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Mycobiota Associated with Wheat Grains, Wheat Flour and Cellulolytic Ability at Taif City, Saudia Arabia

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Abstract Forty species belonging to 20 genera were collected from wheat grains on plates of glucose- (16 genera and 26 species), cellulose- (16 and 25), Czapek's agar, yeast starch- (12 and 19) agar and sabouraud's- (20 and 38) dextrose agar media at 28°C. The most common species were: *Acremonium strictum*, *Alternaria alternata*, *Aspergillus flavus*, *A. niger*., *Cochliobolus lunatus*, *Chrysosporium lucknowense* and *Nectria haematococca*. Forty species belonging to 20 genera were collected from wheat flour on plates of glucose- (16 genera and 32 species), cellulose (16 and 32) - Czapek's agar, yeast starch (15 and 23) agar and sabouraud's (9 and 23) dextrose agar at 28°C. The most common species were: *Acremonium strictum*, *Alternaria altrnata*, *Aspergillus flavus*, *A. fumigatus*, *A. niger*, *Cladosporium cladosporioides*, *Pencillium chrysogenum*, *P. duclauxii*, *Chrysosporium lucknowense* and *Scopulariopsis brevicaulis*. Forty-eight fungal isolates representing 46 species and 1 variety belonging to 24 genera were screened for their abilities to produce both exo- and endo- β-1,4- glucanase (C_1 and C_x, respectively). All isolates could hydrolyze both insoluble and soluble cellulose but with variables degrees. The results show that optimum conditions for maximum production of exo- and endo-β-1,4 glucanase by *Aspergillus niger* and *A. flavus*, respectively were 8 days after incubation at 30°C with incorporation of cellulose, glucose and sodium nitrate as a sole carbon and nitrogen sources in basal medium which is initially adjusted to PH 8.

Keywords: *mycobiota, wheat grains, wheat flour, cellulolytic ability*

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1. Introduction

Cereals and cereal by-products constitute a major part of the daily diet of the human and animal populations. The end products of wheat processing are, other than semolina or flour. However, they may represent a source of compounds with unique physico-chemical, nutritional, and functional properties which may have a high value for human nutrition, too [1]. Wheat (*Triticum aestivum* L.) is one of the most important grains crops providing nearly 20 % of the total world food requirement [2]. It is grown on more acreage than any other crop. In 2010, 653.7 mln tons of wheat was produced in the world, while for two of the other main staple food crops, maize and rice (paddy), 840.3 mln and 696.3 mln tons were produced, respectively [3].

During storage, a change in microflora can occur due to the reduction of the content of product-typical microorganisms or due to the reproduction of the spoilage-indicating microflora, adapted to the storage conditions. The main spoilage indicating moulds are *Penicillium* spp., *Aspergillus* spp., *Scopulariopsis* spp., and *Mucorales* spp. of wheat. Microorganisms causing spoilage are also all species of yeasts [4]. The growth of moulds is greatly influenced by the water content of the substrate. At low moisture content (usually less than 14-16%) most storage fungi do not grow or grow very slowly [5]. [6] evaluate mycoflora and in 53 whole wheat grain samples collected in Southern Brazil during the 2012 crop. He found that for *Fusarium* genera, there was predominance of *Fusarium verticillioides* (34%) and *F. graminearum* (30.2%). For *Aspergillus* species, 37.7% of *Aspergillus flavus* was determined. Regarding the *Penicillium* species, *Penicillium digitatum* (49 %) was the most found species.

Flour is a fine powder made by grinding cereals or other edible starchy plant seeds suitable for grinding. It is most commonly made from wheat. Flour is the key ingredient of bread, which is the stable food in most countries. Bread is the primary food stable for the majority of the Egyptians, who consume 270 million loaves of bread daily on average of 3 loaves per capita [7].

Mycotoxins prevention is very important as, once developed, they become stable at environment temperature and very resistant to thermal changes [8]. In order quality and safety of wheat flour and wheat products to be maintained and prevention from contamination, the objectives of the investigation were to examine molds presence or contamination of wheat flour as well as to identify the isolated species and define the effect storage of wheat flour on presence molds.

Cellulose, a major polysaccharide constituent of plant cell walls, is a 1,4 linked linear polymer of 8000~12000 glucose units. Three major enzymes are involved in the degradation of cellulose to glucose are endoglucanase

(endo-1,4-d-glucanasem EG), cellobiohydrolase (exo-1,4-d-glucanasem CBH) and β-glucosidase (1.4-d-glucosidase, BG). EG acts in random fashion, cleaving linked bonds with in the cellulose molecule; CBH removes cellobiose units from the non reducing ends of the cellulose chain and BG degrades cellobiose and cellooligosaccharides to glucose [9]. The conversion of cellulosic mass to fermentable sugars through biocatalyst cellulase derived from cellulytic organisms has been suggested as a feasible process and offers potential to reduce use of fossil fuels and reduce environmental pollution [10]. Researchers have strong interests in cellulases because of their applications in industries of starch processing, grain alcohol fermentation, malting and brewing, extraction of fruit and vegetable juices, pulp and paper industry, and textile industry [11,12,13,14].

Several investigations have been carried out on the numbers of organic and inorganic nitrogen, carbon materials, pH values, temperatures and incubation periods affecting on the production of cellulase enzyme complex by several fungi [15-23].

The aim of the present investigation is to study the distribution and occurrence of various groups of fungi associated with 50 samples of wheat grains and 50 samples wheat flour collected from different mills in Taif city and cellulolytic activity of some fungal isolates and the effect of some environmental and nutritional factors on cellulase production *Aspergillus niger* and *A.flavus*.

2. Material and Methods

A- Collection of Samples

Fifty Samples of wheat grains (*Triticum vulgare* Vill., Hist. Pl. Dauphinè) and fifty samples of wheat flour (from the same grains) were collected from different mills in Taif city. Each sample was collected in a sterile polyethylene bag and transferred to mycological laboratory for fungal analysis. for isolation of various groups of fungi on glucose, Cellulose Czapek's agar, yeast starch agar [24,25] and Sabouraud's dextrose agar medium [26].

B-Determination of grain borne fungi

1- The dilution plate method:

The dilution plate method was used as employed by [27,28] for the estimation of fungal flora associated with wheat grains. A known weight of of wheat grains was suspended sterilized distilled water to obtain the desired final dilution. One mL of final dilution was transferred to a sterile petridish and poured with melted but cooled agar medium.

