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# Conversion of indene to *cis*-(1*S*),(2*R*)-indandiol by mutants of *Pseudomonas putida* F1

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**Two mutation and selection methods were used to isolate mutants of *Pseudomonas putida* F1 which convert indene to *cis*-(1*S*),(2*R*)-indandiol in a toluene-independent fashion. Using soybean or silicone oil as a second phase to deliver indene to the culture, *cis*-(1*S*),(2*R*)-indandiol, *cis*-(1*R*),(2*S*)-indandiol, 1,2-indenediol (or the keto-hydroxy indan tautomer), and the monooxygenation products 1-indenol and 1-indanone were produced from indene as a function of time. Similarly the enantiomeric excess of the *cis*-(1*S*),(2*R*)-indandiol produced also increased with increasing time. In addition, mutants were isolated which produced *cis*-(1*S*),(2*R*)-indandiol of lower optical purity which corresponded to reduced levels of 1,2-indenediol. These data suggest this toluene dioxygenase produces *cis*-(1*S*),(2*R*)-indandiol of low optical purity and that *cis*-glycol dehydrogenase plays a role in resolving the two *cis*-1,2-indandiol enantiomers.**

**Keywords:** biocatalysis; dioxygenase; *Pseudomonas putida*; *cis*-(1*S*),(2*R*)-indandiol; indene

## Introduction

The catabolism of toluene by *Pseudomonas putida* F1, the genes for which are expressed in the presence of toluene or another suitable inducer, has been exceptionally well studied [for a recent review see Ref. 8]. Germane to this work is the first catabolic step where toluene is dioxygenated to form *cis*-(1*S*),(2*R*)-dihydroxy-3-methylcyclohexa-3,5-diene (*cis*-toluene dihydrodiol) [9]. This dioxygenation reaction is catalyzed by a three-component toluene-dioxygenase enzyme system [6]. Electrons are transferred from NADH through a flavoprotein and a ferredoxin to a two-subunit iron-sulfur protein which incorporates molecular oxygen into the aromatic substrate yielding the corresponding *cis*-dihydrodiol (*cis*-glycol). Experiments with mutants which accumulate the *cis*-toluene dihydrodiol demonstrate that the enantiomeric purity is high in favor of the *cis*-(1*S*),(2*R*)-dihydrodiol [17].

Bioprocesses have been developed for the production of dihydrodiols from various aromatic substrates. The *cis*-dihydrodiol produced from toluene can be used as a chiral synthon for the production of prostaglandin $E_{2a}$ while the *cis*-glycol produced from benzene serves as a good polymerization monomer for the manufacture of films, fibers, and coatings [1,11].

Wackett and co-workers previously demonstrated that toluene-induced *P. putida* F39/D cells (a mutant lacking *cis*-glycol dehydrogenase activity) converted indene to *cis*-(1*S*),(2*R*)-indandiol, and 1-indenol [15]. 1-Indanone also accumulated upon extended incubation as a result of 1-indenol isomerization. The enantiomeric excess of the *cis*-(1*S*),(2*R*)-indandiol produced was approximately 30%. 1-Indenol is not merely a dehydration product of *cis*-1,2-indandiol as it can be formed (via a monooxygenation reaction) *in vitro* with purified dioxygenase components [15]. Moreover, the dehydration product obtained from *cis*-1,2-indandiol upon heating in strong acid is 2-indanone [15].

Our interest in this area stems from the fact that *cis*-(1*S*),(2*R*)-indandiol can be chemically converted with a nitrile in the presence of aqueous acid (ie Ritter reaction) to *cis*-(1*S*)-amino-(2*R*)-indanol (Figure 1) [14] which is a key raw material in the chemical synthesis of the HIV-1 protease inhibitor drug Indinavir Sulfate (Crixivan®). The *cis*-(1*S*),(2*R*)-indandiol enantiomer is crucial for subsequent synthetic reactions leading to a biologically active drug substance and it is the stereospecificity of a biological process that makes it an attractive alternative to the synthetic route.

The requirements of a bioprocess for the production of *cis*-(1*S*),(2*R*)-indandiol from indene are to carry out the conversion in a toluene-independent manner with a high enantiomeric purity and a good overall yield. While indene serves as a substrate for the dioxygenase system, it does not support growth nor does it induce the toluene catabolic genes (*tod* operon). Eliminating the need for toluene would allow for a more productive process since toluene, which would also serve as a competitive substrate, would not have to be added to the fermentation. In addition, elimination of toluene would allow for a process which would be appealing from an environmental and safety point of view.

