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Non-sterile and buffer-free bioconversion of glucose to 2-keto-gluconic acid by using *Pseudomonas fluorescens* AR4 free resting cells

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ABSTRACT

A non-sterile and buffer-free 2-keto-gluconic acid (2KGA) production method was proposed by using resting cells from an industrial strain of *Pseudomonas fluorescens* AR4. 2KGA achieved a maximum production performance of 195.00 g/L, which corresponded to 3.05 g/L h of total productivity and 1.07 g/g of yield under optimal conditions, including resting cell aged 20 h, a cell concentration of 3.0 g/L, a reaction temperature of 30 °C, a glucose concentration of 181.82 g/L, and a reaction volume of 20 mL during the 64-h bioconversion process. *P. fluorescens* AR4 resting cells showed stable bioconversion activity after reuse 3 times or storage for less than 28 d at 4 °C. These findings suggested that the proposed non-sterile and buffer-free bioconversion of glucose to 2KGA using *P. fluorescens* AR4 resting cells had remarkable advantages, including ease handling and low cost, and they are a potent application for industrial production of 2KGA.

1. Introduction

The current world production of erythorbic acid (D-isoascorbic acid, a stereoisomer of ascorbic acid) is estimated at 40,000 tons per annum with a global market in excess of US $20 million [1,2]. Approximately, 80% of erythorbic acid is used as a food antioxidant to prevent food oxidation; inhibit a decrease in color, aroma and flavor; and block the production of the carcinogen ammonium nitrite, based on the Regulations and Standards released by FDA, China, European countries and other international organizations/committees (http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?fr=182.3041; www.codexalimentarius.net/gsfaonline/index.html). The growth of the erythorbic acid market continues to increase after being widely applied in cosmetic products as an antioxidant additive (http://ec.europa.eu/consumers/cosmetics/cosing/index.cfm?fuseaction=search.detailsv2&id=33791). Commercial erythorbic acid production is a two-step process that includes the microbial conversion of glucose to 2-keto-gluconic acid (2KGA) and chemical lactonization of 2KGA to the target product erythorbic acid [3–5].

The conventional 2KGA production method is batch fermentation using free cells [6,7]. Microorganisms from the genera *Pseudogluconobacter*, *Serratia*, *Gluconobacter* and *Acetobacter* have been reported as potential 2KGA producers [8–10]. Some strains, including *Pseudomonas fluorescens* AR4 and *Arthrobacter globiformis* C224, with a high glucose tolerance of over 140 g/L and a 2KGA yield of 92.80% have also been screened by our group and are used in most Chinese erythorbic acid companies [11–13]. Flux studies in *P. fluorescens* and *Pseudomonas putida* revealed that most of the gluconic acid produced from glucose (almost 90%) is transformed into 2KGA [14]. However, 2KGA production by most strains is compromised because approximately 10% of the sugar is utilized for bacterial regeneration and therefore the actual maximum yield of 2KGA is below 90% (mol%).

Resting cells, also known as non-growing cells, refer to non-proliferating but metabolically active cells [15]. In most cases, resting cells are treated not to grow or to grow at a low specific growth rate in inorganic phosphate buffer as the medium [16,17].

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Production stability can be increased by using resting cells, and metabolism will be shifted away from cell growth or by-product formation and towards the formation of the target product [18,19]. For example, the use of resting cells to biotransform phytosterol to androsta-diene-dione (ADD) increased the ADD concentration up to 12,000 mg/L, with a repeated utilization of the cells and a non-sterile culture process [20]. Resting cells of *Rhodococcus ruber* CGMCC3090 have been shown to be effective for producing 5-cyanovaleramide (5-CVAM) by adiponitrile (ADN) hydration with a high conversion efficiency (99.2% of added AND) and a high product purity [21].

As for 2KGA production, immobilized *Pseudomonas aeruginosa* resting cells have been used for the bioconversion of glucose to 2KGA with sodium acetate solution as the buffer [22]. However, the 2KGA production performance was poor under these conditions because only 35 g/L of 2KGA was produced with a maximum productivity of 0.55 g/L h. Generally, addition of sodium acetate buffer salts (100 mM, pH 6.0) and sterilization of the media possibly increased the impurity of the 2KGA, hindered the 2KGA purification process, and led to high equipment, energy, and labor inputs. Hence, to improve 2KGA production performance and economics, the present study will focus on (1) proposing a direct conversion process for 2KGA production using a *P. fluorescens* resting cell culture in a non-sterile and buffer-free condition, (2) evaluating the 2KGA production performance in shaking flask and 5-L fermenter cultures, and (3) evaluating its feasibility for competing with the current 2KGA batch production process.

