Whole-cell biocatalytic production of variously substituted β-aryl- and β-heteroaryl-β-amino acids

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ABSTRACT

Biologically-active β-peptides and pharmaceuticals that contain key β-amino acids are emerging as target therapies; thus, synthetic strategies to make substituted, enantiopure β-amino acids are increasing. Here, we use whole-cell Escherichia coli (OD600 ~35) engineered to express a Pantoea agglomerans phenylalanine aminomutase (PaPAM) biocatalyst. In either 5 mL, 100 mL, or 1 L of M9 minimal medium containing α-phenylalanine (20mM), the cells produced ~1.4 mg mL⁻¹ of β-phenylalanine in each volume. Representative pilot-scale 5 mL cultures, fermentation reactions converted variously substituted α-arylalanines to their (S)-β-aryl-β-amino acids in vivo and were not toxic to cells at mid- to late-stage growth. The β-aryl-β-amino acids made ranged from 0.043 mg (p-nitro-β-phenylalanine, 4% converted yield) to 1.2 mg (m-bromo-β-phenylalanine, 96% converted yield) over 6 h in 5 mL. The substituted β-amino acids made herein can be used in redox and Stille-coupling reactions to make synthetic building blocks, or as biostisomers in drug design.

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1. Introduction

While not abundant in nature, aromatic β-amino acids are found occasionally in pharmacologically important natural products, such as the antibiotic agent andrimid (I) from Pantoea agglomerans (Jin et al., 2006), antifungal and insecticidal agent jasplakinolide (II) from marine sponges (Crowes et al., 1986), and aminopeptidase inhibitor bestatin (III) from Streptomyces olivoreticuli (Chen et al., 2011; Nishizawa et al., 1977) (Table 1). In addition, pharmaceuticals such as the antiglycemic sitagliptin (Savile et al., 2010) (IV) (Merck) and the antiinflammatory paclitaxel/Abraxane® (V) (Cellgene Corp. and Bristol-Myers Squibb) contain moieties derived from β-amino acids. A synthetically-derived agent built from (S)-β-phenylalanine is CCR-5 receptor antagonist maraviroc/Selzentry® (VI) (Pfizer) that treats HIV infection (Hancock-Lewandowski et al., 2008) (Table 1).

β-Amino acids are used also as building blocks for synthetic β-peptide oligomers, which are not as susceptible to cleavage by α-peptidases, hydrolases, and metabolizing enzymes as α-peptides (Frackenpohl et al., 2001; Seebach et al., 1996). β-Peptides can also fold into well-defined, stable conformations in solution to form stable quaternary structures and can form helix-turn and pleated-sheet conformations with as few as four residues in proproteins (Seebach et al., 1999, 1998).

Application of β-amino acids is further seen in the cyclic-β-tetrapeptide (VII), a mimic of the natural peptide hormone somatostatin (regulator of endocrine and nervous system function), that binds the human somatostatin receptors (Gademann et al., 1999) (Table 1). In addition, a new family of nylon-3 polymers (poly-β-peptides) (VIII) shows significant and selective toxicity towards the human fungal pathogen Candida albicans (Liu et al., 2013) (Table 1).

Given the importance of β-amino acids as precursors of pharmaceuticals and peptidomimetics with greater in vivo stability, several methods are described for the stereoselective chemical synthesis of β-amino acids (Lealas and Seebach, 2004; Liu and Sibi, 2002; Weiner et al., 2010). Arndt–Eistert homologation of α-amino acids (Pinho et al., 2014; Seebach et al., 1996; Yuan and Williams, 1997), diastereoselective (Davies et al., 2011; Davies and Ichihara, 1991; Sewald, 1996) and enantioselective (Folberg and Jorgensen, 1996; Sibi and Liu, 2000) conjugate addition reactions, and catalytic asymmetric hydrogenation reactions (Holz et al., 2003; Kadyrov et al., 2005; Lubell et al., 1991) are highlighted among various methods. Oxidative stress potentially caused by silver (Ag⁺) waste (Kim et al., 2009) and safety concerns...
associated with the volatile, carcinogenic diazomethane caution against using Arndt–Eistert homologation. Thus, alternatively employed are catalytic asymmetric-hydrogenation reactions for large-scale enantioselective (>95%) synthesis of β-amino acids. Chiral ligand design and tuning (Holz et al., 2003; Kadyrov et al., 2005) have improved these metal-catalyzed hydrogenation routes; however, these methods still advance through multiple steps. A concern of multistep syntheses at commercial-scale is large-scale chemical waste (Grayson et al., 2011). Thus, currently explored are semisynthetic methods to integrate benign biocatalytic steps in the synthetic pathway towards β-amino acids.

Currently researched are three main biocatalysts for making β-amino acids: lipases (Faulconbridge et al., 2000; Grayson et al., 2011), transaminases (Rudat et al., 2012), and aminomutases (Christenson et al., 2003; Krug and Mueller, 2009; Magarvey et al., 2008; Walker et al., 2004; Wanninayake and Walker, 2013). Principally explored are lipases for industrial-scale enzymatic resolution to semisynthesize β-amino acids (Grayson et al., 2011). Recently, a commercially available Amano lipase PS was used to resolve a racemic mixture of synthetically-derived β-aryl- and heteroaryl-β-amino acid propyl esters with excellent enantioselectivity (98–99% ee) and moderate yields (16–50%, limited at a maximum 50% yield by the 1:1 racemate) (Grayson et al., 2011). In addition, synthesis of the propyl esters needed the corrosive thionyl chloride reagent, with distillation and extraction steps before the two-phase lipase step. Most important, after each batch reaction cycle, fresh enzyme was added to maintain the initial turnover rate and offset the partial inactivation of the Amano lipase PS (Grayson et al., 2011).

