STRATEGIES FOR ENHANCED BIOPRODUCTION OF BENZALDEHYDE USING *PICHIA PASTORIS* IN A SOLID-LIQUID PARTITIONING BIOREACTOR AND INTEGRATED PRODUCT REMOVAL BY IN SITU PERVAPORATION

by

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Abstract

Benzaldehyde (BZA), a biologically derived high-value molecule used in the flavour and fragrance industry for its characteristic almond-like aroma, has also found use in nutraceutical, pharmaceutical, cosmetics, agrochemical, and dye applications. Although, *nature-identical* BZA is most commonly produced by chemical synthesis, biologically derived BZA, whether by plant material extraction or via microbial biocatalysts, commands much higher prices. The bioproduction of high value molecules has often been characterized by low titers as results of substrate and product inhibition. The current work examined a variety of process strategies and the implementation of a solid-liquid bioreactor partitioning system with continuous integrated pervaporation to enhance the bioproduction of BZA using *Pichia pastoris*.

Previous work on two-phase partitioning bioreactors (TPPBs) for the biotransformation of BZA using *Pichia pastoris* has had limitations due to long fermentation times and unutilized substrate in the immiscible polymer phase, contributing to complications for product purification. To reduce fermentation times, a mixed methanol/glycerol feeding strategy was employed and reduced the time required for high-density fermentation by 3.5 fold over previous studies. Additionally, because BZA and not the substrate benzyl alcohol (BA) had been found to be significantly inhibitory to the biotransformation reaction, a polymer selection strategy based on the *ratio* of partition coefficients (PCs) for the two target molecules was implemented. Using the polymer Kraton D1102K, with a PC ratio of 14.9 (BZA:BA), generated a 3.4 fold increase in BZA produced (14.4 g vs. 4.2 g) relative to single phase operation at more than double the volumetric productivity (97 mg L^{-1} h^{-1} vs. 41 mg L^{-1} h^{-1}). This work also confirmed that the solute(s) of interest were taken up by polymers via *absorption*, not *adsorption*.

BZA and BA cell growth inhibition experiments showed that these compounds are toxic to cells and it was their accumulation rather than low enzyme levels or energy (ATP) depletion that caused a reduction in the biotransformation rate. For this reason, the final strategy employed

to enhance the bioproduction of benzaldehyde involved *in situ* product removal by pervaporation using polymer (Hytrel 3078) fabricated into tubing by DuPont, Canada. This aspect was initiated by first characterizing the custom-fabricated tubing in terms BZA and BA fluxes. The tubing was then integrated into an *in situ* pervaporation biotransformation and was shown to be effective at continuous product separation, using 87.4% less polymer by mass in comparison to polymer beads in conventional TPPB operation, and improved overall volumetric productivity by 214% (245.9 mg L^{-1} h^{-1} vs. 115.0 mg L^{-1} h^{-1}) over previous work producing BZA.

Co-Authorship

Chapters 3 and 4 have been accepted or submitted to refereed journals and were co-authored with Dr. Andrew J. Daugulis, who provided editorial and technical advice.

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"For wisdom *is* a defence, *and* money *is* a defence: but the excellency of knowledge *is*, *that* wisdom giveth life to them that have it."

Ecclesiastes 7:12

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Chapter 1

Introduction

1.1 Background

The global production of neutraceutical compounds represents a $151 billion (USD) industry, and includes food and beverage additives such as flavours and fragrances (BCC Research, 2011). Benzaldehyde (BZA), a naturally occurring compound with a bitter almond aroma, is the second most abundantly consumed compound in the flavour and fragrance industry (Gabelman, 1994). *Nature-identical* BZA is commercially produced by chemical synthesis via toluene oxidation; however, biologically derived BZA commands a premium price for its high flavour quality and it is consumer opinion that naturally made neutraceuticals are healthier than chemically processed varieties (Krings and Berger, 1998; Lomascolo *et al.*, 1999). Natural methods of BZA production by plant extraction via steam distillation of fruit pits have a number of disadvantages including the generation of toxic by-products, extensive purification, and dependence on agricultural production (Femenia *et al.*, 1995). Biosynthesis of BZA using isolated enzymes or microorganisms as biocatalysts have shown to be an effective strategy for producing natural BZA (Casey and Dobb, 1992; Duff and Murray, 1989; Kawakami and Nakahara, 1994) and provide an opportunity for industrial developments.

A common limitation in biocatalyst product synthesis is low titers (Straathof *et al.*, 2002), which has been attributed to substrate and product inhibition on the biological system. The accumulation of BZA in the aqueous solution is known to be toxic to whole cells and enzyme systems (Duff and Murray, 1989; Kawakami and Nakahara, 1994; Li and Kubo, 2004), which has been observed by reduced BZA production rate. Furthermore, the production of BZA is also constrained by its low solubility in water (6.55 g L^{-1} at 25 ^{o}C), which also contributes to low

titers. Specially designed bioreactors have shown to significantly reduce substrate and product inhibition resulting in enhanced titers (Gao and Daugulis, 2009; Jain *et al.*, 2010).

The use of two-phase partitioning bioreactors (TPPBs) has been shown to have a positive effect on bioproduction processes (Gao and Daugulis, 2009; Jain *et al.*, 2010; Prpich and Daugulis, 2007). TPPBs are composed of an aqueous phase containing the biocatalyst and an immiscible second phase that sequesters the product based on its partition coefficient (PC), which alleviates product accumulation in the aqueous phase. TPPBs employing biocompatible and non-bioavailable (non-toxic) commodity polymers as the immiscible phase have had several advantages over two-liquid phase systems sequestering BZA: observed higher PC, non-toxicity to cells, ease in handling, and elimination of fugitive aromas (Jain *et al.*, 2010). In the only previous work using polymer-liquid TPPBs, Jain *et al.* (2010) applied a polymer selection approach that chose polymers based on high PC for the substrate and product, which may not be the most effective strategy of polymer selection as it restricts high concentrations of substrate in the polymer phase, and complicates substrate and product purification.

Although TPPBs have been shown to improve BZA volumetric productivity by sequestering BZA in the polymer phase, the constrained volume in the bioreactor limits the total product removal; that is, there is only so much polymer that can be added to a given bioreactor. Methods for continuous product removal by pervaporation have been shown to be very effective for the separation of volatile components from fermentation broths (Baudot and Marin, 1996; Groot *et al.*, 1984). Previous literature on the selection of polymer membrane for pervaporation has been based on using commercially available membranes, and provides an opportunity for selection strategies using solute-polymer affinity, determined by PC, novel to TPPB polymer based selection methodology.

1.2 Objectives

This research was focused on developing a polymer selection strategy for reduced BZA exposure in the aqueous phase using a solid-liquid TPPB system and enhancing system performance. The first objective was to demonstrate (remarkably for the first time since many articles have been published on polymer-based TPPBs) that amorphous polymers sorb solutes based on *absorption*, not *adsorption*. Next, focusing on the biocatalyst, the yeast *Pichia pastoris* the work attempted to determine the degree of substrate and product inhibition during the conversion of benzyl alcohol to benzaldehyde. This was followed by TPPB polymer characterization and selection. The final part of this work examined, for the first time, continuous product removal by integrated pervaporation using tubing fabricated from selected Hytrel polymers.

1.3 References

Baudot A, Marin M. 1996. Dairy aroma compounds recovery by pervaporation. J. Membr. Sci. 120:207–220.

BCC Research. 2011. Global nutraceuticals market to grow to $207 billion by 2016. http://bccresearch.wordpress.com/tag/nutraceuticals/.

Casey J, Dobb R. 1992. Microbial routes to aromatic aldehydes. Enzyme Microb. Technol. 14:739–747.

Duff SJB, Murray WD. 1989. Oxidation of benzyl alcohol by whole cells of *Pichia pastoris* and by alcohol oxidase in aqueous and nonaqueous reaction media. Biotechnol. Bioeng. 34:153–159.

Femenia A, Rossell C, Mulet A, Caiiellas J. 1995. Chemical composition of bitter and sweet apricot kernels. J. Agric. Food Chem. 46:356–361.

Gabelman A. 1994. Bioprocesses Production of Flavor, Fragrance, and Color Ingredients. New York: John Wiley & Sons, Inc.

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. 104:332–9..

Groot WJ, Van den Oever CE, Kossen NWF. 1984. Pervaporation for similtaneous product recovery in the butanol/isopropanol batch fermentation. Biotechnol. Lett. 6:709–714.

Jain AN, Khan TR, Daugulis AJ. 2010. Bioproduction of benzaldehyde in a solid-liquid two-phase partitioning bioreactor using *Pichia pastoris*. Biotechnol. Lett. 32:1649–54.

Kawakami K, Nakahara T. 1994. Importance of solute partitioning in biphasic oxidation of benzyl alcohol by free and immobilized whole cells of *Pichia pastoris*. Biotechnol. Bioeng. 43:918–24.

Krings U, Berger RG. 1998. Biotechnological production of flavours and fragrances. Appl. Microb. Biotechnol. 49:1–8.

Li W, Kubo I. 2004. QSAR and kinetics of the inhibition of benzaldehyde derivatives against *Sacrophaga neobelliaria* phenoloxidase. Bioorg. Med. Chem.12:701–713.

Lomascolo A, Stentelaire C, Asther M, Lesage-meessen L. 1999. Basidiomycetes as new biotechnological tools to generate natural aromatic flavours for the food industry. TIBTECH 17:282–289.

Prpich GP, Daugulis AJ. 2007. Solvent selection for enhanced bioproduction of 3-methylcatechol in a Two-Phase Partitioning Bioreactor. Biotechnol. Bioeng. 97:536–543.

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

Chapter 2

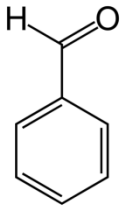
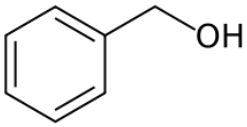
Literature Review

2.1 Introduction

In the US$22 billion Flavour and Fragrance industry, benzaldehyde (BZA), with its bitter-almond aroma, is the second most valuable flavour molecule next to vanillin. Annual production of synthetic and natural BZA is 7000 tons and 100 tons respectively, and the demand for the natural variety has been increasing at a rate of 5% each year (Lomascolo *et al.*, 1999). Natural flavour molecules command a premium price due to flavour potency and consumer opinion that natural chemicals are healthier than synthetic ones (Krings and Berger, 1998).

The cost premium for natural BZA over the synthetized version is roughly 120-460 fold, with a spread in range based on purity and method of production (Dubal *et al.*, 2008; Feron *et al.*, 1996; Krings and Berger, 1998; Lomascolo *et al.*, 1999). Approximately 80 tons/year of natural BZA is produced using natural cinnamaldehyde derived from cassia oil in a retro-aldol reaction (Krings and Berger, 1998). However, there has been controversy as to whether this product can be considered "natural" by the U.S. Food and Drug Administration (FDA) since an acid or base catalyst is required for chemical hydrolysis in the process (Krings and Berger, 1998; Lomascolo *et al.*, 1999). The European Commission legislation established that the BZA produced through this process method must be labeled synthetic (Brenna *et al.* 2009). The other 20 tons/year of natural BZA is produced by steam distillation from kernels of apricots, peaches, cherries, plums, prunes and almonds. Although this process method produces BZA superior in flavour, it forms toxic side products, such as hydrocyanic acid and hydrogen cyanide (Lomascolo *et al.*, 1999). Thus suppliers are in search of alternative routes for production of natural BZA. Table 2-1 shows the structure, and chemical and physical properties for BZA.

Table 2-1: Chemical and physical properties of benzaldehyde and benzyl alcohol (ChemSpider, 2013).

Properties	Benzaldehyde	Benzyl Alcohol
Chemical Structure		
Molecular Weight g mol^{-1}	106.12	108.14
Solubility in water (25^{o}C) g L^{-1}	6.5	42.9
Specific Gravity (25^{o}C)	1.046	1.045
Boiling point ^{o}C	179	205
Vapour pressure (25 ^{o}C) mm Hg	1.01	0.053
Volatilization from water (Henry Law constant) atm m^{-3} mol^{-1}	2.67E-5	3.37E-7
LogP	1.48	1.10

Applications of "White Biotechnologies" have grown in recent years and they are seen to have potential for the synthesis of natural molecules such as BZA. Microbial fermentations in food production have been used since the development of beer, wine, and cheese, and are generally recognized as safe (GRAS) for food (Dubal *et al.*, 2008). The European and US food legislation has classified microbial processes that mimic plant secondary metabolism as a "natural" means for flavour production (Krings *et al.*, 1995; Krings and Berger, 1998; Lomascolo *et al.*, 1999). Applying biotechnological routes for flavour production also provides several advantages: 1) there is a reduced dependence on agricultural production and the effects arising from external conditions that may limit harvest production, such as climate and pesticides; 2) there is flexibility for scale-up operations and reduced restriction to the type of land usage; 3) there is no need to exploit natural resources in developing countries. Furthermore, a biotechnological approach for production of BZA is not limited to a single method of synthesis. Studies synthesizing BZA show numerous species of microbes that can be employed by *de novo*,

bioconversion, biotransformation, and single-step enzymatic methods (Dubal *et al.*, 2008; Krings and Berger, 1998; Lomascolo *et al.*, 1999; Murray and Duff, 1990; Welsh *et al.*, 1989).

2.2 Authentication of Naturally Synthesized Bitter Almond Benzaldehyde

To avoid controversy of origin from products labeled as '*natural*' almond flavour, a study by Debon *et al.* (1997) employed a method using SNIF-NMR on BZA from various sources as a molecular probe. The study was able to determine the origin of the BZA molecule by site specific deuterium isotopes with clearly distinguished groups containing no overlap of isotopic distribution. The SNIF-NMR measurements were capable of differentiating between synthetic or *nature-identical* BZA from toluene and benzal chloride oxidation (Figure 2-1), natural BZA from kernels of apricots, peaches and cherries, and semisynthetic BZA from cinnamaldehyde extract. To determine the detection threshold of the measurements, samples were made containing natural BZA (from kernels of apricots, peaches, and cherries) with synthetic forms of BZA from oxidation of toluene and oxidation of benzal chloride (Debon *et al.*, 1997). The method proved to be effective by detecting synthetic BZA added to the natural BZA at about 10% adulteration (Debon *et al.*, 1997). In another study using $^{2}H/^{1}H$ ratio by pyrolysis isotope ratio mass spectrometry was able to detect the origin of BZA from a variety of sources (Ruff *et al.*, 2000). Commercially available BZA, bitter almond oils, different fruit pulp, kernels, and leaves where all tested and the $\delta^{2}H_{SMOW}$ values show significant differences between the different varieties, most importantly between *nature-identical* and biologically derived BZA. This ability to authenticate a natural BZA from a synthetic one only provides additional value in the marketing of natural BZA. Process methods employing a biological derivate can be verified and would command a premium price.

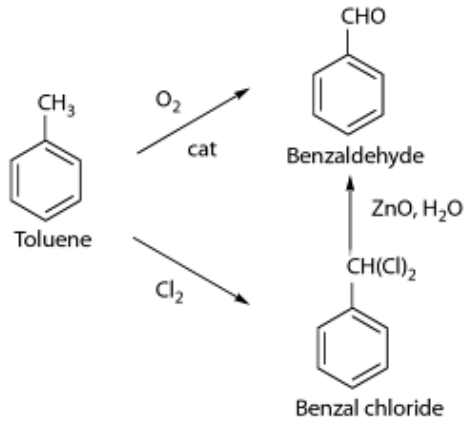


Figure 2-1: Common chemical synthesis of benzaldehyde derived from toluene as the raw material (Brenna *et al.*, 2006).

2.3 Biosynthesis of Benzaldehyde

Currently, there are two biotechnological methods for synthesis of natural BZA: the use of isolated enzymes and the use of microorganisms (which include *de novo* synthesis, bioconversion and biotransformation methods). Isolated enzyme conversion can be applied in situations when a specific enzyme, derived from a cell, is used to perform a single-step conversion for the modification of a precursor molecule. Microorganisms can also be used to produce BZA *de novo* (fermentation starting with growth substrate), by bioconversion (sequential reactions) and by biotransformation (single-step reaction). Studies employing *de novo* synthesis use the primary metabolic pathway in microbial cells to break down substrate for energy, producing only trace amounts of secondary metabolites such as BZA that are not involved in ATP generation in the cell. Microorganisms are able to make specific modifications to precursor molecules by enzymes used in the metabolic pathway *via* bioconversion, a series of enzymatic reactions, or biotransformation, a single-step enzymatic reaction. The following approaches are the most common strategies for natural synthesis of BZA.

2.3.1 Isolated Enzyme Synthesis

Purified enzyme methods are ideally applied in the conversion of a precursor molecule when a single-step reaction can be performed. A number of enzymes are capable of synthesizing BZA such as BZA oxidase (Casey and Dobb, 1992), aminotransferase (Groot and De Bont, 1998), benzoylformate decarboxylase (Park and Jung, 2002), alcohol dehydrogenases (Fabian *et al.*, 2009) and alcohol oxidases (Fabian *et al.*, 2009; Mitarai and Kawakami, 1994; Murray *et al.*, 1989a). Among the available enzymes that can be used to produce BZA, alcohol oxidase isolated from *Pichia pastoris* has reportedly been shown to be the most effective at producing high concentrations of BZA due to its non-specific active site to oxidize C_1-C_6 chained primary alcohols to their corresponding aldehyde forms and its stability at a wide range of operating conditions (Duff and Murray, 1989; Mitarai and Kawakami, 1994). Figure 2-2 shows the enzymatic reaction for the conversion of benzyl alcohol (BA) to BZA with the aid of FADH cofactor and catalase for the oxidation of peroxide to form water and oxygen. One of the advantages of using enzyme synthesis is the ability to more broadly modify operating conditions (temperature, pH and pressure) with respect to whole cell operations for optimal enzyme conversion rates. However, enzymatic processes are typically slow reactions that lose enzyme stability and specific activity with time (Murray *et al.*, 1989a). In addition, the use of cofactors and isolated enzymes is expensive, which may not be practical for industrial applications (Schreier, 1997).

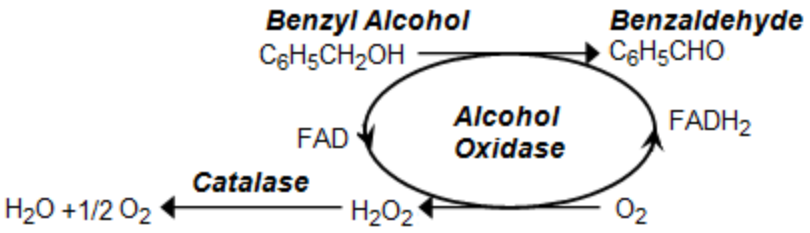


Figure 2-2: Enzymatic benzyl alcohol oxidation by alcohol oxidase coupled with catalase and cofactor FAD, producing H2O and benzaldehyde.

2.3.2 *De novo* Synthesis

Numerous microorganisms exist for *de novo* synthesis of BZA. Most studies employing *de novo* synthesis produced BZA at concentrations below 1 mg L^{-1} due to BZA's toxic properties to cells at very low concentrations. An exception was found using *Ischnoderma benzoinum*, which was capable of synthesizing 6-50 mg L^{-1} of BZA (Krings *et al.*, 1995; Lomascolo *et al.*, 1999). Table 2-2 shows a list of microorganisms used for *de novo* BZA production and their respective titers. The problem with *de novo* synthesis is the low product titer because BZA is produced as a secondary metabolite expending cellular ATP without contributing to generate energy as well as cell inhibition by BZA. Furthermore, previous literature on *de novo* biosynthesis lacks understanding about the mechanisms that control the generation of BZA, this adds to the difficulty of manipulating operating variables for improving titers. As such, *de novo* synthesis does not currently provide a viable method for commercial implementation.

Table 2-2: Studies using de novo synthesis and respective benzaldehyde titer produced in aqueous solution.

Species	Benzaldehyde Titer	Reference
Agaricus bisporus *Agaricus subreferens*	10-100µg L^{-1}	(Chen, C. C. & Wu, 1984)
Armillaria mellea *Armillaria ostoyae*	10µg L^{-1}	(Abraham and Berger, 1994; Gallois *et al.*, 1990)
Dichomitus sualens	250-1000µg L^{-1}	(Gallois *et al.*, 1990)
Ganoderma applanatum	10-100µg L^{-1}	(Abraham and Berger, 1994)
Ischnoderma benzoinum	6-50mg L^{-1}	2.3.2.1.1 (Krings *et al.*, 1995; Lomascolo *et al.*, 1999)
Hericium erinaceus	100-1000µg L^{-1}	(Abraham and Berger, 1994)
Hischioporus pergamenus	50µg L^{-1}	(Gallois *et al.*, 1990)
Kuehneromyces mutabilis	10-100µg L^{-1}	(Abraham and Berger, 1994)
Mycena pura	10-100µg L^{-1}	(Abraham and Berger, 1994)
Pleurotus sapidus	10-100µg L^{-1}	(Abraham and Berger, 1994)
Polyporus sp. *Polyporus tuberaster*	10-100µg L^{-1}	(Abraham and Berger, 1994)
Poria subacida	250-1000µg L^{-1}	(Gallois *et al.*, 1990)
Trametes suaveolens	10-100µg L^{-1}	(Abraham and Berger, 1994)
Tyromyces sambuceus	10-100µg L^{-1}	(Abraham and Berger, 1994)
Sarcodontia setosa	100-1000µ L^{-1}	(Abraham and Berger, 1994)

2.3.3 Bioconversion and Biotransformation Synthesis

Bioconversion and biotransformation approaches are perhaps the most developed methods for the synthesis of BZA. A possible reason for this could be that a bioconversion and biotransformation process uses a precursor molecule, usually cheap and abundant, designed for direct modification of a selected functional group without requiring a system of reaction steps by a metabolic pathway as in *de novo* synthesis. Due to the nature of bioconversion (several-reaction-step conversion) and biotransformation (single-reaction-step conversion) synthesis, it is easier to manipulate operating parameters, such as substrate concentrations, and measure their effects on product yield compared to *de novo* synthesis, which is controlled by a complex reaction system.

A number of studies have shown that L-phenylalanine can be used as a precursor molecule for the bioproduction of BZA in cultures of *Ischnoderma benzoinu, Dichomitus sualen, Polyporus tuberaster, Poria xanthan, Trametes suaveolens, Lactobacillus plantarum* and *Bjerkandera adusta* (Groot and De Bont, 1998; Kawabe and Morita, 1994; Lapadatescu *et al.*, 1997; Lomascolo *et al.*, 2001). In this case, BZA is formed as an intermediate in the decarboxylation of the L-phenylalanine to form 3-phenylpropanol and benzyl alcohol. Table 2-3 shows some microorganism capable of converting L-phenylalanine into BZA and their respective product titers. The final concentrations produced by these microorganisms using L-phenylalanine as the precursor molecule are an improvement compared to *de novo* synthesis; however, the yields are still too low to be of commercialization interest, partially due to reaction rate inhibition by BZA. In addition, the by-products accumulated would require additional separation techniques to isolate BZA.

Table 2-3: Microorganisms used for bioconversion of L-phenylalanine to benzaldehyde.

Species	Benzaldehyde Titer (mg L^{-1})	Reference
Ischnoderma benzoinu	25[a]	(Lapadatescu *et al.*, 1997)
Dichomitus sualen	5[a]	(Lapadatescu *et al.*, 1997)
Bjerkandera adusta	71 and 587[a]	(Lapadatescu *et al.*, 1997)
Trametes suaveolens	33 and 710[b]	(Lomascolo *et al.*, 2001)
Polyporus tuberaster K2606	837	(Kawabe and Morita, 1994)
Lactobacillus plantarum	34	(Groot and De Bont, 1998)
Rhizopus oligosporus	23	(Norliza, A. W. and Ibrahim, 2005)

[a] **Immobilized cells**
[b] **Addition of HP20 resin (styrene divinylbenzene copolymer)**

The low BZA titer using bioconversion was significantly improved when a biotransformation method was applied. A study by Park and Jung (2002) used whole cells of *Pseudomonas putida* to convert benzoylformate to BZA by using the intracellular enzyme benzoylformate decarboxylase. By incorporating encapsulated whole cells in calcium alginate they were able to produce 0.32g L^{-1} BZA in a 4mL reaction mixture. Other studies employing the

methylotrophic yeast, *Pichia pastoris*, containing alcohol oxidase (AOX) have shown to be more effective in the biotransformation of benzyl alcohol (BA) to BZA, reaching titers close to 5 g L^{-1} in a liquid-liquid phase system and a solid-liquid phase bioreactor system, the highest reported in the current literature (Duff and Murray, 1989; Jain *et al.*, 2010).

