Improvement of Poly(γ-glutamic acid) Biosynthesis and Quantitative Metabolic Flux Analysis of a Two-stage Strategy for Agitation Speed Control in the Culture of Bacillus subtilis NX-2

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Abstract In this study, the production of poly(γ-glutamic acid) by Bacillus subtilis NX-2 (PGA) at different agitation speeds was investigated. Based on the analysis of specific cell growth rate (μ) and specific PGA formation rate (qp), a two-stage strategy for agitation speed control was proposed. During the first 24 h, an agitation speed of 600 rpm was used to maintain a high μ for better cell growth, which then reduced to 400 rpm after 24 h to maintain a high qp to enhance PGA production. Using this method, the maximum concentration of PGA reached 40.5 ± 0.91 g/L and the PGA productivity was 0.56 ± 0.012 g/L/h, which was 17.7 and 9.8% higher, respectively, than the best results obtained when a constant agitation speed was used. The flux distributions and the related enzymes of 2-oxo-glutarate could be affected by this two-stage strategy for agitation speed. The activity of isocitrate dehydrogenase and glutamate dehydrogenase at the key node of 2-oxo-glutarate increased, and more flux distribution was directed to glutamate. The flux distribution from extracellular to intracellular glutamate also increased and improved PGA production as the glutamate uptake rates increased using the agitation-shift control method.

Keywords: poly(γ-glutamic acid), two-stage control strategy, agitation speed, metabolic flux, enzyme activity

1. Introduction

Poly(γ-glutamic acid), or PGA, is an anionic polyamide that consists of D- and L-glutamic acid units connected by γ-amide linkages. This biopolymer has been used in a broad range of industrial fields such as food, cosmetics, medicine, and water treatment [1,2]. Due to the potential applications of PGA, many studies have attempted to optimize its fermentation.

The PGA yield can be affected by several parameters, such as medium composition, fermentation conditions, and Bacillus species. The Bacillus species are aerobic microorganisms and oxygen supply is an important factor in their aerobic fermentation, particularly for the production of highly viscous biopolymers [3]. The agitation and aeration rate are associated with the dissolved oxygen level during the fermentation. Bajaj and Singhal [4] investigated the effect of agitation and aeration on the synthesis of PGA in Bacillus licheniformis and showed that a high aeration rate (2 vvm) and agitation speed (750 rpm) enhanced the growth of cells and the maximum PGA production was obtained at an agitation speed of 750 rpm and an aeration rate of 1 vvm. Cromwick et al. [5] found that aeration rate and pH affected PGA production in B. licheniformis and increasing the agitation speed (from 250 to 800 rpm) and aeration rate (from 0.5 to 2.0 L/min) at pH 6.5 yielded a maximum PGA concentration of 24 g/L.

Similar phenomena have been reported for other biopolymers, such as xanthan, pullulan, and hyaluronic acid. Borges et al. [6] found that a high aeration rate (3 vvm) and agitation speed (300 rpm) increased the yield of xanthan, but had no effect on biomass production. Roukas [7] showed that a high aeration rate enhanced cell growth during the synthesis of pullulan, but did not lead to high
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2. Materials and Methods

2.1. Bacterial strain

*B. subtilis* NX-2 (mutant) was isolated from a soil sample [11] and was deposited in the China General Microbiological Culture Collection Center.

2.2. Medium and cultivation conditions

A loopful of *B. subtilis* NX-2 cells were first inoculated into 80 mL of seed medium containing 20 g/L glucose, 15 g/L l-glutamic acid, 5 g/L yeast extract, 2 g/L K₂HPO₄·3H₂O, and 0.1 g/L MgSO₄ in a 500 mL flask, and aerobically incubated at 32 ± 0.5°C for 16 h with shaking at 220 rpm. The seed culture (2% v/v) was then transferred to a 7.5 L BioFlo 110 bioreactor (Rushton-style impeller, 6 cm d.; bioreactor, 17.8 cm i.d., 32.1 cm height, 4.5 L working volume; New Brunswick Scientific, USA) containing 4.5 L of basal medium. The fermentation medium consisted of 60 g/L glucose, 50 g/L glutamate, 8 g/L (NH₄)₂SO₄, 2 g/L K₂HPO₄·3H₂O, 0.1 g/L MgSO₄, and 0.03 g/L MnSO₄, and was incubated at 32 ± 0.5°C. The pH was controlled at 7.0 ± 0.1 by adding 2 N NaOH or 2 N HCl. Since the PGA yield did not change significantly at aeration rates between 0.8 and 1.5 vvm (data not shown), the aeration rate was maintained at 1.2 vvm. The agitation speed was controlled at 200, 400, 600, and 800 rpm during batch fermentation.

2.3. Analytical methods

The PGA concentration was measured using a gel permeation chromatography (GPC) system with an RI-10 refractive-index detector and a Superpose™ 6 column (Shimadzu Co.). 50 mM of a NaCl aqueous acetonitrile solution (4:1, v/v) was used as the mobile phase at a flow rate of 0.7 mL/min. The amount of PGA was calculated from the peak area of the GPC measurements, using purified PGA as a standard.

Ten milliliters of the cell suspension were harvested by centrifugation at 10,000 × g and 4°C for 20 min, washed with distilled water, and dried at 80°C to a constant weight to determine the dry cell weight (DCW). The concentration of glucose and l-glutamic acid remaining in the broth were measured enzymatically using a biosensor (SBA-40C, Shandong Academy of Sciences, China). The CO₂ and O₂ concentrations in the exhaust gas were measured using a gas analyzer (LKM2000A, LOKAS, Korea).

2.4. Assay of enzyme activities

Cells were collected at different stages of fermentation and washed twice with 50 mL of a 0.85% NaCl solution. Harvested cells were suspended in 100 mM Tris-HCl buffer (pH 7.5) and disrupted by sonication for 10 min at 400 W. The cell debris was removed by centrifugation at 12,000 rpm for 10 min. All procedures were conducted at 4°C.

The activity of isocitrate dehydrogenase (ICDH), glutamate dehydrogenase (GDH), and 2-ketoglutarate dehydrogenase (ODHC) in cell extracts was measured as described previously [12]. The activity of these enzymes was determined based on the appearance or disappearance of NADH or NADPH at 340 nm at 32.5°C using an enzyme microplate reader (MK3, Thermo, USA). In all cases, one unit of activity was defined as the amount of enzyme catalyzing 1 µmol of NADH or NADPH per minute. Total protein concentration was determined using the Bradford method [13]. Every experiment was repeated three times, and the experimental errors in these measurements were less than 15%.

2.5. Metabolic flux analysis

The metabolic model for PGA biosynthesis and stoichiometric representations in *B. subtilis* NX-2 was established in our previous study [14]. The observable extracellular metabolites were glucose, l-glutamic acid, biomass, PGA, O₂, and CO₂. Calculations were performed on a personal computer using Matlab 7.0.

3. Results and Discussion

3.1. PGA fermentation at different agitation speeds

The PGA fermentation of *B. subtilis* NX-2 at different agitation speeds (200, 400, 600, and 800 rpm) were investigated using a 7.5 L bioreactor (Figs. 1A, 1B, 1C, and 1D). As shown in Fig. 1B, a maximum PGA concentration of 34.4 ± 0.71 g/L was obtained at 600 rpm. At 800 and 200...