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Improved industrial fermentation of lincomycin by phosphorus feeding

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Abstract

Phosphorus limitation was found in the fermentation production of lincomycin based on the phosphorus elemental analysis. Phosphorus was thus fed into the fermentation system to improve the lincomycin production, and 16 kg fed-phosphorus increased the lincomycin yield by 14.4% compared to that without the phosphorus feeding. As low concentration of dissolved oxygen limited the growth of mycelia, the phosphorus in the base medium and fed-batch were adjusted to give a more reasonable phosphorus distribution. When the phosphorus in the base medium was decreased to 29.4 kg from 33.4 kg and the fed phosphorus was increased from 16.0 kg to 20.0 kg in a 100 m^3 fermenter, the final lincomycin titer increased by 21.6% compared to that the un-fed process. The mycelia growth and lincomycin production rates were also increased at the production stage. The phosphorus feeding and adjustment distribution strategy might be applied to other industrial fermentation processes to improve the process efficiency and productivity.

Keywords: Lincomycin; Industrial fermentation; Phosphorus limitation; Feeding; Productivity

1. Introduction

Lincomycin is a lincosamide antimicrobial agent used as a major antibiotic for the treatment of diseases caused by Gram-positive bacteria [1]. Various efforts have been made to improve the fermentation yield of lincomycin [2–6]. Some major metabolic precursors of lincomycin have been isolated and identified [2], and the biosynthesis pathway and genetic control of lincomycin fermentation were also described recently [3]. Semenova et al. has reported that the batch-type feeding of carbohydrates resulted in an increase of the lincomycin yield by 23–24% compared to the un-fed mode [4]. When olive oil was used as the sole carbon source, the lincomycin yield was 2.0-fold higher than that using the starch medium [5]. Young et al. [6] investigated the effect of phosphorus on the cell growth and lincomycin production of *Streptomyces lincolnensis* in chemically defined media. They found that the increase in phosphorus content significantly increased the cell biomass and the supplement of phosphorus at the logarithmic growth stage suppressed the secondary biosyntheses. However, the effects of phosphorous, an essential element for cell growth, on the lincomycin production is less clear.

Phosphorus is involved in both primary and secondary metabolisms of biosyntheses [7]. The addition of phosphorus into the growth medium led to an increase in biomass [6,8]. Phosphorus is associated with the activation or repression of some enzymes such as phosphatase, protein kinase and phosphoprotein phosphatase [7,9,10]. There are only a few antibiotics including streptomycin, gentamycin and cephalosporin, whose secondary metabolic pathways are negatively affected by inorganic phosphorus [7]. Most other antibiotics including lincomycin are not inhibited by the presence of excess inorganic phosphorus during the production phase [6,7].

Metabolic regulation of carbon, nitrogen and phosphorus is widely used as one of the most effective methods to enhance the productivity of metabolites of interest [7,11]. Several enzymatic reactions in the pentose phosphate cycle and phosphorylation are involved in the biosynthesis of lincomycin [12,13]. It has been reported that the biosynthesis of lincomycin was not inhibited at low levels of free phosphorus [6], indicating the feasibility of improving the production of lincomycin by adjusting the levels of phosphorus during fermentation. However, the exact amount of phosphorous needed during different stages of fermentation is uncertain.

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In this work, we developed a new method to determine the amount of phosphorus to be fed during the fermentation process based on the metabolic flow direction of elemental phosphorus. The effects of phosphorus on lincomycin production were studied in a 100 m^3 industrial fermenter. The phosphorus concentrations in both base media and fed media were carefully adjusted since the oxygen availability in fermentation system became a main limiting factor for the mycelia growth at the log growth stage.

2. Materials and methods

2.1. Strain and media

An industrial strain, *Streptomyces lincolnensis* 2–128, was used. The fermentation of lincomycin involved two stages of the initial seed growth and one stage of lincomycin production. One liter of the primary seed medium contained the following components: 5 g starch, 25 g glucose, 10 g soybean meal, 10 g corn steep liquor (CSL, wet weight), 0.2 g KH_2PO_4, 1 g $(NH_4)_2SO_4$, 3 g $CaCO_3$ and 2.0 ml corn oil.

One liter of the secondary seed medium consisted of the following ingredients: 10 g starch, 40 g glucose, 20 g soybean meal, 10 g corn steep liquor (wet weight), 0.2 g KH_2PO_4, 3 g $(NH_4)_2SO_4$, 5 g $NaNO_3$, 3 g NH_4NO_3, 4 g NaCl, 7 g $CaCO_3$ and 0.65 ml emulsified silicone oil.

The compositions of the base industrial fermentation medium (1 l) were as follows: 20 g starch, 35 g glucose, 20 g soybean meal, 10 g corn steep liquor (wet weight), 0.4 g KH_2PO_4, 1 g $(NH_4)_2SO_4$, 4 g $NaNO_3$, 4 g NaCl, 6 g $CaCO_3$, 0.3 ml corn oil and 0.45 ml emulsified silicone oil. The organic materials such as soybean meal and corn steep liquor were produced by North China Pharmaceutical Corporation.

The starting volumes of the primary seed, the secondary seed, and the basic fermentation media were 1.5 m^3, 10.0 m^3, and 80.0 m^3, respectively. The medium pH value was adjusted within 7.0–7.5 before sterilization.

