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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].

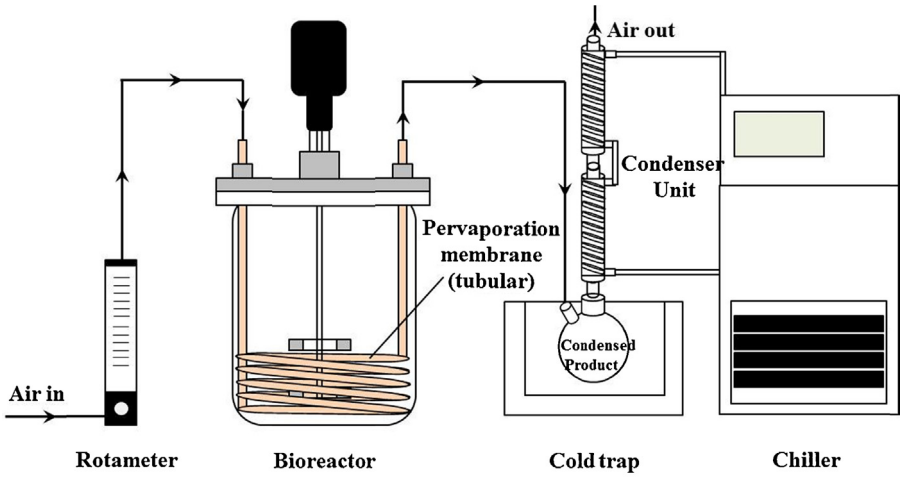

Fig. 1. A schematic diagram of *in situ* pervaporation system.

adding 10 g L^{-1} BA which was sterile-filtered using a 0.2 μm syringe filter. Agitation and aeration were set to 400 rpm and 0.333 vvm to reduce product stripping by aggressive aeration. A pH of 5.5 was maintained, appropriate for cell growth during potential sorbitol substrate consumption, and a constant temperature at 30 °C. To determine if AOX levels were causing reduced biotransformation rates, 1 g L^{-1} methanol was added approximately 10 h into the biotransformation. In a separate experiment to determine whether the depletion of cellular energy source had been limiting the biotransformation rate, 4 g L^{-1} sorbitol, with additional nutrients described above, was added approximately 10 h into the biotransformation. Finally, in a separate experiment to determine if the accumulation of BZA was causing reduced biotransformation rate 600 g of Kraton D1102K polymer was added approximately 9 h after the biotransformation commenced to reduce the aqueous BZA concentration.

2.6. Characterization of pervaporation tubing

2 L MultiGen fermenters (New Brunswick Scientific, Edison, NJ) with 1.5 L working volume and 10 g L^{-1} BA and 3 g L^{-1} BZA were operated at 25 °C, 300 rpm and were used to characterize tubing flux. The Hytrel tubing was custom fabricated by DuPont Canada (Kingston, ON) for our study to very tight tolerances. The pervaporation polymer selected for this work was Hytrel 3078 based on the high tested PC for BZA, and the manufactured tubing had an outer diameter of 6.35 mm, wall thickness of 0.5 mm and a low flexural modulus important for coiling tubing inside the bioreactor.

The permeate gas (air) flow rate and available membrane surface area were used to evaluate pervaporation system effectiveness. Experiments varying permeate gas flow rate were tested at 0.8 L min^{-1}, 2 L min^{-1}, and 4 L min^{-1} operating at a constant available surface area of 220 cm^2 or 1.1 m length of tubing, which is also equivalent to 12.2 g of polymer. Experiments varying available surface area were tested at 220 cm^2, 440 cm^2, and 880 cm^2 operating at a constant permeate gas flow rate of 2 L min^{-1}. 3 mL samples from the aqueous phase were withdrawn periodically and measured for BA and BZA concentration over a period of 24 h.

2.7. Biotransformation with integrated in situ pervaporation operation

Bioreactor operation was as previously described, and included a cell growth phase on methanol/sorbitol, followed by the addition of BA to initiate the biotransformation after substrate depletion had occurred, as determined by a spike in the DO. The growth phase was conducted at pH 5.5, and the biotransformation phase was initiated by shifting the pH to 7.3 using 4 M KOH and by adding 20 g L^{-1} BA which was sterile-filtered using a 0.2 μm syringe filter. A chiller (WKL 230 LAUDA Brinkmann) through which ethylene glycol was circulated was used to condense the permeate products set at an operating temperature of −1.9 °C. Fig. 1 shows a schematic diagram of the *in situ* pervaporation system used for BZA removal in this work.