## Materials and methods

### Cultivation methods

*Pseudomonas putida* F1 (DSM 6899) and mutants thereof were grown and maintained using tryptic soy broth and tryptic soy agar (TSB and TSA respectively, Difco Laboratories, Detroit, MI, USA). All cultures were incubated at 30°C. Plate cultures were stored at 4°C for up to 4 weeks

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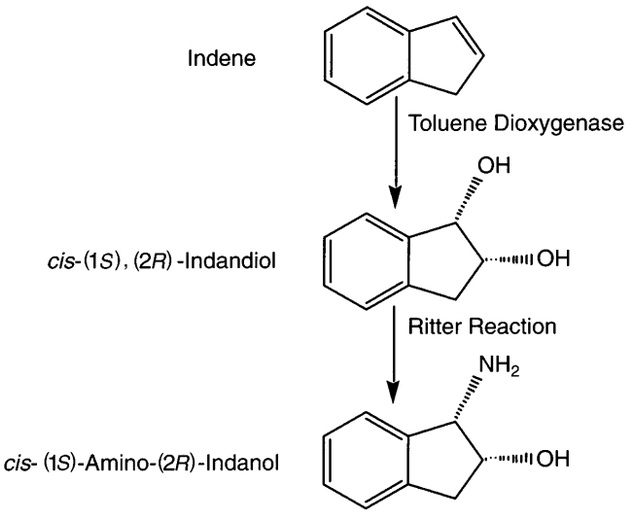


**Figure 1** Combining biocatalysis and chemical synthesis for the production of *cis*-(1*S*)-amino-(2*R*)-indanol. Indene is converted to *cis*-(1*S*),(2*R*)-indandiol by the toluene dioxygenase system of *Pseudomonas putida* F1. The *cis*-(1*S*),(2*R*)-indandiol is treated with a nitrile in the presence of aqueous acid according to the Ritter reaction to produce *cis*-(1*S*)-amino-(2*R*)-indanol.

and were used as sources of inoculum for conversion experiments. Liquid cultures were diluted 1 : 1 with 20% (v/v) glycerol and stored as aliquots at −70°C.

A minimal salts medium, essentially as described by Haigler and Gibson [10], with citrate (MMC) or without carbon source (MM) was also employed and consisted of (per liter): 3[*N*-morpholino]-propanesulfonic acid (MOPS), 20.9 g; sodium citrate $2H_2O$, 2.94 g; $K_2HPO_4$, 2 g; $(NH_4)_2SO_4$, 1 g; $MgSO_4 \cdot 7H_2O$, 0.4 g; $FeSO_4 \cdot 7H_2O$, 10 mg; trace element solution, 2.5 ml; pH 7.2. The trace element solution consisted of (per liter): $H_3BO_3$, 300 mg; $ZnCl_2$, 50 mg; $MnCl_2 \cdot 4H_2O$, 30 mg; $CoCl_2$, 200 mg; $CuCl_2 \cdot 2H_2O$, 10 mg; $NiCl_2 \cdot 6H_2O$, 20 mg; and $Na_2MoO_4 \cdot 2H_2O$, 30 mg. Modifications and additions to this basic formulation are indicated in the text.

Solid culture media were prepared by supplementing media formulations with 20 g $L^{-1}$ agar. To grow cultures on solid medium in the presence of toluene vapors, $100 \times 15$-mm glass petri dishes containing the solid medium of choice were placed in a glass desiccator along with 3–5 ml of toluene in a 15-ml glass test tube and incubated at the appropriate temperature.

### *Indene conversion experiments*

For shake-flask scale conversion experiments, a 250-ml 3-baffled Erlenmeyer flask containing 20 ml of MMC medium, 5 ml of soybean oil or silicone oil (used to deliver indene to the culture), and 2.5 g $L^{-1}$ indene (based on a 25-ml culture volume) was inoculated with 0.2 ml of an overnight TSB culture. Flasks were incubated at 30°C with 180 rpm rotary shaking. Where indicated, additional citrate (10 mM final concentration) was added to flasks mid-cycle (MMC+C process) on or around 8 h of incubation. For time course experiments, several flasks for each culture were inoculated and the entire contents of the flasks were extracted at each time point to determine the *cis*-1,2-indandiol titer and the *cis*-(1*S*),(2*R*)-indandiol enantiomeric excess.

For conversion experiments at the laboratory-fermentor scale, 23-L fermentors (Chemap Inc, South Plainfield, NJ, USA), containing 12 L of MMC medium (without MOPS buffer) and 3 L of soybean oil were sterilized *in situ* and inoculated with 300 ml of an overnight TSB culture. At the time of inoculation, indene was added at a final concentration of 2.5 g $L^{-1}$ (based on a 15-L batch volume). Process conditions for the fermentation were: temperature 30°C; agitation 500 rpm; aeration 0.6 vvm; backpressure 0.6 kg $cm^{-2}$; pH 7.0, maintained with 10% (v/v) sulfuric acid. Carbon dioxide in the off-gas was monitored using a Perkin-Elmer model 1200 mass spectrometer (Perkin Elmer, Norwalk, CT, USA) and a Hewlett Packard model 1000 computer as described previously [2].

Resting-cell cultures were used to investigate the nature of the toluene-independent mutants isolated. Cultures were grown in MMC medium plus soybean oil (30°C, 180 rpm rotary shaking) in the presence of toluene (20 mM), indene (20 mM), or no aromatic substrate. Cells were harvested by centrifugation, washed once with resting-cell medium consisting of 100 mM MOPS buffer (pH 7.2), 10 mM citrate, and 100 mg $L^{-1}$ chloramphenicol, and resuspended at a 2× cell concentration in resting-cell medium. Biomass in the resting cell suspensions was measured by the absorbance at 600 nm using a Hewlett Packard model 8451A spectrophotometer (Hewlett Packard, Palo Alto, CA, USA). Twenty milliliters of resting cell suspension were transferred to 250-ml 3-baffled Erlenmeyer flasks followed by 5 ml of soybean oil and indene to a final concentration of 2.5 g $L^{-1}$ (based on the 25-ml culture volume). Resting-cell cultures were incubated at 30°C with 180 rpm rotary shaking and the *cis*-1,2-indandiol concentration was determined at specified time points and expressed as mg $L^{-1}$ $OD_{600}{}^{-1}$.

