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Nonproteinogenic α-Amino Acid Preparation Using Equilibrium Shifted Transamination

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Abstract:

Microbial α-transaminases such as tyrosine aminotransferase (TAT) and branched chain aminotransferase (BCAT) of *Escherichia coli,* are useful as industrial biocatalysts to prepare nonproteinogenic L-amino acids from α-keto acids and an amino donor. However, they typically yield only 50% product when L-glutamic acid, the preferred amino donor, is used due to accumulated 2-ketoglutaric acid. Accordingly, methods have been sought to increase the reaction yield by the recycle or removal of the keto acid by-product. In this report, we have investigated the biocatalytic coupling of *δ*-transamination with α-transamination to recycle 2-ketoglutaric acid, and thereby increase the yield of aminotransferase reaction products. Ornithine *δ*-aminotransferase (OAT) catalyses the reversible transfer of the *δ*-amino group of L-ornithine to 2-ketoglutaric acid forming L-glutamic acid semialdehyde and L-glutamic acid. The cyclisation of L-glutamic acid semialdehyde to form Δ^1-pyrroline-5-carboxylate under physiological conditions, favours the reaction in the direction of L-glutamic acid formation. The *Bacillus subtilis rocD* gene encoding OAT was cloned and produced at high levels in *E. coli*. Combined cell extracts of separate *E. coli* strains overproducing OAT and *E. coli* tyrosine aminotransferase enabled the synthesis of L-2-aminobutyrate from 2-ketobutyric acid to reach a yield of 92% compared to 50% achievable by TAT alone. Similarly, combined extracts of strains overproducing OAT and *E. coli* branched-chain amino acid aminotransferase synthesised L-*tert*-leucine from trimethylpyruvic acid with a 73% yield compared to 31% with BCAT alone. The use of OAT as a general biocatalytic tool to achieve high yields in aminotransferase reactions is discussed.

Introduction

Nonproteinogenic amino acids are important intermediates for a variety of pharmaceutical classes, most notably in peptidomimetics with antiviral and oncological applications. They are increasingly required at large scale and with high enantiomeric purity as demand for single-enantiomer drugs continues to grow. The stereoselectivity inherent in natural biochemistry has prompted the development of a variety of enzymatic bioprocesses to achieve the enantiospecific synthesis of this family of compounds. These bioprocesses include kinetic and dynamic kinetic resolution of racemates[7,19] and enantio-specific syntheses.[4,9,28] Amongst the latter, aminotransferases have been extensively studied in the large-scale preparation of nonproteinogenic L- or D-α-amino acids[1,9,25] as these enzymes typically display many desirable features of industrial biocatalysts, including high turnover, relaxed substrate specificity, and no requirement for external cofactor recycle.

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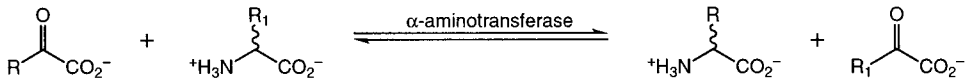

