International Journal of Microbiology and Application
2018; 5(3): 50-55
http://www.openscienceonline.com/journal/ijma
ISSN: 2381-442X (Print); ISSN: 2381-4438 (Online)

Open Science

Scanning Electron Microscopy of Fungi Isolated from Some Cake Samples

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To cite this article

Sherif Mohamed El-Kadi, Husain Abdullah El-Fadaly, El-Said MetwalyEl-Gayar. Scanning Electron Microscopy of Fungi Isolated from Some Cake Samples. *International Journal of Microbiology and Application.* Vol. 5, No. 3, 2018, pp. 50-55.

Received: May 17, 2018; **Accepted**: June 13, 2018; **Published**: July 25, 2018

Abstract

Thirty cake samples of twelve different companies were collected from Damietta and Dakahlia Governorates in September and October 2012. The highest value of total fungal counts was in C12 sample being 90.27×10^2cfu/g and the lowest value was in B27 sample being 0.27×10^2cfu/g. All samples were in production date except one sample (A4) was expired. The validity periods of most samples were between 3 and 6 months except one sample (C7) was one year. All samples were not in conformity with the Egyptian Organization for Standardization and Quality of cake (ES: 4037/2005). Fourteen fungal isolates were obtained from cake samples. Identification was based on the visual observation of fungal isolates grown on different cultivation media. The vegetative and reproductive strictness was observed by digital light microscope and scanning electron microscope (SEM). From these characteristics, fungal isolates were identified as *Rhizopus stolonifer*, *Aspergillus niger*, *Aspergillus oryzae*, *Aspergillus flavus* and *Penicillium* sp.

Keywords

Cake, Fungal Examination, *Rhizopus* sp., *Aspergillus* sp., *Penicillium* sp., Scanning Electron Microscope

1. Introduction

Pastries include cakes and baked shells filled with custard, cream, or sauces. They can be spoiled by microorganisms coming with the ingredients that are added after baking, such as icing, nuts, toppings, and cream. Most products, because of low a_w, allow only molds to grow. However, some materials used as fillings may have high a_w, which allows for bacterial growth [1]. The management of microbial food safety has evolved from mainly relying on product testing to process control approaches such as the implementation of Good Manufacturing Practices (GMP) and the Hazard Analysis Critical Control Point (HACCP) principle [2]. Microbiological risk is managed by governmental standards and regulations on distinct levels of food borne hazards that may not be exceeded. The current level of tolerable or acceptable risk the community is willing to accept is a political decision by risk managers and commonly termed the "Appropriate Level of Protection ALOP" [3]. Microbiological food safety is centered on the production of safer foods and mainly ensured by preventive approaches. Its primary goals are to minimize the risks of food borne pathogens and their toxins, reduce the incidence of human disease as well as facilitating domestic and international trade [4]. *Aspergillus* spp. and *Penicillium* spp. are the common genera of fungi generally isolated from the bakery products. These fungi have been known to produce toxins, which are both acutely and chronically toxic for animal and humans [5]. *A. flavus* and *A. parasiticus* producing aflatoxins were isolated from different Egyptian foods. Fungi can produce their mycotoxins naturally in various agricultural products such cake. Fungi cause a significant yield reduction and economic losses because its commonly contaminate foods and crops. Also, they changes the texture, appearance, odor and taste of food, and it unsafe for human consumption because of there mycotoxins. The consumption of foods which contaminated with mycotoxin has been associated with several cases of human poisoning, sometimes resulting in death. Nowadays, fungi producing mycotoxins have been

receiving worldwide attention and several groups of mycotoxins are known such as ergot, aflatoxins, ochratoxins, citrinin, patulin and fumonisines. *Fusarium*, *Aspergillus* and *Penicillium* were noted to be the major fungal populations in feed and foods [6]. The scanning electron microscope (SEM) is a helpful tool to study fungi and their use in industrial processes. It permits study of several aspects of the morphology such as surface details. This tool has been used since the beginning of the 1970s to study these microorganisms [7]. The purpose of this work was to examine the fungal contamination of some cake samples in local markets. Moreover, the fungal isolates were identified based on the microscopic examination (digital light microscope and scanning electron microscope) and the visual observation on different cultivation media.

2. Material and Methods

2.1. Cake Samples and Collection

Thirty samples of different production companies (12 companies) were collected from Damietta and Dakahlia Governorates in September and October 2012 (Table 1).

