APPLICATIONS OF BIOLUMINESCENCE IN TOXICITY TESTS AND MICROBIAL PHYSIOLOGY

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ABSTRACT

Bioluminescence in general was reviewed before several applications of bacterial luminescence were described in details, which generally include toxicity test and microbial physiology. The first application of bioluminescence was the toxicity testing of end-products from anaerobic and aerobic microbial decolourisation of azo dyes (Sunset Yellow, Orange II, Reactive Black 5 and Acid Red 183). The bacterium *Enterococcus faecalis* reduced selective azo dyes in less than one hour, while the white-rot fungus *Trametes versicolor* needed about 13 days in total for decolourisation. The end products from the bacterial decolourisation were more toxic than the fungi. Toxicity was assessed using the standard *Vibrio fischeri* bioluminescence assay (ToxAlert 100®). The toxicity of naphthol compounds was also assessed using genetic modified bioluminescent *E. coli*, *Ps. putida* as well as marine bacteria *V. fischeri*. These bacteria responded differently to naphthol-based compounds, which are generated from bacterial decolourisation of azo dyes. The second application of bioluminescence was construction of bioluminescent *Campylobacter jejuni,* which is a well-known cause of diarrhoeal disease. A final application of bioluminescence was related to *Enterobacter sakazakii*, and related organisms. These organisms cause bacteraemia, meningitis and other diseases in neonates and immuno-compromised adults. All strains used were previously isolated from dried food. They retained their viability even after begin exposed to stress conditions; desiccation and acidic pH environment. How quick these bacteria recovered from stressed states was unknown, protein synthesis activity during the bacteria recovery from extreme conditions was monitored in this study using bioluminescence and fluorescence markers. *E. sakazakii*, *C. koseri* and related organisms were genetically regrouped by PCR methods prior to the reporter strain construction. Initial transposon modified bioluminescent bacteria were regarded not appropriate for the study as no promoter information was available. Ribosomal protein promoter was used to construct bioluminescent and GFP *E. sakazakii* and *C. koseri,* as the ribosomal protein promoter can represent the ribosomal activity. The result shows that the bacteria required 1-3 hours to recover from the desiccated condition prior to protein synthesis. This was dependent upon the pH. The recovery period for pH 7, 6 and 5 were 2.5, 4.5 and 7 hours, respectively.

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Chapter 1. General Introduction

1.1 Bioluminescence

Bioluminescence is simply light produced by a chemical reaction, which originates in an organism. The organisms generating light include prokaryotes and eukaryotes. Bioluminescent prokaryotes include the marine bacteria *Vibrio fischeri* and *Vibrio harveyi* (Bulich *et al.* 1979) as well as the terrestrial bacterium *Photorhabdus luminescens*, which grows on decaying material (Winson *et al.* 1998). Bioluminescent eukaryotes include *Photinus pyralis* (the fire fly), *Gonyaulax scrippsae* (dinoflagellate), *Pyrophorus plagiophtalamus* (the click beetle), glow-worms, mushrooms and fish (Reynolds *et al.* 2001).

Although precise mechanisms of bioluminescence in many organisms still remain unknown, the genetics, physiology, and biochemistry of prokaryotic bioluminescence have been well studied.

Luciferase is a generic name for any enzyme that catalyses a reaction that results in the emission of light. There are at least two kinds of luciferase system: the *lux* system, which represents the luciferase in the prokaryote kingdom (Hill *et al.* 1997); and the *luc* system, which is the luciferase system of the fire fly *P. pyralis*. Despite the common generation of light, these two bioluminescent systems differ considerably in their biochemical details (Hollis *et al.* 2001).

1.1.1. Physiology and genetics of bioluminescence

Bacterial luminescence is due to the enzyme luciferase, which catalyses the oxidation of reduced flavin mononucleotide ($FMNH_2$) and long chain aliphatic aldehyde, with molecular oxygen. Energy is released as light emission spanning from 420 to 630 nm, with maximum intensity at 490 nm, in the visible region of the spectrum (Equation 1.1.1). It is seen as a green luminescence.

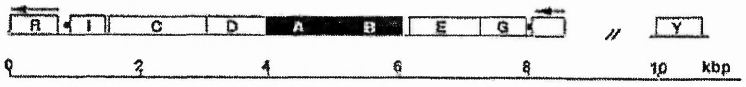
Equation 1.1.1: Bacterial bioluminescent reaction catalysed by luciferase

$$FMNH_2 + O_2 + RCHO \rightarrow FMN + RCOOH + H_2O + hV\ (\sim 490nm)$$

FMN: flavin mononucleotide
$FMNH_2$: reduced flavin mononucleotide
RCHO: long chain aliphatic aldehyde
RCOOH: oxidised long chain aliphatic aldehyde
hV(~490nm): light emission spectrum spans from 420 to 630 nm with an intensity maximum at 490 nm, seen as green luminescence.

All bacterial luciferases are composed of α (40 k Dalton) and β (37 k Dalton) subunits. These are encoded by *lux* A and *lux* B genes, respectively, and both are required for luminescence. In addition to the structural genes (*lux* AB), three other genes (*lux* C, D, and E) are found in all bacterial luminescence systems (Meighen *et al.* 1991). These genes encode three proteins that form the fatty acid reductase protein complex required for the synthesis and recycling of the aldehyde substrate. The *lux* G gene, which lies downstream of the *lux* E gene, is also found in the *lux* operon. The *lux* G is thought to encode proteins involved in the synthesis of reduced flavin.

Figure 1.1.1: *lux* operon from *V. fischeri* (Bluth *et al.* 2004)



All bacterial *lux* systems appear to have a conservation of gene order. Figure 1.1.1 illustrates the *lux* operon from *V. fischeri.* Four genes CDABE were all found in *lux* operons (Meighen *et al.* 1991) together with extensive DNA homology. The *lux* operons from *V. fischeri* and *Ph. luminescens* have been isolated and engineered into many terrestrial Gram-negative bacteria (Hill *et al.* 1997). The expression of the full *lux*CDABE operon synthesizes all the necessary enzymes for the bioluminescence reaction in Gram-negative bacteria (Hill *et al.* 1997).

The firefly uses the luciferase system to emit flashes of light to attract its mate. The eukaryotic luciferase is a 62kDa molecular weight oxygenase but, unlike most other oxygenases, no redox prosthetic group is involved in the reaction. The enzyme requires ATP, molecular oxygen and the heterocyclic compound luciferin to generate light as a yellow luminescence (equation 1.1.2 below).

Equation 1.1.2: Bioluminescent reaction catalysed by luciferase isolated from *Photons payrolls*

Luciferin + ATP +O_2→Oxyluciferin +AMP + PPi + CO_2 +hV (~560nm)

Oxyluciferin: oxidised luciferin;
hV (~560nm): yellow luminescence.

Hollis *et al.* (2000) fused the Eukaryotic luciferase *lac* into the yeast *Saccharomyces cerevisiae*, which showed good luminescence response. Hollis *et al.* later (2001) engineered the bacterial luciferase (*lux*) into both the yeast *S. cerevisiae* and the nematodes *Caenorhabditis elegans*. However, because of the toxicity of luciferin, the yeast and the nematodes showed unstable bioluminescence behaviour. It seems that it is not necessarily a good approach to exchange the Eukaryotic and Prokaryotic luciferases system in the genetically modified organisms. Throughout this report, the applications of bacterial luciferases were emphasised.

Regulation of bioluminescence in most natural luminescent bacteria is due to quorum sensing associated behaviour (Meighen *et al.* 1991). A low constitutive rate of luminescence is produced during the early stages of growth, and *lux* operon expression is induced due to increasing concentrations of the autoinducer in the culture as cells enter the stationary phase. In contrast, in genetically (*lux*) modified bacteria the bioluminescence is dependent upon the promoter to which the *lux*CDABE operon is fused.

Reduced flavin mononucleotide ($FMNH_2$) production depends on the functional electron transport system in bacteria and therefore it is linked to cellular metabolism and viability. Consequently, the intensity of bioluminescence depends upon the metabolic level of the cell. Therefore bioluminescence is used as a biosensor to detect toxic compounds which disrupt cellular metabolism (Bulich *et*

al. 1979). As a biomarker *lux* expression can be used to visualise host-pathogen interactions (Qazi *et al.* 2001).

1.2 Green fluorescence protein

Biological fluorescence is another light related natural phenomenon, and is due to the presence of fluorescent proteins. In contrast with bioluminescence, the fluorescent protein is not a light emitter but an energy transducer. The green fluorescence protein was isolated from *Aequoria victoria* (Pacific jellyfish) by Morin *et al.* (1971). The molecular cloning of GFP (Prasher *et al.* 1992) led to new avenues of investigation in cell, developmental and molecular biology. GFP has been expressed in bacteria (Chalfie *et al.* 1994), yeast (Kahana *et al.* 1995), plants (Casper *et al.* 1996), drosophila (Wang *et al.* 1994), zebrafish (Amsterdam *et al.* 1996), and in mammalian cells (Ludin *et al.* 1996).

The green fluorescent protein is comprised of 238 amino acids. Its absorbance/ excitation peak is at 395 nm with a minor peak at 475 nm. The emission peak is at 508 nm (Sambrook *et al.* 1989). Physical and chemical studies on purified GFP have identified that GFP is very resistant to denaturing treatment, for example, acidic stress down to pH 4.0 and alkaline condition up to pH 12.0 (Ward *et al.* 1982).

Moores *et al.* (1996) reported that GFP tolerated N- and C-terminal fusion to a broad variety of proteins, and still retained native function. Subsequently, the GFP has been widely used as a reporter gene to monitor promoter activity, in which GFP genes hve been fused.

1.3 Luminescence use in toxicity tests

Toxicants perturb cellular metabolism including $FMNH_2$ and hence toxicity can be quantified rapidly by the changes in bacterial bioluminescence. In the past few years, the marine bacterium *V. fischeri* has been well studied as the model organism for bioluminescent bacterial toxicity test and commercialised as

ToxAlert 100® (Merck Ltd.) and MicroTox® (Azur International Ltd.). The intensity of the luminescence output (expressed as Relative Light Units, RLU) depends on several external factors including temperature, pH, osmotic pressure, nature and concentration of the toxicant, exposure time to toxicant, and internal factors including total cell number, cell membrane permeation to the toxin and general cell physiological condition (Juan *et al.* 1987). Bioluminescence in *V. fischeri* is temperature dependent. For every 1°C change in temperature, the light intensity changes by approximately 10% (Bulich *et al.* 1979). Consequently luminometers used for toxicity testing have temperature controlled incubator units, which according to standardised operating is at 15°C (British Standard BS EN ISO 11348-1:1999). Both ToxAlert® and Microtox® are used according to international standardised protocols, with commercial freeze- and liquid- dried preparations of *V. fischeri* distributed by the equipment manufacturers to ensure method standardisation.

The toxic effect on a bioluminescent organism is expressed as the Effective Concentration (EC) value. The EC of a substance is the concentration that causes a defined magnitude of response in a given system (Cronin 1993). In the case of EC_{50}, this is the toxicant concentration causing 50% reduction of maximum bioluminescence for a given exposure period. The exposure period is normally 5 minutes for inorganic toxicants and 15 or 30 minutes for organic toxicants. The exposure time difference is to allow organic compounds sufficient time to diffuse through the bacterial cell wall and elicit an effect. It should be noted that toxicants could perturb cellular metabolism such that $FMNH_2$ concentration increases and subsequently bioluminescence can increase on exposure to a toxicant as well as decrease. Therefore toxicity measurement according to bioluminescence determination is regarded as the change in bioluminescence rather than simply the decrease in bioluminescence.

Using genetic manipulation it is feasible to generate a range of bioluminescent organisms, for example *Escherichia coli* and *Pseudomonas putida*. The latter is well known for degradative ability and hence combined attributes of ring cleavage and toxicity monitoring can be envisaged. Both organisms are easy to grow and can be genetically modified to express the *lux* operon of *Ph. luminescens* for

toxicity testing at 37°C, without a high salt concentration requirement. The high salt concentration is undesirable as it may alter a toxicant's solubility and biological activity (Petanen *et al.* 2003).

1.4 Bioluminescence use as a bioreporter

The intensity of bioluminescence represents the *lux* genes expression level as well as the availability of $FMNH_2$ from intermediary metabolism. Therefore bioluminescence monitors the metabolic activity of the bacterial cell in 'real time'. There are many applications based on these properties of *lux* reaction. For example, Bhattacharyya *et al.* (2005) used *lux* operon under control of metabolic and catabolic promoters to quantify the toxicity. Also Standing *et al.* (2003) used the *lux* reaction to monitor the bacteria growing according nutrient availability. Hence an application of bioluminescence could be monitoring the recovery of stressed cells prior to multiplication.

The bacterial luciferase system has also been used to visualise bacterial pathogen-host interactions. For example, the internalisation of *Staphylococcus aureus* within mammalian cells (Qazi *et al.* 2001) was visualised by using a bacterial *lux* system. A benefit of using the bacterial *lux* operon as a reporter is that very sensitive light detection can be achieved with charged coupled devices with little background luminescence interfering with the signal detection.

1.5 Areas of research

The initial aims of this project were the applications of bioluminescence for toxicity testing, and the project was 50% supported by Westlakes Research Institute (WRI, supervisors Dr M. Dutton, and Prof C. Duggleby). However, at the beginning of the second year (whilst on placement at WRI) the Biotechnology Unit at WRI was closed. This consequently required the project to be redirected. Due to ongoing research at the Nottingham Trent University, the project was modified to include the use of bioluminescence for studying food borne pathogens.

Hence the aims of the project changed during the course of study and are presented here within the seprate chapters.

Construction of bioluminescent *C. jejuni* was proposed to visualise the pathogen's tissue invasion process. However, the Nottingham Trent University restricts the use of hazard group 2 bacteria in tissue culture laboratories for safety reasons. The plan was suspended after the bioluminescent *C. jejuni* was constructed.

There was great interest of bacteria *Enterobacter sakazakii* and *Citrobacter koseri* in the microbiology laboratory, the Nottingham Trent University. Construction of bioluminescent and fluorescent *E. sakazakii* and *C. koseri* was proposed to monitor the protein synthesis recovery from stressed conditions (i.e. desiccated and acidic conditions). Transposon approach was initially considered because of convenient manipulations. However, the results generated could not explain the protein synthesis as proposed because of lacking genetic control information.

As the development of the idea, ribosomal protein promoter was subsequently proposed. Reporter genes under control of ribosomal protein promoter well represented the protein synthesis process under various physiological conditions of the bacteria.

In addition, for epidemiological reasons, genomic typing of *E. sakazakii* and related organisms was also proposed before the construction of genetic modified bacteria. PCR methods were used as main typing methods.

Chapter 2. Bioluminescence used as toxicity tests

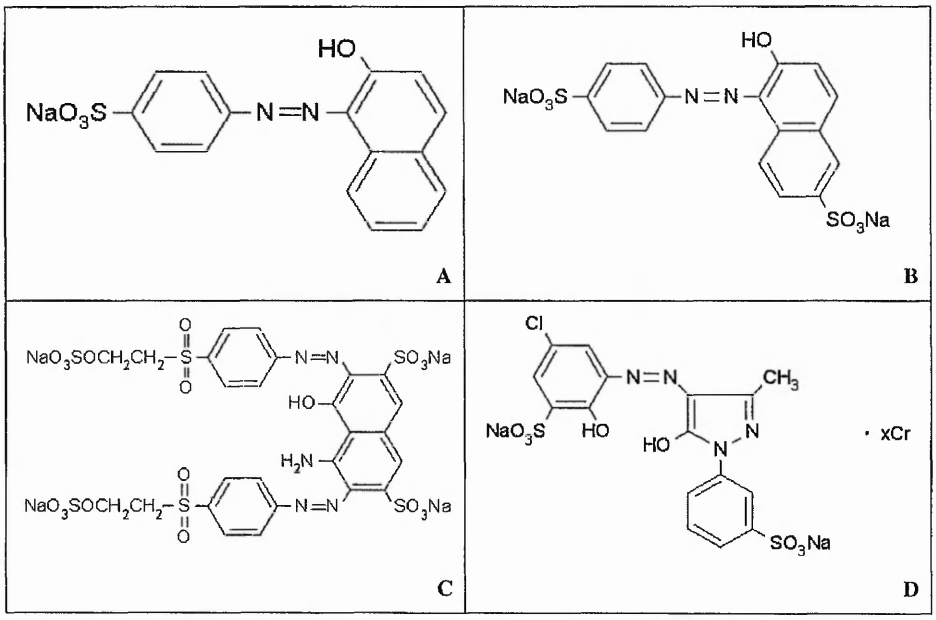
2.1 Introduction

2.1.1. Azo dyes

Textile dyes are the most widely used chemicals nowadays, and about 60-70% of all textile dyes are azo dyes (Carliell *et al.* 1998, O'Neill *et al.* 1999). The reason why azo dyes are the largest and most important group of textile dyes (Carliell *et al.* 1998) is mainly due to their simple synthesis. Consequently over 100,000 kinds of azo dyes are commercially available, and over 7×10^5 tonnes of dyestuff are produced annually in the UK (Banat *et al.* 1996, Rajaguru *et al.* 2000).

There is a considerable diversity in the chemical structure of azo dyes. Nevertheless all azo dyes are generally composed of one or more benzene and naphthalene groups connected by at least one nitrogen-nitrogen double bond (azo bonds, -N=N-). The benzene and naphthalene groups may contain many different substituents such as chloro (-Cl), methyl ($-CH_3$), nitro ($-NO_2$), amino ($-NH_2$), hydroxyl (-OH) and carboxyl (-COOH). A substituent often found in azo dyes is the sulfonic acid group ($-SO_3H$). The chemical structure of selected azo dyes: Acid Red 183, Orange II, Sunset Yellow and Reactive Black 5 are illustrated in figure 2.1.1

Figure 2.1.1: Chemical structure of selected azo-based dyes:



(A) Orange II,
(B) Sunset Yellow,
(C) Reactive Black 5,
(D) Acid Red 183.
reproduced from Sigma-Aldrich handbook of fine chemicals 2003

Reactive dyes are the most widely used members of the azo dyes family. It is a class capable of covalently bonding with fibres and have been widely used to dye cellulose fibres, silk and nylon. Also, reactive dyes are characterized by high degree of chemical stability (O'Neill *et al.* 1999).

2.1.2. Environmental concerns associated with azo dyes

There are a number of environmental concerns associated with dye effluents that have drawn the attention of Environmental Agencies and the public. These problems include:

- The poor fixation of the dye to the textile fibre which may result in coloured effluents, which are considered as polluted and cannot be released to the environment;

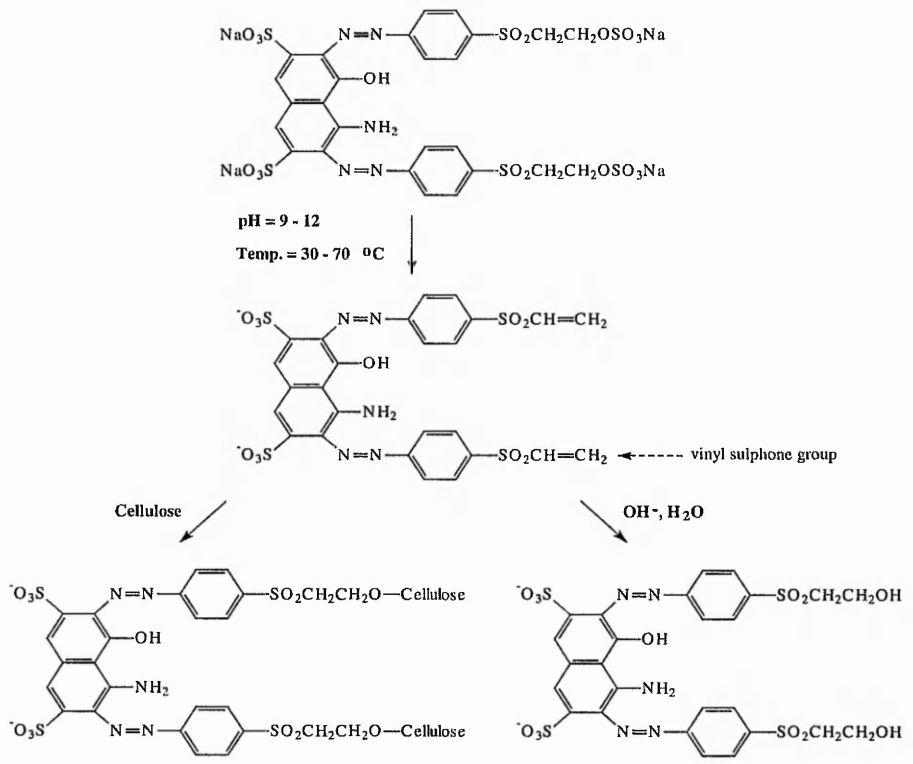
- The used dyes are very likely to be in the hydrolysed form which are difficult to decolourise by conventional treatment methods;
- Due to the diverse chemical structure of dyes, the bacterial decolourisation is rapid but may generate toxic or genotoxic products;
- The bacterial decoloured effluent needs to be treated before being release into the environment.

Hence developments of cheap and effective methods to monitor the toxicity of treated dye effluent have become necessary.

Azo dyes fixation to fibres

During the dyeing process there is a structural change of the azo dye. The vinyl sulphone ($-SO_2-CH=CH_2$) group is activated under the hot, alkaline conditions of a dye-bath, and binds to the fibre (figure 2.1.2). However this activated form of reactive dyes is highly unstable, has a high affinity for hydroxyl ions in water present, and hence may undergo hydrolysis before binding to the fibre can occur (Weber & Stickney 1993). The hydrolysed dye, which is very stable, no longer has any affinity for the fibre and so is lost in the effluent (Hao *et al.* 2000). The average level of fixation of reactive dyes to cotton is between 70 and 80% (Pierce 1994, Waters 1995), but can vary dramatically from 50 to 90%. The higher levels are found with dyes that have two or more reactive groups (Pierce 1994, Easton 1995). An estimated degree of fixation for different dye and fibre combinations is given in table 2.1.1.

Figure 2.1.2: Hydrolysis of the Reactive Azo Dye.



Reactive Black 5 during dyeing (reproduced from Sweeney *et al.* 1994; Gottlieb *et al.* 2003).

Table 2.1.1: Estimated degree of fixation of different dye-fibre combinations: Reproduced from Easton (1995).

Dyeclass	Fiber	Degree of fixation (%)	Loss to effluent (%)
Acid	Polyamide	80-95	5-20
Basic	Acrylic	95-100	0-5
Direct	Cellulose	70-95	5-30
Disperse	Polyester	90-100	0-10
Metal complex	Wool	90-98	2-10
Reactive	Cellulose	50-90	10-50
Sulphur	Cellulose	60-90	10-40
Vat	Cellulose	80-95	5-20

Therefore wastewaters originating from dye-houses using reactive dye processes have created a particular problem. The brightly coloured unfixed dyes are highly water-soluble and are not removed by conventional treatment systems. This is particularly noticeable as the human eye can detect reactive dyes at concentrations as low as 0.005 mg/l in clear river water (Pierce 1994).

In 1992 the Environment Agency (EA), former known as the National Rivers Authority (NRA), conducted a survey that showed that the appearance of colour in natural watercourses was the cause of 500 complaints nationally per year (Pierce 1994, Waters 1995). The appearance of an unnatural colour in a watercourse can be deemed pollution, and must be treated with comparable importance as pollution caused by more traditional pollutants. As a consequence, the EA decided to implement colour consent limits, called River Quality Objectives (RQOs)(Pierce 1994), on effluent discharge to water systems from 1st January 1996 (Easton 1995). These are maximum absorbance values at a series of 50nm wavelength intervals covering the visible spectrum, measured in a 1cm path-length cell after filtration through a 0.45μm filter (Waters 1995). The EA used the absorbance values of samples taken from various watercourses to

calculate the RQOs absorbance consent limits for the contributing sewage treatment works.

2.1.3. Methods of azo dye decolourisation

Since the EA published the RQOs to regulate discharging used dyes into water system, a number of azo decolourisation methods have been developed, generally including chemical, physical and biological methods.

Chemical methods use oxidising agents such as hydrogen peroxide (H_2O_2), H_2O_2+Fe(II) salt (Fentons reagent) and ozonation. These agents may need to be activated by some means, for example, ultra violet light. However there are a number of limitations of these methods:

- First, the chemical decolourisation is sometimes uneconomical and energy demanding especially for large volumes of dyes;
- Second, the chemical decolourisation process may vary depending on the way in which the H_2O_2 is activated, sometimes the decolourisation process remains ineffective;
- Thirdly, chemical oxidising reactions result in aromatic ring cleavage of the dye molecules and hence generate hazardous by-products (Sweeney *et al.* 1994; Gottlieb *et al.* 2003).

Physical methods are mainly adsorption techniques using physical materials (i.e. activated carbon, peat, wood chips, silica gel, fly ash and coal mixture) and biological materials (i.e. bacterial and fungi biomass, waste from the agricultural and seafood industry). Decolourisation by adsorption techniques are influenced by many physio-chemical factors, such as, dye/sorbent interaction, sorbent surface area, particle size, temperature, pH, and contact time. In addition, adsorption methods are normally expensive and the large amount of resultant concentrated sludge creates another disposal problem (Sweeney *et al.* 1994; Gottlieb *et al.* 2003).

Finally, biological dye removal methods have been of considerable interest in recent years, as the biological decolourisation is less costly and more effective.

These biological methods include: anaerobic reduction of azo dyes by bacteria, aerobic decolourisation by lignin-degrading fungi (especially white rot fungi) and aerobic mineralization azo dyes by bacteria.

2.1.4. Biological decolourisation

Bacterial anaerobic reduction of azo dyes

Under anaerobic conditions azo dyes are readily decolourised by bacteria, as a result of the reductive transformation of the azo group. Due to this reduction two or more aromatic amines are formed. The aromatic amines do not absorb light in the visible spectrum and therefore azo dye reduction represents a decolourisation process.

The anaerobic decolourisation of azo dyes was first investigated using intestinal bacteria, and later these compounds were found to become also readily decolourised with various other anaerobic cultures including: *Bacillus* spp., *Clostridium* spp., *Aeromonas* spp., *Rhodococcus* spp., *Plesiomonas* spp., *Enterococcus faecalis* and mixed organisms of unidentified species (Donlon *et al.* 1997, Razo-Flores *et al.* 1997, Beydilli *et al.* 1998, Carliell *et al.* 1998). The exact mechanism of the anaerobic azo dye reduction is not yet clearly understood. Therefore azo dye reduction may involve different mechanisms or locations like enzymatic, non-enzymatic, mediated, intracellular and extracellular (Carliell *et al.* 1995) and various combinations of these mechanisms and cellular locations (Sweeney *et al.* 1994; Gottlieb *et al.* 2003).

A precondition for the reduction of azo dyes is the presence and availability of a co-substrate (Nigam *et al.* 1996), to act as an electron donor. Many different co-substrates can act as electron donors, such as glucose (Carliell *et al.* 1995; Nigam *et al.* 1996), hydrolyzed starch tapioca, yeast extract, a mixture of acetate, butyrate and propionate (Donlon *et al.* 1997; Willetts *et al.* 2000). It also has been observed that the extent of decolourisation of an azo dye varies depending on the co-substrates used, e.g. 82% with glucose, 71% for glycerol and lactose, 51% for starch and 39% for a distillery waste (Nigam *et al.* 1996). Moreover, the rate of

azo-reduction process depends on the type of cosubstrate used and the chemical structure of the azo dyes.

However, azo dyes anaerobically decolourised by bacteria generate aromatic amines and naphthol related compounds that are known to be toxic or genotoxic (Sweeney *et al.* 1994, Gottlieb *et al.* 2003). Hence there is a strong demand for the development of monitoring systems to detect the presence and quantify the toxicity of decolourised effluents.

Decolourisation by white-rot fungi

White rot fungi are able to degrade the woody plant material lignin, which has a complex polymeric structure. In addition to their natural substrate, white-rot fungi are capable of mineralising a diverse range of organic compounds including azo dyes (Reddy 1995).

The decolourisation of azo dyes by white-rot fungi was first reported by Glenn and Gold (1983), who developed a method to measure the ligninolytic activity of *Phanerochaete chrysosporium* based upon the decolourisation of a number of sulfonated polymeric dyes. Subsequently, various white-rot fungi have been assessed for decolourisation and biodegradation of dyes (Field *et al.* 1993). Unfortunately, the detailed degradative pathways utilised by white-rot fungi for decolourisation still remain unclear (Smyth *et al.* 1999). However, it is known that ligninolytic enzymes such as lignin peroxidase (LiP), manganese peroxidase (MnP), and laccase in white-rot fungi play a significant role in dye metabolism (Hattaka 1994). Nevertheless, there is no information on the toxicity of decolourised effluent generated by white rot fungi.

2.1.5. Microbial monitoring system for toxicity

There is very little knowledge available on the end products of decolourisation from the 100,000 kinds of dyes commercially available. Consequently there is an

urgent need for the development of a monitoring system to test the toxicity of various dye-effluents both before and after decolourisation.

Currently there are a number of methods that have been used for the toxicity testing of water. For example, counting the mortality rate of rainbow trout (*Oncorhynchus mikiss*). This method tends to require a long period of time (i.e., 7 days for a chronic test), and is expensive because it requires special neonate fish, which provide the best levels of sensitivity (Banat *et al.* 1996). Another example is to assess the mortality rate of the water flea (*Daphnia magna*), this method though simple is laborious. Both vertebrate and invertebrate organism methods require sample collection, subsequent distribution, testing and report preparation (O'Neill *et al.* 1999). This results in delays in appropriate remedial action to deal with any toxic effluent test result. The total cost of a trout bioassay including laboratory fees and the cost required for sample collection and shipping can become prohibitive when weekly or monthly samples are involved.

Alternative toxicity methods need to be evaluated in terms of cost, sensitivity, speed (turn-around time), accuracy, applicability, and reproducibility. As a result, there is currently a push these days to find cheaper, and faster, bioassay options, and microorganisms seem to be one of the best options.

Microbial toxicity testing systems based on light emission from luminescent bacteria is being applied as a sensitive, rapid and non-invasive assay. It has been successfully commercialised as Microtox® (Azur Environmental) and ToxAlert®(Merck Ltd).

2.1.6. Toxicity testing based on bioluminescent bacteria

$FMNH_2$ production depends on a functional electron transport system in bacteria, which also links to cellular physiology and viability. Hence the strength of bioluminescence depends upon the viability or metabolism level of the cell. The bioluminescence mechanism has been applied to toxicity testing (Boyd *et al.* 1997, Bundy *et al.* 2001 and Belkin 2003). The intensity of the light output depends on

several external factors including temperature (Bulich *et al.* 1979), pH (Sinclair *et al.* 1999), osmotic pressure(Amin-Hanjani *et al.* 1993), nature and concentration of the toxicant, exposure time to toxicant, and internal factors including total cell number, cell membrane permeability to the toxin and general cell physiological condition (Juan *et al.* 1987). *V. fischeri* has been well studied as the model organism for the bioluminescent bacterial toxicity test.

It has been found that for every 1°C change in temperature, the emitted light intensity changes approximately 10% for (Bulich *et al.* 1979). Subsequently luminometers, such as ToxAlert 100®and Microtox® have temperature controlled incubator units. Since *V. fischeri* is a marine bacterium, luciferase isolated from the organism is unstable above 30°C. However, enzymes encoded by *lux*CDABE operon of the terrestrial bacteria *Photorhabdus (Xenorhabdus) luminescens* are functional as high as 45°C (Winson *et al.* 1998).

Bioluminescent whole cells have been used for the bacterial toxicity test. ToxAlert100® (Merck Ltd.) is a commercially available luminometer used to measure the bioluminescence emitted from bacteria. It can regulate both the temperature and exposure time of the *V. fischeri* to the toxicant. The intensity of light given off by bioluminescent cells is described as the Relative Luminescent Unit (RLU).

The effect of toxicity on bioluminescence can be expressed as the Effective Concentration (EC) Value. The EC of a substance is the concentration that causes a defined magnitude of response in a given system (Cronin 1993). In the case of EC_{50}, this is the toxicant concentration causing 50% reduction of maximum bioluminescence.

The mechanisms by which bioluminescence is used for environmental monitoring varies depending on the particular application. Since *V. fischeri* is a marine bacterium, the high salinity conditions are required which could misrepresent terrestrial problems. Hence their application as a toxicity test has been criticised by Amin-Hanjani *et al.* (1993) and Petanen *et al.* (2003). In addition, Bundy *et al.* (1997) and Boyd *et al.* (1997) constructed a bioluminescent *Pseudomonas*

fluorescens strain, which showed a markedly different pattern of response to the organotins than *V. fischeri.*

The gut bacterium *E. coli* and the soil bacterium *Pseudomonas putida* are two terrestrial bacteria that can be easily genetically modified by either plasmid or transposon mutagenesis. For the purpose of monitoring the toxicity of azo dye effluent, *E. coli* and *Ps. putida* may be relevant candidates for human health (gut) and terrestrial pollution issues.

2.1.7. Aims of investigation

The aims of the initial part of this research were to compare bacterial and fungal decolourisation of textile azo dyes. *Enterococcus faecalis* was used for bacterial decolourisation, as it followed the work of Dr Anna Gottlieb, who previously worked in the microbiology research laboratory at the Nottingham Trent University. *Trametes versicolor* was used for fungal decolourisation, as it was followed the work of Dr Martin Dutton, who was the second supervisor of this initial part of the PhD project, originally hosted in the Westlakes Research Institute.

The bacterium *E. faecalis* and the fungi *T. versicolor* were used to compare cost and efficiency of decolourisation of four model dyes. The four chosen dyes were: Orange II, Sunset Yellow, Acid Red 183 and hydrolysed Reactive Black.

Gottlieb *et al.* (2003) argued that aromatic amines and naphthol based compounds were end products of azo dye decolourisation by bacteria. Therefore, in addition to the cost and efficiency of decolourisation by fungi and bacteria, the toxicities of decolourised effluent were to be assessed by using the standard bioluminescence toxicity test, British Standard and International Standard 'BS EN ISO 11348-1:1999'.

Amin-Hanjani *et al.* (1993) criticised the toxicity test standard based on the marine bacterium *V. fischeri*, as it requires high salt environment (2%).

Consequently, bioluminescent *E. coli* and *Ps. putida* are to be constructed to determine "terrestrial" bioluminescence toxicity. Naphthol compounds will be used as model toxicants since these compounds are generated during bacterial decolourisation of textile dyes.

2.2 Materials and Methods

2.2.1. Microbial culture media

All microbial growth media are given below in alphabetical order:

Fahraeus & Reinhammar medium (F&R media)

F&R medium was prepared according to Fahraeus *et al.* (1967) by adding following reagents:

2.5g L-asparagine (DIFCO Laboratories Ltd.),
0.15g D-L-phenylalanine (DIFCO Laboratories Ltd.),
0.0275g adenine (DIFCO Laboratories Ltd.),
50μg thiamine HCl (DIFCO Laboratories Ltd.),
1g potassium dihydrogen phosphate (KH_2PO_4, BDH Ltd),
0.1g sodium phosphate dibasic (Na_2HPO_4, BDH Ltd),
0.5 magnesium sulfate hydrate ($MgSO_4.7H_2O$, BDH Ltd),
0.01g calcium chloride ($CaCl_2$, BDH Ltd),
0.01g ferrous sulfate heptahydrate ($FeSO_4.7H_2O$, BDH Ltd),
1mg manganese(II) sulfate monohydrate ($MnSO_4.4H_2O$, BDH Ltd),
2mg zinc sulfate heptahydrate ($ZnSO_4.7H_2O$, BDH Ltd),
2mg cupric sulfate pentahydrate ($CuSO_4.5H_2O$, BDH Ltd)
10g of glucose (BDH Ltd).

These reagents were dissolved in distilled water, and made up to a final volume of 1 litre. The medium was then autoclaved for 15 min at 121°C.

Luria broth

LB-Broth was prepared by suspending 25 g powdered LB-broth base (Merck product code: 1.10285.0500/5000) in 1 litre of distilled water. The medium was then autoclaved for 15 min at 121 °C. Where necessary, after autoclaving, the

medium was cooled to less than 50°C, and filter sterilized (non-pyrogenic filter, 0.1 μm pore-size Sarstedt Ltd.) ampicillin (100 μg/ml) or kanamycin (40 μg/ml) were added.

Luria agar

Luria agar (LB agar) was prepared by suspending 37g powdered LB-agar base (Merck product code: 1.10283.0500.5000) in 1 litre of distilled water and completely dissolved using a boiling water bath. The medium was then autoclaved for 15 min at 121°C. Where necessary,after autoclaving, the medium was cooled to 50°C, and filter sterilized (non-pyrogenic filter, 0.1 μm pore-size Sarstedt Ltd.) ampicillin (100 μg/ml) or kanamycin (40 μg/ml) were added.

Malt extract agar (MA)

The medium was prepared by dissolving 30g of malt extract and 20g of agar number 3 (both purchased from Oxoid Ltd) in 1 litre of distilled water. The medium was then autoclaved for 15 min at 121°C.

Photobacterium Broth (PB) and Photobacterium Agar (PA)

These media were prepared by adding following reagents:

5.0g tryptone: (DIFCO Laboratories Ltd.),
2.5g yeast extract: (Oxoid Ltd.),
0.3g ammonium chloride (Sigma Ltd.),
0.3g magnesium sulphate (Sigma Ltd.),
0.01g ferric chloride: (BDH Biochemicals Ltd.),
1.0g calcium carbonate (Fischer Scientific UK Ltd.),
3.0g potassium dihydrogen phosphate (KH_2PO_4, Fischer Scientific UK Ltd.),
23.5g sodium glycerophosphate: (Fischer Scientific UK Ltd.),
30.0g sodium chloride (NaCl, Fischer Scientific UK Ltd.),

and in addition, 15g agar number 3 (Oxoid Ltd.) was added when necessary (PA). These reagents were dissolved in distilled water, and made up to a final volume of 1 litre. The pH value was adjusted to 7.0. The medium was autoclaved for 15 min at 121°C.

Tryptone soya broth (TSB)

Tryptone soya broth was prepared by suspending 30g powdered tryptone soya broth base (Oxoid product code: CM0876) in 1 litre of distilled water. The medium was then autoclaved for 15 min at 121 °C. Where necessary, after autoclaving, the medium was cooled to less than 50°C and filter sterilized (non-pyrogenic filter, 0.1 μm pore-size Sarstedt Ltd.) kanamycin (40 μg/ml) was added.

Tryptone soy agar (TSA)

Tryptone soy agar was prepared by suspending 40g powdered tryptone soy agar base (Merck product code: 1.05458.0500) in 1 litre of distilled water and was completely dissolved using boiling water bath. The medium was then immediately autoclaved for 15 min at 121 °C. Where necessary, after autoclaving, the medium was cooled to 50°C and filter sterilized (non-pyrogenic filter, 0.1 μm pore-size Sarstedt Ltd.) kanamycin (40 μg/ml) was added.

Toxicity buffer

In the toxicity assays, the rehydrating solution for bacteria from the desiccated state (according to the British Standard 'BS EN ISO 11348-1:1999') was termed "bacterial toxicity buffer". It was prepared by adding:

7.3g D–glucose (BDH Ltd),

2.035g magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$, Fischer Scientific UK Ltd.),

0.30g potassium chloride (Fischer Scientific UK Ltd.),

11.9g N-(2-hydroxyethyl) piperazine-N-(2-ethanesulfonic acid) (HEPES, Sigma Ltd.).

For *V. fischeri* 20.0g sodium chloride was added, whereas for *E. coli* and *Ps. putida* 3.3g sodium chloride was added. These reagents were mixed in one litre distilled water, and the pH was adjusted to 7.0 with 0.1M sodium hydroxide or 0.1M hydrochloric acid. The bacterial toxicity buffer were divided into aliquots of 15ml in Universal bottles and stored at –20°C until use.

Protective medium for storing *V. fischeri*

Protective medium was used for storing *V. fischeri* at – 70 °C, prior to toxicity test after melting. The protective medium for *V. fischeri* was prepared by adding following reagents:

60.3 g D–glucose ($C_6H_{12}O_6$) (Fischer Scientific UK Ltd.)

4g sodium chloride (NaCl) (Fischer Scientific UK Ltd.)

2g L-histidine (BDH Biochemicals Ltd.)

0.5 g bovine serum albumin, BSA (Sigma Ltd.)

These reagents were dissolved in distilled water (no more than 70 ml) at about 37°C and adjusted to pH7.0 at room temperature with 1mol/L sodium hydroxide or 1mol/L hydrochloric acid as necessary. Distilled water was added to make the final volume to 100 ml.

Protective medium for storing *E. coli*

Preparation of protective medium for *E. coli*, it was used for storing the bacterium at –70 °C, prior to toxicity test after melting. The protective medium for *E. coli* was prepared by adding following reagents:

60.3 g D–glucose ($C_6H_{12}O_6$) (Fischer Scientific UK Ltd.)

1.5g sodium chloride (NaCl) (Fischer Scientific UK Ltd.)

2g L-histidine (BDH Biochemicals Ltd.)

0.5 g bovine serum albumin, BSA (Sigma Ltd.)

These reagents were dissolved in distilled water (no more than 70 ml) at about 37°C and adjusted to pH7.0 at room temperature with 1mol/L sodium hydroxide or 1mol/L hydrochloric acid as necessary. Distilled water was added to the mixture and made up to a final volume at 100 ml.

2.2.2. Bacterial and fungal decolourisation assay

Azo Dyes

Four azo dyes including one hydrolysed reactive dye were used for the decolourisation assays. Acid Red 183(Sigma product code: 201820), Acid Orange 7 (also known as Orange II, Sigma product code: 195235), Sunset Yellow (Sigma product code: 465224) and Reactive Black 5 (Sigma product code: 306452). These were purchased from Sigma-Aldrich Fine Chemicals Ltd.

Preparation of rehydrolysed of Reactive Black

Hydrolysed Reactive Black 5 was prepared as described by Zubrick (1984) and Gottlieb *et al.* (2003). The parent dye was prepared at the concentration of 1g/L with distilled water, and pH was adjusted to 11 with 0.1 M sodium hydroxide. The dye was refluxed for 3 hours by boiling in a round-bottomed flask containing anti-bumping granules with a water-jacketed condenser attached to the top, to minimise water loss by evaporation. The hydrolysed dye was then readjusted to pH7 using 0.1 M hydrochloric acid or 0.1 M sodium hydroxide.

Bacterial decolourisation

The bacterial decolourisation assay was performed as previously described (Gottlieb *et al.* 2003). The bacterium *Enterococcus faecalis* (ATCC 51299) was from the bacterial culture collection of the Nottingham Trent University. *E. faecalis* was maintained at 37°C under anaerobic conditions. One single colony of *E. faecalis* from a TSA plates was used to inoculate 10ml TSB. Then 100µl of *E. faecalis* was used to inoculate a second 10ml of TSB and was incubated at 37°C for 6 hours. The culture was then used to inoculate 400ml TSB, which was incubated overnight at 37°C. The cells were harvested by centrifugation for 20 minutes at 12°C and 800g. The cell pellet was re-suspended in 100mM potassium phosphate buffer (containing 10g/L glucose) at pH7 to a final concentration of about 5g/L (cell wet weight). Filter sterilized (non-pyrogenic filter, 0.1 µm pore-size, Sarstedt Ltd) dyes were added to the cell suspension buffer to a final

concentration of 100mg/L; a negative control was included by substituting 100 mM phosphate buffer (containing 10g/L of glucose) for the appropriate dye.

Fungal decolourisation

The white-rot fungus *Trametes versicolor* (ATCC 20869) was obtained from the culture collection of the Westlakes Scientific Consulting Ltd. The fungus was maintained on malt extract agar plates, and the dyes were decolourised in fungal nitrogen limiting Fahraeus & Reinhammar (F&R) medium. In order to decolourise the dye in the exponential growth phase period (maximum ligninolytic enzyme activity and decolourisation ability), the growth curve of the white-rot fungus was determined by weighing the freeze dried fugal mycelia from F&R medium daily (Reddy 1995). Fungal decolourisation was achieved by adding filter-sterilized dyes into the growing fungus culture, and the decolourisation process was quantified by measuring the optical density at each dye's maximum absorbance value (λ_{max}).

T. versicolor was sub-cultured on a weekly basis by cutting a malt extract agar disc (1cm diameter) containing fungal mycelia from the growing margin of the culture and centrally inoculating a fresh malt extract agar plate which was then incubated at 25°C. Where necessary for liquid culture, the malt extract agar disc (1cm diameter) containing fungal mycelia from the growing edge of the culture, was used to inoculate 100ml conical flasks containing 30ml of F&R medium which was incubated without shaking at 25°C. The growth curve of the *T. versicolor* was obtained by weighing freeze-dried fungal biomass daily. The biomass was collected by filtering the total flask contents (30ml) of the liquid culture into a 50 ml conical flask by using filter paper (Whatman® 15cm diameter). The mycelia retained by the filter were freeze-dried (the freeze-dryer model RV8 A654-01-903, Edwards high vacuum international, Ltd. England) and weighed.

