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Screening for Extracellular Lipase Enzymes with Transesterification Capacity in Mucoromycotina Strains

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Summary

In this study, 169 zygomycetes fungal strains including some cold-tolerant isolates were screened for their extracellular lipolytic activity towards tributyrin. Nineteen of them were outstanding in their enzyme production as they developed the largest lipolytic halo around the colonies in plate tests. *Mortierella alpina*, *M. echinosphaera*, *Mucor corticolus*, *Rhizomucor miehei*, *Rhizopus oryzae*, *Rh. stolonifer*, *Umbelopsis autotrophica*, *U. isabellina*, *U. ramanniana* var. *angulispora* and *U. versiformis* were selected for further studies to characterise their lipolytic enzyme production in detail. In these assays, effect of Tween 80 and palm, soybean, sunflower, olive, extra virgin olive, wheat germ, corn germ, sesame seed, pumpkin seed and cottonseed oils on the enzyme activities was investigated, and wheat bran-based submerged and solid-state fermentations were also tested. Tween 80 and olive oil proved to be efficient inductors for lipolytic enzyme production, which was also enhanced when wheat bran was used as support. Addition of mineral salts and olive oil to the solid fermentation medium resulted in at least 1.5-fold increment in the enzyme activities of the crude extracts. Organic synthesis was also assayed by the selected lipases, in which enzymes from the fungi *R. miehei*, *Rh. stolonifer* and *M. echinosphaera* gave the best yields during transesterification reactions between *p*-nitrophenyl palmitate and ethanol.

Key words: zygomycetes, tributyrin, wheat bran, *p*-nitrophenyl palmitate, transesterification

Introduction

Lipase enzymes (EC 3.1.1.3) catalyse the hydrolysis of insoluble triacylglycerols to generate free fatty acids, diacylglycerols, monoacylglycerols and glycerol. The enzymes may catalyse not only the hydrolysis, but also the synthesis of various long carbon chain acylglycerol molecules. Since lipase enzymes are able to substitute and translocate ester linkages, transesterification reactions can also be conducted under certain conditions. Furthermore, certain lipases may catalyse a range of regio-, enantio- and stereoselective transformations as well (*1*). Due to these excellent catalytic properties, lipases find promising applications in a wide range of biotechnological and industrial processes including flavour enhancement in food processing, biodiesel production and pharmaceutical processing (*2*). Lipases can also be used to accelerate the degradation of fatty waste and polyurethane (*3*,*4*).

Lipases are produced by various organisms including plants, animals and microorganisms such as bacteria and fungi. Most of the current commercial enzymes are derived from microbial sources due to high productivity, genetic modifiability as well as decreased prime costs (*5*). Due to the fact that lipases from various microorganisms may have various substrate specificities, temperature and pH optima, screening for extracellular enzyme activities has special importance in finding novel lipases for industrial process development purposes. Several methods have been described to screen for lipase production. These approaches generally involve culturing

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on solid or in liquid media, which frequently contain some inducer substrates such as Tween 80, vegetable oil or tributyrin (*6–8*).

Filamentous fungi are known to be good lipase producers and numerous fungal enzymes are utilised in various food industrial processes (*9*). Since lipases produced by filamentous fungi are mainly extracellular, extraction and purification of them are relatively easy. This reason may also contribute to the fact that fungal lipases belong to the most important groups of commercial enzymes. Large number of factors determines the lipase production of filamentous fungi in a culture medium; often the presence of an inducer (mostly oil) and the appropriate physiological parameters such as pH, temperature and oxygen levels are the most important factors (*5*). However, to achieve the best yield for extracellular enzyme production, proper selection of the cultivation conditions is even more essential. Moreover, a single lipase showing various distinct biochemical properties can also be obtained if different fermentation procedures are applied for the same fungus (*10*).

Zygomycetes fungi belonging to the Mucoromycotina subphylum have been assumed to play an important role in the decomposition of plant and other organic materials due to their effective extracellular enzyme production. Several members of them are used in different biotechnological applications for the large scale production of industrial enzymes. Investigation of the lipase production by *Rhizomucor* and *Rhizopus* fungi is an intensively studied area wherein lipolytic activity of many strains has been described and some enzymes have been isolated and characterised (*1*,*5*). However, there are only few reports on the lipolytic activity of oleaginous species such as the members of the genera *Mortierella* and *Umbelopsis* (*11*,*12*).

Although some studies have demonstrated that oil-based materials affect the lipase production by zygomycetes strains (*13*,*14*), searching for new inductors able to enhance the enzyme yield is still going on. Similarly, despite the fact that significant microbial lipase production can be achieved when various agro-industrial by-products are applied as substrate, few studies have been carried out on the effect of these substrates on the productivity (*15*,*16*). Wheat bran is a good inducer of lipase production by filamentous fungi; additionally, it also contains adequate amounts of proteins, fats, amino acids and minerals that are essential for the appropriate enzyme production and fungal growth (*17*).

Therefore, the objectives of the present study are to detect and identify lipase activities in Mucoromycotina strains belonging to the orders Mucorales and Mortierellales, and to examine the effect of various inductor oils and wheat bran on the yield of the enzyme production.

A biotechnologically important feature of lipases is the capability to catalyse esterification and transesterification in organic solvents (*18*). During these reactions, synthetic compounds such as aroma esters, phenyl esters and different polymers may be formed. Herein, we also evaluate the transesterification activity of selected lipase-producing isolates under non-aqueous conditions using organic solvent as reaction medium.

