Journal of the Serbian Chemical Society
JSCS-info@shd.org.rs • www.shd.org.rs/JSCS
J. Serb. Chem. Soc. 76 (8) 1081–1092 (2011)
JSCS–4186
UDC *Pseudozyma aphidis*:577.15+544.35–039.7:66.022.362
Original scientific paper

Production of lipase from *Pseudozyma aphidis* and determination of the activity and stability of the crude lipase preparation in polar organic solvents

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(Received 28 April, revised 7 June 2011)

Abstract: The production of lipase from *Pseudozyma aphidis* (DSM 70725) was determined in six different media. The highest lipase production was observed in a medium with glucose as the sole carbon source, and yeast extract and sodium nitrate as the nitrogen sources. The time course studies of growth and lipase production in the optimal medium revealed that the highest lipase production was achieved at the end of the log phase of growth, reaching the value of 35.0 U cm^{-3} in the fifth day of cultivation. The effects of various polar, water-miscible, organic solvents on the activity and stability of the crude lipase produced by *P. aphidis* were evaluated. The hydrolytic activity of the crude lipase towards *p*-nitrophenyl palmitate (*p*-NPP) in aqueous media and in organic solvents was determined, using the same spectrophotometric assay in both the aqueous and organic media. The crude lipase preparation exhibited activity towards *p*-NPP only in acetone and acetonitrile, while the lipase was stable only in acetone, with 23 % residual activity after 24 h of incubation. These results suggested that lipase from *P. aphidis* can be used as a biocatalyst for potential applications in such organic solvents.

Keywords: lipase; activity; stability; organic solvents.

INTRODUCTION

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) are serine hydrolases that catalyze the hydrolysis of triacylglycerols to glycerol and free fatty acids. They also catalyze a variety of synthesis reactions under reduced aqueous conditions (*e.g.*, esterification, transesterification, alcoholysis, acidolysis, aminolysis,

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doi: 10.2298/JSC110428096D

acylation and resolution of racemic mixtures).[1,2] Thus, lipases have become important for biotechnological and industrial applications.[3]

Biocatalysis in organic solvents offers several advantages over biocatalysis in aqueous media, due to the increased solubility of hydrophobic compounds, the ability to perform new reactions which are kinetically or thermodynamically restricted in water, suppression of undesirable side reactions, control or modification of enzyme selectivity, the possibility of recovery of some products by employment of low-boiling-point organic solvents and an increased enzyme thermostability in organic solvents.[2,4] Although enzymes exhibit many advantages, they do not always meet the desired levels of activity, productivity and, most importantly, stability in organic solvents.[5] Generally, hydrophobic solvents lead to higher enzymatic activity and stability than hydrophilic solvents, which strip water required for the enzymatic function, and lower the catalytic activity.[4,6] Thus, lipases that are active and stable in polar organic solvents would enable new applications in biotechnological processes involving polar substrates. For these reasons, it is necessary to find lipases that are active and stable in polar organic solvents.[2]

A considerable number of bacterial and fungal lipases have been commercially produced, the latter being preferable because fungi generally produce extracellular enzymes, which facilitate recovery of the enzyme from the fermentation broth.[7] *Pseudozyma* (*Candida*) *antarctica* is one of the most important sources of lipases for industrial applications.[8,9] Lipases from *P. antarctica* have many excellent characteristics and they are widely used for preparative purposes in organic synthesis in many industrial applications and scientific research projects.[10–13] However, the aim of research dealing with enzymes in non-aqueous media is to determine the optimal conditions for a specific application.[14] Very few studies have been devoted to direct comparisons of lipase activity in aqueous and organic media.[15–17] This comparison is made difficult by the fact that, generally, different reactions are used in both media for the activity assay.[14] A simple colorimetric assay based on the hydrolysis of *p*-nitrophenyl fatty acids esters was introduced by Pencreac'h and Berrati in order to compare the hydrolytic activity of thirty two commercial lipase preparations in water and heptane.[14,17] The main advantage of this assay is the use of the same hydrolytic reaction in both aqueous and organic media, which facilitates the activity comparison.[17] Hitherto, the hydrolytic activity of commercial lipase preparations was determined only in a non-polar organic solvent (heptane).[14] However, commercial lipase preparations usually contain non-protein additives that can greatly increase the enzyme activity in organic media and cause activity differences among different preparations of the same enzyme.[14]

