Development and evaluation of an alkane bioconversion process using genetically modified *Escherichia coli*

by

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ABSTRACT

Alkanes can be used as an inexpensive feedstock to produce more valuable alcohols. The biotransformation of alkanes to alcohols provides an alternative to conventional chemical procedures.

The scope of this research was to develop a process utilising a biocatalyst to catalyse the oxidation of an alkane to its corresponding alcohol on a larger scale than had been reported on in previous research. The research utilised a recombinant *E. coli* BL21(DE3) cell, containing the CYP153A6 operon in pET 28 vector, as the biocatalyst. The CYP153A6 enzyme catalyses the oxidation of octane to 1-octanol. The principle objective of the research was to determine the amount of 1-octanol that can be produced by a system utilising this strain of recombinant *E. coli* as a biocatalyst on a three orders of magnitude larger scale than what had previously been reported on for this reaction system. An additional objective was to model the 1-octanol production performance in the bioreactor.

Bioconversion batch reactions, with excess octane used as a substrate, were conducted in 30ml McCartney bottles and in a 7.5L BioFlo 110 Modular Benchtop Fermentor (New Brunswick). The McCartney bottles were not equipped to actively control process conditions.The bioreactor was equipped to control process conditions such as temperature, pH and dissolved oxygen concentration. Experiments in the bioreactor were therefore described as being performed under controlled conditions. The procedures used to grow, maintain and harvest the biocatalyst cells were based on those developed by the Department of Microbial, Biochemical and Food Biotechnology at the University of the Free State. The product and substrate concentrations were determined through gas chromatography (GC) analysis.

The McCartney bottle bioconversion reactions, with a 1.33ml reaction volume, produced 1.88 mg 1-octanol per gram of dry cell weight per hour. The bioreactor under controlled conditions, with a 2L reaction volume, produced 14.89 mg 1-octanol per gram of dry cell weight per hour. The formation of a secondary product, octanoic acid, was observed for the bioreactor under controlled conditions experiment at a production of 1.12 mg per gram of dry cell weight per hour. The McCartney bottle experiments did not produce any by-products.

The 1-octanol production performance in the bioreactor experiments was empirically modelled. The empirical rate law was based on the form of the Monod equation, with the addition of a product inhibition term. The model achieved an average Root Mean Square Error of less than

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5% when compared to experimental data, and was therefore concluded to be accurate within the range of experimental data and conditions tested for.

The principal finding of the research is that the cells produced an order of magnitude more product in the bioreactor than in the McCartney bottles. The literature on this reaction system, however, reports only on smaller scale research than that performed in the bioreactor. The improved production results in the bioreactor therefore give the first insight into the potential that this technology has for being scaled up.

Of equal significance is the finding that a secondary product developed during the biotransformations performed in the bioreactor. This refutes the assumption that the biocatalyst cells are unable to catalyse any secondary reactions. This aspect of the cells' performance must be addressed before the biocatalyst cell strain can be considered to be a viable option for utilisation in large-scale processes.

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OPSOMMING

Alkane kan gebruik word as ‘n bekostigbare bron om meer waardevolle alkohol te produseer. Die biotransformasie van alkane na alkohol bied dus ‘n alternatief vir konvensionele chemiese prosedure.

Die oogmerk en omvang van hierdie navorsing was om ‘n proses te ontwikkel waarin ‘n biokatalisator gebruik word om die oksidasie van ‘n alkaan tot sy ooreenstemmende alkohol te kataliseer, en om vas te stel hoeveel 1-oktanol vervaardig kan word deur ‘n herverenigde *E. coli* as katalisator gebruik. ‘n Rekombinante *E. coli* BL21(DE3) sel, wat die CYP153A6 operon in pET 28 vector bevat, is as biokatalisator gebruik. Die CYP153A6 ensiem kataliseer die oksidasie van oktaan na 1-oktanol.

Biokonversie lot-reaksies, met oormatige oktaan wat as substraat gebruik word, is in 30ml McCartney bottels en in 7.5L BioFlo 110 Modular Benchtop Fermentor (New Brunswick) uitgevoer. Die bioreaktor was toegerus om kondisies van die proses soos temperatuur, pH and opgeloste suurstof-konsentrasie te kontroleer. Die prosedures wat gebruik is om die groei, onderhoud en oes van die biokatalisator selle te bewerkstellig, is gebaseer op prosedures wat ontwikkel is deur the Department van Microbiese, Biochemiese and Voedsel Biotegnologie van die Universiteit van die Vrystaat. Die produk- en substraat-konsentrasies is vasgestel deur gas-chromatografie (GC) ontleding.

Die McCartney bottel biokonversie-reaksie met ‘n 1.33ml reaksie-volume het 1.88 mg 1-oktanol per gram droeë-sel gewig opgelewer. Die bioreaktor, wat onder beheerde toestande ‘n 2L reaksie-volume het, het 14.89 mg 1-octanol per gram droeë-sel gewig gelewer. Onder beheerde eksperimentele kondisies is die vorming van ‘n sekondere produk, oktanol-suur, by die bioreaktor waargeneem teen 1.23 mg per gram droeë-sel gewig per uur. Die McCartney bottel eksperimente egter het geen newe-produkte opgelewer nie.

Die ontwikkeling van die 1-oktanol in die bioreaktor-ekperimente is empiries gemodelleer. Die empiriese ‘rate law’ is gebaseer op ‘n vorm van die Monod- vergelyking, met byvoeging van ‘n produk-inhiberingsterm. Die model het ‘n gemiddelde vierkantswortel foutvariansie van minder as 5% opgelewer, vergeleke met die eksperimentele data, en word dus binne die rykwydte van die eksperimentele data, en die kondisies waarvoor getoets is, as akkuraat beskou.

Die belangrikste bevinding is dat die selle in die bioreaktor ‘n orde van grootte meer produk gelewer het as die selle in die McCartney bottels. Die literatuur oor hierdie reaksie-sisteem berig

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egter slegs oor kleiner skaalse navorsing as wat in die bioreaktor gedoen is. Die verbeterde opbrengsresultate van die bioreaktor dui daarop dat laasgenoemde tegnologie die potensiaal inhou om opgegradeer te word.

Die bevinding dat 'n sekondere produk in die biotransformasie in die bioreaktor gevorm het, is beduidend. Dit weerspreek die aanname dat die biokatalisator-selle nie sekondere reaksies kataliseer nie. Hierdie aspek moet aangespreek word alvorens die biokataliseer-selle oorweeg kan word as 'n lewensvatbare alternatief vir gebruik in grootskaalse prosesse.

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NOMENCLATURE

C_P	Product concentration
C_P^*	Maximum product concentration
C_S	Substrate concentration
E	Enzyme
E•S	Enzyme-substrate complex
K_1	Reaction constant
K_S	Monod reaction constant
P	Product
S	Substrate
μ	Specific growth rate
μ_{max}	Maximum specific growth rate

Abbreviations and acronyms

BR	Bioreactor
BRM	Bioconversion Reaction Mixture
CSTR	Continuous Stirred Tank Reactor
Da	Dalton
DCW	Dry Cell Weight
dO_2	Dissolved Oxygen
FID	Flame Ionisation Detector
GC	Gas Chromatography
GCMS	Gas Chromatography Mass Spectroscopy
IPTG	Isopropyl-β-D-thiogalactopyranoside
LB medium	Lysogeny Broth medium
LBRM	Litre Bioconversion Reaction Mixture
LPM	Litres Per Minute

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MBR	Membrane Bioreactor
MC	Main Culture
MGR	Membrane Gradostat Reactor
MWCO	Molecular Weight Cut-Off
OD	Optical Density
PCU	Primary Control Unit
PSSH	Pseudo-Steady-State Hypothesis
Std	Standard
Strain A	*E. coli* BL21(DE3)pLysE containing CYP153A6 operon in pET 28 vector
Strain B	*E. coli* BL21(DE3) containing CYP153A6 operon in pET 28 vector
RMSE	Root Mean Square Error
UFS	University of the Free State
VVM	Vessel Volumes per Minute
WCW	Wet Cell Weight
δALA	δ Aminolevulinic acid

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GLOSSARY

Growth incubation period	The period between the inoculation of the main culture and the addition of δ-ALA and $FeCl_3$
Induction incubation period	The period between the growth incubation period and the time at which the biocatalyst cells are harvested for bioconversion reactions
Inoculation	The addition of biocatalyst cells to a culture medium
Reaction mechanism	The sequence of reaction steps that describe the entire bioconversion process
Recombinant cell	A cell that has been genetically modified

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1. INTRODUCTION

1.1 Rationale

Enzymatic bioconversion processes are increasingly being used in the production and transformation of raw materials. These types of processes have found a wide range of applications in various fields, including the food industry, fine chemical synthesis and environmental applications (Rios *et al.*, 2004). The development of a technology for the production of commercially valuable chemicals from hydrophobic substrates such as alkanes presents a lucrative opportunity to utilise a relatively inexpensive feedstock. Fujii *et al.* (2006) states that region-specific oxidations of hydrocarbons have attracted much interest, because of the many valuable chemicals that can be produced from these types of reactions.

Alkanes are an inexpensive carbon feedstock that is often utilised as fuel to produce energy and low-value carbon oxides. A more efficient method of utilising this feedstock would be to produce more valuable alcohols through direct activation of the alkanes. Unfortunately, chemical conversions require an often expensive metal catalyst and reaction temperatures of 200°C - 600°C. Furthermore, the poor selectivity under these conditions and the inherently low reactivity of alkanes lead to poor production results (Ayala & Torres, 2004).

The biotransformation of alkanes to alcohols provides a promising alternative to conventional chemical procedures. The advantages of biological processes stem from the biocatalyst's ability to catalyse complex reactions at physiological conditions with high selectivity and the possibility of no side reactions (Ayala & Torres, 2004). Also, processes of this kind are often more environmentally friendly and safer than their chemical counterparts. Biotransformation processes therefore have the potential to work at lower production costs than comparative chemical processes, with the added benefit of being less harmful to the environment.

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1.2 Scope of the project

This project was conducted in combination with the Department of Microbial, Biochemical and Food Biotechnology at the University of the Free State (UFS) under the umbrella of the Paraffin Programme in the Centre of Excellence (CoE) in Catalysis. The purpose of this research project was to develop a bioconversion process to provide an innovative alternative for utilising an inexpensive hydrocarbon feedstock in an efficient manner.

The first step in developing the bioconversion process was to select an appropriate biocatalyst for the project. The UFS research team was responsible for the development of the two biocatalyst cell strains that this project was obliged to use to perform alkane hydroxylation bioconversion reactions. Cytochrome P450 is a class of oxidative enzyme, which is able to catalyse the oxidation of alkanes to their corresponding alcohols with a high degree of selectivity (Ayala & Torres, 2004). The recombinant *E. coli* cells, which were made available to the project by the UFS research team, were genetically modified to contain the CYP153A6 enzyme, a member of the microbial P450 family. The enzyme is capable of catalysing the oxidation of C6 to C11 alkanes with a regiospecificity of 95% for the terminal carbon position (Randall, 2010). A choice also had to be made as to which process options to implement: using whole cells as opposed to using enzymes, and which biocatalyst cell strain to select. The factors that were taken into account in the selection process are detailed in Section 2.2 of the Literature Review section.

After having selected an appropriate biocatalyst for the project, it was necessary to develop procedures to grow, maintain and harvest the biocatalyst cells. The procedures were adapted from Randall (2010). Experimental work was conducted in an effort to optimise some of the procedures, such as investigating the effect of the induction incubation period on the 1-octanol production performance.

The project aimed to reproduce the octane bioconversion results reported in Gudiminchi *et al.* (2012), who used the same biocatalyst cell as the one chosen for this project, to perform octane bioconversion reactions in 40ml amber vials with a reaction volume of 1.25ml – 1.33ml. For this purpose, octane bioconversion reactions with a 1.33ml reaction volume were performed in 30ml McCartney bottles. Thereafter, the reaction volume was scaled up by an order of magnitude and octane bioconversions were conducted in a 7.5 litre BioFlo 110 Modular Benchtop Fermentor (New Brunswick) setup. The motivation for using the New Brunswick bioreactor setup is detailed in Section 2.1 of the Literature Review.

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An additional project aim was to model the 1-octanol production performance in the bioreactor under controlled conditions, by theoretical or empirical means. The theoretical model was based on the cytochrome P450 reaction mechanism, while the empirical model was based on the results of octane bioconversion experiments performed in the bioreactor setup. The accuracy of the models was to be evaluated by performing a Root Mean Squared Error (RMSE) analysis.

The first six months of experimental work were conducted with a batch of *E. coli* BL21(DE3) biocatalyst cells that were made available at the start of the experimental phase of the project. The results of this experimental work are described in Section 4.1 to Section 4.3. A second batch of the same *E. coli* cell strain was made available six months into the experimental phase of the project. This provided an opportunity to test the reproducibility of the 1-octanol production between different batches of the *E. coli* BL21(DE3) strain.

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2. LITERATURE REVIEW

2.1 Background

The initial idea for this research was based on the Master's thesis findings of Charlene Randall of the Department of Microbial, Biochemical and Food Biotechnology at the University of the Free State. Randall (2010) successfully expressed the CYP153A6 operon in pET 28 vector in *E. coli* BL21(DE3)pLysE and performed whole cell bioconversions of octane with a reaction volume of 1.33ml. Although Randall (2010) performed octane bioconversions, the major focus of her thesis was on the expression of the CYP153A6 enzyme in the *E. coli* host cell. The bioconversions were used as a method of proving that the enzyme had successfully been expressed. Less research was undertaken to optimise the reaction for maximum 1-octanol production.

Rama K. Gudiminchi, a researcher at the same department as C. Randall, continued working and building on the research performed by Randall (2010). His research expressed the CYP153A6 operon in pET 28 vector in *E. coli* BL21(DE3)pLysE and in *E. coli* BL21(DE3). The focus of this research was on optimising the octane bioconversion reaction to achieve maximum 1-octanol production. To do this he performed octane bioconversion reactions in 40ml amber vials with a reaction volume of 1.25ml – 1.33ml. The findings of his research are published in Gudiminchi *et al.* (2012) and discussed in Section 4.6.

The UFS research team developed the two biocatalyst cell strains which were made available to this project. Their research intended to develop a resting recombinant *E. coli* cell which would essentially act like a bag of enzymes. The resting recombinant *E. coli* cell would therefore have no residual activity with respect to unwanted secondary metabolic reactions.

A major aim of the research for this current project was to reproduce the octane bioconversion results reported in Gudiminchi *et al.* (2012), and thereafter to scale up the reaction volume by an order of magnitude. The scaled-up octane bioconversions would be performed in a 7.5 litre BioFlo 110 Modular Benchtop Fermentor (New Brunswick) setup. An additional project aim was to model the 1-octanol production performance in the New Brunswick bioreactor setup, by theoretical or empirical means.

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One of the prerequisites to performing the experimental work detailed above was to develop procedures to grow, maintain and harvest the biocatalyst cells. The procedures were to be adapted from Randall (2010). There exists little documentation in literature on the optimisation of such procedures and experimental work would therefore be conducted in an effort to improve some of the procedures, such as investigating the effect of the induction incubation period on the 1-octanol production performance. The reproducibility of the 1-octanol production between different batches of the same strain was also investigated.

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2.2 Biocatalyst selection and considerations

Choosing the most appropriate biocatalyst to use in the project was essential to the research. This section details the factors that were taken into account in the selection process.

Cytochrome P450 is a class of oxidative enzyme which is able to catalyse the oxidation of alkanes to their corresponding alcohols with a high degree of selectivity. The enzyme uses NAD(P)H as a co-factor and the two-component system usually consists of a hydroxylase and a reductase (Ayala & Torres, 2004). Funhoff *et al.* (2007) states that the first CYP153 enzyme was discovered in *Acinetobacter* sp. EB104 (CYP153A1), after growth was observed on a minimal medium with hexadecane as the sole carbon source. The CYP153A1 enzyme was not expressed in the host unless the cells were grown in the presence of alkanes, biphenyl, indene and phenanthrene. Several additional alkane-degrading *Proteobacteria*, such as *Alcanivorax borkumensis* and *Mycobacterium paraffinicum*, were also tested for the presence of CYP153s. Subsequent cloning and expression studies of 11 genes resulted in the functional expression of seven CYP153s (CYP153A1-A7) in *Pseudomonas putida* GPo12. The enzymes CYP153A6 and CYP153A7 showed the ability to catalyse a broad range of substrates, ranging from medium-chain length aliphatic alkanes to more bulky cyclic compounds. The very low dissociation constants for medium-chain length alkanes suggested that these are their natural substrates, indicating that alkane hydroxylation is the physiological function of CYP153A6 and CYP153A7 (Funhoff *et al.*, 2007).

The CYP153A6 enzyme, a member of the microbial P450 family, is able to catalyse the oxidation of C6 to C11 alkanes with a regiospecificity of 95% for the terminal carbon position Randall (2010). The research undertaken focused on the bioconversion of octane to 1-octanol, therefore the CYP153A6 enzyme was an appropriate choice for the biocatalyst to be used.

A choice also had to be made as to which process options to implement: using whole cells as opposed to using enzymes, and which biocatalyst cell strain to select.

2.2.1 Whole cells *vs.* enzymes

The choice of whether to use a whole cell or enzyme biocatalyst is dependent on the properties of the reaction system. In general, systems implementing isolated enzymes require an investment upstream of the reactor, while whole cell systems require a downstream investment (Woodley, 2006). The whole cell systems are more likely to utilise a product purification step,

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while the isolated enzyme systems require an enzyme preparation step prior to the reaction procedure.

Systems that use enzymes which require expensive co-factors, such as NAD(P)H, often necessitate the need for a recycle. For whole cell systems a recycle of this kind may be implemented by supplying a co-substrate to make use of *in vivo* secondary enzyme activity (Woodley, 2006). The implementation of a co-factor recycle system is more complex and expensive for isolated enzyme systems. For such cases, isolated enzyme systems would require the addition of a second enzyme, or the use of an electrochemical method (Woodley, 2006). Fuji *et al.* (2006) states that since co-factors and their regeneration are often necessary for enzymatic hydroxylation reactions of hydrocarbons, whole cell biotransformations are the preferred method of biocatalysis for alkane substrates. *E. coli* cells are the most popular microbial hosts for biotechnological applications, because extensive molecular genetic resources exist and *E. coli* cells are fully amenable to genetic manipulation (Fujita *et al.*, 2009).

This study utilises a recombinant *E. coli* cell that has been genetically engineered to contain the CYP153A6 enzyme. A whole cell system was chosen, because the CYP153A6 enzyme uses NAD(P)H as a co-factor. The implementation of an isolated enzyme system is likely to be more complex and expensive.

2.2.2 Biocatalyst cells strain selection

The University of the Free State (UFS) developed the recombinant *E. coli* cells that were used in this project. Their research expressed the CYP153A6 operon in pET 28 vector in *E. coli* BL21(DE3)pLysE (Strain A) and in *E. coli* BL21(DE3) (Strain B).

The *E. coli* BL21(DE3)pLysE strain carries the pLysE plasmid, which strongly regulates the overproduction of the CYP153A6 protein. The plasmid thereby increases the likelihood of the protein being folded correctly, but reduces the amount of protein produced. The *E. coli* BL21(DE3) strain, on the other hand, has no such regulation and therefore produces a larger quantity of the CYP153A6 protein Randall (2010).

Both of the strains that were used in the project carried a gene sequence coding for the CYP153A6 protein chain of amino acids. For functional activity of CYP450s, the peptide chain must be properly folded around the heme. Heme is naturally synthesized by the host cell, but the amount of intracellular heme is not enough to make active CYP153A6. This leads to

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synthesized polypeptide chains without heme, which are non-functional. To avoid this, δ Aminolevulinic acid (δALA), a precursor molecule for heme synthesis, was introduced into the culture medium to enhance the intracellular heme synthesis and thereby increase the amount of active CYP153A6 enzyme produced Randall (2010).

The synthesis of the heme requires Fe^{3+} ions. Native *E. coli* cells do not produce many heme-containing enzymes and therefore have a very slow metabolic rate of heme synthesis, which only requires small amounts of Fe^{3+} ions. The Fe^{3+} ions were obtained from medium ingredients. When the CYP153A6 protein is over-expressed in *E. coli,* the rate of recombinant protein synthesis is several folds higher than the native protein synthesis. Under these conditions the cell needs high quantities of heme to synthesise the active CYP153A6 protein and therefore requires an increased amount of Fe^{3+} ions. This demand was satisfied by supplying the cells with $FeCl_3$ as a source of Fe^{3+} ions to be incorporated into the heme Randall (2010).

No comparison between the two strains being used on a similar reaction system could be found in the literature, however it was suspected that the larger quantity of active CYP153A6 protein production inherent to the BL21(DE3) strain, would make it the more suitable option of the two available strains. Comparative experimental work between the *E. coli* BL21(DE3)pLysE strain and the *E. coli* BL21(DE3) strain was conducted to determine which biocatalyst cell strain to use in the project.

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2.2.3 Unwanted secondary metabolic activity

One of the primary advantages of using a biological process stems from the biocatalyst's ability to catalyse complex reactions at physiological conditions with high selectivity and the possibility of no side reactions (Ayala & Torres, 2004). The ability to catalyse only a specific reaction and not catalyse any unwanted secondary reactions can have a significant effect on the productivity of a reaction system.

E. coli BL21(DE3) cells are unable to metabolise octane or 1-octanol in their natural state. The cells were genetically modified to express the CYP153A6 operon in pET 28 vector for the purpose of catalysing the conversion of octane to 1-octanol. Any secondary reactions are unwanted, because they would negatively affect the production of the desired product. Since the *E. coli* BL21(DE3) cells are unable to catalyse any unwanted secondary reactions in their natural state, this ability would have to be given to the cell through genetic manipulation. Since the cells were genetically modified for the purpose of maximising the production of 1-octanol, the ability to catalyse any further reactions should not have been given to the cells. It was therefore expected that no production of secondary products would be observed during bioconversion experimental work.

2.2.4 Expected period of biocatalyst cell activity

The period for which a biocatalyst cell can remain active in a reaction system is an important factor in determining whether the biocatalyst cell is a suitable candidate for a large-scale production process. If the period of activity is too short, the use of the biocatalyst cell may be unfeasible on a larger than bench top scale.

Gudiminchi *et al.* (2012) performed bench top scale octane bioconversion reactions using the same biocatalyst cells that were utilised in this research. He reported no loss of active CYP153A6 enzyme concentration during a 48-hour whole cell biotransformation reaction with an initial concentration of 0.15 μmoles CYP153A6 per gram of dry cell weight (DCW). It was therefore expected that the biocatalyst cells would remain active under controlled conditions in the bioreactor setup used in this research, for a reaction period of at least 48 hours.

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2.3 Bioconversion kinetics

An enzyme is a protein catalyst which increases the rate of a chemical reaction by lowering the activation energy needed to convert a substrate to a product. The drop in activation energy required for a conversion reaction is achieved by an enzyme temporarily binding to a substrate molecule. As with chemical catalysts, enzymes do not undergo a chemical change during the reactions they catalyse (Du Preez, 2008).

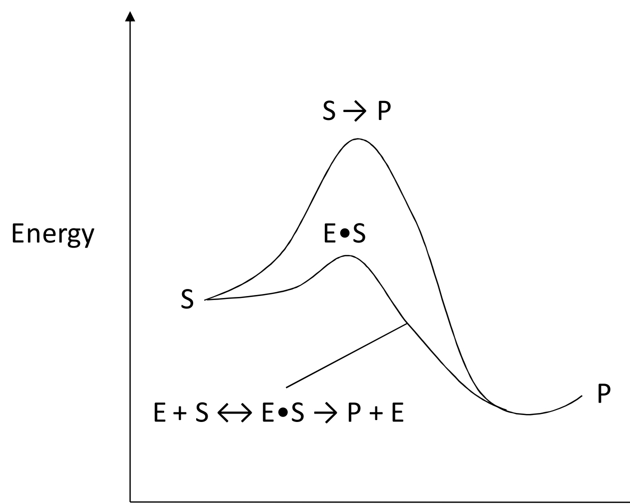

Figure 1: Reaction pathway for enzyme catalysis (redrawn from Fogler, 2006)

The conversion reaction of a substrate molecule (S) to a corresponding product (P) can take one of two reaction pathways (see Figure 1). The un-catalysed reaction pathway requires a significantly larger activation energy than the catalysed reaction pathway. The catalysed reaction pathway proceeds through an active intermediate called the enzyme-substrate complex (E•S) (Fogler, 2006).

$$E + S \leftrightarrow E \bullet S \rightarrow P + E \qquad \textbf{Equation 1}$$

Where E is the enzyme

S is the substrate

P is the product

E•S is the enzyme-substrate complex

The lower activation energy required by the catalysed reaction pathway allows for significantly higher reaction rates.

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The catalytic activity of an enzyme may be influenced by a number of factors such as pH, temperature, fluid forces, chemical agents, irradiation, etc. In general, the change from the enzyme's natural environment should be small to prevent deactivation (Baily & Ollis, 1986).

The effect that pH and temperature have on enzymes is discussed in Section 2.3.1 and Section 2.3.2 respectively. The New Brunswick bioreactor setup used in this research is capable of controlling the pH and temperature of the reaction system, and can therefore influence the performance of the enzymes. The other factors that may have an effect on the enzymes are not discussed in further detail, because the New Brunswick bioreactor setup is not capable of controlling them.

2.3.1 Effect of pH on enzymes

Enzymes consist of various amino acids which have basic, neutral or acidic groups. At any given pH an enzyme may contain both positively or negatively charged groups, which are often part of the active site. It is therefore often necessary for the ionisable groups in the active site to possess a particular charge to allow for the appropriate acid or base catalysation to occur i.e. the enzyme is only catalytically active in one particular ionization state. This implies that the fraction of catalytically active enzyme of the total enzyme present is dependent on the pH (Baily & Ollis, 1986).

Baily & Ollis (Biochemical Engineering Fundamentals, 1986) state that the catalytic activity of an enzyme reaches a maximum at the optimum pH and then decreases when the pH is altered. pH Values far removed from the optimum pH may cause the enzyme to denature.

2.3.2 Effect of temperature on enzymes

Enzymes catalyse reactions by colliding with substrate molecules and then binding at the active site. The temperature of a system is directly related to the kinetic energy of the molecules in the system. A rise in temperature leads to an increase in the kinetic energy of the molecules in the system, causing an increase in enzyme-substrate collisions. An increase in temperature will therefore lead to an increase in the reaction rate (Du Preez, 2008).

Baily & Ollis (Biochemical Engineering Fundamentals, 1986) state that many enzymes begin to denature severely at temperatures approaching 55°C. An increase in temperature causes an increase in the kinetic energy of the atoms of the enzyme molecule. If the kinetic energy of the

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enzyme atoms becomes large enough, the atoms can overcome the weak interactions holding the globular protein structure together. This leads to the denaturisation of the enzyme. The ideal temperature for an enzyme should therefore be as high as possible for high reaction rates, without it causing the enzyme to denature.

2.3.3 Effect of enzyme concentration

There is very little published work on the reaction system and biocatalyst strain used in this research. Only Gudiminchi *et al.* (2012) and Olaofe *et al.* (2013) have published research using the reaction system that is utilised in this research. The effect that the enzyme concentration in a reaction system has on the productivity of that system is dependent on the type of enzyme that is used. Different enzymes can interact differently within the same reaction system, which makes it difficult to predict how the concentration of an untested enzyme will affect the productivity of a reaction system. Because of this, an attempt was made to predict how the concentration of the enzyme utilised in this study would affect the 1-octanol production.

An increase in the CYP153A6 enzyme concentration was expected to result in a proportionally large increase in the 1-octanol production. This expectation was based on the assumption that an increase in the active CYP153A6 enzyme concentration would result in the availability of more active enzyme sites to catalyse the hydroxylation of the octane substrate. This would then result in an increase in the rate of reaction and 1-octanol production.

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2.4 Literature comparison

The pool of literature data for benchmarking of experimental results is relatively small due to the nature of genetically modified *E. coli* cells. There exist large varieties of CYP153 enzymes that have been introduced into *E. coli* cells to perform bioconversions with various alkane substrates. Direct comparisons are only possible between the same enzyme strains and alkane substrate. Furthermore, any difference in the methodology of growing the catalyst cells could have a significant effect on the performance of cells in bioconversion reactions. This meant that any direct comparison became relatively meaningless unless the catalyst cell growth and reaction conditions were the same. Since the list of conditions that needed to be the same was a long one, the number of possibilities for direct comparison diminished significantly. For this reason, the experimental results of this study were compared only to the experimental results of studies done, on the same subject matter, by researchers in the Paraffin Activation Programme of the Centre of Excellence in Catalysis (South Africa) (see Section 4.6).

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2.5 Reaction modelling

Reaction systems are mathematically modelled, because models can predict what factors influence a system, and so can be used as a tool for optimisation. Reactions can be modelled by theoretical or empirical means.

2.5.1 Theoretical models

Theoretical models are developed by translating the characteristic properties of a reaction system into mathematical equations. The strength of such models is that once they have been validated, they can give insight into factors affecting the reaction system's performance, without the need for experimental work. A validated theoretical model is therefore able to predict the performance of a reaction system, outside of the range over which experimental measurements have been made. However, in general, the more complex the reaction mechanism is, the more difficult it is to model the reaction theoretically (Du Preez, 2008).

The first step in developing a theoretical model for a bioconversion reaction is to develop a rate law based on the theory of how the reaction proceeds. The sequence of reaction steps, which describe the entire bioconversion process, is called the reaction mechanism. The reaction mechanism can be described in terms of elementary reactions. The theoretical rate law is therefore described in terms of the elementary reactions of the reaction mechanism. The cytochrome P450 reaction mechanism, upon which the theoretical rate law for this research was based, is introduced in Section 2.5.3 below.

The theoretical rate law can be expressed in terms of measurable quantities through algebraic manipulation and by applying the pseudo-steady-state hypothesis (see Section 2.5.4) to the rate equations derived from the elementary reactions of the reaction mechanism. Once this has been achieved, the performance in a reactor can be theoretically modelled.

