J. Serb. Chem. Soc. 72 (8–9) 757–765 (2007) UDC **Candida utilis*+663.15:547.963.2:547.392.4
JSCS–3609 *Original scientific paper*

Effect of fermentation conditions on lipase production by *Candida utilis*

SANJA Ž. GRBAVČIĆ, SUZANA I. DIMITRIJEVIĆ–BRANKOVIĆ[#], DEJAN I. BEZBRADICA[#], SLAVICA S. ŠILER–MARINKOVIĆ[#] and ZORICA D. KNEŽEVIĆ*[#]

Faculty of Technology and Metallurgy, Department of Biotechnology and Biochemical Engineering, Karnegijeva 4, 11000 Belgrade, Serbia

(Received 5 October 2006, revised 6 March 2007)

Abstract: A wild yeast strain isolated from spoiled soybean oil and identified as *Candida utilis* initially presented rather low lipase activity (approximately 4 IU dm^{-3}) in submerged culture in a universal yeast medium containing 2 % malt extract. Studies were undertaken to improve the lipase production. The best yields of lipase were obtained with a medium supplemented with caprylic and oleic acids as inducers, but higher concentrations of the former (> 0.5 %) had a negative effect on the lipase production and cell growth. The type of nitrogen source seemed also to be very important. The highest lipolytic activity of 284 IU dm^{-3} was achieved after 5 days of fermentation in a medium containing oleic acid and hydrolyzed casein as carbon and nitrogen sources, respectively, and supplemented with Tween 80®. It was shown that optimization of the fermentation conditions can lead to a significant improvement in the lipase production (more than 70-fold higher compared to the initial value obtained in the non-optimized medium).

Keywords: *Candida utilis*, lipase production, media optimization, hydrolyzed casein, oleic acid.

INTRODUCTION

Lipases (triacylglycerol acylhydrolases EC 3.1.1.3) are a class of hydrolases which catalyze the hydrolysis of triglycerides to glycerol and free fatty acids on an oil–water interface.[1,2] In addition, lipases catalyze the hydrolysis and transesterification of other esters,[3] the syntheses of esters and exhibit enantioselective properties.[4] Their ability to perform very specific chemical transformations (biotransformations) has made them increasingly popular in industries where less specific chemical processes produce unwanted by-products, such as the food, detergent, cosmetic and pharmaceutical industries. In recent years, increasing attention has been paid to the conversion of processing industry wastes by microbial lipases or their use as biosensors.[5,6]

* Corresponding author. E-mail: zknez@tmf.bg.ac.yu
[#] Serbian Chemical Society member.
doi: 10.2298/JSC0709757G

The rapidly increasing market for compounds produced by lipases has resulted in a growing demand to identify lipases with novel and specific properties. Each application requires unique lipase properties with respect to specificity, stability, temperature and pH. Often an industrial process can be modified to accommodate the limitations of an enzyme but this is costly and a better approach is to find an enzyme more suited to the existing process. Therefore, the screening of microorganisms with lipolytic activity could enable the discovery of a lipase with convenient properties for its potential industrial application.[1]

Microbial commercial lipases are mainly produced from *Pseudomonas*,[7] *Mucor*,[4] *Geothricum*,[6] *Rhizopus*[8] and *Candida* sp.[9,10,11] In a number of studies, it has been reported that the production of lipase can be significantly increased by the careful selection of the nitrogen and carbon sources for microbial growth and the optimization of the composition of the growth medium.[9,10,12] Most importantly, since lipase is an inducible extracellular enzyme, by application of a proper inducer the lipase production can be considerably increased.[13] Although fermentation conditions have been widely studied and documented, only limited research was directed towards *Candida utilis* lipase. On the other hand, the great nutrition value of this microorganism has been well recognized.[14] It appears that among the nine different microorganisms isolated in this study from spoiled soybean oil, it was the best lipase producer. Therefore, the use of this microorganism could lead to the development of a process for achieving high lipolytic activity and gaining a nutritionally valuable biomass. In this work an attempt was made to select a suitable fermentation medium and optimize its components to increase the production of lipase by the yeast *Candida utilis*.

EXPERIMENTAL

Materials

The substrate solution for the determination of the lipase activity was an emulsion of triolein (Sigma, St. Louis MO) in Triton X–100® (Sigma, St. Louis MO). Malt extract, yeast extract, peptone, meat extract and maltose were obtained from Torlak (Institute of Immunology and Virology, Serbia). Bovine serum albumin and Folin–Ciocalteu`s Phenol Reagent, used for the determination of the protein concentration, were products of Sigma (St. Louis, USA). Oleic acid, Tween 80®, dodecane and palmitic acid were also obtained from Sigma (St. Louis, USA). KH_2PO_4, $MgSO_4 \cdot 7H_2O$ and NaOH were products of Lachema (Neratovice, Czech Republic). All other employed chemicals were of analytic grade.

