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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 20g/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) which is illustrated in Figure 1.

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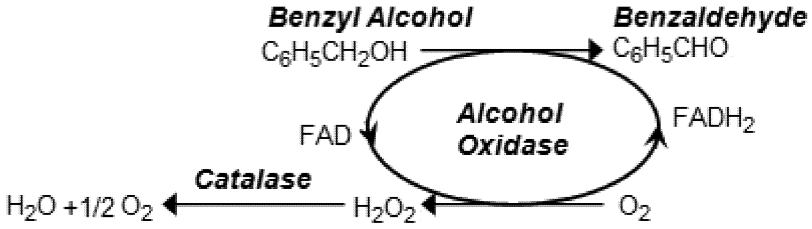

Fig. 1 Enzymatic benzyl alcohol oxidation by alcohol oxidase coupled with catalase and cofactor FAD, producing H_2O and benzaldehyde.

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 benzyl alcohol concentrations greater than 20g/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 rational polymer selection and single and TPPB transformation of benzyl alcohol to benzaldehyde.

2. MATERIALS AND METHODS

2.1 Chemicals and polymers

All chemicals were purchased from Sigma-Aldrich (Canada) or Fisher Scientific (Canada). The yeast *P. pastoris* was purchased from ATCC (ATCC 28485). Table 1 shows the properties and sources of the polymers tested.

2.2 Medium formulation and culture preparation

The shake flask medium was formulated according to Duff and Murray (1989). When applying mixed feed strategies, the total carbon source used in the batch-growth phase was equal to the amount used by Jain et al. 2010 for comparison purposes. To prepare the inoculum, 60µL of frozen *P. pastoris* stock culture was added to six 125mL shake flasks containing 50mL medium with 10g/L glycerol and incubated for 30 h at 30°C and 180rpm.

2.3 Analytics

Cell Biomass Measurement

Optical density readings were measured at 600 nm using a Biochrom Ultraspec spectrophotometer. The readings were converted to cell dry weight (g/L) using a calibration curve.

Chemical Concentration Measurements

During the batch growth-phase, methanol and glycerol concentrations were measured by HPLC- Refractive Index detection at 0.7 ml/min 5mM H_2SO_4 mobile phase, with a Varian, HiPlex H 250 x 7mm column at 55°C. Samples from the reactor were pre-filtered by a 0.2 µm syringe filter, and 20µL of filtered sample was injected in the HPLC. Throughout the biotransformation, 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 5µ C18-A 150 x 4.6 mm column, using the method as described by Jain et al. (2010). HPLC measurements were quantified by peak area calibration to known analytical standards.

2.4 Polymer partition coefficients

Six polymers were tested for partition coefficients for benzaldehyde and benzyl alcohol using the method described by Isaza and Daugulis (2009). The volume fraction of polymer-phase to aqueous solution mimicked the 10% used in the reactor. Twenty millilitres of stock solutions containing 5, 10, 20g/L benzyl alcohol and 0.75, 1.5, 3g/L benzaldehyde in reverse osmosis water were contacted with 2 g polymer beads for 24 h with shaking at room temperature, before analysis and estimation of partition coefficient by mass balance.

2.5 Reactor operation

After 30h incubation, the inoculum flasks were added to a 5L BioFlo III bioreactor (New Brunswick Scientific, Edison, NJ) containing 3L of medium with 10g/L glycerol and 10g/L methanol. When the glycerol and methanol were consumed, deduced from upturns in dissolved oxygen (DO) trace, the biotransformation phase was started.
Single Phase Operation. 3L of medium with 5g/L glycerol was sterilized in the autoclave and 5g/L methanol was added aseptically. The equipment and operating conditions used are as described by Jain et al., 2010 with minor adjustments made in response to any potential oxygen transfer limitations during methanol consumption (i.e. agitation and aeration were set to 500 rpm and 4 L/min). The growth-phase was found to reach the end point when DO trace spiked for the second time at approximately 18 h, and the biotransformation phase was initiated by sterile-filtering 20g/L benzyl alcohol into the reactor vessel using a 0.2 μm syringe filter. Agitation and aeration were set to 400 rpm and 1 L/min to reduce product stripping by aggressive aeration.
Two-Phase Operation. The operating conditions during the cell growth phase for the TPPB system were the same as for the single phase reactor. The biotransformation was initiated by adding benzyl alcohol to a concentration of 20g/L and 300g of polymer beads were added to the reactor vessel to achieve a 10% (w/v) polymer phase ratio.