Materials and Methods

Strains

Fungal strains involved in the study were deposited in the Szeged Microbiological Collection (SZMC, Szeged, Hungary). Isolates of the genera *Dissophora* (1), *Gamsiella* (1), *Gilbertella* (16), *Mortierella* (27), *Mucor* (32), *Rhizomucor* (27), *Rhizopus* (59) and *Umbelopsis* (6) were involved in the study.

Preliminary screening of lipolytic fungi using tributyrin

For screening of lipolytic fungi, a tributyrin plate assay was applied. Volumes of 20 μL of sporangiospore suspensions (10^6 spores/mL) were dropped on the centre of a Petri dish containing 20 mL of culture medium (in %: peptone 0.5, yeast extract 0.3, agar 1) supplemented with 0.1 % tributyrin (Sigma-Aldrich, Seelze, Germany) (*8*). The inoculated plates were incubated for 7 days at 20, 25 or 37 °C depending on the culturing requirements of the tested strain. In the case of cold-tolerant *Mucor* isolates, lipase production was also analysed at 5 and 10 °C. The level of lipase production was evaluated by measuring the width of the clear zones (halo) that formed around the colonies in consequence of the hydrolysis of tributyrin. The halo and diameter of the colonies were measured in millimetres daily.

Effect of lipid materials on lipase production

Effect of different lipid materials on the lipase production was tested in submerged cultures where 10^6 sporangiospores were inoculated into 20 mL of minimal medium (in %: $(NH_4)_2SO_4$ 0.15, Na-L-glutaminate 0.15, yeast nitrogen base 0.05) supplemented with 1 % Tween 80 and palm, soybean, sunflower, olive, extra virgin olive, wheat germ, corn germ, sesame seed, pumpkin seed or cottonseed oil as sole carbon source. In each case, a culture was also incubated with 1 % anhydrous glucose as sole carbon source. Cultures were grown in 100-mL Erlenmeyer flasks under continuous shaking (200 rpm) at 20, 25 or 37 °C for 7 days. For sample preparation, 700-μL samples were collected daily and filtered to remove insoluble particles. The filtrates were centrifuged at 16 200×*g* for 30 min and the supernatant was used for enzyme activity measurements.

Wheat bran-based submerged and solid-state fermentations

To study the effect of other substrates and conditions on the extracellular lipase production during submerged fermentation, mineral growth medium (in %: NaH_2PO_4 0.12, KH_2PO_4 0.2, $(NH_4)_2SO_4$ 0.1, $MgSO_4{\cdot}7H_2O$ 0.03, $CaCl_2$ 0.025) (*7*) supplemented with 2 % anhydrous glucose or wheat bran was used. Both media were also complemented with 2 % olive oil as additional carbon source. Fermentations were carried out on an orbital shaker at 200 rpm in 100-mL Erlenmeyer flasks containing 20 mL of each medium. After inoculation with 10^6 sporangiospores, cultures were incubated at 20, 25 or 37 °C and time course of enzyme production was monitored during 7 days. Sample preparation was performed daily using a similar procedure as mentioned above.

Wheat bran-based solid-state fermentation (SSF) assays were carried out under two culture conditions to monitor the effect of the presence or absence of mineral salts and inductor oil on extracellular lipase production. A mass of 5 g of wheat bran was transferred into 250-mL Erlenmeyer flasks and moistened with 5 mL of distilled water (SSF medium 1) or 9.5 mL of mineral salt medium (SSF medium 2) containing (in %): $(NH_4)_2SO_4$ 0.75, NH_2CONH_2 0.34, NaH_2PO_4 1.8, KH_2PO_4 0.3, $MgSO_4 \cdot 7H_2O$ 0.045, $CaCl_2$ 0.0375, glucose 1.5 and olive oil 1.5. Moisture of 65 % was used in SSF medium 2 as previously optimised by Falony *et al.* (*7*). After sterilisation, the flasks were inoculated with 10^6 sporangiospores and incubated at 20, 25 or 37 °C, fixing the fermentation time to 7 days. For crude enzyme preparation, the fermented medium was extracted with 30 mL of 0.1 M sodium acetate buffer (pH=6.0) by incubating at 4 °C for 3 h. The extracts were then squeezed through a cloth and 1 mL of the filtrates was clarified by centrifugation at 16 200×*g* for 10 min. The resulting supernatants were assayed for lipase activity. All fermentation tests were carried out in three independent experiments.

Assay of lipolytic activity

Lipolytic activity was determined spectrophotometrically by using *p*-nitrophenyl palmitate (*p*NPP; Sigma-Aldrich) as substrate. *p*NPP stock solution (3 mM) was prepared in dimethyl sulphoxide and equal volume of potassium phosphate buffer (pH=6.8) was added. A volume of 50 μL of buffered *p*NPP solution was added to 50 μL of extract, and incubated for 30 min at 25 or 37 °C depending on whether the enzyme was obtained from mesophilic or thermophilic isolate, respectively. The reaction was stopped by adding 25 μL of 0.1 M sodium carbonate, and the *p*-nitrophenol (*p*NP) release was monitored at 405 nm in 96-well microdilution plates using an ASYS Jupiter HD microplate reader (ASYS Hitech GmbH, Cambridge, UK). A blank sample was always used containing distilled water instead of enzyme solution. The molar absorption coefficient of *p*NP (ε=1.2475·10^4 M^{-1} cm^{-1}) was estimated from the absorbance of *p*NP standard solutions measured at 405 nm. One enzymatic unit was defined as the amount of the enzyme that releases one μmol of *p*NP in one minute under the assay conditions.

