

Improved Reactor Performance and Operability in the Biotransformation of Carveol to Carvone Using a Solid–Liquid Two-Phase Partitioning Bioreactor

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ABSTRACT: In an effort to improve reactor performance and process operability, the microbial biotransformation of (–)-*trans*-carveol to (R)-(–)-carvone by hydrophobic *Rhodococcus erythropolis* DCL14 was carried out in a two phase partitioning bioreactor (TPPB) with solid polymer beads acting as the partitioning phase. Previous work had demonstrated that the substrate and product become inhibitory to the organism at elevated aqueous concentrations and the use of an immiscible second phase in the bioreactor was intended to provide a reservoir for substrates to be delivered to the aqueous phase based on the metabolic rate of the cells, while also acting as a sink to uptake the product as it is produced. The biotransformation was previously undertaken in a two liquid phase TPPB with 1-dodecene and with silicone oil as the immiscible second phase and, although improvement in the reactor performance was obtained relative to a single phase system, the hydrophobic nature of the organism caused the formation of severe emulsions leading to significant operational challenges. In the present work, eight types of polymer beads were screened for their suitability for use in a solid–liquid TPPB for this biotransformation. The use of selected solid polymer beads as the second phase completely prevented emulsion formation and therefore improved overall operability of the reactor. Three modes of solid–liquid TPPB operation were considered: the use of a single polymer bead type (styrene/butadiene copolymer) in the reactor, the use of a mixture of polymer beads in the reactor (styrene/butadiene copolymer plus Hytrel[®] 8206), and the use of one type of polymer beads in the reactor (styrene/butadiene copolymer), and another bead type (Hytrel[®] 8206) in an external column through which fermentation medium was recirculated. This last configuration achieved the best reactor performance with 7 times more substrate being added throughout the biotransformation relative to a single aqueous phase benchmark reactor and 2.7 times more substrate being added relative to the best two liquid TPPB case. Carvone was quantitatively recovered from the polymer beads via single stage extraction into methanol, allowing for bead re-use.

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KEYWORDS: carvone; solid–liquid two phase partitioning bioreactor; polymer beads; *Rhodococcus erythropolis* DCL14

Introduction

The worldwide flavor and fragrance industry is commercially significant and was estimated to be valued at US\$ 19,910,000,000 in 2007 (www.leffingwell.com). Many flavor and fragrance compounds come from natural sources such as the essential oils of plants and seeds; correspondingly, because of the limited supply, there are only about 400 aromas that are manufactured on a scale greater than 1 ton per year (Krings and Berger, 1998) and many of these can be very costly. There is significant consumer interest in the natural production of these valuable compounds due to increasing health- and nutrition-conscious lifestyles (Abraham et al., 1994), and the rising demand for natural products used in food applications has increased the necessity for the production of natural flavor compounds (Etschmann and Schrader, 2006). An alternative production method for flavor/aroma chemicals is via microorganisms (Gatfield, 1988) whose products are considered to be natural. The natural label is important for the profitability of microbiologically produced flavors/aromas (Krings and Berger, 1998).

Carvone is a common flavor and fragrance compound 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. The essential oil containing carvone is produced primarily by caraway (*Carum carvi*), dill (*Anethum graveolens*) and spearmint (*Mentha spicata*) plants (Ravid et al.,

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1992). Both carvone enantiomers, especially (*R*)-(-)-carvone, which has a spearmint flavor, are widely used by the flavor and fragrance industry (Welsh et al., 1989). Carvone can be used for numerous applications, namely in food additives, as antimicrobial/antifungal agents and as a potato sprout inhibitor.

The oil yield obtained from caraway, spearmint, and dill plants varies based on production conditions such as harvesting date and cultivar or population of the various plants (Bailer et al., 2001; Galambosi and Peura, 1996). There are also several factors that can influence the production of plant-based essential oils, including the time required for plant development, weather conditions, soil and fertilizer composition and available micro-nutrients (de Carvalho and da Fonseca, 2006). Other natural limitations include competition with weeds, insects, and plant disease (de Carvalho and da Fonseca, 2006).

The production of carvone by microbial biotransformation offers several advantages including the ability to overcome the seasonality, essential oil content in the plant and other agriculturally related problems (de Carvalho and da Fonseca, 2002). The single-step microbial biotransformation of *trans*-carveol to (*R*)-(-)-carvone is carried out by whole cells of *Rhodococcus erythropolis* DCL14. When grown on limonene as the sole carbon source, *R. erythropolis* DCL14 exhibits carveol dehydrogenase (CDH) activity (de Carvalho and da Fonseca, 2002). The CDH activity allows the stereoselective conversion of the substrate's *trans*-isomer to (*R*)-(-)-carvone, resulting in a second product of isomerically resolved *cis*-carveol (de Carvalho et al., 2005). When *R. erythropolis* DCL14 is grown on limonene, the enzyme activity is NAD-dependant (de Carvalho and da Fonseca, 2002), however 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. A major bottleneck in the microbial production of carvone from carveol arises from the fact that both the substrate and the product are inhibitory to cells of *R. erythropolis* DCL14 (Morrish and Daugulis, 2008), greatly affecting the volumetric productivity of the system.

Previously, this biotransformation has been successfully undertaken in a single aqueous phase reactor (Morrish and Daugulis, 2008) and a two liquid phase partitioning bioreactor (TPPB) (Morrish et al., 2008). *R. erythropolis* DCL14 is a highly hydrophobic organism and has been known to produce very strong emulsions in the presence of hydrophobic liquids, which result in severe operational challenges. To overcome these negative aspects, as well as to provide partitioning of the inhibitory substrate and product, a two phase partitioning bioreactor with polymer beads as the second phase is being proposed. To our knowledge, the present study is only the second to have examined the use of solid-liquid TPPBs for the bioproduction of high value nutraceuticals, the first being the production of 3-methylcatechol (Prpich and Daugulis, 2007). Polymer beads are an excellent candidate for use as the second phase

in a TPPB because it is well known that polymers are able to absorb organic molecules (Amsden et al., 2003) and they are capable of partitioning substrates to the aqueous phase in response to the metabolic demand of the organisms (Amsden et al., 2003). Unlike most organic solvents, polymers are low-cost, non-volatile, biocompatible, non-biodegradable, reusable, easy to handle and allow for simple recovery from the bioreactor. The most important advantage is that the polymer structure can be tailored to enhance selective absorption of the desired target molecule(s) (Prpich and Daugulis, 2006). It is important to choose the most suitable polymer in order to achieve improved reactor performance and maximize productivity. A rational strategy for selecting polymers for use in solid-liquid TPPBs has recently been described (Rehmann et al., 2007). In the specific case of using polymer-based TPPBs for the bioproduction of flavor/fragrance compounds polymers also offer the advantage over organic solvents of not imparting any flavor or fragrance to the desired product thus maintaining organoleptic quality.

