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Biocatalyst preparation from *Pseudomonas putida* SM-6 for conversion of DL-lactate to pyruvate

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Abstract

The production of pyruvate using enzymatic methods is a valuable process for its cheap substrate, high conversion ratio, simple composition of reaction, and convenience of recovery. Cell-free extracts from *Pseudomonas putida* SM-6 that contain both lactate oxidase (LOD) and catalase were employed as catalysts for biocatalysis. In order to obtain an efficient and cost-effective biocatalyst, the cultivation of *P. putida* SM-6 both in shake flask and at bioreactor level was investigated. On enzyme production, the suitability of glucose instead of lactate as a carbon source was studied. Maximum LOD production occurred when a mixture of lactate and glucose was used as a carbon source. Lactate was assimilated first and the increase of nitrogen concentration had a positive effect on cell growth and glucose assimilation. The optimum cultivation temperature was 29–31 °C, and the neutral or basic initial medium pH was in favor of both cell growth and LOD production. The interrelation between various process parameters was evaluated for the cultivation of *P. putida* SM-6 in submerged culture. Using a fed-batch cultivation strategy in a 2-l bioreactor, an increase in cell mass was achieved but without a concomitant increase in LOD production compared with cultivation in shake flask. Bioconversion of lactate to pyruvate was carried out using cell-free extract acquired by cell disruption of *P. putida* SM-6. Ethylenediaminetetraacetic acid (EDTA) was added into the biocatalytic system to prohibit the degradation of pyruvate by other enzymes in the cell-free extract. As a result, on a preparative scale, cell-free extract which contained 3.04 mg/ml crude enzyme (10.0 U/ml LOD, 877 U/ml catalase) produced pyruvate of 423 mM from DL-lactate of 660 mM in a reaction period of 22 h. The molar conversion yield of pyruvate on the basis of the amount of lactate consumed was 0.80 g/g.

Keywords: Pyruvate; Lactate oxidase; Bioconversion; Enzyme production; Lactate; Glucose

1. Introduction

Pyruvate not only is employed as a starting material in the biosynthesis of pharmaceuticals [1,2], but also plays an important role in reducing fat, because it can accelerate the metabolism of fatty acids in the human body [3]. Compared with other small non-chiral building blocks, pyruvate is relatively expensive for its increasing commercial demand. On an industrial scale, pyruvate is produced by the dehydration and decarboxylation of tartaric acid [4]. Although this process is simple, the substrate is expensive and the conversion yield is low [5]. Direct fermentative production of pyruvate from a carbon source (such as glucose) has merits in terms of cost-effectiveness. However, as pyruvate is located at a vital junction of cell metabolism, it is usually difficult to obtain strains that can accumulate large amounts of pyruvate extracellularly [6]. In addition, the recovery of pyruvate from such complex fermentation broth is generally difficult and expensive to perform [7]. Lactate oxidase (LOD) catalyzes the direct formation of pyruvate from lactate without requiring NAD^+ as a cofactor [8–12].

$$\text{lactate} + O_2 \leftrightarrow \text{pyruvate} + H_2O_2 \quad (1)$$

Therefore, production of pyruvate by enzymatic methods is considered to be advantageous for its simple composition of reaction, cheap raw materials (racemic as well as stereochemically pure forms of lactate are much cheaper than

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pyruvate), and the convenience of recovery. In this paper, an efficient bioconversion of DL-lactate to pyruvate by lactate oxidase from a bacterial strain *Pseudomonas putida* SM-6 was described and the conditions of lactate oxidase preparation were also investigated.

2. Materials and methods

2.1. Microorganism

P. putida SM-6 used throughout this work was isolated by us from soil sample in Shandong province, China. The strain was deposited in the culture collection of State Key Laboratory of Microbial Technology, Shandong University.

