A STUDY OF POLYMER-SMALL MOLECULE INTERACTIONS FOR SOLID-LIQUID TWO PHASE PARTITIONING BIOREACTORS WITH EMPHASIS ON THE BIOPRODUCTION OF 2-PHENYLETHANOL

by

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Abstract

Biphasic systems have been studied for *in situ* product removal (ISPR), and have shown improvements in bioreactor performance. With immiscible solvents, concerns associated with solvent biocompatibility, bioavailability and operation have been identified. One alternative is a solid-liquid system in which polymer beads are used, absorbing and removing target compounds from the aqueous phase while maintaining equilibrium conditions. In such systems, the capability of a polymer to absorb the compound of interest is an important parameter. This work has identified polymer properties that may be important to the interaction between polymers and target compounds for selected biotransformation molecules including 2-phenylethanol, cis-1,3-indandiol, iso-butanol, succinic acid and 3-hydroxybutyrolactone. Furthermore, the biotransformation from L-phenylalanine to 2-phenyethanol, an important aroma compound in industry, was examined in detail.

It was found that relatively hydrophobic compounds tend to be absorbed by polymers better than hydrophilic ones based on partition coefficient tests. Since all of the biotransformation molecules tested have polar functional groups such as alcohol, acid and lactone, polar polymers such as Hytrel® performed better than non-polar polymers such as Kraton® possibly due to the hydrogen-bonding interaction between the polymer and the solute. Crystallinity and intermolecular hydrogen-bonding were also found to be important polymer properties.

Hytrel® 8206 was identified as the best working polymer to absorb 2-phenylethanol. A solid-liquid batch mode two phase partitioning bioreactor (TPPB) with 500 g Hytrel® generated an overall 2-PE concentration of 13.7 g/L, the highest reported in the current literature. This was based on a polymer phase concentration of 88.74 g/L and aqueous phase concentration of 1.2 g/L. Better results were achieved via contact with more polymers with the aqueous phase applying a semi-continuous reactor configuration. In this system, a final 2-PE concentration (overall) of 20.4 g/L was achieved. The overall productivities of these two reactor systems were 0.38 g/(L-h) and 0.43 g/(L-h), respectively. This experiment successfully demonstrated that with the appropriate selection of polymer, solid-liquid TPPB systems were able to greatly enhance bioproductions associated with end product inhibition in terms of final product concentration and productivity. The ease of operation is also attractive compared to two liquid phase systems.

Co-Authorship Statement

Chapter 3 and Chapter 4 are comprised of scientific articles that either have been accepted or are submitted for publication. In the preparation of these two manuscripts, Dr. Andrew Daugulis has edited, suggested revisions and provided technical advice.

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Table of Contents

List of Tables

List of Figures

Chapter 1

Introduction

1.1 Background

The most complex chemical reactions occur in living organisms thanks to the power of enzymes. The high selectivity, fast reaction rate, coordinated multiple steps and the ability to function at ambient conditions together make enzymes the best choices as catalyst in many cases [Schmid *et al.,* 2001]. Technologies for utilizing living cells and therefore accessing their enzymes allow us to take advantages of the remarkable catalysts created by nature. Compared to animal and plant cell cultures, microbial cell culture is easier to handle, cheaper to operate, and more adaptable to large-scale applications. Consequently, using living microorganisms as biocatalysts has been widely practiced in industry.

The development of new biosynthetic processes has increased rapidly for the past several decades from fewer than 10 industrial processes in the 1960s to more than 130 in 2002, not including traditional fermentation processes [Straathof *et al.*, 2002]. These processes include applications in the pharmaceutical, flavour and fragrance, agrochemical industries [Straathof *et al.,* 2002] and also in the emerging biorefinery opportunities.

However, compared to chemical synthesis, bioprocesses often suffer from relatively low product concentration and low productivity. One of the main causes is due to end product inhibition. Since many biosynthetic products are not produced in high quantity in the natural environment, they may become toxic to cells once exceeding a

certain threshold. This will result in the termination of a biotransformation and large liquid volume with low product concentration leaving bioreactors.

The production of an aroma compound 2-phenylethanol (2-PE) is one such example. 2-PE is an aroma compound that has a rose-like smell. Its bioproduction suffers from end product inhibition. 2-PE becomes toxic to the producing yeast species *Kluyveromyces marxianus* at 2 g/L [Fabre *et al.,* 1998]. Therefore, in an aqueous medium, the maximum product concentration possible is 2 g/L or lower, which limits the large scale bioproduction of this important flavour and fragrance ingredient.

One promising technique in solving such a problem is *in situ* product removal (ISPR). The concept is that as soon as the product is formed, it gets removed in the same operating unit. Among various ISPR approaches, the two phase partitioning bioreactor (TPPB) has shown great enhancement in reactor performance including increased final product concentration, volumetric productivity, and yields. In the presence of a second phase, either an immiscible organic solvent or solid polymer beads, removing the target compound from aqueous phase, the cells are exposed to a low concentration in the aqueous phase that is not toxic to cell growth while the product is accumulated in the sequestering phase. One of the most important factors in such a system is to what extent the sequestering phase can remove the compound of interest from the aqueous phase, which involves an understanding of the interaction between the sequestering phase (solvent or solid polymer) and the compound of interest.

1.2 Objectives

This research has focused on the possibility of applying solid-liquid TPPB systems to various biotransformation processes across different industrial sectors by

examining the extraction of these compounds by solid polymer beads. The work aims to better understand what polymer properties may be important to the uptake/absorption of such compounds. This is evaluated based on the partition coefficient, a measurement of the polymer phase concentration over the aqueous phase concentration of a certain molecule.

The second part of the research involves the bioproduction of one of the selected compounds, 2-phenylethanol to examine bioreactor performance and operation of this specific application using a solid-liquid two phase partitioning bioreactor and the yeast species *Kluyveromyces marxianus*.

1.3 References

Schmid A. Dordick JS, Hauer B, Kiener A, Wubbolts M and Witholt B. 2001. Industrial biocatalysis today and tomorrow. Nature **409**: 258-268.

Straathof A. J.J, Panke S, and Schmid A. 2002. The production of fine chemicals by biotransformations. Current Opinion in Biotechnology **13**: 548-556.

Fabre CE, Blanc PJ, and Goma G. 1998. Production of 2-phenylethyl alcohol by Kluyveromyces marxianus. Biotechnology Progress **14**: 270-274.

Chapter 2
Literature Review

2.1 Living Microorganisms as Biocatalysts

Living cells possess a wide range of enzymes. Some are responsible for catabolic activities in which large molecules are broken down to small building blocks and release energy, while some can do the opposite to synthesize complex molecules by anabolic activities to meet their biological needs. Likewise, in industry, microorganisms can be used to degrade compounds that are toxic to the environment, and can also be used to synthesize value-added products that are of commercial interests. In this review, the main focus will be on biosynthesis which will be discussed in more detail.

Biocatalysts can either be used as isolated enzymes, whole cells, or in combination, and they exist either as free components or immobilized within a bioreactor. Isolated enzymes are usually preferred when few reaction steps are involved such as hydrolytic or isomerization reactions [Schmid *et al.*, 2001]. However, if the reaction requires multiple steps or involves cofactors which must be regenerated through metabolically active cells, then whole cell reactions are preferred over isolated enzymes [Schmid *et al.,* 2001].

2.2 Biosynthesis in Industry

Compared to biodegradation, biosynthesis is a more "value-added" process in that simple precursor molecules are converted to valuable commercial products by metabolic activities. According to a survey of the fine chemical industry done by Straathof *et al.* (2002), more than 50% of biosynthetic applications are in the pharmaceutical sector, followed by food, agrochemical, and other industrial sectors. They also concluded that

44% of the biotransformations used hydrolase type of enzymes and 30% of the processes used redox biocatalysts. Various classes of chemicals can be produced biologically including alcohols, amino acids/peptides, acids, sugars and complex natural products such as antibiotics [Panke *et al.,* 2005].

Recently, the concept of biorefinery has gained much attention because it is a potential solution to the current "oil crisis" around the world. "Biorefinery" refers to the use of biomass as feedstocks to produce transportation fuels, valuable chemical building blocks (platform chemicals) and commodity products. Unlike the petroleum-based industries in which chemical conversion is dominant, many of the biorefinery processes explore fermentation and enzymatic bioconversions of the feedstocks to products. The production scale of commodity chemicals in this category is much larger than that in the fine chemical industry.

To compete with well established chemical processes, bioprocesses must find a way to lower their costs. The critical technical parameters that affect the cost include the volumetric productivity, product concentration, yield and biocatalyst consumption [Straathof, 2003]. In the fine chemical sectors, product concentrations are usually above 2 g/L and typically over 30 g/L with a volumetric productivity range from 0.0002 g/(L-h) to over 50 g/(L-h) [Straathof *et al.,* 2002]. In the biorefinery sector, the proposed minimum productivity of chemical building blocks should be well above 2.5 g/ (L-h) [DOE Report, 2004]. The number should be higher for applications in transportation fuels and commodity chemicals derived from biorefinery processes.

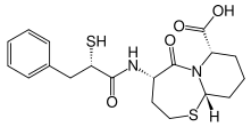
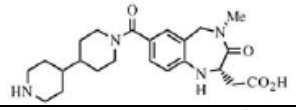
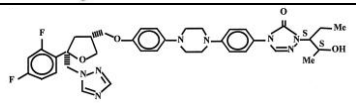
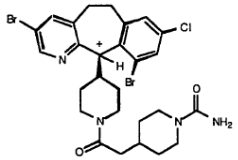
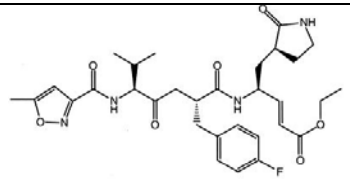
2.3 Advantages of Biosynthesis and Applications

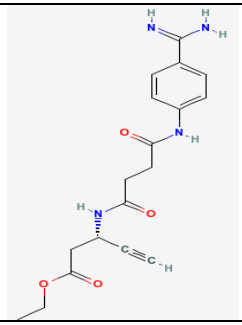
Biocatalysts have many potential advantages over traditional catalysts resulting in industry's renewed interests. These advantages can be summarized as: the ability to interact with complex substrates to form complex products; the high stereo-, regio- and chemo- selectivity; the ambient reaction conditions; and the use of renewable feedstocks [Schmid *et al.,* 2001]. These advantages can be further illustrated by examples from different industrial sectors.

2.3.1 Pharmaceutical Industry

The pharmaceutical industry benefits from biosynthesis because enzymes can interact with complex substrates to form complex products, such as in the production of antibiotics. More commonly, this industry prefers bioprocesses because of the high enantio- and regio- selectivity that they possess. Pharmaceuticals reaching the market are often structurally complex with multiple chiral centres [Lye and Woodley, 1997]. Enantiomerial purity is often critical to the quality of a drug, since higher specificity means fewer side effects and greater safety to the patients. The highly specific biotransformations save the difficult steps of separating racemic enantiomers in downstream which is usually complex and costly. In many cases, the enantio- selectivity has been achieved by biosynthesis of enantiomerially pure precursor molecules. In fact, in 2000, 35% of pharmaceutical intermediates were chiral and this number is expected to increase to 70% by 2010 [Pollard and Woodley, 2006]. Examples of pharmaceuticals that have been formed through biotransformations are listed in Table 2-1. All of these compounds are chiral molecules while some have multiple chiral centres.

Table 2-1: **Examples of pharmaceuticals formed by biological processes in industry**

Name of Compound	Purpose	Structure	Annual Production (kg)	[Product](g/L)	Productivity[g/(L-h)]
Omapatrilat	Treats hypertension		200	41	1.6
Lotrafiban	Platelets inhibitor, treats thrombosis		>100	25	10
SCH 56592	antifungal agent		>30	70	14
SCH 66336	a farnesyl transferase inhibitor in development for cancer therapy		25	3	0.1
AG7088	Human Rhinovirus inhibitor, antivirus		15	40	13.2

Xemilofiban	inhibits platelet aggregation, treats cardiovascular disease		8	35	1.6

Adapted from Straathof, Panke and Schmid. 2002. The production of fine chemicals by biotransformation. Current Opinion in Biotechnology **13:** 548-556.

2.3.2 Flavour and Fragrance Industry

Many flavour and fragrance products can be synthesized by chemical routes, however, biological routes are becoming more and more attractive. The driving forces for this increase include consumers' demand for naturally produced ingredients, mild reaction conditions to preserve unstable products, and high selectivity towards highly specific precursors [Schrader *et al.,* 2004]. Some industrial examples in the flavour and fragrance industry include the biosynthesis of vanillin [Priefert *et al.,* 2001], the most widely produced flavour chemical by far [Schrader *et al.,* 2004]; γ-decalactone, an oily-peachy flavour and fragrance ingredient [Pagot *et al.,* 1997], several types of carboxylic acids (propionic acid, butyric acid, isobutyric acid, 2-methylbutyric acid, and isovaleric acid) [Rabenhorst *et al.,* 2001], many different types of esters, and 2-phenylethanol, a rose-like aroma compound [Etschmann *et al.,* 2002]. The production scale in this sector ranges from several hundred to several thousand tons [Schrader *et al.,* 2004], and the final product concentration ranges from just over 10 g/L to nearly 100 g/L [Schrader *et al.,* 2004].

2.3.3 Emerging Biorefinery

In 2004, the Department of Energy of the U.S published a report which listed 12 "value-added" chemicals that can be derived from biomass [DOE Report, 2004]. These 12 chemical building blocks have the potential to form the majority of chemicals that are currently being synthesized from petroleum-based feedstocks. Out of the 12 compounds, 7 can be produced biologically and 1 is preferred to be produced biologically. In addition, bioprocesses to produce transportation fuel such as ethanol and butanol from biomass are also a promising technology to benefit the environment, and related projects are currently

underway. Commodity products can be derived from biomass feedstocks through biotransformation as well, such as the production of citric acid, itaconic acid and lactic acid.

2.4 Limitations of Biosynthesis Processes

Despite the superior advantages of biocatalysts, their industrial applications are still limited compared to chemical processes. There are historical reasons, since chemical processes have been developed and adapted for a long time, and the switch to bioprocesses is perceived to be costly. Poor production efficiency in bioprocesses including low final product concentration, and low productivity is also a major concern. Low product concentration results in large, dilute liquid volumes which add to the cost of downstream product recovery and wastewater treatment. Low productivity is an indication of low efficiency which is often not acceptable for commercial applications. The toxic effect caused by end products and/or substrates is one of the major causes of these problems. If biological processes can be improved in terms of productivity, product concentration and yield, the cost of switching from chemical to bioprocessing may become a minor issue compared with the benefits gained.

2.4.1 Product and/or Substrate Inhibition

Many of the compounds of commercial interest are not produced naturally by microorganisms or are only produced at low concentrations. However, for economically feasible productions, high product concentration and productivity are essential. The conflict between nature and industrial requirements create a significant challenge for biotechnology. That is, the high concentration of the “unnatural” compound will most often cause an inhibitory or toxic effect to cells. In addition, inhibition can also occur

when end product is accumulated in the culture medium at high concentrations, which results in feedback control of the metabolic pathway to reduce the reaction rate. Inhibitory effects have also been observed for substrates.

In some cases, the biotransformation product causes inhibition to the cells in similar ways that an organic solvent does. For organic solvents, the major cause of molecular toxicity is the disruption of cell membranes imposed by a molecule [Heipieper *et al.*, 1994]. A relatively hydrophobic substance can dissolve in the cell membrane which also has hydrophobic component, and disturbs the integrity of the membrane leading to cell permeabilization [Lilly *et al*., 1987]. This may result in enzyme inhibition, protein deactivation, or a breakdown of transport mechanisms [Lilly *et al*., 1987]. One such example of a biotransformation product is 2-phenylethanol, an aromatic alcohol.

The toxicity effect can also be caused by the deactivation of certain enzymes. For example, in the production of a pharmaceutical intermediate, L-phenylacetylcarbinol, one of the substrates benzaldehyde can cause serious deactivation of the most important enzyme in this biotransformation, pyruvate decarboxylase [Sandford *et al.*, 2005]. This substrate inhibition can occur at a benzaldehyde concentration as low as 0.5 g/L [Long and Ward, 1989].

The inhibitory and toxic effects caused by products and/or substrates greatly affect biotransformation processes, because they limit the final product concentration to be below the toxic level, and the accumulation of end product also limits the forward reaction rate and lowers the productivity of the system.

2.4.2 Other Case Specific Problems

Other problems may not be as common as the previous one, but are very critical for some specific applications. These may include the oxygen transfer limitation encountered by aerobic biotransformations, the destructive sheer stress caused by mixing for mammalian cell cultures, and the mutation of cell cultures when the reaction is run for a long period of time. Minimizing these problems is also important for improving biosynthesis process, but is not the main focus of this review.

2.5 In situ Product Removal

To overcome or minimize the problem imposed by product inhibition, the idea of *in situ* product removal (ISPR) has been developed. Many approaches have been reported in the literature and industry for ISPR.

2.5.1 Two-phase *in situ* Product Removal

One of the promising approaches involves a two-phase partitioning bioreactor (TPPB) system which often contains an aqueous phase and an immiscible organic phase, either organic solvents or solid polymer beads. This system has been demonstrated to be very effective in the delivery of toxic pollutants for their subsequent degradation by microorganisms in the environmental field. Recently, it has also shown encouraging results in the biotransformation field for ISPR purposes [Prpich and Daugulis, 2007; Morrish and Daugulis, 2008]. In these applications, the organic phase acts as a sink, extracting toxic compounds from the aqueous phase into the organic phase. This dynamic equilibrium can bring the product concentration in the aqueous medium down to a level that is not inhibitory to cell functioning. Any increase in aqueous concentration will shift the equilibrium and result in more extraction as long as the sequestering phase is not saturated. It also manages the toxic effect caused by substrates by delivering them from

the organic phase in a controlled manner so that cells get the substrates only when they need them. The organic phase can also act as a carrier to deliver sparingly soluble substrate to cells therefore increase the bioavailability of the substrate.

Solvent selection is very important for two phase systems which may require many considerations and provide a balance between the properties of solvents. Two very important things to consider are the biocompatibility and the extraction capability for a given solvent. In addition to the abovementioned molecular toxicity that may be caused by non-biocompatible solvent, phase toxicity may also be present [Bar, 1987]. Immiscible organic solvents can form coats around cells and block nutrient diffusion or disrupt the cell wall [Bar, 1987]. The molecular and phase toxicity act together can result in inhibitory effect or even death of the microbial culture. Therefore, the test for biocompatibility is the most important aspect in solvent selection [Bruce and Daugulis, 1991]. The other aspect to consider is the extraction capability of a solvent towards the target product. This can be expressed by the partition coefficient which is defined as the ratio of the product concentration in the organic phase to the product concentration in the aqueous medium [Bruce and Daugulis, 1991].

