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Mucor griseocyanus Lipase: Production, Characterization and Study of Some Catalytic Properties of the Immobilised Enzyme

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Summary

The aim of this work is to study the production of extracellular lipase by *Mucor griseocyanus* 55.1.1 strain on different substrates in order to select the ideal one for lipase synthesis. The carbon sources used were: olive oil, glycerol, coconut oil, sunflower oil, glucose, starch and sucrose. The obtained results indicate that the synthesis of the enzyme was possible in the presence of all substrates. Lipase activities in the range of 0.04 to 0.1 IU/mL were obtained. It was found that the most suitable carbon source for the production of the enzyme was a combination of coconut oil and sucrose at 0.5 and 1.5 % (*m*/*V*), respectively, and the level of activity reached under this condition was 0.113 IU/mL. The optimum pH and temperature for enzymatic extract activities were identified in a pH range of 4 to 6 and at a temperature of 60 °C. Enzymatic extract was stable for a period of 5 h in neutral and weakly acidic media (pH=6) at moderate temperatures between 20 and 40 °C. Studies on the catalytic properties (stereoselectivity and enantioselectivity) of the immobilized lipase using the esters of methyl phenyl glycinic and (*R*,*S*)-methyl mandelic acid showed excellent properties of the enzyme compared to commercial lipases tested. *M. griseocyanus* lipase exhibited a greater stereoselectivity towards the *R*-isomer of methyl phenyl glycinic acid ester. However, with the esters of methyl mandelic acid, the enzyme showed a certain preference toward the *S*-isomer and it was hydrolysed 20 times faster than the *R*-isomer.

Key words: Mucor griseocyanus, lipase, submerged cultures, selective hydrolysis

Introduction

A large number of lipases are produced on industrial scale for application in food and detergent industries (*1*) due to the fact that they are extracellular enzymes, secreted by both fungi and bacteria. The ready availability of lipases has created an enormous spin-off with respect to the enantioselective hydrolysis and formation of carboxyl esters. Bearing in mind that the natural substrates for lipases are glycerides, which possess a chiral alcohol moiety, it is understandable that they can be particularly useful for the resolution or asymmetrization of esters bearing such alcohol moiety.

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Nowadays, very good selectivities have been reported for many biotransformations catalyzed by soluble lipases under complex conditions (*2*). However, these conditions are very different when these biotransformations are carried out by immobilized lipase. In an immobilized form, in macroaqueous systems closed and open structures of lipases are in partial equilibrium. Furthermore, immobilization is a suitable approach that allows biocatalyst reuse, easier product recovery, ability to enhance resistance against inactivation by different denaturants and possibility to manipulate several properties of lipases for potential industrial applications (*3*). Even the adsorption on hydrophobic supports, like octyl agarose, allows separation of lipases from other enzymes present in the fermentative broth (*4*).

Most commercially useful lipases are of microbial origin, including bacteria, yeasts, fungi and actinomyces. Among fungal species, the most widely employed are *Aspergillus*, *Rhizopus*, *Penicillium* and *Mucor*. In the last case, only the species *miehei*, *javanicus*, *circinelloides*, *hiemalis* and *racemosus* have been reported as lipase producers (*1*).

This work deals with a preliminary study on the characteristics of lipase produced by the strain *Mucor griseocyanus* 55.1.1, not reported before as a lipase producer, with special emphasis on the general behaviour and applicability of this enzyme on selective hydrolysis reactions.

Materials and Methods

Microorganism and maintenance of the culture

The strain *Mucor griseocyanus* 55.1.1 obtained from the microbial culture collection of the Sugar Cane Derivatives Institute (ICIDCA, Cuba) was used, and the stock culture was maintained on potato dextrose agar at 4 °C. This microorganism was isolated from a lysine sample in 1985.

Growth media

The mineral growth medium contained (in g/L): NaH_2PO_4 12, KH_2PO_4 2, $MgSO_4 \cdot 7H_2O$ 0.3, $CaCl_2$ 0.25, and $(NH_4)_2SO_4$ 1 % (*m*/*V*) as a nitrogen source and 2 % (*m*/*V*) olive oil as a carbon source. The initial pH was adjusted to 6. Other carbon sources, like sunflower oil, coconut oil, starch, glycerol and glucose, were used at 2 % (*m*/*V*). At the same time, the effect of different combinations of coconut oil and glucose on lipase synthesis was analyzed following the feed scheme presented in the results and discussion. Finally, in the best combination, the glucose was substituted by sucrose in the medium at the same concentration.

