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Biogenic silver nanoparticles of resistant *Aspergillus flavus* AUMC 9834 against some pathogenic microorganisms and its synergistic effect with the antifungal fluconazole

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Abstract: The aim of this study was the biosynthesis of silver nanoparticles (AgNPs) by *Aspergillus flavus* AUMC 9834 isolated from oral suspension of β-lactam antibiotic Cefpodoxime proxetil. Antimicrobial activity and the synergistic effect of AgNPs in combination with the antibiotic fluconazole against resistant fungus were investigated. The synthesized AgNPs from cell-free filtrate were characterized by using UV-VS spectrophotometer analysis, Fourier Transform Infrared Spectroscopy (FTIR), X-ray diffraction (XRD) and Transmission Electron Microscopy (TEM). UV-VS spectrophotometer analysis showed a peak at 420 nm corresponding to the plasmon absorbance of silver nanoparticles and FTIR analysis showed the potential biomolecule responsible for the reduction of silver. The structural properties of silver nanoparticles were confirmed using XRD technique, while TEM micrographs revealed that the silver nanoparticles are dispersed and aggregated, and mostly having spherical shape within the size range between 5 and 19 nm. The synthesized silver nanoparticles plus fungal filtrate exhibited a varied growth inhibition activity against the tested pathogenic bacteria. A significant increase in the area of growth inhibition was observed when a combination of silver nanoparticles and fluconazole was applied. The current results revealed that the synthesized silver nanoparticles produced by *A. flavus* strain are promising for the use in medical therapy, due to their broad spectrum against some pathogenic bacteria and resistant tested fungus.

Key words: *A. flavus* strain, biosynthesis, silver nitrate nanoparticles, synergism with fluconazole, antimicrobial activity.

Introduction

Green nanotechnology is the utilization of microbial resources for the fabrication of nanoparticles. Nanoparticles have been synthesized using various fungi (Ahmad *et al*. 2003, Bhainsa and D'souza 2006), bacteria (Nanda and Saravanan 2009) and plants (Kumar and Yadav 2009). When comparing with chemical and physical methods, biological synthesis of nanoparticles has proved to be free of any limitations associated with production of hazardous by-products, and it is simple and cost effective (Singh *et al*. 2011). Microbiological synthesis can take place either intracellularly (Seshadri *et al*. 2012) or extracellularly (Bhambure *et al*. 2009) .The mechanism underlying the synthesis of silver nanoparticles (AgNPs) by fungi can also be predicted. Biosynthesis is basically associated with the reducing mechanism of the cellular components (Bhattacharyya *et al*. 2009). The specific position of reductase enzymes was typically cited (Mukherjee *et al*. 2001, Bhainsa and D'souza 2006). The silver ions were subsequently reduced to AgNPs by enzymes present at the fungal cell surfaces (Bhainsa and D'souza 2006, Jain *et al*. 2011, Mukherjee *et al*. 2001). The reduction process was also facilitated by certain extracellular enzymes like naphthoquinones and anthraquinones (Ahmad *et al*. 2003). Metal nanoparticles of silver, copper, and gold have been found to be active against certain pathogenic bacteria and fungi (Kim *et al*. 2008, Rai *et al*. 2012). Comparatively, AgNPs have been intensely studied owing to their distinct properties such as, chemical stability, nonlinear optical behaviour, and bactericidal activity (Vaidyanathan *et al*. 2009, Kollef *et al*. 2011, Rai *et al*. 2012). These properties make them suitable for use as a microbial disinfectant. The production of AgNPs is relatively inexpensive, and the addition of these particles into goods such as plastics, clothing, creams, and soaps increase their market value (Allahverdiyev *et al*. 2011). New classes of compounds that include nanoparticle–antibiotic conjugates are undergoing clinical evaluations (Li *et al*. 2005, Fayaz *et al*. 2010, Sekhon 2010, Kollef *et al*. 2011, Pissuwan *et al*. 2011, Rai *et al*. 2012). The combination of antibiotics and metal nanoparticles could increase the antibiotics' efficacy against resistant pathogens (Li *et al*. 2005, Naqvi. *et al*. 2013). Moreover, nanoparticle antibiotic conjugates lower the amount of both agents in the dosage, which reduces noxiousness and increases antimicrobial properties. These conjugates are effective

against resistant bacteria. Additionally, due to this conjugation, the concentrations of antibiotics are increased at the place of antibiotic–microbe contact (Fayaz *et al*. 2010, Li *et al*. 2005).

The present research was carried out in response to the significance of biological synthesis of nanoparticles and the implications of their use in controlling pathogenic microbes. Biogenic silver nanoparticles of fluconazole-resistant *Aspergillus flavus* AUMC 9834 isolated from oral suspension of beta-lactam antibiotic Cefpodoxime proxetil was evaluated against some pathogenic microorganisms as well as its synergistic effect with the antifungal fluconazole towards the resistant fungus.

Materials and Methods

Microorganism

The strain of *A. flavus* isolated from oral suspension finished product (cepodem 40mg/5 ml dry mix) was used for the synthesis of nanoparticles. Cepodem contains the active ingredient Cefpodoxime proxetil which is a semi-synthetic beta-lactam antibiotic belonging to the third generation of oral cephalosporin group produced by T3A pharmaceutical company, Assiut, Egypt. The fungus was isolated on Sabouraud Dextrose Agar medium (SDA), characterized and identified according to Raper and Fennell (1965) and Moubasher (1993) and was maintained at 4°C at Assiut University Mycological Center (AUMC 9834).

Assay for the synthesis of nanoparticles

The mycogenesis of AgNPs was performed using *A. flavus* AUMC 9834. The fungal biomass was cultivated aerobically in a broth medium of the following composition (g / l): KH_2PO_4 7; K_2HPO_4 2; $MgSO_4.7H_2O$ 0.1; $(NH_2)SO_4$ 1; yeast extract 0.6; glucose 10; pH 5.8 (Fayaz *et al*. 2010) and incubated at 22°C for 72 h in an orbital shaker at 200 rpm. At the end of incubation period, the fungal biomass was filtered using filter paper Whatman no 1. The cultural filtrate was later used for nanoparticle synthesis. About 200 ml of mycelium-free cultural filtrate containing 0.1 M precursor salt AgNO3 were disposed in a 500 ml Erlenmeyer flask. The flask was incubated in dark condition at 28°C on a shaker at 150 rpm for 72 hours (Bhainsa and D'souza 2006).

Characterization of silver nanoparticles
Visual observations

The formation of AgNPs was followed by visual observation of colour change into reddish color that indicates the reduction reaction (Mukherjee *et al*. 2001).

UV-Vs spectral analysis:

The reduction of pure Ag+ was further confirmed by the sharp peak given by scanning the AgNPs of the reacting solution using Perkin-Elmer Lamda-45 spectrophotometer, in a 1 cm path quartz cell at a resolution of 1 nm from 300 to 800 nm (Vahabi *et al*. 2011).

Fourier transforms infrared spectroscopy (FTIR)

FTIR spectra of dried synthesized AgNPs were recorded using FTIR Nicolet Avatar 660 FTIR spectrometer (Jeevan *et al*. 2012).

X-ray diffraction (XRD) analysis:

The synthesized AgNPs were dried and the powder form of the sample was subjected for XRD analysis using X-ray diffractometer (Model PW 1710 control unit Philips, Anode material Cu, 40 KV, 30 M.A, optics: automatic divergence slit) with Cu Kα radiation λ=1.540562 °A (Prema and Raju 2009).

Transmission electron microscopy (TEM) analysis

The morphology of the AgNPs was investigated by TEM using JEOL- JEM-100 CXII instrument in the electron microscope unit in Assiut University, by drying a drop of the washed colloidal dispersion onto a copper grid covered with a conductive polymer (Li *et al*. 2011).

Antimicrobial activity of fungal filtrate and AgNPs by agar well-diffusion method

Silver nanoparticles (AgNPs) synthesized from *A. flavus* AUMC 9834 and mixed with fungal filtrate were tested for their antimicrobial activity by the well-diffusion method against various pathogenic microorganisms. The tested microorganisms were used, namely gram-negative bacteria (*Escherichia coli* ATCC 8739 and *Pseudomonas aeruginosa* ATCC 9027) and gram-positive bacterium (*Staphylococcus aureus* ATCC 6538P), and the fungal strains of *Candida albicans* ATCC 10231 and *Aspergillus niger* ATCC 16404. Each strain was spread over the surface of nutrient agar using sterile cotton swab and wells of size 6 mm diameter were made with the help of a sterilized cork borer. Different volumes (50, 25, 15 µl) of the AgNPs and the fungal filtrate mixture were loaded separately into wells and the plates were incubated at 35 C° for 24 h in case of bacterial strains and at 28º C for 72 h for fungal strains. The zones of inhibition were measured and the lowest concentration of AgNPs and fungal filtrate mixture that inhibited the growth of each of the test microorganisms was determined as the minimum inhibitory concentration (MIC) (Kareem *et al*. 2008; Silambarasan and Jayanthi 2013).

Evaluation of synergistic effects of AgNPs combined with Fluconazol antibiotic

The resistant strain *A. flavus* AUMC 9834 was also used for studying the synergism between synthesized AgNPs and fluconazole. Well-diffusion method on nutrient agar plates was used to assess the synergistic effect. Different concentrations of the tested antifungal (200, 150, 100, 50, 25 and 12.5 µg /ml) were prepared to determine the MIC. A twenty five µl of freshly prepared AgNPs and fungal filtrate mixture, and 25 µl of each concentration of the antifungal were loaded into each well as the final content of 50 µl of AgNPs and antifungal per well (Silambarasan and Jayanthi 2013). After incubation at 28°C for 24 hours, the inhibition zones were measured; the assays were performed in triplicates.

Statistical analysis

Results were compared statistically using Graphpad Prism v5.00 statistical software. The results are presented as mean ± standard deviation (S.D.). Statistical significance was set up at $p < 0.05$.

Results

Characterization of silver nanoparticles

Visual observation

The cultural filtrate of the filamentous fungus *Aspergillus flavus* AUMC 9834 was used for the biosynthesis of silver nanoparticles using 0.1 M silver nitrate salt. The colour changes to reddish after 24 h of incubation in the dark indicating the nanoparticles biosynthesis, comparable to the control (fungal filtrate without silver nitrate) which shows no color change (Fig. 1).

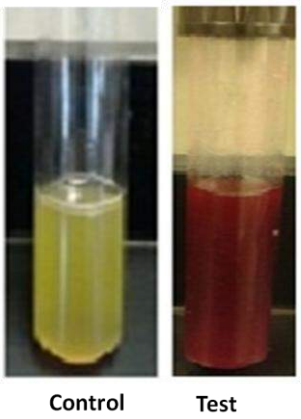

Fig 1: Biosynthesis of AgNPs by *A. flavus* AUMC 9834: Colour change test.

UV-VS spectral analysis

Bioreduction of silver nitrate ions was confirmed by the sharp peak given by the AgNPs in the visible region from UV–VS spectrum of the reaction solution at 420 nm (Fig 2). The spectrum showed a strong surface Plasmon absorption band indicating the presence of AgNPs**.**

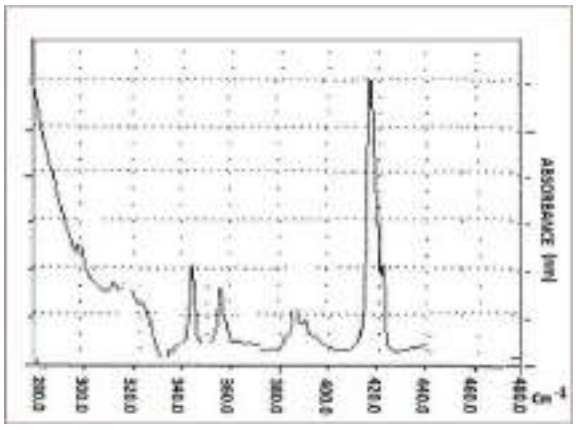

Fig 2: UV-VIS spectral analysis of AgNPs absorption band around 420 nm.

FTIR analysis of Ag NPs

The nature of the biomolecules involved in the reduction and formation of AgNPs was studied by FTIR (Fig 3). The FTIR signals of AgNPs were observed at 1072, 1365, 1648, 2145, 2350, 2933 and 3260 cm^{-1}. FTIR measurement was carried out to identify the potential biomolecule in enzyme filtrate responsible for the reduction of silver ions and capping agent responsible for the stability of the bioreduced silver nanoparticles**.** The FTIR spectra in 1400-1700 cm^{-1} region provide information about the presence of -C=O and -N=H groups which is responsible for the reduction of $AgNO_3$ to Ag. The bands in the region between 3260 and 2145 cm^{-1} were assigned to O-H stretching of alcohols and phenol compounds, and aldehyde -C-H-stretching of alkanes. The peaks in the region 1072, 1365 and 1648 cm^{-1} correspond to N-H of primary and secondary amides and –C-N-stretching vibrations of amines or –C-O-stretching of alcohols, ethers, carboxylic acids and anhydrides (Ninganagouda *et al.* 2013).

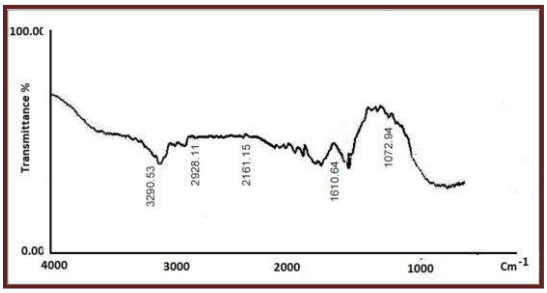

Fig. 3: FTIR spectra of synthesized AgNPs from *A. flavus* AUMC 9834.

X-ray diffraction (XRD) analysis:
The crystalline nature of the particles was confirmed using XRD. Figure 4 shows x-ray powder diffraction patterns of the synthesized AgNPs at 80º C. The line positions are consistent with metallic silver (Mc Clune 1993). The peaks assigned in the AgNPs sample were 108, 132, 206, 222 and 316 Aº, number of Bragg reflections with 2Ɵ values of 27.76°, 32.18°, 46.17°, 38.08°, 46.17°, 54.82°, 57.41°, 67.48°, 74.56° and 76.75Aº (ASTM No. 04-014-6889).

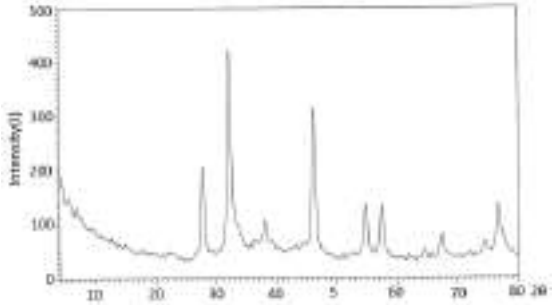

Fig 4: X-ray diffraction (XRD) pattern show silver nanoparticles synthesized from *A. flavus* AUMC 9834.

Transmission Electron Microscopy (TEM) analysis
The TEM image of AgNPs (Fig 5) shows that the particles were spherical in shape and the size of the AgNPs was found to be in the range from 5 to 19 nm.

Fig 5: Transmission Electron Microscopy (TEM) of AgNPs synthesized from *A. flavus* AUMC 9834.

Antimicrobial activity and Synergistic effect of AgNPs in combination with fluconazole

The silver nanoparticles mixed with fungal filtrate exhibited good antibacterial activity against Gram-negative bacteria, the inhibition zone in case of *E. coli* ATCC 8739 was 13.33±2.30 mm, while in *P. aeruginosa* ATCC 9027 was 15.00 ±2.00 mm. In case of Gram-positive bacterium, *S. aureus* ATCC 6538P, they showed inhibition zone of 12.66 ±1.15 mm. They also showed antifungal activity against *C. albicans* ATCC 10231 of 18.33 mm±0.57 and *A. niger* ATCC 16404 of 19.33 ±1.52 mm. The inhibition activity of fungal filtrate combined with silver nanoparticles against test strains were significantly promoted by the fold increase ranging from 25 - 66.2 %. On the other hand, biosynthesized AgNPs induced significantly higher antifungal activity when combined with fluconazole antifungal against resistant *A. flavus* AUMC 9834 than that of the fungal filtrate or fluconazole antifungal alone (Table 1).

Discussion

The scope of the present study was based upon exploring the capability of *A. flavus* AUMC 9834 to synthesize AgNPs and its possible biomedical application in controlling infection. The data showed a great deal of capability of synthesizing AgNPs. In addition, these nanoparticles alone and in conjugation with fluconazole proved to be effective in inhibiting the growth of the resistant fungus and some pathogenic bacteria. Fluconazole inhibits cytochrome P_{450}-dependent enzymes (particularly C-14- demethylase) involved in the biosynthesis of ergosterol, by altering the cell membrane function and permeability, resulting in cell dysfunction (Al-Badriyeh *et al.* 2009).

When the reaction mixture was incubated under dark conditions, the colour of the liquid mixture changed to reddish after 24 hours of reaction as the first indication of AgNPs biosynthesis. The change in color intensity (absorbance) was also monitored through ultraviolet-visible spectroscopy; peaks of the reaction mixture were obtained around 420 nm (Fig 2). It was reported that the reduction of Ag^+ to atomic silver Ag^o corresponds to absorption at 420 nm (Li *et al.* 2005, Fayaz *et al.* 2010). Various reports have provided evidence of extracellular generation of AgNPs by XRD and TEM images (Prema and Raju 2009, Narasimha *et al.* 2011). Also, several studies have proposed that AgNPs may attach to the surface of the cell membrane disturbing permeability and respiration functions of the microbial cell **(**Singh and Raja 2012, Rai *et al.* 2014). Smaller AgNPs having the large surface area available for interaction would give more bactericidal effect than the larger AgNPs **(**Singh and Raja 2012, Rai *et al.* 2014**).**

The mechanism of action of AgNPs is still not well defined. Many proposed mechanisms about antibacterial effect of silver ions have been presented by several researchers (Davies and Etris 1997, Li *et al.* 2005, Fayaz *et al.* 2010, Kollef *et al.* 2011, Rai *et al.* 2012). Being positively charged, they attack negative charges of transmembrane proteins and could destroy the cell membrane and block the transport channels (Davies and Etris 1997). It might be possible

that they penetrate inside the microbial cell and disrupt cellular activities like transportation, protein synthesis, and nucleic acid functioning (Senapati *et al.* 2005). It has also been proposed that silver ions penetrate the cell, intercalate themselves between pyrimidine and purine, and denature the DNA molecule (Rai *et al.* 2012).

When the AgNPs, have large surface area, were combined with the antifungal antibiotic fluconazole, the fungicidal effect is enhanced by interaction between active groups like hydroxyl and amino groups present in this antifungal with AgNPs by chelation (Batarseh 2004). As a result, antifungal–AgNP conjugate is formed in which an AgNPs core is surrounded by antibiotic molecules. Thus, the antimicrobial concentration is increased at the focal site, which leads to increased disruption of peptidoglycan of bacteria (Fayaz *et al.* 2010, Li *et al.* 2005). Likewise, fluconazole in combination with positively charged AgNPs both inhibited and disrupted cell-wall synthesis (Sekhon 2010, Herman and Herman 2014). More studies are needed to find out the exact mechanism of action to develop a novel potent antimicrobial drug against resistant fungus.

Table 1: Antimicrobial activity of the fungal filtrate, fungal AgNPs, fluconazole and fluconazole + AgNPs against some microorganisms (expressed as zone of inhibition in mm ± SD).

Test microorganisms	Fungal filtrate only[a]	Fungal filtrate AgNPs Only[b]	Fluconazole only (MIC)[c]	[Fluconazole (MIC)+filtrate AgNPs][d]	Fold increase%*	P value
	(50 µl)	(50 µl)	(50 µl)	25 µl + 25 µl		
E. coli ATCC 8739	8.00±1.00	13.33±2.30	-	-	66.25	p < 0.05
P. aeruginosa ATCC 9027	10.33±0.75	15.00±2.00	-	-	45.63	
S. aureus ATCC 6538P	9.33±1.52	12.66±1.15	-	-	35.69	
C.Albicans ATCC 10231	14.66±0.57	18.33±0.57	-	-	25.03	
A.niger ATCC 16404	12.66±1.56	19.33±1.52	-	-	52.68	
A.flavus AUMC 9834 (Resistant strain)	6.3±1.52	8.61±0.57	-	-	36.66	
			8±1.00	11.6±1.52	45	

MIC of Fluconazole = 200 µg/ml; *Percentage fold increases of antimicrobial activities were calculated using the formula ((b − a)/a) × 100 or (d − c)/d) × 100 (Fayaz *et al*, 2010).

Conclusion: Antifungal-resistant fungi have been continuously increasing over the past decade; hence, there is a need to detect another method to overcome this problem. In the present scenario, AgNPs have appeared as a promising antimicrobial candidate in the medical field. Hence it could be potentially applied in the fabrication of silver impregnated antimicrobial materials for biomedical applications. The present results demonstrated the synthesis of silver nanoparticles by *A. flavus* AUMC 9834 at room temperature. Within 24 hours of time, spherical-shaped nanoparticles below 20 nm size were formed. The method described is highly efficient and cost effective to produce silver nanoparticle. The activity of antifungal antibiotic fluconazole against resistant *Aspergillus flavus* AUMC 9834 was augmented when impregnated with AgNPs.

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In-vitro susceptibility testing of dermatophytes isolated in Cairo, Egypt against eight antifungal agents by broth microdilution and disk diffusion methods

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Abstract: Nine dermatophytic strains belonging to 7 species, namely *Microsporum canis* (1), *Trichophyton tonsurans* (1), *Trichophyton rubrum* (2), *Trichophyton mentagrophytes* (1), *Trichophyton violaceaum* (1), *Arthroderma* sp. (1), and *Epidermophyton floccosum* (2) isolated from clinical specimens in Cairo, Egypt were tested for their susceptibility toward eight antifungal drugs using broth microdilution and disk diffusion methods. The antifungals used were Amphotericin B, Itraconazole, Voriconazole, Fluconazole, Terbinafine, Ketoconazole, Griseofulvin and Caspofungin. Results of this study demonstrate that the broth-microdilution method is still the preferable method since it is reliable and helps in quantitative MICs determination. The *in-vitro* testing revealed also that Voriconazole, Itraconazole, Ketoconazole, Griseofulvin as well as Caspofungin (echinocandin) were the most active antifungal drugs against the dermatophytic strains tested.

Keywords: antifungal susceptibility, dermatophytes, broth microdilution, disk diffusion, Egypt.

Introduction

Dermatophytes are filamentous keratinophilic fungi commonly involved in cutaneous infections of human. They are classified into three genera, *Epidermophyton*; *Microsporum*, and *Trichophyton* (Perez *et al.* 2010)).

