JMBFS

Journal of Microbiology, Biotechnology and Food Sciences

International peer-reviewed scientific online journal

Published by Faculty of Biotechnology and Food Sciences

FUNGAL DIVERSITY ASSOCIATED WITH PEARL MILLET *PENNISETUM GLAUCUM* L.) GRAINS FROM TAIZ GOVERNORATE, YEMEN AND THEIR AMYLASE PRODUCTION

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doi: 10.15414/jmbfs.2017.7.2.118-123

ARTICLE INFO

Received 25. 10. 2016
Revised 4. 5. 2017
Accepted 13. 8. 2017
Published 1. 10. 2017

Regular article

OPEN ACCESS

ABSTRACT

In Yemen, this is the first record on fungal diversity associated with millet grains. Grain- borne fungi were tested for NaOCl- treated and non- treated samples of millet grain collected from Taiz Governorate, Yemen using direct plate method on Czapek's (Cz) and Czapek's supplemented with 40% sucrose (Cz40S) agar media. A total of 48 species belonging to 20 genera were isolated. The highest count and number of genera and species were recorded in non- treated grains on Cz40S medium. This means that the majority of fungi associated with grains were osmotolerant/osmophilic. The highest frequencies were represented by *Aspergillus flavus*, *A. niger* aggregate, *A. vadensis*, *Eurotium amstelodami*, *Pencillium duclauxii* and *Rhizopus stolonifer*. Among 109 isolates screened for their ability to produce amylase enzyme, 81.65% could produce the enzyme, of which *Aspergillus homomorphus* (a new record in Egypt), *Emericella nidulans*, *Fusarium oxysporum* and *Penicillium griseofulvum* were the best producers. Hence, these fungi may cause degradation of cell walls and spoilage of grains. Moreover, it is important to determine which organisms might be associated with seeds and grains in storage causing quality loss through their growth and enzyme production. The early detection of these organisms is required to prevent their harmful effects.

Keywords: Millet, fungal diversity, amylase, Czapek's agar, Czapek's 40% sucrose, treated and untreated grains

INTRODUCTION

Millets are a group of highly variable small-seeded grasses, broadly grown as cereal crops for fodder and human food. Pearl millet (*Pennisetum glaucum* L.) is an important food and forage crop, mainly cultivated in Yemen, Nigeria, Ghana, Cameroon, Sudan, India, Pakistan and other countries in Asia and South Africa. About 50 - 60% of the cultivated area in Yemen is represented by sorghum and millet **(Reddy *et al.*, 2004).** According to the data of United States Department of Agriculture, about 80 thousand million tons of pearl millet were annually produced. Millet is rich in starch, nutrients, vitamins, minerals, fats and organic compounds that can significantly boost human health in various ways. It is gluten- free, so Celiac sufferers can turn to millet as their source of grains, instead of wheat. Millet provides energy, has a higher protein content and better amino acid balance than sorghum **(Nkama and Ikwelle, 1998).**
Storage fungi are involved in deterioration of seeds and grains especially at high moisture contents. Somewhat, more than 50 fungal species have been isolated from seeds and grains, principally *Aspergillus*, *Penicillium*, *Eurotium* spp, with increasing moisture content above 15%, of which, *Aspergillus candidus*, *A. ochraceus*, *A. flavus*, *A. versicolor* and *A. tamarii* were the most encountered species **(Christensen, 1957).** Grain mold is a fungal disease can reduce grain germination or seedling emergence and consequently, reduce the quality of the grain, but, planting early will minimize yield and grain quality losses because it allows the crop to mature before disease developing. *Fusarium chlamydosporum*, *F. semitectum*, *F. moniliforme*, *F. solani*, *Alternaria* spp., *Aspergillus flavus*, *Cladosporium herbarum*, *Curvularia lunata*, *C. pallescens*, *Drechslera longirostrata*, *D. spicifer*, *D. terramera*, *Mortierella exigua*, *Mucor* spp., *Penicillium oxalicum*, *Penicillium* spp., *Pythium* sp., *Stachybotrys chartarum*, *Torula herbarum*, *Syncephalastrum racemosum*, and *Rhizopus* spp. were isolated previously from millet and sorghum in Egypt or from millet in USA, India and Eritrea **(Moubasher, 1993; Naqvi *et al.*, 2013; Khairnar, 2014; Zohri *et al.*, 2014; Mousa *et al.*, 2015).**
Microbial amylases have immense applications on various fields in world market because of their wide applications in industries including food, brewing, distilling industry, textile, paper, pharmaceutical and bioconversion of solid wastes **(Lall *et al.*, 2015).** Several filamentous fungi have proven to be an important source of industrial enzymes, due to their diversity **(Ogbonna *et al.*, 2015).** Many species of *Aspergillus* (*A. flavus*, *A. fumigatus*, *A. niger* aggregate, *A. ochraceus*, *A. oryzae* and *A. terreus*), *Emericella nidulans*, *Mucor racemosus*, *Mycosphaerella tassiana*, *Penicillium chrysogenum*, *Rhizopus oligosporus* and *R. stolonifer* are used as sources of fungal α- amylases **(Irfan *et al.*, 2012; Singh *et al.*, 2014).**
The objective of the current study was designed to assess the fungal diversity of millet grains collected from Taiz Governorate, Yemen. Also, amylase production by isolated fungi was evaluated.

