Mycobiota of Wheat Flour and Detection of α- Amylase and L-Asparaginase Enzymes

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Abstract: Infection of stored wheat flour with fungi can be an extremely serious problem. This study was conducted to isolate and identify the fungal species, which contaminated the stored flour in Riyadh region in Saudi Arabia. The present results revealed that the total fungal counts which were recorded on three medium types were ranged from 33200 to 35300 per gram of wheat flour. The most predominant genus was *Aspergillus* with high frequency (85.7% - 89.3%). *Aspergillus* was represented by 8 species, *A.flavus* showed maximum frequency (60.7% - 71%) and minimum frequency exhibited by *A.clavatus*, *A.terreus*, *A.ochraceous* and *A.tamarii* (3.5%). *Penicillium* and *Eurotium* were the second dominant genera with frequency (50%). The results revealed that the *Aspergillus* genus was the most active producer of α- amylase (25-27mm) . 12 fungal strains include (3 isolates) for both *Aspergillus flavus* and *A. flavus var.columnaris,* (1 isolate) for *A.niger* , *Fusarium proliferatum*, *F.semitectum*, *Penicillium chrysogenum*, *P.crustosum* and *P.olsonii* exhibited high activity in production of L- asparaginase . Three isolates of *P.olsonii* and one isolate for *A.flavus var.columnaris* , *A.niger*, *Penicilliun aurantiogriseum* ,*P.citrinum* and *Rhizopus rhizopodiformis* showed moderate activity in production of L-asparaginase.
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Key Words: Wheat flour, *Aspergillus* , *Penicillium*, Enzymes, α-amylase , L-Asparaginase .

1. Introduction

Wheat (*Triticum aestivum* L. em Thell.), family *Poaceae* (*Gramineae*) is the most important staple food of about two billion people (36% of the world population). Worldwide, wheat provides nearly 55% of the carbohydrates and 20% of the food calories consumed globally. Among various factors that affect seed health, the most important are the seed borne fungi that not only lower seed germination, but also reduce seed vigor resulting in low yield. Healthy seed plays an important role not only for successful cultivation but also for increasing yield of crop (Wiese, 1984).

Wheat flour is an ingredient used in many foods and is one of the most important foods in European and American culture. Bread, pasta, crackers, and many cakes, among other foods and cooking recipes, are made using flour or including this as an ingredient. Flour is the cleanest end product of the milling process and is generally regarded as a microbiologically safe product as it is a low-activity commodity.

However, pathogens that contaminate flour may survive for extended periods (Berghofer *et al*., 2003; Cabanas *et al*., 2008).

Microorganisms in particular have been regarded as treasure of useful enzymes. There is a great variation between various genera as to their ability to produce a specific enzyme varies with the particular medium and pH (Akpan *et al*., 2009). In recent years the potential of using microorganisms as biotechnological sources of industrially relevant enzymes has stimulated interest in the exploration of extracellular enzymatic activity in several microorganisms (Abu EA *et al*., 2005) .

The first enzyme produced industrially was an amylase from a fungal source in 1894, which was used for the treatment of digestive disorder (Crueger and Crueger, 1984). At present *Aspergillus* and *Rhizopus* species are considered to be the most important sources of industrial amylases. Amylases are among the most important enzymes and are of great significance in present - day biotechnology, having approximately 25% of the enzyme market. Agricultural substrate for the production of amylase from amylolytic *Aspergillus spp.* Amylases represent a group of enzyme of great importance to the food industry and other needs of life. Although amylases can be obtained from several sources, such as plant and animals, the enzyme from microbial sources generally meet industrial demand. *Asparaginase* is manufactured by pure culture fermentation of a genetically modified strain of *A.niger* that contains multiple copies of the asparaginase gene derived from *A. niger*. *A. niger* is a filamentous fungus that commonly occurs in the environment and is considered to be nonpathogenic (OECD, 1992).

Wheat flours may carry a significant mycological load acquired via cultivation, postharvest processing practices, and milling process. Their use in bread production and other food industries could cause accelerated spoilage or illness, if pathogens are present. So, this work aimed

(1) To Survey of natural fungi contaminated wheat flour samples collected from different localities in Riyadh , Saudia Arabia .
(2) Identification of all fungal colonies appeared.
(3) Calculated both percentage of total fungal colonies and fungal frequencies which contaminated these samples.
(4) To analyze specifically the occurrence of α-amylase and L- asparaginase enzymes in isolated fungi .

2. Material and Methods

A- Collection of wheat flour samples :

Thirty samples of wheat flour were randomly collected from different markets in Riyadh City Saudi Arabia. Samples were kept individually in clear plastic bags and stored in a cool place till mycological analysis.

B- Mycological analysis of wheat flour samples

The dilution plate method was used for isolation of fungal species as described by Johnson and Curl (1972). A dilution rate of 1: 200 (w/v) was mostly appropriate for obtaining reasonable number of fungal colonies per plate. Aliquots of one ml of the final dilution were poured in 9 cm diameter Petri plates followed by the addition of 20 ml of warm agar medium. Plates were rotated by hand to allow dispersal of flour solution in the agar medium before solidification. Cultures were then incubated at 28°C for 7-15 days to allow growth of fungal colonies. The developing fungi were identified and counted. The average number of colonies per 5 dishes was multiplied by the dilution factor to obtain the number per gm in the original sample and the number of colony forming units (CFU_s) was calculated per gm dry sample.

