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Bioconversion of limonene to α-terpineol by immobilized *Penicillium digitatum*

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Abstract Bioconversion of (4*R*)-(+)-limonene to (4R)-(+)-α-terpineol by immobilized fungal mycelia of *Penicillium digitatum* was investigated in batch, repeated-batch and continuously fed systems. The fungi were immobilized in calcium alginate beads. These beads remained active for at least 14 days when they were stored at 4 °C. Three different aeration rates were tested. The highest yield was obtained at a dissolved oxygen level of 50.0 μmol/l. α-Terpineol production by this fungus was 12.83 mg (g beads)$^{-1}$ day^{-1}, producing a 45.81% bioconversion of substrate. Repeated-batch bioconversion showed yield decreases in the second and the third cycles. Regeneration with nutrient media after the third cycle improved the bioconversion yields. With continuous bioconversion, the half-life was dependent on the aeration. The optimum conditions with a continuous reactor were at an aeration rate of 0.3 standard l/min and a dilution rate of 0.0144 h^{-1}.

Introduction

Most volatile aroma chemicals are synthetically produced (Layman 1984). Drawbacks in chemical synthesis of these compounds are non-specificity of the final products and an increased consumer perception that "natural" food additives are better. Bioconversion processes provide potential methods for the selective production of "natural" flavors and fragrances.

Limonene ($C_{10}H_{16}$) is the most commonly utilized natural terpene. The limonenes are used as fragrances in household products and as components of artificial "essential oils." They are components of more than 300 essential oils, in concentrations ranging from a high of 90%–95% (lemon, orange, mandarin) to a low of 1% (palmarosa). The most commonly used isomeric form is (+)-limonene, followed by the racemic mixture and then (−)-limonene (Arctander 1969). By far the largest quantities are employed as raw materials for the manufacture of terpene alcohols and ketones (Bauer and Garbe 1985). (+)-Limonene is also utilized as a starting material for the chemical syntheses of terpene resin adhesives and flavor chemicals. Other applications include uses as solvents in waterless hand cleaners, pet shampoos and degreasing agents (Matthews and Braddock 1987). The primary sources of (+)-limonene are the process streams from citrus juice production. The annual production of (+)-limonene is about 50×10^6 kg and is dependent on the quantity of oranges processed in the major citrus growing regions (Braddock and Cadwallader 1995).

α-Terpineol ($C_{10}H_{18}O$) is probably the most important of the monocyclic monoterpene alcohols. It is a colorless, crystalline solid with a lilac odor. Although α-terpineol is found in many "essential oils," only small quantities are commercially produced. Major uses are in various flavor compositions, such as berry, lemon, lime, nutmeg, orange, ginger, anise, peach, etc. Its annual consumption in flavors has been estimated at over 13 000 kg, which places it among the top 30 commonly used flavor compounds (Welsh et al. 1989). It is commonly produced by hydration, with aqueous mineral acid, of pinene or turpentine oil to the *cis*-terpin hydrate, followed by partial dehydration to α-terpineol (Bauer and Garbe 1985).

That racemic limonene is selectively and specifically converted to (4*R*)-(+)-α-terpineol by *Penicillium digitatum* (NRRL 1202) has been reported in previous study (Tan et al. 1998). In this study, an immobilized fungal cell reactor was designed and operated, in different modes, to optimize this bioconversion of limonene to α-terpineol.

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Materials and methods

Chemicals

(*R*)-(+)-, (*S*)-(−)-Limonene and authentic α-terpineol were purchased from the Aldrich Chemical Company (Milwaukee, Wis., USA). The purities of α-terpineol, (*R*)-(+)- and (*S*)-(−)-limonene were 98%, 97% and 95% respectively. Prior to use, these compounds were further purified by silica gel chromatography. The final purities of these terpenes, determined by a SGE 7959 fused-silica capillary chiral column (Cydex B, 50 m length × 0.22 mm inner diameter × 0.25 µl film thickness, SGE Scientific Pty, Australia) on HP 5890 GC, were (4*R*)-, (4*S*)-limonene and α-terpineol, 98.8%, 96.1% and 99.3% respectively. Sodium alginate (low viscosity), used for fungal immobilization, was purchased from the Sigma Chemical Co. (St. Louis, Mo., USA). All other chemicals or solvents were of the best available commercial grade.