The synthesis of AOX enzymes in methylotrophic yeast is of interest for the oxidation of primary alcohols. AOX1 and AOX2 are the two genes in methylotrophic yeasts such as *P. pastoris* that code for the expression of AOX found inside the peroxisome organelle. AOX1 is a tightly regulated gene that has much greater influence on alcohol oxidase activity in the cell than AOX2, and is induced by methanol. Of the methylotrophic yeast, *Pichia naganishii*, *Pichia angusta*, *Hansenula polymorpha*, and *Candida boidinii*, and *P. pastoris*, it was found that *P. pastoris* had the highest alcohol oxidase activity, which contributes to its fast consumption of methanol substrate (Murray *et al.*, 1990). In the first step of the methanol metabolic pathway methanol is oxidized to formaldehyde producing hydrogen peroxide as a by-product as shown in Figure 2-3 (Duff and Murray, 1989). To avoid hydrogen peroxide inhibition, this reaction step occurs in the peroxisome isolating hydrogen peroxide accumulation from the rest of the cell. Due to the low substrate specificity of AOX, primary alcohols of C_2 to C_6 can also be oxidized to their representative aldehyde form in a single-step biotransformation. Table 2-4 shows a list of primary alcohols used for biotransformation to aldehydes by *P. pastoris* in organic-aqueous system composed of 5g L^{-1} cells. Some of the biotransformations have oxidized 100% of the precursor molecule to the respective product molecule. Importantly, BA can undergo a biotransformation to high quality BZA as previously shown in Figure 2-2. The very nature of *Pichia pastoris*, its versatility to harsh operating conditions and ability for high cell densities, as well as the AOX low substrate specificity when activated, make this approach an attractive means for generation of BZA. This provides us with an opportunity for a fermentation route to a premium BZA product.

Table 2-4: Primary alcohols oxidized by *Pichia pastoris* and respective yields in 12 h batch period (Murray *et al.*, 1989b).

Substrate	Product	Initial Substrate ($g\ L^{-1}$)	Final Product ($g\ L^{-1}$)
Propanol	Propionaldehyde	50	25.4
Butanol	Butyraldehyde	50	25.1
Isobutanol	Isobutyraldehyde	50	16.5
Pentanol	Valeraldehyde	20	18.7
Isoamyl alcohol	Isovaleraldehyde	20	6.2
2-Methyl butanol	2-methyl butyraldehyde	20	6.7
Hexanol	Hexanal	5	5.0
Benzyl alcohol	Benzaldehyde	20	5.9

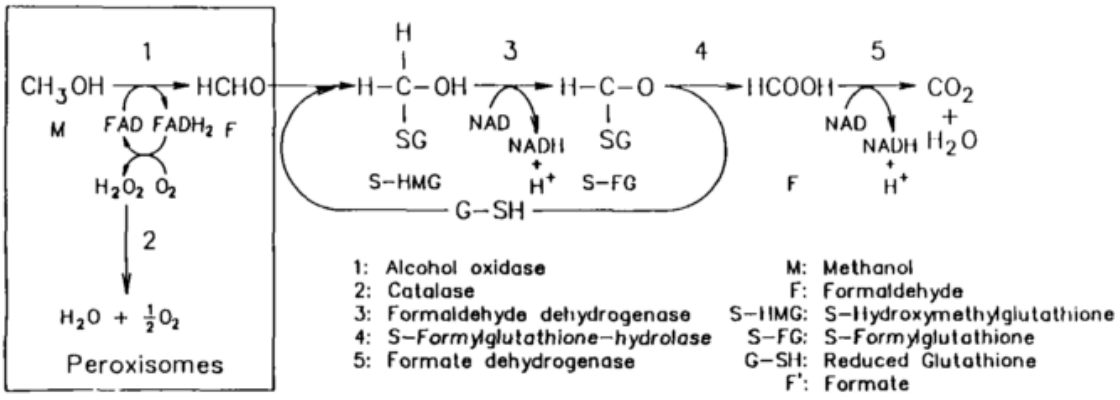


Figure 2-3: Metabolic pathway for the dissimilation of methanol (Duff and Murray, 1989).

There are many approaches for the biosynthesis of high quality BZA; however, only a select number of these approaches have the potential to be implemented as a 'white biotechnology'. Biotransformation using *P. pastoris* to convert BA, a cheap and readily available precursor molecule, to BZA is perhaps the most advanced approach for natural production of premium BZA. Before biotransformation of BA by *P. pastoris* becomes a viable approach for industrial production of natural BZA, successful studies need to produce BZA that is comparable to industrial yields. In addition, a greater understanding of BA and BZA effects on cell inhibition with regard to enzyme and microbial kinetics, as well as AOX activity needs to be developed.

Additionally, as *P. pastoris* derives no energy from BA oxidation, it is important to determine if the *P. pastoris* cells are running out of ATP and essential cofactors during prolonged biotransformations. The low solubility of BZA in water at room temperature is also a concern, and many of the previous biotransformation approaches have been limited by low BZA concentrations.

2.4 Microorganism: *Pichia pastoris* ATCC 28485

Pichia pastoris is commonly used in the expression of heterologous proteins. Due to the regulation of methanol induction for alcohol oxidase, it can also be used as a biocatalyst for primary alcohol oxidation as mentioned previously (Jain *et al.* 2010; Mitarai & Koei Kawakami 1994; Kawakami & Nakahara 1994; Duff & Murray 1989). Although the purpose of expressing protein by *P. pastoris* may be different from that of biotransformation, the literature for operating conditions of *P. pastoris*, such as temperature, pH, and dissolved oxygen (DO), still applies for the cultivation process.

2.4.1 Oxygen Consumption during Biotransformation

The metabolic demand for oxygen by *P. pastoris* varies depending on the substrate. In a fermentation, oxygen consumption during each phase (growth, induction, and biotransformation), and can be monitored using dissolved oxygen (DO), an indicator of when cells are starved of methanol and prompting the start of the biotransformation (Jain *et al.* 2010). If the cells are oxygen limited, the fermentation could take long periods and it is possible that the biotransformation rates could be negatively affected. Previous studies employing *P. pastoris* as a means for biotransformation maintained a constant aeration intensity for both the growth and biotransformation phase (Duff and Murray, 1989; Jain *et al.*, 2010). In the study by Jain *et al.* (2010), it was noted that the final BZA product was partially evaporated from the aqueous phase, which was a contribution of mixing speed and aeration. Subsequently, the importance of high

oxygen demand during the growth phase on methanol is an important consideration and potentially can be reduced during biotransformation to decrease evaporative losses of BZA.

2.4.2 Growth and Induction Phase pH Shift to Biotransformation Phase

The pH for growth of *P. pastoris* can range from 3 to 7, with the optimum pH for growth depending on the strain (Cos *et al.*, 2006; Potvin *et al.*, 2012). The biotransformation pH for BA to BZA in *P. pastoris* has been done at 7.3 (Jain *et al.* 2010). This provides an opportunity to strategically choose distinct pH values for the growth and induction phases that would maximize cell growth, followed by enhanced biotransformation.

2.4.3 Feeding Strategies

Feeding strategies are important in biotransformations producing high cell densities, providing a sufficient supply of ATP and cofactors for high AOX levels, and maintaining a high AOX activity. A number of different feeding strategies exist for the growth of *P pastoris*. The three main operations for feeding *P. pastoris* employ batch, mixed substrate fed-batch and continuous fermentations. Fermentations using *P. pastoris* for heterologous protein expression usually operate by fed-batch and continuous methods for the purpose of optimizing specific growth rate in order to achieve longer fermentation periods for maximum protein productivity. Fermentations using *P. pastoris* for biotransformation have been limited to only batch operation due to the inability of the cells to grow and perform the biotransformation of BA to BZA continuously because the biotransformation is done at higher pH levels than the cells could withstand. Continuously operating fermentations for the biotransformation of *P. pastoris* could have advantages over batch fermentations such as longer biotransformation periods, which could provide higher BZA titers. By determining whether a substrate can provide cell growth and energy at or near the biotransformation pH could potentially allow a continuous or fed-batch feeding strategy to be employed, which could provide ATP and cofactors during biotransformation.

A study done by Duff & Murray *et al.* (1988) performed a comparison between the three types of fermentations using methanol as the sole carbon source and on specific AOX activity. From the batch trials they found that methanol can be fully metabolized only up to concentrations of 20 g L^{-1} producing biomass concentrations below 2.5 g L^{-1}. The biomass yield coefficient $Y_{x/s}$ reached its maximum at 0.18 g biomass/g methanol when 10 g L^{-1} methanol was used and decreased as the initial methanol concentration was increased (Duff and Murray, 1988). Similarly, the specific AOX activity was also observed to decrease with increased methanol concentrations from 10 g L^{-1}. In the fed-batch and continuous fermentation greater biomass concentrations were achieved. After 5 cycles of intermittent methanol addition the overall biomass productivity was increased by 3.5 fold compared to the batch fermentation. The advantage to using fed-batch and continuous fermentations feeding strategy is that high cell densities can be achieved leading to a high production rate of protein (Cos *et al.*, 2006; d'Anjou and Daugulis, 2001; Dietzsch *et al.*, 2011; Potvin *et al.*, 2010). Another way to overcome low biomass concentrations is to introduce a second substrate which has a greater biomass yield coefficient than methanol (Dietzsch *et al.*, 2011; Valentini and Gattás, 2011). Therefore, in order to obtain a sufficiently high cell density using *P. pastoris* a substrate with a high growth coefficient should be used before methanol induction of AOX.

The biomass of *P. pastoris* must be increased to generate high cell density cultures, which is difficult by using methanol substrate alone (Cos *et al.*, 2006). The use of a mixed feeding strategy was found to increase the biomass concentration and energy supply for cells, thereby reducing methanol induction time and increasing volumetric productivity in protein expression (Potvin *et al.* 2010; Dietzsch *et al.* 2011; Duff *et al.* 1989; Jahic *et al.* 2002; Z. Wang *et al.* 2010; Zhu *et al.* 2011b; d' Anjou & Daugulis 2001). Glycerol has been the substrate of choice for achieving high cell densities before the methanol induction phase; however, excess glycerol is known to repress the AOX1 promoter stopping methanol metabolism. A study by

Thorpe *et al.* (1999) used a glycerol-methanol mixed substrate feed strategy and found that the ratio of the substrates was important for the protein production rate. Another study that used a mixture of equal amounts of glycerol-methanol feed (one bolus addition of both substrates) found that there was a reduction in time needed for induction of AOX compared to the transition from glycerol to methanol as sole carbon sources (Jungo *et al.*, 2007). It is believed that a feed of mixed glycerol-methanol substrate shortens the time cells need to adapt during the transition of substrate and reduces transient methanol accumulation, which can overload methanol dissimilation pathway by exerting a toxic accumulation of formaldehyde (Jungo *et al.*, 2007). Furthermore, it was found that the AOX1 gene may not be derepressed in any carbon source, but results show that non-limiting glycerol and carbon starvation cause some derepression of the AOX1 promoter (Tschopp *et al.*, 1987). By using a mixed substrate feeding strategy of glycerol and methanol it could potentially serve two purposes to improve the fermentation process using *P. pastoris*: high cell density on glycerol and strong induction of AOX1 on methanol.

Another substrate that can be used as a carbon source is sorbitol, which is non-repressive on the AOX1 promoter. A study by Thorpe *et al.* (1999) compared fed-batch growth of glycerol-methanol to sorbitol-methanol and found that though sorbitol had lower biomass yield, it produced higher specific product rates. Sreekrishna *et al.* (1997) used sorbitol-methanol mixed feed strategy in a batch reactor to preform several cycles of MMP-2 expression. The results showed a reduction in overall methanol required since sorbitol is also a growth substrate and when feeding on sorbitol it was found that methanol can be added at any time to instigate induction of AOX1 (Sreekrishna *et al.*, 1997). Furthermore, the addition of sorbitol as a co-feed during methanol induction increased average specific growth rate of *P pastoris* when compared to methanol induction alone (Zhu *et al.* 2011b; Z. Wang *et al.* 2010). This provides us with an opportunity to apply a strategy for the induction phase of the fermentation.

At the moment, the application of biotransformation systems in fed-batch and continuous fermentation for the production of BZA has not been reported in the literature. A possible reason may be due to lack of cell growth throughout the biotransformation as a result of high pH, mentioned previously. During the biotransformation process, cells are likely operating on reserve energy to oxidize the precursor molecule (BA). Hence, fermentation prior to the biotransformation should fuel the cells with energy, essential co-factors (FAD NADH) and provide a high specific AOX activity. However, it is also possible to consider the use of a fed-batch or continuous fermentation strategy if a non-repressing substrate, such as sorbitol, can be metabolized in unison with the biotransformation of BA. A greater understanding of how sorbitol will affect the biotransformation in *P. pastoris* is required before it can be added with BA substrate during the biotransformation.

2.4.4 Host Strains of *Pichia pastoris*

P. pastoris has three types of host strains that require much less methanol to induce expression of peroxisome, which have never reportedly been used in the biotransformation of BA to BZA. These strains are different in their ability to consume methanol due to alternations of the AOX1 and AOX2 genes. The wild type *P. pastoris* has the highest rate of metabolism for methanol containing both AOX1 and AOX2. The Methanol Utilization positive (Mut^+) phenotype holds both AOX1 and AOX2 genes and metabolizes methanol at the same rate as the wild type. Methanol Utilization slow (Mut^s) has AOX1 gene deleted and relays on the transcriptionally weaker AOX2 gene for AOX, which causes slower growth on methanol. The Methanol Utilization negative (Mut^-) phenotype has both AOX genes and cannot grow on methanol. Although studies on the biotransformation of BZA using the different host strains could potentially be useful, this work focused on “process” rather than microbiological issues, and as a consequence the wild strain of *P. pastoris* was used in this research.

2.4.5 Benzyl Alcohol and Benzaldehyde Effects on *P. pastoris*

As mentioned previously, studies using *P. pastoris* as a biocatalyst in the biotransformation of BA to BZA have experienced product and substrate inhibition (Jain *et al.* 2010; Mitarai & Koei Kawakami 1994; Murray & Duff 1990; Duff & Murray 1989; Koei Kawakami & Furukawa 1997). Biotechnological strategies have been proposed to reduce substrate inhibition (Dubal *et al.*, 2008). Duff and Murray (1989) and found that the biotransformation of BA by free whole cells in aqueous phase reaction followed Michaelis-Menten kinetics at BA concentrations of 20 g L^{-1} and lower. At BA concentrations greater than 20 g L^{-1} there was strong inhibition and decreased conversion rate of BA to BZA. Table 2-5 shows the kinetic parameters for the oxidation of BA by AOX in whole cells and enzymes. Furthermore, it was noted that complete product inhibition in free whole cells occurred at 3 g L^{-1} BZA (Duff and Murray, 1989). Previous studies employing the biotransformation of BA to BZA all claim that there is product inhibition; however, there is no record in the literature that shows to what degree product inhibition occurs. Therefore, a better understanding of BA and BZA effects on microbial and biotransformation kinetics needs to be undertaken.

Table 2-5: Kinetic parameters for oxidation of benzyl alcohol by alcohol oxidase enzyme in whole cells and as isolated enzymes.

Phase	K_s or K_m *(Apparent)*	V_{max} *(Apparent)*	Reference
Whole cells *P. pastoris*: Aqueous phase	20 g L^{-1}	3.62 g L^{-1} h^{-1}	(Duff and Murray, 1989)
Whole cells *P. pastoris*: Organic-aqueous phase (95% hexane)	7.41 g L^{-1}	5.26 g L^{-1} h^{-1}	(Duff and Murray, 1989)
Enzyme: AOX from *Candida sp.* in aqueous phase	7.2 g L^{-1}	3.48 g unit-s^{-1}	(Mitarai and Kawakami, 1994)
Enzyme: AOX from *Candida sp.* in Organic-aqueous phase (95% butyl acetate)	8.4 g L^{-1}	3.12 g unit-s^{-1}	(Mitarai and Kawakami, 1994)
Enzyme: AOX from *Candida sp.* in Organic-aqueous phase (95% Xylene)	7.3 g L^{-1}	3.43 g unit-s^{-1}	(Mitarai and Kawakami, 1994)
Enzyme: AOX from *Candida sp.* in Organic-aqueous phase (95% n-Decane)	7.4 g L^{-1}	3.16 g unit-s^{-1}	(Mitarai and Kawakami, 1994)

2.5 Detoxification using Tris Buffer

An extensive study was done by Murray & Duff (1989) with the use of Tris buffers to reduce product inhibition by chelating acetaldehyde and BZA. One study applying the biotransformation for acetaldehyde by free enzyme AOX compared the aqueous single phase reaction to the biotransformation in varying Tris buffer concentrations and found a 200% improvement (Duff *et al.*, 1989). The acetaldehyde forms a Schiff base with an amine group of the Tris buffer molecule making the acetaldehyde nonreactive. This method requires the use of an additional buffer to counteract for the pH reduction associated with the chelating step, which also maintains the pH of the bioreactor system (Murray *et al.*, 1989b). A similar study using free enzyme AOX for the biotransformation of BZA employed Tris buffer with an aqueous-organic system and found a 200% improvement in yield compared to the aqueous phase (Duff and Murray, 1989). The limitation of Tris buffer is that it preforms best at pH 8.1; however, the biotransformation of BA to BZA in whole cell *P. pastoris* occurs at pH 7.3, which is not ideal.

Furthermore, Tris buffers are expensive and may not be economical from an industrial process perspective, and for these reasons Tris buffers will not be considered in this thesis.

2.6 Detoxification by Immobilization of Cells in Organic Media

The immobilization of whole cells in organic media can be applied to fermentations to provide a potential means for continuous operation and to stabilize the biocatalyst. Kawakami & Nakahara (1994) examined the effectiveness of immobilized whole cell *P. pastoris* in a mixed matrix composed of silicone polymer and Ca alginate gel operating in an aqueous-organic system and compared it to free cell operation. It was observed that excess alginate immobilized cells exhibited lower production rate of BZA compared to the free cells and the excess silicone immobilized cells showed no significant titer improvements over free cells in the same organic media. The organic solvent acts as a reservoir to reduce the inhibitory effects and the use of immobilized cells reduces the exposure to the organic phase (Kawakami & Nakahara 1994). Other studies applying immobilization of whole cells that found minor improvements in product titer, but had major diffusion limitations, large biomass limitations for immobilization, and problems with the ability to scale up for industrial commercialization (Kawakami & Nakahara 1994; Lapadatecu *et al.* 1997; Kawakami & Furukawa 1996; Couderc & Barratti 1980; Park & Jung 2002). For these reasons the approach of immobilization of whole cell in organic media will not be considered for this thesis.

2.7 Detoxification using Liquid-Liquid Two-Phase Partition Bioreactors

The technology platform known as Two-Phase Partitioning Bioreactors (TPPBs) has seen a number of environmental and biotechnological applications, and is based on the sorption ability of a target molecule(s) by a second phase within the bioreactor itself. In a TPPB system, the cells are present in the aqueous phase which is mixed with an immiscible second phase (liquid or solid) that usually has a high affinity for hydrophobic molecules (Daugulis 2001). The hydrophobic

molecules in the system then partition between the aqueous and sequestering phase until a thermodynamic equilibrium is reached (Daugulis 2001).

The addition of a second phase in TPPBs can lead to a number of applications. In cases in which the goal is to increase biomass production, the second phase can act as a substrate reservoir for growth of microorganisms (Déziel *et al.*, 1999). More specific applications exist using the secondary phase as a sustained release reservoir for microbial degradation of toxic substrates (Daugulis *et al.*, 2011; Déziel *et al.*, 1999; Isaza *et al.*, 2010). Furthermore, the secondary phase can also be applied for removal of high value products synthesized by microbial biocatalysts in the aqueous phase (Déziel *et al.*, 1999).

Two-phase liquid-liquid reaction systems help to alleviate product inhibition by sequestering solute molecules for extraction from the aqueous into an immiscible organic phase. A number of studies have been done to optimize organic solvent selection for a aqueous-organic reactor system (Mitarai & Koei Kawakami 1994; Duff & Murray 1989; Kawakami & Nakahara 1994; Murray & Duff 1990; Duff *et al.*, 1989; Murray *et al.*, 1990). Murray & Duff (1990) have applied this strategy in a number of their studies to recover flavour molecules, acetaldehyde, BZA and hexanal at higher concentrations compared to operating in a single aqueous phase (Duff *et al.*, 1989; Duff and Murray, 1989; Murray and Duff, 1990). However, there are number of disadvantages and constraints to using aqueous-organic reactor systems: 1) There are limitations for the solvent that can be used for the system because of solvent toxicity to the cells and the potential to inhibit the biocatalyst; 2) There are concerns with the selection of a solvent for a flavour and fragrance in the final product. It is undesirable for the organic solvent to reside in the final product creating contamination; 3) There are concerns with the flavour and fragrance molecule exhibiting a high affinity for the organic solvent, which requires additional processing steps for the separation of the flavour and fragrance molecule from the organic solvent. This reduces the quality of the flavour and fragrance; 4) There are concerns with the ability to recover

the organic solvents for reuse because additional processing steps are required for flavour and fragrance recovery and organic solvents can be very expensive to use, especially in large volumes. Potential solutions to these disadvantages exist through the use of biocompatible solid-liquid two-phase partitioning systems.

2.8 Detoxification using Solid-liquid Two Phase Partitioning Bioreactors

An important feature of solid-liquid TPPBs is the ability to tailor polymer selection for individual or collective target molecule(s) sorption in the presence of cells in aqueous solution. As mentioned earlier, polymer selection for a TPPB system can be established using a variety of criteria, but the partition coefficient (PC), the mass or concentration ratio for the affinity of a target molecule to partition in the polymer phase over the aqueous phase, usually has the greatest impact for high value products, such as BZA (Jain *et al.*, 2010; Khan & Daugulis, 2010; Khan & Daugulis, 2011). Table 2-6 shows some experimental PCs for BZA and BA for a wide variety of polymers.

Table 2-6: Polymer properties and experimental partition coefficients for benzaldehyde and benzyl alcohol (Jain *et al.*, 2010; Khan & Daugulis, 2010).

Polymer	Type	Glass Transition Temperature (^{o}C)	Partition Coefficient for BA	Partition Coefficient for BZA	Partition Coefficient Ratio (BZA: BA)
Hytrel 8206	Poly(butylene terephthalate) and butylene ether glycol terephthalate block copolymer	-59	10.6	24.9	2.3
Hytrel G3548L		-45	12.1	39.6	3.3
Hytrel G4078W		-30	8.3 ± 0.6	35.0 ± 5	4.2
Zytel 42A	polyamide 66	70	0.3	0.6	2.0
Pebax 2533	Poly(amide-12-b-ethylene oxide)	-65	10.9	43.3	4.0
Elvax 40W	40% vinyl alcohol (copolymer with ethylene)	N/A	3.5	35.4	10.1
Elvax 3175	28% Vinyl acetate – ethylene copolymer	-38.6	3 ± 4	1.4 ± 0.2	0.5
Desmopan 453	Polyurethane thermoplastic elastomer	-34	5.8 ± 0.3	32.0 ± 9	5.5
Kraton SBR, D4150K	Styrene/butadiene linear triblock copolymer, 28% styrene	N/A	0.5	15.9	31.8
Nylon 6-6	Polyamide 66 (crystalline)	50	2.2 ± 0.3	2.0 ± 0.7	0.9

Jain et al (2010) conducted a study on the bioconversion of BZA by *P pastoris* comparing performance between fermentation experiments done in a single-phase aqueous reactor to solid-liquid TPPB. By using the polymer Elvax 40W as the solid phase it was possible to reduce production inhibition, resulting in a 300% improvement in the overall volumetric productivity of BZA generated compared to that of the aqueous single phase bioconversion. The polymer used in the TPPB system was chosen due to its high partition coefficient (PC) for BZA and its ability to deliver the precursor molecule BA at a constant concentration to overcome substrate inhibition. This method of applying inexpensive, biocompatible (non-toxic), readily

available commodity polymer has a number of advantages over traditional aqueous-organic liquid-liquid systems and will be an approach used to reduce production inhibition throughout this study.

