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Xylanase and cellulase production under extreme conditions in submerged fermentation by some fungi isolated from hypersaline, alkaline lakes of Wadi-El-Natrun, Egypt

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Abstract: Xylan was extracted from oat spelts and confirmed using UV spectrophotometry. Nine hundred and fifty-five isolates of fungi from air, water, mud and soil samples from hypersaline, alkaline lakes of Wadi-El-Natrun were screened for their ability to produce xylanase along with carboxymethyl cellulase (CMCase) using sucrose-free Czapek's agar medium supplemented with 1 % xylan as carbon source. Three hundred and twenty-two isolates out of 955 were xylanase producers (142 high, 108 moderate and 72 low producers). From the high producers, the most active 8 strains were selected and screened for their capabilities of production of extracellular xylanase and CMCase in submerged fermentation using sucrose-free-Cz broth amended with 1 % xylan. pH, nitrogen source and temperature were optimized for the maximum production of xylanase and CMCase by these strains. The most active eight strains showed species and strain variations in their optimal culture conditions. Four strains showed their maximum xylanase production at pH 11 including *A. fumigatus* AUMC 10333, *A. fumigatus* AUMC 10334 at pH 7, *A. flavus* at pH 9, *Corynascus sepedonium* at pH 5, and *A. fumigatus* AUMC 10332 at pH 3. On the other hand, the three strains of *A. fumigatus* and *A. oryzae* exhibited their maximum production of CMCase at pH 11, two strains at pH 7, one strain at pH 9 and one strain at pH 3. With regard to the nitrogen source, the eight strains showed also various capabilities for the maximum production of the two enzymes. Some strains preferred yeast extract or peptone while others preferred $NaNO_3$, $NaNO_2$ or NH_4Cl. The most active two strains, *Aspergillus oryzae* and *A. flavus* were screened for xylanase and cellulase production at different incubation periods, NaCl concentrations and xylan from different origins. The addition of NaCl promoted the production of the two enzymes to a maximum in case of xylanase production at 3.5 % NaCl by *A. flavus*, and at 2 % by *A. oryzae*, and at 4.5 % and 5 % in case of CMCase production by *A. flavus* and *A. oryzae* respectively. Xylan from birchwood was the best source for the yield of xylanase and from wheat bran was the best for cellulose whereas xylan from alkali-treated corn cobs was superior for both enzymes

Key words: Xylanase, cellulase, extreme habitats, Wadi-El-Natrun lakes.

Introduction

xylan is one of the major components of hemicelluloses in plant cell walls, and is the second most abundant polysaccharide after cellulose (Dhiman *et al*. 2008) comprising up to 30 % in angiosperms, up to 10 % in gymnosperms (Singh *et al*. 2003), < 30 % in annual plants (Fang *et al*. 2010, Ghosh *et al*. 2012) and up to 35 % of the renewable organic carbon on Earth (Collins *et al*. 2005).

Due to its heterogeneity and complexicity, the complete hydrolysis of xylan requires a large variety of cooperatively acting enzymes (Puls *et al*. 1987, Biely 1985, Subramaniyan and Prema 2002). Endo1,4-β-D-xylanases randomly cleave the xylan backbone, β-D-xylosidases cleave xylose monomers from the non-reducing end of xylo-oligosaccharides and xylobiose while removal of the side groups is catalyzed by α-L-arabinofuranosidases, α-D-glucuronidases, acetylxylan esterases, ferulic acid esterases and *p*-coumaric acid esterases (Collins *et al*. 2005).

Microbial xylanases have mesmerized researchers because of their prospected applications in industrial processes such as: biobleaching of pulp in paper industry, conversion of biomass waste to fermentable sugars for production of biofuel and other chemical and biomedical products, animal feed quality improvement, clarification of fruit juices and other drinks, detergents, starch, food, textile, baking and leather (Ghosh *et al*. 2012). Filamentous fungi are of special interest among microbial xylanase producers, because of their easy cultivation and secretion of greater amounts of xylanase into the culture medium than do bacteria. Xylanase produced by many filamentous fungi have special attribute like pH stability, high temperature optima and thermostability (Maheshwari *et al*. 2000).

This study aimed at screening of the fungal strains isolated from different saline, alkaline habitats of Wadi-El-Natrun for their ability to produce xylanase along with cellulase and to study the effect of different cultural and nutritional factors on their production by the most active strains.

Materials and Methods

Extraction of xylan from oat spelts

Alkaline extraction of xylan from oat sample was performed following the method of Puls *et al*. (2006). A hundred gram of the fine-milled oat spelts (C. Hanne Muhlenwerke GmbH & Co. Germany; marketed in Egypt) were soaked in one liter of 5 % NaOH. The mixture was steamed at 90° C for 60 min. The mixture was centrifuged at 5.000 xg for 30 min and the supernatant was collected. Methanol was added to the supernatant with a ratio of 2: 1 to precipitate the xylan without neutralization to facilitate the recovery of NaOH.

The residual lignin had to be degraded by peroxide (H_2O_2) bleaching and a subsequent washing using methanol. After centrifugation, the xylan was allowed to air dry before drying in hot air oven at 55°C. The pellets were weighed and powdered in a mixer (Puls *et al.* 2006). The prepared xylan was used for screening and production of xylanase.

UV-Spectrophotometry determination of the extracted xylan

Phenol-sulfuric-acid method described by Albalasmeh *et al*. (2013) for rapid determination of carbohydrate and total carbon concentrations using UV spectrophotometry was used. Two ml of a carbohydrate solution is mixed with one ml of 5 % aqueous solution of phenol in a test tube. Subsequently, 5 ml of concentrated sulfuric acid were added rapidly to the mixture. After allowing the test tubes to stand for 10 min, they were vortexed for 30s and placed for 20 min in a water bath at room temperature for color development. Then, light absorption at 490 nm was recorded on a spectrophotometer (T60 UV-Visible spectrophotometer, PG Instruments).