Eleven days after the fungal inoculation in the liquid culture (Preliminary experiments showed that the fungus was in the exponential phase of growth eleven days after inoculation), filter sterilized (non-pyrogenic filter, 0.1 μm pore-size, Sarstedt Ltd) dyes: Orange II, Acid Red 183, Sunset Yellow and hydrolysed

Reactive Black were added to eleven day old fungal at a final concentration of 100 mg/L.

In order to quantify the dye decolourisation process, the maximum absorbance value (λ_{max}) of each dye was determined by using scanning spectrometer (HP, Ltd.). Calibration curves were then constructed by measuring maximum absorbance values of each dye at serial dilutions (highest concentration was 100mg/L); hydrolysed Reactive Black was problematic. Since the 100mg/L concentration was too dark to be measured by spectrometer, a 1:100 dilution of distilled water (dark blue appearance) was used for both the scanning spectrometer and the calibration curve plot.

The decolourisation process was monitored daily by measuring the absorbance at the λ_{max} for each dye (486nm Sunset Yellow, 480nm Orange II, 494nm for Acid Red 183 and 597nm for hydrolysed Reactive Black) against same age control media. Fungal mycelia were removed prior to absorbance measurement.

2.2.3. Preparation of *V. fischeri* cells for toxicity testing

The liquid dried *V. fischeri* cells were provided by Dr Julie Jones, Merck Ltd. Frozen *V. fischeri* cells for toxicity test were prepared in house at the microbiology research laboratory, the Nottingham Trent University.

V. fischeri (NRRLB-11177) was from the bacterial culture collection of the Nottingham Trent University. Because the bioluminescence reaction in *V. fischeri* requires autoinduction, in-house frozen *V. fischeri* cells were prepared according to the following steps:

Step one: A fresh single colony of *V. fischeri* from a Photobacterium agar plate was used to inoculate 50ml Photobacterium broth in an Erlenmeyer flask. This was then grown for 21 hours at 20°C with shaking at 180 rev/min;

Step two: A second 50 ml of *Photobacterium* broth in an Erlenmeyer flask was inoculated with 1 ml of 'preculture' cells from step one and grown for 21 hours at 20°C with shaking at 180 rev/min;

Step three: Cells from step 2 were then harvested by centrifuging the 50 ml overnight culture at 4 °C in a pre-cooled refrigerated centrifuge for 15 min at 6,000g. The cell pellets was kept in an ice bath until required;

Step four: The harvested *V. fischeri* cell pellet was mixed with 100ml protection medium in an ice bath;

Step five: The ice-cold *V. fischeri* cell, in the protection medium was stored in aliquots of 100 µl at –70°C for future use.

2.2.4. Preparation of luminescent *E. coli* and *Ps. putida* cells for toxicity testing

E. coli HB101 containing the multi-copy plasmid pUCD607 (encoding the *lux*CDABE operon, ampicillin and kanamycin resistance genes) was from the bacterial culture collection of the University of Aberdeen and kindly provided by Professor L. Anne Glover. The luminescence production of *E. coli* (HB101, pUCD607) does not exhibit quorum sensing associated behaviour.

E. coli (HB101, pUCD607) was maintained either in Luria broth or Luria agar supplemented with kanamycin and ampicillin.

Luminescent *E. coli* for toxicity was prepared according to the following steps:

Step one: 10ml LB containing ampicillin and kanamycin in a Universal bottle was inoculated with a fresh single colony on LB agar plate and incubated at 25°C overnight without shaking;

Step two: 0.1ml of the overnight culture was used to inoculate 50ml of LB containing ampicillin and kanamycin in an Erlenmeyer flask. This was incubated for 15 hours at 25°C with shaking at 180 rev/min;

Step three: Bacterial cells were harvested by centrifuging the 50 ml overnight culture at 4°C in a pre-cooled refrigerated centrifuge for 15 minutes at 6,000g. Cell pellets were kept on an ice bath for no longer than one hour before the following steps;

Step four: The cell pellets were dispensed in 50ml protection media in an ice bath for no longer than one hour;

Step five: The ice-cold *E. coli* (HB101, pUCD607) cells in protection media were then stored in aliquots of 100 µl at temperature of –70°C for further use.

2.2.5. Construction and preparation of luminescent *Ps. putida* cells for toxicity testing

The *Ps. putida* conjugation methods were as previously described by Winson *et al.* (1998). The plasmid DNA reception strains (i.e. *Ps. putida*) and the conjugative DNA donor strain *E. coli* (S-17-1, contains plasmid) were mated on agar plates. Transconjugatant strains were isolated by a selective medium as described below.

E. coli S-17-1 (954 pUT Km2 *lux* CDABE) and *E. coli* S-17-1 (909 pUT Km1 *lux* CDABE) were used for *Ps. putida lux* construction. They were kindly provided by Dr. Philip J. Hill at the University of Nottingham. These conjugative strains contained a mini-Tn5 transposon with the *lux* CDABE cassettes and a kanamycin resistance gene. *Ps. putida* (ATCC29607) was from culture collection of Nottingham Trent University. They were maintained on LB agar plates supplemented with kanamycin where appropriate.

The design of selective medium for transconjugants positive *Ps. putida* was achieved by preliminary anti-microbial susceptibility screening using the

following antibiotic discs: ampicillin 10μg, (Oxoid product code: CT0003); carbenicillin 100μg, (Oxoid product code: CT0006); chloramphenicol 30μg, (Oxoid product code: CT0013); erythromycin 15μg, (Oxoid product code: CT0020); gentamicin 10μg, (Oxoid product code: CT0024); kanamycin 5μg, (Oxoid product code: CT0025); streptomycin 10μg, (Oxoid product code: CT0047) and tetracycline 30μg, (Oxoid product code: CT0054).

The anti-microbial susceptibility tests showed that all three *E. coli* strains: S-17-1, S-17-1 (954 pUT Km2 *lux* CDABE) and S-17-1 (909 pUT Km1 *lux* CDABE) were sensitive to chloramphenicol while *Ps. putida* was resistance to it. In contrast, kanamycin significantly inhibited the growth of wild type *Ps. putida*, while two *E. coli* donor strain: S-17-1 (954 pUT Km2 *lux* CDABE) and S-17-1 (909 pUT Km1 *lux* CDABE) contained a kanamycin resistance gene. It was proposed that the combination use of kanamycin and chloramphenicol could inhibit transconjugatants negative reception strain (*Ps. putida*), and the *E. coli* DNA donor strains (954 pUT Km2 *lux* CDABE and S-17-1 909 pUT Km1 *lux* CDABE), while only transconjugatant *Ps. putida* would grow.

Therefore it was decided to use kanamycin and chloramphenicol containing LB agar plates to select for transconjugatant *Ps. putida.*

Bi-parental plate mating between the *Ps. putida* strains and the two *E. coli* donor strains (S-17-1, 954 and 909) was carried out by mixing a loopful (about 10μl) of each strain on non-selective LB agar plate. The plates were incubated at 25°C for 48 hours.

The mixed cells (*E. coli* and *Ps. putida*) were then resuspended in 5ml sterilized 0.9 (w/v) sodium chloride. Aliquots (100 μl) of the cell suspension were spread on LB agar plates containing kanamycin (40 μg/ml) and chloramphenicol (20 μg/ml) and incubated at 30°C for 18 hours. Bright luminescent colonies were observed in a dark room. Luminescent colonies were then purified by re-streaking at least three times on kanamycin and chloramphenicol containing LB agar plates and further confirmed by oxidase tests (positive) and Gram staining.

To identify transconjugant strains, in which the mini-Tn5 *lux*CDABE was inserted into the *Ps. putida* chromosome downstream from an active promoter, the transconjugants were grown in kanamycin and chloramphenicol contained LB broth (50ml) in Erlenmeyer flasks for 16 hours at 30°C at 180 rev/min. The intensity of bioluminescence of 500µl cell aliquots was quantified using the ToxAlert® luminometer.

Growth rates of bioluminescent *Ps. putida* strains were determined using impedance microbiology (Silley and Forsythe 1996). This was a necessary precaution since the *lux* insertion could affect key genes related to cell growth and cause polar mutations.

Frozen *Ps. putida* cells for use in toxicity testing assays were prepared as previously described for *E. coli* HB101 (pUCD607) (section 2.2.4).

2.2.6. Bacterial and yeast bioluminescence toxicity assessment

All bacterial bioluminescence measurements related to toxicity assessment were performed by using the ToxAlert® 100 (Merck Ltd.) system according to the manufacturers' instructions and the British and International standard BS EN/ISO 11348 1999.

The bacterial toxicity testing buffer was thawed in room temperature and well shaken by hand to ensure sufficient oxygen was dissolved in the buffer. This was necessary for the bioluminescent reaction in bacteria. Aliquots (100 µl) of the frozen, stock, luminescent bacteria (*V. fischeri*, *E. coli* and *Ps. putida* cells) prepared as above (section 2.2.4) were pipette into the bacterial toxicity testing buffer (15ml) maintained at 15°C and homogenized by gentle shaking. As required by the British and International standard BS EN/ISO 11348 1999, a minimum waiting time of 30 minutes was used to reduce the variation of bioluminescent behaviour of bacteria.

While the azo dyes were decolourised by bacteria within hours, white-rot fungi on the other hand, need several days to completely remove the colour. The liquid dried *V. fischeri* cells were used for bioluminescence toxicity assessment of bacterial and fungal decolourised dyes and fungal semi-decolourised dyes was also included.

The luminescent bacteria (*V. fischeri*, *E. coli* and *Ps. putida*) were also used to test the toxicity of various naphthol based compounds. These represented possible end products of bacterial decolourisation. The naphthol related compounds were supplied by Aldrich Ltd., with the exception of H-acid which was supplied by Kodak and 1-amino-2-naphthol-6-sulphonate which was synthesised in the School of Biomedical and Natural Sciences at Nottingham Trent University.

When luminescent *V. fischeri* was used, the test compounds were dissolved in 2% NaCl whereas with luminescent *E. coli* and *Ps. putida* the compounds prepared in distilled water.

All samples (1.5 ml) were pipetted into 3 ml transparent cuvettes (purchased from Sarsted Ltd.) and temperature equilibrated incubated at 15°C for 15 minutes. The luminescent bacteria in the toxicity testing buffer were also incubated in 15°C for 15 minutes.

In all the toxic tests, the intensity of bioluminescence (RLU) was monitored at time zero as well as after a known exposure time usually15 minutes. All assays were preformed three times in duplicate. All test samples were tested over a dilution range. The pH of all samples were measured, and if the sample pH was outside the range of 6-8.5, then the pH was adjusted to 7.0 with 0.1M sodium hydroxide or 0.1M hydrochloric acid.

Yeast

S. cerevisiae cells were cultured overnight, harvested by centrifugation at 600g, and washed with 0.1M KCl twice.

The pH of 0.9% sodium chloride was adjusted to 5.5 with 0.1M sodium hydroxide or 0.1 M hydrochloric acid, then 450µl pH5.5 sodium chloride solution was added to 1ml cuvette. The final volume was made up to 500µl by adding 50µl of diluted *S. cerevisiae* cells. After a contact time of 10 minutes, 500µl of pH2.6 citrate phosphate buffer (0.1M) containing luciferin potassium salt (final concentration of luciferin being 0.1mM per cuvette) was added. The luciferin is unstable and was only prepared as a solution when required. The intensity of bioluminescence was measured using the luminometer ToxAlert 100®.

2.2.7. Data evaluation

The RLU reductions against increased concentration of putative toxicants was measured using the luminometer ToxAlert 100® were analysed by the supplied WinTox software (Merck Ltd.) The inhibition value and EC values were calculated according to the following mathematical methods:

Inhibitory effect on luminescent bacteria

Because of biological variations, the correction factor for each individual samples was expressed as equation 2.2.1:

Equation 2.2.1: Correction factors of bacterial bioluminescence.

$$f_{kt} = I_{kt} / I_0$$

Where:
f_{kt} is the correction factor for the contact time in minutes;
I_{kt} is the luminescence intensity in the control sample after the contact time, in relative luminescence units;
I_0 is the luminescence intensity of the control test suspension, immediately before the addition of the diluent, in relative luminescence units.

Corrected bioluminescence intensity was expressed as equation 2.2.2:

Equation 2.2.2: Corrected bioluminescence intensity.

$$I_{ct} = I_0 \, \overline{f_{kt}}$$

Where:
$\overline{f_{kt}}$ is the mean of f_{kt};
I_0 is the luminescence intensity of the control test suspension, immediately before the addition of the diluent, in relative luminescence units;
I_{ct} is the corrected value of I_0 for test sample cuvettes immediately before the addition of test sample.

Inhibition effects on bioluminescent bacteria were expressed as equation 2.2.3:

Equation 2.2.3: Inhibition effect on bioluminescent bacteria.

$$H_t = (I_{ct} - I_{Tt})/I_{ct} \times 100$$

Where:
H_t is the inhibitory effect of a test sample after the contact time, in percent;
I_{ct} is the corrected value of I_0 for test sample cuvettes immediately before the addition of test sample;
I_{Tt} is the luminescence intensity of the test sample after the contact time, in relative luminescence units.

Determination of EC values

For evaluation of concentration-effect relationships, the gamma value was determined for each dilution level as shown equation 2.2.4:

Equation 2.2.4: Gamma value of toxicant.

$$\Gamma_t = H_t / (100 - H_t)$$

Where:
Γ_t is the gamma value of the test sample after the contact time;
H_t is the inhibitory effect of a test sample after the contact time, in percent.

Standard linear regression analyse was used to determine the concentration-effect relationship for each exposure time. The concentration-effect relationship at a given exposure time were described by the following equation 2.2.5:

Equation 2.2.5: The concentration-effect relationship at a given exposure time.

$$\lg c = b \lg \Gamma t + \lg a$$

Where:
Γ_t is the gamma value of the test sample after the contact time;
C_t is the portion of the water sample within the test sample, in percent;
$C_t = EC_{20}$ at $\Gamma t = 0.25$;
$C_t = EC_{50}$ at $\Gamma t = 1$;
Γ_t is the gamma value of the test sample after the contact time;
b: is the value of the slope of the described line.

Colour correction

Luminescent bacteria normally emit light at a maximum wavelength of 490 nm. However the light may be absorbed by some colour compounds such as azo dyes and hence given false inhibition values. Therefore a colour correction was required prior to determine the toxicity value. This was achieved by using the correction procedure of Azur Environmental for the *V. fischeri* toxicity tests.

The absorbance (ABS_x) at 490nm was measured against a diluent blank using a spectrophotometer for each test dilution.

The contribution to absorbance (A_x) was calculated using the absorbance readings taken for each sample concentration using equation2.2.6:

Equation 2.2.6: Contribution towards absorbance.

$$A_x = 2.303 \times ABS_x$$

Where

ABS_x is the absorbance value at 490nm

A_x is the contribution to absorbance

The A_x was then used to calculated the transmittance (T_x) for each sample concentration using equation 2.2.7:

Equation 2.2.7: Transmittance of colorant.

$$T_x = (1-e^{-Ax})/A_x$$

Where

T_x is the transmittance

A_x is the contribution to absorbance

The absorbance corrected I_0 was then calculated using the equation 2.2.8:

Equation 2.2.8: Colour corrected initial luminescence intensity.

$$ACI_0 = I_0 \times T_x$$

Where:

ACI_0 is the corrected I_0 values;

I_0 is the luminescence intensity of the control test suspension, immediately before the addition of the diluent, in relative luminescence units;

T_x is the transmittance.

The corrected I_0 values (ACI_0) and the measured I_5, I_{15} and I_{30} light levels were then entered manually into the WinTox software.

2.3 Results

Four selected dyes: Orange II, Acid Red 183, Sunset Yellow and hydrolysed Reactive Black were tested for decolourisation by the fungus *T. versicolor* and the bacterium *E. faecalis*. Although *E. faecalis* was not able to decolourise Acid Red 183, the other dyes were decolourised more rapidly than by the fungus. However, the bacterial decolourisation generated more toxic end products.

Bioluminescent *E. coli* (pUCD607) was obtained from the University of Aberdeen, and bioluminescent *Ps. putida* were successfully constructed by using full *lux* operon contained mini-tn5 transposon. Both of these two genetically modified bacteria showed sufficient bioluminescence for toxicity testing and could be compared with the international standard test organism *V. fischeri* used in the British and International Standard (EN ISO 11348-1:1999) test procedures.

Subsequently, three bioluminescent bacteria: *V. fischeri, E. coli* and *Ps. putida* were used to test the toxicity of naphthol-based compounds. *V. fischeri*, which is a marine bacterium, showed the highest sensitivity to the naphthol based compounds.

2.3.1. Bacterial and fungal decolourisation

Orange II and Sunset Yellow were decolourised by cell suspension of an overnight culture of *E. faecalis* within 10 minutes, whereas hydrolysed Reactive Black required one hour to be completely decolourised. Acid Red 183, however, was not decolourised within 24 hours. These bacterial decolourisation experiments were at 37°C and the pH was approximately at 7 before and after the decolourisation. In contrast with bacterial decolourisation, the white-rot fungus *T. versicolor* required 11 days to reach the pre-stationary growth phase (prior to the dye addition) and 7 days after the dyes were added, before the majority of the colour was removed.

Fungal pre-stationary growth phase (with maximum ligninolytic enzyme activity) is believed to be optimal for decolourisation (Reddy 1995). Therefore a growth

curve of *T. versicolor* was determined by weighing the whole fungal biomass daily after freeze drying. Every experiment was performed in triplicate. However as the fungal growth showed considerable biological variations, error bars are not always indicated in this thesis. This variation is also evident in many previous publications on fungal decolourisation (Wang *et al.* 1998, Kapdan *et al.* 2000 and Sumathi *et al.* 2000). Figure 2.3.1 shows the growth curve of *T. versicolor*. The biomass dry weight is the average value from triplicate samples.

Figure 2.3.1: The growth curve of *T. versicolor*.

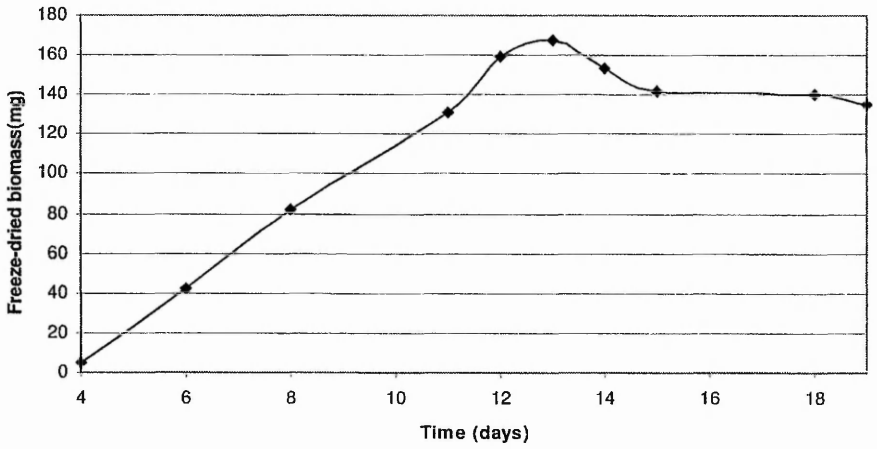


Figure 2.3.1 shows that *T. versicolor* was grown in liquid medium according to Materials and Methods section 2.2.2

Figure 2.3.1 shows that four days after the fungi inoculation, the fungi biomass was rapidly increasing. The fungi were in a pre-stationary growth phase for about 11 days before entering stationary growth phase.

In order to quantify the decolourisation process, the optical density of each dye's maximum absorbance (λ_{max}) was determined by scanning spectrometer. The λ_{max} of Sunset Yellow was 486nm, for Orange II 480nm, and Acid Red 183 was 494nm. Initially it was difficult to determine the maximum absorbance of hydrolysed Reactive Black, as the dye was too intensely pigmented to be measured by the spectrometer, subsequently the dye was diluted to 1mg/l, and the maximum absorbance values was then detected (λ_{max}=597nm). Afterwards, the

linear relationship (calibration curves) between the concentration of dyes and absorbance values at λ_{max} were constructed for further analysis.

Calibration curves of Sunset Yellow, Orange II, and Acid Red 183 are shown in figure 2.3.2:

Figure 2.3.2: Calibration curves of Sunset Yellow, Orange II and Acid Red 183.

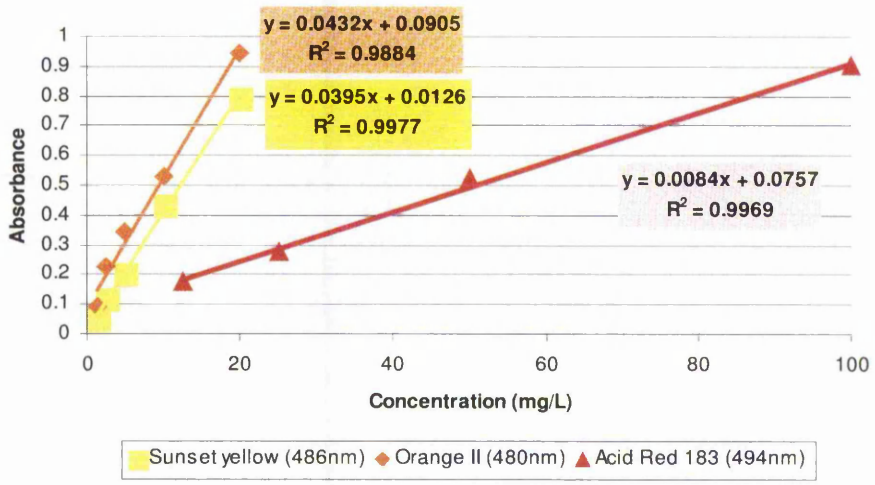


Figure 2.3.2 shows the Calibration curves of Sunset Yellow, Orange II and Acid Red 183 were constructed using spectrometer reading according to Materials and Methods section 2.2.2.
A calibration curve for hydrolysed Reactive Black is described in figure 2.3.3.

Figure 2.3.3: Calibration curves of hydrolysed Reactive Black λmax 597nm.

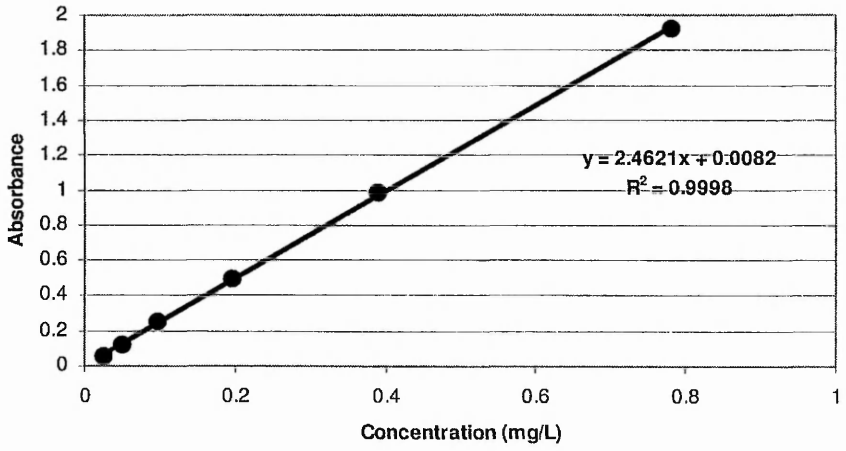


Figure 2.3.3 shows the Calibration curve of Reactive Black was constructed by using spectrometer reading according to Materials and Methods section 2.2.2.

Fungal decolourisation:

T. versicolor required several days to completely remove the colour from the culture flasks. This is in contrast with the bacterial decolourisation, which was too fast to be quantified accurately.

The decolourisation processes of the four dyes were presented in figures 2.3.4-2.3.7:

1. Decolourisation progress of Sunset Yellow:

Figure 2.3.4: Fungal decolourisation progress of Sunset Yellow.

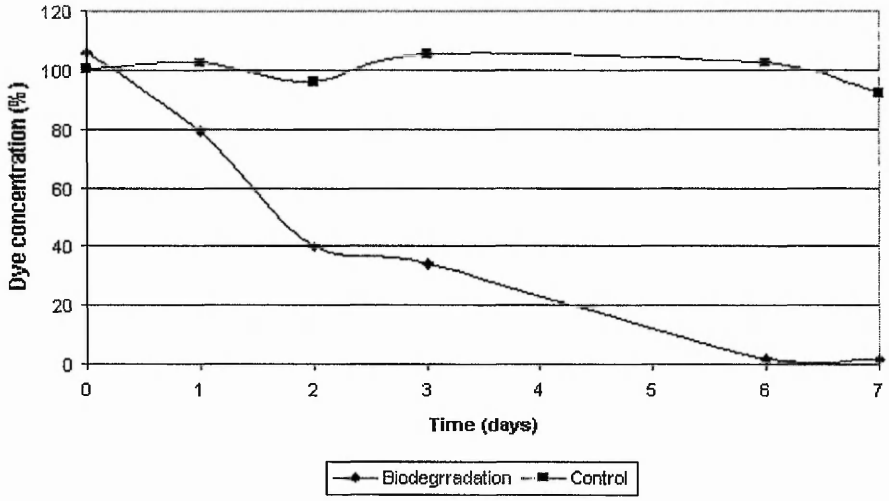


Figure 2.3.4 shows the Sunset Yellow was decolourised by *T. versicolor* according to Materials and Methods section 2.2.2

Figure 2.3.4 shows that Sunset Yellow was decolourised by the white rot fungus. Sunset Yellow was added to the liquid culture of white rot fungus, when it had just entered the pre-stationary growth phase The colour removal progress was expressed by measuring reduction of optical density (λ_{max} of Sunset Yellow is 486nm). In the first day, the absorbance values had decreased by 20%. By the second day, only about 40% of the absorbance values were still present in the culture medium. After about 6-7 days, there were no absorbance values detectable with the spectrometer blank using control fungi culture medium. By eye, the colour in the liquid culture medium decreased day by day, and after 6-7 days, there was no colour difference distinguishable from the control fungal culture without dye.

There was a possibility that the adsorption effect of the fungal biomass was significant enough to influence the decolourisation results. Consequently, the colour of the fungal biomass was additionally observed and recorded.

When the fungi were grown in an Erlenmeyer flask in liquid medium without dye, the biomass was a layer which floated on the liquid medium, the upper surface

(aerobic side) was white; whereas the lower surface in contact with the liquid medium (anaerobic side) was light yellow.

The fungal biomass grown with Sunset Yellow did not show any apparent morphological difference from the biomass grown without dyes. The colour of the biomass however changed according the decolourisation stage. There was no immediate difference with biomass colour after the dye was added to the media. However after 24 hours, the lower surface of fungal biomass was obviously yellow. This colour paled day by day, until 6-7 days later, it was hard to visually distinguish the control samples.

2. Fungal decolourisation progress of Acid Red 183:

Figure 2.3.5: Fungal decolourisation of Acid Red 183.

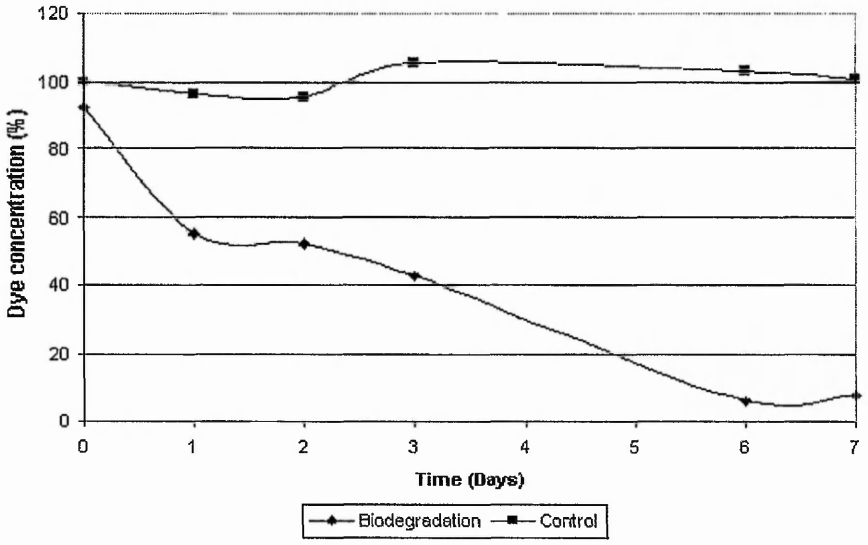


Figure 2.3.5 shows the Acid Red183 was decolourised by *T. versicolor* according to Materials and Methods section 2.2.2

Figure 2.3.5 shows that Acid Red 183 was decolourised by the white rot fungus. As previously, the decolourisation was monitored by measuring the maximum absorbance (λ_{max} 494nm) values daily. In the first day the absorbance values had decreased more than 40%. By the third day, further 20% of the absorbance values were reduced. After about 6-7 days, there was no detectable absorbance using the spectrophotometer blanked against control fungi culture media. By eye, the medium colour paled day by day. It was initially red, then orange, later showed light yellow and finally colour completely disappeared.

One day after Acid Red 183 was added into the fungal media, the biomass was red in the lower surface. This colour decreased day by day, and finally after 6-7 days, the biomass showed obviously darker yellow (and light brown) compared with the control sample.

3. Fungal decolourisation progress of Orange II:

Figure 2.3.6: Fungal decolourisation of Orange II.

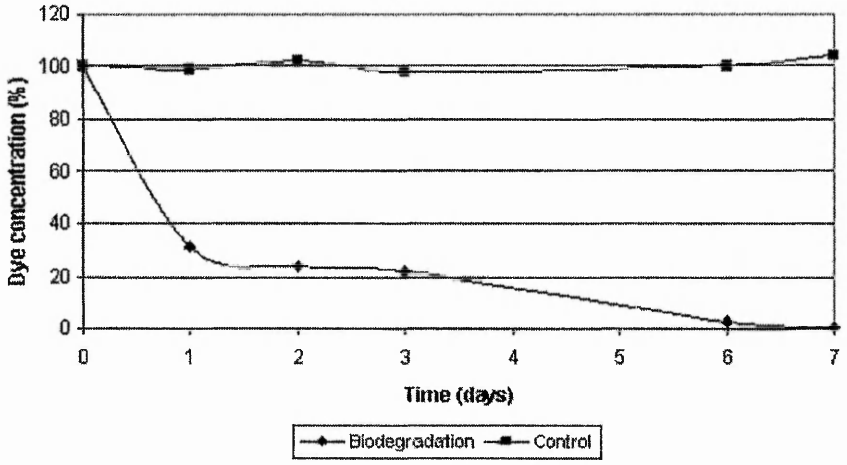


Figure 2.3.6 shows the Orange II was decolourised by *T. versicolor* according to Materials and Methods section 2.2.2.

Figure 2.3.6 shows that Orange II was decolourised by the white rot fungus. As previously, the decolourisation was monitored by measuring the maximum absorbance (λ_{max} 480nm) value daily. In the first day, about 70% of maximum absorbance values were lost; and by day three about 80% of the absorbance were lost; after about 6-7 days, the colour in the culture medium was completely removed. By eye, the colour in the liquid culture medium decreased day by day, finally showed light yellow and later colour completely disappeared.

One day after Orange II was added into the fungi media, the biomass was orange on the lower surface. This colour paled day by day and finally, after 6-7 days, the biomass was obviously dark yellow (and light brown) compared with the control sample.

4. Fungal decolourisation progress of hydrolysed Reactive Black:

Figure 2.3.7: Fungal decolourisation of hydrolysed Reactive Black.

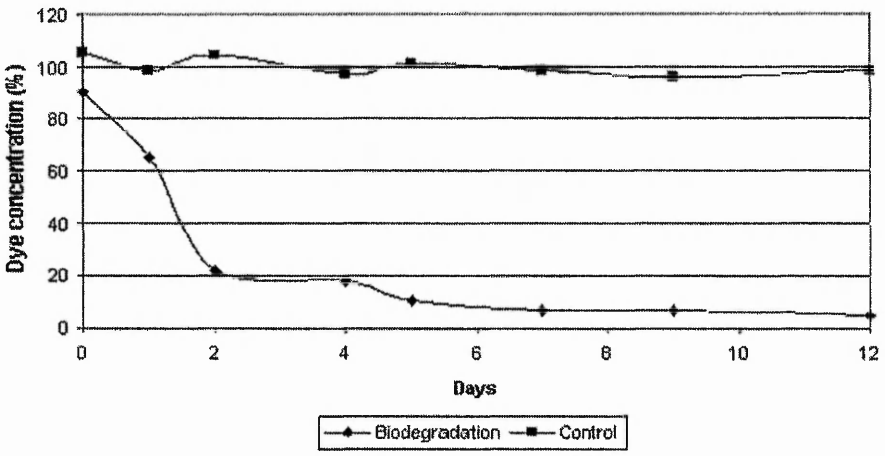


Figure 2.3.7 shows the Reactive Black was decolourised by *T. versicolor* according to Materials and Methods section 2.2.2.

Figure 2.3.7 shows that hydrolysed Reactive Black was decolourised by the white rot fungus. The decolourisation was monitored by measuring the maximum absorbance (λ_{max} 597nm) values regularly. In the first day, about 50% of the dye had been decolourised. By the second day, about 80% of the absorbance had been reduced. After about 6-7 days, the absorbance values in the culture media were steadily at about 5% of the original until 12 days incubation. By eye, the colour in the liquid culture medium was initially black. It paled day by day and finally showed blue or light blue. In the last few days, the colour changed to light brown.

After one day inoculation with hydrolysed Reactive Black the fungal media, the biomass was black in the lower surface. The colour paled day by day, and finally after 10 days, the biomass was obviously brown compared with the control sample.

Figures 2.3.4 to 2.3.7 show the decolourisation of the selected azo-dyes Orange II, Acid Red 183, Sunset Yellow and hydrolysed Reactive Black by *T. versicolor*. It was notable that the majority of the colour was removed within the first 24 hours. The absorbance values of tested dyes at λ_{max} were all approaching zero after 7 days of dye addition. However it was easy to observe by naked eye that there were

slight colour differences between fungi decolourised culture medium and the control medium. This was most evident for the hydrolysed Reactive Black, where a light brown colour was present in the culture medium even 21 days after the dye was added, despite very little difference being detected by the scanning spectrophotometer.

The change in pH during the fungi decolourisation process was not determined and could not practically be controlled. However, the pH value of sterile culture media was 6.5, and the decolourised medium was within pH of 5.5-6.5, and eighteen day old fungal culture media was around 6.0.

All absorbance values at λ_{max} were determined in triplicate using three individual samples. This resulted in large biological variations in the data obtained. Subsequently for clarity, error bars are not indicated in figures 2.3.4-2.3.7. This is also found in many previous publications on fungal decolourisation (Wang *et al.* 1998, Kapdan *et al.* 2000 and Sumathi *et al.* 2000).

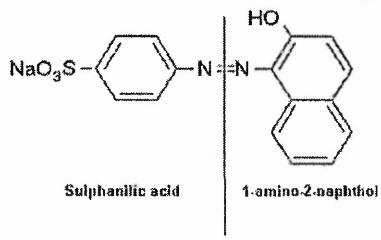
Calibration curves of the dyes made it possible to quantify the decolourisation progress of *T. versicolor*. However, it was noted that after the first few days of fungal decolourisation, the fungal biomass was obviously coloured, and the colour of the biomass disappeared along with the fungal culture medium. Therefore there were two colour removal processes, biomass adsorption and enzymatic decolourisation. However, currently there is no convenient technique available to quantify the colour associated with fungal biomass.

2.3.2. Toxicity of bacterial decolourised azo dyes

The toxicity of *E. faecalis* decolourised Orange II and Sunset Yellow was monitored using the ToxAlert 100®. EC_{50} values are given in tables 2.3.1 and 2.3.2 for 15 and 30 minute exposure periods. The decolourisation experiments

were performed by triplicate on separate occasions, and each toxicity test was performed in duplicate for each replicate.

Table 2.3.1: Structure of Orange II (A) and comparison of EC_{50} values (mg/L) for *E. faecalis* decolourised Orange II (B). *Data source Sweeney, 1999.



A

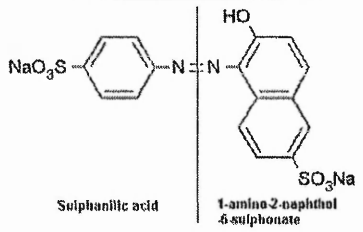
	Reduced Orange II	Sulphanilic acid*	1-amino-2-naphthol
EC_{50} 15min (mg/L)	0.8±0.2	38	0.15±0.04
EC_{50} 30min (mg/L)	0.7±0	38	0.13±0.035

B

Table 2.3.1 shows the Orange II was decolourised by *E. faecalis,* and toxicity tests was according to Materials and Methods section 2.2.3 and 2.2.6

Bacteria can rapidly decolourises certain azo dyes (Section 2.3.1), but often generate toxic end-products (Sweeney *et al.* 1994; Gottlieb *et al.* 2003). Table 2.3.1 (A) shows the chemical structure of Orange II was broken at the azo bond yields sulphanilic acid and 1-amino-2-naphthol. The EC_{50} values of sulphanilic acid showed relatively low toxicity, however the EC_{50} of 1-amino-2-naphthol was about 0.15 mg/L, which is highly toxic. The EC_{50} value of decolourised Orange II was about 0.8 mg/L, which is also highly toxic.

Table 2.3.2: Structure of Sunset Yellow (A) and comparison of EC $_{50}$ values (mg/L) for *E. faecalis* decolourised Sunset Yellow (B). *Data source Sweeney, 1999.



A

	Reduced Sunset Yellow	Sulphanilic acid*	1-amino-2-naphthol-6-sulphonate (colour corrected)
EC_{50} 15min (mg/L)	37± 8	38	783.8
EC_{50} 30min (mg/L)	25± 5	38	>600

B

Table 2.3.2 shows the Sunset Yellow was decolourised by *E. faecalis*, and toxicity tests was performed according to Materials and Methods section 2.2.3 and 2.2.6

Table 2.3.2 A shows the chemical structure of Sunset Yellow and that azo fission generates sulphanilic acid and 1-amino-2-naphthol-6-sulphonate. The EC_{50} values of both sulphanilic acid and 1-amino-2-naphthol-6-sulphonate indicated relatively low toxicity.

2.3.3. Toxicity of fungal decolourised azo dyes

Orange II, Sunset Yellow and Acid Red 183 were decolourised by *T. versicolor* after seven days incubation. During the fungal decolourisation process, toxicity of fungal decolourised culture media were monitored daily. In addition to the International Standard bioluminescence toxicity assay using *V. fischeri,* inhibition of fungal growth was also assessed during the decolourisation period.

Toxicity of fungal decolourised azo dyes as determined using *V. fischeri* bioluminescence

The bioluminescence toxicity assay was expressed as the percent of inhibition effect compared with the control (figure 2.3.8) since insufficient toxicity was detected to be expressed in conventional EC_{50} values. Appropriate fungal culture medium was used as the negative control for toxicity assessment. For example, 13 day-old fungal culture medium was used as control for culture medium sample taken 2 days after the dye was added. This equals to 11 days for the pre-stationary growth phase and 2 days decolourisation. Similarly 16 day old fungi media was used as the negative control for decolourised media 5 days after the dye was added. Since the toxicity test organism was *V. fischeri*, both control sample and the decolourised media were adjusted to 2% NaCl before assessment. The bioluminescent inhibition effect was colour corrected when required using the methods described in section 2.2.7. The decolourisation processes and toxicity assessment were performed in triplicate, and each sample was analysed in duplicate.

Figure 2.3.8: RLU inhibition effect caused by Orange II during fungal decolourisation.

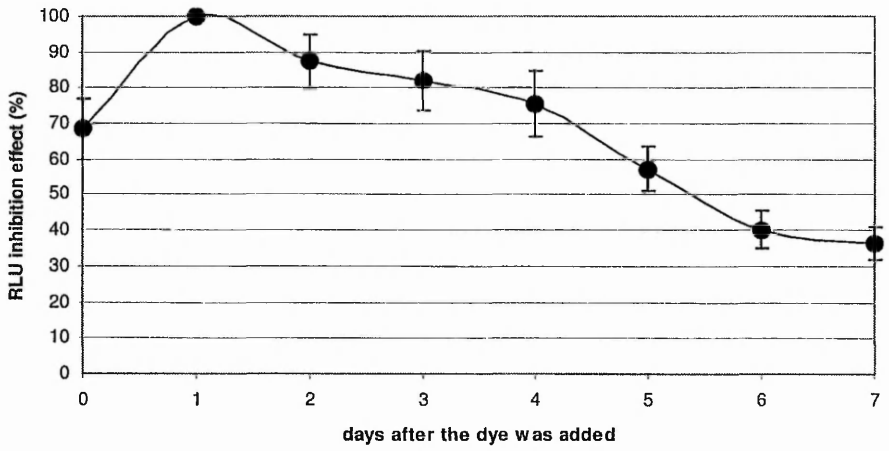


Figure 2.3.8 shows the RLU inhibition effects caused by fungal decolourised Orange II according to Materials and Methods 2.2.2

The toxicity of Orange II was monitored daily as shown in figure 2.3.8. Immediately after Orange II was added, the inhibition effect was 68.5%. Whereas twenty-four hours later, bioluminescence was inhibited 100% due to semi-decolourised Orange II and resultant end products. The decolourised medium showed relatively high toxicity. The RLU inhibition gradually decreased until the seventh day, which had a 36.5% RLU inhibition effect. As figure 2.3.6 shows that the fungus gradually removed the colour in the culture medium, until after seven days when the colour had completely disappeared.

Figure 2.3.9: RLU inhibition effect caused by Sunset Yellow.

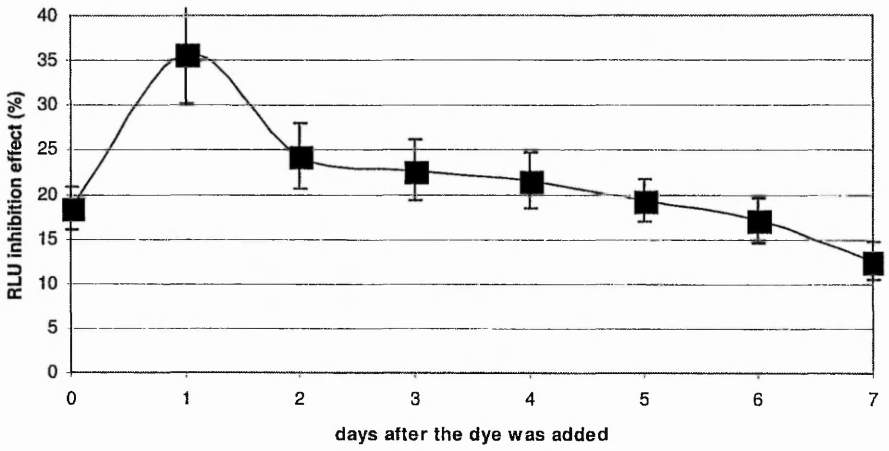


Figure 2.3.9 shows the RLU inhibition effects caused by fungal decolourised Sunset Yellow according to Materials and Methods 2.2.2

Figure 2.3.9 shows that in comparison with the control sample, the toxicity of Sunset Yellow during decolourisation was less than that OrangeII. After Sunset Yellow was added to the culture medium, the bioluminescence inhibition was 18.4%. Whereas after 24 hours, 35.7% of bioluminescence was inhibited by toxicity of semi-decolourised Sunset Yellow. From the second day, the bioluminescence inhibition decreased until the seventh day, with about 12.6% inhibition. As figure 2.3.4 shows, the fungus gradually removed the colour in the culture medium, until after seven days when the colour had completely disappeared.

Figure 2.3.10: RLU inhibition effect caused by Acid Red 183

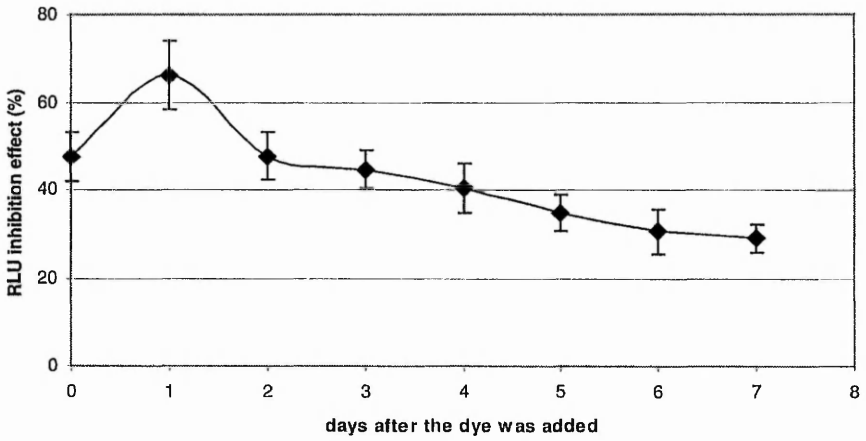


Figure 2.3.10 shows the RLU inhibition effects caused by fungal decolourised Acid Red 183 according to Materials and Methods 2.2.2

Figure 2.3.10 shows the toxicity of semi-decolourised Acid Red 183 over the incubation period. Immediately after Acid Red 183 was added to the culture medium, the RLU inhibition increased to 47.3% and after 24 hours it had further increased to 66.3%. After there the RLU inhibition decreased and on the seventh day, showed about 29.1% RLU inhibition effect.

