Comparison of Two Medium According to Mould Enumeration and Recovered Species from Wheat and Feed

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Received: 14 February 2007
Accepted: 21 April 2007

Abstract

In this study, 15 retail and bulk wheat and 7 animal feed samples from feed factory yielded from Balikesir region were examined in 2002–2006. Standard methods were used for food-borne fungi accepted by the international meetings on food mycology and selective mediums for isolation of potential mycotoxin producing strains were determined. Fungal genera and species were identified by macroscopic and microscopic characters according to taxonomic keys for each genus. Three principal genera of filamentous fungi were *Aspergillus*, *Penicillium* and *Fusarium*.

Totally 307 isolates were obtained from 22 samples. Thirty-two species in 158 isolates recovered from DRBC Agar and 37 species in 149 isolates recovered from DG18 Agar were identified. Two medium according to enumeration and isolation of fungal growth were compared. Fungi recovered from two media were determined; the mean values of populations recovered from DRBC and DG18 were discussed. Distribution of fungal species and potential mycotoxin producer species were evaluated.

Key words: Wheat, Feedstuff, DG18, DRBC, Fungi

INTRODUCTION

The safety of the storage food is of great importance due to fungal contamination and their secondary metabolite, threatens food quality. Inevitable interspesific and intraspesific interactions will happen among fungi due to the nutritional status of the grain and the prevailing environmental conditions. These environmental factors may cause a selective pressure influencing the community structure and the dominance of individual, especially, mycotoxigenic species [1].

The incidence of grain commodity contaminations by fungi are high in tropical and subtropical regions under suitable climatic conditions for fungal development [2]. More than 100.000 fungal species are able to contaminate feeds and foods and have the ability to synthesise mycotoxin molecules [3].

Aspergillus, Penicillium, *Fusarium* and *Alternaria* are important contaminants of cereal grains [4]. These fungal contaminants cause fungal growth and ability to produce mycotoxin on some commodity. Some factors such as moisture content and water activity provide a suitable situation for fungal development and mycotoxin production [5, 6].

Dichloran Medium Base with Rose Bengal (DRBC) Agar is a selective medium that supports good growth of yeasts and moulds. DRBC, is formulated as described by King et al. [7], is a modification of Rose Bengal Chloramphenicol Agar [8]. The substances in the DRBC are dichloran (is added to the medium to reduce colony diameters of spreading fungi), rose bengal (suppresses the growth of bacteria and restricts the size and height of colonies of the more rapidly growing moulds) and chloramphenicol (is included in this medium to inhibit the growth of bacteria present in environmental and food samples). The reduced pH of the medium from 7.2 to 5.6 helps inhibition of the spreading fungi [8].

Dichloran Glycerol Agar Base (DG-18), is also medium for enumeration of fungal growth. It is recommended for dried and semi-dried foods, including fruits, spices, cereals, nuts, meat, and fish products, is based on the formulation by Hocking and Pitt [9].

Aflatoxins, are produced by three closely related species *Aspergillus flavus* Link, A. *parasiticus* Speare, A *wentii* Wehmer and *A. nomius* Kurtzman et al. [10-11], are carcinogenic metabolites produced by *A. flavus* and probably bring up infection by A. flavus in drought stressed plants [12]. Aflatoxin B1 is accepted carcinogen by International Agency of Research on Cancer (IARC) under group I [13].

Aspergillus ochraceus, an ochratoxin producing fungus, can be found on stored cereal grains [14]. Ochratoxin A, under IARC, group 2B, [13] is also known as an important mycotoxin produced by *Aspergillus ochraceus* K. Wilh, *A. carbonarius* (Bainier) Thom., *A. tubingensis* Mosseray and *A. niger* van Tieghem.