Calabrò *et al.* (2002) developed a theoretical model of the behaviour of a hollow fiber membrane bioreactor with entrapped biocatalyst. The model was based on the numerical solutions of the equations governing mass transfer within the different regions of the reaction system. The reaction kinetics were based on a modified form of the Michaelis-Menten rate equation to incorporate the possible effects of substrate and product inhibition. The results of the theoretical model allowed the research to determine the optimal permeate flux in the hollow fiber membrane bioreactor. Furthermore, Calabrò *et al.* (2002) stated that their model

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was quite general and could therefore be applied to different reaction systems to evaluate how the catalyst position within a specific support could affect their performances. This is an illustration of one of the strengths of theoretical models: the ability to be applied to reaction systems other than the system the model was based on.

No theoretical reaction models could be found in literature that could be directly applied to the reaction system utilised in this research. The theoretical model was therefore developed according to the elementary reaction steps that govern the cytochrome P450 reaction mechanism.

2.5.2 Empirical models

Whilst theoretical models are based on reaction mechanisms that can be described mathematically, empirical models are derived purely from experimental observations. Empirical models are based solely on the inputs and outputs of the reaction system, ignoring the mechanism and any other inherent properties of the reaction system.

The strength of empirical models is that they can model complex reaction systems that cannot be modelled theoretically. However, empirical models are only valid for the specific reaction system under investigation and over the range of experimental conditions tested for.

De Jager *et al.* (2009) used an empirical model to model the growth kinetics of *Streptomyces coelicolor* A3(2) in a pressurised membrane gradostat reactor (MGR). Their study states that although empirical modelling of microbial growth kinetics plays an important role in the design and optimisation of MGR systems, their model cannot provide insight into what factors control the microbial growth.

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Monod equation

The Monod equation is an example of an empirical model. The equation represents a mathematical model that is commonly used to describe the growth of microorganisms (Perry, 1999). An example of a typical Monod equation plot is given in Figure 2.

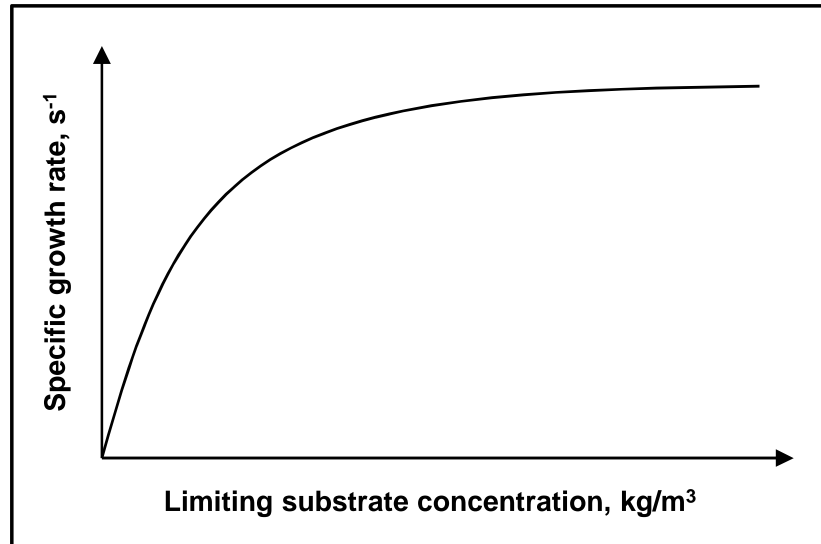

Figure 2: Monod equation plot – Specific growth rate profile against the concentration of a limiting substrate

The Monod equation, in its standard form, is given below:

$$\mu = \mu_{max} \frac{S}{S + K_S} \qquad \text{Equation 2}$$

Where μ – Specific growth rate [1/s]

μ_{max} – Maximum specific growth rate [1/s]

S – Limiting substrate concentration [kg/m^3]

K_s – Monod reaction constant [kg/m^3]

The Monod equation can be used to model production results that resemble the profile of the specific growth rate of the Monod equation when plotted against the concentration of the limiting substrate. Furthermore, the equation can be adapted to include a product inhibition term if such a phenomenon is observed. An example of an adapted Monod equation with a product inhibition term is given below:

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$$\frac{dC_P}{dt} = K_1 \ \frac{C_S}{C_S + K_S} \left(1 - \frac{C_P}{C_P^*}\right)^n \qquad \text{Equation 3}$$

Where C_P - 1-Octanol concentration [mg/g DCW]

C_P^* – Limiting 1-octanol concentration [mg/g DCW]

C_S – Octane concentration [mg/g DCW]

n – Poisoning order of the rate law

K_1 – Reaction constant [mg/g DCW]

K_S – Monod reaction constant [mg/g DCW]

The reaction constant K_1 in Equation 3 is the equivalent of the maximum specific growth rate μ_{max} in Equation 2. The octane concentration C_S in Equation 3 is subbed in for the limiting substrate concentration S in Equation 2. A product inhibition term has been added to Equation 3 in the form of (1- C_P/C_P^*). The poisoning order of the rate law is described by the reaction constant n.

2.5.3 Cytochrome P450 reaction mechanism

Cytochrome P450 is a class of oxidative enzyme which is capable of catalysing the oxidation of alkanes to their corresponding alcohols. The enzyme uses NADH as a co-factor and the two-component system usually consists of a hydroxylase and a reductase (Ayala & Torres, 2004). The typical reaction catalysed by the P450 enzyme may be summarised as follows:

$$\mathbf{RH + O_2 + 2H^+ + 2e^- \rightarrow ROH + H_2O} \qquad \text{Equation 4}$$

The RH in Equation 4 represents a large variety of different chain length alkanes. One molecule of oxygen is consumed for every alkane substrate molecule converted to its corresponding alcohol. One of the oxygen atoms is inserted into the product and the other forms a molecule of water.

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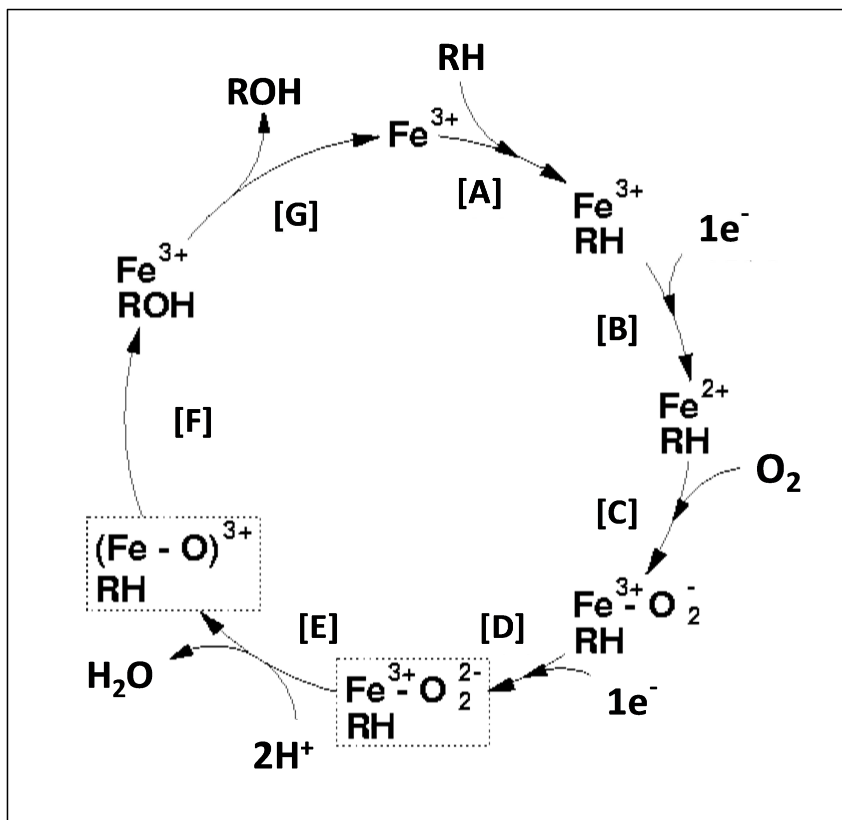

Figure 3: Cytochrome P450 catalytic reaction cycle (redrawn from Segall, 1997)

The cytochrome P450 catalytic reaction cycle can be summarised in seven steps (see Figure 3) (Segall, 1997):

A. The alkane substrate binds to a P450 enzyme, which lowers the redox potential by approximately 100mV. This makes the transfer of an electron from its redox partner NADH favourable.

B. The Fe^{3+} ion is reduced by an electron transferred from NADH via an electron transfer chain.

C. An oxygen molecule then rapidly binds to the Fe^{2+} ion.

D. A second reduction in the catalytic reaction cycle then occurs to fulfil the requirements of the stoichiometry of the reaction.

E. The oxygen reacts with two protons from the surrounding solvent and breaks the O-O bond. The oxygen-cleaving reaction produces water and an $Fe\text{-}O^{3+}$ complex.

F. The alcohol product is formed by the Fe-ligated oxygen atom being transferred to the alkane substrate.

G. The alcohol product is released from the active site of the cytochrome P450 enzyme. The enzyme then returns to its initial state.

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2.5.4 Pseudo-Steady-State Hypothesis

Theoretical rate laws usually involve a number of elementary reactions and at least one active intermediate. An active intermediate is a high-energy molecule that reacts almost at the instant that it is formed. The concentration of an active intermediate is therefore very low and difficult to measure. To simplify theoretical rate laws that have been developed in terms of active intermediates, the rate of formation of the intermediate is assumed to equal its rate of reaction. This means that the net rate of formation of the active intermediate is zero. This assumption is referred to as the Pseudo-Steady-State Hypothesis (PSSH) and it may be described algebraically as follows (Fogler, 2006):

$$r_A = \sum_{i=1}^{n} r_{iA} = 0 \qquad \text{Equation 5}$$

Where r_A – Rate of reaction of an active intermediate

n – Number of reactions in which the active intermediate appears

The PSSH is used to simplify theoretical rate law equations so that they may be described in terms of measurable quantities only.

2.5.5 Root Mean Square Error analysis

The 'fit' of a model is evaluated according to how accurately it represents experimental data. The accuracy of the model can be evaluated by performing a Root Mean Squared Error (RMSE) analysis. The RMSE is a measure of the difference between values predicted by a model and the values of the actual experimental data. The RMSE is calculated as follows:

$$RMSE = \sqrt{(Predicted\ value - \ Experimental\ value)^2} \qquad \text{Equation 6}$$

The smaller the RMSE, the more accurately the model represents the experimental data.

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2.6 Hypotheses

The hypotheses listed below pertain to the Literature Review section and to the development of the Materials, Cultures and Experimental Procedures section. The hypotheses are divided into two categories: biotransformation experiments and reaction modelling.

2.6.1 Biotransformation experiments

1. ***E. coli* BL21(DE3) containing the CYP153A6 operon in pET 28 vector is a more suitable biocatalyst cell strain for 1-octanol production than *E. coli* BL21(DE3)pLysE containing the CYP153A6 operon in pET 28 vector.**

   The *E. coli* BL21(DE3)pLysE strain carries the pLysE plasmid, which strongly regulates the overproduction of the CYP153A6 protein. The plasmid thereby increases the likelihood of the protein being folded correctly, but reduces the amount of protein produced. The *E. coli* BL21(DE3) strain has no such regulation and therefore produces a larger quantity of the CYP153A6 protein Randall (2010). The larger quantity of active CYP153A6 protein production is likely to make the BL21(DE3) strain more suitable for 1-octanol production.

   To test the hypothesis, bioconversion batch experiments with a 1.33ml reaction volume were conducted in 30ml McCartney bottles to evaluate which of the two strains was the most suitable biocatalyst cell strain. The two strains were assessed according to enzyme production, 1-octanol production and pH stability during the bioconversion process.

2. **The active CYP153A6 enzyme concentration is directly related to the 1-octanol production achieved in biotransformation reactions. An increase in the CYP153A6 concentration will result in a proportionally large increase in the 1-octanol production.**

   An increase in the active CYP153A6 enzyme concentration should result in an increase in the rate of reaction, based on the fact that there are more active enzyme sites available to catalyse the hydroxylation of the octane substrate.

   The 1-octanol production results of McCartney bottle batch experiments, with differing active CYP153A6 enzyme concentrations were compared to test the hypothesis.

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3. **The CYP153A6 enzyme, expressed in *E. coli* BL21(DE3), is also capable of catalysing the oxidations of decane ($C_{10}H_{22}$), undecane ($C_{11}H_{24}$) and dodecane ($C_{12}H_{26}$) to their corresponding alcohols.**

   Randall (2010) states that the CYP153A6 enzyme, a member of the microbial P450 family, is able to catalyse the oxidation of C6 to C11 alkanes with a regiospecificity of 95% for the terminal carbon position.

   The hypothesis was tested by performing McCartney bottle batch experiments with the three different alkane substrates.

4. **Octane biotransformation reactions, utilising recombinant *E. coli* BL21(DE3) cells as a catalyst, will produce no unwanted by-products as a result of secondary reactions.**

   E. coli BL21(DE3) cells are unable to metabolise octane or 1-octanol in their natural state. The cells were genetically modified to express the CYP153A6 operon in pET 28 vector for the purpose of catalysing the conversion of octane to 1-octanol. Any secondary reactions are unwanted, because they would negatively affect the production of the desired product. Since the *E. coli* BL21(DE3) cells are unable to catalyse any unwanted secondary reactions in their natural state, this ability would have to be given to the cell through genetic manipulation. Since the cells were genetically modified to specifically maximise the production of 1-octanol, the ability to catalyse any further reactions should not have been given to the cells.

   The hypothesis would be refuted if the production of any secondary products was observed during the biotransformation reactions.

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5. 1-octanol production performance is improved under controlled conditions in a bioreactor.

The New Brunswick bioreactor setup has a number of advantages over the McCartney bottle experimental setup; these advantages have the potential of improving 1-octanol production performance:

i. The New Brunswick bioreactor setup is able to control process conditions such as pH and dissolved oxygen concentration. This type of system control is not possible in the McCartney bottle experimental setup.
ii. The degree of mixing in the New Brunswick bioreactor setup is better than that of the 1.33ml reaction volume in the McCartney bottles. The increased degree of mixing in the New Brunswick bioreactor setup has the potential to improve 1-octanol production performance.
iii. Differences in experimental methodology expose the cells in the bioreactor setup to less centrifugal stress and more nutrients than the cells in the 1.33ml reaction volume in the McCartney bottles. Both of these factors have the potential to contribute to a more stable biocatalyst cell, which in turn can improve the 1-octanol production performance.

The 1-octanol production results of both experimental setups, utilising biocatalyst cells grown from the same main culture, were compared to test the hypothesis.

6. The biocatalyst cells will remain active under controlled conditions in a bioreactor for a reaction period of up to 48 hours.

Gudiminchi *et al.* (2012) reported no loss of active CYP153A6 enzyme concentration during a 48-hour whole cell biotransformation reaction with an initial concentration of 0.15 µmoles CYP153A6 per gram of dry cell weight (DCW).

The product and substrate concentrations in the bioreactor under controlled conditions experiments were monitored throughout an extended reaction period, to make confirmation or refutation of the hypothesis possible.

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2.6.2 Reaction modelling

7. **The 1-octanol production under controlled conditions in a bioreactor can be modelled by determining the theoretical rate of reaction, based on seven elementary reactions derived from the seven steps of the cytochrome P450 reaction cycle.**

   The first step in modelling the 1-octanol production in the New Brunswick bioreactor is to develop a theoretical rate law. The theoretical rate of reaction is based on seven elementary reactions, with seven active intermediates, derived from the seven steps of the cytochrome P450 catalytic reaction cycle.

   The theoretical rate law can then be expressed in terms of measurable quantities only through algebraic manipulation, and by applying the pseudo-steady-state hypothesis to the rate equations derived from the seven elementary reactions. Once this has been achieved, the 1-octanol production in the New Brunswick reactor can be modelled. A theoretical rate law, based on the principles detailed above, was developed.

8. **The 1-octanol production under controlled conditions in a bioreactor can be modelled by determining the rate of reaction by empirical means.**

   An empirical rate law, based on the 1-octanol production results observed in the New Brunswick bioreactor experiments, was developed.

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3. MATERIALS, MEDIA, CULTURES, EQUIPMENT AND EXPERIMENTAL PROCEDURES

3.1 Materials

All of the chemicals and consumables used in the project were acquired from Sigma Aldrich SA, Merck, Kimix and United Scientific unless otherwise stated. A list of the chemicals and equipment used in the project may be found in Appendix C in Table 7 and Table 8 respectively. The potassium phosphate buffers used in the bioconversion experiments were prepared according to the method described in Appendix C.

3.2 Media components and preparation

The experimental work utilised Lysogeny Broth (LB) medium for pre-cultures and Auto-induction medium for main cultures. The preparation for the two media is given below in Table 1 and Table 2 respectively. The constituents of the respective media were added to distilled water in the desired concentrations. The auto-induction medium consisted of four different solutions, which were sterilised separately before being aseptically added together in the desired volumetric amounts (see Table 2). The method of sterilisation for each of the solutions is given in brackets. The LB medium was sterilised in an autoclave.

Table 1: Lysogeny Broth medium preparation

Lysogeny Broth medium (autoclave)		
The medium ingredients are dissolved in distilled water in the concentrations listed.		
Yeast extract	5	g/l
Tryptone	10	g/l
NaCl	10	g/l

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Table 2: Auto-induction medium preparation

Auto-induction medium		
The medium ingredients are dissolved in distilled water in the concentrations listed.		
Solution 1	928	ml
Solution 2	50	ml
Solution 3	20	ml
Solution 4	2	ml
Solution 1 (autoclave)		
Tryptone	10	g/l
Yeast extract	5	g/l
Solution 2 (autoclave)		
$(NH_4)_2SO_4$	66.1	g/l
KH_2PO_4	136.1	g/l
Na_2HPO_4	142.0	g/l
Solution 3 (filter sterilisation)		
Glycerol	250	g/l
Glucose	25	g/l
α-lactose	100	g/l
Solution 4 (autoclave)		
$MgSO_4$	120.4	g/l

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3.3 Culture maintenance

The University of the Free State (UFS) developed the recombinant *E. coli* cells that were used in this project. Their research expressed the CYP153A6 operon in pET 28 vector in *E. coli* BL21(DE3)pLysE (Strain A) and in *E. coli* BL21(DE3) (Strain B). The CYP153A6 enzyme is capable of catalyzing the oxidation of C6 to C11 alkanes with a regiospecificity of 95% for the terminal carbon position (Randall, 2010).

The procedures used to grow, maintain and harvest the biocatalyst cells were based on those used in Randall (2010). The recombinant *E. coli* cells were developed to withstand the antimicrobial effects of the antibiotic Kanamycin. All of the media in which cells were grown contained Kanamycin to ensure that only the target *E. coli* cells could survive in the cultures. This reduced the probability of cell culture contamination.

A 30ml McCartney bottle containing 5ml of the LB medium supplemented with $30\mu g.ml^{-1}$ Kanamycin was inoculated with *E. coli* BL21 (DE3) culture from an LB agar plate, in a laminar airflow cabinet (ClearFlow). The 5ml culture was then incubated on a rotary shaker (Labcon) at 130rpm at 30°C for 3-4 hours. Once the optical density (OD) of the cell culture was in the range of 0.6 – 0.8, it could be used to make glycerol stock for long-term storage. A spectrophotometer (Varian Cary 1E) was used to determine the OD of the cell cultures. Unless otherwise stated, pure LB medium was used as a blank and the OD measurement was taken at a wavelength of 600nm, using glass cuvettes.

The glycerol stock was made by adding 900µl of the cell culture and 100µl 80% glycerol solution to a 1.5ml Eppendorf. Thereafter, the glycerol stock solution was vortexed for 30 seconds and then stored at -80°C (Gudiminchi *et al.*, 2012). Freezing *E. coli* BL21(DE3) glycerol stock units at -80°C should maintain the integrity of the biocatalyst cells for approximately two years (Rama K. Gudiminchi, personal communication).

Additional stock cultures were made by thawing a glycerol stock unit on ice for 15 minutes. A 30ml McCartney bottle containing 5ml of the LB medium supplemented with $30\mu g.ml^{-1}$ Kanamycin was inoculated with 20µl of the thawed glycerol stock unit. A glycerol stock unit was then made and stored according to the procedure described above.

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3.4 Equipment – New Brunswick Bioreactor

Scaled-up octane bioconversions were performed in a 7.5 litre BioFlo 110 Modular Benchtop Fermentor (New Brunswick) setup. Motivation for using the New Brunswick bioreactor setup included the following:

1. The New Brunswick bioreactor setup is able to control process conditions such as pH and dissolved oxygen concentration. This type of system control is not possible in the 1.33ml bioconversion batch experiments conducted in 30ml McCartney bottles.
2. The degree of mixing in the New Brunswick bioreactor setup is better than that of the 1.33ml reaction volume setup. The increased degree of mixing in the New Brunswick bioreactor setup has the potential to improve 1-octanol production performance.
3. Differences in experimental methodology expose the cells in the bioreactor setup to less centrifugal stress and more nutrients than the cells in the 1.33ml reaction volume setup. Both of these factors have the potential to contribute to a more stable biocatalyst cell, which in turn can improve the 1-octanol production performance.
4. The New Brunswick bioreactor setup allows for the possible addition of a membrane separating unit and for the continuous operation of the reaction system.

3.4.1 Experimental setup

Bioconversion batch and semi-batch experiments, with a 2l reaction volume, were conducted in a BioFlo 110 Modular Benchtop Fermentor (New Brunswick). Figure 29 and Figure 30 in Appendix A give a schematic representation of the side view of the New Brunswick bioreactor and a top view the bioreactor's head plate respectively. The non-jacketed vessel has a volume of 7.5 litres and was equipped with a temperature, pH and dissolved oxygen probe. All of the probes were connected to the Primary Control Unit (PCU), which served as the operator interface. The PCU was used to set all of the system parameters and it was responsible for all process control.

The fermentor vessel was suspended from a stainless steel stand with four rubber feet to provide stability. The head plate clamped the vessel onto the stainless steel stand and it provided several ports for the various fittings needed to measure and control the system parameters. A baffle assembly was placed inside the vessel and an impeller was positioned 3cm from the bottom of the agitation drive shaft to ensure sufficient mixing of the vessel's contents. Oxygen was supplied to the system by sparging filtered (Pall Corporation 0.2µm PTFE filter) air into the system. The sparger tube was placed at the bottom of the fermentation vessel and

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attached to the head plate. A thermowell tube was inserted into the head plate and filled with glycerol prior to the temperature probe being inserted. The pH probe was calibrated prior to autoclaving with pH buffer standards of 4.0 and 7.0 respectively and inserted into the head plate. An exhaust condenser was attached to the head plate with a 0.2μm PTFE filter connected to the exit of the condenser. A cooling coil and a dissolved oxygen (dO_2) probe was inserted into the vessel and attached to head plate. A septum fitting was attached to the head plate to allow small amounts of solutions to be added easily and aseptically to the vessel.

A fitting that allowed the PCU to pump a basic solution into the vessel for pH control was attached to the head plate. All unused ports in the head plate were sealed with plug fittings. The autoclavable components of the auto-induction media were transferred into the vessel prior to the sterilisation procedure. All other components were added aseptically after the setup had been autoclaved. A thin layer of vacuum grease was applied to each of the assembly pieces attached to the head plate, to ensure that the seal on each port was airtight.

The sampling system consisted of a sampling tube that was connected to a sampler bottle holder, that had two entry sites, via a silicone tube. A syringe was used to suck air out of the sampler bottle through the one entry site, thereby extracting a sample, from the fermentor vessel, through the other entry site. All silicone tubes connected to the sampler bottle holder were clamped shut when no samples were being taken.

3.4.2 Sterilisation procedure

Once the setup had been assembled, the entire bioreactor was placed in an autoclave. The following four objectives had to be met before the sterilisation process could be started (New Brunswick Scientific Co., 2007):

1. Minimize the pressure difference throughout the sterilisation process by allowing air to move freely between the inside and outside of the vessel
2. Protect susceptible vessel equipment from steam damage
3. Prevent liquid from being expelled from the vessel, due to minor pressure differences, by clamping off all tube fittings that go below the liquid level
4. Prevent condensation from blocking hydrophobic filters.

The first objective was met by leaving the exhaust condenser exit open, the second by using protective caps on all probes and bearings. The third objective was met by clamping off all

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flexible silicon tubing attached to submerged tube fittings. All filters were wrapped with a protective cap of aluminium foil to meet the final sterilisation preparation objective.

After the preparation for the sterilisation had been completed, the setup was autoclaved for 30 minutes at 121°C. Once the temperature had dropped to below 90°C, the autoclave pressure was slowly released over a period of 60 minutes. The setup was then taken out of the autoclave and allowed to cool for 30 minutes. The bioreactor setup was assumed to be sterile on completion of the 30-minute cooling period.

3.4.3 Experimental preparation

Once the New Brunswick bioreactor setup had been assembled and sterilised, the bioreactor could be prepared for biotransformation experimental work. After sterilisation, the water and gas lines were connected to the cooling coil and sparger tube respectively. The flow of water to the cooling coil was regulated by a solenoid valve that was automatically controlled by the PCU. The airflow to the sparger was manually set with a rotameter. All protective caps were then removed from the various probes, bearings and filters. The temperature, pH and dO_2 probes were then attached to the PCU. The agitator motor was installed by positioning the motor sleeve on the bearing house. The heating blanket was then connected to the PCU and wrapped around the vessel in such a way that one of the two viewing holes was facing forward. A bottle containing a basic solution of 2M NaOH was connected, by means of a silicone tube, via a PCU controlled peristaltic pump to the pH control fitting on the head plate. The exhaust condenser was utilised in a counter-current configuration. A manually controlled Manostat® peristaltic pump was used to pump cooling water from an ice bath, in a closed circuit, to the condenser. The flow rate of cooling water to the condenser was kept constant at 300ml/min throughout the operation of the bioreactor. The pH, temperature and air flow rate settings were then set on the operator interface and rotameter respectively.

The dissolved Oxygen (dO_2) probe was calibrated once the media in the vessel had reached the desired operating temperature. The dO_2 probe was calibrated by establishing a reference 0% and 100% dO_2 signal. The 0% reference point was determined by sparging the system with nitrogen until the dO_2 raw input stopped decreasing. The 100% reference point was established by sparging the system with air at the operating air flow rate.

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3.4.4 Control systems

The Primary Control Unit (PCU) was used to set all of the system parameters and it was responsible for all process control. The temperature of the system was measured by a temperature probe that was inserted into a thermowell filled with glycerol. The PCU maintained the desired operating temperature by controlling a heating blanket and a solenoid valve on the cooling coil's water line. The PCU controlled the pH of the system by pumping a basic solution of 2M NaOH into the vessel when the pH of the main culture dropped below its set point. The dO_2 concentration in the system was maintained by cascading the agitation rate control on the dO_2 concentration set point.

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3.5 Experimental procedures

Experimental work was completed on reaction volumes differing by three orders of magnitude. The smaller scale experiments were conducted in 30ml McCartney bottles with a 1.33 ml reaction volume and were referred to as the McCartney bottle experiments. The larger scale experimental work was conducted in a 7.5l BioFlo 110 Modular Benchtop Fermentor (New Brunswick) with a 2 L reaction volume.

The experimental procedures used to grow, maintain and harvest the biocatalyst cells were based on those used in Randall (2010) and were divided into the following four categories:

1. Preparation of the inoculum (Section 3.5.1)
2. Growing recombinant *E. coli* cells (Section 3.5.2)
3. Cell harvesting and alkane bioconversion (Section 3.5.3)
4. Analyses (Section 3.5.4)

The McCartney bottle and New Brunswick bioreactor experiments essentially followed the same procedure, with modifications as explained. These procedures are described in detail in sections 3.5.1 to 3.5.4. Sections 3.5.1 to 3.5.3 each consist of two subsections with titles starting with either the words “McCartney bottle” or “New Brunswick bioreactor”. This has been done to indicate whether the experimental procedures of the small or the large scale experiments are being referred to. All of the equipment that was used in the growing, maintaining and harvesting of the biocatalyst cells was sterilised prior to coming into contact with the cells. All transfers were conducted aseptically in a laminar flow cabinet (ClearFlow).

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3.5.1 Preparation of the inoculum

3.5.1.1 McCartney bottle inoculum

A 30ml McCartney bottle containing 5ml of the LB medium supplemented with $30µg.ml^{-1}$ Kanamycin was inoculated, in a laminar airflow cabinet, with 20µl of glycerol stock (final glycerol stock concentration of 0.4%). The 5ml pre-culture was then incubated on a rotary shaker at 130rpm at 30°C for 16 hours (Step 1 in Figure 9).

3.5.1.2 New Brunswick bioreactor inoculum

The New Brunswick bioreactor experiments with a 2l reaction volume utilised a three-stage inoculation procedure. A 5ml pre-culture was used to inoculate 100ml of the LB medium supplemented with $30µg.ml^{-1}$ Kanamycin in a 500ml Erlenmeyer flask. The 100ml pre-culture was then incubated on a rotary shaker at 130rpm at 30°C for 16 hours, before being used to inoculate 200ml of the LB medium supplemented with $30µg.ml^{-1}$ Kanamycin in a 1000ml Erlenmeyer flask. The 200ml pre-culture was then incubated on a rotary shaker at 130rpm at 30°C for 3.5 hours (Step 1 in Figure 10). The three-stage inoculation procedure was used to ensure that the main culture would be inoculated with *E. coli* cells in the exponential growth phase, thus minimising the lag time in the main culture.

