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ORIGINAL RESEARCH PAPER

Inhibitory effects of substrate and product on the carvone biotransformation activity of *Rhodococcus erythropolis*

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Abstract The aqueous substrate and product toxicity thresholds in the microbial biotransformation of (−)-*trans*-carveol to the fragrance/flavor compound (*R*)-(−)-carvone by *Rhodococcus erythropolis* were determined. Above aqueous phase concentrations of approx. 500 mg carveol/l and 200–600 mg carvone/l, the biotransformation activity of the biocatalyst was inhibited. This biotransformation was undertaken in a single aqueous phase 3 reactor in which a total of 5 ml carveol (mixture of isomers) was added before the biotransformation rate decreased significantly. The carvone volumetric productivity was 31 mg/lh. Although the growth of the organism post-exposure was not affected, dramatic morphological changes in response to the accumulation of the inhibitory substrate and product were observed.

Keywords Biotransformation · Carvone · Morphology · *Rhodococcus erythropolis* DCL14 · Toxicity

Introduction

Carvone is widely used to flavor various food products, pharmaceuticals and even toothpaste (Baysal and Starmans 1999). Carvone also exhibits antimicrobial/antifungal properties (Carson and Riley 1995) and is used as a potato sprout inhibitor in the Netherlands under the name "Talent" (Hartmans et al. 1995). Carvone exists as two enantiomers, (*R*)-(−)-carvone which has a spearmint aroma and (*S*)-(+)-carvone which has a caraway aroma. Most commonly, (*R*)-(−)-carvone is produced in the essential oils of spearmint (*Mentha spicata*) and dill (*Anethum graveolens*) plants whereas (*S*)-(+)-carvone is produced primarily in the essential oil of caraway (*Carum carvi*) plants (Ravid et al. 1992). Carvone is traditionally extracted from the aforementioned plants through hydrodistillation, steam distillation and solvent extraction (Kallio et al. 1994). The product content and quality in the plants depends largely on several agricultural factors including climate and soil conditions.

In order to overcome agriculturally related variabilities and limitations, carvone can also be produced by the microbial biotransformation of (−)-*trans*-carveol to (*R*)-(−)-carvone by *Rhodococcus erythropolis*. The enzyme responsible for the biotransformation (carveol dehydrogenase) must be induced by limonene, which, in this case is also the carbon source. The biotransformation also requires NAD^+ as a cofactor which is regenerated in situ through cell growth. The biotransformation substrate, carveol, is added as a mixture of *cis*- and *trans*-isomers, and through the enantioselective biotransformation, *trans*-carveol is converted to (*R*)-(−)-carvone and *cis*-carveol is isomerically resolved and accumulates as a secondary product.

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The accumulation of the substrate (*cis*-carveol) and product [(*R*)-(−)-carvone] in the aqueous phase become inhibitory to the biotransformation activity of the biocatalyst.

Previously, de Carvalho and da Fonseca (2002) and de Carvalho et al. (2005) attempted to quantify the relative toxicities of carveol and carvone in two phase systems with *n*-dodecane as the second phase. The results of their study were equivocal because the substrate and product toxicity thresholds were reported in terms of concentration in the organic phase rather than in the aqueous phase to which the cells would be exposed, and no partitioning coefficients were provided from which an aqueous concentration could be calculated. The data presented in this previous work therefore seemingly apply only to a two phase system that uses *n*-dodecane as the second phase.

Tecelão et al. (2001) also previously performed this biotransformation in a single aqueous phase assay with a 20 ml working volume. The flask was aerated, but no carbon source was supplied throughout the biotransformation which is necessary for cofactor regeneration. The aqueous concentrations of substrate and product throughout the biotransformation were not reported, nor were the volumetric productivities.

The present study addresses the need for quantification of toxicity thresholds in the aqueous phase and the time course of aqueous substrate and product concentrations throughout the biotransformation in a bioreactor in which the conditions provide for cofactor regeneration.

Materials and methods

Microorganism, medium and inoculum preparation

The organism used to carry out the biotransformation was *Rhodococcus erythropolis* DCL14. The biotransformation medium is a modified version of that used by Wiegant and de Bont (1980) and is (added to 1 l tap water): 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 inoculum was prepared by the addition of 100 μl *R. erythropolis* stock culture to 50 ml medium containing glucose. The inoculum, which consisted of two flasks, was incubated at 30°C for 48 h at 180 rpm.

Bioreactor preparation

A 5 l New Brunswick Scientific BioFlo III, with a working volume of 3 l sterile glucose-free medium, was used to grow cells for the toxicity tests and for the benchmark biotransformation. The temperature and agitation were maintained at 28°C and 350 rpm, respectively. The pH was maintained at 7.0 using 6 M KOH and the dissolved O_2 level was monitored to ensure the system was not oxygen 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 was 200 ml/min and was also equipped with a 0.2 μm sterile filter.