Three medium types were used for isolation and identification of fungi contaminating wheat flour namely:

1. Czapek's glucose agar

This medium contained (g/l): Glucose, 30 gm; $NaNO_3$, 3 gm; K_2HPO_4, 1.0 gm; KCl, 0.5 gm; $MgSO_4.7H_2O$, 0.5 gm; $FeSO_4.7H_2O$, 0.01gm; rosebengal, 0.025 mg and Agar 15 gm; and distilled water 1000 ml, Final PH was 6.2 (Samson *et al*., 2004). This medium is suitable for isolation of common mesophilic fungi requiring simple sugars as a carbon source.

2. Czapek's cellulose agar

Contains the same components as above with microcrystalline cellulose (30 gram) replacing glucose. The medium is suitable for isolation of cellulose decomposing fungi.

3. Czapek's 40% sucrose agar

Contains the same constituents of Czapek's glucose agar with 400 gram sucrose replacing glucose. The high sucrose content allows good growth of osmophilic fungi.

C- Identification of fungi:

Identification of the isolated fungi was carried out using the morphological and microscopic features with the aid of the following references : Raper and Fennell (1965), Pitt (1979), Moubasher (1993), Leslie and Summerell (2006), Moustafa (2006), Domsch *et al* (2007), Pitt and Hocking (2009).

Amylase production by fungi Growth medium:

Fungi were cultured in liquid yeast-starch medium of Emerson (1941) which contains (g/l): Difco powdered yeast extract, 4.0g; K_2HPO_4, 1.0g; $MgSO_4.7H_2O$, 0.5g and soluble starch, 15.0g. The pH was adjusted to 7.0. Before autoclaving, the medium was dispensed in conical flasks of 100 ml capacity containing 50 ml aliquots of this medium. After cooling the medium in flasks was inoculated with disks of hyphae and spores of the most potent fungal strain then incubated for7 days at 28°c. At the end of the incubation period, the contents of each flask were filtered through Whatman No .1 filter paper and the filtrate was used for assay of amylase activity.

Amylase activity was assayed on yeast-starch agar medium (Emerson, 1941) using the cup plate technique. Cups (one/plate) of 10 mm diameter were made in the solidified yeast-starch medium. Aliquots of crude enzyme preparation (0.1ml) were introduced into each cup. The assay plates were incubated for 24 hours at 30°C followed by flooding with 5 ml of (0.02 N) iodine solution. The diameter of transparent zone around cavities were measured in mm and recorded as a positive reaction.

E- L-asparaginase production by fungi

The methodology was based on Gulati *et al*. (1997) with the incorporation of phenol red in a stock solution prepared in ethanol (2.5% in ethanol 95% .pH 6.2). The medium contained (g/l): glucose, 2.0; L-asparagine, 10.0; K_2PO_4 1.52; KCl, 0.52; $MgSO_4 7H_2O$ traces of $FeSO_4.7H_2O$. The final pH was adjusted to 6.2.

After inoculation cultures were incubated at 28°C for 7 days after which results were read. Production of L-asparaginase was observed as red coloration under the growing fungi due to the release of ammonia resulting from degradation of the amino acid L- asparagine. Phenol red at acidic pH is yellow and at alkaline pH it turns pink, thus a pink zone is formed around microbial colonies producing L-asparaginase (De Jong, 1972)

L- sparagines $\xrightarrow{\text{L- asparaginase}}$ L –aspartate + ammonia

3. Results

In the present work from thirty samples of wheat flour only 28 samples with percent (93.3%) were contaminated with fungi. The fungal species were isolated and identified by using three medium types.

Table (1) showed that the total fungal counts in case of Czapek's glucose agar medium were 33200 (colonies/gram) of wheat flour. The mycological analysis of 28 samples revealed the isolation of 24 species belonging to 16 genera of fungi. *Aspergillus* was the most common genus being recovered from 25 samples matching 89.2% of flour wheat samples and 56.8% of total fungal population. It was represented by 4 species of which *A.flavus*, *A.niger*, *A.fumigatus* and *A.candidus* were the most dominant as in Fig.(1). These species appeared respectively in 71.4%, 25 %,25% and 17.8% of flour samples contributing 27.4% , 10.5% ,13.2% and 4% of the total fungal count. *Penicillium* was recovered from 35.7 % of flour samples accounting for 9 % of total fungal counts. It was represented by 3 species of which *P.aurantiogriseum* and *P.chrysogenum* were of low incidence (4.5 % and 3.76 %)of total samples, respectively. *Penicillium citrinium* was rarely encountered from flour samples 0.9%.

Mucor spp. and Alternaria *spp.* were found to colonize (17.8% and 14.2%, respectively) of flour samples and recorded (19.8% and 4.5%) of total fungal counts.