In the present study, the objective was to produce a crude lipase synthesized by *P. aphidis* and to examine the hydrolytic activity and stability of the lipase in

polar, water-miscible organic solvents from five organic classes (ketone, nitrile, ether, alcohol, sulphoxide) using the same spectrophotometric method in aqueous and organic media. *P. aphidis* is closely related to *P.* (*Candida*) *antarctica*, but is considered a separate species.[18] To the best of our knowledge, this is the first time that the hydrolytic activity and stability of lipase produced by *P. aphidis* has been determined in organic solvents.

EXPERIMENTAL

All employed chemicals were of the highest available purity, purchased from Sigma, Merck, Bio-Rad or Aldrich.

Microorganism

Pseudozyma aphidis DSM 70725 was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ). Stock cultures were cultivated for 2 days at 25 °C on agar medium containing 3 g dm^{-3} yeast extract, 3 g dm^{-3} malt extract, 5 g dm^{-3} peptone from soybeans and 10 g dm^{-3} glucose. The culture was stored at 4 °C and renewed every 4 weeks.

Media preparation and culture conditions

Seed cultures were prepared by inoculating cells grown on slants into a growth medium (4 g dm^{-3} glucose, 2 g dm^{-3} sodium nitrate ($NaNO_3$), 0.2 g dm^{-3} magnesium sulphate ($MgSO_4 \cdot 7H_2O$), 0.2 g dm^{-3} potassium dihydrogen phosphate (KH_2PO_4) and 1 g dm^{-3} yeast extract) at 25 °C on a rotary shaker (300 rpm) for 2 days.

Seed cultures (1 cm^3) were transferred into six 250 cm^3 Erlenmeyer flasks containing 100 cm^3 of six different cultivation media containing various carbon (glucose, glycerol, soybean oil or rapeseed oil) and nitrogen sources (yeast extract, peptone or sodium nitrate ($NaNO_3$) and ammonium chloride (NH_4Cl)) and incubated at 25 °C on a rotary shaker (300 rpm) for 6 days.[19,20] Detailed descriptions of the compositions of the cultivation media composition are given in Table I. After six days of cultivation, the lipase activity in the cultivation media was determined.

TABLE I. Media composition (g dm^{-3}) for *P. aphidis* cultivation

Component	Medium					
	M1	M2	M3	M4	M5	M6
Glucose	10	10	–	120	–	40
Glycerol	–	–	6	–	–	
Rapeseed oil	5	–	5	–	–	
Soybean oil	–	5	–	–	40	
Yeast extract	10	10	10	1	1	1
Peptone	10	10	10	–	–	
NH_4Cl	5	5	5	–	–	
$MgSO_4 \cdot 7H_2O$	–	–	–	0.3	0.3	0.3
$NaNO_3$	–	–	–	–	–	3
KH_2PO_4	–	–	–	0.3	0.3	0.3

Time course of P. aphidis *cultivation in the optimal medium for lipase production*

After the optimal medium for lipase production was determined, the seed culture (1 cm^3) was transferred into three 250-cm^3 Erlenmeyer flasks containing 100 cm^3 of optimal (M6) me-

dium (triplicate), and incubated at 25 °C on a rotary shaker (300 rpm) for 7 days. Aliquots were withdrawn each day and the following parameters were determined: pH; absorbance at 610 nm; wet biomass; protein concentration, C_p (mg cm^{-3}); lipase concentration, C_e (U cm^{-3}), and specific activity, S_p (U mg^{-1} of protein). The mean value of each parameter was calculated from the data obtained in the triplicate trials.

Determination of growth parameters

The absorbance at 610 nm was measured. Additionally for biomass determination, 1 cm^3 of fermentation broth was centrifuged at 14,000×*g* for 15 min, the supernatant removed and the remaining biomass was dried for 15 min at room temperature and weighed.

