EXTRACELLULAR LIPASE PRODUCTION OF ZYGOMYCETES FUNGI ISOLATED FROM SOIL

MIKLÓS TAKÓ[1], ALEXANDRA KOTOGÁN[1], BRIGITTA NÉMETH[1], ISIDORA RADULOV[2], LUCIAN DUMITRU NIŢĂ[2], DORIN TĂRĂU[3], DANIEL DICU[3], BEÁTA TÓTH[4], TAMÁS PAPP[1], CSABA VÁGVÖLGYI[1]

[1]Department of Microbiology, Faculty of Science and Informatics, University of Szeged, H-6726 Szeged, Közép fasor 52, Hungary; tako78@bio.u-szeged.hu
[2]Banat University of Agricultural Sciences and Veterinary Medicine, 300645 Timişoara, Calea Aradului 119, Romania
[3]Office of Pedological and Agrochemical Studies, 300516 Timişoara, Calea Şagului, 140A, Romania
[4]Cereal Research Non-profit Ltd., H-6726 Szeged, Alsó kikötő sor 9, Hungary

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, 20µl from 10^6 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 inoculation, plates were incubated at 20 °C or 25 °C for 7 days.

For the induction of the lipase production in submerged culture, 10^6 sporangiospores ml^{-1} were inoculated into 30 ml minimal medium (0.15% $(NH_4)_2SO_4$, 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, *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 µl of buffered *p*NPP 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 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[a]	Source	Cultivation temperature (°C)	Diameter of halo (mm)[b]
Dissophora ornata	**SZMC 11221**	**Forest soil/Columbia**	**25**	**5**
Mortierella gemmifera	SZMC 11201	Pine forest soil/UK	20	1.5
Mortierella longicollis	**SZMC 11208**	**Sandy soil/Australia**	**25**	**6**
Mortierella alpina	**SZMC 11213**	**Sandy soil/Australia**	**20**	**5**
Mortierella humilis	**SZMC 11220**	**Pine forest soil/Mexico**	**20**	**5**
Mortierella parvispora	SZMC 11225	Soil/Germany	20	3
Mortierella verticillata	SZMC 11205	Tundra soil/USA	25	2
Mortierella antarctica	SZMC 11217	Soil, glacier/Antarctica	20	2
Mortierella polygonia	SZMC 11203	Soil/Netherlands	20	1
Mortierella gamsii	SZMC 11215	Soil/Netherlands	20	2
Mortierella schmuckeri	SZMC11207	Soil (pH6.7)/Mexico	25	2
Mortierella beljakovae	SZMC 11232	Soil/Ukraine	20	2
Mortierella camargensis	SZMC 11227	Sandy soil/France	20	3
Mortierella clonocystis	SZMC 11238	Soil/Spain	20	3
Mortierella cystojenkinii	**SZMC 11229**	**Agricultural soil/ Netherlands**	**20**	**5**
Mortierella epicladia	SZMC 11247	Soil/Spain	20	0.5
Mortierella microzygospora	SZMC 11248	Soil/Japan	20	3.5
Mortierella minutissima var. *dubia*	SZMC 11235	Soil/Germany	20	1.5
Mortierella rostafinskii	SZMC 11249	Soil/USA	20	0.5
Mortierella verticillata	SZMC 11233	Soil/France	20	1.5
Mortierella verticillata	SZMC 11236	Forest soil/China	20	2
Mortierella verticillata	SZMC 11230	Forest soil/Germany	20	2
Umbelopsis angularis	SZMC 11252	Soil/ Netherlands	25	5
Mortierella angusta	SZMC 11254	Podzol soil/UK	20	3
Mortierella exigua	SZMC 11257	Soil/India	20	2
Mortierella gamsii	SZMC 11258	Forest soil/Germany	20	2
Mortierella gamsii	SZMC 11259	Forest soil/Germany	20	4
Mortierella lignicola	SZMC 11265	Soil/Germany	20	3
Mortierella parvispora	SZMC 11266	Soil/Germany	20	2.5
Mortierella claussenii	SZMC 11268	Soil/Switzerland	20	2
Mortierella paraensis	SZMC 11272	Rain forest soil/Brazil	20	3
Mortierella rishikesha	SZMC 11273	Forest soil/India	20	2
Mortierella sarnyensis	SZMC 11274	Soil/Ukraine	20	4
Mortierella stylospora	SZMC 11275	Sandy soil/Australia	20	1.5
Mortierella globulifera	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. cystojenkinii* (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.