Lipase production in submerged fermentation (SmF)

The medium was inoculated with 10^7 conidia transferred from the stock culture to 100-mL Erlenmeyer flasks containing 20 mL of sterile growth medium. The flasks were incubated at 30 °C in a shaker operating at 120 rpm for a period of 8 days. The fungal growth was measured in terms of dry mass of the mycelium in g/L. The fungal biomass was filtered under vacuum and later centrifuged at 12 000 rpm for 5 min. The clarified supernatant was used as the source of enzyme.

Lipase assay

Lipase activity was assayed by the addition of 0.1 mL of lipase solution to 2.5 mL of the substrate, *viz*. 0.4 mM *p*-nitrophenyl propionate (*p*NPP) at pH=7 and 37 °C. The increase of absorbance at 348 nm, due to the release of *p*-nitrophenol, was measured (*4*). One unit was defined as the amount of enzyme necessary to hydrolyze 1 μmol of *p*NPP per min under the previously described conditions.

Enzyme characterization

The effect of temperature was evaluated by the measurement of lipase activity in the hydrolysis reaction of *p*NPP at various temperatures ranging from 20 to 80 °C at pH=7. Crude enzyme and substrate were incubated at different reaction temperatures before starting the experiment and the enzyme assay was performed as described earlier. For the estimation of enzyme thermostability, the enzymatic extract was incubated at 20, 30, 40, 50 and 60 °C for 24 hours at pH=7. After incubation, the enzyme was immediately cooled in an ice bath for 15 min and the residual activity was determined (considering the initial activity of the enzyme as 100 %). The effect of pH was studied by measuring the activity at different pH in a range of 4 to 10, at 30 °C. To ensure the pH stability, the enzymatic extract was incubated in 0.05 M buffer solutions of the above pH values (sodium acetate for pH=4 and 5, sodium phosphate for pH=6 and 7, and sodium carbonate for pH=8, 9 and 10) for 24 h at 30 °C. The residual activity was assayed after incubation.

Immobilization of lipase

M. griseocyanus lipase was immobilized on octyl agarose 4BCL according to the procedures described for interfacial adsorption on hydrophobic supports bearing octyl chains (*4*). The immobilized enzyme was quantified by determination of total protein by dye binding assay (*5*). The octyl agarose 4BCL was supplied by Pharmacia Biotech, Uppsala, Sweden.

Study of selective hydrolysis

Evaluations of hydrolytic activities was performed using 10 mL of 10 mM (*R*,*S*)-methyl mandelate ester ((*R*,*S*)-MME) in 25 mM sodium phosphate buffer at pH= =7.0 and 25 °C under mechanical stirring. The same experiment was also carried out at different pH values and temperatures. The degree of hydrolysis was analyzed by HPLC with C18 ODS column and monitored with a UV detector at 225 nm. A mixture of acetonitrile (60 %) and water (40 %) was used as mobile phase with a flow rate of 1.0 mL/min. Both the decrease in the peak area corresponding to the ester and the increase in the peak area corresponding to the acids were analyzed. At different conversion degrees, the enantiomeric excess of the remaining esters was analyzed by Chiral Reverse Phase HPLC. The column was a Chiracel OD-R, and the mobile phase was a mixture of 40 % acetonitrile and 60 % water at a flow rate of 0.5 mL/min. Enantioselectivity was expressed as E value calculated from the enantiomeric excess of the remaining ester according to the formula previously reported by Chen *et al*. (*6*).

In addition, the stereoselectivity of the immobilized lipase on 4BCL agarose gel was studied by the hydrolysis reactions of methyl phenyl glycine ester (MPGE). In this case, the mobile phase was a mixture of 25 % (*V*/*V*) methanol in 50 mM sodium phosphate buffer at a flow rate of 1 mL/min. The enzyme stereospecificity was determined as the ratio between hydrolysis rates of *R*- and *S*-isomers of the ester.