Protein determination

The amount of protein was estimated by the Bradford dye-binding method, using bovine serum albumin (BSA) as the standard.[21]

Preparation of crude P. aphidis *lipase*

In order to produce the crude lipase preparation for the determination of the activity and stability of lipase in organic solvents, 1 cm^3 of the seed culture (prepared as described above) was transferred into 100 cm^3 of optimal (M6) media, and cultivated at 25 °C on a rotary shaker (300 rpm). After five days of cultivation, when the highest lipase production was reached, the cells were removed by centrifuging (3000 rpm, 30 min) and the supernatant was lyophilized and used as the crude lipase preparation. The specific activity of the crude lipase preparation was 705.88 U mg^{-1} of proteins.

Lipase activity in aqueous media

The lipase activity in aqueous media was measured spectrophotometrically using an assay based on the hydrolysis of *p*-nitrophenyl palmitate (*p*-NPP), as described previously, with modifications.[22] The substrate was prepared as follows: 0.1586 g *p*-NPP, 0.0017 g sodium dodecyl sulphate (SDS) and 0.1 g Triton X-100 were dissolved in 10 cm^3 of 0.1 M Tris-HCl buffer pH 8.2 (the final concentration of *p*-NPP was 0.0042 M) mixed vigorously and heated at 80 °C for 15 min.[22] The enzyme and substrate were mixed and the increase of absorbance at 410 nm was monitored every 30 s for 3 min. The reaction rate was calculated from the slope of the curve absorbance *versus* time using a molar extinction coefficient of 13,000 dm^3 mol^{-1} cm^{-1} for *p*-nitrophenol (*p*-NP). This value was determined from the absorbance of standard solutions of *p*-NP in the reaction medium. One enzyme unit was the amount of enzyme liberating one μmol of *p*-NP per minute under the above conditions.

Lipase activity in organic solvents

The hydrolytic activities of the lipase preparation in organic media were determined according to Pencreac'h and Baratti with modifications.[17] A crude lipase preparation dissolved in water was transferred to Eppendorf tubes containing 0.0042 M *p*-NPP dissolved in different organic solvents (acetone, acetonitrile, dimethyl sulphoxide, *t*-butanol or 1,2-dimethoxyethane), so that the final concentrations of the organic solvents in the assay were 95 %. The mixture was incubated for 10 s at 25 °C under reciprocal agitation at 1000 strokes per min. Mixing was stopped and after 30 s the enzyme precipitated, since its is insoluble in organic solvents and the clear supernatant was transferred to a cuvette containing 0.1 M Tris-HCl buffer pH 8.2 and the absorbance was read at 410 nm. The same was repeated in the next 30 min at regular time intervals. The reaction rate was calculated from the slope of the curve absorbance *versus* time using the molar extinction coefficients of *p*-NP in different organic solvents. The values of extinction coefficients were determined from the absorbance of standard solutions of *p*-NP

in the appropriate organic solvent, treated in the same way as in the assay. The extinction coefficients of *p*-NP were determined in organic solvents in which lipase activity was detected, *i.e.*, in acetone and acetonitrile, and they were 880 and 810 $dm^3\ mol^{-1}cm^{-1}$, respectively, under the assay conditions. One enzyme unit was the amount of enzyme liberating one μmol of *p*-NP per min under the above conditions. In order to compare the activity in water and in organic solvents, the $R_{O/A}$ value was introduced. $R_{O/A}$ is the ratio between the activity in the organic solvent and the activity in the aqueous medium.[14]

Lipase stability in organic solvents

0.025 cm^3 of enzyme solution (*P. aphidis* lipase lyophilizate dissolved in water in an appropriate concentration) and 0.475 cm^3 of an organic solvent (the same solvents used for the examination of the lipase activity) were added into Eppendorf tubes, making the final concentration of organic solvent 95 %. The solutions were incubated at 25 °C for 24 h. In order to examine the denaturation kinetics of lipase, the remaining activity in the different organic solvents was determined at different time intervals. The remaining activity in the organic solvents was calculated from the absorbance change at 410 nm, considering the enzyme activity at zero time as 100 %.

