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Overexpression and Characterization of a ω-Transaminase from *Pseudomonas putida* KT2440 for Biocatalytic Synthesis of Norephedrine

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Abstract. Norephedrine (NE) is an industrially important plant alkaloid produced mainly using chemo-catalytic strategies. Considering the need for cost effective and eco-friendly synthesis routes, enzymatic synthesis of NE using transaminase has recently been reported to be a feasible alternative. In this context, present study involves characterization of the transaminase PP2799 from *Pseudomonas putida* KT2440 for enzymatic synthesis of NE. Chemical characterization studies including HPLC, LC-MS/MS and chiral HPLC analysis clearly indicated the ability of transaminase PP2799 for stereoselective synthesis of (1R,2S)-NE, one of the widely used enantiomers of NE. Further time course studies for the synthesis of (1R,2S)-NE using cell lysate have shown 90% substrate conversion within 24 hours. Similar studies using the whole cells as catalyst results in lower substrate conversion but have an advantage of fewer steps involved in catalyst preparation. Overall, this study indicates the potential of using transaminase PP2799 for designing (1R,2S)-NE synthesis process following green chemistry principles.

Keywords: Chiral amine synthesis, norephedrine, whole cell biocatalysis, transaminase, green chemistry.

1. Introduction

Biocatalysis is one of the major areas in bioprocess engineering that involves enzyme or whole cell catalyzed synthesis of different products useful for the welfare of mankind. Compared to the chemo-catalytic routes, biocatalytic routes are eco-friendly and results in designing the processes which follow green chemistry principles. In addition, the structural complexity of certain molecules makes biocatalysis a first choice owing to difficulties in chemical synthesis. To make the biocatalytic processes more efficient, strategies employed includes screening of the enzymes with enhanced properties; which is followed by optimization and engineering at enzyme, strain or process level [1], [2].

A variety of bulk and fine chemicals including pharmaceuticals are produced by biocatalytic route. In this context, biocatalytic synthesis of the chiral amines is a focus area of recent interest. This synthesis route involves transaminases as a stereoselective biocatalyst for chiral amine synthesis [3]. NE is one such industrially important chiral amine that has potential for implementation of the biocatalytic strategies. This compound is used as a drug as well as a chiral auxiliary in organic synthesis. The presence of two chiral centres in NE gives rise to the difficulties in its chemical synthesis such as low enantioselectivity and requirement of multiple synthesis steps involving harsh reaction conditions. To overcome these problems in chemical synthesis of NE, biocatalytic synthesis using transaminase has been reported in previous studies [4], [5]. However, there is a need for screening and characterization of the enzymes with better stability, activity, and enantioselectivity.

In this context, we evaluated the suitability of transaminase PP2799 from *Pseudomonas putida* KT2440 for enzymatic synthesis of (1R,2S)-NE (Fig. 1). This transaminase has been previously reported [6] for

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efficient consumption of the substrate for NE synthesis; but there has been no characterization of the reaction product (NE) or detailed study on biocatalytic process for NE synthesis using this enzyme as cell lysate or whole cells. This paper reports functional overexpression of transaminase PP2799 in *Escherichia coli*, which is followed by systematic molecular characterization of the enzymatically synthesized (1R,2S)-NE. Further, biocatalytic potential of the cell lysate and whole cells expressing transaminase PP2799 is also studied; in order to assess the applicability of this enzyme for synthesis of (1R,2S)-NE.