Aminomutase catalysis provides an alternative biocatalytic route that involves fewer steps than the enzymatic resolution effort. A phenylalanine aminomutase from P. agglomerans (PaPAM) catalyzes the isomerization of (2S)-α-phenylalanine to (3S)-β-phenylalanine (>99.9% ee) (Fig. 1) on the pathway to the antibiotic andrimid (Magarvey et al., 2008; Ratnayake et al., 2011). PaPAM uses several α-phenylalanine analogs with substituents variously positioned on the phenyl ring (fluoro, chloro, bromo, methyl, methoxy and nitro) and heteroaromatic analogs demonstrating an extended substrate scope (Ratnayake et al., 2014).

Considering the high enantioselectivity, atom economy, and expanded substrate specificity of PaPAM, herein, we evaluated the kinetics of recombinant E. coli whole-cells harboring the papam gene. This recombinantly-expressed enzyme was employed in an independent, earlier biocatalysis study that examined the conversion of only the natural substrate α-phenylalanine (Hidesaki, 2009). The present analysis includes 21 surrogate substrates to further understand the relationship among the substrate specificity, substituent effects, time-dependent production rates of the whole-cell biocatalyst, and the in vitro-catalyzed reactions with purified PaPAM (Ratnayake et al., 2014). In addition, this study also evaluated metabolite partitioning between the culture medium and E. coli cells, assessed the effects of various physical parameters (e.g., buffer type, pH, and temperature) on the kinetics. Also explored were amino acid toxicity and multiple batch reaction cycles to gauge the sustainability of the whole-cell biocatalyst. Assessment of these parameters added information on the flux parameters of the PaPAM whole-cell biocatalyst and established a foundation for batch-cell recycling for large-scale biosynthesis.

### 2. Materials and methods

#### 2.1. Substrates, authentic standards and reagents

(S)-α-, p-methoxy-(S)-α-, p-nitro-(S)-α-, and p-chloro-(R/S)-β-phenylalanine and (trimethylsilyl) diazomethane (2.0 M in diethyl ether) were purchased from Sigma–Aldrich–Fluka (St. Louis, MO). Racemic p-nitro-β-phenylalanine was purchased from Oakwood Products, Inc. (West Columbia, SC), and o-methoxy-(S)-α-, m-methoxy-(S)-α-, o-nitro-(S)-α-, m-nitro-(S)-α-, o-methoxy-(S)-β-, m-methoxy-(S)-β-, o-nitro-(S)-β-, and m-nitro-(S)-β-phenylalanine were purchased from Chem-Impex International, Inc. (Wood Dale, IL). All other (S)-α- and β-amino acids were purchased from PepTech Corporation (Burlington, MA). All chemicals were used without further purification, unless noted.

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**Table 1**

<table>
<thead>
<tr>
<th>Bioactive natural products, pharmaceuticals, and β-peptides based on β-amino acids.</th>
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</thead>
<tbody>
<tr>
<td><strong>Andrimid</strong> (I) (Antibiotic)</td>
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<tr>
<td><img src="image" alt="Andrimid" /></td>
</tr>
<tr>
<td>(3S)-β-phenylalanine</td>
</tr>
<tr>
<td>(2R,3S)-phenylisoserine</td>
</tr>
<tr>
<td>Paclitaxel (Abraxane) (V) (Anticancer)</td>
</tr>
<tr>
<td>Poly-β-peptide (VIII) (Antifungal)</td>
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\[ y = 40, 50, 60, 70, 80 or 90; x + y = 100 \]
2.2. Bacterial strains, plasmids, and culture media

BL21(DE3) E. coli bacterial strain transformed with expression vector pET24b(+) was used for the whole-cell biotransformations with PaPAM. E. coli cells were grown in M9 minimal medium [NaH₂PO₄, 7.2H₂O (12.8 g L⁻¹), KH₂PO₄ (3 g L⁻¹), NaCl (0.5 g L⁻¹), NH₄Cl (1 g L⁻¹), MgSO₄, (2 mM), CaCl₂ (0.1 mM), 100X Basal Medium Eagle vitamins (Sigma–Aldrich, St. Louis, MO; 10 mM L⁻¹), and glucose (20%; pH 7.4)] supplemented with kanamycin (Gold Biotechnology Inc., St. Louis, MO; 50 μg mL⁻¹). Optical density measurements of cell suspensions were obtained in 1.5 mL polystyrene cuvettes (General Laboratory Supply, Pasadena, TX) using a UV–vis spectrophotometer (Beckmann DU 640, Beckmann Coulter, Brea, CA).

