THE BIOPRODUCTION OF L-PHENYLACETYLCARBINOL USING SOLID-LIQUID TWO PHASE PARTITIONING BIOREACTORS

by

Tanya Razia Khan

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Abstract

Biphasic systems such as two-phase partitioning bioreactors (TPPBs) have been used to alleviate biological inhibition by sequestering inhibitory compounds within an immiscible phase. The use of solid polymer beads as this auxiliary phase provides a fully biocompatible alternative to commonly used yet potentially toxic organic solvents. This work focused on the application of solid-liquid TPPBs to the bioproduction of the pharmaceutical precursor L-phenylacetylcarbinol (PAC), a biotransformation which suffers from substrate (benzaldehyde), product (PAC), and by-product (benzyl alcohol) inhibition, and simple strategies to improve TPPB performance in general.

A wide range of commercially available, biocompatible, and non-bioavailable polymers were screened for their affinity for benzaldehyde, PAC, and benzyl alcohol. Hytrel G3548L demonstrated the highest affinity for all three target compounds and was subsequently used in solid-liquid TPPBs for PAC production. Using 15% v/v polymer beads, PAC concentration was increased by 104% and benzyl alcohol concentration decreased by 38% over the single phase control. The delivery of benzaldehyde from polymer beads demonstrated only a 6-8% reduction in mass productivity with improved operational simplicity and reduced operator intervention.

The final objective of this work was to independently investigate various aspects of the aqueous phase composition and determine how each factor affects the partition coefficient of benzaldehyde in Hytrel G3548L. Temperature and pH were observed to have no significant effect on partitioning. Salt and glucose additions increased the partition coefficient by 173% and 30% respectively compared to RO water, while ethanol was found to decrease the partition coefficient from 44 (±1.6) to 1 (±0.3). These findings may be applied to solid-liquid TPPBs to increase or decrease partitioning as required, leading to improved bioreactor performance.

This work has successfully shown that with careful polymer selection, solid-liquid TPPBs can be used to increase the productivity of a biotransformation without the associated biocompatibility problems that have sometimes been observed with organic solvents. The delivery of inhibitory substrate from the polymer phase was successfully accomplished, which is a novel demonstration in the field of solid-liquid TPPBs for biocatalysis. Finally this work contributes a range of simple strategies to improve the partitioning behavior of solid-liquid TPPBs using the aqueous phase composition.

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Chapter 1
Introduction

1.1 Background

The use of microorganisms to synthesize valuable compounds is becoming increasingly important in industrial processes (Straathof, et al. 2002). By using metabolic pathways contained in microorganisms, desired products can be produced with minimal by-product formation and with a high level of stereoselectivity, providing a significant advantage over chemical synthesis methods that are often plagued by by-product formation and low stereoselectivity (Woodley, et al. 2008). The pharmaceutical industry is the main industry that has been exploiting the stereoselectivity of metabolic pathways to produce high value compounds (Woodley 2008). Due to the rapidly increasing number of chiral pharmaceuticals and intermediates, the need for commercial scale biosynthesis is projected to increase (Straathof, et al. 2002).

One of the main limitations surrounding the implementation of biocatalysis in industry is a low productivity compared to chemical synthesis methods (Straathof, et al. 2002). Although the metabolic pathways being used in the biotransformation may be native to the microorganism, in high concentrations the microbe often experiences substrate and/or product inhibition. This limits the final product concentration, reducing the productivity of the process. Therefore, while the purity of the final product may be high, the low concentration (and possibly low rate of formation) may limit the ability of the process to meet industrial requirements. It is for this reason that methods to reduce substrate and product inhibition have been of high interest in both industrial research and academia.

Without the use of genetic modification of microorganisms, techniques to improve on process yields have focused on bioreactor design. The design of bioreactors in multiple phases such that the exposure of microbes to the inhibitory compounds is decreased has been

demonstrated to reduce inhibitory effects and improve system productivity (Woodley, et al. 2008). One of the most promising reactor designs is the two-phase partitioning bioreactor (TPPB).

TPPBs consist of an organism-containing aqueous phase and an immiscible second phase, which is either an organic solvent or polymer, and is selected to have a high affinity for the inhibitory compounds. This affinity causes the target molecules to partition into the immiscible phase, effectively creating a reservoir for the inhibitory compounds. With respect to inhibitory substrate, more will transfer from the sequestering phase to the aqueous phase to restore the thermodynamic equilibrium as it is consumed by the microbes. As products are formed, equilibrium is achieved by product molecules partitioning into the immiscible phase. The microbes are thus exposed to only low levels of the inhibitory compounds, allowing the overall system productivity to increase. Therefore, one of the most important aspects of second phase selection is the affinity towards the target compounds. However, as this phase is still in contact with the microbes, any biological interactions (such as toxicity or bioavailability) between the second phase and the microbes must be screened for and avoided in the design. TPPBs are industrially appealing not only because of the improved productivity that is possible, but also because the sequestering phase contains products in high concentrations and can act as the first stage in purification.

1.2 Objectives

This research was focused on examining the application of TPPBs to the bioproduction of an important pharmaceutical precursor, L-phenylacetylcarbinol (PAC), used to produce the drugs ephedrine and pseudoephedrine. PAC production is commercially done through biotransformation using yeast with benzaldehyde and glucose as substrates. However, the system is subject to substrate (benzaldehyde), product (PAC) and by-product (benzyl alcohol) inhibition. Recent studies in the literature have demonstrated the use of octanol as the sequestering phase in a TPPB. However, although octanol has a high affinity for the inhibitory compounds, it is also

toxic to the yeast performing the biotransformation. While organic solvents such as octanol are often toxic to microbes, polymers have recently been shown to provide a fully biocompatible sequestering phase in other biosynthesis processes (Gao and Daugulis 2009; Morrish and Daugulis 2008; Prpich and Daugulis 2007). Therefore, the first goal of this research was to identify a commercially available polymer that demonstrated a high affinity for the inhibitory compounds in the PAC system. Bioreactor performance and operation were then explored with the selected polymer as the sequestering phase in a TPPB.

In order to expand on knowledge in the field of solid-liquid TPPBs, benzaldehyde was selected as a target molecule to evaluate the effect of pH, temperature, and salt, glucose, and ethanol concentrations on the partition coefficient. By identifying how medium composition may alter the equilibrium distribution of compounds, some simple strategies to improve the performance of a solid-liquid TPPB have also been suggested.

1.3 References

Gao F, Daugulis AJ. 2009. Bioproduction of the aroma compound 2-phenylethanol in a solid-liquid Two-Phase Partitioning Bioreactor system by Kluyveromyces marxianus. Biotechnol Bioeng 104: 332-339.

Morrish JL, Daugulis AJ. 2008. Improved reactor performance and operability in the biotransformation of carveol to carvone using a solid-liquid two-phase partitioning bioreactor. Biotechnol Bioeng 101: 946-956.

Prpich GP, Daugulis AJ. 2007. A novel solid-liquid two-phase partitioning bioreactor for the enhanced bioproduction of 3-methylcatechol. Biotechnol Bioeng 98:1008-1016.

Straathof AJJ, Panke S, Schmid A. 2002. The production of fine chemicals by biotransformations. Curr Opin Biotechnol 13:548-556.

Woodley JM. 2008. New opportunities for biocatalysis: making pharmaceutical processes greener. Trends Biotechnol 26: 321-327

Woodley JM, Bisschops M, Straathof AJJ, Ottens M. 20.08. Future directions for in-situ product removal ISPR. Journal of Chemical Technology & Biotechnology 83:121-123.

Chapter 2
Literature Review

2.1 Microbes as biocatalysts

Microbes, such as bacteria and fungi, possess a wide range of enzymes that allow the cells to carry out complex metabolic processes. A microbe's metabolism is the sum of its catabolic and anabolic pathways. Catabolism is the breakdown of large molecules into their constituents, while anabolism is the synthesis of new molecules from simple building blocks. Within these pathways, each step is catalyzed by an enzyme that produces the required product from a given substrate with a high degree of accuracy, creating extremely efficient and reliable pathways. It is this efficiency that makes microbes of growing interest in a wide range of industrial applications. The use of microbes to degrade toxic compounds, known as biodegradation, has become an important aspect of environmental engineering, while the ability to use these naturally occurring metabolic pathways to synthesize valuable compounds is of growing commercial interest in biotechnology.

2.2 Commercial Biosynthesis

Biosynthesis has been used at a commercial level in several industrial sectors, including pharmaceuticals, food, feed, agrochemical, cosmetics and polymers (Straathof, et al. 2002). Synthesis of high value products using biological pathways not only minimizes by-product formation compared to chemical synthesis, but also provides a high level of stereoselectivity, and within these categories, almost 90% of processes are being used to produce chiral compounds (Straathof, et al. 2002). Biosynthetic processes are also of interest from an environmental perspective, where petrochemical based substrates may be replaced with renewable resources (FitzPatrick, et al. 2010).

The main limitations surrounding the industrial implementation of biological processes are often low final product yields, concentrations, or rates of production. While the microbe

possesses the pathway to perform the biotransformation, in order to meet commercial requirements, high concentrations of substrate and product can be present in the system and may inhibit or even cause complete toxicity to enzymes or whole cells (Pollard and Woodley 2007). This requirement of biocatalysts to operate outside their intrinsic metabolic capabilities has been the topic of extensive research in the operation and design of bioreactors, many of which will be discussed later in this review (Section 2.5).

Although a discussion of the current market can extend into several fields, this section will focus on the pharmaceutical industry as the most relevant to this review. 50 out of the top 100 selling pharmaceuticals of 1997 were single isomers with multiple chiral centres (Lye and Woodley 1999). In 2000, 35% of pharmaceutical intermediates were chiral, a value that is projected to increase to 70% of intermediates by 2010 (Pollard and Woodley 2007). This rapid growth rate in high value chiral compounds emphasizes the potential niche available to be occupied by biosynthesis at the commercial scale. Table 2-1 provides several examples of biotransformations currently in use in the pharmaceutical industry.

Table 2-1: Pharmaceuticals produced by biotransformation in industry. Adapted from Straathof, et al. 2002.

Compound	Company	Biocatalyst	Product (g/L.h)	Yield[¥] (%)
Lotrafiban	Glaxo	*Candida antarctica* lipase B	10	42
Paclitaxel	Bristol-Myers Squibb	*Pseudomonas lipase* AK, *P. cepacia* lipase PS-30	0.1	45
Xemilofiban	Monsanto	*Escherichia coli* penicillin acylase	1.6	43
Renin inhibitor	Hoffman La Roche	*Bacillus licheniformis* subtilisin	1	43
ACE Inhibitor	Ciba-Geigy	*Staphylococcus epidermis* D-lactate dehydrogenase	6.9	95
Omapatrilat	Bristol –Myers Squibb	*Thermoactinomyces intermedius* phenylalanine dehydrogenase	1.6	91
2-Quinoxaline-carboxylic acid	Pfizer	*Aspergillus repens* monooxygenase	0.003	57

[¥]Product yield based on total substrate consumed

While Table 2-1 does not provide an exhaustive list, it is important to note the diverse range of biocatalysts, productivities and yields. While in the fine chemical industry a high yield is generally required to be commercially valuable, in the pharmaceutical industry lower yields

and productivities may be acceptable as companies have additional considerations such as difficult chemical synthesis routes and tight time schedules to be competitive (Straathof, et al. 2002). As previously mentioned, pharmaceutical intermediates may also be subject to stereochemistry requirements. A well known example of this is L-phenylacetylcarbinol (PAC), a precursor to the drugs ephedrine and pseudoephedrine that is also commercially produced through biotransformation and is the main focus of this review.

2.3 Ephedrine

Since the commercial interest for PAC production is as an intermediate in the production of the pharmaceuticals ephedrine and pseudoephedrine, a brief overview of the ephedrine market is provided here.

2.3.1 History as a pharmaceutical

(-)-Ephedrine (1-methylamino-ethyl-benzyl alcohol, or 2-methylamino-1-phenyl-1-propanol) and (+)-pseudoephedrine were first discovered as extracts of plants in the genus *Ephedra*, which are indigenous to temperate and subtropical regions in Asia, Europe, and North and Central America (Abourashed, et al. 2003). The structures of ephedrine and pseudoephedrine are shown in Figure 2-1. Ephedrine is a stereoisomer of pseudoephedrine.

HO CH_3 R S $NHCH_3$

(–)-Ephedrine

CH_3 S S HO $NHCH_3$

(+)-Pseudoephedrine

Figure 2-1: Chemical structures of ephedrine and pseudoephedrine, extracts from the Ephedra plant. R and S denote configuration at the chiral carbon. (Abourashed, et al. 2003)

As pharmaceuticals, ephedrine and pseudoephedrine act as α- and β- adrenergic receptor stimulators. This results in enhanced cardiac output, peripheral vasoconstriction, bronchodilation, and central nervous system stimulation, making them common ingredients in flu medication as nasal decongestives (Abourashed, et al. 2003) .

The effects on the central nervous system are of particular importance and have lead to use/misuse in obesity treatment, improved endurance training, and body building. Ephedra alkaloids (which include ephedrine, pseudoephedrine and other extracts from the ephedra plant, such as maokonine or N-methylbenzylamine) have been the subject of investigation after being blamed for serious adverse effects in the 1990s. A review of 140 patient cases decided that only 31% of these were definitely or probably related to ephedra alkaloid containing substances (additionally 31% were possibly related) (Abourashed, et al. 2003). In February of 2000, the FDA withdrew rules banning sales of ephedra products in certain states. However, as of January 1, 2010, the World Anti-Doping Agency has added pseudoephedrine to its list of banned substances in athletic competition at levels above 150 μg/mL urine (World Anti-Doping Agency 2010). In Canada, Health Canada has constraints for nasal decongestant doses of 8 mg of (-)-ephedrine every 6-8 hours, max 32 mg/day (Abourashed, et al. 2003).

2.3.2 Production

China has historically been the main supplier of the ephedra plant. Production of ephedrine from plant material begins with treatment of powdered plant material with a strong base. The base is then extracted with chloroform and distilled off. The remaining material is dissolved in dilute acid and carbon filtered. The filtrate is alkalinized and the alkaloids are extracted with diethyl ether. The solvents are evaporated and recrystallized from hot water leaving pure (-)-ephedrine (Abourashed, et al. 2003).

The most economical and popular method for ephedrine synthesis is through fermentation with benzaldehyde and molasses. The fermentation step produces the intermediate molecule L-phenylacetylcarbinol (PAC), which is then converted to ephedrine or pseudoephedrine by treatment with CH_3NH_2 and H_2/Pt results as shown in Figure 2-2.

Figure 2-2: Production of ephedrine and pseudoephedrine via PAC intermediate (Shukla and Kulkarni 2000).

This research focuses on the production of high purity L-PAC through biological methods.

2.4 PAC Production through Biocatalysis

2.4.1 Stereochemistry of PAC

Phenylacetylcarbinol can exist as two stereoisomers. As only L-phenylacetylcarbinol (L-PAC) acts as a precursor for the production of ephedrine and pseudoephedrine (Shin and Rogers 1996), the use of a biocatalyst is appealing to provide stereoselectivity that is not possible with chemical synthesis methods (Rani, et al. 1995). L-PAC refers to the D/L Fisher projection system, which is structurally equivalent to R-PAC on the R/S light rotation system. PAC will be used to refer to L-PAC/R-PAC for simplicity.

2.4.2 Whole cell versus purified enzyme biocatalysis

Both active microbes as well as purified enzymes can be used as biocatalysts, with whole cell biotransformation remaining more industrially appealing. Whole microbes contain the cellular machinery and enzymes required to perform complex biotransformations. Purified enzymes may be used to catalyze a single step conversion, but as reaction complexity increases, more enzymes and cellular components must be added to the medium. Additionally, whole cells require less catalyst preparation than enzymatic processes as the required enzymes do not need to be purified. However, enzymatic processes offer the benefit of reducing by-product formation by controlling the active enzymes present in the system, whereas whole cell processes may have by-products formed in alternative metabolic pathways present in the microbe. Whole cells may also limit

access of enzymes to substrate in the medium, but this may also serve to protect enzymes from inhibition.

PAC production has been studied with both whole cells and purified enzymes. While this work focuses on whole cell biocatalysis, a brief review of advancements made in PAC production through enzymatic processes is provided in Section 2.5.1.

2.4.3 Pyruvate Decarboxylase (PDC)

The enzyme that catalyzes PAC production, pyruvate decarboxylase (PDC), is present in some filamentous fungi, yeast, and wheat germ. Though the enzyme performs the same function in these organisms, the structures vary between organisms. PDC has a molecular weight of 230-250 kDa for yeast and *Zymomonas mobilis* and 275 kDa for wheat germ (Shin and Rogers 1996). Yeast and wheat germ PDC have two dimeric subunits, α_2 and β_2, which are of slightly different chain lengths. *Z. mobilis* PDC has 4 identical subunits, α_4 (Shin and Rogers 1996). The holoenzyme structure includes thiamine pyrophosphate (TPP) and Mg^{2+} ions as obligatory cofactors (Shin and Rogers 1996).

2.4.4 Microorganisms available as biocatalysts

Although the industrial process uses the yeast *Saccharomyces cerevisiae* to perform the biotransformation, several species of yeast as well as filamentous fungi possess the required pathway and have demonstrated PAC production ability in the literature.

Screening of a wide range of yeast species demonstrated successful PAC production at the shake-flask level (Netrval and Vojtisek 1982). *Candida utilis* was one of the top producing strains of yeast shown and has subsequently been used in many studies on PAC production. In a screening study on purified PDC from 105 different strains of yeast, all but 7 could perform the biotransformation from benzaldehyde and pyruvate. 5 out of those 7 strains were shown to not ferment glucose, indicating no carboligase activity (Rosche, et al. 2003). Throughout the

literature, *S. cerevisiae* and *C. utilis* remain the most studied microbes for PAC production and have been used for both whole cell and purified enzyme studies.

Screening of filamentous fungi revealed PDC activity in some strains, noting that in oxygen limited conditions, these fungi could produce ethanol from sugars (Rosche, et al. 2001). While this study did not test PAC production ability, later work testing 14 filamentous fungi extracts demonstrated PAC production adding benzaldehyde and pyruvate to all 14 extracts (Rosche, et al. 2001). The highest final PAC concentration was produced by the *Rhizopus javanicus* and *Fusarium sporotrichioides* strains, producing 11.7-12.6 g/L PAC in 20 h using benzaldehyde and pyruvate as substrates. This reaction had 90-93% enantiomeric selection for L-PAC (Rosche, et al. 2001).

2.4.5 Metabolic Pathway

Glycolysis in yeast generates pyruvate from glucose. Under aerobic conditions, pyruvate can be further utilized in the Citric Acid Cycle, but anaerobic conditions cause the accumulation of pyruvate and PDC. When benzaldehyde is added to the system, PDC catalyzes the reaction of benzaldehyde and pyruvate to PAC. The reaction mechanism is shown in Figure 2-3.

Figure 2-3: The PDC catalyzed conversion of benzaldehyde and pyruvate to PAC. The structures of by-products benzyl alcohol, acetaldehyde, and acetoin are also shown (Rosche, et al. 2005).

During normal ethanol fermentation, PDC decarboxylates pyruvate to acetaldehyde using thiamine pyrophosphate (TPP) and Mg^{2+} as cofactors. The TPP bound "active" acetaldehyde then

takes up a proton and is released. In the presence of benzaldehyde, PDC reacts with "active acetaldehyde" and benzaldehyde and produces PAC (Rosche, et al. 2001).

2.4.6 By-product formation

The major problem with whole cell biotransformation is the production of benzyl alcohol (structure shown in Figure 2-3) from a side reaction with alcohol dehydrogenase (ADH) or other oxidoreductases (Long and Ward 1989; Shin and Rogers 1995). Using whole cells, ADH cannot be removed as this enzyme is required to regenerate NAD+ cofactors. (Kostraby, et al. 2002).

Additional low concentration by-products are acetoin and acetaldehyde (structures shown in Figure 2-3), but can also include diacetyl, acetylbenzoyl, and 2-hydroxy-1-phenyl-propanon (Goetz, et al. 2001). The ADH enzyme has also been shown to convert PAC to PAC-diol (Shukla and Kulkarni 2000). Benzaldehyde losses from the system can also occur due to volatilization (Sandford, et al. 2005) and oxidation to benzoic acid (Goetz, et al. 2001).

2.4.7 Substrate Selection

As Figure 2-3 showed, benzaldehyde must be used as a substrate in both the enzymatic and the whole cell biotransformations. As PDC reacts with "active acetaldehyde" to produce PAC, there are several compounds that may be used as co-substrates. Most simply, glucose may be used with the microbes operating under anaerobic conditions as glucose will be converted to pyruvate via glycolysis. Under circumstances where the glycolytic pathway is not available to the microbes (for example by using purified enzymes) pyruvate may be used instead of glucose. However, several disadvantages are associated with the use of pyruvate: 1) pH shift occurs due to the decarboxylation process; 2) CO_2 gas formation may interfere with enzyme activity; 3) production of acetaldehyde from pyruvate is significantly faster than the carboligation with benzaldehyde, leading to greater quantities of the by-product acetaldehyde 4) pyruvate is expensive compared to glucose (Goetz, et al. 2001). Acetaldehyde may be used instead of pyruvate as a more cost effective substrate, but has been shown to have inhibitory effects on PDC (Rosche, et al. 2004).

Substrate selection also has an impact on by-product formation in the system. While glucose is an inexpensive substrate, anaerobic conditions are required for the accumulation of pyruvate in the microbe. Other enzymes active under anaerobic conditions, such as alcohol dehydrogenase (ADH), can react with benzaldehyde to produce benzyl alcohol, which is the most significant by-product formed in the industrial process (Shin and Rogers 1995). Studies where ADH was inhibited in the yeast have been done to demonstrate that benzyl alcohol formation can be avoided, though pyruvate must be used as a substrate (Kostraby, et al. 2002), which is associated with an increase in acetaldehyde formation (Goetz, et al. 2001). It is for this reason that glucose remains a preferred substrate, and purification methods to remove by-product are also of interest.

2.4.8 System Inhibition

PAC production poses an interesting challenge due to substrate, product and by-product inhibition of *C. utilis*.

