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ε-Poly-L-lysine production by immobilized cells of *Kitasatospora* sp. MY 5-36 in repeated fed-batch cultures

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ABSTRACT

The production of ε-PL by *Kitasatospora* sp. MY 5-36 through entrapment or adsorption on bagasse, synthetic sponge, macroporous silica gel, and loofah sponge was investigated in shake flask cultures, and immobilization on loofah sponge gave the highest ε-PL production. Repeated fed-batch cultures for ε-PL production were also carried out in a stirred bioreactor and final ε-PL concentrations and productivity of 34.11 g L^{-1} and 9.34 g L^{-1} d^{-1}, respectively were achieved by cells immobilize in loofah sponge. These values exceeded those for cultures with free cells (22.53 g L^{-1} and 3.30 g L^{-1} d^{-1}). The immobilized cells were reused five times over a period of 526 h. These results suggest that the immobilization approach is promising for industrial applications.

1. Introduction

Poly-ε-lysine (ε-PL) is a homo-poly-amino acid characterized by a peptide bond between the ε-amino and α-carboxyl groups of L-lysine. It is non-toxic (Hiraki et al., 2003), biodegradable, water soluble, and can be synthesized independent of petroleum-based resources. In addition, this homo-poly-amino acid has antimicrobial activities (Shima et al., 1984; Hiraki, 2000), removes endotoxin selectively (Hirayama et al., 1999), and has anti-obesity properties by inhibiting intestinal absorption of dietary fat (Tsujita et al., 2006). It is also used as a food preservative in many countries, including Japan, Korea, and the United States (Yoshida and Nagasawa, 2003; Hamano et al., 2005).

ε-PL is manufactured commercially through fermentation with a *Streptomyces albulus* mutant, but the production of ε-PL is unstable and dependent on cell density in some producer strains (Hirohara et al., 2006), which can pose problems such as high viscosity and low oxygen transfer efficiency. Although these problems can be solved by increased agitation speeds, the arising shear stresses can cause undesired effects on mycelial morphology, product formation, and product yields (Kahar et al., 2002). In addition, ε-PL fermentations last about 7–10 days (Kahar et al., 2001, 2002; Shima et al., 1983; Hirohara et al., 2006; Shih and Shen, 2006) and is therefore very energy consuming. Bioprocesses using immobilized cells in inert supports can increase overall productivity and minimize production costs (Pinheiro and Facciotti, 2008), and immobilization of actinomycetes, mycetes, and bacteria has been done in bubble column and airlift bioreactors in batch, repeated-batch, and continuous modes to produce antibiotics and antimicrobial substances like cephamycin C (Devi and Sridhar, 2000; Cruz et al., 2004), chlortetracycline (Dalili and Chau, 1988), neomycin (Adinarayana et al., 2004), daunorubycin (Takashima et al., 1987), clavulanic acid (Lavarda et al., 2006), neomycin (Srinivasulu et al., 2002), gibberellic acid (Meleigy and Khalaf, 2009), and 3-phenyllactic acid (Mu et al., 2009).

ε-PL production has been achieved in batch and fed-batch systems (Shima et al., 1983; Kahar et al., 2001, 2002; Hirohara et al., 2006; Shih and Shen, 2006), and it was produced by Hiraki and Suzuki (1999) with cells immobilized in gel, embedded in porous ceramic and other supports, but without a significant increase in production.

In this study, we immobilized a ε-PL-producing mutant strain *Kitasatospora* sp. MY 5-36 which previously described (Ouyang et al., 2006) on various support materials, and after cultivation in flasks, we selected the most promising material, loofah sponge, derived from bagasse, synthetic sponge, macroporous silica gel, and loofah sponge for cultivation in a 5-L bioreactor. The results were compared with those obtained with free cells. To our knowledge, this is the first report on the immobilization of ε-PL-producing cells on loofah sponge.

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2. Methods

2.1. Microorganism

The ε-PL-producing mutant strain *Kitasatospora* sp. MY 5-36 was isolated from the soil and maintained in our laboratory.

2.2. Culture medium and inoculum preparation

Agar slant medium (AS), containing 10 g L^{-1} glucose, 5 g L^{-1} yeast extract, 5 g L^{-1} beef extract, 0.5 g L^{-1} $MgSO_4 \cdot 7H_2O$, 1 g L^{-1} K_2HPO_4, and 20 g L^{-1} agar, along with pH 7.0. The medium 3G (M3G), composed of 5 g L^{-1} yeast extract, 50 g L^{-1} glucose, 10 g L^{-1} $(NH_4)_2SO_4$, 0.03 g L^{-1} $FeSO_4 \cdot 7H_2O$, 0.5 g L^{-1} $MgSO_4 \cdot 7H_2O$, 0.04 g L^{-1} $ZnSO_4 \cdot 7H_2O$, 0.8 g L^{-1} K_2HPO_4, 1.36 g L^{-1} KH_2PO_4, was the one chosen by researchers who previously investigated the production of ε-PL by *S. albulus* species and *Kitasatospora* sp. (Kahar et al., 2001; Hiraki et al., 1998; Ouyang et al., 2006). For experiments on the cell growth and ε-PL production, *Kitasatospora* sp. MY 5-36 was cultivated in M3G. In every case, the glucose was separately autoclaved. The culture was grown and maintained on AS. One-week old fully grown slants were used for inoculum preparation on a one-tube per flask (500 mL), every flask containing 100 mL of M3G, and incubated at 28 °C for 24 h with 200 rpm rotary shaking.