2.2. Preparation of Cake Samples for Microbiological Examination

Ten gram of each examined sample mentioned above was suspended in 90.0 mL sterilized tap water then serial dilutions were prepared. Each sample was well mixed to be homogenized solution using a Vortex (VM-300 power: 220 VAC, 50Hz, 0.16A/Made in Taiwan-Associated with Cannic, Inc U.S.A.). One mL of each dilution was put into Petri dish, and then melted suitable cultivation medium was poured and mixed well then left to solidify. These Petri dishes were then incubated at the suitable temperature for appropriate period of time (Table 2) and obtained developed single microbial colonies of different morphologies in all used cultivation media were picked-up. Colonies were transferred onto suitable media plates for sub culturing to obtain pure microbial isolates [7].

2.3. Total Fungal Count

One mL of each dilution was transferred to a Petri dish in three triplicates; 15.00mL of melted potato dextrose agar medium was added [8]. The plates were then left to harden and placed Petri dish upturned and incubated at 25°C for 7 days. Obtained results of developed fungal colonies were counted [9]. Single fungal colonies of different morphologies developed on PDA medium were picked-up. Colonies were transferred onto PDA plates for sub-culturing to obtain pure isolates.

2.4. Isolation and Identification of Obtained Fungal Isolates

Isolation was done on PDA medium. Identification was based on the visual observation of fungal isolates grown on PDA and Czapek yeast extract agar media [8], micro-morphological studies in slide culture and the taxonomic keys were followed [10-12].

2.5. Maintenance of Fungal Isolates

Fungal isolates were maintained on potato dextrose agar (PDA) medium slants at 5°C till use. Before use, the fungal strains were sub-cultured on new slants of PDA and incubated at 25°C for 5 days.

2.6. Microscopic Examination

The vegetative and reproductive strictness were observed by digital microscope eye piece with resolution of 10x WF.

2.7. Electron Microscopic Examinations

The SEM technique was carried out at Electron Microscope Unit, Mansoura Univ., Mansoura, Egypt as following: Cultured cells on an agar surface, Fixation, Washing, Post-fixation, Washing again, Dehydration, Critical Point Drying (CPD), Mounting Dried Specimen on SEM Stub, Metal coating and Sputter coating [7, 13, 14 and 15].

3. Results and Discussion

3.1. Total Fungal Count of Cake Samples

The cake samples were differed in size, shape, quality, taste and fillings. The examined cake samples were chocolate cake filling with chocolate; cake with chocolate and other additions; cake with cream and other additions and cream cake. These samples were divided into four groups (Table 1). The first group was chocolate cake, that contained of nine samples. Tow samples (A3 and A5) were imported abroad and seven samples (A1, A2, A4, A8, A14, A15 and A25) were produced in local companies. It was observed that, one sample (A4) was expired and the other eight samples were in the validity period. Total fungal counts were also shown in Table1. The highest value was in A4 sample being 59.13×10^2cfu/g while the lowest value was A5 sample being 0.35×10^2cfu/g. These results are in the same pattern of [16] who tested the presence of yeast and fungi in chocolate cake samples and they found that, the total fungal count was $>10^3$cfu/g.

Table 1 also shown the second group (6 samples) was chocolate cake and other additives. One sample (B6) was imported abroad and five samples (B10, B18, B19, B22 and B27) were produced by local factories. It was observed that, all samples were in the validity period. The total fungal count in samples B6, B10, B18, B19, B22 and B27 were 14.10×10^2, 44.37×10^2, 9.00×10^2, 1.40×10^2, 0.70×10^2 and 0.27×10^2cfu/g, respectively. The highest contaminated samples were B6, B10 and B18. As with bread, mold growth is the predominant spoilage problem for pastries. However, the pastry filling or topping may be more susceptible to microbial growth than cereal product.