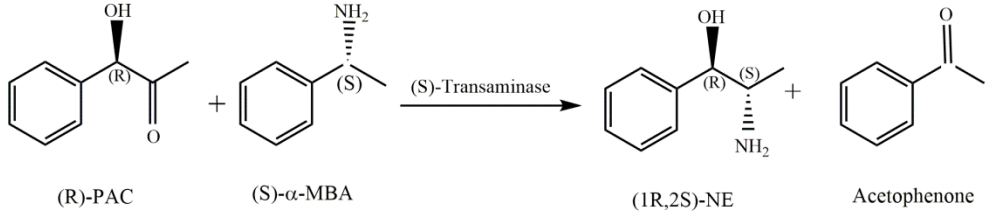

Fig. 1: Transaminase catalyzed synthesis of (1R, 2S)-NE, (Abbreviations: (R)-PAC: (R)-phenyl acetyl carbinol, (S)-α-MBA: (S)-α-methylbenzylamine)

2. Materials and Methods

Chemicals: All HPLC grade chemicals except ethanol were purchased from Merck; ethanol (HPLC grade) was from Fisher Scientific; (R)-PAC and all enantiomers of NE were gifts from Embio Research Centre, India; (S)-α-MBA and methanesulfonic acid were from Sigma Aldrich and pyridoxal phosphate (PLP) and HEPES buffer were from Sisco Research Laboratories, India.

Strains and plasmids: Strains and plasmids used in this study are listed in Table 1.

Cloning and expression of transaminase: All DNA manipulations were performed in *E. coli* DH5α according to the standard procedures [7]. For all studies, *E. coli* was grown in Luria Bertani (LB) medium with 100 µg/mL ampicillin if required. For cloning of the transaminase PP2799 into pET43.1b, a 1.34 kb fragment of gene PP2799 was PCR amplified from *Pseudomonas putida* KT2440 genomic DNA using TA2F(CGACATATGAGCACCCACTCTTCAACCGTTCAGAACG) as a forward primer and TA2R(TGACTCGAGTCACAGCAGGTGCTGTGAGCGCGC) as reverse primer. The resulting PCR fragment was digested with *NdeI* and *BamHI* enzymes and ligated into a pET43.1b vector that had been cut with the same enzymes, thus creating the vector pET43.1b-PP2799. For transaminase expression, vectors pET43.1b and pET43.1b-PP2799 were transformed into *E. coli* BL21(DE3); which gives the strains *E. coli*-pET 43.1b and *E. coli*-PP2799. Overnight grown cultures of these strains were inoculated in 50 mL LB broth containing 100 µg/mL ampicillin at OD600 of 0.1. Transaminase expression was induced by the addition of 50 µM IPTG at OD600 in the range 0.4-0.6. These cultures were incubated at 26 ℃, 200 rpm for next 6 hours for sufficient enzyme expression to occur. After this step, biomass was harvested by centrifugation at 6000 rpm, 4 ℃ and 10 minutes, washed with buffer (100 mM HEPES, 200 µM PLP, pH 7.5) and used for the further experiments.

Table 1: Plasmids and strains used in this study

Strain or plasmid	Description	Source
pET 43.1b	T7 promoter based expression vector for *E. coli*	Lab stock
pET-PP2799	pET vector containing PP2799 gene under T7 promoter	This work
E. coli DH5α	F-ϕ80lacZOM15 O(lacZYA-argF) U169 deoR recA1 endA1 hsdR17	Lab stock
E. coli BL21 (DE3)	F^- ompT gal dcm lon $hsdS_B(r_B^- m_B^-)$ λ(DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5])	Lab stock
E. coli-pET 43.1b	*E. coli* BL21 (DE3) containing pET 43.1b	This work
E. coli-PP2799	*E. coli* BL21 (DE3) containing pET PP2799	This work
Pseudomonas putida KT2440	-	ATCC

Cell disruption and SDS-PAGE analysis: Washed biomass from the previous step was resuspended in 2 mL lysis buffer (100 mM HEPES, 200 µM PLP, 0.5 mM PMSF, pH 7.5) and disrupted using ultrasonication for 5 cycles of 10 seconds followed by 20 cycles of 0.5 seconds at 10 % amplitude. Supernatant and pellet were separated by centrifugation at 12000 rpm, 4 ℃ and 15 minutes. The supernatant was used as soluble fraction for SDS PAGE analysis and also as lysate for the transamination reaction. Pellet was resuspended in lysis buffer and washed by centrifugation. Washed pellet was used as insoluble fraction for SDS-PAGE analysis. Standard procedure [7] was used for the SDS-PAGE analysis and gels were subsequently stained by Coomassie Brilliant Blue R-250.