2.3. General instrumenttion: GC/EI-MS analysis

GC–MS analysis was performed on an Agilent 6890N gas chromatograph equipped with a capillary GC column (30 m × 0.25 mm × 0.25 μM; HP-5MS; J&W Scientific) with He as the carrier gas (flow rate, 1 mL/min). The injector port (at 250 °C) was set to splitless injection mode. A 1-μL aliquot of each sample was injected using an Agilent 7683 auto-sampler (Agilent, Atlanta, GA). The column temperature was increased from 50 to 110 °C at 30 °C/min, then increased by 10 °C/min to 250 °C (total run time of 16 min), and returned to 50 °C over 5 min, with a 5 min hold. The gas chromatograph was coupled to a mass selective detector (Agilent, 5973 inert) operated in electron impact mode (70 eV ionization voltage). All spectra were recorded in the mass range of 50–400 m/z.

2.4. General procedure for whole–cell biocatalytic incubations

BL21(DE3) E. coli cells (50 mL, OD₆₀₀ ~1.0) transformed to express the papam gene from pET24b(+) were used to inoculate M9 minimal medium (1L) supplemented with kanamycin (50 μg/mL). The cells were grown at 37 °C to OD₆₀₀ ~0.6, isopropyl-β-D-thiogalactopyranoside (100 μM) was added to induce the expression of papam, and the cultures were incubated for 16 h at 16 °C. The next steps were performed at 4 °C, unless indicated otherwise. The cells were harvested by centrifugation at 3230 × g (10 min), and the cell pellet was resuspended separately in three different whole-cell feeding media (M9 minimal medium, 50 mM phosphate buffer at pH 7, and 50 mM phosphate buffer at pH 8), each typically adjusted separately to OD₆₀₀ ~35. For analyses at higher biomass, the OD₆₀₀ was adjusted to ~70 and 280 in M9 minimal medium. The α-amino acid substrate (1 mM) was added to the cell suspension and incubated separately at 16, 25 and 30 °C. In control experiments, transformed E. coli cells were incubated without substrate added. Additional control experiments included assays with E. coli transformed with an empty pET-24 vector incubated with or without the substrate. All the biotransformation feeding assays were done in triplicate.

2.5. Derivatization and quantification of amino acids

At the end of each incubation, the reaction medium was separated from the cells by centrifuging at 3230 × g for 10 min, and the supernatant (1 mL) from each assay was basified to pH 10 (6 M NaOH). Internal standards m-fluoro-β-phenylalanine, p-methyl-β-phenylalanine, and β-phenylalanine at 20 μM were added, respectively, to quantify three sets of biosynthetic β-phenylalanine products—Set 1: β-phenylalanine; o-, m-, and p-methyl-β-phenylalanine; and m- and p-nitro-β-phenylalanine; Set 2: o- and p-fluoro; m-, and p-bromo-β-phenylalanine; and (2-thienyl)- and (2-thienyl)-β-alanine; and Set 3: m-fluoro-β-phenylalanine. Each solution was treated with ethylchlorofloromate (50 μL) for 10 min, basified again to pH 10, and reacted with a second batch of ethylchlorofloromate (50 μL) for 10 min. The solutions were acidified to pH 2–3 (6 M HCl) and extracted with diethyl ether (2 × 2 mL). The organic fraction was removed under vacuum, and to the resulting residue dissolved in ethyl acetate:methanol (3:1, v/v) (200 μL) was added (trimethylsilyl) diazomethane until the yellow color persisted. The derivatized aromatic amino acids were quantified by GC/EI-MS. The peak area was converted to concentration by solving the linear equation obtained from the standard curves constructed with the corresponding authentic standards.

2.6. Assessing production rate of β-phenylalanine by the whole–cells in different volumes

The effect of incubation volume on whole-cell biocatalysis was assessed by adding 20 mM of α-phenylalanine to a cell suspension of E. coli (OD₆₀₀ ~35) engineered to express papam at 16 °C, in 5, 100, or 1000 mL of M9 minimal medium. The cultures were incubated, respectively, in 50–mL, loosely-capped conical centrifuge tubes (Corning Incorporated Life Sciences, Tewksbury, MA), 250–mL Erlenmeyer flasks, and 2.8-L Fernbach flasks with agitation on a shaker (225 rpm, MaxQ 5000, Thermo Scientific, Waltham, MA) for 6 h. The cells were removed by centrifugation, and the β-phenylalanine in the culture medium of each assay was derivatized and quantified by GC/EI-MS.

2.7. Analysis of substrate uptake and product release by E. coli cells

To E. coli cells expressing PaPAM incubated in M9 minimal medium (45 mL, OD₆₀₀ ~35) was added α-phenylalanine (1 mM) at 16 °C. Aliquots (5 mL) were withdrawn at 0.5, 1, 2, 4, 6, and 8 h, the cells were harvested by centrifugation (3230 × g, 5 min), and the culture medium was twice serially basified (6 M NaOH) and treated with ethyl chlorofloromate. The solution was acidified (pH 2–3, 6 M HCl), extracted with diethyl ether (2 × 2 mL), concentrated in vacuo, and the amino acids were methyl esterified with (trimethylsilyl) diazomethane for quantification by GC/EI-MS analysis. In parallel, the cell pellet was resuspended in 50 mM sodium phosphate buffer (pH 8.0), the cells were lysed by a Misonix XL 2020 sonicator (Misonix Inc, Farmingdale, NY) to release the soluble amino acids, and the cellular debris was removed by centrifugation (3230 × g, 30 min). The supernatant was decanted and the amino acids therein were derivatized by the same reactions used for the culture medium and analyzed by GC/EI-MS.

