The Journal of Microbiology (2011) Vol. 49, No. 5, pp. 753-758
DOI 10.1007/s12275-011-1155-5

Production of Cephalosporin C Using Crude Glycerol in Fed-Batch Culture of *Acremonium chrysogenum* M35

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(Received March 28, 2011 / Accepted June 17, 2011)

In this study, cephalosporin C production by *Acremonium chrysogenum* M35 cultured with crude glycerol instead of rice oil and methionine was investigated. The addition of crude glycerol increased cephalosporin C production by 6-fold in shake-flask culture, and also the amount of cysteine. In fed-batch culture without methionine, crude glycerol resulted only in overall improvement in cephalosporin C production (about 700%). In addition, *A. chrysogenum* M35 became highly differentiated in fed-batch culture with crude glycerol, compared with the differentiation in batch culture. The results presented here suggest that crude glycerol can replace methionine and plant oil as cysteine and carbon sources during cephalosporin C production by *A. chrysogenum* M35.

***Keywords*:** cysteine, cystathionine-γ-lyase activity, Acremonium chrysogenum M35, methionine, cephalosporin C, crude glycerol

β-Lactam antibiotics, such as cephalosporins, belong to one of the largest-selling classes of drugs worldwide, with an annual production of 2,500 tons (Schmitt *et al*., 2004). Today, β-lactam antibiotics, particularly penicillin and cephalosporins, represent a major biotechnology product with worldwide sales of 15 billion U.S. dollars, which is 65% of the total world market for antibiotics (Elander, 2003).

Cephalosporin C production by *Acremonium chrysogenum* on an industrial scale involves cultivating the fungus in complex medium containing glucose and soybean oil as the main carbon sources (Revin *et al*., 1991). However, the price of soybean oil in the U.S. averaged $1,376 per metric ton in 2008, which was more than double the average of $684 in 2007 (Biodiesel magazine, 2008). Therefore, the development of efficient methods of producing cephalosporin C from low-priced carbon sources instead of soybean oil has received much attention (Brakhage *et al*., 2004).

Biodiesel production in the U.S. has increased dramatically from 500,000 gallons in 1999 to 70 million gallons in 2005 (National Biodiesel Board, 2008). The principal byproduct of biodiesel production is crude glycerol, which constitutes about 10% of vegetable oil. Crude glycerol derived from biodiesel production is of very low value due to impurities. In previous work (Shin *et al*., 2010) it was confirmed that utilization of pure glycerol as a carbon source strongly stimulated differentiation of *A. chrysogenum* M35, and led to enhancement of cephalosporin C production in shake-flask culture. Use of less expensive, low-grade crude glycerol could decrease the cost of cephalosporin C production in the growing global market.

Methionine is a well-known inducer of cephalosporin production by *A. chrysogenum* (Demain and Wolfe, 1987; Zhou *et al*., 1992). One of the limiting steps in cephalosporin C biosynthesis is the availability of cysteine formed from either sulfate by the autotrophic pathway (Döbeli and Nüesch, 1980) or methionine by the reverse trans-sulfuration pathway (Martin and Demain, 2002). Methionine is usually produced by chemical methods. The chemical processes involved in methionine production are generally expensive, use hazardous chemicals, and produce a racemic mixture of methionine that requires a separate resolution process (Araki and Ozeki, 1991; Geiger *et al*., 1998).

The aim of the present study was to reduce the cost of cephalosporin C production by *A. chrysogenum* M35 by using crude glycerol as a carbon and cysteine source instead of plant oils and methionine. In addition, cysteine content and cystathionine-γ-lyase activity were investigated.