[15-17]. Fumonisins are produced by *Fusarium verticilloides* (Sacc.) Nirenberg, *F. proliferatum* (Matsush.) Nirenberg, *F.* nygamai Burgess & Trimboli and *F. oxyporum* Schlecht.:Fr. [18-19]. There are excessive amount of evidence about morphological, cellular and biochemical damage in farm animals fed on fumonisin-contaminated diets [20]. Placinta et al. [21] declared that cereal grains and animal feed can be exposed to the risk of multi-contamination with fumonisin, zearalenone (ZEN) and trichothecenes by Fusarium species all over the world. ZEN is of relatively low toxicity. There are lots of reports notified on trichothecenes, deoxynivalenol (DON) and nivalenol (NIV), contamination in cereal grains from Poland, Germany, New Zealand, Japan and America [1, 4].

The aims of the study are to determine fungal contamination of wheat and feed, to comparing both media, DRBC and DG18, as a general enumeration medium, to investigate fungal growth and research isolated and identified fungi coming from two media to determine their species diversity and ascertain potential mycotoxin producer species within both media.

MATERIALS AND METHODS

A total of twenty-two samples were collected and all of them were not less than 1-2 kilogram-size. Fifteen collected wheat samples were taken from big sacks in grain depots, bazaars and outlets, at random, representing wheat lots. Seven feed samples were taken from feed factory in Balikesir, Turkey. This study was carried out at following steps; samples were collected between 2002 and 2004, isolations were performed, fungi associated with wheat and feed products were identified. Distribution of fungal species and their potential mycotoxin producing capability were evaluated.

Preparation of samples

All the samples were held –20 ºC for 72 h to remove mites and insects.

Media

Two different media, Dichloran Rose Bengal Chloramphenicol Agar (DRBC), (Difco 0587) [7-8] and Dichloran 18% Glycerol Agar (DG18) [9] for xerophylic fungi were used for the isolation and enumeration of fungi from wheat and feed. Czapex dox agar (CZ) (Oxoid CM97), Malt extracts agar (MEA) (Oxoid CM59) and Potato Dextrose agar (PDA) (Merck 110130) were used for identification. DRBC Agar was prepared by adding Chloramphenicol, (50 mg per liter before autoclaving) and chlortetracycline (50 mg per liter filtered to sterilize and added just before pouring to the plates) [22-23]. The plates were dried overnight before inoculating.

Isolations and Identifications of Fungi

Sub samples were prepared to form representative sample for each. 50 g of sub sample was weighted, sample were put into sterile a beaker and disinfected by adding 1% commercial chlorine bleach for 2 minutes by hurling in both sides delicately, then rinsed with sterile distilled water for a few times to remove chlorine and wheat was dried in sterilized towel paper and feed was on filter paper.

Enumeration of fungal propagates was done on DRBC and DG18 agar, using the surface spreading method by blending 50 g portion of each sample with 450 ml sterilized distile water. Serial dilutions were made from 10^{-2} to 10^{-5} from each sample and 0.1 ml aliquots were inoculated in duplicate on DRBC and DG18 agar surface.

All the plates were incubated at 27 ºC for 3-5 days for DRBC and DG18 agar respectively. Fungal colonies were cultured on Czapex Dox agar; Malt extracts agar and Potato Dextrose agar for identification. Fungal colonies were selected for identification according to the methods proposed for each fungus. Fungal genera and species were identified by macroscopic and microscopic characters according to taxonomic keys for each genus [11, 24-33].

Relative density (RD) was calculated for each species or genus in each group as the number of isolates or genus / Total number of fungi isolated x 100 [34].

RESULTS

Wheat and feed samples were collected from Balikesir, Turkey during 2002-2004. As demonstrated in Table 1, the isolated fungi were broadly divided into filamentous fungi. Totally 307 isolates were obtained from 22 samples.

Thirty-two species in 158 isolates were recovered from DRBC Agar and 37 species in 149 isolates were recovered from DG18 Agar and identified. Twenty-four common species were determined in both media. Species diversity except common species was 8 in DRBC and 13 in DG18. The results

Table 1. Distribution of mould genera within 22 samples.