Microorganisms and growth media

The microorganism used in this study was yeast previously isolated from spoiled soybean oil. The yeast was identified as *Candida utilis* according to the identification scheme of Lodder.[15] As a part of the culture collection (Microbiological Laboratory of the Faculty of Technology and Metallurgy, Belgrade), the yeast was maintained on agar slanted at 4 °C. The yeast strain, grown in a medium containing 2 % of malt extract for 24 h at 30 °C (approximately 1.2×10^7 cells cm^{-3}), was used for the inoculum. In the study of the effect of carbon source on lipase production, a medium containing 2 % of malt extract was also used.

The following basal medium was used for lipase production (per dm^3): yeast extract 30.0 g; KH_2PO_4 10.0 g; $MgSO_4 \cdot 7H_2O$ 1.0 g; maltose 5.0 g and Tween 80® 1 cm^3 with the pH adjusted to 6.0. Optimization of enzyme production was carried out by altering the nitrogen sources at a concentration of 3.0 % (w/v). All samples were in duplicates and the experiments were repeated at least twice to ensure reproducibility.

Lipase production

The culture was grown in 100 cm^3 Erlenmeyer flasks containing 50 cm^3 of fermentation media inoculated with 1 % (v/v) (0.5 cm^3) of cell suspension and incubated at 28 °C on a rotary shaker (120 rpm) for 120 h.

Determination of lipase activity

Extracellular lipase activity was measured in culture supernatants after centrifugation (3200 rpm for 20 min). The substrate solution for lipase activity determination was an emulsion of triolein in Triton X-100®. The assay mixture consisting of 3 cm^3 of substrate solution, 2.5 cm^3 of distilled water, 1 cm^3 of Tris–HCl buffer (pH 7.7) and 1 cm^3 of culture supernatant was incubated for 60 min. at 37 °C. The reaction was stopped by the addition of 3 cm^3 of methanol. The fatty acids released were determined by titration with 0.5 M NaOH in the presence of phenolphthalein as indicator. For each test run, a blank control was done separately in which case the reaction was stopped with methanol (3 cm^3) immediately after the addition of the supernatant to the assay mixture. One unit was defined as the enzyme required for releasing 1 μmol of fatty acid per minute at 37 °C.

Determination of cell growth

Determination of yeast cell growth was performed by spreading a suitably diluted cell suspension on malt agar plates and counting the yeast cell colonies after 48 h of incubation at 30 °C.

Determination of protein concentration

The protein concentration was determined according to Lowry method using bovine serum albumin as the standard.[16] The protein concentration was determined from a standard curve which was constructed for each measurement.

RESULTS AND DISCUSSION

Preliminary study

The yeast strain isolated from spoiled soybean oil presented the highest lipolytic activity among several different microorganisms after 5 days of incubation at 28 °C on agar plates containing tributyrine. It was identified as *Candida utilis* by its morphology and biochemical properties.[15]

In a preliminary study, the effects of other conditions of the fermentation process, such as temperature, initial pH of the medium and inoculum concentration, on the yield of lipase were investigated in a medium containing 2 % malt extract. It seems that the optimal temperature and pH range was 27 – 29 °C and 5.9–6.1, respectively, whereas the inoculum concentration did not significantly affect the enzyme yield (data not shown). Therefore, the effect of the composition of the fermentation media and the concentrations of the components was investigated with the temperature and pH fixed at their optimal values (28 °C and pH 6.0) with an inoculum concentration of 1 % (v/v).

Optimization of the composition of the culture medium

Production of the lipase by *Candida utilis* can be optimized by designing the growth medium or conditions which have a positive effect on the genetic regulation of the synthesis of the enzyme. There are two major regulatory mechanisms involved in lipase synthesis: carbohydrate catabolite repression and induction by lipase substrates and products (fatty acids and lipids). Accordingly, the choice of fermentation medium is of crucial importance for the elimination or reduction of catabolite repression and induction of lipase biosynthesis.

Effect of carbon sources and inducers. Studies on the fermentation conditions for the production of extracellular lipases by many microorganisms, such as *Candida rugosa, Yarrowia lipolytica*, showed that the addition of lipid substances enhanced the level of lipase produced. Fatty acids are generally considered to be the most effective inducers for lipase biosynthesis.[12,17] In initial experiments, the fermentation of lipase by *Candida utilis* in batch cultures with different carbon sources was studied in order to determine the influence of different inducers on the production of lipase. Different fatty acids, as well as the other possible inducers, were added at a concentration of 0.1 % (w/v). The obtained data is shown in Fig. 1.