Previous studies using solid polymer-aqueous TPPB systems to alleviate substrate and product inhibition in yeasts from aroma molecules have found large improvements compared to the aqueous single phase experiments. Work done by Gao & Daugulis (2009) used *Kluyveromyces marxianus* in the bioconversion of L-phenylalanine and glucose to 2-phenylethanol. Gao & Daugulis (2009) screened six polymers to find the highest PC for 2-phenylethanol. Hytrel® 8206 was used as the solid phase in the TPPB system for the bioconversion of L-phenylalanine and glucose to 2-phenylethanol. When a semi-continuous reactor configuration was applied there was a 1460% improvement in the overall volumetric productivity of 2-phenylethanol over the single aqueous phase bioconversion trial. A study by Khan & Daugulis (2010) used *Candida utilis* for the bioconversion of BZA and glucose to L-phenylacetylcarbinol. Khan & Daugulis (2010) tested ten polymers for their PCs to find one with the highest PC for L-phenylacetylcatbinol. Hytrel® G3548L at 9% volume partitioning phase was used as the solid polymer in the TPPB during the bioconversion of BZA and glucose to L-phenylacetylcarbinol. It was found that there was a 190% improvement in the product titer compared to the single aqueous phase experiment at the same working volume. Another study by Jain & Daugulis (2010) used *P. pastoris* for the biotransformation of BA to BZA. There was six polymers selected for PC testing, and the polymer rational for selection was high BZA PC and low BA PC to deliver as much substrate as possible to the aqueous phase. Elvax® 40W was used as the second phase in the TPPB system for the biotransformation of BA to BZA. Unlike the bioconversion of L-phenylalanine and glucose to 2-phenylethanol by *Kluyveromyces marxianus* and BZA and glucose to L-phenylacetylcarbinol by *Candida utilis*, the biotransformation of BA to BZA by *P. pastoris* consumes cofactors without regeneration, as shown in Figure 2-3.

2.9 Detoxification using External Pervaporation

The extraction of BZA during fermentation by pervaporation provides a method of continuous product extraction to combat product inhibition. A study by Lamer et al (1996) cultivated *Bjerkandera adusta* for the bioproduction of BZA using a pervaporation strategy showed improvements over single-phase runs. Pervaporation removes solute molecules from the aqueous phase based on their affinity for the membrane, and which diffuse into the polymer matrix, and then evaporate in a "sweep" gas phase. The two membranes studied by Lamer *et al.* (1996) were a GFT-LeCarbone Lorraine composite made of an active top layer comprised of polydimethylsiloxane (PDMS) on polyacrylonitrile and non-woven polyester and a homogeneous PDMS membrane. Lamer (1996) tested a number of physicochemical characteristics of the composite membrane with BZA and BA, such as diffusion, flux of pervaporation, selectivity, and solubility coefficient in PDMS. The study found that the homogenous membrane had higher fluxes of BZA at the same PDMS thickness as well as greater selectivity for BZA. Using the homogenous PDMS membrane in pervaporation, BZA at 132 mg L^{-1} was formed in the fermenter and 6200 mg L^{-1} was the highest concentration reached in the pervaporate, which had a concentration factor of 100 for BZA over aqueous phase operation. This strategy of employing pervaporation has shown to be effective for the extraction of BZA; however, there are still some limitations including the regulation between downstream pressures, which must be low for a high flux of the permeate, and the use of high fluid velocities to avoid biomass accumulating on surface and membrane fouling (Vane, 2005).

2.9.1 Detoxification using Integrated Pervaporation

Integrated pervaporation provides a method for the continuous removal of volatile products from fermentation cultures, and allows for the ability to maintain product concentrations below inhibitory levels, not feasible in batch or fed-batch operations (Schügerl, 2000). The use of pervaporation for product recovery also serves a number of advantages for product separation and

purification over more traditional methods such as distillation, which are energy intensive and expensive (Vane, 2005). Previous work on the removal of alcohols (ethanol and butanol) in fermentation media using integrated pervaporation has been shown to improve product recovery (Groot *et al.*, 1984; Mulder *et al.*, 1983; Nakao *et al.*, 1987; O'Brien and Craig Jr., 1996). A verity of material compositions in the form of flat membranes have been studied for recovery of alcohols including silicone rubber (Groot *et al.*, 1984; Mulder *et al.*, 1983), silicone composites (Strathmann and Gudernatsch, 1987), poly(vinyl alcohol) (Park *et al.*, 1994), poly(tetrafluoroethylene) (Calibo *et al.*, 1989), and chitosan-poly(acrylonitrile) (Watanabe and Kyo, 1992). Although, these membranes are selected based on affinities for the alcohol and trans-membrane diffusion rate, PC measurements for solute-polymer affinity have never been applied in the selection of pervaporate membranes. This provides us with an opportunity to examine various polymers containing high selectively for BZA and not for BA as a potential membrane for pervaporation.

The geometry of the pervaporation membrane is important for fermentations that take long periods and have high biomass concentrations. Flat membranes are vulnerable to fouling of biomass material on the membrane surface area (Sae-Khow and Mitra, 2010; Vane, 2005). A study using tubular shaped membrane for different membrane filtration processes has shown to reduce biomass fouling with high cell density, thereby improve polymer mass fluxes (Harscoat *et al.*, 1999). For this reason, the use of tubular membranes will be further explored. Although, a number of parameters influence the operation of integrated pervaporation, this work will focus on the selection of polymer material for high selectivity of BZA over BA and polymer processing ability for tubing extrusion. Subsequently, the polymer membrane selected will need to be characterized in terms of its BZA and BA flux before it can be evaluated for continuous product separation in pervaporation.

2.10 Scope of Thesis

The literature discussed in this review has highlighted the potential to enhance BZA biotransformation using *P. pastoris*. In this work, improvements in the overall fermentation process were achieved using a mixed substrate feeding strategy to reduce time during the growth phase. The mode of action (*absorption vs. adsorption*) of polymers in TPPBs was determined, and polymers were selected based on their impact on substrate and product inhibition (enzymatically and on cell growth) during biotransformation. The reason behind the inevitable decrease in biotransformation rate was also determined to be product inhibition, rather than a decreasing AOX activity or ATP levels. Finally, integrated *in situ* pervaporation operation using custom-fabricated tubing with a selected polymer was evaluated for its ability to continuously removal BZA from the aqueous phase, and its impact on overall product titer and volumetric productivity was determined.

2.11 References

Abraham BG, Berger RG. 1994. Higher Fungi for Generating Aroma Components through Novel Biotechnologies. J. Agric. Food Chem.:2344–2348.

Baudot A, Marin M. 1996. Dairy aroma compounds recovery by pervaporation. J. Membr. Sci. 120:207–220.

Brenna E, Fronza G, Fuganti C, Gatti G. F, Serra S. 2006. Chap. 3. Biotechnological tools to produce natural flavors and methods to authenticate their origin. In: . Innovation in Food Engineering: CRC Press, pp. 81–106.

Calibo RL, Matsumura M, Kataoka H. 1989. Continuous ethanol fermentation of concentrated sugar solutions coupled with membrane distillation using a PTFE module. J. Ferment. Bioeng. 67:40–45.

Casey J, Dobb R. 1992. Microbial routes to aromatic aldehydes. Enzyme Microb. Technol. 14:739–747.

ChemSpider. 2013. *Royal Society of Chemistry*. http://www.chemspider.com/.

Chen, C. C. & Wu CM. 1984. Studies on the Enzymic Reduction of 1-Octen-3-one in Mushroom (*Agaricus bisporus)*. J. Agric. Food Nutr. 32:1342–1344.

Cos O, Ramón R, Montesinos JL, Valero F. 2006. Operational strategies, monitoring and control of heterologous protein production in the methylotrophic yeast *Pichia pastoris* under different promoters: A review. Microb. Cell Fact. 5:17.

d'Anjou MC, Daugulis AJ. 2001. A rational approach to improving productivity in recombinant *Pichia pastoris* fermentation. Biotechnol. and Bioeng. 72:1–11.

Daugulis AJ. 2001. Two-phase partitioning bioreactors: a new technology platform for destroying xenobiotics. Trends Biotechnol. 19:457–62.

Daugulis AJ, Tomei MC, Guieysse B. 2011a. Overcoming substrate inhibition during biological treatment of monoaromatics: recent advances in bioprocess design. Appl. Microbio. and Biotechnol. 90:1589–608.

Daugulis AJ, Jain A, Khan TR, Dafoe JT, Craig TC. 2011b. Bioproduction of benzaldehyde in a solid-liquid two-phase partitioning bioreactor using *Pichia pastoris*. Chem. Eng. Transac. 24.

Debon AA, Martin GG, Remaud G, Martin Y, Martin G. 1997. Authentication of Bitter Almond Oil and Cinnamon Oil : Application of the SNIF-NMR Method to Benzaldehyde. J. Agric. Food Sci. 45:4042–4048.

Déziel E, Comeau Y, Villemur R. 1999. Two-liquid-phase bioreactors for enhanced degradation of hydrophobic/toxic compounds. Biodegrad. 10:219–33.

Dietzsch C, Spadiut O, Herwig C. 2011. A dynamic method based on the specific substrate uptake rate to set up a feeding strategy for *Pichia pastoris*. Microb. Cell Fact. 10:14.

Dubal SA, Tilkari YP, Momin SA, Borkar I V. 2008. Biotechnological routes in flavour industries. Advan. Biotechnol. :20–31.

Duff SJB, Murray WD. 1988. Production and Application of Methylotrophic Yeast. Biotechnol. Bioeng. 31:44–49.

Duff SJB, Murray WD. 1989. Oxidation of benzyl alcohol by whole cells of *Pichia pastoris* and by alcohol oxidase in aqueous and nonaqueous reaction media. Biotechnol. Bioeng. 34:153–159.

Duff SJB, Murray WD, Overend RP. 1989. Factors affecting the yeast-mediated conversion of ethanol to acetaldehyde in batch reactors. Enzyme Microb. Technol. 11:770-775.

Duff SJB, Murray WD, Overend RP. 1991. Oxygen and temperature effects on acetaldehyde-induced catabolite inactivation in *Pichia pastoris*. Appl. Microb. Biotechnol. 36:82–86.

Fabian WMF, Mautner B, Vries JG De, Orbegozo T, Kroutil W. 2009. Biocatalytic oxidation of benzyl alcohol to benzaldehyde via hydrogen transfer. Tetrahedron 65:6805–6809.

Feron G, Bonnarme P, Durand A. 1996. Prospects for the microbial production of food flavours. Trend. Food Sci. & Technol. 71:285–293.

Gallois A, Gross B, Langlois D, Spinnler H-E, Brunerie P. 1990. Influence of culture conditions on production of flavour compounds by 29 ligninolytic Basidiomycetes. Mycol. Research 94:494–504.

Groot MNN, De Bont JAM. 1998. Conversion of Phenylalanine to Benzaldehyde Initiated by an Aminotransferase in *Lactobacillus plantarum*. Appl. Environ. Microbiol. 64:3009–3013.

Groot WJ, Van den Oever CE, Kossen NWF. 1984. Pervaporation for similtaneous product recovery in the butanol/isopropanol batch fermentation. Biotechnol. Lett. 6:709–714.

Harscoat C, Jaffrin MY, Bouzerar R, Courtois J. 1999. Influence of fermentation conditions and microfiltration processes on membrane fouling during recovery of glucuronane polysaccharides from fermentation broths. Biotechnol. Bioeng. 65:500–11.

Isaza PA, Daugulis AJ, Karan K. 2010. Mass Transport and Thermodynamic Analysis of PAHs in Partitioning Systems in the Presence and Absence of Ultrasonication. AIChE J. 56:2717-2726.

Jahic M, Martinelle M, Hult K, Enfors S. 2002. Modeling of growth and energy metabolism of *Pichia pastoris* producing a fusion protein. Biosyst. Eng. 24:385–393.

Jain AN, Khan TR, Daugulis AJ. 2010. Bioproduction of benzaldehyde in a solid-liquid two-phase partitioning bioreactor using *Pichia pastoris*. Biotechnol. Lett. 32:1649–54.

Jungo C, Marison I, Stockar U Von. 2007. Regulation of alcohol oxidase of a recombinant *Pichia pastoris* Mut + strain in transient continuous cultures. J. Biotechnol. 130:236–246.

Kawabe T, Morita H. 1994. Production of benzaldehyde and benzyl alcohol by the mushroom *Polyporus tuberaster* K2606. J. Agric. Food Chem. 42:2556–2560.

Kawakami K, Nakahara T. 1994. Importance of solute partitioning in biphasic oxidation of benzyl alcohol by free and immobilized whole cells of *Pichia pastori*s. Biotechnol. Bioeng. 43:918–24.

Kawakami K, Furukawa S. 1997. Alcohol-Oxidation Activity of Whole Cells of *Pichia pastoris* Entrapped in Hybrid Gels Composed of Ca-Alginate and Organic Silicate. Appl. Biochem. Biotechnol. 67:27-31.

Khan TR, Daugulis AJ. 2010. Application of solid-liquid TPPBs to the production of L-phenylacetylcarbinol from benzaldehyde using *Candida utilis*. Biotechnol. Bioeng. 107:633–41.

Khan TR, Daugulis AJ. 2011a. The effects of polymer phase ratio and feeding strategy on solid-liquid TPPBs for the production of L-phenylacetylcarbinol from benzaldehyde using *Candida utilis*. Biotechnol. Lett. 33:63–70..

Khan TR, Daugulis AJ. 2011b. Medium composition effects on solute partitioning in solid-liquid two-phase bioreactors. J. Chem. Technol. & Biotechnol. 86:157–160.

Krings U, Abraham BG, Berger RG. 1995. Plant impact volatiles from higher fungi : a biotechnological perspective. Perfumer Flavorist 20:79–86..

Krings U, Berger RG. 1998. Biotechnological production of flavours and fragrances. Appl. Microb. Biotechnol. 49:1–8.

Lamer T, Spinnler HE, Souchon I, Voilley A. 1996. Extraction of benzaldehyde from fermentation broth by pervaporation. Process Biochem.31:533–542.

Lapadatescu C, Vergoignan C, Durand A. 1997. Influence of cell immobilization on the production of benzaldehyde and benzyl alcohol by the white-rot fungi *Bjerkandera adusta*, *Ischnoderma benzoinum* and *Dichomitus squalens*. Appl. Microbiol.:708–714.

Li S-Y, Srivastava R, Parnas RS. 2011. Study of in situ 1-butanol pervaporation from A-B-E fermentation using a PDMS composite membrane: validity of solution-diffusion model for pervaporative A-B-E fermentation. Biotechnol. Prog. 27:111–20.

Lomascolo A, Asther M, Navarro D, Antona C, Delattre M, Lesage-Meessen L. 2001. Shifting the biotransformation pathways of L-phenylalanine into benzaldehyde by *Trametes suaveolens* CBS 334.85 using HP20 resin. Lett. Appl. Microbiol. 32:262–7..

Lomascolo A, Stentelaire C, Asther M, Lesage-meessen L. 1999. Basidiomycetes as new biotechnological tools to generate natural aromatic flavours for the food industry. TIBTECH 17:282–289.

Mitarai H, Kawakami K. 1994. Kinetic analysis of benzyl alcohol oxidation by alcohol oxidase in aqueous—organic two-phase systems. J. Chem. Technol. Biotechnol. 60:375–380..

Mulder MHV, Hendrikman JO, Hegeman H, Smolders CA. 1983. The application of pervaporation in biotechnology. J. Membr. Sci. 16:269–284.

Murray WD, Duff SJB. 1990. Applied Microbiology Biotechnology Bio-oxidation of aliphatic and aromatic high molecular weight alcohols by *Pichia pastori*s alcohol oxidase. Biotechnol. Bioeng.:202–205.

Murray WD, Duff SJB, Lanthier PH. 1989a. Induction and stability of alcohol oxidase in the methylotrophic yeast *Pichia pastoris*. Appl. Microbiol. Biotechnol. 32:95–100.

Murray WD, Duff SJB, Lanthier PH. 1989b. Production of natural flavour aldehydes from natural source primary alcohols C2-C7. US 4,871,669.

Murray WD, Duff SJB, Lantl PH. 1990. Biological Upgrading of Dilute Ethanol Streams to Acetaldehyde for Low-Temperature Recovery. Science 23:229–239.

Nakao S, Saitoh F, Asakura T, Kiyoshi T, Kimura S. 1987. Continuous ethanol extraction from a membrane bioreactor by pervaporation. J. Membr. Sci. 30:273–287.

Norliza, A. W. and Ibrahim CO. 2005. The production of Benzaldehyde by *Rhizopus oligosporus* USM R1 in a Solid State Fermentation (SSF) System of Soy Bean Meal : rice husks. Malaysian J.Microbiol., 1:17–24.

O'Brien DJ, Craig Jr. JC. 1996. Ethanol production in a continuous fermentation/membrane pervaporation system. Appl. Microbiol. Biotechnol. 44:699–704.

Park HC, Meertens RM, Muldef MH V. 1994. Pervaporation of alcohol-toluene mixtures through polymer blend membranes of poly (acrylic acid) and poly (vinyl alcohol). J. Membr. Sci. 90:265–274.

Park JK, Jung JY. 2002. Production of benzaldehyde by encapsulated whole-cell benzoylformate decarboxylase. Enzyme Microb. Technol. 30:726 –733.

Potvin G, Ahmad A, Zhang Z. 2012. Bioprocess engineering aspects of heterologous protein production in *Pichia pastoris*: A review. Biochem. Eng. J. 64:91-105.

Ruff C, Hor K, Weekerle B, Schreier P. 2000. 2H/1H Ratio Analysis of Flavor Compounds by On-Line Gas Chromatography Pyrolysis Isotope Ratio Mass Spectrometry (HRGC-P-IRMS): Benzaldehyde. J. High Resol. Chromatogr. 23:357–359.

Sae-Khow O, Mitra S. 2010. Pervaporation in chemical analysis. J. Chromatogr. A 1217:2736–46.

Schreier P. 1997. Enzymes and Flavour Biotechnology. Advances in biochemical engineering/biotechnology 55:51–72.

Schügerl K. 2000. Integrated processing of biotechnology products. Biotechnol. Adv. 18:581–99.

Scott Parent J, Capela M, Dafoe JT, Daugulis AJ. 2012. A first principles approach to identifying polymers for use in two-phase partitioning bioreactors. J. Chem. Technol. Biotechnol.87:1059-1065.

Sreekrishna K, Brankamp RG, Kropp KE, Blankenship DT, Tsay JT, Smith PL, Wierschke JD, Subramaniam a, Birkenberger L a. 1997. Strategies for optimal synthesis and secretion of heterologous proteins in the methylotrophic yeast *Pichia pastoris*. Gene 190:55–62.

Strathmann H, Gudernatsch W. 1987. Continuous removal of ethanol from fermentation broths by pervaporation. In: Verall, M, Hudson, M, editors. Seperations for Biotechnol. Chichester: Ellis Horwood.

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. 44:7254–9.

Tschopp JF, Brust PF, Cregg JM, Stillman CA, Gingeras TR. 1987. Expression of the lacZ gene from two methanol-regulated promoters in *Pichia pastons*. Nucleic Acids Res. 15:3859–3876.

Valentini SR, Gattás EAL. 2011. Optimal Conditions for Biomass and Recombinant Glycerol Kinase Production Using the Yeast Pichia pastoris. Food Technol. Biotechnol. 49:329–335.

Vane LM. 2005. A review of pervaporation for product recovery from biomass fermentation processes. J. Chem. Technol. Biotechnol. 80:603–629.

Wang Y, Wang Z, Xu Q, Du G, Hua Z, Liu L, Li J, Chen J. 2009. Lowering induction temperature for enhanced production of polygalacturonate lyase in recombinant *Pichia pastoris*. Process Biochem. 44:949–954.

Wang Z, Wang Y, Zhang D, Li J, Hua Z, Du G, Chen J. 2010. Enhancement of cell viability and alkaline polygalacturonate lyase production by sorbitol co-feeding with methanol in Pichia pastoris fermentation. Bioresource Technol. 101:1318–23.

Watanabe K, Kyo S. 1992. Pervaporation performance of hollow-fiber chitosan-polyacrylonitrile composite membrane in dehydration of ethanol. J. Chem. Eng. Japan 25:17–21.

Welsh FW, Murray WD, Williams RE. 1989. Microbioloical and enzymatic production of flavour and frangance chemcials. Crit. Rev. Biotechnol. 9:105–169.

Zhu T, You L, Gong F, Xie M, Xue Y, Li Y, Ma Y. 2011. Combinatorial strategy of sorbitol feeding and low-temperature induction leads to high-level production of alkaline β-mannanase in Pichia pastoris. Enzyme Microb. Technol. 49:407–412.

Chapter 3

Polymer characterization and optimization of conditions for the enhanced bioproduction of benzaldehyde by *Pichia pastoris* in a Two-Phase Partitioning Bioreactor

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3.1 Preface

As stated in Chapter 2, the potential of using polymers to enhance benzaldehyde (BZA) production using *Pichia pastoris* in a solid-liquid two phase partitioning bioreactor (TPPB) is proposed in this work. The biotransformation of BZA to benzyl alcohol (BA) by *Pichia pastoris* has been characterized by low titers as a result of substrate and product inhibition. Solid-liquid TPPBs provide a method to alleviate substrate and product inhibition by sequestering target molecules in the immiscible polymer phase, potentially increasing process performance.

As also mentioned in Chapter 2, previous work on TPPBs for the biotransformation of BZA using *Pichia pastoris* had a number of limitations such as long fermentation times, large amounts of unused substrate at the end of the biotransformation, and excessive substrate in the polymer phase with the product, thus complicating purification. Examining potential strategies using a mixed substrate feed and improved polymer selection provides a means of enhancing the bioproduction of BZA using a TPPB.

The current chapter examined the degree of substrate and product inhibition during the conversion of BA to BZA. Subsequently, amorphous polymers used in polymer-based TPPBs were characterized to determine their sorption mechanism (*absorption* or *adsorption*) for solutes. In addition, the effectiveness of a mixed substrate feeding strategy was evaluated based on its ability to reduce the total fermentation process time during the growth phase. Finally, the performance of the selected polymer strategy and mixed substrate feed strategy were evaluated in a solid-liquid TPPB biotransformation and compared to previous literature values.

3.2 Abstract

Benzaldehyde, with its apricot and almond-like aroma, is the second most abundantly used molecule in the flavour industry, and is most commonly produced via chemical routes, such as by the oxidation of toluene. Biologically produced benzaldehyde, whether by extraction of plant material or via microbial biotransformation, commands a substantial price advantage, and greater consumer acceptance. Methylotrophic yeast, such as *Pichia pastoris*, contain the enzyme alcohol oxidase (AOX), which, in the presence of alcohols other than methanol, are able to yield aldehydes as dead-end products, for example, benzaldehyde from benzyl alcohol. In this work, we have determined that benzaldehyde, and not benzyl alcohol, is inhibitory to the transformation reaction by *P. pastoris*, prompting the development of a selection strategy for identifying sequestering polymers for use in a Partitioning Bioreactor that was based on the *ratio* of partition coefficients (PCs) for the 2 target molecules. Additionally, we have now confirmed for the first time, that the mechanism of solute uptake by amorphous polymers is via absorption, not adsorption. Finally, we have adopted a common strategy used for the production of heterologous proteins by *P. pastoris*, namely the use of a mixed methanol/glycerol feed for inducing the required AOX enzyme, while reducing the time required for high density biomass generation. All of these components were combined in a final experiment in which 10 % of the polymer Kraton D1102K, whose PC ratio of benzaldehyde to benzyl alcohol was 14.9, was used to detoxify the biotransformation in a 5 L Partitioning Bioreactor, resulting in a 3.4 fold increase in benzaldehyde produced (14.4 g vs. 4.2 g) relative to single phase operation, at more than double the volumetric productivity (97 mg L^{-1} h^{-1} vs. 41 mg L^{-1} h^{-1}).