2.8. Analysis of enzyme release to culture medium

To measure the relative percentage of the PaPAM released in the medium over 6 h at 16 °C, a 5–mL cell suspension (OD₆₀₀ ~35) of E. coli engineered to express papam was incubated without α-phenylalanine. The culture was centrifuged (3230 × g, 5 min), and the supernatant was decanted. The cells were resuspended in lysis buffer (5 mL of 50 mM sodium phosphate buffer (pH 8.0)) and sonicated (Misonix Inc, Farmingdale, NY) to release the soluble PaPAM. The cellular debris was removed by centrifugation (3230 × g, 30 min), and the supernatant was decanted. To measure the relative extracellular PaPAM activity, an aliquot (250 μL) of each supernatant was incubated separately with [15N-α-phenylalanine (1 mM) for 2 h at 31 °C. The amino acids were derivatized and quantified by GC/EI-MS analysis.
2.9. Effect of temperature, time, and culture medium type

After expressing the *papam* gene in *E. coli* for 16 h at 16 °C in M9 minimal medium (12 × 1 L), the cultures were centrifuged, the supernatants were decanted, and the cells from each batch were resuspended separately in M9 minimal medium or 50 mM phosphate buffer (each at pH 7 or 8) to OD_{600} ~ 35. α-Phenylalanine (1 mM) was added to 45-mL cell suspensions in each medium, and the feeding studies were conducted at 16, 25 and 30 °C. Aliquots (5 mL) were withdrawn from each suspension at 0.5, 1, 2, 4, 6, 8, 10, and 12 h. The supernatants were clarified by centrifugation, and separately treated with derivatizing reagents to form the N-(ethoxycarbonyl) α- and β-phenylalanine methyl esters, and the cinnamic acid methyl ester, which were quantified by GC/EI-MS.

2.10. Effect of substrate concentration

To a cell suspension of *E. coli* (OD_{600} ~ 35), engineered to express *papam* in 5 mL of M9 minimal medium was added separately 1, 5, 10, 15, 20, and 25 mM of α-phenylalanine. The cultures were incubated for 6 h at 16 °C. The cells were removed by centrifugation, and the β-phenylalanine in the culture medium of each assay was quantified after derivatization and analysis by GC/EI-MS.

2.11. Effect of the biocatalyst amount

The *E. coli* cells harboring *papam* were resuspended in M9 minimal medium to an OD_{600} of 35, 70 or 280. α-Phenylalanine was added to a final concentration of 1, 5, 10, 15, 20, and 25 mM in separate assays at each cell density, and the assays (5 mL) were incubated for 6 h at 16 °C. The culture medium was clarified by centrifugation, and the β-phenylalanine in the supernatant (1 mL aliquot) of each assay was derivatized for and quantified by GC/EI-MS analysis.

2.12. Assessing the substrate scope of the biocatalytic system

α-Phenylalanine, its analogs (ortho/meta/para-methyl, -methoxy, -fluoro, -chloro, -bromo, and -nitro), and 2- and 3-thienylalanine were separately incubated in a cell suspension of engineered *E. coli* (OD_{600} ~ 35) in M9 minimal medium (5 mL) at 16 °C for 6 h. The cells were pelleted by centrifugation, and an aliquot (1 mL) from each supernatant was separately treated with derivatizing reagents to form the N-(ethoxycarbonyl) methyl esters of the α-arylalanines and biosynthetic β-arylalanines, and the methyl esters of the biosynthetic ary lacrylates for quantification by GC/EI-MS.

2.13. Assessing amino acid toxicity on whole cells at stationary- and mid-log phase

To calculate the colony forming units (CFU) of *E. coli* BL21(DE3) cells at stationary-phase growth at OD_{600} ~ 35, an aliquot (50 mL) from each cell suspension incubated with α-phenylalanine was diluted 10^8-fold with the culture medium (in triplicate). An aliquot (100 μL) of the diluted culture suspension was spread on an agar plate supplemented with kanamycin (50 μg/mL) and incubated for 16 h at 37 °C. The colonies on each plate were counted, and the log(CFU mL^-1) was calculated.

To assess the toxicity of the amino acids used in this study on *E. coli* at mid-log stage growth, cells were grown at 37 °C to OD_{600} 0.6 in 250-mL shake-flask in LB medium (100 mL) containing kanamycin (50 μg/mL). Each set of two LB agar plates, supplemented with kanamycin (50 μg/mL), contained one (at 1 mM) of the 18 amino acids used in this study. An aliquot (1 mL) from the bacterial cell suspension was diluted 10^2-fold with the culture medium (in duplicate). The diluted culture suspension (100 μL) was spread on each agar plate and incubated at 37 °C for 18 h. The colonies on each plate were counted, and the log(CFU mL^-1) were calculated.

