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A Novel Solid–Liquid Two-Phase Partitioning Bioreactor for the Enhanced Bioproduction of 3-Methylcatechol

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ABSTRACT: The bioproduction of 3-methylcatechol from toluene via *Pseudomonas putida* MC2 was performed in a solid–liquid two-phase partitioning bioreactor with the intent of increasing yield and productivity over a single-phase system. The solid phase consisted of HYTREL™, a thermoplastic polymer that was shown to possess superior affinity for the inhibitory 3-methylcatechol compared to other candidate polymers as well as a number of immiscible organic solvents. Operation of a solid–liquid biotransformation utilizing a 10% (w/w) solid (polymer beads) to liquid phase ratio resulted in the bioproduction of 3-methylcatechol at a rate of 350 mg/L-h, which compares favorably to the single phase productivity of 128 mg/L-h. . HYTREL™ polymer beads were also reconstituted into polymer sheets, which were placed around the interior circumference of the bioreactor and successfully removed 3-methylcatechol from solution resulting in a rate of 3-methylcatechol production of 343 mg/L-h. Finally, a continuous biotransformation was performed in which culture medium was circulated upwards through an external extraction column containing HYTREL™ beads. The design maintained sub lethal concentrations of 3-methylcatechol within the bioreactor by absorbing produced 3-methylcatechol into the polymer beads. As 3-methylcatechol concentrations in the aqueous phase approached 500 mg/L the extraction column was replaced (twice) with a fresh column and the process was continued representing a simple and effective approach for the continuous bioproduction of 3-methylcatechol. Recovery of 3-methylcatechol from HYTREL™ was also achieved by bead desorption into methanol.

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KEYWORDS: biotransformation; 3-methylcatechol; in situ recovery; solid/liquid two-phase system; product toxicity

Introduction

Bioproduction of substituted catechols via whole cell biocatalysis has been shown to be susceptible to inhibition associated with the substrate and/or product (Held et al., 1999b; Meyer et al., 2003). Toxic effects have resulted in diminished yields and low productivity and therefore to enhance performance these systems often require the partitioning of toxic compounds away from the biocatalyst. The two most commonly employed systems for the partitioning of toxic compounds included biphasic systems employing an immiscible organic liquid or solid adsorbent (Held et al., 1999b; Husken et al., 2001b; Tao et al., 2005; Wery et al., 2000).

Immiscible organic solvents have been successfully employed in biphasic systems for the removal of toxic catechols from solution (Husken et al., 2001b; Rojas et al., 2004). In these systems, the presence of organic solvents allows for increased yields as well as higher rates of productivity when compared to single-phase systems. A limitation often associated with these systems is the possible toxicity of the organic solvent towards the biocatalyst, which may affect the viability and/or metabolic activity of the biocatalyst. This has been shown to result in lengthy lag phases as well as premature termination of the bioprocess possibly due to the cumulative toxic effect of both product and solvent (Husken et al., 2001b). Attempts to avoid solvent toxicity have included the identification and design of solvent tolerant biocatalysts (Neumann et al., 2005; Rojas et al., 2004; Wery et al., 2000). Additionally, physical barriers have been implemented in an attempt to separate organic solvent from the biocatalyst. Examples include the use of hollow fiber membrane technology (Husken et al., 2002) as well as solvents immobilized within a polymer matrix (Serp et al., 2003). These systems have demonstrated modest increases in bioproduction, however, due to the ability of organic solvents to leach across the polymeric membrane the systems still suffer from toxicity-related inhibition.

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The phenomenon of solvent toxicity as well as the parameters governing the design of non-toxic biphasic systems is well understood (Daugulis et al., 1987; Inoue and Horikoshi, 1991; Laane et al., 1987; Schmid et al., 1998). To this end, selective screening processes for the effective employment of non-toxic solvents in biphasic systems have been developed and utilized with successful results (Prpich and Daugulis, 2006b).

Solid adsorbents, such as polymeric resins (Amberlite™ XAD) or activated carbon, represent an alternative to organic solvents in biphasic systems (Held et al., 1999a,b; Lynch et al., 1997). These materials have been shown to successfully remove toxic catechols from solution thus resulting in higher overall yields and productivities. A limitation of these materials may be the non-specific adsorption of organic compounds from solution. This results in the removal of both product and substrate alike leading to decreased product removal efficiencies as well as increased substrate loadings to compensate for sorbed materials. In addition, polymeric resins, due to their porous and highly cross linked nature, are fragile and therefore are often not able to withstand the vigorous agitation that accompanies mechanically agitated systems. Separating the absorbent materials from the hostile environment of the bioreactor by way of external extraction columns has been shown to be an effective strategy (Held et al., 1999b).

An alternative to both immiscible organic solvents and solid-phase adsorbents may be thermoplastic polymers. Uptake of small molecules into thermoplastic polymers is analogous to that of uptake into organic solvents, which operate on an absorption mechanism, while solid adsorbents operate on a surface adsorption phenomenon. These polymers have been successfully used to partition and deliver toxic organic molecules in biphasic systems, specifically for the biodegradation of phenols (Amsden et al., 2003; Prpich and Daugulis, 2004, 2006a). The object of this work was to develop an integrated fermentation/recovery system for the bioproduction of 3-methylcatechol (3MC). A thermoplastic polymer was used to recover toxic product from the cell broth and the study investigated different operation strategies demonstrating the flexibility of the solid–liquid system.

