Biotechnol Lett (2011) 33:63–70
DOI 10.1007/s10529-010-0408-4

ORIGINAL RESEARCH PAPER

The effects of polymer phase ratio and feeding strategy on solid–liquid TPPBs for the production of L-phenylacetylcarbinol from benzaldehyde using *Candida utilis*

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Received: 24 August 2010 / Accepted: 31 August 2010 / Published online: 29 September 2010

Abstract
Purpose of Work To increase the bioproduction of L-phenylacetylcarbinol (PAC), a precursor molecule in the synthesis of the decongestants ephedrine and pseudoephedrine and which suffers from substrate, product, and by-product inhibition, by ensuring that the delivery of the substrate, benzaldehyde, is maintained within a strict concentration window.

Beads of the commercial polymer, Hytrel G3548L, can act as a sequestering phase to reduce inhibitory effects to cells of *Candida utilis* while creating a reservoir for high concentrations of products. In this work we varied the polymer phase volume ratio (from 3 to 15%), and modified the benzaldehyde feeding strategy to further improve on system performance, resulting in greater than 100% increase in the PAC productivity relative to a single phase control, as well as robust operation of the two-phase bioreactor with minimal operator intervention.

Keywords Benzaldehyde · L-Phenylacetylcarbinol · Polymer beads · Solid–liquid two-phase partitioning bioreactors

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Introduction

L-Phenylacetylcarbinol (PAC), a precursor to the decongestant drugs, L-ephedrine and pseudoephedrine, is commercially produced through microbial biotransformation using yeast with benzaldehyde and glucose as substrates (Shin and Rogers 1995). The transformation relies on the anaerobic accumulation of pyruvate and activation of the enzyme pyruvate decarboxylase (PDC). Other oxidoreductases present during anaerobic respiration catalyze the formation of benzyl alcohol from benzaldehyde, which is the major by-product of the industrial process (Long and Ward 1989; Shin and Rogers 1995). While still maintaining a high stereoselectivity and efficiency compared to chemical synthesis methods, this biotransformation is subject to substrate (benzaldehyde), product (PAC) and by-product (benzyl alcohol) inhibition, making it an interesting system for reactor design studies.

Immobilizing cells and encapsulating *Saccharomyces cerevisiae* to reduce exposure to aqueous benzaldehyde, PAC and benzyl alcohol, was one of the first reactor designs implemented for the PAC system (Mahmoud et al. 1990). Performance of immobilized cells was improved using liquid–liquid two-phase partitioning bioreactors (TPPBs), which reduced the exposure of yeast (*Candida utilis*) to inhibitory compounds by sequestering such species into an immiscible phase (Rosche et al. 2005). This system used octanol as the sequestering phase, which

resulted in an inhibitory effect to the *C. utilis* cells. In order to avoid toxicity problems, Zhang et al. (2008) used biocompatible polyethylene-glycol to create a cloud point system, which exists in two phases when operated at the cloud point temperature. This system was able to produce 8 g PAC/l and 4 g benzyl alcohol/l (Zhang et al. 2008). Recent work by Khan and Daugulis (2010) demonstrated that solid polymer beads could be used as a biocompatible partitioning phase for the PAC production system to achieve overall concentrations of 11 g PAC/l and 4.5 g benzyl alcohol/l.

Now that a demonstration of concept has been provided in our earlier work, the objective of the current study was to examine the effect of varying the polymer to aqueous phase ratio on system performance. The delivery of substrate from the polymer phase was then explored, a novel demonstration in the field of solid–liquid TPPBs for biocatalysis. This reduced manual intervention (i.e. manual feeding of substrate) resulting in simplified operation and a reduced risk of contamination.

Materials and methods

Chemicals and polymers, and medium formulation

The sources of all chemicals used, and the medium formulation employed, were as described by Khan and Daugulis (2010). *Candida utilis* 70940 was purchased from the University of New South Wales Culture Collection (World Directory of Culture Collections No. 248). Cylindrical Hytrel G3548L beads (4 × 3 × 2 mm) were graciously donated by DuPont Canada.