2.4.8.1 Substrate Inhibition

Substrate inhibition refers to decreased activity of the biocatalyst (either purified enzymes or whole cells) in the presence of high substrate concentrations. In some cases, the substrate may be so toxic to the organism that activity is completely stopped. Therefore, there exists an optimal level of substrate that can be fed to the system such that productivity is maximized (Long and Ward 1989; Rogers, et al. 1996; Shin and Rogers 1995).

Although benzaldehyde is a necessary substrate for whole cell biotransformation, benzaldehyde is inhibitory to PDC and is toxic to *C. utilis* (Rogers, et al. 1996). There is a discrepancy in literature values for the inhibitory level of benzaldehyde in this biotransformation. Values as low as 1.1 g/L and as high as 5.3 g/L (Rogers, et al. 1996; Shin and Rogers 1995) have been reported as the concentration before inhibition begins.

Glucose has not been shown as inhibitory to *C. utilis*. Additionally, pyruvate has been shown to reduce the inhibitory nature of benzaldehyde on PDC, and two hypotheses have been generated

as to why this may occur: 1) Pyruvate may bind to PDC causing a conformation change that protects the active site or 2) Pyruvate may provide protection to the active site during pyruvate conversion (Rosche, et al. 2005). Since cells will be producing pyruvate from glucose via glycolysis, as long as the cells are fed and under anaerobic conditions, there should always be pyruvate present *in vivo* to, in theory, add to PDC stability. This may be a contributing reason for the discrepancy between inhibitory benzaldehyde concentrations in the literature.

2.4.8.2 Product Inhibition

Product inhibition is analogous to substrate inhibition in that high levels of product in the system can be toxic to the biocatalyst. In the case of PAC production, PAC as well as the by-products benzyl alcohol and ethanol, are inhibitory to *C. utilis*. Proposed inhibition concentrations for these products are 4.1, 5, and 39 g/L respectively (Rogers, et al. 1996)

2.4.8.3 End of the biotransformation

One aspect important to the implementation of an appropriate bioreactor design for a biotransformation is understanding what causes the process to stop. Three possible factors have been proposed for the PAC production system: 1) Significant reduction in PDC activity due to substrate or product inhibition; 2) Pyruvate limitation; 3) Cell viability loss due to extended exposure to benzaldehyde (and/or PAC and benzyl alcohol) (Shin and Rogers 1995). It is important to understand which of these factors are occurring in order to develop strategies to improve the lifespan of the bioprocess.

2.5 Bioreactor design for PAC production

To make a biosynthesis process economically feasible, a high product concentration is required. In the case of PAC production, the inhibitory nature of the substrate and products is the main limitation on productivity. This section outlines the current literature on PAC production, with a focus on whole cell biotransformation studies. A brief review of purified enzyme PAC production is provided for completeness.

2.5.1 Production of PAC using purified enzymes

The upstream processing required to prepare purified enzymes for biotransformation is a costly and time consuming step. To partially purify proteins from cell homogenate, ammonium sulphate fractionation is used. PDC precipitates at 45-55% ammonium sulphate saturation. The product can then be desalted using dialysis in 40 mM potassium phosphate pH 6 buffer. Chromatography with columns containing the PDC cofactors thiamine pyrophosphate (TPP) and Mg^{2+} (in the form of $MgSO_4$) allows PDC to be purified further (Shin and Rogers 1996). The purified enzyme may be used with benzaldehyde and pyruvate as substrates to produce PAC, which has been extensively studied. A brief summary of advancements in this field is provided in Table 2-2.

Table 2-2: Short list of results in enzymatic biotransformation for PAC production

Reference	PDC Source Organism	Contribution
Shin and Rogers, 1996	*C. utilis*	• Optimal conditions for biotransformation determined as 4°C, pH 7 containing 2.0 M ethanol (to improve benzaldehyde solubility)
Rosche, et al., 2001	Various filamentous fungi	• Screening of 14 filamentous fungi extracts for PAC production capability • Highest PAC concentrations reached with *Rhizopus javanicus* and *Fusarium sporotrichioides*
Goetz, et al., 2001 and Iwan, et al., 2001	*Zymomonas mobilis*	• Continuous enzyme-membrane bioreactor
Rosche, et al., 2002	*C. utilis* and *R. javanicus*	• Biphasic octanol/aqueous process • *C. utilis* outperformed *R. javanicus*
Rosche, et al., 2004	*Z. mobilis*	• Demonstrated use of acetaldehyde rather than pyruvate and benzaldehyde as substrates in a biphasic octanol/aqueous system

Though purified enzyme processes can be costly, the results of the studies provided in Table 2-2 showed high concentration of PAC production without benzyl alcohol as a by-product. With respect to the reactor configurations employed with purified enzymes, the biphasic

octanol/aqueous systems can also be applied to whole cell biotransformations, and will be further discussed in Section 2.5.2.2. The use of a membrane bioreactor was effective for enzymatic biotransformations, but can be problematic when implemented for whole cells due to clogging of the membrane. Therefore, whole cell membrane bioreactors often have a micro-/ultrafiltration step before the product is removed to preserve the integrity of the membrane (Stark and von Stockar 2003). Membrane bioreactors could in theory be applied to whole cell PAC production, but due to the increased complexity of the process are not of high interest.

2.5.2 Production of PAC using whole cells

PAC is currently industrially produced using yeast culture fermentation by the biotransformation of benzaldehyde and molasses (Shin and Rogers 1995), giving industrial relevance to research into whole cell biotransformation for PAC production. This section examines the reactor designs that have been used for whole cell PAC production in the literature.

2.5.2.1 Immobilized cells

The most frequently used method to immobilize cells is encapsulation in calcium alginate beads. This reduces the exposure of the microbe to the inhibitory compounds by keep the cells isolated away from the aqueous media. Yeasts have been immobilized in an effort to enhance PAC production by reducing toxic effects sustained from benzaldehyde, PAC, and benzyl alcohol.

S. cerevisiae were immobilized in calcium alginate beads, which was found to increase the tolerance to benzaldehyde to be used for 7 cycles of fed-batch fermentation and over 200 h of operation (Mahmoud, et al. 1990). This experiment reports an overall concentration of 10 g/L PAC produced, which is fivefold higher than the single phase fermentation.

In a 1995 study, Shin and Rogers used immobilized *C. utilis* in calcium alginate beads to produce PAC. The benzaldehyde level was controlled at 2 g/L in this study and a final PAC concentration of 15.2 g/L (in 22 h) was reported (Shin and Rogers 1995). This study provided an important quantification of the tolerance levels of *C. utilis* to benzaldehyde. The immobilized

cells were shown to tolerate up to 7.4 g/L benzaldehyde before substrate inhibition, while free cells could only tolerate up to 5.3 g/L. The immobilization of yeast was successful at increasing tolerance to benzaldehyde, as was predicted to occur with immobilization.

While immobilizing cells in matrices such as calcium alginate is experimentally simple and inexpensive, the commercial use of immobilized cells is limited. Over time, beads wear down and break and the immobilization process must be redone. Additionally, the presence of sodium ions in the medium can reduce the stability of the calcium alginate matrix (Martinsen, et al, 1989).

2.5.2.2 Two-phase partitioning bioreactors (TPPBs)

TPPBs consist of a microorganism containing aqueous growth medium and an immiscible second phase, selected to have a high affinity for the inhibitory compounds present in the system. As a result, the target compounds will preferentially partition into the immiscible phase, reducing exposure of the microbes to toxic compounds. TPPBs are further described in general in Section 2.6.

TPPBs have been applied to PAC production, with *C. utilis* being used as the biocatalyst (Rosche, et al. 2005). Octanol was selected as the sequestering phase for its affinity for benzaldehyde and PAC; however, it was observed to inhibit glucose metabolism in *C. utilis.* As a result, the microbes could not convert glucose to pyruvate, and pyruvate had to be used as a substrate. This study demonstrated a 26% increase in the molar yield of PAC on benzaldehyde, 58 g/L PAC concentration (a 3.9 fold increase), and a 3.1 fold volumetric productivity increase over single phase PAC production. This is a promising improvement, although with the metabolic limitation requiring the use of pyruvate, there is room to improve performance given a biocompatible second phase.

Most recently in the field of PAC production, a cloud point system using the polymer polyethylene glycol (PEG) was developed in an effort to use the principles of the octanol/aqueous TPPB but with a biocompatible polymer as the sequestering phase (Zhang, et al. 2008). The

cloud point refers to the phase separation temperature; therefore, when operated at or above this temperature the reactor is biphasic. While the biocompatibility of PEG allowed glucose to be used as a substrate reducing costs compared to pyruvate, this system produced only 8 g/L of PAC and 4 g/L of benzyl alcohol. While this removed biocompatibility issues surrounding the use of octanol, the door is left open to improve performance with further investigations into the PAC system using a higher affinity, biocompatible sequestering phase.

2.6 Two-phase partitioning bioreactors (TPPBs)

As previously described in Section 2.5.2.2, TPPBs are used to sequester inhibitory compounds into an auxiliary immiscible phase selected to have a high affinity for the target compounds in the system. This affinity causes the compounds to partition into the immiscible phase, characterized by the partition coefficient. The partition coefficient is the ratio of the concentration of the target compound in the sequestering phase relative to its concentration in the aqueous phase at equilibrium. Therefore, a high partition coefficient corresponds to a high affinity of the target compound towards the sequestering phase. This allows the sequestering phase to act as a reservoir for high levels of substrate, delivering only low concentrations to the aqueous phase or extracting products as the biotransformation progresses creating a high concentration of product in the immiscible phase.

The use of the immiscible phase to deliver substrate has been predominantly focused on the biodegradation of toxic compounds. The extraction of products into the second phase is also known as *in situ* product removal (ISPR), and has been a frequently employed method to reduce product inhibition in biological systems.

The success of a TPPB requires that the sequestering phase be selected to have certain characteristics. Aside from a high affinity for the target compounds, the sequestering phase must also not cause any inhibitory effects to the microorganism (it must be biocompatible) and must not be consumed by the microorganism (it must be non-bioavailable). This section will describe

these characteristics, as well as other additional considerations associated with design, for both liquid-liquid and solid-liquid TPPBs.

2.6.1 Liquid-liquid TPPBs

Organic solvents are traditionally used as the extractive phase in TPPBs, and have been successfully applied to both biodegradation and biosynthesis studies. With the large variety of organic solvents available, finding an organic solvent with affinity for given target molecules is often possible. Categories of organic solvents include aliphatic alcohols (e.g. octanol), long chain alkanes (e.g. nonane) and carboxylic acids (e.g. oleic acid) (Prpich and Daugulis 2007b). Solvent selection strategies for use in liquid-liquid TPPBs must not only screen for the partition coefficient of the target molecule to demonstrate a high affinity, but must also screen biocompatibility and bioavailability to ensure that the organic solvent is effectively inert in the system.

2.6.1.1 Biocompatibility

Despite the wide variety available, a common limitation with organic solvents is their potential toxicity towards the microorganism (Bruce and Daugulis 1991; Laane, et al. 1987). If the solvent is toxic to the microbe, this can inhibit the active metabolic pathways required for the biotransformation and the solvent is considered not biocompatible.

The biocompatibility of a solvent is dependent on the species of microbe performing the biotransformation. The logarithm of a solvent's partition coefficient in a standard octanol: water mixture (log P) acts as a polarity index that can be used to predict the tolerance of a given microbial species to a particular solvent. A relationship exists between the log P of a solvent and its biocompatibility with a specific organism. This parameter is known as the critical log P (log P_{crit}) of the organism. Solvents with a log P below the log P_{crit} are usually inhibitory, while solvents above the log P_{crit} are generally biocompatible (Bruce and Daugulis 1991; Laane, et al. 1987). Some common organic solvents and their log P values are shown in Table 2-3.

Table 2-3: Some common organic solvents and their log P values

Solvent	Log P*
Octanol	2.9
Hexane	3.5
Octane	4.5
Nonane	4.8
1-dodecanol	5.0
Decane	5.6
Dodecane	6.6
Oleyl Alcohol	7.5
Oleic acid	7.7
Hexadecane	8.2

*log P values from Syracuse Research Cooperation log P database (http://www.srcinc.com)

Some common microbes and their limiting log P values for growth are shown in Table 2-4.

Table 2-4: Some common microbes and their log P_{crit} values

Microorganism	log P_{crit}
Escherichia coli IFO 3806	3.8 (Inoue and Horikoshi 1991)
Bacillus subtilis AHU 1219	4.9 (Inoue and Horikoshi 1991)
Pseudomonas putida MC2	3.1 (Prpich and Daugulis 2007b)
Pseudomonas aeruginosa IFO 3924	3.4 (Inoue and Horikoshi 1991)
Rhodococcus erythropolis DCL14	5 (Morrish, et al. 2008)
Saccharomyces cerevisiae NCYC 716	5-6 (Bruce and Daugulis 1991)

The information provided in Tables 2-3 and 2-4 is not exhaustive. It is meant to demonstrate the species dependency of log P_{crit} values, as well as the approximate range of log P_{crit} values compared to the available range of organic solvents. It also must be noted that the log P_{crit} method for predicting biocompatibility is not valid for solvent mixtures (Bruce and Daugulis 1991) .

2.6.1.2 Bioavailability

Because TPPBs have direct contact between the fermentation medium and the immiscible phase, bioavailability of this second phase must also be considered. In this work, bioavailability refers to the sequestering phase being metabolizable by the microbes present in the system. Microbes will consume whatever is present in the medium that is most readily available based on their metabolisms. Therefore, in order to ensure that biocatalysts are only performing the desired reaction, the substrate available to the biocatalysts must be controlled. This means ensuring that

the sequestering phase selected is not readily metabolized by the specific biocatalyst for that process (Rehmann, et al. 2007).

Bioavailability of organic solvents is a common problem when microbial consortia are used as biocatalysts, but must still be considered when using a pure strain of microorganism for the biotransformation (Rehmann, et al. 2007). The bioavailability of a particular class of solvent to a microorganism has been demonstrated previously in work with *Mycobacterium* PYR-1 (MacLeod and Daugulis 2003). Therefore, with some screening work on a particular microorganism, some predictability about which classes of organic solvents may be suitable is possible.

2.6.2 Solid-liquid TPPBs

Advances in the field of TPPBs have demonstrated that solid polymer beads may be used as the sequestering phase to replace organic solvents (Amsden, et al. 2003). Desirable polymer characteristics include: commercially available at a low cost; non-hazardous; biocompatible; non-bioavailable; not promoting biofilm formation; high affinity for the target compounds; thermally stable to allow for sterilization; stable in aqueous medium at reaction conditions; and stable in the medium used to load polymer with target compounds (Rehmann and Daugulis 2007; Rehmann, et al. 2007). This section will briefly discuss issues related to the use of polymer beads and then review the current literature in solid-liquid TPPBs.

2.6.2.1 Biocompatibility and Bioavailability

Biocompatibility problems between polymer beads and microorganisms have not been observed in solid-liquid TPPBs, providing an immediate relief to the major limitation associated with the use of organic solvents. Bioavailability is a concern, as there are many commercially available biodegradable polymers. However, with the use of non-biodegradable polymers, both biocompatibility and bioavailability problems are avoided.

2.6.2.2 Biofilm formation

The aggregation of microbes into a film around the surface of the polymer bead must also be determined. The addition of a film layer around the beads has the potential to interfere with the partitioning of target compounds between phases. This is a concern with hydrophobic microbes, such as *Rhodococcus erythropolis*, which has been evaluated previously for use in solid-liquid TPPBs (Morrish and Daugulis 2008). Biofilm formation is best evaluated on a case by case basis with polymer screenings for individual microbial species.

2.6.2.3 Rate of Release and Uptake

Solid-liquid TPPBs have additional mass transfer considerations that are not associated with liquid-liquid TPPBs where the organic solvent disperses as droplets when agitated. The rate of release of target molecules from the polymer beads is most often a consideration when using polymer beads to deliver substrates. If the rate of release is too slow to meet the metabolic demands of the microbes, the microbes will function under starved conditions, which may not be optimal for the productivity of the system. Factors reducing the rate of release can include a low diffusivity of the polymer, a long path length for diffusion, or a low concentration gradient. Strategies to reduce mass transport limitations have included increasing polymer bead surface area to volume ratio and applying ultrasonic waves to the system (Isaza and Daugulis 2009; Rehmann and Daugulis 2006).

2.6.2.4 The use of polymers for drug delivery

Polymers are of great interest in the field of drug delivery. Polymers allow controlled release of drugs implanted in the body; however biodegradable polymers are of more interest for drug delivery than inert polymers. Therefore, this field will not be discussed, as non-bioavailable polymers are of greatest concern to this work.

2.6.2.5 The use of polymers for biodegradation

Although biodegradation is not the focus of the present research, biodegradation has been more extensively examined than synthesis in solid-liquid TPPBs, and the basic concepts

surrounding polymer selection and bioreactor operation remain the same. Therefore, examination of biodegradation experiments can provide valuable guidelines for designing a synthesis reaction.

The polymer phase is used to sequester the compound being degraded such that the microorganisms in the aqueous phase are exposed to low levels of toxic compounds. Polymers provide the additional benefit over the use of organic solvents of easy management and separation of the sequestering phase. For remediation purposes, the polymer can be applied directly to a contaminated site, such as soil or water, to absorb the target compounds. The loaded beads are then added to a bioreactor containing microbes that are able to degrade the target molecule. As the microbes consume the target compound in the aqueous phase, more of the substrate will partition from the polymer to the aqueous phase to maintain equilibrium. Therefore, over time, the target compounds are consumed, while the microbes are not exposed to inhibitory concentrations at any point. Examination of loading strategies in biodegradation studies can be used as a starting point for biosynthesis processes with substrate delivery from polymer beads.

Degradation studies in solid-liquid bioreactors have included: the delivery of benzene to *Alcaligenes xylosoxidans* (Daugulis, et al. 2003); degradation of a phenolic mixture by microbial consortium (Prpich and Daugulis 2005)**;** degradation of biphenyl by *Burkholderia xenovorans* (Rehmann and Daugulis 2007), and remediation of PAH contaminated soils (Rehmann, et al. 2008).

2.6.2.6 The use of polymers for biosynthesis

The use of solid-liquid TPPBs for synthesis of high value compounds is a relatively new field that has been pioneered by the Daugulis group, with the bioproduction 3-methylcatechol (Prpich and Daugulis 2007a), carvone (Morrish and Daugulis 2008) and 2-phenylethanol (Gao and Daugulis 2009).

The production of 3-methylcatechol from toluene using *Pseudomonas putida* MC2 in a solid-liquid bioreactor evaluated both the geometry of the sequestering phase as well as the recirculation of the aqueous phase in an external loop (Prpich and Daugulis 2007a). Submersed

polymer beads and polymer beads that had been reconstituted into flat sheets lining the internal wall of the bioreactor were both evaluated. The partition coefficient of the polymer sheets was observed to be higher than that of the original beads, however the productivity was lower. This may be due to a mass transfer limitation of 3-methylcatechol transferring into the polymer sheets, because of their lower surface area than beads (Prpich and Daugulis 2007a). The implementation of an external loop containing polymer beads demonstrates the ability of a solid-liquid TPPB to run continuously, an additional advantage over the use of organic solvents.

Investigations on the production of carvone from carveol using *Rhodococcus erythropolis* DCL14 examined the effects of polymer mixtures on volumetric productivity (Morrish and Daugulis 2008). This system contained both an inhibitory substrate and product, and therefore, both compounds needed to be sequestered. When only styrene/butadiene co-polymer (SBR) was used as a second phase in a fed-batch experiment, a volumetric productivity of 102 mg/L-h was achieved. However, this polymer has a partition coefficient of 118 for carvone and only 6 for carveol. The polymer Hytrel 8206 demonstrated partition coefficients of 49 for carvone, and 36 for carveol. This provided the opportunity to use a polymer mixture of SBR, for its affinity for carvone, and Hytrel 8206, for its affinity for carveol. The volumetric productivity increased to 106 mg/L-h. While only demonstrating a 4% increase in productivity, this study opens the door for the polymer screening to select a polymer mixture in systems with multiple inhibitory compounds.

Most recently, a study on the bioproduction of 2-phenylethanol (2-PE) by *Kluyveromyces marxianus* from L-phenylalanine demonstrated the use of a solid-liquid TPPB to achieve the highest 2-PE concentration reported in the current literature (Gao and Daugulis 2009). This study demonstrated not only two polymer phase ratios, but also recirculation of the aqueous phase in an external column of polymer beads to allow the process to run continuously. This can be used to provide helpful guidelines for the design of batch and continuous solid-liquid TPPBs.

2.6.2.7 Additional considerations for scale up and commercial use

Although solid-liquid polymer systems do not display any foaming or viscosity difficulties, such as those associated with the use of silicone oil, there are some operational factors to consider for industrial scale work. Accumulation of polymer beads behind bioreactor internals has been observed in solid-liquid systems, and 20% polymer phase by volume has been experimentally suggested as an upper limit (Boudreau and Daugulis 2006). Recent studies have shown that polymers may also influence oxygen transfer properties of the system by increasing oxygen transfer rate (Littlejohns and Daugulis 2007). Scale-up of solid-liquid TPPBs to an industrial level, therefore, must account for the effects of polymer bead mixing and oxygen transport.

Another consideration is the recovery of the sequestering phase. When the sequestering phase is inside the bioreactor, the system must be operated in batch to allow the removal of the organic solvent or polymer beads after the biotransformation is complete. Organic solvent separation requires the use of aspiration, while the recovery of solid polymers is simple using sieving. Recently, polymer beads have been magnetized for easy recovery after use in solid-liquid TPPBs (Yeom, et al. 2010). With the use of an external loop containing the polymer phase, the system may be operated continuously, which could be advantageous on an industrial scale. Additionally, the reuse of the second phase is also highly desirable to reduce operating costs. The reuse of polymers in synthesis reactions has been demonstrated (Prpich and Daugulis 2007a), adding to the potential for solid-liquid TPPBs to be used in commercial processes. Polymers can be cleaned by washing the beads in a solvent such as methanol, leaving the beads to be reused. This method allows target compounds to be extracted into the solvent phase for further downstream processing.

