THE KOREAN JOURNAL OF MYCOLOGY
Vol. 25, No 2, p91-100 June 1997

Printed in S. KOREA

Production of Alkaline Carboxymethyl Cellulase and Xylanase by Batch and Fed-batch Cultures of Alkalophilic *Cephalosporium* sp. RYM-202

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호알카리성 *Cephalosporium* sp. RYM-202의 회분 및 유가배양에 의한 Alkaline Carboxymethyl Cellulase와 Xylanase의 생산

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ABSTRACT: Production of alkaline carboxymethyl cellulase (CMCase) and xylanase by batch and fed-batch cultures of alkalophilic *Cephalosporium* sp. RYM-202 was investigated. Of carbon sources tested, wheat bran gave the highest production of those enzymes. The high levels of CMCase on carboxymethyl cellulose and xylanase on birchwood xylan suggest that the biosynthesis of CMCase and xylanase in *Cephalosporium* sp. RYM-202 is regulated separately at the level of enzyme induction. The temperature and pH for maximal production of those enzymes was 20 °C and 9.0, respectively. High concentration of wheat bran in batch fermentation resulted in the lower and delayed production of the enzymes by catabolite repression. In fed-batch fermentation with controlled feeding of 5% final wheat bran concentration, the highest activities of CMCase and xylanase were 0.39 and 9.2 units/ml, respectively, and 1.22 and 1.36 times higher respectively than those in batch fermentation on 5% wheat bran.

KEYWORDS: Alkalophilic *Cephalosporium* sp., Alkaline carboxymethyl cellulase (CMCase), Alkaline xylanase, Batch fermentation, Fed-batch fermentation

The structural polysaccharides, cellulose and hemicellulose, together are considered greater than 50% of plant biomass and are consequently the most abundant terrestrial organic molecules (Kuhad and Singh, 1993). For the same reason, hydrolysis of cellulose and hemicellulose by cellulolytic and hemicellulolytic enzymes have attracted the continuing interest in exploitation of plant biomass (Gilbert and Hazlewood, 1993; Wong and Saddler, 1992).

In addition to application to utilization and treatment of biomass in form of wastes, the uses of alkaline cellulase for laundry detergents and xylanase for biopulping processes have attracted interest as the most promising industrial applications of the microbial enzymes (Ito *et al.*, 1989; Senior *et al.*, 1991; Herbert, 1992; Hoq and Deckwer, 1995). Cellulases and xylanases occur widely in bacteria and fungi. However, the great majority of these enzymes usually are most active at acidic or neutral pH. Recently, there are several reports on the alkaline cellulases and xylanases from alkalophilic or alkalotolerant bacterial strains including *Bacillus* spp. (Dey *et al.*, 1992; Ratto *et al.*, 1992; Nakamura *et al.*, 1993) and *Streptomyces* sp. (Rhyum *et al.*,

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1993), but rather scarce attention has been paid to the alkaline enzymes of fungal origin.

Cephalosporium sp. RYM-202 is an alkalophilic fungal strain that produces multiple carboxymethyl cellulases (CMCases) and xylanases exhibiting high activities and stabilities at highly alkaline pH (Kang *et al.*, 1993, 1995, 1996). Moreover, these CMCases and xylanases fulfill the essential requirements for use as additive in laundry detergents and for biopulping applications, respectively (Kang and Rhee, 1995; Kang *et al.*, 1996). These properties make this fungus attractive in view of its application as a potential candidate for the production of alkaline CMCase and xylanase.

Economic analysis indicated that production of cellulase and xylanase would be a major cost factor hindering the wide spread use of these enzymes in industries, and it is therefore essential to improve yields and productivity of the enzymes to make the process economically feasible (Gomes *et al.*, 1992; Herbert, 1992). To enhance production of cellulase and xylanase by microorganisms and reduce production costs several efforts such as mutation, gene cloning, protoplast fusion and optimization of medium composition and environmental factors have been made (Brown *et al.*, 1987; Lejeune and Baron, 1995, Warzywoda and Pourquie, 1983). Moreover, enzyme production has been markedly increased by the mode of fermentation, eg. continuous, batch, and fed-batch culture (Gomes *et al.*, 1993; Hendy *et al.*, 1984). In this paper, we describe some culture conditions and fermentation processes that affect the production of CMCase and xylanase during batch and fed-batch cultures of *Cephalosporium* sp. RYM-202.