Dermatophytoses are spreading easily and rapidly, especially in lower economic classes of populations. Topical formulations of antifungal drugs such as Ketoconazole, Clotrimazole, and Terbinafine were often used for their treatment (Singh *et al*. 2007).

Oral antifungal drugs such as Itraconazole, Griseofulvin, and Terbinafine are often used to treat severe cases of dermatophytoses such as tinea capitis and tinea ungium. Some other compounds, such as Posaconazole, Voriconazole and Ravuconazole are also effective (Fernandez-Torres *et al*. 2002).

In the last two decades, numerous studies discussed the development of standardized antifungal susceptibility testing methods for dermatophytes (Jessup *et al*. 2000, Ghannoum *et al*. 2004, Esteban *et al*. 2005, Fernandez – Torres *et al*. 2002, 2006, and Singh *et al*. 2007). The Clinical and Laboratory Standards Institute (CLSI) had approved the microdilution method (M38A2) as a reference technique for antifungal susceptibility testing of moulds and dermatophytes (CLSI 2008).

Broth-microdilution, disk-diffusion, and E-test methods are the most frequently techniques used for *in vitro* antifungal susceptibility of dermatophytes (Fernandez – Torres *et al*. 2003, Singh *et al*. 2007, and Nweze *et al*. 2010).

Although, the incidence of dermatophytoses among Egyptian populations was considerably high, studies devoted to the antifungal susceptibility testing of dermatophytes are rare (Girgis *et al*. 2006, Abdel Aal *et al* 2007, and Zaki *et al*. 2009).

The present study aimed at testing the susceptibility of some strains of dermatophytes isolated in Cairo, Egypt toward eight antifungal drugs by using broth-microdilution and disk-diffusion methods.

Materials and Methods

Dermatophytic strains

Nine strains of clinical origin were tested for their susceptibility toward some selected antifungal agents. These strains were identified by traditional and molecular methods (Zaki *et al*. 2009). They were deposited at Assuit University Mycological Center (AUMC), Assuit, Egypt. The test strains included: *Microsporum canis* AUMC 4328, *Trichophyton tonsurans* AUMC 4330, *T. rubrum* AUMC 4332, *T. rubrum* AUMC 9063, *T. mentagrophytes* AUMC 4333, *T. violaceum* AUMC 9064, *Arthroderma* sp. AUMC 9065, *Epidermophyton floccosum* AUMC 4329, and *E. floccosum* AUMC 5497.

Antifungal agents

The following drugs were obtained in powder form, Amphotericin B (Bristol Myers Squibb Woerden, The Netherlands), Itraconazole (Apex pharma., Egypt), Voriconazole (Pfizer,

Egypt), Fluconazole and Terbinafine (Novartis, Egypt), Ketoconazole (Ramedia, Egypt), Griseofulvin (Kahira pharm & Chem. Ind. Co., Egypt), and Caspofungin (Merck, Rahway, NJ, USA). A starting dose of 32 µg/ml for each of Amphotericin B, Itraconazole, Voriconazole, Ketoconazole and Terbinafine antifungals was prepared by weighing 3.2 mg powder and dissolving in 1 ml dimethyl sulfoxide (DMSO). In case of Griseofulvin and fluconazole, 6.4 mg powder were weighed and dissolved individually in 1 ml DMSO to get 64 µg/ml starting dose. Caspofungin was prepared at a higher dose where a total of 25.6 mg powder was dissolved in 1 ml sterile water for 256 µg/ml. All prepared stock solutions were maintained at -20^{o}C until needed. A working solution of each drug was prepared by making 1:10 dilution in DMSO or sterile water as appropriate.

For disk-diffusion method, four antifungal drugs were obtained as ready to use as disks, these were Amphotericin B (100 µg), Voriconazole (1 µg), Fluconazole (25 µg), and Ketoconazole (50 µg) (Biorad, USA). For the other four drugs a stock solution of each drug was prepared according to antifungal disks potency standard of Rosco Diagnostica Company (Neo-Sensitabs, Denmark) using DMSO or sterile water for Caspofungin, as follows: Griseofulvin, 0.8 mg/ml; Caspofungin 0.25 mg/ml, Terbinafine, 50 µg/ml, Itraconazole, 0.4 mg/ml. Taxo™ Blank paper disks (6 mm diameter) were loaded with 20 µl of the prepared stock solutions to obtain the desired drug concentration per disk (16 µg/disk for Griseofulvin, 5 µg/disk for Caspofungin, 1 µg/disk for Terbinafine and 8 µg/disk for Itraconazole). Disks were allowed to dry up at room temperature and then stored at 4°C in a refrigerator.

Medium

Broth microdilution method was performed in RPMI 1640 medium (Sigma-Aldrich, Mississauga, Ontario, Canada) with L-glutamine but without sodium bicarbonate and buffered with 0.165M morpholinepropanesulfonic acid (MOPS) (Sigma-Aldrich) at pH 7.0 (CLSI, 2008). The disk diffusion method was performed on Mueller-Hinton (MH) agar medium (Remel, KS) (Espinel-Ingroff *et al*. 2007).

Test procedure

Broth-microdilution method: The CLSI M38-A2 guidelines (CLSI, 2008) were followed. Fungal strains were grown on Sabouraud dextrose agar for 7-14 days at 35°C while the quality control strain (QC) *Candida albicans* NCPF 8154 was grown for 24h before testing. Each culture was gently swabbed with a cotton tip applicator to dislodge the conidia from the hyphal mat. The spore suspension was transferred to a sterile tube where the volume was adjusted to 5 ml with sterile normal saline and shaked well. Spores were counted with a hemocytometer and then diluted into RPMI 1640 broth medium to a concentration of 2x 10^3 CFU /ml. The QC strain cell suspension was prepared by taking a swab from 24 h culture into 5 ml of sterile distilled water and then adjusting the concentration using the 0.5 McFarland standard tubes to give a yeast suspension of 10^6 CFU/ml. A working suspension was then prepared in sterile distilled water to yield 10^3 CFU/ml. A sterile microdilution plate (96-flat bottomed wells) was used for each strain so that 100 µl of RPMI medium were added to each well then another 100 µl of the working solution of each antifungal drug were pipetted to a well in the 1st row. Two-fold serial dilutions were made using the multichannel pipette so that rows 1-10 contain series of drug dilutions in 100 µl volumes. The final concentrations of the antifungal agents were 0.06-64 mg/l for Griseofulvin and Fluconazole, 0.25–256 mg/l for Caspofungin and 0.03–32 mg/l for the rest of the drugs. Aliquots of fungal spore suspension (100 ml each) were then added to each well. The 11th row which served as the positive control contained 100 µl of inoculum suspension and 100 µl of drug free medium whereas the 12th row which served as negative control contained only 200 µl of RPMI broth. Microdilution trays were incubated at 35^{o}C. Minimum inhibitory concentrations (MICs) were determined after 4-7 days. MIC was defined as the concentration at which there was 100 % inhibition of fungal growth as compared with control when read visually in the microtitre plates.

Disk-diffusion method: Fungal cell suspensions were prepared as mentioned above and adjusted to a concentration of 10^6 CFU/ml. Sterile cotton swabs were loaded with the fungal inocula were individually streaked on the surface of MH agar plates in 4 different directions (at 90 degree angles) to cover the entire surface. Using a flamed sterilized forceps, disks loaded with the antifungal agents were applied separately onto the surface of the inoculated agar and pressed lightly to ensure complete contact with the culture which were then incubated at 35^{o}C for 4-7 days. The inhibition zone diameters (IZDs) were around disks measured to the nearest millimeter (Espinel-Ingroff *et al*. 2007).

Quality control: The quality control strain *Candida albicans* NCPF 8154 was included in each set of experiments for comparison.

Data analysis: MICs and IZDs breakpoints were analyzed according to the values presented in

Tables 1 and 2. The categorical agreement between the results of broth microdilution method as a reference method and the disk diffusion assay was calculated, within the same pattern of susceptibility. Errors were ranked as very major (false-susceptible result by disk diffusion), major (false-resistance by disk diffusion) and minor (intermediate by disk diffusion), resistant or susceptible in broth microdilution method) (Espinel-Ingroff *et al.* 2007).

Table 1: The minimum inhibitory (MIC) or minimum effective concentrations (MEC) breakpoints of tested antifungal agents.

Antifungal agent	MIC or MEC			Reference
	S	I	R	
Amphotericin B	≤ 1 µg/ml	2 µg/ml	≥ 4 µg/ml	Espinel-Ingroff *et al*. (2007)
Itraconazole	≤ 1 µg/ml	2 µg/ml	≥ 4 µg/ml	Espinel-Ingroff *et al*. (2007)
Voriconazole	≤ 1 µg/ml	2 µg/ml	≥ 4 µg/ml	Espinel-Ingroff *et al*. (2007)
Caspofungin	≤ 1 µg/ml	2 µg/ml	≥ 4 µg/ml	Espinel-Ingroff *et al*. (2007)
Ketoconazole	< 4 µg/ml	-	4-16 µg/ml	Ellis (2012)
Fluconazole	< 8 µg/ml	16-32 µg/ml	> 64 µg/ml	Ellis (2012)
Terbinafine	< 1 µg/ml	1 µg/ml	> 1 µg/ml	Ghannoum *et al.* (2011)
Griseofulvin	< 2 µg/ml	2 µg/ml	> 2 µg/ml	Fachin *et al.* (1996)

S=Susceptible; I=Intermediate; R=Resistant

Table 2: The inhibition zone diameters (IZDs) breakpoints of tested antifungal agents

Antifungal agent	IZDs			Reference
	S	I	R	
Amphotericin B	> 15 mm	13-14 mm	≤ 12 mm	Espinel-Ingroff *et al*. (2007)
Itraconazole	≥ 17 mm	14-16 mm	≤ 13 mm	Espinel-Ingroff *et al*. (2007)
Voriconazole	≥ 17 mm	14-16 mm	≤ 13 mm	Espinel-Ingroff *et al*. (2007)
Caspofungin	≥ 17 mm	14-16 mm	≤ 13 mm	Espinel-Ingroff *et al*. (2007)
Ketoconazole	≥ 30 mm	23-29 mm	≤ 22 mm	Pakshir *et al*. (2009)
Fluconazole	≥ 21 mm	15-22 mm	≤ 14 mm	Pakshir *et al*. (2009)
Terbinafine	≥ 20 mm	12-19 mm	≤ 11 mm	Pakshir *et al*. (2009)
Griseofulvin	≥ 10 mm	-	0 mm	Pakshir *et al*. (2009)

S=Susceptible; I=Intermediate; R=Resistant

Results

The minimum inhibitory concentrations (MICs) of the *in vitro* susceptibility testing of nine dermatophytic strains toward eight antifungal drugs are shown in Table 3. Results indicated that both Voriconazole and Ketoconazole showed antifungal activities against all tested strains except *Trichophyton mentagrophytes* AUMC 4333. The MICs of Voriconazole against susceptible strains ranged from 0.25–1 µg/ml while those of Ketoconazole ranged from 0.25-2 µg/ml. Terbinafine showed high activity against all tested strains except *Trichophyton violaceum*. The MIC for Terbinafine against *T. mentagrophytes* AUMC 4333, *T. tonsurans* AUMC 4330 and *T. rubrum* AUMC 4332 was 0.125 µg/ml which means that these fungal strains are susceptible to this antifungal agent. In case of *Microsporum canis* AUMC 4328, *Arthroderma* sp. AUMC 9065, *Trichophyton rubrum* AUMC 9063 and the two *Epidermophyton floccosum* strains the MIC of Terbinafine (1 µg/ml) was in the intermediate susceptibility range. Itraconazole, Griseofulvin and Caspofungin showed antifungal activities against seven strains but they were not active against *T. rubrum* AUMC 9063 and *T. violaceaum* AUMC 9064. Amphotericin B and Fluconazole exhibited a narrow spectrum of antifungal activity where Amphotericin B had activities against four strains at 0.5 µg/ml while, Fluconazole was active against two strains at 16 µg/ml.

As shown in Table (3), *Arthroderma* strain AUMC 9065 showed sensitivity toward all drugs used whereas the two strains of *Epidermophyton floccosum* and *T. rubrum* (AUMC 4332) were sensitive to seven drugs but resistant only to Fluconazole. Both *M. canis* and *T. tonsurans* showed sensitivity towards the tested antifungal agents except Amphotericin B and Fluconazole. *T. mentagrophytes* showed sensitivity to 4 drugs whereas, *T. rubrum* AUMC 9063 and *T. violaceum* were only sensitivite to Voriconazole, Ketoconazole and Terbinafine.

The inhibition zone diameters (IZDs) of the *in-vitro* susceptibility testing of the nine dermatophytic strains to eight antifungal drugs are shown in Table 4. The results indicated that Voriconazole and Ketoconazole exhibited high activities against all tested strains except *T. mentagrophytes* AUMC 4333. Itraconazole, Griseofulvin and Caspofungin showed almost high antifungal activities against seven strains but they were not effective against *T. rubrum* AUMC 9063 and *T. violaceum* AUMC 9064. Terbinafine exhibited high to intermediate activity against six strains but it displayed no visible activity towards *M. canis* AUMC 4328, *T. violaceum* AUMC 9064 and *Arthroderma* sp. AUMC 9065. Amphotericin B demonstrated activity against five fungal strains only, whereas Fluconazole (25 µg/disk) was not effective against all tested strains (Table 4).

T. rubrum AUMC 4332 and the two tested strains of *E. floccosum* showed high sensitivity to all antifungal drugs except Fluconazole. *T. tonsurans* and *Arthroderma* sp. came next showing sensitivity towards six drugs and resistance to two drugs. *M. canis* and *T. mentagrophytes* were susceptible to five and four drugs respectively. Both *T. rubrum* AUMC 9063 and *T. violaceum* AUMC 9064 responded to only three drugs but it displayed no visible activity towards *M. canis* AUMC 4328, *T. violaceum* AUMC 9064 and *Arthroderma* sp. AUMC 9065 (Table 4).

The MICs of *Candida albicans* NCPF 8154 (control strain) ranged from 0.06 to 16 µg/ml where the lowest concentration was observed with Itraconazole and Voriconazole and the highest with Griseofulvum. Considering the overall range of inhibition zone diameters (IZDs) exihibited by the different antifungal agents, the present data in Table 4 revealed that Voriconazole, followed by Ketokonazole and Caspofungin were the most effective against the tested dermatophytes showing IZDs ranging from 10-55 mm. Similarly, Griseofulvin, Itraconazole and Amphotericin-B exhibited IZDs ranging from 9-45 mm. The lowest range of IZD was observed with Terbinafine (13-21 mm) whereas Fluconazole displayed no visible inhibition as mentioned before.

In this study, a categorical agreement between the broth microdilution and disk diffusion methods was found for all antifungal agents tested. No major errors were detected, but minor errors were observed, ranging from 1 (11.1%) for Amphotericin B, to 2 (22.2%) for each of Fluconazole and Terbinafine. Minor errors shown between the results of the two methods for amphotericin B were due to the categorization of *T. violaceum* as resistant by the broth microdilution method and its categorization within the intermediate range by disk diffusion method. For fluconazole, *T. violaceum* and *Arthroderma* sp. were categorized as susceptible (dose- dependent) by broth microdilution method while they were within the resistance range by the disk diffusion method. The two minor errors detected for Terbinafine included the *M. canis* and *Arthroderma* sp. strain that were categorized as susceptible (dose-dependent by broth microdilution method) while they were within the resistance range by the disk diffusion method. The overall levels of agreement between the results of broth microdilution and disk diffusion methods came in the following sequence: 100% for each of Itraconazole, Voriconazole, Ketoconazole, Griseofulvin and Caspofungin, 89% for Amphotericin B and 78 % for Fluconazole, and Terbinafine.

Table 3: *In-vitro* susceptibility of dermatophytes strains toward 8 antifungal drugs using broth microdilution method.

Strain	MIC (µg/ml) for antifungal agent							
	AMB	ITC	VRC	FLC	KTC	TRB	GRS	CAS
M. canis AUMC 4328	16	0.5	0.5	64	0.5	1	1	1
T. tonsurans AUMC 4330	8	0.125	0.5	64	0.5	0.125	0.5	1
T. rubrum AUMC 4332	0.5	0.125	0.25	64	0.25	0.125	0.25	0.5
T. rubrum AUMC 9063	16	16	1	64	1	1	32	128
T. mentagrophytes AUMC 4333	8	0.25	8	64	8	0.125	0.5	2
T. violaceum AUMC 9064	4	16	1	16	2	8	16	128
Arthroderma sp. AUMC 9065	0.5	0.125	0.25	16	0.25	1	0.25	0.5
E. floccosum AUMC 4329	1	0.25	1	64	0.5	1	0.5	2
E. floccosum AUMC 5497	1	0.25	1	64	0.5	1	0.5	2
MIC range	0.5-16	0.125-16	0.25-8	16-64	0.25-8	0.125-8	0.25-32	0.5-128
Candida albicans NCPF 8154	0.5	0.06	0.06	0.125	16	0.5	16	0.5

MIC: Minimum Inhibitory Concentration; AMB, amphotericin B; ITC, itraconazole; VRC, voriconazole; FLC, fluconazole; KTC, ketoconazole; TRB, terbinafine; GRS, griseofulvin; CAS, caspofungin. AUMC, Assuit University Mycological Center. NCPF: National Center for Pathogenic Fungi, England.

Table 4: *In-vitro* susceptibility of dermatophytes strains toward 8 antifungal drugs using disk diffusion method.

Strain	Mean value of inhibition zone diameter, IZDs (mm)							
	AMB 100 µg	**ITC 8 µg**	**VRC 1 µg**	**FLC 25 µg**	**KTC 50 µg**	**TRB 1 µg**	**GRS 16 µg**	**CAS 5 µg**
M. canis AUMC 4328	0	17	22	0	36	0	21	30
T. tonsurans AUMC 4330	10	25	27	0	36	21	38	38
T. rubrum AUMC 4332	32	41	55	0	50	20	45	49
T. rubrum AUMC 9063	0	0	17	0	24	13	0	0
T. mentagrophytes AUMC 4333	9	21	0	0	15	20	31	18
T. violaceum AUMC 9064	14	12	25	0	40	0	0	10
Arthroderma sp. AUMC 9065	20	35	55	0	50	0	45	52
E. floccosum AUMC 4329	28	35	50	0	55	15	42	55
E. floccosum AUMC 5497	19	37	55	0	50	13	40	55
Range of inhibition zone in resported strains	0-32	0-41	0-55	0	15-55	0-21	0-45	0-55
Candida albicans NCPF 8154	16	25	35	35	40	0	0	23

IZD: Inhibition Zone Diameter; AMB, amphotericin B; ITC, itraconazole; VRC, voriconazole; FLC, fluconazole; KTC, ketoconazole; TRB, terbinafine; GRS, griseofulvin; CAS, caspofungin. AUMC, Assuit University Mycological Center. NCPF: National Center for Pathogenic Fungi, England.

Discussion

Dermatophyte infections are frequently recurrent or chronic and mandate long-term treatment with antifungal agents. However, poor compliance and emergence of antifungal drug resistance account for the increasing disease prevalence and rates of treatment failures. This makes it imperative to conduct in-vitro antifungal susceptibility testing for the commonly used and newly introduced drugs (Pfaller, 2000). Many antifungal agents have been recently introduced but their susceptibility patterns and imperative criteria are lacking (Espinel-Ingroff et al. 2007). In addition to treatment failure problems, selection of an appropriate antifungal drug is extremely necessary to evade liver and cardiac side effects as well as possible drug interactions following prolonged administration, as in the case of Terbinafine and azole-like compounds (Bao et al. 2012).

In this study, eight antifungal agents including Amphotericin B (a member of polyene antibiotcs), Itraconazole, Voriconazole, Fluconazole Itriazoles), Ketoconazole (imidazole), Terbinafine (allylamines), Caspofungin (echinocandin), and Griseofulvin were tested against nine dermatophytic strains of clinical origin using broth microdilution and disk diffusion methods.

The MIC and IZD results using the broth microdilution and disk diffusion methods support and extend the findings presented in previous reports of the in-vitro activity of Voriconazole, Ketoconazole, Itraconazole, and Griseofulvin (Perea et al. 2001, Fernandez-Torres et al. 2002, and Esteban et al. 2005).

The newly introduced echinocandin drug (Caspofungin) in the Egyptian market showed good activity against dermatophytic strains, similar to that obtained obtained by Bao et al. (2012).

In the present study, a range of MICs for Terbinafine (0.125-8 µg/ml) which was slightly higher than by those reported by Perea *et al.* (2001). *Arthroderma* sp. and *M. canis* were intermediately affected by Terbinafine in the broth microdilution, but they showed no inhibition zones upon using disk diffusion method. This error might be attributed to the use of Terbinafine at a low concentration of 1µg/disk instead of 30µg/disk used in the other studies.

Five of the 9 strains tested showed resistance to Amphotericin B and this is in agreement with the findings of Abdel Aal *et al.* (2007) whereas *T. rubrum* AUMC 4332, *Arthroderma* sp. AUMC 9065, and *E. floccosum* AUMC 4329 and AUMC 5497 were susceptible to this drug. *T. violaceum* demonstrated resistance in broth microdilution, but its response was within the intermediate range when disk diffusion method was used. This was regarded as a minor error, possibly because of the high Amphotericin B disk content in disk diffusion test (100 µg/disk).

Resistance of dermatophytic strains to Fluconazole was also reported previously by Pakshir *et al*. (2009).

In conclusion: the current results demonstrated that the broth-microdilution method employed for antifungal susceptibility of dermatophytes is preferred because it is simple, cheap and reliable. Moreover, it helps in quantitative MICs determination. It is also concluded that *in-vitro* testing showed that Voriconazole, Itraconazole, Ketoconazole and Caspofungin were the most active drugs against dermatophytes tested. It is also highly recommended to encourage dermatologists to cosult mycologists for isolation, identification of pathogenic fungi and performing *in-vitro* sensitivity tests to choose the most effective antifungal drugs needed for successful treatment of fungal infections.

