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A STUDY OF THE GROWTH OF *PSEUDALLESCHERIA BOYDII* ISOLATES FROM SEWAGE SLUDGE AND CLINICAL SOURCES ON TRIBUTYRIN, RAPESEED OIL, BIODIESEL OIL AND DIESEL OIL

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Abstract: The study compared the growth of *Pseudallescheria boydii* isolates from sewage sludge and from clinical sources on tributyrin, rapeseed oil, biodiesel oil and diesel oil agars. The isolates grew on all substrates tested. The highest growth was observed on rapeseed oil agar, while the lowest on diesel agar. On tributyrin agar, hydrolysis zones were observed around or underneath the colonies. On rapeseed oil agar, no hydrolysis zones were formed, while most isolates formed such a zone on biodiesel oil agar. Rapeseed oil and biodiesel oil stimulated the growth of *P. boydii* isolates, while tributyrin inhibited fungal growth. The stimulation or inhibition effect of diesel oil was dependent on the specified strain. In clinical isolates, fungal growth and activity were found to be more variable compared to sludge isolates. The data suggest that contamination of the environment with these oils could favor the growth of *P. boydii*. However, no association was found between the growth and oil utilization by this fungus and its pathogenicity.

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Key words: ***Pseudallescheria boydii*, growth, hydrolysis, rapeseed oil, biodiesel oil, diesel oil.**

INTRODUCTION

Emerging infections have become an important public health problem over the last three decades. Although emerging viral and bacterial infections have been the central focus of most reports, the emerging mycotic diseases are an important group of infections receiving increased attention. Among the emerging fungal pathogens, *Pseudallescheria boydii* (Shear) McGinnis *et al.* (anamorph *Scedosporium apiospermum* (Sacc.) Sacc.) is of special public health importance. This fungus causes localized or disseminated infections called *hyalohyphomycosis*, which is characterized by the growth of non-pigmented septate hyphae in the infected tissue. *P. boydii* shows high morphological, physiological and molecular variability. The fungus is also resistant to many antimycotic drugs. Therefore, precise identification of this pathogen is important from the treatment point of view [6, 11, 13].

The increasing number of *P. boydii* infections could be associated with the increasing occurrence of this fungus in the environment. It has been shown that the occurrence of *P. boydii* is much more abundant in urban and industrial areas and in agricultural soil compared to habitats with low human activity [12]. The fungus has also been found to occur in abundance in sewage sludge [15]. However, there is still no consensus as to the environmental factors affecting the incidence of *P. boydii* in the environment.

Markovetz *et al.* [9] (after Bilai and Kowal [2]) and April *et al.* [1] demonstrated that *P. boydii* isolates were able to degrade oil alkanes. Subsequently, Clauβen and

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Schmidt [3, 4] showed that this fungus also utilized aromatic compounds (phenol, *p*-cresol and phenylbenzoate with its derivatives) as carbon and energy sources. The *P. boydii* abilities to degrade chain and aromatic hydrocarbons have also been stressed by Prenafeta-Boldú *et al.* [1]. It is not surprising, therefore, that *P. boydii* was found to occur abundantly in soil heavily contaminated with petroleum wastes [16]. In available literature, however, no data have been found on the ability of *P. boydii* to grow on fatty substrates and fuels such as diesel and biodiesel oils; being common environmental pollutants. This study compared the growth of *P. boydii* isolates from sewage sludge and from clinical sources on tributyrin, rapeseed oil, biodiesel oil and diesel oil agars.

MATERIAL AND METHODS

The isolates IETU 151, IETU 185, IETU 187, IETU 188, IETU 190 and IETU 192 were isolated from sewage sludges sampled at wastewater treatment plants in Upper Silesia, Poland. All samples were excess sludges, after extended aeration (without primary settling tank) and the integrated biological process for C, N and P removal, dewatered with a drying belt or centrifuge and in drying beds. The isolates were stored on Sabouraud 1:10 + mineral salts slants [14] under paraffin in the fungus collection at the Institute for Ecology of Industrial Areas (IETU), Katowice, Poland. Subsequently, the isolates FMR 4167 (otitis), FMR 7884 (transplant), FMR 8356 (leukemia), FMR 8357 (cystic fibrosis), FMR 8358 (cystic fibrosis) and FMR 8623 (cystic fibrosis) were isolated from human patients and stored in the Facultat de Medicina Reus (FMR) fungus collection at the Department of Microbiology, Universitat Rovira i Virgili, Spain.

