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ИССЛЕДОВАНИЕ НЕКОТОРЫХ КАТАЛИТИЧЕСКИХ СВОЙСТВ ИММОБИЛИЗОВАННОЙ ЛИПАЗЫ ИЗ *MUCOR GRISEOCYANUS*

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Грибы *Mucor griseocyanus*, штамм 55.1.1, использовали для получения липазы с различными субстратами в качестве основных источников углерода и сульфата аммония в качестве источника азота. На четвертый день процесса ферментации было получено около 0,1 МЕ/мл липолитической активности. Оптимальные значения pH для грубого экстракта, содержащего фермент, находились в диапазоне от 4 до 6, оптимальная температура 60°C. Экстракт был стабилен при средней температуре от 20 до 40°C и при pH 6. Исследование каталитических свойств иммобилизованной липазы, таких, как стерео- и энантиоселективность, с использованием метилфенилглюцината и (R,S)-метилманделата показало, что полученный нами фермент по каталитическим характеристикам превышает коммерчески доступные липазы. Липаза из *M. griseocyanus* имела лучшую стереоселективность по отношению к R-изомеру метилфенилглюцината. В случае метилманделата фермент предпочтительно гидролизовал S-изомер эфира, и скорость гидролиза S-изомера была в 20 раз выше, чем R-изомера.

INTRODUCTION

A large number of fat-cleaving enzymes, lipases are produced on an industrial scale for application in food and detergents industries [1]. This is facilitated by the fact that these enzymes are formed extracellularly by both fungi and bacteria. The ready availability has created an enormous spin-off with respect to the enantioselective hydrolysis and formation of carboxyl esters [2]. Bearing in mind that the natural substrates for lipases are glycerides and they possess a chiral alcohol moiety, it is understandable that lipases are particularly useful for the resolution or asymmetrization of esters bearing a chiral alcohol moiety.

Nowadays, under complex conditions very good selectivities have been reported for many biotransformations catalyzed by soluble lipases [3]. However when these biotransformations are carried out by immobilized lipase, the enzyme is working under very different conditions. In immobilized system, the lipases in macro aqueous systems display their closed structure in a partial equilibrium with the open one. Further, immobilization is a suitable approach that allows biocatalyst reuse, makes product recovery easier, able to enhance resistance against inactivation by different denaturants and possible to manipulate several properties of lipases for potential industrial applications [4]. Even the adsorption on hydrophobic supports like octyl agarose allows separating lipases from other enzymes present in the fermentative broth [5].

This work deals about a preliminary study on characteristics of lipase produced by a fungal strain, with special emphasis on the general behaviour and applicability of this enzyme on selective hydrolysis reactions.

MATERIAL AND METHODS

Microorganism and growth medium

The strain *Mucor griseocyanus* 55.1.1 from the microbial culture collection of the Sugar Cane erivatives Institute, of Havana Cuba, was used for lipase production. A mineral medium with the following composition (g/l): NaH_2PO_4 12, KH_2PO_4 2, $MgSO_4.7\ H_2O$ 0.3, $CaCl_2$ 0.25 and 1% of ammonia sulphate as nitrogen source, and olive oil at 2% as carbon source was used. Other substrates were assayed as carbon sources. The initial pH was adjusted to 6. The medium was inoculated with 10^7 conidia transferred from the stock culture to 100 ml erlenmeyer flasks cointaning 20 ml of sterile growth medium. The flasks were incubated at 30°C in a shaker operating at 120 rpm during 8 days.

Enzyme extract was collected on fourth day of fermentation. The fungal biomass was filtered under vacuum and later centrifuged at 12000 rpm during 5 min. The clarified supernatant was used as the source of enzymes.