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Production of bioethanol from sugarcane bagasse using recombinant *Saccharomyces cerevisiae* strain

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Abstract: An improvement in bioethanol production from glucose-xylose sugars has been achieved by a recombinant xylose fermenting *Saccharomyces cerevisiae* SK-NY. The recombinant strain was used to ferment sugars hydrolyzed from hemicellulose combined with enzymatically saccharified cellulose of sugarcane bagasse. The main components of bagasse were cellulose, hemicellulose, lignin and ash representing 37.3%, 30.5%, 30.2% and 1.99% of total weight respectively. Hemicellulose hydrolysis conditions were optimized by pretreatment of bagasse with 1% sulfuric acid at 135ºC for 25 min. Delignification was accomplished by three oxidation-bleaching cycles of treatment using sodium chlorite and acetic acid at 70 ºC for one hour per cycle. Liberation of integrated lignin takes place in the final oxidation cycle by the swelling action of sodium bicarbonate. Simultaneous saccharification of cellulignin and cellulose with cofermentation (SSCF) of sugars hydrolyzed from hemicellulose were compared using cellulase enzyme and recombinant *S. cerevisiae* SK.NY. Released glucose-xylose sugars were successfully fermented and the productivity of ethanol was raised from 13.7 g/l to 18.9 g/l from cellulignin and cellulose respectively. Enzymatic saccharification of cellulose showed 37% higher production of glucose compared with that produced from cellulignin. Consequently, the bioethanol production from sugar obtained by SSCF of cellulose increased to 28%.

Keywords: sugarcane bagasse, bioethanol, delignification, simultaneous saccharification and cofermentation (SSCF), recombinant *Saccharomyces cerevisiae.*

Introduction

Sugarcane (*Saccharum officinarum*) bagasse is one of the lignocellulosic materials that composed of up to 75% carbohydrates. Small amounts of pectin, extractives and ashes were also included in biomass composition. Bagasse has high tensile strength crystalline cellulose fibers, embedded in an amorphous matrix of cellulose, hemicellulose and lignin. Lignin acts as a physical barrier and lowers the biodegradability of both cellulose and hemicelluloses (Fox *et al.* 1987, Bidlack *et al.* 1992, Pandey *et al.* 2000, Jørgensen *et al.* 2007). High costs of conversion of cellulose and hemicellulose to fermentable sugars is a bottleneck to be applied on an industrial scale (Ragauskas *et al.* 2006). A number of different pretreatment strategies have been envisioned to convert biomass into fermentable sugars. These pretreatment technologies are being under developments using physical, chemical, biological or combinations of those methods. Multiple steps of pretreatment are needed to overcome the hardness of bagasse e.g. hydrolysis of hemicelluloses and/or removing lignin to increase surface area and decrease the crystallinity of cellulose in order to improve enzyme saccharification and prevents the formation of byproducts that can inhibit the fermentation process. Pretreatment also has great potential for improving the efficiency and lowering the costs of fermentation (Lee *et al.* 1994, Lynd 1996). Researchers are going on to reduce the costs of pretreatments via mechanical pulverization, pyrolysis as well as addition of acid, alkali, hydrogen peroxide, ammonia fiber explosion, wet-oxidation, lime, CO2 explosion and organic solvent treatment. Each of these pretreatment methods has its distinct advantages and disadvantages (Martín *et al.* 2002, Ramos and Fontana 1996).

On the other hand, finding the robust microorganism strains that have the ability to ferment hydrolyzed sugars efficiently was the other obstacle for production of bioethanol from biomass. Intensive studies were done to modify the microorganisms genetically in order to efficiently ferment the hydrolyzed sugars of biomass to bioethanol. Many investigations have indicated that native *Saccharomyces cerevisiae* ferment significant amounts of glucose in adverse conditions and tolerate the action of inhibitors present in the medium, but it cannot utilize xylose, which is the second most abundant sugar in biomass after glucose. *S. cerevisiae* acquired the ability of efficiently ferment xylose via some genetic strategies. One of them was recycling cofactors NADPH/NAP^+ between *Pichia stipitis* xylose reductase (PsXR) and xylitol dehydrogenase (PsXDH) genes via genetic and protein engineering (Khattab *et al.* 2011 a & b). Chromosomal integration of overexpressed endogenous *S. cerevisiae*

xylulokinase ScXK with PsXR and PsXDH was also done to boost the production of bioethnol (Khattab *et al.* 2013). Furthermore, the production of bioethanol from glucose and xylose mixture was improved by recombinant *S. cerevisiae* SK-NY in which NADPH-dependent aldose reductase (GRE3) and ScXK of *S. cerevisiae* genes combined with mutated $NADP^+$-dependent PsXDH gene were cloned in overexpression state to chromosomal DNA (Khattab and Kodaki 2014).

In this study, a combination of diluted acid hydrolysis and oxidation bleaching pretreatments prior to simultaneous saccharfication and cofermentation (SSCF) of hydrolzed bagasse by a recombinant *S. cerevisiae* SK-NY was carried out for enhancing production of bioethanol.

Material and Methods

Sugarcane bagasse and analytical methods

Sugarcane bagasse used in this study was obtained from a local squeezer in Damietta Governorate, Egypt, during April 2013. The bagasse was dried under sunlight, pulverized in domestic machine (0.1 to 25 mm long) and kept in a plastic bag in dry place until use. Moisture content, cellulose, hemicellulose, lignin and ash of bagasse were analyzed according to the National Renewable Energy Laboratory (NREL) standard procedure (Ehrman 1994 a & b, Ehrman 1996, Sluiter 2008). Glucose and xylose of liquid hydrolyzates were analyzed and determined using HPLC (JASCO, Tokyo) equipped with an Aminex HPX-87H column (Bio-Rad Laboratories, Hercules, CA, USA).

Hydrolysis of hemicellulose

Different sulfuric acid concentrations 0.5, 1~2.5 % combined with three different temperatures 110º, 121º and 135ºC for 25 minutes were used to determine the best conditions for hydrolysis of hemicellulose. Hydrolysis was achieved using 1/10 W/V (bagasse/sulfuric acid) in TOMY LSX 500 high-pressure steam sterilizer. Optimal conditions of hydrolysis were determined based on monitoring the maximum sugars produced using HPLC as described by Khattab *et al.* (2013). Liquid hydrolyzates of hemicellulose were collected by filtration, using glass microfiber filter GF/A 110 mm diameter. The percent of digestibility was calculated as following:

Digestibility % = [Total sugar(s) concentration g/l × volume] / [biomass weight (g)] × 100

Neutralization process

Neutralizing liquid hydrolyzates of first treatment during acid hydrolysis was achieved by calcium hydroxide (powder- Wako Company) added gradually to the hydrolyzed hemicellulose liquid fraction- A (Fig.1) in order to react with excess H_2SO_4 and produce calcium sulphate (Ranatunga *et al.* 2000; Mohagheghi *et al.* 2006). Calcium sulfate (gypsum) was precipitated by overliming with keeping temperature at 40ºC for 30 min using METTLER TOLEDO pH meter and IKA® C-MAG HS4 magnetic stirrer. After gypsum (calcium sulfate) had been precipitated, it was separated by filtration using vacuum pressure ULVAC MDA-006 and glass microfiber filter GF/A 110mm diameter. Neutralized liquid fraction (Fraction A) was kept at 4ºC for further use during SSCF.

Delignification Process

Oxidation bleaching using sodium chlorite acidified by acetic acid was used in delignification of cellulignin (Residual part; RP after dilute sulfuric acid pretreatment) according to the modified methods of Hubbell *et al.* (2010) and Kahar (2013). Cellulignin was suspended in 1/30 W/V of 0.2 M acetate buffer (pH 3.0) and three oxidation/bleaching cycles using gentle swirling in glacial acetic acid (0.04 ml/g RP) and sodium chlorite (0.2 g/g RP) per cycle/h at 70ºC. Sodium bicarbonate (0.1g/g RP) was added in the final cycle in order to swell cellulose fiber and liberate integrated lignin. Cellulose fiber was self-separated from hydrolyzed lignin fraction by repeated filtration cycles via porcelain Büchner funnel without filter paper, then washed by 10 % acetone solution and dried.

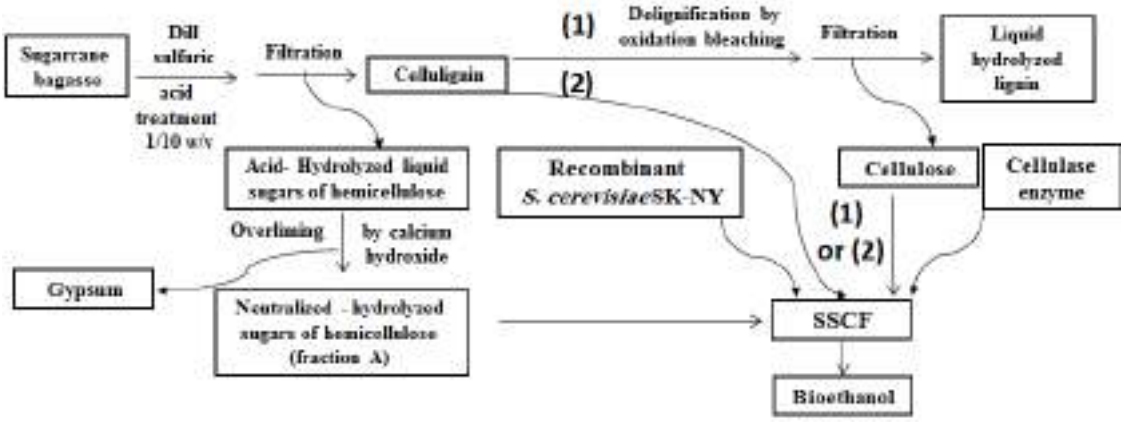

Figure 1: Schematic representation of sugarcane bagasse pretreatments prior to simultaneous saccharfication and cofermentation (SSCF) of cellulignin or cellulose by a recombinant *S. cerevisiae* SK-NY for production of bioethanol.

Simultaneous saccharification and cofermentation of pretreated sugarcane biomass

Cellulignin and cellulose powders (Fig. 1) were individually suspended in fraction A (1/20 W/V) buffered in 50 mM of citrate. Two doses of cellulose enzyme (168 and 350 EGU) obtained from Sigma-Aldrish, novozyme (*Trichoderma reesei* ATCC 26921 which contained ≥700 units/gram) were tested during simultaneous saccharification (SS) and SSCF, where one unit of enzyme liberates one µmole of glucose from carboxymethyl cellulose (CMC) in one hour at pH 5.0 and 37 ºC. Additionally, one mg of Almond β-glucosidase enzyme (11 CBU/mg protein) was added to each 84 U of cellulase enzyme SSCF.

Recombinant *S. cerevisiae* SK-NY (Khattab and Kodaki 2014), which has the ability to ferment glucose/xylose mixtures, was cultivated in liquid yeast peptone dextrose (YPD) medium with 150 rpm of shacking for 24 h at 30 ºC. Cell pellets were collected by centrifugation at 3000g for 4 min then washed twice by YP medium prior to SSCF process (Fig. 1). The reaction of 100 ml was performed in 300 ml Erlenmeyer flasks and incubated at 30 ºC under continuous agitation (150 rpm). The initial cell density in the fermentation culture was approximately adjusted to an optical density of 1.0 before addition to slurry mixture. All growth rates were monitored using spectrophotometer (DEN-1B McFarland Densitometer). For SSCF, concentration of sugars and ethanol were measured at 0, 3, 6, 12, 24, 48, 72, 96, 120, 144, 168, 192h. In case of SS, replicated flasks were prepared without *S. cerevisae* SK-NY in order to determine sugar production rate during saccharification. Samples were centrifuged and then filtered via 0.45 µm millipore filter prior to high performance liquid chromatography (HPLC) analysis. Concentrations of ethanol, glucose and xylose were determined using HPLC as described in a previous work (Khattab *et al.* 2013). To assess the effects of removing lignin, comparative studies of SSCF using cellulose and cellulignin were achieved (Fig. 1).

Results and Discussion

Sugarcane bagasse analysis

The main components of sugarcane bagasse analyzed in this study included cellulose, hemicellulose, lignin and ash. These substances represented respectively 37.3, 30.5, 30.2 and 1.98 % of dry mass (Table 1). Reports from Brazil showed that these components accounted for 38.8 % to 45.5% in case of cellulose; 25.2 % - 27.0 % for hemicelluloses, 19.1 % - 32.4 % for lignin and 1.0 % - 2.8 % for ash (Table 1).

According to Hatfield and Fukushima (2005), variations in the chemical composition of sugarcane bagasse could be attributed to several factors including sugarcane species, the hybrids, environmental conditions, harvesting system, crop age and the analytical methods employed.

Effect of pretreatment of bagasse by dilute sulfuric acid

Pretreatments are the most expensive steps of converting biomass to fermentable sugars and then to ethanol due to complexity of biomass, crystallinity of cellulose and degree of polymerization. Furthermore, lignin that covers and bind cellulose-hemicellulose network lowers the process of the hydrolysis (Binod *et al.* 2012). Hydrolysis of bagasse's hemicellulose was achieved by sulfuric acid. The effects of sulfuric acid concentrations 0.5~ 2.5 % at three different temperatures were investigated (Table 2). Results showed that the total amount of released sugars increased with increasing acid concentrations to 1% and temperatures 135 ºC, then the amount of sugars declined by the action of excess acid. Autoclaving 1/10 w/v bagasse in 1% sulfuric acid at 135 ºC for 25 min was found to be the best conditions to achieve maximum sugars concentrations (Table 2). Data also show that xylose was the main constituent of hemicellulose-hydrolyzed sugars (18.55 g/l) matching 15.16 % of total weight of bagasse. Decrease in glucose and xylose concentrations was accompanied with excess acid concentration and/or rise in temperature during the first pretreatment certainly by decomposition of xylose and glucose to furfural and hydroxy methyl furfural (Rodríguez-Chong *et al.* 2004). A maximum concentration of glucose produced from hemicellulose hydrolysis in the first pretreatment was 8.11 g/l (Table 2).

Table 1: Chemical composition (% w/w, dry basis) of sugarcane bagasse compared with other references

Component (%)	Brienzo *et al.* (2009)	da Silva *et al.* (2010)	Canilha *et al.* (2011)	Rabbelo *et al.* (2011)	Rocha *et al.* (2011)	Present study*
Cellulose	42.4	38.8	45.0	38.4	45.5	37.3
Hemicellulose	25.2	26.0	25.8	23.2	27.0	30.5
Lignin	19.6	32.4	19.1	25.1	21.1	30.2
Ash	1.6	2.8	1.0	1.5	2.20	1.98

* The values are the average of three independent experiments.

Table 2: Effect of pretreatment of bagasse by different concentrations of sulfuric acid at three different temperatures for 25 minutes of treatments on glucose and xylose release.

Sulfuric acid concentration (%)	Temperature (ºC)	Glucose (g/l)	Xylose (g/l)
0.5	110	6.31±0.31	13.55±0.32
	121	7.11±0.12	15.55±0.37
	135	7.71±0.26	15.85±0.11
1	110	7.51±0.08	16.85±0.18
	121	7.81±0.32	17.04±0.20
	135	8.11±0.22	18.55±0.35
1.5	110	7.01±0.14	16.55±0.08
	121	7.45±0.42	17.65±0.16
	135	7.67±0.36	17.11±0.19
2	110	7.78±0.31	17.32±0.24
	121	7.89±0.12	16.75±0.29
	135	7.98±0.20	14.45±0.31
2.5	110	6.23±0.19	16.66±0.33
	121	6.11±0.21	14.36±0.25
	135	5.70±0.22	13.33±0.27

The values of glucose and xylose concentrations are average ± SD, n=3.

Numerous attempts have been done to investigate effective methods to alleviate harmful effect of hydrolyzates (furfural and hydroxymethylfurfural) in order to improve fermentation efficiency (Gong *et al.* 1993, Rodríguez-Chong *et al.* 2004). Direct neutralization was found to be somewhat effective in removing sulfate anions, although sulfate toxicity is considerably less than that of acetic acid (Ranatunga *et al.* 2000). Overliming was reported as an effective method of reducing toxicity of various hydrolyzates (Ranatunga *et al.* 2000; Mohagheghi *et al.* 2006). As a consequent of overliming by calcium hydroxide for 30 minutes at 40ºC and pH 6, calcium sulfate (Gypsum) precipitated and completely

hydrolyzed sugars were successfully fermented to ethanol. Diluted sulfuric acid pretreatments effectively hydrolyzed hemicelluloses, nonetheless only 0.85% (w/w) of lignin was removed from the bagasse. Furthermore, cellulose has been well cited as being more resistant to attack by diluted acids than hemicelluloses and needs more pretreatment processes (Lee *et al.* 1994; Mussatto and Roberto 2006).

Acid oxidation for bleaching lignin

Lignin represents a physical barrier to accessible cellulose chains by cellulases enzymes. It adsorbs cellulose hydrolyzing enzymes. In addition, depolymerized lignin acts as an inhibitor for saccharification process (Converse *et al.* 1990). For this reason, a second pretreatment was proposed to hydrolyze and separate lignin from cellulignin using thermo-chemical acid oxidation-bleaching processes before enzymatic saccharification. Treatment of cellulignin by sodium bicarbonate changes the properties of cellulose surface by swelling cellulose microfibers and cellulose matrix for further removal of the integrated lignin (Kahar 2013). Bleaching by acidified sodium chlorite after hydrolysis of hemicellulose prevents degradation of glucan or xylan where chlorite is a strong oxidant and acts selectively on lignin (Ahlgren and Goring, 1971). These double pretreatments of diluted acid hydrolysis and delignification increased pure cellulose content and decreased lignin content (Yasuda *et al.* 2013).

Simultaneous saccharification and cofermentation

Residual cellulose and cellulignin powder were individually saccharified by cellulase enzyme in liquid hydrolyzates of hemicellulose (fraction A) and simultaneously fermented the released sugars to bioethanol using recombinant *S.cerevisiae* SK-NY (Fig. 1). Seventy-four grams of cellulose were obtained from double pretreated 200g crude bagasse. At saccharification dose of 0.18g cellulase enzyme/ g cellulose, a maximum released glucose was reached 22.05 g/l (data not showed in figures). Simultaneous fermentation of these released sugars produced 15.12 g/l ethanol (Table 3). By increasing saccharification enzyme dose to 0.39 g cellulase / g cellulose, a maximum released glucose was increased to 29.46 g/l (Fig. 3) and simultaneous fermentation the released sugars produced 18.9 g/l ethanol represented 0.151 g ethanol / g of bagasse (Fig. 2 & Table 3). Surprisingly, there were no improvements in SS or SSCF at higher enzymes loading than 0.39g cellulase/g of cellulose. Furthermore, there were no improvements in SS or SSCF by addition of β-glucosidase enzyme with 0.39g cellulase / g cellulose, where the accumulated cellobiose was depleted after 72 h of saccharification (data not shown in figures), where cellulase cocktail hydrolyzed cellulose to cellobiose and then to glucose through the combined action of endo-exoglucanases and β-glucosidase enzymes.

The effects of delignification from cellulignin were also evaluated during SS and SSCF at saccharification dose 0.39 g cellulase enzyme/ g cellulignin and cellulose. A maximum released glucose from cellulignin was reached (18.75 g/l) while that from cellulose was reached (29.46 g/l) (Fig. 3). Simultaneous fermentation of these released sugars produced 13.7 g/l and 18.9 g/l ethanol respectively. The potential effects of delignification process were clarified by 37% improvement in saccharification and 28% higher ethanol production respectively; nonetheless, 41 % of cellulose was rested, for unknown reason, without hydrolysis to fermentable sugars (Figure 3). Tan *et al.* (2013) recovered 92.3% of theoretical glucose by acid–alkali combination (1% H_2SO_4, 150º C, 10 min, followed by 1% NaOH, 80ºC and 60 min) and enzymatic saccharification. Fermentation efficiency for released glucose and xylose from sugarcane bagasse by recombinant *S. cerevisiae* SK-NY reached 77 % of theoretical released sugars. Khattab and Kodaki (2014) reported its fermentation efficiency to mixture of glucose-xylose sugars by 85.7% of the theoretical sugars. This is the first study using recombinant *S. cerevisiae* SK-NY to ferment hydrolyzed sugars from bagasse or any kind of biomass. Furthermore, the positive effects of delignification on both saccharification and fermentation process were an emphasized by this study where the total amount of released glucose increased from 18.75 g/l to 29.46 g/l and bioethanol production increased from 13.7 g/l to 18.9 g/l from cellulignin and cellulose respectively. Effectiveness of removing lignin by alkali-pretreatment prior to simultaneous saccharification of glucan and fermentation by native *S. cerevisiae* was also reported by Yasuda *et al.* (2013); nevertheless there was a lower production of bioethanol than the results reported in this study, probably due to fermentation of xylose by recombinant *S. cerevisiae* SK-NY.

Table 3: Simultaneous saccharification and cofermentation of cellulignin and cellulose suspended in hydrolyzed hemicellulose sugars of sugarcane bagasse using cellulase enzyme and recombinant *Saccharomyces cerevisiae* SK-NY.

Enzyme concentration (g / g Biomass)	Cellulase 0.18 g / cellulose			Cellulase 0.39 g / cellulignin			Cellulase 0.39 g / cellulignin		
Time (h)	Glucose	Xylose	Ethanol	Glucose	Xylose	Ethanol	Glucose	Xylose	Ethanol
0	8.11±0.31	18.55±0.31	0.00±0.00	8.11±0.31	18.55±0.31	0.0±0.0	8.11±0.31	18.55±0.31	0.00±0.00
3	1.21±0.11	17.87±0.21	4.30±0.10	1.22±0.25	17.77±0.23	4.14±0.20	1.40±0.21	17.56±0.21	4.76±0.10
6	0.31±0.06	16.91±0.11	6.97±0.22	0.21±0.31	16.75±0.24	5.98±0.31	0.52±0.11	16.72±0.19	6.89±0.20
12	0.21±0.01	15.21±0.21	7.00±0.24	0.19±0.01	15.62±0.40	6.84±0.24	0.44±0.02	15.35±0.20	8.24±0.21
24	0.21±0.01	12.78±0.31	8.88±0.34	0.17±0.03	12.45±0.34	7.21±0.41	0.32±0.02	12.25±0.27	10.21±0.21
48	0.15±0.01	9.25±0.33	10.01±0.10	0.15±0.02	10.1±0.28	8.05±0.31	0.25±0.01	9.78±0.21	12.01±0.29
72	0.14±0.01	7.82±0.24	11.20±0.21	0.13±0.01	7.78±0.15	8.99±0.39	0.20±0.01	6.89±0.18	14.22±0.30
96	0.12±0.01	4.35±0.28	12.31±0.09	0.11±0.02	4.74±0.25	9.79±0.44	0.16±0.01	4.68±0.15	15.01±0.18
120	0.10±0.01	2.64±0.16	13.46±0.22	0.08±0.0l	2.56±0.30	10.88±0.26	0.10±0.0l	2.79±0.10	16.20±0.22
144	0.09±0.01	1.46±0.17	14.52±0.20	0.06±0.01	1.85±0.26	12.03±0.21	0.08±0.00	1.81±0.05	17.18±0.19
168	0.07±0.01	0.88±0.11	14.75±0.24	0.05±0.01	0.83±0.28	13.57±0.23	0.04±0.00	0.98±0.01	18.09±0.20
196	0.07±0.01	0.42±0.02	15.12±0.31	0.04±0.01	0.30±0.18	13.70±0.33	0.03±0.00	0.32±0.01	18.90±0.23

The values of glucose and xylose and ethanol concentrations are average ± SD, n=3.