In this work, the biotransformation of carveol to carvone has been undertaken in a TPPB using solid polymer beads as the second phase. Eight different polymer types were examined for potential use in this system based on a variety of considerations including partition coefficients for the biotransformation substrate and product. Three different reactor configurations have been employed with the aim of improving reactor performance. A single polymer used in situ, a mixture of two polymers used in situ, and a polymer mixture with one polymer in situ and one in an external extraction column were explored. In addition to quantifying the performance of these systems, the operability of solid-liquid TPPBs was assessed relative to two liquid TPPBs, as was the recovery of the carvone product from the polymer beads.

Materials and Methods

Chemicals and Polymers

(*R*)-(-)-carvone (CAS 6485-40-1) and (-)-carveol, mixture of *trans*- and *cis*-isomers (CAS 99-48-9) were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). (+)-limonene (CAS 5989-27-5), ethyl acetate (CAS 141-78-6) and methanol (CAS 67-56-1) were purchased from Fisher Scientific (Oakville, ON, Canada). The polymers that were tested for use as the second phase included: KRATON[®] D4150K, styrene/butadiene ABA block copolymer, TONE[™] P787, Desmopan 453, Elvax[®] 770, Nucrel[®] 925, ethylene/vinyl alcohol copolymer and Hytre[®] 8206. The properties of each of the polymers are summarized in Table I.

Microorganism, Medium and Inoculum Preparation

R. erythropolis DCL14 was originally obtained from a sediment sample in Reeuwijk, The Netherlands (van der

Table 1. Candidate polymer details.

Polymer				
Commercial or common name	Chemical name	Provider	Glass transition temperature (T_g , °C)	Density (g/mL)
KRATON [®] D4150K	Styrene/butadiene linear triblock copolymer	Kraton	Styrene: 90; butadiene: -90	0.92
Styrene/butadiene	Styrene/butadiene, ABA block copolymer	Scientific Polymer Products Inc.	Styrene: 90; butadiene: -90	0.94
Hytre [®] 8206	Polyether-ester	Dupont	-59	1.17
TONE [™] P787	Linear polycaprolactone polyester	Dow Chemical	-69	1.15
Desmopan 453	Thermoplastic polyurethane	Bayer Material Science	-34	1.22
Elvax [®] 770	Ethylene/vinyl acetate	Dupont	30	0.93
Nucrel [®] 925	Ethylene/methacrylic acid copolymer	Dupont	228	0.94
EVA	Ethylene/vinyl alcohol	Scientific Polymer Products Inc.	55	1.14

Werf et al., 1999) and 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.

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), K₂HPO₄ (1.55 g), NaH₂PO₄·H₂O (0.85 g), NH₄Cl (3 g), MgCl₂·6H₂O (0.075 g), (NH₄)₂SO₄ (1 g) and 0.2 mL trace element as described by Vishniac and Santer (1957). The medium used for fermentations contained no glucose. The medium was autoclaved at 121°C for 45 min.

For all shake flask experiments and fermentations, the inoculum was prepared by adding 100 µL *R. erythropolis* DCL14 stock culture to 50 mL growth medium. The inoculum flasks were incubated at 30°C for 48 h at 180 rpm. For fermentations, two 50 mL inoculum flasks were added to the reactor, which contained 3 L sterile medium.

Analytics

Gas Chromatography

Gas chromatography was used to identify and quantify carvone as well as the individual carveol isomers contained within aqueous samples. The 5 mL aqueous samples were added to 5 mL ethyl acetate. The samples were then vortexed for 10 s, twice. After phase separation, a 1 µL sample of the ethyl acetate layer was then injected into the Varian GC which was equipped with 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 flowrate of 30 mL/min. The hydrogen and air flowrates 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°C 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. The Varian GC was also used to examine methanol samples used in the extraction of the product from the polymer beads. It

was not possible to use ethyl acetate as the extractant as it caused the polymers to dissolve.

Biomass Quantification

Biomass samples were quantified using cell dry weight measurements. The aqueous samples of known volume were dried in pre-weighed metal dishes in a 90°C oven overnight.

Polymer Selection

Bioavailability

Eight polymers were tested for their bioavailability in the presence of *R. erythropolis* DCL14. To separate 125 mL flasks, 50 mL of sterile carbon-free medium, 5 g of the polymer being tested and 1 mL inoculum were added. To reduce the chance of contamination, the polymer beads were placed under a UV lamp for 5 h before being added to the flasks. A positive control with glucose as the carbon source and a negative control with no polymer beads were also prepared. The flasks were incubated at 30°C for 96 h at 180 rpm. After incubation, the aqueous phase was centrifuged, washed with distilled water and the optical density was measured at 650 nm and compared to the controls.

Partitioning Coefficients

The partition coefficients of carvone and carveol were determined for eight different polymers. A partition coefficient is calculated as the ratio of concentration of solute in the polymer phase divided by the concentration of the solute in the aqueous phase at equilibrium. For each polymer sample, 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. Once the samples were well mixed, 5 g of the polymer being tested was added to the vial and the samples were mixed at 180 rpm for 24 h. After 24 h, 5 mL of the aqueous phase was removed to be tested using the GC.