Materials and Methods

Cultivation of fungal strain and media

A. chrysogenum M35, a UV-induced mutant of *A. chrysogenum* (ATCC 20339) with increased cephalosporin C production capability, was cultured in basal seed medium consisting of 2.5% (w/v) sucrose, 1.0% (w/v) glucose, 2.5% (v/v) corn steep liquor, and 0.4% (w/v) $(NH_4)_2SO_4$. Addition of 3.0% (w/v) soy bean meal, and 1.0% (w/v) cotton seed flour to the basal seed medium improved morphological differentiation. Primary culture medium contained 2% (w/v) glucose, 5% (v/v) corn steep liquor, 0.8% (w/v) $(NH_4)_2SO_4$, 0.3% (w/v) KH_2PO_4, 0.5% (w/v) K_2HPO_4, 0.5% (w/v) DL-methionine, and 0.4% (w/v) trace element solution (Lee *et al*., 2010). Sugar and $(NH_4)_2SO_4$ were separately sterilized from other components. Before 0.5% (w/v) $CaCO_3$ was added to the medium, the pH was adjusted to 7.0 using a 1 M solution of NaOH prior to sterilization. The effects of crude glycerol on cystathionine-γ-lyase activity and cysteine content were

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evaluated by addition of 4% (v/v) crude glycerol (DanSuk Industry corporation) to the primary culture medium. The cultures were incubated in 500 ml Erlenmeyer flasks containing 50 ml of medium, at 300 rpm, and at 27°C, for 5 days.

Batch and fed-batch culture

Suspended cells from slants were added to the initial inoculum flask (2,000 ml) containing 300 ml of basal seed medium. The seed culture was incubated at 27°C for 60 h, on a rotary shaker at 300 rpm. The main cultures were grown in a 5 L stirred tank bioreactor filled with 3 L of primary culture medium containing 4% crude glycerol, at 27°C, for 120 h. The primary culture medium was without methionine. The aeration rate and agitation rate were 1.2 vvm and 300 rpm, respectively. Samples were withdrawn every 24 h during cultivation.

The glycerol from the main culture is consumed; 40 ml/L crude glycerol and 4 ml/L trace element solution were fed to the fed-batch culture at 120 ml/h to enhance cephalosporin C biosynthesis.

Cell extraction and cystathione-γ-lyase activity assay

Frozen mycelia (1.0 g) collected every 24 h were ground in a mortar containing liquid nitrogen. The ground mycelia were then resuspended in 100 mmol/L sodium phosphate buffer (pH 7.3) containing 1 mmol/L EDTA and 0.1 mmol/L pyridoxal 5′-phosphate. Cell debris was removed by centrifugation at 15,000×g for 20 min at 4°C. Cystathione--lyase activity was analyzed by determining the conversion rate of L-cystathionine into L-cysteine and α-ketobutylate. The reaction mixture was composed of 4 mmol/L L-cystathione, 5.5×10^{-2} mmol/L pyridoxal 5′-phosphate, 7 mmol/L EDTA, 2 mmol/L dithiothreitol, and 0.1 mg of protein in a final volume of 0.5 ml. The reaction mixtures were incubated for 30 min at 30°C. The reactions were then stopped by the addition of 1 ml of Gaitonde's reagent and boiled for 5 min. The precipitated proteins were removed by centrifugation, after which the amount of cysteine in 1 ml of supernatant was determined by acid ninhydrin assay (Gaitonde, 1967). Total protein content was measured by the Bradford method with bovine serum albumin as a standard.

Determination of cysteine in *A. chrysogenum* M35

A reaction mixture containing 100 mg of protein from *A. chrysogenum* M35 was made with 0.5 ml of acetic acid and 0.5 ml of acid ninhydrin reagent. The tubes were covered with aluminum caps or glass marbles and then heated in a boiling water bath for 10 min. Tubes were then rapidly cooled in tap water, and the contents of the tubes were diluted to 5 or 10 ml with 95% ethanol. The amount of cysteine was calculated according to a calibration curve at an extinction of 560 nm (Gaitonde, 1967).

Analytical methods

Cephalosporin C was measured by high-performance liquid chromatography (HPLC) using a μ-Bondapak C-18 reverse-phase column and a 254 nm UV detector. The mobile phase consisted of 98% (v/v) phosphate buffer (pH 7.0) and 2% (v/v) acetonitrile applied at a flow rate of 0.9 ml/min. Cephalosporin C zinc salt (Sigma, USA) was used as a standard.