3.5.2 Growing recombinant *E. coli* cells

3.5.2.1 McCartney bottle main culture

A 100ml auto-induction medium was prepared in a 500ml Erlenmeyer flask (see Table 2 for medium preparation details) and supplemented with $30µg.ml^{-1}$ Kanamycin. The auto-induction medium was then inoculated with 2ml of a 5ml pre-culture and incubated on a rotary shaker at 130rpm at 30°C (Step 2 in Figure 9). Once the OD of the main culture was in the range of 0.8 – 1.0, it was supplemented with 0.5mM δ-Aminolevulinic acid (δ-ALA), a precursor molecule for heme synthesis, and $1mg.ml^{-1}$ $FeCl_3$ to compensate for the lack of iron in *E. coli* (Steps 3 & 4 in Figure 9). The main culture was then incubated on a rotary shaker at 130rpm at 23°C for 20 hours (Step 6 in Figure 9). Samples for CO difference spectra analysis were taken from the main culture after the incubation period (Step 5 in Figure 9).

The period between the inoculation of the main culture and the addition of δ-ALA and $FeCl_3$ was defined as the growth incubation period. The period between the addition of the δ-ALA and $FeCl_3$ and the harvesting of the biocatalyst cells was defined as the induction incubation period.

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The incubation temperature was dropped from 30°C to 23°C in the transition from the growth to the induction incubation period to ensure that the CYP153A6 proteins were folded correctly. At an incubation temperature of 30°C the rate of metabolic activity is higher than at 23°C, and an increase in biomass is achieved more quickly. However, if the cells are incubated at 30°C during the expression of the CYP153A6 proteins, large amounts of inactive proteins may be produced, because the protein cannot be folded rapidly enough. Gudiminchi *et al.* (2012) advise that the induction incubation temperature should be between 20°C and 25°C. The incubation temperature was therefore dropped to 23°C. At this incubation temperature the production of CYP153A6 is slow enough to allow the protein to be folded correctly.

Cell growth is clearly visible after the induction incubation period (see Figure 4).

Figure 4: 100ml auto-induction main culture before (left) and after (right) inoculation and incubation

3.5.2.2 New Brunswick bioreactor main culture

A 1800ml auto-induction medium solution was prepared in a 7.5l fermentor vessel and supplemented with 30μg.ml^{-1} Kanamycin. The auto-induction medium solution was then inoculated with 200ml pre-culture and maintained at 30°C (Step 2 in Figure 10). The 1800ml auto-induction medium solution was prepared to compensate for the diluting effect of the additional 200ml inoculum, to ensure the correct final concentration of the auto-induction medium. The pH of the main culture was automatically controlled, with a 2M NaOH solution, to a set point of 7.0, and the percentage of dissolved oxygen in the main culture was set to 20%. The agitation rate control was cascaded to the dissolved oxygen set point with a minimum and

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maximum agitation rate of 50rpm and 500 rpm respectively. The air sparging rate was set to 1.6 litres per minute on a rotameter, which translated to 0.8 vessel volumes per minute (VVM).

Once the OD of the main culture was in the range of 0.8 – 1.0, it was supplemented with 0.5mM δ-ALA and 1mg.ml^{-1} $FeCl_3$ (Steps 3 & 4 in Figure 10). The main culture was then incubated at 23°C until its OD plateaued (approximately 15 hours) (Step 6 in Figure 10). Samples for CO difference spectra analysis were taken from the main culture after the incubation period (Step 5 in Figure 10).

3.5.3 Cell harvesting and alkane bioconversion

The length of the induction incubation period is based on the time it takes for the biocatalyst cells to reach the early stationary phase of their growth profile (Rama K. Gudiminchi, personal communication). Once the cells reach this phase in their growth profile, they metabolise only the amount of nutrients necessary to maintain the functions and structural integrity of the cells. The cells are then harvested to be used in bioconversion reactions.

The bioconversion temperature and pH operating ranges were based on the UFS research group's experience of working with the biocatalyst cell. The CYP153A6 protein is a mesophylic protein and these types of proteins usually have an optimal temperature in the region of 30°C. A lower bioconversion temperature of 23°C was chosen as a trade-off between an increased reaction rate and the desire to run the experiment at mild conditions to reduce the strain on the cell and to reduce the energy requirements of the bioconversion reaction. Running the bioconversion reaction at a higher temperature increases the kinetic energy of the molecules in the system, which places strain on the structural integrity of the cell and may compromise the cell's ability to catalyse the alkane hydroxylation reaction. The operating pH was defined as 7.0, because the optimal pH for CYP153A6 was expected to be close to this value (Rama K. Gudiminchi, personal communication).

3.5.3.1 McCartney bottle bioconversions

Sterile centrifuge tubes were pre-weighed to allow for the subsequent determination of the wet cell weight. The main culture was then transferred to the centrifuge tubes and centrifuged for 10 minutes at 4000xg at 4°C (Eppendorf Centrifuge 5702 R). The cell pellet was clearly visible after centrifugation (see Figure 5 below).

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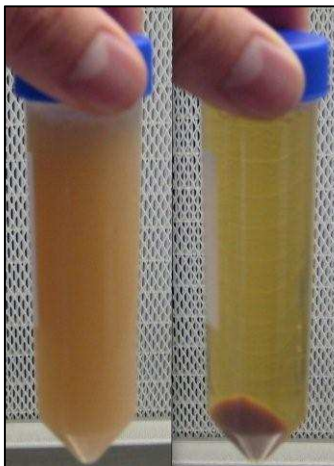

Figure 5: 100ml auto-induction medium main culture before and after centrifugation for 10 minutes at 4000xg at 4°C

The main culture's supernatant was discarded and the centrifuge containers were placed on ice. The wet cell weight was determined by subtracting the weight of the pre-weighed empty tube from the weight of the tube with the cell pellet.

After the cells were harvested the pellet was resuspended in a solution consisting of the following components (Step 7 in Figure 9):

- Potassium phosphate buffer: 5ml 200mM buffer, with a pH of 7.0, per gram of wet cell weight
- Glucose: 40mM final solution concentration
- Glycerol: 0.8% (v/v) final solution concentration
- $FeSO_4$: 100μg per 1ml final solution

The resulting mixture is referred to as the bioconversion reaction mixture. The glucose and glycerol were added to the reaction mixture to regenerate NADH from NAD^+. The glycerol was considered to have a stabilising effect on *E. coli* cells for P450 reactions (Fujita *et al*., 2009). Gudiminchi el al (2012) performed whole cell biotransformations of n-octane using *E. coli* cells expressing CYP153A6 to determine the role of glucose and glycerol in NADH regeneration. Their research found that *E. coli* BL21(DE3) utilises glucose more efficiently than glycerol for the regeneration of NADH.

The bioconversion reaction mixture was vortexed for two minutes. 1ml of the reaction mixture and 333μl of octane were then added to a 30ml McCartney bottle (Step 8 in Figure 9) for batch type bioconversion experiments to give an initial octane concentration of 176mg octane/ml.

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The bioconversion experiment was then incubated on a rotary shaker at 130rpm at 23°C for 25 hours (Step 9 in Figure 9). The bioconversion reaction was stopped by the addition of 190µl of 1M HCl to the 1333µl of the reaction solution.

The pH of the bioreaction was monitored, in a separate parallel experiment, by adding 7ml of reaction mixture and 2.3ml of octane to a 200ml Erlenmeyer flask and taking several measurements with a pH probe throughout the experiment. The reason for the increased reaction volume was to ensure that the pH probe could be completely immersed in the reaction medium. The pH measurements were taken at predetermined times to determine the pH profile of the reaction medium throughout the reaction period.

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3.5.3.2 New Brunswick bioreactor bioconversions

Once the OD of the main culture had plateaued, the bioconversion reaction was initiated. The bioconversion temperature and pH were set to 23°C and 7.0 respectively. All other bioreactor settings remained unchanged from the values described in Section 3.5.2.2. The compounds listed below were added to the main culture to make up the desired final solution concentrations:

- Glucose: 40mM final solution concentration
- Glycerol: 0.8% (v/v) final solution concentration
- $FeSO_4$: 100µg per 1ml final solution

The amounts of glucose, glycerol and $FeSO_4$ required to make up the desired final solution concentrations were dissolved in 100ml of distilled water. The solution was then autoclaved and allowed to cool to room temperature. The sterile 100ml solution was then added aseptically to the main culture in the bioreactor through an appropriate fitting in the reactor head plate (Step 7 in Figure 10).

The substrate (octane) was then added to the bioreactor to start the bioconversion reaction. The octane addition to the bioreactor experiment was scaled up proportionally relative to the ratio of grams wet cell weight versus millilitres octane added to the McCartney bottle experiments. The determination of the initial octane concentration is therefore dependent on the wet cell weight of the main culture in the bioreactor. The equations used to determine the initial octane addition are given below.

$$Cell\ conc.\ for\ McCartney\ bottle\ experiment = \frac{1g\ WCW}{5ml\ reaction\ mixture} \qquad \text{Equation 7}$$

$$Octane\ addition\ for\ McCartney\ bottle\ experiment = \frac{0.33\ ml\ octane}{1ml\ reaction\ mixture} \qquad \text{Equation 8}$$

$$1g\ WCW \times \frac{5ml\ reaction\ mixture}{1g\ WCW} \times \frac{0.33\ ml\ octane}{1ml\ reaction\ mixture} = 1.67ml\ octane \qquad \text{Equation 9}$$

The initial octane addition for a main culture wet cell weight of 65.7g was thereby determined to be 109.5ml (Step 8 in Figure 10). This translated into an initial substrate concentration of 38mg octane/ml reaction medium.

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The initial octane concentration in the McCartney bottle experiment was 176 mg/ml, whereas the initial concentration in the New Brunswick bioreactor experiment was 38 mg/ml. The difference in the initial octane concentration was a result of the cell concentration in the McCartney bottle experiment being approximately 5.5 times higher than that of the bioreactor experiment. The octane addition was scaled up according to the absolute mass of cells in the respective experiments and this led to the different initial octane concentrations. The initial octane concentration in both experiments was meant to be in excess. Although the initial octane concentration in the bioreactor was lower than in the McCartney bottle experiments, it was still expected to be in excess, due to the very low degree of octane conversion seen in the McCartney bottle experiments.

The production results were reported as mg 1-octanol per gram dry cell weight. This allowed for comparison between the production results achieved in the McCartney bottle experiments and New Brunswick bioreactor experiments. The dry cell weight to wet cell weight conversion was determined by preparing a 100ml main culture and determining the wet cell weight, following the methods described in Section 3.5.2.1 and 3.5.3.1. The dry cell weight was then determined by placing the wet cell pellet in its open container in a vacuum oven for 24 hours at 60°C. The dry cell weight was determined to be 11.6% of the wet cell weight. The results of the wet cell weight to dry cell weight experiment may be found in Table 9 in Appendix D.

Bioconversion samples were taken throughout the experimental run to determine the rate and magnitude of 1-octanol production. The agitation rate was set to 250rpm during bioconversion sampling, to ensure that the reaction mixture was well mixed. The bioconversion reaction in the samples was stopped by the immediate addition of 140µl of 1M HCl to the 1000µl of sample.

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3.5.4 Analyses

The product and substrate concentrations were determined through gas chromatography (GC) analysis. The enzyme concentration was determined through CO-difference spectra analysis.

3.5.4.1 Quantification of product and substrate concentration

Samples for GC analysis were prepared by an organic extraction. The organic solvent used to extract the product and substrate was ethyl acetate. 1-Undecanol was added to the ethyl acetate to act as an internal standard.

The entire bioconversion sample and 500µl - 1000µl of ethyl acetate with 1.6mM - 3.5mM 1-undecanol was added to a 2ml Eppendorf tube (Step 10 in Figure 9& Figure 10). The sample was then vortexed for 10 minutes prior to being centrifuged (Mini Spin plus Centrifuge 6305) for 10 minutes at 13200 x g. Three distinct layers were formed by the centrifugation process (see Figure 6). A layer of cells separates the top organic layer from the bottom inorganic layer. The top organic layer was extracted with a pipette and could then be used in the GC analysis.

Figure 6: Ethyl acetate organic extraction after centrifugation in preparation for Gas Chromatography analysis

The GC analysis was performed on 1µl samples using a Varian 3400 gas chromatograph equipped with a flame ionisation detector (FID) and a ZP5 60m x 0.32mm x 0.25µm ZB-5 capillary column. The GC parameter settings are given in Table 3.

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Table 3: Gas chromatography (GC) parameter settings for product and substrate analysis

Gas chromatography settings		
Initial column temperature	50	˚C
Initial column hold time	5	min
Temperature ramp rate	20	˚C/min
Final column temperature	250	˚C
Final column hold time	9	min
Injector temperature	250	˚C
Detector temperature	280	˚C
Run time	24	min
Carrier gas	H_2 gas	
Split flow	12	ml/min

3.5.4.2 Quantification of enzyme concentration

Carbon monoxide-difference (CO-difference) spectra analysis was used to quantify the enzyme concentrations of the various main cultures produced for experimental work. The analysis is based on the fact that the reduced P450 enzyme forms a complex with carbon monoxide to produce a unique absorption peak at 450nm. This spectral property was used to quantify the P450 enzyme concentration in the respective main cultures (Randall, 2010).

The P450 enzymes in the main culture samples may be reduced by the addition of sodium dithionite. A wavelength scan between 400nm and 500nm is conducted on the samples before and after they are exposed to carbon monoxide. The difference between the peak heights of the reduced sample, before and after being exposed to carbon monoxide, is used to determine the P450 enzyme concentration. The P450 enzyme concentration of the main culture sample is determined by using a formula (see Equation 10) that incorporates the change in absorbance at 450nm relative to the change in absorbance at 490nm, an extinction coefficient of $91mM^{-1}.cm^{-1}$ and the cuvette path length (Randall, 2010; Van Beilen *et al.*, 2004).

$$C_{P450}[\mu l] = \frac{\Delta A_{450} - \Delta A_{490}}{\epsilon . l} \times 1000 \qquad \text{Equation 10}$$

Where C_{P450} is the P450 enzyme concentration

ΔA_{450} is the difference in absorbance at 450nm before and after CO exposure

ΔA_{490} is the difference in absorbance at 490nm before and after CO exposure

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ε is the extinction coefficient

l is the cuvette path length in cm

The ΔA_{490} should theoretically equal zero, because there should be no difference in the peak heights of the reduced sample, before and after being exposed to carbon monoxide, at a wavelength of 490nm. The parameter is included in Equation 10 to compensate for any inherent error caused by the spectrophotometer.

Figure 7 shows an example of a wavelength scan for the CO-difference spectra analysis. The ΔA_{490} is almost equal to zero and the ΔA_{450} indicates that there is active CYP153A6 protein.

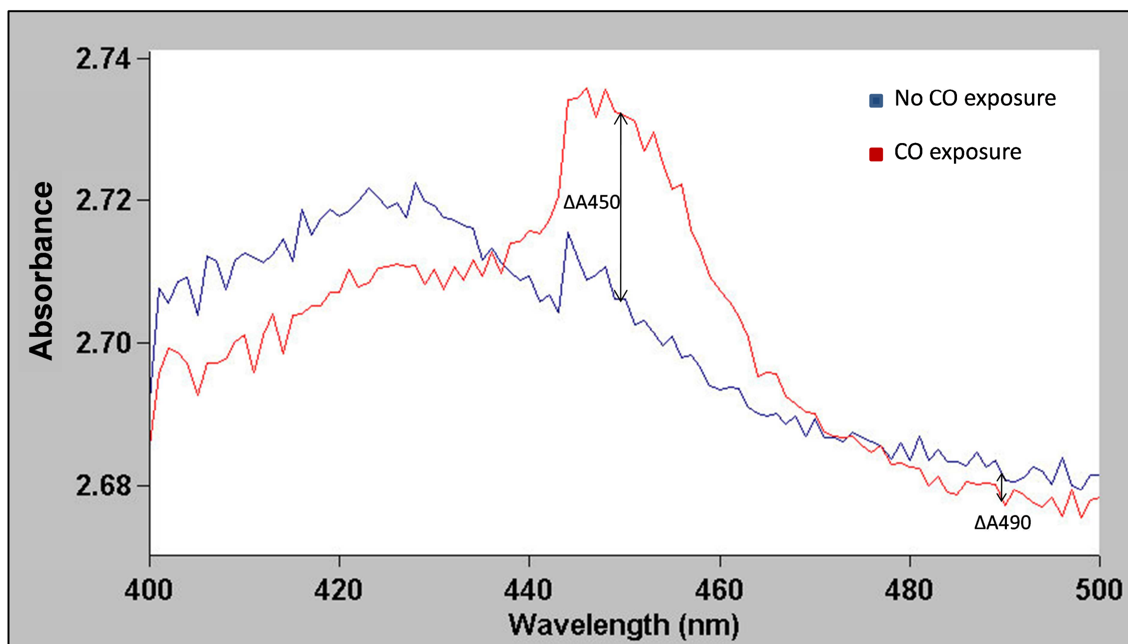

Figure 7: Example of a wavelength scan for CO-difference spectra analysis

The first step in performing the CO-difference spectra analysis on a main culture was to take two 2ml main culture samples. The two 2ml samples were transferred into Eppendorf tubes and then centrifuged (Eppendorf Centrifuge 5702) for 5 minutes at 4000xg at 4°C.

The supernatant of the two centrifuged samples was discarded and the cell pellets were placed on ice. 1ml of 50mM potassium phosphate buffer with a pH of 7.4 was then added to each of the cell pellets. The samples were then vortexed for 2 minutes and 15 mg of sodium dithionite was added to each of the samples using a micro spatula. An OD scan was then performed on one of

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the two samples for a wavelength range of 400nm – 500nm and a cuvette path length of 1cm. The 50mM potassium phosphate buffer with a pH of 7.4 was used as a blank. The two samples were then transferred into a single glass test tube (15mm diameter, 150mm length) and exposed to CO for 5 minutes by bubbling the gas through the combined sample (see Figure 8). The CO sparger consisted of a pipe with a pipette tip attached to its end. An analogous OD scan was then performed on the CO exposed sample for a wavelength range of 400nm – 500nm using the same blank as in the previous scan. The data obtained from the two OD scans was then used to determine the enzyme concentration of the main culture.

Figure 8: Carbon monoxide exposure of biocatalyst cell sample for CO-difference spectra analysis

3.5.5 Flow diagrams of experimental procedures

The bioconversion procedures for the McCartney bottle and bioreactor experiments are summarised in flow diagrams in Figure 9 and Figure 10 respectively. Each step in the flow diagrams is described in detail in the experimental procedures described in Section 3.5.1 to Section 3.5.4.

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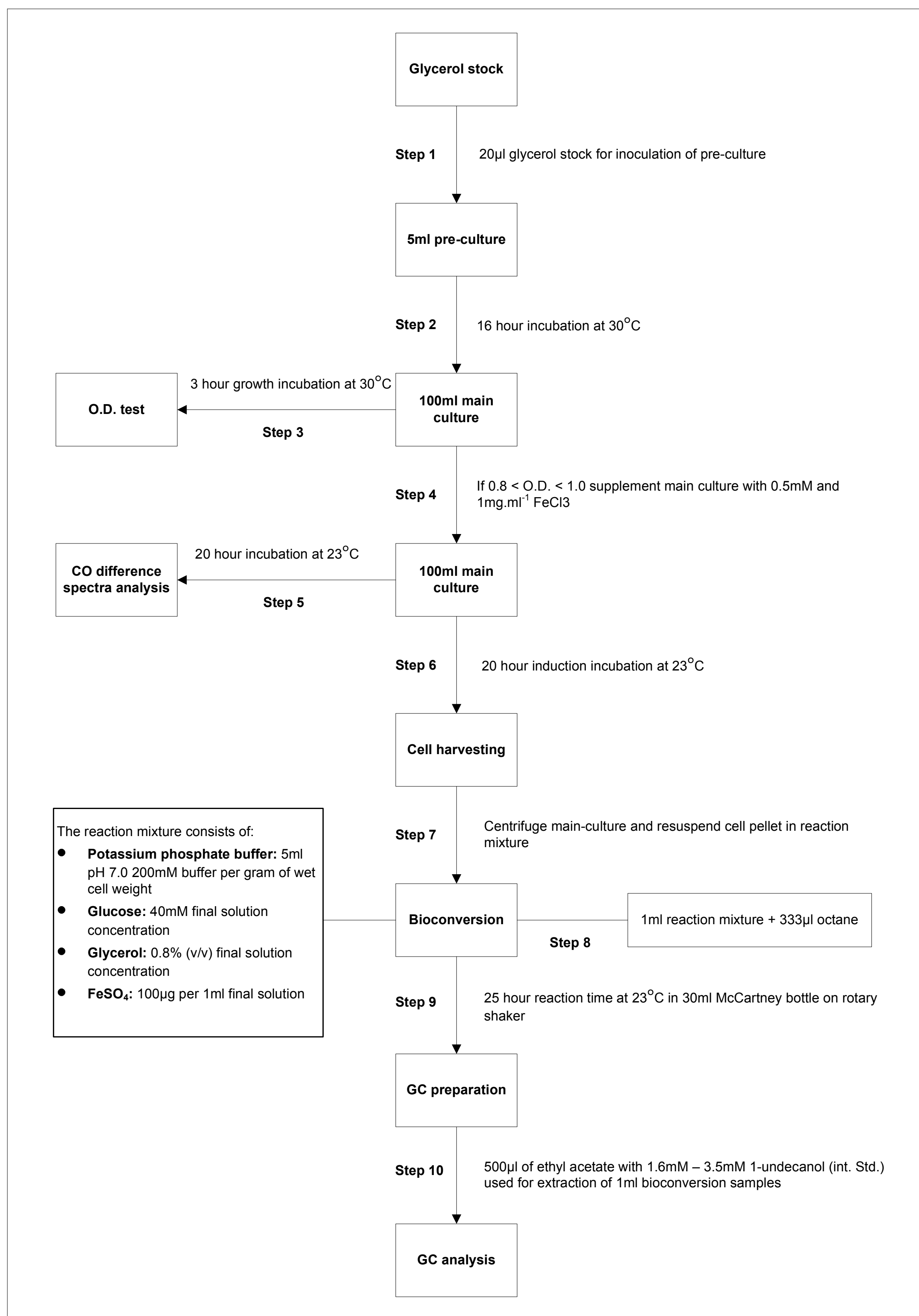

Figure 9: Flow diagram of experimental procedure for McCartney bottle experiments

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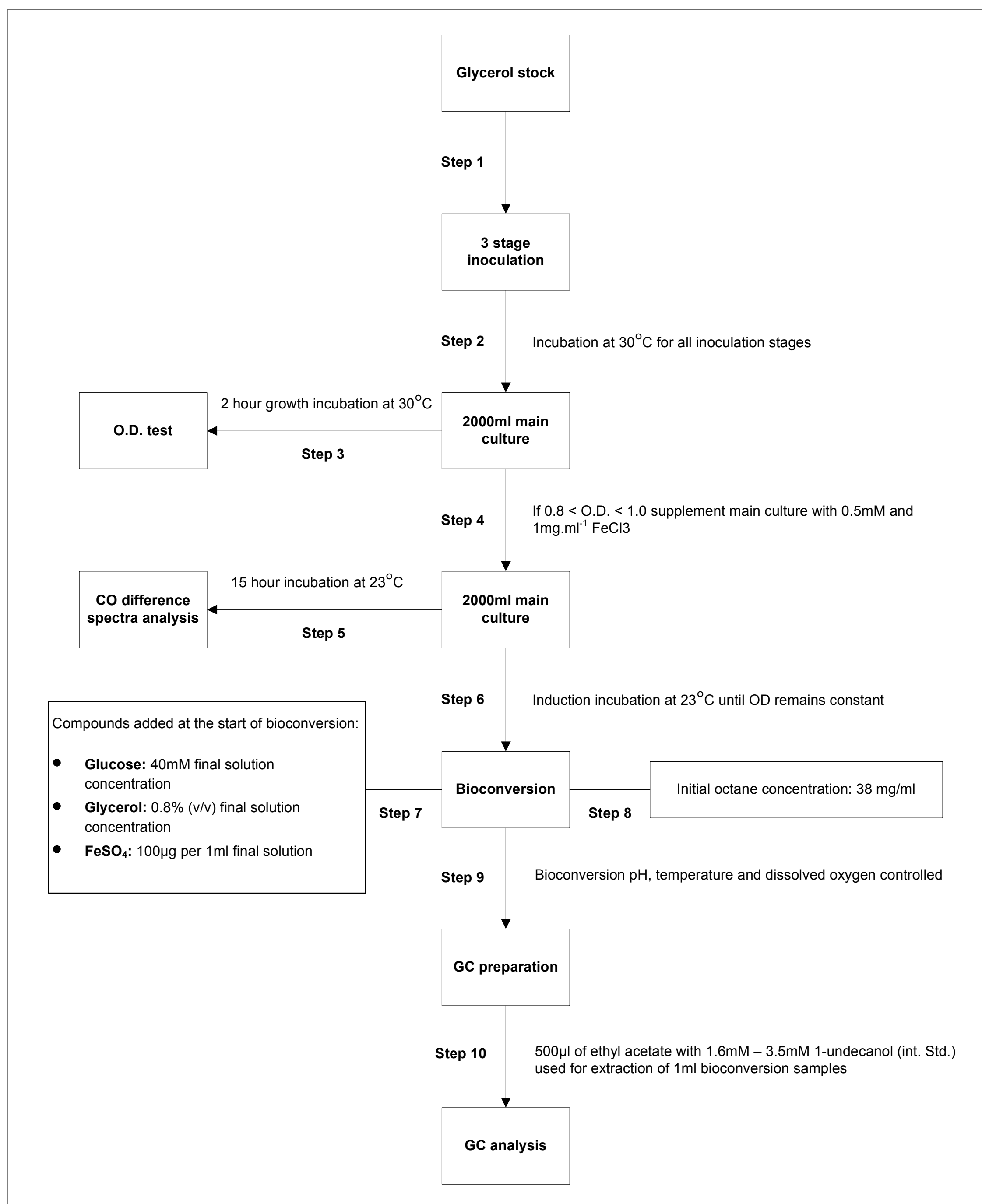

Figure 10: Flow diagram of experimental procedure for New Brunswick bioreactor experiments

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4. RESULTS AND DISCUSSION

In this section, the results of the experimental work that was conducted to address the "Biotransformation experiments" hypotheses listed in Section 2.6.1 are discussed.

4.1 Biocatalyst cell strain selection

As reported, the two biocatalyst cell strains made available to the project by the University of the Free State were:

1. *E. coli* BL21(DE3)pLysE containing CYP153A6 operon in pET 28 vector (Strain A)
2. *E. coli* BL21(DE3) containing CYP153A6 operon in pET 28 vector (Strain B)

Bioconversion batch experiments, with a 1.33ml reaction volume, were conducted in 30ml McCartney bottles to evaluate which of the two strains should be used in the project. The two strains were assessed according to enzyme production, 1-octanol production and pH stability during the bioconversion process.

Three 100ml shake flask cultures were prepared to compare the two strains. Two of the cultures were inoculated with Strain A and the remaining culture was inoculated with Strain B. The cells of one of the two Strain A main cultures (MC2) were washed with a physiological saline solution after being harvested to test how this would affect the pH stability of the reaction mixture. The effect that washing the cells before adding them to the reaction mixture was assumed to be the same for both strains of cells, which meant that it was not necessary to test both Strain A and Strain B. This assumption was based on the cell itself not being responsible for an initial pH drop of the reaction mixture. The pH drop would instead be caused by the low pH incubation medium liquid that is retained in the cell pellet when it is harvested. The cell pellet was therefore washed in an attempt to neutralize this affect.

The initial pH of all of the reaction mixtures was buffered to 7.0 and the bioconversion temperature was maintained at 23°C.

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4.1.1 Active enzyme production

The active CYP153A6 enzyme concentration achieved by each of the three main cultures was quantified, prior to cell harvesting, with CO-difference spectra analysis. The experimental data for Figure 11 may be found in Table 10 in Appendix D.

Strain B achieved an active enzyme concentration of 0.065 µmoles CYP153A6 per gram of dry cell weight (DCW) during the induction incubation period (see Figure 11). Strain B thereby achieved a 35%-40% larger enzyme concentration than Strain A. Gudiminchi *et al.* (2012) conducted similar whole-cell biotransformations of n-octane with *E. coli* BL21(DE3)pLysE and *E. coli* BL21(DE3), both containing the CYP153A6 operon in pET 28 vector. Their research found that the BL21(DE3)pLysE strain (Strain A) achieved a lower active enzyme concentration of 0.15 µmoles CYP153A6 / g DCW compared to the BL21(DE3) strain (Strain B) which produced an active enzyme concentration of 0.5 – 1.0 µmoles CYP153A6 / g DCW. The finding, based on the results represented in Figure 11, that Strain B was able to produce a larger CYP153A6 enzyme concentration than Strain A were therefore verified by the experimental work performed by Gudiminchi *et al.* (2012).

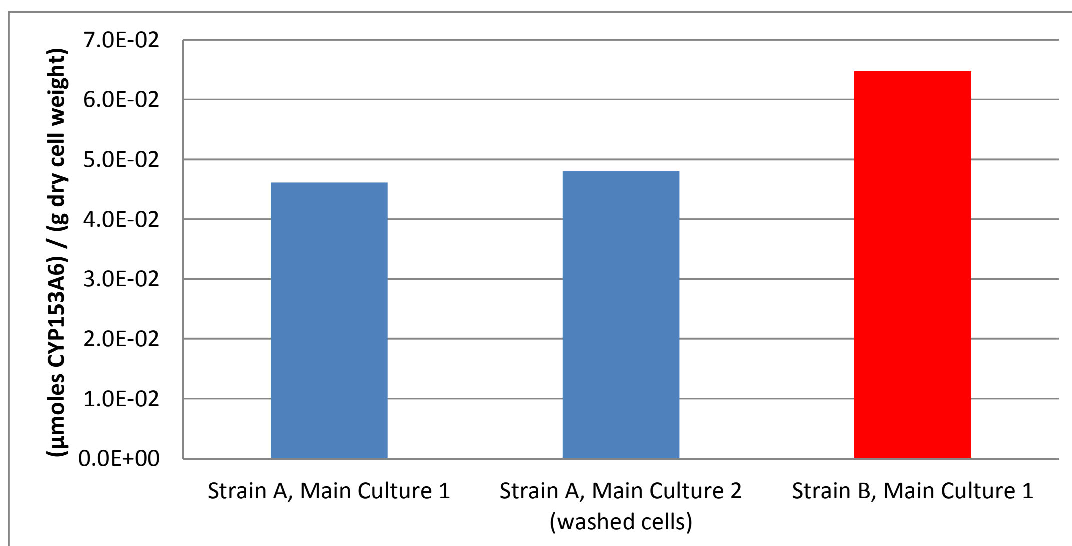

Figure 11: Enzyme concentrations achieved by Strain A and Strain B at 23°C and an initial reaction pH of 7.0

Both Gudiminchi *et al.* (2012) and this research found that the *E. coli* BL21(DE3) strain (Strain B) produced a larger active enzyme concentration in comparison to the *E. coli* BL21(DE3)pLysE strain (Strain A). However, it should be noted that the strains utilised in Gudiminchi *et al.* (2012) produced an order of magnitude larger concentration of active

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enzymes in comparison to this study, in spite of the fact that the same strains were used in the two studies. The reason for this discrepancy could not be determined. When Gudiminchi later duplicated some of our experiments, he obtained the same results as reported in this thesis.

