ARTICLE

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Polymer Characterization and Optimization of Conditions for the Enhanced Bioproduction of Benzaldehyde by *Pichia pastoris* in a Two-Phase Partitioning Bioreactor

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

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Introduction

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

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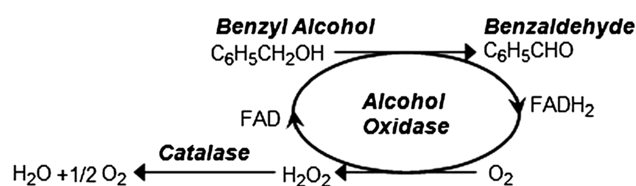

Figure 1. Enzymatic benzyl alcohol oxidation by alcohol oxidase coupled with catalase and cofactor FAD, producing H2O and benzaldehyde.

before the biotransformation step (Jain et al., 2010; Murray et al., 1989). Mixed substrate feeding of glycerol and methanol for cell growth and induction of AOX1 and AOX2 genes has shown to be effective for generating high cell density and allow for rapid induction of the AOX enzyme (d'Anjou and Daugulis, 2001; Jungo et al., 2007).

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

A few previous studies using *P. pastoris* for the biotransformation have introduced a second auxiliary phase to sequester benzaldehyde and alleviate inhibition in the aqueous phase, as well as to overcome benzaldehyde's low solubility in water (6.55 g L^{-1}; Duff and Murray, 1989; Jain et al., 2010). In this context, biocompatible and non-bioavailable commodity polymers as the sequestering phase in two-phase partitioning bioreactors (TPPBs) have been shown to have a positive effect with respect to two-liquid phase systems sequestering benzaldehyde due to observed higher partition coefficient (PC) in polymers over liquid solvents, elimination of potentially toxic effects of organic solvents to cells, ease of process operation and handling, and the elimination of possible fugitive aromas which could negatively affect the quality of the flavor/fragrance (Jain et al., 2010). In the only previous work using polymer-based TPPBs for benzaldehyde production, Jain et al. (2010) utilized a polymer selection approach based on identifying polymers possessing high PC for both benzyl alcohol and benzaldehyde. However, this may not be the most effective strategy of finding the most appropriate polymer for this biotransformation, as significant residual benzyl alcohol could remain in the polymer at the end of the transformation, complicating benzaldehyde purification.

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

Materials and Methods

Chemicals and Polymers

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

Medium Formulation and Culture Preparation

P. pastoris ATCC 28485 was used in this work. The medium for the bioreactor and shake flasks was formulated according

Table I. Physical properties, and benzyl alcohol and benzaldehyde partition coefficient for six candidate polymers.

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

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

Shake Flask Inhibition Experiments

One hundred twenty-five milliliter Erlenmeyer flasks with 50 mL medium containing 5 g L^{-1} glycerol and 5 g L^{-1} methanol were prepared containing different concentrations of benzyl alcohol: 2, 4, 8, and 14 g L^{-1} and benzaldehyde 0, 0.75, 1.5, and 3 g L^{-1}. The flasks were inoculated and the concentrations of benzyl alcohol and benzaldehyde were determined periodically over a 14 h period.

Polymer Characterization

Sorption Mechanism

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

Beads of the three polymers were cut in half to obtain two different surface areas for each polymer, and the surface area was measured assuming that Pebax 2533 and Elvax 40W maintain a spherical shape, and that Hytrel 8206 maintains a cylindrical shape (Fig. 2). The diameter and length were measured using Tuff Grade 6″ Caliper with an LCD Screen. The dimensions of twenty-five beads for each polymer were measured for both the as-received polymer beads and the cut polymer beads. The mass of 50 beads of polymer was determined and the average mass of one bead was calculated.

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

Polymer PCs of Selected Polymers

Six polymers were tested for PC for benzaldehyde and benzyl alcohol using the method described previously (Isaza and Daugulis, 2009).

Single- and Two-Phase Reactor Operation

The cell growth phase was conducted in a 5 L BioFlo III bioreactor (New Brunswick Scientific, Edison, NJ) containing 3 L of medium with 10 g L^{-1} glycerol and 10 g L^{-1} methanol and operated at 500 rpm, 4 L min^{-1} aeration and pH 5.5. When the glycerol and methanol were consumed, deduced from characteristic increases in dissolved oxygen (DO), the single-phase biotransformation phase was started by shifting the pH to 7.3 using 4 M KOH and by adding 20 g L^{-1} benzyl alcohol which was sterile-filtered using a 0.2 μm syringe filter. Agitation and aeration were set to 400 rpm and 1 L min^{-1} to reduce product stripping by aggressive aeration.

