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Effects of oxygen-vectors on the synthesis of epsilon-poly-lysine and the metabolic characterization of *Streptomyces albulus* PD-1

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ABSTRACT

The production of epsilon-poly-lysine (ε-PL) was enhanced by adding oxygen-vectors to the culture broth of *Streptomyces albulus* PD-1. ε-PL concentration reached a maximum of 30.8 ± 0.46 g/L and the dry cell weight was 33.8 ± 0.29 g/L when 0.5% *n*-dodecane was added to fed-batch fermentation. This improvement in ε-PL production and cell mass production can be related to 0.5% *n*-dodecane that could maintain dissolved oxygen concentration >32% saturation throughout the course of the ε-PL production phase. The transcription level of key genes and enzyme activities were analyzed. Results showed that the metabolic pathway responsible for ε-PL synthesis was strengthened when *n*-dodecane was added. The change in intracellular nucleotide levels was also investigated. Higher ATP level and a lower NADH/NAD^+ ratio were obtained with 0.5% *n*-dodecane in broth, thereby providing more energy for ε-PL synthesis. These results indicated that ε-PL synthesis was strengthened from carbon metabolism and energy metabolism with 0.5% *n*-dodecane in broth.

1. Introduction

Epsilon-poly-lysine (ε-PL) is a homo-poly-amino acid discovered by Shima and Sakai when they screened Dragendorff-positive substances [1]. With L-lysine residues linked by bonds between α-carboxyl and ε-amino groups, ε-PL is used in many fields, such as food, medicine, cosmetics, and electronics, because this substance exhibits good properties, including antimicrobial activity, biodegradability, water solubility, edibility, and non-toxicity to humans and environment [2–4]. For instance, ε-PL is used as a food preservative in Japan, USA, South Korea, and other countries. Despite this broad application, ε-PL can only be produced microbiologically.

Streptomyces albulus is a common ε-PL-producing strain. Several fermentation conditions, including pH control, carbon source selection, airlift bioreactor application, and cell immobilization, have been optimized to produce ε-PL [5–8]. Those studies have mainly focused on optimizing the parameters of fermentation; however, metabolic changes in ε-PL-producing strain have been rarely reported. Fermentation kinetics performed under different aeration and agitation conditions has shown that dissolved oxygen (DO) level is a significant parameter in ε-PL production [9]. However, DO level in broth is difficult to improve because of high oxygen consumption and mycelial biomass. This problem can be partially solved by increasing agitation speeds and aeration, but shear stress can cause undesired effects on mycelial morphology, product formation, and product yields. What is more, the increasing aeration also leads to high power consumption [8]. Thus, an effective approach should be developed to improve DO level in broth.

Oxygen-vectors are auxiliary liquids immiscible in aqueous phase; these liquids can solve the limitations of oxygen transfer in fermentation. Volumetric oxygen transfer coefficient is significantly increased by the addition of a non-aqueous liquid phase in culture broth. There are several possible mechanisms of oxygen-vectors action on oxygen transfer in broth. The most plausible is that assuming oxygen-vectors adsorption to the air bubbles surface, with or without the formation of a continuous film, the oxygen diffusion from air to microorganisms occurs through an oxygen-vector and then through an aqueous phase [10]. What is more, the effects of oxygen-vectors on oxygen transfer also depend on broths and biomass characteristics, specific power input, and superficial air velocity [11,12]. High oxygen solubility, non-toxicity to microbes, antifoaming action, and low energy consumption make hydrocarbons, perfluorocarbons, and various oils the most sought after

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oxygen-vectors used in aerobic fermentation. For example, the production of L-asparaginase activity and biomass increased when oxygen-vectors, such as liquid paraffin, *n*-dodecane, and silicone oil, were added to broth [13]. Oleic acid and *n*-hexane can significantly enhance L-sorbose production of immobilized cells in flask cultures [14]. The production of poly(γ-glutamic acid), another homo-poly-amino acid, has also been improved with the presence of oxygen-vectors in broth [15]. However, no research on oxygen-vectors in ε-PL fermentation has been conducted.

S. albulus PD-1 is a high ε-PL-producing strain screened from soils. In our previous study, the genome sequence of this strain was presented [16], in which much information may be used to investigate several metabolic pathways implicated in ε-PL synthesis, such as gene transcriptional expressions, enzyme activities, and intracellular nucleotide levels. In this study, a high level of ε-PL production by *S. albulus* PD-1 was obtained with 0.5% *n*-dodecane added to broth. Gene transcriptional expressions, key enzyme activities, and intracellular nucleotide levels were investigated to elucidate the effect of oxygen-vectors on ε-PL synthesis.

2. Method

2.1. Microorganism and culture media

S. albulus PD-1 was isolated from soils and deposited in China Center for Type Culture Collection (Accession No. M2011043). Agar slant medium contained glucose (10 g/L), yeast extract (5 g/L), beef extract (5 g/L), $MgSO_4{\cdot}7H_2O$ (0.5 g/L), K_2HPO_4 (1 g/L), and agar (20 g/L). The pH of the medium was adjusted to 7.0 by using 2 M NaOH; the medium was then sterilized in an autoclave at 121 °C for 30 min. Medium 3G (M3G) was used as a fermentation medium [5,6]. M3G consisted of glucose (50 g/L), $(NH_4)_2SO_4$ (10 g/L), yeast extract (5 g/L), $FeSO_4{\cdot}7H_2O$ (0.03 g/L), $MgSO_4{\cdot}7H_2O$ (0.5 g/L), $ZnSO_4{\cdot}7H_2O$ (0.04 g/L), K_2HPO_4 (0.8 g/L), and KH_2PO_4 (1.36 g/L). The initial pH of M3G was adjusted to 6.8 with ammonia. The medium was sterilized in an autoclave for 15 min at 121 °C, and the glucose was separately autoclaved for 10 min at 118 °C.