Inhibition of fungal growth

Inhibition of fungal growth was recorded by adding three azo dyes to 11-day old fungal cultures. The suppression in fungal growth was probably due to the toxicity of the semi-decolourised dyes. The three semi-decolourised dyes slightly inhibited fungal growth especially in the first 24 hours after the dye was added compared with the control fungal biomass (Figure 2.3.11 to 2.3.13). Every measurement was performed triplicate.

Figure 2.3.11: *T. versicolor* growth inhibition caused by addition of Orange II.

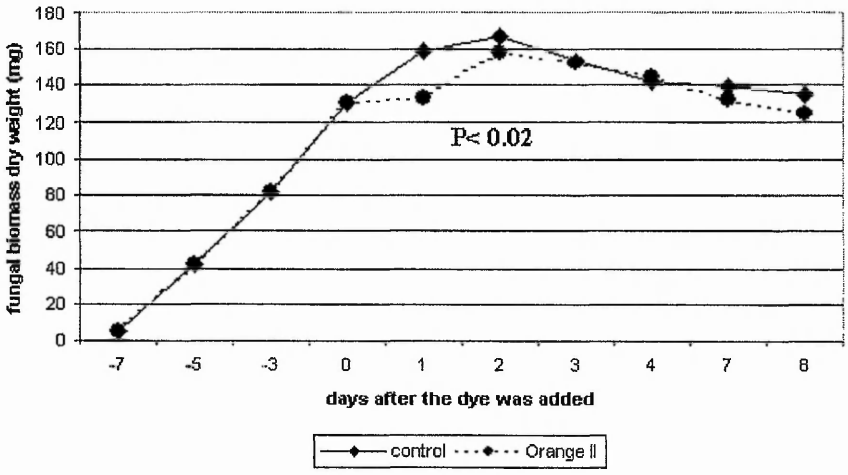


Figure 2.3.11 shows the fungal growth inhibition caused by semi decolourised Orange II according to Materials and Methods 2.2.2. Analysis of Variance used fungal growth without dye as reference, while semi-decolourised dyes showed significance (P) inhibition value.

Figure 2.3.11 showed that the first day after the Orange II was added, the growth of *T.versicolor* was reduced compared with the control.

Figure 2.3.12: *T. versicolor* growth inhibition caused by addition of Sunset Yellow.

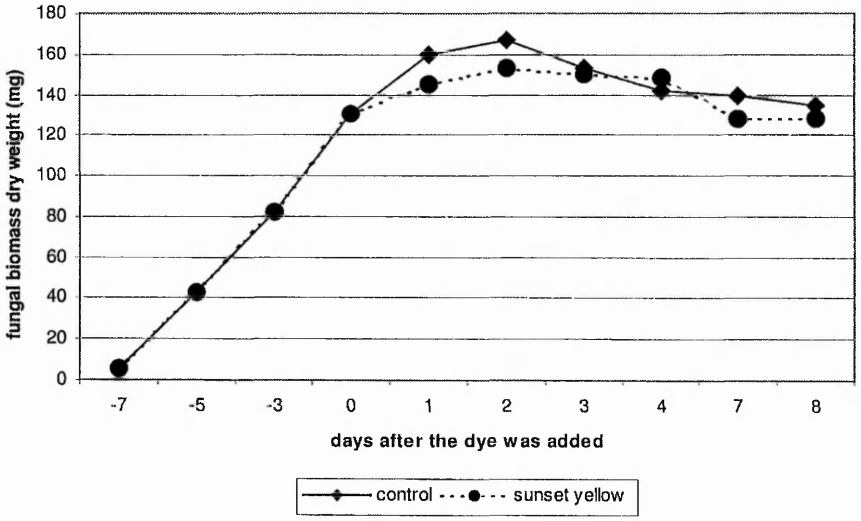


Figure 2.3.12 shows the fungal growth inhibition caused by semi decolourised Sunset Yellow according to Materials and Methods 2.2.2.

Figure 2.3.12 shows that the first two days after the Sunset Yellow was added, the growth of *T. versicolor* was reduced compared with the control samples.

Figure 2.3.13: *T. versicolor* growth inhibition caused by addition of Acid Red 183.

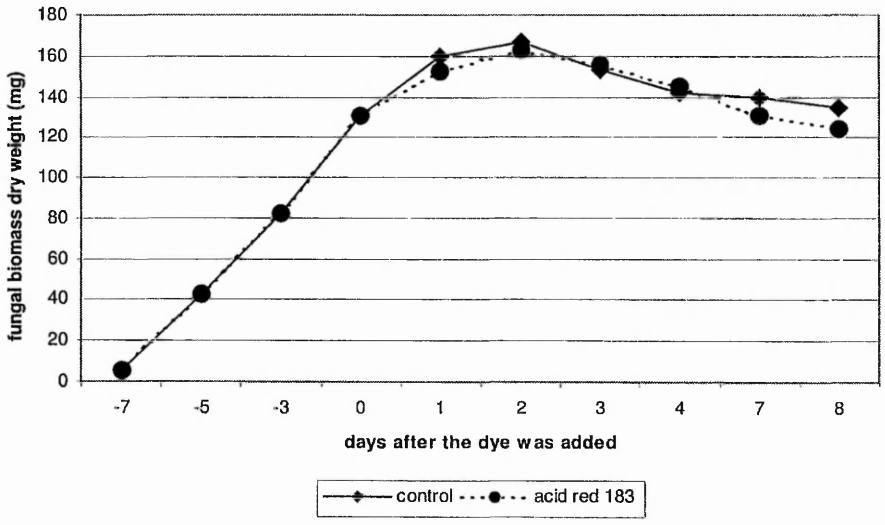


Figure 2.3.13 shows the fungal growth inhibition caused by semi decolourised Acid Red 183 according to Materials and Methods 2.2.2.

Figure 2.3.13 shows that the first day after the Acid Red 183 was added, the growth of *T. versicolor* was reduced compared with control samples.

A summary of the inhibition of *T. versicolor* following dye addition is presented in figure 2.3.14.

Figure 2.3.14: The growth inhibition caused by semi decolourised dyes.

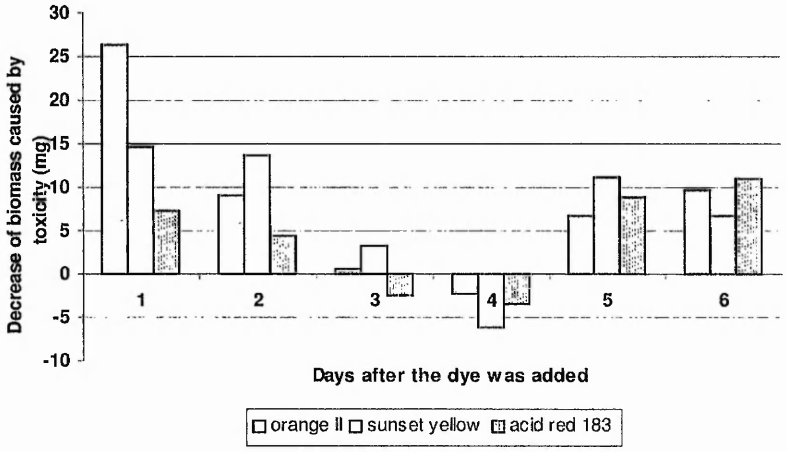


Figure 2.3.14 shows the fungal growth inhibition caused by semi decolourised dyes according to Materials and Methods 2.2.2. Where:
Y-axis shows the decrease in fungal biomass, compared with the control.
X-axis showed days after the dye was added into the fungal effluent.

Fungal decolourisation of Orange II showed the highest toxicity effect, as was also found in the bacterial decolourisation studies (of table 2.3.1). However the toxicity was not as great when expressed as % decrease in bioluminescence rather than as an EC_{50} determination.

2.3.4. Bioluminescent behaviour of naturally occurring and genetically modified organisms

The bioluminescence outputs of commercial available (freeze dried) and frozen in-house preparations of *V. fischeri* were compared for toxicity testing. Also, the bioluminescence of in-house prepared *V. fischeri* was compared with genetically modified bacteria *E. coli* (*lux*) and *Ps. putida*(*lux*).

Rehydrated commercial preparations (liquid dried, Merck™) of *V. fischeri* produced more than 300,000 RLU after 15 minutes rehydration time (as according to manufacturer's instructions). Similar levels of bioluminescence were recorded for in-house preparations of *V. fischeri* cells which had been stored frozen prior to testing.

Both liquid cultures and single colonies on agar plates of *V. fischeri*, *E. coli* HB101 containing *lux* operon plasmid pUCD607 and *Ps. putida* XL2 produced enough light to be easily detected by naked eye in a dark room. Cell suspensions in the testing buffer produced enough RLU for toxicity testing at the temperature of 15°C and pH 7. Under similar physiological conditions (i.e. similar bacterial age and cell density in culture) the naturally bioluminescent organism *V. fischeri* produced more light (~300,000) than *E. coli* HB101 (pUCD607) and *Ps. putida* XL2 (100,000 and 70,000 RLU respectively).

Since the three bioluminescent bacteria produced quantifiable amounts of light, they were compared for their response to the naphthol like compounds; as it would be generated from the azo fission during the decolourisation of textile dyes.

2.3.5. Toxicity of naphthol-related compounds

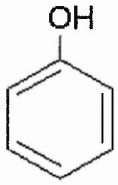
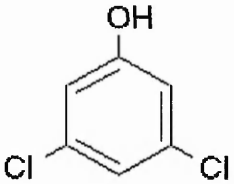
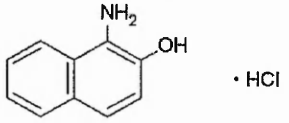
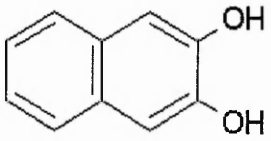
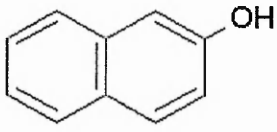
The aims of this part of the project were:

1. Test the toxicity of naphthol-based compounds, which represent products from bacterial azo dye decolourisation;

2. Compare the bioluminescent toxic test of approved, commercially available ToxAlert100®, (Merck™) with in-house prepared luminescent bacteria. The latter would be prepared according to the British Standard EN ISO 11348-1:1999.

Naphthol based compounds are the likely end products of bacterial decolourisation of textile dyes and therefore their toxicity needed to be assessed. The toxicity (EC_{50} values) of seven naphthol-based compounds together with two model organic toxicants phenol and 3,5-dichlorophenol were tested. Table 2.3.3 and 2.3.4 shows the EC_{50} values for 15 and 30 minutes exposure periods. In general EC_{50} values of commercial preparations of *V. fischeri* were lower than in-house cells (Table 2.3.3 and 2.3.4). All toxicity tests were performed in duplicate with three cell batches.

Table 2.3.3: Comparison of EC50 values (mg/L) for commercial and in-house preparation of *V. fischeri* with phenol and naphthol-based compounds (15 minutes contact time).

Chemicals	EC_{50} values (mg/L)	
	In-house *V. fischeri* cell preparation	Merck™ Liquid-dried *V. fischeri*
 Phenol	43.33±10.2	32 ± 9
 3,5-dichlorophenol	7.11± 0.69	2.3±0.17
 1-amino-2-naphthol	0.99± 0.3	0.15±0.04
 2,3-dihydroxynaphthalene	3.89± 0.38	0.93±0.15
 2-naphthol	1.11± 0.77	0.28±0.09

(to be continued on the next page)

(Table 2.3.3 continued)

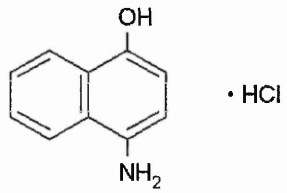
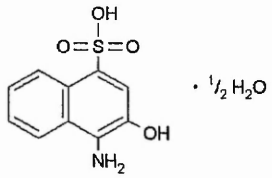
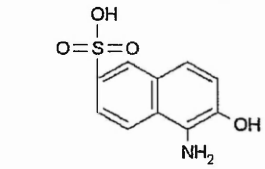
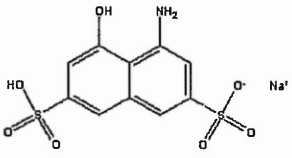
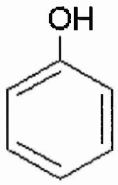
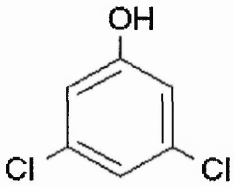
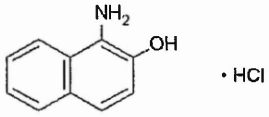
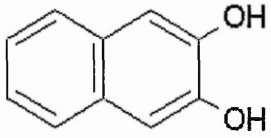
Chemicals	EC_{50} values (mg/L)	
	In-house *V. fischeri* cell preparation	Merck™ Liquid-dried *V. fischeri*
 4-amino-1-naphthol	1.57± 1.05	0.09±0.005
 1-amino-2-naphthol-4-sulphonic acid	46.91	40.04
 1-amino-2-naphthol-6-sulphate	>600	>600
 H-acid	>600	>600

Table 2.3.3 shows EC_{50} values (mg/L) for commercial and in-house preparation of *V. fischeri* with phenol and naphthol-based compounds (15 minutes contact time). According to Materials and Methods section 2.2.6

Table 2.3.4 Comparison of EC_{50} values (mg/L) for commercial and in-house preparation of *V. fischeri* with phenol and naphthol-based compounds (30 minutes contact time).

Chemicals	EC_{50} values (mg/L)	
	In-house *V. fischeri* cell preparation	Merck™ Liquid-dried *V. fischeri*
 Phenol	58.33 ± 10.5	32 ± 9
 3,5-dichlorophenol	7.66 ± 0.66	2.2±0.2
 1-amino-2-naphthol	0.88± 0.28	0.13±0.035
 2,3-dihydroxynaphthalene	4.9± 0.4	1.13±0.15

(To be continued on the next page)

(Table 2.3.4 continued)

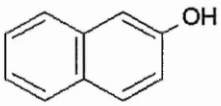
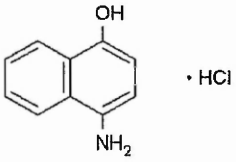
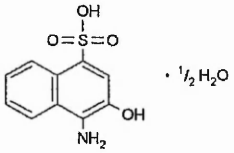
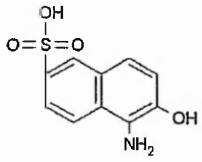
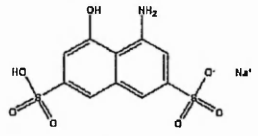
Chemicals	EC_{50} values (mg/L)	
	In-house *V. fischeri* cell preparation	Merck™ Liquid-dried *V. fischeri*
 2-naphthol	1.23± 0.7	0.29±0.07
 4-amino-1-naphthol	0.23± 0.033	0.10±0.005
 1-amino-2-naphthol-4-sulphonic acid	48.21	41.33
 1-amino-2-naphthol-6-sulphate	>600	>600
 H-acid	>600	>600

Table 2.3.3 shows EC_{50} values (mg/L) for commercial and in-house preparation of *V. fischeri* with phenol and naphthol-based compounds (30 minutes contact time). According to Materials and Methods section 2.2.6.

Generally the EC_{50} values of commercial preparations of *V. fischeri* were lower than in-house prepared cells (Table 2.3.3 and 2.3.4). In addition, as expected, the

shorter incubation time of 15 minutes showed relative lower EC_{50} values than longer incubation time of 30 minutes.

2.3.6. Toxicity comparison of naphthol based compounds using three luminescent bacteria

It has been described in previous sections (2.3.3 to 2.3.5) that decolourised azo dyes are toxic to the marine bacterium *V. fischeri.* However *V. fischeri* is a psychrotrophic bacterium, and hence toxicity is assessed at 15°C, additionally it requires at least 20g/L sodium chloride. There was no publication describe its toxicity in contrast to terrestrial and mesophilic bacteria, as this information may address pollution problems that human beings are facing. As part of this investigation, terrestrial bioluminescent biosensors were used to assess toxicity of naphthol compounds on terrestrial organisms.

As a standard procedure for bioluminescent toxicity testing, with *V. fischeri* all agents contained 20g/L sodium chloride. Whereas no sodium chloride was required in assays using bioluminescent *E. coli* and *Ps. putida.*

All three bioluminescent bacteria, *V. fischeri, E. coli* (HB101, pUCD607) and *Ps. putida* (XL2), were tested in the standard pH environment. These bacteria were suspended in buffer solution at pH 7 to guarantee the whole toxicity test was under stable pH environment.

E. coli and *Ps. putida* are mesophiles and grow optimally at 37°C and 30°C, respectively. Therefore initial experiments were carried out using a toxicity testing temperature of 37°C for *E. coli* and 30°C for *Ps. putida.* However the luminescence output by *E. coli* and *Ps. putida* at higher temperatures showed unstable luminescence intensity. Additionally the temperature and sodium chloride concentration modification do not comply with the British and International testing Standard (BS EN ISO 11348-1:1999). Subsequently 15°C

was used as universal toxicity testing temperature for all the bacteria as more reproducible results were generated.

Seven naphthol-based compounds, which represent putative toxic products generated from bacterial decolourisation, were tested for RLU inhibition of the three prokaryotic organisms: *V. fischeri, E. coli* and *Ps. putida.* Two-model organic toxicants phenol and 3,5-dichlorophenol were use as positive controls.

The intensity of bioluminescence was monitored after 15 and 30 minutes exposure time. However for presentation purposes, only 15 minute exposure time results are presented in this thesis. As expected, the inhibition effect increased with increasing toxicant concentration. All experiments were repeated at least three times, and the standard deviations of each experiment are indicated as error bars.

The error bars indicate that the *Ps. putida* has the most variable bioluminescent behaviour in response to toxicants, *E. coli* (HB101) showed less variable results than *Ps. putida* and *V. fischeri.* The least variable *V. fischeri* has relatively most stable bioluminescent behaviour among three bacteria against toxicants.
Bioluminescence response of *V. fischeri, E. coli* and *Ps. putida* on exposure to the model organic toxicant phenol are described in figure 2.3.15.

Figure 2.3.15: Toxicity effect of phenol after 15 minute contact time.

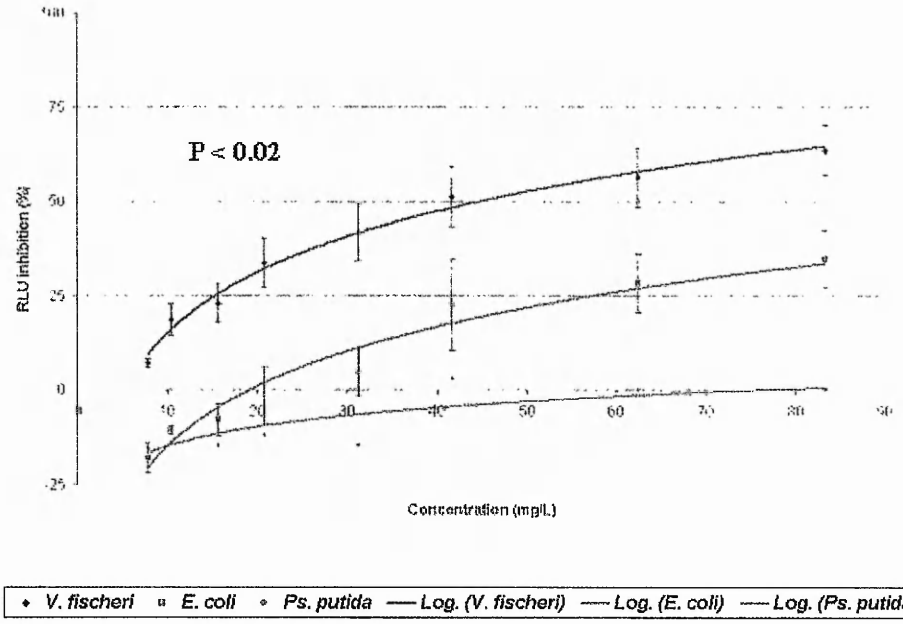


Figure 2.3.15 shows that three bacteria *V. fischeri, E. coli* and *Ps. putida* were treated with phenol according to Materials and Methods section 2.2.6 and 2.2.7. Analysis of Variance used *V. fischeri* as reference, while *E. coli* and *Ps. putida* showed significance (P) inhibition value.

Phenol (Table 2.3.3) is a recognised organic toxicant, and is also the standard organic toxicant used for bioluminescence assay of *V. fischeri.* It has been recommended by Azur Environmental Ltd for assessing variation in *Vibrio* sensitivity to toxicants.

The intensity of bioluminescence of the three prokaryotic organisms, *V. fischeri, E. coli* and *Ps. putida*, decreased on exposure to phenol, and it was concentration dependent. For *V. fischeri*, the bioluminescence was inhibited 50% while the phenol concentration reached 43mg/L. Hence, the EC_{50} value of phenol to the *V. fischeri* was about 43mg/L. The EC_{50} value of *E. coli* was higher than 90mg/L. *Ps. putida* showed relative low bioluminescence change on exposure to phenol.

It should be noted that phenol can perturb cellular metabolism such that enhances [$FMNH_2$] and subsequently bioluminescence can increase on exposure to phenol

as well as decrease. The bioluminescence intensity generated from *E. coli* and *Ps. putida* was increased in certain phenol concentrations. Figure 2.3.15 showed that *E. coli* and *Ps. putida* on exposure to phenol showed negative bioluminescence inhibition effect (at concentration of 20mg/L and 80mg/L respectively). Therefore toxicity measurement according to bioluminescence determination is regarded as the change in bioluminescence rather than simply the decrease in bioluminescence.

The bioluminescence response of *V. fischeri, E.coli* and *Ps. putida* on exposure to the model organic toxicant 3,5-dichlorophenol are shown in figure 2.3.16.

Figure 2.3.16: Toxicity effect of 3,5-dichlorophenol after 15 minute contact time.

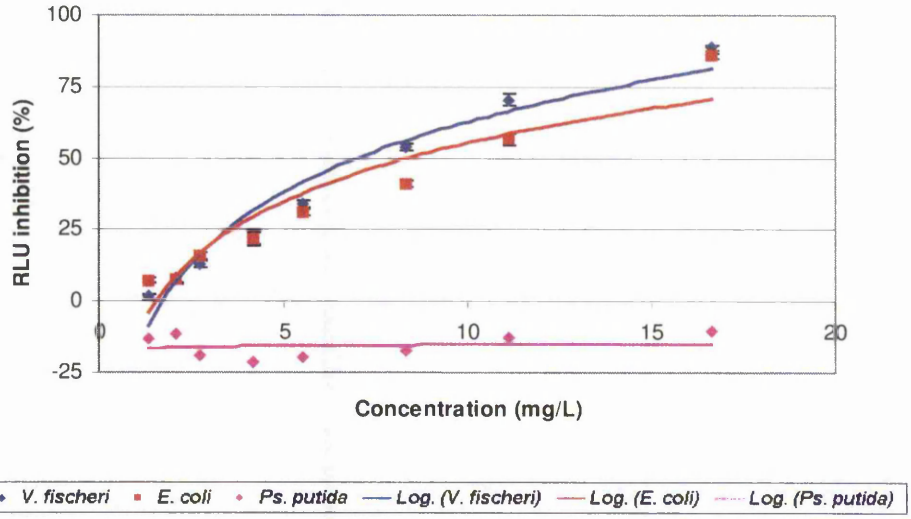


Figure 2.3.16 shows that three bacteria *V. fischeri, E. coli* and *Ps. putida* were treated with 3,5-dichlorophenol according to Materials and Methods section 2.2.6 and 2.2.7.

3,5-dichlorophenol (table 2.3.3) is also a reference organic toxicant for bioluminescence assay of *V. fischeri.* It has been recommended by Merck Ltd for assessing variation in sensitivity to toxicants.

As expected, the bioluminescence intensity decreased on exposure to the 3,5-dichlorophenol. Figure 2.3.16 shows the bioluminescence response of *V. fischeri* and *E. coli*, were inhibited on exposure to 3,5-dichlorophenol. The EC_{50} value of 3,5-dichlorophenol to the *V. fischeri* was about 7.1mg/L, and to *E. coli* was about 8.37mg/L.

The bioluminescence intensity generated from *Ps. putida* was generally increased on exposure to 3,5-dichlorophenol. Figure 2.3.16 shows that the bioluminescence

inhibition effect was negative when *Ps. putida* was exposured to 3,5-dichlorophenol. It should also be regarded as a toxicity effect to *Ps. putida*.

The bioluminescence responses of *V. fischeri, E. coli* and *Ps. putida* on exposure to 1-amino-2-naphthol are shown in figures 2.3.17 and 2.3.18.

Figure 2.3.17: Toxicity effect of 1-amino-2-naphthol on *V. fischeri* after 15 minute contact time.

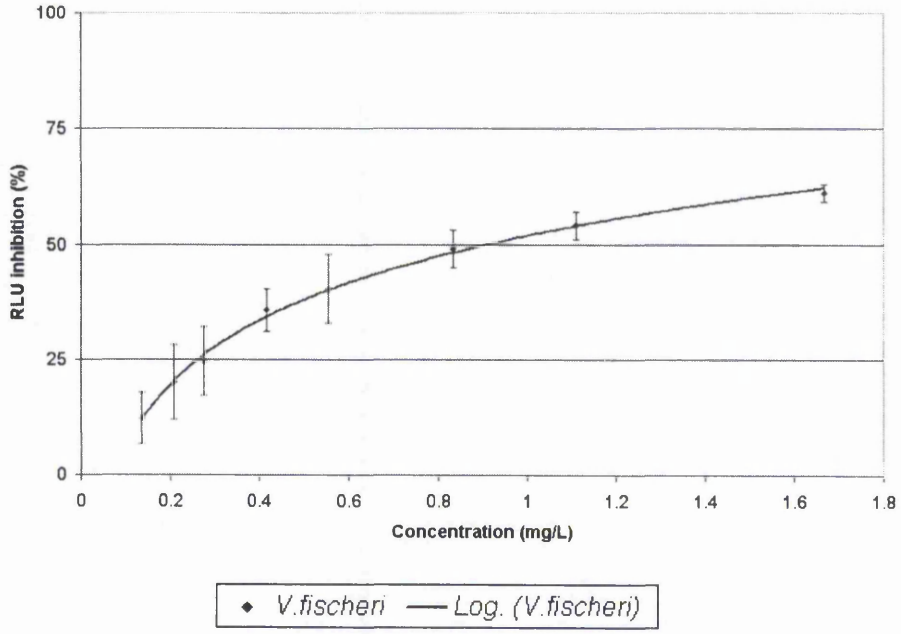


Figure 2.3.17 shows that bacterium *V. fischeri* was treated with 1-amino-2-naphthol according to Materials and Methods section 2.2.6 and 2.2.7.

1-amino-2-naphthol (table 2.3.3) is a simple non-coloured naphthol based compound. Figure 2.3.17 shows that the bioluminescence of *V. fischeri,* was inhibited on exposure to low concentration 1-amino-2-naphthol. Specifically, the *V. fischeri* EC_{50} value of 1-amino-2-naphthol was about 0.99mg/L.

Toxicity effects of 1-amino-2-naphthol to *E. coli* and *Ps. putida* is described in figure 2.3.18 due to the difference in concentration range compared with *V. fischeri*.

Figure 2.3.18: Toxicity effect of 1-amino-2-naphthol on *E. coli* and *Ps. putida* after 15 minute contact time.

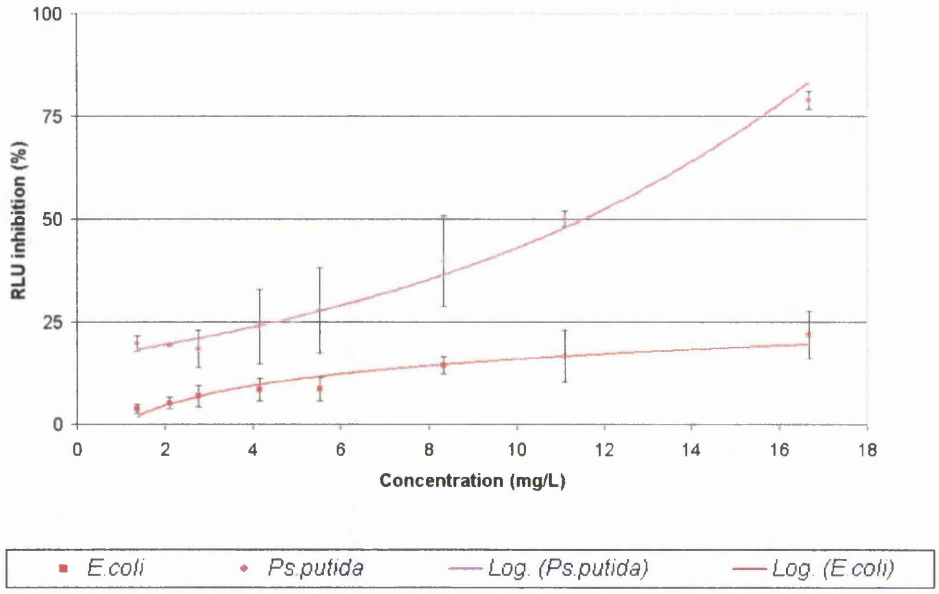


Figure 2.3.18 shows that bacteria *E. coli* and *Ps. putida* were treated with 1-amino-2-naphthol according to Materials and Methods section 2.2.6 and 2.2.7.

1-amino-2-naphthol was much less toxic to *E. coli* and *Ps. putida* than *V. fischeri* (of figure 2.3.17 and 2.3.18). The EC_{50} value of 1-amino-2-naphthol to the *Ps. putida* was about 11mg/L whereas for 1-amino-2-naphthol, very limited toxicity was detected by the bacterium *E. coli*.

Bioluminescence responses of *V. fischeri, E. coli* and *Ps. putida* on exposure to 2, 3 dihydroxynaphthalene are described in figure 2.3.19.

Figure 2.3.19: Toxicity effect of 2,3 dihydroxynaphthalene after 15 minute contact time.

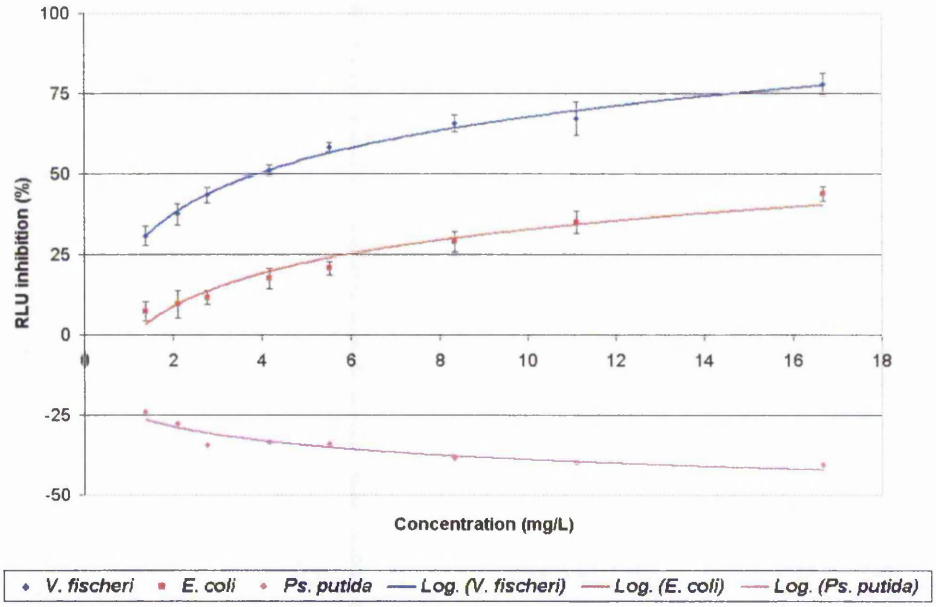


Figure 2.3.19 shows that three bacteria *V. fischeri, E. coli* and *Ps. putida* were treated with 2,3 dihydroxynaphthalene according to Materials and Methods section 2.2.6 and 2.2.7.

Figure 2.3.19 shows that the intensity of bioluminescence of *V. fischeri, E. coli* and *Ps. putida*, changed on exposure to 2, 3 dihydroxynaphthalene. The EC_{50} value of 2, 3 dihydroxynaphthalene to the *V. fischeri* was about 3.89mg/L. 3,5-dichlorophenol showed much less toxicity to *E. coli,* its EC_{50} value was more than 16mg/L.

Figure 2.3.19 showed that the bioluminescence of *V. fischeri* and *E. coli* was decreased on exposure to 2, 3 dihydroxynaphthalene, whereas *Ps. putida* increased its bioluminescence. Furthermore, the bioluminescence generated from *Ps. putida* increased with the increased concentration of 2, 3 dihydroxynaphthalene.

Bioluminescence responses of *V. fischeri, E. coli* and *Ps. putida* on exposure to the 4-amino-1-naphthol are described in figure 2.3.20.

Figure 2.3.20: Toxicity effect of 4-amino-1-naphthol after 15 minute contact time.

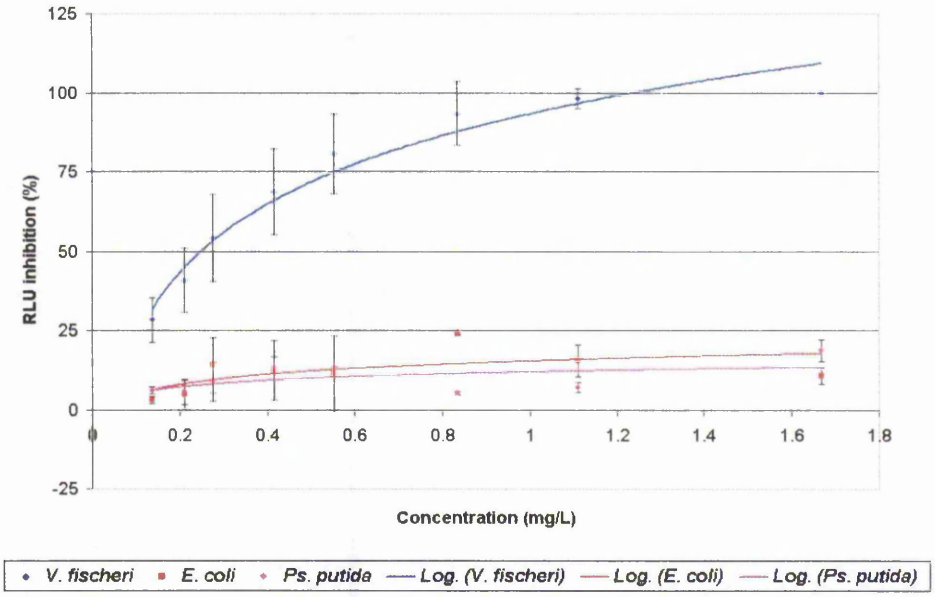


Figure 2.3.20 shows that three bacteria *V. fischeri, E. coli* and *Ps. putida* were treated with 4-amino-1-naphthol according to Materials and Methods section 2.2.6 and 2.2.7.

Figure 2.3.20 shows that the intensity of bioluminescence of *V. fischeri, E. coli* and *Ps. putida*, was changed on exposure to 4-amino-1-naphthol. The EC_{50} value of 4-amino-1-naphthol to the *V. fischeri* was about 1.57mg/L. 4-amino-1-naphthol had much less affection on bioluminescence production of *E. coli* and *Ps. putida.*

Bioluminescence responses of *V. fischeri, E. coli* and *Ps. putida* on exposure to 2-naphthol are described in figure 2.3.21.

Figure 2.3.21: Toxicity effect of 2-naphthol after 15 minute contact time.

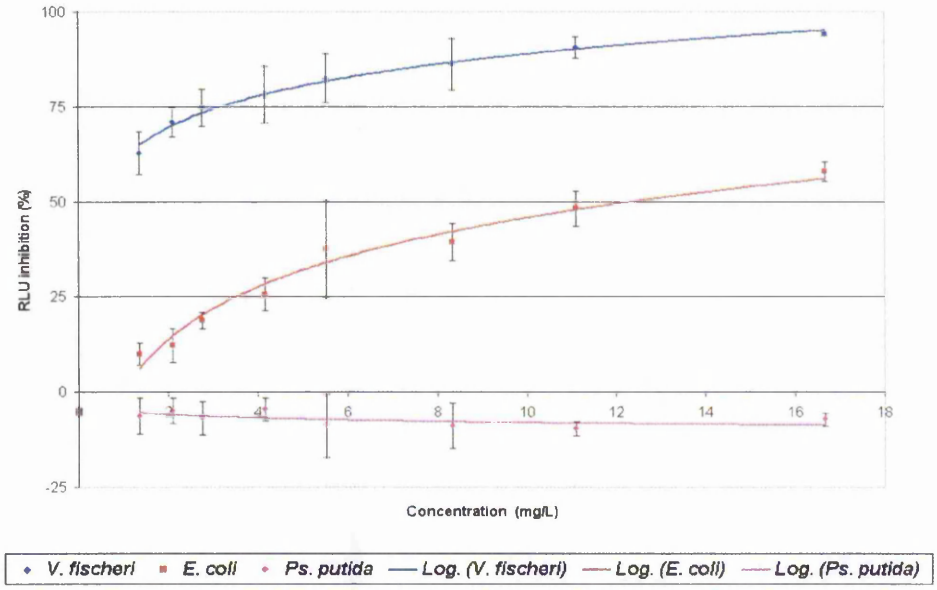


Figure 2.3.21 shows that three bacteria *V. fischeri, E. coli* and *Ps. putida* were treated with 2-naphthol according to Materials and Methods section 2.2.6 and 2.2.7.

Figure 2.3.21 shows that the intensity of bioluminescence of *V. fischeri, E. coli* and *Ps. putida*, was changed on exposure to 2-naphthol. The EC_{50} value of 2-naphthol to the *V. fischeri* was about 1.1mg/L. 2-naphthol showed much less toxicity towards *E. coli,* the EC_{50} value of 2-naphthol to the *E. coli* was about 12 mg/L.

Figure 2.3.21 showed that the bioluminescence generated from *V. fischeri* and *E. coli* decreased on exposure to 2-naphthol, whereas *Ps. putida* increased its bioluminescence production on exposure to 2-naphthol. Furthermore, the bioluminescence generated from *Ps. putida* remained relatively stable on exposure of 2-naphthol.

Bioluminescence responses of *V. fischeri, E. coli* and *Ps. putida* on exposure to the coloured naphthol compound H-acid are described in figure 2.3.22.

Figure 2.3.22: Toxicity effect of H-acid after 15 minute contact time.

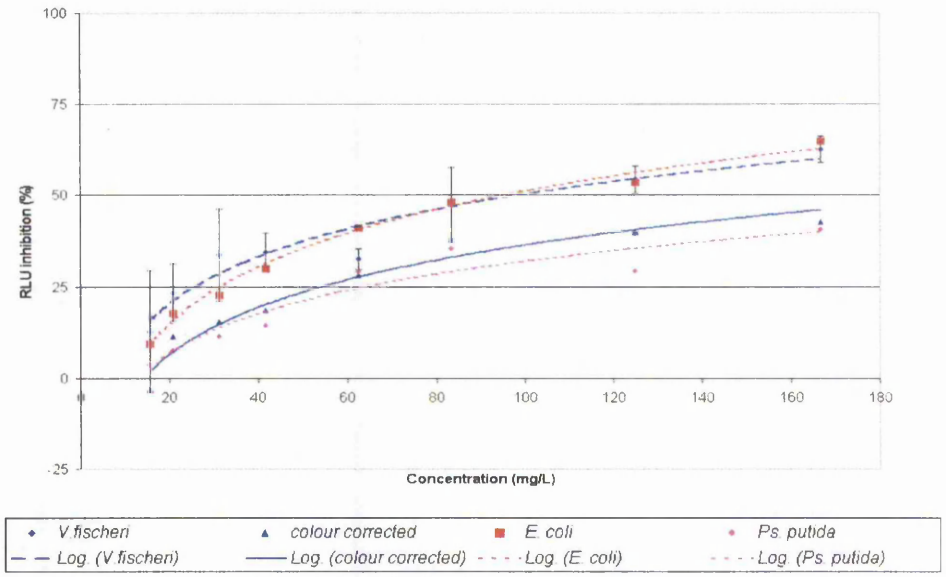


Figure 2.3.22 shows that three bacteria *V. fischeri, E. coli* and *Ps. putida* were treated with H-acid according to Materials and Methods section 2.2.6 and 2.2.7.

As expected, the luminescence intensity decreased on exposure to H-acid.

It has been noticed that H-acid was a coloured compound, and bacterial luminescence were in the spectrum spanning from 420 to 630 nm with intensity maximum at 490 nm. Also 490nm is the detection wavelength for the bioluminescence toxicity test by the luminometer ToxAlert 100.

Coloured compounds present in the bacteria extra-cellular environment. Light emitted by bioluminescent bacteria may be absorbed by the colour compounds at 490nm, and therefore cannot be monitored by a luminometer. Consequently, regardless of the maximum absorbance values of the coloured compounds, absorbance at 490 nm was measured to deduct the interference of the colour. For detailed methods please refer to the materials and methods section 2.2.7. This colour correction method was suggested by Azur Environmental Ltd.

Figure 2.3.22 shows that the bioluminescence behaviour of *V. fischeri, E. coli* and *Ps. putida*, changed on exposure to H-acid. On which, the *V. fischeri* toxicity

effect have been colour corrected. The EC50 values of *V. fischeri* was greater than 200mg/L, and *E. coli* showed similar toxicity effect as *V. fischeri*. Again, *Ps. putida* showed insensitive to h-acid.

Bioluminescence responses of *V. fischeri, E. coli* and *Ps. putida* on exposure to the coloured naphthol compound 1-amino-2-naphthol-4-sulfonic acid are described in figure 2.3.23.

Figure 2.3.23: Toxicity effect of 1-amino-2-naphthol-4-sulfonic acid after 15 minute contact time.

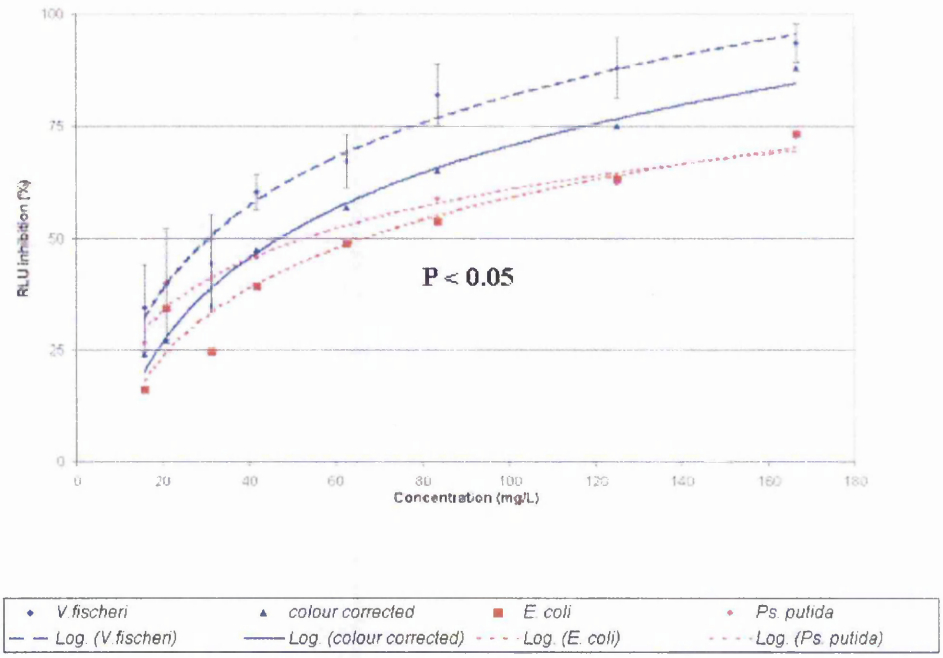


Figure 2.3.23 shows that three bacteria *V. fischeri, E. coli* and *Ps. putida* were treated with 1-amino-2-naphthol-4-sulfonic acid according to Materials and Methods section 2.2.6 and 2.2.7. Analysis of Variance used *V. fischeri* as reference, while *E. coli* and *Ps. putida* showed significance (P) inhibition value.

1-amino-2-naphthol-4-sulfonic acid was another coloured naphthol based compounds. Figure 2.3.23 shows the bioluminescence responses of *V. fischeri, E. coli* and *Ps. putida*, changed on exposure to 1-amino-2-naphthol-4-sulfonic acid. The *V. fischeri* toxicity effects have been colour corrected. The EC_{50} values of *V.*

fischeri was 46.9mg/L. Bacteria *E. coli* and *Ps. putida* showed less sensitivity to 1-amino-2-naphthol-4-sulfonic acid.

