ARTICLE

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Enhanced Bioproduction of Carvone in a Two-Liquid-Phase Partitioning Bioreactor With a Highly Hydrophobic Biocatalyst

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ABSTRACT: The microbial biotransformation of (−)-*trans*-carveol to the flavor and fragrance compound (*R*)-(−)-carvone by *Rhodococcus erythropolis* DCL14 was carried out in a 3 L two phase partitioning bioreactor with an immiscible liquid second phase in an effort to improve upon the reactor performance achieved in a single aqueous phase system. The purpose of employing the liquid second phase is to minimize biotransformation rate inhibition due to the accumulation of the toxic substrate (*cis*-carveol) and product (carvone) in the aqueous phase. 1-Dodecene was chosen as the solvent for this application because it is biocompatible, non-biodegradable and has a superior affinity for the target product (carvone) relative to the other solvents tested. However, when 1-dodecene was used in the biotransformation, the extremely hydrophobic *R. erythropolis* DCL14 created an emulsion with the organic solvent with significant sequestering of the cells into the organic phase and negligible substrate conversion. To overcome these operational difficulties, silicone oil, which is considered a liquid polymer, was used with the aim of preventing emulsification and sequestration of cells in the non-aqueous phase. Although some emulsification of the water-silicone oil was again created by the cells, operability was improved and, in fed-batch mode, the system was able to convert approximately 2½ times more carveol than a benchmark single aqueous phase system before substrate/product toxicity caused the biotransformation to stop. This study has demonstrated enhancement of a microbial biotransformation for the production of a high value nutraceutical compound via the use of a second partitioning phase, along with operational challenges arising from the use of a highly hydrophobic organism in such systems.

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KEYWORDS: carvone; substrate toxicity; product toxicity; two phase partitioning bioreactor; emulsion; *Rhodococcus erythropolis* DCL14

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Introduction

Carvone is a common terpenoid that is produced by over 70 different plants (Burdock, 1995) and exists as two enantiomers: (*R*)-(−)-carvone which has a spearmint aroma and (*S*)-(+)-carvone which has a caraway aroma. Both carvone enantiomers, especially (*R*)-(−)-carvone, are natural flavors widely used by the flavor and fragrance industry (Welsh et al., 1989). The essential oil containing carvone is produced primarily by caraway (*Carum carvi*), dill (*Anethum graveolens*), and spearmint (*Mentha spicata*) plants (Ravid et al., 1992). The essential oils of these plants (containing carvone) are obtained using conventional extraction methods such as hydrodistillation, steam distillation, and solvent extraction (Kallio et al., 1994). It is important to note that crop-processing operations, including premature harvesting and extended storage, can cause a loss of aroma due to the volatile nature of these products (Krings and Berger, 1998). It is for this reason that the production of essential oils from natural plant materials be as efficient as possible. The essential oil yield and composition (or quality) varies based on the quality of the plant and the extraction method used.

The carvone enantiomers can be used in many applications; namely as food additives to flavor such things as pickles and bread (Baysal and Starmans, 1999) as well as ice cream, candy, baked goods, meats, cheese, condiments, soft drinks, and alcoholic beverages. Carvone has also been used to flavor pharmaceutical products and toothpaste (Baysal and Starmans, 1999). Carvone, like most other oxygenated monoterpenes, exhibits some degree of antimicrobial and/or antifungal capability (Carson and Riley, 1995). Carvone is currently used as a sprout inhibitor to maintain the quality of stored potatoes in the Netherlands under the name of "Talent" (Hartmans et al., 1995). The extensive applications of carvone as a fragrance and flavor compound, antimicrobial agent and potato sprouting inhibitor justify research aimed at providing improved production methods (de Carvalho and da Fonseca, 2006).

