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Determination of Some Fungal Metabolite as Influenced by Temperature, Time, pH and Sugars by Bioassay Method

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Abstract: This study investigated the fungal biomass and inhibition of *Bacillus megaterium* DSM 32 with extracts of *Fusarium equiseti*, *Penicillium italicum, Penicillium digitatum, Aspergillus candidus* and *Aspergillus parasiticus* in modified Czapek Dox Broth (mCDB) as affected by temperature (6, 15, 25 °C), incubation time (up to 4 wk), pH (3.0, 5.0 and 8.0) and glucose the and fructose (4.0, 6.0 and 9.0%). The biomass production was greatest at 25 °C. In general, biomass production was greatest in media containing an addition of 4.0% glucose or 6.0% fructose; however, bacterial inhibition production was reduced by elevated sugars in mCDB over a 4-wk incubation period.

Key Words: *Fusarium sp., Aspergillus sp., Penicillium sp.*, Fungal metabolite.

Farklı Sıcaklık, Süre, pH ve Şeker Varlığında Bazı Fungus Metabolitlerinin Biyolojik Ölçüm Metodu ile Belirlenmesi

Özet: Bu çalışmada, modifiye Czapek Dox buyyonda, *Fusarium equiseti*, *Penicillium italicum, Penicillium digitatum, Aspergillus candidus* ve *Aspergillus parasiticus* ekstraktlarının farklı sıcaklık (6, 15, 25 °C), inkübasyon süresi (4 hafta), pH (3.0, 5.0, 8.0), glukoz ve fruktoz (% 4.0, 6.0, 9.0) içeren ortamda *Bacillus megaterium* DSM 32'nin gelişmesinin engellenmesi ve biyomas oluşumu incelendi. Biyomas miktarının 25 °C'de en fazla olduğu belirlendi. Genel olarak biyomas oluşumu %4.0 glukoz ve %6.0 fruktoz içeren ortamda daha fazla bulundu. Bakteri gelişmesinin engellenmesi, 4 hafta sonunda, fazla şeker ilave edilen mCDB'da genel olarak azaldığı tespit edildi.

Anahtar Sözcükler: *Fusarium sp., Aspergillus sp., Penicillium sp.*, Fungus Metabolitleri.

Introduction

Many of the moulds capable of producing mycotoxins are also frequent contaminants of agricultural commodities (seeds, grains, fruits, vegetables, etc.). Mycotoxins are secondary metabolites produced by certain strains of fungi. The presence of these toxins has become a major topic of research since 1961, when the carcinogenicity of aflatoxins was detected. Hundreds of species of more than a dozen fungal genera are known to be toxigenic (1) and these fungi may grow on standing crops or stored feeds (2).

Moulds, which are of importance in commodities because of potential mycotoxin production, include members of the genera *Aspergillus, Penicillum, Fusarium, Alternaria* and *Cladosporium*. These organisms are capable of growing on a variety of substrates and under a diversity of conditions of moisture, pH and temperature (3). Many types of seeds, grains, fruits and vegetables are susceptible to fungal invasion before and during harvest, transport or storage. If mould growth occurs, there is always the concomitant possibility of mycotoxin production (4). The production of mycotoxins, a group of secondary fungal metabolites, is reportedly dependent on the physico-chemical environment where the mould develops (5).

The economic loss resulting from fungal and mycotoxin contamination of nuts is difficult to estimate. However, judging from the widespread occurrence of fungal and mycotoxin contamination and the large number of nuts affected, one can assume that such losses must be large (6). These losses constitute direct nut losses, human illness and reduced productivity, and livestock losses due to deaths and lower growth rates. Additional economic losses include the indirect costs of various systems for the control of mycoflora and mycotoxins in nuts, the reduced value of rejected nuts, the costs of detoxification to recover acceptable products, and, occasionally, from the loss of export markets (7,8).

The object of the study reported here was to investigate the effects of temperature, time, pH and simple sugars on biomass and toxic metabolite production with five fungal species.

Material and Methods

Fungi investigated

Fusarium equiseti, *Penicillium italicum, Penicillium digitatum, Aspergillus candidus* and *Aspergillus parasiticus* were obtained from the University of Uludağ, Faculty of Arts and Science Department of Microbiology, Bursa Turkey. Stock cultures were maintained on malt extract agar (MEA).

