Annals of Parasitology 2016, 62(3), 201–208
doi: 10.17420/ap6203.55

Original papers

The hydrolytic enzymes produced by fungi strains isolated from the sand and soil of recreational areas

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ABSTRACT. The pathogenicity of fungi depends on, *inter alia,* the secretion of hydrolytic enzymes. The aim of this study was to determine the enzymatic activity of yeasts and yeast-like fungi isolated from children's recreation areas, and compare the results with literature data of strains obtained from patients with mycoses. The enzymatic activity of 96 strains was assessed using an API ZYM kit (bioMerieux, France) and their biotypes were established. The fungal species were found to produce from 16 to 19 hydrolases: the most active were: leucine arylamidase (e_5), acid phosphatase (e_{10}), alkaline phosphatase (e_1), naphthol-AS-BI-phosphohydrolase (e_{11}), esterase – C4 (e_2), β-galactosidase (e_{13}) and β-glucosidase (e_{16}). In addition, 13 biotypes characteristic of particular species of fungi were defined. Most strains could be categorized as biotypes C_2 – 39.5% and A – 26%. The examined fungal strains isolated from recreational areas have selected biochemical characteristics i.e. production of hydrolases, which demonstrate their pathogenicity. They produce a number of enzymes which are also present in strains isolated from patients with mycoses, including: leucine arylamidase (e_5), acid phosphatase (e_{10}), naphthol-AS-BI-phosphohydrolase (e_{11}) and alkaline phosphatase (e_1). The biotypes identified in the course of this study (A, B_3, B_4, C_1, C_6 and D_3) have been also reported in cases of fungal infection. Therefore, the fungi present in the sand and soil of recreational have pathogenic properties and are possible factors of fungal infection among children.

Key words: *Candida*, enzyme, fungi, pathogenicity, soil

Introduction

Approximately 1.5 million species of fungi are believed to exist on Earth and about 600 of them are human pathogens [1–3]. They are present in all parts of the biosphere, and so readily induce infections, especially among patients who are immunocompromised or those who have factors which promote a fungal infections (such as exposure to broad-spectrum antibacterial agents, corticosteroids or prolonged use of catheters). Serious invasive fungal infections (IFI) can be caused by, among others, *Candida* spp., *Cryptococcus* spp., *Aspergillus* spp., *Trichosporon* spp. and *Rhodotorula* spp. The incidence of invasive mycoses is still increasing and many are life-threatening [4].

In order to colonize, infect and evade host defense mechanisms, fungi possesses such virulence factors as adapted cell morphology, adhesion factors, phenotypic switching, biofilm formation and enzymatic activity. One particularly effective factor is the production of extracellular hydrolytic enzymes, which facilitate the destruction of the cellular membranes and thus allow the fungal cells to penetrate host tissue. The high activity and large number of different hydrolases may be responsible for the high incidence of infections seen in humans [5–7].

Children are particularly at risk of developing fungal infection and are often exposed to fungi, often when playing in contaminated soil, sandpits or playgrounds. In one study performed from 2010 to 2012 in Lodz alone, 66 genera and 112 species of potentially pathogenic fungi were isolated from 44 sites based around the sandboxes and soil of recreation areas [8].

The aim of present study was to determine the enzymatic activity of the yeasts and yeast-like fungi isolated with high prevalence from these recreation areas, one of their pathogenecity factors and compare the results with literature data concerning the characteristics of strains obtained from patients with mycoses.

Materials and Methods

Ninety-six strains obtained with high prevalence from recreation places were classified into 7 species: *Cryptococcus neoformans*, *C. albidus*, *C. laurentii*, *Candida lusitaniae*, *C. guilliermondii*, *Trichosporon cutaneum* and *Rhodotorula glutinis* were used. The isolation and identification of the strains were carried out as described previously [8].