The other issue is bioavailability because many organic solvents can also act as a carbon source for microbial cells. If the cell culture is a consortium comprised of diverse groups of microbes, there may exist some microorganisms that can feed on the organic solvent and grow more rapidly than the others, which will also result in a reduction in the amount of the second phase present in the system. Therefore, the selected solvent must be non-biodegradable. Other things to consider in solvent selection include the costs,

whether it forms emulsion with the aqueous phase, the availability of the solvent in bulk quantity and safety issues (flammable or not) [Bruce and Daugulis, 1991].

An alternative to the use of organic solvents is polymers. The use of polymers as a second phase for biosynthesis has not yet been studied widely. Recently, Prpich and Daugulis (2007) demonstrated that by using Hytrel® polymer beads, the overall productivity for the biosynthesis of 3-methylcatechol was increased from 128 mg/ (L-h) to 350 mg/ (L-h). Polymers are interesting as an alternative to organic solvent because they are usually more biocompatible than organic solvents and easy to work with for large scale application (small volume, non-flammable, easy to recycle). The problems associated with bioavailability when solvents are used are not significant for polymers because polymer beads cannot be readily utilized by microorganisms [Prpich and Daugulis, 2004]. For some special applications, such as the production of aroma compounds and food ingredients, the odour of any residual solvent is problematic and will significantly affect the product grade and lower its market value. In these cases, polymer beads are promising alternatives as they normally do not have an unpleasant odour. Furthermore, polymers can be modified to take up a target compound through monomer selection, functionalization, copolymerization, cross-linking and processing [Prpich and Daugulis, 2004]. Limitations of using polymer beads may include less efficient mass transfer and poor thermostability towards autoclaving. Nevertheless, the use of polymers as a second phase for TPPBs is a relatively new but promising technology, and there is a great deal to explore in this area. Polymer factors include: what structures, functionalities and properties dictate the uptake of a certain group of compounds and how to formulate a polymer to extract a target compound.

2.5.2 Other Approaches

Other approaches for ISPR include the use of adsorption, pervaporative extraction, reactive extraction, immobilized solvents in resin, extraction with supercritical fluid, among others [Straathof, 2003]. Please refer to this review article, Straathof *et al.* (2003), for further details.

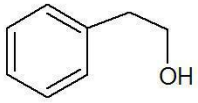
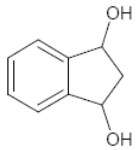
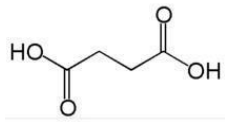
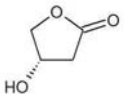
2.6 Applications of ISPR in This Thesis

To demonstrate the broad spectrum of ISPR applications, five compounds from different industrial sectors are reviewed in detail. Some useful information about these compounds is listed in Table 2-2.

The compounds selected differ in their molecular complexity, market value, and production scale. They cover different industrial sectors from commodity goods to high value chemicals. The demonstration of how their production can benefit from ISPR in terms of product concentration and productivity will show the potential applications of this technology to a wide range of products. Also, due to the different functional groups in their chemical structures, searching for the best second phase will require an understanding of target compound-second phase interaction, especially for the use of polymers.

The first compound examined, 2-phenylethanol (2-PE), is from the flavour and fragrance industry. As mentioned before, many compounds from this sector can be chemically synthesized. However, the products made biologically have the advantage of being marketed as “natural”. Taking 2-phenylethanol as an example, the “natural” product sells at 300 times the price of chemically formed 2-PE [Etschmann *et al.*, 2002].

Table 2-2: **A list of properties of the selected compounds**

Name of compound	Industrial application	Structure	CAS number	K_{ow}	Melting point (^oC)	Solubility in water
2-phenylethanol	Flavour and fragrance ingredient		60-12-8	37.15	-27	20 g/L @25^oC
cis-1,3 -indandiol	A structural isomer of a pharmaceutical intermediate		172977-38-7	6.31	107-108	66 g/L*
iso-butanol	Potential transportation fuel		78-83-1	5.89	-108	75 g/L @30°C
succinic acid	Biorefinery chemical building block		110-15-6	0.18	185	1000 g/L water @ 100^oC
3-hydroxybutyrolactone	Biorefinery chemical building block		58081-05-3	0.23	N/A	N/A

Note: * data taken from Lendon N. Pridgen, J Org.Chem. 1974. **39:** 3059-306

The second compound examined is a structural isomer (cis-1,3-indandiol) of a pharmaceutical intermediate, cis-1,2-indandiol. cis-1,2-indandiol is a chiral intermediate for a commercial drug developed by Merck & Co. for treating HIV [Buckland *et al.*, 1999]. Compounds from this category can be characterized as being complicated to synthesize, possess high market value, and are produced at a relatively small scale.

Butanol as a transportation fuel has gained much attention recently. Compared to ethanol, butanol has many advantages, such as high energy content [Qureshi and Blaschek, 1999], low vapour pressure, and better miscibility with gasoline [Ladisch, 1991]. Like ethanol, butanol can be produced from biomass. The production scale of butanol as a transportation fuel will be large, in the order of multi-million tons/year.

Potential biorefinery building block compounds are selected from hundreds of candidate chemicals based on the product market, chemical properties and industrial experience [DOE Report, 2004]. Among them, succinic acid and 3-hydroxybutyrolactone have been selected to further investigate the possibility for ISPR. Important derivatives from succinic acid include butanediol (BDO), tetrahydrofuran (THF) and gamma-butyrolactone (GBL) via selective hydrogenation/reduction of this 4-C carboxylic acid. 3-hydroxybutyrolactone is currently being used as a pharmaceutical intermediate [DOE Report, 2004]. It can also be used as a precursor to synthesize acrylate-lactone, potential use for this derivative may include new polymers [DOE Report, 2004].

2.7 Flavour and Fragrance - 2-Phenylethanol

2-phenylethanol (2-PE) is an aromatic alcohol that has a rose-like odour. This highly popular fragrance makes 2-PE a commonly-used ingredient in cosmetic and food industries [Stark *et al.*, 2003 (2)]. The vast majority of 2-PE currently in use is produced chemically via three major pathways: Friedel-Craft reaction, catalytic reduction of styrene oxide and as a by-product of the production of propylene oxide [Etschmann *et al.,* 2002]. A major problem encountered in chemical synthesis is the costly product purification process. Quite often, the solvents and by-products impart off-odours in the final product and therefore influence the grade of 2-PE. Only after costly removal of these solvents and by-products, can the product be sold as prime quality [Etschmann *et al.,* 2002].

2-PE has been known to be a microbial metabolite, and therefore an alternative option is to produce 2-PE via biological routes. By taking advantage of a highly-selective biocatalyst, not only can the influence of by-products be minimized, but also the final product can be classified as a naturally-derived product which adds to its marketing advantages. Especially for food applications, consumers are very sensitive to the label of artificial or natural, and it is one of the reasons why bioproduction of flavours is becoming increasingly attractive [Stark *et al.,* 2002].

In the 1990s, the world's annual production of 2-PE was estimated to be 7,000 tons [Somogyi, 1996] out of which the biologically-produced quantity is only about 0.5 ton/year [Etschmann *et al.*, 2002]. 2-PE produced chemically is

available at a price around $3.5 / kg while the same material labeled “natural” can be sold for around $1,000 / kg [Etschmann *et al.,* 2002].

2.7.1 Bioproduction

It is not surprising that 2-PE can be extracted from rose petals; however, the extremely low concentration in one petal of the flower makes it very challenging for industrial applications. Plant cell cultures of *Rosa damascena* have also been studied, but the yield is low (0.013 mg 2-PE/kg) and the production can take several weeks to complete [Banthorpe *et al.*, 1988].

Filamentous fungi have also been reported to be able to produce 2-PE. Fabre *et al.* (1996) studied *Ischnoderma benzoinum* and reported a final product concentration of 280 mg/L in 20 days. Studies on *Geotrichum penicillatum* by Janssens *et al.* (1988) found that in 99 hours, a final 2-PE concentration of only 0.0142 mg/L was generated. Lomascolo *et al.* (2001) had shown that in the presence of L-phenylalanine (L-Phe), an amino acid precursor, filamentous fungi *Aspergillus niger* was able to produce 2-PE with a concentration of 1.375 g/L and a productivity of 0.153 g/ (L-d) which was considered high compared with other fungi species.

The bacteria *Microbacterium sp*. and *Brevibacterium linens* were found to produce 2-PE, however, the concentrations were in the range of a few mg/L [Jollivet *et al.*, 1992].

The most commonly studied and used microorganisms that produce 2-PE are yeasts. Etschmann *et al.* (2003) screened 14 different types of yeast to study their ability to produce 2-PE in molasses medium, including different strains of

Kluyveromyces marxianus, different strains of *Saccharomyces cerevisiae, Zygosaccharomyces rouxii, Clavispora lusitaniae, Pichia anomala, Pichia membranaefaciens, and Schizosaccharomyces pombe*. They concluded that the most productive strains were *Kluyveromyes marxianus* CBS 600 and CBS 397.

2-PE is a normal metabolite of microorganisms especially in yeasts; however, the low concentration produced by regular fermentation processes is very low. This problem can be managed by adding the amino acid L-Phe as a precursor to the medium [Albertazzi *et al.,* 1994]. L-Phe is converted to 2-PE in yeast cells by transamination of the amino acid to phenylpyruvate, decarboxylation to phenylacetaldehyde and reduction to 2-PE [Etschmann *et al.,* 2002]. The pathway is shown by Figure 2-1.

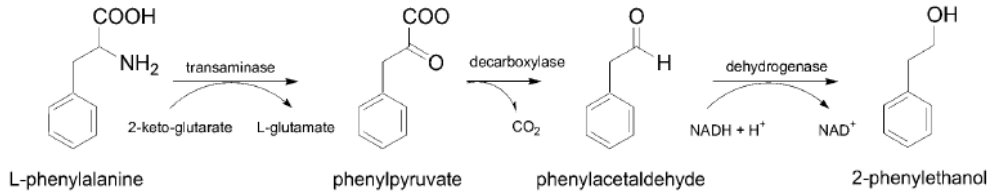


Figure 2-1: Ehrlich pathway in yeast cells which converts L-phenylalanine to 2-phenylethanol [Etschmann *et al.* 2002].

This metabolic pathway was first described by Ehrlich in 1907 and is therefore called the Ehrlich pathway [Etschmann *et al.,* 2002]. Etschmann and Schrader (2006) reported that by providing L-Phe as the sole nitrogen source in high excess, the product concentration and productivity could be enhanced. L-Phe is the raw material of this synthesis, and the fact that it is also a microbial-derived product allows the production of 2-PE to be called a "natural" product

[Etschmann *et al.*, 2002]. The biotransformation of L-Phe to 2-PE is strictly growth-associated and the maximum conversion occurs during the exponential growth phase [Stark *et al.,* 2002]. However, Wittmann *et al.* (2002) showed that although the highest product formation rate was under the cell growth phase, during the stationary phase of *Kluyveromyces marxianus* growth, there was still some accumulation of 2-PE. Therefore, they concluded that Ehrlich pathway was also active under the non-growing conditions to a small extent.

2.7.2 Product Inhibition

Like many other biological processes, the biotransformation of L-Phe to 2-PE suffers from end product inhibition. 2-PE is an aromatic alcohol with a K_{ow} (the partition coefficient of a substance between octanol and water) value of 37.15. The primary site of its toxic effect is the cell membrane, whose structure can be altered by 2-PE causing cell leakage and disruption of the transport system [Etschmann *et al.,* 2002]. Different yeast strains were found to have different tolerances toward this toxic effect. Fabre *et al.* (1998) found that 2.0 g/L of 2-PE was able to cause complete growth inhibition for *Kluyveromyces marxianus*, while the Stark group reported an aqueous toxic concentration of 4 g/L for *Saccharomyces cerevisiae* growth [Stark *et al.,* 2002].

The bioconversion to 2-PE has been improved over the past several years in single-phase batch production; however, there exist small variations in the final 2-PE concentration reported using the same yeast species. Fabre *et al.* (1998) found a maximum product concentration of 1.4 g/L at the end of a batch culture using *K. marxianus*. Using the same species but different stains, Wittmann *et al.*

(2002) obtained 1.84 g/L of 2-PE in a single phase system, and Etschmann *et al.* (2003) reported 0.89 g/L of 2-PE as the final concentration for screening purposes. *S. cerevisiae* strains were reported to be able to produce higher concentration of 2-PE at 2.6 g/L [Seward *et al.,* 1996]. The highest concentration obtained from a single-phase batch reactor reported by Stark *et al.* (2001) was 3.8 g/L using *S. cerevisiae*, which is the concentration determined to stop cell growth completely due to 2-PE toxicity. These data indicated that despite the efforts made, without an effective product removal strategy, the bioconversion of L-Phe to 2-PE would be limited by end product inhibition.

Another technical difficulty for 2-PE biotransformation is that not only the end product is inhibitory, but the by-product, ethanol also causes an inhibitory effect. Many Crabtree positive yeasts can produce ethanol even under aerobic conditions, and the formation of ethanol in 2-PE biotransformation had been found to have a synergistic toxic effect for Crabtree positive yeast strains such as *Saccharomyces cerevisiae* [Seward *et al.,* 1996]. This problem can be minimized by either choosing Crabtree negative yeasts such as *K. marxianus* from the beginning, or controlling the feed of glucose below a critical level so that the production of ethanol can be prevented or minimized [Etschmann *et al.,* 2002].

Based on the published results regarding to the biotransformation of L-Phe to 2-PE, there was no indication that the substrate L-Phe could cause any toxic effect even at its saturation aqueous concentration.

2.7.3 ISPR Approaches

Since the 2-PE concentration obtained from only aqueous phase is likely too low to justify an industrial process, researchers have explored many options for ISPR so that it can be removed as soon as it forms.

2-PE is an aromatic alcohol which opens the option for hydrophobic solvent extraction. Liquid-liquid solvent extraction is the most commonly studied ISPR technique for 2-PE biotransformation because it has the advantages of being cheap, simple and easy to scale up [Stark *et al.,* 2002]. Etschmann *et al.* (2003) used oleyl alcohol when they screened yeasts for the production of 2-PE and increased the overall product concentration from 0.89 g /L (aqueous phase only) to 3g/L (aqueous and oleyl alcohol phase). The Stark group reported an overall 2-PE concentration of 12.6 g/L using oleic acid as the second liquid phase and *Saccharomyces cerevisiae* as the biocatalyst [Stark *et al.,* 2002]. The number was obtained based on an aqueous phase concentration of 2.1 g/L and an oleic acid phase concentration of 24 g/L [Stark *et al.,* 2002]. Etschmann and Schrader (2006) achieved the highest overall 2-PE concentration reported by using polypropylene glycol 1200 (PPG 1200) as the second phase, and *Kluyveromyces marxianus* CBS 600 as the yeast strain. At the end, 26.5 g/L and 0.3 g/L of 2-PE were obtained in the PPG1200 phase and aqueous phase, respectively. The reported overall productivity was 0.33 g/ (L-h) [Etschmann and Schrader, 2006]. The group improved the process by providing the precursor L-Phe from the very beginning so that the bioconversion occurs simultaneously with yeast growth, and also by providing L-Phe as the sole nitrogen source in large excess. They compared 4 different types of organic solvents for their extraction capability

toward 2-PE, and found that PPG 1200 was the best one with a partition coefficient > 30. The other solvents and their corresponding partition coefficients were: oleyl alcohol 15, Miglyol (caprylic/capric triglyceride) 6, and isopropylmyristate 5 [Etschmann and Schrader, 2006]. The other conclusion they made was that 18 g/L of ethanol formed in the aqueous phase did not cause a synergistic toxic effect to the cells [Etschmann and Schrader, 2006]. However, an important disadvantage of using PPG 1200 was that the solvent formed an emulsion with the aqueous phase upon stirring. This can be problematic when measuring real-time cell density using optical density method. Also, according to the data provided by the group (0.3 g/L in the aqueous phase; 26.5 g/L in the organic phase), the partition coefficient should be as high as 88, instead of the reported value of >30 with PPG 1200.

While the results from applying two phase system to produce 2-PE are encouraging, only organic solvents as the second phase have been explored. 2-PE is an aroma compound which is also used as food ingredient, therefore, its product grade can be affected significantly by the odour of solvent residues.

Other ISPR techniques such as immobilization of solvent in polymeric matrix to form a chemically and mechanically stable resin [Serp *et al.,* 2003]; microcapsulation of solvent to prevent the solvent toxic effect [Stark *et al.,* 2003 (1)]; organophilic pervaporation with a polyoctylmethylsiloxane membrane [Etschmann *et al.,* 2005] have also been reported. All of these approaches have their own advantages, such as being non-toxic, possessing high mass transfer rate and being easy to handle. However, the overall product concentrations obtained

from these processes were lower than liquid-liquid two phase approach, and the cost may be too high for industrial feasible processes.

2.8 Chiral Pharmaceutical Intermediate - cis-1,2-indandiol

The development of antiviral agents, a complicated undertaking, is one of the most rapidly growing areas in chiral pharmaceutical synthesis [Buckland *et al.*, 1999]. CRIXIVAN is a drug that interrupts the replication of the HIV virus by inhibiting the function of protease, an enzyme required for the post-translational processing to form mature viral proteins [Buckland *et al.*, 1999]. This chiral drug contains five asymmetric centers which results in 32 possible stereoisomers; however, only one of them, in enantiomeric purity is the active ingredient of CRIXIVAN [Buckland *et al.*, 1999]. Currently, CRIXIVAN is synthesized at a multi-ton scale by a route that joins two enantiomerically pure intermediates, (S)-piperazine-1-carboxamide and (-)-cis-aminoindanol [Buckland *et al.*, 1999]. The chemical synthesis of one of the compounds, (-)-cis-aminoindanol is highly demanding technically and is one of the most difficult parts of the synthetic pathway to CRIXIVAN [Buckland *et al.,* 1999; Amanullah *et al.,* 2002 (1)]. The literature has been focusing on biological routes for its synthesis because it is possible to control the chirality of the final product and increase the efficiency of the process to cut purification costs [Amanullah *et al.,* 2002 (1)]. (-)-cis-aminoindanol can be directly synthesized by using either cis-(1S,2R) or trans-(1R,2R) indandiol which are chiral compounds that can be produced biologically using indene as the substrate [Wackett *et al.,* 1988]. Therefore, the

biotransformation process to form cis-(1S,2R) or trans-(1R,2R) indandiol is of high interest.

2.8.1 Bioproduction

The transformation of indene to both cis- and trans-1,2 indandiol by mammals was first recorded in 1956 by Brooks and Young. The microbial transformation was reported by Wackett *et al.* (1988) and they found that the enzyme dioxygenase activity can be induced in *Psudomonas putida* by toluene. The three-component toluene-dioxygenase enzyme (TDO) system can transform both toluene and indene to their dihydrodiols form [Gibson, *et al.* 1970; Wackett, *et al.* 1988]. The pathway is shown by Figure 2-2.

