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PRODUCTION, RELEASE AND THERMAL CHARACTERIZATION OF CELLULOLYTIC ENZYME FROM *CELLULOMONAS* sp. STRAIN "O"

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Abstract

Cellulase production by a *Cellulomonas* sp., isolated in 1985 from forest humus soil along the border of the Caspian Sea in Iran, was investigated. This strain secreted endo-and exo-cellulases in the culture medium, but some of the enzymes produced remained cell membrane bound. Cell bound enzymes were released by various treatments. The highest amount of endo-glucanase (up to 95%) and exo-glucanase (62%) was released when the cells were treated with 1% Tween 80 for 300 and 60 minutes, respectively. No extracellular or cell membrane bound β-glucosidase was obtained. Optimum temperature for the endo-glucanase and exo-glucanase activities was 50°C and optimum pH was 7 for both enzymes. Thermal stability of enzymes was measured at 30 to 50°C and was compared with that of endo- and exo-glucanses from mesophilic fungi such as *Neurospora crassa, Trichoderma viride* and *Pencillium funiculosum*. It was found that the thermal stability of endo- and exo-glucanases from *Cellulomonas* sp. strain "O" was the same as that of fungal enzymes up to 37°C, however, at higher temperatures these enzymes lost their activities faster than the fungal cellulases.

Introduction

Cellulose is the most abundant carbohydrate waste. It consists of a linear chain of β-1, 4-linked D-glucose residues. Its enzymatic hydrolysis is thought to require the action of endo-glucanases (β-1,4-glucan-glucanohydrolase), exo-glucanases (β-1,4-glucan cellobiohydrolase) and β-glucosidase. A synergistic action of these enzymes is necessary for the complete hydrolysis of crystalline cellulose [11]. Microbial cellulase production has been studied extensively in various microorganisms [5,12], and it has been shown that *Penicillium occitanis* and *Trichoderma reesei* secreted extracellular cellulases with reasonable levels of endo- and exo-glucanase activities and a high level of β-glucosidase activity [3, 10, 12, 20].

Among bacteria, *Cellulomonas* species have been given the most attention for cellulolytic enzymes production by many investigators. *C. flavigena* [1], *C. fradia* [5], *C. fimi* [14] and *C. fermentans* [1] and characterization and specificity of these enzymes have been reported [5,9,18]. This paper reports on the production, release and thermal characterization of cellulolytic enzymes of *Cellulomonas* sp. strain "O" which have already been

Keywords: Cellulolytic enzymes; *Cellulomonas* sp.; Characterization; Production; Release

described [13].

Materials and Methods

Microorganisms, Culture Media and Enzymes

Cellulomonas sp. strain "O" was isolated from forest humus soil along the border of the Caspian sea, Iran, and identified as described earlier [13]. The lyophilized bacterium was first cultured on peptone yeast extract glucose medium (PYG) containing 2% agar and incubated at 30°C for 120 hours. For the production of cellulases the strain was grown in a 500 ml flask containing 100 ml of 0.5% microcrystalline cellulose, 0.2% yeast extract, 0.1% $NaNO_3$, 0.1% K_2HPO_4, 0.05% KCl, 0.05% $MgSO_4$ and 0.1% Tween 80. Cellulases from *T. viride* and *Penicillium funiculosum* were purchased from Sigma (USA) company. Cellulase from *N.crassa* was prepared according to Yazdi *et al.* [23]. All other materials were prepared from Sigma or Merck (Germany) Companies.

Protein Measurement

Protein was measured according to Bradford [2]. The method was standardized by fraction V bovine serum albumin, and a standard curve was drawn for protein concentrations between 10-100 µg/ml and was used for protein estimation.

Enzyme Assay

Carboxymethylcellulose (CMC), low viscosity, was used as substrate for measurement of endo-glucanase activity, and filter paper Whatman No.1 was used for estimation of exo-glucanase activity. Para-nitrophenyl-β-D-glucopyranoside (Fluka) was the substrate for measurement of β-glucosidase activity. The methods used for the measurement of enzyme activities were according to Yazdi *et al.* [23,24].

