ARTICLE

BIOTECHNOLOGY and BIOENGINEERING

Application of Solid–Liquid TPPBs to the Production of L-Phenylacetylcarbinol From Benzaldehyde Using *Candida utilis*

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

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

Introduction

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

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

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

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

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

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

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

Materials and Methods

Chemicals and Polymers

Benzaldehyde, benzyl alcohol, glucose, oleyl alcohol, octane, 1-dodecanol, and oleic acid were purchased from Sigma–Aldrich Canada (Oakville, ON, Canada). $(NH_4)_2SO_4$, KH_2PO_4, $MgSO_4$, $CaCl_2$, $FeSO_4$, $ZnSO_4$, $MnSO_4$, $CuSO_4$, hexadecane, decane, dodecane, hexane, and linoleic acid were purchased from Fisher Scientific Canada (Oakville, ON, Canada). Butanol, tetradecene, nonane, 1-octadecene, hexadecane, 1-dodecene, and octanol were purchased from Acros Organics (through Fisher Scientific Canada). Aldehyde C-14 was purchased from Givaudan (Mississauga, ON, Canada). Because PAC is not available commercially, a small amount (approximately 0.5 g, enough for calibration) was obtained by chemical synthesis adapted from Rani et al. (1995). Polymers tested in this study and their properties are listed in Table I.

Medium Formulation and Culture Preparation

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

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

Cell Measurement

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

Analytics

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

Solvent Biocompatibility and Bioavailability

The method to determine $\log P_{crit}$ was adapted from Prpich and Daugulis (2007b). Fifty milliliters of 24-h old stock

Table I. Properties of polymers tested for potential use in the PAC TPPB.

	Grade	Supplier	Hardness	T_g (°C)[a]	T_m (°C)[b]	Specific gravity	Description
Amides							
PEBAX	2533	Arkema (Philadelphia, PA)	25D	−65	134	1	Polyether block amide
Zytel	42A	DuPont	60 (Rockell M)	70	262	1.15	Polyamide 66
Nylon	6.6	DuPont	N/A	N/A	N/A	N/A	
Vinyl acetates							
EVA	40W	DuPont	40 (Shore A)	N/A	N/A	0.965	40% vinyl alcohol (co-polymer with ethylene)
	3175		N/A	N/A	69	0.95	28% vinyl alcohol
Other							
Hytrel	G3548L	DuPont	35D	−45	156	1.16	Co-polymer of poly(butylene terephthalate) and polyether
	G4078W		40D	N/A	170	1.18	
	5544		55	−35	215	1.22	
	6108		61	N/A	168	1.25	
	8238		82	−50	223	1.28	
	8206		35–40[c]	−59	180	1.19	
Nucrel	925	DuPont	N/A	228	N/A	0.94	Ethylene/methacrylic acid co-polymer
Kraton SBR	D4150K	Kraton	N/A	Styrene: 90; butadiene: −90	N/A	0.92	Styrene/butadiene linear triblock copolymer, 28% styrene

[a]Glass transition temperature.
[b]Melting point.
[c]Approximated in personal correspondence with DuPont.

culture was used in each system to ensure equivalent starting ODs when 5 mL of organic solvent was added. The log *P* values of all solvents in this study were from the Syracuse Research Cooperation (SRC) log *P* database (http://www.srcinc.com). Percent metabolic activity was determined by comparison of the final OD of each system to a positive control (a shake flask without solvent) after 24 h.

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

Polymer Partition Coefficients

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

Batch Reactor Operation

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

Two-Phase Batch Reactor Operation

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

Product Recovery From Polymer

Every 2 h over the course of the biotransformation period, two 1 g polymer samples were collected and tested with the extraction technique described by Gao and Daugulis

(2009) using methanol. Two methanol washes were used per sample. Additional washes were demonstrated to only increase recovery by approximately only 1%. Using this method, the polymer beads are able to act as the first step in downstream purification operations, which in other systems could require high-cost chromatographic recovery methods.

Results and Discussion

Characterization of *C. utilis*

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

From Figure 1, it is evident that *C. utilis* is not inhibited when exposed to 1-dodecanol ($\log P$ of 5), while nonane ($\log P$ of 4.8) prevented growth. Therefore, the $\log P_{crit}$ of *C. utilis* can be approximated as 4.8. Though the critical $\log P$ is organism dependent, there exists comparable behavior between similar species (Inoue and Horikoshi, 1991). Previous work with yeasts showed *S. cerevisiae* having a $\log P_{crit}$ between 5 and 6 (Bruce and Daugulis, 1991). Therefore, a $\log P_{crit}$ of 4.8 seems reasonable.