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The production of carvone by microbial biotransformation offers several advantages over agricultural methods, including the ability to overcome the seasonality, and the modest essential oil content in plants (de Carvalho and da Fonseca, 2002a). The single-step microbial biotransformation is carried out by whole cells of *Rhodococcus erythropolis* DCL14 which, when grown on limonene as the sole carbon source, exhibit carveol dehydrogenase (CDH) activity (de Carvalho and da Fonseca, 2002a). The microbial biotransformation substrate is a mixture of *cis*- and *trans*-carveol isomers. The CDH activity allows for the stereoselective conversion of the *trans*-isomer to *R*-(−)-carvone, resulting in a second product of isomerically resolved *cis*-carveol (de Carvalho et al., 2005). Since monooxygenases are dependent on cofactors, the successful production of carvone by microbial biotransformation involves the regeneration of the required cofactor (Mihovilovic et al., 2002). When *R. erythropolis* DCL14 is grown on limonene, the enzyme activity is NAD-dependant (de Carvalho and da Fonseca, 2002a). The NAD cofactor can be regenerated through cell growth and as such, the carbon source (limonene) and oxygen must be supplied throughout the biotransformation period.

The major limitation of this biotransformation system is that at certain concentrations in the aqueous phase, the product (carvone) and substrate (*cis*-carveol) become toxic to the cells and inhibit the biotransformation rate. In an effort to overcome these inhibition effects, the use of a two phase partitioning bioreactor (TPPB) which is comprised of an aqueous phase containing the biomass and a second, immiscible phase designed to partition the inhibitory compounds (Malinowski, 2001) has been investigated. In order to improve reactor performance, it is important to follow a rational solvent selection regime as developed by Bruce and Daugulis (1991). An appropriate solvent is chosen based on several factors including biocompatibility, biodegradability, partitioning capacity and selectivity, low volatility, low cost and safety.

In this work, both 1-dodecene and silicone oil were used in a TPPB in an effort to improve reactor performance, which was compared to the performance of a benchmark single aqueous phase system (Morrish and Daugulis, 2008). The amount of substrate (mixture of isomers) that could be provided to the systems before the onset of inhibition was used as a measure of process performance, as was the operability, which was affected by the highly hydrophobic nature of the test organism.

Materials and Methods

Chemicals and Microorganism

(*R*)-(−)-carvone (CAS 6485-40-1) and (−)-carveol, mixture of isomers (CAS 99-48-9, *trans*—55.2% and *cis*—44.8%) were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada) (+)-limonene (CAS 5989-27-5), methylene chloride (CAS 75-09-2), alcohol reagent (89–91% ethanol) (CAS 64-17-5) and ethyl acetate (CAS 141-78-6) were purchased from Fisher Scientific (Ottawa, ON, Canada). The organic solvents that were tested for use as the second phase in a TPPB included: 1-dodecene (CAS 112-41-4), 1-octanol (CAS 111-87-5), decane (CAS 124-18-5), *n*-dodecane (CAS 112-40-3), 1-tetradecene (CAS 1120-36-1), hexadecane (CAS 544-76-3) and 1-octadecene (CAS 112-88-9) and were purchased from Fisher Scientific, with the exceptions of silicone oil (CAS 63148-62-9, viscosity = 5 cSt), undecane (CAS 1120-21-4) and octane (CAS 111-65-9) purchased from Sigma-Aldrich Canada Ltd. and bis(2-ethylhexyl)phthalate (CAS 117-81-7) purchased from Alfa Aesar (Ward Hill, MA).

R. erythropolis DCL14 was originally obtained by the Division of Industrial Microbiology of the Wageningen Agricultural University, Wageningen, The Netherlands. The strain was isolated from a sediment sample in Reeuwijk, The Netherlands (van der Werf et al., 1999). *R. erythropolis* DCL14 was received as a donation from the Center for Biological and Chemical Engineering of the Technical University of Lisbon. The stock culture was stored at −77°C in 5% dimethyl sulfoxide solution.

Medium and Inoculum Preparation

The medium formulation is a modified version of that used by Wiegant and de Bont (1980). The growth medium formulation is (added to 1 L tap water): glucose (10 g), yeast extract (0.1 g), K_2HPO_4 (1.55 g), $NaH_2PO_4 \cdot H_2O$ (0.85 g), NH_4Cl (3 g), $MgCl_2 \cdot 6H_2O$ (0.075 g), $(NH_4)_2SO_4$ (1 g) and 0.2 mL trace element as described by Vishniac and Santer (1957). The medium used in fermentations and biodegradability experiments did not contain glucose. The medium was autoclaved at 121°C for 45 min.

