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Strategies for improved bioproduction of benzaldehyde by *Pichia pastoris* and the use of hytrel as tubing material for integrated product removal by *in situ* pervaporation

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ABSTRACT

Benzaldehyde (BZA), with its almond-like aroma, is an important additive in the food, fragrance and nutraceutical industries, and biologically derived BZA by microbial catalysts provides many advantages over the traditional methods of plant material extraction. The methylotrophic yeast *Pichia pastoris* induces alcohol oxidase (AOX) in the presence of methanol, which is highly nonspecific to other primary alcohols, and can oxidize them to their aldehyde form without further degradation, such as benzyl alcohol (BA) to BZA. In this work BA and BZA inhibition on *P. pastoris* cell growth was determined with an IC_{50} at 0.98 g L^{-1} for BZA and 2.95 g L^{-1} for BA, which are low levels and are undesirable for high biotransformation rates. Product detoxification, and not low AOX and low cellular energy source, was found to most improve the biotransformation rates, which prompted the application of *in situ* product removal (ISPR) using the thermoplastic polymer Hytrel 3078, fabricated into pervaporation tubing by DuPont, Canada. Hytrel 3078, chosen for its high partition coefficient (PC) for BZA and its low PC for BA, was characterized by available tubing surface area and permeate gas flow rate in terms of its BZA/BA flux, showing that permeate gas flow rate has a greater positive influence on flux. Finally, the integrated *in situ* pervaporation biotransformation was effective at continuous product separation, using 87.4% less polymer in comparison to studies with the use of polymer beads, and improved overall volumetric productivity of 214% (245.9 mg L^{-1} h^{-1} *vs.* 115.0 mg L^{-1} h^{-1}) over previous studies producing BZA.

1. Introduction

Benzaldehyde (BZA), a biologically produced aromatic molecule of high commercial value, widely used in the flavour and fragrance industry, has also gained acceptance for use in nutraceutical, pharmaceutical, cosmetics, agrochemical, and dye applications [1–8]. The extraction of BZA from various fruit pits, including cherries, apricots and peaches, generates cyanate, a toxic by-product, which requires additional processing steps and purification that can negatively influence product quality [6,9]. Microbial biocatalysts provide a potential route for producing natural BZA at a large scale without the generation of toxic by-products, extensive purification, and dependence on agricultural production [10–12].

The methylotrophic yeast *Pichia pastoris*, has been shown to effectively produce BZA [13–15] utilizing alcohol oxidase (AOX) and catalase employed in the first step of the methanol utilization pathway (MUP). As a result of methanol induction of the genes encoding AOX enzymes, high expression and non-specificity towards primary alcohol substrates enables it to oxidize alcohols other than methanol to their respective aldehyde form [16] without further degradation since the subsequent enzyme in the pathway is highly specific to its natural substrate, formaldehyde [17]. Previous work using *P. pastoris* to produce BZA has been limited by reduced BZA conversion rates, however, after elevated product concentrations were reached in the aqueous phase.

Studies using *P. pastoris* for heterologous protein production have shown that feeding strategies based on improving AOX expression in the MUP after long-term fermentations have been effective at increasing protein titre [18–22] although no previous literature has explored AOX conversion rates for the bioproduction of BZA. Previous work has shown that BZA production rates decrease as BZA accumulates in the medium, and it is possible that the addition of methanol, which is AOX inducing, and/or sorbitol, which is AOX non-repressing could potentially enhance AOX synthesis and/or provide a cellular energy source, thereby increasing BZA production rates. Alternatively, recent work done by us [13] using solid–liquid two-phase partitioning bioreactors (TPPBs) for *in situ* product removal (ISPR) during the biotransformation of benzyl alcohol (BA) to BZA has shown that BZA has a strong inhibitory

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effect on the conversion rate, and thus it is possible that additional product removal needs to occur to enhance BZA production.

Although solid polymer TPPBs used for the biotransformation of BZA by *P. pastoris* have shown to be an effective strategy for alleviating end-product inhibition, the extent of product removal is limited due to the confined volume in the bioreactor (*i.e.* by how much polymer can be added). Methods using integrated product recovery, such as by pervaporation, have shown to be very effective for the continuous separation of volatile products from fermentation cultures with relatively high product removal fluxes [23–29]. Previous studies employing pervaporation for separation have used a variety of commercially available membranes consisting of silicone rubber, silicone composites, poly(vinyl alcohol), poly(tetrafluoroethylene), and chitosan-poly(acrylonitrile), selected based on affinities or activity coefficients of the target molecule in the membrane, and trans-membrane diffusion rates [24–26,30–34]. We have shown that solute–polymer affinity can be determined by partition coefficient (PC) measurements, which have been useful for selecting TPPB polymers based on their PCs for BZA and BA [13,14,35,36]. Polymer selection using this methodology, followed by the fabrication of custom pervaporation membranes, could be a useful strategy for enhancing continuous ISPR that would also exploit the physical property differences (affinity and volatility) of BA and BZA.