2. Materials and methods

2.1. Microorganisms and media

The bacteriophage-resistant mutant strain of *P. fluorescens* AR4 was screened and kept in our laboratory [23,24]. The medium for preparing the stock culture consisted of glucose 10.0 g/L, beef extract 5.0 g/L, NaCl 5.0 g/L and agar 20.0 g/L. The seed culture was prepared by transferring the stock culture to the seed medium in a 500 mL Erlenmeyer flask containing glucose 20.0 g/L, corn steep liquor 10.0 g/L, urea 2.0 g/L, KH_2PO_3 2.0 g/L, $MgSO_4{\cdot}7H_2O$ 0.5 g/L, $CaCO_3$ 5.0 g/L and incubated at 30 °C for 18 h. The media for preparing seed cultures and resting cells was sterilized separately at 121 °C for 15 min.

The buffer-free bioconversion media contained glucose at various concentrations and $CaCO_3$ for balancing the broth pH to 5.0–6.5. The bioconversion process started when *P. fluorescens* AR4 resting cells were added into the non-sterile bioconversion media.

2.2. Resting cell preparation

Seed cultures (80 mL/flask) were grown to mid-late log phase and centrifuged at 4000 × *g* for 10 min. Resting cells were harvested by removing the supernatant and resuspending the pellet in buffer-free bioconversion solution for catalyzing glucose to 2KGA.

2.3. Experimental design

To investigate the effect of resting cell concentration on 2KGA production, the harvested *P. fluorescens* cells were diluted to a concentration of 1.5–6.0 g cells (dry weight)/L. Temperatures ranging from 18 to 42 °C, glucose concentrations from 110.0 to 255.0 g/L, and medium volumes from 20 to 80 mL were used to evaluate the 2KGA production performance.

To evaluate the number of times *P. fluorescens* resting cells were reused, the cells were collected by centrifugation at 4000 × *g* at the end of the bioconversion process and washed twice with sterilized water for further use in 48-h bioconversion. Storage times of resting cells at 4 °C from 4 to 44 d were investigated for their effect on 2KGA bioconversion performance.

A bioconversion process was also carried out in a 5 L GRCB lab scale mechanically stirred fermenter equipped with three 6-bladed disc impellers and oxygen and pH electrodes (GRCB, Green Bio-engineering Co., Ltd, Zhenjiang, China) under the following conditions: a working medium volume of 3-L, an inoculation volume of 10% (v/v), a culture temperature of 30 °C, a pressure of 0.04 MPa and an agitation speed of 450 rpm. The experiments continued until 2KGA production peaked. Samples were withdrawn from the fermenter every eight hours for 2 KGA testing, the residual glucose concentration, pH and cell concentration.

2.4. Analytical methods

The 2KGA concentration was determined and calculated on the basis of glucose concentration using the polarimetry method developed by our group [23]. The procedure is described briefly as follows: a sample of cultured broth was centrifuged at 4000 × *g* for 20 min, 25 mL of resulting supernatant was mixed with 20 mL of deionized water, and the pH was adjusted to 1.5 by adding 1N HCl, then diluted to 100 mL with deionized water. The degree of optical rotation of the final sample solution was determined with a WZZ-1SS Digital Automatic Polarimeter (Precision Instrument Co., Ltd., Shanghai, China). The 2KGA concentration was calculated with the equation:

$$Y = -0.88X_1 + 0.5275X_2$$

where Y is the optical rotation degree of broth (°), X_1 the 2KGA concentration (g/L); X_2 the glucose concentration (g/L), which was determined with Biosensor Analyzer (Shandong Academy of Sciences Institute of Biology, Jinan, China) at 25 °C; −0.88 represents the optical rotation degree of a 10 g/L 2KGA solution (°) and 0.5275 represents the optical rotation degree of 10 g/L of glucose solution (°).

The cell growth was measured by optical density at 650 nm ($OD_{650\,nm}$), or the dry cell weight (DCW) was computed from a curve relating $OD_{650\,nm}$ (Biospec-1601 spectrophotometer, Shimadzu) to dry weight. The standard curve equation was:

$$CDW(g/L) = 0.46OD_{650nm} - 0.02.$$

An $OD_{650\,nm}$ of 1.0 represented 0.44 g cell dry weight per liter.

The 2KGA production performance was evaluated based on the 2KGA concentration, productivity, and yield from glucose. The 2KGA productivity was defined as the amount of 2KGA produced per hour per liter. The 2KGA yield was calculated by dividing the amount of 2KGA produced by the amount of glucose consumed. The glucose consumption ratio was calculated by dividing the amount of glucose consumed by the initial amount of glucose.

2.5. Statistical analysis

Each experiment was repeated three times using duplicate samples. The results were expressed as means ± standard deviations. Statistical comparisons were made by one-way analysis of variance (ANOVA), followed by Duncan's multiple-comparison test using the SAS System (SAS Institute, Cary, NC, USA). Differences were considered significant when the P-values were <0.05.

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