Keywords: benzaldehyde, whole-cell biotransformation, *Pichia pastoris*, polymer sorption, Two-Phase Partitioning Bioreactor (TPPB)

3.3 Introduction

Benzaldehyde is a biologically derived C_7 aromatic compound, and is the primary component of bitter almond oil (Surburg and Panten, 2006). It occurs in various essential oils and is most abundantly used in aroma compositions, and as a raw material for the production of araliphatic fragrance and flavour products. *Nature-identical* benzaldehyde is commercially produced by chemical synthesis, however, truly natural flavour forms command a premium price due to their flavour potency and consumer opinion that naturally derived neutraceuticals are healthier than synthetic ones (Krings and Berger, 1998; Lomascolo *et al.*, 1999). Microbial biocatalysts can be used for the synthesis of benzaldehyde and provide an opportunity to produce naturally pure benzaldehyde on an industrial scale. Methylotrophic yeast such as *Pichia pastoris* can be induced to synthesize alcohol oxidase (AOX) and catalase, which aid in the dissimilation pathway to mineralize methanol as a substrate. The AOX enzyme, highly non-specific towards primary alcohol substrates, can also oxidize primary alcohols other than methanol to aldehydes, however the dissimilation pathway is terminated because the next enzyme in the sequence is specific to formaldehyde formation. Thus the biotransformation of benzyl alcohol to benzaldehyde in *Pichia pastoris* has shown to be a potentially promising production strategy as outlined in Figure 3-1 (Duff and Murray, 1989; Jain *et al.*, 2010; Kawabe and Morita, 1994) . Nevertheless, a limitation of previous work using *Pichia pastoris* has been the long fermentation periods for cell growth and induction of AOX1 and AOX2 gene expression using methanol as the sole substrate, which needs to occur before the biotransformation step (Jain *et al.*, 2010; Murray *et al.*, 1989). Mixed substrate feeding of glycerol and methanol for cell growth and induction of AOX1 and AOX2 genes has shown to be effective for generating high cell density and allow for rapid induction of the AOX enzyme (d'Anjou and Daugulis, 2001; Jungo *et al.*, 2007).

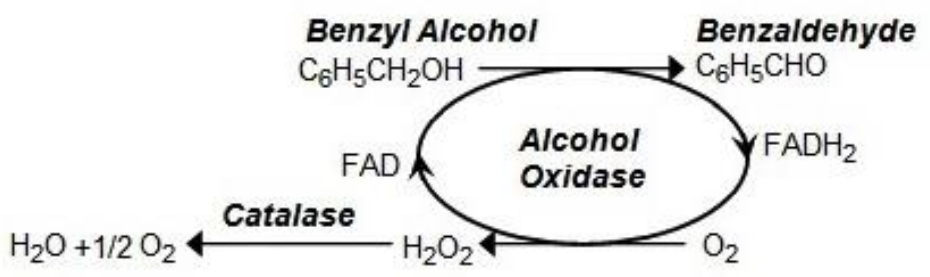

Figure 3-1: Enzymatic benzyl alcohol oxidation by alcohol oxidase coupled with catalase and cofactor FAD, producing H_2O and benzaldehyde.

The biotransformation of benzyl alcohol to benzaldehyde in single aqueous phase batch reactors has also been limited by low titers, which has been attributed to substrate and end-product inhibition by benzaldehyde and to a lesser extent by benzyl alcohol (Kawakami and Nakahara, 1994; Murray *et al.*, 1989). The use of whole cell *Pichia pastoris* as the biocatalysis suggests that benzaldehyde and possibly benzyl alcohol are inhibitors on the reaction conversion rate; however no previous literature has explored the degree to which they affect the process kinetics during biotransformation. Determining this would be useful to develop strategies for avoiding the inhibitory reaction rates, and would be helpful in deciding when to terminate the fermentation when low benzyl alcohol conversion rates arise.

A few previous studies using *Pichia pastoris* for the biotransformation have introduced a second auxiliary phase to sequester benzaldehyde and alleviate inhibition in the aqueous phase, as well as to overcome benzaldehyde's low solubility in water (6.55 g L^{-1}) (Duff and Murray, 1989; Jain *et al.*, 2010). In this context, biocompatible and non-bioavailable commodity polymers as the sequestering phase in Two-Phase Partitioning Bioreactors (TPPBs) have been shown to have a positive effect with respect to two-liquid phase systems sequestering benzaldehyde due to observed higher partition coefficient (PC) in polymers over liquid solvents, elimination of potentially toxic effects of organic solvents to cells, ease of process operation and handling, and

the elimination of possible fugitive aromas which could negatively affect the quality of the flavour/fragrance (Jain *et al.*, 2010). In the only previous work using polymer-based TPPBs for benzaldehyde production, Jain et al (2010) utilized a polymer selection approach based on identifying polymers possessing high PC for both benzyl alcohol and benzaldehyde. However, this may not be the most effective strategy of finding the most appropriate polymer for this biotransformation, as significant residual benzyl alcohol could remain in the polymer at the end of the transformation, complicating benzaldehyde purification.

The purpose of this work was to first identify the degree of substrate and product inhibition during biotransformation of benzyl alcohol to benzaldehyde using *P. pastoris*, to create a starting point for developing a TPPB polymer selection strategy. Next, polymer selection and characterization was undertaken via PC determinations and uptake mechanisms. Third, a mixed substrate feed strategy using glycerol and methanol was implemented with the aim of reducing the fermentation process time during the growth phase, while still ensuring AOX induction for benzaldehyde production. Finally, a TPPB biotransformation was undertaken, incorporating the previous steps to reduce cell inhibition and production time, with the aim of enhancing overall product titres and volumetric productivity.

3.4 Materials and Methods

3.4.1 Chemicals, and polymers

All reagents and medium components were obtained from Sigma-Aldrich. Table 3-1 (where available) shows the properties and sources for the polymers used in this work, many of which were kindly donated by the manufacturers.

Table 3-1: Physical properties, and benzyl alcohol and benzaldehyde partition coefficient for six candidate polymers (This work).

Polymer and Supplier	Glass transition temperature, Tg (^{o}C)	Type	Partition coefficient for benzyl alcohol	Partition coefficient for benzaldehyde	Partition coefficient Ratio (Benzaldehyde: Benzyl alcohol)
Kraton D1102k, Kraton	Styrene: 90; butadiene: -90	Styrene/ butadiene linear block copolymer Poly(dimethylsiloxane)	1.7	25.3	14.9
Silicone Rubber, Mastercraft	-116	Poly(dimethyl siloxane)	1.24	9.9	8.0
Elvax 360, DuPont	-25	25% Vinyl alcohol (copolymer with ethylene)	3.1	24.1	7.8
Desmopan 453, Bayer MSD	-34	Polyurethane thermoplastic elastomer	6.6	42.6	6.5
Hytrel 8171, DuPont	-59	Poly(butylene/poly(alkylene ether)phthalate thermoplastic	14.8	40.9	2.8
Nylon 6-6, DuPont	50	Polyamide 66 (crystalline)	2.9	5.6	1.9

3.4.2 Medium formulation and culture preparation

Pichia pastoris ATCC 28485 was used in this work. The medium for the bioreactor and shake flasks was formulated according to Duff and Murray (1989). When using a mixed feed of glycerol-methanol, the total carbon source for the cell growth phase was equivalent to the total amount of substrate used by Duff and Murray (1989) (who used only methanol) for comparison purposes. To prepare the inoculum, 60 µL of frozen *P. pastoris* stock culture was added to six 125 mL shake flasks containing 50 mL medium with 10 g L^{-1} glycerol and incubated at 30 °C and 180 rpm for 30 h.

3.4.3 Shake Flask Inhibition Experiments

125 mL Erlenmeyer flasks with 50 mL medium containing 5 g L^{-1} glycerol and 5 g L^{-1} methanol were prepared containing different concentrations of benzyl alcohol: 2 g L^{-1}, 4 g L^{-1}, 8 g L^{-1}, and 14 g L^{-1} and benzaldehyde 0 g L^{-1}, 0.75 g L^{-1}, 1.5 g L^{-1}, and 3 g L^{-1}. The flasks were

inoculated and the concentrations of benzyl alcohol and benzaldehyde were determined periodically over a 24 h period.

3.4.4 Polymer Characterization

Sorption Mechanism

Although polymer-based TPPB systems have been studied for several years, the mechanism of solute uptake by amorphous polymers has never been demonstrated (i.e. adsorption vs. absorption) resulting in repeated queries as to whether surface area (adsorption) or mass of polymers (absorption) determines extent of solute uptake. To confirm the mechanism three different polymers (PEBAX 2533, Hytrel 8206 and Elvax 40W), and solutions of two target solutes (benzaldehyde and phenol) were equilibrated using the same mass of polymer, but possessing different surface areas. Phenol was also tested, in addition to benzaldehyde to confirm that whatever uptake mechanism was present was not solute-dependent.

Beads of the three polymers were cut in half to obtain two different surface areas for each polymer, and the surface area was measured assuming that Pebax 2533 and Elvax 40W maintain a spherical shape, and that Hytrel 8206 maintains a cylindrical shape (Figure 3-2). The diameter and length were measured using Tuff Grade 6" Caliper with an LCD Screen. The dimensions of twenty five beads for each polymer were measured for both the as-received polymer beads and the cut polymer beads. The mass of fifty beads of polymer was determined and the average mass of one bead was calculated.

Solutions of either benzaldehyde or phenol were added to 25 mL scintillation vials along with 0.5 g, 0.75 g, 0.875 g, 1 g, 1.125 g, 1.5 g, 1.75 g, and 2 g of each polymer of each size. Each system was allowed to equilibrate on a rotary shaker at 30 $^{\circ}$C for 24 h. After 25 h the solute concentration on the vials was measured, and the PC determined by mass balance.

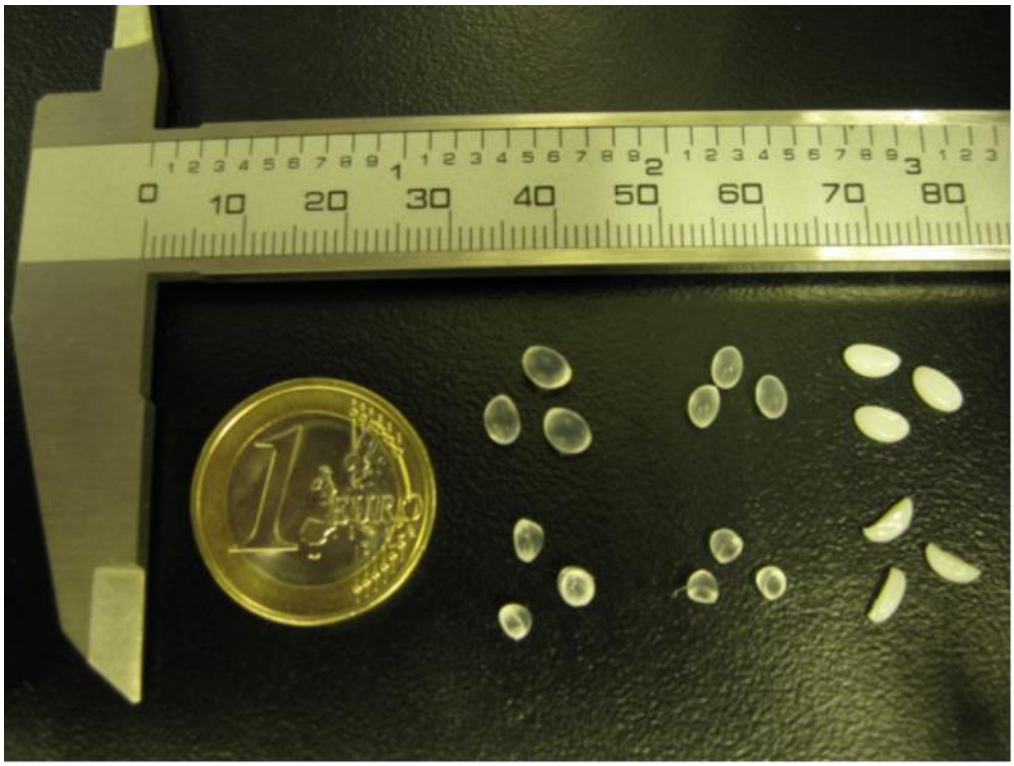

Figure 3-2: Polymers used in sorption study: Top, as received pellets: Elvax 40W surface area 1.74 m^2 kg^{-1}, Pebax 2533 surface area 1.76 m^2 kg^{-1}, Hytrel 8206 surface area 1.89 m^2 kg^{-1}. Bottom, cut pellets: Elvax 40W surface area 2.75 m^2 kg^{-1}, Pebax 2533 surface area 2.15 m^2 kg^{-1}, Hytrel 8206 surface area 2.84 m^2 kg^{-1}. Caliper scale is in centimeters.

3.4.5 Polymer PCs of Selected Polymers

Six polymers were tested for PC for benzaldehyde and benzyl alcohol using the method described previously (Isaza and Daugulis, 2009). The PC results are shown in Table 3-1.

3.4.6 Single-Phase and Two-Phase Reactor Operation

The cell growth phase was conducted in a 5 L BioFlo III bioreactor (New Brunswick Scientific, Edison, NJ) containing 3 L of medium with 10 g L^{-1} glycerol and 10 g L^{-1} methanol and operated at 500 rpm, 4 L min^{-1} aeration and pH 5.5. When the glycerol and methanol were consumed, deduced from characteristic increases in dissolved oxygen (DO), the single-phase biotransformation phase was started by shifting the pH to 7.3 using 4 M KOH and by adding 20 g

L^{-1} benzyl alcohol which was sterile-filtered using a 0.2μm syringe filter. Agitation and aeration were set to 400 rpm and 1 L min^{-1} to reduce product stripping by aggressive aeration.

The operating conditions during the cell growth phase for the TPPB system were the same as the single phase reactor. At the start of the biotransformation 300 g of polymer beads were also added to the reactor vessel to achieve a 10 % (w/w) polymer phase ratio. Various polymer fractions have been used in previous studies (Gao and Daugulis, 2009; Jain *et al.*, 2010; Khan and Daugulis, 2010), and 10% was selected here as a typical value.

3.4.7 Product recovery from polymer

Concentrations of benzyl alcohol and benzaldehyde in the polymer were determined from polymer samples during the biotransformation period by methanol extraction of the polymer beads (Gao and Daugulis, 2009). For each sample three polymer extractions were performed to determine overall target molecule concentration, each allowed to reach thermodynamic equilibrium before the subsequent extraction.

3.4.8 Analytics

Cell Biomass Measurement

Optical density readings were made at 600 nm using a Biochrom Ultraspec spectrophotometer, and readings were converted to cell dry weight (g L^{-1}) using a calibration curve.

Concentration Measurements

Methanol and glycerol concentrations were measured by HPLC and Refractive Index detection at a flow of 0.7 ml min^{-1} 5 mM H_2SO_4 mobile phase, with a Varian, HiPlex H 250 x 7mm column at 55 $^{\circ}$C. Samples from the reactor were pre-filtered by a 0.2 μm syringe filter and 20 μL of filtered sample was injected in the HPLC.

Benzyl alcohol and benzaldehyde concentrations were measured by HPLC-UV detection using a Varian 410 autosampler with an injection volume of 20 μL, a Varian Prostar 325 UV/Vis detector and a Polaris 5μ C18-A 150 x 4.6 mm column, using the method as described by Jain *et al.* (2010).

Phenol concentrations (polymer uptake experiments) where measured using the 4-amionantipyrene method at λ = 510 nm (Vrionis *et al.*, 2002) using an Ultraspec 3000 UV-vis Spectrophotometer.

3.5 Results and Discussion

3.5.1 Substrate and end-product inhibition

The effect of product and substrate inhibition on the reaction rate was investigated by examining the biotransformation of benzyl alcohol to benzaldehyde at various initial concentrations of benzyl alcohol and benzaldehyde (Figure 3-3). Note that this experiment focused only on the biotransformation, and not on cell inhibition, which would need to be considered in a separate study. Due to the volatility of benzaldehyde and its susceptibility for autoxidation, the performance of the system was measured by benzyl alcohol consumption, based on the 1:1 stoichiometry of benzyl alcohol to benzaldehyde. Figure 3-3 shows that the presence of substrate actually seems to accelerate the rate of benzaldehyde production, suggesting that not only is benzyl alcohol significantly less inhibitory on the reaction rate than benzaldehyde over the range of concentrations tested it actually may stimulate the rate of benzyl alcohol oxidation. Previous work by Duff and Murray (1989), demonstrated that the oxidation of benzyl alcohol in single-phase aqueous solution follows Michaelis-Menten kinetics at benzyl alcohol concentrations less than 20 g L^{-1}, from which a K_m and V_{max} value were estimated to be 20 g L^{-1} and 3.7 g L^{-1} h^{-1} respectfully. The high value for K_m suggests that a large concentration of substrate is required to produce high conversion rates. For the product, benzaldehyde, the benzyl alcohol consumption rates were highest when no benzaldehyde was present at all benzyl alcohol

concentrations (Figure 3-3), and as the benzaldehyde concentration added to the system increased from 0 to 3 g L^{-1} there was a strong inhibition on the reaction rate to the biotransformation. These results indicate low benzaldehyde tolerance for this biotransformation and in previous work using single phase bioreactor experiments (Duff and Murray, 1989), suggesting that a benefit could be obtained by removing benzaldehyde from the aqueous phase as it is formed. A number of strategies exist for the removal product/substrate inhibitors, but solid-liquid TPPBs using polymers as target molecule sorbents have shown to be effective for this purpose (Gao and Daugulis, 2009; Jain *et al.*, 2010; Khan and Daugulis, 2010).

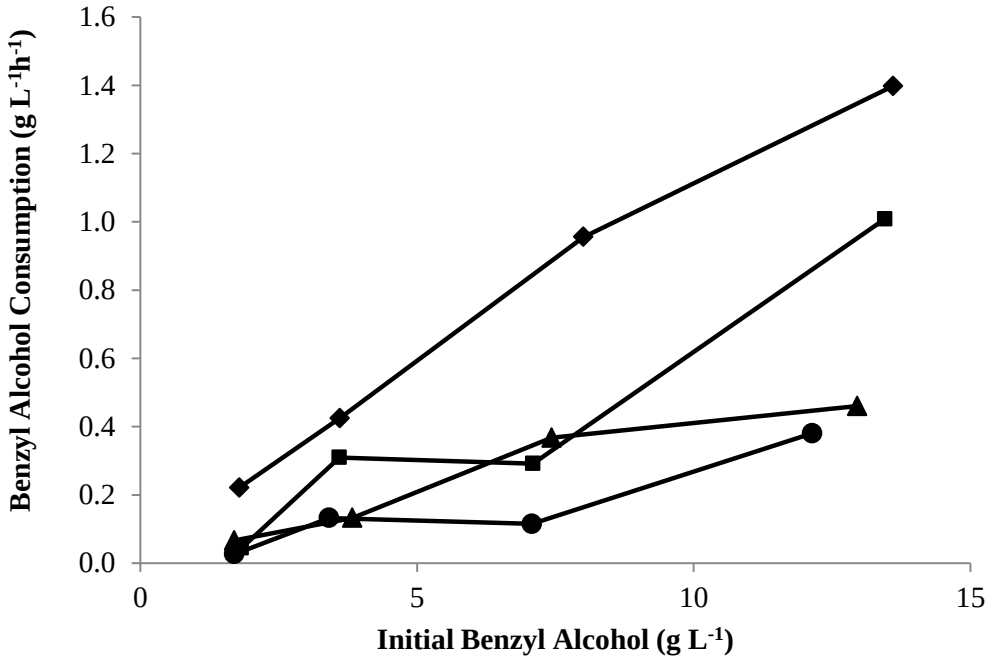

Figure 3-3: Benzyl alcohol consumption as a function of initial benzyl alcohol concentration in the presence of 0 g L^{-1} (♦), 0.75 g L^{-1} (■), 2 g L^{-1} (▲), and 3 g L^{-1} (●) benzaldehyde.

3.5.2 Polymer sorption mechanism

The mechanism of solute sorption by soft, amorphous polymers in the application in TPPBs has not yet been identified in the current literature. Solute sorption/release by amorphous polymers, however, is the basis of controlled drug release, an area that has been studied and demonstrated for more than 30 years (Ulrich *et al.*, 1999). In contrast, the uptake mechanism of solutes using hard, crystalline polymeric resins, is by surface adsorption (Nielsen and Prather, 2009; Qureshi *et al.*, 2005). It is critical to establish whether absorption or adsorption is the fundamental mechanism for polymer uptake in our case, as this affects the strategy used for polymer selection in TPPB systems, and also determines the amount of polymer utilized (i.e. area required, or mass). The average surface areas of the regular polymer beads Hytrel 8206, Elvax 40W and Pebax 2533 were 1.89 m^2 kg^{-1}, 1.74 m^2 kg^{-1} and 1.76 m^2 kg^{-1} respectively, while the reduced polymer sizes had surface areas of 2.84 m^2 kg^{-1}, 2.75 m^2 kg^{-1} and 2.15 m^2 kg^{-1}. Figure 3-4(a) provides a comparison of the PC values at different surface areas for the 3 polymers with benzaldehyde as the solute, and clearly shows that sorption is determined by the mass of polymer (absorption) and not the surface area (adsorption). To demonstrate that this was not peculiar to only benzaldehyde, phenol was used a second target molecule and PCs were determined as shown in Figure 3-4(b). Again it can be seen that varying the surface areas of the polymers had no effect on the PC for that polymer using that specific target molecule, which again confirms that the primary mechanism for polymer uptake is by absorption.

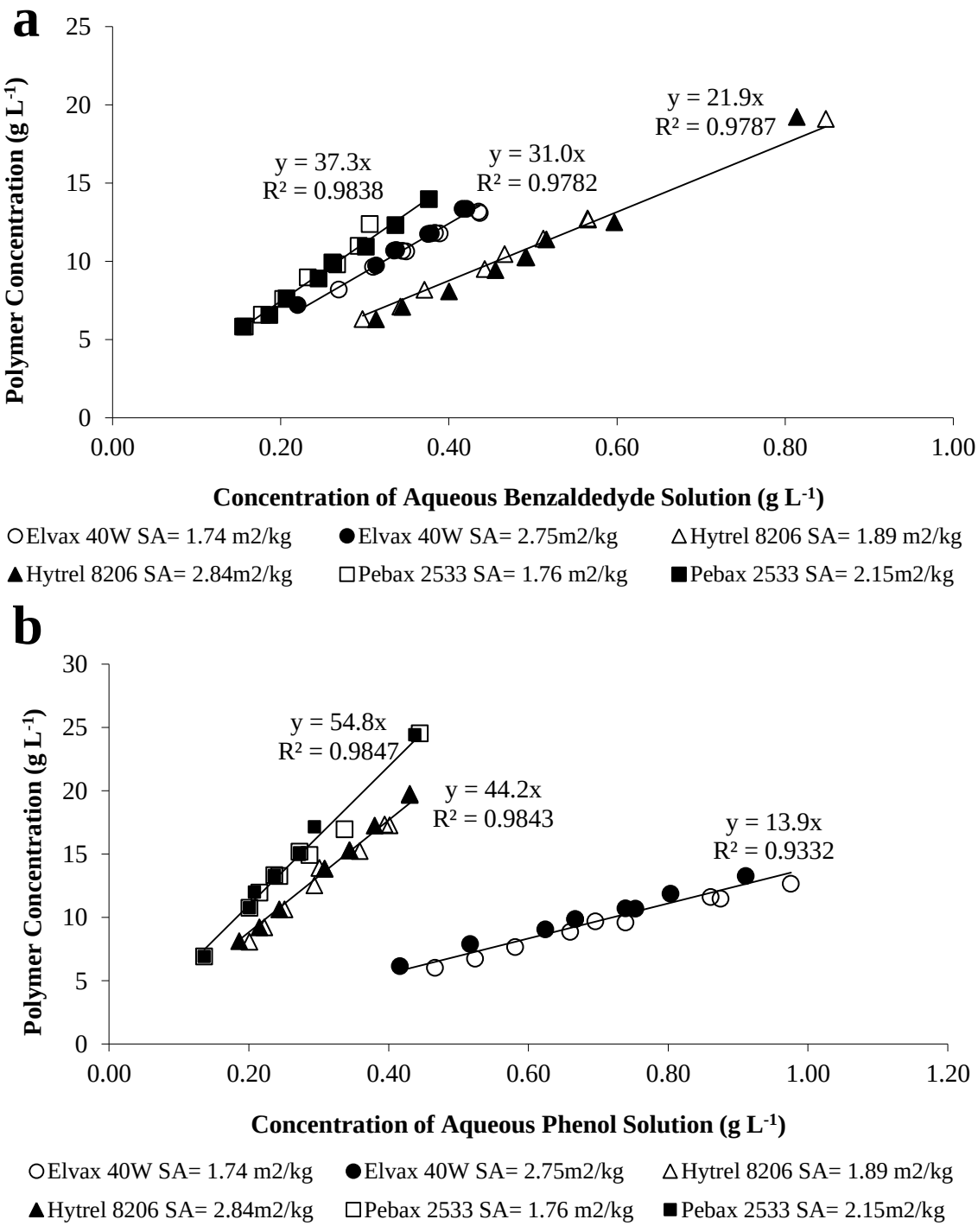

Figure 3-4: Partition coefficients for (a) benzaldehyde uptake and (b) phenol uptake. Elvax, Hytrel and Pebax were tested at different surface areas, but with the same mass of polymer.

