Pak. J. Bot., 46(5): 1911-1914, 2014.

COMPARATIVE STUDIES ON PRODUCTION OF CELLULASES FROM THREE STRAINS OF *ASPERGILLUS NIGER*

MUHAMMAD SOHAIL, AQEEL AHMAD AND SHAKEEL AHMED KHAN*

Department of Microbiology, University of Karachi, Karachi-75270, Pakistan
**Correspondence author e-mail: shakeelkhan@uok.edu.pk; Phone No: +(9221) 99261300 Extension No: 2369*

Abstract

Three strains of *Aspergillus niger* were retrieved from culture collection of the Department of Microbiology, University of Karachi, Karachi, Pakistan and were studied for their ability to produce cellulases. Cultivation at different temperatures and in presence of various carbon sources revealed that all the three strains produced more amounts of endoglucanase, β-glucosidase and filter-paperase activities at 35°C; carboxymethyl cellulose promotes the production of filter paperase and endoglucanase activities whereas salicin induced β-glucosidase activity. Experiments on growth and enzyme production kinetics showed that generation time and hence volumetric rate of biomass production is influenced by the carbon source used in the medium; simple carbon source, such as glucose favored the growth of all the strains. Cellulases from all the strains showed optimum activity at temperature ≥50°C and under acidic range of pH, while melting temperature was 64-65°C. These findings affirm that cellulases from *A. niger* are potential candidates as alternative to *Trichoderma* cellulases.

Key words: *Aspergillus niger*, Endoglucanase, β-glucosidase, Volumetric productivity.

Introduction

Fungi are considered as natural decomposers of organic matter which is because of their ability to penetrate materials deeply and to produce a battery of hydrolytic enzymes. One of the most important hydrolases elaborated by fungi to degrade organic material is a complex set of related enzymes called 'cellulase'. This enzyme hydrolyze the β-1,4-glycosidic linkage of an abundant organic material, cellulose. There are different types of cellulase that act together to bring about complete hydrolysis of cellulosic materials (Bhat, 2000). These include endoglucanase, (1,4-D-glucan-4-glucanohydrolase; EC 3.2.1.4), exocellobiohydrolase (1,4-D-glucan glucohydrolase; EC 3.2.1.74) and β-glucosidase (D-glucoside glucohydrolase; EC 3.2.1.21).

A large number of fungal species have been reported to produce one or more types of cellulases (Malik *et al.,* 2011a; Zia *et al.,* 2012). However, cellulases from the members of the genus *Trichoderma* have particularly been exploited and are commercially available. In spite of its commercial applications, *Trichderma* cellulases contain low levels of β-glucosidase and hence it cannot completely remove cellobiose from the reaction mixture, therefore, search for alternatives is a subject of research these days. There are few fungal strains considered as having equal potential for commercial production of cellulases as that of *Trichoderma*, including *Aspergillus niger* (Gusakov, 2011).

The genus *Aspergillus* is a diversified group of several pathogenic and industrially important species. Members of black aspergilli (including *Aspergillus niger*) are of particular interest (De Vries & Visser, 2001; Malik *et al.,* 2011b) as their several products have been gained the status of GRAS (generally regarded as safe). There are many *Aspergillus* species that can elaborate significant amounts of cell-free cellulase and are capable of hydrolyzing cellulosic mass into fermentable soluble sugars such as glucose. These end products can serve as important raw material in chemical and fermentation industries. The present manuscripts describes cellulases from three strains of *Aspergillus niger* selected after screening a large number of indigenously isolated fungal strains for their cellulolytic potential.

Materials and Methods.

Fungal strains and cellulase production: Strains of *Aspergillus niger* were isolated from soil and spoiled orange juice as described elsewhere (Sohail *et al.,* 2009) and maintained in the culture collection of Department of Microbiology, University of Karachi, Karachi, Pakistan. These strains were screened for cellulase production by inoculating mycelial mass to a tube containing Mandels medium (Mandels *et al.,* 1974) supplemented with ball-milled cellulose and incubated at ambient temperature for one week. The extent of the depth of clearing from the surface was measured and considered as cellulolytic potential of fungal strain.