Dry cell weight of the mycelium was measured as follows: 10 ml of culture broth was filtered through a Whatman GF/C filter, filters

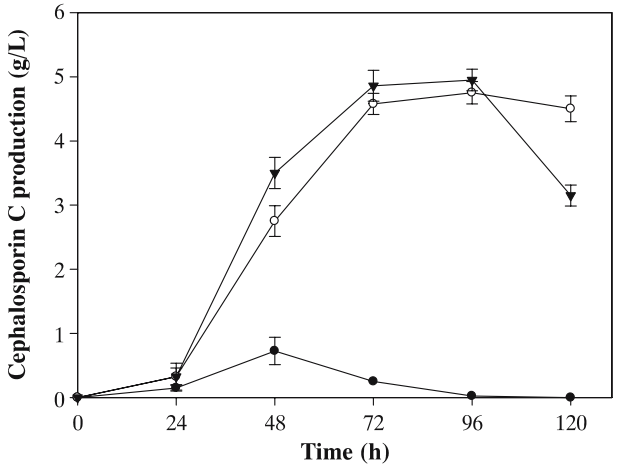

Fig. 1. Effect of glucose (●), rice oil (○), and crude glycerol (▼) on cephalosporin C production by *A. chrysogenum* M35 in shake-flask culture.

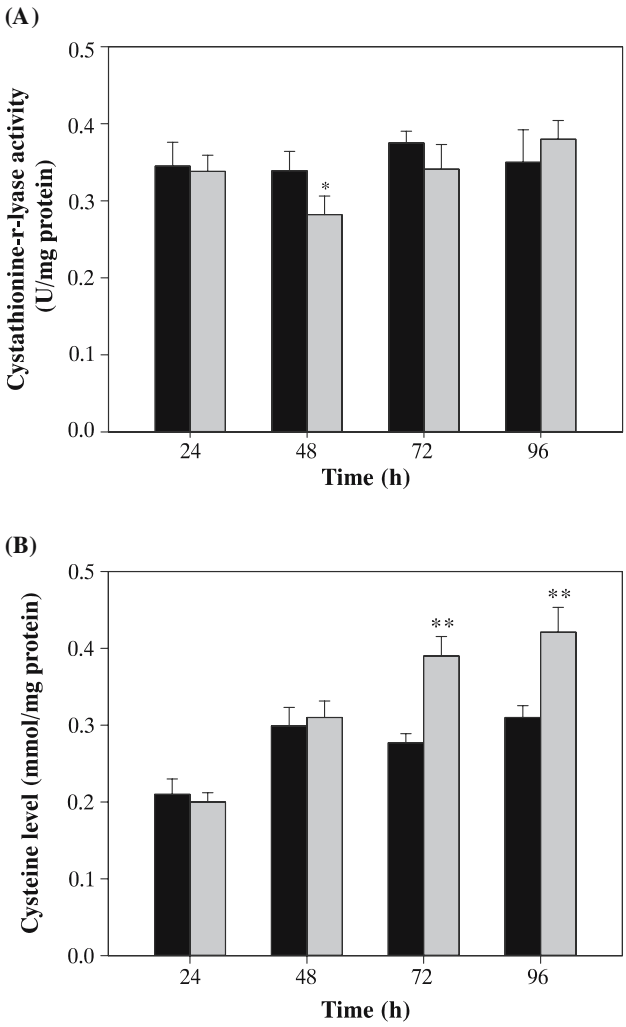

Fig. 2. Cystathionine-γ-lyase activity (A) and level of cysteine (B) in extracts of *A. chrysogenum* M35 grown without (■) or with (■) crude glycerol. *$P<0.05$ Student's t-test, **$P<0.01$ Student's t-test.

with captured solids were washed twice with deionized water, and dried at 80°C for 24 h, then weighed.

Concentrations of crude glycerol were measured by high-performance liquid chromatography (HPLC) using an Aminex HPX-87H column (300×7.8 mm, Bio-Rad, Inc., USA) and a refractive index detector (Shimadzu, Japan). Temperature of the column and detector was maintained at 55°C. Mobile phase was 0.0025 mol/L H_2SO_4 at a flow rate of 0.8 ml/min. Pure glycerol (Daejung Chem., Korea) was used as a standard.

Statistical analysis

The results are expressed as mean ± standard error of the mean (SE), and $p<0.05$ was statistically significant. The significance of measured differences in the levels of cystathione-γ-lyase activity and of cysteine was determined using t-tests (Sigmaplot software).

