

Mycotoxins and Invertase Enzyme of the Mycoflora of Molasses in Upper Egypt

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(Received December 6, 2001)

A total of twenty-nine species and one species variety belonging to 12 genera was isolated from 30 samples of molasses on 1% glucose (10 genera, 22 species and 1 variety) and 50% sucrose (7, 21 and 1) Czapek's agar at 25°C media. *Aspergillus*, *Mucor*, *Mycosphaerella* and *Penicillium* were the most common genera on the two types of media. From the above genera, the most prevalent species were: *Aspergillus flavus*, *A. fumigatus*, *A. niger*, *Mycosphaerella tassiana*, *Penicillium chrysogenum*, *P. oxalicum* and *P. purpurogenum*. Also, some species were only isolated on 50% sucrose such as *Eurotium amstelodami*, *E. chevalieri*, *E. repens*, *Humicola fuscoatra*, *Penicillium aurantiogriseum* and *P. puberulum*. About 65 fungal isolates isolated from 50% sucrose agar were tested for their ability to produce invertase enzyme in liquid medium and 93.8% of the isolates could produce this enzyme. From the positive isolates, 32 showed high invertase activity, 21 had moderate activity and the remaining 8 isolates were of weak activity. Sixty isolates of *Aspergillus*, *Emericella*, *Eurotium*, *Mycosphaerella* and *Penicillium* from the preceding study were screened for the presence of their respective mycotoxins. Larva of brine shrimp (*Artemia salina* L.) were used for toxicity test of the fungal crude extracts. Three isolates out of 60 tested were toxic. Using thin-layer chromatographic technique, 5 different known mycotoxin were detected aflatoxins : B1, B2, G1, G2 and citrinin.

KEYWORDS: Invertase enzyme, Mycoflora of molasses, Mycotoxins

Molasses are considered to be one of the most important consumed food in Upper Egypt. It is one of the most important sugarcane products. Molasses has traditionally been marketed as a popular and public food (meal) especially for kids because of its richment with energy and minerals. Molasses is easily invaded by microorganisms. Scott (1989) considered that prolonged storage could permit mould growth particularly if the package is opened. These moulds exhibit the potential to produce toxic metabolites (Northolt *et al.*, 1995). Therefore, proliferation of the organisms is to be regarded as a potential health hazard. Many studies were made in the uses of microorganisms in transformation and fermentation of molasses into ethanolic and organic acid products (Galazzo and Bailely, 1990; Nigam *et al.*, 1997; Nolan *et al.*, 1994; Parvez *et al.*, 1998; Rohr *et al.*, 1983; Sheroran *et al.*, 1998).

Several fungal isolates are able to produce extracellular enzymes in the substrate such as invertase (Abdel-Sater and Ismail, 1993; Abdel-Sater and Saber, 1999).

Moulds contamination on molasses affects the quality and nutritional value of the products, and molasses may contain toxic moulds metabolites (Barakat *et al.*, 2000) as a result of mould growth. The secondary metabolites of fungi known as mycotoxins have gained considerable importance during the past two decades as health hazards to animals and man (Purchase, 1974). Toxicity and toxin production by members of the genera *Aspergillus*, *Emericella*, *Eurotium*, *Mycosphaerella* and *Penicillium* were extensively investigated in this laboratory (Abdel-Mallek *et*

al., 1993; Abdel-Sater and Saber, 1999; Abdel-Sater *et al.*, 1996; Barakat *et al.*, 2000; El-Kady and Abdel-Hafez, 1981; El-Kady *et al.*, 1994; El-Maraghy and Zohri, 1988).

Studies have not been conducted on fungal flora and mycotoxins of molasses in Upper Egypt. Thus, the objective of this study was to investigate the mycoflora of molasses. In addition, the ability of these fungi for production of invertase enzyme and mycotoxins were examined as well.

Materials and Methods

Collection of samples. Thirty samples of molasses were collected randomly from different markets in Upper Egypt (Sohage, Qena and Aswan) during the winter of 2000. Samples were transferred to the laboratory and kept at 4 °C in a refrigerator till fungal analysis.