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

Product Recovery from Polymer

Concentrations of benzyl alcohol and benzaldehyde in the polymer were determined from polymer samples during the biotransformation period by methanol extraction of

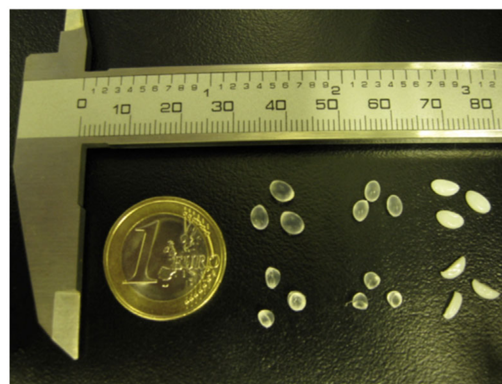

Figure 2. Polymers used in sorption study: Top, as received pellets: Elvax 40 W surface area 1.74 m^2 kg^{-1}, Pebax 2533 surface area 1.76 m^2 kg^{-1}, Hytrel 8206 surface area 1.89 m^2 kg^{-1}. Bottom, cut pellets: Elvax 40 W surface area 2.75 m^2 kg^{-1}, Pebax 2533 surface area 2.15 m^2 kg^{-1},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

the polymer beads (Gao and Daugulis, 2009). For each sample three polymer extractions were performed to determine overall target molecule concentration, each allowed to reach thermodynamic equilibrium before the subsequent extraction.

Analytics

Cell Biomass Measurement

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

Concentration Measurements

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

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

Phenol concentrations (polymer uptake experiments) where measured using the 4-amionantipyrene method at $\lambda = 510$ nm (Vrionis et al., 2002) using an Ultraspec 3000 UV–Vis Spectrophotometer.

Results and Discussion

Substrate and End-Product Inhibition

The effect of product and substrate inhibition on the reaction rate was investigated by examining the biotransformation of benzyl alcohol to benzaldehyde at various initial concentrations of benzyl alcohol and benzaldehyde (Fig. 3). Note that this experiment focused only on the biotransformation, and not on cell inhibition, which would need to be considered in a separate study. Due to the volatility of benzaldehyde and its susceptibility for autoxidation, the performance of the system was measured by benzyl alcohol consumption, based on the 1:1 stoichiometry of benzyl alcohol to benzaldehyde. Figure 3 shows that the presence of substrate actually seems to accelerate the rate of benzaldehyde production, suggesting that not only is benzyl alcohol significantly less inhibitory on the reaction rate than benzaldehyde over the range of concentrations tested it actually may stimulate the rate of benzyl alcohol oxidation. Previous work by Duff and Murray (1989), demonstrated that the oxidation of benzyl alcohol in single-phase aqueous solution follows Michaelis–Menten kinetics at benzyl alcohol concentrations less than $20\,g\,L^{-1}$, from which a K_m and V_{max} value were estimated to be $20\,g\,L^{-1}$ and $3.7\,g\,L^{-1}\,h^{-1}$, respectfully. The high value for K_m suggests that a large concentration of substrate is required to produce high conversion rates. For the product, benzaldehyde, the benzyl alcohol consumption rates were highest when no benzaldehyde was present at all benzyl alcohol concentrations (Fig. 3), and as the benzaldehyde concentration added to the system increased from 0 to $3\,g\,L^{-1}$ there was a strong inhibition on the reaction rate to the biotransformation. These results indicate low benzaldehyde tolerance for this biotransformation and in previous work using single phase bioreactor experiments (Duff and Murray, 1989), suggesting that a benefit could be obtained by removing benzaldehyde from the aqueous phase as it is formed. A number of strategies exist for the removal product/substrate inhibitors, but solid-liquid TPPBs using polymers as target molecule sorbents have shown to be effective for this purpose (Gao and Daugulis, 2009; Jain et al., 2010; Khan and Daugulis, 2010).