Analytics

HPLC–UV detection (Varian, Prostar, Polaris 5μC18-A 150 × 4.6 mm column) was used to quantify benzaldehyde, benzyl alcohol and PAC using the method described by Rosche et al. (2001). HPLC–refractive index detection (Varian, Prostar, HiPlex H 8 μm 300 × 7.7 mm column at 75°C) was used to quantify glucose and ethanol with a mobile phase of 9 mM H_2SO_4 maintained at 0.4 ml/min.

All cell dry weight measurements were made from OD_{600} values.

Batch reactor operation

Inoculum was prepared by adding 40 μl frozen stock culture to each of six 125 ml shake-flasks containing 50 ml sterile growth medium. Cultures were grown at 30°C and 180 rpm for 36 h (to reach an OD of 2.6) and then added to the sterile bioreactor. A 5 l bioreactor (3 l medium) equipped with pH (6 M KOH) and temperature (30°C) controls was used for all reactor runs (New Brunswick Scientific, BioFlo III). Bioreactor operation began with 16 h growth (300 rpm, 1 vvm aeration), followed by enzyme induction for 18 h (300 rpm, 0.1 vvm air aeration). After enzyme induction, benzaldehyde was added manually to maintain the concentration between 1 and 2 g benzaldehyde/l. This range has been demonstrated to result in the highest PAC productivity, notwithstanding some inhibitory effects.

Two-phase batch reactor operation: varying polymer phase ratio

For experiments varying the polymer to aqueous phase ratio, 100 and 500 g of Hytrel G3548L beads ($\rho = 1.15$) were used to correspond to 3% (86.7 ml polymer/3 l aqueous) and 15% (434.7 ml polymer/3 l aqueous) polymer phase by volume. To stay within the aqueous benzaldehyde concentration feeding window, the polymer beads were preloaded with benzaldehyde: the required mass of beads (100 or 500 g) was added to 3 l of sterile medium, and benzaldehyde was added until the aqueous concentration equilibrated to 1 g benzaldehyde/l. The aim was to buffer the lower limit of the feeding window so that subsequent manual additions of benzaldehyde during the biotransformation period would stay in the aqueous phase rather than being absorbed by the polymer. The beads were removed, dried, and refrigerated. The previously described batch reactor procedure was then followed, including manual benzaldehyde addition, with the beads being added to the reactor at the start of the biotransformation. This operation procedure follows the two-phase batch reactor protocol described in Khan and Daugulis (2010) to allow for comparison.

Feeding strategies

Three feeding strategies were evaluated in this study. The first (Strategy 1) was manual delivery of benzaldehyde with preloaded beads as described above. To minimize operator intervention, feeding of benzaldehyde directly from the beads was explored (Strategies 2 and 3). This required high levels of benzaldehyde to be preloaded into the beads. Because of the limited solubility of benzaldehyde (6.3 g/l in RO water), the aqueous preloading strategy described for the phase ratio tests could not be employed. In Strategy 2, two aliquots of 250 g Hytrel G3548L were each incubated with 40 g pure benzaldehyde to provide higher loading. Once benzaldehyde was absorbed (allowing 6 h incubation time), the beads were sealed in a beaker and refrigerated to avoid losses of benzaldehyde through volatilization. Benzaldehyde addition to the bioreactor started by adding the first 250 g aliquot of beads. When PAC production began to slow, the remaining beads were added for a total of 500 g (15% by volume) of polymer phase. This strategy aimed to delivery benzaldehyde at 1 g/l.

Strategy 3 was designed to increase delivery towards the upper value of the feeding window, 2 g benzaldehyde/l. Strategy 3 preloaded 500 g Hytrel G3548L with 100 g pure benzaldehyde. All 500 g preloaded beads were added to the reactor in a single aliquot to commence the biotransformation.

Product sampling from polymer

Every 2 h over the course of the biotransformation period, two 1 g polymer samples were collected and tested with the extraction technique described by Gao and Daugulis (2009) using methanol. Two methanol washes were used per sample. Additional washes were demonstrated to increase recovery by only approx. 1%.