The inoculum for all shake flask experiments and fermentations was prepared by the addition of 100 μL *R. erythropolis* DCL14 stock culture to 50 mL growth medium containing glucose. The flasks were incubated at 30°C for 48 h at 180 rpm.

Analytics

Gas Chromatography

Five milliliters aqueous samples (which were centrifuged at 3,500 rpm at 20°C for 5 min to remove any biomass) were added to 5 mL ethyl acetate (used as the extractant). The samples were then vortexed for 10 s, twice. When the phases had separated, a 1 μL sample of the ethyl acetate layer was then injected into the Varian GC which was equipped with an FID detector and an Agilent/J & W DB-WAX column with internal diameter of 0.53 mm and length of 30 m. The carrier gas was helium and had a flow rate of 30 mL/min. The hydrogen and air flow rates were 45 and 450 mL/min, respectively. The method used is as follows: injector

temperature −250°C, detector temperature −270°C, oven temperature −100°C, hold −0.5 min, ramp to 160 at 50°C/min, hold for 1 min and finally ramp to 175°C at a rate of 50°C/min. The run time for this method is 3 min.

Biomass Quantification in Presence of Solvent

The hydrophobic nature of *R. erythropolis* DCL14 provided challenges with respect to the quantification of biomass in the presence of solvents and as such a washing method was developed using methylene chloride and ethanol to separate biomass from the resulting emulsion. To a 50 mL aqueous sample with 5 mL organic solvent, 10 mL methylene chloride was added and the sample was vortexed for 10 s. The sample was then centrifuged for 5 min at 3,500 rpm and 20°C. This step resulted in the aggregation of the biomass at the aqueous/methylene chloride and solvent interface. Then, most of the aqueous phase was aspirated off being careful not to disturb the biomass at the interface. Next, 30 mL ethanol was added and the sample was vortexed for 10 s. The sample was centrifuged again for 10 min at 3,500 rpm and 20°C which resulted in the formation of a pellet. The supernatant was decanted and the pellet was resuspended in distilled water after which the sample was dried in a pre-weighed metal dish at 90°C oven overnight. If either cycle (addition of methylene chloride or ethanol) did not perform the desired function (i.e., biomass accumulation at the interface or pellet formation), that cycle was repeated with fresh methylene chloride and/or ethanol.

Biocompatibility: Determination of Critical log *P*

Twelve organic solvents with a broad range of log *P*s (1.25–9.04) and silicone oil were tested for their biocompatibility with *R. erythropolis* DCL14. To separate 125 mL flasks, 50 mL of sterile growth medium, 5 mL of the solvent being tested, and 1 mL inoculum were added. To ensure sterility, the solvents were passed through a syringe with a 0.2 μm sterile filter prior to use. A positive control flask which contained no solvent was also prepared. The flasks were incubated at 30°C for 96 h at 180 rpm. After incubation, the extent of biomass growth was measured relative to the control.

Biodegradability

The solvents tested for biodegradability were those that had a log *P* above the critical log *P* of *R. erythropolis* DCL14, and were therefore biocompatible. To separate 125 mL flasks, 50 mL of sterile glucose-free medium, 5 mL of the solvent being tested, and 1 mL inoculum were added. To ensure sterility, the solvents were passed through a syringe with a 0.2 μm sterile filter prior to use. A positive control flask with canola oil and a negative control with no second phase were also prepared in a similar manner. The flasks were incubated at 30°C for 96 h at 180 rpm. After incubation, the biomass was measured relative to the control flasks.

Determination of Solvent Partition Coefficients

The partition coefficients of carvone and carveol were determined for those solvents that were both biocompatible and non-biodegradable. Increasing concentrations of carvone and carveol that were below the aqueous solubility limits (0–1,340 and 0–2,400 mg/L, respectively) were added to 10 mL distilled water in a sealed 20 mL vial. After mixing well, 5 mL of the solvent being tested was added and the samples were agitated at 180 rpm for 24 h. After 24 h, the solvent was aspirated off and 5 mL of the aqueous phase was removed to be tested by gas chromatography.

