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Optimization of cell growth and poly(ε-lysine) production in batch and fed-batch cultures by *Streptomyces albulus* IFO 14147

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Abstract

Streptomyces albulus IFO 14147 was cultivated in a stirred tank reactor; the pH and nutrient effects were evaluated in batch and fed-batch fermentation, particularly the control of pH and glucose feeding for enhancing the cell growth and ε-PL production were demonstrated. It was concluded that high cell growth was obtained and the cells remained stable at pH above 4.5 in the broth, while high ε-PL accumulation was achieved at lower pH; the high rate of cell growth at high pH was accompanied by a high rate of glucose consumption. To enhance the ε-PL production, a two-stage pH control and glucose feeding strategy was employed. At first sage the pH was maintained at its initial 6.8 for 48 h for achieving high cell density; the second stage aimed at increasing the ε-PL accumulation by maintaining pH at 4.0. In addition, pulsed feeding of glucose was carried out when it was depleted from the culture medium. By using this control and feeding strategy, the increase of ε-PL accumulation in 10-days cultivation is 258%. We have demonstrated that pH control and substrate feeding strategy is a feasible method for enhancing ε-PL production by *S. albulus* IFO 14147.

Keywords: Poly-ε-lysine; *Streptomyces albulus* IFO 14147; Fed-batch fermentation; pH control; Batch culture; Stirred tank reactor

1. Introduction

Poly-ε-lysine (ε-PL) is an unusual cationic, naturally occurring homo-polyamide made of L-lysine connected between ε-amino and α-carboxyl groups. It was accidentally discovered as an extra-cellular material produced by filamentous bacterium *Streptomyces albulus* strain 346 as a result of a screening for a Dragendorff's positive substances [1–3]. ε-PL is biodegradable, edible and nontoxic toward humans and the environment. Therefore, potential applications have been shown in the areas such as food, medicine, pesticides, electronic material design [4–6]. ε-PL molecules are cationic, surface active agents due to their positively charged amino groups in water, hence they were shown to have a wide antimicrobial activities against yeast, fungi, gram-positive and gram-negative bacterial species [7,8]. Because of its excellent antimicrobial activity, heat stability and lack of toxicity [7–10], ε-PL has attracted a great deal of attention as a natural food preservative. It is approved for food use in Japan as an antimicrobial preservative [8,9], and has been used in multiple foods, including several staples of the oriental diet. It is used as a preservative on sliced fish, fish sushi, Nimono (Japanese daily dishes), boiled rice, soups, noodles, cooked vegetables, sukiyaki (Japanese beef steak), potato salad, steamed cake and custard cream.

It was shown that the wild strain of *S. albulus* grown in a medium containing glycerol, ammonium sulfate and yeast extract yielded only low amount of ε-PL (0.3 g/L) after cultivation for 48 h at 30 °C and pH 6.8 [1]. In order to have a plentiful supply of ε-PL for increasing usage, investigations have been carried out to optimize its production. Shima et al. [11] has improved the production of ε-PL to a large amount (4–5 g/L) in 8–9 days by implementing a two-step cultivation method in that *S. albulus* was first grown in a mineral medium containing glycerol and yeast extract for 1 day at 30 °C, then the cells were collected by filtration and inoculated into a mineral medium containing glucose, citric acid and ammonium sulfate. Mutation by nitrosoguanidine treatment was also employed to enhance the ε-PL productivity of the wild strain 346. As a result, a *S*-(2-aminoethyl)-L-cysteine (AEC) and glycine-resistant mutant was isolated, which produced four times higher amounts of ε-PL than the wild strain did when it was

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grown in M3G medium at 30 °C for 96 h in reciprocal flask culture [12]. In the same study, it was also found that the accumulation of ε-PL in the broth increased significantly when pH was lower than 4.2, while the cell growth decreased simultaneously suggesting the importance of pH control in ε-PL production. Accordingly, a pH control strategy has successfully enhanced the production of ε-PL by *S. albulus* 410 cultivated in M3G medium in 5-L jar fermentor [13]. In addition, ε-PL production has also been evaluated using an airlift bioreactor (ABR) instead of a stirred tank reactor (STR) [14].

