A^-]$ to HA is 109.64 /110.64 or 99% in cytoplasm)
$[A^-]/[H^+]$ = (109.64 /110.64) x (13.01mM) (HA dissociates to anions and protons)
$[A^-]/[H^+]$= 12.89 Mm

Concentration of undissociated acid [HA] remaining in cytoplasm

$pH = pK_a + \log [A-] / [HA]$
$6.80 = 4.76 + \log [A-] / [HA]$
$109.64 = [A-]/[HA]$ (Ratio of [HA] to $[A^-]$ is 1/109.64 or 1% to in solution)
[HA] = (1/109.64) x (13.01 mM) (HA remaining after dissociation in cytoplasm)
[HA] = 0.11 mM

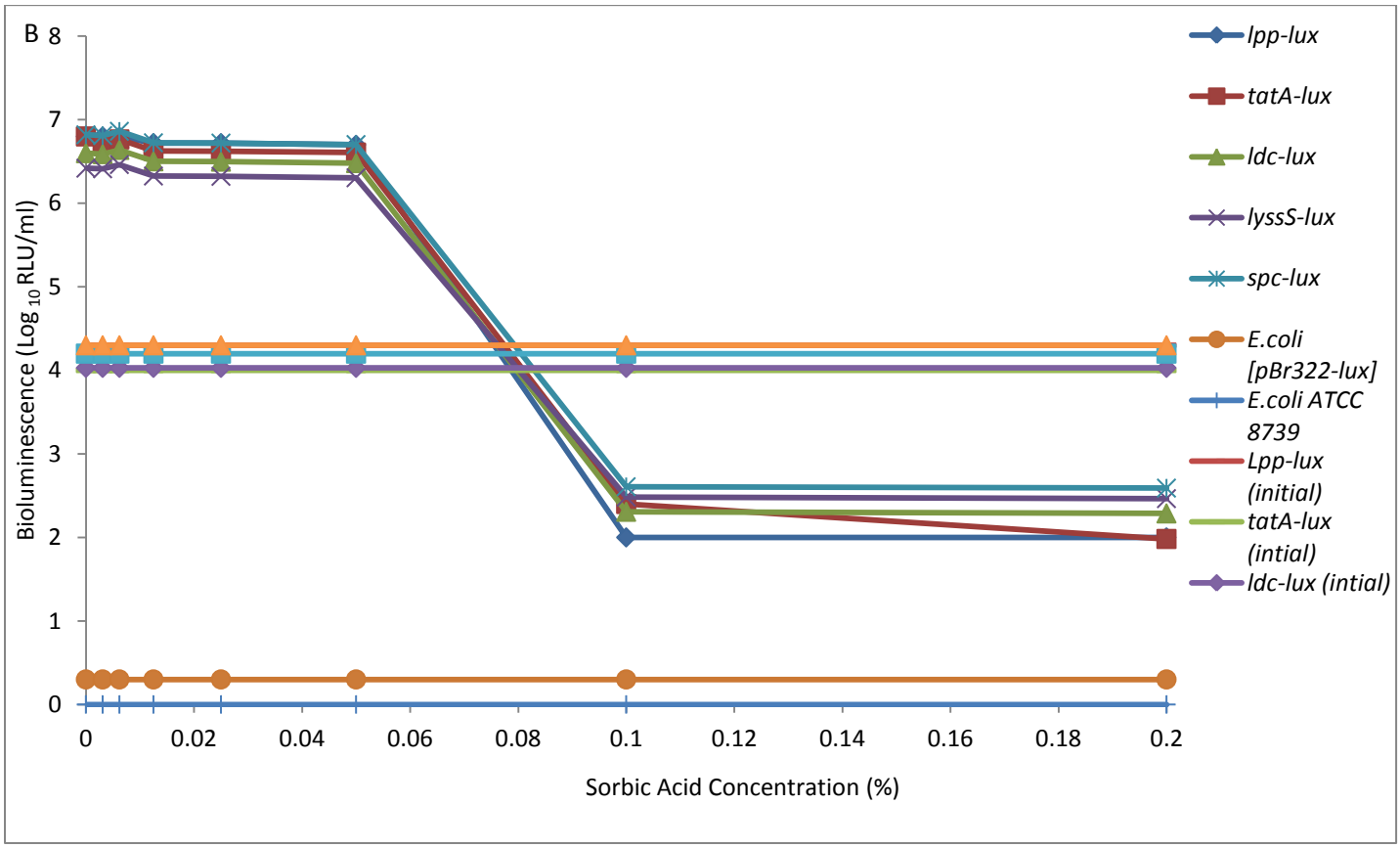
4.5.2 : Minimum Inhibitory Concentration (MIC) of Sorbic Acid

Initially the absorbance of the culture was 0.05 ± 0.07 A.U. for all of the cultures (without SA), whilst the bioluminescence of the biosensor culture was between 4.25 ± 0.08 $\log_{10}$ RLU/ml. After 24 hours of incubation, the culture density was high, at 0.95± 0.07 A.U. when the cells of the *E.coli* strains exposed to sorbic acid concentration at concentrations; 0%, 0.05%, 0.025%, 0.0125%, 0.0062%, and 0.0031%, and significantly (P = 0.001, Tukey *Post Hoc* analysis) at 0.05 ± 0.007 A.U. at 0.1% and 0.2% SA, after 24 hours of incubation. The response to exposure to sorbic acid was the same for each of the biosensor and control strains (Figure 4.3 (a)).



Figures 4.3 (a): Effect of sorbic acid upon culture density (absorbance at 620 nm) after 24 hours of incubation in its presence in order to determine the Minimum Inhibitory Concentration (MIC)
The SEM for each individual biosensor strain in Figures 4.3 (a) did not exceed 0.007

The bioluminescence expressed by each of the biosensor strains was also high, between 6.5 to 7.00 ± 0.10 $\log_{10}$ RLU/ml at sorbic acid concentrations; 0.05%, 0.025%, 0.0125%, 0.0062%, 0.0031%, or 0%, and significantly (P = 0.02, Tukey *Post Hoc* analysis) decreased to 0.75 ± 0.07 $\log_{10}$ RLU/ml at sorbic acid concentrations; 0.1% and 0.2% (Figures 4.3 (b)).



Figures 4.3 (b): Effect of sorbic acid upon bioluminescence after 24 hours of incubation in its presence in order to determine the Minimum Inhibitory Concentration (MIC)
The SEM for each individual biosensor strain in Figures 4.3 (b) did not exceed 0.10

The correlation coefficients for bioluminescence versus absorbance readings for each of the biosensors were as follows: *lpp-lux* R^2 = 0.9798; *tatA-lux* R^2 = 0.9553; *ldc-lux* R^2 = 0.9917; *lysS-lux* R^2 = 0.9748; *spc-lux* R^2 = 0.9727

The control strains, *E. coli* [pBR-322.*lux*] and *E. coli* ATCC 8739 did not exhibit any bioluminescence (Figure 4.3 (b)). Strong correlations were found between the absorbance and the bioluminescence (R^2= 0.9553-0.9917) for each of the 5 biosensor strains exposed to differing sorbic acid concentrations.

The MIC of sorbic acid was determined to be 0.1 % SA. This was confirmed by using plate counts which demonstrated a 99.9% reduction of viable counts from those present in the initial innoculum.

4.5.3 : Bioluminescence and Viable Counts of the Preservative-Free Negative Controls

Immediately after inoculation of the experiments, the bioluminescence was 5.9 ± 0.1 $\log_{10}$ RLU/ml for *lpp-lux*, 5.5± 0.04 $\log_{10}$ RLU/ml for *tatA-lux*, 5.18± 0.1 $\log_{10}$ RLU/ml for *ldc-lux*, 5.02± 0.002 $\log_{10}$ RLU/ml for *lysS-lux* and 5.5± 0.03 $\log_{10}$ RLU/ml for *spc-lux* (Figure 4.4 (a)). The bioluminescence expressed by each biosensor strain decreased by approximately 0.3 $\log_{10}$ RLU/ml between inoculation and day 2 of incubation with no significant differences. On the other hand, between day 7 and day 28 of incubation, the bioluminescence expressed by *lpp-lux* and *lysS-lux* decreased to approximately 3.0 ± 0.1 $\log_{10}$ RLU/ml.

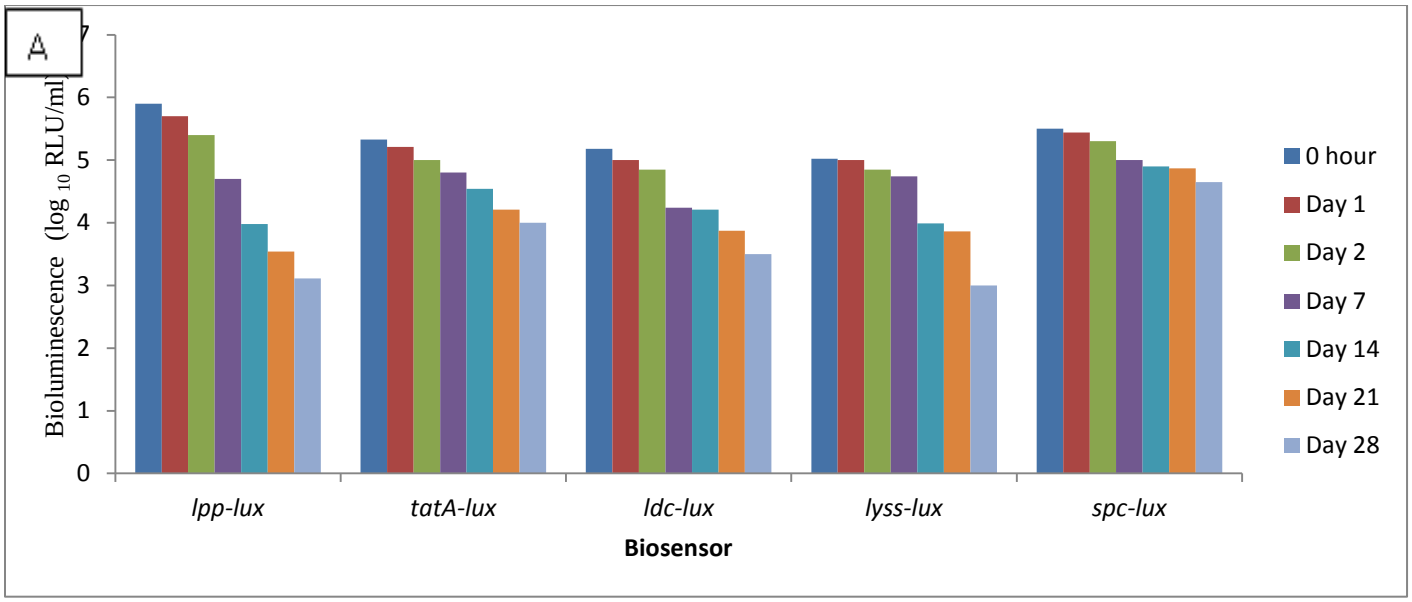


Figure 4.4 (a): Bioluminescence of the biosensor strains were incubated without preservative for up to 28 days, in triplicates n=3

The SEM for each individual biosensor strain in Figures 5.6 (a) did not exceed 0.15

The initial viable counts ($\log_{10}$ CFU/ml) of the five bioreporter strains and wildtype ATCC 8739 strain were 6.0 ± 0.5 $\log_{10}$ CFU/ml, and remained broadly unchanged with up to 2 days of incubation with no significant difference. After 7 days of incubation the viable counts had declined slightly but this was not statistically significant. The viable counts of all bioreporter strains had reduced by approximately 1.00 ± 0.02 $\log_{10}$ CFU/ml at day 14 of the incubation and a further 0.5 ± 0.05 $\log_{10}$ CFU/ml at day 21. Between 21 and 28 days of

incubation, there was no significant difference between (Figure 4.4 (b)). Although the viable counts decreased significantly with incubation, there were no significant differences between the viable counts of the five *E. coli* biosensor strains and the control strains at any given time point between inoculation and 28 days of incubation.

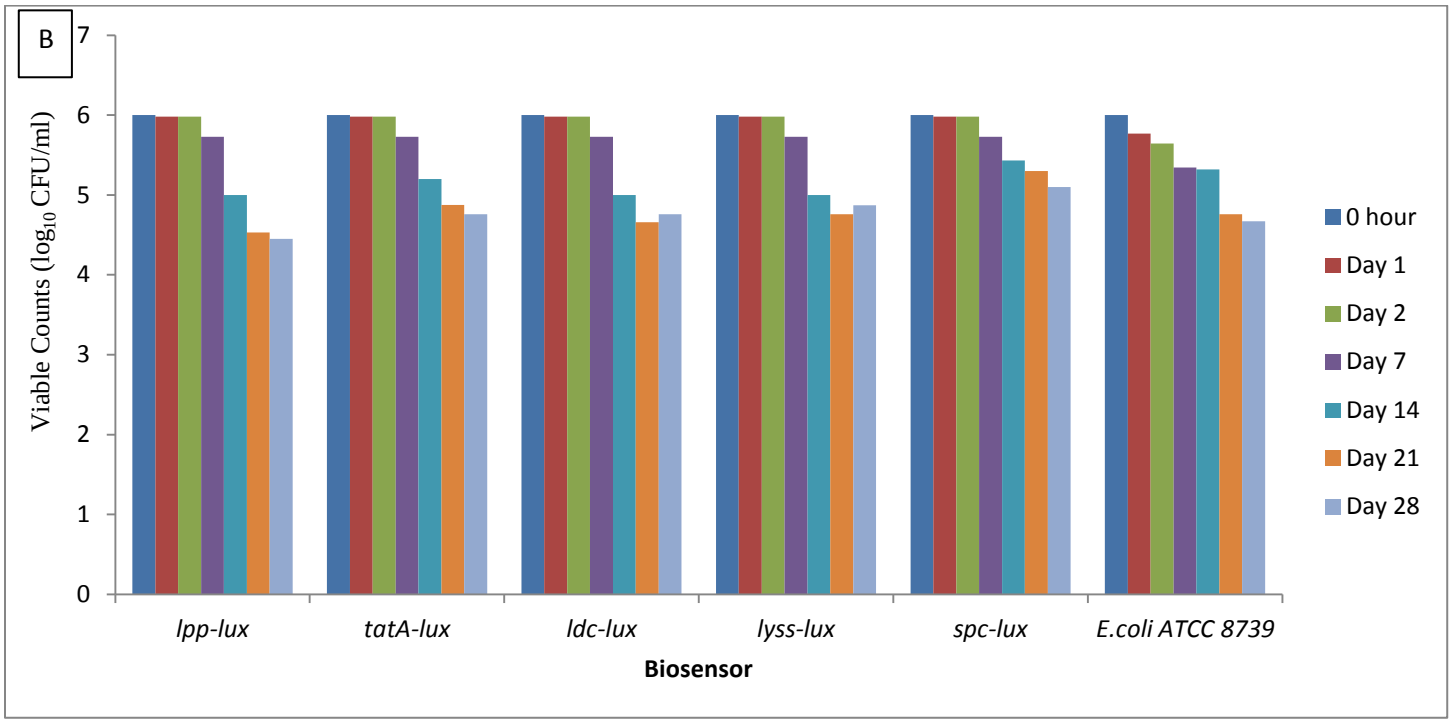


Figure 4.4 (b): Viable counts of the biosensor strains were incubated without preservative for up to 28 days, in triplicates n=3

The SEM for each individual biosensor strain in Figures 5.6 (b) did not exceed 0.10

The bioluminescence per cell (RLU:CFU) was the highest for *lpp-lux* immediately after inoculation (0 hour) (at 0.79 $\pm$ 0.2), followed by *spc-lux* (at 0.31$\pm$ 0.3), *tatA-lux* (at 0.2 $\pm$ 0.4), *ldc-lux* (at 0.15 $\pm$ 0.3), and *lysS-lux* (at 0.10 $\pm$ 0.1). At day 1 of incubation the RLU:CFU was, in descending order, *lpp-lux* (at 0.52$\pm$ 0.1), followed by *spc-lux* (at 0.28 $\pm$ 0.1), *tatA-lux* (at 0.16 $\pm$ 0.1), *ldc-lux* (at 0.10 $\pm$ 0.15), and finally *lysS-lux* (at 0.10 $\pm$ 0.08). At day 2 of incubation the RLU:CFU for *lpp-lux* (at 0.26 $\pm$ 0.1) was similar to that *spc-lux* (at 0.2 $\pm$ 0.09), whilst *tatA-lux*, *ldc-lux*, and *lysS-lux* demonstrated an RLU:CFU of between 0.07 and 0.1. From day 7 to day 28 of

incubation, the RLU:CFU for *spc-lux* from day 7 to day 28 of incubation were between 0.22-0.37. In contrast the RLU:CFU for *ldc-lux* and *tatA-lux* was on average 0.15 ± 0.05, whilst for *lpp-lux* and *lysS-lux* it was on average 0.056 ± 0.05. There was no statistical difference in the bioluminescence expressed by *tatA-lux*, *ldc-lux* and *spc-lux* although it declined significantly in each case (P = 0.002, 0.000, 0.000, Tukey *Post Hoc*).

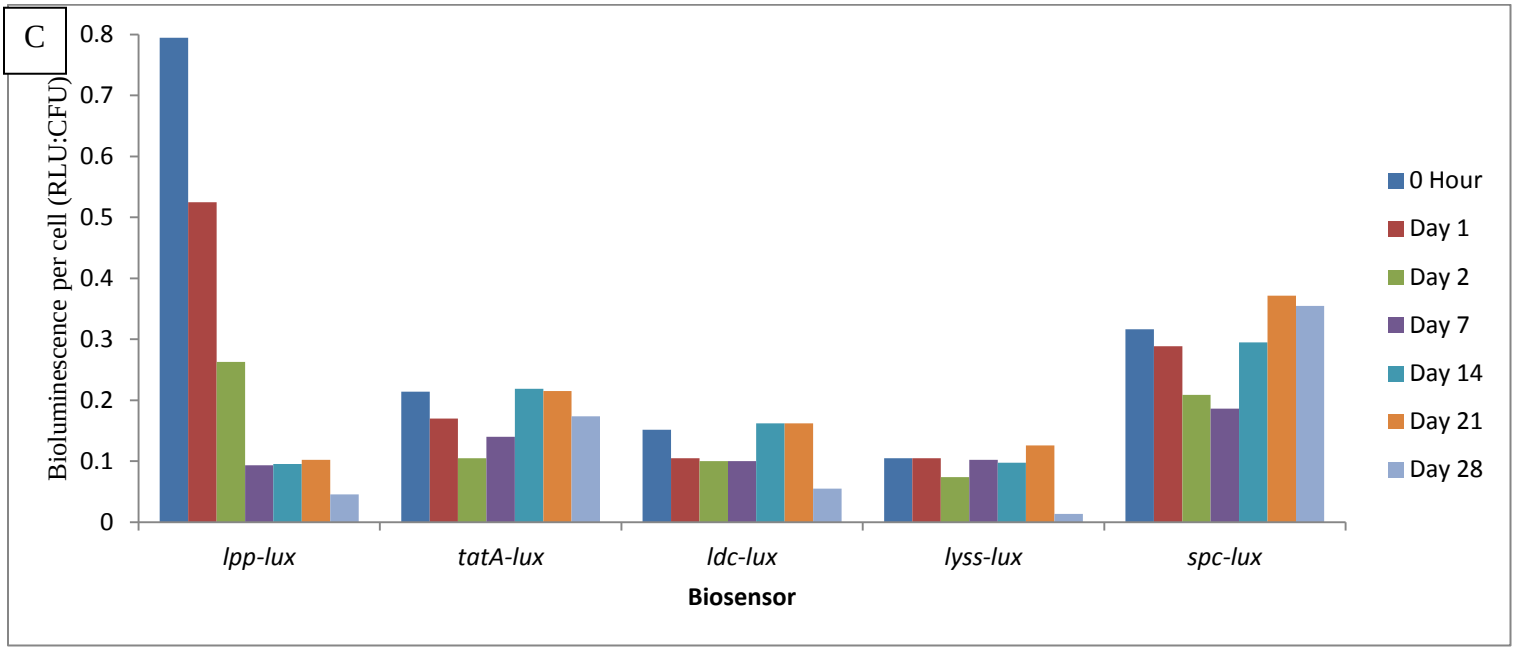


Figure 4.4 (a, b, & c): Bioluminescence per cell (RLU:CFU) when the biosensor strains were incubated without preservative for up to 28 days, in triplicates n=3

The SEM for each individual biosensor strain in Figures 5.6 (c) did not exceed 0.1

4.5.4 : Effect of Sorbic Acid on Bioluminescence, Viable Counts and ATP-Chemiluminescence for the Five Biosensor Strains and the Control Strains of *E. coli* ATCC 8739 in a Preservative Efficacy Test

The bioluminescence expressed by the five bio-reporter strains, and also the intracellular ATP levels (as demonstrated by the chemiluminescence assay), decreased significantly (P = 0.003, 0.001, 0.008, Tukey *Post Hoc* analysis) between inoculation and 24 hours of incubation when cultures were exposed to high concentrations of sorbic acid (0.2%, and 0.1%) (Figures 4.5, 4.6 and 4.8). At sorbic acid concentrations at 0.2% to 0.1%, the reduction in *E. coli* viable counts to undetectable levels between 0 h and day 1 of incubation exceeded the European Pharmacopoeia-A criteria for preservatives in oral, topical ophthalmic and parenteral preparations. The bioluminescence per cell was in ascending orders was; *spc* (0.0018 ± 0.002), *lpp* (0.0005 ± 0.002), *ldc* (0.0001 ± 0.005), *tatA* (0.001 ± 0.006), and *lysS* (0.001± 0.0001) at 0 h in 0.2% SA and remained zero at each of the time points tested up to 28 days of incubation, for the five biosensor strains at 0.2 % SA. Whilst the average bioluminescence per cell for the five biosensors at 0.1% SA at 0 hr of incubation in ascending orders was; *lpp* (0.008 ± 0.001), *spc* (0.0022± 0.002), *ldc* (0.00138 ± 0.002), *tatA* (0.00126 ± 0.003), and *lysS* (0.00126± 0.001) was reduced to zero between 0 hr and 24 hours of incubation, resembling the bioluminescence, ATP-chemiluminescence and plate counts with no significant differences.

When exposed to concentrations of sorbic acid of 0.05%, 0.0125%, 0.0062%, and 0.0031% SA, the bioluminescence expressed by the *lpp-lux* biosensor strain showed a significant reduction (P = 0.01, ANOVA) of at least 5 of magnitude after 7 days of incubation, and there was no subsequent recovery in bioluminescence up to 28 days of incubation (P = 0.01, ANOVA). The bioluminescence per cell were on average 0.00003 ± 0.34 after 7 d for 0.0125%, 0.0062%, and 0.0031%, SA.

The bioluminescence levels (log_{10} RLU/ml), viable counts (log_{10} CFU/ml) and ATP-chemiluminescence (RLU/ml) of each of the *E. coli* biosensor strains except *lpp-lux* reduced significantly (P = 0.00, ANOVA) by 3 orders of magnitude between 2 and 7 days when exposed to 0.05% SA, whilst at 0.025% SA reduction of bioluminescence (log_{10} RLU/ml), viable counts (log_{10} CFU/ml) and ATP-chemiluminescence (RLU/ml) of each of the *E. coli* biosensor strains except *lpp-lux* reduced decreased significantly by at least 3 orders of magnitude between 7 and 14 days of incubation with no significant differences between the methods. The average bioluminescence per cell from the biosensor strains at the reduction of 3 orders of magnitude at concentrations 0.05% and 0.025% SA was as follows (in ascending order): *lysS* (0.01± 0); *ldc* (0.28 ± 0.7); *tatA* (0.44 ± 0.60) and *spc-lux* (0.66 ± 0.22).

When exposed to concentration 0.0125% SA, the bioluminescence levels expressed by *lysS-lux* decreased significantly (P = 0.00, ANOVA) to at least 3 orders of magnitude between 7 and 14 days whilst bioluminescence levels expressed by *ldc-lux* and *tatA-lux* biosensors strains decreased significantly (P = 0.00, ANOVA) between 14 and 21 days of incubation when exposed to 0.0125% SA. Bioluminescence expression by *Spc-lux* reduced by 1.10 ± 0.36 log_{10} RLU/ml from 0 h to 28 days in 0.0125% SA. The bioluminescence per cell obtained at these time points were in ascending order of were *ldc* (0.022 ± 0.07); *lyss* (0.026 ± 0.02); *tatA* (0.03 ± 0.05) and *spc* (0.39 ± 0.05). Plate counts and ATP chemiluminescence readings at 0.0125% SA reduced significantly (P = 0.00, ANOVA) by 1.21 ± 0.20 log_{10} CFU/ml and 1.205 ± 0.25 log_{10} RLU/ml from 0 h to 28 days.

In sorbic acid concentrations of 0.0062% and 0.0031% a significant reduction in bioluminescence expression of at least 3 orders of magnitude was demonstrated by the *tatA-lux, ldc-lux,* and *lysS-lux*

biosensor strains from 0 h to 28 days of incubation, whilst bioluminescence expression by *Spc-lux* reduced on average 1.075 ± 0.36 $\log_{10}$ RLU/ml from 0 h to 28 days in 0.0062% and 0.0031% SA. The bioluminescence per cell obtained at these time points were in ascending order of were *ldc* (0.0018 ± 0.07); *lyss* (0.0018 ± 0.03); *tatA* (0.007 ± 0.09) and *spc* (0.50 ± 0.07). Whilst, the plate counts and ATP chemiluminescence readings at 0.0062% and 0.0031% SA reduced significantly (P= 0.00, 0.000, Tukey *Post Hoc*) on average by 1.00 ± 0.22 $\log_{10}$ CFU/ml and 0.60 ± 0.27 $\log_{10}$ RLU/ml from 0 h to 28 days.

Overall, there is a significant decreased (*P*= 0.146, Tukey *Post Hoc*) between bioluminescence readings exhibited by *lpp-lux* and *lysS-lux* to bioluminescence readings exhibited by *ldc-lux* (P= 0.026, Tukey *Post Hoc), tatA-lux* (P= 0.006, Tukey *Post Hoc*) and *spc-lux* (P= 0.00, Tukey *Post Hoc*) whilst no significant difference between bioluminescence readings exhibited by *spc-lux* to *ldc-lux* (P= 0.299, Tukey *Post Hoc*), and *tatA-lux* (P= 0.882, Tukey *Post Hoc*) across the 28 days.

Importantly, a reduction in viable counts of 3 orders of magnitude correlated significantly with a at least more than 99.9% reduction in bioluminescence for each of the bio-reporter strains ($P \leq 0.05$, ANOVA) (Table 4.3). The correlation coefficients between the viable counts ($\log_{10}$ CFU/ml) and bioluminescence ($\log_{10}$ RLU/ml) were significant ($P \leq 0.05$, Pearson Correlation); *lpp-lux* (R^2=0.55-1.00); *tatA-lux* (R^2=0.886-1.00); *ldc-lux* (R^2=0.833-1.00); *lysS-lux* (R^2=0.811-1.00); and *spc-lux* (R^2=0.879-1.00) (Table 4.3). In addition, the correlation coefficients between the bioluminescence ($\log_{10}$ RLU/ml) and ATP-chemiluminescence ($\log_{10}$ RLU/ml) were; *lpp-lux* (R^2=0.676-1.00); *tatA-lux* (R^2=0.806-1.00); *ldc-lux* (R^2=0.854-1.00); *lysS-lux* (R^2=0.827-1.00); and *spc-lux* (R^2=0.8733-1.00) (Table 4.3).

In table 4.3 illustrates the tabulation for suitability of the range of sorbic acid tested in this study in accordance to EP-A criteria. This revealed that sorbic acid at concentrations; 0.2% and 0.1% were proved to be effective for oral, parenteral, and topical pharmaceutical preparations, whilst at concentration 0.05% SA was suitable for oral and tropical preparation and finally at concentration 0.025% was suitable for only oral preparations. On the other hand, concentrations of SA at 0.0125%, 0.0062%, and 0.0031% were not effective for any pharmaceutical preparations. The *E.coli* strains demonstrate the same effect against sorbic acid concentrations.

The overall standard error of means was lower for whole-cell bioluminescence readings by over an order of magnitude (0.011), in comparison to CFU (0.127) and was approximately half that of ATP-bioluminescence (0.056). The recommended statistical method of comparing the precision is the application of the F-test. The variance of each method is estimated, and the ratio of the largest to the smallest variance is calculated and compared to the tabulated values for an F distribution. The calculated ratio (by statistical programme, SPSS) is $F_{(258, 35, 0.05)} = 1.404$, which less than the critical value of 1.62, indicates no significant difference exists between the precision of the methods (PDA, Technical Report No. 33) between the whole-cell bioluminescence compared to traditional plate count, and ATP chemiluminescence method.

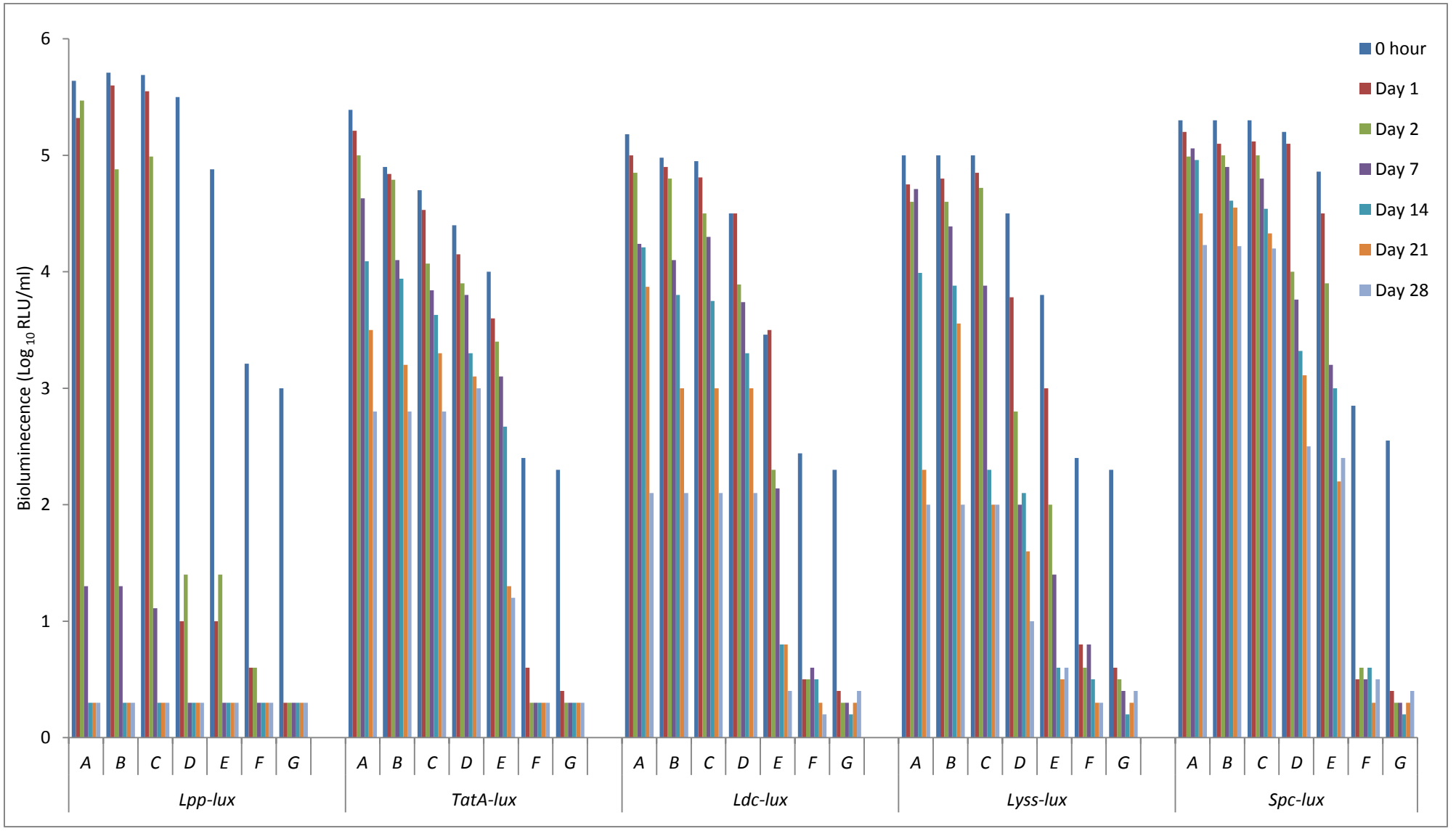


Figure 4.5 Bioluminescence expression (log_{10} RLU/ml) by the five biosensor strains when challenged with sorbic acid (the concentrations of sorbic acid are presented in ascending order (A to G) from left to right, and were: 0.0031%; 0.0062%; 0.0125%; 0.025%; 0.05%; 0.1%; and 0.2% for each biosensor strain) at pH 5.0 over 28 days of incubation. # The overall SEM is 0.011 at 95% confidence interval.

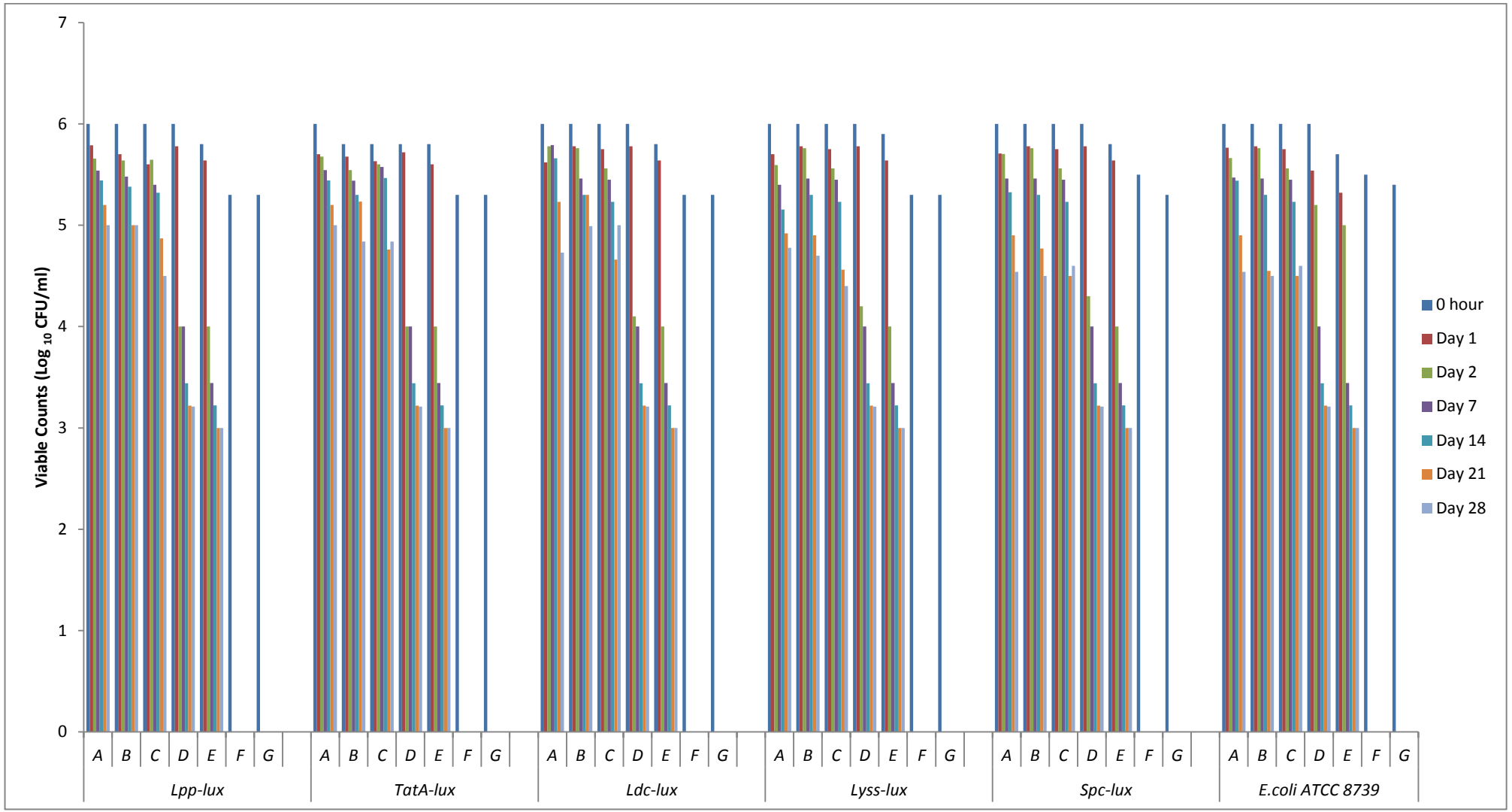


Figure 4.6: Viable counts (log_{10} CFU/ml) for the five biosensor strains and also the control strain when challenged with sorbic acid (the concentrations of sorbic acid are presented in ascending order (A to G) from left to right, and were: 0.0031%; 0.0062%; 0.0125%; 0.025%; 0.05%; 0.1%; and 0.2% for each biosensor strain) at pH 5.0 over 28 days of incubation. # The overall SEM is 0.127 at 95% confidence interval

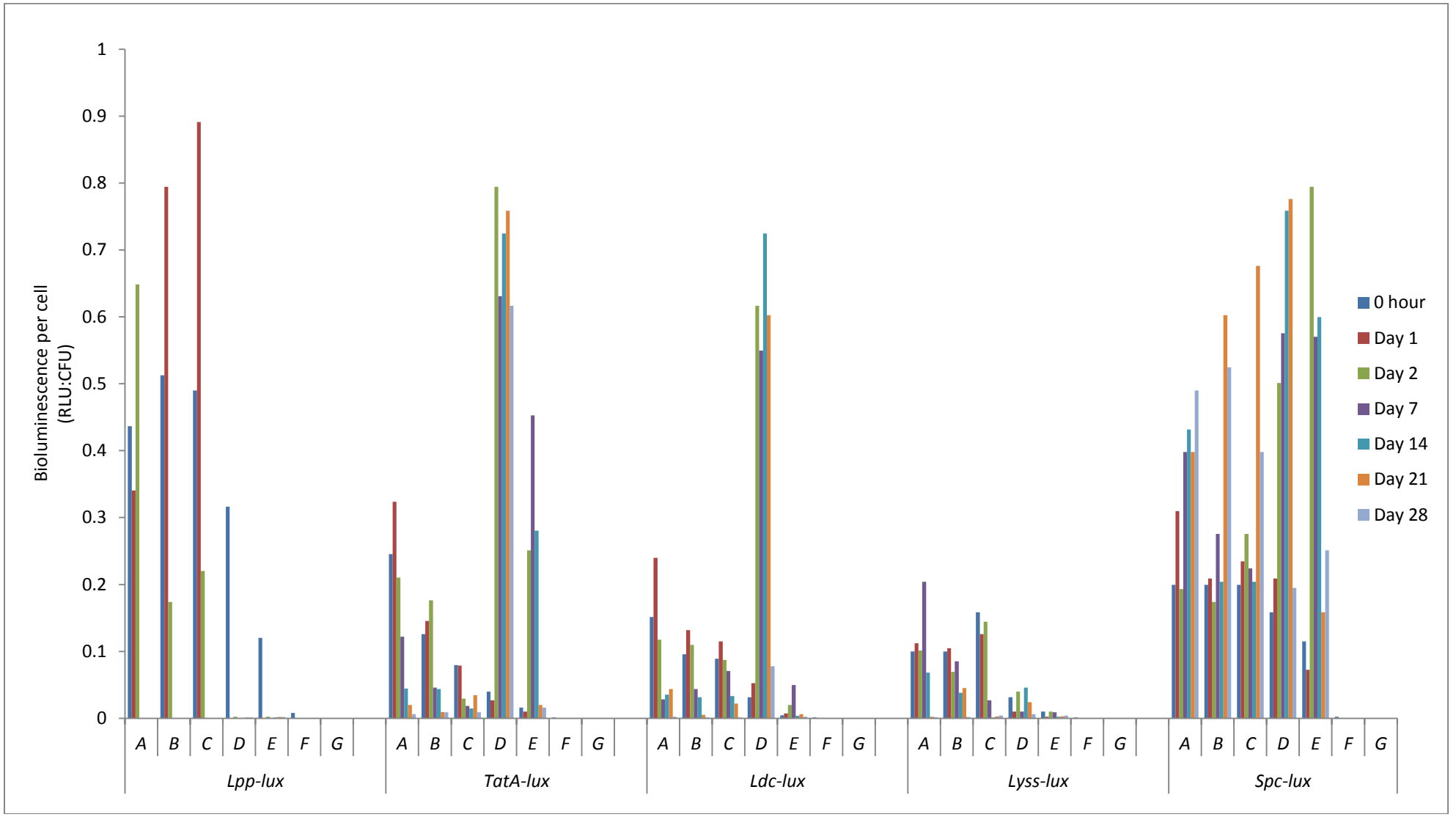


Figure 4.7: Bioluminescence per cell (RLU:CFU) for the five biosensor strains when challenged with sorbic acid (the concentrations of sorbic acid are presented in ascending order (A to G) from left to right, and were 0.0031%; 0.0062%; 0.0125%; 0.025%; 0.05%; 0.1%; and 0.2% for each biosensor strain) at pH 5.0 over 28 days. # The overall SEM is 0.012 at 95% confidence interval

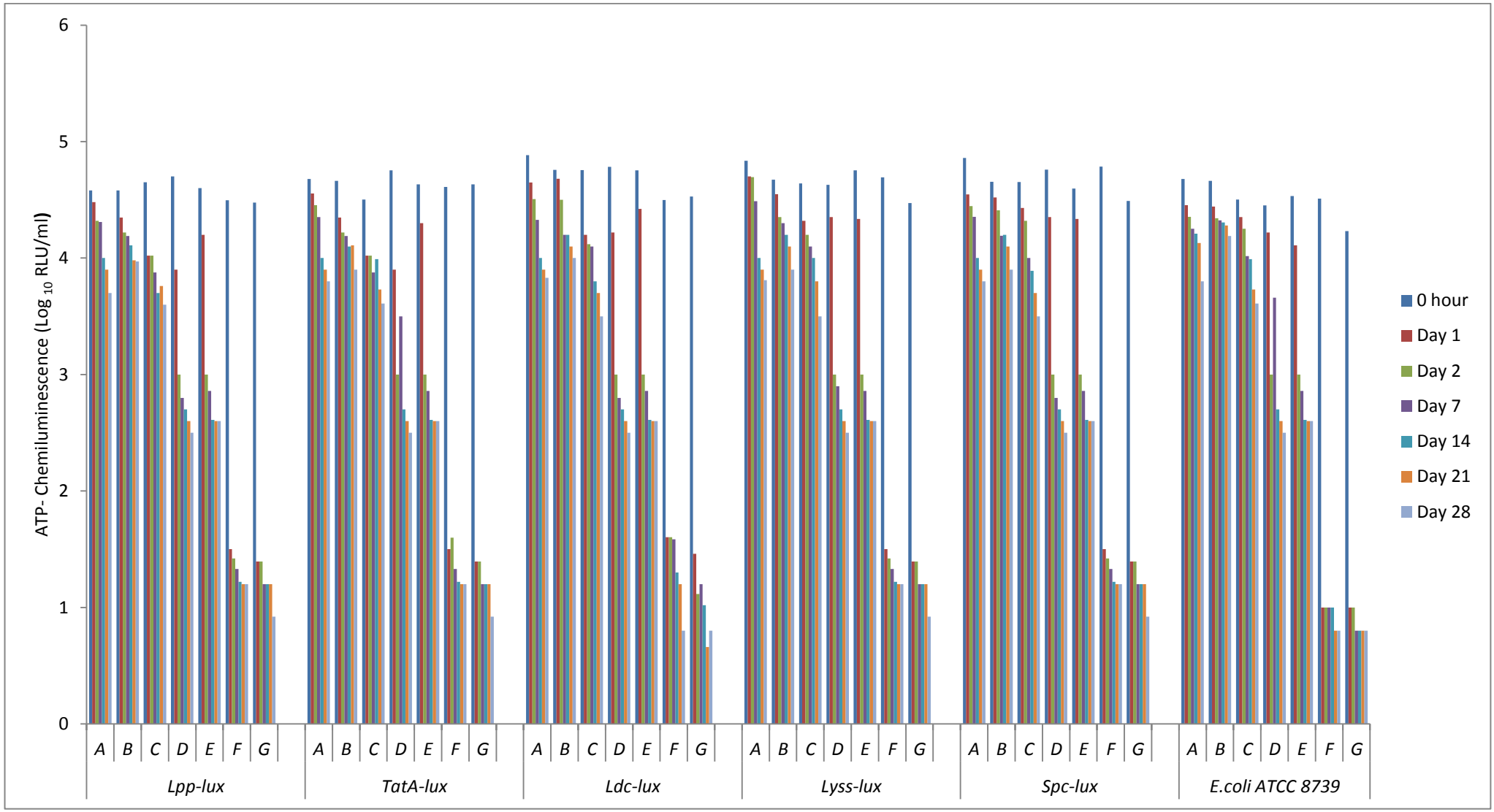


Figure 4.8: ATP-chemiluminescence (log_{10} RLU/ml) measurements for the five biosensor strains and also the control strain when challenged with sorbic acid (the concentrations of sorbic acid are presented in ascending order (A to G) from left to right, and were 0.0031%; 0.0062%; 0.0125%; 0.025%; 0.05%; 0.1%; and 0.2% for each biosensor strain) at pH 5.0 over 28 days of incubation. # The overall SEM is 0.056 at 95% confidence interval

Table 4.3: Analysis of the viable counts, ATP-chemiluminescence, bioluminescence and the suitability of sorbic acid for different pharmaceutical preparations in accordance to European and British Pharmacopeia

Biosensor strain	[Sorbic acid] (%)	Incubation time required to achieve more than 3 orders of magnitude reduction in viable counts (days)	Incubation time required to achieve more than 3 orders of magnitude reduction in ATP-chemi-luminescence (days)	Incubation time required to achieve more than 3 orders of magnitude reduction in bio-luminescence (days)	% reduction in bio-luminescence when viable counts reduced by more than 3 orders of magnitude	Correlation coefficient (R^2) between RLU and CFU	Correlation coefficient (R^2) between CFU and ATP	Correlation coefficient (R^2) between RLU and ATP	Suitability of sorbic acid for different pharmaceutical preparations, as determined from the Pharmacopoeia[#]
Lpp-lux	0.2	1	1	1	99.9	1.000	1.000	1.000	O, P, T
	0.1	1	1	1	99.9	1.000	1.000	1.000	O, P, T
	0.05	7	7	1	99.9	0.790	0.973	0.961	O, T
	0.025	14	14	1	99.9	0.558	0.958	0.676	O
	0.0125	n/a	n/a	7	99.9	0.745	0.968	0.748	Non-effective
	0.0062	n/a	n/a	7	99.9	0.796	0.885	0.742	Non-effective
	0.0031	n/a	n/a	7	99.9	0.684	0.850	0.720	Non-effective
tatA-lux	0.2	1	1	1	99.9	1.000	1.000	1.000	O, P, T
	0.1	1	1	1	99.9	1.000	1.000	1.000	O, P, T

Biosensor strain	[Sorbic acid] (%)	Incubation time required to achieve more than 3 orders of magnitude reduction in viable counts (days)	Incubation time required to achieve more than 3 orders of magnitude reduction in ATP-chemi-luminescence (days)	Incubation time required to achieve more than 3 orders of magnitude reduction in bio-luminescence (days)	% reduction in bio-luminescence when viable counts reduced by more than 3 orders of magnitude	Correlation coefficient (R^2) between RLU and CFU	Correlation coefficient (R^2) between CFU and ATP	Correlation coefficient (R^2) between RLU and ATP	Suitability of sorbic acid for different pharmaceutical preparations, as determined from the Pharmacopoeia[#]
tatA-lux	0.025	14	14	14	99.9	0.929	0.977	0.937	O
	0.0125	n/a	n/a	21	n/a	0.877	0.870	0.837	Non-effective
	0.0062	n/a	n/a	28	n/a	0.886	0.807	0.806	Non-effective
	0.0031	n/a	n/a	28	n/a	0.966	0.955	0.919	Non-effective
ldc-lux	0.2	1	1	1	99.9	1.000	1.000	1.000	O, P, T
	0.1	1	1	1	99.9	1.000	1.000	1.000	O, P, T
	0.05	7	7	7	99.9	0.973	0.906	0.962	O, T
	0.025	14	14	14	99.9	0.988	0.909	0.962	O
	0.0125	n/a	n/a	21	n/a	0.903	0.863	0.984	Non-effective
	0.0062	n/a	n/a	28	n/a	0.948	0.992	0.930	Non-effective

Biosensor strain	[Sorbic acid] (%)	Incubation time required to achieve more than 3 orders of magnitude reduction in viable counts (days)	Incubation time required to achieve more than 3 orders of magnitude reduction in ATP-chemi-luminescence (days)	Incubation time required to achieve more than 3 orders of magnitude reduction in bio-luminescence (days)	% reduction in bio-luminescence when viable counts reduced by more than 3 orders of magnitude	Correlation coefficient (R^2) between RLU and CFU	Correlation coefficient (R^2) between CFU and ATP	Correlation coefficient (R^2) between RLU and ATP	Suitability of sorbic acid for different pharmaceutical preparations, as determined from the Pharmacopoeia[#]
	0.0031	n/a	n/a	28	n/a	0.833	0.985	0.854	Non-effective
lysS-lux	0.2	1	1	1	99.9	1.000	1.000	1.000	O, P, T
	0.1	1	1	1	99.9	1.000	1.000	1.000	O, P, T
	0.05	7	7	7	99.9	0.82	0.822	0.981	O, T
	0.025	14	14	14	99.9	0.811	0.925	0.914	O
	0.0125	n/a	n/a	14	99.9	0.803	0.959	0.949	Non-effective
	0.0062	n/a	n/a	28	n/a	0.886	0.949	0.956	Non-effective
	0.0031	n/a	n/a	28	n/a	0.963	0.901	0.827	Non-effective
spc-lux	0.2	1	1	1	99.9	1.000	1.000	1.000	O, P, T
	0.1	1	1	1	99.9	1.000	1.000	1.000	O, P, T
	0.05	7	7	7	99.9	0.82	0.822	0.981	O, T
	0.025	14	14	14	99.9	0.871	0.925	0.914	O

Biosensor strain	[Sorbic acid] (%)	Incubation time required to achieve more than 3 orders of magnitude reduction in viable counts (days)	Incubation time required to achieve more than 3 orders of magnitude reduction in ATP-chemi-luminescence (days)	Incubation time required to achieve more than 3 orders of magnitude reduction in bio-luminescence (days)	% reduction in bio-luminescence when viable counts reduced by more than 3 orders of magnitude	Correlation coefficient (R^2) between RLU and CFU	Correlation coefficient (R^2) between CFU and ATP	Correlation coefficient (R^2) between RLU and ATP	Suitability of sorbic acid for different pharmaceutical preparations, as determined from the Pharmacopoeia[#]
E.coli [pBR-322.lux]	0.0062	n/a	n/a	n/a	n/a	0.896	0.939	0.954	Non-effective
	0.0031	n/a	n/a	n/a	n/a	0.963	0.921	0.927	Non-effective
	0.2	1	1	n/a	n/a	n/a	0.945	n/a	O, P, T
	0.1	1	1	n/a	n/a	n/a	0.973	n/a	O, P, T
	0.05	7	7	n/a	n/a	n/a	0.990	n/a	O, T
	0.025	14	14	n/a	n/a	n/a	0.874	n/a	O
	0.0125	n/a	n/a	n/a	n/a	n/a	0.882	n/a	Non-effective
	0.0062	n/a	n/a	n/a	n/a	n/a	0.805	n/a	Non-effective
	0.0031	n/a	n/a	n/a	n/a	n/a	0.945	n/a	Non-effective
E.coli ATCC	0.2	1	1	n/a	n/a	n/a	0.945	n/a	O, P, T

Biosensor strain	[Sorbic acid] (%)	Incubation time required to achieve more than 3 orders of magnitude reduction in viable counts (days)	Incubation time required to achieve more than 3 orders of magnitude reduction in ATP-chemi-luminescence (days)	Incubation time required to achieve more than 3 orders of magnitude reduction in bio-luminescence (days)	% reduction in bio-luminescence when viable counts reduced by more than 3 orders of magnitude	Correlation coefficient (R^2) between RLU and CFU	Correlation coefficient (R^2) between CFU and ATP	Correlation coefficient (R^2) between RLU and ATP	Suitability of sorbic acid for different pharmaceutical preparations, as determined from the Pharmacopoeia[#]
E.coli ATCC 8739	0.1	1	1	n/a	n/a	n/a	0.973	n/a	O, P, T
	0.05	7	7	n/a	n/a	n/a	0.991	n/a	O, T
	0.0125	n/a	n/a	n/a	n/a	n/a	0.886	n/a	Non-effective
	0.0062	n/a	n/a	n/a	n/a	n/a	0.813	n/a	Non-effective
	0.0031	n/a	n/a	n/a	n/a	n/a	0.847	n/a	Non-effective

O, P and T denote the effectiveness of sorbic acid according to Pharmacopeia guidelines of Oral, Parenteral and ophthalmic, Topical preparation

n/a: not applicable

4.6 : Discussions

4.6.1 : Minimum Inhibitory Concentration (MIC) of Sorbic Acid

Organic acids have a long history of being utilized as food additives and preservatives for preventing food deterioration and extending the shelf life of food. These compounds primarily include saturated straight–chain monocarboxylic acids, often referred to as fatty acids, volatile fatty acids and weak acids (Ricke, 2003). Sorbic acid is one of the most widely used antimicrobial agents for food preservation worldwide, and it is used to preserve food, animal feed, pharmaceuticals, and cosmetics (Sofos et al., 1986).

