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Optimization of β-alanine production from β-aminopropionitrile by resting cells of *Rhodococcus* sp. G20 in a bubble column reactor using response surface methodology

Lu-Yi Liang, Yu-Guo Zheng *, Yin-Chu Shen

Institute of Bioengineering, Zhejiang University of Technology, Hangzhou 310014, People's Republic of China

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ABSTRACT

Resting cells of *Rhodococcus* sp. G20 were used for the transformation of β-aminopropionitrile to β-alanine, an important beta amino acid. A 2^3 central composite experimental design was performed with the purpose of optimizing the β-alanine production in a bubble column reactor with 200 mL working volume using response surface methodology (RSM). The individual and interactive effects of three independent variables (cells loading, substrate concentration, airflow rate) on β-alanine production were investigated. A quadratic polynomial predictive model was obtained after statistical analysis to predict the optimum biotransformation conditions. The optimum bioconversion conditions of β-aminopropionitrile in a batch operation for β-alanine production were as follows: cells loading of 16.50 g_{ww}/200 mL, substrate concentration of 1.29% (v/v), and airflow rate of 86.56 L/h, under which an overall 40.6% increase in productivity of β-alanine was obtained. The influences of the temperature and pH on the conversion were also studied, and the optimums were 30 °C and pH 7.5. The measured activation energy (E_a) was found to be 22,199 J/mol, thus indicating the presence of diffusional resistance.

1. Introduction

Nitriles are widely used in organic synthesis as precursors for compounds such as amides and organic acids. However, chemical conversion of nitriles presents several problems: reactions require either strongly acidic or basic media; energy consumption is high; and unwanted by-products (toxic substances or large amount of salts) are formed [1]. Nitrile-hydrolyzing enzymes, including nitrilase (EC3.5.5.1) [2,3] that transforms the nitriles directly into acids, nitrile hydratase (EC4.2.1.84) [4,5] and amidase (EC3.5.1.4) [6,7] that convert nitriles into acids following a two-step reaction via amides as intermediate, have great potential as catalysts for converting nitriles to higher value amides and acids on an industrial scale.

β-Alanine is the only naturally occurring beta amino acid, which are amino acids in which the amino group is at the β-position from the carboxylate group. The IUPAC name for β-alanine would be 3-aminopropionic acid. Unlike its normal counterpart, L-α-alanine, β-alanine has no chiral center. As a component of the naturally occurring peptides carnosine and anserine, and also of pantothenic acid (Vitamin B-5) which itself is a component of coenzyme A [8], β-alanine plays an important role in fine chemical and pharmaceutical synthesis.

Currently, the methods used by the industrial production of β-alanine are mainly concentrated on chemical conversion. The treatment of acrylonitrile with ammonia under heat and pressure could synthesize β-alanine [9]. The process for formation of β-alanine from β-aminopropionitrile in the presence of barium hydroxide under heat is also a chemical method [10]. However, there was only one report relating to β-alanine production from β-aminopropionitrile by microorganisms, which were *Alcaligenes* sp. OMT-MY14 and *Aminobacter aminobrance* ATCC 23314 [11].

Rhodococcus spp. are members of the nocardioforms and actinomycetes, and belong to a group of bacteria with a diverse spectrum of carbon and energy compounds [12,13]. To our knowledge, there were a lot of reports relating to nitrilase, nitrile hydratase, and amidase produced by *Rhodococcus* spp., for example, the nitrile hydratase and amidase of *Rhodococcus equi* TG328 have been used to produce 2-aryl propionic acids from the corresponding nitriles [14]; *Rhodococcus* sp. N-774 was selected as efficient catalysts for the production of acrylamide [15,16]; *Rhodococcus erythropolis* NCIMB 11540 was found to have a highly active nitrile hydratase/amidase enzyme system which could accept the nitrile function of alpha-hydroxynitriles (cyanohydrins) as substrates [17]; *Rhodococcus rhodochrous* J1 was found to have a more powerful ability to produce acrylamide, and could produce

* Corresponding author. Tel.: +86 571 88320614; fax: +86 571 88320630.
E-mail address: zhengyg@zjut.edu.cn (Y.-G. Zheng).

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acrylic acid and methacrylic acid when induced [18,19]; *Rhodococcus* sp. ZJUT-N595 could convert glycolonitrile to glycolic acid [20]. This paper reports on the β-aminopropionitrile bioconversion into β-alanine using resting cells of microorganism, which has been identified as *Rhodococcus* sp. based on the characteristics of morphology, physiology and biochemical tests, Biolog GP2 identification, and 16S rDNA sequence analysis. As far as we know, this is the first report of biotransformation of β-aminopropionitrile to β-alanine by *Rhodococcus* sp.

Bioreactors are used as a means of supporting or immobilizing, and hence applying, the biocatalysts in the biotransformation systems [21]. There are several types of bioreactors designed for bioconversion, for example, packed bed bioreactor [22], external-loop fluidized bed airlift bioreactor [23], stirred tank reactor [24], membrane bioreactor [25], microlitre/millilitre shaken bioreactor [26]. A bubble column reactor was tentatively selected for our experimental investigation of β-aminopropionitrile-converting with batch operations, and in the process of conducting such an experiment, we found that the duration of β-aminopropionitrile conversion was short and there was no risk of microbial contamination due to lack of culture medium.

Response surface methodology (RSM), an empirical modeling technique used to estimate the relationship between a set of controllable experimental factors and observed results [27], is an effective tool for optimizing the process. This method has been successfully applied in the optimization of medium compositions, conditions of enzymatic hydrolysis, and fermentation processes [28–31].