Reactor Operation

A 5 L New Brunswick Scientific BioFlo III bioreactor was used for all fermentations. The temperature and agitation were maintained at 28°C and 350 rpm, respectively. The pH was maintained automatically at 7.0 using 6M KOH and a Broadley James FermProbe. A Broadley James D100 Series Oxyprobe was used to track the dissolved oxygen (DO) level to ensure the system was not oxygen transfer limited. The air flow rate into the reactor was 1 L/min and was passed through a 0.2 μm sterile filter. The carbon source, which was also used as the enzyme induction source (+)-limonene, was supplied by passing an air stream through a sparger contained in a flask of liquid (+)-limonene. The limonene-saturated air flow rate into the reactor (200 mL/min) was also equipped with a 0.2 μm sterile filter. The reactor containing 3 L carbon-free medium was sterilized at 121°C for 65 min prior to use. The inoculum was prepared as outlined above and two 50 mL inoculum flasks were added to 3 L sterile medium. When the biomass concentration had reached approximately 500 mg/L, the sterile second phase (0.5 L) was added and the biotransformation was initiated by the addition of carveol. The selected solvents and substrate were added to the reactor at this time to ensure that the biomass concentration for each reactor would be consistent and that the metabolic state of the cells was similar. For the reactor with 1-dodecene as the second phase, the solvent could not be autoclaved due to its low flash point and was instead passed through a sterile 0.2 μm filter prior to use. For the reactor that used silicone oil as the second phase, the reactor and the solvent were autoclaved separately. The air and (+)-limonene flows were continuously supplied throughout the duration of the transformation. The transformation substrate (−)-carveol, was supplied in liquid form. The product and substrate concentrations of the microbial biotransformation were monitored frequently using the GC and when the *trans*-carveol isomer was near depletion, more substrate was added such that its concentration in the reactor was below the aqueous solubility and so as not to unduly inhibit the

cells present in the aqueous phase. These substrate additions were provided once the *trans*-carveol isomer was near depletion until the transformation ceased due to the accumulation of the toxic substrate and product.

Results and Discussion

Solvent Selection

Biocompatibility: Determination of Critical log P

A biocompatible solvent ensures that its presence will not be inhibitory to the organism or otherwise affect its metabolic activity. Biocompatibility is usually evaluated based on the log *P* of the solvent and the critical log *P* of the organism. The critical log *P* of an organism is such that the organism will grow in the presence of solvents with log *P*s greater than its critical log *P* and will not grow in solvents with log *P*s lower than its critical log *P*. Although log *P* is a strong indicator of biocompatibility in the selection of solvents, the process of identifying a biocompatible solvent can be more involved. A solvent is usually deemed non-biocompatible because it has the ability to accumulate in the cytoplasmic membrane and affect the integrity of the cell membrane by limiting the ability of the cell to perform essential biochemical reactions (de Bont, 1998). Recently, the adaptability of specialized bacteria to tolerate the presence of a toxic solvent phase has been explored (Heipieper et al., 2007; Neumann et al., 2005). Neumann et al. (2005) note that certain organisms can adapt to the presence of a solvent by the modification of the membrane and/or cell surface properties as well as the activation and/or induction of an active transport system for eliminating solvents from the cell membrane. Though the adaptability of certain organisms has recently become an important factor to consider when determining the biocompatibility of a solvent, the present work uses log *P* as the only biocompatibility selection criterion.

The critical log *P* of *R. erythropolis* DCL14 was determined by exposing the cells to a selection of twelve organic solvents with log *P*s ranging from 1.25 to 9.04 as well as a ‘‘liquid polymer,’’ silicone oil. Figure 1 clearly demonstrates that in the presence of solvents with a log *P* below 5 the growth of the organism was inhibited, and above 5, it was not. For this reason, the critical log *P* of *R. erythropolis* DCL14 is estimated to be 5, and is in reasonable agreement with the reported critical log *P* of *R. erythropolis* of 6 (Inoue and Horikoshi, 1991). The slightly different critical log *P* values may be due to the different strains of this organism employed. Figure 1 does not include a data point for silicone oil since there is currently no reported log *P* value for this material, although its biocompatibility with a variety of organisms has been previously documented (Guieysse et al., 2001; Marcoux et al., 2000). The final biomass concentration for the silicone oil flask was 1,276 mg/L and since this concentration is in the range of the positive control flask (916 mg/L), silicone oil was also considered to be biocompatible.