3.5.3 Polymer selection

In light of the results above indicating that benzaldehyde is inhibitory to the biotransformation, and that the substrate, benzyl alcohol, is not, polymers were tested to determine not only their PC values for each of these 2 compounds, but also the *ratio* of the PC values. That is, the current strategy was to maximize the ratio of the PC of benzaldehyde, relative to that of benzyl alcohol, to target the compound that is exerting an inhibitory effect and to remove it, while minimizing the amount of substrate that would be sorbed into the polymer. As noted earlier, this in contrast to the work of Jain et al (2010) in which the strategy was to have high PC values for both target molecules. Of the six polymers tested for their PCs for benzyl alcohol and benzaldehyde as shown in Table 3-1, Kraton D1102K had a polymer PC ratio of 14.9, the highest for substrate to product and was used for the biotransformation.

3.5.4 Mixed substrate feed

In addition to removing product inhibition by benzaldehyde sorption into a polymer, the biotransformation of benzyl alcohol to benzaldehyde by *P. pastoris* could also be potentially improved by reducing the cell growth period of the process. Previous work employing *P. pastoris* for the biotransformation of benzyl alcohol to benzaldehyde used methanol as the sole substrate for cell growth due to its ability to induce the AOX1 and AOX2 promoter, however the growth periods were very long and achieved relatively low cell densities (Duff and Murray, 1989; Jain *et al.*, 2010; Kawakami and Nakahara, 1994). Therefore, here we used glycerol as the carbon source for shake flask inoculum, and glycerol and methanol mixed feed for the growth and induction phase of the fermentation process. Figure 3-5(a) displays the time course for the growth and induction phase using a 10 g L^{-1} glycerol and 10 g L^{-1} methanol mixed feed strategy. The DO reading provided a good real-time indicator of the substrate transition from glycerol consumption to methanol, shown at the first DO spike, as well as the initiation for the biotransformation, at the second DO spike. Although, glycerol does repress the AOX1 and AOX2 promoter, the methanol

dissimilatory pathway is not utilized until the glycerol is consumed, a feature of diauxic growth. In the first 15 h the methanol concentration is observed to decrease due to stripping and not consumption (Jungo *et al.*, 2007). Overall by applying the mixed feed strategy in the fermentation process the time required before the biotransformation was initiated had been reduced by 3.4 fold from previously reported data (Jain *et al.*, 2010).

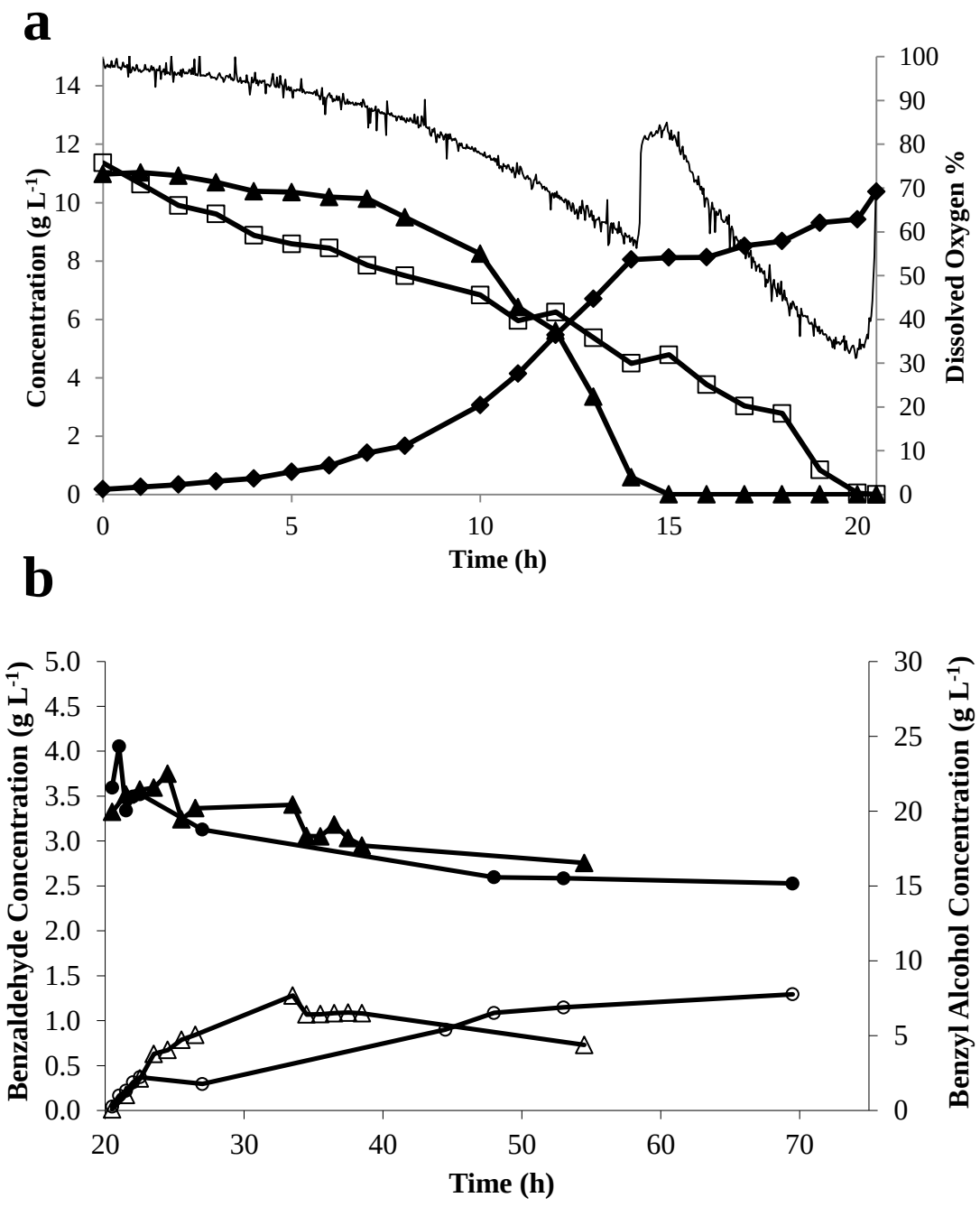

Figure 3-5: (a) Cell growth and AOX induction period with dry cell weight (♦), glycerol (▲) and methanol (□) concentrations, and dissolved oxygen trace (line) as a function of time with 10 g L^{-1} glycerol and 10 g L^{-1} methanol. (b) Single phase biotransformation concentrations of benzyl alcohol (▲) and benzaldehyde (Δ). TPPB aqueous phase concentrations of benzyl alcohol (●) and benzaldehyde (○).

3.5.5 Comparison of single and two phase systems

The biotransformation for both systems started at 20.5 h and extended to 54.5 h for the single phase run and 69.5 h for the TPPB system as shown in Figure 3-5(b). In both cases the highest aqueous benzaldehyde concentration reached was 1.3 g L^{-1}, which suggests that product inhibition may have been the reason for the cessation of the biotransformation.

In the single phase biotransformation the maximum benzaldehyde concentration was 1.3g L^{-1} (Figure 3-5(b)) at 35 h. The consumption of benzyl alcohol is proportional to benzaldehyde production by the stoichiometric ratio of benzyl alcohol to benzaldehyde conversion, however, in Figure 3-5 (b) the benzaldehyde concentration decreased over time, which is likely due to benzaldehyde volatilization (the aroma was very strongly smelled) or autoxidation with air (Beek, 1928).The total mass of benzaldehyde produced in the single phase system was 4.2 g and based on benzyl alcohol stoichiometry it was 9.9 g, shown in Figure 3-6, with a volumetric productivity of 41 mg L^{-1} h^{-1}, based on the amount of benzaldehyde measured. In comparison, the total mass of benzaldehyde produced based on the measured amount was less than half of the total mass of benzaldehyde based on stoichiometry, which shows the effect of significant volatilization losses and autoxidation in the single phase biotransformation.

The TPPB system used a 10 % (w/w) polymer phase ratio consisting of Kraton D1102K and the same operating conditions as the single phase. The two phase biotransformation shows a gradual increase in benzaldehyde with a slight plateau that occurs at 48 h with approximately 1.1 g L^{-1} benzaldehyde (Figure 3-5(b)). The total mass of benzaldehyde produced using the TPPB system was 14.4 g and based on benzyl alcohol stoichiometry it was 16.9 g, shown in Figure 3-6, and a volumetric productivity of 97 mg L^{-1} h^{-1}, based on the amount of benzaldehyde measured.

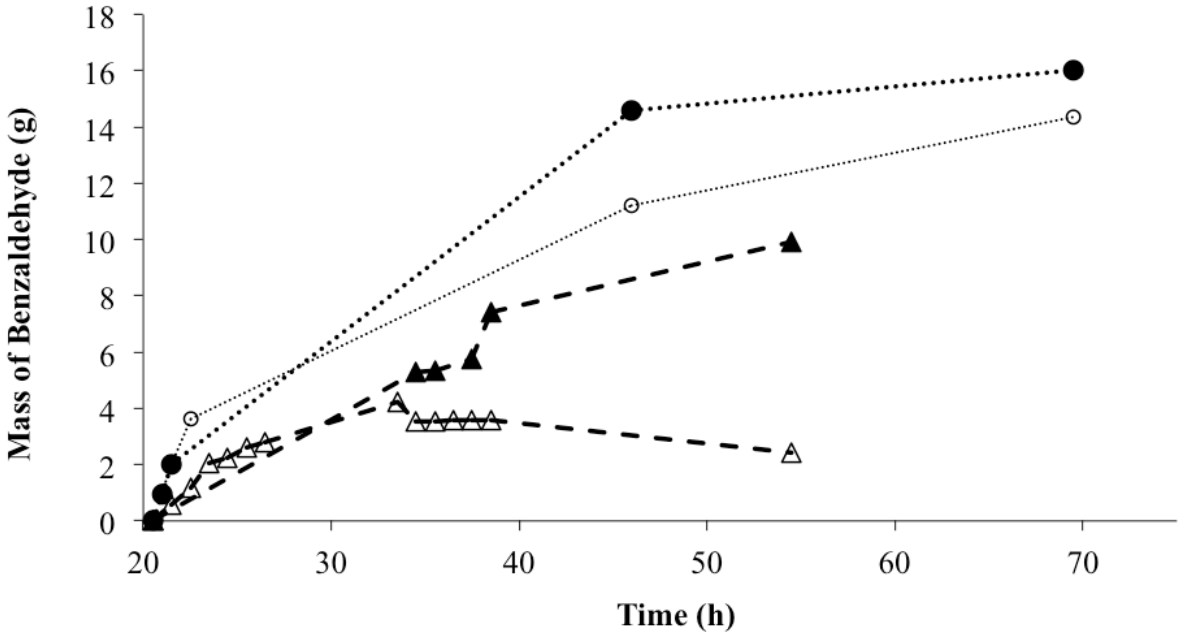

Figure 3-6: Single phase total mass of benzaldehyde based on benzyl alcohol stoichiometry (▲) and benzaldehyde measured (∆). TPPB aqueous phase total mass of benzyl alcohol (●) and benzaldehyde (○) as a function of time.

In comparing the total mass of benzaldehyde measured and that determined by benzyl alcohol stoichiometry the results show that there was a 118 % reduction in volatilization losses and autoxidation when applying TPPBs with the biotransformation than compared to the single phase biotransformation. Thus it appears as though TPPB operation may not only reduce inhibition, but may also reduce losses of volatile target molecules. To confirm this, Henry's Law calculations were undertaken for the 2 systems, assuming that the aqueous and vapour phases were in equilibrium, and by using the trapezoid rule to determine cumulative benzaldehyde losses due to stripping. It was found that in the single phase case, 3.1 g of benzaldehyde (73.8% of the total benzaldehyde produced) was lost due to stripping, while in the TPPB case, only 4.2 g, or 29.2 % of the benzaldehyde, was lost.

A comparison of the single phase and two phase system using Kraton D1102K as well as the TPPB system using Elvax 40W from work by Jain et al (2010) is shown in Table 3-2. The TPPB system using Kraton D1102K initially had 21.6 g L^{-1} benzyl alcohol in the aqueous phase and below 10g L^{-1} in the polymer phase, which was the rationale applied to achieve high benzyl alcohol consumption rates. In work by Jain et al (2010) the Elvax 40W polymer beads were preloaded with benzyl alcohol such that when they were added to the system the target benzyl alcohol concentration was 10 g L^{-1}. For the TPPB system with Elvax 40W the total time required to complete the biotransformation was 143 h, and the time required for the TPPB system with Kraton D1102K was 69.5 h, a significant reduction. The benzaldehyde volumetric productivity for the system with Elvax 40W was 70 mg L^{-1} h^{-1} and using Kraton D1102K it was 97 mg L^{-1} h^{-1}. The difference between the benzaldehyde produced based on stoichiometry and the measured benzaldehyde is more significant in the single phase biotransformation than with the TPPB system, which strongly suggests that using TPPBs may reduce product volatilization and autoxidation as noted above.

Table 3-2: Comparison of single phase and TPPB system with Kraton D1102K polymer beads and TPPB system with Elvax 40W.

Criteria for Comparison	Single phase (This work)	TPPB with Elvax 40W (Jain *et al.*, 2010)	TPPB with Kraton D1102K (This work)
Operating Variables			
Initial aqueous benzyl alcohol concentration (g L^{-1})	21.1	7.8	21.6
Total benzyl alcohol mass in system (g)	63.3	54.5[i]	70.7
Total Polymer mass in system (g)	NA	300	300
Final Cell Density (g biomass L^{-1})	10.4	4.10	11.8
Fermentation Time			
Elapsed time for growth & induction phase (h)	20.5	72	20.5
Elapsed time for biotransformation phase (h)	34.0	71	49.0
System Performance			
Total benzaldehyde produced (g)	4.2	15.0	14.4
Benzaldehyde volumetric productivity (mg L^{-1} h^{-1})	41.0	70.0	97.0
Total benzyl alcohol consumed (g)	9.9	-	16.9

[i] Polymers were preloaded with benzyl alcohol.

3.6 Conclusion and Future Work

This paper has demonstrated that benzaldehyde has a strong inhibition effect on the reaction rate during the biotransformation of benzyl alcohol to benzaldehyde, and the advantages of introducing an auxiliary phase for product removal. It has also shown that benzyl alcohol does not exert significant substrate inhibition at low concentrations. The polymer surface area experiments have confirmed that absorption is the dominating mechanism for solute sorption. Applying a mixed substrate feed reduced the total fermentation time required for the biotransformation of benzyl alcohol to benzaldehyde by 3.4 fold compared to previous work done in this area. The use of Kraton D1102K in the TPPB contributed to high initial benzyl alcohol consumption rates and increased productivity relative to the single phase operation.

Future work will be conducted on using a fundamental approach to polymer selection based on Solubility Parameters to identify polymers with higher PC values for benzaldehyde. In addition the use of non-AOX-repressing substrates for the generation of ATP may also provide an

opportunity to enhance benzaldehyde production via a substrate fed-batch strategy before the biotransformation phase to reduce overall fermentation time, and during the biotransformation should ATP be required.

3.7 References

d'Anjou MC, Daugulis AJ. 2001. A Rational Approach to Improving Productivity in Recombinant *Pichia pastoris* Fermentation. Biotechnol. Bioeng. 72:1-11.

Beek PAAVD. 1928. The autoxidation of benzaldehyde. Recueil des Travaux Chimiques des Pays-Bas 47:286-300.

Duff SJB, Murray WD. 1989. Oxidation of Benzyl Alcohol by Whole Cells of *Pichia pastoris* and by Alcohol Oxidase in Aqueous and Nonaqueous Reaction Media. Biotechnol. Bioeng. 34:153-159.

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. 104:332-9.

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. 2010. Bioproduction of benzaldehyde in a solid-liquid two-phase partitioning bioreactor using *Pichia pastoris*. Biotechnol. Lett. 32:1649-54.

Jungo C, Marison I, Stockar UV. 2007. Mixed feeds of glycerol and methanol can improve the performance of *Pichia pastoris* cultures : A quantitative study based on concentration gradients in transient continuous cultures. J Biotechnol. 128:824-837.

Kawabe T, Morita H. 1994. Production of Benzaldehyde and Benzyl Alcohol by the Mushroom *Polyporus tuberaster* K2606. J. Agric. food Chem. 42:2556-2560.

Kawakami K, Nakahara T. 1994. Importance of solute partitioning in biphasic oxidation of benzyl alcohol by free and immobilized whole cells of *Pichia pastoris*. Biotechnol. Bioeng. 43:918-24.

Khan TR, Daugulis AJ. 2010. Application of solid-liquid TPPBs to the production of L-phenylacetylcarbinol from benzaldehyde using *Candida utilis*. Biotechnol. Bioeng. 107:633-41.

Krings U, Berger RG. 1998. Biotechnological production of flavours and fragrances. Appl Microbiol. Biotechnol. 49:1-8.

Lomascolo A, Stentelaire C, Asther M, Lesage-meessen L. 1999. Basidiomycetes as new biotechnological tools to generate natural aromatic flavours for the food industry. TIBTECH 17:282-289.

Murray WD, Duff SJB, Lanthier PH. 1989. Induction and stability of alcohol oxidase in the methylotrophic yeast Pichia pastoris. Appl. Microbiol. Biotechnol. 32:95-100.

Nielsen DR, Prather KJ. 2009. In situ product recovery of n-butanol using polymeric resins. Biotechnol. Bioeng. 102:811-21.

Qureshi N, Hughes S, Maddox IS, Cotta MA. 2005. Energy-efficient recovery of butanol from model solutions and fermentation broth by adsorption. Bioprocess. Biosys. Eng. 27:215-22.

Surburg H, Panten J. 2006. Common Fragrance and Flavor Materials: Preparation, Properties and Uses 5th ed. Weinheim: WILEY-VCH Verlag GmbH & Co. KGaA.

Uhrich, KE, Cannizzaro SM, Langer RS. 1999. Polymeric Systems for Controlled Drug Release. Chem. Rev. 99:3181-3198.

Vrionis HA, Kropinski AM, Daugulis AJ. 2002. Enhancement of a two-phase partitioning bioreactor system by modification of the microbial catalyst: demonstration of concept. Biotechnol. Bioeng. 79:587-94.

Chapter 4

Strategies for Improved Bioproduction of Benzaldehyde by *Pichia pastoris* and the Use of Hytrel as Tubing Material for Integrated Product Removal by *in situ* Pervaporation

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With minor editorial changes to fulfill formatting requirements, this chapter is substantially as it has been submitted to: *Biotechnology and Bioengineering* (2013)

4.1 Preface

The work in Chapter 3 demonstrated that benzaldehyde (BZA) and not benzyl alcohol (BA) is substantially inhibitory to the transformation reaction by *P. pastoris*, initiating the development of a polymer selection strategy for use in a Partitioning Bioreactor. Additionally, it was demonstrated for the first time that amorphous polymers sorb solutes based on *absorption*, not *adsorption*. Finally, a strategy employing mixed methanol/glycerol feed was used to reduce the time required for high-density biomass generation. All of these components were combined in a final TPPB experiment, which increased total BZA produced relative to single-phase operation at more than double the volumetric productivity. However, a difficulty associated with the use of polymer beads as means of sequestering product is the inevitable accumulation of BZA in the aqueous phase at the end of the biotransformation, which could have caused the biotransformation to stop.

The current chapter investigated why biotransformation rates were reduced during the transformation by examining the possible impact of low AOX, low ATP levels and high BZA levels. Product detoxification using specially fabricated polymer tubing for integrated *in situ* pervaporation was characterized in terms of BZA and BA fluxes. The performance of the integrated pervaporation system was then evaluated based on its ability to continuously separate product from the aqueous phase and its performance in the bioproduction of BZA.

4.2 Abstract

Benzaldehyde (BZA), with its almond-like aroma, is an important additive in the food, fragrance and nutraceutical industries, and biologically derived BZA by microbial catalysts provides many advantages over the traditional methods of plant material extraction. The methylotrophic yeast *Pichia pastoris* induces alcohol oxidase (AOX) in the presence of methanol, which is highly nonspecific to other primary alcohols, and can oxidize them to their aldehyde form without further degradation, such as benzyl alcohol (BA) to BZA. In this work BA and BZA inhibition on *P. pastoris* cell growth was determined with an IC_{50} at 0.98 g L^{-1} for BZA and 2.95 g L^{-1} for BA, which are low levels and are undesirable for high biotransformation rates. Product detoxification, and not low AOX and low ATP levels, was found to most improve the biotransformation rates, which prompted the application of *in situ* product removal using the thermoplastic polymer Hytrel 3078, fabricated into pervaporation tubing by DuPont, Canada. Hytrel 3078, chosen for its high partition coefficient (PC) for BZA and its low PC for BA, was characterized by available tubing surface area and permeate gas flow rate in terms of its BZA/BA flux, showing that permeate gas flow rate has a greater positive influence on flux. Finally, the integrated *in situ* pervaporation biotransformation was effective at continuous product separation, using 87.4% less polymer in comparison to studies with the use of polymer beads, and improved overall volumetric productivity of 214% (245.9 mg L^{-1} h^{-1} vs. 115.0 mg L^{-1} h^{-1}) over previous studies producing BZA.

Keywords: Benzaldehyde, *Pichia pastoris*, Whole-cell Biotransformation, *in situ* Pervaporation, *in situ* Product Removal, Flavour and Fragrance

4.3 Introduction

Benzaldehyde (BZA), a biologically produced aromatic molecule of high commercial value, widely used in the flavour and fragrance industry, has also gained acceptance for use in nutraceutical, pharmaceutical, cosmetics, agrochemical, and dye applications (Ariyoshi-Kishino *et al.*, 2010; Chen *et al.*, 2010; Femenia *et al.*, 1995; Kochi M, Takeuchi S, Mizutani T, Mochizuki K, Matsumoto Y, 1980; Liu *et al.*, 2008; Mathanghi, 2012; Wilson *et al.*, 1999; Ziegler and Ziegler, 1998). The extraction of BZA from various fruit pits, including cherries, apricots and peaches, generates cyanate, a toxic by-product, which requires additional processing steps and purification that can negatively influence product quality (Femenia *et al.*, 1995; Lomascolo *et al.*, 1999). Microbial biocatalysts provide a potential route for producing natural BZA at a large scale without the generation of toxic by-products, extensive purification, and dependence on agricultural production (Cheetham, 1993; Hagedorn and Kaphammer, 1994; Straathof *et al.*, 2002).