### *Extraction and analytical methods*

For *cis*-1,2-indandiol titer determinations by reverse phase chromatography, the entire contents of a shake-flask or a well-mixed 25-ml fermentor sample (both containing 20 ml of medium and 5 ml of oil) were mixed with 40 ml of isopropanol (equal to two volumes of medium) for 30 min. The mixture was centrifuged to separate the oil from the aqueous/alcohol phase which was analyzed for the presence of *cis*-1,2-indandiol by reverse-phase HPLC. To prepare samples for *cis*-(1*S*),(2*R*)-indandiol enantiomeric excess analysis, the oil phase was separated by centrifugation and the aqueous phase was extracted with an equal volume of ethyl acetate. The ethyl acetate layer was removed, evaporated to dryness and the residue dissolved in 3 ml of hexane : ethanol (80 : 20).

For the reverse-phase determination of *cis*-1,2-indandiol titers, 20 $\mu$l of culture supernatant or isopropanol extract were chromatographed on a Zorbax RX-C8 column (5-$\mu$m particle, 4.6 mm i.d. × 25 cm, Mac Mod Analytical Inc, Chadds Ford, PA, USA) using a gradient method. With a flow rate of 1 ml $min^{-1}$ and a column temperature of 30°C, the acetonitrile : 0.1% (v/v) phosphoric acid in water ratio was increased linearly over 20 min (post-injection) from 15 : 85 to 85 : 15. Ultraviolet absorption of the eluant at

220 nm was monitored and *cis*-1,2-indandiol or monooxygenated compounds (1-indenol and 1-indanone) were identified and quantitated by comparison to pure standards. 1,2-Indenediol (or the keto-hydroxy indan tautomer) had been identified previously by LC-MS (J Ballard *et al*, Merck & Co, unpublished results) and was monitored by area counts.

To determine the *cis*-(1*S*),(2*R*)-indandiol enantiomeric excess, 20 $\mu$l of the redissolved extract were chromatographed on a Chiralpak AD column (250 × 4.6 mm, Chiral Technologies Inc, Exton, PA, USA) employing a mobile phase of hexane : ethanol (80 : 20) at a flow rate of 1 ml $min^{-1}$. Ultraviolet absorption of the eluant at 220 nm was monitored and the *cis*-(1*S*),(2*R*)-indandiol and *cis*-(1*R*),(2*S*)-indandiol enantiomers were identified by comparison to standards. The areas of peaks were used to calculate the *cis*-(1*S*),(2*R*)-indandiol enantiomeric excess, which was expressed as a percentage of the difference of the two enantiomers [(1*S*,2*R*) − (1*R*,2*S*)] divided by the sum of the two enantiomers [(1*S*,2*R*) + (1*R*,2*S*)].

### *Methods for isolating toluene-independent cultures*

Two mutation and screening procedures were employed to generate mutants of *P. putida* F1 capable of converting indene to *cis*-1,2-indandiol without requiring toluene in the culture medium. Central to both screening procedures is the ability of many aromatic hydrocarbon-utilizing microbes to convert indole to indigo (blue pigment) [3,5]. Toluene dioxygenase converts indole to *cis*-indole 2,3 dihydrodiol [3]. Spontaneous elimination of water forms indoxyl, followed by air oxidation to produce indigo [5].

Colonies of *P. putida* F1 grown on minimal media containing indole in the presence of toluene vapors become dark blue within 18–24 h while colonies incubated in a normal air atmosphere (with a non-inducing carbon source in the medium) require up to 36 h to obtain a light blue color (data not shown) [7]. Thus the conversion of indole to indigo ('indigo marker') serves as a useful biochemical marker for dioxygenase and *tod* operon activity.

The 'direct screening' procedure for isolating toluene-independent cultures utilized the 'indigo marker' to identify directly cultures able to carry out this conversion in the absence of toluene (ie, inducer). The 'toluene-positive revertant' screening procedure relied on reverting toluene-negative strains (identified using the 'indigo marker') to toluene-positive as a means for isolating cultures with altered *tod* operon regulation. This approach was successfully employed by Worsey and co-workers for isolating TOL plasmid regulatory mutants which expressed the genes in the operon in a constitutive fashion [16].

### *Direct screening*

For the direct screening procedure, an overnight culture of *P. putida* F1 grown in TSB was diluted $10^{-4}$ with phosphate buffer (100 mM, pH 7.2). Several 18 × 150-mm test tubes containing 3 ml of MMC medium plus 0 (control), 10, 20, 30, 40, 50, 75, or 100 mg $L^{-1}$ ICR 191 (acridine mutagen) were inoculated with 0.1 ml of the diluted inoculum. Test tube cultures were incubated in the dark at 30°C with 220 rpm rotary shaking. After a 16- to 18-h incubation, the culture displaying low but significant levels of growth was harvested by centrifugation, washed twice with phosphate buffer, and diluted appropriately to yield a workable plating density.