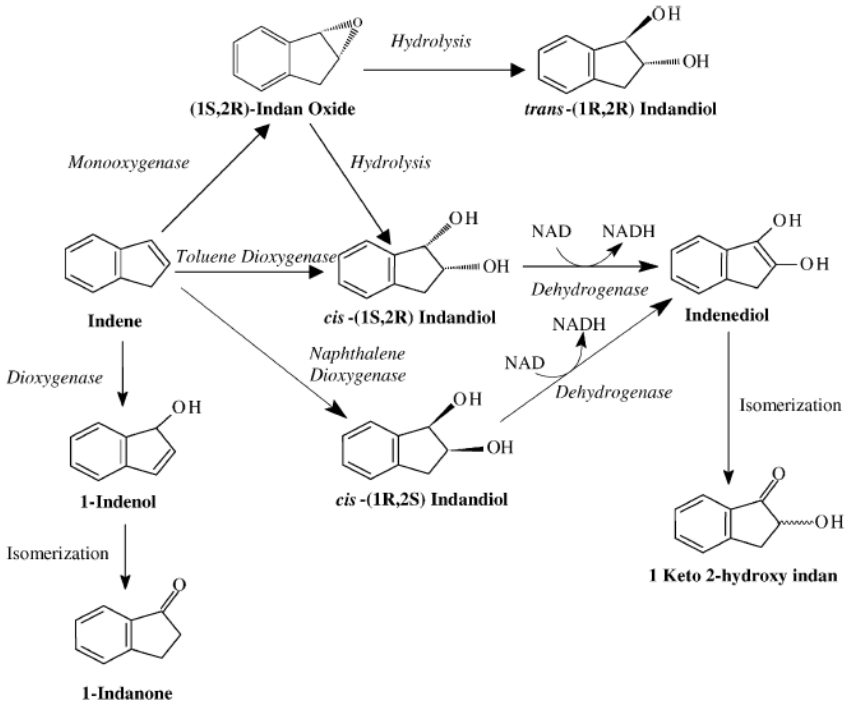


Figure 2-2: Biochemical pathway of converting indene to cis-indandiol [Amanullah *et al.* 2002 (2)]

Many microbial isolates from hydrocarbon-contaminated site were also found to have the ability to convert indene to indandiol among which, *Rhodococcus sp*. cultures are the most active ones [Buckland *et al.,* 1999]. Compared with *P. putida*, the biotransformation carried out by *Rhodococcus sp*. is found to have low yields with the production of various undesirable monooxygenation by-products such as 1-indenol and 1-indanone [Amanullah *et al.,* 2002 (2)]. However, the enantiomeric excess was high when *Rhodococcus sp.* is used [Buckland *et al.,* 1999].

Recombinant *E. coli* with incorporated TDO genes from *P. putida* had also been demonstrated to convert indene to cis-1S,2R-indiandiol [Reddy *et al.,* 1999].

2.8.2 Substrate/Product Inhibition

Wild type of *P. putida* requires toluene as the inducer for the transformation of indene to indandiol, however, toluene can be severely toxic to *P. putida* above its 50% saturation in the aqueous phase [Brazier and Lily, 1990]. According to a toxicity study done by Amanullah and coworkers using fluorescent probes, the substrate indene showed toxic effects to Rhodococcus sp. above 0.0368 g indene/g dry cell. [Amanullah *et al.,* 2002 (1)].

2.8.3 Two phase approaches

Efforts have been made to solve this problem in two ways: 1) two-phase systems to minimize the toxic effect 2) isolation of a mutant that is able to activate TDO in the absence of toluene [Buckland *et al.,* 1999]. Buckland *et al.* (1999) applied an aqueous/silicone oil two phase system. The results showed that

the addition of 20% of silicone oil allowed the cells to grow at a toluene level much above its toxic level (50% of water saturation). These encouraging results led to the construction of a pilot plant fermentation scale of 800 L, and 1000 g of purified cis-1S, 2R-indandiol was produced [Buckland *et al.,* 1999]. In order to further simplify the fermentation process, the group searched for mutant strains of *P. putida* that do not require the addition of toluene to induce the activity of TDO. They successfully isolated a *P. putida* strain 421-5 which produced high concentrations of the desired product with high enantiomeric excess in the absence of toluene [Buckland *et al.,* 1999].

The toxic effect posed by the substrate indene has been reported by all the literature studies on the bioconversion of indene to indandiol. Two phase systems and controlled-feeding in single phase reactor strategies had been applied to minimize the inhibitory effect caused by indene. The result showed that with two phase system (silicone oil as the second phase), a higher concentration was obtained (4 g/L) compared with single phase production (0.8-1.2 g/L) [Amanullah *et al.*, 2002 (2)]. In addition, the yield based on biomass utilization was significantly improved from 0.02-0.04 g product/dry cell weight to 0.27 g product/dry cell weight when two phase system was applied. The overall process still needs to be optimized to obtain satisfactory results. One aspect worth exploring when a solid-liquid TPPB is used is to use different polymers to target different substrates/products. For example, a "polymer cocktail" that contains the best working polymers for sequestering each of the two substrates (one for toluene, one for indene), and the best working polymer to remove cis-1,2-

indandiol. Since various types of polymers can be combined in a bioreactor, it is possible to target all of the compounds of interest at once.

2.9 Transportation Fuel – Butanol

The fermentation of carbohydrates to produce solvents such as butanol and acetone was first industrialized by C. Weizman in 1912 [Durre 1998]. However, the rapid development of petroleum-based chemical processes by the 1960s had demonstrated economical advantages over fermentation processes for butanol synthesis, especially with the high price of carbohydrate substrate at the time [Qureshi and Blaschek, 1999; Qureshi and Blaschek, 2001]. As a result, the number of fermentation facilities was dramatically decreased [Qureshi and Balschek 1999].

Butanol is currently used as a feedstock for plastic synthesis, fuel, and as an extraction solvent in the food industry [Qureshi and Blaschek, 1999]. The demand for butanol is in the range of million tons per year worldwide [Qureshi and Blaschek, 2001]. The majority of butanol currently in use is derived from chemical processes; however, when used as a food grade extractant, "naturally-derived" butanol is highly desirable [Qureshi and Blaschek, 2001]. As crude oil is likely to become more and more expensive, and the demand for switching petroleum-based feedstocks to renewable biomass is attracting more attention, using fermentation process to produce large quantity of biobutanol is under extensive consideration. Also, as a result of technology development in metabolic, genetic engineering and the improvement in fermentation process, more efficient bioproduction of butanol can be achieved. Therefore, driven by the benefit of

using a sustainable energy source and guided by the newly developed fermentation processes and microbial strains, bioproduction of butanol has now reached a point that can compete with chemical processes [Qureshi and Blaschek, 2001].

Fuel additives such as ethanol are widely used to reduce emissions caused by incomplete combustion of gasoline. Butanol is one of the promising alcohols to be used as fuel additives and it has several advantages over the others. Firstly, butanol has a lower vapour pressure (0.63 psi @ 50^{o}C) than ethanol (2.25 psi @ 50^{o}C) [Ladisch, 1991]. This reduces the chances for smog formation caused by volatile organic compounds. Secondly, butanol has an energy content that is close to gasoline and is higher than ethanol [Qureshi and Blaschek, 1999]; therefore, consumers face less of a compromise on fuel economy as the portion of blended fuel additives increases. Thirdly, butanol is more miscible with gasoline and diesel fuels than ethanol and is less miscible with water which leads to a higher blended concentration than ethanol and prevents water from mixing with the fuel. As a result, producing butanol as a transportation fuel additive is attracting significant investments.

2.9.1 Bioproduction

For a fermentation process, acetone and ethanol are usually produced along with butanol, and a typical ratio of acetone, butanol and ethanol is 3:6:1 [Qureshi and Blaschek, 2001]. The two primary microbial species used to produce butanol are *Clostridium acetobutylicum* and *Clostridium beijerinckii* [Ezeji *et al.*, 2007]. Other strains that have higher enzymatic activities for butanol production

such as *Clostridium beijerinckii* BA101 are genetically modified by treating the microbial culture using N-methyl-N'-nitro-N-nitrosoguanidine and selective enrichment [Annous and Blaschek, 1991]. Researchers had suggested that *Clostridium beijerinckii* species may have greater industrial potential to produce butanol because they can utilize a wide range of substrates, adapt broader pH optimum for growth and solvent production [Ezeji *et al.,* 2007]. Genomic information for *Clostridium beijerinckii* can enable the examination and manipulation of genes that control the expression of relevant enzymatic activity, and further create strains that are more efficient at producing butanol.

2.9.2 Product Inhibition

Another aspect of improving process efficiency relies on the upstream and downstream operations. One major problem of bioproduction of butanol is the toxicity effect caused by the accumulation of butanol. The addition of 12-16 g/L of butanol totally inhibited the growth of *Clostridium acetobutylicum* [Barton and Daugulis, 1992; Ezeji *et al.,* 2007], and no more than 60 g/L of sugar can be added to boost the production due to end product inhibition in a non-integrated process (without product removal) [Ezeji *et al.*, 2004]. The problems associated with it are the low productivity, low product concentration and yield which leave a large volume of dilute solution to both upstream and downstream processes.

2.9.3 ISPR Approaches

Several fermentation technologies were investigated to improve the process. A fed-batch reactor configuration had been used in combination with *in situ* product removal techniques such as gas stripping [Qureshi and Maddox,

1992] and pervaporation [Groot *et al.,* 1984]. Other methods such as an immobilized-cell continuous reactor [Qureshi *et al.,* 2000] and membrane cell recycle reactors [Pierrot *et al.,* 1986] have also been shown to improve the butanol production process, however, deactivation of cells in the immobilized reactor and fouling of the membrane with the fermentation broth remain problematic [Ezeji *et al*., 2004].

Liquid-liquid extraction had also been studied. Corn oil, oleyl alcohol [Roffler *et al.*, 1987] and poly(propylene glycol) 1200 [Barton and Daugulis, 1992] were tested for their extraction power and biocompatibility, and all had gained better results compared with a single phase reactor. The extractant potential toxicity is still a major limitation for liquid-liquid systems. Pervaporation where ABE diffuses through a solid membrane leaving behind nutrients, sugar, and microbial cells is another *in situ* product removal approach. The composition and selectivity of the membrane play an important role in controlling the material flow [Ezeji *et al.,* 2004]. One promising membrane chosen by many researchers is polyether-block-amide (PEBA) (with the trade name PEBAX®). It was shown to have relatively good selectivity and permeability when extracting n-butanol from the dilute aqueous phase [Liu *et al.,* 2005]. This membrane material has a high affinity toward butanol, and at high temperature (around 80^{o}C), the polymer can be dissolved in butanol [Liu *et al.,* 2005]. Boddeker *et al.* (1990) also compared different block polymeric membrane such as polyurethane, PDMS and PEBA, and found that with PEBA membrane,

the pervaporation flux was more than two times higher compared to the other two types of membranes.

2.10 Biorefinery Building Blocks – Succinic Acid and 3 - Hydroxybutyrolactone

Although it is the traditional energy source that feeds the world, petroleum use is neither sustainable nor environmentally friendly. Among many types of alternative energy sources, such as solar and nuclear engery, biomass is the only one that can not only provide energy, but can also be used to produce useful goods. The dependency on petroleum-based feedstocks can be greatly reduced by exploring the potential of bio-based feedstocks. Currently, only a tiny portion of biomass produced yearly is used for biological production processes, and only 3-3.5% of this amount is used in the non-food area, such as the synthesis of chemical building blocks [Kamm and Kamm, 2007]. There is considerably more that can be done to utilize biomass to restructure the chemical industry.

The biorefinery is built on the similar concept as current chemical refineries. Just as a variety of materials are synthesized based on petroleum-based feedstocks, many different compounds can also be derived from biomass. Among these compounds, some simple-to-handle (safe, non-hazardous) and well-defined chemically pure materials [Kamm and Kamm, 2007] can form platform chemicals, or building blocks for other intermediate products, and more sophisticated synthetic products.

2.10.1 Bioproduction

Succinic acid can be derived from fermentations to overproduce 4-carbon diacids from Krebs cycle pathways [DOE Report, 2004]. This compound has the ability to supply 2.7×10^8 kg industrial products per year [Werpy *et al.* 2006] including: 1,4 butandiol, tetrahydrofuran, adipic acid and 2-pyrrolidone. The basic chemistry of succinic acid is similar to that of the petrochemically derived maleic acid /anhydride. It can be synthesized through fermentation using *Anaerobiospirillum succiniciproducens* and recently, recombinant *E.coli* [DOE Report, 2004].

The current fermentation process usually operates at a pH well above the pKa of succinic acid, otherwise the cells would not survive in the culture medium. However, only in their un-dissociated form, is the acid useful as platform chemical. The conversion of the salt to the free acid adds significant costs to the whole process [DOE Report, 2004], so ideally, the fermentation should be run at a lower pH. Therefore, the other technical barrier is to find a microorganism strain or genetically engineered strain that can adapt low pH fermentation.

The other platform compound 3-hydroxybutyrolactone (3-HBL) is in itself an intermediate for high value pharmaceutical compounds, and its derivatives have the potential to form solvents and amino analogs to lycra fibers [DOE Report, 2004]. The chemical route to produce 3-HBL is available, but it involves multi-steps and is difficult. Although a biological route has not yet been found, the direct bioconversion from sugar all the way to 3-HBL is the future of 3-HBL synthesis [DOE Report, 2004], and is being pursued by a number of groups working on the area of synthetic biology.

2.10.2 ISPR Approaches

The technical challenge for the production of all the platform chemicals is to improve the yield and productivity of the bioprocess. According to the report prepared by the U.S Department of Energy, a minimum productivity of 2.5 g/ (L-h) must be achieved. From a kinetic point of view, the fermentation rate can be increased if the final product is removed simultaneously as it is produced. Therefore, ISPR is a suitable technology to test to increase the productivity of the bioprocess.

Extraction with conventional solvents such as ketones and alcohols is not effective for succinic acid due to the low partition coefficient [Jun *et al.,* 2007]. Jun *et al.* (2007) tested a technology called reactive extraction where they used 1-octanol solution of tri-n-octylamine (TOA) to form a complex with succinic acid and take it out of the fermentation broth through a hydrophobic microporous membrane. Kim *et al.* (2007) used the same method and found that the extraction power is higher as the pH in the aqueous phase is higher. In the fermentation product, succinic acid coexists with acetic acid. Hong *et al.* (2000) adapted the reactive extraction technique to separate the binary mixture, and the highest extraction efficiency of succinic acid was achieved when the combination of 1-octanol and TOA was used. They concluded that amine groups interact with organic acid strongly. Another method to extract acids from fermentation broth is using sorbents. Davison *et al.* (2004) tested 25 different sorbents and the highest capacity was 0.06 g succinic acid / g resin (at a moderate succinic acid concentration of 1-5 g/L). They successfully concentrated the effluent from 10 g/L to >100 g/L. However, this is not an ISPR approach because the adsorption

did not occur in the same operating unit as the production, and the recovery of the product requires hot water. There is little information about the extraction of 3-hydroxybutyrolactone from an aqueous system.

Succinic acid is mainly in its dissociated form at neutral pH which imposes great challenges for ISPR using an organic second phase. However, functionality in polymer beads may open the door for molecular interactions between the acid and the polymer and consequently extract the acid molecule into the organic phase. This can result in the shift of the dissociation equilibrium of the acid, and drive the equilibrium towards the formation of the acid molecule. ISPR of 3-HBL has not yet been studied. 3-HBL has a ring structure and is soluble in both water and organic solvents. This means that it is possible to use an organic/aqueous two phase system to extract 3-HBL from the fermentation broth. As a result, the investigation of using two-phase system to extract these two compounds is possible and worthwhile.

2.11 Scope of Thesis

All of the abovementioned bioprocesses can benefit from ISPR technologies, either to solve the problem of end product inhibition, or to act as an initial concentrating step. So far, many ISPR approaches have been studied, however, no study had ever explored the potential of a solid-liquid TPPB system in which polymer beads are used as a sequestering phase. Such a system had shown promising results in terms of reactor performance and operability for the biotransformations to form 3-methylcatechols, [Prpich and Daugulis, 2007] and from carveol to carvone [Morrish and Daugulis, 2008].

This work will examine the possibilities of five biotransformation (or potential biotransformation) products that would benefit from solid-liquid TPPB systems. The compounds are strategically selected from different industrial sectors. They are different in molecular size and functional group. The exploration of their interaction with different polymer beads would help better understand the underlining cause for polymer-small molecule interactions, and what polymer properties dictate the absorption between the sequestering phase and the compound of interest. Finally, the biotransformation from L-phenylalanine to 2-phenylethanol by *Kluyveromyces marxianus* will be studied in more detail for reactor performance and operability.

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Chapter 3

An assessment of polymer-solute interactions for their application in solid-liquid two phase partition bioreactor systems

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3.1 Preface

As can be seen from the examples in Chapter 2, ISPR technology can be applied to many biotransformation processes as a way to solve end product inhibition problems, or act as an initial separating or concentrating step for downstream processing. Two phase partitioning systems are one of the ISPR approaches that are relatively easy to implement while showing promising results. Although many immiscible organic solvents have been studied for their ability to sequester products or deliver substrates, the research on polymers as the second phase is still limited.

One important aspect of study is the extraction capability of polymer beads towards a target compound for solid-liquid TPPBs for ISPR purposes. Polymers that have high extraction capability can absorb and remove compounds of interest to a great extend from the aqueous phase. Understanding of important polymer properties and the interaction between polymers and target compounds may facilitate the selection of the best working polymer for a certain application.

This chapter will focus on the study of the extraction capability of different polymers towards various biotransformation products mentioned in

Chapter 2, aiming to understand some of the important polymer properties that affect the interaction between polymers and the compounds of interest.

3.2 Abstract

BACKGROUND: Biphasic systems with immiscible solvents have been studied for *in situ* product removal, and have shown improvements in bioreactor performance. However, problems associated with solvent biocompatibility, bioavailability and operation have been identified. One alternative is the solid-liquid system in which polymer beads are used, absorbing and removing target compounds from the aqueous phase while maintaining equilibrium conditions. This work aims to identify polymer properties that may be important in polymer selection for selected biotransformation molecules including 2-phenylethanol, cis-1,3-indandiol, iso-butanol, succinic acid and 3-hydrobybutyrolactone.

RESULTS: Relatively hydrophobic compounds tend to be absorbed by polymers better than hydrophilic ones based on partition coefficient tests. Due to the presence of polar functional groups on these compounds, polar polymers such as Hytrel® performed better than non-polar polymers such as Kraton® possibly due to the hydrogen-bonding interactions between the polymer and the solute. Crystallinity and intermolecular hydrogen-bonding were also found to be important polymer properties.

CONCLUSION: This work examined the application of solid-liquid biphasic systems to selected biotransformation processes. Polymers showed excellent results in absorbing hydrophobic compounds such as aromatic alcohols, and positive results in absorbing hydrophilic compounds but to a lesser extent. Grafting hydrophilic functional groups onto polymers may be a promising

approach for extending polymer uptake capabilities and is currently being undertaken in Daugulis Group.