2.2. Cultivation conditions

A loop of 1-week-old fully grown spore was inoculated into 100 mL of M3G medium in a 500 mL Erlenmeyer flask and then incubated at 30 °C with 200 rpm rotary shaking for 24 h. For batch fermentations, 300 mL of pre-cultured seed was inoculated in 2.7 L of sterilized M3G in a 5 L bioreactor (diameter = 17.0 cm, height = 23.4 cm; KoBio Tech Co., Ltd., Korea). Agitation was operated at 300 rpm by using a standard six-blade impeller (diameter = 6.7 cm) and aeration was provided by a ring sparger at 3 vvm [17]. As fermentation proceeded for approximately 12 h, the pH of the broth declined sharply and 30% ammonia was added to maintain the pH level at 4.0. Fed-batch fermentations were conducted with a two-stage pH control method as described in a previous paper [5]. During the first stage and the second stage, pH was maintained at 6.0 to favor cell growth and at 4.0 to stimulate ε-PL production. After the residual glucose concentration of the broth was decreased to approximately 10 g/L, the feeding solution containing glucose (500 g/L) and ammonium sulfate (50 g/L) was pumped into the broth to maintain a residual glucose concentration at approximately 10 g/L.

Compared with the control group, sterilized oxygen-vectors (oleic acid, Tween 80, *n*-hexane, *n*-dodecane) at concentrations of 0.2, 0.5, 1, and 3% (v/v) were added to the medium at the beginning of fermentation.

2.3. Analytical methods

2.3.1. Determination of biomass growth, residual glucose concentration, pH, and DO concentration

Biomass growth was detected by subjecting the broth to centrifugation, collecting the precipitate, washing with distilled water twice, drying at 65 °C until constant weight was achieved, and measuring dry cell weight. Residual glucose concentration was determined using a biosensor (SBA-40C, Shandong Science Academy, China). pH was measured using a pH probe of the bioreactor. An oxygen electrode (Oxyferm 225, Hamilton, Switzerland) with rapid response time (t98% <60 s) was used to monitor the DO level of the culture broth. DO concentrations under different conditions were expressed in terms of DO saturation level (%), where 100% corresponds to actual DO saturation from air supplied at 30 °C and 1 atm. Each experiment was repeated thrice, and experimental errors were <5%.

2.3.2. Determination of ε-PL concentration in broth

ε-PL concentration was determined in accordance with a previously described method [18]. Approximately 0.5 mL of the sample was added to 2 mL of MeO solutions (1 mM MeO and 50 mM sodium phosphate, pH 7.0) and then vortexed. After the sample was allowed to stand for 30 min, mixtures were centrifuged at 1700 × *g*; the supernatants were diluted 10-fold and the resulting dilutions were evaluated at 470 nm.

2.3.3. qRT-PCR analysis

As fed-batch fermentation reached 110 h, the maximum specific ε-PL production rate was reached. Bacterial RNA was then extracted from *S. albulus* PD-1 cultures by using an RNeasy Mini Kit (TaKaRa Biotechnology Company, Dalian, China). Reverse transcription reactions were performed using a PrimeScript RT Reagent Kit (TaKaRa Biotechnology Company, Dalian, China). qRT-PCR was carried out by mixing SYBR Premix Ex*Taq* (10 μL), forward primer (5 pmol), reverse primer (5 pmol), ROX (0.4 μL), and cDNA (0.4 μL); a final volume of 20 μL was prepared by adding ddH_2O. The mixture was amplified using a StepOne Plus Real-Time PCR system (Applied Biosystems, USA) under the following parameters: 10 min at 94 °C, followed by 35–45 two-temperature cycles (15 s at 94 °C and 1 min at 60 °C) [19]. All of these reactions were repeated thrice and qRT-PCR results were subjected to $2^{-\Delta\Delta Ct}$ method for relative quantification with *hrdB* as endogenous control gene [20]. Primers were designed according to the sequences from the *S. albulus* PD-1 genome. Table 1 lists all of the primers mentioned in this study.

2.3.4. Enzyme assay

As fed-batch fermentation reached 110 h, the mycelia from 10 mL of broth were harvested by centrifugation, washed twice with 0.85% (w/v) saline, and suspended in 1 mL of buffer. The suspended mycelia were disrupted by sonication. Cell debris was removed by centrifugation at 16,000 × *g* for 20 min, and the supernatant was collected as a crude extract for enzymatic activity assays [21].

The enzyme activities of glucose-6-phosphate dehydrogenase (G6PD: EC: 1.1.1.49), phosphoenolpyruvate carboxylase (PPC: EC: 4.3.1.1), aspartate kinase (ASK: EC: 2.7.2.4), isocitrate dehydrogenase (ICDH: EC: 1.1.1.42), malate dehydrogenase (MDH: EC: 1.1.1.37), and ε-PL synthetase (Pls) were determined. The G6PD activity in crude cell extracts was evaluated spectrophotometrically by determining the increase in NADPH. To quantify G6PD activity, we used a reaction mixture containing $MgCl_2$ (10 mM), KCl (200 mM), Tris/HCl (100 mM, pH 7.5), 6-G-P (4 mM), $NADP^+$ (2 mM), and 50 μL of cell extract with a total volume of 1 mL [22]. PPC activity was determined spectrophotometrically by monitoring NADH oxidation in a coupled assay with MDH, as described by