The (*R*,*S*)-MME and the pure species of MPGE were purchased from Sigma Aldrich and Aldrich Chemical Co., Inc., respectively.

Results and Discussion

Production of the enzyme using different substrates

Growth kinetics and lipase expression showed by *M. griseocyanus* on different substrates are outlined in Fig. 1. The growth of the microorganism was very distinct. The major values (20 g/L) were reached in coconut oil at the 8th day of fermentation, followed by (in g/L): sunflower oil 14, olive oil 10 and glucose 8. However, when starch and glycerol were used, the biomass yield was only 4 and 7 g/L, respectively, therefore neither of these substrates was considered suitable as a carbon source for *M. griseocyanus* growth. Lipase activities of 0.04–0.1 IU/mL were observed at the third day of fermentation in the presence of all substrates. This is the reason why the production of lipase is carried out in a constitutive manner by *M. griseocyanus*. This finding makes it possible to envisage the substitution of olive oil with coconut oil in the medium.

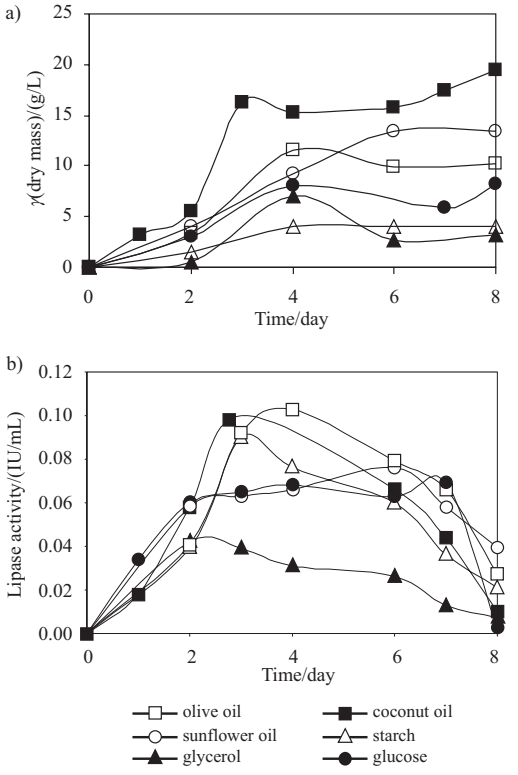

Fig. 1. Production of lipase by *Mucor griseocyanus* cultures on different carbon sources in the concentration of 2 % (*m*/*V*) at 30 °C and 120 rpm: (a) microbial growth, (b) lipase activity

Experimental results were analyzed to evaluate the tested carbon sources from the point of view of the energy they supply and how this energy is used for growth and expression of metabolites. The yields of the biomass/substrate for each carbon source independently of the employed microorganism can be determined by applying the mass balance approach to the system, assuming that all energy supplied is used for growth. According to the values reported by Erickson *et al.* (*7*), and taking into consideration that the maximum yield observed for growth is 0.7, the theoretical biomass/substrate yield ($\eta_{x/s}$) for some of the studied substrates can be calculated. Since the concentration of each substrate in the medium was 20 g/L, it is possible to estimate the maximum feasible growth for this microorganism, assuming a maximal value of biomass/substrate yield ($\eta_{x/s}$) and 100 % substrate conversion (AS=S_o), considering that the glucose was totally consumed at the second day. Table 1 outlines a comparison between theoretical and real biomass/substrate yields for the studied substrates.

Table 1. Comparison between theoretical and real biomass/substrate yields

Substrate	$\eta_{x/s(t)}$	γ_t g/L	γ_r g/L	Efficiency %
Starch	0.63	12.6	3.5	27.8
Glycerol	0.63	12.6	7.0	55.6
Glucose	0.56	11.2	8.0	71.4

t – theoretical, r – real

In all cases, the obtained growth was lower than the theoretical. The difference between theoretical and real growth was more significant in the case of starch as a carbon source. However, the major efficiency of glucose as a carbon source is about 71.4 %. It can be assumed that the energy supplied by starch was mainly employed for the synthesis of the enzyme. In the presence of starch the lipase activity levels on the third day were similar to the levels observed in cultures where coconut and olive oil were added. Olive oil in particular is a good inducer of lipases, as indicated by many researchers (*1*,*8*,*9*). In the case of glucose, utilisation was achieved by the microorganism mainly for growth than for enzyme synthesis.