RESULTS AND DISCUSSION

Production of P. aphidis *lipase and the cultivation time course*

The production of lipase from *P. aphidis* was monitored in six different media.[19,20] After six days of cultivation, the lipolytic activity of *P. aphidis* in the media supplemented with a lipid substrate (rapeseed oil or soybean oil) was between 1.5 and 2.5 U cm^{-3} (Fig. 1), which is in accordance with the results obtained by other authors.[23] However, a higher production of lipase was detected in the media supplemented with rapeseed oil (M1 and M3 in Fig. 1) than in the media supplemented with soybean oil (M2 and M5 in Fig. 1). This was expected, since it was reported that rapeseed oil induces lipase production by *P. aphidis.*[20] Generally, a slightly higher production of lipase was observed in nutrient-rich media (M1, M2 and M3 in Fig. 1) with both types of nitrogen sources (organic and mineral) and two carbon sources (carbohydrate/glycerol and oil) as also reported by other authors.[20] The lowest values of lipolytic activity were obtained in the M4 medium, in which glucose was the sole carbon source and yeast extract was the only nitrogen source. This is in accordance with a previous report, where the lowest values of lipolytic activity were obtained in media supplemented with carbohydrates as the sole carbon sources.[23] The highest value of lipase production was detected in the M6 medium (Fig. 1), which was similar in composition to the medium used for seed preparation. It is interesting that the only difference between the medium with the highest (M6) and lowest (M4) lipase production was the presence of an inorganic nitrogen source ($NaNO_3$) in the M6 medium. This interesting observation indicates that selection of an inorganic nitrogen source plays an important role in lipase production, although in some cases, it could reduce lipase production.[24] Factors controlling lipase synthesis and transport have been investigated in only a few cases, *e.g.*, the production of lipase by

Pseudomonas sp. was shown to be strongly induced by triacylglycerols and detergents and not repressed by glucose or glycerol.[25,26] However, lipase production is strongly strain-dependent, for example Dalmau *et al*. indicated the request for lipid substrates and the inhibition effect of glucose in the production of lipase by *Candida rugosa.*[23,27,28]

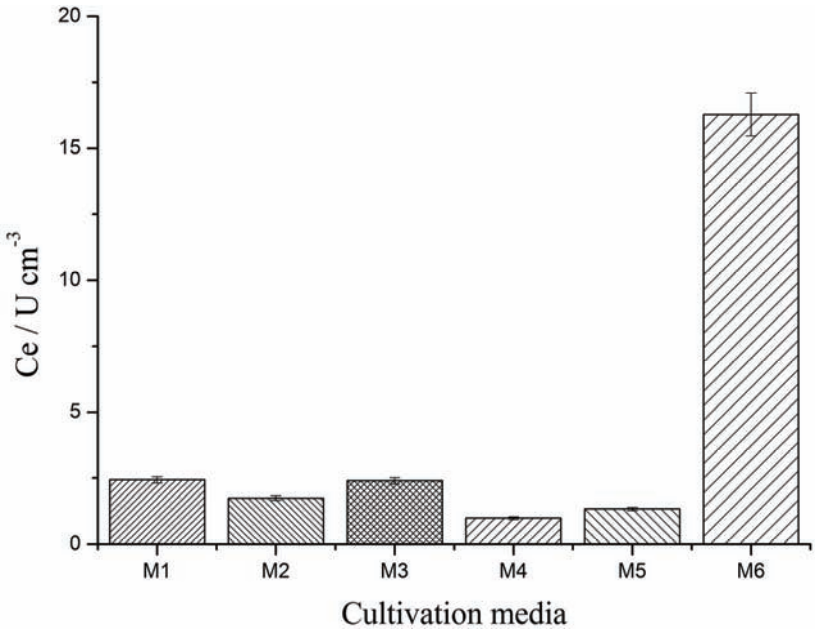

Fig. 1. Lipolytic activity of *P. aphidis* in different cultivation media after 6 days of cultivation.

