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Stimulation of Cephalosporin C Production by *Acremonium chrysogenum* M35 with Fatty Acids

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Abstract Supplementation with rice oil and its major components (oleic acid and linoleic acid) was found to have a significant influence on cephalosporin C (CPC) production and cell growth by *A. chrysogenum* M35 in shake flask cultures. Five percent (v/v) rice oil had the most robust effect and 5% (v/v) oleic acid was the second most efficient on cell growth, whereas 3% (v/v) linoleic acid was found to be optimal for CPC production. Rice oil, oleic acid, and linoleic acid also significantly improved the rates of glucose consumption. When glucose was almost consumed, CPC production was initiated and, on the addition of rice oil, lipase activity increased steadily to 1.56 U/ml for 4 days. These results suggest that rice oil and fatty acids are used as carbon source to produce CPC by *A. chrysogenum* M35. Moreover, a mixture, composed of 40% (v/v) oleic acid and 60% (v/v) linoleic acid, had the strongest stimulatory effect on CPC production, due to a synergistic effect of the two fatty acids. Consequently, the maximum CPC titer (7.44 g/l) was improved about 4.5-fold.

Key words: *Acremonium chrysogenum* M35, cephalosporin C, fatty acids, lipase activity, rice oil

Since their introduction into clinical practice in the 1960s, cephalosporins have become key agents in the antibacterial armamentarium, because of their favorable pharmacokinetic activities and safety profiles [14]. Over the years, modifications of the basic cephem structure have led to the evolution of several generations of cephalosporins, which are classified according to their activity spectra [8]. Cephalosporins are produced by *Acremonium chrysogenum* (also called *Cephalosporium acremonium*) and *Streptomyces clavuligerus*.

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It has been reported that the cell growth and microbial metabolite production are influenced by the medium composition in the fermentation process [2, 7, 20], and CPC is produced by *A. chrysogenum* on a complex medium containing glucose and different plant oils as the major carbon sources [19]. Many investigators have reported that plant oil and fatty acids may be successfully used to produce fungal metabolites [17, 21]. In addition, similar observations on other antibiotic fermentations have also been reported [3, 5, 18]. Although many investigators have reported positive results for several plant oils and fatty acids, the optimal addition level of various plant oils and fatty acids has just recently been obtained. Thus, the biochemical background of their beneficial effects on CPC production still remains not fully understood.

Rice oil from rice (*Oryza sativa*) bran, which is the main byproduct produced by the milling of rice, has high oil, protein, and carbohydrate content [16]. As shown by Gunstone [4], oleic acid and linoleic acid make up about 80% (w/w) of rice oil. In our previous studies, rice oil was fed to a culture broth of *C. acremonium* M25 to improve CPC production [8, 9, 13]. In this study, rice oil, oleic acid, and linoleic acid were supplemented to medium, in order to elucidate whether rice oil stimulates CPC production and cell growth. In addition, we investigated the effects of different ratios of fatty acid.

Materials and Methods

Strain

A strain that produces CPC, namely, *A. chrysogenum* M35, was used in this work. This strain was developed from *C. acremonium* M25 at our laboratory, using the same procedure used to produce *C. acremonium* M25. In our

previous work, *C. acremonium* ATCC 20339 was mutagenized with UV light, and a mutant *C. acremonium* strain, M25, was finally selected by agar diffusion [12].

Media and Culture Condition

Stock cultures were maintained by monthly transferring the organism onto potato dextrose agar slants. The basal seed medium was composed of 2.5% sucrose, 1.0% glucose, 2.5% corn steep liquor, and 0.4% $(NH_4)_2SO_4$. To improve morphological differentiation, 3.0% soy bean meal, 1.0% cotton seed flour, and 0.5% $CaCO_3$ were added to the basal seed medium [11]. The main medium consisted of 1.95% glucose, 5% corn steep liquor, 0.8% $(NH_4)_2SO_4$, 0.3% KH_2PO_4, 0.5% K_2HPO_4, 0.5% DL-methionine, and 0.4% trace element solution [12]. Sugars and $(NH_4)_2SO_4$ were sterilized separately from the other components. The pH was adjusted to 7.0 with 1 N NaOH prior to sterilization, and $CaCO_3$ was added after the pH adjustment. Seed and main cultures were carried out in 250-ml Erlenmeyer flasks containing 15 ml of medium. Flask cultures were operated at 300 rpm at 27°C on a rotary shaking incubator.