Results and discussion

Effect of varying polymer phase ratio

The first objective of this study was to examine the effect of varying the polymer phase ratio on system performance. The polymer volumes tested were 3, 9, and 15%, which correspond to phase ratios that have been used in the literature (Gao and Daugulis 2009; Prpich and Daugulis 2007a). Manual feeding to maintain the concentration between 1 and 2 g benzaldehyde/l was used resulting in a "saw-tooth" concentration profile for benzaldehyde. As manual feeding may cause some variation in results, the reproducibility of these experiments was verified using a replicate run of the 9% case (Fig. 1). Figure 1 demonstrates that notwithstanding minor variation, the performance of the replicate system was the same for both PAC and benzyl alcohol. Therefore, no replicate experiments were performed and the data presented in subsequent figures and tables are from single bioreactor experiments.

Figure 2 demonstrates that varying the phase ratio does not have a significant impact on the aqueous concentration profiles, with some variation being attributed to differences in benzaldehyde concentration due to manual feeding.

Analyzing the total mass produced (accounting for the mass present in both phases) allows for a better comparison between systems, as the quantity of reservoir is taken into account. The total mass of PAC in the system at the end of the biotransformation is the highest in the 15% case, which produced 40 g PAC, a 134% increase over the single phase control and an 11% increase over the 9% case.

System performance can also be evaluated in terms of by-product formation (Fig. 3). The formation of by-product is not only undesirable due to the loss of substrate, but in this system, by-product also contributes to inhibition. Figure 3 shows a 26, 34,

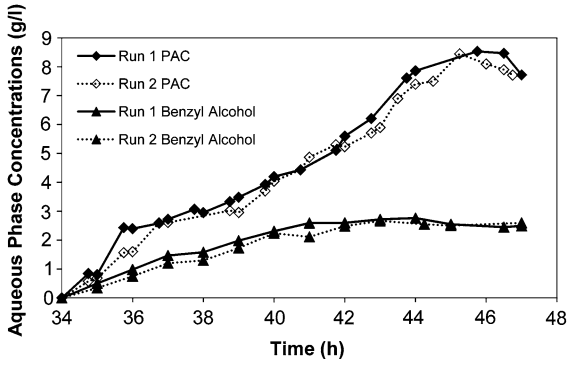

Fig. 1 Replicate aqueous phase concentration profiles for PAC and benzyl alcohol for the 9% case to demonstrate the reproducibility of experiments in this study

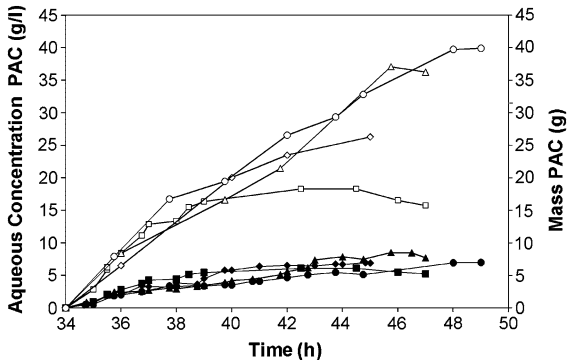

Fig. 2 Aqueous phase concentration and total system mass of PAC for reactors varying phase ratio: *filled square*—single phase concentration, *filled diamond*—3% case aqueous concentration, *filled triangle*—9% case aqueous concentration, *filled circle*—15% case aqueous concentration. *Open symbols* represent the data points for the total mass produced in the corresponding system. Benzaldehyde addition began at 34 h. Single phase and 9% polymer phase data are from Khan and Daugulis (2010)

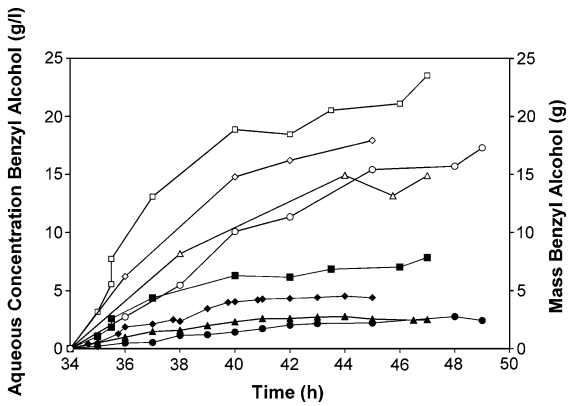

Fig. 3 Aqueous phase concentration and total system mass of benzyl alcohol for reactors varying second phase ratio: *filled square*—single phase concentration, *filled diamond*—3% case aqueous concentration, *filled triangle*—9% case aqueous concentration, *filled circle*—15% case aqueous concentration. *Open symbols* represent the data points for the total mass produced in the corresponding system. Benzaldehyde addition began at 34 h. Single phase and 9% polymer phase data are from Khan and Daugulis (2010)