Bioluminescence responses of *V. fischeri, E. coli* and *Ps. putida* on exposure to the coloured naphthol compound 1-amino-2-naphthol-6-sulphate are described in figure 2.3.24.

Figure 2.3.24: Toxicity effect of 1-amino-2-naphthol-6-sulphate after 15 minute contact time.

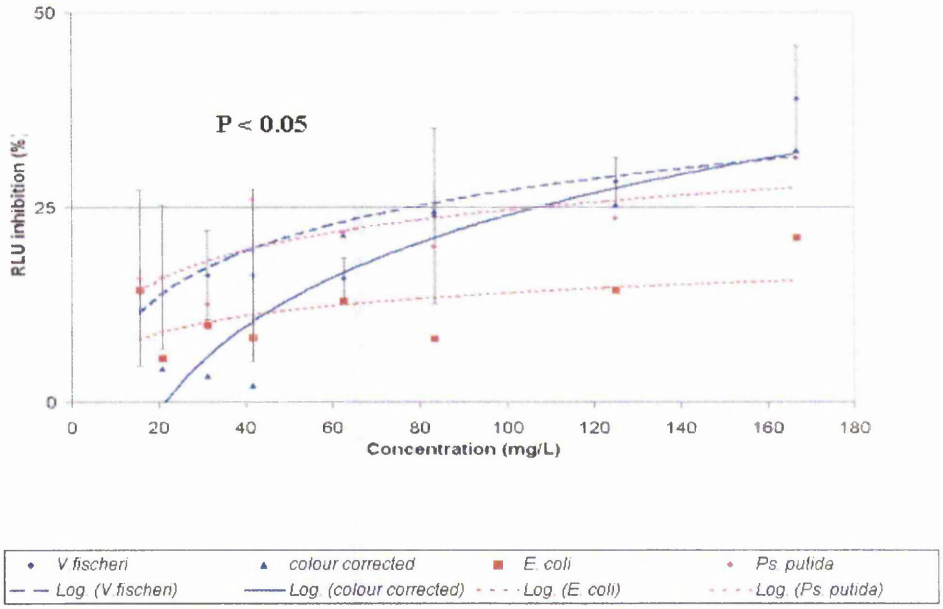


Figure 2.3.24 shows that three bacteria *V. fischeri, E. coli* and *Ps. putida* were treated with 1-amino-2-naphthol-6-sulphate according to Materials and Methods section 2.2.6 and 2.2.7. Analysis of Variance used *V. fischeri* as reference, while *E. coli* and *Ps. putida* showed significance (P) inhibition value.

1-amino-2-naphthol-6-sulphate was another coloured naphthol based compound. Figure 2.3.24 shows the bioluminescence responses of *V. fischeri, E. coli* and *Ps. putida*, changed on exposure to 1-amino-2-naphthol-6-sulphate. The *V. fischeri* toxicity effects have been colour corrected. The EC_{50} value of *V. fischeri* was greater than 200mg/L. Bacteria *E. coli* and *Ps. putida* showed less sensitivity to 1-amino-2-naphthol-6-sulphate.

2.4 Discussion

2.4.1. Bacterial and fungal decolourisation

Overnight cultures of *E. faecalis* rapidly decolourised the azo dyes. This bacterial decolourisation was so fast that the colours disappeared within a few minutes. Whereas using the fungus *T. versicolor*, 11 days were required for the organism to complete the pre-stationary growth phase, which was then followed by 7 days of decolourisation. This comparative study clearly showed that the bacterium *E. faecalis* can decolourise equal concentrations of azo dyes much more quickly than *T. versicolor*. The bacterial decolourisation progress in this investigation was too fast to be quantified. Whereas using the white-rot fungus *T. versicolor*, the decolourisation could be measured.

An obvious benefit of using the bacterium *E. faecalis* is the high rate of multiplication. The growth curves (cross reference to section 2.3.1 and figure 2.3.1) of *T. versicolor* indicated that the eukaryotic organism *T. versicolor* needed several days before it was in the appropriate growth phase.

Although bacterial decolourisation was fast (section 2.3.1), the mechanism of bacterial decolourisation is still uncertain. It has been widely believed that the decolourisation by *E. faecalis* is due to the reduction of the azo-bond in the dye. Subsequently aromatic amines will be generated. These organic chemicals are known as toxicants and genotoxic (Sweeney *et al.*, 1994; Gottlieb *et al.*, 2003).

In this study, *E. faecalis* was able to decolourise Orange II, Sunset Yellow and Reactive Black but the bacterium was not able to decolourise Acid Red 183 (section 2.3.1). Jarosz-Wilkolazka *et al* (2002) found that a number of fungal strains also could not decolourise Acid Red 183. These studies may suggest the chemical structure of the dye plays a key role for the ability of decolourisation enzymes. Fortunately *T. versicolor* is able to decolourise Acid Red 183 and generate non toxic products. *T. versicolor* is described as one of the most effective

fungal dye degrader, due to the non-substrate specific ligninolytic enzyme system (Jarosz-Wilkolazka *et al.*, 2002).

2.4.2. Toxicity of decolourised textile dye containing effluent

Due to the specific azo dye fission activity, without ring cleavage, it can be predicted that bacterial decolourisation is more likely to generate toxic effluent than fungal decolourisation. For example, EC_{50} values of bacterial decolourised Orange II at 15 minutes contact time was 0.8 mg/L, while the toxicity of fungal decolourised Orange II was too low to be expressed as an EC_{50} value (see Table 2.3.1). Instead it was expressed as a bioluminescence inhibition vaue, which was lower than 40% of the control. This inhibition effect normally can be regarded as low toxicity.

100mg/L of fungi decolouised orange II only caused 40% of bioluminescence reduction (figure 2.3.8). In another words, bacterially decolourised Orange II was 156 times more toxic than when it was decolourised by the fungi. Similarly, bacterial decolourised Sunset Yellow generated about 10 times more toxicity than the fungi (table 2.3.2 and figure 2.3.10).

Although cellular toxicity is normally detected as a decrease in bioluminescence, under certain conditions such as low toxicant concentrations, the amount of light emitted can increase. This is presented as "negative % inhibition" values. This is a recognised phenomenon and is due the toxicant affecting cellular metabolism. For example, bioluminescent *Ps. putida* showed negative bioluminescence inhibition on exposure to phenol (figure 2.3.15), 3,5-dichlorophenol(figure 2.3.16), 2-3 dihydroxynaphthalene (figure 2.3.19) and 2-naphthol (figure 2.3.21). Bioluminescent *E. coli* also showed negative bioluminescence inhibition on exposure to phenol (figure 2.3.15).

2.4.3. Mechanism of decolourisation:

The precise mechanism of bacterial decolourisation is still in dispute, and scientific methods to prove the mechanism of bacterial decolourisation remain limited. However the most widely accepted theory is that anaerobic bacterial decolourisation is due to azo bond fission (Sweeney *et al.* 1994; Gottlieb *et al.* 2003). And the chemical naphthalene could not be mineralised by anaerobic reduction (Artz *et al.* 2002).

The toxicity of bacterial decolourised azo dyes showed similar toxicity as corresponding naphthol based compounds and aromatic amines. For example, the EC_{50} value at 15 minutes contact time of bacterial decolourised Orange II is 0.8mg/L, and EC_{50} values of their azo bond broken products 1-amino-2-naphthol and sulphanilic acid was 0.15mg/L and 38mg/L respectively. Other examples, EC_{50} of bacterial decolourised Sunset Yellow was 37mg/L, and their corresponding end-product of sulphanilic acid was 38mg/L, while 1-amino-2-naphthol-6-sulphonate showed very little toxicity. The toxicity of reduced Sunset Yellow was probably due to the sulphonate group which lowers cell wall permeability and hence toxicity. These results further support the theory that decolourisation by anaerobic bacteria reduction is due to the azo bond fission.

White rot fungi on the other hand, due to their ligninolytic enzymes were able to mineralise the dye and hence yield relatively low toxicity products. Spadaro *et al.* (1992) proposed that decolourisation of azo dyes by ligninolytic enzymes is an oxidative process that can result in complete mineralise the dye molecule to H_2O and CO_2. Although no general pattern has been established between the structure of dyes and decolourisation, it can be inferred from research so far that the dyes that may have the chemical shape similar to the natural substrate will be decolourised faster and more efficiently (Pasti-Grigsby *et al.* 1992).

In the fungal decolourisation process, the toxicity (expressed as bioluminescence inhibition effect) of semi-decolourised effluent decreased daily for seven days, by which time the majority of the colour was removed. The fungal decolourisation

process was very active in the first 24 hours. For example with Orange II about 70% of the colour was removed in the first day, the following second day only removed 10% of the colour and after about 6-7 days, the colour in the culture media was completely removed. Hydrolysed reactive black was also decolourised by about 50% in the first day.

The toxicity of fungal decolourised dyes also corresponded with the toxicity of naphthol and aromatic amines. As will be discussed further, the toxicity was demonstratable not only by bioluminescence (figures 2.3.8, 2.3.9 and 2.3.10), but also, the growth of fungal biomass (figures 2.3.11 to 2.3.14).

Semi-decolourised dye inhibited bioluminescence production. For example the toxicity of Orange II in table 2.3.1 (1-amino-2-naphthol, 0.15mg/L; and sulphanilic acid, 38mg/L). These are higher than Sunset Yellow as shown in table 2.3.2 (sulphanilic acid, 38mg/L; 1-amino-2-naphthol-6-sulphonate, none toxic). Similarly, in the first day of fungal decolourisation, semi-decolourised Orange II caused more than 25% of bioluminescence inhibition (figure 2.3.8); by contrast, Sunset Yellow caused less than 15% bioluminescence inhibition (figure 2.3.9).

Semi-decolourised dyes also inhibited fungal growth. For example, semi-decolourised Orange II decreased the accumulation of the fungal biomass by more than 20mg (figure 2.3.11) in the first day, and Sunset Yellow caused about 10mg biomass decrease (figure 2.3.12) in the first day.

Nitrogen limiting medium (F&R medium) and the pre-stationary growth phase were used to maximise the ligninolytic enzyme production. These enzymes actively involved into dye molecule cleavage soon after the dye was added when the fungi were in pre-stationary growth phase.

The cleavage of the dye molecule resulted in changes of molecule polarity and water solubility, and hence further toxic organic compounds may be generated. These mixed organic compounds could alter physical attractions between molecules and consequently denature proteins. These physical attractions including: hydrogen bond, Van-der-waals attraction, and hydrophobic interaction,

which maintain the three dimension structure and biological function of both ligninolytic enzymes and enzymes required for fungal growth. The toxicities of organic compounds to fungi was evidenced by Weitz *et al.* (2002). Natural bioluminescent fungi were exposed to the model organic compounds 3,5-dichlorophenol, before measurement of bioluminescence reduction.

The ligninolytic enzymes are associated with secondary metabolism. However, the fungus was able to express these enzymes and grow at the same time. Possibly the mineralisation of toxicants was rapid enough to maintain fungal growth conditions.

In addition to enzymatic degradation, it was also noticed that in the initial days of fungal decolourisation, the fungal biomass was obviously coloured (section 3.2.1). This phenomenon may suggest that physical adsorption is a contributor to fungal decolourisation. These adsorbed dyes were also degraded by ligninolytic enzymes, as dyes adsorbed in the fungi biomass decolourised along with the dye effluent.

Since ligninolytic enzymes are regarded as the main mechanism in fungal decolourisation, enhancing the production of these enzymes may greatly improve the azo dye decolourisation.

Rodriguez *et al.* (1999) described non-specific fungal oxidative enzymes (lignin peroxidase, manganese peroxidase, laccase), which attack a wide range of azo dyes outside the cell. An example of this approach was described by Williams *et al.* (1991). who applied biotechnological techniques to enhance production of polyphenol oxidase by *T. versicolor.* Polyphenol oxidase converts o-diphenois to o-diquinones and oligomerizes syringic acid. Because *T. versicolor* can be batch cultured, overproduction and enhanced secretion of these enzymes by molecular genetics (Williams *et al.*, 1991). This technology should be applicable for the overproduction of more commercially important cellulases and ligninases (Desoretz, 1993). In this connection, Kammermeyer and Clark (1989) published an extensive monograph regarding foreign gene insertion and cell trans-formation. MnP was also successfully expressed in *Aspergillus oryzae* (Stewart *et al.*, 1996). The recombinant MnP was secreted into culture media in the active form. This

would provide large quantities of pure enzymes suitable for decolourisation studies.

There are no previous studies on the toxicity of fungal decolourised dyes; hence these approaches need to be further studied.

2.4.4. Bioluminescent behaviour of different bacteria

The bioluminescence of three bacteria: *V. fischeri E. coli* (HB101, pUCD607), and *Ps. putida* (XL2) was easily detected by the naked eye and quantified using a luminometer. The natural bioluminescent organism *V. fischeri* showed the highest light output, and genetically modified *E. coli* (HB101, pUCD607) shows stronger light output than *Ps. putida* (XL2).

Natural bioluminescent bacteria produced the best luminescent intensity compared with the genetically engineered bacteria. The level of luminescence depends on several factors including: the luciferases gene expression level, temperature, and the luciferase substrates abundance (oxygen, and reduced flavin monomucleotide $FMNH_2$). Overall, the bioluminescence reaction is a dynamic process, and responds to the metabolic ability of the cell.

The luciferase naturally expressed in *V. fischeri*, is linked to quorum sensing. Whilst in the genetic modified bacteria, the luciferase expression level largely depended on the activity of regulatory promoter to which the *lux* operon was fused. In *E. coli* HB101 as modified by plasmid pUCD607, there was no promoter information available. Luminescent *Ps. putida* were genetically modified using transposon insertion, which enables the *lux* operon to randomly insert into the genome. Unfortunately, again there was no information with respect to the promoter activity.

The growth temperature of *V. fischeri* is 25°C. Whereas *E. coli* and *Ps. putida* are mesophiles, and grow optimally at 37°C and 30°C respectively. However

luminometers specifically manufactured for bioluminescence-based toxicity assays are pre-set to operate at 15°C as required by the ISO method. Therefore the main comparison between the toxicity results between the organisms was to use of high salt (2%) with *V. fischeri.* It was possible to cancel the temperature control, however the lack of temperature stability influenced the reliability of bioluminescence data. This issue needs to be more fully addressed in order to fully use the more thermostable *lux* system of *Ph. luminescens*, which has been engineered into *Ps. putida.* This temperature standard is obviously low for *E. coli* and *Ps. putida* and may account for the slower response.

Bioluminescence is an energy consuming reaction. In eukaryotic luciferase system (*luc*), ATP is the substrate. While in prokaryotic luciferase system (*lux*), $FMNH_2$ is the substrate of the bacterial luciferase, and $FMNH_2$ generated from the electron transport chain is oxidized to an excited form of flavin monomucleotide (FMN), a carrier molecule derived from riboflavin that emits light as it returns to its unexcited state. In this later process, phosphorylation reactions are bypassed, and less ATP is generated. In other words, ATP was indirectly consumed by bacterial luciferase for producing light. It has been known that the luminescence enables *V. fischeri* to enter a symbiotic relation with squid (*Euprymna scolopes*). However, there was no obvious function for *E. coli* and *Ps. putida*, as a result necessary energy of *E. coli* and *Ps. putida* cells were drained by luminescence reaction.

E. coli and *V. fischeri*, are facultative anaerobes whereas *Ps. putida* is an aerobe. Oxygen is poorly dissolved in water; and therefore limited resources for bacteria to remain viable and bioluminescence. The luciferase reaction requires as much as 10-20% of the total available oxygen (Hastings *et al.* 1985) This competition for oxygen between luciferase and other metabolic enzymes has been previously identified as a possible source of complex luminescence behaviour (King *et al.* 1990). As this investigation shows *Ps. putida*, which has the highest oxygen requirements, showed relatively lower bioluminescence.

2.4.5. Toxicity testing using in-house made and commercially available liquid dried *V. fischeri*

Toxicity assays based on *V. fischeri* bioluminescence can provide a rapid assessment of chemical toxicity, and it has been successfully applied to toxicity assessment in azo-dye decolourisation (Gottlieb *et al.* 2003). Two sources of *V. fischeri* cells have been compared in this study: commercial available cells from Merck and frozen cells prepared in-house following the British Standard 'BS EN ISO 11348-1:1999'.

The price of commercial available liquid dried *V. fischeri* is much higher than the laboratory prepared bacteria. Bacteria for bioluminescent toxicity test from commercial sources cost at least three sterling pounds per test, while with the in-house method, a 50ml overnight liquid culture stored with glucose generated enough cells for 100 tests. In addition, in-house prepared frozen cells showed more stable bioluminescent behaviour than the commercially provided bacteria. Obviously commercial liquid dried bacteria are more convenient and save time and labour compared with in-house prepared bacteria.

The data presented in this study clearly showed that both bacterial preparations were sensitive to naphthol-related compounds and model organic toxicants: phenol and 3,5dichlorphenol. These two methods have no significant difference by means of toxicity values (EC_{50}), and they are all reliable methods of toxicity test.

Nevertheless, it has been noted that liquid dried *V. fischeri* provided by Merck showed lower EC_{50} values than frozen *V. fischeri* prepared from the standard procedures 'BS EN ISO 11348-1:1999'. It may be possible that the instantly rehydrated bacteria from liquid dried status were more vulnerable than frozen cells, hence more sensitive to the toxicity attack.

2.4.6. Development of bioluminescent *E. coli* and *Ps. putida* for toxicity test and comparison with *V. fischeri*

V. fischeri was the most sensitive as well as stable biosensor to dye related organic compounds. *Ps. putida* remained least sensitive biosensor to dye related organic compounds among tested three bioluminescent bacteria. Bhattacharyya *et al.* (2005) also used bioluminescent *V. fischeri*, *E. coli* HB101 (pUCD 607) and *Ps. putida* to test the toxicity effect of organic compounds Chlorinated aliphatic hydrocarbons, Bhattacharyya *et al.* (2005) also found *V. fischeri* was more sensitive than *E. coli* HB101, in addition, a trichloroethene induced bioluminescent *Ps. putida* strain showed limited response to organic compounds.

Bioluminescent changes and toxicity effect

Three bioluminescent bacteria *V. fischeri, E. coli* and *Ps. putida* were tested with two model organic toxicants (phenol, and 3,5-dichlorophenol) and other seven naphthol-related compounds. All three bacteria showed changes in bioluminescence on exposure to these toxicants.

Generally the intensity of bioluminescence (RLU) generated from *V. fischeri* decreased on exposure to the increased concentration of organic toxicant. Bacterial bioluminescence inhibition was expressed, unless otherwise stated as EC_{50} values, which corresponds to the toxicant concentration causing 50% reduction of maximum bioluminescence output Cronin (1993).

It is important to note that toxicants can perturb cellular metabolism and subsequently enhances $FMNH_2$ concentrations. Subsequently bioluminescence can increase on exposure to toxicant as well as decrease. The bioluminescence intensity generated from *E. coli* and *Ps. putida* was increased in certain toxicants concentrations, for example with phenol and 3,5-dichlorophenol. As shown in figure 2.3.15 and 2.3.16, there are negative bioluminescence inhibition effects on exposure to these toxicants. Therefore toxicity measurement according to bioluminescence determination is regarded as the change in bioluminescence rather than simply the decrease in bioluminescence.

Cell wall permeability

In this investigation, exposure times between bacteria and toxicants were 15 and 30 minutes. The reason for using the longer contact time was because only organic toxicants were tested in this investigation. It is widely believed organic chemical need longer contact time than inorganic toxicants to cross cell wall to cause a toxic affect. Consequently 5 minutes contact time is not recommended by most bioluminescent toxicity testing manufactures including Merck™. Therefore 5 minutes contact time was not considered in this investigation.

The mechanism of toxicity is not identified using this method but would be expected to include disruption to membrane function, and central metabolism.

Frozen stored *V. fischeri* complying with the testing standard BS EN ISO 11348-1:1999 was used as the standard control for overall studies. Compared with overnight culture of *E. coli* and *Ps. putida*, the frozen *V. fischeri* was more sensitive to toxicants. After freezing condition, the cell wall and membrane is partially ruptured.

V. fischeri, *E. coli* and *Ps. putida* are all Gram negative bacteria. They have relatively thin, inner layer of peptidoglycan and an outer membrane of phospholipids, lipopolysaccharide, and lipoprotein. However their cell wall permeability probably differed at the test temperature.

In this investigation, 15°C was used for overall testing temperature for all bacteria. The reason to do so not only because of the toxicity testing standard EN ISO 11348-1:1999 defines 15°C for bioluminescence toxicity testing standard, but also, many preliminary experiments proved that it was not possible to test the bioluminescence inhibition of *E. coli* and *Ps. putida* in higher temperatures (for example 30°C and 37°C) primarily because the instability of bioluminescence. It was also evidence that 15°C generated relatively stable bioluminescence output for *E. coli* and *Ps. putida*, which is essential condition for the toxicity test.

Future work

It has been speculated that the cell wall and membrane permeability is one of the most important factors influencing the toxicity testing. For the well-recognized toxicants phenol and 3,5-dichlorophenol, *E. coli* and *Ps. putida* showed different bioluminescent behaviour to the marine bacterium *V. fischeri*. Consequently, it would be necessary to further investigate the bioluminescence behaviour of *E. coli* and *Ps. putida* on exposure to these organic toxicants.

This investigation has demonstrated that bioluminescent toxicity test of *E. coli* and *Ps. putida* cannot be performed at higher temperatures.

It is well known that the sensitivity of toxicity test can be improved if the cell wall/membrane permeability can be increased. Consequently it was proposed to generate mutant strains to increase membrane permeability for *E. coli* and *Ps. putida*.

Chapter 3. Bioluminescent *C. jejuni*

3.1 Introduction

C. jejuni is one of the leading causes of bacterial food-borne diarrhoeal disease throughout the development world. Cases of campylobacter gastroenteritis reported in 1999 were more than three times the numbers due to *Salmonella* (Central Public Health Service, 2000) and thus *C. jejuni* is a major economic and social problem in the United Kingdom. *C. jejuni* enteritis is generally a self-limiting, unpleasant and debilitating illness, in certain cases the infection also can result in serious neurological sequel.

In contrast with *Salmonella* and many other enteric pathogens, *C. jejuni* is relatively difficult to study in the laboratory. First, it grows only under microaerophilic conditions and over a very limited growth temperature range of 30 to 42°C; second, the bacterium is refractory to traditional genetic analysis, for example transposon mutagenesis (Wren *et al.* 2001). Finally, most *C. jejuni* strains can be transformed efficiently with homologous DNA, but not with heterologous DNA (Vliet *et al.* 1998)

Although the mechanism of *C. jejuni* pathogenesis has been reviewed (Ketley 1997, Wren *et al.* 2001, and Park 2002), there are no reports visualising the *C. jejuni* invasion of human epithelial tissues.

It is believed that bacterial invasion of epithelial cells *in vivo* ultimately results in cellular injury and consequent loss of cellular function and diarrhoea. Therefore, epithelial tissue invasion has been proposed as an important pathogenic mechanism for *C. jejuni*. Hence the construction of bioluminescent-labelled *C. jejuni* was proposed to investigate the attachment and invasion of host cells.

Aims of this investigation will include following issues:

- First, development of transformation procedure for *C. jejuni*;

- Second, growth phase dependent bioluminescent behaviour will be studied to understand luminescent promoter activity in *C. jejuni*;
- Finally, luminescent microscopy will be used to visualise tissue invasion process by luminescent *C. jejuni*.

3.2 Materials and methods:

3.2.1. Overview

Preliminary experiments used various methods to introduce the plasmid pRY*lux*CDABE into *C. jejuni* including heat shock and electroporation. Subsequently it was shown that the plasmid pRY*lux*CDABE could be transformed into *C. jejuni* by conjugation. Essentially the plasmid, pRY*lux*CDABE was isolated from its host strain *E. coli* DH5α (pRY*lux*CDABE), and transformed into *E. coli* strain s17-1. This strain contains conjugative genes capable of transferring the plasmid into the *C. jejuni*. The conjugation between *E. coli* S17-1 and *C. jejuni* was on blood agar plates. Finally, a designed selective plate distinguished transconjugant *C. jejuni* strains.

3.2.2. Microbial culture media

All culture media used in this investigation are listed in alphabetical order:

Blood agar was prepared by suspending 40g of powdered blood agar base No.2 (Oxoid product code: CM0271) in 1 litre of distilled water and completely dissolved using a boiling water bath. The medium was then autoclaved for 15 min at 121 °C, cooled at room temperature to 45-50°C before 7% sterile defibrinated Horse Blood, (Oxoid product code: SR0050) was added and well mixed. Where necessary, additional filter sterilized (non-pyrogenic filter, 0.1 μm pore-size Sarstedt Ltd.) kanamycin (40 μg/ml) was added.

Campylobacter blood-free selective agar was prepared by suspending 22.75g of powdered Campylobacter blood-free selective agar base (Oxoid product code:

CM739) in 500ml of distilled water and completely dissolved by using a boiling water bath. The medium was then autoclaved for 15 min at 121 °C, and cooled down at room temperature to 50°C. One vial of CCDA Selective Supplement (Oxoid product code: SR0155) was aseptically added to the medium. Where necessary for additional selection, filter sterilized (non-pyrogenic filter, 0.1 µm pore-size) kanamycin (40 µg/ml) was added.

For Luria broth and agar details refer section 2.2.1.

Nutrient broth number 2 was used as the liquid culture media for *Campylobacter jejuni* as previously described (Griffiths *et al.* 1993). Nutrient broth No.2 powder 12.5 g (Oxoid product code: CM0067) was dissolved in 475ml of distilled water and sterilised by autoclaving at 121°C for 15 minutes.

3.2.3. Bacterial strains

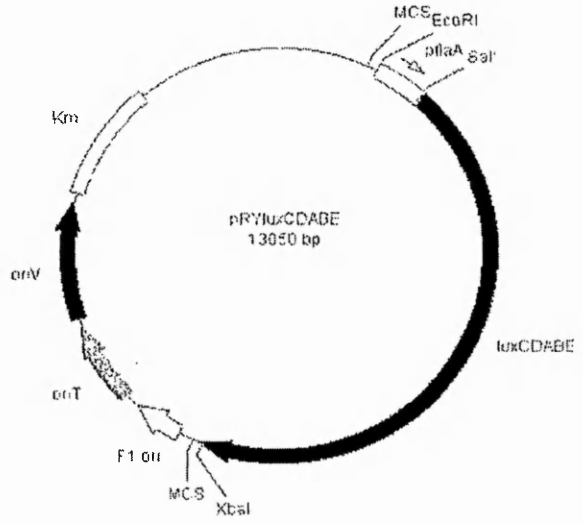
Two *C. jejuni* strains, NCTC 11168 and 11351, were obtained from the NTU microbiology culture collection. They were identified by oxidase (positive), catalase (positive) and Gram staining (Gram negative) and confirmed by restreaking several times on the respective blood agar plate. *C. jejuni* cells were always grown at 37°C under microaerophilic conditions.

The plasmid host strain, *E. coli* DH5α (pRY*lux*CDABE) was received from Kevin James Allen, at the Department of Food Science, University of Guelph, Canada. *E. coli* DH5α (pRY*lux*CDABE) were grown overnight on kanamycin contained selective media LB agar.

Conjugative positive strain, *E. coli* S-17-1 was kindly provided by Dr. Philip J. Hill from food microbiology unit, University of Nottingham. *E. coli* S-17-1 was maintained on none selective Luria media.

3.2.4. Plasmid

Figure 3.2.1: Plasmid pRYluxCDABE



The plasmid pRY*lux*CDABE was isolated from the host strain *E. coli* DH5α (pRY*lux*CDABE) using plasmid mini-preparation kits ("DNA purification system", Promega Product Code #A1330). Plasmid DNA concentration was determined by absorbance at 260nm (OD_{260}). The plasmid pRY*lux*CDABE was confirmed by restriction analysis using three restriction enzymes: EcoRI, XbaI, and SalI (supplied by Promega).

The restriction analysis experiments followed the instructions of Promega. One or more restriction endonucleases were well mixed with a given buffer in 1.5ml sterile plastic tube before the plasmid DNA was added. The mixture were then incubated in 37°C water bath. The incubation period was optimised between one hour to 24 hours in order to achieve the best results. The DNA fragments were then loaded into 1% agarose gel for electrophoresis analysis.

3.2.5. Design of selective media for *C. jejuni* transformants

Conjugation experiments were between the plasmid donor strain *E. coli* S-17-1 (pRY*lux*CDABE) and the recipient strain *C. jejuni* NCTC 11168 or 11351. Therefore a selective medium was needed to distinguish the transconjugants positive *C. jejuni* strains (pRY*lux*CDABE) from the donor and parent recipient strains.

E. coli S-17-1 and *E. coli* S-17-1 (pRY*lux*CDABE) were not able to grow on Campylobacter blood-free selective agar. *C. jejuni* was not able to grow on kanamycin selective media. Hence it was decided to use kanamycin containing Campylobacter blood-free selective agar to select the kanamycin resistant transconjugant *C. jejuni.*

3.2.6. Transformation of *C. jejuni*

Initial experiments with electroporation were not successful in introducing the plasmid pRY*lux*CDABE into *C. jejuni.* Although the method used had been previously described by Vliet *et al.* (1998). Fortunately, the transformation was later achieved by conjugation using *E. coli* S-17-1 (pRY*lux*CDABE). The plasmid pRY*lux*CDABE was transformed into conjugative *E. coli* S-17-1 by heat shock in calcium chloride; following the standard protocol of Sambrook *et al.* (1989). The transformant *E. coli* S-17-1 (pRY*lux*CDABE) strain was purified by restreaking single colonies at least 3 times on selective LA plates. The strain was stored at -80°C until further use.

For mating experiments, the *E. coli* S-17-1 (pRY*lux*CDABE) was grown overnight on non-selective LA plates since preliminary experiments had shown that transfer rate was reduced using *E. coli* S-17-1 pre-grown on kanamycin contained agar. The *C. jejuni* cells were grown on blood agar plates under microaerophilic conditions for about 30 hours prior to mating. Two loopfuls (about 20µl) of *C. jejuni* cells were harvested and spread on a fresh blood agar plate and mixed with one loopful (about 10µl) of overnight *E. coli* S-17-1

(pRY*lux*CDABE). The mixed culture was incubated at 37°C under microaerophilic conditions overnight. It was then harvested using a loop and spread onto *Campylobacter* selective agar containing kanamycin. The transconjugant *C. jejuni* were confirmed by oxidase (positive), catalase (positive) and Gram staining (Gram negative) and purified by restreaking three times on kanamycin blood agar plates.

3.3 Results and discussion

The plasmid pRY*lux*CDABE was confirmed by restriction mapping before its transformation into *C. jejuni*. Unfortunately despite many attempts, electroporation was unsuccessful in introducing the plasmid pRY*lux*CDABE into *C. jejuni* NCTC 11168 and 11351. Nevertheless (and despite many initial failures) the plasmid was finally transformed using conjugation into *C. jejuni* NCTC 11168 and 11351. Phenotypically these transconjugants showed bioluminescence. However analysis of the *lux* promoter was not successful. The growth of the transconjugatants was difficult to quantify and their luminescent output was hard to quantify by classic methods. Tissue invasion was also not successfully carried out, it was postponed due to university safety regulations, and finally, it was decided to work on other aspect of bioluminescence (chapter 4).

3.3.1. The plasmid restriction mapping

The plasmid pRY*lux*CDABE was digested with restriction endonucleases. Apart from lacking the EcoRI restriction site, other restriction fragments were of correct size. Consequently it was decided to transform the plasmid into *C. jejuni*.

A restriction analysis was carried out routinely of the plasmid pRY*lux*CDABE. According to Kevin James Allen's information, there is one restriction site for each of endonucleases EcoRI and SalI; there are two XbaI restriction sites, one located in the central of *lux*CDABE, the other located in the end of *lux* operon. The plasmid is 13050bp and the *lux* operon is 6960bp(as shown figure 4.2.1).

The restriction digestion experiments followed Promega instructions; with the exception that the incubation time was optimised to 1hour 20min and the use of restriction buffer D instead of buffer A instructed. The DNA agarose electrophoresis gels are shown in the figures 3.3.1 and 3.3.2.

Figure 3.3.1: Restriction mapping of pRY*lux*CDABE

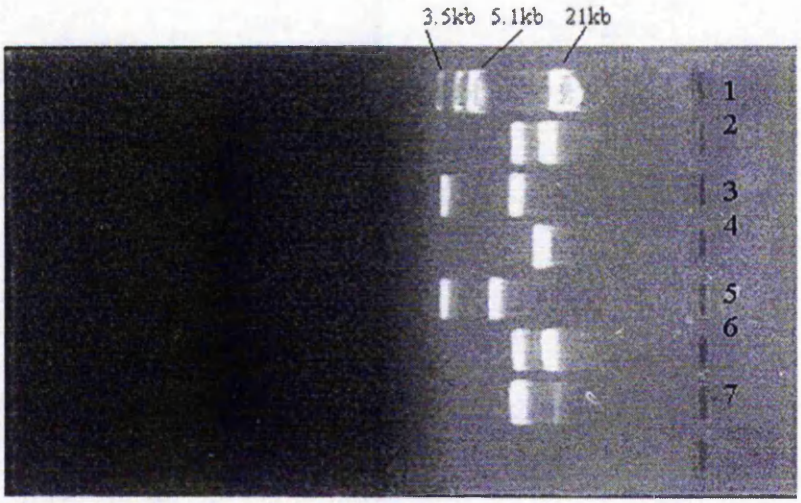


Figure 3.3.1 shows the restriction mapping of the plasmid pRYluxCDABE according to Materials and Methods section 2.3.3, where the lanes were:

1. Lambda DNA/EcoR I + Hind III Markers
2. pRY*lux*CDABE digest with EcoRI
3. pRY*lux*CDABE digest with XbaI
4. pRY*lux*CDABE digest with SalI
5. pRY*lux*CDABE digest with SalI + XbaI
6. pRY*lux*CDABE digest with EcoRI
7. plasmid pRY*lux*CDABE (uncut)

Figure 3.3.1 showed that the restriction site EcoRI did not exist in the received plasmid pRY*lux*CDABE. Further experiments were carried out in which EcoRI was used in combination with the other restriction enzymes XbaI and SalI. Neither of them showed EcoRI restriction site.

As expected that XbaI digestion of the plasmid yielded two bands at about 3500bp and 9500bp, SalI digestion yielded a band between 5100bp to 21000bp, the DNA fragment supposed to be 13050bp.

Figure 3.3.2: Restriction mapping of pRY*lux*CDABE.

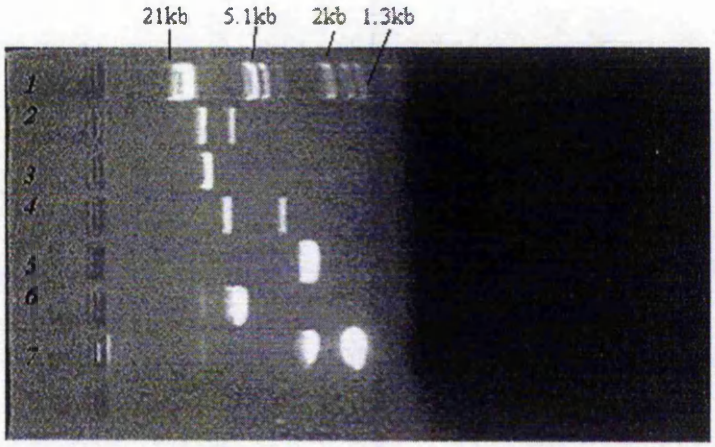


Figure 3.3.1 shows the restriction mapping of the plasmid pRYluxCDABE according to Materials and Methods section 2.3.3, where the lanes were

1. Lambda DNA/EcoR I + Hind III markers
2. pRY*lux*CDABE digest with EcoRI
3. pRY*lux*CDABE digest with SalI + EcoRI
4. pRY*lux*CDABE digest with XbaI + EcoRI
5. Plasmid pUC19 digest with EcoRI
6. Plasmid pRY*lux*CDABE
7. Plasmid pUC19

This confirms that the restriction site EcoRI does not exist in pRY*lux*CDABE. Therefore a control plasmid pUCD19 was used to confirm that the enzyme EcoRI was active(lane 5 figure 3.3.2).

There are no previous reports of the shuttle vector pRY*lux*CDABE being used between *E.coli* and *C. jejuni*. Wren *et al.* (2001) reported that *C. jejuni* strains may have unique genetic codon system. Therefore it is possible that the EcoRI restriction site was modified by *C. jejuni* codon usage.

3.3.2. Transformation and bioluminescence of *C. jejuni* transconjugants

Wren *et al.* (2001) described that *C. jejuni* was refractory to traditional genetic analysis methods for example transposon. Vliet *et al.* (1998) also found that *C. jejuni* was deficiently transformed by heterologous DNA. Therefore the initial difficult of this research task was that there was very limited literature available describing the transformation procedure of *C. jejuni*. Various methods were used to introduce the plasmid pRY*lux*CDABE into *C. jejuni*. These methods included heat shock and electroporation, which were unsuccessful. However the plasmid pRY*lux*CDABE was finally transformed into *C. jejuni* by conjugation.

Two transconjugant strains *C. jejuni* NCTC 11168 (pRY*lux*CDABE) and *C. jejuni* NCTC 11351(pRY*lux*CDABE), were grown on kanamycin containing blood agar plate, under microaerophilic conditions (5% oxygen). Their bioluminescence output was lower than *Ps. Putida, E. coli* (section 2.3.4), *E. sakazakii* and *C. koseri* (section 4.3.2 and 4.3.3). Nevertheless allowing for vision adaptation in a dark room for 3 minutes, bioluminescent *C. jejuni* colonies were visible on agar plates.

It was difficult to test the *lux* promoter activity, which controlled the *lux* operon in *C. jejuni*. The bacteria could be grown in liquid culture medium (ferric enriched nutrient broth number 2) as described by Griffiths (1993). However the growth of *C. jejuni* could not be quantified as the biomass precipitated under microaerophilic conditions. Therefore the optical absorbance value did not represent the bacterial growth. In addition, the bioluminescence of *C. jejuni* was too low to be detected by normal luminometer, as majority of growth was under microaerophilic conditions, whilst, the bioluminescence is oxygen requiring.

It was not possible to carry out the *C. jejuni* epithelial tissue invasion studies due to the Nottingham Trent University safety restrictions on the use of hazard group 2 bacteria in tissue culture laboratories. As a consequence, the bioluminescence research was redirected to another aspect.

Chapter 4. Construction and application of bioluminescent *E. sakazakii* and *C. koseri*

4.1 Introduction:

Due to changes in supervisory provision, another application of bioluminescence as a bioreporter for foodborne pathogens was studied. The principal organisms were the emergent pathogens *Enterobacter sakazakii* and the related organism *Citrobacter koseri*. The expression of the bioluminescence reporter genes was used to investigate bacterial recovery from stress (ie. desiccated) states.

4.1.1. The bacteria *Enterobacter sakazakii* and *Citrobacter koseri*

Enterobacter sakazakii, a Gram negative rod shaped bacterium, has been well described as a newly emerged infective organism of neonates. It causes bacteraemia, necrotizing enterocolitis (NEC), a rare but often fatal (mortality rate of 40 to 80 per cent) form of neonatal meningitis (Iversen *et al.* 2003). It also has been isolated from infections of elderly adult patients.

The first two reported cases of neonatal meningitis caused by *E. sakazakii* were in England (Urmenyi *et al.* 1961) and both of the infants died from infection. Monroe *et al.* (1979) described the first case of neonatal bacteraemia without meningitis associated with *E. sakazakii*. Several outbreaks of *E. sakazakii*, in Neonatal Intensive Care Units (NICUs), have been reported in many countries including England, Netherlands, Greece, US and Canada. In Canada, two incidents of neonatal meningitis caused by *E. sakazakii* were reported in two Canadian hospitals (1990, 1991). It should be noted that healthy infants might not always be immune to *E. sakazakii* infections. It has been reported that in Iceland, a healthy, full-term, newborn infant became ill prior to hospital discharge and suffered permanent neurological sequelae as a result of an *E. sakazakii* infection.

The *E. sakazakii* infections reported in adults have mainly been in elderly patients with serious underlying medical conditions. Pribyl *et al.* (1985) isolated *E. sakazakii* along with two other organisms from a diabetic foot ulcer. The patient was treated with aztreonam with a good response.

Monroe *et al.* (1979) reported the first case of neonatal bacteraemia without meningitis associated with *E. sakazakii*. This infection was treated successfully with ampicillin. Lai *et al.* (2001) described 4 adult *E. sakazakii* infections that were resistant to ampicillin. Dennison *et al.* (2002) reported that *E. sakazakii* is generally more resistance to antibiotics than other *Enterobacter* species. Nevertheless, there is no literature describing the genetic plasticity of the *E. sakazakii*, for example, conjugation or plasmid phenomenon associated with antibiotic resistance genes.

Similar as *E. sakazakii*, *C. koseri* is also Gram negative bacterium, DNA-DNA hybridisation studies showed *E. sakazakii* was about 50% similar to *Citrobacter koseri*. There are numerous literature describing the clinical symptoms caused by *C. koseri* are similar as those caused by *E. sakazakii*. *C. koseri* is responsible for many cases of neonatal bacteraemia and meningitis, often causing multiple brain abscesses (Townsend *et al.* 2003). Kline (1988) described that infants with *C. koseri* meningitis develop brain abscesses at a frequency of 77%. The *C. koseri* caused meningitis are also fatal at a mortality rate up to 50%, those survivors 75% have severe neurological impairment such as seizures, hearing loss, or mental retardation (Townsend *et al.* 2003). Similar to *E. sakazakii*, immunocompromised mature individuals are also susceptible to *C. koseri* infections (Tang *et al.* 1994).

4.1.2. Identification of *E. sakazakii*

Historically the organism was known as "yellow pigmented *Enterobacter cloacae*" until Farmer *et al.* (1980) assigned the name *E. sakazakii*. DNA-DNA hybridisation studies showed it was 51% similar to *E. cloacae* (and *Citrobacter koseri*) and only 40% similar to *Citrobacter freundii* the defining strain of the

Citrobacter genera. In addition, *E. cloacae* has emerged as an important pathogen causing neonatal sepsis (Shi *et al.* 1996) and *C. koseri* is also a well-known cause of neonatal meningitis (Kline 1988).

E. sakazakii presents a special, well-recognised problem in immune compromised cases. In such examples, strain typing might help in identifying the source of infection (for example, a particular batch of foodstuffs), and therefore assist in limiting the further spread of infection.

Bacterial typing means strain-specific identification and grouping of similar strains. The objective of typing studies is to ascertain if two or more strains are derived from a single parent organism. Such investigations are usually performed to clarify whether two isolates from different sources represent the same strain or distinct ones, in order to provide essential data for epidemiological investigations.

The US Food and Drug Administration (FDA) have published an isolation method for *E. sakazakii* in early 2002. However Iversen *et al.* (2004b) have questioned the sensitivity of the FDA isolation method, as it is not specific for *E. sakazakii*. Consequently a chromogenic medium (Druggan-Forsythe-Iversen agar) specific for *E. sakazakii* has been commercialised by Oxoid Ltd UK (Oxoid product code: CM1055). More recently Oh *et al.* (2004) developed fluorogenic selective and differential medium for isolation of *E. sakazakii*.

However, isolation and identification methods specific to pathogenic *E. sakazakii* have not been validated. Current research at NTU has shown that the identification methods for *E. sakazakii* and related organisms are flawed (data not presented for confidentiality reasons). This is a major issue due to the implementation of current detection methods, i.e. FDA protocol, and powdered infant milk formula recalls are based in USA and other countries.

It should be noted that *C. koseri* has been probably been mis-assigned to the *Citrobacter* genus. Iversen *et al.* (2004d) studied the phylogenetic relationships of *E. sakazakii, C. koseri* and other related organisms using 16S ribosomal DNA (rDNA) and *hsp60* sequencing. The studies reveal that the *E. sakazakii* type strain

16S rDNA sequence was 97.8% similar to that of *C. koseri*, 97.0% similar to *E. cloacae* and *C. freundii* was 96.0% related. The studies suggest that further investigations are warranted to clarify their relationship. Additionally *E. sakazakii, C. koseri*, and other related organisms (including *E. cloacae* and *C. fruendii*) have been isolated from powdered infant milk.

There are numerous phenotypic typing methods applied to *E. sakazakii*, including: morphology, the DFI medium, API microbial identification strips, and growth on antimicrobial susceptibility profiling. However these phenotypic typing methods show low discriminatory power; do not index genotype, which often associated with pathogenesis. Nowadays molecular typing methods are often used.