In the present study, response surface method was applied to study the combined effects of various factors such as cells loading, substrate concentration, and airflow rate, which would affect the process of β-aminopropionitrile bioconversion into β-alanine, to obtain optimum β-alanine productivity. In addition, the influences of temperature and pH on the bioconversion, the time course experiments, and the stability of system were also studied. This study will assist further in determining the suitable bioconversion conditions for industrial production.

2. Materials and methods

2.1. Microorganism

A bacterial strain namely G20, which could convert β-aminopropionitrile into β-alanine, was isolated and screened from soil samples collected mainly from hill area in Hangzhou. Based on the characteristics of morphology, physiology and biochemical tests, Biolog GP2 identification, and 16S rDNA sequence analysis, strain G20 was identified as *Rhodococcus* sp. The bacterial colonies on the plate appeared round, dry, convex and pale orange-yellow in color after 48 h incubation. The cells were long rod-like (0.3–0.8) μm × (2–5) μm.

2.2. Media and culture conditions

The composition of the culture medium was as follows: 10 g/L glucose, 3 g/L yeast extract, 1 g/L KH_2PO_4, 2 g/L Na_2HPO_4, 0.4 g/L $MgCl_2$, 3 mL/L β-aminopropionitrile. No attempt was made to control the pH of the medium. The bacterium was grown at 30 °C in shaken flasks (500 mL flasks containing 100 mL broth) in a rotary shaker (150 rpm). After 48 h incubation at 30 °C, the cells were harvested by centrifugation at 13,000 rpm for 10 min at 4 °C, and then washed twice with 50 mM Na_2HPO_4/NaH_2PO_4 buffer (pH 7.0) and re-suspended in the same buffer. The cells suspension was either used as biocatalysts for bioconversion or stored until use at 4 °C.

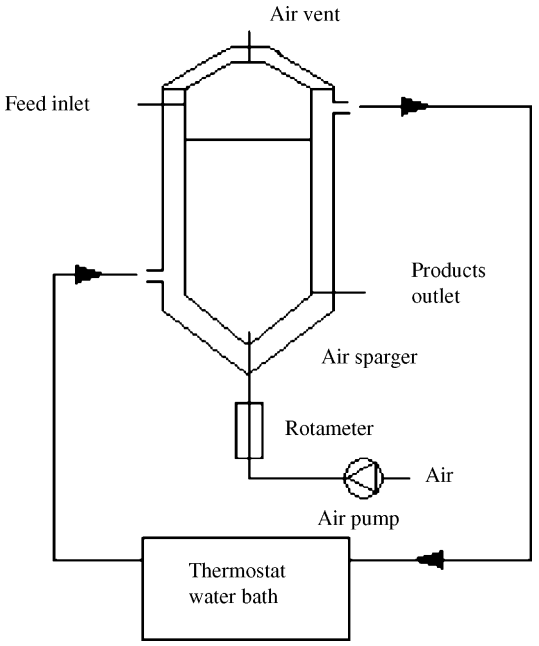

Fig. 1. Schematic presentation of the bubble column reactor used for biotransformation.

2.3. Derivatization with 2,4-dinitrofluorobenzene

Our samples, in order to be able to detect them by using high performance liquid chromatography with ultra-violet detection, were derivatized with 2,4-dinitrofluorobenzene (Sanger's reagent). The derivatization was done according to the method as follows: 1 mL $NaHCO_3$ (0.5 mol/L in distilled water) was added to 100 μL of sample in a test tube. The mixture was thoroughly mixed. To this was added 400 μL of 2, 4-dinitrofluorobenzene solution (3% (v/v) in acetonitrile) and 1 mL distilled water in turn. The mixture was incubated in the dark in a water bath for 30 min at 60 °C. After that, 7.5 mL distilled water was added to the test tube which contained the mixture to obtain 2,4-dinitrofluorobenzene derivatives for HPLC analysis.

2.4. HPLC-UV operating conditions

The concentrations of β-alanine, β-aminopropionamide and β-aminopropionitrile in the reaction mixture were measured by HPLC (LC-10Avp Shimadzu, Japan), equipped with an UV detector (SPD-10A Shimadzu, Japan). The HPLC column Elite C_{18} (250 mm × 4.6 mm), 5 μm particle size with a precolumn was used. Analysis was performed at ambient temperature. As a mobile phase 55% of methanol and 45% of acetic acid/sodium acetate solution (0.05 mol/L) was used. The amount of sample injected was 20 μL, and the flow rate was 1 mL/min. 2,4-Dinitrofluorobenzene derivatives were detected with an UV detector, set at λ = 362 nm. The retention times were 4.3, 4.9, and 5.4 min for β-alanine, β-aminopropionamide, and β-aminopropionitrile, respectively.

2.5. Bioreactor operation

Batch experiments were performed in a jacketed glass bubble column of 200 mL working volume (Fig. 1). The reaction temperature was controlled by circulating water through the reactor jacket from a temperature-controlled water bath. The reaction medium was mixed and aerated by introduction of air into the bottom. Substrate solutions were prepared from a concentrated solution of β-aminopropionitrile dissolved in a 50 mM Na_2HPO_4/NaH_2PO_4 buffer (pH 7.0).

Table 1
Coded and actual values of the experimental variables

Variables	Symbol	Coded values				
		−1.58	−1	0	1	1.58
Cells loading (g_{ww}/200 mL)	x_1	7.12	10.00	15.00	20.00	22.88
Substrate concentration (% (v/v))	x_2	0.21	0.50	1.00	1.50	1.79
Airflow rate (L/h)	x_3	13.77	40.00	90.00	140.00	166.23