To study the effect of temperature, the lipase activity in the reaction medium was measured at various temperatures ranging from 20 to 90°C. To determine the opti-

mum pH, the activity was measured at different pH in the range of 4 to 10. To study the thermostability, the enzyme extract was incubated at 20, 30, 40, 50 and 60°C for 24 hours. After incubation, the enzyme was immediately cooled in ice bath for 15 minutes and the residual activity was determined (the initial activity of the enzyme was taken as 100%). To determine the pH stability of the enzyme, the samples of soluble enzyme were incubated in 0.05 M buffer of different pH values from 4 to 10 at 30°C for 24 hours. After that, the residual activity was assayed.

Lipase assay

Lipase activity was assayed by addition of 0.1 ml of lipase solution to 2.5 ml of substrate, *viz.*,0.4 mM *p*-nitrophenyl propionate at pH 7 and 37°C. The increase in the absorbance at 348 nm, due to the release of p-nitrophenol was measured [5]. One unit was defined as the amount of enzyme necessary to hydrolyze 1ґmol of pNPP per minute under the previously described conditions.

Immobilization of lipase

The lipase of *M. griseocyanus* was immobilized on octyl agarose 4BCL by following the procedures described for interfacial adsorption on hydrophobic supports bearing octyl chains [5]. The immobilized enzyme was quantified by determination of total protein by dye binding assay [6].

Study of selective hydrolysis

Evaluations of hydrolytic activities was performed by using 10 ml of 10 mM (R,S)-Methyl mandelate esters in 25 mM sodium phosphate buffer at pH 7.0 and 25°C under mechanic stirring. Same experiment was also carried out under different pH and temperatures. The degree of hydrolysis was analyzed by HPLC with C18 O S column and monitored with an 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 an 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 O-R, and the mobile phase was a mixture of 40% acetonitrile and 60% water at a flow 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. [7]. In addition, the stereoselectivity of the immobilized lipase on 4BCL agarose gel was studied by the hydrolysis reactions of Methyl phenyl glycine ester. In this case, the mobile phase was a mixture of 25% methanol in 50 mM Sodium Phosphate buffer and at a flow rate of 1 ml/min. The enzyme stereospecificity (Q) was determined as the ratio between hydrolysis rates of R and S species of the ester.

RESULTS AND DISCUSSION

Production of the enzyme

Growth kinetics and lipase expression of *M. griseocyanus* on different culture media are presented in Fig. 1.

The growth pattern of the fungal strain differed with substrates. The highest growth, 16 g/l, was reached with coconut oil, followed by sunflower oil 14 g/l, olive oil 10 g/l and glucose 8 g/l. However, when starch and glycerol sources were added to the culture medium, the biomass was 3.7 and 2.1 times less than to the values obtained with coconut oil. But synthesis of the enzyme was observed with all substrates. Lipase activity between 0.1 and 0.04 IU/ml was observed at the second, third and fourth day of growth. The results demonstrated that the production of lipase by this fungal strain was constitutive and as higher growth and activity was observed with coconut oil, it was used as carbon source for further studies. These reported results for *M. griseocyanus* was entirely different to the results observed for *A. niger* and *A. fumigatus* cultures [8].

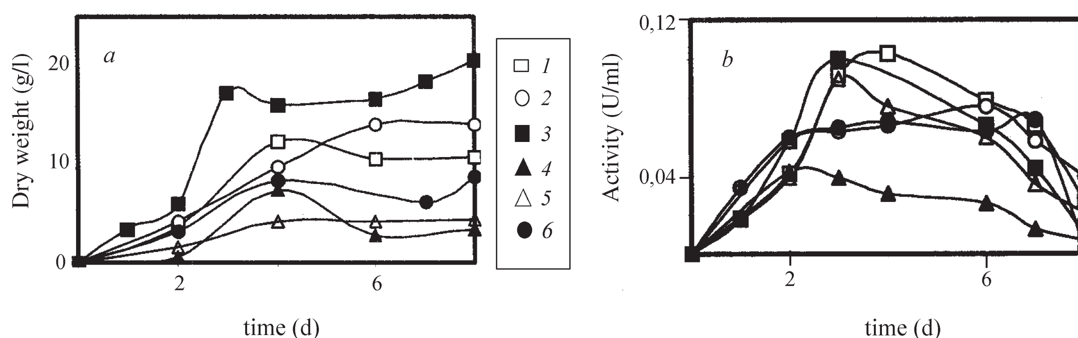

Fig. 1. Microbial growth (*a*) and lipase activity expressed (*b*) by *Mucor griseocyanus* on different carbon sources added at 2 %: Olive oil (*1*), Sunflower oil (*2*), Coconut oil (*3*), Glycerol (*4*), Starch (*5*), Glucose (*6*)