Transesterification assay

Transesterification activity in the crude extracts was measured through a slightly modified spectrophotometric method using *p*NPP (Sigma-Aldrich) in *n*-heptane (*19*). An aliquot of 300 μL from each crude extract was lyophilised for 12 h and then mixed with 450 μL of *n*-heptane containing 10 mM *p*NPP. After the addition of 50 μL of absolute ethanol, reaction mixtures were suspended by pipetting and vortexing and were incubated at 40 °C under continuous shaking for 30 min. After incubation, the lipase and other unresolved materials were settled for a minute. To extract the liberated *p*NP, 200 μL of the clear supernatant were taken and then mixed with 2 mL of 0.1 M sodium carbonate in a 3-mL cuvette. The extracted *p*NP was monitored at 405 nm against a blank without enzyme using Jenway 6800 Double Beam spectrophotometer (Jenway, Bibby Scientific Ltd, Stone, Staffordshire, UK). To avoid undesired hydrolysis, traces of water were removed by sodium sulphate from all reagents and solvents. For background control, a reaction mixture was also prepared without the addition of absolute ethanol. Enzyme activities were calculated from a calibration curve of *p*NP obtained after solubilisation in *tert*-butyl methyl ether (Sigma-Aldrich) and dilution in *n*-heptane (Scharlau Chemie S.A., Barcelona, Spain). Extraction and detection of *p*NP were performed using the same procedure mentioned above. One unit of lipase transesterification activity was defined as the amount of enzyme that releases one μmol of *p*NP in one minute under the assay conditions.

Estimation of the total protein content

Total protein content in the culture filtrates and crude extracts was estimated colorimetrically using Bradford reagent (Sigma-Aldrich) according to the manufacturer's instructions. Bovine serum albumin was used to calibrate the standard curves.

Statistical analysis

Standard deviations of mean values were calculated using Microsoft Office Excel 2007 function. Enzyme activity values are average values counted from three independent measures.

Results and Discussion

Screening of zygomycetes for lipolytic activity using tributyrin

In a previous screening of 35 *Dissophora*, *Mortierella* and *Umbelopsis* strains isolated from soil, *Dissophora ornata*, *Mortierella longicollis*, *M. alpina*, *M. humilis* and *M. cystojenkinii* showed outstanding lipolytic activity in comparison with the other tested isolates, showing about 5- to 6-mm haloes after 7-day cultivation on tributyrin containing solid medium (*20*). Continuing these investigations, 169 zygomycetes strains belonging to the genera *Dissophora*, *Gamsiella*, *Gilbertella*, *Mortierella*, *Mucor*, *Rhizomucor*, *Rhizopus* and *Umbelopsis* were also screened with respect to their ability to hydrolyse tributyrin. The investigated strains had been isolated from different sources including soil, fruit and compost; some isolates originated from mucormycoses. Lipolytic activity of the tested strains showed high variability, but those of 19 isolates had the largest lipolytic haloes around the colonies on tributyrin agar (Table 1). However, it is very important to notice that some strains showed intensive growth and the halo produced by the hydrolysis of tributyrin could be covered by the mycelium. Unfortunately, due to the problematic visibility, this disadvantage of the method causes some difficulties when comparing the data obtained on plate tests. In particular, many *Gilbertella* and *Rhizopus* isolates displayed fast growth on this medium, wrapping the total surface of the agar plates after 6 days. For this reason, results obtained on the 5th day of incubation were compared (Table 1). On the contrary, growth of several isolates (especially of some *Mortierella* and *Umbelopsis* strains) was fairly slow on this medium;

Table 1. Fungal strains that showed the best lipolytic activity towards tributyrin during growth on tributyrin agar

Isolate	Code	Lipolytic activity* *t*(incubation)/day			Cultivation temperature	Origin
		3	5	7	°C	
Gilbertella persicaria	SZMC 11091	1.9±0.08	5.1±0.2	n.d.	25	peach / California, USA
Mortierella echinosphaera	CBS 575.75	1.5±0.1	4.1±0.2	6.1±0.1	20	begonia / Netherlands
Mucor piriformis	SZMC 12077	2.1±0.08	5.0±0.1	9.0±0.2	25	unknown
Rhizomucor miehei	NRRL 5282	5.1±0.2	9.2±0.1	12.0±0.1	37	peppermint compost / India
R. miehei	NRRL 5901	5.0±0.2	7.2±0.2	13.9±0.1	37	cow placenta / Dakota, USA
R. miehei	ETH M4918	2.7±0.2	7.8±0.2	12.9±0.09	37	compost / Switzerland
R. miehei	CBS 370.71	5.1±0.1	10.2±0.2	18.4±0.3	37	sputum / Netherlands
R. miehei	CBS 360.92	2.3±0.2	10.1±0.2	16.3±0.2	37	human mycosis / Australia
R. pusillus	NRRL A-23448	2.8±0.2	5.2±0.2	8.2±0.2	37	unknown
R. pusillus	ETH M4920	2.9±0.2	6.1±0.08	8.1±0.1	37	trachea / Switzerland
Rhizopus niveus	CBS 403.51	1.6±0.1	6.9±0.2	n.d.	37	unknown / Japan
Rh. oryzae	NRRL 1526	5.3±0.2	10.2±0.2	n.d.	37	tempeh / Illinois, USA
Rh. oryzae	NRRL 1472	4.3±0.2	10.8±0.2	n.d.	25	unknown / Illinois, USA
Rh. stolonifer	SZMC 13609	2.2±0.2	6.1±0.08	n.d.	25	nectarine / California, USA
Rh. stolonifer var. *stolonifer*	SZMC 13601	2.1±0.1	8.3±0.2	n.d.	25	peach / California, USA
Umbelopsis autotrophica	CBS 310.93	4.2±0.2	8.1±0.3	12.0±0.3	20	soil / UK
U. isabellina	NRRL 1757	3.9±0.08	5.2±0.2	9.2±0.2	25	soil / USA
U. ramanniana var. *angulispora*	CBS 222.29	5.2±0.2	6.8±0.2	8.1±0.1	25	unknown / Russia
U. versiformis	CBS 473.74	4.6±0.2	7.3±0.2	12.3±0.2	20	soil / Australia