2.7 Scope of Thesis

The above literature points towards the potential to improve biological PAC production using the sequestering capabilities of a TPPB. As liquid-liquid TPPBs with octanol as the immiscible phase have been applied in the literature to this system with sustained inhibitory effects on *C. utilis*, one avenue for improvement would be the use of a fully biocompatible sequestering phase. Recent studies using polymer beads as an inert but highly effective second phase are encouraging in that the screening of commercially available polymers can yield an appropriate sequestering phase.

This work examined the application of a solid-liquid TPPB to the production of PAC from benzaldehyde using *C. utilis*. The first objective of this work was to determine the biocompatibility and bioavailability of a variety of solvents with *C. utilis*, with the aim of providing guidelines for future liquid-liquid TPPB studies. The potential of various commercially available polymers to sequester benzaldehyde, PAC and benzyl alcohol was also explored, with the most effective polymer being used in a solid-liquid TPPB.

Once the solid-liquid TPPB concept was successfully applied to the PAC system, the effect of varying the phase ratio was also evaluated to obtain a deeper understanding of the performance of TPPBs. The possibility of delivering benzaldehyde from the polymer phase was also explored in order to reduce manual intervention for simplified operation and reduced risk of contamination.

Finally, solute partitioning into the polymer phase was evaluated using some common elements of medium composition that may change during biotransformation. By identifying how medium composition may alter the equilibrium distribution of compounds, some simple strategies to improve the performance of a solid-liquid TPPB have also been suggested.

2.8 References

Abourashed EA, El-Alfy AT, Khan IA, Walker L. 2003. Ephedra in perspective – a current review. Phytother Res 17:703-712.

Amsden BG, Bochanysz J, Daugulis AJ. 2003. Degradation of xenobiotics in a partitioning bioreactor in which the partitioning phase is a polymer. Biotechnol Bioeng 84:399-405.

Boudreau NG, Daugulis AJ. 2006. Transient performance of two-phase partitioning bioreactors treating a toluene contaminated gas stream. Biotechnol Bioeng 94:448-457.

Bruce LJ, Daugulis AJ. 1991. Solvent Selection Strategies for Extractive Biocatalysis. Biotechnol Prog 116-124.

Daugulis AJ, Amsden BG, Bochanysz J, Kayssi A. 2003. Delivery of benzene to *Alcaligenes xylosoxidans* by solid polymers in a two-phase partitioning bioreactor. Biotechnology Letters 25:1203-1207.

FitzPatrick M, Champagne P, Cunningham M, Whitney, R. 2010. A biorefinery processing perspective: Treatment of lignocellulosic materials for the production of value-added products. Bioresource Technol 101:8915-8922.

Gao F, Daugulis AJ. 2009. Bioproduction of the aroma compound 2-phenylethanol in a solid-liquid Two-Phase Partitioning Bioreactor system by Kluyveromyces marxianus. Biotechnol Bioeng 104: 332-339.

Goetz G, Iwan P, Hauer B, Breuer M, Pohl M. 2001. Continuous production of (R)-Phenylacetylcarbinol in an enzyme-membrane reactor using a potent mutant of pyruvate decarboxylase from *Zymomonas mobilis*. Biotechnol Bioeng 74:317-325.

Inoue A, Horikoshi K. 1991. Estimation of solvent-tolerance of bacteria by the solvent parameter log P. J Ferment Bioeng 71:194-196.

Isaza PA, Daugulis AJ. 2009. Ultrasonically enhanced delivery and degradation of PAHs in a polymer-liquid partitioning system by a microbial consortium. Biotechnol Bioeng 104:91-101.

Iwan P, Goetz G, Schmitz S, Hauer B, Breuer M, Pohl M. 2001. Studies on the continuous production of (*R*)-(-)-phenylacetylcarbinol in an enzyme-membrane reactor. Journal of Molecular Catalysis B: Enzymatic 11:387-396.

Kostraby MM, Smallridge AJ, Trewhella MA. 2002. Yeast-mediated preparation of *l*-PAC in an organic solvent. Biotechnol Bioeng 77:827-831.

Laane C, Boeren S, Vos K, Veeger C. 1987. Rules for optimization of biocatalysis in organic solvents. Biotechnol Bioeng 30:81-87.

Littlejohns JV, Daugulis AJ. 2007. Oxygen transfer in a gas–liquid system containing solids of varying oxygen affinity. Chem Eng J 129:67-74.

Long A, Ward O. 1989. Biotransformation of benzaldehyde by Saccharomyces cerevisiae: Characterization of the fermentation and toxicity effects of substrates and products. Biotechnol Bioeng 34:933-941.

Lye GJ, Woodley JM. 1999. Application of in situ product-removal techniques to biocatalytic processes. Trends Biotechnol 17:395-402.

MacLeod C, Daugulis A. 2003. Biodegradation of polycyclic aromatic hydrocarbons in a two-phase partitioning bioreactor in the presence of a bioavailable solvent. Appl Microbiol Biotechnol 62:291-296.

Mahmoud WM, El-Sayed A, Halim M.M., Coughlin RW. 1990. Production of L-phenylacetylcarbinol by immobilized yeast cells: II. Semicontinuous fermentation. Biotechnol Bioeng 36:55-63.

Martinsen A, Skjak-Braek G, Smidsrsd O. 1989. Alginate as Immobilization Material: I. Correlation between Chemical and Physical Properties of Alginate Gel Beads. Biotechnol Bioeng 33-79-89.

Morrish JL, Brennan ET, Dry HC, Daugulis AJ. 2008. Enhanced bioproduction of carvone in a two-liquid-phase partitioning bioreactor with a highly hydrophobic biocatalyst. Biotechnol Bioeng 101:768-775.

Morrish JL, Daugulis AJ. 2008. Improved reactor performance and operability in the biotransformation of carveol to carvone using a solid-liquid two-phase partitioning bioreactor. Biotechnol Bioeng 101: 946-956.

Netrval J, Vojtisek V. 1982. Production of Phenylacetylcarbinol in Various Yeast Species. European Journal of Applied Microbiology and Biotechnology 16:35-38.

Pollard DJ, Woodley JM. 2007. Biocatalysis for pharmaceutical intermediates: the future is now. Trends Biotechnol 25:66-73.

Prpich GP, Daugulis AJ. 2007a. A novel solid-liquid two-phase partitioning bioreactor for the enhanced bioproduction of 3-methylcatechol. Biotechnol Bioeng 98:1008-1016.

Prpich GP, Daugulis AJ. 2007b. Solvent selection for enhanced bioproduction of 3-methylcatechol in a two-phase partitioning bioreactor. Biotechnol Bioeng 97:536-543.

Prpich GP, Daugulis AJ. 2005. Enhanced biodegradation of phenol by a microbial consortium in a solid–liquid two phase partitioning bioreactor. Biodegradation 16:329-339.

Rani BR, Ubukata M, Osada H. 1995. Reduction of Arylcarbonyl Using Zinc Dust in Acetic Acid. Bull Chem Soc Jpn 68:282-284.

Rehmann L, Daugulis AJ. 2007. Biodegradation of biphenyl in a solid–liquid two-phase partitioning bioreactor. Biochem Eng J 36:195-201.

Rehmann L, Daugulis AJ. 2006. Biphenyl degradation kinetics by Burkholderia xenovorans LB400 in two-phase partitioning bioreactors. Chemosphere 63:972-979.

Rehmann L, Prpich GP, Daugulis AJ. 2008. Remediation of PAH contaminated soils: Application of a solid–liquid two-phase partitioning bioreactor. Chemosphere 73:798-804.

Rehmann L, Sun B, Daugulis AJ. 2007. Polymer selection for biphenyl degradation in a solid-liquid two-phase partitioning bioreactor. Biotechnol Prog 23:814-819.

Rogers P, Shin H, Wang B. 1996. Biotransformation for L-Ephedrine Production. Advances in Biochemical Engineering Biotechnology 56:33-60.

Rosche B, Sandford V, Breuer M, Hauer B, Rogers PL. 2002. Enhanced Production of R-phenylacetylcarbinol (R-PAC) through enzymatic biotransformation. Journal of molecular catalysis B: Enzymatic 109-115.

Rosche B, Breuer M, Hauer B, Rogers PL. 2005. Role of pyruvate in enhancing pyruvate decarboxylase stability towards benzaldehyde. Journal of Biotechnology 115:91-99.

Rosche B, Sandford V, Breuer M, Hauer B, Rogers P. 2001. Biotransformation of benzaldehyde into (R)-phenylacetylcarbinol by filamentous fungi or their extracts. Appl Microbiol Biotechnol 57:309-315.

Rosche B, Breuer M, Hauer B, Rogers PL. 2005. Cells of Candida utilis for in vitro (R)-phenylacetylcarbinol production in an aqueous/octanol two-phase reactor. Biotechnol Lett 27:575-581.

Rosche B, Breuer M, Hauer B, Rogers PL. 2004. Biphasic aqueous/organic biotransformation of acetaldehyde and benzaldehyde by *Zymomonas mobilis* pyruvate decarboxylase. Biotechnol Bioeng 86:788-794.

Rosche B, Breuer M, Hauer B, Rogers PL. 2003. Screening of yeasts for cell-free production of (*R*)-phenylacetylcarbinol. Biotechnology Letters 25:841-845.

Sandford V, Breuer M, Hauer B, Rogers P, Rosche B. 2005. (R)-phenylacetylcarbinol production in aqueous/organic two-phase systems using partially purified pyruvate decarboxylase from *Candida utilis*. Biotechnol Bioeng 91:190-198.

Shin H, Rogers P. 1995. Biotransformation of benzeldehyde to l-phenylacetylcarbinol, an intermediate in l-ephedrine production, by immobilized Candida utilis. Appl Microbiol Biotechnol 44:7-14.

Shin HS, Rogers PL. 1996. Production of L-phenylacetylcarbinol (L-PAC) from benzaldehyde using partially purified pyruvate decarboxylase (PDC). Biotechnol Bioeng 49:52-62.

Shukla V, Kulkarni P. 2000. L-Phenylacetylcarbinol (L-PAC): biosynthesis and industrial applications. World Journal of Microbiology and Biotechnology 16:499-506.

Stark D, von Stockar U. 2003. In Situ Product Removal (ISPR) in Whole Cell Biotechnology During the Last Twenty Years. Advances in Biochemical Engineering Biotechnology 80:149-176.

Straathof AJJ, Panke S, Schmid A. 2002. The production of fine chemicals by biotransformations. Curr Opin Biotechnol 13:548-556.

World Anti-Doping Agency. 2010. WADA 2010 Prohibited List Now Published.

Yeom SH, Daugulis AJ, Lee SH. 2010. Bioremediation of Phenol-Contaminated Water and Soil Using Magnetic Polymer Beads. Process Biochemistry 45: 1582-1586.

Zhang W, Wang Z, Li W, Zhuang B, Qi H. 2008. Production of l-phenylacetylcarbinol by microbial transformation in polyethylene glycol-induced cloud point system. Appl Microbiol Biotechnol 78:233-239.

Chapter 3

Application of Solid-Liquid TPPBs to the Production of L-phenylacetylcarbinol from benzaldehyde using *Candida utilis*

Tanya R. Khan and Andrew J. Daugulis

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3.1 Preface

As noted in Chapter 2, the production of PAC from benzaldehyde using *C. utilis* is an industrially important process, but suffers from complications arising from substrate (benzaldehyde), product (PAC), and by-product (benzyl alcohol) inhibition. Two-phase partitioning bioreactors (TPPBs) provide a method to reduce inhibitory effects by sequestering target compounds in an immiscible phase.

Selection of an appropriate immiscible phase requires the consideration of several factors in addition to affinity for the target compounds, including biocompatibility and non-bioavailability to the microbe. Traditionally, organic solvents have been used as this auxiliary phase, but are often associated with toxicity to the biocatalyst (non-biocompatibility). In the recent literature, octanol was used in a TPPB for PAC production, but inhibited glucose metabolism in the yeast being used for the biotransformation. The use of solid polymer beads instead of organic solvents has been demonstrated in the literature to provide a fully biocompatible and non-bioavailable sequestering phase, opening the door to improve PAC production through the use of an appropriate second phase.

This chapter first analyzes the behaviour of *C. utilis* when exposed to a variety of organic solvents with respect to biocompatibility and bioavailability of the solvents, with the aim of providing guidelines for any future work with *C. utilis* and organic solvents. Screening of various commercially available polymers for affinity for benzaldehyde, PAC and benzyl alcohol was then performed to identify an appropriate biocompatible sequestering phase. The performance of the selected polymer was then evaluated in a solid-liquid TPPB.

3.2 Abstract

The biotransformation of benzaldehyde and glucose to L-phenylacetylcarbinol (PAC) using *C. utilis* was demonstrated in a solid-liquid two-phase partitioning bioreactor (TPPB) with the aim of reducing substrate, product and by-product toxicity via sequestration. Previous work in the field had used octanol as the sequestering phase of liquid-liquid TPPBs but was limited by the toxic effects of octanol on *C. utilis*. To improve solvent selection in any future studies, the critical log P of *C. utilis* was determined in the current study to be 4.8 and can be used to predict biocompatible solvents. Bioavailability tests showed alkanes and alkenes to be non-bioavailable. A wide range of commercially available polymers known to be biocompatible and non-bioavailable was screened and it was demonstrated that polymer softness plays a key role in absorptive capability. The polymer Hytrel G3548L was selected as the second phase to sequester benzaldehyde, PAC and benzyl alcohol, with partition coefficients of 35, 7.5, and 10 respectively. With a 9% by volume partitioning phase, 13.6 g/L biomass of *C. utilis* achieved an overall PAC concentration of 11 g/L, a 1.9 fold improvement over the single phase case. Benzyl alcohol concentration was 4.5 g/L, a 1.6 fold reduction. The volumetric productivity was 0.85 g/L.h, a 1.2 fold improvement over the single phase system. These results demonstrate a promising starting point for solid-liquid TPPBs for PAC production.

Key words: L-phenylacetylcarbinol; benzaldehyde; solid-liquid two-phase partitioning bioreactors; polymer beads; whole cell biotransformation

3.3 Introduction

L-phenylacetylcarbinol (PAC) is a precursor to the drugs L-ephedrine and pseudoephedrine, which are used as decongestants. Though PAC can be chemically synthesized, the purity of the final product is low because of both substrate impurities and by-product formation. Commercial production is done through microbial biotransformation using yeast with benzaldehyde and glucose as substrates (Shin and Rogers 1995). While this process produces a significant by–product, benzyl alcohol, the overall purity and stereoselectivity is far greater than that of the chemical method.

Research in PAC production has focused mainly on *Candida utilis* or *Saccharomyces cerevisiae* as the microorganism to perform the biotransformation, although many strains of yeast and filamentous fungi contain the necessary metabolic pathways to produce PAC (Netrval and Vojtisek 1982; Rosche, et al. 2001). Under anaerobic conditions, the enzyme pyruvate decarboxylase (PDC) converts benzaldehyde and pyruvate to PAC, while alcohol dehydrogenase (ADH) (or other oxidoreductases) produces benzyl alcohol from benzaldehyde as a by-product (Long and Ward 1989). Although the metabolic pathway is intrinsic to the microorganism, the system suffers from substrate (benzaldehyde, BZA), product (PAC), and by-product (benzyl alcohol, BOH) inhibition (Long and Ward 1989). In order to shield the yeast from the inhibitory compounds, immobilized cells (with *S. cerevisiae*) (Mahmoud, et al. 1990), cloud point systems (with *S. cerevisiae*) (Zhang, et al. 2008), and liquid-liquid two-phase partitioning bioreactors (TPPBs) (with *C. utilis*) (Rosche, et al. 2005) have been employed to date, with TPPBs demonstrating the highest final PAC system concentration.

TPPBs consist of a microorganism containing aqueous growth medium and an immiscible second phase, selected to have a high affinity for the inhibitory compounds. As a result, the target compounds will preferentially partition into the second phase, characterized by their respective partition coefficients. Organic solvents are often used as the immiscible phase,

but can be toxic to the microorganisms (non-biocompatible). A relationship exists between the logarithm of the standard octanol-water partition coefficient (log P) of a solvent and its biocompatibility with a specific organism. This parameter is known as the critical log P (log P_{crit}) of the organism. Solvents with a log P below the log P_{crit} are inhibitory, while solvents above the log P_{crit} are generally biocompatible (Bruce and Daugulis 1991; Laane, et al. 1987).

The organic solvent used in previous TPPBs for PAC production has been octanol. It was noted in work by Rosche et al., (2005), that exposing *C. utilis* to octanol immediately stopped glucose uptake (and subsequent glycolytic conversion of glucose to pyruvate) (Rosche, et al. 2005). Consequently pyruvate had to be directly fed to the system. This suggests that the log P of octanol (2.9) may have been lower than the log P_{crit} of *C. utilis*, which has not been reported in the literature to date. While system performance was high, the cost of pyruvate compared to glucose cannot be ignored for its economic impact in commercial situations, as well as other limitations associated with the use of pyruvate described Goetz (2001) (Goetz, et al. 2001). To avoid biocompatibility difficulties, polymers, either liquid or solid, may be used in place of organic solvents (Barton and Daugulis 1992; Amsden, et al. 2003). With the wide array of polymers commercially available, there is great potential to find a polymer with high affinity for the inhibitory target compounds without toxicity effects such as those sustained by exposure to octanol.

Although solid-liquid polymer systems do not display any foaming or viscosity difficulties, such as those associated with the use of silicone oil, there are some operational factors to consider for industrial scale work. Accumulation of polymer beads behind bioreactor internals has been observed in solid-liquid systems, and 20% polymer phase by volume has been experimentally suggested as an upper limit (Boudreau and Daugulis 2005). Recent studies have shown that polymers may also influence oxygen transfer properties of the system by increasing oxygen transfer rate (Littlejohns and Daugulis 2006). Scale-up of solid-liquid TPPBs to an

industrial level, therefore, must account for the effects of polymer bead mixing and oxygen transport.

The first objective of this work was to determine the biocompatibility and bioavailability of a variety of solvents with *C. utilis*, with the aim of providing guidelines for future liquid-liquid TPPB studies. The potential of various commercially available polymers to sequester benzaldehyde, PAC and benzyl alcohol was also explored, with the most effective polymer being used in a solid-liquid TPPB. Since polymers are generally biocompatible and non-bioavailable, glucose was used as a substrate making the system more industrially applicable than systems requiring the use of expensive pyruvate.

3.4 Materials and Methods

3.4.1 Chemicals and Polymers

Benzaldehyde (>99%), benzyl alcohol (>99%), glucose, oleyl alcohol, octane, 1-dodecanol, and oleic acid were purchased from Sigma Aldrich Canada (Oakville, ON, Canada). $(NH_4)_2SO_4$, KH_2PO_4, $MgSO_4$, $CaCl_2$, $FeSO_4$, $ZnSO_4$, $MnSO_4$, $CuSO_4$, hexadecane, decane, dodecane, hexane, and linoleic acid were purchased from Fisher Scientific Canada (Oakville, ON, Canada). All salts were of Certified ACS grade and solvents are of HPLC grade. Butanol, tetradecene, nonane, 1-octadecene, hexadecane, 1-dodecene, and octanol were purchased from Acros Organics. Aldehyde C-14 was purchased from Givaudan (Mississauga, ON, Canada). Because PAC is not available commercially, a small amount (approximately 0.5 g, enough for calibration), was obtained by chemical synthesis adapted from Rani, et al. (1995) (Synthesized by the Jessop Group at Queen's University). Polymers tested in this study and their properties are listed in Table 3-1.

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Table 3-1: Properties of polymers tested for potential use in the PAC TPPB

	Grade	Supplier	Hardness	T_g (°C)‡	T_m (°C)¥	Specific Gravity	Description
Amides							
PEBAX	2533	Arkema	25D	-65	134	1	Polyether block amide
Zytel	42A	DuPont	60 (Rockwell M)	70	262	1.15	Polyamide 66
Nylon	6,6	DuPont	N/A	N/A	N/A	N/A	
Vinyl Acetates							
EVA	40W	DuPont	40 (Shore A)	N/A	N/A	0.965	40% vinyl alcohol (co-polymer with ethylene)
	3175		N/A	N/A	69	0.95	28% vinyl alcohol
Other							
Hytrel	G3548L	DuPont	35D	-45	156	1.16	Co-polymer of poly(butylene terephthalate) and polyether
	G4078W		40D	N/A	170	1.18	
	5544		55	-35	215	1.22	
	6108		61	N/A	168	1.25	
	8238		82	-50	223	1.28	
	8206		35-40 †	-59	180	1.19	
Nucrel	925	DuPont	N/A	228	N/A	0.94	Ethylene/ methacrylic acid co-polymer
Kraton SBR	D4150K	Kraton	N/A	Styrene: 90 Butadiene: -90	N/A	0.92	Styrene/ butadiene linear triblock copolymer, 28% styrene

†Approximated in personal correspondence with DuPont
‡ Glass transition temperature
¥ Melting point

3.4.2 Medium formulation and culture preparation

The yeast strain *Candida utilis* 70940 was purchased from the University of New South Wales Culture Collection (World Directory of Culture Collections No. 248). The medium formulation was adapted from the minimal medium described by Rosche, et al. (2005), with $MnSO_4$ being substituted for $MnCl_2$ in the trace element solution.

40 µL of frozen stock culture were added to 125 mL shake flasks containing 50 mL sterile medium to prepare inoculum. Flasks were grown at 30°C and 180 rpm for 36 hours (to reach an OD of 2.6), after which 6 flasks were added to the sterile bioreactor.

3.4.3 Cell measurement

A cell dry weight versus optical density (OD) calibration curve was prepared for *C. utilis* at 600 nm wavelength (Biocrom Ultraspec). All cell measurements were determined using OD.

3.4.4 Analytics

HPLC-UV detection (Varian, Prostar, Model # PS325, Polaris 5u C18-A 150 x 4.6 mm column) was used to quantify benzaldehyde, benzyl alcohol and PAC using the method described by Rosche, et al., (2001). HPLC- refractive index detection (Varian, Prostar, Model # PS356, HiPlex H 8 µm 300 x 7.7 mm column at 75°C) was used to quantify glucose and ethanol. The mobile phase of 9 mm H_2SO_4 was maintained at a flow rate of 0.4 mL/min.