Figure 1. **General α-aminotransferase reaction.**

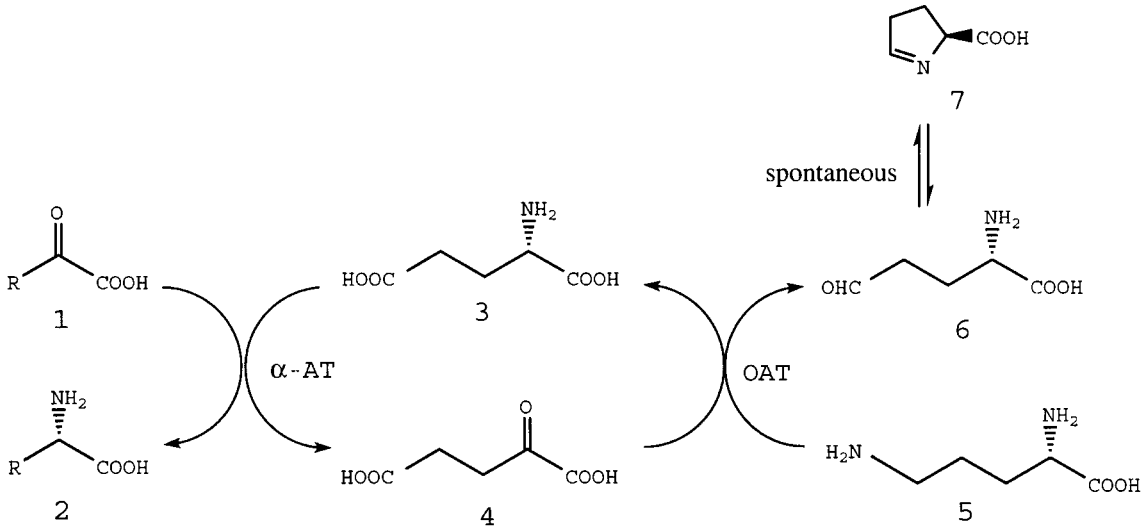

Figure 2. **Coupled reactions involving an α-aminotransferase and ornithine-*δ*-aminotransferase. 1: α-Keto acid. 2: L-Amino acid, 3: L-Glutamic acid. 4: α-Ketoglutaric acid. 5: L-Ornithine., 6: L-Glutamate-*γ*-semialdehyde. 7: Δ^1-Pyrroline-5-carboxylic acid.**

Aminotransferases catalyse the pyridoxal 5′-phosphate-dependent reversible transfer of an amino and keto group between an amino acid and keto acid substrate pair yielding new amino acid and keto acid products as shown in Figure 1. In *E. coli* and most other bacteria studied, aminotransferases are involved in the biosynthesis of many α-amino acids from their corresponding α-keto acids with L-glutamic acid serving as the preferred amino donor.[8,9,16] Due to the relaxed substrate specificity of microbial aminotransferases such as the tyrosine aminotransferase (TAT) and branched-chain aminotransferase (BCAT) of *E. coli*, these enzymes are also efficient in the enantiospecific preparation of nonproteinogenic α-amino acids such as L-homophenylalanine, L-2-aminobutyrate and L-*tert*-leucine from their corresponding α-keto acids.[9,31] However, the reversible transamination reaction typically results in a 50% conversion of substrates to products. This low yield and corresponding low product purity is not feasible for economical large-scale production and has prompted efforts to overcome this limitation of an otherwise attractive biocatalytic process.

Principally, this has been addressed by the use of aspartate, rather than glutamate, as the amino donor since aspartate yields the unstable *β*-keto acid product oxaloacetate instead of the relatively stable α-ketoglutarate.[16] Rapid decarboxylation of oxaloacetate to form pyruvate under biotransformation conditions favours aspartate consumption, thus increasing product yield significantly beyond 50%.[9] This is not a complete solution as residual pyruvate is often transaminated to L-alanine which also compromises product yield and purity. Additionally many aminotransferases do not accept aspartate as amino donor.[16] Although multienzyme systems have been devised to address these problems,[9,14] an alternative approach which enables a versatile high-yielding transamination from L-glutamate is preferable to eliminate undesired L-alanine as a by-product and to employ the generally preferred amino donor for this enzyme class. L-Ornithine *δ*-aminotransferase (OAT) [EC 2.6.1.13] catalyses the pyridoxal 5′-phosphate-dependent, reversible transamination of the *δ*-amino group from L-ornithine to α-ketoglutarate, producing L-glutamate-*γ*-semialdehyde and L-glutamic acid. The formation of L-glutamic acid from α-ketoglutarate is strongly favoured by the spontaneous cyclisation of L-glutamate-*γ*-semialdehyde to form Δ^1-pyrroline-5-carboxylate,[2] a precursor of L-proline in plants[27] and a product of arginine catabolism in microorganisms.[10,16] Ornithine aminotransferases have been found in a number of microbial isolates, many of them *Bacilli*.[11,15,17,20,23,35] This report describes the use of OAT, produced from the *Bacillus subtilis rocD* gene cloned in *E. coli*, in a coupled transamination, as shown in Figure 2, to recycle α-ketoglutarate generated in α-aminotransferase reactions in order to increase the production of specific nonproteinogenic amino acids.