In the initial run, 2 L min^{-1} permeate gas flow was used at an available surface area of 600 cm^2 scaled up from membrane characterization experiments to maintain a length per volume ratio of 1 and estimated flux removal rate of 5 g m^{-2} h^{-1}. In a second run the initial gas flowrate was 8 L min^{-1}, which was increased to 16 L min^{-1} to try to prevent the aqueous phase BZA concentration reached highly inhibitory levels.

The outlet tubing material (connecting the pervaporation bioreactor to the condenser) was tested for PC values (Table 1) to insure that insignificant amounts of BZA permeate were absorbed in the outlet lines. Masterflex Norprene Food Grade ¼ in. outer diameter tubing was used for the all tubing lines involving the permeate gas flow except for the connection from the bioreactor to the condenser, where poly propylene tubing was used as all such tubing had a low experimental PC for BZA and BA.

3. Analytics

3.1. Cell biomass measurement

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

3.1.1. Concentration measurements

BA and BZA 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 mm × 4.6 mm column [13,14]. For the *in situ* pervaporation operation BZA concentrations were also determined from aqueous phase concentrations using Henry's Law constant as used by Poleo and Daugulis [38].

4. Results and discussion

4.1. Substrate and product inhibition on cellular growth

The degree to which BZA and BA are inhibitory to cell growth is important for maintaining high cell activity during the

biotransformation. A previous study on the impact of elevated BA and BZA levels on the enzymatic conversion reaction (not on cell growth) showed that BZA is highly inhibitory, whereas high BA concentrations actually increase the enzymatic conversion rate [13]. In this study the effect of BA and BZA inhibition on cellular growth was examined as shown in Fig. 2. Fig. 2(a) and (b) shows that even at very low concentrations of BZA, 0.5 g L^{-1}, and BA 2.0 g L^{-1} the cell biomass concentration decreases significantly relative to the control. Inhibition of cell growth as a function of substrate and product concentration at 30 h is also seen in Fig. 3, which shows that cell growth is very sensitive to both BZA and BA at low concentrations. The half maximal inhibition concentration (IC_{50}) was determined using Fig. 3, which shows that for BZA the IC_{50} was at 0.98 g L^{-1} and for BA the IC_{50} was at 2.95 g L^{-1}. It can be seen that at all concentrations of BA it is better tolerated by the cells even at high concentrations as the cell inhibition plateaus, appropriate for the biotransformation, compared to the high cell inhibition shown by BZA. These findings suggest that in order for *P. pastoris* cells to grow in the presence of BZA and BA, the BZA concentration must be kept low, which is contrary to the aim of producing large amounts of BZA, and the BA concentration is tolerable to the cells at moderate concentrations.

4.2. Strategies for enhanced biotransformation rates

4.2.1. Maintaining high AOX activity

Methanol induces AOX, but in the presence of alternative primary alcohols such as BA this is not the case, as the substrate is not compatible with the MUP. This was our motivation for exploring if adding methanol to enhance AOX activity would lead to