*The presented values are averages calculated from the data of three independent experiments. Lipolytic activities are represented by the width of haloes (mm)±standard deviation; n.d.=not detectable, surface of the agar plates was wrapped during growth
CBS=Centraalbureau voor Schimmelcultures, ETH=Swiss Federal Institute of Technology Culture Collection, NRRL=Agricultural Research Service Culture Collection, SZMC=Szeged Microbiological Collection

however, the detected halo was considerably broad in some cases.

Evaluation of the results presented in Table 1 indicates that isolates having the best lipolytic activity could be identified among the *Rhizomucor* and *Rhizopus* strains. Out of them, *Rhizomucor miehei* CBS 370.71 and *Rhizopus oryzae* NRRL 1472 displayed the highest enzyme activity, having 10.2- and 10.8-mm haloes after 5 days, respectively. The hydrolytic haloes exhibited by the *Rhizopus* isolates were greater than by the 12 strains studied by Rapp and Backhaus (*21*) using tributyrin agar. In connection with these fungal groups, it should be noted that certain strains isolated from mycoses proved to be especially good enzyme producers (Table 1). This may be related to the fact that secreted fungal lipases can play an important role in the virulence (*22*), supporting the growth and adhesion of these opportunistic pathogenic fungi. However, these fungi were omitted from further fermentation studies because their potential application in the industry is rather limited.

Among the *Gilbertella* and *Mucor* strains, *Gilbertella persicaria* SZMC 11091 and *Mucor piriformis* SZMC 12077 were the most promising enzyme producers with 5.1 and 5 mm wide haloes on the 5th day of incubation, respectively (Table 1). Alves *et al.* (*23*) presented the screening of several *Mucor* strains, in which a *Mucor genevensis* isolate showed the highest lipolytic activity using agar plates containing Tween 20 as substrate. In our experiments, *M. genevensis* isolates also revealed a good potential for lipase production having hydrolysis haloes about 4–5 mm on the 7th day (data not shown). Furthermore, it is worth mentioning that the high variability of lipase activity of the *Mucor* isolates corresponds well with our previous work performed with 27 strains representing 10 different species using an API-ZYM test (*24*).

Table 1 shows the tributyrin hydrolysing capacity of one *Mortierella* and four *Umbelopsis* isolates, which were identified as the best lipase producers within these genera. Additional data about the lipolytic capacity of other *Mortierella* and *Umbelopsis* isolates on tributyrin are available in our previous report (*20*). Although certain species belonging to the aforementioned genera have great potential in the industry due to their ability to produce different oil compounds in high amounts (*25*), relatively few data are available about their lipolytic activity. The majority of the selected fungi had been isolated from various soil samples and were characterised by well-defined hydrolysis haloes that varied from 6.1 to 12.3 mm after 7 days. Comparative evaluation of the results, taking into account the colony diameter, indicates that the best producers belong to the *Umbelopsis* genus. The haloes of these strains after 7 days (8.1 to 12.3 mm) were similar to those detected for the *Rhizomucor* isolates discussed above (8.1 to 18.4) (Table 1).

Screening of cold-tolerant zygomycetes with lipolytic activity using tributyrin

Cold-active lipolytic enzymes cover a wide spectrum of biotechnological and industrial applications including fermentation procedures in food processing, environmental bioremediations, biotransformations and pharmaceutical processing. Such enzymes have been reported in some psychrophilic and psychrotrophic filamentous fungi and yeasts (*26*), but cold-tolerant zygomycetes fungal strains have rarely been investigated in this regard. To explore new cold-active lipase-producing fungi, enzyme production was tested in some cold-tolerant *Mucor* strains, which are able to survive at temperatures near 5 °C.

Among the 14 cold-tolerant strains selected for the enzyme production studies, 9 exhibited appropriate growth within the 7-day incubation period at 5 and 10 °C (Fig. 1). The *M. racemosus* f. *chibinensis* isolate gave the highest activity with haloes of 3 and 4.1 mm at 5 and 10 °C, respectively. In the case of the most tested strains, lipolytic activity at 10 °C was higher than that observed at 5 °C.