MATERIAL AND METHODS

Collection of samples

Twenty samples of millet grains were collected from different markets, (local stores), in Taiz Governorate, Yemen. The samples were put in clean polyethylene plastic bags and brought to the Mycological Laboratory and keep at 5ºC till fungal analysis.

Determination of moisture content (MC%)

The moisture content was estimated using the method described by **Abdel-Hafez *et al.*, (2014)** and expressed as percentage of the dry-weight.

Germinability (Grain germination) test

Ten millet grains from each sample were first surface- sterilized by shaking in 2% sodium hypochloride solution for 5 minutes, rinsed three times by sterilized distilled water. Thereafter it was incubated at 25°C over a pad of moist sterile filter paper, then, placed in a sterile Petri dish for 7-10 days. The grain with healthy roots and plumules was counted and expressed as percentage according to the formula recommended by **Gummert (2011)**. Germination percentage (GP) = Number of germinated seeds/total number of seeds x 100.

Isolation and identification of fungi

The direct- plating technique was used to determine grain- borne fungi of millet. Czapek's (Cz) and Czapek's supplemented with 40% sucrose (Cz40S) agar media were used for isolation and identification of fungi. Two methods were used for isolation, grains were either placed directly on the surface of agar plate (non- treated) or surface- sterilized by 1% sodium-hypochlorite (NaOCl) (treated) **(Pitt *et al.*, 1992).** Five grains from each sample were put on the surface of agar plate and four replicates were used. All plates were incubated at 28°C for 7 days and the developing fungi were counted, isolated, identified and calculated as colony forming units (CFUs) per 20 grains for each sample. Pure cultures were cultivated on appropriate media (Czapek´s agar, Czapek yeast autolysate agar and potato dextrose agar) and incubated at 28ºC for identification. Microscopic examination of preparations of the different fungal species stained with lactophenol cotton blue was carried out. To distinguish *A. candidus* from other members of Section *Candidi* especially *A. tritici*, the isolates were grown for 7 days as 3-point inoculations on Czapek yeast autolysate agar (CYA) at 37ºC. *Aspergillus* species were identified phenotypically using the standard media (e.g. Czapek agar, CYA and MEA at 25C and on CYA at 37C) as recommended by Raper and Fennell (1965), Samson and Varga (2007) and Pitt and Hocking (2009), whereas, *Penicillium* species using the standard methodology (3 media and 3 incubation temperatures) recommended by Pitt (1979) and Pitt and Hocking (2009), *Fusarium* species using standard media and temperatures and following the keys and descriptions of Leslie and Summerell (2006) and Ismail *et al.*, (2015) and for other genera following the descriptive and dichotomous keys of Ellis (1971); Domsch *et al.*, (2007); Moubasher (1993); Pitt and Hocking (2009). Moreover, a doubtful isolate related to Section *Nigri* was identified molecularly by DNA sequencing using ITS1 ((5' - TCC GTA GGT GAA CCT GCG G - 3') and ITS4 (5'- TCC TCC GCT TAT TGA TAT GC -3') primers in SolGent company (Daejeon, Stouth Korea). The sequence obtained from the isolate was further analyzed using BLAST from the National Center of Biotechnology Information (NCBI) website and identified as *Aspergillus homomorphus*.

Screening for α- amylase production

One hundred and nine isolates of 44 fungal species related to 20 genera collected from millet grains were assayed for their ability to produce α- amylase enzyme according to the method of **Bridge (1985).** Fungal isolates were cultured on modified Czapek's agar medium (starch, 30 g; $NaNO_3$, 3 g; KH_2PO_4, 1.0 g; $MgSO_4.7H_2O$, 0.5 g; KCL, 0.5 g; $FeSO_4$, 0.01 g; agar, 15 g and distilled water, 1000 ml). The medium was inoculated with fungal isolates and incubated at 28°C, then flooded with iodine solution (iodine, 0.2 ml; potassium iodide, 0.4 ml and distilled water, 100 ml). A clear zone around fungal growth indicated the production of amylase **(Cowan 1974).** Enzyme index (EI) was calculated according to **Ho and Foster (1972)** as follows:

$$\text{Enzyme index (EI)} = \frac{\text{Diameter of the outer limited of the clear zone}}{\text{Diameter of the fungal colony}}$$

RESULTS AND DISCUSSION

Moisture contents (MC%)

The moisture contents (MC%) of all millet grain samples ranged from 9.4 – 12.9%. Sample numbers 16 (from ALaamor village, Taiz, Yemen), 18 and 20 (from 26 September street, Taiz, Yemen) followed by no 19 (from 26 September street) and 17 (from ALaamor village) recorded the highest MC%, on contrast, samples numbers 1 and 2 (from Alshenany, , Taiz, Yemen) were the lowest in their moisture contents (Table 1).