Materials and Methods

Microorganism and media

Cephalosporium sp. RYM-202 isolated from a soil sample was used (Kang *et al.*, 1993). The fungus was grown on potato dextrose agar containing 0.5% sodium carbonate at 30°C for 5 days and then stored at 4°C. Stock cultures were transferred to fresh medium every 6~7 weeks and incubated under the same conditions. The culture media used were based on a basal medium in which the concentrations of different components were changed to evaluate their effect on growth and enzyme production. The basal medium contained (W/V): 1, 2 or 5% wheat bran, carboxymethyl cellulose (CMC) or birchwood xylan; 0.3% $NaNO_3$; 0.1% K_2HPO_4; 0.05% $MgSO_4 \cdot 7H_2O$; 0.05% KCl; 0.001% $FeSO_4 \cdot 7H_2O$; and 0.015% chloramphenicol. The initial pH of the medium was adjusted to 10.0 by addition of 0.5% (W/V) sterilized sodium carbonate.

Shaking culture experiments.

The conidia from potato dextrose agar plates were inoculated into the medium which reached 5×10^5 per m*l* in shake cultures unless otherwise stated. Shake cultures were carried out in 250 m*l* erlenmeyer flasks containing 50 m*l* of basal medium. The inoculated flasks were shaken continuously on an orbital shaker at 200 rpm and 30°C. To investigate the effects of carbon sources on the enzyme production, glucose-grown mycelium which was a 18 h old shake-flask preculture was suspended in the medium containing different carbon substrate.

Fermentation conditions

All batch fermentation experiments were carried out in a 5 L jar fermentor (Korea Fermentation Co.) containing 2.5 L of the basal medium which was the same as that described above, except that it contained different concentration of wheat bran as a carbon source. The conidia of 1×10^6 per m*l* medium were inoculated into the medium and cul-

tivated at 30°C. The pH was controlled at 9.5 automatically with 3 N sodium carbonate. The air flow rate was 1.5 vvm and agitation was set at 400 rpm during the whole fermentaton period. Antifoam agent (SAG-471, Union Carbide) was treated when severe foam was generated. Fed-batch cultures were conducted in 5 L jar fermentor with a working volume of 2.5 L. Each fermentation was initiated with 1% wheat bran. In fed-batch culture, intermittent feeding of wheat bran (equivalent to 0.5% of the medium volume) began after 24 h of cultivation and the feeding was repeated every 6 h, raising the final wheat bran concentration to 5%.

Enzyme assays

CMCase activity was measured in the reaction mixture consisting of 0.9 m*l* of 0.5% (W/V) carboxymethyl cellulose in 50 mM glycine-NaOH buffer (pH 9.5) and 0.1 m*l* of suitably diluted enzyme solution. The reaction mixture was then incubated in a shaking water bath at 40°C for 1 h. At the end of this reaction period, 1 m*l* of dinitrosalicylic acid solution was added and the mixture was heated in boiling water bath for 5 min. After then 2 m*l* of distilled water was added and absorbance of the sample was measured at 575 nm. Xylanase assays were identical to CMCase assay, except that birchwood xylan in 50 mM sodium phosphate buffer (pH 7.5) was used as the substrate and the reaction mixture was incubated at 40°C for 15 min. One unit of CMCase and xylanase activity was defined as the amount of enzyme which liberates 1 mol of reducing sugar, expressed as glucose or xylose, per minute under the reaction conditions. Reducing sugar production by enzymatic hydrolysis and free sugar in culture broth was quantified by the method of Miller (1959). Productivity of the enzymes was calculated by relating the enzyme activity obtained over a particular time period and expressed as units/L/h (Brown *et al.*, 1987).

Results and Discussion

Effect of carbon sources on enzyme production

To find the most favorable conditions for the production of CMCase and xylanase, equal portion of mycelium harvested from preincubation of *Cephalosporium* sp. RYM-202 in modified Czapek broth (pH 10.5) for 18 h, was inoculated into the medium containing different carbon sources. The carbon sources examined were: CMC (1%), xylan (1%), mixture of CMC (0.5%) and xylan (0.5%), and wheat bran (1%). Of the carbon sources tested, wheat bran gave the highest production of CMCase and xylanase (Fig. 1). The significant levels of CMCase and xylanase were also observed in the medium containing CMC and xylan, respectively. However, CMC-xylan mixture was commonly less effective for the induction of CMCase and xylanase. The production of CMCase on xylan and xylanase on CMC was nearly negligible. The present results suggest that the induction of CMCase and xylanase in *Cephalosporium* sp. RYM-202 depends on the type of carbon source in the medium and that the biosynthesis of CMCase and xylanase is regulated independently. Previous reports have indicated the synergistic effects of carbon sources on the biosynthesis of cellulase and xylanase systems in many fungal strains such as *Aspergillus nidulans* (Lee *et al.*, 1989), *A. niger* (Roh *et al.*, 1990), *Trichoderma longibrachiatum* (Royer and Nakas, 1990), and an acidophilic NC-11 (Cauchon and LeDuy, 1985). In these organisms, mixtures of cellulose and xylan were much more effective not only for the enhancement of the biosynthesis of cellulase and xylanase complexes but also for the balanced production of these enzyme components than individual sub-