As far as the contribution of the nitrogen source is concerned, the following data can be taken into account: fungi contain about 30 % of proteins on dry mass basis (*10*), nitrogen represents 16 % of proteins (*10*), and ammonium sulphate contains 21.2 % of nitrogen. Due to the presence of 10 g of ammonium sulphate in the culture medium, each culture litre contains 2.12 g of nitrogen. Assuming that all nitrogen is transformed into proteins, it is possible to obtain 13.25 g of proteins and 44.17 g of biomass. Therefore, the theoretical potential to produce biomass using 1 % (*m*/*V*) of this nitrogen source is vastly different, compared to those obtained using the other studied cultures. This result can be attributed to two facts. The first one relates to the physiological and genetic characteristics of the microorganism responsible

for the consumption and efficient use of the nitrogen source employed in the culture medium. The second one relates to the concentration of the nitrogen source employed in the medium, due to the fact that it would not respond to what is considered an optimal concentration for adequate conditions for microorganism growth.

In biotechnological processes, specifically in relation to the fermentation steps, raw materials have an important role in the production of any protein. The cost of the raw material has been estimated to be in the range of 30 to 80 % of total costs (*11*). Since raw materials have a major influence on the physiological state of microorganisms, it is very important to study the effect of different nutrients, especially carbon sources, on the culture media.

Production of the enzyme using combinations of substrates

The results obtained on the third day of fermentation for combinations of coconut oil and glucose, and coconut oil and sucrose are presented in Table 2. These substrates were added to the *M. griseocyanus* cultures in order to improve the medium from an economical standpoint. The second and fifth experiments yielded the best results (about 0.1 IU/mL of lipase activity), differing mainly in the growth and productivity reached in the medium. Similar values of lipase activities were achieved despite using 4 times smaller coconut oil concentration of 0.5 % (*m*/*V*) in the second than in the fifth experiment. Such a result is very interesting as it allows the substitution of olive oil with coconut oil, as well as reduction of the concentration of coconut oil in the culture medium, without affecting the lipase expression.

The growth obtained in the presence of 0.5 % (*m*/*V*) of coconut oil and 1.5 % (*m*/*V*) of glucose on the third day of fermentation is an intermediate value relative to the growth reported for each individual substrate (Fig. 1a). This result is a good indication that microorganisms use the oil source more efficiently for growth. Nevertheless, the behaviour of the lipase activity in the combined medium was similar to the lipase activity observed in the medium containing coconut oil. The enzymatic activity decreased until the end of the experiment after the third day for both media even when the microorganism growth continued. The decrease of the enzymatic activity is attributable to the decrease in pH as a consequence of the fatty acids that are liberated to the medium from the oil hydrolysis. The pH values in the culture medium on the third and last day of fermentation were 2.23 and 1.56, respectively.

Interesting results were obtained when the glucose added to the medium was substituted with sucrose following the scheme presented in Table 2. The lipase activity obtained with sucrose combined with coconut oil (0.113 IU/mL) was slightly superior to the lipase activity obtained when coconut oil was combined with glucose as the carbon source. For these reasons it can be concluded that it is possible to produce lipase by replacing the glucose with sucrose while reducing the coconut oil concentration from 2 to 0.5 % (*m*/*V*). This result could contribute to establishing a more profitable process from an economic standpoint.

The analysis of variance of the results presented in Table 2 demonstrates that there are significant statistical differences among the average values of dry mass, lipase activity and productivity (p-value for F-test less than 0.05). The analysis showed that the highest dry mass was obtained using coconut oil (20 %, *m*/*V*).

Regarding lipase activity values, two homologous groups were obtained with significant statistical differences between them. Second, fifth and sixth experimental runs form one of these groups, which means that there are not differences among their activity values at a 95 % reliability level. This is in agreement with the conclusions expressed above.

