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Biotransformation of aromatic compounds by immobilized bacterial strains in barium alginate beads

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In the present study, we have investigated the biotransformation of different aromatic compounds to their cis-*dihydrodiols (*cis-*diols) with immobilized* Pseudomonas putida *UV4 cells using barium alginate as the immobilization medium for the cells in a fluidized-bed reactor. The reactions have been performed in a biphasic system containing 20% (v/v) tetradecane. The bacterial beads were fluidized using a flow of pure oxygen at a flow rate of 100 ml min^{-1}. The production of toluene* cis-*diol under the same conditions by the recombinant organism* Escherichia coli *JM109 (pKST11) was compared. The production of toluene* cis-*diol with* E. coli *JM109 (pKST11) was more than 24 times higher that the values reported in the literature using similar recombinant non-immobilized organisms. The capacity of immobilized* E. coli *JM109 (pKST11) to yield* cis-*diols from different aromatic substrates in small-scale biotransformations was also investigated.* © *1998 Elsevier Science Inc.*

Keywords: Biotransformation: *Pseudomonas putida* UV4; *Escherichia coli* JM109 (pKST11); alginate beads; recombinant organism

Introduction

The *cis*-3,5-cyclohexadien-1,2-diols (*cis*-diols) are valuable chiral synthons used in the pharmaceutical and chemical industries to prepare a wide range of valuable products.[1] The use of bacterial strains compared with purely chemical routes to the synthesis of these molecules offers significant advantages that include enantiospecificity, high yields, low economic cost, and environmental considerations. The conversion of aromatic molecules to nonaromatic compounds is a very difficult reaction to perform due to the stability of the aromatic ring, requiring severe conditions and the use of chemical reagents and/or toxic, metal-derived components,[2,3] moreover, there is no single chemical reaction which permits the direct oxidation of aromatic rings to such valuable chiral synthons as the *cis*-diols.[4] Biotransformations, on the other hand, take place under very mild conditions (30°C and atmospheric pressure) and offer an environmentally friendly alternative in tune with the current tendency toward clean chemical technologies.

Immobilized cells have been used extensively for production of useful chemicals and the degradation of wastewater pollutants.[5] Cell immobilization is one of the most attractive methods to avoid the problems inherent in the use of free bacteria including ease of handling and cell separation.[6] The main advantages in the use of immobilized whole microorganisms are their higher operational stability,[7] their ease of use in continuous reactors, high-cell density, and ability to scale up.[8,9]

Following our successful development of a new immobilization methodology of bacteria using barium alginate and its use to produce toluene *cis*-diol,[10] an investigation of the general utility of the method was undertaken. The aim of the present investigation was not only to increase the number of products capable of being produced with this immobilization methodology but also to apply the immobilization technique to the entrapment of different *cis*-diol-producing microorganisms and to compare their relative effectiveness. We report here for the first time the production of toluene *cis*-diol with an entrapped recombinant microorganism, *Escherichia coli* JM109 (pKST11) expressing toluene dioxygenase. The capacity of the recombinant to produce diol was compared with the entrapped mutant *Pseudomonas putida* UV4 and with the reported data available in the literature for this reaction using similar non-immobilized recombinant microorganisms.

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Materials and methods

Materials

Sodium alginate from *Laminaria hyperborea* was obtained from Fluka (Fluka Chemicals, Gillingham-Dorset, England). All other chemicals used were analytical-grade quality and were obtained commercially from the usual suppliers.

Microorganisms and growth conditions

Unless stated otherwise, all growth conditions and analytical methods were performed as described previously.[10]

P. putida UV4 was obtained from Zeneca, Biological Products Business, (Billingham, U.K.). *E. coli* pKST11, derived from parent strain JM109, was obtained from our collection of cultures.[11]

Growth conditions in shake flask culture of *E. coli* JM109 (pKST11). *E. coli* JM109 containing the plasmid pKST11 was maintained on Luria-Bertani agar plates containing 0.2% of a 50 mg ml^{-1} solution of ampicillin in water. A 250-ml flask containing 50 ml of Luria-Bertani broth and 0.2% of a 50 mg ml^{-1} solution of ampicillin in water was inoculated with *E. coli* JM109 (pKST11) from the culture plates. The cells were grown on a rotary shaker at 30°C for 12 h. Culture (10 ml) was used to inoculate a 2-l flask containing 500 ml of Luria-Bertani Broth and 0.2% of a 50 mg ml^{-1} solution of ampicillin in water. After 4 h of growth in an orbital shaker at 30°C, 5 ml of 0.1 M isopropyl-β-D-thiogalactopyranoside (IPTG) solution in water was added to the flask for induction of the dioxygenase. The bacteria were harvested after 1.5 h of further incubation at 30°C in an orbital shaker and used directly in the biotransformation reaction.