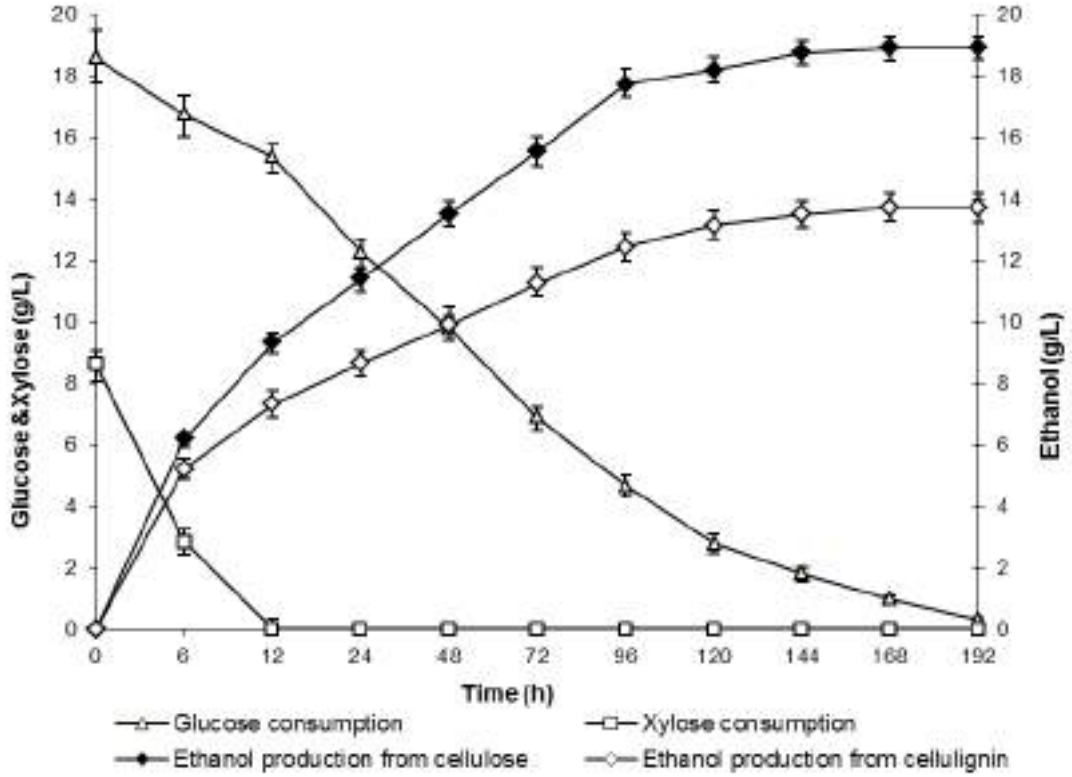

Figure 2: Simultaneous saccharification of cellulignin and cellulose suspended in liquid hydrolyzed hemicellulose (Fraction A) of sugarcane bagasse using 0.39g cellulase/ g biomass and fermentation of released sugars by recombinant *S. cerevisiae* SK-NY.
Values are average ± SD, n=3.
Fermentation efficiency % = [(Ethanol produced g/l/ total consumed sugars g/l) * 0.51] * 100

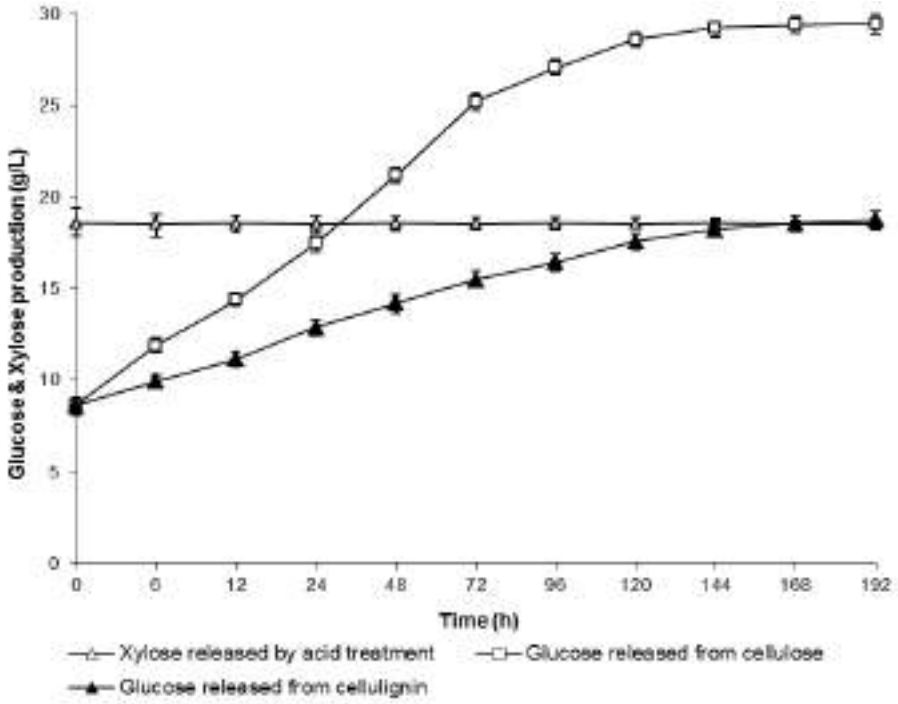

Figure 3: Total released glucose and xylose during saccharification of cellulignin and cellulose suspended in liquid hydrolyzed hemicellulose (Fraction A) of sugarcane bagasse using 0.39g cellulase/ g biomass.
Values are average ± SD, n=3.

Conclusion: The main components of Egyptian sugarcane bagasse were matched with other ratios in the literatures where cellulose, hemicellulose, Klason lignin and ash. Xylose was the dominant sugar in the hemicelluloses fraction and the second most abundant sugar after glucose in the total hydrolyzates, which represented 15.16 % of total weight. Double pretreatments were integrated to overcome the complexity structures of bagasse prior to SSCF. First, hemicellulose was thermo-chemically hydrolyzed using 1% sulfuric acid at 135 °C for 25 min. Second, lignin was bleached by acid oxidation. The potential effect of delignification process was clarified by 37% and 28% improvements in released glucose and produced ethanol respectively compared with those of cellulignin. Bioethanol production during SSCF was increased from 0.110 to 0.151 g ethanol / g of bagasse from cellulignin and cellulose respectively. Fermentation efficiency of saccharified glucose and xylose sugars using recombinant *S. cerevisiae* SK-NY reached 77 % of released sugars during SSCF of acid pretreated and delignified sugarcane bagasse.

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Osmophilic/osmotolerant and halophilic/halotolerant mycobiota of soil of Wadi El-Natrun region, Egypt

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Abstract: Ninety- six samples of newly reclaimed soil were collected from around the main 8 lakes of Wadi El-Natrun region. Chemical analysis of these samples revealed that they are alkaline with pH 8.2–9.05, organic matter content from 0.3 to 2.0% and total soluble salts from 1.7 - 6.3%. Soil collected from Al Gaar Lake showed the highest levels of total soluble salts, potassium, sodium, calcium, magnesium carbonate, bicarbonate, and chlorides. On the other hand, some parameters showed their peaks in other lakes, such as sodium (11.1 mg/g) in Umm Risha, calcium (0.2 mg/g) in Hamra and magnesium (0.04 mg/g) in AL Beida. A total number of genera (31) and species and varieties (84+3) were recorded from soil samples collected from all lakes during the study on the three isolation media used, with the widest spectrum of species being isolated on Czapek´s medium (60+2) and the narrowest on 10% NaCl medium (41+1), whereas 40% sucrose medium was intermediate (49+2). *Aspergillus* (17+2 species varieties), *Fusarium* (14), *Myrothecium* (3), *Stachybotrys* (2), *Penicillium* (8) and *Emericella* (4) were regularly the most dominant genera possessing the highest proportions of propagules on the three isolation media. *Aspergillus niveus*, *Chaetomium globosum*, *C. olivaceum*, *C. monocereus*, *Fusarium poae*, *Scopulariopsis* (4 species), *Scytalidium lignicola* and *Thermoascus aurantiacus* were isolated only on 10% NaCl medium. *Aspergillus* showed its count peak in Khadra Lake on the control medium, in Fasida on 40% sucrose and 10% NaCl medium. *A. flavus*, *A. fumigatus*, *A. niger*, *A. ochraceus*, *A. sydowii* and *A. terreus*, in addition to *P. chrysogenum* and *F. solani* were common on all isolation media. *Emericella* and *Eurotium* were the most common genera on 40% sucrose. The data of the present study showed that the majority of fungal species recovered from soil around the 8 lakes of Wadi El-Natrun are osmotolerant/osmophilic and halotolerant/halophilic.

Key words: Alkaline soil around 8 lakes, Wadi El-Natrun, osmotolerant/osmophilic and halotolerant/halophilic fungi.

Introduction

Saline lakes are confined to dry regions of the world where evaporation exceeds precipitation. Wadi El- Natrun is considered among the important depressions in the Western Desert (El Ghani *et al.* 2014). Zahran and Willis (2009) reported the presence of 8 principal lakes for a distance of about 30 km; from south to north: Fasida, Umm Risha, Rosetta, Abu Gubara, Hamra, El Zugm, Al Beida, Khadra and Al Gaar, noting that Abu Gubara and Hamra form one lake in the summer. Wadi El-Natrun lakes are the native source of natron salt, which has been used in mummification techniques of ancient Egyptians.

Microbial mats commonly line the margin of saline lakes of Wadi El-Natrun (Taher 1999). Most microbial diversity studies in hypersaline environments (salterns) have focused on halophilic Archaea bacteria of the order Halobacteriales (Oren 2002). Other organisms such as algae, protozoa, eubacteria and even fungi are also found in the salterns (Cantrell *et al*. 2006).

Halophiles are salt-loving organisms that inhabit hypersaline environments. They can be loosely classified according to their salt requirement into 3 categories: slight halophiles, grow optimally at 0.2–0.85 mol/l (2–5%) NaCl, moderate halophiles grow optimally at 0.85–3.4 mol/l (5–20%) NaCl and extreme halophiles grow optimally above 3.4–5.1 mol/l (20–30%) NaCl (DasSarma and Arora 2001) . Halotolerant organisms can grow both in high salinity and in the absence of a high concentration of salts. *Gymnascella marismortui* was the first halophilic fungus to be isolated from the Dead Sea (Buchalo *et al.* 1999). The black yeasts and closely related dematiaceous *Cladosporium* were among the first halophilic fungi to have been isolated from salterns in Secovlje, Slovenia-Adriatic (Butinar *et al.* 2005). The isolation of other filamentous fungi from salterns (Cantrell *et al.* 2006), as well as from coastal environments of Arctics (Gunde-Cimerman *et al*. 2005) has followed.

Halophilic mycobiota were isolated from Mandovi estuary and dominated by *Aspergillus* and *Penicillium* species. *Aspergillus penicillioides* was isolated as obligate halophile.

All the isolates of *Penicillium, Cladosporium* and *Eurotium* were facultative halophiles (Gonsalves *et al.* 2012). Numerous studies have been made on species composition of fungi in saline soils of different regions and on their ability to grow on media with high salt concentration, which reported many halotolerant/halophilic species of the genera, namely *Aspergillus, Penicillium, Cladosporium, Fusarium, Geotrichum, Rhizopus, Mucor, Phoma* and *Trichoderma* (Ruiz-Suárez 2004, Steiman *et al.* 2004, Gunde-Cimerman *et al*. 2005 and Gashgari and Al- Hzmi 2006). The mycobiota of the saline Arubotaim Cave (on the southwestern shore of Dead Sea) contained a total of 68 species of 28 genera, where *Aspergillus* (17 species), *Penicillium* (9) and *Chaetomium* (8) were the most prevailing genera (Grishkan *et al.* 2003).

Osmophiles are microorganisms adapted to environments with high osmotic pressures, such as high sugar concentrations. Osmophiles are similar to halophiles because a critical aspect of both types of environment is their low water activity. High sugar concentrations represent a growth-limiting factor for many microorganisms, yet osmophiles protect themselves against this high osmotic pressure by the synthesis of osmoprotectants such as alcohols and amino acids (Fadl-Allah and Sayed 1991). Hamada and Yamada (1991) recorded *Wallemia sebi*, *Aspergillus restrictus* and *Eurotium* spp. as the most abundant osmophilic mycobiota of house dust.

Soil fungi in Egypt are generally osmophilic or osmotolerant (recovered on up to 50% sucrose agar), halophilic or halotolerant (recovered on 5% or more sodium chloride agar) (Moubasher *et al.* 1990). They may increase the concentration of sodium and chloride ions, and accumulate proline as an osmoregulator (Fadl-Allah and Sayed 1991).

The objective of this work is to highlight on the osmophilic/osmotolerant and halophilic/halotolerant fungi that may be found in the newly reclaimed soil around Wadi El-Natrun lakes.

Materials and Methods

Collection of soil samples

Samples of reclaimed soil, were collected during January 2006 – May 2007, from around the eight main lakes (Fasida, Umm-Risha, Rosetta, Hamra, El Zugm, Al Beida, Khadra, Al Gaar) of Wadi El-Natrun depression, Egypt. Ninety- six samples (12 from each lake) were collected randomly and put directly into clean plastic bags and brought into the laboratory and kept in a cold place (5°C) till chemical and fungal analysis.

Chemical analysis of soil samples

pH value: A pH meter (Orior Research Model GOHL Digital Ionalyzer) was used for the determination of soil pH. The electrode was immersed directly in the soil suspension with a ratio 1:5 (w /v) (Jackson 1958).

Organic matter content (OM%): A semi-quantitative method was used for the determination of organic matter involves the heated destruction of all organic matter in the soil (Astem 2000). OM% is calculated as the difference between the initial and final sample weights divided by the initial sample weight times 100%.

Total soluble salts (TSS): The specific electrical conductance was measured in the soil extract using the conductance meter (YSI, model 35). The percentage TSS in the samples were estimated using this equation: % TSS in the dry sample = 0.064 × EC × extract ratio. The conversion factor to percentage salts (0.064) fairly applied for solutions extracted from the soil (Jackson 1958).

Sodium and potassium (Na^+ & K^+): Flame photometer method (Williams and Twin 1960) using Carl Zeiss flame photometer was used for the determination of Na^+ and K^+ cations.

Carbonate and bicarbonate: Total carbonate and bicarbonate was determined directly in the soil through hydrochloric acid digestion according to the method described by Jackson (1958).

Calcium and magnesium (Ca^{+2} & Mg^{+2}): The versene (disodium dihydrogen ethylene diamine tetraacetic acid) titration method as recommended by Schwarzenbach and Biedermann (1948) was employed for Ca^{+2} and $Ca^{+2} + Mg^{+2}$ determination.

Chloride (Cl^-): Soluble chloride was estimated by applying the silver nitrate titration method using potassium chromate as an indicator (Jackson 1958).

Isolation of fungi from soil samples

The dilution- plate method was used for enumeration of different fungal species as described by Johnson and Curl (1972). One ml of the desired dilution was transferred aseptically into each of 5 Petri-dishes and ~20 ml / plate of one of the three media used cooled to just above solidifying temperature were poured. The dishes are rotated by hand in a broad swirling motion, so that the dilution soil is dispersed in the agar. After agar solidification, the plates were incubated at 28ºC for 3-4 weeks

during which the developing fungi were counted and isolated for further identification and the number of colony forming units (CFUs) was calculated per g soil sample.

The three types of media used for isolation of fungi were: 1) Modified Czapek's agar in which glucose (10 g/l) replaced sucrose, of the following composition (g/l) sodium nitrate 3.0, potassium dihydrogen phosphate 1.0, magnesium sulphate 0.5, potassium chloride 0.5, ferrous sulphate 0.01, glucose 10.0, agar 15.0), to which rose bengal (1/15000) and chloramphenicol (25 µg/ml) were added as bacteriostatic agents. This medium was used as a control medium; 2) Czapek's agar supplemented with 40% sucrose for isolation of osmophilic and osmotolerant fungi and 3) Czapek's agar medium supplemented with 10% sodium chloride was used for isolation of halophilic and halotolerant fungi.

Identification of fungi

The identification of fungal genera and species was based on macroscopic and microscopic features following the keys and descriptions of Ellis (1971), for Dematiaceous Hyphomycetes; Pitt (1979), for *Penicillium* and its teleomorphic states; Raper and Fennell (1965), for *Aspergillus* species; Leslie and Summerell (2006) and Ismail *et al.* (2015), for *Fusarium* species; Moubasher (1993) and Domsch *et al.* (2007), for fungi in general.

Statistical analysis

Hierarchical clustering analysis using free online software statistical analysis (www.wessa.nit.com) was.

Results and Discussion

Chemical analysis of soil samples

Chemical analysis of soil samples collected during January 2006 – May 2007 from around the 8 main lakes of Wadi El-Natrun showed that pH was alkaline ranging from 8.2 (in Khadra) to 9.05 (in Al Gaar) (Table 1). This is in agreement with the previous results obtained by Taher (1999) and Moussa *et al.* (2009) in Wadi El-Natrun Lakes (pH 8.51-9.45) and by Steiman *et al.* (2004) of Mono Lake of California (pH 9.4-9.8). El Ghani *et al.* (2014) reported lower levels of pH in soil around Wadi El- Natrun Lakes (7.65-7.75), but Grant *et al.* (2004) found that soil samples with soda crust collected from Umm Risha, Al Beida and Khadra Lakes had higher pH range (10-10.3). Organic matter (OM%) ranged from 0.3 in Al-Gaar to 2% in El Zugm Lake. These results showed almost higher values in organic matter content compared with the results obtained from different types of soil in Egypt and in some other Arab countries (Moubasher *et al.* 1990, El-Said and Saleem 2008). Total soluble salts (TSS%) was much lower in soil than in water (Moubasher *et al.* 2013), ranging from 1.7 (in Hamra) to 6.3% (in El-Zugm). In the study of Taher (1999) the concentration of TSS ranged from 2.83 g/l in Khadra to 557 g/l in Al- Beida Lake. The Dead Sea (Steiman *et al.* 1997) and the Great Salt Lake (Utah Geological Survey 2001) have similar salinities to those of Wadi El-Natrun lakes water, but their chemical composition is different.

Sodium cations ranged from 3.5 (mg/g) ± 2.3 in Rosetta to 11.1 ± 8.36 in Umm-Risha Lake; results disagreed with those reported by Moussa *et al.* (2009) who found higher sodium concentration of 954.0 mmol/l (=41.5 mg/ml) and Taher (1999) (30.2- 295.2 g/l). Potassium cation (K^+ mg/g. dry wt) fluctuated between 0.1 ± 0.01 (in Rosetta Lake) and 0.3 ± 0.1 in Al Gaar Lake. Carbonate (CO_3^{-2} mg/g. dry wt) anions ranged from 0.01 ± 0.2 in Al Beida Lake to 0.3 ± 0.03 in Al-Gaar Lake. Bicarbonate (HCO_3^- mg/g dry wt) ranged from 0.07 ± 0.04 in Umm Risha to 0.5 ± 0.5 in Al Gaar Lake. Calcium cation (Ca^{+2} mg/g dry wt) varied from 0.008 in Fasida to 0.2 ± 9.7 x 10^{-3} in Hamra Lake. Magnesium cations (Mg^{+2} mg/g dry wt) ranged from 0.01 in Khadra and Fasida to 0.04 ± 0.2 in Al Beida Lake. Chloride anions (Cl^- mg/g dry wt) ranged from 0.7 ± 0.1 in Al Hamara to 3.6 ± 2.5 in Al Gaar. Moubasher *et al.* (1990) recorded that the amount of carbonate, bicarbonate and chlorides in the soil samples collected from Egypt ranged between 0.07~5.4%, 0.14~5.88% and 0.18~5.94%, respectively.

From the above results, it is obvious that soil samples collected from Al Gaar Lake possessed the highest values of pH, total soluble salts, sodium, potassium, carbonate, bicarbonate and chloride compared to those collected from the other 7 lakes of Wadi El-Natrun. On the other hand, other parameters showed their peaks in different lakes e.g. organic matter (2.0±2.4) in El Zugm Lake, calcium (0.2±9.7x10^{-3}) in Hamra Lake, magnesium (0.04±0.2) in Al Beida and sodium (11.1±8.4 mg/g) in Umm Risha (Table 1). Compared with our findings, the results of El Ghani *et al.* (2014) revealed higher organic matter (2.17-6.55%), carbonates (2.93-21 mg/g), calcium (2.56-5.13 mg/g), magnesium (0.89-1.94 mg/g), sodium (4.16-47.66 mg/g), potassium (0.12-6.49 mg/g), bicarbonate (0.68-1.18 mg/g) as well as, chloride ions (3.11-35.59 mg/g).

Table 1: Chemical analysis of soil samples collected from around Wadi El-Natrun lakes.

Lake	Al-Gaar	Khadra	Al-Beida	El-Zugm	Hamra	Rosetta	Umm-Risha	Fasida
pH	9.05	8.2	8.7	8.5	8.7	8.4	8.5	8.3
OM%	0.3	1.2	1.4	2.0	1.4	1.3	1.7	0.7
TSS%	3.5	1.9	2.7	6.3	1.7	4.9	5.7	4.9
Na^{+}*	4.9±3.1	3.9±3.4	9.9±8.7	4.7±2.7	7.4±8.9	3.5±2.3	11.1±8.36	10.1±9.7
K^{+}*	0.3±0.1	0.14±0.05	0.15±0.1	0.16±0.08	0.25±0.1	0.1±0.01	0.13±0.03	0.2±0.3
CO_3^{-2}*	0.3±0.03	0.04±0.02	0.01±0.2	0.07±0.07	0.26±0.17	0.2±0.3	0.1±0.1	0.2±0.3
HCO_3^{-}*	0.5±0.5	0.1±0.07	0.2±0.2	0.1±0.1	0.1±0.08	0.26±0.3	0.07±0.04	0.2±0.2
Ca^{+2}*	0.01±0.01	0.03±0.03	0.03±0.02	0.01±0.1	$0.2\pm9.7\times10^{-3}$	0.02±0.01	0.01±0.01	0.008±0.0
Mg^{+2}*	0.023±0.01	0.01±0.01	0.04±0.2	0.02±0.01	0.03±0.02	0.02±0.01	0.03±0.03	0.01±1.4
Cl^{-} *	3.6±2.5	0.7±0.1	3.4±2.5	2±2.2	1±0.3	2.0±2.5	0.9±0.5	1.6±0.8

OM= Organic matter and TSS= Total soluble salts as percentage of dry soil, * = mg/g dry soil

Fungi isolated on Czapek's **agar**

Eighty-one species and 2 varieties related to 28 genera were recovered on Czapek´s agar (as control medium). *Aspergillus* (15 species), *Fusarium* (12), *Myrothecium* (3) *Stachybotrys* (2), *Penicillium* (7) and *Emericella* (5) were the most common genera. They accounted for between 5.75% - 46.38% of total fungi (Tables 2, 5). The highest total count was noted in soil around Umm Risha lake, while, the lowest was recorded in Fasida (Table 2).