Determination of fungi. The dilution-plate method was used for determination of molasses moulds (Johnson and Curl, 1972). Two types of media were used : glucose- and 50% sucrose Czapek's Dox agar for isolation of glucophilic and osmophilic (or osmotolerant) fungi, respectively. Rose bengal (30 mg/l) and chloramphenicol (200 mg/l) were added to the above media as bacteriostatic agents. Six plates were used for each examined samples (3 plates for each medium). Plates were incubated at 25 °C for 7 days in the case of glucose and 15 days for sucrose plates. The developing fungal colonies were counted, identified and the numbers were calculated per ml of molasses.

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Screening for invertase production. Sucrose hydrolysis by the isolates recovered on 50% sucrose were tested on liquid medium of 50% sucrose-Czapek's. After the incubation at 28°C for 10 days, the sucrose (invertase) activity was determined in the culture filtrate using fehling's solution. The positive result was indicated by a yellow, green or brown precipitate.

Cultivation and extraction of the fungal toxins. The test fungi were collected from the molasses samples on 50% sucrose. All isolates were cultivated on Czapek's liquid medium fortified by 2 g yeast extract and 10 g pep-

tone and incubated at 28°C for 10 days at static cultures. After the incubation period, the content of each flask (medium + mycelium) was homogenized for 5 min in a high speed blender (1600 rpm) with 100 ml chloroform. The extract procedure was repeated three times. The combined chloroform extracts were washed with equal volume of distilled water, dried over anhydrous sodium sulphate, filtered then concentrated under vacuum to near dryness, and diluted to 1 ml with chloroform.

Thin-layer chromatographic separation of mycotoxins. For qualitative identification, a thin-layer chromatographic

Table 1. Total counts (TC per ml), number of cases of isolation (NCI, out of 30 samples), occurrence remark (OR) and percentage frequency (F) of fungal genera and species recovered from molasses on glucose and 50% sucrose-Czapek's agar at 25 °C

Genera and species	1% Glucose				50% Sucrose			
	TC	% F	NCI & OR	% F	TC	% F	NCI & OR	% F
<i>Aspergillus</i>	12675	79.1	25H	83.3	8150	53.4	25H	83.3
<i>A. flavus</i> Link	6325	39.5	22H	73.3	3600	23.6	25H	83.3
<i>A. fumigatus</i> Fresenius	1175	7.3	17H	56.7	925	6.1	18H	60
<i>A. niger</i> Van. Tieghem	4025	25.1	20H	66.7	3125	20.5	21H	70
<i>A. ochraceus</i> Wilhelm	125	0.78	4L	13.3	—	—	—	—
<i>A. sydowii</i> (Bain & Sart.) Thom & Church	275	1.7	4L	13.3	75	0.5	2R	6.7
<i>A. terreus</i> Thomt	375	2.3	5L	16.7	250	1.6	6L	20
<i>A. terreus</i> var. <i>aureus</i> Thom	125	0.8	4L	13.3	50	0.3	2R	6.7
<i>A. ustus</i> (Bainier) Thom & Church	125	0.8	4L	13.3	—	—	—	—
<i>A. versicolor</i> (Vuill.) Tiraboschi	125	0.8	4L	13.3	125	0.8	2R	6.7
<i>Emmericella nidulans</i> (Edam.) Vuill	150	0.94	3R	10	250	1.6	5L	16.7
<i>Eurotium</i>	—	—	—	—	1250	8.2	16H	53.3
<i>E. amstelodami</i> Mangin	—	—	—	—	250	1.6	6L	20
<i>E. chevalieri</i> Mangin	—	—	—	—	925	6.1	16H	53.3
<i>E. repens</i> De Bary	—	—	—	—	75	0.5	3R	10
<i>Humicola fuscoatra</i> Traaen	—	—	—	—	550	3.6	2R	6.7
<i>Mucor</i>	325	2.02	8M	26.7	200	1.3	8M	26.7
<i>M. circinelloides</i> Van Tiegh	175	1.1	5L	16.7	100	0.7	4L	13.3
<i>M. racemosus</i> Fresenius	150	0.94	4L	13.3	100	0.7	4L	13.3
<i>Mycosphaerella tassiana</i> (de Not.) Johanson	350	2.2	8M	26.7	2125	13.9	9M	30
<i>Penicillium</i>	1975	12.3	19H	63.3	2750	18	19H	63.3
<i>P. aurantiogriseum</i> Dierckx	—	—	—	—	100	0.7	3R	10
<i>P. chrysogenum</i> Thom	800	4.9	12M	40	1825	11.9	11M	36.7
<i>P. citrinum</i> Thom	100	0.62	4L	13.3	—	—	—	—
<i>P. corylophilum</i> Dierckx	475	2.9	5L	16.7	175	1.1	5L	16.7
<i>P. funiculosum</i> Thom	—	—	—	—	75	0.5	3R	10
<i>P. oxalium</i> Currie & Thom	225	1.4	8M	26.7	200	1.3	8M	26.7
<i>P. puberulum</i> Bainier	—	—	—	—	150	0.98	4L	13.3
<i>P. purpurogenum</i> Stoll	375	2.3	9M	30	225	1.5	5L	16.7
<i>Rhizopus stolonifer</i> (Fhrenb) Lindt	200	1.2	3R	10	—	—	—	—
<i>Scopulariopsis brevicaulis</i> (Sacc.) Bainier	100	0.62	3R	10	—	—	—	—
<i>Sterile mycelia</i> (white & dark colour)	75	0.5	2R	6.7	—	—	—	—
<i>Torula herbarum</i> (Pers.) link	25	0.2	1R	3.3	—	—	—	—
<i>Trichoderma viride</i> Pers. ex S. F. Gray	150	0.94	3R	10	—	—	—	—
Total counts			16025				15275	
Number of genera = 12 genera			10				7	
Number of species = 29 + 1 var.			22 + 1				21+1	