The methylotrophic yeast *Pichia pastoris*, has been shown to effectively produce BZA (Craig and Daugulis, 2013; Duff and Murray, 1989; Jain *et al.*, 2010) utilizing alcohol oxidase (AOX) and catalase employed in the first step of the methanol utilization pathway (MUP). As a result of methanol induction of the genes encoding AOX enzymes, high expression and non-specificity towards primary alcohol substrates enables it to oxidize alcohols other than methanol to their respective aldehyde form (Couderc *et al.*, 1980) without further degradation since the subsequent enzyme in the pathway is highly specific to its natural substrate, formaldehyde (Patel *et al.*, 1983). Previous work using *P. pastoris* to produce BZA has been limited by reduced BZA conversion rates, however, after elevated product concentrations were reached in the aqueous phase.

Studies using *P. pastoris* for heterologous protein production have shown that feeding strategies based on improving AOX expression in the MUP after long-term fermentations have

been effective at increasing protein titer (Cos *et al.*, 2006; d'Anjou and Daugulis, 2001; Lee *et al.*, 2003; Thorpe *et al.*, 1999; Zhu *et al.*, 2011) although no previous literature has explored AOX conversion rates for the bioproduction of BZA. Previous work has shown that BZA production rates decrease as BZA accumulates in the medium, and it is possible that the addition of methanol, which is AOX inducing, and/or sorbitol, which is AOX non-repressing could potentially enhance AOX synthesis and/or provide ATP, thereby increasing BZA production rates. Alternatively, recent work done by us (Craig and Daugulis, 2013) using solid-liquid Two-Phase Partitioning Bioreactors (TPPBs) for the biotransformation of benzyl alcohol (BA) to BZA has shown that BZA has a strong inhibitory effect on the conversion rate, and thus it is possible that additional product removal needs to occur to enhance BZA production.

Although solid polymer TPPBs used for the biotransformation of BZA by *P. pastoris* have shown to be an effective strategy for alleviating end-product inhibition, the extent of product removal is limited due to the confined volume in the bioreactor (i.e. by how much polymer can be added). Methods using integrated product recovery, such as by pervaporation, have shown to be very effective for the continuous separation of volatile products from fermentation cultures with relatively high product removal fluxes (Baudot and Marin, 1996; Groot *et al.*, 1984; Li *et al.*, 2011; Vane, 2005). Previous studies employing pervaporation for separation have used a variety of commercially-available membranes consisting of silicone rubber, silicone composites, poly(vinyl alcohol), poly(tetrafluoroethylene), and chitosan-poly(acrylonitrile), selected based on affinities or activity coefficients of the target molecule in the membrane, and trans-membrane diffusion rates (Baudot and Marin, 1996; Calibo *et al.*, 1989; Groot *et al.*, 1984; Lamer *et al.*, 1996; Li *et al.*, 2011; Watanabe and Kyo, 1992). We have shown that solute-polymer affinity can be determined by partition coefficient (PC) measurements, which have been useful for selecting TPPB polymers based on their PCs for BZA and BA (Craig and Daugulis, 2013; Jain *et al.*, 2010; Khan and Daugulis, 2010; Khan and Daugulis, 2011). Polymer selection using this methodology,

followed by the fabrication of custom pervaporation membranes, could be a useful strategy for enhancing continuous *in situ* product removal (ISPR) that would also exploit the physical property differences (affinity, volatility) of BA and BZA.

In this study, BA and BZA inhibition on *P. pastoris* cell growth, as well as the possible impact of low AOX and low ATP levels were investigated to determine why biotransformation rates decreased during the bioproduction of BZA. As a result of these findings, product detoxification was further addressed by selecting a thermoplastic polymer with a high PC for BZA and a low PC for BA, and having the polymer fabricated into pervaporation tubing, which was further characterized in terms of its flux. Finally, an integrated *in situ* pervaporation biotransformation was conducted to demonstrate continuous product separation, high product recovery, and increased overall product titer and volumetric productivity.

4.4 Materials and Methods

4.4.1 Chemicals and Polymers

All medium components were obtained from Fisher Scientific, Guelph, ON, Canada, and BZA (≥ 99.5%) and BA (99.8%) were acquired from Sigma-Aldrich, Oakville, ON, Canada. Table 4-1 shows the properties and sources for the polymers used in this work (where available), many of which were kindly donated by the manufacturers. The polymers were selected based on polymer processing ability for tubing extrusion (flex modulus), and polymer physical properties. The different grades of Hytrel were subsequently custom-extruded as tubing by DuPont Canada for the purposes of this work.

Table 4-1: Thermal properties, physical properties, and experimental PC results for benzyl alcohol and benzaldehyde applied for the selection of pervaporation tubing.

Polymer	Glass Transition Temperature, Tg (^{o}C)	Specific Gravity	Flexural Modulus[a], MPa (psi)	Partition Coefficient[b] for Benzyl Alcohol	Partition Coefficient[b] for Benzaldehyde
Kraton D1102K, Kraton	Styrene: 90 Butadiene: -90	0.94	-	1.7[c]	25.3[c]
Hytrel 8206, DuPont	-59	1.17	80 (11,600)	8.6 ± 0.3	26.4 ± 0.7
Hytrel 8171, DuPont	N/A	N/A	~30 (4400)	9.3 ± 0.3	27.6 ± 1.0
Hytrel G4078W, DuPont	-37	1.18	65.5 (9,500)	8.4 ± 0.5	35.1 ± 0.2
Hytrel G3548W, DuPont	-40	1.15	32.4 (4,700)	10.4 ± 0.2	40.8 ± 1.0
Hytrel 3078, DuPont	-60	1.07	28 (4,000)	9.2 ± 0.2	45.0 ± 1.0
Masterflex Norprene Food Grade (A60 F), Cole Palmer	-	-	-	0.2 ± 0.1	8.2 ± 0.4
Polypropylene, Cole Palmer	-	-	-	0.8 ± 0.9	1.1 ± 0.6

[a]ASTM D790 (ISO 178), at room temperature
[b]Experimental PC of cut-up tubing (This study)
[c]Experimental PC value of Kraton D1102K beads published in Craig and Daugulis, (2013)

4.4.2 Medium Formulation and Culture Preparation

Pichia pastoris was obtained from the American Type Culture Collection, Rockville, MD. (ATCC 28485). The medium for the bioreactor and shake flasks was formulated according to Duff and Murray (1989) and Craig and Daugulis (2013) except 5 g L^{-1} glycerol and 5 g L^{-1} methanol were used in the bioreactor during the cell growth phase, unless otherwise indicated, and culture preparation was as described by Craig and Daugulis (2013). Immediately before the biotransformation a nutrient supplement was added also formulated according to Duff & Murray (1989) equivalent for 10 g L^{-1} methanol, except with no carbon source.

4.4.3 Shake Flask Growth Inhibition Experiments

These experiments were intended to evaluate the degree of BZA and BA inhibition on cell growth, as we had already shown the effect of BA and BZA on enzymatic biotransformation rates by *P. pastoris*. The growth inhibition experiments were conducted using 20 x 125 mL Erlenmeyer flasks with 50 mL medium containing the same formulation as described above only with 10 g L^{-1} sorbitol as the carbon source. The flasks contained different concentrations of BA: 2 g L^{-1}, 4 g L^{-1}, 8 g L^{-1}, and 16 g L^{-1}, and BZA 0.5 g L^{-1}, 1.0 g L^{-1}, 1.5 g L^{-1}, and 2 g L^{-1}. Three replicate runs were done for the control and all the BZA and BA concentrations except for 2 g L^{-1} BA, 4 g L^{-1} BA, and 2 g L^{-1} BZA (No error bars on these points). The flasks were inoculated and the biomass concentrations were determined over a period of 30 h.

4.4.4 Polymer Partition Coefficient Measurements

To determine if extruding the Hytrel polymers into tubing affected their PCs for BZA and BA, polymer pellets and tubing partition coefficients were determined using the method described previously (Isaza and Daugulis, 2009). The polymer tubing was cut into pieces such that they fit in the 20 mL scintillation vials used in the experiments.

4.4.5 Determining Why the Biotransformation Stops

Cells were grown in a 5 L BioFlo III bioreactor (New Brunswick Scientific, Edison, NJ) containing 3 L of medium with 5 g L^{-1} glycerol and 5 g L^{-1} methanol operating at 30 ^{o}C, 500 rpm, 4 L min^{-1} aeration and pH 5.5. The dissolved oxygen (DO) was used to indicate when to initiate the biotransformation phase (substrate depletion and a rise in DO), by adding 10 g L^{-1} BA which was sterile-filtered using a 0.2 μm syringe filter. Agitation and aeration were set to 400 rpm and 1 L min^{-1} to reduce product stripping by aggressive aeration. The pH was maintained at 5.5 which is optimum for cell growth. To determine if AOX levels were causing reduced biotransformation rates, 1 g L^{-1} methanol was added approximately 10 h into the biotransformation. In a separate

experiment to determine whether ATP had become depleted, 4 g L^{-1} sorbitol, with additional nutrients described above, was added approximately 10 h into the biotransformation. Finally, in a separate experiment to determine if the accumulation of BZA was causing reduced biotransformation rate 600 g of Kraton D1102K polymer was added approximately 10 h after the biotransformation commenced to reduce the aqueous BZA concentration.

4.4.6 Pervaporation Polymer Membrane Characterization

Abiotic Experiments

2 L MultiGen fermenters (New Brunswick Scientific, Edison, NJ) with 1.5 L working volume and 10 g L^{-1} BA and 3 g L^{-1} BZA were operated at 25 ^{o}C, 300 RPM and were used to characterize tubing flux. The Hytrel tubing was custom fabricated by DuPont Canada (Kingston, ON) for our study to very tight tolerances. The pervaporation polymer selected for this work was Hytrel 3078 based on the high tested PC for BZA, and the manufactured tubing had an outer diameter of 6.35 mm, wall thickness of 0.5 mm and a low flexural modulus important for coiling tubing inside the bioreactor.

The permeate gas (air) flow rate and available membrane surface area were used to evaluate pervaporation system effectiveness. Experiments varying permeate gas flow rate were tested at 0.8 L min^{-1}, 2 L min^{-1}, and 4 L min^{-1} operating at a constant available surface area of 220 cm^2 or 1.1 m length of tubing, which is also equivalent to 12.2 g of polymer. Experiments varying available surface area were tested at 220 cm^2, 440 cm^2, and 880 cm^2 operating at a constant permeate gas flow rate of 2 L min^{-1}. 3 mL samples from the aqueous phase were withdrawn periodically and measured for BA and BZA concentration over a period of 24 h.

4.4.7 *In situ* Pervaporation Operation

Bioreactor operation was as previously described, and included a cell growth phase on methanol/sorbitol, followed by the addition of BZ to initiate the biotransformation after substrate depletion had occurred, as determined by a spike in the DO. The growth phase was conducted at

pH 5.5, and the biotransformation phase was initiated by shifting the pH to 7.3 using 4 M KOH and by adding 20 g L^{-1} BA which was sterile-filtered using a 0.2μm syringe filter. A chiller (WKL 230 LAUDA Brinkmann) through which ethylene glycol was circulated was used to condense the permeate products. Figure 4-1 shows the schematic of the *in situ* pervaporation system of BZA removal.

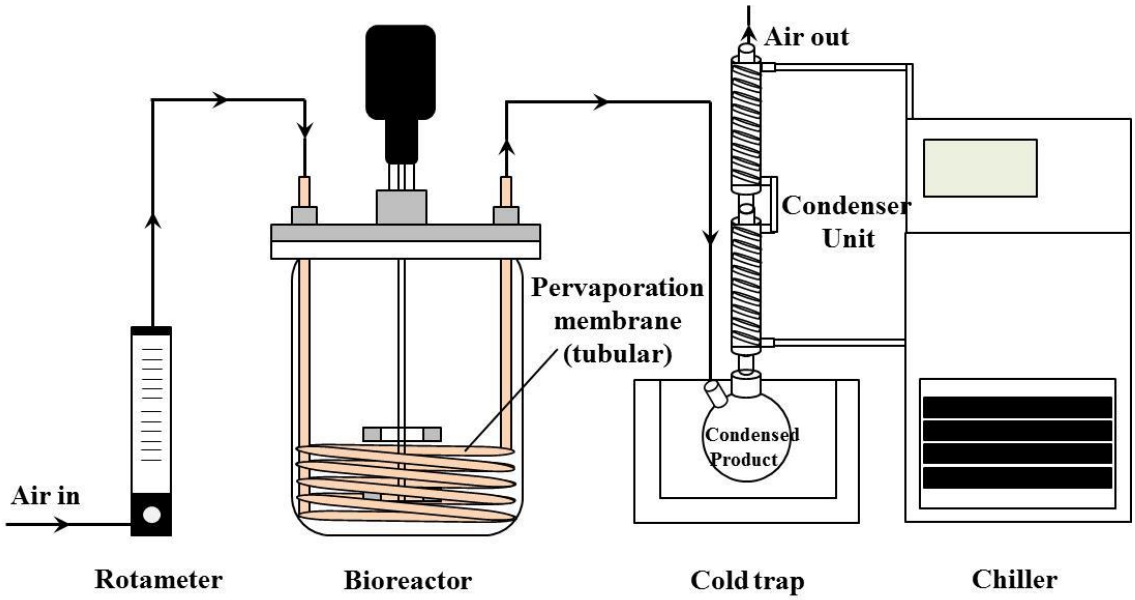

Figure 4-1: Schematic of in situ pervaporation system.

In the initial run, 2 L min^{-1} permeate gas flow was used at an available surface area of 600 cm^2 scaled up from membrane characterization experiments to maintain a length per volume ratio of 1 and estimated flux removal rate of 5 g m^{-2} h^{-1}. In a second run the initial gas flowrate was 8 L min^{-1}, which was increased to 16 L min^{-1} to try to prevent the aqueous phase BZA concentration reached highly inhibitory levels.

The outlet tubing material (connecting the pervaporation bioreactor to the condenser) was tested for PC values (Table 4-1) to insure that insignificant amounts of BZA permeate were absorbed in the outlet lines. Masterflex Norprene Food Grade ¼ inch OD tubing was used for the

all tubing lines involving the permeate gas flow except for the connection from the bioreactor to the condenser, where poly propylene tubing was used as all such tubing had a low experimental PC for BZA and BA.

4.4.8 Analytics

Cell Biomass Measurement

Optical density readings were made at 600 nm using a Biochrom Ultraspec spectrophotometer, and readings were converted to cell dry weight (g-biomass L^{-1}) using a calibration curve.

Concentration Measurements

BA and BZA concentrations were measured by HPLC-UV detection using a Varian 410 autosampler with an injection volume of 20 μL, a Varian Prostar 325 UV/Vis detector and a Polaris 5μ C18-A 150 x 4.6 mm column (Craig and Daugulis, 2013; Jain *et al.*, 2010). For the in situ pervaporation operation BZA concentrations were also determined from aqueous phase concentrations using Henry's Law constant as used by Poleo and Daugulis (2013).

4.5 Results and Discussion

4.5.1 Substrate and Product Inhibition on Cellular Growth

The degree to which BZA and BA are inhibitory to cell growth is important for maintaining high cell activity during the biotransformation. A previous study on the impact of elevated BA and BZA levels on the enzymatic conversion reaction (not on cell growth) showed that BZA is highly inhibitory, whereas high BA concentrations actually increase the enzymatic conversion rate (Craig and Daugulis, 2013). In this study the effect of BA and BZA inhibition on cellular growth was examined as shown in Figure 4-2. Figure 4-2 (a) and (b) show that even at very low concentrations of BZA, 0.5 g L^{-1}, and BA 2.0 g L^{-1} the OD decreases significantly relative to the control. Inhibition of cell growth as a function of substrate and product

concentration is also seen in Figure 4-3, which shows that cell growth is very sensitive to both BZA and BA at low concentrations. The half maximal inhibition concentration (IC_{50}) was determined using Figure 4-3, which shows that for BZA the IC_{50} was at 0.98 g L^{-1} and for BA the IC_{50} was at 2.95 g L^{-1}. At high concentrations of BA, above 8 g L^{-1}, it can be seen that BA is better tolerated by the cells as the cell inhibition plateaus, appropriate for the biotransformation, compared to the high cell inhibition shown by BZA. These findings suggest that in order for *P. pastoris* cells to grow in the presence of BZA and BA, the BZA concentration must be kept low, which is contrary to the aim of producing large amounts of BZA, and the BA concentration is tolerable to the cells at moderate concentrations.

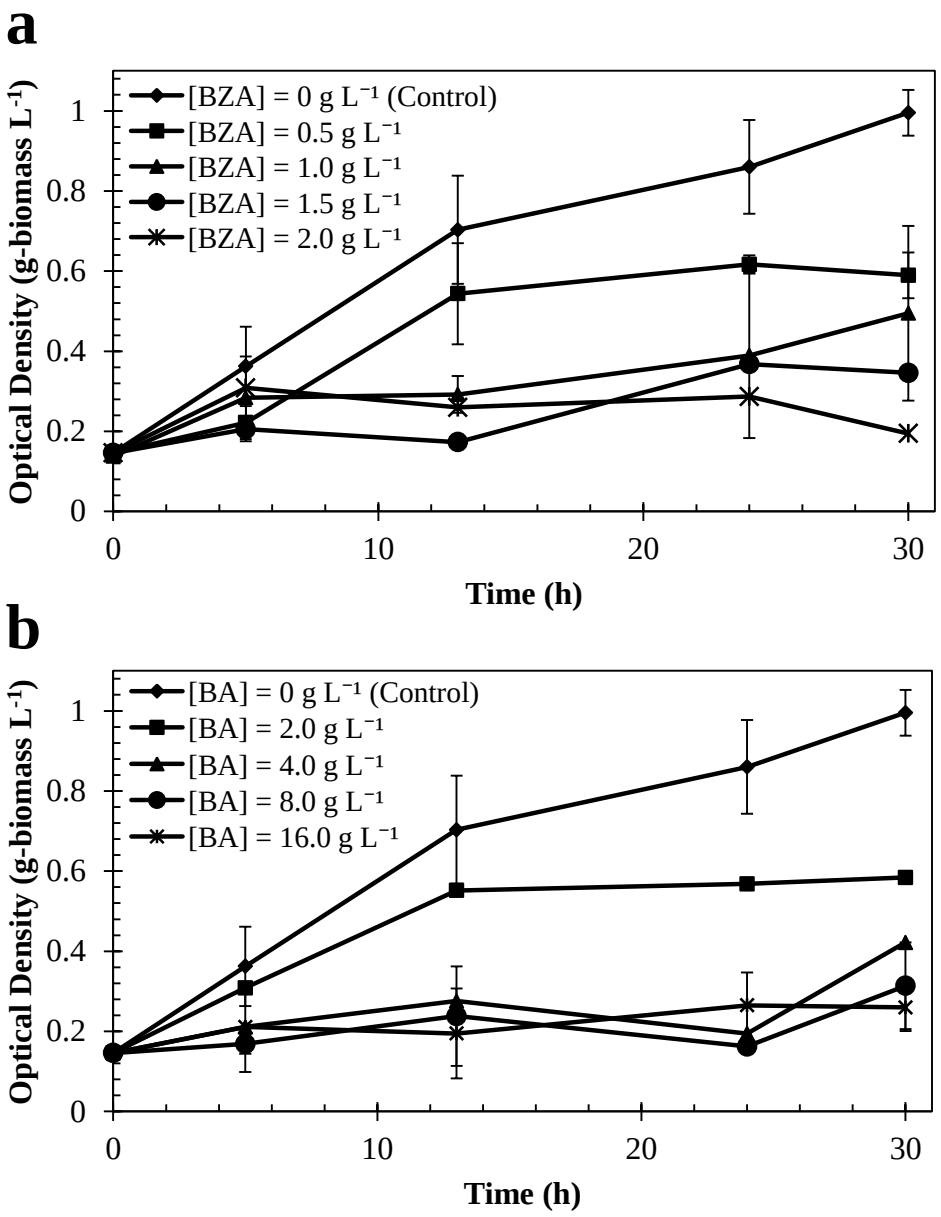

Figure 4-2: Optical density as a function of time with 10 g L^{-1} sorbitol as the sole carbon source in the presence of varying benzaldehyde concentrations from 0.5 g L^{-1} to 2 g L^{-1} shown in (a) and varying benzyl alcohol concentrations from 2 g L^{-1} to 16 g L^{-1} represented in (b).

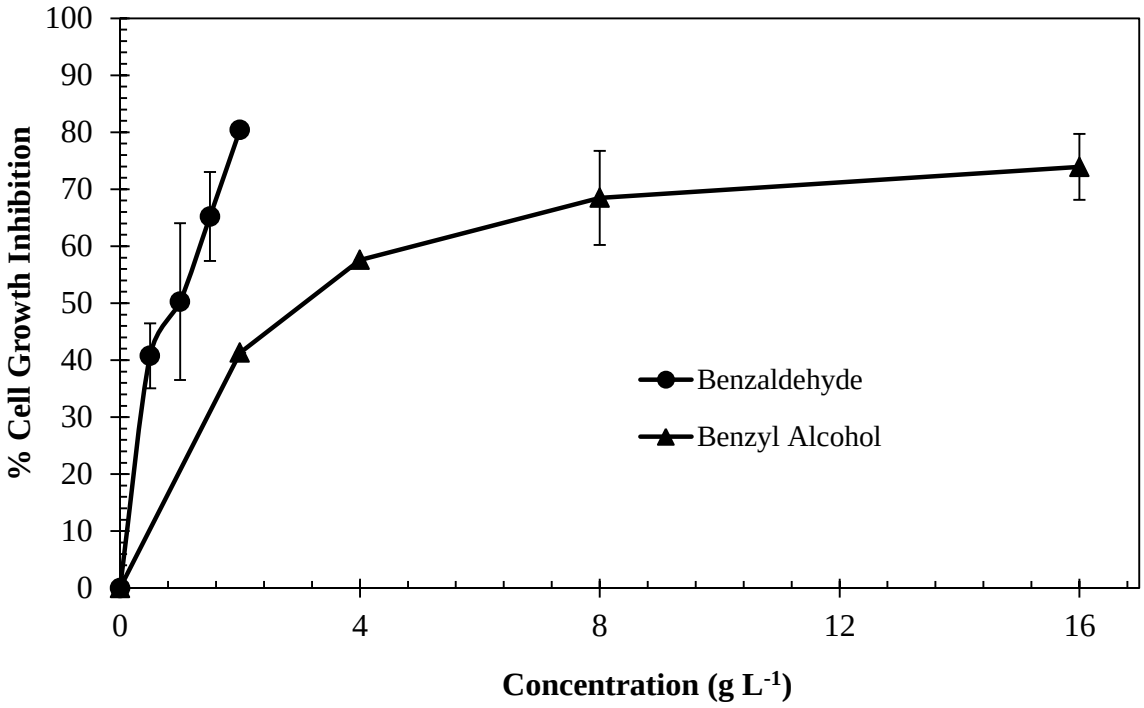

Figure 4-3: The % cell growth inhibition by benzaldehyde and benzyl alcohol at various substrate and product concentrations at 30 h.

4.5.2 Strategies for Enhanced Biotransformation Rates

Maintaining High AOX Activity

Methanol induces AOX, but in the presence of alternative primary alcohols such as BA this is not the case, as the substrate is not compatible with the MUP. This was our motivation for exploring if adding methanol to enhance AOX activity would lead to increased BZA production during the biotransformation. The methanol bolus of 1 g L^{-1} did not appear to improve the biotransformation rate (Fig. 4(a)) even though it was observed that the methanol was indeed consumed (and therefore AOX was produced) as seen by a DO drop and an increase in OD. However, due to the competition between methanol and BA at this time, methanol was naturally the preferred substrate for cell metabolism, which may explain why no BZA was produced after methanol addition. Furthermore, it can confirmed that no BZA was produced by observing that no

BA was consumed, since the biotransformation is based on a 1:1 stoichiometry of BA to BZA (Craig and Daugulis, 2013). The reason for the decrease in aqueous BZA can be explained by BZA's susceptibility to volatilization.