Substrate and product toxicity experiment

Two sets of 125 ml flasks sealed with rubber stoppers were prepared for the carvone and carveol toxicity tests and 50 ml of culture broth from the inoculum bioreactor, prepared as described above and operated for 72 h, were added to each flask. For the carvone set, varying amounts of carvone were added to five flasks in the range of 100–1,000 mg/l and 500 mg carveol/l was added to each of the carvone flasks to start the biotransformation. For the carveol set, increasing concentrations of carveol were added in the amount of 500–1,200 mg/l. These concentration ranges were chosen to be below the substrate and product water solubilities, which are 2,890 mg carveol/l and 1,350 mg carvone/l. The flasks were incubated for 2 h at 30°C and 180 rpm, after which samples were centrifuged to remove the biomass and the substrate and product concentrations were determined using gas chromatography to measure the extent of biotransformation taking place. To test the effect of the substrate and product levels on cell growth, the biomass pellet from each sample was also resuspended in distilled water and a small amount was plated on glucose-containing agar medium and incubated at 30°C for 48 h. After 48 h, the cell growth on the surface of the plate was assessed by visual observation.

Single aqueous phase biotransformation

The biotransformation was initiated approximately 44 h after inoculation when the biomass concentration had reached 280 mg CDW/l in the bioreactor. The air and (+)-limonene flows were continuously supplied throughout the duration of the transformation to provide conditions favorable for cofactor regeneration. The transformation substrate, carveol, was supplied in a fed-batch manner and the product and substrate concentrations in the reactor were monitored frequently using gas chromatography. When the *trans*-carveol isomer was near depletion, more substrate was added such that its concentration in the reactor was below the aqueous saturation solubility and so as not to unduly inhibit the cells. These substrate additions continued until the biotransformation rate decreased significantly due to the accumulation of the inhibitory substrate and product.

Analytics

Each aqueous sample (5 ml) was added to 5 ml ethyl acetate and vortexed for 10 s, twice. After phase separation, a 1 μl sample of the ethyl acetate layer was injected into a gas chromatograph equipped with an Agilent/J & W DB-WAX column (ID = 0.53 mm, length = 30 m). The carrier gas was helium at 30 ml/min. 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 50°C/min. The run time for this method is 3 min. The biomass concentration was monitored throughout the biotransformation using cell dry weight (CDW) measurements by drying a 5 ml aqueous sample in a pre-weighed metal dish at 90°C overnight.

Results and discussion

Inhibitory effect of substrate and product on biotransformation capacity

In order to assess the relative toxicities of carveol and carvone, assays were carried out in shake flasks to determine their effect on the biotransformation capacity, cell growth and cell morphology of *Rhodococcus erythropolis*. The most general means of quantifying toxicity is via evaluation of cell growth post-exposure to toxic concentrations; the concentration at which the cells' reproduction ceases is identified as the toxic threshold. However, in this case it is more important to consider the inhibitory effects of the substrate and product on the biotransformation capacity of the cells since the end goal is to maintain a high biotransformation rate and not to produce biomass.

Figure 1 shows the effect of carvone toxicity in terms of *trans*-carveol isomer that was not transformed during the 2 h test period. It is evident that the biotransformation rate is affected above an initial carvone concentration of 200–600 mg/l. Over the 2 h test period, the cells in the flasks containing lower initial carvone concentrations (100 and 200 mg/l) completely transformed the available *trans*-carveol isomer (approximately 275 mg/l) into carvone. At higher initial carvone concentrations (600, 800, 1000 mg/l), approximately half of the available *trans*-isomer remained unconverted suggesting that the biotransformation rate is significantly depressed above a carvone concentration of 200–600 mg/l since the available *trans*-carveol was not completely transformed in the 2 h time period.

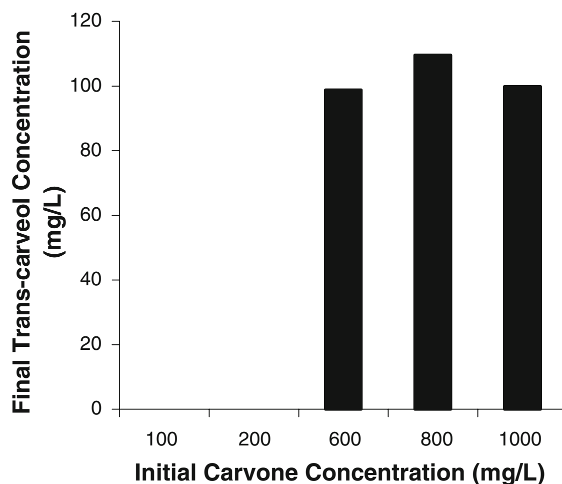

Fig. 1 Inhibition effects of initial carvone concentration on the biotransformation capacity of *Rhodococcus erythropolis*. Five flasks containing 50 ml of cell suspension were subjected to increasing concentrations of carvone. To induce the biotransformation, 500 mg carveol/l (mixture of *trans*- and *cis*-isomers) was also added to each flask. After a 2 h incubation period, the final carvone, *trans*-carveol and *cis*-carveol concentrations were tested