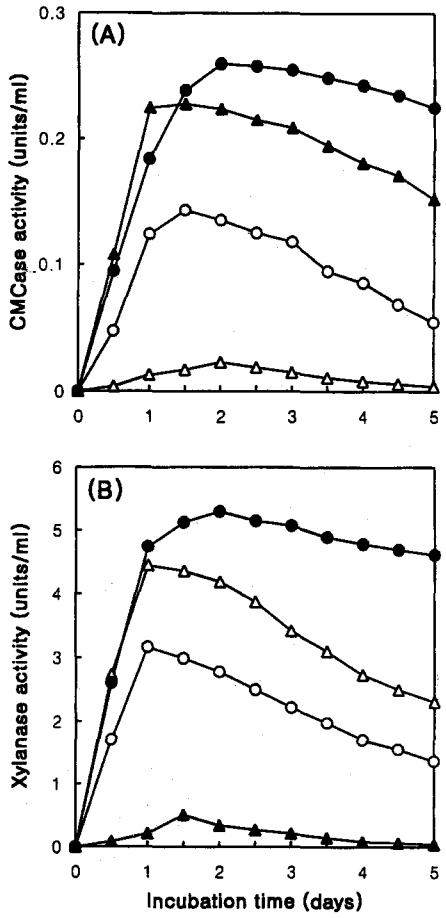

Fig. 1. Effects of different carbon sources on production of extracellular CMCase (A) and xylanase (B) by *Cephalosporium* sp. RYM-202 in shake flask cultures. Symbols; ▲, CMC (1%, w/v); △, xylan (1%, w/v); ○, CMC-xylan (0.5: 0.5%, w/v); ●, wheat bran (1 %, w/v).

strate. On the other hand, Gomes *et al.*, (1992) reported that cellulase and xylanase of *T. viride* were produced irrespective of the carbon source such as Avicel or xylan. In addition, Berenger *et al.*, (1985) reported that both of CMCase and xylanase were highly produced when *Clostridium stercorarium* was grown on xylan and cellulose, but surprisingly more xylanase was formed on cellulose than on xylan. In contrast, the effective inducers for CMCase production by *Cephalosporium* sp. RYM-202 were wheat bran and CMC, while xylanase was effectively induced separately on wheat bran and xylan. Very poor induction of CMCase on xylan and xylanase on CMC was found. From these results, biosynthesis of cellulase and xylanase in *Cephalosporium* sp. RYM-202 was considered to be regulated separately at the level of enzyme induction. The induction patterns of cellulase and xylanase in *Cephalosporium* sp. RYM-202 were very similar with those of *A. terreus* (Hrmova *et al.*, 1989, 1991). The induction specificity of these enzymes derived from *Cephalosporium* sp. RYM-202 is advantageous to differential production of CMCase or xylanase by using the corresponding inducer for each enzyme.

Effect of nitrogen sources on enzyme production

The nature of nitrogen source, either organic or inorganic, in medium is reported to affect the cell growth and enzyme production (Brown *et al.*, 1987; Chaabouni *et al.*, 1995). The effect of nitrogen source on the production of CMCase and xylanase by *Cephalosporium* sp. RYM-202 was evaluated in shake flask culture using the basal medium containing 2% wheat bran as carbon source and a range of nitrogen sources with different concentrations. As shown in Fig. 2, *Cephalosporium* sp. RYM-202 produced the enzymes well with the various nitrogen sources even with different concentrations. Among the nitrogen sources tested, nitrate and urea were more effective for the production of both enzymes. When nitrate was the nitrogen source, increasing of concentration from 0.5 to 20 g/L did not show a significant increase in either CMCase or xylanase activities. By contrast to nitrate, however, higher concentrations of urea exceeding 2 g/L were not favorable and markedly inhibited the production of the enzymes. Less difference in enzyme activities