There is some scatter in the biomass concentrations for those solvents whose log *P* values are above the critical log *P*. This variability is due to the difficulty of separating the biomass from the resulting emulsion which occurred in all flasks with solvents that have a log *P* greater than 5. It has been suggested that emulsion formation in the presence of solvents is linked to cell growth (Bredholt and Eimhjellen, 1999) which confirms that the emulsion formation observed in the flasks with solvents with log *P*s greater than 5 was a result of biomass growth. The formation of an emulsion can

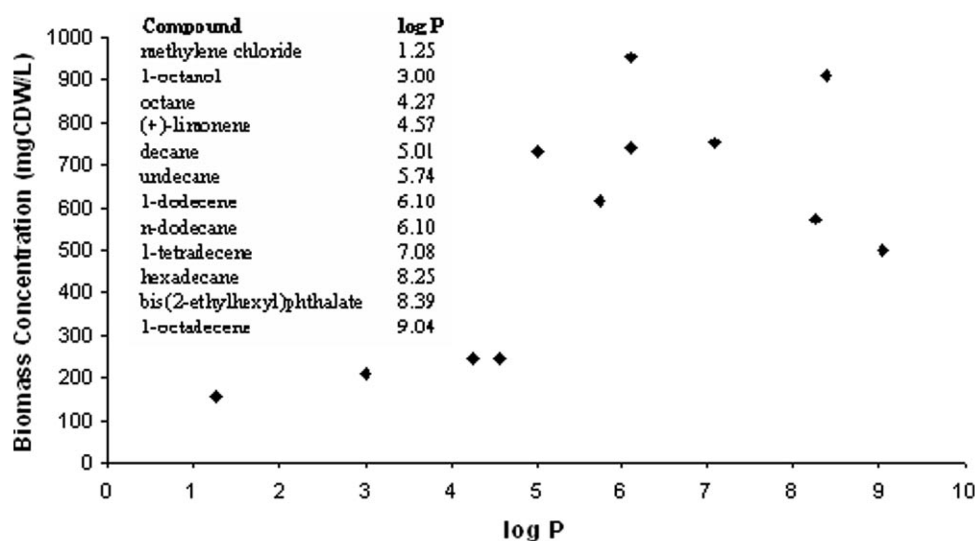

Figure 1. Effect of solvents with varying log *P*s on the growth of *R. erythropolis* DCL14.

be attributed to the ability of some organisms to secrete biosurfactants to increase the availability of a hydrophobic solvent (Madigan et al., 2000).

The biocompatible solvents (1-dodecene, *n*-dodecane, 1-tetradecene, hexadecane, BEHP, 1-octadecene and silicone oil) were further tested for biodegradability. Two biocompatible solvents, decane ($\log P = 5.01$) and undecane ($\log P = 5.74$), were not included in further testing because their $\log P$s are too close to the critical $\log P$ and could potentially affect cell activity.

Biodegradability

Another very important factor in solvent selection is biodegradability of the solvent. It is important that the organism not metabolize the solvent because this can cause loss of solvent (with increased cost for replacement), production of undesirable by-products and can also lead to substrate competition where the solvent is preferentially metabolized relative to the desired biotransformation substrate.

The seven biocompatible solvents were evaluated based on biodegradability, and Figure 2 represents the relative biodegradabilities of the selected solvents with respect to a positive control sample containing corn oil. The values represented by the columns in Figure 2 provide a comparison of the final biomass concentration in each flask, relative to that of the positive control to provide an estimate of the biodegradability of each solvent. It is not clearly evident whether or not a particular chemical class is biodegradable to *R. erythropolis* DCL14, as has been seen elsewhere (MacLeod and Daugulis, 2005), although it appears that alkanes are inherently biodegradable. The biodegradability of the even-chained alkenes is not consistent through the test samples. As expected, the molecularly complex silicone oil is relatively non-biodegradable (perhaps due to the xenobiotic character of the Si–C and Si–O bonds) and BEHP, a large branched-chain ester is also non-biodegradable.

