CHEMICAL ENGINEERING TRANSACTIONS Volume 24, 2011
Editor Sauro Pierucci
 ISBN 978-88-95608-15-0 ISSN 1974-9791
DOI: 10.3303/CET1124141

Bioproduction of Benzaldehyde in a Solid-Liquid Two-Phase Partitioning Bioreactor using *Pichia pastoris*

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Neutraceutical compounds represent an $86 billion (USD) industry and include additives such as flavours and fragrances, which can often be derived from plant sources, but which can also be produced via microbial biotransformations, providing a means to generate naturally produced, and hence higher priced, products on an industrial scale. Benzaldehyde, with an almond-like aroma, is the second most abundantly used molecule in the flavour industry, and can be produced via microbial transformation. Although the methylotrophic yeast *Pichia pastoris* can oxidize a variety of alcohols to their aldehyde form (e.g. benzyl alcohol to benzaldehyde) at benzyl alcohol concentrations greater than 20 g/l, there is strong inhibition of the reaction by the substrate. Benzaldehyde also has a strong negative effect on the reaction, as it is a potent inhibitor of alcohol oxidase. Bioreactors employing two phases have been shown to be very successful at eliminating substrate/product toxicity by preferential partitioning, suggesting that the use of two-phase partitioning bioreactors (TPPBs) may be a viable approach for microbial benzaldehyde production. Although TPPBs using immiscible organic solvents as a sequestering phase may be effective, their limitations can include potential toxicity of the solvent to the biocatalyst and their possible biodegradability by the organism(s) being used. It is possible to replace immiscible organic solvents in TPPBs with solid commodity polymers, which are chemically inert, and inexpensive. In this work, the biotransformation of benzyl alcohol to benzaldehyde using *Pichia pastoris* was investigated using rational polymer selection to identify the sequestering phase in a solid-liquid TPPB.

1. Introduction

Benzaldehyde is commercially produced via chemical synthesis or through extraction from various fruits where benzaldehyde is naturally found (Gabelman 1994). Microbial biosynthesis of benzaldehyde provides an opportunity to produce natural, and hence higher priced, benzaldehyde under industrially controlled conditions. Methylotrophic yeasts possess the enzyme alcohol oxidase, which catalyzes the oxidation of methanol to formaldehyde, and additional enzymes, which completely convert methanol to carbon dioxide. By adding alcohols other than methanol, the yeasts are able to convert the alcohols to their aldehyde form, with the metabolic pathway stopped at this point (and the accumulation of the aldehyde) due to the specificity of the next enzyme in the pathway, formaldehyde dehydrogenase. The yeast *Pichia pastoris* has been shown to be effective in transforming benzyl alcohol to benzaldehyde in a one-step bioconversion (Duff and Murray 1989).
Previous work using a single aqueous phase has produced benzaldehyde at relatively low yields and rates (e.g. 100 mg/l and 1.4 mg/l-h) (Norliza and Ibrahim 2005). At

Please cite this article as: Daugulis A.J., Jain A., Khan T., Dafoe J.T. and Craig T., 2011, Bioproduction of benzaldehyde in a solid-liquid two-phase partitioning bioreactor using pichia pastoris, Chemical Engineering Transactions, 24, 841-846
DOI: 10.3303/CET1124141

benzyl alcohol concentrations greater than 20 g/l, there is strong inhibition of the reaction by the substrate. Benzaldehyde also has a strong negative effect on the reaction as it is a potent inhibitor of alcohol oxidase. Two-liquid phase systems have used an immiscible organic solvent as a sequestering phase for benzaldehyde (Kawakami and Nakahara 1993; Duff and Murray 1989) and have shown initially promising results with increases total benzaldehyde concentrations achieved relative to single phase systems. In recent work with other biotransformations affected by product inhibition, we have confirmed the efficacy of commodity polymers (which are biocompatible, non-bioavailable, and inexpensive) as the sequestering phase in TPPBs (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 *Pichia pastoris* was investigated via: demonstration of the mechanism of benzaldehyde uptake by polymers, rational polymer selection, and single and TPPB transformation of benzyl alcohol to benzaldehyde.

2. Materials and Methods

2.1 Chemicals and polymers

Benzyl alcohol and all medium components were purchased from Sigma-Aldrich. The sources and properties of the tested polymers are shown in Table 1.

2.2 Medium formulation and culture preparation

P. pastoris ATCC 28485 was grown on the medium formulation of Duff and Murray (1989). The inoculum was prepared by adding 60 µl of frozen *P. pastoris* stock culture to 50 ml medium with 20 g/l methanol to eight 125 ml shake flasks. After 72 h, the cells were centrifuged resuspended in 50 ml of fresh medium. These cells were then added to the reactor vessel containing 3 l of sterile medium with a concentration of 20 g/l methanol to increase cell concentration to approximately 5 g/l and upon depletion of the methanol the cells were ready to perform the biotransformation.