The obtained results were analyzed in terms of energy provided by the substrates and used by the fungal strain for its growth and activity. By applying mass balance to this system and considering that energy supplied by carbon sources have been used only for growth and to express metabolites; it is possible to obtain biomass/substrate yields for any microorganism.

According to values reported by Erickson et al. [9], and considering the maximum observed energetic yield for growth of 0.7, we calculated the theoretical biomass?substrate yield ($Y_{x/s}$) for some of the substrates. The concentration of each substrate in the medium was 20 g/l, we estimated the maximum possible growth by the test fungi with an assumption of maximum yield of biomass/substrate ($Y_{x/s}$) and a 100% of substrate conversion ($AS = S_o$). A comparison between theoretical and actual biomass/substrates yield for some of the substrates tested in this study are given in Table.

In all cases obtained growth was lower than the expected ones. The difference between theoretical and the actual growth was more significant with starch as carbon source, and higher efficiency was with glucose (71,4%). The less growth on starch could be related to the higher synthesis of lipases with this substrate,as the lipase activity levels were similar to the values obtained with olive and coconut oil as substrates, both of which are recognized as good inducers of lipases.In the case of glucose, the energy in the substrate was used mainly for growth than for synthesis of enzymes.

For the analysis on the effect of the nitrogen source we had three following assumptions. a) fungi have about 30% of protein on dry base, b) nitrogen represents 16% of proteins in microorganisms and c) ammonium sulphate contains 21.2% of nitrogen.

We added 10 g of ammonium sulphate to the culture medium, which meant there was 2.12 g of nitrogen for each liter of medium. Assuming all nitrogen is transformed to protein, we could obtain 13.25 g of protein and 44.17 g of biomass. Then the possible theoretical biomass yield with 1% (*w/v*) of this nitrogen source is very far to the obtained ones in the studied cultures. This result can be attributed to two facts, one of them associated to the physiological and genetic characteristics of the fungal strain used in the study and the concentration of the nitrogen employed in the medium, because it did not meet the optimum concentration needed for the growth of the test fungal strain.

Enzyme characterization

The influence of the temperature and pH on enzyme activity of *M. griseocyanus* is presented in Fig. 2.

Optimum temperature for the *M.griseocyanus* lipase activity was 60°C and the activity decreased drastically at temperatures above 60°C and it was almost zero at 70°C.Most lipases were active between 25 to 40°C [10, 11].In case of pH, *M. griseocyanus* lipase was found to be most active in a pH range between 4 and 7. Similar pH range was reported for optimum lipase activity by *Rhizopus rhizopodiformis* [12]. A review of literature indicated that there are no reports so far about the production of lipase by *M. griseocyanus.*

However, this enzyme was very unstable at a wide range of temperature and pH. It retained only about 80% of its initial activity at 20, 30 and 40°C for 5 hours, and later the residual activity was decreased considerably (Fig. 3, *a*).Thermal stability of alipase is obviously related with its structure [13] and also is 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 [14].

The enzyme was very stable at pH 6 and it retained almost 80% of its initial activity for 24 hours. Nevertheless it was rapidly inactivated when incubated at pH 9 and 10, and lost about 30 and 40% of its initial activity in half an hour (Fig. 3, *b*). Similar behaviour was reported for afungal lipase expressed by a *Penicillium* strain [15].