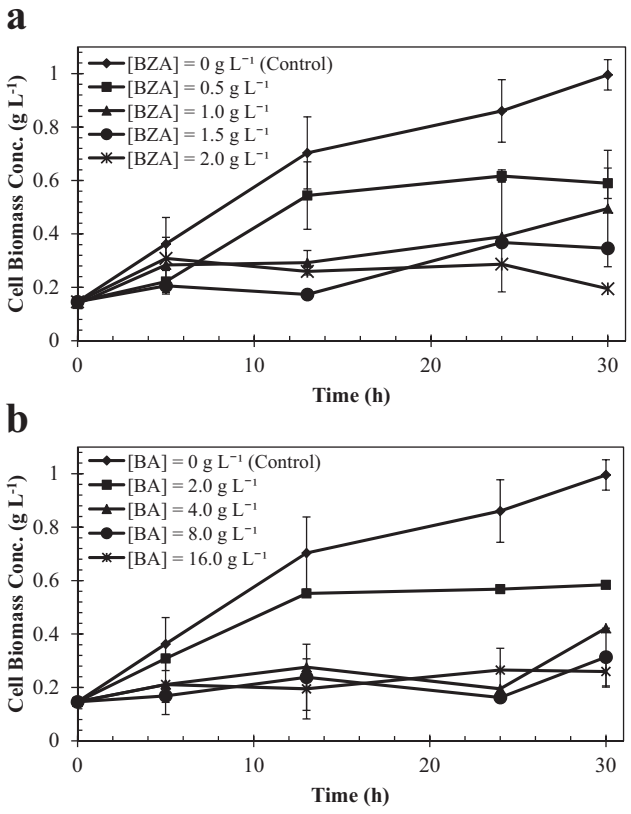

Fig. 2. Cell biomass concentration as a function of time with 10 g L^{-1} sorbitol as the sole carbon source in the presence of varying benzaldehyde concentrations from 0.5 g L^{-1} to 2 g L^{-1} shown in (a) and varying benzyl alcohol concentrations from 2 g L^{-1} to 16 g L^{-1} represented in (b).

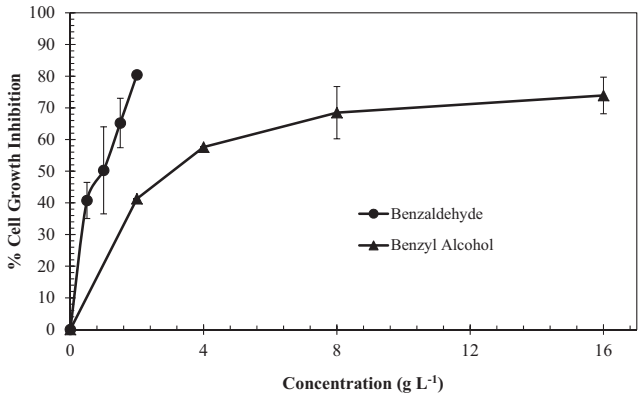

Fig. 3. The % cell growth inhibition by benzaldehyde and benzyl alcohol at various substrate and product concentrations at 30 h.

increased BZA production during the biotransformation. Methanol addition of 1 g L^{-1} did not appear to improve the biotransformation rate (Fig. 4(a)) even though it was observed that the methanol was indeed consumed (and therefore AOX was produced) as seen by a DO drop and an increase in OD (data not shown). However, due to the competition between methanol and BA at this time, methanol was naturally the preferred substrate for cell metabolism, which may explain why no BZA was produced after methanol

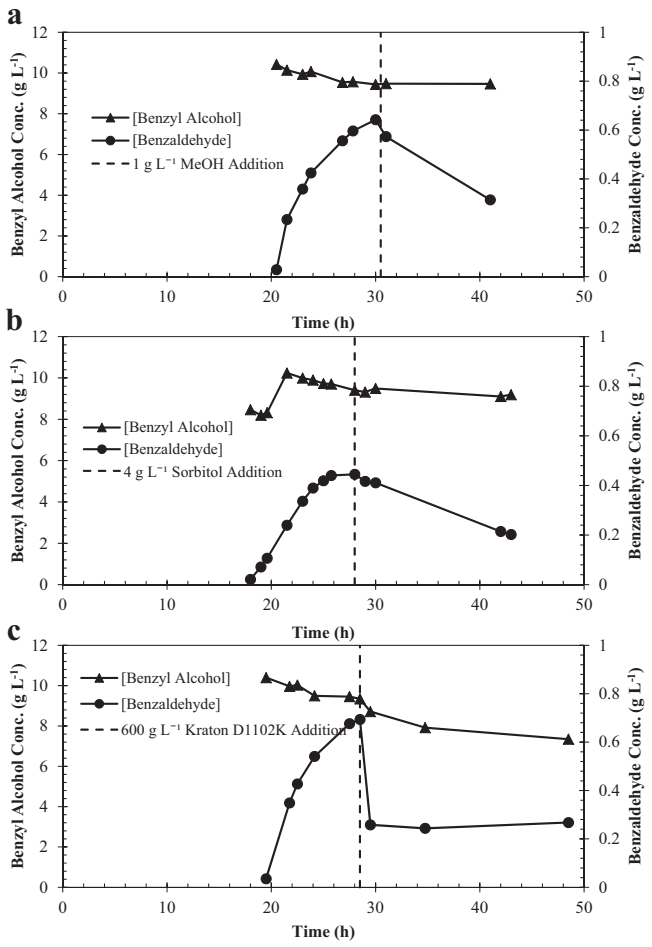

Fig. 4. Strategies for enhanced bioproduction reaction rates of benzaldehyde (a) AOX activation by 1 g L^{-1} methanol addition, (b) cellular energy source cycling by 10 g L^{-1} sorbitol addition, and (c) detoxification of benzaldehyde by addition of 600 g Kraton D1102K polymer.