Since lipase production was the highest in the M6 medium (Fig. 1), time course studies were conducted in order to determine the growth and lipase production characteristics of *P. aphidis* in the optimal medium. Different parameters were determined, including growth (biomass and absorbance at 610 nm), pH, C_p, C_e and S_p at regular intervals (24 h) during seven days. The results obtained are shown in Fig. 2. The highest lipase production (35.0 U cm^{-3}) in the M6 medium was achieved after five days of cultivation. This value was over four times higher than in previous reports dealing with *P. aphidis.*[23,20] From the values of the biomass and absorbance at 610 nm, it is clear that after five days of cultivation *P. aphidis* reached a stationary phase of growth. Thus, the maximum of lipase production was reached at the end of the log-phase. As *P. aphidis* entered the stationary phase, the lipase production decreased and the protein concentration increased, probably as a result of cell death.[25] From Fig. 2, it is clear that the specific activity was the highest at the end of the log-phase and significantly decreased in the stationary phase of growth, due to a decrease of

lipase activity and an increase of the protein concentration. Although it was reported that lipase from *C. antarctica* showed the highest activity at pH 8.0 and the enzyme was stable in the pH range of 7–9, the pH values during the cultivation of *P. aphidis* ranged from 5–6 (Fig. 2).[23]

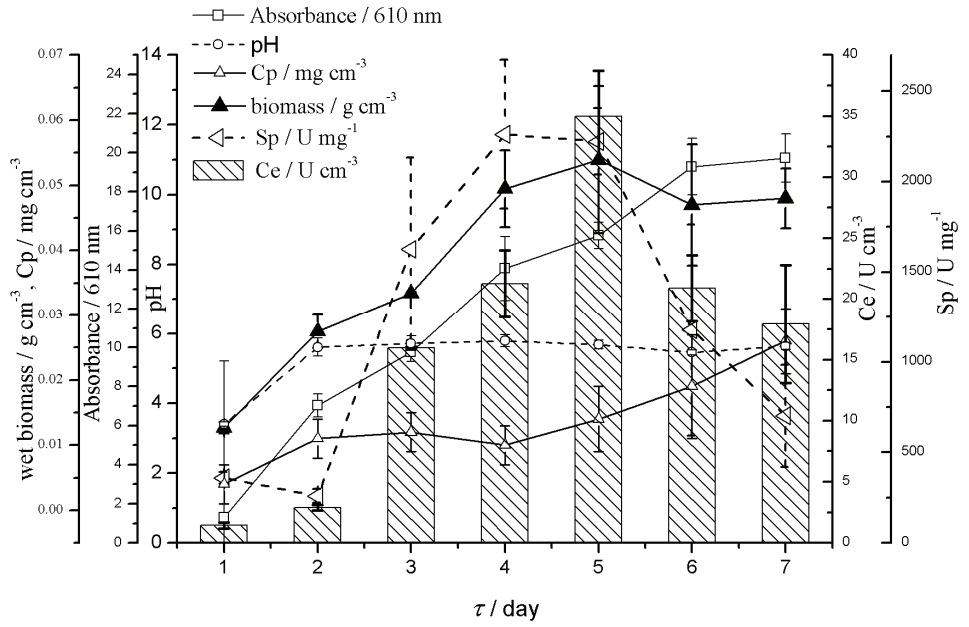

Fig. 2. Time course of the growth and lipase production of *P. aphidis* in the optimal (M6) medium.

Lipase activity in aqueous medium and in organic solvents towards p-*NPP hydrolysis*

Pencreac'h and Baratti proposed a method to compare the activity of lipases towards *p*-NPP in aqueous and organic medium.[14] Since the same reaction was used in both aqueous and organic media, the authors introduced the activity ratio $R_{O/A}$ to characterize the activity in organic media. Using this method, Pencreac'h and Baratti compared the hydrolytic activity of 32 commercial lipase preparations towards *p*-NPP in aqueous media and in *n*-heptane.[14] Since polar organic solvents have technological advantages, such as low toxicity, low boiling points, low costs and offer the possibility of using polar substrates for novel reactions, the method proposed by Pencreac'h and Baratti was modified in the present and used to compare the activity of crude *P. aphidis* lipase in aqueous media and in various, polar, water-miscible organic solvents (Table II).[2] The activity of *P. aphidis* lipase towards *p*-NPP in an aqueous medium was considered as 100 %. Among the organic solvents, lipase activity towards *p*-NPP was only observed in