Determination of Solvent Partition Coefficients

The non-biodegradable solvents that were considered further include 1-tetradecene, 1-dodecene, BEHP and silicone oil. Even though *n*-dodecane is slightly biodegradable (Fig. 2) it was tested for partitioning capacity because this was the solvent used in previous work by Tecelão et al. (2001), de Carvalho and da Fonseca (2002a,b) and de Carvalho et al. (2005). Also, though BEHP and 1-tetradecene were found to be non-biodegradable, their partitioning capacities were not determined because they formed an emulsion with the aqueous phase in the abiotic partitioning experiment and were eliminated to avoid further emulsion-related complications. The partition coefficients for carvone and carveol as well as the other selection criteria used to rationally select an appropriate solvent (i.e., volatility, cost, and safety) for 1-dodecene, *n*-dodecane, and silicone oil are summarized in Table I. It is important to note that only one partition coefficient is provided for carveol (representing both isomers) as the partition coefficients of *trans*-carveol and *cis*-carveol were similar for each solvent. Considering the above solvent selection, 1-dodecene was chosen as an appropriate solvent for use as the second phase in this TPPB.

The selection of an organic phase for use in a two phase reactor was also previously undertaken by de Carvalho and da Fonseca (2002a). In this case, the solvent selection criteria included the maintenance of cell viability and biotransformation activity in the presence of the solvent. It was determined that *n*-dodecane ($\log P = 6.01$) satisfied both of these criteria, however, it is important to note that all of the other solvents that were tested by de Carvalho and da Fonseca (2002a) had a $\log P$ lower than the critical $\log P$ of

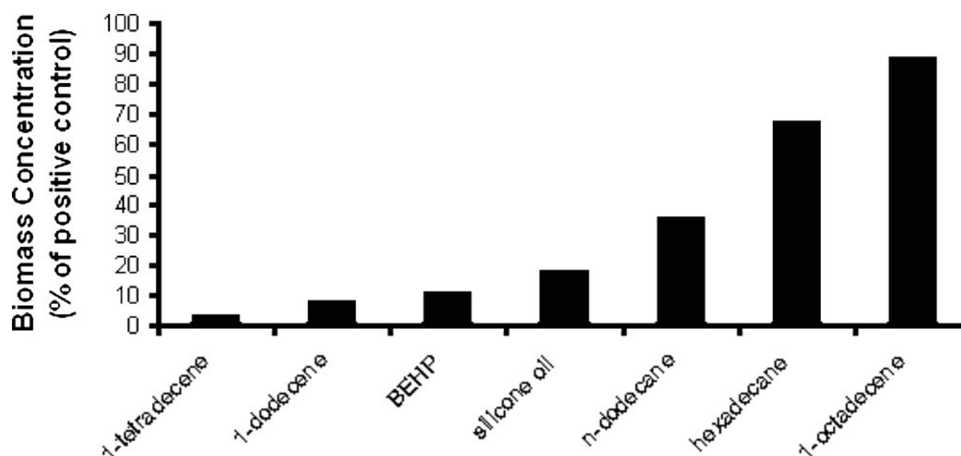

Figure 2. The biodegradabilities of seven biocompatible solvents represented by the biomass concentration of each sample relative to the positive control containing corn oil.

Table I. Solvent properties used in rational solvent selection.

Solvent	PC for carvone	PC for carveol	log *P*	Boiling point (°C)	Cost[a] ($/L)	Identified hazards[b]
1-dodecene	38	8	6.10	213	230	Flash point is 77°C
n-dodecane	31	6	6.10	217	438	None
Silicone oil	12	1	—[c]	>140	308	None

PC, partition coefficient.
[a]Approximate costs in Canadian dollars from company websites.
[b]All solvents have the potential to cause eye and skin irritation, digestive and respiratory irritation if exposed.
[c]There is currently no reported log *P* for silicone oil.

the organism, and as expected, resulted in low biotransformation activity and low cell viability.