Materials and Methods

Chemicals and Microorganisms

All chemicals used in the study were of reagent grade and were purchased from Fisher Scientific (Ottawa, ON, Canada). The polymers used were received from Bayer Material Science (Pittsburgh, PA; Desmopan™ 9370A), Dupont, Kingston, ON, Canada (HYTREL™ 8206, EVLVAX 360, Nylon 6), Kraton, Houston, TX (styrene butadiene copolymer, 28% w/w styrene) and Dow Chemical Canada, Inc., Sarnia, ON, Canada (TONE™ P-787). HYTREL™ 8206, an experimental blend, is a polyether ester thermoplastic (PEEP) that consists of cylindrical shaped pellets approximately 3.5 mm long with a maximum diameter of 4 mm. The melting point of the polymer is 180°C, the glass transition temperature is −60°C and the density is 1.17 g/cm^3. The equilibrium moisture content of this particular family of HYTREL™ was 1% w/w (Dupont personal communication).

Pseudomonas putida MC2, a recombinant strain designed specifically to overproduce 3MC, was the biocatalyst used in the study. The construction of *P. putida* MC2 has been previously described (Husken et al., 2001a) and was received as a gift from the laboratory of Professor J Tramper.

Culture Conditions

P. putida MC2 cultures were cultivated from frozen stock as described previously (Prpich and Daugulis, 2006b). Cultures were incubated for 18 h at 30°C after which time cells were centrifuged at 2,370*g*, supernatant was removed and the cell pellet was harvested and re-suspended in 50 mL of fresh mineral salts medium.

Analytics

Cell concentrations were measured at 650 nm with a Biocrom Ultraspec 3000 UV/Vis spectrophotometer, and a calibration curve was prepared to convert optical density into cell dry weight (CDW). Analysis of 3MC concentrations were performed using the Arnow (1937) Method, a colorimetric test described previously. Gaseous toluene concentrations were measured by gas chromatograph using a method described previously (Prpich and Daugulis, 2006b). Analysis of glucose concentration was performed using the method described by (Miller, 1959). Sample preparation required removal of 3MC and the method used has been described previously (Prpich and Daugulis, 2006b).

Measurement of 3MC Partitioning Into Polymer Beads

Four grams of polymer beads were added to 125 mL Erlenmeyer shake flasks containing 50 mL of mineral salt medium and a predetermined concentration of 3MC ranging from 0 to 3,000 mg/L. The shake flasks were sealed with rubber stoppers, agitated at 180 rpm and maintained at 30°C for a period of 24 h after which time aqueous samples were analyzed for the presence of 3MC. A mass balance on the system was performed whereby initial and final 3MC concentrations were used to determine the mass of 3MC absorbed into the polymer beads. A control containing 3MC and no polymer beads was used to show that 3MC did not physically degrade over the 24 h period, and that any disappearance could be attributed to absorption.

The partition coefficient for 3MC between aqueous and polymeric phases was determined using varying initial 3MC concentrations and by letting each system attain equilibrium

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over the course of 24 h. Correlating the mass of 3MC absorbed into the polymeric matrix to the mass of 3MC remaining in the aqueous phase yielded a linear relationship and regression of the plotted data generated the value of the partition coefficient.

Single-Phase Control Biotransformation

A single–single phase biotransformation was performed to provide a comparison to biphasic operations. The biotransformation was performed in a 5 L Bioflo III bioreactor (New Brunswick Scientific, Edison, NJ). The bioreactor contained a working volume of 3 L of mineral salts medium, and 8 g/L glucose. Prior to inoculation the system was sterilized for 45 min at 121°C.

The bioreactor was maintained at a pH of 6.0, a temperature of 30°C and was agitated at a rate of 600 rpm. Toluene was introduced to the system via the air inlet stream. The toluene air stream was created by sparging air through pure toluene (contained in a flask) and then mixing the toluene vapor with a fresh air inlet stream. Toluene loading rates were maintained at approximately 500 mg/L-h. Prior to inoculation toluene was allowed to achieve equilibrium between the aqueous and air phases after which time the system was inoculated with 75 mg CDW/L. Biomass, 3MC and toluene concentrations were measured periodically throughout the duration of the experiment as described previously. Dissolved oxygen (DO) levels were monitored using a polarographic DO probe (Broadley James Corp, Irvine, CA) coupled with a data acquisition system. DO levels were maintained above 20% saturation in order to prevent an oxygen limited environment.

Solid–Liquid Two-Phase Biotransformation Using Polymer Beads

Solid–liquid biotransformations were performed in an identical manner as previously described for single-phase operation, apart from the addition of 300 g of HYTREL^{TM} beads. The polymers were added prior to sterilization and were autoclaved together with the reactor medium. Desorption tests were performed on the polymer matrices upon completion of the biotransformation. A mass of polymer was placed in 125 mL Erlenmeyer flasks containing 50 mL of mineral salts medium. The flasks were sealed and agitated at 180 rpm and maintained at 30°C for a period of 24 h after which time 3MC levels were quantified in the aqueous phase. The aqueous volume was removed and replaced with fresh minimal medium that was placed back in the shaker for 24 h. This process was repeated until a negligible mass of 3MC was detected in the aqueous phase. The total mass of 3MC removed from the beads constituted the extractable fraction and was used to determine the partition coefficient of the system during operation.