Results

Cloning and Expression of Aminotransferase Genes. Plasmid pTL17 carries the cloned *rocD* gene of *B. subtilis* 168 encoding the 44 kD OAT protein.[15] Cell extracts prepared following induction of *rocD* expression and analysed by SDS-PAGE indicated a new 44 kD band. The

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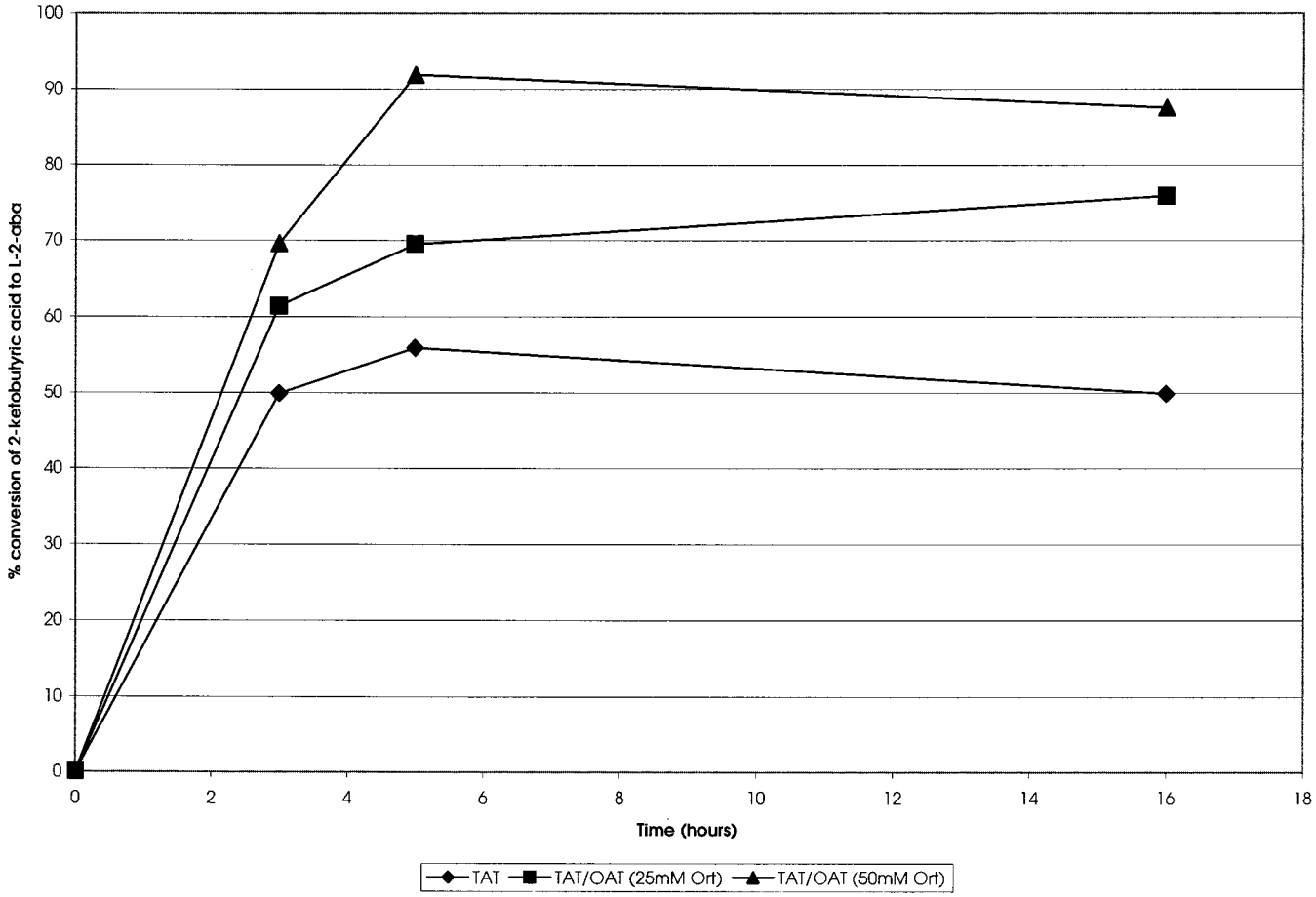