Comparison between theoretical and real biomass/substrates yields

Substrate	$Y_{x/s\ (t)}$	Growth $_{(t)}$ g/l	Growth $_{(r)}$ 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.

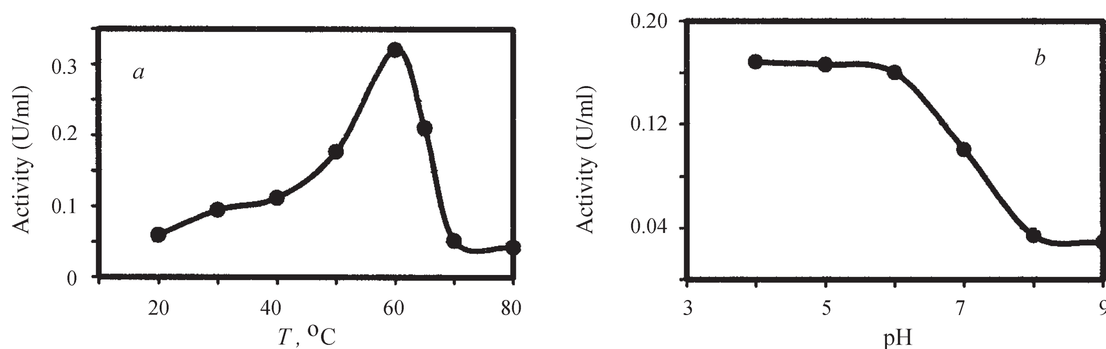

Fig. 2. Effect of the temperature at pH 7.0 (*a*) and the pH at 30°C (*b*) on the lipase activity

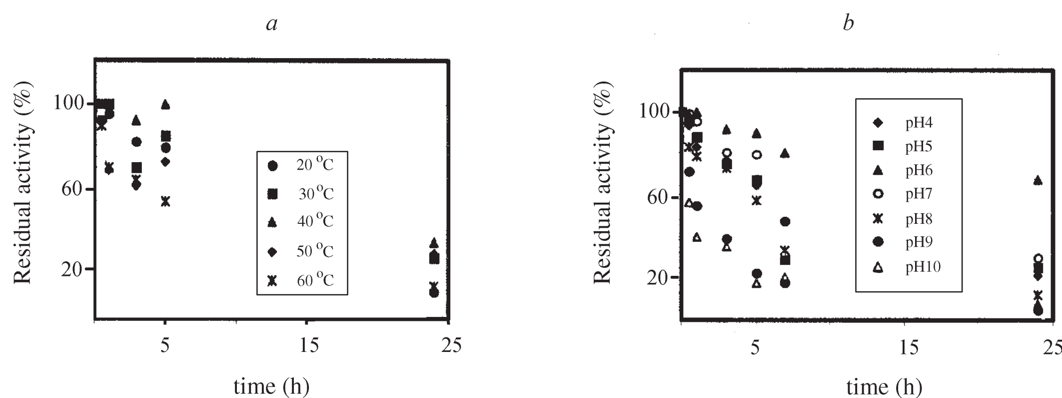

Fig. 3. Stability profile of *M.griseocyanus* lipase in relation to the temperature, without addition of buffer at pH 7 (*a*) and in relation to pH with addition of buffer (*b*) at 30°C

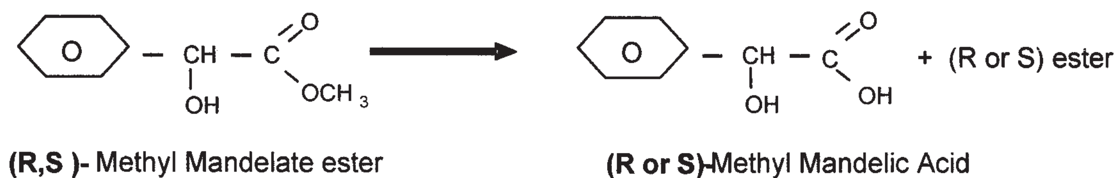

Fig. 4. Enantioselective hydrolysis of (R,S)-methyl mandelate ester catalyzed by *M. griseocyanus* lipase