Solid–Liquid Two-Phase Biotransformation Using Alternative Polymeric Geometry

A solid–liquid biotransformation was performed whereby polymer beads were reconstituted into polymer sheets and placed around the circumference of the bioreactor. The dimensions of the polymer sheets were $200 \times 200 \times 3$ mm. The objective of this study was to demonstrate that polymer beads could be transformed into other useful shapes (reactor internals, for example) while still maintaining their ability to absorb 3MC. Placing raw, fresh polymer beads into a metal mold and then subjecting them to 220°C and 5,000 psi for 15 min formed the polymer sheets. Four polymer sheets were created having a total mass of 441 g. The 3MC biotransformation was performed in an identical fashion as the one described previously. To ensure that the total surface area of the polymer sheets were in contact with aqueous phase the working volume was increased to 3.7 L. Upon completion of the biotransformation a slab of the polymer sheet was desorbed in a 1 L Erlenmeyer shake flask containing 500 mL of mineral salts solution. The shake flask was agitated at 180 rpm and maintained at 30°C for a period of 24 h after which time the an aqueous sample was analyzed for the presence of 3MC. The partition coefficient, or mass of extractable 3MC, was determined using the method described in the previous section during operation.

Continuous Solid–Liquid Two-Phase Biotransformation (Via Recirculation of Media)

A continuous biotransformation was performed whereby reactor medium was circulated through an external extraction column containing HYTREL^{TM} polymer beads which absorbed 3MC thus removing it from the aqueous phase. The system was set up and operated in an identical fashion as that described in the previous section for the solid–liquid batch system.

The extraction column consisted of a glass cylinder with barbed hose fittings on either end. A mass of 250 g of polymer beads was loaded through the top of the column (by way of a removable barbed fitting) and a copper mesh at the bottom of the column prevented beads from exiting the bottom of the column. Medium was circulated through the bottom of the column at a rate of 3 L/h via a peristaltic pump and the effluent was returned to the bioreactor. The retention time of the column was approximately 5 min. Three columns were assembled and sterilized and were used sequentially when the aqueous 3MC concentrations reached 500 mg/L, at which time the column was removed and aseptically replaced with an unloaded, sterile column. Glucose levels were periodically monitored to ensure the presence of this carbon source throughout operation. After removing each column from the extraction circuit, a desorption test (as described previously) was performed on the contents of the extraction column to determined the mass of 3MC absorbed by the polymer beads. The results of this test were used to determine the actual mass of 3MC

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produced by the system as well as to calculate the partition coefficient of 3MC between aqueous and polymeric phases during operation.

Recovery of 3MC From HYTREL™

Upon completion of a biotransformation and subsequent removal of polymers from the system, recovery of the 3MC product was undertaken. Because mineral salts medium, used previously to determine the mass of 3MC absorbed into the polymer beads would not result in low, highly concentrated extractant volumes, methanol, a more favorable organic extractant was examined. 3MC was recovered from HYTREL™ by desorbing the polymer beads into methanol using a method identical to that used to determine the partition coefficient of 3MC between HYTREL™ and water. Arnow's method was used to measure 3MC in methanol.

Results

Selective Screening of Candidate Polymers

A pre-screening of candidate polymers from those available in our laboratory, resulted in the selection of six polymers for further study. Of the six polymers five were selected with the expectation that they would demonstrate an affinity for 3MC while one, styrene butadiene copolymer (SBS), was chosen to demonstrate a polymer possessing little or no affinity for 3MC. Hydrogen bond interactions have been hypothesized to play an important role in the uptake of 3MC by immiscible organic solvents (Leon et al., 1998). Based on this premise, it was expected that polymers possessing the functionality capable of participating in hydrogen bond interactions would demonstrate an affinity for 3MC. Table I contains a list of the candidate polymers and gives both the chemical and commercial names of each product.

Of the six candidate polymers selected five possessed the appropriate functionality (i.e., ester, amine or hydroxyl groups) while SBS did not. The results of the experiments to determine the partition coefficient of 3MC between aqueous and polymeric phases are given in Table I and as expected the candidate polymers possessing the appropriate functionality demonstrated some degree of affinity for 3MC while SBS, devoid of hydrogen bonding potential, did not. The justification for including SBS, a potentially negative result, was to demonstrate that not all polymers are effective for this application and a rational selection strategy for polymer selection is required. Based on the success of HYTREL™, a poly(ether-ester) block copolymer, to absorb 3MC it was chosen as the polymeric phase use in all two-phase biocatalytic experiments.

Table I. List of candidate polymers and partition coefficients for 3MC between aqueous and respective polymeric phases. Experiments carried out at 30°C.

Polymeric material	Partition coefficient K
Polyether-ester (HYTREL™)	58
Polyurethane (Desmopan™)	28
Polycaprolactone (TONE™787)	12
Ethylene vinylacetate (ELVAX™)	2.5
Polycaprolactam (Nylon 6)	2
Styrene butadiene styrene copolymer (SBS)	0

95% Confidence Interval = ± 8%.

Solid–Liquid Two-Phase Biotransformation With Polymer Beads

Prior to solid–liquid operation, a single-phase biotransformation was performed to illustrate the performance achievable in single-phase operation and to characterize the effects of 3MC toxicity. The results of the biotransformation are shown in Figure 1. From the results it is apparent that bioproduction ends once 3MC concentrations reach approximately 1,000 mg/L. Furthermore, 3MC was observed to inhibit growth of *P. putida* MC2 at levels of approximately 500 mg/L. The single-phase system produced 2,895 mg of 3MC at a rate of 128 mg/L-h.