ATP Generation

Previous studies using *P. pastoris* for the biotransformation of alcohols reported that AOX enzyme activity decreases rapidly during a 24 h biotransformation, which could be a result of cells potentially depleting ATP reserves without replenishment during a biotransformation, since no carbon source is available for cellular metabolism (Murray and Duff, 1990). As shown in the previous strategy for generating high AOX activity, methanol cannot be introduced for ATP production during biotransformation because methanol would also generate AOX, thus confounding the results. For this reason a non-repressing substrate, sorbitol, was added as a potential strategy to increase ATP (d'Anjou and Daugulis, 2001; Inan and Meagher, 2001; Thorpe *et al.*, 1999), the results of which are seen in Figure 4-4(b). It can be observed that after the addition of 10 g L^{-1} sorbitol at approximately 10 h after the start of the biotransformation, the production rate of BZA continued to decrease.

Product Detoxification

Product detoxification was the final strategy employed to determine the reason for a declining conversion rate during the biotransformation. Solid-liquid TPPBs using polymers specifically for this system have been shown to positively affect the production of BZA and reduce product inhibition (Craig and Daugulis, 2013). Since it is important to maintain a high concentration of BA for high reaction rate conversion, the polymer Kraton D1102K was selected due to its high PC ratio of BZA relative to BA, removing the inhibitory molecule while minimizing the amount of substrate absorbed in the polymer (Craig and Daugulis, 2013). A bolus of 600g Kraton D1102K was added and the aqueous BZA concentration dropped from 0.70 g L^{-1} to 0.26 g L^{-1} in less than an hour, which shows the detoxification method was effective at quickly

removing BZA from the aqueous phase (Figure 4-4c). After the initial drop in BZA in the aqueous phase there was a notable increase in BZA concentration. The aqueous concentration of BA also decreased from 9.46 g L^{-1} to 8.72 g L^{-1} within the first hour of the polymer addition. Studies on the mass transport properties using semi-crystalline amorphous polymers have found that polymer-liquid solute equilibrium is reached very quickly, in 1-2 h (Amsden *et al.*, 2003; Fam and Daugulis, 2012), indicating that after the addition of Kraton D1102K the observed decrease in BA was due to transformation by AOX. Product detoxification using bolus polymers addition was thus effective at removing BZA from the aqueous phase, thereby allowing for the AOX conversion to resume. Consequently, a strategy of continuous BZA removal by pervaporation was our next approach.

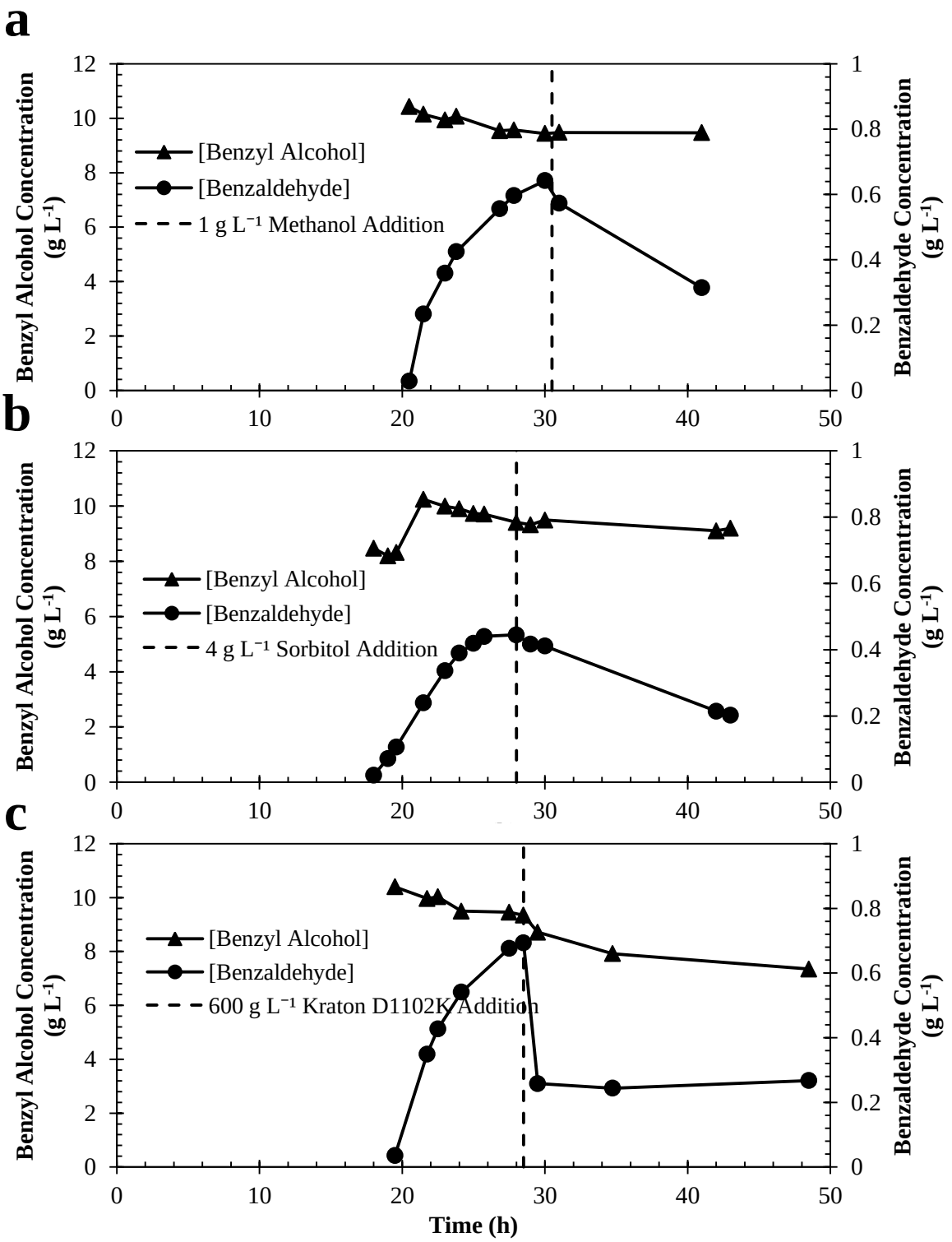

Figure 4-4: Strategies for enhanced bioproduction reaction rates of benzaldehyde (a) AOX activation by 1 g L^{-1} methanol addition (b) ATP cycling by 10 g L^{-1} sorbitol addition (c) Detoxification of benzaldehyde by addition of 600 g Kraton D1102K polymer.

4.5.3 Characterization of Pervaporation Tubing

Although the above results showing that product removal by polymer addition was successful at alleviating BZA toxicity, continuing to add polymer beads to a bioreactor is not a practical option, and therefore an integrated *in situ* pervaporation method was developed using Hytrel polymer tubing for continuous product recovery. After considering the various polymer properties shown in Table 4-1, the polymer selected for this study was a thermoplastic polyester elastomer Hytrel 3078 because it had the highest tested PC for BZA, low water absorption, and lowest flexural modulus for ease of coiling in the bioreactor. Traditional pervaporation methods applied in fermentation processes have tended to separate components using a flat membrane external to the bioreactor while the membrane is under vacuum for the vapour phase to permeate by evaporation. However, in this study, *in situ* pervaporation was performed with tubing membrane internal to the bioreactor with the driving force controlled by the gas flow rate, where permeate evaporates after permeation through the membrane. Overall, in comparison with traditional pervaporation using flat membranes, this integrated method of pervaporation allows for greater flexibility in operation by control of the gas flow rate, increased availability for mass transfer area, and instantaneous product removal.

The parameters tested for the characterization of the Hytrel 3078 tubing membrane were the permeate gas flow rate and available membrane surface area shown in Figure 4-5. The results from permeate gas flow rate, 5(a) show that as the permeate flow rate increased from 0.8 L min^{-1} to 4 L min^{-1} the concentration of BA decreased due to initial absorption into the polymer and plateaued at all three permeate flow rates. The BZA concentration in the aqueous phase decreased at a greater rate than BA partly due to the higher polymer affinity for BZA and BZA's greater evaporation rate into the gas phase. As the permeate flow rate increased from 0.8 L min^{-1} to 4 L min^{-1} the average flux removal of BZA increased from 2.1 g $m^{-2}h^{-1}$ to 3.3 g $m^{-2}h^{-1}$. The surface areas tested were 220 cm^2, 440 cm^2 and 880 cm^2 at a permeate flow rate of 2 L min^{-1} shown in

Figure 4-5(b). It can be observed that as the available surface area increased so did the mass of polymer for available absorption of the solute molecules. The aqueous BA concentrations decrease primarily due to polymer absorption and plateaued at all three available surface areas. The aqueous BZA concentration initially decreases due to polymer absorption as the available surface area or mass of polymer increased the amount of BZA removed initially increased. However, as the available surface area increased for pervaporation there was not a significant increase in the flux removal rate of BZA, indicating that there could potentially be some diffusion limitations at higher available membrane surface areas. Available membrane surface area, also affecting polymer mass, seems to have the greatest effect on BZA removal at the beginning of the biotransformation, where the permeate flow rate has the greatest effect on the latter part of the biotransformation. Therefore, an effective strategy that could be applied for *in situ* pervaporation using tubular membrane is to use high permeate gas flow rates with a membrane surface area that fits the constraints of the bioreactor size and does not impede the functional performance of the bioreactor, such as oxygen delivery to cells and homogeneous mixing.

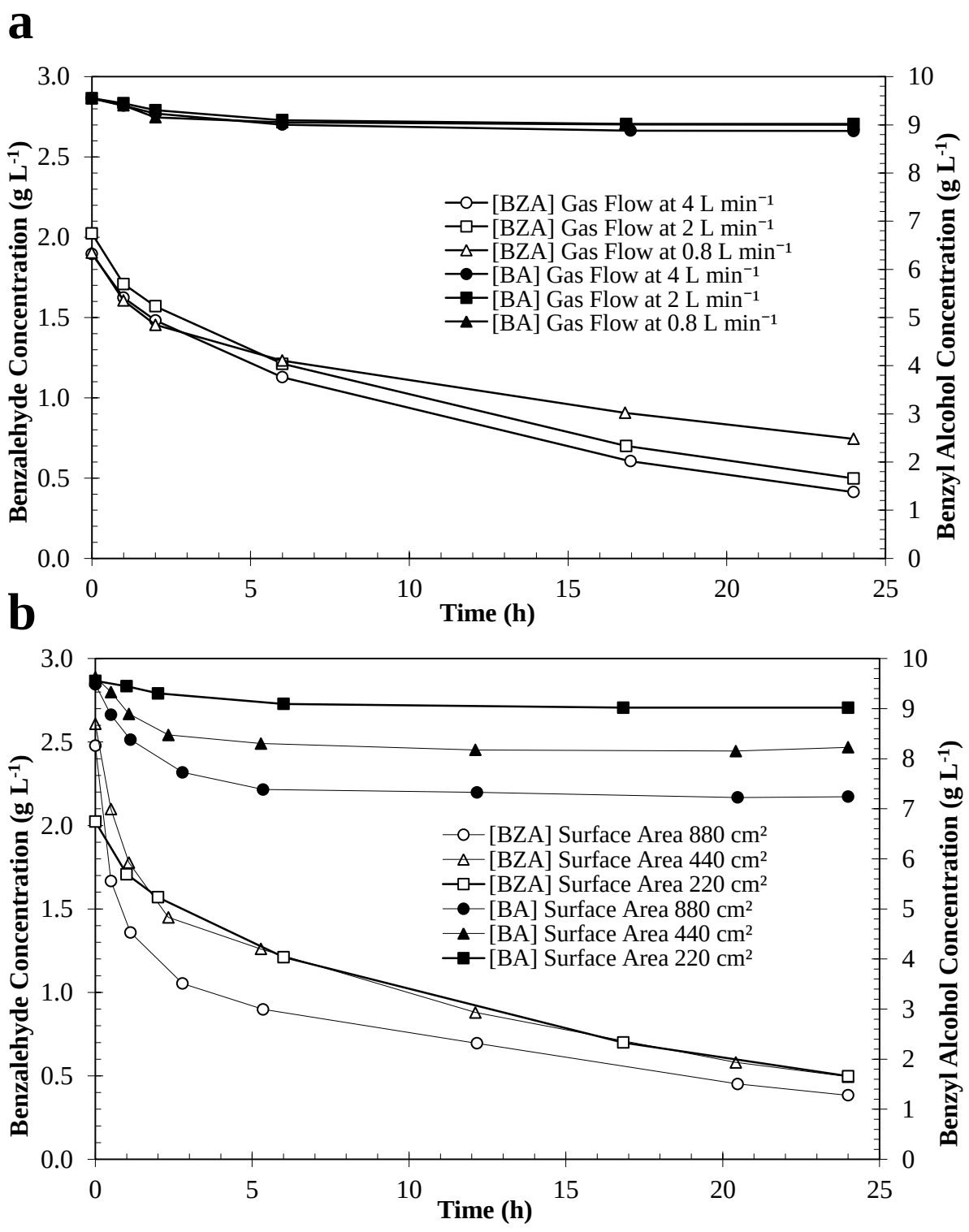

Figure 4-5: Characterization of pervaporation membrane tubing (a) Varying permeate gas flow rates of 0.8 L min^{-1}, 2.0 L min^{-1}, 4.0 L min^{-1} at a constant surface area of 220 cm^2. (b) Varying surface areas 220 cm^2, 440 cm^2, and 880 cm^2 at a constant flow rate of 2 L min^{-1}.

4.5.4 Biotransformation with Integrated *in situ* Pervaporation

Since it was BZA accumulation rather than reduced AOX or ATP levels that was determined to be the cause of a diminishing biotransformation rate, experiments were conducted with a fixed amount of tubing (having a surface area of 600 cm^2) in the bioreactor with varying pervaporation air flowrates to try to keep aqueous BZA levels low. As a point of comparison, an *in situ* pervaporation run was done at a low permeate gas flow rate and one at a higher permeate gas flow rate with the same operating conditions as mentioned above, except at pH 7.3 instead of the growth pH 5.5, a pH which enhances the biotransformation rate. The biotransformation for both the low and high pervaporation flow rate started approximately around the same time 19.5 h and 19.4 h, respectively, that is, at the end of methanol/glycerol growth period when both substrates had become depleted. The biotransformation for the low pervaporation run was extended until the aqueous BA concentration started to plateau and the high permeate gas flow run was stopped as the aqueous BZA concentration was approaching zero shown in Figure 4-6.

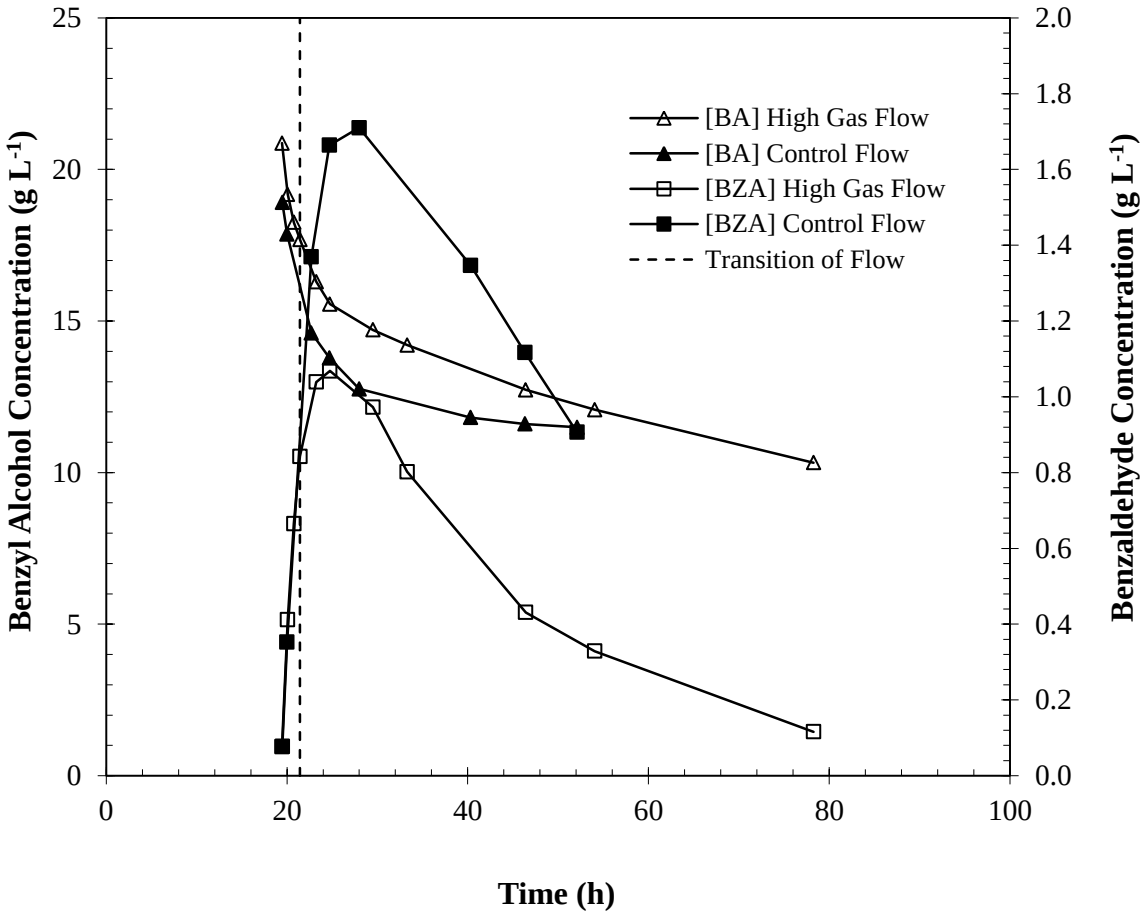

Figure 4-6: Aqueous benzaldehyde and benzyl alcohol concentration during biotransformation for *in situ* pervaporation of control run with permeate gas flow rate at 2 L min^{-1} and high permeate gas flow rate at 8 L min^{-1} transitioning to 16 L min^{-1}.

At the low pervaporation flowrate, the maximum aqueous BZA concentration was 1.71 g L^{-1} at 28 h. Since the stoichiometric ratio of BA to BZA conversion is 1:1, when the BA concentration began to plateau the decrease in BZA can be explained by pervaporation removal, and to a lesser degree evaporation from the bioreactor. The total mass of BA consumption was 22.4 g, at a volumetric productivity of 228.0 mg L^{-1}h^{-1}. A mass balance was done to quantify the BZA in the system and the effectiveness of product removal. The extraction of BZA using *in situ* pervaporation was able to remove 53.6% of the BZA produced, 19.6% was lost due to

volatilization (calculated based on Henry's Law), 13.4% was retained in the polymer phase, and 13.4% remained in the aqueous phase. It is also interesting to note that only 3.8% of the total BA was removed by pervaporation, which shows the high selectivity of the pervaporation polymer.

The high permeate gas flow rate biotransformation was done at gas flow rates of 8 L min^{-1} and 16 L min^{-1} compared to 2 L min-1 for the low flow case. The high permeate gas flow rate biotransformation follows a similar reaction rate of BZA production; however, it reached a maximum aqueous BZA concentration of 1.07 g L^{-1} at 23.25 h (Fig. 6), confirming that the changed operating condition was able to reduce BZA concentrations to below about 1 g L^{-1}, concentrations above which are known to reduce conversion rates significantly (Craig and Daugulis, 2013). The aqueous BA concentration continued to decrease indicating that the biotransformation was still operational even though the BZA concentration in the aqueous phase was decreasing, which is due to a greater rate of BZA removal from the system than production. The total mass of BA consumed was 31.3 g, at a volumetric productivity of 177.4 mg L^{-1} h^{-1}. Although the volumetric productivity decreased over time due to reduced reaction rate, the high permeate gas flow rate run showed improved results for BZA production over the low flow run since at 34.7 h there was 25.6 g L^{-1} BA consumed, equivalent to a volumetric productivity of 245.9 mg L^{-1} h^{-1}. A mass balance based on BA consumed was used to effectively quantify BZA at the end of the biotransformation and showed that 83.5% was removed by pervaporation, 9.4% was lost due to volatilization (calculated by Henry's Law), 6.0% was retained in the polymer phase, leaving 1.2% remaining in the aqueous phase. Importantly, the high permeate gas flow rate had less losses of BZA due to volatilization, which could be a result of reduced concentrations of BZA in the aqueous phase. The total removal of BA by pervaporation was 3.3%, suggesting that the pervaporation polymer is still very selective of BZA even at high flow rates.

4.5.5 Comparison of *in situ* Pervaporation and Two-Phase Systems

A performance comparison of the low and high permeate gas flow rate pervaporation systems, and a two phase system using Kraton D1102K beads from work by Craig and Daugulis (2013) is shown in Table 4-2. In the present pervaporation study we used a mass of only 37.9 g of polymer compared to 300 g in the TPPB system, showing the effectiveness of the pervaporation system in continuous rather than batch removal of BZA, using 87.4% less polymer. The TPPB system with Kraton D1102K had a maximum aqueous BZA concentration of 1.29 g L^{-1}, compared to 1.71 g L^{-1} in the low flow pervaporation system and 1.07 g L^{-1} in the high gas flow pervaporation, which shows that *in situ* pervaporation was an effective strategy in reducing BZA toxicity.

Table 4-2: Comparison of process operation: in situ pervaporation (this study) to TPPB system with Kraton D1102K.

Criteria for Comparison	**TPPB Kraton 4105K** (Craig & Daugulis, 2013)	**Pervaporation Low Flow Rate** (This study)	**Pervaporation High Flow Rate** (This study)
Operation Variables			
Initial aq. BA conc. (g L^{-1})	21.6	18.9	20.9
Total polymer mass in system (g)	300	37.9	37.8
OD growth-phase (g-biomass L^{-1})	11.8	6.1	5.9
Fermentation Time			
Growth & induction phase (h)	20.5	19.5	19.4
Biotransformation phase (h)	49.0	32.6	58.8 (34.7)[d]
System Performances			
Maximum aq. BZA conc. (g L^{-1})	1.29	1.71	1.07
Total BA consumed (g)	16.9	22.3	31.3 (25.6)[d]
Volumetric prod. BZA (mg L^{-1} h^{-1})	115.0	228.0	177.4 (245.9)[d]
Total BZA removal by pervap. (%)	-	53.6	83.5
Total BA removal by pervap. (%)	-	3.8	3.3

[d]Closest time point to the end of control pervaporation run for comparison purposes

The biotransformation using Kraton D1102K as a second phase was able to recover 14.4 g of BZA and produce a total of 16.9 g BZA, based on BA consumption, while the *in situ*

pervaporation systems had a BA consumption of 22.3 g (low flow) and 31.3 g (high flow). The BZA volumetric productivity, based on BA consumption, for the Kraton D1102K system was 115.0 mg L^{-1} h^{-1}, and using *in situ* pervaporation it was 228.0 mg L^{-1} h^{-1} for the low flow, and 245.9 mg L^{-1} h^{-1} for high gas flow rate, which is a 198% and 214% improvement in system performance.

4.6 Conclusion

This study has shown that low BZA and BA concentrations in the presence of *P. pastoris* are required in order to reduce cell inhibition, even though high concentrations of BA are advantageous to maintain a high enzymatic biotransformation rate. It was also shown that product detoxification, and not AOX or low ATP levels, had the most influence on improving the bioconversion reaction rate. By carefully selecting a thermoplastic polymer for high BZA and relatively low BA affinity, high performance pervaporation tubing was prepared by DuPont Canada, and BA/BZA flux was characterized in terms of tubing surface area and permeate gas flow rate, the latter having a greater influence on flux. The use of *in situ* pervaporation for the biotransformation of BZA provided continuous product removal and a significant increase in volumetric productivity over previous studies employing the *P. pastoris* system for BZA bioproduction.

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4.7 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.

