Biotechnol Lett (2010) 32:1649–1654
DOI 10.1007/s10529-010-0353-2

ORIGINAL RESEARCH PAPER

Bioproduction of benzaldehyde in a solid–liquid two-phase partitioning bioreactor using *Pichia pastoris*

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Received: 7 June 2010 / Accepted: 2 July 2010 / Published online: 18 July 2010

Abstract The bioproduction of benzaldehyde from benzyl alcohol using *Pichia pastoris* was examined in a solid–liquid two-phase partitioning bioreactor (TPPB) to reduce substrate and product inhibition. Rational polymer selection identified Elvax 40W as an effective sequestering phase, possessing partition coefficients for benzyl alcohol and benzaldehyde of 3.5 and 35.4, respectively. The use of Elvax 40W increased the overall mass of benzaldehyde produced by approx. 300% in a 5 l bioreactor, relative to a single phase biotransformation. The two-phase system had a molar yield of 0.99, indicating that only minor losses occurred. These results provide a promising starting point for solid–liquid TPPBs to enhance benzaldehyde production, and suggest that multiple, targeted polymers may provide relief for transformations characterized by multiple inhibitory substrates/product/by-products.

Keywords Benzaldehyde · Benzyl alcohol · Microbial biotransformation · *Pichia pastoris* · Solid–liquid two-phase partitioning bioreactor

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Introduction

Benzaldehyde, with an almond-like aroma, is the second most abundantly used molecule in the flavour industry. Production is currently via chemical synthesis or through the breaking of glycosidic linkages in various fruits where benzaldehyde is naturally found (Gabelman 1994). The use of microbial biocatalysts to generate benzaldehyde provides a potential option from which naturally produced, and hence higher priced, benzaldehyde can be obtained on an industrial scale. Methylotrophic yeasts contain the enzyme alcohol oxidase, which allows for the oxidation of methanol to formaldehyde, as well as additional enzymes which completely degrade methanol to carbon dioxide. In the presence of alcohols other than methanol, the yeasts are able to oxidize the alcohols to their aldehyde form, with the metabolic pathway stopped due to the specificity of the next enzyme in the pathway, formaldehyde dehydrogenase, for formaldehyde. The yeast, *Pichia pastoris*, is effective in transforming benzyl alcohol to benzaldehyde in a one-step bioconversion (Duff and Murray 1989).

Previous work using a single aqueous phase and various microorganisms has produced benzaldehyde at relatively low yields and rates (e.g. 100 mg/l and 1.4 mg/l h) (Kawabe and Morita 1994; Norliza and Ibrahim 2005). Benzyl alcohol above 20 g/l strongly inhibits the reaction. Benzaldehyde also has a strong negative effect on the reaction as it is a potent inhibitor of alcohol oxidase. In addition, benzaldehyde has a

low solubility in water, approx. 3.84 g/l at 25°C (Duff and Murray 1989).

Studies employing two phases have found greater success, indicating that the use of two-phase partitioning bioreactors (TPPB) may be a viable approach to benzaldehyde production. Previously, two-liquid phase systems have used an immiscible organic solvent as a sequestering phase for benzaldehyde (Kawakami and Nakahara 1994; Duff and Murray 1989). These studies have shown initially promising results with an increase in total benzaldehyde concentration achieved compared to that of single phase systems. However, limitations associated with the use of organic solvents, including potential toxicity to the biocatalyst as well as the negative effect on the flavour/fragrance compound due to fugitive aromas, have led to the use of commodity polymers, which are biocompatible, non-bioavailable as the sequestering phase, chemically inert, and inexpensive (Gao and Daugulis 2009; Khan and Daugulis 2010; Morrish and Daugulis 2008; Prpich and Daugulis 2007). In this work, the biotransformation of benzyl alcohol to benzaldehyde using *P. pastoris* was investigated using rational polymer selection for sequestering phase selection in a solid–liquid TPPB.

Materials and methods

Chemicals and polymers

Benzyl alcohol and all medium components were purchased from Sigma-Aldrich. The tested polymers included Elvax 40W, Hytrel 8206, Hytrel G3458, Pebax 2533, styrene/butadiene rubber (SBR), and Zytel 42A; the sources and properties of these polymers are shown in Table 1.