Tributyrin (glyceryl tributyrate), rapeseed oil, biodiesel oil (fatty acid methyl esters) and diesel oil were tested as substrates. Tributyrin was provided by Sigma-Aldrich, while rapeseed oil was a commercially available food product. Biodiesel oil and diesel oil were provided by one of the Polish oil refineries. Two media with controls were used in the experiment.

The first medium contained Bactopeptone (Difco) – 5 g, yeast extract (Difco) – 3 g, tributyrin – 10 g, Bactoagar (Difco) – 15 g and redistilled water – 1,000 ml was used for evaluation of fungal growth and tributyrin hydrolysis activity. The final pH of the medium was 6.5. The same medium without tributyrin served as control.

The second medium contained Bactopeptone (Difco) – 10 g, NaCl – 5 g, rapeseed oil, biodiesel oil or diesel oil – 10 g, polyvinyl alcohol (PVA) – 1.5 g, Nile blue – 0.04 g, Bactoagar (Difco) – 15 g and redistilled water – 1,000 ml was used for evaluation of fungal growth and hydrolytic activity on oils. In this medium, polyvinyl alcohol served as the emulsion stabilizer, while Nile blue indicated the pH decrease caused by liberated fatty acids. The final pH of these media was 7.4. The same medium without oil served as control. The media were homogenized, sterilized by triple pasteurization at 24-hour intervals and poured into standard (9-cm diam) Petri dishes. Ten-day fungal cultures on MEA at 25°C were used as inoculum. Small aerial mycelium/spore pieces were picked up from this medium with a syringe needle and inoculated in the centre of the test media dishes. The inoculated dishes were then incubated at 30°C for 5 days in the dark.

The experiments were performed in triplicate.

On tributyrin agar, the hydrolytic activity was observed as a clear zone around the colony. On rapeseed oil and

Table 1. Growth and hydrolytic activity of *P. boydii* isolates on tributyrin agar.

Isolates	Hydrolysis zone diameter [mm]	Colony diameter on tributyrin agar [mm]	Hydrolysis activity index	Colony diameter on control agar [mm]	Growth stimulation/ inhibition index
IETU 151	10 ± 0*	10 ± 0*	1 ± 0*	28 ± 0*	0.36 ± 0*
IETU 185	15.5 ± 0.7	13 ± 1.4	1.2 ± 0.08	32.5 ± 0.7	0.4 ± 0.05
IETU 187	15 ± 1.4	13 ± 0	1.15 ± 0.11	30.5 ± 0.7	0.43 ± 0.01
IETU 188	14 ± 1.4	12.5 ± 0.7	1.12 ± 0.05	29.5 ± 0.7	0.42 ± 0.03
IETU 190	15 ± 2.8	12.5 ± 2.1	1.2 ± 0.02	28.5 ± 0.7	0.44 ± 0.09
IETU 192	16 ± 0	16 ± 0	1 ± 0	31 ± 0	0.52 ± 0
FMR 4167	17.5 ± 0.7	16.5 ± 0.7	1.03 ± 0.04	33.5 ± 2.1	0.49 ± 0.01
FMR 7884	22 ± 0	18 ± 0	1.22 ± 0	31.5 ± 0.7	0.57 ± 0.01
FMR 8356	8.5 ± 0.7	8.5 ± 0.7	1 ± 0	18.5 ± 0.7	0.46 ± 0.02
FMR 8357	10 ± 0	10 ± 0	1 ± 0	18.5 ± 0.7	0.54 ± 0.02
FMR 8358	14 ± 1.4	14 ± 1.4	1 ± 0	26 ± 1.4	0.54 ± 0.08
FMR 8623	19.5 ± 3.5	16.5 ± 2.1	1.18 ± 0.07	27 ± 0	0.61 ± 0.08