Results

Effect of crude glycerol on cephalosporin C production

The optimal concentration of pure glycerol for cephalosporin C production by *A. chrysogenum* M35 in shake-flask culture was found to be 4% in previous work (Shin *et al*., 2010). Therefore, 4% crude glycerol was selected for the current tests.

The effects of crude glycerol as a carbon source during cephalosporin C production by *A. chrysogenum* M35 were investigated in shake-flask cultures incubated for 5 days. The addition of 3% rice oil to the primary culture medium as a positive control enhanced cephalosporin C production. When 4% crude glycerol was added to the primary culture medium, cephalosporin C production increased from 0.72 to 4.92 g/L (Fig. 1). Therefore, crude glycerol could be used as

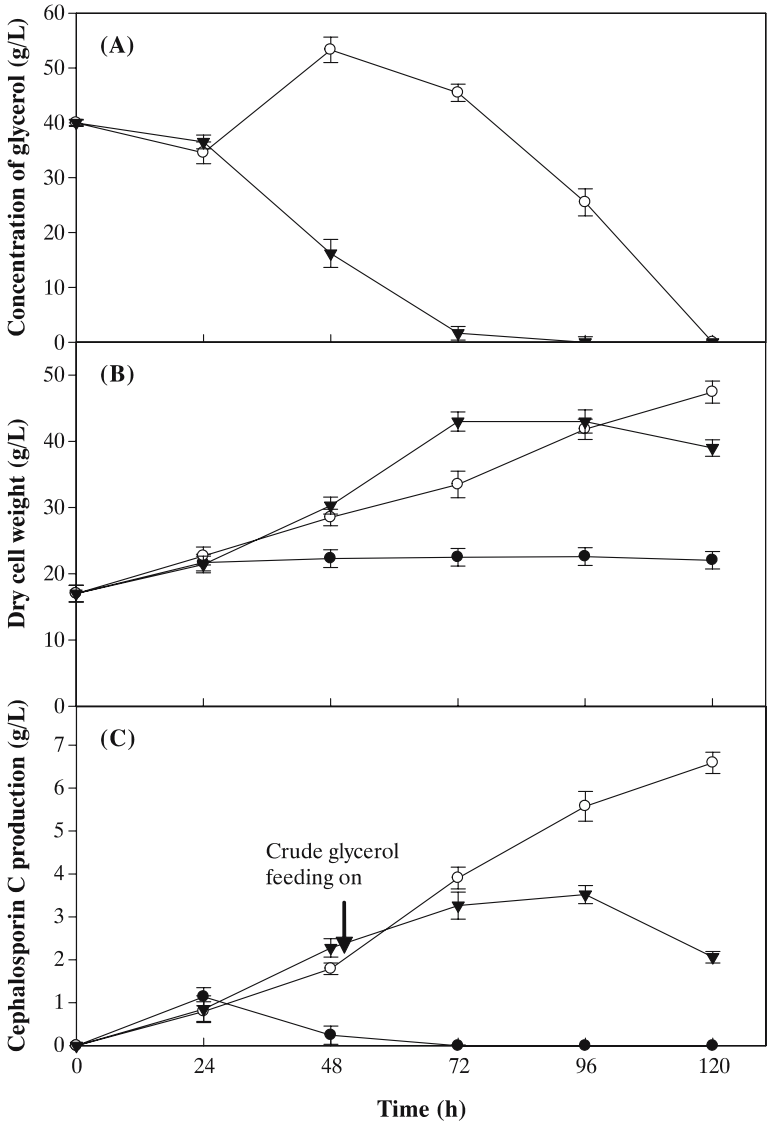

Fig. 3. Production of cephalosporin C by crude glycerol without methionine in batch (▼) and fed-batch (○) *A. chrysogenum* M35 cultures. Glucose was used as a control (●). (A) Consumption of glycerol, (B) dry cell weight of *A. chrysogenum* M35, and (C) production of cephalosporin C during fermentation. Culture was carried out in a 5 L stirred tank bioreactor (27°C, 300 rpm, and 1.2 vvm). Feeding of crude glycerol (120 ml/h) was performed at 48 h in fed-batch culture.

an alternative carbon source for cephalosporin C production by *A. chrysogenum* M35.