4.1.2 1-Octanol production

The substrate and product analysis had not been refined at this stage of the project and it was therefore not possible to quantify the amounts of 1-octanol produced by the two strains. The 1-octanol production analysis was therefore limited to the following basic qualitative observations based on a rudimentary GC analysis:

- The cells that were washed in a physiological saline solution did not produce any visible 1-octanol peaks
- The 1-octanol peaks produced by Strain A and Strain B were in the same order of magnitude, with Strain B's peaks the larger by visual inspection

4.1.3 Stability of reaction mixture pH

The pH of a 100ml shake flask culture, inoculated with Strain A, was monitored during its entire incubation period. The experimental data for Figure 12 may be found in Table 11 in Appendix D.

The pH of the shake flask culture dropped from a starting value of 6.6 to a final value of 5.7 (see Figure 12). It is suspected that the *E. coli* cells metabolised the carbon sources in the auto-induction medium and produced compounds which caused the pH to drop.

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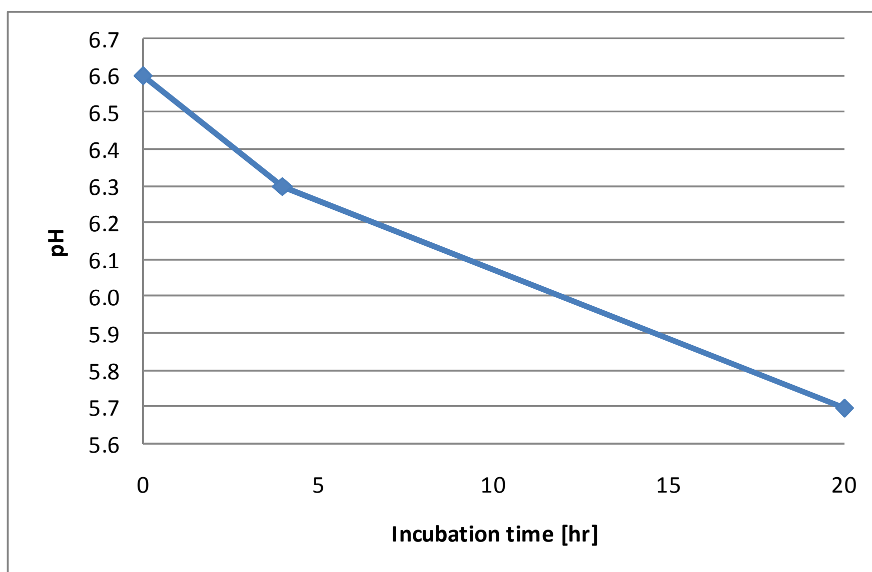

Figure 12: pH profile of 100ml auto-induction medium shake flask culture during incubation at 23°C

The reaction mixture, in which the harvested cells were re-suspended, was buffered to a pH of 7.0. The harvested *E. coli* cells were therefore at a lower pH than the buffer in the reaction mixture. Consequently, the addition of the harvested cells to the buffered reaction mixture could drop the pH value from the desired set point. In an attempt to prevent this phenomenon from occurring, a second main culture (MC2) inoculated with Strain A was prepared. The cells of this main culture were washed with a physiological saline solution after being harvested.

The pH of the reaction mixtures of each of the three main cultures was monitored throughout the reaction period. The results of the pH monitoring are displayed in Figure 13 and Table 4 respectively.

The addition of the biocatalyst cells to the buffered reaction mixture caused a pH drop of at least 0.5 units for both strains (see Table 4). This initial pH drop was not avoided by washing the cells with a physiological saline solution. Furthermore, the addition of the substrate octane did not affect the pH of the reaction mixture.

The cells used in the bioconversion reactions were harvested while they were in the early stationary phase of their growth profile. The cells were then placed in a solution with nutrients and octane to create the bioconversion reaction mixture (BRM). The BRM is not rich enough in nutrients to push the biocatalyst cells into an exponential growth phase. This means that while the recombinant *E. coli* cells performed their function as catalyst for the n-octane biotransformation reaction, the biocatalyst cells would only have metabolised enough

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of the nutrients in the BRM to maintain the functions and structural integrity of the cells. The cells would therefore have produced only small amounts of by-products, such as pyruvic acid, that could cause the pH of the reaction mixture to drop. Furthermore, the BRM was pH buffered to prevent any pH changes caused by the production of small amounts of by-products. In spite of this, the pH of all of the reaction mixtures continued to drop after cell and substrate addition (see Figure 13). The pH drop must therefore be attributed to the cells having residual activity with respect to other unwanted metabolic reactions, the products of which caused the pH of the reaction mixture to decrease. Since the bioconversion performance of the cells can be affected by changes in the pH of the reaction system, the pH control of the system needed to be improved.

An additional observation to be made from Figure 13 is that the rate and magnitude of the pH drop was almost identical for the two strains tested and that the pH change after a 5-hour reaction period was negligibly small.

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Table 4: pH data of reaction mixture in McCartney bottles at 23°C

Bioconversion pH study				
Time [hr]	**Description**	**pH**		
		Strain A Main culture 1	**Strain A Main culture 2 (washed cells)**	**Strain B Main culture 1**
0	Reaction mixture	7.0	7.0	7.0
0	+ cells	6.3	6.3	6.5
0	+ octane	6.3	6.3	6.5
1		5.9	5.8	6.2
2		5.7	5.5	5.8
3		5.4	5.3	5.7
4		5.4	5.3	5.7
5		5.3	5.2	5.5
18		5.3	5.2	5.4
19		5.3	5.2	5.4

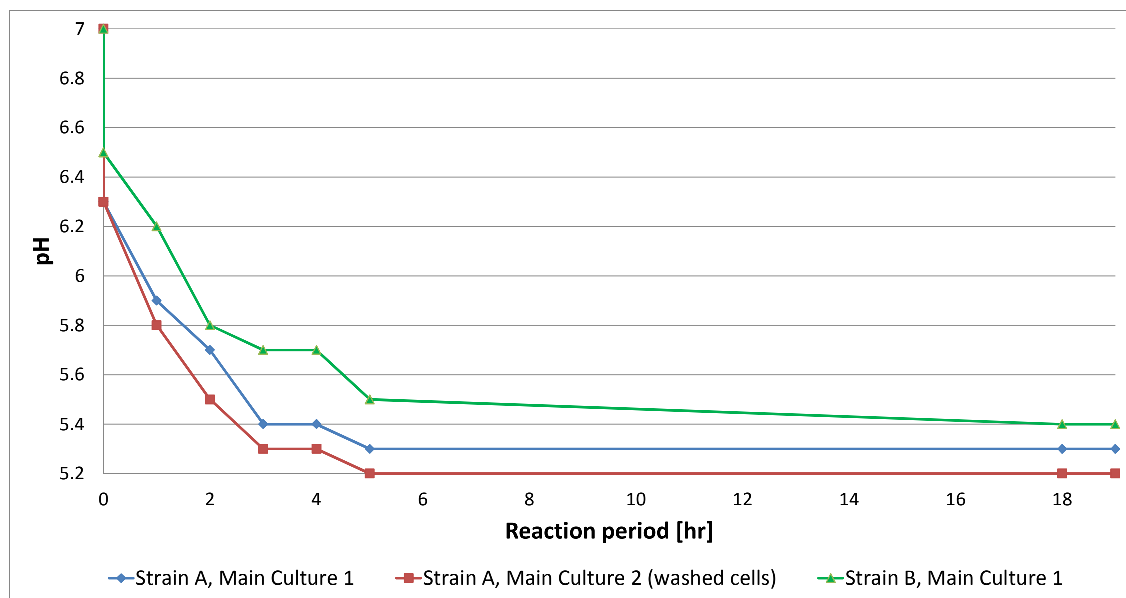

Figure 13: pH profile of reaction mixture in McCartney bottles at 23°C

The results of the pH study indicate that the pH of the reaction system is not stable throughout the reaction period. Increasing the strength of the pH buffer was not considered, because an increase in the buffer concentration would place strain on the biocatalyst cell. Genetically modifying the biocatalyst cell places strain on the cell's structural integrity and any additional strain is therefore undesirable. A different method of maintaining the pH in the reaction system at the desired set point needs to be implemented.

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4.1.4 Strain selection criteria

The biocatalyst cell strain selection was based on the following observations:

- Strain B produced a 35%-40% higher active CYP153A6 enzyme concentration
- Strain B produced more 1-octanol according to a visual inspection of a rudimentary GC analysis
- Neither strain showed a significant advantage over the other in terms of pH stability and 1-octanol production

E. coli BL21(DE3) containing CYP153A6 operon in pET 28 vector (Strain B) was chosen as the biocatalyst cell strain for the project. The principle reason for the decision was the higher active CYP153A6 enzyme concentration produced in comparison to Strain A. Furthermore, it was suspected that a more accurate GC analyses would have shown Strain B to have produced more 1-octanol than Strain A.

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4.2 Relationship between enzyme concentration and 1-octanol production

The relationship between the enzyme concentration and the 1-octanol production achieved was investigated by monitoring the 1-octanol production results of bioconversion reactions performed with different concentrations of active CYP153A6 enzyme per gram of dry cell weight.

The method used to produce biocatalyst cells with different active CYP153A6 enzyme concentrations was to vary the induction incubation period. As described in Section 3.5.2, the start of the induction incubation period was defined as the time when the main culture was supplemented with 0.5mM δ-Aminolevulinic acid and 1mg.ml^{-1} $FeCl_3$. The optimal induction incubation period for bioconversion reactions had not yet been determined in literature, however the UFS research team suggested an induction incubation period of 16 – 20 hours for the 1.33ml reaction volume experiments based on their experience of working with the biocatalyst cell strain used in this research. The shortest (16 hours) and longest (20 hours) induction incubation periods within the range suggested by the UFS research team were therefore chosen to produce differing active enzyme concentrations.

Bioconversion batch experiments with a 1.33ml reaction volume were conducted in 30ml McCartney bottles to investigate the relationship between the enzyme concentration and the 1-octanol production achieved. Four identical 100ml main cultures were prepared for the assessment. Two of the main cultures (2 and 4) were harvested after a 16 hour induction period and the remaining two (1 and 3) were harvested after a 20-hour induction period. The bioconversion temperature and pH were set to 23°C and 7.0 respectively.

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4.2.1 Active enzyme production

The enzyme concentration of the four main cultures was monitored by performing CO-difference spectra analyses on samples taken during the induction incubation period. The experimental data for Figure 14 may be found in Table 12 in Appendix D.

Incubating the main culture for more than 16 hours did not significantly improve the active enzyme concentration produced (see Figure 14). The difference between the average enzyme concentration produced after 16 and 20 hours respectively was less than 5%.

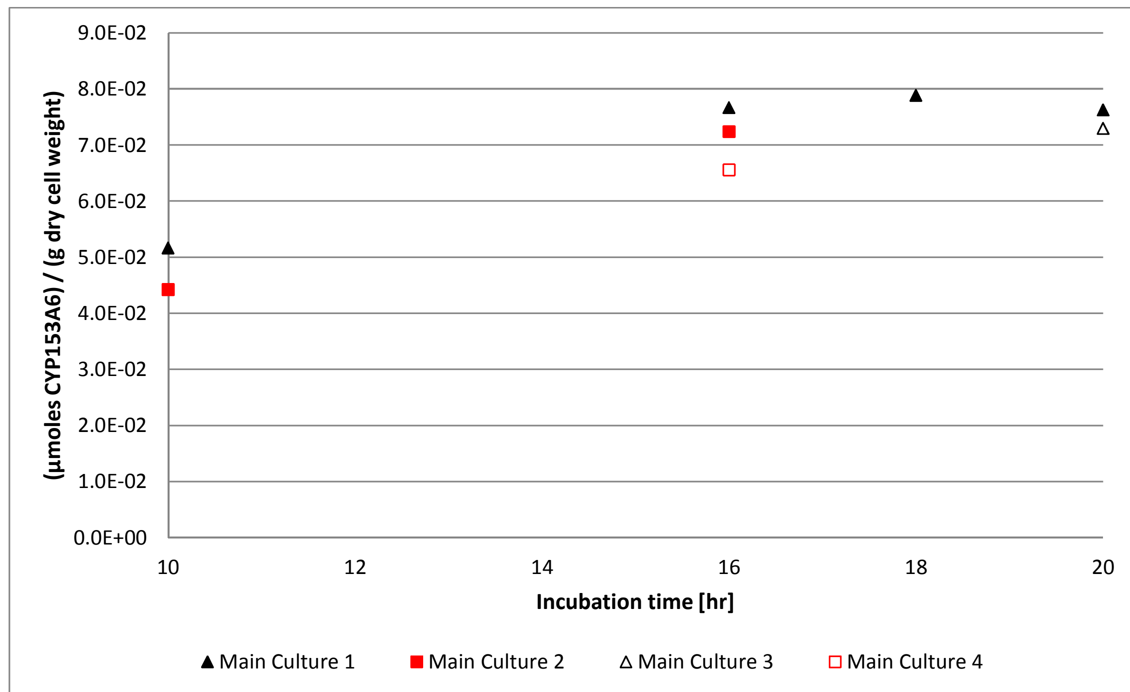

Figure 14: Active enzyme concentration profile during induction incubation period at 23°C and an initial pH of 7.0

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4.2.2 1-Octanol production

The cells of the four main cultures were used as biocatalysts in octane bioconversion reactions, by performing batch experiments with a 1.33ml reaction volume in 30ml McCartney bottles. The 1-octanol production per gram dry cell weight achieved by the bioconversion reactions was monitored throughout the reaction period. The experimental data for Figure 15 may be found in Table 14 in Appendix D.

The cells incubated for 20 hours produced an average of 32% more 1-octanol per gram dry cell weight, after a 15-hour reaction period, than the cells incubated for 16 hours (see Figure 15). This result was somewhat unexpected, because the concentration of the active enzymes was essentially the same for both incubation periods. The relationship between 1-octanol production and active enzyme concentration was thereby shown to be nonlinear. In line with this observation, Gudiminchi *et al.* (2012) found that a high CYP153A6 enzyme concentration does not necessarily translate into high octanol production.

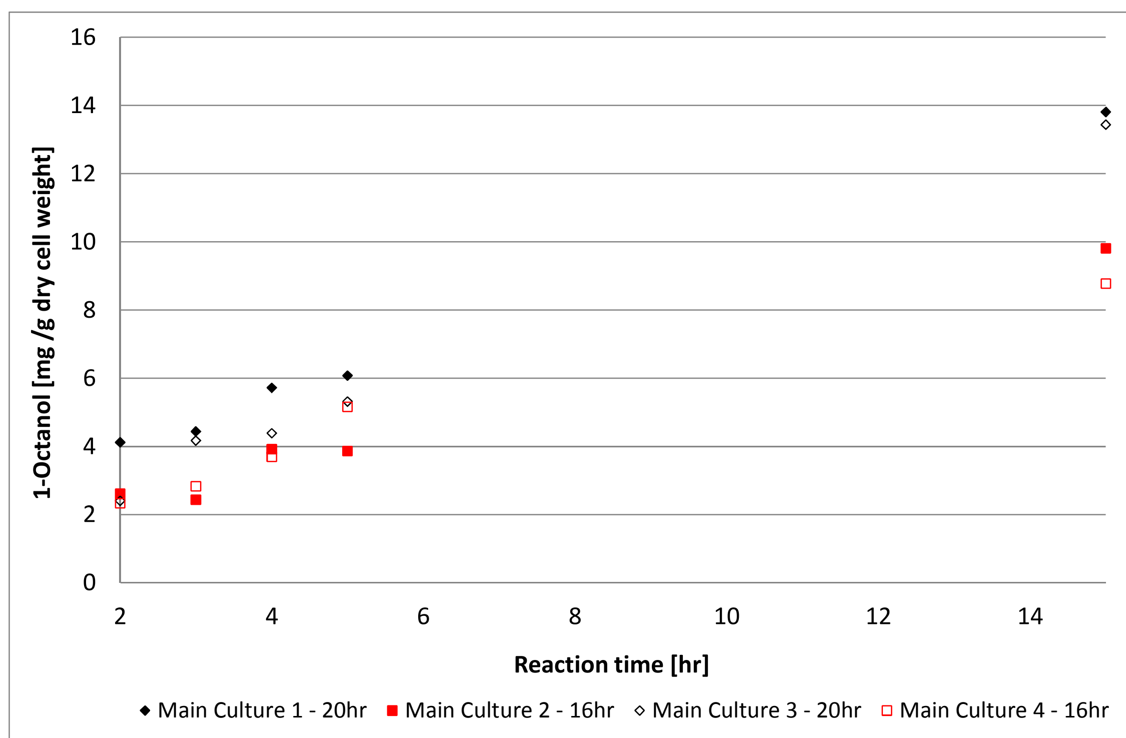

Figure 15: 1-Octanol production for 1.33ml McCartney bottle batch reaction at 23°C and an initial pH of 7.0

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4.2.3 Induction incubation period optimisation

The cell growth of the *E. coli* cells was monitored to determine the effect of the induction incubation period on the cell concentration produced. The cell growth was related to an optical density profile. The profile was produced by performing OD measurements on several samples taken during the induction incubation period. The experimental data for Figure 16 may be found in Table 13 in Appendix D.

The cell growth rate was constant for the first ten hours of the induction incubation period and then started to decrease (see Figure 16). This was an indication of the cells being in the late exponential or early stationary phase from as early as the 10th hour of the induction incubation period. Cell growth from 16 to 20 hours was negligibly small, which indicated that the cells were in the stationary phase. This observation indicated that no advantage could be gained in terms of cell concentration production by increasing the induction incubation period from 16 hours to 20 hours.

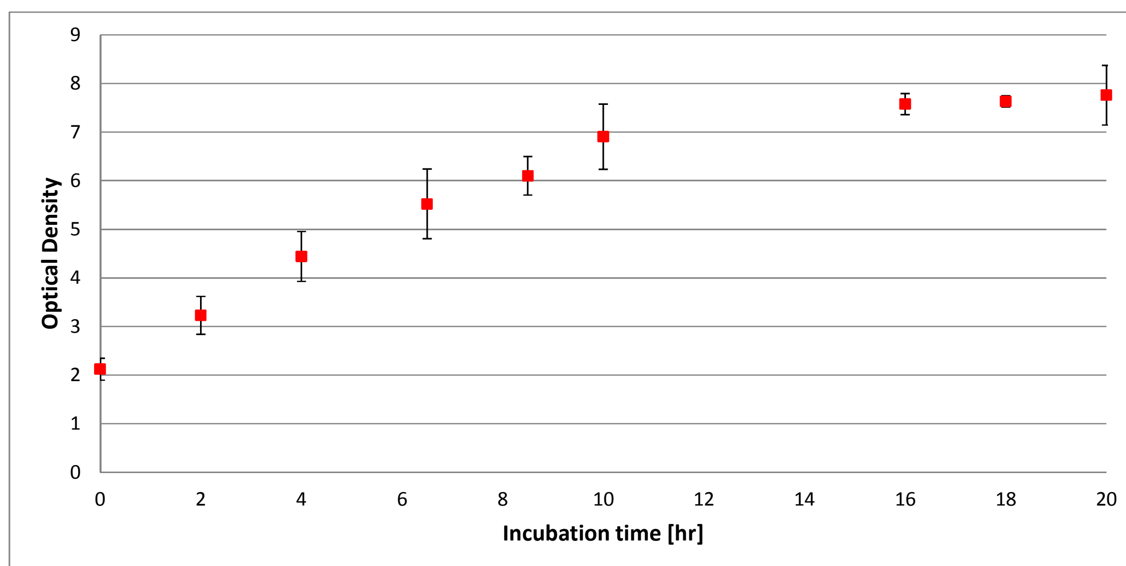

Figure 16: Cell growth profile of *E. coli* BL21(DE3) in auto-induction medium during induction incubation period at 23°C

The experimental work performed to determine the relationship between the enzyme concentration and the 1-octanol production achieved gave some insight into the optimisation of the induction incubation period. Incubating the main culture for 20 hours improved the 1-octanol production by an average 32%, for a 15-hour reaction period, in comparison to a 16 hour induction incubation period (see Section 4.2.2). A 20-hour induction incubation period

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was therefore chosen for all further bioconversion batch experiments conducted in 30ml McCartney bottles to maximise 1-octanol production.

4.3 Substrate screening

The longer the hydrocarbon chain length being converted to its corresponding alcohol, the more valuable the final product. Randall (2010) states that the biocatalyst cell strain used in this project is capable of catalysing the oxidation of C6 to C11 alkanes.

Bioconversion batch experiments with a 1.33ml reaction volume were conducted in 30ml McCartney bottles to determine whether the biocatalyst cell strain was capable of catalysing longer chain length hydrocarbons. The following substrates were tested for:

- Decane ($C_{10}H_{22}$)
- Undecane ($C_{11}H_{24}$)
- Dodecane ($C_{12}H_{26}$)

The bioreactions were conducted at 23°C and an initial pH of 7.0. The GC analysis of reaction samples taken after a 22-hour reaction period found no product peaks for any of the substrates tested.

The lack of product peaks was unexpected for the reactions that used decane and undecane as the alkane substrate, because Randall (2010) states that the CYP153A6 enzyme is able to catalyse the oxidation of C6 to C11 alkanes with a regiospecificity of 95% for the terminal carbon position.

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4.4 Reproducibility of biocatalyst cell performance

The first six months of experimental work were conducted with a batch of *E. coli* BL21(DE3) biocatalyst cells that were made available at the start of the experimental phase of the project. The results of this experimental work are described in Section 4.1 to Section 4.3. A second batch of the same *E. coli* cell strain was made available six months into the experimental phase of the project. This provided an opportunity to test the reproducibility of the 1-octanol production between different batches of the *E. coli* BL21(DE3) strain.

The first batch of *E. coli* BL21(DE3) biocatalyst cells is referred to as 'old' and the second batch of cells of the same strain is referred to as 'fresh' for ease of reference. Bioconversion batch experiments, with a 1.33ml reaction volume, were conducted in 30ml McCartney bottles to assess the reproducibility of the 1-octanol production performance for the 'old' and for the 'fresh' biocatalyst cells. Six 100ml main cultures were prepared for the assessment. Three of the main cultures were inoculated with the 'old' cells and the remaining three main cultures were inoculated with the 'fresh' cells. The bioconversion temperature and pH were set to 23°C and 7.0 respectively.

The average enzyme concentration achieved by the three 'old' cell main cultures was 0.075±0.002 μmoles CYP153A6 per gram DCW. The three 'fresh' cell main cultures achieved an average concentration of 0.152±0.007 μmoles CYP153A6 per gram DCW - a 102% increase.

The cells of each of the respective main cultures were used as biocatalysts in octane bioconversion reactions. The 1-octanol production per gram dry cell weight achieved by the bioconversion reactions was monitored throughout the reaction period. The experimental data for Figure 17 may be found in Table 15 in Appendix D.

The average 1-octanol production with the 'fresh' biocatalyst cells was significantly larger than the production achieved with the 'old' cells (see Figure 17). After a 25-hour reaction period the 'fresh' cell bioconversions achieved an average concentration of 45±2 mg 1-octanol per gram DCW in comparison to the 'old' cell bioconversions which produced an average of 17±2 mg 1-octanol per gram DCW - a 170% increase in production. This large difference in production indicated that the 'old' and 'fresh' cells were not equivalent biocatalyst cells.

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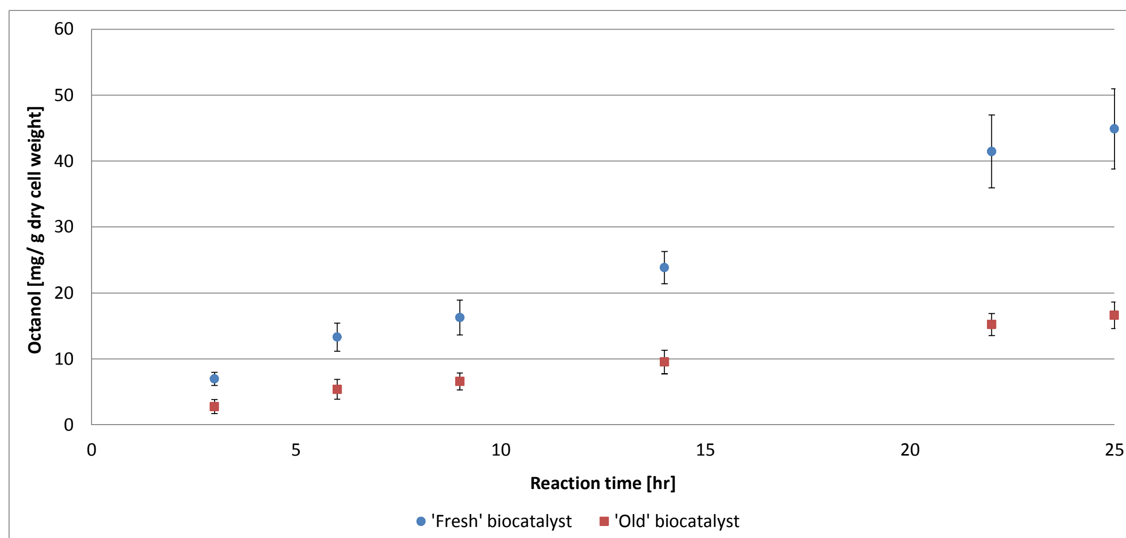

Figure 17: 1-Octanol production for 1.33ml McCartney bottle batch reaction utilising 'old' and 'fresh' biocatalyst cells at 23°C and an initial pH of 7.0

A number of factors may have caused the lack of reproducibility in 1-octanol production across the two batches of the same strain. The six-month storage period that the 'old' cells were exposed to may have placed strain on the cells and therefore have been the cause for the lack of reproducibility. In addition, minor differences between the two batches of cells may have inadvertently been introduced during the genetic modification of the 'fresh' cells. Any differences in the genetic modification of the 'fresh' cells could have had a significant effect on the 1-octanol production performance of the biocatalyst cells. Unfortunately, the McCartney bottle experiments that were conducted could not determine which factors were responsible for the lack of reproducibility in 1-octanol production between the 'old' and 'fresh' biocatalyst cells. All that could be determined was that the 'fresh' cells performed significantly better than the 'old' cells in terms of 1-octanol production.

Utilising biological catalysts poses a number of unique challenges. One of these is to ensure reproducibility across different batches of the same strain of cells. Although it was important to determine the reason for the 'old' and the 'fresh' cells not achieving the same production results, it was decided that this avenue of research was not in the direct scope of the project. All further bioconversion reactions in the project utilised the 'fresh' biocatalyst cells, because of the increased 1-octanol production in comparison to the 'old' cells.

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4.5 Bioreactor under controlled conditions

4.5.1 Batch bioreactor under controlled conditions

A bioconversion batch experiment was performed in a New Brunswick BioFlo 110 Modular Benchtop Fermentor. The purpose of the experiment was to investigate the performance of the biocatalyst cell in a larger reaction volume and in an environment that could control process conditions such as pH and dissolved oxygen concentration. The following factors were monitored and investigated during the batch reactor experiment:

- Cell growth during entire incubation period
- Substrate and product concentrations of the bioconversion reactions
- Comparison of 1-octanol production between bioreactor and McCartney bottle experiments

A three-stage inoculation procedure was used to prepare a 2l main culture as described in Section 3.5.1. A cumulative total of 100ml in samples were taken throughout the main culture growth incubation and induction incubation periods, for cell growth and CO difference spectra analysis data points. A further 100ml sample was taken at the end of the induction incubation period. The cells of this sample were harvested and used to conduct McCartney bottle experiments, following the methods described in Section 3.5.3.1, for the purpose of comparing their bioconversion results with those achieved by the bioreactor. A 100ml glucose and glycerol solution was added to the New Brunswick bioreactor, prior to octane addition in accordance with the experimental methods described in Section 3.5.3.2.

The octane addition to the bioreactor experiment was scaled up proportionally relative to the ratio of grams wet cell weight versus millilitres octane added to the McCartney bottle experiments. The wet cell weight of the 100ml main culture sample was used to estimate the wet cell weight of the remaining 1800ml of the main culture. The equation used to calculate the octane addition to the bioreactor, given below, was based on the equations given in Section 3.5.3.2.

$$1800 ml\ culture \times \frac{3.57 g\ WCW}{100 ml\ culture} \times \frac{1.67 ml\ octane}{1 g\ WCW} = 107.2\ ml\ octane \qquad \text{Equation 11}$$

The initial octane addition for the main culture wet cell weight of 64.3g was thereby determined to be 107.2ml. This translated into an initial substrate concentration of 37.5 mg octane/ml in

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the bioreactor. The McCartney bottle experiments were prepared as described in Section 3.5.3.1 with an initial octane concentration of 176mg octane/ml.

The reaction temperature and pH was set to 23°C and 7.0 respectively for the McCartney bottle and bioreactor experiments. The dissolved oxygen set point in the bioreactor was set at 20% to ensure sufficient dissolved oxygen for the biocatalyst cells.