Thermal Stability of Polymers

In order to determine whether or not the candidate polymers would be resistant to sterilization via autoclaving, 5 g of each type of polymer bead was added to a 125 mL flask and covered with water. The flasks were autoclaved at 121°C for 65 min (same autoclave conditions as for the reactors). After the autoclave cycle had finished, the resistance of the polymer beads was determined based on visual observation of the sample.

Biofilm Formation on Polymer Beads

Due to complications involving the hydrophobic nature of the organism, it was important to determine whether *R. erythropolis* DCL14 would produce a biofilm on the surface of the polymer beads which could inhibit the partitioning of the substrate and product between the solid polymer and aqueous phases. The experiment was performed in 125 mL flasks containing 50 mL sterile growth medium, 2 g of the bead being tested and 1 mL inoculum. There were also negative control flasks containing 50 mL sterile growth medium and 2 g of beads. A positive control containing no polymer beads was also prepared. To reduce the chance of contamination due to the addition of the polymer beads, they were placed under a UV lamp for 5 h before being added to the flasks. The optical density (650 nm) of the flasks was measured every 24 h for 96 h. Biofilm formation was measured by comparison of the biomass concentration in the bulk suspension (minus the OD of the negative control flasks) relative to the positive control flask. If the biomass concentration in the flasks with the polymers was lower than that of the control, it was assumed that biomass had formed a biofilm on the surface of the polymer.

Reactor Operation

A 5 L New Brunswick Scientific BioFlo III was used for all biotransformations. 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 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 induction source, (+)-limonene, was supplied by passing an air stream through a sparger contained in a flask of liquid (+)-limonene as previously described by de Carvalho and da Fonseca (2002). The limonene-saturated air flow rate into the reactor was also equipped with a 0.2 µm sterile filter and was 200 mL/min. The reactor containing the carbon-free medium was sterilized at 121°C for 65 min. The inoculum was prepared as outlined above. The transformations began when the biomass concentration had reached approximately 700 mg CDW/L (CDW = cell dry weight). The air and

(+)-limonene flows were continuously supplied throughout the duration of the biotransformation. The biotransformation substrate, (–)-carveol, was supplied in liquid form and the product and substrate concentrations of the microbial biotransformation were monitored frequently using gas chromatography and when the *trans*-carveol isomer was near depletion, more substrate was added such that *trans*-carveol was always available for biotransformation. These substrate additions were supplied once the *trans*-carveol isomer was near depletion until the transformation ceased due to the accumulation of the toxic substrate and product.

Each of the biotransformations had an aqueous volume of 3 L sterile glucose-free medium. In the first TPPB, 0.57 L styrene/butadiene copolymer beads (532 g) were used as the second phase for a total working volume of 3.57 L. In the second TPPB, a mixture of 0.25 L styrene/butadiene copolymer (235 g) and 0.25 L Hytrel[®] 8206 polymer beads (293 g) was used as the second phase for a total working volume of 3.50 L. For the final TPPB configuration, 0.57 L styrene/butadiene copolymer (532 g) was used as the second phase in the reactor and 0.26 L Hytrel[®] 8206 polymer beads (300 g) were packed into a glass external extraction column for a total working volume of 3.83 L. The reactors and the beads were autoclaved separately (the beads were covered with tap water) and the beads were decanted and added just before the transformation started. To sterilize the Hytrel[®] 8206 polymer beads within the extraction column, they were autoclaved inside the glass column that contained approximately 10 mL water (to allow for steam formation). The ends of the glass column were fitted with a short length of rubber tubing that was loosely clamped to allow for pressure to escape. The addition of the polymers just before the biotransformation was initiated (by the addition of carveol) ensured that each reactor would have consistent biomass concentration and that the metabolic state of the biomass would be similar. For the continuous reactor, the recirculation of the medium through sterile tubing using a peristaltic pump operating at a rate of 30 mL/min was started after the addition of carveol.

Product Recovery From Polymer Beads

In order to demonstrate that it is possible to recover carveone from the polymer beads, methanol was used as an extractant. Isolation of flavor compounds from fermentation fluid is typically undertaken via extraction into organic solvents, distillation, or extractive distillation (Cheetham, 1997; Eikani et al., 2005; Gatfield, 1997). Once the biotransformation was complete, a small mass of beads was dried and was then added to 10 mL methanol in a sealed 20 mL vial and placed in a rotary shaker for 24 h at 180 rpm and 30°C. After 24 h, the product concentration in the methanol was measured using the GC. To ensure that all of the product had been extracted in the first methanol aliquot the beads were subsequently added to 10 mL fresh methanol for another 24 h after which the methanol was assayed for carveone using the GC.

Results and Discussion

Polymer Selection

As with choosing an appropriate solvent for use in a liquid–liquid TPPB (Bruce and Daugulis, 1991), a rational selection protocol was followed when choosing a suitable polymer. Candidate polymers may be chosen based on chemical structure (i.e., monomer selection, functional groups, copolymerization and crosslinking) which influences partitioning capacity, as well as bioavailability, thermal resistance to sterilization and a resistance to biofilm formation. A comprehensive list of desirable polymer characteristics is detailed by Rehmann et al. (2007) which include the above characteristics among others. It is generally not necessary to verify biocompatibility of polymers because it is well known that synthetic polymers of the type employed in this study are biocompatible.

Bioavailability

Due to the complexity of their long chains, polymers are generally not susceptible to degradation by microorganisms. In order to confirm this fact for the candidate polymers, the bioavailabilities of eight polymer beads were assessed. It was evident that none of the polymers were bioavailable since the final biomass concentrations relative to a positive control were negligible (data not shown). Although these results indicate that the polymers are not bioavailable, the relative biomass concentrations were not zero. This may be due to either the presence of residual processing materials (i.e., powder coating) on the surface of the polymers that could have leached into solution during the 96 h incubation period and/or the varying hardness of the respective polymers that could have resulted in small particulates being broken off the surface of the polymer beads throughout the 96 h incubation period.

