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Solvent Selection For Enhanced Bioproduction of 3-methylcatechol in a Two-Phase Partitioning Bioreactor

George P. Prpich, Andrew J. Daugulis

Department of Chemical Engineering, Queen's University, Kingston, Ontario, K7L 3N6, Canada; telephone: 613-533-2784; fax: 613-533-6637; e-mail: daugulis@chee.queensu.ca

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ABSTRACT: The biotransformation of toluene to 3-methycatechol (3MC) via *Pseudomonas putida* MC2 was used as a model system for the development of a biphasic process offering enhanced overall volumetric productivity. Three factors were investigated for the identification of an appropriate organic solvent and they included solvent toxicity, bioavailability of the solvent as well as solvent affinity for 3MC. The critical log P (log P_{crit}) of the biocatalyst was found to be 3.1 and log *P* values were used to predict a solvent's toxicity. The presence of various functional groups of candidate solvents were used to predict the absorption of 3MC and it was found that solvents possessing polarity showed an affinity towards 3MC. Bis (2-ethylhexyl) sebecate was selected for use in the biphasic system as it fulfilled all selection criteria. A two-phase biotransformation with BES and a 50% phase volume ratio, achieved an overall volumetric productivity of 440 mg 3MC/L-h, which was an improvement by a factor of approximately 4 over previously operated systems. Additional work focused on reducing the toluene feed in order to minimize possible toxicity and decrease loss of substrate (toluene), a result of volatilization. Toluene losses were reduced by a factor of 4, compared to previously operated systems, without suffering an appreciable loss in overall volumetric productivity.

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Introduction

Whole cell biotransformations of organic substrates to molecules of higher value are often limited by the toxicity of the product and/or substrate. Due to such constraints, many biotransformations performed in single phases are susceptible to low yields that render the systems economically unattractive. To improve productivities, specifically in batch mode, an assortment of approaches has been suggested to reduce the toxic effect of substrate/product, the most popular of which has been the addition of an immiscible second phase (Chartrain et al., 1998; Ereldinger et al., 2000; Husken et al., 2001b).

A second, immiscible phase acts as a reservoir, delivering or absorbing excess concentrations of toxic product/substrate from the aqueous phase. The partitioning of the target compounds reduces the biocatalyst's exposure to these materials and allows for an increase in product yield. A number of such model systems have been successfully described in the literature (Chartrain et al., 1998; Husken et al., 2001b; Rojas et al., 2004; Wubbolts et al., 1996).

Organic solvents are the most commonly employed immiscible phase for use in biphasic systems due to their variety and the sheer numbers available. Examples of organic solvents that have been used include aliphatic alcohols such as octanol (Wery et al., 2000), long chain alkanes such as dodecane (Janikowski et al., 2002) and esters such as bis (2-ethylhexyl) phthalate (Panke et al., 2000; Tao et al., 2005). A limitation associated with organic solvents is the potential toxicity that they may possess towards the biocatalyst. In order to minimize the toxic effects of a solvent, researchers have utilized solvent tolerant strains of bacteria (Faizal et al., 2005; Neumann et al., 2005; Wery et al., 2000; Wierckx et al., 2005), alternative bioreactor designs (Husken et al., 2002b) and have implemented intelligent solvent selection based on the octanol-water partitioning coefficient (log P) of solvents (Brink and Tramper, 1985; Bruce and Daugulis, 1991; Laane and Tramper, 1990). Of the techniques attempted to reduce solvent toxicity, rational solvent selection represents the most direct approach. The relationship between the log *P* value of a solvent and the bioactivity of a cell has been

Correspondence to: A.J. Daugulis

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described previously (Bruce and Daugulis, 1991; Laane et al., 1985). The log P_{crit} of a cell, which must be determined experimentally, is a measure of the sensitivity of cells to the presence of organic solvents and is a common means of quantifying solvent-cell biocompatibility (Bruce and Daugulis, 1991). Solvents with log *P* values above the log P_{crit} of a cell are invariably biocompatible with the organism.

The research conducted in this work seeks to improve the performance of a model biotransformation in which toluene is converted to 3-methylcatechol (3MC) by *Pseudomonas putida* MC2, a recombinant strain. This biotransformation has been described previously (Husken et al., 2001a) and is an excellent candidate for use in a biphasic system as both the product and the substrate are toxic to the biocatalyst. A biphasic system has previously been utilized in which octanol served as the immiscible phase (Husken et al., 2001b). Octanol has been demonstrated to offer excellent uptake of target compounds for many biotransformations, but as a result of its relatively low log *P* value (2.8), solvent toxicity is an issue (Husken et al., 2001b, 2002a; Neumann et al., 2005; Rojas et al., 2004; Wery et al., 2000).