Polymer Sorption Mechanism

The mechanism of solute sorption by soft, amorphous polymers in the application in TPPBs has not yet been identified in the current literature. Solute sorption/release by amorphous polymers, however, is the basis of controlled drug release, an area that has been studied and demonstrated for more than 30 years (Uhrich et al., 1999). In contrast, the uptake mechanism of solutes using hard, crystalline polymeric resins, is by surface adsorption (Nielsen and Prather, 2009; Qureshi et al., 2005). It is critical to establish whether absorption or adsorption is the fundamental

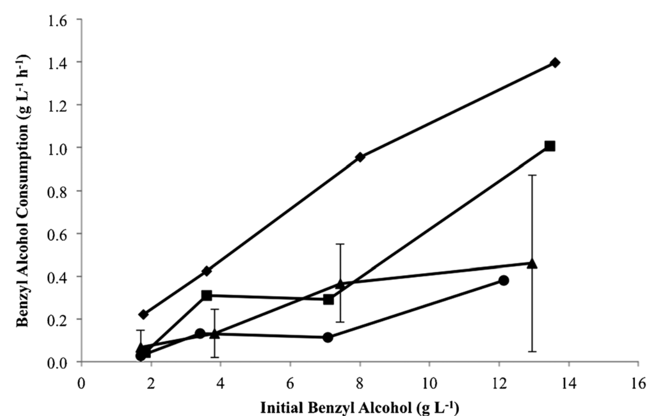

Figure 3. Benzyl alcohol consumption as a function of initial benzyl alcohol concentration in the presence of $0\,gL^{-1}$ (◆), $0.75\,gL^{-1}$ (■), $2\,gL^{-1}$ (▲), and $3\,gL^{-1}$ (●) benzaldehyde.

mechanism for polymer uptake in our case, as this affects the strategy used for polymer selection in TPPB systems, and also determines the amount of polymer utilized (i.e., area required, or mass). The average surface areas of the regular polymer beads Hytrel 8206, Elvax 40W and Pebax 2533 were 1.89, 1.74, and 1.76 $m^2 kg^{-1}$, respectively, while the reduced polymer sizes had surface areas of 2.84, 2.75, and 2.15 $m^2 kg^{-1}$. Figure 4a provides a comparison of the PC values at different surface areas for the three polymers with benzaldehyde as the solute, and clearly shows that sorption is determined by the mass of polymer (absorption) and not the surface area (adsorption). To demonstrate that this was not peculiar to only benzaldehyde, phenol was used a second target molecule and PCs were determined as shown in Figure 4b. Again, it can be seen that varying the surface areas of the polymers had no effect on the PC for that polymer using that specific target molecule, which again confirms that the primary mechanism for polymer uptake is by absorption.

Polymer Selection

In light of the results above indicating that benzaldehyde is inhibitory to the biotransformation, and that the substrate, benzyl alcohol, is not, polymers were tested to determine not only their PC values for each of these two compounds, but also the *ratio* of the PC values. That is, the current strategy was to maximize the ratio of the PC of benzaldehyde, relative to that of benzyl alcohol, to target the compound that is