Medium formulation and culture preparation

P. pastoris ATCC 28485 was grown on the medium of Duff and Murray (1989). The inoculum was prepared by adding 60 µl frozen *P. pastoris* stock culture to 50 ml medium with 20 g methanol/l to eight 125 ml shake-flasks. After 72 h, the cells were centrifuged for 30 min at 3,000×*g* and resuspended in 50 ml fresh medium. These cells were then added to the reactor vessel containing 3 l sterile medium with 20 g methanol/l to increase cell concentration to approx. 5 g/l. Upon depletion of the methanol [confirmed through dissolved oxygen (DO) measurements] the cells were ready to perform the biotransformation.

Analyses

The cell dry weight was calculated from the OD_{600} value.

Benzyl alcohol and benzaldehyde concentrations were measured using HPLC. Samples were filtered using 0.2 µm syringe filters and 10 µl was passed through an HPLC column (MetaChem Polaris, C18 4.6 × 150 mm). Benzyl alcohol was detected at 263 nm and benzaldehyde at 283 nm. The column was kept at room temperature and eluted with acetonitrile/water (30:70 v/v) at 1 ml/min.

Polymer partition coefficients

Partition coefficients for the six different polymers were determined for benzyl alcohol and benzaldehyde

Table 1 Properties and partition coefficients for benzyl alcohol and benzaldehyde for 6 candidate polymers

Polymer	Supplier	Glass transition temperature, T_g (°C)	Specific gravity	Type	Partition coefficient for benzyl alcohol	Partition coefficient for benzaldehyde
Hytrel 8206	DuPont	−59	1.19	Poly(butylene terephthalate) and poly ether block copolymer	10.6	24.9
Hytrel G3548L	DuPont	−45	1.16		12.1	39.6
Kraton SBR, D4150K	Kraton	N/A	0.92	Styrene/butadiene linear triblock copolymer, 28% styrene	0.5	15.9
Zytel 42A	DuPont	70	1.15	Polyamide 66	0.3	0.6
Pebax 2533	Arkema	−65	1	Polyether block amide	10.9	43.3
Elvax 40W	DuPont	N/A	0.965	40% Vinyl alcohol (copolymer with ethylene)	3.5	35.4

using the method described by Isaza and Daugulis (2009). A stock solution of consisting of 10 g benzyl alcohol/l and 3 g benzaldehyde/l was used with polymer masses varying between 1 and 4 g.

Reactor operation

Single phase operation

A 5 l BioFlo III bioreactor (New Brunswick Scientific, Edison, NJ) was used, with temperature, agitation and aeration (with air) maintained at 30°C, 400 rpm and 1 l/min, respectively. The reactor, containing 3 l medium, was sterilized, and methanol was then added to 20 g/l. During the cell growth phase on methanol, the pH was maintained at 5.5 and during the biotransformation at 7.3 using 3 M KOH. Upon depletion of the methanol, based on a distinct increase in the DO reading, at approximately 72 h, benzyl alcohol was added to 10 g/l for the single phase run and reactor conditions were maintained as described above.

Two-phase operation

For the solid–liquid two-phase run, 300 g polymer was preloaded with benzyl alcohol by equilibrating the beads in 3 l medium with continued benzyl alcohol addition until 10 g benzyl alcohol/l was present in the aqueous phase at equilibrium. The intended use of these polymers was to deliver the substrate, at an initial aqueous phase concentration of approx. 10 g/l when added to the bioreactor, as well as sequester the product. Once the cell growth phase in the bioreactor had ended following the same procedure as that used in single phase operation, the pre-equilibrated polymers were added and samples were taken at regular intervals, passed through a 0.2 μm syringe filter and analyzed.

Product recovery from polymer beads

Over the course of the biotransformation period, 1 g polymer samples were periodically collected and tested for benzyl alcohol and benzaldehyde concentrations using the methanol extraction technique described by Gao and Daugulis (2009), in which the polymer beads are extracted repeatedly with methanol and the concentrations of target molecules determined from mass balances and polymer density.

Results and discussion

Polymer selection

A polymer with a high partition coefficient towards benzaldehyde and a lower partition coefficient towards benzyl alcohol was sought. This would allow for maximum sequestration of the product, thereby avoiding end-product inhibition of the catalyst, as well as providing substrate in the aqueous phase available for the catalyst. The results of the partition coefficient experiments are shown in Table 1, and the values reflect those reported earlier for the absorption of other important biomolecules (Gao and Daugulis 2010) in which partition coefficients were found to range from relatively low values (<1) to relatively high ones (>40). Two factors were taken into account when selecting a polymer for use in the TPPB: the absolute value of the partition coefficient towards benzaldehyde was important as the higher the value, the greater the amount of product that could be sequestered from the aqueous phase. In addition, the relative values of the partition coefficients were considered, as a polymer with a higher relative affinity for benzaldehyde over benzyl alcohol would likely lead to a higher purity end product upon desorption. On this basis, Elvax® 40W was chosen as an appropriate polymer based on its partition coefficients.