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

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

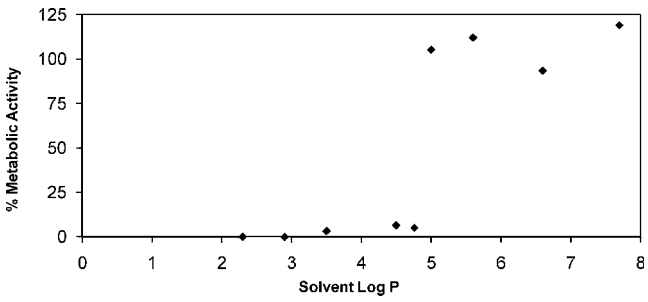


Figure 1. Log *P* of *C. utilis* using solvents of known log *P*'s: aldehyde C-14 (log *P* 2.3), octanol (log *P* 2.9), hexane (log *P* 3.5), octane (log *P* 4.5), nonane (log *P* 4.8), 1-dodecanol (log *P* 5.0), decane (log *P* 5.6), dodecane (log *P* 6.6), and oleic acid (log *P* 7.7). A control shake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

Table II. Bioavailability of some common solvents to *C. utilis*.

Solvent	Log *P*	Bioavailability (+/−)
Alkanes		
Decane	5.6	−
Dodecane	6.6	−
Hexadecane	8.2	−
Alcohols		
Dodecanol	5	+
Oleyl alcohol	7.5	+
Alkenes		
Dodecene	6.1	−
Tetradecene	7.1	−
1-Octadecene	9.0	−
Carboxylic acids		
Oleic acid	7.7	+
Linoleic acid	7.5	+
Other		
Bis-2-ethylhexyl sebacate	8.2	+

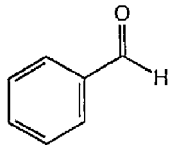
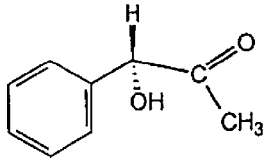
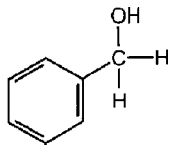
PAC partition coefficients have been tested in solvent screening by Rosche et al. (2004) and Sandford et al. (2005) for use with purified enzymes. Therefore, biocompatibility was not used as a selection criterion. Although now with the $\log P_{crit}$ determined, the biocompatibility of these solvents can be predicted. The $\log P_{crit}$ suggests that only two biocompatible solvents have been tested previously, hexadecane ($\log P$ of 8.2) (Rosche et al., 2004) and dodecane ($\log P$ of 6.1) (Sandford et al., 2005). These provided PAC partition coefficients of <1, while octanol ($\log P$ of 2.9) provided the best partition coefficient of 4 (Sandford et al., 2005). Thus, biocompatible solvents had low partition coefficients, while octanol, which was subsequently used with whole cells of *C. utilis* in a TPPB due to its moderate partition coefficient, was ultimately found to be toxic to *C. utilis* (Rosche et al., 2005). To avoid the compromise between toxicity and extractive capability of the sequestering phase, it is now possible to re-evaluate the PAC system using polymers instead of octanol.

Polymer Selection for PAC Production

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

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

Table III. Chemical properties of species of interest.

	Molecular weight	Structure	K_{ow}	Solubility in water (25°C) (g/L)
Benzaldehyde (C_6H_5COH)	106.12		21[a]	6.55
PAC ($C_9H_{10}O_2$)	150.17		4[a]	Not available
Benzyl alcohol (C_7H_8O)	108.14		12.6[b]	42.9

[a]Sandford et al. (2005).
[b]The Good Scents Company (2010).

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

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

To determine whether polymer functionality or softness had a more significant effect on partition coefficients, data from Table IV were analyzed with polymer property data from Table I. Comparing the amide polymers tested, it is clear that only PEBAX 2533 demonstrated a high affinity for the target molecules, while the other amide polymers Zytel and Nylon had minimal absorption. A significant difference between these polymers is their hardness, with PEBAX 2533 being the softest. This supports the previous trend seen in

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

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

the Hytrel family, which demonstrated increased absorption as a function of softness (Gao and Daugulis, 2010).

