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The enhancement of rapamycin production using *Streptomyces hygroscopicus* through a simple pH-shifted control

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ABSTRACT

Rapamycin, a ring macrolide compound, is recognized as a potent immunosuppressive drug which works by inhibiting T-cell activation and proliferation and is also characterized as having cancer-fighting and anti-aging functions. The pellet morphology of *Streptomyces hygroscopius* was suggested to have the high rapamycin production titer. The change of culturing pH could alter the morphology of actinomycetes. Therefore, the influence of pH level on rapamycin production was examined. No significant effects of initial pH on rapamycin production were observed in the pH range of 6–8 in the flask trials. Nevertheless, a two-stage pH control strategy was proposed in a bench-scale fermenter, with no pH control in the 1st stage and with the pH controlled at 5.5 in the 2nd stage after the pH rebounded from the bottom. A recorded high rapamycin level of 537 ± 23 mg/L was obtained in a simple batch operation, far higher than the 45 ± 5 and 98 ± 5 mg/L levels in the batches with the pH maintained at a fixed level of 7.0 and without the pH controlled, respectively. The strategy of a two-stage pH control was simple to operate and potentially applied in the scaled-up production of rapamycin.

1. Introduction

Rapamycin, a ring macrolide compound, was originally discovered as an antifungal antibiotic, produced by *Streptomyces hygroscopicus*, which is first isolated from the soils of Easter Island [1]. It also displays potent immunosuppressive activity by inhibiting T-cell activation and proliferation. It has been approved in the United States for use in combination with cyclosporine and corticosteroids for various clinical treatments [2,3]. More recently it has been suggested that rapamycin may even assist in the treatment of heart attacks, and act as a tumor-suppressing drug [2]. Rapamycin has been reported to be capable of slowing aging in yeast and invertebrate species, and it has also recently been found to increase the life span of rodents [4]. In addition, rapamycin has demonstrated impressive effects in rodents modeling age-associated human diseases.

The current commercial production of rapamycin is achieved as a secondary metabolite from the fermentation of *S. hygroscopicus* cells [5]. So far, the study of the rapamycin biosynthesis pathway in *S. hygroscopicus* indicates that adding precursors (acetate, propionate, shikimate, L-methionine and L-lysine), carbon sources and nitrogen sources could have significant effects on rapamycin production by *S. hygroscopius* [6,7]. In the fermentation of rapamycin produced by *S. hygroscopicus*, chemically defined media was adopted for the greatest portion of the cultivation. The best combination of two carbon sources for rapamycin production was 2% fructose and 0.5% mannose [8]. Of the nitrogen sources studied, 40 mM ammonium sulfate was best suited to the formation of rapamycin, and supported cell growth comparable to the organic nitrogen sources used in the chemically defined control medium [9].

Besides the effects of nutrients, the culture pH value was a well-known factor which might affect the growth of microorganisms and the following production of metabolites. Sometimes, the optimum pH value for the cells growth and the production of metabolites might not be at the same level. Therefore, a two-stage pH control was often adopted in the fermentation process to simultaneously achieve the highest biomass and the highest metabolite concentration [10,11]. A novel two-stage pH control strategy was adopted in the batch fermentations by *Streptomyces* sp. M-Z18 for ε-poly-L-lysine production. Based on the analysis of the time course of specific cell growth rate and specific ε-poly-L-lysine formation rate, a two-stage pH control strategy of the culture pH changed from 3.5 to 3.8 after 36 h of cultivation was performed, which led to about 16.6% increase of final ε-poly-L-lysine concentration [12].

Even though the medicinal function of rapamycin has attracted much attention, the examination of rapamycin production has not appeared much in the related literature [13,14]. This study aimed

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to investigate the effects of initial pH level on rapamycin production. Ultimately, a two-stage pH control strategy for enhancing the production of rapamycin was proposed.

2. Methods and materials

2.1. Strain and medium preparation

The original strain used in the experiment, *S. hygroscopicus*, was purchased from the Bioresource Collection and Research Center, Taiwan, under catalog number BCRC 16270. The strain had been mutated by UV induction method [14] to select a high rapamycin production strain for the trials of this study. A spore suspension was used for the inoculation of *S. hygroscopius* cultivation. The spore suspension aliquot was prepared with agar plates containing the sporulation medium of 4 g/L dextrose, 20 g/L agar, 4 g/L yeast extract, and 10 g/L malt extract. These were plated out with the prepared spore solution and incubated at 28 °C for about 14–21 days. After black spores were observed spreading on the surface of the agar plate, 1 ml of sterilized water was added to the agar plates to harvest the spore suspension, and then preserved as frozen stock (stored in 25% glycerine at −20 °C). The count of the prepared spore suspension was about 10^8 spores/ml as measured in the colony forming unit.

2.2. Cultivation methods

A seed culture was initiated by adding l ml of the thawed cell suspension to a 250-ml Erlenmeyer flask containing 50 ml of seed medium (4 g/L dextrose, 4 g/L yeast extract, 10 g/L malt extract) at an initial pH of 7.3 for 48 h. A 5 ml aliquot of the seed culture was transferred into a 50 ml fermentation medium containing 25 g/L glucose, 4 g/L yeast extract, and 10 g/L malt extract in a 250 ml flask. This was incubated at 28 °C and shaken at 150 rpm for 7 days.