Cystathione-γ-lyase activity and cysteine content in *A. chrysogenum* M35

The level of cysteine was increased in *A. chrysogenum* M35 by pure glycerol in shake-flask cultures (Shin *et al*., 2011). Crude glycerol contained potassium, chloride, sodium, ash, and MONG (Matter of Organic Non Glycerin). These components could affect cystathionine-γ-lyase activity and cysteine level during cephalosporin C production. Therefore, cystathionine-γ-lyase activity and cysteine level in the presence of crude glycerol were measured especially since these may be linked to the production of β-lactam antibiotics by *A. chrysogenum*. Figure 2 shows the cystathionine-γ-lyase activity of *A. chrysogenum* M35 in cultures with or without crude glycerol.

The addition of crude glycerol did not result in increased cystathionine-γ-lyase activity during 24-96 h of cultivation (Fig. 2A), whereas the cysteine level of *A. chrysogenum* M35 was increased (Fig. 2B). Thus, crude glycerol was converted into cysteine or cysteine intermediates.

Production of cephalosporin C using crude glycerol in batch and fed-batch culture

Utilization of crude glycerol during cephalosporin C production was investigated in fed-batch cultures of *A. chrysogenum* M35 without methionine, since cysteine content was increased in crude glycerol-fed media. Crude glycerol was consumed more slowly than glucose and led to less catabolic repression in *A. chrysogenum* M35 during cephalosporin synthesis (Fig. 3A). The dry cell weight of crude glycerol in the culture without methionine was higher than that of glucose in the culture with methionine (Fig. 3B). In addition, cephalosporin C production from crude glycerol in the culture without methionine was about 6-fold greater than that from glucose in the culture containing methionine (Fig. 3C). These results suggest that crude glycerol as a carbon and cysteine source contributed to cephalosporin C production either directly or indirectly.

Morphological differentiation of *A. chrysogenum* M35 in a batch/fed-batch culture containing crude glycerol without methionine

A. chrysogenum cultures show various morphological forms: hyphae, conidia, arthrospores, and germlings (Hoff *et al*., 2005). Morphological differentiation of *A. chrysogenum* M35 grown in a shake-flask culture containing crude glycerol without methionine was observed for cephalosporin C production. Figure 4 shows a comparison of the differentiation of *A. chrysogenum* M35 between batch and fed-batch culture containing crude glycerol. The level of arthrospores increased during 72-120 h of cultivation, when crude glycerol was added to the medium (Figs. 4D-F and G-I), while many hyphal fragments were observed in the glucose-fed cultures (Figs. 1A-C). The filaments were wide, swollen, and highly vacuolated after 72 h (Fig. 4G-I), in fed-batch culture containing crude glycerol without methionine. *A. chrysogenum* M35 was more obviously differentiated into arthrospores after addition of crude glycerol to