Determination of Polymer Bead Partition Coefficients

The partition coefficients of the candidate polymers were determined and are summarized in Table II along with partition coefficients for selected liquid second phases for comparison purposes. The candidate polymers were purposely chosen to include a “negative control” to demonstrate that not all polymers will have an affinity for the target molecules. Table II shows that KRATON[®] D4150K and styrene/butadiene copolymer have a superior partitioning capacity for carvone. Both of these polymers are styrene/butadiene structures with KRATON[®] D4150K having 31% styrene content and styrene/butadiene copolymer having 28% styrene content. Their affinity for carvone may be associated with their relative polarities. Since ketones (carvone) are less polar than alcohols (carveol) and the copolymers are non-polar, this may explain the higher affinity exhibited by these polymers in a case of “like dissolves like.” Most of the polymers that were tested have a

Table II. Partition coefficients for carvone and carveol in various solvents and polymer beads.

Polymer	PC ^a for carvone	PC ^a for carveol
KRATON [®] D4150K	121	6
Styrene/butadiene copolymer	118	5
Hytrel [®] 8206	49	36
TONE [™] P787	40	19
Desmopan 453	29	14
Elvax [®] 770	7	2
Nucrel [®] 925	2	1
Ethylene/vinyl alcohol	0	0
1-Dodecene ^b	38	8
Silicone oil ^b	12	1

^aPartition coefficient.

^bMorrish et al. (2008).

much higher affinity for carvone than they do for carveol, the exceptions being Hytrel[®] 8206, TONE[™] P787 and Desmopan 453 all of which contain esters as a functional group. An explanation for their added affinity for carveol might be the possibility of hydrogen bonding that the ester functional groups provide between the double-bonded oxygen atom in the ester functional group and the alcohol functional group on the carveol molecule. As expected, the polymer chosen to serve as a “negative control,” ethylene/vinyl alcohol, did not exhibit any affinity for either carvone or carveol. Ethylene/vinyl alcohol is a polar molecule and may be expected to attract the polar carveol molecule, however the glass transition temperature (T_g) of this polymer (44% ethylene) is 55°C which means that the polymer configuration is rigid at 30°C (the temperature at which the experiments were run). Since carveol and carvone are relatively large molecules, their diffusion into the polymer would require the cooperative movement of the polymer chains and since the experiment was run at a temperature below the T_g , it is expected that the polymer chains are very rigid and would not move to facilitate the absorption of the target molecules. Based on the determined partition coefficients, the polymers with the highest affinity for carvone were KRATON[®] D4150K and styrene/butadiene copolymer and those with the highest affinity for *cis*-carveol (the secondary product) are Hytrel[®] 8206, TONE[™] P787 and Desmopan 453.

Thermal Stability of Polymers

Since the biotransformation of (–)-*trans*-carveol to (R)-(–)-carvone uses a pure culture of *R. erythropolis* DCL14, it is essential that the polymer beads are sterilized before they are used in the reactor to reduce the chance of contamination. The simplest and most effective means of sterilization is through high temperatures (via autoclaving) and thus the polymer beads were tested to see if they would maintain their shape and remain as separate pellets so that the surface area available for absorption would be consistent. The results indicated that the only polymer beads suitable to be sterilized by autoclaving were Hytrel[®] 8206, Desmopan

453 and styrene/butadiene copolymer as the sterilization process did not affect the integrity of their polymer shapes. In order to confirm that the absorption capability of the polymers is not affected by autoclaving, absorption tests have been performed which showed that autoclaving the polymers does not change the uptake capacity of the beads (data not shown).

Biofilm Formation on Beads

Due to the previous operational problems that were encountered as a result of the extreme hydrophobicity of *R. erythropolis* DCL14 in the presence of organic solvents (Morrish and Daugulis, 2008), it was considered necessary to determine whether a biofilm would form on the hydrophobic surface of the polymer beads that could potentially inhibit the transfer of substrate and product between the aqueous and polymer phases. The only two polymers tested were styrene/butadiene copolymer and Hytrel[®] 8206 because they met all the polymer selection criteria of being: non-bioavailable, having good affinity for product and substrate (respectively) and being able to withstand sterilization in the autoclave. Desmopan 453 was not considered for further testing because its affinity for the target molecules did not compare to those of styrene/butadiene copolymer and Hytrel[®] 8206 for carvone and carveol, respectively. It was concluded that no biofilm formed on the surface of the polymer beads because the suspended cell concentrations of the two samples containing polymer beads were very close to that of the control sample

which did not contain polymer beads (data not shown). Though this method is an indirect measure of biofilm formation, Amsden et al. (2003) used SEM to evaluate biofilm formation on similar polymer beads and confirmed that there was no evidence of cell adhesion to the beads.

After completion of the polymer bead selection protocol, styrene/butadiene copolymer was selected for use as the second phase in the two phase biotransformation primarily because of its high affinity for carvone, the product. Another candidate polymer, Hytrel[®] 8206 having a high affinity for the unconverted substrate isomer (*cis*-carveol) was also retained for further consideration.

Two Phase Biotransformation With Styrene/Butadiene Copolymer

Once the biomass concentration in the bioreactor had reached approximately 700 mg CDW/L, the polymer beads were added and the biotransformation was initiated by the addition of carveol. The carveol isomer mixture was added in a fed-batch manner when the *trans*-carveol isomer was near depletion, and until the aqueous carveol concentration had approached the previously determined toxicity threshold (200 ± 25 mg carveol/L) (Morrish and Daugulis, 2008) as is evident in Figure 1. It can be seen that the biotransformation rates decrease with each substrate addition due to the accumulation of carvone and *cis*-carveol in the reaction system. The final aqueous concentrations of carvone and *cis*-carveol were 179 and 462 mg/L, respectively, which are in reasonable agreement