Analytical Methods

The dry cell weight of mycelium was measured as follows; 10 ml of culture broth was filtered through a preweighed Whatman glass-microfiber filter GF/C under suction. After washing twice with deionized water, cells were dried at 80°C for 24 h.

CPC was measured by high-performance liquid chromatography (HPLC) using a reverse-phase column of μ Bondapak C-18 and a 254 nm UV detector. The mobile phase was an acetonitrile-phosphate buffer, and the elution mixture was a 98% (v/v) phosphate buffer and 2% (v/v) acetonitrile with a flow rate of 0.9 ml/min. Cephalosporin C zinc salt (Sigma, U.S.A.) was used as a standard.

Glucose levels were measured using a glucose assay kit (Glucose Kit, Youngdong Co. Ltd., Korea), which is based on an enzymatic method. After reaction, absorbance of red substance generated during the reaction was measured at 505 nm.

Assay of Lipase Activity

Culture broth was centrifuged at 10,000 rpm for 10 min, and supernatant that contained lipase was then collected. Ten ml of isooctane containing 10% (w/v) rice oil was added to 10 ml of water containing 1 ml of the supernatant, and the reaction mixture was heated with shaking in a water bath at 37°C and 150 rpm for 30 min. The reaction was stopped by adding 1 ml of 6 M HCl and agitated vigorously for 30 sec. Two ml of the upper layer was then placed in a test tube. Cupric acetate-pyridine reagent (0.5 ml) was added, and free fatty acids liberated and dissolved in isooctane were quantified by UV spectrometry at 715 nm. One unit of lipase activity was defined as the amount of enzyme that liberated 1 μmol of free fatty acid per min [10].

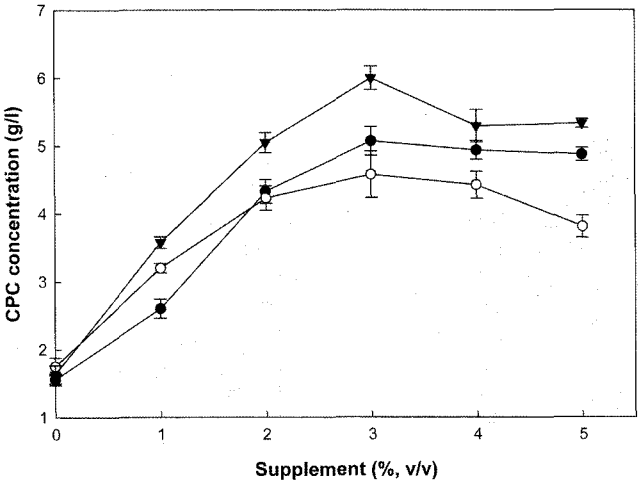

Fig. 1. Effect of rice oil (●), oleic acid (○), and linoleic acid (▼) on CPC production by *A. chrysogenum* M35 in shake flask cultures.
Each culture was carried out in duplicate at 27°C and 300 rpm for 7 days using a 10% (v/v) inoculation.