2- The grain-plate method:

Four wheat seeds were placed on the surface of each glucose-, cellulose-, yeast starch- Czapek's agar and sabouroud's dextrose agar media. Four plates were used for each type of media. Plates were incubated at 28°C for 7 days and the developing fungi were counted and identified. The numbers were calculated per 16 seeds for each sample. The relative importance value (RIV) for each genus and species recovered was also calculated [29].

C- Estimation of Extracellular Enzymes Produced by Fungi

Forty-six species and 1 species variety belonging to 24 genera were screened for their abilities to produce cellulase, on solid media. The most active isolates were selected for further studies dealing with the effect of different environmental and nutritional factors on enzyme production.

I- Cellulase production:

1- Screening of fungal isolates for cellulase production:

Forty-six species and 1 species variety belonging to 24 genera were screened for their abilities to produce exo- and endo-β-1,4-glucanase (C_1 and C_x enzymes, respectively). Isolates were cultured on medium [30] of the following composition (g/L): $(NH_4)_2SO_4$, 0.5; L-asparagin, 0.5; KH_2PO_4, 1.0; KCL, 0.5; $MgSO_4.7H_2O$, 0.2; $CaCl_2$, 0.2; yeast extract, 0.5; cellulose microcrystalline, 10; agar, 20. pH was adjusted to 5.4 using acetate buffer. Using sterile cork borer, 10 mm diameter, discs were cut to inculate 50 ml sterile liquid medium (in 250 ml Erlenmeyer flasks) of medium [30] for exo-glucanase production and medium [31] for endo-glucanase. The later medium contained the following ingredients (g/L): NH_4NO_3, 2.1; KH_2PO_4, 1.0; $MgSO_4$. $7H_2O$, 0.5; carboxymethyle cellulose (CMC), 10.0. Cultures were incubated at 28°C for 7 days. After 7 days of incubation at 28°C the cultures were filtered and the filtrates were used to detect the activity of the enzymes as follows:

a- Detection of exo-β-1, 4-glucanas (C_1 enzyme):

Using a sterile cork borer 3 cavities (10 mm diameter) were made in plates containing solid medium [30]. A 0.1 ml of culture filtrate was dropped in each of these cavities followed by incubation at 28°C for 24 hours, then the plates were flooded with chloroiodide of zinc solution and the uncolored zone gave a measure of cellulolytic power of isolates.

b- Detection of endo-β-1.4-glucanase (C_x enzyme):

Ten mm cavities were cut in plates containing solid medium of [32] of the following composition (g/L): carboxymethyle cellulose (CMC), 10; agar, 17; pH 5.4. A 0.1ml filtrate obtained from 7 days old fungal cultures grown on medium [31] was dropped in each cavity. After 24 hours of incubation at 28°C plates were flooded with chloroidide of zinc solution and the clear zone around cavities were measured.

2- Factors affecting cellulase production:

The effect of different ecological and nutritional factors on production of cellulase enzymes (C_1 and C_x) by *Aspergillus niger* and *A. flavus*, respectively. Since these species were found to be highly active in cellulase production so these species were used for this study. The previous isolates were grown on medium containing (g/L): $NaNO_3$, 5.0; KH_2PO_4, 1.0; $MgSO_4$. $7H_2O$, 0.5; $FeCl_3$, 1.0 mg; Zn SO_4. $7H_2O$, 0.9 mg; $MnSO_4$. H_2O, 0.4 mg; thiamine, 100 mg; biotin, 10 mg and cellulose powder, 10 g [33]. Fifty ml of the medium were dispensed into each 250 ml Erlenmeyer flask and each flask was inoculated with an agar mycelial disc (10- mm diameter) of the mould obtained from 7 days old fungal cultures growing on the solid basal medium. Experiments were done to indicate the best conditions which produce a good deal of the of the enzyme as well as of the best expence.

a- Effect of temperature and time course:

The inoculated flasks were incubated at 20, 30 and 40°C for 14 days and harvested at 48 hours intervals. Culture fluid were filtered and centrifuged at 5000 r.p.m. for 10 min. the clear supernatants were assayed for enzyme activity.

b- Effect of pH values:

The test isolates (*A. niger* and *A. flavus*) were grown on the basal medium of [33]. The initial pH of the medium was adjusted with 0.1N NaOH or 0.1N HCL to different values ranging from 2 to 12. After inoculation with *A. niger* and *A. flavus*, cultures were incubated at 20°C for 8 days for C_1 and C_x, respectively. At the end of incubation period the cultures were filtered, centrifuged at 5000 r.p.m. for 10 min. and the clear supernatants were assayed for cellulase activity.

c- Effect of different carbon sources:

The basal medium [33] with pH 8 (The best pH for cellulase production) was supplemented with 1% of one of the following carbon sources: glucose, fructose, lactose, sucrose, cellulose, starch and carboxymethyl cellulose. The flasks were inoculated with *A. niger* and *A. flavus* and incubated at 20°C (the best temperature of C_1 and C_x enzymes production) for 8 days (the best incubation periods for C_1 and C_x enzymes, respectively) and the cultures were filtered. After centrifugation the filtrate was used to detect the cellulase activity.

d- Effect of various nitrogen sources:

To determine the effect of nitrogen source on cellulase production, sodium nitrate (2 g/L) in the basal medium was replaced by the same amount of various nitrogen compounds such as; sodium nitrate, potassium nitrate, yeast extract, ammonium sulphate, ammonium nitrate and peptone in addition to sodium nitrate as a control. Cultures were incubated at 20°C for 8 days after 8 days cultures were filtered, centrifuged and the filtrates were used for detection of cellulase activity.

3- Assay for cellulase activity (C_1 and C_x enzymes):

The method described by [34] and modified by [35] was emplyed as follows: each of 50 mg of filter paper (Watmann No. 1) and 1 ml of 1% CMC were added separately to 1 ml of acetate buffer (pH 6) and 1 ml of each culture filtrate. The mixture was incubated for 30 min. at 25°C for assaying activity of C_1 and C_x enzymes, respectively. Similar reaction mixtures using heated inactive enzyme solution were also prepared as controls and water with reagents as a blank. 3 ml of Nelson's solution were added and the reaction mixtures, were shaken and placed in a boiling water bath for 15 min. After cooling, 3 ml of the arsenomolybdate solution was added, mixed throughly and then diluted to 10 ml with distilled water. The whole mixture was centrifuged to remove any turbidity. The amount of reducing sugars produced was estimated by determining the optical density (absorption spectrum) at 700 nm wave length with a spectrophotometer model (Spectronic ® Genesys ™ 2PC USA). A standard curve was plotted using aqueous solution of D-glucose.