A solid–liquid two-phase biotransformation was performed with HYTREL™ polymer beads as the immiscible phase. The biphasic system employed a phase volume ratio of 10% (w/w), which is identical to the phase ratios used in previously operated liquid-liquid systems (Husken et al., 2001b; Prpich and Daugulis, 2006b). From the results (Fig. 2), 3MC began to accumulate in the aqueous phase immediately upon introduction of the biocatalyst. As 3MC concentrations increased in the aqueous phase it was assumed that 3MC was partitioning into the polymer

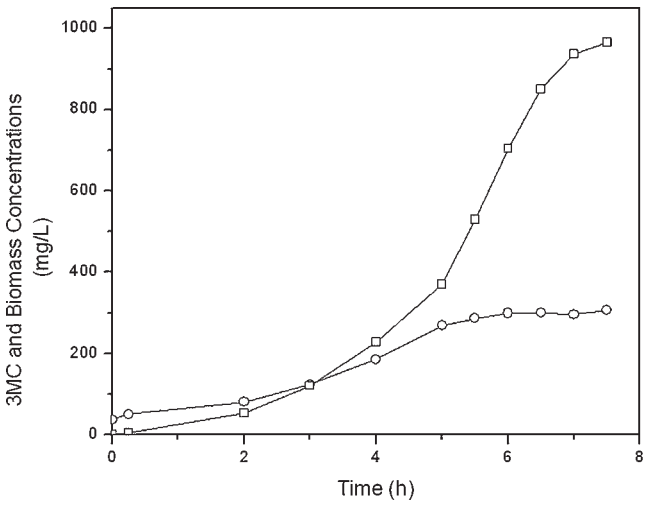

Figure 1. Single-phase bioproduction of 3MC. Squares represent 3MC concentration and circles represent biomass.

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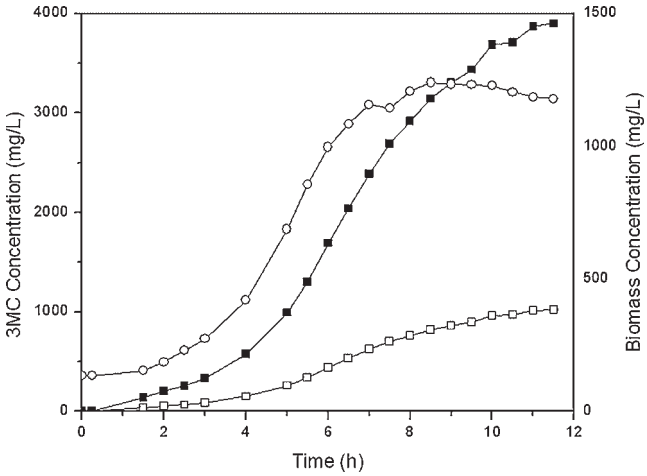

Figure 2. Bioproduction of 3MC in a two-phase bioreactor employing HYTREL™ polymer beads, in a 10% (w/w) phase ratio, as the immiscible phase. Circles represent biomass, hollow squares represent aqueous 3MC concentrations, and solid squares represent the total 3MC concentration in the system.

beads at a concentration corresponding to the previously determined partition coefficient (Table I). Biomass concentrations increased exponentially until $t = 7$ h at which time growth of the biocatalyst stopped corresponding to an aqueous 3MC concentration of approximately 533 mg/L. This phenomenon was expected as previous studies have observed a similar inhibitory behavior once aqueous 3MC concentrations achieved levels of approximately 500 mg/L (Husken et al., 2002; Prpich and Daugulis, 2006b). Production of 3MC continued until $t = 11$ h at which time aqueous 3MC concentrations reached lethal levels (1,020 mg/L) and production ceased. Polymer desorption tests confirmed a 3MC concentration of 32 mg/g in the beads corresponding to a partition coefficient of 44 during operation and a volumetric productivity of 353 mg 3MC/L-h. The final overall concentration of 3MC in the bioreactor was 3,888 mg/L. This value was determined based on the total mass of 3MC in both the polymer and aqueous phases divided by the total volume (liquid + solid) of the system.

Upon completion of the biotransformation (circa $t = 12$ h) the polymer beads were removed, desorbed of all 3MC and then reused. The replicate biotransformation (data not shown) mirrored the first biotransformation in terms of mass of 3MC produced and productivity thus demonstrating the re-usability of the polymer beads. In addition, polymer beads were inspected at the conclusion of each biotransformation for the formation of biofilms. Biofilm formation was not observed and this is in agreement with previous studies (Amsden et al., 2003).

Solid–Liquid Two-Phase Biotransformation With Alternative Geometries

A demonstration of concept was performed that took advantage of HYTREL™'s thermoplastic nature whereby HYTREL™ beads were melted and re-formed into polymer sheets. The polymer sheets were then placed around the inner circumference of the bioreactor and the aim of this demonstration was to highlight the versatility of solid–liquid TPPB whereby the bioreactor walls' were constructed of HYTREL™.

The results of the biotransformation that employed polymer sheets are given in Table II. The biotransformation performed in an identical manner as the previous batch biotransformation with polymer beads. Product accumulated in the aqueous phase immediately upon introduction of the biocatalyst, growth of the biocatalyst continued until approximately $t = 7$ h when aqueous concentration of 3MC reached 550 mg/L and 3MC production continued until $t = 9.5$ h at which time 3MC levels became lethal and bioproduction ended. It was suspected that due to the short operational time of the biotransformation ($t = 9.5$ h rather than $t = 12$ h), mass transfer of 3MC into the polymer sheets may have limited the system by not being able to remove 3MC from the aqueous phase as quickly as it was being produced. The system was maintained until $t = 26$ h to ensure that the aqueous phase and polymer sheets had reached equilibrium, at which time a portion of the polymer sheet was removed from the bioreactor and desorbed in mineral salts medium. The partition coefficient of 3MC between HYTREL™ and aqueous medium was found to be 47 and the overall 3MC concentration of the system was 3,264 mg/L (Table II).