Enzymatic activity was assessed using API ZYM test (bioMerieux, France), which is a semiquantitative micromethod for evaluating following enzymes: alkaline phosphatase (e_1), esterase – C4 (e_2), esterase lipase – C8 (e_3), lipase – C14 (e_4), leucine arylamidase (e_5), valine arylamidase (e_6), cystine arylamidase (e_7), trypsin (e_8), α-chymotrypsin (e_9), acid phosphatase (e_{10}), naphthol–AS-BI-phosphohydrolase (e_{11}), α-galactosidase (e_{12}), β-galactosidase (e_{13}), β-glucuronidase (e_{14}), α-glucosidase (e_{15}), β-glucosidase (e_{16}), N-acetylo-β-glucosyloaminidase (e_{17}), α-mannosidase (e_{18}), α-fucosidase (e_{19}). The procedure was conducted according to the manufacturer's instructions. Visible changes in the color of the medium were considered positive. The intensity of the color reflected the concentration of the degraded substrate produce by the enzyme and enzyme activity was expressed in nmol of hydrolyzed substrate according to the scale provided in the kit: 1 corresponded to 5 nmol, 2 to 10, 3 to 20, 4 to 30 i 5 to 40 and above. All tests were repeated 3 times for each strain.

Strain biotyping was conducted according to Kurnatowska [9], as presented in Table 1.

Results

All strains from 6 out of 7 the examined species produced acid phosphatase (e_{10}); slightly fewer (6) were found to produce alkaline phosphatase (e_1), esterase – C4 (e_2) and five of them naphthol-AS-BI-phosphohydrolase (e_{11}). The strains of 3 species produced esterase lipase – C8 (e_3), and α-galactosidase (e_{12}), two species produced leucine arylamidase (e_5) and β-galactosidase (e_{13}) and one of them – N-acetylo-β-glucosyloaminidase (e_{17}).

Table 1. Biotypes of fungi strains based on enzymatic activity [9]

Group	Biotype	Valine arylamidase (e_6)	Naphthol-AS-BI-phosphohydrolase (e_{11})	α-glucosidase (e_{15})	N-acetylo-β-glucosyloaminidase (e_{17})
A	A	+	+	+	+
B	B_1	–	+	+	+
	B_2	+	–	+	+
	B_3	+	+	–	+
	B_4	+	+	+	–
C	C_1	–	–	+	+
	C_2	–	+	–	+
	C_3	–	+	+	–
	C_4	+	–	–	+
	C_5	+	–	+	–
	C_6	+	+	–	–
D	D_1	–	–	–	+
	D_2	–	–	+	–
	D_3	–	+	–	–
	D_4	+	–	–	–
E	E	–	–	–	–