addition. Furthermore, it can confirmed that no BZA was produced by observing that no BA was consumed, since the biotransformation is based on a 1:1 stoichiometry of BA to BZA [13]. The reason for the decrease in aqueous BZA can be explained by BZA's susceptibility to volatilization.

4.2.2. Maintaining high cellular energy source

Previous studies using *P. pastoris* for the biotransformation of alcohols reported that AOX enzyme activity decreases rapidly during a 24 h biotransformation, which could be a result of cells potentially depleting cellular energy source reserves without replenishment during a biotransformation, since no carbon source is available for cellular metabolism [39]. As shown in the previous strategy for generating high AOX activity, methanol cannot be introduced as a cellular energy source during biotransformation because methanol would also generate AOX, thus confounding the results. For this reason a non-repressing substrate, sorbitol, was added as a potential strategy to increase cellular energy [18,19,40], the results of which are seen in Fig. 4(b). It can be observed that after the addition of 10 g L^{-1} sorbitol at approximately 10 h after the start of the biotransformation, the production rate of BZA continued to decrease.

4.3. Product detoxification

Product detoxification was the final strategy employed to determine the reason for a declining conversion rate during the biotransformation. An ISPR approach employing solid–liquid TPPBs using polymers specifically for this system have been shown to positively affect the production of BZA and reduce product inhibition [13]. Since it is important to maintain a high concentration of BA for high reaction rate conversion, the polymer Kraton D1102K was selected due to its high PC ratio of BZA relative to BA, removing the inhibitory molecule while minimizing the amount of substrate absorbed in the polymer [13]. After the addition of 600 g Kraton D1102K the aqueous BZA concentration dropped from 0.70 g L^{-1} to 0.26 g L^{-1} in less than an hour, which shows the detoxification method was effective at quickly removing BZA from the aqueous phase (Fig. 4(c)). After the initial drop in BZA in the aqueous phase there was a slight notable increase in BZA concentration. The aqueous concentration of BA also decreased from 9.46 g L^{-1} to 8.72 g L^{-1} within the first hour of the polymer addition. Studies on the mass transport properties using semi-crystalline amorphous polymers have found that polymer–liquid solute equilibrium is reached very quickly, in 1–2 h [41,42], indicating that after the addition of Kraton D1102K the observed decrease in BA (after 30 h) was due to transformation by AOX. The stoichiometric conversion ratio of BA:BZA, which is 1:1, suggests that BZA was produced at this time (due to the drop in BA concentration), although due to Kraton D1102K polymer affinity for BZA, most of the BZA produced was sequestered into the polymer phase. Hence only a slight increase in the aqueous phase BZA is observed. Product detoxification using polymer addition was thus effective at removing BZA from the aqueous phase, thereby allowing for the AOX conversion to resume. Consequently, a strategy of continuous BZA removal by pervaporation was our next approach.

4.4. Characterization of pervaporation tubing

Although the above results showing that product removal by polymer addition was successful at alleviating BZA toxicity, continuing to add polymer beads to a bioreactor is not a practical option, and therefore an integrated *in situ* pervaporation method was developed using Hytrel polymer tubing for continuous product recovery. After considering the various polymer properties shown in Table 1, the polymer selected for this study was a thermoplastic polyester elastomer Hytrel 3078 because it had the highest tested PC for BZA, low water absorption, and lowest flexural modulus for ease of coiling in the bioreactor. Traditional pervaporation methods applied in fermentation processes have tended to separate components using a flat membrane external to the bioreactor while the membrane is under vacuum for the vapour phase to permeate by evaporation. However, in this study, *in situ* pervaporation was performed with tubing membrane internal to the bioreactor with the driving force controlled by the gas flow rate, where permeate evaporates after permeation through the membrane. Overall, in comparison with traditional pervaporation using flat membranes, this integrated method of pervaporation allows for greater flexibility in operation by control of the gas flow rate, increased availability for mass transfer area, and instantaneous product removal.