Enzyme activities are expressed in units, defined as µmol products (reducing sugar as glucose for endo- and exo-glucanase and para-nitro-phenol for β-glucosidase) liberated by 1 ml of enzyme in either 15 min (for endo-glucanase and β-glucosidase) or 60 min (for exo-glucanase).

Release of Cell Membrane Bound Enzymes

Experiments for the isolation of cell membrane bound enzymes were performed according to the method of Penefsky *et al.*[16]. In this procedure, strain "O" was grown in 100 ml of the production medium without Tween 80. After 120 hours, when there was nearly no cellulose left in the medium, cells were removed by centrifugation at 10,000 rpm for 10 min at 4°C, the pellet was washed twice with 10 ml of 0.1 M phosphate buffer pH7, resuspended in 100 ml of 0.1 M phosphate buffer pH7 and used as the source for releasing the cell membrane bound enzymes.

To investigate the effect of detergents, 1 ml Tween-80 was added to the 100 ml of cell suspension and incubated at 30°C with shaking at 150 rpm. Samples were taken after 60, 180 and 300 min of incubation and cellulolytic activities were measured in the supernatant of the samples after removing cells by centrifugation at 10,000 rpm for 10 min.

To find the effect of sonication on the cell membrane bound enzymes, 100 ml of the cell suspension in a 500 ml flask was sonicated at 100 W while the flask was placed in ice to prevent thermal inactivation of the enzymes. The activities of released enzymes were measured after 4,8 and 12 min.

To find the effect of solvents (acetone, ethanol and butanol), a washed pellet of 100 ml culture media was suspended in 100 ml phosphate buffer (pH 7,0.1 M). To this 100 ml of cold acetone, ethanol or butanol was added and the mixture was kept at -20°C. After 1 hour the suspensions were centrifuged for 10 minutes at 10,000 rpm, supernatants were discarded and pellet (cells and precipitated released enzymes) were resuspended in 100 ml phosphate buffer 0.1 M, pH 7. After 1 hour cells were separated from solved enzymes by centrifugation as above. The activities of enzymes were measured. In all experiments Gram stain and microscopic examination were used to ensure cell integrity. It was found that the conditions were quite suitable and all released enzymes can be considered as cell membrane bound enzymes.

For comparison of thermal stability of the cellulolytic enzymes of *Cellulomonas* sp. strain "O" with those of mesophilic fungi, solutions of the cellulase enzymes with nearly the same concentrations of protein (600-900 µg/ml) and enzyme activities (endo-glucanase 39-52 U/ml, exo-glucanase 15-21 U/ml) were incubated at 30, 37, 40, 45 and 50°C for 48 hours. Samples were taken at 1, 2, 3, 6, 12, 24 and 48 hour intervals, and enzyme activities were measured.

Results and Discussion

β-glucosidase, endo- and exo-glucanase activities were measured in the supernatant after removing the microorganisms and unused cellulose by centrifugation at 10,000 rpm for 10 min. For five days, 3 ml samples were taken daily for the measurements. As shown in Figure 1, the isolate secretes both endo- and exo-glucanases, while no β-glucosidase was found in the culture medium, the production of intra cellular β-glucosidase was evident. Extracellular and cell membrane bound production of endo- and exo-glucanase and intracellular production of β-glucosidase have been shown in many microorganisms such as *C. fimi*[15]; *Microbispora bispora*[22]; and *Ruminococcus flavefaciens* [7].