4.5.1.1 Cell growth during incubation

The cell growth of the *E. coli* cells in the New Brunswick bioreactor was related to an optical density profile. The profile was produced by performing OD measurements on several samples taken throughout the entire incubation period. The experimental data for Figure 18 and the system conditions throughout the bioreactor run may be found in Table 16 in Appendix D.

Only a short lag time was observed in the bioreactor main culture (see Figure 18). This indicated that the three-stage incubation procedure successfully inoculated the main culture with cells close to or in the exponential growth phase. The cells were supplemented with δ-ALA and $FeCl_3$ after 2 hours and 45 minutes and the stationary phase was reached approximately 8 hours later.

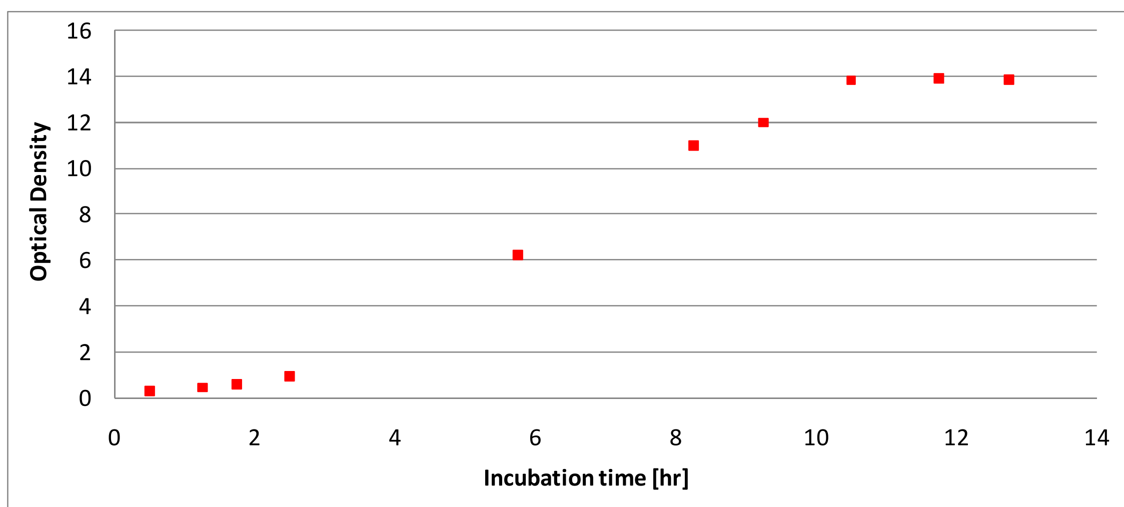

Figure 18: *E. coli* cell growth in New Brunswick bioreactor during incubation period at 23°C and a pH of 7.0

The biocatalyst cells used in the New Brunswick bioreactor and the McCartney bottle bioreactions had an induction incubation period of 10 hours. The enzyme concentration achieved in this time was 0.0134 µmoles CYP153A6 per gram DCW.

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4.5.1.2 Bioconversion under controlled conditions

A bioconversion experiment was performed in a 7.5l BioFlo 110 Modular Benchtop Fermentor (New Brunswick). The substrate concentration and 1-octanol production per gram dry cell weight achieved by the bioconversion reaction was monitored throughout the reaction period. The experimental data for Figure 19 may be found in Table 17 in Appendix D.

The 1-octanol concentration increased for the first 19 hours of the bioreaction and then dropped for the rest of the reaction period (see Figure 19). The 1-octanol concentration started decreasing when the octane in the system had been almost entirely depleted. The formation of an unwanted secondary product was observed during the reaction period, suggesting the possibility of residual endogenous activity. Gas Chromatography Mass Spectroscopy (GCMS) analysis identified the secondary product as octanoic acid. The octanoic acid concentration continued to increase after a 19-hour reaction period in the absence of a significant octane concentration in the system. The production of octanoic acid in the absence of significant amounts of octane suggested that 1-octanol was being converted to octanoic acid.

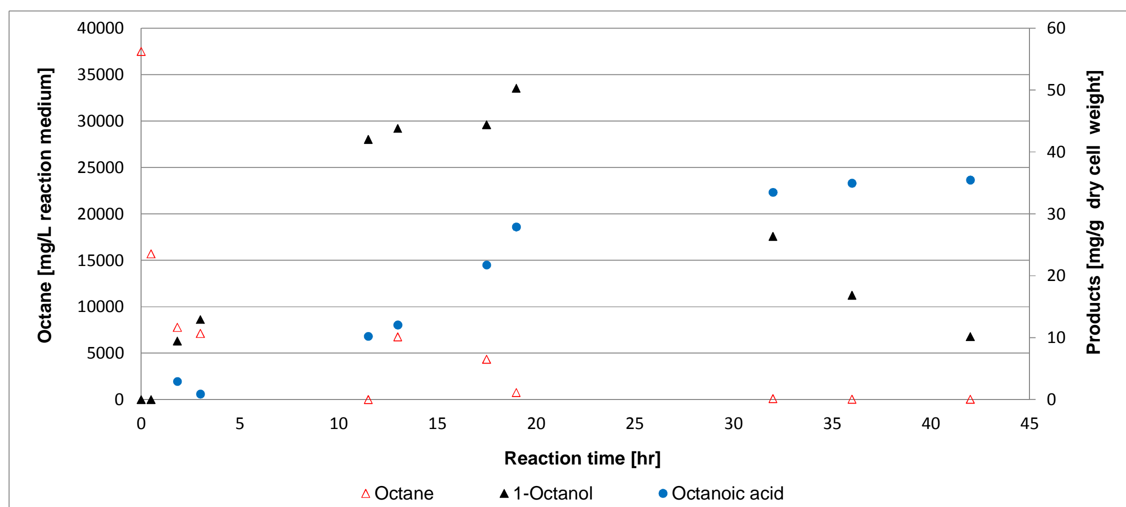

Figure 19: Substrate (octane) and product (1-octanol and octanoic acid) concentrations for New Brunswick bioreactor batch reaction at 23°C and a pH of 7.0

The formation of the unwanted secondary product octanoic acid was a negative finding. The genetically modified *E. coli* cells should not have been able to catalyse the conversion of 1-octanol to octanoic acid. The secondary reaction resulted in a decrease in the desired product concentration. The production of octanoic acid at the expense of 1-octanol was the second negative finding that had been caused by the biocatalyst cells having residual activity with respect to other unwanted metabolic reactions, the first negative finding being that the pH of

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the reaction mixtures could not be maintained at a desired set point due to the unwanted and unexpected metabolic activity of the cells (see Section 4.1.3).

The octane concentration dropped rapidly in the first 3 hours of the bioreaction and was almost completely depleted after a 19-hour reaction period. The drop in octane concentration could have occurred through conversion to products and by-products or through volatilisation and subsequent exiting of the bioreactor system through the ice-cooled exhaust condenser. The 1-octanol and octanoic acid production was too small to explain the large drop in octane concentration. It was therefore suspected that large amounts of the substrate were volatilising and exiting the system before the octane could participate in the bioreaction.

McCartney bottle experiments were conducted with cells grown from the same main culture that was used in the bioreactor experiment for the purpose of comparing the bioconversion results of the two experimental setups. The 1-octanol production per gram dry cell weight achieved in the 1.33ml McCartney bottle batch reaction was monitored throughout the reaction period. The experimental data for Figure 20 may be found in Table 17 in Appendix D.

The 1-octanol production in the 1.33ml McCartney bottle batch reactions was significantly smaller than the production achieved by the New Brunswick bioreactor (see Figure 20). After a 17.5-hour reaction period the New Brunswick bioreactor achieved a concentration of 44.4 mg 1-octanol per gram DCW in comparison to the McCartney bottles which produced 10.9 mg 1-octanol per gram DCW - a 307% increase in production. The higher production of 1-octanol in the New Brunswick bioreactor indicated a faster rate of reaction in the larger reaction environment. The increased rate of reaction was promoted by the increased mixing and aeration in the bioreactor setup in comparison to the McCartney bottle batch reaction.

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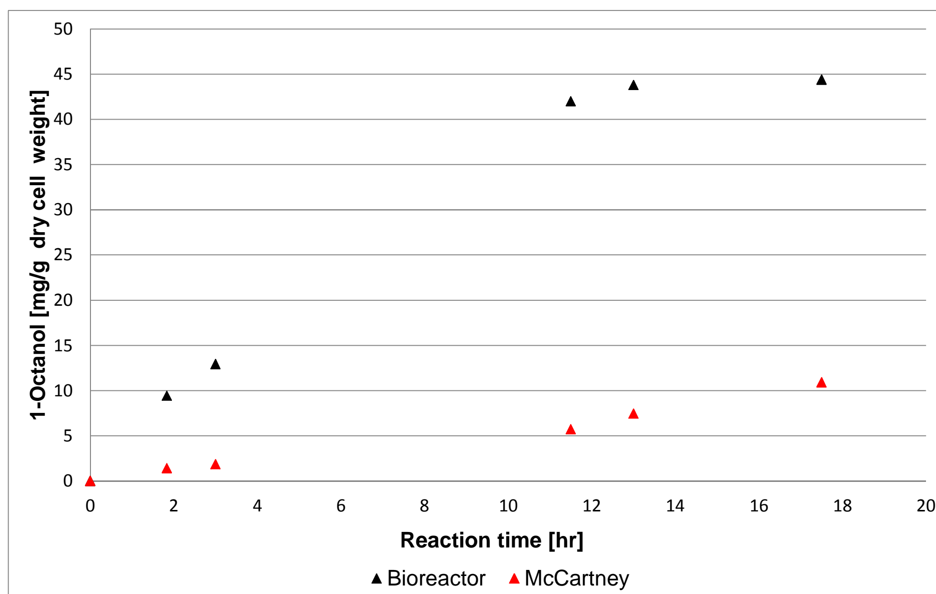

Figure 20: 1-Octanol concentration for New Brunswick bioreactor and 1.33ml McCartney bottle batch reactions at 23°C and a pH of 7.0

The cells used in the McCartney bottle reactions were centrifuged out of the auto-induction medium, in which they were grown, and re-suspended in a pH buffered reaction medium. The cells used in the bioreactor setup were kept in the complex auto-induction medium for the bioreaction. The cells in the bioreactor setup were therefore not exposed to the stress of being centrifuged out of the auto-induction medium. Furthermore, the cells in the bioreactor setup had exposure to the remaining nutrients in the auto-induction medium after the growth induction and induction incubation periods. Both of these factors may have contributed to a more stable biocatalyst cell, which contributed to an increased rate of reaction in the bioreactor compared with that of the McCartney bottle reactions.

The 1.33ml McCartney bottle batch reactions did not produce any by-products. The cells in the 1.33ml reaction were exposed to an excess of octane throughout the reaction period, because the reaction system was closed and no octane could volatilise and exit the system. In the New Brunswick bioreactor batch reaction, the octanoic acid production rate increased when the octane concentration was low (see Figure 19). The availability of excess octane in the 1.33ml reactions may have been a reason for no by-products being produced.

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4.5.2 Quantification of octane volatilisation and mitigating actions

The observations made on the bioreactor batch experiment performed in Section 4.5.1 gave rise to the theory that large amounts of octane was volatilising and exiting the system before the substrate could participate in the bioreaction. This theory needed to be tested and the loss of octane due to volatilisation quantified so that mitigating actions could be taken to ensure that the substrate remained in excess in the system throughout the reaction period.

4.5.2.1 Quantification of octane volatilisation

An identical bioreactor setup to the one described in Section 4.5.1 was prepared to quantify the octane and 1-octanol loss in the system due to volatilisation. The only difference in the experimental setup was that the auto-induction medium in the bioreactor was not inoculated with the biocatalyst *E. coli* cells. This meant that any drop in the initial concentration of the octane and 1-octanol added to the system would be due to the effects of volatilisation. Octane and 1-octanol were added to the system for an initial concentration of 38.1 mg/ml and 4.1 mg/ml respectively. The initial concentrations were chosen in the same order of magnitude as the values obtained in the New Brunswick bioreactor batch experiment with cells. In addition, the conditions in the reactor were maintained at the same levels as the bioreactor batch experiment. The temperature and pH was set to 23°C and 7.0 respectively. The air sparging rate was maintained at 1.6 litres per minute and the agitation rate was set to 250rpm. The agitation rate was based on the average agitation rate in the bioreactor batch experiment. The experimental data for Figure 21 may be found in Table 18 in Appendix D.

The octane concentration profile in the experiment was almost identical to the profile observed in the analogous experiment with cells. The octane concentration dropped rapidly in the first 3 hours of the experiment and was almost completely depleted after a 24-hour reaction period (see Figure 21). As there was no reaction taking place in the bioreactor, the drop in octanol concentration was a clear indication of the volatilisation of the compound. The vapour pressure of octane at 23°C is relatively high at 1.66 kPa (Shuzo, 2009), which was one of the reasons for the rapid volatilisation of the compound. Furthermore, the air sparging and agitation in the system would also have contributed to the rapid volatilisation of the octane.

The 1-octanol concentration did not change significantly throughout the experimental period (see Figure 21). The vapour pressure of 1-octanol at 25°C is 0.019 kPa (www.sigmaaldrich.com, 2011), which is two orders of magnitude smaller than the vapour pressure of octane at the same temperature. The effect of volatilisation on the 1-octanol concentration was therefore

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determined to be negligible. This finding meant that the drop in 1-octanol concentration in the New Brunswick bioreactor batch experiment (see Figure 19) was a result of the 1-octanol being converted to the unwanted secondary product octanoic acid.

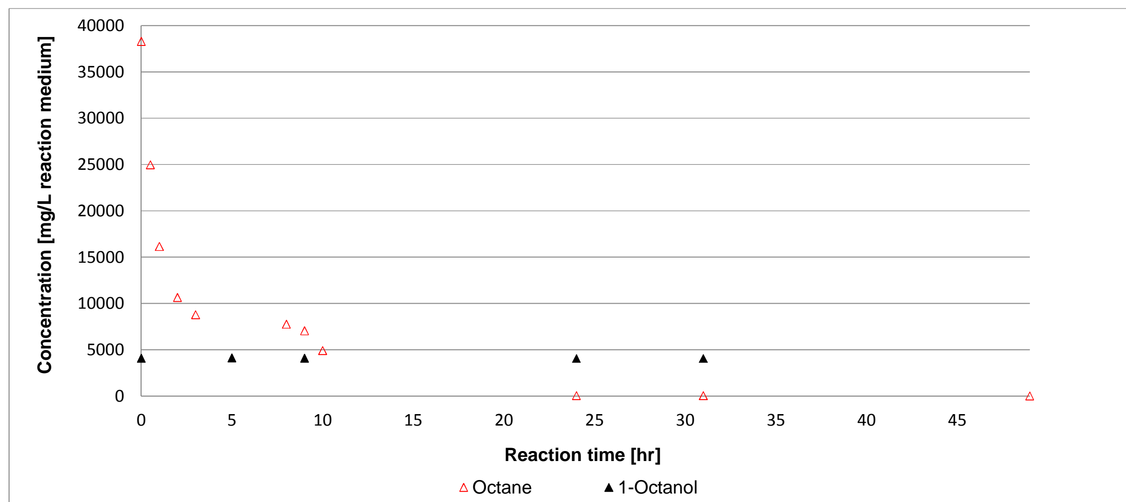

Figure 21: Octane and 1-octanol concentration in New Brunswick bioreactor batch setup without cells at 23°C and a pH of 7.0

The experiment did not test whether octanoic acid would volatilise to a significant degree in the New Brunswick batch reactor setup. The vapour pressure of octanoic acid at 20°C is 2.67 Pa (www.sciencelab.com, 2011), which is an order of magnitude smaller than the vapour pressure of 1-octanol at the same temperature. A compound with a lower vapour pressure is less likely to volatilise. It was therefore assumed that the octanoic acid concentration in the batch reactor setup would not be significantly affected by volatilisation.

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4.5.2.2 Determination of continuous octane feed rate

The volatilisation of octane in the New Brunswick bioreactor setup caused the majority of the substrate to leave the system before it could participate in the bioreaction. The ice-cooled condenser on the exhaust attachment of the New Brunswick bioreactor setup was unable to condense the octane vapours before they could exit the reaction system.

Instead of attempting to prevent the volatilised octane from exiting the system, a constant octane feed was added to the bioreactor setup to maintain a minimum substrate concentration in the reaction system. A fitting that allowed the Primary Control Unit (PCU) to pump octane into the bioreactor vessel was attached to the head plate bioreactor. A Schott bottle containing octane was connected, with a silicone tube, via a PCU controlled peristaltic pump to the octane feed fitting on the head plate. Octane could then be fed into the system to ensure that the substrate concentration remained in excess throughout the reaction period.

An experiment was performed in a 7.5l BioFlo 110 Modular Benchtop Fermentor (New Brunswick) to determine the rate at which the substrate should be fed to ensure that the octane concentration remained in excess throughout the reaction period. The concentration at which the octane was defined as being in excess was 10mg/ml reaction medium. This set point was based on the results of the batch reaction performed in the same bioreactor setup (see Section 4.5.1.2) where the 1-octanol concentration increased while the octane concentration was between 5mg/ml and 10mg/ml (see Figure 19).

The experiment was performed with a 2l auto-induction medium solution prepared in accordance with the experimental methods described in Section 3.5, with the exception of the inoculation of the medium with the biocatalyst *E. coli* cells. Octane was added to the system for an initial concentration of 8.8 mg/ml. In addition, the conditions in the reactor were maintained at the same levels as the bioreactor batch experiment described in Section 4.5.1. The temperature and pH were set to 23°C and 7.0 respectively. The air sparging rate was maintained at 1.6 litres per minute and the agitation rate was set to 250rpm.

The maximum continuous octane feed, at a pump speed of 100%, was 110 g/hr. The octane feed pump speed was varied between 5%, 10%, 15% and 20% to determine at which feed rate the octane concentration in the system could be maintained in excess in spite of the effects of volatilisation. The experimental data for Figure 22 may be found in Table 19 in Appendix D.

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Figure 22 is divided into four sections according to the pump speed of the octane feed pump during the respective time periods. A linear trend line was drawn for each period to determine the rate of change for the octane concentration in the system. The rate of change was determined by taking the derivative of the trend line equation for each of the respective octane feed periods. The rate of change for a 5% and 10% pump speed feed was calculated to be -1.65 g/l/hr and 0.58 g/l/hr respectively. By interpolating between the two pump speed points it was determined that a 9% pump speed feed rate would result in a negligibly small rate of change in the octane concentration in the system. However, the 9% feed pump speed only accounted for the loss of octane caused by volatilisation in the system. A 10% feed pump speed, 11.0 g/hr, was therefore chosen to compensate for the loss of octane in the bioreactor system due to the projected conversion of octane to its corresponding products and for losses caused by volatilisation.

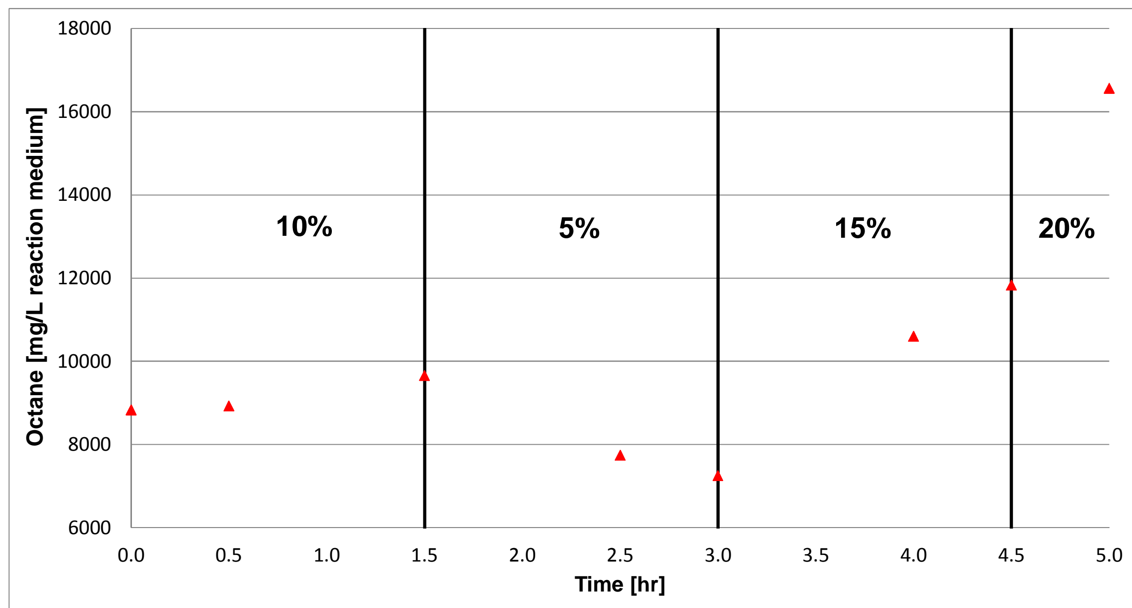

Figure 22: Octane concentration profile for varied feed pump speeds in New Brunswick bioreactor setup without cells at, 23°C and a pH of 7, with an agitation rate of 250rpm and an air sparging rate of 1.6l/min

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4.5.3 Bioconversion under controlled conditions with continual octane feed

Two identical semi-batch bioconversion reactions were performed in a BioFlo 110 Modular Benchtop Fermentor (New Brunswick). The purpose of the experiment was to investigate the performance of the biocatalyst cell in an environment that could control process conditions such as pH and dissolved oxygen concentration, while a constant octane feed into the reaction system ensured that the substrate concentration was maintained above 10g/l.

A three-stage inoculation procedure was used to prepare a 2l main culture, as described in Section 3.5.1. A cumulative total of 100ml samples were taken throughout the main culture growth incubation and induction incubation periods, for cell growth and CO difference spectra analysis data points. A 100ml glucose and glycerol solution was added to the bioreactor prior to octane addition in accordance with the experimental methods described in Section 3.5.3.2.

Octane was added to the system at the start of the bioconversion reaction for an initial concentration of 10g/l. A continuous octane feed of 11.0g/hr was maintained throughout the reaction period. The reaction temperature and pH were set to 23°C and 7.0 respectively and the dissolved oxygen set point in the bioreactor was set at 20% to ensure sufficient oxygen for the biocatalyst cells.

The biocatalyst cells had an induction incubation period of 16 hours and the enzyme concentration achieved in this time was 0.020±0.002 µmoles CYP153A6 per gram DCW. The 1-octanol and octanoic acid production per gram dry cell weight produced by the bioconversion reaction was monitored throughout the reaction period. The experimental data for Figure 23 may be found in Table 20 in Appendix D.

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The two analogous semi-batch bioconversion experiments produced almost identical 1-octanol production results (see Figure 23). The second bioconversion experiment had a 13.5-hour longer reaction period than the first experiment. The 1-octanol concentration of the second experiment levelled off at approximately 600 mg/g dry cell weight, possibly indicating that the reaction was inhibited by the product concentration.

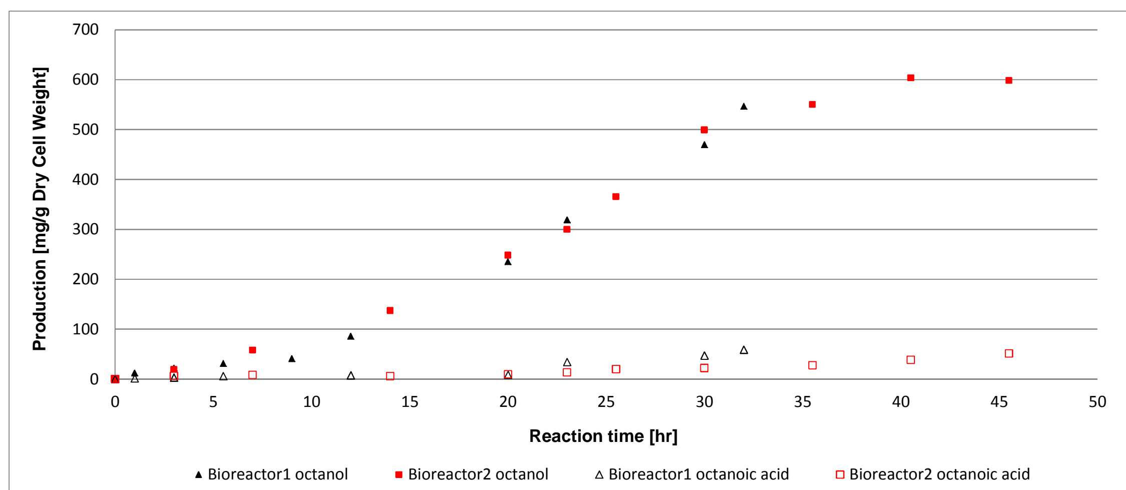

Figure 23: Product (1-octanol and octanoic acid) concentrations for New Brunswick bioreactor semi-batch reaction at 23°C and a pH of 7.0

The 1-octanol production in the semi-batch New Brunswick bioreactor was significantly larger than the best production results achieved by the 1.33ml McCartney bottle batch reactions. The highest 1-octanol concentration achieved by the McCartney bottle reactions was 45±2 mg 1-octanol per gram DCW (see Section 4.4) after a 25-hour reaction period. The semi-batch New Brunswick bioreactor achieved a 1-octanol concentration of 365.4 mg/g DCW after a reaction period of 25.5 hours - an order of magnitude increase in production.

The production of the unwanted secondary product was again observed. The octanoic acid concentration increased throughout the reaction period and reached a maximum concentration of 51.1 mg/g dry cell weight in 45.5 hours at a rate of 1.12 mg/g dry cell weight / hr. The continuous production of the secondary product indicated that the biocatalyst cells remained active for 45.5 hours. In spite of the biocatalyst cells remaining active throughout the reaction period, the production of 1-octanol plateaued once a product concentration of approximately 600 mg/g dry cell weight was reached. This observation supported the theory that the reaction was being inhibited by the product concentration.

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The semi-batch bioreactor setup achieved a significant improvement in the production rate and maximum 1-octanol concentration in comparison to the batch reactor setup with the same reaction conditions (see Figure 24). The semi-batch reaction achieved a production rate of 12.4 mg/g dry cell weight/hr over the first 20 hours of the reaction compared to the 2.6 mg/g dry cell weight/hr over the first 19 hours for the batch reaction. Furthermore, the semi-batch reaction achieved a maximum 1-octanol concentration of 603.4 mg/g dry cell weight at a rate of 14.89 mg/g dry cell weight / hr in comparison to the 50.3 mg/g dry cell weight for the batch reaction. An order of magnitude increase in the maximum 1-octanol concentration produced in the bioconversion reaction was achieved by ensuring that the octane concentration was maintained in excess throughout the reaction period.

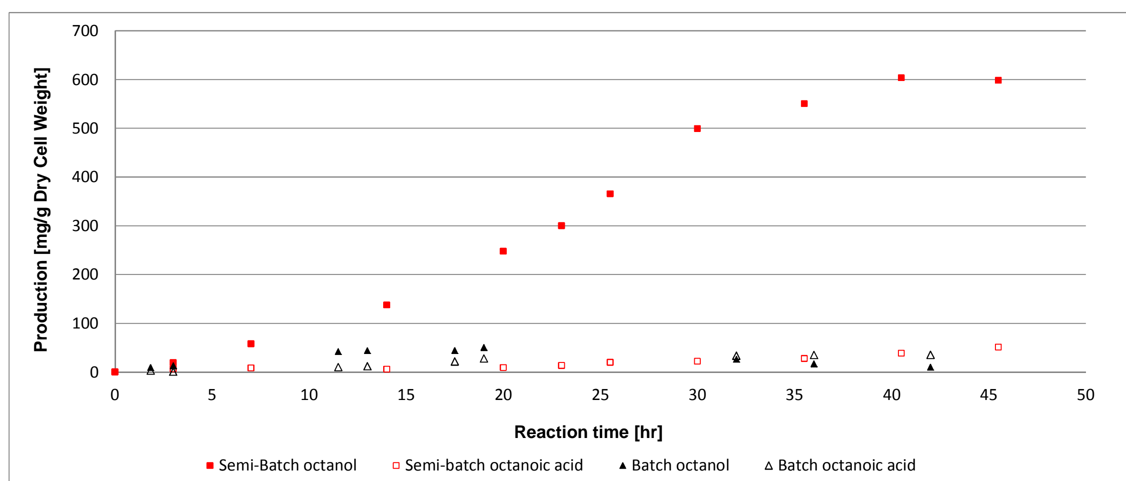

Figure 24: Comparison of product (1-octanol and octanoic acid) concentrations achieved by New Brunswick bioreactor semi-batch and batch reactions at 23°C and a pH of 7.0

The semi-batch reaction produced octanoic acid at a rate of 0.95 mg/g dry cell weight/hr over the first 40.5 hours of the reaction compared to 0.84 mg/g dry cell weight/hr over the first 42 hours for the batch reaction. The similar rate of octanoic acid production was expected because neither of the reactions was limited by a low 1-octanol concentration. The two reactor setups provided identical conditions for the conversion of 1-octanol to octanoic acid, which resulted in similar octanoic acid concentration profiles.

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4.6 Literature comparison

The experimental results of this study were compared only to the experimental results of studies done, on the same subject matter, by researchers in the Paraffin Activation Programme of the Centre of Excellence in Catalysis (South Africa). These researchers utilised the same biocatalyst cells used in this study to perform analytical scale whole-cell biotransformations of n-octane and so were directly comparable. The reasons for limiting the pool of comparative literature are detailed in Section 2.4.

The results and reaction conditions of the biotransformation reactions performed by Gudiminchi *et al.* (2012), Olaofe *et al.* (2013) and this research (under the heading Roux (2011)) are summarised in Table 5.