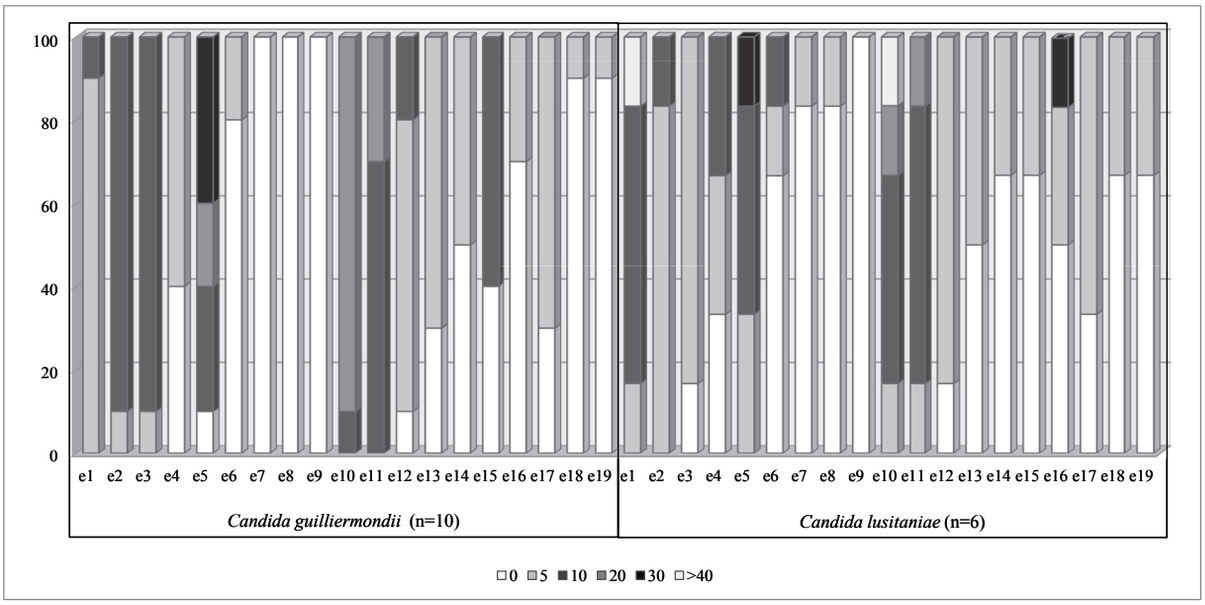

Fig. 1. The percentage of *Candida guilliermondii* (n=10) and *Candida lusitaniae* (n=6) strains showing activity of particular enzymes (in nmol divide substrate) in API ZYM test (bioMérieux)

Four of the seven examined strains did not produce any α-chymotrypsin (e_9), while two did not produce trypsin (e_8), and only single strains did not produce cystine arylamidase (e_7), α-glucosidase (e_{15}), α-mannosidase (e_{18}) or α-fucosidase (e_{19}). Detailed data regarding the activities of particular enzymes based on fungal strain, according to the API ZYM test are presented in Figs 1–3.

The test results indicate that the isolated fungal species produced from 16 to 19 hydrolases. The most active were leucine arylamidase (e_5) and acid phosphatase (e_{10}), produced by 5 species; alkaline phosphatase (e_1) and naphthol-AS-BI-phosphohydrolase (e_{11}) by 2 species; and esterase – C4 (e_2), β-galactosidase (e_{13}) and β-glucosidase (e_{16}) by one species.

Thirteen biotypes characteristic of particular species of fungi were defined based on their ability to release certain hydrolytic enzymes. Most strains could be categorized as biotypes C_2 – 39.5% and A