The high correlations obtained between bioluminescence and culture density (absorbance) in the MIC assay described above (R^2: 0.9553-0.9917; Figures 4.3 (a) & (b)) suggest that each of the five constructs biosensor strains were equivalent to measuring growth by determining the turbidity of culture. Furthermore, on the basis of these results, each of the five biosensor strains could be considered for use in preservative MIC assays and to screen preservatives in preservative efficacy tests by measuring the emission of bioluminescence instead of turbidity.

The broth dilution assay is one of the earliest and most commonly used antimicrobial susceptibility testing methods (Wiegand et al., 2008). The advantages of using either turbidity or bioluminescence to estimate the effect of a preservative are the ability to produce rapid quantitative results using them. However, the principal disadvantage of measuring turbidity is the inability to determine the metabolic activity of the target cell(s). Mixtures of broth and high concentrations of certain preservatives (*e.g.* benzalkonium chloride) can result in a cloudy solution, and so the measurement of turbidity could be

yield a false positive result. However, the turbidity or otherwise of the culture medium is not a significant issue when measuring bioluminescence and so an assay based upon it would not be particularly affected by medium turbidity.

The MIC of sorbic acid was found to be at 0.1% in this study. This correlates well with previous work in which *E. coli* ML308-225 was challenged with sorbic acid at pH 5.0 and resulted in an MIC of 0.112% (Eklund, 1983). The same MIC was found for each of the biosensor and control strains in the work described in this thesis, which implies that the genetic manipulation of *E. coli* did not affect its susceptibility to sorbic acid. Sorbic acid is a six-carbon monocarboxylic acid, unsaturated at position 2 and 4. Other six-carbon acids, alcohols and aldehydes analogues have been found to have similar MIC to sorbic acid, despite different levels of saturation (Stratford & Anslow, 1998). Consequently, it is possible that these bioluminescent biosensor strains could be applied to preservative efficacy testing for all six-carbon sorbic acid analogues.

The work described in this thesis represents, to the best of this author's knowledge, the first attempt to investigate the use of *E. coli* transformed to a bioluminescent phenotype, using bioluminescent bioreporter constructs with constitutive promoters, as a rapid microbiological method for preservative efficacy testing (PET). Sorbic acid was prepared at pH 5.0 due to the potential for increased oxidation of sorbic acid at lower pH values (Stopforth et al., 2005) and also to mimic the pH often used in pharmaceutical preparations, food and cosmetic products. The *E. coli* biosensor strains, as well as control strains were, challenged with sorbic acid and their response was studied by monitoring bioluminescence in comparison with two methods recommended by the British and European

Pharmacopeias' (*i.e.* plate count and ATP chemiluminescence) to evaluate the effectiveness of sorbic acid as a preservative.

4.6.2 : Calculation of Undissociated and Dissociated levels of Sorbic Acid by Henderson-Hasselbalch Equation

The log acid dissociation ($-\log_{10} K_a$) (pK_a) represents the degree of dissociation for a weak acid; sorbic acid is a weak acid, and its pK_a is 4.76 (Cowles, 1941; Cerruti et al., 1990; Freese et al., 1973; Hoffamn et al., 1944; Sofos & Busta, 1981; Rahn & Conn, 1944; Pethybridge et al.,1983). This means that the concentration of undissociated sorbic acid molecules is equimolar to the dissociated form at a pH of 4.76. Whilst the concentration of undissociated molecules increases with decreasing pH, in contrast the concentration of undissociated molecules decreases with increasing pH levels (Simon & Beevers, 1951). Acetic acid has a pK_a value of 4.76, which is the same sorbic acid. However, the MIC required to inhibit the growth of *Saccharomyces cerrevisiae* by acetic acid was 30 times higher than the MIC of sorbic acid (Stratford & Anslow 1998). This indicates that sorbic acid has more than one mode of action, for example it may act as a membrane-active compound (Stratford & Anslow, 1998) in addition to the weak acid effect of reducing intracellular pH.

The undissociated form of sorbic acid ($C_6H_{11}COOH$) is membrane permeable by passive diffusion, and at the pH of 5.0 used in the current experiments 37% of the sorbic acid molecules would be in the undissociated form at pH 5.0 (Booth & Kroll, 1989). However, within the cytoplasm of a bacterial cell, the pH is approximately pH 6.8, the increased pH will favour the dissociation of the undissociated acid molecules entering the cell. Within the cytoplasm, 99% of the acid molecules will be in the dissociated form, releasing protons (H_3O^+) and anions ($C_6H_{11}COO^-$) according to the Henderson-Hasselbalch

equation (Table 4.1). The calculated percentage of undissociated molecules of sorbic acid (pH 5.0) in table 4.1 agrees with Sofos and Busta (1981). Since the vast majority of the intracellular sorbic acid will be in the dissociated form, but 37% of the extracellular acid will be in the undissociated form, a concentration gradient will ensue and lead to further diffusion of the undissociated form into the cell.

Acidification of the cytoplasm due to the release of protons, due to the dissociated sorbic acid, to prevent *E. coli* growth (Salmond et al., 1984). Both the undissociated and dissociated forms of acid cause the intracellular pH to fall (Salmond et al., 1984). However, on the contrary, Eklund (1983) proposed that growth inhibition requires the undissociated form of acid, which is approximately 10-600 times more effective as an inhibitor than the dissociated acid. Yet, the principle of inhibitory effect of weak acids is to reduce pH_i, which leads to the translocation of protons using H^+-ATPase which drains cells' energy (Salmond et al., 1984). The accumulation of the 99% of protons from the 37% undissociated SA entering the cytoplasm causes further acidification.

4.6.3 : Effect of Sorbic Acid on Bioluminescence, Viable Counts and ATP-Chemiluminescence of Biosensor and Control *E. coli* ATCC 8739 Strains in Preservative Efficacy Test (PET)

When challenged with 0.2% and 0.1% sorbic acid, the populations of *E.coli* biosensor strains and also the control strains (wildtype *E. coli* ATCC 8739 and *E. coli* [pBR322.*lux*]) had been reduced to undetectable levels between 0 h and 1 day of exposure (Figure 4.6). This related to the high concentrations calculated of undissociated form of SA calculated by the Handerson-Hasselbalch equation which dissociates to protons and anions in the cytoplasm (Table 4.1) resulting in cell death between 0 h and day 1. The high intracellular levels of dissociated sorbic acid leads to a pH difference

across the cell membrane (ΔpH) and forms a proton potential (Δp). Consequently the cell is required to expend energy to restore the intracellular pH to normal physiological levels. Under weak acid stress, ATPase activity requires 40% to 60% of total cellular ATP to restore internal pH of the cytoplasm (Holyoak et al., 1996; Serrano, 1991) in contrast to normal growth, the H^+-ATPase is estimated to use 10% to 15% of the total ATP produced. In addition, Plumridge et al. (2004) monitored NMR spectra to demonstrate rapid depletion of intracellular ATP under sorbic acid stress. Furthermore, it has been suggested that the concentration of intracellular anions can also lead to an osmotic imbalance within the cell and thus lead to an increase in cell turgor pressure (McLaggan et al., 1994). The combined effects of both accumulated protons and acid anions lead to the lethal effect of sorbic acid upon *E. coli* (Brown & Booth, 1991). Furthermore, the inhibition of metabolic enzymes such as fumarase and aspartase may be an indirect effect of sorbic acid, contributing to its antimicrobial activity (Beales, 2004; Liewen & Marth, 1985) at concentrations of 0.2%, and 0.1%SA. Sorbic acid has been demonstrated to covalently bind with, and inactivate, the sulphydryl groups of these enzymes (Denyer & Stewart, 1998; Eklund, 1989). Additionally, sorbic acid can also result in: interference with nutrient transport; cytoplasmic membrane damage; and disruption of outer membrane permeability (Beales, 2003; Freese et al., 1973; Liewen & Marth, 1985; Startford & Anslow, 1996). Finally, changes in pH_i can also affect control of the cell cycle (Anand & Prasad, 1989) resulting in decreased rates of DNA and RNA synthesis (Madshus, 1988). Thus sorbic acid can have multiple inimical effects upon the microbial cell.

The significant decrease in bioluminescence expression by the five biosensor strains when exposed to high concentration of SA (0.2% and 0.1%) was the result of exhaustion of the intracellular ATP levels in an attempt to restore of internal pH of the cell via the export of protons by the membrane H^+-ATPase (Holyoak et al., 1996) with undetectable levels of viable counts and low ATP-chemiluminescence

(Figures 4.6 & 4.8)). Furthermore, strong correlations between bioluminescence versus ATP chemiluminescence; and bioluminescence versus viable counts at these concentrations (R^2: 1.00). This reflects the light emission by the bioreporter strains is an indicative of the active state of cells whilst a reduced metabolism reflects on decreased bioluminescence (Unge et al., 1999). Consequently, dead bacteria do not produced light (Hastings et al., 1985).

Data tabulation of at least 3 orders of magnitude of viable counts (log_{10} CFU/ml), ATP-chemiluminescence (log_{10} RLU/ml) and bioluminescence (log_{10} RLU/ml) were employed in this study in compliance with the Pharmacopoeia requirements. The decrease of viable counts upon exposure to 0.2% and 0.1% SA between 0 h and 24 h for all *E.coli* strains exceeded the European Pharmacopoeia-A criteria and these concentrations are effective in oral, topical ophthalmic and parenteral preparations.

In 0.05% and 0.025% SA exposure, the dissociation of sorbic acid resulted in a decrease of viable counts of more or at least 3 or more orders of magnitude between 2 and 7 days in 0.05% SA whilst between 7 and 14 days respectively for all five constructs and control strains (wildtype *E. coli* ATCC 8739 and *E. coli* [pBR322.*lux*]).The antimicrobial activity at 0.05% and 0.025% SA took a longer time for a 99.9% decrease in viable counts. This suggests that, the driven H^+-ATPase activity was able to adequately pump protons, for restoration of internal pH at 0.05% and 0.025% SA, however the depletion of energy within the cells fail to sustain the activity at day 7 and 14 respectively for all five constructs and control strains (wildtype *E. coli* ATCC 8739 and *E. coli* [pBR322.*lux*] in which case wasn't able to pump protons out of the cell adequately).

The bioluminescence expressed by the *lpp-lux* strain was reduced by at least 3 orders of magnitude after 1 day of exposure. However, it was not until 7 days of exposure to 0.05% sorbic acid, and 14 days of exposure to 0.025% sorbic acid, that there was a corresponding reduction in bioluminescence expression of at least 3 orders of magnitude by the *tatA-lux, ldc-lux, lysS-lux* and *spc-lux* biosensor strains. There was a simultaneous decrease in the bioluminescence (log_{10} RLU/ml), viable counts (log_{10} CFU/ml), and ATP-chemiluminescence (log_{10} RLU/ml) from the *tatA-lux, ldc-lux, lysS-lux* and *spc-lux* biosensor strains at sorbic acid concentrations of 0.05 % and 0.025%. It has previously been reported that sorbic acid did not appear to have a major effect on the total glycolytic or respiratory flux, under any of the conditions tested, as the total CO_2 production and O_2 consumption were not affected (Holyoak et al., 1996). Consequently, it is likely that the intracellular supply of substrates to drive the bioluminescence pathway is independent of any energy depletion caused by the sorbate weak acid effect. This suggests that these biosensor strains with the appropriate constitutive promoter could be perfectly effective for a PET assay and that bioluminescence is not affected by SA and so can be used as a proxy for viable counts in PET (Ellison et al., 1994 a & b, Hill et al., 1993; Marincs, 2000; Steward, 1990, 1993; Stewart & Williams, 1992, 1993; Stewart et al., 1991, 1993, 1996, 1997).

However, at low concentrations of sorbic acid (*i.e.* 0.0125%, 0.0062%, and 0.0031%), the concentrations of dissociated SA (Table 4.1) from the undissociated form were proved to be less effective to *E. coli* populations for all five biosensor strains as well as the wildtype *E. coli* strains. This suggests that, at lower concentrations of sorbic acid, the H^+-ATPase activity of *E.coli* may have been able to pump protons out from the cell to a sufficient extent to control the internal pH of *E. coli* cells (Eraso & Gancedo, 1987; Salmond et al., 1984; Errano, 1980; Serrano, 1984; Plumridge et al., 2004).

The threshold concentration of intracellular undissociated sorbic acid that would result in a reduction of viable counts of at least 3 or more orders of magnitude was 0.81mM or 0.009% (Table 4.1).

The bioluminescence (log_{10} RLU/ml) expressed by the *lpp-lux* biosensor strain was decreased by more than 3 orders of magnitude after 7 days of exposure. In contrast, the viable counts did not decline by more than 3 orders of magnitude, even by the 28^{th} day of exposure to these concentrations of sorbic acid (Table 4.3) for all *E.coli* strains. At a sorbic acid concentration of 0.0125%, the bioluminescence expressed by the *lysS-lux* biosensor strain had decreased by 3 orders of magnitude between 7 and 14 days of exposure. In contrast, at an sorbic acid concentration of 0.0125% it took 14 and 21 days of exposure before the bioluminescence expressed by the *ldc-lux*, and *tatA-lux* biosensor strains had decreased by 3 orders of magnitude. At a sorbic acid concentration of 0.0031% it took 28 days of exposure for the bioluminescence expressed by *lysS-lux*, *ldc-lux*, and *tatA-lux* to be reduced by more than 3 orders of magnitude. This suggests that the metabolic activity of the *lysS-lux*, *ldc-lux* and *tatA-lux* biosensor strains decreased more rapidly than the viable counts. Never the less, the correlations between the bioluminescence and viable counts for these three biosensors were high (*tatA-lux*; R^2=0.877-0.966; *ldc-lux*; R^2=0.833-0.948, *lysS-lux*; R^2=0.803-0.963). Hence, the lowest SA concentration to inhibit *E.coli* growth is 0.1% which coincides with previous study by Eklund (1983) which reiterates that below this concentration is unable to cause a 3 orders of magnitude reduction. This agrees with the reduction of viable counts at 0.0125%, 0.0062%, and 0.0031% in this study. This implies that *lpp, lysS, ldc,* and *tatA* promoter constructs yielded a quicker bioluminescence reduction which could likely that the differences between the bioluminescence expressed by the five biosensor strains were brought about as a result of using the different constitutive promoters to control bioluminescence expression, and this will be discussed further in Section 4.7.3.

In contrast, at concentrations; 0.0125%, 0.0062%, and 0.0031% sorbic acid, the bioluminescence expressed by the *spc-lux* biosensor strains did not demonstrate a reduction of 3 orders of magnitude at any time over the 28 days of incubation, which matches the observation for viable counts and ATP chemiluminescence. Moreover, the bioluminescence per cell (RLU:CFU) was significantly higher at low SA concentrations expressed by *spc-lux* biosensor strain, as compared to *tatA-lux, ldc-lux, lysS-lux* and *lpp-lux* biosensor strains . It seems reasonable that a higher expression of bioluminescence per cell would offer greater flexibility for antimicrobial testing due to the wider working range of bioluminescence between maximal bioluminescence expression and zero. Consequently, it would seem reasonable that *spc-lux* would be a particularly good candidate for further evaluation in "real-life" preservative efficacy testing, more so than the other biosensor strains tested here. This conclusion is further supported by the excellent correlation coefficients between bioluminescence and viable counts obtained (R^2= 0.879-1.00) in the trial of sorbic acid PET.

It is noteworthy that a residual low level of bioluminescence was observed from the five biosensor strains even when viable counts were undetectable in the presence of 0.2%, and 0.1% sorbic acid, which suggests two potential possibilities. The first is that there may be a small population that is below the MDL limit whilst a large population of injured cells that are luminescing sub-maximally (Dodd et al., 1997). The residual ATP measured within the cells (Figure 4.5) could promote the residual bioluminescence, but be insufficient for growth. A second explanation is that the residual luminescence may represent viable but non culturable (VBNC) state of *E. coli* cells at high SA concentrations. Previous efficacy studies of pharmaceutical oils have demonstrated that *E. coli* 8739 enters into a viable but non culturable (VBNC) state which can be demonstrated by measurement of a signal using solid-

phase cytometry (SPC), whilst viable counts are undetectable (Prijck et al., 2008). In addition, Duncan et al. (1994) was able to detect the presence and activity of viable but non culturable cells utilizing luminescence genes *luxAB*. Although, VBNC cells show very limited metabolic activity (Oliver, 2005), and therefore there may not be sufficient FMNH available, or aldehyde produced, to drive measurable bioluminescence.

It is recommended by both the European and British Pharmacopeias that *E. coli* ATCC 8739 only be used PET assays for oral preparations. However, the United States Pharmacopeia still permits *E. coli* ATCC 8739 to be used for PET assays of different pharmaceutical preparations. Sorbic acid was effective at reducing both the bioluminescence and viable counts of all of the *E. coli* ATCC 8739 biosensor strains by more than 3 orders of magnitude at concentrations of 0.2%, 0.1%, 0.05% and 0.025%. The viable counts and bioluminescence expressed by the *lpp-lux*, *tatA-lux*, *ldc-lux*, *lysS-lux* and *spc-lux* biosensor strains was reduced by 99.9% (3 orders of magnitude) at sorbic acid concentrations of 0.2%, 0.1%, 0.05% and 0.025% concentrations which demonstrates that bioluminescence yields results that are directly comparable with the Pharmacopoeial requirement for a reduction in viable counts of at least 3 orders of magnitude.

4.6.4 : The Overall Comparisons between the Constitutive Promoters Expression Bioluminescence to the ATP-Chemiluminescence and Viable Counts Methods

F values result signifies the equivalence and precision of the whole-cell bioluminescence as a rapid microbiological method in comparisons to the pharmacopeia certified methods; plate counting and ATP chemiluminescent method. This implies that the success of whole-cell bioluminescence method to monitor *E.coli* viability in diverse preservatives. The SEM obtained for the whole-cell bioluminescent

method was an order lower than the plate counting method, and half that of to ATP chemiluminescence. These SEM obtained for the three methods were in line with the SEM which were less than 0.3 (PDA, 2000). This further justifies the application of the whole-cell bioluminescence method as a rapid real time microbiological method instead of viable counts for PET.

Previously ATP measurements have been used as an alternative to viable counts (Kremer et al., 2008), and this has subsequently been validated and incorporated into the Pharmacopoeia as the first alternative method for PET that has been accepted by the regulatory authorities. ATP-chemiluminescence has been demonstrated to yield equivalent results to traditional viable counting when *E. coli* ATCC 8739 was challenged with methyl parahydroxybenzoate (MHB) (Kramer et al., 2008). Similarly, in the current experiments, ATP-chemiluminescence demonstrated excellent correlations (R^2 = 0.806-1.00) with viable counts for each of the biosensor and control strains for all of the concentrations of sorbic acid tested, from 0.2% to 0.031%. Moreover, in this study, there was excellent correlation (R^2 = 0.806-1.00) between the bioluminescence expressed by the *tatA-lux*, *ldc-lux*, *lysS-lux* and *spc-lux* biosensor strains and ATP-chemiluminescence measured using Pharmacopoeial methods which indicates the equivalence of these two methods for PET assays of sorbic acid. However, *lpp-lux* biosensor strain yield a lower correlation coefficient (R^2:0.676-1.00) between ATP-chemiluminescence and bioluminescence.

In addition to bioluminescence versus ATP-chemiluminescence, the correlation between bioluminescence and viable counts was also excellent for each of the biosensor strains (for *tatA-lux*, R^2 = 0.886-1.00; for *ldc-lux*, R^2 = 0.833-1.00; for *lysS-lux* R^2 = 0.811-1.00; and for *spc-Lux* R^2 = 0.879-1.00). Furthermore, the result of F test implies equivalence in precision of the whole-cell bioluminescent

method to the traditional plate count method and ATP chemiluminescence as they are regarded as valid microbial enumeration methods for PET as defined by the Pharmacopeia.

Although sorbic acid presents a substantial inimical challenge to the target cells, and this was hypothesised to affect intracellular ATP levels due to the requirement to excrete protons, the bioluminescence was found to correlate well with both the viable counts and the ATP-chemiluminescence The results found in this study prove otherwise. On the other hand, at low concentrations of sorbic acid, the viable counts, bioluminescence and ATP chemiluminescence decreased proportionally and were strongly-correlated amongst the methods except for *lpp-lux.*

Amongst the five biosensor strains tested, that using the *lpp* promoter to drive *luxCDABE* did not exhibit any bioluminescence after day 2 of exposure to even relatively low concentrations of sorbic acid, whilst *ldc-lux*, *lysS-lux* and *spc-lux* all exhibited bioluminescence at lower sorbic acid concentrations. Studies have indicated increased expression of *Slp* (lipoprotein), (Arnold et al., 2001) and *pal* (lipoprotein associated with peptidoglycan) (Maurer et al., 2005) by *E. coli* cells under acidic conditions at pH 5.0. These genes all function to protect against protein damage which is caused by the intracellular dissociation of weak organic acids that have diffused into the cell in the undissociated form (Mates et al., 2007). In this study, it is possible that *lpp* expression was down-regulated under sorbic acid stress. This is suggested by the decrease in when exposed to low concentrations of sorbic acid, even when the viable counts and ATP-chemiluminescence levels remained relatively unchanged. Production of an alternative lipoprotein using the *slp* gene product, is up-regulated to provide outer membrane lipoprotein when cells are stressed by starvation (Mates et al., 2007) and its role is to limit penetration of organic acids across the outer membrane or as part of a signal transduction mechanism that activates an organic

acid protection system (Castanie-Cornet et al., 2006). It has been noted that the maintenance of pH homeostasis under mild acid shock induces changes in the composition of outer membrane proteins and also to cell surface hydrophobicity (Dilworth and Glenn, 1999). Hence, due to the possible up-regulated products of outer membrane lipoprotein as demonstrated in previous studies, it is likely that the expression of *lpp* would have been switched off under starvation stress, as seen in the drastic decrease in bioluminescence.

4.6.5 Bioluminescence Expression in Relation to the Promoters Employed

Under acidic condition, it has been revealed previously that the stationary phase sigma factor RpoS ($\sigma^{38/S}$), is required for weak acid tolerance induced by microbial growth at nonlethal acidic pH (Arnold et al., 2001). The RpoS ($\sigma^{38/S}$) regulon constitutes of a large regulatory network with a hierarchical (cascade-like) expression of regulatory genes under the control of RpoS ($\sigma^{38/S}$) in acidic conditions (pH 5) (Richard & Foster, 2003; Weber et al., 2005).

A virtual foot printing analysis (Regulon DB) was carried out, and this indicated that a *crp* binding site was present in the negative strand of the *lpp* promoter within the -10 regions (Score: 5.07) *(*Appendix 4) A PWM's score is the sum of log-likelihoods, which corresponds to the presence of *crp* binding site present. In addition, two *crp* binding sites were found located on the negative strand of the *lysS* promoter sequence (Scores: 6.00, and 6.55). When bound at tandem binding sites within the UP (-60 and -40 positions) elements, *crp* functions synergistically with cyclic adenosine monophosphate (cAMP) as an activator when there is an upstream *crp* binding site located in contact with α-CTD which contributes to the stability of the RNA polymerase (Czarniekci et al., 1997; Gaal et al., 1996; Tang et al., 1994). However, when there is/are *crp* binding site (s) that overlaps or located within the downstream of the transcription sites between the -10 and +1 regions, this acts a repressor (s) (Busby &Ebright, 1994; Lee

& Busby, 2012; Weber et al., 2005). This suggests that the location of the *crp* regulators within the -10 elements results in a possibility of down-regulation in promoter activity of *lpp* and *lysS* in the presence of repressor (Busby & Ebright, 1994). Marques et al (2006) reveals the obstacles concerning the presence of regulons which leads to repression of bioreporter gene expressions in whole-cell biosensor. Hence, the use of the virtual foot printing software could detect possible regulons for future constructs of biosensors for possible reasoning for lower/higher promoter strength.

Despite finding two *crp* loci within the *lysS* promoter, as opposed to only one in the *lpp* promoter, bioluminescence was not so strongly affected by acid stress as it was in *lpp*. However, Rhodius and Mutalik (2010) have described a scoring system position weight matrix (PMW) that could predict inaccurate transcription binding sites where the interactions are weak or inactive under physiological conditions, and false predictions (Rhodius & Mutalik, 2010). Consequently, virtual foot printing tool can be used to identify potential presence of repressors within the promoter sequences of biosensors which could explain the lowered expression levels under stress (*i.e.* nutrients and acid). However, further studies to identify these repressors.

4.6.6 : Selected Promoters under Acidic Conditions at pH 5.0

The apparent down-regulation of *lpp* during the sorbic acid challenge experiments described here suggests that the *lpp* promoter is not suitable for use in a biosensor for PET assays. Amongst the four other promoters tested, expression of bioluminescence by *lysS* was the weakest which resulted in a significantly lower bioluminescence expression under sorbic acid challenge than was observed for the other bioreporter strains. It is possible that the *crp* locus within the promoter region may have influenced this behavior. In contrast, both the *tatA*-*lux* and *ldc*-*lux* constructs both yielded bioluminescence levels per cell that were almost 100-1000 times higher than *lpp*, when challenged with low concentrations (0.0125%, 0.0062%, and 0.0031%) of sorbic acid at pH 5.0. Previous studies have shown that an RpoS-dependent mechanism regulates *ldc* in the stationary phase (Kikuchi et al., 1998). However, the expressions of bioluminescence per cell of *ldc* was significantly lower to *spc*. Whilst, *tatA* demonstrated approximately half the intensity significantly of bioluminescence per cell at low SA concentrations (0.0125%, 0.0062%, and 0.0031%) compared to *spc* In addition, the increased levels of bioluminescence per cell were observed in alkaline pH (Chapter 3) demonstrates a potential of the application of *tatA* constitutive promoter in alkaline preservatives. Therefore, it is concluded that *spc* is the most appropriate promoter, from amongst the five tested, for use in sorbic acid, pH 5 from 0.2% to 0.0031%, since the bioluminescence expressed per cell was 10-1000 times greater than that exhibited by the other four promoters. The *spc*-*lux* biosensor demonstrates a stronger correlation in with the consensus sequences the extended -10 region and -10 region of *spc* is the exact consensus sequence of RpoD (σ^{70}) (Cowing, 1985) and only a -8 nucleotide difference to the consensus of RpoS (σ^{38}) (Becker & Hengge-Aronis, 2001). These resemblance of the *spc* nucleotide in the promoter regions to the consensus sequences of RpoD (σ^{70}) and RpoS (σ^{38}) could suggest that these consensus sequences contributes to a high bioluminescence per cell under sorbic acid regulation.

Moreover, the fact that there were no significant differences between the bioluminescence results, and the viable counts and ATP-chemiluminescence levels, demonstrates that bioluminescence shows great potential for application in preservative efficacy studies. Rapid methods to determine preservative efficacy are also in high demand in areas of monitoring, where results need to be supplied in minutes. Consequently, the application of *lux* constructs provides much more than a convenient bioreporter for gene expression but also an alternative rapid method to the conventional plate counting method. The ability to monitor in real-time microbial population dynamics means that the time taken to determine the activity of antimicrobials in food, cosmetic and pharmaceutical products can be significantly reduced.

4.7 : Conclusions

1) There was a strong correlation between the bioluminescence expressed by the *spc-lux*, *tatA-lux*, *lysS-lux* and *ldc-lux* biosensor strains and the viable count and ATP-chemiluminescence compliance methods from by the Pharmacopoeias.
2) There did not appear to be any factors in the *tatA*, *ldc* and *spc* promoters that might affect the expression of bioluminescence, whilst potential repressor sequences were identified in both *lpp* and *lysS*.
3) Expression of the *lpp* promoter was found to be down-regulated under acidic conditions, since bioluminescence was significantly reduced after 7 days of exposure to low concentrations of sorbic acid whilst the viable counts and ATP-chemiluminescence were unchanged.
4) The *spc* promoter is the best candidate for further testing of bioluminescence as a rapid whole-cell method for PET assays, since there was a 10-1000 times greater bioluminescence per cell than that exhibited by the other four promoters, when challenged in sorbic acid, pH 5.0.

Chapter 5

5 Evaluation of Whole-Cell Bioluminescence Using Benzalkonium Chloride (BAK)

5.1 : Benzalkonium Chloride as a Preservative

Quaternary ammonium compounds (QACs) such as benzalkonium chloride (BAK) are used in a wide range of applications such as disinfectants, pharmaceutical antiseptics, cosmetics, oral, parenteral, nasal, and ophthalmic products. The aims of its use are to kill microorganisms (bactericidal effect) and/or prevent microbial growth (bacteriostatic effect) (Brown & Norton, 1965; Eriksen, 1970).

BAK is also used in alcohol-free hand sanitizers (Dyer et al., 1998). The use of alcohol in hand sanitizers frequently causes dryness and can lead to subsequent micro abrasions to the skin (Dyer et al., 1998). This therefore, increases the susceptibility of the skin surface to infection by members of the transient microflora. The use of BAK complements, rather than compromises, the natural barrier function of the skin much better than alcohol (Dyer et al., 1998). In addition, the use of BAK as an antiseptic causes less of a 'burning' sensation on any wounds than alcohol or hydrogen peroxide do. BAK is effective in inhibiting the growth of bacteria, yeast, and moulds (Dyer et al., 1998).

Velandia et al. (1995) demonstrated the inhibition of HIV particles by 0.05% BAK. Formulations using BAK and other QAC derivatives have also been shown to possess antiviral properties. Overall, the use of BAK is favoured for skin sanitizers and disinfectants due to its neutral to slightly alkaline nature, non-metal corrosive and non-flammable characteristics. In addition, it is safe to use on washable surfaces.

BAK has been used to preserve ophthalmic medications since the late 1940s. Microbial contamination has been found to be present in approximately 29% of all in-use ophthalmic containers (Geyer et al., 1995; Schein et al., 1992). Therefore, BAK is commonly added to ophthalmic care products to prevent contamination by microbes that might potentially cause physicochemical deterioration of a multi-dose ophthalmic solution, or pose a risk of further infection to the patient. Such potential contamination may occur either during the preparation of a medication or during its application into the eye (Furrer et al., 2001). However, the use of BAK for sanitisation of soft contact lenses is limited due to binding of BAK to the lens material which can lead to ocular irritation (Doughty, 1994). Overall, the maximum concentration of BAK recommended for ophthalmic and parenteral procedures is 0.02% (Furrer et al., 2001).

5.1.1 : Mode of Action

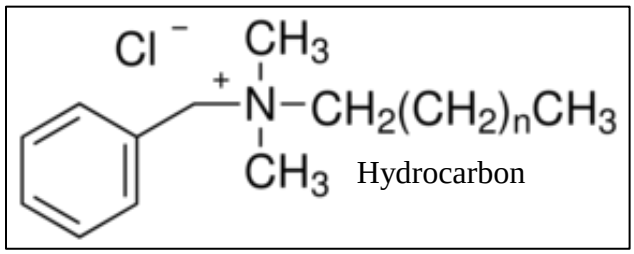


Figure 5.1: The chemical structure of BAK.

Chemically, benzalkonium chloride (BAK) is a mixture of alkylbenzyldimethylammonium chloride which is strongly positively charged and has a slight hydrophobic property which is contributed by the hydrocarbon chain (Figure 5.1). The inimical activity of BAK is initiated by the attraction of opposite charges between BAK and the bacterial cell. The structure of the outermost layer of bacterial cells universally carries a net negative charge associated with the cytoplasmic membrane and also the lipopolysaccharide of the cell wall of Gram negative bacteria. The attraction of charges results in a high

binding affinity and the effect of BAK leads to destabilisation of the cell membrane, resulting in a loss of proton motive force (pmf) and also the leakage of cell contents resulting in growth arrest as depiced in Figure 5.2. Bacterial cell walls are often stabilized by the presence of divalent cations such as Mg^{2+} or Ca^{2+} (Figure 5.2). Consequently the action of BAK can be enhanced by the addition of chelating agents such as EDTA that perturb the membrane structure through the sequestration of stabilizing metal cations (Gilbert & Moore, 2005). Therefore, synergistic relationship between BAK and EDTA that can enhance the antimicrobial activity of BAK has been reported.

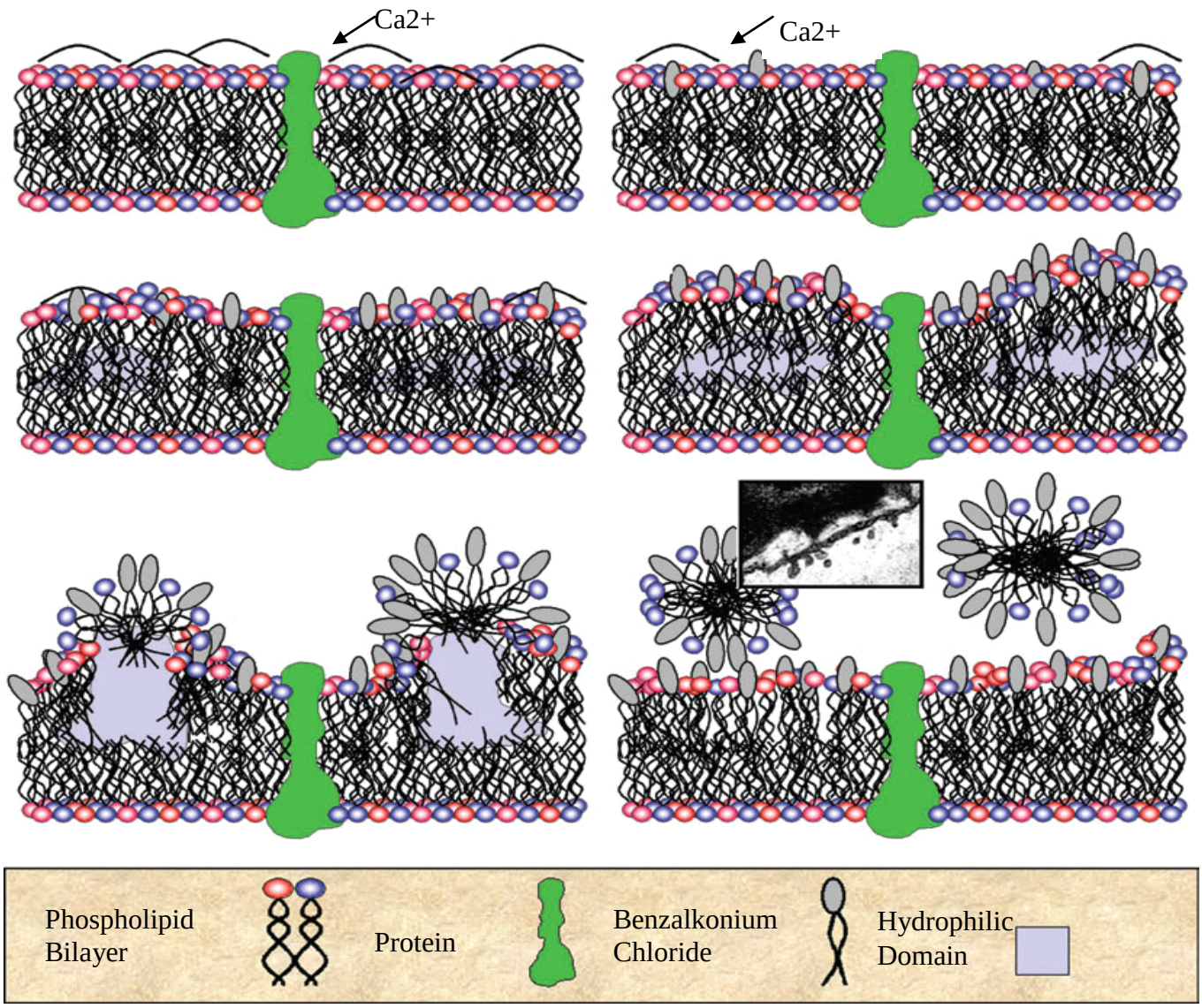
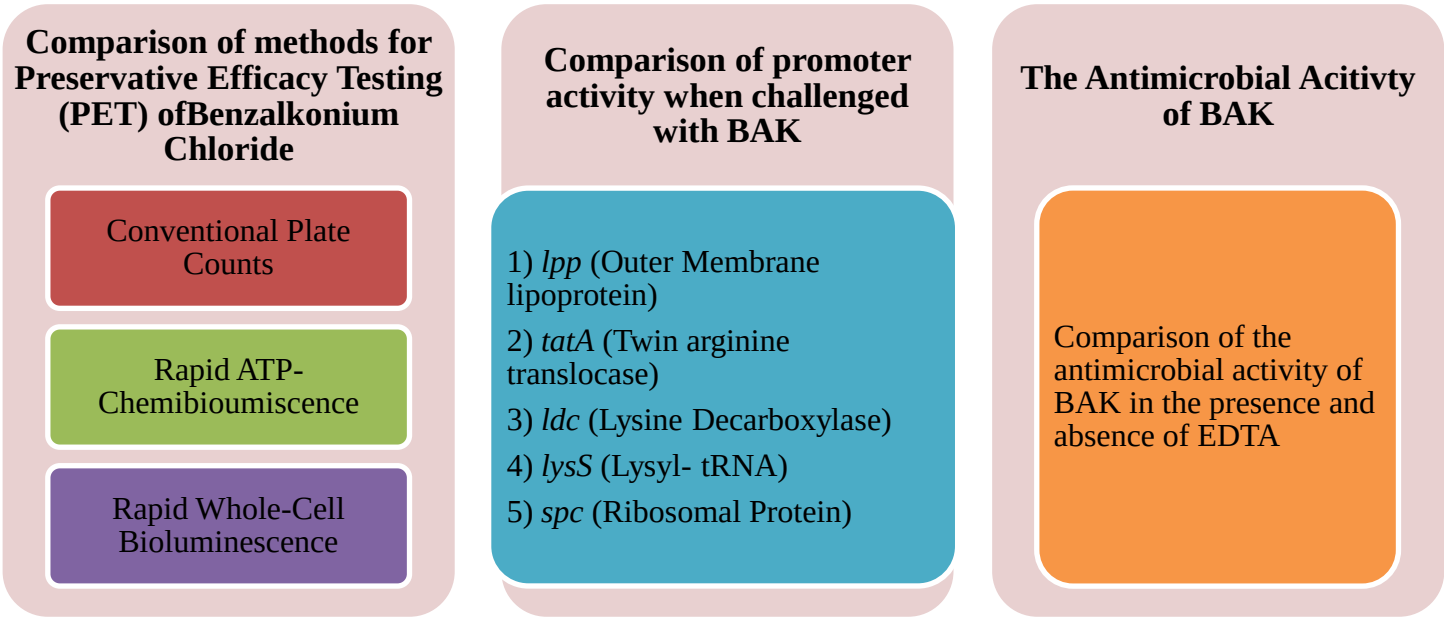


Figure 5.2: The progressive adsorption of the quaternary head group of acidic phospholipids into the bacterial cell membrane, leading to the decreased fluidity of the bilayer, and to the creation of hydrophilic voids within the membrane. Protein function is also perturbed by BAK, and eventually phospholipids and proteins will bud off into phospholipid micelles that will lead to cell lysis (adapted from Gilbert & Moore, 2005).

5.2 : Rationale of Chapter 5

This chapter describes an investigation into the application of a whole-cell biolumiscent biosensor as an alternative rapid microbiological method to test the efficacy of a quanternary ammonium compound preservative. In this chapter, the whole-cell bioluminescence method was employed to screen a range of concentrations of benzalkonium chloride (BAK) from 0.00125% to 0.00039% at a pH of 7.0, in accordance with the requirements set out by the British & European Pharmacopoeias. The reductions in the viable counts of the experimental organisms, when exposed to the preservatives tested, were also determined in accordance with the requirements of the British & European Pharmacopoeias. Furthermore the whole-cell bioluminescent biosensors were also used to investigate the synergistic relationship between EDTA and BAK in the work described in this chapter. In addition to monitoring the viable counts of *E. coli* in the presence of the preservative using bioluminescence, viable counts and ATP-chemiluminescence were also used to monitor viability as described by the pharmacopoeias. The aim of the work described in this chapter was to determine the accuracy and reproducibility of whole-cell bioluminescence as a biosensor to screen the activity of BAK in comparison with the currently-accepted methods.

This chapter covers the following comparisons:



5.3 : Objectives

1) To compare the response of the 5 bioluminescent reporter strains to exposure to benzalkonium chloride at a range of concentrations from 0.00125% to 0.00039% at a pH of 7.0, and in the presence or absence of EDTA.
2) To compare the response of the bioluminescent reporter strains to benzalkonium chloride, at a range of concentrations from 0.00125% to 0.00039% at a pH of 7.0 and with the addition of EDTA, to the existing methods prescribed by the British and European pharmacopoeias.
3) To compare the antimicrobial activity of BAK in the presence and absence of EDTA.

5.4 : Materials and Methods

5.4.1 : Preparation of Bacterial Initial Inoculum and Preservative Solutions

The initial inocula of the five biosensors and also the control strains were prepared as described in Section 4.5.1.

5.4.2 : Preparation of Preservative Solutions

5.4.2.1 : Preparation of buffer solution without BAK

2.50 g of Sodium Chloride (Fisher Scientific), 2.50g of Potassium Chloride (Fisher Scientific), 2.50 ml of Glycerol (Sigma-Aldrich), 1.25 ml of Propylene Glycol (Sigma-Aldrich) and volumes of BAK as indicated in 5.2.2.2 were dissolved in 150.0 ml of deionized water. The pH of the solution was adjusted to neutrality (pH 6.9-7.1) by the dropwise addition of 0.1M HCl. The solution was then topped up with deionized water to a final volume of 250.0 ml (Table 5.1). This buffer solution composition was based on moisturizing and lubricating eye solution without addition of preservatives.

Table 5.1: Composition of the buffer solution for the PET assays

Chemical	Concentration in Percentage (%)
Sodium Chloride	1.0 w/v
Potassium Chloride	1.0 w/v
Glycerol	1.0 v/v
Propylene Glycol	0.5 v/v

5.4.2.2 : BAK solutions

1.0g of benzalkonium chloride (Sigma-Aldrich) was weighed out and dissolved in 10ml of sterile distilled water to obtain a 10.0% stock solution which kept at 4°C until use. To prepare the working solution of BAK, 15.6µl of the 10% BAK stock was added to the buffer solution indicated in Section 5.2.2 for the final concentration of the BAK was 0.0062%.

For the working concentrations of 0.0031% (v/v), 0.0016% (v/v) (round up to 2 significant figures), 0.00078% (v/v) and 0.00039% (v/v)– 78.1 µl, 39.1 µl, 19.5 µl and 9.7 µl of BAK 10% – were added to 250 ml of buffer solution to yield the concentrations noted above. The volume of BAK stock solution, concentration of BAK stock solution and final working concentration of BAK solution produced are as indicated in Table 5.2.

Table 5.2: Amounts of BAK required for respective final working concentrations

Volume of stock BAK solution (µl)	Concentration of stock BAK solution (% v/v)	Final working concentration of BAK (% v/v)
15.6	10	0.0062
78.1	10	0.0031
39.1	10	0.0016
19.5	10	0.00078
9.7	10	0.00039

The aliquots of BAK stock solution were individually added to buffer solutions, and then filtered through 0.22 µm Millipore Minisart syringe filters to produce the sterile working solutions.

5.4.2.3 : Benzalkonium chloride with 0.03% EDTA Formulations

7.5g of EDTA (Sigma-Aldrich) was added to 250 ml buffer solutions to yield a final concentration of 0.03% (w/v). The 0.03% EDTA in basal salt formulation was then used to prepare the BAK solutions as described in Table 5.2. The final BAK concentrations used in combination with 0.03% EDTA were 0.0062% (v/v), 0.0031% (v/v), 0.0016% (v/v), 0.00078% (v/v) and 0.00039% (v/v).

5.4.3 : Preservative Efficacy Test

Preservative efficacy testing of the various preservative solutions, prepared as described in section 5.2.2, was carried out as described previously in section 4.5.2.

5.4.4 : Determination of the Minimum Inhibitory Concentration (MIC) ofBenzalkonium Chloride (BAK), BAK +0.03% EDTA, and EDTA

Stock concentrations of the benzalkonium chloride, and also the initial bacterial inocula, for the experiments to determine the MIC of BAK were made up as described in Section 4.5.1. Initially, a ten-fold dilution was made in TSB from the 1% BAK stock solution to yield an initial working concentration of 0.1% BAK and then two-fold serial dilutions were subsequently carried out to give final working concentrations of BAK of 0.05%, 0.025%, 0.0125%, 0.0063%, 0.0031%, 0.0016%, 0.00078%, and 0.00039%. In a second set of MIC tests, 0.03% EDTA was added to the TSB and a ten-fold of 0.1%, and then two-fold serial dilutions were subsequently carried out to give final working concentrations of BAK with 0.03% EDTA of 0.05%, 0.025%, 0.0125%, 0.0063%, 0.0031%, 0.0016%, 0.00078%, and 0.00039%. In a third set of MIC tests EDTA alone was used, 900µl of the 1% stock solution was added to a 99.1 ml volume of TSB, and then three-fold dilutions were performed to achieve the final working concentrations of 0.09%, 0.03%, and 0.01% EDTA.

The initial optical density of the bacterial suspension, the bioluminescence throughout the course of the experiment, and the MIC were all determined as described in Section 4.5.3. The viable counts of the bacterial cultures were determined before addition of the preservatives and also from the concentration determined to be the MIC.

5.4.5 : Statistical Analysis

Statistical analyses were performed as described in Section 4.5.3

5.5 : Results

This results section contains three experimental parts.

