

## 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, monoacylglycerols and glycerol. There is a growing interest in microbial lipase production due to its great potential for various industrial applications. Zygomycetes 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 zygomycetous fungi is also limited. The main objective of this work was the screening of 35 soil isolated strains belonging to the genera *Mortierella*, *Dissophora* and *Umbelopsis* with potential to produce lipases. For detection of extracellular lipase production, culturing 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 *Dissophora ornata*, *Mortierella longicollis* and *Umbelopsis angularis* strains proved to be outstanding in their enzyme producing ability. The *M. longicollis* were selected to investigate the effects of various inductor oils on the enzyme production using submerged culture fermentation systems.

**Keywords:** Zygomycetes, 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 catalyse 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 (PETRUCCIOLI and FEDERICI, 1992), industrial reagents (JAEGER and REETZ, 1998) and stain removers, as well as for medical applications (KAZLAUSKAS and BORNSCHEUER, 1998). Lipases can also be used to accelerate the degradation of fatty waste and polyurethane (MASSE et al, 2001; TAKAMOTO 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). Zygomycetes 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*, *Dissophora* 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, 20 $\mu$ l from 10<sup>6</sup> sporangiospores ml<sup>-1</sup> 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 inoculation, plates were incubated at 20 °C or 25 °C for 7 days.

For the induction of the lipase production in submerged culture, 10<sup>6</sup> sporangiospores ml<sup>-1</sup> were inoculated into 30 ml minimal medium (0.15% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.15% Na-L-glutamate, 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, 700 $\mu$ l 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, *p*NPP). Three mM concentration of *p*NPP stock solution was prepared in dimethyl sulfoxide (DMSO) and equal volume of potassium phosphate buffer (pH 6.8) was added. Fifty  $\mu$ l of buffered *p*NPP solution was given to 50 $\mu$ l diluted extract, and incubated for 30 min. at 25 °C. The reaction was stopped by 25 $\mu$ l 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  $\mu$ mol 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 Hitech) microplate reader. Enzyme activities were determined in three independent experiments.

## RESULTS

This work evaluated the extracellular lipase activity of 35 strains representing the Zygomycetes genera *Mortierella*, *Dissophora* 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)**

| Isolate  | Code <sup>a</sup> | Source                               | Cultivation temperature (°C) | Diameter of halo (mm) <sup>b</sup> |
|--|-------------------|--------------------------------------|------------------------------|------------------------------------|
| <i>Dissophora ornata</i>                         | <b>SZMC 11221</b> | <b>Forest soil/Columbia</b>          | <b>25</b>                    | <b>5</b>                           |
| <i>Mortierella gemmifera</i>                     | SZMC 11201        | Pine forest soil/UK                  | 20                           | 1.5                                |
| <b><i>Mortierella longicollis</i></b>            | <b>SZMC 11208</b> | <b>Sandy soil/Australia</b>          | <b>25</b>                    | <b>6</b>                           |
| <i>Mortierella alpina</i>                        | <b>SZMC 11213</b> | <b>Sandy soil/Australia</b>          | <b>20</b>                    | <b>5</b>                           |
| <i>Mortierella humilis</i>                       | <b>SZMC 11220</b> | <b>Pine forest soil/Mexico</b>       | <b>20</b>                    | <b>5</b>                           |
| <i>Mortierella parvispora</i>                    | SZMC 11225        | Soil/Germany                         | 20                           | 3                                  |
| <i>Mortierella verticillata</i>                  | SZMC 11205        | Tundra soil/USA                      | 25                           | 2                                  |
| <i>Mortierella antarctica</i>                    | SZMC 11217        | Soil, glacier/Antarctica             | 20                           | 2                                  |
| <i>Mortierella polygonia</i>                     | SZMC 11203        | Soil/Netherlands                     | 20                           | 1                                  |
| <i>Mortierella gamsii</i>                        | SZMC 11215        | Soil/Netherlands                     | 20                           | 2                                  |
| <i>Mortierella schmuckeri</i>                    | SZMC11207         | Soil (pH6.7)/Mexico                  | 25                           | 2                                  |
| <i>Mortierella beljakovae</i>                    | SZMC 11232        | Soil/Ukraine                         | 20                           | 2                                  |
| <i>Mortierella camargensis</i>                   | SZMC 11227        | Sandy soil/France                    | 20                           | 3                                  |
| <i>Mortierella clonocystis</i>                   | SZMC 11238        | Soil/Spain                           | 20                           | 3                                  |
| <b><i>Mortierella cystojenkinii</i></b>          | <b>SZMC 11229</b> | <b>Agricultural soil/Netherlands</b> | <b>20</b>                    | <b>5</b>                           |
| <i>Mortierella epicladia</i>                     | SZMC 11247        | Soil/Spain                           | 20                           | 0.5                                |
| <i>Mortierella microzygospora</i>                | SZMC 11248        | Soil/Japan                           | 20                           | 3.5                                |
| <i>Mortierella minutissima</i> var. <i>dubia</i> | SZMC 11235        | Soil/Germany                         | 20                           | 1.5                                |
| <i>Mortierella rostafinskii</i>                  | SZMC 11249        | Soil/USA                             | 20                           | 0.5                                |
| <i>Mortierella verticillata</i>                  | SZMC 11233        | Soil/France                          | 20                           | 1.5                                |
| <i>Mortierella verticillata</i>                  | SZMC 11236        | Forest soil/China                    | 20                           | 2                                  |
| <i>Mortierella verticillata</i>                  | SZMC 11230        | Forest soil/Germany                  | 20                           | 2                                  |
| <i>Umbelopsis angularis</i>                      | SZMC 11252        | Soil/ Netherlands                    | 25                           | 5                                  |
| <i>Mortierella angusta</i>                       | SZMC 11254        | Podzol soil/UK                       | 20                           | 3                                  |
| <i>Mortierella exigua</i>                        | SZMC 11257        | Soil/India                           | 20                           | 2                                  |
| <i>Mortierella gamsii</i>                        | SZMC 11258        | Forest soil/Germany                  | 20                           | 2                                  |
| <i>Mortierella gamsii</i>                        | SZMC 11259        | Forest soil/Germany                  | 20                           | 4                                  |
| <i>Mortierella lignicola</i>                     | SZMC 11265        | Soil/Germany                         | 20                           | 3                                  |
| <i>Mortierella parvispora</i>                    | SZMC 11266        | Soil/Germany                         | 20                           | 2.5                                |
| <i>Mortierella claussenii</i>                    | SZMC 11268        | Soil/Switzerland                     | 20                           | 2                                  |
| <i>Mortierella paraensis</i>                     | SZMC 11272        | Rain forest soil/Brazil              | 20                           | 3                                  |
| <i>Mortierella rishikeshia</i>                   | SZMC 11273        | Forest soil/India                    | 20                           | 2                                  |
| <i>Mortierella sarnyensis</i>                    | SZMC 11274        | Soil/Ukraine                         | 20                           | 4                                  |
| <i>Mortierella stylospora</i>                    | SZMC 11275        | Sandy soil/Australia                 | 20                           | 1.5                                |
| <i>Mortierella globulifera</i>                   | SZMC 11260        | Soil (pH6.4)/Germany                 | 20                           | 4                                  |

