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Original Research

Extracellular Enzyme Profiles of Xerophilic Fungi Isolated from Dried Materials of Medicinal Plants

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Abstract

Fungal extracellular enzymes may play a role in biodeterioration of dried materials of medicinal plants and in propagation of toxigenic and pathogenic fungal strains. However, no data on enzymatic activities of xerophilic fungi contaminating these materials have been found in the literature. The objective of the study was to determine extracellular enzyme profiles of slow-growing fungi, i.e. *Eurotium amstelodami, E. chevalieri, E. herbariorum* and *Aspergillus versicolor* isolated from dried materials of medicinal plants from herbal shops of Szczecin, Poland. Solid media and API ZYM® test were used to determine enzymatic activities. The highest colony diameters were observed in *A. versicolor* on gelatin, cellulose, tributyrin, rapeseed oil, biodiesel oil and diesel oil agars, and in *E. herbariorum* on milk and starch agars. *A. versicolor* also showed the highest hydrolytic activity on milk, gelatin, starch and tributyrin agars. No hydrolysis zones were formed on cellulose, rapeseed oil and biodiesel oil agars, but the stimulation effect of the oils on fungal growth was clearly observed. The effect was the highest in *E. amstelodami*, and considerably increased during a 21-day incubation period. In addition, *E. amstelodami* and *A. versicolor* showed high catalase, urease and DNA-se activities. *A. versicolor* had higher pectate lyase activity compared to *E. amstelodami*. Of the fungi examined, *E. amstelodami* showed the highest hydrolase activity in the API ZYM® test. *A. versicolor* and *E. amstelodami* were found to be the two species with the highest biodeterioration potential for dried materials of medicinal plants. Xerophilic fungi isolated from this environment could also be used in bioremediation.

Keywords: xerophilic fungi, *Eurotium amstelodami, E. chevalieri, E. herbariorum, Aspergillus versicolor*, extracellular enzymes, solid media, API ZYM®, dried materials of medicinal plants

Introduction

Medicinal plants are widely used for prophylaxis or treatment of many diseases, usually to complete chemical treatment methods. Poland is the leader on the medicinal plant market [1]. However, the problem of plant contamination with microscopic fungi is still poorly understood. Most attention has been paid to the contamination of medicinal plants with mesophilic fungi, e.g. *Aspergillus flavus, A. parasiticus, A. ochraceus* and their mycotoxins, e.g. aflatoxins and ochratoxin A [2-6]. Thermophilic and thermotolerant, actidione-resistant and xerophilic and xerotolerant fungi have also been isolated from these plants [7, 8].

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Xerophilic fungi, including *Eurotium* species and *A. versicolor*, are able to grow at a wide range of water activity in many types of environments. The fungi are ubiquitous and found in most foods with low and intermediate moisture contents, including spices, tea and medicinal plants [6, 9, 10]. The mycobiota of these plants have been studied in many countries all over the world [11-15]. Much attention has been paid to plant decontamination methods [16, 17]. However, the enzymatic activity of xerophilic fungi is poorly characterized [18, 19]. No data on the activity of the fungi isolates from dried materials of medicinal plants has been found in the literature. Fungal extracellular enzymes may play a role in biodeterioration of these plants, in propagation of toxigenic and pathogenic strains, and may be useful in biotechnology.

Table 1. Colony diameters of fungal species from dried materials of medicinal plants after a 21-day incubation at 25ºC on agar media containing different substrates.

Fungal species	Colony diameter [mm] on agar media containing:							
	Milk	Gelatin	Starch	Cellulose	Tributyrin	Rapeseed oil	Biodiesel oil	Diesel oil
E. amstelodami	41.3 ± 4.9*	38.9 ± 5.5	43.2 ± 7.9	16.1 ± 3.6	30.5 ± 4.4	26.9 ± 2.9	34.8 ± 3	14.1 ± 3.2
E. chevalieri	25 ± 0	31.5 ± 19.1	50 ± 0	10 ± 1.4	30 ± 0	21.5 ± 0.7	25.5 ± 0.7	10.5 ± 0.7
E. herbariorum	53.8 ± 7	41.8 ± 3.8	50.3 ± 16.3	30.2 ± 23.9	29.5 ± 1.2	25.5 ± 6.3	33.2 ± 4.3	15.3 ± 5.2
A. versicolor	52.5 ± 6.7	42 ± 11.7	49 ± 8	52.6 ± 10.1	38.4 ± 5.7	29.3 ± 2.3	34.9 ± 4.9	22.1 ± 2.7

* - mean ± standard deviation

Table 2. Hydrolytic activity indices of fungal species from dried materials of medicinal plants during incubation at 25ºC on agar media containing different substrates.