* mean for three Petri dishes ± standard deviation; IETU – Institute for Ecology of Industrial Areas, fungus collection; FMR – Facultat de Medicina Reus, fungus collection.

biodiesel oil agars, the hydrolytic activity was observed as the medium colour change from salmon pink to green or blue due to the liberation of fatty acids and pH decrease. Colony diameters and hydrolysis zones were measured after 5 days of incubation. The hydrolytic activity index was computed as the hydrolysis zone diameter/colony diameter ratio [7, 8]. Subsequently, the growth stimulation/inhibition index was computed as the colony diameter on fatty substrate agar/colony diameter on control agar ratio. When the 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 and correlation analyses were used for statistical analysis of the data obtained. Statistical tests were performed at $p \leq 0.05$.

RESULTS AND DISCUSSION

All *Pseudallescheria boydii* isolates grew on tributyrin agar and produced esterases, which catalyze the hydrolysis of this substrate (Tab. 1). Also, high variability in hydrolysis zone and colony diameters characterized clinical isolates compared to the low variability observed in sludge isolates. The clinical isolates FMR 7884, FMR 4167 and FMR 8623 showed hydrolytic activity even higher than the sludge isolates. Subsequently, among the isolates tested, FMR 8356 displayed the lowest hydrolytic activity on tributyrin. The hydrolysis zone diameter correlated well with the colony diameter on tributyrin agar ($r = 0.95$). However, hydrolysis zone diameters were only slightly higher than colony diameters. Therefore, hydrolysis activity indices were found to be only slightly >1.

No data on the toxicity of tributyrin to fungi have been found in the literature. Glycerol and butyric acid are both the products of tributyrin hydrolysis. Glycerol has been found to inhibit some fungal enzymes [5]. Toxic properties of butyric acid to many fungi, including filamentous species have also been demonstrated [18]. In the light of these data, the inhibition of the growth of *P. boydii* isolates on tributyrin agar seems to be understandable. In general, the inhibition of sludge isolates on tributyrin agar was slightly higher compared to the inhibition of clinical isolates. The mean values of the inhibition index were 0.4 and 0.5 for sludge and clinical isolates, respectively. This difference was statistically significant at $p \leq 0.05$.

The *P. boydii* isolates grew on rapeseed oil agar, with no hydrolysis zones observed (Tab. 2). Although free fatty acids liberated during hydrolysis of triglycerides may strongly inhibit the growth of some filamentous fungi [17], except for FMR 8356 considerable stimulation of the growth of *P. boydii* isolates was observed on rapeseed oil agar (Fig. 1). This suggests that this oil or/and the products of its degradation could have been used by these isolates for biomass production. Similarly to fungal growth on tributyrin, the clinical isolates showed higher variability when growing on rapeseed oil compared to the sludge isolates. The growth of FMR 4167 on rapeseed oil agar was found to be the highest among the isolates tested. The lowest growth on this substrate was observed for FMR 8357.

Table 2. Growth and hydrolytic activity of *P. boydii* isolates on rapeseed oil agar.

Isolates	Colony diameter on rapeseed oil agar [mm]	Colony diameter on control agar [mm]	Growth stimulation/ inhibition index
IETU 151	13.5 ± 0.7*	8 ± 0*	1.69 ± 0.09*
IETU 185	17.5 ± 0.7	10.5 ± 0.7	1.67 ± 0.04
IETU 187	19.5 ± 0.7	10 ± 1.4	1.97 ± 0.35
IETU 188	17 ± 1.4	9 ± 0	1.89 ± 0.16
IETU 190	17.5 ± 0.7	7 ± 0	2.5 ± 0.1
IETU 192	15 ± 2.8	9 ± 0	1.67 ± 0.31
FMR 4167	22.5 ± 10.6	5 ± 0	4.5 ± 2.12
FMR 7884	15 ± 0	6.5 ± 0.7	2.32 ± 0.25
FMR 8356	7 ± 0	6.5 ± 0.7	1.08 ± 0.12
FMR 8357	4.5 ± 0.7	3 ± 0	1.5 ± 0.24
FMR 8358	14 ± 1.4	9.5 ± 0.7	1.47 ± 0.04
FMR 8623	12 ± 1.4	8.5 ± 0.7	1.42 ± 0.28