Growth medium

Modified Czapek Dox broth (mCDB) was used as the basal medium for culturing the fungi. (9). The composition of the medium consisted of glucose, 30.0; Yeast extract, 1.0; $NaNO_3$, 2.0; KH_2PO_4, 1.0; $MgSO_3.7H_2O$, 0.5; KCl, 0.5 and $FeSO_4.2H_2O$ 0.01 (g/l distilled water). Streptomycin (20 µg/ml) and rose bengal (30 µg/ml) were used as bacteriostatic agents (10,11). For experiments designed to determine the effect of pH on biomass and toxic metabolite production, the medium was adjusted to pH 3.0 and 5.0 with 0.05N HCl, and pH 8.0 with 0.05N NaOH. Glucose and fructose (4.0, 6.0 and 9.0 %, w/v) were added to the basal medium (pH 5.0) in tests designed to determine the effects of sugars; 50-ml quantities in 100 ml Erlenmeyer flasks served as substrates in all the experiments (12).

Inoculation and Incubation procedure

The fungal species were cultured on MEA at 25 °C for 8 days. Conidial suspensions were prepared by flooding the surface of the cultures with sterile water and gently rubbing with an inoculation needle; 0.1 ml of a dense suspension was used as the inoculum for each 50 ml quantity of mCDB.

Inoculated mCDB (pH 5.0) was incubated at 25 °C for 1, 2 and 4 wk to determine the effect of time on biomass and toxin production. The effect of temperature was determined by incubating mCDB (pH 5.0) cultures at 6, 15 and 25 °C for 4 wk. The influence of pH (3.0, 5.0 and 8.0) on biomass and toxin production was studied at 25 °C; 4.0, 6.0, and 9.0 % sugar (glucose and fructose) were determined in mCDB (pH 5.0) incubated at 25 °C for 4 wk. (11,12). The cultures were incubated in darkness since light is known to adversely affecte mycotoxin production by fungi. The cultures were shaken once daily to break up the mycelial mat. All the experiments were conducted with three replicates.

Extraction procedure

At the end of the incubation periods, mycelia were recovered by filtration. The mycelia were dried at 85 °C for 3 h to determine biomass weight. Thirty milliliters of each sample was extracted three times with ethyl acetate (1 media:1 ethyl acetate) using a separator funnel. The ethyl acetate extract was dried over anhydrous sodium sulfate, filtered and then evaporated under vacuum to near dryness (5,8,9). The residue was diluted with ethyl acetate to two ml. The determination of fungal metabolites was carried out according to the methods of biological measurements.

Bioassay procedure

The microorganisms (*Bacillus megaterium* DSM 32) tested in this study were from the culture collections of the Microbiology Laboratory of the Science & Arts Faculty of the University of Fırat, in Elazığ, Turkey. The extracts of samples thus obtained were injected into empty sterilized antibiotic discs with a diameter of 6 mm (Schleicher & Shüll No: 2668, Germany) in amounts of 100 µl (13). The discs injected with only ethyl acetate were used as the controls.

B. megaterium DSM 32 was incubated at 30±0.1 °C for 24 h by injection into Nutrient Broth (Difco). Mueller Hinton Agar (MHA) (oxoid), sterilized in a flask and cooled to 45-50 °C, was distributed in sterilized petri dishes with a diameter of 9 cm using pipettes in 15 ml quantities after injecting cultures of bacteria prepared as mentioned above in 0.01 ml quantities (10^5 bacteria per ml), and ensuring that the distribution of Mueller Hinton Agar medium in the petri dishes was homogeneous. The discs injected with extracts were located on the MHA medium by pressing slightly. The petri dishes were kept at 4 °C for 2 h, and were then incubated for 15 h at 37 ± 0.1 °C (14,15,16). At the end of the period, inhibition zones which had formed on the MHA medium were measured in millimeters (mm).

Results and Discussion

Fusarium equiseti and *P. digitatum* grew more rapidly than the other fungi on mCDB at 25°C (Table 1). Extracts from the *P. italicum, A. candidus* and *A. parasiticus* cultures, however, were not toxic after 1 and 2 wk of incubation but produced metabolites toxic to *B. megaterium* after 4 wk of incubation. El-Maraghy and Salem (17) reported that *Fusarium spp.* produced zearalenone and T2-toxin. *Fusarium* strains isolated from poultry and animal feeds were toxigenic. Sterigmatocystin was detected in the extracts of *Aspergillus fumigatus* while citrinin

was produced by *A. terreus.* These two species isolated from poultry feed and litter in Ohio were found to be toxigenic (1).