As it was suspected that 3MC toxicity became lethal at $t = 9.5$ h, when the aqueous concentration has reached lethal levels, it is reasonable to assume that no more 3MC was produced after this time. This assumption is well supported by previous single-phase operations (data not shown) as well as a single-phase toxicity experiment described later, which show that the system is not able to regain the ability to synthesis 3MC even if 3MC concentrations are reduced to sub-lethal levels. Based on this assumption, the overall concentration of 3MC in the system at $t = 26$ (3MC in both aqueous and polymeric phases) would be equivalent to the overall concentration of 3MC in the system at $t = 9.5$ h. The transfer of 3MC, from the aqueous to the polymeric phase, may therefore explain the change in 3MC aqueous

Table II. Performance data for the bioproduction of 3MC in the three studied modes of biphasic operation.

Biotransformations	Partition coefficient (K)	3MC produced (mg)	Productivity (mg/L-h)
Single phase	—	2,895	128
Batch mode—beads	44	12,636	353
Batch mode—beads replicate	42	13,050	350
Batch mode–sheets	47	13,319	344
Continuous operation			
First column	32	5,397	225
Second column	32	3,755	419
Third column	32	3,629	420
Overall	32	12,781	270

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concentrations over the period of $t = 9.5$ to $t = 26$ h. We are unable to account for the distribution of 3MC between the aqueous and polymeric phases prior to $t = 9.5$ h, as the system was likely not in equilibrium, however, based on the known final overall 3MC concentration, we were able to provide a suggested concentration profile of 3MC in the polymeric phase between $t = 9.5$ h and $t = 26$ h by performing a mass balance on the system (Fig. 3).

Continuous Solid–Liquid Biotransformation (Via Recirculation of Media)

A single-phase toxicity experiment was initially performed (data not shown) to develop a strategy for operation of the continuous system. The objective of this work was to identify the fate of the cells once 3MC levels had reached lethal concentrations (circa 1,000 mg/L) as well as to determine if the cells could be rejuvenated once these 3MC levels were reached. In addition, this work looked to identify an appropriate 3MC concentration for exchange of extraction columns. In the single-phase biotransformation 3MC was allowed to reach lethal concentrations (circa 1,000 mg/L) at which time biocatalytic activity ceased. Polymer beads were added to the bioreactor thus reducing aqueous 3MC concentrations to sub-lethal levels, however the cells were unable to regain biocatalytic activity. Furthermore, from previously operated batch fermentations (Figs. 2 and 3) as well as the literature, deleterious effects due to 3MC toxicity have been observed in systems at concentrations of approximately 500 mg/L (Husken et al., 2001b; Prpich and Daugulis, 2006b). At this level 3MC inhibits growth of *P. putida* MC2 although biocatalytic activity continues. From these findings it was concluded that exchange of the extraction columns should be carried out once aqueous levels of 3MC in the bioreactor reached the growth inhibitory level of 500 mg/L.

The continuous biotransformation was operated using three extraction columns and the results are illustrated in Figure 4. Circulation of medium through the extraction column commenced at $t = 2.75$ h at which time the system was determined to have exited the lag phase. At $t = 7$ h 3MC concentrations in the aqueous medium approached 500 mg/L. The recirculation pump was stopped, the extraction column was removed and aseptically exchanged with a fresh column, the pump was re-started and bioproduction was allowed to continue. Immediately upon exchange of the column the system experienced a decrease in 3MC concentration from 550 mg/L to 490 mg/L. 3MC levels appeared to plateau from $t = 7$ to 8.5 h after which time levels began to increase and approach 500 mg/L. The extraction column was again replaced with the last remaining fresh column at $t = 10$ h and behavior similar to the previous column was observed. Production of 3MC continued until $t = 12.5$ h at which time 3MC levels reached the 500 mg/L level and the biotransformation was stopped. Polymer beads removed from the extraction columns were desorbed and the results were used to determine actual partition coefficients, yields and productivity (Table II).

Discussion

3MC has been shown to impart toxic effects on the biocatalyst, *P. putida* MC2, leading to inhibition of growth

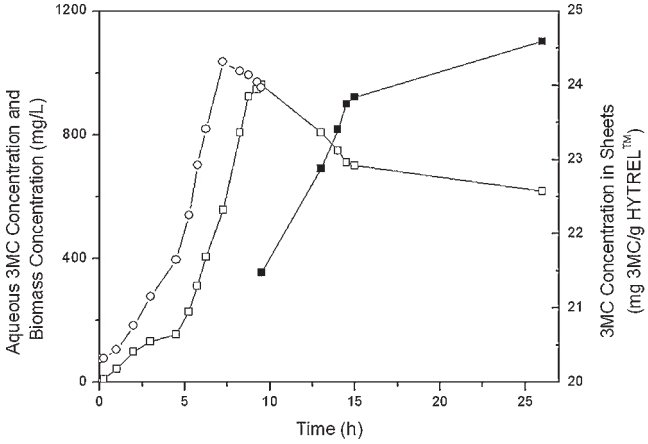

Figure 3. Bioproduction of 3MC in which HYTREL™ polymers constitute the internals of the bioreactor. The final concentration of 3MC in the system was determined by polymer desorption at $t = 26$ h and it was assumed that this mass was unchanged from $t = 9.5$ h. The final overall 3MC concentration was 3,264 mg/L and based from this value a mass balance was performed and a suggested concentration of 3MC in the sheets was determined. Circles represent biomass concentration, hollow squares represent aqueous 3MC concentration and solid squares represent the suggested 3MC concentration in the polymer sheets.