Single phase biotransformation

The single phase biotransformation was performed as a benchmark with the results shown in Fig. 1. Benzaldehyde production peaked at 21 h at 1.63 g/l, and between 21 and 72 h the amount of product in the system steadily decreased. Since benzyl alcohol is not catabolized by the yeast, it is likely that the transformation stopped because of a lack of cellular energy. The maximum total amount of benzaldehyde produced was 4.89 g at a rate of 0.078 g/l h. In addition, the 10 g benzyl alcohol/l initially added appears to have been only slightly too high for the given mass of cells to transform completely into benzaldehyde via the oxidase enzyme, as approx 70% of the added substrate was converted. The molar yield was found to be 0.31, indicating that there were significant losses from the system as this biotransformation has a one-to-one stoichiometric ratio relative to substrate.

The likely cause for the losses was volatilization of the benzaldehyde, which is known to occur easily at room temperature. An additional cause for loss of product may be due to the fact that benzaldehyde can be oxidized to benzoic acid when exposed to air (Smith and Hendlin 1953).

Two-phase biotransformations

A 10% (w/w) polymer phase ratio was employed and the polymer beads, which had been pre-loaded with benzyl alcohol, were used to provide an initial bolus of approx. 10 g/l to the aqueous phase, equivalent to the single phase case, thus providing similar levels of substrate inhibition. The aqueous phase concentrations of both the substrate and product are also shown in Fig. 1. The initial increase in benzyl alcohol concentration is due to the release of benzyl alcohol from the loaded polymer as it established equilibrium with the reactor contents to near the desired aqueous target of 10 g/l, which is below the inhibitory level. This release is very rapid, occurring within 1–2 h, as has been seen in other polymer-aqueous TPPB systems (Tomei et al. 2009). The production of benzaldehyde occurred immediately after benzyl alcohol was introduced into the system, and reached a plateau at 48 h at an aqueous concentration of just under 0.7 g/l. The aqueous phase concentration of benzaldehyde was expected to be relatively low given the high partitioning coefficient of the polymer.

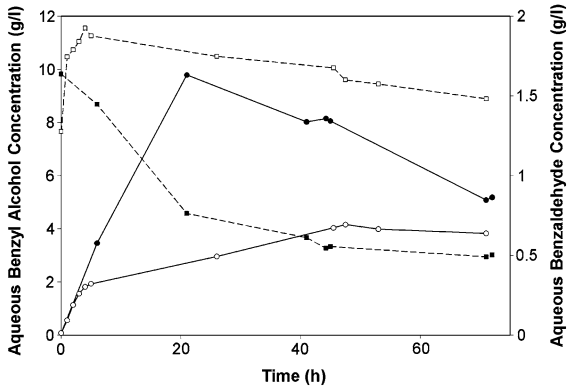

Fig. 1 Aqueous phase concentrations of benzyl alcohol and benzaldehyde during the single phase and two phase biotransformations. *Squares* represent benzyl alcohol and circles represent benzaldehyde. *Closed symbols* are for the single phase run and *open symbols* are for the two phase run

The concentrations of the substrate and product within the polymer phase are shown in Fig. 2. The polymer phase concentration of benzaldehyde reached a plateau of 41.8 g/l at about 71 h, with the substrate concentration also stabilizing near this time. In contrast to the single phase case in which the majority of the benzyl alcohol had been consumed, it is clear from the amount of substrate remaining in the polymer that this system still had the potential to produce substantially more benzaldehyde product if an energy source had been available to the cells. That is, the polymer system clearly has the capacity to sequester large amounts of substrate, potentially available for biotransformation, while maintaining aqueous phase concentrations at sub-inhibitory levels.