Organism and growth conditions

Stock cultures of *Penicillium digitatum* (NRRL 1202) were maintained on potato/dextrose/agar (PDA, Difco) slants. After sporulation, they were stored at 4 °C. Spores were collected from 2-week-old PDA plates inoculated from stock cultures and maintained at 25 °C. The growth medium contained 10 g glucose/l, 10 g peptone/l, 20 g malt extract/l and 3 g yeast extract/l. The pH of the medium was adjusted to pH 7.0 with NaOH (40%, w/v) prior to sterilization. Aliquots of spore suspensions (5 ml; 1×10^7–3×10^7 spores/ml) were transferred aseptically into 250-ml conical flasks containing 50 ml growth medium and incubated for 12 h. This culture was transferred to fresh medium, in a 1:10 (v/v) ratio, 12 h prior to use in bioconversion studies. The cultures were grown on a rotary shaker (NBS model G25-KC rotary shaker, NBS Co., Edison, N.J.) at 28 °C and 100 rpm.

Bioconversion activity

Bioconversion activity was determined by withdrawing aseptically for analysis 5-ml samples from the cultures. To each 5-ml analytical sample, 50 µl limonene was added and the mixture vortexed for 30 s. This reaction mix was incubated, with shaking at 100 rpm, for 12 h at 28 °C and then extracted with 5 ml re-distilled diethyl ether. An internal standards solution (50 µl) containing two standards (tetradecane and 1-decanol) was added to each reaction mixture prior to extraction. Tetradecane and 1-decanol were used as standards for limonene and α-terpineol respectively. Both chemicals were prepared in methanol as 5000 ppm stock solutions. The samples were vortexed for 30 s, and then anhydrous sodium sulfate (4 g) was added to break the emulsion that formed. The ether fraction was separated, dried over anhydrous Na_2SO_4 and concentrated to 0.5 ml under a stream of nitrogen prior to gas chromatography.

Immobilization of *P. digitatum* on calcium alginate

Suspensions of germinated *P. digitatum* spores (30 ml; early log phase, 6 h growth) containing 24.5 mg dry weight/ml fungi were transferred into 200 ml fresh medium and then allowed to grow for 6 h at 100 rpm and 28 °C. This culture broth was suspended into 230 ml 10% (w/v) sodium alginate and dissolved in the same medium to produce 5% (w/v) of sodium alginate in the final mixture. The mixture was pumped dropwise through a tube of 0.25 cm diameter into 400 ml cold 0.2 M $CaCl_2$ solution. After the beads had formed, they were aged for 1 h in the $CaCl_2$ solution. The beads (about 0.4 cm diameter) were removed by filtration and washed twice with a 0.9% sterile NaCl solution, once with sterile water and twice with sterile 0.01 M pH 7 citrate/phosphate buffer. They were stored wet at 4 °C until use.

Calcium alginate beads storage stability

Samples of 3 g beads were stored at −20 °C and 4 °C. The bioconversion activity of these samples was tested at 0, 2, 4, 6, 8, 10, 12 and 14 days. Each bioconversion test required adding 3 ml 0.01 M pH 7.0 citrate/phosphate buffer and 50 µl limonene to the sample and then incubating for 1 day at 100 rpm and 28 °C prior to extraction. The extraction and analytical methods are described above.

Bioconversion of immobilized cells with batch and repeated-batch form

Batch bioconversion was conducted by suspending 30 g immobilized beads into each 250-ml flask containing 100 ml 0.01 M pH 7.0 citrate/phosphate buffer. A 1% (v/v) solution of limonene and 1% Tween 80 (v/v) were added, and then the flasks were placed on the 28 °C shaker. Samples of 5 ml were withdrawn every 24 h for product determination. Bioconversion was complete after 4 days. The beads were separated from the spent reaction medium and washed three times with 0.9% (w/v) NaCl solution and twice with buffer before new reaction medium [100 ml 0.01 M pH 7.0 citrate/phosphate buffer with 1% limonene (v/v) and 1% Tween 80 (v/v)] was added. This procedure was repeated until the activity significantly dropped. The beads were then regenerated for 3 days in fresh growth medium and reused. This procedure was repeated five times.

Bioconversion of immobilized beads in a continuously fed air-lift bioreactor

A continuously fed, upflow air-lift bioreactor was used for the continuous bioconversion of limonene to α-terpineol. The reactor was a 580-ml Kontes air-lift bioreactor (Kontes Life Science Products, Vineland, N.J.) containing 500 ml reaction mix and 150 g immobilized calcium alginate beads. The feed was 1% (v/v) limonene and 1% (v/v) Tween 80 in sterile 0.01 M citrate/phosphate buffer (pH 7.0). The air flow was regulated using an in-line direct-reading air flow meter (65-MM, Cole-Parmer Instrument Co., Niles, Ill.). The air was pre-filtered by passage through two sterile Whatman Hepa-vent filters (Whatman Inc., Clifton, N.J.).