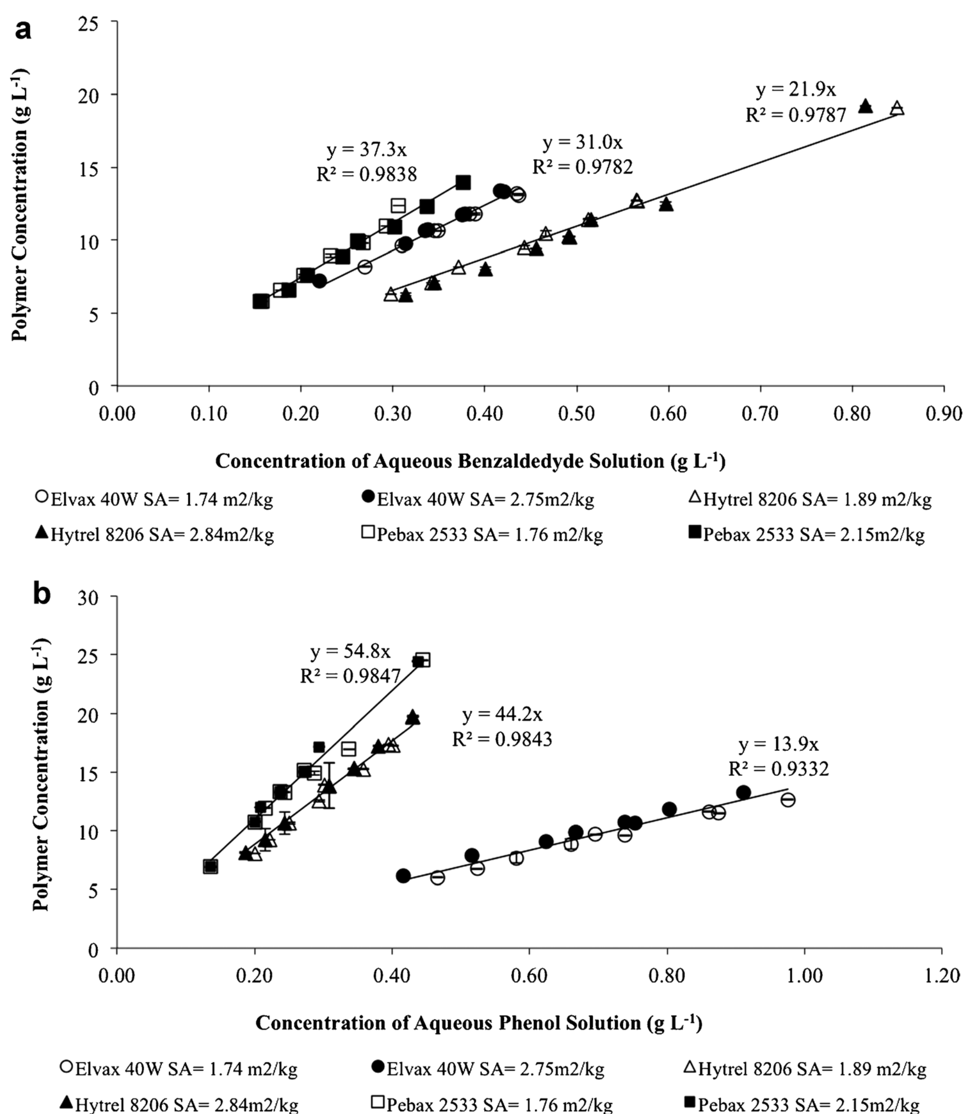

Figure 4. Partition coefficients for (**a**) benzaldehyde uptake and (**b**) phenol uptake. Elvax, Hytrel, and Pebax were tested at different surface areas, but with the same mass of polymer.

exerting an inhibitory effect and to remove it, while minimizing the amount of substrate that would be sorbed into the polymer. As noted earlier, this in contrast to the work of Jain et al. (2010) in which the strategy was to have high PC values for both target molecules. Of the six polymers tested for their PCs for benzyl alcohol and benzaldehyde as shown in Table I, Kraton D1102K had a polymer PC ratio of 14.9, the highest for substrate to product and was used for the biotransformation.

Mixed Substrate Feed

In addition to removing product inhibition by benzaldehyde sorption into a polymer, the biotransformation of benzyl alcohol to benzaldehyde by *P. pastoris* could also be potentially improved by reducing the cell growth period of the process. Previous work employing *P. pastoris* for the biotransformation of benzyl alcohol to benzaldehyde used methanol as the sole substrate for cell growth due to its ability to induce the AOX1 and AOX2 promoter, however the growth periods were very long and achieved relatively low cell densities (Duff and Murray, 1989; Jain et al., 2010; Kawakami and Nakahara, 1994). Therefore, here we used glycerol as the carbon source for shake flask inoculum, and glycerol and methanol mixed feed for the growth and induction phase of the fermentation process. Figure 5a displays the time course for the growth and induction phase using a 10 g L^{-1} glycerol and 10 g L^{-1} methanol mixed feed strategy. The DO reading provided a good real-time indicator of the substrate transition from glycerol consumption to methanol, shown at the first DO spike, as well as the initiation for the biotransformation, at the second DO spike. Although, glycerol does repress the AOX1 and AOX2 promoter, the methanol dissimilatory pathway is not utilized until the glycerol is consumed, a feature of diauxic growth. In the first 15 h, the methanol concentration is observed to decrease due to stripping and not consumption (Jungo et al., 2007). Overall by applying the mixed feed strategy in the fermentation process the time required before the biotransformation was initiated had been reduced by 3.4-fold from previously reported data (Jain et al., 2010).