The results of the experiments designed to determine the effect of temperature on biomass and toxic metabolite production are summarized in Table 2. The highest amounts of biomass were produced at 25 °C. Toxic metabolite production by all fungi was greatest at 25 °C. *P. italicum* and *P. digitatum* did not inhibit *B. megaterium* growth when incubated at 6 °C.

Jimenez et al. (5) reported that the patulin (The patulin-producing fungal species were *Penicillium expansum* and *P. urticae*) concentrations in the mycelium and the broth increased markedly between the 20th and 30th day, after which they remained virtually unchanged. Little mycotoxin was then produced in the laboratory cultures during the vigorous growth phase;

Table 1. Biomass and toxic metabolite production by fungi cultured in mCDB (pH 5.0) at 25 °C for 1,2, and 4 wk (Data are average of three replicates).

Fungal species	Biomass (mg/50 ml mCDB)			Zone of inhibition (mm dia)		
	1 wk	2 wk	4 wk	1 wk	2 wk	4 wk
F. equiseti	36	87	164	8	17	29
P. italicum	39	54	99	-a	-	10
P. digitatum	44	89	179	8	13	21.5
A. candidus	24	46	112	-	-	13
A. parasiticus	29	42	121	-	-	11

[a]No inhibition.

Table 2. Biomass and toxic metabolite production by fungi cultured in mCDB at 6, 15 and 25 °C for 4 wk (Data are average of three replicates).

Fungal species	Biomass (mg/50 ml mCDB)			Zone of inhibition (mm dia)		
	6 °C	15 °C	25 °C	6 °C	15 °C	25 °C
F. equiseti	16	105	164	10	12	29
P. italicum	11	56	99	-a	9	10
P. digitatum	19	109	179	-	10	21.5
A. candidus	25	124	112	8	11.5	13
A. parasiticus	20	118	121	7	11	11

[a]No inhibition.

Table 3. Biomass and toxic metabolite production by fungi cultured in mCDB (pH 3.0, 5.0 and 8.0) at 25 °C for 4 wk (Data are average of three replicates).

Fungal species	Biomass (mg/50 ml mCDB)			Zone of inhibition (mm dia)		
	pH 3.0	pH 5.0	pH 8.0	pH 3.0	pH 5.0	pH 8.0
F. equiseti	7	164	185	-a	29	14
P. italicum	3	99	134	-	10	8.5
P. digitatum	9	179	170	-	21.5	10
A. candidus	5	112	129	-	13	7
A. parasiticus	4	121	138	-	11	9.5

[a]No inhibition.

however, as nutritional factors diminished and growth was limited as a result, the biosynthesis of this toxin was accelerated. The higher incubation temperature (28 °C) was found to promote more extensive growth and intensive patulin biosynthesis than the lower one (20 °C). The amounts of patulin produced on incubation in the culture broth at 28 and 20 °C were 11.9 and 7.1 mg/100 ml of medium.

The effects of pH on the biomass and toxic metabolite production by the fungi used in this study are shown in Table 3. Biomass production was clearly retarded at pH 3.0. The growth of all the test species was greatest at pH 8.0; however, the highest quantities of toxic metabolite production were observed at pH 5.0. Extracts from *F. equiseti* had the greatest effect on *B. megaterium*.

The effects of supplementing mCDB with glucose and fructose on biomass and toxic metabolite production are shown in Table 4. The growth of all the test strains was enhanced in mCDB containing 6.0% glucose. No apparent correlation between biomass and toxic metabolite production was noted for the fungi used in this study.

The results of this investigation indicate that growth and toxic metabolite production by the fungi used in this study were influenced by the interacting effects of temperature, pH and simple sugars. Many raw and processed fruits and fruit products appear to represent suitable substrates for toxin production. Further studies are needed to determine the effects of oxygen availability and common food preservatives on growth and toxin production by some fungi on these products as they are stored and marketed by the food industry.

Table 4. Biomass and toxic metabolite production by fungi cultured in mCDB (pH 5.0) supplemented with glucose and fructose at 25 °C for 4 wk (Data are average of three replicates).