Figure 3. **Influence of OAT on the yield of L-2-aminobutyrate (L-2-aba) synthesised from 2-ketobutyric acid by TAT. Reaction 1 (♦) contained TAT in extract of W3110/pME64. Reactions 2 (■) and 3 (▲) additionally contained OAT in cell extract of W3110/pTL17 and 25 or 50 mM ornithine (Ort), respectively.**

intensity of the band represented approximately 40% of the releasable cell protein. The aminotransferase specific activity of the crude extract was 17 units/mg protein. Plasmid pME64 carries the cloned *E. coli* K12 *tyrB* gene[13] encoding the aromatic aminotransferase. Whole cells of strain W3110/pME64 have previously been shown to efficiently catalyse the L-2-aminobutyrate/L-glutamate transamination reaction.[14] Expression of the *tyrB* gene from its native promoter on plasmid pAT153,[33] is deregulated by the repressor titration effect of the very high copy number plasmid vector.[34] Plasmid pIF328 carries the cloned *ilvE* gene encoding the *E. coli* K12 branched-chain aminotransferase (BCAT) which has previously been shown to catalyse the L-*tert*-leucine/L-glutamate transamination reaction.[31] The *ilvE* gene is expressed from the *E. coli* K12 *pckA* promoter[22] region introduced immediately upstream on the vector.

Biosynthesis of L-2-Aminobutyric Acid. The synthesis of L-2-aminobutyrate was catalyzed by tyrosine aminotransferase using the crude cell extract of W3110/pME64. The effect on the reaction yield of coupling this reaction to ornithine δ-transamination was investigated in three reactions as shown in Figure 3. The reactions were sampled over the course of 16 h for amino acid analysis by HPLC. Reaction 1 is the α-transamination by TAT in the absence of OAT which contained 3.7 mg of the soluble protein from W3110/pME64, 50 mM 2-ketobutyric acid and 50 mM L-glutamic acid. Samples at 3, 5, and 16 h contained L-2-aminobutyrate at 24.93, 27.96, and 24.95 mM, respectively. The OAT transamination was incorporated into the other two reactions. Reaction 2 contained 3 mg of the soluble protein from W3110/pTL17 and 25 mM of L-ornithine in addition to the components of reaction 1. Samples of reaction 2 at 3, 5 and 16 h contained L-2-aminobutyrate at 30.71, 34.74, and 37.97 mM, respectively. In reaction 3 the concentration of ornithine was 50 mM, while everything else remained the same as reaction 2. Samples of reaction 3 at 3, 5 and 16 h contained L-2-aminobutyrate at 34.8, 45.96, and 43.79 mM, respectively.

Reaction 1 appeared to have reached equilibrium following incubation for 3 h. The final yield of reaction 1 was 49.9%, the equilibrium of the reversible TAT transamination. The yields of reaction 2 and 3 after 16 h incubation were 75.9 and 87.6%, respectively. Reaction 3 showed a 92% conversion of 2-ketobutyric acid to L-2-aminobutyrate after 5 h. When coupled with OAT reaction, there was a further increase in product yield as the amount of ornithine was increased.

Biosynthesis of L-*tert*-Leucine. The synthesis of L-*tert*-leucine was catalyzed by the branched-chain aminotransferase from the crude extract of W3110/pIF328. The effect of coupling to the OAT reaction was also investigated in three reactions similar to those for L-2-aminobutyrate prepa-