3.3 Introduction

The development of new biosynthetic processes has increased rapidly from fewer than 10 industrial processes in the 1960s to more than 130 in 2002 [Straathof *et al.*, 2002]. These processes include applications in the pharmaceutical, flavour and fragrance, and agrochemical industries [Straathof, *et al.* 2002] as well as in emerging biorefinery opportunities. The advantages of biocatalysts can be summarized as: the ability to interact with complex substrates to form complex products; the high stereo-, regio- and chemo- selectivities; the ambient reaction conditions; and the use of renewable feedstock [Schmid *et al.,* 2001]. However, compared to chemical processes, biological processes often suffer from low productivity and low final product concentration. One major cause is the toxicity effect imposed by products and/or substrates. Biphasic systems have been investigated as a possible solution for substrate delivery [Daugulis, 2001] and *in situ* product removal (ISPR) [Lye and Woodley, 1999]. Applying immiscible organic solvents as the delivery and/or sequestering phase has been the main focus of many studies.

Although two liquid phase systems have demonstrated improvements in reactor performance, there are drawbacks. The toxicity effect that may arise from the use of immiscible organic solvents can be found at both the molecular and phase level [Lilly et al. 1987], which results in inhibition or even death of the cell culture; bioavailability is also of concern because of the possible preferential uptake of the solvent at the cost of the target substrate [Amsden *et al.* 2003], especially when a microbial consortium is used. In operational aspects, organic

solvents can be flammable, cause emulsions [Etschmann and Schrader, 2006], and the residual odours may affect product quality, especially for fragrance and flavour products.

A promising alternative to overcome these problems involves the use of solid polymer beads as an immiscible second phase, taking advantages of polymers as being biocompatible, non-bioavailable, and easy to recycle. The idea was developed by Amsden *et al.* (2003) for the biodegradation of phenol. Polymer beads were added to absorb phenol and reduce its aqueous phase concentration to a sub-inhibitory level, and then deliver the substrate (phenol) to the cells as the aqueous phase concentration decreases due to metabolic demand [Amsden *et al.* 2003]. The polymer used in their study was poly(ethylene-co-vinyl acetate) (Elvax®), and the selection was made based on the polymer(Elvax®)-solute(phenol) interaction parameter [Amsden et al. 2003]. This work showed that solid polymer beads can be used as the delivery agent in a biphasic system in a similar fashion as organic solvents, but with improved operability and almost no effect on the cell culture. Prpich and Daugulis (2004) employed a more rigorous selection for candidate polymers for the delivery of phenol, and showed that a copolymer of poly(butylene terephthalate) and polyether (with the trade name Hytrel®) performed the best with both improved specific loading and diffusivity relative to EVA [Prpich and Daugulis, 2004]. Among the 8 polymers tested in this study, only 3 showed affinity for phenol which indicates that polymers need to be carefully selected for a given application.

Rehmann *et al.* (2007) investigated the absorption of biphenyl, a more hydrophobic substance than phenol, by different types of polymers. They showed that the partition mechanism of hydrophobic substances between water and polymers is similar to the mechanism between water and organic solvents [Rehmann *et al.* 2007]. This suggests that the uptake of a hydrophobic substance by polymers is primary through absorption instead of adsorption. The concept of using polymers as a sequestering phase for ISPR applications was first introduced by Prpich and Daugulis (2007) when they studied the bioproduction of 3-methylcatechol from toluene. The challenge imposed by end product inhibition in this biotransformation was overcome by the addition of a selected polymer sequestering phase resulting in enhanced reactor performance (product concentration and productivity) [Prpich and Daugulis, 2007]. The work by Morrish and Daugulis (2008) also showed the advantages of using polymer beads to solve problems associated with multiple inhibitions (both the substrate and product were inhibitory). During the polymer selection process, they identified different types of polymers for the substrate and the product as the most suitable ones, and by combining the two, they obtained better results than when only one type of polymer was used [Morrish and Daugulis, 2008].

For ISPR applications, it is important to know the extraction capability that a polymer possesses towards the compound of interest. High extraction capability often translates to better removal of the compound from the aqueous phase, and higher overall product concentration (taking both phases into account). Selecting the most suitable polymer among candidates can be challenging. This

work aims to better understand the polymer properties that may be important for the uptake/absorption of selected compounds based on an evaluation of the partition coefficient, which is a measure of the solute in the polymer phase relative to the aqueous phase at equilibrium. The compounds selected in this study differ in terms of their molecular complexity, market value, and production scale, and cover a wide range of sectors from commodity goods to high value chemicals. Investigating the possibility of using polymer extraction as an ISPR method for these compounds can provide valuable information about broader applications of the solid-liquid two phase partition bioreactor (TPPB) technology. Important information about these compounds is summarized in Chapter 2, Table 2-2.

3.4 Materials and Methods

3.4.1 Chemicals and Polymers

2-phenylethanol (2-PE), succinic acid and phosphoric acid were purchased from Sigma Aldrich Canada (Oakville, ON, Canada). Magnesium sulfate, dipotassium phosphate, potassium dihydrogen phosphate and ammonium sulphate were purchased from Fisher Scientific Canada (Oakville, ON, Canada). cis-1,3-indandiol was purchased from TopChem Laboratories (Dublin, Ireland). Iso-butanol was purchased from SAFC (St. Louis, USA). 3-Hydroxybutyrolactone (3-HBL) was purchased from TCI America (Portland, OR).

Information about the polymers used in this study is listed in Table 3-1. We thank DuPont Canada, Arkema Canada and Bayer Canada for their kind donations of these polymers.

3.4.2 Analytics

All concentration measurements in this study were performed with High Performance Liquid Chromatography (Varian, Prostar). The columns used for each of the compounds are listed below. The detection was performed by either a UV-vis detector (Varian, Prostar, Model #: PS325) or a Refractive Index (RI) detector (Varian, Prostar, Model #: PS356). The detailed method for each of the compound is listed in Table 3-2.

3.4.3 Partition coefficient tests

All partition coefficient tests were performed using a typical medium to simulate bioreactor conditions. The composition of this generic medium contained the following components (g/L): $MgSO_4$: 0.5; K_2HPO_4: 1.6; KH_2PO_4: 12.5; $(NH_4)_2SO_4$: 6. A carbon source was not included to prevent microbial growth over an extended period of time. The pH of this simulated medium was 5.5. In the case of succinic acid, the pH of the samples was adjusted to 3 using concentrated phosphoric acid prior to partition coefficient testing.

2-PE. Samples with 2-PE concentrations (g/L) of 1, 2, 3, 4 and 5 were prepared by dissolving the appropriate amount of 2-PE in 10 mL of the simulated medium in 20 mL scintillation vials. Triplicates were prepared for each concentration to account for experimental errors. The amount of polymer beads added to each vial was approximately 0.46 g. The vials were then put onto a shaker for mixing with polymers overnight. After equilibrium was established, the aqueous phase 2-PE concentration was analyzed by HPLC. The amount of 2-PE absorbed by polymer beads was back calculated based on mass balance between the two phases. The partition coefficient was obtained by plotting the polymer

phase concentration (g of 2-PE/ kg of polymer) as a function of aqueous phase concentration (g of 2-PE/ L). The slope of the linear fit of the data was the partition coefficient for this polymer. Three slopes were obtained using the triplicate data points. The average of the three was taken to be the mean partition coefficient of the polymer, and the standard deviation was represented as the error.

cis-1,3-indandiol. Since cis-1,3-indandiol is more hydrophilic than 2-PE, it was expected that the absorption by polymers would be less significant than 2-PE. In order to accurately measure the concentration change before and after the addition of polymer for every sample, a single low initial concentration was used. Therefore, instead of varying cis-1,3-indandiol concentrations, different polymer loadings were used. Initially, 10 mL of 0.8 g/L of cis-1,3-indandiol were prepared using the simulated medium in 20 mL scintillation vials. Polymer beads from 0.5 g to 2 g in 0.5 g increments were then added to each of the vials. Triplicate samples of 0.8 g/L cis-1,3-indandiol without polymer were also prepared as the blank control and to account for experimental errors. All of the other procedures were the same as the method for 2-PE.

iso-butanol. The experimental procedure was similar to that of cis-1,3-indandiol. Samples with initial butanol concentration of 6 g/L, 39 g/L, and 65 g/L were prepared. The amounts of polymer added ranged from 1 g/L to 3 g/L in 0.5 g/L increments.

Table 3-1: Information about the polymer beads used in this study

Trade Name	Grade	Supplier	Tg	Melting Point (^{o}C)	Chemical Composition	Specific Gravity
Hytrel®	G3548L	DuPont Canada	-45	156	copolymer of poly(butylene terephthalate) and polyether	1.16
	5544		-35	215		1.22
	6108		N/A	168		1.25
	8238		-50	223		1.28
	8206		-59	180		1.19
Zytel®	7304 NC010	DuPont Canada	N/A	220	Polyamide 6	
	42 NC010		70	262	Polyamide 66	1.15
	158 NC010		N/A	217	Polyamide 612	1.06
	RSLC1000 BK385		N/A	203	Polyamide 1010	1.05
PEBAX®	2533	Arkema Canada	-65	134	Polyether block amide	1
	4033		-65	160		1
	7033		N/A	172		1.01
Desmopan®	453	Bayer Canada	-34	145	Thermoplastic polyurethane	1.22
Kraton®	D4150K	Kraton	N/A	N/A	Styrene/butadiene block copolymer	0.92
Elvax®	3175	DuPont Canada	N/A	69	Ethylene/vinyl alcohol	0.95
	770		N/A	96		0.93

Note: The shape of all polymer beads used is approximately spherical with a diameter about 2-5 mm.

Table 3-2: **HPLC methods for the detection of the compounds in this study**

Compound	Column (Varian Inc.)	Mobile Phase	Flow Rate (mL/min)	Detection	Temperature
2-phenylethanol	Polaris C18-A 250*4.6 mm	Water/Methanol: 65: 35	1	UV-vis @ 216 nm	Room Temperature
cis-1,3-indandiol	Pursuit XRs C-8 250*4.6 mm	Acetonitrile/ water (10 mM of KH_2PO_4); the concentration ingredient of acetonitrile increased from 10% to 40% over 15 min	1	UV-vis @ 220 nm	Room Temperature
iso-butanol	PL - Hi Plex H 8um 300*7.7 mm	9 mM of H_2SO_4 water solution	0.7	Refractive Index	75^oC
succinic acid	PL - Hi Plex H 8um 300*7.7 mm	9 mM of H_2SO_4 water solution	0.7	UV-vis @ 220 nm	75^oC
3-hydroxybutrolactone	PL - Hi Plex H 8um 300*7.7 mm	9 mM of H_2SO_4 water solution	0.7	Refractive Index	65^oC

Succinic acid The experimental procedure was similar to that of cis-1,3-indandiol. The initial concentration used was 2 g/L, and the amount of polymer beads added ranged from 1 g/L to 3 g/L in 0.5 g/L increments. The pH of all the samples was adjusted to 3 using phosphoric acid prior to conducting partition coefficient tests. Due to the small amount of polymer uptake, the partition coefficients were obtained by calculating an average partition coefficient for each partitioning test.

3-hydroxybutyrolactone The experimental procedure was similar to that of the succinic acid. The initial concentration used was 10 g/L, and the amount of polymer beads added ranged from 1 g/L to 3 g/L with 0.5 g/L increments. Similar to succinic acid, the partition coefficients were obtained by calculating an average partition coefficient of every sample.

3.5 Results and Discussion

3.5.1 2-Phenylethanol

The first compound examined is from the flavour and fragrance industry, 2-phenylethanol (2-PE). Biologically derived 2-PE is commercially attractive because it can be marketed as a "natural" product. During its production by the yeast *Kluyveromyces marxianus*, it can become toxic as the aqueous phase concentration approaches 2 g/L. Solid-liquid biphasic systems are a better option for this application than solvent-aqueous systems because emulsion formation has

been reported for the latter [Etschmann and Schrader, 2006] and the odour from solvent residue may compromise the product quality.

The polymers used to test the uptake of 2-PE were a class of Hytrel® polymers. The selection was made based on the fact that 2-PE has a hydroxyl functional group, and it has been suggested that hydrogen-bonding might be important for polymer uptake [Leon *et al.* 1998; Prpich and Daugulis, 2004]. Because the two monomers (polyester and polyether) comprising Hytrel® both have the potential for hydrogen-bonding with an aqueous phase solute, Hytrel® was expected to be a good candidate for the uptake of 2-PE. The results of the partition coefficient of different grades of Hytrel® towards 2-PE is shown in Figure 3-1.

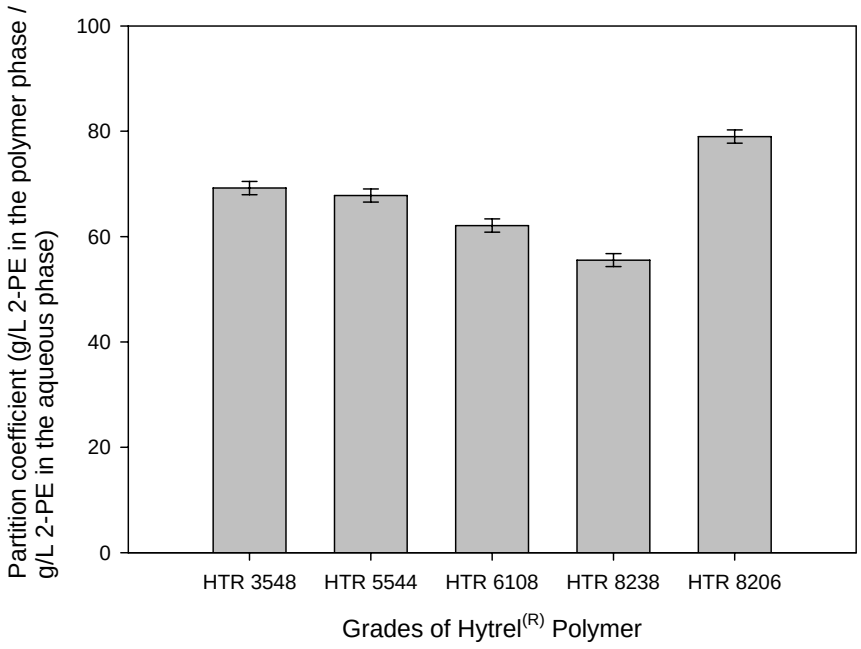

Figure 3-1: Partition coefficients of different grades of Hytrel® towards 2-phenylethanol

As shown by Figure 3-1, all of the Hytrel® grades tested showed high partition coefficients. In fact in a parallel study, a two phase partitioning bioreactor (TPPB) was applied to the production of 2-PE using *Kluyveromyces marxianus* and Hytrel® polymer beads as the sequestering phase. The results of this study were compared to other work in the literature in which immiscible organic solvents were used, this study showed significantly better results [Gao and Daugulis, 2009]. The high partition coefficients lead to the hypothesis that one important aspect in the absorption of 2-PE by polymers is the interaction of functional groups between 2-PE and the polymer, in this particular case, through hydrogen-bonding interactions. This is consistent with what has been proposed by Prpich and Daugulis (2004) for the absorption of phenol and the absorption of 3-methycatechols [Prpich and Daugulis, 2007].

The difference between the various grades of Hytrel® is the composition of the two monomers. The proportion of polybutylene ester increases in the series of Hytrel 3548 < Hytrel 5544 < Hytrel 6108 < Hytrel 8238. The degree of hardness also increases in the same fashion. An interesting trend shown by Figure 3-1 is that except for Hytrel 8206, the partition coefficient decreased as the hardness of Hytrel® increased. The moisture absorption data reported by DuPont also followed the same trend. The percentage of water absorption by the polymers tested in 24 h time span is: G3548L: 5%, G5544: 1.5% and G8238: 0.3%. In

Hytrel® copolymer polybutylene ester makes up the crystalline region (the "hard" segment) while polyether makes up the amorphous region in the polymer chains (the "soft" segment). Therefore, by varying the composition of the two, the crystallinity, hence the hardness, of Hytrel® can be altered. It is well known that the crystallinity of polymers can affect the diffusion of small molecules into polymers [Gavara *et al.*, 1996]. In the amorphous regions of a polymer, both the chain mobility and the free space are larger than in the crystalline regions. Therefore, they can accommodate molecules that entered the chains better than the crystalline regions. The data obtained from the 2-PE/Hytrel® system and the water/Hytrel® system agreed well with this hypothesis, and showed that polymers with low crystallinity are often preferred for small molecule absorption relative to polymers with high crystallinity. Hytrel® 8206 is a special polymer grade designed especially for moisture and polar compound permeation (personal communication with DuPont Canada). Therefore, it did not follow the trend as all the other general grades of Hytrel®. The special features of Hytrel® 8206 are unknown at this point due to proprietary reasons. Hytrel® 6108 is a polymer grade with a modified structure. The terphthalic acid in the polyester segment was replaced by isophthalic acid (personal communication with DuPont Canada). The incorporation of this isophthalic acid disrupted the original regularity in the polyester and reduced the crystallinity of the polyester phase. This modification changed the appearance of the polymer beads, but it was not sufficient to

outperform the grades with lower hardness in terms of absorbing small molecules according to the data shown in Figure 3-1.

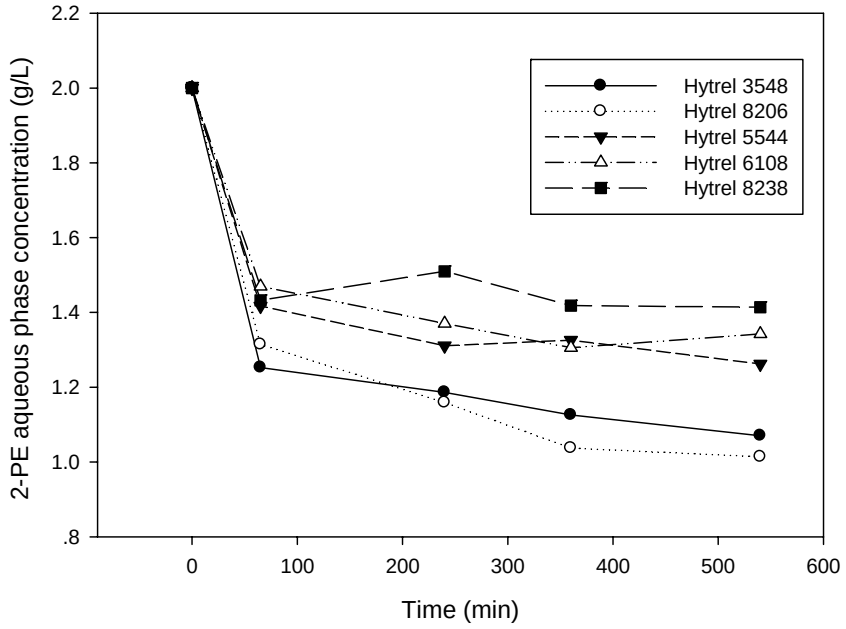

Figure 3-2: The decrease of 2-PE concentration in the aqueous phase as a function of time

In order to obtain more insights for the parallel study on the bioproduction of 2-PE in a solid-liquid TPPB system, the rate of absorption was also measured for each polymer tested, shown in Figure 3-2. In a bioreactor system, in addition to the partition coefficient, the rate that polymer beads can take up the target compound is also of great interest. Fast diffusion is preferred because it shortens the time span that cells are exposed to the toxic compound. As shown in Figure 3-2, the two grades of Hytrel® that had the highest partition coefficient towards 2-

PE also showed faster uptake, possibly due to their high moisture absorption ability. It also showed that for all polymer tested, the major reduction of aqueous 2-PE concentration occurred within the initial 100 mins. It is expected that under more vigorous agitation in a bioreactor, the absorption could be faster. The abovementioned characterization of 2-PE uptake (extent and rate) by polymers formed the basis of the successful demonstration of enhanced 2-PE production by ISPR in a solid-liquid TPPB with Hytrel® 8206[Gao and Daugulis, 2009].