In this study, BA and BZA inhibition on *P. pastoris* cell growth, as well as the possible impact of low AOX and low cellular energy source levels were investigated to determine why biotransformation rates decreased during the bioproduction of BZA. As a result of these findings, product detoxification was further addressed by selecting a thermoplastic polymer with a high PC for BZA and a low PC for BA, and having the polymer fabricated into pervaporation tubing, which was further characterized in terms of its flux. Finally, an integrated *in situ* pervaporation biotransformation was conducted to demonstrate continuous product separation, high product recovery, and increased overall product titre and volumetric productivity.

2. Materials and methods

2.1. Chemicals, and polymers

All medium components were obtained from Fisher Scientific, Guelph, ON, Canada, and BZA (≥99.5%) and BA (99.8%) were acquired from Sigma–Aldrich, Oakville, ON, Canada. Table 1 shows the properties and sources for the polymers used in this work (where available), many of which were kindly donated by the manufacturers. The polymers were selected based on polymer processing ability for tubing extrusion (flex modulus), and polymer physical properties. The different grades of Hytrel were subsequently custom-extruded as tubing by DuPont Canada for the purposes of this work.

2.2. Medium formulation and culture preparation

P. pastoris was obtained from the American Type Culture Collection, Rockville, MD (ATCC 28485). The medium for the bioreactor and shake flasks was formulated according to Duff and Murray [15] and Craig and Daugulis [13] except 5 g L^{-1} glycerol and 5 g L^{-1} methanol were used in the bioreactor during the cell growth phase, unless otherwise indicated, and culture preparation was as described by Craig and Daugulis [13]. Immediately before the biotransformation a nutrient supplement was added also formulated according to Duff and Murray [15] equivalent for 10 g L^{-1} methanol, except with no carbon source.

2.3. Shake flask growth inhibition experiments

These experiments were intended to evaluate the degree of BZA and BA inhibition on cell growth, as we had already shown the effect of BA and BZA on enzymatic biotransformation rates by *P. pastoris*. The growth inhibition experiments were conducted using 20 × 125 mL Erlenmeyer flasks with 50 mL medium containing the same formulation as described above only with 10 g L^{-1} sorbitol as the carbon source at 30 °C and 180 rpm. The flasks contained different concentrations of BA: 2 g L^{-1}, 4 g L^{-1}, 8 g L^{-1}, and 16 g L^{-1}, and BZA 0.5 g L^{-1}, 1.0 g L^{-1}, 1.5 g L^{-1}, and 2 g L^{-1}. Three replicate runs were done for the control and all the BZA and BA concentrations except for 2 g L^{-1} BA, 4 g L^{-1} BA, and 2 g L^{-1} BZA (no error bars on these points). The flasks were inoculated and the biomass concentrations were determined over a period of 30 h.

2.4. Polymer partition coefficient measurements

To determine if extruding the Hytrel polymers into tubing affected their PCs for BZA and BA, polymer pellets and tubing partition coefficients were determined using the method described previously [37]. The polymer tubing was cut into pieces such that they fit in the 20 mL scintillation vials used in the experiments.

2.5. Determining why the biotransformation stops

Cells were grown in a 5 L BioFlo III bioreactor (New Brunswick Scientific, Edison, NJ) containing 3 L of medium with 5 g L^{-1} glycerol and 5 g L^{-1} methanol operating at 30 °C, 500 rpm, 1.33 vvm aeration and pH 5.5 as previously reported in Craig and Daugulis [13]. The dissolved oxygen (DO) was used to indicate when to initiate the biotransformation phase (substrate depletion and a rise in DO), by

Table 1
Thermal properties, physical properties, and experimental PC results for benzyl alcohol and benzaldehyde applied for the selection of pervaporation tubing.

Polymer	Glass transition temperature, T_g (°C)	Specific gravity	Flexural modulus[a], MPa (psi)	Partition coefficient[b] for benzyl alcohol	Partition coefficient[b] for benzaldehyde
Kraton D1102K, Kraton	Styrene: 90 Butadiene: −90	0.94	–	1.7[c]	25.3[c]
Hytrel 8206, Dupont	−59	1.17	80 (11,600)	8.6 ± 0.3	26.4 ± 0.7
Hytrel 8171, Dupont	N/A	N/A	~30 (4400)	9.3 ± 0.3	27.6 ± 1.0
Hytrel G4078W, Dupont	−37	1.18	65.5 (9500)	8.4 ± 0.5	35.1 ± 0.2
Hytrel G3548W, Dupont	−40	1.15	32.4 (4700)	10.4 ± 0.2	40.8 ± 1.0
Hytrel 3078, Dupont	−60	1.07	28 (4000)	9.2 ± 0.2	45.0 ± 1.0
Masterflex Norprene Food Grade (A60 F), Cole Palmer	–	–	–	0.2 ± 0.1	8.2 ± 0.4
Polypropylene, cole palmer	–	–	–	0.8 ± 0.9	1.1 ± 0.6

[a] ASTM D790 (ISO 178), at room temperature.
[b] Experimental PC of cut-up tubing (this study).
[c] Experimental PC value of Kraton D1102K beads published in Craig and Daugulis [13].