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Enhanced bioconversion rate and released substrate inhibition in (*R*)-phenylephrine whole-cell bioconversion via partial acetone treatment

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ABSTRACT

An approach was developed to enhance the efficiency for the bioconversion of 1-(3-hydroxyphenyl)-2-(methyamino)-ethanone to (*R*)-phenylephrine. The strain *Serratia marcescens* N10612, giving the benefit of 99% enantiomeric excess in (*R*)-PE conversion, was used. The fermentation was devised to harvest cells with high hydrophobic prodigiosin content inside the cells. Then, the partial acetone extraction was applied to remove prodigiosin from the cells. The treatment was found to increase the cells conversion rate without loss of the cells NADPH redox system. When using 50% (v/v) acetone for 5 min, the processed cells can give a specific conversion rate of 16.03 μmol/h/g-cells. As compared the treated cells with cells under the basal medium, the maximum reaction rate (V_{max}) increased from 6.69 to 10.27 (μmol/h/g-cells), the dissociation constant (K_m) decreased from 0.236 to 0.167 mM and the substrate inhibition constant (K_{Si}) increased from 0.073 to 1.521 mM. The 20-fold increase in substrate inhibition constant referred to a great release from the substrate inhibition for the use of *S. marcescens* N10612 in the bioconversion, which would greatly benefit the bioconversion to be industrialized.

1. Introduction

The application of fermentation to collect the whole cells as biocatalyst is a common practice in bioconversion to convert precursors into active ingredients [1,2]. There are many advantages for bioconversion via the aid of whole cells, such as high specificity, mild reaction conditions, no need of enzyme separation and being easy for product recovery [3–5]. In the literature, the whole cells bioconversion process is more or less related to the NADH-dependent enzymes in the energy regeneration system within the cells. Peng et al. reported that the short-chain dehydrogenase/reductase isolated from *Serratia marcescens* BCBC 10948 could be used as the biocatalyst for (*R*)-PE production from 1-(3-hydroxyphenyl)-2-(methyamino)-ethanone (HPMAE); whereas, in this reaction, NADPH must be added as the chiral activity for the bioconversion [6]. Thus, the energy regeneration system is considered to be a key factor to produce racemic active pharmaceutical ingredients, such as chiral alcohols [7–10]. In convention, chiral alcohols were produced by the organic synthesis with the aid of catalysts plus the optical resolution from the racemic mixtures [5,11]. On the other hand, whole cells could be applied as the biocatalysts for chiral alcohols production. The whole cells techniques are superior to the conventional catalysts with the advantages such as chemo-specificity, regio-specificity and stereo-selectivity [5]. However, the low conversion efficiency was a major drawback for the bioconversion to be realized in industrial applications.

A *Serratia marcescens* N10612 strain, capable of converting the drug precursor HPMAE into pharmaceutical active compound (*R*)-phenylephrine [(*R*)-PE], was screened and isolated in our lab [12–14]. It is of interest to find that this strain can give a high stereo-selectivity with an enantiomeric excess value of more than 99% in (*R*)-form PE. Meanwhile, this strain was found to be able to produce red pigment prodigiosin under certain conditions [15]. The whole cells bioconversion process was known to create a high conversion yield (more than 90%); however, the low conversion rate sets a limit for the process to be industrialized. The low permeability for the substrate to pass through the cell membrane was considered to be a main barrier for the bioconversion. For *S. marcescens*, the hydrophobic prodigiosin embedded in the cell's membrane is

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expected to bring about the penetration resistance for substrate HPMAE in the (*R*)-PE bioconversion.

In this study, an attempt was made for the first time to enhance the efficiency of bioconversion of (*R*)-PE by processing the whole cells of *S. marcescens* N10612. To increase cell membrane's permeability, an extraction approach was devised. In the process, the high prodigiosin-accumulated cells were produced via the fermentation. Then, an extraction process was applied to partially remove prodigiosin inside cells. The main issue is to improve cells membrane permeability without loss of cells NADPH redox system. Studies have proposed that the acetone-dried cells would lose the NADPH redox system. Therefore, NADPH must be exogenously added for the ketones reduction process when using the acetone-dried cells [16,17]. However, this kind of approach was considered inefficient and costly. In this study, the partial extraction process was devised. Under proper treatment condition, the processed *S. marcescens* cells were expected to retain the NADPH redox system and perform the high efficient (*R*)-PE bioconversion. A comparison of conversion by the original cells and the processed cells was carried out. It was interesting to find the issue of substrate inhibition of the cells can be greatly released. Kinetics data were obtained to illustrate the mechanism on the enhanced bioconversion process.

2. Materials and methods

2.1. Chemicals

Standard (*R*)-PE and other chemicals were purchased from the Sigma–Aldrich Co., USA. All other reagents and solvents were of analytical grade and purchased from the Sigma–Aldrich Co., USA. The precursor, HPMAE, was supplied by the Industrial Technology Research Institute (Hsinchu, Taiwan).