Transamination reactions using cell lysate or whole cells: For transamination reaction with the cell lysate, final protein concentration of the supernatant (soluble fraction) was adjusted to 5 mg/mL using HEPES buffer containing 200 µM PLP. Reaction was started by the addition of 200 µL of this 5 mg/mL stock solution to 800 µL assay buffer (12.5 mM R-PAC, 12.5 mM S-α-MBA in 100 mM HEPES, 200 µM PLP, pH 7.5). This reaction mixture was incubated at 26 ℃ for different time intervals (mentioned in results and discussion). For whole cell biocatalysis experiment, conditions similar to the reaction with cell lysate were used by replacing cell lysate with the washed cells of final optical density (OD 600) 2.5.

Chemical characterization of NE: a) HPLC analysis of NE was carried out using C18 column (HiQ Sil C18W-Kya Tech, Japan) and water:acetonitrile:trifluoroacetic acid (90:10:0.3) as mobile phase at 0.5 mL/min flow rate and 25 ℃ column oven temperature with UV detection at 210 nm.

b) LC-MS analysis of NE was carried out using Agilent MS Q-TOF G6550A system using a C18 column (HiQ Sil C18W- Kya Tech, Japan) as stationary phase. Samples were extracted into diethyl ether, dried at room temperature overnight and dissolved in the corresponding mobile phase. Appropriate dilutions were used for injection into MS detector. Mobile phase used for this analysis was water:acetonitrile:formic acid (700:30:0.1) at a flow rate of 0.4 mL/min. Column oven temperature was maintained at 25 ℃ throughout the analysis. Positive electrospray ionisation technique was used for the molecule fragmentation.

c) Separation of all NE stereoisomers was carried out using the chiral HPLC. Sample preparation procedure was similar to that used for LC-MS. Conditions for chiral HPLC includes Chiralpak-ADH (Daicel) column as the stationary phase and n-Hexane:Ethanol:methanesulphonic acid (90:10:0.1) as mobile phase at 0.5 mL/min flow rate and 25 ℃ column oven temperature. UV Detection of the NE stereoisomers was carried out at wavelength 210 nm.

3. Results and Discussion

3.1 Cloning and Overexpression of Transaminase PP2799

The gene PP2799 was successfully cloned into vector pET 43.1b to create the clone pET-PP2799. This clone was confirmed by restriction digestion analysis and DNA sequencing. The transaminase expression was further confirmed using SDS-PAGE analysis. When expressed using pET system under the conditions reported in previous study [6] (30 ℃, 1 mM IPTG, and 200 rpm), no visible band corresponding to the transaminase was obtained in soluble fraction (data not shown). This could be due to the fast overexpression of transaminase resulting in formation of the inclusion bodies. Considering the need for soluble enzyme for efficient biocatalysis, transaminase expression conditions were optimized further. Optimized conditions for transaminase expression were 26 ℃, 200 rpm and 50 µM IPTG concentrations (data not shown). Under these expression conditions, an extra band of size around 50 kDa corresponding to transaminase PP2799 was obtained in both soluble and insoluble fraction (Fig. 2, lane 3 and 5), as compared to the control (Fig. 2, lane 2 and 4). Thus, expression of transaminase PP2799 in *E. coli* was confirmed at the DNA and protein level.

3.2 NE Synthesis Using Transaminase PP2799

The reverse phase HPLC analysis was used as a first step towards the confirmation and characterization of NE synthesis using transaminase PP2799. For these studies, the (R)-PAC transamination reaction was carried out for 24 hr using 1 mg/ml crude cell extract. Under the reaction conditions mentioned in materials and methods, 9.1 mM NE was synthesized within 24 hours. In addition, a non-detectable level NE in control reaction indicated that the native transaminases from *E. coli* BL21 (DE3) were not capable of catalyzing

(R)-PAC transamination. This HPLC analysis demonstrated that, the transamination of (R)-PAC using PP2799 enzyme results in synthesis of NE.