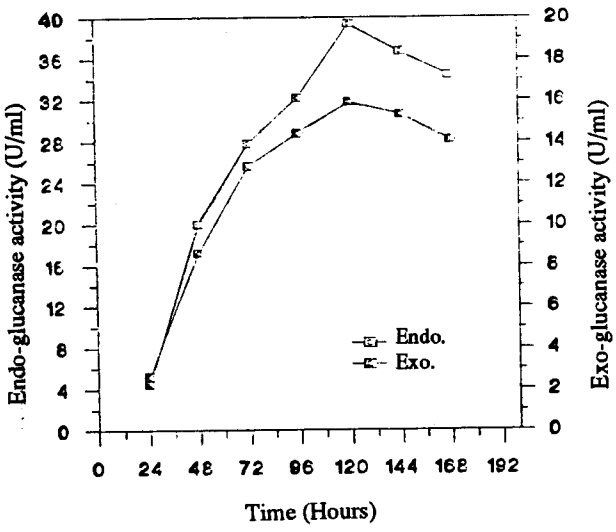

Figure 1. Production of cellulolytic enzymes by *Cellulomonas* sp. strain "O" in liquid media

As shown in Tables 1, 2 and 3, fractions of the enzymes produced by *Cellulomonas* sp. Strain "O" remained cell membrane bound and they were released by various chemical and physical treatments. Tween-80 was the best for releasing endo- and exo-glucanases and about 95% of endo- and 62% of exo-glucanase were released by 1% Tween 80 after 300 and 60 minutes respectively. This enzyme releasing effect of Tween-80 may be the reason for the positive effect of Tween-80 on the production of cellulase by *Cellulomonas* sp. and other cellulolytic microorganisms [19,23]. The amount of the enzymes released by organic solvents was not as high as that of Tween-80, probably due to some denaturation brought on by the solvents. Although sonication released the enzymes easily from the cell membrane, most of them were rapidly denatured because of the heat which is inevitably produced during sonication.

A concentrated solution of produced cellulase with 60% W/V ammonium sulphate (protein 630 µg/ml, endo-glucanase 46.5 U/ml, and exo-glucanase 18.8 U/ml) was used to determine the optimum pH and temperature. It was found that optimum temperature and pH for endo- and exo-glucanases activities were 50°C and 7 for both enzymes, respectively (Figs. 2 &3). These data were similar to those of mesophilic fungi [4, 6, 8, 25].

Thermal stability of the concentrated enzymes was also determined. Results are shown in Figures 4 and 5. Endo -and exo-glucanases of *Cellulomonas* sp. strain "O" exhibited high stabilities and nearly retained their activities up to 85% at 37°C and 100% at 30°C for 48 hours. At higher temperatures, the activities of endo- and exo-glucanases decreased rapidly.

As shown in Figures 6 and 7, the stability of

Table 1. Activities of the cell membrane bound enzymes in the presence of 1% Tween 80 after 60, 180 and 300 minutes

Fractions	Endo-glucanase U/ml - %	Exo-glucanase U/ml - %
Supernatant	14.60 - 100%	1.75 - 100 %
Cell suspension	0.0 0.0	0.0 0.0
" " 60 min	10.27 70.3	1.1 62.8
" " 190 min	12.50 85.6	0.90 51.4
" " 300 min	13.88 95.0	0.90 51.4

Table 3. Activities of the cell membrane bound enzymes after release by organic solvents treatments

Fractions	Endo-glucanase U/ml - %	Exo-glucanase U/ml - %
Supernatant	4.5 - 100%	1.52 - 100 %
Cell suspension	0.0 0.0	0.0 0.0
Released enzymes with acetone	9.24 63.7	0.86 56.5
Ethanol	7.60 52.4	0.48 31.5
Butanol	4.22 29.1	0.78 51.3

Table 2. Activities of the cell membrane bound enzymes after 4,8 and 12 minutes of sonication

Fractions	Endo-glucanase U/ml - %	Exo-glucanase U/ml - %
Supernatant	14.60 - 100%	1.73 - 100 %
Cell suspension	0.0 0.0	0.0 0.0
After 4 min sonication	9.0 62.1	0.67 38.7
" 8 " "	9.5 65.5	0.31 17.9
" 12 " "	7.11 49.0	0.22 12.7