RESULTS

Effect of Rice Oil and Its Major Components on CPC Production and Cell Growth

The influence of rice oil and of its major components (oleic acid and linoleic acid) on the CPC production of *A. chrysogenum* M35 was investigated in shake flask cultures for 7 days. As shown in Fig. 1, rice oil, oleic acid, and linoleic acid were added to the medium at 1–5% (v/v). When linoleic acid at 1–5% (v/v) was supplemented to the

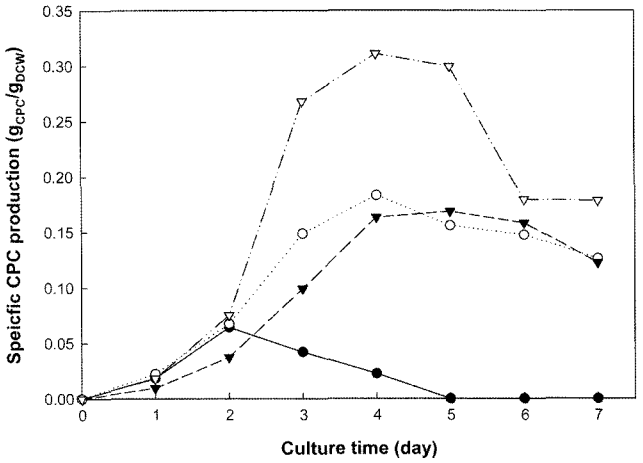

Fig. 2. Specific CPC production in the absence (●) or presence of 3% (v/v) rice oil (○), 3% (v/v) oleic acid (▼), or 3% (v/v) linoleic acid (▽) by *A. chrysogenum* M35 in shake flask cultures.
Each culture was carried out in duplicate at 27°C and 300 rpm for 7 days using a 10% (v/v) inoculation.

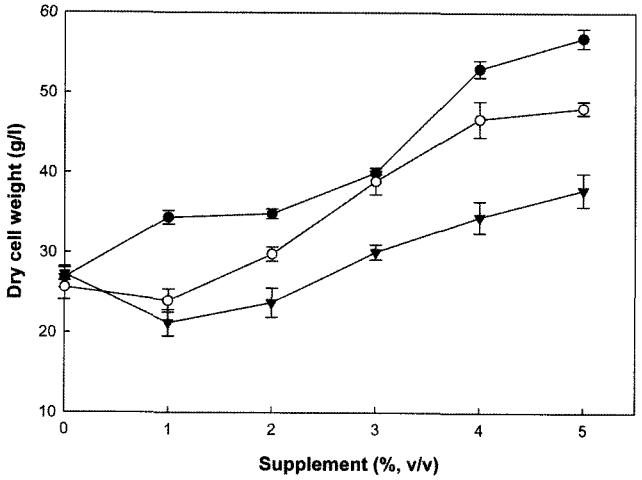

Fig. 3. Effects of rice oil (●), oleic acid (○), or linoleic acid (▼) on the cell growth of *A. chrysogenum* M35 in shake flask cultures.
Each culture was carried out in duplicate at 27°C and 300 rpm for 7 days using a 10% (v/v) inoculation.

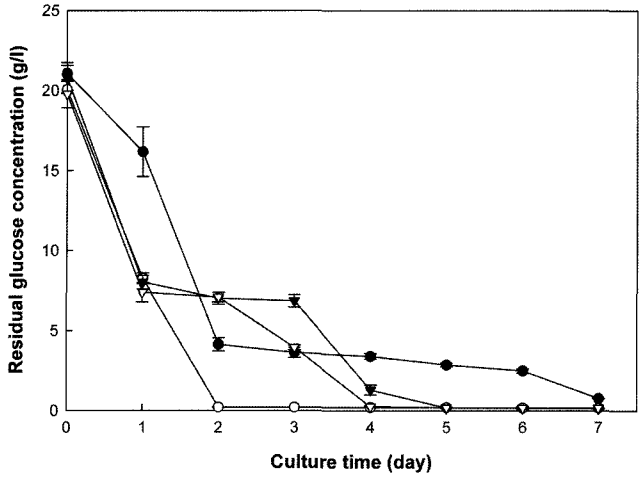

Fig. 4. Glucose consumption by *A. chrysogenum* M35 in the absence (●) or presence of 3% (v/v) rice oil (○), 3% (v/v) oleic acid (▼), or 3% (v/v) linoleic acid (▽) in shake flask cultures.
Each culture was carried out in duplicate at 27°C and 300 rpm for 7 days using a 10% (v/v) inoculation.

medium, the maximum CPC production increased remarkably from 1.64 to 3.57–6.00 g/l, with an optimum at 3% (v/v). Rice oil and oleic acid also had strong stimulatory effects on CPC production. In accordance with the result shown in Fig. 1, the stimulatory effect of rice oil on CPC production might be attributed to the fact that its main components are oleic acid and linoleic acid. Increased CPC production cannot simply be explained by increased dry cell weight, since stimulation of specific CPC production was shown after 2 days (Fig. 2). Karaffa *et al.* [6] obtained similar results.