One-milliliter aliquots of the mutagenized population were spread on solid indole indicator medium in 24.5 × 24.5-cm bioassay dishes and incubated at 30°C for 18–24 h. The indicator medium consisted of modified MMC medium (0.040 g $L^{-1}$ $FeSO_4 \cdot 7H_2O$) with 1 mM indole as the indicator. Candidate colonies (small, blue, and less than 24 h old) were identified and confirmed by re-streaking on indicator medium. After confirmation, these cultures were transferred to TSA and evaluated for their ability to convert indene to *cis*-1,2-indandiol in the absence of toluene in the shake-flask bioconversion process.

### *Screening of toluene-positive revertants*

For screening of toluene-positive revertants, an aliquot of a *P. putida* F1 overnight culture grown in TSB was harvested by centrifugation and washed twice with citrate buffer (100 mM, pH 5.5). *N*-Methyl-*N*′-nitro-*N*-nitrosoguanidine (NTG) was added to a final concentration of 50 mg $L^{-1}$ and the cells were incubated at room temperature with occasional mixing. After a 30-min incubation, cells were washed twice with phosphate buffer (100 mM, pH 7.2) and diluted appropriately.

Aliquots of the mutated population (0.1 ml) were spread on solid indole indicator medium in 100 × 15-mm glass petri dishes and incubated at 30°C in the presence of toluene. White colonies (no indigo production, dioxygenase and toluene-negative) were transferred to TSA. Single dioxygenase-negative colonies were inoculated into 250-ml 3-baffled Erlenmeyer flasks containing minimal medium (MM) with silicone oil as the second phase and no citrate. Toluene was introduced into the culture in the silicone oil phase at final overall concentration of 5 g $L^{-1}$. Within 48–72 h of incubation at 30°C with 180 rpm rotary shaking, spontaneous, toluene-positive revertants began to grow out. Colonies from these revertant cultures and re-isolates thereof were evaluated for toluene-independent conversion of indene to *cis*-1,2-indandiol in the shake-flask fermentation process.

### *Isolation of toluene-negative strains producing reduced amounts of 1,2-indenediol (keto-hydroxy indan)*

The procedure used to isolate toluene-negative strains was essentially that described by Finette and co-workers [6]. A 4-ml culture of mutant 421-5 was mutagenized with NTG as outlined above. After washing them with phosphate buffer (100 mM, pH 7.2), the cells were tranferred to a 250-ml 3-baffled Erlenmeyer flask containing 20 ml of minimal medium with toluene (5 g $L^{-1}$ final concentration) and 5 ml of silicone oil. Following a 3-h incubation at 30°C with 180 rpm rotary shaking, ampicillin and *D*-cycloserine were added to the culture at final concentrations of 200 and 100 mg $L^{-1}$ respectively. The culture was incubated for an additional 3 h after which the cells were harvested, washed twice with phosphate buffer, and diluted appropriately.

Aliquots of the toluene-negative enriched population (0.1 ml) were spread on solid TTC (triphenyltetrazolium chloride) indicator medium in glass petri dishes and incubated at 30°C in the presence of toluene vapors. TTC indi-

cator medium consisted of MM medium supplemented with 1.25 g $L^{-1}$ tryptone and 0.025 g $L^{-1}$ TTC as the redox indicator. Under these conditions, toluene-positive cells grow as large red colonies while toluene-negative isolates are small and white. Small, white colonies were transferred to TSA and evaluated in the shake-flask process for reduced 1,2-indenediol (or the keto-hydroxy indan tautomer) production based on relative areas of peaks from HPLC compared to the control (toluene-independent parent).

## Results

### *Isolation of toluene-independent cultures—direct screening*

This direct procedure had a high throughput and allowed for the screening of 150 000 colonies; 16 candidates (small, blue, and less than 24 h old) were identified. After confirmation by re-streaking on indicator medium, these candidate cultures were evaluated for their ability to convert indene to *cis*-1,2-indandiol in the absence of toluene in shake-flask cultures. One of the isolates tested, culture 421-5 (ATCC 55687) produced 235 mg $L^{-1}$ compared to 12 mg $L^{-1}$ for the *P. putida* F1 parent.

The conversion of indene to *cis*-1,2-indandiol by culture 421-5 was performed at the 23-L fermentor scale with the MMC process (Figure 2). Using $\%CO_2$ in the off-gas as an indication of growth, it is apparent that the majority of product formation occurs during the active growth phase (duration = 4–4.5 h). Moreover, the major product formed during the conversion is 1-indenol with a maximum titer of approximately 350 mg $L^{-1}$ compared to a 200 mg $L^{-1}$ maximum titer for *cis*-1,2-indandiol. 1-Indanone is produced in a slow but linear fashion throughout the course of the cycle. These results are consistent with the findings of Wackett and co-workers for toluene-induced *P. putida* cells [15].