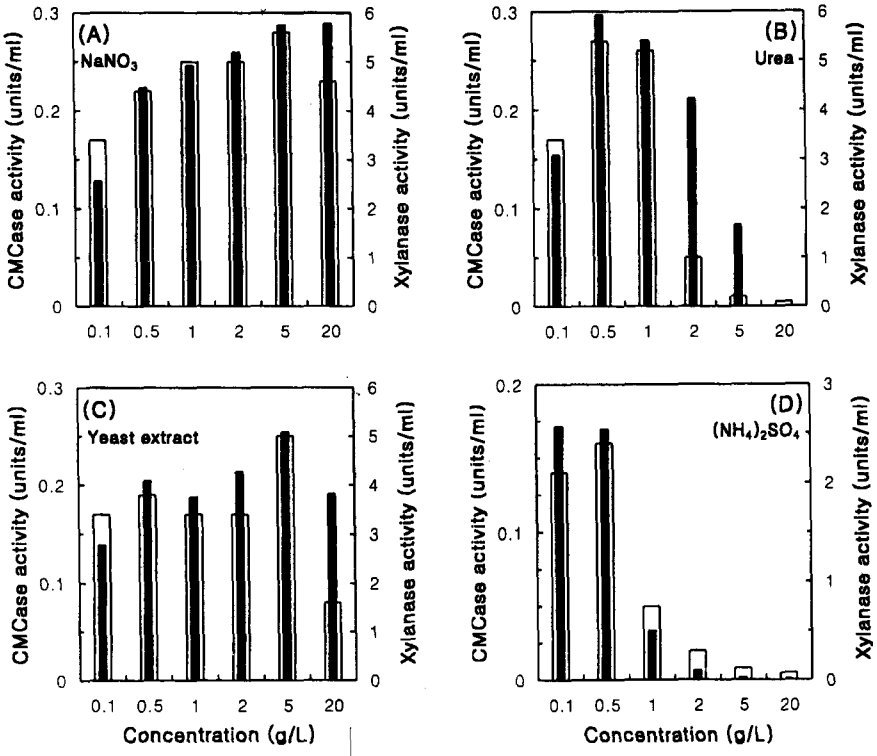

Fig. 2. Effects of different nitrogen sources on production of extracellular CMCase and xylanase by *Cephalosporium* sp. RYM-202 in shake flask cultures. Wheat bran (2%) was the carbon source and the basic mineral composition of Czapek broth was used except nitrogen source. The pH was adjusted to 10.0 with sodium carbonate. After 72 h of cultivation at 30°C, the enzyme activities were assayed. Symbols: □, CMCase; ■, xylanase.

between yeast extract and inorganic nitrogen sources indicates that amino acids are not essential for the enzyme production in *Cephalosporium* sp. RYM-202. Gomes *et al.* (1992) reported that the effective production of cellulase and xylanase by *T. viride* requires high concentrations of essential amino acids.

Effects of temperature and pH on enzyme production

The production of CMCase and xylanase by *Cephalosporium* sp. RYM-202 was examined at different temperatures. Although xylanase and CMCase, respectively, were maximally produced at 20°C and 30°C, there was no remarkable difference in enzyme activities within the temperature ranges from 20 to 30°C (Fig. 3). Neither of cell growth nor enzyme ac-

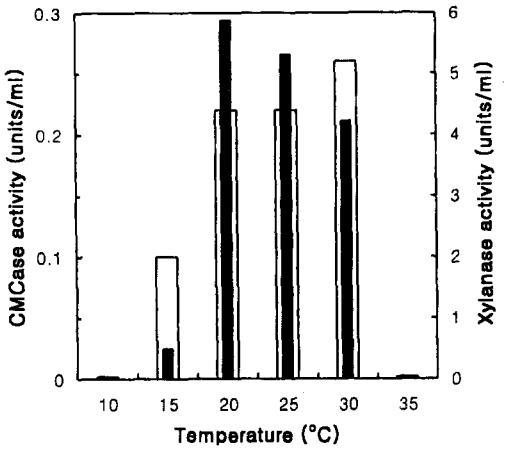

Fig. 3. Effect of temperature on CMCase and xylanase production. After 72 h of cultivation in the medium (pH 10.0) containing 2% wheat bran, the enzyme activities were assayed. Symbols: □, CMCase; ■, xylanase.

