Development of a bioconversion process for production of cis-1S,2R-indandiol from indene by recombinant Escherichia coli constructs

Abstract Recombinant Escherichia coli cells expressing the toluene dioxygenase (TDO) genes from Pseudomonas putida convert indene to cis-1S,2R-indandiol, a potentially important intermediate for the chemical synthesis of the HIV-1 protease inhibitor, Crixivan. A bioconversion process was developed through optimization of medium composition and reaction conditions at the shake-flask and 23-l fermentor scales. A cis-1,2-indandiadiol productivity of approx. 1000 mg/l was achieved with construct TDO123, which represents a 50-fold increase over the initial titer. Varying the bioconversion conditions did not change the enantiomeric excess (e.e.) for the 1S,2R enantiomer from about 30%, suggesting that toluene dioxygenase intrinsically converts indene to 1S,2R- and 1R,2S-indandiadiols at a ratio of 2:1. Further inclusion of the Pseudomonas dehydrogenase gene in construct D160-1 led to the production of chirally pure cis-1S,2R-indandiol (e.e. > 99%) as a result of the selective degradation of the 1R,2S enantiomer, with the overall yield (650 mg/l) proportionally reduced. A single stage process was developed for D160-1 and scaled up to the 23-l fermentor, achieving a cis-1S,2R-indandiol titer of 1200 mg/l.

Introduction

Bacterial aromatic ring dioxygenases are multicomponent enzyme systems capable of catalyzing the initial incorporation of molecular oxygen and two hydrogen atoms into the aromatic substrate to produce the cis-diene diol. One such enzyme system is toluene dioxygenase (TDO) from Pseudomonas putida F1, the genes of which have been given the designation tod (Zylstra et al. 1988). TDO consists of three protein components: reductase_{tol} (todA), ferredoxin_{tol} (todB), and a terminal oxygenase, which is an iron-sulfur protein, i.e. ISP_{tol} (todC1 and -C2). The three protein components essential to the toluene dioxygenase activity have been purified and their properties extensively characterized (Gibson et al. 1979). TDO functions by transferring electrons from NADH through a flavoprotein (reductase_tol) and a ferredoxin to the two-subunit iron-sulfur protein that incorporates molecular oxygen into the aromatic substrates forming the cis-dihydrodiol (Kobal et al. 1973; Gibson et al. 1979; Gibson and Subramanian 1979). In P. putida F1, the cis-toluene dihydrodiol produced from toluene by TDO is dehydrogenated in a NAD^{+}-dependent reaction by cis-toluene dihydrodiol dehydrogenase (todD) to form 3-methylcatechol, which is cleaved by 3-methylcatechol 2,3-dioxygenase (toddE) to form 2-hydroxy-6-oxo-2,4-heptadienoate (Gibson et al. 1979). Figure 1 shows the organization of the toluene dioxygenase multienzyme complex and the reactions used by P. putida F1 to metabolize toluene (Zylstra and Gibson 1989).

Interest in the reactions catalyzed by TDO and other dioxygenases stems from their ability to produce enantioselectively pure cis-diols from an extremely wide range of substrates (Ziffer et al. 1973; Gibson and Subramanian 1984; Gibson et al. 1990), and from the availability of recombinant DNA technology for quickly optimizing the activity of specific enzymes in whole cells. Knowledge of the nucleotide sequence of the tod genes was employed by Zylstra and Gibson (1989) to construct clones of Escherichia coli JM109 which, upon induction with isopropyl β-d-thiogalactopyranoside (IPTG), overproduced toluene dioxygenase, which oxidized toluene to the dihydrodiol. Stephens et al. (1989) cloned the genes encoding toluene dioxygenase from P. putida NCIB 11767 into E. coli HB101 and the recombinant strain produced indigo from indole. Wahbi et al. (1997) isolated toluene dioxygenase genes from P. putida and subcloned them to produce high levels of toluene cis-glycol. Wackett et al. (1988) have shown that toluene-induced cells of P. putida F39/D (a mutant lacking cis-glycol dehydrogenase activity) converted indene to a mixture of cis-1S,2R-indandiol and cis-1R,2S-indandiol. The enantiomeric excess (e.e.) of the cis-1S,2R-indandiol produced was approximately 30%.
cis-1S,2R-Aminoindanol is a key precursor in the chemical synthesis of the HIV protease inhibitor Indinavir Sulfate (Crixivan). The current chemical route includes the synthesis of cis-1S,2R-aminoindanol from indene (Reider 1997). As an alternative to the rather difficult steps in the chemical synthesis (Senanayake et al. 1995; Hughes et al. 1997), biotransformation was explored to convert indene to cis-1S,2R-aminoindanol by means of the Ritter reaction (Senanayake et al. 1995). From P. putida F1, a region of the tod operon encoding TDO was cloned into commercial plasmids by Merck scientists and transformed into E. coli hosts. This paper describes the development and optimization of the process for conversion of indene to cis-1,2-indandiols using recombinant E. coli constructs harboring TDO genes, focusing on the product yield and purity for the desired 1S,2R enantiomer.

