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Copper sulfate improves pullulan production by bioconversion using whole cells of *Aureobasidium pullulans* as the catalyst

Dahui Wang [1], Xiaomin Ju [1], Gaochuan Zhang, Donghua Wang, Gongyuan Wei *

School of Biology and Basic Medical Sciences, Soochow University, Suzhou 215123, PR China

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ABSTRACT

The effects of mineral salts on pullulan production by bioconversion using whole cells of *Aureobasidium pullulans* CCTCC M 2012259 as the catalyst were investigated. Copper sulfate ($CuSO_4$) improved pullulan production by 36.2% and 42.3% when added at the optimum concentration of 0.2 mg/L to the bioconversion broth or seed medium, respectively, as compared with controls without $CuSO_4$ addition. Pullulan production was further enhanced when $CuSO_4$ was added to both seed medium and bioconversion broth simultaneously. In order to probe the mechanism of $CuSO_4$ improvement, cell viability, membrane integrity, intracellular adenosine triphosphate (ATP) levels and the activities of key enzymes involved in pullulan biosynthesis were determined. As a result, $CuSO_4$ increased the activities of key biosynthetic enzymes, maintained intracellular ATP at a higher level, and accelerated the rate of pullulan secretion, all of which contributed to improved pullulan production by bioconversion.

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1. Introduction

Pullulan is a linear homopolysaccharide composed of repeating maltotriose units jointed through α-1, 6 linkages (Leathers, 2003; Singh, Saini, & Kennedy, 2008). Owing to its good adhesive properties and capacity to form fibers, pullulan is widely used in foods, cosmetics, and the biomedical and pharmaceutical industries (Cheng, Demirci, & Catchmark, 2011; Oğuzhan and Yangilar, 2013). Moreover, pullulan and its derivatives are also regarded as promising biomaterials with potential for use in medical applications such as tissue engineering, wound healing as well as targeted drug and gene delivery (Mishra, Vuppu, & Rath, 2011; Prajapati, Jani, & Khanda, 2013). Hence, the demand for this non-toxic, non-mutagenic, non-immunogenic and non-carcinogenic biopolymer is ever-increasing (Farris, Unalan, Introzzi, Fuentes-Alventosa, & Cozzolino, 2014).

Pullulan is most commonly produced by fermentation using the yeast-like fungus *Aureobasidium pullulans* under aerobic conditions (Chi et al., 2009; Gaur, Singh, Gupta, & Gaur, 2010). Many studies have focused on strain screening, process optimization, operation methods selection and bioreactor design to improve the efficiency of fermentative pullulan production (Cheng et al., 2011; Leathers, 2003; Mishra and Suneetha, 2014; Prajapati et al., 2013; Singh et al., 2008; Shingel, 2004). Optimization of mineral salts in the medium can improve cell growth and pullulan production (Gao, Kim, Chung, Li, & Lee, 2010), and metal ions such as Fe^{3+}, Mn^{2+} and Zn^{2+} have been reported to enhance pullulan production by *A. pullulans* ATCC 42023 (West and Reed-Hamer, 1992). In contrast, Reeslev and Jensen (1995) found that Fe^{3+} and Zn^{2+} had an inhibitory effect on cell growth and morphology, as well as pullulan elaboration, while Mn^{2+}, Ca^{2+} and Cu^{2+} had no effect. Nevertheless, Gadd and Griffiths (1980) had reported that the presence of Cu^{2+} during cell growth had a profound effect on morphology of the strain.

Instead of fermentative method, pullulan production can also be accomplished by direct bioconversion from glucose using *A. pullulans* cells in a whole-cell biocatalyst approach (Ju, Wang, Zhang, Cao, & Wei, 2015). Compared with batch fermentation for pullulan production, the cost of raw materials for bioconversion decreased

* Corresponding author at: School of Biology and Basic Medical Sciences, Soochow University, 199# Ren'ai Road, Suzhou 215123, PR China.
E-mail address: weigy@suda.edu.cn (G. Wei).
[1] Xiaomin Ju and Dahui Wang contributed equally to this work.

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~20%, and pullulan production increased by more than 86% within a comparable cultivation cycle (Ju et al., 2015). According to the literature, α-phosphoglucose mutase (PGM, EC 5.4.2.2), uridine diphosphate glucose pyrophosphorylase (UGP, EC 2.7.7.9) and glucosyltransferase (FKS, EC 2.4.1.34) are the three key enzymes involved in pullulan biosynthesis (Cheng et al., 2011; Duan, Chi, Wang, & Wang, 2008), and increasing the activity of these enzymes is likely to improve pullulan biosynthesis in a whole-cell bioconversion.

Metal ions can improve the activity of enzymes involved in the biosynthesis of polysaccharides (Miletić, Nastasović, & Loos, 2012; Zhao, Chai, Li, Chen, & Tang, 2014), and although the effects of some metal ions on pullulan fermentation have been previously described, no literature is available on how pullulan biosynthesis is influenced by metal ions during bioconversion. In this study, the effects of several mineral salts on pullulan production by bioconversion were investigated. Special attention was paid to the role of copper sulfate ($CuSO_4$) on pullulan biosynthesis, and insight was gained into the mechanism by which Cu^{2+} supplementation improved pullulan production.

2. Materials and methods

2.1. Microorganism

A strain of *A. pullulans* stored in China Center for Typical Culture Collection with the serial number of CCTCC M 2012259 was used as the whole-cell catalyst for direct bioconversion of glucose to pullulan (Ju et al., 2015). The strain was maintained in seed medium containing 20% (w/v) potato juice and 20 g/L glucose, and stored in 1 mL Eppendorf tubes at −70 °C with 15% (v/v) glycerol.