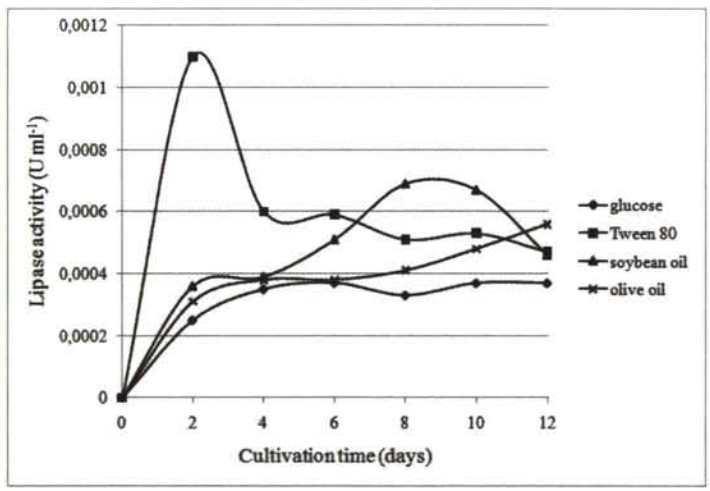

Figure 1. Extracellular lipase production of *M. longicollis* in submerged fermentation using different carbon sources

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.

ACKNOWLEDGEMENTS

The project is co-financed by the European Union through the Hungary-Romania Cross-Border Co-operation Programme (SOILMAP, HURO/0901/058/2.2.2).

REFERENCES

ALVES, M.H., CAMPOS-TAKAKI, G.M., PORTO, A.L.F., MILANEZ, A.I. (2002): Screening of *Mucor* spp. for the production of amylase, lipase, polygalacturonase and protease. Brazilian Journal of Microbiology, Volume 33. pp. 325-330.

CERTÍK, M., BALTÉSZOVÁ, L., SAJBIDOR, J. (1997): Lipid formation and γ-linolenic acid production by Mucorales fungi grown on sunflower oil. Letters in Applied Microbiology, Volume 25. pp. 101-105.

COLEN, G., JUNQUEIRA, R.G., MORAES-SANTOS, T. (2006): Isolation and screening of alkaline lipase-producing fungi from Brazilian savanna soil. World Journal of Microbiology and Biotechnology, Volume 22. pp. 881-885.

COSTA, M.A., PERALTA, R.M. (1999): Production of lipase by soil fungi and partial characterization of lipase from a selected strain (*Penicillium wortmanii*). Journal of Basic Microbiology, Volume 39. pp. 11-15.

GRIEBELER, N., POLLONI, A.E., REMONATTO, D., ARBTER, F., VARDANEGA, R., CECHET, J.L., DI LUCCIO, M., DE OLIVEIRA, D., TREICHEL, H., CANSIAN, R.L., RIGO, E., NINOW, J.L. (2011): Isolation and screening of lipase-producing fungi with hydrolytic activity. Food and Bioprocess Technology, Volume 4. pp. 578-586.

JAEGER, K.E., REETZ, M.T. (1998): Microbial lipases form versatile tools for biotechnology. Trends in Biotechnology, Volume 16. pp. 396-403.

KAZLAUSKAS, R.J., BORNSCHEUER, U.T. (1998): Biotransformations with lipases. In: REHM, H.J., REED, G., PÜHLER, A., STADLER, P.J.W., KELLY, D.R. (ed.) Biotechnology-Series. Wiley-VCH, Weinheim. Volume 8a. pp. 37-191.

LIMA, N., TEIXERIA, J.A., MOTA, M. (1991): Deep agar-diffusion test for preliminary screening of lipolytic activity of fungi. Journal of Microbiological Methods, Volume 14. pp. 193-200.

MASSE, L., KENNEDY, K.J., CHOU, S.P. (2001): The effect of an enzymatic pretreatment on the hydrolysis and size reduction of fat particles in slaughterhouse wastewater. Journal of Chemical Technology and Biotechnology, Volume 76. pp. 629-635.

NOEL, N., COMBES, D. (2003): *Rhizomucor miehei* lipase: differential scanning calorimetry and pressure/temperature stability studies in presence of soluble additives. Enzyme and Microbial Technology, Volume 33. pp. 299-308.

PETRUCCIOLI, M., FEDERICI, R.G. (1992): A note on the production of extracellular hydrolytic enzymes by yeast-like fungi and related microorganisms. Annali di Microbiologia ed Enzimologia, Volume 42. pp. 81-86.

SHARMA, R., CHISTI, Y., BANERJEE, U.C. (2001): Production, purification, characterization and applications of lipases. Biotechnology Advances, Volume 19. pp. 627-662.

TAKAMOTO, T., SHIRASAKA, H., UYAMA, H., KOBAYASHI, S. (2001): Lipase-catalyzed hydrolytic degradation of polyurethane in organic solvent. Chemistry Letters, Volume 6. pp. 492-493.