From *Aspergillus, A. terreus, A. niger* and *A. flavus* were the most dominant *Aspergillus* species comprising 18.82, 12.07 and 8.29% of total fungi respectively and were reported in all lakes (Table 2). The highest count of *Aspergillus* was recorded in soil around Khadra Lake, while, the lowest was recorded in Rosetta lake. Other species, such as *A. deflectus* (from Rosetta), *A. parasiticus* (El Zugm), *A. parvulus* (Khadra), *A. pulverulentus, A. punicus* and *A. sydowii* (Hamra) were isolated from only one lake (Table 2). Ismail and Abdullah (1977) found that the most common fungi from alkaline (pH 8.1-8.6) soil around Basrah, Iraq belong to *Aspergillus* and *Penicillium*. Of *Aspergillus, A. niger, A. ochraceus, A. carneus, A. oryzae, A. wentii, A. versicolor, A. nidulans, A. terreus, A. flavus* and *A. fumigatus* were recorded. Also, Gomes *et al*. (2008) found also that *A. flavus, A. janus, A. japonicus, A. niger, A. sydowii* and *A. terreus* were the most common from the sand and water samples taken from alkaline soil at Casa Caiada and Bairro Novo beaches, Brazil. Abdel-Hafez *et al.* (2009) isolated *Aspergillus* and *Penicillium* in high frequency of occurrence from salt marsh soils in Egypt and found *A. flavus, A. fumigatus, A. niger* and *A. ochraceus* to be the most common.

The peak of *Fusarium* was detected in soil around Umm Risha. *F. solani* (4.19% of total fungi) was recorded from all lakes. *F. equiseti* (from El Zugm), *F. subglutinans* (Umm Risha) and *F. tumidum* (from Hamra) were isolated each from one lake. In agreement with our results, Abdel-Hafez *et al.* (2009) isolated 17 *Fusarium* species from salt marsh soils, of which *F. solani* was the most common. Gomes *et al.* (2008) isolated *Fusarium solani, F. dimerum, F. equiseti* and *F. oxysporum* from alkaline soil in Brazil, of which *F. solani* was also the most common.

Stachybotrys (*S. chartarum* and *S. kampalensis*) was recorded from all lakes accounting for 6.74% of total fungi. The peak of *Stachybotrys* was recorded from Al Gaar Lake. In this respect, *S. chartarum* was isolated from the alkaline soil (pH 8.1-8.6) around Basrah, Iraq (Ismail and Abdullah 1977) and was of high frequency of occurrence in the sandy soil of Egyptian beaches (Migahed 2003).

Penicillium (5.75% of total fungi) and its dominant species (*P. chrysogenum,* 2.42%) were recorded from all lakes except Fasida. Migahed (2003) isolated *P. chrysogenum* and *P. citrinum* with high frequency from the sandy soil of Egyptian beaches, while Ismail and Abdullah (1977) isolated *Penicillium notatum, P. lilacinum, P. corymbiferum* and *P. crustosum* from the alkaline soil around Basrah, Iraq.

Emericella (7.92% of total fungi) was recorded from all lakes, while its dominant species, *E. nidulans* (3.7%), was detected from all lakes except Al Beida and *E. quadrilineata* (3.52%) from all lakes except Khadra and Umm Risha Lakes. *E. lata* was recorded from three lakes (Khadra, El Zugm and Rosetta), while *E. acristata* was recorded from 2 lakes (Table 2). *Emericella nidulans* is a typical soil fungus and was isolated from desert and saline soil (Moustafa 1975). However, it was isolated in rare frequency from Casa Caiada and Bairro Novo beaches, Brazil (Gomes *ei al.* 2008). *Myrothecium verrucaria* was isolated from all

lakes, while *M. roridum* was recorded from 4 lakes and *M. striatisporum* from 3 lakes.

The remaining genera were less commonly isolated from 5 lakes (*Acremonium*, *Alternaria*, *Humicola*), 4 lakes (*Cochliobolus* and *Setosphaeria*), 2 lakes (*Rhizopus*, *Chaetomium*, *Scopulariopsis*, *Trichoderma*) or one lake (*Phialophora* sp., *Phoma* sp., *Talaromyces* sp. and *Ulocladium botrytis*) (Table 2,5). In this respect, Ismail and Abdullah (1977) isolated *Acremonium strictum*, *Alternaria alternata*, *Botryotrichum piluliferum*, *Drechslera spicifera*, *Myrothecium roridum*, *Stachybotrys chartarum*, *Ulocladium botrytis*, *U. atrum* and *U. chartarum* from alkaline soil around Basrah, Iraq. Steiman *et al*. (1997) found species of Ascomycetes, *Sporormiella minima*, *S. minimoides*, and the hyphomycetes *Acremonium strictum*, *Alternaria alternata*, *A. chlamydospora*, *Aphanocladium album*, *Aspergillus fumigatus*, *A. niger*, *Botryotrichum piluliferum*, *Botrytis cinerea*, *Cladosporium cladosporioides*, *Gliocladium roseum*, *Penicillium aurantiacum*, *P. chrysogenum*, *P. griseoroseum*, *P. purpurogenum*, *P. simplicissimum* in both oases and in desert zones of Ein Gedi and Einot Zugim of Dead Sea area. Also, Abdel-Hafez *et al*. (2009) isolated species of *Botryotrichum*, *Chaetomium*, *Cladosporium*, *Curvularia*, *Cylindrocarpon*, *Humicola*, *Rhizopus*, *Setosphaeria*, *Stachybotrys*, *Stemphylium*, *Thamnidium*, *Trichothecium* and *Ulocladium* from salt marsh soil, in Egypt. *Chaetomium* was also significantly represented in soil of the Dead Sea area (13 species, Steiman *et al*. 1997) and in the salt marshes of Kuwait (8 species, Moustafa, 1975).

Fungi isolated on Czapek´s agar supplemented with 40% sucrose or 10% NaCl

It should be admitted that the present results do not draw a sharp demarcation between osmotolerant and osmophilic or between halotolerant and halophilic fungi. Therefore they are treated as one category viz. osmotolerant / osmophilic or halotolerant / halophilic compared to those on Czapek (Cz). Fifty-eight species and 2 varieties assigned to 18 genera of osmotolerant/osmophilic fungi were recovered on Czapek's + 40% sucrose agar compared to 44 species and one variety related to 16 genera of halotolerant/halophilic fungi recovered on Czapek's + 10% NaCl agar were identified from soil around 8 lakes of Wadi El-Natrun. These results when compared to 81 species and 2 varieties on Czapek's (Cz) related to 28 genera reveal that the majority of fungi of Wadi El-Natrun are osmotolerant/osmophilic and halotolerant/halophilic. They are able to accommodate themselves to exist under conditions of water stress of the two media but a lower number can tolerate water stress when combined with the toxic effect of excessive concentration of Na^+ and Cl^- ions. The most significant observations of this part (Tables 2, 3, 4 and 5) were:

1. ***Aspergillus*** **(17 spp. + 2 varieties)** was consistently the most frequent genus (100% frequency), and the most dominant, regularly contributing the highest CFU's on the three media, but Czapek's + 40% sucrose agar sustained the highest count and Cz + 10 NaCl the lowest. *A. terreus*, *A. flavus* , *A. níger*, *A. ochraceus*, *A. sydowii*, *A. terreus* var. *africanus*, *A. ustus* and *A. fumigatus* were represented on the three media, but *A. terreus* was regularly the most dominant contributing the highest numbers on the three media but the optimum one was Cz + 40% sucrose. *A. flavus* showed its highest frequency on Cz agar, but *A. níger* and *A. fumigatus* on 40% sucrose.
2. ***Eurotium*** **(2 spp.)** the real osmophilic and halophilic genus was regularly encountered on the three media, but the optimum medium was 40% sucrose for the highest frequency (81.3%) and *E. chevalieri* was the dominant species of the genus.
3. ***Emericella*** **(4 spp.)** was absent on 10% NaCl and its maximum frequency and count were recorded on 40% sucrose. *E. nidulans* and *E. quadrilineata* were the dominant species on 40% sucrose.
4. ***Fusarium*** **(14 spp.)** was regularly recovered on all media but its highest frequency on 40% sucrose and the best count on 10% NaCl. *F. solani*, *F. oxysporum*, *F. sambucinum* and *F. semitectum* were regularly isolated on the three media. *F. solani*, *F. oxysporum* and *F. semitectum* were more frequent on Cz, but *F. sambucinum* was more frequent on 40% sucrose. *F. poae* was only encountered on 10% NaCl.
5. ***Penicillium*** **(8 spp.)** was identified on all media but the best frequency and count were registered on 40% sucrose. *P. chrysogenum* exhibited its best frequency and count on 40% sucrose, but was absent on 10% NaCl.
6. ***Scopulariopsis*** **(6 spp.)** was isolated on the three media, but its highest frequency and count were recorded on 10% NaCl. *S. brevicaulis* was encountered on the three media but its highest frequency on 40% sucrose. *S. brumptii* exhibited its highest frequency on NaCl but was absent on Cz. *S. halophilica* was recovered on NaCl only.
7. ***Cladosporium*** **(3 spp.)** was recovered on the three media; it showed its best frequency on 40% sucrose. *C. cladosporioides* was the

most frequent species on Cz and 40% sucrose, but was absent on 10% NaCl, whereas *C. oxysporum* was regularly recovered on the three media.

8. ***Acremonium* (4 spp.)** was regularly isolated on the three media. *A. furcatum* exhibited its highest frequency on 10% NaCl, followed by 40% sucrose and Cz. *A. strictum* showed its highest frequency on 10% NaCl followed by the control medium and absent on 40% sucrose. *Acremonium* was with relatively higher frequency in the soil of Wadi El-Natrun than in the other surveys in Egypt in the last century (Moubasher, 1993).
9. **On Cz** the highest frequency (100%) among genera was recorded for *Aspergillus* and the runner-up was *Stachybotrys* (80.2%), followed by *Fusarium* (76%) and *Penicillium* (62.5%). The highest records for species frequency were held to *A. terreus* (85.4%), *A. flavus* (83.3%), *S. chartarum* (80.2%) and *A. niger* (78.1%).

   On 40% sucrose, the highest record of frequency was held to *Aspergillus* (95.8%), *Eurotium* (81.3%), *Fusarium* (79.2%), *Emericella* (78.1%), *Penicillium* (68.8%), *Stachybotrys* (42.7%). The highest frequencies among species were recorded for *A. terreus* (89.6%), *A. niger* (81.3%), *S. chartarum* (80.2%), *A. flavus* (79.2%), *Eurotium chevalieri* (77.1%), *E. nidulans* (59.4%) and *P. chrysogenum* (58.3%).

   On 10% NaCl, the runner–up of *Aspergillus* (46.9% frequency) was *Penicillium* (36.5%), followed by *Scopulariopsis* (34.4%), *Eurotium* (30%) and *Fusarium* (22.9%). The most prevalent species was *A. terreus* (44.8%), followed by *A. sydowii* (34.4%), *Eurotium chevalieri* (30.0%), *S. brumptii* (29.2%) and *S. halophilica* (22.9%).

   Cz sustained the broadest spectra of genera (26) and species (60), and 10% NaCl the narrowest (16 and 41 respectively).

   In the literature a great number of fungi recorded in the present investigation on 40% sucrose and 10% NaCl media were also identified in different parts of the world on high sugar concentrations, from sand dunes and house dust samples in Saudi Arabia (Bakhary 1999); sand soil of Egyptian beaches (Migahed 2003); and dry fruits (Srivastava *et al.* 2014); and on high salt concentrations from Mayaguez Bay shoreline, western Puerto Rico (Rusi-Suarez 2004), saline soils (Steiman *et al*. 1997), the Arubotaim Caves, one of the Holocene Karst Caves developed within the salt body of Mount Sodom, the south western part of the Dead Sea (Grishkan *et al.* 2004) and hypersaline Dead Sea shore (Grishkan *et al.* 2003), the Red Sea shore (Abol-Nasr 1981), salt marsh soil in Egypt (Moubasher *et al.* 1990), solar salterns (Cantrell *et al.* 2006), saline environments in Slovenia (Butinar *et al.* 2005), hypersaline environment of solar salterns and hypersaline lakes worldwide (Gunde-Cimerman *et al.* 2005), and Mondovi estuary (Gunde-Cimerman *et al.* 2009 and Gonsalves *et al.* 2012).

Cluster analysis of the diversity of fungal species of the soil around the eight lakes

On Czapek´s agar (Figure 1), the analysis showed that Rosetta and Al Gaar lakes were clustered together in group (A), which possessed the highest values of TSS% (4.9 and 3.5% respectively). Also Fasida and Al Beida were clustered in group (B) and closely related to group (A). Both lakes had the same content of HCO_3^- (0.2 mg/g) and almost similar Na^+ concentrations (10.1 and 9.9 mg/g respectively) (Table 1).

On Czapek's 40% sucrose agar, The cluster analysis classified lakes into four groups: group (A), included Umm Risha and Khadra Lakes which possessed almost similar contents of K^+ (0.13 and 0.14 mg/g respectively). While group (B), included El Zugm and Al Gaar Lakes, which had almost similar Na^+ content (4.7, 4.9 mg/g respectively) and group (C), including Fasida and Al Beida Lakes which are closely related (Figure 2).

On Czapek's 10% NaCl, the cluster analysis in Figure (3) showed two closely-related groups including Rosetta, El Zugm and Fasida Lakes (group A), Cl^- contents very-close ranging from 1.6 to 2.0 mg/g and Al Beida and Al Gaar Lakes (group B) with Cl^- contents of 3.4-3.6 mg/g (Table 1). In contrast, Khadra Lake was separated from the other lakes in a separate clade which recorded the least pH value (8.2) and Cl^- content (0.7 mg/g).

Table 2: Percentage total counts (TC%) and Percentage frequencies (F%, out of 12 samples) of fungal taxa recorded from soil samples collected from each of the 8 Lakes of Wadi El- Natrun on Czapek´s agar at 28°C.

Fungal taxa	Al Gaar		Khadra		Al Beida		El Zugm		Hamra		Rosetta		Umm Risha		Fasida		Total	
	TC%	F%	TC%	F%	TC%	F%	TC%	F%	TC%	F%	TC%	F%	TC%	F%	TC%	F%	TC%	F%
Aspergillus	36.32	100	64.55	100	54.55	100	36.99	100	42.38	100	32.42	100	51.57	100	52.22	100	46.38	100
A. terreus	23.63	100	30.78	83.3	11.91	33.3	11.02	100	14.69	83.3	13.64	83.3	36.00	100	8.87	100	18.82	85.4
A. flavus	6.98	83.3	0.71	50	17.98	100	18.89	66.7	1.95	83.3	5.42	83.3	6.98	100	7.39	100	8.29	83.3
A. niger	4.07	83.3	2.86	33.3	19.93	83.3	4.86	66.7	18.87	83.3	8.57	100	8.34	75	29.06	100	12.07	78.1
A. fumigatus	0.47	16.7			3.37	33.3	0.44	50	0.65	50	2.06	33.3					0.87	22.9
A. ochraceus	0.47	16.7			1.35	16.7			3.68	50			0.26	25			0.72	13.5
Stachybotrys	14.09	100	3.46	100	11.13	50	9.34	100	2.62	83.3	6.87	83.3	4.43	75	1.97	50	6.74	80.2
S. chartarum	14.09	100	3.46	100	11.13	50	9.34	100	2.62	83.3	6.51	83.3	4.43	75	1.97	50	6.69	80.2
Fusarium	8.15	33.3	8.54	83.3	12.74	66.7	15.49	100	18.68	100	21.01	100	27.57	75	2.96	50	14.39	76.0
F. solani	1.63	33.3	0.76	33.3	5.99	50	0.59	33.3	9.82	100	11.50	83.3	0.26	25	2.96	50	4.19	51.0
Fusarium spp.	1.40	16.7	6.86	33.3	6.44	33.3	11.13	83.3	0.01	33.3	5.50	100					3.92	37.5
F. oxysporum	3.49	16.7					0.20	33.3	1.31	33.3	1.07	50	5.70	75			1.47	26.0
F. semitectum			0.38	33.3					0.22	33.3	0.18	33.3	16.17	75			2.12	21.9
Penicillium	1.86	50	8.39	83.3	16.45	83.3	4.68	83.3	4.04	83.3	6.68	66.7	3.91	50			5.75	62.5
P. chrysogenum	0.47	16.7	0.38	33.3	7.42	16.7	2.31	50	1.78	66.7	3.96	50	3.06	33.3			2.42	33.3
P. funiculosum			6.52	50					0.22	33.3	0.41	33.3	0.17	25			0.91	17.7
P. duclauxii			0.57	33.3	0.34	16.7	0.89	33.3					0.68	33.3			0.31	14.6
Myrothecium	15.37	33.3	6.78	100	2.25	16.7	1.42	50	3.29	83.3	1.38	50	2.38	75	14.78	50	5.96	57.3
M. verrucaria	15.37	33.3	6.39	100	2.25	16.7	0.83	50	3.00	83.3	0.28	33.3	0.34	50	4.43	50	4.11	52.1
M. roridum			0.19	33.3			0.59	33.3	0.29	33.3			2.04	75			0.39	21.9
Emericella	7.22	33.3	3.05	33.3	0.34	16.7	21.48	100	6.69	83.3	9.10	50	0.26	25	15.27	100	7.92	55.2
E. nidulans	4.42	33.3	2.86	33.3			13.52	100	1.87	66.7	3.19	50	0.26	25	3.45	50	3.70	44.8
E. quadrilineata	2.79	16.7			0.34	16.7	3.32	66.7	4.17	66.7	5.72	50			11.82	100	3.52	39.6
Humicola							3.20	83.3	6.53	33.3	1.68	50	0.26	25	8.87	100	2.57	36.5
H. grisea							3.20	83.3			1.68	50	0.26	25	4.93	100	1.26	32.3
Gliocladium roseum	2.33	16.7	0.95	33.3	0.45	16.7			2.18	33.3	3.69	50	0.77	25	0.49	50	1.36	28.1
Alternaria	1.16	16.7	0.57	33.3	0.22	16.7	0.49	33.3	1.31	33.3			1.28	25			0.63	19.8
Total counts	85900		209860		88967		135083		183811		145411		352500		81200		1282733	
No. of genera	11		10		11		15		16		16		13		10		26	
No. of species+varieties	16+1		21+1		15		26+1		35		26		25		15+1		60+2	

Results of genera and species recovered from one or two lakes only were omitted from the table.

Table 3: Percentage total counts (TC%) and Percentage frequency (F%, out of 12 samples) of fungal taxa recorded from soil samples collected from each of the 8 Lakes of Wadi El- Natrun on Czapek´s agar supplemented with 40% sucrose (40%S) at 28°C.

Fungal taxa	Al Gaar		Khadra		Al Beida		El Zugm		Hamra		Rosetta		Umm Risha		Fasida		Total	
	TC%	F%	TC%	F%	TC%	F%	TC%	F%	TC%	F%	TC%	F%	TC%	F%	TC%	F%	TC%	F%
Aspergillus	39.14	66.7	59.55	100	55.49	100	28.52	100	52.53	100	40.20	100	58.80	100	67.74	100	51.81	95.8
A. terreus	29.95	50	28.71	100	30.29	83.3	18.03	100	16.79	100	20.63	83.3	38.51	100	38.02	100	24.76	89.6
A. niger	3.59	66.7	6.30	100	9.92	66.7	4.27	50	27.80	100	15.56	66.7	11.18	100	20.74	100	12.01	81.3
A. flavus	5.06	33.3	2.20	83.3	10.18	83.3	2.83	66.7	5.26	83.3	2.94	83.3	8.70	100	2.76	100	5.50	79.2
A. fumigatus	0.53	16.7	2.06	50	2.11	16.7	3.39	33.3			0.43	33.3	0.41	25	3.46	100	1.37	34.4
A. sydowii			8.23	50	1.58	33.3					0.64	33.3			1.38	50	1.58	20.8
Eurotium	12.78	100	8.48	83.3	5.09	66.7	15.71	83.3	16.50	83.3	13.34	83.3	6.00	100	0.46	50	9.28	81.3
E. chevalieri	11.18	83.3	7.48	83.3	4.04	66.7	12.21	83.3	4.30	66.7	10.14	83.3	6.00	100	0.46	50	6.91	77.1
E. amstelodami	1.60	33.3	1.00	33.3	0.35	16.7	3.05	33.3	12.20	33.3	3.20	33.3					2.25	22.9
Fusarium	11.72	83.3	7.73	83.3	12.47	83.3	5.29	66.7	1.45	33.3	5.79	83.3	16.36	100	8.29	100	9.74	79.2
F. solani	5.86	33.3	2.09	66.7	6.50	50	1.02	33.3			4.83	83.3	1.04	50	6.91	50	2.91	45.8
F. verticillioides	2.59	50	4.64	83.3	0.26	16.7			1.45	33.3			1.24	25			1.20	26.0
F. sambucinum			0.50	33.3	5.71	33.3					0.32	33.3	0.83	25			0.94	15.6
Emericella	24.76	83.3	8.38	83.3	8.78	50	36.98	100	13.31	83.3	16.53	100	2.17	25	20.74	100	13.46	78.1
E. quadrilineata	16.51	83.3			5.97	33.3	12.00	83.3	11.32	83.3	11.93	83.3	0.62	25	11.06	100	6.68	61.5
E. nidulans	8.25	66.7	6.13	83.3	2.46	33.3	9.16	66.7	2.00	50	4.61	50	1.55	25	8.29	100	4.57	59.4
Penicillium	9.34	100	6.28	66.7	13.17	66.7	3.46	50	9.53	83.3	19.16	83.3	13.77	100			10.24	68.8
P. chrysogenum	6.66	83.3	6.28	66.7	3.51	33.3	2.44	50	3.43	66.7	1.28	66.7	9.01	100			4.95	58.3
P. puberulum	1.33	33.3			0.70	16.7	1.02	33.3	5.01	66.7	0.64	33.3	2.69	100			1.59	35.4
P. funiculosum					0.70	16.7			0.36	33.3	0.64	33.3	0.72	50			0.38	16.7
P. citrinum	0.01	16.7			0.35	16.7					14.97	33.3	1.35	50			2.13	14.6
Stachybotrys chartarum			4.49	66.7			5.29	50	1.13	50	0.51	50	1.45	75	0.46	50	1.85	42.7
Cladosporium	1.20	16.7	1.00	50			1.36	33.3	1.09	66.7	1.60	50	2.03	25	0.46	50	0.71	36.5

Fungal taxa	Al Gaar		Khadra		Al Beida		El Zugm		Hamra		Rosetta		Umm Risha		Fasida		Total	
	TC%	F%	TC%	F%	TC%	F%	TC%	F%	TC%	F%	TC%	F%	TC%	F%	TC%	F%	TC%	F%
C. cladosporioides	1.20	16.7	0.50	33.3			1.36	33.3	0.36	33.3			2.03	25	0.46	50	0.38	20.8
Total Counts	75114		160413		113900		117959		110167		125079		289800		86801		1079233	
No. of genera	7		12		8		10		11		11		9		10		18	
No. of species+varieties	19		22+1		24		17		21+1		21		23		18		49+2	

Results of genera and species recovered from one or two lakes only are omitted from the table.