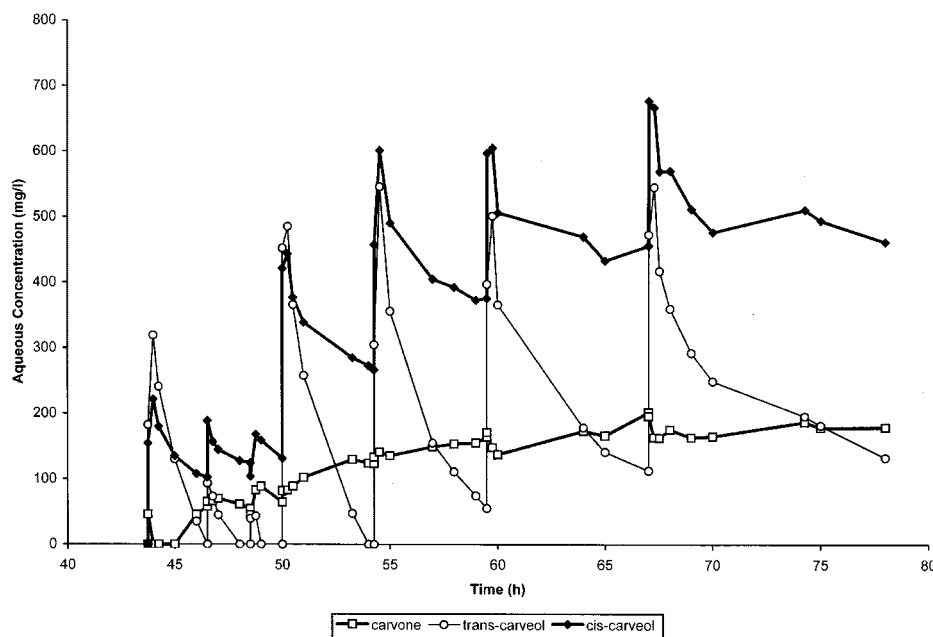


Figure 1. The time course of the substrate and product concentrations throughout the duration of the two phase biotransformation with styrene/butadiene copolymer as the second phase. The symbols represent the product, carvone (\square), and the two substrate isomers, *cis*-carveol (\blacklozenge) and *trans*-carveol (\circ).

with the previously reported inhibition thresholds of 200–600 and 500 mg carveol/L in the aqueous phase (Morrish and Daugulis, 2008). The biotransformation rate was not inhibited by cofactor depletion (air and carbon source were supplied throughout the biotransformation period), nor by oxygen limitation (DO was monitored and was never limiting).

In this configuration, a total of 25 mL of carveol was added over a 34.25 h biotransformation period to obtain a carvone volumetric productivity of 102 mg/Lh. The volumetric productivity was calculated by dividing the final system carvone mass by the total system working volume (aqueous plus polymer) and the biotransformation time. Each of these reactor performance criteria (volume of substrate added before transformation termination, obtainable biotransformation time, and carvone volumetric productivity) were greatly improved compared to those achieved in the single aqueous phase reactor (5 mL carveol, 15.25 h of biotransformation and a product volumetric productivity of only 31 mg/Lh) (Morrish and Daugulis, 2008) and the two liquid phase reactor (13 mL carveol, 28.75 h of biotransformation and a product volumetric productivity of 29 mg/Lh) using silicone oil as the immiscible liquid phase (Morrish et al., 2008).

Closer examination of the *cis*-carveol curve in Figure 1 suggests that the carveol concentration does not plateau after each substrate addition as would be expected since the *cis*-carveol is not consumed in the biotransformation. Instead, after each substrate addition, there is a peak followed by a gradual decrease in aqueous concentration. Since this decrease is not due to the biotransformation of *cis*-carveol it is assumed that this gradual decrease is a consequence of the slow diffusion of *cis*-carveol into the polymer beads. It is well known that equilibrium in a liquid–liquid system is established almost instantaneously whereas equilibrium between a solid and liquid phase can be delayed due to diffusion into the solid polymer matrix (Rehmann and Daugulis, 2007). The diffusion rates of carvone and carveol into styrene/butadiene copolymer have not been determined; however the diffusivities of similarly sized molecules (benzene and phenol) into styrene/butadiene copolymer, Hytrel[®] 8206 and ethylene/vinyl acetate (EVA) have been determined. The diffusion rates of benzene into EVA and styrene/butadiene copolymer were determined by Daugulis et al. (2003) to be 4.3×10^{-6} and 2.23×10^{-6} cm²/s, respectively. The diffusion rates of phenol into Hytrel[®] 8206 and EVA are similar and are reported by Prpich and Daugulis (2004) to be 1.54×10^{-7} and 3.73×10^{-9} cm²/s, respectively. Slower uptake of the target molecules may also be due to the surface area available for transfer in the solid–liquid system being less than that for the liquid–liquid system because the polymer beads are larger than the minute solvent droplets formed as a result of vigorous agitation in TPPB bioreactors.

Aside from the improved volumetric productivity, increase in substrate added and increase in biotransformation time, this reactor configuration offers several operational advantages over the two liquid phase bioreactor

(Morrish et al., 2008). First and foremost is the lack of emulsion formation in the presence of the polymer beads which had caused the cells to partition into the organic liquid phase in the two liquid TPPBs, and had made estimation of process variable concentrations (cells, substrate, product) and thus material balances extremely difficult. It is also interesting to note that there were no dramatic morphological changes to the cells observed as had been seen in the single phase bioreactor configuration (Morrish and Daugulis, 2008).

Two Phase Biotransformation With a Mixture of Styrene/Butadiene Copolymer and Hytrel[®] 8206 Polymer Beads

In the previous fed-batch two phase biotransformation with styrene/butadiene copolymer beads as the second phase, the ability of the organism to carry out the biotransformation was ultimately affected by inhibitory concentrations of substrate and product, as anticipated. Since for styrene/butadiene the partition coefficient for the substrate, carveol, is much lower than that for the product, carvone, carveol accumulated more quickly in the aqueous phase likely causing acute inhibition. In an effort to balance the partition coefficients of carvone and carveol in the reactor, a two phase biotransformation was conducted in which two types of polymer beads were employed. The choice for the second polymer was Hytrel[®] 8206 because of its affinity for carveol (Table II). The goal of this biotransformation was to demonstrate the ability to tailor a mixture of polymer beads in the reactor to affect the ratio of inhibitory substances in the aqueous phase. This simple adjustment is an effect that is unique to the case in which polymer beads are used as the second phase in a TPPB. Previous work by Bruce and Daugulis (1991) had demonstrated the ability to use mixtures of organic solvents as the extractant phase in a TPPB, whereby a non-biocompatible solvent with a higher affinity for a target molecule was mixed in small proportions with a biocompatible solvent with a lower partition coefficient. The selection process for the co-solvents was quite involved, however, and did not always provide reliable outcomes, particularly with regard to the overall biocompatibility of the solvent mixture. Using mixtures of polymer beads to influence the overall partitioning of target molecules is much more straightforward as biocompatibility is not a consideration (since the majority of polymers are inherently biocompatible) and as such the only selection criterion for choosing the polymer mixtures would be their partitioning capability. In addition, the separation of the polymer types for re-use is much easier than is the separation of solvent mixtures.