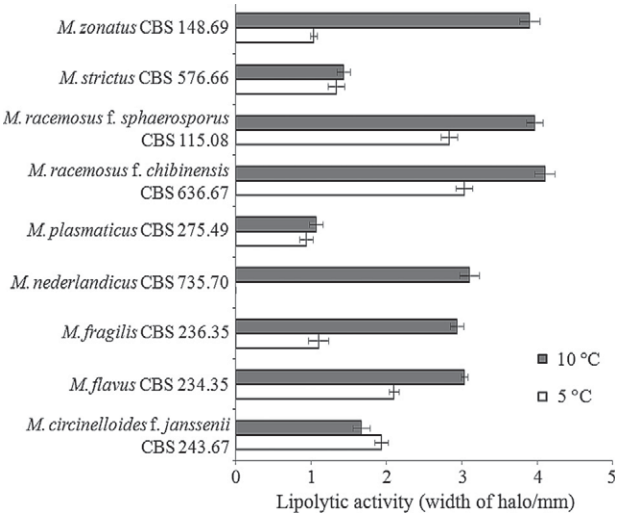

Fig. 1. Width of halo (mm) representing the tributyrin-hydrolysing capacity of cold-tolerant *Mucor* strains growing at 5 and 10 °C for 7 days. The presented values are averages of three independent measurements. Error bars indicate standard deviations

One possible explanation of this phenomenon may be that the former temperature is closer to the optimal growth conditions of these fungi, which is about 20 °C. On the other hand, the produced enzymes may have the optimum temperature for their activity at higher temperatures. Anyway, production of cold-active lipases is considered to be temperature dependent and thermolabile (*27*). Regarding the other tested strains, *M. amphibiorum*, *M. microsporus*, *M. guillermondii*, *M. indicus* and *M. minutus* started to grow at the end of the predetermined incubation period; however, hydrolysis of tributyrin by these fungi could also be observed at both temperatures (data not shown).

Effect of lipid sources on lipase production

A range of different oils and oil-based materials were tested for their ability to support the lipolytic enzyme production of 10 zygomycetes strains selected on the basis of their tributyrin-hydrolysing capacity. Although all *M. corticolus* strains developed only about 0.5-mm clear zones on tributyrin agar plates, due to their intensive growth, one isolate (SZMC 12031) was also involved in these studies. For comparison, a culture from each isolate was also incubated with 1 % anhydrous glucose; however, no or very low activity towards *p*NPP could be detected when this sugar was used as sole carbon source, suggesting that the presence of an inducer may be crucial to produce filamentous fungal lipases in high amount. This finding corresponds well to that reported on the lipase production of other filamentous fungi (*7*,*28*).

Lipid materials that induced the best enzyme activities by each isolates are presented in Table 2. In general, Tween 80 proved to be a good inductor for lipase production since most of the investigated fungi displayed high enzyme activity when this substrate was applied. This enhancing effect was strain-independent as Tween 80 induced the lipolytic activity of representatives of each tested species. Several reports on the production of fungal lipases indicated that the enzyme yield and activity have been influenced differently by various types of

Table 2. Lipolytic activity of zygomycetes strains in submerged fermentations using different inductor oils

Isolate	Inductor oil	Lipolytic activity* U per mL of medium
Mortierella alpina	soybean oil	26.7
	extra virgin olive oil	19.2
M. echinosphaera	Tween 80	4.7
	olive oil	1.8
Mucor corticolus SZMC 12031	Tween 80	118.7
	olive oil	9.6
Rhizomucor miehei NRRL 5282	extra virgin olive oil	1336.0
	sesame seed oil	1169.1
Rhizopus oryzae NRRL 1526	Tween 80	918.5
	olive oil	668.0
Rh. oryzae NRRL 1472	Tween 80	1003.1
	palm oil	106.2
Rh. stolonifer SZMC 13609	Tween 80	16.1
	palm oil	12.0
Umbelopsis autotrophica	olive oil	2.8
	Tween 80	2.7
U. isabellina	olive oil	8.0
	Tween 80	7.1
U. ramanniana var. *angulispora*	extra virgin olive oil	3.1
	cottonseed oil	1.8
U. versiformis	Tween 80	4.5
	soybean oil	2.3

*Presented activities were measured on the 7th day of incubation except for *Rh. oryzae* NRRL 1526 and *R. miehei* NRRL 5282 (activities were determined on the 3rd day) and *M. echinosphaera* and *U. autotrophica* (activities were determined on the 4th day). Lipolytic activity was measured colorimetrically using *p*-nitrophenyl palmitate as substrate. In each case, the two inductor oils that induced the highest activities are presented

Tween substrates (*29*,*30*); however, increased yields were achieved in most cases. Furthermore, Tween substrates are often used to determine the hydrolytic activity of lipolytic enzymes (*31*). Tween 80 also had positive effect on the extracellular lipase production of *Mortierella vinacea* (*11*). Other lipid materials such as olive, extra virgin olive, soybean, sesame seed and cottonseed oils also enhanced the enzyme production. The highest volumetric activity was achieved by using extra virgin olive oil as a lipid source during fermentation with *R. miehei* NRRL 5282 (Table 2). Olive oil as a good inducer for lipolytic activity of various fungal sources, such as *Penicillium aurantiogriseum* (*28*) and *Rh. chinensis* (*14*), has been documented. It can also be noted that the *Umbelopsis* isolates formed a rather large clear zone on tributyrin agar (Table 1), while they produced only an average activity towards the lipid materials during shake-flask cultivation. On the contrary, *M. alpina* revealed relatively high activity on soybean oil (26.7 U/mL) in submerged tests, but the size of its halo on tributyrin agar was smaller than those of the other *Mortierella* and *Umbelopsis* isolates.