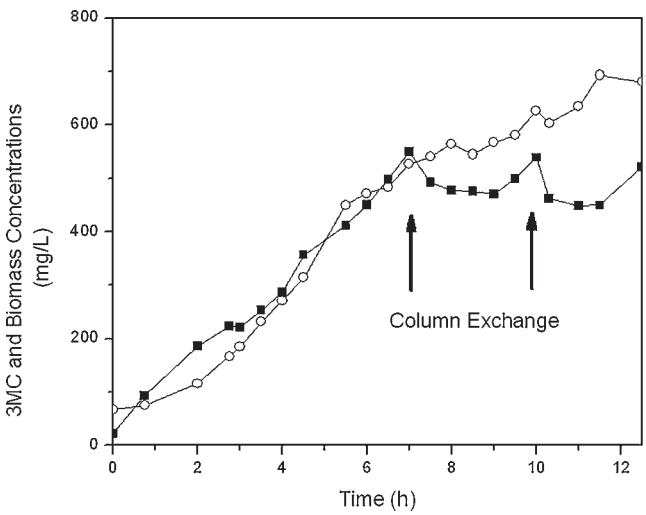

Figure 4. Bioproduction of 3MC in a continuous solid–liquid bioreactor. Circulation of medium through column was initiated at $t = 2.75$ h. Column exchanges occurred at $t = 7$ h and $t = 10$ h. The biotransformation was terminated at $t = 12.5$ h at which time aqueous 3MC concentrations reached 500 mg/L. Circles represent biomass and the squares represent aqueous 3MC concentration.

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followed by biocatalytic inactivity. Initial efforts aimed at alleviating 3MC toxicity employed octanol in a two-liquid phase system, however, due to toxicity of the octanol towards the biocatalyst the system experienced extended lag phases as well as diminished rates of 3MC production (Husken et al., 2001b). We found that by using a biocompatible solvent phase (Prpich and Daugulis, 2006b) solvent toxicity may be avoided thus eliminating lag phase and enhancing 3MC production by a factor of 5. The present research was intended to replace organic solvents completely by employing inert polymeric materials.

Attempts to predict the affinity that a polymer may possess towards a given target solute have been described previously (Prpich and Daugulis, 2004; Rzeszutek and Chow, 1998; Schumack and Chow, 1987). For relatively soluble aromatic compounds, such as phenols, it has been postulated that hydrogen bond interactions play a key role in molecule uptake (Rzeszutek and Chow, 1998). Based on this premise it would be expected that 3MC, a more soluble compound than phenol, possessing two hydroxyl groups, would perform in a similar manner. Therefore we predicted that candidate polymers for the uptake of 3MC must possess the molecular functionality to participate in hydrogen bond interactions. Organic solvents possessing ester functionality, and thus hydrogen bonding potential, have been shown to absorb 3MC from solution (Prpich and Daugulis, 2006b). In addition to molecular structure, physical characteristics, such as polymeric chain mobility have also been shown to affect uptake of small organic molecules (Hoshi et al., 1999). It was predicted that HYTREL^{TM}, a polyether-ester thermoplastic, would demonstrate superior uptake of 3MC as it possessed the appropriate molecular functionality (ester groups) and desired chain mobility (low glass transition temperature). In contrast, SBS copolymer was selected to represent a polymer incapable of 3MC uptake. Although SBS possesses sufficient chain mobility it does not have hydrogen bonding potential hence its inability to absorb 3MC from solution. Inclusion of SBS in the selective screening process strengthens the argument for the requirement of hydrogen bond interaction potential and the necessity of a rational strategy for polymer selection. Partition data between aqueous and polymeric phases (Table I) confirm our predictions.

The uptake of 3MC into the polymer matrix is analogous to the absorption of 3MC into organic solvents and results in a linear partition coefficient over the operational range of the system. The abiotic partition coefficient of 3MC in HYTREL^{TM} was found to be 58, which is far higher than those of organic solvents such as octanol and bis(2-ethylhexyl) sebacate which possess partition coefficients for 3MC of 22 and 9 respectively. The much greater capacity for 3MC in HYTREL^{TM} compared to organic solvents, in combination with the relatively inert nature of the polymer, leads to enhanced production of 3MC in a solid–liquid system. Operation of a batch biotransformation employing HYTREL^{TM} at a 10% phase ratio resulted in an overall productivity of 353 mg/L-h and a final 3MC yield of 12,636 mg. These results represent a 60% increase in productivity over that of a similarly operated system employing an identical phase ratio of bis(2-ethylhexyl) sebacate (Prpich and Daugulis, 2006b) and a substantial increase of the use of a moderately toxic liquid extractant (octanol) (Husken et al., 2001b). For the purpose of this study overall volumetric productivity was used to describe the performance of the system. It could be argued that specific rates would offer an alternative description of performance, however, specific rates of the examined systems would be affected by the concentrations of 3MC to which the cells were exposed, and the times of exposure, which also varied between the various contacting schemes. In addition, the effects of 3MC on biocatalytic activity and cell growth could be different, making estimation of specific product formation rates challenging. Nevertheless, such an investigation could provide useful insights into the microbial aspects of these systems.

Desorption of 3MC from the beads showed the partition coefficient of 3MC during operation to be 44. This value is less than the abiotic partition coefficient of 58 and may be a result of the biotic system not being permitted the sufficient time to reach equilibrium.

