Brazilian Journal of Microbiology 46, 4, 1269-1277 (2015)
ISSN 1678-4405
DOI: http://dx.doi.org/10.1590/S1517-838246420140958

Research Paper

Enhanced alkaline cellulases production by the thermohalophilic *Aspergillus terreus* AUMC 10138 mutated by physical and chemical mutagens using corn stover as substrate

George Saad Isaac, Medhat Ahmed Abu-Tahon

Biological and Geological Sciences Department, Faculty of Education, Ain Shams University, Cairo, Egypt.

Submitted: November 19, 2014; Approved: May 6, 2015.

Abstract

A thermohalophilic fungus, *Aspergillus terreus* AUMC 10138, isolated from the Wadi El-Natrun soda lakes in northern Egypt was exposed successively to gamma and UV-radiation (physical mutagens) and ethyl methan-sulfonate (EMS; chemical mutagen) to enhance alkaline cellulase production under solid state fermentation (SSF) conditions. The effects of different carbon sources, initial moisture, incubation temperature, initial pH, incubation period, inoculum levels and different concentrations of NaCl on production of alkaline filter paper activity (FPase), carboxymethyl cellulase (CMCase) and β-glucosidase by the wild-type and mutant strains of *A. terreus* were evaluated under SSF. The optimum conditions for maximum production of FPase, CMCase and β-glucosidase were found to be the corn stover: moisture ratio of 1:3(w/v), temperature 45 °C, pH range, 9.0-11.0, and fermentation for 4, 4 and 7 day, respectively. Inoculum levels of 30% for β-glucosidase and 40% for FPase, CMCase gave the higher cellulase production by the wild-type and mutant strains, respectively. Higher production of all three enzymes was obtained at a 5% NaCl. Under the optimized conditions, the mutant strain *A. terreus* M-17 produced FPase (729 U/g), CMCase (1,783 U/g), and β-glucosidase (342 U/g), which is, 1.85, 1.97 and 2.31-fold higher than the wild-type strain. Our results confirmed that mutant strain M-17 could be a promising alkaline cellulase enzyme producer employing lignocellulosics especially corn stover.

Key words: thermohalophilic fungus, *Aspergillus terreus*, alkaline cellulase, solid state fermentation, corn stover.

Introduction

Soda lakes are widely distributed all over the world and represent the major type of naturally occurring highly alkaline environments in which the indigenous microflora is subjected to a number of extreme ecological pressures. The lakes represent the most stable high-pH environments on Earth, where large amounts of carbonate minerals can generate pH values higher than 11.5 (Grant and Jones, 2000). These environments exhibit extreme temperature, pH and salt content, and enzymes produced by microorganisms have evolved to function optimally under harsh conditions (Zhang *et al.*, 2006). One of such environmental niches which has not been studied in detail is represented by the Wadi El-Natrun soda lakes in northern Egypt. The ecosystem of Wadi El-Natrun area is considered a rich source for isolation of various extremophiles, including alkalophilic microorganisms.

Cellulase enzymes, which can hydrolyze cellulose forming glucose and other commodity chemicals, can be divided into three types: endoglucanase (endo-1,4-β-D-glucanase, EG, EC 3.2.1.4); cellobiohydrolase (exo-1,4-β-D-glucanase,CBH, EC 3.2.1.91) and β-glucosidase (1,4-β-D-glucosidase, BG, EC 3.2.1.21) (Hong *et al.*, 2001; Li *et al.*, 2006). Lignocellulosic biomass, especially agricultural

Send correspondence to G.S. Isaac. Biological and Geological Sciences Department, Faculty of Education, Ain Shams University, Roxy, Heliopolis, P.C.11757, Cairo, Egypt. E-mail: dr.georgesaad@gmail.com.

wastes, is known to be an excellent carbon source for microbial enzyme production; and lignocellulosic biomass, such as corn cob, corn stover and wheat bran, are very abundant, cheap and easily available. Various agricultural substrates-byproducts and microbial cultures have been successfully used in the solid state fermentation (SSF) for cellulase production (Yang *et al.*, 2006).

Cellulase production has been described for many *Aspergillus* species (Ong *et al.*, 2004; Wang *et al.*, 2006; Gao *et al.*, 2008). *A. terreus* has been shown to be the best producer of cellulase as compared to other microorganisms (Mizaakhmedov *et al.*, 2007), although only few reports available on production of cellulase from *A. terreus*, especially under SSF conditions (Emtiazi *et al.*, 2001). Also, cellulase research has focused on halophilic enzymes only to a very limited extent (Wang *et al.*, 2006).

Strain improvement is usually achieved by mutating the microorganism that produces the enzyme by classical mutagenesis, generally involves exposing the microbe to physical mutagens (*e.g.*, X-rays, γ-rays, UV-rays, etc.), and/or chemical mutagens (*e.g.*, nitroglycerin, ethyl methanesulfonate (EMS), etc.; Parekh *et al.*, 2004; Adsul *et al.*, 2007; Raghuwanshi *et al.*, 2014). Hydrolysis of cellulose to soluble sugars makes it available as a feedstock for alcoholic fermentation, single cell protein production, and other industrial processes (Louime and Uckelmann, 2008). The utilization of cellulosic biomass for ethanol production continues to be a subject of worldwide interest in view of fast depletion of oil reserves and food shortages (Singh *et al.*, 2009). In the present study, we evaluated the potential of *A. terreus* AUMC 10138, an alkalophilic and salt-tolerant fungus, isolated from the Wadi El-Natrun soda lakes in northern Egypt, for cellulase production and attempted to improve the enzyme yield by physical (γ-rays and UV-rays) and chemical (EMS) mutagenesis of the microorganism. We also optimized culture conditions for the mutant and wild-type strains using corn stover as a substrate under solid state fermentation.