2.14. Sustainability of the biocatalytic system

*E. coli* cells (at OD_{600} ~ 35) engineered to express *papam* were resuspended in M9 minimal medium (5-mL), and α-phenylalanine at 1 and 5 mM was added separately to different batches of cell suspensions. The cells were incubated for 6 h at 16 °C, the culture medium was clarified by centrifugation at 3230 × g (10 min). The cell pellets were serially washed (2 × 5 mL) with M9 minimal medium and clarified by centrifugation between each wash to remove residual substrate/product from the cells. The washed cell pellets were resuspended in the culture medium (5 mL), a new batch of α-phenylalanine substrate was added to each suspension, and the biotransformation reactions were incubated for 6 h at 16 °C. The supernatant from each reaction was twice serially basified (6 M NaOH) and treated with ethyl chloroformate. The solution was acidified (pH 2–3, 6 M HCl), extracted with diethyl ether (2 × 2 mL), concentrated in vacuo, and treated with (trimethylsilyl) diazomethane to methyl esterify the amino acids for quantification by GC/EI-MS. Cell harvesting, cell pellet washing, incubation, and GC/EI-MS quantification of the synthetically derivatized β-phenylalanine from the supernatant were repeated for 4 cycles.

3. Results and discussion

3.1. General assay conditions

*E. coli* (OD_{600} ~ 35) expressing *papam* incubated in different volumes (5 mL, 100 mL, or 1 L) of minimal medium containing α-phenylalanine (20 mM) produced ~1.4 mg mL^{-1} of β-phenylalanine in each volume. Thus, 5-mL pilot-scale whole-cell reactions were representative of larger-scale conversion rates of α-arylalanines to their β-isomers in vivo. The host strain used for whole-cell biocatalysis was grown in M9 minimal medium, lacking the α-amino acids found in rich growth media. The latter could potentially confound the analysis of the β-aryl-β-alanines made in vivo by the aminomutase (Strom et al., 2012).

3.2. Assessment of the whole-cell biocatalytic properties of PaPAM

Precursor biotransformations by a whole-cell organism are designed ideally to release the products with low impendence into the medium without cell disruption (Chen, 2007). *E. coli* cells uptake aromatic amino acids through their inner membrane by five distinct active-transporters (Chye et al., 1986; Piperno and Oxender, 1968; Whipp and Pittard, 1977). An AroP permease actively transports arylalanines (Chye et al., 1986), while PheP permease transports phenylalanine (Cosgriff and Pittard, 1997; Pi and Pittard, 1996). A permissive ATP-binding cassette type transporter, LIV-ILS system, primarily conveys branched-chain amino acids yet also occasionally transports phenylalanine (Koyanagi et al., 2003). Thus, these transporters likely play a central role in amino acid transfer into the bacteria cells used herein. In this study, α-phenylalanine (benchmark substrate) entered the cells (OD_{600} ~ 35), and β-phenylalanine accumulated in the medium (0.4 mg/5 mL) and in the cells (0.016 mg/40 mg wet wt) after 8 h (Fig. 2). The log(CFU mL^{-1}) in the *E. coli* samples incubated with α-phenylalanine at 1 mM during biocatalysis remained constant (Fig. 2), suggesting no time-dependent accumulation of dead and/or viable but nonculturable cells at stationary growth phase.
Aliquots of the clarified growth medium did not convert $^{15}$N-$\alpha$-to $^{15}$N-$\beta$-phenylalanine, suggesting that the PaPAM enzyme did not release to the medium from dead cells over a 6-h incubation. Thus, biocatalysis did not occur fortuitously in the culture medium. The labeled $\alpha$-phenylalanine enabled distinction of de novo biosynthetic $^{15}$N-$\beta$-phenylalanine and unlabeled $\alpha$- and $\beta$-phenylalanine isomers made endogenously. The crude, soluble fraction isolated from mechanically-lysed cells, containing active PaPAM enzyme, catalyzed labeled $\beta$-phenylalanine from $^{15}$N-$\alpha$-phenylalanine (see Supplementary Fig. 1).

### 3.3. Enantiomeric excess of the biosynthetic (S)-$\beta$-phenylalanine

The synthesis of pharmaceutically active drugs from chiral building blocks continues the drive to develop industrially viable methods for making enantiomerically pure compounds (Petrovic et al., 2002). Hence, optimizing the enantiomeric excess of a catalytic process is an important goal. To analyze the enantioselectivity of the whole-cell biocatalyst used herein, biosynthetic $\beta$-phenylalanine (as the reference compound) was converted to its $N$-[(S)-camphanoyl] methyl ester. The derivatized diastereomeric $\beta$-amino acid eluted at a retention time (14.43 min) identical with that of authentic N-[(S)-camphanoyl]-3S-phenylalanine (14.43 min) (Fig. 3), confirming the 3S-product stereochemistry (>99.9% ee) as described in earlier in vitro assays with PaPAM (Magarvey et al., 2008; Ratnayake et al., 2011). No biosynthetic (3R)-$\beta$-derivative (14.10 min) was detected.

