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The main byproducts and metabolic flux profiling of γ-PGA-producing strain *B. subtilis* ZJU-7 under different pH values

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ABSTRACT

Poly-γ-glutamic acid (γ-PGA) is an extracellular anionic polymer with many potential applications. Although microbial fermentation is the common approach to produce γ-PGA, the broth at the latter stage usually becomes very viscous and severely influences the metabolites producing pattern of target microbe. In this study, acetoin and 2,3-butanediol were confirmed to be the main byproducts of γ-PGA-producing strain *B. subtilis* ZJU-7 (*B. subtilis* CGMCC1250), and their effects on the cell growth and γ-PGA biosynthesis were further investigated in shake flasks. The outcome indicated that both acetoin and 2,3-butanediol showed clear impairment on γ-PGA production of *B. subtilis* ZJU-7. Moreover, the extracellular metabolites profiles of fermentation under three different pH values were acquired and the metabolic flux redistribution of pathways related to γ-PGA biosynthesis was calculated based on the collected data. As a result, the metabolic flux favored to distribute toward glycolytic pathway at pH 6.5, in which the ingestion rate of extracellular glutamic acid was higher and the subsequent γ-PGA biosynthesis was enhanced. The present work provided us a deep insight into the metabolic flux control of γ-PGA biosynthesis, which will stimulate some novel metabolic engineering strategies to improve the productivity of γ-PGA in future.

1. Introduction

Poly (γ-glutamic acid) (γ-PGA) is composed of D- and L-glutamic acid units via amide linkages between α-amino and γ-carboxylic acid groups. Several members of *Bacilli* species are able to synthesize γ-PGA in vivo, such as *B. subtilis, B. licheniformis*, and *B. amyloliquefaciens* (Birrer et al., 1994; Goto and Kunioka, 1992; Liu et al., 2011). Possessing several beneficial characteristics such as water soluble, anionic, non-toxic, biocompatible, biodegradable and edible, γ-PGA has presented great potential in its industrial applications (Bajaj and Singhal, 2011). For fermentative production of γ-PGA, *B. licheniformis* and *B. subtilis* have been normally chosen because of their easy cultivation and general biosafety (GRAS). Intensive studies of these strains regarding enzymatic synthesis regulations, nutrient requirements, and cultivation conditions were carried out to improve cell growth, γ-PGA production and desirable ratio in chain D/L-repeat unit composition (Ashiuchi et al., 2004; Shih and Van, 2001). The metabolic pathways related to the γ-PGA biosynthesis were also studied (Wu et al., 2008). According to the origin of basal unit glutamate, γ-PGA producing strains are divided into two groups: one needs the addition of external L-glutamate and the other can self-synthesize γ-PGA without supply of L-glutamate (Kunioka, 1997; Zhang et al., 2012).

The fermentation of γ-PGA biosynthesis is generally an aerobic process, but the high viscosity of broth at the latter stage of fermentation seriously hampers the transfer of dissolved oxygen and thus forms the anaerobic-like environment affecting the metabolites producing pattern of microbes. It was reported that *B. subtilis* could grow under the anaerobic fermentation condition by respiration with nitrate as the terminal electron acceptor

Abbreviations: G6P, glucose-6-phosphate; F6P, fructose-6-phosphate; R5P, ribulose-5-phosphate; T3P, triose-3-phosphate pool; Xyl5P, xylulose-5-phosphate; Rib5p, ribose-5-phosphate; E4P, erythrose-4-phosphate; S7P, seduheptulose-7-phosphate; PG3, 3-phosphoglycerate; C1, methyl group bound to tetrahydrofolate; PEP, phosphoenolpyruvate; Pyr, pyruvate; AcCoA, acetyl coenzyme A; OAA, oxaloacetate; αKG, α-ketoglutaric acid; FUM, fumarate; MAL, maleic acid; Glu, intracellular glutamic acid; exGlu, extracellular glutamic acid; PGAex, extracellular poly γ-glutamic acid; 3H2B, intracellular acetoin; 3H2Bex, extracellular acetoin; 23BDOex, extracellular 2,3-butanediol.

