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Ultrasound-assisted (R)-phenylephrine whole-cell bioconversion by *S. marcescens* N10612

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ABSTRACT

The strain *Serratia marcescens* N10612 is used to perform the bioconversion of 1-(3-hydroxyphenyl)-2-(methyamino)-ethanone (HPMAE) to (R)-phenylephrine ((R)-PE), which is an ephedrine drug substitute. The use of an ultrasound approach is found to improve the efficiency of the (R)-PE bioconversion. The optimization of the (R)-PE bioconversion is carried out by means of statistical experiment design. The optimal conditions obtained are 1.0 mM HPMAE, 18.68 g/L glucose and ultrasound power of 120 W, where the predicted specific rate of the (R)-PE bioconversion is 31.46 ± 2.22 (ìmol/h/g-cells) and the experimental specific rate is 33.27 ± 1.46 (ìmol/h/g-cells), which is 3-fold higher than for the operation under ultrasound power of 200 W (11.11 ìmol/h/g-cells) and 4.3-fold higher than for the shaking operation (7.69 ìmol/h/g-cells). The kinetics study of the bioconversion also shows that under the ultrasound operation, the optimal rate (V_{max}) of the (R)-PE bioconversion increases from 7.69 to 11.11 (μmol/h/g-cells) and the substrate inhibition constant (K_{Si}) increases from 1.063 mM for the shaking operation to 1.490 mM for ultrasound operation.

1. Introduction

Ephedrine and its derivatives have long been used in a wide variety of medical and clinical therapies. However, ephedrine molecules stimulate the central nervous system and result in addictive effects, as compared to other adrenergic agents [1]. As a result, substitutes for ephedrine derivatives with similar structures and efficacy are necessary. Of these, (R)-phenylephrine [(R)-PE] is a commonly used substitute for ephedrine. It is a racemic compound of chiral alcohols produced by the reduction of the chiral ketone precursor, and as it does not stimulate the central nervous system, insomnolence, anxiety, testiness, restlessness and other side effects are minimized [2,3]. For these reasons, (R)-PE is generally selected as the substitute for ephedrine.

In the pharmaceutical industry, chiral alcohols are produced via organic asymmetric reduction processes, including hydrogenation and optical resolution separation [4,5]. The chiral alcohol products have to be converted from the precursors via a series of organic synthesis processes [6]. Biocatalysis has been applied to the chiral alcohols production using biocatalysts [7]. As compared to organic synthesis, biocatalysis has many advantages, including high specificity, mild reaction conditions, renewability, and economy [6,8,9]. Biocatalysis, also known as bioconversion, is carried out using microorganism whole cells or enzymes as catalysts to convert the raw materials to biomedical products [10,11]. In the literature, the bioconversion process was always carried out via the reactions relating to NADH-dependent enzymes and the energy regeneration systems within the cells, which are keys to the production of the racemic products [12–15]. In our lab, the strain *Serratia marcescens* N10612 was screened and found to be able to convert the drug precursor 1-(3-hydroxyphenyl)-2-(methyamino)-ethanone (HPMAE) to phenylephrine [16]. The product was further identified as (R)-PE, a bioactive compound used in pharmacology [17].

Although bioconversion is the most feasible and specific method for racemic compound production, the efficiency of the product conversion has some limitations, the mass transfer of the substrate through the cell membrane is among the main barrier for high bioconversion efficiency [18]. As a result, some approaches have been applied to enhance the efficiency of the reaction. Methods such as the addition of auxiliary agents were employed to improve the permeability of the cell membrane. For example, the solubility of the hydrophobic substrate was increased by applying an aqueous-organic two-phase system [19,20]. In our lab, some treatments have been taken to enhance the bioconversion. Various

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surfactants were added to the reaction medium to improve (R)-PE bioconversion. However, no significant enhancement was observed. Interesting to find that a positive effect for the bioconversion was observed when ultrasound operation was adopted. There is no report concerning about the enhancement of *S. marcescens* whole-cell bioconversion by ultrasound. As a result, ultrasound was applied to test its effect on (R)-PE bioconversion.

In this study, the bioconversion and kinetics models of (R)-PE from HPMAE by *S. marcescens* N10612 are discussed. The effect of ultrasound on (R)-PE production is evaluated. The factors of the compositions within the reaction substrates are investigated. Statistical experiment designs, such as Box–Behnken design and response surface methodology (RSM), are applied as the means for obtaining the optimal conditions and comparing them with the experimental results.