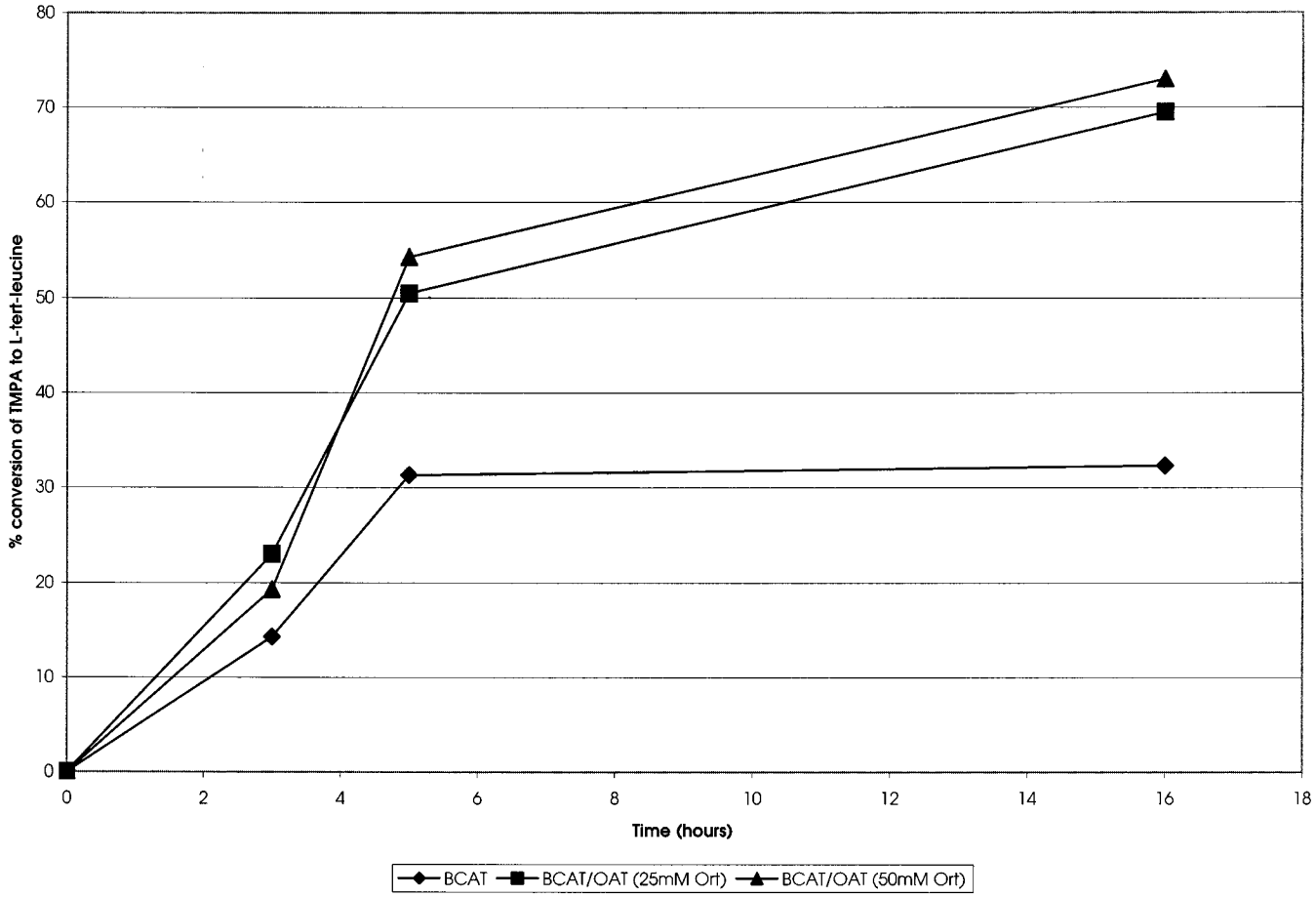

Figure 4. **Influence of OAT on the yield of L-*tert*-leucine synthesised from trimethylpyruvic acid (TMPA) by TAT. Reaction 1 (♦) contained TAT in extract of W3110/pIF328. Reactions 2 (■) and 3 (▲) additionally contained OAT in cell extract of W3110/pTL17 and 25 or 50 mM ornithine (Ort), respectively.**

ration as shown in Figure 4. Reaction 1 included 3.7 mg of the extracted soluble protein from W3110/pIF328, 40 mM trimethylpyruvic acid (TMPA) and 40 mM L-glutamic acid. Samples at 3, 5, and 16 h contained L-*tert*-leucine at 5.72, 12.53, and 12.92 mM, respectively. Reaction 2 contained 3 mg of soluble protein from W3110/pTL17 and 25 mM L-ornithine in addition to everything in reaction 1. Samples of reaction 2 at 3, 5 and 16 h contained L-*tert*-leucine at 9.2, 20.21, and 27.79 mM, respectively. In reaction 3 the concentration of ornithine was 50 mM, while everything else remained the same as reaction 2. Samples of reaction 3 at 3,5 and 16 h contained L-*tert*-leucine at 7.71, 21.72, and 29.19 mM, respectively. Reaction 1 did not progress further after 5 h incubation at 37 °C with a yield of 31.3% of L-*tert*-Leucine. Reactions 2 and 3 did not stop at 5 h. Yields for these two reactions at 16 h reached 69.5 and 73%, respectively.