When the moisture contents of seeds and grains are raised artificially, the seed- and grain-borne fungi grow competitively to colonize and invade the seed. Several fungi could significantly increase their numbers at the different levels of moisture content. The degree of dominance among fungi differed according to the moisture level **(Moubasher *et al.*, 1980). Abdel-Hafez *et al.*, (2014)** noticed that the moisture contents of cereal grains (maize and sorghum) ranged between 8.75 – 16.76% in maize and 7.16 – 13.63% in sorghum.

Germination capacity of millet grains

The results in Table (1) revealed that the percentage germination of 20 grain samples ranging between 50 – 100% of the tested grains.

In general, in most samples there is a reverse correlation between moisture content and germination ability of the grain; for example, samples Nos. 1, 3, 4, 5 with relatively low MC% (9.4-10.4%) showed the highest G% (90-100%). On contrast, samples Nos. 16, 18, 19, 20 with relatively high MC% (12.7-12.9%) showed the lowest G% (50-70%), whereas, high moisture content enhance fungal growth on the grains. These results are in agreement with the finding of **Moubasher *et al.* (1980).** They stated that under low moisture content, fungi cannot grow and invade peanut seeds with slight loss in germinability and the seeds fell off with increasing moisture content. Also, at high levels of relative humidity (92 – 100% RH), the loss of germination capacity is serious and leads to complete mortality due to increasing in moisture contents of grains **(*Mazen et al.*, 1993).**

Fungi recovered in the present investigation

A total of 48 species and one species variety belonging to 20 fungal genera were isolated and identified from both NaOCl- treated and non- treated millet grains on Czapek's (Cz) and Czapek's supplemented with 40% sucrose (Cz40S) agar at 28°C. There is a remarkably high incidence of diverse fungal contamination of the analyzed samples. The genera of highest occurrence and their respective number of species were *Aspergillus* (15 spp.) and *Eurotium* (5 spp.). The total viable counts of fungi in all samples were 67 and 103 CFUs/400 of treated grains on Cz and Cz40S respectively and 384 and 449 CFUs/400 of untreated grains on Cz and Cz40S respectively (Tables 2,3) (Figure 1). Figure (1) showed that the highest total fungal count was recorded in non- treated grains on Cz40S medium (449), followed by that isolated on Cz (384 CFUs per 400 grains). Also the highest number of genera and species were detected in non- treated grains on Cz40S (33 species and 15 genera) (Figure 2). Isolates of *A. candidus* showed no growth on CYA at 37ºC and this differentiate it from *A. tritici* (Varga *et al.* 2007).

Table 1 Localities, moisture content (MC%) and germinability (G%) of 20 millet grain samples tested.

Sample No	Locality	MC%	G%
1	**Alshenany**	**9.4**	**90**
2	**Alshenany**	**9.6**	**60**
3	**Alshenany**	**10.4**	**100**
4	**Alshenany**	**10.4**	**100**
5	**Alshenany**	**10.4**	**100**
6	**Alsamsara**	**11.6**	**70**
7	**Alsamsara**	**11.6**	**90**
8	**Alsamsara**	**11.5**	**90**
9	**Alsamsara**	**11.7**	**90**
10	**Alsamsara**	**11.4**	**100**
11	**Alsamsara**	**11.2**	**70**
12	**Bearbasha**	**11.3**	**90**
13	**Bearbasha**	**11.3**	**80**
14	**Bearbasha**	**11.1**	**80**
15	**ALaamor village**	**11.4**	**80**
16	**ALaamor village**	**12.9**	**60**
17	**ALaamor village**	**12.6**	**90**
18	**26 September Street**	**12.9**	**50**
19	**26 September Street**	**12.7**	**70**
20	**26 September Street**	**12.9**	**70**

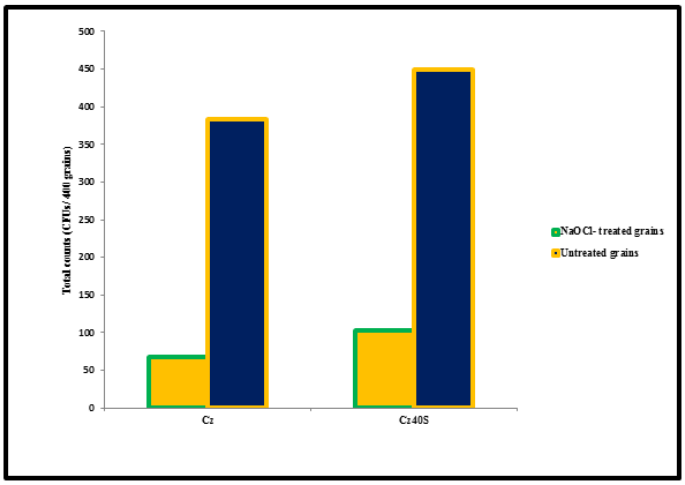

Figure 1 Total counts of fungi isolated from NaOCl- treated and untreated millet grains (per 400 grains) on Cz and Cz40S agar media