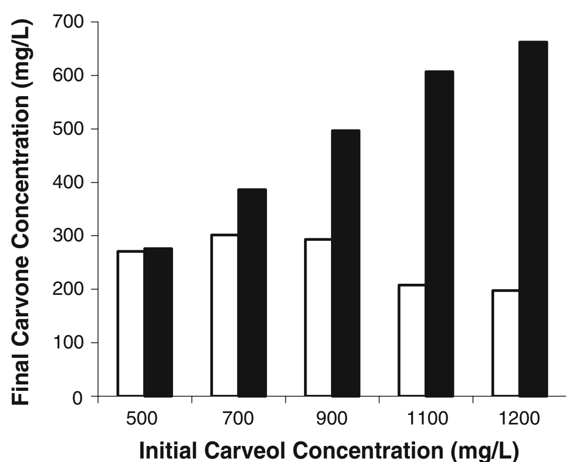

Fig. 2 Inhibition effects of initial carveol concentration on the biotransformation capacity of *Rhodococcus erythropolis* in terms of carvone produced. Symbols indicate the theoretical amount of carvone that could be produced (■) and the actual amount of carvone produced (□). Five flasks containing 50 ml of cell suspension were subjected to increasing concentrations of carveol (mixture of *trans*- and *cis*-isomers). The biotransformation began immediately after the addition of the substrate and after a 2 h incubation period, the final carvone, *trans*-carveol and *cis*-carveol concentrations were tested

Figure 2 shows the carvone produced in the carveol toxicity test after the 2 h incubation period. The resulting final carvone concentrations on their own do not provide conclusive evidence of the toxic threshold of the initial carveol concentration because the amount of carvone produced is dependant on the initial amount of carveol present. Thus, the final carvone concentrations in Fig. 2 must be compared to the theoretical amount of carvone that could have been produced based on the amount of *trans*-carveol initially present. For the lowest initial carveol concentration (500 mg/l), it is evident that all of the available *trans*-carveol isomer was consumed since the final carvone concentration is almost equal to the expected theoretical amount of carvone. Above 500 mg carveol/l the biotransformation capacity appears to be inhibited since less carvone was produced despite the fact that there was initially more *trans*-carveol present in those flasks.

Our results indicate that the toxicity thresholds, here defined as a reduction in biotransformation activity, are approximately 200–600 mg carvone/l and 500 mg carveol/l.

Previous studies were undertaken by de Carvalho and da Fonseca (2002) and de Carvalho et al. (2005) aimed at quantifying the relative toxicities of carvone and carveol in two phase systems with *n*-dodecane as an immiscible organic second phase. The toxicity thresholds from their study are reported in terms of the concentration in the organic phase, and no partitioning coefficients were provided to allow calculation of the corresponding aqueous concentrations. For this reason, the results obtained in their study cannot be directly compared to the current results. It is also important to note that in their results, even though partition coefficients were not provided the reported aqueous substrate and product concentrations were above the aqueous solubility, which would have exposed the cells in each flask to the same aqueous concentrations (saturated).

To further characterize the toxic effects of substrate and product on the cells, the potential for cell growth after the 2 h exposure was investigated. Post-exposure, samples were plated on agar medium containing glucose and incubated for 48 h. Every plate displayed substantial cell growth which indicates that in the cases where the substrate and product concentrations were sufficient to inhibit biotransformation capacity, the cells are still viable and able to replicate. This result is confirmed by an observation made by de Carvalho et al. (2005) suggesting that increasing carvone concentrations affect primarily the enzymatic system of the cells and only at higher concentrations will the carvone concentration have an effect sufficient enough to cause cell death.

Rhodococcus erythropolis normally grows as a suspension of individual cells throughout the medium but has been known to exhibit morphological changes when exposed to high concentrations of toxic compounds (de Carvalho and da Fonseca 2005). A typical representation of the morphological effect at the highest substrate concentration is shown in Fig. 3 which suggests that carveol is one such toxic compound. It has been proposed that the cells aggregate in an effort to protect the bulk of the population (on the inside of the formation) from the inhibitory concentrations of substrate and product in the aqueous phase (de Carvalho and da Fonseca 2005).