The third samples group was cream cake (6 samples and

presented in Table 1). All samples were obtained from local factories and it occur in the validity period. The following four samples D24, D26, D28 and D30 were 0.48×10^2, 1.91×10^2, 3.02×10^2 and 1.2×10^2cfu/g, respectively. Samples D9 and D16 gave 81.42×10^2 and 25.32×10^2cfu/g, respectively. Nine samples of cream cake with other additives samples (the fourth group) are listed in Table 1. Tow samples (C7 and C21) were imported abroad and seven samples (C11, C12, C13, C17, C20, C23 and C29) were obtained by local companies. All samples were in the validity period. The highest value of total fungal counts was in C12 sample being 90.27×10^2cfu/g and the lowest value was in C23 samples being 0.44×10^2cfu/g

Table 1. *Total fungal count and collection of cake samples from different companies.*

Sample No.	Examined cake samples	Productioncompany Code	Date of Production	Expiration	Collection	Validity period (month)	Total fungal count (cfu/g)
A1	Chocolate cake (Frist group)	CO.1	19/6/2012	18/9/2012	9/2012	3	2.25×10^2
A2		CO.1	18/6/2012	17/9/2012	9/2012	3	1.94×10^2
A3*		CO.11	6/2012	12/2012	9/2012	6	1.57×10^2
A4**		CO.5	24/3/2012	25/7/2012	9/2012	4	59.13×10^2
A5*		CO.11	26/5/2012	25/11/2012	9/2012	6	0.35×10^2
A8		CO.6	2/8/2012	1/12/2012	9/2012	4	15.05×10^2
A14		CO.7	6/9/2012	5/1/2013	9/2012	4	20.03×10^2
A15		CO.2	12/9/2012	11/12/2012	9/2012	3	3.19×10^2
A25		CO.8	21/7/2012	20/10/2012	10/2012	3	0.76×10^2
B6*	Chocolate cake and other additives (Second group)	CO.11	25/6/2012	24/2/2013	9/2012	8	14.10×10^2
B10		CO.4	12/7/2012	11/10/2012	9/2012	3	44.37×10^2
B18		CO.1	14/9/2012	13/12/2012	9/2012	3	9.00×10^2
B19		CO.2	2/9/2012	1/12/2012	9/2012	3	1.40×10^2
B22		CO.9	11/6/2012	10/10/2012	9/2012	4	0.70×10^2
B27		CO.2	25/9/2012	23/1/2013	10/2012	4	0.27×10^2
D9	Cream cake (Third group)	CO.3	27/8/2012	26/12/2012	9/2012	4	81.24×10^2
D16		CO.1	29/8/2012	28/11/2012	9/2012	3	25.32×10^2
D24		CO.3	19/9/2012	18/1/2013	10/2012	4	0.48×10^2
D26		CO.2	12/8/2012	10/12/2012	10/2012	3	1.91×10^2
D28		CO.2	25/9/2012	23/1/2013	10/2012	4	3.02×10^2
D30		CO.1	19/9/2012	18/12/2012	10/2012	3	1.20×10^2
C7*	Cream cake and other additives (Four group)	CO.12	4/2012	4/2013	9/2012	12	4.19×10^2
C11		CO.4	12/7/2012	11/10/2012	9/2012	3	12.17×10^2
C12		CO.10	22/7/2012	21/10/2012	9/2012	3	90.27×10^2
C13		CO.1	14/7/2012	13/10/2012	9/2012	3	24.75×10^2
C17		CO.2	10/9/2012	9/3/2013	10/2012	6	31.90×10^2
C20		CO.2	12/9/2012	11/12/2012	9/2012	3	2.38×10^2
C21*		CO.11	28/8/2012	27/2/2013	9/2012	6	2.50×10^2
C23		CO.1	18/9/2012	17/12/2012	10/2012	3	0.44×10^2
C29		CO.3	3/9/2012	2/1/2013	10/2012	4	19.08×10^2

* These products were imported abroad.
**Expired sample

Generally, the highest value of total fungal counts was in C12 sample being 90.27×10^2cfu/g and the lowest value was in B27 samples to be 0.27×10^2cfu/g. All samples were in production date except one sample (A4) was in expired date. The validity periods of most samples were between 3 and 6 months except one sample (C7) was in one year. All samples were not in conformity with the standard specifications of the Egyptian cake No. 4037 [17].

3.2. Isolation and Identification of Cake Samples Contaminating Fungi

Fourteen fungal isolates were obtained from twelve different cake production companies as shown in the Table 2. Fungal isolates were obtained from either chocolate cake or cream cake that divided into five different groups according to the microscopic examination.