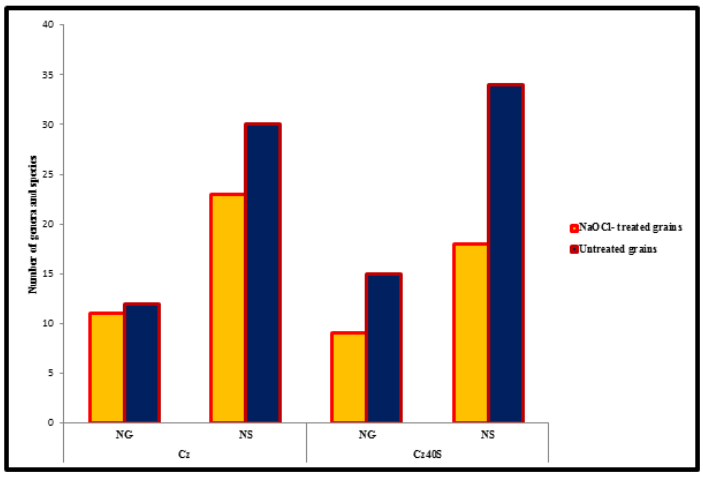

Figure 2 Number of genera (NG) and species (NS) of fungi isolated from NaOCl- treated and untreated millet grains (per 400 grains) on Cz and Cz40S agar media

Fungi isolated from NaOCl- treated grains

Twenty-two identified species + 1 species variety and 18 species appertaining to 11 and 9 genera were collected from NaOCl- treated millet grains on Cz and Cz40S at 28ºC respectively (Table 2).

On Cz agar medium, *Aspergillus* (5 species + 1 variety) was consistently the most frequent genus (50% of total samples) and had the highest percentage total counts (28.36% of total fungi). From the genus, *A. flavus* (20% of total samples and 10.45% of total fungi) and *A. vadensis* (20%, 5.97%) were the most common species. The remaining species were isolated in rarely from 5 – 10% of total samples, matching collectively about 12% of total fungi. The runner- up is black sterile mycelia which represented 40% of total samples and 22.38% of total fungi, followed by *Drechslera halodes* (30% and 13.4%) and *Curvularia* (2 species, 25%, 11.94%). The remaining genera and their respective species were isolated in rare frequency (Table 2).

On Cz40S agar medium, *Eurotium* (4 spp.) and *Aspergillus* (5 spp.) recorded the highest frequencies (60% and 45% of total samples respectively), comprising 33.98% and 23.3% of total fungi respectively. Moderate and low frequencies were represented by black sterile mycelia (35% of total samples and 19.4% of total fungi), *Cladosporium* (25% and 5.83%) and *Curvularia* (15% and 8.74%). The most prevalent species were *Aspergillus flavus* (35%), *Eurotium amstelodami* (30%), *E. rubrum* (25%), *E. repens*, *A. niger* and *Cladosporium cladosporioides* (20% each) (Table 2).

This is the first report on the occurrence and diversity of fungi in millet grains cultivated in Yemen, but there are some other studies from different countries. Overall, mold load was significantly higher in 16 samples tested of fonio millet (range = 2.30–4.88, mean = 4.12 ± 0.64 log10CFU/g) than in 17 samples tested of sesame (range = 2.48–3.98, mean = 2.97 ± 1.09 log10CFU/g) in Plateau State, Nigeria **(Ezekiel *et al.*, 2014)**. **Khairnar (2014)** isolated 23 fungal species belong to 12 genera from seeds of eight different cultivars by treated and untreated seeds from pearl millet in Maharashtra, India.

A great number of fungi that recorded in the present investigation from pearl millet grains were previously isolated from different localities of the world. According to **Agrios (1978),** the most common storage fungi are *Aspergillus* and *Penicilium* species. Seed infestation by microorganisms is a common and widespread phenomenon which has been variously reported. **Amadi and Adeniyi (2009)** isolated *A. terreus, A. flavus, A. niger, P. italicum, P. spinulosum, R. stolonifer* and *Fusarium* species from stored rice, maize and millet grains surface sterilized in NaOCl from Niger State, Nigeria, but *A. terreus, A. flavus* were detected only in finger millet grains. Various fungi (*Alternaria alternata, Aspergillus candidus, A. flavus, A. fumigatus, A. niger, A. sydowii, A. tamarii, A. versicolor, C. cladosporioides, C. herbarum, E. amstelodami, F. moniliforme, F. oxysporum* and others) were recorded on Czapek´s agar from paddy grains collected from different Governorates in Egypt ***(Mazen et al.*, 1993). Abe *et al.*, (2015)** recorded *Fusarium verticillioides* as the most frequently species on maize grains and *Aspergillus flavus* was the second most diverse, but *Cladosporium cladosporioides, Epicoccum nigrum* and *Mucor* sp. were rarely isolated. A total of 158 fungal isolates were cultured and identified from 83 surface sterilized mouldy millet grain samples studied in the state. Ten genera of fungi namely *Aspergillus* (70 isolates), *Penicillium* (43), *Fusarium* (23), *Rhizopus* (6), *Mucor* (5), *Syncephalastrum* (4), *Phoma* (4), *Cladosporium* (1), *Arthroconidia* (1) and *Helminthosporium* (1) were the identified fungal contaminants of surface sterilized millet grains in the Niger State, Nigeria **(Makun *et al.*, 2007).**

Table 2 Total counts (TC, calculated per 400 grains in all samples), percentage total counts (TC%) and percentage frequency (F%, calculated per 20 samples) of fungi isolated from NaOCl- treated millet grain samples on Czapek's and Czapek's supplemented with 40% sucrose agar media at 28 ℃ .