### 3.4. Effect of temperature on $\beta$-phenylalanine production

The activity, stability, and selectivity of a whole-cell biocatalyst are important for industrial scale biosynthesis (Zhou et al., 2011). To examine the thermal stability of the whole-cell biocatalyst, the cell cultures were incubated at 16, 25 or 30 °C. Therefore, herein, aliquots of the supernatant from each sample were withdrawn over time, and $\beta$-phenylalanine in the medium was measured (Fig. 4). Cultures incubated at 16 °C maximally produced $\beta$-phenylalanine (0.092 mg mL$^{-1}$) at 6h, yet cultures incubated at 25 and 30 °C produced $\beta$-phenylalanine at ~1.5-fold lower maximum (~0.065 mg mL$^{-1}$) at 1h (Fig. 4A). We suspect that increased temperatures affected one or more unknown physiological parameters in E. coli at stationary phase, such as gene expression (Gadgil et al., 2005), enzyme activity, substrate binding, $\alpha$-phenylalanine flux toward post-exponential Pex protein synthesis (Kolter et al., 1993), and/or amplified phenylalanine catabolism (Teufel et al., 2010). Thus, considering the slightly greater conversion rate at 16 °C, this temperature was used to incubate the recombinant E. coli biocatalyst for the non-natural $\alpha$-arylalanine substrates.

At all three temperatures, the $\beta$-phenylalanine decreased markedly after the equilibrium phase beyond 8-h incubation. Analysis of the product distribution at each time point at 16 °C revealed that the concentration of cinnamic acid (a 5% by-product from
α-phenylalanine during PaPAM catalysis) increased over time, while the amount of α-phenylalanine rapidly decreased (Fig. 5). An increase in cinnamic acid suggested that the occurrence of the reverse reaction catalyzed by PaPAM (Fortin et al., 2007) is converting α-phenylalanine to cinnamate as the β-phenylalanine concentration increased over that of α-phenylalanine in the medium (Fig. 5). α-Phenylalanine did not equilibrate with β-phenylalanine in the reaction medium, likely due to its partitioning to other metabolic pathways.

3.5. Effect of reaction medium-type on α- to β-phenylalanine isomerization

The influence of pH and buffer type on the stability and activity of the whole-cell biocatalyst was evaluated in phosphate buffer (50 mM, pH 7 and 8) and M9 minimal medium (pH 7.4). The M9 minimal medium contains sources of nitrogen, carbon, phosphorous, sulfur, cations (K+, Ca2+, Mg2+, and Fe2+), and BME-vitamins needed for bacterial cell survival and growth. By comparison, the phosphate buffer lacks these nutrients. The production rate of β-phenylalanine by the whole-cell biocatalyst (OD600 ~35) in M9 minimal medium was only slightly higher (~0.062 mg mL−1 h−1) than the initial rate (~0.059 mg mL−1 h−1) in 50 mM phosphate buffer (pH 7 and 8) over 1 h. While β-phenylalanine then slowly increased in minimal medium between 2 and 8 h, it decreased in phosphate buffer (pH 7 and 8) (Fig. 4B). α-Phenylalanine depleted ~2-fold faster in 50 mM phosphate buffer (0.024 mg mL−1 h−1 and 0.028 mg mL−1 h−1 at pH 7 and 8, respectively), compared to when the bacteria was incubated in M9 minimal medium (0.012 mg mL−1 h−1) (Fig. 6). We also observed that α-phenylalanine disappeared from the phosphate buffer at a rate greater than β-phenylalanine was exported into the medium over 8 h (Fig. 6). This suggested that the biocatalyst metabolized the exogenous α-phenylalanine (and likely β-phenylalanine), directly or via cinnamate, for viability in the nutrient-deprived phosphate buffers. Thus, the moderately fortified M9 minimal medium was used for other whole-cell PaPAM biocatalyst assays.

3.6. Effect of medium-type on cell viability at stationary-phase growth

In addition, to assess viable and culturable cells in the different media, log(CFU mL−1) were calculated for bacteria incubated in M9 minimal medium (pH 7.4) and phosphate buffer (pH 7) containing α-phenylalanine (1 mM). The log(CFU mL−1 = 11.6) of E. coli incubated for 4 h in minimal medium was only slightly higher than for cells [log(CFU mL−1 = 11.4)] incubated in phosphate buffer (pH 7). E. coli viability in minimal medium did not change significantly [log(CFU mL−1 = 11.2)] in phosphate buffer without α-phenylalanine supplementation. Thus, the exogenous amino acid and minimal media likely provided only a small mesotrophic effect for the bacteria, and alone does not account for the differences in the production profiles in the various media (Fig. 4B). Nonetheless, M9 medium was used in all incubation studies described herein.

3.7. The effect of α-phenylalanine concentration on cell viability at stationary-phase growth

α-Phenylalanine (1–25 mM) was used as the benchmark for all the substrates tested to examine its effect on β-phenylalanine production and E. coli whole-cell viability. β-Phenylalanine production in the whole-cells increased 0.09–1.2 mg mL−1 with increasing substrate concentration in 6-h incubations at 16 °C (Fig. 7A). The production approached a maximum at ~1.3 mg mL−1 for substrate concentrations >25 mM. The production approached maximum turnover at higher substrate concentrations either because mass transfer to the biocatalyst was limiting or the substrate became toxic to the cells. Therefore, substrate toxicity was analyzed by incubating E. coli cells at stationary phase growth with increasing substrate concentration. Calculating the log(CFU mL−1) of cultures at increasing substrate concentration revealed that the cells remain viable and cultivable even at 25 mM α-phenylalanine (Fig. 7A).