3. Results and Discussion

A- Wheat Grains Fungi

Thirty-one species belonging to 16 genera were collected from wheat grains on plates of glucose - (16 genera and 26 species), cellulose- (16 species and 25 genera) Czapek's agar, yeast-starch agar (12 species and 19 genera) and Sabouraud's - dextrose agar media (22 species and 10 genera) at 28°C. The most common genera on four types of media were: *Alternaria* (2 species), *Aspergillus* (10), *Candida* and *Chrysosporium* (3). They were emerged in 26-88% of the samples comprising 5.4-65% of total fungi and had RIV's 34.5-105.9. From the above genera the most prevalent species were: *Alternaria alternata*, *Aspergillus flavus*, *A. niger* and *Chrysosporium luknowense* (Table 1 and Table 2).

Table 1. Total counts, (calculated per 48 seeds), number of cases of isolation (NCI, out of 50), occurrence remarks (OR) and the relative importance value (RIV) of fungal genera and species from grans of *Triticum vulgare* on glucose, cellulose-Czapek's agar and starch at 28°C

Genera & Species	Glucose			Cellulose			Starch		
	TC	NCI&OR	RIV	TC	NCI&OR	RIV	TC	NCI&OR	RIV
Acremonium	130	22M	52.3	151	21M	55	30	11L	23.8
A.fusidioides (Nicot) W.Gams	70	12M	26.6	82	10L	28.2			
A.strictum W.Gam	60	12M	25.7	69	12M	26.9	30	11L	23.8
Alternaria	249	41H	103.6	257	38H	105.5	53	17M	36.6
A.alternata (Fr) Keissler	205	40H	99.5	245	37H	94.4	53	17M	36.6
A.citri Elli & Pierce apud Pierce	44	12M	24.2	12	4R	11.2			
Aspergillus	213	36H	90.2	184	34H	88.3	527	44H	105.9
A.flavo-furcatis Batista Maia	4	2R	4.4						
A.flavus Link	133	30H	72.6	103	28H	70.2	182	35H	92.8
A.fumigatus Fresenius	10	5R	10.9	21	6L	12.1	75	20M	49.4
A.giganteus Wehmer	6	3R	10.6						
A.melleus Yukawa	12	3R	11.1				6	3R	10.8
A.niger Van Tieghem	43	16M	34.1	51	18M	45.1	188	34H	63.5
A.niveus Blachwitz							11	4R	11.4
A.parasiticus Speare							23	8L	22.9
A.sydowii (Bain. & Sart.) Thom & Church	5	2R	4.5	9	2R	4.9			
A.terreus Thom							42	8L	25.3
Genera & Species	Glucose			Cellulose			Starch		

	TC	NCI&OR	RIV	TC	NCI&OR	RIV	TC	NCI&OR	RIV
Candida sp.									
Chaetomium globosum Kunze & Steud									
Chrysosporium									
C.lucknowense Garg									
C.pseudomerdarium Van Oorschot, sp. nov									
C.sulfureum (Fiedl.) van Oorschot & Samson, comb. nov									
Cladosporium	5	2R	4.5	4	2R	4.4			
C.cladosporioides (Fresen) de Vries	5	2R	4.5	4	2R	4.4			
C.sphaerospermum Penzig									
Cochliobolus	59	12M	25.6	63	14M	36.3	24		
C.lunatus Nelson & Haasis				2	1R	2.2	24		
C.spicifer Nelson	59	12M	25.6	61	13M	36.1			
Emericella nidulans (Eidam) Vuillemin	3	2R	4.3	6	2R	4.6	41	10L	25.1
Epicoccum purpurascens Ehrenb. ex Schlecht	14	6L	11.3	11	4R	11.1	3	1R	2.4
Fusarium oxysporum Schlecht	51	4R	14.8	27	6L	12.7	14	6L	11.8
Melanopsamma pomiformis (Pers ex Fr.) Sacc	1	1R	2.1	8	3R	10.8	13	4R	11.6
Mucor hiemalis Wehmer	4	2R	4.4	10	2R	5.0	31	11L	23.9
Nectria haematococca Berkeley & Brown	212	29H	80.1	173	26H	67.2			
Papulaspores immersa Hotson	2	1R	2.2	1	1R	2.1			
Penicillium	25	8L	22.4	22	8L	22.2	30	8L	238
P.chrysogenum Thom	5	4R	10.5	1	1R	2.1	18	4R	12.3
P.citrinum Thom				2	1R	2.2			
P.corylophilum Dierckx	4	1R	2.4	2	1R	2.2	12	4R	11.5
P.duclauxi Delacroix	16	4R	11.5	17	6L	11.7			
P.steckii Zaleski									
Scopulariopsis brevicaulis (Sacc.) Bainier	10	4R	10.9	4	2R	10.4	15	6L	11.9
Setosphaeria rostrata Leonord							19	5R	12.4
Sterile mycelia (dark & white color)	32	8L	23	17	10	21.7			
Trichothecium roseum (Pers.) Link ex Gray									
Ulocladium alternariae (Cooke) Simmons	30	12M	52.8	45	13M	34.3			
Gross total count	1053			1006			800		
Number of genera 20	16			16			12		
Number of species 40	26			25			19		

Occurrence remarks: OR (out of 24 samples) H=high occurrence from 12-24 cases, M= moderate occurrence from 6 – 11 case, L = low occurrence from 3 – 5 cases and R = rare occurrence from 1 – 2 cases

Table 2. Total counts, (calculated per 48 seeds), number of cases of isolation (NCI, out of 50), occurrence remarks (OR) and the relative importance value (RIV) of fungal genera and species from grans of *Triticum vulgare* on sabouraud's dextrose at 28°C