Ariyoshi-Kishino K, Hashimoto K, Amano O, Saitoh J, Kochi M, Sakagami H. 2010. Tumor-specific cytotoxicity and type of cell death induced by benzaldehyde. Anticancer Res. 30:5069–76.

Baudot A, Marin M. 1996. Dairy aroma compounds recovery by pervaporation. J. Membr. Sci. 120:207–220.

Calibo RL, Matsumura M, Kataoka H. 1989. Continuous ethanol fermentation of concentrated sugar solutions coupled with membrane distillation using a PTFE module. J. Ferment. Bioeng. 67:40–45.

Cheetham PSJ. 1993. The use of biotransformations for the production of flavours and fragrances. Trends Biotechnol. 11:478–488.

Chen H, Ji H, Zhou X, Wang L. 2010. Green synthesis of natural benzaldehyde from cinnamon oil catalyzed by hydroxypropyl- b -cyclodextrin. Tetrahedron 66:9888–9893.

Cos O, Ramón R, Montesinos JL, Valero F. 2006. Operational strategies, monitoring and control of heterologous protein production in the methylotrophic yeast *Pichia pastoris* under different promoters: A review. Microb. Cell Fact. 5:17.

Couderc R, Baratti J, Biochimie C De. 1980. Immobilized yeast cells with methanol oxidase activity : preparation and enzymatic properties. Biotechnol. Bioeng. 22:1155–1173.

Craig T, Daugulis AJ. 2013. Polymer characterization and optimization of conditions for the enhanced bioproduction of benzaldehyde by *Pichia pastoris* in a two-phase partitioning bioreactor. Biotechnol. Bioeng. 110:1098–105.

d'Anjou MC, Daugulis AJ. 2001. A rational approach to improving productivity in recombinant *Pichia pastoris* fermentation. Biotechnol. Bioeng. 72:1–11.

Duff SJB, Murray WD. 1989. Oxidation of benzyl alcohol by whole cells of *Pichia pastoris* and by alcohol oxidase in aqueous and nonaqueous reaction media. Biotechnol. Bioeng. 34:153–159.

Fam H, Daugulis AJ. 2012. Substrate mass transport in two-phase partitioning bioreactors employing liquid and solid non-aqueous phases. Bioprocess Biosyst. Eng. 35:1367–74.

Femenia A, Rossell C, Mulet A, Caiiellas J. 1995. Chemical composition of bitter and sweet apricot kernels. J. of Agric. Food Chem. 46:356–361.

Groot WJ, Van den Oever CE, Kossen NWF. 1984. Pervaporation for similtaneous product recovery in the butanol/isopropanol batch fermentation. Biotechnol. lett. 6:709–714.

Hagedorn S, Kaphammer B. 1994. Microbial biocatalysis in the generation of flavor and fragrance chemicals. Annu. Rev. Microbiol. 48:773–800.

Inan M, Meagher MM. 2001. Non-repressing carbon sources for alcohol oxidase (AOX1) promoter of *Pichia pastoris*. J. Biosci. Bioeng. 92:585–9.

Isaza P a, 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. 2010. Bioproduction of benzaldehyde in a solid-liquid two-phase partitioning bioreactor using *Pichia pastoris*. Biotechnol. lett. 32:1649–54.

Khan TR, Daugulis AJ. 2010. Application of solid-liquid TPPBs to the production of L-phenylacetylcarbinol from benzaldehyde using *Candida utilis*. Biotechnol. Bioeng. 107:633–41.

Khan TR, Daugulis AJ. 2011. Medium composition effects on solute partitioning in solid-liquid two-phase bioreactors. J. Chem. Technol. Biotechnol. 86:157–160.

Kochi M, Takeuchi S, Mizutani T, Mochizuki K, Matsumoto Y SY. 1980. Antitumor activity of benzaldehyde. Cancer Treat. Rep. 64:21–3.

Lamer T, Spinnler HE, Souchon I, Voilley A. 1996. Extraction of benzaldehyde from fermentation broth by pervaporation. Process Biochem. 31:533–542.

Lee CY, Nakano A, Shiomi N, Lee EK, Katoh S. 2003. Effects of substrate feed rates on heterologous protein expression by *Pichia pastoris* in DO-stat fed-batch fermentation. Enzyme Microb. Technol. 33:358–365.

Li S-Y, Srivastava R, Parnas RS. 2011. Study of in situ 1-butanol pervaporation from A-B-E fermentation using a PDMS composite membrane: validity of solution-diffusion model for pervaporative A-B-E fermentation. Biotechnol. Prog. 27:111–20.

Liu Y, Sakagami H, Hashimoto K, Kikuchi H, Amano O, Ishihara M, Kanda Y, Kunii S, Kochi M, Zhang W, Yu G. 2008. Tumor-specific cytotoxicity and type of cell death induced by beta-cyclodextrin benzaldehyde inclusion compound. Anticancer Res. 28:229–36.

Lomascolo A, Stentelaire C, Asther M, Lesage-meessen L. 1999. Basidiomycetes as new biotechnological tools to generate natural aromatic flavours for the food industry. TIBTECH 17:282–289.

Mathanghi SK. 2012. Nutraceutical properties of great millet-*Sorghum vulgare*. Int. J. Food, Agric. Vet. Sci. 2:40–45.

Patel RN, Hou CT, Derelanko P. 1983. Microbial oxidation of methanol : purification and properties of formaldehyde dehydrogenase from a *Pichia sp* . NRRL-Y-11328. Arch. Biochem. Biophys. 221:135–142.

Poleo EE, Daugulis AJ. 2013. Simultaneous biodegradation of volatile and toxic contaminant mixtures by solid-liquid two-phase partitioning bioreactors. J. Hazard. Mat. 254-255:206–13.

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

Thorpe ED, d'Anjou MC, Daugulis AJ. 1999. Sorbitol as a non-repressing carbon source for fed-batch fermentation of recombinant *Pichia pastoris*. Biotechnol. lett. 21:669–672.

Vane LM. 2005. A review of pervaporation for product recovery from biomass fermentation processes. J. Chem. Technol. Biotechnol. 80:603–629.

Watanabe K, Kyo S. 1992. Pervaporation performance of hollow-fiber chitosan-polyacrylonitrile composite membrane in dehydration of ethanol. J. Chem. Eng. Jpn. 25:17–21.

Wilson CL, Solar JM, Ghaouth A El, Fravel DR. 1999. Benzaldehyde as a soil fumigant, and an apparatus for rapid fumigant evaluation. HortScience 34:681–685.

Zhu T, You L, Gong F, Xie M, Xue Y, Li Y, Ma Y. 2011. Combinatorial strategy of sorbitol feeding and low-temperature induction leads to high-level production of alkaline β-mannanase in *Pichia pastoris*. Enzyme Microb. Technol. 49:407–412.

Ziegler E, Ziegler H. 1998. Flavourings: production, composition, applications, regulations. *New York*. Weinheim; New York; Chichester; Brisbane; Singapore; Toronto: Wiley-VCH.

Chapter 5

Conclusions and Recommendations for Future Work

5.1 Conclusion

The current work investigated benzaldehyde (BZA) and benzyl alcohol (BA) inhibition on the alcohol oxidation reaction using the biocatalysis *Pichia pastoris*. The shake flask inhibition experiments showed that the performance of the system, based on BA consumption rate, seems to accelerate in the presence of BA, suggesting that BA is significantly less inhibitory on the reaction rate than BZA at concentrations from 2 to 14 g L^{-1}. The highest rates for biotransformation were found when no BZA was present at all concentrations of BA, and as the BZA concentration increased (0 to 3 g L^{-1}) there was a significant inhibition to the biotransformation reaction rate. These findings demonstrate the degree of BZA inhibition on the biotransformation using *P. pastoris* and the need for product removal strategies, which prompted the motivation for subsequent aspects of this study.

Additionally, as confirmation for the first time, the mechanism of solute sorption by soft amorphous polymers in the application of TPPBs was demonstrated. The testing of the partition coefficients (PCs) for BZA and phenol with polymer beads Hytrel 8206, Elvax 40W, Pebax 2533 at different surface areas determined that the sorption was a function of the mass of polymer, *absorption*, and not the surface area, *adsorption*. These results also validate previous articles published on polymer-based TPPBs and also provide important information for polymer selection strategies.

Subsequently, the current work improved the overall fermentation time using *P. pastoris* for the biotransformation of BZA and system performance for the bioproduction of BZA using TPPBs. Previous research using *P. pastoris* for the production of BZA in TPPBs (Jain *et al.*, 2010) experienced long growth phase when the cells were grown on methanol alone and unutilized substrate in the polymer phase. This work demonstrated a 3.5 fold improvement in reduced time required before the biotransformation via mixed substrate feed strategy containing glycerol and methanol: glycerol providing fast cell growth and methanol inducing the AOX1 and AOX2 promoter. Furthermore, a polymer selection strategy was developed to reduce the unutilized substrate in the polymer phase, and provide a high biotransformation reaction rate while reducing inhibitory product. PC tests for BZA and BA in different polymers focused on the *ratio* (BZA:BA) of the two compounds. The polymer Kraton D1102K was selected based on a high BZA:BA PC *ratio* for the removal of the inhibitory compound while minimizing the amount of substrate absorbed into the polymer. These findings applied in a TPPB showed improved BA consumption rates, 3.4 fold increases in total BZA (14.4 g vs. 4.2 g) relative to single phase operation, and more than double the volumetric productivity compared to previous studies using TPPBs for BZA production.

Additionally, substrate and product inhibition on *P. pastoris* cellular growth, and the impact of low AOX and low ATP levels were investigated to determine why the biotransformation stops. The shake flask cell growth inhibition experiments showed that both BA and BZA are significantly toxic to *P. pastoris*. The half maximal inhibition concentration was found to be 0.98 g L^{-1} for BZA and 2.95 g L^{-1} for BA. Although, BA was found to be better tolerated by the cells at higher concentrations than BZA, low BA concentrations are required for

P. pastoris cell growth, which defeats the purpose of using high BA concentrations for producing large amounts of BZA. The use of substrate feeding (methanol or sorbitol) during the biotransformation showed that the cells were not limited with respect to low ATP and low AOX, but by product toxicity, which provided motivation for work on continuous BZA removal by pervaporation.

The use of Hytrel 3078 tubing, selected for its high PC for BZA, low water absorption and flexural modulus, for integrated pervaporation was demonstrated and characterized for the first time. The Hytrel 3078 tubing, fabricated by DuPont Canada, was assessed in terms of its BA and BZA fluxes based on tubing surface area and permeate gas flow rate, and showed permeate gas flow rate has a greater influence on the flux over long periods compared to tubing surface areas. Furthermore, the polymer's high selectivity of BZA over BA was not influenced by tubing surface area and permeate gas flow rate, which was important for continuous product removal by pervaporation.

Finally, the use of integrated *in situ* pervaporation for the biotransformation of BZA was demonstrated for continuous product removal. In comparison to previous studies using TPPBs for the biotransformation of BZA using *P. pastoris*, the integrated pervaporation approach used 87.4% less polymer (37.9 g *vs.* 300 g) compared to the TPPB system employing Kraton D1102K as polymer beads. Furthermore, the TPPB system reached a maximum aqueous BZA concentration of 1.29 g L^{-1} with a total mass of 16.9 g BZA produced, based on BA consumption, in comparison to the integrated pervaporation reaching a maximum BZA concentration at 1.07 g L^{-1} using the high gas flow pervaporation with a total mass of 31.3 g BZA produced. Consequently, the use of continuous product removal by integrated pervaporation improved the

volumetric productivity by 214% over previous studies employing the *P. pastoris* system for BZA bioproduction.

5.2 Recommendations for Future Work

Although the current work has demonstrated enhanced bioproduction of BZA using *P. pastoris* by integrated pervaporation, there is still further research that can be done using factorial design in enhancing tubing performance in the pervaporation system. There are many polymer tubing parameters, such as length to volume ratio, tubing dimensions inner diameter and thickness, and permeate flow rates, that can be optimized to potentially provide performance improvements. As an example, using polymer tubing with smaller thickness lengths could potentially improve the mass transfer diffusion rates, thus provide higher flux for BZA removal from the aqueous phase, reducing the need for high permeate flow rates and benefiting the downstream recovery process. The downstream recovery of BZA could also be optimized as the current scheme for BZA recovery is significantly low, which could be potentially due to high permeate flow rates stripping BZA from the condenser system before it can be condensed and even as it is condensed. Future work should also consider the condenser design and real-time methods for measuring BZA concentration to evaluate the pervaporation performance.

Additionally, as mentioned in Chapter 2, there are three types of host strains of *P. pastoris* that could potentially improve the biotransformation for BZA production. The use of methanol utilization deficient strains is employed for heterologous protein production and can also be induced to express AOX production at high levels from the AOX1 promoter. The methanol utilization plus phenotype could potentially be useful because it has a similar growth rate on methanol as the wild type and contains both the AOX1 and AOX2 genes.

In Chapter 3 the polymers selected for testing target molecule PC in polymer had been by a somewhat random "off-the-shelf" approach. Future work in the area of solute and polymer selection would potentially be useful and should be investigated using a more rigorous first principles' approach as previously done for liquid-liquid partitioning bioreactors (Bruce and Daugulis, 1991). A recent study using first principles' thermodynamic methods Hildebrand solubility parameter, Hansen solubility parameter, and infinite dilution activity coefficient predictions in comparison to measured PC values has shown some promise for rational polymer selection (Poleo and Daugulis, 2013), however a detailed polymer strategy using such methods is still lacking.

5.3 References

Bruce LJ, Daugulis a J. 1991. Solvent selection strategies for extractive biocatalysis. Biotechnol. Prog. 7:116–24.

Jain AN, Khan TR, Daugulis AJ. 2010. Bioproduction of benzaldehyde in a solid-liquid two-phase partitioning bioreactor using *Pichia pastoris*. Biotechnol. Lett. 32:1649–54.

Poleo EE, Daugulis AJ. 2013. A comparison of three first principles methods for predicting solute-polymer affinity, and the simultaneous biodegradation of phenol and butyl acetate in a two-phase partitioning bioreactor. J.Chem.Technol. Biotechnol.:In Press.

Appendix A

Partition Coefficient for Additional Polymers Tested

Table A- 3: Addition list of copolymers tested for benzaldehyde and benzyl alcohol partition coefficients.

Polymer	Partition Coefficient for Benzyl Alcohol	Partition Coefficient for Benzaldehyde
Desmopan 9370A	9.7	41.4
Hytrel G3548W	12.1	39.6
RiteFlex 425	8.4	39.3
Hytrel G4078W	8.3	35
Elvax 360	3.1	24.1
Kraton D1184 KT-N-P	0.6	18.7
Kraton D0243 KT-N-P	0.5	17.4
Kraton SBR, D4150K	0.5	15.9
Silicone Rubber	1.24	9.9
Kraton G1657	0.1	9.8
Nylon 6-6	2.9	5.6
LDPE 8200	0.0	5.4
Flexomer DFDA 1086 NT (LLDPE)	0.0	4.3
Elvax 3175	3	1.4
Zytel 42A	0.3	0.6

Table A- 4: Addition list of homopolymers tested for benzaldehyde and benzyl alcohol partition coefficients.

Polymer	Partition Coefficient for Benzyl Alcohol	Partition Coefficient for Benzaldehyde
poly(trimethylene adipate)	8.5	35.6
Poly vinyl acetate	8.2	28.7
poly(ethylene azelate)	4.2	24.3
poly(1,4-butylene adipate)	5.4	23.2
poly(caprolactone)	3.9	23.2
Poly(trimethylene succinate)	5.4	22.9
poly(ethylene adipate)	5.0	22.7
poly(caprolactone)	2.6	15.4
poy(ethylene succinate)	2.2	8.4
polyisoprene	0.9	7.3
Ethylene, propylene, diene terpolymer	0.7	7.0
poly(acetal)	0.1	0.4

Appendix B

Calibration Curves

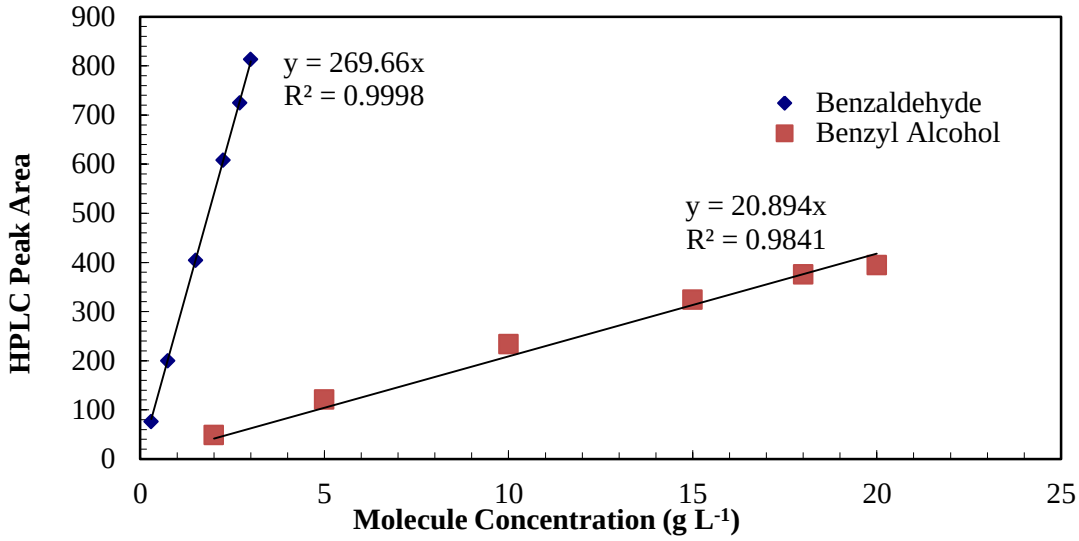

Figure B- 5: Calibration curve generated for benzaldehyde and benzyl alcohol. Linear regression was used to evaluate the trend and the corresponding equation shown.

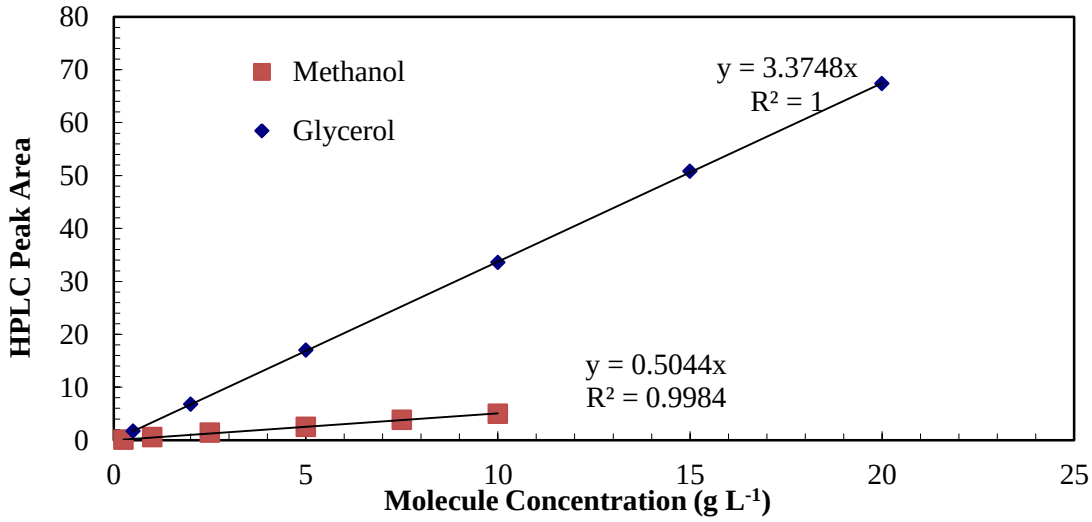

Figure B- 6: Calibration curve generated for methanol and glycerol. Linear regression was used to evaluate the trend and the corresponding equation shown.

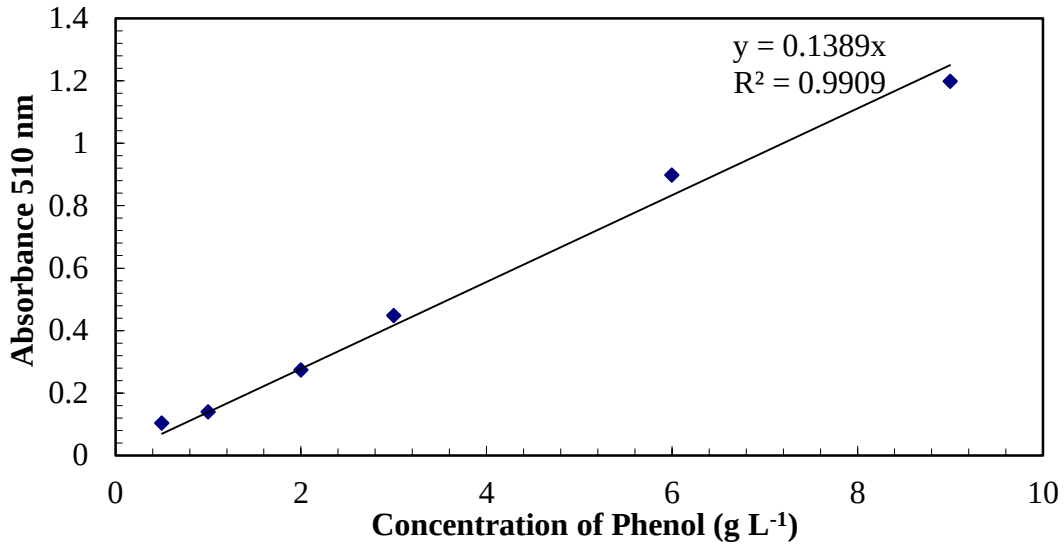

Figure B- 7: Calibration curve generated for phenol. Linear regression was used to evaluate the trend and the corresponding equation shown.

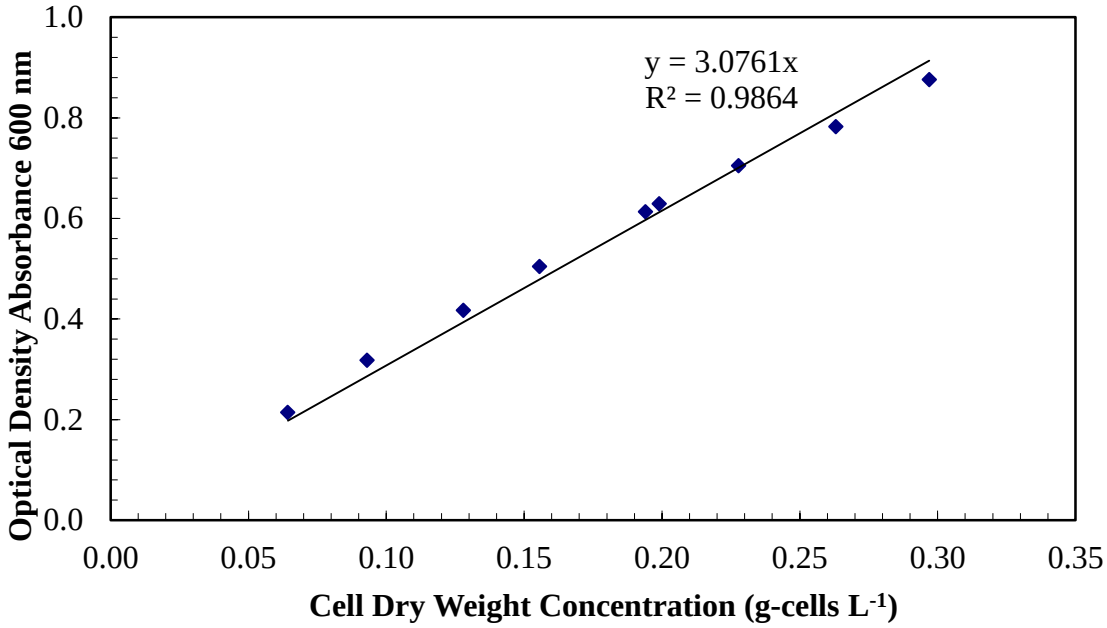

Figure B- 8: Calibration curve generated for phenol. Linear regression was used to evaluate the trend and the corresponding equation shown.