Immobilization of cells

Cells (40 ml) from the culture broth were harvested by centrifugation at 2,500 *g* for 6 min. The supernatant was removed and the bacterial pellet mixed with 60 ml of an autoclaved 3% (w/v) sodium alginate solution in water. The mixture was then added, dropwise, to a 500 ml chilled solution of 0.05 M $BaCl_2$. The resulting gel beads (diameter, 2–3 mm; cell load, $6–13 \times 10^9$ cells g^{-1} bead) were hardened for 15 min at 4°C. The biocatalyst solution was then filtered through a No. 1 Whatman filter and washed with 500 ml of distilled water.[10]

Biotransformation conditions

Small-scale biotransformations. Biotransformations were done in 250-ml flasks shaken at 200 rpm and maintained at 30°C. The reaction medium comprised 0.1 M Tris-HCl buffer pH 7.0 containing 0.5% (w/v) glucose as a carbon source to allow cofactor regeneration and 10% (v/v) minimal salts medium[12] in a total volume of 50 ml. Substrate (1 ml) or 1 g in the case of solid substrates (7.45–11.19 mmol) was mixed with 10 ml of tetradecane and added directly to the reaction medium. Beads (5 g) prepared as described above were added to the flask. Each flask was filled with pure oxygen and sealed with a Suba Seal (W. H. Freeman, Barnsley, U. K.).

Large-scale biotransformations. Approximately 15 g of beads (accurately weighed) were packed in a 250-ml glass-jacketed column (3 cm × 30 cm) maintained at 30°C. The reaction medium consisted of 0.1 M Tris-HCl buffer pH 7.0, 0.5% (w/v) glucose, and 10% (v/v) minimal salts medium and was pumped from the bottom to the top of the column with a peristaltic pump at a flow rate of 20 ml min^{-1}. The beads were fluidized by an oxygen flow at a flow rate of 100 ml min^{-1}. The medium was recirculated through a reservoir where the pH and temperature were controlled at 7.0 and 30°C, respectively. The total volume of medium in circulation was 1,500 ml and 30 ml of substrate (30 g in the case of naphthalene and benzo[b]thiophene) were mixed with 300 ml tetradecane and added directly to the reaction medium.

Analytical methods

Diol production. The production of diols was measured by HPLC. Samples from the aqueous phase were diluted with H_2O/MeOH (50/50, v/v) and analyzed with a Zorbax ODS column (250 × 4.6 mm; Hichrom Ltd., Reading, U.K.) at a flow rate of 0.8 ml min^{-1}. The mobile phase was water/methanol with a linear gradient of $t = 0$ min, 60/40 (v/v); $t = 20$ min, 30/70 (v/v); $t = 21$ min, 0/100 (v/v); $t = 30$ min, 0/100 (v/v); $t = 40$ min, 60/40 (v/v). The diode array detector was set at 265 nm and the column was operated isothermally at room temperature. The area of each peak was measured by the integrator and transformed to concentration using a standard curve of pure diols in water/methanol (50/50, v/v). Absorbance readings were correlated to dry cell weight using a standard curve. The ^{1}H-NMR spectra of the $CDCl_3$-dissolved crude of reaction from the ethyl acetate extraction of the aqueous phase from the biotransformation with benzo[b]thiophene were obtained with a General Electric GE300 NMR spectrometer.

Cell concentration. Free cell concentration (cells ml^{-1}) was determined using both a particle analyzer apparatus, (CellFacts, Microbial Systems Ltd., Warwick Science Park, U.K.) and optical density measurements at 600 nm of the culture broth and were correlated to dry cell weight (dcw) using a standard curve. The dry cell weight was obtained by drying pure water-washed centrifuged cells at 120°C over a 24-h period to constant weight and weighing the resultant material on a balance (Sartorius 2006 MP, Sartorius, Epsom, U.K.) to an accuracy of 0.1 mg. No charring of cells was observed during the drying process.

Results and discussion

Production of cis*-diols with* P. putida *UV4 in a fluidized-bed reactor*

The production of *cis*-diols from different aromatic substrates with alginate-immobilized cells of *P. putida* UV4 in a fluidized-bed reactor is shown in *Figure 1*. In the case of fluorobenzene, a mixture of the *cis*- and *trans*-isomers was obtained (60% e.e.; configuration 1S, 2S).[13,14] Immobilized *P. putida* UV4 is thus capable of producing *cis*-diols from a wide variety of aromatic compounds that have been previously reported as substrates for this biotransformation with non-immobilized *P. putida* UV4 to yield the same final products.[13–16]