Four aeration rates [0, 0.2, 0.3 and 0.4 standard liters/min (sl/min)] were compared. During each run, the amount of dissolved O_2 consumed by immobilized cells inside the column was monitored by measuring the dissolved oxygen (DO) difference between the feed and the eluate. This was measured using a dissolved oxygen meter (YSI model 58 with YSI 5730 dissolved-oxygen probe; Yellow Springs Instrument Co. Inc., Yellow Springs, Ohio). Two feed rates (0.007 h^{-1} and 0.014 h^{-1}, or 100 ml/day and 200 ml/day) were tested. Because batches of beads may vary, 30 g beads from the same batch as that in the bioreactor were used as a control. The control beads were suspended into 100 ml of the same feed to the column, in a 250-ml flask, and maintained at the same temperature as the bioreactor. The α-terpineol productivity from eight runs was averaged on the basis of productivity relative to their control.

Results

Optimization of bioconversion conditions with immobilized mycelia

Aeration

Oxygen accelerated bioconversion; however, excess oxygen decreased yields. Three different oxygen concentrations, 24.1, 50.0 and 94.7 µmol/l, were tested. The highest bioconversion activity with immobilized cells

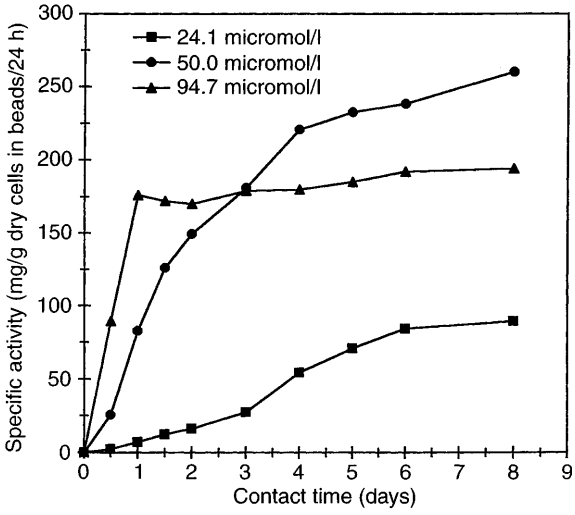

Fig. 1 Bioconversion activity with time of immobilized *Penicillium digitatum* mycelia at different aeration rates

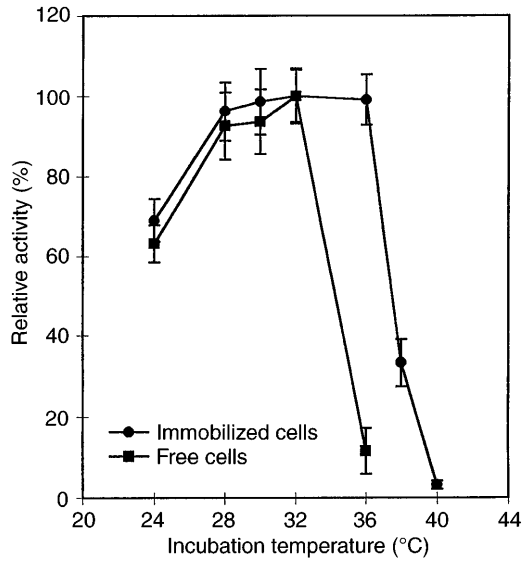

Fig. 2 Temperature optima for bioconversion by *P. digitatum* with free and immobilized mycelia. Error bars show SD. Values are mean values of three trials

was 188.2 mg α-terpineol produced in 24 h/g dry cells at 50.0 μmol/l DO. Activity at 94.7 μmol/l DO was 51% lower. The lowest activity was 9% of the highest, at 24.1 μmol/l DO.

At a DO level of 94.7 μmol/l, bioconversion activity by immobilized cells peaked 24 h after contact with substrate (Fig. 1). The α-terpineol concentration remained unchanged for the next 7 days, indicating no further production and no metabolism of the product (Fig. 1). A similar observation was made by Cadwallader et al. (1989) on a bacterial limonene/α-terpineol conversion system. At the 50.0 μmol/l DO level, the α-terpineol content increased for 8 days after limonene addition. The quantity of α-terpineol produced was greater than that produced at 94.7 μmol/l DO after 3 days (Fig. 1).