acetone and acetonitrile (Table II), giving values of $R_{O/A}$ of 0.006 and 0.002, respectively. In all other tested solvents, no activity was detected. It is clear that lipase activity was detected in solvents with similar log *P* values. However, the activity of lipase in acetone was three times higher than in acetonitrile (Table II), indicating that a specific functional group also has an effect on the enzyme activity, as was also observed in the case of *C. antarctica* lipase.[29] From Table II, it is obvious that the $R_{O/A}$ values for acetone and acetonitrile were smaller than one. This was not surprising since reported activities in organic media are usually lower than those observed in aqueous media.[7,14] There are several possible explanations for the low activity in organic solvents. First, the amount of available water, necessary for the hydrolysis reaction, could be too low to allow good reaction rates. Second, the structural flexibility of the enzyme molecules is lower in an organic solvent than in water, which may reduce its activity. Third, the heterogeneous catalysis, which is a consequence of enzyme insolubility in organic solvents, may lead to reduced activity due to limited substrates and/or product diffusion.[14]

TABLE II. Activity of crude lipase from *P. aphidis* in 95 % organic solvents

Solvent	Log *P* (o/w)	Activity, %	$R_{O/A}$
Water	–	100	1
Acetone	–0.24	0.62	0.006
Acetonitrile	–0.15	0.21	0.002
Dimethyl sulphoxide	–1.22	0	0
t-Butanol	0.35	0	0
1,2-Dimethoxyethane	0.09	0	0

Lipase stability in organic solvents

The stability in organic solvents is an important characteristic of lipases. It can determine whether the enzyme can be used to catalyze synthetic reactions and also to predict which solvent would be better for performing the reaction.[29] Lipases are diverse in their sensitivity to solvents, but there is a general agreement that polar water-miscible solvents ($-2.5 < \log P < 0$) are more destabilizing than water immiscible solvents ($2 < \log P < 4$).[30] According to Klibanov, hydrophilic solvents exhibit a higher affinity to water and hence it is more likely that they will strip essential water from enzyme molecules, than hydrophobic solvents.[31] Although in most cases, lipases have good stability in water immiscible organic solvents and are highly denatured in hydrophilic solvents, it was reported that *A. niger* lipase exhibited better activity in acetone than in hexane and heptane, indicating that the effect of water immiscible organic solvents on enzyme stability differs from lipase to lipase.[7,30,32,33] However, in general, water immiscible organic solvents are not appropriate for reactions with polar substrates or in cases where the two substrates greatly differ in terms of polarity.[34]

Thus, lipases that are active and stable in polar solvents would open new opportunities in biocatalysis with polar substrates.[2]

Therefore, the stability of the crude lipase preparation produced by *P. aphidis* in five water-miscible, 95 % organic solvents, from five organic classes was studied. In zero time of incubation in 95 % organic solvents, the enzyme was stable in all organic solvents except in dimethyl sulphoxide (DMSO), indicating that enzyme deactivation in DMSO was immediate. Low enzyme stability and a highly destabilizing effect of DMSO were also reported by other authors.[29] After 1 h of incubation, the enzyme was stable only in acetone, with a residual activity of 82.2 %. In all other solvents, the stability was reduced drastically after 1 h. The kinetics of denaturation of the lipase in acetone is given in Fig. 3. Although, the enzyme had lost almost 50 % of its activity after 3 h, it remained active even after 24 h (Fig. 3).