TC = Total counts, NCI = Number of cases of isolation, H = High occurrence, between 15-30 cases (out of 30), M = Moderate occurrence, between 8-14, L = Low occurrence, between 4-7, R = Rare occurrence, between 1-3.

technique was employed, and mycotoxins were identified by comparison with appropriate reference standards (Gimeno, 1979; Moss, 1971; Van Egmond *et al.*, 1980; Wilson, 1971).

Bioassay method for mycotoxins. The immature brine shrimp (*Artemia salina* L.) was used for mycotoxins bioassay. The test has been used for aflatoxins (Biji *et al.*, 1981) and for other mycotoxins (Scott *et al.*, 1980).

Results and Discussion

Twenty-nine species and one variety of *A. terreus* var. *aureus* belonging to 12 genera were collected from 30 samples of molasses on 1% glucose and 50% sucrose Czapek's agar at 25°C (Table 1). In this respect, Barakat *et al.* (2000) isolated 56 species and 3 varieties appertaining to 25 genera from 36 Gallab samples on 1% glucose and 40% sucrose Czapek's agar at 28°C. In this investigation, the most common genera were *Aspergillus* (8 species + 1 var.), *Mucor* (2), *Mycosphaerella* (1) and *Penicillium* (8) on the two types of media. They were encountered in 26.7–83.3% and 26.7–83.3% of the samples comprising 2.02–79.1% and 1.3–53.4% of total fungi, respectively (Table 1). These results agreed with those observed by Higgy *et al.* (1977), Sandhu and Sidhu (1980), Olufolaji (1986), Abdel-Sater and Ismail (1993), Abdel-Hafez *et al.* (1995), Muhsin and Abdel-Kader (1995), and Abdel-Sater and Saber (1999). Barakat *et al.* (2000) recorded that *Aspergillus*, *Mucor*, *Mycosphaerella* and *Penicillium* were the most prevalent genera in Gallab in Upper Egypt using 1% glucose and 40% sucrose-Czapek's agar at 25°C. The most prevalent species from the above genera were: *Aspergillus flavus*, *A. fumigatus*, *A. niger*, *Mucor circinelloides*, *M. racemosus*, *Mycosphaerella tassiana*, *Penicillium chrysogenum*, *P. oxalicum* and *P. purpurogenum*. They occurred in 13.3–73.3% and 13.3–83.3% of the samples constituting 1.1–39.5% and 0.7–23.6% of the total fungi on the two types of media, respectively (Table 1).