Table 5: Literature comparison of whole-cell biotransformations of n-octane

Reference	Olaofe *et al.* (2013)	Gudiminchi *et al.* (2012)		Roux (2011)	
Culture medium	LB medium induced with IPTG	LB medium induced with IPTG	Auto-induction	Auto-induction	
Experiment reference number	1	2	3	4	5
Reaction vessel	40ml vial	40ml vial	40ml vial	30ml McCartney bottle	7.5L New Brunswick Bioreactor
Reaction volume, ml	1.33	1.25 - 1.33	1.25 - 1.33	1.33	2000
Reaction pH	7.2	7.2	7.2	7.0	7.0
Growth temperature, °C	25	37	37	30	30
Incubation temperature, °C	20	20	20	23	23
Bioconversion temperature,°C	20	20	20	23	23
Biomass concentration, g DCW/L	3-5	7	7	17.5	3.7
Max octanol production, mg/g DCW	1200	-	-	47	603
Time max octanol, hrs	48	-	-	25	40.5
1-Octanol productivity, mg/g DCW/h	25	-	-	2	15
1-Octanol productivity, g/L/h	0.16	0.46±0.01	0.24±0.01	0.0313±0.0014	0.0552
CYP153A6 conc., μmol/g DCW	0.20	0.5-1.0	0.5-1.0	0.15	0.02

The results documented in Table 5 will be discussed in the section below. Each of the experiments in Table 5 has been assigned an arbitrary reference number from one to five for ease of comparison.

Gudiminchi *et al.* (2012) performed biotransformations with cells grown from auto-induction medium and with cells from LB cultures induced with isopropyl-β-D-thiogalactopyranoside (IPTG). The scale of these biotransformations was comparable to the McCartney bottle experiments performed in this study. Gudiminchi *et al.* (2012) used a reaction mixture with 100mM glucose in comparison to the 40mM used in this research. The specific active CYP153A6 content was between 0.5 and 1.0 μmoles CYP153A6/g DCW for both culture mediums. The cells grown from the auto-induction medium achieved a volumetric production rate of 0.24±0.01

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g/LBRM/h in comparison to the 0.46±0.01 g/LBRM/h achieved by the cells from the LB cultures induced with IPTG, in comparative experiments between the two culture mediums. The auto-induction experiments performed by Gudiminchi *et al.* (2012) thereby achieved an order of magnitude higher 1-octanol productivity than the McCartney bottle and New Brunswick bioreactor experiments performed in this study. The CYP153A6 enzyme concentration achieved by the auto-induction biotransformations (Experiment 3) was roughly 3 – 6 times larger than that of the New Brunswick bioreactor reactions and an order of magnitude larger than the McCartney bottle reactions (see Table 5). The large difference in 1-octanol productivity and CYP153A6 enzyme concentration between the McCartney bottle experiments and Experiment 3 was unexpected, because the reaction conditions were similar, and the reaction methodology of this study was based directly on the methods used in Gudiminchi *et al.* (2012).

The difference in the glucose concentration of the respective reaction mixtures used in this research and that of Gudiminchi *et al.* (2012) may have been a significant factor in this research, achieving reduced 1-octanol production results. The glucose was added to the reaction mixture to regenerate NADH from NAD^+. The smaller glucose concentration in the reaction mixtures used in this research may therefore have reduced the amount of NADH available for the catalysation of the octane hydroxylation.

Olaofe *et al.* (2013) performed biotransformations with cells grown from LB cultures induced with IPTG. The scale of these biotransformations was comparable to the McCartney bottle experiments performed in this study. The biotransformations were performed at varied reaction temperatures, with a 1-octanol production of 16 mg/g DCW/hr being achieved at 20°C. The 1-octanol production achieved by this research's McCartney bottle experiments was significantly lower at 2 mg/g DCW/hr. The New Brunswick reactor performance achieved almost the same production at 15 mg/g DCW/hr.

Table 5 shows that even within the c*Change Paraffin Activation research group we find different conditions for growing the same biocatalyst cells and performing the biotransformations. Until the incubation and reaction conditions are standardised, the task of comparing results of different researchers will remain a difficult one. Additionally, the method of reporting results of biotransformation reactions has not been standardised, which creates further difficulties in making direct comparisons between different pieces of research.

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5. OUTCOMES OF REACTION MODELLING

This section will discuss the reaction modelling work that was conducted to address the "Reaction modelling" hypotheses listed in Section 2.6.2. The purpose of this section was to determine a rate law to model the 1-octanol production in the New Brunswick bioreactor. Theoretical and empirical methods of determining the 1-octanol rate of reaction were attempted.

5.1 Theoretical rate law development

The first step in modelling the 1-octanol production of the bioconversion reaction was to develop a theoretical rate law. The first attempt at developing the theoretical rate of reaction was based on the reaction mechanism described in Section 2.5.3. Seven elementary reactions with seven active intermediates were set up, based on the cytochrome P450 catalytic reaction cycle.

Reaction mechanism

1. P450 + $(NADH/H^+)$ ↔ $(NADH/H^+/P450)$
2. $(NADH/H^+/P450)$ + R-H ↔ $(NADH/H^+/P450/R\text{-}H)$
3. $(NADH/H^+/P450/R\text{-}H) + O_2$ ↔ $(NADH/H^+/P450/R\text{-}H/O_2)$
4. $(NADH/H^+/P450/R\text{-}H/O_2)$ ↔ $H_2O + (NAD^+/P450/R\text{-}OH)$
5. $(NAD^+/P450/R\text{-}OH)$ ↔ R-OH + $(NAD^+/P450)$
6. $(NAD^+/P450)$ ↔ (NAD^+) + P450
7. $(NAD^+) + H_2$ ↔ $(NADH/H^+)$

The round brackets denote the active intermediates in the elementary reactions.

The following assumptions were made in regards to the seven elementary reactions that describe the cytochrome P450 catalytic reaction cycle:

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1. The forth elementary reaction proceeds in only the forward direction. Water is released as a product in the forward direction. The likelihood that the water will react with the (NAD^+/P450/R-OH) active intermediate is very small. The reverse reaction is therefore assumed to be negligibly small.
2. The fifth elementary reaction proceeds in only the forward direction. The alcohol product is released from the active site of the cytochrome P450 enzyme. It is very unlikely that the product will rebind itself to the active site once it has been released. The reverse reaction was therefore assumed to be negligibly small.
3. The seventh elementary reaction proceeds in only the forward direction. The enzyme is returned to its initial state in the forward reaction by reacting with hydrogen. Once the enzyme is in its initial state it is very unlikely that the enzyme will spontaneously react with itself to go back to its depleted form. The reverse reaction was therefore assumed to be negligibly small.

The following rate equations were derived from the seven elementary reactions to determine the theoretical rate law:

$r_1 = k_1 [P450][NADH/H^+]$ $\qquad r_{-1} = k_{-1} [P450][NADH/H^+/P450]$

$r_2 = k_2 [NADH/H^+/P450][R\text{-}H]$ $\qquad r_{-2} = k_{-2} [NADH/H^+/P450/R\text{-}H]$

$r_3 = k_3 [NADH/H^+/P450/R\text{-}H][O_2]$ $\qquad r_{-3} = k_{-3} [NADH/H^+/P450/R\text{-}H/O_2]$

$r_4 = k_4 [NADH/H^+/P450/R\text{-}H/O_2]$

$r_5 = k_5 [NAD^+/P450/R\text{-}OH]$

$r_6 = k_6 [NAD^+/P450]$ $\qquad r_{-6} = k_{-6} [NAD^+][P450]$

$r_7 = k_7 [NAD^+][H_2]$

The overall total enzyme concentration was assumed to be constant and could therefore be described as follows:

$$[P450]_{total} = X = [P450] + [NADH/H^+/P450] + [NADH/H^+/P450/R\text{-}H] + [NADH/H^+/P450/R\text{-}H/O_2] + [NAD^+/P450/R\text{-}OH] + [NAD^+/P450]$$

Where the concentration of the P450 enzymes that were free in the solution and, therefore, not actively partaking in the reaction was denoted as [P450], and where X was defined as a constant. The theoretical rate law could then be determined through algebraic manipulation

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and by applying the pseudo-steady-state hypothesis to the rate equations derived from the seven elementary reactions. The calculations for the development of the rate equation can be found in Appendix E.

$$r_{R-OH} = \frac{([P450]_{total} - [P450])\, k_2 k_3 k_4 k_5 k_6 k_7 [O_2][H_2][R{-}H]}{\begin{gathered}(k_{-3} + k_4) k_2 k_5 k_6 k_7 [H_2][RH] + k_2 k_3 k_5 k_6 k_7 [O_2][H_2][R{-}H] + \\ k_2 k_3 k_4 k_5 k_6 k_7 [O_2][H_2][R{-}H] + (k_{-6} [P450] + k_7 [H_2]) k_2 k_3 k_4 k_5 [O_2][RH] + \\ (k_{-2} + k_3 [O_2])(k_{-3} + k_4) k_5 k_6 k_7 [H_2] + k_{-3} k_3 k_5 k_6 k_7 [O_2][H_2]\end{gathered}}$$

Equation 12

The theoretical rate law was successfully expressed in terms of only measurable quantities. Unfortunately, the rate law was described in terms of nine different reaction constants, which made the 1-octanol production almost impossible to model. Further simplifications of the reaction model were attempted, but no significant reduction in the complexity of the theoretical rate law could be achieved. The topic of developing a theoretical rate law, based on the reaction mechanism described in Section 2.5.3, was discussed at the 2011 c*Change Symposium, in Johannesburg, by the Paraffin Activation research group. The consensus view was that this method of developing a rate law was unable to reduce the complexity of the rate law to such an extent that the 1-octanol production rate could be successfully modelled. It was therefore decided that a different method of modelling the 1-octanol production needed to be developed.

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5.2 Empirical rate law development

The empirical model was based on the octane substrate concentration and 1-octanol production results of the New Brunswick semi-batch bioreactor experiments. It was observed that the profile of the 1-octanol production results (see Figure 24) resembled that of the specific growth rate of the Monod equation when plotted against the concentration of the limiting substrate (see Figure 2).

The biocatalyst cells remained active throughout the reaction period, but the production of 1-octanol plateaued once a product concentration of approximately 600 mg/g dry cell weight was achieved. A product inhibition term was therefore added to the adapted Monod equation. The final empirical model is described by Equation 2 (see Section 2.5.2) and is given again below for ease of reference:

$$\frac{dC_P}{dt} = K_1 \frac{C_S}{C_S + K_S} \left(1 - \frac{C_P}{C_P^*}\right)^n \qquad \text{Equation 2}$$

Where C_P - 1-Octanol concentration [mg/g DCW]

C_P^* – Limiting 1-octanol concentration [mg/g DCW]

C_S – Octane concentration [mg/g DCW]

n – Poisoning order of the rate law

K_1 – Reaction constant [mg/g DCW]

K_S – Monod reaction constant [mg/g DCW]

The empirical rate law was therefore expressed in terms of four reaction constants instead of the nine constants of the theoretical rate law based on the cytochrome P450 catalytic reaction cycle. This made the determination of the reaction constants and the modelling of the 1-octanol production significantly less complex.

A MATLAB function was developed by Lidia Auret, a lecturer in the Chemical Engineering Department of the University of Stellenbosch, to determine the value of the four reaction constants of the empirical rate law, and thereby model the 1-octanol production. The values of the four reaction constants were determined by minimizing the sum of squares error between the actual and the model predicted 1-octanol production. The full function, with comments, can

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be viewed in Appendix F. The function required the following inputs to model the 1-octanol production:

- Experimental data of 1-octanol production concentration–time profile
- Function of octane substrate concentration–time profile
- An estimation of the poisoning order (n) of the rate law
- An estimation of the limiting 1-octanol concentration
- An estimation of the reaction constants K_1 and K_s

The function then generated the following outputs:

- A graph of the 1-octanol concentration–time profile predicted by the model with the experimental data also plotted on the graph
- Values for the four reaction constants; K_1, C_P^*, n, K_S
- The cumulative least squares prediction error of the model
- A variable containing the model prediction values in spread sheet format, for the purpose of exporting the information to Excel.

The MATLAB modelling function also required that the initial lag period in the 1-octanol production concentration-time profile be removed (see Figure 25). The initial lag period was, therefore, not accounted for in the model. When comparing the results of multiple experiments, the same amount of lag period had to be removed from each of the 1-octanol production concentration–time profiles.

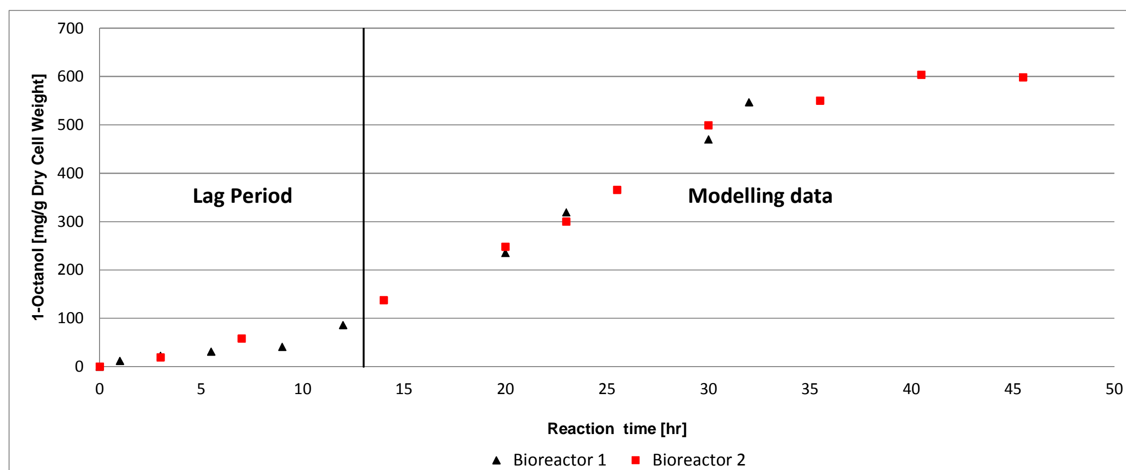

Figure 25: Manipulation of 1-octanol production data used for MATLAB model of New Brunswick semi-batch bioreactor at 23°C and a pH of 7.0

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A 13-hour lag period was removed from the 1-octanol production concentration–time profiles of the two identical New Brunswick semi-batch bioreactor experiments that were conducted (see Figure 26). The 1-octanol production results of the second semi-batch bioreactor experiment were fed into the MATLAB function to model the 1-octanol production. The experimental data for Figure 26 may be found in Table 21 in Appendix D.

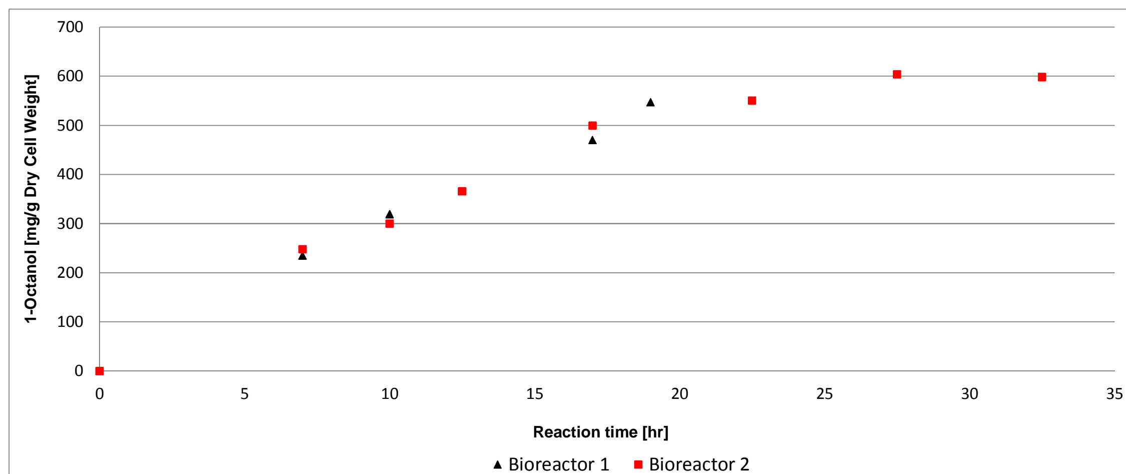

Figure 26: 1-Octanol production of New Brunswick semi-batch bioreactor at 23°C and a pH of 7.0 with lag period removed

The octane feed rate function for the second semi-batch bioreactor experiment was determined by plotting experimental data of octane concentration measurements taken throughout the reaction period (see Figure 27). A linear trend line was then plotted to determine the octane substrate feed rate function. The feed rate function could then be entered into the MATLAB function to model the 1-octanol production. The experimental data for Figure 27 may be found in Table 22 in Appendix D.

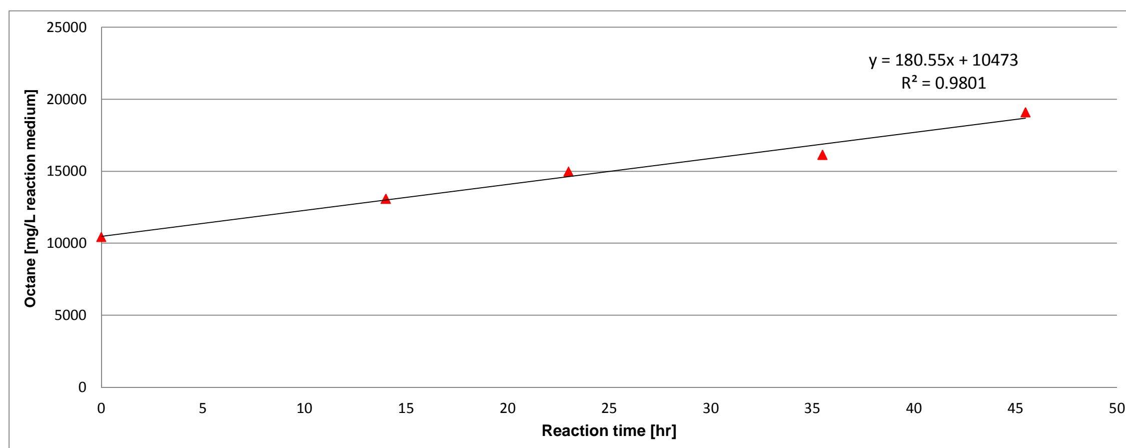

Figure 27: Octane feed to New Brunswick semi-batch bioreactor at 23°C and a pH of 7.0

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The limiting 1-octanol concentration estimate for the MATLAB modelling function was based on the highest concentration achieved in the second New Brunswick semi-batch bioreactor experiment (see Figure 26). The poising order (n) and reaction constants k_1 and k_s were assigned arbitrary values. The input variables fed into the modelling function are given below:

- Maximum 1-octanol concentration, C_P^* = 603.4 [mg/g dry cell weight]
- Poising constant, n = 1
- Reaction constant K_1 = 100 [mg/g dry cell weigh]
- Reaction constant K_S = 100 [mg/g dry cell weight]

The output results of the modelling function are given below:

- Maximum 1-octanol concentration, C_P^* = 598.4 [mg/g dry cell weight]
- Poising constant, n = 0.42
- Reaction constant K_1 = 39.7 [mg/g dry cell weight]
- Reaction constant K_S = 276.3 [mg/g dry cell weight]
- Cumulative least squares error = 1.415e+3

The 1-octanol concentration–time profile predicted by the empirical model gave a close approximation of the actual 1-octanol production performance achieved by the two identical New Brunswick semi-batch bioreactor experiments (see Figure 28). The 1-octanol prediction results produced by the empirical model may be found in Table 23 in Appendix D.

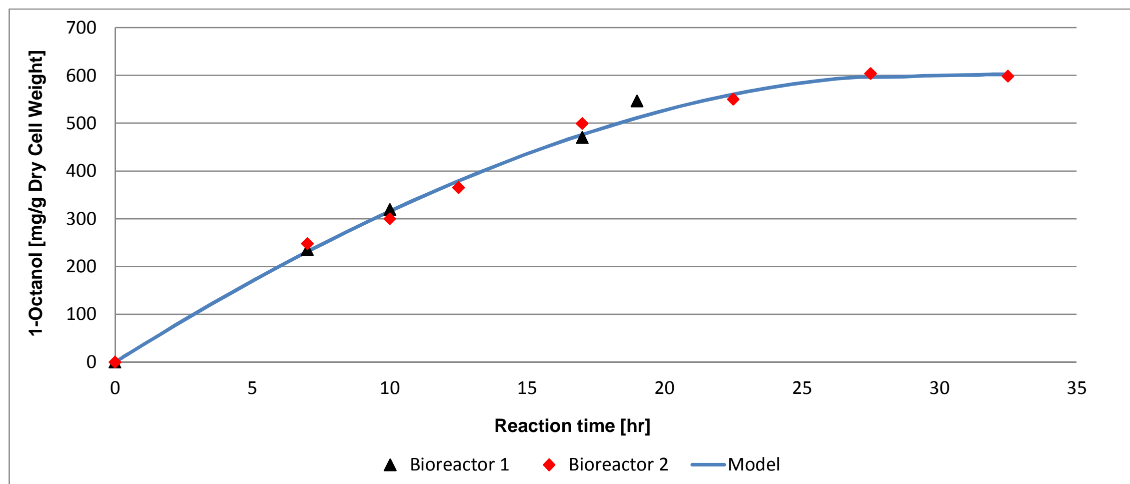

Figure 28: Empirical model 1-octanol prediction results compared to experimental results of two identical New Brunswick semi-batch bioreactors at 23°C and a pH of 7.0

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The 'fit' of the empirical model was evaluated by calculating the average percentage Root Mean Square Error (RMSE) between the predicted and the actual 1-octanol production values. The average RMSE was 2.6% and 3.5% for the first and second New Brunswick semi-batch experiments respectively (see Table 24 and Table 25 in Appendix D for calculations). A variance of up to 5% was expected on the experimental 1-octanol production data, due to the cumulative effects of minor errors inherent in sampling and analysing reaction samples. For this reason, the average RMSE values for both of the New Brunswick semi-batch reactions were determined to be acceptably small. The modelling function was therefore judged to be accurate in its representation of the 1-octanol production within the range of experimental data and conditions tested for.

The final empirical model is given in equation form below.

$$\frac{dC_P}{dt} = 39.7\ \frac{C_S}{C_S + 276.3}\left(1 - \frac{C_P}{598.4}\right)^{0.42} \qquad \text{Equation 13}$$

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6. CONCLUSIONS

The conclusions arrived at are discussed, and their significance considered, under the same headings and in the same order as the hypotheses were introduced in Section 2.6, namely biotransformation experiments and reaction modelling. An additional subsection was added to deal with the experimental challenges that were experienced. The following conclusions were derived from the results and discussion section:

6.1 Biotransformation experiments

1. ***E. coli* BL21(DE3) containing the CYP153A6 operon in pET 28 vector is a more suitable biocatalyst cell strain for 1-octanol production than *E. coli* BL21(DE3)pLysE containing the CYP153A6 operon in pET 28 vector.**

   The suitability of the two strains was assessed according to enzyme production, 1-octanol production and pH stability during the bioconversion process. The final conclusion was based on the following observations:

   - *E. coli* BL21(DE3) produced a 35%-40% higher active CYP153A6 enzyme concentration
   - *E. coli* BL21(DE3) produced more 1-octanol based on the qualitative observations of a rudimentary gas chromatography (GC) analysis
   - Neither strain showed a significant advantage over the other in terms of pH stability and 1-octanol production.

   The *E. coli* BL21(DE3) biocatalyst cell strain was determined to be the most suitable strain for 1-octanol production on the grounds of the higher active CYP153A6 enzyme concentration produced, in comparison to the E. coli BL21(DE3)pLysE strain, so supporting the hypothesis. Furthermore, it was suspected that a more accurate GC analyses would have shown the *E. coli* BL21(DE3) strain to produce more 1-octanol than the *E. coli* BL21(DE3)pLysE strain.

   This finding is significant, because very little information exists in literature in regards to comparisons between the two strains that were investigated in this research.

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2. **The active CYP153A6 enzyme concentration is directly related to the 1-octanol production achieved in biotransformation reactions. An increase in the CYP153A6 concentration will result in a proportionally large increase in the 1-octanol production.**

   McCartney bottle batch experiments with induction incubation periods of 16 hours and 20 hours produced average CYP153A6 enzyme concentrations differing by less than 5%. However, the cells incubated for 20 hours produced an average of 32% more 1-octanol per gram dry cell weight, after a 15-hour reaction period, than the cells incubated for 16 hours. If the relationship between 1-octanol production and CYP153A6 enzyme concentration had been directly proportional, the two McCartney bottle experiments should have produced similar amounts of 1-octanol. Since this was not observed, the relationship between 1-octanol production and active CYP153A6 enzyme concentration was determined to be nonlinear.

   The implication of a nonlinear relationship between 1-octanol production and enzyme concentration is that it is difficult to predict how the reaction system will be affected by changes in the enzyme concentration without performing further experimental work. The finding is significant in that it refutes the simplifying assumption that the relationship between the 1-octanol production and enzyme concentration is linear, and therefore points to the necessity of determining the actual relationship between the two factors.

3. **The CYP153A6 enzyme, expressed in *E. coli* BL21(DE3), is also capable of catalysing the oxidations of decane ($C_{10}H_{22}$), undecane ($C_{11}H_{24}$) and dodecane ($C_{12}H_{26}$) to their corresponding alcohols.**

   McCartney bottle batch experiments with the three different alkane substrates were performed to test the hypothesis. The gas chromatography (GC) analysis of reaction samples taken after a 22-hour reaction period found no product peaks for any of the substrates tested. The hypothesis was thereby refuted.

   The implication of this finding is that Randall's (2010) statement that the CYP153A6 enzyme is able to catalyse the oxidation of C6 to C11 alkanes with a regiospecificity of 95% for the terminal carbon position could not be confirmed. However, not enough experimental work was conducted to conclusively disprove the statement.

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4. **Octane biotransformation reactions, utilising recombinant *E. coli* BL21(DE3) cells as a catalyst, will produce no unwanted by-products as a result of secondary reactions.**

The 1.33ml McCartney bottle batch reactions did not produce any by-products in any of the experimental work that was conducted. However, the formation of an unwanted secondary product was observed for reactions conducted in the New Brunswick bioreactor, suggesting the possibility of residual activity with respect to other unwanted metabolic reactions. A gas chromatography mass spectroscopy (GCMS) analysis identified the secondary product as octanoic acid. Further experimental work determined that the octanoic acid in the system was the result of the 1-octanol product being converted to the secondary product.

The formation of the unwanted secondary product octanoic acid, at a maximum concentration of 51.1 mg/g DCW in 45.5 hours, was a major finding of this research. The recombinant biocatalyst cells had been developed to catalyse only the conversion of octane to 1-octanol and should therefore not have been able to catalyse any secondary reactions. However, the production of octanoic acid indicated that this assumption was false. Furthermore, the unwanted secondary reaction resulted in a decrease in the desired product concentration.

The major implication of this finding is that there is room for improvement in the development of the biocatalyst cell strain. The biocatalyst cell's residual activity with respect to other unwanted metabolic reactions is an issue that needs to be addressed before the biocatalyst cell strain can be considered a viable option for utilisation in large-scale processes.

5. **1-octanol production performance is improved under controlled conditions in a bioreactor.**

E. coli BL21(DE3) cells grown from the same main culture were used in New Brunswick and McCartney bottle batch bioconversion experiments, to compare the 1-octanol production performance in the two setups. The 1-octanol production in the New Brunswick bioreactor was significantly larger than the production achieved by the 1.33ml McCartney bottle batch reactions. After a 17.5-hour reaction period, the New Brunswick bioreactor achieved a concentration of 44.4 mg 1-octanol per gram DCW in comparison to the McCartney bottles, which produced 10.9 mg 1-octanol per gram DCW - a 307% increase in production.

The volatilisation of octane in the New Brunswick bioreactor setup caused the majority of the substrate to leave the system before it could participate in the bioreaction. The New

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Brunswick bioreactor setup was therefore modified by the addition of a constant octane feed to counteract the effects of volatilisation on the substrate concentration in the system. The 1-octanol production in the semi-batch New Brunswick bioreactor was significantly larger than the best production results achieved by the 1.33ml McCartney bottle batch reactions. The highest 1-octanol concentration achieved by the McCartney bottle reactions was 45±2 mg 1-octanol per gram dry cell weight (DCW) after a 25-hour reaction period. The semi-batch New Brunswick bioreactor achieved a 1-octanol concentration of 365.4 mg/g DCW after a reaction period of 25.5 hours - an order of magnitude increase in production.

The order of magnitude increase of 1-octanol production achieved by the bioreactor under controlled conditions compared to the 1.33ml McCartney bottle batch reactions was a major finding of this research. At the time of writing, bioconversion reactions utilising the biocatalyst cell strain and reaction system used in this research had not been reported on as large a scale in any of the literature studied. The improved production results achieved in the bioreactor therefore gave the first insight into the potential that this technology holds for scaling up into larger scale processes.

6. **The biocatalyst cells will remain active under controlled conditions in a bioreactor for a reaction period of up to 48 hours.**

The octanoic acid concentration in the New Brunswick batch and semi-batch bioreactor experiments increased throughout the reaction periods of 42 hours and 45.5 hours respectively. The biocatalyst cells therefore remained active throughout the reaction period, but the production of 1-octanol plateaued once a product concentration of approximately 600 mg/g dry cell weight was produced.

The period for which a biocatalyst cell can remain active in a reaction system is an important factor in determining whether the biocatalyst cell is a suitable candidate for a large-scale production process. If the period of activity is too short, the biocatalyst cell can be screened out as a potential candidate for use in such a process. The finding of this hypothesis was that the biocatalyst cell strain could remain active for a minimum of approximately two days; the maximum period of activity was not established. Based on the finding that the biocatalyst cell strain used in this research remained active for at least 45.5 hours, this strain cannot be discounted for potential use in a larger scale process.

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6.2 Reaction modelling

7. The 1-octanol production under controlled conditions in a bioreactor can be modelled by determining the theoretical rate of reaction, based on seven elementary reactions derived from the seven steps of the cytochrome P450 reaction cycle.