Temperature

There was no difference in bioconversion temperature optima between free and immobilized cells. A wider temperature range for activity was found with immobilized mycelia (Fig. 2). High α-terpineol yields were found over a range from 28 °C to 36 °C. Free cells achieved high yields only over a temperature range of 28–32 °C. Similar results have been reported with other immobilized enzyme systems (Gaikwad and Deshpande 1992).

pH

Immobilized cells showed bioconversion activity across the pH range from 3.5 to 8. Two distinct activity peaks were identified with immobilized cells, the highest activity being found at pH 7. Compared with the pH profile for free cells (Tan et al. 1998), immobilization shifted the pH optimum in the acidic direction.

Substrate concentration

Completeness of bioconversion was tested over a substrate range from 1.2 mmol/l (0.02%, v/v) to 494.1 mmol/l (8%, v/v) (Fig. 3). The amount of α-terpineol produced increased with substrate concentration,

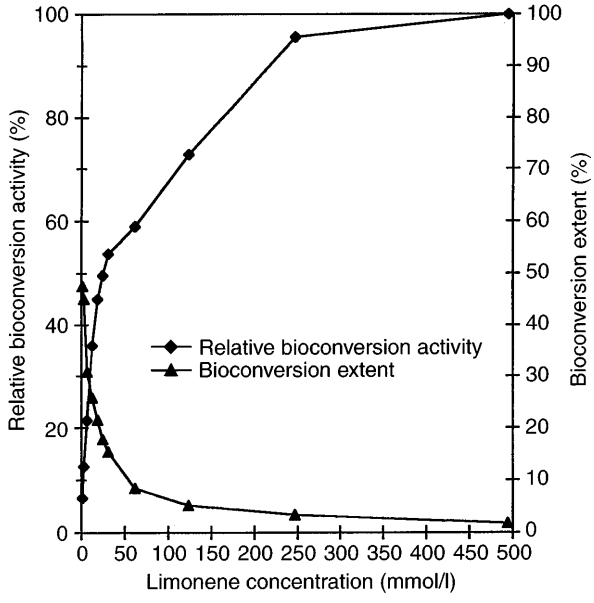

Fig. 3 Effect of substrate concentration on bioconversion activity for immobilized *P. digitatum* mycelia. Each assay was conducted at 28 °C for 24 h. The extent of bioconversion was calculated by the ratios of the amount of α-terpineol formed to the amount of limonene added

up to 247.1 mmol/l (4%, v/v) limonene. No significant change in product yield was found between substrate concentrations of 247.1 mmol/l and 494.1 mmol/l. A substrate concentration of 247.1 mmol/l (4%, v/v) was chosen as the optimum for immobilized cell conversion.

Stability of immobilized beads

The storage stability of immobilized beads was tested at −20 °C and 4 °C. Bioconversion activity of immobilized cells stored at −20 °C was rapidly lost over time. The activity loss was almost a straight line with a negative slope where 12.25% of the activity was lost per day. All activity was lost after 10 days storage. In contrast, activity increased slightly in beads stored at 4 °C. The activity of the beads after 14 days was 137 ± 20% of its activity when they were freshly made.

Bioconversion of limonene with immobilized fungal cells

Batch and repeated-batch bioconversions

The highest α-terpineol bioconversion rate (12.83 mg g beads^{-1} day^{-1}) was obtained 3 days after limonene addition. The bioconversion extent was 45.81%. Repeated-batch experiments were carried out to determine the reusability of immobilized cells (Table 1). The specific productivity of α-terpineol dropped from 346.5 mg/day to 101.8 mg/day from the first to second usage. The productivity dropped even more dramatically after the second usage. The average productivity of the third use was only 6% that of the first use. According to some reports (Schlosser and Schmauder 1991; Vidyarthi and Nagar 1994), the activity of immobilized cells can be improved by a regeneration process after several uses. Regeneration was conducted after the third use, and the batch bioconversions were repeated. Comparing the beads after regeneration (the fourth use) with the batch before (third use), the average productivity of α-terpineol increased three-fold from 18.77 mg/day to 59.02 mg/day. In the fifth use, the average productivity remained at 87.08 mg/day.