Materials and Methods

Strain isolation and identification

Soil samples were collected from different regions of the Wadi El-Natrun soda lakes in northern Egypt. Isolation of alkalophilic fungi was carried out using carboxymethyl cellulose (CMC) medium containing (g/L); yeast extract, 2.5 g; tryptone, 2.5 g; $(NH_4)_2SO_4$, 1.0 g; KH_2PO_4, 0.5 g; K_2HPO_4, 0.5 g, $MgSO_4$, 0.2 g; CMC (Sigma, USA), 5 g, and supplemented with 5% NaCl and 1.5% agar (w/v). pH was adjusted to 9.0 using 1 M KOH. Briefly, 10 g of a soil sample was added to 90 mL of sterile water, agitated for 1 h at 250 rpm, and then 1.0 mL of the suspension was spread on a CMC agar plate. All plates were incubated at 45 °C until colonies with a distinct clearing zone was visible. A colony of *A. terreus* (designated as AUMC10138) with the largest halo zone was picked and purified for further study (data not shown). The ability of the isolate to secrete cellulase was evaluated on a Congo red plate according to Kasana *et al.* (2008). The purified isolates were maintained on potato dextrose agar (PDA) at 4 °C and were subcultured at monthly intervals. The identification was carried out by the Assiut University Mycological Centre (AUMC), Egypt.

Fungal strain and media

A. terreus AUMC 10138 was maintained on PDA medium at 45 °C for 7 days for spore production. Conidial suspensions were prepared by washing slant cultures with 10 mL of a sterilized 0.9% NaCl (w/v) solution. A spore suspension was counted at 10^5-10^6 spores/mL by a haemocytometer.

Fungal strain improvement by mutagenesis

Spores of *A. terreus* (10^5-10^6 spores/mL) were harvested from a 7 day-old spores grown on PDA medium following exposure to different doses (0.25~2.5 kGy) of Co^{60} γ-rays emitted by an Indian gamma cell located at the National Center for Radiation Research and Technology (NCRRT), Nasr City, Cairo, Egypt. Survivors were grown by spreading 1.0 mL of the treated spores on CMC medium and incubating at 45 °C for 7 days. Mutants with the largest clear zones around the colonies and cellulase production were selected and re-irradiated with Co^{60}γ-rays. The best surviving mutant was then exposed to UV-radiation (power: 30W, wavelength: 260 nm at a distance of 20 cm for 2~30 min). Selected UV mutants were treated with EMS (20 mg were added to 10 mL of suspension containing 10^5-10^6 spores/mL). The mutant spores were washed four times with a saline solution and spread on CMC medium as described above. A mutant displaying high cellulase activity after the mutagenic treatments was selected, and stability of the enzyme production was studied for nine generations by successive inoculations of the strain on cellulase production medium.

Substrates

The lignocellulosic substrates, namely, corn cob, corn stover, saw dust, sugar cane bagasse and wheat straw, were all obtained locally. They were dried and chopped into small pieces, then ground into smaller particles in a hammer mill, and finally separated using a 0.45 mm (40 mesh) sieve. The fraction that passed through the sieve was used for SSF medium preparation.

Medium and cultivation conditions

A dry carbon source (5 g) was amended with 10 mL of a mineral salt solution containing (g/L); $(NH_4)_2SO_4$, 3.5 g; KH_2PO_4, 3 g; $MgSO_4.\ 7H_2O$, 0.5 g, $CaCl_2$, 0.5g and

supplemented with 5% NaCl, (pH 9.0). The mixture was placed in 250 mL Erlenmeyer flask, mixed homogenously and sterilized at 121 °C for 15 min (Juhasz *et al.*, 2005). One milliliter of prepared spores from wild and selected mutant strain M17 was inoculated and incubated at 45 °C under static conditions. The best solid substrate was selected and used in subsequent experiments.

Optimization of SSF

SSF was carried out to study the effects of various physico-chemical parameters required for the maximal cellulase production by *A. terreus* AUMC10138 and its most potent mutant strain M-17. The parameters that were optimized were the substrate (corn cob, corn stover, saw dust, sugar cane bagasse, wheat straw), initial moisture (1:1, 1:2, 1:3, 1:4 and 1:5 w/v, where v and w represent water and water + dried corn stover, respectively), incubation temperature (25~55 °C), initial pH (7~12), incubation period (2~9 days), inoculum level (10~50%, v/w) and concentrations of NaCl (0~15.0%). Each experiment was performed in triplicate.

Enzyme extract

The solid substrate culture broth was prepared by adding 10-fold (v/w) of distilled water and shaking the mixture (200 rpm) at 30 °C for 60 min. Then, the solid materials and fungal biomass were separated by centrifugation (10,000 x *g*, 15 min), and the clarified supernatant was used for enzyme assays.