3.5.2 cis-1,3-indandiol

The second compound considered is from the pharmaceutical industry, cis-1,2-indandiol. cis-1,2-indandiol is a chiral intermediate for a commercial drug developed by Merck & Co. for treating HIV [Buckland *et al.*, 1999]. Since cis-1,2-indandiol is not commercially available, the close structural isomer cis-1,3-indandiol was selected.

As shown in Table 2-2, cis-1,3-indandiol contains an aromatic ring and two hydroxyl groups. The candidate polymers included Kraton®, Hytrel® (G3524), Desmopan® and Zytel®. The rationale behind the polymer selection was to test which functional group is more important in the absorption of this compound, the aromatic ring or the hydroxyl groups. The results of partition coefficient tests are shown in Figure 3-3. It can be seen that within the 4 types of polymers tested, only Hytrel® 3524 showed uptake with a partition coefficient significantly lower compared to the results for 2-PE uptake. Hydrophobic

polymers with only aromatic functionality such as Kraton® (styrene-co-butadiene) did not work well in this case. The results suggested that π-interactions between the aromatic rings of the polymer and cis-1,3-indandiol were not likely the mechanism for absorption in this case. On the other hand, a relatively polar polymer such as Hytrel® showed uptake suggesting that the hydroxyl groups in cis-1,3-indandiol interact with polyether and polyester in the polymer.

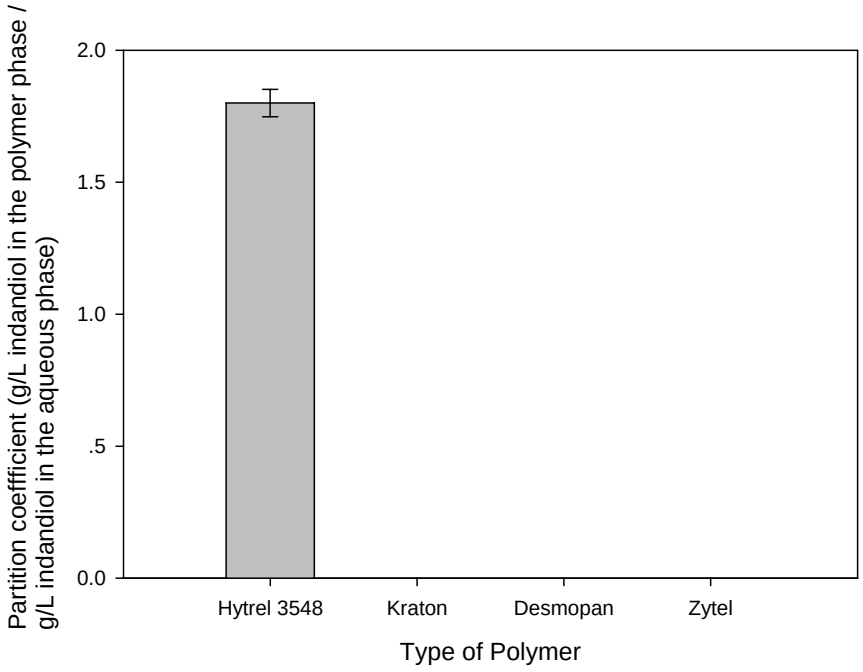

Figure 3-3: Partition coefficients of candidate polymers towards cis-1,2-indandiol

K_{ow} is the partition coefficient of a compound between octanol and water, which is a relative measurement of hydrophobicity of a certain substance. K_{ow} has been a good predictor of how much a polymer phase can extract a certain substance from the aqueous phase. For example, the partition coefficient of

biphenol (K_{ow} = 5623) with Kraton® was as high as 7072 [Rehmann *et al.* 2007], while the partition coefficient of 3-methylcatechol (K_{ow} = 37.15) with Hytrel® was 58. [Prpich and Daugulis, 2007]. This correlation has also been reported by Saitoh *et al.* (1999) when they studied the polymer-mediated extraction of hydrophobic organic compounds. It is interesting to note that the K_{ow} of cis-1,2-indandiol was 33% of the K_{ow} of 2-PE, however, the partition coefficient was only 3% of 2-PE in the same polymer. The result may not be surpring because octanol has been arbitrarily chosen as the extraction phase for the estimation of K_{ow} and polymers have a much more complicated internal structure and chain entanglements compared to the simple 8-C chain solvent. For example, steric hinderance can become important in polymer extraction. cis-1,3-indandiol is larger than 2-PE in molecular size and has an additional ring structure (non-aromatic) that adds to the strain of the molecule. The two hydroxyl groups on this ring have less freedom to move and bond with functional groups in the polymer. These reasons may explain the lower partition coefficient of cis-1,3-indandiol/Hytrel® system relative to 2-PE/Hytrel® system.

3.5.3 iso-butanol

Butanol as a transportation fuel additive has gained attention recently due to its lower vapour pressure, high energy content, and better miscibility with gasoline and diesel relative to ethanol. Like ethanol, butanol can be produced

from biomass, and the production scale of butanol as a transportation fuel will be large, in the order of multi-million tons/year.

Similar to 2-PE, iso-butanol is an alcohol, but is more hydrophilic than 2-PE because of the lack of an aromatic ring. Based on several studies on the use of polymer membranes for the pervaporation of butanol [Liu *et al.* 2005; Jonquieres *et al.* 2002], polyether block amide (with the trade name PEBAX®) was found to be among the best working polymers in transporting butanol from aqueous solution across a polymer membrane. In fact at elevated temperature, butanol can dissolve PEBAX® 2533 [Liu *et al.* 2005]. Therefore, 3 grades of PEBAX® were selected as the candidate polymers together with 2 grades of Elvax®, a copolymer of polyethylene and polyvinyl acetate. Since Elvax® had performed well in absorbing phenol [Amsden *et al.* 2003], it was thought that the acetate group may facilitate hydrogen-bonding with butanol. The results are shown in Figure 3-4.

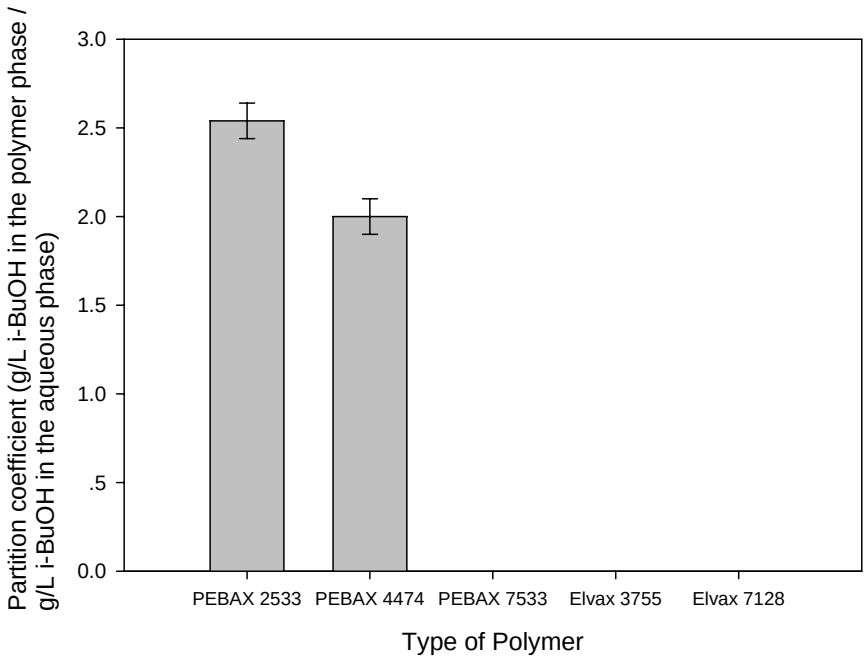

Figure 3-4: Partition coefficients of candidate polymers towards iso-butanol

Similar to Hytrel®, the difference between the various grades of PEBAX® is the proportion of the two monomers (polymer amide and polyether). The proportion of polyamide (the "hard" segment) increases as PEBAX 2533 < PEBAX 4474 < PEBAX 7533, and the percentage of the other monomer, polyether (the "soft" segment) decreased in the same manner. Therefore, PEBAX® 2533 is the softest and the least crystalline of the three grades. A similar trend as the 2-PE/Hytrel® system can be seen from the results in Figure 3-4 in which the softer, amorphous grade showed better absorption than the hard, crystalline grade. The most crystalline grade PEBAX 7533 showed no uptake of iso-butanol at all. Elvax® also did not show any uptake, possibly because of the

crystalline polyethylene content. It has been suggested by Gavara *et al.* (1996) that iso-butanol has a much lower solubility coefficient in ethylene vinyl alcohol (EVOH) polymers than n-butanol, possibly due to the branched structure of this molecule.

Another ISPR approach that has been studied in the literature involves the adsorption of butanol using polymeric resins. The particle size of these resins is usually smaller compared to polymer beads and they often possess high surface area. The mechanism of extraction in this case is through surface adsorption [Nielsen and Prather, 2009] instead of whole polymer absorption as is the case in our work. Although the isotherm reported for poly(styrene-co-DVB) type based resins showed higher uptake than whole polymer beads [Nielsen and Prather, 2009], this type of resin can impose great challenges in reactor operation. These resins are often brittle and fragile, therefore, under the shear stress introduced by agitation in a bioreactor, they can be easily broken down [Prpich and Daugulis, 2007]. This can cause the loss of the extraction phase with time. The other drawback of adsorption resin is that once the surface area is all occupied, any increase in aqueous phase concentration, or driving force, will not result in more uptake. In contrast, the uptake by polymer beads is through absorption which utilizes the entire mass of the beads, therefore, under increased driving force, the beads can respond and take up more solute. In this study, three different butanol starting concentrations were tested, and the results showed a linear relationship

between the specific loading (g of butanol/ kg of polymer) and the initial butanol concentration in the aqueous phase (g of butanol / L) (data not shown). Also, the cost of polymeric resins can be more than 30 times higher than that of the polymer beads used in this study [Nielsen and Prather, 2009].

3.5.4 Succinic acid

Potential building block compounds for the emerging biorefinery concept have been identified from hundreds of candidate compounds [DOE Report, 2004]. Among them, succinic acid and 3-hydroxybutyrolactone have been selected in this study to further investigate the potential for polymer extraction.

Succinic acid is a dicarboxylic acid with a pKa_1 of 4.21. It was expected that the dissociated ions of succinic acid would be difficult to extract because the ions would be solvated by water molecules. Therefore, the experiments were performed at a pH that is well below its pKa_1. In fact, the bioproduction of succinic acid at low pH is the future direction of this process [DOE Report, 2004] and research of this nature is currently being undertaken in Daugulis Group. Different grades of Zytel® (polyamide) were first selected to be the candidates for the extraction of undissociated succinic because polyamide is a relatively polar polymer. However, the results showed no uptake for all the grades of Zytel® tested. The reason may be that although polyamide has polar amide linkages that can interact with the carboxyl group in succinic acid, the polymer sites are mostly occupied by intermolecular hydrogen-bonding within the polymer itself,

therefore, leaving less possibility to bond with external molecules. The intermolecular hydrogen-bonding between polymer chains not only reduces the polar sites for external hydrogen-bonding, but also makes the chain structure more regular and symmetric hence increasing the crystallinity of the polymer. Other polymers such as PEBAX® and Hytrel® were then tested and the results are shown by Figure 3-5.

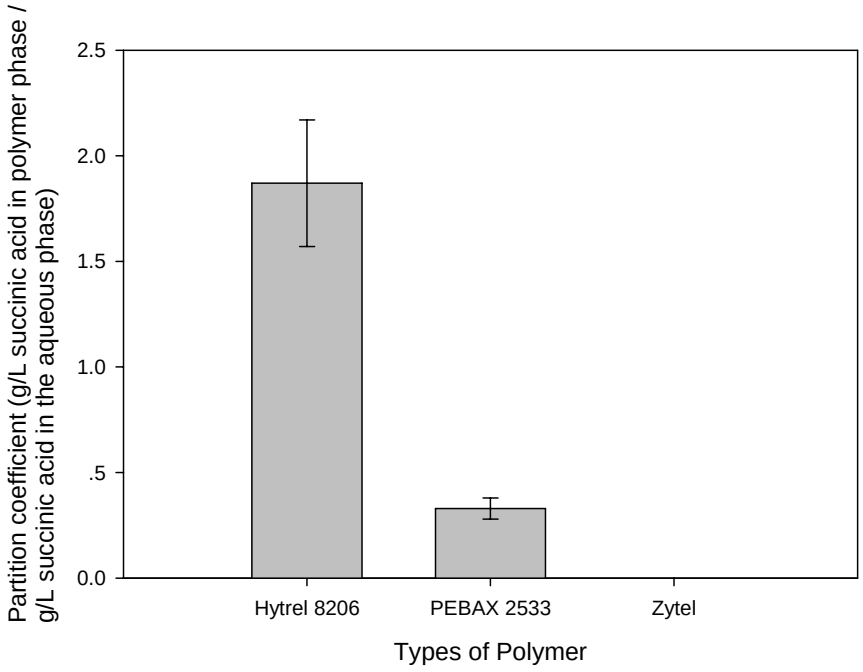

Figure 3-5: Partition coefficients of candidate polymers towards succnic acid

PEBAX® showed better results than Zytel® possibly because it is a copolymer of polyamide and polyether. The presence of the "soft" segment polyether in PEBAX® reduces the crystallinity of the the polymer compared to purely polyamide (Zytel®). Hytrel® showed even better results than PEBAX®

because compared to PEBAX®, both of the monomers in Hytrel® are open for hydrogen-bonding and are easy to access.

3.5.5 3-hydroxybutyrolactone (3-HBL)

As can be seen in Table 2-2, 3-HBL is a fairly polar compound with K_{ow} < 1. This posed great challenges for possible polymer absorption. The candidate polymers included Hytrel®, PEBAX®, Zytel® and Kraton®. The results are shown by Figure 3-6. The only polymer that showed positive results was Hytrel® with a partition coefficient of 1.6. All of the other polymers showed no signs of absorption towards 3-HBL. It can be expected that for fairly hydrophilic substance such as succinic acid and 3-HBL, polymer extraction can be challenging because of the hydrophobic nature of polymer beads. Grafting hydrophilic functional groups onto the hydrophobic backbones can be an option to improve the results. Shantora and Huang (1981) have shown that by grafting hydrophilic functional groups such as acrylic acid and methacrylic acid onto an EVOH based polymer membrane, its permeability towards polar compounds such as methanol were greatly improved compared to the commercially available EVOH membranes. Bulk polymer beads can be treated as a dense polymer membrane, and grafting suitable functional groups that meet the need of polymer-solute interaction may show promising results in the future. Research in this direction is currently undertaking in Daugulis Group.

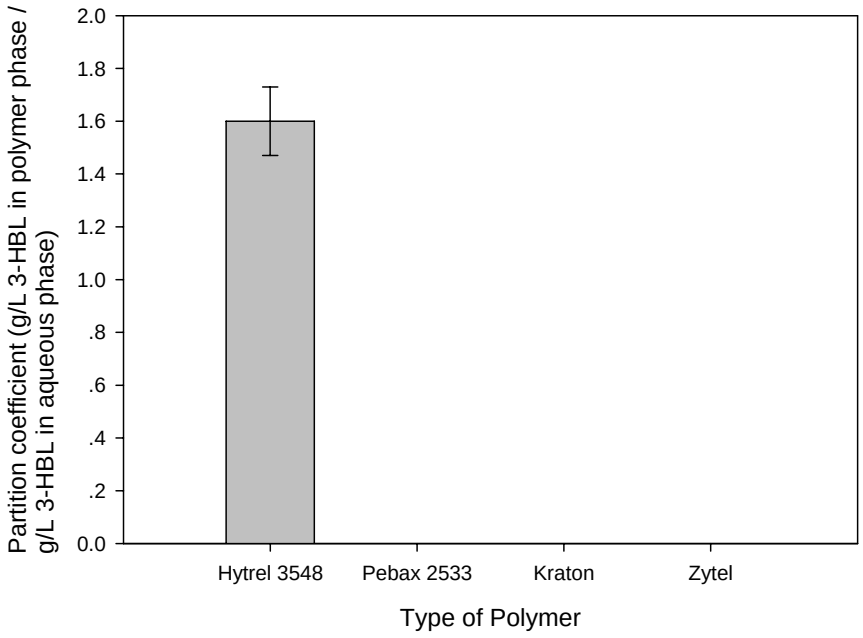

Figure 3-6: Partition coefficients of candidate polymers towards 3-HBL

3.6 Conclusion

This study showed that in a copolymer, the composition of the "hard" and "soft" segment can affect the absorption of small organic compounds. Copolymers that have a larger proportion of the "soft" segment tend to have higher extraction power when other aspects are equal. This is preferred in a solid-liquid two phase bioreactor system in which polymers act as the sequestering phase for the products. Hydrogen-bonding between functional groups of small molecules and polymers is important for their interactions and possibly is the main mechanism in polymer absorption of relatively polar molecules. However, intermolecular hydrogen-bonding inside the polymer chains is not desirable

because it occupies sites available with external molecules for hydrogen-bonding and increases the crystallinity of the polymer. Compounds with low K_{ow}, such as succinic acid and 3-HBL pose great challenge for polymer extraction compared to compounds with higher K_{ow}, such as 2-PE. However, the fact that relatively polar polymer, such as Hytrel® showed positive results for their extraction is a promising first step. One advantage of using polymers as the extraction phase is the versatility of polymers which can be tailored for specific applications. Grafting functional groups onto the backbone of bulk polymers seems to be a good option and related research is currently underway in Daugulis Group.

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Chapter 4

Bioproduction of the aroma compound 2-phenylethanol in a solid-liquid Two-Phase Partitioning Bioreactor system by *Kluyveromyces marxianus*

Fang Gao and Andrew. J. Daugulis

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4.1 Preface

The partition coefficient tests performed in Chapter 3 showed positive results for all compounds tested, especially for the relatively hydrophobic compound such as 2-phenylethanol, the partition coefficient was as high as 79 with Hytrel® 8206. To the best of our knowledge, this is the highest partition coefficient currently reported in literature. Based on the physical testing results obtained through Chapter 3, both the partition coefficient and the diffusion rate, it was proposed that Hytrel® 8206 would be a promising sequestering phase in the bioproduction of 2-phenyethanol.