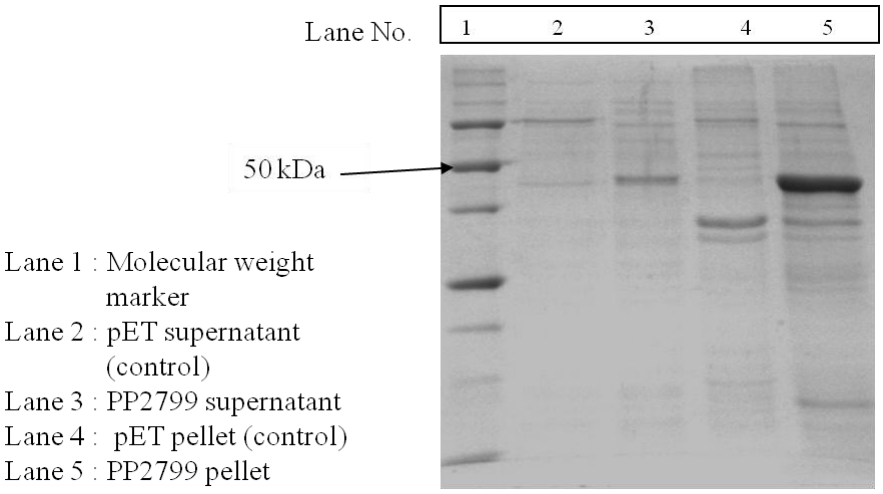

Fig. 2: SDS-PAGE analysis for expression of transaminase PP2799

3.3 Chiral Analysis of Enzymatically Synthesized NE

Chiral purity is a major factor in various applications of NE, which depends on selectivity of the catalyst and subsequent purification strategies used. In this study, S-selectivity of the transaminase PP2799 had been proved using chiral HPLC analysis. Retention time of NE synthesized by transaminase PP2799 was 35.1 min, which was close to the retention time of (1R,2S) NE (35.3 min). This clearly indicated that only (1R,2S) – NE was formed during this reaction in detectable amount. Presence of a amino group in (S)- orientation in (1R,2S)-NE confirmed the (S)- specificity of transaminase PP2799 towards NE synthesis, as predicted through the sequence analysis in previous studies [6]. Whereas, absence of the peaks corresponding to NE stereoisomers in control reaction proved inability of the native *E. coli* transaminase for R-PAC transamination. From the peak areas (data not shown) for corresponding NE enantiomers, calculated enantiomeric excess (ee) and diastereomeric excess (de) values for PP2799 mediated NE synthesis were close to 100 %. These ee and de values reported were considering the lower detection limit of the HPLC system.

3.4 Confirmation of Chemical Identity of NE by LC-MS/ MS

The chemical identity of enzymatically synthesized NE was confirmed by LC-MS and LC MS/MS using electrospray ionization technique (ESI) in positive mode.

a. LC-MS analysis: Liquid chromatogram for the most abundant mass species (m/z value 134.09) of NE after fragmentation is shown in Fig. 3. Both standard and enzymatically synthesized NE has retention time of 6.0 minutes. Absence of this mass species in the control (pET) indicated the inability of native *E. coli* transaminase for NE synthesis. Major mass species obtained after the fragmentation of NE standard have m/z values 134.09, 117 and 91.06. A similar fragmentation pattern was obtained for the NE synthesized using transaminase PP2799. Additionally, this fragmentation pattern was similar to the literature reports [8].

b. LC-MS/MS analysis: To further confirm the identity of the enzymatically synthesized NE, MS/MS analysis was carried out. This was achieved by fragmentation of the most abundant mass species obtained in first fragmentation step (m/z: 134.09). Major fragments after the second fragmentation of this species have m/z values of 117.05, 115 and 91.06 (data not shown). This fragmentation pattern was found similar for the standard and enzymatically synthesized NE.

Similar fragmentation pattern of standard and enzymatically synthesized NE for LC-MS and MS/MS analysis proved the chemical identity of NE synthesized using transaminase PP2799.