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

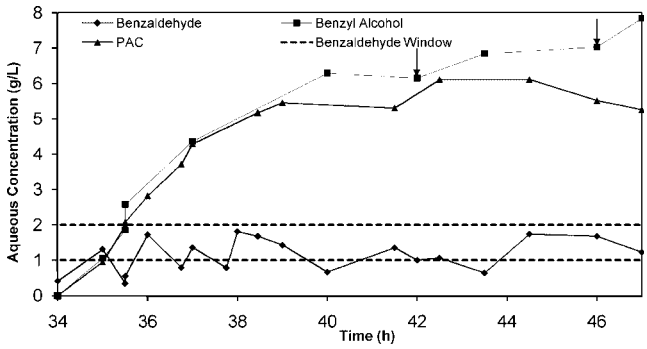


Figure 3. PAC, benzyl alcohol formation, and benzaldehyde concentration for the single-phase benchmark fermentation. The arrow represents the addition of 60 g of dissolved glucose.

Single-Phase Benchmark Fermentation

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

At the start of the biotransformation ($t = 34$ h), ethanol and biomass concentrations were 20 and 13 g/L, respectively, with negligible glucose remaining in the system. To ensure that this did not result in a pyruvate limitation, a 60 g bolus of glucose (in the form of a 500 g/L solution) was added to the reactor when the biotransformation was initiated. The time course of the biotransformation period (34 h onward) is shown in Figure 3.

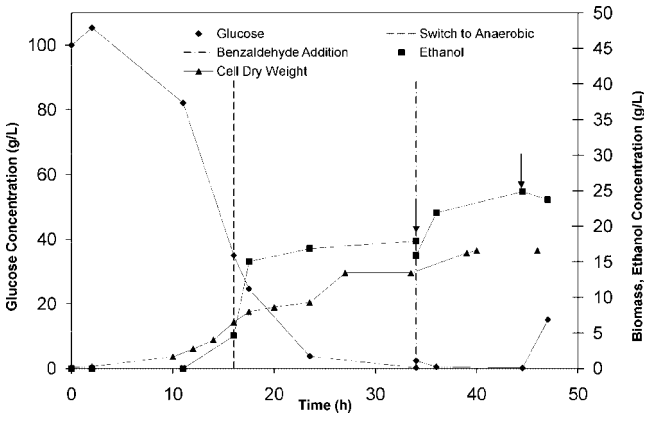


Figure 2. Cell density, glucose consumption, and ethanol production for the single-phase benchmark fermentation. The arrows represent the addition of 60 g of dissolved glucose. The aerobic growth period was 0–16 h, the anaerobic enzyme induction period was 16–34 h, and benzaldehyde addition began at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

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

The end of the biotransformation was taken to be the point at which PAC production stopped, which is at approximately 8 h into the transformation. Shin and Rogers (1995) provided three reasons for the transformation stopping: (1) reduction in PDC activity due to inhibitory effects, (2) pyruvate limitation, (3) cell viability loss due to exposure to benzaldehyde, benzyl alcohol, or PAC. Pyruvate limitation was tested by the addition of glucose. PAC concentration was not able to increase. Five milliliters of the reactor contents was used to inoculate 50 mL of fresh medium and growth was observed. This suggests that the system was likely not pyruvate limited, and that cells were still viable. Therefore, the most likely cause of stoppage was enzyme inhibition due to sustained exposure to inhibitory compounds.

Two-Phase Bioreactor With 300 g Hytrel G3548L

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

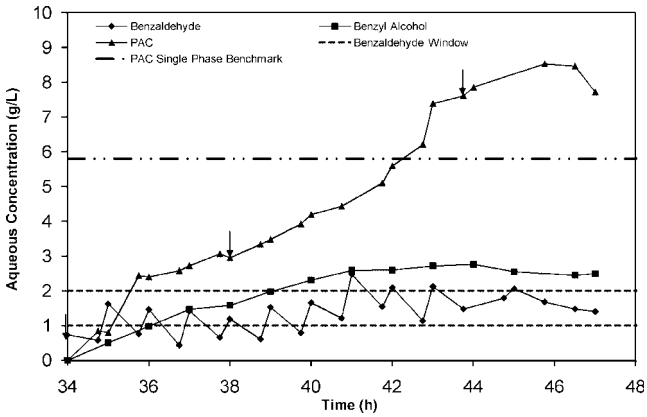


Figure 4. Aqueous PAC, benzyl alcohol, and benzaldehyde concentrations when 300 g of Hytrel G3548L was used as a second phase. Arrows represent the addition of 60 g of dissolved glucose.