Recently, we have applied response surface methodology (RSM) to optimize ε-PL production in a shake flask culture by *S. albulus* IFO 14147 [15]. The yield of ε-PL by *S. albulus* IFO 14147 was increased significantly by 984% when the strain was cultivated in the optimal medium developed by RSM as compared to conventional medium M3G used in the literature. In this study, the possibility to enhance cell growth and ε-PL production of *S. albulus* IFO 14147 was evaluated in a STR; the pH and nutrient effects were studied in batch and fed-batch fermentation, particularly the control of pH and glucose feeding for enhanced production of ε-PL were demonstrated.

2. Materials and methods

2.1. Chemicals and microorganism

Reagents for cultivation such as yeast extract, malt extract, agar, peptone were purchased from DIFCO Laboratories Michigan, USA. D-Glucose, K_2HPO_4, KH_2PO_4, $FeSO_4{\cdot}7\ H_2O$, $MgSO_4{\cdot}7\ H_2O$, $ZnSO_4{\cdot}7H_2O$, $(NH_4)_2SO_4$, and NaCl were obtained from Sigma Chemical, USA. All other reagents used were of the highest grade available unless otherwise indicated. *S. albulus* IFO 14147 used in this study was obtained from the Culture Collection and Research Center (CCRC), Taiwan.

2.2. Culture medium and inoculum preparation

Medium 3G (M3G), composed of yeast extract 5 g/L, glucose 50 g/L, $(NH_4)_2SO_4$ 10 g/L, $FeSO_4{\cdot}7H_2O$ 0.03 g/L, $MgSO_4{\cdot}7H_2O$ 0.5g/L, $ZnSO_4{\cdot}7H_2O$ 0.04g/L, K_2HPO_4 0.8 g/L, KH_2PO_4 1.36 g/L, was a medium of choice for researchers previously investigating production of ε-PL by *S. albulus* species [12,13]. RSM medium, composed of yeast extract 2.8 g/L, glucose 28 g/L, $(NH_4)_2SO_4$ 1.3 g/L, $FeSO_4{\cdot}7H_2O$ 0.03 g/L, $MgSO_4{\cdot}7H_2O$ 0.5 g/L, $ZnSO_4{\cdot}7H_2O$ $7H_2O$ 0.04 g/L, K_2HPO_4 0.8 g/L, KH_2PO_4 1.36 g/L, was an optimal medium developed by RSM in shake flask culture for enhanced production of ε-PL by *S. albulus* IFO 14147 [15]. For initial experiments on the cell growth and ε-PL production, *S. albulus* IFO 14147 was cultivated in M3G. *S. albulus* IFO 14147, obtained as a lyophilized powder in a glass ampoule sealed under vacuum, was first cultured on ISP2 solid culture which contains agar (15 g/L), yeast extract (4 g/L), malt extract (10 g/L) and glucose (4 g/L). After 24 h incubation (30 °C, pH 6.8), colonies that appeared on the plates were picked up (1-cm^2) and inoculated into 300 mL of liquid broth (LB) composed of peptone (10 g/L), yeast extract (5 g/L), NaCl (5 g/L), and incubated at 30 °C for 2 days with shaking at 160 rpm.