Fungal isolates tested

Nine hundred fifty-five fungal isolates recovered from air, water, mud and newly reclaimed soil samples collected from the biggest 8 hypersaline, alkaline lakes of Wadi-El-Natrun, Egypt (Ismail *et al.* 2017) were used (Table, 1).

Cultivation medium

Sucrose-free Czapek's medium supplemented with 1 % xylan as carbon source was the initial medium used for xylanase production and has the following composition of (g/l): xylan, 10; Na_2NO_3, 2; K_2HPO_4, 1; KCl, 0.5; $MgSO_4.7H_2O$, 0.5; $FeSO_4$, 0.01; $ZnSO_4$, 0.01; $CuSO_4$, 0.005, agar, 15.

Screening for xylanase production

Xylanase production was detected on sucrose-free Czapek's agar medium amended with xylan (extracted from oat spelts) as a sole carbon source. Fifty µl of spore suspension from 7-day-old culture of the tested fungal isolates were added to each well (5 mm diameter) on the agar plates. The inoculated plates were incubated for 2 days at 30ºC. The clear zones formed around the wells were more visible when the plates were flooded with 0.25 % (w/v) aqueous iodine solution. The diameters of the clear zones against the purple-blue colour of the test medium indicating enzyme production were measured (Fig. 1).

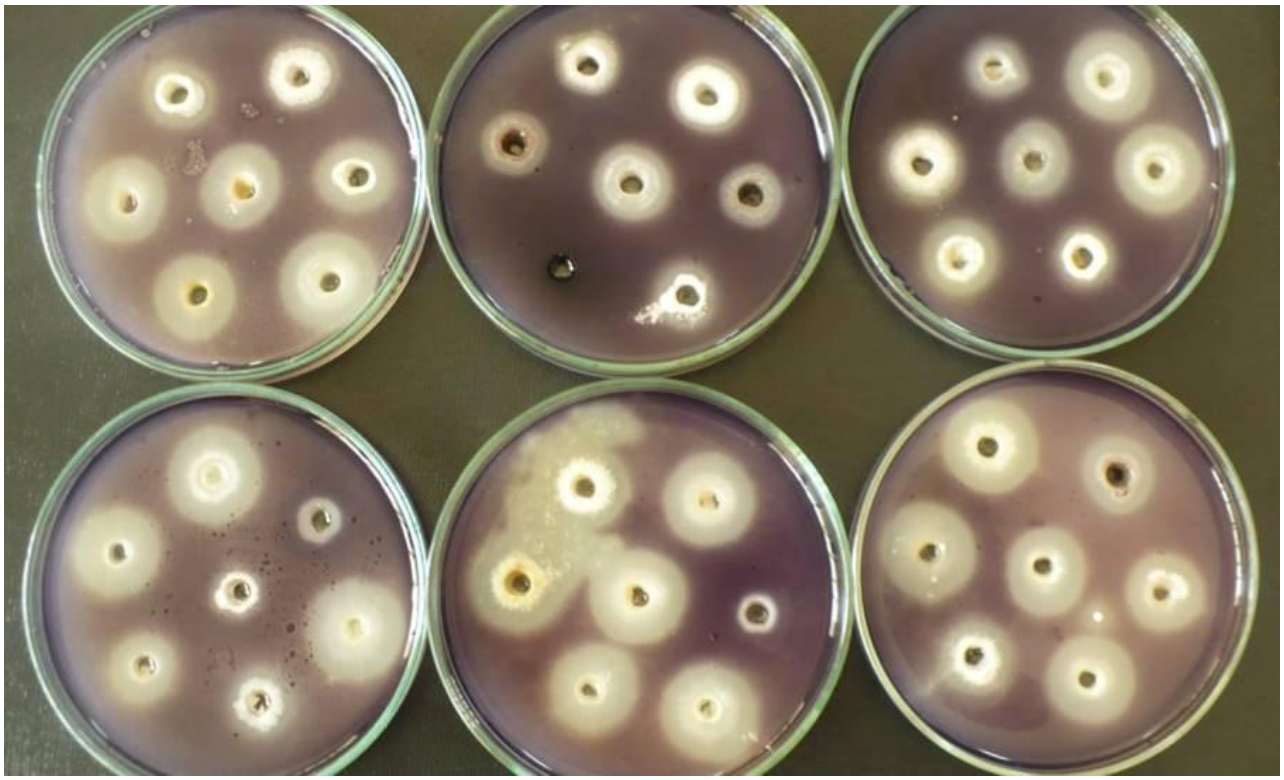

Figure 1: Xylanolytic activity of some fungal isolates tested on 1 % xylan-Cz agar after 48 h at 30º C showing the clear zones against purple-blue colour of the test medium.

Growth of isolates under submerged fermentation

The 322 positive fungal isolates were grown each in a 250 ml Erlenmeyer conical flask containing 50 ml Czapek's broth medium supplemented with 1 % previously prepared xylan from oat spelts. The flasks were inoculated each with 1 ml spore suspension of 7-day-old colony. The flasks were incubated at 30ºC in shaking incubator at 120 rpm for 7 days. After incubation, the medium was filtered through Whatman No.1 filter paper and the filtrate was centrifuged at 10.000 xg for 10 min at 4ºC. The clear supernatant was used as a source of xylanase and cellulase.