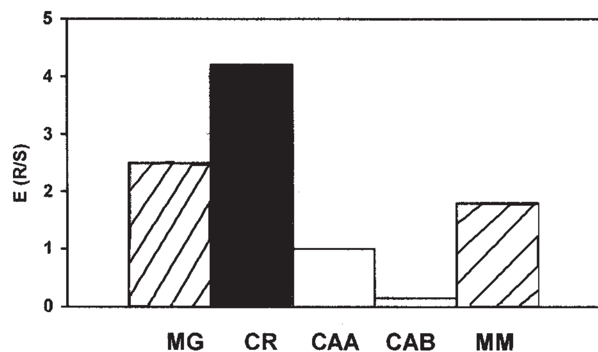

Fig. 5. Enantioselectivity of *M. griseocyanus* lipase and commercial lipases on hydrolysis of (R,S)-Methyl mandelate ester at 25°C and at pH 7, MG: *M. griseocyanus,* CR: *C. rugosa,* CAA: *C. antarctica* A, CAB, *C. antarctica* B, MM, *M. miehei*

Immobilization of lipase

A moderate amount of lipases (2 mg of protein/mL of activated supports) was applied to the activated supports.Around 95% of the applied lipase was immobilized on the support. Guisan et al., 2001 [16] obtained similar results for the immobilization of commercial lipases and showed that it was possible to purify the enzyme too. Although we did not do electrophoretic analysis of the immobilized lipase of *M. griseocyanus*, we demonstrated in our previous work that it is possible to selectively separate immobilized lipase from other enzymes in fermentation broth [5].

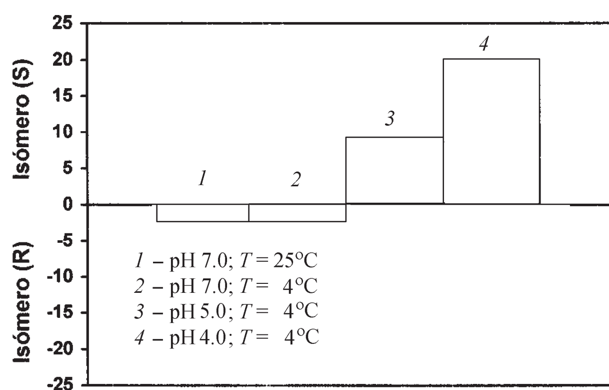

Fig. 6. Effect of the reaction conditions on the selective hydrolysis of (R,S)-Methyl mandelate ester by *M. griseocyanus* lipase

Selective hydrolysis reactions

Optically pure isomers of R and S mandelic acid and their esters are very useful in organic synthesis of the antibiotic cefamandole [17] and optically pure acids are used in the resolution of racemates [18]. 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, in certain conditions when lipase exhibits a very high enantioselectivity (E200) both optically pure ester and pure acid can be obtained. The schematic diagram of the hydrolysis reaction of methyl mandelate ester is given in Fig. 4. The enantioselectivity of different immobilized lipases towards the hydrolysis of (R,S)-Methyl mandelate esters is given in Fig. 5.

The fungal lipase showed acertain enantiopreference for the hydrolysis of the R-isomer and its value was superior to some values obtained for commercial enzymes. But this derivative was sensitive to the changes in reaction conditions (pH and temperature) and the results are shown in Fig 6.

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 slightly increased its enantiopreference for the hydrolysis of the S-isomer when the pH was lowered from 5 to 4 (from 9 to 20). It suggested that it is possible to improve the enantioselectivity of the enzyme by changing the reaction conditions [16].

The stereoespecificity of immobilized lipase from *M. griseocyanus* strain was tested with pure species of Methyl phenyl glycine ester at 4єC and at pH 5 and the reaction system is given in Fig. 7.