Ulocladium chartarum, Eurotium *amstelodami*, *Emericella nidulans, Acremonium strictum, Cladosporium cladosporiodies, Rhizopus rhizopodiformis, Scytalidium hyalinum, Stemphylium botryosum, Trichoderma harzianum,Graphium penicillioide* and *Corynascus sepedonium* were infrequently recovered from wheat flour samples. And the percentage of total counts of these species was ranged from (0.3% – 4.8%). Dark hyphomycetes and sterile hyphae representing 0.6% of total fungal population Table(1).

The total fungal counts by culturing wheat flour samples on cellulose-Czapek's agar medium was 35300 (colonies/gram) of wheat flour. A total of 23 species of fungi belonging to 13 genera were isolated. *Aspergillius* was the most dominant genus isolated from 24 samples matching 85.7% of flour wheat samples and 60.6% of total fungal counts. *Aspergillius* was represented by 6 species, *A.flavus, A.fumigatus ,A.candidus, A.niger, A.ochraceus* and *A.clavatus.* The frequency of these species were (71.4%, 46.4%, 17.8%, 21.4%, 3.5% a and 3.5%, respectively) as shown in Fig.(1) and contributing 31.2%, 19.5%, 4.3%, 3.7%, 1.7% and 0.3% of the total fungal counts.

Penicillium was the second dominant genus isolated from 50% of samples accounting for 14.4% of total count. *Penicillium* represented by 3 species, *P.chrysogenum*, *P.citrinium* and *P.aurantiogriseum* with frequencies (25%, 17.8% and 10.7 %, respectively) and contributing 4.2%, 9% and 1.1% of total counts. The frequencies of *Alternaria spp.*and *Mucor spp.* were 28.5% and 14.2% with 5.4% and 3.1% of total counts.

The total counts of *Ulocladium chartarum,Fusarium proliferatum, Sterile dark hyphae, Cochliobolus spicifer, Cladosporium cladosporioides, Trichoderma harzianum, Emericella nidulans, Acremonium strictum, Graphium penicillioide* and *Rhizopus rhizopodiformis* were ranged from (0.3%-8.5%) with low frequencies ranged from (3.5% -14.2%) as shown in Table(2).

35200(colonies/gram) of wheat flour were obtained by culturing wheat flour samples on 40% sucrose-Czapek's agar medium. 20 species of fungi belonging to 10 genera were isolated. *Aspergillius* was also the most common genus isolated from 25 samples matching 89.3% of wheat flour samples and 53.1% of total fungal counts. *Aspergillius* was represented by *A.flavus*, *A.fumigatus*, *A.candidus*, *A.niger*, *A.ochraceus*, *A.terreus* and *A.tamarii*. The frequency of these species was (60.7%, 32.1%,32.1%,17.8%,3.5%, 3.5% and 3.5%, respectively) Fig.(1) and contributing 25.3%, 17.8%, 5.1%, 3.9%, 0.28% , 0.28 % and 0.28% of the total counts.

Eurotium spp. was the second dominant genus isolated from 50% of samples accounting for 15.9 % of total count. *Europium* include two species, *Eurotium sp* .with frequency 35.7% and *E. amstelodami* with frequency 28.5% and contributing (8.5 % and 7.3%, respectively) of total fungal counts.

Penicillium isolated from 42.8% of samples accounting for 14.2 % of total counts. *Penicillium* represented by 2 species, *P.olsonii* and *P.crustosum* with frequency(25% and 17.8% respectively) and contributing 6.25 % and 7.59 % of total counts.

Acremonium strictum and *Cladosporium cladosporioides* were isolated from 25% of samples accounting for 7.1 % and 3.1 % of total counts.

Frequency of *Mucor spp.* and *Alternaria spp.* were low 10.7% and 7.1% with 2.5 % and 1.9% of total counts. The total counts of *Hypomyces sp.*, *Scopulariopsis brumptii* and sterile hyphae were ranged from (0.28 - 1.1%) with low frequency ranged from (3.5 %- 7.1%) as shown in Table (3).

In the present study, twenty fungal isolates representing 11 fungal species related to 5 genera were numerated according to AUMC as shown in table (4). These strains were screened for their ability to release extracellular enzymes; α – amylase and L-asparaginase

From the data, all of the tested strains have ability to produce both enzymes with varying strength. All strains of Aspergilli genus exhibited high activity in production of α - amylase (25-27mm) except (*A.flavus var.columnaris* AUMC 5549) which has weak activity in production of this enzyme.

Fusarium proliferatum, Penicillium aurantiogriseum and *P.olsonii*(AUMC 5563) were also showed maximum activity in the production of α – amylase (20-25mm). Only three strains, *Penicillium crustosum*, *P.olsonii* (AUMC 5553) and *P.olsonii* (AUMC 5546) exhibited moderate activity in the production α- amylase (16-17mm) . *Fusarium semitectum, Penicillium citrinium, P.chrysogenum, P.olsonii* (AUMC 5572) and *Rhizopus rhizopodiformis* were very weak in the production of α-amylase (12-13mm).

Twelve fungal strains include (3 isolates)for both *Aspergillus flavus* and *A.flavus var.columnaris* , (1 isolate) for *A.niger , Fusarium proliferatum, F.semitectum, Penicillium chrysogenum, P.crustosum* and *P.olsonii* exhibited high activity in production of L- asparaginase .