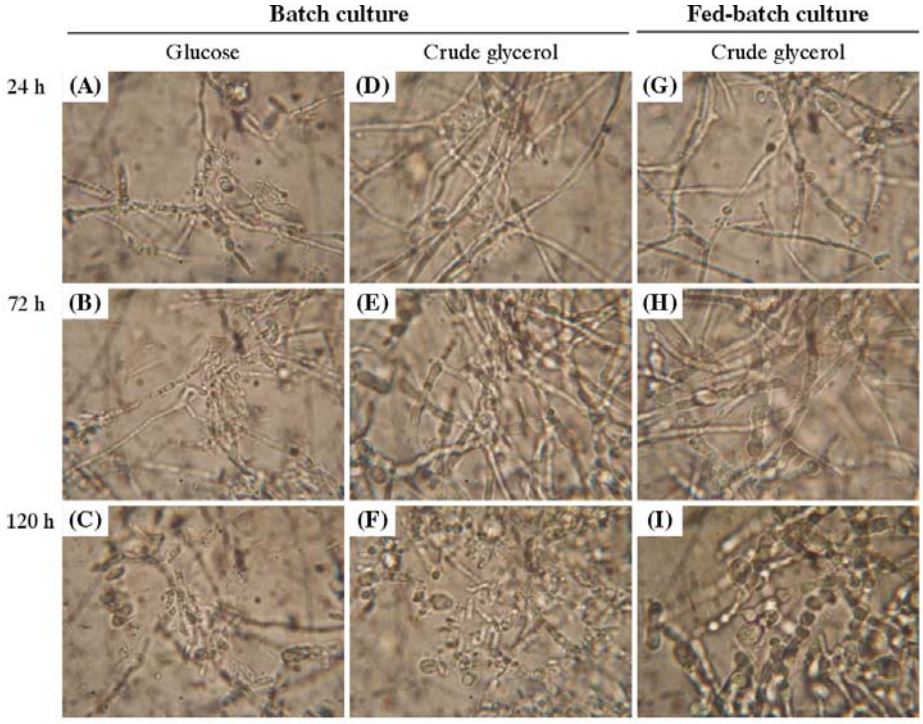

Fig. 4. Comparison of morphological differentiation of *A. chrysogenum* M35 between batch and fed-batch culture without methionine. (A-C) and (D-F) were time courses of batch culture containing glucose and crude glycerol, respectively. (G-I) were time courses of the fed-batch culture.

the medium in a fed-batch culture, compared with a batch culture (Figs. 4D-F).

Discussion

Various carbon sources such as glucose, sucrose, and plant oils have been adopted as the major carbon sources in industrial-scale fermentation (Revin *et al*., 1991). Previously, it was found that addition of 3% rice oil with methionine to a culture of *A. chrysogenum* M35 enhances cephalosporin C production by about 4.5 g/L (Kim *et al*., 2006). Alternative carbon sources, including soybean oil and rice oil, are used in commercial antibiotic production to minimize catabolic repression. Addition of a low solubility carbon source such as oil can also avoid carbon catabolic repression. However, it has been reported that the use of oils in media is restricted to environments in which there are higher residual oil levels and oxygen requirements compared to use of carbohydrates (Bader *et al.*, 1984). Crude glycerol can be applied to cephalosporin C production due to advantages such as production yield, and less expensive purification steps on an industrial scale. In addition, crude glycerol is widely available due to the increased production of biodiesel in response to the need for renewable fuels. Since crude glycerol can be utilized as a carbon source in industrial microbiological processes, its availability as a by-product of biodiesel production contributes to its competitiveness.

A rate-limiting step in cephalosporin C biosynthesis is the availability of cysteine formed from methionine by the reverse transsulfuration pathway (Paszewski *et al*., 2000). The production of cysteine by cystathionine hydrolysis is believed to be the main source of cysteine for cephalosporin biosynthesis (Nüesch *et al*., 1987). Therefore, cystathionine-γ-lyase activity is required for high-level cephalosporin production in methionine-supplemented medium. Crude glycerol is utilized in a similar metabolic pathway during the production of β-lactam antibiotics. Glycerol is readily metabolized into 3-phosphoglycerate through the glycolysis pathway (Liepins *et al*., 2006). Then, 3-phosphoglycerate and pyruvate are converted into cysteine by *Penicillium chrysogenum* (Usher *et al*., 1992). A glycerol derivative is directly changed to precursors of β-lactam antibiotic as clavulanic acid by *Streptomyces clavuligerus* (Salowe *et al*., 1990). Therefore, it was expected that *A. chrysogenum* M35 would convert crude glycerol into precursors of cephalosporin C and cysteine during cephalosporin C production in this study.

Recently, related research has shown that physiological differentiation results from cultivation in the presence of methionine, which stimulates the formation of arthrospores (Sándor *et al*., 2001). *A. chrysogenum* differentiates into arthrospores with a high rate of cephalosporin C biosynthesis (Nash and Huber, 1971; Bartoshevich *et al*., 1990). Arthrospores have more lipid-containing vacuoles than do hyphal fragments (Queener and Ellis, 1975). Additionally, the vacuoles may play an ancillary role in the supply of precursor amino acids, and in the storage of intermediates when cephalosporin C is synthesized from α-aminoadipate, cysteine, and valine (Kamp *et al*., 1999). Furthermore, lipid accumulation must affect the physicochemical properties of the cell during the fermentation, including membrane fluidity and nutrient transport, since accumulation of cellular lipids is widely recognized as an important factor in membrane fluidity of *A. chrysogenum* (Keweloh *et al*., 1991).