The parameters tested for the characterization of the Hytrel 3078 tubing membrane were the permeate gas flow rate and available membrane surface area shown in Fig. 5. The results from permeate gas flow rate, Fig. 5(a) show that as the permeate flow rate increased from 0.8 L min^{-1} to 4 L min^{-1} the concentration of BA decreased due to initial absorption into the polymer and plateaued at all three permeate flow rates. The BZA concentration in the aqueous phase decreased at a greater rate than BA partly due to the higher polymer affinity for BZA and BZA's greater evaporation rate into the gas phase. As the permeate flow rate increased from 0.8 L min^{-1} to 4 L min^{-1} the average flux removal of BZA increased from 2.1 g m^{-2} h^{-1} to 3.3 g m^{-2} h^{-1}. The surface areas tested were 220 cm^2, 440 cm^2 and 880 cm^2 at a permeate flow rate of 2 L min^{-1} shown in Fig. 5(b). It can be observed that as the available surface

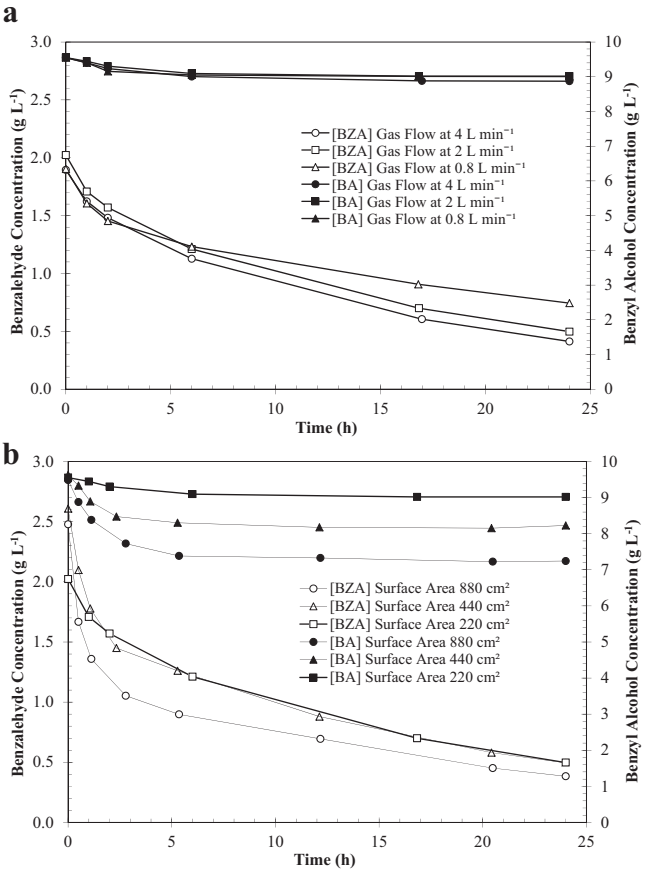

Fig. 5. Characterization of pervaporation membrane tubing (a) Varying permeate gas flow rates of 0.8 L min^{-1}, 2.0 L min^{-1}, 4.0 L min^{-1} at a constant surface area of 220 cm^2 and (b) varying surface areas 220 cm^2, 440 cm^2, and 880 cm^2 at a constant flow rate of 2 L min^{-1}.

area increased so did the mass of polymer for available absorption of the solute molecules. The aqueous BA concentrations decrease primarily due to polymer absorption and plateaued at all three available surface areas. The aqueous BZA concentration initially decreases due to polymer absorption as the available surface area or mass of polymer increased the amount of BZA removed initially increased. However, as the available surface area increased for pervaporation there was not a significant increase in the flux removal rate of BZA, indicating that there could potentially be some diffusion limitations at higher available membrane surface areas. Available membrane surface area, also affecting polymer mass, seems to have the greatest effect on BZA removal at the beginning of the biotransformation, where the permeate flow rate has the greatest effect on the latter part of the biotransformation. Therefore, an effective strategy that could be applied for *in situ* pervaporation using tubular membrane is to use high permeate gas flow rates with a membrane surface area that fits the constraints of the bioreactor size and does not impede the functional performance of the bioreactor, such as oxygen delivery to cells and homogeneous mixing.