Xylanase assay

Xylanase production was determined by mixing 0.9 ml of 1 % xylan from oat spelts (Fluka), prepared in 50 mM Na-citrate buffer, pH 5.4) with 0.1 ml of filtered crude enzyme and the mixture was incubated at 50°C for 5 min (Bailey *et al*., 1992). The reaction mixture was stopped by addition of 1.5 ml of 3, 5-dinitrosalicylic acid (DNS) (Miller, 1959) and the contents were boiled in water bath for 10 min. After cooling, absorbance of the colour developed was measured at 540 nm (T60 UV-Visible spectrophotometer, PG Instruments). The amount of reducing sugar liberated was quantified using standard curve of xylose. One unit of xylanase is defined as the amount of enzyme that liberates 1 µmol of xylose equivalent per minute under the previous assay conditions (Ghose and Bisaria 1987), and xylanase concentration is calculated as follows:

$$\text{Xylanase concentration} = \frac{\text{xylose concentration (g/l)}}{0.00015} \quad \text{IU/l}$$

Cellulase assay

Cellulase concentration was determined as mentioned in xylanase assay but carboxymethyl cellulose replaced xylan and glucose replaced xylose for standard curve. One unit of CMCase is defined as the amount of enzyme that liberates 1 µmol of glucose equivalent per minute under the previous assay conditions (Ghose and Bisaria 1987), and cellulase concentration is calculated as follows:

$$\text{Cellulase concentration} = \frac{\text{glucose concentration (g/l)}}{0.00018} \quad \text{IU/l}$$

The most active 8 producing strains (based on submerged fermentation results) were selected for optimization of pH, nitrogen source and temperature for the best production of xylanase and cellulase and these strains were deposited in Assiut University Mycological Centre Culture Collection (AUMC) (Table, 1).

1) Optimization of pH

Five pH values (3, 5, 7, 9 and 11) were tested on xylanase and CMCase production. pH 3 and 5 were prepared using citrate buffer while pH 7, 9 and 11 were prepared using phosphate buffer solution. Erlenmeyer conical flask (250 ml) containing 50 ml of sucrose-free Czapek's solution amended with 1 % xylan from oat spelts adjusted at the desired pH value was inoculated with 1 ml of 10^6 spore suspension (prepared in 10 % tween 80) of the tested fungal strains. The flasks were incubated at 30ºC and 120 rpm for 7 days. The amount of reducing sugars liberated was quantified using xylose or glucose as standard. The enzyme concentration was determined.

2) Optimization of nitrogen source

The effect of five nitrogen sources, namely peptone, yeast extract, sodium nitrate, sodium nitrite and ammonium chloride on xylanase and CMCase production at the optimized pH for each fungal strain was determined. Sodium nitrite (1.621 g/l) and ammonium chloride (1.257 g/l) were used as sodium nitrate (2 g/l) equivalent.

3) Optimization of temperature

The effect of five temperature degrees, 25, 30, 35, 40 and 45º C on xylanase and CMCase production was determined at the optimal pH and the nitrogen source for each fungal strain.

The most potent xylanase producing strains, *A. oryzae* and *A. flavus* were confirmed genotypically (using the method described in Moubasher *et al*. 2016) and selected for further optimization of xylanase and cellulase at the previously optimized pH, nitrogen source and temperature (Table 1).

4) Optimization of incubation period

The effect of incubation period of 1, 2, 3, 4, 5, 6 and 7 days at 120 rpm was tested. The amount of reducing sugar liberated was quantified using xylose and glucose as standard.

5) Effect of NaCl concentration

Ten concentrations of NaCl (0.5-5 %) in 0.5 % increment were tested after 7 days of incubation at the optimized pH, nitrogen source and temperature conditions.

6) Effect of different types of xylan

Birchwood xylan, xylan extracted from oat spelts, corn cobs, alkali-treated corn cobs, wheat bran or corn stalks was used as the sole carbon source at 1 % rate in Cz-solution for production of xylanase and cellulase enzymes by the 2 active strains under submerged fermentation at the optimized conditions of pH, nitrogen source and temperature.

Table 1: Assiut University Mycological Centre (AUMC) accession number of the highest xylanase producing 2 strains with their accession GenBank numbers given together with the closest match in the GenBank data base and sequence similarity percent to the match as inferred from blastn searches of ITS sequences.

Source	AUMC	GenBank accession No	bp	Closest match	Similarity (%)	Identification
Mud	10329	KX531010	598	NR_111041=ATCC 16883 NR_135395=NRRL 447^T	589/595(98.99) 574/577(99.48)	*A. oryzae*
Soil	10331	KX531011	599	AD-B3 = HQ285520 NR_111041=ATCC 16883^T	599/599(100) 593/596(99.49)	*A. flavus*

Results and Discussion

Extraction and determination of xylan from oat spelts

The oat spelts were milled before the extraction process to facilitate the penetration of the NaOH. This treatment also enables the separation of the residual starch granules from the spelts (Puls *et al*., 2006). They found that the arabinoxylan composition of pretreated and sifted oat spelts was: glucose (31.9 %); was completely derived from cellulose and no starch could be detected, xylose (28.1 %) and arabinose (3.5 %). Because of the presence of 21.3 % lignin in the oat spelts there was a requirement for elevated temperature (90αC) for an effective extraction of xylan. Saake *et al*. (2004) and Puls *et al*. (2006) found that extraction with 5 % (w/v) NaOH at 90° C for 60 min. was optimal for xylan recovery. Using UV analysis, our results revealed that after alkaline treatment and methanol precipitation, about 40 g of crude product (contained 22.77 g = 56.92 % xylan) was obtained from 100 g sifted oat spelts. In this respect, Hettrich *et al*. (2006) reported that in oat spelts, xylan comprises up to 35-40 % of the total mass.