Table 2. *Fungal isolates obtained from cake samples and their sources.*

Isolates Group	Isolates No.	Cake Production Companies												∑
		Co1	Co2	Co3	Co4	Co5	Co6	Co7	Co8	Co9	Co10	Co11	Co12	
G1	14, 55		*									*		2
G2	6, 5, 11	*		*		*								3
G3	54, 24, 25		*	*								*		3
G4	53, 41, 42	*	*	*										3
G5	40, 43, 56				*		*	*						3
	∑	2	3	3	1	1	1	1				2		14

* The isolates which chosen for the identification.

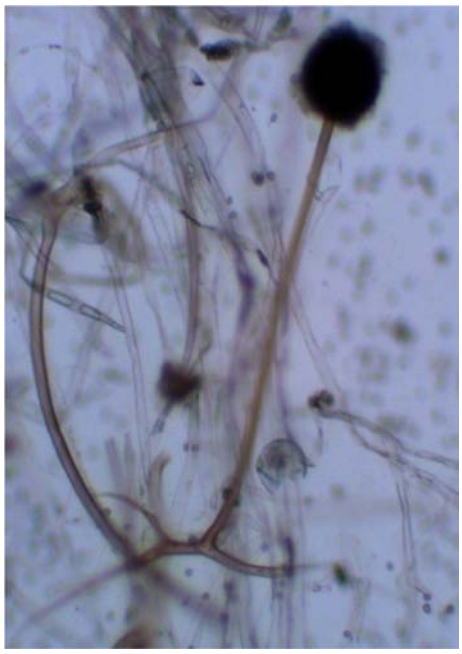

Figure 1. *Morphological characteristics of Rhizopus stolonifer by light microscope (x400).*

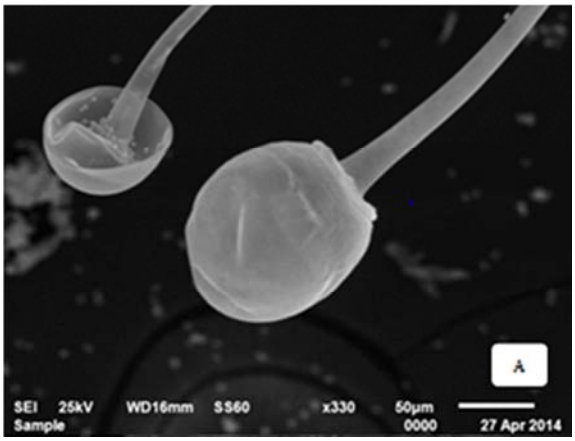

Figure 2. *Morphological characteristics of Rhizopus stolonifer using scanning electronmicroscope.*

Colonies of isolates No. 14 and 55 (G1) grew very rapidly, filled the Petri dish, and mature in 4 days. The texture was typically cotton-candy like on PDA medium. From the edge, the color of the colony was white initially and turns grey to yellowish brown in time. The reverse was white to pale. Non septate or sparsely septate broad hyphae, sporangiophores, rhizoids (root-like hyphae), sporangia, and sporangiospores are visualized (Figures1 and 2). Sporangiophores were brown in color and usually unbranched. They can be solitary or form clusters. Rhizoids are located at the point where the stolons and sporangiophores meet. Sporangiospores are unicellular, round to ovoid in shape, hyaline to brown in color, and smooth or striated in texture. From these characteristics, fungal isolates No. 14 and 55 were identified as *Rhizopus stolonifer* according to the protocol [10].

[5] reported that *Rhizopus stolonifer* was common to breads, biscuits, cakes, patties and buns; *Penicillium oxalicum* was common to breads, cakes, pastries and patties. Also, *Alternaria tenuissima* and *Mucor* sp. were common to breads and pastries. Mould spoilage of bakery products has been the subject of many studies, and a number of species have been implicated. The most widespread and most important in bakery products are species of *Eurotium* sp., *Aspergillus* sp. and *Penicillium* sp.[18]. *Aspergillus* sp. and *Penicillium* sp. are the main spoilage fungi in bakery products [19]. In bakery products with coatings, fillings, or ingredients such as nuts, raisins, jam, or jelly that may be added after baking, contamination with *S. cerevisiae* and more often with xerotolerant yeasts (*Zygosaccharomyces rouxii, Zygo. bisporus, Schizosaccharomyces pombe, Schizo. octosporus*) may cause fermentative spoilage [20].