### *Isolation of toluene-independent cultures—screening of toluene-positive revertants*

Within 48–72 h of incubation, 40 of the 43 toluene-negative strains isolated spontaneously reverted to the toluene-positive phenotype in liquid culture with toluene as the sole carbon source. TSA cultures from these flasks were generated and evaluated for toluene-independent conversion of indene to *cis*-1,2-indandiol in the shake-flask fermentation process in the absence of toluene. Of the 40 revertant cultures tested, culture 419-11 produced significantly more *cis*-1,2-indandiol than the *P. putida* F1 parent (83 mg $L^{-1}$ *vs* 9 mg $L^{-1}$ respectively) using the MMC bioconversion process.

At the 23-L fermentor scale a pattern of results similar to those for culture 421-5 was obtained for culture 419-11 (Figure 3). 1-Indenol was again the major indene conversion product (225 mg $L^{-1}$ *vs* 150 mg $L^{-1}$ for *cis*-1,2-indandiol) and 1-indanone was produced in a linear fashion. In contrast to culture 421-5, culture 419-11 continued to produce a fair amount of product after the peak $\%CO_2$ was reached (≈3.5–4 h). This result indicates an inherent difference in the two strains.

### *Conversion of indene to cis-1,2-indandiol by resting cells*

A resting-cell conversion system was employed to clarify whether cultures 421-5 and 419-11 were constitutively expressing the dioxygenase enzyme system or were being induced by indene. Growing cultures 421-5, 419-11, and *P. putida* F1 in MMC medium in the presence of toluene, indene, or no aromatic substrate, followed by a bioconversion period in the presence of a protein synthesis inhibitor (chloramphenicol) would determine the difference between constitutive and indene-inducible. Control experiments demonstrated that 100 mg $L^{-1}$ chloramphenicol was capable of inhibiting growth of *P. putida* F1.

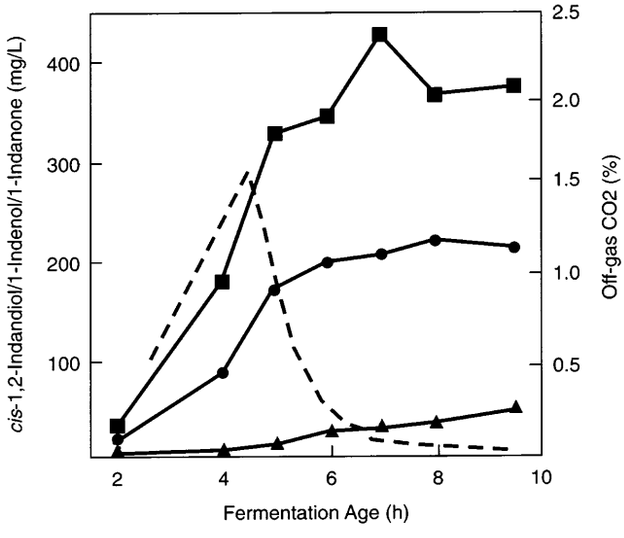


**Figure 2** Conversion of indene to *cis*-1,2-indandiol (—●—), 1-indenol (—■—), and 1-indanone (—▲—) by toluene-independent culture 421-5. The fermentation was carried out at the 23-L scale using the MMC process as described in Materials and Methods. Off-gas $CO_2$ (– – – –) was used as a measure of culture growth and overall metabolic activity.

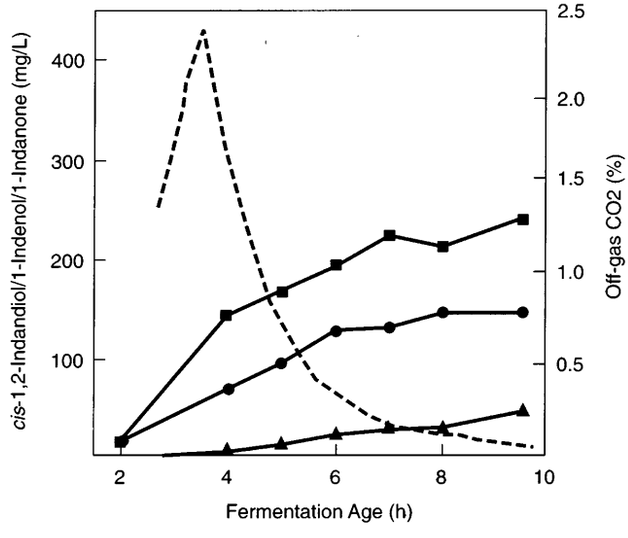


**Figure 3** Conversion of indene to *cis*-1,2-indandiol (—●—), 1-indenol (—■—), and 1-indanone (—▲—) by toluene-independent culture 419-11. The fermentation was carried out at the 23-L scale using the MMC process as described in Materials and Methods. Off-gas $CO_2$ (– – – –) was used as a measure of culture growth and overall metabolic activity.