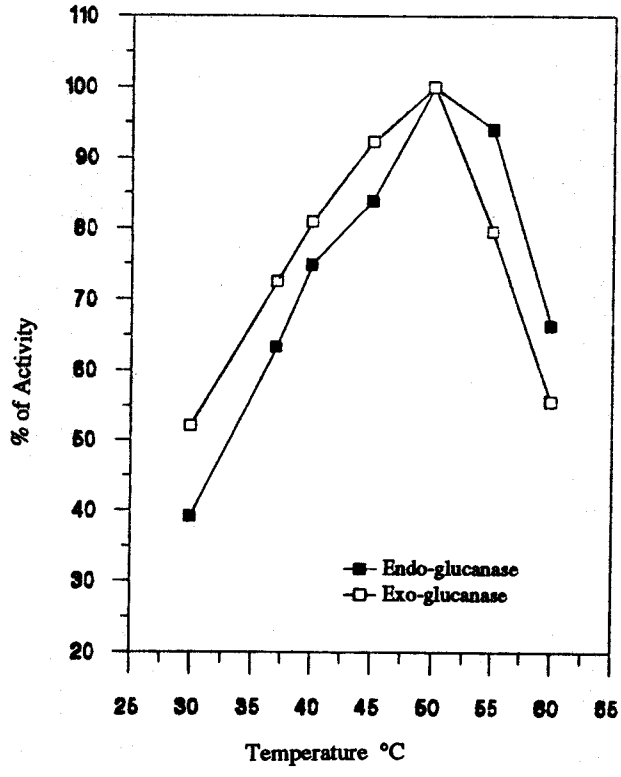

Figure 2. Activities of exo-glucanase and endo-glucanase from *Cellulomonas* sp. strain "O" at various temperatures

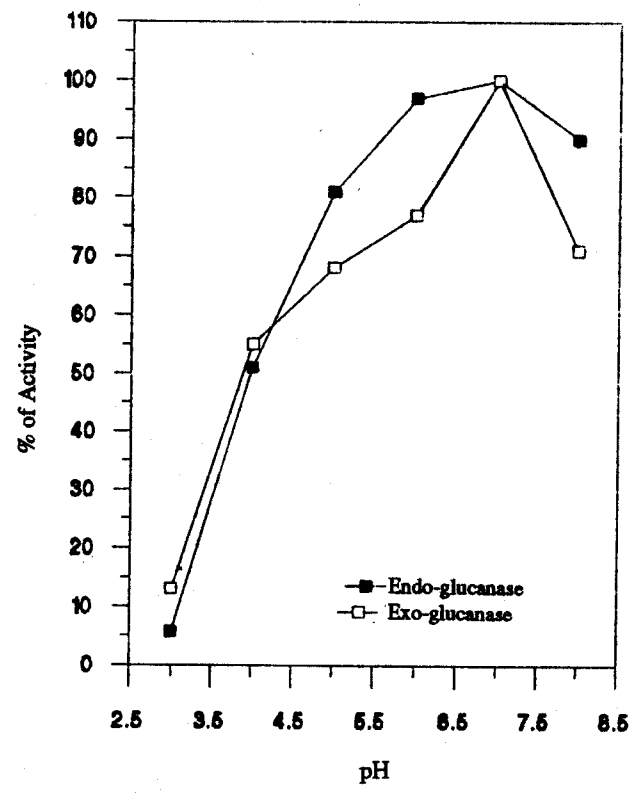

Figure 3. Activities of exo-glucanase and endo-glucanase from *Cellulomonas* sp. strain "O" at various pH