A theoretical rate law of the 1-octanol production in the New Brunswick bioreactor was developed according to the seven steps of the cytochrome P450 reaction mechanism. The theoretical rate law was successfully expressed in terms of only measurable quantities through algebraic manipulation and by applying the pseudo-steady-state hypothesis to the rate equations derived from the seven elementary reactions. Unfortunately, the rate law was described in terms of nine different reaction constants, which made the 1-octanol production almost impossible to model.

Further simplifications of the reaction model were attempted, but no significant reduction in the complexity of the theoretical rate law could be achieved. The consensus view of the c*Change Paraffin Activation research group was that this method of developing a rate law was incapable of reducing the complexity of the rate law to such an extent that the 1-octanol production rate could be successfully modelled.

8. The 1-octanol production under controlled conditions in a bioreactor can be modelled by determining the rate of reaction by empirical means.

The empirical production model was based on the octane substrate concentration and 1-octanol production results of the New Brunswick semi-batch bioreactor experiments. The empirical rate law was based on the form of the Monod equation, with the addition of a product inhibition term.

The empirical rate law was expressed in terms of four reaction constants. A MATLAB function developed by Lidia Auret (see Section 5.2) was used to determine the value of the reaction constants, and thereby model the 1-octanol production. The values of the four reaction constants were determined by minimizing the sum of squares error between the actual and the model predicted 1-octanol production.

The 'fit' of the empirical model was evaluated by calculating the average percentage Root Mean Square Error (RMSE) between the predicted and the actual 1-octanol production values. The average RMSE was 2.6% and 3.5% for the first and second New Brunswick semi-

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batch experiments respectively. A variance of up to 5% was expected on the experimental 1-octanol production data, due to the cumulative effects of minor errors inherent in sampling and analysing reaction samples. For this reason, the average RMSE values for both of the New Brunswick semi-batch reactions were determined to be acceptably small. The modelling function was therefore determined to be accurate in its representation of the 1-octanol production within the range of experimental data and conditions tested for.

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7. RECOMMENDATIONS

What follows are recommendations to guide future research.

7.1 Opportunities for future research

1. **The New Brunswick bioreactor setup could be expanded to include a membrane-separating unit.**

   A membrane bioreactor (MBR) setup would allow for continuous operation of the bioreactor setup. The biocatalyst cells would be retained or immobilised within the system, while reaction products could pass through the membrane and then exit the system. The product inhibition problems that were encountered with the semi-batch reactor system used in this research could be negated by manipulating a recycle stream in the continuous MBR system. The recycle stream would be used to maintain the product concentration below the point where it could inhibit the bioconversion reaction. Furthermore, a continuous reaction system is likely to be more attractive in terms of being scaled up to an industrial size system, because of the larger production capacity in comparison to batch and semi-batch systems.

   The original scope of this research had included the use of a membrane bioreactor to investigate the potential of this technology. However, as the project progressed, it became clear that the time available to the project would not be sufficient to develop a working MBR system. As a result of this, the addition of a membrane separating unit to the New Brunswick bioreactor setup was regrettably excluded from the final scope of the project.

   Before the decision to move away from an MBR system was taken, a methodology on how to develop a MBR system had already been completed. The methodology was divided into the four main sections listed here:

   i. Reactor configuration
   ii. Membrane selection
   iii. Biocatalyst considerations
   iv. Immobilisation technique

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As this research no longer fell directly within the scope of the project, the methodology was placed in Appendix B.

2. **Research could be conducted to improve the current biocatalyst cell strain and to find other strains that can catalyse the hydroxylation of alkanes.**

One of the most exciting aspects of bioprocessing engineering is the potential for finding new strains of biocatalyst cells and enzymes that have the inherent ability to catalyse reactions that are complex and/or expensive to perform chemically. The potential for reducing the complexity and cost of hydroxylating alkanes with biocatalysts is significantly large enough for researchers to continue to screen for strains of cells that might have the ability to do so.

The biocatalyst cell used in this research catalysed a secondary reaction to produce the unwanted by-product octanoic acid during experimental work. Any secondary reactions were unwanted, because they negatively affected the production of the desired product. For this reason, it is recommended that further research be conducted to genetically modify the current biocatalyst cell strain so that it does not have the ability to catalyse any secondary reactions. Alternatively, research could be conducted on screening different strains of cells in the hope of finding a better biocatalyst cell strain than the one used in this project.

3. **A standardised methodology for performing bioconversion reactions and reporting results could be developed.**

One of the difficulties experienced while performing this research was finding literature data with which to compare the results of this study. Direct comparisons between results were only possible with research groups that utilised the same incubation methodology, biocatalyst cell strain and alkane substrate. Since the list of conditions that needed to be the same was a long one, the number of possibilities for direct comparison diminished significantly. Furthermore, the methodology of reporting results was found to vary significantly between research groups. This added to the difficulty of making comparisons between the results reported in literature.

It is recommended that members within the same research group make an effort to standardise the methodology for performing bioconversion reactions and reporting results. This would allow for simple comparisons between results and prevent confusion that could lead to duplication of work. Until this is achieved, the task of comparing results of different researchers will remain a difficult one.

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7.2 Challenges related to experimental work that need to be addressed

1. Stability of reaction mixture pH

The pH of the reaction mixture in the McCartney bottle bioconversion experiments was expected to remain stable throughout the reaction period. However, this expectation proved to be false as a stable pH could not be maintained in the McCartney bottle experiments. This was a significant finding, because the observation disproved the UFS research team's assumption that the pH of this reaction system would remain constant.

The implication of this finding is that future research on this reaction system will need to develop a method of maintaining a stable pH in experimental setups that do not have the ability to actively monitor and control the pH. Alternatively, future research could utilise a bioreactor that can control the pH of the reaction system, similar to the bioreactor that was used in this study.

2. Reproducibility of biocatalyst cell performance

The reproducibility of the 1-octanol production performance of different batches of the *E. coli* BL21(DE3) strain was investigated. It was expected that the 1-octanol production performance would be reproducible between different batches of the same strain of biocatalyst cell. However, this expectation proved to be false as differing 1-octanol production performances were observed for the different batches of the same strain of biocatalyst cell.

The investigation could not conclusively prove what had caused the difference in 1-octanol production performance, but the observation was a significant finding nonetheless. The finding refutes the assumption that 1-octanol production performance is reproducible across different batches of the same strain.

The implication of this finding is that future research will have to investigate the reasons for the lack of reproducibility between different batches of the same biocatalyst cells. The procedures to grow, maintain and store the biocatalyst cells may then have to be modified to avoid factors that could affect the performance of the biocatalyst cell.

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3. Octane volatilisation in bioreactor setup

The research highlighted the issue of octane volatilisation in bioreactor setup. Prior to performing the octane bioconversion experimental work in the bioreactor setup, it was expected that the ice-cooled condenser would be able to prevent the volatilised octane substrate from leaving the system. However, this expectation proved to be false, and a constant octane feed had to be added to the bioreactor setup to maintain the octane substrate concentration at an excess. The constant octane feed was able to perform its function satisfactorily, but this came at a heavy cost in terms of volatilised octane that exited the system without being involved in the desired conversion reaction.

The implication of this finding is that future research on this reaction system will need to develop a more cost effective method of preventing the volatilised octane substrate from leaving the system.

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APPENDIX A: NEW BRUNSWICK BIOREACTOR SCHEMATICS

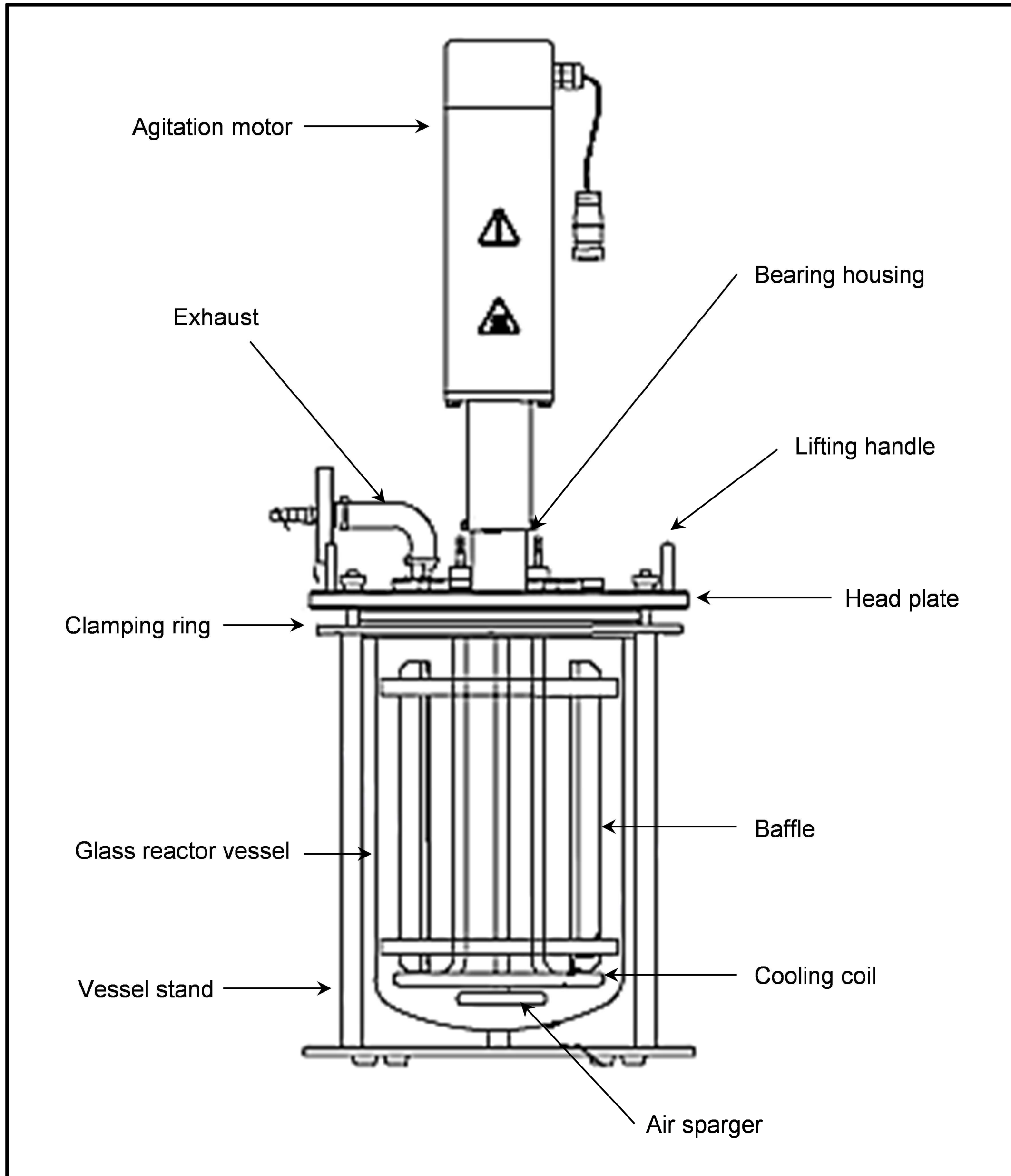

Figure 29: Side view schematic of New Brunswick bioreactor (redrawn from New Brunswick Scientific Co., 2007)

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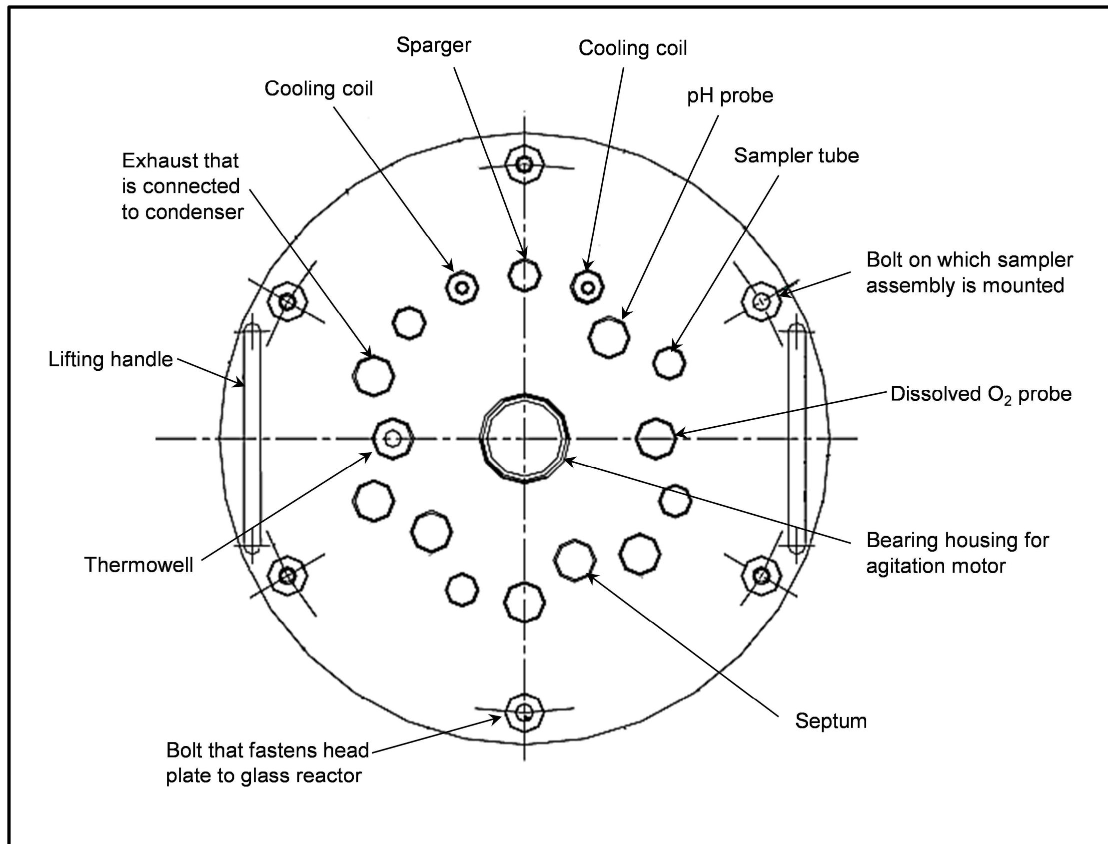

Figure 30: Top view schematic of head plate of New Brunswick bioreactor (redrawn from New Brunswick Scientific Co., 2007)

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APPENDIX B: DEVELOPMENT OF MEMBRANE BIOREACTOR

A membrane bioreactor (MBR) integrates the properties of membranes and biocatalysts such as enzymes or whole cells. MBRs have a wide variety of applications and have been utilised in the production of bio-fuels, foods, vitamins, proteins, antibiotics, amino acids, fine chemicals, *etc.* (Nagy & Kulcsár, 2009). Hollow-fibre MBRs work on the premise that a biocatalyst is immobilised on the surface of or within the porous support structure of an ultrafiltration membrane. A bioconversion reaction may then be achieved by feeding a substrate solution to the immobilised biocatalyst in the membrane. The interest in hollow-fibre MBRs stems mainly from their large specific surface area for biocatalyst immobilisation and their ability to integrate reaction and separation procedures (Nagy & Kulcsár, 2009). The factors that should be considered when developing an MBR system are outlined below.

The basic structure of a hollow fibre membrane bioreactor is sketched in Figure 31.

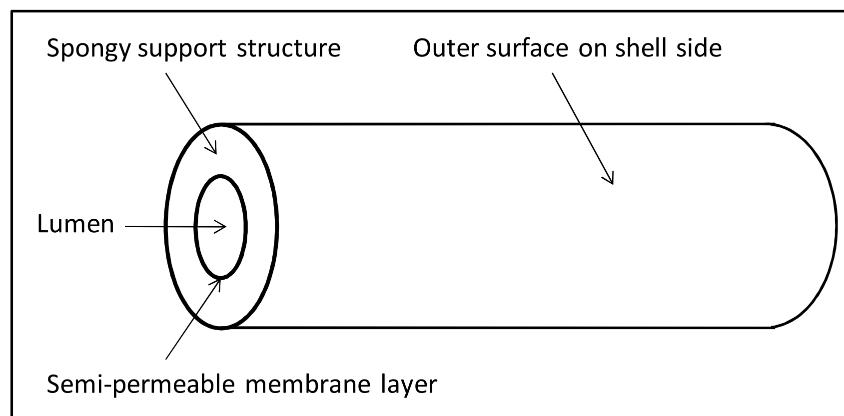

Figure 31: Basic structure of hollow fibre membrane bioreactor (redrawn from Bunch, 1988)

A number of factors should be taken into account when developing an MBR. The development has been divided into the four main sections listed here:

i. Reactor configuration
ii. Membrane selection
iii. Biocatalyst considerations
iv. Immobilisation technique.

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B.1 Reactor configuration

Membrane bioreactors generally utilise biocatalysts in one of two ways. In the first method, the biocatalyst is suspended in a reaction vessel, typically a continuous stirred-tank reactor (CSTR), and retained by a membrane separating unit. The bioreaction takes place mainly in the reaction vessel and the product and cells are then separated by the ultrafiltration membrane unit (see Figure 32).

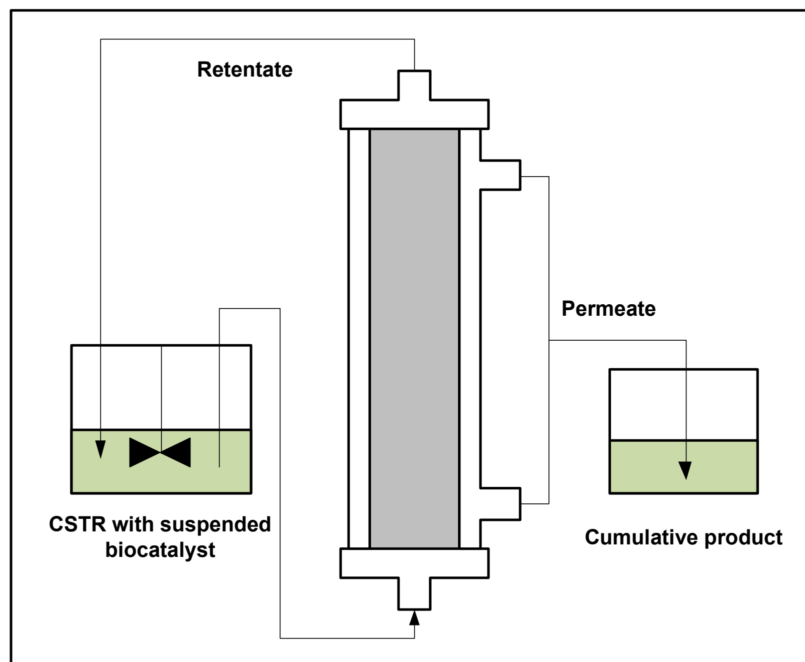

Figure 32: Membrane bioreactor setup with retained biocatalyst

Cantarella *et al.* (2007) utilised a membrane bioreactor setup with retained biocatalyst cells to produce amidase-catalysed nicotinic acid. After optimisation of the residence time they were able to achieve high conversions of up to 88%. Cánovas *et al.* (2002) utilised a hollow fiber membrane reactor to retain biocatalyst cells for continuous production of l-carnitine using *Proteus* sp. Their system was able to remain stable for long periods (140-200h) and it achieved relatively high conversions of 35-50%.

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The second type of membrane bioreactor setup is characterised by the biocatalyst being immobilised on the surface of or inside of the porous support structure of an ultrafiltration membrane (see Figure 33). The reaction and separation steps of the process occur simultaneously when the biocatalyst is immobilised within the ultrafiltration membrane (Giorno & Driol, 2000).

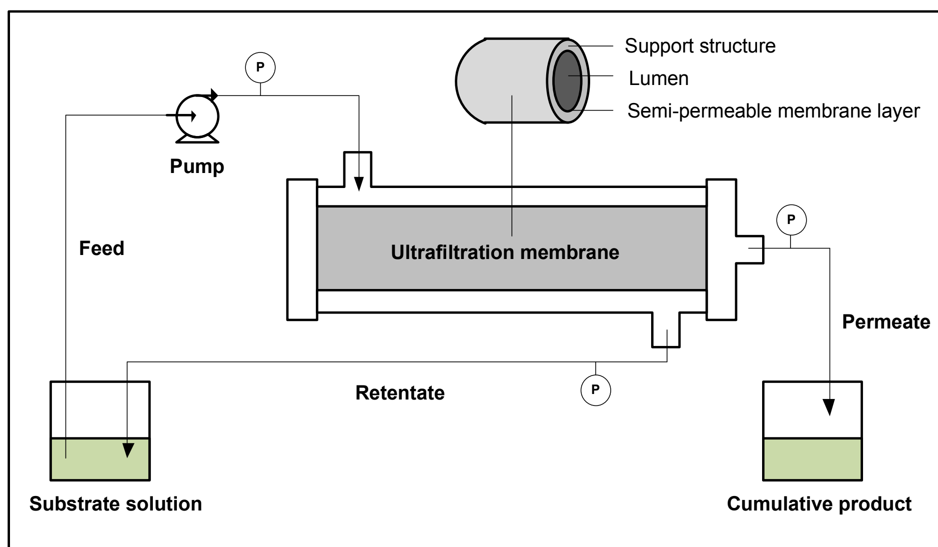

Figure 33: Membrane bioreactor setup with immobilised biocatalyst

A number of research groups have utilised hollow-fibre MBRs in a shell and tube reactor configuration where the substrate is recycled through the shell side and the permeate is collected from the lumen (Raihan *et al.*, 1997; Lloyd & Bunch, 1995; Edward *et al.*, 2003). This mode of operation is referred to as the "shell-to-lumen" mode. The biocatalyst is physically immobilised within the porous support structure of an ultrafiltration membrane which has its molecular weight cut off on the lumen side. An applied trans-membrane pressure allows the substrate to permeate the membrane and thereby come into contact with the biocatalyst (Du Preez, 2008).

There exists no one reactor configuration which is suitable for all types of MBR applications. The choice of which configuration to use is dependent on the production goals of the project and the properties of the reaction system (Giorno & Driol, 2000). A lab-scale project may favour a batch type system, while an industrial-scale project with the same reaction system may require a configuration that supports continuous production.

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B.2 Membrane selection

Membrane selection is based on the following factors:

B.2.1 Molecular Weight Cut-Off (MWCO)

Membranes are characterised in terms of their ability to retain molecules of a specified molecular weight. The Molecular Weight Cut-Off (MWCO) defines the size of the molecules that are retained to a degree of approximately 90% (Lee & Cho, 2003). The molecular weight cut-off must therefore be smaller than the biocatalyst used so that the catalyst may be retained by the membrane. The recombinant *E. coli* cells used in this research have approximate dimensions of 2μm length and 0.5μm diameter. The experimental setup was to have utilised membranes with MWCOs of 8 kDa or 15 kDa, which compares closely to Lloyd & Bunch (1995), who used a hollow-fibre MBR with a 10kDa MWCO to immobilise *Escherichia coli* B SPAO.

B.2.2 Membrane material

The membrane material must be able to withstand operating temperatures of up to 40°C and a pH value in the range of 6 - 8. The membrane must also be able to withstand a trans-membrane pressure of up to 2.2 bars and be stable against the sterilizing agents used in the cleaning procedures.

A number of membrane materials have been successfully used in immobilising different types of biocatalysts:

- Fluoro-polymers (Cantarella *et al.*, 2007; Cantarella *et al.*, 2005; Cantarella *et al.*, 1998)
- Polysulphones (Kabaivanova *et al.*, 2004; Cánovas *et al.*, 2002; Cantarella *et al.*, 1998)
- Ceramics (Magnan *et al.*, 2004; Coronas & Santamaría, 1999).

A significant part of the research that has been done in the MBR field focuses on the use of polymeric membranes. The high cost of ceramic membranes (3 to 5 times that of polymeric membranes) may often be the deciding factor in choosing polymeric membranes above ceramic membranes. This has been the case in spite of the fact that continued exposure to mechanical and thermal stresses, caused by cell growth and the fermentation process, may cause polymeric membranes to rupture or distort due to their inherent lack of mechanical strength (Liu, Liu, &

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Tan, 2010). The advantages that ceramic membranes have over polymeric membranes may be summarised as follows:

- Ceramic membranes are physically superior to polymeric membranes and can therefore be used for longer periods of operation (Lee & Cho, 2003)
- Ceramic membranes exhibit higher permeability than equivalent polymeric membranes (Lee & Cho, 2003; Grezeoekowiak-Przywecka & Slomińska, 2005)
- Ceramic membranes are absolutely inert, are able to withstand severe chemical environments with pH-values of 0-14 and are solvent resistant (www.sterlitech.com)
- Ceramic membranes can resist temperatures of up to 350°C (www.sterlitech.com) and operate at pressures of up to 10 MPa (Garmash *et al.*, 1995)
- Ceramic membranes are sterilisable (Kolsch *et al.*, 2002; Liu *et al.*, 2010).

In light of the above it is felt that the use of ceramic membranes may be justified, in spite of the higher initial capital cost , due to the overall durability of the material.

B.2.3 Support structure

The support structure of ultrafiltration membranes can either be isotropic or asymmetric. Isotropic membranes have a uniform matrix structure with a typical thickness of 10µm - 30µm. Isotropic membranes are designed not to admit the biocatalyst cells. Asymmetric membranes have a semi-permeable membrane layer of only 0.1µm – 0.5 µm thick and are surrounded by a spongy support structure which has a much larger MWCO. The biocatalyst cells are small enough to enter the spongy structure, but cannot pass through the semi-permeable layer (Bunch, 1988). The cells can therefore be immobilised in the spongy support structure of asymmetric membranes. Asymmetric membranes are more susceptible to damage caused by mechanical strain, but they exhibit less mass transfer resistance (Du Preez, 2008).

The experimental setup was to have utilised an asymmetric membrane, because it is less mass transfer resistant. The danger of mechanical damage would have been reduced by using a ceramic membrane.

B.2.4 Membrane geometry

A variety of membrane geometries have been used in MBR systems to date. Cantarella *et al.* (2007) used a flat sheet membrane to retain biocatalyst cells, while Bhatia *et al.* (2004) were able to successfully immobilise enzymes with a hollow-fibre MBR in their research. The main

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advantages associated with using a hollow-fiber MBR may be summarised as follows (Nagy & Kulcsár, 2009; Raihan *et al.*, 1997):

- Large specific surface area for biocatalyst immobilisation
- Capacity for high cell density
- Integration of reaction and separation procedures
- Good mass transfer capabilities
- Biocatalyst is not exposed to harsh immobilisation procedures
- Immobilised biocatalyst may be reused as long as it stays active.

The experimental setup was to have utilised a hollow-fibre membrane geometry to make use of the advantages listed above.

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B.3 Biocatalyst selection and considerations

As set out in Section 2.2, cytochrome P450 is a class of oxidative enzyme which is able to catalyse the oxidation of alkanes to their corresponding alcohols with a high degree of selectivity. The enzyme uses NAD(P)H as a co-factor and the two-component system usually consists of a hydroxylase and a reductase (Ayala & Torres, 2004). The CYP153A6 enzyme, a member of the microbial P450 family, is able to catalyse the oxidation of C6 to C11 alkanes with a regiospecificity of 95% for the terminal carbon position (Randall, 2010). This research was to focus on the bioconversion of octane to octanol, which makes the CYP153A6 enzyme an ideal candidate for the biocatalyst to be used.

A decision also had to be made as to which process option to implement: using whole calls as opposed to using enzymes, and which biocatalyst cell strain to choose.

B.3.1 Whole cells *vs.* enzymes

See Section 2.2.1 of the literature study in the main body of the thesis.

B.3.2 Immobilised cells *vs.* retained cells

Biocatalyst cells can be immobilised on the surface of or within the porous structure of an ultrafiltration membrane. Systems that utilise immobilised cells have a number of advantages over free cell system:

- The biocatalyst is retained within the system with simultaneous product removal
- The overall stability of the biocatalyst may be expected to improved (Nasratun *et al.*, 2009)
- The biocatalyst is more resistant to toxic shocks (Raihan *et al.*, 1997)

However, there have been reports of cases where immobilised biocatalysts have had a reduction in activity when compared to the activity of retained cells (Nasratun *et al.*, 2009). Nevertheless, it was felt that problems of this type could be overcome with careful membrane selection, based on the system requirements.

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B.4 Immobilisation technique

Sato & Tosa (1999) define immobilised biocatalysts as biocatalysts "physically confined or localized in a certain defined region of space with retention of their catalytic activities, and which can be used repeatedly and continuously". The techniques used for the immobilisation of the biocatalyst can be categorised into four basic groups:

1. Carrier binding – the biocatalyst is bound to water-insoluble carriers such as synthetic polymers and porous glass (Sato & Tosa, 1999)
2. Cross-linking – the biocatalyst is immobilised by intermolecular cross-linking by means of bi- or multi-functional reagents such as glutaraldehyde (Sato & Tosa, 1999)
3. Entrapping – the biocatalyst is incorporated into the lattice of a semipermeable gel by enclosing the biocatalyst in a semipermeable membrane (Sato & Tosa, 1999)
4. Membrane immobilisation – the biocatalyst is immobilised within the porous support structure of a semipermeable membrane.

The first three methods immobilise the biocatalyst on the surface of the membrane. To do this, it is often necessary to alter the biocatalyst or its micro-environment to retain the biocatalyst effectively. Such alterations may lead to a decrease in biocatalyst activity or selectivity (Du Preez, 2008). Physical immobilisation of the biocatalyst in the porous support structure of an ultrafiltration membrane has no effect on the form of the biocatalyst. This reduces the risk of a drop in biocatalyst activity and selectivity caused by the membrane immobilisation procedure.

Table 6 compares the immobilisation technique properties of methods that immobilise the biocatalyst on the membrane surface with methods that immobilise the biocatalyst in the porous support structure of the membrane (Kragl *et al.*, 1999).

Table 6: Comparison of advantages/disadvantages of immobilisation technique

Biocatalyst fixed on membrane surface	**Biocatalyst retained within membrane**
Continuous and batch operation	Continuous and batch operation
Improved stability of biocatalyst	Lower biocatalyst stability
Immobilisation decreases activity	Immobilisation does not affect activity
Immobilsation costs	No immobilisation costs
Modification required for different biocatalysts	No modification required for different biocatalysts

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The research project would have immobilised the biocatalyst within the porous support structure of a semipermeable membrane. This immobilisation technique is relatively inexpensive and easy to implement, while offering a large degree of versatility.