Toluene is the substrate which yielded the highest production of *cis*-diol. This may be explained by the fact that the methyl group has an electron-donating effect upon the aromatic ring that facilitates the oxidation of the aromatic group by electrophilic attack of oxygenases.[17] It can also be observed that in the group of monocyclic aromatic compounds, the higher the electron-removing effect of the substituent (fluoro), the lower the yield of *cis*-diol (0.7 mol *cis*-diol g^{-1} dcw). The higher yield of *cis*-diol from toluene (6.1 mol *cis*-diol g^{-1} dcw) may also be due to the 3D structure of the active site of oxygenase that has evolved to bind and use toluene as the prime substrate. In the case of the bicyclic unsaturated rings, naphthalene and benzo[b]thiophene, steric hindrance seems to be an

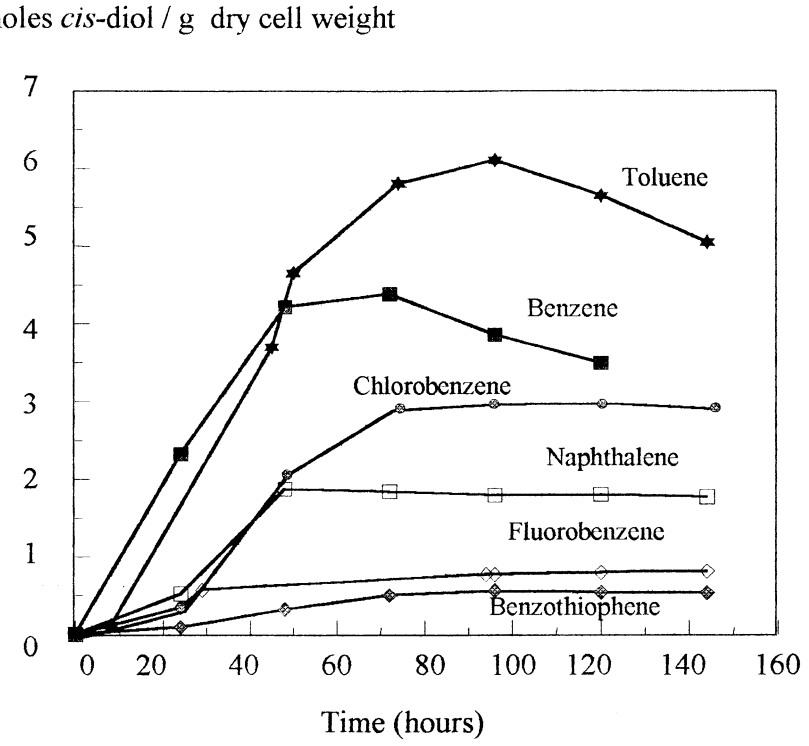

Figure 1 Production of *cis*-diols in a fluidized-bed reactor (1,500 ml) fed with oxygen at a flow rate of 100 ml min^{-1}. The flow rate of medium was 20 ml min^{-1}. Reaction medium was 0.1 M Tris-HCl buffer pH 7.0 containing 0.5% glucose, 10% minimal salts medium, and 20% tetradecane. Total recirculating volume was 1,500 ml. Beads (15 g) were put in the reactor. Substrate starting concentrations: benzene, 7.46 mM; benzothiophene, 4.97 mM; toluene, 6.26 mM; naphthalene, 5.20 mM; fluorobenzene, 7.11 mM; and chlorobenzene, 6.55 mM. Reactions were stopped when the diol concentration diminished in at least two consecutive analyses.

important factor that prevents the diffusion of the substrates through the alginate matrix, leading to relatively low yields (naphthalene, 1.8 mol *cis*-diol g^{-1} dcw; benzo[b]thiophene, 0.56 mol *cis*-diol g^{-1} dcw). The higher yield obtained with naphthalene could be related to its higher log *P* value (3.3) than benzo[b]thiophene (3.1).[18] The toxicity of an organic molecule upon biocatalysts can be expressed as a function of its log *P*; the lower the log *P*, the higher the toxicity. This toxicity can be explained by the ability of the organic solvent to distort the essential layer around the biocatalyst,[19] moreover, small differences in log *P* can have dramatic effects on the activity of the biocatalyst.[20]

In an attempt to determine the effect that the immobilization may have on the qualitative production of *cis*-diols, we chose to study the biotransformation of benzo[b]thiophene. It has been reported previously to form both the 2,3- and 4,5-*cis*-diols when non-immobilized *P. putida* UV4 was used.[15] The ^{1}H-NMR analysis of the combined organic phases from the ethyl acetate extraction of the biotransformation aqueous phase shows that the relative proportion of both products, 75% of 2,3-diol and 25% of 4,5-diol, is almost identical to the data reported in the literature when the same non-immobilized organism was used.[15] This result seems to indicate that the toluene dioxygenase complex inside the cells remained unaffected by the process of immobilization with barium alginate.