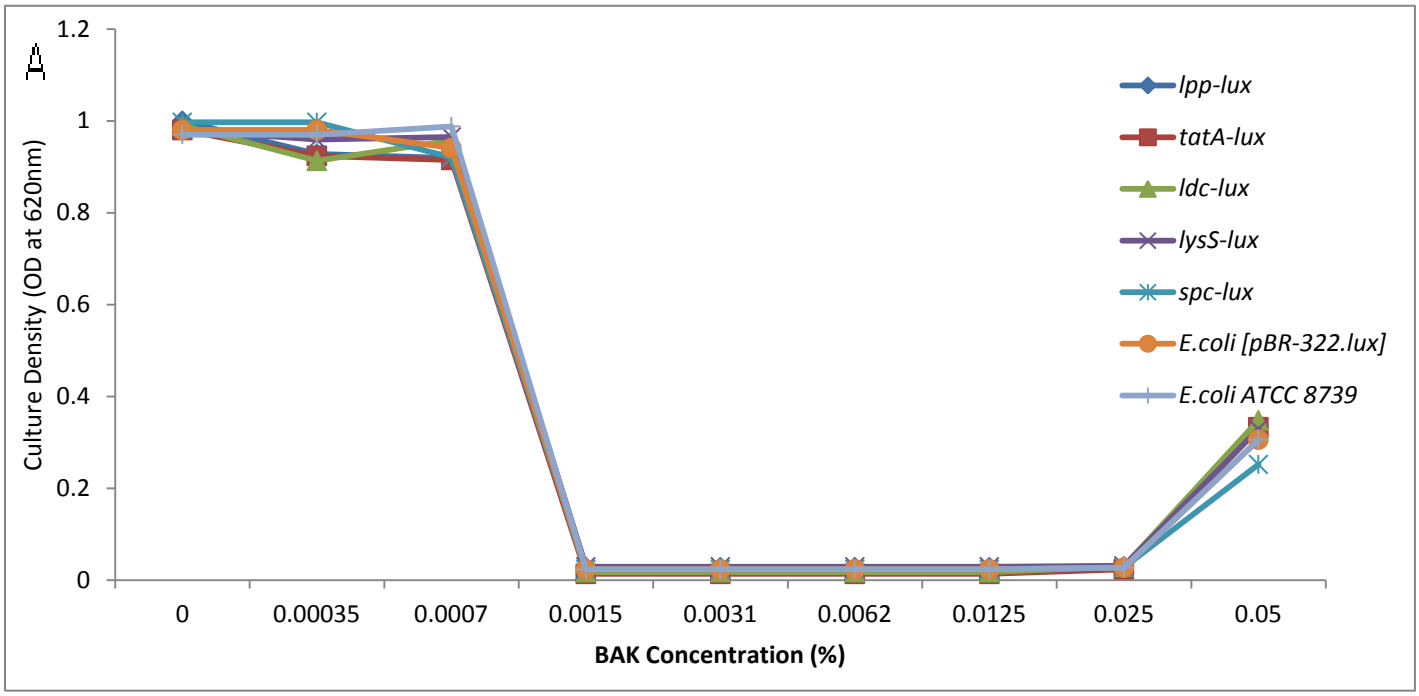
The first experimental part, described in section **5.3.1** presents the MIC of benzalkonium chloride (BAK) alone, BAK with 0.03% EDTA and of EDTA alone evaluated using both the bioluminescence and the turbidity method. The second experimental part, described in sections **5.3.2, 5.3.3** and **5.3.4** evaluates BAK in the preservative efficacy test (PET) using bioluminescence, viable counts and ATP-chemiluminescence. The final experimental part, described in sections **5.3.5** and **5.3.6**, evaluates the combination of BAK and EDTA in the preservative efficacy test (PET) using bioluminescence, viable counts and ATP-chemiluminescence.

5.5.1 : Minimum Inhibitory Concentration (MIC) Benzalkonium Chloride (BAK)

The mean + SEM (viable count of the initial inoculum before the addition of preservatives was approximately 2 ± 0.1 x 10^6 CFU/ml for all *E.coli* strains. The mean $\pm$ SEM absorbance of the initial inoculum was 0.05 ± 0.01 A.U. across BAK concentrations ranging from 0.00039% to 0.025%, whilst for 0.05% BAK it was significantly higher at 0.3 ± 0.02 AU. The mean $\pm$ SEM initial bioluminescence readings were between 6.79 to 4.25 ± 0.10 $\log_{10}$ RLU/ml (this was done as described in Section 4.6.2).

After 24 h exposure to the BAK, the mean $\pm$ SEM culture density was 0.96 ± 0.05 A.U. with no statistical significant differences at BAK concentrations of 0%, 0.00039% and 0.00078%, and was significantly lower with a mean of 0.02 ± 0.70 A.U. at BAK concentrations of 0.0016%, 0.0031%, 0.0063%, 0.0125% and 0.025% (P = 0.00, ANOVA) (Figure 5.3(a)). However, the mean $\pm$ SEM absorbance of the culture after exposure to 0.05% BAK (0.37 ± 0.06 A.U.) was significantly higher than

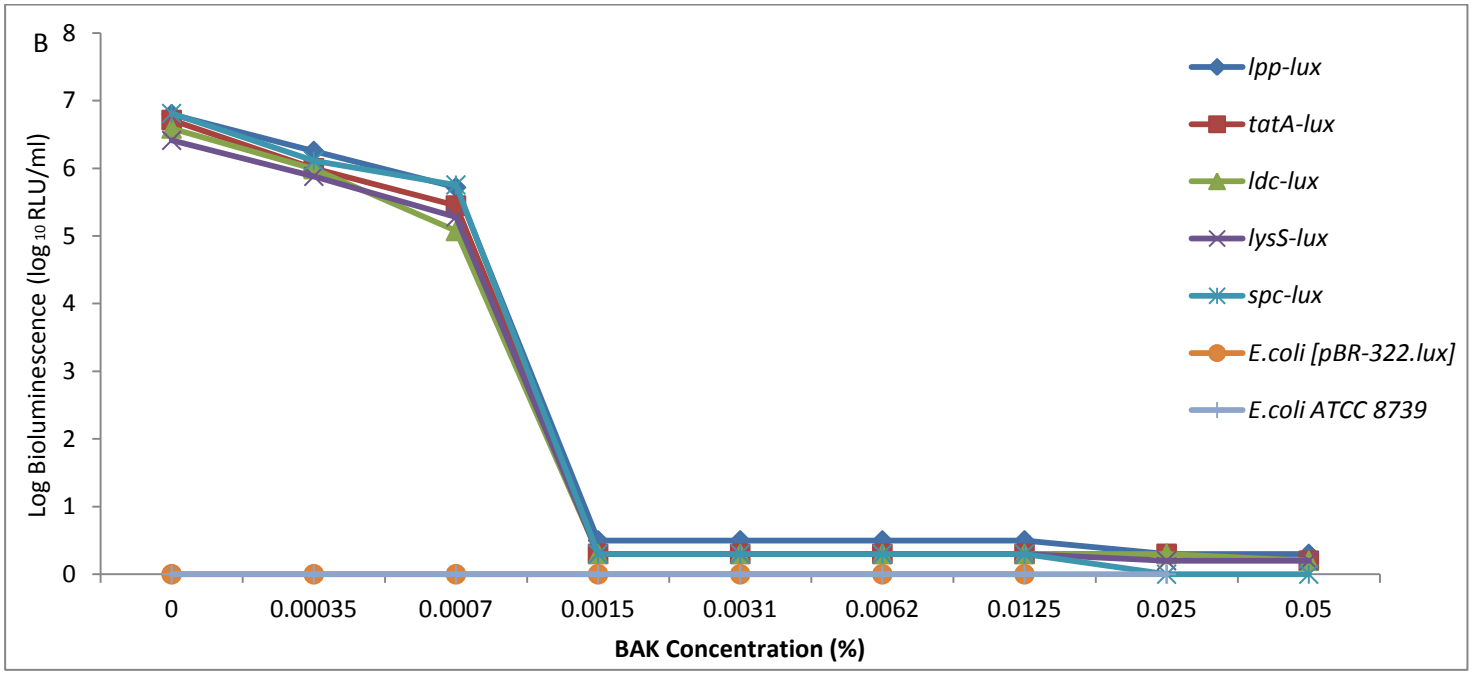
after exposure to 0.025% BAK (0.05 ± 0.01 A.U.) (*P* = 0.00, Tukey Kramer *Post Hoc*) (Figure 5.3(a)). The same effect was observed for each of the *E.coli* strains.



Figures 5.3 (a): Effect of Benzalkonium Chloride upon culture density after 24 hours of incubation in its presence, in order to determine the Minimum Inhibitory Concentration (MIC)
The SEM for each individual biosensor strain in Figure 5.3 (a) did not exceed 0.005

The bioluminescence ± SEM was between 5.04 to 6.69 ± 0.2 $\log_{10}$ RLU/ml at 0% BAK, 0.00039% BAK and 0.00078% BAK after 24 h incubation. The mean ± SEM bioluminescence was significantly lower after 24 hours of incubation with BAK concentrations of 0.0016%, 0.0031%, 0.0063%, 0.0125%, 0.025% and 0.05%, than with 0%, 0.00039% or 0.00078% (*P*= .000, ANOVA) (Figure 5.3b). Neither of the control strains, *E.coli* [pBR-322. *lux*] and *E.coli* ATCC 8739, expressed any bioluminescence. Strong significant correlations were found between the absorbance and the bioluminescence (R^2 = 0.9362-0.9774) (*P*= 0.001) in the determination of the BAK MIC.

The MIC of BAK was determined to be 0.0016 %, from the change in bioluminescence expression as well as the OD, and this was confirmed by the plate counts with a statistically significant (*P* =0.000, ANOVA) ≥ 99.9% reduction in the viable counts from a mean of 6.2 ± 0.3 CFU/ml in the initial inocula to a final value of approximately 2 ± 0.1 x 10^3 CFU/ml at 0.0016% BAK and greater (*P* =0.000, ANOVA).

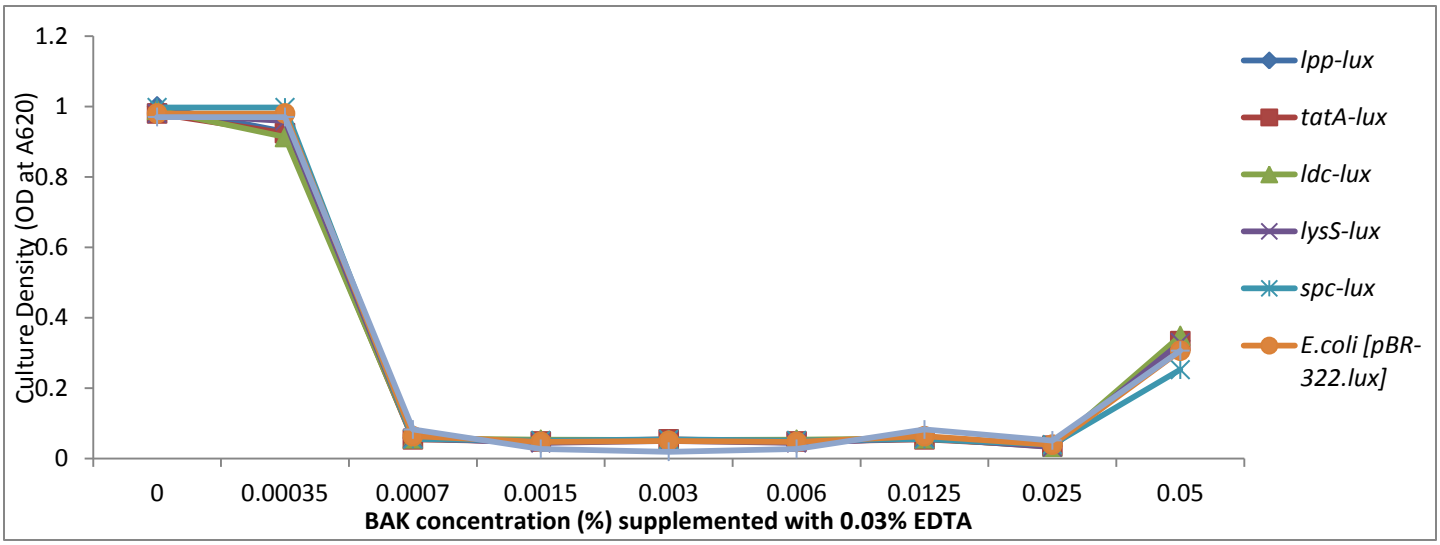


Figures 5.3 (b): Effect of Benzalkonium Chloride upon bioluminescence, after 24 hours of incubation in its presence, in order to determine the Minimum Inhibitory Concentration (MIC)
The SEM for each individual biosensor strain in Figure 5.3 (b) did not exceed 0.11
Correlation coefficients between bioluminescence and absorbance readings were: *lpp-lux* R^2 = 0.9774; *tatA-lux* R^2 = 0.9362; *ldc-lux* R^2 = 0.9497, *lysS-lux* R^2 = 0.9564; *spc-lux* R^2 = 0.9752

5.5.2 : Minimum Inhibitory Concentration (MIC) of Benzalkonium Chloride (BAK) when Supplemented with 0.03% EDTA

When the cultures were exposed to 0% and 0.00039% BAK supplemented with 0.03% EDTA the mean ± SEM cell density, at 0.96 ± 0.05 A.U. was statistically significantly higher than when exposed to 0.00078%, 0.0016%, 0.0031%, 0.0063%, 0.00125% and 0.025% after 24 hour incubation (P = 0.002, ANOVA) (Figure 5.4 (a)). The initial O.D and bioluminescence were as mentioned in Section 5.3.1.

When cultures were exposed to 0.05% BAK supplemented with 0.03% EDTA the mean cell density was 0.278 ± 0.14 A.U. which was statistically significantly higher than for cultures exposed to 0.025% BAK supplemented with 0.03% EDTA when the mean cell density was 0.025 ± 0.01 A.U. after 24 hours of incubation (P = 0.02, Tukey-Kramer *post hoc* analysis) (Figure 5.4 (a)).

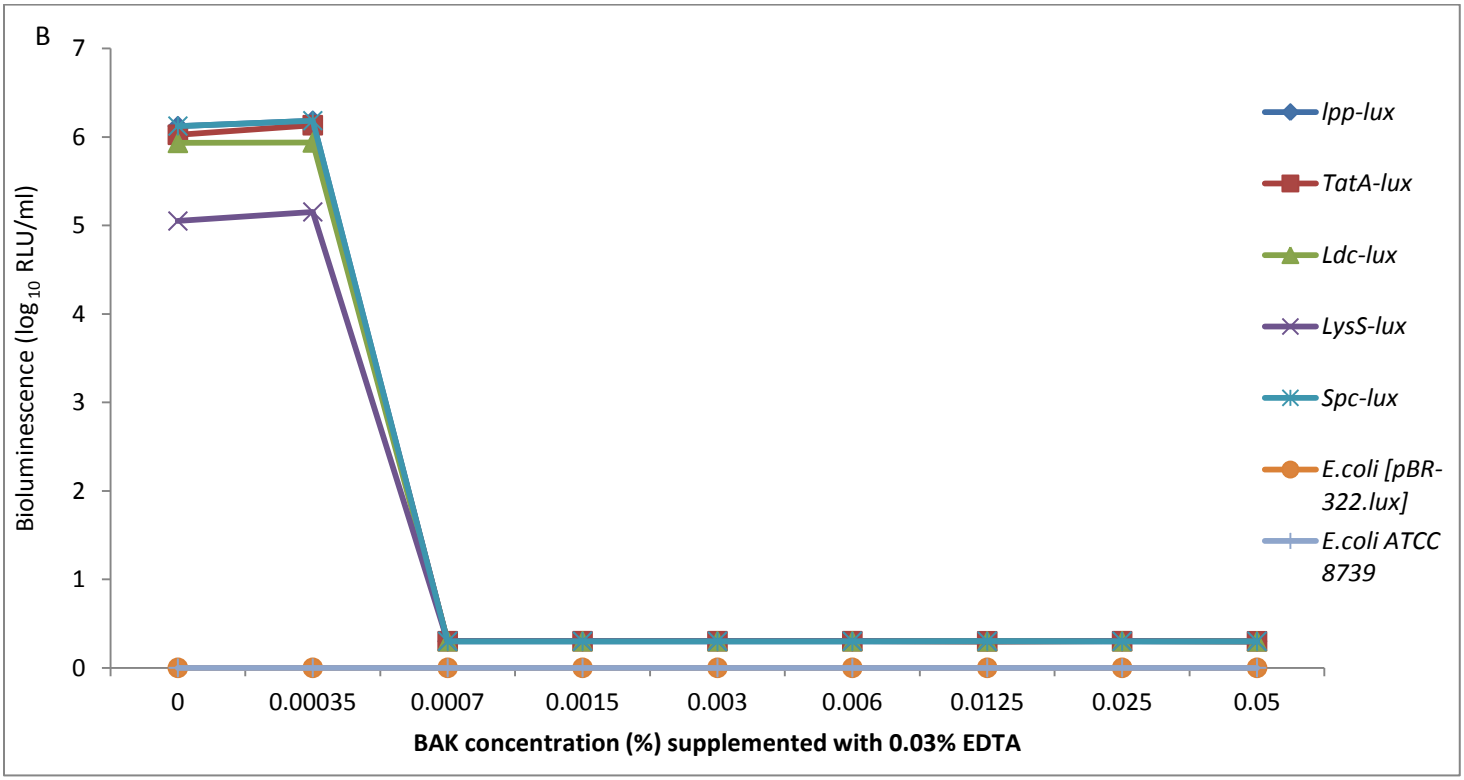


Figures 5.4 (a): Effect of Benzalkonium Chloride upon culture density when supplemented with 0.03% EDTA, after 24 hours of incubation in their presence in order to determine the Minimum Inhibitory Concentration (MIC)
The SEM for each individual biosensor strain in Figures 5.4 (a) did not exceed 0.003.

The bioluminescence ± SEM from the cultures exposed to 0.00078%, 0.0016%, 0.0031%, 0.0063%, 0.00125%, 0.025% and 0.05% BAK supplemented with 0.03% EDTA was between significantly lower (P=

0.002, ANOVA) than that from the cultures exposed to 0% or 0.00035% BAK supplemented with 0.03% EDTA, for all five biosensor (Figure 5.4 (b)).

The mean bioluminescence expressed by the cultures exposed across the concentrations of BAK, supplemented with 0.03% EDTA, showed a significant statistical correlation (P = 0.001) with the cell density (R^2= 0.9556-0.9855).



Figures 5.4 (b): Effect of Benzalkonium Chloride upon bioluminescence, when supplemented with 0.03% EDTA, after 24 hours of incubation in their presence in order to determine the Minimum Inhibitory Concentration (MIC)
The SEM for each individual biosensor strain in Figures 5.4 (b) did not exceed 0.09.
Correlation coefficients between bioluminescence and absorbance readings were: *lpp-lux*,R^2= 0.9645; *tatA-lux* R^2 = 0.9644, *ldc-lux*-R^2 = 0.9247, *lysS-lux* R^2 = 0.971; s*pc-lux* R^2 = 0.960

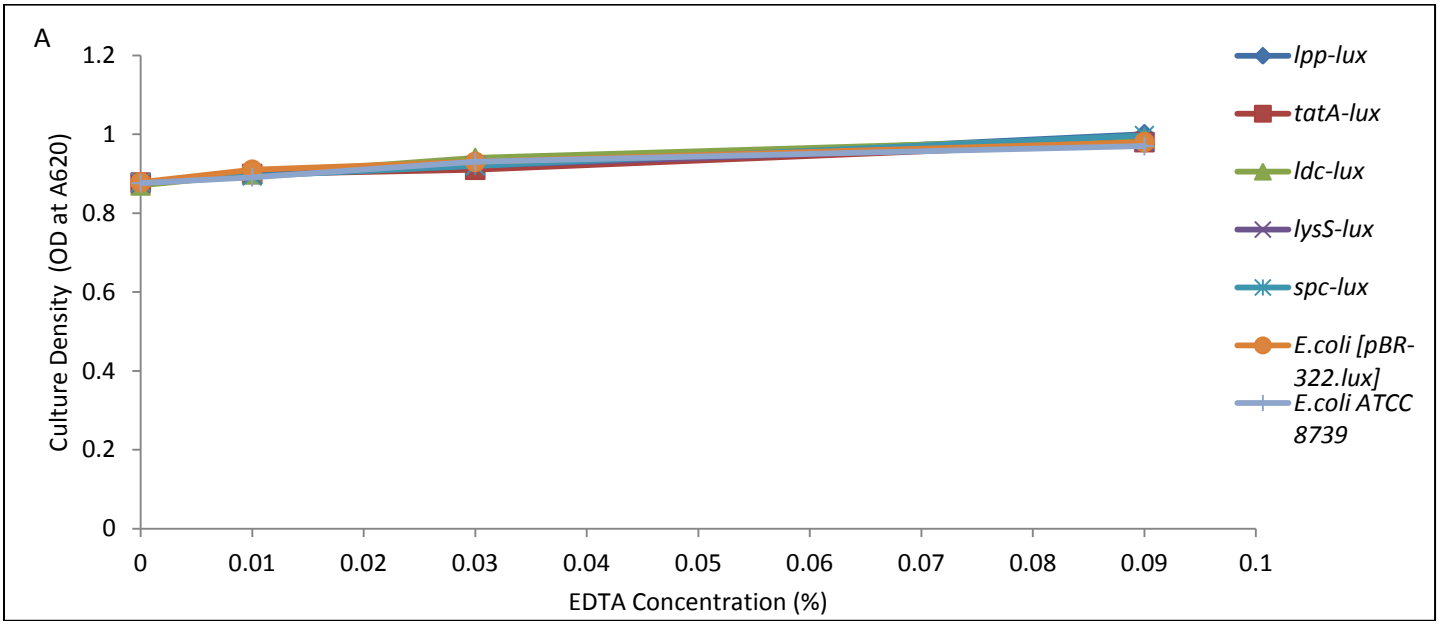
The MIC of BAK, when supplemented with 0.03% EDTA, was determined from the change in bioluminescence expression as well as the OD to be 0.00078 % BAK. This was confirmed by the change in the viable counts which demonstrated a 99.9% reduction of viable counts from a mean of 6.2 $\pm$ 0.3 CFU/ml

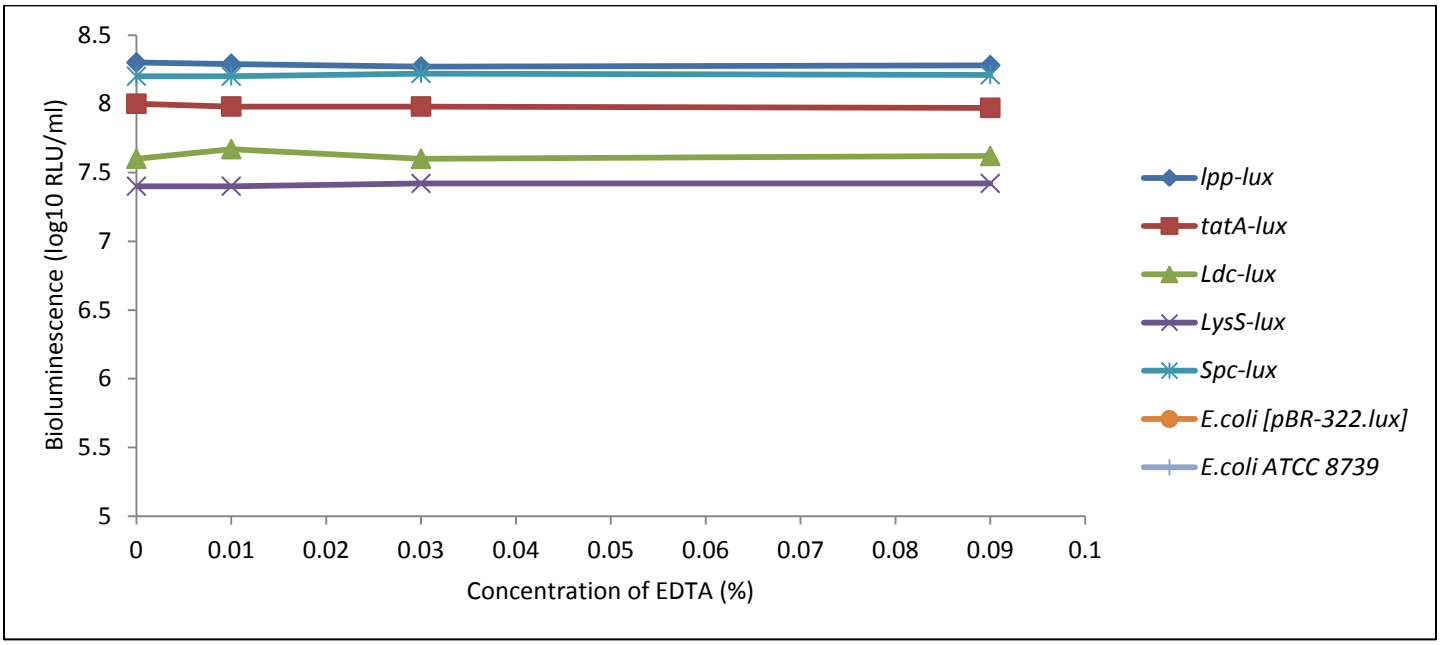
in the initial inocula to a final value of approximately 3.4 ± 0.3 log $_{10}$ CFU/ml at 0.00078% BAK and greater (P = 0.000, ANOVA).

5.5.3 : Minimum Inhibitory Concentration (MIC) of EDTA

The initial bioluminescence and O.D readings before the addition of EDTA are as follows from Section 5.3.1.

There were no significant differences between the mean densities of the cultures exposed for 24 h to any of the concentrations of EDTA tested (*P* = 1.00 in each case, Tukey-Kramer *post hoc* analysis). The mean ± SEM culture density in the presence of 0% EDTA after 24 h incubation was 0.90 A.U. ± 0.005., whilst at 0.01% EDTA it was 0.92 ± 0.001 A.U., at 0.03% EDTA it was 0.93 ± 0.015 A.U., and at 0.09% EDTA it was 0.97 ± 0.02 A.U. The bioluminescence expressed by the five biosensors after 24 hour incubation with EDTA showed a significant correlation with the culture density (*P*= 0.000) (R^2 = 0.8414-0.99), with a OD ± SEM of between 0.92-0.97 ± 0.01 A.U. and bioluminescence ± SEM of between 7.4 to8.3 ± 0.5 $\log_{10}$ RLU/ml being recorded (Figures 5.5 (a) & (b)). The bioluminescence expression by either of the control strains - *E.coli* [pBR-322.*lux*] or *E.coli* ATCC 8739 – was 0 $\log_{10}$ RLU/ml.

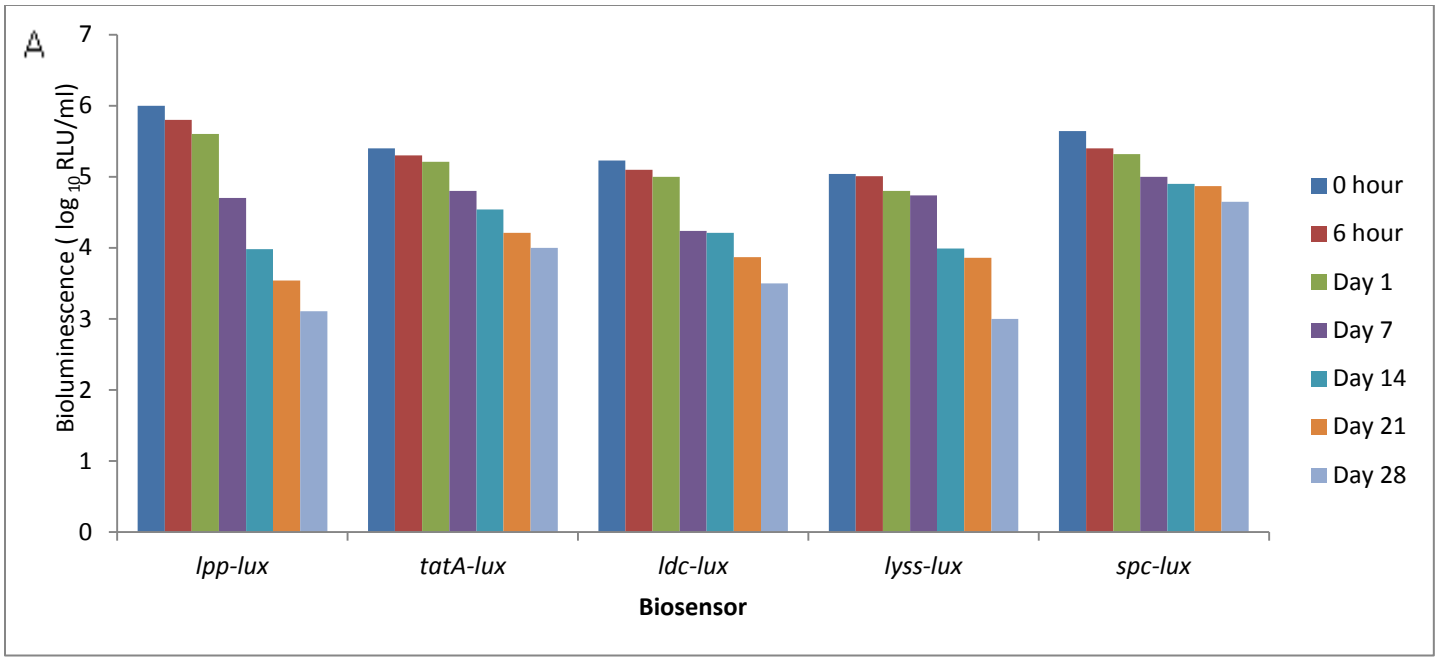




Figures 5.5 (a & b): Effect of EDTA upon culture density (absorbance measured at 620 nm) and bioluminescence after 24 hours of incubation in its presence in order to determine the Minimum Inhibitory Concentration (MIC), n= 3.
The SEM for each individual biosensor strain in Figures 5.5 (a) did not exceed 0.001.
The SEM for each individual biosensor strain in Figures 5.5 (b) did not exceed 0.10.
Correlation coefficients between bioluminescence and absorbance readings were: *lpp-lux* R^2 = 0.9717; *tatA-lux* R^2 = 0.9554; *ldc-lux* R^2 = 0.9746; *lysS-lux* R^2 = 0.9899; *spc-lux* R^2 = 0.9702

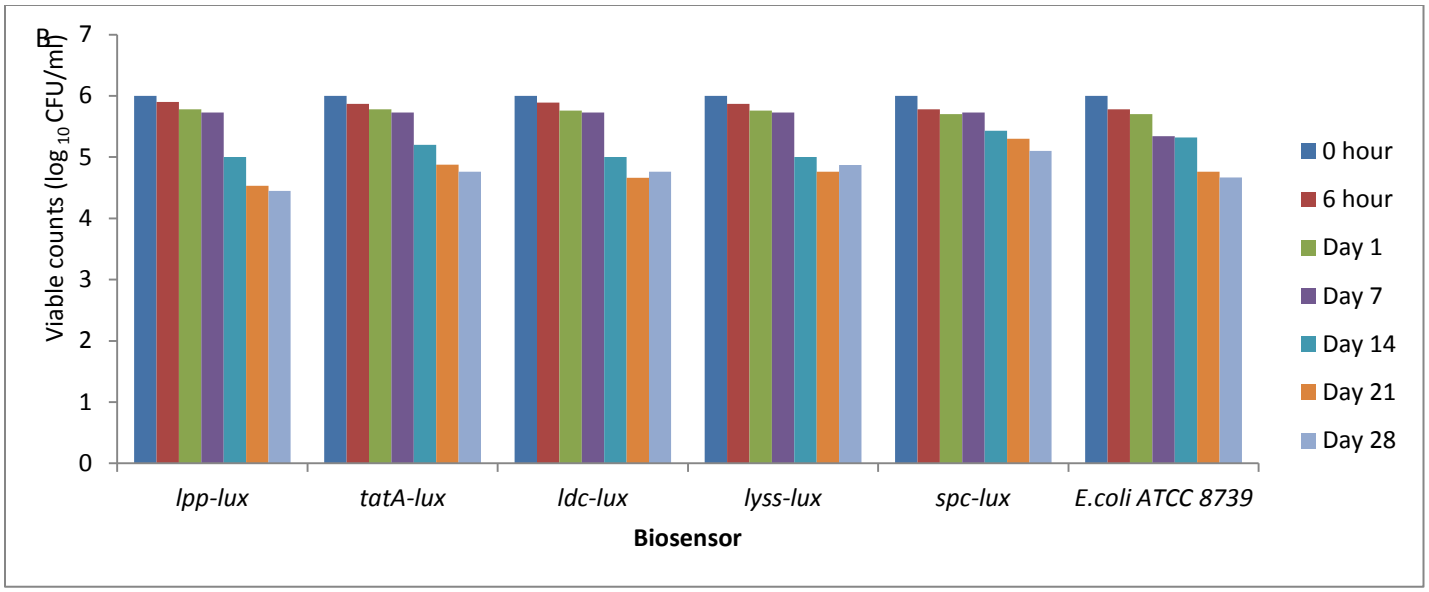
5.5.4 : Bioluminescence and Viable Counts of the Preservative-Free Negative Controls

The mean ± SEM bioluminescence expressed by the cultures, immediately after inoculation, was as follows: 6 ± 0.002 $\log_{10}$ RLU/ml for *lpp-lux*; 5.4 ± 0.006 $\log_{10}$ RLU/ml for *tatA-lux*; 5.23 ± 0.002 $\log_{10}$ RLU/ml for *ldc-lux*; 5.04 ± 0.008 $\log_{10}$ RLU/ml for *lysS-lux*; and 5.644 ± 0.01 $\log_{10}$ RLU/ml for *spc-lux* (Figure 5.6 (a). The bioluminescence expressed by the cultures decreased relatively linearly, by approximately 0.2 $\log_{10}$ RLU/ml across 6 h and 24 h on incubation immediately after inoculation up to 1 day of incubation with no statistical differences between any of the strains. However, between 7 days and 28 days of incubation, the mean ± SEM bioluminescence expressed by *lpp-lux* and *lysS-lux* decreased significantly from *lpp-lux*; 5.4 ± 0.006; 5.04 ± 0.008 $\log_{10}$ RLU/ml for *lysS-lux*to 3 ± 0.023 $\log_{10}$ RLU/ml ($P \leq 0.05$, Tukey-Kramer *post hoc* analysis) whilst there was no statistical difference in the mean bioluminescence expressed by *tatA-lux*, *ldc-lux* and *spc-lux* although it declined significantly ($P \leq 0.05$ in each case, Tukey-Kramer *post hoc* analysis). The bioluminescence levels remained significantly lower for both *lpp-lux* and *lysS-lux* than any of the other biosensor strains, after 28 d of incubation ($P = 0.05$, Tukey-Kramer *post hoc* analysis).



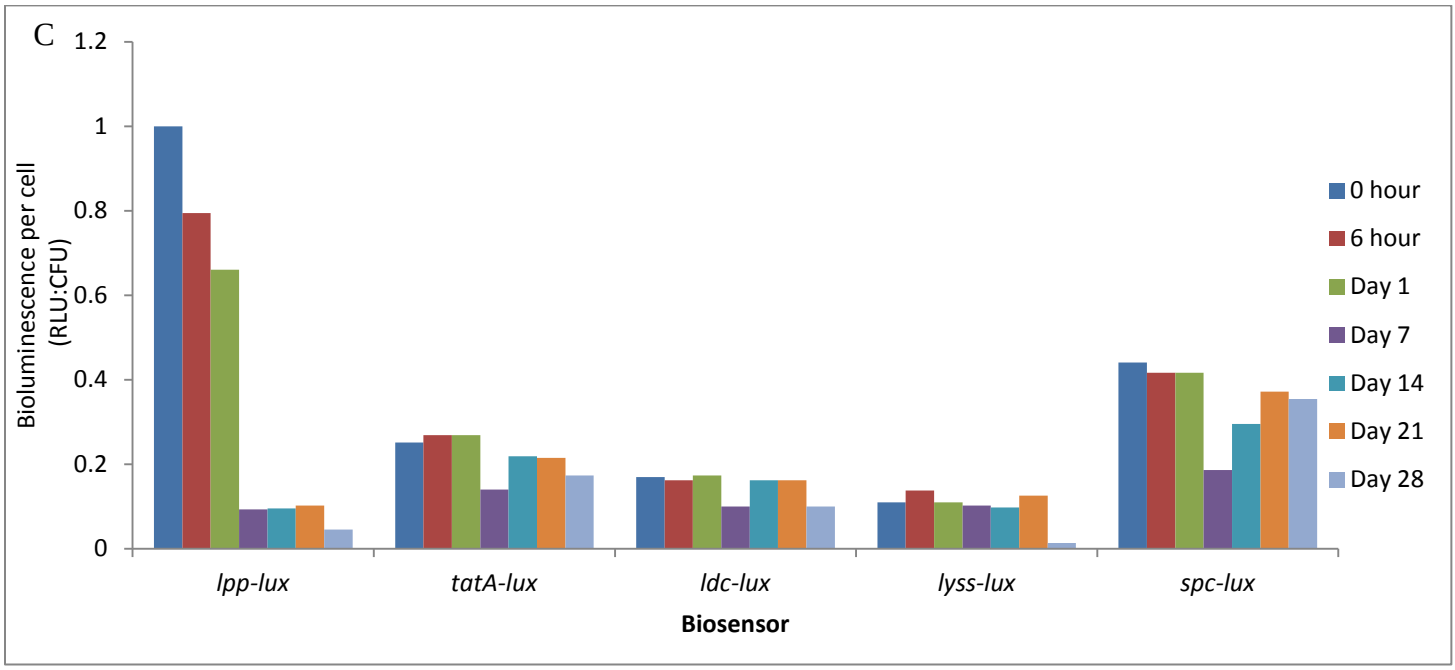
Figures 5.6 (a): Bioluminescence, viable counts, and bioluminescence per cell (RLU:CFU) when the biosensor strains were incubated without preservative for up to 28 days, in triplicates n=3
The SEM for each individual biosensor strain in Figures 5.6 (a) did not exceed 0.15.

The mean ± SEM initial plate counts (CFU/ml) of the five biosensor strains, and also the wildtype ATCC 8739 strain, were 6.2 ± 0.3 $\log_{10}$ CFU/ml (Figure 5.6 (b)). The viable counts of each of the cultures showed a small, but statistically insignificant, decrease after 7 days of incubation in each case. The mean ± SEM viable counts of each of the *E. coli* strains had declined by approximately 1 ± 0.5 $\log_{10}$ CFU/ml after 14 days of incubation, and a further 0.5 ± 0.01 $\log_{10}$ CFU/ml after 21 days of incubation. The mean ± SEM viable counts for each of the strains remained above approximately 4.5 ± 0.8 $\log_{10}$ CFU/ml (Figure 5.6 (b). There were no significant differences between the viable counts of the five *E. coli* biosensor strains and the control strains at any given time point between inoculation and 28 days of incubation.



Figures 5.6 (b): Bioluminescence, viable counts, and bioluminescence per cell (RLU:CFU) when the biosensor strains were incubated without preservative for up to 28 days, in triplicates n=3
The SEM for each individual biosensor strain in Figures 5.6 (b) did not exceed 0.10

The bioluminescence per cell (RLU:CFU) was the highest for *lpp-lux* at 0 hour (at a ratio of 1.00), and this was followed by *spc-lux* (at 0.44 ± 0.03), *tatA-lux* (at 0.25 ± 0.02), *ldc-lux* (at 0.16 ± 0.01), and *lysS-lux* (at 0.10 ± 0.02). After 6 hours of incubation, the RLU:CFU for *lpp-lux* was 0.80 ± 0.03, whilst for *spc-lux* it was 0.41 ± 0.009, for *tatA-lux* it was 0.26 ± 0.01, for *ldc-lux* it was 0.16 ± 0.01, and for *lysS-lux* it was 0.13 ± 0.02. After 1 day of incubation the RLU:CFU of *lpp-lux* was 0.66 ± 0.05, whilst for *spc-lux* it was 0.41 ± 0.04, for *tatA-lux* it was 0.26 ± 0.04, for *ldc-lux* it was 0.17 ± 0.01 and for *lysS-lux* it was 0.10 ± 0.06. From day 7 to day 28, the bioluminescence per cell expressed by *lpp-lux* and *lysS-lux* decreased significantly to 0.04 ± 0.03 and 0.013 ± 0.001 respectively ($P \leq 0.05$ in both cases, Tukey-Kramer *post hoc* analysis), whilst for *ldc-lux*, *tatA-lux* and s*pc -lux* it was 0.1 ± 0.006, 0.17 ± 0.006, and 0.35 ± 0.007 respectively (Figure 5.6 (c)). There was no statistical difference in the bioluminescence expressed by *tatA-lux*, *ldc-lux* and *spc-lux* although it declined significantly ($P \leq 0.05$ in each case, Tukey-Kramer *post hoc* analysis).



Figures 5.6 (c): Bioluminescence, viable counts, and bioluminescence per cell (RLU:CFU) when the biosensor strains were incubated without preservative for up to 28 days, in triplicates n=3

The SEM for each individual biosensor strain in Figures 5.6 (c) did not exceed 0.1.

5.5.5 : Effect of Benzalkonium Chloride (BAK) on Bioluminescence, Viable Counts and ATP-Chemiluminescence for the Five Biosensor Strains and also the Control Strains of *E. coli* ATCC 8739 in a Preservative Efficacy Test

The bioluminescence expressed by the five biosensor strains (figure 5.7), and also the intracellular ATP levels (as demonstrated by the chemiluminescence assay) (figure 5.10) and the viable counts (figure 5.8) decreased significantly between inoculation and 6 hours of incubation when the cultures were exposed to high concentrations of benzalkonium chloride (0.0062%, 0.00031 and 0.00016%) (P = 0.008, 0.000, 0.001, Tukey Post Hoc analysis) The bioluminescence per cell at between 0 to 6 h was (in ascending order); *lpp* (0.229 ± 0.02), *tatA* (0.028 ± 0.07), *ldc* (0.017 ± 0.04), *spc* (0.016 ± 0.04), *lysS* (0.0014 ± 0.04) and remained zero at each of the time points tested up to 28 days of incubation, for the five biosensor strains (Figure 5.8).

When exposed to BAK concentrations of 0.0016%, 0.00078% and 0.00039 %, the bioluminescence expressed by the *lpp-lux* biosensor strain had been reduced significantly by 5 orders of magnitude between 24 h to 7 d of incubation ($P \leq 0.05$, ANOVA) with no subsequent recovery in bioluminescence up to 28 days of incubation. The bioluminescence per cell was on average 0.00008 ± 0.0004 with no significant difference after 7 d for 0.0031%, 0.0016%, 0.00078%, and 0.00039% BAK.

When exposed to a benzalkonium chloride concentration of 0.00078% BAK, the bioluminescence ($\log_{10}$ RLU/ml) expressed by *ldc-lux* decreased significantly ($P \leq 0.05$, ANOVA) by at least 3 orders of magnitude between 14 and 21 days from initial levels whilst bioluminescence readings expressed by *lysS-lux* decreased significantly ($P \leq 0.05$, ANOVA) by at least 3 orders of magnitude between 7 and 14

days of incubation respectively. The bioluminescence expressed by both *spc-lux* and *tatA-lux* was reduced significantly by at least 2 orders of magnitude when these cultures were sampled at the 28th day of incubation ($P \leq 0.05$, ANOVA). In addition, the viable counts (log_{10} CFU/ml) and the ATP-chemiluminescence (log_{10} RLU/ml) were only reduced significantly ($P \leq 0.05$, ANOVA) by 1.77± 0.05, 1.71± 0.12, respectively in orders of magnitude for the five biosensor strains from 0 h to 28 days with no statistical differences over 28 days between two methods. The bioluminescence per cell obtained at the reduction of more or at least 3 orders of magnitude for *lysS* and *ldc* were 0.004 ± 0.01 between 7 and 14 days and 0.16 ± 0.01 between 14 and 21 days respectively at 0.00078%. Whilst the bioluminescence per cell for *tatA* and *spc* measured between 21 and 28 days of incubation were 0.24 ± 0.05 and 0.211 ± 0.06. At the point of 3 order of magnitude of bioluminescence reduction by the biosensors, the bioluminescence per cell expressed by *tatA-lux* and *spc-lux* were approximately 1000 times higher to bioluminescence per cell expressed by *lpp-lux*, 10 times higher to *lysS-lux*, and approximately twice higher to *ldc-lux.*

When exposed to 0.00039% BAK, the bioluminescence expressed by the *spc-lux* and *tatA-lux* biosensor strains was reduced significantly by at least 1.85 orders of magnitude after 28 d of incubation from initial bioluminescent readings ($P \leq 0.05$, ANOVA). In contrast, the bioluminescence expressed by the *ldc-lux* biosensor strain was reduced significantly by at least 3 orders of magnitude between 14 and 21 days of incubation ($P \leq 0.05$, ANOVA) whilst the bioluminescence readings by *lysS-lux* was reduced significantly by at least 3 orders of magnitude between 7 and 14 days of incubation ($P \leq 0.05$, ANOVA). The viable counts (log_{10} CFU/ml) and the ATP-chemiluminescence (log_{10} RLU/ml) were only reduced significantly ($P \leq 0.05$, ANOVA) by 1.33, 1.31, respectively in orders of magnitude for the five biosensor strains from 0 h to 28 days with no statistical differences over 28 days between the methods.

The bioluminescence per cell obtained at the reduction of 3 orders of magnitude for *lysS* and *ldc* was 0.06 ± 0.01 between 7 and 14 days of incubation and 0.10 ± 0.01 between 14 and 21 days of incubation respectively. Whilst the bioluminescence per cell for *tatA* and *spc* at 28 days of incubation, were 0.211 ±0.05 and 0.233 ±0.06.

At the point of 3 order of magnitude of bioluminescence reduction by the biosensors, the bioluminescence per cell expressed by *spc-lux* and *tatA-lux* were approximately 100 times higher to bioluminescence per cell expressed by *lpp-lux*, 5 times higher to *lysS-lux*, *ldc-lux* and *tatA-lux.*

The comparison between the biosensors revealed that bioluminescent and bioluminescent per cell readings by *lpp-lux* and *lysS-lux* were significantly lower ($P \geq 0.05$, Tukey *post hoc*) compared to the bioluminescence and the bioluminescence per cell readings by *ldc-lux*, *tatA-lux* and *spc-lux* across all concentrations and all time points. Whilst no significant difference between bioluminescence readings exhibited by *ldc-lux*, *spc-lux* and *tatA-lux* when challenged with 0.0125%, 0.0062%, 0.0001 %, 0.0016%, 0.00078% and 0.00039% across the 28 days of incubation.

Importantly, in each case when there was a reduction in viable counts of 3 orders of magnitude there was also a reduction in bioluminescence of at least 99.9% for each of the biosensor strains (Table 5.3). The correlation coefficients between the viable counts ($\log_{10}$ CFU/ml) and bioluminescence ($\log_{10}$ RLU/ml) were generally very good significant; *lpp-lux* (R^2= 0.603-1.00); *tatA-lux* (R^2= 0.934-1.00); *ldc-lux* (R^2= 0.907-1.00); *lysS-lux* (R^2= 0.896-1.00); and *spc-lux* (R^2= 0.918-1.00) (Table 5.3). In addition, the correlation coefficients between the bioluminescence ($\log_{10}$ RLU/ml) and ATP-chemiluminescence ($\log_{10}$ RLU/ml) were generally very good; *lpp-lux* (R^2= 0.651-1.00); *tatA-lux* (R^2= 0.85-1.00); *ldc-lux*

(R^2= 0.906-1.00); *lysS-lux* (R^2= 0.851-1.00); *spc-lux* (R^2= 0.838-1.00); *E.coli* [pBR 322.lux] (R^2= 0.943-0.992) and *E.coli (*R^2=0.961-0.994) (Table 5.3). Table 5.3 illustrates the tabulation for suitability of the range of benzalkonium chloride tested in this study in accordance to European Pharmacopeia-A criteria. BAK concentrations of 0.0062%; and 0.0031% were found to be suitable for oral, parenteral, and topical pharmaceutical preparations, when the data were related to the EP-A criteria, whilst BAK concentrations of 0.00078% and 0.00039% were not found to be effective for any pharmaceutical preparations in the current experiments. The *E.coli* strains demonstrate the same level effectiveness against benzalkonium chloride concentrations (Table 5.3).

The standard error of means for all the biosensor strains was lower for bioluminescence readings (0.106), compared to plate counts (0.156), and was slightly higher than ATP chemiluminescence (0.102). In addition, the F value obtained by ANOVA between the bioluminescence, viable counts and ATP chemiluminescence data was $(_{159,\ 50,\ 0.05})$ = 0.59, does not exceed the F critical value of 1.57, which indicates no significance difference were found between whole-cell bioluminescent method compared to traditional plate count, and ATP chemiluminescence method over the PET time points and BAK concentrations.

Additionally, there is no significant difference in bioluminescence per cell levels for *lpp*, *ldc* and *lyss* to expressions of *tatA* and *spc* in BAK PET test.