Fig. 2 demonstrates that when one half of the glucose supplied to the medium was consumed (day one), the enzyme production had already begun. This proves once again that the enzyme synthesis carried out by *M. griseocyanus* is done in a constitutive manner. This result contrasts the results obtained using *Aspergillus niger* and *Aspergillus fumigatus* cultures, as previously reported (*12*). However, it should be noted that similar results were reported for *C. rugosa* strain when it was fermented in different concentrations of olive oil and glucose (*13*).

Enzyme characterization

Fig. 3 shows the influence of temperature and pH on the enzyme activity expressed by *Mucor griseocyanus*.

Table 2. *M. griseocyanus* cultures grown using combinations of coconut oil, glucose and saccharose with different concentrations at 30 °C and 120 rpm. Growth and lipase activity were measured on the third day of fermentation. The productivity was calculated by dividing the lipase activity value by the time. Each experimental condition was carried out in triplicate

No.	Carbon source (%, *m*/*V*)	$\frac{\gamma(\text{dry mass})}{\text{g/L}}$		$\frac{\gamma(\text{lipase activity})}{\text{IU/mL}}$		$\frac{\text{Productivity}}{\text{IU/(mL·day)}}$
		Average	SD	Average	SD	
1	Coconut oil (0)-Glucose (2)	8.98	0.05	0.069	0.0002	0.023
2	Coconut oil (0.5)-Glucose (1.5)	11.80	0.04	0.096	0.0004	0.032
3	Coconut oil (1)-Glucose (1)	13.54	0.07	0.061	0.0002	0.020
4	Coconut oil (1.5)-Glucose (0.5)	15.67	0.04	0.071	0.0001	0.023
5	Coconut oil (2)-Glucose (0)	16.35	0.09	0.100	0.0003	0.033
6	Coconut oil (0.5)-Sucrose (1.5)	13.50	0.03	0.113	0.0001	0.038

SD – standard deviation

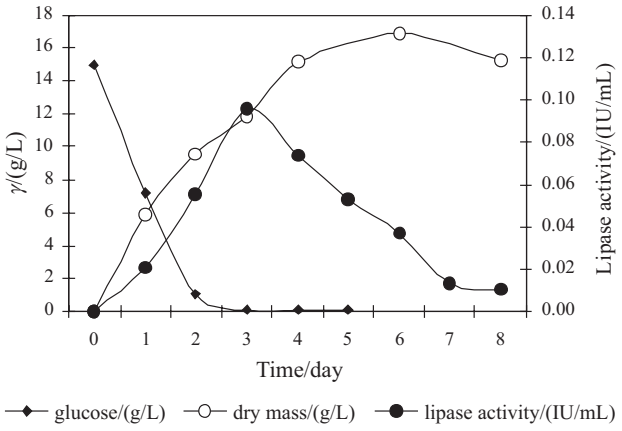

Fig. 2. Lipase production and glucose consumption rate in *M. griseocyanus* cultures in the presence of coconut oil 0.5 % and glucose 1.5 % (*m*/*V*) in the medium at 30 °C and 120 rpm

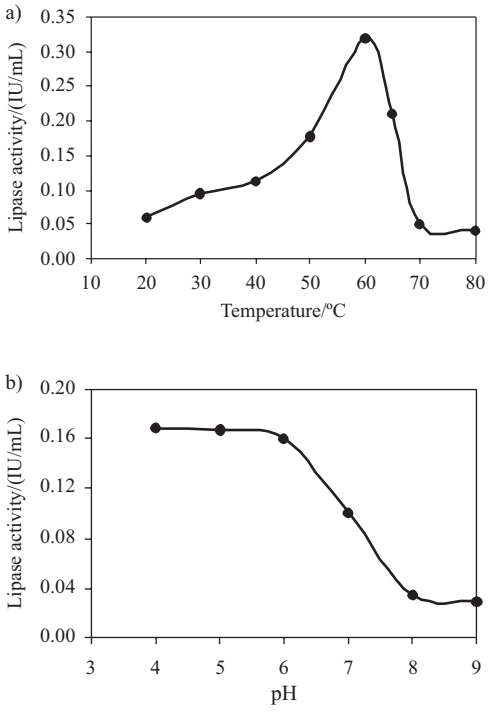

Fig. 3. Behaviour of the enzymatic activity of *M. griseocyanus* lipase: (a) effect of temperature at pH=7, (b) effect of pH at 30 °C