2.2. Strains and cultivation conditions

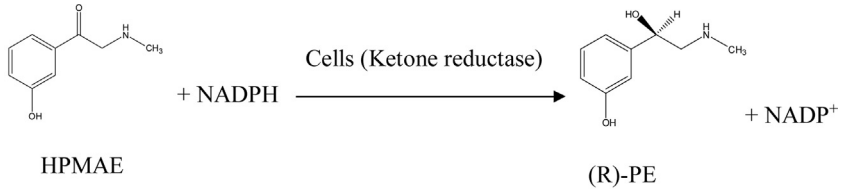
The strain, *S. marcescens* N10612, was isolated and identified via 16S-rDNA [15]. This strain was activated in a complete medium (yeast extract 10 g/L, peptone 10 g/L, sucrose 5 g/L and NaCl 5 g/L) at pH 7.0, 28 °C, and 150 rpm for 24 h. The medium containing (yeast extract 2 g/L, peptone 2 g/L, sucrose 10 g/L and NaCl 1 g/L) was defined as the basal medium. Seed culture was carried out in a 50 ml flask containing 10 ml basal medium and incubated at 28 °C, pH 7.0 and 150 rpm for 24 h. To study the nutrient effect, the fermentation was carried out by inoculating 1.0% (v/v) of the seed culture into 100 ml of the tested medium and operated at 28 °C, pH 7.0 and 150 rpm for 24 h.

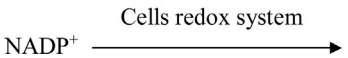
2.3. Cells preparation

For the preparation of high-prodigiosin cells, the fermentation was conducted by inoculating 1.0% (v/v) of the seed culture into 100 ml of the medium containing yeast extract 5.6 g/L, peptone 6.9 g/L, sucrose 31.6 g/L and NaCl 1 g/L and operated at 28 °C, pH 7.0 and 150 rpm for 24 h to obtain the prodigiosin-enriched cell culture. In the second step, various contents of acetone (5, 10, 20, 50, 75%) were added directly to the 24 h culture broth, followed by stirring at 150 rpm for 5 min. The broth was centrifuged at 12,000 × *g* for 10 min. The obtained pellets were washed with 10% (v/v) glycerin solution and applied to the (*R*)-PE bioconversion as described in Section 2.4.

2.4. Bioconversion of (R)-PE

The whole cells conversion from HPMAE to (*R*)-PE coupled with NADPH regeneration was described as follows:





In order to examine the ability of (*R*)-PE bioconversion from HPMAE by the whole cells, the bioconversion was performed by re-suspending 1 g of wet cells pellet in 10 ml of solution containing 1 mM HPMAE, 20 g/L glucose in 100 mM, pH 7.0 sodium phosphate buffer and operated at 28 °C for 24 h [18]. After 24 h reaction, the mixture was centrifuged at 12,000 × *g* for 10 min and the HPMAE and (*R*)-PE contents were assayed via the HPLC analysis.

2.5. Assays

To detect the extracted prodigiosin, the supernatant was sampled and analyzed via HPLC (PU-1580, JASCO, Japan) equipped with an RP-18 column (5 μm, 18 mm × 100 mm). Detection was conducted with a UV–vis detector (UV-1575, JASCO, Japan) set at 535 nm. The mobile phase was composed of pH 6.0 with 10 mM triethylamine in methanol (1/19, v/v) with a flow rate of 1.0 ml/min [19].

Detection of HPMAE and (*R*)-PE was carried out via the HPLC system equipped with a reverse phase C-18A column (5 mm, 18 mm × 100 mm Hypersil, Thermo Fisher Scientific Inc., USA). The mobile phase was composed of methanol/sodium acetate (0.5%, pH 5.5) at the ratio of 5/95 with a flow rate of 0.8 ml/min. Detection was conducted with a UV–vis detector set at 215 nm [18]. The estimated activity of the NADH regeneration system inside the cells was measured according to the assay proposed by Ando et al. [20].

2.6. Kinetics study

To study the enhanced effects of (*R*)-PE bioconversion by the treated cells, the kinetics study was carried out. The mechanism of reaction with the substrate inhibition of the (*R*)-PE bioconversion was described as follows [21,22]:

$$\begin{array}{l} E + S \overset{K_m}{\rightleftharpoons} ES \overset{k_2}{\rightarrow} E + P \\ \quad K_{Si} \Updownarrow + S \\ \quad\quad ES_2 \end{array} \tag{1}$$

With the assumption of rapid equilibrium, the model is expressed in the following:

$$V = \frac{V_{max}[S]}{K_m + [S] + \frac{[S]^2}{K_{Si}}} \tag{2}$$

where V_{max} is the maximum specific rate, K_m is the dissociation constant of [ES], K_{Si} is the substrate inhibition constant and [*S*] is the concentration of the substrate. The equation can be fitted with the double reciprocal approach. Under a low substrate concentration, it can be reduced as follows:

$$\frac{1}{V} = \frac{1}{V_{max}} + \frac{K_m}{V_{max}[S]} \tag{3}$$

Under a high substrate concentration, it can be reduced to the equation as follows:

$$\frac{1}{V} = \frac{1}{V_{max}} + \frac{[S]}{V_{max}K_{Si}} \tag{4}$$

V_{max}, K_m and K_{Si}, can be obtained by linear regression from Eqs. (3) and (4), respectively.

3. Results and discussion

3.1. Fermentation process

Prodigiosin is a membrane anchorage compound with high hydrophobic property. This compound might exert a resistance to prevent the substrate HPMAE from penetrating the cells membrane. To study this effect, the fermentation was carried out