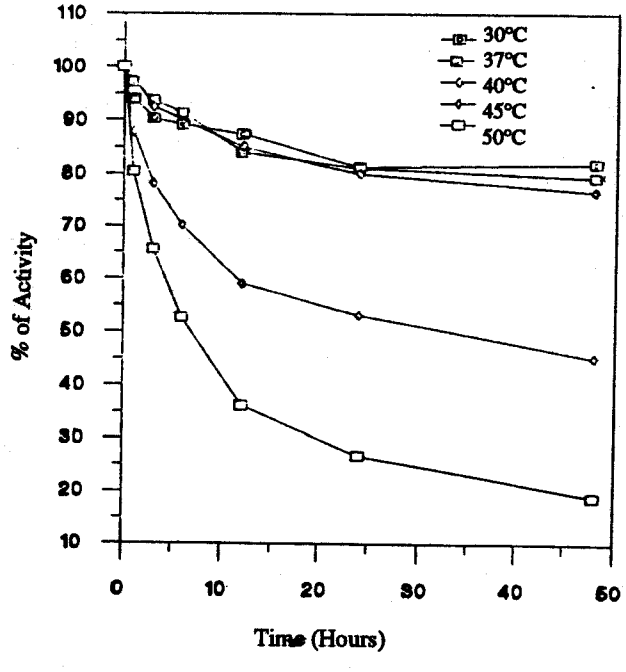

Figure 4. Thermal stability of endo-glucanases in *Cellulomonas* sp. strain "O"

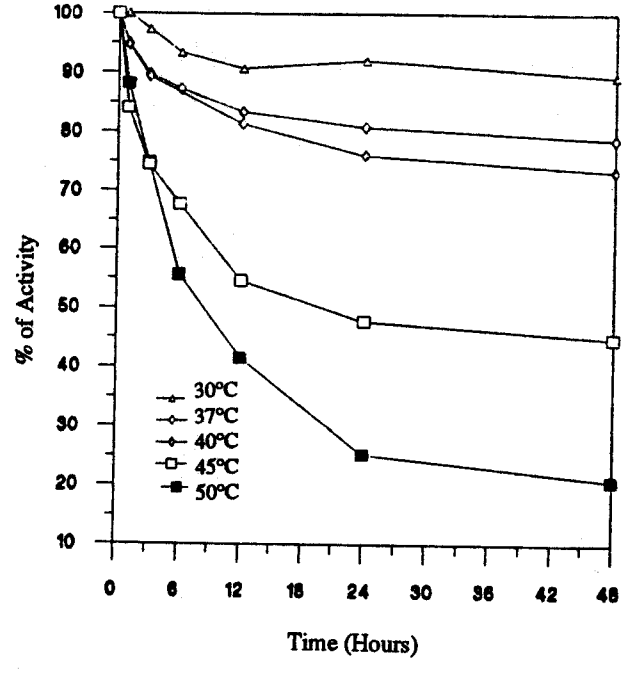

Figure 5. Thermal stability of exo-glucanases in *Cellulomonas* sp. strain"O"

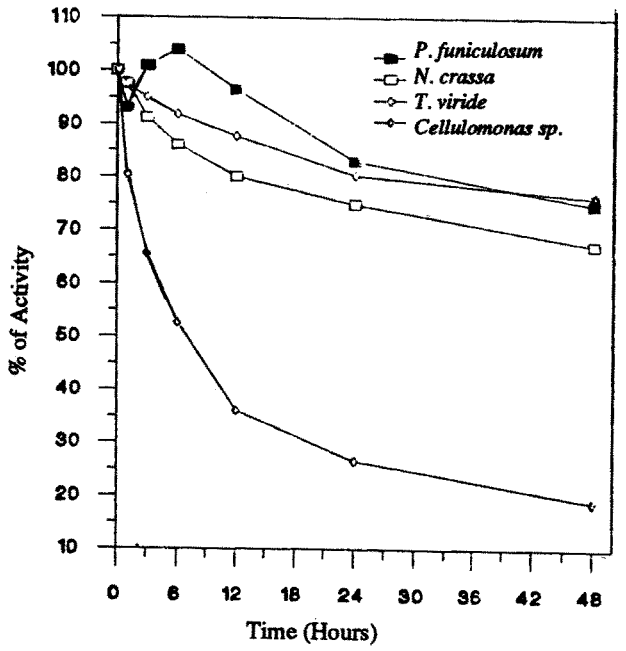

Figure 6. Comparison of thermal stability of endo-glucanases in *Cellulomonas* sp. strain "O" and some mesophilic fungi at 50°C