Enzyme production: Cultures were grown on Sabouraud's Dextrose Agar (SDA) for one week and spores were collected. Spore suspensions were prepared in sterile saline and were inoculated in Mandels medium containing carboxymethyl cellulose (CMC), ball-milled cellulose (BMC), phosphoric acid swollen cellulose (PASC), filter paper (FP), Sigmacell (SC), salicin, cellulose acetate (CA) and glucose; and in Sabauraud's dextrose broth (SDB). Growth and enzyme production kinetics were studied in MSM supplemented with CMC, salicin or glucose and in SDB. Growth was determined gravimetrically and enzyme assays were performed in the aliquots collected periodically.

Enzyme assays and characterization of enzymes: Endoglucanase, β-glucosidase, filter-paperase and cotton cellulase activities were performed as described by Sohail *et al*., (2011). Optimum temperature, pH and melting temperatures of cellulases; and effect of metallic ions were studied by adopting previously described methods (Sohail *et al.,* 2011).

Results and Discussion

Aspergillus niger was found as one of the most frequently isolated fungi when attempts were made to isolate cellulolytic microorganisms (Gautam *et al.,* 2012). Kadarmoidheen *et al*., (2012) referred *A. niger* as next to *Trichoderma viridae* to hydrolyze cellulosic contents of paddy straw, sugarcane bagasse and banana stalk. An earlier study has also revealed that *A. niger*, is not only a commonly found soil-borne fungus, but there are a number of strains belonged to this genus can over-produce cellulases (Sohail *et al.,* 2009). In this context, three strains of *A. niger* were selected for further studies available in the culture collection of the Department of Microbiology, University of Karachi, Karachi; *A. niger* MS19 and MS177 were isolated from soil, whereas MS156 was isolated from a spoiled orange juice sample. All the strains were initially screened for their cellulolytic potential in tubes containing ball-milled cellulose by observing a clear zone under the growth. Shake-flask experiments revealed that in presence of cellulosic substrates these strains elaborate higher endoglucanase, β-glucosidase, and filter-paperase activities when cultivated at 35°C compared to 30°C (data not shown), therefore, further experiments were conducted by growing fungal strains at 35°C. No cellulytic activities were noted when the strains were cultivated at 40°C.

To find out better inducers for individual cellulase components, the strains were cultivated in MSM supplemented with cellulosic materials and glucose (Table 1). It was revealed that in presence of CMC much higher titers of International Filter Paper Unit (IFPU) and endoglucanase were produced by all the strains while, salicin appeared as a better inducer for the production of β-glucosidase. None of the cellulolytic components were present when the MSM supplemented with glucose or SDB medium was used. This finding was more or less similar with Jatinder *et al*., (2006) where *Scytalidium thermophilum* produced higher titers of endoglucanase than β-glucosidase in CMC containing medium, nonetheless a down regulation was noted in the presence of glucose. In an earlier study, Jahangeer *et al*., (2005) reported that the strains of *A. niger, A. terreus* and *A. nidulans* produced $\geq$ 0.3 units of IFPU at 30°C. Juhasz *et al*., (2005) obtained even a lesser titer of >1.2 FPU (0.111 IFPU) from *Trichoderma reesei* on steam pretreated lignocellulosics at 30°C. Amongst the fungal strains under study, *A. niger* MS19 produced as much as 0.148 IFPU when it was grown on CMC supplemented with MSM.

Cotton-cellulase activity (sometimes referred to as C_1 component of cellulase system) is responsible for the degradation of highly crystalline cellulose and has not been cited commonly. The cotton-cellulase activity was only noted when the strains MS156 and MS177 were grown at 30°C but, MS19 produced this activity at 30°C as well as at 35°C.