Fungal species	Incubation time [day]	Hydrolytic activity indices on agar media containing:			
		Milk	Gelatin	Starch	Tributyrin
E. amstelodami	5	0	0.4 ± 0.9	0.9 ± 0.1	1.4 ± 0.2
	9	0	nd	0.9 ± 0.1	1.4 ± 0.1
	13	0	nd	0.9 ± 0.1	1.4 ± 0.1
	17	0	nd	0.9 ± 0.1	1.5 ± 0.2
	21	0	0.5 ± 0.9	0.9 ± 0.1	1.4 ± 0.2
E. chevalieri	5	0	0	1 ± 0	1.3 ± 0
	9	0	nd	0.9 ± 0.1	1.4 ± 0
	13	0	nd	0.9 ± 0	1.4 ± 0.1
	17	0	nd	1 ± 0	1.3 ± 0.1
	21	0	0	1 ± 0	1.3 ± 0
E. herbariorum	5	0	0.6 ± 0.6	0.6 ± 0.1	0.8 ± 0.6
	9	0.3 ± 0.5	nd	0.8 ± 0.1	0.7 ± 0.6
	13	0.4 ± 0.6	nd	0.8 ± 0.1	1.2 ± 0.1
	17	0.4 ± 0.6	nd	1 ± 0.1	1.3 ± 0.2
	21	0.4 ± 0.6	0.7 ± 1.1	1 ± 0.1	1.4 ± 0.2
A. versicolor	5	1.2 ± 0.1	2.7 ± 1	1.1 ± 0.3	1.7 ± 0.4
	9	1.3 ± 0.2	nd	1.4 ± 0.3	1.7 ± 0.4
	13	1.3 ± 0.2	nd	1.4 ± 0.3	1.7 ± 0.3
	17	1.3 ± 0.1	nd	1.4 ± 0.2	1.7 ± 0.3
	21	1.3 ± 0.1	2.2 ± 0.5	1.3 ± 0.2	1.7 ± 0.2

* - mean ± standard deviation
nd – not done

Table 3. Inhibition/stimulation indices on fatty substrates for fungal species from dried materials of medicinal plants.

Substrate	Incubation time [day]	Inhibition/stimulation index for species:			
		E. amstelodami	*E. chevalieri*	*E. herbariorum*	*A. versicolor*
Tributyrin	5	1 ± 0.4*	1 ± 0	1.2 ± 0.1	0.9 ± 0.2
	9	1.2 ± 0.3	1 ± 0.1	1.3 ± 0.1	1.1 ± 0.1
	13	1.4 ± 0.3	1.2 ± 0	1.3 ± 0.3	1.2 ± 0.1
	17	1.4 ± 0.2	1.3 ± 0	1.4 ± 0.3	1.4 ± 0.2
	21	1.5 ± 0.3	1.7 ± 0	1.5 ± 0.4	1.4 ± 0.3
Rapeseed oil	5	1.3 ± 0.2	1.1 ± 0.1	1 ± 0.2	1.3 ± 0.2
	9	1.4 ± 0.2	1.3 ± 0	1.1 ± 0.2	1.5 ± 0.3
	13	1.7 ± 0.3	1.1 ± 0	1.2 ± 0.2	1.5 ± 0.3
	17	2 ± 0.2	1.3 ± 0	1.3 ± 0.1	1.6 ± 0.2
	21	2.3 ± 0.3	1.7 ± 0.1	1.5 ± 0.1	1.6 ± 0.4
Biodiesel oil	5	1.9 ± 0.4	1.3 ± 0.1	1.3 ± 0.2	1.5 ± 0.2
	9	2.3 ± 0.4	2.1 ± 0.3	1.7 ± 0.3	1.6 ± 0.4
	13	2.4 ± 0.6	1.9 ± 0.2	1.8 ± 0.2	1.7 ± 0.2
	17	2.6 ± 0.5	2 ± 0.1	2 ± 0.4	1.8 ± 0.2
	21	2.9 ± 0.4	2 ± 0.1	2 ± 0.3	1.9 ± 0.5
Diesel oil	5	1 ± 0.2	0.7 ± 0.1	0.6 ± 0	0.8 ± 0.1
	9	0.9 ± 0.1	0.8 ± 0	0.7 ± 0.1	1 ± 0.3
	13	1 ± 0.2	0.8 ± 0.1	0.7 ± 0.1	1.1 ± 0.2
	17	1.1 ± 0.2	0.8 ± 0.2	0.8 ± 0.1	1.1 ± 0.2
	21	1.2 ± 0.2	0.8 ± 0.1	0.9 ± 0.1	1.2 ± 0.2

* - mean ± standard deviation

The objective of the study was to determine extracellular enzyme profiles of slow-growing xerophilic fungi, i.e. *Eurotium amstelodami, E. chevalieri, E. herbariorum* and *Aspergillus versicolor*, isolated from dried materials of selected medicinal plants. Solid media and API ZYM® test were used to determine fungal enzymatic activities.