To evaluate if the mass transfer limits could be extended, the biocatalyst amount was increased from OD600 ~35 to 280. The 8-fold increase in cell biomass did not significantly change the β-phenylalanine production when the substrate was between 1 and 10 mM yet did increase the product ~33% over the lower density cells at 25 mM (Fig. 7B). At OD600 280 the substrate concentration per cell is diluted ~8-fold compared to that of cells at OD600 35, and this dilution accordingly reduces the turnover per cell. The linear regression fit for β-phenylalanine production at higher OD (Fig. 7B) extrapolates to a production level of ~3.2 mg mL−1 at ~40 mM substrate, which is ~8-fold higher than production for the OD600 35 cells incubated with 5 mM substrate. In addition, the production of β-phenylalanine is projected also to reach a maximum product level 8-fold higher (estimated at ~10 mg mL−1) than in the OD600 35 reaction; thus, the biocatalyst reaction was deemed scalable for increased β-amino acid production at OD600 280.
3.8. Substrate scope of the recombinant E. coli whole-cell biocatalyst

Limited substrate scope is commonly a main disadvantage of enzyme biocatalysis (Reetz, 2013). PaPAM, however, has a broad substrate scope in vitro (Ratnayake et al., 2014), and thus was anticipated to perform similarly in vivo. The PaPAM whole-cell biocatalyst was probed with 21 α-arylanalines, including two heteroaromatic compounds. The productive and inactive substrates were similar to those identified earlier for PaPAM in vitro (Ratnayake et al., 2014). The transport of amino acids across the E. coli cell membrane can depend on their steric and electronic properties (Silhavy et al., 2010) and aromatic amino acid: H⁺ −symporter permeases (PheP and AroP) (Brown, 1970; 1971; Haney and Oxender, 1992; Pi et al., 1991; Willshaw and Tristram, 1972). AroP was shown earlier to bind p-fluoro-α-phenylalanine and 3-(2-thieryl)-α-alanine (Brown, 1971; Willshaw and Tristram, 1972), also used as substrates herein. These influx transporters likely contribute significantly to the flux of the α-arylanalines into the whole-cell biocatalyst in the present feeding study.

In general, the whole-cell PaPAM biocatalyst preferentially isomerized meta- and para-substituted substrates over their ortho-substituted isomers. The near complete conversion of five α-arylanaline substrates (m-bromo (96%), p-chloro (93%), p-bromo (92%), 3-thienyl (92%), and m-methyl (90%)) (based on the 1 mM of substrate added) by whole-cell biocatalysis to their α-arylanalines suggested that efflux transporters selectively pumped these β-amino acid products into the extracellular space over 6 h. This ∼9:1 β:α-arylanaline ratio greatly exceeds the intrinsic $K_{eq}$ values (typically ∼1:2, where the $\beta:\alpha$-arylanaline ratio is close approximately 1:1) for other amino acids in the PaPAM family (Chesters et al., 2012; Mutatu et al., 2007). As mentioned earlier herein, the whole-cell biocatalyst at OD$_{600}$ ~35 in both 5-mL and 1-L media made ~1.4 mg mL$^{-1}$ β-phenylalanine from α-phenylalanine. Thus, the production levels of β-amino acids from the surrogate substrates are reported with units of *mg L$^{-1}$* (Table 2). m-Bromo (1) accumulated in the medium (235 mg L$^{-1}$) more than any other substrate tested. It is interesting to note that the in vitro catalytic rate ($k_{cat}$) of PaPAM for m-bromo (1) coincidentally was among the fastest of the substrates tested in an earlier study (Ratnayake et al., 2014). In addition, the log(CFU mL$^{-1}$) for E. coli cells at log phase growth showed that each substrate at 1 mM was not toxic (see Supplementary Table 1).

In vitro biocatalysts are governed by cofactors (if any), physical conditions (temperature, pH, ionic strength), and the intrinsic properties of the enzymes when reconstituted in buffer. In vivo whole-cell biocatalysts are also dependent on other factors such as mass transfer across cell membranes, cellular metabolism, protein synthesis, stimulation and inactivation of microbial cell growth, toxicity, and by-product formation (Schrewe et al., 2013). The effects of influx and efflux permeases, in part, may have caused the various α- and β-arylanalines to move at different rates through the E. coli membrane. These factors, combined with other physiological pathways, are imagined to affect the production of each β-arylanaline by the whole-cell biocatalyst. As a result, the rank-order correlation between the in vitro catalytic rates ($k_{cat}$) and the in vivo production rate of the whole-cell biocatalyst was largely redistributed for the substrates tested (Table 2 and see Supplementary Fig. 2).

Thus, the intrinsic $k_{cat}$ of PaPAM, described in an earlier study (Ratnayake et al., 2014), could not alone predict the turnover of the whole-cell system for each substrate (Ratnayake et al., 2014). For example, the intrinsic $k_{cat}$ values of PaPAM in vitro for m-chloro (8) and m-bromo (1) (ranked 1st and 2nd), respectively, were followed by the natural substrate α-phenylalanine (11) (ranked 3rd) as described in an earlier study (Ratnayake et al., 2014). By comparison, the product accumulation by the whole-cell biocatalyst for
Table 2
Production levels of PaPAM whole-cell biocatalyst for various α-arylalanine substrates.