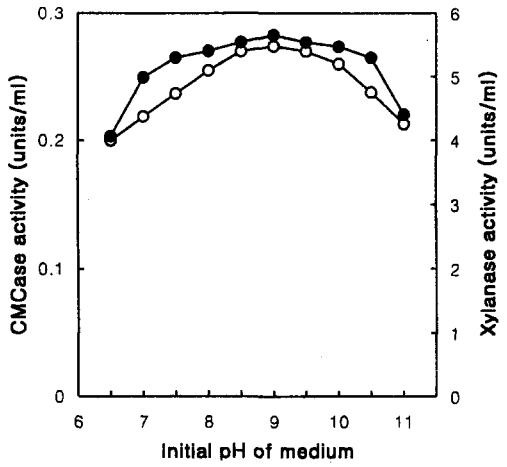

Fig. 4. Effect of initial pH of medium on CMCase and xylanase production. The initial pH of medium was adjusted by addition of sodium carbonate. After 72 h of cultivation, the enzyme activities were assayed. Symbols: ○, CMCase; ●, xylanase.

tivities could be detected at 35°C. The effect of initial pH of culture broth on the production of the enzymes was also examined (Fig. 4). The highest activity of both enzymes was obtained at pH 9.0. The CMCase and xylanase activities more than 90% of maximum activity took place over a pH range between 8.0 and 10.5. These results were very similar with the pH-profiles of cell growth of *Cephalosporium* sp. RYM-202 as described in previous report (Kang *et al.*, 1993).

Batch fermentation

To examine the effect of substrate concentration on the production of CMCase and xylanase by *Cephalosporium* sp. RYM-202, batch cultures in a jar fermentor were carried out using optimized culture conditions with varing amounts of wheat bran as a carbon source. Progress of time course of a typical batch fermentation in the basal medium (pH 9.0) containing 1%, 2% or 5% wheat bran is shown in Fig. 5. The highest activities of CMCase and xylanase increased with increasing substrate concentration. On 5%

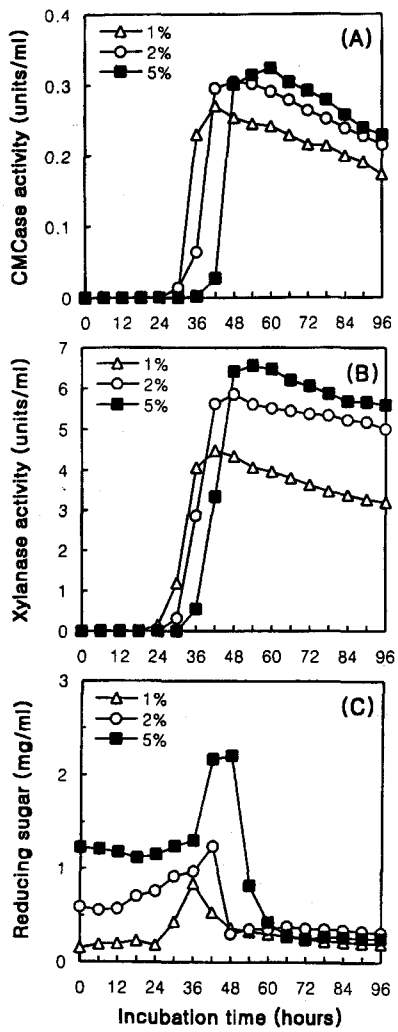

Fig. 5. Time courses of batch cultures of *Cephalosporium* sp. RYM-202 in a jar fermentor with different concentrations of wheat bran as a carbon source. The pH of medium was controlled at 9.5 during whole fermentation period.

wheat bran concentration, the maximum CMCase and xylanase activity was 0.324 and 6.46 units/ml, respectively. However, the increase of substrate concentration resulted in an extension of lag phase, and thus delayed the time at which the enzymes appeared in the culture medium. Synthesis of CMCase and xylanase commenced after 30~36 h on 1% wheat bran and 36~42 h on 5% wheat bran,

and the activities of both enzymes reached maxima around 42 h on 1% wheat bran and 54~60 h on 5% wheat bran. After reaching maxima, the enzyme activities declined to 67~87% of the maximum level after 96 h of incubation. The delay in appearance of enzyme activity in the medium may be due to catabolite repression (Godden *et al.*, 1989; Pinaga *et al.*, 1994) by the high concentration of reducing sugars which are originally included in the wheat bran (approximately 10 μg of reducing sugar per gram of substrate). Moreover, the delayed appearance of enzyme synthesis on higher substrate concentration resulted in lower productivity (units/L/h) of both enzymes. The maximum productivities of CMCase and xylanase on 5% wheat bran were 5.4 and 107 units/L/h, respectively, compared with 6.4 and 122 units/L/h, respectively, on 1% wheat bran. These results suggested that low level feeding of wheat bran by fed-batch culture would be more effective than batch culure for the production of CMCase and xylanase by *Cephalosporium* sp. RYM-202.