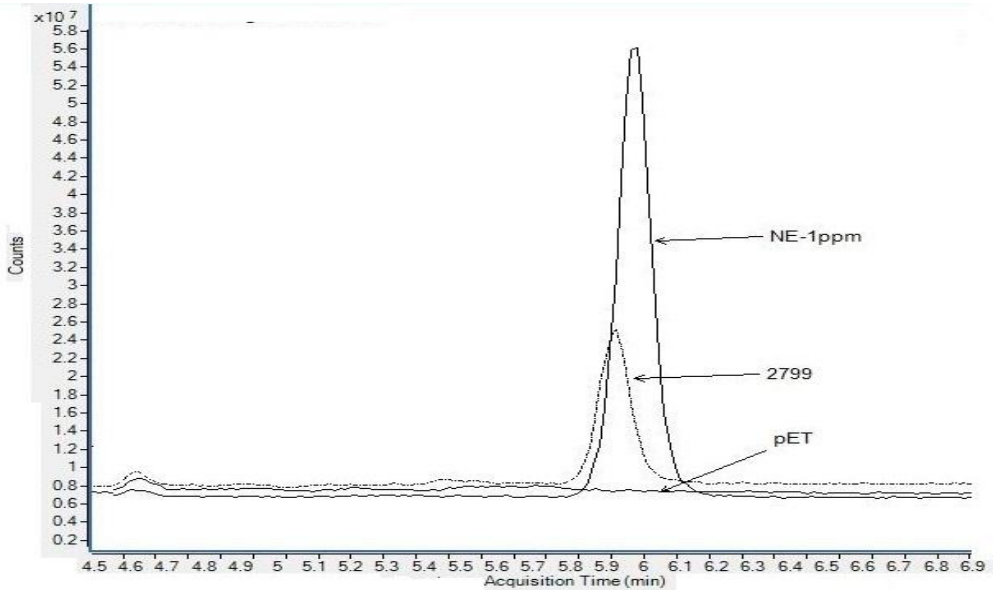

Fig. 3: Chromatogram for MS analysis of NE synthesised using transaminase PP2799 for major mass fragment (134); pET: negative control, NE : norephedrine standard, 2799: transaminase .

3.5 Reaction Kinetics for (1R,2S)-NE Synthesis Using Cell Lysate

Detailed insight into (1R,2S)-NE synthesis catalyzed by PP2799 was obtained by the time course study with cell lysate. As shown in Fig. 4, 90 % substrate conversion was obtained before 24 hrs. The reaction was continued to 36 hrs and no considerable increase in the (R)-PAC conversion was observed. In addition, almost complete substrate conversion with constant (1R,2S)-NE concentration over long time period (from 24 hrs to 36 hrs) proved that the produced NE was stable under reaction conditions used, without its significant enzymatic or non-enzymatic degradation. The control reaction showed absence of (1R,2S)-NE at each time point (data not shown).

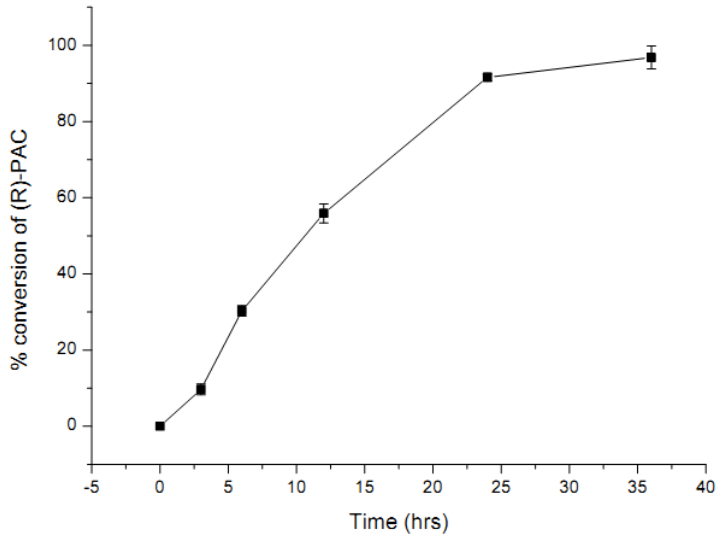

Fig. 4: Reaction progress curve for (1R,2S)-NE synthesis using *E. coli* cell lysate expressing PP2799