Screening of fungi for xylanase production

Out of 955 isolates, 322 isolates were positive xylanase producers using clear zone test. The latters were screened quantitatively using submerged fermentation test, and were classified into low (72), moderate (108) and high (142). From high producers, the most active 8 strains were selected for further optimization conditions of xylanase and CMCase production (Tables 2, 3-5). The 8 strains were related to *Aspergillus fumigatus* (3 strains), *A. flavus*, *A. oryzae*, *Corynascus sepedonium*, *Penicillium chrysogenum* and *Trichoderma koningii* (1 strain each). Similar results were obtained by Kadowaki *et al*. (1995), Das and Nanda (1995) and Carmona *et al*. (1997) who reported that *Aspergillus* species were the most dominant as xylanase and cellulase producers. All strains produced xylanase along with cellulase in submerged fermentation and this might be because of the presence of cellulose in the crude xylan prepared. It was found that both xylanase and cellulase were produced when cellulose and hemicellulose were used together as carbon source (Kulkarni *et al*. 1999). Haltrich *et al*. (1996) also reported that xylan-degrading organisms are often cellulolytic and secrete complex mixtures of xylanase and cellulase. Among the mesophilic fungi, species of *Aspergillus* and *Trichoderma koningii* are predominant in xylanase production (Polizeli *et al*. 2005). Sales *et al*. (2011) in their study found that all *Aspergillus* strains tested produced cellulase and xylanase in varied levels. In harmony with the current results, Abdel-Sater and El-Said (2001) found also members of *Aspergillus*, *Penicillium* and *Trichoderma* to be the most prevalent fungi isolated from rice straw and were xylan decomposing fungi.

Table 2: Preliminary screening using clear zone test for positive xylanase producers (322 isolates out of 955 isolates) and quantitative screening using submerged fermentation test for the positive strains.

Taxa	No. of isolates	No. of + ve isolates			
		Preliminary screening	Submerged fermentation		
			H	M	L
Acremonium Link	**70**	**10**		**4**	**6**
A. alabamensis Morgan-Jones	1	0			
A. alternatum Link	1	0			
A. curvulum W. Gams	2	1		1	
A. thermophilum W. Gams & J. Lacey	2	1		1	
A. zonatum (Sawada) W. Gams	21	2			2
Acremonium spp.	43	6		2	4
Alternaria	**7**	**1**			**1**
A. alternata (Fries) Keissler	5	1			1
A. chlamydosporigena Woudenberg & Crous	2	0			
Aspergillus P. Micheli ex Link	**302**	**150**	**77**	**42**	**31**
A. aegyptiacus Moubasher & Moustafa	2	1		1	
A. brasiliensis Varga *et al*.	17	5		3	2
A. candidus Link	1	1		1	
A. deflectus Fennell & Raper	3	0			
A. flavipes (Bainier & Sartory) Thom & Church	17	4	1	1	2
A. flavus Link	54	29	17	8	4
A. flavus var. *columnaris* Raper & Fennell	9	6	5		1
A. flocculosus Frisvad & Samson	1	1		1	
A. foetidus (Nakaz.) Thom & Raper	2	0			
A. fumigatus Fresenius	54	30	18	5	7
A. insulicola Montemayor & Santiago	3	2		1	1
A. lacticoffeatus Frisvad & Samson	2	1	1		
A.neoafricanus Samson, Peterson, Frisvad & Varga	5	2	2		

Taxa	No. of isolates	No. of + ve isolates			
		Preliminary screening	Submerged fermentation		
			H	M	L
A. niger van Tieghem	18	8	5	2	1
A. ochraceus Wilhelm	16	8	4	2	2
A. oryzae (Ahlburg) E. Cohn	1	1	1		
A. parasiticus Speare	12	8	3	4	1
A. petrakii Vörös- Felkai	3	2			2
A. roseoglobulosus Frisvad & Samson	2	0			
A. sclerotiorum G. A. Huber	3	2	1	1	
A. sulphureus (Fresen.) Wehmer	6	1		1	
A. sydowii (Bainier & Sartory) Thom & Church	16	8	3	1	4
A. tamarii Kita	1	1	1		
A. terreus Thom	46	25	14	9	2
A. terricola E. J. Marchal	1	1			1
A. turcosus Hong, Frisvad & Samson	1	1		1	
A. unguis (Emile-Weil & Gaudin) Thom & Raper	1	1			1
A. versicolor (Vuillemin) Tirab.	1	0			
A. viridi-nutans Ducker & Thrower	3	0			
Aspergillus sp.	1	1	1		
Botryotrichum piluliferum Saccardo & Marchal	**2**	0			
Chaetomium Kunze	**6**	**0**			
C. globosum Kunze	3	0			
C. nigricolor L.M. Ames	1	0			
C. thermophile La Touche	2	0			
Chordomyces antarcticum Bilanenko, Georgieva, Gum-Grzhimaylo	2	0			
Cladosporium Link	**15**	**1**		**1**	
C. cladosporioides (Fresenius) de Vries	4	0			
C. sphaerospermum Penzig	8	0			
Cladosporium spp.	3	1		1	
Clonostachys Corda	**4**	**1**		**1**	
C. rosea (Link) Schroers, Samuels, Seifert & W. Gams	3	0			
C. solani (Harting) Schroers & W. Gams	1	1		1	
Cochliobolus tuberculatus Sivanesan	15	1			1
Corynascus sepedonium (C.W.Emmons) Arx	4	4	4		
Emericella Berkeley & Broome	**76**	**38**	**22**	**14**	**2**
E. acristata Fennell & Raper	1	0			
E. dentata (D.K.Sandhu & R.S.Sandhu) Y. Horie	4	1	1		
E. lata Subramanian	3	0			
E. nidulans (Eidam) Vuillemin	33	14	8	6	
E. quadrilineata (Thom & Raper) C.R. Benj	32	21	13	7	1
E. rugulosa (Thom & Raper) C.R. Benj	2	1		1	
E. variecolor var. *astellata* (Fennell & Raper) Benjamin	1	1			1
Eurotium	**5**	**3**		**3**	
E. amstelodami (Mangin) Thom & Church	2	0			
E. rubrum Thom & Church	3	3		3	
Exerohilum rostratum (Drechsler) Leonard & Suggs	**5**	1		1	
Fusarium Link	**69**	**17**	**2**	**12**	**3**
F. oxysporum Schlechtendahl & Hansen	7	4		4	
F. sambucinum Füchel	6	0			
F. semitectum Berkeley & Ravenel	8	1		1	
F. solani (Mart.) Appel & Wollenw. emend. Sny. & Hans.	47	12	2	7	3
F. thapsinum Klittich, Leslie, Nelson & Marasas	1	0			
Gliocladium Corda	**3**	**1**		**1**	
G. catenulatum Gilman & Abbott	1	0			
G. penicillioides Corda	2	1		1	