As Figure 4 illustrates, growing culture 421-5 in the presence of toluene or indene resulted in active resting-cell cultures. When 421-5 was grown in MMC medium (with no aromatic substrate), no significant resting-cell activity was detected. Compared to *P. putida* F1, where active resting cells were obtained only after growth in the presence of toluene (but not indene), the TDO system in culture 421-5 was clearly induced by the presence of indene in the culture medium during growth. In contrast, all three growth conditions for culture 419-11 resulted in active resting-cell cultures. Since no aromatic inducer (ie, toluene or indene) was necessary for generating active resting cells, this culture is clearly constitutive.

### *Increase in the 1S, 2R-enantiomeric excess as a function of time*

Using a toluene/indene co-oxidation process with *P. putida* F1 (parent), it was determined that the 1S,2R-enantiomeric excess was low at the beginning of the conversion period and increased linearly to >90% by the end of the cycle even in the absence of additional indene-conversion activity (data not shown). To determine if a similar phenomenon was taking place with the toluene-independent strains, culture 421-5 was evaluated in the MMC shake-flask fermentation process (Figure 5). Production of *cis*-1,2-indandiol began at about 6 h and increased sharply to 210 mg $L^{-1}$ by 10 h. The rate of conversion dropped off dramatically between 10 and 20 h reaching a maximum titer of 250 mg $L^{-1}$ which fell to 220 mg $L^{-1}$ by 24 h. The *cis*-(1*S*),(2*R*)-indandiol enantiomeric excess also increased with time—starting at 30% when conversion began and increasing to 95% at the end of the fermentation. As had been observed earlier with the parent culture, the *cis*-(1*S*),(2*R*)-indandiol enantiomeric excess increased between 20 and 24 h which corresponded to a decrease in the *cis*-1,2-indandiol titer.

Since the increase of the *cis*-(1*S*),(2*R*)-indandiol enantiomeric excess likely occurred during a period of carbon starvation, the mid-cycle (8 h post inoculation) addition of 10 mM citrate (MMC+C process) was evaluated to determine if adding additional carbon to the fermentation would affect the kinetics of the *cis*-(1*S*),(2*R*)-indandiol enantiomeric excess increase (Figure 5). The additional citrate resulted in a maximum *cis*-1,2-indandiol titer of 400 mg $L^{-1}$ being achieved in 12 h. As was seen for the MMC process, the (1*S*),(2*R*)-enantiomeric excess also increased with time. However, the addition of citric acid at 8 h resulted in the levelling off of the (1*S*),(2*R*)-enantiomeric excess at 50% until 12 h into the cycle at which point the value rose to 92% by the end of the cycle.

### *Isolation of toluene-negative strains producing reduced amounts of 1,2-indenediol*

The combination of NTG mutagenesis and ampicillin/*D*-cycloserine enrichment resulted in a 4-log reduction in the viable cell population. As a result of the enrichment procedure, the viable population had a significant percentage of toluene-negative cells (data not shown) which were evaluated in the MMC shake-flask process. The *cis*-1,2-indandiol titers and *cis*-(1*S*),(2*R*)-indandiol enantiomeric excesses for four cultures producing roughly 50% of the 1,2-indenediol (or the keto-hydroxy indan tautomer, data

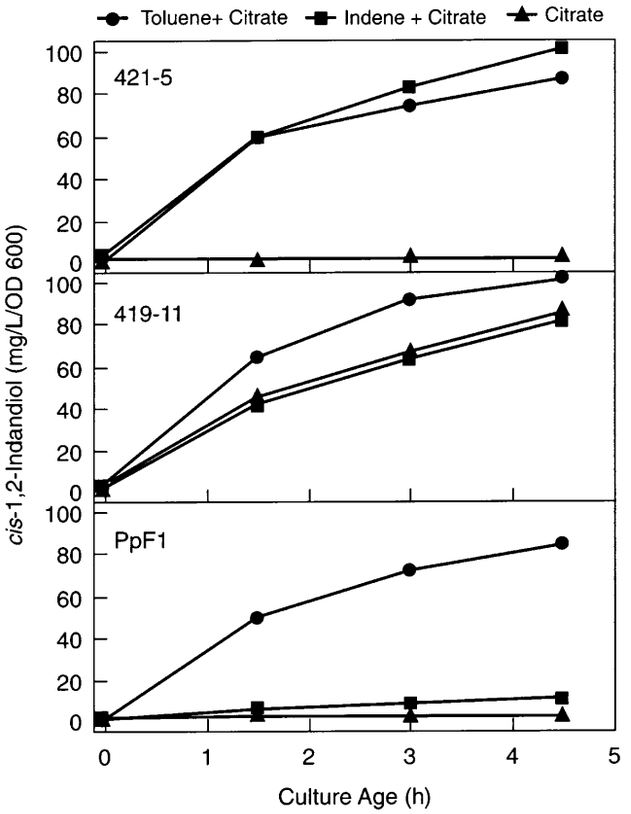


**Figure 4** Effect of aromatic-substrate inducer on the conversion of indene to *cis*-1,2-indandiol by cultures 421-5, 419-11 and *P. putida* F1. Each culture was grown in MMC medium (citrate as the main carbon source) with toluene (20 mM), indene (20 mM), or no additional carbon source added. Resting-cultures were prepared and allowed to convert indene to *cis*-1,2-indandiol as described in Materials and Methods.