* mean for three Petri dishes ± standard deviation; IETU – Institute for Ecology of Industrial Areas, fungus collection; FMR – Facultat de Medicina Reus, fungus collection.

Table 3. Growth of *P. boydii* isolates on biodiesel oil agar.

Isolates	Hydrolysis zone diameter [mm]	Colony diameter on Biodiesel agar [mm]	Hydrolysis activity index	Growth stimulation/ inhibition index
IETU 151	0	15 ± 0*	0	1.88 ± 0*
IETU 185	15 ± 0*	15 ± 0	1 ± 0*	0.75 ± 1.06
IETU 187	16 ± 1.4	16 ± 1.4	1 ± 0	1.61 ± 0.09
IETU 188	11 ± 0	11 ± 0	1 ± 0	1.22 ± 0
IETU 190	13.5 ± 2.1	13.5 ± 2.1	1 ± 0	1.93 ± 0.3
IETU 192	0	13.5 ± 2.1	0	1.5 ± 0.24
FMR 4167	13 ± 0	13 ± 0	1 ± 0	2.6 ± 0
FMR 7884	0	16.5 ± 0.7	0	2.56 ± 0.39
FMR 8356	3.5 ± 4.9	6 ± 1.4	0.5 ± 0.71	0.94 ± 0.32
FMR 8357	0	3.5 ± 0.7	0	1.17 ± 0.24
FMR 8358	0	17 ± 0	0	1.79 ± 0.13
FMR 8623	0	17 ± 0	0	2.01 ± 0.17

* mean for three Petri dishes ± standard deviation; IETU – Institute for Ecology of Industrial Areas, fungus collection; FMR – Facultat de Medicina Reus, fungus collection.

All isolates grew on biodiesel oil agar (Tab. 3). However, the isolates differed in their ability to hydrolyze fatty acid methyl esters. In general, the clinical isolates had much lower hydrolytic (esterase) activity on biodiesel oil agar compared to the sludge isolates. The mean hydrolysis zone diameter ranged between 0–16 and 0–13 mm, with the means of 8.7 and 2.8 mm for sludge and clinical