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APPENDIX C: CHEMICALS, EQUIPMENT & BUFFER PREPARATION

C.1 Chemicals

Table 7: List of chemicals used in the project

Item Name	Supplier	Catalogue No.	Remarks
Yeast Extract Powder	Merck	Art No: BX6	Lysogeny Broth medium and Auto-induction medium ingredient
Sodium chloride	Merck	5822320 EM	Lysogeny Broth medium ingredient
Pancreatic Digest of Casein	Merck	HG000BX4.250	Auto-induction medium ingredient
Potassium Dihydrogen Orthophosphate	Merck	5043600 EM	Phosphate buffer ingredient for reaction mixture in McCartney bottle experiments
di-Potassium hydrogen phosphate	Merck	1.05104.1000	Phosphate buffer ingredient for reaction mixture in McCartney bottle experiments
1-Octanol for synthesis	Merck	8.20931.0100	For Gas Chromatography analysis standard curve determination
Ethyl acetate uniLAB	Merck	SAAR2235000LC	For organic extraction of bioconversion experimental samples in preparation for Gas Chromatography analysis
Ferric sulphate hydrate uniLAB	Merck	SAAR2340800EM	Auto-induction medium ingredient
Ammonium sulphate uniVAR	Merck	SAAR1124020EM	Auto-induction medium ingredient
di-Sodium hyd orthophosphate anhyd	Merck	SAAR5822870EM	Reduces CYP153A6 enzyme in preparation for CO difference spectra analysis
Lactose uniLAB	Merck	SAAR3862000EM	Auto-induction medium ingredient
Glycerol uniVAR	Merck	SAAR2676520LC	Ingredient for medium that is used for long term cell storage
Octane	Fluka	74822	Alkane substrate used in bioconversion experiments
1-Undecanol	Fluka	94061	Internal standard for Gas Chromatography analysis
Octane Fraction purum	Fluka	74830	For Gas Chromatography analysis standard curve determination
Octane, free of olefins	Fluka	74822	For Gas Chromatography analysis standard curve determination
Kanamycin sulfate, from Streptomyces Kanamyceticus	Sigma Aldrich	K4000-5G	Antibiotic that was added to all cultures to help prevent cell culture contamination
δ Aminolevulinic acid	Sigma Aldrich	A3785	A precursor molecule for heme synthesis which is added to the cell culture at the start of the Induction Incubation period
Caprylic acid free acid	Sigma Aldrich	C2875 - 10ml	For Gas Chromatography analysis standard curve determination
Ferric chloride hexahydrate	Saarchem	2340530	Reaction mixture ingredient to promote enzyme synthesis by delivering ions to make heme

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C.2 Equipment

Table 8: List of equipment used in the project

Equipment	Manufacturer	Use
Rotary shaker incubator	Labcon	Incubation and bioconversion environment for various experimental uses
Spectrophotometer	Varian	Optical density scans and individual wavelength measurements
Centrifuge 6305	Eppendorf	Centrifuge for 0.5ml-2ml samples
Centrifuge 5702 R	Eppendorf	Temperature controlled centrifuge work
Pipettes	Eppendorf	Precision volumetric additions
Peristaltic pump	Manostat	Cooling water pump to condensor in bioreactor setup
Varian 3400 Gas chromatograph	Varian	Product and substrate analysis
BioFlo 110 Modular Benchtop Fermentor	New Brunswick	Bioreactor experiments
Laminar airflow cabinet	ClearFlow	Sterile working environement
McCartney bottles 30ml		McCartney bottle experiments
Centrifuge tubes 50ml		Centrifuge work
Fume extraction cabinet		To remove CO gas during CO difference spectra analysis
Autoclave		Sterilisation of cultures and equipment
Fridge (-80°C, -20°C & -4°C)		Storage
Vortex mixer		Mixing
Eppendorf tubes 1.5ml & 2.0ml		Sampling work
Glass test tubes (15mm diameter, 150mm length)		Used during CO difference spectra analysis

C.3 Potassium phosphate buffer preparation

The following two stock solutions were made up to make the pH buffers used in the project:

- 1M K_2HPO_4 solution (174.17g of K_2HPO_4 per litre of distilled water)
- 1M KH_2PO_4 solution (136.09 g of K_2HPO_4 per litre of distilled water)

The Henderson-Hasselbalch equation was used to determine the ratio in which the two stock solutions needed to be added to obtain the desired pH values.

$pH = pK_a + \log([acid]/[base])$

with $pK_a = 7.21$

Where pK_a is defined as the negative log of the acid dissociation constant

The calculations for a pH of 7.0 are given below:

61.5ml 1M K_2HPO_4 + 38.5ml 1M KH_2PO_4

Solution volume = 0.1 L

Number of moles of base = 0.0615 moles

Number of moles of acid = 0.0385 moles

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[base] = 0.0615 moles / 0.1 L = 0.615 M

[acid] = 0.0385 moles / 0.1 L = 0. 385 M

pH = 7.21 + log ([0.615]/[0.385]) = 7.0

The following 1M buffer solutions were made according to the ratios determined from the Henderson-Hasselbalch equation:

- pH 7.0: 61.5ml 1M K_2HPO_4 + 38.5ml 1M KH_2PO_4 Equation A
- pH 7.4: 80.2ml 1M K_2HPO_4 + 19.8ml 1M KH_2PO_4 Equation B

A 200mM solution was prepared by adding 400ml distilled water to the 100ml solution in Equation A. A 50mM was prepared by adding 1900ml distilled water to the 100ml solution in Equation B.

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APPENDIX D: EXPERIMENTAL RESULTS

Table 9: Wet cell weight to dry cell weight conversion data

WCW vs. DCW results							
Container Name	MC volume	Container	WCW + Container	WCW	DCW + Container	DCW	DCW/WCW
[#]	[ml]	[g]	[g]	[g]	[g]	[g]	[%]
1	30	14.065	14.919	0.854	14.170	0.105	12.3%
2	30	14.090	15.071	0.980	14.199	0.108	11.0%
3	20	14.052	14.649	0.596	14.120	0.067	11.3%
4	20	14.085	14.845	0.760	14.175	0.091	11.9%
						Average	11.6%
						Std deviation	0.6%
						Std deviation %	4.9%

Table 10: CO difference spectra analysis results for Strain A and Strain B at 23°C

Strain A, Main culture 1			
Wavelength	**no CO**	**CO**	**Difference**
450nm	2.698	2.722	0.024
490nm	2.691	2.692	0.001
Strain A, Main culture 2			
Wavelength	**no CO**	**CO**	**Difference**
450nm	2.703	2.722	0.019
490nm	2.691	2.691	0
Strain B, Main culture 1			
Wavelength	**no CO**	**CO**	**Difference**
450nm	2.712	2.745	0.033
490nm	2.680	2.68	0
Summary			
	Dry cell weight	**450nm-490nm**	**Enzyme conc.**
	[g]	**-**	**[µmoles / g WCW]**
Strain A, MC1	0.274	0.023	4.61E-02
Strain A, MC2	0.217	0.019	4.80E-02
Strain B, MC 1	0.280	0.033	6.47E-02

Table 11: pH of 100ml shake flask culture during incubation at 23°C

Auto-induction media pH study		
Time [hr]	**Description**	**pH**
0	Auto-induction media	6.6
0	+ cells to inoculate	6.6
4		6.3
4	+ δ ALA & FeSO4	6.3
20		5.7

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Table 12: Enzyme concentration data for induction incubation period at 23°C and an initial pH of 7.0

CO difference spectra analysis		
Incubation time [hr]	**Main Culture [#]**	**Enzyme concentration [µmoles / g WCW]**
10	1	5.16E-02
	2	4.42E-02
16	1	7.67E-02
	2	7.24E-02
	4	6.56E-02
18	1	7.88E-02
20	1	7.62E-02
	3	7.29E-02

Table 13: Cell growth data of *E. coli* in auto-induction medium during induction incubation period at 23°C

Optical density					
Reaction time [hr]	**Main Culture 1**	**Main Culture 2**	**Main Culture 3**	**Average**	**Standard Deviation x 3**
0.00	2.07	2.21	2.08	2.12	0.23
2.00	3.09	3.35	3.24	3.23	0.39
4.00	4.25	4.59	4.49	4.44	0.51
6.50	5.28	5.76	5.52	5.52	0.72
8.50	5.98	6.24	6.08	6.10	0.40
10.00	6.68	6.92	7.12	6.90	0.67
16.00	7.60	7.50	7.64	7.58	0.22
18.00	7.63	7.60	7.67	7.63	0.12
20.00	7.59	7.99	7.70	7.76	0.61

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Table 14: 1-Octanol production data for 1.33ml bioconversion batch experiments at 23°C and an initial pH of 7.0

Gas Chromatography analysis				
	Octanol produced [mg/g DCW]			
Time [hr]	Main Culture 1 - 20hr	Main Culture 2 - 16hr	Main Culture 3 - 20hr	Main Culture 4 - 16hr
2	4	3	2	2
3	4	2	4	3
4	6	4	4	4
5	6	4	5	5
15	14	10	13	9

Table 15: 1-Octanol production data for 1.33ml bioconversion batch experiments utilising 'old' and 'fresh' biocatalyst cells at 23°C and an initial pH of 7.0

Gas Chromatography analysis										
	Octanol produced [mg/g DCW]									
	Old cells					Fresh cells				
Time [hr]	1	2	3	Average	Std Dev x 3	1	2	3	Average	Std Dev x 3
3	3	3	3	3	1	7	7	7	7	1
6	6	5	5	5	1	14	12	14	13	2
9	7	6	6	7	1	17	16	16	16	3
14	10	10	9	10	2	24	24	23	24	2
22	15	16	15	15	2	42	39	43	41	6
25	16	17	17	17	2	45	43	47	45	6

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Table 16: *E. coli* cell growth and system conditions of New Brunswick bioreactor batch experiment

System Conditions							
	Time [hrs]	OD	O2 %	Agitation	pH	T	lpm
Growth incubation period	0.00		60.0	50	7.0	24.9	1.6
	0.25		48.0	50	7.0	24.8	1.6
	0.50	0.30	33.0	50	7.0	24.7	1.6
	1.25	0.45	14.2	121	7.0	24.8	1.6
	1.50		20.0	145	7.0	24.9	1.6
	1.75	0.60	17.0	185	7.0	25.0	1.6
	2.25		20.6	203	7.0	25.0	1.6
	2.50	0.95	21.7	228	7.0	24.5	1.6
	2.75		20.0	242	7.0	24.5	1.6
Induction incubation period	4.00		24.9	323	7.0	24.9	1.6
	4.50		16.7	350	7.0	25.0	1.6
	5.00		14.0	370	7.0	25.0	1.6
	5.50		16.8	388	7.0	24.8	1.6
	5.75	6.21	30.0	408	7.0	24.8	1.6
	8.25	10.98	17.1	398	7.0	24.2	1.6
	9.25	11.97	17.8	384.0	7.0	24.2	1.6
	9.75		16.5	411	7.0	24.2	1.6
	10.50	13.81	20.8	379	7.1	23.9	1.6
	11.75	13.90	18.4	298	7.2	23.6	1.6
	12.75	13.82	22.1	256	7.3	23.5	1.6
Bioconversion period	13.75		19.6	322	7.2	23.9	1.6
	14.25		25.0	303	7.1	23.7	1.6
	14.75		21.0	280	7.0	23.0	1.6
	15.58		17.2	245	7.0	22.0	1.6
	16.75		19.8	137	7.0	22.8	1.6
	18.75		25.3	250	7.0	22.3	1.6
	25.25		13.4	160	7.0	19.0	1.6
	25.75		22.0	125	7.0	23.1	1.6
	26.75		16.0	173	7.0	23.0	1.6
	31.25		25.6	247	7.0	23.0	1.6
	32.75		18.1	202	7.0	23.0	1.6
	45.75		34.5	282	7.1	23.0	1.6
			18.6	146	7.0	23.0	1.6
	49.75		19.3	50	7.0	23.0	1.6
	55.75		62.1	50	7.0	23.0	1.6

The rows highlighted in grey in Table 16 indicate the times at which samples were taken for GC analysis.

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Table 17: Substrate and product concentration data for New Brunswick bioreactor and 1.33ml McCartney bottle batch reactions at 23°C and a pH of 7.0

Gas Chromatography analysis				
Time [hr]	Octanol		Octanoic acid	Octane
-	[mg/g DCW]		[mg/g DCW]	[mg/L]
	McCartney	Bioreactor	Bioreactor	Bioreactor
0.0				37499.16
0.5				15690.82
1.8	1.43	9.45	2.90	7765.65
3.0	1.86	12.93	0.88	7105.20
11.5	5.73	42.00	10.21	
13.0	7.46	43.79	12.02	6738.42
17.5	10.92	44.39	21.71	4331.99
19.0		50.28	27.87	741.14
32.0		26.35	33.45	81.00
36.0		16.83	34.92	12.99
42.0		10.16	35.45	12.74

Table 18: Octane and 1-octanol concentration data in bioreactor batch setup without cells at 23°C and a pH of 7.0

Gas Chromatography analysis		
Time	1-Octanol	Octane
[hr]	[mg/ml]	[mg/ml]
0.0	4079	38282
0.5		24965
1.0		16145
2.0		10635
3.0		8760
5.0	4105	
8.0		7751
9.0	4089	7034
10.0		4910
24.0	4055	44
31.0	4053	44
49.0		11

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Table 19: Octane concentration data for varied feed pump speeds in New Brunswick bioreactor setup without cells at 23°C and a pH of 7 with an agitation rate of 250rpm and an air sparging rate of 1.6l/min

Pump Speed Analysis					
Time [hours]	Pump Speed [%]	Octane feed [mg/hr]	Octane concentration [mg/l]	Rate of change [mg/l/hr]	
0.0			8823		10%
	10%	11004			
0.5			8916	578	
	10%	11004			
1.5			9651		
1.5			9651		5%
	5%	5502			
2.5			7734	-1649	
	5%	5502			
3.0			7244		
3.0			7244		15%
	15%	16507			
4.0			10595	3095	
	15%	16507			
4.5			11823		
4.5			11823		20%
	20%	22009		9473	
5.0			16559		

Table 20: Product concentration data for New Brunswick bioreactor semi-batch reaction at 23°C and a pH of 7.0

New Brunswick Semi Batch Experiments				
	Bioreactor 1		Bioreactor 2	
Time [hr]	1-Octanol [mg/g DCW]	Octanoic acid [mg/g DCW]	1-Octanol [mg/g DCW]	Octanoic acid [mg/g DCW]
0.0	0.0	0.0	0.0	0.0
1.0	12.0	1.2		
3.0	22.1	2.8	19.0	6.3
5.5	31.2	5.3		
7.0			57.9	8.2
9.0	40.8			
12.0	86.0	7.4		
14.0			137.3	5.7
20.0	235.1	8.2	247.9	9.2
23.0	319.0	34.1	299.8	13.2
25.5			365.4	19.9
30.0	469.6	46.8	499.0	22.2
32.0	546.6	58.6		
35.5			550.0	27.7
40.5			603.4	38.6
45.5			598.1	51.1

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Table 21: 1-Octanol production data for New Brunswick semi-batch bioreactor at 23°C and a pH of 7.0 with lag period removed

	Bioreactor 1	Bioreactor 2
Time	1-Octanol	1-Octanol
[hr]	[mg/g DCW]	[mg/g DCW]
0.0	0.0	0.0
7.0	235.1	247.9
10.0	319.0	299.8
12.5		365.4
17.0	469.6	499.0
19.0	546.6	
22.5		550.0
27.5		603.4
32.5		598.1

Table 22: Octane concentration data for 2l semi-batch bioreactor at 23°C and a pH of 7.0

Octane concentration date	
Time	Octane
[hr]	[mg/L]
0.0	10421
14.0	13069
23.0	14968
35.5	16132
45.5	19080

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Table 23: Empirical model 1-octanol prediction results for New Brunswick semi-batch bioreactor at 23°C and a pH of 7.0

Empirical Model Results				
Time [hr]	1-Octanol [mg/g DCW]		Time [hr]	1-Octanol [mg/g DCW]
0.01	0.31		14.09	416.00
0.01	0.47		14.90	433.72
0.02	0.63		15.71	450.69
0.02	0.78		16.52	466.88
0.04	1.57		17.00	475.91
0.07	2.35		17.34	482.28
0.09	3.14		18.15	496.87
0.11	3.92		18.96	510.63
0.22	7.83		19.77	523.55
0.33	11.73		20.59	535.59
0.43	15.62		21.40	546.73
0.54	19.50		22.21	556.94
1.08	38.73		22.50	560.24
1.63	57.71		23.02	566.20
2.17	76.42		23.84	574.46
2.71	94.87		24.65	581.64
3.52	122.01		25.46	587.71
4.34	148.54		26.27	593.45
5.15	174.46		27.09	596.71
5.96	199.74		27.50	596.65
6.77	224.39		27.90	596.60
7.00	231.08		28.71	596.97
7.59	248.40		29.39	599.15
8.40	271.75		30.07	600.14
9.21	294.43		30.74	600.23
10.02	316.44		31.42	600.86
10.84	337.77		31.69	601.61
11.65	358.40		31.96	602.05
12.46	378.32		32.23	602.06
13.27	397.53		32.50	602.23

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Table 24: Root mean square error analysis of 1-octanol production for New Brunswick semi-batch bioreactor 1 compared to empirical model data

Root Mean Square Error analysis - New Brunswick Semi-Batch Reactor 1				
	Experimental data	**Model data**	**Root Mean Square Error analysis**	
Time	1-Octanol	1-Octanol	Absolute error	Percentage error
[hr]	[mg/g DCW]	[mg/g DCW]	[mg/g DCW]	[-]
7.0	235.1	231.1	4.0	1.7%
10.0	319	316.4	2.6	0.8%
17.0	469.6	475.9	6.3	1.3%
19.0	546.6	510.6	36.0	6.6%
			Average	2.6%

Table 25: Root mean square error analysis of 1-octanol production for New Brunswick semi-batch bioreactor 2 compared to empirical model data

Root Mean Square Error analysis - New Brunswick Semi-Batch Reactor 2				
	Experimental data	**Model data**	**Root Mean Square Error analysis**	
Time	1-Octanol	1-Octanol	Absolute error	Percentage error
[hr]	[mg/g DCW]	[mg/g DCW]	[mg/g DCW]	[-]
7.0	247.9	231.1	16.8	6.8%
10.0	299.8	316.4	16.6	5.6%
12.5	365.4	378.3	12.9	3.5%
17.0	499	475.9	23.1	4.6%
22.5	550	560.2	10.2	1.9%
27.5	603.4	596.7	6.7	1.1%
32.5	598.1	602.2	4.1	0.7%
			Average	3.5%

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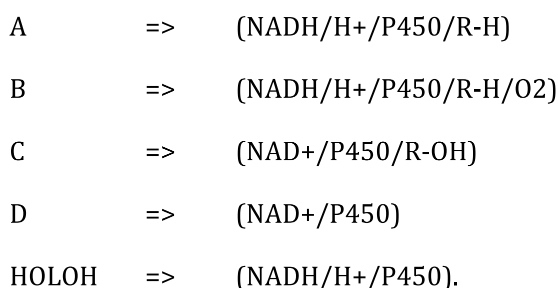
APPENDIX E: DEVELOPMENT OF THEORETICAL RATE EQUATION

The theoretical rate law could be determined through algebraic manipulation and by applying the pseudo-steady-state hypothesis to the rate equations derived from the seven elementary reactions that were based on the reaction mechanism described in Section 2.5.3. The calculations for the development of the rate equation are given below.

Reaction Mechanism

Refer to Section 5.1 for the seven elementary reactions that describe the reaction mechanism.

Let:



A => (NADH/H+/P450/R-H)

B => (NADH/H+/P450/R-H/O2)

C => (NAD+/P450/R-OH)

D => (NAD+/P450)

HOLOH => (NADH/H+/P450).

Therefore:

1. P450 + ($NADH/H^+$) ↔ HOLOH
2. HOLOH + R-H ↔ A
3. A + O_2 ↔ B
4. B ↔ H_2O + C
5. C ↔ R-OH + D
6. D ↔ (NAD^+) + P450
7. (NAD^+) + H_2 ↔ ($NADH/H^+$).

Rate equations

Refer to Section 5.1 for the rate equations that were derived from the seven elementary reactions to determine the theoretical rate law.

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Pseudo-Steady-State-Hypothesis

$r_A = 0 = r_2 - r_{-2} - r_3 + r_{-3}$

$0 = k_2 [HOLOH][RH] - k_{-2} [A] - k_3[A][O_2] + k_{-3} [B]$ (Equation I)

$[A] = (k_2 [HOLOH][RH] + k_{-3} [B]) / (k_{-2} + k_3 [O_2])$ (Equation II)

$r_B = 0 = r_3 - r_{-3} - r_4$

$0 = k_3 [A][O_2] - k_{-3} [B] - k_4 [B]$ (Equation III)

$[B] = (k_5 [A][O_2]) / (k_{-3} + k_4)$ (Equation IV)

$r_C = 0 = r_4 - r_5$

$0 = k_4 [B] - k_5 [C]$ (Equation V)

$[C] = (k_4 / k_5) [B]$ (Equation VI)

$r_D = 0 = r_5 - r_6 + r_{-6}$

$0 = k_5 [C] - k_6 [D] + k_{-6} [P450][NAD^+]$ (Equation VII)

$[D] = (k_5 [C] + k_{-6} [P450][NAD^+]) / (k_6)$ (Equation VIII)

$r_{NAD+} = 0 = r_6 - r_{-6} - r_7$

$0 = k_6 [D] - k_{-6} [P450][NAD^+] - k_7 [NAD^+][H_2]$ (Equation IX)

$[NAD^+] = (k_6 [D]) / (k_{-6} [P450] + k_7 [H_2])$ (Equation X)

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$r_{HOLOH} = 0 = r_1 - r_{-1} - r_2 + r_{-2}$

$0 = k_1 [P450][NADH/H^+] - k_{-1} [HOLOH] - k_2 [HOLOH][RH] + k_{-2} [A]$ (Equation XI)

$[HOLOH] = (k_1 [P450][NADH/H^+] + k_{-2} [A]) / (k_{-1} + k_2 [RH])$ (Equation XII)

$r_{NADH/H+} = 0 = r_7 - r_{-7} - r_1 + r_{-1}$

$0 = k_7 [NAD^+][H_2] - k_1 [P450][NADH/H^+] + k_{-1} [HOLOH]$ (Equation XIII)

$[NADH/H^+] = (k_7 [NAD^+][H_2] + k_{-1} [HOLOH]) / (k_1 [P450])$ (Equation XIV)

$r_{ROH} = r_5 = k_5 [C]$ (Equation XV)

Manipulations

Product reaction rate:

$r_{R\text{-}OH} = k_5 [C]$ (Equation XV)

The overall total enzyme concentration was assumed to be constant and could therefore be described as follows:

$[P450]_{total} = X = [P450] + [NADH/H^+/P450] + [NADH/H^+/P450/R\text{-}H] +$
$[NADH/H^+/P450/R\text{-}H/O_2] + [NAD^+/P450/R\text{-}OH] + [NAD^+/P450]$

OR

$[P450]_{total} = X = [P450] + [HOLOH] + [A] + [B] + [C] + [D]$ (Equation XVI)

We need to describe [HOLOH], [A], [B] and [D] in terms of [C].

For [B], from Equation V:

$[B] = (K_5 / k_4) [C]$ (Equation XVII)

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For [A], substitute Equation XVII into Equation IV:

$(k_5 / k_4) [C] = (k_3 [A][O_2]) / (k_{-3} + k_4)$

$[A] = ((k_{-3} + k_4) k_5 [C]) / (k_3 k_4 [O_2])$ (Equation XVIII)

For [HOLOH], substitute Equations XVII & XVIII into Equation II:

$((k_{-3} + k_4) k_5 [C]) / (k_3 k_4 [O_2]) = (k_2 [HOLOH][RH] + k_{-3} (k_5 / k_4) [C]) / (k_{-2} + k_3 [O_2])$

$k_2 [HOLOH][RH] + k_{-3} (k_5 / k_4) [C] = ((k_{-2} + k_3 [O_2]) (k_{-3} + k_4) k_5 [C]) / (k_3 k_4 [O_2])$

$[HOLOH] = ((k_{-2} + k_3 [O_2]) (k_{-3} + k_4) k_5 [C]) / (k_2 k_3 k_4 [O_2][RH]) - (k_{-3} k_5 [C]) / (k_2 k_4 [RH])$

(Equation XIX)

For [D], substitute Equation X into Equation VII:

$0 = k_5 [C] - k_6 [D] + k_{-6} [P450] (k_6 [D]) / (k_{-6} [P450] + k_7 [H_2])$

$[D] = k_5 [C] (k_{-6} [P450] + k_7 [H_2]) / (k_6 k_7 [H_2])$ (Equation XX)

For [C], substitute [HOLOH], [A], [B] and [D] into Equation XVI:

$[P450]_{total} = ((k_{-2} + k_3 [O_2]) (k_{-3} + k_4) k_5 [C]) / (k_2 k_3 k_4 [O_2][RH]) - (k_{-3} k_5 [C]) / (k_2 k_4 [RH]) + [P450] + [C] + ((k_{-3} + k_4) k_5 [C]) / (k_3 k_4 [O_2]) + (K_5 / k_4) [C] + (k_5 [C] (k_{-6} [P450] + k_7 [H_2]) / (k_6 k_7 [H_2]))$

$[C] = \{ [P450]_{total} - [P450] \} / \{ (k_{-3} + k_4) k_5 / k_3 k_4 [O_2] + k_5 / k_4 + 1 + k_5 (k_{-6} [P450] + k_7 [H_2]) / k_6 k_7 [H_2] + + (k_{-2} + k_3 [O_2]) (k_{-3} + k_4) k_5 / k_2 k_3 k_4 [O_2][RH] - k_{-3} k_5 / k_2 k_4 [RH] \}$

Product reaction rate:

$r_{R-OH} = k_5 [C]$ (Equation XV)

Substitute [C] into the production reaction rate:

$r_{R-OH} = \{ ([P450]_{total} - [P450]) k_2 k_3 k_4 k_5 k_6 k_7 [O_2][H_2][R-H] \} / \{ (k_{-3} + k_4) k_2 k_5 k_6 k_7 [H_2][RH] + k_2 k_3 k_5 k_6 k_7 [O_2][H_2][R-H] + k_2 k_3 k_4 k_5 k_6 k_7 [O_2][H_2][R-H] + (k_{-6} [P450] + k_7 [H_2]) k_2 k_3 k_4 k_5 [O_2][RH] + (k_{-2} + k_3 [O_2])(k_{-3} + k_4) k_5 k_6 k_7 [H_2] + k_{-3} k_3 k_5 k_6 k_7 [O_2][H_2] \}$

The theoretical rate law was thereby successfully expressed in terms of only measurable quantities.

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APPENDIX F: MATLAB PROGRAMMING SCRIPT

The following MATLAB function was developed by Lidia Auret, a lecturer in the Chemical Engineering Department of the University of Stellenbosch, for the purpose of modelling the 1-octanol production of the New Brunswick semi-batch bioconversion reaction experiments.

```
function out = Octanol(expdata)
% units in terms of mg/g DCW
%% Rate constant estimation
% Inputs:
% Experimental data (1- octanol concentration-time profile): expdata
% Octane concentration equation (dC/dt = f_C(r))
% Rate equation (r = f_r(k))
% Lower and upper bounds for k

% Bounds for k
%%bounds = [6e-08 6e-06];

% Relevant time span
tspan = expdata(:,1);

% Experimental concentration %X in this case is concentration of octanol
Xexp = expdata(:,2);

% Bounds and initial guess for k
%%lb = bounds(1);
%%ub = bounds(2);

k0 = [100 603.4 1 100];
%k0(1) = initial guess for K1;
%k0(2) = initial guess for Cp*;
%k0(3) = n poisoning order;
%k0(4) = Ks

% Wrapper function for minimization function
PredictionErrork = @(k)
PredictionError(Xexp,ConcentrationPrediction(tspan,k));

% Minimization
[out.kOpt,out.predErrOpt,out.exitflag,out.output] =
fminsearch(PredictionErrork,k0);

% Calculute profile for optimimum k
% Wrapper to pass constant k value
ConcentrationEquationConstantk = @(t,X)
ConcentrationEquation(t,X,out.kOpt);
[tpredOpt,XpredOpt] = ode45(ConcentrationEquationConstantk,[tspan(1)
tspan(end)],0);

% Plots
figure;
% Predicted profile
plot(tpredOpt,XpredOpt,'-');
hold on;
```

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```
% Actual data
plot(tspan,Xexp,'rx');

legend('Predicted profile','Actual data');

%To get Matlab data into excel
out.tpredOpt = tpredOpt;
out.XpredOpt = XpredOpt;

end

function dX = ConcentrationEquation(t,X,k)
% Change of concentration with time

% Constant parameters
% Octane concentration function [mg / g DCW]
Cs = (10473+180.5*t)/3.708;

% Differential equation / Rate equation
dX = k(1)*((1-X/k(2))^k(3))*Cs/(k(4)+Cs);  %X in this case is concentration
of octanol

end

function Xpred = ConcentrationPrediction(tspan,k)
% Predicting the concentration values at specific time points (tspan),
% for a given choice of the rate constant k

% Initial conditions for concentration
X0 = 0; %X in this case is concentration of octanol

% Wrapper to pass constant k value
ConcentrationEquationConstantk = @(t,X) ConcentrationEquation(t,X,k);

% Ordinary differential equation solving
[temp,Xpred] = ode45(ConcentrationEquationConstantk,tspan,X0);

end

function predErr = PredictionError(Xexp,Xpred)
% Sum of squared errors
% for prediction associated with a certain k value
predErr = sum((Xexp-Xpred).^2);

end
```

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