Taxa	No. of isolates	No. of + ve isolates			
		Preliminary screening	Submerged fermentation		
			H	M	L
Gymnascella hyalinospora (Kuehn, Orr & Ghosh) Currah	**2**	0			
Humicola	**17**	**1**		**1**	
H. grisea var. *thermoidea* Cooney & Emerson	8	1		1	
H. fusco-atra Traaen	9	0			
Isaria felina (DC.) Fries	1	0			
Malbranchea cinnamomea (Lib.) Oorschot & de Hoog	24	6	3	2	1
Microascus manginii (Loubière) Curzi	2	0			
Mucor Fresenius	**8**	**0**			
M. circinellioides van Tieghem	4	0			
M. hiemalis Wehmer	4	0			
Myriococcum albomyces Cooney & Emerson	23	5	3	1	1
Myrothecium verrucaria (Albertini & Schweinitz) Ditmer ex Steudel	1	0			
Paecilomyces Bainier	**10**	**3**		**1**	**2**
P. inflatus (Burnside) J. W. Carmich.	1	1			1
P. variotii Bainier	5	1			1
P. zollerniae Stolk & Samson	1	0			
Paecilomyces sp.	3	1		1	
Papulaspora irregularis H.H. Hotson	2	0			
Paracremonium inflatum Lombard & Crous	1	0			
Penicillium Link	**123**	**41**	**13**	**15**	**13**
P. aurantiogriseum Dierckx	10	5	2	1	2
P. brevicompactum Dierckx	4	0			
P. chrysogenum Thom	49	24	10	8	6
P. citrinum Thom	3	1		1	
P. corylophilum Dierckx	1	0			
P. crustosum Thom	7	3		2	1
P. dendriticum Pitt	1	0			
P. donkii Stolk	2	1		1	
P. duclauxii Delacroix	3	1			1
P. funiculosum Thom	8	2		1	1
P. griseofulvum Dierckx	1	0			
P. italicum Wehmer	1	0			
P. janthinellum Biourge	1	0			
P. melinii Thom	2	2			2
P. olsonii Bainier & Sartory	2	0			
P. oxalicum Currie & Thom	4	0			
P. puberulum Bainier	3	0			
P. raistrickii G.Sm.	1	0			
P. sublateritium Biourge	1	0			
P. waksmanii K.M. Zalessky	1	0			
Penicillium spp.	18	2	1	1	
Phaeoacremonium sp.	5	0			
Phialophora sp.	3	1			1
Phoma glomerata (Corda) Wollenw. & Hochapfel	2	0			
Plectosphaerella oligotrophica Liu, Hu, Liu & Cai	2	0			
Purpureocillium lilacinum (Thom) Luangsa-ard, Houbraken, Hywel-Jones & Samson	2	0			
Ramophialophora sp.	1	1		1	
Rhizomucor pusillus (Lindt) Schipper	10	2	1	1	
Sarocladium Gams & Hawksworth	**10**	**0**			
S. kiliense (Grütz) Summerbell	4	0			
S. strictum (W. Gams) Summerbell	5	0			
S. subulatum Giraldo, Gené & Guarro	1	0			
Scopulariopsis Bainier	**13**	**5**		**3**	**2**

Taxa	No. of isolates	No. of + ve isolates			
		Preliminary screening	Submerged fermentation		
			H	M	L
S. acremonium (Delacroix) Vuillemin	1	0			
S. brevicaulis (Saccardo) Bainier	3	2		2	
S. brumptii Salv. -Duval	5	2		1	1
S. carbonaria Morton & G. Sm.	1	0			
S. fusca Zach	2	1			1
S. halophilica Tubaki	1	0			
Scytalidium thermophilum (Cooney & Emerson) Austwick	16	6	4		2
Stachybotrys Corda	**4**	**0**			
S. dichroa Grove	1	0			
S. ramosa Udaiyan	1	0			
S. verrucispora Matsushima	1	0			
S. virgata Krzemien. & Badura	1	0			
Stilbella fimetaria (Pers.) Lindau	1	0			
Talaromyces thermophilus Stolk	18	1			1
Thermoascus aurantiacus var. *levisporus* Upadhyay, Farmelo, Goetz & Melan	2	1		1	
Thermomyces Tsikl	**43**	**14**	**8**	**1**	**5**
T. ibadanensis Apinis & Eggins	9	2	1		1
T. lanuginosus Tsikl	34	12	7	1	4
Trichoderma Pers.	**19**	**5**	**5**		
T. harzianum Rifai	12	1	1		
T. koningii Oudem	4	4	4		
T. longibrachiatum Rifai	3	0			
Ulocladium Preuss	**5**	**2**		**2**	
U. atrum Preuss	2	0			
U. botrytis Preuss	1	1		1	
U. lanuginosum (Harz.) Simmons	1	1		1	
U. tuberculatum E.G. Simmons	1	0			
Total	**955**	**322**	**142**	**108**	**72**
No. of genera	**(44)**				
No. of species and variety	**(129 + 4 varieties)**				