2.6 Product recovery from polymer

Concentrations of benzyl alcohol and benzaldehyde in the polymer were determined from polymer samples during the biotransformation period. Methanol extraction of the polymer beads as described by Gao and Daugulis (2009) was used to determine the concentrations of substrate and product in the polymer. The volume of methanol used for this extraction was maximized to minimize head space in the 20 mL scintillation vial as benzaldehyde and benzyl alcohol are volatile compounds. For each sample three polymer extractions were performed to determine overall target molecule concentration, each allowed to reach thermodynamic equilibrium before the subsequent extraction.

3. RESULTS AND DISCUSSION

Table 1 shows the partition coefficients of the polymers tested towards benzaldehyde and benzyl alcohol. Kraton D1102K, the polymer exhibiting the highest selectivity for benzaldehyde relative to benzyl alcohol, indicated by the partition coefficient ratio, was chosen as the product sequestering phase to maintain a high concentration of benzyl alcohol in the aqueous phase, as the alcohol oxidase enzyme has a relatively high Km value for benzyl alcohol of approximately 20g/L (Duff and Murray 1989).
A mixed feed strategy of diauxic growth on glycerol and methanol was employed during the growth and induction phases which achieved a high density of induced cells in less than one-third the time required when supplying methanol alone by Jain et al. 2010, (data not shown). Figure 2 shows the time course for the single and two-phase biotransformation periods, while Figure 3 shows the total mass of benzaldehyde produced in each system. For the single phase biotransformation benzaldehyde production reached a maximum at 13 h at 1.66g/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 5.01g at a rate of 0.13g/L-h. The initial decrease in benzyl alcohol concentration is due to the uptake of some benzyl alcohol by the polymer as it established equilibrium with the aqueous phase to near the desired aqueous target of 20g/L, which is below the inhibitory level. The production of benzaldehyde occurred immediately after benzyl alcohol was introduced into the system, and reached a plateau at 49 h at an aqueous concentration of 1.29g/L. The aqueous phase concentration of benzaldehyde in the two-phase system was expected to be relatively low given the high partitioning coefficient of the polymer.

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

Polymer	Supplier	Glass transition temperature, Tg (°C)	Specific gravity	Type	Partition coefficient for benzyl alcohol	Partition coefficient for benzaldehyde	Partition coefficient Ratio (Benzaldehyde: Benzyl alcohol)
Kraton D1102k	Kraton	Styrene: 90; butadiene: -90	0.94	Styrene/ butadiene linear block copolymer Poly(dimethylsiloxane)	1.7	25.3	14.9
Silicone Rubber	Mastercraft	-116	0.991	Poly(dimethyl siloxane)	1.24	9.9	8.0
Elvax 360	DuPont	-25	0.948	25% Vinyl alcohol (copolymer with ethylene)	3.1	24.1	7.8
Desmopan 453	Bayer MSD	-34	1.22	Polyurethane thermoplastic elastomer	6.6	42.6	6.5
Hytrel 8171	DuPont	-59	1.17	Poly(butylene/poly(alkylene ether)phthalate thermoplastic	14.8	40.9	2.8
Nylon 6-6	DuPont	50	1.24	Polyamide 66 (crystalline)	2.9	5.6	1.9