Three isolates of *P.olsonii* and one isolate for *A.flavus var.columnaris, A.niger, Penicilliun aurantiogriseum, P.citrinum* and *Rhizopus rhizopodiformis* showed moderate activity in the production of L-asparaginase (Table 4).

Table (1): Counts (colonies/g) of fungi isolated from wheat flour on glucose –Czapek's agar.

Fungi taxa	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Acremonium strictum																	
Aspergillus candidus						100				100	500	100					
Aspergillus flavus	400	1500	100	200	100		700	200	100	100	200	500	400	500		200	100
Aspergillus fumigatus				100							100			400	800	1200	
Aspergillus niger		1500	600	100								500					
Alternaria alternata	500																100
A lternaria Chlamydospora	400																
Cladosporium cladosporioides	400																
Dark hyphomycete								200									
Emericella nidulans																	
amstelodami Eurotium																	100
Graphium penicillioide																	
Corynascus sepedonium										100							
Mucor himealis		1000						100					500				
Mucor racemosus																	
Penicillium chrysogenum						100											
Penicillium citrinium									200		100						
Penicillium aurantiogriseum									300		100			200		200	
Rhizopus rhizopodiformis																	
Scytalidium hyalinum	100																
Stemphylium botryosum	100																
Trichoderma harzianum				100													
Uloclodium chartarum	100	100															
Sterile hyphae	200																
No of genera	7	3	1	2	1	2	1	3	2	2	2	1	2	2	1	2	3
No of species	8	4	2	4	1	2	1	3	3	3	5	3	2	3	1	3	3
Total count	2200	4100	700	500	100	200	700	500	600	300	1000	1100	900	1100	800	1600	300

Table (1):Continued

Fungi taxa	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**	**TC**	**TC%**	**F**	**F%**
Acrmonium strictum										300		300	0.9	1	3.5L
Aspergillus candidus										500		1300	4	5	17.8L

Aspergillus flavus			500	100			1900	300	100		1000	9100	27.4	20	71.4H
Aspergillus fumigatus							800				1000	4400	13.2	7	25M
Aspergillus niger		100	400						300			3500	10.5	7	25 M
Alternaria alternata	400	100										1100	3.3	4	14.2L
Alternaria chlamydospora												400	1.2	1	3.5 L
Cladosporium cladosporioides												400	1.2	1	3.5 L
Dark hyphomycete												200	0.6	1	3.5 L
Emericella nidulaus							100					100	0.3	1	3.5 L
Eurotium amstelodami		100								100		300	0.9	3	10.7L
Graphium penicillioide							100					100	0.3	1	3.5 L
Corynascus sepedonium												100	0.3	1	3.5 L
Mucor himealis												1600	4.8	3	10.7L
Mucor racemosus						400			300			5000	15	2	7.1 L
Penicillium chrysogenum					100	100		400		600		1200	3.76	4	14.3 L
Penicillium citrinium												300	0.9	2	7.1L
Penicillium aurantiogriseum									600			1500	4.5	5	17.8L
Rhizopus rhizopodiformis			1500									1500	4.5	1	3.5L
Scytalidium hyalinum												100	0.3	1	3.5L
Stemphylium botryosum												100	0.3	1	3.5L
Trichoderma harzianum												100	0.3	1	3.5L
Uloclodium chartarum			100									300	0.9	3	10.7L
Sterile hyphae		100										200	0.6	1	3.5L
No. of genera	1	4	3	1	1	2	3	2	3	3	1				
No. of species	1	4	4	1	1	2	4	2	4	4	2				
Total count	400	400	2500	100	100	500	2900	700	1300	1500	2000	33200			

Table (2): Counts (colonies/g) of fungi isolated from wheat flour on cellulose –Czapek`s agar.

Fungi taxa	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Acrmonium strictium								200	100	100	100						
Aspergillus candidus						100					200	200					
Aspergillus clavatus												100					
Aspergillus flavus	1000	1000	600	200	200		400		200	1100	400	200	400	500	100	400	
Aspergillus fumigatus			300				300	100	400	1300		300		300	900	1000	
Aspergillus niger		400	200	400						100							
Aspergillus ochraceus																	
Alternaria alternata	100			100				200				100					

Alternaria chlamydospora	200	300	200														
Cladosporium cladosporioides									100					100			
Cochliobolus spicifer		200															
Emericella nidulans	200													100			
Fusarium proliferatum						100											
Graphium penicillioide									100					100			
Mucor himealis													200				
Mucor racemosus																	
Penicillium chrysogenum		200			300			100					200				100
Penicillium aurantiogriseum										100	100	200					
Penicillium citrinium							500				300	200					
Rhizopus rhizopodiformis	1000																
Trichoderma harzianum							100				100				200		
Uloclodiumc hartarum		100															
Sterile hyphae dark											1			1			
No. of genera	5	4	2	2	2	2	3	3	3	2	5	4	3	5	2	1	1
No. of species	5	6	4	3	2	2	4	4	5	4	7	7	3	6	3	2	1
Total count	2500	2300	1300	700	500	200	1300	600	900	2700	1300	1300	800	1200	1200	1400	100