Enzyme assay

The activities of total cellulase (filter paper activity, endoglucanase and β-glucosidase were determined as reported earlier (Grajek, 1987). FPase activity was assayed by incubating 1 mL of a suitably diluted enzyme solution with Tris-HCl buffer (20 mM, pH 9.0) containing Whatman No. 1 filter paper (50 mg, 1 x 6 cm). The reaction mixture was incubated at 60 °C for 30 min. Endoglucanase (CMCase, endo-1,4-β-D-glucanase; EC 3.2.1.4) activity was measured in a total reaction volume of 1 mL containing 0.5 mL of suitably diluted enzyme and 0.5 mL of 1% (w/v) carboxymethyl cellulose (CMC) solution in Tris-HCl buffer (20 mM, pH 9.0). The mixture was incubated at 60 °C for 30 min. The release of reducing sugars was determined by the 3,5-dinitrosalicylic acid (DNS) method (Miller, 1959). β-Glucosidase (β-D-glucoside, glucohydrolase; EC3.2.1.21) activity was estimated using *p*-nitrophenyl-β-Dglucopyranoside (pNPG) a substrate. An assay mixture (1 mL) consisting of 0.9 mL of *p*NPG (1 mM) and 0.1 mL of suitably diluted enzyme was incubated at 60 °C for 30 min. The *p*-nitrophenol liberated was measured at 420 nm after developing the color in the presence of 2 mL of sodium carbonate (2 M).One unit (U) of enzyme activity was defined as the amount of enzyme required to liberate 1 μmol of glucose or *p*-nitrophenol from the appropriate substrates per minute under the assay conditions.

Statistical analysis

The data obtained were statistically analyzed with SPSS (Scientific Package for Scientific Social Studies, version 20), in which the equations of the hypothesis tests, including the mean, standard deviation, T-statistics value and probabilities (p) were used.

Results and Discussion

Screening of mutants for cellulase

The use of mutagenesis and selection has been found effective in increasing cellulase production by microorganisms (Vu *et al.*, 2011; Raghuwanshi *et al.*, 2014). In the present study, wild-type strain *A. terreus* AUMC10138 was subjected to mutagenesis by using Co^{60} γ-rays, UV-irradiation and EMS (data not shown). Six mutants were selected (M-14, M-15, M-16, M-17, M-18 and M-19) which showed increased production of FPase, CMCase and β-glucosidase. Mutant M-17 was selected on the basis of improved enzyme production and the largest clear zone. *A. terreus* M-17 showed a 1.98, 2.43 and 2.35-fold increase in

Table 1 - Alkaline cellulase production under liquid state cultivation by *A. terreus* AUMC 10138 and its most potent mutants.

Strain		Zone of clearance (mm)	Enzyme activity (U/mL)		
Wild	Mutant		CMCase	FPase	β-Glucosidase
AUMC 10150	-	18	1.11 ± 0.12	0.46 ± 0.17	0.23 ± 0.10
	M14	23	1.53 ± 0.15	0.66 ± 0.11	0.33 ± 0.09
	M15	21	1.67 ± 0.11	0.72 ± 0.13	0.37 ± 0.15
	M16	21	1.85 ± 0.17	0.84 ± 0.15	0.42 ± 0.18
	M17	24	2.20 ± 0.24	1.12 ± 0.13	0.54 ± 0.10
	M18	21	1.93 ± 0.10	0.97 ± 0.12	0.39 ± 0.14
	M19	20	1.70 ± 0.18	0.68 ± 0.27	0.26 ± 0.15

FPase, CMCase and β-glucosidase activities, respectively, compared to the wild-type strain (Table 1).

Genetic stability of the selected mutant strain

The stability of cellulase production by selected mutant M-17 was studied by successive subculturing of the strain for nine generations. After each subculture, the mutant was tested for its stability to produce cellulase. The mutant maintained the same production yields after being subcultured nine times; indicating hereditary stability of the mutation has (Table 2).

Effect of carbon source

Cellulase production was found to be dependent on the nature of the carbon source used in the culture medium (Gao *et al.*, 2008). The influence of various carbon sources on the cellulase production by both wild-type and mutant strains of *A. terreus* is shown in Table 3. Corn stover was the best carbon source for FPase, CMCase and β-glucosidase production among the tested lignocellulosic materials. This might be attributed to its hemicellulose nature, favorable degradability, and the presence of certain nutrients in corn stover (Senthilkumar *et al.*, 2005). Corn stover is composed of approximately 39.54% of cellulose, 25.76% of hemicellulose, 17.49% of lignin, and 5.04% of ash (Yang, 2001). Besides, the efficiency of enzyme production also depends on the chemical composition of a raw material, accessibility of various components and their chemical or physical associations, corn stover has been known as an ideally suitable substrate for cellulase production (Gao *et al.*, 2008; Liu *et al.*, 2011). In this study corn stover induced maximum FPase (140 U/g), CMCase (330 U/g) and β-glucosidase (66 U/g) production by the wild-type strain of *A. terreus*. In the case of mutant strain M-17, the maximum FPase (267 U/g), CMCase (561 U/g) and β-glucosidase (132 U/g) production was also obtained on corn stover as a carbon source.