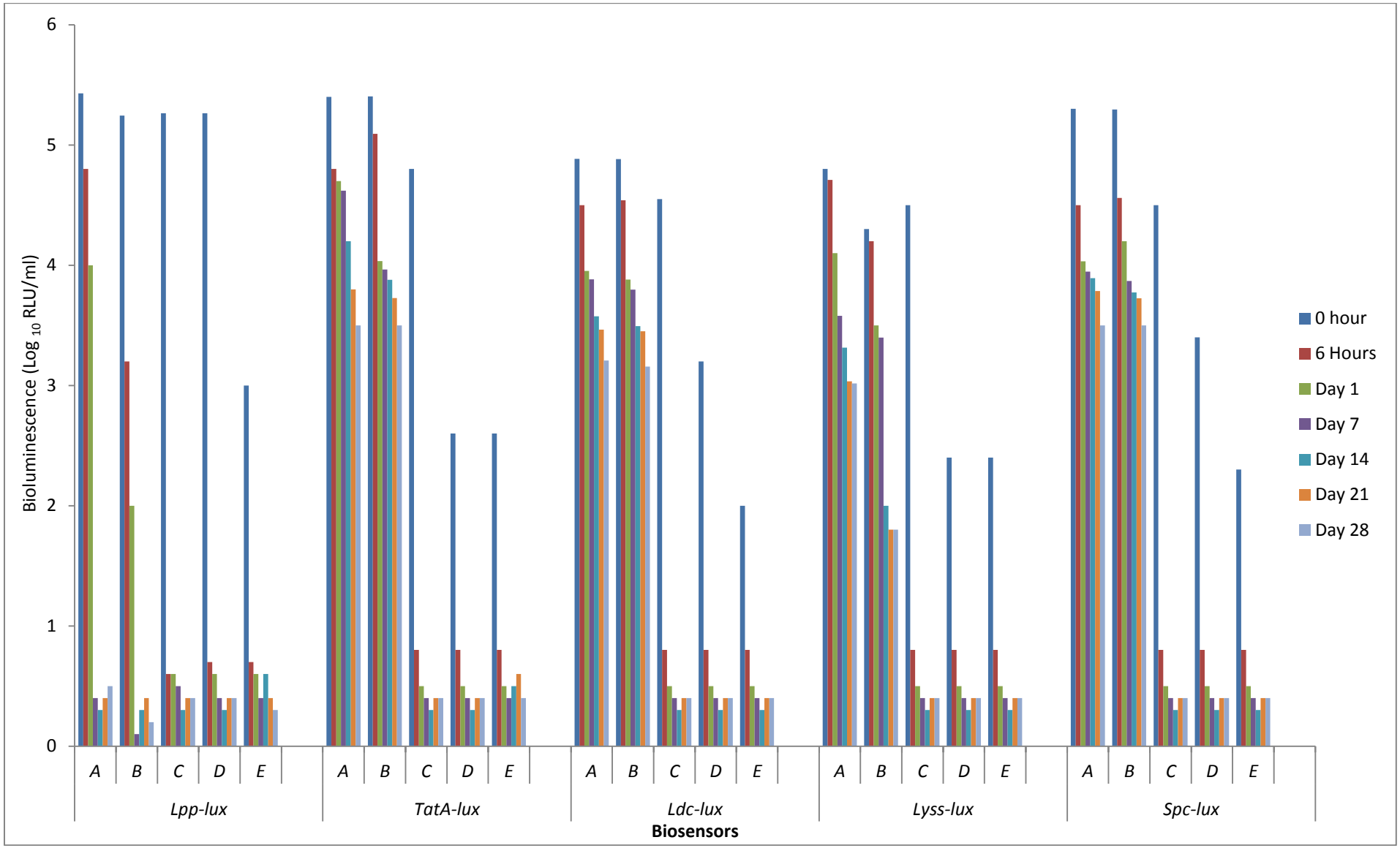


Figure 5.7: Bioluminescence expression (log_{10} RLU/ml) by the five biosensors when challenged with benzalkonium chloride (BAK) at concentrations, presented in ascending order (A to E) from left to right, of: 0.00039%; 0.00078%; 0.0016%; 0.0031%; and 0.0062% at pH 7.0 over 28 days of incubation. # Overall the SEM is 0.106 at 95% confidence interval

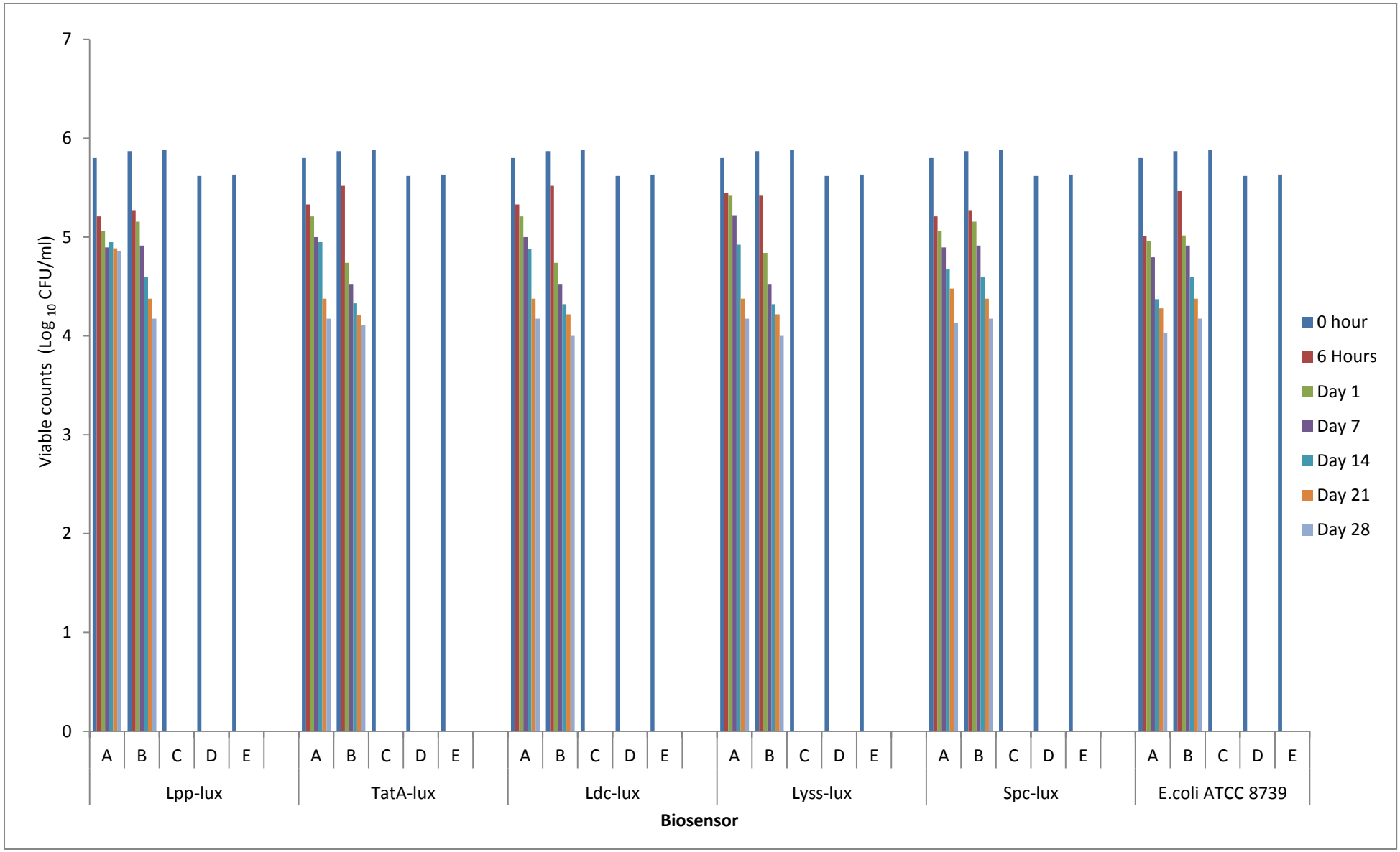


Figure 5.8: Viable counts (log_{10} CFU/ml) for the five biosensor strains and also the control strain when challenged with benzalkonium chloride (BAK) at concentrations, presented in ascending order (A to E) from left to right, of: 0.00039%; 0.00078%; 0.0016%; 0.0031%; and 0.0062% at pH 7.0 over 28 days. # Overall the SEM is 0.154 at 95% confidence interval

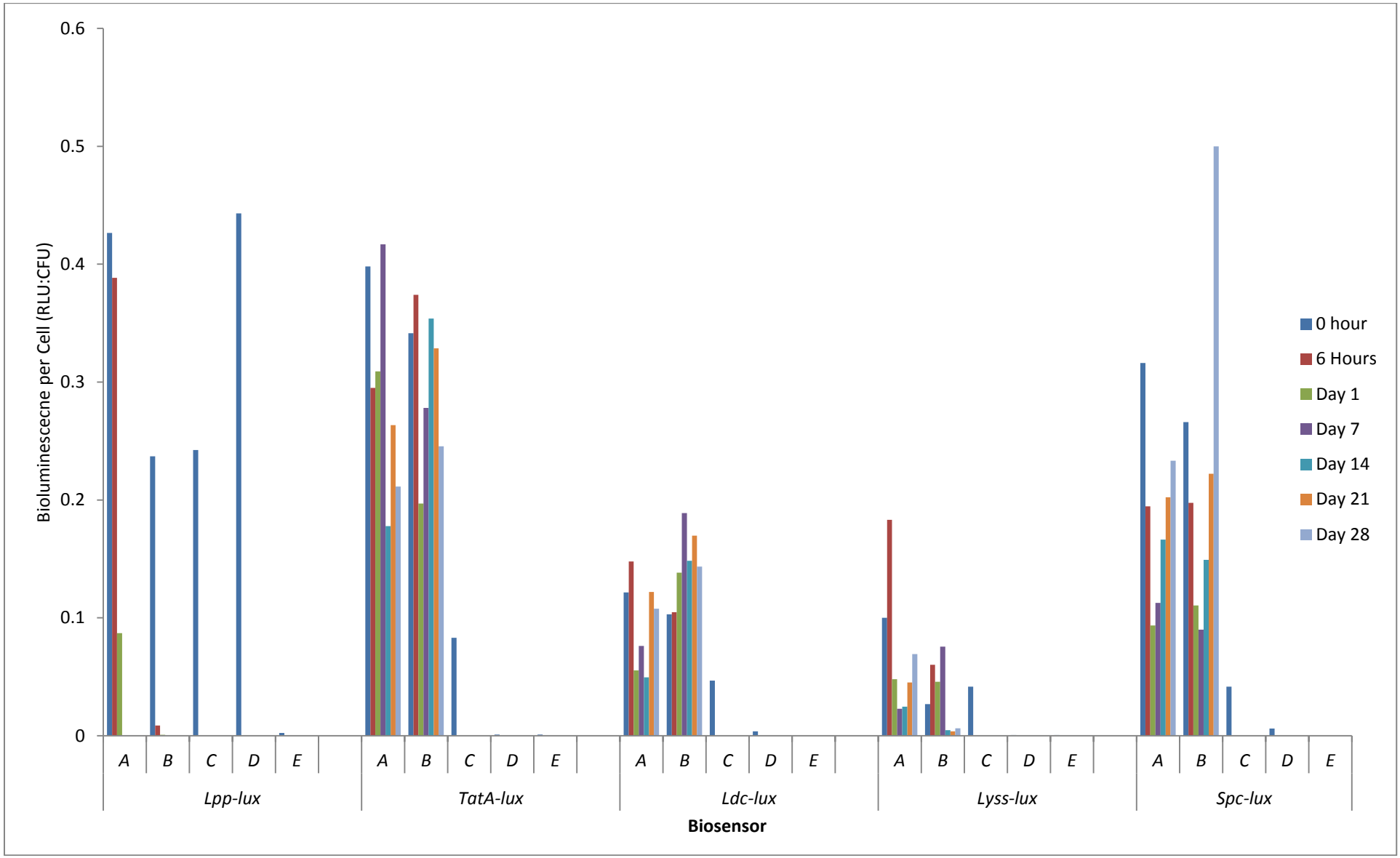


Figure 5.9: Bioluminescence per cell (RLU:CFU) for the five biosensor strains when challenged with benzalkonium chloride (BAK) at concentrations, presented in ascending order (A to E) from left to right, of: 0.00039%; 0.00078%; 0.0016%; 0.0031%; and 0.0062% at pH 7.0 over 28 days # Overall the SEM is 0.017 at 95% confidence interval

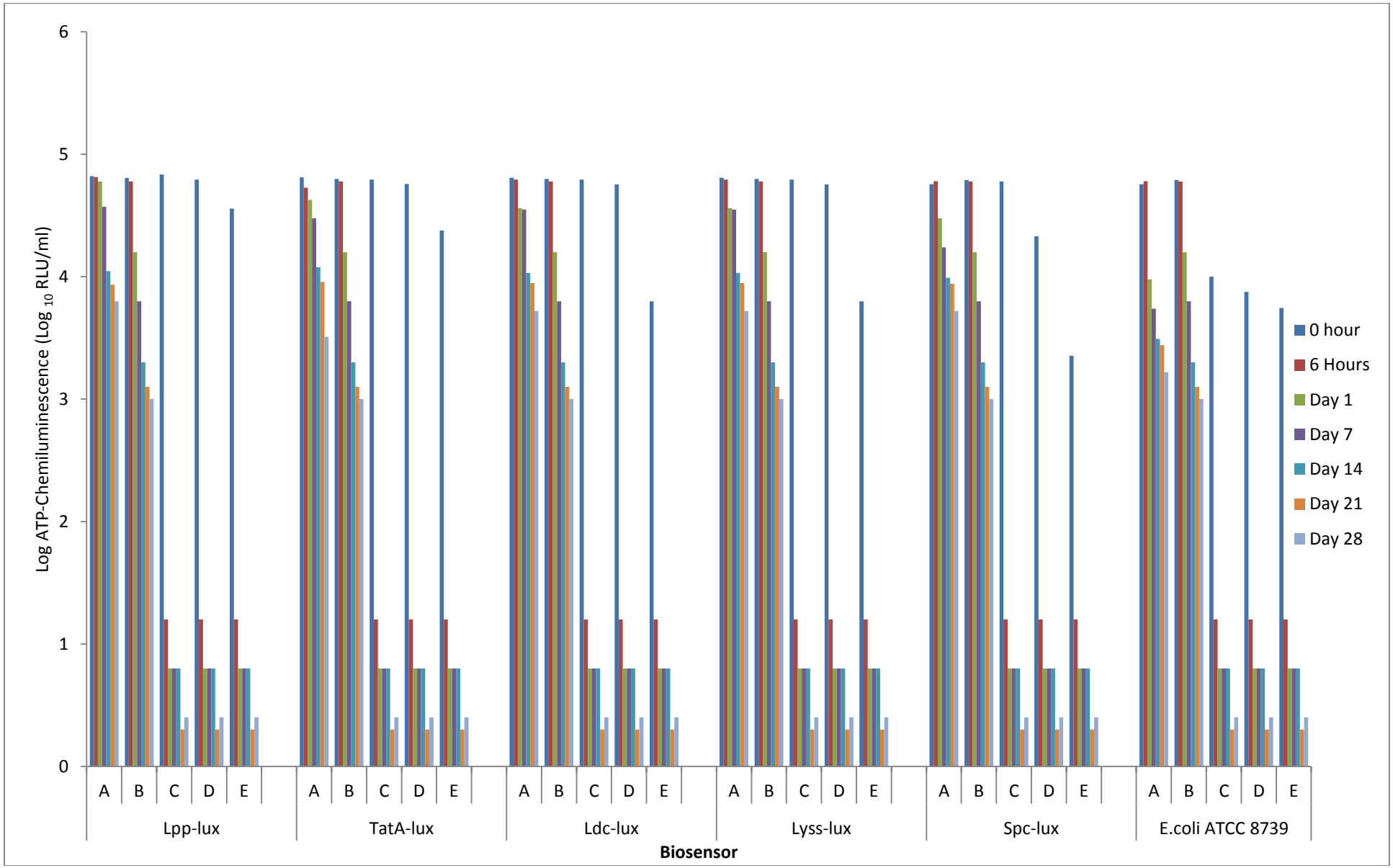


Figure 5.10: ATP chemiluminescence measurements (log_{10} RLU/ml) for the five biosensor strains and the control strain when challenged with benzalkonium chloride (BAK) at concentrations, presented in ascending order (A to E) from left to right, of: 0.000037%; 0.000078%; 0.00016%; 0.00031% and 0.00062% at pH 7.0 over 28 days. # Overall the SEM is 0.102 at 95% confidence intervals.

Table 5.3: The viable counts, ATP-chemiluminescence, and bioluminescence from the biosensor strains when exposed to benzalkonium chloride, and an analysis of the suitability of this preservative for use in different pharmaceutical preparations in accordance with the guidelines set out in the European and British Pharmacopoeias

Biosensor strain	[BAK] (%)	Incubation time required to achieve a reduction in viable counts of greater than 3 orders of magnitude (days)	Incubation time required to achieve a reduction in ATP-chemi-luminescence of greater than 3 orders of magnitude (days)	Incubation time required to achieve a reduction in bio-luminescence of greater than 3 orders of magnitude (days)	% reduction in bio-luminescence when the viable counts had been reduced by more than 3 orders of magnitude	Correlation coefficient (R^2) between RLU and CFU	Correlation coefficient (R^2) between CFU and ATP	Correlation coefficient (R^2) between RLU and ATP	Suitability of BAK for different pharmaceutical preparations [#]
lpp-lux	0.0062	6 Hr	6 Hr	6 Hr	99.9	0.989	0.837	0.851	O,P,T
	0.0031	6 Hr	6 Hr	6 Hr	99.9	0.803	0.806	0.947	O,P,T
	0.0016	14	14	6 Hr	99.9	0.603	0.955	0.651	O,P,T
	0.00078	n/a	n/a	7	99.9	0.873	0.965	0.758	Non-Effective
	0.00039	n/a	n/a	7	99.9	0.851	0.956	0.816	Non-Effective
tatA-lux	0.0062	6 Hr	6 Hr	6 Hr	99.9	0.996	0.928	0.968	O,P,T
	0.0031	6 Hr	6 Hr	6 Hr	99.9	0.998	0.806	0.969	O,P,T
	0.0016	14	14	14	99.9	0.983	0.955	0.997	O,P,T
	0.00078	n/a	n/a	28	n/a	0.934	0.965	0.8543	Non-Effective
	0.00039	n/a	n/a	n/a	n/a	0.929	0.956	0.996	Non-Effective
ldc-lux	0.0062	6 Hr	6 Hr	6 Hr	99.9	0.998	0.928	0.973	O,P,T
	0.0031	6 Hr	6 Hr	6 Hr	99.9	0.985	0.806	0.963	O,P,T
	0.0016	14	14	14	99.9	0.907	0.955	0.997	O,P,T

Biosensor strain		Incubation time required to achieve more than 3 orders of magnitude reduction in viable counts (days)	Incubation time required to achieve more than 3 orders of magnitude reduction in ATP-chemi-luminescence (days)	Incubation time required to achieve more than 3 orders of magnitude reduction in bio-luminescence (days)	% reduction in bio-luminescence when viable counts reduced by more than 3 orders of magnitude	Correlation coefficient (R^2) between RLU and CFU	Correlation coefficient (R^2) between CFU and ATP	Correlation coefficient (R^2) between RLU and ATP	Suitability of BAK acid for different pharmaceutical preparations, as determined from the Pharmacopoeia[#]
	0.00078	n/a	n/a	14	99.9	0.927	0.934	0.942	Non-Effective
	0.00039	n/a	n/a	21	n/a	0.989	0.965	0.906	Non-Effective
lysS-lux	0.0062	6 Hr	6 Hr	6 Hr	99.9	0.992	0.928	0.969	O,P,T
	0.0031	6 Hr	6 Hr	6 Hr	99.9	0.896	0.806	0.972	O,P,T
	0.0016	Day 14	Day 14	Day 14	99.9	0.968	0.955	0.851	O,P,T
	0.00078	n/a	n/a	Day 14	n/a	0.841	0.965	0.986	Non-Effective
	0.00039	n/a	n/a	Day 21	n/a	0.858	0.956	0.929	Non-Effective
spc-lux	0.0062	6 Hr	6 Hr	6 Hr	99.9	0.918	0.928	0.970	O,P,T
	0.0031	6 Hr	6 Hr	6 Hr	99.9	0.991	0.806	0.961	O,P,T
	0.0016	Day 14	Day 14	Day 14	99.9	0.969	0.955	0.995	O,P,T
	0.00078	n/a	n/a	28	n/a	0.983	0.965	0.891	Non-Effective
	0.00039	n/a	n/a	n/a	n/a	0.999	0.956	0.838	Non-Effective
E.coli [pbr-322.lux]	0.0062	6 Hr	6 Hr	n/a	n/a	n/a	0.990	n/a	O,P,T
	0.0031	6 Hr	6 Hr	n/a	n/a	n/a	0.992	n/a	O,P,T
	0.0016	Day 1	Day 1	n/a	n/a	n/a	0.963	n/a	O,P,T

Biosensor strain		Incubation time required to achieve more than 3 orders of magnitude reduction in viable counts (days)	Incubation time required to achieve more than 3 orders of magnitude reduction in ATP-chemi-luminescence (days)	Incubation time required to achieve more than 3 orders of magnitude reduction in bio-luminescence (days)	% reduction in bio-luminescence when viable counts reduced by more than 3 orders of magnitude	Correlation coefficient (R^2) between RLU and CFU	Correlation coefficient (R^2) between CFU and ATP	Correlation coefficient (R^2) between RLU and ATP	Suitability of BAK acid for different pharmaceutical preparations, as determined from the Pharmacopoeia[#]
E.coli ATCC 8739	0.00078	n/a	n/a	n/a	n/a	n/a	0.982	n/a	Non-Effective
	0.00039	n/a	n/a	n/a	n/a	n/a	0.943	n/a	Non-Effective
	0.0062	6 Hr	6 Hr	n/a	n/a	n/a	0.994	n/a	O,P,T
	0.0031	6 Hr	6 Hr	n/a	n/a	n/a	0.996	n/a	O,P,T
	0.0016	Day 1	6 Hr	n/a	n/a	n/a	0.968	n/a	O,P,T
	0.00078	n/a	Day 1	n/a	n/a	n/a	0.986	n/a	Non-Effective
	0.00039	n/a	n/a	n/a	n/a	n/a	0.961	n/a	Non-Effective

O, P and T indicate the effectiveness of BAK or use inOral, Parenteral and ophthalmic, and Topical preparations, determined in accordance with the pharmacopoeial guidelines

n/a: not applicable according to Pharmacopeia guidelines of

5.5.6 : Effect of Benzalkonium Chloride (BAK) with the addition of 0.03% EDTA on Bioluminescence, Viable Counts and ATP-Chemiluminescence for the Five Biosensor Strains and the Control Strains of *E. coli* ATCC 8739 in a Preservative Efficacy Test

The bioluminescence expressed by the five biosensor strains (figure 5.11), and also the intracellular ATP levels determined by ATP chemiluminescence assay (figure 5.14), and viable counts (figure 5.12) decreased significantly between inoculation and 6 hours of incubation when the cultures were exposed to concentrations of benzalkonium chloride of 0.0062%, 0.0031% and 0.0016%, supplemented with 0.03% EDTA (P= 0.000, 0.002, 0.000, Tukey *Post Hoc analysis*) (Figures 5.11, 5.12, & 5.14). The average bioluminescence per cell between 0 and 6 h was (in descending order): *lpp* (0.13 ± 0.02); *spc* (0.018 ±0.05); *ldc* (0.006±0.04); *tatA* (0.004±0.07); *lysS* (0.004±0.04). The bioluminescence expressed by each biosensor strain in the presence of 0.0062%, 0.0031% and 0.0016%, BAK remained at effectively zero at each of the time points after 6 hours of incubation up to 28 days of incubation.

When exposed to BAK concentrations of 0.0016%, 0.00078%, and 0.00039%, supplemented with 0.03% EDTA, the bioluminescence expressed by the *lpp-lux* biosensor strain was reduced significantly by 5 orders of magnitude between 24 h to 7 days of incubation ($P \leq 0.05$, ANOVA) and showed no subsequent increase in bioluminescence. The bioluminescence per cell was on average 0.03 ± 1.41 between day 7 and 28 d when exposed to 0.00078% and 0.00039% BAK, supplemented with 0.03% EDTA.

When exposed to a benzalkonium chloride of concentration 0.00078%, *lysS-lux, ldc-lux, tatA-lux,* and *spc-lux* all showed a 3 orders of magnitude reduction in bioluminescence between the 6^{th} and 24^{th} hour

of incubation whilst the intracellular ATP levels and viable counts decreased significantly ($P \leq 0.05$, ANOVA) at the same time.

When exposed to a benzalkonium chloride concentration of 0.00039% BAK, the bioluminescence (log_{10} RLU/ml) expressed by *lysS-lux* decreased significantly by at least 3 orders of magnitude between 7 and 14 days of incubation ($P \leq 0.05$, ANOVA), whilst the bioluminescence expressed by *ldc-lux* decreased significantly (by at least 3 orders of magnitude between 21 and 28 days of incubation ($P \leq 0.05$, ANOVA). Bioluminescence expression by *spc-lux* and *tatA-lux* was reduced significantly by at least 2.11 magnitude after 28 days of incubation with 0.00039% BAK ($P \leq 0.05$, ANOVA).

The viable counts (log_{10} CFU/ml) and the ATP-chemiluminescence (log_{10} RLU/ml) were only reduced significantly ($P \leq 0.05$, ANOVA) by 2.00 ± 0.15, 2.34± 0.11, respectively in orders of magnitude for the five biosensor strains from 0 h to 28 days with no statistical differences over 28 days between the methods at 0.00039% BAK. The bioluminescence per cell obtained at 3 orders of magnitude for *lysS* and *ldc* was 0.006±0.21 between 7 to 14 days and 0.005 ±0.03 between 21 and 28 days respectively. The bioluminescence per cell for *tatA* and *spc* at 28 days of incubation were 0.04 ±0.05 and 0.23 ±0.06 at 0.00039% BAK.

At 0.00039% BAK, the bioluminescence per cell expressed by *spc* were approximately 100 times higher to bioluminescence per cell expressed by *lpp-lux,* 5 times higher to *tatA-lux* and 10 times higher to *lysS-lux, ldc-lux.*

The comparison between the biosensors revealed that bioluminescent and bioluminescent per cell readings

Overall Tukey Post Hoc analysis revealed that there is a significant decreased ($P \leq 0.05$, Tukey Post Hoc) between bioluminescence and bioluminescence per cell readings by *lpp-lux* and *lysS-lux* to *ldc-lux*, *tatA-lux* and *spc-lux* whilst no significant difference between bioluminescence readings exhibited by *ldc-lux, spc-lux* and *tatA-lux* when challenged with 0.0062%, 0.0031 %, 0.0016%, 0.00078% and 0.00039% with the addition of 0.03% EDTA to each BAK concentration across the 28 days of incubation. Overall analysis revealed that bioluminescence levels were significantly lower expressed by *lpp* to *lyss,ldc, spc* and *tatA*

Importantly, a reduction in viable counts of 3 orders of magnitude correlated significantly with a at least more than 99.9% reduction in bioluminescence for each of the bio-reporter strains ($P \leq 0.05$, ANOVA) (Table 5.3). The correlation coefficients between the viable counts ($\log_{10}$ CFU/ml) and bioluminescence ($\log_{10}$ RLU/ml) were significantly good; *lpp-lux* (R^2=0.75-1.00); *tatA-lux* (R^2=0.904-1.00); *ldc-lux* (R^2=0.87-1.00); *lysS-lux* (R^2=0.87-1.00); and *spc-lux* (R^2=0.94-1.00) (Table 5.4). In addition, the correlation coefficients between the bioluminescence ($\log_{10}$ RLU/ml) and ATP-chemiluminescence were significantly good ($\log_{10}$ RLU/ml) were; *lpp-lux* (R^2=0.86-1.00); *tatA-lux* (R^2=0.85-1.00); *ldc-lux* (R^2:0.87-1.00); *lysS-lux* (R^2=0.90-1.00); and *spc-lux* (R^2=0.83-1.00); *E.coli* [pBR 322.lux] (R^2=0.808-0.991) and *E.coli* (*(*R^2=0.820-0.991) (Table 5.5). In table 5.4 illustrates the tabulation for suitability of the range of benzalkonium chloride with 0.03% EDTA tested in this study in accordance to European Pharmacopeia-A criteria. The effectiveness of 0.0062%; 0.0031%; 0.00016; and 0.00078% were suitable for oral, parenteral, and topical pharmaceutical preparations whilst at concentrations 0.000037% BAK

effective against topical pharmaceutical preparations. The *E.coli* strains demonstrate the same effect against benzalkonium chloride with 0.03 % EDTA concentrations (Table 5.4).

The standard error of means for all the biosensor strains across all PET time points and BAK concentrations by the bioluminescence method was lower for whole-cell bioluminescence readings (0.095), compared to plate counts (0.164), and ATP chemiluminescence (0.107). The F value obtained ($_{155,\ 54,\ 0.05}$) = 0.994, does not exceed the F critical value of 1.57, indicates no significance difference were found between whole-cell bioluminescent method and compedial methods.

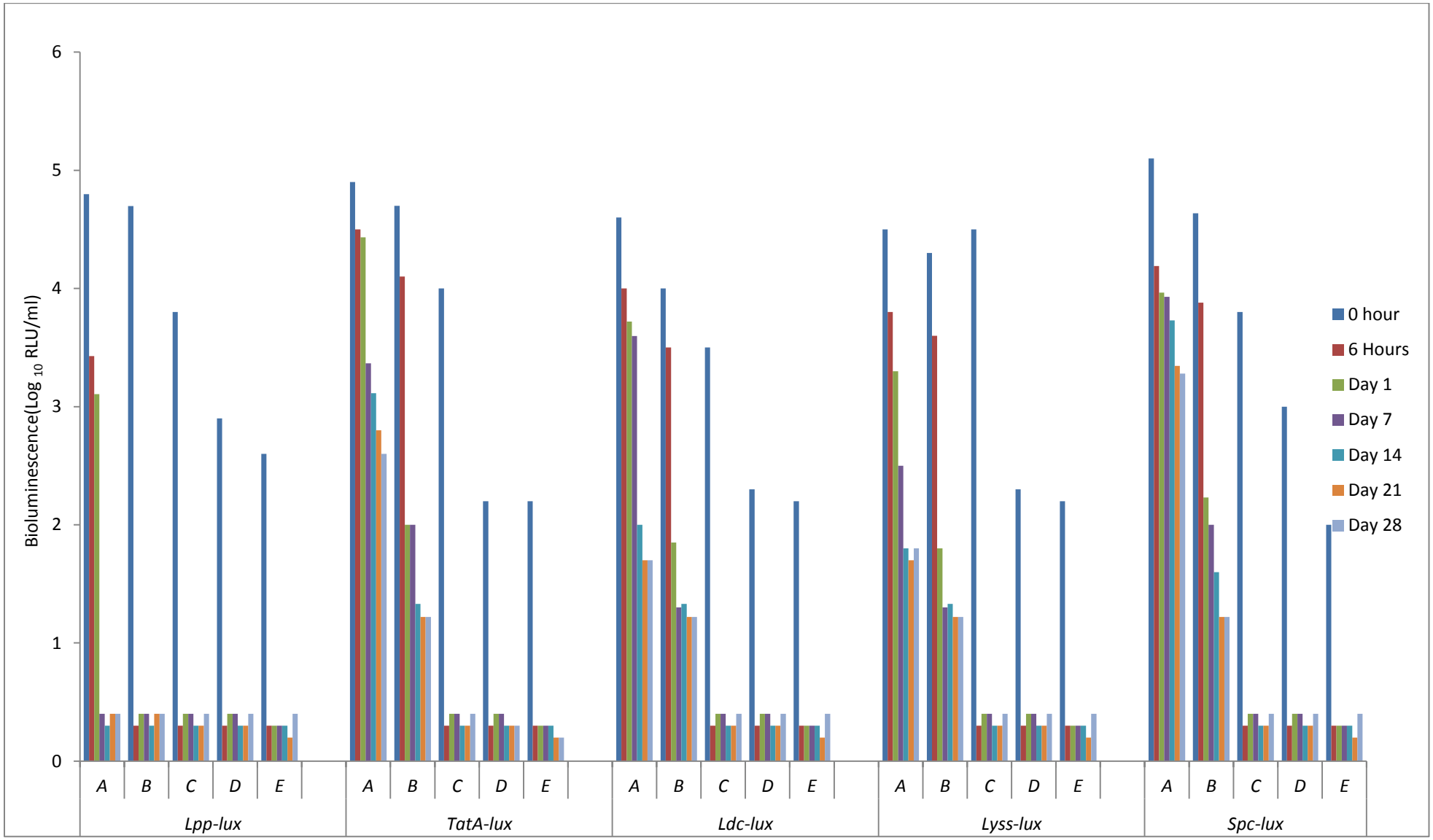


Figure 5.11: Bioluminescence levels (log $_{10}$ RLU/ml) expressed by the five biosensor strains challenged with benzalkonium chloride (BAK) + 0.03% EDTA presented in ascending concentrations (A to E) from left to right: 0.00039%; 0.00078%; 0.0016%; 0.0031%; and 0.0062% of each biosensor, pH 7.0 over 28 days. # Overall the SEM is 0.095 at 95% confidence interval

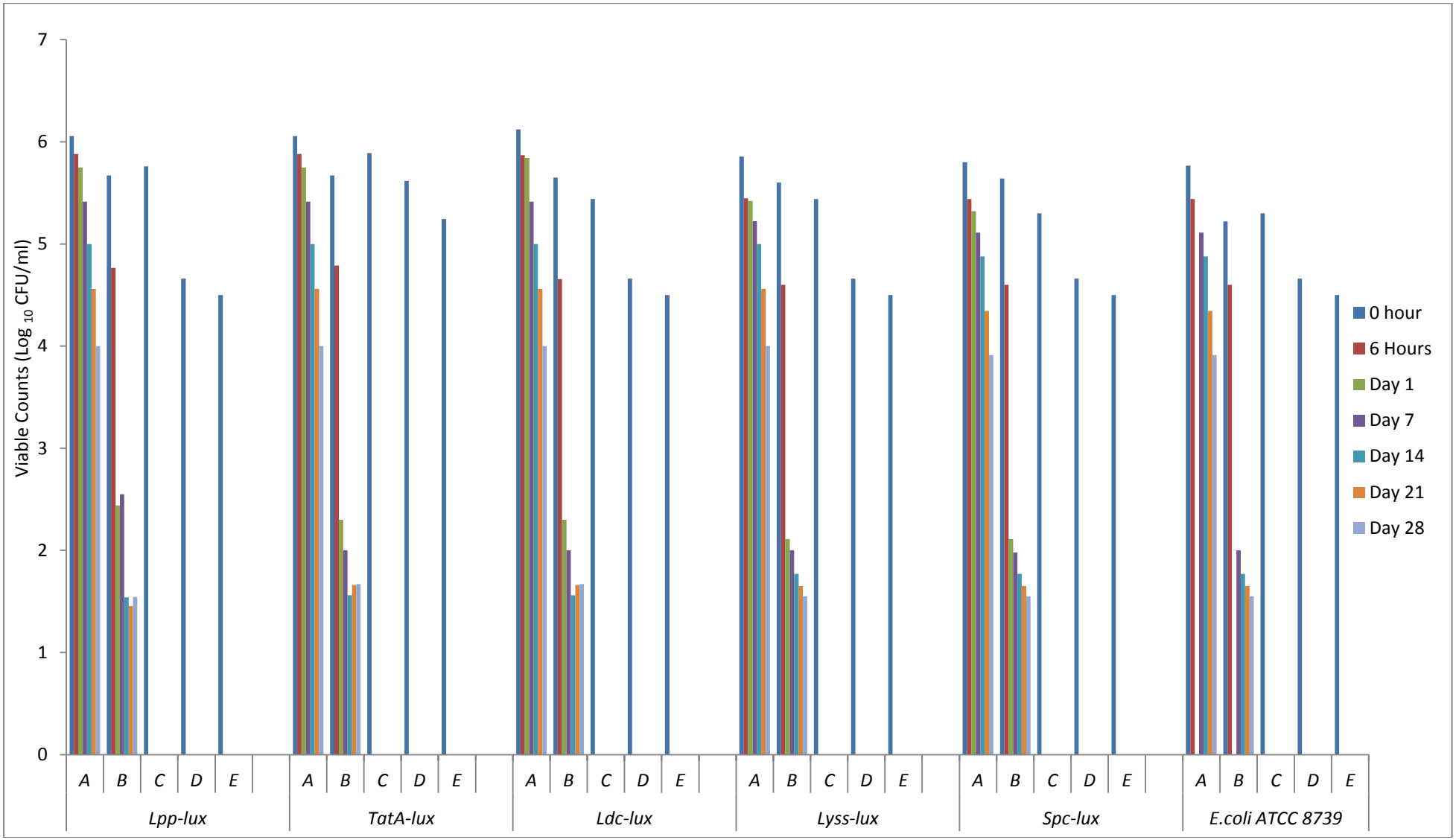


Figure 5.12: Viable counts (log_{10} CFU/ml) expressed by the five biosensors with benzalkonium chloride (BAK) + 0.03% EDTA presented in ascending concentrations (A to E) from left to right: 0.00039%; 0.00078%; 0.0016%; 0.0031%; and 0.0062%. # Overall the SEM is 0.164 at 95% confidence interval

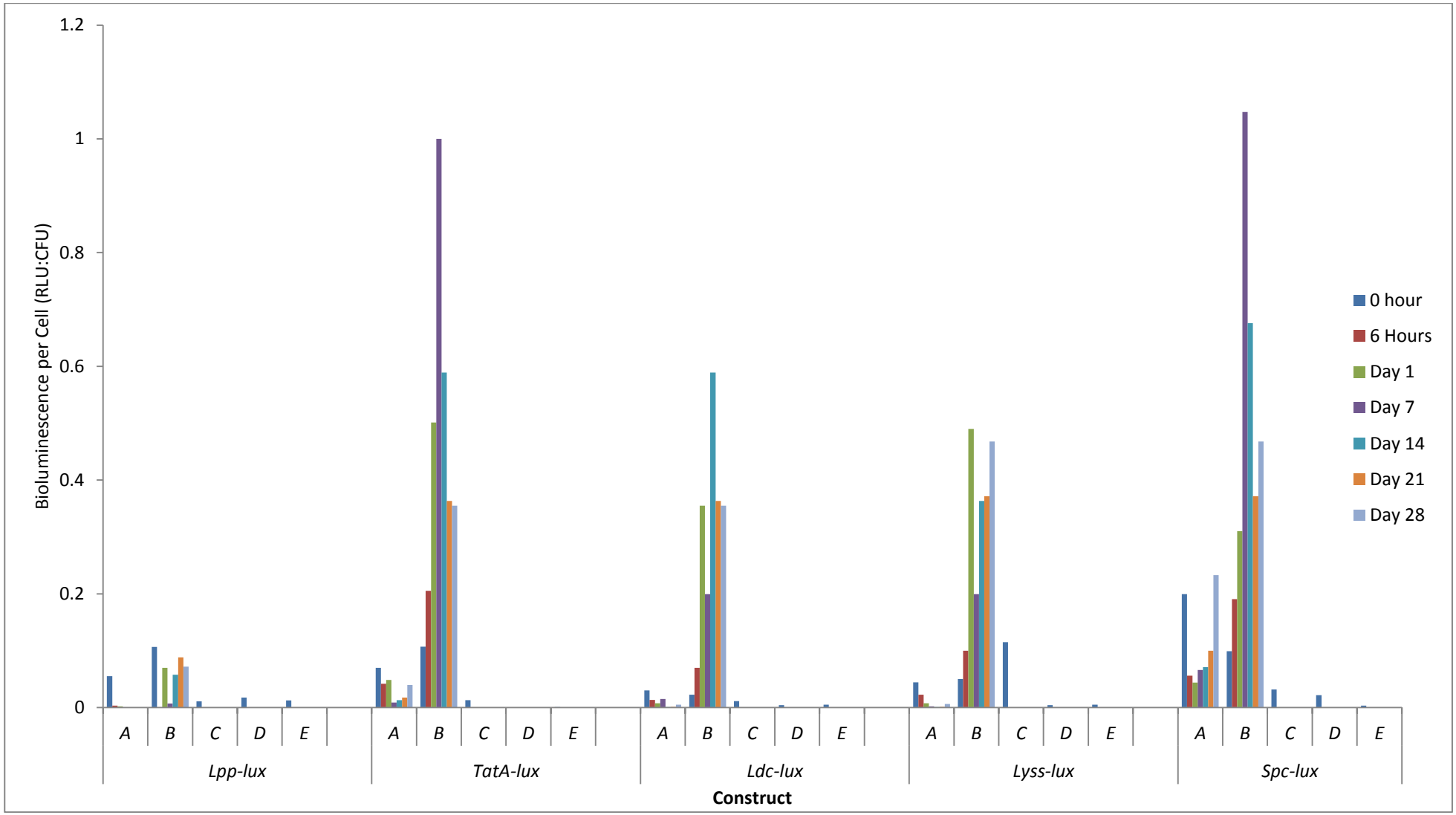


Figure 5.13: Bioluminescence per cell (RLU:CFU) for the five biosensor challenged with benzalkonium chloride (BAK) + 0.03% EDTA presented in ascending concentrations (A to E) from left to right: 0.00039%; 0.00078%; 0.0016%; 0.0031%; and 0.0062% pH 7.0 over 28 days. # Overall the SEM is 0.017 at 95% confidence interval

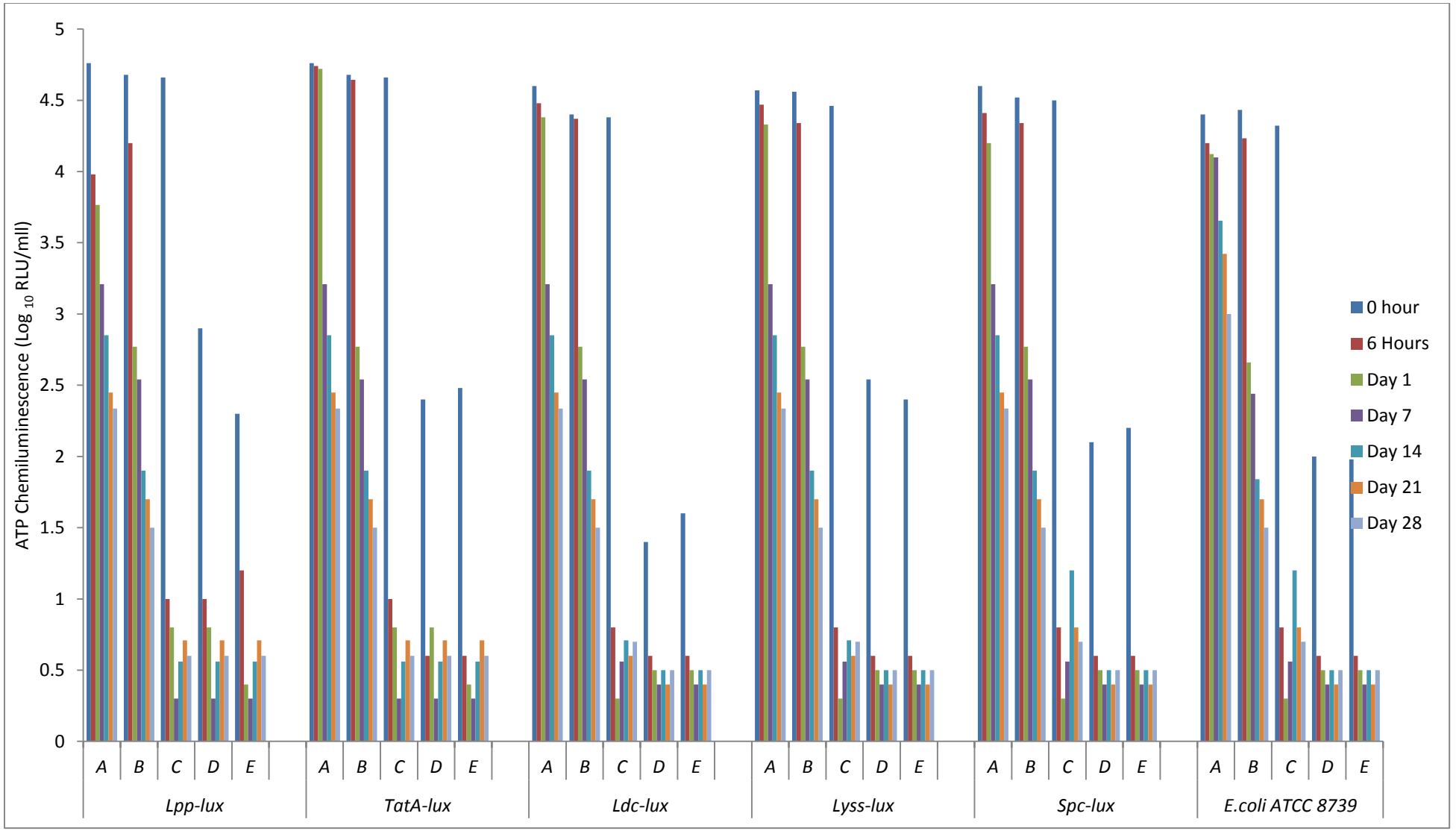


Figure 5.14: ATP chemiluminescence levels (log_{10} RLU/ml) of the five biosensors challenged with benzalkonium + 0.03% EDTA presented in ascending concentrations (A to E) from left to right: 0.00039%; 0.00078%; 0.0016%; 0.0031%; and 0.0062% pH 7.0 over 28 days. # Overall the SEM is 0.107 at 95% confidence interval

Table 5.4: Analysis of the viable counts, ATP-chemiluminescence, and bioluminescence from the biosensor strains when exposed to benzalkonium chloride with 0.03% EDTA, and an analysis of the suitability of this preservative for use in different pharmaceutical preparations in accordance with the guidelines set out in the European and British Pharmacopoeias

Biosensor strain	[BAK] (%)	Incubation time required to achieve more than 3 orders of magnitude reduction in viable counts (days)	Incubation time required to achieve more than 3 orders of magnitude reduction in ATP-chemi-luminescence (days)	Incubation time required to achieve more than 3 orders of magnitude reduction in bio-luminescence (days)	% reduction in bio-luminescence when viable counts reduced by more than 3 orders of magnitude	Correlation coefficient (R^2) between RLU and CFU	Correlation coefficient (R^2) between CFU and ATP	Correlation coefficient (R^2) between RLU and ATP	Suitability of BAK+ 0.03% EDTA for different pharmaceutical preparations, as determined from the Pharmacopoeia[#]
Lpp-lux	0.0062	6 Hr	6 Hr	6 Hr	99.9	0.996	0.992	0.993	O,P,T
	0.0031	6 Hr	6 Hr	6 Hr	99.9	0.997	0.992	0.99	O,P,T
	0.0016	6 Hr	6 Hr	6 Hr	99.9	0.996	0.860	0.863	O,P,T
	0.00078	1	1	6 Hr	99.9	0.917	0.973	0.922	O,P,T
	0.00039	n/a	n/a	6 hr	99.9	0.754	0.990	0.758	T
tatA-lux	0.0062	6 Hr	6 Hr	6 Hr	99.9	0.994	0.995	0.994	O,P,T
	0.0031	6 Hr	6 Hr	6 Hr	99.9	0.977	0.995	0.983	O,P,T
	0.0016	6 Hr	6 Hr	6 Hr	99.9	0.975	0.953	0.850	O,P,T
	0.00078	1	1	1	99.9	0.979	0.904	0.991	O,P,T
	0.00039	n/a	n/a	n/a	99.9	0.917	0.944	0.965	T
ldc-lux-	0.0062	6 Hr	6 Hr	6 Hr	99.9	0.994	0.987	0.988	O,P,T
	0.0031	6 Hr	6 Hr	6 Hr	99.9	0.974	0.980	0.986	O,P,T

Biosensor strain		Incubation time required to achieve more than 3 orders of magnitude reduction in viable counts (days)	Incubation time required to achieve more than 3 orders of magnitude reduction in ATP-chemi-luminescence (days)	Incubation time required to achieve more than 3 orders of magnitude reduction in bio-luminescence (days)	% reduction in bio-luminescence when viable counts reduced by more than 3 orders of magnitude	Correlation coefficient (R^2) between RLU and CFU	Correlation coefficient (R^2) between CFU and ATP	Correlation coefficient (R^2) between RLU and ATP	Suitability of BAK+ 0.03% EDTA for different pharmaceutical preparations, as determined from the Pharmacopoeia[#]
	0.0016	6 Hr	6 Hr	6 Hr	99.9	0.968	0.851	0.924	O,P,T
	0.00078	1	1	1	99.9	0.871	0.952	0.872	O,P,T
	0.00039	n/a	n/a	28	99.9	0.934	0.978	0.973	T
lyss-lux	0.0062	6 Hr	6 Hr	6 Hr	99.9	0.994	0.995	0.994	O,P,T
	0.0031	6 Hr	6 Hr	6 Hr	99.9	0.974	0.996	0.984	O,P,T
	0.0016	6 Hr	6 Hr	6 Hr	99.9	0.966	0.857	0.820	O,P,T
	0.00078	1	1	1	99.9	0.87	0.960	0.841	O,P,T
	0.00039	n/a	n/a	28	99.9	0.891	0.971	0.900	T
spc-lux	0.0062	6 Hr	6 Hr	6 Hr	99.9	0.993	0.994	0.992	O,P,T
	0.0031	6 Hr	6 Hr	6 Hr	99.9	0.982	0.993	0.991	O,P,T
	0.0016	6 Hr	6 Hr	6 Hr	99.9	0.978	0.863	0.877	O,P,T
	0.00078	1	1	1	99.9	0.975	0.967	0.985	O,P,T
	0.00039	n/a	n/a	n/a	99.9	0.948	0.978	0.911	T
E.coli [pBr-322.lux]	0.00062	6 Hr	6 Hr	n/a	n/a	n/a	0.993	n/a	O,P,T

Biosensor strain	[BAK] (%)	Incubation time required to achieve more than 3 orders of magnitude reduction in viable counts (days)	Incubation time required to achieve more than 3 orders of magnitude reduction in ATP-chemi-luminescence (days)	Incubation time required to achieve more than 3 orders of magnitude reduction in bio-luminescence (days)	% reduction in bio-luminescence when viable counts reduced by more than 3 orders of magnitude	Correlation coefficient (R^2) between RLU and CFU	Correlation coefficient (R^2) between CFU and ATP	Correlation coefficient (R^2) between RLU and ATP	Suitability of BAK+ 0.03% EDTA for different pharmaceutical preparations, as determined from the Pharmacopoeia[#]
E.coli ATCC 8739	0.0031	6 Hr	6 Hr	n/a	n/a	n/a	0.993	n/a	O,P,T
	0.0016	6 Hr	6 Hr	6 Hr	n/a	n/a	0.863	n/a	O,P,T
	0.00078	1	1	n/a	n/a	n/a	0.922	n/a	O,P,T
	0.00039	n/a	n/a	n/a	n/a	n/a	0.808	n/a	T
	0.0062	6 Hr	6 Hr	n/a	n/a	n/a	0.994	n/a	O,P,T
	0.0031	6 Hr	6 Hr	n/a	n/a	n/a	0.983	n/a	O,P,T
	0.0016	6 Hr	6 Hr	6 Hr	n/a	n/a	0.820	n/a	O,P,T
	0.00078	1	1	n/a	n/a	n/a	0.991	n/a	O,P,T
	0.00039	n/a	n/a	n/a	n/a	n/a	0.965	n/a	T

+O, P and T denote the effectiveness of benzalkonium chloride according to Pharmacopeia guidelines of Oral, Parental and ophthalmic, Topical preparation

n/a; not applicable

5.6 : Discussions

5.6.1 : The Effect of Benzalkonium Chloride (BAK) on Biosensor and Control Strains

Benzalkonium chloride (BAK) is a single positively charged quaternary ammonium compound and is classified as a monoquaternary ammonium compound (QAC). BAK is used as an active ingredient in disinfectants, and as a preservative in pharmaceutical antiseptics, cosmetics, oral, parenteral, nasal, and ophthalmic products. BAK is bactericidal when cells are exposed to sufficiently high concentrations, and/or it can also have bacteriostatic activities at lower concentrations preventing exponential increase of bacterial growth (Brown & Norton, 1965; Eriksen, 1970). As demonstrated previously, in chapter 4 of this thesis, whole-cell bioluminescence can be used to monitor *E. coli* viability in preservative efficacy tests and in MIC screening of weak acid preservatives. Since BAK acts by perturbation of lipid bilayer membranes, which is a different mode of action from weak acids, it was decided to test whether the biosensor strains could be used to determine the MIC of BAK and then subsequently to monitor the viability of the bacteria in a preservative efficacy test (Gilbert & Moore, 2005). Consequently, this would help to show the potential of the bioluminescent biosensor strains, employed in the current research, to monitor the viability of cells exposed to a diverse range of preservatives.