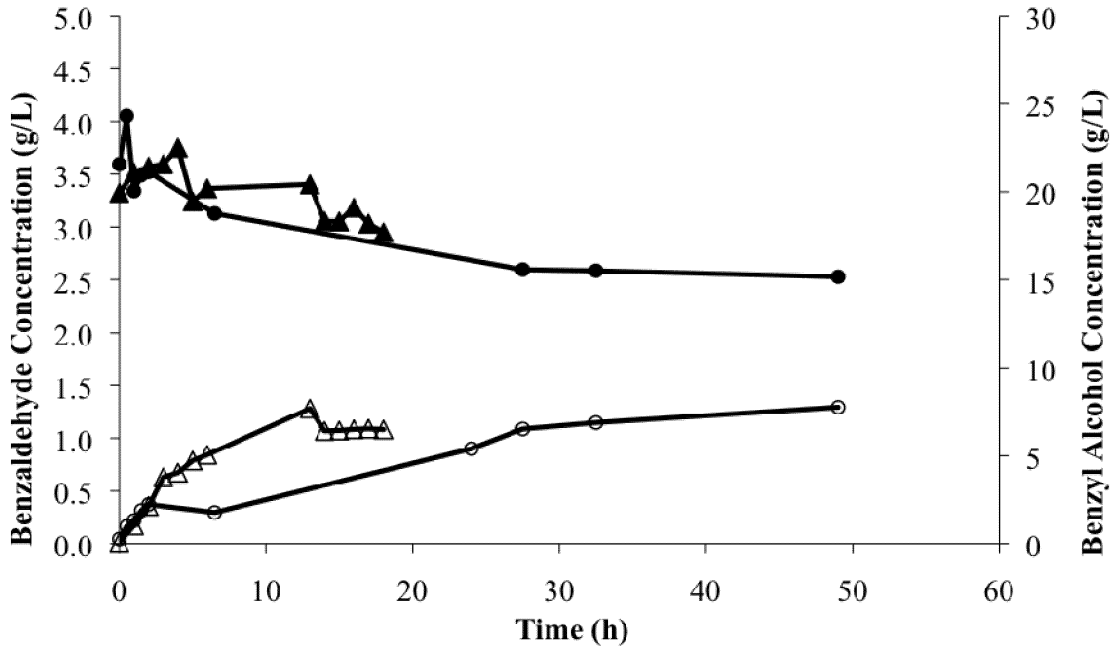

Fig. 2: Single phase concentration of benzyl alcohol (▲) and benzaldehyde (Δ). TPPB aqueous phase concentration of benzyl alcohol (●) and benzaldehyde (○) as a function of time.

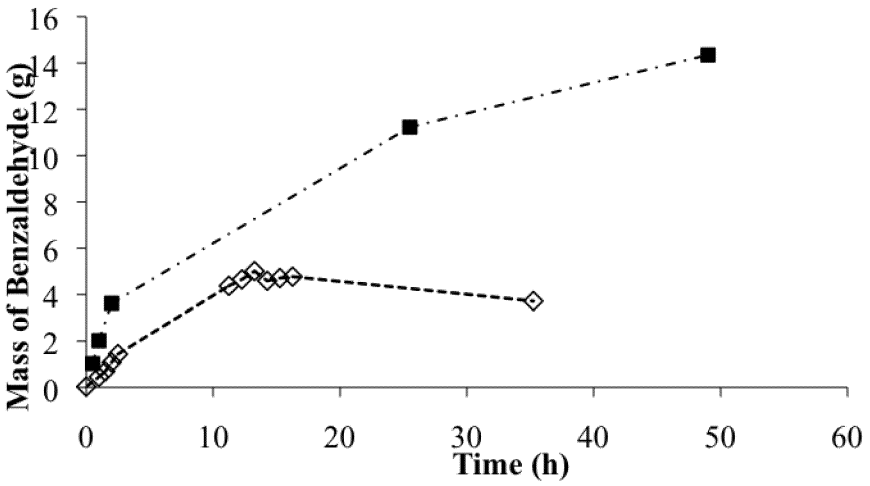

Fig. 3: Total benzaldehyde production in the single-phase (◊) and two-phase system (■) as a function of time.

From the comparison of the overall performance of the systems based on total mass of benzaldehyde produced, shown in Figure 2, it can be seen that mass production in the two-phase system using Kraton D1102k beads was 290% higher than that achieved in a single phase system. The molar yield calculated was 0.91 in the Kraton D1102k system, which is an increase from the single phase case, which had a yield of 0.85 (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.

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