The biotransformation was started with the addition of the carveol isomer mixture when the biomass concentration had reached 700 mg CDW/L. As before, the carveol isomer mixture was added in a fed-batch manner when the *trans*-carveol isomer was near depletion and until the aqueous

carvone concentration had approached the previously determined toxicity threshold (200 ± 25 mg carvone/L) (Morrish and Daugulis, 2008) as is evident in Figure 2. The final carvone and *cis*-carveol concentrations were 198 and 294 mg/L, respectively resulting in a product volumetric productivity of 106 mg/L.h. The volumetric productivity was again calculated by dividing the final system carvone mass by the total system working volume (aqueous plus polymers) and the biotransformation time (24 h). The final *cis*-carveol concentration was lower than that found through the toxicity test (500 mg carveol/L) (Morrish and Daugulis, 2008), but it is apparent that immediately after each substrate addition (before it is absorbed into the polymers) the aqueous phase (and hence biomass) is exposed to concentrations of *cis*-carveol of approximately 600 mg/L which could be sufficient to inhibit the biotransformation activity of the cells.

Figure 2 shows the time course data for the substrate and product throughout the fed-batch biotransformation. In this case, compared to the previous result in a TPPB with styrene/butadiene copolymer used as the second phase, it is clear that the combined partitioning capacity of styrene/butadiene copolymer and Hytrel[®] 8206 is inferior. After only five substrate additions (a total of 15 mL carveol), as compared to the seven substrate additions in the previous reactor (totaling 25 mL), the reaction rate had decreased significantly as seen in the carvone accumulation curve. The styrene/butadiene copolymer beads have a very strong affinity for carvone (partition coefficient of 118), thus when half of the volume of these beads were replaced with Hytrel[®]

8206, with a much lower affinity for carvone (partition coefficient of 49), there was ultimately more carvone present in the aqueous phase. Despite the fact that only 15 mL of substrate was added over a period of 24 h, the data are successful in demonstrating that by changing the fractions of polymer in the reactor, the concentration of the target compound(s) in the aqueous phase can be manipulated. Through further polymer selection, it may be possible to identify a polymer that has a comparable affinity for carveol as styrene/butadiene copolymer has for carvone which would maintain low concentrations of both substrate and product in the aqueous phase. Again, it is interesting to note the slow diffusion of *cis*-carveol into the polymer beads as seen by the gradual decrease from the initial concentrations after each substrate addition.

Two Phase Continuous Biotransformation With Styrene/Butadiene Copolymer and Hytrel[®] 8206

The strategy in this experiment was to address the accumulation of the *cis*-isomer in the aqueous phase. In the two phase reactor with styrene/butadiene copolymer as the second phase, the biotransformation rate was inhibited by the accumulation of the *cis*-carveol isomer, and to mitigate this accumulation, an external column packed with Hytrel[®] 8206 polymer beads was employed along with styrene/butadiene copolymer beads in the reactor. This configuration ensures that the polymer phase in the reactor (styrene/butadiene copolymer) will have the full extraction

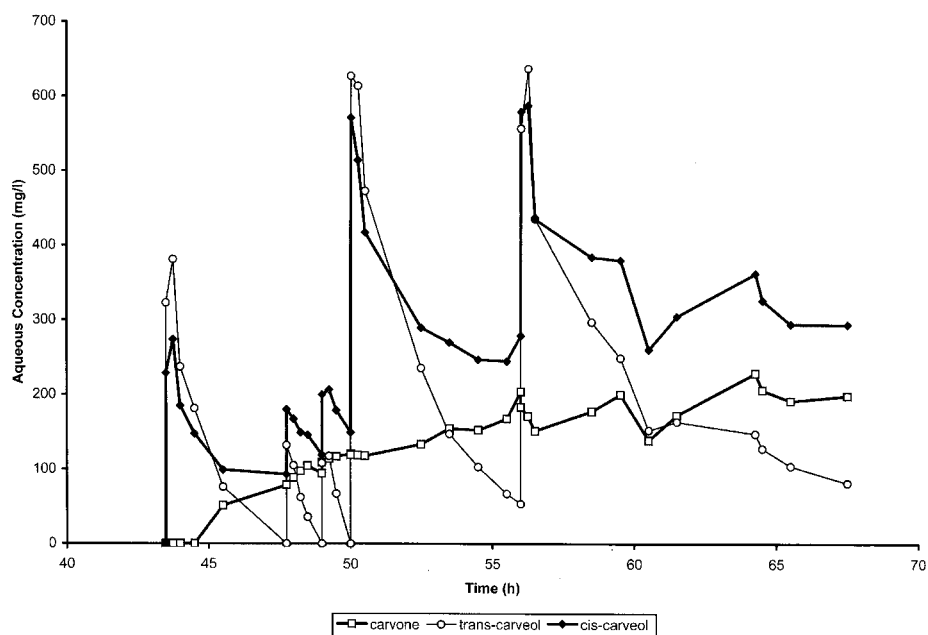


Figure 2. The time course of the substrate and product concentrations throughout the duration of the two phase biotransformation with a mixture of styrene/butadiene copolymer and Hytrel[®] 8206 as the second phase. The symbols represent the product, carvone (□), and the two substrate isomers, *cis*-carveol (◆) and *trans*-carveol (○).

potential for carvone (as did the first reactor configuration) and the Hytrel[®] 8206 containing column will act to reduce the accumulating *cis*-carveol isomer present in the aqueous phase.