Studies on growth kinetics revealed that the duration of the lag- and log-phase and hence generation time 'g' is influenced by the composition of the medium particularly by the carbon source present in the medium (Table 2). A longer lag-phase followed by a moderately shorter log-phase was observed when a complex carbon source (*i.e.* CMC or salicin) was supplied to the strains. Except for the strain MS19, the values of 'g' were generally 2-5 times higher in CMC or salicin containing MSM compared to MSM with glucose or in SDB. However, the rate of biomass production (Q_x) remained unaffected by the composition of the medium. Shorter lag- and longer log-phases when the fungal strains were grown in SDB or in glucose supplemented MSM can be attributed to the fact that simple nutritional sources are assimilated easily and promote growth rapidly as noted by Walter and Schrempf (1996). The production of endoglucanase of β-glucosidase was initiated when the strains were entered in exponential phase of their growth in CMC or salicin containing media and attained a peak in early or late stationary phase; the titers of endoglucanase were found 1.5-2 folds higher than β-glucosidase in CMC containing medium. These findings show that *A. niger* produced cellulase enzyme as primary metabolites as reportedly noted in some other organisms (Tamburini *et al.,* 2004; Jang & Chang 2005).

In terms of volumetric productivity (Table 3), the strain MS156 was found to be a promising strain as it gave 2-6 times higher productivity of endoglucanase (0.899 $IU.L^{-1}.h^{-1}$) and β-glucosidase (1.147 $IU.L^{-1}.h^{-1}$) compared to the other two strains in CMC or salicin supplemented MSM, respectively. Consequently, the same strain also showed higher specific productivity.

Table 1. Cellulase production from selected strains of *Aspergillus niger* in different media.

Strains	Enzyme activity	Enzyme activities in MSM supplemented with						
		CMC	BMC	PASC	FP	CA	SC	Sal
MS19	IFPU	0.148	0.10	0.12	0.12	0.06	0.10	0.10
	EGL	0.55	0.24	0.31	0.34	0.22	0.31	0.32
	BGL	0.10	0.07	0.08	0.05	0.03	0.10	0.22
	CCase	0.10	0.10	0.10	0.08	0.04	0.08	0.04
MS156	IFPU	0.12	0.10	0.11	0.12	0.06	0.10	0.11
	EGL	0.65	0.46	0.40	0.52	0.22	0.32	0.52
	BGL	0.15	0.11	0.16	0.10	0.08	0.10	0.42
	CCase	0	0	0	0	0	0	0
MS177	IFPU	0.082	0.07	0.08	0.08	0.05	0.072	0.08
	EGL	0.23	0.16	0.18	0.17	0.10	0.14	0.22
	BGL	0.21	0.13	0.11	0.10	0.06	0.08	0.21
	CCase	0	0	0	0	0	0	0

*Carboxymethyl cellulose (CMC), Ball-milled cellulose (BMC), Phosphoric acid-swollen cellulose (PASC), Filter-paperase (FP), Cellulose acetate (CA), Sigmacell (SC) and Salicin (Sal)

Table 2. Comparative growth kinetics of three strains of *Aspergillus niger*.

Strain	Substrate/ medium	Duration (h)		g (h)	Q_x (mg. h^{-1})
		Lag-phase	Log-phase		
A. niger MS19	CMC	20	60	2.267	0.423
	Salicin	20	40	1.513	0.660
	Glucose	5	60	2.657	0.447
	SDB	5	55	1.875	0.501
A. niger MS156	CMC	20	40	7.181	0.141
	Salicin	20	40	7.889	0.149
	Glucose	25	25	3.810	0.263
	SDB	20	45	1.345	0.644
A. niger MS177	CMC	50	40	12.344	0.081
	Salicin	50	40	13.065	0.095
	Glucose	5	65	1.864	0.232
	SDB	20	90	2.308	0.536

*Volumetric biomass production (Q_x), generation time (g), Carboxymethyl cellulose (CMC), Sabouraud's dextrose broth (SDB)

Table 3. Product formation parameters, of endoglucanase (EG) and β-glucosidase (BGL) from the three strains.