Genera& Species	Sabouraud		
	TC	NCI&OR	RIV
Acremonium	15	6L	11.9
A.fusidioides (Nicot) W.Gams	8	3R	11.0
A.strictum W.Gam	7	3R	10.9
Alternaria	385	41H	127.5
A.alternata (Fr) Keissler	363	38H	1.2
A.citri Elli & Pierce apud Pierce	22	4R	12.7
Aspergillus	196	32H	84.2
A.flavo-furcatis Batista Maia			
A.flavus Link	105	23M	63.0
A.fumigatus Fresenius			
A.giganteus Wehmer			
A.melleus Yukawa	3	1R	2.4
A.niger Van Tieghem	67	22M	48.3
A.niveus Blachwitz	3	1R	2.4
A.parasiticus Speare	3	1R	2.4
A.sydowii (Bain. & Sart.) Thom & Church	6	1R	2.7
A.terreus Thom	9	3R	11.1
Genera& Species	Sabouraud		

	TC	NCI&OR	RIV
Candida sp.	44	13M	35.4
Chaetomium globosum Kunze & Steud			
Chrysosporium	80	17M	39.3
C.lucknowense Garg	59	14M	37.3
C.pseudomerdarium Van Oorschot, sp. nov	10	6L	11.2
C.sulfureum (Fiedl.) van Oorschot & Samson, comb. nov	11	4R	11.4
Cladosporium	2	1R	2.2
C.cladosporioides (Fresen) de Vries	2	1R	2.2
C.sphaerospermum Penzig			
Cochliobolus			
C.lunatus Nelson & Haasis			
C.spicifer Nelson			
Emericella nidulans (Eidam) Vuillemin			
Epicoccum purpurascens Ehrenb. ex Schlecht			
Fusarium oxysporum Schlecht	11	2R	5.4
Melanopsamma pomiformis (Pers ex Fr.) Sacc			
Mucor hiemalis Wehmer	15	3R	11.9
Nectria haematococca Berkeley & Brown	9	3R	11.1
Papulaspores immersa Hotson			
Penicillium	29	12	23.6
P.chrysogenum Thom	16	8L	22.0
P.citrinum Thom			
P.corylophilum Dierckx	4	1R	2.5
P.duclauxi Delacroix	9	3R	11.1
P.steckii Zaleski			
Scopulariopsis brevicaulis (Sacc.) Bainier	24	10L	23.0
Setosphaeria rostrata Leonard			
Sterile mycelia (dark & white color)			
Trichothecium roseum (Pers.) Link ex Gray			
Ulocladium alternariae (Cooke) Simmons			
Gross total count	810		
Number of genera 20			
Number of species 38	`		

[36] could isolated 117 different species belonging to 31 fungal genera contributed the mycobiota of stored wheat on glucose- Czapek's agar medium. The most common species isolated in decreasing orders of frequency were: *Aspergillus flavus* (72.5%), *A. niger* (71.3%), *P. chrysogenum* (47.5%), *P. cirinum*, *P. oxalicum*, (40% for each), *A. sydowii*, *A. terreus* (35% for each), *A. flavus* var. *columnaris*, *Rhizopus stolonifer* (35% for each), *A. ochraceus* (27.5%), *A. flavipes* (26.3%) and *Alternaria altrnata* (25%). The data obtained by [37] indicated that wheat grains were contaminated with many fungal and the most common genera were: *Aspergillus*, *Fusarium* (*F. graminiarium* and *F. verticillioides*) and *Penicillium*. [38] found five pathogens to be wide spread on commercial barely seeds on plates of Sabouraud's - dextrose agar medium, and these were: *Drechslera teres*, *D. graminea*, *Septoria nodorum*, *D. sorokiniana* and *Fusarium nivale*. [39] studied the mycoflora of Algerian wheat. The commonly isolated fungi on dichloran rose-bengal chlramphenicol agar medium were species of *Aspergillus*, *Fusarium*, *Penicillium*, *Alternaria* and *Mucor*. *Aspergillus* was the genus most detected at higher frequency. Among the *Aspergillus* species isolated *A. flavus*, *A. niger* and *A. versicolor*. [40] isolated ten fungal species belonging to seven different genera from wheat grains and these were: *Aspergillus*, *Alternaria*, *Cladosporium*, *Fusarium* and *Penicillium* of the Phylum Ascomycota; *Mucor* and *Rhizopus* of the Phylum Zygomycota. The frequency of *Aspergillus. niger*, *A. fumigatus* and *Alternaria alternate* were higher (33.76 to 40.34%) than the other fungal species identified. The frequency of *A. niger* was highest which is quite alarming because this strain can produce ochratoxins. Out of the *Penicillium* species isolated, the commonly found species were *P. expansum*, and *P. citrinum* with comparatively low percentage. [41] found the most common moulds contaminated the wheat storage (silos) in Golestan Provine, North of Iran were *Alternaria* spp. 26.7%, *Aspergillus niger* 21.4%, *Fusarium* spp.17.8%, *Aspergillus flavus* 10.7%, *Clasdosporium* spp.10.7%, *Penicillium* spp. 8.9% and *Rhizopus* spp.3.5%. [42] found that Fungi associated with wheat grain samples collected from five different Gaza governorates were were *Aspergillus flavus* 84%, *Aspergillus parasiticus* 72%, *Fusarium oxysporum* 64%, *Aspergillus niger* 48%, *Alternaria alternate* 36%, *Penicillium* 22%, *Aspergillus ochraceus* 20% and *Aspergillus versicolor* 4%.

B-Wheat Flour Fungi

Thirty-three species belonging to 17 genera were collected from wheat flour on plates of glucose- (16 genera and 32 species), cellulose- (16 genera and 32 species) Czapek's agar, yeast-starch agar medium (15 genera and 23 species) and Sabouraud's - dextrose agar media (9 genera and 23 species) at 28°C. The most common genera on four types of media were: *Aspergillus* (10 species), *Chrysosporium* (3), *Cladosporium* (1) and

Penicillium (5); emerging in 28-100% of the samples comprising 16.2-44.5% of total fungi. From the above genera the most common species were: *Aspergillus flavus*, *A. fumigatus*, *A. niger*, *Chrysosporium lucknowense*, *Cladosporium cladosporioides*, *Pencillium chrysogenum* and *P. duclauxii* (Table 3 and Table 4).