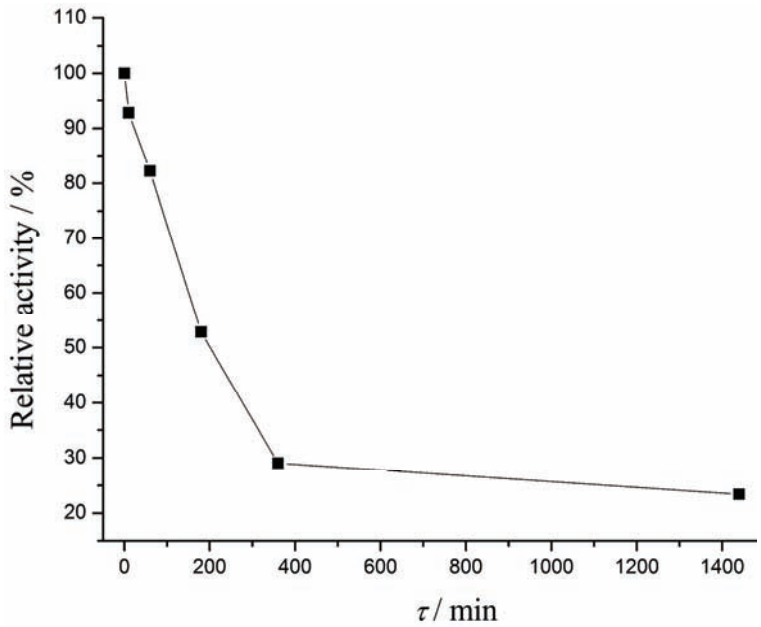

Fig. 3. Denaturation kinetics of *P. aphidis* lipase in acetone.

The low stability of the crude *P. aphidis* lipase in water-miscible organic solvents was the consequence of enzyme denaturation, due to stripping of water from the enzyme surface, as described above. The high relative activity of *P. aphidis* lipase (82.2 %) in acetone after 1 h of incubation corresponds to the results obtained by other authors.[2] Only a few reports show a high stability of lipases in acetone after a prolonged period. For example, the lipase produced by *Pseudomonas* sp. had relative activity values from 100 to 110 % after 15 h at

room temperature in acetone, and the lipase from *Mucor javanicus* exhibited high stability and an increased activity after 2 h of incubation at 25 °C in acetone.[35,36]

CONCLUSIONS

The production of lipase from *P. aphidis* was the highest in the medium with glucose as the carbon source, and yeast extract and $NaNO_3$ as the nitrogen sources. It was shown that the nitrogen source, especially an inorganic nitrogen source, plays an important role in lipase production. The highest lipase production (35.0 U cm^{-3}) in the optimal medium was achieved at the end of the log phase of growth during the fifth day of cultivation. The crude lipase preparation exhibited activity towards *p*-NPP in acetone and acetonitrile. The activity in acetone was three times higher. The results obtained indicate that besides the log *P* values, specific functional groups of the solvent affect the enzyme activity. Of the solvents tested, the lipase was stable only in acetone, even after 24 h. The stability and activity of the crude *P. aphidis* lipase preparation in acetone justifies the search for potential applications of the enzyme in biocatalysis in such a medium.

Acknowledgements. This work was supported by the Ministry of Science and Technological Development of the Republic of Serbia (projects: 172049 and 046010) and FP7 RegPot FCUB ERA GA No 256716.

ИЗВОД

ПРОИЗВОДЊА ЛИПАЗЕ ИЗ *Pseudozyma aphidis* И УТВРЂИВАЊЕ АКТИВНОСТИ И СТАБИЛНОСТИ ЛИПАЗЕ У ПОЛАРНИМ ОРГАНСКИМ РАСТВАРАЧИМА

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Производња липазе из *Pseudozyma aphidis* утврђена је у шест различитих медијума. Највиша производња уочена је у медијуму где је глукоза била извор угљеника, а екстракт квасца и натријум-нитрат извори азота. Праћењем динамике раста и производње липазе у оптималном медијуму, уочено је да се највиша производња липазе достиже пред крај логаритамске фазе раста, и достиже вредност од 35 U cm^{-3} у петом дану култивације, што је четри пута већа производња од оне до сада пријављене у литератури. Утврђен је ефекат различитих поларних органских растварача, мешљивих са водом, на активност и стабилност липазе из *P. aphidis*. Хидролитичка активност липазе према пара-нитрофенил-палмитату (*p*-NPP-у) у воденој средини и органским растварачима утврђена је употребом истог спектрофотометријског теста. Показано је да липаза има активност према *p*-NPP-у само у ацетону и ацетонитрилу, док је ензим стабилан једино у ацетону и задржава 23 % активности након 24 часа инкубације. Добијени резултати указују да липаза из *P. aphidis* може бити коришћена као биокатализатор за потенцијалне примене у ацетону као медијуму.

(Примљено 28. априла, ревидирано 7. јуна 2011)

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