2.3 Analytics

Cell Measurement

A cell dry weight versus optical density calibration curve was used to convert OD readings at 600 nm to biomass concentrations.

Concentration Measurements

Benzyl alcohol and benzaldehyde concentrations were measured by filtering samples and using HPLC with a MetaChem Polaris™, C18 4.6 x 150 mm column. Benzyl alcohol was detected by a UV-Visible Detector at 263 nm and benzaldehyde detected at 283 nm. The column was kept at room temperature with 70% (v/v) sterile water and 30% acetonitrile pumped isocratically at 1 ml/min.

2.4 Uptake of benzaldehyde by polymers

To determine whether adsorption (surface area dependence) or absorption (mass dependence) was the mechanism for benzaldehyde uptake, a fixed aqueous concentration was contacted with different masses of whole, and cut, polymers and allowed to equilibrate for 24 hours, followed by assaying for aqueous benzaldehyde concentration and mass balance to determine partition coefficients.

2.5 Polymer partition coefficients

Partition coefficients for the six different polymers were determined for benzyl alcohol and benzaldehyde as described by Isaza and Daugulis (2009). A stock solution consisting of 10 g benzyl alcohol/l and 3 g benzaldehyde/l was used with polymer masses varying between 1 and 4 g.

2.6 Reactor operation

Single Phase Operation. A 5 l BioFlo III bioreactor (NBS, Edison, NJ) was used, with temperature, agitation and aeration maintained at 30ºC, 400 rpm and 1 L/min, respectively. The reactor, with 3 l of medium, was sterilized, and methanol added to 20 g/l. During cell growth on methanol, the pH was kept at 5.5, and at 7.3 for the biotransformation. Upon depletion of the methanol, based on an increase in the DO, at about 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. 300 g of polymer was preloaded with benzyl alcohol by equilibrating the beads in 3 l of medium with continued benzyl alcohol addition until a 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 approximately 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.

3. Results and Discussion

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

Table 2 shows the uptake of benzaldehyde by 3 polymers of various surface areas and confirms that the uptake of target molecules by polymers is the same mechanism as that for immiscible organic solvents: absorption. Therefore surface area is not important for equilibrium uptake in solid-liquid TPPBs, although it may be for uptake rate.

Figure 1 shows the data for Elvax® 40W, the polymer ultimately selected for TPPB operation, confirming the absorptive uptake mechanism of benzaldehyde by polymers. Table 2 shows the uptake of benzaldehyde by a variety of polymers, demonstrating a wide rage of affinities for this target molecule. To allow for maximum uptake of the

product (avoiding end-product inhibition), as well as to deliver substrate to the aqueous phase a polymer with a high partition coefficient towards benzaldehyde and a lower partition coefficient towards benzyl alcohol was sought. Based on the partition coefficients shown in Table 1 Elvax® 40W was chosen
as an appropriate polymer for TPPB operation.

Table 2. *Uptake of benzaldehyde by polymers of various surface areas.*

Polymer Name	Surface Area (m^2/kg)	Partition Coefficient in Benzaldehyde	Correlation Coefficient for Benzaldehyde
Elvax 40W	2.75	31.6	0.993
Elvax 40W	1.74	30.4	0.993
Hytrel 8206	2.84	21.6	0.965
Hytrel 8206	1.89	22.2	0.995
Pebax 2533	2.15	36.7	0.997
Pebax 2533	1.76	38.1	0.977

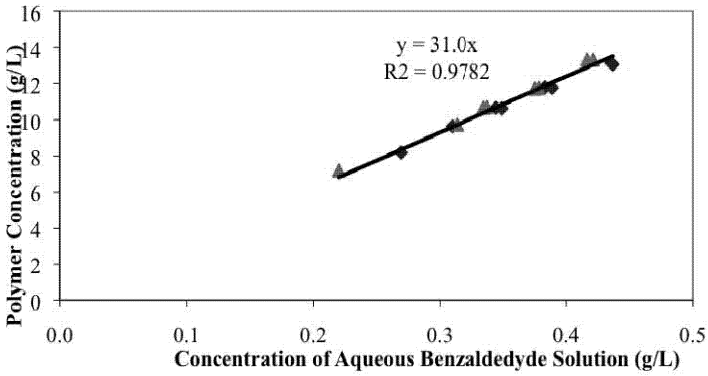

Fig. 1: Uptake of benzaldehyde by Elvax beads with 2 different surface areas. Diamonds are 1.74 m^2/kg and triangles are 2.75 m^2/kg.