Effect of initial moisture content

The initial moisture content plays an important role in biosynthesis and secretion of many kinds of enzymes, especially cellulases (Vu *et al.*, 2011). Therefore, the effect of moisture content on the cellulase production by *A. terreus* AUMC10138 and mutant M-17 was analyzed. The results showed that an increase in the initial moisture ratio from 1:1 to 1:3 (w/v) greatly enhanced the production of all three cellulases. The substrate to moisture ratio of 1:3 resulted in the maximum production of FPase (163 U/g), CMCase (390 U/g) and β-glucosidase (78 U/g) by the wild-type strain. Similarly, mutant strain M-17 also produced FPase (293 U/g), CMCase (702 U/g) and β-glucosidase (178 U/g) maximally at a substrate to moisture ratio of 1:3(w/v). Any further increase in the moisture level in SSF led to decreased enzyme production (Table 4). This might be due to

Table 2 - Alkaline cellulase activities of most potent mutant *A. terreus* M-17 for nine generations.

Cellulolytic enzymes	Enzyme activity (U/mL) in nine generations								
	1st	2nd	3rd	4th	5th	6th	7th	8th	9th
CMCase	2.20	2.13	2.10	2.12	2.15	2.18	2.22	2.22	2.24
	± 0.20	± 0.02	± 0.10	± 0.12	± 0.05	± 0.30	± 0.26	± 0.34	± 0.31
FPase	1.10	1.12	1.11	1.12	1.13	1.10	1.12	1.11	1.13
	± 0.30	± 0.13	± 0.02	± 0.05	± 0.06	± 0.31	± 0.13	± 0.09	± 0.08
β-glucosidase	0.54	0.54	0.53	0.53	0.55	0.56	0.54	0.55	0.55
	± 0.10	± 0.09	± 0.18	± 0.14	± 0.11	± 0.16	± 0.09	± 0.12	± 0.22

Table 3 - Effect of different carbon sources on alkaline cellulase production by wild-type *A. terreus* AUMC 10138 and its mutant strain M-17 under solid state fermentation.

Carbon source (g)	Enzyme yield (U/g dry carbon source)					
	CMCase		FPase		β-Glucosidase	
	Wild	Mutant	Wild	Mutant	Wild	Mutant
Corn cob	282 ± 3	479 ± 9	97 ± 5	191 ± 4	30 ± 6	69 ± 2
Corn stover	330 ± 5	561 ± 11	140 ± 4	267 ± 3	66 ± 7	132 ± 5
Saw dust	266 ± 4	500 ± 9	76 ± 9	134 ± 2	34 ± 3	78 ± 4
Sugarcane bagasse	163 ± 10	326 ± 8	48 ± 7	86 ± 3	19 ± 6	45 ± 2
Wheat straw	215 ± 5	451 ± 8	63 ± 2	153 ± 9	27 ± 8	71 ± 8

Table 4 - Effect of initial moisture content on alkaline cellulase production by wild-type *A. terreus* AUMC 10138 and its mutant strain M-17 under SSF.

Substrate: moisture ratio	Enzyme yield (U/g dry carbon source)					
	CMCase		FPase		β-Glucosidase	
	Wild	Mutant	Wild	Mutant	Wild	Mutant
1:1	301 ± 9	511 ± 3	120 ± 5	210 ± 6	48 ± 4	86 ± 8
1:20	330 ± 5	561 ± 7	140 ± 4	267 ± 3	66 ± 7	132 ± 5
1:3	390 ± 7	702 ± 9	163 ± 6	293 ± 8	78 ± 2	178 ± 6
1:4	308 ± 8	616 ± 6	126 ± 3	230 ± 4	58 ± 6	125 ± 8
1:5	250 ± 4	475 ± 8	100 ± 3	201 ± 2	41 ± 7	93 ± 3

steric hindrance of the cell growth, which results in reduction in the solid matrix porosity which interferes with oxygen transfer that in turn influences cell growth and metabolism, thus inhibiting the enzyme production (Xin and Geng, 2010). Similar results were obtained by Raghuwanshi *et al.* (2014) who reported that the maximum FPase, CMCase and β-glucosidase production by *Trichoderma asperellum* RCK2011and mutant SR1-7 was observed under SSF at a substrate to moisture ratio of 1:2.5.

Effect of incubation temperature

Temperature was the most important physical variable in the SSF. The wild-type strain showed the maximum production of FPase (163 U/g), CMCase (390 U/g) and β-glucosidase (78 U/g) at 45 °C. Likewise, mutant strain M-17 produced the maximum levels of FPase (293 U/g), CMCase (702 U/g) and β-glucosidase (160 U/g) at 45 °C. As soon as the temperature was above 45 °C, cellulase production markedly declined in both wild-type and mutant strains (Figure 1). The maximal cellulase production occurred at 45 °C which was in the range of the temperature of thermophilic fungi (Maheshwari *et al.*, 2000). Our results are also in agreement with those by Gao *et al.* (2008) who reported that the optimum temperature range for cellulase production by *A. terreus* under SSF to be 35-45 °C.

Effect of initial pH

The pH of the medium is one of the most critical factors affecting fungal growth, enzyme production and transport of various components across the cell membrane (Juhasz *et al.*, 2004). The wild-type strain showed the maximum production of FPase (163 U/g) at pH 9.0, and the maximum production of CMCase (470 U/g) and β-glucosidase (92 U/g) at pH 10.0. However, mutant strain M-17 exhibited the maximum FPase (378 U/g) and CMCase (893 U/g) production at pH 10.0, whereas the maximum β-glucosidase production (195 U/g) was observed at the initial pH 11.0 (Figure 2). These results clearly confirmed the alkalophilic nature of the cellulases produced by *A. terreus* and its mutant strain M-17.