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(Hartig et al., 2006). The nuclear magnetic resonance scan analysis of in vivo metabolites indicated that *B. subtilis* had performed mixed acid-butanediol fermentation without oxygen, and hardly with any formate detected (Earl et al., 2008). Further metabolome analysis implied that pyruvate was probably not metabolized by pyruvate formate lyase (PFL) to generate acetyl coenzyme A (CoA) and formate, but oxidatively decarboxylated by pyruvate dehydrogenase (PDH) to produce butanediol. The fermentation metabolites produced by wild-type *B. subtilis* include acetate, ethanol, lactate, 2,3-butanediol (2,3-BDO), and a small portion of acetoin (Nakano et al., 1998; Ramos et al., 2000). In addition, acetate and 2,3-BDO were the major byproduct identified during the γ-PGA production by *B. licheniformis* 9455a (Birrer et al., 1994).

Over the last decade, genome scale metabolic models have been widely established and are available for more than 50 organisms. Among them, metabolic flux balancing is a valuable tool to gain insight into the complex responses and capabilities of whole cellular metabolism (Dauner et al., 2001). The model of center carbon metabolism pathways in *B. subtilis* CGMCC0833 using glucose and glutamate has been proposed (Wu et al., 2008). However, the effects of such byproducts on the fermentation process have not been investigated yet. In our previous work, *B. subtilis* strain ZJU-7 (*B. subtilis* CGMCC1250) showed great capacity to produce γ-PGA as high as 80 g/l, with yeast extract as an alternative nitrogen source to reduce the cost of γ-PGA production (Chen et al., 2010). In the present work, we focused on the measurement of main metabolic byproducts in *B. subtilis* ZJU-7 and tried to evaluate the effects of metabolic byproducts and pH control strategies on cell growth and PGA production. Especially, the metabolic flux redistribution under different pH values was adopted to explore the strategy to improve γ-PGA productivity.

2. Material and methods

2.1. Cultivation of B. subtilis ZJU-7 in shake flask

B. subtilis ZJU-7 was isolated from fermented bean curd and reserved in our lab (Shi et al., 2006a). It was deposited in CGMCC (China General Microbiological Culture Collection Center) and named as *B. subtilis* CGMCC1250. The strain was cultured in 250 ml flask containing 20 ml seed medium (per liter: 10 g tryptone, 5 g beef extract, and 5 g NaCl) and incubated at 37 °C and 200 rpm for overnight. Three milliliters of such seed culture was then inoculated into 30 ml production medium (initial pH 6.5) that comprised of 80 g/l L-glutamic acid, 10 g/l NaCl, 1 g/l $CaCl_2$, 1 g/l $MgSO_4$, 80 g/l glucose, and 40 g/l yeast extract. *B. subtilis* ZJU-7 was cultivated at 37 °C and 200 rpm for 48 hours to produce γ-PGA (Shi et al., 2006b).

To investigate the effects of main byproducts on cell growth of *B. subtilis* ZJU-7 and γ-PGA production, different concentrations of acetoin (0, 2, 5, 15 g/l, respectively) and 2,3-BDO (0, 10, 20, 30 g/l, respectively) were added into the medium at the beginning of the cultivation and the beginning of stationary phase, respectively. After 6 hours growth, samples were taken every 4 hours until the exhaustion of carbon source.