Discussion

The use of *B. subtilis* ornithine aminotransferase (OAT) to couple L-ornithine ω-transamination to L-glutamate α-transamination enabled the latter reaction to greatly exceed the typical 50% product yield in the biosynthesis of two high-value amino acids. The yield of L-2-aminobutyrate produced by *E. coli* tyrosine aminotransferase increased from 50 to 92% when OAT was included in the biotransformation. Similarly the yield of L-*tert*-leucine produced by the *E. coli* branched-chain aminotransferase increased from 31 to 73% when OAT was included. Since neither 2-ketobutyric acid nor trimethylpyruvic acid is a substrate for OAT, the results in this report demonstrate that OAT can efficiently regenerate L-glutamate as the amino donor for the α-aminotransferase reaction. The improved reaction yields were due to the reduced reversibility of the OAT reaction under conditions where L-ornithine is transaminated to L-glutamate semialdehyde and spontaneously cyclised to Δ^1-pyrroline-5-carboxylate. In previous reports of preparative aminotransferase reactions the use of aspartic acid as the amino donor has been the only general strategy to enable the reaction to progress beyond the normal conversion of 50%. However, this often leads to accumulation of L-alanine which is difficult to remove and decreases the overall yield by creating an alternate pathway converting L-aspartate directly to L-alanine. Conversely, ornithine aminotransferase creates no alternate pathway, and the removal of residual L-ornithine and L-glutamate by precipitation or chromatographic approaches is facilitated by their charged nature. Selective crystallisation or precipitation can be used as appropriate to remove Δ^1-pyrroline-5-carboxylate on a case-specific basis. Future work will examine the optimal conditions for this bioprocess, including the minimum catalytic amount of L-glutamate required, operation at very high substrate concentrations, and

the coordinated maximal expression of the enzymes. Additionally further cost reduction could be achieved by replacing L-ornithine with L-lysine, a very low-cost feedstock amino acid whose transamination product also generates a cyclic imine, or by incorporation of arginase to produce ornithine directly from arginine.[3,5,32] The coupling of α- and ω-transamination offers a general method to enhance the overall applicability of these enzymes as biocatalysts in the preparation of unnatural amino acids at high enantiomeric purity. Since none of the enzymes used in this approach require active cellular metabolism for prolonged use as a biocatalyst, the process is appropriate for immobilisation of crude enzyme or whole cells. In this way, the production and handling of the enzymes can be greatly simplified, and recovery and recycling of the biocatalysts are improved, thereby reducing costs and minimising the effect on downstream processing and product isolation. The high expression of the OAT enzyme from the cloned *rocD* gene of *B. subtilis* again illustrates the growing potential to construct multienzyme biotransformations in recombinant strains of *E. coli* for scalable production of valuable compounds.

Experimental Section

General DNA Manipulation. General DNA handling including PCR, restriction analysis, DNA recovery, chromosomal and plasmid DNA preparations, and *E. coli* transformation were carried out using standard methods as described previously.[12] Restriction enzymes and DNA ligase were purchased from New England Biolabs (Beverly, MA). PCR was carried out using ClonTech HF PCR kits (Palo Alto, CA). Molecular biology grade reagents were purchased from Sigma-Aldrich (Milwaukee, WI).

