EXTRACELLULAR LIPASE PRODUCTION OF ZYGOMYCETES FUNGI ISOLATED FROM SOIL

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ABSTRACT
Filamentous fungi are well known by their ability to secrete biotechnologically important enzymes into the environment. Lipase enzymes catalyze the hydrolysis of triacylglycerols to give free fatty acids, diacylglycerols, monoaoylglycerols and glycerol. There is a growing interest in microbial lipase production due to its great potential for various industrial applications. Zygomyces are good producers of lipases, however, representatives of the order Mortierellales are poorly characterized from this aspect. Our knowledge in reference to the activity and production of the enzymes by soil isolated zygomycotous fungi is also limited. The main objective of this work was the screening of 35 soil isolated strains belonging to the genera Mortierella, Dissoaphora and Umbelopsis with potential to produce lipases. For detection of extracellular lipase production, culture media containing tributyrin was used and the level of the lipase production was evaluated by measuring the diameter of the halo around the colonies. The halo was formed in consequence of the lipase activity and measured daily during the incubation period. The lipase production of the tested strains showed high variability and several isolates showing high enzyme activity were detected in each genus. Among the tested isolates, the Dissoaphora ornata, Mortierella longioscellis and Umbelopsis angularis strains proved to be outstanding in their enzyme producing ability. The M. longioscellis were selected to investigate the effects of various inducer oils on the enzyme production using submerged culture fermentation systems.

Keywords: Zygomyces, lipase, tributyrin, microorganism screening, submerged culture fermentation

INTRODUCTION
Lipases (glycerol ester hydrolases; EC 3.1.1.3) have multiple applications in a wide range of biotechnological processes. These enzymes catalyze the hydrolysis of triacylglycerols, which are the major constituents of fats and oils, to produce free fatty acids, glycerol and partial acylglycerols (SHARMA et al, 2001). This reaction is reversible, so that these enzymes also catalyze the formation of acylglycerols from glycerol and free fatty acids. There is a growing interest in microbial lipase production due to its great potential for industrial applications such as food additives (PETRUCCO et al. and FEDERICI, 1992), industrial reagents (JAEGER and REETZ, 1998) and stain removers, as well as for medical applications (KAULASMEIS and BORNSCHEUER, 1998). Lipases can also be used to accelerate the degradation of fatty waste and polyurethane (MASSE et al, 2001; TAMAKOMO et al, 2001). Filamentous fungi are well known by their ability to secrete biotechnologically important enzymes into the environment, e.g. mainly proteases and lipases. Filamentous fungi able to produce lipase enzymes can be found in some agro-industrial wastes, deteriorated foods as well as different soil samples (COLEN et al, 2006; GRIEBELER et al, 2011). Zygomyces are good producers of lipases and some Mucor, Rhizomucor and Rhizopus lipases have been isolated and utilized in the industry (SHARMA et al, 2001; NOEL and COMBES, 2003). However, representatives of the order Mortierellales are poorly characterized from this aspect, and our knowledge in reference to the activity and production of the enzymes by other soil-borne zygomycetous fungi is also limited. Therefore, 35 Mortierella, Dissoaphora and Umbelopsis strains isolated from different soil samples have been screened for their secreted lipase activity in order to find new producer isolates potentially applicable in further basic studies and biotechnological applications.

MATERIAL AND METHOD

Culture conditions
For the detection of lipase activity in plates, 20µl from 106 sporangiospores ml-1 suspension of the isolates were inoculated on the centre of the Petri-dish containing 20 ml culture media (0.5% peptone, 0.3% yeast extract, 1% agar) supplemented with 0.1% tributyrin (Sigma) (LIMA et al, 1991). After the incubation, plates were incubated at 20 °C or 25 °C for 7 days.

For the induction of the lipase production in submerged culture, 106 sporangiospores ml-1 were inoculated into 30 ml minimal medium (0.15% (NH4)2SO4, 0.15% Na-L-glutaminate, 0.05% yeast nitrogen base) supplemented with 1% glucose, Tween 80, palm-, soybean-, sunflower-, olive-, extra virgin olive-, wheat germ-, corn germ-, sesame seed-, pumpkin seed- or cottonseed oil as sole carbon source and incubated under continuous shaking (200 rpm) at 25 °C for 12 days.

Detection of the lipase activity
For sample preparation from submerged culture, 700µl of the filtrates were collected every second day and after filtration centrifuged at 16.200 x g for 30 min and the supernatant was stored at -20 °C. Enzyme activity was assayed by using p-nitrophenyl palmitate (Sigma, pNPP). Three mM concentration of pNPP stock solution was prepared in dimethyl sulfoxide (DMSO) and equal volume of potassium phosphate buffer (pH 6.8) was added. Fifty µl of buffered pNPP solution was given to 50µl diluted extract, and incubated for 30 min. at 25 °C. The reaction was stopped by 25µl of 0.1 M sodium carbonate, and the p-nitrophenol release was measured at 405 nm. One enzymatic unit was defined as the amount of enzyme that releases 1 µmol of p-nitrophenol in 1 minute under the assay conditions. Enzyme activities were measured in 96-well microtiter plates using an ASYS Jupiter HD (ASYS Hitoch) microplate reader. Enzyme activities were determined in three independent experiments.