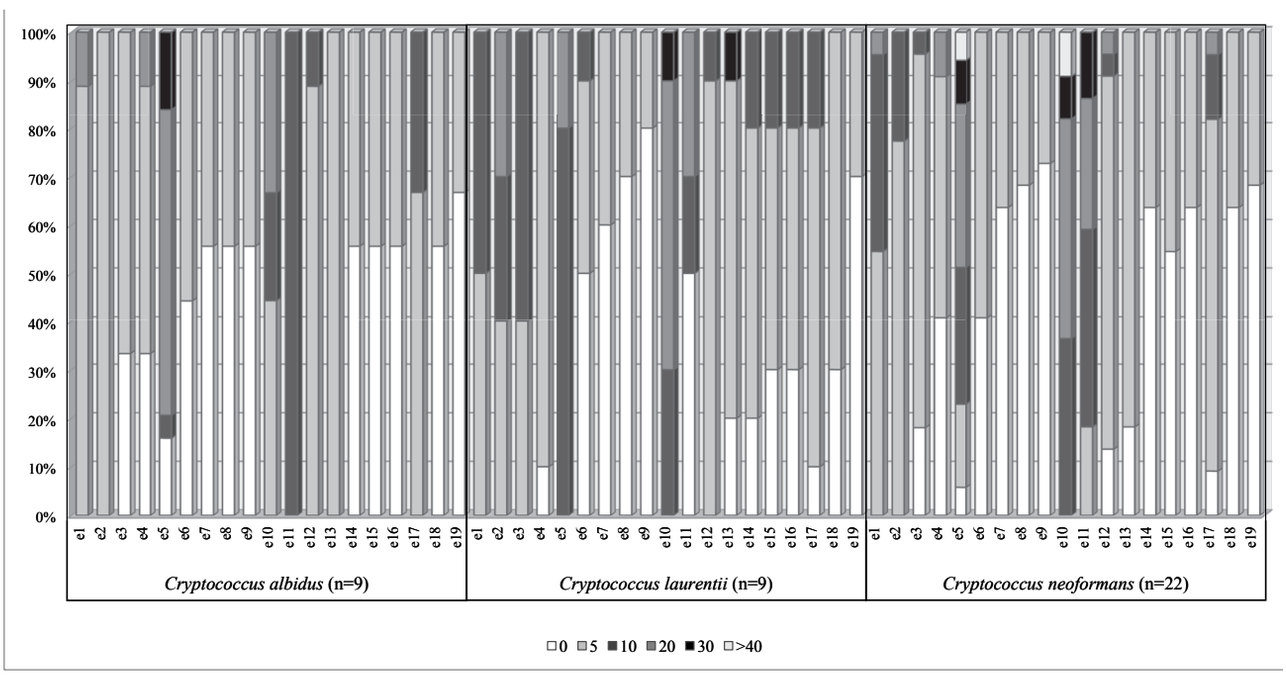

Fig. 2. The percentage of *Cryptococcus albidus* (n=9), *Cryptococcus laurentii* (n=10) and *Cryptococcus neoformans* (n=22) strains showing activity of particular enzymes (in nmol divide substrate) in API ZYM test (bioMérieux)

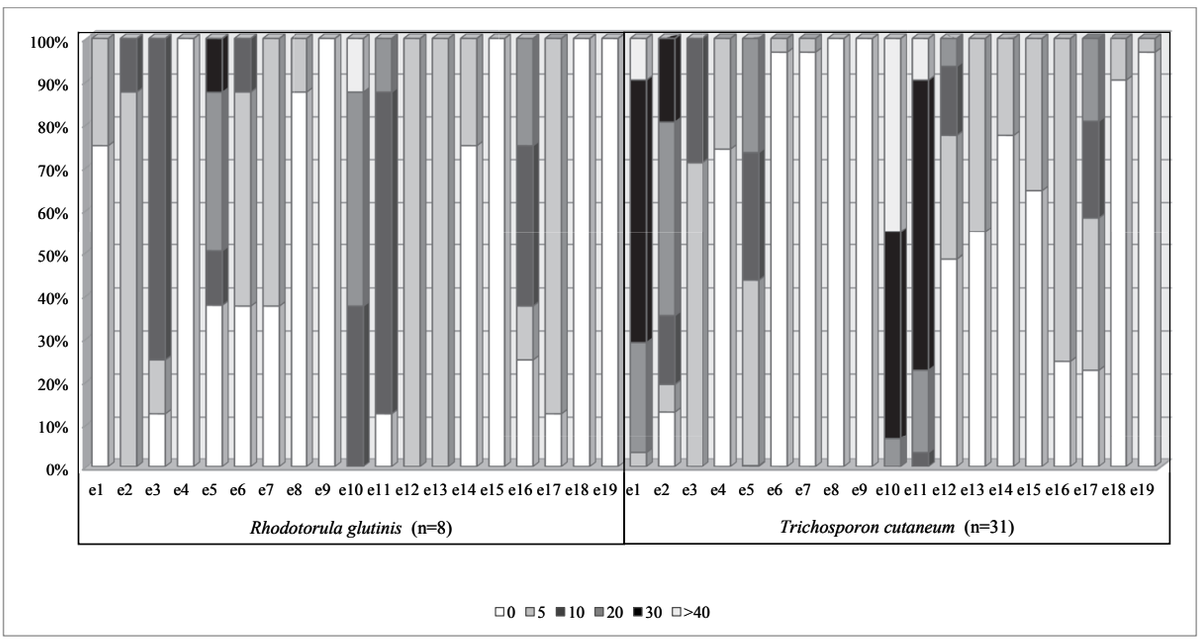

Fig. 3. The percentage of *Rhodotorula glutinis* (n=8) and *Trichosporon cutaneum* (n=31) strains showing activity of particular enzymes (in nmol divide substrate) in API ZYM test (bioMérieux)