Lipase obtained from *M. griseocyanus* was the most active at 60 °C. The activity decreased drastically at higher temperatures, becoming almost nonexistant at 70 °C. It has been reported that the most lipases are active between 25 and 40 °C (*14*,*15*). *M. griseocyanus* lipase was found to be most active in a pH range of 4 to 6. This is the same pH range reported for a lipase expressed by *Rhizopus rhizopodiformis* (*16*). There is not much literature data in relation to the research on production of lipase by *M. griseocyanus*.

The stability profile of the enzyme regarding temperature is shown in Fig. 4a. This enzyme was very unstable at different temperatures and pH values assayed. It only retained about 80 % of its initial activity at 20, 30 and 40 °C for 5 h. Beyond this time, the residual activity decreased considerably.

Results regarding the effect of pH on the stability of the enzyme activity are shown in Fig. 4b. Enzyme activity was more stable at pH=6, retaining almost 80 % of its initial activity for 24 h. Nevertheless, it rapidly became inactivate when it was incubated at pH values of 9 and 10 and lost about 30 to 40 % of its initial activity within half an hour. Similar behaviour was reported for the fungal lipase expressed by a *Penicillium* strain (*17*).

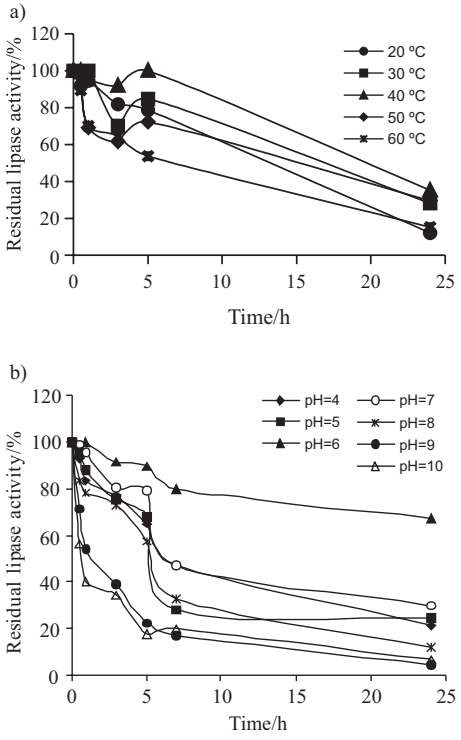

Fig. 4. Stability profile of *M. griseocyanus* lipase: (a) effect of temperature at pH=7, (b) effect of pH at 30 °C

Thermal stability of a lipase is obviously related with its structure and is also influenced by environmental factors such as pH and the presence of metal ions. At least in some cases, thermal denaturation appears to occur through the intermediate state of unfolding of the polypeptide. It has been reported that the thermal and operational stability of many lipases can be significantly enhanced by immobilization (*18*).

Immobilization of lipase and selective hydrolysis reactions

A moderate amount of lipases (2 mg of protein/mL of activated supports) was applied to the activated supports, around 95 % of which was immobilized. Fernández-Lorente *et al*. (*19*) obtained similar results for the immobilization of commercial lipases and they demonstrated that it was possible to purify the enzyme too. Although electrophoretic analysis was not made for immobilized lipase of *M. griseocyanus*, it had been demonstrated in a previous work that it was possible to selectively separate immobilized lipase from the other enzymes in fermentation broth (*20*).

Optically pure *R*- and *S*-isomers of mandelic acid and their esters are very useful in organic synthesis of cefamandole and optically pure acids are used in the resolution of racemates (*19*). An optically pure nonhydrolyzed ester can be easily obtained by enantioselective hydrolysis of the other ester, even with immobilized lipases showing moderate enantioselectivity (*e.g.* 20–40). Moreover, under certain conditions when lipase exhibits a very high enantioselectivity (E 200), both optically pure ester and pure acid can be obtained. The schematic diagram of the hydrolysis reaction of (*R,S*)-MME is given in Fig. 5.