Table(2): Continued

Fungi taxa	18	19	20	21	22	23	24	25	26	27	28	TC	TC%	F	F%
Acrmonium strictum												500	1.4	4	14.2 L
Aspergillus candidus								100			800	1500	4.3	5	17.8L
Aspergillus clavatus												100	0.3	1	3.5L
Aspergillus flavus			700	200	100		1200	100		500	1600	11000	31.2	20	71.4H
Aspergillus fumigatus					700		800	100		300	800	6900	19.5	13	46.4M
Aspergillus niger			100						100			1300	3.7	6	21.4L
Aspergillus ochraceus											600	600	1.7	1	3.5L
Alternaria alternata	400	200										1100	3.1	6	21.4
Alternaria chlamydospora		100										800	2.3	4	14.2L
Cladosporium cladosporioides		100										300	0.8	3	10.7L
Cochliobolus spicifer												200	0.6	1	3.5L
Emericella nidulans							100					400	1.1	3	10.7L
Fusarium proliferatum												100	0.3	1	3.5L
Graphium penicillioide							100			300		600	1.7	4	14.2L
Mucor himealis												200	0.6	1	3.5L
Mucor racemosus						400		100	400			900	2.5	3	10.7L
Penicillium chrysogenum								400		200		1500	4.3	7	25M
Penicillium aurantiogriseum												400	1.1	3	10.7L
Penicillium citrinium					300	200		400	900			3200	9.1	5	17.8L
Rhizopus rhizopodiformis			2000									3000	8.5	2	7.1L
Trichoderma harzianum												400	1.1	3	10.7L
Uloclodium chartarum												100	0.3	1	3.5L
Sterile hyphae dark												200	0.6	2	7.1L
No. of genera	1	3	2	1	2	2	3	4	3	3	2				
No. of species	1	3	3	1	3	2	4	6	3	4	4				
Total count	4	4	28	2	11	6	22	12	14	13	38	35300			

Table (3): Counts (colonies/g) of fungi isolated from wheat flour on 40% sucrose –Czapek`s agar.

Fungi taxa	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**
Acremonium strictum						300				600		500					
Aspergillus candidus		200				200		100			400						

Aspergillus flavus	800	300	700	500			800		100	200		100	200	500	300		100
Aspergillus fumigatus				200			100						200	400	1000	600	
Aspergillus niger		100	400				100										500
Aspergillus ochraceus																	
Aspergillus terreus	100																
Aspergillus tamarir																	
Alternaria alternata	300																
Alternaria chlamydospora	200																
Cladosporium cladosporioides	300		200	400					200								
Eurotium amstelodami					100						700	100				200	
Eurotium sp.		600			100			200			300	100					
Hypomyces sp.												100					
Mucor himealis								100									
Mucor racemosus																	
Penicillium olsonii	600	200	300						100								
Penicillium crustosum										1300							
Scopulariapsis brumptii						200											
Uloclodium chartarum																	
Sterile hyphae	100	100															
No of genera	5	4	3	2	2	2	1	3	3	2	3	4	1	1	1	2	1
No of species	7	6	4	3	2	3	3	3	3	3	3	5	2	2	2	2	2
Total; count	2400	1500	1600	1100	200	700	1000	400	400	2100	1400	900	400	900	1300	800	600

Table (3): Cont.

Fungi taxa	18	19	20	21	22	23	24	25	26	27	28	TC	TC%	F	F%
Acremonium strictum	200	600								600	500	2500	7.1	7	25 M
Aspergillus candidus			100					300	200	100	200	1800	5.1	9	32.1M
Aspergillus flavus		400			600	100	900	1000	100	1100	1200	8900	25.3	17	60.7H
Aspergillus fumigatus					600		900	1100			1800	6300	17.8	9	32.1M
Aspergillus niger			300				200					1400	3.5	5	17.8 L
Aspergillus ochraceus											100	100	0.28	1	3.5L
Aspergillus terreus												100	0.28	1	3.5L
Aspergillus tamarii					100							100	0.28	1	3.5L
Alternaria alternata												300	0.85	1	3.5L
Alternaria chlamydospora			200									400	1.1	2	7.1L
Cladosporium cladosporioides	300	100					800					1100	3.1	7	25M
Eurotium amstelodami	100	100	1200								300	2600	7.3	8	28.5M
Eurotium sp.			700	100			500		100	300		3000	8.5	10	35.7M
Hypomyces sp.												100	0.28	1	3.5L
Mucor himealis												100	0.28	1	3.5L
Mucor racemosus						400		400				800	2.2	2	7.1L
Penicillium olsonii			300	500						200		2200	6.25	7	25M
Penicillium crustosum					400	400		300	1200			2800	7.95	3	10.7L
Scopulariapsis brumptii												200	0.56	1	3.5L
Sterile hyphae												400	1.1	2	7.1 L
No. of genera			1	1		3	3	3	3	3	3				
No. of species	3	3	6	3	3	3	5	5	4	5	6				
Total count	300	400	700	300	400	900	3300	3100	1600	2300	4100	35200			