The objective of this work was to demonstrate, through rational solvent selection, alternative immiscible liquids that offer similar, or improved uptake of 3MC, while remaining completely biocompatible and enhancing overall volumetric productivities. In addition, this work suggests the possibility of using novel second phases such as poly(propylene glycol), a liquid polymer, and solid polymer beads.

Materials and Methods

Microorganism and Chemicals

All chemicals were of reagent grade and were purchased from Fisher Scientific (Canada). *Pseudomonas putida* MC2 was the microorganism used in the study. Construction and characterization of the strain have been described previously (Husken et al., 2001a).

Culture Conditions

Pseudomonas putida MC2 cultures were taken from frozen stock (−80°C) and cultivated on solid LB medium containing 50 μg/L of kanamycin. For inoculum preparation, a single colony was used to inoculate a liquid mineral salt medium (Hartmans et al., 1989) with glucose (14.4 g/L) serving as the carbon source. Sodium salicylate (160 mg/L) was added to the culture medium to induce the genes responsible for 3MC production and kanamycin (50 μg/L) was added to maintain selection pressure (Husken et al., 2001a).

For experiments involving the biotransformation of toluene to 3MC, the pH was adjusted to 6.0 from 7.0 by balancing the ratio of K_2HPO_4 and NaH_2PO_4. Cells, grown as inoculum for the biotransformation experiments, were cultured in 125 mL Erlenmeyer flasks containing 50 ml of mineral salts medium, glucose, kanamycin and salicylate at concentrations described previously. The inocula were incubated for 18 h at 30°C after which time the cells were centrifuged at 2370 g, re-suspended and concentrated in 50 mL of fresh mineral salts medium containing no glucose.

Analytics

Cell concentrations were measured at 650 nm with a Biocrom Ultraspec 3000 UV/Vis spectrophotometer, and a calibration curve was prepared to convert the optical density into a cell dry weight (CDW). 3MC concentrations in the aqueous phase were measured using a colorimetric test as previously described (Arnow, 1937), following centrifugation of samples for 10 min at 2370 g. For two-phase samples the organic layer was removed after centrifugation. The concentration of 3MC contained within the organic phase was estimated using the previously determined partition coefficient of 3MC between the aqueous and organic phases. 3MC in the organic phase was periodically confirmed using an HPLC (Waters 2487) with an ODS2 Spherisorb analytical column (4.6 × 250 mm) (Waters), a UV/Vis detector (207 nm) and eluent consisting of water—methanol in a 1:1 ratio at a flow of 0.8 ml/min. HPLC analysis of aqueous samples was also periodically used to validate the results of the colorimetric test and based on the results there appeared no significant difference between the two methods.

Aqueous phase glucose concentrations were measured using the method described by (Miller, 1959). Dissolved 3MC in the aqueous phase interfered with the glucose assay so samples obtained during the bioproduction of 3MC were pretreated by injecting 3ml of aqueous volume into a Waters Oasis HLB pretreatment column. The column removed 3MC from the aqueous sample while glucose, contained within the effluent, was analyzed. The sample pre-treatment method was validated using known concentrations of glucose and 3MC (data not shown).

Gaseous toluene concentrations were measured using a Perkin Elmer gas chromatograph (GC) equipped with a flame ionization detector and connected to a J&W Scientific DB-5 GC column. Gas samples were taken from the headspace of the reactor and the air/toluene inlet gas feed stream. Based on a Henry's law ratio of 3.8:1, the concentration of toluene in the aqueous phase was calculated. The flow rate of toluene into the bioreactor was determined based on the concentration of toluene in the feed stream multiplied by the flow rate of air from both the air inlet and toluene feed streams.

Biotransformations

Single-phase biotransformation were carried out in a 1.5 L Bioflo I bioreactor (New Brunswick Scientific) with a working volume of 1 L. The bioreactor was agitated at 700 rpm and aerated at 600 mL/min. Temperature was

maintained at 30°C and pH was varied between 6.7 and 6.0, depending on experimental requirements. For single-phase biotransformation experiments, sparging air into toluene, contained within a flask, and then mixing the saturated toluene vapor stream with makeup air generated the toluene feed stream.