Previous studies [5], [9] on (1R,2S)-NE done at similar substrate concentration (10 mM) showed that the (1R,2S)-NE synthesis reaction progress was controlled by thermodynamic equilibrium of the reaction. In contrast, almost complete conversion of (R)-PAC to (1R,2S)-NE by transaminase PP2799 indicated the kinetically controlled behaviour of this reaction. This is attributed to the use of (S)-α- MBA as amine donor instead of L-alanine in previous studies. However, a major drawback of using (S)-α- MBA as amine donor is its cost. Overcoming this problem requires screening of the cheap amine donor which will be efficiently accepted by transaminase PP2799. It should also be noted that in spite of the optimization of solubility of the

transaminase, reaction progress obtained was similar to the previous studies [6]. This indicated the independence of NE synthesis reaction on transaminase solubility. There may be possible involvement of some other factors like substrate inhibition for controlling reaction progress for (1R,2S)-NE synthesis.

3.6 (1R,2S)-NE Synthesis Using Whole Cells

The recombinant whole cells are desirable biocatalysts as compared to cell lysate or purified enzyme, owing to their ease of preparation and enhanced stability [10]. In order to study the possibility of using a whole cell catalyst for (1R,2S)-NE synthesis, (R)-PAC transamination reaction was carried out with the *E. coli* whole cells expressing transaminase PP2799. Whole cells expressing PP2799 had a capability of NE synthesis but only 32% of (R)-PAC was converted into (1R,2S)-NE within 24 hours. Absence of (1R,2S)-NE in the control reaction confirmed inability of the native transaminase from *E. coli* whole cells to catalyze (R)-PAC transamination. Compared to the cell lysate or purified enzyme, use whole cells as catalyst has the advantage of bypassing cell disruption and purification steps. However, the (R)-PAC conversion obtained using whole cell catalysis was lower as compared to the use of cell lysate. This may be either due to the low transaminase activity or mass transfer limitation of the substrate or products across the cell membrane.

3.7 Overall Discussion: Biocatalytic Potential of Transaminase PP2799 for (1R,2S)-NE Synthesis

Biocatalytic synthesis of (1R,2S)-NE in this study indicates potential advantages of the transaminase based process over existing chemo-catalytic route [11], [12]. As compared to the chemo-catalytic route which is a multistep, transaminase based process involves single step. This is beneficial in terms of reduction of the reagents and process equipments required. Another advantage of the biocatalytic process is the use of mild reaction conditions in terms of pH and temperature. In contrast, chemical process needs harsh reaction conditions and costly metal catalysts. Additionally, excellent stereoselectivity of the enzyme PP2799 for (1R,2S)-NE synthesis has potential to bypass the costly chiral separation steps involved in chemo-catalytic route. Thus, a biocatalytic process is expected to be cost effective and eco-friendly compared to chemical catalysis.

It should also be noted that compared to chemo-catalysis, biocatalytic strategies have certain limitations including slower rate of reaction and less stability under process conditions [13], [14]. In context of the present work, further biocatalyst characterization studies are desirable. Additionally, insights from well established transaminase based processes such as sitagliptin production [15] will be useful to identify and overcome the bottlenecks in efficient biocatalytic synthesis of NE.

4. Conclusion

This study reports the suitability of the transaminase PP2799 for stereoselective biocatalytic synthesis of (1R,2S) NE. Additionally, the use of recombinant *E. coli* cell lysate or whole cells as a biocatalyst for NE synthesis is demonstrated. Application of this transaminase can also be further extended to the synthesis of other industrially relevant chiral amines.

5. Acknowledgements

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6. References

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