Table 3. Average total counts, maximum values (calculated per g fresh weight flours in every samples), number of cases of isolation (NCI, out of 50) and occurrence remarks (OR) of fungal genera and species recovered from flour of *Triticum vulgare* on glucose, cellulose- Czapek's agar and starch at 28°C

Genera & Species	Glucose		Cellulose		Starch	
	ATC±SD (MV)	NCI&OR	ATC±SD (MV)	NCI&OR	ATC±SD (MV)	NCI&OR
Acremonium	485±31.45 (165)	19M	255±15.65(52)	15M	360±30.27(125)	9L
A.fusidioides (Nicot) W.Gams	245±13.14(75)	10L	185±15.47(60)	11L		
A.strictum W.Gam	240± 2 (95)	10L	70±2.88(20)	4R	360±30.27(125)	9L
Alternaria	285±40.28(105)	16M	455±45.16(165)	25H	145±8.53(45)	5R
A.alternata (Fr.) Keissler	245±33.0(85)	14M	395±35.44(135)	21M	145±8.53(45)	5R
A.citri Elli & Pierce apud Pierce	40±8.16(20)	3R	60±10.80(30)	6L		
Aspergillus	9390±522.43(2685)	49H	7985±484.25(2315)	50H	4940±46.47(285)	45H
A.flavo-furcatis Batista Maia						
A.flavus Link	5440±309.56(1565)	47H	4455±311.85(1325)	46H	1315±77.5(440)	30H
A.fumigatus Fresenius	600±77.13(230)	20M	870±96.39(320)	25H	1330±11.93(84)	34H
A.giganteus Wehmer	175±15.47(65)	6L	235±19.73(75)	6L		
A.melleus Yukawa	720±40.82(230)	7L	680±21.06(190)	7L		
A.niger Van Tieghem	1885±163.31(610)	38H	1260±113.65(420)	35H	1570±18.43(99)	38H
A.niveus Blachwitz	105±9.46(40)	2R	150±21.79(55)	3R	90±2.88(25)	2R
A.parasiticus Speare	190±23.27(75)	2R	170±47.87(110)	1R	395±16.52(115)	8L
A.sydowii (Bain. & Sart.) Thom & Church	245±29.26(95)	5R	135±17.01(50)	5R		
A.terreus Thom	30±8.66(20)	2R	30±2.88(10)	4R	240±19.57(80)	8L
Candida sp.						
Chaetomium globosum Kunze ex Steud	15±4.78(10)	1R	10±2.88(5)	1R	120±10.80(45)	4R
Chrysosporium						
C.lucknowense Garg						
C.pseudomerdarium Van Oorschot, sp. nov						
C.sulfureum (Fiedl.) van Oorschot & Samson, comb. nov						
Cladosporium	5280±241.45(1605)	35H	6565±177.35(1805)	41H	1795±48.19(495)	14M
C.cladosporioides (Fresen) de Vries	5215±225.55(1565)	34H	6375±218.49(1765)	40H	1795±48.19(495)	14M
C.sphaerospermum Penzig	65±17.01(40)	1R	190±49.74(120)	1R		
Cochliobolus	240±20.81(85)	13M	215±14.36(70)	12M	105±8.53(35)	4R
C.lunatus Nelson & Haasis	110±13.22(45)	7L	120±6.45(40)	8L	105±8.53(35)	4R
C.spicifer Nelson	130±8.66(40)	8L	85±14.36(30)	5R		
Emericella nidulans (Eidam) Vuillemin	30±10.80(30)	1R	20±0(5)	3R	155±4.78(45)	4R
Epicoccum purpurascens Ehrenb. ex Schlecht	65±13.14(35)	5R	70±8.66(30)	5R	90±4.1(30)	2R
Fusarium oxysporum Schlecht	50±9.57(20)	3R	55±4.78(20)	4R	160±4.08(45)	5R
Melanopsamma pomiformis (Pers ex Fr.) Sacc	60±6.45(25)	4R	35±4.78(15)	4R	20±9.12(60)	1R
Mucor hiemalis Wehmer	265±13.72(110)	7L	160±11.54(50)	6R	270±18.48(90)	11L
Nectria haematococca Berkeley & Brown	370±5(100)	12M	340±8.16(85)	13M		
Papulaspores immersa Hotson						
Penicillium	4755±148.28(1355)	44H	5095±164.64(1410)	38H	2275±22.69(138)	26H
P.chrysagenum Thom	1630±44.81(470)	27H	480±19.57(140)	12M	1560±91.74(495)	18M
P.citrinum Thom	190±9.57(55)	4R	300±23.45(105)	6L	195±6.29(55)	1R
P.corylophilum Dierckx	1195±62.09(355)	7L	1800±87.27(565)	8L	60±9.12(25)	3R
P.duclauxi Delacroix	805±93.30(300)	17M	1610±102.51(490)	19M	460±30.82(150)	6L
P.steckii Zaleski	935±42.69(270)	3R	900±26.77(260)	2R		
Scopulariopsis brevicaulis (Sacc.) Bainier	145±19.31(55)	12M	115±24.28(65)	9L	345±11.08(95)	19M
Setosphaeria rostrata Leonord					150±6.45(45)	4R
Sterile mycelia (dark & white color)	70±6.45(25)	5R	10±2.88(5)	1R	200±9.12(60)	7L
Trichothecium roseum (Pers.) Link ex Gray					5±2.5(5)	1R
Ulocladium alternariae (Cooke) Simmons	10±2.88(5)	1R	30±6.45(15)	3R		
Gross total count	21530 ± 443.52(5800)		21425 ±494.80(5855)		11135±68.34(632)	
Number of genera 20	16		16		15	
Number of species 40	32		32		23	

ATC±SD (MV) = average total count in every sample ± standard deviation (and in brackets, the maximum values in all cases). Occurrence remarks: OR (out of 50 samples) : H = high occurrence from 25 – 50 cases, M = moderate occurrence from 12 – 24 cases, L - low occurrence from 6 - 11 cases and R = rare occurrence 1 – 5 cases.