The enantioselectivity of different immobilized lipases towards the hydrolysis of (*R,S*)-MME is given in Fig. 6. The fungal lipase showed a certain enantiopreference for the hydrolysis of the *R*-isomer and its value was similar to the values obtained for commercial enzymes (Fig. 6). But this derivative was sensitive to changes in the reaction conditions (pH and temperature) and the results are shown in Fig. 7.

It was possible to modify the enantiopreference of the enzyme when the pH of the medium was changed from 7 to 5, where the enantioselectivity of the enzyme totally changed toward *S*-isomer. *M. griseocyanus* lipase immobilized on octyl agarose support highly increased its enantiopreference for the hydrolysis of the *S*-isomer from 9 to 20 when the pH was lowered from 5 to 4. It has been suggested that it is possible to improve the enantioselectivity of the enzyme by changing the reaction conditions.

The stereospecificity of immobilized lipase from *M. griseocyanus* strain was tested with pure species of MPGE and the reaction system is given in Fig. 8.

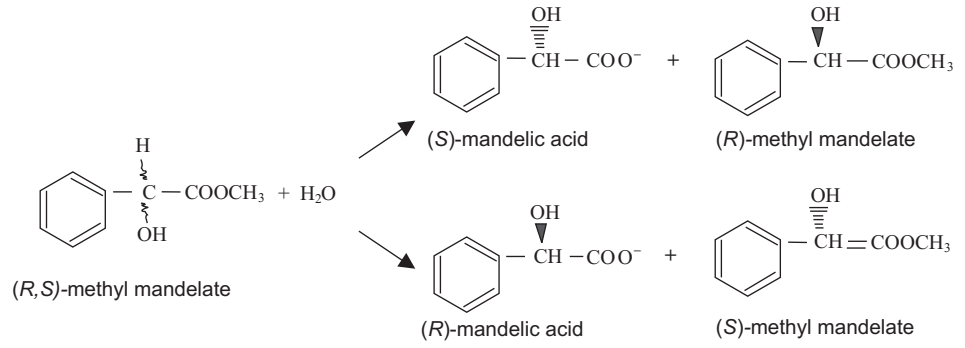

Fig. 5. Enantioselective hydrolysis of (*R,S*)-methyl mandelate ester catalyzed by *M. griseocyanus* lipase

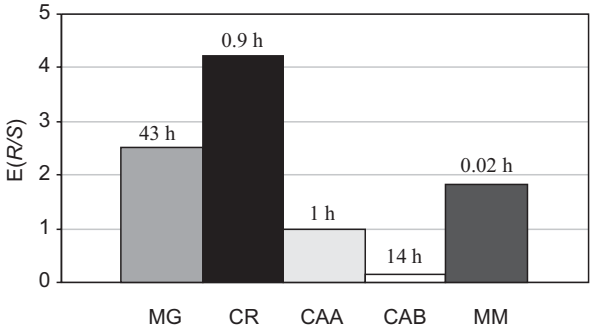

Fig. 6. Enantioselectivity of *M. griseocyanus* lipase and commercial lipases on hydrolysis of (*R,S*)-methyl mandelate ester at 25 °C and pH=7, MG: *M. griseocyanus*, CR: *C. rugosa*, CAA: *C. antarctica* A, CAB: *C. antarctica* B, MM: *M. miehei*

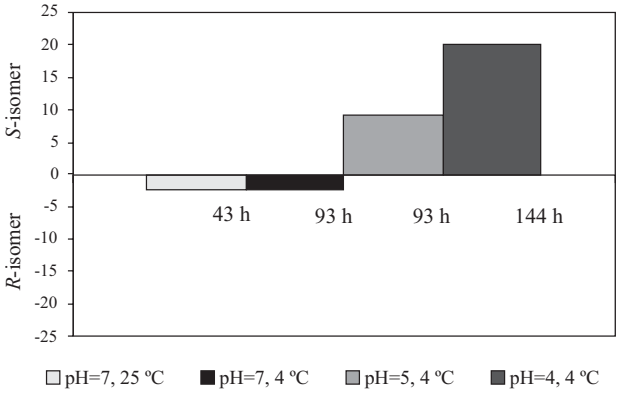

Fig. 7. Effect of the reaction conditions on the selective hydrolysis of (*R,S*)-methyl mandelate ester by *M. griseocyanus* lipase