The use of solid polymer absorbents also improved handling and re-use. Separation of culture medium and beads was uncomplicated, requiring a simple mesh screen, and the replicate biotransformation with re-used beads performed as expected with production duplicating the performance of the initial biotransformation.

To demonstrate the operational versatility of a solid–liquid TPPB, HYTREL^{TM} beads were used to create polymer sheets, which were then used as bioreactor internals. The motivation behind this experiment was to demonstrate an operational advantage over that of other comparable solid–liquid systems featuring rigid, unchangeable solid adsorbents such as Amberlite^{TM} XAD-4. This work reveals an immense potential for future solid–liquid TPPB design, as it is reasonable to suppose that polymers may be used to create other bioreactor internals, such as baffles and/or impellors that may be used to assist in product recovery.

The biotransformation successfully demonstrated the ability of the polymeric bioreactor internals to remove 3MC from solution and thus increase the overall yield of 3MC, although based on the mass of HYTREL^{TM} present and abiotic partitioning data for 3MC it was expected that a much greater mass of 3MC would have been present in the system. The fact that 3MC levels did not attain target values leads to the conclusion that the system may have succumbed to a mass transfer limitation. In this scenario it would be expected that the rate of 3MC productivity exceeded the rate of 3MC absorption thus leading to culture toxification due to the inability of the polymer to remove toxic product fast enough. This notion is supported by the disappearance of 3MC from the aqueous phase over the period $t = 9.5$ to $t = 26$ during which time 3MC levels were observed to decrease approximately 30% from 906 mg/L to 620 mg/L. If no mass transfer limitation existed the system should have

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been in equilibrium at $t = 9.5$ h when 3MC levels became lethal, rather the system was not in equilibrium thus, further absorption of 3MC during the period of extended operation was observed. Assuming abiotic losses did not exceed 3% (Husken et al., 2001a), the partition coefficient at $t = 26$ h was calculated to be 47, which is statistically similar to the partition coefficients of two the previously operated batch biotransformations (Table II). The fact that the polymer sheets do not absorb 3MC as rapidly as polymer beads may be attributed to the decreased surface area of the polymer. Although volumetric productivity was used as a common measure to compare the performance of the various systems, it would also be possible to normalize the productivity values with respect to the polymer surface areas. This would pose some difficulties with the polymer configurations used in the present work, however, as the beads were of somewhat varying size and shape (an industrial grade of HYTREL^{TM} was used) and the sheets were non-uniform in shape and roughness. Future work will investigate approaches to mass transfer limitation such as improved construction of bioreactor internals, for example, increasing the liquid contact area of the polymer, and improved control over the rate of 3MC production.

External extraction loops have been used for the containment of sorbent materials such as solid adsorbents and immobilized organic solvents (Held et al., 1999b; Serp et al., 2003). The use of external loops in these systems is made necessary due to the fragile nature of these materials. We have successfully demonstrated that polymer beads are able to withstand the rigorous mixing conditions within a bioreactor, however, placement of polymers within an external extraction column enables continuous product removal through multiple column exchanges thus extending the life of normal system operation. Although only three cycles were performed in this experiment, it may be expected that extended operation with additional exchanges of the polymer beads columns would provide enhanced performance.

The results of the continuous biotransformation successfully illustrated the potential of this mode of continuous operation. The overall productivity of the system was calculated to be 270 mg/L-h, which is a decrease in performance compared to previous solid–liquid batch mode operation (Table I). Closer inspection of performance reveals that if startup is neglected and performance assessed over the final two column exchanges rates of 419 and 420 mg/L-h can be maintained. These levels of productivity represent a 20% increase in performance over the previously performed batch biotransformation. With a single start-up period, and prolonged operation in continuous mode, it can be expected that enhanced productivity would result.

Due to the toxic nature of the product it is necessary to operate at sub lethal 3MC concentrations. This was achieved by replacing extraction columns at an upper 3MC limit of approximately 500 mg/L. The continued growth of the cells as well as 3MC production confirmed that bioactivity of the system was maintained throughout the biotransformation. From our results (Fig. 4) however, it appears that 3MC begins to inhibit microbial growth at concentrations lower than previously reported. At concentrations of 400–450 mg/L, 3MC was observed to negatively affect growth, as biomass concentrations were reduced from exponential growth to a linear increase. This is indicative of product inhibition (Held et al., 1999b) and future work with this system will investigate the effects of biocatalyst toxification and methods to overcome this apparent limitation.

The polymer beads have been demonstrated to provide a means of capturing the product from solution as it is formed but in order to be a useful and reusable tool for recovery of the product further processing is required. In order to show that the beads would be desorbed of 3MC and re-used in a bioreactor we were able to demonstrate that 3MC can be desorbed from HYTREL^{TM} using a minimal salts medium. However, the high partition coefficient of 3MC in favor of HYTREL^{TM} (58) requires a large volume of extractant. In addition, 3MC in an aqueous environment has been shown to be susceptible to auto oxidation (Wery et al., 2000) and will result in decreased yields. Therefore, desorption of 3MC from HYTREL^{TM} into a more favorable extractant, such as methanol, was shown to be feasible. The partition coefficient for 3MC between HYTREL^{TM} and methanol was 2.3 and thus 25 times less volume would be required than that of water for the same extent of 3MC recovery. In addition to reduced extraction volumes organic solvents have been shown to protect 3MC from auto oxidation thus increasing its stability (Wery et al., 2000).