Effect of incubation period

The effect of incubation period on cellulase production under SSF was evaluated. The incubation periods of

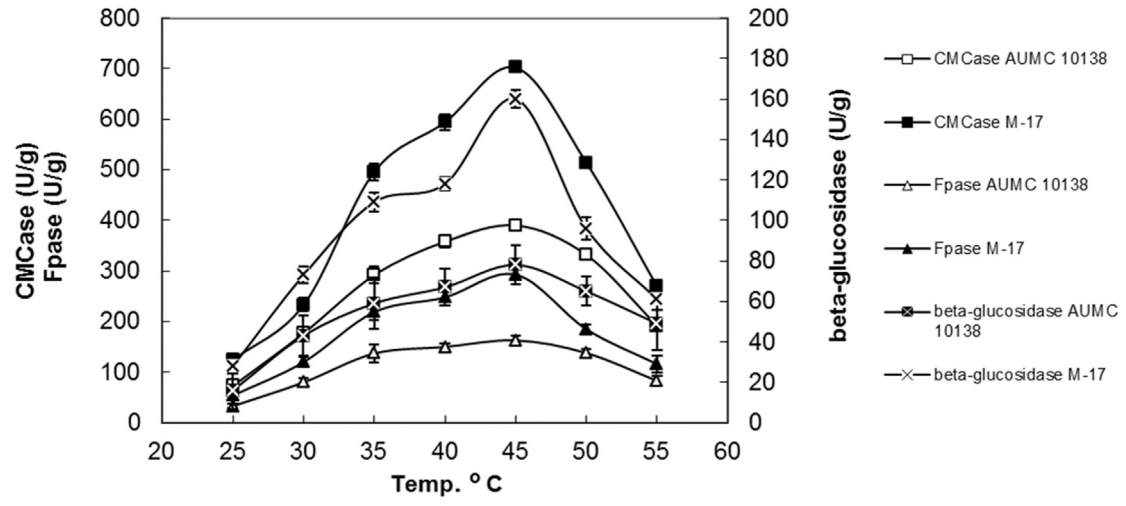

Figure 1 - Effect of the incubation temperature on alkaline cellulase production by wild-type *A. terreus* AUMC 10138 and its mutant strain M-17.

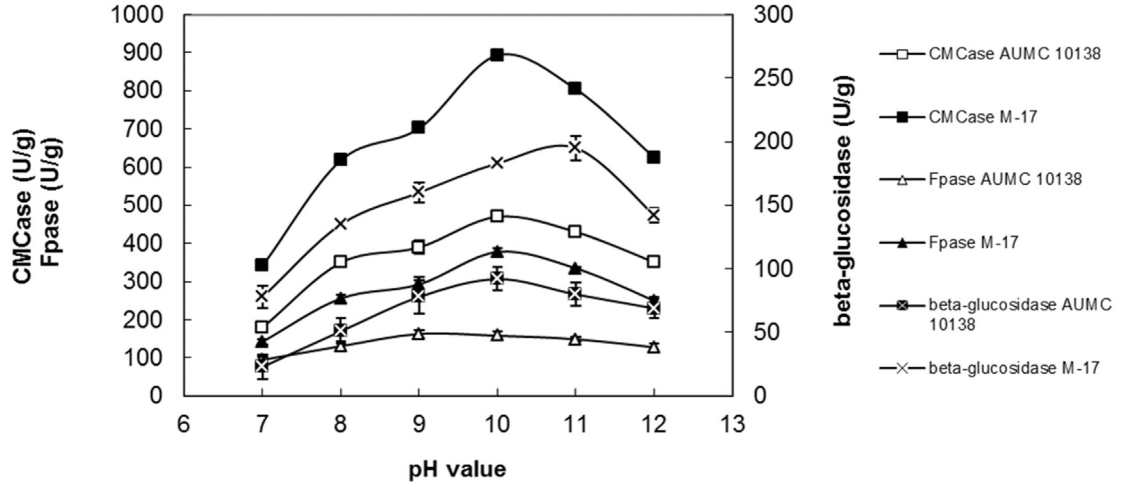

Figure 2 - Effect of the initial pH value on alkaline cellulase production by wild-type *A. terreus* AUMC 10138 and its mutant strain M-17.

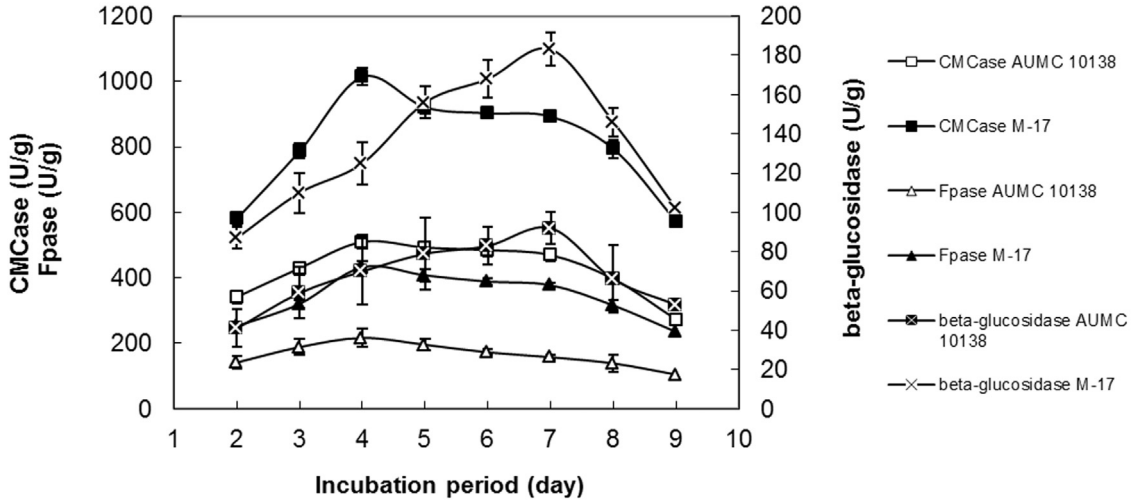

Figure 3 - Effect of the incubation periods on alkaline cellulase production by wild-type *A. terreus* AUMC 10138 and its mutant strain M-17.