PCR identification

Recently, PCR-based fingerprinting has become the preferred method of identification, since it provides a more stable determination of isolate identity (Kostman *et al.* 1992). It has been used for assessing the genetic diversity of many microorganisms. Depending on the primers and amplification conditions employed, the results allow discrimination between organisms at the level of genera, species, or strains.

One method is PCR based on intergenic length polymorphisms, termed as tDNA-PCR. The rationale of the tRNA intergenic length polymorphism molecular strategy for taxonomic studies relies on the observation that bacterial tRNA genes contain sequence motifs that exhibit a high level of phylogenetic conservation, while tRNA intergenic regions exhibit a higher degree of variation (Welsh *et al.* 1991). Indeed, the PCR fingerprints of tRNA intergenic regions generated by using consensus tRNA gene primers generally produce species-specific patterns which have been successfully used to discriminate species belonging to the same genus, as reported for *Acinetobacter* sp., *Streptococcus* sp., *Staphylococcus* sp., and *Enterobacter* sp. (Ehrenstein *et al.* 1996, Maes *et al.* 1997, De Gheldre *et al.* 1999, and Clementino *et al.* 2001). Moreover, application of tDNA-PCR analysis to strains validly placed in the same species has also allowed discrimination of

strain clusters showing distinct PCR fingerprints, as demonstrated for *Enterobacter cloacae, Bacillus stearothermophilus*, and *Bacillus licheniformis* (Borin, *et al.* 1997, Daffonchio *et al.* 1998, Clementino *et al.* 2001).

A PCR method based on the Intergenic Transcribed Spacer regions (ITS) between the 16S and 23S rRNA genes is termed "ITS PCR". It has received a great deal of attention for strain typing, since such regions have greater polymorphism than adjacent genes (Jenson *et al.* 1993 and Gürtler *et al.* 1996). Furthermore, because of the increasing number of ITS sequences that are being deposited in databases, a database specific for ITS, the Ribosomal Internal Spacer Sequence Collection (RISSC, http://ulise.umh.es/RISSC/), has been developed (Garcia-Martinez *et al.* 2001). Polymorphism in ITS is quite often due to the presence of tRNA genes, since such genes are responsible for intrastrain length and sequence polymorphisms (Gürtler 1999). This polymorphism can be used for discriminating closely related strains in species that harbour multiple ribosomal operons that differ in length and sequence (Jensen *et al.* 1996 and Baudart *et al.* 2000).

Another important PCR-based fingerprinting technique used for typing a wide range of bacteria is random amplified polymorphic DNA (RAPD) analysis developed by Williams *et al.* (1990) and Welsh *et al.* (1990). The RPAD PCR is based on polymorphism of whole genomes, it has been used successfully to differentiate species and strains belonging to the genus Enterobacter using oligonucleotide primers having arbitrary sequences (Clementino *et al.* 2001).

Hence these three PCR methods (tDNA, RAPD, ITS) offered potential identification and discrimination methods for *E. sakazakii, C. koseri* and related organisms.

4.1.3. Epidemiology review

E. sakazakii has been isolated from a diverse range of foods and clinical materials. Foods include powdered infant formula (IFM) cheese, fermented bread, tofu, sour tea, cured meats, minced beef and sausage meat. The organism has also been

isolated from various clinical materials, such as cerebrospinal fluid, blood, skin, wounds, respiratory tract (sputum, throat, and nose), digestive tract, and urine (Iversen *et al.* 2004a). However the natural habitat and epidemiology of this organism remains a mystery.

E. sakazakii has recently isolated from the gut of *Stomoxys calcitrans* (Hamilton *et al.* 2003), which are blood-feeding insects that primarily feed on cattle, horses, dogs, pigs and humans. They may also feed on reptiles and birds. *Stomoxys* spp are found in cowsheds. Therefore, it is not surprising that *E. sakazakii* could be spread to milk and meat products by blood transmitting insects. In addition to *Stomoxys* spp, *E. sakazakii* also isolated from the guts of Mexican fruit flies, *Anastrepha ludens* (Kuzina *et al.* 2001) and the housefly, *Musca domestica* (Hamilton *et al.* 2003). The insects thus might be an environmental reservoir for *E. sakazakii.* Consequently, reducing arthropod presence in food manufacturing environments and hospital could result in a substantial reduction in the transmission of *E. sakazakii.*

Neonatal infection with *E. sakazakii* has been associated with the use of contaminated powdered infant formula (IFM). A number of cases of neonatal deaths related to the infective organism in IFM have been reported in the literature (Muytjens *et al.* 1990). The *E. sakazakii* have been isolated from IFM from a number of different manufacturers in 13 countries (Muytjens *et al.* 1988).

World Helath Organization (WHO) is drawing attention to the fact that IFM are not commercially sterile products. Unlike liquid formulas, which are subjected to sufficient heat to render them commercially sterile, powdered infant formulas are not processed to achieve commercial sterility.

Ready-to-use liquid infant formulas are available only in a commercially sterile form and often suggested for normal weight neonates. A number of formulas, including formulas for infants with metabolic conditions are only available in powdered form. Powdered soy-based infant formulas may also become contaminated with *E. sakazakii* through improper cleaning of production lines and may, therefore, pose a safety hazard.

Kandhai *et al.* (2004) studied the presence of *E. sakazakii* in nine food-processing factories and 16 households, and from this it is evident that *E. sakazakii* widely exists in these area (Table 4.1.1).

Table 4.1.1: Presence of *E. sakazakii* in food-processing factories and households, reproduced from Kandhai *et al.* (2004).

	Number of samples analysed	Number of samples positive for *E sakazakii* (%)	95% CI
Origin			
Milk powder factory	23	2 (9%)	0·01–0·27
Milk powder factory	26	9 (35%)	0·19–0·53
Milk powder factory	11	1 (9%)	0·002–0·404
Milk powder factory	8	2 (25%)	0·03–0·64
Chocolate factory	8	2 (25%)	0·03–0·64
Cereal factory	9	4 (44%)	0·15–0·78
Potato flour factory	15	4 (27%)	0·08–0·53
Pasta factory	26	6 (23%)	0·10–0·42
Spice factory	5	0	0–0·52
Households	16	5 (31%)	0·12–0·57

High temperature spray drying is often used to dry the milk powder in industry. Nazarowec-White *et al.* (1997) described *E. sakazakii* as one of the most thermo-tolerant *Enterobacteriaceae* members found in dairy products (Iversen *et al.* 2004c).

The *D* value is a term describes microbial resistance to sterility such as heat or saturated steam under pressure. The *D* value is normally understood as the time (e.g. seconds) required to reduce a microbial population by 90%. The *D*-values of *E. sakazakii* and other *Enterobacteriaceae* members were evaluated using batch methods by Nazarowec-White (*et al.* 1997), and are shown in figure 4.1.1 and 4.1.2.

Figure 4.1.1: Comparison of D 58°C Values for Different Enterobacteriaceae (reproduced from Nazarowec-White *et al.* 1997).

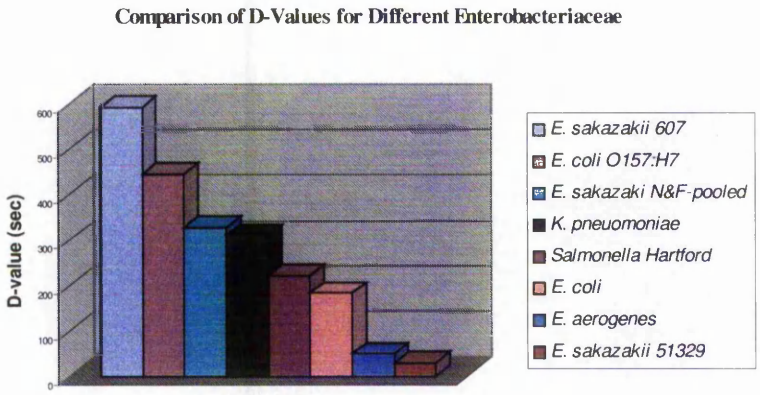
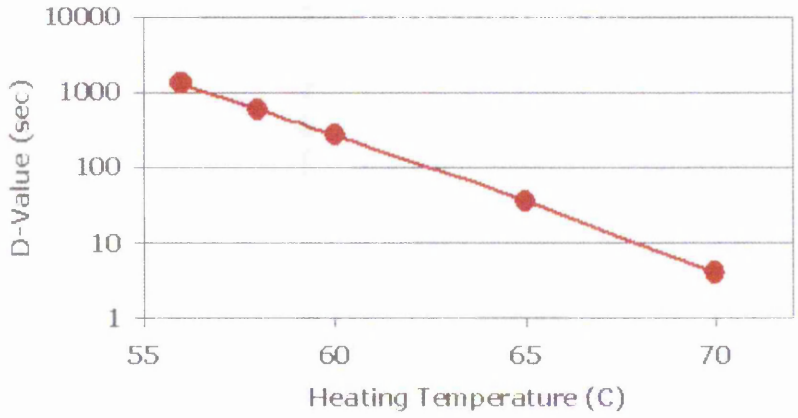
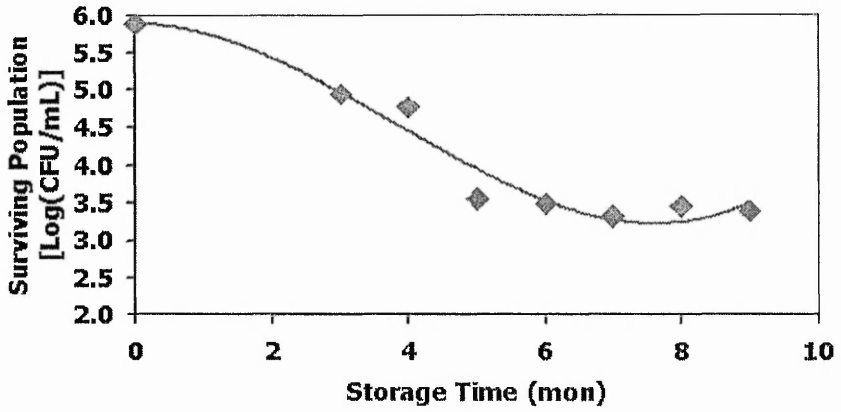


Figure 4.1.2: Effect of Heating Temperature on D-Value for *E. sakazakii* 607 (reproduced from FDA).



In addition to thermal-resistance, *E. sakazakii* also reported resistance to dehydration for up to nine months (figure 3.1.3).

Figure 4.1.3: *E. sakazakii* is resistance to dehydration.



E. sakazakii is powdered IFM is able to survive rehydration with hot (45-60°C) water (Nazarowec-White *et al.* 1997). How the organism can survive the heat shock and recover quickly enough to colonise the infant's gut and subsequently cause bacteraemia and meningitis is unknown.

The presence of *E sakazakii* in arthropods, food-processing factories, foods and domestic environments, strongly indicates that it is a widespread bacterium. However, the source and mode of transmission are still poorly understood. Additionally the pathogenicity and virulence factors of this organism are also not known.

4.1.4. Virulence hypothesis

In order to establish a successful infection, *E. sakazakii* probably produces toxins that subvert host cellular processes allowing efficient colonisation and evasion of the host cells.

Bacterial toxins are either lipopolysaccharides, which are associated with the cell walls of Gram-negative bacteria, often referred to endotoxins, which are released from bacterial cells and may act at tissue sites removed from the site of bacterial growth, and are often referred as exotoxins.

Exotoxins are typically soluble proteins secreted by living bacteria during exponential growth. The production of the toxin is generally species independent. Usually, virulent strains of the bacterium produce the toxin while non-virulent strains do not, and the toxin is the major determinant of virulence. Both Gram-positive and Gram-negative bacteria produce soluble protein toxins.

Pathogen–host interactions are especially based on factors that are located on the bacterial surface or are secreted into the extracellular environment. In this respect, protein secretion systems play a crucial role by delivering these effector molecules to their place of action.

The processes by which protein toxins are assembled and secreted by bacterial cells are also variable. Many of the classic exotoxins are synthesized with a leader signal sequence consisting of a few (1-3) charged amino acids and a stretch (14-20) of hydrophobic amino acids. The signal sequence may bind and insert into the cytoplasmic membrane during translation such that the polypeptide is secreted while being synthesized. The signal peptide is cleaved as the toxin (protein) is released into the periplasm. Alternatively, the toxin may be synthesized intracytoplasmically, then bound to a leader sequence for passage across the membrane. Frequently, chaperone proteins are required to guide this process. Some multicomponent toxins, such as the cholera toxin, have their subunits synthesized and secreted separately into the periplasm where they are assembled. In Gram-negative bacteria, the outer membrane poses an additional permeability barrier that a protein toxin usually has to mediate if it is to be released in a soluble form. It has been proposed that some Gram-negative exotoxins (e.g. *E. coli* ST enterotoxin) might be released in membrane vesicles composed of outer membrane components. Since these vesicles presumably would possess the outer membrane associated attachment factors, they could act as smart bombs capable of specifically interacting with and possibly entering target cells to release their contents of toxin.

E. sakazakii remains viable for long periods of time in the desiccated states, and must synthesize proteins after rehydration, protein synthesis activity must be sufficiently tolerant the low pH in the stomach.

4.1.5. Problems of *Enterobacter* in the United Kingdom

The Health Protection Agency's Communicable Disease Surveillance Centre (CDSC) reviewed the bacterial species from blood specimens from England, Wales and Northern Ireland. In 2003, there were 2354 reports of bacteraemias related to *Enterobacter* infections. Seventy-five per cent of reports of were attributed to *E. cloacae,* followed by *E. aerogenes* (9%) and *E. sakazakii* (3%), while 13% were not specified.

The rate of *Enterobacter* spp. bacteraemia reporting in England, Wales, and Northern Ireland was 4.35/100,000. Regional offices in England received the majority of these reports (2160) followed by Wales, (116) and Northern Ireland (78). Within the English regions, rates varied from 3.41/100,000 in the North West to 7.36/100,000 in the North East.

There were 634 bacteraemia reports of *Citrobacter* spp. Over half (56%) of these reports were attributed to *C. freundii* followed by *C. koseri (diversus)* with 24%. One hundred and twenty-five reports (20%) of *Citrobacter* isolates were not speciated.

The rate of *Citrobacter* spp bacteraemia in England, Wales, and Northern Ireland was 1.17/100,000. Reports submitted to regional offices in England accounted for the majority of these reports (572) followed by Wales (36), and Northern Ireland (26). Within the English regions, rates varied from 0.67/100,000 in the North West to 1.89/100,000 in Yorkshire and Humberside.

4.1.6. Aims of investigation

The general aim of this section was the construction of bioluminescent and fluorescent *E. sakazakii* and *C. koseri* to monitor the recovery from stressed conditions. In more detail, the investigation including following aspects:

Due to current situation on the identification of *E. sakazakii* and related organisms, strains will be subjected to molecular profiling. Three most commonly used genomic DNA typing methods will be used: analysis of the tRNA intergenic spacer region (tDNA-PCR), the 16S-23S internal transcribed spacer region (ITS-PCR) and random amplified polymorphic DNA (RAPD-PCR). These methods have previously been used for similar purposes with the related organism *E. cloacae* (Clementino *et al.* 2001).

Genomic DNA of many strains, which have been previously phenotypically identified as *E. sakazakii*, *E. cloacae, C. koseri* and *C. freundii*, will be clustered following PCR typing methods.

As successful opportunistic pathogens, *E. sakazakii* and related organisms have to resist many stressed conditions (i.e. heat and desiccated condition), and then survive the lower pH environment in stomach and gut. Luminescent and GFP expressing bacteria will be constructed to help understanding metabolic changes (i.e.$FMNH_2$ levels) of cell recovery from stressed condition. Fluorescent GFP expressing bacteria will be used to report protein synthesis activities on cell recovery from stressed condition.

4.2 Materials and methods

4.2.1. Microbial culture media

Microbial media used in this investigation are given in alphabetic order:

Brilliant green agar was prepared by suspending 40 g powdered brilliant green agar base (Oxoid product code: PO0171) in 1 litre of destilled water and completely dissolved by using boiling water bath for 30 minutes, no autoclaving was necessary for this medium. For selective purposes, the medium was cooled to 50°C, and filter sterilized (non-pyrogenic filter, 0.1 µm pore-size Sarstedt Ltd.) kanamycin (40 µg/ml) was added.

Druggan-Forsythe-Iversen (DFI) medium (Iversen *et al.* 2004b) was prepared by suspending 43g powdered DFI medium base (Oxoid product code: CM1055) in 1 litre of distilled water and completely dissolved by using a boiling water bath. The medium was then autoclaved for 15 minutes at 121°C. Where necessary, after autoclaving, the medium was cooled to 50°C, and filter sterilized (non-pyrogenic filter, 0.1 µm pore-size Sarstedt Ltd.) kanamycin (40 µg/ml) was added.

Freeze-dry protective medium was prepared by mixing 0.8 g powdered nutrient broth base (Merck product code: 1.07884) and 5 g *myo*-inositol (Sigma product code: S860360) in 100 ml of distilled water. The medium was then autoclaved for 15 minutes at 121°C and stored at room temperature until required.

For description of Luria broth and agar, please refer to section 2.2.1.

Tryptone soya broth (TSB) and tryptone soya agar (TSA) are already described in section 2.2.1. Where TSB was used for acidic stress experiments, the broth was acidified to pH 6,5 or 4, respectively, by using 0.1M hydrochloric acid prior the autoclaving.

4.2.2. PCR methods to discriminate *E. sakazakii* from related organisms

Bacterial strains

Strains used in this study included the type strain *E. sakazakii* 822 and various related organisms. These included food and clinical isolates. The strains are listed in table 4.2.1.

Table 4.2.1: *E. sakazakii* and related organisms used in PCR typing.

Bacteria Strains	Sources:
Enterobacter sakazakii (822) (Type strain)	National Collection for Type Cultures (NCTC) 11467
Enterobacter sakazakii (825)	American type culture collection (ATCC) 51329
Enterobacter sakazakii (sk90)	Clinical isolation, Hospital for Sick Children Toronto, Canada
Enterobacter sakazakii (LB)	Clinical isolation, St Joseph Hospital London Ontario, Canada
Enterobacter sakazakii (LA)	Clinical isolation, St Joseph Hospital London Ontario, Canada
Enterobacter sakazakii (BF006BB)	Curly Organic Puffs, Baby Food isolation
Enterobacter cloacae (NTU 831)	Oxoid Culture Collection (OCC) 641
Enterobacter cloacae (NTU 830)	OCC 118
Enterobacter cloacae (NTU 832)	ATCC 13047
Enterobacter cloacae (NTU 833)	OCC 1067
Enterobacter cloacae (MP0056W 326)	Milk Powder isolates
Enterobacter cloacae (IFMK6W 254)	Infant Formula Milk from Korea
Enterobacter cloacae (JOHVY 370)	Herbal food isolation
Citrobacter koseri (4277)	Clinical isolates, Children's Hospital Los Angeles, USA
Citrobacter koseri (4036)	Clinical isolates, Children's Hospital Los Angeles, USA
Citrobacter koseri (JB62)	Clinical isolates, Children's Hospital Los Angeles, USA
Citrobacter koseri (SMT319)	Clinical isolates, Children's Hospital Los Angeles, USA
Citrobacter fruendiii (FF08BK 340)	Fresh food isolation

ITS, t-DNA, and RAPD PCR analysis

These methods were based on the *E. cloacae* studies by Clementino *et al.* (2001). All strains were streaked on tryptone soya agar plates at least three times for purity checking. Approximately one loop of fresh biomass (about 10 µl) was scraped off the agar plates, suspended in 200 µl of sterile distilled water, boiled for 10 minutes by using boiling water bath, and then the boiled cells directly used as the template DNA for PCR. All PCR reagents including reaction buffer, DNA

polymerase (taq), and dNTPs were from Promega. PCR primers were from Sigma Ltd. T-DNA, ITS and RAPD PCR running conditions were described by Clementino *et al.* (2001) with optimisation as described in the table 4.2.2. The PCR experiments were evaluated in triplicate with at least two bacterial lysates of each strain. DNA was amplified in separate PCR. DNA molecular markers for this study used a 100bp DNA ladder (Promega product code: G2101). The banding patterns were highly reproducible after visual and automated analysis. The gel image was analysed using the software Phoretix 1D v5.20 (NonLinear Dynamics Ltd, UK).

Table 4.2.2: PCR running conditions.

PCR	Primers		PCR condition	PCR Program
T-DNA	T5A	5'-AGTCCGGTGCTCTAACCAACTGAG-3'	20 mM Tris-HCl, pH 8.4; 50 mM KCl; 3 mM $MgCl_2$; 200 µM concentration (each) of dATP, dCTP, dGTP, and dTTP; 0.8 µM concentration of each primer; 1 U of *Taq* DNA polymerase; and 5 µl of DNA.	Initial denaturing for 2 minutes at 94°C, followed by 94°C for 30 s, annealing at 50°C for 30 s, and extension at 72°C for 1 minute for 30 cycles, with an additional extension at 72°C for 10 minutes. then standby at 4 °C
	T3B	5'-AGGTCGCGGGTTCGAATCC-3'		
ITS	L1	5'-CAAGGCATCCACCGT-3'	20 mM Tris-HCl, pH 8.4; 50 mM KCl; 3 mM $MgCl_2$; 200 µM concentration (each) of dATP, dCTP, dGTP, and Dttp; 0.5 µM concentration of each primer; 1.5 U of *Taq* DNA polymerase; and 5 µl of DNA.	Initial denaturing for 2 minutes at 94°C, followed by 94°C for 30 s, annealing at 50°C for 30 s, and extension at 72°C for 1 minute for 30 cycles, with an additional extension at 72°C for 10 minutes. then standby at 4 °C
	G1	5'-GAAGTCGTAACAAGG-3'		
RAPD	RAPD 1	5'-GGTGCGGGAA-3'	20 mM Tris-HCl, pH 8.4; 50 mM KCl; 3 mM $MgCl_2$; 200 µM concentration (each) of dATP, dCTP, dGTP, and dTTP; 1 µM of the primer; 2.5 U of *Taq* DNA polymerase ; and 5 µl of DNA.	Initial denaturing for 2 minutes at 94°C, followed by 94°C for 1 minute, annealing at 36°C for 1 minute, and extension at 72°C for 2 minutes for 45 cycles, with an additional extension at 72°C for 7 minutes. then standby at 4 °C

4.2.3. Design of selective medium for transconjugant *E. sakazakii* and *C. koseri*

The selective medium for transconjugant positive strains was designed following preliminary anti-microbial susceptibility tests towards *E. coli* (S-17-1), *E. sakazakii* and *C. koseri* strains. Antibiotic discs used were: ampicillin 10µg, (Oxoid product code: CT0003); carbenicillin 100µg, (Oxoid product code: CT0006); chloramphenicol 30µg, (Oxoid product code: CT0013); erythromycin 15µg, (Oxoid product code: CT0020); gentamicin 10µg, (Oxoid product code: CT0024); kanamycin 5µg, (Oxoid product code: CT0025); streptomycin 10µg, (Oxoid product code: CT0047) and tetracycline 30µg, (Oxoid product code: CT0054).

The result of anti-microbial susceptibility tests on *E. coli* (S-17-1), *E. sakazakii* and *C. koseri* showed that it was difficult to inhibit the DNA donor strain *E. coli* S-17-1 from the recipients. Subsequently, brilliant green agar was used for the selection of *C. koseri*, *C. koseri* formed brilliant green colonies on this agar, whilst *E. coli* colonies were white. Druggan-Forsythe-Iversen (DFI) medium supplemented with kanamycin was used for selection of *E. sakazakii*, *E. sakazakii* forms blue-green colony on DFI agar.

4.2.4. Construction of luminescent *E. sakazakii* and *C. koseri* by using transposon mutagenesis

Transposons (pUTmini-Tn5 *lux*CDABE Km2 and pUTmini-Tn5 *lux*CDABE Km1) in the conjugation strain *E. coli* S-17-1 were used to modify *E. sakazakii* and *C. koseri* strains. The method was as previously described by Winson *et al.* (1998) with the exception that the mating temperature was 25°C and the incubation time was optimised as 48 hours for *E. sakazakii* strains and 33 hours for *C. koseri* strains. One loopful (about 10µl) of the DNA donor strain (*E. coli* S-17-1 with plasmid) was mixed with either *E. sakazakii* or *C. koseri* on TSA plates. The mixed cells were then resuspended with 5ml sterilized 0.9mg/ml sodium chloride, and spread onto respective selective media.

Selective kanamycin DFI medium was used for the selection of *E. sakazakii.* Transconjugants formed blue-green colonies. Selective kanamycin brilliant green

agar was used for transconjugants selection of *C. koseri*, which formed green colonies. All isolated strains were purified by re-streaking at least three times on the respective selective agar plate and confirmed by negative oxidase tests, Gram staining and API20 biochemical profiles. Every strain was inoculated into 10ml TSB (containing 50 µg/ml kanamycin) and grown overnight for bioluminescence assay. Strains producing strong luminescence were selected for storage and further study.

Luminometry was used to study the relationship between bacterial growth and bioluminescence behaviour of *lux* modified *E. sakazakii* and *C. koseri*. The absorbance at 405nm was used to measure of optical density to avoid light emission (420 to 630 nm) due to bacterial bioluminescence. The bacteria were inoculated into TSB and luminescence measured using a thermal stable (30°C) luminmotor Lucy1® at Sutton Bonington Campus, University of Nottingham. Plots of $\log_{10}$ (RLU/optical density) against optical density were constructed to show the variation of bioluminescence of the bacterium at different phases of growth.

4.2.5. Construction of *lux* and GFP *E. sakazakii* and related organisms by using ribosomal protein promoter

The plasmids were modified from pDEST™ R4-R3 (Invitrogen Life Technologies product code: 12537023), which is a multiple copy plasmid designed to express none-toxic proteins for *E. coli*. The reporter genes (including full *lux* operon and GFP) were inserted under control of an *E. coli* ribosomal small subunit protein transcription promoter rpsJ, and followed by a strong transcription terminator ($rrnBT_1T_2$). Detailed plasmid information are described in the following figures 3.2.1 to 3.2.3:

Figure 4.2.1: pDEST™ *lux* operon reporter plasmid.

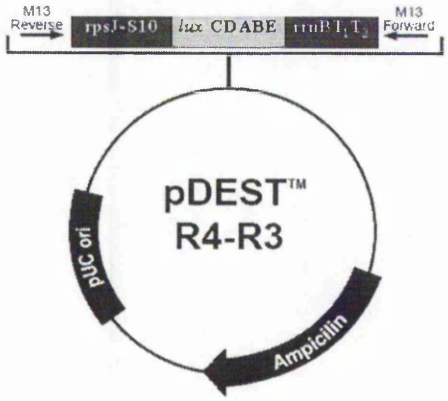


Figure 4.2.2: pDEST™ GFP operon reporter plasmid.

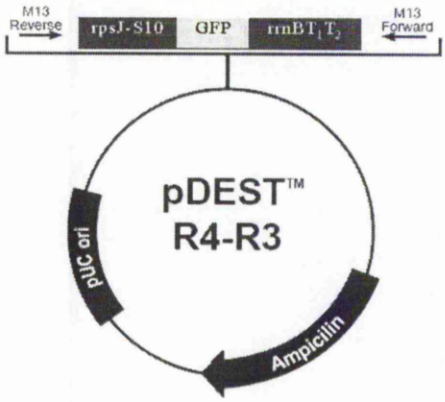
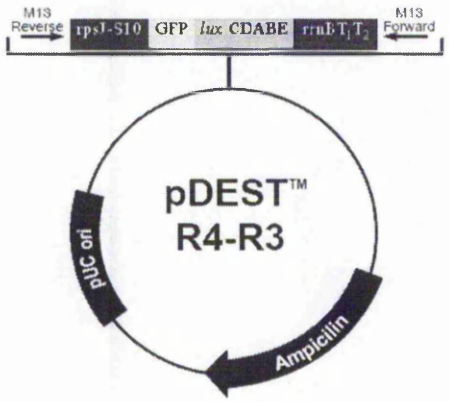


Figure 4.2.3: pDEST™ GFP-*lux* dual operon reporter plasmid.



These plasmids were commercial products with minor modifications by the second supervisor of this research project Dr Phil Hill, hence they were considered to be from a reliable source. Restriction analyses were not performed before transformation into *E. sakazakii* and related organisms.

Electroporation

One hundred micro-litres of overnight *E. sakazakii* or *C. koseri* culture were used to inoculate 50ml of TSB in an 150ml Erlenmeyer flask and incubated at 37°C with shaking at 180 rev/min. The optical density at 600nm (OD_{600}) was measured every 15 minutes until it reached 0.4. Immediately, the cells were incubated on ice for 10 minutes and harvested by centrifugation at 4°C and 800g for 20 minutes. The cells were washed twice and finally resuspended in 0.2ml of sterilised 10% (v/v) glycerol.

Forty microlitres of freshly prepared competent bacteria (*E. sakazakii* and *C. koseri*) were mixed with 2µl of the DNA (20ng) in a 0.2cm gap electroporation cuvette (Bio-Rad product code 165-2095) and left on ice for 10 minutes. The cells were electroporated by using an electroporation apparatus (Gene Pulser, Bio-Rad®). The electroporation apparatus was set to deliver an electrical pulse of 25µF capacitance, 2.5kV, and 200 ohm resistance. Cells were then immediately removed from the electroporation cuvette, added to 1ml of TSB broth and

incubated at 37°C for one hour without shaking. One hundred microlitres aliquots of cells were plated on TSA plates containing 100µg/ml ampicillin. The plates were then incubated overnight at 37°C for identification of transformants.

Identification of transformants

Selective TSB plates with colonies were screened for bioluminescent phenotype by using either an Arugs-100 photo-imaging camera (Hamamatsu Photonics) or Luminograph LB980 photon video camera (E.G. and G. Berthold). Fluorescent phenotype were screened by identifying bright yellow/green colonies under visible blue / violet light in a dark room.

Preparing bacterial cells for bioluminescent and fluorescent assay

Freeze-dried luminescence and fluorescence cells were prepared by the following steps: 100µl of overnight culture were inoculated into 50ml of TSB (contained 100 µg/ml ampicillin) in an 150ml Erlenmeyer flask and incubated at 37°C and shaken at 180 rev/min. The optical density at 450nm (OD_{450}) of the bacteria culture was measured every 15 minutes until it reached 0.4. The cells were then placed on ice for at least 10 minutes before were harvesting by centrifugation for 20 minutes at 4°C and 800g and washed twice in freeze-dry protective medium. The cells were then resuspended in 10ml freeze-dry protective medium (as described in section 3.2.1); and frozen (-70°C) before freeze drying (freeze-dryer model RV8 A654-01-903 by Edwards High Vacuum International, Ltd. England). The freeze-dried cells were kept sealed at room temperature until further use.

The freeze-dried cells were resuspended in 10 ml TSB. Immediately 20µl of resuspended cells were diluted with 10ml TSB (containing 100 µg/ml ampicillin) and analysed for luminescence or fluorescence by using the Tecan® GENios Pro automated analyse instrument. The excitation wavelength was 485nm and 535nm was used as emission wavelength for the fluorescence assay.

Fresh bacteria for luminescence and fluorescence assays were prepared as follows: 100µl of overnight culture was used to inoculated 50ml of TSB (contained 100 µg/ml ampicillin) in an 150ml Erlenmeyer flask and incubated at 37°C and shaken at 180 rev/min. Optical density at 450nm (OD_{450}) was measured every 15 minutes until it reached 0.4. Immediately 100µl of the cells was diluted with 10ml TSB (containing 100 µg/ml ampicillin) and used for luminescence / fluorescence assay as previously described for freeze-dried bacteria.

Test the viability of freeze-dried bacteria

Although freeze-dried bacteria were resuspended in a recognized free-drying protection medium, determine the viability of freeze-dried bacteria was essential for the bioluminescence and fluorescence recovery study. Subsequently the viability of the bacteria after freeze-drying was determined by plate counting using TSA agar plate.

When the bacterial growth reached OD_{450}=0.4, 1ml of the culture in a 1.5 ml microtube was placed on ice for at least 10 minutes. The cells were harvested using bench centrifuge at 4°C and 13,000 rpm for 15 minutes. Two hundred microlitres of freeze-dry protection medium was well mixed with the cell pellet before the freeze-drier process. The freeze-dried bacterial cells in 1.5 ml microtubes were resuspended in 1ml of TSB. Plate counts experiments were carried out after the cells were serial diluted by sterile 0.9% sodium chloride solution. A control sample was included, which was chilled bacteria at the growth stage OD_{450}=0.4.

4.3 Results

The phylogenetic relationship of *E. sakazakii* and related organisms was studied by PCR amplification of their genomic DNA. The studied organisms included: *E. sakazakii*, *E. cloacae*, *C. koseri* and *C. fruendii*, which are listed in table 3.2.1. The phylogenetic study ensured the correct identification of representative *E. sakazakii* strains, which were subsequently selected for bioluminescence marking.

Selected *E. sakazakii* and *C. koseri* strains were mutated by mini-Tn5 transposons insertion, which encoded the full *lux* operon and kanamycin resistance gene. Apart from the strain *E. sakazakii* London B, the other *E. sakazakii* and *C. koseri* strains showed detectable luminescent intensity. However, subsequent photometry and luminometry study showed that their growth dependent luminescent behaviours were irregular in that they expressed the *lux* operon in different growth phases. The irregular luminescence behaviour was probably due to the random mutation nature of the transposon insertion causing the *lux* operon to be fused with different promoters, thus resulting in different expression at various phases of growth.

There was no promoter information for the transposon generated luminescent *E. sakazakii* and *C. koseri*. These mutants were subsequently regarded as of limited value to understand bacterial recover from stressed conditions. Consequently, the ribosomal protein promoter (rpsJ) was considered to construct *lux* and GFP marked *E. sakazakii* and *C. koseri*.

Ribosomal protein promoter (rpsJ) followed by the reporter genes full *lux* operon and GFP was inserted into the commercially available plasmid pDEST®. The plasmids were then transformed into *E. sakazakii* and related strains by using electroporation. The transformants showed the phenotype of luminescence or fluorescence while growing in ampicillin containing culture media.

The ribosomal protein promoter driven luminescent and fluorescent *E. sakazakii* mutants showed very similar growth dependent promoter activities. These

transformants were deemed appropriate models to study cell protein synthesis activity during the recovery from desiccated and acidic conditions.

4.3.1. PCR typing of *E. sakazakii* related organisms

Eighteen candidate strains were analysed by PCR methods. Six strains of *E. sakazakii*, seven *E. cloacae* strains, four *C. koseri* and one *C. freundii* strains. All these strains were listed in table 3.2.1.

Three genomic DNA typing methods were used to identify the *E. sakazakii* and related organisms. These methods included: analysis of the tRNA intergenic spacer region (tDNA-PCR), the 16S-23S internal transcribed spacer region (ITS-PCR) and random amplified polymorphic DNA (RAPD-PCR).

The genomic DNA of candidate strains was screened by PCR, and the subsequent DNA fragments were analysed using agarose gel electrophoresis. The agarose gel were photo printed by a UV camera and phylogenetic relations of candidates were interpreted by computer based cluster analysis.

These three PCR analysis results are described below:

1) T-DNA PCR

Figure 4.3.1: T-DNA PCR patterns of *E. sakazakii* and related organisms.

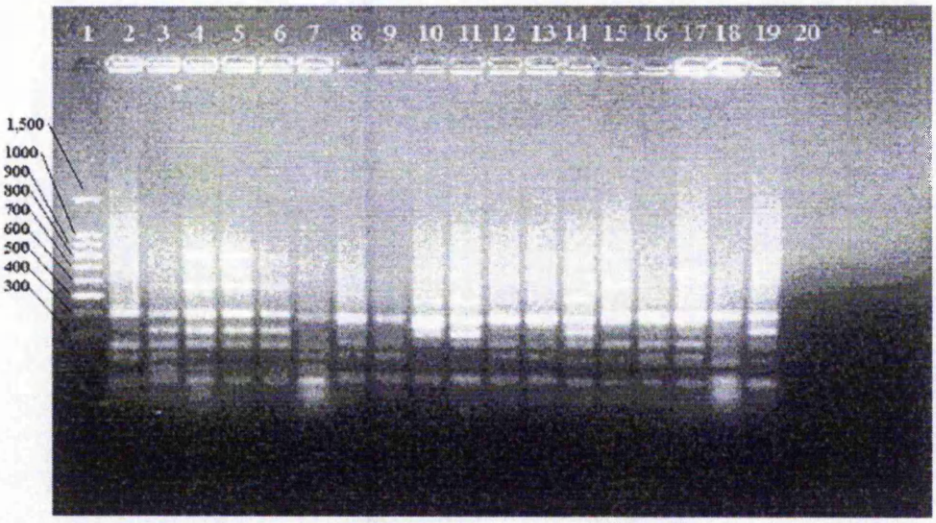


Figure 4.3.1 shows that various bacteria strains analysised by T-DNA PCR and 2% agarose gel according to Materials and Methods section 4.2.2, where lanes:
1. 100bp DNA marker **2**.*E. sakazakii* (825) **3**.*E. sakazakii* (822) **4**.*E. sakazakii* (sk90) **5**.*E. sakazakii* (LB) **6**.*E. sakazakii* (LA) **7**.*E. sakazakii*(BF006BB) **8**.*E. cloacae* (NTU 831, OCC641) **9**.*E. cloacae* (NTU 830, OCC118) **10**.*E. cloacae* (NTU 832 OCC 766) **11**.*E. cloacae* (NTU 833 OCC1067) **12**.*E. cloacae* (MP0056W 326) **13**.*E. cloacae* (IFMK6W 254) **14**.*C. koseri*(4277) **15**.*C. koseri*(4036) **16**.*C. koseri*(JB62) **17**.*C. koseri*(SMT319) **18**.*E. cloacae* (JOHVY 370) **19**.*C. fruendii*(FF08BK 340).

The tDNA-PCR profiles of *E. sakazakii* and related species showed DNA fragments ranging in size from 70 to 1,000 bp that distinguished the species by a number of specific DNA fragments.

Figure 4.3.1 shows that all eighteen strains produced a 400bp band, though *E. cloacae*, *C. koseri* and *C. fruendii* strains showed stronger 400bp band than *E. sakazakii* strains.

Regardless of the bacterium designated name, the strains were re-grouped according differentiated PCR banding profiles. The *E. sakazakii* type strain 822 (figure 4.3.1 lane 3) was used as reference strain for this typing study. Strain *E.*

sakazakii 825 (figure 4.3.1 lane 2) and *E. sakazakii* BF006BB (figure 4.3.1 lane 7) lacked the 300bp bands in comparison with other *E. sakazakii* strains (figure 4.3.1 lane 3, 4, 5 and 6).

The clinical isolates *E. sakazakii* sk90, London A and London B (figure 4.3.1 lane 4, 5 and 6) produced two extra bands (800 and 600bp) compared with the *E. sakazakii* type strain.

Many *E. cloacae* strains (831, 830, MP0056W326 and IFMK6W254) (figure 4.3.1 lane 8,9, 12 and 13) produced similar DNA bands profiles to *E. sakazakii* 825(figure 4.3.1 lane 2).

E. cloacae 832 and 833 (figure 4.3.1 lane 10 and 11) produced an extra 500bp band compared with *E. sakazakii* type strain (figure 4.3.1 lane 3).

C. koseri strains (figure 4.3.1 lane 14-19) produced stronger 400bp bands in comparison with *E. sakazakii* type strain 822 (figure 4.3.1 lane 3). In addition, within *C. koseri* strains, *C. koseri* 4036, JB62, SMT319 and JOHVY 370 (figure 4.3.1 lane 15-18) lacked a 300bp band; *C. koseri* 4277 (figure 4.3.1 lane 14) produced an extra 500bp band; whereas *C. koseri* 4036 SMT319 and *C. freundii* FF08BK 340 (figure 4.3.1 lane 15, 17 and 19) produced an extra 600bp band.

2) ITS-PCR

Figure 4.3.2: ITS PCR patterns of *E. sakazakii* and related organisms. DNA patterns analysed in 2% agarose gel.

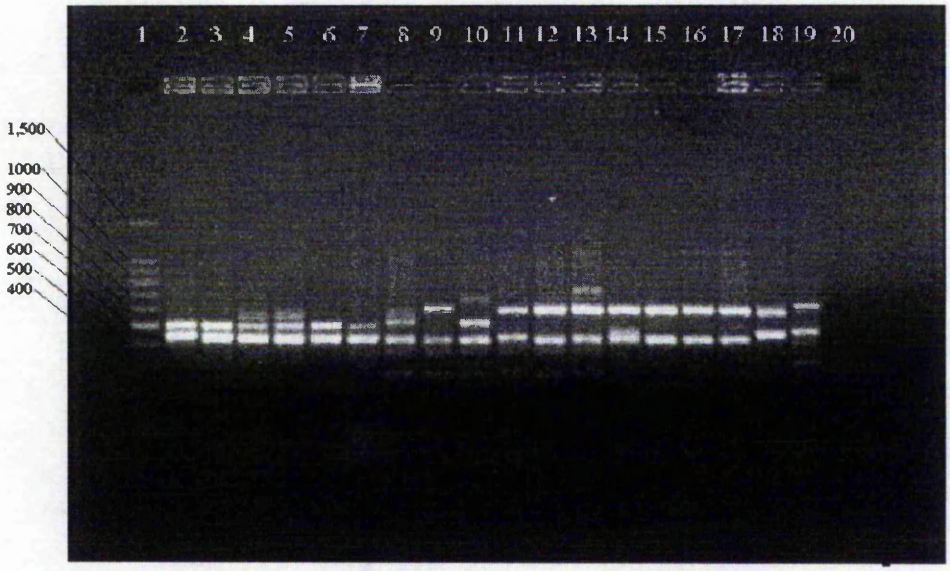


Figure 4.3.2 shows that various bacteria strains analysised by ITS PCR and 2% agarose gel according to Materials and Methods section 4.2.2, where lanes:
1. 100bp DNA marker **2**.*E. sakazakii* (825) **3**.*E. sakazakii* (822) **4**.*E. sakazakii* (sk90) **5**.*E. sakazakii* (LB) **6**.*E. sakazakii* (LA) **7**.*E. sakazakii*(BF006BB) **8**.*E. cloacae* (NTU 831, OCC641) **9**.*E. cloacae* (NTU 830, OCC118) **10**.*E. cloacae* (NTU 832 OCC 766) **11**.*E. cloacae* (NTU 833 OCC1067) **12**.*E. cloacae* (MP0056W 326) **13**.*E. cloacae* (IFMK6W 254) **14**.*C. koseri*(4277) **15**.*C. koseri*(4036) **16**.*C. koseri*(JB62) **17**.*C. koseri*(SMT319) **18**.*E. cloacae*(JOHVY 370) **19**.*C. freundii*(FF08BK 340).

The ITS-PCR profiles of *E. sakazakii* and related species showed DNA fragments ranging in size from 300 to 1,000 bp that distinguished the species by a number of specific DNA fragments.

Eighteen tested strains all produced a 450bp band. All *E. sakazakii* strains (figure 4.3.2 lane 2-7) produced 500bp bands, the strength of the 500bp bands of the type strain 822 similar with *E. sakazakii* 825; while those clinical isolated *E. sakazakii* strains sk90, London B, London A (figure 4.3.2 lane 4, 5 and 6) and baby food isolates BF006BB (figure 4.3.2 lane 7) produced a relative weak 500bp band.

E. cloacae NTU831 OCC 641 (figure 4.3.2 lane 8) produced a similar 500bp band similar to *E. sakazakii* strains. While all other *E. cloacae* and *C. koseri* strains produce a 600bp band instead of the *E. sakazakii* 500bp bands (figure 4.3.2 lane 9, lane 11-19). In addition, *E. cloacae* NTU 832 (figure 4.3.2 lane 10) showed extra 450 and 650bp bands compared with other *E. cloacae*.

All *E. cloacae* strains were distinguished from other species due to the presence of a 300bp band.

3) RAPD-PCR

Figure 4.3.3: RAPD PCR patterns of *E. sakazakii* and related organisms. DNA patterns analysed in 2% agarose gel.