	DRBC		DG18	
Genus	**Frekans**	**%RD**	**Frekans**	**%RD**
Aspergillus	31	19.74	35	23.48
Fusarium	19	12.1	21	14.09
Penicillium	18	11.46	17	11.4
Acremonium	16	10.12	10	6.71
Rhizopus	14	8.91	10	6.71
Cladosporium	9	5.73	6	4.02
Trichoderma	7	4.4	9	6.04
Mucor	4	2.53	3	2.01
Absidia	4	2.53	7	4.69
Aerobasidium	3	1.89	3	2.01
Alternaria	2	1.26	6	4.02
Other	10	6.36	5	3.35
Unidentified	21	13.36	17	11.4
Total	158		149	
General Total	307			
Total species	32		37	

Table 2. Species diversity and common species recovered DRBC and DG18 Agar from wheat and feed samples.

	Species recovered from wheat and feed samples				
No	**Species from DRBC agar**	**Frequency**	**No**	**Species from DG18 agar**	**Frequency**
1	*Absidia corymbifera*	4	1	*Absidia corymbifera*	7
2	*Acremonium fusidioides*	4	2	*Acremonium fusidioides*	1
3	*Acremonium sordidudilum*	4	3	*Acremonium sordidudilum*	1
4	*Acremonium strictum*	8	4	*Acremonium strictum*	8
5	*Aerobasidium pullulans* var. *melanigrium*	3	5	*Aerobasidium pullulans* var. *melanigrium*	3
6	*Alternaria alternata*	2	6	*Alternaria alternate*	6
7	*Aspergillus candidus*	5	7	*Aspergillus aculatus**	1
8	*Aspergillus flavus*	13	8	*Aspergillus candidus*	3
9	*Aspergillus flavus* var. *columnaris*	2	9	*Aspergillus ficuum**	5
10	*Aspergillus niger*	7	10	*Aspergillus flavus*	12
11	*Aspergillus penicilloides**	1	11	*Aspergillus flavus* var. *columnaris*	2
12	*Aspergillus pulverulentus**	2	12	*Aspergillus foetidus* var. *pallidus**	2
13	*Aspergillus wentii*	1	13	*Aspergillus niger*	2
14	*Cladosporium cladosporoides*	6	14	*Aspergillus parasiticus**	2
15	*Cladosporium herbarum*	2	15	*Aspergillus terreus**	1
16	*Cladosporium macrocarpum**	1	16	*Aspergillus tubingensis**	2
17	*Epicoccum sp.**	4	17	*Aspergillus wentii*	2
18	*Fusarium culmorum*	8	18	*Byssochlamys sp**	1
19	*Fusarium oxyporum*	11	19	*Cladosporium cladosporoides*	2
20	*Mucor hiemalis*	4	20	*Cladosporium herbarum*	4
21	*Neosortaria sp**	1	21	*Fusarium culmorum*	8
22	*Nigrospora musae**	5	22	*Fusarium oxyporum*	13
23	*Penicillium crustosum*	5	23	*Mucor hiemalis*	2
24	*Penicillium charlesii**	2	24	*Mucor racemosus**	1
25	*Penicillium expansum*	4	25	*Penicillium corylophylum**	2
26	*Penicillium funiculosum*	1	26	*Penicillium crustosum*	3
27	*Penicillium nalgiovense**	3	27	*Penicillium expansum*	6
28	*Penicillium oxalicum*	2	28	*Penicillium funiculosum*	1
29	*Penicillium stoloniferum*	1	29	*Penicillium ochraceum**	1
30	*Rhizopus oligosporus*	8	30	*Penicillium oxalicum*	3
31	*Rhizopus oryzae*	6	31	*Penicillium stoloniferum*	1
32	*Trichoderma viridae*	7	32	*Rhizoctonia sp.**	4
	Unidentified	21	33	*Rhizopus oligosporus*	5
			34	*Rhizopus oryzae*	5
			35	*Trichoderma harzianum**	1
			36	*Aspergillus clavatus**	1
			37	*Trichoderma viridae*	8
				Unidentified	17
	Total	158		Total	149

Species diversity was shown by "*".