In order to demonstrate the benefits gained from using solid-liquid TPPB systems, in addition to the physical testing, bioreactor performance and operability must also be assessed. Therefore, in this chapter, a solid-liquid TPPB was further examined in detail for a biotransformation that converts L-phenylalanine to 2-phenylethanol applying the best performing polymer Hytrel® as the second immiscible phase and *Kluyveromyces marxianus* CBS 600 as the

biocatalyst. The reactor performances of a single phase bioreactor, two-phase batch mode bioreactors and a two phase semi-continuous bioreactor with an external column were compared in the study.

4.2 Abstract

The rose-like aroma compound 2-phenylethanol (2-PE) is an important fragrance and flavor ingredient. Several yeast strains are able to convert L-phenylalanine (L-phe) to 2-PE among which *Kluyveromyces marxianus* has shown promising results. The limitation of this process is the low product concentration and productivity primarily due to end product inhibition. This study explored the possibility and benefits of using a solid-liquid Two-Phase Partition Bioreactor (TPPB) system. The system applies polymer beads as the sequestering immiscible phase to partition 2-PE and reduce the aqueous 2-PE concentration to non-inhibitory levels. Among 6 polymers screened for extracting 2-PE, the DuPont polymer Hytrel® 8206 performed best with a partition coefficient of 79. The desired product stored in the polymer was ultimately extracted using methanol. A 3L working volume solid-liquid batch mode TPPB using 500 g Hytrel® as the sequestering phase generated a final overall 2-PE concentration of 13.7 g/L, the highest reported in the current literature. This was based on a polymer phase concentration of 88.74 g/L and aqueous phase concentration of 1.2 g/L. Even better results were achieved via contact with more polymers (approximately 900 g) with the aqueous phase applying a semi-continuous reactor configuration. In this system, a final 2-PE concentration (overall) of 20.4 g/L was achieved with 1.4 g/L in the aqueous and 97 g/L in the polymer phase. The overall productivities of these two reactor systems were 0.38 g/L-hr and 0.43 g/L-

hr, respectively. This is the first report in the literature of the use of a polymer sequestering phase to enhance the bioproduction of 2-PE, and exceeds the performance of two-liquid phase systems in terms of productivity as well as ease of operation (no emulsions) and ultimate product recovery.

Key Words: 2-phenylethanol, biotransformation, two-phase partitioning bioreactor, polymer, *Kluyveromyces marxianus*

4.3 Introduction

2-phenylethanol (2-PE) is an important fragrance and flavour ingredient in the cosmetic and food industry, mainly because of its popular rose-like smell. Most of the 2-PE currently in use is produced chemically (Etschmann *et al.,* 2002). The major problem in its chemical synthesis is the costly product purification process. Additionally, the solvents and by-products can impart off-odours in the final product and therefore influence the grade of 2-PE (Etschmann *et al.*, 2002). In the last two decades, consumers have tended to favour "natural" ingredients in products and this behavior has driven up the market demand for naturally produced 2-PE. Because products derived from bioprocesses starting with natural substrates are in principle defined as "natural" if they have already been identified in plants or other natural sources (Schrader *et al.,* 2004), an option of obtaining natural 2-PE is to use microbial biocatalysts.

Yeasts have shown great ability to produce higher alcohols and esters as aromas (Etschmann *et al.,* 2002). When provided with L-phenylalanine as the precursor, several food grade yeasts are able to convert it to 2-PE via the Ehrlich pathway (Albertazzi *et al.,* 1994). The yeast strains *Kluyveromyces marxianus* and *Saccharomyces cerevisiae* have shown promising results in producing 2-PE (Etschmann *et al.,* 2003; Fabre *et al.,* 1998; Stark *et al.*, 2002). Even with the efforts to optimize medium and choose the best-producing microorganisms, this biological system is still limited by low 2-PE concentration and low productivity

primarily due to end product inhibition. 2-PE at even low concentrations can be used as a biocide because it is toxic to yeast cultures once it exceeds a critical inhibitory level. Fabre *et al.* (1998) reported that 2.0 g/l of 2-PE could cause complete growth inhibition for *K. marxianus*. Several *in situ* product removal (ISPR) approaches for reducing product inhibition have been attempted. Such a technique allows the aqueous phase 2-PE to be removed as it forms *in situ* by an extractant. Liquid-liquid solvent extraction is the most commonly studied ISPR technique for 2-PE biotransformation. Solvents such as oleyl alcohol (Etschmann *et al.,* 2003), oleic acid (Stark *et al.,* 2002), and polypropylene glycol 1200 (PPG 1200) (Etschmann and Schrader, 2006) have been tested and were able to enhance the system performance. Etschmann and Schrader (2006) achieved the highest 2-PE concentration (10.2 g/L overall) reported by using *K. marxianus* CBS 600. Other ISPR techniques such as microcapsulation (Stark *et al.*, 2003 (1); Serp *et al.*, 2003); and organophilic pervaporation (Etschmann *et al.*, 2005) have also been reported.

There are several drawbacks of the liquid-liquid two phase system. First, organic solvents, especially those with high carbon content, are hard to evaporate and remove. Therefore, their residual odor could affect the quality of the final product. Second, organic solvents may exhibit cytotoxicity and affect cell viability (Bruce and Daugulis, 1991). Third, although PPG 1200 showed the highest partition coefficient in extracting 2-PE from the aqueous phase, it formed

emulsions with the aqueous phase upon stirring (Etschmann and Schrader, 2006), causing problems for OD measurements and separation of the two phases. Compared with two liquid phase systems, the solid-liquid Two-Phase Partitioning Bioreactor (TPPB) is a new technique for ISPR. There have been several successful demonstrations in the environmental biotechnology field (Prpich and Daugulis, 2006; Rehmann and Daugulis, 2007; Littlejohns and Daugulis, 2008), and two biotransformation applications have also shown promising results (Morrish and Daugulis, 2008; Prpich and Daugulis, 2007). In such a system, polymers (usually in the form of polymer beads) act as the sequestering phase to absorb and store the desired product, not only targeting the product inhibition problem, but also acting as the initial concentrating step in downstream processing. Polymers have several advantages over organic solvents (Prpich and Daugulis, 2004; Rehmann *et al.* 2007) in that they are generally biocompatible, non-biodegradable, non-volatile, inexpensive, and for this particular application, polymers will not compromise the product quality by affecting its organoleptic property.

In this paper, a solid-liquid TPPB system has been investigated for the production of 2-PE by *K. marxianus* CBS 600. Several polymers were screened to select the best sequestering phase. The polymer that showed the best results was then used in a TPPB system. A single-phase biotransformation, biotransformations with different amounts of polymer loading, and a semi-

continuous solid-liquid reactor configuration were studied. The reactor performances were quantified with regard to the final product concentration, productivity, and yields; reactor operability and the recovery of the desired product from the polymer were also assessed.

4.4 Materials and Methods

4.4.1 Chemicals and polymers

$MgSO_4$, K_2HPO_4, citric acid and glucose, and the solvents 1-dodecene and 1-dodecane were purchased from Fisher Scientific. L-phenylalanine, 2-phenylethanol, 2-phenylethylacetate (2-PEA) and Polypropylene Glycol 1000 (PPG 1000) were purchased from Sigma-Aldrich. Yeast Nitrogen Base (YNB) without amino acids and was purchased from BD Difco™. Polymers with the trade name: Hytrel ® 8206, Nylon 6,6 ®, Elvax® were kindly donated by DuPont Canada. Other polymers used for screening purpose were Desmopan® (Bayer), High Density Polyethylene (Sigma-Aldrich) and Kraton® (Styrene/butadiene copolymer, Bayer).

4.4.2 Medium formulation and culture preparation

The yeast strain *Kluyveromyces marxianus* CBS 600 was obtained from the American Type Culture Collection (ATCC 200965). The medium formulation was adapted from Etschmann *et al.* (2004) and Wittmann *et al.* (2002). Defined media were used throughout the inoculum preparation and bioreactor runs. For preparing inocula, the following medium formulation was used (g/L): L-phe 7,

glucose 30, $MgSO_4$ 0.5, citric acid 10.3, K_2HPO_4 27.1, YNB without amino acid and ammonium sulphate 6.8. For bioreactor medium formulation, glucose and L-phe concentrations were increased to 70 g/L and 26 g/L, respectively for TPPB systems, and only glucose concentration was increased to 70 g/L for the single phase system.

Seven flasks each containing 50 mL medium were inoculated with 20 µL cells. One flask was used for tracking cell OD. Once it reached 1.2 (approximately after 24 hrs of incubation), the remaining six flasks were added to the bioreactor. All operations were conducted aseptically.

4.4.3 Analytics

Cell measurement

A cell dry weight vs. optical density (OD) calibration curve was used. Samples taken from the reactor were measured immediately for their OD at 600 nm (Biocrom Ultraspec).

Concentration measurements

High performance Liquid Chromatography (HPLC) was used to quantify 2-PE, 2-PEA and L-phe. After measuring cell OD, the mixture was centrifuged at 3,500 rpm for 15 min at 4^{o}C. The supernatant was then passed through a 0.45-µm syringe filter (Waters) prior to the injection into the HPLC (Waters 2478). 20µL sample was injected and passed through a RP-C18 column (Spherisorb ODS-2 column), and the compounds were detected by a Waters UV–Visible Detector at

258 nm. 35% sterile water and 65% methanol was pumped isocratically through a Waters 515 pump at 1ml/min. The column was kept at room temperature. Ethanol concentration was tracked by an enzymatic kit purchased from R-Biopharm. Glucose concentration was measured by a DNS assay adapted from Miller (1959).

4.4.4 Polymer partition coefficient

Six 20 mL scintillation vials were used to test partition coefficient. One vial containing 10 mL of 4 g/L 2-PE solution was used as the control with no polymer, and the other five vials contained 2-PE solution with concentrations (g/L) 2, 3, 4, 5, and 6. Equal amount of polymers (approximately 0.33 g) were added to each of the 5 vials, and the vials were put onto a shaker for mixing overnight. After equilibrium was established, the aqueous phase 2-PE concentration was analyzed by HPLC. The amount left in the polymer phase was back calculated using mass balance. The control showed no detectable change of the 2-PE concentration. The slope of the straight line of polymer phase concentrations vs. aqueous phase concentrations of 2-PE was the partition coefficient of that polymer towards 2-PE. The same tests were performed with other molecules such as 2-PEA and L-phe. Similar method was applied to test partition coefficient using organic solvents.

4.4.5 Batch Reactor Operation

A 5L bioreactor (3L medium) equipped with pH, temperature and dissolved oxygen control was used for all reactor runs (New Brunswick Scientific,

BioFlo III). The pH was controlled automatically by adding 6M KOH. The aeration rate was controlled at 1 vvm (3L/min) and the agitation was set to 600 rpm for single phase and 700 rpm for TPPB runs. The temperature was controlled at 35^{o}C. Dissolved oxygen was monitored to ensure the reactor was non-oxygen limited. Two trials of solid-liquid TPPB were performed with 100 g of Hytrel® and 500 g of Hytrel®. The glucose concentration was tracked online after hour 18 to closely monitor the depletion of the carbon source. Saturated glucose solutions were added at various time points during the bioreactor runs, and the glucose concentration was maintained in this fashion between 10 g/L and 30 g/L after the initial rapid consumption.

4.4.6 Semi-continuous Reactor Operation

A glass column (0.5 L; length: 30cm; Diameter: 4.6cm) filled with polymer beads was connected to the reactor via sterile tubing and a peristaltic pump. The contact between the aqueous phase and the polymer phase was achieved by circulating the broth (containing cells) through the external column packed with polymer beads at a rate of 40 mL/min. The polymer beads were around 2 mm in diameter, therefore, the column was not tightly packed due to the void space between polymer beads. Therefore, blockage caused by cells did not occur in the column. The assembly and preparation of this reactor was adapted from Morrish and Daugulis (2008). Three glass columns were used each containing approximately 300 g of polymer beads. Other reactor conditions were

kept the same as the batch TPPB operations. Besides glucose addition, substrate L-phe was also added at time 29 hours.

4.4.7 Product Recovery from Polymer

An extraction procedure with methanol was performed after the polymers were recovered from the reactor. In a 20 mL vial, 10 ml of methanol was added together with approximately 1 g of polymer randomly selected from the recovered polymers. The mixture was put onto a shaker to equilibrate overnight. After 24 hours, the methanol was decanted completely to a new vial. 10 mL of fresh methanol was then added to the vial that contained the polymers for a second round of extraction overnight. The procedure was repeated several times until there was no detectable 2-PE in methanol. The total amount of 2-PE stored in the polymer was calculated by adding the amount of 2-PE extracted into methanol from all rounds of methanol extractions.

4.5 Results and Discussion

4.5.1 Polymer Screening

Initial efforts were made to find a polymer that had the highest partition coefficient towards 2-PE. Two important functional groups in the 2-PE chemical structure are the aromatic ring and the hydroxyl group. Therefore, polymers that can attract 2-PE through π interaction and/or through hydrogen bonding may be considered as promising absorbants. Previously, PPG 1200, Oleic acid, and Oleyl

alcohol were used and showed good results. Generally speaking, these solvents were polar solvents with no aromatic functionality.

A similar trend was found in the polymer partition coefficient tests shown in Figure 4-1. Hytrel® 8206 possessed the highest affinity toward 2-PE among all polymers tested. Hytrel® is a block copolymer of polybutylene ester and polyether (Prpich and Daugulis, 2004). Both ester and ether linkages open the possibility for hydrogen bonding, a strong intermolecular force, between the polymer and the hydroxyl group in 2-PE. By the same mechanism, polyurethane also showed good results in attracting 2-PE. Nylon®6,6 also had hydrogen-bonding potential due to its polyamide linkage in the structure; however, it appeared to have a lower partition coefficient. The reason for this is that Nylon 6,6 can form intermolecular hydrogen bonding inside the polymer chains, and it reduces the sites available for bonding with 2-PE. This phenomenon is also reflected in its high degree of crystallinity because the intermolecular hydrogen bonding holds and arranges the chains in a regular and ordered way. A high degree of crystallinity is undesirable when polymers are used in a TPPB system. In order to absorb the target compound, not only attraction between the polymers and 2-PE has to occur, but also the polymer needs to possess large free volume to accommodate the target compound. This requires the polymer to have a large portion of its structure in an amorphous condition instead of being crystalline. As a result, Nylon® 6,6 was not as good a candidate as Hytrel®. Elvax ®, a type of

ethylene-vinyl acetate, had modest affinity to 2-PE because the acetate group can hydrogen-bond with 2-PE. However, due to the presence of a large portion of ethylene making the polymer chains packed in an ordered pattern, its structure has a high degree of crystallinity. High density polyethylene (HDPE), showed even lower affinity, and the highly aromatic polymer styrene butadiene rubber (SBR) showed no measurable affinity. This observation led to the hypothesis that the primary uptake mechanism of 2-PE by polymer beads is through hydrogen bonding interaction instead of aromatic π interaction. Similar results can also be found when organic solvents were tested. The most polar solvent PPG 1000 had a partition coefficient of 31 while the non-polar solvents 1-dodecene and 1-dodecane showed almost no affinity for 2-PE.

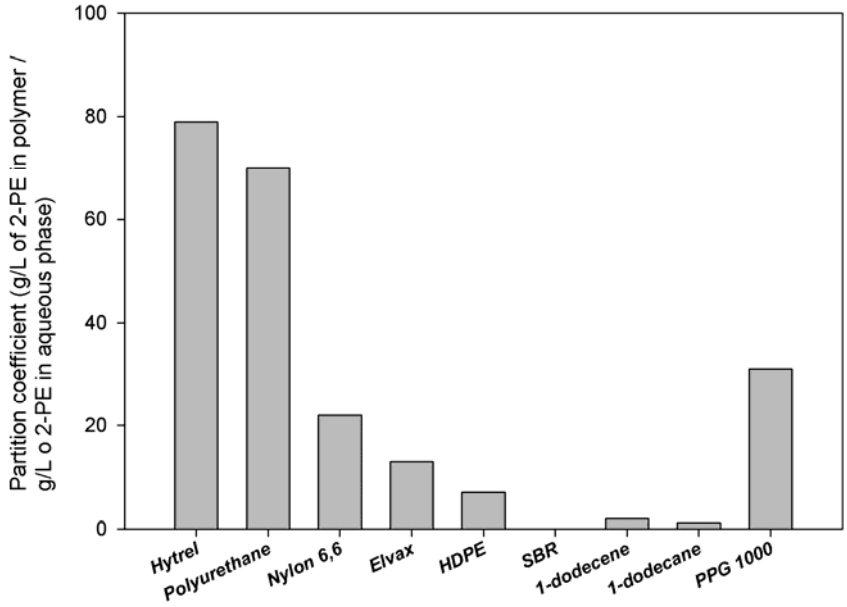

Figure 4-1: Partition coefficient screening of all polymers and organic solvents tested. The number was obtained based on the linear relationship (slope) between the aqueous phase 2-PE concentration and polymer phase 2-PE concentration. The former one was from direct measurement while the latter one was calculated by mass balance.

The best polymer Hytrel® 8206 was then tested for its affinity towards L-phe and 2-PEA (2-phenylethylacetate). L-phe was mainly in its dissociated form (pKa for carboxylic acid = 2.59) in the medium (pH = 5). The tests showed no measureable uptake of L-phe by Hytrel®. Hytrel® was also tested for its affinity towards 2-PEA, a valuable fragrance by-product in this biotransformation. The partition coefficient was found to be 50. Hytrel® is an FDA compliant polymer for use in the food and fragrance industry (personal communication, DuPont Canada). It is odorless and its properties were not affected by autoclaving at

121^{o}C. Hytrel® was also determined to be non-bioavailable and biocompatible, and was thus a good choice for this biotransformation.

4.5.2 Batch reactor runs with a single phase and a solid-liquid TPPB system

Prior to solid-liquid TPPB operation, a single phase run with only aqueous medium was performed to provide a benchmark. The time course graph of cell growth and product accumulation is illustrated by Figure 4-2. The arrows represent the time points when glucose was added (same for Figure 3, 4, and 6). A final cell density of 5.8 g/L was obtained at the end of 42.5 hours with 1.4 g/L of 2-PE in the aqueous broth. The results were similar but with slightly lower numbers than Wittmann's results which found 7 g/L of biomass and 1.83 g/L of 2-PE in a single phase bioreactor (Wittmann *et al.,* 2002). However, although the two studies both used *K. marxianus*, the specific strains were different. Using the same strain of *K. marxianus* CBS 600, Etschmann *et al.* (2003) had accumulated 0.89 g/L of 2-PE in a single phase reactor with non-optimal medium and carbon source. Even though the reported toxic threshold of 2-PE for *K. marxianus* was 2 g/L, it is difficult to reach that concentration in a reasonable time period. By the end of the biotransformation, there was 14 g/L of glucose and 4.2 g/L of L-phe left which indicated sufficient carbon and nitrogen source, and the termination of the biotransformation was due to product inhibition. To ensure this was the case, at the end of the run, 50 mL of broth was taken from the reactor followed by the addition of 10 g of Hytrel® beads, and was put into an incubator. After 6 hours,

the optical density increased indicating the resumption of cell growth. Furthermore, this test indicated that during a short period of time, the damage to the cells caused by 2-PE toxicity was not permanent, and cell growth can be restored.