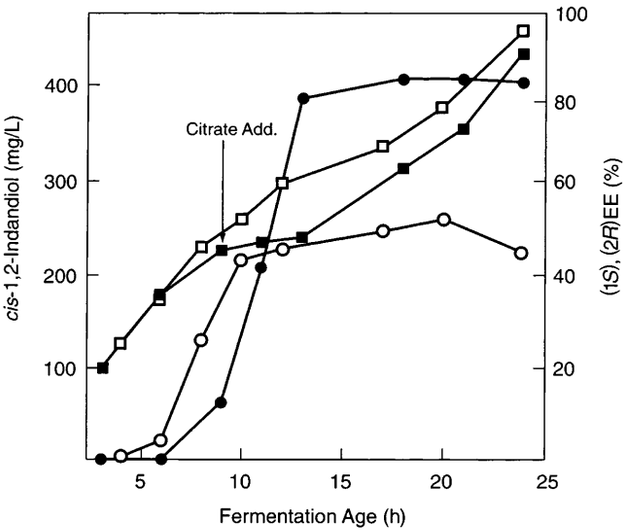


**Figure 5** Kinetics of *cis*-1,2-indandiol (—●—, —○—) and (1*S*),(2*R*)-enantiomeric excess (—■—, —□—) increase. Culture 421-5 was utilized in the MMC shake-flask process with no additional citrate being added (open symbols) or additional citrate being added after 8 h post inoculation (closed symbols).

**Table 1** Evaluation of mutants producing reduced amounts of 1,2-indenediol (keto-hydroxy indan)

| Strain | *cis*-1,2-indandiol (mg $L^{-1}$) | (1*S*),(2*R*) EE (%) |
|---|---|---|
| 421-5 (parent) | 220 | 95 |
| 519-33 | 390 | 53 |
| 526-6 | 447 | 55 |
| 526-19 | 237 | 66 |
| 526-30 | 417 | 65 |

not shown) compared to the 421-5 parent culture are shown in Table 1. Three of the four cultures produced 75–100% more *cis*-1,2-indandiol than the 421-5 parent. However, the *cis*-(1*S*),(2*R*)-indandiol enantiomeric excesses produced ranged from 53 to 66% for the four mutants compared to 95% for the 421-5 parent strain.

## Discussion

Figure 6 summarizes the comparative metabolism of toluene and indene by *P. putida* F1 and mutants thereof. At the 23-L fermentor scale, cultures 421-5 (ATCC 55687) and 419-11 produced *cis*-1,2-indandiol in a toluene-independent manner. LC-MS and NMR results (Ballard *et al*, unpublished results) demonstrated that 1,2-indenediol and the keto-hydroxy indan tautomer were also accumulated in the culture medium during the conversion. Both cultures, however, produced 1-indenol as the major conversion product from which 1-indanone arises as a function of time via chemical isomerization [15]. Since no enzymatically-produced monooxygenation products are produced for toluene (DT Gibson, University of Iowa, personal communication), it can be concluded that the formation of 1-indenol is the result of an 'improper fit' of indene in the active site of the dioxygenase.

Culture 419-11 continued to convert indene during the period of respiratory decline which indicated an inherent difference between this strain and culture 421-5 whose conversion activity was mainly growth associated. Based on the resting-cell activity data, culture 421-5 is indene-inducible while culture 419-11 expresses the TDO enzyme system constitutively. It is plausible that the mutation that was introduced in culture 421-5 allowed the TDO system to be better induced by indole and since their structures are similar, by indene as well. It is not clear where the mutation/reversion lies which resulted in the generation of culture 419-11. However, it is noteworthy that Finette and Gibson isolated pleiotropic-negative mutants with high frequency indicating that the *tod* operon is likely to be positively regulated [4,7].