Two-phase biotransformations were carried out in a 5 L Bioflo III bioreactor (New Brunswick Scientific) with a total working volume of 3 L. The bioreactor controlled both temperature and pH, which were maintained at 30°C and 6.0 respectively. Oxygen levels were monitored via a polarographic dissolved oxygen probe (Broadley James Corp.) coupled with a data acquisition system. The reactor was agitated at 650 rpm and aerated at 800 mL/min. Gaseous toluene was provided as described above.

For all bioreactor experiments, both single and two-phase, air and toluene were introduced prior to inoculation to ensure toluene saturation of the aqueous phase. All bioreactor experiments were inoculated to provide the same initial cell concentration of 75 mg CDW/L. Aqueous samples for analysis of 3MC, biomass and glucose were drawn in 5ml aliquots. The samples were centrifuged for 10 minutes at 2370 g and stored at 4°C until analysis was performed.

Partitioning of 3MC between Aqueous and Immiscible Phases

Measurements of the partitioning of 3MC between the aqueous and immiscible phases was performed in 15mL test tubes containing 9mL of sterile culture medium, a pre-determined concentration of 3MC and 1ml of immiscible liquid. The solvent was contacted with the aqueous phase for 3 hours during which time the contents were vortexed for 30 s every half hour. The contents were centrifuged at 2370 g and the immiscible liquids were removed by aspiration. The aqueous phase was analyzed for 3MC concentration and based on an initial 3MC measurement, as well as a control, a partition coefficient for each immiscible liquid was calculated.

Toxicity and Bioavailability of Organic Solvents

Toxicity tests were initially conducted to screen for candidate immiscible liquids. Tests were conducted in 125 mL Erlenmeyer shake flasks containing 50mL of culture medium with glucose and 5 mL of immiscible liquid. The flasks were inoculated with 1mL of 1-day-old culture of *Pseudomonas putida* MC2 and incubated for 24 hours at 30°C and 180 rpm. After 24 h, samples were withdrawn, centrifuged at 2370 g for 10 min the immiscible liquid was removed, and CDW analysis of the aqueous phase was performed. Using a positive control containing no immiscible phase and a negative control containing no inoculum the extent of growth was determined and normalized with respect to the positive control. Further assessment of the candidates involved evaluating the bioavailability of those immiscible liquids that proved to be non-toxic. Tests were performed in a similar fashion as previously described except that the medium contained no glucose. Canola oil, a bioavailable immiscible liquid, was used as a positive control. The CDWs of the flasks were determined and normalized with respect to the positive control.

Results

Single Phase Operating Conditions

The initial objective of this work was to improve upon the operating parameters for the single-phase bioproduction of 3MC, as reported in the literature, and to provide a "best case" benchmark against which two-phase operation would be compared. The aim was to reduce the pH of the culture medium, thereby providing a more stable environment for 3MC, and to decrease inlet toluene feed concentrations in order to avoid possible inhibitory effects as well as to minimize loss due to volatilization.

3MC has been shown to be sensitive to chemical degradation in aerated systems at pH values greater than 7 (Knackmuss, 1996). As the pH of the culture medium declines there has been an observed increase in product stability with percent losses decreasing from 60% to 3% for pH values of 8 and 5.8 respectively (Husken et al., 2001a). As the pH of the culture medium was originally maintained at 6.7 and previous work using glucose as the carbon source recommended an initial pH of 7.0 (Husken et al., 2002a) it was hypothesized that a reduction of culture medium pH to 6.0 would create an environment in which 3MC would be more stable. Fermentations, in which toluene was not present were performed at pH 6.7 and 6.0 (data not shown) to assess the impact that a change in pH may have on the metabolic activity of *Pseudomonas putida* MC2. Maximum specific growth rates (μ_{max}) were calculated for each system resulting in a μ_{max} of 0.45 1/h for growth at pH 6.7 and 0.47 1/h for growth at pH 6.0. From the results, a pH shift from 6.7 to 6.0 did not appear to affect the maximum specific growth rate of *Pseudomonas putida* MC2, therefore 6.0 was chosen as the medium pH for all subsequent experiments.