Submerged fermentation [L = low producers (> 0 – 2 mg/ml xylose), M = moderate (> 2 – 5 mg/ml), and H = high (> 5 mg/ml)].

Optimization of pH

pH 11 was the optimum for xylanase production by 4 strains: *A. oryzae*, *A. fumigatus* AUMC 10333, *P. chrysogenum* and *T. koningii*. pH 3 was for *A. fumigatus* AUMC 10332, pH 5 for *C. sepedonium*, pH 7 for *A. fumigatus* AUMC 10334 and pH 9 for *A. flavus* (Table 3). CMCase reached its maximum production with pH 11 in 4 strains, the three *A. fumigatus* strains and *A. oryzae*. *P. chrysogenum* and *T. koningii* gave their highest production at pH 7 while pH 9 was optimum for *A. flavus* and pH 3 for *C. sepedonium* (Table 3). These results showed the optimum pH for xylanase production varied greatly with the strain. *A. fumigatus* strains (3 strains) varied in their pH optima from 3 for *A. fumigatus* AUMC 10332, 7 for AUMC 10334 and 11 for AUMC 10333. Many researchers have reported acidic pH (5–6.5) as the optimum for xylanase production from fungi (Gupta *et al*. 2009, Murthy and Naidu 2010). In agreement with the present study, Ruckmanl and Rajendran (2001) found that *A. flavus* and *A. fumigatus* showed sufficient alkalitolerance and capability to grow and produce xylanase at pH 10. Bailey and Viikari (1993) found also that a pH below 3.0 was found to be essential for efficient production of xylanase by *A. fumigatus* on beech xylan. Maximum CMCase produced by *A. fumigatus* was recorded at pH 4, whereas maximum xylanase production was observed at pH 5 (Sarkar and Aikat 2014). Cellulase and xylanase production by *Trichoderma* spp. was found to be gradually increased as the pH value increased from 4-5.5 and reached its optimum level of pH 5.5 (Bilal *et al*. 2015).

Table 3: Xylanase and CMCase production by the most active 8 strains in submerged fermentation culture medium with different pH values at 30° C.

Taxa	AUMC	pH	Xylanase (IU/l)	CMCase (IU/l)
Aspergillus flavus	10331	3	3760	500
		5	10666	616.7
		7	18666	1894
		9	**24800**	**1944**
		11	14600	1444
Aspergillus fumigatus	10332	**3**	**10300**	833
		5	4426	905
		7	6000	372
		9	4360	1233
		11	5466	**1944**
Aspergillus fumigatus	10333	3	2740	1094
		5	6000	1477
		7	8400	2222
		9	10666	2611
		11	**12760**	**2777**
Aspergillus fumigatus	10334	3	4780	1333
		5	6000	511
		7	**17000**	2611
		9	14800	2777
		11	14066	**3333**
Aspergillus oryzae	10329	3	5666	611
		5	7000	372
		7	13706	1744
		9	15666	1350
		11	**20333**	**2000**
Corynascus sepedonium	10335	3	1133	**2388**
		5	**8366**	1572
		7	4666	1888
		9	3166	1388
		11	5833	2333
Penicillium chrysogenum	10330	3	533	350
		5	566	255
		7	653	**1172**
		9	573	1083
		11	**700**	905
Trichoderma koningii	10328	3	1666	488
		5	5026	905
		7	9840	**1488**
		9	9840	1361
		11	**13640**	1388

Optimum pH for each strain for maximum production of xylanase and CMCase appears in bold.

Optimization of nitrogen source

Xylanase production by *A. flavus* AUMC 10331 and *A. fumigatus* AUMC 10334 was enhanced when sodium nitrate was used as a nitrogen source. Yeast extract was found to be the best source for *A. fumigatus* AUMC 10333 and *T. koningii*. Ammonium chloride was the best for xylanase production by *C. sepedonium* and *P. chrysogenum*. *A. fumigatus* AUMC 10332 gave its peak in xylanase production with peptone while sodium nitrite was the best nitrogen source for *A. oryzae* (Table 4). On the other hand, Olanbiwoninu and Odunfa (2016) found that the supplementation of organic nitrogen (peptone, urea and yeast extract) yielded higher xylanase production by *A. terreus* than the inorganic (ammonium sulfate and sodium nitrate) nitrogen sources.

CMCase gave its maximum production with peptone for the three *A. fumigatus* strains, *P. chrysogenum* and *T. koningii*. It was reported that the maximum cellulase production by *A. fumigatus* was obtained with peptone, tryptone, or yeast extract (Sarkar and Aikat 2014). On the other hand, sodium nitrite was the best nitrogen source for production of CMCase by *A. flavus*, *A. oryzae* and *C. sepedonium* (Table 4). In accordance with the current results, some studies have reported inorganic nitrogen sources resulting in higher cellulase production by *Thermoascus aurantiacus* (Kalogeris *et al*. 2003) while some others have reported organic nitrogen sources gave the highest yield from *A. terreus* (Olanbiwoninu and Odunfa 2016). Ghanem *et al*. (2000) stated that addition

of yeast extract always lead to an increase in cellulase and xylanase production by *A. terreus*.