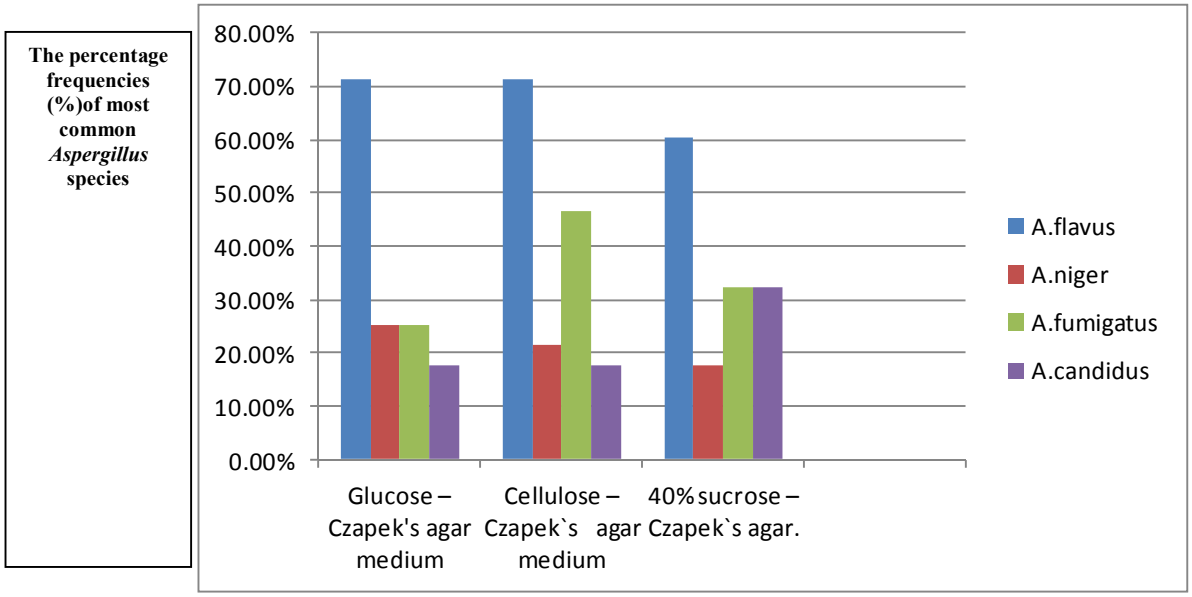

Figure (1): The frequencies of most common species of *Aspergillus* genus on three medium types (Glucose Czapek's agar, Cellulose Czapek's agar and 40% Czapek's)

Table (4): Activity of L- asparaginase(color density of pH indicator) and α-amylase(clear zone in mm) produced by fungal species isolated from wheat flour.

Strain No. (AUMC No.)	Fungal species	L- asparaginase (color density)	α-amylase (clear zone in mm)
5535	*Aspergillus flavus*	+++ H	26 H
5574	*A. flavus*	+++ H	27 H
5568	*A. flavus*	+++ H	27 H
5569	*A. flavus*var. *columnaris*	+++ H	27 H
5598	*A. flavus*var.*columnaris*	+++ H	27 H
5585	*A. flavus*var. *columnaris*	+++ H	25 H
5549	*A. flavus*var.*columnaris*	++ M	12 W
5547	*A. niger*	+++ H	27 H
5530	*A. niger*	++ M	25 H
5594	*Fusarium proliferatum*	+++ H	25 H
5556	*F. semitectum*	+++ H	12 W
5565	*Penicillium aurantiogriseum*	++ M	20 H
5591	*P. citrinum*	++ M	12 W
5571	*P. chrysogenum*	+++ H	13 W
5566	*P. crustosum*	+++ H	17 M
5563	*P. olsonii*	+++ H	24 H
5546	*P. olsonii*	++ M	16 M
5572	*P. olsonii*	++ M	13 W
5553	*P. olsonii*	++ M	16 M
5536	*Rhizopus rhizopodiformis*	++ M	12 W

L-asparaginase activity: H= High (+++), M= Moderate (++).
α-amylase activity: H= High (≥20 mm); M= Moderate (15 – 19 mm) and W: Weak (≤ 14 mm)

4. Discussion

Wheat flour is an ingredient used in many foods and is one of the most important foods in European and American culture. Bread, pasta, crackers, and many cakes, among other foods and cooking recipes, are made using flour or including this as an ingredient. Flour is the cleanest end product of the milling process and is generally regarded as a

microbiologically safe product as it is a low-activity commodity.

However, pathogens that contaminate flour may survive for extended periods (Berghofer *et al*., 2003; Cabanas *et al*., 2008).

Microorganism propagates get on grain in different ways, most often with dust from soil, from the surface of plant remnants during harvesting, transportation, storage, and processing (Klich, 2002). Mold spores present in flour survive for several years, and therefore, care should be taken in the storage of flour (Christensen and Cohen, 1950).