It has been reported that disruption of the cell membrane by BAK leads to increased levels of highly reactive oxygen species, within the cell affected, which could affect the electron-transport chain (Bore et al., 2006). Accumulation of intracellular reactive oxygen species (R.O.S) has been demonstrated to cause depletion of NADPH (Bore et al., 2006), which is a requirement in the synthesis of aldehyde as a substrate for bioluminescence (Meighen, 1991). Consequently, there is a danger that perturbation of the cytoplasmic membrane could have a negative impact upon bioluminescence levels. However, there were significant correlations between the simultaneous

decrease in bioluminescence (RLU/ml), viable counts (CFU/ml) and ATP chemiluminescence (RLU/ml) that was observed in the work described in this thesis.

Benzalkonium chloride exhibited strong antibacterial properties at concentrations from 0.05% to 0.0016% since it was able to inhibit *E.coli* growth completely demonstrated by bioluminescence measurements. The attraction of charges between BAK and the outer membrane of *E. coli* results in high binding affinity between the two. Binding of BAK leads to destabilisation of the cell membrane, resulting in a loss of proton motive force (p.m.f.) and leakage of cell contents in which leads to growth arrest (Gilbert & Moore, 2005). In this study, the MIC of BAK was determined to be 0.0016% using bioluminescence as well as viable counts and optical density, which is very similar to previous estimates of the MIC of BAK against *E. coli* ATCC 8739 as being 0.0016% determined using optical density (Kamysz & Turecka, 2005). On the other hand, Bore et al. in 2007 found that the MIC of BAK against *E. coli* K-12 was much higher, at 0.008 - 0.009%, than in either the current study or that of Kamysz & Turecka (2005). However, the K-12 *E.coli* strain has adapted to BAK, and become more resistant to it than the ATCC 8739 strain, through increased expression of multi-drug efflux pumps (Li & Nikaido, 2004; Tikhonova & Zgurskaya, 2004). Thus the *E. coli* ATCC 8739 biosensor and control strains used in the study described in this thesis demonstrated a clear susceptibility to BAK by both bioluminescence and viable counts with an MIC of 0.0016%, which is comparable to the literature.

Further analysis of the viability of the biosensor strains in preservative efficacy test (PET) assays demonstrated that the bioluminescence ($\log_{10}$ RLU/ml), viable counts ($\log_{10}$ CFU/ml), and ATP-chemiluminescence ($\log_{10}$ RLU/ml) of the *E.coli* biosensor strains and also the control strains were all reduced significantly between inoculation and 6 hours of incubation when the cultures were exposed to BAK at concentrations of 0.0062%, 0.0031% and 0.0016% (Figures 5.7, 5.8, 5.9 and

5.10). This finding agrees with the results of the MIC and PET assays with benzalkonium chloride and *E.coli* ATCC 8739 published by Kamysz & Turecka (2005). Consequently, it is possible to determine the minimum concentration of BAK that is required for the preparation of ophthalmic, parenteral and topical products, following the recommendations set out in the Pharmacopoeias. Concentrations of 0.0062%, 0.0031%, and 0.0016% BAK all led to a reduction in the *E. coli* viable counts to undetectable levels and a significant decrease in bioluminescence for all biosensor strains between inoculation and the 6^{th} hour of incubation, which exceeds the European Pharmacopoeia-A requirements and so any of these concentrations would provide effective antimicrobial preservation for oral, topical ophthalmic and parenteral preparations.

The bioluminescence expressed by the *lpp-lux* biosensor was reduced significantly by more than 5 orders of magnitude after 7 days of exposure to BAK concentrations of 0.0016%, 0.00078% and 0.00039%. This reduction in bioluminescence from *lpp-lux* occurred significantly quicker than it did for any of the other four biosensors (Figures 5.7 and 5.9). A similar effect was observed when *lpp-lux* was exposed to BAK concentrations of 0.0016%, 0.00078% or 0.00039% when supplemented with 0.03% EDTA. It is possible that the *lpp* promoter is down regulation in BAK alone or in the presence of EDTA, as was also observed in this study in the presence of sorbic acid. Previous studies by Moen et al (2012) have demonstrated increased expression of *osmB* (lipoprotein) in non-resistant *E.coli* K12. expression of the OmpA, OmpF, and OmpT outer membrane proteins has been demonstrated in BAK-sensitive *E. coli* strains, whereas expression of OmpC was increased in BAK adapted strains (Bore et al., 2007; Ishikawa et al.,. 2002). This suggests that expression of *lpp* may have decreased to minimal levels whilst expression of the *ompA*, *ompF*, and *ompT* outer membrane protein genes was increased at BAK concentrations of 0.0016%, 0.00078% and 0.00039 % . However, direct evidence for down regulation of *lpp* was not sought in the current study, due to time constraints, and so further analyses using quantitative reverse transcription PCR comparing *lpp*

expression levels with that of other genes such as *osmB*, *ompA*, *ompF* and *ompT* could provide valuable insights.

When exposed to low concentrations of BAK (0.00078% and 0.00039%), the viable counts (log_{10} CFU/ml) and the ATP-chemiluminescence (log_{10} RLU/ml) were only reduced significantly ($P \leq 0.05$, ANOVA) by less than 2 orders of magnitude when exposed to 0.00078% and 0.00039% BAK for the five biosensor and control strains from 0 h to 28 days with no statistical differences over 28 days between two methods. It is clear from the results that low BAK concentrations (0.00078% and 0.00039%), were not effective for use in oral, topical ophthalmic and parenteral preparations since the reductions in viable counts and bioluminescence did not comply with the requirements of the Pharmacopoeias. Moreover, the low BAK concentration failed to cause perturbation in cells which is linked to a loss of osmoregularotory to cause cell death/arrest (Gilbert & Moore, 2005) and since BAK is known to disrupt the membrane it is possible that the potential involvement of the H^+-ATPase activity, whereby was able to pump protons out from the cell controlling sustaining the internal cytoplasm pH of *E. coli.*

There was a reduction in the bioluminescence of the biosensor strains *ldc-lux, lysS-lux* of 3 or more orders of magnitude by 28 days of incubation in both the low concentrations. It is likely that the earlier loss of bioluminescence expression from these two strains in low concentrations of BAK was due to their weaker constitutive promoters leading to a loss of bioluminescence to base-line sooner than for *tatA-lux* and *spc-lux.* When exposed to 0.00078% BAK, the bioluminescence expressed by the *ldc-lux* and *lysS-lux* biosensor strains was reduced by 3 orders of magnitude between 7 and 14 days; and 14 to 21 days of incubation, respectively. On the other hand it was not until between 21 and 28 days of incubation in 0.00078% BAK that the bioluminescence expressed by the *tatA-lux* and *spc-lux* biosensor strains was reduced by 3 orders of magnitude. Previous work by Srikantha et al. (1995) has demonstrated that fusion of the *lux* operon to a weaker constitutive promoter resulted in

less transcription of the operon and so lower expression of bioluminescence which resulted in a quicker reduction in bioluminescence to baseline when the cells were metabolically challenged. This implies that *lysS-lux* biosensor strain represents the weakest promoter whereby the reduction of 3 or more orders of magnitude in bioluminescence in PET assays.

The bioluminescence expressed by both the *tatA-lux* and *spc-lux* biosensor strains was reduced by at least 2 orders of magnitude when exposed to concentrations of 0.00078% and 0.00039 % BAK. The change in bioluminescence expressed by these two strains showed an excellent significant correlation with both the viable counts and the ATP-chemiluminescence indicating that these three measures yielded equilvalent results. The bioluminescence per cell expressed by both the *tatA-lux* and *spc-lux* biosesnsors was approximately twice that expressed by *ldc-lux*, approximately 10 times greater than that expressed by *lysS-lux*, and approximately 1000 times greater than that expressed by the *lpp-lux* biosensor strain. Consequently, this suggests that the biosensor strains *tatA-lux* and *spc-lux* would be particularly good candidates for further evaluation in "real-time" preservative efficacy testing, since a high level of bioluminescence expression per cell offers the potential for improved reliability in antimicrobial testing (Stewart, 1990).

There was a low level of residual bioluminescence expression from all of the biosensor strains, even when there were no detectable viable counts from them at concentrations of BAK (without EDTA) of 0.0062% to 0.00039%. One possible explanation for this is that there may have been a small population of uninjured cells present, which was below the minimum detection limit (MDL) of the viable counting technique employed, mixed in with a much larger population of injured cells that were luminescing sub-maximally (Dodd et al.,1997). A second explanation for this observation is that there could be a small sub-population of VBNC *E.coli* present that could metabolise and bioluminescent at a low level (Rowan, 2004), but which could not be found by the viable counting

method employed. also observed when the biosensor strains were exposed to concentrations of benzalkonium chloride and also in sorbic acid PET.

5.6.2 : The Effect of 0.03 % EDTA and EDTA on the Biosensor and Control Strains upon Bioluminescence, Viable counts and ATP-Chemiluminescence

When the biosensor strains were challenged with BAK at 0.0062% and 0.0031% supplemented with 0.03% EDTA in the PET assays, the bioluminescence , viable counts and intracellular ATP levels all decreased significantly between inoculation and 6 hours of incubation ($P \leq 0.05$, ANOVA) (Figures 5.11, 5.12 & 5.14). When benzalkonium chloride at concentrations of 0.0062% and 0.0031 % was supplemented with 0.03% EDTA, the reduction in the *E. coli* population density to undetectable levels, as demonstrated by bioluminescence, viable counts and ATP-chemiluminescence, between inoculation and the 6 hours of incubation was in excess of the requirements of the European Pharmacopoeia for preservatives to be used in oral, topical ophthalmic and parenteral preparations.

When exposed to 0.00078% BAK supplemented with 0.03% EDTA, the time taken, after inoculation, for the bioluminescence expression by *lysS-lux, ldc-lux, tatA-lux,* and *spc-lux* to be reduced by 3 orders of magnitude was in agreement with the ATP-chemiluminescence and viable counts. Hence, the interactions of EDTA and BAK synergistically enhance the disruption of membrane layers is supported by Gilbert and Moore, (2005) as it lowers the minimum inhibitory concentration of BAK. The data presented in this thesis indicate that the MIC of BAK, when supplemented with 0.03% EDTA, was 0.00078% for all of the *E. coli* strains, whether the change in bioluminescence, viable counts or optical density was taken into account, which is significantly lower than the MIC of BAK alone by half. Previous studies have also noted similar synergistic effects for the combination of BAK and EDTA (Dantas et al.,2000).

It is known that EDTA is a chelating agent, which acts to destabilise the lipopolysaccharide outer membrane of *E. coli* by sequestering divalent cations, such as Ca^{2+} (Singer & Nicolson, 1972). EDTA contains four carboxylates and two amines group consisting of three pairs of lone pair electrons (donor). This lone pair of electrons would attract an electron-pair acceptor for coordination bonding (Rao, 1982). The divalent cations found in lipopolysaccharides, such as Mg^{2+} or Ca^{2+}, interact with the carboxylate groups of EDTA forming stable coordination compounds (Rao, 1982). The enhanced killing effect of 0.00078% BAK when supplemented with 0.03% EDTA, demonstrated by reduction a in viable counts, bioluminescence and ATP- chemiluminescence readings of at least 3 orders of magnitude, suggests that loss of magnesium and calcium ions from the destabilisation of the outer membrane leads to a greater cell death with the formation of stable EDTA complex (Rao, 1982). Furthermore, Alakomi et al. (2006) have demonstrated, by using atomic force microscopy (AFM), the release of lipopolysaccharide from the surface of cells exposed to BAK supplemented with EDTA, at lower concentrations than when BAK was used alone, which resulted in large and irregular-shaped pits where the peptidoglycan layer was exposed.

However, at 0.00039% BAK with the additional of 0.03% EDTA, a 2 order magnitude of was achieved in plate counts (log_{10} CFU/ml) and the ATP-chemiluminescence (log_{10} RLU/ml) for the five biosensor and control strains from 0 h to 28 days with no statistical differences over 28 days between the methods, whilst bioluminescence expression by *spc-lux* and *tatA-lux* decreased 2 orders of magnitude from the 0 h to 28 days. The bioluminescence expressed by *spc-lux* and *tatA-lux* agreed with the two pharmacopeia certified method therefore signifies excellent choice of constitutive promoters for the evaluation of viability in BAK PET. However, the bioluminescence expressed by the *tatA-lux* biosensor was not statistically different from that expressed by the *spc-lux* biosensor when exposed to BAK alone, but when exposed to BAK supplemented with EDTA the expression of

bioluminescence by the *tatA-lux* strain was barely 20% of that from the *spc-lux* strain and this suggests that *spc* would be a better choice of constitutive promoter PET assays, certainly for BAK and EDTA, BAK, and sorbic acid. In contrast, the bioluminescence expressed by the *lysS-lux* and *ldc-lux* biosensor strains had decreased by 3 orders of magnitude before the 28 days of incubation had been completed, indicating that these promoters of the two strains were weaker than the *spc-lux* and *tatA-lux* biosensor strains.

The addition of EDTA was found to enhance the antimicrobial activity of BAK. However it is known that EDTA can potentially act as a quencher for the ATP-chemiluminescence assay and therefore decrease the bioluminescence detected. Consequently, there is a danger that perturbation to the cytoplasmic membrane could have a negative impact upon bioluminescence levels. Nevertheless, there were significant strong correlations between the changes in ATP chemiluminescence (RLU/ml) and both bioluminescence (RLU/ml) and viable counts for each of the *tatA-lux, ldc-lux, lysS-lux,* and *spc-lux* biosensor strains when they were exposed to 0.03% EDTA with significant correlation coefficient values of (R^2: 0.82-1.00) indicating no divergent to the population (CFU/ml) and ATP chemiluminescence (RLU/ml) readings despite the depletion effect of BAK and BAK with 0.03% EDTA . Previous studies have found that concentrations of EDTA ranging from 0.05% to 0.58% decreasing ATP- chemiluminescence significantly (Wen et al., 2001). The concentration of EDTA used in combination with BAK in this study was 0.03%, and showed showed no significant quenching of the ATP-chemiluminescence, which concurs with Wen et al. (2001). This denotes the potential limitation of ATP chemiluminescence method in concentrations a more or at least 0.05% to 0.58% EDTA contained preservative system. In addition, the luciferin-luciferase interaction with intracellular ATP extracted occurs extracellularly in which the effects of EDTA and other ions such as copper (II), zinc (II), calcium (II) (Wen et al., 2001) can act as quenchers thereby the utilizations of the concentrations are limited. In contrast, the emission of

bioluminescence is intracellular within the genetically altered *E.coli* cells linked to metabolic activity and promoter expression which is less likely to be affected by quenchers as demonstrated by this study.

It was not unreasonable to supplement the BAK with EDTA at a concentration of 0.03% in the current experiments, as this falls within the concentration of EDTA permitted to be added to eye care products (Furrer et al., 2001) and to food products (FSA). Moreover, it has become increasingly common for medicines to be formulated with a combination of preservatives to protect against microbial spoilage rather than to use just a single one (Denyer & King, 1988). The reason for this change in practice is twofold: firstly a single agent added to a product may not have a sufficiently broad spectrum of antimicrobial activity meaning that preservation can be incomplete; and secondly this makes it possible to reduce the concentration of the individual agents used without loss of efficacy overall. It is also necessary to achieve acceptable levels of product protection with only a relatively limited choice of agents available, and so efforts are being made to capitalise on potential synergistic interactions between preservatives, whilst at the same time cutting down on the concentrations used so as to minimise the risk of adverse reactions (Hodges et al., 1996). EDTA has been demonstrated to act synergistically in combination with either benzalkonium chloride or potassium sorbate (Hart, 1984). EDTA has previously been reported to reverse the resistance of Gram negative organisms to some antimicrobial agents, and to enhance the effect of several cosmetic preservatives, such as the parabens, imidazolidinyl urea, and tert-butyl hydroxyanisole (Hart, 1984). Furthermore, EDTA is a safe, inexpensive, and effective product and its addition to cosmetic and toiletry formulations maintains clarity, protects the fragrance of the components, and stabilizes the coloring agents (Hart, 1984). Hence, by adding EDTA to benzalkonium chloride, this may prevent a lower concentration of BAK to be effective and hence demonstrating successful monitoring of viability by the biosensors.

However, EDTA alone does not appear to inhibit *E.coli* growth since neither the cell density nor the bioluminescence of the biosensor strains was significantly altered in the presence of EDTA in the current experiments (Figures 5.5 a & b). Previous studies by Leive (1968) demonstrated that concentrations of EDTA between 5.8% and 17.5% were bactericidal to *E.coli* cells. However, since the concentrations of EDTA used in this study did not exceed 0.09% it is clear that they would have been unable to inhibit growth (Leive, 1968).

Absorbance readings at 0.05% BAK (with and without EDTA) were significantly higher than 0.025% BAK (with and without EDTA) in Figures 5.3 (a) & 5.4 (a) before and after 24 hour of incubation). It is most likely that this increase in absorbance is a result of the interaction of the 0.05% BAK halide anions (Cl^-) with tryptone soy broth at these high concentrations causing slight precipitation of and thus cloudiness of the medium, leading to a false increase. This was reinforced by the high control absorbance readings without the addition of *E.coli* and checked by plate counts. However, the bioluminescence from the *E. coli* biosensor strains was minimal in 0.05% BAK, indicating that the *E. coli* had been inhibited. It is clear from these data that monitoring bioluminescence is more effective than attempting to determine culture density by absorbance when determine MIC of preservatives as absorbance remains as one of the oldest method in determining MIC. Spectrophotometry does not differentiate between live and dead cells, whereas bioluminescence does since it relies upon functioning metabolism in *E. coli* and thus should provide both a more sensitive and a more reliable method to monitor the effect of preservatives and other toxins upon cells.

5.6.3 : Overall Comparison of the Bioluminescent Biosensors with the Viable Counts and ATP-Chemiluminescence Methods in Preservative Efficacy Testing

The bioluminescence per cell (RLU:CFU) expressed by the *spc-lux* and *tatA-lux* biosensor strains was significantly higher at a reduction of a 3 or more magnitude reduction of bioluminescence than the bioluminescence per cell expressed by the *ldc-lux, lysS-lux,* and *lpp-lux* biosensor strains in the BAK PET assay. Furthermore, in the second series of PET assays when BAK was supplemented with 0.03% EDTA, the bioluminescence per cell expressed by *spc* was on average five times greater than that produced by *tatA* at the lower concentration of BAK tested (0.00078% and 0.00039%). Stewart, noted in 1990 that a high level of bioluminescence expression per cell offers the potential for improved reliability in antimicrobial testing, hence by validating the biosensors have concluded that *spc* represents the best biosensor as high bioluminescence per cell was yielded.

It is possible that the statistically greater level of bioluminescence expressed by the *spc-lux* biosensor strain, as compared with the *ldc-lux*, *lpp-lux* and *lysS-lux* biosensor strains, is the result of a closer match between the *spc-lux* promoter regions and the consensus sequences for the -10 and extended -10 regions, which would afford greater affinity for binding by RpoS ($\sigma^{38/S}$) and RpoD ($\sigma^{70/D}$).

Under benzalkonium chloride stress, RpoS ($\sigma^{38/S}$) and RpoD ($\sigma^{70/D}$) both play important roles in combating the oxidative stress that can result from it (Bore et al., 2007; Martinez & Kolter, 1997; Martinez-Martinez et al., 2000) as similar roles were discovered in weak acid stress discussed in chapter 4. The major global regulatory gene, *dps* (DNA binding protein) is known to regulate the expression of a variety of stress-response genes when bacterial cells are challenged with BAK (Bore et al., 2007; Martinez & Kolter, 1997; Martinez-Martinez et al., 2000). Dps has been shown to be regulated by *OxyR*, RpoS ($\sigma^{38/S}$) and RpoD ($\sigma^{70/D}$) (Altuvia et al., 1994, Azam et al., 1999;

Lomovskaya et al., 1994) which is involved in the defence mechanism against oxidative damage by BAK (Almiron et al., 1992; Choi et al., 2000; Martinez & Kolter, 1997; Martinez-Martinez et al., 2000).

It is clear, therefore, that the nucleotide sequence of the promoter region is important as it can have an influence on the binding affinity of sigma factors as this is directly influence the expression of bioluminescence. Yet, a promoter that matches the consensus sequence for a promoter in all the conserved regions is undesirable because it actually reduces transcriptional activity for a promoter use as by having too many contacts impedes the transition from the open complex to promoter clearance and elongation (Grana et al., 1988) restricting opportunity for regulation. Furthermore, by recognizing the consensus sequence of a strong constitutive promoter, this can be implemented for promoter designing for future biosensor designs for monitor viability PET and MIC screening. Furthermore, the choice of a strong promoter with a good match to the consensus sequence would be advantageous in the design of future bioluminescent biosensors for both MIC screening and PET assays.

5.7 : Conclusions

1) There was a strong correlation between the bioluminescence expressed by the *spc-lux*, *tatA-lux*, *lysS-lux* and *ldc-lux* biosensor strains with both the viable counts and the ATP-chemiluminescence methods, which are currently accepted by the Pharmacopoeias, when challenged with benzalkonium chloride with or without EDTA.
2) EDTA acted synergistically with BAK to enhance its bactericidal activity.
3) Expression of the *lpp* promoter was found to be down-regulated under BAK and BAK with EDTA conditions, since bioluminescence was significantly reduced after day 1 of exposure to low concentrations of BAK whilst the viable counts and ATP-chemiluminescence were unchanged.
4) The bioluminescent biosensor incorporating the *spc* promoter is likely to be the best candidate for further development, since it led expressed greater levels of bioluminescence per cell than biosensors based upon the other four promoters, when challenged with benzalkonium chloride.

Chapter 6

6 : General Discussion and Conclusion

The microbiological analysis of food, cosmetics, and pharmaceutical products is an integral part of their microbiological safety management. However, both the conventional and current alternative microbiological testing methods used in these industries can require a long incubation time, highly skilled personnel, expensive reagents and expensive equipment to achieve useable results. A method which circumvents the undesirable traits of conventional and current rapid methods, but which retains their sensitivity and specificity, would be advantageous in rapid microbiological testing. The study described in this thesis investigates the application of five individual plasmid-borne biosensors constructed from constitutive *Escherichia coli* ATCC 8739 promoters in transcriptional fusion with the *Photorhabdus luminescence luxCDABE* operon which were then transformed into *E. coli* ATCC 8739 challenged with two different kinds of preservative.

6.1 : Whole-cell Bioluminescence as a Rapid Method

A major benefit of the *lux* system is that it can act as a real-time reporter to monitor microbial growth and death kinetics. This was successfully demonstrated in the microbial growth and survival profiles of chapter 3, and during the preservative efficacy testing studies described in chapters 4 and 5. The decay of bioluminescence with time is a first-order process and reflects the rate of turnover of the luciferase enzyme and aldehyde substrate under the challenged conditions. The energy for the bioluminescence reaction is supplied via the oxidation of reduced $FMNH_2$, consequently, the *in vivo* production of FMNH is an essential prerequisite for a bioluminescent phenotype. Since FMNH production is coupled in aerobic organisms to the electron transport chain *via* an NADH and FMN-reductase couple, when given a non-limiting supply of the enzyme luciferase a limiting availability of FMNH will be reflected in variable and sub-maximal *in vivo* bioluminescence. Previous studies have suggested that stress responses that affect the production of intracellular FMNH, either directly

or indirectly, can be monitored in *lux* recombinant bacteria as changes in light output per cell (Ellison et al., 1994a &b; Hill., 1993; Steward, 1990, 1993,1997; Stewart et al., 1991, 1993, 1996, 1997; Stewart & Williams, 1992, 1993).

The naturally bioluminescent bacteria, *Vibrio fisheri* have proven and established their potential in toxicity testing (Kahru, 1993; Kahru et al., 1994; Loibner et al., 2004; Mortimer et al., 2008). Kinetic format of the *V. Fisheri* test (Microtox^{TM}) – A Flash Away has been recently standardized (ISO, 2010). In a comparative study of toxicity chemicals between *V.fisheri* and two bioluminescent *E.coli* strains; *E.coli* MC1061(pSLlux) and *E.coli* NC1061(pDNlux) found no statistical differences and reasonable correlation values obtained (Kurvet at al., 2011). In addition, studies from Deryabin and Aleshina, (2008) demonstrated the factors affecting luminescent expressions for bacterial strains; *E.coli* and *V.fisheri* were universal.

Therefore, this establishes the five bioluminescent *E.coli* ATCC 8739 strains used in this study were in accordance to the standardized bacterium, *V. fisheri*. Furthermore, *lux* CDABE of *V.fisheri* is less thermostable as compared to *P. Luminescence* (Meighen, 1991), hence the novel *E.coli* strains could also serve as a improved replacement to *V.fisheri* for antimicrobial screening.

The aim of this study was to investigate the potential of the bioluminescence method as a novel rapid method to replace traditional plate counting methods for use in preservative efficacy testing (PET). The five biosensor strains described in this thesis were challenged with two different preservatives to evaluate their potential for use in PET. Sorbic acid and benzalkonium chloride were the preservatives chosen for these preliminary studies because they have unique antimicrobial modes of action. Moreover, these preservatives are commonly used either singly or in combination with other preservatives in many food, cosmetic and pharmaceutical products.

Under sorbic acid and benzalkonium chloride challenge the whole-cell biosensors *tatA-lux, ldc-lux, lysS-lux* and *spc-lux* demonstrated high correlations with the two Pharmacopoiea-recommended methods (*i.e.* ATP Bioluminescence and conventional plate counting) for all of the concentrations tested and at all of the time points stipulated except for *lpp-lux* which has a significantly lower correlation coefficient values compared to the other four biosensors. This could imply that measuring the metabolic activity of cells is a desirable way of indicating cell viability and a quicker indication/prediction of viability status, a real time attribute of the *lux CDABE* genes as a bioreporter.

Furthermore, the disadvantage of the ATP-chemiluminescence method lies in the reactions that occur with the firefly luciferase-luciferin assay. The reaction takes place *outside* of the cell and presence of salts, ions, and anions, interferes with the assays as demonstrated by Wen et al (2001). Moreover, the ATP extraction process requires a sterile environment to prevent contamination with exogenous ATP-rich cells, which can be difficult to maintain (Sharma & Malik, 2012). In contrast, the emission of bioluminescence is intracellular within the genetically altered *E.coli* cells linked to metabolic activity and promoter expression which is less likely to be affected by quenchers. In addition, the whole-cell bioluminescence does not require extraction process which is time and reagent consuming.

Overall, the accuracy, precision and the linearity of their response to selected preservatives, have been successfully demonstrated for five *E. coli* biosensor strains which had a minimum detection limit of 10^3 CFU/ml, a detection range of 6 orders magnitude of, and yielded equivalent results to methods currently recommended by the pharmacopoeias (British Pharmacopoeia Volume 5 SC IV L, European Pharmacopoeia 5.1.6). In comparison with the methods tested concurrent with bioluminescence in the work described in this thesis (*i.e.* ATP-bioluminescence, fluorescence

spectrometry, epifluorescence microscopy, and conventional plate counting) it is evident that the whole-cell bioluminescence method has significant advantages (Chapter 2) as the response time of bioluminescence is short, it is cost effective, and less laborious.

6.2 : Selection of the Best overall Constitutive Promoter

Across the 28 days of incubation, the significant reduction in bioluminescence from initial incubation time is explained by a reduction in metabolic flux as the cells senesce. Statistical analysis demonstrated that there is no significant difference between the bioluminescence readings, viable counts, adenylate energy charge (AEC) and plasmid copy number (PCN) supported with strong correlation values.

However, *lpp-lux* demonstrated a significant reduction in bioluminescence expression as the population progressed further into stationary phase and the level of bioluminescence it expressed was significantly lower than any of the other biosensor strains in PET assays. Moreover, the AEC was not significantly different between the 5 biosensor strains when compared at intervals through the growth curve. Consequently, it was hypothesized that the plasmid copy number for the *lpp-lux* strain had declined, since there have been reports of genetic instability in bioluminescent strains (Turdean, 2011). However, further analysis demonstrated that the PCN did not differ significantly through the growth phase, neither from its peak nor between the different strains. Moreover, there was no significant difference between viable counts across all incubation time to biosensor strains.

There is evidence in the literature that an alternative starvation outer membrane lipoprotein is expressed under stress conditions (Alexander & St John, 1999; Bore et al., 2007; Mates et al., 2007) which could correlate to the significant reduction in expression of *lpp* in late stationary phase or in the presence of the preservatives tested. In addition, a second promoter has been identified upstream of the *lpp* promoter obtained by the Regulon DB database. Originally, expression of the *lpp* gene was

considered to be constitutive (Nakamura and Inouye, 1982). However, later workers have found that a σ^E promoter is located upstream of the *lpp* gene, and in the opposite orientation to the gene, which may lead to repression bioluminescence under stress conditions (Horton & Kanehisa, 1992) Furthermore, a CRP binding site located on the negative strand of the promoter within the -10 regions (Score: 5.07) of *lpp* was found using virtual footprinting software indicating the effect of this CRP binding site is to repress transcription of the *lux* cassette. To the best of this author's knowledge, this study presents the first evidence to hypothesize the down-regulation of the *lpp* promoter under the stressful conditions encountered in late stationary phase and in preservatives. However, this proposes future work in quantitating mRNA levels of *lpp* by reverse transcriptase qPCR.

Indirectly, this suggests that the outer membrane lipoprotein, which serves as a defence system for *E.coli*, is of great importance to the cell. Alternative outer membrane genes are known to be expressed differentially to combat the various stressful conditions that a cell may encounter (Alexander & St John, 1999; Mates et al., 2007; Maurer et al., 2005). Consequently, it can be concluded that this promoter would be inappropriate for the construction of a constitutive biosensor since its expression could break down under stress conditions.

Amongst the other four other promoters tested in the bioluminescent biosensor constructs, *lysS* was the weakest promoter in PET assay, as the bioluminescence expressions levels reduced more than 3 orders of magnitude significantly quicker than *ldc, tatA* and *spc* in PET and across the extended stationary phase. There are two CRP binding sites located on the negative strand of the *lysS* promoter sequence which could potentially act as a repressor which was found via virtual foot printing database and also contribute to the initial lower levels of bioluminescence expressed. Future

experiments to prove the presence of CRP within this region would be a new discovery to the claimed constitutive characteristic attribute of *lysS* (Clark & Neidhardt, 1990).

The bioluminescent biosensor constructed with the *ldc* promoter exhibited approximately two-fold less bioluminescence per cell (RLU:CFU) compared bioluminescence expressions exhibited by biosensor constructed with *spc* constitutive promoter when challenged in sorbic acid, BAK, BAK with 0.03% EDTA PET assays. However, previous studies have shown *ldc* expression is a RpoS-dependent regulatory mechanism during stationary phase (Kikuchi et al., 1998; Van Dyk et al., 1998). Never the less, bioluminescence per cell by exhibited by *ldc*-*lux* was significantly lower to *spc*-*lux*, hence, would not be the best candidate for PET assays in sorbic acid and BAK.

The bioluminescence per cell expressed by the biosensor incorporating the *tatA* promoter was significantly twofold lower in sorbic acid PET assay than that expressed by the *spc*-*lux* biosensor in the PET assays. However, statistical analysis revealed no significant difference in the significance of bioluminescecen per cell for *tatA* and *spc* in BAK and BAK+ EDTA PET assays despite lower levels of levels bioluminescence per cell of *tatA*. Hence, the *tatA*-*lux* would not be suggested as the best candidate for PET assays due to the lower promoter strength *tatA* exhibits in acidic conditions. Nevertheless, there could be a potential use for *tatA* in testing of alkaline preservatives, since it expressed greater bioluminescence under the significant increase of pH 8.9 from neutrality alkaline conditions of extended stationary phase than the other biosensors.

On the other hand, the bioluminescent biosensor incorporating the *spc* promoter exhibited significantly greater levels of bioluminescence per cell which was 10-1000 times greater than that exhibited by the other four biosensor strains under stress conditions. The plasmid copy number of the vector encoding *spc*-*lux* was similar to that for the other biosensor plasmids, therefore it is seems

likely that the strength of bioluminescence intensity was the result of the *spc* promoter. The *spc-lux* construct has great reliability and linearity with viable counts over a very wide range of with and without preservative challenge, a sensitive range in detection whereby is able to response towards over a wide range of preservatives concentrations, robust in both acidic and alkaline conditions, and in addition was not found have any repressor sequences. Therefore, from the strains tested in this study, the *spc-lux* biosensor strain is the best candidate for further development as a bioluminescent biosensor to use in PET assays.

Although the bioluminescence per cell for the other strains were significantly lower to *spc-lux* across the concentrations for use in PET assays, the other biosensor strains could prove useful as screening tools in some circumstances, since the strains are theoretically capable of reflecting growth as observed in the minimum inhibitory studies (MIC) after 24 hour incubation. However, the analysis duration of the *lpp* construct should be within the 48^{th} hour of stationary phase, as there was no significant difference between the bioluminescence expressions (Chapter 3).

In addition, the comparison of the nucleotide sequences to the consensus sequences within promoter region demonstrated that the activity of the promoter is not reliant on any single particular conserved region, but the sum of the interactions between the RNA polymerase with all the conserved regions. In this study, the presence of a sequence similar to the consensus sequence in the extended -10 region, -10 region of RpoD ($\sigma^{70/D}$), along with A+T% about 40% in the UP regions and close to consensus spacer length between -35 and -10 regions, in *spc* could together have resulted in relatively high expression levels from this promoter over a 28 days of incubation, both under starvation and preservative stress. Promoters that have the consensus of the extended -10 regions have demonstrated strong expression (Campbell et al., 2002), following the mutagenesis studies by Inouye and Inouye (1985) demonstrating increased expressions of promoters with the consensus of -

10 region and Harley and Reynolds, (1987) demonstrated that inter region spacing of 16 and 18 bp should not affect the promoter activity compared to the consensus sequence which is 17 bp. These were promoter regions found to be important factors for greater bioluminescence expressions under stress conditions is potentially linked to the binging affinity to RpoD ($\sigma^{70/D}$) and RpoS ($\sigma^{38/S}$). In contrast, the small number of spacers, only 13 nucleotides between the -35 and -10 regions could have resulted in the low levels of bioluminescence expression by *lysS-lux*. It is important to appreciate the relationship of the conserved promoter regions with the level of gene expression in of the selection of a suitable promoter for an efficient whole-cell biosensor, where high levels of expression are required.

Nevertheless, a fully consensus promoter is undesirable in several ways (Hook-Barnard & Hinton, 2007). The numerous contacts actually reduce transcriptional activity for a promoter, because they prevent the transition from the open complex to promoter clearance and elongation (Grana et al., 1988).

However, some potential disadvantages have been suggested for whole-cell biosensors by Turdean (2011). Turdean (2011) stated that the disadvantages include a lack of genetic stability, requirement for long incubation times (usually more than 30 minutes), and sensitivity in bioluminescence intensity production in changing experimental conditions. Nevertheless, the results of the study described in this thesis show that genetic instability is unlikely to be a problem in the types of PET assays performed here since there is no significant loss of plasmids from the host cells throughout long-term stationary phase under alkaline conditions at the maximum of pH 8.9. Quantitative real time PCR (qPCR) was used to quantify plasmid copies present in the cells demonstrated that there was no significant plasmid loss in the long term stationary phase from 7 days to 28 days which proved that the bioluminescence expression expressed in the PET assays reflects the metabolic status

of the cell. There were no significant differences between adenosine groups and adenosine energy charge (AEC) levels between the whole-cell biosensors and control strains across 28 days demonstrating the genetically modification did not amend the metabolic activity of the biosensors. This signifies that the bioluminescence expressed in the PET assays were not significant to the metabolic activity, hence demonstrating a true reflection the effect of the preservatives. In addition, bioluminescence per cell (RLU:CFU) exhibited by the *tatA-lux, ldc-lux* and *spc-lux* were relatively high across a very wide range of concentrations during the PET time frame and extended growth phase over 28 days of incubation.

However, biosensors cells need to be grown to stationary phase, which takes at least 7 hours of incubation time. Never the less, this inconvenience can be avoided by lyophilized cells by freeze drying cells (Pellinen et al., 2004; Tauriainen et al., 1999). In addition, genetically engineered biosensor cells as genetically modified organisms (GMOs)' make them difficult to be commercialized due to official regulations in the European countries.

6.3 : Future work

To circumvent the incubation time of biosensor cells, lyophilisation of cells could be an alternative way to minimize incubation time, the utilization of nutrient broth, incubation space and possibly glassware. For the consideration of long shelf life of biosensors, a buffer could be added to lyophilized cells in multiwall plates to 'activate' the cells in specifically designed experiments for PET. Although lyophilisation and 'activation' of luminescent cells could not be tested in the current work due to time constraints, this could potentially be a fruitful area for further research. The whole-cell bioluminescence method offers a speed of response and adequately monitored of bioluminescence bacteria to the action of antimicrobial which reinforce the prospect of real-time microbial assays for antimicrobials (Marincs, 2000; Stewart, 1989) and possibility as a commercial

kit. Furthermore, the application of the whole-cell bioluminescent method could be used as a challenged microorganism in actual pharmaceutical products to evaluate the effectiveness of the product. As majority of pharmaceutical product contains active ingredients could also be investigated for defining the limits for testing; this also includes optically dense or colored products. The development of the whole-cell bioluminescence method aims for complete rapid automation in a multi well plate for high throughput samples. This is directed to large capacity testing in labs or large companies in-house labs. The main advantage of this bioluminescent method is that it is simple and user-friendly in production and interpretation of the results.

In addition, *lpp* was hypothesised to be downregulated with a few possible scenarios; the first is an alternative lipoprotein is expressed abundantly under stress and preservative exposure. The second is the presence of CRP binding sites within the -10 region of the promoter, and thirdly the speculation of another promoter which is transcribed in the opposite direction of transcription under stress response. In order to confirm all these theories this proposes reserve transcription- qPCR of targets on *lpp* and alternative lipoproteins, *slp*, *pal,* and *osmB* in quantifying expression levels in starvation and preservative stress. Secondly to target CRP binding sites by PCR under stress and preservative stress suggesting that potentially the regulation of CRP within the -10 promoter regions is be triggered by stress levels. Similarly, the promoter region of *lysS* was found to have two CRP binding sites. And lastly to identify the presence of the second *lpp* promoter.

6.4 : Conclusion

In conclusion, the use of whole-cell bioluminescence as an alternative method to replace traditional viable counting in preservative efficacy testing has shown great potential. Bioluminescent biosensors proved to be more rapid, more sensitive and easier to operate traditional viable counts which are laborious, and time consuming with the results being delayed by several days. Amongst the five biosensors tested in the current work *spc-lux* would be the best choice of biosensors for sorbic acid in the range of 0.2% to 0.031%, at pH 5.0; for benzalkonium chloride in the range of 0.0062% to 0.00039%, at pH 7.0; and also for BAK with the addition of 0.03% EDTA.

Therefore, this concludes that by selecting the appropraite constitutive promoter, this reflects the ability of the whole-cell bioluminescence method in monitoring viability and growth in PET and MIC assays.

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List of Appendices

Appendix Chapter 2

The Validation of the five E.coli bioluminescent reporter strains- as a Rapid Real Time Microbiological Testing Method

Table 2.0: Recoveries values of 5 *E.coli* biosensors at dilution factors; 75%, 50%, 25%, and 10%

Genetically Modified*E.coli* Strains	Dilution Factor (%)	Recovery (%) in High Bacterial Suspension		Recovery (%) in Low Bacterial Suspension	
		Bioluminescence Method	Conventional Method	Bioluminescence Method	Conventional Method
Lpp-lux	75	70.77 (±0.09)	113.23(±0.0004)	77.14(±0.19)	121.34(±0.08)
	50	70.02(±0.006)	109.03 (±0.01)	88.45(±0.008)	193.53(±0.10)
	25	116.78(±0.02)	79.11(±0.03)	70.19 (±0.04)	124.72(±0.18)
	10	91.14(±0.008)	97.03(±0.04)	99.27(±0.02)	108.78(±0.16)
TatA-lux	75	116.61 (±0.006)	99.08(±0.04)	112.75(±0.003)	102.31(±0.04)
	50	95.78(±0.03)	100.34(±0.05)	155.94(±0.061)	190.77(±0.07)
	25	167.30(±0.009)	101.99(±0.02)	73.93(±0.035)	152.05(±0.04)
	10	113.83(±0.05)	93.84(±0.07)	108.11(±0.01)	111.56(±0.14)
Ldc-lux	75	70.26(±0.009)	86.21(±0.02)	70.61(±0.036)	98.82(±0.05)
	50	70.17(±0.013)	92.58(±0.08)	70.89(±0.06)	169.18(±0.05)
	25	99.52(±0.05)	82.91(±0.10)	104.99(±0.06)	137.83(±0.08)
	10	106.89(±0.02)	88.90(±0.06)	71.03(±0.18)	109.42(±0.09)
LysS-lux	75	183.74(±0.03)	81.78(±0.02)	70.24(±0.05)	97.13(±0.09)
	50	85.91(±0.017)	85.61(±0.08)	78.13(±0.10)	136.11(±0.01)
	25	132.74(±0.03)	83.10(±0.09)	96.32(±0.05)	124.47(±0.04)
	10	138.37(±0.04)	77.91(±0.07)	70.14(±0.13)	99.85(±0.05)
Spc-lux	75	179.36((±0.02)	117.18(±0.02)	72.89(±0.06)	117.18(±0.02)
	50	199.75(±0.05)	112.61(±0.06)	72.49(±0.005)	177.63(±0.06)
	25	190.43(±0.02)	110.02(±0.05)	89.49(±0.12)	104.82(±0.01)
	10	161.10(±0.08)	11.02(±0.06)	91.94(±0.07)	102.60(±0.08)
Pbr322-lux	75	n/a	138.78(±0.07)	n/a	138.78(±0.02)
	50	n/a	142.48(±0.09)	n/a	113.13(±0.08)
	25	n/a	113.3(±0.03)	n/a	131.05(±0.05)
	10	n/a	138.21(±0.03)	n/a	136.51(±0.08)
WildtypeE.coli	75	n/a	102.02(±0.019)	n/a	104.49(±0.05)
	50	n/a	107.91(±0.05)	n/a	107.54(±0.06)
	25	n/a	94.43(±0.11)	n/a	94.19(±0.13)
	10	n/a	100.74(±0.07)	n/a	102.60(±0.07)

Recovery values of each dilution of Bioluminescence and Viable Counts from Section 2.5.1

Table 2.1 (a): Statistical analysis of bioluminescence and conventional plate count method for the constructs

Statistical Analysis between Recovery values from section 2.5.1 of bioluminescence and conventional plate counts

No significant difference found

Multiple Comparisons

Tukey HSD

Dependent Variable	(I) Biosensor	(J) Biosensor	Mean Difference (I-J)	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
RLU	lpp	tatA	-34.0550	.448	-108.6404	40.5304
		Ldc	2.0950	1.000	-72.4904	76.6804
		Lyss	-21.8600	.765	-96.4454	52.7254
		Spc	.6500	1.000	-73.9354	75.2354
	tatA	lpp	34.0550	.448	-40.5304	108.6404
		Ldc	36.1500	.401	-38.4354	110.7354
		Lyss	12.1950	.958	-62.3904	86.7804
		Spc	34.7050	.433	-39.8804	109.2904
	Ldc	lpp	-2.0950	1.000	-76.6804	72.4904
		tatA	-36.1500	.401	-110.7354	38.4354
		Lyss	-23.9550	.709	-98.5404	50.6304
		Spc	-1.4450	1.000	-76.0304	73.1404
	Lyss	lpp	21.8600	.765	-52.7254	96.4454
		tatA	-12.1950	.958	-86.7804	62.3904
		Ldc	23.9550	.709	-50.6304	98.5404
		Spc	22.5100	.748	-52.0754	97.0954
	Spc	lpp	-.6500	1.000	-75.2354	73.9354
		tatA	-34.7050	.433	-109.2904	39.8804
		Ldc	1.4450	1.000	-73.1404	76.0304
		Lyss	-22.5100	.748	-97.0954	52.0754
CFU	lpp	tatA	-.6000	1.000	-108.1271	106.9271
		Ldc	10.0750	.994	-97.4521	117.6021
		Lyss	20.0600	.935	-87.4671	127.5871
		Spc	11.6750	.990	-95.8521	119.2021
	tatA	lpp	.6000	1.000	-106.9271	108.1271
		Ldc	10.6750	.993	-96.8521	118.2021
		Lyss	20.6600	.929	-86.8671	128.1871
		Spc	12.2750	.988	-95.2521	119.8021
	Ldc	lpp	-10.0750	.994	-117.6021	97.4521

	tatA	-10.6750	.993	-118.2021	96.8521
	Lyss	9.9850	.995	-97.5421	117.5121
	Spc	1.6000	1.000	-105.9271	109.1271
	lpp	-20.0600	.935	-127.5871	87.4671
	tatA	-20.6600	.929	-128.1871	86.8671
Lyss	Ldc	-9.9850	.995	-117.5121	97.5421
	Spc	-8.3850	.997	-115.9121	99.1421
	lpp	-11.6750	.990	-119.2021	95.8521
	tatA	-12.2750	.988	-119.8021	95.2521
Spc	Ldc	-1.6000	1.000	-109.1271	105.9271
	Lyss	8.3850	.997	-99.1421	115.9121

Table 2.2: F value obtained between Bioluminescence and Viable counts in section 2.5.2

a) Lpp-lux biosensor

Levene's Test of Equality of Error Variances[a]

Dependent Variable: RLU

F	df1	df2	Sig.
1.717	4	18	.018

b) *tatA-lux* biosensor

Levene's Test of Equality of Error Variances[a]

Dependent Variable: RLU

F	df1	df2	Sig.
1.715	4	18	0.15

c) *ldc-lux* biosensor

Levene's Test of Equality of Error Variances[a]

Dependent Variable: RLU

F	df1	df2	Sig.
1.849	4	18	0.12

d) *lysS-lux* biosensor

Levene's Test of Equality of Error Variances[a]

Dependent Variable: RLU

F	df1	df2	Sig.
2.656	4	18	.009

e) *spc-lux* biosensor

Levene's Test of Equality of Error Variances[a]

Dependent Variable: RLU

F	df1	df2	Sig.
1.884	4	18	.10

Table 2.3: Pearson Correlation Coefficients between RLU, CFU, TVC, ATP and RFU readings

a) Outer membrane lipoprotein (LPP)

		RLU	CFU	ATP	RFU	Epi
RLU	Pearson Correlation	1	.976**	.993**	.978**	.976**
	Sig. (2-tailed)		.000	.000	.000	.000
	N	8	8	8	8	8
CFU	Pearson Correlation	.976**	1	.965**	.936**	1.000**
	Sig. (2-tailed)	.000		.000	.001	.000
	N	8	8	8	8	8
ATP	Pearson Correlation	.993**	.965**	1	.995**	.965**
	Sig. (2-tailed)	.000	.000		.000	.000
	N	8	8	8	8	8
RFU	Pearson Correlation	.978**	.936**	.995**	1	.936**
	Sig. (2-tailed)	.000	.001	.000		.001
	N	8	8	8	8	8
Epi	Pearson Correlation	.976**	1.000**	.965**	.936**	1
	Sig. (2-tailed)	.000	.000	.000	.001	
	N	8	8	8	8	8

**. Correlation is significant at the 0.01 level (2-tailed).

b) Twin Arginine Translocase (TATA)

Correlations

		RLU	CFU	ATP	RFU	Epi
RLU	Pearson Correlation	1	.988**	.976**	.948**	.988**
	Sig. (2-tailed)		.000	.000	.000	.000
	N	8	8	8	8	8
CFU	Pearson Correlation	.988**	1	.954**	.914**	1.000**
	Sig. (2-tailed)	.000		.000	.001	.000
	N	8	8	8	8	8
ATP	Pearson Correlation	.976**	.954**	1	.993**	.954**
	Sig. (2-tailed)	.000	.000		.000	.000
	N	8	8	8	8	8

	Pearson Correlation	.948**	.914**	.993**	1	.914**
RFU	Sig. (2-tailed)	.000	.001	.000		.001
	N	8	8	8	8	8
	Pearson Correlation	.988**	1.000**	.954**	.914**	1
Epi	Sig. (2-tailed)	.000	.000	.000	.001	
	N	8	8	8	8	8

**. Correlation is significant at the 0.01 level (2-tailed).

c) Lysine decarboxylase (LDC)

Correlations

		RLU	CFU	ATP	RFU	Epi
	Pearson Correlation	1	.968**	.957**	.900**	.968**
RLU	Sig. (2-tailed)		.000	.001	.006	.000
	N	7	7	7	7	7
	Pearson Correlation	.968**	1	.917**	.856*	1.000**
CFU	Sig. (2-tailed)	.000		.004	.014	.000
	N	7	7	7	7	7
	Pearson Correlation	.957**	.917**	1	.988**	.917**
ATP	Sig. (2-tailed)	.001	.004		.000	.004
	N	7	7	7	7	7
	Pearson Correlation	.900**	.856*	.988**	1	.856*
RFU	Sig. (2-tailed)	.006	.014	.000		.014
	N	7	7	7	7	7
	Pearson Correlation	.968**	1.000**	.917**	.856*	1
Epi	Sig. (2-tailed)	.000	.000	.004	.014	
	N	7	7	7	7	7

**. Correlation is significant at the 0.01 level (2-tailed).

d) Lysyl t-RNA Synthetase (lYSS)

Correlations

		RLU	CFU	ATP	RFU	Epi
	Pearson Correlation	1	.963**	.955	.826*	.963**
RLU	Sig. (2-tailed)		.000	.029	.022	.000
	N	7	7	7	7	7
	Pearson Correlation	.963**	1	.9239	.766*	1.000**
CFU	Sig. (2-tailed)	.000		.038	.045	.000
	N	7	7	7	7	7
ATP	Pearson Correlation	.955	.939	1	.884**	.92
	Sig. (2-tailed)	.029	.038		.008	.0384
	N	7	7	7	7	7

	Pearson Correlation	.826*	.766*	.884**	1	.766*
RFU	Sig. (2-tailed)	.022	.045	.008		.045
	N	7	7	7	7	7
	Pearson Correlation	.963**	1.000**	.92	.766*	1
Epi	Sig. (2-tailed)	.000	.000	.0038	.045	
	N	7	7	7	7	7

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

e) Ribosomal Protein (SPC)

Correlations

		RLU	CFU	ATP	RFU	Epi
	Pearson Correlation	1	.971**	.907**	.884**	.971**
RLU	Sig. (2-tailed)		.000	.005	.008	.000
	N	7	7	7	7	7
	Pearson Correlation	.971**	1	.881**	.851*	1.000**
CFU	Sig. (2-tailed)	.000		.009	.015	.000
	N	7	7	7	7	7
	Pearson Correlation	.907**	.881**	1	.998**	.881**
ATP	Sig. (2-tailed)	.005	.009		.000	.009
	N	7	7	7	7	7
	Pearson Correlation	.884**	.851*	.998**	1	.851*
RFU	Sig. (2-tailed)	.008	.015	.000		.015
	N	7	7	7	7	7
	Pearson Correlation	.971**	1.000**	.881**	.851*	1
Epi	Sig. (2-tailed)	.000	.000	.009	.015	
	N	7	7	7	7	7

f) E.coli [pBR322.lux]

Correlations

		RLU	CFU	ATP	RFU	Epi
	Pearson Correlation	.[a]	.[a]	.[a]	.[a]	.[a]
RLU	Sig. (2-tailed)		.	.	.	.
	N	7	7	7	7	7
	Pearson Correlation	.[a]	1	.848*	.864*	1.000**
CFU	Sig. (2-tailed)	.		.016	.012	.000
	N	7	7	7	7	7
ATP	Pearson Correlation	.[a]	.848*	1	.997**	.848*
	Sig. (2-tailed)	.	.016		.000	.016
	N	7	7	7	7	7

	Pearson Correlation	.[a]	.864[*]	.997[**]	1	.864[*]
RFU	Sig. (2-tailed)	.	.012	.000		.012
	N	7	7	7	7	7
	Pearson Correlation	.[a]	1.000[**]	.848[*]	.864[*]	1
Epi	Sig. (2-tailed)	.	.000	.016	.012	
	N	7	7	7	7	7

*. Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).

a. Cannot be computed because at least one of the variables is constant.

g) Wildtype E.coli

Correlations

		RLU	CFU	ATP	RFU	Epi
	Pearson Correlation	.[a]	.[a]	.[a]	.[a]	.[a]
RLU	Sig. (2-tailed)		.	.	.	.
	N	7	7	7	7	7
	Pearson Correlation	.[a]	1	.848[*]	.864[*]	1.000[**]
CFU	Sig. (2-tailed)	.		.016	.012	.000
	N	7	7	7	7	7
	Pearson Correlation	.[a]	.848[*]	1	.997[**]	.848[*]
ATP	Sig. (2-tailed)	.	.016		.000	.016
	N	7	7	7	7	7
	Pearson Correlation	.[a]	.864[*]	.997[**]	1	.864[*]
RFU	Sig. (2-tailed)	.	.012	.000		.012
	N	7	7	7	7	7
	Pearson Correlation	.[a]	1.000[**]	.848[*]	.864[*]	1
Epi	Sig. (2-tailed)	.	.000	.016	.012	
	N	7	7	7	7	7

*. Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).