Eurotium (3 species) was frequently recovered only on 50% sucrose, emerging in 53.3% of the samples contributing 8.2% of total fungi (Table 1). *E. chevalieri* (53.3% of the samples and 6.1% of total fungi) was the most common species, while *E. amstelodami* (20% and 1.6%) was isolated in low frequency of occurrence. *E. repens* (10% and 0.5%) was rarely isolated (Table 1). These results almost agree with those obtained by Abdel-Sater and Ismail (1993), Abdel-Sater and Saber (1999) and Barakat *et al.* (2000). They reported that *E. amstelodami* and *E. chevalieri* were two most common *Eurotium* species on biscuits, dried fruits and gallab. This genus was also isolated with variable frequencies and populations from substrates with high osmotic potential and using sucrose or sodium chloride-Czapek's agar as an isolation medium

Table 2. Ability of fungal species isolated from molasses to produce invertase

Organisms	NI	P	H	M	W
<i>Aspergillus flavus</i>	10	8	3	4	1
<i>A. fumigatus</i>	8	8	5	3	–
<i>A. niger</i>	10	9	7	2	–
<i>A. sydowii</i>	1	1	1	–	–
<i>A. terreus</i>	4	3	2	1	–
<i>A. terreus</i> var. <i>aureus</i>	1	1	1	–	–
<i>A. versicolor</i>	1	1	–	–	1
<i>Emericella nidulans</i>	1	1	–	1	–
<i>Eurotium amstelodami</i>	2	2	2	–	–
<i>E. chevalieri</i>	6	6	3	2	1
<i>E. repens</i>	1	1	1	–	–
<i>Humicola fuscoatra</i>	2	2	1	1	–
<i>Mucor circinelloides</i>	1	1	–	1	–
<i>M. racemosus</i>	1	1	–	1	–
<i>Mycosphaerella tassiana</i>	3	3	1	1	1
<i>Penicillium aurantiogriseum</i>	1	1	–	–	1
<i>P. chrysogenum</i>	5	5	3	1	1
<i>P. corylophilum</i>	1	1	–	1	–
<i>P. funiculosum</i>	1	1	–	1	–
<i>P. oxalicum</i>	1	1	–	–	1
<i>P. puberulum</i>	3	2	2	1	–
<i>P. purpurogenum</i>	1	1	–	–	1
Total isolates	65	61	32	21	8

NI = Number of isolates tested; P = positive isolates; H = high producers; M = moderate producers; W = weak producers.

(Abdel-Hafez *et al.*, 1995; Hocking and Pitt, 1980; Khayria Abdel-Gawad and Zohri 1993; Kozakiewicz, 1989; Nasar, 1986).

The remaining genera and species were isolated in low or rare frequency of occurrence (Table 1).

Sixty five isolates, representing 21 species and one variety of *A. terreus* var. *aureus* were tested for their ability to produce invertase enzyme in liquid medium. It was observed that the invertase production was greatly variable not only among different species, but also among different isolates of the same species (Table 2). Of the isolates tested, about 93.8% (61 isolates) could produce invertase enzyme. From the positive isolates, 61 isolates belonging to *Aspergillus flavus*, *A. fumigatus*, *A. niger*, *A. terreus*, *Eurotium amstelodami*, *E. chevalieri*, *Penicillium chrysogenum* and *P. puberulum* (52.5%) had high invertase activity. 34.4% of the tested isolates showed moderate invertase activity which related to *Aspergillus flavus*, *A. fumigatus*, *A. niger* and *Eurotium chevalieri* and some other isolates. The remaining 8 isolates (13.1%) were found to be weak invertase-producers and these were related to *Aspergillus flavus* and *Mycosphaerella tassiana* and other isolates (Table 2). Abdel-Sater and Ismail (1993) tested 69 isolates, recovered from food materials (biscuits), for invertase production and noticed that all isolates could produce this enzyme. Also, Abdel-Sater and Saber (1999) tested 92 isolates recovered from dried fruit