– 26%. The greatest variety was demonstrated for *Cryptococcus neoformans* (7 different biotypes), *Candida guilliermondii* (6 different biotypes), *Cryptococcus laurentii* and *Trichosporon cutaneum* (each with 5 different biotypes), *Candida lusitaniae* (4 different biotypes) and *Cryptococcus albidus* (3 different biotypes). All strains of *Rhodotorula rubra* were classified as C_6 biotype. Datailed results are presented in Table 2.

Discussion

Most of the assessed hydrolytic enzymes (14–19) were produced by the strains examined in this study. It is difficult to compare our present findings with literature data, because most of the studies are based on *C. albicans* and other species of *Candida*. Nevertheless, in most cases, the highest activity was shown by leucine arylamidase (e_5), acid phosphatase (e_{10}) and naphthol-AS-BI-phospho-

Table 2. The percentage of particular species classified to different biotypes

Species	Biotype												
	A	B_1	B_2	B_3	B_4	C_2	C_3	C_4	C_5	C_6	D_1	D_2	D_3
Cryptococcus neoformans	36.4	4.54		13.6	4.54	31.8				4.54			4.54
C.albidus	44.4			11.2		44.4							
C.terreus	25.0			50.0								25.0	
C.laurentii		20.0	40.0			20.0	10.0	10.0					
Candida lusitaniae	14.3				28.6	42.9							14.3
C.guilliermondii	10.0	20.0			10.0	30.0	20.0						10.0
Geotrichum sp.						50.0					50.0		
Trichosporon cutaneum		14.3				57.1	22.9		2.85				2.85

Table 3. Literature data concerning enzymatic activity of fungi and their biotypes

	Enzymes		The most common biotype*	Genus/species Download site	Author
	the highest activity	inactive			
1	1,10,11	8,9,19		*Candida* sp. Oral cavity	Majewska et al. [23]
2	1,5,10,11	9,14,19		*Candida albicans* Oral cavity	Drozdowska [10]
3	2,5,10	8,9		*Candida albicans* Oral cavity	Anees et al. [26]
4	1,2,5,10,13	12,16		*Candida krusei* Oral cavity	
5	5,10,11	8,9,13,14,19	A (49.2%) B_4 (42.9%)	*Candida albicans* Oral cavity	Pytko-Polonczyk et al. [18]
6	5,10,11	9,18	D_3 (22.5%) C_6 (17.5%) A (15%)	*Candida* sp. Oral cavity	Moqbil and Kurnatowski [15]
7	2,3,5,7	14,19	A (48,9%) B_4 (42%)	*Candida albicans* Oral cavity	Lukaszuk et al. [32]
8	2,3,5	19	A (44%) B_4 (26.3%)	*Candida albicans* Oral cavity	Batura-Gabryel and Mlynarczyk [20]
9	2,3,5,10	8,9,14,18,19		Non-*C. albicans* Oral cavity	
10	2,3,5,6,16	8,9,19		*Candida* sp. Different biological materials	Batura-Gabryel et al. [21]
11	5,10	1,4,6-9,11-19		*Candida albicans* Different biological materials	Mamos and Kurnatowska [24]
12			C_6 (36.4%) B_3 (24.2%) C_1 (24.2%)	*Candida albicans* sputum	Brajer and Batura-Gabryel [31]
13	5,10,11	4,8,12-14,18,19	A (18%) C_3 (16%) C_6 (22%) D_3 (18%)	*Candida albicans* Voice prosthesis	Nowak [16]
14	5,10,11	4,7-9,12-19	D_3 (91%) C_6 (9%)	*Candida glabrata* Voice prosthesis	
15	5,17	12,13,16,19	A (59%) D_1 (10.6%) D_3 (9.1%)	*Candida* sp. Urethra	Krajewska-Kułak et al. [11]
16	2,3,5,10,11, 17	8,12,13,19	B_4 (35%) A (30%) C_6 (20%)	*Candida* sp. Vagina	
17	1,5,10,17	4,8,9,12-16,18, 19		*Candida* sp. Vagina	Ogrodziński and Kurnatowska [25]
18	5,10	4,8,9,12,14,18, 19	A (52.2%) B_4 (30.4%) B_3 (17.5%)	*Candida albicans* Different biological materials	Dąbkowska [22]
19	5,10	4,7-9,12-16, 18,19		*Candida* sp. Oral cavity	Kurnatowski and Tyczkowska-Sieron [13]