Continuous bioreactor

Continuous bioconversion by immobilized fungal beads was tested in a bioreactor. Since aeration enhanced α-terpineol production, the bioreactor was tested using four different aeration rates (0, 0.2, 0.3, and 0.4 sl/min). Each aeration rate was tested at two flow rates (0.0072 h^{-1} and 0.0144 h^{-1} dilution). Productivities were

Table 1 Repeated-batch bioconversion activity

Cycle	Running time (days)	Specific activity (mg g beads^{-1}day^{-1})	α-Terpineol productivity (mg/day)	Bioconversion extent (%)
1	0	0	0	0
	1	9.39	281.7	33.53
	2	11.39	341.7	40.67
	3	12.83	384.9	45.81
	4	12.31	369.3	43.97
2	0	0	0	0
	1	1.23	36.9	4.39
	2	2.83	84.9	10.10
	3	4.09	122.7	14.59
	4	4.24	127.2	15.16
3	0	0	0	0
	1	0.61	18.3	2.19
	2	0.65	19.5	2.33
	3	0.66	19.8	2.35
	4	0.58	17.4	2.07
Regeneration of the beads with growth medium for 3 days				
4	0	0	0	0
	1	0.57	17.1	2.02
	2	1.18	35.4	4.22
	3	2.25	67.5	8.04
	4	2.92	87.6	10.44
5	0	0	0	0
	1	2.43	72.9	8.67
	2	2.80	84.0	10.00
	3	3.18	95.4	11.37
	4	3.20	96.0	11.43

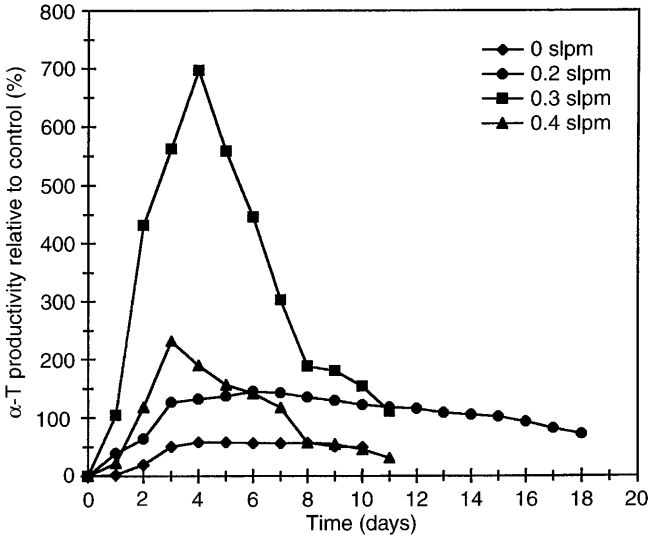

Fig. 4 Comparison of different aeration rates on α-terpineol productivity relative to control of immobilized mycelia with flow rate of 0.0072 h^{-1} in an air-lift bioreactor. All runs were conducted at room temperature

calculated on the basis of the productivity relative to a flask control containing 30 g of the same batch of beads used in the bioreactor. Figures 4 and 5 show the bioconversion activities relative to the controls at two flow rates and four different aeration rates.

At a fixed (0.0072 h^{-1}) dilution rate, the highest α-terpineol productivity was obtained with an aeration rate of 0.3 sl/min. Productivity was 2.6-fold higher than the control. The second highest productivity was obtained with 0.2 sl/min aeration, 1.2-fold that of the control. The lowest productivity was obtained with 0 aeration with a productivity of 45.8% of that of the control. The half-life of the column was about 20 days at an aeration of 0.2 sl/min. A half-life of 6 days was found with 0.3 sl/min and 0.4 sl/min (Fig. 4). At 0.0144 h^{-1} dilution rate and 0.3 sl/min aeration, the productivity was almost four times that of the control. The half-life of the column at this aeration rate was 6 days (Fig. 5), which was unchanged by doubling the dilution rate.

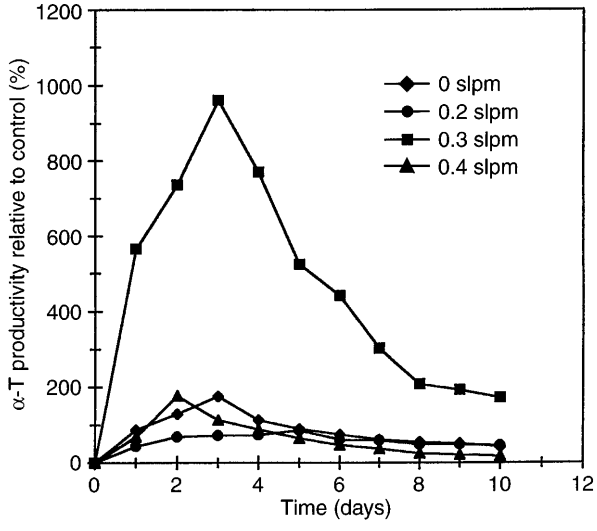

Fig. 5 Comparison of different aeration rates on α-terpineol productivity relative to control of immobilized mycelia with flow rate of 0.0144 h^{-1} in an air-lift bioreactor. All runs were conducted at room temperature