Fed-batch fermentation

Since fed-batch culture system has proved successful for many fermentations where carbon limitation is a requirement (Yamane and Shimizu, 1984), we attempted to use this fermentation mode. The fermentation was initiated as a conventional batch culture at 30°C using 0.5% wheat bran. At 24 h, when the level of reducing sugar was observed to be decreasing, the substrate level was raised to 1% by addition of wheat bran equivalent to 0.5% of total volume. This addition was repeated every 6 h, raising the final substrate concentration to 5%. The time course of a typical fed-batch fermentation is shown in Fig. 6. The enzyme activities were observed within 30~36 h and increased very rapidly reaching the enzyme titers of 0.34 units/m*l* for CMCase and 8.2 units/m*l* for xylanase at 42 h. After this the enzyme production increased slightly but steadily with the progress of fermentation and reached to maximum values (CMCase, 0.39 units/m*l* at 66 h; xylanase, 9.2 units/m*l*). The maximum activities of CMCase and xylanase in the fed-batch of fermentation on a 5% final wheat bran were 1.22 and 1.36 times higher respectively than those obtained in batch fermentation on 5% wheat bran. The fed-batch fermentation of *Trichoderma* spp. with controlled nutrient feeding has been an effective approach for high production of cellulase and xylanase (Hendy *et al.*, 1984; Gomes *et al.*, 1992). In these cases, the enzyme production was enhanced by 30-300% in comparison with the batch fermentation processes. Moreover, the simultaneous addition of cellulose and ammonia during fed-batch cultivation of *T. viride* was reported to be useful for the enhancement of cellulase production by 40% (Gottvaldova *et al.*, 1982). However, a fed-batch culture of *Cephalosporium* sp. RYM-202 in which wheat

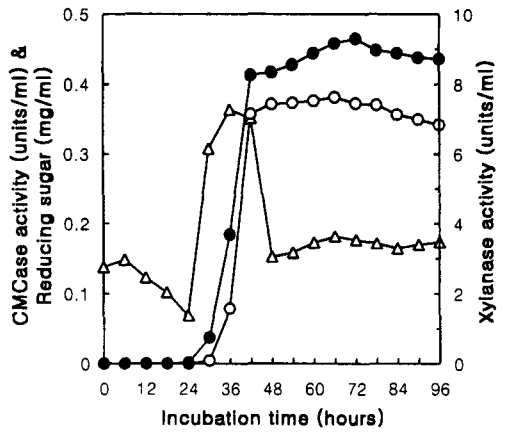

Fig. 6. Experimental results for the production of CMCase and xylanase in fed-batch fermentation of *Cephalosporium* sp. RYM-202. Intermittent feeding of wheat bran was repeated every 6 h after 24 h of cultivation, raising the final substrate concentration to 5%. Symbols: ○, CMCase; ●, xylanase; △, reducing sugar

bran was added intermittently together with other nutrients (minerals) in 100 ml water showed very similar enzymes titers as compared with the fed-batch culture without any addition of the minerals (data not shown). Even though, in this study, it was proven that fed-batch fermentation of *Cephalosporium* sp. RYM-202 is more useful than batch fermentation for the production of CMCase and xylanase, the overall productivities of these enzymes by this strain were relatively lower than those by cellulase- and xylanase-hyperproducing strains that have been mostly referenced in many literatures (Warzywoda and Pourquie, 1983; Cauchon and LeDuy, 1985; Gomes *et al.*, 1992, 1993; Adamsen *et al.*, 1995), and should be increased from an industrial viewpoint. It is still expected that further optimization of fed-batch conditions and medium composition will improve the production of the enzymes.

적 요

회분배양과 유가배양을 이용하여 호알칼리성 *Cephalosporium* sp. RYM-202로부터 alkaline carboxymethyl cellulase (CMCase)와 xylanase의 생산을 위한 배양조건에 대하여 조사하였다. 조사한 탄소기질 중에서 밀기울이 두 효소의 생산에 가장 효율적이었다. 또한 CMCase는 carboxymethyl cellulose (CMC)를 탄소기질로 첨가한 배양액에서, 반면에 xylanase의 경우에는 xylan을 기질로 하였을 때 높은 생산량을 나타냄으로서 유도기질 특이성을 보였는 바, 이 결과는 *Cephalosporium* sp. RYM-202에서의 CMCase와 xylanase의 생합성이 효소 유도 수준에서 독립적으로 조절됨을 시사해 준다. 조사된 질소원 중에서는 무기질소원인 $NaNO_3$가 효소생산에 효과적이었으며, 이들 효소의 최대 생산을 위한 배양온도와 pH는 각각 20°C와 9.0인 것으로 나타났다. 한편, 발효조에서의 회분배양을 통한 효소생산의 경우, 밀기울의 농도를 5%까지 증가시킴에 따라 효소생산량은 증가되었으나 catabolite repression에 의해 효소생산의 지연과 생산력의 감소를 초래하였다. 이러한 문제점은 탄소원의 간헐적 공급에 의한 유가배양을 통해서 어느 정도 해결될 수 있는 것으로 나타났으며, 밀기울의 최종농도가 5% 되게 공급된 유가배양시 CMCase와 xylanase의 최대 효소생산량은 각각 0.39 및 9.2 units/ml이었으며, 이는 같은 농도의 밀기울을 함유하는 회분배양시 획득된 효소활성에 비해 각각 1.22배와 1.36배 증가된 것이다.