In conclusion, the effects of crude glycerol on cephalosporin C production by *A. chrysogenum* M35 were evaluated in methionine-unsupplemented cultures. It was found that crude glycerol stimulated cephalosporin C production by increasing cysteine content in *A. chrysogenum* M35 cultured without methionine. Additionally, crude glycerol utilization led to significantly higher cephalosporin C production compared to use of rice oil. Overall, these results indicate that crude glycerol was utilized as a carbon and cysteine source in cephalosporin C production by *A. chrysogenum* M35. Thus, the results presented here suggest that low-priced crude glycerol can be used as an alternative to plant oils and methionine for cephalosporin C biosynthesis on an industrial scale.

Acknowledgements

This work was supported by the Advenced Biomass R&D Center (ABC) of Korea Grant funded by the Ministry of Education, Science and Technology (ABC-2010-0029799) and the core Environmental Technology Development project for Next Generation funded by the Ministry of Environment, Republic of Korea (No.032-091-019).

References

Araki, K. and T. Ozeki. 1991. Amino acid. *Kirk-Othmer Encycl. Chem. Technol.* 9, 504-571.

Bader, F.G., M.K. Boekeloo, H.E. Graham, and J.W. Cagle. 1984. Sterilization of oils: Data to support the use of a continuous point-of-use sterilizer. *Biotechnol. Bioeng.* 26, 848-856.

Bartoshevich, Y.E., P.L. Zaslavskaya, M.J. Novak, and O.D. Yudina. 1990. *Acremonium chrysogenum* differentiation and biosynthesis of cephalosporin. *J. Basic Microbiol.* 30, 313-320.

Biodiesel magazine. 2008. http://www.biodieselmagazine.com/article.jsp?article_id=2881&q=&page=all. Accessed on August 28, 2010.

Brakhage, A.A., P. Sprote, Q. Al-Abdallah, A. Gehrke, H. Plattner, and A. Tuncher. 2004. Regulation of penicillin biosynthesis in filamentous fungi. *Adv. Biochem. Eng. Biotechnol.* 88, 45-90.

Demain, A.L. and S. Wolfe. 1987. Biosynthesis of cephalosporins. *Dev. Ind. Microbiol.* 27, 175-182.

Döbeli, H. and J. Nüesch. 1980. Regulatory properties of O-acetyl-L-serine sulfhydrylase of *Cephalosporium acremonium*: evidence of an isoenzyme and its importance in cephalosporin C biosynthesis. *Antimicrob. Agents Chemother.* 18, 111-117.

Elander, R.P. 2003. Industrial production of β-lactam antibiotics. *Appl. Microbiol. Biotechnol.* 61, 385-392.

Gaitonde, M.K. 1967. A spectrophometric method for the direct determination of cysteine in the presence of other naturally occurring amino acids. *Biochem. J.* 104, 627-633.

Geiger, F., B. Halsberghe, H.J. Basselbach, K. Hentchel, K. Humacher, M. Korfer, S.P. Mannsfeld, and *et al*. 1998. Process for the preparation of D,L-methionine or the salt thereof. US patent No. 5770769.

Hoff, B., E.K. Schmitt, and U. Kück. 2005. CPCR1, but not its interacting transcription factor AcFKH1, controls fungal arthrospore formation in *Acremonium chrysogenum*. *Mol. Microbiol.* 56, 1220-1233.

Kamp, M., A.J. Driessen, and W.N. Konings. 1999. Compartmentalization and transport in beta-lactam antibiotic biosynthesis by filamentous fungi. *Antonie van Leeuwenhoek* 75, 41-78.

Keweloh, H., R. Diefenbach, and H.J. Rehm. 1991. Increase of phenol tolerance of *Escherichia coli* by alterations of the fatty acid composition of the membrane lipids. *Arch. Microbiol*. 157, 49-53.

Kim, J.C., S.W. Kang, J.S. Lim, Y.S. Song, and S.W. Kim. 2006. Stimulation of cephalosporin C production by *Acremonium chrysogenum* M35 with fatty acid. *J. Microbiol. Biotechnol.* 16, 1120-1124.