Two Phase Biotransformation With 1-Dodecene

Despite completing the rational solvent selection protocol, the hydrophobic nature of *R. erythropolis* DCL14 caused several operational difficulties when attempting to complete the biotransformation in a two-phase system in which 1-dodecene was the second phase. These included enormous difficulties in biomass quantification, sequestration of biomass into the organic phase, formation of a third phase (due to emulsion formation) and difficulty in quantifying the amount of substrate and product in the system due to emulsions. In the presence of 1-dodecene, as soon as the solvent was added to the bioreactor, an emulsion began to form and a significant portion of the biomass partitioned into the second phase as determined by the change in color of the second phase (from transparent to translucent) and confirmed by microscopic examination of the organic phase. The sequestration of biomass into the second phase is cause for concern because the second phase is meant to act as a reservoir for inhibitory concentrations of the toxic compounds. Thus, the biomass that is contained within the organic phase is unable to carry out the biotransformation efficiently which can greatly decrease the performance of the system. Nonetheless, the experiment was continued but the results were inconclusive. Throughout the experiment, a third phase was produced, potentially due to the formation of polysaccharides secreted by the organism in an effort to emulsify the solvent (Madigan et al., 2000).

Two Phase Biotransformation With Silicone Oil

To overcome the operational difficulties associated with the extensive emulsion formation and biomass sequestration that occurred when using 1-dodecene as the second phase, a biotransformation was carried out using silicone oil. Although possessing lower partition coefficients for carveol and carvone than 1-dodecene, silicone oil was selected as being less "organic" than the other solvents tested arising from its structure of repeating silicone-oxygen bonds with only methyl side groups. However, silicone oil is not necessarily an ideal candidate due to its fixed molecular structure which does not allow any opportunity to enhance substrate or product affinity (Prpich and Daugulis, 2006), as is the case when selecting from a broad range of organic solvents.

As soon as the silicone oil was added to the reactor, the agitation was temporarily stopped and it was immediately apparent that the biomass had started to partition into the silicone oil phase as it changed from transparent to translucent, and by the end of the biotransformation the silicone oil phase was opaque. Despite the observation of cell sequestering behavior, microscopic examination confirmed that the extent of sequestration was much less than that observed when the second phase was 1-dodecene which improved the overall operability of the reactor. As well, a procedure was developed to remove the biomass from the solvent/aqueous interface to obtain reasonable biomass estimations and is described in Materials and Methods Section.

With improved operability, the biotransformation was carried out and the results are shown in Figure 3. The substrate was added in a fed-batch manner to ensure that the *trans*-carveol isomer was always available and the substrate additions were stopped once the biotransforma-

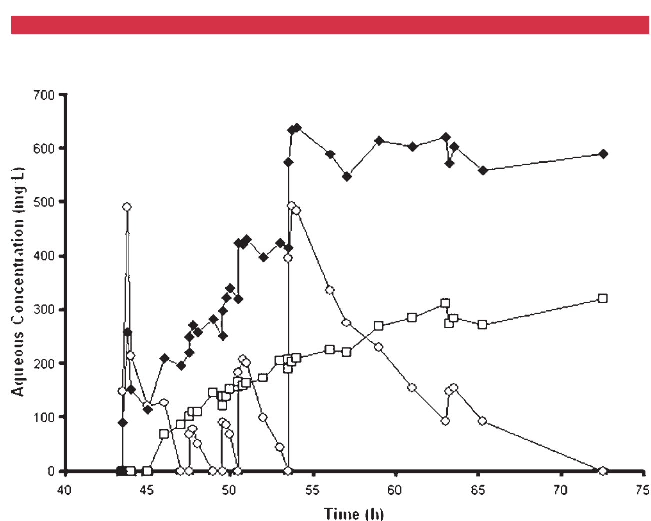

Figure 3. The time course of the substrate and product concentrations throughout the duration of the two phase biotransformation with silicone oil as the second phase. The symbols represent the product, carvone (□), and the two substrate isomers, *cis*-carveol (◆) and *trans*-carveol (○). The volume and time of each carveol addition are as follows: 3 mL (43.5 h), 1 mL (47.5 h), 1 mL (49.5 h), 2 mL (50.5 h), 5 mL (53.5 h), and 1 mL (63.25 h).