Toluene is provided to the bioreactor by feeding a dilute toluene feed stream at a rate that would maintain a predetermined concentration of toluene in the aqueous phase. Previous work has employed a toluene loading that achieved an aqueous toluene concentration of 92 mg/L (Husken et al., 2001b). Under such single-phase conditions, toluene may be inhibiting microbial activity but more importantly much of the toluene introduced to the bioreactor is lost due to volatilization. In order to minimize possible toxic effects and toluene losses, tests were performed in which feed concentrations were reduced and overall volumetric productivities were calculated.

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An initial experiment was performed to assess the mass of toluene lost during a biotransformation in which the loading maintained an aqueous toluene concentration of approximately 92 mg/L. Toluene was fed to the system at an average loading of 1350 mg toluene/L-h and upon completion of the experiment it was calculated, from a mass balance, that 10760 mg of toluene was lost due to volatilization. Work was then performed to reduce the mass of toluene lost in the off gas stream and assess possible effects to the overall volumetric productivity of 3MC.

Toluene loading rates were initially reduced by a factor of 1.5 to 900 mg toluene/L-h and then reduced further by a factor of approximately 4 to 350 mg toluene/L-h. The results of the biotransformations are given in Table I, and it can be seen that reducing the inlet concentration of toluene does not have a negative effect on the final 3MC concentrations nor the overall volumetric productivities. This suggests that at the initial toluene loading (1350 mg/L-h), substrate inhibition in the single phase may not have inhibited the performance of the system. Although no apparent kinetic benefits were observed from decreasing inlet substrate concentrations, toluene losses due to volatilization were reduced by approximately 75% and the loading of 350 mg toluene/L-h was adopted for all subsequent biotransformations. Throughout all the biotransformations toluene was detected in the headspace and was therefore never considered a limiting factor.

Selection of an Appropriate Immiscible Liquid

The first stage of the selection process involved determining the critical octanol-water partition coefficient (log P_{crit}) of *Pseudomonas putida* MC2. As the objective of this study is to enhance 3MC production it is important to select a solvent that does not inhibit microbial activity thus allowing immediate production of 3MC. As result, toxicity tests were performed over a 24 h period which was deemed ample time to demonstrate both toxicity and biocompatibility. In order to determine the log P_{crit} a number of organic solvents were chosen that spanned a broad range of log *P* values. From the results in Figure 1, it can be seen that the log P_{crit} of *Pseudomonas putida* MC2 is approximately 3.1. This result is in agreement with the literature, which has reported log P_{crit} values for *Pseudomonas putida* of 3.2 (Vrionis et al., 2002) and 3.1 (Inoue and Horikoshi, 1989). Based on this result, immiscible liquids that possess a log *P* value greater than 3.1 should not have a negative impact on the growth of the microorganism while the use of immiscible liquids with log *P* values below 3.1 would be expected to impair microbial activity.

Table I. Single-phase bioproduction of 3MC.

Toluene Feed Rate (mg toluene/L-h)	Volumetric Productivity (mg 3MC/L-h)	Final $[3MC]_{aq}$ (mg/L)	Toluene Loss (mg)
1350	110	925	10922
900	116	930	6265
350	111	890	2569

Results are based on a reduction of toluene loading.

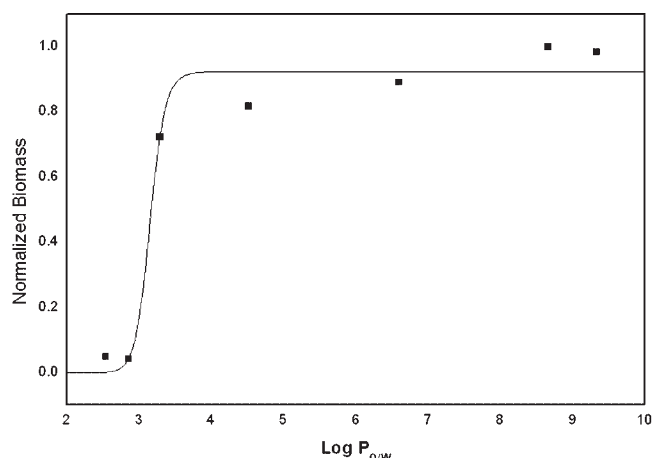

Figure 1. Determination of the log P_{crit} of *Pseudomonas putida* MC2. Organic solvents of varying log *P* values were used. In order of increasing log P they were: toluene, octanol, hexane, dodecanol, dodecane, hexadecane, octadecene, BES.