Table V. Final system characteristics comparison of single- and two-phase fermentations for PAC production.

	Single phase	300 g
Time to completion (h)	8	13
Cell density (g/L)	13.4	13.6
Aqueous PAC end point (g/L)	5.7	7.6
Aqueous BOH end point	7.4	2.5
Polymer PAC (g/L)	—	50.5[b]
Polymer BOH (g/L)	—	28.5[b]
Overall PAC (g/L)[a]	5.7	11.0
Overall BOH (g/L)[a]	7.4	4.5
$Y_{PAC/BZA\ (mol/mol\ consumed)}$	0.34	0.41
$Y_{BOH/BZA\ (mol/mol\ consumed)}$	0.61	0.24
Selectivity (g/L PAC/g/L BOH)	0.77	2.44
PAC volumetric productivity (g/L h)	0.71	0.85
PAC mass productivity (g PAC/g cells/h)	0.160	0.203

[a]Determined using the total mass divided by the total system volume (3 L for the single phase, 3.26 L for the TPPB).
[b]Obtained from average two random polymer samples of 1 g desorbed using methanol washing procedure.

PAC concentration in the single phase is shown for comparison.

Figure 4 shows that the two-phase system achieved final aqueous concentrations of 7.6 and 2.5 g/L of PAC and benzyl alcohol, respectively. Polymer beads were sampled every 2 h and desorbed to provide a polymer concentration profile, as shown in Figure 5.

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

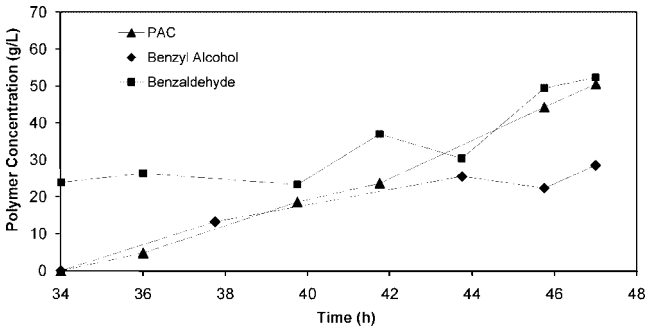


Figure 5. Polymer concentrations of PAC, benzyl alcohol, and benzaldehyde during the two-phase biotransformation.

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

The reduction of by-product (concentration and yield) in the TPPB may be due to polymer sequestration. The cells, and consequently the active enzymes, are exposed to lower concentrations of inhibitory species. This may allow PDC to stay active for longer periods, or maintain a higher specific activity for benzaldehyde than the oxidoreductases used for benzyl alcohol production. Previous work in solid–liquid TPPBs suggests that the aqueous concentration should reach the same known inhibitory value as the single-phase case (Gao and Daugulis, 2009; Morrish and Daugulis, 2008; Prpich and Daugulis, 2007a). However, this system has an inhibitory by-product present, which introduces effects not previously explored. The inhibition of PDC could be a cumulative effect of PAC, benzyl alcohol, and benzaldehyde, and therefore a reduction in benzyl alcohol concentration could allow an increase in PAC concentration.

To compare the present work with the PAC literature, similar metrics of performance should be considered.

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

Though the current work did not outperform the liquid–liquid TPPB described by Rosche et al. (2005), with respect to overall concentrations and productivities, it is important to note that this was not the overall goal. The goal was to re-evaluate the potential for TPPBs to be used in the PAC system now that a biocompatible and high-affinity sequestering phase is available. Zhang et al. (2008) aimed to improve the Rosche et al. (2005) system by using a polyethylene glycol-induced cloud point system instead of octanol. Though this system was able to use glucose as a substrate, the PAC concentration reached only 8 g/L and a benzyl alcohol concentration of 4 g/L (Zhang et al., 2008). A recent study using non-ionic surfactant extraction was able to achieve 4.1 g/L PAC and 1.8 g/L of benzyl alcohol, also without sequestering phase toxicity problems (Xue et al., 2010). Therefore, the current work has managed to balance the high performance elements of a liquid–liquid TPPB with the biocompatibility aspects of the cloud point and non-ionic surfactant systems, generating what may be considered a more promising industrial process.

Conclusions and Future Work

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

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

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