Single aqueous phase biotransformation

The biotransformation of *trans*-carveol to (*R*)-(−)-carvone was conducted in a single aqueous

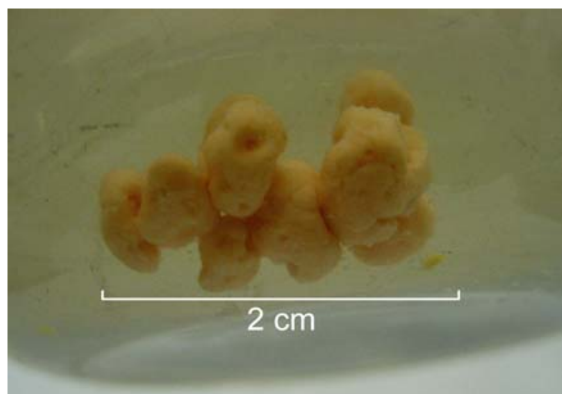

Fig. 3 Aggregation of *Rhodococcus erythropolis* after 2 h of exposure to a high concentration of carveol. In response to the high substrate concentration, the cells in a uniform cell suspension aggregated into multiple clumps and the surrounding medium became completely clear (free of cells)

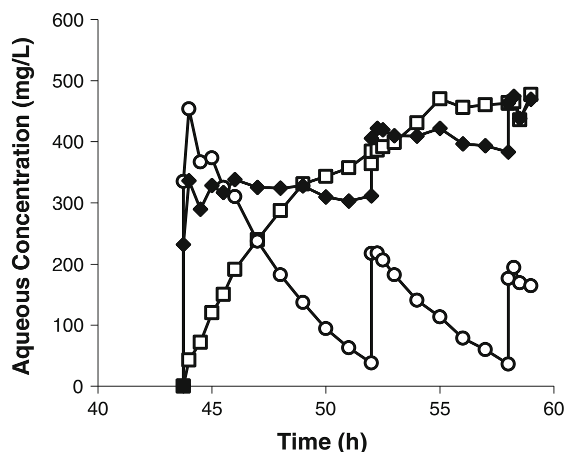

Fig. 4 Time course of the aqueous substrate and product concentrations throughout the benchmark biotransformation. Symbols indicate the desired product, carvone (□), and the two substrate isomers, *cis*-carveol (♦) and *trans*-carveol (○). Substrate was added in a fed-batch manner whenever the *trans*-carveol isomer was near depletion

phase reactor to provide a benchmark for the biotransformation system with performance being quantified by total amount of substrate added and overall carvone volumetric productivity. Figure 4 shows that the *trans*-carveol isomer is transformed into carvone, and the *cis*-isomer is isomerically resolved and remains present as a secondary product. It is evident that after each substrate addition, the biotransformation rate decreases as seen by the decrease in rate of carvone production. The final aqueous concentrations of carvone and *cis*-carveol were 478 mg/l and 470 mg/l respectively and are consistent with the results of the toxicity tests. The total amount of (*R*)-(−)-carvone produced was 1,434 mg.

Previous work by Tecelão et al. (2001) suggested that the decrease in biotransformation rates could be due to accumulation of the unconverted *cis*-isomer or cofactor depletion. In the present study, to maintain conditions favorable for cofactor regeneration, the carbon substrate and biotransformation inducer (limonene) and air were supplied throughout the duration of the fermentation. For this reason, it is evident that cofactor depletion is not the cause of the decrease in reaction rate. As well, in our work, the dissolved O_2 (DO) was never limiting and the biomass concentration throughout the biotransformation did not decrease (data not shown) which means that the decrease in biotransformation rate is not due to oxygen limitation or loss of biomass, but in fact decreased as a result of the inhibitory substrate and product concentrations. Further, as the inhibitory substrate and product accumulated in the aqueous phase in the biotransformation experiment, the biomass began to aggregate (as in Fig. 3) resulting in a non-uniform cell suspension which suggests the view that substrate/product inhibition are the cause of process cessation. In terms of performance, Tecelão et al. (2001) added carveol in a fed-batch manner in four aliquots of 10.4 µl (a total of 39.85 mg), with the total carvone produced being 16.14 mg. From the reported data, it is not possible to calculate a volumetric productivity for comparison purposes since the duration of the biotransformation was not given. The time course data that are reported in Fig. 4 are the first ever reported for a single phase system. Overall, with the addition of 5 ml carveol, the volumetric productivity of the single phase system was 31 mg/lh.

In summary, toxicity thresholds of the substrate and product determined here (500 mg/l and 200–600 mg/l, respectively), are consistent with those concentrations subsequently obtained in the fed-batch reactor and represent the upper limit of reactor performance for a single aqueous phase reactor. In order to improve reactor performance in terms of total substrate added and product volumetric productivity, a two phase partitioning bioreactor (TPPB) with a second phase used to deliver the toxic substrate and uptake the toxic product is currently being investigated.

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