and 29% decrease in the total mass of benzyl alcohol in the system at the end of the biotransformation for the 3, 9, and 15% cases respectively, when compared to the single phase control. As benzaldehyde concentration was being manually maintained within the feeding window, this change in benzyl alcohol production can largely be attributed to the performance of the TPPB, with minimal contribution arising from biological effects caused by variation in benzaldehyde feeding. This reduction in inhibitory by-product formation is a novel demonstration in the field of TPPBs and appears in two aspects of by-product formation: (1) the final aqueous concentration of benzyl alcohol decreases with higher polymer phase ratios and (2) the total mass of benzyl alcohol produced in the system decreases with higher polymer phase ratios. However, there is no improvement in benzyl alcohol formation in the 15% case over the 9% case. This indicates that the by-product effect does not indefinitely reduce by-product formation. This is likely due to the fact that because yeast are continuously exposed to 1–2 g benzaldehyde/l in the aqueous phase, the enzymes producing benzyl alcohol will always have some minimum access to benzaldehyde. The underlying cause of the by-product effect may be due to the ability of PDC to maintain a higher activity when exposed to lower aqueous concentrations of benzyl alcohol (and PAC). The active oxidoreductases in the system can convert some benzaldehyde in the system to benzyl alcohol, but if PDC activity is high, the system may demonstrate a preference for PAC production.

Analysis of the final system parameters shown in Table 1 expands our understanding of the effect of varying the phase ratio on system productivity. The overall system concentrations shown in Table 1 account for the PAC and benzyl alcohol present in both phases. The 3, 9, and 15% cases correspond to 44, 93, and 104% increases in overall PAC concentration and 28, 39, and 38% reductions in overall benzyl alcohol concentration respectively, relative to the single phase control. This further demonstrates the improvement made to the system with respect to both final PAC and final benzyl alcohol concentrations.

An interesting expansion to current knowledge in the field of TPPBs is the increase in molar yield of PAC with respect to benzaldehyde with increasing phase ratio. Recent literature in the field of liquid–liquid TPPBs demonstrated a 29% increase in molar yield of PAC on benzaldehyde with a 50:50 ratio of organic solvent to aqueous phase (Rosche et al. 2005). When solid–liquid TPPBs were first investigated for PAC production, a 9% increase in molar yield was observed with a 9% phase ratio. This improvement was assumed to be less than the liquid–liquid system due to the lower phase ratio (Khan and

Table 1 System parameters for reactors of varying polymer phase ratio

	Single phase[a]	3%	9%[a]	15%
Time to completion (h)	8	10	13	14
Cell density (g biomass/l)	13.4	13.4	13.6	12.1
Aqueous PAC end point (g PAC/l)	5.7	6.6	7.6	7.0
Aqueous BOH end point (g BOH/l)	7.4	4.5	2.5	2.6
Polymer PAC (g PAC/l polymer)	–	65.1[b] (60.5, 69.7)	50.5[b] (46.6, 54.4)	43.7[b] (42, 45.3)
Polymer BOH (g BOH/l polymer)	–	34.1[b] (31.1, 37.2)	28.5[b] (25.5, 31.5)	18.8[b] (15, 22.6)
Overall PAC (g PAC/l)	5.7	8.2	11.0	11.6
Overall BOH (g BOH/l)	7.4	5.3	4.5	4.6
$Y_{PAC/BZA}$ (mol/mol consumed)	0.34	0.39	0.37	0.51
$Y_{BOH/BZA}$ (mol/mol consumed)	0.61	0.35	0.21	0.28
PAC mass productivity (g PAC/h)	2.14	2.53	2.76	2.85
Benzaldehyde partition coefficient	–	31	39	38
Benzyl alcohol partition coefficient	–	7.4	11.4	7.8
PAC partition coefficient	–	9.8	7.1	6.2

[a] Data from Khan and Daugulis (2010)

[b] Average value of the 2 × 1 g random polymer samples shown in parentheses

PAC L-phenylacetylcarbinol, *BOH* benzyl alcohol

Daugulis 2010). However, this work demonstrates a 50% increase in molar yield of PAC for the 15% case. This significant improvement over recent literature is most reasonably caused by the ability for polymer beads to retain benzaldehyde more effectively than organic solvents, reducing losses to volatilization or oxidation.