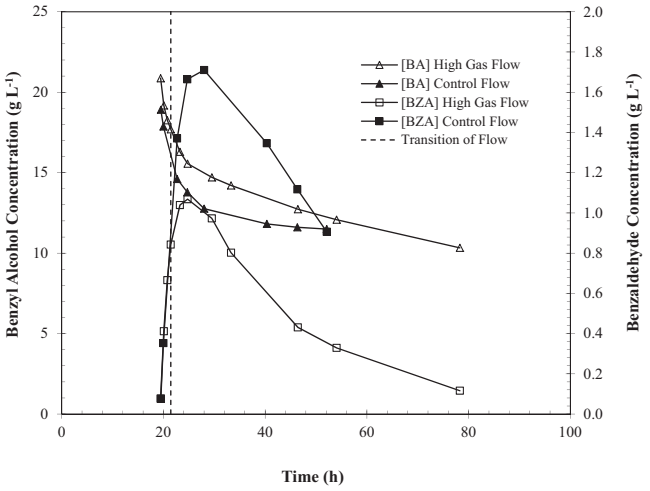

Fig. 6. Aqueous benzaldehyde and benzyl alcohol concentration during biotransformation for *in situ* pervaporation of control run with permeate gas flow rate at 2 L min^{-1} and high permeate gas flow rate at 8 L min^{-1} transitioning to 16 L min^{-1}.

4.5. Biotransformation with integrated in situ pervaporation

Since it was BZA accumulation rather than reduced AOX or cellular energy source levels that was determined to be the cause of a diminishing biotransformation rate, experiments were conducted with a fixed amount of tubing (having a surface area of 600 cm^2) in the bioreactor with varying pervaporation air flowrates to try to keep aqueous BZA levels low. As a point of comparison, an *in situ* pervaporation run was done at a low permeate gas flow rate and one at a higher permeate gas flow rate with the same operating conditions as mentioned above, except at pH 7.3 instead of the growth pH 5.5, for comparison to previous work [13,14]. In addition, work done by Khan and Daugulis [31] examined BZA PC with Hytrel 3548L at a pH range from 5 to 9 and confirmed that this pH range does not have a significant impact on PC. The biotransformation for both the low and high pervaporation flow rate started approximately around the same time 19.5 h and 19.4 h, respectively, that is, at the end of methanol/glycerol growth period when both substrates had become depleted. The biotransformation for the low pervaporation run was extended until the aqueous BA concentration started to plateau and the high permeate gas flow run was stopped as the aqueous BZA concentration was approaching zero shown in Fig. 6.

At the low pervaporation flowrate, the maximum aqueous BZA concentration was 1.71 g L^{-1} at 28 h. Since the stoichiometric ratio of BA to BZA conversion is 1:1, when the BA concentration began to plateau the decrease in BZA can be explained by pervaporation removal, and to a lesser degree evaporation from the bioreactor. The total mass of BA consumption was 22.4 g, at a volumetric productivity of 228.0 mg L^{-1} h^{-1}. A mass balance was done to quantify the BZA in the system and the effectiveness of product removal. The extraction of BZA using *in situ* pervaporation was able to remove 53.6% of the BZA produced, 19.6% was lost due to volatilization (calculated based on Henry's Law), 13.4% was retained in the polymer phase, and 13.4% remained in the aqueous phase. It is also interesting to note that only 3.8% of the total BA was removed by pervaporation, which shows the high selectivity of the pervaporation polymer.