Strains	Substrate	Product formation parameters			
		Q_p (IU. $L^{-1}.h^{-1}$) (IU.L.h^{-1})		$Y_{p/x}$ (IU.mg^{-1} of cells)	
		EG	BGL	EG	BGL
MS19	CMC	0.386	0.302	0.14	0.109
	Salicin	0.412	0.483	0.13	0.152
MS156	CMC	0.899	0.721	0.933	0.748
	Salicin	0.769	1.147	0.7035	1.049
MS177	CMC	0.216	0.183	0.654	0.553
	Salicin	0.1735	0.217	0.417	0.521

*Volumetric productivity (Q_p) and specific productivity ($Y_{p/x}$)

Table 4. Optimum temperature (T_{opt}), pH (pH_{opt}) and melting temperature (T_m) of endoglucanase (EG) and β-glucosidase (BGL) from strains MS19, MS156 and MS177 of *A. niger*.

Strain	T_{opt} (^oC)		pH_{opt}		T_m (^oC)	
	EG	BGL	EG	BGL	EG	BGL
MS19	60	45-50	7.0	4.0	64	65
MS156	50	50	4.5	4.5	65	64
MS177	60-65	55	5.0	4.5	64	65

The optimum temperature for the activity of endoglucanase (Table 4) from the strains MS19 and MS177 were higher (60-65^{o}C) compared to the strain MS156 (50^{o}C). β-glucosidases from all the strains, however, showed its activity optima at 50-55^{o}C. Except for endoglucanase of MS19 strain, the optimum pH for all the other endoglucanases and β-glucosidases was found to range between pH 4-5.5. The endoglucanase from MS19 showed its maximum activity at neutral pH. Singh *et al*., (1990) reported that endoglucanase activity from an indigenous strain of *A. niger* showed its maximum activity at 60^{o}C and at pH 5.5. An optimum temperature of 65^{o}C had also been reported for endoglucanase activity from a strain of *A. fumigatus* (Grigorevski-Lima *et al.,* 2009). Cellulases working optimally under acidic environment are regarded as potential candidate in fruit- and vegetable-juice extraction process together with other macerating enzymes (Bhat, 2000).

Interestingly, the melting temperature, a parameter of assessing thermal stability, was found to be almost the same (64-65^{o}C) for endoglucanase and β-glucosidase regardless of the strain. Many authors reported that the T_m of several cellulases were lower than noted during the present study for instance, a T_m of 52^{o}C for β-glucosidase (Rajoka *et al.,* 2004) and endoglucanase (Rajoka *et al.,* 2003) of *Cellulomonas biazotea*.

Since cellulases along with xylanases are applied on crude preparations of lignocellulosics that may contain metallic ions in residual concentrations therefore, effect of these chemicals on cellulases from the strains was also studied. It was found that there was some improvement in β-glucosidase activity of *A. niger* MS19 in the presence of 20 mM $Ca^{2+}$and Mg^{2+}, which was further enhanced when the concentration increased to 50 mM (data not shown). The endoglucanase activity from this strain was also slightly increased in presence of 50mM Mg^{2+}. On contrary, the presence of 20 mM Ca^{2+} and Mg^{2+} did not affect either endoglucanase or β-glucosidase activity obtained from MS156 and MS177 strains of *A. niger*; nevertheless, a slight increase in the enzyme activity when the concentration was increased to 50 mM.

These findings clearly indicate that *A. niger* strains produce different components of cellulases in good yield. Parameters for optimum activity and thermal stability also show that these strains can be the potential candidates for biotechnological applications in food industries.

Acknowledgements

Authors acknowledge the funding from Higher Education Commission of Pakistan and Dean Faculty of Science.

References

Bhat, M.K. 2000. Cellulases and related enzymes in biotechnology. *Biotechnol. Adv.,* 18: 355-383.

De Vries, R.P. and J. Visser. 2001. *Aspergillus* enzymes involved in degradation of plant cell wall polysaccharides. *Microbiol. Molec. Biol. Rev.,* 65: 497-522.

Gautam, S.P., P.S. Bundela, A.K. Pandey, Jamaluddin, M.K. Awasthi and S. Sarsaiya. 2012. Diversity of cellolytic microbes and the biodegradation of municipal waste by a potential strain. *Int. J. Microbiol.,* 2012: Article ID 325907, 12 pages, 2012. View at Publisher · View at Google Scholar · View at Scopus.