Table 3. Toxin produced by different isolates of *Aspergillus*

Species	No. of isolates tested	Toxicity*				No. of isolates produced mycotoxin	Mycotoxin produced
		High	Moderate	Low	None		
<i>Aspergillus flavus</i>	10	1	2	7	0	1	aflatoxin B1, B2, G1, G2
<i>A. terreus</i>	8	1	1	2	4	2	and citrinin

*Toxicity to brine shrimp (*Artemia salina* L.). High toxicity = More than 75% mortality of larve tested; Moderate toxicity = More than 50-75% mortality of larve tested; Low toxicity = More than 25-49% mortality of larve tested; None toxicity = Less than 25% mortality of lar ve tested.

for invertase production and noticed that 80 isolates could produce this enzyme. Many plant species infected with pathogenic fungi have been shown to have significant increase in invertase activity (Benhamou *et al.*, 1991; Long *et al.*, 1975). In respect, Bhaskaran and Smith (1993) noticed that, in the absence of the substrate, the pathogen may not produce the enzyme. When the pathogen is studied in association with its host plant, extracellular release of the enzyme by the pathogen into the host tissues can be mistaken for an enzyme of plant origin. Also, in the absence of the host plant but in the presence of defined carbohydrates, it has been clearly shown that invertase is produced by the fungus tested.

Sixty different isolates of fungi belonging to *Aspergillus* 24 (*A. flavus* 10 isolates; *A. fumigatus*, 4; *A. terreus*, 8 and *A. versicolor*, 2), *Emericella nidulans* 8 isolates, *Eurotium* 10 isolates (*E. amstelodami* 4; *E. chevalieri*, 4 and *E. repens*, 2); *Mycosphaerella tassiana* 7 isolates and *Penicillium* 12 isolates (*P. chrysogenum*, 6; *P. corylophilum*, 2; *P. puberulum*, 2 and *P. purpurogenum*, 2) were screened for their ability to produce mycotoxins. These fungi were collected in this study from molasses samples. Table 3 indicated that the crude extrate of one isolate out of ten tested contained aflatoxin B1, B2, G1 and G2. The crude extracts of two out of eight of *A. terreus* contained citrinin as revealed from the thin layer chromatographic analysis. The crude extract of one isolate of *A. flavus* was highly toxic to the brine shrimp larva. Those of 2 and 7 were moderate and low toxicity, respectively. On the other hand, the crude extract of 1, 1, 2 and 4 isolates of *A. terreus* tested were high, moderate, low and none toxic to the larva tested, respectively (Table 3). From the above results, *A. flavus* and *A. terreus* produced aflatoxin B1, B2, G1, G2 and citrinin. This result agrees with all results obtained previously by (Abdel-Mallek *et al.*, 1993; Abdel-Sater and Saber 1999; Barakat *et al.*, 2000; El-Kady *et al.*, 1994; Sargeant *et al.*, 1961; Youssef, 1986). Of the known mycotoxins, the most important from the viewpoint of direct hazard to human health are aflatoxins (Scott, 1973). They are also the most extensively investigated mycotoxins. Aflatoxins are mutagenic, carcinogenic, teratogenic and actually toxic to most experimental and domesticated animals and man (Davis and Dienes, 1978).

In conclusion, Several saprobes such as *Aspergillus*,

Euratum, *Penicillium* and others are associated with molasses causing deterioration of molasses. In addition, mycotoxins specially aflatoxins, one of the most carcinogenic substance, were identified. Therefore, it is important to take a consideration of different methods for prevention of the fungal growth and mycotoxin accumulation which cause a hazard to the health.

Acknowledgement

The authors are deeply indebted to Prof. S. I. I. Abdel-Hafez (Professor of Microbiology, Faculty of Science, Assiut University) for their kind help.

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