3.4.5 Solvent biocompatibility and bioavailability

The method to determine log P_{crit} was adapted from Prpich and Daugulis (2007b). 50 mL of 24 hour old stock culture were used in each system to ensure equivalent starting ODs when 5 mL of organic solvent were added. The log P values of all solvents in this study were from the Syracuse Research Cooperation (SRC) Log P Database (http://www.srcinc.com). Percent metabolic activity was determined by comparison of the final OD of each system to a positive control (a shake flask without solvent) after 24 hours.

The bioavailability of a solvent was assessed by using a glucose-free medium (50 mL in 125 mL shake flasks) with 5 mL of solvent, and inoculating with 40 µL of frozen stock. Growth after 48 hours was monitored using OD and increases in OD were attributed to the solvent being used as a carbon source (bioavailable).

3.4.6 Polymer partition coefficients

Partition coefficients were determined using the method described by Isaza and Daugulis (2009). 10 mL of stock solution were incubated with 1-5 g of polymer for each test. Stock solution was generated using the contents of a single phase reactor (centrifuged and filtered to remove cells) in order to obtain a high purity sample of PAC, with benzaldehyde and benzyl alcohol spiked to concentrations of 3 g/L and 10 g/L respectively.

3.4.7 Batch reactor operation

A 5L bioreactor (3L medium) equipped with pH (6M KOH) and temperature (30°C) controls was used for all reactor runs (New Brunswick Scientific, BioFlo III). Bioreactor operation began with a 16 hour growth period for biomass accumulation (300 rpm, 1 vvm air aeration), followed by an 18 hour enzyme induction period, (300 rpm, 0.1 vvm air aeration), adopted from Chen, et al. (2005). After the 18 hour enzyme induction period, benzaldehyde was added manually to maintain the concentration between 1 and 2 g/L. This range of benzaldehyde has been demonstrated to have the highest PAC productivity with some sustained inhibitory effects (Long and Ward 1989; Rogers, et al. 1996; Shin and Rogers 1995).

3.4.8 Two-phase batch reactor operation

Because aqueous substrate concentration was to be maintained between 1 and 2 g/L, the polymer beads were preloaded with benzaldehyde, as follows: 300 g of Hytrel G3548L were added to 3L of sterile medium and benzaldehyde was added until the aqueous concentration equilibrated to 1 g/L. The aim was to buffer the lower limit of the feeding window so that manual additions of benzaldehyde would stay in the aqueous phase rather than being absorbed by the polymer. The beads were removed, dried, and refrigerated. The previously described batch reactor procedure was then followed, including manual benzaldehyde addition, with the beads being added to the reactor at the start of the biotransformation.

3.4.9 Product recovery from polymer

Every two hours over the course of the biotransformation period, two 1 g polymer samples were collected and tested with the extraction technique described by Gao and Daugulis (2009) using methanol. Two methanol washes were used per sample. Additional washes were demonstrated to only increase recovery by approximately only 1%. Using this method, the polymer beads are able to act as the first step in downstream purification operations, which in other systems could require high-cost chromatographic recovery methods.

3.5 Results and Discussion

3.5.1 Characterization of C. utilis

The log P_{crit} for *C. utilis* is not available in the literature and was determined in this study to facilitate any future liquid-liquid TPPB work with *C. utilis*. Tests were conducted on organic solvents chosen to have a broad range of log P values. Figure 3-1 shows the metabolic activity of *C. utilis* when exposed to these solvents.

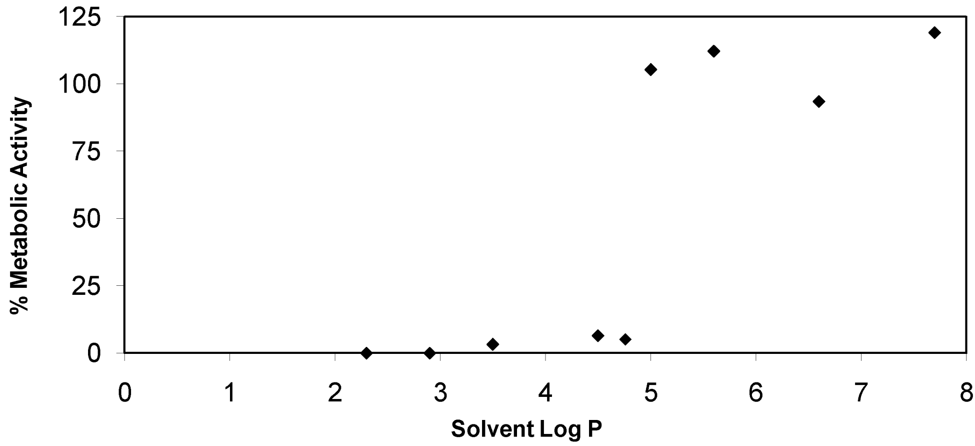

Figure 3-1: Log P of *C. Utilis* using solvents of known Log Ps: aldehyde C-14 (Log P 2.3), octanol (Log P 2.9), hexane (Log P 3.5), octane (Log P 4.5), nonane (Log P 4.8), 1-dodecanol (Log P 5.0), decane (Log P 5.6), dodecane (Log P 6.6), oleic acid (Log P 7.7). A control shake flask without solvent was used to determine 100% metabolic activity.

From Figure 3-1, it is evident that *C. utilis* is not inhibited when exposed to 1-dodecanol (log P of 5), while nonane (log P of 4.8) prevented growth. Therefore, the log P_{crit} of *C. utilis* can

be approximated as 4.8. Though the critical log P is organism dependent, there exists comparable behavior between similar species (Inoue and Horikoshi 1991). Previous work with yeasts showed *S. cerevisiae* having a log P_{crit} between 5-6 (Bruce and Daugulis 1991). Therefore, a log P_{crit} of 4.8 seems reasonable.

Once a solvent is concluded to be biocompatible (log P >4.8), its bioavailability to *C. utilis* must also be assessed as part of rational solvent selection. Table 3-2 presents some common classes of solvents and their bioavailability to *C. utilis*. All solvents were chosen to have a log P greater than 4.8 to ensure biocompatibility.

Table 3-2: Bioavailability of some common solvents to *C. utilis*

Solvent	Log P*	Bioavailability (+/-)
Alkanes		
Decane	5.6	-
Dodecane	6.6	-
Hexadecane	8.2	-
Alcohols		
Dodecanol	5	+
Oleyl Alcohol	7.6	+
Alkenes		
Dodecene	6.1	-
Tetradecene	7.1	-
1-octadecene	9.0	-
Carboxylic Acids		
Oleic Acid	7.7	+
Linoleic Acid	7.5	+
Other		
Bis-2-ethylhexyl Sebacate	8.2	+

* Data from Log P Database (http://www.srcinc.com).

Examination of the bioavailable solvents allows some general conclusions to be drawn. The bioavailability of a particular class of solvent to a microorganism has been demonstrated previously in work with *Mycobacterium* PYR-1 (MacLeod and Daugulis 2003). Table 3-2 suggests that as a class, alkanes and alkenes are not likely to be bioavailable, compared to alcohols and fatty acids, and therefore could be considered for use in liquid-liquid TPPBs with *C. utilis*.

PAC partition coefficients have been tested in solvent screening by Rosche et al. (2004) and Sandford et al. (2005) for use with purified enzymes. Therefore, biocompatibility with whole cell *C. utilis* was not used as a selection criterion. Although now with the log P_{crit} determined, the biocompatibility of these solvents can be predicted. The log P_{crit} suggests that only two biocompatible solvents have been tested previously, hexadecane (log P of 8.2) (Rosche, et al. 2004) and dodecane (log P of 6.1) (Sandford, et al. 2005). These provided PAC partition coefficients of less than one, while octanol (log P of 2.9) provided the best partition coefficient of 4 (Sandford, et al. 2005). Thus, biocompatible solvents had low partition coefficients, while octanol, which was subsequently used with whole cells of *C. utilis* in a TPPB due to its moderate partition coefficient, was ultimately found to be toxic to *C. utilis* (Rosche, et al. 2005). To avoid the compromise between toxicity and extractive capability of the sequestering phase, it is now possible to re-evaluate the PAC system using polymers instead of octanol.

3.5.2 Polymer selection for PAC production

With the diverse selection of polymers available, ideally three unique polymers would be found, each having affinity for only the substrate, product, or by-product independently to assist downstream purification steps. However, due to the structural similarity between the three target compounds (Table 3-3), this goal was unattainable.

Table 3-3: Chemical properties of species of interest

	Molecular Weight	Structure	K_{ow}	Solubility in Water (25°C) (g/L)
Benzaldehyde C_6H_5COH	106.12		21[¥]	6.55
PAC $C_9H_{10}O_2$	150.17		4[¥]	Not Available
Benzyl Alcohol C_7H_8O	108.14		12.6[*]	42.9

¥(Sandford, et al. 2005)
* (The Good Scents Company 2010)

All target compounds contain an aromatic ring with varying side groups. Benzaldehyde is the most hydrophobic species due its non-polar functional groups, reflected in its higher octanol: water partition coefficient (K_{ow}) relative to the other compounds. Both PAC and benzyl alcohol have alcohol functional groups making them significantly more hydrophilic, which in combination with their structural similarity indicates that both compounds may interact similarly with polymers.

For polymer selection, two criteria were explored. One strategy was to try polymers with a high degree of softness in hopes that the higher permeability would improve sorption. Table 3-1 presents polymer hardness data available from the polymer suppliers. Hardness is a measure of a given polymer's ability to withstand deformation, and several measurement methods exist, such as Rockwell and Shore hardness, to characterize this property. A lower hardness value indicates a softer polymer. The second strategy was to test polymers with functional groups that have hydrogen bonding potential (amides, alcohols) in an attempt to interact with the hydroxyl group of PAC and benzyl alcohol. This strategy was recommended by Gao and Daugulis (2010) for 2-phenylethanol, a compound containing both aromatic and hydroxyl functionality. The resulting partition coefficients are shown in Table 3-4.

Table 3-4: Partition coefficients determined using reactor product where ranges are determined by the 95% confidence interval from linear regression.

	Benzaldehyde	PAC	Benzyl Alcohol
Octanol: Aqueous (Table 3-3)	21	4	13
Amides			
PEBAX 2533	30 (±8)	9.5 (±2)	13 (±0.6)
Zytel 42A	2 (±0.2)	0.5 (±0.2)	1.7 (±1)
Nylon 6,6	2 (±0.7)	0.7 (±0.9)	2.2 (±0.3)
Vinyl Acetates			
EVA 40W	46 (±0.8)	5.6 (±1)	6.5 (±2.5)
EVA 3175	1.4 (±0.2)	0.6 (±0.3)	3 (±4)
Other			
Hytrel G3548L	35 (±5)	7.5 (±1.5)	10 (±2.7)
Hytrel G4078W	35 (±5)	6.8 (±0.5)	8.3 (±0.6)
Hytrel 8206	11.4 (±0.6)	5.3 (±2)	7.6 (±0.5)
Desmopan	32 (±9)	4.8 (±1.8)	5.8 (±0.3)
Kraton SBR	18 (±3.5)	1.4 (±0.9)	4.75 (±1)

From Table 3-4 it can be seen that the extraction performance of octanol could be met and surpassed by PEBAX 2533 and Hytrel G3548L, with close performance by EVA 40W, Hytrel G4078W, and Desmopan for all compounds. This underscores the fact that by being biocompatible and non-bioavailable, there is a wide range of commercial polymers available for use in solid-liquid TPPBs. The best performance for the three targets was with PEBAX 2533, and also with Hytrel G3548L, which displayed overlapping 95% confidence intervals for all compounds. As anticipated, none of the polymers tested showed discrimination between PAC and benzyl alcohol. Therefore, one polymer was selected to provide the best uptake for all three target species. It was found that PEBAX 2533 would melt when autoclaved making it difficult to work with, therefore, Hytrel G3548L was selected as the partitioning phase.

To determine whether polymer functionality or softness had a more significant effect on partition coefficients, data from Table 3-4 were analyzed with polymer property data from Table 3-1. Polymer softness may be reflective of the crystallinity (and associated T_g) and free space in a polymer, and was used to represent these aspects of polymers as it is the most complete data set available from Table 3-1. Comparing the amide polymers tested, it is clear that only PEBAX 2533 demonstrated a high affinity for the target molecules, while the other amide polymers Zytel and Nylon had minimal absorption. A significant difference between these polymers is their hardness, with PEBAX 2533 being the softest. This supports the previous trend seen in the Hytrel family, which demonstrated increased absorption as a function of softness (Gao and Daugulis 2010).

Absorption was consistently higher for benzaldehyde over PAC and benzyl alcohol. This preference to absorb the more hydrophobic target molecule indicates that hydrophobic interactions, possibly between the benzene ring and the polymer backbone, are the main factors involved in the absorption. This also suggests that hydrogen bonding between PAC or benzyl alcohol and the polar functional groups of polymers was not able to improve performance. These findings suggest that it is polymer softness, over functionality, that may provide a key role in

absorption. This may be due to softer polymers having more available sites for hydrophobic interaction and/or allowing better permeability of bulky functional groups such as a benzene ring.

3.5.3 Single Phase Benchmark Fermentation

The objective of the single phase benchmark fermentation was to consolidate previous knowledge in the field so that improvements to the system using TPPBs could be isolated from any biological variability. For this work, after an aerobic growth phase, the enzyme induction period (18 hours) was maintained as closely as possible to the base case provided by Chen et al. (2005) (Chen, et al. 2005), which demonstrated that PDC activity reached a maximum in 15 hours, with little change after that point. The profile of glucose, ethanol and biomass in the current work is shown in Figure 3-2.

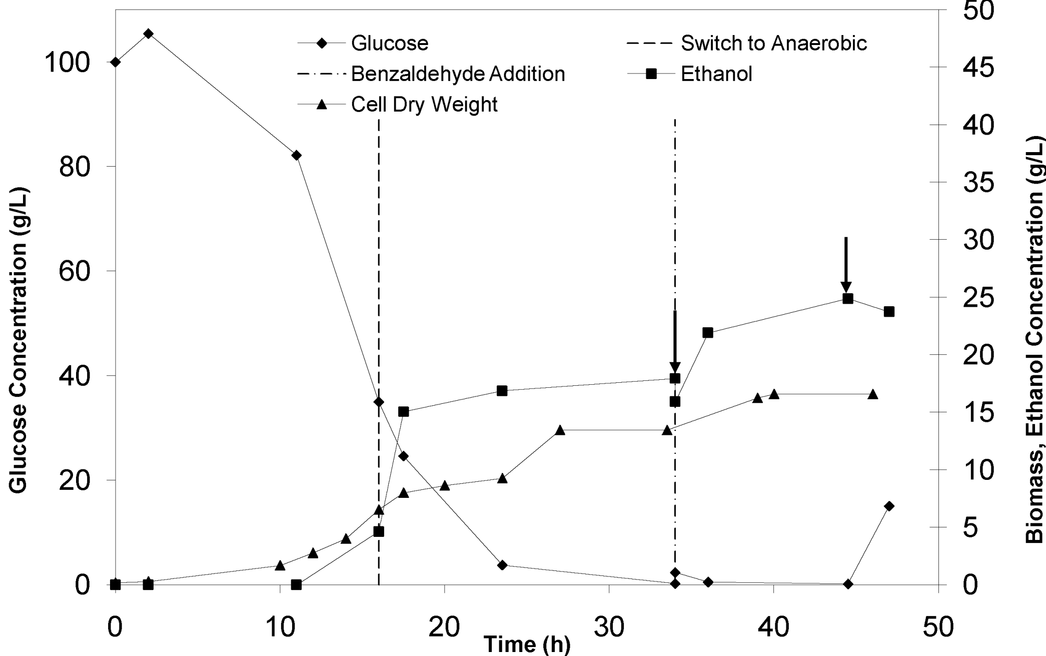

Figure 3-2: Cell density, glucose consumption and ethanol production for the single phase benchmark fermentation. The arrows represent the addition of 60 g of dissolved glucose. The aerobic growth period was 0-16 hours, the anaerobic enzyme induction period was 16-34 hours, and benzaldehyde addition began at 34 hours.

At the start of the biotransformation (t=34 hours), ethanol and biomass concentrations were 20 g/L and 13 g/L respectively, with negligible glucose remaining in the system. To ensure

that this did not result in a pyruvate limitation, a 60 g bolus of glucose (in the form of a 500 g/L solution) was added to the reactor when the biotransformation was initiated. The time course of the biotransformation period (34 hours onward) is shown in Figure 3-3.

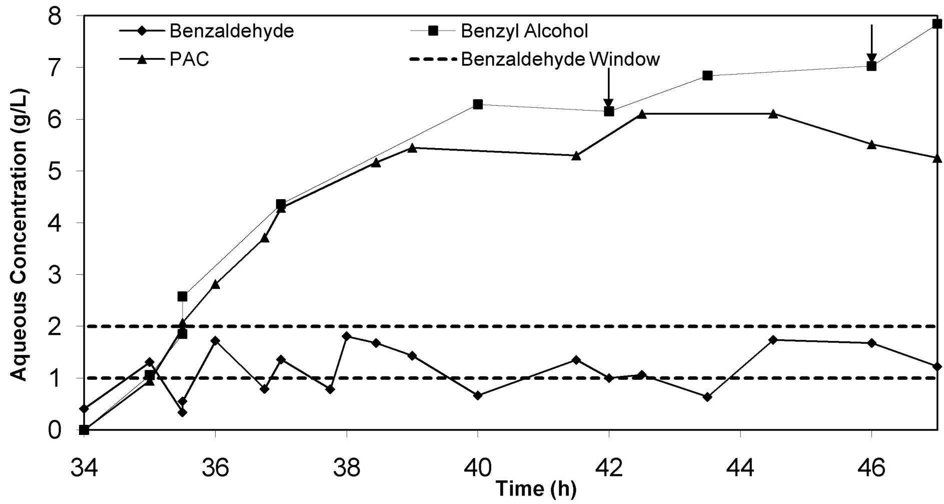

Figure 3-3: PAC, benzyl alcohol formation, and benzaldehyde concentration for the single phase benchmark fermentation. The arrow represents the addition of 60 g of dissolved glucose.

The most dramatic increase in both PAC and benzyl alcohol concentrations was in the first 5 hours of the transformation. From Figure 3-3, the final system concentrations of 5.7 g/L PAC and 7.4 g/L benzyl alcohol were determined. Final PAC concentrations with whole cell *C. utilis* in the literature have varied with reactor designs, but the base level shake flask design by Netrval and Vojtisek (1982) demonstrated 3.6 g/L PAC. Shin and Rogers (1995) observed no further PAC or benzyl alcohol production when benzyl alcohol reached 6-7 g/L. These values indicate that the current system achieved comparable single phase results.

The end of the biotransformation was taken to be the point at which PAC production stopped, which is at approximately 8 hours into the transformation. Shin and Rogers (1995) provided three reasons for the transformation stopping: 1) reduction in PDC activity due to inhibitory effects, 2) pyruvate limitation, 3) cell viability loss due to exposure to benzaldehyde, benzyl alcohol, or PAC. Pyruvate limitation was tested by the addition of glucose. PAC

concentration was not able to increase. 5 mL of the reactor contents were used to inoculate 50 mL of fresh medium and growth was observed. This suggests that the system was likely not pyruvate limited, and that cells were still viable. Therefore, the most likely cause of stoppage was enzyme inhibition due to sustained exposure to inhibitory compounds. However, it should be noted that the growth of microbes in the fresh medium only suggests that some cells remained viable and that an approximation using viabilitiy staining methods would more conclusively demonstrate that cells remain viable.

3.5.4 Two-Phase Bioreactor with 300 g Hytrel G3548L

To isolate the improvements of a TPPB over a single phase reactor, the same reactor conditions were employed. The growth period and enzyme induction period profiles were the same as the single phase and therefore are not shown. Figure 3-4 shows the aqueous phase concentrations in the TPPB after the start of the biotransformation. The final PAC concentration in the single phase is shown for comparison.

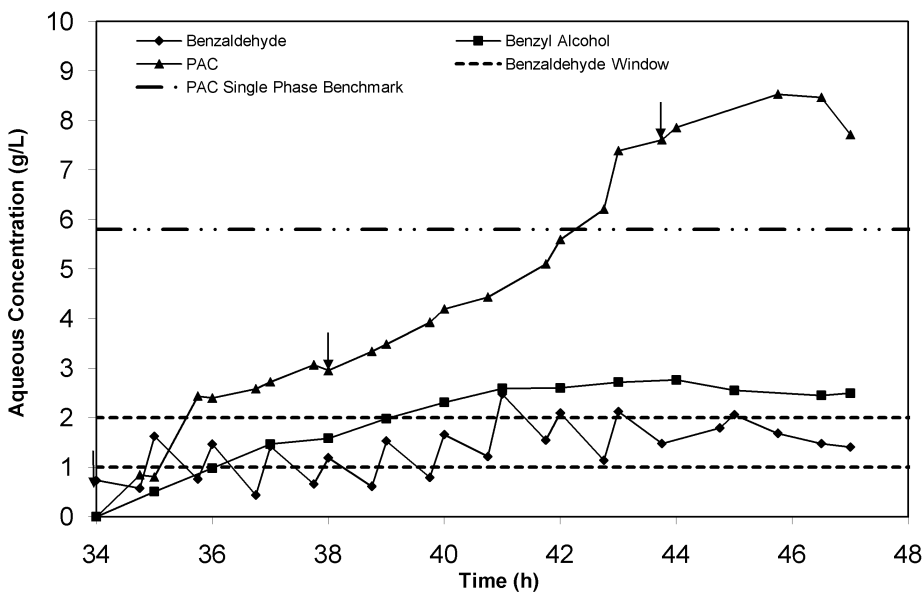

Figure 3-4: Aqueous PAC, benzyl alcohol and benzaldehyde concentrations when 300 g of Hytrel G3548L were used as a second phase. Arrows represent the addition of 60 g of dissolved glucose.

Figure 3-4 shows that the two phase system achieved final aqueous concentrations of 7.6 g/L and 2.5 g/L of PAC and benzyl alcohol respectively. Polymer beads were sampled every two hours and desorbed to provide a polymer concentration profile, shown in Figure 3-5.