Solid polymer absorbents, such as HYTREL^{TM}, when employed in a biphasic system, have been shown to enhance bioproduction of 3MC by 60% over similarly operated two liquid systems. Taking advantage of HYTREL^{TM}'s thermoplastic nature permits alteration to the geometry of the polymer and increases the operational versatility of the biphasic system. Finally, a packed bed of HYTREL^{TM} beads in an extraction column was shown to offer prolonged bioproduction of 3MC thus demonstrating continuous fed-batch production of 3MC.

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References

Amsden B, Bochanysz J, Daugulis AJ. 2003. Degradation of xenobiotics in a partitioning bioreactor in which the partitioning phase is a polymer. Biotechnol Bioeng 84:399–405.

Arnow L. 1937. Colorimetric determination of the components of 3,4-dihydroxyphenylalanine tyrosine mixtures. J Biol Chem 118:531–537.

Daugulis AJ, Swaine DE, Kollerup F, Groom CA. 1987. Extractive fermentation integrated reaction and product recovery. Biotechnol Lett 9:425–430.

Held M, Panke S, Kohler PE, Feiten HJ, Schmid A, Wubbolts MG, Witholt B. 1999a. Solid phase extraction for biocatalytic production of toxic compounds. Bio World 5:2–9.

Held M, Schmid A, Kohler HPE, Suske W, Witholt B, Wubbolts MG. 1999b. An integrated process for the production of toxic catechols from toxic phenols based on a designer biocatalyst. Biotechnol Bioeng 62:641–648.

Hoshi M, Ieshige M, Saitoh T, Nakagawa T. 1999. Separation of aqueous phenol through polyurethane membranes by pervaporation. II. Influence of diisocyanate and diol compounds and crosslinker. J Appl Pol Sci 71:439–448.

Husken LE, Beeftink R, de Bont JAM, Wery J. 2001a. High-rate 3-methylcatechol production in *Pseudomonas putida* strains by means of a novel expression system. Appl Microb Biotechnol 55: 571–577.

Husken LE, Dalm MCF, Tramper J, Wery J, de Bont JAM, Beeftink R. 2001b. Integrated bioproduction and extraction of 3-methylcatechol. J Biotechnol 88:11–19.

Husken LE, Oomes M, Schroen K, Tramper J, de Bont JAM, Beeftink R. 2002. Membrane-facilitated bioproduction of 3-methylcatechol in an octanol/water two-phase system. J Biotechnol 96:281–289.

Inoue A, Horikoshi K. 1991. Estimation of solvent-tolerance of bacteria by the solvent parameter log-P. J Ferment Bioeng 71:194–196.

Laane C, Boeren S, Vos K, Veeger C. 1987. Rules for optimization of biocatalysis in organic-solvents. Biotechnol Bioeng 30:81–87.

Leon R, Fernandes P, Pinheiro HM, Cabral JMS. 1998. Whole cell biocatalysis in organic media. Enz Microb Technol 23:483–500.

Lynch RM, Woodley JM, Lilly MD. 1997. Process design for the oxidation of fluorobenzene to fluorocatechol by *Pseudomonas putida*. J Biotechnol 58:167–175.

Meyer A, Held M, Schmid A, Kohler HPE, Witholt B. 2003. Synthesis of 3-tert-butylcatechol by an engineered monooxygenase. Biotechnol Bioeng 81:518–524.

Miller GL. 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal Chem 31:426–428.

Neumann G, Kabelitz N, Zehnsdorf A, Miltner A, Lippold H, Meyer D, Schmid A, Heipieper HJ. 2005. Prediction of the adaptability of *Pseudomonas putida* DOT-T1E to a second phase of a solvent for economically sound two-phase biotransformations. Appl Environ Microb 71:6606–6612.

Prpich GP, Daugulis AJ. 2004. Polymer development for enhanced delivery of phenol in a solid-liquid two-phase partitioning bioreactor. Biotechnol Progr 20:1725–1732.

Prpich GP, Daugulis AJ. 2006a. Biodegradation of a phenolic mixture in a solid-liquid two-phase partitioning bioreactor. Appl Microb Biotechnol 72:607–615.

Prpich GP, Daugulis AJ. 2006b. Solvent selection for enhanced bioproduction of 3-methylcatechol in a two-phase partitioning bioreactor. Biotechnol Bioeng DOI:10.1002/bit.21257.

Rojas A, Duque E, Schmid A, Hurtado A, Ramos JL, Segura A. 2004. Biotransformation in double-phase systems: Physiological responses of *Pseudomonas putida* DOT-T1E to a double phase made of aliphatic alcohols and biosynthesis of substituted catechols. Appl Environ Microb 70:3637–3643.

Rzeszutek K, Chow A. 1998. Extraction of phenols using polyurethane membrane. Talanta 46:507–519.

Schmid A, Kollmer A, Mathys RG, Witholt B. 1998. Developments toward large-scale bacterial bioprocesses in the presence of bulk amounts of organic solvents. Extremophiles 2:249–256.

Schumack L, Chow A. 1987. Extraction of aromatic organic compounds by polyurethane foam. Talanta 34:957–962.

Serp D, von Stockar U, Marison IW. 2003. Enhancement of 2-phenylethanol productivity by *Saccharomyces cerevisiae* in two-phase fed-batch fermentations using solvent immobilization. Biotechnol Bioeng 82:103–110.

Tao Y, Bentley WE, Wood TK. 2005. Phenol and 2-naphthol production by toluene 4-monooxygenases using an aqueous/dioctyl phthalate system. Appl Microb Biotechnol 68:614–621.

Wery J, da Silva DIM, de Bont JAM. 2000. A genetically modified solvent-tolerant bacterium for optimized production of a toxic fine chemical. Appl Microb Biotechnol 54:180–185.

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