Figure 3 shows the effects of rice oil, oleic acid, and linoleic acid as well as their levels on cell growth for 7 days. Rice oil, oleic acid, or linoleic acid stimulated cell growth, and rice oil was the most impressive of the three with an optimum level of 3% (v/v) on dry cell weight, which significantly increased dry cell weight from 26.88 to 56.84 g/l, by 0 to 5% (v/v) addition, and oleic acid was more effective on cell growth than linoleic acid.

As shown by these data, linoleic acid promoted CPC production, and oleic acid affected cell growth. It is highly likely that the fatty acid mixture has the strongest stimulatory effect on cell growth.

Glucose Consumption and Lipase Activities

Supplementation of either 3% (v/v) rice oil, oleic acid, or linoleic acid was the most effective on CPC production. The addition of 3% (v/v) rice oil, 3% (v/v) oleic acid, and 3% (v/v) linoleic acid increased the rate of glucose consumption. As shown in Fig. 4, at 1 day, rice oil or fatty acid supplemented flasks contained only about half of the glucose concentration, compared with those not supplemented. However, glucose was almost consumed at day 2 after the addition of 3% (v/v) rice oil, whereas glucose concentrations decreased slowly after the addition of 3% (v/v) oleic acid or linoleic acid.

Figure 5 shows temporal courses of lipase activities at 3% (v/v) rice oil, oleic acid, or linoleic acid. In the cases of supplementation with 3% (v/v) oleic or linoleic acid, lipase activities showed an almost similar level to the control. However, after adding 3% (v/v) rice oil, lipase activities were expressed significantly. In particular, 1.35 U/ml of lipase activity was obtained, when almost all the glucose was consumed at day 3.

Rapid glucose consumption was observed in these experiments after addition of rice oil, oleic acid, or linoleic

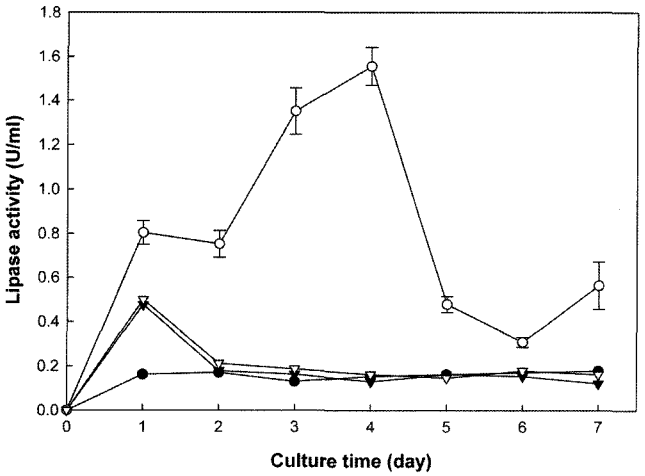

Fig. 5. Lipase activity of *A. chrysogenum* M35 in the absence (●) or presence of 3% (v/v) rice oil (○), 3% (v/v) oleic acid (▼), or 3% (v/v) linoleic acid (▽) in shake flask cultures.
Each culture was performed in duplicate at 27°C and 300 rpm for 7 days using a 10% (v/v) inoculation.