RESULTS

This work evaluated the extracellular lipase activity of 35 strains representing the Zygomyces genera Mortierella, Dissoaphora and Umbelopsis. The investigated strains had been isolated from different soil samples (Table 1).

Detection of lipase production in plates
The culturing media contained tributyrin to monitor the lipase activity. Incubation was performed at the optimal temperature conditions (20 °C or 25 °C) of each isolate. The level
of the lipase production was evaluated by measuring the diameter of the halo around the colonies that formed in consequence of the hydrolysis of tributyrin. The halo was measured in millimeters daily during the incubation period. The enzyme activity of the tested strains showed high variability and several isolates showing high activity were detected (Table 1).

Growth of several isolates was fairly low on this media; unlike the small diameter of the colonies, the detected enzyme production was considerably high in some cases.

Table 1. The investigated strains and the average diameter of halo representing the lipase activity of each isolate (best producers are highlighted with bold characters)

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Code</th>
<th>Source</th>
<th>Cultivation temperature (°C)</th>
<th>Diameter of halo (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dis sophora ornata</td>
<td>SZMC 11221</td>
<td>Forest soil/Columbia</td>
<td>25</td>
<td>5</td>
</tr>
<tr>
<td>Mortierella genniforma</td>
<td>SZMC 11201</td>
<td>Pine forest soil/UK</td>
<td>20</td>
<td>1.5</td>
</tr>
<tr>
<td>Mortierella longicollis</td>
<td>SZMC 11208</td>
<td>Sandy soil/Australia</td>
<td>25</td>
<td>6</td>
</tr>
<tr>
<td>Mortierella alpina</td>
<td>SZMC 11213</td>
<td>Sandy soil/Australia</td>
<td>20</td>
<td>5</td>
</tr>
<tr>
<td>Mortierella hamulii</td>
<td>SZMC 11220</td>
<td>Pine forest soil/Mexico</td>
<td>20</td>
<td>5</td>
</tr>
<tr>
<td>Mortierella parvispora</td>
<td>SZMC 11225</td>
<td>Soil/Germany</td>
<td>20</td>
<td>3</td>
</tr>
<tr>
<td>Mortierella verticillata</td>
<td>SZMC 11205</td>
<td>Tundra soil/USA</td>
<td>25</td>
<td>2</td>
</tr>
<tr>
<td>Mortierella antarctica</td>
<td>SZMC 11217</td>
<td>Soil, glacier/Antarctica</td>
<td>20</td>
<td>2</td>
</tr>
<tr>
<td>Mortierella polygonia</td>
<td>SZMC 11203</td>
<td>Soil/Netherlands</td>
<td>20</td>
<td>1</td>
</tr>
<tr>
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<td>SZMC 11215</td>
<td>Soil/Netherlands</td>
<td>20</td>
<td>2</td>
</tr>
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<td>SZMC 11207</td>
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<td>Mortierella belgavene</td>
<td>SZMC 11232</td>
<td>Soil/Ukraine</td>
<td>20</td>
<td>2</td>
</tr>
<tr>
<td>Mortierella camargarus</td>
<td>SZMC 11227</td>
<td>Sandy soil/France</td>
<td>20</td>
<td>3</td>
</tr>
<tr>
<td>Mortierella clenocytis</td>
<td>SZMC 11238</td>
<td>Soil/Spain</td>
<td>20</td>
<td>3</td>
</tr>
<tr>
<td>Mortierella cytopoenikii</td>
<td>SZMC 11229</td>
<td>Agricultural soil/</td>
<td>20</td>
<td>5</td>
</tr>
<tr>
<td>Mortierella epichlia</td>
<td>SZMC 11247</td>
<td>Soil/Spain</td>
<td>20</td>
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<tr>
<td>Mortierella microxygospora</td>
<td>SZMC 11248</td>
<td>Soil/Japan</td>
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<td>Mortierella minitissima var. dubia</td>
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<td>Soil/France</td>
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<td>Mortierella verticillata</td>
<td>SZMC 11236</td>
<td>Forest soil/China</td>
<td>20</td>
<td>2</td>
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<td>Mortierella verricella</td>
<td>SZMC 11230</td>
<td>Forest soil/Germany</td>
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<td>2</td>
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<tr>
<td>Umbelopsis angulata</td>
<td>SZMC 11252</td>
<td>Soil/Netherlands</td>
<td>20</td>
<td>5</td>
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<td>Mortierella angusta</td>
<td>SZMC 11254</td>
<td>Popud soil/UK</td>
<td>20</td>
<td>3</td>
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<td>SZMC 11257</td>
<td>Soil/India</td>
<td>20</td>
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<td>Mortierella gamsii</td>
<td>SZMC 11258</td>
<td>Forest soil/Germany</td>
<td>20</td>
<td>2</td>
</tr>
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<td>Mortierella gamsii</td>
<td>SZMC 11259</td>
<td>Forest soil/Germany</td>
<td>20</td>
<td>4</td>
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<td>Mortierella lignicornia</td>
<td>SZMC 11265</td>
<td>Soil/Germany</td>
<td>20</td>
<td>3</td>
</tr>
<tr>
<td>Mortierella parvispora</td>
<td>SZMC 11266</td>
<td>Soil/Germany</td>
<td>20</td>
<td>2.5</td>
</tr>
<tr>
<td>Mortierella clausenii</td>
<td>SZMC 11268</td>
<td>Soil/Switzerland</td>
<td>20</td>
<td>2</td>
</tr>
<tr>
<td>Mortierella paraisis</td>
<td>SZMC 11271</td>
<td>Forest soil/Brasil</td>
<td>20</td>
<td>3</td>
</tr>
<tr>
<td>Mortierella spirokheka</td>
<td>SZMC 11273</td>
<td>Forest soil/India</td>
<td>20</td>
<td>2</td>
</tr>
<tr>
<td>Mortierella sarmynesis</td>
<td>SZMC 11274</td>
<td>Soil/Ukraine</td>
<td>20</td>
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<td>Mortierella stylasporc</td>
<td>SZMC 11275</td>
<td>Sandy soil/Australia</td>
<td>20</td>
<td>1.5</td>
</tr>
<tr>
<td>Mortierella globulifera</td>
<td>SZMC 11260</td>
<td>Soil (pH 6.4)/Germany</td>
<td>20</td>
<td>4</td>
</tr>
</tbody>
</table>