FPase, CMCase and β-glucosidase production were shown in Figure 3. The wild-type strain exhibited the maximum FPase (217 U/g) and CMCase (510 U/g) on 4th day of incubation, while β-glucosidase production reached maximum (92 U/g) on 7th day. Mutant M-17 behaved similarly to the wild-type and reached the peak enzyme production on 4th day with FPase (433 U/g) and CMCase (1,014 U/g), while β-glucosidase production reached maximum (183 U/g) on 7th day of incubation. Various fungi have been reported to produce maximum cellulase at different times; therefore direct comparison of time-dependent changes is not possible (Singhania *et al.*, 2010). Our results are in accordance with those by Raghuwanshi *et al.* (2014), who recorded that maximum FPase and CMCase were exhibited on 4th day of incubation, while β-glucosidase production reached maximum on 7th day of incubation by *Trichoderma asperellum* RCK2011 and mutant strain SR1-7 under SSF. Similar results were obtained by Gao *et al.* (2008) who stated that the maximum FPase and CMCase production was shown by *A. terreus* M11 under SSF after 4 days.

Effect of inoculum level

The effect of the inoculum level on cellulase production under SSF was evaluated at the corn cob to moisture ratio of 1:3. The temperature was kept at 45 °C, the initial pH for the wild-type and mutant strains was 10 and the incubation period was 4 days for FPase and CMCase production and 7days for β-glucosidase production for both strains. The inoculum size (10~50%, v/w) showed noticeable effect on cellulase production by *A. terreus* AUMC 10138 and M-17, under SSF (Table 5). The results showed that cellulase production by the wild-type and mutant strains of *A. terreus* increased with the increase of the initial inoculum

Table 5 - Effect of the inoculum level on alkaline cellulase production by wild-type *A. terreus* AUMC 10138 and its mutant strain M-17 under SSF.

Inoculum level (v/w)	Enzyme yield (U/g dry carbon source)					
	CMCase		FPase		β-Glucosidase	
	Wild	Mutant	Wild	Mutant	Wild	Mutant
10%	425 ± 2	850 ± 4	177 ± 3	375 ± 4	80 ± 2	166 ± 5
20%	510 ± 4	1,014 ± 11	217 ± 4	433 ± 2	92 ± 3	183 ± 4
30%	612 ± 3	1,150 ± 8	278 ± 2	460 ± 5	148 ± 8	242 ± 9
40%	905 ± 6	1,783 ± 7	395 ± 6	729 ± 7	126 ± 5	217 ± 5
50%	831 ± 2	1,662 ± 5	334 ± 5	644 ± 3	113 ± 6	203 ± 8

level from 10~50% (v/w). The wild-type strain showed the maximum production of FPase (395 U/g) and CMCase (905 U/g) at the 40% (v/w) inoculum level, whereas β-glucosidase production (148 U/g) peaked at the 30% (v/w) inoculum level. Also, mutant strain M-17 produced maximum FPase (729 U/g) and CMCase (1,783 U/g) at the 40% (v/w) inoculum level and maximum β-glucosidase (242 U/g) at the 30% (v/w) inoculum level. The results revealed the importance of the inoculum level in SSF. Higher inoculum levels increased the spore numbers per gram of solid substrate, as well as the water content in the medium. Both factors acted to hinder the penetration of oxygen into the solid medium, thus preventing inhibition of fungal growth and cellulase production. At the sametime, a lower inoculum level required more time for the fermentation process to complete in a batch culture (Vu *et al.*, 2011). Studies carried out by several other authors exhibit different levels of inocula for efficient induction of cellulase. This shows that a balance between nutrients and growing cells is necessary for optimum enzyme production (Matkar *et al.*, 2013).

Effect of salinity

The effect of salinity on cellulase production under was evaluated under the previously optimized SSF conditions. The salinity of the culture medium strongly influenced the extracellular enzyme production by halophiles (Li and Yu, 2013). As shown in Table 6, the effect of different concentrations of NaCl on the cellulase production by the wild-type and mutant strains of *A. terreus* was evaluated. The wild strain showed optimal FPase, CMCase and β-glucosidase production (395, 905 and 148 U/g, respectively) at 5% NaCl. Our results are in accordance with those by Wang *et al.* (2009) who recorded the maxium cellulase production by halophilic bacterium *Salinivibrio* sp. NTU-05 isolated from soil samples taken near Szutsau saltern, in southern Taiwan was obtained in a saline medium containing 5% NaCl. Likewise, the strain M-17 exhibited the maxium FPase, CMCase and β-glucosidase production (729, 1,783 and 342 U/g respectively) at 5% NaCl. These results clearly revealed the halophilic nature of *A. terreus* AUMC 10138 and mutant M-17, for which salt appeared to be a prerequisite for enzyme production. Halophiles can survive in hypersaline habitats because of their ability to maintain osmotic balance. They accumulate salts such as sodium or potassium chloride (NaCl or KCl) up to concentrations that are isotonic with the environment. Accordingly, exoenzymes from halophiles must tollerate with high salt concentrations (Kamekura, 1998).