The high permeate gas flow rate biotransformation was done at gas flow rates of 8 L min^{-1} and 16 L min^{-1} compared to 2 L min^{-1} for the low flow case. The high permeate gas flow rate biotransformation follows a similar reaction rate of BZA production; however, it reached a maximum aqueous BZA concentration of 1.07 g L^{-1} at 23.25 h (Fig. 6), confirming that the changed operating condition was able to reduce BZA concentrations to below about 1 g L^{-1}, concentrations above which are known to reduce conversion rates significantly [13]. The aqueous BA concentration continued to decrease indicating that the biotransformation was still operational even though the BZA concentration in the aqueous phase was decreasing, which is due to a greater rate of BZA removal from the system than production. The total mass of BA consumed was 31.3 g, at a volumetric productivity of 177.4 mg L^{-1} h^{-1}. Although the volumetric productivity decreased over time due to reduced reaction rate, the high permeate gas flow rate run showed improved results for BZA production over the low flow run since at 34.7 h there was 25.6 g L^{-1} BA consumed, equivalent to a volumetric productivity of 245.9 mg L^{-1} h^{-1}. A mass balance based on BA consumed was used to effectively quantify BZA at the end of the biotransformation and showed that 83.5% was removed by pervaporation, 9.4% was lost due to volatilization (calculated by Henry's Law), 6.0% was retained in the polymer phase, leaving 1.2% remaining in the aqueous phase. Importantly, the high permeate gas flow rate had less losses of BZA due to volatilization, which could be a result of reduced concentrations of BZA in the aqueous phase. The total removal of BA by pervaporation was 3.3%, suggesting that the pervaporation polymer is still very selective of BZA even at high flow rates.

4.6. Comparison of in situ pervaporation and two-phase systems

A performance comparison of the low and high permeate gas flow rate pervaporation systems, and a two phase system using Kraton D1102K beads from work by Craig and Daugulis [13] is shown in Table 2. In the present pervaporation study we used a mass of only 37.9 g of polymer compared to 300 g in the TPPB system, showing the effectiveness of the pervaporation system in continuous rather than batch removal of BZA, using 87.4% less polymer. The TPPB system with Kraton D1102K had a maximum aqueous BZA concentration of 1.29 g L^{-1}, compared to 1.71 g L^{-1} in the low flow pervaporation system and 1.07 g L^{-1} in the high gas flow pervaporation, which shows that *in situ* pervaporation was an effective strategy in reducing BZA toxicity.

The biotransformation using Kraton D1102K as a second phase was able to recover 14.4 g of BZA and produce a total of 16.9 g BZA, based on BA consumption, while the *in situ* pervaporation systems had a BA consumption of 22.3 g (low flow) and 31.3 g (high flow). The BZA volumetric productivity, based on BA consumption, for the Kraton D1102K system was 115.0 mg L^{-1} h^{-1}, and using *in situ* pervaporation it was 228.0 mg L^{-1} h^{-1} for the low flow, and

Table 2
Comparison of process operation: *in situ* pervaporation (this study) to TPPB system with Kraton D1102K.

Criteria for comparison	TPPB Kraton 4105K [13]	Pervaporation low flow rate (this study)	Pervaporation high flow rate (this study)
Operation variables			
Initial aq. BA conc. ($g\,L^{-1}$)	21.6	18.9	20.9
Total polymer mass in system (g)	300	37.9	37.8
OD growth-phase (g-biomass L^{-1})	11.8	6.1	5.9
Fermentation time			
Growth and induction phase (h)	20.5	19.5	19.4
Biotransformation phase (h)	49.0	32.6	58.8 (34.7)[a]
System performances			
Maximum aq. BZA conc. ($g\,L^{-1}$)	1.29	1.71	1.07
Total BA consumed (g)	16.9	22.3	31.3 (25.6)[a]
Volumetric prod. BZA ($mg\,L^{-1}\,h^{-1}$)	115.0	228.0	177.4 (245.9)[a]
Total BZA removal by pervap. (%)	–	53.6	83.5
Total BA removal by pervap. (%)	–	3.8	3.3

[a] Closest time point to the end of control pervaporation run for comparison purposes.

245.9 $mg\,L^{-1}\,h^{-1}$ for high gas flow rate, which is a 198% and 214% improvement in system performance.

5. Conclusion

This study has shown that low BZA and BA concentrations in the presence of *P. pastoris* are required in order to reduce cell inhibition, even though high concentrations of BA are advantageous to maintain a high enzymatic biotransformation rate. It was also shown that product detoxification, and not low AOX and cellular energy source levels, had the most influence on improving the bioconversion reaction rate. By carefully selecting a thermoplastic polymer for high BZA and relatively low BA affinity, high performance pervaporation tubing was prepared by DuPont Canada, and BA/BZA flux was characterized in terms of tubing surface area and permeate gas flow rate, the latter having a greater influence on flux. The use of *in situ* pervaporation for the biotransformation of BZA provided continuous product removal and a significant increase in volumetric productivity over previous studies employing the *P. pastoris* system for BZA bioproduction.

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