2.3. Selection of solid supports used as immobilized carrier

Four solid supports, including bagasse, synthetic sponge, macroporous silica gel, and loofah sponge, were collected from the local market. The loofah sponge, used as an immobilization matrix, was obtained from the ripened dried fruit of *Luffa cylindrical* and cut into pieces with approximately 2.5 cm diameter and 2–4 mm thickness. The bagasse, synthetic sponge, and macroporous silica gel supports were cut into 1-cm cubic pieces, boiled in water for 30 min, thoroughly washed under tap water, and left for 12 h in distilled water. After soaking, the solid supports were oven-dried at 80 °C to constant weight and stored in a desiccator (EI-Naggar et al., 2003).

Two milliliters of pre-cultured seed was inoculated into 250 mL flask containing 50 mL of the autoclaved M3G with four immobilized carriers (about 1 g of each immobilized carrier). A flask without immobilized carrier in the medium was also provided for the free biomass controls. The inoculated flasks were shaken at 200 rpm at 28 °C. After 4 days, both free and immobilized biomass of *Kitasatospora* sp. MY 5-36 were harvested from the medium and washed with distilled water. The dry weight of the biomass was determined by weighing the oven-dried (80 °C overnight) immobilized carrier before and after the bacterial growth.

2.4. Actinomycete biomass immobilized loofah sponge bioreactor (ABILS) construction

The design details and operating conditions of the ABILS used in this study were as following: internal diameter, 8 cm; height, 56 cm; working volume, 5 L; airflow rate 3.5 vvm, and temperature, 28 °C. The reactor containing 3 L of culture medium was inoculated with 300 mL inoculum. A piece of loofah sponge, with 20 × 18 × 0.2 cm dimensions, and affixed to the baffles and probes of the bioreactor. Fermentation was carried out in a fully instrumented and computer-controlled bioreactor, equipped with a pH, antifoam and a temperature probe.

2.5. Bioreactor cultures

Three hundred milliliters of pre-cultured seeds was inoculated into 2.7 L of M3G with an initial pH of 6.8. The agitation was done by a standard six-blade impeller operated at 350 rpm, while the aeration was provided by a ring sparger with a rate maintained at 3.5 vvm. To control the pH at a set level, 10% (v/v) NH_4OH solution was automatically added to the culture broth. Generally, foaming appeared in the culture medium after 20 h of cultivation. The anti-foaming agent KM-70 was automatically added to the culture broth by antifoam probe online.

The fermentation temperature was maintained using a re-circulating water bath at 28 °C. During fermentation, the pH was monitored online, while the cell growth, residual glucose, and $(NH_4)_2SO_4$ were analyzed offline as described in Section 2.7.

2.6. Performance of fed-batch and repeated fed-batch operations

The fed-batch cultures both in the free cell fermentation system and the ABILS were started separately when the glucose concentration in the culture medium was exhausted (below 10 g L^{-1}). The feeding solution, composed of glucose (500 g L^{-1}) and $(NH_4)_2SO_4$ (100 g L^{-1}), for fed-batch cultures was pumped into the bioreactor using a peristaltic pump. This feeding was repeated to set the glucose level at nearly 10 g L^{-1}. For the repeated fed-batch experiments, the mixed culture from the first batch was used as the inoculums for the second batch. When the residual glucose of the culture was exhausted (below 5 g L^{-1}), 90% of the culture was withdrawn and the medium was replenished with the same amount of fresh, autoclaved culture medium.

2.7. Analytical methods

The production of ε-PL was monitored by means of high-performance liquid chromatography (HPLC), following the previously reported Kahar et al. (2001) method. A ε-PL standard, obtained from Chisso Co. of Japan, was used to construct a calibration curve. Cell growth was monitored by optical density (OD) measurement at 660 nm, and biomass was determined as dry cell weight. The harvested culture sample was filtered; the mycelia were washed and dried at 95 °C until a constant weight. Residual glucose concentration was analyzed by the 3,5-dinitro salicylic acid (DNS) method (Miller, 1959). Ammonium sulfate was analyzed by means of a colorimetric method using the Nessler reagent (AOAC International, 1995). All experiments were carried out at least in duplicate, and the results were averaged.

3. Results and discussion

3.1. Utilization of different immobilized materials for ε-PL fermentation

The suitable substrate for immobilized carrier for ε-PL fermentation in the shake flask was evaluated to improve the production of the process. As shown in Table 1, with the exception of bagasse, the immobilized carriers showed a higher ε-PL production than in the control. It was noticed that the total cell weight of loofah sponge immobilized carrier culture was higher as compared to the rest of the cultures, reaching 12.5 g L^{-1} and 1.27-fold of that obtained in control.

Recently, the viability of using loofa sponge as a carrier for microbial cells was studied successfully (Vignoli et al., 2006; Parag et al., 2008; Samir and Mahmoud, 2009). Especially, use the loofa sponge for the immobilized carrier to immobilized actinomycetes has significant effect. The loofah sponge is made up of interconnecting voids with an open network of fibrous support giving the