Table 4: Percentage total counts (TC%) and Percentage frequencies (F%, out of 12 soil samples) of fungal taxa recorded from soil samples collected from each of the 8 Lakes of Wadi El- Natrun on Czapek´s agar supplemented with 10% NaCl at 28°C.

Fungal taxa	Al Gaar		Khadra		Al Beida		El Zugm		Hamra		Rosetta		Umm Risha		Fasida		Total	
	TC%	F%	TC%	F%	TC%	F%	TC%	F%	TC%	F%	TC%	F%	TC%	F%	TC%	F%	TC%	F%
Aspergillus	55.38	50	5.91	33.3			69.79	33.3	20.09	66.7	72.58	66.7	46.86	75	91.42	50	34.49	46.9
A. terreus	19.69	33.3	3.73	33.3			64.64	33.3	11.48	66.7	41.21	66.7	42.39	75	39.28	50	23.91	44.8
A. sydowii	1.84	16.7	1.16	33.3			1.74	33.3	0.39	50	2.77	66.7	0.38	75			0.84	34.4
A. flavus	27.56	50					2.85	16.7	1.45	50	25.27	50	2.35	50	11.06	50	4.16	33.3
A. ochraceus	1.05	16.7	0.87	33.3			0.37	33.3	5.51	50	0.51	50	1.36	50			1.86	29.2
A. carneus	2.62	16.7					0.19	16.7	0.55	33.3	2.82	33.3	0.38	25			0.50	15.6
Penicillium	3.41	33.3	0.70	33.3					0.49	66.7	5.13	83.3	0.38	75			0.85	36.5
P. puberulum	3.41	33.3							0.49	66.7	2.77	66.7	0.30	50			0.48	27.1
Scopulariopsis	33.60	33.3			100.00	50	24.24	33.3	3.36	50	16.10	33.3	0.15	25	4.90	50	5.26	34.4
S. brumptii	24.15	33.3			62.07	50	20.51	33.3	2.46	33.3	10.76	33.3			4.90	50	3.79	29.2
S. halophilica	9.45	33.3			36.55	50	3.73	33.3	0.90	33.3	0.51	33.3					1.10	22.9
Eurotium	1.57	16.7	0.29	33.3			1.12	33.3	0.98	50	1.28	33.3	0.38	25	3.69	50	0.75	30.2
E. chevalieri	0.79	16.7	0.29	33.3			0.75	33.3	0.57	50	0.77	33.3	0.38	25	3.69	50	0.60	30.2
E. amstelodami	0.79	16.7					0.37	33.3	0.41	33.3	0.51	33.3					0.16	14.6
Total Counts	762		6874		290		1072		4877		1561		7926		1139		24486	
No. of genera	7		4		1		5		9		6		12		3		16	
No. of species+varieties	16		6+1		3		12		16		18		24		5		41+1	

Results of genera and species recovered from one or two lakes only are omitted from the table.

Table 5: Percentage total counts (TC%) and percentage frequencies (F%, out of 96 soil samples) of fungal taxa recorded from 96 soil samples collected from 8 lakes on Czapek's agar (Cz), Czapek´s supplemented with 40% sucrose or 10% NaCl at 28°C.

Fungal taxa	Cz		40% S		10% NaCl	
	TC%	F%	TC%	F%	TC%	F%
Acremonium Link	1.05	27.1	0.51	16.7	2.47	11.5
A. curvulum W. Gams	0.05	4.2				
A. furcatum Moreau & Moreau ex Gams	0.14	4.2	0.40	6.3	0.14	7.3
A. kiliense Gütz	0.05	4.2				
A. strictum W. Gams	0.16	4.2			0.16	7.3
Acremonium sp.	0.64	18.8	0.11	12.5	2.18	4.2
Alternaria Nees: Fries	0.63	19.8			0.14	5.2
A. alternata (Fries) Keissler	0.12	7.3			0.07	3.1
A. tenuissima (Kunze: Fries) Wiltshire	0.06	3.1			0.07	5.2
Alternaria sp.	0.45	14.6				
Aspergillus P. Micheli ex Link	46.38	100	51.81	95.8	34.49	46.9
A. aureolatus Munt., Cvet. & Bata	0.16	8.3				
A. candidus Link			0.04	4.2		
A. carneus Blochwitz			0.06	2.1	0.50	15.6
A. deflectus Fennell & Raper	0.34	4.2				
A. flavus Link	8.29	83.3	5.50	79.2	4.16	33.3
A. flavus var. c*olumnaris* Raper & Fennell	0.05	6.3				
A. fumigatus Fresenius	0.87	22.9	1.37	34.4	0.08	2.1
A. niger van Tieghem	12.07	78.1	12.01	81.3	0.05	4.2
A. niveus van Tieghem					0.09	4.2
A. ochraceus Wilhelm	0.72	13.5	0.26	12.5	1.86	29.2
A. parasiticus Speare	0.15	4.2				
A. parvulus Smith	0.02	4.2				
A. pulverulentus (Mcalpine) Thom	0.03	4.2				
A. puniceus Kwon & Fennell	0.05	4.2				
A. sydowii (Bainier & Sartory) Thom & Church	0.19	9.4	1.58	20.8	0.84	34.4
A. terreus Thom	18.82	85.4	24.8	89.6	23.91	44.8
A. terreus var. *africanus* Fennell & Raper	3.18	10.4	5.99	4.2	1.09	6.3
A. ustus (Bainier) Thom & Church	1.43	10.4	0.11	6.3	1.91	6.3
A. versicolor(Vuillemin) Tiraboschi			0.14	6.3		
Botryotrichum sp.	0.04	4.2				
Chaetomium Kunze	0.63	14.6			1.00	3.1
C. globosum Kunze					0.44	3.1
C. olivaceum Cooke & Ellis					0.56	3.1
Chaetomium sp.	0.63	14.6				
Cladosporium Link	2.07	25.0	0.71	36.5	0.08	7.3
C. cladosporioides (Fresenius) de Vries	0.54	9.4	0.38	20.8		
C. oxysporum Berkeley & Curtis	0.44	7.3	0.07	8.3	0.03	4.2
C. sphaerospermum Penzig	0.06	6.3			0.05	3.1
Cladosporium spp.	1.03	8.3	0.26	6.3		
Cochliobolus Drechsler	0.32	18.8	0.33	14.6	0.07	4.2
C. australiensis (Tsuda & Ueyama) Alcorn	0.05	4.2	0.33	14.6		
C. monocereus Alcorn					0.03	4.2

Fungal taxa	Cz		40% S		10% NaCl	
	TC%	F%	TC%	F%	TC%	F%
C. spicifer Nelson	0.23	12.5				
C. tuberculatus Sivanesan	0.05	4.2			0.03	4.2
Curvularia lunata var. *aeria* (Balista, Lima & Vasconcelos) M. B. Ellis			0.04	4.2		
Cylindrocarpon sp.			0.06	2.1		
Emericella Berkeley & Broome	7.92	55.2	13.46	78.1		
E. acristata (Fennell & Raper) Subrananiam	0.30	8.3	0.28	10.4		
E. lata Subramanian	0.33	12.5	1.26	12.5		
E. nidulans (Eidam) Vuillemin	3.70	44.8	4.57	59.4		
E. quadrilineata (Thom & Raper) Benjamin	3.52	39.6	6.68	61.5		
Emericella sp.	0.07	4.2	0.67	12.5		
Eurotium Link	1.11	15.6	9.28	81.3	0.75	30.0
E. amstelodami Mangin			2.25	22.9	0.16	14.6
E. chevalieri Mangin	0.10	9.4	6.91	77.1	0.60	30.0
Eurotium spp.	1.01	6.3	0.12	6.3		
Fusarium Link	14.39	76.0	9.74	79.2	0.07	22.9
F. chlamydosporum Wollenweber & Reinking	0.19	8.3			36.30	22.9
F. equiseti (Corda) Saccardo	0.20	6.3				
F. lateritium Nees			0.07	4.2	0.07	4.2
F. oxysporum Schlechtendal	1.47	26.0	0.22	7.3	6.78	7.3
F. poae (Peck) Wollenweber					1.63	4.2
F. proliferatum (Matsushima) Nirenberg	0.07	6.3	0.22	3.1		
F. sambucinum Fuckel	0.77	10.4	0.94	15.6	25.49	12.5
F. semitectum Berk. & Rav	2.12	21.9	3.24	16.7	1.59	9.4
F. solani (Martius) Saccardo	4.19	51.0	2.91	45.8	0.67	14.6
F. sporotrichioides Sherbakoff			0.30	6.3		
F. subglutinans (Wollenweber & Reinking) Nelson *et al*	0.62	9.4	0.06	3.1		
F. tricinctum (Corda) Saccardo	0.06	9.4				
F. tumidum Sherb.	0.35	4.2				
F. verticillioides (Saccardo) Nirenberg	0.43	17.7	1.20	26.0		
Fusarium spp.	3.92	37.5	0.58	10.4	0.07	2.1
Gliocladium Corda.	1.36	28.1	0.11	10.4		
G. catenulatum Gilman & Abbott			0.04	6.3		
G. roseum Bainier	1.36	28.1	0.07	4.2		
Humicola Traaen.	2.57	36.5	0.04	6.3		
H. fuscoatra Traaen.	1.31	16.7				
H. grisea Traaen.	1.26	32.3	0.04	6.3		
Myrothecium Tode	5.96	44.8	0.59	29.2	5.72	4.2
M. roridum Tode	0.39	21.9				
M. striatisporum Preston	1.45	14.6	0.15	10.4		
M. verrucaria (Albertini & Schweinitz) Ditmar	4.11	44.8			5.72	4.2
Nectria sp.	0.04	2.1	0.44	18.8		
Paecilomyces variotii Bainier			0.14	6.3		
Penicillium Link	5.75	62.5	10.24	68.8	0.85	36.5
P. chrysogenum Thom	2.42	33.3	4.95	58.3		
P. citrinum Thom			2.13	14.6	0.06	7.3

Fungal taxa	Cz		40% S		10% NaCl	
	TC%	F%	TC%	F%	TC%	F%
P. duclauxii Delacroix	0.31	14.6	0.20	6.3		
P. expansum Link			0.07	2.1		
P. funiculosum Thom	0.91	17.7	0.38	16.7		
P. pinophilum Hedgcock	0.05	4.2	0.46	10.4		
P. puberulum Bainier	0.13	8.3	1.59	35.4	0.48	27.1
P. purpurgenum Stoll	0.08	4.2	0.17	10.4		
Penicillium spp.	1.85	33.3			0.31	10.4
Phialophora sp.	0.05	4.2				
Phoma sp.	0.22	4.2	0.28	6.3		
Rhizopus Ehrenberg	0.12	10.4	0.50	20.8		
R. oryzae Went & Prinsen-Geerligs	0.08	6.3	0.23	16		
Rhizopus sp.	0.04	4.2	0.27	4.2		
Scopulariopsis Bainier	0.83	10.4	0.29	22.9	5.26	34.4
S. brevicaulis (Saccardo) Bainier	0.31	6.3	0.07	10.4	0.25	7.3
S. brumptii Salvanet- Duval			0.19	10.4	3.79	29.2
S. candida (Gueguen) Vuillemin					0.02	3.1
S. carbonaria Morton & Smith					0.02	2.1
S. halophilica Tubaki					1.10	22.9
S. spherospora Zach					0.08	4.2
Scopulariopsis sp.	0.52	4.2	0.02	4.2		
Scytalidium lignicola Pesante					0.52	8.3
Setosphaeria Leonard & Suggs	0.49	18.8	0.31	14.6		
S. holmii (Luttr.) K.J. Leonard & Suggs	0.14	4.2				
S. rostrata Leonard	0.27	20.8	0.13	10.4		
Setosphaeria sp.	0.08	4.2	0.18	6.3		
Stachybotrys Corda	6.74	80.2	1.85	42.7	12.25	3.1
S. chartarum (Ehrenberg) Hughes	6.69	80.2	1.85	42.7	12.25	3.1
S. kampalensis Hansf.	0.05	4.2				
Sterile mycelia	0.72	12.5				
Syncephalastrum sp.	0.06	2.1				
Talaromyces sp.	0.03	4.2				
Thermoascus aurantiacus Miehe					0.02	2.1
Torula sp	0.06	6.3				
Trichoderma sp.	0.09	9.4				
Ulocladium Preuss						
U. botrytis Preuss	0.06	2.1				
U. consortiale (Thümen) Simmons	0.06	2.1			0.07	3.1
Yeasts	0.35	2.1				
Total counts	1282733		1079233		24486	
No. of genera (31)	26		18		16	
No. of species (84+3 varieties)	60+2		49+2		41+1	

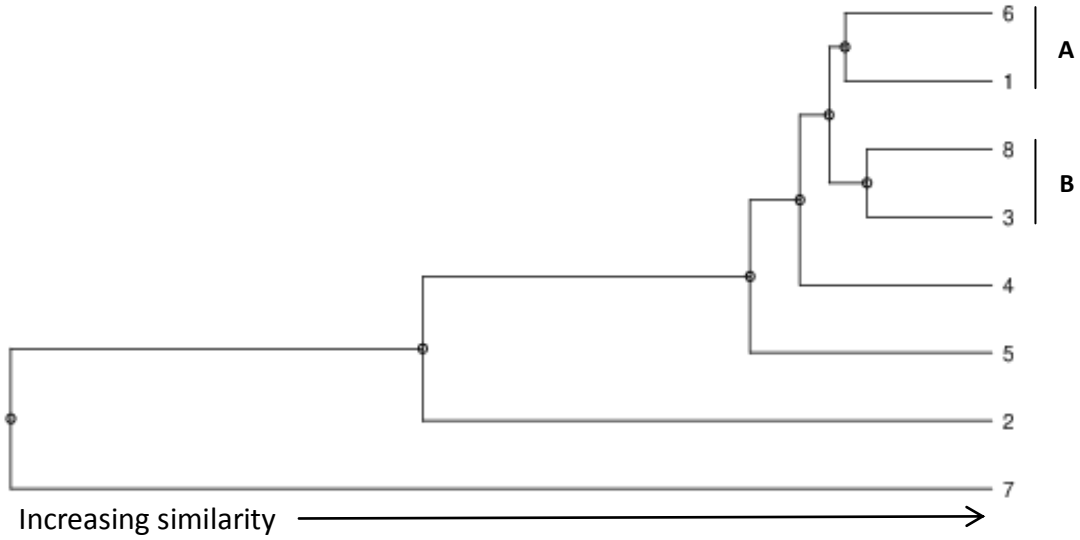

Figure 1. Cluster analysis of 8 lakes; Al Gaar (1), Khadra (2), Al Beida (3), El Zugm (4), Hamra (5), Rosetta (6), Umm Risha (7) and Fasida (8), based on the similarity of their fungal species diversity isolated on Czapek's dox agar medium at 25ºC.

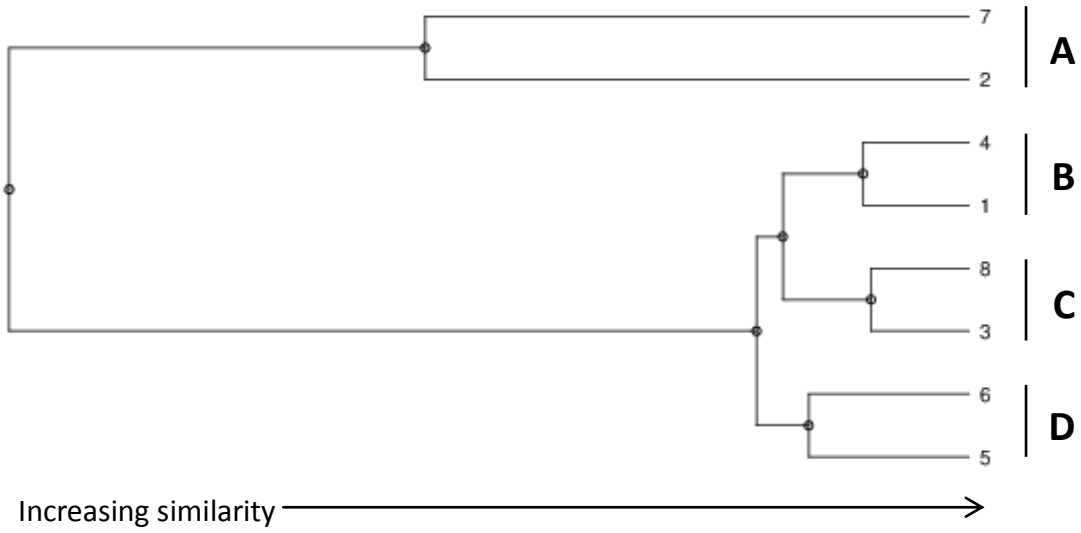

Figure 2: Cluster analysis of 8 lakes; Al Gaar (1), Khadra (2), Al Beida (3), El Zugm (4), Hamra (5), Rosetta (6), Umm Risha (7) and Fasida (8), based on the similarity of their fungal species diversity isolated on Czapek's 40% sucrose agar medium at 25ºC.

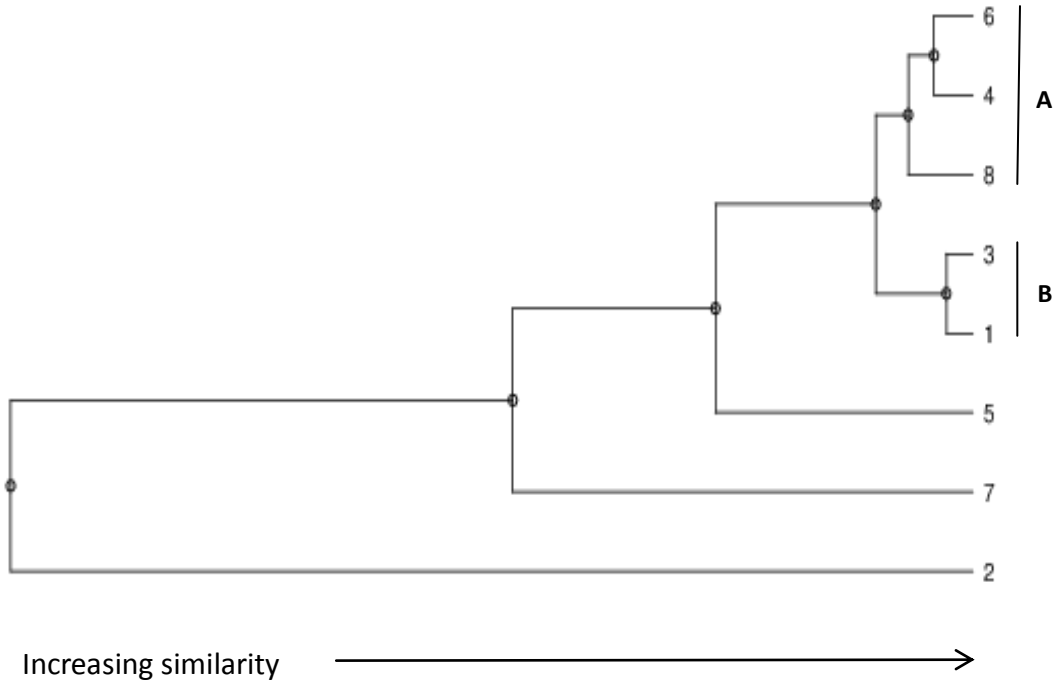

Figure 3: Cluster analysis of 8 lakes; Al Gaar (1), Khadra (2), Al Beida (3), El Zugm (4), Hamra (5), Rosetta (6), Umm Risha (7) and Fasida (8), based on the similarity of their fungal species diversity isolated on Czapek's 10% NaCl agar medium at 25ºC.

Conclusion: In the present investigation a total of 104 species and 3 varieties were collected from soil around 8 lakes of Wadi El- Natrun, from which 60 species and 2 varieties; 49 species and 2 varieties; 41 species and 1 variety were isolated on Czapek´s, 40% sucrose and 10% NaCl agar media respectively. These resultes indicate that the majority of soil fungi of Wadi El- Natrun are osmotolerant/osmophilic and halotolerant/halophilic. *Aspergillus*, *Fusarium*, *Penicillium*, *Emericella* were the most predominant genera on the three media.

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Incidence of entomopathogenic fungi of the oat bird-cherry aphid, *Rhopalosiphum padi* L. (Homoptera: Aphididae) infesting wheat plants in Assiut, Egypt

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Abstract: Four genera of entomopathogenic fungi and two hyphomycetes were identified from the cadavers of the aphid infesting wheat plants in the two growing seasons of 2013-2014. Entomophthorales was represented by four species belonging to three families; Ancylistaceae represented by *Conidiobolus*, Entomophthoraceae by *Entomophthora planchoniana* and *Pandora neoaphidis* and Neozygitaceae by *Neozygites fresenii*. The hyphomycetes fungi were represented by two species *Beauveria bassiana* and *Verticillium lecanii* which belong to the family Moniliaceae, order Moniliales. Data show that the aphid began to infest wheat plants early during the middle of January when wheat plants were in the stem-elongation stage. Thereafter, numbers of aphids increased gradually to reach a peak, when the plants were at the flowering stage during the third week of February; during the next three weeks the number of the oat aphid declined sharply. Mortality rate with the fungal pathogens was observed from the end of January up to the end of March. The number of cadavers increased gradually to reach the maximum level during the end of March.

Key words: Oat bird-cherry aphid, *Rhopalosiphum padi* L, wheat, entomopathogens, Assiut.