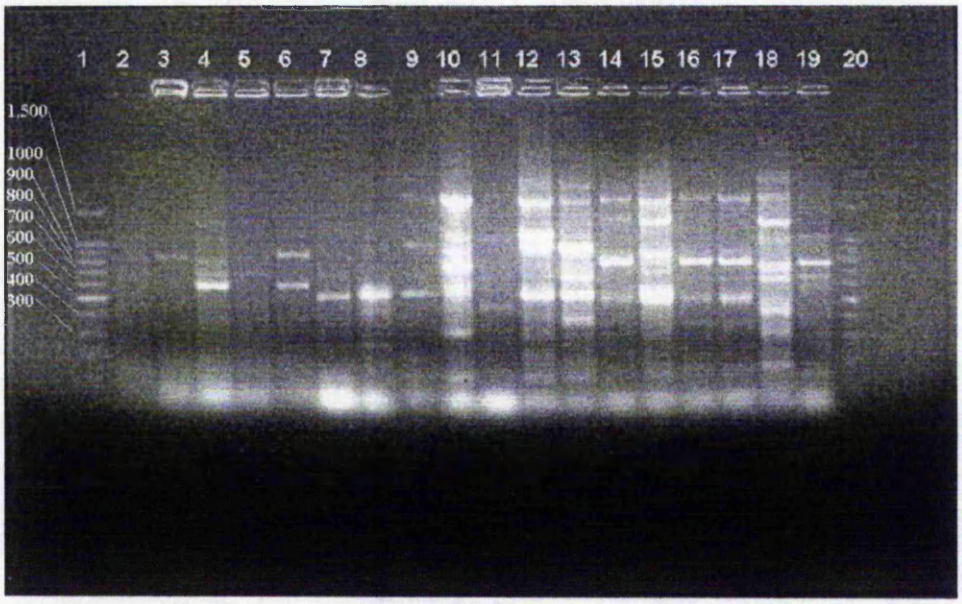


Figure 4.3.3 shows that various bacteria strains analysised by RAPD PCR and 2% agarose gel according to Materials and Methods section 4.2.2, where lanes:
1. DNA marker **2**.*E. sakazakii* (825) **3**.*E. sakazakii* (822) **4**.*E. sakazakii* (sk90) **5**.*E. sakazakii* (LB) **6**.*E. sakazakii* (LA) **7**.*E. sakazakii*(BF006BB) **8**.*E. cloacae* (NTU 831, OCC641) **9**.*E. cloacae* (NTU 830, OCC118) **10**.*E. cloacae* (NTU 832 OCC 766) **11**.*E. cloacae* (NTU 833 OCC1067) **12**.*E. cloacae* (MP0056W 326) **13**.*E. cloacae* (IFMK6W 254) **14**.*C. koseri*(4277) **15**.*C. koseri*(4036) **16**.*C. koseri*(JB62) **17**.*C. koseri*(SMT319) **18**.*E. cloacae* (JOHVY 370) **19**.*C. freundii* (FF08BK 340).

The RAPD-PCR profiles of *E. sakazakii* related species showed DNA fragments ranging in size from 70 to 1,800 bp. The profiles distinguished the species by a number of specific DNA fragments. It was different from ITS PCR and t-DNA PCR as there was no common band universal to all candidate strains.

All the *E. sakazakii* strains produced only a few DNA bands in RAPD PCR analysis.

E. cloacae strains were divided into two groups by RAPD PCR. First three strains: NTU 830, NTU 831 and NTU 833 showed relatively weak DNA bands, while

other *E. cloacae* strains (NTU 832, MP0056W 326, IFMK6W 254, JOHVY 370) showed relatively strong DNA bands.

C. koseri strains (figure 4.3.3 lane 10, 12-18) are distinguished from *E. sakazakii* and some *E. cloacae* strains by many strong DNA bands in RAPD PCR analysis. 1800 bp and 500 bp bands were commonly produced in tested *C. koseri* strains.

C. freundii(FF08BK 340) also produced weak DNA bands in RAPD PCR analysis.

These three PCR methods showed that many *C. koseri, E. cloacae* strains and one *C. freundii* strain were similar to the *E. sakazakii* type strain cluster. This may due to the close relationship of *E. sakazakii* with these organisms which cannot be easily distinguished using conventional microbiological methods.

Different genetic analysis methods gave different analysis results. T-DNA, RAPD and ITS-PCR were based on polymorphism of tRNA intergenic spacer, the 16S-23S internal transcribed spacer and genomic DNA of the bacteria respectively. The disagreement of phenotypes and genotypes of these species seem to be more heterogeneous than previously believed. The identification methods currently used need to be reviewed by their epidemiological value (i.e. virulence of the bacteria). Also the results have implications for the reliability of clinical data due to misidentification of neonatal infections.

4.3.2. Transposon modification and bioluminescence analysis of *E. sakazakii* and related organism

Several *E. sakazakii* strains and *C. koseri* strains were used for *lux* marking. The *E. sakazakii* strains included: London A, 822 (type), capsulated, and sk90. The *C. koseri* strains were 4036, 4277, SMT319, JB62 and JOHVY. These strains were labelled using transposon insertion following conjugation. At least twenty transconjugants (selected by antibiotic resistance) from each parent strain were purified for bioluminescent assessment. Only strong luminescence output transconjugants were kept for further study. These were named as parent strain name plus mutant number, for example, *E. sakazakii* London A mut 1.

The liquid cultures and single colonies of *E. sakazakii* and *C. koseri* transconjugants strains produced enough light to be easily detected by either naked eye in a dark room or the luminometer ToxAlert 100®.

For an unknown reason, *lux* marking of the strain *E. sakazakii* London B was not successful. The conjugation mating time was varied between 6 and 96 hours, but no transconjugant positive strains were isolated. This may because the London B strain is resistant to transposon or plasmid conjugation. It may also be because London B has special restriction endonuclease systems, which cleaves the heterologous DNA.

In *E. sakazakii* strains, modified by *lux*-transposons, the promoterless *lux* operon was fused into the genome randomly. Hence the luminescence output depended on the strength of the promoter to which the *lux* operon was fused. Bacterial gene promoters can be affected by many factors, for example, growth phase, pH, growth substrates and heat shock. The relationship between bioluminescent behaviour and bacterial growth phase of nine strains of genetically modified *E. sakazakii* was determined by luminometry and photometry studies. These nine strains were selected due to their overnight culture produced relatively high luminescence intensity. Figure 4.3.4 shows that these mutants had different luminescent behaviours during growth. For comparison Figure 4.3.5 shows the growth curve (OD_{405}) of *E. sakazakii* 822. *E. sakazakii* cap-909mut1 showed very active luminescence in the initial stage of growth (initial 200 minutes of growth). While sk90-mut1 showed active luminescence in later stage of growth (late log phase at 300-500 minutes growth time).

Figure 4.3.4: Growth phase dependent bioluminescence behaviour *of E. sakazakii* related mutants.

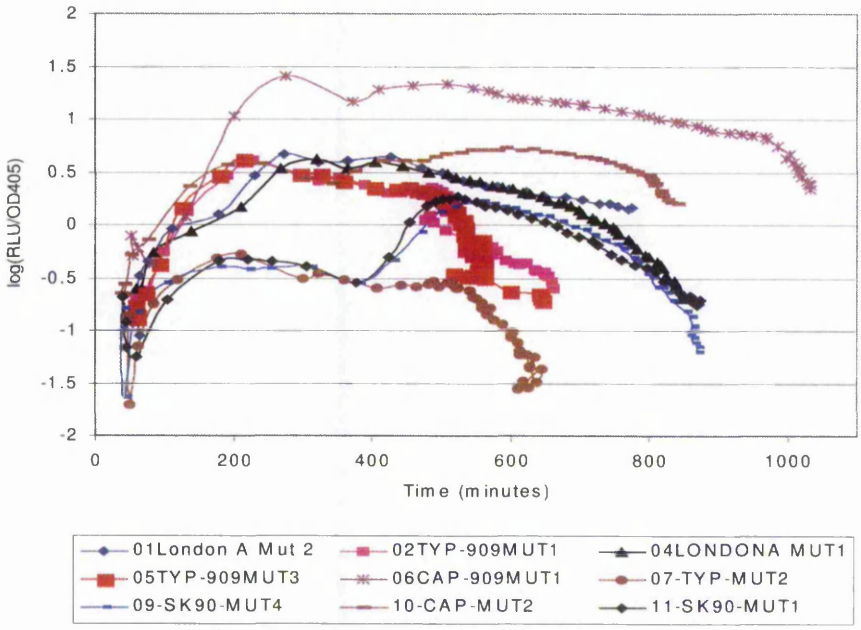


Figure 4.3.4 shows that the luminescent behaviours of various mutants were analysised by luminometers according to Materials and Methods section 4.2.4.

Figure 4.3.5: Growth curve of *E. sakazakii* 822.

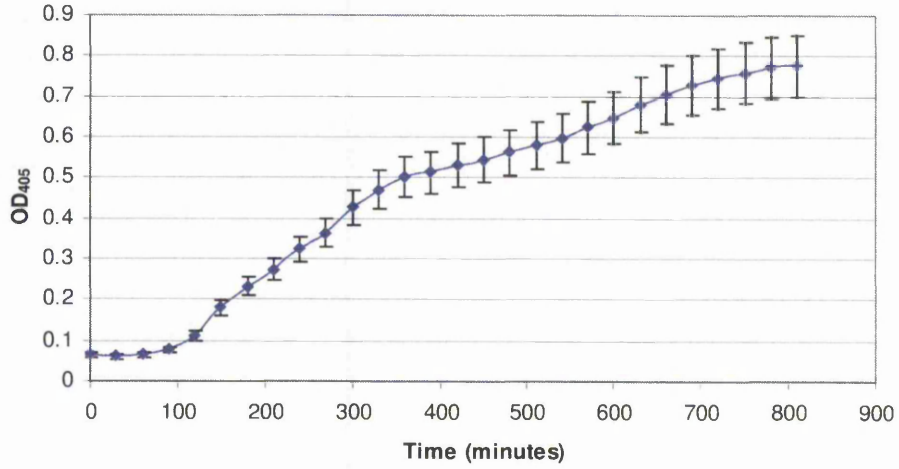


Figure 4.3.5 shows that the growth of bacteria type strain 822 analysised according to Materials and Methods section 4.2.4

The main purposes of this study was to construct luminescent bacteria to monitor protein synthesis activities during bacterial recovery process from stressed condition (desiccated and acidic stress). Because of the result of the bioluminescence, it was not possible to study the protein recovery process, since there was no promoter information available. Subsequently, the ribosomal protein promoter (rpsJ) was included in the construction of bioluminescent bacteria, as this promoter is known to be active in early stages of growth.

4.3.3. Bioluminescence and fluorescence modification of *E. sakazakii* and related organism by ribosomal promoter

As successful opportunistic pathogens, *E. sakazakii* and related organisms survive stressed conditions such as heat and desiccation during milk powder production, and the acidic pH environment of the stomach. In order to understand this aspect of bacterial virulence, protein synthesis activity was assessed during cell recovery from stressed conditions. The reporter genes were fused under the control of ribosomal protein promoter (rpsJ) to monitor the recovery process.

Previous PCR typing results showed that two *E. sakazakii* clinical isolates London A and sk90 were phenotypically and genotypically related to the *E. sakazakii* type strain 822. Therefore these strains were selected as representative *E. sakazakii* strains for *lux* and GFP marking. In addition to *E. sakazakii*, one clinical isolate of *C. koseri* (JB62) was also selected for *lux* and GFP marking for comparative purposes.

The plasmid pDEST® modified with the insertion of ribosomal protein promoter (rpsJ) plus *lux* operon, GFP gene or dual operon GFP-*lux*. The resultant plasmids were transformed into *E. sakazakii* and *C. koseri* by electroporation. The transformants were grown on ampicillin culture medium, and showed bioluminescence or fluorescence.

Single colonies were isolated and purified using at least three subcultures. The *lux* strains produced enough light to be observed by naked eye in a dark room. In order to ensure very bright colonies were to be identified, an Arugs-100 photo-imaging camera (Hamamatsu Photonics) were used to detect the brightest colonies. Their luminescence was quantified by the luminometer ToxAlert 100® or Tecan® GENios.

For the GFP transformants, the fluorescent colonies were easily identified under visible blue / violet light in a dark room. The GFP expression in liquid cultures was quantified using a fluorescence detecting device Tecan® GENios.

For unknown reasons, not all transformants showed appropriate phenotype. The parent strains and the phenotype of transformants were given as following table 4.3.1:

Table 4.3.1: Phenotype of reporter transformants constructed by rpsJ.

Parent strains	Reporters	Ampicillin resistance	RLU	RFU
E. sakazakii London A	GFP-*lux*	+	+	-
	GFP	+		-
	lux	+	+	
E. sakazakii sk90	GFP-*lux*	+	-	-
	GFP	+		+
	lux	+	+	
C. koseri jb62	GFP-*lux*	+	+	-
	GFP	+		+
	lux	+	+	

Table 4.3.1 shows the phenotype of various strains that were transformed with the plasmid (pDEST®) according to Materials and Methods section 4.2.5, RLU means relative luminescent units, and RFU means relative fluorescent units; "+" means phenotype positive, "-" means phenotype negative.

It was noticed that although the *E. sakazakii* sk90 *lux* transformants (pDEST® *lux*) grew and showed bioluminescence in ampicillin medium, this required 3-4 days. Consequently *E. sakazakii* sk90 *lux* transformants (pDEST® *lux*) were excluded from the growth recovery of desiccated cells study.

Viable plate counts of freeze-dried bacteria

Freeze-dried bacteria were stored in the recommended freeze-drying protection medium, as the viability was essential for the recovery study. The viability of the bacteria after freeze-drying was confirmed by viable plate counting.

The viable bacteria density was determined using plate counts as described in table 4.3.2.

Table 4.3.2: comparison viability of fresh and freeze dried bacteria.

Parent strains	Reporters	Fresh bacteria	Freeze-dried bacteria
E. sakazakii London A	GFP-*lux*	2.8×10^{7}	3.2×10^{7}
	GFP	1.6×10^{7}	2.0×10^{7}
	lux	2.3×10^{7}	2.1×10^{7}
E. sakazakii sk90	GFP-*lux*	2.3×10^{7}	3.7×10^{7}
	GFP	3.4×10^{7}	2.9×10^{7}
	lux	N/A	N/A
C. koseri jb62	GFP-*lux*	1.9×10^{7}	2.7×10^{7}
	GFP	1.8×10^{7}	1.9×10^{7}
	lux	2.9×10^{7}	2.6×10^{7}

Table 4.3.2 shows the viability of fresh and freeze dried transformants by plate counting according to Materials and Methods section 4.2.5

Table 4.3.2 shows that the bacterial viability was well protected during freeze drying process, and their viable plate count result was very similar with the control samples.

4.3.4. Monitoring of growth after rehydration of *E. sakazakii* London A using luminescence

As previously stated luminescent and fluorescent reporter operons (GFP-*lux* dual reporter, *lux* and GFP operon) driven by a ribosomal protein promoter rpsJ, and transcriptional terminator, were separately inserted into the plasmid pDEST® and then transformed into clinical isolate *E. sakazakii* London A. These transformants were grown in ampicillin-contained media and showed luminescence, however no fluorescence was detected in any *E. sakazakii* London A transformants.

The transformants were desiccated by freeze-drying in an inositol containing bacterial freeze-drying protective medium. The results showed that these bacteria

were well protected during freeze drying, and their viable plate count result was very similar to fresh cultures.

The freeze-dried transformants were rehydrated in broth and immediately analysed for growth and luminescence. Control samples were included. These were transformants, which had not been desiccated, that were grown in the ampicillin medium.

There was a concern about bacterial age during the experimental design. The viable plate count ensured that the viability of the freeze-dried bacteria was well protected. In addition, young cells were used for rehydration and control samples. Both the freeze-dried bacteria and control sample were grown to optical density OD_{405}=0.4. Then, a low inoculate amount were used for recovery study.

Absorbance (405nm) was used for bacterial growth assessment. Luminescent intensity was detected with the luminometer Tecan®, and presented by two methods. First, RLU by time with luminescence intensity produced by the whole batch of bacteria according to incubation period. Secondly RLU/Absorbance (405nm) by absorbance (405nm) with luminescence intensity produced by every individual bacterial unit changed with biomass of bacteria.

E. sakazakii London A with GFP and *lux* operon

As previously stated this strain did not express fluorescence (table 4.3.1). The measurements of both growth and luminescence were performed in triplicate. The growth dependent luminescent behaviour of rehydrated and control *E. sakazakii* London A (pDEST® GFP-*lux* dual reporters) are shown in Figure 4.3.6 with error bars to indicate standard deviation values (n=3).

Figure 4.3.6: Growth dependent luminescent behaviour of rehydrated *E. sakazakii* London A, (pDEST® GFP-*lux*).

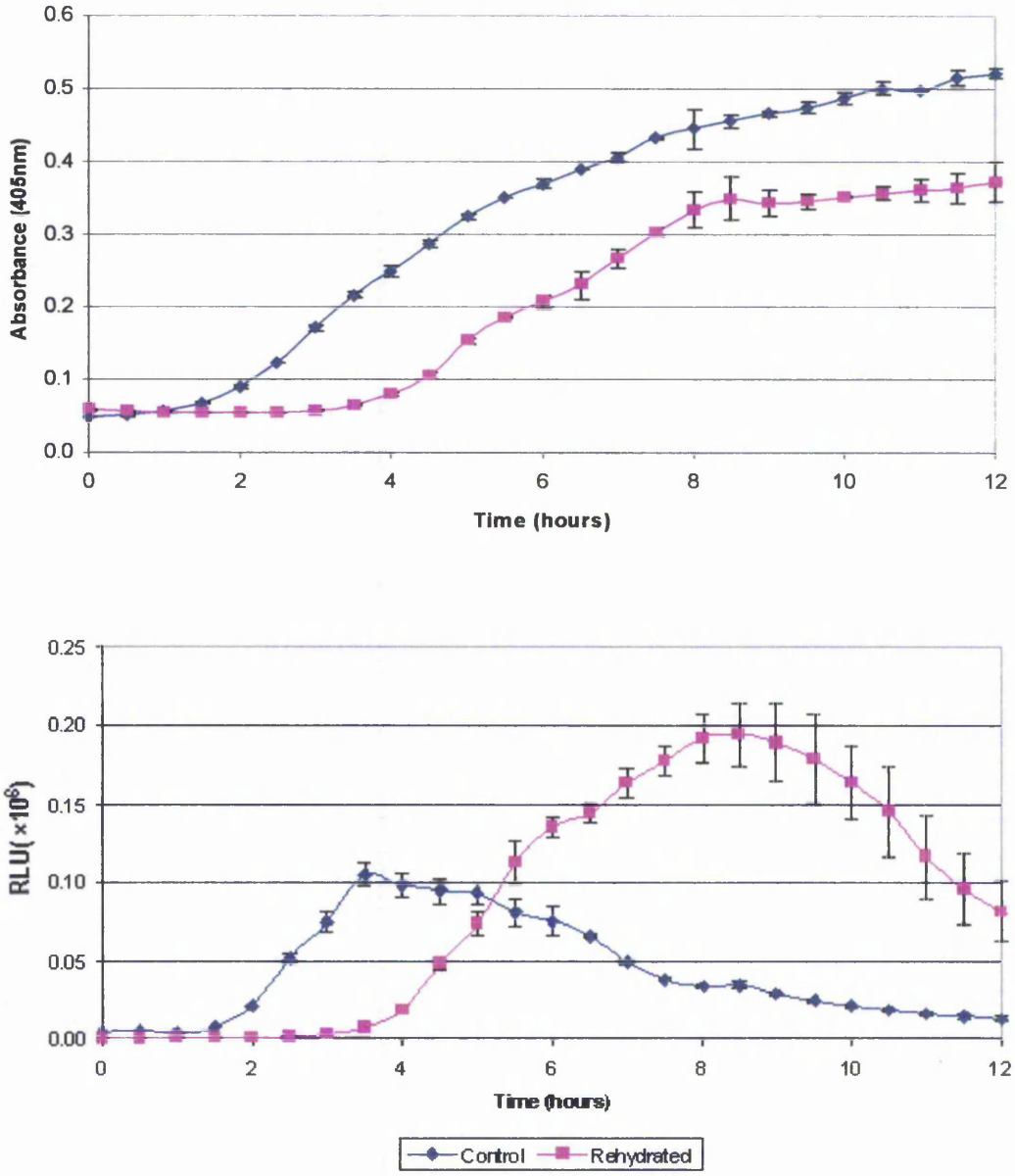


Figure 4.3.6 shows the growth and luminescence behaviour of *E. sakazakii* London A, (pDEST® GFP-*lux*) assessed by luminometer according to Materials and Methods section 4.2.5.

Figure 4.3.6 shows that the rehydrated bacterium *E. sakazakii* London A (pDEST® GFP-*lux*) had longer lag phase than the fresh control samples. The lag phase of fresh *E. sakazakii* London A (pDEST® GFP-*lux*) was less than one hour, while the lag phase of the rehydrated strain was delayed by 2.5 hours.

In contrast, Figure 4.3.6 also shows that fresh and rehydrated *E. sakazakii* London A(pDEST® GFP-*lux*), had detectable luminescence after about 1.5 hours and 3.5 hours, respectively. Their luminescence intensity rapidly increased until after 6 and 8 hours, which correlates with the late log phase of the bacterial growth curve.

A preliminary experiment demonstrated that the freeze-drying process did not reduce the cell viability. Consequently it was certain that the delay of the growth and induction of bioluminescence was primarily due to the physiological recovery from desiccated condition and not due to reduced viable counts.

Figure 4.3.7: Biomass dependent luminescent behaviour of rehydrated *E. sakazakii* London A (pDEST® GFP-*lux*).

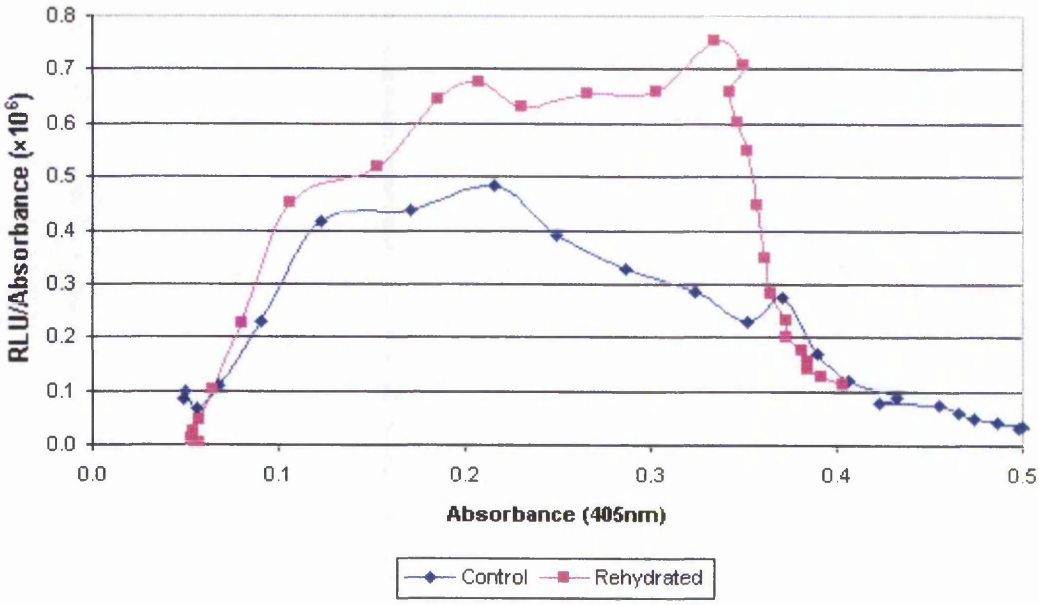


Figure 4.3.7 shows the growth and luminescence behaviour of *E. sakazakii* London A, (pDEST® GFP-*lux*) assessed by luminometer according to Materials and Methods section 4.2.5.

Although Figure 4.3.6 shows that there was a lag phase in luminescence production for the whole batch of bacteria, above Figure 4.3.7 shows that the luminescence output from every individual bacterial unit was actively increased throughout their early growth stages. The activity of luminescence production did not decrease until the bacterial density late log phase (refer figure 4.3.7).

E. sakazakii London A (pDEST® *lux* reporters)

The growth dependent luminescent behaviour of rehydrated and control *E. sakazakii* London A (pDEST® *lux*) are shown in Figure 4.3.8 with error bars to indicate standard deviation values (n=3).

Figure 4.3.8: Growth dependent luminescent behaviour of rehydrated *E. sakazakii* London A (pDEST® *lux*).

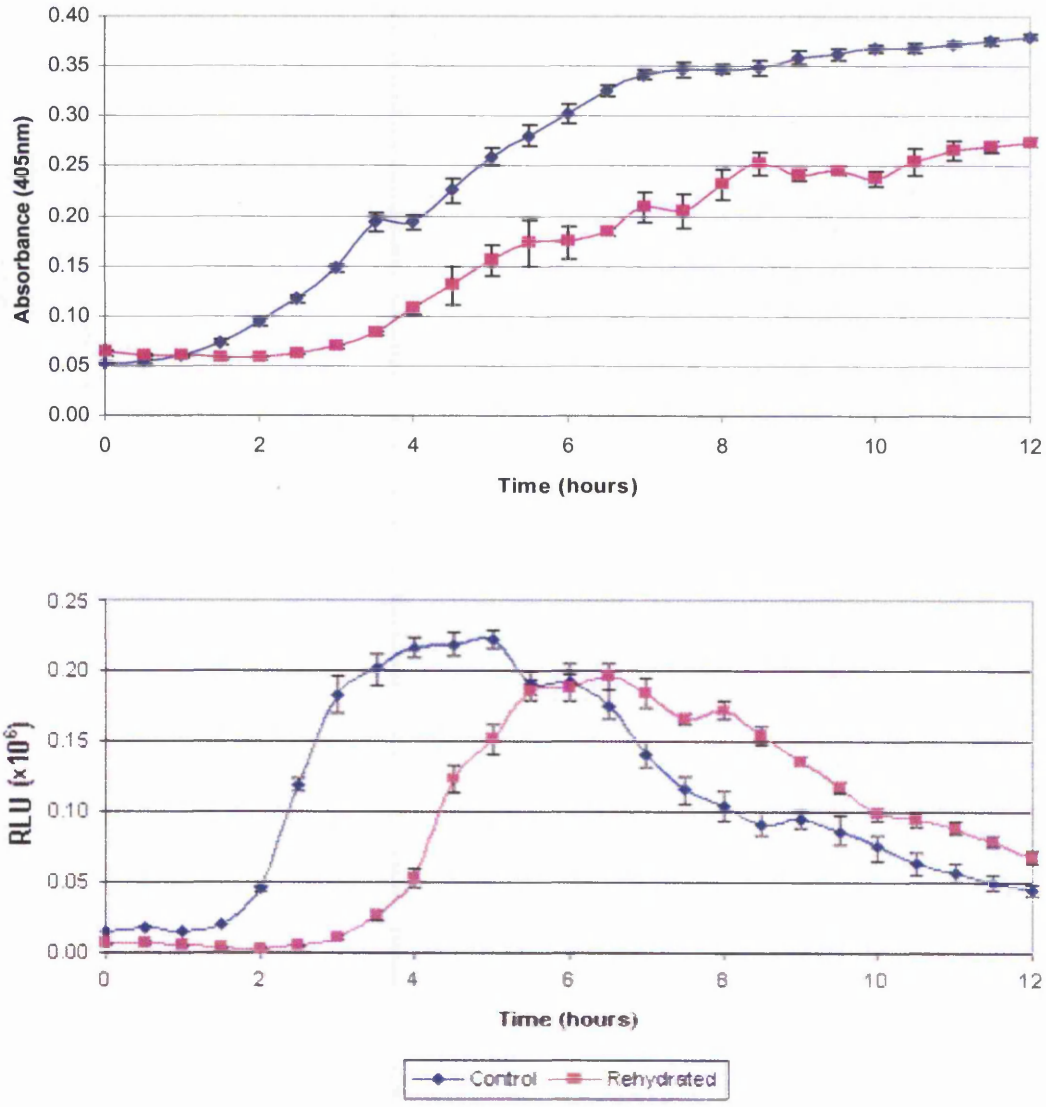


Figure 4.3.8 shows the growth and luminescence behaviour of *E. sakazakii* London A, (pDEST®*lux*) assessed by luminometer according to Materials and Methods section 4.2.5.

Figure 4.3.8 shows that the growth and luminescent behaviour of *E. sakazakii* London A (pDEST® *lux*), is similar to *E. sakazakii* London A (pDEST® GFP-*lux*). The lag phase of fresh bacteria was less than one hour, whereas the lag phase of the rehydrated was delayed by 2.5 hours.

The luminescent behaviour of *lux* transformants was also similar to GFP-*lux* transformants London A strain. The fresh and rehydrated *E. sakazakii* London A (pDEST® *lux*), initialled their luminescence production after about 1.5 hours and 3 hours respectively. Their luminescence intensity rapidly increased until 5 and 7 hours time, which corresponds to late log phase of the bacteria growth curve.

A preliminary experiment showed that the freeze-drying process did not reduce the cell viability. Consequently it was certain that the delay of the growth and induction of bioluminescence was primarily due to the physiological recovery from the desiccated condition.

Figure 4.3.9: Biomass dependent luminescent behaviour of rehydrated *E. sakazakii* London A (pDEST®*lux*)

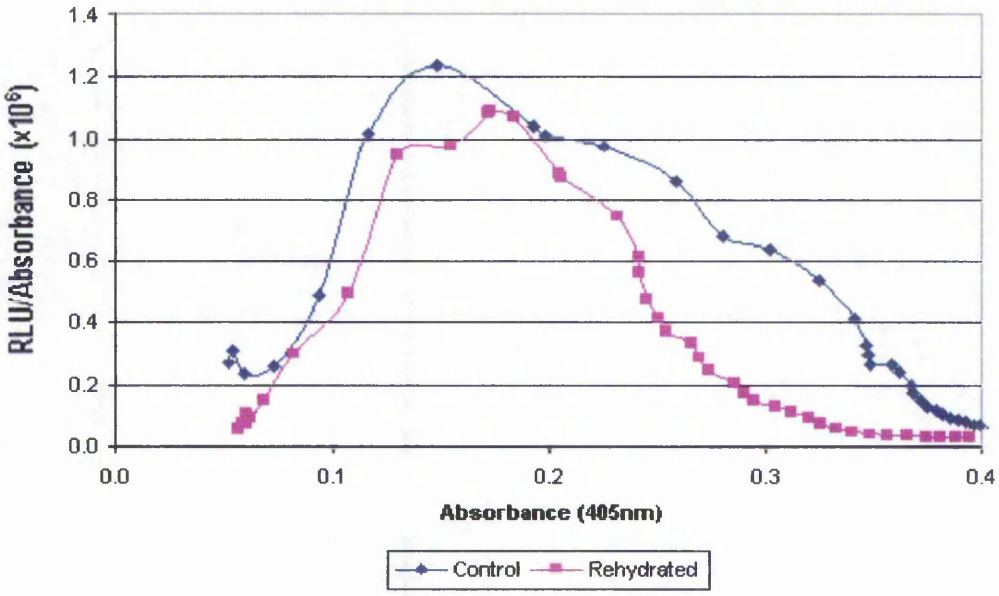


Figure 4.3.9 shows the growth and luminescence behaviour of *E. sakazakii* London A, (pDEST®*lux*) assessed by luminometer according to Materials and Methods section 4.2.5.

Although Figure 4.3.8 shows that there was a lag phase in luminescence production for the whole batch of bacteria, Figure 4.3.9 shows that the luminescence output from every individual bacterial unit was actively increased throughout their early growth stages. This was most notable for rehydrated bacteria, which after a delay, showed a strong increase in bioluminescence. The fresh bacteria, the luminescence did not decreased until the bacterial density reached a certain level (normally late log phase, refer to figure 4.3.9).

E. sakazakii London A GFP operon

There was no fluorescence detectable in any *E. sakazakii* London A transformants. Hence only the growth of the GFP transformant is presented in figure 4.3.10, error bars are indicated as standard deviation of triplicate experiments.

Figure 4.3.10: Growth and recovery of rehydrated *E. sakazakii* London A (GFP).

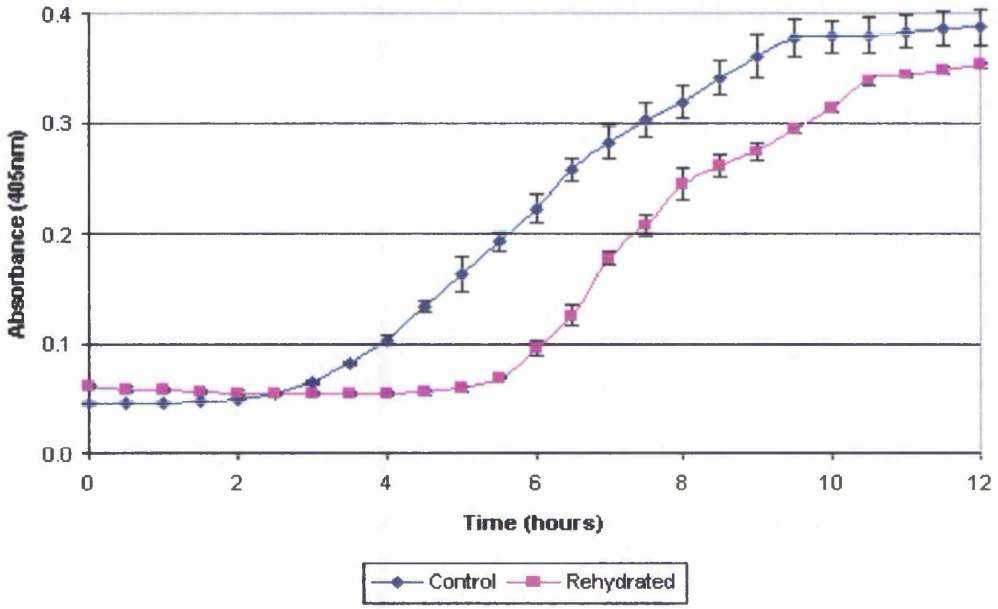


Figure 4.3.10 shows the growth *E. sakazakii* London A, (pDEST® GFP) assessed by absorbance 405nm according to Materials and Methods section 4.2.5.

Figure 4.3.10 shows that, on the rehydration desiccated *E. sakazakii* London A (pDEST® GFP), had a longer lag phase than the fresh control. The lag phase of control was about 1.5 hours, while the lag phase of the rehydrated delayed by 3 hours.

E. sakazakii London A GFP transformants did not show fluorescence. In addition, it cost more time to initial their growth than other London A transformants, which showed luminescent phenotype.

4.3.5. Monitoring of growth after rehydration of *E. sakazakii* sk90 using fluorescence

As previously described in section 3.2.5, the plasmid pDEST® with luminescent and fluorescent reporter operons (GFP-*lux* dual reporter, *lux* and GFP operon) driven by ribosomal protein promoter rpsJ, and transcriptional terminator were also individually transformed into the clinical isolate *E. sakazakii* sk90. All these transformants were able to grow in ampicillin-contained media.

E. sakazakii sk90 (pDEST® *lux*) needed much longer time to grow (3-4days) in ampicillin-contained media, and it was not possible to compare its growth and luminescence behaviour with other mutant strains within a 24 hour period. In addition, only *E. sakazakii* sk90 (pDEST® GFP) showed fluorescence, while transformants of GFP-*lux* operon did not show sufficient fluorescence and luminescence. Subsequently, only the strain *E. sakazakii* sk90 (pDEST® GFP) was used for fluorescence and growth studies. The GFP-*lux* transformant was used for growth recovery analysis only.

The transformants were freeze-dried in the inositol protective medium (materials and methods section 3.2.1). An initial viable plate count experiment was carried out to assess the cell viability after cells have been freeze-dried. The results showed that these bacteria survived freeze drying, as their viable plate count result was very similar with the control samples (fresh culture).

The freeze-dried transformants were rehydrated in appropriate ampicillin broth. Immediately, the rehydrated bacteria were analysed for growth and fluorescence. Control samples were included, which were transformants grown in ampicillin medium that had not been desiccated.

Absorbance (405nm) was used for bacterial growth assessments. While the fluorescence was detected by the Tecan® fluorescence detector.

It was noticed that the fluorescence of the bacterial culture medium interfered with GFP fluorescence in initial growth stages. Consequently biomass dependent fluorescence behaviour, RFU/Absorbance (405nm) by absorbance (405nm), was found not to be appropriate for the fluorescence assay.

E. sakazakii sk90 GFP operon

The assessment of both growth and fluorescence were performed in triplicate. The growth dependent fluorescent behaviour of rehydrated and control *E. sakazakii* sk90 (pDEST® GFP reporters) were described as Figure 4.3.10 with error bars to indicate standard deviation values.

E. sakazakii sk90 with the GFP reporter

Figure 4.3.11: Growth dependent fluorescent behaviour of rehydrated *E. sakazakii* sk90 (pDEST® GFP).

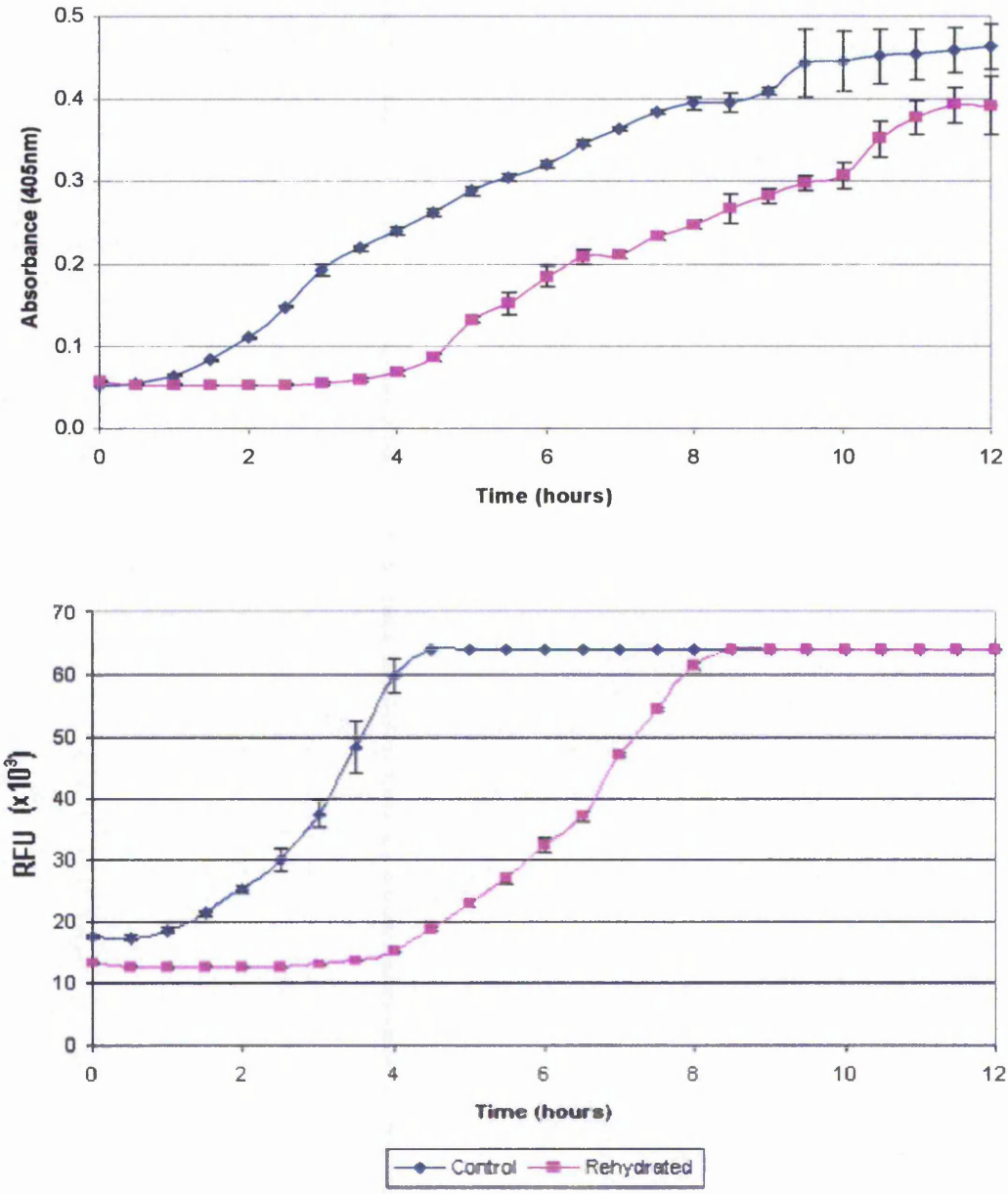


Figure 4.3.11 shows the growth and fluorescence behaviour of *E. sakazakii* sk90 (pDEST® GFP) assessed by fluorescence meter according to Materials and Methods section 4.2.5.

Figure 4.3.11 shows that the rehydrated *E. sakazakii* sk90 (GFP) had a longer lag phase than the freshly inoculated control samples. The lag phase of fresh *E.*

sakazakii sk90 (GFP) was about one hour time, while the lag phase of rehydrated bacteria was about 3.5 hours. Hence the recovery of cell proliferation rate in the lag phase of rehydrated bacteria was approximately 2.5 hours longer than the fresh bacteria.

Figure 4.3.11 also shows that fresh and rehydrated *E. sakazakii* sk90 (pDEST® GFP) initialled their fluorescence production after about 1 hours and 3.5 hours time respectively. Their expression of fluorescence proteins rapidly increased and accumulated to the maximum fluorescence detection scale of Tecan® at RFU 64,000.

Preliminary experiments had ensured that the freeze-drying process did not damage the cell viability. Consequently it was certain that the delay in growth and induction of fluorescence was primarily due to the physiological recovery from the desiccated condition.

Growth recovery of *E. sakazakii* sk90 with dual reporter system

Figure 4.3.12: Recovery of rehydrated *E. sakazakii sk90* (pDEST® GFP-*lux*).

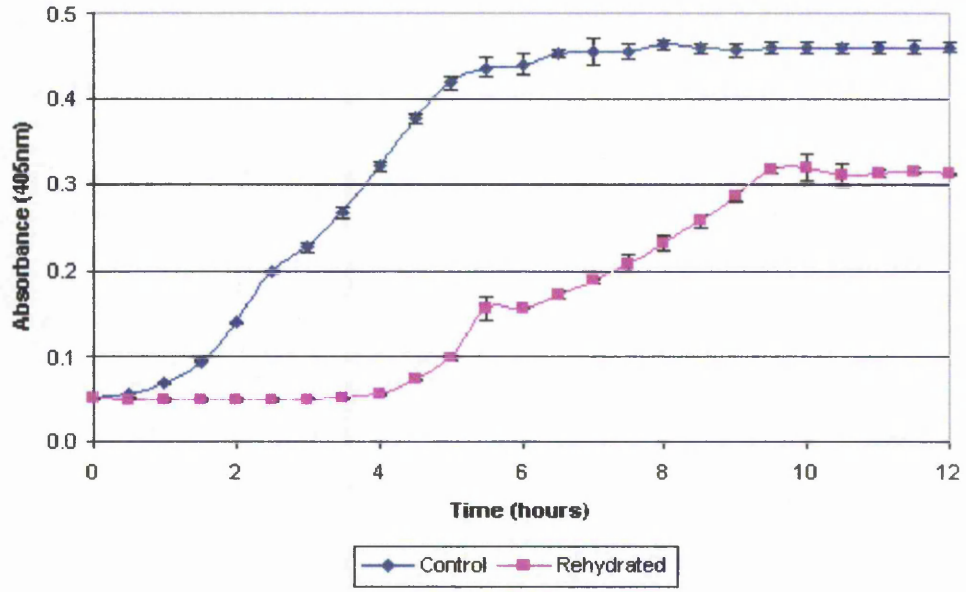


Figure 4.3.12 shows the growth of *E. sakazakii* sk90, (pDEST® GFP-*lux*) assessed by absorbance at 405nm according to Materials and Methods section 4.2.5.

Figure 4.3.12 shows that the freeze-dried *E. sakazakii* (GFP-*lux*) had a longer lag phase than the freshly inoculated control samples. The lag phase of fresh sk90 (GFP-*lux*) was less than one-hour time, while the lag phase of the rehydrated cells was delayed by 3.5 hours.

Preliminary experiments had ensured that the freeze-drying process did not damage the cell viability. Consequently it was speculate that the delay in growth and induction of bioluminescence was primarily due to the physiological recovery from the desiccated condition.

4.3.6. Monitoring of growth after rehydration of *C. koseri* jb62 using luminescence and fluorescence

As previously described in section 3.2.5, the plasmid pDEST® modified with luminescent and fluorescent reporter operons (GFP-*lux* dual reporter, *lux* and GFP operon) driven by ribosomal protein promoter rpsJ, and transcriptional terminator were transformed into the clinical isolate *C. koseri* jb62. The resultant transformants were able to grow in ampicillin-containing media.

The *E. sakazakii* sk90 transformants with plasmids pDEST® GFP-*lux* and pDEST® *lux* showed bioluminescence, and pDEST® GFP showed fluorescence, while for unknown reasons, pDEST® GFP-*lux* transformants did not show any fluorescence.

In order to study growth, luminescent and fluorescent behaviour following rehydration, the transformants were desiccatied by freeze-drying in an inositol containing protective medium (see materials and methods in section 3.2.1). An initial viable plate count experiment was carried out to assess the cell viability after the cells have been freeze-dried. The results showed that the bacteria survived freeze drying, as their viable plate count result was very similar to the control samples i.e.fresh culture.

Further experiments used the freeze-dried transformants. These were rehydrated in ampicillin broth. And immediately, the rehydrated bacteria were analysed for growth, fluorescence and luminescence. Control samples were included, which were transformants grown in ampicillin media, and had not been desiccated.