Increasing the polymer phase ratio appears to have increased the lifespan of the biotransformation (Table 1). This is likely due to the polymer phase lowering the aqueous concentration, reducing system inhibition. The mass productivities of the 3, 9, and 15% cases demonstrated an 18, 29, and 33% increase in productivity over the single phase performance. With the increased lifespan of the 15% case, more PAC was produced, resulting in a significant improvement in system productivity compared to the single phase, as well as the lower phase ratio TPPBs. This is analogous to observations made in liquid–liquid systems of varying phase ratio, for up to 50% by volume (Prpich and Daugulis 2007b). However, it should be noted that achieving a 50% polymer phase would be difficult due to reductions in mixing caused by the accumulation of polymer beads behind reactor internals (Boudreau and Daugulis 2006).

As polymers sequester PAC with a high affinity, the PAC concentration in the polymers is substantially higher than in the aqueous phase, and depends on the value of the partition coefficient. The partition coefficients of the target compounds toward the polymer (the ratios of polymer concentration to aqueous concentration) are listed in Table 1. There is some variation between the partition coefficients in Table 1 and those previously reported, which were 39, 11, and 7 for benzaldehyde, benzyl alcohol, and PAC respectively (Khan and Daugulis 2010). As the same polymer was used as the sequestering phase in both experiments, the most likely cause of this variation is changes to the medium composition during biotransformation. The effect of medium composition on partition coefficients is an area of work currently being investigated.

With respect to overall PAC concentration, all three TPPBs investigated were able to surpass the performance of the recent work using a cloud point system for PAC production, which obtained 8 g PAC/l (Zhang et al. 2008). However, the Zhang et al. (2008) system produced only 4 g benzyl alcohol/l. Considering the best performing TPPB from the current work, the 15% case, the benzyl alcohol produced was higher, reaching a total concentration

of 4.6 g benzyl alcohol/l. However, it is important to note that this can still be considered an overall improvement, as the ratio of total product to by-product concentrations is 2.5:1, while Zhang et al. (2008) achieved a ratio of 2:1. Therefore, the 15% phase ratio TPPB was able to provide better selectivity for PAC production over by-product formation compared to the recent literature.

Effect of feeding strategy

A delicate balance exists in the PAC production system between preferential formation of the by-product benzyl alcohol at low benzaldehyde concentrations and inhibition of the biocatalyst at high benzaldehyde concentrations (Shin and Rogers 1995). Maintaining benzaldehyde inside the 1–2 g/l feeding window normally requires frequent operator intervention, which not only complicates operation but leads to potential sources of error and contamination. Delivery of benzaldehyde from the sequestering phase of a TPPB for the PAC system has not been demonstrated for liquid–liquid TPPBs, likely due to the more complicated nature of work with organic solvents. The sequestering phase must be loaded, recovered from the loading procedure, and stored, which would be more difficult when working with hazardous liquids, such as octanol, rather than inert polymer beads. This study explored the possibility of preloading polymer beads to deliver benzaldehyde concentrations within the optimum feeding window.

Three feeding strategies are compared in this section: Strategy (1) manual feeding (corresponding to the 15% phase ratio case previously described), Strategy (2) delivery of 80 g benzaldehyde separated into two bolus additions of beads, and Strategy (3) delivery of 100 g benzaldehyde in one addition of beads. For Strategies 2 and 3, 500 g (15% polymer phase by volume) of Hytrel G3548L were preloaded with benzaldehyde. The amount to be loaded was determined to account for the 1 g benzaldehyde/l aqueous phase concentrations required at the end of the biotransformation as well as the mass of benzaldehyde that was likely to be consumed (based on the results of the 15% case used as Strategy 1), with some additional benzaldehyde to account for any unexpected losses, such as volatilization. However, with a benzaldehyde partition coefficient of approximately 38, the 80 g loaded in Strategy 2 could have resulted in a theoretical aqueous concentration of 4.8 g benzaldehyde/l, which is significantly above the feeding window. Therefore, we added the polymers to the reactor in two 250 g additions. The first addition of 250 g of polymer (containing 40 g total benzaldehyde) was used to begin the biotransformation. The second addition of beads was done when PAC production began to plateau.