	Enzymes		The most common biotype*	Genus/species Download site	Author
	the highest activity	inactive			
20	1,2,5,10,11	8-9,13,14,16,19	D_3 (40.4%) C_6 (10.6%) B_3 (8.51%)	*Candida albicans* Oral cavity	Kurnatowskiet al. [14]
21	N.E.	12-14,18,19	C_6 (30.4%) B_4 (22.3%) A (16.1%)	*Candida* sp. Digestive tract	Kurnatowski et al. [12]
22	2,3,5,10,11, 17	4,8,9,12-14,18,19	B_3 (35.3%) B_1 (17.6%) D_3 (11.8%)	*Candida* sp. Different biological materials	Plomer-Niezgoda et al. [17]
23	2,5,10,11	8,14,19		Nails Immunosupression	Weglowska et al. [6]
24	2,5,10	4,7-9,12,14,18, 19		Nails Immunocompetence	
25	5,10,11	4,8,9,12-14		*Candida* sp. skin	Skóra et al. [19]
26	5,10,11	4,8,9, 12-14, 17-19		*Rhodotorula* sp. skin	
27	10,11,16	1,7-9,12,14,18, 19		*Cryptococcus* Different biological materials	Leone et al. [28]
28	1,3,5,10,11	4,8,9,13,14,18		*Cryptococcus* Different biological materials	Garcia-Martos et al. [27]
29	2,3,10,14,15	8,9,12,13,19		*Cryptococcus* Different biological materials	Vidotto et al. [29]

N.E. – not estimated

hydrolase (e_{11}), also noted previously by Drozdowska [10], Krajewska-Kulak et al. [11], Kurnatowski et al. [12–14], Moqbil and Kurnatowski [15], Nowak [16], Plomer-Niezgoda et al. [17], Pytko-Polonczyk et al. [18], Skora et al. [19] and Weglowska et al. [6].

C. guilliermondii strains did not produce cystine arylamidase (e_7), trypsin (e_8) or α-chymotrypsin (e_9), while *C. lusitaniae* produced only α-chymotrypsin (e_9). Previous studies indicated that the following enzymes are not produced by most *Candida* strains: trypsin (e_8), α-chymotrypsin (e_9), as in our study, and α-galactosidase (e_{12}), β-galactosidase (e_{13}), β-glucuronidase (e_{14}), α-mannosidase (e_{18}) and α-fucosidase (e_{19}) [6,12–14, 16–25]. Interestingly, α-chymotrypsin (e_9) was not found in the present study. Anees [26] reports that *C. albicans,* one of the most common human pathogenic fungi, is able to produce all the hydrolases estimated by the API ZYM test. The literature values of fungal enzyme production are included in Table 3.

In present study the *Rhodotorula* strains were characterized by the activity of 14 enzymes with the highest production being of leucine arylamidase (e_5), acid phosphatase (e_{10}) and β-glucosidase (e_{16}). It should be noticed that lipase – C14 (e_4), α-chymotrypsin (e_9), α-glucosidase (e_{15}), α-mannosidase (e_{18}) and α-fucosidase (e_{19}) were not secreted. Of the enzymes secreted by strains examined by Skora [19], the highest production was found for leucine arylamidase (e_5), acid phosphatase (e_{10}) and naphthol-AS-BI-phosphohydrolase (e_{11}); however nine enzymes were not produced: lipase – C14 (e_4), trypsin (e_8), α-chymotrypsin (e_9), α-galactosidase (e_{12}), β-galactosidase (e_{13}), β-glucuronidase (e_{14}), N-acetylo-β-glucosyloaminidase (e_{17}), α-mannosidase (e_{18}), α-fucosidase (e_{19}). Detailed results are in Table 3.