Fungal Taxa	Czapek's agar			Czapek's 40% sucrose agar		
	TC	TC%	F%	TC	TC%	F%
***Acremonium* W. Gams**	**2**	**2.99**	**10**			
***A. blochii* (Matruchot) W.Gams**	**1**	**1.5**	**5**			
***A. strictum* W. Gams**	**1**	**1.5**	**5**			
***Alternaria* Nees: Fries**	**2**	**2.99**	**5**	**1**	**0.97**	**5**
***A. alternata* (Fr.) Keissler**	**1**	**1.5**	**5**			
***A. chlamydospora* Mouchacca**	**1**	**1.5**	**5**			
***A. citri* Ellis and Pierce emend. Bliss & Fawcett**				**1**	**0.97**	**5**
***Aspergillus* P. Micheli ex Link**	**19**	**28.36**	**50**	**24**	**23.3**	**45**
***A. flavus* Link**	**7**	**10.45**	**20**	**11**	**10.7**	**35**
***A. flavus* var. *columnaris* Raper & Fennell**	**3**	**4.5**	**10**			
***A. homomorphus* Steiman, Guiraud, Sage & Seigle-Mur.**	**1**	**1.5**	**5**			
***A. niger* aggregate**	**3**	**4.5**	**10**	**5**	**4.8**	**20**
***A. ochraceus* Wilhelm**				**2**	**1.94**	**10**
***A. sydowii* (Bain. & Sart.) Thom & Church**	**1**	**1.5**	**5**			
***A. terreus* Thom**				**1**	**0.97**	**5**
***A. vadensis* Samson,de Vries, Frisvad & Visser**	**4**	**5.97**	**20**	**5**	**4.8**	**10**
***Cladosporium* Link**	**2**	**2.99**	**10**	**6**	**5.83**	**25**
***C. cladosporioides* (Fresenius) de Vries**	**1**	**1.5**	**5**	**5**	**4.8**	**20**
***C. herbarum* (Persoon) Link**	**1**	**1.5**	**5**	**1**	**0.97**	**5**
***Cochliobolus spicifer* Nelson**	**1**	**1.5**	**5**			
***Curvularia* Boedijn**	**8**	**11.94**	**25**	**9**	**8.74**	**15**
***C. lunata* (Wakker) Boedijn**	**4**	**5.97**	**15**	**4**	**3.9**	**10**
***C. ovoidea* (Hiroe and Watan.) Muntanole**	**4**	**5.97**	**20**	**5**	**4.8**	**5**
***Drechslera halodes* (Drechsler) Subram. and Jain**	**9**	**13.4**	**30**	**1**	**0.97**	**5**
***Emericella* Berkeley and Broome**	**2**	**2.99**	**10**			
***E. nidul ans* (Eidam) Vuillemin**	**1**	**1.5**	**5**			
***E. rugulosa* (Thom & Raper) Benjamin**	**1**	**1.5**	**5**			
***Eurotium* Mangin**	**4**	**5.97**	**15**	**35**	**33.98**	**60**

Table 2 Continued.

Fungal Taxa	Czapek's agar			Czapek's 40% sucrose agar		
	TC	TC%	F%	TC	TC%	F%
***E. amstelodami* Mangin**				**15**	**14.6**	**30**
***E. intermedium* Blaser**				**1**	**0.97**	**5**
***E. pseudoglaucus* Blochwitz**	**3**	**4.5**	**10**			
***E. repens* de Bary**	**1**	**1.5**	**5**	**4**	**3.9**	**20**
E. rubrum* Konig *et al.				**15**	**14.6**	**25**
***Fusarium* Link**	**2**	**2.99**	**5**	**1**	**0.97**	**5**
***F. sambucinum* Fuckel**	**1**	**1.5**	**5**	**1**	**0.97**	**5**
***F. verticillioides* (Sacc.) Nirenberg**	**1**	**1.5**	**5**			
***Penicillium griseofulvum* Dierckx**				**2**	**1.94**	**10**
***Scytalidium* sp. Pesante**				**4**	**3.9**	**5**
Sterile mycelia (black)	**15**	**22.38**	**40**	**20**	**19.4**	**35**
***Ulocladium alternariae* (Cooke) Simmons**	**1**	**1.5**	**5**			
Total count		**67**			**103**	
No. of genera (13)		**11**			**9**	
No. of species (30 + 1 variety)		**22 + 1 var.**			**18**	

Fungi isolated from untreated grains

Twelve and 15 genera including 29 species + 1 variety, and 33 species + 1 variety were isolated from 400 untreated millet grains on Cz and Cz40S agar media respectively (Tables 3).