The present results revealed that the total fungal counts which were recorded on three medium types were ranged from 33200 to 35300 per gram of wheat flour. The most predominant genus was *Aspergillus* with high frequency (85.7% - 89.3%). *Aspergillus* was represented by 8 species, *A.flavus* showed maximum frequency (60.7%-71%) and minimum frequency exhibited by *A.clavatus, A.terreus, A.ochraceous* and *A. tamarii* (3.5%). These results are contradict the results recorded by Weidenbo¨ rner *et al*. (2000) and Kumar *et al*. (2008) who investigated the mycobiota of two German wheat flours and reported that *Aspergillus candidus* was the most frequently encountered mold. Also, they indicated that *A. flavus* was isolated to a lesser degree. *Penicillium* and *Eurotium* were the second dominant genera with frequency (50%). The frequency of *Eurotium amstelodami* was 35.7% followed by *Penicillium chrysogenum* and *P.olsonii* (25%). *P. citrinium* rarely isolated with frequency 0.9%. *Aspergillus* and *Penicillium* have been recorded among the most prevalent in flours by many authors.

Megalla *et al*. (1985) studied the fungal flora of wheat flour and baladi bread in upper Egypt. They reported that most of the isolated fungal species belonged to the genus *Aspergillus*. *Aspergillus* was the genus most detected at high frequency in all of the wheat flour samples from Algeria (Riba *et al*., 2008). Roige´ *et al*. (2009) reported that *Penicillium* (70%), and *Aspergillus* (34%) were the most frequent fungi isolated from corn and *Penicillium*(42%) was the most frequently recovered genera from wheat in Argentina. Usuoge *et al*.(2011) found that *Penicillium* species was the most common in the storage wheat flour. This finding corresponding with previous studies that *Penicillium* species are among the dominant fungi in wheat and wheaten flour. Kent-Jones and Amos (1967) reported about 90% *Penicillium* of the total mould isolated from white flour and Weidenborner *et al*.(2000) also reported 15% *Penicillium* of the numerous mould counts(1.730 x 10^3 cfu/g) of white wheat flour. For maize flour the isolation rate was 3.2% for *A.flavus*, *A.ochraceous* and *Penicillium sp*.(Simpanya *et al.,* 2001).

Alternaria spp. showed moderate frequency (28.5%) on cellulose-Czapek's agar medium. The frequency of *Alternaria* species of wheat flour according to Doolotkeldieva(2010) was 14%. On 40% sucrose-Czapek's agar medium *Acremonium strictum* and *Cladosporium cladosporioides* exhibited moderate frequency (25%) . These results are not consistent with the results recorded by Doolotkeldieva (2010) where as the *Cladosporium* species was recorded frequently and made a maximum contribution (43.7%). Graves and Hesseltine (1965) found that the frequency of *Alternaria spp*. and *Cladosporium cladosporoidies* in samples of wheat flour was very low (0.8%).

The frequency of *Mucor spp.* in all three medium types was very low ranged from (10.7% - 17.8%).But the frequency of *Mucor* species was maximum in other previous study (43%) (Doolotkeldieva, 2010).

Ulocladium chartarum, *Emericella nidulans, Rhizopus rhizopodiformis, Fusarium proliferatum, Cochliobolus spicifer, Scytalidium hyalinum, Stemphylium botryosum, Trichoderma harzianum, Graphium penicillioide Corynascus sepedonium, Hypomyces sp. Scopulariopsis brumptii* , Dark hyphomycetes and sterile hyphae were infrequently recovered from wheat flour samples(3.5%-10.7%) and contributed (0.28% - 1.7%) to the total counts. According to Halt *et al*.,(2004) *Cladosporium*, *Fusarium*, *Absidia*, *Rhizopus* and *Trichoderma* contributed less than 1% of the total mould count that were isolated from wheat flour.

The wheat flour is very rich with nutrition compounds, starch (67%- 70%), protein (12%-14%), fat (3%), ash(2%) and fiber (10%-13 %). Starch and protein is a major components and considered as good substrates for fungi that contaminated wheat flour. The present work concerned on the study of two fungal degradation enzymes, amylase for starch degradation and L- asparaginase for amino acid (L-asparagine).

Amylases are enzymes that participate in the hydrolytic degradation of starch are collectively referred to as amylolytic enzymes or amylases. Specific enzymes classified within this group include α-amylase, β-amylase, gluco-amylase (also known as amyloglucosidase), pullulanase and insoamylase . Amylases are, classified into two categories, endoamylases and exoamylases (Gupta *et al*., 2008). Endoamylases catalyse hydrolysis in a random manner in the interior of the starch molecule. This action causes the formation of linear and branched oligosaccharides of various chain lengths. Exoamylases hydrolyse from the non-reducing end, successfully resulting in short end products. A large array of amylases, are involved in the complete

breakdown of starch. Enzymatic degradation of starch yields glucose, maltose and other low molecular weight sugars (Gupta *et al*., 2008).

Amylase is of very wide occurrence in living organism ranging from human saliva to several species of fungi and bacteria (Reed, 1996). Amylase is a very important commercial enzyme having found use in the conversion of starch to varied product (Forgarty and Kelly, 1979).Amylases are important enzymes employed in the starch processing industries for the hydrolysis of polysaccharides such as starch into simple sugar constituents (Akpan *et al.,* 1999b; Omemu *et al*., 2004). Starch degrading enzymes like amylase have received great deal of attention because of their perceived technological significance and economic benefits. Among the microorganisms, many fungi had been found to be good sources of amylolytic enzymes (Omemu *et al., 2004).*

α -Amylase activity is widely distributed in nature. α-Amylase is an endo-acting enzyme, catalyzing the random hydrolysis of internal α-1,4 glycosidic linkages present in the starch substrate. However, α - amylases which are in most demand hydrolyses the α- 1,4 glycosidic bond in the interior of the molecule. These enzymes are incapable of hydrolyzing α-1,6 glycosidic linkages present at branch points of amylopectin chains(Gupta *et al*., 2008).