A comparison of the overall performance of the systems, based on total mass of benzaldehyde produced, is shown in Fig. 3, and further additional

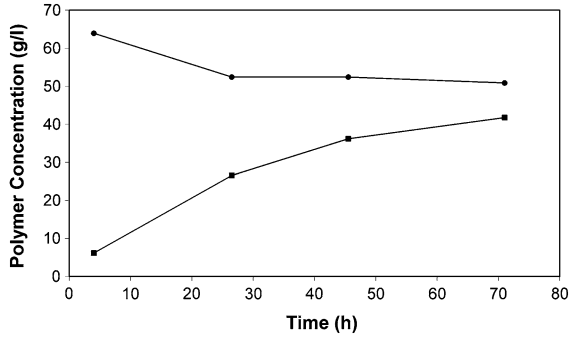

Fig. 2 Benzyl alcohol and benzaldehyde concentrations in the polymer phase of the TPPB system. *Circles* represent benzyl alcohol and *squares* represent benzaldehyde

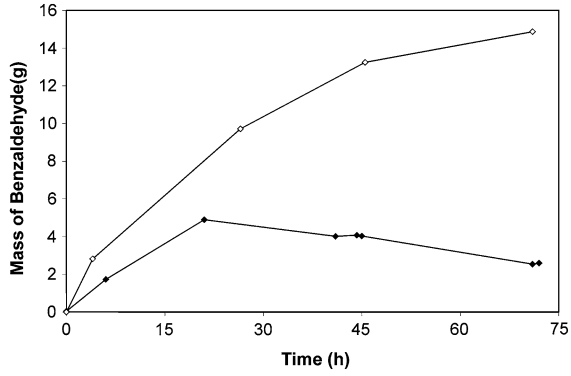

Fig. 3 Total mass of benzaldehyde produced in the single and two-phase systems as a function of time. *Open diamonds* represent the two-phase system, and *closed diamonds* the single phase case

Table 2 Comparison of bioreactor performance using a single phase and TPPB system with Elvax 40W polymer beads

	Single phase	Two phase using Elvax 40W
Time to completion (h)	21	71
Cell density (g/l)	4.58	4.10
Aqueous benzaldehyde end point (g/l)	1.63	0.64
Aqueous benzyl alcohol end point (g/l)	4.58	8.90
Polymer benzaldehyde end point (g/l)	–	41.75
Polymer benzyl alcohol end point (g/l)	–	50.85
Overall benzaldehyde (g)	4.89	15.0
Yield (mol/mol)	0.31	0.99
Benzaldehyde volumetric oroductivity (g/l h)	0.078	0.063
Benzaldehyde nass productivity ($g_{benzaldehyde}/g_{cells}/h$)	0.017	0.017

comparisons are shown in Table 2, in which various metrics are provided to contrast the one and two phase biotransformation systems. The performance in the two-phase system using Elvax 40W beads is about 300% higher than that achieved in a single phase system. The molar yield calculated was 0.99 in the Elvax 40W system, which is a significant increase from the single phase case, which had a yield of 0.31. The increase in molar yield is important as the problem of losses, potentially due to the high volatility of benzaldehyde, is essentially eliminated by using the two-phase approach and an appropriate polymer.

This work has demonstrated that by employing polymers as an immiscible sequestering phase, the yield and total mass production of a bioreactor system producing benzaldehyde from benzyl alcohol can be greatly improved. The use of such commercial polymers, which are extremely easy to handle, completely biocompatible, non-bioavailable, and inexpensive, represents a significant advantage over the use of organic solvents as sequestering/delivery/uptake phases in TPPBs. The ability to maintain a high overall system loading of substrate by using the polymer as a buffer is particularly beneficial, as it enhances total loading of the substrate, while maintaining low (sub-inhibitory) aqueous phase concentrations with no requirement for operator intervention to provide multiple feedings. By-product formation also appears to have been avoided in addition to overcoming product inhibition and solubility limitations. Purification of the final product from the polymer can be done easily, through methanol extraction of the polymer beads followed by distillation.

Current work is aimed at exploiting the large reservoir of substrate absorbed by the polymer to produce benzaldehyde by operating at higher cell densities while also inducing alcohol oxidase activity. *P. pastoris* lends itself to high cell density growth on easily metabolized substrates (e.g. glycerol, sorbitol) and cycling with intermittent methanol feeding to maintain alcohol oxidase activity (d'Anjou and Daugulis 2000; Thorpe et al. 1999). In the area of polymer selection/development, we are currently seeking to identify a polymer with equivalent partition coefficients for the 2 target molecules, given the one-to-one molar yield of the biotransformation, which could result in complete substrate desorption coupled with equivalent product uptake. Alternatively, we are also considering the use of 2 polymers with substantially different partition coefficients, which would more completely sequester the substrate and the product, providing better discrimination and no loss of product in the "substrate polymer" with minimal contamination of the "product polymer" by the substrate.

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