On Cz agar medium, the highest frequencies of fungal genera and their species were represented by *Aspergillus* (11 spp. + 1 var.) and *Fusarium* (4 spp.), followed by *Curvularia* (2 spp.), *Drechslera* (1 sp.), *Rhizopus* (1 sp.) and black sterile mycelia. They were detected in 95%, 45%, 35%, 35%, 35%, and 35% of the grain samples, comprising 70.3%, 4.7%, 3.13%, 1.82%, 3.91% and 7.03% of total fungi respectively. The best counts were recorded by *Aspergillus* (70.3% of total fungi), followed by sterile mycelia (7.03%) and *Fusarium* (4.7%). The most common species were *A. flavus* (75% of the samples), *A. niger* (60%), *A. vadensis* (50%), *A. flavus* var. *columnaris* (40%), *Drechslera halodes*, *Rhizopus stolonifer* (35% each), *A. terreus*, *E. nidulans*, *Fusarium verticillioides* and *M. circinelloides* (30% each) and *Curvularia lunata* (25%) constituting 1.82%-28.4% of total fungi (Table 3).

On Cz40S agar medium, *Aspergillus* (12 spp. + 1 var.) was recovered from all samples constituting 58.4% of total fungi. The most prevalent species were *A. flavus* (80% of total samples, 21.6% of total fungi), *A. niger* aggregate (55%, 9.8%) and *A. vadensis* (55%, 13.6%). The second higher incidence rate was *Eurotium* (80% of the samples and 29.65% of total fungi) and the commonest species was *E. amstelodami* (45%, 14.7%). The third common fungi on Cz40S were black sterile mycelia (30% of total samples and 2.23% of total fungi), followed by *Penicillium* (2 spp., 25% and 1.6% respectively). From *Penicillium*, *P. duclauxii* (15%, 1.11%) was the most common (Table 3).

Table 3 Total counts (TC, calculated per 400 grains in all samples), percentage total counts (TC%) and percentage frequency (F%, calculated per 20 samples) of fungi isolated from non- treated millet grain samples on Czapek's and Czapek's supplemented with 40% sucrose agar media at 28ºC.

Fungal Taxa	Czapek's agar			Czapek's 40% sucrose agar		
	TC	TC%	F%	TC	TC%	F%
***Acremonium blochii* (Matruchot) W.Gams**	**1**	**0.26**	**5**			
***Alternaria* Nees: Fries**				**2**	**0.45**	**10**
***A. chlamydospora* Mouchacca**				**1**	**0.22**	**5**
***A. citri* Ellis and Pierce emend. Bliss & Fawcett**				**1**	**0.22**	**5**
***Aspergillus* P. Micheli ex Link**	**270**	**70.3**	**95**	**262**	**58.4**	**100**
***A. brasiliensis* Varga, Frisvad & Samson**	**5**	**1.3**	**10**	**6**	**1.34**	**10**
***A. candidus* Link**				**2**	**0.45**	**10**
***A. flavipes* (Bain. & Sart.) Thom and Church**				**1**	**0.22**	**5**
***A. flavus* Link**	**109**	**28.4**	**75**	**97**	**21.6**	**80**
***A. flavus* var. *columnaris* Raper & Fennell**	**30**	**7.8**	**40**	**28**	**6.24**	**35**
***A. fumigatus* Fresenius**	**2**	**0.52**	**10**			
***A. homomorphus* Steiman, Guiraud, Sage & Seigle-Mur.**	**2**	**0.52**	**10**	**8**	**1.8**	**15**
***A. niger* aggregate**	**36**	**9.4**	**60**	**44**	**9.8**	**55**
***A. parasiticus* Speare**	**5**	**1.3**	**10**	**3**	**0.7**	**5**
***A. sydowii* (Bain. & Sart.) Thom & Church**	**7**	**1.82**	**20**	**7**	**1.6**	**30**
***A. tamarii* Kita**	**5**	**1.3**	**20**	**1**	**0.22**	**5**
***A. terreus* Thom**	**22**	**5.7**	**30**	**3**	**0.7**	**15**
***A. vadensis* Samson,de Vries, Frisvad & Visser**	**46**	**11.97**	**50**	**61**	**13.6**	**55**
***A. versicolor* (Vuillemin) Tiraboschi**	**1**	**0.26**	**5**	**1**	**0.22**	**5**
***Botryotrichum* sp. Saccardo & Marchal**				**3**	**0.7**	**10**
***Cladosporium cladosporioides* (Fresenius) de Vries**	**4**	**1.04**	**20**	**10**	**2.23**	**30**
***Curvularia* Boedijn**	**12**	**3.13**	**35**	**2**	**0.45**	**10**
***C. lunata* (Wakker) Boedijn**	**10**	**2.6**	**25**	**1**	**0.22**	**5**
***C. ovoidea* (Hiroe and Watan.) Muntanole**	**2**	**0.52**	**10**	**1**	**0.22**	**5**
***Drechslera halodes* (Drechsler) Subram and Jain**	**7**	**1.82**	**35**	**2**	**0.45**	**10**
***Emericella* Berkeley and Broome**	**7**	**1.82**	**30**	**2**	**0.45**	**10**
***E. nidulans* (Eidam) Vuillemin**	**6**	**1.6**	**30**	**2**	**0.45**	**10**
***E. rugulosa* (Thom & Raper) Benjamin**	**1**	**0.26**	**5**			
***Epicoccum nigrum* Link**				**1**	**0.22**	**5**
***Eurotium* Mangin**	**5**	**1.3**	**5**	**134**	**29.65**	**80**
***E. amstelodami* Mangin**	**5**	**1.3**	**5**	**66**	**14.7**	**45**
***E. pseudoglaucum* (Blochwitz) Malloch & Cain**				**8**	**1.8**	**5**
***E. repens* de Bary**				**28**	**6.24**	**20**