In the present study *Cryptococcus* strains were able to produce all the examined hydrolases: the highest concentrations were noticed for leucine arylamidase (e_5) and acid phosphatase (e_{10}). According the literature data the most strains of the

genus *Cryptococcus* are characterized by the following enzymes: esterase C4 (e_2), esterase lipase C8 (e_3), leucine arylamidase (e_5), acid phosphatase (e_{10}), naphthol-AS-BI-phosphohydrolase (e_{11}). However, they do not produce alkaline phosphatase (e_1), cystine arylamidase (e_7), trypsyne (e_8), α-chymotrypsyne (e_9), α-galactosidase (e_{12}), β-galac - tosidase (e_{13}), β-glucuronidase (e_{14}), α-mannosidase (e_{18}) and α-fucosidase (e_{19}) [27–30].

Interestingly, Vidotto et al. [29] report that only 9 enzymes were produced by *Cryptococcus neoformans* strains: alkaline phosphatase (e_1), esterase – C4 (e_2), esterase lipase – C8 (e_3), leucine arylamidase (e_5), acid phosphatase (e_{10}), naphthol-AS-BI-phosphohydrolase (e_{11}), β-glucuronidase (e_{14}), α-glucosidase (e_{15}), β-glucosidase (e_{16}). The most active were: acid phosphatase (e_{10}), naphthol-AS-BI-phosphohydrolase (e_{11}) and β-glucosidase (e_{16}). In later studies [30] they compare the enzymatic activities of *Cryptococcus neoformans* in different countries. Chymotrypsin (e_9) and α-fucosidase (e_{19}) were not detected in any case. The number of extracellular enzymes differ from 9 to 15, depending on the country. The most active were found to be: esterase (e_2), esterase lipase (e_3), leucine arylamidase (e_5) and phosphatase acid (e_{10}) in the majority of the strains from different countries (95–100%). Detailed data is given in Table 3.

Thirteen different biotypes were found; the most common being A (0–44.4%), B_1 (0–20.0%), C_2 (0–57.1%) and D_3 0–14.3%) based on the 4 enzymes: valine arylamidase (e_6), naphthol-AS-BI-phosphohydrolase (e_{11}), α-glucosidase (e_{15}) and N-acetylo-β-glucosyloaminidase (e_{17}). The literature data given in Table 3 suggests that fungi isolated from patients are characterized mainly as biotypes A (0–59.0%), B_3 (0–35.3%), B_4 (0–42.9%), C_1 (0–24.2%), C_6 (0–36.4%) and D_3 (0–91.0%) [11–18, 20–22, 31, 32]

Conclusions

Examined fungal strains isolated from recreational areas have selected biochemical characteristics namely hydrolases productions, which demonstrate their pathogenicity.

They produce a number of enzymes which are also present in strains isolated from patients with mycoses including: leucine arylamidase (e_5), acid phosphatase (e_{10}), naphthol-AS-BI-phosphohydrolase (e_{11}) and alkaline phosphatase (e_1).

The biotypes identified in the course of this study (A, B_3, B_4, C_1, C_6 and D_3) have been also reported in cases of fungal infection.

Fungi present in the sand and soil of recreational areas have pathogenic properties and are possible factors of fungal infection among children.

Acknowledgements

Study was supported by Ministry of Science and High Education of Poland: N N304 006539.

Presented study comply with the current laws of Poland. There are no conflicts of interest.

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Received 21 June 2016
Accepted 23 August 2016