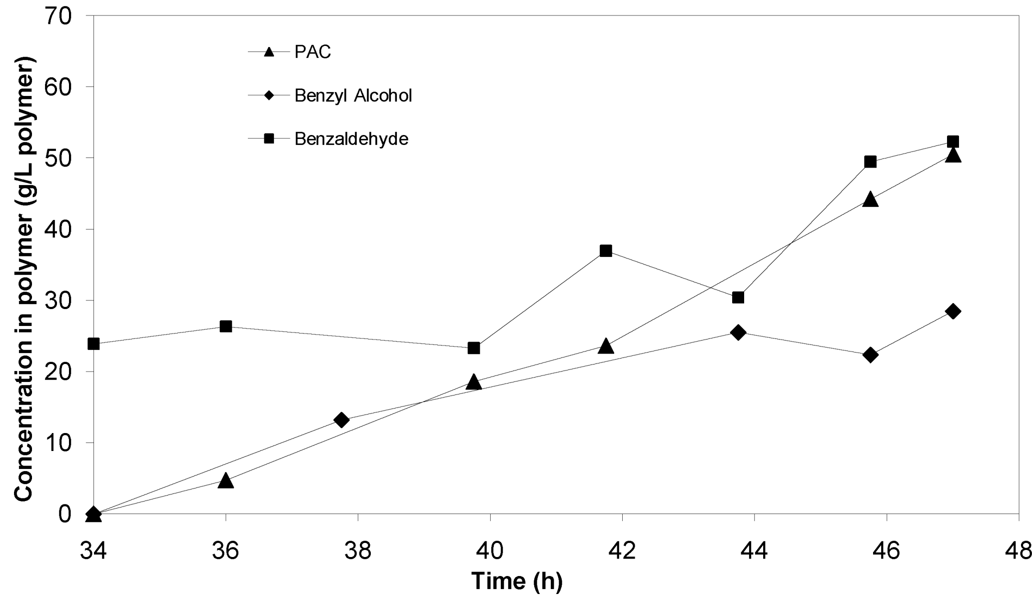

Figure 3-5: Polymer concentrations of PAC, benzyl alcohol, and benzaldehyde during the two-phase biotransformation.

From Figure 3-5 it is evident that product concentrations are an order of magnitude larger than those in the aqueous phase. As is expected, the PAC and benzyl alcohol concentrations increase over the course of the fermentation, however, benzaldehyde concentration also increases despite the fact that it is converted in the reaction. This is because upon manual addition of benzaldehyde to the aqueous phase some will partition into the beads to re-establish equilibrium, causing an increase in benzaldehyde concentration in the polymer phase. A comparison of the single and two phase systems is shown in Table 3-5.

Table 3-5: Final system characteristics comparison single and two phase fermentations for PAC production

	Single Phase	300g
Time to completion (h)	8	13
Cell Density (g/L)	13.4	13.6
Aqueous PAC end point (g/L)	5.7	7.6
Aqueous BOH end point	7.4	2.5
Polymer PAC (g/L)	--	50.5‡
Polymer BOH (g/L)	--	28.5‡
Overall PAC (g/L) ¥	5.7	11.0
Overall BOH (g/L) ¥	7.4	4.5
$Y_{PAC/BZA}$ (mol/mol consumed)	0.34	0.41
$Y_{BOH/BZA}$ (mol/mol consumed)	0.61	0.24
Selectivity (g/L PAC/ g/L BOH)	0.77	2.44
PAC Volumetric productivity (g/Lahr)	0.71	0.85
PAC mass productivity (g PAC/g cells/h)	0.160	0.203

‡Obtained from average 2 random polymer samples of 1 g desorbed using methanol washing procedure

¥ Determined using the total mass divided by the total system volume (3L for the single phase, 3.26 L for the TPPB)

The overall concentrations of PAC and benzyl alcohol reported in Table 3-5 demonstrate that the sequestering phase was able to increase the overall PAC concentration by 1.9 fold, and decrease the overall benzyl alcohol concentration by 1.6 fold. The combination of these two effects results in a 3.2 fold increase in selectivity for PAC over benzyl alcohol. The two-phase system demonstrated partition coefficients of 39, 7, and 11 for benzaldehyde, PAC and benzyl alcohol respectively, consistent with their 95% confidence intervals from Table 3-4. PAC yield on benzaldehyde increased, which is consistent with the improvement observed in a liquid-liquid TPPB previously noted by Rosche et al. (2005). Losses of benzaldehyde in PAC production systems in the literature have been attributed to volatilization of benzaldehyde, as well as production of several minor by-products (Goetz, et al. 2001; Sandford, et al. 2005). Further metabolism of PAC to PAC-diol has also been noted (Shukla and Kulkarni, 2001).

The reduction of by-product (concentration and yield) in the TPPB may be due to polymer sequestration. The cells, and consequently the active enzymes, are exposed to lower concentrations of inhibitory species. This may allow PDC to stay active for longer periods, or

maintain a higher specific activity for benzaldehyde than the oxidoreductases used for benzyl alcohol production. Previous work in solid-liquid TPPBs suggests that the aqueous concentration should reach the same known inhibitory value as the single phase case (Gao and Daugulis, 2009; Morrish and Daugulis, 2008; Prpich and Daugulis, 2007a). However, this system has an inhibitory by-product present, which introduces effects not previously explored. The inhibition of PDC could be a cumulative effect of PAC, benzyl alcohol and benzaldehyde, and therefore a reduction in benzyl alcohol concentration could allow an increase in PAC concentration.

To compare the present work with the PAC literature, similar metrics of performance should be considered. Rosche et al. (2005) performed the biotransformation with whole cells of *C. utilis* for PAC production in an octanol-aqueous TPPB, and were able to increase product concentration 3.9 fold over the single phase case. Volumetric productivity was improved 3.1 fold and catalyst efficiency (g PAC/ g cell dry weight) improved 6.9 fold (Rosche, et al. 2005). In the current work, volumetric productivity increased 1.2 fold comparing the two-phase and single phase cases. The catalyst efficiency improved 2 fold (2.6 g PAC/g cells TPPB / 1.3 g PAC/g cells single phase). These improvements are not as large as those reported by Rosche et al. (2005) as a significant amount of benzaldehyde was used to produce benzyl alcohol, which was not present in their system. This may also be due to the fact that the volume phase ratio of the current work is 0.087: 1, while Rosche et al. (2005) used a phase ratio of 1:1. A larger sequestering volume could significantly improve overall PAC concentrations, and is an area of future work being explored for the PAC system. The concentration in the polymer compared to single phase performance was 8.9 fold higher (g/L polymer / g/L single phase), compared to a 6.9 fold improvement shown by Rosche et al. (2005). This is likely due to the significantly higher partition coefficient for PAC in Hytrel G3548L compared to octanol (7.5 compared to 4).

Though the current work did not outperform the liquid-liquid TPPB described by Rosche et al. (2005), with respect to overall concentrations and productivities, it is important to note that this was not the overall goal. The goal was to re-evaluate the potential for TPPBs to be used in

the PAC system now that a biocompatible and high affinity sequestering phase is available. Zhang et al. (2008) aimed to improve on the Rosche et al. (2005) system by using a polyethylene glycol-induced cloud point system instead of octanol. Though this system was able to use glucose as a substrate, the PAC concentration reached only 8 g/L and a benzyl alcohol concentration of 4 g/L (Zhang, et al. 2008). A recent study using nonionic surfactant extraction was able to achieve 4.1 g/L PAC and 1.8 g/L of benzyl alcohol, also without sequestering phase toxicity problems (Xue, et al. 2010). Therefore, the current work has managed to balance the high performance elements of a liquid-liquid TPPB with the biocompatibility aspects of the cloud point and non-ionic surfactant systems, generating what may be considered a more promising industrial process.

3.6 Conclusions and Future Work

This work has expanded on the knowledge of *C. utilis* and its use in liquid-liquid TPPBs by demonstrating a critical log P of 4.8 and a group of bioavailable solvents. Strategies for polymer selection have been expanded to include the importance of polymer softness and hydrophobic interactions on absorptive capabilities. Using Hytrel G3548L as the sequestering phase in a solid-liquid TPPB, a 1.9 fold improvement on PAC concentration over single phase concentration was demonstrated, as well as a 1.6 fold decrease in by-product (benzyl alcohol) concentration.

Areas of future work already underway with the PAC system include testing the phase volume ratio and simplifying the manually fed system by delivering benzaldehyde from polymers with sustained release, a potentially novel demonstration in the field. Future work in our group to expand the use of solid-liquid TPPBs is the design and fabrication of specifically tailored polymers for greater discrimination between target compounds.

3.7 Acknowledgements

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3.8 References

Amsden BG, Bochanysz J, Daugulis AJ. 2003. Degradation of xenobiotics in a partitioning bioreactor in which the partitioning phase is a polymer. Biotechnol Bioeng 84:399-405.

Boudreau NG, Daugulis AJ. 2006. Transient Performance of Two-Phase Partitioning Bioreactors Treating a Toluene Contaminated Gas Stream. Biotechnol Bioeng 94: 448-457.

Bruce LJ, Daugulis AJ. 1991. Solvent Selection Strategies for Extractive Biocatalysis. Biotechnol Prog 116-124.

Chen AKL, Breuer M, Hauer B, Rogers PL, Rosche B. 2005. pH shift enhancement of Candida utilis pyruvate decarboxylase production. Biotechnol Bioeng 92:183-188.

Gao F, Daugulis AJ. 2010. Polymer solute interactions in solid liquid two-phase partitioning bioreactors. Journal of Chemical Technology & Biotechnology 85:302-306.

Gao F, Daugulis AJ. 2009. Bioproduction of the aroma compound 2-phenylethanol in a solid-liquid Two-Phase Partitioning Bioreactor system by Kluyveromyces marxianus. Biotechnol Bioeng 104: 332-339.

Goetz G, Iwan P, Hauer B, Breuer M, Pohl M. 2001. Continuous production of (R)-Phenylacetylcarbinol in an enzyme-membrane reactor using a potent mutant of pyruvate decarboxylase from *Zymomonas mobilis*. Biotechnol Bioeng 74:317-325.

Inoue A, Horikoshi K. 1991. Estimation of solvent-tolerance of bacteria by the solvent parameter log P. J Ferment Bioeng 71:194-196.

Isaza PA, Daugulis AJ. 2009. Ultrasonically enhanced delivery and degradation of PAHs in a polymer-liquid partitioning system by a microbial consortium. Biotechnol Bioeng 104: 91-101.

Laane C, Boeren S, Vos K, Veeger C. 1987. Rules for optimization of biocatalysis in organic solvents. Biotechnol Bioeng 30:81-87.

Littlejohns JV, Daugulis AJ. 2007. Oxygen transfer in a gas–liquid system containing solids of varying oxygen affinity. Chemical Engineering Journal 129: 67-74.

Long A, Ward O. 1989. Biotransformation of benzaldehyde by Saccharomyces cerevisiae: Characterization of the fermentation and toxicity effects of substrates and products. Biotechnol Bioeng 34:933-941.

MacLeod C, Daugulis A. 2003. Biodegradation of polycyclic aromatic hydrocarbons in a two-phase partitioning bioreactor in the presence of a bioavailable solvent. Appl Microbiol Biotechnol 62:291-296.

Mahmoud WM, El-Sayed A, Halim M.M., Coughlin RW. 1990. Production of L-phenylacetyl carbinol by immobilized yeast cells: II. Semicontinuous fermentation. Biotechnol Bioeng 36:55-63.

Morrish JL, Daugulis AJ. 2008. Improved reactor performance and operability in the biotransformation of carveol to carvone using a solid-liquid two-phase partitioning bioreactor. Biotechnol Bioeng 101: 946-956.

Netrval J, Vojtisek V. 1982. Production of Phenylacetylcarbinol in Various Yeast Species. European Journal of Applied Microbiology and Biotechnology 16:35-38.

Prpich GP, Daugulis AJ. 2007a. A novel solid-liquid two-phase partitioning bioreactor for the enhanced bioproduction of 3-methylcatechol. Biotechnol Bioeng 98:1008.

Prpich GP, Daugulis AJ. 2007b. Solvent selection for enhanced bioproduction of 3-methylcatechol in a two-phase partitioning bioreactor. Biotechnol Bioeng 97:536-543.

Rani BR, Ubukata M, Osada H. 1995. Reduction of Arylcarbonyl Using Zinc Dust in Acetic Acid. Bull Chem Soc Jpn 68:282-284.

Rogers P, Shin H, Wang B. 1996. Biotransformation for L-Ephedrine Production. Advances in Biochemical Engineering Biotechnology 56:33-60.

Rosche B, Sandford V, Breuer M, Hauer B, Rogers P. 2001. Biotransformation of benzaldehyde into (R)-phenylacetylcarbinol by filamentous fungi or their extracts. Appl Microbiol Biotechnol 57:309-315.

Rosche B, Breuer M, Hauer B, Rogers PL. 2005. Cells of Candida utilis for in vitro (R)-phenylacetylcarbinol production in an aqueous/octanol two-phase reactor. Biotechnol Lett 27:575-581.

Rosche B, Breuer M, Hauer B, Rogers PL. 2004. Biphasic aqueous/organic biotransformation of acetaldehyde and benzaldehyde by *Zymomonas mobilis* pyruvate decarboxylase. Biotechnol Bioeng 86:788-794.

Sandford V, Breuer M, Hauer B, Rogers P, Rosche B. 2005. (R)-phenylacetylcarbinol production in aqueous/organic two-phase systems using partially purified pyruvate decarboxylase from *Candida utilis*. Biotechnol Bioeng 91:190-198.

Shin H, Rogers P. 1995. Biotransformation of benzaldehyde to l-phenylacetylcarbinol, an intermediate in l-ephedrine production, by immobilized Candida utilis. Appl Microbiol Biotechnol 44:7-14.

Shukla V, Kulkarni P. 2001. Process parameters and reusability of the free cell mass of Torulaspora delbrueckii for the production of L-phenylacetylcarbinol (L-PAC). World Journal of Microbiology and Biotechnology 17:301-306.

The Good Scents Company. 2010. Benzyl Alcohol. 2010: thegoodscentscompany.com

Xue Y, Qian C, Wang Z, Xu J, Yang E, Qi H. 2010. Investigation of extractive microbial transformation in nonionic surfactant micelle aqueous solution using response surface methodology. Appl Microbiol Biotechnol 85:517-524.

Zhang W, Wang Z, Li W, Zhuang B, Qi H. 2008. Production of l-phenylacetylcarbinol by microbial transformation in polyethylene glycol-induced cloud point system. Appl Microbiol Biotechnol 78:233-239.

Chapter 4

The effects of polymer phase ratio and feeding strategy on solid-liquid TPPBs for the production of L-phenylacetylcarbinol from benzaldehyde using *Candida Utilis*

Tanya R. Khan and Andrew J. Daugulis

With minor editorial changes to fulfill formatting requirements, this chapter is substantially as it appears in: *Biotechnology Letters*. Accepted 20 Aug 2010. Article BILE897.

4.1 Preface

Chapter 3 demonstrated the use of a biocompatible and non-bioavailable commercial polymer to improve the PAC production in a TPPB over traditionally used organic solvents. By using a biocompatible polymer, glucose was able to be used as a substrate rather than more expensive pyruvate, maintaining industrial relevancy. The results obtained hinted towards greater potential to improve the system by increasing the sequestering phase ratio. Additionally, simplifying the benzaldehyde feeding strategy from manual addition to release from pre-loaded beads was also proposed in Chapter 3 to increase the practicality of the process.

The current chapter examines the effect of varying the phase ratio of the polymer phase (from 3% to 15% by volume) on system productivity. Strategies to pre-load and deliver benzaldehyde from the polymer beads were also investigated to provide simultaneous substrate delivery and product extraction with minimal operator intervention.

4.2 Abstract

The bioproduction of L-phenylacetylcarbinol (PAC), a precursor molecule in the synthesis of the decongestants ephedrine and pseudoephedrine, suffers from substrate, product, and by-product inhibition, and requires that the delivery of the substrate, benzaldehyde, be maintained within a strict concentration window. We have previously shown that beads of the commercial polymer Hytrel G3548L can act as a sequestering phase to reduce inhibitory effects to cells of *Candida utilis* while creating a reservoir for high concentrations of products. In this work we varied the polymer phase volume ratio (from 3 to 15%), and modified the benzaldehyde feeding strategy to further improve on system performance, resulting in greater than 100% increase in the PAC productivity relative to a single phase control, as well as robust operation of the two-phase bioreactor with minimal operator intervention.

Key Words: benzaldehyde; L-phenylacetylcarbinol; polymer beads; solid-liquid two-phase partitioning bioreactors

4.3 Introduction

L-phenylacetylcarbinol (PAC), a precursor to the decongestant drugs L-ephedrine and pseudoephedrine, is commercially produced through microbial biotransformation using yeast with benzaldehyde and glucose as substrates (Shin and Rogers 1995). The transformation relies on the anaerobic accumulation of pyruvate and activation of the enzyme pyruvate decarboxylase (PDC). Other oxidoreductases present during anaerobic respiration catalyze the formation of benzyl alcohol from benzaldehyde, which is the major by-product of the industrial process (Long and Ward 1989; Shin and Rogers 1995). While still maintaining a high stereoselectivity and efficiency compared to chemical synthesis methods, this biotransformation is subject to substrate (benzaldehyde), product (PAC) and by-product (benzyl alcohol) inhibition, making it an interesting system for reactor design studies.

Immobilizing cells, encapsulating the yeast (*Saccharomyces cerevisiae*) to reduce their exposure to aqueous benzaldehyde, PAC and benzyl alcohol, was one of the first reactor designs implemented for the PAC system (Mahmoud, et al. 1990). Performance of immobilized cells was improved using liquid-liquid two-phase partitioning bioreactors (TPPBs), which reduced the exposure of yeast (*Candida utilis*) to inhibitory compounds by sequestering such species into an immiscible phase (Rosche, et al. 2005). This system used octanol as the sequestering phase, which resulted in inhibitory effect to the *C. utilis* cells. In order to avoid toxicity problems, Zhang et al. (2008) used biocompatible polyethylene-glycol to create a cloud point system, which exists in two phases when operated at the cloud point temperature. This system was able to produce 8 g PAC/L and 4 g benzyl alcohol /L (Zhang, et al. 2008). Recent work by Khan and Daugulis (2010) demonstrated that solid polymer beads could be used as a biocompatible partitioning phase for the PAC production system to achieve overall concentrations of 11 g PAC/L and 4.5 g benzyl alcohol/L.

The objective of the current work was to study the effect of varying the polymer to aqueous phase ratio on system performance. The possibility of delivering benzaldehyde from the

sequestering phase was then explored in order to reduce manual intervention for simplified operation and reduced risk of contamination.

4.4 Materials and Methods

4.4.1 Chemicals and Polymers, and Medium Formulation

The sources of all chemicals used, and the medium formulation employed, were as described by Khan and Daugulis (2010). The yeast strain *Candida utilis* 70940 was purchased from the University of New South Wales Culture Collection (World Directory of Culture Collections No. 248). Cylindrical Hytrel G3548L beads (4x3x2 mm) were graciously donated by DuPont Canada.

4.4.2 Analytics

HPLC-UV detection (Varian, Prostar, Model # PS325, Polaris 5u C18-A 150 x 4.6 mm column) was used to quantify benzaldehyde, benzyl alcohol and PAC using the method described by Rosche, et al. 2001. HPLC- refractive index detection (Varian, Prostar, Model # PS356, HiPlex H 8 μm 300 x 7.7 mm column at 75°C) was used to quantify glucose and ethanol with a mobile phase of 9 mm H_2SO_4 maintained at 0.4 mL/min.

All cell dry weight measurements were made using a cell dry weight versus optical density (OD) calibration curve for *C. utilis* at 600 nm (Biocrom Ultraspec).

4.4.3 Batch reactor operation

Inoculum was prepared by adding 40 μL of frozen stock culture to each of six 125 mL shake flasks containing 50 mL of sterile growth medium. Flasks were grown at 30°C and 180 rpm for 36 hours (to reach an OD of 2.6) and then added to the sterile bioreactor. A 5L bioreactor (3L medium) equipped with pH (6M KOH) and temperature (30°C) controls was used for all reactor runs (New Brunswick Scientific, BioFlo III). Bioreactor operation began with a 16 hour growth period (300 rpm, 1 vvm air aeration), followed by an 18 hour enzyme induction period (300 rpm, 0.1 vvm air aeration). After enzyme induction, benzaldehyde was added manually to maintain the

concentration between 1 and 2 g benzaldehyde/L. This range has been demonstrated to result in the highest PAC productivity, notwithstanding some inhibitory effects.

4.4.4 Two-phase batch reactor operation: Varying polymer phase ratio

For experiments varying the polymer to aqueous phase ratio, 100 g and 500 g of Hytrel G3548L beads (ρ=1.16) were used to correspond to 3% (86.7 mL polymer/ 3L aqueous) and 15% (434.7 mL polymer / 3L aqueous) polymer phase by volume. To stay within the aqueous benzaldehyde concentration feeding window, the polymer beads were preloaded with benzaldehyde: the required mass of beads (100 g or 500 g) was added to 3L of sterile medium, and benzaldehyde was added until the aqueous concentration equilibrated to 1 g benzaldehyde/L. The aim was to buffer the lower limit of the feeding window so that subsequent manual additions of benzaldehyde during the biotransformation period would stay in the aqueous phase rather than being absorbed by the polymer. The beads were removed, dried, and refrigerated. The previously described batch reactor procedure was then followed, including manual benzaldehyde addition, with the beads being added to the reactor at the start of the biotransformation. This operation procedure follows the two-phase batch reactor protocol described in Khan and Daugulis (2010) to allow for comparison.

4.4.5 Feeding Strategies

Three feeding strategies were evaluated in this study. The first (Strategy 1) was manual delivery of benzaldehyde with preloaded beads as described above. To minimize operator intervention, feeding of benzaldehyde directly from the beads was explored (Strategies 2 and 3). This required high levels of benzaldehyde to be preloaded into the beads. Because of the limited solubility of benzaldehyde (6.3 g/L in RO water), the aqueous preloading strategy described for the phase ratio tests could not be employed. In Strategy 2, two aliquots of 250 g Hytrel G3548L were incubated with 40 g of pure liquid benzaldehyde each to provide higher loading. Once benzaldehyde was absorbed (allowing 6 hours of incubation time), the beads were sealed in a

beaker and refrigerated to avoid losses of benzaldehyde through volatilization. Benzaldehyde addition to the bioreactor started by adding the first 250 g aliquot of beads. When PAC production began to slow, the remaining beads were added for a total of 500 g (15% by volume) of polymer phase. This strategy aimed to delivery benzaldehyde at 1 g/L.