Fungal Taxa	Czapek's agar			Czapek's 40% sucrose agar		
	TC	TC%	F%	TC	TC%	F%
E. rubrum* Konig *et al.				**32**	**7.13**	**30**
***Fusarium* Link**	**18**	**4.7**	**45**	**4**	**0.9**	**10**
***F. oxysporum* Schlechtendal**	**2**	**0.52**	**10**			
***F. solani* (Martius) Saccardo**	**1**	**0.26**	**5**			
***F. sambucinum* Fuckel**	**5**	**1.3**	**10**	**1**	**0.22**	**5**
***F. verticillioides* (Sacc.) Nirenberg**	**10**	**2.6**	**30**	**3**	**0.7**	**10**
***Humicola fuscoatra* Traaen**				**1**	**0.22**	**5**
***Mucor circinelloides* van Tieghem**	**11**	**2.84**	**30**	**4**	**0.9**	**10**
***Penicillium* Link**	**6**	**1.6**	**20**	**7**	**1.6**	**25**
***P. duclauxii* Delacroix**	**1**	**0.26**	**5**	**5**	**1.11**	**15**
***P. funiculosum* Thom**	**3**	**0.8**	**15**			
***P. griseofulvum* Dierckx**	**2**	**0.52**	**5**	**2**	**0.45**	**10**
***Rhizopus stolonifer* (Ehrenberg) Vuillemin**	**15**	**3.91**	**35**	**2**	**0.45**	**5**
***Scopulariopsis candida* (Gueguen) Vuill.**				**3**	**0.7**	**5**
Sterile mycelia (black)	**27**	**7.03**	**35**	**10**	**2.23**	**30**
***Syncephalastrum racemosum* Cohn ex Schroter**	**1**	**0.26**	**5**			
Total count		**384**			**449**	
No. of genera (17)		**12**			**15**	
No. of species (40 + 1 variety)		**29 + 1 var.**			**33 + 1 var.**	

It is worthy to mention that *Acremonium strictum, Alternaria alternata, Cochliobolus specifer* and *Ulocladium alternariae* were recorded only from treated grains on Cz agar medium. On the other hand, *Aspergillus ochraceus, Eurotium intermedium* and *Scytalidium* sp. were isolated only from treated grains on Cz40S agar medium. Some other species were detected only from non- treated grains on either Cz (*A. fumigatus, F. oxysporum, F. solani, P. funiculosum* and *Syncephalastrum racemosum*), or on Cz40S (*A. candidus, A. flavipus, Botryotrichum* sp., *Epicoccum nigrum, Humicola fuscoatra* and *Scopulariopsis candida*) (Tables 2,3).

Also the highest number of genera and species in grains, were detected in untreated grains on Cz40S (33 species and 15 genera) (Figure 2).This means that the majority of fungi associated with millet grains are either osmotolerant or osmophilic, thus, pearl millet grains have higher carbohydrate content (67.5 g/ 100 g) than maize (24.6 g/ 100 g) and the main sugar of the grain is sucrose **(NIN 2003; Nambiar *et al*., 2011).** It is worthy to mention that, stored grains have low water activity and this enables xerophilic fungi to grow in this condition **(Atanda *et al*., 2011)**. Sixteen fungal species were obtained from maize grains from Colombo **(Abe *et al.*, 2015).** Thirteen fungal genera were recorded on maize (8 genera) and sorghum (7) grains, lentil (5) and sesame (9) seeds using the seed/grain-plate method **(Abdel-Hafez *et al*., 2014).**

Ezekiel *et al*., (2014) isolated species of *Alternaria, Aspergillus* (*A. flavus* and *A. tamarii*), *Fusarium* and *Penicillium* from fonio millet and sesame kernels in Plateau State, Nigeria. In a study on mycobiota associated with maize, sorghum, lentil and sesame seeds, *Aspergillus* was isolated in high frequency from all samples from all substrates with *A. flavus* and *A. niger* were the most frequent **(Abdel-Hafez *et al*., 2014).** *Penicillium* was also isolated from all substrates but more frequent in lentil and sesame seeds. *Alternaria* was also isolated from all substrates but in low frequency from sorghum and lentil and in rare frequency from maize and sesame. *Aspergillus flavus, A. terreus, Emericella nidulans, F usarium oxysporum, F. moniliforme* and *Penicillium griseofulvum* were detected previously from finger millet in Andhra Pradesh of India **(Penugonda *et al*., 2010).**

A. homomorphus, the first record in Egypt, was isolated once from treated millet grain on Cz and also it was recorded herein from untreated grains on both isolation media.

α- Amylase production

Among the 109 isolates tested, 89 (81.65% of the total isolates) were able to produce amylase enzyme. *A. homomorphus, E. nidulans, F. oxysporium* and *P. griseofulvum* exhibited the highest amylase production, while *C. cladosporioides, C. herbarum, E. nigrum, F. solani, F. verticillioides, P. duclauxii, P. funiculosum* and *S. racemosum* were weak producers (Table 4). The negative enzyme producers (20 isolates) were related to *Botryotrichum* sp. (1), *Eurotium amstelodami* (5), *E. intermedium* (3), *E. pseudoglaucum* (1), *E. repens* (5) and *E. rubrum* (5) (Table 4).