Table 2.4: Tukey Statistical Analysis between Bioluminescence per cell to Biosensors at 24 hours

Multiple Comparisons

Dependent Variable: Ratio

	(I) Biosensor	(J) Biosensor	Mean Difference (I-J)	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Tukey	lpp	tatA	6.6000*	.000	6.3040	6.8960
		Ldc	7.0133*	.000	6.7173	7.3093
		Lyss	7.1000*	.000	6.8040	7.3960
		Spc	-1.6000*	.000	-1.8960	-1.3040
	tatA	lpp	-6.6000*	.000	-6.8960	-6.3040
		Ldc	.4133*	.007	.1173	.7093
		Lyss	.5000*	.002	.2040	.7960
		Spc	-8.2000*	.000	-8.4960	-7.9040
	Ldc	lpp	-7.0133*	.000	-7.3093	-6.7173
		tatA	-.4133*	.007	-.7093	-.1173
		Lyss	.0867	.865	-.2093	.3827
		Spc	-8.6133*	.000	-8.9093	-8.3173
	Lyss	lpp	-7.1000*	.000	-7.3960	-6.8040
		tatA	-.5000*	.002	-.7960	-.2040
		Ldc	-.0867	.865	-.3827	.2093
		Spc	-8.7000*	.000	-8.9960	-8.4040
	Spc	lpp	1.6000*	.000	1.3040	1.8960
		tatA	8.2000*	.000	7.9040	8.4960
		Ldc	8.6133*	.000	8.3173	8.9093
		Lyss	8.7000*	.000	8.4040	8.9960
	lpp	tatA	6.6000*	.000	6.3996	6.8004
		Ldc	7.0133*	.000	6.8129	7.2137
		Lyss	7.1000*	.000	6.8996	7.3004
		Spc	-1.6000*	.000	-1.8004	-1.3996
		lpp	-6.6000*	.000	-6.8004	-6.3996

Table 2.5: Costing of the individual methods that was tested

Fluorescence spectrometry and Epifluorescence method	ATP-bioluminescence method	Plate count method	Whole-cell bioluminescence
Reagents/kit; Cost (£)			
LIVE/DEAD Baclight kit; £232 Tryptone soy broth; £42.2 Buffered peptone Water: £28.5	ATP Bioluminescence assay kit HS II; £259.40 Tryptone soy broth; £ 42.2 Buffered peptone Water: £28.5	Tryptone soy broth: £42.2 Buffered peptone water; £28.5 Tryptone soy agar; £51.20	Tryptone soy broth: £42.2 Buffered peptone water; £28.5
Total: £302.7	Total: £330.1	Total: £121.9	Total: £192.6
Approximately 300 assays	Approximately 500 assays	Approximately 1000 assays	Approximately 1000 assays

7 Sequencing output

Sequence 1.0: Ribosomal Protein (SPC)

ctgcgtatgcaggctgcaagtggccagctgcaacagtctcacctgttgaagcaagtgcgtcgcgatg
tcgcacgcgttaagactttactgaacgagaaggcgggtgcgtaatgaccgataaaatccgtactctg
caaggtcgcgttgttagcgacaaaatggagaaatccattgttgttgctatcgaacgttttgtgaaac
acccgatctacggtaaattcatcaagcgtacgaccaaactgcacgtacatgacgagaacaacgaatg
cggtatcggtgacgtggttgaaatccgcgaatgccgtccgctgtccaagactaaatcctggacgctg
gttcgcgttgtagagaaagcggttctgtaatacagtacactctctcaatacgaataaacggctcaga
aatgagccgtttatttTTTCTAcccatatccttgaagcggTGTTATAATgccgcgccctcgatatgg
ggatttttaacgacctgattttcgggtctcagtagtagttgacattagcggagcactaaaatgatcc
aagaacagactatgctgaacgtcgccgacaactccggtgcacgtcgcgtaatgtgtatcaaggttct
gggtggctcgcaccgtcgctacgcaggcgtaggcgacatcatcaagatcaccatcaaagaagcaatt
ccgcgtggtaaggtcaaaaaaggtgatgtgctgaaggcggtagtggtgcgcaccaagaagggtgttc
gtcgcccggacggttctgtcattcgcttcgatggtaatgcttgtgttcttctgaacaacaacagcga
gcagcctatcggtacgcgtatttttgggccggtaactcgtgagcttcgtagtgagaagttcatgaaa
attatctctctggcaccagaagtactctaaggagcgaatcatggcagcgaaaatccgtcgtgatgac
gaagttatcgtgttaaccggtaaagataaaggtaaacgcggtaaagttaagaatgtcctgtcttccg
gcaaggtcattgttgaaggtatcaacctggttaagaaacatcagaagccggttccggccctgaacca
accgggtggcatcgttgaaaaagaagccgctattcaggtttccaacgtagcaatcttcaatgcggca
accggcaaggctgaccgtgtaggctttagattcgaagacggtaaaaaagtccgtttcttcaagtcta
acagcgaaactatcaagtaatttggagtagtacgatggcgaaactgcatgattactacaaagacgaa
gtagttaaaaaactcatgactgagtttaactacaattctgtcatgcaagtccctcgggtcgagaaga
tcaccctgaacatgggtgttggtgaagcgatcgctgacaaaaaactgctggataacgcagcagcaga
cctggcagcaatctccggtcaaaaaccgctgatcaccaaagcacgcaaatctgttgcaggcttcaaa
atccgtcagggctatccgatcggctg

REDrpsQ
YELLOWrplN
GreenrplX
Blue -35
Grey -10
The orange nucleotides represent the -40 to -60 regions
The green nucleotides represent the inter region of -10 to -35
The purple nucleotides represent the -10 to +1 region

Sequence 2.0: Lysyl-tRNA synthetase (LYSS)

cggcgcgggcggtcagcacgttaaccgtaccgaatctgcggtgcgtattacccacatcccgaccggg
atcgtgacccagtgccagaacgaccgttcccagcacaagaacaaagatcaggccatgaagcagatga
aagcgaagcTTTATGaactggagatgcaGAAGAAAAAtgccgagaaacaggcgatggaagataacaa
atccgacatcggctggggcagccagattcgttcttatgtccttgatgactcccgcattaaagatctg
cgcaccggggtagaaacccgcaacacgcaggccgtgctggacggcagcctggatcaatttatcgaag
caagtttgaaagcagggttatgaggaaccaacatgtctgaacaacacgcacagggcgctgacgcggt
agtcgatcttaacaatgaactgaaaacgcgtcgtgagaagctggcgaacctgcgcgagcaggggatt
gccttcccgaacgatttccgtcgcgatcatacctctgaccaattgcacgcagaattcgacggcaaag
agaacgaagaactggaagcgctgaacatcgaagtcgccgttgctggccgcatgatgacccgtcgtat
tatgggtaaagcgtctttcgttaccctgcaggacgttggcggtcgcattcagctgtacgttgcccgt
gacgatctcccggaaggcgtttataacgagcagttcaaaaaatgggacctcggcgacatcctcggcg
cgaaaggtaagctgttcaaaaccaaaaccggcg
GreenprfB peptide chain release factor
YellowLysSlysyl-tRNAsynthetase
Blue -35 box
Grey -10 box
The orange nucleotides represent the -40 to -60 regions
The green nucleotides represent the inter region of -10 to -35
The purple nucleotides represent the -10 to +1 regions

Sequence 3.0: Twin Arginine translocase (TATA)

cattcttgttggtcagccgacctgaatgggggctgatgcccggctggttaatggcaggtggtctgat
cgcctggtttgtcggttgcgcaaaacacgctgattttTTCATCgctcaaggcgggccgtgtaACGT
ATAATgcggctttgtttaatcatcatctaccacagaggaacatgtatgggtggtatcagtatttggc
agttattgattattgccgtcatcgttgtactgctttttggcaccaaaaagctcggctccatcggttc
cgatcttggtgcgtcgatcaaaggctttaaaaaagcaatgagcgatgatgaaccaaagcaggataaa
accagtcaggatgctgattttactgcgaaaactatcgccgataagcaggcggatacgaatcaggaac
aggctaaaacagaagacgcgaagcgccacgataaagagcaggtgtaatcc
YELLOW - tatA
Grey -10
Blue -35

The orange nucleotides represent the -40 to -60 regions
The green nucleotides represent the inter region of -10 to -35
The purple nucleotides represent the -10 to +1 regions

Sequence 4.0: Outer membrane Lipoprotein (LPP)

tattgcttttgtgaattaatttgtatatcgaagcgccctgatgggcgctttttttaTTTAATcgata
accagaagcaATAAAAAATcaaatcggatttcactatataatctcactttatctaagatgaatccga
tggaagcatcctgttttctctcaatttttttatctaaaacccagcgttcgatgcttctttgagcgaa
cgatcaaaaataagtgccttcccatcaaaaaaaataTTCTCAacataaaaaactttgtgtAATACTtg
taacgctacatggagattaactcaatctagagggtattaataatgaaagctactaaactggtactgg
gcgcggtaatcctgggttctactctgctggcaggttgctccagcaacgctaaaatcgatcagctgtc
ttctgacgttcagactctgaacgctaaagttgaccagctgagcaacgacgtgaacgcaatgcgttcc
gacgttcaggctgctaaagatgacgcagctcgtgctaaccagcgtctggacaacatggctactaaat
accgcaagtaatagtacctg
The orange nucleotides represent the -40 to -60 regions
The green nucleotides represent the inter region of -10 to -35
The purple nucleotides represent the -10 to +1 regions
GreenpykF
YellowlppPurple -35 (lpp)
Dark green -10 (lpp)

Sequence 5.0: Lysine Decarboxylase (LDC)

cgtgttaagcactgaagatttaaaaaatcgtcgttatcagcgcctgatgagctacggttacgcgtaa
ttcgcaaaagttctgaaaaagggtcacttcggtggccctTTTTTAtcgccacggtttgagcaGGCTA
TGATtaaggaaggattttccaggaggaacacatgaacatcattgccattatgggaccgcatggcgtc
ttttataaagatgagcccatcaaagaactggagtcggcgctggtggcgcaaggctttcagattatct
ggccacaaaacagcgttgatttgctgaaatttatcgagcataaccctcgaatttgcggcgtgatttt
tgac
Red - accA gene
Yellow - ldcC gene
Blue -35
Grey -10
The orange nucleotides represent the -40 to -60 regions
The green nucleotides represent the inter region of -10 to -35

The purple nucleotides represent the -10 to +1 regions

Appendix Chapter 3

The Comparisons of Bioluminescence, Growth, Adenylate Energy Charge (AEC), Plasmid copy number over extended period of time

Table 3.1: Growth curve statistical analysis from Section 3.7.1

No significance difference between biosensors across 48 hours for the 5 biosensor

RLU

	Biosensor	N	Subset	
			1	2
Tukey HSD[a,b]	ldc	50	7.1891	
	lyss	50	7.2747	
	tat	50	7.3217	
	lpp	50		7.6922
	spc	50		7.7153
	Sig.		.149	.993

Table 3.20: Tukey Analysis of bioluminescence readings of the five constructs across 48 hours

No significant differences between bioluminescence of the five strains

Multiple Comparisons

Dependent Variable: RLU

Tukey HSD

(I) Biosensor	(J) Biosensor	Mean Difference (I-J)	Sig.	95% Confidence Interval	
				Lower Bound	Upper Bound
lpp	tat	.37044	.374	-.1941	.9350
	ldc	.50306	.106	-.0615	1.0676
	lyss	.41745	.254	-.1471	.9820
	spc	.00429	1.000	-.5575	.5661
tat	lpp	-.37044	.374	-.9350	.1941
	ldc	.13263	.967	-.4319	.6972
	lyss	.04702	.999	-.5175	.6115
	spc	-.36614	.381	-.9279	.1956
ldc	lpp	-.50306	.106	-1.0676	.0615
	tat	-.13263	.967	-.6972	.4319
	lyss	-.08561	.994	-.6501	.4789
	spc	-.49877	.108	-1.0605	.0630
lyss	lpp	-.41745	.254	-.9820	.1471
	tat	-.04702	.999	-.6115	.5175
	ldc	.08561	.994	-.4789	.6501
	spc	-.41316	.259	-.9749	.1486
spc	lpp	-.00429	1.000	-.5661	.5575
	tat	.36614	.381	-.1956	.9279
	ldc	.49877	.108	-.0630	1.0605

lyss	.41316	.259	-.1486	.9749

Table 3.3: Standard Mean error RLU growth curve across 48 hours

Grand Mean

Dependent Variable: RLU

Mean	Std. Error	95% Confidence Interval	
		Lower Bound	Upper Bound
7.439	.065	7.311	7.566

Table 3.4: Standard Error CFU growth curve across 48 hours

Grand Mean

Dependent Variable: CFU

Mean	Std. Error	95% Confidence Interval	
		Lower Bound	Upper Bound
9.342	.083	9.179	9.504

Table 3.5: F Values in comparison for RLU to CFU values across 48 hours for the five biosensors

Levene's Test of Equality of Error Variances[a]

Dependent Variable: RLU

F	df1	df2	Sig.
3.888	228	21	.000

Table 3.6: Standard error RLU in extended growth phase across 28 days for the five biosensors

Grand Mean

Dependent Variable: RLU

Mean	Std. Error	95% Confidence Interval	
		Lower Bound	Upper Bound
4.903[a]	.005	4.893	4.914

Table 3.7: Standard error for CFU in extended growth phase across 28 days for the five biosensors

Grand Mean

Dependent Variable: CFU

Mean	Std. Error	95% Confidence Interval	
		Lower Bound	Upper Bound
7.726[a]	.014	7.695	7.757

Table 3.8 (a & b): Multiple comparisons of RLU, CFU. RLU: CFU, ATP, Time in extended growth phase across 28 days for the five biosensors

Multiple Comparisons

Tukey HSD

Dependent Variable	(I) biosensor	(J) biosensor	Mean Difference (I-J)	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
RLU	lpp	tatA	-1.3061*	.000	-1.5513	-1.0609
		Ldc	-1.3261*	.000	-1.5713	-1.0809
		Lyss	-1.4989*	.000	-1.7441	-1.2537
		spc	-1.4683*	.000	-1.7136	-1.2231
	tatA	lpp	1.3061*	.000	1.0609	1.5513
		Ldc	-.0200	.999	-.2652	.2252
		Lyss	-.1928	.190	-.4380	.0524
		spc	-.1622	.350	-.4074	.0830
	Ldc	lpp	1.3261*	.000	1.0809	1.5713
		tatA	.0200	.999	-.2252	.2652
		Lyss	-.1728	.287	-.4180	.0724
		spc	-.1422	.484	-.3874	.1030
	Lyss	lpp	1.4989*	.000	1.2537	1.7441
		tatA	.1928	.190	-.0524	.4380
		Ldc	.1728	.287	-.0724	.4180
		spc	.0306	.997	-.2147	.2758
	spc	lpp	1.4683*	.000	1.2231	1.7136
		tatA	.1622	.350	-.0830	.4074
		Ldc	.1422	.484	-.1030	.3874
		Lyss	-.0306	.997	-.2758	.2147
RLUCFU	lpp	tatA	-.0219*	.000	-.0241	-.0198
		ldc	-.0141*	.000	-.0163	-.0120
		lyss	-.0052*	.000	-.0074	-.0031
		spc	-.0061*	.000	-.0083	-.0040
	tatA	lpp	.0219*	.000	.0198	.0241
		ldc	.0078*	.999	.0056	.0099

		lyss	.0167*	.190	.0146	.0188
		spc	.0158*	.350	.0137	.0179
		lpp	.0141*	.000	.0120	.0163
	ldc	tatA	-.0078*	.999	-.0099	-.0056
		lyss	.0089*	.287	.0068	.0111
		spc	.0080*	.484	.0059	.0102
		lpp	.0052*	.000	.0031	.0074
	lyss	tatA	-.0167*	.190	-.0188	-.0146
		ldc	-.0089*	.287	-.0111	-.0068
		spc	-.0009	.997	-.0030	.0012
		lpp	.0061*	.000	.0040	.0083
	spc	tatA	-.0158*	.350	-.0179	-.0137
		ldc	-.0080*	.484	-.0102	-.0059
		lyss	.0009	.997	-.0012	.0030
		tatA	.0000	1.000	-.0234	.0234
	lpp	ldc	.0000	1.000	-.0234	.0234
		lyss	.0000	1.000	-.0234	.0234
		spc	.0000	1.000	-.0234	.0234
		lpp	.0000	1.000	-.0234	.0234
	tatA	ldc	.0000	1.000	-.0234	.0234
		lyss	.0000	1.000	-.0234	.0234
		spc	.0000	1.000	-.0234	.0234
		lpp	.0000	1.000	-.0234	.0234
	ldc	tatA	.0000	1.000	-.0234	.0234
CFU		lyss	.0000	1.000	-.0234	.0234
		spc	.0000	1.000	-.0234	.0234
		lpp	.0000	1.000	-.0234	.0234
	lyss	tatA	.0000	1.000	-.0234	.0234
		ldc	.0000	1.000	-.0234	.0234
		spc	.0000	1.000	-.0234	.0234
		lpp	.0000	1.000	-.0234	.0234
	spc	tatA	.0000	1.000	-.0234	.0234
		ldc	.0000	1.000	-.0234	.0234
		lyss	.0000	1.000	-.0234	.0234

Multiple Comparisons

Tukey HSD

Dependent Variable	(I) tIME	(J) tIME	Mean Difference (I-J)	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
RLU		48 H	1.1660*	.000	1.1324	1.1996
	24 H	168 H	2.2687*	.000	2.2351	2.3022
		336 H	3.0967*	.000	3.0631	3.1302

		504 H	2.6307*	.000	2.5971	2.6642
		672 H	3.7973*	.000	3.7638	3.8309
		24 H	-1.1660*	.000	-1.1996	-1.1324
		168 H	1.1027*	.000	1.0691	1.1362
	48 H	336 H	1.9307*	.000	1.8971	1.9642
		504 H	1.4647*	.000	1.4311	1.4982
		672 H	2.6313*	.000	2.5978	2.6649
		24 H	-2.2687*	.000	-2.3022	-2.2351
		48 H	-1.1027*	.000	-1.1362	-1.0691
	168 H	336 H	.8280*	.000	.7944	.8616
		504 H	.3620*	.000	.3284	.3956
		672 H	1.5287*	.000	1.4951	1.5622
		24 H	-3.0967*	.000	-3.1302	-3.0631
		48 H	-1.9307*	.000	-1.9642	-1.8971
	336 H	168 H	-.8280*	.000	-.8616	-.7944
		504 H	-.4660*	.000	-.4996	-.4324
		672 H	.7007*	.000	.6671	.7342
		24 H	-2.6307*	.000	-2.6642	-2.5971
		48 H	-1.4647*	.000	-1.4982	-1.4311
	504 H	168 H	-.3620*	.000	-.3956	-.3284
		336 H	.4660*	.000	.4324	.4996
		672 H	1.1667*	.000	1.1331	1.2002
		24 H	-3.7973*	.000	-3.8309	-3.7638
		48 H	-2.6313*	.000	-2.6649	-2.5978
	672 H	168 H	-1.5287*	.000	-1.5622	-1.4951
		336 H	-.7007*	.000	-.7342	-.6671
		504 H	-1.1667*	.000	-1.2002	-1.1331
RLUCFU		48 H	-.0167*	.000	-.0191	-.0142
		168 H	.0008	.921	-.0016	.0033
	24 H	336 H	.0028*	.016	.0004	.0053
		504 H	-.0244*	.000	-.0269	-.0219
		672 H	-.0066*	.000	-.0091	-.0042
		24 H	.0167*	.000	.0142	.0191
		168 H	.0175*	.000	.0150	.0199
	48 H	336 H	.0195*	.000	.0170	.0219
		504 H	-.0077*	.000	-.0102	-.0053
		672 H	.0100*	.000	.0076	.0125
		24 H	-.0008	.921	-.0033	.0016
		48 H	-.0175*	.000	-.0199	-.0150
	168 H	336 H	.0020	.175	-.0005	.0045
		504 H	-.0252*	.000	-.0277	-.0228
		672 H	-.0075*	.000	-.0099	-.0050

		24 H	-.0028*	.016	-.0053	-.0004
		48 H	-.0195*	.000	-.0219	-.0170
	336 H	168 H	-.0020	.175	-.0045	.0005
		504 H	-.0272*	.000	-.0297	-.0248
		672 H	-.0095*	.000	-.0119	-.0070
		24 H	.0244*	.000	.0219	.0269
		48 H	.0077*	.000	.0053	.0102
	504 H	168 H	.0252*	.000	.0228	.0277
		336 H	.0272*	.000	.0248	.0297
		672 H	.0178*	.000	.0153	.0202
		24 H	.0066*	.000	.0042	.0091
		48 H	-.0100*	.000	-.0125	-.0076
	672 H	168 H	.0075*	.000	.0050	.0099
		336 H	.0095*	.000	.0070	.0119
		504 H	-.0178*	.000	-.0202	-.0153
		48 H	2.0267*	.000	1.9999	2.0535
		168 H	2.2367*	.000	2.2099	2.2635
	24 H	336 H	2.2400*	.000	2.2132	2.2668
		504 H	2.7233*	.000	2.6965	2.7501
		672 H	3.3633*	.000	3.3365	3.3901
		24 H	-2.0267*	.000	-2.0535	-1.9999
		168 H	.2100*	.000	.1832	.2368
	48 H	336 H	.2133*	.000	.1865	.2401
		504 H	.6967*	.000	.6699	.7235
		672 H	1.3367*	.000	1.3099	1.3635
		24 H	-2.2367*	.000	-2.2635	-2.2099
		48 H	-.2100*	.000	-.2368	-.1832
	168 H	336 H	.0033	.999	-.0235	.0301
CFU		504 H	.4867*	.000	.4599	.5135
		672 H	1.1267*	.000	1.0999	1.1535
		24 H	-2.2400*	.000	-2.2668	-2.2132
		48 H	-.2133*	.000	-.2401	-.1865
	336 H	168 H	-.0033	.999	-.0301	.0235
		504 H	.4833*	.000	.4565	.5101
		672 H	1.1233*	.000	1.0965	1.1501
		24 H	-2.7233*	.000	-2.7501	-2.6965
		48 H	-.6967*	.000	-.7235	-.6699
	504 H	168 H	-.4867*	.000	-.5135	-.4599
		336 H	-.4833*	.000	-.5101	-.4565
		672 H	.6400*	.000	.6132	.6668
	672 H	24 H	-3.3633*	.000	-3.3901	-3.3365
		48 H	-1.3367*	.000	-1.3635	-1.3099

	168 H	-1.1267*	.000	-1.1535	-1.0999
	336 H	-1.1233*	.000	-1.1501	-1.0965
	504 H	-.6400*	.000	-.6668	-.6132

Table 3.9: Multivariate Analysis of RLU, CFU, AEC, and PCN between the biosensors

No significant difference between the parameters

Tests of Between-Subjects Effects

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	RLU	5.427[a]	4	1.357	.410	.800
	CFU	.412[b]	4	.103	.048	.996
	PCN	22.686[c]	4	5.671	.016	.999
	AEC	.001[d]	4	.000	.008	1.000
Intercept	RLU	1094.520	1	1094.520	330.947	.000
	CFU	2144.089	1	2144.089	990.423	.000
	PCN	40528.029	1	40528.029	115.112	.000
	AEC	14.716	1	14.716	492.080	.000
Biosensor	RLU	5.427	4	1.357	.410	.800
	CFU	.412	4	.103	.048	.996
	PCN	22.686	4	5.671	.016	.999
	AEC	.001	4	.000	.008	1.000
	RLU	99.217	30	3.307		

Tables 3.20 (a) : Concentration of genomic DNA (gDNA) and plasmid DNA (pDNA) was extracted at the following time points; 0 hours, 4 hours, 6 hours, 24 hours, 168 hours, 336 hours, 504 hours, and 672 hours for all *E.coli* biosensor strains.
Genomic DNA extract of Tat-lux *Esherichia.coli 8739*

Samples (Hours)	OD 230	OD 260	OD 280	OD 320	DNA purity	DNA conc (µg/ml)	DNA Concentration (µg)
0	1.292	0.91	0.767	0.531	1.605932	37.9	3.79
4	1.104	0.851	0.702	0.472	1.647826	37.9	3.79
6	1.492	1.518	1.061	0.41	1.701997	110.8	11.08
24	2.352	2.943	1.942	0.54	1.71398	240.3	24.03
168	2.064	3	1.8	0.584	1.986842	241.6	24.16
336	1.1	0.882	0.624	0.32	1.848684	56.2	5.62
504	1.263	0.845	0.696	0.5	1.760204	34.5	3.45

672	1.645	0.94	0.78	0.6	1.888889	34	3.4

Table 3.2.1: Plasmid DNA extract of Tat-lux *Escherichia.coli 8739*

time	OD 230	OD 260	OD 280	OD 320	DNA purity	DNA conc (µg/µl)	DNA Concentration (µg)
0	1.106	1.106	0.468	0.403	1.59633	17.4	1.74
4	1.21	1.21	0.616	0.531	1.664063	21.3	2.13
6	1.572	1.572	1.229	0.904	1.730337	77	7.7
24	1.396	1.396	1.23	0.78	1.729335	106.7	10.67
168	1.62	1.62	0.845	0.66	1.711538	44.5	4.45
336	1.141	1.141	0.85	0.612	1.815068	53	5.3
504	1.23	1.23	0.743	0.555	1.737255	44.3	4.43
672	1.2	1.2	0.6	0.5	1.714286	24	2.4

DNA molecules absorb UV light strongly at 260nm whilst aromatic amino acid present in protein absorbs UV at 280nm. To evaluate DNA purity by spectrometry, measurement of absorbance from 230nm to 320nm in order to detect other possible contaminants present in DNA solution. Strong absorbance reading at 230nm indicates that organic compounds or chaotropic salts are present in the purified DNA. A reading of 320nm indicated turbidity in the solution, another indication of possible contamination. DNA purity (260nm/280nm) ratio between 1.7-2.0 is generally accepted as representative of a high quality DNA sample. The ratio is calculated upon the subtraction of readings at 320nm

Table 3.22: Genomic DNA extract of Ldc-lux *Escherichia.coli 8739*

Samples (Hours)	OD 230	OD 260	OD 280	OD 320	DNA purity	DNA conc (µg/ml)	DNA Concentration (µg
0	0.182	0.537	0.47	0.361	1.614679	17.6	1.76
4	0.005	0.547	0.419	0.21	1.61244	33.7	3.37
6	0.336	1.4979	0.902	0.166	1.809647	133.19	13.319
24	0.167	0.657	0.373	0.012	1.786704	64.5	6.45
168	0.174	0.877	0.554	0.124	1.751163	75.3	7.53

336	1.52	1.577	1.316	1.22	3.71875	35.7	3.57
504	2.5	2.1	1.855	1.32	1.457944	78	7.8
672	2	2.2	1.8	1.3	1.8	90	9

Table 3.23: Plasmid DNA extract of Ldc-lux *Escherichia.coli 8739*

Hours	OD 230	OD 260	OD 280	OD 320	DNA purity	DNA conc	DNA Concentration (µg
0	0.374	0.913	0.839	0.731	1.685185	9.1	0.91
4	0.291	0.85	0.692	0.478	1.738318	18.6	1.86
6	0.07	0.632	0.444	0.233	1.890995	19.95	1.995
24	0.748	1.55	1.103	0.566	1.832402	49.2	4.92
168	0.385	1.108	0.773	0.352	1.795724	37.8	3.78
336	0.058	0.671	0.475	0.238	1.827004	21.65	2.165
504	1.244	2.215	1.653	0.933	1.780556	64.1	6.41
672	1.544	1.397	1.2	0.879	1.613707	25.9	2.59

Table 3.24: Genomic DNA extract of Lyss-lux Escherichia.coli 8739

Hours	OD 230	OD 260	OD 280	OD 320	DNA purity	DNA conc (µg/µl)	DNA Concentration (µg
0	1.188	0.567	0.469	0.322	1.666667	24.5	2.45
4	1.393	0.758	0.637	0.443	1.623711	31.5	3.15
6	2.053	1.3	0.905	0.4	1.782178	90	9
24	2.1	1.776	1.193	0.5	1.84127	127.6	12.76
168	1.661	1.125	0.804	0.42	1.835938	70.5	7.05
336	2.304	1.145	0.969	0.75	1.803653	39.5	3.95
504	1.6	1.2	0.8	0.325	1.842105	87.5	8.75

672	1.6	1.311	0.8	0.32	1.904583	99.1	9.91

Table 3.25 Plasmid DNA extract of lyss-lux Escherichia.coli 8739

Hours	OD 230	OD 260	OD 280	OD 320	DNA purity	DNA conc (µg/µl)	DNA Concentration (µg
0	0.977	0.303	0.242	0.152	1.677778	15.1	1.51
4	1.466	0.744	0.63	0.457	1.65896	28.7	2.87
6	1.603	1.68	1.036	0.22	1.789216	146	14.6
24	2.739	2.965	1.953	0.403	1.652903	256.2	25.62
168	1.999	1.72	1.136	0.319	1.71481	140.1	14.01
336	2.281	2.249	1.465	0.335	1.693805	191.4	19.14
504	1.772	1.312	0.863	0.306	1.806104	100.6	10.06
672	1.319	1.2	0.519	0.353	5.10241	84.7	8.47

Table 3.26 Genomic DNA extract of Spc-lux Escherichia.coli 8739

Hours	OD 230	OD 260	OD 280	OD 320	DNA purity	DNA conc (µg/ml)	DNA Concentration (µg
0	1.14	0.488	0.422	0.3	1.540984	18.8	1.88
4	0.39	0.18	0.148	0.09	1.551724	9	0.9
6	2.591	1.938	1.419	0.7	1.721836	123.8	12.38
24	2.449	2.783	1.766	0.4	1.74451	238.3	23.83
168	1.92	1.864	1.265	0.5	1.783007	136.4	13.64
336	2.502	1.388	1.145	0.75	1.61519	63.8	6.38
504	2.037	1.3	0.99	0.6	1.794872	70	7
672	2.168	1.2	0.98	0.67	1.709677	53	5.3

Table 3.27 Plasmid DNA extract of Spc-lux Escherichia.coli 8739

Hours	OD 230	OD 260	OD 280	OD 320	DNA purity	DNA conc (µg/ml)	DNA Concentration (µg
0	1.496	0.754	0.649	0.491	1.664557	26.3	2.63
4	1.305	0.491	0.397	0.262	1.696296	22.9	2.29
6	2.206	2.448	1.556	0.45	1.80651	199.8	19.98
24	2.311	2.135	1.44	0.6	1.827381	153.5	15.35
168	1.562	2.08	1.273	0.3	1.829394	178	17.8
336	2.409	2.261	1.5	0.413	1.700092	184.8	18.48
504	1.892	1.061	0.789	0.308	1.565489	75.3	7.53
672	1.9	1	0.7	0.3	1.75	70	7

Table 3.28: genomic DNA of lpp-lux Escherichia coli ATCC 8739

Hours	OD 230	OD 260	OD 280	OD 320	DNA purity	DNA conc (µg/ml)	DNA Concentration (µg
0	0.671	0.264	0.213	0.141	1.708333333	12.3	1.23
4	1.322	0.839	0.625	0.333	1.732876712	50.6	5.06
6	1.643	1.661	1.059	0.306	1.799468792	135.5	13.55
24	2.187	2.434	1.634	0.586	1.763358779	184.8	18.48
168	2.127	2.335	1.464	0.277	1.733782645	205.8	20.58
336	1.736	1.757	1.142	0.346	1.772613065	141.1	14.11
504	2.22	1.65	1.145	0.49	1.770992366	116	11.6
672	0.55	1.223	0.871	0.335	1.656716418	88.8	8.88

Table 3.29: Plasmid DNA of lpp-lux Escherichia coli ATCC 8739

Hours	OD 230	OD 260	OD 280	OD 320	DNA purity	DNA conc (µg/ml)	DNA Concentration (µg
0	1.671	1.29	1.164	1.011	1.823529412	13.95	1.395
4	2.753	2.22	1.937	1.544	1.720101781	33.8	3.38
6	2.539	2.3	1.964	1.484	1.7	40.8	4.08
24	2	2.99	2.536	2.035	1.906187625	47.75	4.775
168	1.805	2.3	1.553	0.59	1.775700935	85.5	8.55
336	1.16	1.64	1.08	0.44	1.875	60	6
504	1.293	1.6	1.15	0.6	1.818181818	50	5
672	0.533	1.5	0.987	0.32	1.769115442	59	5.9

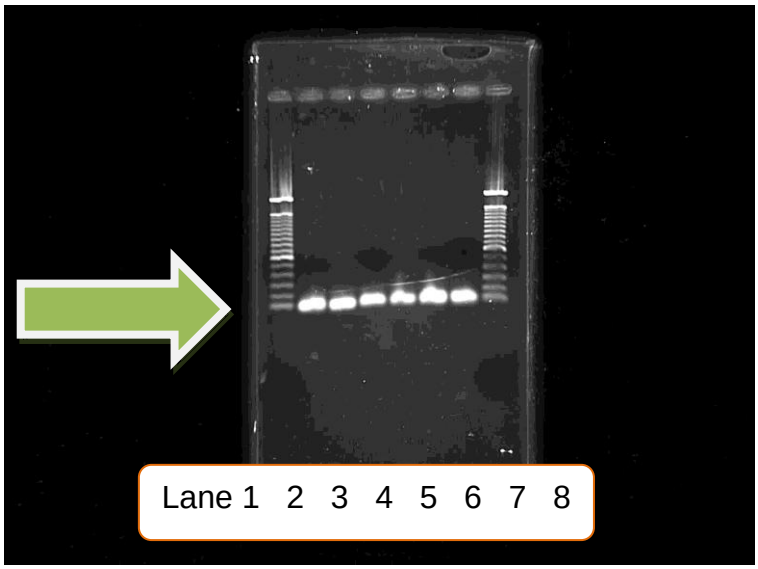


Figure3.1 (a): Deoxy-D-Xylulose Phosphate (DXS) pcr product (113bp,1.0% gel) observed with total DNA from respective 5 biosensors and control. (Lanes 1&8; 100bp ladder, 2: Lpp, 3:Tat, 4:ldc; 5:Lys, 6: Spc, 7:Pless

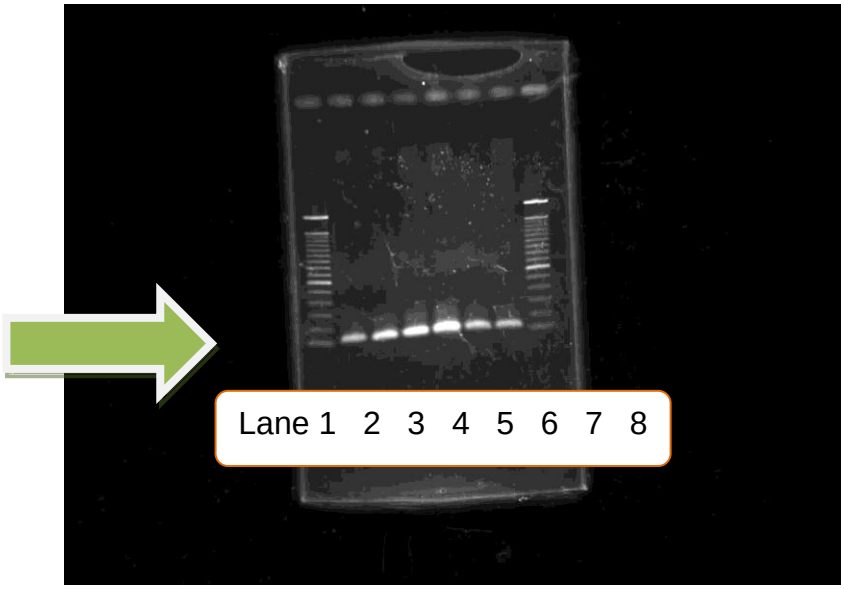


Figure 3.1 (b): Ampilicin (BLA) pcr product (81bp, 1.0% gel) observed with total DNA from respective 5 biosensors and control. (Lanes 1&8; 100bp ladder, 2: Lpp, 3:Tat, 4:ldc; 5:Lys, 6: Spc, 7:Pless) At annealing temperature 58 degrees

Calculations

Tables 3.3 (a, b, c, d, and e): The tabulation of the plasmid copy numbers based on the copies of *dxs* and *bla*

Table 3.5 (a) : Tabulation of ct values, coefficient of variance, copies of target, and plasmid coy numbers (PCN) for Lpp-lux

Hours	Target	Standard curve Equations	Correlation Coefficients Between copies and Ct values	Ct values (n=3)	Coefficient of variance (CV)	Copies	Plasmid copy number (per cell)
0	*Dxs*	y= -2.835x+ 42.649	0.88	22.67	0.029554	7.09E+06	35
0	*Bla*	y = -2.53x + 42.28	0.89	21.07	0.003322	2.52E+08	
4	*Dxs*	y= -2.51x+ 44.927	0.98	23.1	0.007498	4.18E+07	27
4	*Bla*	y=-2.711x+41.919	0.99	17.366	0.022989	1.14E+09	
6	*Dxs*	y=-2.863x+ 42.783	0.98	23.47	0.024505	5.57E+06	67
6	*Bla*	y= -2.812x+ 44.094	0.97	20	0.022703	3.70E+08	
24	*Dxs*	y= --3.82x+ 45.983	0.994	21.3	0.015471	2.03E+06	54
24	*Bla*	y= -3x+ 43.983	0.996	19.8	0.040404	1.09E+08	
168	*Dxs*	y = -3.121x + 38.31	0.92	22.3	0.013453	7.08E+07	26
168	*Bla*	y = -3.247x + 41.8	0.99	21	0.071429	1.89E+09	
336	*Dxs*	y = -3.158x + 40.30	0.90	26.5	0.018868	1.11E+06	14
336	*Bla*	y = -3.044x + 46.84	0.99	23.5	0.021277	1.53E+07	
504	*Dxs*	y= -3.4x+ 35.9	0.95	26.4	0.011858	1.82E+05	15
504	*Bla*	y = -3.256x + 41.38	0.99	28.65	0.011517	2.65E+06	
672	*Dxs*	y = -3.329x + 43.21	0.93	26	0.023002	1.59E+07	16
672	*Bla*	y = -3.4x + 45.364	0.95	28.45	0.015817	2.53E+08	

Table 3.31 (b): Tabulation of ct values, coefficient of variance, copies of target, and plasmid coy nu(PCN

Hours	Target	Standard curve Equations	Correlation Coefficients Between copies and Ct values	Ct values (n=3)	Coefficient of variance (CV)	DNA Copies	Plasmid copy number (per cell)
0	*Dxs*	y= -2.4332x + 43.46	0.95	22.87	0.003103	1.51E+10	38
0	*Bla*	y= -2.5813x + 43.26	0.87	17.85	0.00855	5.74E+11	
4	*Dxs*	y= -3.174x + 53.972	0.99	23.15	0.001247	7.45E+08	22
4	*Bla*	y=-2.857x + 50.47	0.95	19.5	0.00782	1.62E+10	
6	*Dxs*	y=-4x + 29.899	0.96	25.62	0.00137	1.18E+08	65
6	*Bla*	y= -2.5105x + 32.16	0.9	21.24	0.023232	7.77E+09	
24	*Dxs*	y= -3.17x + 42.882	0.994	22.67	0.000509	2.38E+06	50
24	*Bla*	y= -3.428x + 48.804	0.996	21.1	0.045116	1.21E+08	
168	*Dxs*	y = -3.005x + 53.37	0.99	30.25	0.001252	2.79E+08	25
168	*Bla*	y = -3.316x + 50.89	0.96	18.22	0.013458	7.00E+09	
336	*Dxs*	y= -3.395x + 58.89	0.91	33.69	0.020627	2.65E+07	15
336	*Bla*	y = -3x + 48.56	0.98	22.69	0.057278	4.20E+08	
504	*Dxs*	y= -3.693x + 55	0.95	24.88	0.005052	1.42E+08	25
504	*Bla*	y= -3.2x+ 43.478	0.92	19.4	0.005386	3.90E+09	
672	*Dxs*	y = -3.693x + 48	0.95	32.45	0.025447	5.42E+08	22
672	*Bla*	y = -3.2x + 37.13	0.92	19.33	0.012746	1.19E+10	

Table 3.32 (c) : Tabulation of ct values, coefficient of variance, copies of target, and plasmid coy numbers (PCN) for Ldc-lux

Hours	Target	Standard curve Equations	Correlation Coefficients Between copies and Ct values	Ct values (n=3)	Coefficient of variance (CV)	DNA Copies	Plasmid copy number (per cell)
0	*Dxs*	y= -2.422x+24.86	0.97	24.77	0.026372	2.43E+05	41
0	*Bla*	y= -2.628x+22.24	0.95	17.85	0.00855	1.00E+07	
4	*Dxs*	y= -3.01+38.6999	0.97	25.56	0.00984	1.49E+06	18
4	*Bla*	y=-2.8+36.778	0.95	21.014	0.002892	2.68E+07	
6	*Dxs*	y=-2.64x+31.65	0.97	23.2	0.004336	1.62E+06	55
6	*Bla*	y= -3.105x + 35	0.93	20.63	0.012788	7.85E+07	
24	*Dxs*	y= -3.17x + 42.882	0.994	23.21	0.006848	1.61E+06	49
24	*Bla*	y= -3.387x +47.36	0.996	20.63	0.001454	7.85E+07	
168	*Dxs*	y= -2.752x +35.13	0.93	24.66	0.023755	8.51E+04	28
168	*Bla*	y= -2.504x+ 33.30	0.94	20.14	0.002498	2.40E+06	
336	*Dxs*	y= -2.5x+ 30	0.99	25.66	0.009783	4.30E+05	16
336	*Bla*	y= -2.54x+ 27.8	0.98	20.34	0.006412	6.85E+06	
504	*Dxs*	y= -3.23x+ 34	0.98	24.88	0.006901	1.42E+08	28
504	*Bla*	y= -2.67x+ 30.784	0.90	19.4	0.023136	3.90E+09	
672	*Dxs*	y= -2.8x + 41.182	1	25.26	0.016565	7.01E+08	25
672	*Bla*	y= -2.8x + 40.1	0.96	20.266	0.031723	1.75E+10	

Table 3.33 (d) : Tabulation of ct values, coefficient of variance, copies of target, and plasmid coy numbers for lyss

Hours	Target	Standard curve Equations	Correlation Coefficients Between copies and Ct values	Ct values (n=3)	Coefficient of variance (CV)	DNA Copies	Plasmid copy number (per cell)
0	*Dxs*	y= -2.568x+ 33	0.97	21.3	0.014085	9.77E+07	49
0	*Bla*	y= -2.804x+ 34.97	0.95	17.45	0.020257	4.83E+09	
4	*Dxs*	y= -2.993x+ 33.1	0.97	23.78	0.003364	1.36E+06	22
4	*Bla*	y=-2.473x+ 30.364	0.93	19.36	0.019107	2.95E+07	
6	*Dxs*	y=-3.315x+ 37.52	0.98	24.88	0.033811	2.83E+06	61
6	*Bla*	y= -3.125x+ 38.34	0.92	20.85	0.012788	1.72E+08	
24	*Dxs*	y= --3x+ 42.75	0.994	23	0.007498	3.83E+06	53
24	*Bla*	y= -2.95x+ 45.548	0.996	21.02	0.000951	2.06E+08	
168	*Dxs*	y = -3.041x + 46.66	0.99	24.85	0.003628	1.28E+07	22
168	*Bla*	y = -3.446x + 46.60	0.94	17.5	0.028571	2.79E+08	
336	*Dxs*	y = -2.750x + 42.85	0.93	25.83	0.003067	1.56E+06	20
336	*Bla*	y = -2.732x + 41.60	0.94	21.12	0.005682	3.14E+07	
504	*Dxs*	y = -3.596x + 50.74	0.99	22.1	0.006479	9.24E+07	20
504	*Bla*	y = -3.41x + 53.167	0.98	21.54	0.025527	1.88E+09	
672	*Dxs*	y = -2.837x + 40.8	0.90	27.46	0.020086	1.32E+07	15
672	*Bla*	y = -3.0x + 45	0.96	27.33	0.012075	2.05E+08	

Table 3.34 (e): Tabulation of ct values, coefficient of variance, copies of target, and plasmid coy numbers (PCN) for spc-lux

Hours	Target	Standard curve Equations	Correlation Coefficients Between copies and Ct values	Ct values (n=3)	Coefficient of variance (CV)	DNA Copies	Plasmid copy number (per cell)
0	*Dxs*	y= -2.585x+ 31.649	0.92	23.3	0.004453	4.19E+05	34
0	*Bla*	y = -2.5x + 33.75	0.98	21.85	0.036697	1.46E+07	
4	*Dxs*	y= -2.56x+ 37.87	0.94	25.33	0.013028	5.32E+06	32
4	*Bla*	y=-2.57x+ 36	1.0	19.55	0.028133	1.68E+08	
6	*Dxs*	y=-3.257x+ 38.68	0.99	25.02	0.001221	9.37E+05	67
6	*Bla*	y= -3.22x+ 40.12	0.99	20.68	0.015965	6.32E+07	
24	*Dxs*	y= -3.322x+ 45.983	0.994	21.33	0.015471	2.84E+06	58
24	*Bla*	y= -3x+ 43.983	0.996	19.33	0.017072	1.65E+08	
168	*Dxs*	y = -3.121x + 42.91	0.97	24.33	0.013564	9.01E+05	29
168	*Bla*	y = -3.747x + 50.41	0.99	22.65	0.028481	2.57E+07	
336	*Dxs*	y = -3.458x + 40.30	0.95	22.4	0.013453	1.93E+07	10
336	*Bla*	y = -3.544x + 41.5	0.99	19.5	0.046064	1.90E+08	
504	*Dxs*	y= -3.4x+ 37.75	0.97	25.24	0.026369	8.30E+05	22
504	*Bla*	y = -3.256x + 42.88	0.99	26.55	0.004143	1.82E+07	
672	*Dxs*	y = -3.329x + 33.82	0.89	28.06	0.007036	2.18E+04	21
672	*Bla*	y = -3.4x + 36.5	0.99	26.1	0.013814	4.63E+05	