Absorbance (405nm) was used for bacterial growth measurements. While the luminescent and fluorescent intensities were detected by Tecan® as previously described.

C. koseri jb62 (pDEST® GFP-*lux*)

The measurements of both growth and luminescence were performed in triplicate. The growth dependent luminescent behaviour of rehydrated and control *C. koseri* jb62 (pDEST® GFP-*lux*) transformants are shown in Figure 4.3.13 with error bars to indicate standard deviation values.

C. koseri jb62 with dual reporter system

Figure 4.3.13: Growth dependent luminescent behaviour of rehydrated *C. koseri* jb62 (pDEST® GFP-*lux*).

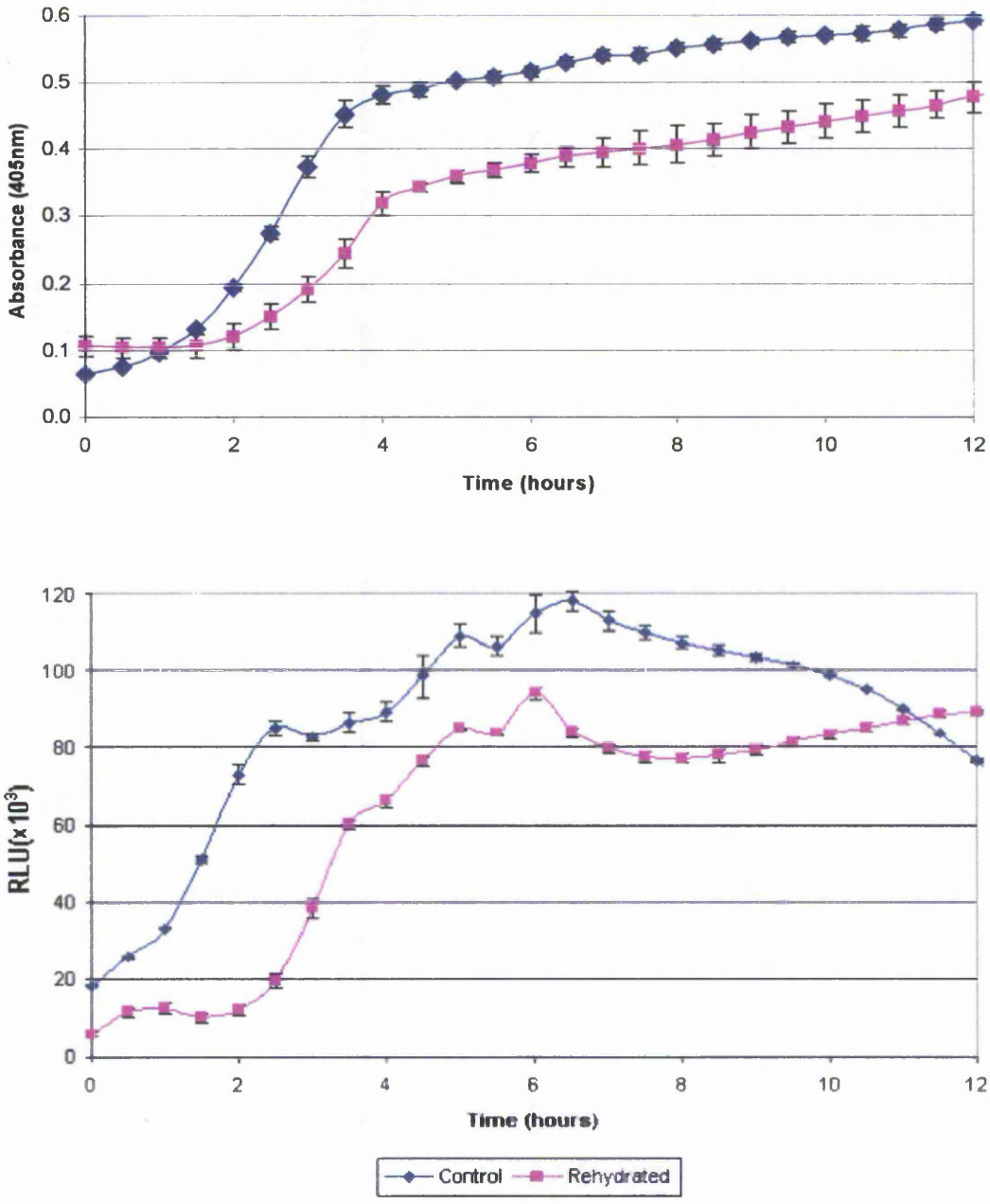


Figure 4.3.13 shows the growth and luminescence behaviour of *C. koseri* jb62, (pDEST® GFP-*lux*) assessed by luminometer according to Materials and Methods section 4.2.5.

Figure 4.3.13 shows that the rehydrated *C. koseri* with dual reporter system had a longer lag phase than the freshly inoculated control samples. The lag phase of fresh *C. koseri* jb62 with dual reporter system was less than one-hour, while the lag phase of rehydrated bacteria was approximately one hour longer than the fresh bacteria.

Figure 4.3.13 also shows that fresh and rehydrated *C. koseri* jb62 showed detectable luminescence after about 0.5 and 2 hours respectively. Their luminescence intensity rapidly increased for 6 hours, which corresponds with the late log growth phase.

Preliminary experiments ensured that the freeze-drying process did not reduce the cell viability. Consequently it was speculated that the delay in growth and induction of bioluminescence was primarily due to the physiological recovery from desiccated condition and not due to decreased viability.

Figure 4.3.14: biomass dependent luminescent behaviour of rehydrated *C. koseri* jb62 (pDEST® GFP-*lux*).

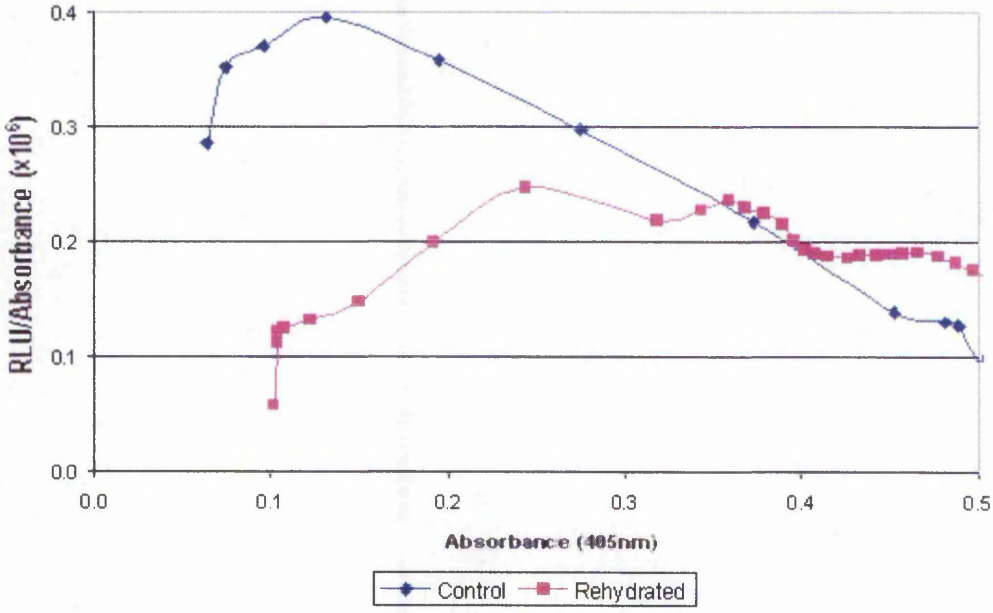


Figure 4.3.14 shows the growth and luminescence behaviour of *C. koseri* jb62, (pDEST® GFP-*lux*) assessed by luminometer according to Materials and Methods section 4.2.5.

Figure 4.3.14 shows that the luminescence output from every individual bacterial unit actively increased in the early growth stages. The fresh inoculated bacteria showed higher biomass dependent luminescence production. The rehydrated bacteria, however, still showed very high luminescent activity in early growth stages, and luminescence production did not decreased until the bacterial biomass reached late log phase.

C. koseri jb62 with *lux* reporter

Figure 4.3.15: Recovery of rehydrated *C. koseri* jb62 (*lux*).

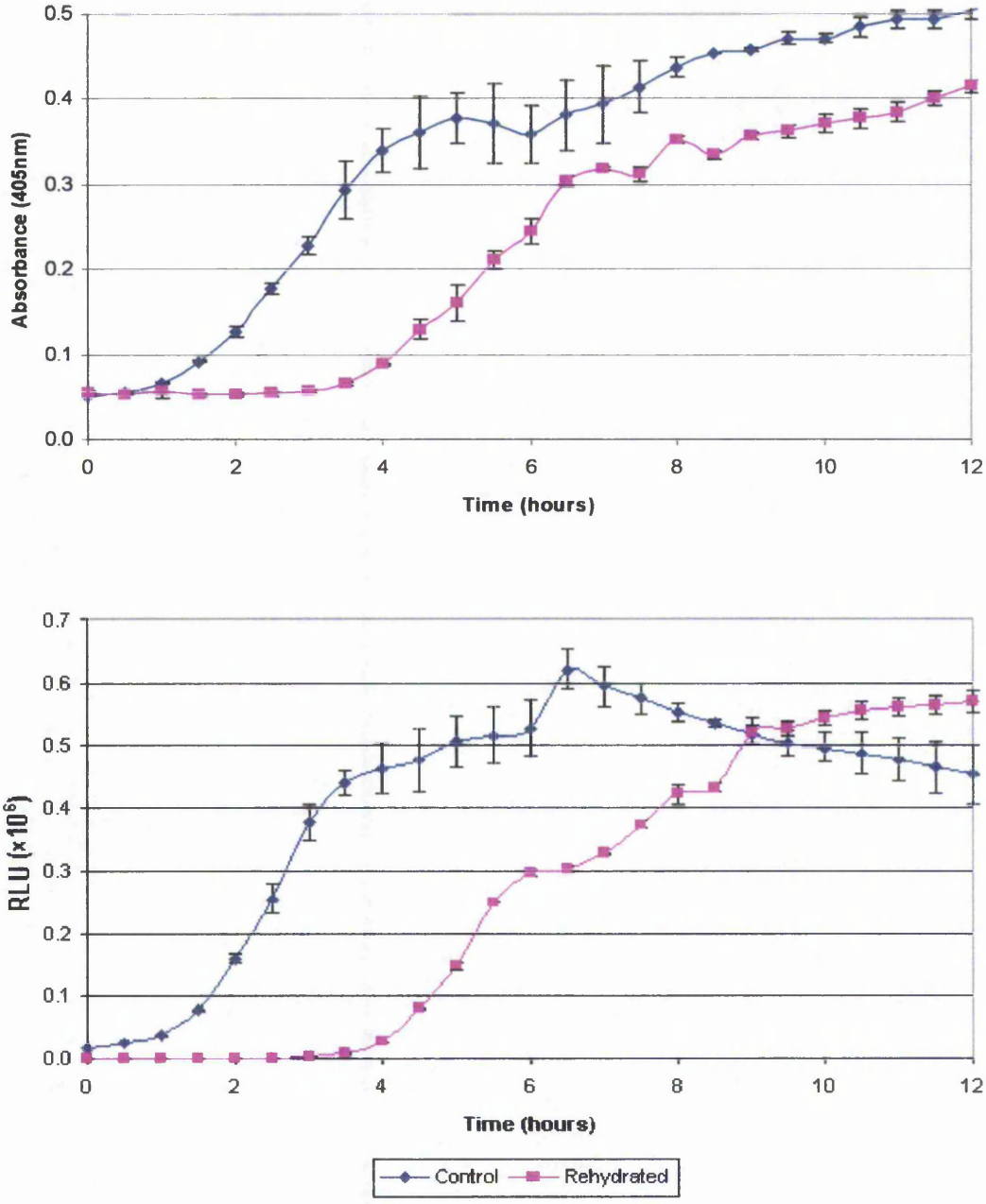


Figure 4.3.15 shows the growth and luminescence behaviour of *C. koseri* jb62, (pDEST® *lux*) assessed by luminometer according to Materials and Methods section 4.2.5.

Figure 4.3.15 shows that the rehydrated *C. koseri* with the dual reporter had longer lag phase than the freshly inoculated control samples. The lag phase of fresh fresh *C. koseri* jb62 with the dual reporter system was less than one-hour, while the lag phase of rehydrated bacteria approximately three hours later.

Figure 4.3.15 also shows that fresh and rehydrated *C. koseri* jb62, had detectable luminescence after about 0.5 hours and 3.5 hours respectively. Their luminescence intensity then rapidly increased until 6 hours time, which corresponds with the late log phase of the bacteria growth curve.

A preliminary experiment ensured that the freeze-drying process did not damage the cell viability. Consequently it was speculated the delay of the growth and induction of bioluminescence was primarily due to the physiological recovery from desiccated condition.

Figure 4.3.16: Biomass dependent luminescence behaviour of rehydrated *C. koseri* jb62 (pDEST®*lux*).

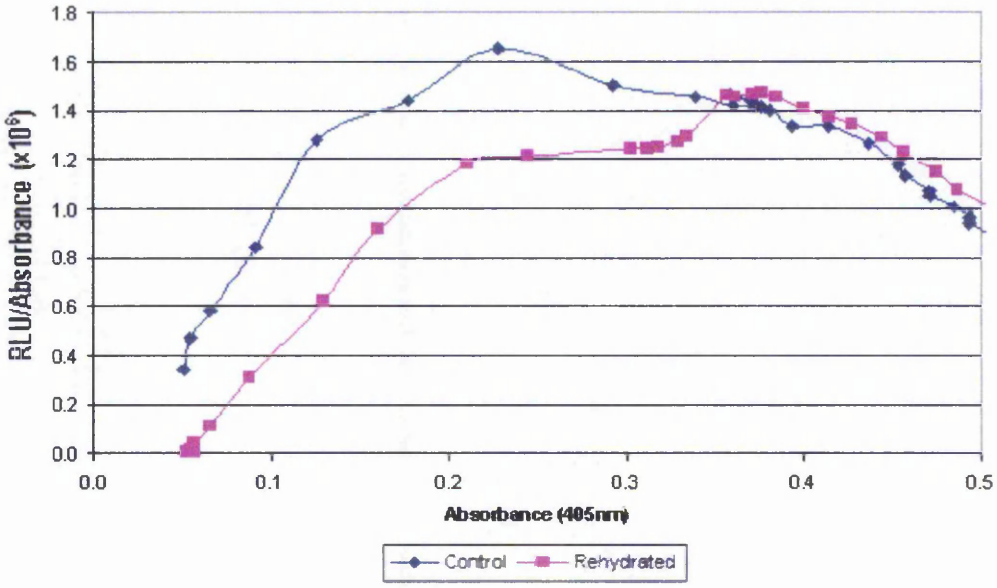


Figure 4.3.16 shows the growth and luminescence behaviour of *C. koseri* jb62, (pDEST® *lux*) assessed by luminometer according to Materials and Methods section 4.2.5.

Figure 4.3.16 shows that the luminescence output according to biomass, was actively increased in their early growth stages. The fresh inoculated bacteria showed higher biomass dependent luminescence production. The freeze dried bacteria, however still showed very high luminescent activity in early growth stages. The level of luminescence production did not decrease until the bacterial biomass reached a certain level in the late log phase.

C. koseri jb62 with GFP reporter

Figure 4.3.17: Growth dependent fluorescent behaviour of rehydrated *C. koseri* jb62 (pDEST® GFP).

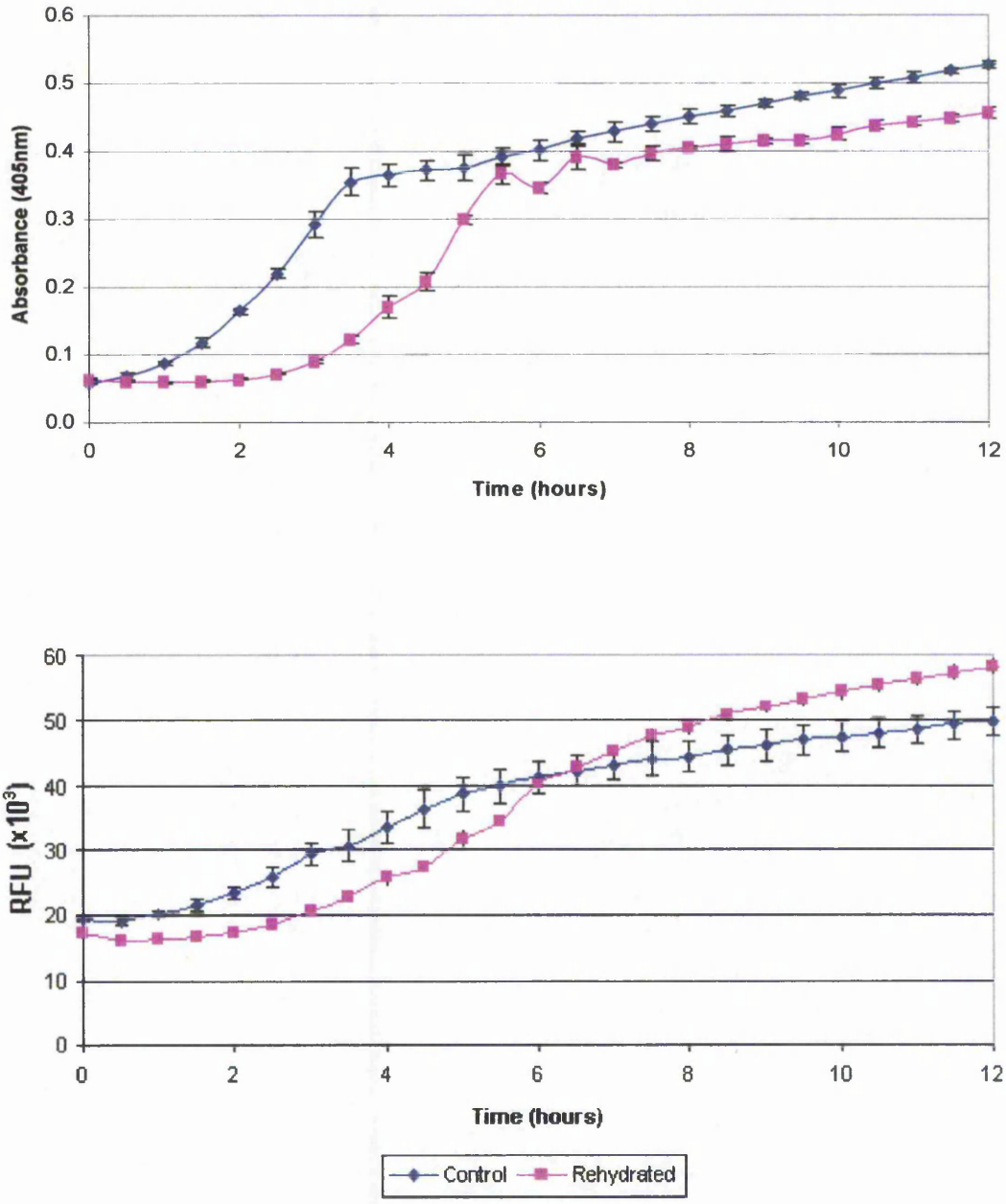


Figure 4.3.17 shows the growth and fluorescence behaviour of *C. koseri* jb62, (pDEST® GFP) assessed by fluorescence meter according to Materials and Methods section 4.2.5.

Figure 4.3.17 shows that the rehydrated *C. koseri* jb62 (pDEST® GFP) also had longer lag phase than the freshly inoculated control samples. The lag phase of fresh *C. koseri* jb62 (pDEST® GFP) was less than one hour time, while the lag phase of rehydrated bacteria were approximately 2 hours later.

Figure 4.3.17 also shows that fluorescence production in fresh and rehydrated *C. koseri* jb62 (pDEST® GFP) was after about 1 hours and 2 hours respectively. Their expression of fluorescence proteins rapidly increased and accumulated to the maximum fluorescence detection scale for the Tecan® at RFU 64,000.

Preliminary experiments ensured that the freeze-drying process did not damage the cell viability. Consequently it was certain the delay of the growth and induction of fluorescence was primarily due to the physiological recovery from desiccated condition.

4.3.7. Growth dependent luminescent and fluorescent behaviours of *E. sakazakii* London A under acidic stress conditions

Luminescent and fluorescent reporter operons (GFP-*lux* dual reporter, *lux* and GFP operon) driven by a ribosomal protein promoter rpsJ, and transcriptional terminator, were separately inserted into the plasmid pDEST® and then transformed into the clinical isolates *E. sakazakii* London A. These transformants were grown in ampicillin-contained medium and showed luminescence, but no fluorescence was detected in any *E. sakazakii* London A transformants.

These transformants were inoculated into ampicillin broth at pH 4 to 7. Protein expression and metabolism under these pH conditions was assessed using fluorescence and bioluminescence respectively.

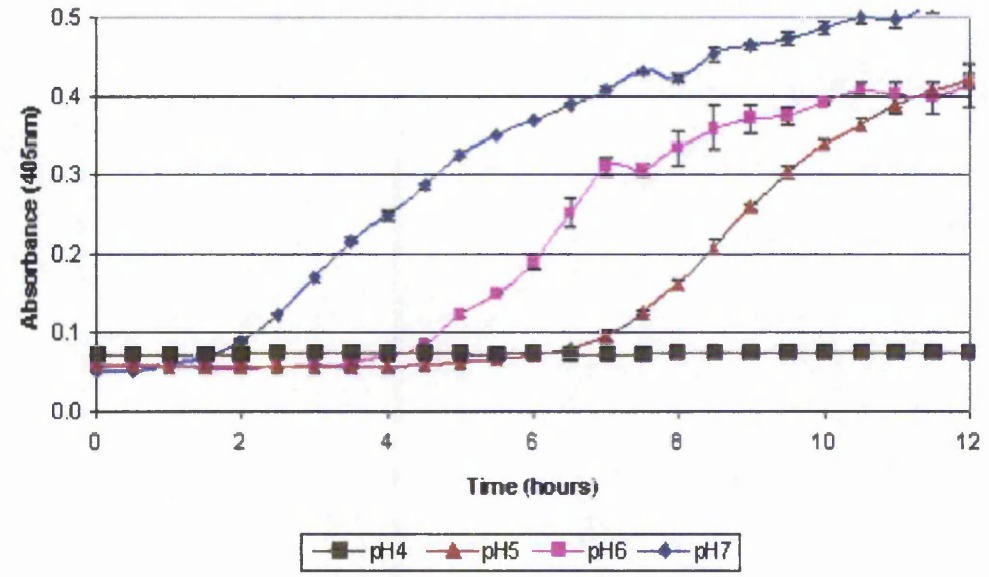
As previously described, absorbance (405nm) was used for bacterial growth assessment. While, the luminescent behaviour of bacteria presented as time dependent and biomass dependent.

Growth dependent luminescence behaviour of *E. sakazakii* London A

E. sakazakii London A with dual reporter system

E. sakazakii London A (pDEST® GFP-*lux*) was grown in various pH conditions. Absorbance (405nm) was used for bacterial growth assessments and luminescent interpretation as previously described. Every growth and luminescent measurement was performed in triplicate as described in the following figure 4.3.18 with error bars to indicate standard deviation values.

Figure 4.3.18: Growth dependent luminescence of *E. sakazakii* London A (pDEST® GFP-*lux*) under acidified conditions.



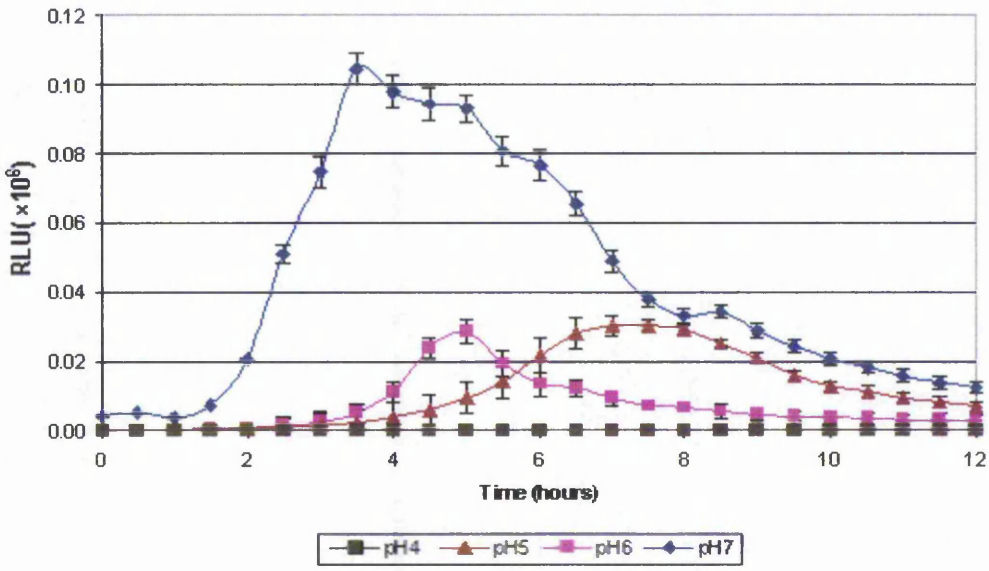


Figure 4.3.18 shows the growth and luminescence behaviour of *E. sakazakii* London A, (pDEST® GFP-*lux*) assessed by luminometer according to Materials and Methods section 4.2.5.

Figure 4.3.18 shows that the lag phase of *E. sakazakii* London A, (pDEST® GFP-*lux*) was extended under low pH conditions. The lag phase was: pH 7, 1.5 hours; pH 6, 4.5 hours; pH 5, 7 hours and at pH 4 the bacteria did not grow.

E. sakazakii London A (pDEST® GFP-*lux*) produced maximum luminescence under neutral pH conditions. Whereas an increase in the acidity caused the luminescence output to be delayed.

Figure 4.3.19: biomass dependent luminescent behaviour of *E. sakazakii* London A (pDEST® GFP-*lux*) under acidified conditions.

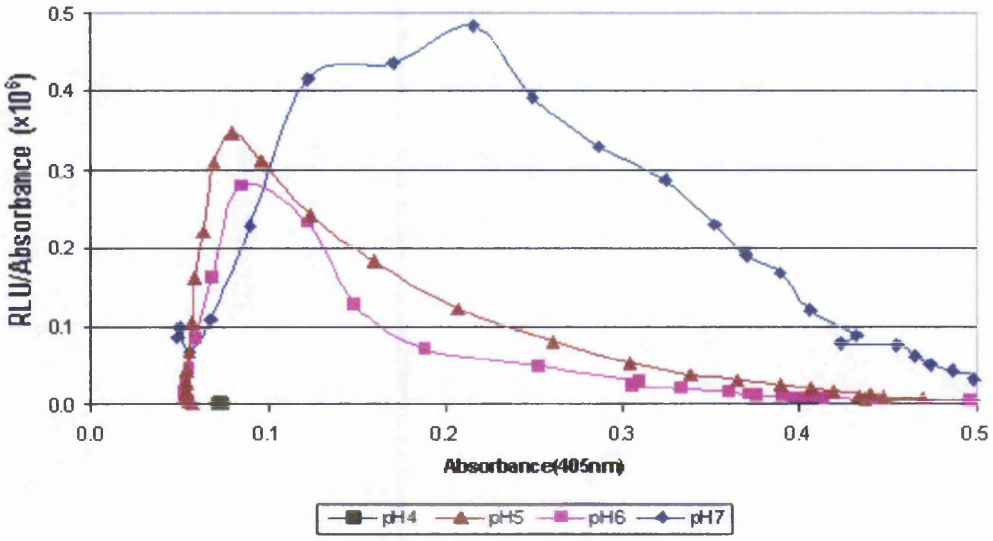


Figure 4.3.6 shows the growth and luminescence behaviour of *E. sakazakii* London A, (pDEST® GFP-*lux*) assessed by luminometer according to Materials and Methods section 4.2.5.

The luminescent output of *E. sakazakii* London A, (pDEST® GFP-*lux*) was greatest under neutral pH conditions. Less luminescence per unit of biomass was produced at pH6 and 5, except at low biomass values (absorbance <0.1 units).

E. sakazakii London A with *lux* operon

E. sakazakii London A (pDEST® *lux*) was grown in various pH conditions. Absorbance (405nm) was used for bacterial growth assessments and luminescent interpretation as previously described. Every growth and luminescent assessment was performed in triplicate as described in the following figure 4.3.20 with error bars to indicate standard deviation values.

Figure 4.3.20: Growth dependent luminescence of *E. sakazakii* London A (pDEST® *lux*) under acidified conditions.

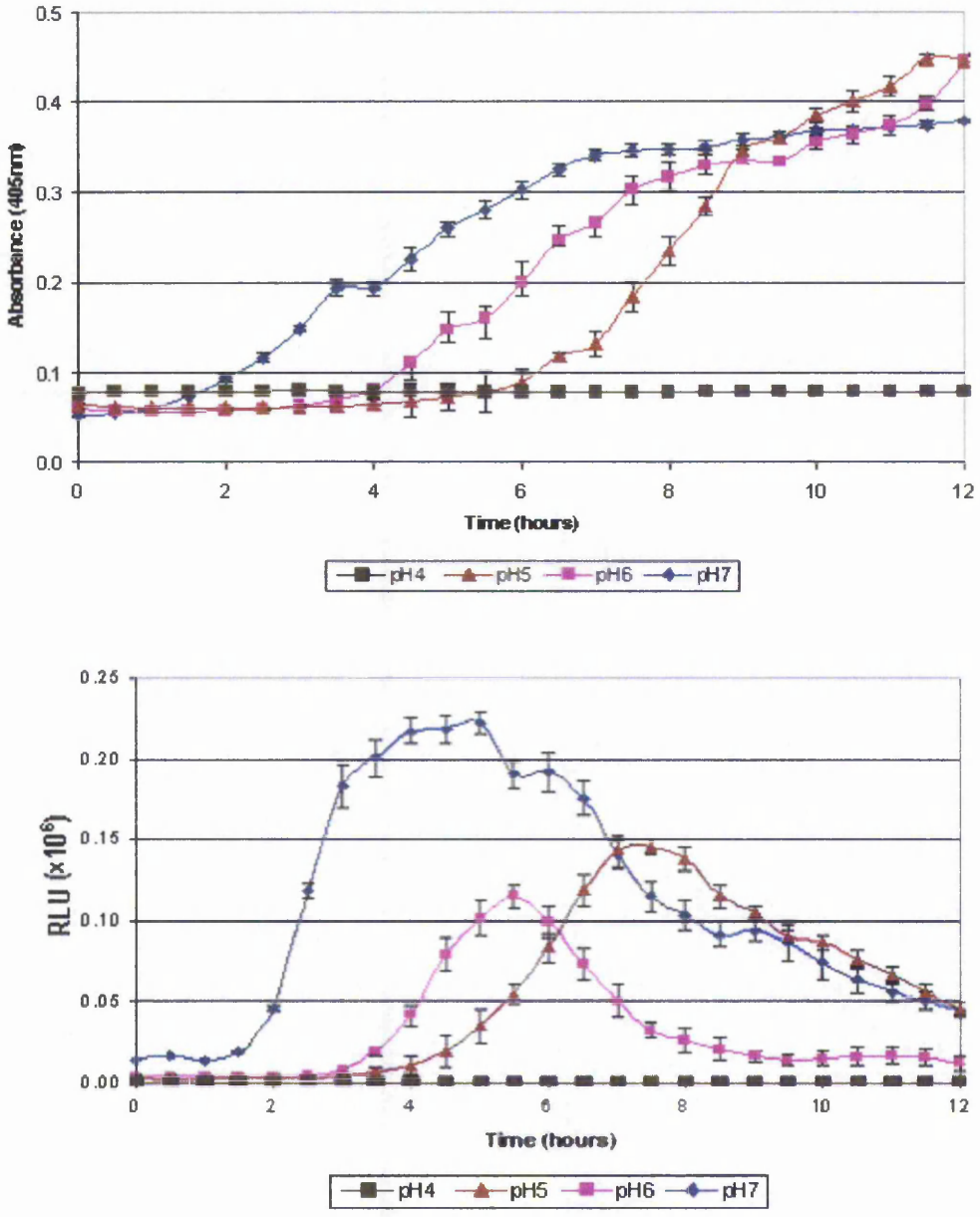


Figure 4.3.20 shows the growth and luminescence behaviour of *E. sakazakii* London A, (pDEST® *lux*) assessed by luminometer according to Materials and Methods section 4.2.5.

Figure 4.3.20 shows that the lag phase of *E. sakazakii* London A (pDEST® *lux*) was prolonged at low pH conditions. The lag phase was: pH 7, 1.5 hours; pH 6, 3.5 hours; pH 5, 6 hours and at pH 4 there was no growth.

E. sakazakii London A (pDEST® *lux*) produced maximum luminescence under neutral pH conditions. Whereas an increase in the acidity caused the luminescence output to be reduced.

Figure 4.3.21: biomass dependent luminescent behaviour of *E. sakazakii* London A (pDEST® *lux*) under acidified conditions.

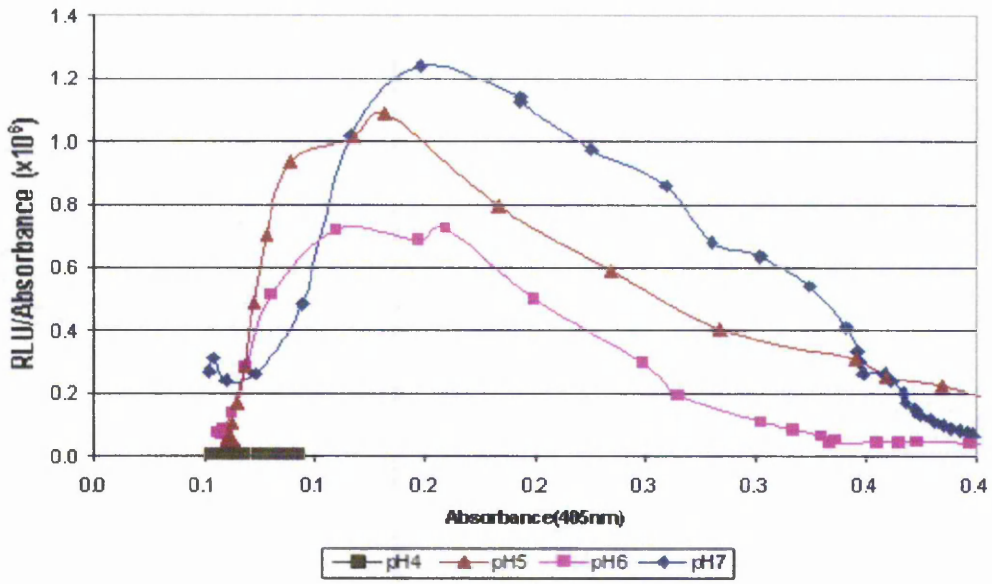


Figure 4.3.21 shows the growth and luminescence behaviour of *E. sakazakii* London A, (pDEST® *lux*) assessed by luminometer according to Materials and Methods section 4.2.5.

The luminescent output of *E. sakazakii* London A (pDEST® *lux*) was greatest under neutral pH conditions. As previously shown with *E. sakazakii* London A (pDEST® GFP-*lux*, Figure 4.3.18), less luminescence per unit of biomass was produced at pH6 and 5, except at low biomass values (absorbance <0.1 units).

E. sakazakii London A with GFP operon

E. sakazakii London A (pDEST® GFP) was grown in various pH conditions. Absorbance (405nm) was used for bacterial growth measurement. Fluorescence was not detected in this transformants. All growth measurements were performed in triplicate and in the following graphs the error bars indicate standard deviation values.

Figure 4.3.22: Growth of *E. sakazakii* London A (GFP) under acidified conditions.

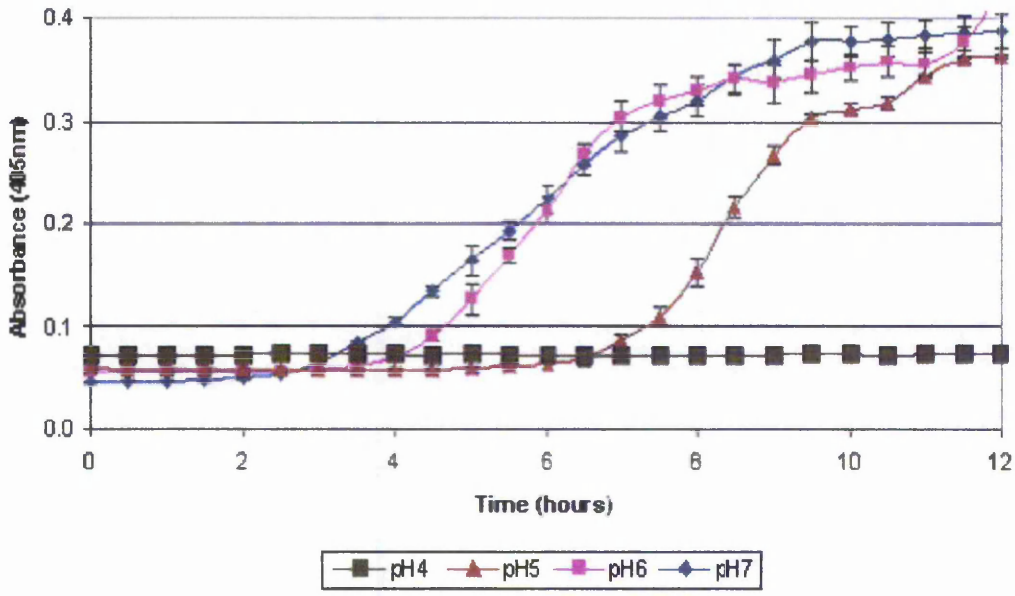


Figure 4.3.22 shows the growth of *E. sakazakii* London A, (pDEST® GFP) assessed by absorbance at 405nm according to Materials and Methods section 4.2.5.

Figure4.3.22 shows that the lag phase of *E. sakazakii* London A, (pDEST® GFP) was prolonged at low pH conditions. The lag phase was: pH 7, 2.5 hours; pH 6, 4.5 hours; pH 5, 7 hours and at pH 4 three was no growth.

4.3.8. Growth dependent luminescent and fluorescent (ribosomal protein promoter) behaviours of *E. sakazakii* sk90 under acidified conditions

Luminescent and fluorescent reporter operons (GFP-*lux* dual reporter system, *lux* and GFP operon) driven by a ribosomal protein promoter rpsJ, and transcriptional terminator, were separately inserted into the plasmid pDEST® and then transformed into the clinical isolates *E. sakazakii* sk90. The *lux* transformants *E. sakazakii* sk90 (pDEST® *lux*) showed very slow growth rates, requiring a few days (3-4 days) before growth and luminescence were detectable. Consequently *E. sakazakii* sk90 *lux* transformants (pDEST® *lux*) were excluded from the growth and luminescence recovery study. Also, there was no obvious luminescence and fluorescence detected from the dual reporter strain *E. sakazakii* sk90 (pDEST® GFP-*lux*). The gene expression activity of the strain *E. sakazakii* sk90 was shown as fluorescence with the GFP transformants only (pDEST® GFP).

E. sakazakii sk90 (pDEST® GFP) was inoculated into ampicillin broth at different pH values, (pH at 7, 6, 5 and 4). Protein expression and metabolism under various pH conditions then was assessed by using fluorescence.

As previously described absorbance (405nm) was used for bacterial growth assessment. While, the fluorescence behaviours of the bacteria were presented as growth dependent manner.

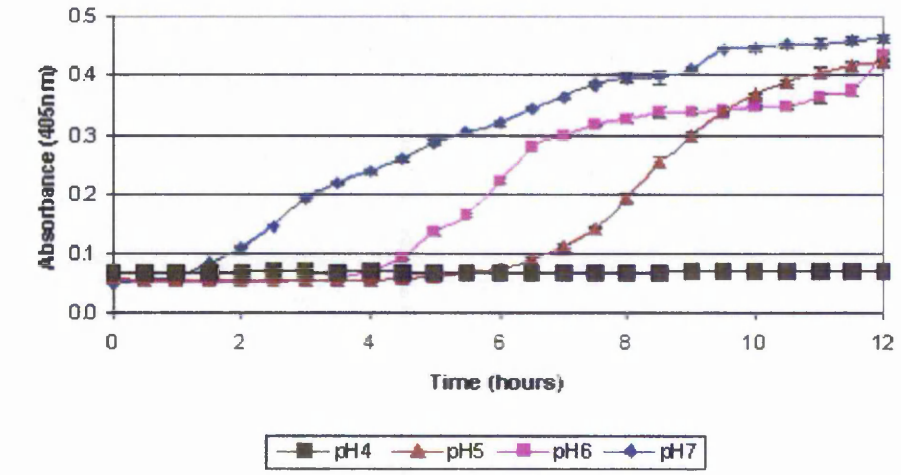
Growth dependent fluorescence behaviour of *E. sakazakii* sk90

E. sakazakii sk90 (pDEST® GFP) was grown under various pH conditions. Absorbance (405nm) was used for bacterial growth assessments and fluorescence measurement as previously described. All growth and fluorescent measurement

were in triplicate, and described in the following graphs with error bars to indicate standard deviation values.

E. sakazakii sk90 with GFP reporter

Figure 4.3.23: Growth dependent luminescence of *E. sakazakii* sk90 (pDEST® GFP) under acidified conditions.



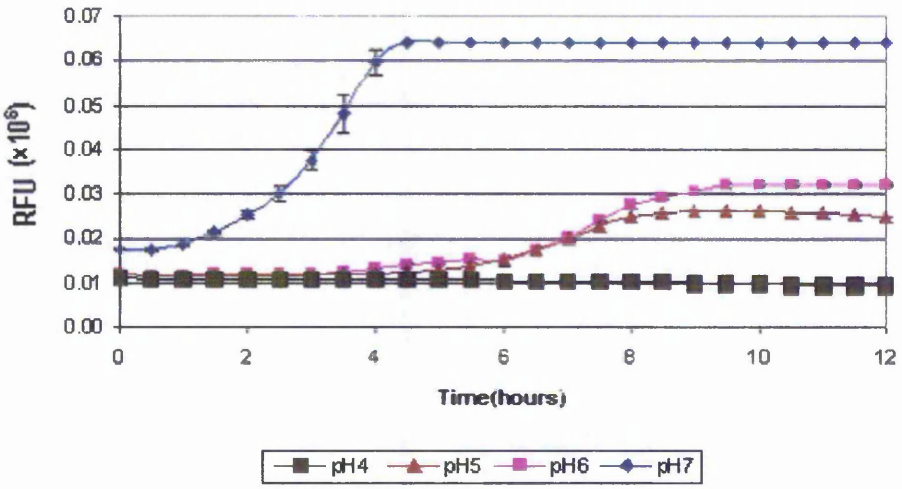


Figure 4.3.23 shows the growth and fluorescence behaviour of *E. sakazakii* sk90, (pDEST® GFP) assessed by fluorescence meter according to Materials and Methods section 4.2.5.

Figure 4.3.23 shows that the lag phase of *E. sakazakii* sk90 (pDEST® GFP) was extended under low pH conditions. The lag phase was: pH 7, 0.5 hours; pH 6, 4 hours; pH 5, 6 hours and at pH 4 there was no growth.

In contrast with the growth, *E. sakazakii* sk90 (pDEST® GFP) produced maximum fluorescence under neutral pH conditions. Whereas or increase in the acidity caused the fluorescence output to be reduced.

The growth of *E. sakazakii* sk90 with dual reporter system

Neither luminescence nor fluorescence were detected in the transformants *E. sakazakii* sk90 (pDEST® GFP-*lux*).

Figure 4.3.24: Growth of *E. sakazakii* sk90 (pDEST® GFP-*lux*) under acidified conditions.

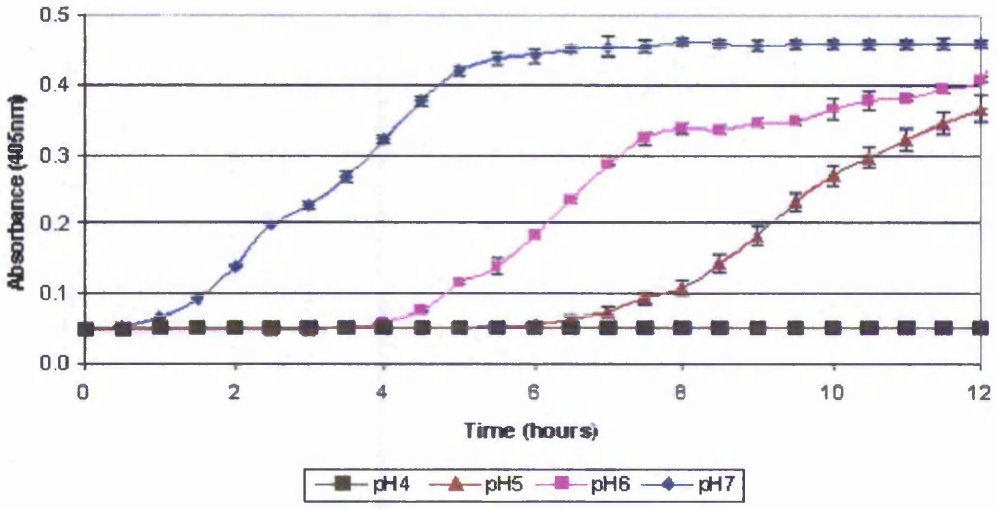


Figure 4.3.24 shows the growth of *E. sakazakii* sk90, (pDEST® GFP-*lux*) assessed by absorbance at 405nm according to Materials and Methods section 4.2.5.