As shown in Table 2, *R. miehei* NRRL 5282 and *Rh. oryzae* NRRL 1472 and NRRL 1526 isolates displayed outstanding lipase-producing abilities with 1336.0, 1003.1 and 918.5 U/mL volumetric activities, respectively. These volumetric activities are significant compared to those of other filamentous fungal lipases presented previously (*5*). Maximal enzyme concentrations were reached after three days of culturing at *R. miehei* NRRL 5282 and *Rh. oryzae* NRRL 1526 isolates. Decrease of mycelium mass was not observed at this point so it might be unlikely that these lipases in the fermentation broth originated from cell lysis instead of enzyme secretion. Degradation of mycelium had been described by Hiol *et al.* (*32*) after four days during a lipase-producing submerged fermentation test with *Rh. oryzae*. In our study, *Rh. oryzae* NRRL 1472 had its maximal enzyme activity after seven days. Although mycelial decrease was not observed, it cannot be excluded that part of the released lipases might derive from cell lysis.

Wheat bran-based submerged fermentation

To study the effect of wheat bran on the lipase production in submerged culture, fermentation with the selected zygomycetes strains was carried out. Since olive oil generally supplied high enzyme yield during previous tests, the media used here were enriched with 2 % olive oil as additional carbon source. Interestingly, the *Rh. oryzae* NRRL 1526, NRRL 1472 and *U. autotrophica* strains had notable lipolytic activity on glucose-supplemented media, showing 76.9, 96.7 and 171.6 U/mg lipase activities, respectively, which may be due to the presence of the olive oil in the medium.

Table 3 shows the best volumetric and specific lipolytic activity values reached during wheat bran-based liquid fermentations. Since it is difficult to separate the mycelia from the culture medium under the applied fermentation conditions, specific activity data were given referring to the protein concentration measured in the supernatant. Except for *M. alpina*, *R. miehei*, *U. isabellina*, *M. corticolus* and both *Rh. oryzae* isolates, submerged fermentation in wheat bran containing mineral medium resulted in higher volumetric activities than those in minimal cultures supplemented with oil-based materials as sole carbon source. This indicates that the application of wheat bran in liquid cultures does not always increase the enzyme production of zygomycetes. Nevertheless, it can be concluded that significant increase in the enzyme production could only be found by *Rh. stolonifer* compared to minimal medium exhibiting a maximum lipolytic activity of 84 U/mL at 24 h post inoculation (Table

Table 3. Lipolytic activity of lipase-producing zygomycetes fungi in wheat bran-based liquid and solid-state fermentations with the addition of olive oil and mineral salts

Isolate	Lipolytic activity			
	Submerged fermentation[a]		Solid-state fermentation[b]	
	U per mL of medium	U per mg of protein	U per mL of crude extract	U per mg of protein
Mortierella alpina	5.4±0.6	33.8±10.2	10.5±1.2	34.7±2.7
M. echinosphaera	11.6±2.3	248.1±75.5	13.6±0.5	56.1±4.8
Mucor corticolus SZMC 12031	61.3±8.4	827.4±79.5	288.9±25.6	1325±117.3
Rhizomucor miehei NRRL 5282	371.7±116.3	3682.6±371.7	735.8±50.8	3618.6±114.5
Rhizopus oryzae NRRL 1526	113.1±9.6	1099.6±102.7	92.9±2.9	534.12±34.9
Rh. oryzae NRRL 1472	126.6±3.8	1013.8±264.8	34.9±1.2	131.2±6.5
Rh. stolonifer SZMC 13609	84.1±4.8	776.9±80.3	25.5±1.1	98.3±5.9
Umbelopsis autotrophica	17.8±1.1	223.2±33.9	69.4±4.6	284.8±6.1
U. isabellina	7.1±0.4	101.2±7.6	11.2±1.9	41.3±7.9
U. ramanniana var. *angulispora*	13.3±1.9	128.1±4.3	59.3±1.9	215.8±14.9
U. versiformis	9.6±1.6	94.8±16.6	47.8±1.7	164.7±1.5

[a]Presented activities were measured on the 1st day of incubation by *Rhizopus*, 2nd day by *M. corticolus*, 4th day by *M. echinosphaera* and *R. miehei*, 5th day by *U. isabellina* and *U. ramanniana* var. *angulispora* and 7th day by *M. alpina*, *U. autotrophica* and *U. versiformis* isolates; [b]presented activities were measured on the 7th day of the fermentation by each isolate; values are averages calculated from the data of three independent measurements±standard deviation

3). Under these conditions, enzyme production of *Rh. stolonifer* is comparable to that presented by both *Rh. oryzae* isolates.

Comparing the best results obtained using wheat bran-based liquid fermentations, it can be observed that the *p*NPP hydrolysis in the supernatant of *R. miehei* was also superior (371.7 U/mL) to that measured by the *Rhizopus* isolates (Table 3). Specific activity of *R. miehei* was also higher than those of the *Rhizopus* isolates. Among the *Umbelopsis* isolates, *U. autotrophica* proved to be the best producer (223.2 U/mg). Similarly, maximum specific activity of 248.1 U/mg was detected in the case of *M. echinosphaera*. It is worth mentioning that unlike the *Mortierella* and *Umbelopsis* isolates, enzyme production of *Rhizopus* and *M. corticolus* strains generally reached its maximum during the first phase of fermentation. Similarly, Mateos Diaz *et al.* (*10*) published a maximum extracellular lipase activity after 22 h during submerged culture fermentation of *Rh. homothallicus*. The authors of this study showed that enzyme activity markedly decreased after reaching its maximum, which is comparable to our findings when using these strains (data not shown).