Strategy 3 was designed to increase delivery towards the upper value of the feeding window, 2 g benzaldehyde/L. Strategy 3 preloaded 500 g of Hytrel G3548L with 100 g of pure liquid benzaldehyde. All 500 g of preloaded beads were added to the reactor in a single aliquot to commence the biotransformation.

4.4.6 Product sampling from polymer

Every two hours over the course of the biotransformation period, two 1 g polymer samples were collected and tested with the extraction technique described by Gao and Daugulis (2009) using methanol. Two methanol washes were used per sample. Additional washes were demonstrated to increase recovery by only approximately 1%.

4.5 Results and Discussion

4.5.1 Effect of varying polymer phase ratio

The first objective of this study was to see the effect of varying the polymer phase ratio on system performance. The polymer volumes tested were 3%, 9%, and 15%, which correspond to phase ratios that have been used in the literature (Gao and Daugulis 2009; Prpich and Daugulis 2007a). Manual feeding to maintain the concentration between 1-2 g benzaldehyde/L was used resulting in a “saw-tooth” concentration profile for benzaldehyde. As manual feeding may cause some variation in results, the reproducibility of these experiments was verified using a replicate run of the 9% case (Figure 4-1).

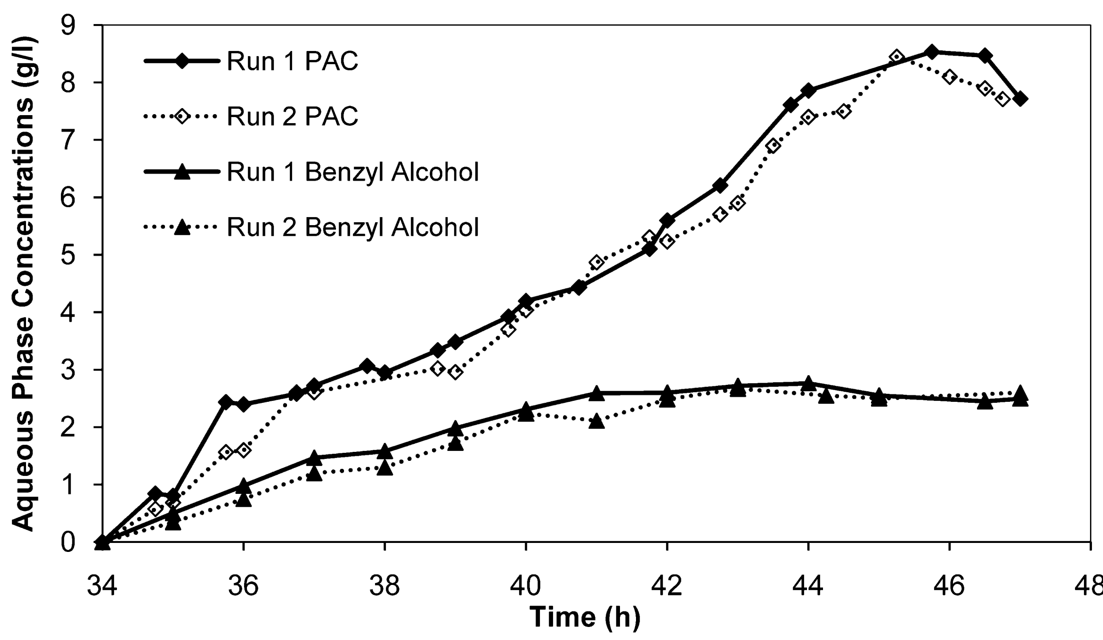

Figure 4-1 Replicate aqueous phase concentration profiles for PAC and benzyl alcohol for the 9% case to demonstrate the reproducibility of experiments in this study.

Figure 4-1 demonstrates that notwithstanding minor variation, the performance of the replicate system was the same for both PAC and benzyl alcohol. Therefore, no replicate experiments were performed and the data presented in subsequent figures and tables are from single bioreactor experiments. Figure 4-2 displays the PAC aqueous concentrations and total mass present in the system for the various single bioreactor cases.

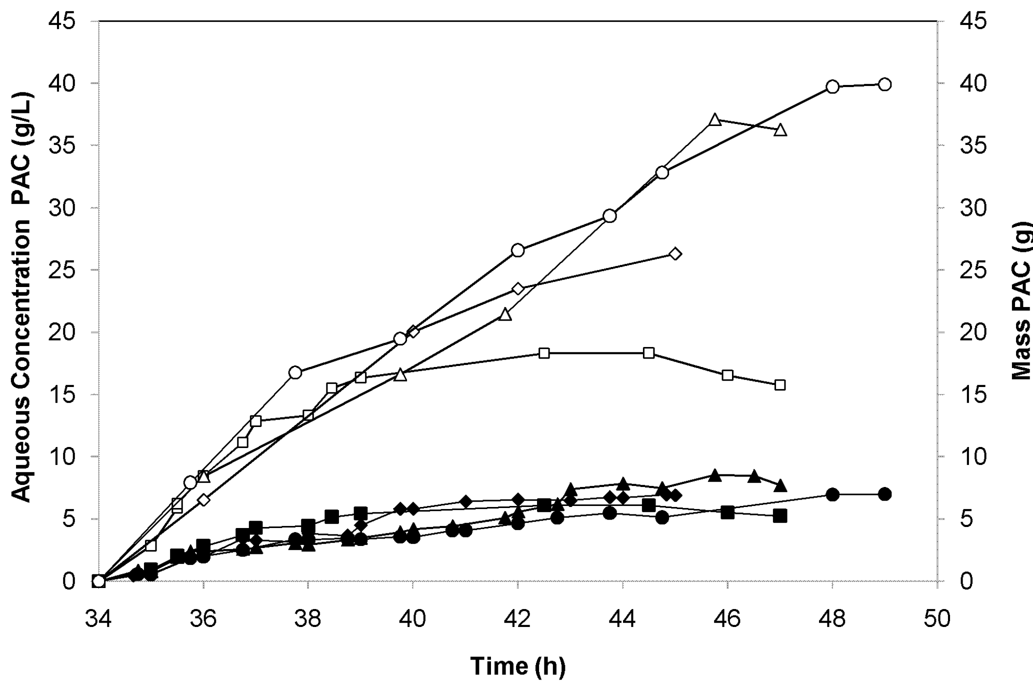

Figure 4-2: Aqueous phase concentration and total system mass of PAC for reactors varying phase ratio: ■ single phase concentration, ♦ 3% case aqueous concentration, ▲9% case aqueous concentration, ●15% case aqueous concentration. Open symbols represent the total mass produced in the corresponding system. Single phase and 9% polymer phase data are from Khan and Daugulis (2010).

Figure 4-2 demonstrates that varying the phase ratio does not have a significant impact on the aqueous concentration profiles, with some variation being attributed to differences in benzaldehyde concentration due to manual feeding. Analyzing the total mass produced (accounting for the mass present in both phases) allows for better comparison between systems, as the quantity of reservoir is taken into account. The total mass of PAC in the system at the end of the biotransformation is the highest in the 15% case, which produced 40 g of PAC, a 134% increase over the single phase control and an 11% increase over the 9% case. System performance can also be evaluated in terms of by-product formation (Figure 4-3).

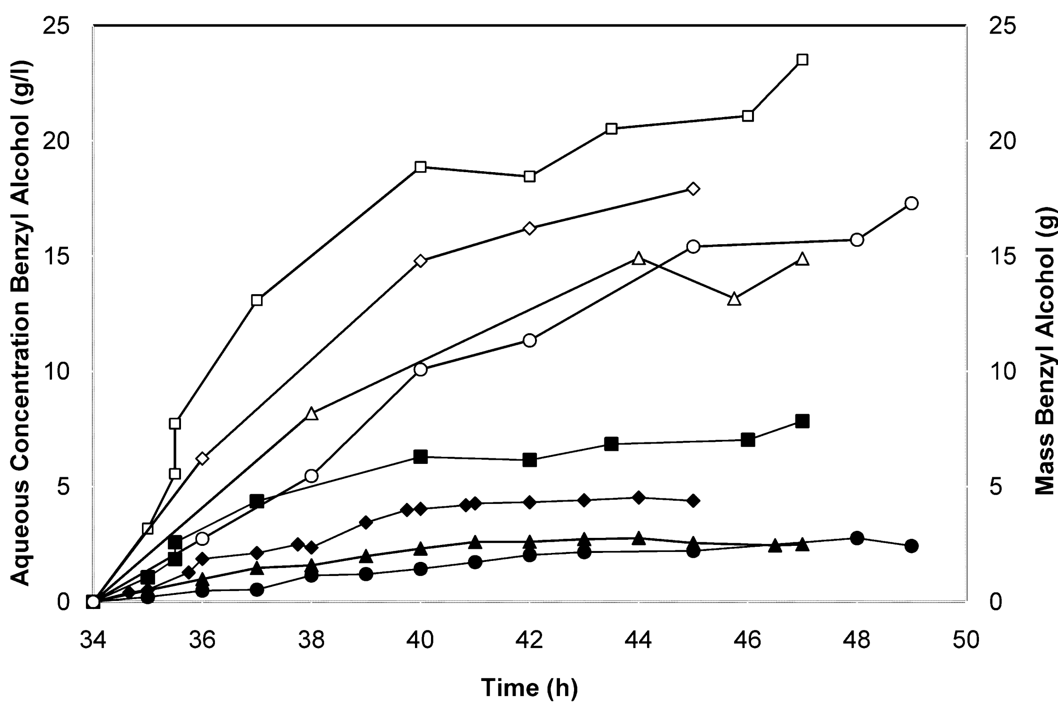

Figure 4-3: Aqueous phase concentration and total system mass of benzyl alcohol for reactors varying second phase ratio: ■ single phase concentration, ♦ 3% case aqueous concentration, ▲9% case aqueous concentration, ●15% case aqueous concentration. Open symbols represent the total mass produced in the corresponding system. Single phase and 9% polymer phase data are from Khan and Daugulis (2010).

The formation of by-product is not only undesirable due to the loss of substrate, but in this system, by-product also contributes to inhibition. Figure 4-3 shows a 26%, 34%, and 29% decrease in the total mass of benzyl alcohol in the system at the end of the biotransformation for the 3%, 9%, and 15% cases respectively, when compared to the single phase control. As benzaldehyde concentration was being manually maintained within the feeding window, this change in benzyl alcohol production can largely be attributed to the performance of the TPPB, with minimal contribution arising from biological effects caused by variation in benzaldehyde feeding. This reduction in inhibitory by-product formation is a novel demonstration in the field of TPPBs and appears in two aspects of by-product formation: 1) the final aqueous concentration of benzyl alcohol decreases with higher polymer phase ratios and 2) the total mass of benzyl alcohol produced in the system decreases with higher polymer phase ratios. However, there is no

improvement in benzyl alcohol formation in the 15% case over the 9% case. This indicates that the by-product effect does not indefinitely reduce by-product formation. This is likely due to the fact that because yeast are continuously exposed to 1-2 g benzaldehyde/L in the aqueous phase, the enzymes producing benzyl alcohol will always have some minimum access to benzaldehyde. The underlying cause of the by-product effect may be due to the ability of PDC to maintain a higher activity when exposed to lower aqueous concentrations of benzyl alcohol (and PAC). The active oxidoreductases in the system can convert some benzaldehyde in the system to benzyl alcohol, but if PDC activity is high, the system may demonstrate a preference for PAC production.

Analysis of the final system parameters shown in Table 4-1 expands our understanding of the effect of varying the phase ratio on system productivity.

Table 4-1 System parameters for reactors of varying polymer phase ratio

	Single Phase*	3%	9%*	15%
Time to completion (h)	8	10	13	14
Cell Density (g biomass/l)	13.4	13.4	13.6	12.1
Aqueous PAC end point (g PAC/l)	5.7	6.6	7.6	7.0
Aqueous BOH end point (g BOH/l)	7.4	4.5	2.5	2.6
Polymer PAC (g PAC/l polymer)	--	65.1‡ (60.5,69.7)	50.5‡ (46.6,54.4)	43.7‡ (42,45.3)
Polymer BOH (g BOH/ l polymer)	--	34.1‡ (31.1,37.2)	28.5‡ (25.5,31.5)	18.8‡ (15,22.6)
Overall PAC (g PAC/ l)	5.7	8.2	11.0	11.6
Overall BOH (g BOH /l)	7.4	5.3	4.5	4.6
$Y_{PAC/BZA}$ (mol/mol consumed)	0.34	0.39	0.37	0.51
$Y_{BOH/BZA}$ (mol/mol consumed)	0.61	0.35	0.21	0.28
PAC Mass Productivity (g PAC/h)	2.14	2.53	2.76	2.85
Benzaldehyde Partition Coefficient	--	31	39	38
Benzyl Alcohol Partition Coefficient	--	7.4	11.4	7.8
PAC Partition Coefficient	--	9.8	7.1	6.2

*Data from Khan and Daugulis (2010)

‡Average value of the 2x1g random polymer samples shown in parentheses

The overall system concentrations shown in Table 4-1 account for the PAC and benzyl alcohol present in both phases. The 3%, 9%, and 15% cases correspond to 44%, 93%, and 104% increases in overall PAC concentration and 28%, 39%, and 38% reductions in overall benzyl alcohol concentration respectively, relative to the single phase control. This further demonstrates the improvement made to the system with respect to both final PAC and final benzyl alcohol concentrations.

An interesting expansion to current knowledge in the field of TPPBs is the increase in molar yield of PAC on benzaldehyde with increasing phase ratio. Recent literature in the field of liquid-liquid TPPBs demonstrated at 29% increase in molar yield of PAC on benzaldehyde with a 50: 50 ratio of organic solvent to aqueous phase (Rosche, et al. 2005). When solid-liquid TPPBs were first investigated for PAC production, a 9% increase in molar yield was observed with a 9% phase ratio. This improvement was assumed to be less than the liquid-liquid system due to the lower phase ratio (Khan and Daugulis, 2010). However, this work demonstrates a 50% increase in molar yield of PAC for the 15% case. This significant improvement over recent literature is most reasonably caused by the ability for polymer beads to retain benzaldehyde more effectively than organic solvents, reducing losses to volatilization or oxidation.

Increasing the polymer phase ratio appears to have increased the lifespan of the biotransformation (Table 4-1). This is likely due to the polymer phase lowering the aqueous concentration, reducing system inhibition. The mass productivities of the 3%, 9%, and 15% cases demonstrated an 18%, 29%, and 33% increase in productivity over the single phase performance. With the increased lifespan of the 15% case, more PAC was produced, resulting in a significant improvement in system productivity compared to the single phase, as well as the lower phase ratio TPPBs. This is analogous to observations made in liquid-liquid systems of varying phase ratio, for up to 50% by volume (Prpich and Daugulis 2007b). However, it should be noted that achieving a 50% polymer phase would be difficult due to reductions in mixing caused by the accumulation of polymer beads behind reactor internals (Boudreau and Daugulis 2006).

As polymers sequester PAC with a high affinity, the PAC concentration in the polymers is substantially higher than in the aqueous phase, and depends on the value of the partition coefficient. The partition coefficients of the target compounds toward the polymer (the ratios of polymer concentration to aqueous concentration) are listed in Table 4-1. There is some variation between the partition coefficients in Table 4-1 and those previously reported, which were 39, 11, and 7 for benzaldehyde, benzyl alcohol, and PAC respectively (Khan and Daugulis 2010). As the same polymer was used as the sequestering phase in both experiments, the most likely cause of this variation is changes to the medium composition during biotransformation. The effect of medium composition on partition coefficients is an area of work currently being further investigated.

With respect to overall PAC concentration, all three TPPBs investigated were able to surpass the performance of the recent work using a cloud point system for PAC production, which obtained 8 g PAC/L (Zhang, et al. 2008). However, the Zhang et al. (2008) system produced only 4 g benzyl alcohol/L. Considering the best performing TPPB from the current work, the 15% case, the benzyl alcohol produced was higher, reaching a total concentration of 4.6 g benzyl alcohol/L. However, it is important to note that this can still be considered an overall improvement, as the ratio of total product to by-product concentrations is 2.5:1, while Zhang et al (2008) achieved a ratio of 2:1. Therefore, the 15% phase ratio TPPB was able to provide better selectivity for PAC production over by-product formation compared to the recent literature.

4.5.2 Effect of Feeding Strategy

A delicate balance exists in the PAC production system between preferential formation of the by-product benzyl alcohol at low benzaldehyde concentrations and inhibition of the biocatalyst at high benzaldehyde concentrations (Shin and Rogers 1995). Maintaining benzaldehyde inside the 1-2 g/L feeding window normally requires frequent operator intervention, which not only complicates operation but leads to potential sources of error and contamination. Delivery of

benzaldehyde from the sequestering phase of a TPPB for the PAC system has not been demonstrated for liquid-liquid TPPBs, likely due to the more complicated nature of work with organic solvents. The sequestering phase must be loaded, recovered from the loading procedure, and stored, which would be more difficult when working with hazardous liquids, such as octanol, rather than inert polymer beads. This study explored the possibility of preloading polymer beads to deliver benzaldehyde concentrations within the optimum feeding window.

Three feeding strategies are compared in this section: Strategy 1) manual feeding (corresponding to the 15% phase ratio case previously described), Strategy 2) delivery of 80 g of benzaldehyde separated into two bolus additions of beads, and Strategy 3) delivery of 100 g of benzaldehyde in one addition of beads. For strategies 2 and 3, 500 g (15% polymer phase by volume) of Hytrel G3548L were preloaded with benzaldehyde. The amount to be loaded was determined to account for the 1 g benzaldehyde/L aqueous phase concentrations required at the end of the biotransformation as well as the mass of benzaldehyde that was likely to be consumed (based on the results of the 15% case used as Strategy 1), with some additional benzaldehyde to account for any unexpected losses, such as volatilization. However, with a benzaldehyde partition coefficient of approximately 38, the 80 g loaded in Strategy 2 could have resulted in a theoretical aqueous concentration of 4.8 g benzaldehyde/L, which is significantly above the feeding window. Therefore, it was decided to add the polymers to the reactor in two 250 g additions. The first addition of 250 g of polymer (containing 40 g total benzaldehyde) was used to begin the biotransformation. The second addition of beads was done when PAC production began to plateau.

When it was determined that Strategy 2 did not reach the feeding window, remaining around 0.5 g benzaldehyde/L during the biotransformation, the third feeding strategy was designed. Beads were added to the reactor in one addition, and a slightly higher mass of benzaldehyde was loaded (100 g), with the aim being to reach the upper end of the feeding

window. The deliveries achieved for the feeding strategies tested in this study are shown in Figure 4-4.

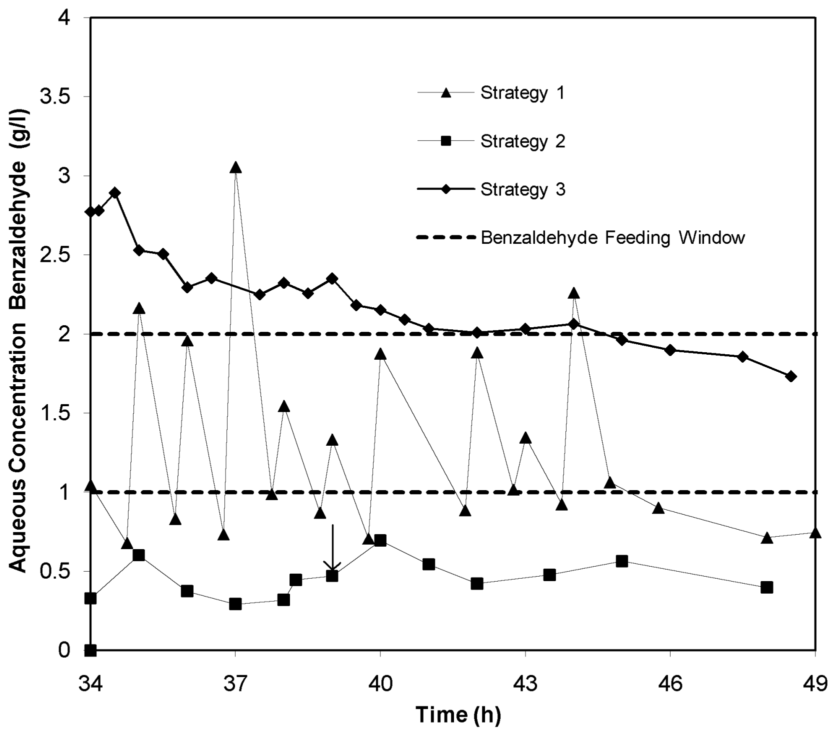

Figure 4-4: Aqueous benzaldehyde concentrations for 3 delivery strategies. The arrow represents the addition of the second aliquot of polymer beads for Strategy 2.

From Figure 4-4, it is evident that delivery of benzaldehyde in two separate aliquots of beads was ineffective as benzaldehyde concentration was not able to meet the target. This is likely a result of the time required for benzaldehyde to diffuse from the polymer beads into the aqueous phase. Although the theoretical aqueous concentration would be above the inhibitory window, the yeast began to consume benzaldehyde as soon as it was present in the aqueous phase, resulting in a decrease in aqueous phase concentration.

The objective of Strategy 3 was to deliver benzaldehyde towards the higher concentrations of the target window. With 100 g of benzaldehyde loaded in the polymer, the theoretical aqueous concentration would be 6 g benzaldehyde/L (based on a partition coefficient of 38). For this strategy, the delivery rate was first tested abiotically (Figure 4-5).