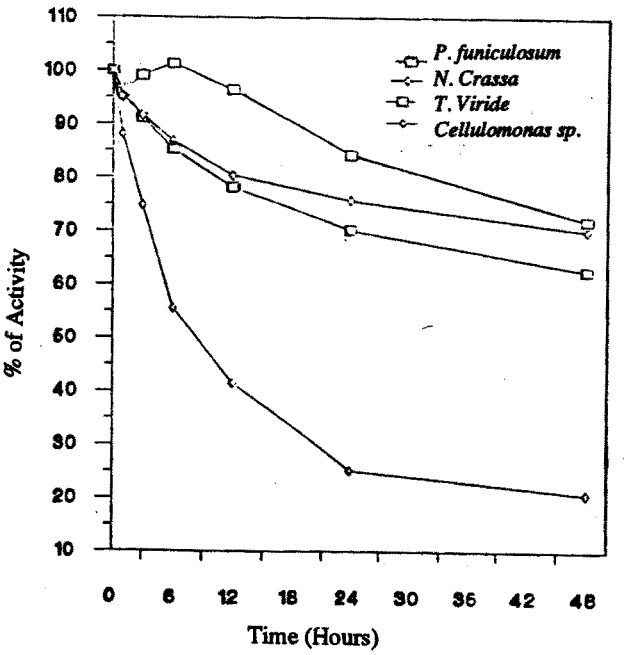

Figure 7. Comparison of thermal stability of exo-glucanases in *Cellulomonas* sp. strain "O" and some mesophilic fungi at 50°C

Cellulomonas sp. enzymes was nearly the same as that of mesophillic fungi up to 40°C for 48 hours, however, they were less stable at higher temperatures. More than 85% of the activities of endo- and exo-glucanases from *Cellulomonas* sp. strain "O" was lost at 50°C in 48 hours, while the cellulase activity of the fungi was nearly stable up to 50°C and only about 25-30% of their activity was lost under this condition.

Acknowledgements

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References

1. Bagnara, C., Gaudina, C. and Belaich, J. P. Purification and partial characterization of two extracellular endo-glucanases from *Cellulomonas fermentans*. *Biochem. and Biophys. Res. Commun.*, **140**, 219-229, (1986).
2. Bradford, M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Anal. Biochem.*, **144**, 142-146, (1976).
3. Chaabouni, E., Belguith, H. and Hassairi, I. Optimization of cellulase production by *Penicillium occitanis*. *Appl. Microbiol. Biotechnol.*, **43**, 267-269, (1995).
4. Durand, K., Soucaille, P. and Tiraby, G. Comparative study of cellulase and hemicellulase from fungi. *Enzy. Microb. Technol.*, **6**, 175-180, (19840).
5. Enari, T.M. Microbial cellulases. In *Microb. enzymes and biotechnol.*, (ed. W. M. Fogarty) pp. 183-220. Applied Sciences, Barking, Essex, (1983).
6. Fahnrick, P. and Irrgang, K. Some characteristics of the cellulolytic enzyme system of *Chaetomium cellulolyticum*. *Biotechnol. Lett.*, **4**, 519-524, (1982).
7. Gardner, R.M., Doewer, K.C., and White, B.A. Purification and characterization of exo-β-, 1, 4-glucanase from *Ruminococcus flavefaciens* FD-1. *J. Bacteriol.*, **169**, 4581-4588, (1987).
8. Hagedal, B., Ferchak, J.D. and Pye, E.K. Saccharification of cellulose by cellulolytic enzyme system of *Thermomonospora* sp. I. Stability of cellulolytic activities with respect to time, temperature and pH. *Biotech. Bioeng.*, **22**, 15/5-1526, (1980).
9. Haggett, K.D., Choi, W.Y. and Dunn, N.W. Mutants of *Cellulomonas* which produce increased levels of β-glucosidase. *Eur. J. Appl. Microbiol. Biotechnol.*, **6**, 189-191, (1978).
10. Jain, S., Pariche, M., Durand, H. and Tisaby, G. Production of polysaccharides by a cellulase pectinase hyper producing mutant (pol 6) of *Penicillium occitanis*. *Enzyme Microb. Technol.*, **12**, 691-696, (1990).
11. Kosaric, N., Wieczorek, A., Cosentino, G. P., Magee, R.J. and Prenosil, J. E. Ethanol fermentation. In *Biotechnology. A comprehensive treatise in eight volumes*. Vol. 3 (Ed. H. Dellweg). VCH, (1983).
12. Kubicek, C.P., Eveleigh, D.E., Esterbauer, H., Steiner, W. and Kubicek-Pranz, E.M. *Trichoderma cellulases*: Biochemistry, genetics, physiology and application. The

Royal Chemical Society, Cambridge, U.K. (1991).