Tables 3.35 : Tukey Post Hoc test of PCN values

Multiple Comparisons

Dependent Variable: PCN

Tukey HSD

(I) Time	(J) Time	Mean Difference (I-J)	Sig.	95% Confidence Interval	
				Lower Bound	Upper Bound
	4 Hour	-5.2000	.996	-27.9347	17.5347
	6 h	-29.5333*	.003	-52.2681	-6.7986
	24 h	-19.2667	.158	-42.0014	3.4681
0 Hour	168 h	7.8000	.962	-14.9347	30.5347
	336 h	18.8000	.181	-3.9347	41.5347
	504 h	12.0000	.723	-10.7347	34.7347
	672 h	13.8667	.556	-8.8681	36.6014
	0 Hour	5.2000	.996	-17.5347	27.9347
	6 h	-24.3333*	.027	-47.0681	-1.5986
	24 h	-14.0667	.538	-36.8014	8.6681
4 Hour	168 h	13.0000	.635	-9.7347	35.7347
	336 h	24.0000*	.031	1.2653	46.7347
	504 h	17.2000	.278	-5.5347	39.9347
	672 h	19.0667	.168	-3.6681	41.8014
	0 Hour	29.5333*	.003	6.7986	52.2681
	4 Hour	24.3333*	.027	1.5986	47.0681
	24 h	10.2667	.852	-12.4681	33.0014
6 h	168 h	37.3333*	.000	14.5986	60.0681
	336 h	48.3333*	.000	25.5986	71.0681
	504 h	41.5333*	.000	18.7986	64.2681
	672 h	43.4000*	.000	20.6653	66.1347
	0 Hour	19.2667	.158	-3.4681	42.0014
	4 Hour	14.0667	.538	-8.6681	36.8014
	6 h	-10.2667	.852	-33.0014	12.4681
24 h	168 h	27.0667*	.009	4.3319	49.8014
	336 h	38.0667*	.000	15.3319	60.8014
	504 h	31.2667*	.001	8.5319	54.0014
	672 h	33.1333*	.001	10.3986	55.8681
	0 Hour	-7.8000	.962	-30.5347	14.9347
	4 Hour	-13.0000	.635	-35.7347	9.7347
	6 h	-37.3333*	.000	-60.0681	-14.5986
168 h	24 h	-27.0667*	.009	-49.8014	-4.3319
	336 h	11.0000	.802	-11.7347	33.7347
	504 h	4.2000	.999	-18.5347	26.9347
	672 h	6.0667	.991	-16.6681	28.8014
336 h	0 Hour	-18.8000	.181	-41.5347	3.9347

	4 Hour	-24.0000*	.031	-46.7347	-1.2653
	6 h	-48.3333*	.000	-71.0681	-25.5986
	24 h	-38.0667*	.000	-60.8014	-15.3319
	168 h	-11.0000	.802	-33.7347	11.7347
	504 h	-6.8000	.982	-29.5347	15.9347
	672 h	-4.9333	.997	-27.6681	17.8014
	0 Hour	-12.0000	.723	-34.7347	10.7347
	4 Hour	-17.2000	.278	-39.9347	5.5347
	6 h	-41.5333*	.000	-64.2681	-18.7986
504 h	24 h	-31.2667*	.001	-54.0014	-8.5319
	168 h	-4.2000	.999	-26.9347	18.5347
	336 h	6.8000	.982	-15.9347	29.5347
	672 h	1.8667	1.000	-20.8681	24.6014
	0 Hour	-13.8667	.556	-36.6014	8.8681
	4 Hour	-19.0667	.168	-41.8014	3.6681
	6 h	-43.4000*	.000	-66.1347	-20.6653
672 h	24 h	-33.1333*	.001	-55.8681	-10.3986
	168 h	-6.0667	.991	-28.8014	16.6681
	336 h	4.9333	.997	-17.8014	27.6681
	504 h	-1.8667	1.000	-24.6014	20.8681

Table 3.4 (a, b, & c): Concentrations of ATP, ADP, and AMP converted from chemiluminescence readings to mg/ml from the standard curve from Standards of Figure 3.13 in Section 3.7.4

	ATP concentrations (mg/ml)						
Time (Hour)	*Lpp-lux*	*tatA-lux*	*Ldc-lux*	*Lyss-lux*	*Spc-lux*	*E.coli [pBR322.lux]*	*E.coli ATCC 8739*
0	0.810402	0.810643	0.808429	0.806528	0.803313	0.835995	0.835995
6	0.809812	0.809812	0.802895	0.802863	0.802302	0.802739	0.814619
24	0.8478	0.841881	0.844011	0.842302	0.846406	0.848997	0.85364
168	0.838705	0.843026	0.845035	0.848706	0.846469	0.836203	0.83724
336	0.842399	0.848415	0.847054	0.844566	0.848207	0.843907	0.844651
504	0.847412	0.847737	0.848309	0.849699	0.848054	0.847936	0.845769
672	0.843692	0.846068	0.845583	0.846582	0.846485	0.847327	0.85163
	ADP concentrations (mg/ml)						
Time (Hour)	*Lpp-lux*	*tatA-lux*	*Ldc-lux*	*Lyss-lux*	*Spc-lux*	*E.coli [pBR322.lux]*	*E.coli ATCC 8739*
0	0.835995	0.829081	0.826276	0.827064	0.826276	0.823868	0.848997
6	0.750077	0.835995	0.831138	0.829813	0.829844	0.832178	0.830147
24	1.06678	0.858377	0.855856	0.852507	0.857258	0.858042	0.85364
168	1.006005	0.829086	0.833122	0.832018	0.832272	0.83246	0.835995

336	1.002912	0.834257	0.831652	0.829573	0.830391	0.828528	0.831545
504	1.001922	0.838876	0.838275	0.836633	0.836998	0.836668	0.824743
672	1.001435	0.830611	0.832267	0.830652	0.828286	0.829565	0.827187

	AMP concentrations (mg/ml)						
Time (Hour)	*Lpp-lux*	*tatA-lux*	*Ldc-lux*	*Lyss-lux*	*Spc-lux*	*E.coli [pBR322.lux]*	*E.coli ATCC 8739*
0	0.845025	0.845318	0.845318	0.847534	0.845318	0.859639	0.859265
6	0.845261	0.847546	0.847546	0.845075	0.848763	0.858217	0.858713
24	0.862496	0.856191	0.858002	0.856939	0.859673	0.860503	0.860093
168	0.839732	0.85135	0.846985	0.850576	0.855062	0.83634	0.839229
336	0.848871	0.8523	0.847165	0.844677	0.848225	0.84321	0.846886
504	0.848997	0.848233	0.8495	0.85343	0.849067	0.848149	0.846346
672	0.84323	0.841916	0.845152	0.847844	0.847034	0.854938	0.852776

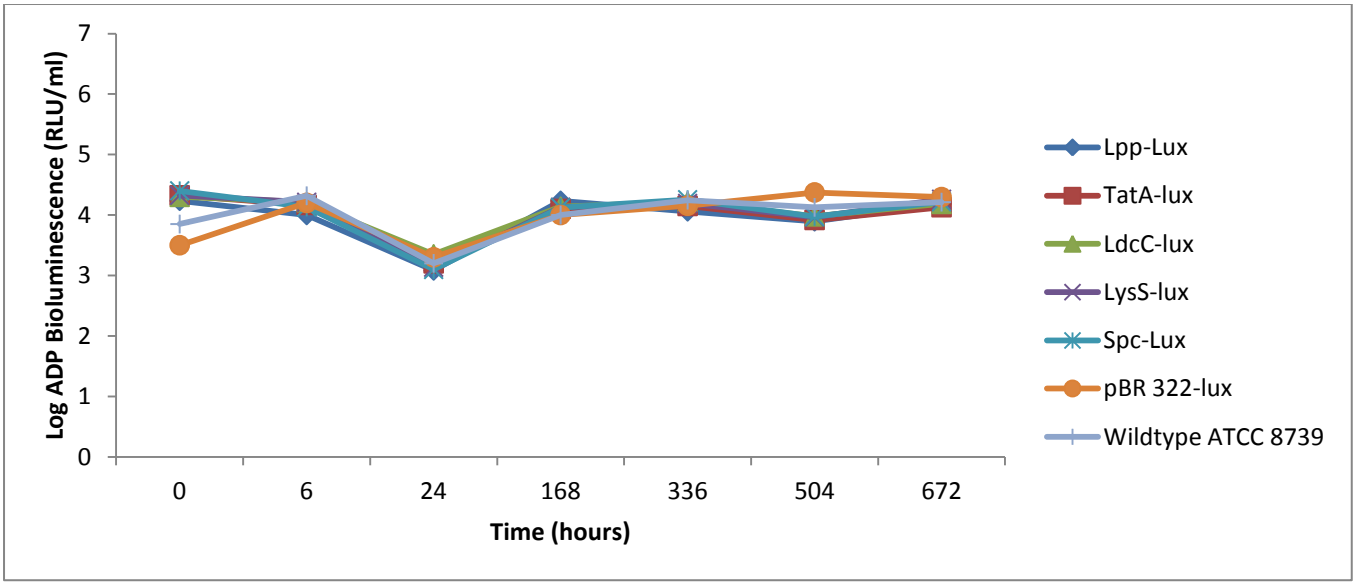


Figure 3.7 (a): The ADP levels of five biosensors and control strains over extended period of time

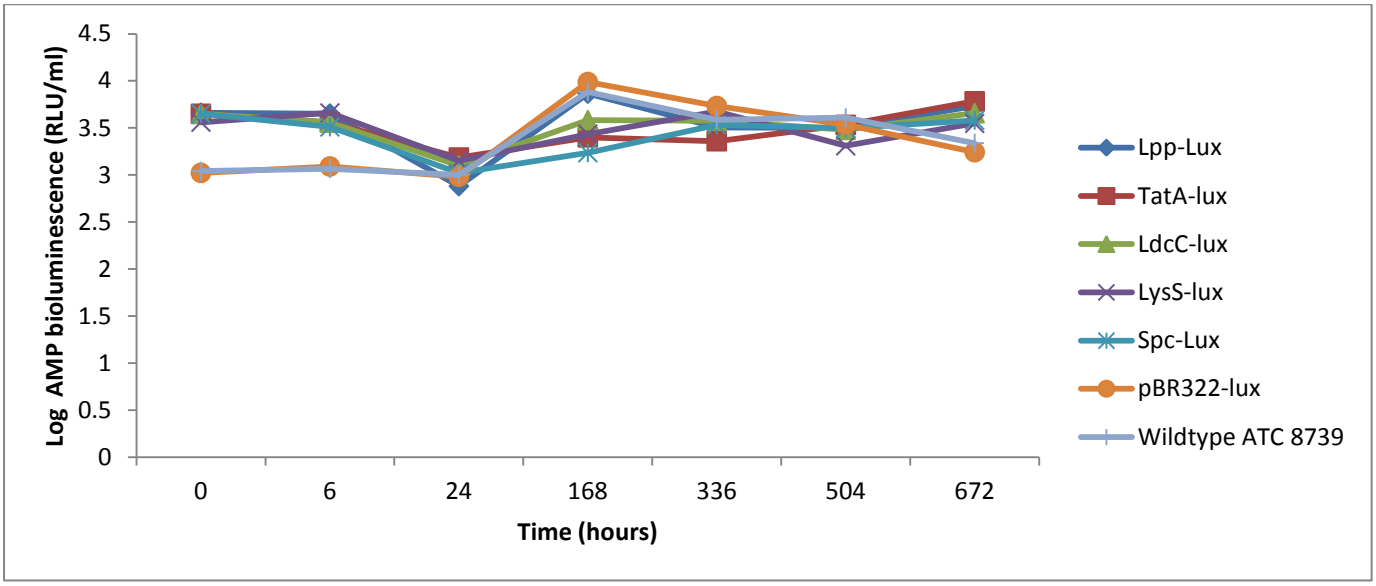


Figure 3.7 (b): The AMP levels of five biosensors and control strains over extended period of time

Table 3.4: ANOVA analysis for ATP, ADP, and AMP readings

No significance difference was found

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
ATP	Between Groups	1359920183 6118.500	6	2266533639 353.084	.419	.862
	Within Groups	2271725765 01678.700	42	5408870869 087.588		
	Total	2407717783 37797.200	48			
ADP	Between Groups	1178735920 499.357	6	1964559867 49.893	.348	.907
	Within Groups	2371945751 1671.790	42	5647489883 73.138		
	Total	2489819343 2171.145	48			
AMP	Between Groups	7124123929 66.786	6	1187353988 27.798	.663	.680
	Within Groups	7519614253 864.255	42	1790384346 15.816		
	Total	8232026646 831.041	48			

Table 3.4.1: Standard mean error for ATP readings from Section 3.7.4.1

Grand Mean

Dependent Variable: ATP

Mean	Std. Error	95% Confidence Interval	
		Lower Bound	Upper Bound
3.929	.006	3.918	3.940

Table 3.4.2: Tukey analysis for ATP readings from section 3.7.4.1

No significance difference was found

ATP

Tukey HSD[a,b,c]

Biosensor	N	Subset		
		1	2	3
wt	21	3.7886		
pless	21		3.8692	
Spc	21		3.9300	3.9300
lpp	25			3.9524
Lyss	21			3.9534
tatA	21			3.9738
Ldc	21			3.9895
Sig.		1.000	.067	.078

Table 3.4.3: Standard Mean error for AEC readings across 28 days

Grand Mean

Dependent Variable: AEC

Mean	Std. Error	95% Confidence Interval	
		Lower Bound	Upper Bound
.654	.003	.648	.660

Table 3.4.4: Tukey analysis for AEC readings across 28 days

No significance difference was found

AEC

Tukey HSD[a,b,c]

Biosensor	N	Subset		
		1	2	3
wt	21	.6187		
lpp	21	.6304	.6304	

pless	21	.6490	.6490	.6490
tatA	21		.6629	.6629
Spc	21			.6645
Ldc	21			.6648
Lyss	21			.6695
Sig.		.088	.054	.498

Table 3.4.5: Tukey Analysis for time vs AEC values

Multiple Comparisons

Dependent Variable: AEC

Tukey HSD

(I) Time	(J) Time	Mean Difference (I-J)	Sig.	95% Confidence Interval	
				Lower Bound	Upper Bound
0 hr	6hr	-.06029*	.015	-.1128	-.0077
	24 hr	.11234*	.000	.0598	.1649
	168 hr	.29828*	.000	.2457	.3508
	336 hr	.31247*	.000	.2599	.3650
	504 hr	.31528*	.000	.2627	.3678
	672 hr	.32566*	.000	.2731	.3782
6hr	0 hr	.06029*	.015	.0077	.1128
	24 hr	.17263*	.000	.1201	.2252
	168 hr	.35857*	.000	.3060	.4111
	336 hr	.37276*	.000	.3202	.4253
	504 hr	.37557*	.000	.3230	.4281
	672 hr	.38595*	.000	.3334	.4385
24 hr	0 hr	-.11234*	.000	-.1649	-.0598
	6hr	-.17263*	.000	-.2252	-.1201
	168 hr	.18595*	.000	.1334	.2385
	336 hr	.20014*	.000	.1476	.2527
	504 hr	.20294*	.000	.1504	.2555
	672 hr	.21332*	.000	.1608	.2659
168 hr	0 hr	-.29828*	.000	-.3508	-.2457
	6hr	-.35857*	.000	-.4111	-.3060
	24 hr	-.18595*	.000	-.2385	-.1334
	336 hr	.01419	.980	-.0384	.0667
	504 hr	.01700	.951	-.0356	.0695
	672 hr	.02737	.675	-.0252	.0799
336 hr	0 hr	-.31247*	.000	-.3650	-.2599
	6hr	-.37276*	.000	-.4253	-.3202
	24 hr	-.20014*	.000	-.2527	-.1476
	168 hr	-.01419	.980	-.0667	.0384
	504 hr	.00281	1.000	-.0497	.0554
	672 hr	.01318	.986	-.0394	.0657
504 hr	0 hr	-.31528*	.000	-.3678	-.2627

	6hr	-.37557*	.000	-.4281	-.3230
	24 hr	-.20294*	.000	-.2555	-.1504
	168 hr	-.01700	.951	-.0695	.0356
	336 hr	-.00281	1.000	-.0554	.0497
	672 hr	.01038	.996	-.0422	.0629
	0 hr	-.32566*	.000	-.3782	-.2731
	6hr	-.38595*	.000	-.4385	-.3334
	24 hr	-.21332*	.000	-.2659	-.1608
672 hr	168 hr	-.02737	.675	-.0799	.0252
	336 hr	-.01318	.986	-.0657	.0394
	504 hr	-.01038	.996	-.0629	.0422

*. The mean difference is significant at the 0.05 level.

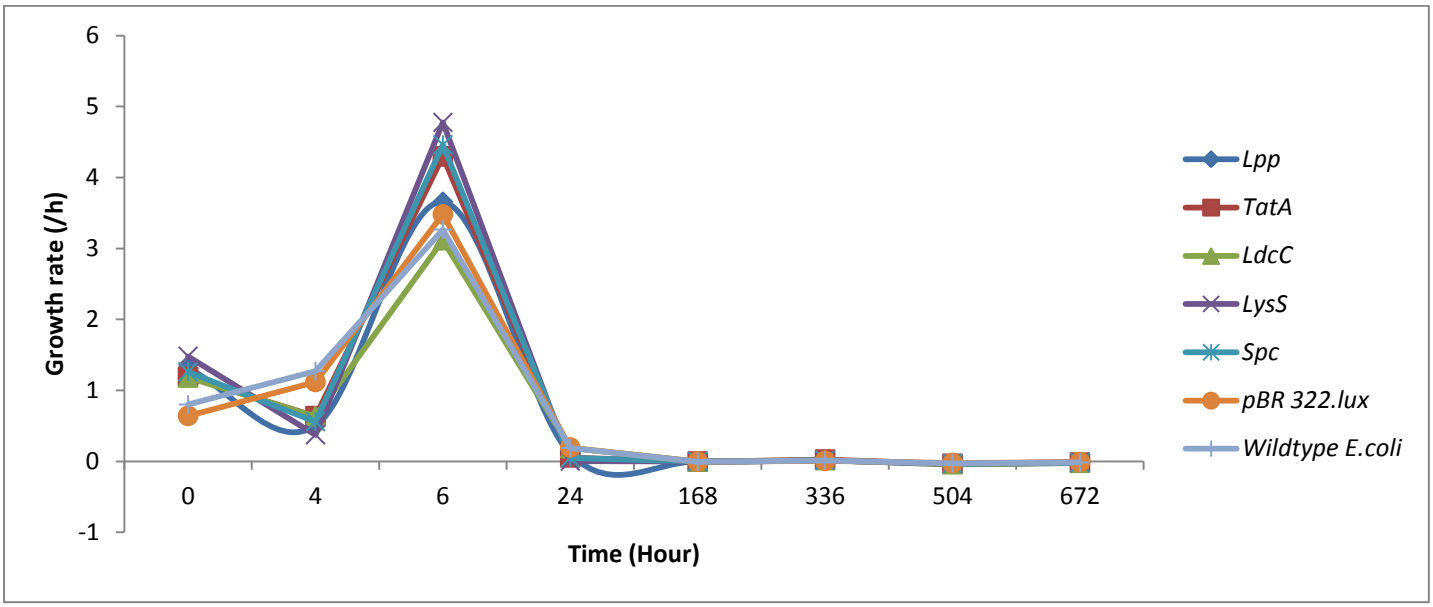


Figure 3.8: Growth rate of all seven *E.coli* strains across 28 days

Formulation of growth rate ;

$$= \frac{t(time\ interval\ in\ hours)}{3.3\log(CFUv/CFUi)}$$

= v is CFU counts at the end of the time interval.

= i is CFU counts at the beginning of the time interval

No statistical differences were found across the *E.coli* strains

Appendix 4

Whole-Cell Bioluminescence evaluation using Sorbic Acid

Table 4.1: Tukey Post Hoc Analysis on Preservative-free vs Biosensors, Time in Section 4.6.3

Multiple Comparisons

Dependent Variable: RLU

Tukey HSD

(I) Biosensor	(J) Biosensor	Mean Difference (I-J)	Sig.	95% Confidence Interval	
				Lower Bound	Upper Bound
LPP	tatA	.1243	.005	-.1151	.3637
	ldc	.4981*	.000	.2587	.7375
	lyss	.5129*	.810	.2735	.7522
	spc	-.0933	.000	-.3327	.1460
tatA	LPP	-.1243	.595	-.3637	.1151
	ldc	.3738*	.000	.1344	.6132
	lyss	.3886*	.000	.1492	.6279
	spc	-.2176	.002	-.4570	.0218
ldc	LPP	-.4981*	.000	-.7375	-.2587
	tatA	-.3738*	.000	-.6132	-.1344
	lyss	.0148	1.000	-.2246	.2541
	spc	-.5914*	.000	-.8308	-.3521
lyss	LPP	-.5129*	.000	-.7522	-.2735
	tatA	-.3886*	.800	-.6279	-.1492
	ldc	-.0148	1.000	-.2541	.2246
	spc	-.6062*	.810	-.8456	-.3668
spc	LPP	.0933	.000	-.1460	.3327
	tatA	.2176	.002	-.0218	.4570
	ldc	.5914*	.000	.3521	.8308
	lyss	.6062*	.000	.3668	.8456

Time Analysis

Multiple Comparisons

Dependent Variable: RLU

Tukey HSD

(I) TIME	(J) TIME	Mean Difference (I-J)	Sig.	95% Confidence Interval	
				Lower Bound	Upper Bound
0 hour	6 hr	-.0387	1.000	-.3457	.2684
	24 hr	.0727	.991	-.2344	.3797
	7 days	.5587*	.000	.2516	.8657
	14 days	.9780*	.000	.6709	1.2851
	21 days	.8700*	.000	.5629	1.1771

	28 days	1.5713*	.000	1.2643	1.8784
24 hr	0 hour	.0387	1.000	-.2684	.3457
	24 hr	.1113	.926	-.1957	.4184
	7 days	.5973*	.000	.2903	.9044
	14 days	1.0167*	.000	.7096	1.3237
	21 days	.9087*	.000	.6016	1.2157
	28 days	1.6100*	.000	1.3029	1.9171
48 hr	0 hour	-.0727	.991	-.3797	.2344
	6 hr	-.1113	.926	-.4184	.1957
	7 days	.4860*	.000	.1789	.7931
	14 days	.9053*	.000	.5983	1.2124
	21 days	.7973*	.000	.4903	1.1044
	28 days	1.4987*	.000	1.1916	1.8057
7 days	0 hour	-.5587*	.000	-.8657	-.2516
	24 hr	-.5973*	.000	-.9044	-.2903
	48 hr	-.4860*	.000	-.7931	-.1789
	14 days	.4193*	.002	.1123	.7264
	21 days	.3113*	.045	.0043	.6184
	28 days	1.0127*	.000	.7056	1.3197
14 days	0 hour	-.9780*	.000	-1.2851	-.6709
	6 hr	-1.0167*	.000	-1.3237	-.7096
	24 hr	-.9053*	.000	-1.2124	-.5983
	7 days	-.4193*	.002	-.7264	-.1123
	21 days	-.1080	.935	-.4151	.1991
	28 days	.5933*	.000	.2863	.9004
21 days	0 hour	-.8700*	.000	-1.1771	-.5629
	24 hr	-.9087*	.000	-1.2157	-.6016
	48 hr	-.7973*	.000	-1.1044	-.4903
	7 days	-.3113*	.045	-.6184	-.0043
	14 days	.1080	.935	-.1991	.4151
	28 days	.7013*	.000	.3943	1.0084
28 days	0 hour	-1.5713*	.000	-1.8784	-1.2643
	24 hr	-1.6100*	.000	-1.9171	-1.3029
	48 hr	-1.4987*	.000	-1.8057	-1.1916
	7 days	-1.0127*	.000	-1.3197	-.7056
	14 days	-.5933*	.000	-.9004	-.2863
	21 days	-.7013*	.000	-1.0084	-.3943

Table 4.2: Standard Error for Bioluminescence (RLU/ml) readings in Sorbic acid

Grand Mean

Dependent Variable: RLU

Mean	Std. Error	95% Confidence Interval	
		Lower Bound	Upper Bound
2.389[a]	.011	2.363	2.415

a. Based on modified population marginal mean.

Table 4.3: Standard Error for population (CFU/ml) readings in Sorbic acid

Grand Mean

Dependent Variable: CFU

Mean	Std. Error	95% Confidence Interval	
		Lower Bound	Upper Bound
3.868[a]	.127	3.556	4.179

a. Based on modified population marginal mean.

Table 4.4: Standard Error for RLU:CFU readings in Sorbic acid

Grand Mean

Dependent Variable: RLUCFU

Mean	Std. Error	95% Confidence Interval	
		Lower Bound	Upper Bound
.121	.012	.097	.146

Table 4.1.4: Standard Error for ATP- Bioluminescence (RLU/ml) readings in Sorbic acid

Grand Mean

Dependent Variable: ATP

Mean	Std. Error	95% Confidence Interval	
		Lower Bound	Upper Bound
3.278[a]	.056	3.141	3.416

Table 4.5: Levene`s test between RLU and two compendia method

Levene's Test of Equality of Error Variances[a]

Dependent Variable: RLU

F	df1	df2	Sig.
1.404	258	35	.113

Table 4.6: Statistical analysis of RLU, CFU, ATP-chemiluminescence for biosensor stains Sorbic acid analysis in Sections 4.6.4

Multiple Comparisons

Tukey HSD

Dependent Variable	(I) Biosensor	(J) Biosensor	Mean Difference (I-J)	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
RLU	lpp	tatA	-1.1353*	.006	-2.0391	-.2315
		Ldc	-.9808*	.026	-1.8847	-.0770
		Lyss	-.7583	.146	-1.6621	.1456
		Spc	-1.6186*	.000	-2.5224	-.7147
	tatA	lpp	1.1353*	.006	.2315	2.0391
		Ldc	.1545	.990	-.7493	1.0583
		Lyss	.3770	.781	-.5268	1.2809
		Spc	-.4833	.582	-1.3871	.4206
	Ldc	lpp	.9808*	.026	.0770	1.8847
		tatA	-.1545	.990	-1.0583	.7493
		Lyss	.2226	.961	-.6813	1.1264
		Spc	-.6378	.299	-1.5416	.2661
	Lyss	Lpp	.7583	.146	-.1456	1.6621
		tatA	-.3770	.781	-1.2809	.5268
		Ldc	-.2226	.961	-1.1264	.6813
		Spc	-.8603	.071	-1.7641	.0435
	Spc	Lpp	1.6186*	.000	.7147	2.5224
		tatA	.4833	.582	-.4206	1.3871
		Ldc	.6378	.299	-.2661	1.5416
		Lyss	.8603	.071	-.0435	1.7641
CFU	lpp	tatA	-.0030	1.000	-1.2593	1.2533
		Ldc	-.0266	1.000	-1.2829	1.2297
		Lyss	.0340	1.000	-1.2223	1.2904
		Spc	.0279	1.000	-1.2284	1.2842
	tatA	lpp	.0030	1.000	-1.2533	1.2593
		Ldc	-.0236	1.000	-1.2799	1.2327
		Lyss	.0370	1.000	-1.2193	1.2934
		Spc	.0309	1.000	-1.2254	1.2872
	Ldc	lpp	.0266	1.000	-1.2297	1.2829
		tatA	.0236	1.000	-1.2327	1.2799
		Lyss	.0607	1.000	-1.1956	1.3170
		Spc	.0545	1.000	-1.2018	1.3108
	Lyss	lpp	-.0340	1.000	-1.2904	1.2223

		tatA	-.0370	1.000	-1.2934	1.2193
		Ldc	-.0607	1.000	-1.3170	1.1956
		Spc	-.0061	1.000	-1.2625	1.2502
		lpp	-.0279	1.000	-1.2842	1.2284
	Spc	tatA	-.0309	1.000	-1.2872	1.2254
		Ldc	-.0545	1.000	-1.3108	1.2018
		Lyss	.0061	1.000	-1.2502	1.2625
		tatA	-.0424	1.000	-.7565	.6717
	lpp	Ldc	-.1086	.994	-.8227	.6055
		Lyss	-.1183	.991	-.8324	.5958
		Spc	-.1017	.995	-.8158	.6124
		lpp	.0424	1.000	-.6717	.7565
	tatA	Ldc	-.0662	.999	-.7803	.6479
		Lyss	-.0759	.998	-.7900	.6382
		Spc	-.0593	.999	-.7734	.6548
		lpp	.1086	.994	-.6055	.8227
		tatA	.0662	.999	-.6479	.7803
ATP	Ldc	Lyss	-.0097	1.000	-.7238	.7044
		Spc	.0069	1.000	-.7072	.7210
		lpp	.1183	.991	-.5958	.8324
		tatA	.0759	.998	-.6382	.7900
	Lyss	Ldc	.0097	1.000	-.7044	.7238
		Spc	.0166	1.000	-.6975	.7307
		lpp	.1017	.995	-.6124	.8158
		tatA	.0593	.999	-.6548	.7734
	Spc	Ldc	-.0069	1.000	-.7210	.7072
		Lyss	-.0166	1.000	-.7307	.6975
		tatA	-.0292	.942	-.1352	.0768
		Ldc	.0160	.994	-.0901	.1220
	lpp	Lyss	.0677	.402	-.0383	.1737
		Spc	-.1549*	.001	-.2610	-.0489
		lpp	.0292	.942	-.0768	.1352
		Ldc	.0452	.767	-.0609	.1512
	tatA	Lyss	.0969	.091	-.0091	.2029
RLUCFU		Spc	-.1257*	.011	-.2317	-.0197
		lpp	-.0160	.994	-.1220	.0901
		tatA	-.0452	.767	-.1512	.0609
	Ldc	Lyss	.0517	.665	-.0543	.1578
		Spc	-.1709*	.000	-.2769	-.0648
	Lyss	lpp	-.0677	.402	-.1737	.0383
		tatA	-.0969	.091	-.2029	.0091

Spc	Ldc	-.0517	.665	-.1578	.0543
	Spc	-.2226*	.000	-.3287	-.1166
	lpp	.1549*	.001	.0489	.2610
	tatA	.1257*	.011	.0197	.2317
	Ldc	.1709*	.000	.0648	.2769
	Lyss	.2226*	.000	.1166	.3287

Table 4.7: Statistical analysis of RLU, CFU, ATP-chemiluminescence against time Sorbic acid analysis

Multiple Comparisons

Tukey HSD

Dependent Variable	(I) time	(J) time	Mean Difference (I-J)	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
RLU	0 hr	24 hr	.9737	.003	-.1834	2.1308
		48 hr	1.2383*	.027	.0812	2.3954
		168 hr	1.8397*	.000	.6826	2.9968
		336 hr	2.2057*	.000	1.0486	3.3628
		504 hr	2.5536*	.000	1.3965	3.7106
		672 hr	2.7840*	.000	1.6269	3.9411
	24 hr	0 hr	-.9737	.163	-2.1308	.1834
		48 hr	.2646	.994	-.8925	1.4217
		168 hr	.8660	.285	-.2911	2.0231
		336 hr	1.2320*	.029	.0749	2.3891
		504 hr	1.5799*	.001	.4228	2.7369
		672 hr	1.8103*	.000	.6532	2.9674
	48 hr	0 hr	-1.2383*	.027	-2.3954	-.0812
		24 hr	-.2646	.994	-1.4217	.8925
		168 hr	.6014	.716	-.5557	1.7585
		336 hr	.9674	.169	-.1897	2.1245
		504 hr	1.3153*	.015	.1582	2.4724
		672 hr	1.5457*	.002	.3886	2.7028
	168 hr	0 hr	-1.8397*	.000	-2.9968	-.6826
		24 hr	-.8660	.285	-2.0231	.2911
		48 hr	-.6014	.716	-1.7585	.5557
		336 hr	.3660	.965	-.7911	1.5231
		504 hr	.7139	.525	-.4432	1.8709
		672 hr	.9443	.192	-.2128	2.1014

	336 hr	0 hr	-2.2057*	.000	-3.3628	-1.0486
		24 hr	-1.2320*	.029	-2.3891	-.0749
		48 hr	-.9674	.169	-2.1245	.1897
		168 hr	-.3660	.965	-1.5231	.7911
		504 hr	.3479	.973	-.8092	1.5049
		672 hr	.5783	.752	-.5788	1.7354
	504 hr	0 hr	-2.5536*	.000	-3.7106	-1.3965
		24 hr	-1.5799*	.001	-2.7369	-.4228
		48 hr	-1.3153*	.015	-2.4724	-.1582
		168 hr	-.7139	.525	-1.8709	.4432
		336 hr	-.3479	.973	-1.5049	.8092
		672 hr	.2304	.997	-.9267	1.3875
	672 hr	0 hr	-2.7840*	.000	-3.9411	-1.6269
		24 hr	-1.8103*	.000	-2.9674	-.6532
		48 hr	-1.5457*	.002	-2.7028	-.3886
		168 hr	-.9443	.192	-2.1014	.2128
		336 hr	-.5783	.752	-1.7354	.5788
		504 hr	-.2304	.997	-1.3875	.9267
CFU	0 hr	24 hr	1.6824*	.034	.0740	3.2907
		48 hr	2.1770*	.001	.5687	3.7853
		168 hr	2.3434*	.000	.7351	3.9518
		336 hr	2.5203*	.000	.9120	4.1286
		504 hr	2.7325*	.000	1.1242	4.3408
		672 hr	2.8352*	.000	1.2269	4.4436
	24 hr	0 hr	-1.6824*	.034	-3.2907	-.0740
		48 hr	.4946	.970	-1.1137	2.1030
		168 hr	.6611	.884	-.9473	2.2694
		336 hr	.8379	.713	-.7704	2.4462
		504 hr	1.0501	.454	-.5582	2.6584
		672 hr	1.1529	.337	-.4555	2.7612
	48 hr	0 hr	-2.1770*	.001	-3.7853	-.5687
		24 hr	-.4946	.970	-2.1030	1.1137
		168 hr	.1664	1.000	-1.4419	1.7748
		336 hr	.3433	.996	-1.2650	1.9516
		504 hr	.5555	.947	-1.0528	2.1638
		672 hr	.6582	.886	-.9501	2.2666
	168 hr	0 hr	-2.3434*	.000	-3.9518	-.7351
		24 hr	-.6611	.884	-2.2694	.9473
		48 hr	-.1664	1.000	-1.7748	1.4419
		336 hr	.1769	1.000	-1.4315	1.7852
		504 hr	.3891	.991	-1.2193	1.9974

		672 hr	.4918	.971	-1.1165	2.1001
		0 hr	-2.5203*	.000	-4.1286	-.9120
		24 hr	-.8379	.713	-2.4462	.7704
		48 hr	-.3433	.996	-1.9516	1.2650
	336 hr	168 hr	-.1769	1.000	-1.7852	1.4315
		504 hr	.2122	1.000	-1.3961	1.8205
		672 hr	.3149	.997	-1.2934	1.9233
		0 hr	-2.7325*	.000	-4.3408	-1.1242
		24 hr	-1.0501	.454	-2.6584	.5582
		48 hr	-.5555	.947	-2.1638	1.0528
	504 hr	168 hr	-.3891	.991	-1.9974	1.2193
		336 hr	-.2122	1.000	-1.8205	1.3961
		672 hr	.1027	1.000	-1.5056	1.7111
		0 hr	-2.8352*	.000	-4.4436	-1.2269
		24 hr	-1.1529	.337	-2.7612	.4555
		48 hr	-.6582	.886	-2.2666	.9501
	672 hr	168 hr	-.4918	.971	-2.1001	1.1165
		336 hr	-.3149	.997	-1.9233	1.2934
		504 hr	-.1027	1.000	-1.7111	1.5056
ATP		24 hr	1.0927*	.008	.1785	2.0069
		48 hr	1.5063*	.000	.5921	2.4205
		168 hr	1.5443*	.000	.6301	2.4585
	0 hr	336 hr	1.7070*	.000	.7928	2.6212
		504 hr	1.7571*	.000	.8429	2.6713
		672 hr	1.9070*	.000	.9928	2.8212
		0 hr	-1.0927*	.008	-2.0069	-.1785
		48 hr	.4136	.829	-.5006	1.3278
		168 hr	.4516	.762	-.4626	1.3658
	24 hr	336 hr	.6143	.417	-.2999	1.5285
		504 hr	.6644	.320	-.2498	1.5786
		672 hr	.8143	.116	-.0999	1.7285
		0 hr	-1.5063*	.000	-2.4205	-.5921
		24 hr	-.4136	.829	-1.3278	.5006
		168 hr	.0380	1.000	-.8762	.9522
	48 hr	336 hr	.2007	.995	-.7135	1.1149
		504 hr	.2508	.983	-.6634	1.1650
		672 hr	.4007	.849	-.5135	1.3149
		0 hr	-1.5443*	.000	-2.4585	-.6301
		24 hr	-.4516	.762	-1.3658	.4626
		48 hr	-.0380	1.000	-.9522	.8762
	168 hr	336 hr	.1627	.998	-.7515	1.0769
		504 hr	.2128	.993	-.7014	1.1270
		672 hr	.3627	.901	-.5515	1.2769

	336 hr	0 hr	-1.7070*	.000	-2.6212	-.7928
		24 hr	-.6143	.417	-1.5285	.2999
		48 hr	-.2007	.995	-1.1149	.7135
		168 hr	-.1627	.998	-1.0769	.7515
		504 hr	.0501	1.000	-.8641	.9643
		672 hr	.2000	.995	-.7142	1.1142
	504 hr	0 hr	-1.7571*	.000	-2.6713	-.8429
		24 hr	-.6644	.320	-1.5786	.2498
		48 hr	-.2508	.983	-1.1650	.6634
		168 hr	-.2128	.993	-1.1270	.7014
		336 hr	-.0501	1.000	-.9643	.8641
		672 hr	.1499	.999	-.7643	1.0641
	672 hr	0 hr	-1.9070*	.000	-2.8212	-.9928
		24 hr	-.8143	.116	-1.7285	.0999
		48 hr	-.4007	.849	-1.3149	.5135
		168 hr	-.3627	.901	-1.2769	.5515
		336 hr	-.2000	.995	-1.1142	.7142
		504 hr	-.1499	.999	-1.0641	.7643
RLUCFU	0 hr	24 hr	-.0161	1.000	-.1519	.1196
		48 hr	-.0509	.923	-.1866	.0849
		168 hr	-.0117	1.000	-.1474	.1241
		336 hr	-.0089	1.000	-.1446	.1269
		504 hr	-.0051	1.000	-.1408	.1306
		672 hr	.0388	.979	-.0969	.1746
	24 hr	0 hr	.0161	1.000	-.1196	.1519
		48 hr	-.0347	.988	-.1705	.1010
		168 hr	.0045	1.000	-.1313	.1402
		336 hr	.0073	1.000	-.1285	.1430
		504 hr	.0110	1.000	-.1247	.1468
		672 hr	.0550	.891	-.0808	.1907
	48 hr	0 hr	.0509	.923	-.0849	.1866
		24 hr	.0347	.988	-.1010	.1705
		168 hr	.0392	.978	-.0966	.1749
		336 hr	.0420	.969	-.0937	.1778
		504 hr	.0458	.953	-.0900	.1815
		672 hr	.0897	.439	-.0460	.2255
	168 hr	0 hr	.0117	1.000	-.1241	.1474
		24 hr	-.0045	1.000	-.1402	.1313
		48 hr	-.0392	.978	-.1749	.0966
		336 hr	.0028	1.000	-.1329	.1386

		504 hr	.0066	1.000	-.1292	.1423
		672 hr	.0505	.925	-.0852	.1863
		0 hr	.0089	1.000	-.1269	.1446
		24 hr	-.0073	1.000	-.1430	.1285
	336 hr	48 hr	-.0420	.969	-.1778	.0937
		168 hr	-.0028	1.000	-.1386	.1329
		504 hr	.0038	1.000	-.1320	.1395
		672 hr	.0477	.943	-.0881	.1834
		0 hr	.0051	1.000	-.1306	.1408
		24 hr	-.0110	1.000	-.1468	.1247
	504 hr	48 hr	-.0458	.953	-.1815	.0900
		168 hr	-.0066	1.000	-.1423	.1292
		336 hr	-.0038	1.000	-.1395	.1320
		672 hr	.0439	.961	-.0918	.1797
		0 hr	-.0388	.979	-.1746	.0969
		24 hr	-.0550	.891	-.1907	.0808
	672 hr	48 hr	-.0897	.439	-.2255	.0460
		168 hr	-.0505	.925	-.1863	.0852
		336 hr	-.0477	.943	-.1834	.0881
		504 hr	-.0439	.961	-.1797	.0918

Table 4.8: Statistical analysis of RLU, CFU, ATP-chemiluminescence against Concentrations Sorbic acid analysis

Multiple Comparisons

Tukey HSD

Dependent Variable	(I) Concentration	(J) Concentration	Mean Difference (I-J)	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
RLU		0.1	.9737	.163	-.1834	2.1308
		0.05	1.2383*	.027	.0812	2.3954
		0.025	1.8397*	.000	.6826	2.9968
	0.2	0.0125	2.2057*	.000	1.0486	3.3628
		0.0062	2.5536*	.000	1.3965	3.7106
		0.0031	2.7840*	.000	1.6269	3.9411
		0.2	-.9737	.163	-2.1308	.1834
		0.05	.2646	.994	-.8925	1.4217
	0.1	0.025	.8660	.285	-.2911	2.0231
		0.0125	1.2320*	.029	.0749	2.3891
		0.0062	1.5799*	.001	.4228	2.7369

		0.0031	1.8103*	.000	.6532	2.9674
		0.2	-1.2383*	.027	-2.3954	-.0812
		0.1	-.2646	.994	-1.4217	.8925
		0.025	.6014	.716	-.5557	1.7585
	0.05	0.0125	.9674	.169	-.1897	2.1245
		0.0062	1.3153*	.015	.1582	2.4724
		0.0031	1.5457*	.002	.3886	2.7028
		0.2	-1.8397*	.000	-2.9968	-.6826
		0.1	-.8660	.285	-2.0231	.2911
		0.05	-.6014	.716	-1.7585	.5557
	0.025	0.0125	.3660	.965	-.7911	1.5231
		0.0062	.7139	.525	-.4432	1.8709
		0.0031	.9443	.192	-.2128	2.1014
		0.2	-2.2057*	.000	-3.3628	-1.0486
		0.1	-1.2320*	.029	-2.3891	-.0749
		0.05	-.9674	.169	-2.1245	.1897
	0.0125	0.025	-.3660	.965	-1.5231	.7911
		0.0062	.3479	.973	-.8092	1.5049
		0.0031	.5783	.752	-.5788	1.7354
		0.2	-2.5536*	.000	-3.7106	-1.3965
		0.1	-1.5799*	.001	-2.7369	-.4228
		0.05	-1.3153*	.015	-2.4724	-.1582
	0.0062	0.025	-.7139	.525	-1.8709	.4432
		0.0125	-.3479	.973	-1.5049	.8092
		0.0031	.2304	.997	-.9267	1.3875
		0.2	-2.7840*	.000	-3.9411	-1.6269
		0.1	-1.8103*	.000	-2.9674	-.6532
		0.05	-1.5457*	.002	-2.7028	-.3886
	0.0031	0.025	-.9443	.192	-2.1014	.2128
		0.0125	-.5783	.752	-1.7354	.5788
		0.0062	-.2304	.997	-1.3875	.9267
CFU		0.1	1.6824*	.034	.0740	3.2907
		0.05	2.1770*	.001	.5687	3.7853
		0.025	2.3434*	.000	.7351	3.9518
	0.2	0.0125	2.5203*	.000	.9120	4.1286
		0.0062	2.7325*	.000	1.1242	4.3408
		0.0031	2.8352*	.000	1.2269	4.4436
		0.2	-1.6824*	.034	-3.2907	-.0740
	0.1	0.05	.4946	.970	-1.1137	2.1030

		0.025	.6611	.884	-.9473	2.2694
		0.0125	.8379	.713	-.7704	2.4462
		0.0062	1.0501	.454	-.5582	2.6584
		0.0031	1.1529	.337	-.4555	2.7612
		0.2	-2.1770*	.001	-3.7853	-.5687
		0.1	-.4946	.970	-2.1030	1.1137
	0.05	0.025	.1664	1.000	-1.4419	1.7748
		0.0125	.3433	.996	-1.2650	1.9516
		0.0062	.5555	.947	-1.0528	2.1638
		0.0031	.6582	.886	-.9501	2.2666
		0.2	-2.3434*	.000	-3.9518	-.7351
		0.1	-.6611	.884	-2.2694	.9473
	0.025	0.05	-.1664	1.000	-1.7748	1.4419
		0.0125	.1769	1.000	-1.4315	1.7852
		0.0062	.3891	.991	-1.2193	1.9974
		0.0031	.4918	.971	-1.1165	2.1001
		0.2	-2.5203*	.000	-4.1286	-.9120
		0.1	-.8379	.713	-2.4462	.7704
	0.0125	0.05	-.3433	.996	-1.9516	1.2650
		0.025	-.1769	1.000	-1.7852	1.4315
		0.0062	.2122	1.000	-1.3961	1.8205
		0.0031	.3149	.997	-1.2934	1.9233
		0.2	-2.7325*	.000	-4.3408	-1.1242
		0.1	-1.0501	.454	-2.6584	.5582
	0.0062	0.05	-.5555	.947	-2.1638	1.0528
		0.025	-.3891	.991	-1.9974	1.2193
		0.0125	-.2122	1.000	-1.8205	1.3961
		0.0031	.1027	1.000	-1.5056	1.7111
		0.2	-2.8352*	.000	-4.4436	-1.2269
		0.1	-1.1529	.337	-2.7612	.4555
	0.0031	0.05	-.6582	.886	-2.2666	.9501
		0.025	-.4918	.971	-2.1001	1.1165
		0.0125	-.3149	.997	-1.9233	1.2934
		0.0062	-.1027	1.000	-1.7111	1.5056
ATP		0.1	1.0927*	.008	.1785	2.0069
		0.05	1.5063*	.000	.5921	2.4205
		0.025	1.5443*	.000	.6301	2.4585
	0.2	0.0125	1.7070*	.000	.7928	2.6212
		0.0062	1.7571*	.000	.8429	2.6713
		0.0031	1.9070*	.000	.9928	2.8212
		0.2	-1.0927*	.008	-2.0069	-.1785
	0.1	0.05	.4136	.829	-.5006	1.3278
		0.025	.4516	.762	-.4626	1.3658

		0.0125	.6143	.417	-.2999	1.5285
		0.0062	.6644	.320	-.2498	1.5786
		0.0031	.8143	.116	-.0999	1.7285
		0.2	-1.5063*	.000	-2.4205	-.5921
		0.1	-.4136	.829	-1.3278	.5006
	0.05	0.025	.0380	1.000	-.8762	.9522
		0.0125	.2007	.995	-.7135	1.1149
		0.0062	.2508	.983	-.6634	1.1650
		0.0031	.4007	.849	-.5135	1.3149
		0.2	-1.5443*	.000	-2.4585	-.6301
		0.1	-.4516	.762	-1.3658	.4626
	0.025	0.05	-.0380	1.000	-.9522	.8762
		0.0125	.1627	.998	-.7515	1.0769
		0.0062	.2128	.993	-.7014	1.1270
		0.0031	.3627	.901	-.5515	1.2769
		0.2	-1.7070*	.000	-2.6212	-.7928
		0.1	-.6143	.417	-1.5285	.2999
	0.0125	0.05	-.2007	.995	-1.1149	.7135
		0.025	-.1627	.998	-1.0769	.7515
		0.0062	.0501	1.000	-.8641	.9643
		0.0031	.2000	.995	-.7142	1.1142
		0.2	-1.7571*	.000	-2.6713	-.8429
		0.1	-.6644	.320	-1.5786	.2498
	0.0062	0.05	-.2508	.983	-1.1650	.6634
		0.025	-.2128	.993	-1.1270	.7014
		0.0125	-.0501	1.000	-.9643	.8641
		0.0031	.1499	.999	-.7643	1.0641
		0.2	-1.9070*	.000	-2.8212	-.9928
		0.1	-.8143	.116	-1.7285	.0999
	0.0031	0.05	-.4007	.849	-1.3149	.5135
		0.025	-.3627	.901	-1.2769	.5515
		0.0125	-.2000	.995	-1.1142	.7142
		0.0062	-.1499	.999	-1.0641	.7643
		0.1	-.0161	1.000	-.1519	.1196
		0.05	-.0509	.923	-.1866	.0849
	0.2	0.025	-.0117	1.000	-.1474	.1241
		0.0125	-.0089	1.000	-.1446	.1269
RLUCFU		0.0062	-.0051	1.000	-.1408	.1306
		0.0031	.0388	.979	-.0969	.1746
	0.1	0.2	.0161	1.000	-.1196	.1519
		0.05	-.0347	.988	-.1705	.1010
		0.025	.0045	1.000	-.1313	.1402

	0.0125	.0073	1.000	-.1285	.1430
	0.0062	.0110	1.000	-.1247	.1468
	0.0031	.0550	.891	-.0808	.1907
	0.2	.0509	.923	-.0849	.1866
	0.1	.0347	.988	-.1010	.1705
0.05	0.025	.0392	.978	-.0966	.1749
	0.0125	.0420	.969	-.0937	.1778
	0.0062	.0458	.953	-.0900	.1815
	0.0031	.0897	.439	-.0460	.2255
	0.2	.0117	1.000	-.1241	.1474
	0.1	-.0045	1.000	-.1402	.1313
0.025	0.05	-.0392	.978	-.1749	.0966
	0.0125	.0028	1.000	-.1329	.1386
	0.0062	.0066	1.000	-.1292	.1423
	0.0031	.0505	.925	-.0852	.1863
	0.2	.0089	1.000	-.1269	.1446
	0.1	-.0073	1.000	-.1430	.1285
0.0125	0.05	-.0420	.969	-.1778	.0937
	0.025	-.0028	1.000	-.1386	.1329
	0.0062	.0038	1.000	-.1320	.1395
	0.0031	.0477	.943	-.0881	.1834
	0.2	.0051	1.000	-.1306	.1408
	0.1	-.0110	1.000	-.1468	.1247
0.0062	0.05	-.0458	.953	-.1815	.0900
	0.025	-.0066	1.000	-.1423	.1292
	0.0125	-.0038	1.000	-.1395	.1320
	0.0031	.0439	.961	-.0918	.1797
	0.2	-.0388	.979	-.1746	.0969
	0.1	-.0550	.891	-.1907	.0808
0.0031	0.05	-.0897	.439	-.2255	.0460
	0.025	-.0505	.925	-.1863	.0852
	0.0125	-.0477	.943	-.1834	.0881
	0.0062	-.0439	.961	-.1797	.0918

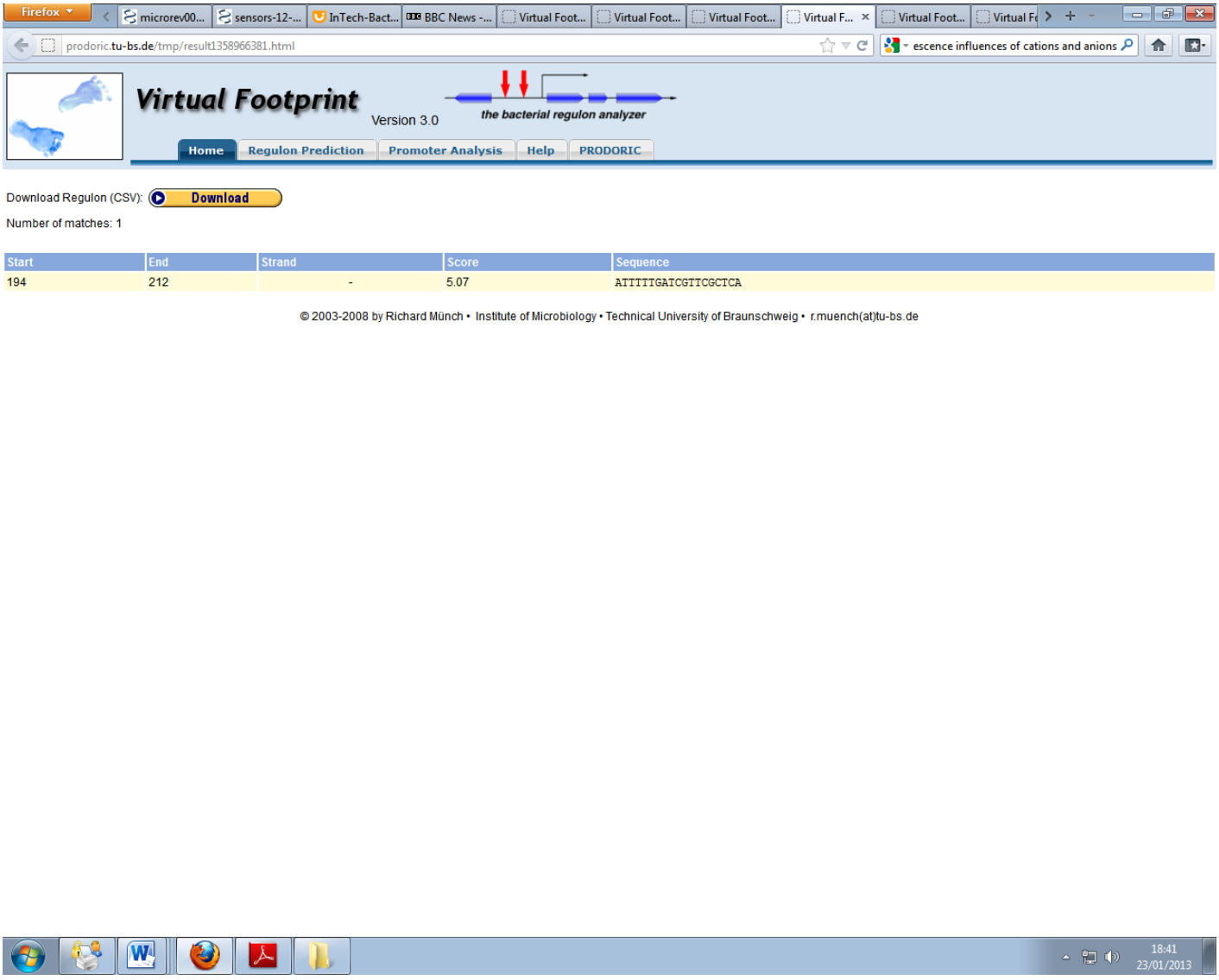


Figure 4.1: Virtual Foot printing analysis for Lpp sequences. Crp locus is found in the blue font color below

tattgcttttgtgaattaatttgtatatcgaagcgccctgatgggcgctttttttaTTTAATcgataaccagaagcaATAAAAAATcaaat
cggatttcactatataatctcactttatctaagatgaatccgatggaagcatcctgttttctctcaatttttttatctaaaacccagcgttcgatgcttctttga
gcgaacgatcaaaaataagtgccttcccatcaaaaaaataTTCTCAacataaaaaacttgtgtAATACTtgtaacgctacatggagatta
actcaatctagagggtattaataatgaaagctactaaactggtactgggcgcggtaatcctgggttctactctgctggcaggttgctccagcaacgct
aaaatcgatcagctgtcttctgacgttcagactctgaacgctaaagttgaccagctgagcaacgacgtgaacgcaatgcgttccgacgttcaggct
gctaaagatgacgcagctcgtgctaaccagcgtctggacaacatggctactaaataccgcaagtaatagtacctg

The light blue fonts are where the *crp* locus was identified based on the start and end positions.