Introduction

The oat bird-cherry aphid, *R. padi* is considered one of the most serious cereal aphids attacking wheat plants in Assiut, Egypt. Its damage to the plants is manifested through loss of sap by sucking, reaction of plant tissues stimulated by aphid saliva; in different ways (change of color, curling of leaves and stem… etc.), excreting liquid, viscous honeydew, that is very harmful and on it sooty-moulds usually develop and, finally aphid transmission of virus diseases to plants (El-Hariry 1979, Abdel-Rahman 1997, El-Lathy 1999, and El-Fatih 2000 and 2006).

Entomopathogenic fungi are now being considered as biological alternatives to chemical control (Roberts and Yendol 1971, Samson *et al*. 1988). Entomopathogenic fungi are frequently reported as major factors suppressing populations of cereal aphids and can cause sudden decline of dense populations (Feng *et al*. 1991). Drastic reduction in the populations of various cereal aphids due to infection with Entomopathorales fungi was observed by Dedryver (1983).

The aim of the present study was to seek further information on mycopathogens of the oat bird-cherry aphid, *R. padi* L. infesting wheat plants in Assiut, Egypt.

Materials and Methods

The present investigation was carried out in Assiut Governorate (Abnoub district) about 15 Km Northeast Assiut City during 2013 and 2014 wheat-growing seasons. An area of about 2100 m^2 (about half of feddan) was cultivated with wheat (cultivar Sids 1) normally at mid-November in both cultivated seasons. The normal conventional agricultural practices were normally performed and no chemical control (insecticides or fungicides) was used during the study period. Weeds were removed by hand.

During the two seasons, the oat bird-cherry aphid numbers (all forms) were counted and recorded on 200 randomly selected seedlings or main tillers, later in the season, when the aphid numbers increase on the plants, the numbers of examined tillers were reduced to 100 tillers. Four replicates each were taken weekly from the beginning of January, when the migration of aphids onto wheat crop, at the stage of tillering or early stem-elongation stage from

overwintering sites began, and continued through the time when aphid population declined to low or undetectable levels. At the same time number of infected aphids was also counted and recorded.

Cadavers (dead aphids) were recorded, placed in 1x5 cm vials and stored at 5ºC. Aphid cadavers were examined under a dissecting microscope as soon as possible after collected to observe external symptoms and fungal reproductive structures if produced *in situ* on the plant. Desiccated and fresh cadavers were placed in a moist chamber for about 20 hrs to allow hyphae and reproductive structures to develop. Individual aphids were mounted in cotton blue or aceto-orcein and observed under a compound microscope. Identification of fungi was based on external symptoms and the morphology of spores and sporulating structures (Waterhouse and Brady, 1982), and new revision of the classification of Entomophthorales (Humber 1989 and 1991) was followed. Fungi identified as known aphid pathogens were considered to be the cause of death of their host.

Statistical analysis

Data were statistically analyzed using analysis of variance (F test) and means were compared according to Duncan's multiple range test.

Percentage of infection (mortality %) caused by entomopathogenic fungi was calculated in each sampling date according to Feng *et al*. (1992) as follows:

$$\text{Mortality \%} = \frac{\text{Number of infected aphids}}{(\text{Total number of alive aphids}) + \text{No. of cadavers}} \times 100$$

Results and Discussion

1-Entomopahogens identified from the aphid

From the survey studies through 2013 and 2014 wheat growing seasons, five species and one genus of entomopathogens, including four entomophthorales and two hyphomycetes were identified from the oat bird-cherry aphid, *R. padi*, infesting wheat plants. Entomophthorales was represented by four species belonging to three families: Ancylistaceae was represented by one genus, *Conidiobolus*, Order: Entomophthoraceae by *Entomophthora planchoniana* and *Pandora neoaphidis*, and Neozygitaceae by *Neozygites fresenii*. The hyphomycetes fungi were represented by *Beauveria bassiana* and *Verticillium lecanii* from order Moniliales.

These fungi mentioned above are surveyed worldwide as they are well-known species as biological control agents of cereal aphids (Feng *et al*. 1990, 1991 and 1992, Abdel-Rahman 2001 and Hammam 2003, 2009, Moubasher *et al*. 2010). Mycopathogens are considered to be the best means for biological control of aphids (Latge and Papierok, 1988), and numerous accounts of cereal aphids killed by entomophthoralean fungi were documented in Europe (Dean and Wilding 1971, 1973, Dedryver 1983, Papierok and Havukkala 1986) and South America (Lazzari 1985). Regional lists of aphid pathogenic fungi have been published in Australia (Milner *et al*. 1980) and Finland (Papierok 1989). Five entomopathogenic fungi were reported from 34 aphid hosts in eastern Canada and the United States (Remaudiere *et al*. 1978, Humber and Soper 1986).

2-Incidence of entomopathogens recorded

Data in Table 1 show the relative incidence of six entomopathogens which infect the oat aphid, namely *Conidiobolus* sp., *E. planchoniana*, *P. neoaphids*, *N. fresenii*, *B. bassiana* and *V. lecanii* during 2013 and 2014 wheat growing seasons.

In 2013 season, 147 cadavers were collected from *R. padi* naturally-infected with entomopathogens. The six species were identified.

Statistical analysis showed that *E. planchoniana* followed by *P. neoaphidis* were the most dominant species encountered in 38.26%, and 33.20% of samples respectively. Three species: *N. fresenii*, *B. bassiana* and *V. lecanii* showed a moderate level of infection, while *Conidiobolus* sp. was scarce.

In 2014 season, 659 cadavers of the oat aphids were collected during the whole season. The six species were also collected with the same sequence of dominance in 2013 season.

3- Entomopathogens and their host, *R. padi*

Data in table 2 show that in 2013 season, the aphid began to appear on wheat plants (0.02 individual / tiller) during the first week of January. Its population reached a peak of 4.10 aphids / tiller during the first week of March, then sharply declined to reach 0.80 aphids / tiller during the end of March. The cadavers appeared on wheat plants during the period extended from the beginning of February up to the end of March. The percentage of infection was relatively low, generally <6% during the first of March. Then the level of infection dramatically increased as the aphid population increased. Maximum infection (26.52%) was recorded during the second week of March correlated with 0.31 aphid / tiller.

In 2014 season, the aphid first appeared on wheat plants during the first week of January up

to the end of March with a peak of abundance (6.70 individuals / tiller) during the first half of March. The cadavers were detected after three weeks of aphids observed at the end of January (0.01 cadaver / tiller). Percentage of mycosis increased from 0.79% correlated with 1.25 aphids / tiller during the first week of February to 3.43% correlated with 1.04 aphids / tiller during the end of March. Maximum infection (41.51%) was markedly higher in the season of 2013 and was recorded during the middle of March correlated with 3.10 aphids / tiller.

In general, the mean data of the two seasons showed that the first case of infection by the entomopathogens was observed at the beginning of February with a percent mortality of 0.43% up to the end of March with a percent mortality of 8.45%. Maximum mortality (38.52%) was detected on March 17th.

Several species of entomopathogenic are known to cause fatal diseases in aphids, including *Conidiobolus* sp. *V. lecanii*, various species of *Beauveria*, *P. farinosus* (Holm ex S.F. Gery) Brown & Smith (Roberts & Yendol 1971, Samson *et al*. 1988). Entomopathogenic fungi are frequently reported as major factors suppressing populations of cereal aphids and can cause sudden decline of dense populations (Feng *et al*. 1991). Three entomopathoralean fungi species killed 65-80% of common cereal aphids in in eastern England (Dean and Wilding 1973). Drastic reduction in the populations of various cereal aphids due to infection with Entomopathorales fungi was observed by Dedryver (1983).

Members of order Entomophthorales are excellent candidates for biological control of aphids (Latge and Papierok 1988). Worldwide, *P. neoaphidis* is the most common and frequently the dominant-pathogen of aphids (Waterhouse and Brady 1982). This fungus can cause collapse of unmanaged aphid population within few weeks of the onset of disease (Feng *et al*. 1990). Without management however, the fungus is not effective as control agent alone because it does not attack until the aphid population has peaked and has already caused considerable damage (Feng *et al*. 1991).

Table 1: Numbers and percentages of entomopathogens naturally infecting the oat bird-cherry aphid in the field during 2013 and 2014 wheat-growing seasons.

Fungi species	2013		2014		Total	
	No.	(%)	No.	(%)	No	(%)
Conidiobolus sp.	2c	1.54	3c	0.45	5c	0.62
Entomophthora planchoniana Cornu	56a	38.26	291a	44.16	347a	43.05
Pandora neoaphidis (Remaudierel & Hennebert) Humber	49a	33.20	230a	34.90	279a	34.62
Neozygites fresenii (Nowakowski) Batko	14b	9.53	50b	7.59	64b	7.94
Beauveria bassiana (Balsamo) Vuill.	11b	7.35	34b	5.16	45b	5.58
Verticilium lecanii (Zimmermann) A.W. Viegas	15b	10.12	51b	7.74	66b	8.19
Total	147	100	659	100	806	100

Means vertically followed by the same letter are not significantly different < 0.05 level of probability.

Table 2: Average number of the oat bird-cherry aphid on wheat plants and natural infection rate with entomopathogens in the field during 2013 and 2014 wheat growing seasons.

Inspection date	Growth stage (ZGS)*	2013 season			2014 season		
		No / tiller		Infection (%)	No / tiller		Infection (%)
		Aphids	Cadavers		Aphids	Cadavers	
Jan. 6	28	0.02	0	0	0.02	0	0
13	30	0.07	0	0	0.18	0	0
20	32	0.21	0	0	0.34	0	0
27	33	1.05	0	0	1.25	0.01	0.79
Feb. 3	37	1.62	0.01	0.61	3.19	0.04	1.24
10	39	2.31	0.04	1.60	4.31	0.07	1.59
17	43	3.40	0.09	2.57	4.97	0.10	1.97
24	57	3.87	0.13	3.05	4.99	0.24	4.59
March 3	65	4.10	0.22	5.09	4.13	1.30	23.94
10	71	1.64	0.46	21.90	6.70	2.00	22.99
17	73	0.97	0.35	26.52	3.10	2.20	41.51
24	75	0.31	0.11	26.19	1.51	0.52	25.62
31	77	0.80	0.06	6.98	1.04	0.11	9.57
Total	----	20.37	1.47	6.73	35.73	6.59	15.57

*(ZGS) = A decimal code for growth stage of cereal (Zadoks *et al*. 1974)

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Mycobiota associated with weaning dried foods consumed in Taïz City, Republic of Yemen

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Abstract: A total of thirty-eight fungal species and one variety belonging to 16 genera were recovered from local traditional weaning dried food (12 samples: 27 spp., 11 genera, and cereal-legumes ingredients (40 samples: 34 spp., 15 genera). The total number of CFU of fungi, including yeasts, clearly showed that the local weaning food was seriously contaminated with fungi (45263, calculated per gm in each of the 12 samples comparable to 3068 per gm in each of the cereal legumes). On the other hand, the 12 samples of imported commercial weaning food collected from the pharmacies were fungus-free, which means that the manufacture processes of production eliminate all fungi from their ingredients. *Aspergillus*, *Fusarium* and *Penicillium* were regularly the most common genera, and possessed the broadest spectrum of species during the study, of which the most dominant species were *Aspergillus flavus, A. niger*, *Fusarium oxysporum* and *Penicillium chrysogenum* and *P. corylophilum.*

Key words: Fungi, weaning foods, cereals, legumes, mycobiota.

Introduction

The word weaning means the process of gradually replacing mother's milk or milk substitute with usual family diet (WHO 1989 & 2000, IFIS 2009). Weaning foods include imported commercial and traditional weaning foods products. Commercial weaning foods are easy to prepare, hygienic provided it is packaged, but expensive and not available everywhere locally. On the contrary the traditional weaning foods are cheaper, always available locally (Castel and Wijngaart 2005). Weaning foods made from ingredients do not differ from those for adult foods so that the same types and levels of microorganisms would occur on these ingredients naturally (ACMSF 2006). Traditional weaning foods in Yemen are cereals-legumes blending, composed of staple cereals, mostly: wheat, maize, barley, red and white sorghum, millet and rice blending in equal quantities, besides adequate amount of one of these legumes: black or red lentil or both and sometimes fewer quantities of beans. Since the major components of weaning foods are cereals and legumes, moulds that contaminate cereal and legume grains are divided into two groups, field fungi and storage fungi. Field fungi invade grains in the field that includes species of *Alternaria*, *Cladosporium*, *Fusarium*, *Helminthosporium*, *Curvularia*, *Epicoccum*, *Nigrospora*, and *Stemphylium*. Storage fungi invade grain in storage that includes species of *Aspergillus*, *Penicillium*, *Absidia*, *Chaetomium*, *Mucor*, *Paecilomyces*, *Rhizopus*, and *Scopulariopsis* (Meronuck 1987). Soil, water, air, dust, insects, rodents, birds, animals, humans, storage and shipping containers, and handling and processing equipment are the most important sources of the contaminated cereal grains (Bullerman and Bianchini 2009).

Sayed (2004) tested 30 random samples of locally-manufactured dried baby foods with milk base in Assiut City, Egypt; the average count for yeast and mould was 5 CFU/g. Amusa *et al*. (2005) isolated *Aspergillus niger, A. flavus, A. tamarii, Penicillium oxalicum, Macrophomina phaseolina, Fusarium oxysporum* and *Rhizopus* sp. from dried ogi (fermented weaning food in Africa) and soy-ogi. Olorunfemi *et al*. (2006) reported isolation of *Aspergillus flavus, A. niger, Mucor mucedo, Rhizopus stolonifer* and *Saccharomyces* sp. from different dried milled mixtures of weaning foods with maize or sorghum base. Ismail *et al*. (2008) isolated 42 species belonging to 21 genera in addition to some unidentified fungi from baby food products imported into Uganda. The most predominant fungi were *Cladosporium sphaerospermum, Fusarium tricinctum* and *Penicillium oxalicum.* Aydin *et al*. (2009) tested 142 wheat flour samples and found that the mean of mould counts were 7.4×10 - 1.8×10^4 CFU/g. Ajima *et al*. (2011) isolated 10 species of fungi belonging to 8 genera from ogi and observed that *A. niger, Penicillium* sp. and *Saccharomyces* sp. were the predominant fungal isolates. Oluwafemi and Ibeh (2011) investigated the fungal population in the locally-made weaning foods (one sample) and commercial weaning foods (seven samples). They noted that the locally-made weaning food had greatly higher fungal count (6500 CFU/g)

than the commercial one where the fungal count ranged from 10-50 CFU/g. They isolated *A. niger, A. flavus* and *A. glaucus* from the locally-weaning food; also *Cladosporium* sp., and *Penicillium* sp. from commercial weaning foods. They also pointed out that the low counts in commercial weaning foods are probably due to good food-handling and good manufacture practices. Abanno *et al*. (2012) recorded that the total fungal counts for pap "ogi" and breadfruit flour "seeds of a tree *Treculia africana*" were 1.3×10^1 CFU/ml and 1.0×10^1 CFU/ml respectively. *Rhizopus* spp. were recovered from the dried ogi and breadfruit flour in addition to *Candida* that was isolated from the breadfruit flour only. Adebayo-Tayo *et al*. (2012) found that the total counts of fungi ranged from 1.0 - 4.0×10^3 CFU/g in cereal-based baby foods sold in Nigeria. *A. niger, A. fumigatus, A. terreus, A. flavus, A. glaucus, Rhizopus stolonifer, Penicillium* sp. *F. semithectum, F. proliferatum* and *F. sacchari* were isolated from these samples. Mbakwem-Anitbo and Udemgba (2012) isolated species of *Aspergillus, Rhizopus, Fusarium* and *Saccharomyces* from ogi. The total fungal counts were 3.5×10^6 to 1.35×10^7CFU/g. They also observed there was a great decrease in the fungal counts of salt-treated ogi comparable with untreated one. Ismail *et al*. (2012) mentioned that *Aspergillus, Penicillium, Fusarium* and *Cladosporium* were the most predominant genera in 50 samples of five baby food products mainly made of cereal flour produced in Uganda. Nwogwugwu *et al*. (2012) found that the highest fungal load in wet "Dupap" (novel weaning food composed from pap and African yam bean "Odudu") was 3.2×10^5 CFU/ml, where they isolated *Aspergillus, Rhizopus, Penicillium* and *Candida.*

The present work was designed to evaluate the mycological status of weaning dried food samples (commercial and traditional) consumed in Taïz city, Yemen.

Materials and Methods

A total of 64 random weaning dried food samples (commercial and traditional) were collected from local markets and pharmacies in Taïz city, including 12 commercial weaning food samples, 12 traditional weaning food samples and 40 cereal and legume samples (wheat, barley, corn, rice, millet, red sorghum, white sorghum, black lentil, red lentil and bean) as a total of 4 samples from each kind of cereal and legume. Each sample was put in a sterile polyethylene bag and transferred to the laboratory, where they were prepared for the mycological examination.

Estimation of fungi

The mycobiota of samples was determined using the flour dilution-plate method **(**Pitt and Hocking 2009). Rose-Bengal chloramphenicol agar medium (Oxoid 549) according to Harrigan and McCance (1976) was used. The plates were incubated at 28^{o}C for 7-10 days and the developing fungi were counted and identified (based on macro-and microscopic characteristics) according to the following references: Raper and Thom (1949), Raper and Fennell (1965), Booth (1971), Ellis (1971&1976), Pitt (1979), Domsch *et al*. (2007), Ramirez (1982), Sivanesan (1984), Pitt and Hocking (2009), Koz akiewicz (1989), Moubasher (1993), Firsvad and Filtingborg (1995) and Samson *et al*. (1995).

Results and Discussion

1. Fungi recovered from weaning food samples

A total of 38 species and one variety belonging to 16 genera in addition to some other unidentified species were isolated from 24 commercial and traditional weaning food samples and 40 cereal and legume samples (Table 1). It was noticed that the 12 samples of the imported commercial weaning foods were completely free from any fungi (moulds or yeasts), which means that the manufacture processes eliminate all fungi. However, Ismail *et al*. (2008) identified 42 species of 21 genera in addition to some unidentified fungi from baby food products imported into Uganda on dichloran rose bengal chloramphenicol agar (DRBC). Also, Ismail *et al*. (2012) isolated 16 genera and 48 species on Dichloran 18% glycerol agar (DG18) and 36 genera and 65 species DRBC from baby food products mainly made of cereal flour. Oluwafemi and Ibeh (2011) noticed that the total fungal counts ranged from 10 – 50 CFU/g in the commercial weaning foods samples in Nigeria, greatly lower than in the locally-made weaning food (6500 CFU/g). Sayed (2004) found that the manufactured dried baby foods with milk base in Assiut City, Egypt was 5 CFU/g.

There was high incidence of diverse fungal genera and species in the traditional mix weaning foods (TWF). The total count of fungi in TWF samples was 229105 CFU/g in all samples. Twenty-seven species belonging to 11 genera were recorded from 12 TWF samples on rose-bengal chloramphenicol agar at 28^{o}C (Table 1).

The count of fungal propagules in weaning food samples was broadly varied ranging between 1-4 $x10^3$CFU/g in the flours of cereal base in Nigeria (Adebayo-Tayo *et al.* 2012), 3.5 $x10^6$- 1.35 x 10^7 CFU/g in the ogi (Mbakwem-Anitbo and Udemgba

2012), $3.2x10^5$ CFU/ml in Dupap (Nwogwugwu *et al*. 2012) and $1\text{-}1.3x10^1$ CFU/ml in pap "ogi" and breadfruit flour (Abanno *et al*. 2012).

Aspergillus, *Fusarium* and *Penicillium* were the most common fungi (isolated in high frequency of occurrence). *Aspergillus* was the most predominant genus; it was encountered in 75 % of the samples comprising 6.77 % of total filamentous fungi. Our results agreed with those of Ajima *et al*. (2011), who reported that *Aspergillus* (*A. niger*) was the most common (in addition to other fungi) from ogi. Also, Ismail *et al*. (2012) reported that *Aspergillus* was the most frequent genus recovered from locally-made baby food products in Uganda.

Aspergillus was represented by 9 species of which *A.niger* and *A. flavus* were the most common occurring in 25% of the samples contributing 47.3 % and 1.3% of total *Aspergillus* and 3.2% and 0.09% of total counts of fungi respectively. *A. fumigatus* and *A. niveus* were isolated in low frequency, emerging in 16.7 % of the samples constituting 23.65% and 2.8% of total *Aspergillus* and 1.6% and 0.19% of total fungi respectively. The remaining *Aspergillus* species were isolated from one sample only. *A. niger* and *A. flavus* were recorded from weaning dried foods in some countries by several workers (Amusa *et al*. 2005, Olorunfemi *et al*. 2006 and Oluwafemi and Ibeh 2011, Ajima *et al*. 2011 and Adebayo-Tayo *et al*. 2012).

Ismail *et al*. (2012) isolated *A. flavus* in high frequency, *A. niger* and *A. ochraceus* in moderate frequencies, *A. fumigatus*, *A. oryzae*, *A. tamarii* and *A. versicolor* in low frequencies and *A. parasiticus*, *A. sydowii* and *A. terreus* in rare frequencies from cereal baby foods locally produced in Uganda.

Fusarium and *Penicillium* occupied the second place in the number of cases of isolation in high frequency. *Fusarium* occurred in 66.7% of the samples comprising 3.54% of total fungi. Almost similar results were obtained from locally-prepared cereal-based infant weaning food in Jos, Nigeria (Ikah *et al.* 2001) and from the ogi (Mbakwem-Anitbo and Udemgba 2012). From *Fusarium*, six species were identified of which *F. oxysporum* was recovered in low frequency, occurring in 19% of samples constituting 28.4% of total *Fusarium.* This species was previously recovered from dried ogi and soy-ogi (Amusa *et al*. 2005) and from baby food products imported or produced in Uganda (Ismail *et al*. 2012). *F. avenaceum*, *F. culmorum*, *F. solani*, *F. sporotrichiodes* and *F. verticillioides* were rarely recovered comprising 0.06%, 0.01%, 0.01%, 1.63% and 0.82% of total fungi respectively. Most of these species were previously recovered from baby food products in Uganda (Ismail *et al*. 2008, 2012).

Penicillium occurred in 66.7% of samples contributing 2.45 % of total fungi. Several studies reported the isolation of *Penicillium* spp. from baby food samples (Ajima *et al*. 2011, Oluwafemi and Ibeh 2011, Adebayo-Tayo *et al*. 2012 and Nwogwugwu *et al*. 2012). In the present investigation, it was represented by six species of which *P. chrysogenum* and *P. corylophilum* were the most common (16.7% of samples) comprising 4.8% and 6.55% of total *Penicillium* respectively. *P. citrinum*, *P. funiculosum*, *P. glabrum* and *P. nalgiovense* were rarely isolated. Our results agreed with those of Ismail *et al*. (2008) who reported six species of which *P. citrinum* and *P. corylophilum* were recorded from the imported baby foods into Uganda. Also, Ismail *et al*. (2012) identified *P. chrysogenum*, *P. corylophilum* and *P. citrinum* in the studied samples from cereal baby food samples produced in Uganda.