Table 4. Average total counts, maximum values (calculated per 50 g weight flours in every samples), number of cases of isolation (NCI, out of 50) and occurrence remarks (OR) of fungal genera and species recovered from flour of *Triticum vulgare* on sabouraud's dextrose at 28°C

Genera&Species	Sabouraud's	
	ATC±SD (MV)	NCI&OR
Acremonium	10±2.88(5)	1R
A.fusidioides (Nicot) W.Gams		
A.strictum W.Gam	10±2.88(5)	1R
Alternaria	55±2.5(15)	1R
A.alternata (Fr) Keissler	35±6.29(15)	3R
A.citri Elli & Pierce apud Pierce	20±4.08(10)	1R
Aspergillus	2780±183(920)	43H
A.flavo-furcatis Batista Maia	15±4.78(10)	1R
A.flavus Link	2155±167.2(695)	35H
A.fumigatus Fresenius	60±9.12(25)	2R
A.giganteus Wehmer	5±2.88(5)	1R
A.melleus Yukawa	25±21.6(15)	1R
A.niger Van Tieghem	135±19.31(55)	5R
A.niveus Blachwitz	230±40.31(115)	12M
A.parasiticus Speare	120±16.83(55)	3R
A.terreus Thom	35±7.5(15)	2R
Candids sp.	585±84.69(260)	6L
Chrysosporium	1300±66.20(395)	27H
C.lucknowense Garg	1020±82.15(340)	25H
C.pseudomerdarium Van Oorschot, sp. nov	190±18.48(70)	8L
C.sulfureum (Fiedl.) van Oorschot & Samson, comb. nov	90±18.48(40)	5R
Cladosporium	325±8.53(90)	12M
C.cladosporioides (Fresen) de Vries	325±8.53(90)	12M
Epicoccum purpurascens Ehrenb. ex Schlecht	35±2.5(10)	1R
Mucor hiemalis Wehmer	75±14.36(40)	4R
Penicillium	2315±76.41(645)	37H
P.chrysagenum Thom	640±42.5(185)	16M
P.citrinum Thom	355±19.31(105)	6L
P.corylophilum Dierckx	150±20.20(55)	4R
P.duclauxii Delacroix	1190±48.73(345)	17M
Sterile mycelia (dark & white color)	130±28.43(75)	5R
Gross total count	7610±346.65(2345)	
Number of genera 9	9	
Number of species 23	23	

ATC±SD (MV) = average total count in every sample ± standard deviation (and in brackets, the maximum values in all cases). Occurrence remarks: OR (out of 50 samples) : H = high occurrence from 25 – 50 cases, M = moderate occurrence from 12 – 24 cases, L - low occurrence from 6 - 11 cases and R = rare occurrence 1 – 5 cases.

[43] made mycological examination on two German wheat flours and collected 45 different species from whole wheat flour and 49 species from white flour on dichloran rose-bengal chloramphenicol (DRBC) agar medium. The most common genera on whole wheat flour were: *Aspergillus* (83.7%), *Penicillium* (7.6%), *Eurotium* (2.9%) and *Alternaria* (2.5%). The white flour contained *Aspergillus* (77.3%), fungi of the genera *Penicillium* (15%) and *Cladosporium* (4.1%) were of minor importance. The most common species from two types were: *Aspergillus candidus*, *Alternaria alternata*, *Aspergillus flavus*, *Cladosporium cladosporioides*, *C. herbarum* and *Penicillium aurantiogriseum*. [44] made mycological examination on wheat flours in a local super - markets in Egypt. Forty two different species were collected. The most common isolated molds on Czapek's – dextrose agar media and potato dextrose agar (PDA) medium belong to the genera *Aspergillus*, *Alternaria*, *Penicillium* and *Cladosporium*. *Aspergillus flavus* was the most dominating mold followed by *Penicillium duclauxii*, *Alternaria alternata*, and *Cladosporium cladosporioide.*

[45] found that the most prevalent genera recovered from white wheat flour on plates of malt extract agar and Czapek's -dextrose agar media were *Aspergillus* (73.7%), *Penicillium* (5.6%), *Eurotium* (3.9%) and *Alternaria* (1.5%). [46] were isolated *Absidia, Alternaria, Aspergillus, Cladosporium Epicoccum, Eurotium, Fusarium, Mucor, Penicillium* and *Rhizopus* from wheat flour mill in

Argentina. [47] were isolated *Aspergillus*, *Acremonium*, *Alternaria*, *Fusarium*, *Mucor*, *Penicillium* and *Cladosporium* spp. from consumed flour in the bakeries of Tabriz city. [48] were isolated *Aspergillus*, *Penicillium*, *Fusarium*, *Cladosporium*, *Alternaria*, *Mucor*, *Rhizoctonia*, *Trichoderma*, *Rhizopus*, *Nigrospora*, *Bipolaris*, *Macrophomina* from wheat flour samples by using PDA (Potato dextrose Agar) medium according to decreasing frequency.

Table 5. Activity of exo-B-1,4glucanase (C_1) (calculated as average diameter of clear zone in mm) of the fungal isolates

Fungal isolates	Diameter of clear zone (mm)
Acremonium fusidioides	50 M
A.strictum	70 H
Alternaria alternata	50 M
A.citri	55 M
A.dianthi	53 M
Aspergillus flavo-furcatis	45 M
A.flavus	67 H
A.flavus var.columnaris	60 H
A.fumigatus	67 H
A.giganteus	32 W
A.melleus	55 M
A.niger	71 H
A.niveus	13 W
A.parasiticus	57 M
A.sydowii	47 M
A.terreus	47 M
Chaetomium globosum	45 M
Cladosporium cladosporioides	47 M
C.sphaerospermum	42 M
Cochliobolus lunatus	60 H
C.spicifer	27 W
Cunninghamella echinulata	34 W
C.elegans	40 M
Emericella nidulans	57 M
Epicoccum purpurascens	47 M
Fusarium oxysporum	70 H
Melanopsamma pomiformis	45 M
Mucor circinelloides	38 W
M.hiemalis	30 W
Myrothecium verrucaria	42 M
Nectria haematococca	50 M
Paecilomyces variottii	35 W
Papulaspores immerse	35 W
Penicillium chrysogenum	45 M
P.citrinum	37 W
P.corylophilum	52 M
P.duclauxi	51 M
P.funiculosum	54 M
P.steckii	62 H
Rhizopus stolonifer	45 M
Scopulariopsis brevicaulis	57 M
Setosphaeria rostrata	50 M
Stachybotrys parvispora	40 M
Sterile mycelia (dark&white color)	27 W
Trichoderma hamatum	62 H
T.viride	64 H
Trichothecium roseum	35 W
Ulocladium alternariae	37 W

Degree of C_1 activity: high activity, H: from 60-71 mm; moderate activity, M = 40-59 mm and weak activity, W = less 39 mm.