Figure 4.3.24 shows that the lag phase of *E. sakazakii* sk90 (pDEST® GFP-*lux*) was prolonged at low pH conditions. The lag phase was: pH 7, 0.5 hours; pH 6, 4 hours; pH 5, 5.5 hours and at pH 4 there was no growth.

4.3.9. Growth dependent luminescent and fluorescent (ribosomal protein promoter) behaviours of *C. koseri* jb62 under acidified conditions

Luminescent and fluorescent reporter operons (GFP-*lux* dual reporter, *lux* and GFP operon) driven by a ribosomal protein promoter rpsJ, and transcriptional terminator, were separately inserted into the plasmid pDEST® and then transformed into the clinical isolates *C. koseri* jb62. These transformants were grown in ampicillin-contained media. All the transformants were able to phenotypically show reporter genes, except the dual reporter transformant *C. koseri* jb62 (pDEST® GFP-*lux*), which was able to produce luminescence, but no fluorescence was detected.

These transformants were inoculated into ampicillin broth at different pH values, (pH at 7, 6, 5 and 4). Protein expression and metabolism under various pH conditions then were assessed using fluorescence and bioluminescence measurement.

As previously described absorbance at 405nm was used for bacterial growth assessments. The luminescent and fluorescent behaviours of bacteria are presented as previously described.

C. koseri jb62 with dual reporter system

C. koseri jb62 (pDEST® GFP-*lux*) were grown in various pH conditions, absorbance (405nm) was used for bacterial growth assessments and luminescent measurement as previously described. All growth and luminescent assessments were performed in triplicate and error bars indicate standard deviation values:

Figure 4.3.25: Growth dependent luminescence of *C. koseri* jb62 (pDEST® GFP-*lux*) under acidified conditions.

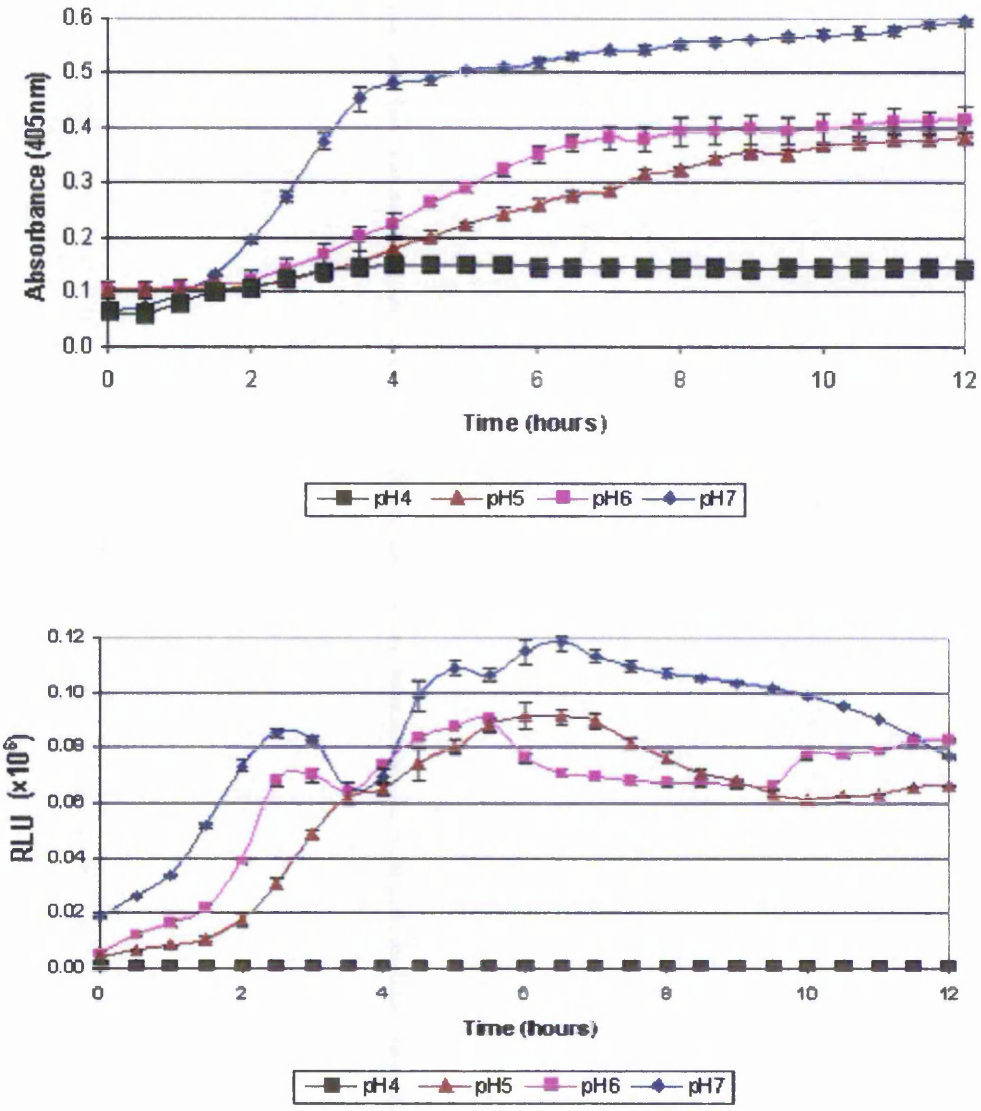


Figure 4.3.25 shows the growth and luminescence behaviour of *C. koseri* jb62, (pDEST® GFP-*lux*) assessed by luminometer according to Materials and Methods section 4.2.5.

Figure 4.3.25 shows that the lag phase of *C. koseri* jb62 (pDEST® GFP-*lux*) was extended in low pH conditions. The lag phase was pH 7, 0.5 hours; pH 6, 2 hours; pH 5, 2.5 hours and at pH 4 there was no growth.

C. koseri jb62 (pDEST® GFP-*lux*) produced maximum luminescence under neutral pH conditions. Whereas an increase in the acidity caused the luminescence output to be decreased.

Figure 4.3.26: biomass dependent luminescent behaviour of *C. koseri* jb62 (pDEST® GFP-*lux*) under acidified conditions.

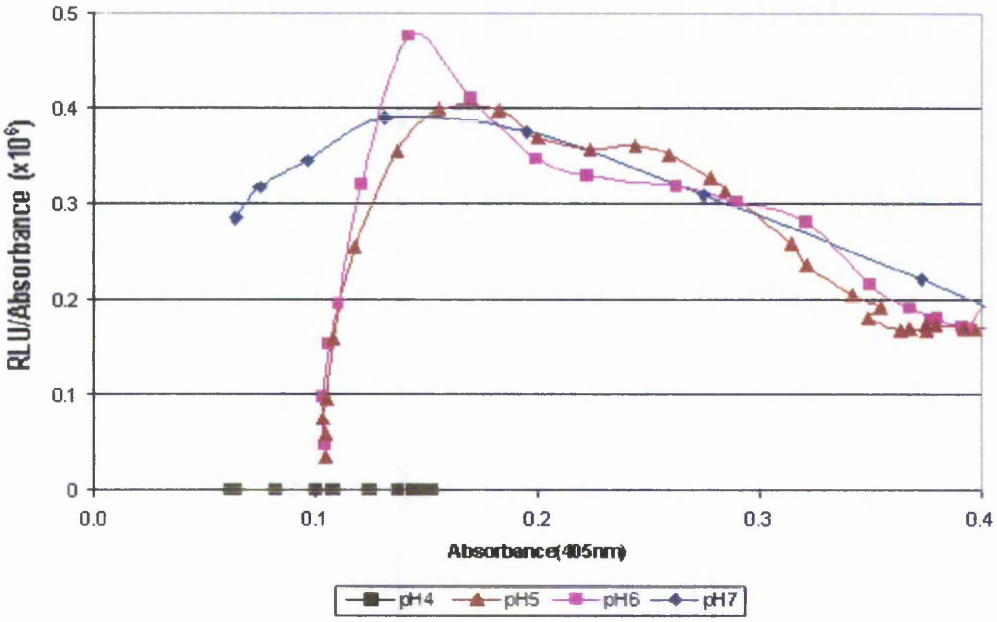


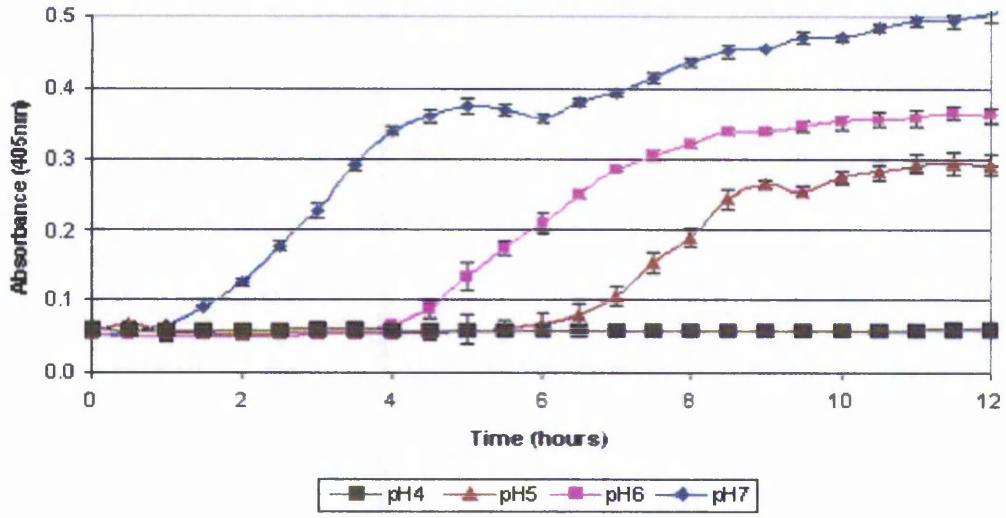
Figure 4.3.26 shows the growth and luminescence behaviour of *C. koseri* jb62, (pDEST® GFP-*lux*) assessed by luminometer according to Materials and Methods section 4.2.5.

The luminescent output of *C. koseri* jb62 (pDEST® GFP-*lux*) was greatest under neutral pH conditions. Less luminescence per unit of biomass was produced at pH6 and 5, except at low biomass values (absorbance <0.1 units).

C. koseri jb62 *lux* reporter

C. koseri jb62 (pDEST® *lux*) were grown at various pH conditions. Absorbance (405nm) was used for bacterial growth assessments and luminescent measurement as previously described. Every growth and luminescent ameasurement was performed in triplicate and shown in the following graphs with error bars to indicate standard deviation values.

Figure 4.3.27: Growth dependent luminescence of *C. koseri* jb62 (pDEST® *lux*) under acidified conditions.



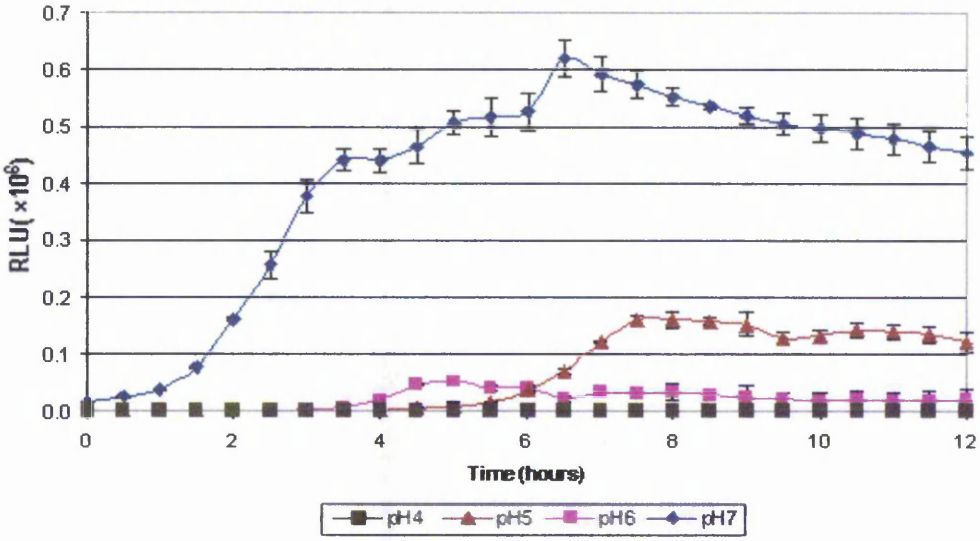


Figure 4.3.27 shows the growth and luminescence behaviour of *C. koseri* jb62, (pDEST® *lux*) assessed by luminometer according to Materials and Methods section 4.2.5.

Figure 4.3.27 shows that the lag phase of *C. koseri* jb62 (pDEST® *lux*) was extended in low pH conditions. The lag phase was pH 7, 1 hours; pH 6, 4 hours; pH 5, 6 hours and at pH 4 the bacteria never grew.

In contrast with the growth, *C. koseri* jb62 (pDEST® *lux*) produced maximum luminescence under neutral pH conditions. Whereas an increase in the acidity caused the luminescence output to be delayed.

Figure 4.3.28: biomass dependent luminescent behaviour of *C. koseri* jb62 (pDEST® *lux*) under acidified conditions.

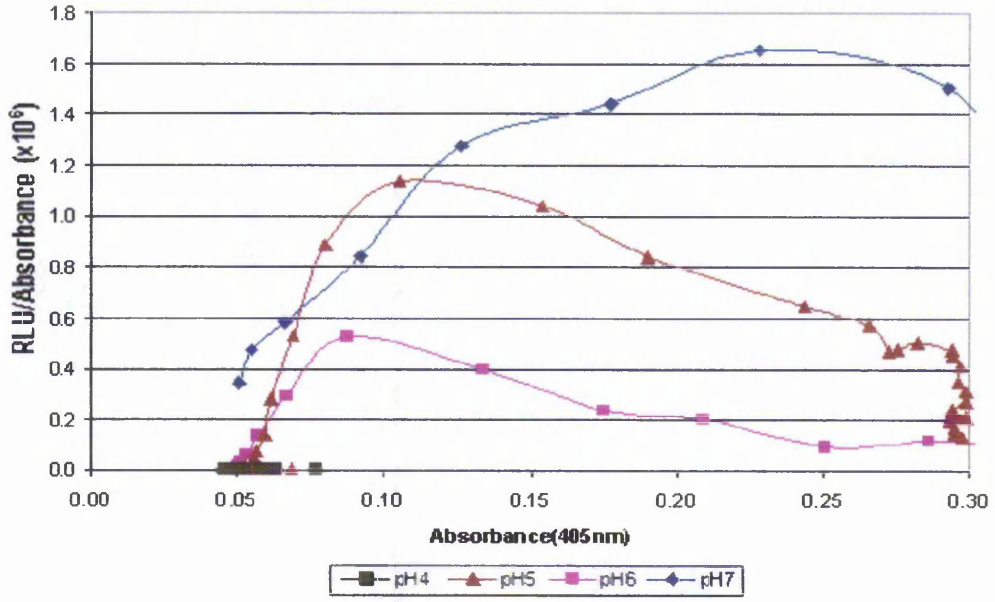


Figure 4.3.28 shows the growth and luminescence behaviour of *C. koseri* jb62, (pDEST® GFP-*lux*) assessed by luminometer according to Materials and Methods section 4.2.5.

The luminescent output of *C. koseri* jb62 (pDEST® *lux*) was greatest under neutral pH conditions. Less luminescence per unit of biomass was produced at pH6 and 5, except at low biomass values (absorbance <0.1 units).

C. koseri jb62 with GFP reporter

C. koseri jb62 (pDEST® GFP) was grown in various pH conditions, absorbance (405nm) was used for bacterial growth assessments and luminescent measurement as previously described. Every growth and luminescent measurement was performed in triplicate, and shown in the following graphs with error bars to indicate standard deviation values:

Figure 4.3.29: Growth dependent fluorescence of *C. koseri* jb62 (pDEST® GFP) under acidified conditions.

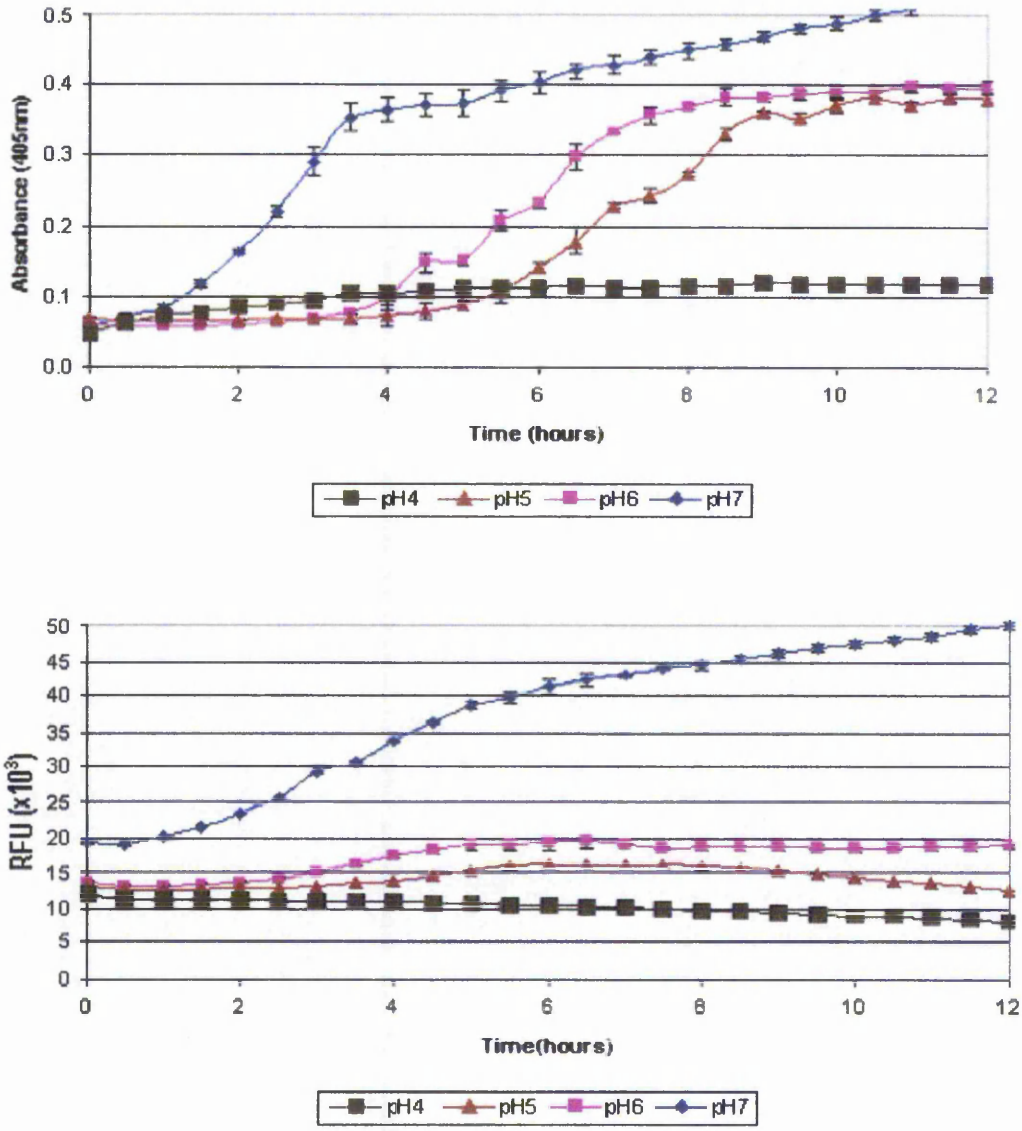


Figure 4.3.29 shows the growth and fluorescence behaviour of *C. koseri* jb62, (pDEST® GFP) assessed by fluorescence meter according to Materials and Methods section 4.2.5.

Figure 4.3.29 shows that the lag phase of *C. koseri* jb62 (pDEST® GFP) was extended respectively in low pH conditions. The lag phase was pH 7, 1 hour; pH 6, 3.5 hours; pH 5, 4.5 hours and at pH 4 the bacteria never grew.

In contrast with the growth, *C. koseri* jb62 (pDEST® GFP) produced maximum fluorescence under neutral pH conditions. Whereas increased acidity caused the luminescence output to be decreased.

4.4 Discussion

4.4.1. Identification of *E. sakazakii*

Epidemiological investigations are reliant upon the accurate identification of bacteria from clinical, food and environmental specimens and assist in the diagnosis of disease. Therefore determining the relatedness of isolates has become increasingly important, as for example in outbreaks of *E. sakazakii*. *E. sakazakii* infection can be life threatening, and is one of a number of nosocomial pathogens that can infect the most vulnerable individuals such as premature babies and senior patients.

In this study, genomic DNA of *E. sakazakii* and related organisms was extracted to study their phylogenetic relationship. Three PCR methods (tDNA, RAPD, ITS) offered potential reliable identification and discrimination methods for these bacteria. These PCR methods differentiated *Enterobacter* and *Citrobacter* species at the strain level and the banding pattern indicated their relatedness.

tDNA-PCR

PCR based on intergenic length polymorphism, termed as tDNA-PCR. The rationale of the tRNA intergenic length polymorphism molecular strategy for taxonomic studies relies on the observation that bacterial tRNA genes contain sequence motifs that exhibit a high level of phylogenetic conservation, while tRNA intergenic regions exhibit a higher degree of variation (Welsh *et al.* 1991).

Indeed, the PCR fingerprints of tRNA intergenic regions generated by using consensus tRNA gene primers generally produce species-specific patterns which have been successfully used to discriminate species belonging to the same genus, as reported for *Acinetobacter* sp., *Streptococcus* sp., *Staphylococcus* sp., and *Enterobacter* sp. (Ehrenstein *et al.* 1996, Maes *et al.* 1997, De Gheldre *et al.* 1999,

and Clementino *et al.* 2001). Moreover, application of tDNA-PCR analysis to strains validly placed in the same species has also allowed discrimination of strain clusters showing distinct PCR fingerprints, as demonstrated for *Enterobacter cloacae, Bacillus stearothermophilus*, and *Bacillus licheniformis* (Borin, *et al.* 1997, Daffonchio *et al.* 1998, Clementino *et al.* 2001).

However, in this investigation, it was noticed that many strains, which phenotypically have been assigned as *C. koseri,* showed similar tRNA intergenic regions to *Enterobacter* strains, in particular *E. cloacae.*

On the other hand, it was also discovered that in contrast with other *E. sakazakii* strains, 825 and London B (figure 4.3.1 lane 2 and 5) were not closely related to the *E. sakazakii* type strain (figure 4.3.1 lane 3). This may suggest that the strains London B and 825 exhibit higher degrees of variation in the tRNA intergenic regions than the *E. sakazakii* reference strain (type strain 822).

It should be noted that *E. sakazakii* 825 was isolated with FDA suggested methods, confirmed by biochemical profile (API) test (data not shown), and its phenotype is close to the *E. sakazakii* type strain. However phylogenetic analysis based on 16S-rRNA sequence (data not shown) also separates the *E. sakazakii* type strain cluster and strain 825. Strain London B was also special, in contrast with other *E. sakazakii* and *C. koseri* strains, London B was resistant to mini-Tn5 transposon insertion during construction of *lux*-modified *E. sakazakii.*

Clinical *E. sakazakii* isolates sk90, London A and London B (figure 4.3.1 lane 4, 5 and 6) produced extra 800 and 600bp bands compare with the *E. sakazakii* type strain 822. However, there was no evidence link these DNA bands and their pathogenesis.

ITS-PCR

Intergenic transcribed spacer regions (ITS) between the 16S and 23S rRNA genes have received a great deal of attention for strain typing, since such regions have greater polymorphism than adjacent genes (Jenson *et al.* 1993 and Gürtler *et al.*

1996). Furthermore, because of the increasing number of ITS sequences that are being deposited in databases, a database specific for ITS, the Ribosomal Internal Spacer Sequence Collection (RISSC, http://ulise.umh.es/RISSC/), has been developed (Garcia-Martinez *et al.* 2001). Polymorphism in ITS is quite often due to the presence of tRNA genes, since such genes are responsible for interoperonic and intrastrain length and sequence polymorphisms (Gürtler 1999). This polymorphism can be used for discriminating closely related strains in species that harbour multiple ribosomal operons that differ in length and sequence (Jensen *et al.* 1996 and Baudart *et al.* 2000).

In this study, it was found that the strains had considerable polymorphism of the intergenic transcribed spacer regions. All *E. sakazakii* (figure 4.3.2 lane 2-7) produced a similar 500bp band, which was different from t-DNA PCR profiles, the type strain 822 ITS profile was similar to *E. sakazakii* 825. While the clinical *E. sakazakii* strains sk90, London A and London B (figure 4.3.2 lane 4, 5 and 6) and baby food isolate BF006BB (figure 4.3.2 lane 7) produced relative weak 500bp band compared with *E. sakazakii* type strain 822.

E. cloacae NTU 831 OCC641 (figure 4.3.2 lane 8) also produced a 500bp band as the *E. sakazakii* strains. While all other *E. cloacae* and *C. koseri* strains produced a 600bp instead (figure 4.3.2 lane 9 and 11-19). In addition, *E. cloacae* NTU 832 (figure 4.3.2 lane 10) showed different DNA bands to other strains, as it produced extra 450 and 650bp bands.

RAPD-PCR

RAPD-PCR fingerprinting based on polymorphism of whole genomes, it has been used successfully to differentiate species and strains belonging to the genus *Enterobacter* using oligonucleotide primers having arbitrary sequences (Clementino *et al.* 2001). In this study, the primer (RPAD 1) was used under low-stringency conditions to amplify the genomic DNA of *E. sakazakii* and related organism.

The RAPD PCR patterns did not distinguish many *E. cloacae* and *C. koseri* strains from *E. sakazakii*. Previously Farmer *et al.* (1980) studied DNA-DNA hybridisation of these species, the study showed that the *E. cloacae* and *C. koseri* hybridisation value with *E. sakazakii* was 51%, only 40% for the *C. freundii*. Under the PCR condition of low stringency strength and short PCR primer, the results suggest that the RAPD PCR band patterns showed *E. cloacae* and *C. koseri* and *E. sakazakii* have highly conserved polymorphism of whole genomic DNA.

The different genetic analysis methods show different typing results. T-DNA, RPAD and ITS-PCR were based on polymorphism of t-RNA intergenic spacer, the 16s-23s internal transcribed spacer and genomic DNA of the bacteria respectively. The disagreement of phenotypes and genotypes of these species seem to be more heterogeneous than previously believed. The identification methods currently being used need to be reviewed for their epidemiological value (i.e. virulence of the bacteria). Also the results have implications for the reliability of clinical data due to misidentification of the neonatal infections.

4.4.2. Genetics of antibiotic susceptibility

E sakazakii is described as more susceptible to antibiotics than any other *Enterobacter* species (Dennison *et al.* 2002). Historically Muytjens *et al.* (1986) conducted comparative *in vitro* susceptibility testing of *Enterobacter* species, including 195 isolates of *E sakazakii*. Their work revealed that *E. sakazakii* was susceptible to all agents tested except cephalothin and sulfamethoxazole. *E. sakazakii* was the only *Enterobacter* species tested that was sensitive to ampicillin. Subsequently Willis *et al.* (1988) suggested the combined use of ampicillin and gentamicin for treatment of *E. sakazakii* meningitis in neonates. However Lai in 2001 described four adult *E. sakazakii* infections resistant to ampicillin. In addition, Hawkins *et al.* (1991) reported *E. sakazakii* was resistant to cephalothin. During 1987-1991, antibiotic resistance strain of *E sakazakii* increased. It is now classified among those Gram-negative bacteria most likely to produce β-lactamases capable of inactivating broad-spectrum penicillins and cephalosporins (Lai 2001).

Some *Enterobacter* strains have developed broad-spectrum resistance to the newer β lactam antibiotics in part due to increased use of cephalosporin in hospitals. The mechanism of antibiotic-resistance of *Enterobacter* species may be because of the production of inactivation enzymes, and may be encoded on the bacterial chromosome (Korfmann *et al.* 1988). Nevertheless, how these antibiotic resistance genes are mobilised unknown.

This study indicates that *E. sakazakii* and *C. koseri* strains can become ampicillin and kanamycin resistance through inter-species conjugation.

The suicide transposon containing kanamycin and ampicillin resistance genes was from a conjugative *E. coli* s-17 donor strain. When the donor strain was mated with *E. sakazakii* and *C. koseri*, they become antibiotic resistant. As previously described, *E. sakazakii* and related organisms are widely distributed, and it is widely believed that the inter-species conjugation phenomenon can happen outside of the laboratory. Therefore conjugation easily offers the bacteria the opportunity to acquire these antibiotic resistance genes.

I also proved that *E. sakazakii* and *C. koseri* could host various plasmids, which may contain antibiotic resistance genes. In this study, I used commercially available plasmids including pDEST® to modify *E. sakazakii* and *C. koseri*. The plasmid was originally isolated with origin of replication in *E. coli*. I have seen that these plasmids remained stable and most of the antibiotic resistance genes were expressed.

In addition, it was noticeable in this study that the ampicillin sensitivity was strain specific. *E. sakazakii* sk90 (pDEST® *lux*) showed extremely slow growth rate (3-4 days) in ampicillin-contained culture media. While other similar transformants of *E. sakazakii* sk90 needed only a few hours to initial the growth.

4.4.3. Bioluminescent mutants constructed by transposon mutagenesis

The suicide transposon, encoding kanamycin resistance gene and full *lux* operon, was randomly inserted into the *E. sakazakii* and *C. koseri* genome, and offers many opportunities for further studies. It is possible that the transposon insertion would result in a mutated gene, which may play important role in housekeeping activity in the bacteria. Also, the transposon might insert downstream of any promoter, may be inactivated and hence affect the gene expression activity under different growth environment.

After the conjugative transposon mutation of *E. sakazakii* and *C. koseri* strains, several mutant libraries were produced. These mutants were all kanamycin resistance strains, and mean that the transposon was active under a promoter. However, due to large variations of the activity of the promoter, less than 1/20 of the mutated strains showed detectable bioluminescence, and only 1/50 of the mutated strains showed sufficient bioluminescence for viability test.

These mutant strains showed irregular bioluminescent behaviour. The different luminescent intensity was primarily due to the differences of the promoter activity. Since the promoter controlling the bioluminescence was random; the activities of promoter would be affected by various conditions including nutrient abundance, environmental stress, pH, temperature. It was evident that, the bioluminescence behaviour of majority of the mutants was not a quorum sensing associated behaviour.

Nevertheless, the primary goal to construct bioluminescent mutant *E. sakazakii* was to understand protein synthesis during recovery process from stressed conditions. The transposon approach was subsequently deemed not appropriate for this purpose. Consequently ribosomal protein promoters were considered as appropriate to construct bioluminescent mutants *E. sakazakii* and related strains.

4.4.4. Rehydrated bacteria growth and metabolism

The previous results section recorded the growth of *E. sakazakii* and *C. koseri* strains containing pDEST® plasmids. Figure 4.3.6 to Figure 4.3.17 illustrated that, in contrast with freshly inoculated bacteria, all of the rehydrated bacteria showed delays in growth. Therefore, it was concluded that rehydrated *E. sakazakii* and *C. koseri* were able to grow, though a few hours recovery were needed.

The recovery of growth from the desiccated condition will include several activities such as nutrient uptake and macromolecule synthesis, which are necessary for their virulence. Table 3.4.1 shows the time before the early log phase growth phase by the luminescent and fluorescent bacteria, as modified by pDEST® plasmids.

Table 4.4.1: Time for rehydrated pDEST® plasmids transformants to enter early log growth phase.

Bacteria strains		Log phase (hours)		Delay (hours)
		Fresh	Rehydrated	
E. sakazakii London A	GFP-*lux*	1	3.5	2.5
	GFP	1.5	5.5	4
	lux	1	3.5	2.5
E. sakazakii SK90	GFP-*lux*	1	4.5	3.5
	GFP	1	4.5	3.5
	lux	2-3days	2-3days	Not detectable
C. koseri JB62	GFP-*lux*	1	1.5	1
	GFP	0.5	2.5	2
	lux	0.5	3.5	3

Table 4.4.1 shows the length of log phase required for luminescent and fluorescent transformants. It was summarised from Result section 4.3.4-4.3.6.

It was noticed that the same bacteria strain with different plasmids showed various log phases. For example, *C. koseri* JB62 with plasmid pDEST® GFP-*lux* reporter required 1 (for fresh culture) and 1.5 (for rehydrated culture) hours to enter early log growth phase, while with GFP reporter plasmid the values were 0.5 and 2.5 hours respectively. These delays could be due to two reasons:

Firstly, the reporter protein synthesis interfered with housekeeping gene expression. Protein synthesis requires energy and nutrient resources to synthesize and, if necessary, transport reporter proteins. Also, as the pDEST® reporter

plasmids were induced by ribosomal protein promoter, the regulation of the reporter gene was either at the transcription or translation level. Consequently, the expression of the reporter proteins competed for gene regulation resources such as DNA binding proteins and ribosomal binding. For a more detailed discussion please refer section 4.4.6. Additionally, further energy is needed to support the luminescent reaction, of the luciferase activity.

As the experiments were performed in batch culture, bacteria have to balance their energy and nutrient requirement, between reporter genes and their housekeeping genes. The latter are important for both basic metabolism as well as cell proliferation. Alteration in gene expression and metabolism would significantly change the length of the lag phase.

Secondly, the plasmid nature and the antibiotic resistance may change the growth of the bacteria. The antibiotic sensitive profiles show that *E. sakazakii* sk90 was the strain most sensitive to ampicillin compare with other *E. sakazakii* strains. *E. sakazakii* sk90 pDEST® *lux* reporter did not show detectable growth in ampicillin contained media within 24 hours. While other sk90 strains seems grow well in the ampicillin media. The problem was not the plasmid stability because it was stable in other strains, not in the plasmid promoter because it works well on GFP and dual operons, neither was it the *lux*CDABE operon because it works well in other transformants luminescent sk90 strains.

The precise reasons fore the growth curve variations remain unknown, and was considered out of the scope of this study.

4.4.5. Bacterial growth and metabolism under acidic stress

The growth and expression of reporter genes in *E. sakazakii* and *C. koseri* containing pDEST® was followed under acidic conditions. Figure 4.3.18 to Figure 4.3.29 show the bacterial growth curves from neutral to pH 4. It was evidence that the *E. sakazakii* and related organisms can resist growth conditions down to pH4

with longer recovery time under low pH condition. As previously described rehydrated cells had prolonged lag phases to adapt to acidic conditions.

The bacteria took a few hours to adapt to acidic conditions, and cells were then actively proliferated entered the early log phase. Table 3.4.2 lists the time required to enter early log growth phase by the luminescent and fluorescent bacteria, as modified by pDEST® plasmids.

Table 4.4.2: Lag time (h) of *E. sakazakii* and *C. koseri* pDEST® transformants.

Bacteria strains		pH7	pH6	pH5	pH4
E. sakazakii London A	GFP-*lux*	1.5	4.5	7	>18
	GFP	2.5	4.5	7	>18
	lux	1.5	3.5	6	>18
E. sakazakii SK90	GFP-*lux*	0.5	4	5.5	>18
	GFP	0.5	4	6	>18
	lux	>24	>24	>24	>24
C. koseri JB62	GFP-*lux*	0.5	2	2.5	>18
	GFP	1	4	6	>18
	lux	1	3.5	4.5	>18

Table 4.4.2 shows the length of log phase required for luminescent and fluorescent transformants. It was summarised from Result section 4.3.7-4.3.9.

It is apparent that as occurred with desiccated cells, under acidic conditions, the same bacteria strain with pDEST® and different reporters showed considerable variation in the lag phase. Again, I believe that this was mainly due to the resources drain caused by the reporter genes, as well as dynamic interactions of antibiotics and plasmids, (section 4.4.4).

4.4.6. Bioluminescence behaviour driven by rpsJ

All organisms have evolved mechanisms to regulate ribosome synthesis so that rapidly dividing cells dedicate a larger fraction of their mass and energy to manufacturing ribosomes than do more slowly growing cells.

The regulation of ribosomal protein synthesis is best understood in *E. coli.* However it is reasonable to believe that the regulation is similar to other bacteria including *E. sakazakii* and *C. koseri.* In *E. coli,* ribosomal proteins are encoded in the chromosomes in multigene operons that facilitate production of the more than 50 ribosomal proteins. Most ribosomal protein operons contain a gene whose product functions not only as a ribosomal protein but also as a repressor of the expression of the operon. The ribosomal proteins and rRNA are synthesised independently and then assembled into mature ribosomal. Nevertheless, there is never an excess of either free ribosomal proteins or free rRNA in the cell, suggesting that synthesis is somehow coordinated. Possiblly the rate of ribosomal protein synthesis is adjusted to match the rate of rRNA synthesis.

Ribosomal protein L4 from *E. coli* specifically regulates the S10 operon, which codes for 11 r-proteins including L4 itself and S10. When ribosomal protein S10 production exceeds rRNA synthesis, free regulatory ribosomal proteins L4 accumulate and each represses expression of its own operon, usually by inhibiting translation of the S10 operon mRNA.

This autogenous control mechanism is believed to coordinate the production of rRNA and ribosomal protein and to balance the expression of individual ribosomal protein operons.

Regulation of rRNA synthesis depends on the global regulatory nucleotide ppGpp. The ppGpp accumulates to different cellular levels at amino acid deprivation or during different growth rates. It changes the activity of RNA polymerase to transcribe from rRNA promoters.

In this study, several reporter plasmids were used to monitor the ribosomal protein synthesis activity shown as bioluminescence and fluorescence. The *lux* and GFP was under control of an S10 promoter. In another words, the activity of the reporter gene (shown as bioluminescence and fluorescence) was controlled by overall metabolism. This indicated ribosomal synthesis, and therefore protein synthesis as required for virulence.

S10 promoter was encoded in a plasmid, which when the nutrient level is high (i.e. more free radical amino acid acquired), the global regulatory nucleotide ppGpp is stimulated. This results in up regulated rRNA synthesis. Subsequently, the S10 promoter in *E. sakazakii* and *C. koseri* genome will be activated. The S10 promoter (rpsJ) encoded on the pDEST® plasmid would also be up regulated; the *E. sakazakii* and *C. koseri* cells would show luminescent and fluorescent.

The tandem T1T2 transcription terminator, derived from the rrnB ribosomal RNA operon of *E. coli*, is an efficient and commonly used transcription terminator.

I noticed in this investigation, that the ribosomal promoter is active in early growth phase, and the activity decreased in later log phase. The ribosomal protein promoter is not under a quorum sensing associated response.

Following rehydration of desiccated cells, the recovery of protein transcription is quicker than cell proliferation (refer to table 4.4.1).

4.4.7. Bioluminescence and fluorescence behaviour of rehydrated bacteria

There was an obvious retardation of growth, luminescence and fluorescence induction of desiccated bacteria following rehydration. Nevertheless the ribosomal protein promoter driven luminescence and fluorescence behaviour indicated overall protein synthesis activity during the bacterial recovery process.

The bioluminescence and fluorescence activity depends on several activities:

Primarily, the luminescence and fluorescence depends on the expression of bacterial luciferases or the green fluorescence protein respectively. Which were linked to the ribosomal protein promoter. In addition to the promoter, availability of substrates for the luciferase also determined the activity of bioluminescence reaction. The abundance of reduced flavin mononucleotide, long chain aliphatic aldehyde and oxygen are co-factors affecting the bioluminescence reaction. It should be noted that all our bioluminescence assessments were performed with bacterial batch cultures. All the metabolites required as substrates for the bioluminescence reaction were limited, and hard to quantify. However, there was every reason to believe that these luciferase substrates were in very low level after late log phase. Consequently, it was concluded that the induction of the bioluminescence indicated initial active metabolism activity.

4.4.8. Ribosomal protein promoter approach to monitoring cellular metabolism

Protein secretion is a universal process of fundamental importance for various aspects of cell physiology including the infection of a host organism by a bacterial pathogen. Many Gram-negative pathogens export virulence-associated proteins across one or two cell membranes to their place of action using a wide plethora of secretory pathways with the objective of infecting the host.

Protein secretion in bacteria is a remarkably complex biological process that includes substrate recognition followed by delivery of the substrate across the membrane barrier. In Gram-negative bacteria, proteins have to cross both the cytoplasmic membrane and outer membrane during their journey across the bacterial cell envelope in order to reach the extracellular environment. To achieve export of virulence factors such as pili, degradative enzymes, adhesins and toxins, bacteria have evolved numerous complex multimeric transporter systems.

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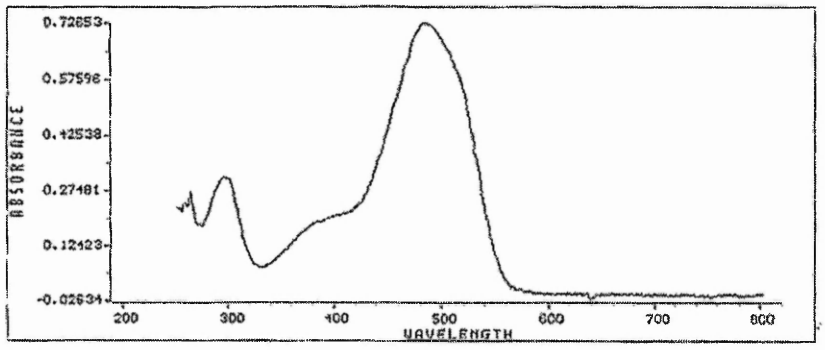
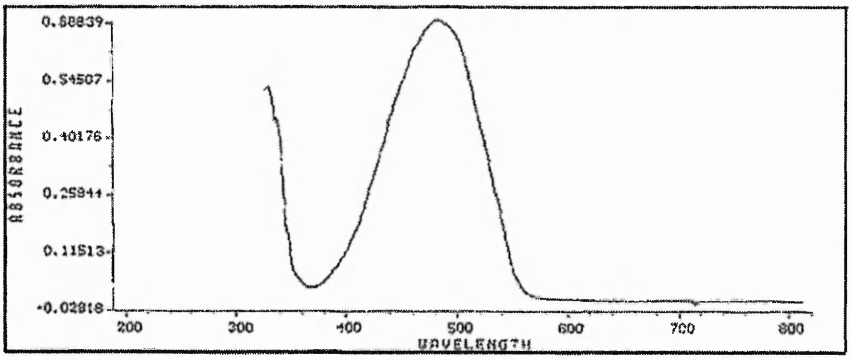
Appendix

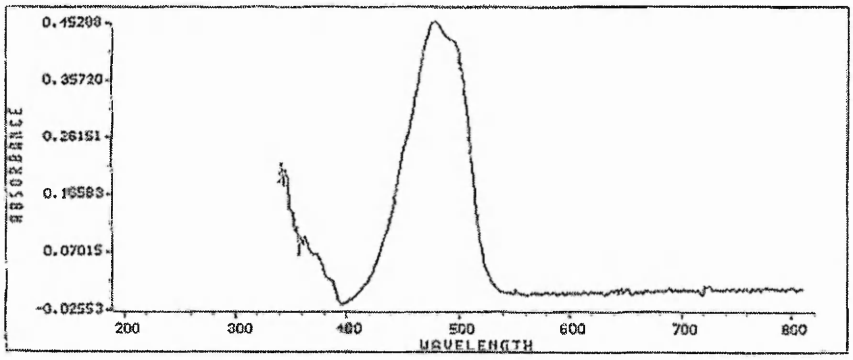
The maximum absorbance value (λ_{max}=494nm) of Acid Red 183 was determined by using scanning spectrometer (HP, Ltd.) according to Materials and Methods section: 2.2.2. Calibration curves were then constructed by measuring maximum absorbance values of each dye at serial dilutions (Results section 2.3.1).



The maximum absorbance value (λ_{max}=480nm) of Orange II was determined by using scanning spectrometer (HP, Ltd.) according to Materials and Methods section: 2.2.2. Calibration curves were then constructed by measuring maximum absorbance values of each dye at serial dilutions (Results section 2.3.1).



The maximum absorbance value (λ_{max}=486nm) of Sunset Yellow was determined by using scanning spectrometer (HP, Ltd.) according to Materials and Methods section: 2.2.2. Calibration curves were then constructed by measuring maximum absorbance values of each dye at serial dilutions (Results section 2.3.1).



The maximum absorbance value (λ_{max}=597nm) of Reactive Black 5 was determined by using scanning spectrometer (HP, Ltd.) according to Materials and Methods section: 2.2.2. Calibration curves were then constructed by measuring maximum absorbance values of each dye at serial dilutions (Results section 2.3.1).