When it was determined that Strategy 2 did not reach the feeding window, remaining around 0.5 g benzaldehyde/l during the biotransformation, the third feeding strategy was designed. Beads were added to the reactor in one addition, and a slightly higher mass of benzaldehyde was loaded (100 g), with the aim being to reach the upper end of the feeding window. The deliveries achieved for the feeding strategies tested in this study are shown in Fig. 4.

From Fig. 4, it is evident that delivery of benzaldehyde in two separate aliquots of beads was ineffective as benzaldehyde concentration was not able to meet the target. This is likely a result of the time required for benzaldehyde to diffuse from the polymer beads into the aqueous phase. Although the theoretical aqueous concentration would be above the inhibitory window, the yeast began to transform benzaldehyde as soon as it was present in the aqueous phase, resulting in a decrease in aqueous phase concentration.

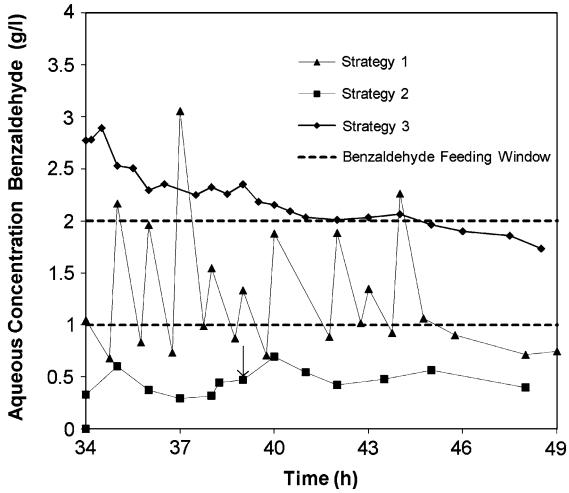

Fig. 4 Aqueous benzaldehyde concentrations for three delivery strategies. The *arrow* represents the addition of the second aliquot of polymer beads for Strategy 2

The objective of Strategy 3 was to deliver benzaldehyde towards the higher concentrations of the target window. With 100 g of benzaldehyde loaded in the polymer, the theoretical aqueous concentration would be 6 g benzaldehyde/l (based on a partition coefficient of 38). For this strategy, the delivery rate was first tested abiotically (Fig. 5). Figure 5 demonstrates that benzaldehyde concentration increased to 2.7 g/l in approximately 20 min and then remained constant. The theoretical 6 g benzaldehyde/l was not reached. Polymer release work by Rehmann et al. (2007) demonstrated that polymers can deliver only to the aqueous phase solubility limit (Rehmann et al. 2007), which from Fig. 5 observed to be 2.7 g benzaldehyde/l at 30°C (compared to 6.3 g/l, experimentally determined in RO water in this study). As this is only 35% above the target of 2 g benzaldehyde/l, the release profile for Strategy 2 was encouraging in that delivery of benzaldehyde may be met with consumption by the microbes to avoid surpassing the feeding window. The resulting release profile from Fig. 4 showed that microbial activity was not able to reduce the benzaldehyde concentration to within the feeding window until 6 h into the biotransformation. As a result, the yeast were exposed to higher than desired concentrations of benzaldehyde for more than half of the biotransformation. The impact of delivery strategy on system performance is shown in Table 2.