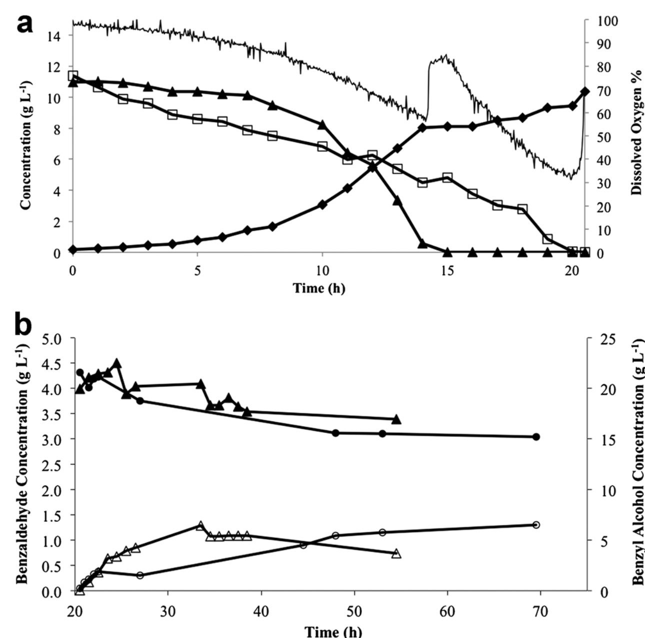

Figure 5. **a**: Cell growth and AOX induction period with dry cell weight (◆), glycerol (▲), and methanol (□) concentrations, and dissolved oxygen trace (line) as a function of time with 10 gL^{-1} glycerol and 10 gL^{-1} methanol. **b**: Single phase biotransformation concentrations of benzyl alcohol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

Comparison of Single and Two Phase Systems

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

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

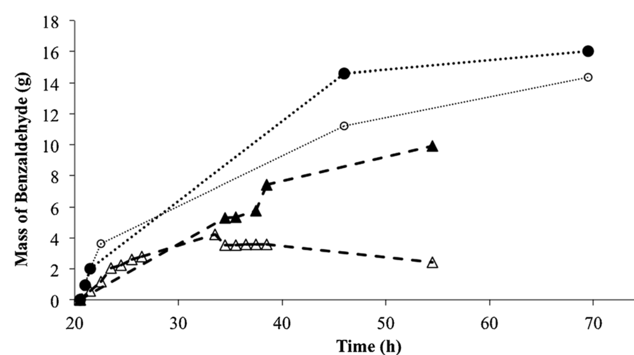

Figure 6. Single phase total mass of benzaldehyde based on benzyl alcohol stoichiometry (▲) and benzaldehyde measured (Δ). TPPB aqueous phase total mass of benzyl alcohol (●) and benzaldehyde (○) as a function of time.

mass of benzaldehyde produced based on the measured amount was less than half of the total mass of benzaldehyde based on stoichiometry, which shows the effect of significant volatilization losses and autoxidation in the single phase biotransformation.

The TPPB system used a 10% (w/w) polymer phase ratio consisting of Kraton D1102K and the same operating conditions as the single phase. The two phase biotransformation shows a gradual increase in benzaldehyde with a slight plateau that occurs at 48 h with approximately 1.1 $g L^{-1}$ benzaldehyde (Fig. 5b). The total mass of benzaldehyde produced using the TPPB system was 14.4 g and based on benzyl alcohol stoichiometry it was 16.9 g, shown in Figure 6, and a volumetric productivity of 97 $mg L^{-1} h^{-1}$, based on the amount of benzaldehyde measured. In comparing the total mass of benzaldehyde measured and that determined by benzyl alcohol stoichiometry the results show that there was a 118% reduction in volatilization losses and autoxidation when applying TPPBs with the biotransformation than compared to the single phase biotransformation. Thus it appears as though TPPB operation may not only reduce inhibition, but may also reduce losses of volatile target molecules. To confirm this, Henry's Law calculations were undertaken for the two systems, assuming that the aqueous and vapor phases were in equilibrium, and by using the trapezoid rule to determine cumulative benzaldehyde losses due to stripping. It was found that in the single phase case, 3.1 g of benzaldehyde (73.8% of the total benzaldehyde produced) was lost due to stripping, while in the TPPB case, only 4.2 g, or 29.2% of the benzaldehyde, was lost.