Nine immiscible liquids, representing an extensive range of organic solvents, were selected as possible candidates for use in a biphasic biotransformation and are listed in Table II. The list consists of a number of aliphatic solvents, chosen based on their various structures, availability and price. Aliphatic alcohols have demonstrated an affinity towards 3MC and although octanol does not possess an appropriately high log *P* value it was chosen for comparison purposes because of its use in prior studies (Husken et al., 2001b; Wery et al., 2000). Dodecanol was chosen as an aliphatic alcohol that possesses a log *P* value higher than octanol. The alkanes were chosen to see if branching or chain length would affect affinity for 3MC, and also based on the fact that their log *P* values were greater than log P_{crit}. Esters were also investigated due to their polar functional groups as well as their high log *P* values, while silicone oil

Table II. Partition coefficient for uptake of 3MC and log *P* values of candidate immiscible liquids.

Immiscible Liquid	Partition coefficient $K_{(solvent/aqueous\ phase)}$	Log *P*
1-octanol	22	2.8
1-dodecanol	14	4.77
BES	9	10.08
BEHP	4.5	8.39
silicone oil	0	*
decane	0	5.25
iso-octane	0	4.09
dodecane	0	6.23
1-dodecene	0	6.10

*A log *P* value for silicone oil was not found but silicone oil has been successfully demonstrated as an immiscible liquid for a number of two-phase applications. Guieysse et al. (2001); Villemur et al. (2000).

was selected as it has been shown to be an effective immiscible liquid in two-phase systems (Tikhomiroff et al., 2002; Villemur et al., 2000) demonstrating both biocompatibility and non-bioavailability.

Of the 9 immiscible liquids studied, prediction of 3MC uptake by a solvent was further assessed based on the consideration of a solvent's functionality as well as the structure of the target molecule. It was expected that solvents with polar functionality would be capable of 3MC uptake while the less polar solvents would not. From the results (Table II), four organic solvents demonstrated an affinity for 3MC: Octanol, 1-dodecanol and two di-esters, bis (2-ethylhexyl) sebecate (BES) and bis (2-ethylhexyl) pthalate. Under the given conditions, the alkanes did not absorb an appreciable mass of 3MC. Partitioning coefficients of 0 were given for those liquids unable to absorb greater than 5% of the original mass of 3MC.

Comparing the four candidate solvents that expressed an affinity towards 3MC, BES was selected for use as the immiscible liquid in the two-phase system. In contrast to octanol, BES has a more favorable log P value for use in a biological system (Fig. 1). On the other hand, 1-dodecanol possesses a log P value sufficiently high enough to support biological activity, but due to its relatively high melting point (24°C) it is often difficult to work with and was therefore not selected. A final test to determine whether BES may be available to *Pseudomonas putida* MC2 as a carbon/energy source proved negative (data not shown).

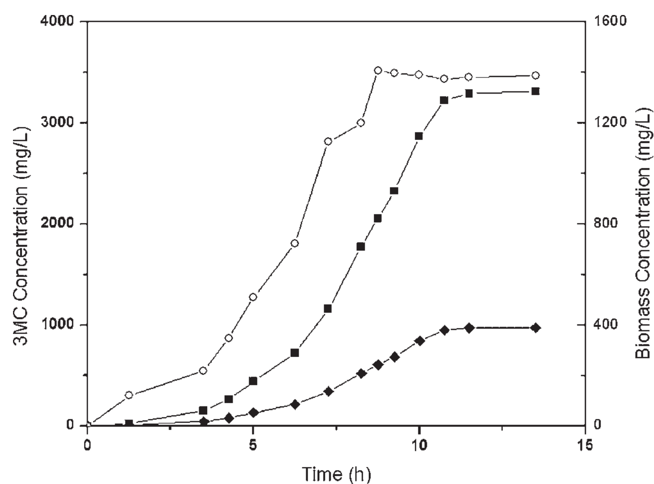

Figure 2. Two-phase biotransformation of 3MC using a phase ratio of 30% v/v BES. Squares represent 3MC concentration in BES, the diamonds represent 3MC concentration in the aqueous phase and the circles represent biomass concentration.