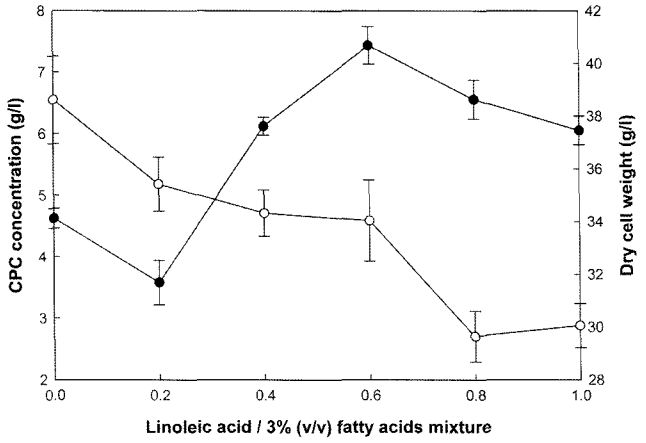

Fig. 6. Effect of 3% (v/v) mixture on CPC production (●) and the cell growth (○) of *A. chrysogenum* M35 in shake flask cultures.
Total 3% (v/v) mixture of oleic acid and linoleic acid was made up at different ratios. Each culture was performed in duplicate at 27°C and 300 rpm for 7 days using a 10 % (v/v) inoculation.

acid, which suggests that *A. chrysogenum* M35 consumes glucose first and then the rice oil by lipase. Therefore, rice oil stimulates glucose consumption more than oleic acid or linoleic acid. Furthermore, improved CPC production by rice oil or fatty acids occurred when all the glucose was almost consumed, thus indicating that rice oil or fatty acids are likely to serve as carbon sources for the production of CPC. However, when rice oil was supplemented into the medium, *A. chrysogenum* M35 produced lipase to utilize the rice oil as an energy source, showing that the fatty acids are more effective sources than the rice oil.

Effect of Different Ratios of Oleic Acid/Linoleic Acid

Because rice oil was found to be most effective for enhancing the cell growth, it was considered that a fatty acid mixture could more effectively improve CPC production. Thus, a different ratio of fatty acids, i.e., linoleic acid to oleic acid where the total fatty acid level was held at 3% (v/v), was supplemented into the medium. Figure 6 shows the effect of such mixtures on CPC production and cell growth. When the ratio of linoleic acid to total fatty acid was 0.6, the maximum CPC concentration obtained was 7.44 g/l. When the ratio of linoleic acid was increased, dry cell weight decreased. It is highly likely that linoleic acid promotes CPC production and oleic acid affects cell growth, suggesting that synergy between the two fatty acids improved CPC production. When the ratio of linoleic acid was higher than 0.6, the maximum CPC concentration decreased because of low cell growth.

Discussion

The supplementation of rice oil, oleic acid, and linoleic acid improved the production of CPC and cell growth by *A. chrysogenum* M35 in shake flask cultures. Such improvement could mainly be attributed to the effects on membrane structure and its permeability [15, 17, 21], or improved oxygen uptake by the medium through interfacial effects or defoaming properties [5]. However, considering the time courses of glucose consumption and cephalosporin C production, rice oil, oleic acid, and linoleic acid appear to be major sources of cephalosporin C. Such results were also reported by Karaffa *et al.* [6]. This conclusion is supported by the lipase activities, which suggest that cysteine and valine, which are CPC precursors, are synthesized from fatty acids through acetyl-CoA or pyruvate, after degradation of rice oil [1]. In the present study, linoleic acid was comparatively effective for CPC production, whereas oleic acid was related to cell growth. In addition, when the medium was supplemented with rice oil, the rice oil was used as an energy source to produce lipase. Consequently, linoleic acid more effectively promoted CPC production than rice oil. In particular, the fatty acid mixture had the strongest stimulatory effect on CPC production. In conclusion, fatty acid mixtures were found to be the most effective for enhancing CPC production during the submerged culture of *A. chrysogenum* M35. Furthermore, it is also likely that other fungal metabolites can effectively be produced using fatty acid mixtures. Rice oil is hydrolyzed to fatty acids and glycerol by lipase, and glycerol is then also used as the carbon source. Further study is needed to investigate the effect of glycerol on the CPC production and cell growth of *A. chrysogenum* M35.

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