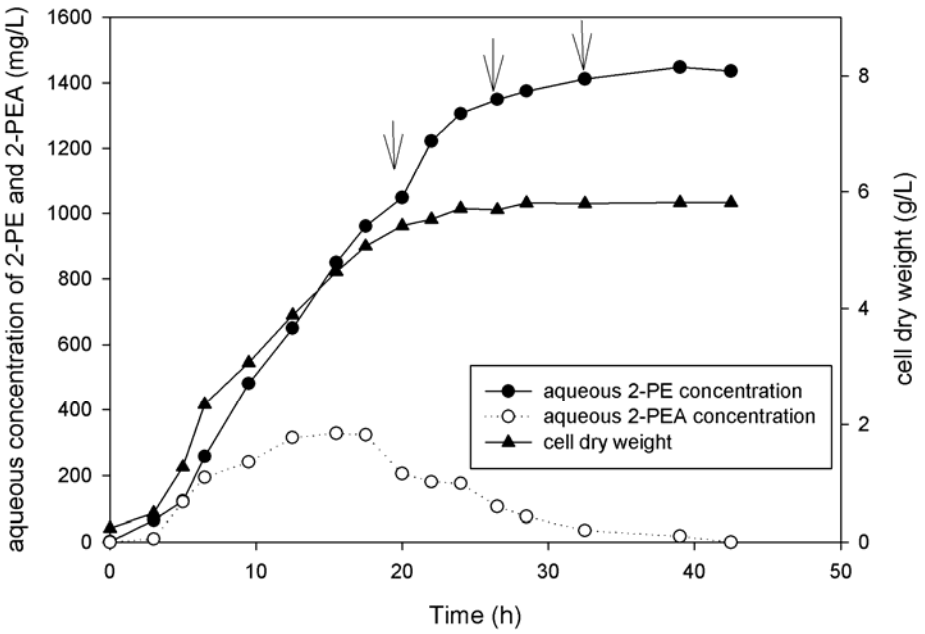

Figure 4-2: Aqueous phase 2-PE and 2-PEA concentration and cell density as a function of time in a single phase reactor. The time points marked with arrows indicate the addition of 30 mL of saturated glucose solution (600 g/L).

Two batch trials of the solid-liquid TPPB with Hytrel® polymer beads (100g, 500g) were performed and the results are shown in Figure 4-3 and Figure 4-4, respectively. The amount of 2-PE in the polymer beads was measured via the desorption test in methanol. After the third round of extraction, there was no detectable 2-PE in the methanol. The 1st wash was able to extract 80% of the total

2-PE stored in the polymer beads. A final partition coefficient was calculated and the results agreed well with the partition coefficient tests obtained earlier. It is obvious that by adding the selected polymer as the sequestering phase, nearly all the important measurements of reactor performance improved significantly (Table 4-1). The total 2-PE concentration in the TPPB system was 2.6 times (100 g Hytrel® run) and 9 times (500 g Hytrel® run) that of the single phase and 3 g/L (500 g Hytrel® run) more than the highest reported 2-PE production using *K. marxianus* (Etschmann and Schrader, 2006). The overall productivity using the solid-liquid TPPB system was 2 times (100 g Hytrel® run) and 6 times (500 g Hytrel® run) that of its single phase counterpart. The polymer appeared to have no affinity for L-phe and glucose, therefore the substrates were delivered to the cells in the same manner as the single phase system. The fact that aqueous 2-PE accumulation curve was not affected by adding the sequestering phase showed that the two-phase system was kinetically limited instead of being mass transfer limited, which means that the diffusion and absorption of 2-PE by Hytrel® beads were fast enough to accommodate 2-PE production by the cells. One additional observation is that in the single phase system, the 2-PEA concentration reached 0 at the end of the fermentation while in the TPPB system did not. The single phase 2-PEA profile agreed with Wittmann's results when they analyzed the metabolic physiology of *K. marxianus* (Wittmann *et al.,* 2002). However in the TPPB system, a portion of 2-PEA produced at the beginning of the fermentation was

likely stored in the polymer. When the aqueous 2-PEA concentration started to decrease, 2-PEA stored in the polymer diffused out of the polymer driven by the increased concentration gradient between the two phases, and a new equilibrium was established. Therefore, the 2-PEA concentration in the aqueous phase never reached 0. This is also consistent with Etschmann's observation (Etschmann and Schrader, 2006).

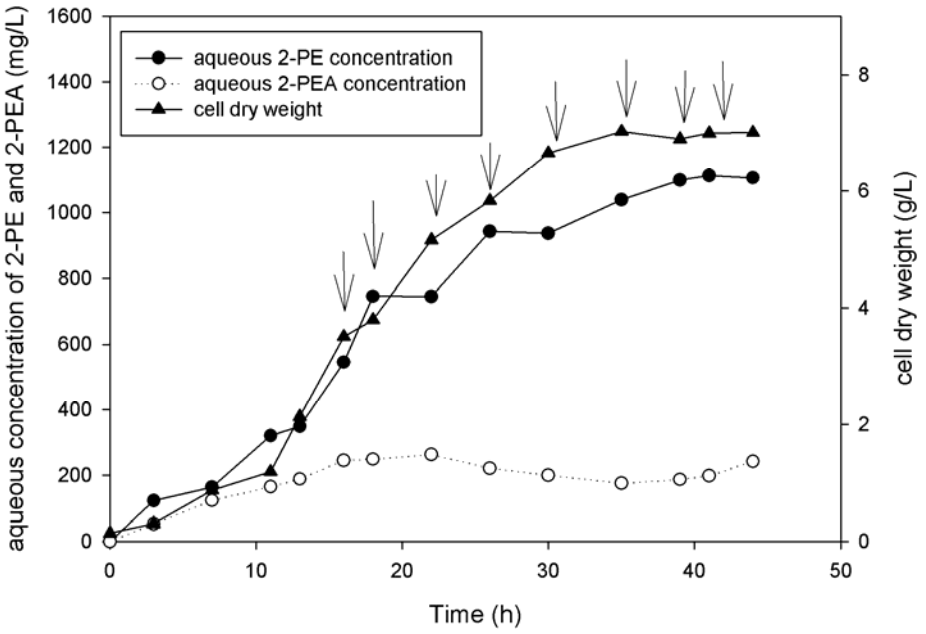

Figure 4-3: Aqueous phase 2-PE and 2-PEA concentration and cell density as a function of time in a TPPB batch system in which 100 g of Hytrel® polymer was added as the sequestering phase. The time points marked with arrows indicate the addition of 60 mL of saturated glucose solution (600 g/L).

The starting glucose concentrations were around 70 g/L for all reactor runs to provide enough carbon source. Initially it was consumed rapidly and at around 20 hours, its concentration dropped below 10 g/L. Glucose was then fed to the

reactor at the time points marked by arrows in Figure 4-3 and Figure 4-4, and by applying this feeding strategy, the glucose level was maintained between 10 g/L – 30 g/L after initial consumption. The ethanol concentration was kept below 10 g/L for all reactor runs (data not shown). Ethanol was produced at a faster rate initially at higher glucose concentration, and as the glucose was utilized by cells, the production of ethanol slowed and stopped. As glucose approached depletion, the ethanol concentration dropped because cells used ethanol as a carbon source, consistent with Wittmann's finding (Wittmann *et al.,* 2002). The primary substrate L-phe was also provided as the sole nitrogen source for the cells. Shown in Figure 4-5, its consumption was significantly enhanced by the presence of Hytrel®. This is expected since the increase in cell density required more nitrogen, and more importantly, the sequestration of the product by the polymer drove the biotransformation towards the formation of more 2-PE and therefore the consumption of L-phe was increased.

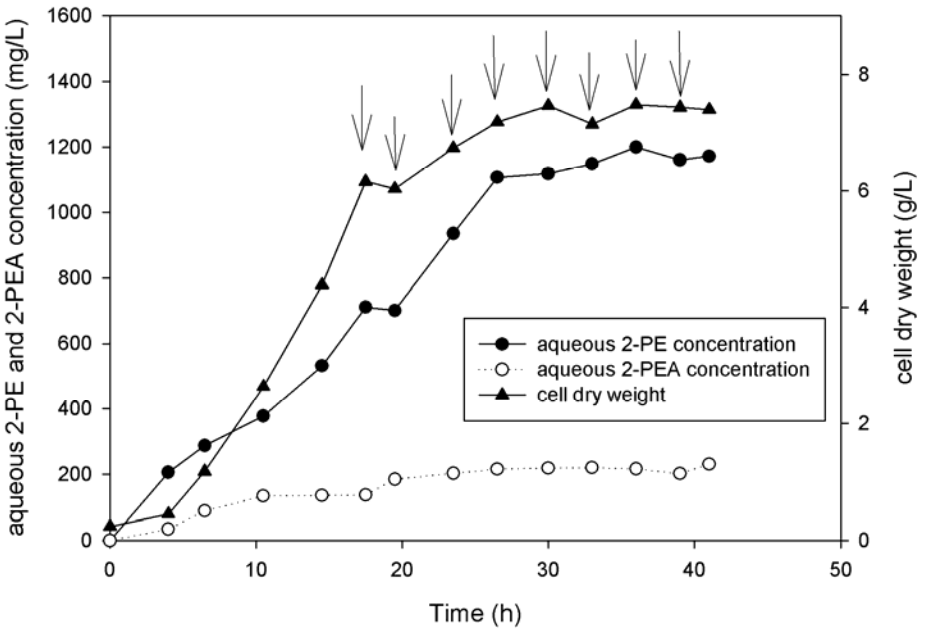

Figure 4-4: Aqueous phase 2-PE and 2-PEA concentration and cell density as a function of time in a TPPB batch system in which 500 g of Hytrel® polymer was added as the sequestering phase. The time points marked with arrows indicate the addition of 60 mL of saturated glucose solution (600 g/L).

The reactor operation was trouble-free for all cases. Since solid polymer beads were used the potential problems of forming emulsions upon stirring were not encountered. The presence of 500 g of Hytrel® beads in 3L of liquid volume did not cause any mechanical problems associated with stirring, aeration, sampling and operation in general. The oxygen transfer was sufficient under 700 rpm agitation and 1 vvm aeration. With more vigorous aeration, major product loss might be expected due to the high volatility of this aroma compound. A condenser was attached to the bioreactor and at the exhaust end of the condenser,

there was slight rose-like smell even under 1vvm aeration rate indicating minor product loss via evaporation.

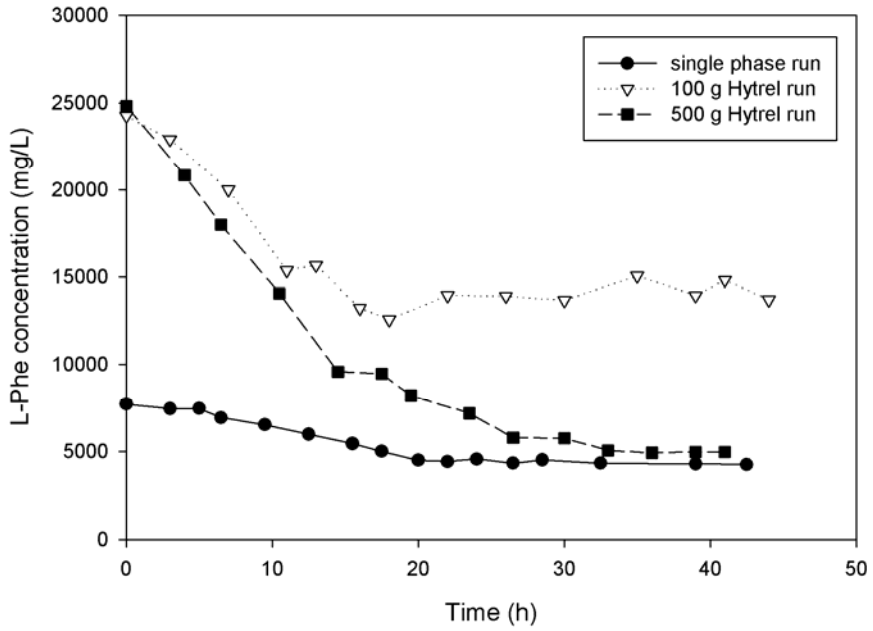

Figure 4-5: Substrate L-phe consumption curve as a function of time for three different reactor runs.

Methanol was chosen because of its complete miscibility with 2-PE and therefore high extraction power. Also, the separation of methanol from 2-PE can be achieved by distillation given the large difference in boiling point of the two compounds. However, a less toxic solvent such as ethanol, which is also miscible with 2-PE, could be a better choice, and its potential should be further explored.

4.5.3 Semi-continuous solid-liquid TPPB reaction System

It can be seen from the batch mode runs that by adding more polymer beads, the reactor performance was greatly enhanced. However, there exists a

maximum practical amount of beads that can be added, limited by the physical operation of the reactor. The compromise between adequate bead loading and good operability will limit the reactor performance. However, this problem can be solved by the use of an external column loaded with polymer beads through which the bioreactor contents are circulated. Once the beads are fully loaded, the column can be taken offline and a new column with fresh beads can be attached. This configuration not only makes it possible to bring large amount of polymer into contact with the aqueous broth, but also makes the process semi-continuous. Two column changes were made at hour 20 and hour 38. The decision about when to change columns was based on previous experience and online tracking of 2-PE concentration. The cell viability can be greatly reduced when the 2-PE concentration reached 0.89 g/L (Etschmann *et al.,* 2003), and in order to preserve reasonable cell viability, the first column change was made when 2-PE concentration was just below 1 g/L. The new column contained fresh beads; therefore, the concentration gradient at the time of column change was high. As the biotransformation progressed, the driving force decreased until the beads were saturated and no absorption would occur. This change was monitored by tracking 2-PE concentration in the aqueous phase and once it plateaued, it was time for the second column change. The progress is shown in Figure 4-6. As shown, after the second column change, the 2-PE concentration was brought down to 1 g/L from

1.3 g/L. The cells kept producing 2-PE and kept growing due to the less toxic level of 2-PE.

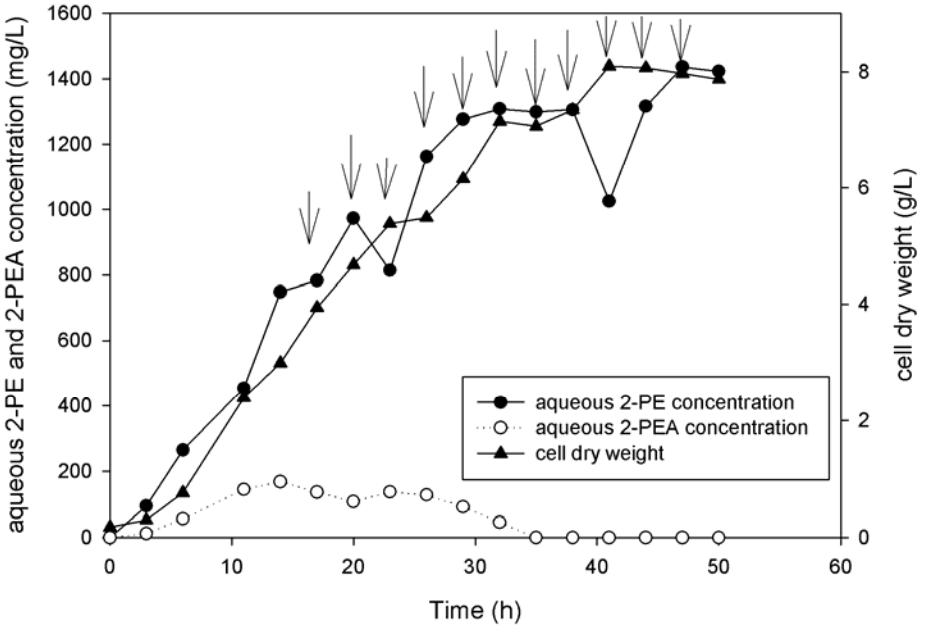

Figure 4-6: Aqueous phase 2-PE and 2-PEA concentration and cell density as a function of time in a TPPB semi-continuous system with approximately 900 g of Hytrel® polymer. The time points marked with arrows indicate the addition of 60 mL of saturated glucose solution (600 g/L).

The overall reactor performance of all bioreactor runs is summarized by Table 4-1. The polymer phase 2-PE concentration was calculated based on the average of all three columns used. Since more beads (over 900 g) were brought into contact with the aqueous broth, the overall 2-PE concentration and overall productivity were greatly improved compared with the 500 g Hytrel® run. It is worth noting that the 2-PE/L-phe yield was also increased when comparing the

TPPB system with the single phase system. It has been reported that about 65.2% of the L-phe consumed is transformed to 2-PE in a single-phase setting (Wittmann *et al.* 2002). Although the reason for the increase in the product/substrate to 93% by adding the sequestering phase (500 g and 900 g Hytrel®) is unclear, our initial hypothesis is that the higher starting L-phe concentration in the TPPB system favoured the Ehrlich pathway to make 2-PE over the Cinnamate pathway. Etschmann's group also showed that by supplying L-phe initially at high concentration, the biotransformation achieved better results (Etschmann and Schrader, 2006).

Table 4-1: **Comparison of reactor performance of the biotransformation process carried out in a single phase system, TPPB batch system, and TPPB semi-continuous system**

	Single phase	**100 g Hytrel**	**500 g Hytrel**	**External column**	
Final C.D.W (g/L)	5.81	7.01	7.40	7.90	
[Aquous 2-PE] (g/L)	1.45	1.12	1.20	1.40	
[Polymer 2-PE] (g/L)	N/A	80.45	88.74	97.00	(average)
[Polymer 2-PEA] (g/L)	N/A	12.10	11.50	N/A	
[2-PE] overall (g/L)	1.45	3.82	13.70	20.38	
Partition coefficient	N/A	71.19	73.95	79.23	(average)
Overall productivity (g/L-h)	0.05	0.10	0.38	0.43	
Y2-PE/L-Phe (mol/mol)	0.60	0.55	0.93	0.91	

1. [] represents the concentration of 2-PE

2. The overall 2-PE concentration was calculated based on the total amount of 2-PE in the aqueous phase and the polymer phase divided by the total reactor volume which include both the aqueous medium and the polymer beads

3. The concentration of 2-PEA in the polymer phase was calculated based on the partition coefficient test and the aqueous phase 2-PEA concentration at the end of the reactor run

4. The concentration unit in the polymer phase (g/L) was based on the mass of polymer used and its particle density (the volume does not contain void space).

This investigation showed that by selecting an appropriate polymer, a solid-liquid TPPB system can greatly enhance the biotransformation process and achieve high final 2-PE concentration and high productivity. Hytrel® showed a high partition coefficient towards 2-PE, however, the issue of selectivity has not been addressed yet. The methanol extraction showed that although 2-PE is the most dominant component in the polymer, small quantities of other by-products from the biotransformation were also extracted. Further polymer screening may identify an even more effective sequestering phase than Hytrel®. Alternatively, the use of mixtures of polymers may provide the ability to preferentially absorb mixtures of molecular species from fermentation media. Nonetheless, this solid-liquid TPPB system showed very promising results in removing a toxic product as an ISPR technique and as an initial concentrating step for downstream processing. Efforts are currently underway to explore the application of solid-liquid TPPBs for producing other flavor and fragrance compounds.