2.2. Fermentation conditions

The cultivation of the primary seed was carried out in a 2 m^3 seed fermenter containing 1.5 m^3 sterile medium with inoculation of 400 ml cultures from shake flasks. The system was incubated at 30 °C for 72 h with an aeration rate of 1.1 vvm and an agitation speed of 200 rpm. Then the produced primary seed culture (1.5 m^3) was transferred to 10 m^3 of seed medium in a 15 m^3 seed fermenter for cultivation of the secondary seed. The culture was incubated at 30 °C with agitation at 168 rpm and aeration at a rate of 1.3 vvm for 40 h.

The fermentation was performed in a 100 m^3 fermenter equipped with 4 baffles and 3 six-bladed turbines. The inoculation size was about 14% and the initial volume after inoculation was 80 m^3. The fermentation conditions were as follows: aeration rate at 0.8 vvm; agitation speed at 130 rpm; head pressure at 0.5 bar, temperature at 30 °C; time cycle 200 h. The medium pH value was maintained at 6.6–6.9. The culture samples were collected every 4 h to test pH value, which was controlled by manual addition of ammonia at 32–192 h. Glucose (400 g/l) and ammonium sulfate (150 g/l) solutions were continuously fed in the fed-batch process. The reducing sugars in the broth were maintained at 5–7 g/l from 24 h to the end of the fermentation. No cultures were drawn off during the fermentation and the harvested volume at the end of the fermentation was about 88 m^3.

2.3. Analytical methods

The culture samples taken at different times were centrifuged at 0.67 g for 15 min to collect the mycelia. The lincomycin titer and supernatant pH value were determined, and the measurement was done in triplicate each time. The collected mycelia were washed three times with deionized water, centrifuged and dried at 85 °C for 24 h in a desiccator to measure the dry weight of mycelia (DMW).

The free phosphorus content was determined by the phosphomolybdic acid colorimetric assay [14]. After centrifugation of the culture samples, 2.5 ml of the supernatant was collected and mixed with 5 ml of 100 g/l trichloroacetic acid. The mixture was boiled for 10 min followed by addition of water to a total volume of 50 ml. One ml of the resulting solution was mixed with 1 ml of 28.5 g/l ammonium molybdate and 1 ml of 1.0 g/l metol solutions. The mixture was boiled for 30 min followed by addition of water to a total volume of 10 ml. The formed phospho-molybdenum blue complex was measured at 650 nm using a 721 UV–visible spectrophotometer. The dry mycelia and organic materials such as soybean meal and corn steep liquor were treated by a wet digestion procedure with HNO_3, $HClO_4$ and H_2O_2 [15] before measurement. Their phosphorus contents were then measured following the same procedure.

The dissolved oxygen (DO) in fermenter was expressed as the air saturation (%) and measured using a DO electrode (Ingold, Switzerland). The DO concentration was assumed to be zero after sterilization, and adjusted to 100% air saturation when the medium was fully aerated and agitated before inoculation. During the growth and production stages, the critical oxygen concentration was determined via the curve of the aeration rate against DO [16]. With the aeration rate increasing stepwise, DO was read from the DO electrode. The critical oxygen concentration was at the first inflection point of the curve. The critical DO concentrations were about 27% and 35% air saturation at the growth stage and the production stage, respectively.

The lincomycin concentration was determined by HPLC following the procedures described in [17].

2.4. Stoichiometric analysis of phosphorus feeding

During the lincomycin fermentation, the phosphorus in the medium was not incorporated into the molecular composition of lincomycin ($C_{18}H_{34}N_2O_6S$) but only used to form mycelia. Hence, the macroscopic phosphorus mass balance can be applied to the integrated fermentation system consisting of the primary and secondary seed cultures as well as the main fermentation (Fig. 1). The inflows of elemental phosphorus consisted of the phosphorus in the base fermentation medium, inoculation cultures and feeding medium. The outflows of elemental phosphorus consisted of the phosphorus in the harvested cultures including the final mycelia and supernatant. The amount of phosphorus that flowed from the inoculation cultures into the fermentation medium was exactly the amounts of phosphorus added in the primary and secondary seed media. Thus, the relation between the phosphorus flowing in and out the integrated system can be mathematically described as follows:

$$P_{m3} + P_i + P_f = P_m + P_s \quad (1)$$

$$P_i = P_{m1} + P_{m2} \quad (2)$$

$$P_m = pXV \quad (3)$$

where P_{m1}, P_{m2}, and P_{m3} are the amounts of elemental phosphorus in the primary seed, secondary seed and fermentation media, respectively. P_i, P_m and P_s are the amounts of phosphorus in the inoculation cultures, total harvested

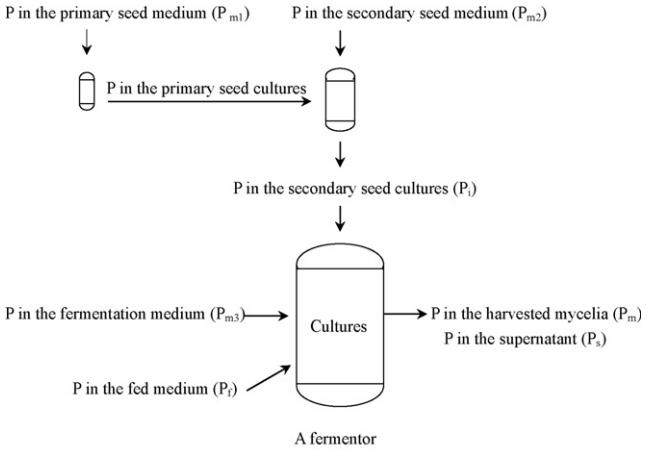

Fig. 1. Schematic representation of phosphorus flows in the fermentation system from the primary seed cultivation to the main fermentation. P represents phosphorus.