Two-Phase Biotransformations with BES

Prior to conducting two-phase biotransformations for the production of 3MC, a two-phase fermentation was performed to confirm that *Pseudomonas putida* MC2 would be unaffected by the presence of BES (data not shown). Bioreactor conditions were maintained identical to those of the single-phase biotransformations (pH 6.0 and a toluene loading of 350 mg/L-h), contained a phase ratio of 10% BES and was inoculated with 75 mg CDW/L. The maximum specific growth rate of *Pseudomonas putida* MC2 in the two-phase system was calculated to be 0.47 1/h, which is identical to the μ_{max} calculated in the previous section involving the study of single-phase operating conditions (0.47 1/h). The result of the experiment demonstrates that the cells do not suffer any deleterious effects due to the presence of BES.

Three biotransformations of varying phase ratios were performed to demonstrate the improved overall productivity offered by a biphasic system at phase volume ratios of 10%, 30% and 50% BES. The two-phase system was operated in a similar manner to the single-phase systems concerning inoculum size and aeration. The cultivation pH was maintained at 6.0 for all two-phase experiments and toluene loading was maintained at 350 mg toluene/L-h.

The results of the two-phase biotransformations were very similar and only the results of the 30% phase volume ratio are shown in Figure 2. It can be seen that production of 3MC was immediate and continued until t = 11 h at which time the aqueous concentration of 3MC reached an inhibitory level of approximately 968 mg 3MC/L. Based on a partition coefficient of 9.0 for 3MC between BES and water, the final concentration of 3MC in BES was calculated to be 8715 mg 3MC/L. With a total working volume of 3 L the overall 3MC concentration within the system was found to be 3292 mg/L, which is approximately 3.2 times greater than the final 3MC concentration for single-phase operation. The results of random BES samples (not shown), analyzed using HPLC confirmed the accumulation and final concentration of 3MC in BES. Furthermore, during the biotransformation, the presence of 3MC in BES was indicated by the coloration of the liquid solvent, which, initially colorless, changed to grey during the biotransformation and ended with a golden brown coloration.

Dissolved oxygen concentration was monitored throughout the biotransformations and was found never to drop below 20% saturation. Glucose levels were also monitored and confirmed that glucose was present during the entire fermentation. During the fermentation it was observed that *Pseudomonas putida* MC2 interacted with BES to form an emulsion although the emulsion did not appear to interfere with uptake of 3MC by BES as partition coefficients between the abiotic and biotic experiments were the same.

The biotransformations where phase volume ratios of 10% and 50% BES v/v, were operated in an identical fashion to the 30% BES biotransformation, with 3MC accumulating in the system until aqueous concentrations achieved inhibitory levels, of 978 mg 3MC/L and 1098 mg 3MC/L for the 10% and 50% phase ratios respectively. The total overall concentration of 3MC within the 3 L systems was determined to be 1762 mg/L and 5494 mg/L respectively.

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Overall volumetric productivities for the 10%, 30% and 50% BES two-phase biotransformations were also calculated. The values were compared to the overall volumetric productivity observed in single-phase operation as shown in Table III.

Discussion

The production of metabolic intermediates via a biocatalyst in a single-phase system may often times suffer from the presence of an inhibitory substrate and/or accumulation of a cytotoxic product. In the case of 3MC production, the literature has reported that *Pseudomonas putida* MC2 will cease conversion of toluene to 3MC once the aqueous concentration approaches 900–1200 mg 3MC/L (Husken et al., 2002a). In order to improve the overall volumetric productivity an immiscible phase may be introduced to partition toxic compounds from the aqueous environment. The initial scope of our work was to refine a single-phase operating system for the bioconversion of toluene to 3MC. The motivation behind the development was to create a model system that best exemplified the most favorable single-phase conditions for production of 3MC in order to offer a fair comparison to a similarly operated two-phase system.

3MC has been shown to be unstable at high pHs resulting in the formation of polymeric chains (Wery et al., 2000). At pHs lower than 7.0, 3MC has shown improved stability (Husken et al., 2001b) with chemical degradation reducing from 60% at pH 8 to 9% for pH 7 and 3% for pH 5.8. Therefore, by reducing the medium pH to 6.0 we have created an environment that would allow small fluctuations in pH without compromising the stability of 3MC. Although a decrease in pH to 6.0 will provide product stability the actual 3MC concentration may be higher than the measured values.