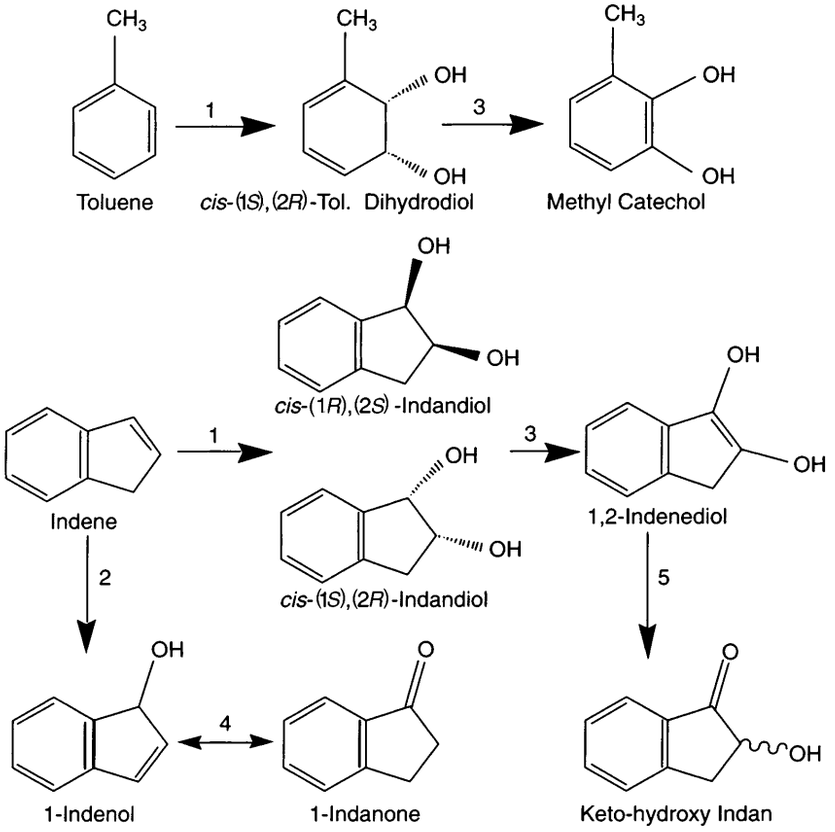


**Figure 6** Comparison of the metabolism of toluene and the proposed metabolism of indene. Numbers (over arrows) indicate the following enzymatic or chemical reactions: (1) dioxygenation of toluene or indene by *P. putida* toluene dioxygenase producing the indicated diols; (2) monooxygenation of indene by *P. putida* toluene dioxygenase forming 1-indenol; (3) oxidation of toluene dihydrodiol or *cis*-1,2-indandiol by *cis*-glycol dehydrogenase (NAD→NADH) forming methyl catechol and 1,2-indenediol respectively; (4) chemical isomerization of 1-indenol to 1-indanone; and (5) tautomerization of 1,2-indenediol to keto-hydroxy indan.

Both methyl catechol (from toluene) and catechol (from benzene) will serve as carbon sources but must be introduced to cultures at a slow rate to avoid toxicity [13]. Based on the toxicity of catechols, it was thought that reducing the amount of 1,2-indenediol (keto-hydroxy indan) produced would allow the culture to continue to convert indene. Three strains were isolated that produced 75–100% more *cis*-1,2-indandiol than the 421-5 parent. However, the *cis*-(1*S*),(2*R*)-indandiol enantiomeric excesses were between 53 and 66% corresponding to a 50% reduction in the level of 1,2-indenediol produced (Table 1). Based on analogous reactions taking place with toluene as the carbon source, these strains should have reduced levels of the *cis*-glycol dehydrogenase which oxidizes *cis*-glycols to catechols (Figure 6) [12]. While reduced levels of 1,2-indenediol correlated with increased levels of *cis*-1,2-indandiol, a reduction in the *cis*-(1*S*),(2*R*)-indandiol enantiomeric excess was also seen.

Using a mutant strain deficient in *cis*-glycol dehydrogenase activity (*P. putida* F39/D), Wackett and co-workers demonstrated the conversion of indene to *cis*-(1*S*),(2*R*)-indandiol of only 30% enantiomeric excess [15]. In the present work, it was demonstrated that the *cis*-(1*S*),(2*R*)-indandiol enantiomeric excess increased as a function of time even when indene conversion had ceased and that adding additional carbon source (in the form of citrate) resulted in a brief delay (approximately 4 h) in the kinetics of the *cis*-(1*S*),(2*R*)-indandiol enantiomeric excess increase (Figure 5). These results suggest that the TDO enzyme system produces a *cis*-(1*S*),(2*R*)-indandiol of low enantiomeric excess (30–40%) and that a *cis*-glycol dehydrogenase plays a role in the kinetic resolution of the (1*S*),(2*R*) and (1*R*),(2*S*) enantiomers.

The first four enzymes of the *tod* operon (toluene dioxygenase enzyme system, *tod*ABC1C2; *cis*-glycol dehydrogenase, *tod*D; methylcatechol 2,3-dioxygenase, *tod*E; and 2-hydroxy-6-oxo-2,4-heptadienoate hydrolase, *tod*F) are coordinately expressed indicating that the increase in the *cis*-(1*S*),(2*R*)-indandiol enantiomeric excess is not the result of an enzyme induction effect [7]. Since the oxidation of *cis*-1,2-indandiol to 1,2-indenediol results in generation of NADH, the cell may utilize this reaction for harnessing energy during a time of carbon starvation. The NADH so produced would then be oxidized via the electron transport system. In the presence of a suitable carbon source (eg citrate) the oxidation/reduction of NAD(H) would occur between the TCA cycle and the electron transport system. At this point it would be argued that the preferred substrate for the *cis*-glycol dehydrogenase would be *cis*-1R,2S-indandiol. This is unusual because the 1R,2S enantiomer is not produced in excess at any point during the conversion and the (1*S*),(2*R*) enantiomer is produced from toluene (natural substrate). Jenkins and co-workers presented evidence that *P. putida* NCIB11767 has two *cis*-glycol dehydrogenases which differ in their sensitivities to inhibitors, substrate affinities, and pH optima [12]. If two *cis*-glycol dehydrogenases exist in *P. putida* F1 (parent strain), then perhaps one isozyme has a relatively higher affinity for *cis*-(1*R*),(2*S*)-indandiol than the other isozyme has for *cis*-(1*S*),(2*R*)-indandiol. This would also suggest that *P. putida* F39/D is a double mutant. The physiological significance of these two isozymes is not readily apparent.

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