2.2. Preparation of whole cells for bioconversion

A 1 mL frozen glycerol stock was precultured in a 500 mL Erlenmeyer flask containing 50 mL potato-dextrose medium and incubated on a rotary shaker at 30 °C and 200 rpm for 24 h. Cultures were inoculated into 500 mL flasks containing 50 mL seed medium at an inoculation rate of 10% (v/v) to prepare whole cells. Seed medium contained 63.97 g/L glucose, 3.57 g/L yeast extract, 0.6 g/L $(NH_4)_2SO_4$, 5.0 g/L K_2HPO_4, 1.0 g/L NaCl, and 0.18 g/L $MgSO_4{\cdot}7H_2O$, at pH 6.5 (Ju et al., 2015). Cells were incubated at 30 °C and 200 rpm on a shaker for 36 h. Sterilized $CuSO_4$ (1 g/L) was added to seed medium as required according to the experimental scheme.

2.3. Whole-cell bioconversion for pullulan production

Wet cells of *A. pullulans* CCTCC M 2012259 were harvested from the seed culture by centrifugation at 12,000*g* and 4 °C for 20 min. Cells were washed twice with deionized water and resuspended in a 500 mL shaking flask containing 50 mL bioconversion broth (50 g/L glucose, 0.2 g/L $MgSO_4{\cdot}7H_2O$, pH 6.8). The initial dry cell density was adjusted to ~10 g/L, and bioconversion of glucose to pullulan was performed at 30 °C and 200 rpm on a rotary shaker for 48 or 72 h. Concentrated salts were sterilized separately and added to the bioconversion broth as required.

2.4. Analytical methods

A 10 mL seed culture was centrifuged at 12,000*g* for 20 min, cells were washed twice with deionized water, and wet cells were dried at 70 °C to a constant weight prior to the biomass assay. Cell viability during the bioconversion process was determined by counting colonies on plates containing seed medium as described by Chesney, Eaton, and Mahoney (1996). Plates were incubated at 30 °C for 48 h and colony forming units (CFU) were counted.

The concentration of pullulan and residual glucose in the bioconversion broth was determined as previously described (Wang, Yu, & Wei, 2013), followed by inactivating the broth at 80 °C in a water bath for 20 min. The cell capacity for pullulan bioconversion was defined as the amount of pullulan generated from glucose by 1 g of cells per hour at 30 °C (Ju et al., 2015). The yields of pullulan to glucose ($Y_{P/S}$) and biomass ($Y_{P/X}$) were defined as the ratio of pullulan generated vs. glucose consumed or cells used during bioconversion, respectively.

Intracellular adenosine triphosphate (ATP) and adenosine triphosphate (ADP) levels were determined by high performance liquid chromatography (HPLC) using a SunFire™ ODS C18 column (4.6 × 250 mm) based on the previous method (Wang, Wang, Wei, & Shao, 2012). Samples were prepared by lysing wet cells using ultrasonication in phosphate buffer (0.2 mol/L, pH 7.0) followed by centrifugation at 8000*g* and 4 °C for 10 min (Wang et al., 2013).

2.5. Enzyme activity assays

A 5 mL bioconversion broth was centrifuged at 12,000*g* and 4 °C for 20 min to harvest wet cells, which were then resuspended in 5 mL phosphate buffer (0.2 mol/L, pH 7.0) prior to ultrasonication. Cell ultrasonication was carried out for 10 min with 10 s active and passive intervals in an ice bath using an ultrasonic processor VCX 750 (Sonics & Materials Inc., Newtown, CT, USA) at 20 kHz. Following centrifugation at 12,000*g* and 4 °C for 10 min, the cell-free extract (supernatant) was used for enzyme assays (Ju et al., 2015).

The activity of FKS in the cell-free extract was assayed as previously described by Duan et al. (2008). The activities of PGM and UGP were measured using commercial enzyme-linked immunosorbent assay kits (ShhcBio, Shanghai, China) according to the manufacturer's instructions.

2.6. Flow cytometric analysis

After bioconversion for 24 or 48 h, yeast cells were harvested and washed twice with 0.1 mol/L phosphate buffer solution (PBS, pH 7.0), then resuspended in PBS immediately and diluted to a cell density of ~10^6 cells/mL. Cells in 100 μL suspension were stained with 1 μL of 2.5 mg/L propidium iodide (PI, Key-GEN, Nanjing, China), incubated in the dark at 37 °C for 50 min, then mixed with 900 μL of 0.1 mol/L PBS before flow cytometric analysis.

Flow cytometric measurement of cell membrane integrity was carried out using a Cytomics™ FC 500 flow cytometer (Beckman Coulter, Fullerton, CA, USA) equipped with an air-cooled Argon ion laser (488 nm, 20 mW output) for excitation and monitoring with a single emission channel (625 nm). Cells were delivered at a flow rate of ~500 events per second. In total, ~10,000 events were detected during each run. Recorded signals were amplified logarithmically and a quad-gate was created in the dot plot to discriminate differential yeast populations. Data were analyzed using CXP analysis 2.2 software (Beckman Coulter).

Cells collected from bioconversion broth not containing $CuSO_4$ served as controls. The PI uptake rate of cells was defined as the ratio of cells stained by PI vs. the total number of cells.

2.7. Statistical analysis

All experiments were performed in triplicate, and data were averaged and expressed as means ± standard deviation (SD). The Student's *t*-test was employed to evaluate statistical differences and samples with $p < 0.05$ were considered to be statistically different.

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