Toluene is toxic to *Pseudomonas putida* MC2 and if introduced to batch cultures at high concentrations will inhibit 3MC production; conversely, if toluene concentrations are too low, substrate availability becomes an issue and this will have a negative impact on 3MC production. To overcome the matters of substrate toxicity as well as availability, a fed-batch feeding strategy, whereby toluene is introduced continually through the feed air stream, has been utilized (Huseken et al., 2001b). Our primary intent in refining this strategy was to reduce toluene feeding while not promoting substrate-limiting conditions in an effort to minimize toxic effects in single-phase operation. We anticipated that the two-phase system would be less susceptible to toluene inhibition arising from the partitioning effect provided by the immiscible organic phase.

Table III. Volumetric productivity and final overall 3MC concentrations in a two-phase system using BES with varying phase volume ratios.

Phase Ratio	Volumetric Productivity (mg 3MC/L-h)	Final Overall $[3MC]_{Total}$ (mg/L)
10%	213	1762
30%	310	3292
50%	440	5494

Additionally, however, toluene is known to be a potentially hazardous environmental pollutant and for the purpose of this study, a valuable and expensive feedstock, therefore it was a second objective to minimize its loss. Previously operated fed-batch systems have based toluene-loading rates on the results of batch biotransformations (Husken et al., 2001b) involving the addition of toluene in finite volumes and allowing it to be converted to 3MC. The result of the study showed that toluene, at an initial aqueous concentration of 92 mg/L, was most favorable for operation in batch systems but maintaining this aqueous toluene concentration in a fed-batch system would result in significant substrate loss. A fed-batch strategy may not require a continuous toluene level of 92 mg/L, rather, it should be introduced at a rate equivalent to or greater than that of 3MC production. As a result, in single-phase operation, we were successful in reducing the toluene loading rates by a factor of 4 without observing a significant decrease in volumetric productivity of 3MC. In two-phase operation, maximum instantaneous volumetric productivities exceeded toluene-loading rates for short periods of time but toluene demands were met by the partitioning of toluene from the immiscible organic phase. In order to fine-tune toluene loading without affecting the production of 3MC more precise control of toluene addition is required as well as a better understanding of microbial kinetics and possibly a change to continuous mode of operation.

Rational solvent selection is an integral part of two-phase bioreactor system design and the principal characteristic used to predict a solvent's suitability in a two-phase system is a solvent's log *P* value. Generally, biological activity in the presence of solvents that possess log *P* values less than 2 is low, for values between 2–4 biological activity is unpredictable and for values greater that 4 biological activity is considered high (Laane et al., 1987). Although many biological systems comply with the aforementioned toxicity categories there are biocatalysts that have been shown to experience toxic effects from solvents possessing log *P* values greater than four (Janikowski et al., 2002; MacLeod and Daugulis, 2003; Rehmann and Daugulis, 2006). As a result, the categories governing solvent toxicity become inadequate and a more precise toxicity parameter (log P_{crit}) is required in order to improve biphasic design. Therefore, immiscible liquids with log *P* values at or above the log P_{crit} value of a microorganism should be considered 'safe' for use with little to no compromise in biological activity. From our results we found the log P_{crit} of *Pseudomonas putida* MC2 to be 3.1, which is comparable to other *Pseudomonas putida* strains and suggests that to avoid solvent toxicity solvents with log *P* values greater than 3.1 must be used. As was reported in the literature (Husken et al., 2001b), Pseudomonas putida MC2 is capable of growth in the presence of solvents with log

P values below that of 3.1. Although growth is possible, the microorganism experienced a significant acclimation period, which led to increased process times and thus reduced productivities. As the present work looks to enhance productivity it is advantageous to minimize lag and therefore, from a design/process standpoint, the log P_{crit} of *Pseudomonas putida* MC2 is appropriate.