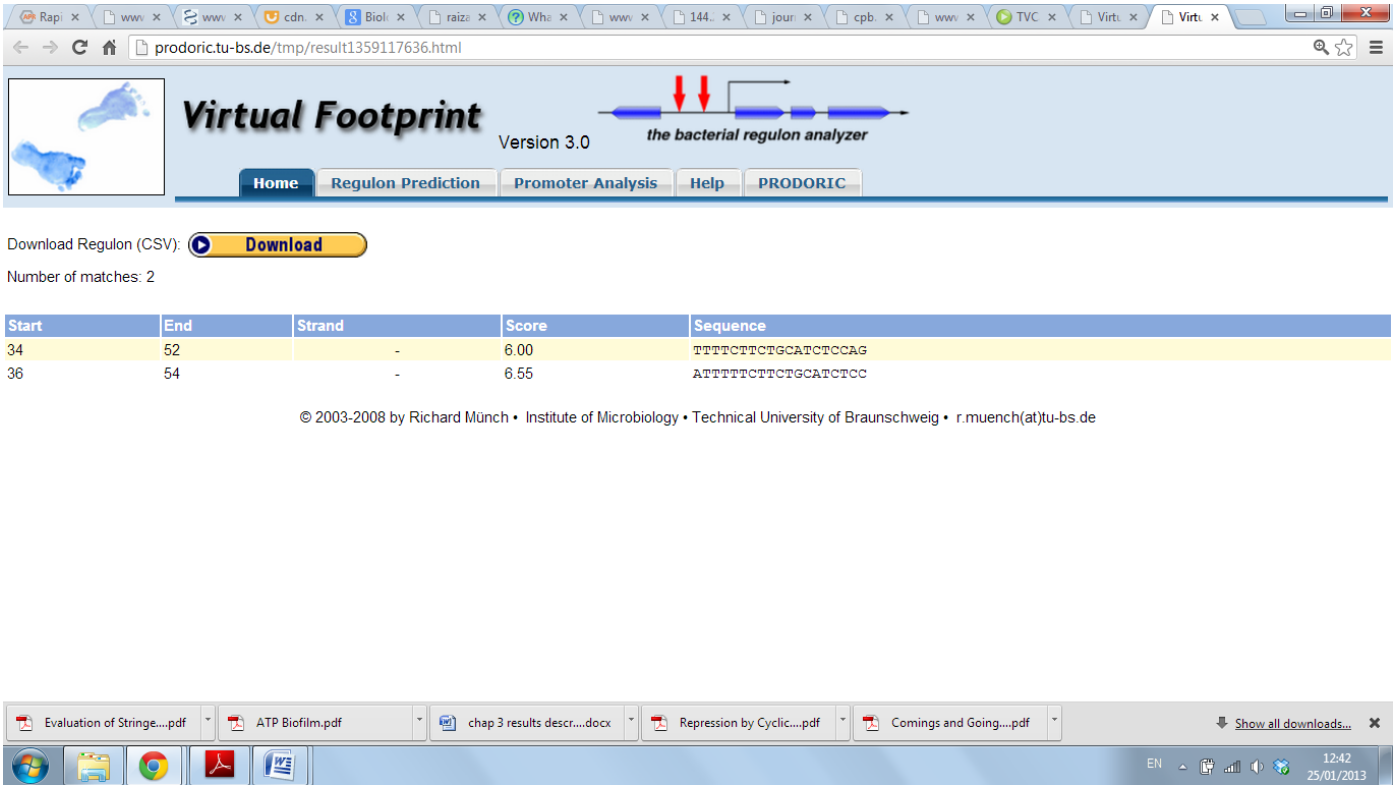


Figure 4.4 : Virtual Foot printing analysis for LysS sequences. Crp locus is found in the negative strand, corresponding complement sequences were not found within the lyss promoter region.

caggccatgaagcagatgaaagcgaagcTTTATGaactggagatgcagAACAAAAAtgccgagaaac
aggcgatggaagataacaaatccgacatcggctggggcagccagattcgttcttatgtccttgatga
ctcccgcattaaagatctgcgcaccggggtagaaacccgcaacacgcaggccgtgctggacggcagc
ctggatcaatttatcgaagcaagtttgaaagcagggttatgaggaaccaaca

The two *crp* locus were found to overlap within the positions indicated in blue fonts

A second lpp promoter was located (RegulonDB)

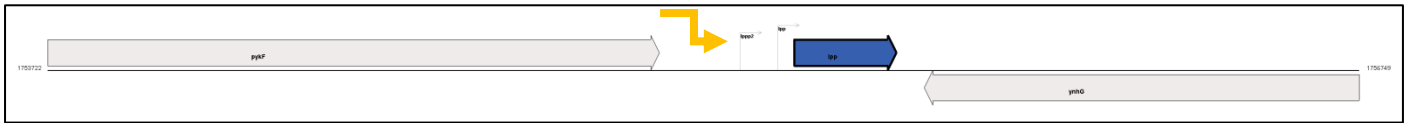


Figure 4.2: Lpp promoter obtained from Regulon DB indicates 2 promoters

Appendix 5

Evaluation of Whole-cell Bioluminescence in Benzalkonium Chloride

Table 5.1: Standard Error for Bioluminescence (RLU/ml) readings in Benzalkonium Chloride

Grand Mean

Dependent Variable: RLU

Mean	Std. Error	95% Confidence Interval	
		Lower Bound	Upper Bound
1.699	.106	1.490	1.908

Table 5.2: Standard Error for plate counts (CFU/ml) readings in Benzalkonium Chloride

Grand Mean

Dependent Variable: CFU

Mean	Std. Error	95% Confidence Interval	
		Lower Bound	Upper Bound
2.628	.154	2.325	2.932

Table 5.3: Standard Error for ATP-Chemiluminescence (RLU/ml) readings in Benzalkonium Chloride

Grand Mean

Dependent Variable: ATP

Mean	Std. Error	95% Confidence Interval	
		Lower Bound	Upper Bound
2.614	.102	2.413	2.814

Table 5.4: F test for RLU against CFU and ATP-bioluminescence

Levene's Test of Equality of Error Variances[a]

Dependent Variable: RLU

F	df1	df2	Sig.
.958	159	50	.590

Table 5.5: Overal SEM for RLU Benzalkonium Chloride +0.03% EDTA

Grand Mean

Dependent Variable: RLU

Mean	Std. Error	95% Confidence Interval	
		Lower Bound	Upper Bound
1.344	.095	1.157	1.530

Table 5.6: Standard Error for plate counts (CFU/ml) readings in Benzalkonium Chloride +0.03% EDTA

Grand Mean

Dependent Variable: CFU

Mean	Std. Error	95% Confidence Interval	
		Lower Bound	Upper Bound
2.120	.164	1.797	2.443

Table 5.7: Standard Error for ATP bioluminescence (RLU/ml) readings in Benzalkonium Chloride +0.03% EDTA

Grand Mean

Dependent Variable: ATP

Mean	Std. Error	95% Confidence Interval	
		Lower Bound	Upper Bound
2.101	.107	1.889	2.312

Table 5.8: F test for RLU against CFU and ATP-bioluminescence readings

Levene's Test of Equality of Error Variances[a]

Dependent Variable: RLU

F	df1	df2	Sig.
.583	155	54	.994

Table 5.9: Tukey for bioluminescence, Viable Counts ATP-chemiluminescence, and bioluminescence per cell readings in BAK preservative

Multiple Comparisons

Dependent Variable: RLU

(I) Biosensor	(J) Biosensor	Mean Difference (I-J)	Sig.	95% Confidence Interval	
				Lower Bound	Upper Bound
lpp	tatA	-.9418*	.011	-1.6690	-.2146
	Ldc	-.7265	.050	-1.4537	.0007
	Lyss	-.5990	.056	-1.3262	.1282
	Spc	-.8974*	.016	-1.6246	-.1702
tatA	lpp	.9418*	.011	.2146	1.6690
	Ldc	.2153	.560	-.5119	.9425
	Lyss	.3428	.354	-.3844	1.0700
	Spc	.0444	.904	-.6828	.7716
Ldc	lpp	.7265	.050	-.0007	1.4537
	tatA	-.2153	.560	-.9425	.5119
	Lyss	.1275	.730	-.5997	.8547
	Spc	-.1709	.644	-.8981	.5563
Lyss	lpp	.5990	.500	-.1282	1.3262
	tatA	-.3428	.354	-1.0700	.3844
	Ldc	-.1275	.730	-.8547	.5997
	Spc	-.2984	.419	-1.0256	.4288
Spc	lpp	.8974*	.016	.1702	1.6246
	tatA	-.0444	.904	-.7716	.6828
	Ldc	.1709	.644	-.5563	.8981
	Lyss	.2984	.419	-.4288	1.0256

Multiple Comparisons

Dependent Variable: CFU

Tukey HSD

(I) Biosensor	(J) Biosensor	Mean Difference (I-J)	Sig.	95% Confidence Interval	
				Lower Bound	Upper Bound
lpp	tatA	-.0025	1.000	-1.4747	1.4696
	Ldc	-.0312	1.000	-1.5034	1.4410
	Lyss	-.0402	1.000	-1.5124	1.4320
	Spc	-.0175	1.000	-1.4897	1.4547
tatA	lpp	.0025	1.000	-1.4696	1.4747
	Ldc	-.0287	1.000	-1.5008	1.4435
	Lyss	-.0377	1.000	-1.5099	1.4345
	Spc	-.0150	1.000	-1.4871	1.4572
Ldc	lpp	.0312	1.000	-1.4410	1.5034
	tatA	.0287	1.000	-1.4435	1.5008
	Lyss	-.0090	1.000	-1.4812	1.4632
	Spc	.0137	1.000	-1.4585	1.4859
Lyss	lpp	.0402	1.000	-1.4320	1.5124
	tatA	.0377	1.000	-1.4345	1.5099
	Ldc	.0090	1.000	-1.4632	1.4812
	Spc	.0227	1.000	-1.4495	1.4949
Spc	lpp	.0175	1.000	-1.4547	1.4897
	tatA	.0150	1.000	-1.4572	1.4871
	Ldc	-.0137	1.000	-1.4859	1.4585
	Lyss	-.0227	1.000	-1.4949	1.4495

Multiple Comparisons

Dependent Variable: ATP

Tukey HSD

(I) Biosensor	(J) Biosensor	Mean Difference (I-J)	Sig.	95% Confidence Interval	
				Lower Bound	Upper Bound
lpp	tatA	.0413	1.000	-1.0149	1.0975
	Ldc	-.0891	.999	-1.1453	.9671
	Lyss	-.0891	.999	-1.1453	.9671
	Spc	.1158	.998	-.9404	1.1720
tatA	lpp	-.0413	1.000	-1.0975	1.0149
	Ldc	-.1305	.997	-1.1867	.9257
	Lyss	-.1305	.997	-1.1867	.9257

Ldc	Spc	.0745	1.000	-.9817	1.1307
	lpp	.0891	.999	-.9671	1.1453
	tatA	.1305	.997	-.9257	1.1867
	Lyss	.0000	1.000	-1.0562	1.0562
Lyss	Spc	.2049	.984	-.8512	1.2611
	lpp	.0891	.999	-.9671	1.1453
	tatA	.1305	.997	-.9257	1.1867
	Ldc	.0000	1.000	-1.0562	1.0562
Spc	Spc	.2049	.984	-.8512	1.2611
	lpp	-.1158	.998	-1.1720	.9404
	tatA	-.0745	1.000	-1.1307	.9817
	Ldc	-.2049	.984	-1.2611	.8512
	Lyss	-.2049	.984	-1.2611	.8512

Multiple Comparisons

Dependent Variable: RLUCFU

Tukey HSD

(I) Biosensor	(J) Biosensor	Mean Difference (I-J)	Sig.	95% Confidence Interval	
				Lower Bound	Upper Bound
lpp	tatA	-.0741*	.015	-.1387	-.0096
	Ldc	.0006	1.000	-.0640	.0651
	Lyss	.0188	.930	-.0458	.0833
	Spc	-.0414	.039	-.1059	.0232
tatA	lpp	.0741*	.015	.0096	.1387
	Ldc	.0747*	.014	.0102	.1392
	Lyss	.0929*	.001	.0284	.1574
	Spc	.0328	.629	-.0317	.0973
Ldc	lpp	-.0006	1.000	-.0651	.0640
	tatA	-.0747*	.014	-.1392	-.0102
	Lyss	.0182	.937	-.0463	.0827
	Spc	-.0419	.383	-.1064	.0226
Lyss	lpp	-.0188	.930	-.0833	.0458
	tatA	-.0929*	.001	-.1574	-.0284
	Ldc	-.0182	.937	-.0827	.0463
	Spc	-.0601	.081	-.1246	.0044
Spc	lpp	.0414	.039	-.0232	.1059
	tatA	-.0328	.629	-.0973	.0317
	Ldc	.0419	.038	-.0226	.1064
	Lyss	.0601	.041	-.0044	.1246

Table 5.10: Tukey for bioluminescence, ATP-chemiluminescence, and bioluminescence per cell readings against time in BAK preservative

Multiple Comparisons

Dependent Variable: RLU

Tukey HSD

(I) time	(J) time	Mean Difference (I-J)	Sig.	95% Confidence Interval	
				Lower Bound	Upper Bound
0 hr	6 hr	1.4470*	.004	.3079	2.5862
	24 hr	2.0160*	.000	.8769	3.1551
	168 hr	2.3331*	.000	1.1939	3.4722
	336 hr	2.5484*	.000	1.4092	3.6875
	504 hr	2.5803*	.000	1.4412	3.7195
	672 hr	2.6687*	.000	1.5296	3.8078
6 hr	0 hr	-1.4470*	.004	-2.5862	-.3079
	24 hr	.5690	.752	-.5702	1.7081
	168 hr	.8860	.241	-.2531	2.0252
	336 hr	1.1013	.065	-.0378	2.2405
	504 hr	1.1333	.052	-.0059	2.2724
	672 hr	1.2217*	.027	.0825	2.3608
24 hr	0 hr	-2.0160*	.000	-3.1551	-.8769
	6 hr	-.5690	.752	-1.7081	.5702
	168 hr	.3171	.982	-.8221	1.4562
	336 hr	.5324	.806	-.6068	1.6715
	504 hr	.5643	.759	-.5748	1.7035
	672 hr	.6527	.613	-.4864	1.7918
168 hr	0 hr	-2.3331*	.000	-3.4722	-1.1939
	6 hr	-.8860	.241	-2.0252	.2531
	24 hr	-.3171	.982	-1.4562	.8221
	336 hr	.2153	.998	-.9238	1.3545
	504 hr	.2473	.995	-.8919	1.3864
	672 hr	.3356	.976	-.8035	1.4748
336 hr	0 hr	-2.5484*	.000	-3.6875	-1.4092
	6 hr	-1.1013	.065	-2.2405	.0378
	24 hr	-.5324	.806	-1.6715	.6068
	168 hr	-.2153	.998	-1.3545	.9238
	504 hr	.0320	1.000	-1.1072	1.1711
	672 hr	.1203	1.000	-1.0188	1.2595
504 hr	0 hr	-2.5803*	.000	-3.7195	-1.4412
	6 hr	-1.1333	.052	-2.2724	.0059
	24 hr	-.5643	.759	-1.7035	.5748
	168 hr	-.2473	.995	-1.3864	.8919
	336 hr	-.0320	1.000	-1.1711	1.1072
	672 hr	.0884	1.000	-1.0508	1.2275

0 hr	-2.6687*	.000	-3.8078	-1.5296
6 hr	-1.2217*	.027	-2.3608	-.0825
24 hr	-.6527	.613	-1.7918	.4864
168 hr	-.3356	.976	-1.4748	.8035
336 hr	-.1203	1.000	-1.2595	1.0188
504 hr	-.0884	1.000	-1.2275	1.0508

Multiple Comparisons

Dependent Variable: CFU

Tukey HSD

(I) time	(J) time	Mean Difference (I-J)	Sig.	95% Confidence Interval	
				Lower Bound	Upper Bound
0 hr	6 hr	3.1692*	.000	1.5680	4.7704
	24 hr	3.4042*	.000	1.8030	5.0054
	168 hr	3.5472*	.000	1.9460	5.1484
	336 hr	3.7170*	.000	2.1158	5.3182
	504 hr	3.9082*	.000	2.3070	5.5094
	672 hr	3.9826*	.000	2.3814	5.5838
6 hr	0 hr	-3.1692*	.000	-4.7704	-1.5680
	24 hr	.2349	.999	-1.3663	1.8362
	168 hr	.3780	.992	-1.2232	1.9792
	336 hr	.5477	.949	-1.0535	2.1489
	504 hr	.7390	.815	-.8622	2.3402
	672 hr	.8134	.737	-.7878	2.4146
24 hr	0 hr	-3.4042*	.000	-5.0054	-1.8030
	6 hr	-.2349	.999	-1.8362	1.3663
	168 hr	.1431	1.000	-1.4582	1.7443
	336 hr	.3128	.997	-1.2884	1.9140
	504 hr	.5040	.966	-1.0972	2.1052
	672 hr	.5784	.935	-1.0228	2.1796
168 hr	0 hr	-3.5472*	.000	-5.1484	-1.9460
	6 hr	-.3780	.992	-1.9792	1.2232
	24 hr	-.1431	1.000	-1.7443	1.4582
	336 hr	.1697	1.000	-1.4315	1.7709
	504 hr	.3610	.994	-1.2402	1.9622
	672 hr	.4354	.984	-1.1658	2.0366
336 hr	0 hr	-3.7170*	.000	-5.3182	-2.1158
	6 hr	-.5477	.949	-2.1489	1.0535
	24 hr	-.3128	.997	-1.9140	1.2884
	168 hr	-.1697	1.000	-1.7709	1.4315
	504 hr	.1912	1.000	-1.4100	1.7925
	672 hr	.2657	.999	-1.3356	1.8669
504 hr	0 hr	-3.9082*	.000	-5.5094	-2.3070
	6 hr	-.7390	.815	-2.3402	.8622
	24 hr	-.5040	.966	-2.1052	1.0972
	168 hr	-.3610	.994	-1.9622	1.2402
	336 hr	-.1912	1.000	-1.7925	1.4100
	672 hr	.0744	1.000	-1.5268	1.6756
672 hr	0 hr	-3.9826*	.000	-5.5838	-2.3814
	6 hr	-.8134	.737	-2.4146	.7878
	24 hr	-.5784	.935	-2.1796	1.0228
	168 hr	-.4354	.984	-2.0366	1.1658
	336 hr	-.2657	.999	-1.8669	1.3356
	504 hr	-.0744	1.000	-1.6756	1.5268

Multiple Comparisons

Dependent Variable: ATP

Tukey HSD

(I) time	(J) time	Mean Difference (I-J)	Sig.	95% Confidence Interval	
				Lower Bound	Upper Bound
0 hr	6 hr	1.4234*	.009	.2280	2.6188
	24 hr	1.9575*	.000	.7620	3.1529
	168 hr	2.0349*	.000	.8395	3.2303
	336 hr	2.2200*	.000	1.0246	3.4155
	504 hr	2.5442*	.000	1.3488	3.7396
	672 hr	2.5970*	.000	1.4016	3.7925
6 hr	0 hr	-1.4234*	.009	-2.6188	-.2280
	24 hr	.5341	.837	-.6614	1.7295
	168 hr	.6115	.730	-.5839	1.8069
	336 hr	.7966	.428	-.3988	1.9921
	504 hr	1.1208	.082	-.0746	2.3162
	672 hr	1.1736	.058	-.0218	2.3691
24 hr	0 hr	-1.9575*	.000	-3.1529	-.7620
	6 hr	-.5341	.837	-1.7295	.6614
	168 hr	.0774	1.000	-1.1180	1.2729
	336 hr	.2626	.995	-.9328	1.4580
	504 hr	.5867	.767	-.6087	1.7822
	672 hr	.6396	.687	-.5559	1.8350
168 hr	0 hr	-2.0349*	.000	-3.2303	-.8395
	6 hr	-.6115	.730	-1.8069	.5839
	24 hr	-.0774	1.000	-1.2729	1.1180
	336 hr	.1851	.999	-1.0103	1.3806
	504 hr	.5093	.865	-.6861	1.7047
	672 hr	.5621	.801	-.6333	1.7576
336 hr	0 hr	-2.2200*	.000	-3.4155	-1.0246
	6 hr	-.7966	.428	-1.9921	.3988
	24 hr	-.2626	.995	-1.4580	.9328
	168 hr	-.1851	.999	-1.3806	1.0103
	504 hr	.3242	.984	-.8713	1.5196
	672 hr	.3770	.966	-.8184	1.5724
504 hr	0 hr	-2.5442*	.000	-3.7396	-1.3488
	6 hr	-1.1208	.082	-2.3162	.0746
	24 hr	-.5867	.767	-1.7822	.6087
	168 hr	-.5093	.865	-1.7047	.6861
	336 hr	-.3242	.984	-1.5196	.8713
	672 hr	.0528	1.000	-1.1426	1.2483
672 hr	0 hr	-2.5970*	.000	-3.7925	-1.4016

6 hr	-1.1736	.058	-2.3691	.0218
24 hr	-.6396	.687	-1.8350	.5559
168 hr	-.5621	.801	-1.7576	.6333
336 hr	-.3770	.966	-1.5724	.8184
504 hr	-.0528	1.000	-1.2483	1.1426

Multiple Comparisons

Dependent Variable: RLUCFU

Tukey HSD

(I) time	(J) time	Mean Difference (I-J)	Sig.	95% Confidence Interval	
				Lower Bound	Upper Bound
0 hr	6 hr	-.0109	1.000	-.0960	.0742
	24 hr	.0539	.491	-.0312	.1390
	168 hr	.0441	.717	-.0409	.1292
	336 hr	.0520	.535	-.0331	.1371
	504 hr	.0366	.861	-.0485	.1217
	672 hr	.0423	.756	-.0428	.1274
6 hr	0 hr	.0109	1.000	-.0742	.0960
	24 hr	.0648	.264	-.0203	.1499
	168 hr	.0550	.465	-.0300	.1401
	336 hr	.0629	.299	-.0222	.1480
	504 hr	.0475	.643	-.0376	.1326
	672 hr	.0532	.508	-.0319	.1383
24 hr	0 hr	-.0539	.491	-.1390	.0312
	6 hr	-.0648	.264	-.1499	.0203
	168 hr	-.0098	1.000	-.0949	.0753
	336 hr	-.0019	1.000	-.0870	.0832
	504 hr	-.0174	.997	-.1025	.0677
	672 hr	-.0116	1.000	-.0967	.0735
168 hr	0 hr	-.0441	.717	-.1292	.0409
	6 hr	-.0550	.465	-.1401	.0300
	24 hr	.0098	1.000	-.0753	.0949
	336 hr	.0079	1.000	-.0772	.0930
	504 hr	-.0076	1.000	-.0927	.0775
	672 hr	-.0018	1.000	-.0869	.0833
336 hr	0 hr	-.0520	.535	-.1371	.0331
	6 hr	-.0629	.299	-.1480	.0222
	24 hr	.0019	1.000	-.0832	.0870
	168 hr	-.0079	1.000	-.0930	.0772
	504 hr	-.0155	.998	-.1006	.0696
	672 hr	-.0097	1.000	-.0948	.0754
504 hr	0 hr	-.0366	.861	-.1217	.0485
	6 hr	-.0475	.643	-.1326	.0376
	24 hr	.0174	.997	-.0677	.1025
	168 hr	.0076	1.000	-.0775	.0927
	336 hr	.0155	.998	-.0696	.1006
	672 hr	.0057	1.000	-.0793	.0908
672 hr	0 hr	-.0423	.756	-.1274	.0428
	6 hr	-.0532	.508	-.1383	.0319
	24 hr	.0116	.02857	-.0735	.0967
	168 hr	.0018	.02857	-.0833	.0869
	336 hr	.0097	.02857	-.0754	.0948
	504 hr	-.0057	.02857	-.0908	.0793

Table 5.10: Tukey for bioluminescence, ATP-chemiluminescence, and bioluminescence per cell readings against concentrations in BAK preservative

Multiple Comparisons

Dependent Variable: RLU

Tukey HSD

(I) Concentration	(J) Concentration	Mean Difference (I-J)	Sig.	95% Confidence Interval	
				Lower Bound	Upper Bound
0.0062	0.0031	1.1612*	.001	.3392	1.9833
	0.0016	2.4880*	.000	1.6660	3.3100
	0.0007	2.6013*	.000	1.7792	3.4233
	0.0003	2.5956*	.000	1.7735	3.4176
0.0031	0.0062	-1.1612*	.001	-1.9833	-.3392
	0.0016	1.3267*	.000	.5047	2.1488
	0.0007	1.4400*	.000	.6180	2.2620
	0.0003	1.4343*	.000	.6123	2.2563
0.0016	0.0062	-2.4880*	.000	-3.3100	-1.6660
	0.0031	-1.3267*	.000	-2.1488	-.5047
	0.0007	.1133	.999	-.7088	.9353
	0.0003	.1076	.999	-.7145	.9296
0.0007	0.0062	-2.6013*	.000	-3.4233	-1.7792
	0.0031	-1.4400*	.000	-2.2620	-.6180
	0.0016	-.1133	.999	-.9353	.7088
	0.0003	-.0057	1.000	-.8277	.8163
0.0003	0.0062	-2.5956*	.000	-3.4176	-1.7735
	0.0031	-1.4343*	.000	-2.2563	-.6123
	0.0016	-.1076	.999	-.9296	.7145
	0.0007	.0057	1.000	-.8163	.8277

Multiple Comparisons

Dependent Variable: CFU

Tukey HSD

(I) Concentration	(J) Concentration	Mean Difference (I-J)	Sig.	95% Confidence Interval	
				Lower Bound	Upper Bound
	0.0062	.2076	.993	-.8546	1.2697
	0.0031	1.4966*	.001	.4345	2.5588
0.0125	0.0016	4.2165*	.000	3.1543	5.2786
	0.0007	4.2143*	.000	3.1521	5.2764
	0.0003	4.2219*	.000	3.1597	5.2840
	0.0125	-.2076	.993	-1.2697	.8546
	0.0031	1.2890*	.008	.2269	2.3512
0.0062	0.0016	4.0089*	.000	2.9468	5.0710
	0.0007	4.0067*	.000	2.9446	5.0688
	0.0003	4.0143*	.000	2.9521	5.0764
	0.0125	-1.4966*	.001	-2.5588	-.4345
	0.0062	-1.2890*	.008	-2.3512	-.2269
0.0031	0.0016	2.7199*	.000	1.6577	3.7820
	0.0007	2.7176*	.000	1.6555	3.7798
	0.0003	2.7252*	.000	1.6631	3.7874
	0.0125	-4.2165*	.000	-5.2786	-3.1543
	0.0062	-4.0089*	.000	-5.0710	-2.9468
0.0016	0.0031	-2.7199*	.000	-3.7820	-1.6577
	0.0007	-.0022	1.000	-1.0643	1.0599
	0.0003	.0054	1.000	-1.0568	1.0675
	0.0125	-4.2143*	.000	-5.2764	-3.1521
	0.0062	-4.0067*	.000	-5.0688	-2.9446
0.0007	0.0031	-2.7176*	.000	-3.7798	-1.6555
	0.0016	.0022	1.000	-1.0599	1.0643
	0.0003	.0076	1.000	-1.0545	1.0697
	0.0125	-4.2219*	.000	-5.2840	-3.1597
	0.0062	-4.0143*	.000	-5.0764	-2.9521
0.0003	0.0031	-2.7252*	.000	-3.7874	-1.6631
	0.0016	-.0054	1.000	-1.0675	1.0568
	0.0007	-.0076	1.000	-1.0697	1.0545

Multiple Comparisons

Dependent Variable: ATP

Tukey HSD

(I) Concentration	(J) Concentration	Mean Difference (I-J)	Sig.	95% Confidence Interval	
				Lower Bound	Upper Bound
	0.0062	.0428	1.000	-.6521	.7377
	0.0031	1.2286*	.000	.5337	1.9235
0.0125	0.0016	3.0513*	.000	2.3563	3.7462
	0.0007	3.1512*	.000	2.4563	3.8461
	0.0003	3.2682*	.000	2.5733	3.9631
	0.0125	-.0428	1.000	-.7377	.6521
	0.0031	1.1858*	.000	.4908	1.8807
0.0062	0.0016	3.0084*	.000	2.3135	3.7033
	0.0007	3.1084*	.000	2.4135	3.8033
	0.0003	3.2254*	.000	2.5304	3.9203
	0.0125	-1.2286*	.000	-1.9235	-.5337
	0.0062	-1.1858*	.000	-1.8807	-.4908
0.0031	0.00016	1.8227*	.000	1.1278	2.5176
	0.0007	1.9226*	.000	1.2277	2.6175
	0.0003	2.0396*	.000	1.3447	2.7345
	0.0125	-3.0513*	.000	-3.7462	-2.3563
	0.0062	-3.0084*	.000	-3.7033	-2.3135
0.0016	0.0031	-1.8227*	.000	-2.5176	-1.1278
	0.0007	.1000	.998	-.5950	.7949
	0.0003	.2169	.947	-.4780	.9118
	0.0125	-3.1512*	.000	-3.8461	-2.4563
	0.0062	-3.1084*	.000	-3.8033	-2.4135
0.0007	0.0031	-1.9226*	.000	-2.6175	-1.2277
	0.0016	-.1000	.998	-.7949	.5950
	0.0003	.1170	.997	-.5779	.8119
	0.0125	-3.2682*	.000	-3.9631	-2.5733
	0.0062	-3.2254*	.000	-3.9203	-2.5304
0.0003	0.0031	-2.0396*	.000	-2.7345	-1.3447
	0.0016	-.2169	.947	-.9118	.4780
	0.0007	-.1170	.997	-.8119	.5779

Multiple Comparisons

Dependent Variable	(I) Biosensor	(J) Biosensor	Mean Difference (I-J)	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
RLU	lpp	tatA	-.4759	.124	-1.0843	.1326
		Ldc	-.3910	.206	-.9994	.2175
		Lyss	-.4425	.153	-1.0509	.1660
		Spc	-.5713	.046	-1.1797	.0372
	tatA	lpp	.4759	.124	-.1326	1.0843
		Ldc	.0849	.783	-.5235	.6934
		Lyss	.0334	.914	-.5750	.6419
		Spc	-.0954	.757	-.7038	.5130
	Ldc	lpp	.3910	.206	-.2175	.9994
		tatA	-.0849	.783	-.6934	.5235
		Lyss	-.0515	.867	-.6600	.5569
		Spc	-.1803	.559	-.7888	.4281
	Lyss	lpp	.4425	.153	-.1660	1.0509
		tatA	-.0334	.914	-.6419	.5750
		Ldc	.0515	.867	-.5569	.6600
		Spc	-.1288	.676	-.7373	.4796
	Spc	lpp	.5713	.046	-.0372	1.1797
		tatA	.0954	.757	-.5130	.7038
		Ldc	.1803	.559	-.4281	.7888
		Lyss	.1288	.676	-.4796	.7373
CFU	lpp	tatA	-.0486	.926	-1.0830	.9858
		Ldc	.0000	1.000	-1.0344	1.0344
		Lyss	-.0486	.926	-1.0830	.9858
		Spc	.0000	1.000	-1.0344	1.0344
	tatA	lpp	.0486	.926	-.9858	1.0830
		Ldc	.0486	.926	-.9858	1.0830
		Lyss	.0000	1.000	-1.0344	1.0344
		Spc	.0486	.926	-.9858	1.0830
	Ldc	lpp	.0000	1.000	-1.0344	1.0344
		tatA	-.0486	.926	-1.0830	.9858
		Lyss	-.0486	.926	-1.0830	.9858
		Spc	.0000	1.000	-1.0344	1.0344
	Lyss	lpp	.0486	.926	-.9858	1.0830
		tatA	.0000	1.000	-1.0344	1.0344
		Ldc	.0486	.926	-.9858	1.0830
		Spc	.0486	.926	-.9858	1.0830
	Spc	lpp	.0000	1.000	-1.0344	1.0344
		tatA	-.0486	.926	-1.0830	.9858

		Ldc	.0000	1.000	-1.0344	1.0344
		Lyss	-.0486	.926	-1.0830	.9858
		tatA	.0000	1.000	-.5325	.5325
	lpp	Ldc	.0000	1.000	-.5325	.5325
		Lyss	.0000	1.000	-.5325	.5325
		Spc	.0000	1.000	-.5325	.5325
		lpp	.0000	1.000	-.5325	.5325
	tatA	Ldc	.0000	1.000	-.5325	.5325
		Lyss	.0000	1.000	-.5325	.5325
		Spc	.0000	1.000	-.5325	.5325
		lpp	.0000	1.000	-.5325	.5325
		tatA	.0000	1.000	-.5325	.5325
ATP	Ldc	Lyss	.0000	1.000	-.5325	.5325
		Spc	.0000	1.000	-.5325	.5325
		lpp	.0000	1.000	-.5325	.5325
		tatA	.0000	1.000	-.5325	.5325
	Lyss	Ldc	.0000	1.000	-.5325	.5325
		Spc	.0000	1.000	-.5325	.5325
		lpp	.0000	1.000	-.5325	.5325
		tatA	.0000	1.000	-.5325	.5325
	Spc	Ldc	.0000	1.000	-.5325	.5325
		Lyss	.0000	1.000	-.5325	.5325
		tatA	-8.0286	.141	-18.7380	2.6809
	lpp	Ldc	-4.3714	.421	-15.0809	6.3380
		Lyss	-10.4857	.055	-21.1951	.2237
		Spc	-9.8286	.072	-20.5380	.8809
		lpp	8.0286	.141	-2.6809	18.7380
	tatA	Ldc	3.6571	.501	-7.0523	14.3666
		Lyss	-2.4571	.651	-13.1666	8.2523
		Spc	-1.8000	.740	-12.5094	8.9094
		lpp	4.3714	.421	-6.3380	15.0809
		tatA	-3.6571	.501	-14.3666	7.0523
RLUCFU	Ldc	Lyss	-6.1143	.261	-16.8237	4.5951
		Spc	-5.4571	.315	-16.1666	5.2523
		lpp	10.4857	.055	-.2237	21.1951
		tatA	2.4571	.651	-8.2523	13.1666
	Lyss	Ldc	6.1143	.261	-4.5951	16.8237
		Spc	.6571	.904	-10.0523	11.3666
		lpp	9.8286	.072	-.8809	20.5380
		tatA	1.8000	.740	-8.9094	12.5094
	Spc	Ldc	5.4571	.315	-5.2523	16.1666
		Lyss	-.6571	.904	-11.3666	10.0523

Table 5.11: Tukey Analysis for BAK + 0.03 % EDTA in terms of RLU, Viable counts, ATP, RLU:CFU

Tukey HSD

Dependent Variable	(I) Concentration	(J) Concentration	Mean Difference (I-J)	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
RLU	0.0062	0.0031	1.1399*	.000	.4027	1.8771
		0.00015	2.0047*	.000	1.2675	2.7419
		0.0007	2.5983*	.000	1.8611	3.3355
		0.0003	2.6783*	.000	1.9411	3.4155
	0.0031	0.0062	-1.1399*	.000	-1.8771	-.4027
		0.0015	.8648*	.013	.1276	1.6020
		0.0007	1.4584*	.000	.7212	2.1956
		0.0003	1.5384*	.000	.8012	2.2756
	0.0016	0.0062	-2.0047*	.000	-2.7419	-1.2675
		0.0031	-.8648*	.013	-1.6020	-.1276
		0.0007	.5935	.177	-.1437	1.3307
		0.0003	.6735	.091	-.0637	1.4107
	0.0007	0.0062	-2.5983*	.000	-3.3355	-1.8611
		0.0031	-1.4584*	.000	-2.1956	-.7212
		0.0016	-.5935	.177	-1.3307	.1437
		0.0003	.0800	.998	-.6572	.8172
	0.0003	0.0062	-2.6783*	.000	-3.4155	-1.9411
		0.0031	-1.5384*	.000	-2.2756	-.8012
		0.0016	-.6735	.091	-1.4107	.0637
		0.0007	-.0800	.998	-.8172	.6572
CFU	0.0062	0.0031	2.5805*	.000	1.4871	3.6740
		0.0016	4.1085*	.000	3.0151	5.2020
		0.0007	4.6941*	.000	3.6006	5.7875
		0.0003	4.7292*	.000	3.6358	5.8227
	0.0031	0.0062	-2.5805*	.000	-3.6740	-1.4871
		0.0016	1.5280*	.002	.4346	2.6215
		0.0007	2.1136*	.000	1.0201	3.2070
		0.0003	2.1487*	.000	1.0553	3.2422
	0.0016	0.0062	-4.1085*	.000	-5.2020	-3.0151
		0.0031	-1.5280*	.002	-2.6215	-.4346
		0.0007	.5855	.579	-.5079	1.6790
		0.0003	.6207	.522	-.4728	1.7141
	0.0007	0.0062	-4.6941*	.000	-5.7875	-3.6006
		0.0031	-2.1136*	.000	-3.2070	-1.0201
		0.0016	-.5855	.579	-1.6790	.5079
		0.0003	.0351	1.000	-1.0583	1.1286
	0.0003	0.0062	-4.7292*	.000	-5.8227	-3.6358
		0.0031	-2.1487*	.000	-3.2422	-1.0553
		0.0016	-.6207	.522	-1.7141	.4728
		0.0007	-.0351	1.000	-1.1286	1.0583
ATP	0.0062	0.0031	.7277*	.005	.1576	1.2978

List of Posters Presented In SGM Conferences 2010, 2011, 2012 And 2013


The study of gene expression in *Escherichia coli* of 5 constitutive promoters.

Choong. M. Y.Y [1], Aldsworth.T [1] and Naseby.D.C [1]

[1] Microbiology, Molecular Biology and Biotechnology Research Group, School of Life Sciences , University of Hertfordshire, Hatfield, Al10 9AB, Hertfordshire

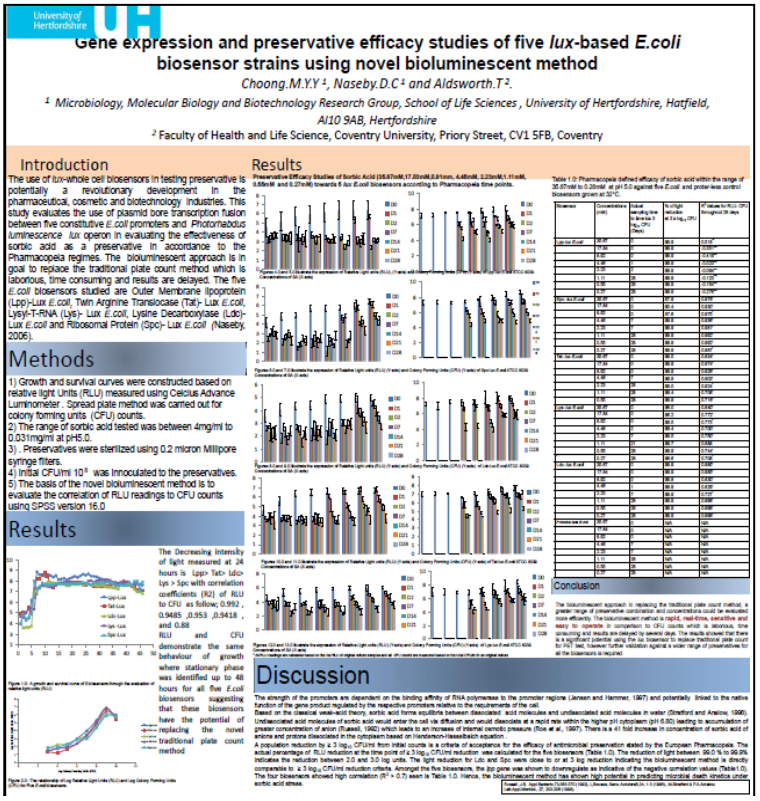
Introduction

Results

Methods

Results and Discussion

Reference


Gene expression and preservative efficacy studies of five *lux*-based *E.coli* biosensor strains using novel bioluminescent method

Choong.M.Y.Y [1], Naseby.D.C [1] and Aldsworth.T [2].

[1] Microbiology, Molecular Biology and Biotechnology Research Group, School of Life Sciences , University of Hertfordshire, Hatfield, Al10 9AB, Hertfordshire

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Introduction

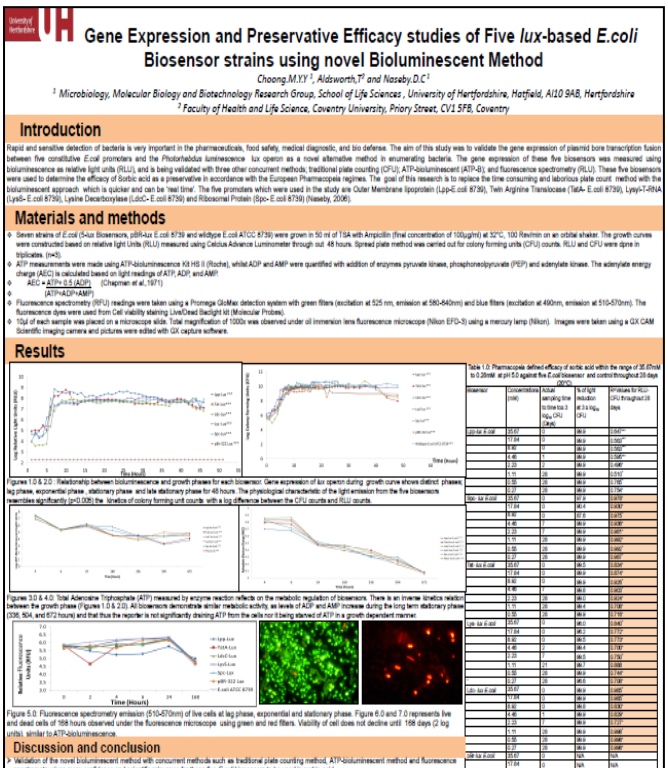
Results

Methods

Results

Discussion

Conclusion


Gene Expression and Preservative Efficacy studies of Five *lux*-based *E.coli* Biosensor strains using novel Bioluminescent Method

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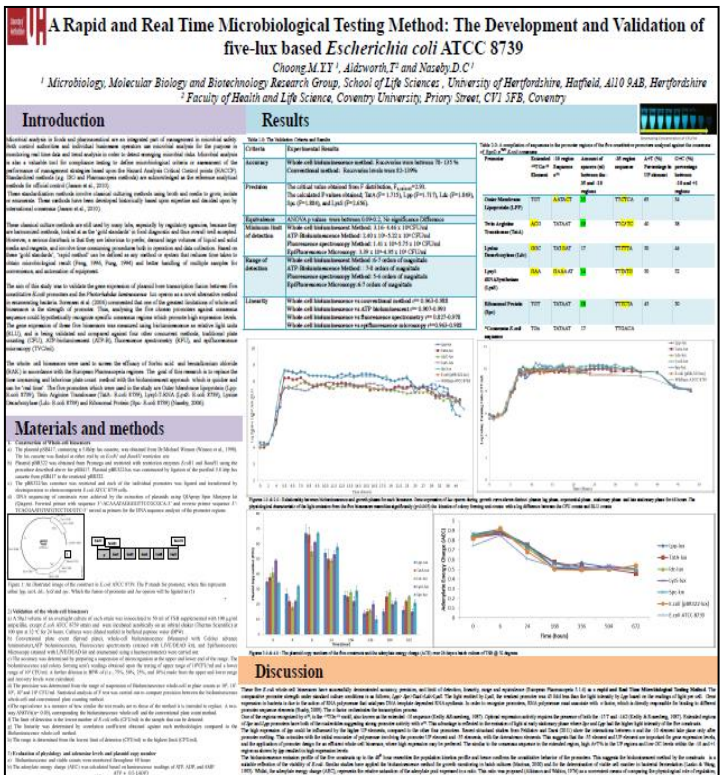
Introduction

Materials and methods

Results

Discussion and conclusion


A Rapid and Real Time Microbiological Testing Method: The Development and Validation of five-lux based *Escherichia coli* ATCC 8739

Choong.M.Y.Y [1], Aldsworth.T [2] and Naseby.D.C [1]

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Introduction

Results

Materials and methods

Discussion