2.3. Lab top fermenter operation

The preparation of seed cultivation and the medium components were the same as described in the previous section, except for the addition of 0.05% antifoam agent in the fermentation medium; this avoided foaming occurring under severe agitation. About 200 ml of seed medium was prepared and inoculated into a 5 L fermenter (model BTF-A, Biotop Ltd., Taiwan) containing a 2 L working volume. The pH control strategy would be described in the text. The fermenter was maintained at 28 °C and the aeration of 1 vvm was adopted. The DO level was automatically controlled by over 30% by adjusting the agitation rate automatically. However, the maximum agitation rate was limited to not higher than 400 rpm to avoid damaging the pellets.

2.4. Analytical methods

Glucose concentration was estimated using the glucose analysis instrument assay (YSI 2300 STAT Glucose Analyzer). Dry biomass was estimated by using the IR-35 moisture analyzer (DENVER) assay; 5 ml of whole broth was centrifuged at 7000 rpm for 5 min to harvest the precipitated biomass. Following this, 1 ml of purified water was put into the tube to complete the aqueous mixing of the biomass. The whole aqueous biomass was transferred onto the IR dryer and evaporation of water by infrared radiation was used to estimate the proportion of dry biomass. The measurement of rapamycin titer was performed by the HPLC method. A 1 ml aliquot of fermentation broth was centrifuged at 7000 rpm for 5 min. The supernatant was carefully transferred by pipette into a test-tube and the precipitate pellet was extracted again by shaking it with 1 ml of methanol for 20 min at room temperature. The extractant was subjected to the analysis of HPLC (HITACHI), using a Vercopak C18 column under the following conditions: mobile phase: methanol/water/acetic acid, 80/20/0.1; flow rate: 2 ml/min; detector: UV 254 nm (this method was suggested by LC laboratories). The standard rapamycin was purchased from LC Laboratories, USA (catalog number 53123-88-9, purity > 99%). Results shown in this study were obtained from triplicate analyses for each sample and were expressed as mean ± standard deviations, unless otherwise stated.

3. Results and discussion

3.1. Effects of initial pH in the flask trials

It was known that the morphology of pellet form can enhance antibiotics production in the cultivation of *S. hygroscopius* [15,16]. The pH control is an important environmental parameter, which might affect the physiological pellet formation. Therefore, the initial pH effects on rapamycin production by *S. hygroscopius* were examined. The initial pH levels of 6, 6.5, 7.3 and 8 were performed in the flask trials. The results shown in Figs. 1 and 2 suggested that the initial pH had no significant effects on rapamycin production, except for slightly less biomass observed in the batch with the initial pH of 8. The final pH would drop to a level of about 5.5 for all batches with different initial pH values in the flask trials. The

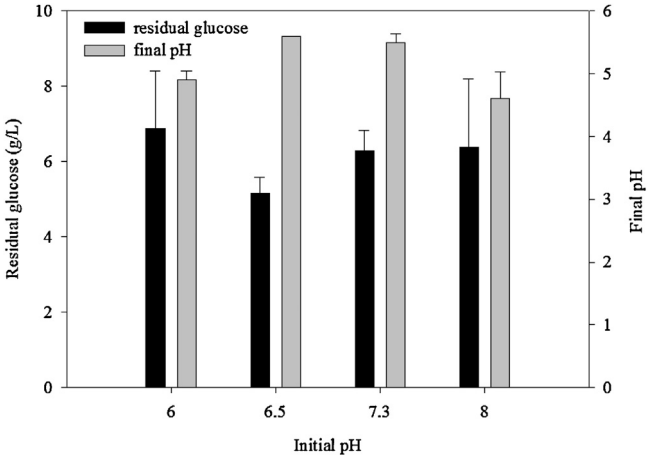

Fig. 1. Effects of initial pH on the residual glucose and the final pH in the flask trials after 7 days cultivation.

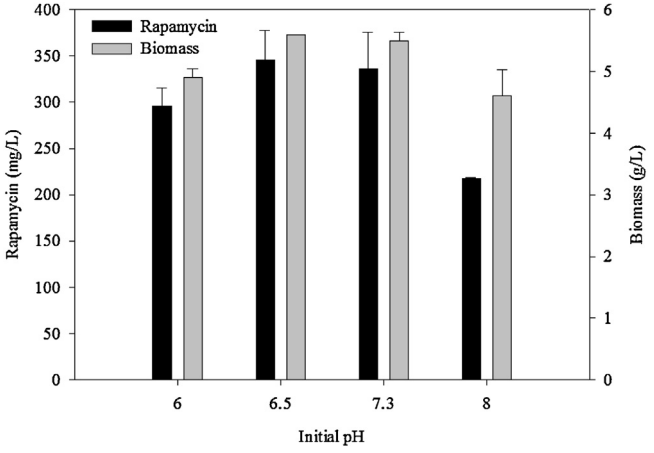

Fig. 2. Effects of initial pH on cells growth and rapamycin production in the flask trials after 7 days cultivation.