Acknowledgements

This work was financially supported by research grants from the Korea Research Foundation (to M.K. Kang) and the Korea Science and Engineering Foundation (to Y.H. Rhee, grant no. 951-0503015-2).

References

Adamsen, A.K., Lindhagen, J. and Ahring, B.K. 1995. Optimization of extracellular xylanase production by *Dictyoglomus* sp. B1 in continuous culture. *Appl. Microbiol. Biotechnol.* **44**: 327-332.

Berenger, J.F., Frixon, C., Bigliardi, J. and Creuzet, N. 1985. Production, purification, and properties of thermostable xylanase from *Clostridium stercorarium*. *Can. J. Microbiol.* **31**: 635-643.

Brown, J.A., Collin, S.A. and Wood, T.M. 1987. Development of a medium for high cellulase, xylanase and β-glucosidase production by a mutant strain (NTG III/6) of the cellulolytic fungus *Penicillium pinophilum*. *Enzyme Microb. Technol.* **9**: 355-360.

Cauchon, N. and LeDuy, A. 1985. Novel process for the production of cellulolytic enzymes. *Biotechnol. Bioeng.* **27**: 456-462.

Chaabouni, S.E., Belguith, H., Hassairi, I., M'Rad, K. and Ellouz, R. 1995. Optimization of cellulase production by *Penicillium occitanis*. *Appl. Microbiol. Biotechnol.* **43**: 267-269.

Dey, D., Hinge, J., Shendye, A. and Rao, M. 1992. Purification and properties of extracellular endoxylanase from alkalophilic thermophilic *Bacillus* sp. *Can. J. Microbiol.* **38**: 436-442.

Gilbert, H.J. and Hazlewood, G.P. 1993. Bacterial cellulases and xylanases. *J. Gen. Mi-*

crobiol. **139**: 87-194.

Godden, B., Legon, T., Helvenstein, P. and Penninckx, M. 1989. Regulation of the production of hemicellulolytic and cellulolytic enzymes by a *Streptomyces* sp. growing on lignocellulose. *J. Gen. Microbiol.* **135**: 285-292.

Gomes, I., Gomes, J., Steiner, W. and Esterbauer, H. 1992. Production of cellulase and xylanase by a wild strain of *Trichoderma viride*. *Appl. Microbiol. Biotechnol.* **36**: 701-707.

Gomes, J., Purkarthofer, H., Hayn, M., Kapplmuller, J., Sinner, M., and Steiner, W. 1993. Production of a high level of cellulase-free xylanase by the thermophilic fungus *Thermomyces lanuginosus* in laboratory and pilot scales using lignocellulosic materials. *Appl. Microbiol. Biotechnol.* **39**: 700-707.

Gottvaldova, M., Kucera, J., and Podrazky, V. 1982. Enhancement of cellulase production by *Trichoderma viride* using carbon/nitrogen double-fed-batch. *Biotechnol. Lett.* **4**: 229-232.

Herbert, R.A. 1992. A perspective on the biotechnological potential of extreamophiles. *TIBTECH.* **10**: 395-402.

Hendy, N.A., Wilke, C.R. and Blanch, H.W. 1984. Enhanced cellulase production in fed-batch culture of *Trichoderma reesei* C-30. *Enzyme. Microbiol. Technol.* **6**: 73-77.

Hoq, M.M. and Deckwer, W.-D. 1995. Cellulase-free xylanase by thermophilic fungi: a comparison of xylanase production by two *Thermomyces lanuginosus* strains. *Appl. Microbiol. Biotechnol.* **43**: 604-609.

Hrmova, M., Biely, P. and Vrsanska, M. 1989. Cellulose and xylan-degrading enzymes of *Aspergillus terreus* and *Aspergillus niger*. *Enzyme Microb. Technol.* **11**: 610-616.