Lee, H.H., H.Y. Shin, E.J. Kim, and S.W. Kim. 2010. Differentiation of *Acremonium chrysogenum* M35 in submerged culture with glass beads or silicone rubbers. *J. Microbiol.* 48, 734-738.

Liepins, J., S. Kuorelahti, M. Penttila, and P. Richard. 2006. Enzymes for the NADPH-dependent reduction of dihydroxyacetone and D-glyceraldehyde and L-glyceraldehyde in the mould *Hypocrea jecorina*. *FEBS J.* 273, 4229-4235.

Martin, J.F. and A.L. Demain. 2002. Unraveling the methionine-cephalosporin puzzle in *Acremonium chrysogenum. Trends Biotechnol.* 20, 502-507.

Nash, C.H. and F.M. Huber. 1971. Antibiotic synthesis and morphological differentiation of *Cephalosporium acremonium*. *Appl. Environ. Microbiol.* 22, 6-10.

National Biodiesel Board. 2008. http://www.biodiesel.org/pdf_files/fuelfactsheets/Production_Graph_Slide.pdf. Accessed on June 16, 2010.

Nüesch, J., J. Heim, and H.J. Treichler. 1987. The biosynthesis of sulfur containing β-lactam antibiotics. *Annu. Rev. Microbiol.* 41, 51-57.

Paszewski, A., R. Natorff, M. Piotrowska, J. Brzywczy, M. Sienko, M. Grynberg, E. Pizzinini, and G. Turner. 2000. Regulation of sulfur amino acid biosynthesis in *Aspergillus nidulans*: physiological and genetical aspects. Paul Haupt, Bern, Switzerland.

Queener, S.W. and L.F. Ellis. 1975. Differentition of mutants of *Cephalosporium acremonium* in complex medium: the formation of unicellular arthrospores and their germination. *Can. J. Microbiol*. 21, 1981-1996.

Revin, V.V., S.A. Kasatkin, G.N. Cherkasova, V.T. Nikolaev, N.S. Iamashkina, M.N. Chabushkina, O.A. Popova, and V.F. Belianina. 1991. Effect of the quality of fat substrate on the dynamics of fatty acid utilization during biosynthesis of cephalosporin C. *Antibiot. Khimioter.* 36, 5-8.

Salowe, S.P., E.N. Marsch, and C.A. Townsend. 1990. Purification and characterization of clavaminate synthase from *Streptomyces clavuligerus*; an unusual oxidative enzyme in natural product biosynthesis. *Biochem.* 29, 6499-6508.

Sándor, E., A. Szentirmai, G.C. Paul, R.T. Colin, L. Pócsi, and L. Karaffa. 2001. Analysis of the relationship between growth, cephalosporin C production, and fragmentation in *Acremonium chrysogenum*. *Can. J. Microbiol.* 47, 801-806.

Schmitt, E.K., A. Bunse, D. Janus, B. Hoff, E. Friedlin, H. Kurnsteiner, and U. Kuck. 2004. Winged helix transcription factor CPCR1 is involved in regulation of beta-lactam biosynthesis in the fungus *Acremonium chrysogenum*. *Eukaryot. Cell* 3, 121-134.

Shin, H.Y., J.Y. Lee, Y.R. Jung, and S.W. Kim. 2010. Stimulation of cephalosporin C production in *Acremonium chrysogenum* M35 by glycerol. *Bioresour. Technol.* 101, 4549-4553.

Shin, H.Y., J.Y. Lee, C. Park, and S.W. Kim. 2011. Utilization of glycerol as cysteine and carbon sources for cephalosporin C production by *Acremonium chrysogenum* M35 in methionine-unsupplemented culture. *J. Biotechnol*. 151, 363-368.

Usher, J.J., D.W. Hughes, M.A. Lewis, and S.J. Chiang. 1992. Determination of the rate-limiting step(s) in the biosynthetic pathways leading to penicillin and cephalosporin. *J. Ind. Microbiol.* 10, 157-163.

Zhou, W., K. Holzhauer-Rieger, M. Dors, and K. Schügerl. 1992. Influence of medium composition on the cephalosporin C production with a highly productive strain *Cephalosporium acremonium*. *J. Biotechnol.* 23, 315-329.