13. Malekzadeh, F., Azin, M., Shahamat, M. and Colwell, R.R. Isolation and identification of three *Cellulomonas* sp. from forest soil. *W. J. of Microbiol. and Biotechnol.*, **9**, 53-55, (1993).
14. Meinke, A., Gilkes, N. R., Kilburn, D. G., Miller, J.R. and Warren, R.A. J. Cellulase binding polypeptides from *Cellulomonas fimi*: endoglucanase D (cen D), a family A β-1, 4-glucanase. *J. Bacteriol.*, **175**, 1910-1918, (1993).
15. Miller, R.C., Gilkes, N.R., Greenberg, N.M., Kilburn, D.G., Langsford, M.L. and Warren, R.A.J. *Cellulomonas fimi*: Cellulases and their genes. In *Biochemistry and genetics of cellulase degradation, FEMS Symposium No. 43*. (ed. J. P. Aubert, P. Beguin, and J. Millet) pp. 235-248. Academic Press, London, (1988).
16. Penefsky, H.S. and Tzagoloff, A. Extraction of water-soluble enzymes and proteins from membranes. In *Methods in enzymology*, Vol. 22, pp. 204-219, Academic Press, London, (1971).
17. Ponce-Noyola, T. de la and Torre, M. Interactions in a mixed culture composed of *Cellulomonas flavigena* and *Xanthomonas* sp. growing in continuous culture on sugar cane bagasse pith. *Appl. Microbiol. Biotechnol.* **40**, 531-534, (1995).
18. Rodriguez, H., Volfova, O. and Klysov, A. Characterization of the cellulase complex from *Cellulomonas* grown on bagasse pith. *Ibid.*, **28**, 394-397, (1988).
19. Sami, A.J. and Akhtar, M. W. Purification of two low molecular weight endoglucanases of *Cellulomonas flavigena*. *Enzyme Microb. Technol.*, **15**, 586-592, (1993).
20. Suominen, P. and Reinikainen, T. *Trichoderma reesei* cellulases and other hydrolases. Enzyme structures, biochemistry, genetics and applications. In *Proceedings of the Tsical 93 Symposium*. June 2-5, Espoo. Finland. (1993).
21. Wakarchuk, W.W., Kilburn, D.G., Miller, J. R. and Warren, R. A. J. The preliminary characterization of the β-glucosidase of *C. fimi*. *J. Gen. Microbiol.*, **130**, 1385-1389, (1984).
22. Yablonsky, M.D., Bartley, T., Elliston, K.O., Kahrs, S. K., Shalita, Z.P. and Eveleigh, D. E. Characterization and cloning of the cellulase complexes of *Microbispora bispora*. In *Biochemistry and genetics of cellulose degradation*, FEMS symposium, No. 43, (ed. J.P. Aubert, P. Beguin and J. Millet), pp. 249-266. Academic Press, London, (1988).
23. Yazdi, M. T., Radford, A. and Woodward, J.R. Optimization of cellulase production by *Neurospora crassa*. *Enzyme Microb. Technol.*, **12**, 116-120, (1990[a]).
24. Yazdi, M. T., Radford, A., Keen, J. N. and Woodward, J. R. Cellulase production by *Neurospora crassa*, Purification and characterization of cellulolytic enzymes. *Ibid.*, **12**, 120-123, (1990[b]).
25. Yazdi, M.T., Radford, A. and Woodward, J. R. The cellulase complex of *Neurospora crassa*: activity, stability and release. *J. Gen. Microbiol.*, **136**, 1313-1319, (1990[c]).