Grigorevski-Lima, A.L., F.N.M. Da Vinha, D.T. Souza, A.S.R. Bispo, E.P.S. Bon, R.R.R. Coelhoand and R.P. Nascimento. 2009. *Aspergillus fumigatus* thermophilic and acidophilic endoglucanase. *Appl. Biochem. Biotechnol*., 155: 18-26.

Gusakov, A.V. 2011. Alternatives to *Trichoderma reesei* in biofuel production. *Trends in Biotechnol.,* doi:10.1016/j.tibtech.2011.04.004.

Jahangeer, S., N. Khan, S. Jahangeer, M. Sohail, S. Shahzad, A. Ahmad and S.A. Khan. 2005. Screening and characterization of fungal cellulases isolated from the native environmental source. *Pak. J. Bot.,* 37:739-748.

Jang, H. and K. Chang. 2005. Thermostable cellulases from *Streptomyces* sp: scale-up production in 50 l fermenter. *Biotech. Lett.,* 27: 239-242.

Jatinder, K. and R. Sharma. 2008. Directed evolution: An approach to engineer enzymes. *Crit. Rev. Biotechnol.*, 26: 165-199.

Juhasz, T., Z. Szengyel, K. Reczey, M. Siika-Aho and L. Viikari. 2005. Characterization of cellulases and hemicellulases produced by *Trichoderma reesei* on various carbon sources. *Process Biochem.,* 40: 3519-3525.

Kadarmoidheen, M., P. Saranraj and D. Stella. 2012. Effect of cellulolytic fungi on the degradation of cellulosic agricultural wastes. *Int. J. Appl. Microbiol.* Sci.,1: 13-23.

Malik, S., T. Iftikhar and I.U. Haq. 2011a. Enhanced amyloglucosidase biosynthesis through mutagenesis using *Aspergillus niger*. *Pak. J. Bot*., 43(1):111-119.

Malik, S., T. Iftikhar and I.U. Haq. 2011b. Optimization of cultural conditions for a potent wild and mutant strain of *Aspergillus niger*. *Pak. J. Bot*., 43(4): 2211-2219.

Mandels, M., I. Hontz and J. Nystrom. 1974. Enzymatic hydrolysis of waste cellulose. *Biotechnol. Bioeng.*, 16: 1471-1493.

Rajoka, M.I., I.S. Durrani and A.M. Khalid. 2004. Kinetics and improved production and thermostability of intracellular β-glucosidase from a mutant-derivative of *Cellulomonas biazotea*. *Biotech. Lett.,* 26: 281-285.

Rajoka, M.I., Y. Ashraf, H. Rashid and A.M. Khalid. 2003. Knietics and thermodynamics of the native and mutated extracellular endoglucanase from *Cellulomonas biazotea. Protein Peptide Lett*., 10: 561-568.

Singh, A., A.K. Agrawal, A.B. Abidi and N.S. Darmwal. 1990. General and kinetic properties of endoglucanase from *Aspergillus niger*. *FEMS Microbiol. Lett*., 71: 221-224.

Sohail, M., A. Ahmad and S.A. Khan. 2011. Production of cellulases from *Alternaria* sp. MS28 and their partial characterization. *Pak. J. Bot.,* 43: 3001-3006.

Sohail, M., S. Naseeb, S.K. Sherwani, S. Sultana, S. Aftab, S. Shahzad, A. Ahmad and S.A. Khan. 2009. Distribution of hydrolytic enzymes among native fungi: *Aspergillus* the pre-dominant genus of hydrolase producer. *Pak. J. Bot.,* 41: 2567-2582.

Tamburini, E., B. Perito and G. Mastromei. 2004. Growth-phase dependent expression of an endoglucanase encoding gene (*egl*S) in *Streptomyces rocei* A2. *FEMS Microbiol Lett.,* 237: 267-272.

Walter, S. and H. Schrempf. 1996. Physiological studies of cellulase (avicelase) synthesis in *Streptomyces reticuli*. *Appl. Env. Microbiol*., 62: 1065-1069.

Zia, M.A., S. Rasul and T. Iftikhar. 2012. Effect of gamma irradiation on *Aspergillus niger* for enhanced production of glucose oxidase. *Pak. J. Bot*., 44(5): 1575-1580.

(Received for publication 24 May 2013)