2.2. Media

Seed medium consisted of 10.0 g sodium DL-lactate, 1.1 g $KH_2PO_4{\cdot}3H_2O$, 0.9 g KH_2PO_4, 1.0 g NH_4Cl, 0.5 g $MgSO_4{\cdot}7H_2O$, 1.0 g yeast extract, 5.0 mg $CaCl_2$, 1.2 ml trace elements, and 1000 ml distilled H_2O; pH 7.0, adjusted with 1 M KOH or 1 M HCl. Trace elements solution consisted of 50 g Na_2EDTA, 20 g $ZnSO_4{\cdot}7H_2O$, 5.5 g $CaCl_2$, 5.0 g $MnCl_2{\cdot}4H_2O$, 1.0 g $(NH_4)_2Mo_7O_{24}{\cdot}4H_2O$, 5.0 g $FeSO_4{\cdot}7H_2O$, 1.5 g $CuSO_4{\cdot}5H_2O$, 1.61 g $CoCl_2{\cdot}6H_2O$, and 1000 ml distilled H_2O. The basal fermentation medium consisted of 1.1 g $KH_2PO_4{\cdot}3H_2O$, 0.9 g KH_2PO_4, 0.5 g $MgSO_4{\cdot}7H_2O$, 1.0 g yeast extract, 5.0 mg $CaCl_2$, 1.2 ml trace elements, and 1000 ml distilled H_2O. The concentration of the carbon and nitrogen resources in the fermentation medium was according to the requirement of each experiment.

2.3. Cultivation

The culture from a slant was inoculated into 300 ml flask with 50 ml seed medium and cultivated for 12 h on a rotary shaker. Then they were inoculated into a 500 ml flask with 100 ml fermentation medium and 2-l automatic jar fermentor (Biostat-2-l, B. Braun Medical Ltd., Germany) containing 2 l fermentation medium, respectively. The inoculum's size was 5% (v/v). In fermentor culture, the temperature, pH and dissolved oxygen (pO_2) of fermentation medium were monitored by Ingold electrode. A fed-batch operation was performed in the meantime of cultivation in 2-l bioreactor to obtain higher cell mass and enzyme production. The feed medium containing 20% (g/g) DL-lactate was supplied at constant speed to maintain the lactate concentration in the culture broth.

2.4. Cell-free extract preparation

The fermentation broth (100 ml) was centrifuged (10,000 rpm, 15 min, 4 °C), and the cells were harvested and then washed twice with 67 mM phosphate buffer (pH 7.4) followed by resuspension in 10 ml of the same buffer. The next step involved disruption of the cells using ultrasonic oscillator (Sonic & Material Inc., Danbury CT, USA) for 5 min at 0 °C and a minimum power of 100 W. After disruption, the cell-free extracts were further centrifuged (12,000 rpm, 15 min, 4 °C); the supernatant was collected and used as an enzyme source.

2.5. Analytical methods

The lactate and glucose concentrations were measured by SBA-40C lactate and glucose analyzer (The Academy of Science in Shandong Province, PR China). Pyruvate estimations were carried out spectrophotometrically as its 2,4-dinitrophenylhydrazone derivative [13]. The concentration of pyruvate and lactate was also determined by P/ACE™ MDQ capillary electrophoresis (Beckman Coulter Co. Ltd., USA). Fused silica capillaries (50 μm × 600 mm, Yongnian Co., Hebei, PR China) were used. Detection was carried out at 195 nm in 20 mM sodium tetraborate buffer (pH 8.0). The concentration of nitrogen in ammonium was determined with the Nessler's reaction [14] by Hanna instrument (Hanna Co. Ltd., Italy). Off-line measurement of the cell concentration was carried out using a spectrophotometer (UV-340, Beckman Co. Ltd., USA) at 660 nm with appropriate dilution. The value of optical density was converted to dry cell weight (DCW) using the calibration equation such that 1 OD_{660} = 0.30 g DCW/l. Protein was assayed by method of Bradford test [15] with bovine serum albumin (Sigma, USA) as the standard.

Lactate oxidase activity was measured by monitoring the forming rate of pyruvate. One unit was defined as the amount of enzyme that causes the formation of 1.0 μmol of pyruvate/min under tested conditions. Catalase activity was quantified by Beers & Sizers' method [16] by following the decrease in absorbance at 240 nm during decomposition of H_2O_2 by the enzyme. One unit of catalase activity was defined as the amount of the crude enzyme that reduced 1.0 μmol H_2O_2/min.

3. Results and discussion

3.1. Evaluation of lactate oxidase and catalase content in cell-free extract from Pseudomonas putida SM-6

Lactate oxidase transforms lactate and oxygen into pyruvate and hydrogen peroxide (Eq. (1)). Catalase could destroy hydrogen peroxide (Eq. (2)) formed by lactate oxidase and limit the nonenzymatic oxidation of pyruvate to acetate and carbonate (Eq. (3)) [17], which stabilizes pyruvate bioconversion.

$$H_2O_2 + H_2O_2 \leftrightarrow O_2 + 2H_2O \text{ (catalase)} \quad (2)$$

$$\text{pyruvate} + H_2O_2 \leftrightarrow \text{acetate} + CO_2 + H_2O \quad (3)$$