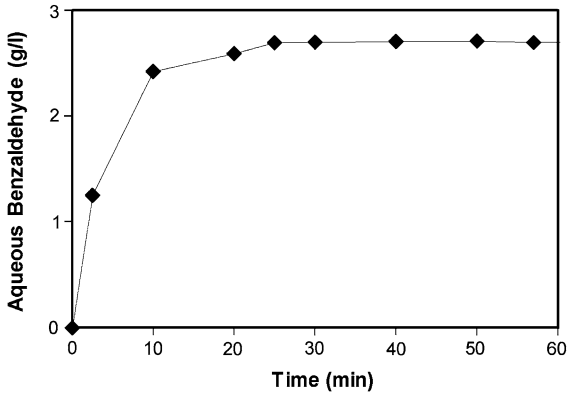

Fig. 5 Abiotic release profile for 100 g benzaldehyde loaded into 500 g Hytrel G3548L (15% phase ratio by volume) and added to 3 l aqueous medium

Table 2 shows Strategy 2 not only had a 31% decrease in overall final PAC concentration, but also demonstrated a 19.5% increase in overall final benzyl alcohol concentration compared to manual feeding. While Strategy 3 provided a 26% reduction in PAC concentration, a 19.5% decrease in benzyl alcohol was observed. This is likely due to the metabolic preference for benzyl alcohol formation at lower benzaldehyde concentrations (Shin and Rogers 1995). It is interesting to note that while Strategies 2 and 3 demonstrated a lower yield of PAC on benzaldehyde,

Table 2 Final system parameters testing benzaldehyde delivery strategy: manual feeding (Strategy 1), delivery of 80 g benzaldehyde from polymers (Strategy 2) and delivery of 100 g benzaldehyde (Strategy 3)

	Single phase[a]	15% Strategy 1[b]	15% Strategy 2	15% Strategy 3
Time to completion (h)	8	14	10	11
Cell density (g biomass/l)	13.4	13.6	12.2	13.0
Aqueous PAC end point (g PAC/l)	5.7	7.0	4.5	4.8
Aqueous BOH end point (g BOH/l)	7.4	2.6	2.4	1.8
Polymer PAC (g PAC/l polymer)	–	44[c] (42,45.3)	32.0[c] (30.5,33)	35[c] (32.9,37.1)
Polymer BOH (g BOH/l polymer)	–	18.8[c] (15,22.6)	27.1[c] (25.1,29.2)	17[c] (16.2,17.3)
Overall PAC (g PAC/l)	5.7	11.6	8.0	8.6
Overall BOH (g BOH/l)	7.4	4.6	5.5	3.7
$Y_{PAC/BZA}$ (mol)	0.34	0.51	0.32	0.32
$Y_{BOH/BZA}$ (mol)	0.61	0.28	0.32	0.19
PAC mass productivity (g/h)	2.14	2.93	2.75	2.69

[a] Data from Khan and Daugulis (2010)

[b] Data from Table 1

[c] Average value of the 2 × 1 g random polymer samples shown in parentheses

PAC L-phenylacetylcarbinol, *BOH* benzyl alcohol

Strategy 3 was able to maintain the selectivity for PAC over benzyl alcohol that was demonstrated with manual feeding. Manual feeding demonstrated a PAC to benzyl alcohol yield ratio of 1.8:1, while Strategy 3 demonstrated a ratio of 1.7:1.

Strategies 2 and 3 had a decreased time required for biotransformation compared to Strategy 1, as listed in Table 2. Strategy 2 is likely stopped by benzyl alcohol inhibition, while Strategy 3 is likely suffering from benzaldehyde inhibition. Despite the overall lower PAC concentrations observed with benzaldehyde delivery from the beads, when evaluated using mass productivity, Strategies 2 and 3 demonstrated only a modest decrease (a 6 and 8%) compared to manual feeding. This could be used to improve commercial applications by increasing operational simplicity.

Conclusions and future work

The phase ratio tests in this work not only improved the PAC system with respect to productivity, but also demonstrated a reduction in by-product formation through the use of solid–liquid TPPBs. Also novel to the field of TPPBs for biosynthesis is the delivery of substrate from the sequestering phase. The ability to deliver substrate from polymer beads adds to the advantages in the use of solid–liquid over liquid–liquid TPPBs.

The partitioning of target molecules in this study showed some variation from literature values reported for the same polymer phase. Therefore, current work with solid–liquid TPPBs is aimed at further investigating the effect of medium composition on the extent of partitioning of target compounds. It would also be of interest to examine other biocatalysis systems with inhibitory by-products in a solid–liquid TPPB to expand on the observed decrease in by-product formation.

Acknowledgments The authors would like to thank the Natural Sciences and Engineering Research Council of Canada and DuPont Canada for financial support.

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