Cladosporium (represented by *C. cladosporioides* was isolated in moderate frequency from 33.3% of samples comprising 29.12% of total fungi. *Phoma sorghina* came behind C. *cladosporioides* in 25% of samples (low frequency) contributing 17.5% of total count of fungi. Our results are in harmony with the findings of Ismail *et al*. (2008, 2012), who recorded *Cladosporium cladosporioides* from baby food products imported into Uganda and *C. cladosporioides* and *Phoma* sp. from baby food produced in Uganda. *Cladosporium* sp. was recovered from ogi (Ajima *et al*. 2011) and from commercial weaning foods (Oluwafemi and Ibeh 2011).

Mucor spp. were isolated in low frequency of occurrence in 16.7% of samples comprising 0.04% of total fungi. *Curvularia pallescens*, *Epicoccum nigrum*, *Rhizopus stolonifer* and *Ulocladium botrytis* were isolated from one sample (8.3 % of samples) matching collectively 0.01% of total fungi except *Epicoccum nigrum* from 0.15% of total fungi. Some studies recorded the isolation of *Rhizopus* spp. such as those of Ikah *et al.* (2001), Amusa *et al*. (2005), Abanno *et al*. (2012), Mbakwem-Anitbo and Udemgba (2012) and Nwogwugwu *et al.* (2012), while others reported the isolation of *Rhizopus stolonifer* such as those recorded by Olorunfemi *et al*. (2006), Adebayo-Tayo *et al*. (2012) and Ismail *et al.* (2008, 2012). Ajima *et al*. (2011) noticed that *Alternaria* sp., *Curvularia* sp. and *Mucor* sp. were found in ogi. Ismail *et al*. (2008) isolated *Curvularia* (*C. ovoidea* and *C. trifolii*), *Geotrichum candidum*, and *Mucor plumbeus* from food products imported into Uganda. *Mucor* (*M. plumbeus* and *M. racemosus*), *Curvularia* sp,, *Geotrichum candidum* and *Epicoccum nigrum* were also recovered from baby food products produced in Uganda (Ismail *et al.* 2012).

2. Fungi recovered from cereal and legume samples

The data in Table (2) show that all samples examined were heavily contaminated with fungi and the highest count (30500 CFU/g) was recorded in red sorghum samples followed by maize (5346 CFU/g), millet (2733 CFU/g), barley samples (293 CFU/g), red lentil (211 CFU/g) and the lowest count was recorded in rice (4 CFU/g).

According to the number of genera, barley was the most contaminated with genera (9 genera) and bean was the least contaminated (3 genera). However, regarding the number of fungal species, white sorghum samples were contaminated with the highest number (12 species) followed by maize, red sorghum and lentil samples (each 10 species) and the lowest number was in rice (4 species).

The gross total count of fungi in all cereal and legume samples was 157771 CFU/g representing 34 species and one variety belonging to 14 genera on rose bengal chloramphenicol agar at 28^{o}C. *Aspergillus*, *Fusarium* and *Penicillium* were the most common (high or moderate frequency of occurrence). These results are in agreement with El-Kady *et al*. (1982) who noticed that the most common genus in some kinds of cereals in Egypt was *Aspergillus* followed by *Fusarium*, *Penicillium* and *Rhizopus*. Trung *et al*. (2001) found that the most frequent fungi in Vietnamese rice were *Aspergillus* (43.75 %), *Fusarium* (21.8%) and *Penicillium* (10.9 %). Ismail *et al*. (2003) observed that *Aspergillus*, *Fusarium*, *Penicillium*, *Khuskia*, *Chaetomium* and *Cladosporium* were the most frequent genera in maize grain in Uganda.

Aspergillus was also the most prevalent genus, occurring in 72.5% of the samples constituting 2.7% of total filamentous fungi. Similarly, *Aspergillus* was the main component of the mycobiota of barley (Abdel-Kader *et al*. 1979), millet (Mishra and Daradhiyar 1991), wheat (Berghofer *et al*. 2003) and maize (Ismail *et al*. 2003 and Trung *et al*. 2008). From this genus, 13 species and one variety were identified, of which *A. niger* was the most common, emerging from 35% of the samples comprising 11.74% of total *Aspergillus* and 0.30% of total fungi. The total counts of this species fluctuated between 1-51 CFU/g showing the highest count in millet samples and the lowest in rice samples. Samples of maize and black lentil were free from this species, but the rest of cereals were with different counts. Our results are in similar trend with other reports on barley (Abdel-Kader *et al*. 1979), cereals (El-Kady *et al*. 1982), sorghum (Hussaini *et al*. 2009). *A. niger* was also reported from pearl millet (Badau 2006), wheat flours (Tahani *et al*. 2008), sorghum (Islam *et al*. 2009) and rice (Gopalakrishnan *et al*. 2010).

A. flavus was isolated from 27.5% of the samples contributing 21.36% of total *Aspergillus* and 0.55% of total fungi. All kinds of studied cereals were contaminated with this species except wheat and bean samples. This species was previously isolated from different kinds of cereals but with different frequencies and counts such as those reported from barley (Abdel-Kader *et al*. 1979), cereals (El-Kady *et al*. 1982, Lee *et al*. 1986 and Jakić-Dimić *et al*. 2009), millet (Mishra and Daradhiyar 1991), rice (Osman *et al*. 1999, Trung *et al*. 2001 and Gopalakrishnan *et al*. 2010), wheat (Tahani *et al*. 2008), maize (Trung *et al*. 2008 and Makun *et al*. 2010) and sorghum (Hussaini *et al*. 2009 and Islam *et al*. 2009).

A. ochraceus was isolated in low frequency of occurrence from 20% of the samples contributing 51.27% of total *Aspergillus* and 1.3% of total fungi. It occurred in samples of maize, millet, red sorghum and bean with counts varied from 5 CFU/g in bean to 467 CFU/g in red sorghum. Mislivec *et al*. (1975) isolated *A. ochraceus* from dried beans, Trung *et al*. (2001) from Vietnamese rice and Trung *et al*. (2008) from maize.

A. candidus and *A. fumigatus* were also isolated in low frequency of occurrence from 10 and 12.5 % of samples contributing 2.8 and 3.5 % of total *Aspergillus* and 0.07 and 0.09 of total fungi respectively. Some reports recorded *A. fumigatus* from different kinds of samples, barley (Abdel-Kader *et al*. 1979), cereals (El-Kady *et al*. 1982), millet (Badau 2006), wheat (Tahani *et al*. 2008) and maize (Makun *et al*. 2010). Trung *et al*. (2008) examined twenty-five samples of maize and isolated *A. flavus, A. niger, A. wentii, A. glaucus, A. ochraceus, A. restrictus, A. ornatus and A. candidus*. The species recorded in the current investigation were infrequently recovered from grains in many parts of the world (Abdel-Kader *et al*. 1979, El-Kady *et al*. 1982, Badau 2006, Tahani *et al*. 2008, Makun *et al*. 2010 and Trung *et al*. 2008). The remaining *Aspergillus* species were isolated from one sample only and these were *A. oryzae A. parasiticus*, *A. sydowii*, *A. terreus*, *A. ustus*, *A. versicolor* and *A. wentii*. They comprised collectively 1.04% of total *Aspergillus* and 0.03% of total fungi. Most of these species were recovered from some types of cereals as reported by many workers as follows: *A. parasiticus* from millet (Mishra and Daradhiyar 1991) and from rice (Osman *et al*. 1999), *A. sydowii* from barley (Kader *et al*. 1979), *A. wentii* from maize (Trung *et al*. 2008), and *A. versicolor* from bean (Mislivec *et al*. 1975) and from Korean cereals (Lee *et al*. 1986).

Fusarium was the second most common genus and was recovered in moderate frequency (50 % of the samples) comprising 18.7% of total fungi. The average total counts of *Fusarium* widely fluctuated between 1 - 5052 CFU/g. The highest count was recorded in maize and the lowest in beans. The dominance of *Fusarium* in maize was also reported from Egypt (El-Kady *et al*.

1982, Moubasher *et al*. 1982), Uganda (Ismail *et al*. 2003) and in rice from Vietnam (Trung *et al*. 2001). Samples of maize, red sorghum and millet were heavily contaminated by *Fusarium*, while bean and wheat were less contaminated. On the other side, samples of rice and black lentil were free from *Fusarium* species. Eight species of the genus were identified of which *F. oxysporum* was the most common, occurring in 30% of the samples constituting 29.1% of total *Fusarium* and 5.4% of total fungi. *F. solani* was isolated in low frequency (15% of the samples) constituting 12.8% of total *Fusarium* and 2.4% of total fungi. Abdel-Kader *et al*. (1979) isolated *F. oxysporum* and *F. solani* from barley. El-Kady *et al*. (1982), Lee *et al*. (1986) and Aran and Eke (1987) recorded *F. oxysporum* from different cereal samples in Egypt, Korea and Turkey respectively. Gopalakrishnan *et al*. (2010) isolated *F. solani* from rice in India. The rest of *Fusarium* species were isolated in rare frequency and these were *F. avenaceum*, *F. graminearum*, *F. culmorum*, *F. semitectum*, *F. sporotrichiodes*, contributing collectively 58.1% of total *Fusarium* and 10.85% of total fungi. Lee *et al*. (1986) identified *F. germinearum* from cereal samples. Abdel-Kader *et al*. (1979), Trung *et al*. (2008), Islam *et al*. (2009) and Gopalakrishnan *et al*. (2010) isolated *F. verticillioides* (*F. moniliforme*) from barley, maize, sorghum, and rice respectively.

Penicillium came behind *Fusarium* and was encountered in 32.5% of the samples comprising 0.96% of total fungi. The counts of this genus widely varied from 1-175 CFU/g and the highest count was recorded in maize, while the lowest was in bean and rice. Samples of maize and millet were the most contaminated and bean and rice were the less contaminated, whereas red sorghum and black lentil were free from this genus. Some reports recorded the isolation of *Penicillium* in the first and/or second order of frequencies of occurrence such as Abdel-Kader *et al*. (1979), Aran and Eke (1987), Mishra and Daradhiyar (1991), and Berghofer *et al*. (2003). Four species of *Penicillium* were identified and these were *P. citrinum*, *P. funiculosum*, *P. glabrum* and *P. corylophilum*. They occurred in 2.5-5% of the samples constituting 46.4%, 26.5 %, 2.19 % and 0.7% of total *Penicillium* and 0.44%, 0.25 %, 0.13% and 0.006% of total fungi respectively. *P. citrinum* was isolated from bean (Mislivec *et al*. 1975), barley (Abdel-Kader *et al*. 1979), and rice (Trung *et al*. 2001), whereas *P. corylophilum* was isolated from cereals (El-Kady *et al*.1982).

Cladosporium cladosporioides and *Mucor* spp. were recovered in low frequencies (each 22.5% of the samples contributing of 8.6% and 1.4% of total fungi respectively) in samples of barley, white and red sorghum, black and red lentil. Jakić-Dimić *et al*. (2009) identified *Mucor* spp. in samples of corn, wheat, bran, silage, barley, soybean, and sorghum in Serbia. The count of this genus fluctuated between 7- 442 CFU/g, showing the highest number in red sorghum and the lowest in black lentil (Table 2). Count of *C. cladosporioides* greatly varied from 1- 3333 CFU/g and the highest count was recorded in red sorghum and the lowest in millet and red lentil, while rice, black lentil and bean samples were completely uncontaminated with this fungus (Table 2). Several studies reported the isolation of *Cladosporium* spp. from cereals (Lee *et al*., 1986 and Aran and Eke 1987), wheat (Berghofer *et al*. 2003) and maize (Ismail *et al*. 2003).

Rhizopus stolonifer was isolated in low frequency from 12.5% of the samples (wheat, barley, red sorghum, and black lentil). Its count fluctuated between 1-10 CFU/g showing the highest in wheat and the lowest in barley and black lentil samples (Table 2). This fungus was isolated from different cereals, namely barley (Abdel-Kader *et al*. 1979), cereals (El-Kady *et al*. 1982 and Aran and Eke 1987), millet (Mishra and Daradhiyar 1991 and Badau 2006) and wheat (Tahani *et al*. 2008).

Alternaria alternata, *Curvularia pallescens* and *Emericella nidulans* were recovered in rare frequency (each 7.5% of samples) contributing 0.09%, 0.02% and 0.05% of total fungi respectively. *A. alternata* occurred in wheat and black lentil samples, *C. pallescens* in barley, millet, and rice. Whereas, *E. nidulans* was isolated from wheat, red and black lentil. *Drechslera* sp. was isolated from 5% of the samples of wheat and sorghum only comprising 0.004% of total fungi. It was isolated from wheat and white sorghum (Table 1). Abdel-Kader *et al*. (1979) isolated *Drechslera* and *Alternaria* from barley grain samples in Egypt. Lee *et al*. (1986) observed presence of *Alternaria* spp., *Drechslera* spp. and *Emericella nidulans* in cereal samples in Korea. *A. alternata* was also isolated by Zur *et al*. (2002) from cereal grains. *E. nidulans* was also recorded by Badau (2006) and Tahani *et al*. (2008) from millet and wheat respectively. *Curvularia* was isolated from millet (Mishra and Daradhiyar 1991) and rice (Gopalakrishnan *et al*. 2010). Also, Islam *et al*. (2009) identified *Curvularia lunata* in 17.4% of sorghum grains.

Epicoccum nigrum, *Geotrichum candidum*, *Moniliella suaveolens* and *Phoma sorghina* were isolated from one sample only (each 2.5% of the samples) contributing 0.002%, 0.002%, 0.06% and 0.002% of total fungi respectively. Lee *et al*. (1986) isolated *Epicoccum* sp. from Korean cereal. Tahani *et al*. (2008) isolated *Geotrichum* from wheat.

Table 1: Total counts (TC, CFU/g in all samples) and percentage frequencies (%F) of fungal genera and species recovered from 12 traditional weaning food and 40 cereals and legumes samples on Rose Bengal chloramphenicol (Oxoid CM 549) agar at 28°C.

Genera and Species	Traditional weaning food		Cereals and legumes	
	TC	%F	TC	%F
Alternaria alternata (Fries) Keissler			140	7.5
Aspergillus	15498	75	4055	72.5
A. awamori Nakazawa	133	8.33	203	5
A. candidus Link	333	8.33	114	10
A. flavus Link	200	25	866	27.5
A. flavus var. *columnaris* Raper & Fennell			133	5
A. fumigatus Fresenius	3666	16.67	142	12.5
A. niger van Tieghem	7334	25	476	35
A. niveus Blochwitz	433	16.67		
A. ochraceus Wilhelm	3333	8.33	2079	20
A. oryzae (Ahlburg) Cohn			20	2.5
A. parasiticus Speare			7	2.5
A. sydowii (Bain. & Sart.) Thom & Church			3	2.5
A. terreus Thom	33	8.33	3	2.5
A. ustus (Bainier) Thom & Church			3	2.5
A. versicolor (Vuillemin) Tiraboschi	33	8.33	3	2.5
A. wentii Wehmer			3	2.5
Cladosporium cladosporioides (Fresenius) de Vries	66710	41.67	13615	22.5
Curvularia pallescens (Tsuda & Ueyama) Sivanesan	33	8.33	39	7.5
Drechslera sp.			6	5
Emericella nidulans (Eidam) Vuill.			73	7.5
Epicoccum nigrum Link	333	8.33	3	2.5
Fusarium	8099	66.67	29469	50
*F. avenaceum (*Corda: Fr.) Saccardo	133	8.33	4034	5
F. culmorum (Smith) Saccardo	33	8.33	1567	2.5
F. graminearum Schwabe			6510	5
F. oxysporum Schlechtendal	2300	25	8562	30
F. semitectum Berkeley & Ravenel			733	2.5
F. solani (Martius) Sacchardo	33	8.33	3780	15
F. sporotrichiodes Sherb.	3733	8.33	4267	2.5
F. verticillioides (Sacchardo) Nirenberg	1867	8.33	13	2.5
Geotrichum candidum Link			3	2.5
Moniliella suaveolens (Lind. & Lind.) V.Arx			100	2.5
Mucor spp.	100	16.67	2222	22.5
Penicillium	5603	66.67	1509	32.5
P. chrysogenum Thom	266	16.67		
P. citrinum Thom	133	8.33	700	5
P. corylophilum Dierckx	367	16.67	10	2.5
P. funiculosum Thom	67	8.33	400	5
P. glabrum (Wehmer) Westling	367	8.33	33	5
P. nalgiovense Laxa	67	8.33		0
Penicillium spp.	4336	25	196	12.5
Phoma sorghina (Sacchardo) Boerema *et al.*	40073	25	3	2.5
Rhizopus stolonifer (Ehrenberg) Vuillemin	33	8.33	79	12.5
Sterile mycelia	39252	50	106455	32.5
Ulocladium botrytis Preuss	33	8.33		
Total count of fungi	229105	100	157771	100
Number of genera (16 genera)	11		15	
Number of species (38 + 1 variety)	27		34+1 variety	

Results of the 12 samples of imported weaning food are omitted because they were, all nil.
%F= Percentage frequency for traditional weaning food, H = High occurrence >50% of total samples, M = Moderate occurrence, >25 – 50%, L = Low occurrence, 16.67 – 25%, R = Rare occurrence < 16.67.

Table 2 : Total counts (TC, calculated CFU/g), and percent frequency (F %) of fungal genera and Species recovered from tested cereals, legumes and weaning foods samples on rose Bengal chloramphenicol agar at 28 °C.

	Wheat		Maize		Barley		Millet		White sorghum		Red sorghum		Rice		Red lentil		Black lentil		Bean	
Genera and species	TC	F%	TC	F%	TC	F%	TC	F%	TC	F%	TC	F%	TC	F%	TC	F%	TC	F%	TC	F%
Alternaria alternata	2	25	0	0	0	0	0	0	0	0	0	0	0	0		0	33	50	0	0
Aspergillus	37	50	36	50	98	100	122	75	43	100	633	75	1	25	20	100	68	75	12	75
A. awamori	0	0	0	0	0	0	0	0	0	0	50	25	0	0	1	25	0	0	0	0
A. candidus	2	25	0	0	2	25	0	0	8	25	17	25	0	0	0	0	0	0	0	0
A. flavus	0	0	17	25	86	50	42	50	18	25	42	25	1	25	11	50	2	25	0	0
A. flavus var. *columnaris*	0	0	0	0	0	0	0	0	0	0	33	50	0	0	0	0	0	0	0	0
A. fumigatus	10	25	0	0	8	25	0	0	8	25	0	0	0	0	0	0	9	50	0	0
A. niger	25	25	0	0	3	25	51	50	2	25	25	25	1	25	8	50	0	0	6	50
A. niveus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
A. ochraceus	0	0	19	50	0	0	29	50	0	0	467	50	0	0	0	0	0	0	5	50
A. oryzea	0	0	0	0	0	0	0	0	5	25	0	0	0	0	0	0	0	0	0	0
A. parasticus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	25	0	0
A. sydwii	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	25	0	0	0	0
A. terreus	0	0	0	0	0	0	0	0	1	25	0	0	0	0	0	0	0	0	0	0
A. ustus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	25	0	0
A. versicolor	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	25
A. wentii	0	0	0	0	0	0	0	0	1	25	0	0	0	0	0	0	0	0	0	0
Cladosporium cladosporioides	3	50	8	25	42	25	1	25	17	50	3333	25	0	0	1	25	0	0	0	0
Curvularia pallescens	0	0	0	0	1	25	8	25	0	0	0	0	1	25	0	0	0	0	0	0
Drechslera sp.	1	25	0	0	0	0	0	0	1	25	0	0	0	0	0	0	0	0	0	0
Emericella nidulans	17	25	0	0	0	0	0	0	0	0	0	0	0	0	1	25	1	25	0	0
Epicoccum nigrum	0	0	0	0	1	25	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Fusarium	11	50	5052	100	62	50	953	100	55	75	1150	50	0	0	84	50	0	0	1	25
F. avnaceum	0	0	42	25	0	0	0	0	0	0	967	25	0	0	0	0	0	0	0	0
F. culmorum	0	0	392	25	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
F. graminearum	3	25	1625	25	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
F. oxysporum	8	25	1558	50	62	50	444	75	52	50	0	0	0	0	17	25	0	0	1	25
F. semitectum	0	0	0	0	0	0	0	0	0	0	183	25	0	0	0	0	0	0	0	0
F. solani	0	0	369	50	0	0	508	50	0	0	0	0	0	0	68	50	0	0	0	0
F. sporotrichiodes	0	0	1067	25	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
F. verticillioides	0	0	0	0	0	0	0	0	3	25	0	0	0	0	0	0	0	0	0	0
Geotrichum candidum	0	0	0	0	0	0	0	0	1	25	0	0	0	0	0	0	0	0	0	0
Moniliella suaveolens	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	25	25	0	0
Mucor spp.	0	0	0	0	8	25	0	0	13	50	442	25	0	0	86	75	7	25	0	0

Genera and species	Wheat		Maize		Barley		Millet		White sorghum		Red sorghum		Rice		Red lentil		Black lentil		Bean	
	TC	F%	TC	F%	TC	F%	TC	F%	TC	F%	TC	F%	TC	F%	TC	F%	TC	F%	TC	F%
Penicillium	4	25	175	25	36	50	100	50	42	25	0	0	1	25	19	75	0	0	1	25
P. chrysogenum	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
P. citrinum	0	0	175	25	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	25
P. corylophilum	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	25	0	0	0	0
P. funiculosum	0	0	0	0	0	0	100	50	0	0	0	0	0	0	0	0	0	0	0	0
P. glabrum	0	0	0	0	0	0	0	0	42	25	0	0	0	0	8	25	0	0	0	0
P. nalgiovense	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Penicillium spp.	4	25	0	0	36	50	0	0	0	0	0	0	1	25	8	25	0	0	0	0
Phoma sorghina	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	25	0	0
Rhizopus stolonifer	10	50	0	0	1	25	0	0	0	0	8	25	0	0	0	0	1	25	0	0
Ulocladium botrytis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Sterile mycelia	0	0	75	25	45	75	1549	75	10	25	24933	75	1	25	0	0	8	25	0	0
Total count of fungi	83	100	5346	100	293	100	2733	100	181	100	30500	100	4	50	211	100	110	100	13	75
Number of genera = 16	8		5		9		6		8		6		4		6		8		3	
Number of species =38+1 var.	9		10		9		8		12		9+1		3		10		9		5	

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