a: SZMC - Szeged Microbiological Collection
b: Values are measured on the seventh day of the cultivation.

Based on the cultivation on plates, Umbelopsis angulata (SZMC 11252-Szeged Microbiological Collection), Mortierella longicollis (SZMC 11208), M. alpina (SZMC 11231), M. hamulii (SZMC 11220), M. cytopoenikii (SZMC 11229) and Dis sophora ornata (SZMC 11221) isolates showed the highest lipase production at the optimal cultivation temperature of each isolate (highlighted with bold characters in Table 1). Significant enzyme production could also be observed by the M. gammaru (SZMC 11259), M. sarmynesis (SZMC 11274) and M. globulifera (SZMC 11260) strains at 20 °C (4 mm of halo). It is worth to mention that ALVES et al. (2002) presented the screening of Macor strains isolated from herbivores dung and considered as good lipase producers. In the referenced work, the halo diameters were found between 4 and 6 mm at most of the isolates.

Enzyme production in submerged cultures

M. longicollis was selected for further submerged culture studies to investigate the effects of different inducers (TWEEN 80, palm-, soybean-, sunflower-, olive-, extra virgin olive-, wheat germ-, corn germ-, sesame seed-, pumpkin seed- or cottonseed oil) on the enzyme activity. To evaluate the effect of lipid material, sporangiophores of the isolate were transferred to minimal medium supplemented with a given inducer and incubated at 25 °C for 12 days. Enzyme activities on each inducer were correlated to data obtained using 1% glucose as sole carbon source. Results show, that the lipase production was enhanced only by Tween 80, soybean- and olive oil, and the maximum level of the activity was on the second day by Tween 80, on the eighth day by soybean oil, and on the tenth day by olive oil (Figure 1). Similar stimulative effects of Tween 80 and different vegetable oils have been described for many bacterial and fungal lipases (SARMA et al., 2001). No difference from the control enzyme activity was observed using palm-, sunflower-, extra virgin olive-, wheat germ-, corn germ-, sesame seed-, pumpkin seed- or cottonseed oil. Interestingly, CERTIK et al. (1997) reported that sunflower oil is good substrate for Mortierella species; however, in our test, no significant enzyme activity was detected using this substrate.

![Figure 1. Extracellular lipase production of M. longicollis in submerged fermentation using different carbon sources](image)

CONCLUSIONS

Soil isolated zygomycetous fungi proved to be good sources of lipase enzymes. Besides other filamentous fungi isolated from soil (COSTA and PERALTA, 1999), the D. ornata, M. longicollis and U. angulata strains also have great potential to produce lipase enzymes into the environment. Different inducer oils to enhance the lipase production of M. longicollis were also investigated. It is proved that Tween 80, soybean- and olive oil are
good lipase inducers at this isolate; this result is similar to findings reported for some other filamentous fungi (Sharma et al., 2001). Analysis and detection of lipases produced by other soil isolated Mortierella and Umbelopsis strains and testing of the enzyme activity on different oils and oil derivatives are in progress.

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REFERENCES