D - Cellulolytic activities of some fungal isolates:

Forty-six species and 1 species variety belonging to 24 genera were screened for their abilities to produce C_1 and C_x enzyme on solid media proved to be active to utilize cellulose, but with different degrees. Two isolates (4.2% of total isolates) showed high cellulolytic activity in both exo- and endo-β-1,4- glucanases and these were: *Aspergillus flavus* and *A. flavus* var. *columnaris*. Ten isolates (20% of total isolate) showed high cellulolytic activities in production of C_1 enzyme only and these were: *Acremonium strictum*, *Aspergillus flavus*, *A. flavus* var. *columnaris*, *A. fumigatus*, *A. niger*, *Cochliobolus lunatus*, *Fusarium oxysporum*, *Penicillium steckii*, *Trichoderma hamatum* and *T. viride*. On the other hand five fungal isolates (10.4% of total isolates) showed high cellulolytic activities for C_x enzyme only, and these were: *Aspergillus flavo-furcatis*, *A. flavus*, *A. flavus* var. *columnaris*, *Nectria haematococca* and *Penicillium citrinum*. Twenty-six and thirty-eight isolates (55% and 79.2% of total isolates) were found to be moderate production of C_1 and C_x enzymes, respectively, while 11 and 5 isolates (25% and 10.4% of total isolates) were of weak cellulolytic activity (Table 5 and Table 6). Most of the above fungal isolates were reported as cellulase producers, but with variable capabilities by several workers [15,17,18,19,22,23,49-57].

Table 6. Activity of endo-B-1,4glucanase (C_x) (calculated as average diameter of clear zone in mm) of the fungal isolates

Fungal isolates	Diameter of clear zone (mm)
Acremonium fusidioides	20 M
A.strictum	32 M
Alternaria alternata	30 M
A.citri	21 M
A.dianthi	24 M
Aspergillus flavo-furcatis	42 H
A.flavus	50 H
A.flavus var.columnaris	43 H
A.fumigatus	23 M
A.giganteus	33 M
A.melleus	25 M
A.niger	22 M
A.niveus	33 M
A.parasiticus	30 M
A.sydowii	19 W
A.terreus	22 M
Chaetomium globosum	27 M
Cladosporium cladosporioides	18 W
C.sphaerospermum	20 M
Cochliobolus lunatus	25 M
C.spicifer	32 M
Cunninghamella echinulata	20 M
C.elegans	23 M
Emericella nidulans	23 M
Epicoccum purpurascens	30 M
Fusarium oxysporum	26 M
Melanopsamma pomiformis	16 W44
Mucor circinelloides	23 M
M.hiemalis	25 M
Myrothecium verrucaria	27 M
Nectria haematococca	39 H
Paecilomyces variottii	28 M
Papulaspores immerse	24 M
Penicillium chrysogenum	20 M
P.citrinum	37 H
P.corylophilum	32 M
P.duclauxi	29 M
P.funiculosum	30 M
P.steckii	22 M
Rhizopus stolonifer	28 M
Scopulariopsis brevicaulis	16 W
Setosphaeria rostrata	20 M
Stachybotrys parvispora	16 W
Sterile mycelia (dark&white color)	30 M
Trichoderma hamatum	32 M
T.viride	33 M
Trichothecium roseum	29 M
Ulocladium alternariae	30 M

Degree of C_X activity: high activity, H: from 35-50 mm; moderate activity, M = 20-34 mm and weak activity, W = less 19 mm.