Material and Methods

The fungal species investigated were *Eurotium amstelodami* (5 isolates), *E. chevalieri* (1 isolate), *E. herbariorum* (3 isolates), and *Aspergillus versicolor* (4 isolates). All isolates were from dried materials of medicinal plants purchased in herbal shops of Szczecin, West Pomeranian Voivodeship, Poland. *E. amstelodami* occurred abundantly in peppermint (*Folium Menthae piperitae*), lemon balm (*Folium Melissae*), St. John's wort (*Herba Hyperici*) and in two herbal mixtures. Chamomile (*Flos Chamomillae*) was the only medicinal plant from which the species was not isolated. Subsequently, *E. herbariorum* and *A. versicolor* occurred in St. John's wort and herbal mixture and in lemon balm, respectively. Finally, only one *E. chevalieri* isolate was recovered from all medicinal plant samples examined [7].

Extracellular enzyme profiles were determined on solid media using the API ZYM® test (bioMèrieux, France). Amylase production was tested on Bacto nutrient agar (Difco) containing 0.2% soluble starch [20]. After incubation the plates were flooded with iodine solution. Yellowish "halo" was shown around, or underneath, the amylase-producing colonies. Casein hydrolysis was detected on the medium containing commercially available defatted and powdered milk [21]. Subsequently, the Frazier medium was used for gelatinase [22]. Clear zones around the casein hydrolase-producing colonies were observed without development, whereas gelatin hydrolysis zones were developed with saturated $HgCl_2$ solution. Cellulolytic activity was tested on Bravery's mineral salts medium supplemented with crystalline cellulose [23]. Hydrolysis zones were observed without development. Media for testing fatty substrates (tributyrin, rapeseed oil, biodiesel oil and diesel oil) were those of Janda-Ulfig et al. [24]. On tributyrin agar clear hydrolysis zones were shown without development. On rapeseed oil (fatty acid glycerol esters) and biodiesel oil (fatty acid methyl esters) agars, the color of the media changed from salmon to green or blue due to the liberation of fatty acids and pH decrease (Nile blue was the pH indicator). On diesel agar, fungal growth was only examined.

Table 4. Catalase, urease, DNA-se, pectate lyase and polygalacturonase activities of fungal species from dried materials of medicinal plants.

Fungal species	Catalase	Urease	DNA-se	Pectate lyase	Polygalacturonase
E. amstelodami	+/+++[1]	+/++[2]	++/+++[2]	-/+[2]	-
E. chevalieri	++	++	++	-	-
E. herbariorum	+/+++	+	++/+++	-/+	-
A. versicolor	+/++	++/+++	+/+++	++/+++	-

[1](+) – weak reaction; (++) – moderate reaction; (+++) – strong reaction.
[2](-) – no activity; (+) – weak activity (hydrolysis zone width <2 mm); (++) – moderate activity (hydrolysis zone width 2-5 mm); (+++) – strong activity (hydrolysis zone width >5 mm).

Table 5. Production of 19 hydrolases by fungal species from dried materials of medicinal plants in the APIZYM® test (bioMèrieux). Rounded up mean values in the test color scale.

Hydrolases	*E. amstelodami*	*E. chevalieri*	*E. herbariorum*	*A. versicolor*	Sum
Phosphatase alkaline	2	-	1	1	4
Esterase (C_4)	1	1	2	1	5
Esterase Lipase (C_8)	2	1	1	1	5
Lipase (C_{14})	-	-	-	-	-
Leucine arylamidase	1	-	1	-	2
Valine arylamidase	-	-	-	1	1
Cystine arylamidase	-	-	-	-	-
Trypsin	-	-	-	-	-
Chymotrypsin	-	-	-	-	-
Phosphatase acid	2	2	3	2	9
Naphthol-AS-BI-phosphohydrolase	2	2	3	1	8
α-galactosidase	-	-	-	-	-
β-galactosidase	-	1	-	-	1
β-glucuronidase	-	-	-	-	-
α-glucosidase	-	-	-	-	-
β-glucosidase	3	2	-	3	8
N-acetyl-β-glucosaminidase	4	2	2	3	11
α-mannosidase	-	-	-	-	-
α-fucosidase	-	-	-	-	-
Sum	17	11	13	13	-

Rapeseed oil was a commercially available product. Biodiesel oil and diesel oil was provided by one of the Polish oil refineries. Controls were media devoid of fatty substrates.