Optimization of temperature

The three *A. fumigatus* showed three different optimum temperatures (25º, 30º and 35ºC) for xylanase production, while 25ºC was the optimum for *C. sepedonium* and *P. chrysogenum*, 30° C for *A. oryzae* and *T. koningii* and 35º C for *A. flavus* (Table 5).

For CMCase production 30° C was the optimum temperature for 6 strains out of 8 tested and these were *A. flavus*, *A. fumigatus* AUMC 10332, AUMC 10333, *A. oryzae*, *P. chrysogenum*, and *T. koningii*. In accordance with the present results, *A. oryzae* exhibited its maximum cellulase and xylanase production at 30º C (Hassan *et al*. 2016). *T. koningii* exhibited its peak for cellulase production at 28º C (Wang *et al*. 2012). Bilal *et al*. (2015) found that the optimum temperature of xylanase and cellulase production by *Trichoderma* spp. was 28º to 32º. It was also observed that cellulase as well as xylanase production by *A. fumigatus* reached their maxima at 30 º C (Sarkar and Aikat 2014). On the other hand, the maximum production of CMCase was recorded for *A. fumigatus* AUMC 10334 at 40º C while for *C. sepedonium* at 45º C (Table 5), and both are thermotolerant.

The highest xylanase producing 2 strains (*A. flavus* AUMC 10331 and *A. oryzae* AUMC 10329) were selected for further optimization of xylanase and cellulase production. Also, the identity of the two strains was confirmed genotypically (Table 1).

Table 4: Xylanase and CMCase production by the most active 8 strains in submerged fermentation with different nitrogen sources at 30° C and optimized pH values.

Taxa	AUMC	N_2- source	Xylanase (IU/l)	CMCase (IU/l)
Aspergillus flavus	10331	Peptone	7400	1977
		Yeast extract	9100	2300
		Na_2NO_3	**35640**	3266
		$NaNO_2$	32800	**3444**
		NH_4Cl	27400	2166
Aspergillus fumigatus	10332	**Peptone**	**11000**	**8944**
		Yeast extract	6666	555
		Na_2NO_3	5766	2111
		$NaNO_2$	9866	7016
		NH_4Cl	3000	2455
Aspergillus fumigatus	10333	**Peptone**	9633	**4444**
		Yeast extract	**31666**	3538
		Na_2NO_3	1266	2277
		$NaNO_2$	1933	1816
		NH_4Cl	3093	2777
Aspergillus fumigatus	10334	**Peptone**	18666	**3138**
		Yeast extract	20666	2316
		Na_2NO_3	**27666**	1944
		$NaNO_2$	24266	2333
		NH_4Cl	24500	1022
Aspergillus oryzae	10329	Peptone	18333	2572
		Yeast extract	13200	2522
		Na_2NO_3	14200	2777
		$NaNO_2$	**35466**	**4722**
		NH_4Cl	19800	2033
Corynascus sepedonium	10335	Peptone	2666	594
		Yeast extract	3733	744
		Na_2NO_3	4780	733
		$NaNO_2$	7700	**833**
		NH_4Cl	**7840**	733
Penicillium chrysogenum	10330	Peptone	5093	**3333**
		Yeast extract	3600	3138
		Na_2NO_3	4500	1944
		$NaNO_2$	1266	1594
		NH_4Cl	**6266**	2555
Trichoderma koningii	10328	**Peptone**	6780	**5000**
		Yeast extract	**15333**	2888
		Na_2NO_3	5480	1788
		$NaNO_2$	2493	1666
		NH_4Cl	11700	2777

Optimum nitrogen source for each strain for maximum production of xylanase and CMCase appears in bold

Table 5: Effect of temperature on xylanase and CMCase production by the most active 8 strains in submerged fermentation with optimum pH and nitrogen sources.

Taxa	AUMC	Temperature °C	Xylanase (IU/l)	CMCase (IU/l)
Aspergillus flavus	10331	25	30933	1755
		30	35640	**3122**
		35	**39333**	2277
		40	13333	1411
		45	4280	777
Aspergillus fumigatus	10332	25	8966	66
		30	11033	**8533**
		35	**14800**	122
		40	9733	0.00
		45	5173	355
Aspergillus fumigatus	10333	**25**	**27866**	1066
		30	3166	**3388**
		35	13040	588
		40	7133	2311
		45	6066	988
Aspergillus fumigatus	10334	**25**	**30666**	1244
		30	27693	1844
		35	23800	1188
		40	12373	**2522**
		45	3666	833
Aspergillus oryzae	10329	25	32966	1311
		30	**35466**	**4522**
		35	12400	388
		40	5333	377
		45	4153	1000
Corynascus sepedonium	10335	**25**	**14833**	92
		30	7833	733
		35	8800	0.00
		40	8866	0.00
		45	8700	**2305**
Penicillium chrysogenum	10330	**25**	**23333**	1388
		30	6333	**2444**
		35	6000	1655
		40	5333	900
		45	4520	988
Trichoderma koningii	10328	25	4780	477
		30	**15333**	**2755**
		35	5833	655
		40	4666	300
		45	4020	644

Optimum temperature for each strain for maximum production of xylanase and CMCase appears in bold.