Discussion

The use of immobilized microbial cells for biotransformations has the advantage of freedom from enzyme extraction and purification, higher operational stability, greater potential for multi-step processes and greater resistance to environmental perturbations (Fukui and Tanaka 1982). Immobilization of fungi is far less common than that of bacteria or yeasts (Koshcheyenko et al. 1983; Ceen et al. 1987). The mycelial morphology complicates the initial immobilization, and subsequent growth is more difficult to accommodate within a support structure (Fukui and Tanaka 1984). Few fungal immobilizations have been reported for preparations of flavor compounds, even though numerous bioconversions have been conducted with free cells (Janssens et al. 1992). This study shows that immobilized *P. digitatum* can be used to convert (4*R*)-(+)-limonene to (4*R*)-(+)-α-terpineol.

Oxygen had been shown to accelerate this bioconversion, but excess O_2 decreased yield (Tan et al. 1998). A comparison of three different oxygen concentrations showed that the highest yield was obtained from 50.0 μmol/l oxygen. However, immobilized cells were less oxygen-sensitive than free cells. Immobilization probably also produced a diffusion barrier to O_2 transfer to the mycelia inside the support. Aeration not only influenced the productivity but also the half-life of the immobilized beads in a continuously fed system. The highest productivity was obtained with 0.3 sl/min aeration. The half-life of the immobilized cells was about 6–7 days. The longest half-life was found with 0.2 sl/min aeration and 0.0072 h^{-1} dilution rate; the half-life was estimated to be about 20 days.

The reusability of the immobilized cells was found to be adequate only if the beads were regenerated after the third use. The regeneration of the catalytic activity by reincubation in a nutrient medium after several uses is common for immobilized systems used in the biotransformations of steroids and alkaloids (Sonomoto et al. 1983; Kren et al. 1989; Schlosser and Schmauder 1991). The beads' reusability and ability to regenerate in this bioconversion were not as good as reported for the bioconversion of 15α-hydroxylation of 13-ethyl-gon-4-ene-3, 17-dione (Schmauder et al. 1991). There, *P. raistrickii* on calcium alginate beads maintained a relative activity of 100% after ten runs, when the beads were regenerated every 24–72 h (Schlosser and Schmauder 1991). The reusability of beads in the bioconversion of progesterone to 11α-hydroxylation of progesterone by

Rhizopus nigricans has also been tested by Vidyarthi and Nagar (1994). Their bioconversion productivity dropped to 30% after the fourth run, in three different supporting materials (agar gel, polyacrylamide and chitosan). The reusability of immobilized beads may be related to the characteristics of the bioconversion as well as to the properties of physical strength of the beads.

Bioconversion of (4*R*)-(+)-limonene to (4*R*)-(+)-α-terpineol by immobilized *P. digitatum* on calcium alginate beads is feasible. Compared with free fungal mycelia, immobilized fungi are somewhat protected from the toxic substrate at high concentration. Immobilization also decreased the substrate affinity by increasing the K_{mapp} (data not shown). Optimum bioconversion temperatures for free and immobilized fungal mycelia were the same, 28 °C, but immobilized fungal mycelia showed better temperature stability. pH optima showed different patterns for free and immobilized fungal mycelia. With immobilized beads, repeated-batch cultivation or continuous feeding in an air-lift bioreactor for bioconversion of limonene may be possible. Nutrient regeneration helped activity maintenance after three bioconversion cycles. The half-life for continuous operation of the bioconversion ranged from 7 to 20 days, depending on the rate of aeration.

The minimal cost for production of 0.45 kg (4*R*)-(+)-α-terpineol through this bioconversion was estimated to be over $35, which is higher than the current selling price of the product. Thus, this process is not profitable at current yield levels and the cost of chemicals. Industrial production of a single flavor substance by microorganisms only becomes economical if the compound has a market value of $200/kg (Welsh et al. 1989). Therefore, this microbial transformation has only potential interest for application in the flavor and fragrance industry under current conditions.

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