Mycelium and colonies of isolates No. 5, 6 and 11 (G2) on PDA medium were white, conidial heads dark brown, greenish black, brownish black to black reverse colourless, conidial heads globose, radiate or splitting into several irregular well-defined columns of conidial chains, conidiophores hyaline to brown and smooth-walled vesicles globose to subglobose hyaline to dark brown (Figures 3 and 4). From these characteristics, these isolates were identified and designated as *Aspergillus niger* [11].

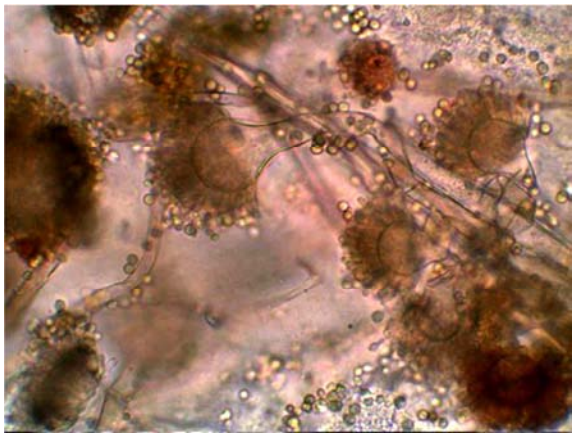

Figure 3. *Morphological characteristics of Aspergillus niger by light microscope (x400).*

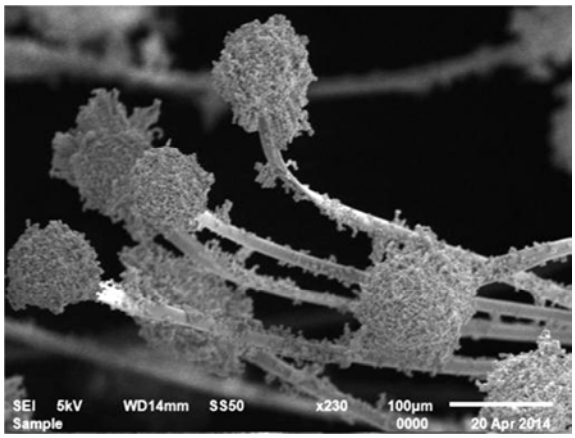

Figure 4. *Morphological characteristics of Aspergillus niger using scanning electronmicroscope.*

Characterization of the isolates No. 24, 25 and 54 (G3) showed that, colonies yellow green on Rose Bengal and PDA media, conidiophores colorless, long, and coarsely roughened. Conidial heads typically radiate conidia globose to subglobose. From these characteristics, those isolates were identified as *Aspergillus oryzae* [11].

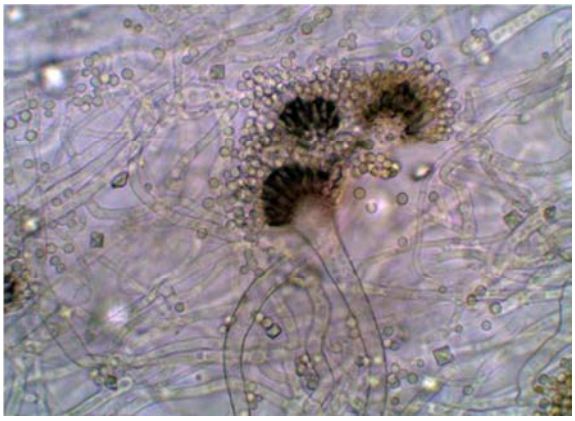

Figure 5. *Morphological characteristics of Aspergillusflavus by light microscope (x400).*

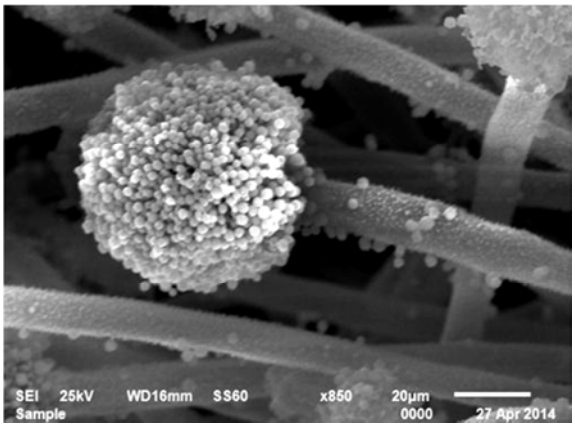

Figure 6. *Morphological characteristics of Aspergillusflavus using scanning electronmicroscope.*