Comparison of cellulases production under SSF

Comparison of cellulase activities produced by *A. terreus* AUMC 10138 and its mutant strain M-17 under

Table 6 - Effect of different concentrations of NaCl on alkaline cellulase production by wild-type *A. terreus* AUMC 10138 and its mutant strain M-17 under SSF.

NaCl (%)	Enzyme yield (U/g dry carbon source)					
	CMCase		FPase		β-Glucosidase	
	Wild	Mutant	Wild	Mutant	Wild	Mutant
0%	633 ± 3	945 ± 4	209 ± 3	413 ± 4	102 ± 2	172 ± 4
2.5%	756 ± 7	1,274 ± 11	355 ±	556 ± 2	127 ± 3	209 ± 3
5%	905 ± 6	1,783 ± 7	395 ± 6	729 ± 7	148 ± 8	342 ± 1
7.5%	777 ± 4	1,511 ± 7	347 ± 6	666 ± 7	131 ± 6	283 ± 5
10.0%	624 ± 6	1,236 ± 5	259 ± 5	511 ± 3	114 ± 7	175 ± 3
12.5%	447 ± 3	1,096 ± 9	187 ± 2	378 ± 5	89 ± 2	153 ± 2

Table 7 - Comparison of cellulases production by wild-type *A. terreus* AUMC 10138 and its mutant strain M-17 with other fungi under SSF.

Microorganism	Carbon source	Enzyme yield (U/g dry carbon source)			Reference
		CMCase	FPase	β-Glucosidase	
A. niger KK2	Rice straw	129	19.5	100	(Kang *et al*., 2004)
A. niger MTCC	Wheat bran	135.4	4.6	21.4	(Sukumaran *et al*., 2009)
Penicillium citrinum YS40-5	Rice bran	180.3	3.8	159.1	(Ng *et al*., 2010)
Trichoderma reesi RUT	Wheat bran	299.6	22.8	4.5	(Sukumaran *et al*., 2009)
Fusarium oxysporum	Corn stover	304	-	0.140	(Panagiotou *et al*., 2003)
A. fumigatus Z5	Corn stover	526.3	144.6	-	(Liu *et al*., 2011)
A. terreus M11	Corn stover	563	231	119	(Gao *et al*., 2008)
Thermoascus aurantiacus	Wheat straw	1,572		101.6	(Kalogeris *et al*., 2003a)
Thermoascus aurantiacus	Wheat straw	1,709	5.5	79	(Kalogeris *et al*., 2003b)
A. terreus AUMC 10138	Corn stover	905	395	148	This study
A. terreus M-17	Corn stover	1,783	729	342	This study

SSF with those reported for other cellulase producing fungi showed that the yields of the cellulases produced by this work were much higher than those produced by other fungi (Table 7). In fact, the comparisons of cellulase activities produced by different laboratories is not readily made in quantitative manner as no standard conditions of cellulase activity assay have yet been adopted. Therefore only relative comparison is provided in this paper.

References

Adsul MG, Bastawde KB, Varma AJ *et al.* (2007) Strain improvement of *Penicillium janthinellum* NCIM 1171for increased cellulase production. Bioresour Technol 98:1467-1473.

Emtiazi G, Naghavi N, Bordbar A (2001) Biodegradation of lignocellulosic waste by *Aspergillus terreus*. Biodegradation 12:259-263.

Gao J, Weng H, Zhu D *et al.* (2008) Production and characterization of cellulolytic enzymes from the thermoacidophilic fungal *Aspergillus terreus* M11 under solid-state cultivation of corn stover. Bioresour Technol 99:7623-7629.

Grajek W (1987) Comparative studies on the production of cellulases by thermophilic fungi in submerged and solid-state fermentation. Appl Microbiol Biotechnol 26:126-129.

Grant WD, Jones BE (2000) Alkaline environments. In: Lederberg J (ed) Encyclopaedia of Microbiology. 2nd ed. Academic Press, New York p.126-133.

Hong J, Tamaki H, Akiba S *et al.* (2001) Cloning of a gene encoding a highly stable endo-b-1,4-glucanase from *Aspergillus niger* and its expression in yeast. J Biosci Bioeng 92:434-441.

Juhasz T, Szengyel Z, Szijarto N *et al.* (2004) Effect of pH on cellulase production of *Trichoderma reesei* RUT C30. Appl Biochem Biotechnol 113-116:201-211.

Juhasz T, Szengyel Z, Reczey K *et al.* (2005) Characterization of cellulases and hemicellulases produced by *Trichoderma reesei* on various carbon sources. Process Biochem 40:3519-3525.

Kalogeris E, Christakopoulos P, Katapodis P *et al.* (2003a) Production and characterization of cellulolytic enzymes from the thermophilic fungus *Thermoascus aurantiacus* under solid state cultivation of agricultural wastes. Process Biochem 38:1099-1104.

Kalogeris E, Iniotaki F, Topakas E *et al.* (2003b) Performance of an intermittent agitation rotating drum type bioreactor for solid-state fermentation of wheat straw. Bioresour Technol 86:207-213.