Although a solvent may be biologically compatible with a given biocatalyst, the solvent may be poor at taking up the target molecule. Our results showed that only the aliphatic alcohols and BES displayed significant affinity towards 3MC, likely a result of their functionality. 3MC is a relatively polar aromatic compound due to the presence of two hydroxyl groups, therefore it is reasonable to suppose that those solvents possessing polar functionality should exhibit an affinity for 3MC. Solvents such as the aliphatic alcohols have been demonstrated to possess a strong affinity for polar target molecules (Wery et al., 2000) and it has been hypothesized that the attraction may be due to the formation of hydrogen bonds between the polar groups of both solvent and target molecule (Leon et al., 1998). Although aliphatic alcohols serve as excellent reservoirs for 3MC, they generally possess low log *P* values, which make them moderately toxic to the biocatalyst. The literature has provided a number of cases where aliphatic alcohols have served as the immiscible phase in a biphasic system for the biotransformation of 3MC (Husken et al., 2002a; Neumann et al., 2005; Rojas et al., 2004; Wery et al., 2000). Although aliphatic alcohols exhibit excellent partitioning of 3MC between the aqueous phase the log *P* values of these compounds, ranging between 2.8 for octanol to 3.78 for decanol, are quite low and observation of these systems reveals that they suffer from lengthy lag phases or periods of adjustment, as well as a reduction in cell viability. By increasing chain length, the log *P* value of aliphatic alcohols increases but solvent solidification, at or near operational temperatures, then becomes a problem as we observed with 1-dodecanol. Our solvent selection process enabled us to identify BES as a possible replacement for aliphatic alcohols. BES possesses a much larger log *P* value than the aliphatic alcohols previously examined, which is an attractive characteristic for use in biological systems and we did not observe a reduction in the metabolic performance of *Pseudomonas putida* MC2. The two ester groups of BES may be responsible for polar functionality that lends itself to the formation of hydrogen bonds thus permitting the absorption of 3MC.

In the literature, a similar biphasic system (Husken et al., 2001b) in which octanol served as the immiscible phase had lag times approximately six times greater than the lag phase observed in our BES/water system. The lengthy lag phase may be a direct result of solvent toxicity and therefore, in replacing octanol with BES we were able to eliminate non-productive lag times and observed an increase in overall volumetric productivity as a result. Our system, containing 50% v/v BES, achieved an overall volumetric productivity of 440 mg 3MC/L-h whereas Husken et al. (2001b), operating a biphasic system consisting of 40% v/v octanol observed an overall volumetric productivity of 111 mg 3MC/L-h. In addition, due to the possibly toxic effects of octanol, the system operated by Husken et al. (2001b), with a phase ratio of 50%, achieved a final overall 3MC concentration of 3115 mg/L, which is well below the expected value of approximately 10 900 mg/L (based on a partition coefficient of 22 for octanol and aqueous 3MC concentration achieving a maximum of 950 mg/L). Although BES possesses a lower affinity for 3MC the BES biphasic system achieved higher overall 3MC concentrations as result of its biocompatible nature.

To reduce the toxic effects of octanol Husken et al. (2002b) implemented a hollow fiber membrane system and although the system experienced an increase in 3MC productivity to 173 mg/L-h difficulties such as solvent toxicity and mass transport still persisted. Further attempts to reduce the effects of solvent toxicity in biphasic system using aliphatic alcohols involve utilizing solvent tolerant microorganisms (Neumann et al., 2005; Rojas et al., 2004). Although this strategy has shown promise, the effort required to search for and construct these microorganisms may be much greater than the effort required to select a more appropriate solvent using rational means. Finally, solid matrices, such as hydrophobic resins, have been used to partition high concentrations of 3MC from the aqueous phase (Held et al., 1999). These systems are effective at immobilizing product but are currently not capable of selective adsorption and as result both substrate and product will vie for available sorption sites limiting final product concentrations.

Preliminary work has been undertaken to locate immiscible liquids that may serve as alternatives to organic solvents for use in biphasic systems. Understanding the predominate role hydrogen bonds may play in the uptake of 3MC we are now focusing on the liquid polymer, PPG, as a possible replacement in a biphasic system. PPG is a viscous, colorless liquid, commonly employed as an anti-foam agent during microbial fermentations. The molecular structure of PPG consists of straight chain propylene segments joined by ether bonds and terminated by hydroxyl groups. The propylene glycol monomer utilized in our preliminary study had a molecular weight of 1100, was not soluble in water and has been shown in the literature to be biocompatible and non-bioavailable (Barton and Daugulis, 1992). It was predicted that PPG, and its polar functional groups, would express an affinity for 3MC and preliminary work has determined that the partition coefficient of 3MC in a PPG/aqueous medium system was 32. Liquid polymers as well as solid polymer beads hold promise as replacements for immiscible organic solvents in biphasic systems. Solid polymer beads have been successfully demonstrated in the literature as an effective non-toxic, inert second phase for the partitioning as well as delivery of toxic aromatic substrates (Amsden et al., 2003; Daugulis et al., 2003). We are currently exploring the possibility of adapting the utilization of liquid polymers, as well as solid polymer beads

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as second phases for use within the biotransformation system.

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