Hrmova, M., Petrakova, E. and Biely, P. 1991. Induction of cellulose- and xylan-degrading enzyme systems in *Aspergillus terreus* by homo- and heterodisaccharides composed of glucose and xylose. *J. Gen. Micribiol.* **137**: 541-547.

Ito, S., Shikata, S., Ozaki, K., Kawai, S., Okamoto, K., Inoue, S., Takei, A., Ohta, Y.I. and Satoh, T. 1989. Alkaline cellulase for laundry detergents: production by *Bacillus* sp. KSM-635 and enzymatic properties. *Agric. Biol. Chem.* **53**: 1275-1281.

Kang, M.K., Park, H.M., Rhee, Y.H., Kim, Y.S. and Kim, Y.K. 1993. Partial purification and some properties of carboxymethyl cellulases from *Cephalosporium* sp. RYM-202. *Kor. J. Mycol.* **21**: 301-309.

Kang, M.K., Kwon, T.I., Yang, Y.K. and Rhee, Y. H. 1995. Purification and characterization of a xylanase from alkalophilic *Cephalosporium* sp. RYM-202. *J. Microbiol.* **33**: 109-114.

Kang, M.K., Maeng, P.J. and Rhee, Y.H. 1996. Purification and characterization of Two xylanases from alkalophilic *Cephalosporium* sp. strain RYM-202. *Appl. Environ. Microbiol.* **62**: 3480-3482.

Kang, M.K. and Rhee, Y.H. 1995. Carboxymethyl cellulases active and stable at alkaline pH from alkalophilic *Cephalosporium* sp. RYM-202. *Biotechnol. Lett.* **17**: 507-512.

Kuhad, R.C. and Singh, A. 1993. Lignocellulose biotechnology:current and future prospects. *Crit. Rev. Biotechnol.* **13**: 151-172.

Lee, J.A., Maeng, J.S., Maeng, P.J. and Rhee, Y. H. 1989. Synergistic effect of substrates on the biosysthesis of cellulase and xylanase complexes from *Aspergillus nidulans*. *Kor. J. Mycol.* **17**: 57-65.

Lejeune, R. and Baron, G.V. 1995. Effect of agitation on growth and enzyme production of *Trichoderma reesei* in batch fermentation. *Appl. Microbiol. Biotechnol.* **43**: 249-258.

Miller, G.L. 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal. Chem.* **31**: 426-428.

Nakamura, S., Wakabayashi, K., Nakai, R., Aono, R. and Horikoshi, K. 1993. Purification and some properties of an alkaline xylanase from alkalophilic *Bacillus* sp. strain 41M-1. *Appl. Environ. Microbiol.* **59**: 2311-2316.

Pinaga, P., Fernandez-Espinar, M.T., Valles, S. and Ramon, D. 1994. Xylanase production in *Aspergillus nidulans*: Induction and carbon catabolite repression. *FEMS Microbiol. Lett.* **115**: 319-324.

Ratto, M., Poutanen, K. and Viikari, L. 1992. Production of xylanolytic enzymes by an alkalitolerant *Bacillus circulans* strain. *Appl. Microbiol. Biotechnol.* **37**: 470-473.

Rho, J.R., Rhee, Y.H. and Chung, J.H. 1990. Influence of substrates on the isozyme patterns of cellulase and xylanase complexes in *Aspergillus niger*. *Kor. J. Mycol.* **18**: 209-217.

Rhyum, S.B., Kang, M.K., Maeng, P.J., Park, H.

M. and Rhee, Y.H. 1993. Purification and characterization of xylanases from alkalophilic *Streptomyces* sp. S-510. *Kor. J. Microbiol.* **31**: 436-444.

Royer, J.C. and Nakas, J.P. 1990. Interrelationship of xylanase induction and cellulase induction of *Trichoderma longibrachiatum*. *Appl. Environ. Microbiol.* **56**: 2535-2539.

Senior, D.J., Mayers, P.R. and Saddler, J.N. 1991. The interaction of xylanases with commercial pulp. *Biotechnol. Bioeng.* **37**: 274-279.

Warzywoda, V.F. and Pourquie, J. 1983. Development of a culture medium for large scale production of cellulolytic enzymes by *Trichoderma reesei*. *Biotechnol. Bioeng.* **25**: 3005-3010.

Wong, K.K.Y. and Saddler, J.N. 1992. *Trichoderma* xylanases, their properties and application. *Crit. Rev. Biotechnol.* **12**: 413-435.

Yamane, T. and Shimizu, S. 1984. Fed-batch techniques in microbial processes. pp. 147-194. *In*: Fiechter, A. Ed. Advances in Biochemical Engineering/Biotechnology. Vol. 30, Springer-Verlag.