2.3. Cell growth and ε-PL production in batch fermentation

Fermentation were carried out in a fully instrumented and computer controlled 10-L stirred tank bioreactor (Major Science, Taipei, Taiwan), equipped with a pH probe (Type InPro 3030; Mettler Toledo) and a dissolved oxygen probe. Two hundred milliliters pre-cultured seed was inoculated into 5 L of M3G or RSM medium, and then cultured for 96–264 h. Agitation was provided by a standard six-blade impeller operated at 300 rpm. Aeration was provided by a ring sparger and aeration rate was maintained at 3 vvm. The pH change during cultivation was monitored by a pH probe attached to a PID controller (Type: CS-787. Chen-Shen Inc., Taipei, Taiwan). To control pH at a set level, 2N NaOH and 2N HCl was automatically added to the culture broth. Generally, foaming appeared in the culture medium after 1 day of cultivation, a situation leading to unstable culture conditions that can be avoided by addition of anti-foaming agent KM-70 (ShinEtsu Chemical Co. Ltd., Tokyo). The fermentation temperature was maintained using a re-circulating water bath at 30 °C, and the initial pH was at 6.8. During the fermentation, the pH and DO was monitored on line, and cell growth, residual glucose and $(NH_4)_2SO_4$ was analyzed off-line by the method described below.

Fed-batch culture was started when the glucose concentration in the culture medium was depleted. The feeding solution, composed of glucose (500 g/L), $(NH_4)_2SO_4$ (100 g/L), for fed-batch cultures was pumped into the fermenter using a peristaltic pump operated at 32 mL/min. The feeding was repeated to give the final glucose level at nearly 10 g/L. All experiments were carried out at least in duplicate, and the results were averaged.

2.4. Analytical methods

The production of the ε-PL was monitored by high performance liquid chromatography (HPLC) following the Kahar's method previously reported [13]. The HPLC system for analysis of ε-PL concentration was composed of a Hitachi L-6200 solvent delivery controller, a Hitachi 4250 UV–vis detector, a Hitachi-D-2500 Chromato-Integrator, and a TSK gel ODS-120T column (Tosho Co. Ltd., Tokyo, 4.6 mm × 250 mm, 10 μm). The injection volume was 20 μL. The sample was eluted with a mobile phase comprising 0.1% H_3PO_4 at a flow rate of 0.4 mL/min. The chromatogram was monitored at 215 nm. ε-PL standard obtained from Chisso Co., Japan was used to construct a calibration curve from which ε-PL concentration in fermentation broth was determined.

Cell growth was monitored by optical density (OD) measurement at 660 nm. Dry cell weight was determined by diluting and filtering 10 mL of culture broth through a 0.45 μm cellulose nitrate membrane (Whatman, Hillsboro, OR), followed by washing and drying at 90 °C to constant weight. Residual glucose was determined by the phenol-sulfuric acid method [16]. Ammonium sulfate was analyzed by a colorimetric method using Nessler reagent [17].

2.5. Purification and characterization of ε-PL

At the end of the cultivation, the whole broth was centrifuged at 10,000 × *g* for 30 min to remove the cells, the supernatant was adjusted to pH 8.5 with 0.1N NaOH and then filtered, the filtrate was applied on an Amberlite IRC-50 column and washed with 0.2N acetic acid and water, successively. After eluated with 0.1N hydrochloric acid, the eluate was neutralized with 0.1N sodium hydroxide to pH 6.5, decolorized with activated charcoal, and evaporated under vacuum to a small volume. Finally the pure substance (hydrochloride) was obtained as white powder by repeated precipitation; first with four volumes of MeOH, then by addition four volumes of an ethanol/diethyl-ether (2:1) mixture to the solution of the first precipitate in 20 mL de-ionized water. The substance was further purified to homogeneity as a single fraction by column chromatography on CM-cellulose and gel filtration on Sephadex column under a constant flow rate and monitoring the UV absorption at 220 nm. The purified material was tested on Dragendorff's, ninhydrin and Cl_2–KI–starch regents (1), further characterized by amino acid analysis. The molecular weight of ε-PL was estimated by gel filtration on a Sephadex column using bradykinin (MW 1060), cytochrome *c* (MW 12,300), myoglobin (MW 17,800), α-chymotrypsinogen A (MW 25,000) as molecular weight marker; the column was developed with 1 M sodium chloride at a flow rate of 0.2 mL/min.

3. Results and discussion

3.1. Batch fermentaion of S. albulus IFO 14147 in M3G or RSM medium

We previously showed that *S. albulus* IFO 14147 produced 0.75 g/L of ε-PL in the culture broth when it was cultivated in