Wheat bran-based solid-state fermentation

Solid-state fermentation (SSF) is a frequently used technique to produce lipases by fungi, and higher product yield can generally be achieved with this method compared to that obtained with submerged fermentations. Most wheat bran-based fermentation studies to enhance the fungal lipase production were carried out in SSF systems; therefore, the objective of our assays was to investigate the lipolytic enzyme-producing capacity of the previously selected zygomycetes in SSF. Additionally, as we know, there has been no previous report on lipase production by *Mortierella* and *Umbelopsis* isolates using SSF techniques. For all SSF cultures, the applied inoculum size was 1 %, which is similar to that identified as the optimal for *Rh. oligosporus* by ul-Haq *et al.* (*33*). Lipolytic activity was detected in the crude extracts after only 168 h of fermentation.

Two fermentation media were compared to evaluate the enzyme production in SSF: a simple medium containing only distilled water to moisturise the wheat bran (SSF medium 1), and a supplemented one (SSF medium 2) optimised for *Aspergillus niger* lipase production in an earlier study (*7*). The hydrolytic activity of the lipolytic enzymes produced by the selected strains in terms of the amount of solid substrate is presented in Fig. 2. The isolates grew well on both media, but the *p*NPP hydrolysis in the crude extracts was considerably higher when mineral salts and olive oil were added to the culture medium (SSF medium 2). These results are in agreement with the data published about other filamentous fungal strains where the enzyme yield during SSF on wheat bran was also increased after the addition of oil or solid materials with high oil content (*34*,*35*). SSF medium 1 also served well for enzyme production, but relatively low hydrolytic activities were achieved compared to medium 2. Nevertheless, the lipolytic activity of *R. miehei* (1006.1 U per g of dry substrate) obtained on medium 1 may be important since this medium did not contain an inducer, which could cause protein aggregation and thus generate problems in the enzyme purification processes (*10*). During fermentation on medium 2, enzyme production by *R. miehei*, *Rh. oryzae* NRRL 1526, *Rh. stolonifer* (Fig. 2a) and *U. versiformis* (Fig. 2b) isolates improved considerably showing at least four times higher enzyme activities than on medium 1. The enzyme yield by *R. miehei* (4415 U per g of dry substrate) was higher than those reported for other filamentous fungi in wheat bran SSF (*33*,*34*,*36*). When mineral salt solution and olive oil were used as supplements, *M. corticolus*, *Rh. oryzae* NRRL 1526, *U. autotrophica*, *U. ramanniana* var. *angulispora* and *U. versiformis* also proved to be promising lipase producers on wheat bran, expressing hydrolytic activities of 1733.4, 559.7, 416.3, 355.7 and 287.1 U per g of dry substrate, respectively.

Table 3 also summarises the volumetric and specific activity values corresponding to the lipolytic activity of the investigated isolates during fermentation on medium 2. These results indicate that SSF provided consid-

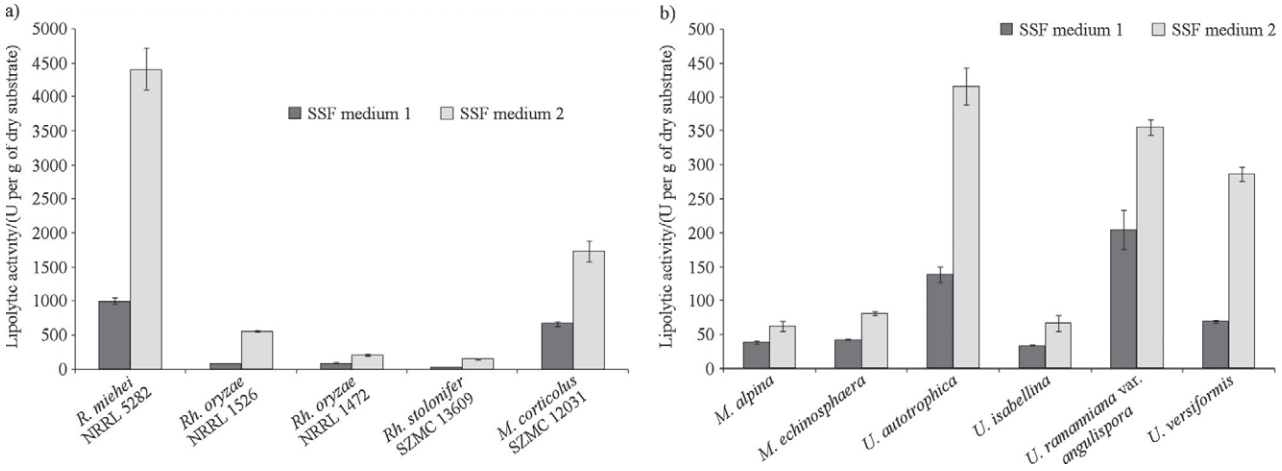

Fig. 2. Comparative evaluation of lipolytic activity of lipase-producing fungi: a) *Rhizomucor*, *Rhizopus* and *Mucor*, and b) *Mortierella* and *Umbelopsis* on wheat bran moisturised with only distilled water (SSF medium 1) or with mineral salt solution and olive oil as supplements (SSF medium 2). The presented activity values are averages of three independent measurements performed on the 7th day of the cultivation. Error bars indicate standard deviations

erably higher enzyme yields by *R. miehei* (735.8 U/mL), *M. corticolus* (288.9 U/mL), *U. autotrophica* (69.4 U/mL), *U. ramanniana* var. *angulispora* (59.3 U/mL) and *U. versiformis* (47.8 U/mL) than submerged fermentation. It is possible, like in the case of some other fungal hydrolases (*37*), that these lipases may be in cell wall-bound form under liquid culture conditions while they are secreted to the medium during solid-state fermentation. Moreover, it had previously been demonstrated that the lipase from *Rh. homothallicus* is more thermostable if it is produced using solid-state fermentation than in submerged culture (*10*). Therefore, it also cannot be excluded that the lipases analysed in this study may have distinct temperature stability parameters depending on which conditions were used for production. This may concern the *U. isabellina* and both *Mortierella* isolates, whose lipolytic activity in SSF showed a slight increase compared to submerged fermentation (Table 3). Less significant increase could be observed in terms of the specific activity values due to the higher concentration of proteins found in the crude extract (Table 3).