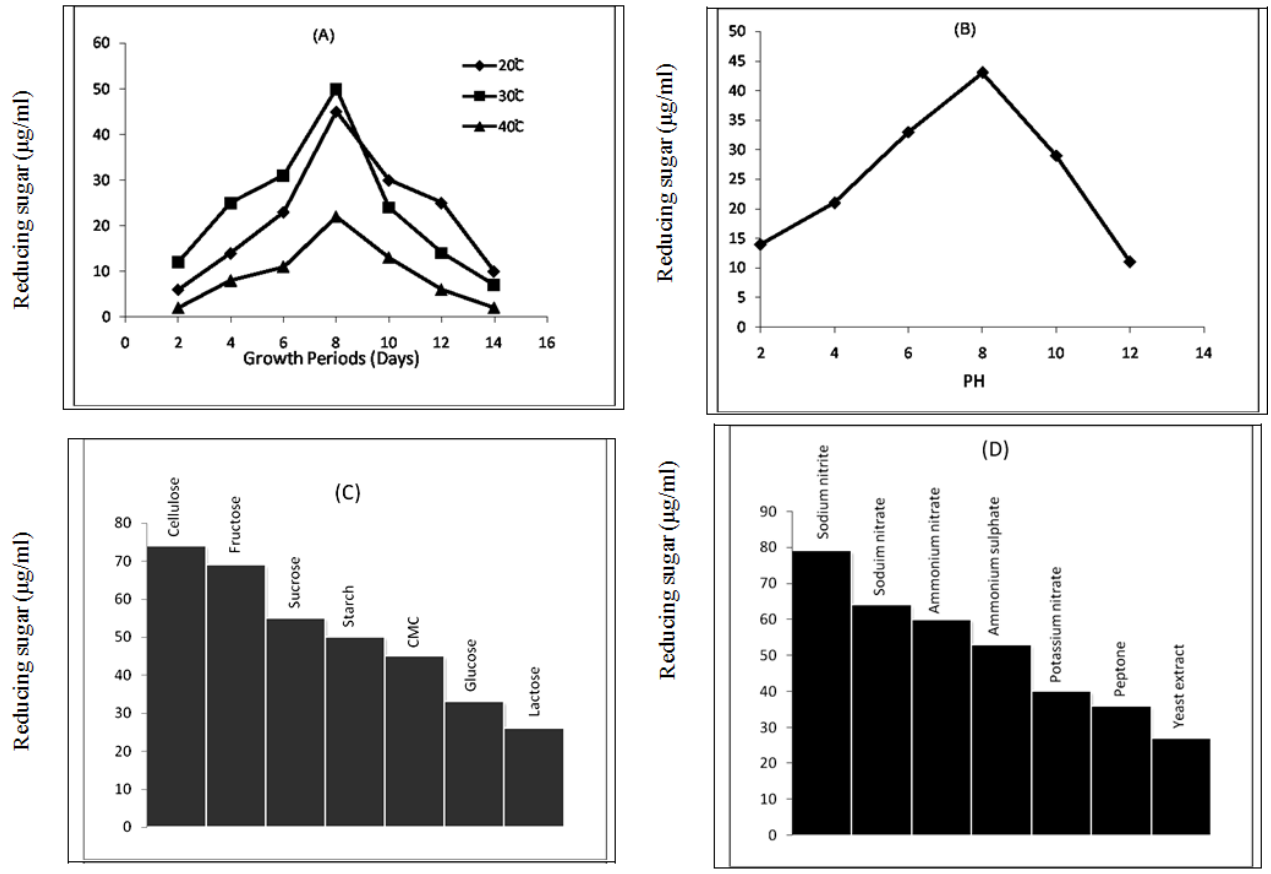

Figure 1. Effect of time course and temperature, pH values, carbon sources and nitrogen sources on production of exo-ß-1,4-glucanase(C_1) by *Aspergillus niger*

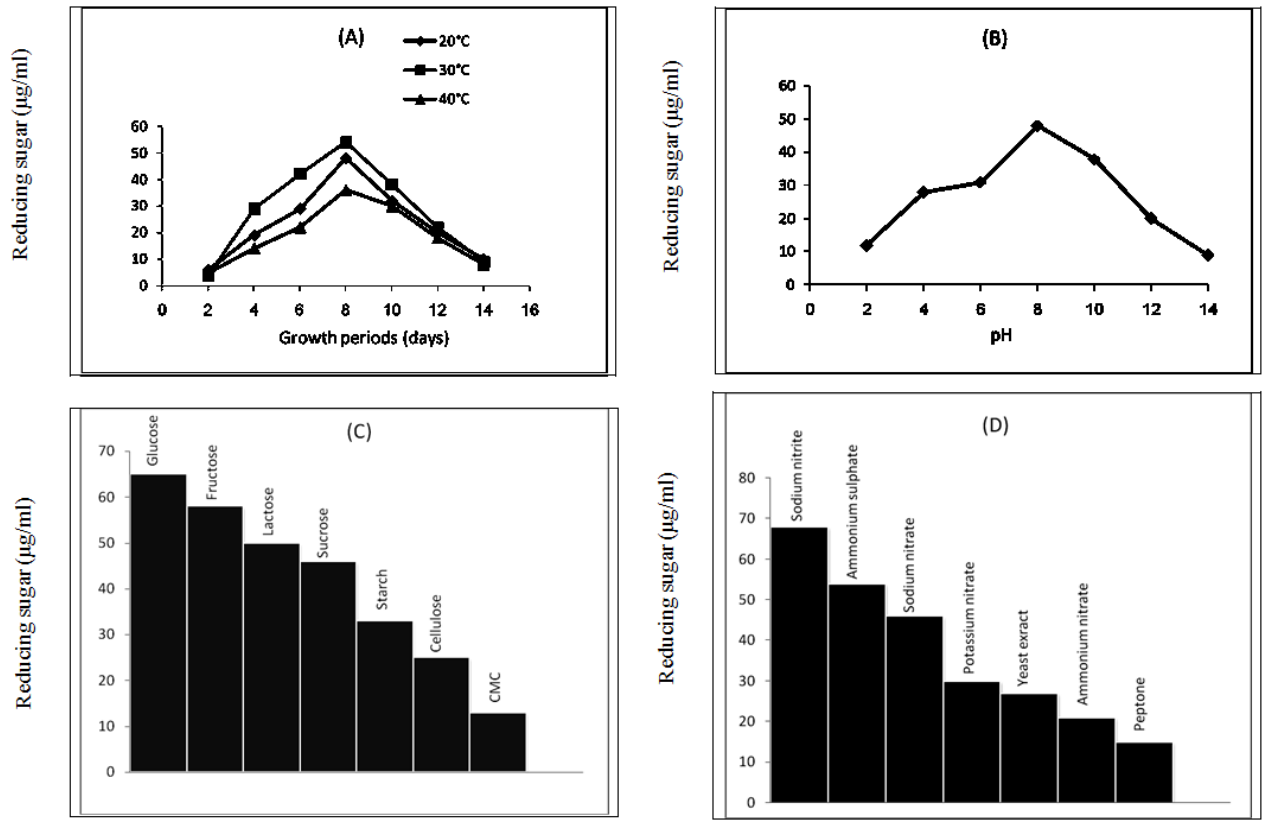

Figure 2. Effect of time course and temperature,pH values, carbon sources and nitrogen sources on production of endo-ß-1,4-glucanase(C_x) by *Aspergillus flavus*

Aspergillus niger and *A. flavus* headed the list of the most active cellulase producers (for C_1 and C_x, respectively), so they were chosen for further studies to achieve the most favourable environmental and nutritional conditions for C_1 and C_x enzymes production. Maximum production of exo and endo-β-1,4-glucanase by *A. niger*

and *A. flavus* were obtained after 8 days of incubation at 30°C with culture media contain cellulose and glucose as a carbon sources and sodium nitrite as nitrogen source and the culture medium was initially adjusted to pH 8 (Figure 1 and Figure 2). These findings are almost in agreement with those reported by [54] studied the effect of environmental factors on production of cellulase enzyme by *Aspergillus fumigatus* and *A. niger*. They reported that the optimum pH for cellulase production 6 to 7 and optimum temperature around 40°C. Also, [18] found that maximum production of endo -ß- 1,4 glucanase by *Cheatomium globosum* was achieved 6 days after incubation at 30°C with incorporation of maltose as carbon source and NH_4NO_3 as nitrogen source in the culture medium which is initially adjusted to pH6. Recently [23] found that maximum production of exo- and endo-ß-1,4 glucanase by *Mucor circinelloides* and *Aspergillus flavus* was achieved 6 days after incubation at 30°C with incorporation of fructose or sucrose as a sole carbon source and potassium nitrate or sodium nitrate as a sole nitrogen source, respectively in the basal medium initially adjusted to pH 6.

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