The method for catalase was that of Bordner and Winter [25]. Urease production was tested on Christensen's urea agar and urea hydrolysis was shown by the appearance of a deep pink color [26]. Deoxyribonuclease activity was detected on DNA-se test medium (Difco). After incubation the plates were flooded with 1M HCl. DNA depolymerization was shown by clear zones surrounding the colonies. Pectate lyase and polygalacturonase activities were tested on the medium by Hankin and Anagnostakis [20]. After incubation the plates were flooded with 1% hexadecyltrimethylammonium bromide aqueous solution to develop hydrolytic zones.

Ten-day fungal cultures on MEA slants at 25ºC were used for preparing propagule suspensions. Five ml of sterile physiological saline was added to each slant. The slants were then vigorously shaken with a Vortex for three minutes.

The plates were each centrally inoculated with 5 µl of the suspension and then incubated at 25ºC in the dark for 21 days. For each strain, production of each enzyme was tested in 3 repetitions (plates). After 5, 9, 13, 17 and 21 days of incubation, colony and hydrolysis zone diameters were measured on starch, milk, gelatin, cellulose, tributyrin, rapeseed oil, biodiesel and diesel agars with a ruler. Two indices of fungal growth and hydrolytic activity were computed:

1. hydrolytic activity index for starch, milk, gelatin, cellulose, tributyrin, rapeseed oil and biodiesel oil (hydrolysis zone diameter/colony diameter ratio); and,
2. growth stimulation/inhibition index for fatty substrates (colony diameter on fatty substrate agar/colony diameter on control agar ratio).

When the growth stimulation/inhibition index value was <1, the given fatty substrate inhibited fungal growth, while the index value was >1, the substrate stimulated the growth.

One-way ANOVA test were used for statistical analysis of the data obtained. The test was performed at $p \leq 0.05$.

Catalase activity was evaluated using the scale:

(+) – weak reaction (single bubbles);
(++) – moderate reaction;
(+++) – strong reaction (abundant bubbles).

The scale for urease, DNA-se, pectate lyase and polygalacturonase activities was as follows:

(-) – no activity;
(+) – weak activity (hydrolysis zone width <2 mm);
(++) – moderate activity (hydrolysis zone width 2-5 mm);
(+++) – strong activity (hydrolysis zone width >5 mm).

The API ZYM® test allows examination of 19 hydrolases (phosphatase alkaline, esterase (C_4), esterase lipase (C_8), lipase (C_{14}), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, chymotrypsin, phosphatase acid, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase, and α-fucosidase). Reading was performed using the API-ZYM® color scale ranging from 0 (no activity) to 5 (maximum activity). Rounded up mean values and the sum of these values were computed for each species and hydrolase.

Results and Discussion

After a 21-day incubation the highest colony diameters were observed in *A. versicolor* on cellulose, tributyrin, rapeseed oil and diesel oil agars (Table 1). The differences between *A. versicolor* and the other species in the colony diameter on the media were statistically significant at $p \leq 0.05$. The colony diameters of *A. versicolor* and *E. herbariorum* were found to be high and similar on milk, gelatin, starch and biodiesel oil agars. Except for starch agar, the lowest colony diameters on the media were observed in *E. chevalieri. E. amstelodami* showed the lowest colony diameter on starch agar. These differences were also statistically significant at $p \leq 0.05$.