Unlike the other fungi tested, in the case of *Rhizopus* strains, much lower volumetric activities could be detected in SSF than in submerged fermentation (Table 3). This common property of these *Rhizopus* isolates is probably due to the fact that the best lipolytic activities in submerged cultures were obtained at 24 h post inoculation while, in the case of SSF, preparation of crude extracts was carried out at 168 h of fermentation. It is assumed that higher enzyme yield could be reached if the extraction is performed at an earlier stage of fermentation. However, further optimisation studies are needed to prove this suggestion. On the other hand, due to their slower growth, *Mortierella* and *Umbelopsis* isolates needed one- or two-week incubation to reach remarkable lipase production under the conditions used in this study. The use of low-cost agro-industrial by-products as a substrate, especially in SSF systems, has numerous economical benefits for high yield production of lipases (*38*).

Screening for transesterification activity

Based on a spectrophotometric method using *p*NPP, we also aimed to screen the transesterification activity of the selected lipase-producing fungi in organic media (with *n*-heptane as a solvent). Since transesterification and esterification activities were found to be related according to Teng and Xu (*19*), we could monitor the whole synthetic capacity of the lipolytic enzymes produced by each isolate.

Data presented in Fig. 3 revealed that transesterification activities are much lower than the hydrolytic enzyme activities (Table 3), which is in agreement with the previously published data (*39*). The maximum amount of the released *p*NP was observed by *R. miehei*, exhibiting 4.8 U/mg of transesterification activity. Additionally, some lipases, which were highly active in *p*NPP hydrolysis, showed moderate activity in synthetic assays or *vice versa*. For instance, lipases from *M. echinosphaera* and *Rh. stolonifer* demonstrated low hydrolytic activity while they were very active in the transesterification assays (see Table 3 and Fig. 3). Big differences were also found in the hydrolytic activities of the *Umbelopsis* enzymes, which were not observed in their organic synthesis capacities (see Table 3 and Fig. 3). A possible explanation for this different behaviour of the investigated lipolytic enzymes would be their different resistance to the inhibiting effect of the organic solvent. Such deactivation effect of organic solvents has been described for other fungal lipases (*40*). The *Umbelopsis* lipolytic enzymes displayed much lower catalysis of transesterification (from 0.38 to 0.54 U/mg) compared to the other enzymes. It is possible that for proper catalysis of organic synthesis reactions by these enzymes, lower temperatures than those used in this study are needed. Nevertheless, as we know, this is the first study about the use of extracellular *Mortierella* and *Umbelopsis* lipases to catalyse synthetic reactions in organic media.

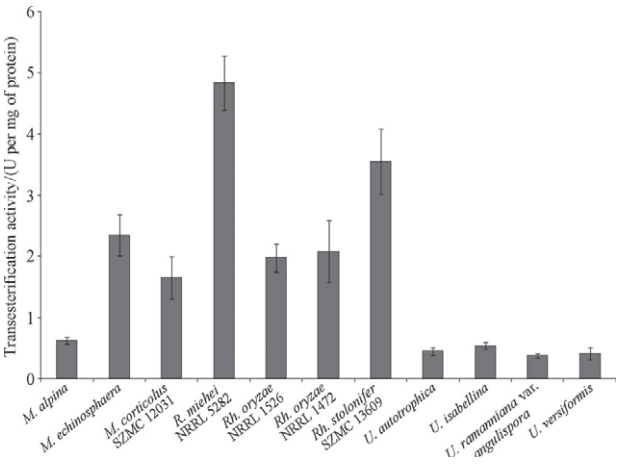

Fig. 3. Transesterification activity exhibited by the lipolytic enzymes selected in this study. Reactions were performed at 40 °C in 0.5 mL of *n*-heptane containing 10 mM of *p*NPP and 50 μL of absolute ethanol. The activity values are averages of three independent measurements. Error bars indicate standard deviations

Conclusions

Lipase-producing fungi were successfully identified from 169 zygomycetes isolates screened in terms of their tributyrin hydrolysing capacity. Most promising lipase producers were in the genera *Rhizomucor*, *Rhizopus*, *Mucor*, *Mortierella* and *Umbelopsis*. Many lipid materials had the potential to induce the enzyme production of selected lipase producers in submerged fermentation tests. Tween 80 and olive oil proved to be the best inducers of most of the analysed strains. It can be concluded that the enzyme yield generally increased by using wheat bran as a supplement in submerged and solid-state fermentations. Moreover, addition of olive oil and use of mineral salts in solid-state fermentation also enhanced the enzyme production. This study also indicates that the lipolytic enzymes produced in solid-state fermentation by selected lipase-producing fungi were able to catalyse transesterification reactions in organic media. However, there was no general correlation between the hydrolytic and the synthetic activities. Our results suggest that enzymes from *R. miehei*, *Rh. stolonifer* and *M. echinosphaera* could be used as effective biocatalysts for organic synthesis. After purification and biochemical characterisation, fur-

ther assays need to be carried out for detailed analysis of the synthetic reactions catalysed by these enzymes.

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