Materials and methods

Microorganisms

E. coli TDO123 (MB5736, deposited in the Merck Culture Collection) was constructed by cloning the todCl, C2, B, and A genes of P. putida F1 into the commercial plasmid vector pBC-ks (Stratagene, La Jolla, Calif.) and transforming them into the E. coli host DH5. First, the 2.6-kb fragment between the KpnI and NotI sites was amplified by the polymerase chain reaction (PCR) and cloned into pBC-ks under control of the tac promoter using DH5 as a host strain. Positive clones were replicated and the plasmid (pTDO12) was isolated from E. coli to serve as a vector for subsequent cloning of the 1.2-kb TDO fragment between NotI and SacI. The second PCR-amplified fragments and the pTDO12 vector, which were cut with NotI and SacI, were ligated and transformed into E. coli DH5 by the CaCl2 method (Maniatis et al. 1982), generating the recombinant strain TDO123.

The entire TDO operon (todCl, C2, B, A, D, E genes) from P. putida chromosomal DNA (Zylstra and Gibson 1989) were cloned as a 6- to 7-kb fragment from a lambda phage sublibrary, which was created by insertion of the DNA digested with EcoRI and XhoI (6–7 kb, sized on agarose gels) into the EcoRI, XhoI cut of lambda ZAPII vector (Stratagene). Recombinant plasmid was packaged and screened by plating on E. coli Blue cells and probing with TDO123 DNA. TDO-positive plaques were obtained and converted to a pBluescript SK II plasmid with the 6- to 7-kb EcoRI, XhoI TDO insert by an in vivo excision reaction, which removed lambda phage DNA, according to the vector manufacturer’s instruction. The resulting plasmid was used to transform the E. coli host NEB316 (New England Biolabs), of which a selected transformant was designated clone D160-1 (MB5735, deposited in Merck Culture Collection).

All the E. coli constructs were grown and maintained in LB medium (Luria broth) and LB medium with 2% (w/v) agar. Plates were incubated at 37 °C. Liquid cultures were stored at 4 °C for up to 4 weeks. Liquid cultures were diluted 1:1 with 20% (v/v) glycerol and stored as 1-ml aliquots at −70 °C.

Media

LB medium was used for inoculum development, while several media including 2XLB, M101/glucose, M101/glucose, 1XTB and its modifications, and the defined DME/glucose, DME/glucose and M9 were tested for growth and bioconversion. LB was composed of 10 g/l Bacto tryptone, 5 g/l Bacto yeast extract and 10 g/l NaCl at pH 7.0. 2XLB had doubled concentrations of each component except NaCl. M101/glucose contained 3.5 g/l K2HPO4, 3.5 g/l KH2PO4, 10 g/l Difco yeast extract, 50 g/l hysoy peptone, 3 g/l (NH4)2SO4, and post-sterilization additions of 20 g/l glucose, 0.002 g/l thiamine and 2 g/l MgSO4 · 7H2O. M101/glyceral contained 20 g/l glycerol instead of glucose. 1XTB medium had 12 g/l Bacto tryptone, 24 g/l Bacto yeast extract, 4 ml/L glycerol, and a post-sterilization addition of phosphates (0.17 M KH2PO4, 0.72 M K2HPO4). When modifications were made to 1XTB, FeSO4 was used at 0.04 g/l, (NH4)2SO4 at 5 g/l and MgSO4 · 7H2O at 1.2 g/l. The FeSO4 and MgSO4 · 7H2O were added after sterilization. DME/glucose contained 3.5 g/l KH2PO4, 3.5 g/l K2HPO4, 3.0 g/l (NH4)2SO4, 5 g/l monosodium glutamate, 15 g/l glycerol, and post-sterilization additions of 0.13 g/l thiamine, 0.52 g/l MgSO4 · 7H2O, and 2 ml/l trace element solution, which was comprised of 27 g/l FeCl3 · 6H2O, 2 g/l ZnCl2 · 4H2O, 2 g/l CoCl2 · 6H2O, 2 g/l Na2MoO4 · 2H2O, 1 g/l CaCl2 · 2H2O, 1.27 g/l CuCl2 · 2H2O and 0.5 g/l H3BO3. DME/glucose contained 15 g/l glucose instead of glycerol. M9 defined medium contained 4 g/l glucose, 12.5 g/l Na2HPO4 · 7H2O, 1 g/l NH4Cl, 0.5 g/l NaCl and 3 g/l KH2PO4.

Culture conditions

The thawed seed from a frozen vial was inoculated into a 250-ml conical flask containing 20 ml LB medium and 50 mg/l chloramphenicol (for TDO123) or ampicillin (for D160-1). After overnight cultivation at 37 °C and 220 rpm (5-cm throw), 2 ml broth was