2. Materials and methods

2.1. Chemicals, bacterial strains and media

(R)-PE and other chemicals were obtained from Sigma–Aldrich (St. Louis, MO., USA). All other reagents and solvents were of analytical grade and purchased from Sigma–Aldrich (St. Louis, MO., USA). HPMAE and (S)-PE were obtained from Industrial Technology Research Institute (Hsinchu, Taiwan). (S)-PE is the asymmetric isomer of phenylephrine which is non-pharmaceutically active and used for comparing with pharmaceutically active (R)-PE. The strain *S. marcescens* N10612 was screened from the soil and identified by 16S-rDNA [16]. To study the bioconversion of (R)-PE from HPMAE, the strain was cultivated in the 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, 150 rpm for 24 h.

2.2. Bioconversion of HPMAE to PE

In order to ensure the strain ability for PE bioconversion from HPMAE, the 24 h-cultivated cells were centrifuged at 12,000×*g* for 10 min. The pellets were resuspended in 10 mL of substrate medium containing 1 mM HPMAE, 20 g/L glucose in 100 mM pH 7.0 sodium phosphate buffer and reacted at 28 °C for 24 h [17]. After the reaction, the supernatant was obtained by centrifugation, followed by filtering through a 0.22 ìm membrane for further HPLC analysis.

2.3. Analysis of HPMAE and PE

The product for the bioconversion reaction was analyzed using an HPLC system (JASCO, Japan) 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 the mixture of methanol and 0.5%, pH 5.5 sodium acetate (5:95) at a flow rate of 0.8 mL/min. A UV–Vis detector (JASCO, Japan) with a wavelength of 215 nm was used. The chirality of PE was analyzed by HPLC equipped with a chiral column (3.2 × 250 mm, CYCLOBOND I2000 AC, Astec Inc., USA). The mobile phase and conditions were the same as above. The values of the enantiomeric excess of (R)-PE were calculated using the following equation [17]: enantiomeric excess (*e.e.*) of (R)-PE = ([(R)-PE] − [(S)-PE])/([(R)-PE] + [(S)-PE]) × 100%.

2.4. Ultrasound operation

In order to study the effect of ultrasound on (R)-PE bioconversion, the reaction was carried out in an ultrasonic tank with a unitary power of 200 W (Branson 5210, U.S.A) and varying powers from 90 to 150 W (Delta DC-150H, Taiwan). The cells were centrifuged at 12,000×*g* for 10 min and harvested. The harvested cell pellets (0.1 g wet weight/mL) were resuspended in 10 mL of substrate medium containing 1 mM HPMAE, 20 g/L glucose in 100 mM pH 7.0 sodium phosphate buffer and reacted at 28 °C with ultrasound for 10 min. After the reaction, the supernatant was obtained by centrifugation and then filtered through a 0.22 ìm membrane for HPLC analysis.

2.5. Kinetics studies on ultrasound effect

To study the enhanced effects of (R)-PE bioconversion, the kinetics data of the bioconversion were obtained. The reaction mechanism with the substrate inhibition of the (R)-PE bioconversion can be described as follows [21,22]:

$$\begin{array}{c} E + S \underset{}{\overset{K_m}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P \\ K_{Si} \Updownarrow + S \\ ES_2 \end{array} \tag{1}$$

By the assumption of rapid equilibrium, the model can be expressed as follows:

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

where V_{max} is the maximum specific rate, K_m is the dissociation constant of [*ES*], [*S*] is the concentration of the substrate and K_{Si} is the substrate inhibition constant. The equation can be further deduced to fit for the linear regression with the double reciprocal approach. When the concentration of the substrate is low, it can be reduced as follows:

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

When the concentration of substrate is high, it can be reduced as follows:

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

The V_{max}, K_m and K_{Si}, as shown in Table 1, were obtained by the linear regression from Eqs. (3) and (4), respectively.

2.6. Optimization of (R)-PE bioconversion by ultrasound

A Box–Behnken design was employed to study the effects of the reaction medium and ultrasound power for the (R)-PE bioconversion. Factors such as HPMAE, glucose and ultrasound power were selected to determine the mutual influences and the optimal conditions for the (R)-PE bioconversion. The designed levels of the variables are shown in Table 2. The experimental data were analyzed via response surface methodology (RSM) using the SAS program.

Table 1
Kinetics data for (R)-PE bioconversion by *S. marcescens* N10612.

Operations	V_{max} (μmol/h/g-cell)	K_m (mM)	K_{Si} (mM)
Shaking	7.92	0.171	1.063
Ultrasound	11.11	0.167	1.490