Using a similar operating policy, the carveol isomer mixture was added in a fed-batch manner when the *trans*-carveol isomer was near depletion and until the aqueous carvone concentration had approached the previously determined toxicity threshold (200 ± 25 mg carvone/L) (Morrish and Daugulis, 2008) as is evident in Figure 3.

From Figure 3 it is clear that this operating strategy was highly successful in improving reactor performance with a total of 35 mL carveol being added to the reactor over 48.75 h before the reaction rate decreased significantly. The final product volumetric productivity was 99 mg/L h, which was calculated by dividing the final carvone mass by the system working volume (aqueous plus polymers) and biotransformation time. The final carvone and *cis*-carveol concentrations in the aqueous phase were 225 and 400 mg/L, respectively.

Although volumetric productivity is a commonly used indicator of reactor performance, in this case it may not adequately represent the performance of these three reactors because of the changing working volumes, the changing biotransformation times, and the mode of operation. The volumetric productivities achieved for each reactor are very similar, however it is important to consider total volume of substrate added to each reactor as well as “on stream”

operation time to get a more complete estimate of reactor performance. Volumetric productivity calculations for fed-batch operation often do not take into account turnaround time for dumping, cleaning, filling and sterilizing which are necessary activities for prolonged operation with multiple batches. In fact, fed-batch systems that are “on stream” for longer periods of time between turnarounds may in fact be penalized in simple volumetric productivity calculations even though they may be superior operationally. Thus, if the volumetric productivities from the three reactor configurations are adjusted to include a 12-h turnaround time, a more balanced comparison, along with the other performance indicators (total volume of substrate added and biotransformation time), emerges and is summarized in Table III.

Product Recovery From Polymer Beads

To demonstrate that it is possible to recover the product from the polymers at the end of the biotransformation, extractions of carvone from the polymer beads were performed into methanol. After 24 h of extraction into one aliquot of methanol, all of the carvone had partitioned from the polymer phase, as a second extraction of the polymers into fresh methanol yielded no additional carvone. As an example of product recovery, after the third biotransformation, the masses of carvone recovered from three styrene/butadiene copolymer beads and three Hytrel[®]

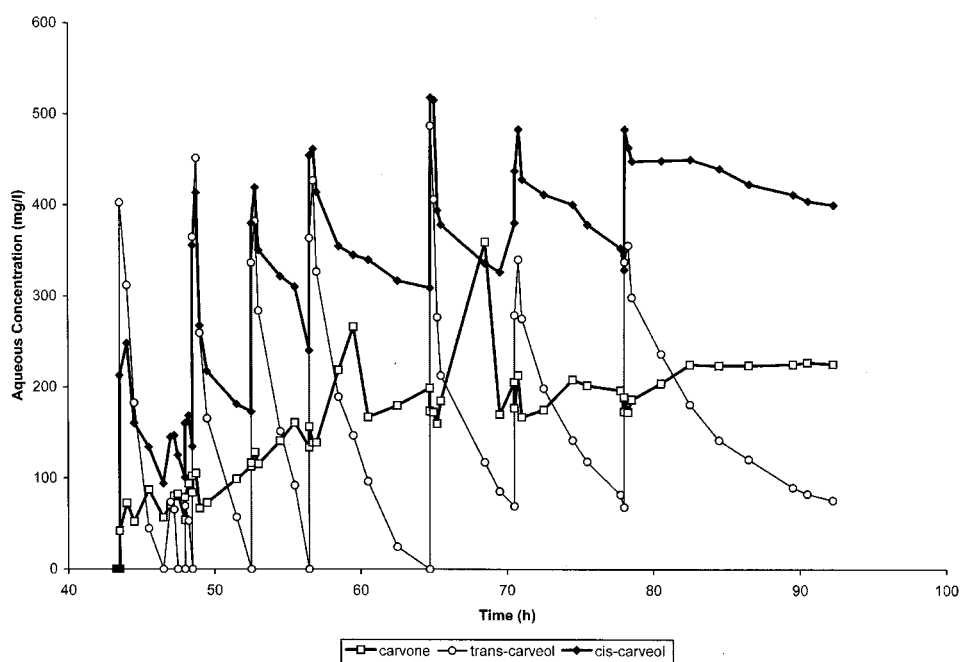


Figure 3. The time course of the substrate and product concentrations throughout the duration of the two phase biotransformation with styrene/butadiene copolymer as the second phase and Hytrel[®] 8206 contained within an external extraction column. The symbols represent the product, carvone (\square), and the two substrate isomers, *cis*-carveol (\blacklozenge) and *trans*-carveol (\circ).

Table III. Summary of reactor performance.

	Carveol added (mL)	Time (h)	Volumetric productivity (mg/L/h)	Volumetric productivity with 12 h turnaround time (mg/L/h)
Single phase ^a	5	15.25	31	17
Liquid-liquid TPPB ^b	13	28.75	29	20
Solid-liquid TPPB 1	25	34.25	102	76
Solid-liquid TPPB 2	15	24	106	71
Solid-liquid TPPB 3	35	48.75	99	79

^aMorrish and Daugulis (2008).^bMorrish et al. (2008).

8206 polymer beads were 1 and 4 mg, respectively, resulting in calculated partitioning coefficients of 104 for styrene/butadiene copolymer and 35 for Hytrel[®] 8206. These values are in reasonable agreement with the abiotic partition coefficients of 118 and 49 in Table II.

The above results demonstrate that in a relatively short time period (24 h) and using a small volume of methanol, it is possible to efficiently recover the product from the beads if an appropriate extractant is used. In addition to product recovery, this downstream step allows for regeneration of the polymer beads for future re-use. Product extraction from polymer beads in this manner may be much easier than the removal of products from a liquid organic extracting phase where distillation of the solvent or back extraction using another immiscible solvent is required.