<table>
<thead>
<tr>
<th>Product (R=)</th>
<th>Production (mg L⁻¹)&lt;()&gt; Yields (%)</th>
<th>Ranking&lt;&gt;()</th>
<th>Product (R=)</th>
<th>Production (mg L⁻¹)</th>
<th>Yield (%)</th>
<th>Ranking&lt;&gt;()</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>In vivo</td>
<td>In vitro</td>
<td>In vivo</td>
<td>In vitro</td>
<td>In vivo</td>
<td>In vitro</td>
</tr>
<tr>
<td>H₃C-</td>
<td>235 ± 3.6</td>
<td>96</td>
<td>1</td>
<td>2</td>
<td>109 ± 9.2</td>
<td>56</td>
</tr>
<tr>
<td>Cl-</td>
<td>224 ± 5.8</td>
<td>92</td>
<td>2</td>
<td>10</td>
<td>96 ± 2.1</td>
<td>60</td>
</tr>
<tr>
<td>Br-</td>
<td>186 ± 4.2</td>
<td>93</td>
<td>3</td>
<td>9</td>
<td>77 ± 2.4</td>
<td>43</td>
</tr>
<tr>
<td>F-</td>
<td>162 ± 7.5</td>
<td>90</td>
<td>4</td>
<td>8</td>
<td>74 ± 3.6</td>
<td>41</td>
</tr>
<tr>
<td>Br-</td>
<td>159 ± 0.84</td>
<td>87</td>
<td>5</td>
<td>14</td>
<td>68 ± 2.2</td>
<td>40</td>
</tr>
<tr>
<td>S-</td>
<td>157 ± 4.6</td>
<td>92</td>
<td>6</td>
<td>6</td>
<td>49 ± 3.2</td>
<td>27</td>
</tr>
<tr>
<td>H₃C-</td>
<td>140 ± 4.3</td>
<td>72</td>
<td>7</td>
<td>4</td>
<td>17 ± 1.4</td>
<td>8</td>
</tr>
<tr>
<td>Cl-</td>
<td>128 ± 6.1</td>
<td>64</td>
<td>8</td>
<td>1</td>
<td>13 ± 0.77</td>
<td>6</td>
</tr>
<tr>
<td>F-</td>
<td>113 ± 1.5</td>
<td>62</td>
<td>9</td>
<td>12</td>
<td>8.5 ± 1.1</td>
<td>4</td>
</tr>
</tbody>
</table>

* The concentrations of biosynthesized β-amino acids are reported in units of mg L⁻¹.  
* Ranking order is based on the β-phenylalanine production from the in vivo system and the catalytic rate (kcat) of the in vitro system.

m-bromo (1) ranked 1st, m-chloro (8) ranked 8th, and the natural substrate (11) ranked 11th. 11 was isomerized in vivo interestingly slower than ten other substrates, likely because it was diverted to other natural pathways in the E. coli cells. The whole-cell biocatalyst did not isomerize o-chloro-, o-bromo- and o-nitro-α-phenylalanine (Table 2) in vivo; likely, caused by ineffective substrate binding to PaPAM as described in the earlier in vitro study (Ratnayake et al., 2014).

The halogenated β-arylalaninines made herein can be used as building blocks for biosstere drug candidates and in Stille- and Suzuki-type reactions to build molecules that are more elaborate (Ahmed et al., 2015).

3.9. Sustainability of the PaPAM whole-cell biocatalytic system

Recycling of recombinant cells is a potential goal when developing a sustainable industrial process for producing fine chemicals (Zhou et al., 2011). To test the operational stability of the recombinant E. coli whole-cell biocatalyst towards recycling, a set of consecutive biocatalytic cycles was performed at 16 °C for 30 h using a single sample of the biocatalyst. After each 6-h reaction cycle, the cells were recovered by centrifugation, washed with M9 minimal medium to remove residual substrate and product, and to reduce cell aggregates (at OD₆₀₀ ~35). The cells were recovered by centrifugation and resuspended in M9 minimal medium before the next reaction in the series. β-Phenylalanine production remained almost constant for whole-cell biocatalyst fed α-phenylalanine at 1 mM and 5 mM (0.075 and 0.9 mg mL⁻¹, respectively) in each of the five batch reaction cycles (Fig. 8). CFU measurements suggested cell viability was maintained even after centrifugation and cell resuspension over at least five reaction cycles (Fig. 8).

4. Conclusions

A PaPAM whole-cell biocatalyst was shown to produce several unnatural (3S)-β-aryl-β-amino acids at >99.9% ee, with the highest turnover rate in M9 minimal medium at 16 °C. The whole-cell biocatalyst biosynthesized 18 β-arylalanines with moderate to excellent converted yields (4-96%) at production levels between 8.5 and 235 mg L⁻¹ over 6 h, respectively. More notably, E. coli cells are reusable over at least five reaction cycles without a noticeable loss in activity and cell viability. Using sustainable whole-cell biocatalysts are attractive over biocatalytic routes employing purified enzymes for in vitro assays, in part, because of the potential higher risk for enzyme denaturation during purification and incubation with the substrate. Further, this biocatalyst offers notable advantages over conventional synthetic methods because of its excellent enantioselectivity, broad substrate scope, single-step conversion,
and sustainability. In addition, the small-scale production yields of the whole-cell biocatalyst, described here, can likely improve by using a bioreactor, increasing the number of bacterial membrane permeases, optimizing the outer membrane permeability (Ni and Chen, 2004), and reducing aromatic amino acid flux through catalytic pathways in engineered E. coli.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jbiotec.2015.10.012.

References