tion rate decreased significantly as seen from the decreasing rate of carvone accumulation. The DO was monitored throughout the biotransformation and it was determined that the system was never oxygen limited as the DO never dropped below 85% of saturation. The final product concentration in the aqueous phase was 320 mg/L and the final *cis*-isomer concentration in the aqueous phase was 589 mg/L. These final aqueous concentrations are consistent with the previously determined toxicity thresholds of 200–600 mg carvone/L and 500 mg carveol/L (Morrish and Daugulis, 2008). The volumetric productivity achieved in this reactor configuration was 29 mg/L h which was calculated from the total product concentration in the system (823 mg/L) and the total time for the biotransformation (28.75 h). This productivity is comparable to that achieved in the benchmark single aqueous phase reactor as previously reported to be 31 mg/L h (Morrish and Daugulis, 2008). Although the volumetric productivities of these two reactors are quite similar, the other performance indicators (volume of substrate added and length of biotransformation) highlight the advantage of using the TPPB over the single aqueous phase reactor. In the TPPB with silicone oil as the second phase, 2½ times more substrate was added to the reactor before the biotransformation rate was significantly inhibited and the biotransformation continued for 13.5 h longer than in the single aqueous phase case. The use of a second phase clearly allows for higher substrate loading which suggests that the system does not require as much monitoring and maintenance (i.e., continual addition of small amounts of substrate) and ensures that the overall mass of product obtained is higher. It is advantageous to have a longer biotransformation time when using a cyclic batch reactor because there will ultimately be fewer biotransformation cycles and therefore less turn around time required, which in turn affects volumetric productivity. The TPPB also offers advantages with respect to downstream product recovery because the product is concentrated in the second phase. Although we have not attempted to recover the product from the solvent phase, we speculate that product recovery from the solvent could be achieved by back-extraction into a buffer or by vacuum distillation. Product recovery from the sequestering phase has been demonstrated in a recently submitted manuscript in which the partitioning phase consists of rationally selected solid polymer beads. It was shown that product recovery from the solid polymer beads could be achieved by a simple one-step extraction into methanol.

In previous work Tecelão et al. (2001) undertook the same biotransformation in a biphasic system in shake flasks with a 20 mL aqueous phase and either a 4 mL *iso*-octane or a 20 mL *n*-dodecane second phase. Since *iso*-octane, has a $\log P$ lower than the critical $\log P$ of *R. erythropolis* DCL14 (4.07 vs. 5.00), it is likely that the metabolic activity of the organism was affected by the non-biocompatible solvent. The biotransformation substrate was supplied to the system by the addition of the loaded solvent phase. Once the *trans*-carveol isomer had been depleted, the solvent phase was removed and replaced with fresh solvent fully loaded with substrate. This strategy was developed in an effort to reduce the build-up of *cis*-carveol in the system. It was observed that after each substrate addition, the biotransformation rate decreased. This decrease was attributed to the lack of co-factor regeneration (no carbon source supplied) as well as a loss of biomass due to the removal of the solvent phase (and resulting emulsion). In an effort to conserve the biomass concentration, a larger solvent volume (20 mL) was applied and after the *trans*-carveol depletion, only a portion of the solvent was removed (and replaced with an equal volume of newly loaded solvent) such that the emulsion (containing cells) would remain intact. At this point the authors also switched the organic solvent from *iso*-octane, to a more biocompatible solvent, *n*-dodecane ($\log P = 6.10$). For the *n*-dodecane reaction system, a total of 1,434 mg carveol (mixture of isomers) was added during the biotransformation and produced a total of 781 mg carvone. It is not possible to determine a volumetric productivity to which the current results can be compared because the length of the biotransformation was not stated.

In summary, a rational solvent selection regime was applied to determine an appropriate solvent to be used as a second phase in a TPPB based on biocompatibility, non-biodegradability and affinity for the target compounds. The extremely hydrophobic nature of *R. erythropolis* DCL14 presented significant operational challenges when the organic solvent (1-dodecene) was used in the bioreactor. However, the use of silicone oil as the second phase improved the overall operability of the liquid–liquid TPPB and despite a few remaining operational difficulties the reactor performance was significantly improved with respect to that achieved in a single aqueous phase reactor. In order to alleviate the operational challenges associated with the use of this highly hydrophobic organism, the use of polymer beads as the partitioning phase (Prpich and Daugulis, 2007) is currently being explored.

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