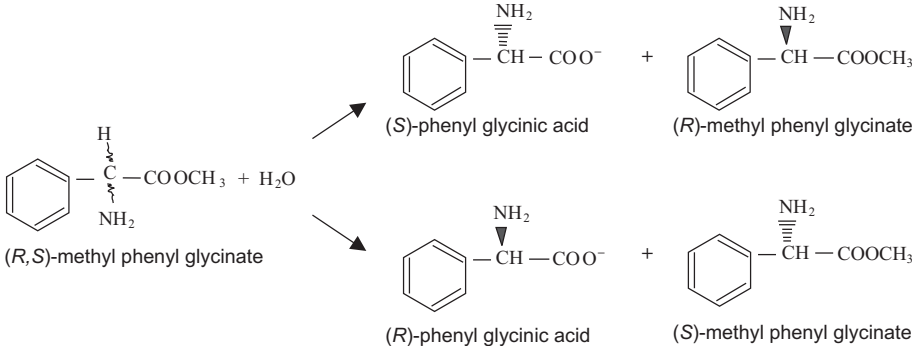

Fig. 8. Selective hydrolysis of pure species of methyl phenyl glycinate ester (MPGE) catalyzed by lipase from *M. griseocyanus*

Results obtained for the stereospecificity of *M. griseocyanus* lipase compared with commercial lipases in the same reaction are given in Fig. 9. The reaction was carried out at 4 °C and pH=5 as demonstrated in previous studies; such conditions improve the selectivity of the enzyme catalyzing asymmetric hydrolysis (*19*).

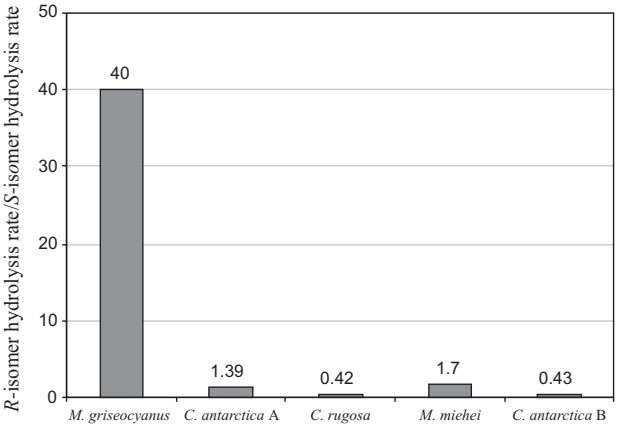

Fig. 9. Stereoespecificity of *M. griseocyanus* lipase and commercial lipases on hydrolysis of the pure species of methyl phenyl glycinate ester at 4 °C and pH=5

The obtained results showed that the hydrolysis rate of the *R*-isomer of MPGE was 40 times higher than the *S*-isomer hydrolysis rate. This value was higher than the values obtained for other immobilized commercial lipases. This is an important result and a starting point for future works, because the *R*-isomer could be employed for ampicillin synthesis.

Conclusions

There has been very little literature documenting lipase expression by *M. griseocyanus* or its characteristics so far. Results of this study demonstrate that *M. griseocyanus* strain 55.1.1 synthesised extracellular lipase in natural way, and the combination of coconut oil at 0.5 % and sucrose at 1.5 % (*m*/*V*) was the most suitable carbon source for the production of the enzyme. *M. griseocyanus* lipase is thermotolerant in nature, and this property could be exploited for application in detergent industries and others. However, more studies are required to improve the stability of the enzyme. The enzyme also exhibited favourable properties in the hydrolysis reactions under standard experimental conditions (pH=7, 25 °C). Furthermore, an additional increase in enantioselectivity of the enzyme was observed by lowering the pH to 4 and temperature to 4 °C. It was also demonstrated that it is possible to modify the selectivity of lipase by changing the reaction conditions and protocols for immobilization.

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