Figure 2 shows the time course for the single and two-phase systems, while Figure 3 shows the total mass of benzaldehyde produced in each system. For the single phase biotransformation benzaldehyde production reached a maximum at 21 h at 1.63 g/l, and steadily decreased thereafter due to evaporation. 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. For the TPPB run a10% (w/w) polymer phase ratio pre-loaded with benzyl alcohol was employed and the polymer beads provided an initial bolus of approximately 10 g/l to the aqueous phase, equivalent to the single phase case, thus providing similar levels of substrate inhibition (Figure 2). 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 aqueous phase 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 hours, 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.

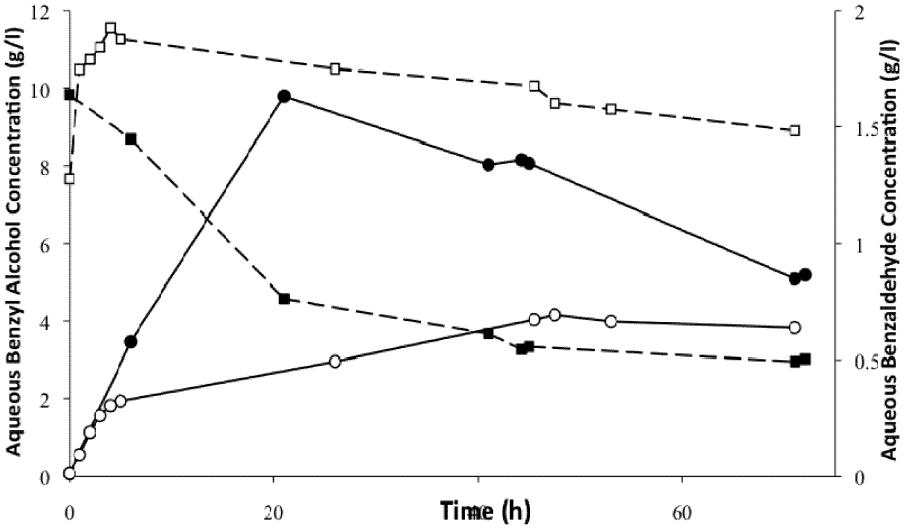

Figure 2. Aqueous concentrations of benzyl alcohol and benzaldehyde in single and two phase biotransformations. Squares are benzyl alcohol and circles are benzaldehyde. Closed symbols are the single phase run and open symbols are the two phase run.

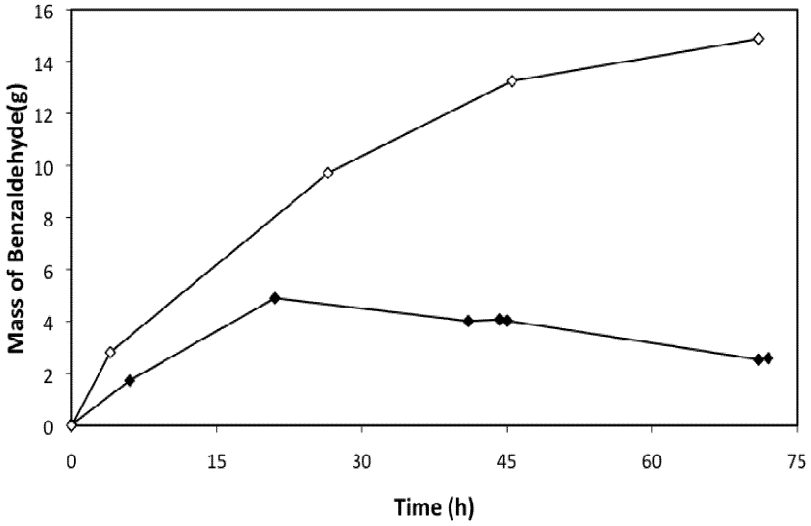

Fig. 3 Total mass of benzaldehyde produced in the single and two-phase systems. Open diamonds represent the two-phase system, and closed diamonds the single phase case.

From the comparison of the overall performance of the systems based on total mass of benzaldehyde produced, shown in Figure 3, it can be seen that mass production in the two-phase system using Elvax® 40W beads was 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 (due to evaporative losses). 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.

4. Conclusion

Commercial polymers have been shown to have a high affinity for toxic substrate and product molecules present in the microbial production of high value neutraceuticals (here, benzaldehyde), and have been used to effectively deliver/sequester these moieties, resulting in enhanced process performance. The use of mixtures of polymers may be an interesting next step to more precisely control the aqueous concentrations of multiple target molecules in solid-liquid TPPBs.

5. References

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