The present study revealed that all twenty fungal isolates which were chosen randomly for studying their activity in the production of α- amylase were positive in the production of this enzyme but with variant potent. All strains of Aspergilli genus exhibited high activity in production of α - amylase (25-27mm) except (*A.flavus var.columnaris* AUMC 5549) which has weak activity in the production of this enzyme. *Fusarium proliferatum, Penicillium aurantiogriseum* and *P.olsonii* (AUMC 5563) were also showed maximum activity in production of α – amylase (20 -25mm) . Only three strains, *Penicillium crustosum*, *P.olsonii* (AUMC 5553) and *P.olsonii* (AUMC 5546) exhibited moderate activity in production α- amylase (16-17mm) . *Fusarium semitectum, Penicillium citrinium, P.chrysogenum, P.olsonii* (AUMC 5572) and *Rhizopus rhizopodiformis* were very weak in the production of amylase (12-13mm). These results are in accord with the results of Kumar and Duhan(2011) who reported that five fungal strains, *Aspergillus candidus* , *A.terreus*, *A.niger*(Mtcc-104), *A.flavus* and *A.allahabadi* which were screened for production of α-amylase were positive . *Aspergillus niger(MTCC-104)* was found to be maximum (i.e. 1249 U/ml) amylase producer while *A.flavus* was least (i.e. 338 U/ml) producer.

Also , Park *et al*. (1995) and Lee *et al*. (2004) observed that from 10 selected fungi identified, N159-1 (KCTC 11927BP), N241-2, and N252-1 were *Aspergillus oryzae* ; N220-1 and N262-1were *A. flavus*, N36-1 was *Talaromyces spectabilis*, N83 was *Paecilomyces variotii*, and N109-2 and N278-1 were *Lichtheimia* sp. *A. oryzae showed* high amylase activity for starch degradation. *Penicillium olsonii* exhibited maximum level of α- amylase (0.7 U/mg protein) at 30°C (Afifi *et al*., 2008).

L-asparagine is a non-essential amino acid, has since been found in a number of different animal and plant sources. Whole grains such as wheat and oats are excellent sources of the compound. L-asparagine of wheat flour is considered as source nitrogen for fungi contaminated flour. L-asparaginase is secreted by fungi to degrade L-asparagine.

L-asparaginase (L-asparagine amidohydrolase, E.C.3.5.1.1) received increased awareness in current years for its anticarcinogenic potential. The important application of the L-asparaginase enzyme is in the treatment of acute lymphoblastic leukemia (mainly in children), L-asparaginase catalyzes the hydrolysis of L-asparagine into L-aspartate and ammonia(McCredie *et al*.,1973).

L-Asparaginase (L-asparagine amidohydrolyses) has been widely found in biological world. Asparaginase activity is widely distributed in plants, animal tissues and microorganisms including bacteria, yeast and fungi. Major genera of microorganisms reported to produce asparaginase. Bacteria species include; *Erwinia carotovora, E. coli, Pseudomonas flourescens, Mycobacterium phlei, Staphylococci, Thermus aquaticus, Tetrahymena pyriformis, Pseudomonas ovalis,* and *Serratia marcescens.* Yeast includes *Rhodotorula sp,* and *Candida utilis* and the fungi like *Aspergillus tamari, Aspergillus terreus, Aspergillus nidulans, Penicillum sp, Fusarium sp,* and *Helminthosporium sp* (Sarquis *et al*.,2004).

Our study revealed that 12 fungal strains include (3 isolates) for both *Aspergillus flavus* and *A.flavus var.columnaris,* (1 isolate) for *A.niger , Fusarium proliferatum, F.semitectum, Penicillium chrysogenum, P.crustosum* and *P.olsonii* exhibit high activity in production of L- asparaginase . Three isolates of *P.olsonii* and one isolate for of *A.flavus var.columnaris, A.niger, Penicilliun aurantiogriseum, P.citrinum* and *Rhizopus rhizopodiformis* showed moderate activity in production of L-asparaginase. There were only a few studies on L-asparaginase production by fungi (Lapmak *et al*.,2010). These have established that filamentous fungi belonging to the genera *Aspergillus*, *Penicillium*, *Fusarium* and some yeast species produce this enzyme (Sarquis *et al*., 2004).

Some fungi such as *Aspergillus tamari* and *Aspergillus terreus* have proved to be beneficial sources of this enzyme (Soni, 1989). Saranya *et al*. (2012) reported that the maximal enzyme production by *A.terreus* 8.3IU/ml was recorded at 35˚c and production was reduced at temperature higher than 35˚c. The temperature normally employed in the range of 25-35˚c and it depends mostly on the growth kinetics of the microorganisms used, and then moderate activity of L-asparaginase ezyme production by *A.flavus* in 7.75IU/ml. The *Penicillium* sp. showed good enzyme activity in cell biomass (Warangkar and khobragade, 2010).

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