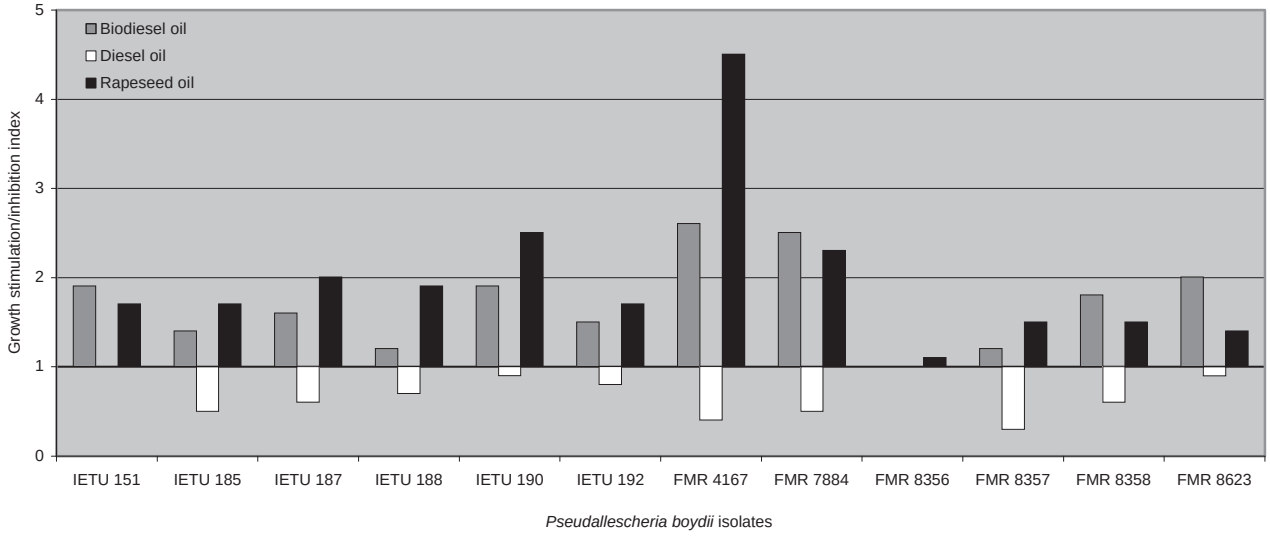

Figure 1. Stimulation or inhibition effects of biodiesel oil, diesel oil and rapeseed oil on *P. boydii* isolates.

isolates, respectively. This difference was statistically significant at $p \leq 0.05$. Four of the 6 clinical isolates showed no hydrolytic activity on this substrate, while no hydrolysis zone was observed in only 2 sludge isolates. Except for FMR 8356, hydrolysis zone diameters were identical with colony diameters. In FMR 8356, the hydrolysis zone diameter was smaller than the colony diameter (hydrolytic activity index <1). Except for FMR 8356, biodiesel oil considerably stimulated the growth of all other *P. boydii* isolates (Fig. 1). This suggests that biodiesel oil could have been used by these isolates for biomass production.

The lack of hydrolysis zones associated with fungal growth on rapeseed oil and biodiesel oil can be explained by ammonification of peptone, which causes alkalization of the medium and masking the acidification effect from liberated fatty acids.

Table 4. Growth of *P. boydii* isolates on diesel oil agar.

Isolates	Colony diameter on diesel oil agar [mm]	Growth stimulation/ inhibition index
IETU 151	8 ± 0*	1 ± 0*
IETU 185	5.5 ± 0.7	0.52 ± 0.03
IETU 187	6 ± 0	0.61 ± 0.09
IETU 188	6.5 ± 0.7	0.72 ± 0.08
IETU 190	6 ± 0	0.86 ± 0
IETU 192	7 ± 0	0.78 ± 0
FMR 4167	8 ± 1.4	1.60 ± 0.28
FMR 7884	9.5 ± 0.7	1.48 ± 0.27
FMR 8356	6.5 ± 0.7	1.01 ± 0.22
FMR 8357	1 ± 0	0.33 ± 0
FMR 8358	6 ± 0	0.63 ± 0.05
FMR 8623	8 ± 0	0.94 ± 0.08

* mean for three Petri dishes ± standard deviation; IETU – Institute for Ecology of Industrial Areas, fungus collection; FMR – Facultat de Medicina Reus, fungus collection.

Among the substrates tested, diesel oil hydrocarbons were found to be the most resistant for biodegradation. It is understandable, therefore, that the growth of *P. boydii* isolates on diesel oil was small (Tab. 4). Except for four isolates, i.e. FMR 4167, FMR 7884, FMR 8356 and IETU 151, diesel oil inhibited fungal growth (Fig. 1). In FMR 8356 and IETU 151, the addition of diesel oil to the medium did not affect fungal growth, while in FMR 4167 and FMR 7884 the stimulation effect of this addition was evident. This finding confirms conclusions of Markovetz *et al.* [9] (after Bilai and Kowal [2]), April *et al.* [1] and other researchers that some *P. boydii* isolates are able to utilize oil alkanes.

The use of biodiesel as the renewable alternative to fossil fuels, including diesel oil is likely to increase in the near future. The data suggest that contamination of the environment with the above-mentioned oils could favour the growth of *P. boydii*. However, no association was found between the growth and oil utilization by this fungus and its pathogenicity.

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