a: SZMC - Szeged Microbiological Collection

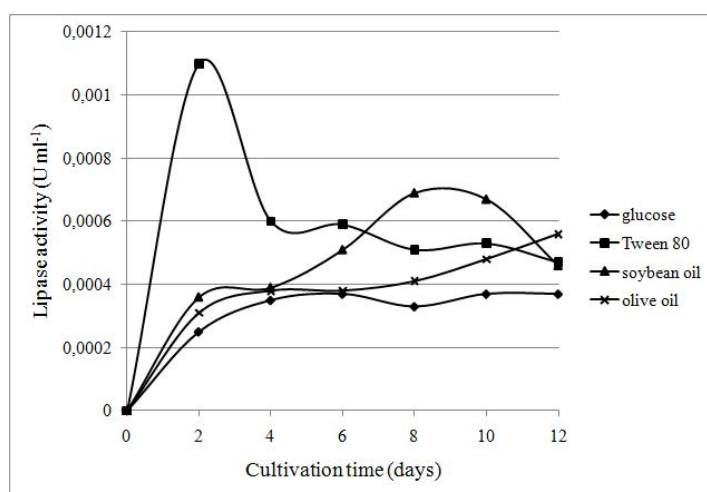
b: Values are measured on the seventh day of the cultivation.

Based on the cultivation on plates, *Umbelopsis angularis* (SZMC 11252-Szeged Microbiological Collection), *Mortierella longicollis* (SZMC 11208), *M. alpina* (SZMC

11213), *M. humilis* (SZMC 11220), *M. cystojenkini* (SZMC 11229) and *Dissophora ornata* (SZMC 11221) isolates showed the highest lipase production at the optimal cultivation temperature of each isolate (highlighted with bold characters in the Table 1). Significant enzyme production could also be observed by the *M. gamsii* (SZMC 11259), *M. sarnyensis* (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 *Mucor* 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 inductors (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, sporangiospores of the isolate were transferred to minimal medium supplemented with a given inductor and incubated at 25 °C for 12 days. Enzyme activities on each inductor 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 (SHARMA 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, CERTÍK et al. (1997) reported that sunflower oil is good substrate for *Mortierella* species; however, in our test, no significant lipase activity was detected using this substrate.



**Figure 1. Extracellular lipase production of *M. longicollis* in submerged fermentation using different carbon sources**

### 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. angularis* strains also have great potential to produce lipase enzymes into the environment. Different inductor 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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