Identification of isolates No. 41, 42 and 53 (G4) showed conidial heads pale to intense yellow green when young, colonies not shifting to brown in age on PDA medium. Conidia definitely echinulate predominance; conidial heads radiate or very loosely columnar, colonies shifting to brownish in age; conidia smooth to roughened; conidiophores arising primarily from the substrate (Figures 5 and 6). From these characteristics, isolates No. 41, 42 and 53 were identified as *Aspergillus flavus* [11].

The colonies of isolates No. 40, 43 and 56 were rapid growing (G5), flat, filamentous, and velvety, woolly, or cottony in texture on PDA medium. The colonies are initially white and become blue green, gray green, olive gray, yellow or pinkish in time. Visualized as globose to elongated sausage-shaped cells that multiply by fission (Figures 7 and 8). From these characteristics, isolates No. 40, 43 and 56 were identified as *Penicillium* sp. [12].

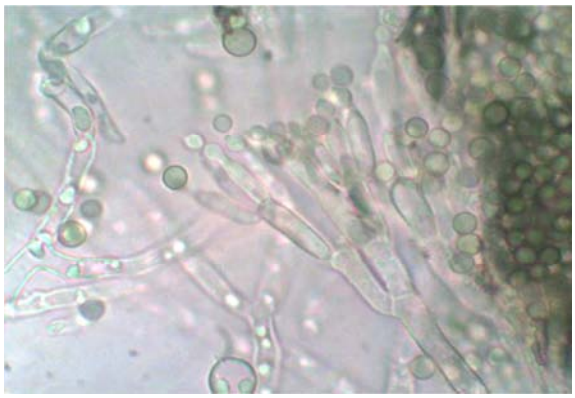

Figure 7. *Morphological characteristics of Penicillium sp. by light microscope (x400).*

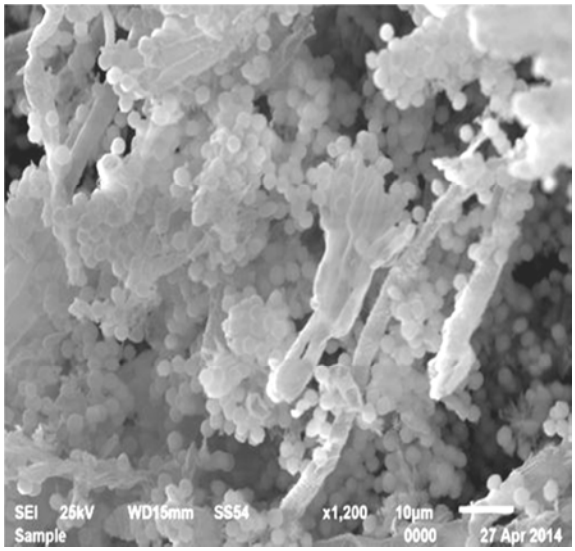

Figure 8. *Morphological characteristics of Penicillium sp. using scanning electronmicroscope.*

All samples were not in conformity with the standard specifications of the Egyptian cake No. 4037 [17] and that is considered a very beg dangerous, because fungi are significant spoilage microorganisms of food, rendering them unfit for human consumption by retarding their nutritive value and sometimes by producing mycotoxins. Fungal growth on cake is a common problem for the cake manufacture. Species of *Penicillium* and *Aspergillus* are common contaminants of cake. By the searching in the medical references, it was observed that, most of this fungi had the ability to human and animal pathogenicity or produced toxins. The growth of toxigenic fungi on cake samples must be considered as a problem of safety for human consumption [21].

4. Conclusion

Generally, the total fungal counts were between 0.27×10^2 and 90.27×10^2cfu/g and in B27 and C12 sample, respectively. All samples were not in conformity with the standard specifications of the Egyptian cake (ES: 4037/2005). The fungal isolates were identified according to

the microscopic examination (digital light microscope and scanning electron microscope) and the visual observation on different cultivation media. Fungal isolates were identified as *Rhizopus stolonifer*, *Aspergillus niger*, *Aspergillus oryzae*, *Aspergillus flavus* and *Penicillium* sp.

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