A comparison of the single phase and two phase system using Kraton D1102K as well as the TPPB system using Elvax 40W from work by Jain et al. (2010) is shown in Table II. The TPPB system using Kraton D1102K initially had 21.6 $g L^{-1}$ benzyl alcohol in the aqueous phase and below 10 $g L^{-1}$ in the polymer phase, which was the rationale applied to achieve high benzyl alcohol consumption rates. In work by Jain et al. (2010) the Elvax 40W polymer beads were preloaded with benzyl alcohol such that when they were added to the system the target benzyl alcohol concentration was 10 $g L^{-1}$. For the TPPB system with Elvax 40W the total time required to complete the biotransformation was 143 h, and the time required for the TPPB system with Kraton D1102K was 69.5 h, a significant reduction. The benzaldehyde volumetric productivity for the system with Elvax 40W was 70 $mg L^{-1} h^{-1}$ and using Kraton D1102K it was 97 $mg L^{-1} h^{-1}$, which is a 140% improvement in system performance. The difference between the benzaldehyde produced based on stoichiometry and the measured benzaldehyde is more significant in the single phase biotransformation than with the TPPB system, which strongly suggests that using TPPBs may reduce product volatilization and autoxidation as noted above.

Conclusion and Future Work

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

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

Table II. Comparison of single phase and TPPB system with Kraton D1102K polymer beads and TPPB system with Elvax 40W.

Criteria for comparison	Single phase (this work)	TPPB with Elvax 40W (Jain et al., 2010)	TPPB with Kraton D1102K (this work)
Operating variables			
Initial aqueous benzyl alcohol concentration ($g L^{-1}$)	21.1	7.8	21.6
Total benzyl alcohol mass in system (g)	63.3	54.5[a]	70.7
Total polymer mass in system (g)	NA	300	300
Final cell density (g biomass L^{-1})	10.4	4.10	11.8
Fermentation time			
Elapsed time for growth and induction phase (h)	20.5	72	20.5
Elapsed time for biotransformation phase (h)	34.0	71	49.0
System performance			
Total benzaldehyde produced (g)	4.2	15.0	14.4
Benzaldehyde volumetric productivity ($mg L^{-1} h^{-1}$)	41.0	70.0	97.0
Total benzyl alcohol consumed (g)	9.9	—	16.9

[a]Polymers were preloaded with benzyl alcohol.

provide an opportunity to enhance benzaldehyde production via a substrate fed-batch strategy during the biotransformation phase to reduce overall fermentation time.

References

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

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

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

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

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

Jain AN, Khan TR, Daugulis AJ. 2010. Bioproduction of benzaldehyde in a solid-liquid two-phase partitioning bioreactor using *Pichia pastoris*. Biotechnol Lett 32:1649–1654.

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

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

Kawakami K, Nakahara T. 1994. Importance of solute partitioning in biphasic oxidation of benzyl alcohol by free and immobilized whole cells of *Pichia pastoris*. Biotechnol Bioeng 43:918–924.

Khan TR, Daugulis AJ. 2010. Application of solid-liquid TPPBs to the production of L-phenylacetylcarbinol from benzaldehyde using Candida utilis. Biotechnol Bioeng 107:633–641.

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

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

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

Nielsen DR, Prather KJ. 2009. In situ product recovery of n-butanol using polymeric resins. Biotechnol Bioeng 102:811–821.

Qureshi N, Hughes S, Maddox IS, Cotta MA. 2005. Energy-efficient recovery of butanol from model solutions and fermentation broth by adsorption. Bioprocess Biosyst Eng 27:215–222.

Surburg H, Panten J. 2006. Common fragrance and flavor materials: Preparation, properties and uses, 5th edn. Weinheim: WILEY-VCH Verlag GmbH & Co. KGaA.

Uhrich KE, Cannizzaro SM, Langer RS. 1999. Polymeric systems for controlled drug release. Chem Rev 99:3181–3198.

Vrionis HA, Kropinski AM, Daugulis AJ. 2002. Enhancement of a two-phase partitioning bioreactor system by modification of the microbial catalyst: Demonstration of concept. Biotechnol Bioeng 79:587–594.