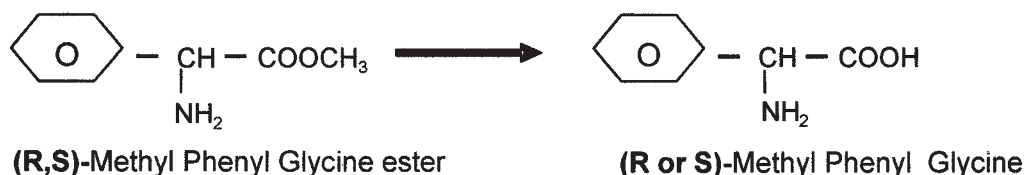

Fig. 7. Selective hydrolysis of pure species of methyl phenyl glycinate ester catalyzed by lipase from *M. griseocyanus*

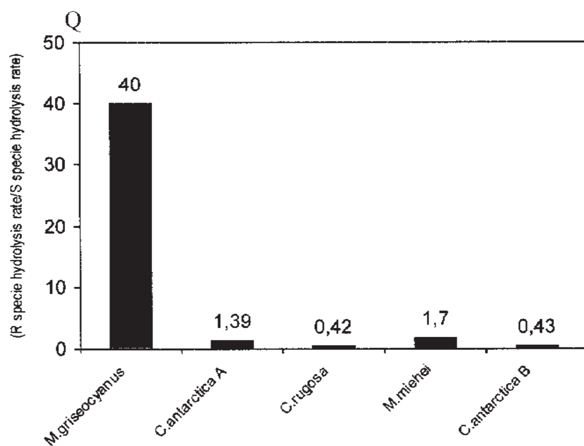

Fig. 8. Stereospecificity of *M.griseocyanus* lipase and commercial lipases on hydrolysis of the pure species of Methyl phenyl glycinate ester at 4°C and at pH 5

Results obtained for the stereospecificity of *M. griseocyanus* lipase showed unique characters when compared to the commercial lipases in the hydrolysis of pure species of analyzed ester (Fig. 8).

Obtained results showed that the hydrolysis rate of R specie of Methyl phenyl glycinate was 40 times higher than S specie hydrolysis rate. This value was higher than the values obtained for other immobilized lipases of commercial origin. This is an important result and a starting point for future works, because the R species could be employed to for ampicillin synthesis.

CONCLUSIONS

Results of this study demonstrated that *Mucor griseocyanus* strain 55.1.1 synthesised lipase extracellular constitutively, and olive and coconut oil at 2% (*w/v*) were

the suitable carbon sources for the production of the enzyme.Both the expression of lipase by this fungal strain and its characteristics have not been previously reported in the literature.

Lipase *M. griseocyanus* was thermophilic in nature, and this property could be exploited for application by detergent industries. However, few studies are required to improve the stability of the enzyme. The enzyme also exhibited unique characteristics on the hydrolysis reactions under standard experimental conditions (pH 7; 25°C). Further, 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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STUDY OF SOME CATALYTIC PROPERTIES OF IMMOBILIZED LIPASE FROM *MUCOR GRISEOCYANUS*

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In this work a fungi, *Mucor griseocyanus* strain 55.1.1 was used for the production of lipase using different substrates as main carbon sources and ammonium sulphate as nitrogen source. About 0,1 IU/mL of lipolitic activity was obtained on fourth day of fermentation process. The optimum pH for the crude enzyme extract was in the range of 4 to 6 and the optimum and temperature was 60°C. The enzyme extract was stable for 5 hours at moderate temperatures between 20 and 40°C and at pH 6. Studies on the catalytic properties (stereoselectivity and enantioselectivity) of the immobilized lipase using Methyl phenyl glycinate and (R,S)-Methyl mandelate esters showed the excellent properties of the enzyme compared to commercial lipases tested. *M. griseocyanus* lipase exhibited a greater stereoselectivity towards the R species of methyl phenyl glycinate ester. However with methyl mandelate ester, the enzyme showed a certain preference toward the S-isomer and it was hydrolysed 20 times faster than the R-isomer.