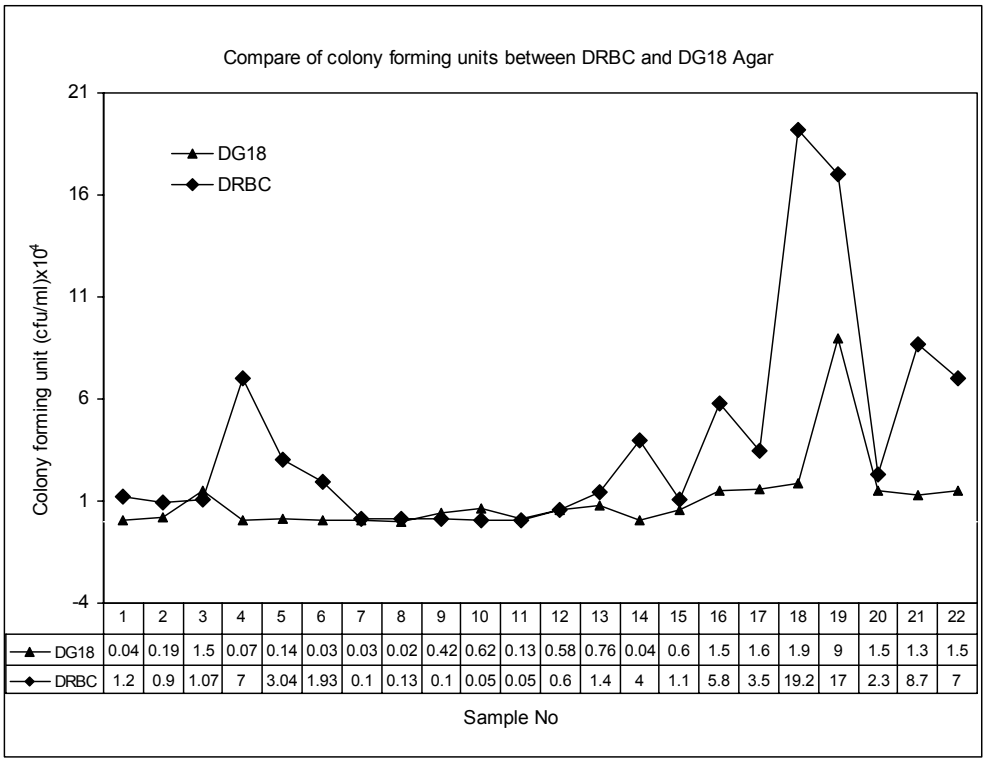
of determined genera dominancy and species diversity in both media were given below (Table 1, Table 2).

Potential mycotoxin producer fungi within 307 isolates from wheat and feed samples were given in the list (Table 3). As shown from the table, 10 species (56 strains) were recovered from DRBC agar and 11 species (55 strains) were recovered from DG18 agar including *Aspergillus candidus* Link: Fries, *Aspergillus flavus* Link, *Aspergillus niger* van Tieghem, *Aspergillus wentii* Wehmer, *Aspergillus parasiticus* Speare, *Penicillium crustosum* Thom, *Penicillium expansum* Link, *Fusarium oxyporum* Schlecht.:Fr., *Fusarium culmorum* (W.G. Smith) Saccardo and *Alternaria alternata,* (Fries) Kiessler.

Total colony forming units (CFU/ml) were calculated. As shown Figure 1, calculating number of colony forming units (CFU) per ml of counts ranged from $0.05x10^4$ to $1.92x10^5$ for DRBC and, $0.2x10^4$ to 9.0×10^4 CFU/ml for DG18.

Table 3. Incidence of potential mycotoxin producing species in wheat and feed samples.