No hydrolysis zones were observed in *E. amstelodami* and *E. chevalieri* on milk agar (Table 2). *E. herbariorum* showed such zones underneath the colonies (caseinase activity index <1). Hydrolytic zones around the colonies were only observed in *A. versicolor* (index >1). After a 21-day incubation period the differences in the caseinase activity index between *A. versicolor* and the other species were statistically significant at $p \leq 0.05$. Gelatin hydrolysis zones were observed underneath the *Eurotium* colonies (index <1). *A. versicolor* displayed gelatinase activity around the colonies (index >2). The differences in the 21-day gelatinase activity index between *A. versicolor* and the other species were statistically significant at $p \leq 0.05$. The *Eurotium* amylolytic activity indices were ≤1. Amylolytic zones around the colonies were observed in *A. versicolor* (index >1). In *E. herbariorum* and, to a much lower extent, in *A. versicolor*, the amylolytic activity increased during incubation. The differences in the 21-day amylolytic activity index between *A. versicolor* and the other species were statistically significant at $p \leq 0.05$. On tributyrin agar hydrolysis zones around the colonies and activity index >1 during the whole incubation period were observed in *A. versicolor*, *E. amstelodami*, and *E. chevalieri. E. herbariorum* showed hydrolytic zones underneath the colonies up to the 9th day, and the zones expanded out of the colonies during further incubation. *A. versicolor* had the highest esterase activity index (1.7) on tributyrin agar. The differences in the 21-day esterase activity index between *A. versicolor* and the other species were statistically significant at $p \leq 0.05$. No zones were observed on cellulose, rapeseed oil and diesel oil agars.

Our finding of high amylase, gelatinase and esterase activities in *A. versicolor* agrees with that by Gobinath et al. [19] for isolates from oil-rich environments. Also, the finding on the lack of proteolytic activity in *E. amstelodami* agrees with that of Ismail [18] for peanut and coconut isolates.

Tributyrin, rapeseed oil, biodiesel oil and/or the products of their hydrolysis stimulated the growth of all fungi examined (Table 3). The inhibition/stimulation index was >1, and increased during incubation. The highest stimulation effects were found in *E. amstelodami* on biodiesel oil and rapeseed oil agars. The differences in the 21-day inhibition/stimulation index between *E. amstelodami* and the other species were statistically significant at $p \leq 0.05$. Diesel oil stimulated the growth of *E. amstelodami* and *A. versicolor* (index mostly >1). However, this substrate and/or the products of its biodegradation inhibited the growth of *E. chevalieri* and *E. herbariorum* (index <1). In *E. herbariorum* the inhibition effect decreased during incubation (index values increased). The differences in the 21-day inhibition/stimulation index between *E. herbariorum* and *E. chevalieri* and the other species were statistically significant at $p \leq 0.05$, whereas no statistically significant differences were found for inhibition/stimulation indices on tributyrin agar.

The *E. amstelodami* and *E. herbariorum* catalase reaction ranged from weak (single bubbles) to strong (abundant

bubbles) (Table 4). The reaction of *E. chevalieri* and *A. versicolor* varied from weak to moderate. The urease activities were found to be moderate to strong, moderate, weak to moderate, and weak in *A. versicolor, E. chevalieri, E. amstelodami* and *E. herbariorum,* respectively. The DNAse activity was moderate to strong in both *E. amstelodami* and *E. herbariorum*, weak to strong in *A. versicolor*, and moderate in *E. chevalieri*. Moderate to strong pectate lyase activity was observed in *A. versicolor*. The species from the genus *Eurotium* showed no or weak activity of this enzyme. No polygalacturonase activity was observed in all isolates examined.

N-acetyl-β-glucosaminidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, esterase lipase (C_8) and esterase (C_4) activities were observed in all species (Table 5). Alkaline phosphatase was not shown in *E. chevalieri,* while β-glucosidase did not occur in *E. herbariorum.* Leucine arylamidase activity was observed in *E. amstelodami* and *E. herbariorum*, while valine arylamidase occurred in *A. versicolor*. *E. chevalieri* was the only species with β-galactosidase activity.

The API ZYM® data complemented the results obtained in solid media. Of the fungi examined, *E. amstelodami* showed the highest hydrolase activity followed by *E. herbariorum, A. versicolor* and *E. chevalieri*. Of the hydrolases tested, N-acetyl-β-glucosaminidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase and β-glucosidase activities were found to be the highest. However, the qualitative differences in hydrolase activities between the species were rather small.

In general, the dried medicinal plant materials differed in the qualitative and quantitative composition of fungal contaminants. Subsequently, the fungi contaminating these materials differed in extracellular enzyme profiles. *A. versicolor* was found to be the species with the highest colony diameter and enzymatic activity on a wide variety of substrates, whereas the highest stimulation effect characterized the growth of *E. amstelodami* on rapeseed and biodiesel oils. The last species had the widest distribution among the medicinal plants examined. The conclusion is that *A. versicolor* and *E. amstelodami* are the species with the highest biodeterioration potential to dried medicinal plant materials. The abilities of both species to produce mycotoxins should also be mentioned [27, 28]. The data indicate that due to the ability to degrade oils and hydrocarbons fungal isolates from medicinal plants can be used for biotechnological purposes, e.g. in air biofiltration and waste or soil bioremediation.

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