Fungal extracellular enzymes may play an important role in biodeterioration of dried seeds and grains of several plants, and in propagation of toxigenic and pathogenic fungal strains. The results indicated that, *Eurotium* spp. failed to grow on starch medium because they are osmophiles and accordingly they were unable to produce the enzyme. Our results were greatly similar with the findings of **Dar *et al*., (2014)** who recorded that *Eurotium rubrum* had least enzyme activity. Our finding on the lack of amylase production by xerophilic fungus, *Eurotium*, did not agree with that of **Ulfig *et al*., (2009).** It was found that fungi isolated from pearl millet in India *A. alternata, A. fumigatus, A. niger, E. nidulans, Cladosporium herbarum, Curvularia lunata, F. oxysporum, P. oxalicum* and *Rhizopus nigricans* have the ability to produce amylase **(Khairnar, 2014)** and *A. flavus* and *Curvularia pallescens* were high producers. The above mentioned fungi were previously recorded as α- amylase producers from various substrates **(Lall *et al*., 2015; Dar *et al*., 2014; Pathak *et al*., 2014).**

Table 4 Screening of fungal isolates recovered from Millet for α- amylase production

Fungal Taxa	NIT	NPI	EI
Acremonium blochii	**1**	**1**	**1.05**
A. strictum	**1**	**1**	**1.1**
Alternaria chlamydospora	**1**	**1**	**1.05**
A. citri	**1**	**1**	**1.1**
Aspergillus brasiliensis	**1**	**1**	**1.1**
A. flavus	**18**	**18**	**1.02 - 1.13**
A. flavus* var. *columnaris	**1**	**1**	**1.03**
A. fumigatus	**1**	**1**	**1.1**
A. homomorphus	**1**	**1**	**1.4**
***A. niger* aggregate**	**7**	**7**	**1.1**
A. ochraceus	**1**	**1**	**1.04**
A. tamari	**1**	**1**	**1.03**
A. terreus	**6**	**6**	**1.04 - 1.15**
A. vadensis	**2**	**2**	**1.1**
A. versicolor	**1**	**1**	**1.05**
***Botryotrichum* sp.**	**1**	**-**	**0**
Cladosporium cladosporioides	**4**	**4**	**1**
C. herbarum	**2**	**2**	**1**
Cochliobolus spicifer	**1**	**1**	**1.3**
Curvularia lunata	**5**	**5**	**1.04 - 1.3**
C. ovoidea	**5**	**5**	**1 - 1.3**
Drechslera halodes	**3**	**3**	**1.2 - 1.3**
Emericella nidulans	**1**	**1**	**1.4**
E. rugulosa	**1**	**1**	**1.3**
Epicoccum nigrum	**1**	**1**	**1**
Eurotium amstelodami	**5**	**-**	**ng**
E. intermedium	**3**	**-**	**ng**
E. Pseudoglaucus	**1**	**-**	**ng**
E. repens	**5**	**-**	**ng**
E. rubrum	**5**	**-**	**ng**
Fusarium oxysporum	**1**	**1**	**1.4**
F. solani	**1**	**1**	**1**
F. sambucinum	**1**	**1**	**1.1**
F. verticillioides	**3**	**3**	**1**
Humicola fuscoatra	**1**	**1**	**1.1**
Mucor circinelloides	**1**	**1**	**1.03**
Pencillium duclauxii	**2**	**2**	**1**
P. griseofulvum	**5**	**5**	**1 - 1.4**
P. funiculosum	**2**	**2**	**1**
Rhizopus stolonifer	**1**	**1**	**1.03**
Scopulariopsis candida	**1**	**1**	**1.05**
***Scytalidium* sp.**	**1**	**1**	**1.04**
Syncephalastrum racemosum	**1**	**1**	**1**
Ulocladium alternariae	**1**	**1**	**1.04**
Total number	**109**	**89**	

NIT = Number of isolates tested, NPI = Number of positive isolates, EI = Enzyme index and ng = No growth.

CONCLUSION

This is the pioneer work on fungal diversity associated with millet, which is the very important crop in Yemen. *Aspergillus flavus*, *A. vadensis* and *D. halodes* were the most common on Cz medium, whereas, on Cz40S *A. flavus*, *Eurotium amstelodami*, *E. rubrum*, *E. repens*, *A. niger* aggregate and *Cladosporium cladosporioides* had the highest frequencies. The highest frequencies of fungi of non- treated grains were *A. flavus*, *A. niger* aggregate, *A. vadensis*, *E. amstelodami*, *P. duclauxii* and *R. stolonifer*. Most fungi isolated from millet have the ability for amylase production which may cause degradation of plant cell walls and spoilage of grains. It is important to know which organisms might cause spoilage problems and the early detection of these organisms is importance to prevent the spoilage. Also, there is urgent need to further studies on fungi associated with millet and other important crops in Yemen and role of their enzyme productions in biodeterioration of stored grains.

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