Effect of incubation period

Xylanase concentration reached its peak after 96 hours in submerged culture by both *A. flavus* and *A. oryzae* (Table 6). On the other hand, cellulase recorded its highest production after 72 hours of incubation for both *A. oryzae* and *A. flavus* (Table 6). Similarly, maximum production of xylanase and cellulase was obtained by *A. oryzae* after 4 days of incubation in the study of Hassan *et al*. (2016), but 2 days of incubation were optimum for *A. flavus* (Guimaraes *et al*. 2013) and *A. terreus* (Sorgatto *et al*. 2012). In accordance with the current results, 96 hours of incubation were the optimum incubation period for production of xylanases and cellulases [filter paper (FPase), endoglucanases and β-glucosidases] by *Penicillium echinulatum* in submerged fermentation (dos Reis *et al*. 2015) while 120 hours were the optimum for xylanase production by *Fusarium solani* F7 (Gupta *et al*. 2009) and 144 hours were the optimum for *Penicillium oxalicum* (Muthezhilan *et al*. 2007). CMCase reached its maximum production through incubation of *A. flavus* for 3 days (Gomathi *et al*. 2012).

Effect of NaCl on xylanase and cellulase production

2 % NaCl induced the highest xylanase production by *A. oryzae*, while 3.5 % NaCl achieved the highest xylanase production by *A. flavus*. On the other hand,

cellulase production by *A. flavus* gave its peak at 4.5 % NaCl, while *A. oryzae* recorded its peak at 5 % NaCl (Table 7). This halophilic/halotolerant tribute is most probably acclimatization of the two organisms for the saline nature of the habitats. Also, Yadav and Jaitly (2011) tested the effect of 0-3 % NaCl on xylanolytic ability of some thermophilic/thermotolerant fungi and found that all isolates tested of *Humicola fusco-atra*, *Humicola grisea*, *Sporotrichum thermophile* showed an increase in their xylanase activity with the increase in salt concentration up to 2 % NaCl. thereafter it decreased and one of them showed double salt optima for xylanase activity, one at 1.5 % and the other at 2.5 % salt level (*Thermoascus aurantiacus*).

Table 6: Xylanase and cellulase production by the most active 2 strains at different incubation periods

Taxa	AUMC	Incubation period (h)	Xylanase (IU/l)	Cellulase (IU/l)
A. flavus	10331	24	15931	12626
		48	25920	11936
		72	21242	**15809**
		96	**26363**	12573
		120	21937	12520
		144	21495	11671
		168	20799	14323
A. oryzae	10329	24	12644	6100
		48	73399	3713
		72	84779	**7108**
		96	**107286**	6100
		120	87245	5517
		144	89015	6578
		168	83831	5782

Optimum incubation period for maximum production of xylanase and CMCase appears in bold

Table 7: Xylanase and cellulase production by the most active 2 strains at different concentrations of NaCl in submerged fermentation

Taxa	AUMC	NaCl conc. (%)	Xylanase (IU/l)	Cellulase (IU/l)
A. flavus	10331	0.5	15109	13475
		1	17512	11671
		1.5	21811	12732
		2	21052	11565
		2.5	17765	12679
		3	15425	11671
		3.5	**39386**	12626
		4	18776	11724
		4.5	25162	**13528**
		5	16500	12414
A. oryzae	10329	0.5	40271	5570
		1	47036	6260
		1.5	71692	4880
		2	**98814**	6153
		2.5	98372	5994
		3	72577	6525
		3.5	73968	6949
		4	46024	7586
		4.5	19535	8169
		5	13466	**8912**

Optimum NaCl concentration for maximum production of xylanase and CMCase appears in bold.

Enzyme production using different xylan sources

Birchwood xylan (sigma) was the best carbon source for xylanase production and wheat bran was the most favorable for cellulase production by *A. flavus*, while alkali-treated corn cobs was the best xylanase ans cellulose production by *A. oryzae* (Table 8). Da Silva *et al*. (2015) revealed that maximum xylanase production by *A. flavus* was obtained when wheat bran and sugarcane bagasse followed by a mixture of wheat bran and corncob were used as carbon source. Many reports have been carried out using agroindustrial residues as carbon sources for xylanase production by *Aspergillus* species (Techapun *et al*. 2003, Facchini *et al*. 2011, Guimaraes *et al*. 2013).

Table 8: Xylanase and cellulase production by the most active 2 strains with different xylan sources in submerged fermentation

Taxa	AUMC	Xylan origin	Xylanase (IU/l)	Cellulase (IU/l)
A. flavus	10331	**Birchwood (Sigma)**	**24276**	11140
		Oat spelts (prepared)	15805	12095
		Corn cobs (prepared)	18776	10822
		Alkali-treated corn cobs	16247	11671
		Wheat bran (prepared)	16247	**12732**
		Corn stalks (prepared)	15868	11034
A. oryzae	10329	Birchwood (Sigma)	31041	5941
		Oat spelts (prepared)	32432	5888
		Corn cobs (prepared)	40145	4668
		Alkali-treated corn cobs	**81428**	**7745**
		Wheat bran (prepared)	35150	5623
		Corn stalks (prepared)	54370	6949

Best xylan type for each strain for maximum production of xylanase and CMCase appears in bold.

Conclusion

Fungi from extreme hypersaline and alkaline habitats of Wadi-El-natrun were isolated and screened for their ability to produce xylanase along with cellulase. Some nutritional and environmental conditions for the most active strains were optimized for the highest xylanase production using medium supplemented with xylan from oat spelts in solid and liquid medium. The most active two strains were selected for co-production of xylanase and cellulase under different NaCl concentrations. These two strains are considered as promising xylanase producers that need more investigation for maximizing production.

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