Mainly capable of potential mycotoxin producing species				
Species	DRBC	DG18	Mycotoxins	References
Aspergillus candidus	5	6	Ochratoxin-A	[36, 37]
Aspergillus flavus	13	7	Aflatoxin B1, B2	[2]
Aspergillus niger	7	2	Ochratoxin-A	[15, 38]
Aspergillus wentii	1	2	Ochratoxin-A, Aflatoxin	[10, 39]
Aspergillus parasiticus		2	Aflatoxin B1, B2, G1, G2	[40]
Aspergillus foetidus var. pallidus	2	3	Ochratoxin A	[41]
Penicillium crustosum	3	3	Ochratoxin-A, Viomellein	[36]
Penicillium expansum	4	6	Patulin	[42-43]
Fusarium oxysporum	11	13	Fumonisin B1, B2, B3, Zeralenon	[5, 18-19]
Fusarium culmorum	8	5	Fumonisin B1, B2, T1 ve T2 toksin, Yavanisin, DON, NIV	[44-45]
Alternaria alternata	2	6	Altertoksin, Alternariol	[46-48]
Total	56	55		



	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
DG18	0.04	0.19	1.5	0.07	0.14	0.03	0.03	0.02	0.42	0.62	0.13	0.58	0.76	0.04	0.6	1.5	1.6	1.9	9	1.5	1.3	1.5
DRBC	1.2	0.9	1.07	7	3.04	1.93	0.1	0.13	0.1	0.05	0.05	0.6	1.4	4	1.1	5.8	3.5	19.2	17	2.3	8.7	7

Figure 1. Compare of colony forming units of samples between DRBC and DG18 Agar

DISCUSSION

When compared two moulds population growing DG18 Agar and DRBC agar, we found lower population means for mould recovered from DG18 Agar than DRBC agar ($p<0.05$). According to our findings on mould enumeration, DG18 gave significantly lower numbers of colonies than DRBC did. We decided that the use of DG18 Agar as a general enumeration medium for food borne moulds may cause lower population counts.

Thirty-two species (158 isolates) recovered from DRBC agar and 37 species (149 isolates) recovered from DG18 agar were isolated and identified. There were no significant differences between DRBC agar and DG18 agar according to isolated and identified species ($t = -0,944$, $p > 0.05$). Eight in 32 species from DRBC and 13 in 37 species from DG18 showed diversity. Especially DG18 is valuable to isolate xerophilic fungi in wheat and feed in reduced water activity. There were no significant differences between DRBC and DG18 according to species diversity ($t = 0.63$, $p >0.05$). We found 24 common species recovered both media. There were no significant differences between DRBC and DG18 agar according to common species ($t = 0.638$, $p >0.05$).

As compare of DRBC colony forming units with DG18, there was a meaningful difference between DRBC and DG18 agar. As demostrated in Figure 1, colony forming units in DRBC agar were significantly higher ($t = 2.39$, $p< 0.05$) than that from DG18.

Other striking fact about the present study was that we couldn't isolated *F. proliferatum* from wheat and feed samples even though we recovered it from maize kernels abundantly in our former research [35].

Fifty-six potential mycotoxin producer strains were recovered from DRBC agar and 55 strains were recovered from DG18 agar [2,5,10,15,18-19,36-38,39-46]. Fusarium is the most important genera producing different mycotoxins such as trichothecene toxins, deoxynivalenol and nivalenol. Although, *F. culmorum* was the dominant species in cooler growing areas [47], this species was isolated in high temperature and moisture growing areas in our study and identified. According to the recent studies, *F. culmorum*, regardless temperature and water availability, was dominant against other grain fungi and this explained of why *F. culmorum* was important during pre and post harvest [1].

Statistics

Significant differences were determined using Students' *t*-test, ($P < 0.05$).

ACKNOWLEDGEMENTS

The author is indebted to Cevza Kızılırmak due to her evaluated English of manuscript and to Arif Sahin for his help on statistics and also grateful to the research funds of BAU 2004/12 financial support.

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