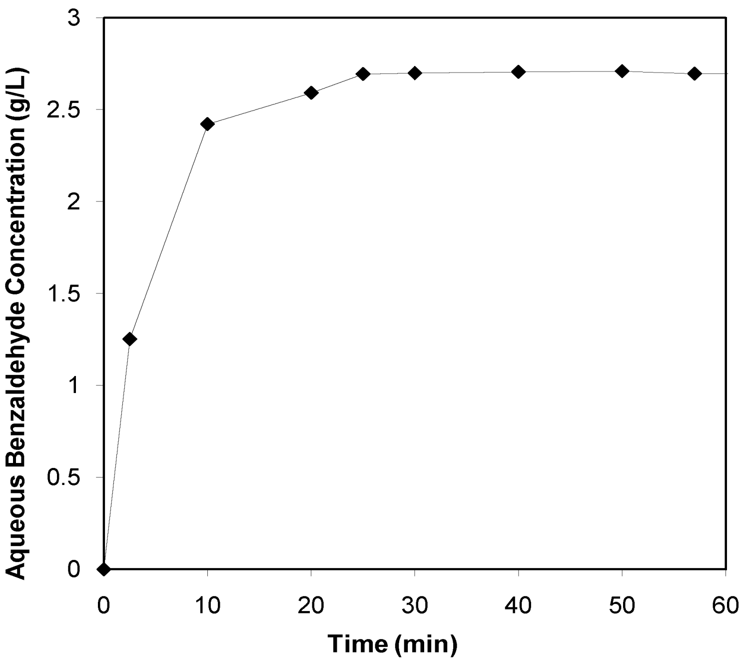

Figure 4-5: Abiotic release profile for 100 g of benzaldehyde loaded into 500 g of Hytrel G3548L (15% phase ratio by volume) and added to 3L aqueous medium.

Figure 4-5 demonstrates that benzaldehyde concentration increased to 2.7 g/L in approximately 20 minutes and then remained constant. The theoretical 6 g benzaldehyde/L was not reached. Polymer release work by Rehmann et al (2007) demonstrated that polymers can deliver only to the aqueous phase solubility limit (Rehmann, et al. 2007), which from Figure 4 observed to be 2.7 g benzaldehyde/L at 30 ˚C (compared to 6.3 g/L, experimentally determined in RO water in this study). As this is only 35% above the target of 2 g benzaldehyde/L, the release profile for Strategy 2 was encouraging in that delivery of benzaldehyde may be met with consumption by the microbes to avoid surpassing the feeding window. The resulting release profile from Figure 3 showed that microbial activity was not able to reduce the benzaldehyde concentration to within the feeding window until 6 hours into the biotransformation. As a result, the yeast were exposed to higher than desired concentrations of benzaldehyde for more than half of the biotransformation. The impact of delivery strategy on system performance is shown in Table 4-2.

Table 4-2 Final system parameters testing benzaldehyde delivery strategy: manual feeding (Strategy 1), delivery of 80 g benzaldehyde from polymers (Strategy 2) and delivery of 100 g benzaldehyde (Strategy 3)

	Single Phase*	15% Strategy 1¥	15% Strategy 2	15% Strategy 3
Time to Completion (h)	8	14	10	11
Cell Density (g biomass/l)	13.4	13.6	12.2	13.0
Aqueous PAC end point (g PAC/l)	5.7	7.0	4.5	4.8
Aqueous BOH end point (g BOH/l)	7.4	2.6	2.4	1.8
Polymer PAC (g PAC/l polymer)	--	44‡ (42,45.3)	32.0‡ (30.5,33)	35‡ (32.9,37.1)
Polymer BOH (g BOH/l polymer)	--	18.8‡ (15,22.6)	27.1‡ (25.1,29.2)	17‡ (16.2,17.3)
Overall PAC (g PAC/l)	5.7	11.6	8.0†	8.6
Overall BOH (g BOH/l)	7.4	4.6	5.5	3.7
$Y_{PAC/BZA\ (mol)}$	0.34	0.51	0.32	0.32
$Y_{BOH/BZA\ (mol)}$	0.61	0.28	0.32	0.19
PAC Mass Productivity (g/h)	2.14	2.93	2.75	2.69

*Data from Khan and Daugulis (2010)
¥Data from Table 1
‡Average value of the 2x1g random polymer samples shown in parentheses

Table 4-2 shows Strategy 2 not only had a 31% decrease in overall final PAC concentration, but also demonstrated a 19.5% increase in overall final benzyl alcohol concentration compared to manual feeding. While Strategy 3 provided a 26% reduction in PAC concentration, a 19.5% decrease in benzyl alcohol was observed. This is likely due to the metabolic preference for benzyl alcohol formation at lower benzaldehyde concentrations (Shin and Rogers 1995). It is interesting to note that while Strategies 2 and 3 demonstrated a lower yield of PAC on benzaldehyde, Strategy 3 was able to maintain the selectivity for PAC over benzyl alcohol that was demonstrated with manual feeding. Manual feeding demonstrated a PAC to benzyl alcohol yield ratio of 1.8:1, while Strategy 3 demonstrated a ratio of 1.7: 1.

Strategies 2 and 3 had a decreased time required for biotransformation compared to Strategy 1, as listed in Table 4-2. Strategy 2 is likely stopped by benzyl alcohol inhibition, while Strategy 3 is likely suffering from benzaldehyde inhibition. Despite the overall lower PAC concentrations observed with benzaldehyde delivery from the beads, when evaluated using mass

productivity, Strategies 2 and 3 demonstrated only a modest decrease (a 6% and 8%) compared to manual feeding. This could be used to improve commercial applications by increasing operational simplicity.

4.6 Conclusions and Future Work

The phase ratio tests in this work not only improved the PAC system with respect to productivity, but also demonstrated a reduction in by-product formation through the use of solid-liquid TPPBs. Also novel to the field of TPPBs for biosynthesis is the delivery of substrate from the sequestering phase. The ability to deliver substrate from polymer beads adds to the advantages in the use of solid-liquid over liquid-liquid TPPBs.

The partitioning of target molecules in this study showed some variation from literature values reported for the same polymer phase. Therefore, future work suggested for solid-liquid TPPBs is to further investigate the effect of medium composition on the extent of partitioning of target compounds. It would also be of interest to examine other biocatalysis systems with inhibitory by-products in a solid-liquid TPPB to expand on the observed decrease in by-product formation.

4.7 Acknowledgements

The authors would like to thank the Natural Sciences and Engineering Research Council of Canada and DuPont Canada for financial support.

4.8 References

Boudreau NG, Daugulis AJ. 2006. Transient performance of two-phase partitioning bioreactors treating a toluene contaminated gas stream. Biotechnol Bioeng 94:448-457.

Gao F, Daugulis AJ. 2009. Bioproduction of the aroma compound 2-phenylethanol in a solid-liquid Two-Phase Partitioning Bioreactor system by Kluyveromyces marxianus. Biotechnol Bioeng 104: 332-339

Khan TR, Daugulis AJ. Application of solid-liquid TPPBs to the production of L-phenylacetylcarbinol from benzaldehyde using *Candida utilis*. Biotechnol Bioeng DOI 10.1002/bit.22839

Long A, Ward O. 1989. Biotransformation of benzaldehyde by Saccharomyces cerevisiae: Characterization of the fermentation and toxicity effects of substrates and products. Biotechnol Bioeng 34:933-941.

Mahmoud WM, El-Sayed A, Halim M.M., Coughlin RW. 1990. Production of L‐phenylacetyl carbinol by immobilized yeast cells: II. Semicontinuous fermentation. Biotechnol Bioeng 36:55-63.

Prpich GP, Daugulis AJ. 2007a. A novel solid-liquid two-phase partitioning bioreactor for the enhanced bioproduction of 3-methylcatechol. Biotechnol Bioeng 98:1008-1013.

Prpich GP, Daugulis AJ. 2007b. Solvent selection for enhanced bioproduction of 3-methylcatechol in a two-phase partitioning bioreactor. Biotechnol Bioeng 97:536-543.

Rehmann L, Sun B, Daugulis AJ. 2007. Polymer selection for biphenyl degradation in a solid-liquid two-phase partitioning bioreactor. Biotechnol Prog 23:814-819.

Rogers P, Shin H, Wang B. 1996. Biotransformation for L-Ephedrine Production. Advances in Biochemical Engineering Biotechnology 56:33-60.

Rosche B, Sandford V, Breuer M, Hauer B, Rogers P. 2001. Biotransformation of benzaldehyde into (R)-phenylacetylcarbinol by filamentous fungi or their extracts. Appl Microbiol Biotechnol 57:309-315.

Rosche B, Breuer M, Hauer B, Rogers PL. 2005. Cells of Candida utilis for in vitro (R)-phenylacetylcarbinol production in an aqueous/octanol two-phase reactor. Biotechnol Lett 27:575-581.

Shin H, Rogers P. 1995. Biotransformation of benzeldehyde to l-phenylacetylcarbinol, an intermediate in l-ephedrine production, by immobilized Candida utilis. Appl Microbiol Biotechnol 44:7-14.

Zhang W, Wang Z, Li W, Zhuang B, Qi H. 2008a. Production of l-phenylacetylcarbinol by microbial transformation in polyethylene glycol-induced cloud point system. Appl Microbiol Biotechnol 78:233-239.

Chapter 5

Medium composition effects on solute partitioning in solid-liquid two-phase bioreactors

Tanya R. Khan and Andrew J. Daugulis

With minor editorial changes to fulfill formatting requirements, this chapter is substantially as it appears in: *Journal of Chemical Technology and Biotechnology* (2010) Accepted 4 Aug 2010. Article JCTB_2508.

5.1 Preface

As can be seen from the partition coefficients for the target compounds shown in Chapters 3 and 4, some discrepancy exists between those obtained from abiotic polymer screening (Chapter 3) and those obtained using bioreactor product medium (Chapters 3 and 4). This difference in partition coefficients has appeared in other studies throughout the literature, though it is not currently understood (Gao and Daugulis, 2009; Prpich and Daugulis, 2007). As polymer properties remain largely constant, the most reasonable source of this variation must be due to the aqueous phase, which will change over the course of the biotransformation and may have some differences between studies.

While TPPB design criteria have traditionally focused on solvent selection strategies, little consideration has been given to the properties of the aqueous phase that may cause deviation between abiotic and fermentation media partition coefficients. This chapter identifies some aspects of medium composition that may change over the course of a biotransformation and examines their respective effects on the partition coefficient of benzaldehyde in the polymer Hytrel G3548L. The ultimate goal of this chapter is to provide simple strategies that may be implemented to improve bioreactor performance using medium composition.

5.2 Abstract

Biphasic systems such as two-phase partitioning bioreactors (TPPBs) have been used to alleviate biological inhibition by sequestering inhibitory compounds within an immiscible phase. The use of solid polymer beads as this auxiliary phase provides a fully biocompatible alternative to potentially toxic organic solvents. While guidelines exist for the rational selection of the polymer phase, the effect of the aqueous phase composition on molecular sequestration has not been explored in the literature. This work aims to identify aspects of medium composition that influence the partitioning of target molecules into the sequestering phase. Using benzaldehyde as the target molecule and Hytrel G3548L (DuPont) as the polymer phase, pH, temperature, salt and glucose concentrations, as well as ethanol concentrations, were examined for their effects on the partition coefficient. pH and temperature were observed to have no significant effect on benzaldehyde partitioning. Salt and glucose additions increased the partition coefficient by 173% and 30% respectively compared to pure RO water, while increasing ethanol concentration was found to decrease the partition coefficient from 44 (±1.6) to 1 (±0.3). Strategic changes to the aqueous phase can be made to improve affinity of the sequestering phase for target molecules. This provides a simple and cost-effective method to potentially improve TPPB system performance.

Keywords: polymer beads; two-phase partitioning bioreactor; partition coefficient; biotransformation

5.3 Introduction

The bioproduction of high-value compounds, such as pharmaceuticals or food additives, has great potential for application in industry due to the high purity and stereoselectivity achieved in the final product (Pollard and Woodley 2007). The major limitation of biological synthesis routes compared to chemical synthesis is low final product concentrations, often caused by toxicity of substrate, product, or by-product to the microorganism performing the transformation. Biphasic systems, such as two-phase partitioning bioreactors (TPPBs), are often employed to alleviate inhibition by sequestering the inhibitory compounds into an auxiliary immiscible phase (Freeman, et al. 1993).

The selection of an appropriate immiscible phase is an essential component of TPPB design. This phase must demonstrate a high affinity for the target compound(s), which is characterized by the partition coefficient(s). The partition coefficient is the ratio of the concentration of the target compound in the sequestering phase relative to its concentration in the aqueous phase at equilibrium. Organic solvents have often been employed as the immiscible phase, but can have limitations such as cytotoxicity, bioavailability, high viscosity, flammability and high cost (Bruce and Daugulis 1991). However, recent advances in the field of TPPBs have demonstrated that solid polymer beads can provide an effective alternative sequestering phase (Amsden, et al. 2003).

Solid-liquid TPPBs have been applied to the production of several high value compounds, such as 3-methylcatechol (Prpich and Daugulis 2007), carvone (Morrish and Daugulis 2008), 2-phenylethanol (Gao and Daugulis 2009), L-phenylacetylcarbinol (Khan and Daugulis, 2010), and benzaldehyde (Jain, et al. 2010). These systems have all shown greater than 100% improvement in performance over single phase systems, demonstrating the potential of solid-liquid TPPBs to approach concentration requirements for industrial applications. The TPPB literature has focused on providing strategies for sequestering phase selection, with little to no

attention being given to the composition of the aqueous phase (Bruce and Daugulis 1991; Gao and Daugulis 2010; Rehmann, et al. 2007).

A difference in partitioning between abiotic and biologically determined partition coefficients has been observed in the solid-liquid TPPB literature, but the cause for this discrepancy is not currently understood. As polymer properties remain largely constant, the most reasonable source of this variation comes from the aqueous phase, which will change from its starting composition over the course of the biotransformation. While many studies observe between a 5 to 30% decrease in the partition coefficient for biological studies compared to abiotic studies (Khan and Daugulis 2010; Gao and Daugulis 2009; Morrish and Daugulis 2008; Prpich and Daugulis 2007), increases in partitioning have also been reported (Yeom, et al. 2010). Partition coefficients are often used in mass balances to account for the target compound in the polymer phase, emphasizing the need to account for variation in partition coefficients over the course of a biotransformation (Gao and Daugulis 2009; Prpich and Daugulis 2006). By independently analyzing factors that may affect partitioning, the discrepancies observed in the literature can be better understood and applied to future systems to allow greater operational control over TPPB partitioning behavior.

The objective of this study was to investigate some common elements of medium composition that may change during biotransformation and their individual effects on the partition coefficient. Benzaldehyde was used as the target molecule with the polymer Hytrel G3548L (DuPont), as there has been recently reported studies in the field of solid-liquid TPPBs with Hytrel G3548L demonstrating affinity for benzaldehyde (Jain, et al. 2010; Khan and Daugulis 2010). By identifying how medium composition may alter the equilibrium distribution of compounds, some simple strategies to improve the performance of a solid-liquid TPPB have also been suggested.

5.4 Experimental

5.4.1 Chemicals and Polymers

For experiments testing growth medium as the aqueous phase, a typical minimal medium with all necessary macronutrients for growth was used with all components (glucose, $(NH_4)_2SO_4$, KH_2PO_4, $MgSO_4$, $CaCl_2$, $FeSO_4$, $ZnSO_4$, $MnSO_4$, $CuSO_4$) purchased from Fisher Scientific Canada (Oakville, ON, Canada) (Khan and Daugulis, 2010)Additional materials for modifying the aqueous phase (KCl, ethanol, H_2SO_4 and KOH) were also purchased from Fisher Scientific Canada. Cylindrical Hytrel G3548L polymer beads (4x3x2 mm) were graciously donated by DuPont Canada. Hytrel G3548L is a co-polymer of poly(butylene terephthalate) and polyether with a specific gravity of 1.16, glass transition temperature of -45˚C, melting point of 156˚C and shore hardness rating of 35D (Khan and Daugulis 2010).

5.4.2 Concentration measurements

HPLC-UV detection (Varian, Prostar, Model # PS325, Polaris 5u C18-A 150 x 4.6 mm column) was used to quantify benzaldehyde at 283 nm. The mobile phase of 30% v/v acetonitrile was maintained at 1 mL/min.

5.4.3 Partition coefficients

Partition coefficients were determined using a previously described method (Isaza and Daugulis 2009). 10 mL of stock solution were incubated (24 hours, 30˚C, 180 rpm) with a set mass of polymer (1-5 g) for each test. Error bars in all figures show the 95% confidence interval obtained from the linear regression to determine the partition coefficient. Medium composition effects were tested using independent stock solutions of RO water with 2 g/L of benzaldehyde. pH was tested at 5, 7 and 9. KCl was tested from 0 to 360 g/L, glucose from 0 to 500 g/L and ethanol from 0 to 100 %v/v. To test the effect of temperature, samples were sealed to avoid losses due to volatilization and incubated at room temperature (23˚C), 30˚C, and 45˚C for 24 hours at 180 rpm.

5.5 Results and Discussion

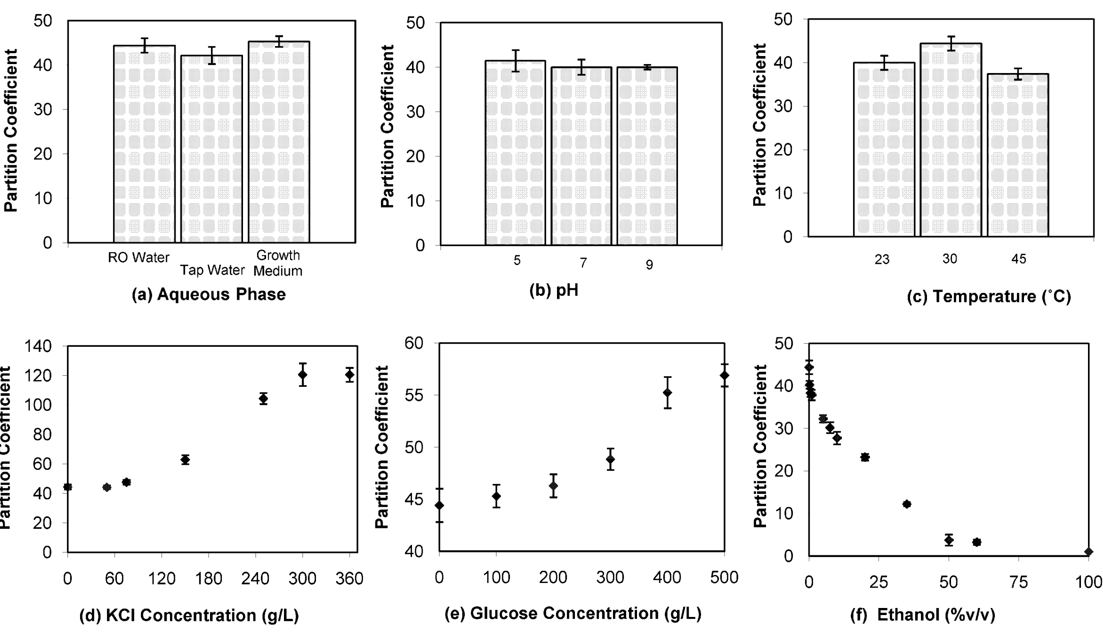

Figure 5-1: Partition coefficient of benzaldehyde toward Hytrel G3548L varying (a) RO water, tap water, growth medium (b) pH (c) Temperature (d) KCl concentration (e) glucose (f) ethanol concentration

5.5.1 The effect of RO water, Tap water, Growth Mediuma

The first objective was to analyze RO water, tap water, and a simple growth medium to see what, if any, effect these had on partitioning, and to select a standard aqueous phase for subsequent testing (Figure 1(a)). Figure 1 (a) shows that within the 95% confident intervals, there is no statistical difference in partition coefficient for RO water, tap water, or the minimal growth medium tested. RO water was selected as the aqueous phase for subsequent tests to avoid any unforeseen interactions between compounds in the aqueous phase and the variable being tested.

When designing the medium composition for biocatalysis, some important aspects that must be considered are pH, salt concentrations, and glucose (or another carbon source) concentration. This study examined these elements of medium composition, and how they may be altered to effect partitioning. Additionally, the effect of ethanol on the partition coefficient

was examined, as ethanol is an example of an inhibitory fermentation product produced in large quantities, often at high concentrations, and is also a common anaerobic fermentation by-product.

5.5.2 Effect of pH

The effect of pH on partitioning of benzaldehyde was examined over a range of pH values typical of biological operation (Jain, et al. 2010; Khan and Daugulis, 2010). Figure 1(b) shows that there is no statistical difference in partitioning of benzaldehyde, which was expected because the functional group of benzaldehyde is an aldehyde, and therefore was unlikely to ionize over this range. It is expected that some molecular change, such as ionization, would be required in order for pH to affect partitioning. However, this effect may be the least broadly applicable of all the variables tested in this study, as it likely depends on the chemical nature of the target molecule and is an area under investigation in our group with a variety of molecules possessing different molecular features.

5.5.3 Effect of temperature

The effect of temperature on partitioning was examined from room temperature (23˚C) to 45˚C, shown in Figure 1(c). This range was selected to avoid experimental complications due to volatilization of benzaldehyde at high temperatures as well as to avoid adding additional power requirements with bioreactor operation to cool the medium to lower temperatures. There is no significant effect of temperature on partitioning shown in Figure 1(c). However, temperature may affect the rate of partitioning due to the temperature dependence of diffusivity, which may be useful to evaluate for a given system but is beyond the scope of this investigation.

5.5.4 Effect of salt concentration

To determine the effect of salt concentration, potassium chloride (KCl) was selected as it is fully ionizable, has been used in similar studies on liquid-liquid partitioning, and may be representative of other dissociable salts present in growth medium (Malinowski and Daugulis 2004). The full range of KCl solubility at 30˚C was tested, and the effect is shown in Figure 1(d).

Salt concentration provided the most significant change in partition coefficient of all variables explored in this study, with a 173% increase over pure RO water with KCl at its solubility limit (precipitated KCl was observed at 360 g/L, 4.8 M). It appears as though low concentrations of KCl have a minimal effect on partitioning, with overlapping 95% confidence intervals seen for up to 75 g/L. However, above this point, the partition coefficient increases, until a plateau is reached when approaching the solubility limit, with negligible differences between 300 g/L (below the solubility limit) and 360 g/L (above the solubility limit). An increase in partitioning with salt addition in liquid-liquid TPPBs has been previously observed and was explained by hydration theory, in which the electrolyte prefers to associate with water molecules, resulting in the effective removal of the water molecules from their role as a solvent (Malinowski and Daugulis 2004). This causes an increase in the activity of nonelectrolyte (in this study, benzaldehyde) in solution corresponding to the increase in partitioning. There exists a limiting water activity below which further improvements in partitioning are not observed (Malinowski and Daugulis 2004), which may explain the plateau observed above 300 g/L KCl.