Plasmid Construction. Plasmid pME64 was constructed as described.[13] Plasmid pIF328 was constructed as follows. The *ilvE* gene encoding the branched-chain aminotransferase was cloned from *E. coli* K12 chromosomal DNA by PCR using the following pair of oligonucleotide primers: 5′-CGCGGATCCACTATGACCACGAAGAAAGCTGATTACATTTGG-3′ and 5′-CAGCGTGCATGCTTATTGATTAACTTGATCTAACCA-3′. The amplified PCR product was cleaved with *Bam*HI and *Sph*I, and ligated to the 3.9 kb fragment resulting from similar cleavage of of pIF306.[12] The resulting plasmid was named pIF307. Plasmid pIF307 was cleaved with *Eco*RI and *Pst*I and the 4.1 kb band was isolated. This was ligated to a 982 bp fragment containing the kanamycin resistance gene from similarly cleaved plasmid pLG338.[29] The resulting plasmid was named pIF312. This plasmid was cleaved with *Eco*RI and *Bam*HI. The vector fragment of 4.97 kb was isolated and ligated to a 0.27 kb DNA fragment containing the *pckA* promoter of *E. coli* K12. This fragment was generated by *Eco*RI/*Bam*HI double digestion of an *E. coli* chromosomal fragment amplified using PCR with the following oligonucleotides as primers: 5′-GACGAATTCACTTTACCGGTTGAATTTGC-3′ and 5′-GACGGATCCTCCTTAGCCAATATGTATTGCC-3′. The resulting plasmid, named pIF313, was cleaved with *Sph*I and *Bsp*EI and the 4.1 kb vector fragment isolated. This fragment was then ligated to a 0.97 kb DNA fragment containing the *par* locus[24] of pLG338 This fragment was generated by *Sph*I and *Bsp*EI digestion of a fragment prepared by PCR from pLG338 as the template and the following oligonucleotides as primers: 5′-GACGCATGCACCATTCCTTGCGGCGGCG-3′ and 5′-GACTCCGGAGGCAAATCGCTGAATATTCC-3′. The resulting plasmid was named pIF328. Plasmid pTL17 was constructed as follows. The *rocD* gene was cloned from *B. subtilis* strain 168 by PCR with the following pair of primers: 5′-GACGGATCCACTATGACAGCTTTATCTAAATCCAAA-3′ and 5′-CAGGGTACCTCATTATGCGTTTCGCAGCACGTG-3′. The 1.2 kb DNA from the PCR was then cleaved with *Bam*HI and *Kpn*I and ligated to the 4.8 kb vector fragment of pPOT3[14] obtained from similar digestion. The resulting plasmid was named as pTL17. In this plasmid, the transcription of *rocD* gene is controlled by an upstream l P_R promoter. This promoter is regulated by a temperature-sensitive cI857 repressor carried on the same plasmid.[26]

Preparation of Enzymes for Biotransformation. Each plasmid was used to transform separate *E. coli* K12 W3110 (ATCC 2735) hosts for enzyme expression. Transformation was carried out by electroporation using a Bio-Rad Gene Pulser. Cell cultures carrying pME64, or pIF328 were prepared by inoculating 50 mL of LB medium with a single colony from an LB plate and were incubated with shaking at 37 °C overnight. Antibiotics, where appropriate, were used at the concentrations of 100 mg/mL ampicillin, 40 mg/mL kanamycin for solid and liquid media. Overnight cultures were then used to inoculate 1 L of LB plus antibiotics. They were grown in a 4-L flask with agitation at 37 °C until the OD_{600} reached 1.0. The cells were then recovered by centrifugation at 10000*g* for 5 min. The culture of W3110/pTL17 was prepared by inoculating 50 mL LB containing 10 mg/mL chloramphenicol with a single colony from an overnight LB agar/chloramphenicol plate. This culture was incubated with shaking at 30 °C overnight. The overnight culture was used to inoculate 1 L of LB plus chloramphenicol in a 4-L flask. The culture was grown to $OD_{600} = 0.6-0.8$, at which point OAT expression was induced by raising the incubator temperature to 42 °C. The incubation at 42 °C was continued for a further 2 h before the cells were recovered by centrifugation. To prepare enzyme extracts for biotransformation, the cells were broken with a French press at a pressure of 1000 lb/in^2, and the insoluble particles were removed by centrifugation at 10000*g* × 30 min.

Enzyme and Protein Assays. Protein concentration was determined using the Bradford assay.[6] Aminotransferase expression levels were analyzed by SDS-PAGE of crude cell extracts using 12% polyacrylamide.[21] The activity of OAT was determined using a standard aminotransferase assay.[30]

Amino Acid Preparation via Coupled Transaminations. All reactions were conducted in 50 mM phosphate, pH 8.5 at 37 °C, in a total volume of 2 mL in 5-mL capped tubes with agitation. After 2, 4, and 5 h of incubation the pH was checked and adjusted to pH 8.5 with 1 N NaOH. Samples of 100 mL were taken at 3, 5, and 16 h, centrifuged to remove cells, and diluted as appropriate for amino acid analysis by quantitative HPLC.

HPLC Analysis of Amino Acids. Amino acid samples and standards were derivatised with *o*-phthaldialdehyde [18] before analysis. The amino acid derivatives were separated on a C18 column (5 mm, 250 mm × 4.6 mm) by a 2−100% acetonitrile gradient in phosphate buffer (15 mM, pH 6.2) at the flow rate of 1.5 mL/min.

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