4.6 References

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Chapter 5
Conclusions and Recommendations for Future Work

5.1 Conclusion

The study of the extraction capability of polymers towards selected biotransformation products have revealed positive results for all compounds tested. For a relatively hydrophobic compound, the aromatic alcohol 2-phenylethanol, polymer Hytrel® with a partition coefficient of 79 was able to outperform all the immiscible solvents that have been tested in the literature. Applying this polymer as the sequestering phase in a solid-liquid TPPB, the biotransformation from L-phe to 2-PE using *Kluyveromyces marxianus* was able to generate the highest overall final product concentration currently reported in the literature. The addition of 500 g of Hytrel® 8206 in a batch mode TPPB resulted in an overall 2-PE concentration of 13.7 g/L, 9 times that of the single phase benchmark. In a semi-continuous bioreactor configuration, 900 g of Hytrel® was brought into contact with the aqueous phase and the system generated an overall 2-PE concentration of 20.38 g/L, 14 times that of the single phase run. Operational challenges found in other studies such as emulsion formation was not encountered in the solid-liquid TPPB system.

These results suggest that solid-liquid TPPB systems are a promising approach for ISPR purposes, especially when the polymer phase possesses

high extraction capability towards the compound of interest. Identifying important polymer properties for extracting small organic molecules may facilitate better selection of a suitable polymer phase and may help modifying an existing polymer or formulating a new type of polymer for a certain application. Some polymer properties that have been found to have an effect on the absorption of small molecules by polymers are summarized below:

- Relatively hydrophobic molecules, such as 2-phenylethanol tend to be absorbed by polymers better than hydrophilic molecules such as succinic acid.
- Polymers that have polar linkages such as polyester and polyether performed better than non-polar polymers such as styrene-butadiene rubber in extracting molecules with polar functional groups such as alcohols, acids and lactones. Hydrogen-bonding was thought to be important in these interactions, especially hydrogen-bonding donors linkage, such as polyether, because they cannot cause the problem of intermolecular hydrogen-bonding between polymer chains.
- Copolymers of the same type but with different monomer composition showed difference in extracting target molecules possibly due to the difference in crystallinity. It was found that polymers with relatively high crystallinity may not be preferred for the use in absorbing small organic molecules.

- Polymers that have a high degree of intermolecular hydrogen-bonding showed poor results in the extraction of target compounds. Possible causes may include the high crystallinity introduced by intermolecular hydrogen-bonding and the fact that fewer sites are available to interact with an external molecule.

Hydrophilic compounds, such as those with a Kow smaller than 1, posed challenge for polymer absorption. Although the results showed positive absorption with partition coefficients greater than 1 for succinic acid and 3-HBL, the extraction capability with commercially available polymers is still limited. A large volume proportion of the second phase may be needed to obtain satisfactory results. The other option is to modify the existing polymers or to formulate new polymers that are tailored for the application of extracting hydrophilic compounds. Nonetheless, this study serves as the initial assessment for polymer absorption towards relatively hydrophilic compounds compared to polyaromatic hydrocarbons or benzene, examined previously in Daugulis Group, and the positive results are considered to be an encouraging first step.

5.2 Recommendations for Future Work

As was noticed in Chapter 3, studies have shown that by grafting polar functional groups onto the polymer backbone, the extraction towards polar compounds, such as methanol, can be improved. Therefore, modification of

commercially available polymers by grafting polar functional groups may facilitate the absorption of hydrophilic molecules in a solid-liquid TPPB system and therefore is recommended as future work for the biotransformation products succinic acid and butanol. Possible options for grafting may include polar molecules such as acrylic acid and maleic anhydride (structurally similar to succinic acid).

For the biotransformation to produce cis-1,2-indandiol, the substrate indene is toxic to cells at a certain concentration. Managing substrate inhibition problem and ISPR can be achieved simultaneously by combining two different polymers that each targets one specific compound. Since indene is much more hydrophobic than cis-1,2-indandiol, non-polar polymers such as styrene butadiene rubber may work well for the absorbing and delivering of indene while polar polymers such as Hytrel® would work well for uptaking cis-1,2-indandiol.

Extracting acids from aqueous phase will normally require the acids to be in their molecular form. Therefore, acid-producing biotransformation that can be operated at a pH lower than the acid dissociation constant needs to be developed. This may involve the searching for low-pH-tolerant strains, or may be overcome by special bioreactor design. Research is this direction is currently being undertaken in Daugulis Group.

Appendix A: Biomass quantification – optical density vs. cell dry weight calibration

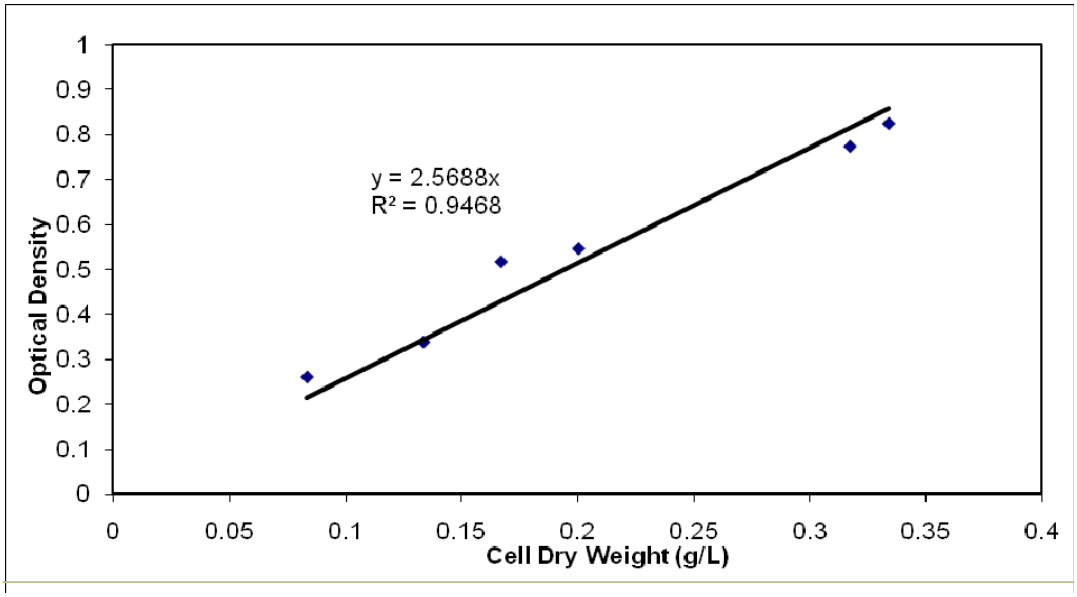

Figure A- 1: Cell dry weight vs. optical density calibration curve (optical density was measured at 600 nm wavelength).

Appendix B: Calibration curve used to quantify glucose concentration using DNS assay

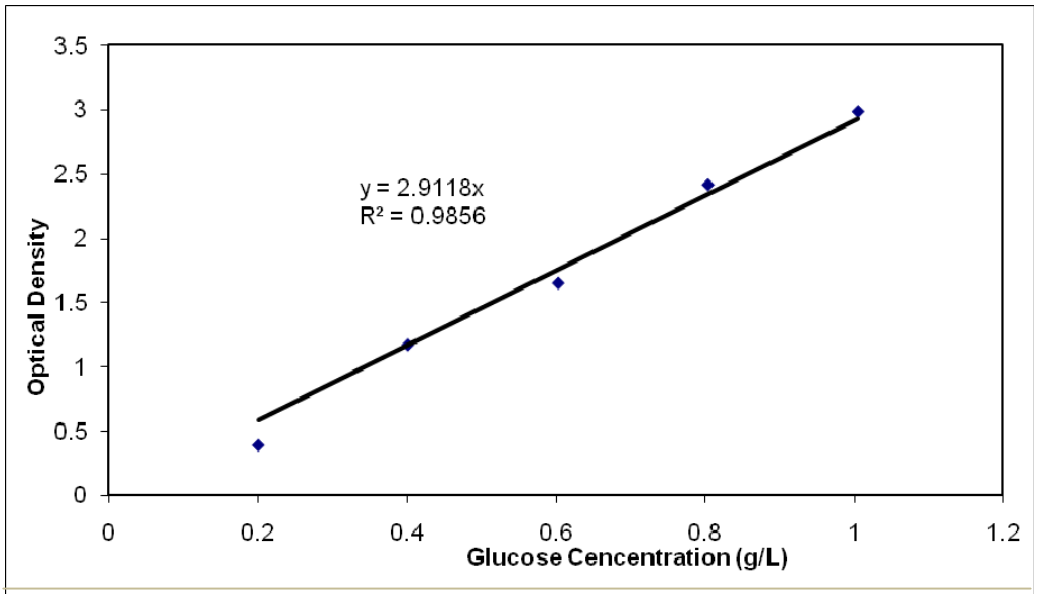

Figure B- 1 : Glucose concentration vs. optical density

Appendix C: Partition Coefficient Tests

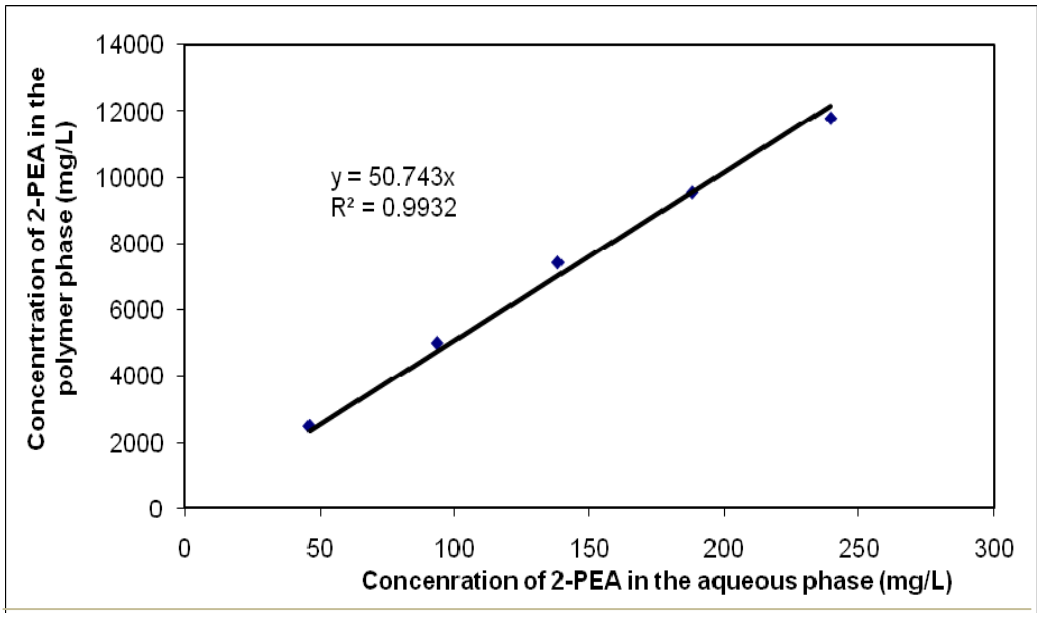

Figure C- 1: Partition Coefficient of 2-PEA in Hytrel 8206

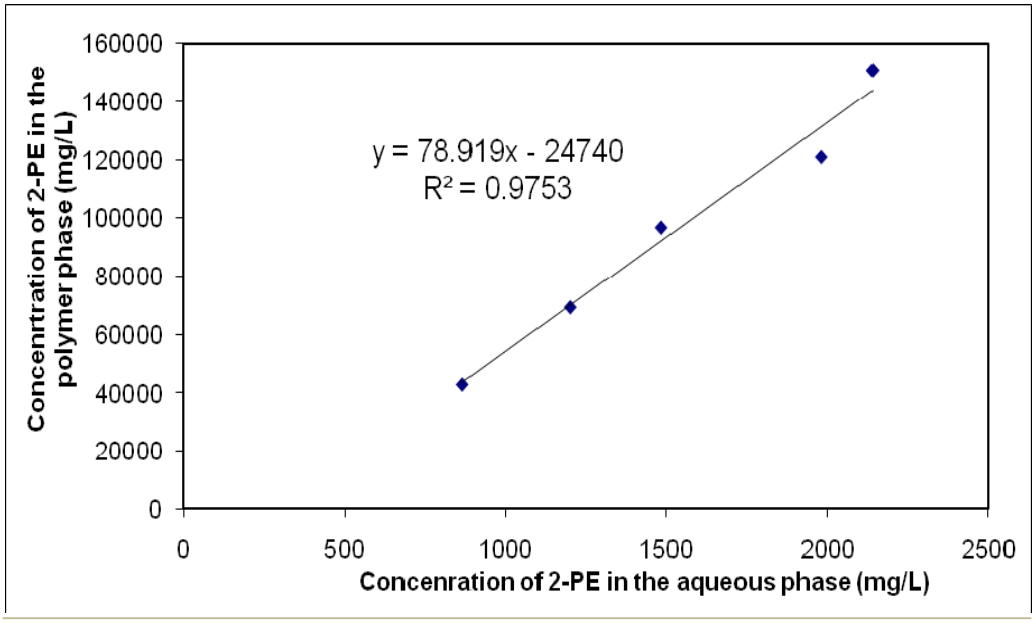

Figure C- 2: Partition Coefficient of 2-PE in Hytrel 8206

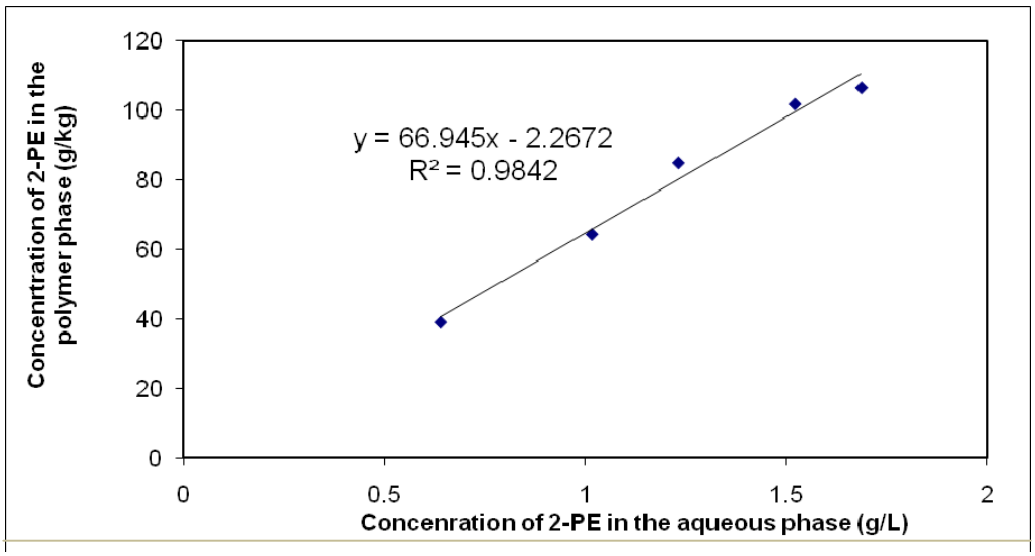

Figure C- 3: Partition Coefficient of 2-PE in Hytrel 3548

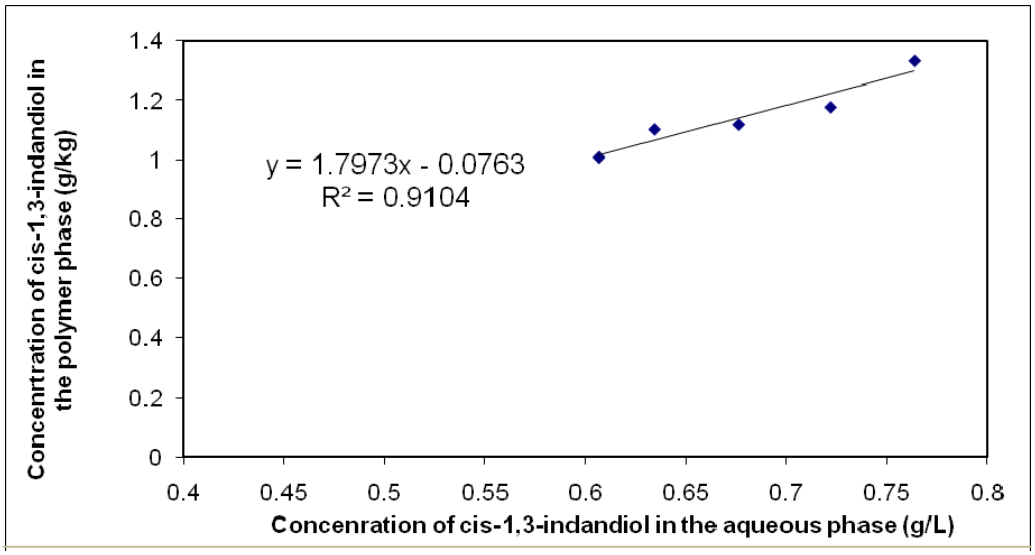

Figure C- 4: Partition Coefficient of cis-1,3-indandiol in Hytrel 3548

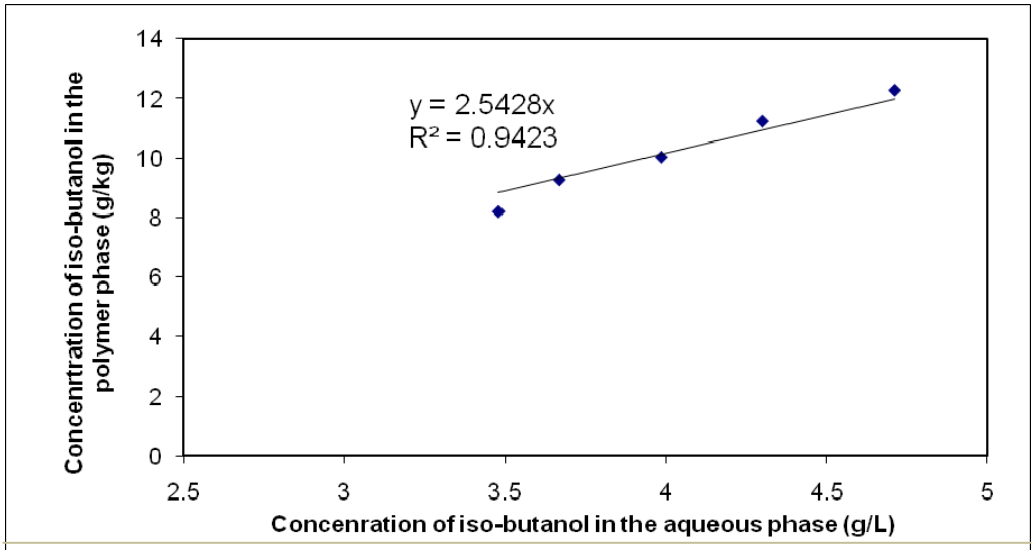

Figure C- 5: Partition Coefficient of iso-butanol in PEBAX 2533.