The salt effect has great potential to improve TPPB performance in a broad range of applications. While the addition of electrolytes at high concentrations may damage active microbes (by osmotic pressure effects), the addition of salt at the end of a biotransformation may provide a way to improve extraction in batch processes.

5.5.5 Effect of glucose concentration

Glucose, when present in a growth medium, can be present at high concentrations, such as those used in high gravity ethanol fermentation (up to 500 g/L (Daugulis, et al. 1994)). This presents a potentially significant change to medium composition. Therefore, the effect of glucose concentration on partitioning was selected as a variable of interest for this study.

Figure 1(e) shows that the effect of changing glucose levels displays the same general shape as the salt effect shown in Figure 1(d). However, the change in partitioning is much less significant, with only a 30% increase over pure RO water at 500 g/L glucose. Based on hydration

theory suggested for the salt effect, an analogous trend would be expected for glucose, since non-electrolytes still cause a reduction in water activity, which, as previously described, would increase partitioning (Malinowski and Daugulis 2004). It should be noted that a statistically significant change in partitioning was not observed until a glucose concentration of 300 g/L was reached. Therefore, partitioning of target molecules into the polymer beads may be improved over the course of the biotransformation by maintaining high concentrations of glucose, which is less likely to disrupt microbial activity than high levels of electrolytes such as KCl.

5.5.6 Effect of ethanol concentration

Fermentation for ethanol production yields ethanol concentrations in excess of 70 g/L (Daugulis, et al. 1994). Traditional acetone-butanol-ethanol fermentation has cumulatively lower yields due to end-product inhibition of approximately 20 g/L (Daugulis, et al. 1994; Friedl, et al. 1991). This may represent a significant change to the medium composition over the course of a biotransformation, and has been presented in the literature as a potential source of discrepancy between abiotic partition coefficients relative to those determined using medium obtained from a biotransformation containing ethanol (Khan and Daugulis 2010).

Figure 1(f) shows a decrease in partition coefficient with increasing ethanol concentration, reducing from 44.4 (±1.6) for pure RO water to 1 (±0.3) for 100% ethanol. The trend displayed in Figure 2(d) shows sharp decreases in partition coefficient for up to 10% ethanol by volume, with the slope declining after this point. This may be due to ethanol increasing the solubility of benzaldehyde in the aqueous phase, reducing the thermodynamic preference of benzaldehyde for the polymer phase, as benzaldehyde is moderately hydrophobic (water solubility of 6.5 g/L). Therefore, during an anaerobic biotransformation, the gradual accumulation of ethanol may result in a reduction of the partition coefficient. In systems in which the product is being sequestered by the polymer beads, this reduced affinity may decrease system productivity. However, in systems where substrate is being delivered from polymer beads, this reduction may facilitate the release of the compounds from the polymers. Previous work in the

field of solid-liquid TPPBs has demonstrated that some systems with hydrophobic target compounds display such a high affinity for the sequestering phase that their release from polymer beads is hindered (Isaza and Daugulis 2009). With a statistically significant decrease in partitioning at 0.5% ethanol by volume, even low levels of ethanol may reduce the partition coefficient enough to help improve their delivery. This concept has been demonstrated to improve substrate release in a solid-liquid TPPB for the biodegradation of phenols, with 0.5% methanol added as a co-solvent (Tomei, et al. 2010).

5.6 Conclusions and Suggestions for enhanced TPPB performance

This work demonstrated a high dependency of the partition coefficient of benzaldehyde on medium composition. Therefore, it is recommended that studies reporting partition coefficients not only describe the medium used for testing, but also use a composition that is as similar as possible to the aqueous phase of any biotransformation involved. Additionally, solid-liquid TPPB studies using partition coefficients in mass balances must account for variation over the course of the biotransformation.

The effects determined in this study provide a broad range of strategies to improve TPPB performance. In a batch biotransformation, the addition of a strong electrolyte, such as KCl, to approach the solubility limit may provide significant improvements in product recovery. For batch or continuous processes, the maintenance of high levels of glucose may provide a moderate increase in partitioning without damage to microbes that could result at high levels of electrolytes. When a biotransformation involves the delivery of a substrate from the polymer phase, the presence of ethanol as a co-solvent may improve performance by facilitating the release of substrate from the polymer.

With the application of these simple changes to medium composition, the performance of a solid-liquid TPPB may show significant improvement as an alternative to designing specifically tailored polymers. Therefore, while rational polymer selection or even customization of polymers are important aspects of solid-liquid TPPB design, the aqueous phase should not be

ignored, as simple alterations may be able to provide relatively high improvements in performance. Current work is investigating other target molecules and polymers to develop heuristics for determination of the aqueous phase composition, as has been done previously for organic solvent and polymer selection, and implement these strategies in fermentation studies (Bruce and Daugulis 1991; Rehmann, et al. 2007).

5.7 Acknowledgements

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5.8 References

Amsden BG, Bochanysz J, Daugulis AJ. 2003. Degradation of xenobiotics in a partitioning bioreactor in which the partitioning phase is a polymer. Biotechnol Bioeng 84:399-405.

Bruce LJ, Daugulis AJ. 1991. Solvent Selection Strategies for Extractive Biocatalysis. Biotechnol Prog 116-124.

Daugulis A, Axford D, Ciszek B, Malinowski J. 1994. Continuous fermentation of high-strength glucose feeds to ethanol. Biotechnol Lett 16:637-642.

Freeman A, Woodley JM, Lilly MD. 1993. In situ product removal as a tool for bioprocessing. Biotechnology 11:1007-1012.

Friedl A, Qureshi N, Maddox IS. 1991. Continuous acetone-butanol-ethanol (ABE) fermentation using immobilized cells of Clostridium acetobutylicum in a packed bed reactor and integration with product removal by pervaporation. Biotechnol Bioeng 38:518-527.

Gao F, Daugulis AJ. 2010. Polymer solute interactions in solidliquid two-phase partitioning bioreactors. Journal of Chemical Technology & Biotechnology 85:302-306.

Gao F, Daugulis AJ. 2009. Bioproduction of the aroma compound 2-phenylethanol in a solid-liquid two-phase partitioning bioreactor system by kluyveromyces marxianus. Biotechnol Bioeng 104: 332-339.

Isaza PA, Daugulis AJ. 2009. Ultrasonically enhanced delivery and degradation of PAHs in a polymer-liquid partitioning system by a microbial consortium. Biotechnol Bioeng 104:91-101

Jain AN, Khan TR, Daugulis AJ. Bioproduction of benzaldehyde in a solid-liquid two-phase partitioning bioreactor using *Pichia pastoris*. Accepted June 23 2010. DOI: 0.1007/s10529-010-0353-2

Khan TR, Daugulis AJ. 2010. Application of solid-liquid TPPBs to the production of L-phenylacetylcarbinol from benzaldehyde using *Candida utilis*. Biotechnol Bioeng DOI 10.1002/bit.22839

Malinowski JJ, Daugulis AJ. 2004. Salt effects in extraction of ethanol, 1-butanol and acetone from aqueous solutions. AICHE J 40:1459-1465.

Morrish JL, Daugulis AJ. 2008. Improved reactor performance and operability in the biotransformation of carveol to carvone using a solid-liquid two-phase partitioning bioreacto*r*. Biotechnol Bioen*g* 101:946-956.

Pollard DJ, Woodley JM. 2007. Biocatalysis for pharmaceutical intermediates: the future is now. Trends Biotechnol 25:66-73.

Prpich GP, Daugulis AJ. 2007. A novel solid-liquid two-phase partitioning bioreactor for the enhanced bioproduction of 3-methylcatechol. Biotechnol Bioeng 98:1008-1016.

Prpich GP, Daugulis AJ. 2006. Biodegradation of a phenolic mixture in a solid–liquid two-phase partitioning bioreactor. Appl Microbiol Biotechnol 72:607-615.

Rehmann L, Sun B, Daugulis AJ. 2007. Polymer selection for biphenyl degradation in a solid-liquid two-phase partitioning bioreactor. Biotechnol Prog 23:814-819.

Tomei MC, Annesini MC, Rita S, Daugulis AJ. 2010. Two-Phase Partitioning Bioreactors Operating with Polymers Applied to the Removal of Substituted Phenols. Environ Sci Technol DOI**:** 10.1021/es903806p

Yeom SH, Daugulis AJ, Lee SH. 2010. Bioremediation of Phenol-Contaminated Water and Soil Using Magnetic Polymer Beads. Process Biochemistry 45: 1582-1586.

Chapter 6
Conclusions and Recommendations for Future Work

6.1 Conclusion

The first objective of this work was to determine the biocompatibility and bioavailability of a variety of organic solvents with *C. utilis.* The biocompatibility study performed determined the critical log P of *C. utilis* to be 4.8, while the bioavailability study found that alkanes and alkenes are generally non-bioavailable. These findings can help any future work with *C. utilis* and organic solvents to predict which solvents will be suitable with respect to both biocompatibility and bioavailability.

The second objective of this work was to apply solid polymer beads as the sequestering phase in a TPPB producing PAC from benzaldehyde using *C. utilis*. To achieve this, a screening of various commercially available polymers was done to determine the partition coefficients of each polymer towards benzaldehyde, PAC, and benzyl alcohol, which are the main inhibitory compounds in the biotransformation. The polymer Hytrel G3548L displayed the highest affinity for each compound with partition coefficients of 35, 7.5, and 10 for benzaldehyde, PAC, and benzyl alcohol respectively. To demonstrate the application of solid-liquid TPPBs to the PAC system, 300 g of Hytrel G3548L were used as the sequestering phase, and showed a 1.9 fold improvement on PAC concentration over the single phase fermentation benchmark, as well as a 1.6 fold decrease in benzyl alcohol concentration of the single phase case.

The third objective of this study was to study the effect of varying the phase ratio and feeding strategy in the PAC system. This work has expanded on the knowledge of solid-liquid TPPBs by demonstrating that increasing polymer phase ratio increases product formation while also reducing overall by-product concentration. Using Hytrel G3548L as the sequestering phase, a 15% polymer phase ratio was able to increase overall PAC concentration by 104% while decreasing benzyl alcohol by 38% over single phase performance. In an effort to simplify

bioreactor operation, reduce the possibility of contamination, and avoid operator intervention, delivery of benzaldehyde from the polymer phase was explored, a novel demonstration in the field of solid-liquid TPPBs for the synthesis of high value compounds, resulting in only a 6-8% reduction in mass productivity compared to manual substrate feeding.

The final objective of this work was to investigate the effect of various aqueous phase constituents on the partition coefficient of benzaldehyde in Hytrel G3548L. The factors investigated were pH, temperature, salt and glucose concentrations, as well as ethanol concentration. pH and temperature were observed to have no significant effect on benzaldehyde partitioning. Salt and glucose additions increased the partition coefficient by 173% and 30% respectively compared to pure RO water, while increasing ethanol concentration was found to decrease the partition coefficient from 44 (±1.6) to 1 (±0.3). The following strategies have been suggested to improve the performance of solid-liquid TPPBs:

- In a batch biotransformation, the addition of a strong electrolyte, such as KCl, to approach the solubility limit may increase the partition coefficient to provide significant improvements in product recovery.
- For batch or continuous processes, the maintenance of high levels of glucose may provide a moderate increase in partitioning without damage to microbes that could result at high levels of electrolytes.
- When a biotransformation involves the delivery of a substrate from the polymer phase, the presence of ethanol as a co-solvent may improve performance by facilitating the release of substrate from the polymer.

The aforementioned findings can be applied in the field of solid-liquid TPPB to aid in both the design (in terms of polymer phase and aqueous composition selection) and operation (in terms of phase ratio and feeding strategy). As PAC is currently produced through biotransformation, the process can likely easily be converted to a TPPB. Additional stages that would be required to retrofit the process include an extraction stage where the product is recovered from the polymer

beads, which may be done using methanol, as was done in this study. This would not only improve the system in terms of PAC productivity, but would also reduce by-product formation as was demonstrated in Chapter 4 of this work. By implementing feeding from the polymer beads, the industrial process would also benefit from reduced operator intervention. This work also contributed strategies to improve solid-liquid TPPBs for other biotransformations. Polymer screening criteria can be expanded to include the importance of polymer softness and hydrophobic interactions between the target compounds and the polymer as the main factors affecting absorption. Additionally, strategies using electrolytes, glucose, or ethanol in the aqueous phase can be applied to improve performance by either increasing or decreasing partitioning as required.

6.2 Recommendations for Future Work

The polymer screening in Chapter 3 successfully found one polymer with a high affinity for all three target compounds. However, the use of three unique polymers each with an affinity for only one of the target compounds is desired, as this would provide a high purity final product ready for downstream processing. As the screening of commercially available polymers was not able to demonstrate this desired affinity, the design of specifically tailored polymers is recommended. With the high value of compounds being synthesized in solid-liquid TPPBs, the expense associated with customized polymers may be offset by the value and purity of the final product achieved.

As was noticed in Chapter 4, increasing the polymer phase ratio appears to not only increase product formation, but also decrease total by-product formation, which is an effect that has not been previously observed in the field of solid-liquid TPPBs. It would be of great interest to study other biocatalysis systems with inhibitory by-product formation to determine if this effect was unique to the PAC system or broadly applicable.

Chapter 5 introduced the concept of simple alterations to the aqueous phase that may enhance bioreactor performance. Recommended future work is to investigate other target

molecules and polymers to develop heuristics to aid in the determination of aqueous phase composition, as has as has been done previously for organic solvent and polymer selection (Bruce and Daugulis 1991; Rehmann, et al. 2007).

The application of the polymer selection criteria established in Chapter 3, the delivery strategy developed in Chapter 4, and the aqueous phase composition strategies suggested in Chapter 5 can be used to improve system productivity over single phase biotransformations while simplifying operation. These may prove to be useful alterations to the processes currently producing high value compounds through biotransformation in industry (Straathof, et al. 2002), and additionally may help biotransformations suffering from low productivity meet commercial requirements.

6.3 References

Bruce LJ, Daugulis AJ. 1991. Solvent Selection Strategies for Extractive Biocatalysis. Biotechnol Prog 116-124.

Rehmann L, Sun B, Daugulis AJ. 2007. Polymer selection for biphenyl degradation in a solid-liquid two-phase partitioning bioreactor. Biotechnol Prog 23:814-819.

Straathof AJJ, Panke S, Schmid A. 2002. The production of fine chemicals by biotransformations. Curr Opin Biotechnol 13:548-556.

Appendix A- Calibration Curves

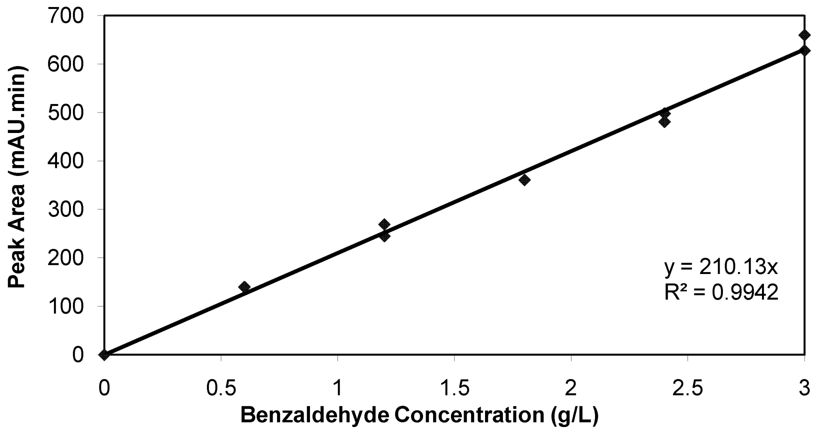

Figure A-1: Peak area versus benzaldehyde concentration calibration curve

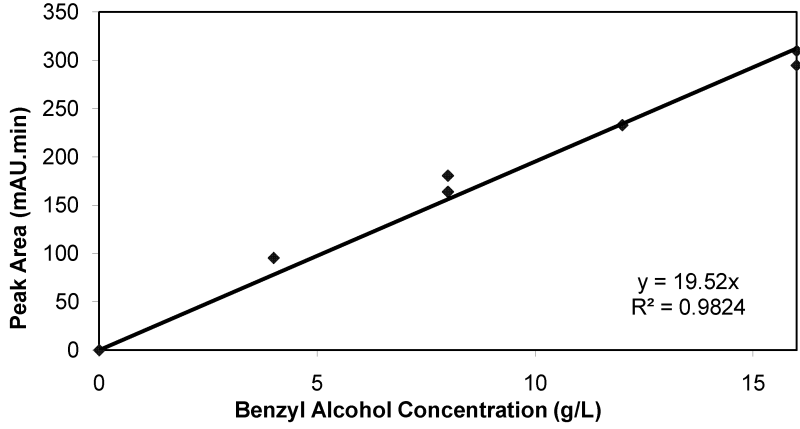

Figure A-2: Peak area versus benzyl alcohol concentration calibration curve

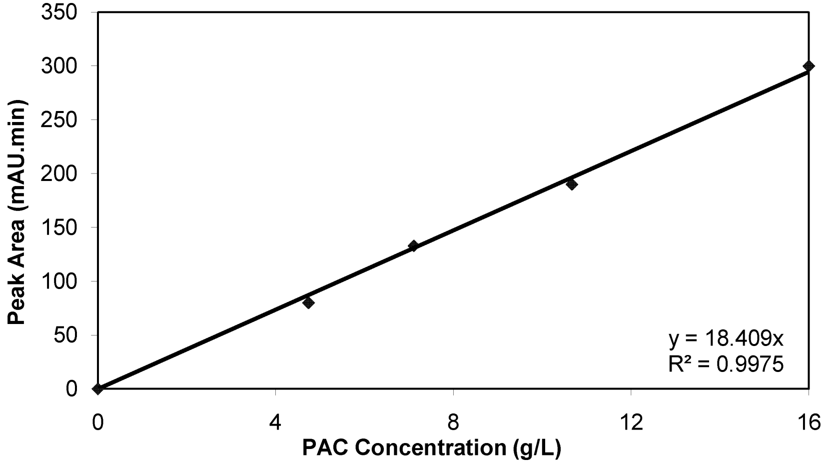

Figure A-3: Peak area versus PAC concentration calibration curve

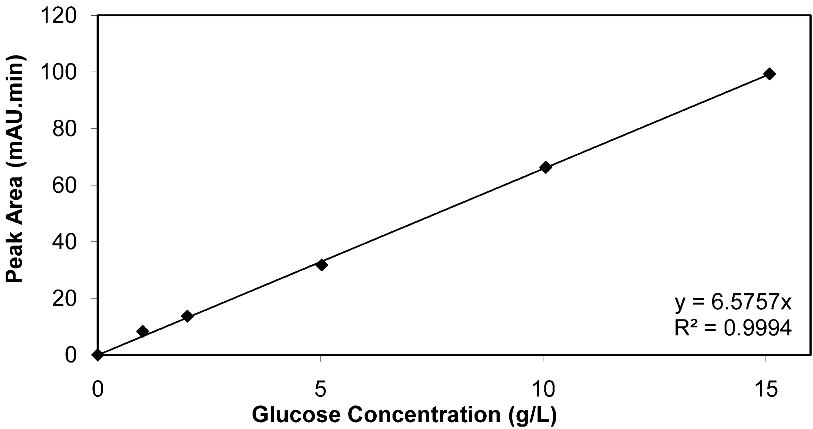

Figure A-4: Peak area versus glucose concentration calibration curve

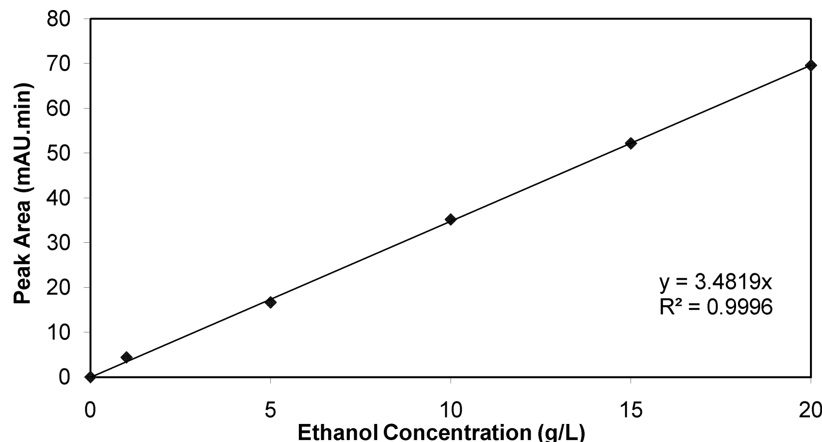

Figure A-5: Peak area versus ethanol concentration calibration curve

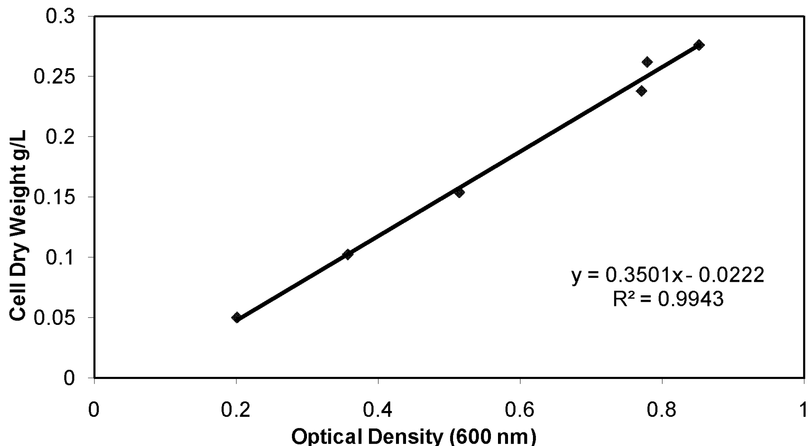

Figure A-6: Cell dry weight versus optical density at 600 nm calibration curve