2.2. Fed-batch fermentation of B. subtilis ZJU-7

For fed-batch fermentation in the fermentors, *B. subtilis* ZJU-7 was first cultured in 250 ml flask containing 30 ml seed medium and incubated at 37 °C and 200 rpm for overnight. Such culture was then inoculated into 500 ml production medium and grew at 37 °C and 200 rpm for 36 h to obtain the mature seed broth. The resulted 500 ml seed broth was transferred into a 10-l fermentor (Shanghai Baoxing Bioengineering Equipment Co., Shanghai, China) containing 5-l production medium (initial concentrations of glucose was 20 g/l, L-glutamate was 40 g/l) for fermentation. The temperature was kept at 37 °C. The pH was automatically controlled at a certain value (5.7, 6.5 or 7.3) with the addition of 25% (v/v) NH_4OH or 1 M HCl. The aeration rate was set at 1 vvm, with agitation rate ranging from 350 to 650 rpm to maintain enough dissolved oxygen. The pulse feeding of 750 g/l glucose solution using a peristaltic pump at the rate of $2\,g\,l^{-1}\,h^{-1}$ was started when the concentration of glucose in the broth was lower than 5 g/l. The pulse interval was manually adjusted to maintain the concentration of glucose in the range of 3–8 g/l. The fermentation process was lasted for about 48–72 h (Huang et al., 2011).

2.3. Analysis of fermentation samples

The concentrations of CO_2 and O_2 in the exhaust gas were measured online using gas analyzer (MultiRAE IR Gas Monitor, RAE Systems). The DO and pH values of broth were measured online using related electrodes (Mettler Toledo).

The cell density of broth was measured at 600 nm by a 752 s UV–Vis spectrophotometer. For determination of cell dry weight, 10 ml culture was centrifuged at 3030g and 4 °C for 20 min using Eppendorf 5810 R Centrifuge. The resulted cell pellets were washed three times with 0.15 M NaCl and resuspended in 20 ml of 0.15 M NaCl. Such mixture was dried at 55 °C to a constant weight; 20 ml of 0.15 M NaCl was also dried to a constant weight as the control to get the final cell dry weight.

For GC analysis, the cell pellet from centrifugation was washed once with 20 mM Tris–HCl (pH 7.6) and resuspended in 5 ml dd H_2O, 4-fold volume of methanol was added to precipitate γ-PGA and other polysaccharides. After centrifugation, the collected supernatant was subjected to GC analysis. An Agilent 6850 GC equipped with split/splittless injector was applied. Helium with a constant flow rate of 1 ml/min was used as carrier gas. The splitless injection was set at 250 °C, and the total run time was 6.5 min. The initial temperature of column oven was set at 60 °C and maintained for 2 min, and then the temperature was raised to 300 °C with the rate of 75 °C/min. The final ramping at 300 °C was kept for 2 min. The authentic samples of ethanol, acetate, lactate, propionate, acetoin and 2,3-BDO were also analyzed by GC to provide standard references for metabolites characterization.

The concentrations of glucose and glutamate were measured enzymatically using a bioanalyzer (SBA-40 C, Shandong Academy of Sciences). The yield of γ-PGA was measured offline by gel permeation chromatography (GPC) system following the previously reported method (Wu et al., 2008) using an Agilent 1100 HPLC system equipped with TSK G4000PXWL column (30 cm × 7.8 mm × 10 μm), RID detector and 0.01 M $NaNO_3$ as the mobile phase. Every experiment was repeated at least three times, and the experimental errors were less than 4%.

2.4. Metabolic flux profiling

The previously described biochemical reaction network of *B. subtilis* (Wu et al., 2008) was augmented with the proposed pathway and shunts involved in the biosynthesis of acetoin and 2,3-BDO (shown in Appendix A). The model constituted reactions from the intermediary metabolisms (including glycolysis, pentose–phosphate pathway, tricarboxylic acid (TCA) cycle), cell mass formation, and biosynthesis of main by-products (acetoin and 2,3-BDO) detected in our experiments. From this network, a stoichiometric matrix containing 30 unknown fluxes and 30 metabolite balances (including 8 balances of the measured substrates and products: biomass, glucose, L-glutamate, γ-PGA, acetoin, 2,3-BDO, CO_2, and O_2) was constructed. Some linear reactions in the model were lumped together for simplification. The cell mass term in the model was constructed by considering all required metabolites,