Fungal species	Sugar		Biomass (mg/50 ml mCDB)	Zone of inhibition (mm dia)
	Type	%		
F. equiseti	glucose	3.0	164	29.0
		6.0	145	15.0
		9.0	278	9.0
	fructose	3.0	80	-a
		6.0	89	-
		9.0	66	15.0
P. italicum	glucose	3.0	99	10.0
		6.0	173	11.0
		9.0	210	7.5
	fructose	3.0	116	8.5
		6.0	157	11.0
		9.0	92	14.5
P. digitatum	glucose	3.0	179	21.5
		6.0	160	15.0
		9.0	198	11.5
	fructose	3.0	228	-
		6.0	256	-
		9.0	191	9.5
A. candidus	glucose	3.0	112	13.0
		6.0	180	9.5
		9.0	208	10.0
	fructose	3.0	103	14.5
		6.0	159	9.0
		9.0	124	8.0
A. parasiticus	glucose	3.0	121	11.0
		6.0	170	9.5
		9.0	214	8.5
	fructose	3.0	66	7.5
		6.0	84	10.0
		9.0	72	7.0

[a]No inhibition.

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References

1. Lovett, J., Toxigenic fungi from poultry feed and litter. Poultry Science, 31: 309-317, 1972.
2. Moharram, A.M., Abdel-Gawad, K.M., Megalla, S.E., Mahmoud, L.E., Fungal flora of poultry feedstuff ingredients. J. Basic Microbiol., 29: 491-499, 1989.
3. Stinson, E.E, Bills, D.D., Osman, S.F., Sciliano, J., Ceponis, M.J., Heisler, E.G., Mycotoxin production by *Alternaria* species grown on apples, tomatoes and blueberries. J. Agric. Food Chem., 28: 960-963, 1980.
4. Zohri, A.A., Abdel-Gawad, K.M., Survey of mycoflora and mycotoxins of some dried fruits in Egypt. J. Basic Microbiol., 33: 279-288, 1993.
5. Jimenez, M., Mateo, R., Mateo, J.J., Huerta, T., Hernandez, E., Effect of the incubation conditions on the production of patulin by *Penicillium griseofulvum* isolated from wheat. Mycopathologia, 63-168, 1991.
6. Stinson, E.E., Osman, S.F., Heisler, E.G., Sciliano, J., Bills, D.D., Mycotoxin production in whole tomatoes, apples, oranges and lemenos. J. Agric. Food Chem., 29: 790-792, 1981.
7. Abdel-Gawad, K.M., Zohri, A.A., Fungal flora and mycotoxins of six kinds of nut seeds for human consumption in Saudi Arabia. Mycopathologia, 124: 55-64, 1993.
8. Logrieco, A., Battolico, A., Solfrizzo, M., Mule, G., Incidence of *Alternaria* species in grains from Mediterranean countries and their ability to produce mycotoxins. Mycologia, 2: 501-505, 1990.
9. Özçelik, S., *Bacillus megaterium* kullanılarak patulinin biyolojik yolla ölçümü. Gıda 10: 281-285, 1985.
10. Mahmoud, L.E., Toxigenic fungi and mycotoxin content in poultry feedstuff ingredients. J. Basic Microbiol., 33: 101-104, 1993.
11. Özçelik, N., Özçelik, S., Biyolojik ölçüm yöntemi ile *Alternaria* toksinlerinin oluşumuna bazı faktörlerin ve suşların etkisinin araştırılması. Tr. J. of Agriculture and Forestry 20: 19-25,1996.
12. Scott, W.T., Bullerman, L.B., Influence of carbohydrate and nitrogen source on patulin production by *Penicillium patulum*. Appl. Microbiol., 30: 850-854, 1975.
13. Buckle, A.E., Sanders, M.F., An appraisal of bioassay methods for the detection of mycotoxins a review. Letters Appl Microbiol, 10: 155-160, 1990.
14. Bağcı, E., Dığrak, M., Antimicrobial activity of essential oils of some Abies (Fir) species from Turkey. Flavour and Fragrance Journal, 11: 251-256, 1996.
15. Bradshaw, L.J., Laboratory Microbiology. Fourth Edition. Printed in USA. 1992, p 435.
16. Collins, C.H., Lyne, P.M., Grange, J.M., Microbiological Methods. Butterworth & Co. Ltd. 1989; p 410.
17. El-Maraghy, S.S.M., Salem DA. Mycoflora and mycotoxins of feedstuff samples collected from milk production farms in Assiut Governorate. Bull. Fac. Sci., 15: 11-23, 1986.