In summary, the use of rationally selected polymer beads as the partitioning phase in TPPBs has resulted in enormously enhanced operability in the bioproduction of carvone by the highly hydrophobic organism *R. erythropolis* DCL14 relative to two-liquid TPPB configurations. This enhanced operability might reasonably be expected in other biotransformations when using hydrophobic and/or surfactant producing organisms. Two phase bioreactors, whether liquid-liquid or solid-liquid, also easily outperformed single phase systems that feature inhibitory substrates/products. The solid-liquid TPPB reactor configuration with an external extraction column packed with polymer beads in addition to in situ polymers, demonstrated that rational polymer selection, including the use of polymer mixtures, can favorably influence aqueous concentrations of target molecules. The product, carvone, was easily and quantitatively recovered from the polymers by single stage extraction into methanol, resulting in the prospect of polymer bead re-use. Significantly, the use of polymers rather than organic solvents in TPPBs, is also advantageous in the microbial bioproduction of flavor/fragrance compounds since the polymers do not impart any fugitive off-aromas that could affect product quality or other organoleptic properties.

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References

- Abraham B, Berger RG, Schulz H. 1994. Oakmoss impact volatiles generated by basidiomycete of the genus *polyporus*. *Flav Frag J* 9: 265–268.
- Amsden BG, 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.
- Bailer J, Aichinger T, Hackl G, de Hueber K, Dachler M. 2001. Essential oil content and composition in commercially available dill cultivars in comparison to caraway. *Ind Crop Prod* 14:229–239.
- Bruce LJ, Daugulis AJ. 1991. Solvent selection strategies for extractive biocatalysis. *Biotechnol Prog* 7:116–124.
- Burdock GA. 1995. Fenaroli's handbook of flavor ingredients, 3rd edn. Boca Raton, FL: CRC Press.
- Cheetham PSJ. 1997. Combining the technical push and the business pull for natural flavours. *Adv Biochem Eng Biotechnol* 55:1–49.
- Daugulis AJ, Amsden BG, Bochanysz J, Kayssi A. 2003. Delivery of benzene to *Alcaligenes xylosoxidans* by solid polymers in a two-phase partitioning bioreactor. *Biotechnol Lett* 25:1203–1207.
- de Carvalho CCCR, da Fonseca MMR. 2002. Maintenance of cell viability in the biotransformation of (–)-carveol with whole cells of *Rhodococcus erythropolis*. *J Mol Catal B: Enzym* 19:389–398.
- de Carvalho CCCR, da Fonseca MMR. 2006. Carvone: Why and how should one bother to produce this terpene. *Food Chem* 95:413–422.
- de Carvalho CCCR, Poretti A, da Fonseca MMR. 2005. Cell adaptation to solvent, substrate and product: A successful strategy to overcome product inhibition in a bioconversion system. *Appl Microbiol Biotechnol* 69:268–275.
- Eikani MH, Golmohammad F, Rowshanzamir S, Mirza M. 2005. Recovery of water-soluble constituents of rose oil using simultaneous distillation-extraction. *Flavour Frag J* 20:555–558.
- Etschmann MMW, Schrader J. 2006. An aqueous-organic two-phase bioprocess for efficient production of the natural aroma chemicals 2-phenylethanol and 2-phenylethylacetate with yeast. *Appl Microbiol Biotechnol* 71:440–443.
- Galamposi B, Peura P. 1996. Agrobotanical features and oil content of wild and cultivated forms of caraway. *J Essent Oil Res* 8:389–397.
- Gatfield IL. 1988. Production of flavor and aroma compounds by biotechnology. *Food Technol* 42:110–120.
- Gatfield IL. 1997. Biotechnological production of flavour-active lactones. *Adv Biochem Eng Biotechnol* 55:221–238.
- Krings U, Berger RG. 1998. Biotechnological production of flavours and fragrances. *Appl Microbiol Biotechnol* 49:1–8.
- Morrish JLE, Daugulis AJ. 2008. Effects of inhibitory substrate and product on the carvone biotransformation activity of *Rhodococcus erythropolis* DCL14. *Biotechnol Lett*, in press. DOI: 10.1007/s10529-008-9686-5.
- Morrish JLE, Brennan ET, Dry HC, Daugulis AJ. 2008. Enhanced bioproduction of carvone in a two liquid phase partitioning bioreactor with a highly hydrophobic biocatalyst. *Biotechnol Bioeng*, in press. DOI:10.1002/bit.21941.
- Ppich 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. 2006. Biodegradation of a phenolic mixture in a solid-liquid two-phase partitioning bioreactor. *Appl Microbiol Biotechnol* 72:607–615.
- Prpich GP, Daugulis AJ. 2007. A novel solid-liquid two-phase partitioning bioreactor for the enhanced bioproduction of 3-methylcatechol. *Biotechnol Bioeng* 98:1008–1016.
- Ravid U, Putievsky E, Katzir I, Weinstein V, Ikan R. 1992. Chiral GC analysis of (S)(+)- and (R)(-)-carvone with high enantiomeric purity in caraway, dill and spearmint oils. *Flavour Frag J* 7:289–292.
- Rehmann L, Daugulis AJ. 2007. Biodegradation of biphenyl in a solid-liquid two-phase partitioning bioreactor. *Biochem Eng J* 36:195–201.
- Rehmann L, Sun B, Daugulis AJ. 2007. Polymer selection for biphenyl degradation in a solid-liquid two-phase partitioning bioreactor. *Biotechnol Progr* 23:814–819.
- van der Werf MJ, Swarts HJ, de Bont JAM. 1999. *Rhodococcus erythropolis* DCL14 contains a novel degradation pathway for limonene. *Appl Environ Microbiol* 65:2092–2102.
- Vishniac W, Santer M. 1957. The thiobacilli. *Bacteriol Rev* 21:195–213.
- Welsh FW, Murray WD, Williams RE. 1989. Microbiological and enzymatic production of flavor and fragrance chemicals. *Crit Rev Biotechnol* 9:105–169.
- Wiegant WM, de Bont JAM. 1980. A new route for ethylene-glycol metabolism in *Mycobacterium*-E44. *J Gen Microbiol* 120:325–331.