Production of cis*-diols by* E. coli *JM109 (pKST11)*

Toluene dioxygenase from *P. putida* has been previously cloned and expressed in *E. coli*.[21] *E. coli* clone pKST11, a strain which accumulates toluene *cis*-diol, was constructed by PCR amplification from published sequence information expressing the toluene dioxygenase enzyme from *P. putida* NCIMB 11767, a wild-type isolate from which the UV4 mutant was derived.[11] In the present study, *E. coli* pKST11 was used under the same conditions as *P. putida* UV4 to produce toluene *cis*-diol in a fluidized-bed reactor. *Table 1* shows the results thus obtained.

The amount of toluene *cis*-diol produced with *P. putida* UV4 is 17 times higher than observed in the recombinant strain with *E. coli* pKST11 based on *cis*-diol produced g^{-1} dry cell weight. This difference is less (4.5-fold) if we contrast the results in terms of production of *cis*-diol per million of bacteria. The reason for this difference is that *E. coli* pKST11 is a heavier organism [1.1×10^{-6} g $(10^6$ cells$)^{-1}$] than *P. putida* UV4 [3.1×10^{-7} g $(10^6$ cells$)^{-1}$]. This is confirmed since the dry cell weight of the non-recombinant parent strain JM109 is 4.1×10^{-7} g $(10^6$ cells$)^{-1}$ and phase-contrast microscopy of IPTG-induced cells of *E. coli* JM109 (pKST11) revealed the presence of inclusion bodies in the bacteria that could be insoluble

Table 1 Toluene *cis*-diol production in a fluidized-bed reactor

Microorganism	Toluene *cis*-diol production (mol *cis*-diol g^{-1} dry cell weight)	[μmol *cis*-diol $(10^6$ bacteria$)^{-1}$]
P. putida UV4	6.1	1.8
E. coli pKST11	0.36	0.4

Flow rate of oxygen was 100 ml min^{-1}. Flow rate of medium was 20 ml min^{-1}. Reaction medium was 0.1 M Tris-HCl buffer pH 7.0 containing 0.5% (w/v) glucose, 10% (v/v) minimal salts medium, and 20% (v/v) tetradecane. Total recirculating volume was 1,500 ml. Beads (15 g) containing immobilized cells were added to the reactor. Results are given after 96 h operation time

Table 2 Toluene *cis*-diol production at different toluene concentrations in small-scale biotransformations at 30°c

Toluene concentration (% v/v)	Toluene *cis*-diol production (mmol *cis*-diol g^{-1} dry cell weight) *P. putida* UV4	*E. coli* JM109 (pKST11)
1	153	149
2	166	129
3	149	18

Reaction medium was 0.1 M Tris-HCl buffer pH 7.0 containing 0.5% (w/v) glucose, 10% (v/v) minimal salts medium, and 20% (v/v) tetradecane. Total volume was 50 ml. Beads (5 g) containing immobilized cells were added to the flask. Each flask was filled with pure oxygen and sealed. Results are given after 24 h reaction time

enzyme formed after induction; nevertheless, *P. putida* UV4 is a superior strain for the production of toluene *cis*-diol due to its higher resistance to organic solvents (*Table 2*) and because recombinant organisms such as *E. coli* JM109 (pKST11) produce a relatively low amount of toluene *cis*-diol probably due to inefficient expression of the dioxygenase genes and product consumption caused by residual toluene *cis*-diol dehydrogenase activity.[22]

Comparing the results using *E. coli* JM109 (pKST11) with the data previously reported for the production of toluene *cis*-diol with other non-immobilized recombinant organism, the production achieved in this study is more than 24 times better than the best result available in the literature.[22–25] This dramatic improvement in toluene *cis*-diol production may be related to the use of optimum biotransformation conditions (substrate addition, concentration of oxygen and minerals in the reaction medium, entrapment conditions, etc) whose operational parameters have been previously optimized by us[10] rather than the superior genetic nature of the bacteria used. We have recently proven how important optimization is when high production of toluene *cis*-diol is desired.[10]

Other aromatic substrates such as naphthalene, ethylbenzene, fluorobenzene, methylbenzoate, and benzylacetate were tested as substrates for the biotransformation with immobilized *E. coli* JM109 (pKST11) to produced *cis*-diols. In all the cases, the bacteria produced the same products as in non-immobilized conditions. Of special interest was the successful production of the 3,4-benzylacetate *cis*-diol, a precursor in the synthesis of pseudosugars, that cannot be performed with the mutant organism *P. putida* UV4.

Conclusions

The biotransformation of organic molecules with immobilized bacterial strains in barium alginate beads is an extremely versatile approach that can be used in the production of different valuable organic molecules for much longer periods of time than with non-immobilized microorganisms. The method due to its excellent productivity, economy, and ease to use though requiring a further development can be suitable for an industrial scaleup. A dramatic increase in the production of toluene *cis*-diol with *E. coli* JM109 (pKST11) was observed when compared with values previously reported in the literature for the production of this compound with non-immobilized recombinant organisms.[22–25]

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