Kamekura M (1998) Diversity of extremely halophilic bacteria. Extremophiles 2:289-295.

Kang SW, Park YS, Lee JS *et al.* (2004) Production of cellulases and hemicellulases by *Aspergillus niger* KK2 from lignocellulosic biomass. Bioresour Technol 91:153-156.

Kasana RC, Salwan R, Dhar H *et al.* (2008) A rapid and easy method for the detection of microbial cellulases on agar plates using gram's iodine. Curr Microbiol 57:503-507.

Li X, Yu HY (2013) Halostable cellulase with organic solvent tolerance from *Haloarcula* sp. LLSG7 and its application in bioethanol fermentation using agricultural wastes. J Ind Microbiol Biotechnol 40:1357-1365.

Li YH, Ding M, Wang J *et al.* (2006) A novel thermoacidophilic endoglucanase, Ba-EGA, from a new cellulosedegrading bacterium, *Bacillus* sp. AC-1. Appl Microbiol Biotechnol 70:430-436.

Liu D, Zhang R, Yang X *et al.* (2011) Thermostable cellulase production of Aspergillus fumigatus Z5 under solid-state fermentation and its application in degradation of agricultural wastes. Int Biodeter Biodegr 65:717-725.

Louime C, Uckelmann H (2008) Cellulosic ethanol: securing the planet future energy needs. Int J Mol Sci 9:838-841.

Maheshwari R, Bharadwaj G, Bhat M (2000) Thermophilic fungi: their physiology and enzymes. Microbiol Mol Biol Rev 64:461-488.

Matkar K, Chapla D, Divecha J *et al.* (2013) Production of cellulase by a newly isolated strain of *Aspergillus sydowii* and its optimization under submerged fermentation. Int Biodeterior Biodegrad 78:24-33

Miller GL (1959) Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal Biochem 31:426-428.

Mirzaakhmedov S, Ziyavitdinov Z, Akhmedova Z *et al.* (2007) Isolation, purification, and enzymatic activity of cellulase

components of the fungus *Aspergillus terreus*. Chem Nat Compd 43:594-597.

Ng IS, Li CW, Chan SP *et al.* (2010) High-level production of a thermoacidophilic beta-glucosidase from *Penicillium citrinum* YS40-5 by solid-state fermentation with rice bran. Bioresour Technol 101:1310-1317.

Ong LG, Abd-Aziz S, Noraini S *et al.* (2004) Enzyme production and profile by *Aspergillus niger* during solid substrate fermentation using palm kernel cake as substrate. Appl Biochem Biotechnol 118:73-79.

Panagiotou G, Kekos D, Macris BJ *et al.* (2003) Production of cellulolytic and xylanolytic enzymes by *Fusarium oxysporum* grown on corn stover in solid state fermentation. Ind Crop Prod 18:37-45.

Parekh S, Vinci VA, Strobel RJ (2004) Improvement of microbial strains and fermentation processes. Appl Microbiol Biotechnol 54:287-301.

Raghuwanshi S, Deswal D, Karp M *et al.* (2014) Bioprocessing of enhanced cellulase production from a mutant of *Trichoderma asperellum* RCK2011 and its application in hydrolysis of cellulose. Fuel 124:183-189.

Senthilkumar SR, Ashokkumar B, Raj KC *et al.* (2005) Optimization of medium composition for alkali-stable xylanase production by *Aspergillus fischeri* Fxn 1 in solid-state fermentation using central composite rotary design. Bioresour Technol 96:1380-1386.

Singh A, Singh N, Bishnoi NR (2009) Production of cellulases by *Aspergillus heteromorphus* from wheat straw under submerged fermentation. Int J CivEnviron Eng 1:23-26.

Singhania RR, Sukumaran RK, Patel AK *et al.* (2010) Advancement and comparative profiles in the production technologies using solid-state and submerged fermentation for microbial cellulases. Enzyme Microb Technol 46:541-549.

Sukumaran RK, Singhania RR, Mathew GM *et al.* (2009) Cellulase production using biomass feed stock and its application in lignocellulose saccharification for bio-ethanol production. Renewable Energy 34:421-424.

Vu VH, Pham TA, Kim K (2011) Improvement of Fungal Cellulase Production by Mutation and Optimization of Solid State Fermentation. Mycobiology 39:20-25.

Wang CY, Hsieh YR, Ng CC *et al.* (2009) Purification and characterization of a novel halostable cellulase from *Salinivibrio* sp. strain NTU-05. Enzyme Microb Technol 44:373-379.

Wang XJ, Bai JG, Liang YX (2006) Optimization of multienzyme production by two mixed strains in solid-state fermentation. Appl Microbiol Biotechnol 73:533-540.

Xin F, Geng A (2010) Horticultural west as the substrate for cellulase and hemicellulase production by *Trichoderma reesei* under solid-state fermentation. Appl Biochem Biotechnol 162:295-306.

Yang S (2001) Chemistry of Plant Fibers. third ed. China Light Industry Press. Beijing.

Yang SQ, Yan QJ, Jiang ZQ *et al.* (2006) High-level of xylanase production by the thermophilic *Paecilomyces themophila* J18 on wheat straw in solid-state fermentation. Bioresour Technol 97:1794-1800.

Zhang YHP, Himmel ME, Mielenz JR (2006) Outlook for cellulase improvement: screening and selection strategies. Biotechnol Adv 24:452-481.

Associate Editor: Miguel Juan Beltran-Garcia