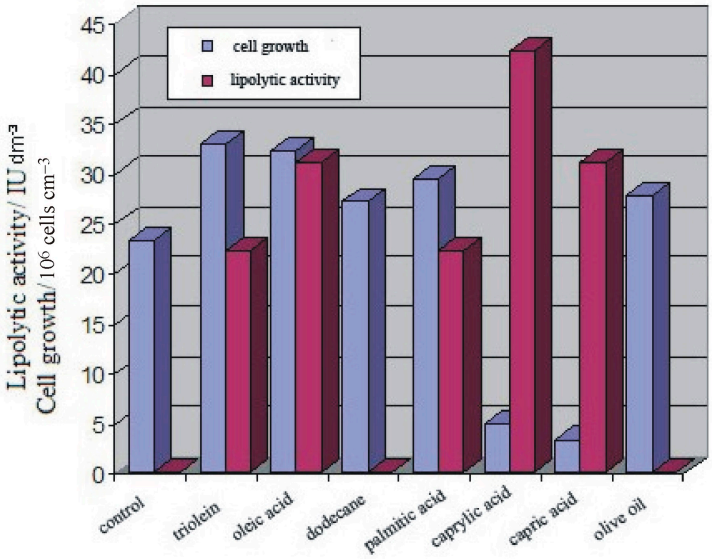

Fig. 1. Effects of carbon sources as inducers on lipase production and cell growth. Fermentation conditions: 2 % malt extract medium, 28 ºC, 120 h.

It was concluded that the highest lipase activity was obtained in a medium supplemented with oleic and caprylic acids. The addition of olive oil and dodecane did not improve the production of lipase. The lipase activity and cell growth were not in strict correlation. Namely, the short-chain inducers (capric and caprylic acid) inhibited cell growth, but improved lipase production.

According to the obtained results, a second experiment was conducted to study the effect of the concentrations of caprylic and oleic acid in the concentration range 0.1–0.9 %. This series of experiments was also performed in 2 % malt extract media. As indicated in Fig. 2, increasing the oleic acid concentration up to 0.5 % enhanced the production of lipase, as well as the total amount of proteins produced and cell growth.

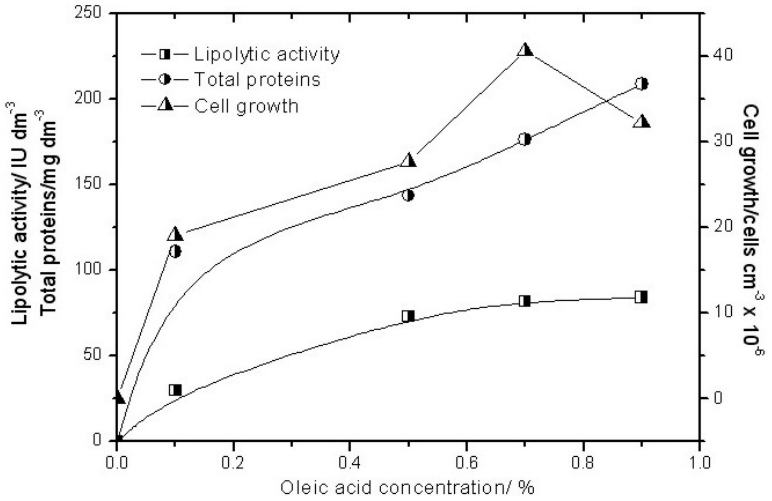

Fig. 2. Effect of oleic acid concentration on lipase production, cell growth and total proteins. Fermentation conditions: 2 % malt extract medium, 28 ºC, 120 h.

It seems that caprylic acid is a better inducer than oleic acid at a concentration of 0.1 % (Fig. 1). However, higher concentrations of caprylic acid (Fig. 3) had a negative effect on lipase production and cell growth, which indicated its possible toxicity and, therefore, caprylic acid was excluded from further experiments. The inhibitory effect of caprylic acid at higher concentrations was previously registered for other yeasts, such as *C. rugosa*.[13]

Increasing the oleic acid concentration had a favorable effect only up to 0.5 %, and a further increase did not lead to an enhancement of the yield of lipolytic activity (Fig. 2). This may be due to the reduced oxygen transfer into the medium caused by the low solubility of oleic acid at high concentration. Low oxygen supplies can alter the yeast metabolism and, consequently, production of lipase.

It is a well known fact that surfactants can increase permeability of the yeast membrane, facilitating the export of various compounds, including enzymes, out of the cell.[12] Therefore, the effect of the addition of the surfactant Tween 80® to the growth medium containing 1 % of oleic acid was investigated. It was evidenced that the yield of lipolytic activity was two times higher in the presence than in the absence of the surfactant. Additionally, it was shown that in the presence of Tween 80®, increasing the oleic acid concentration up to 6 % led to a con-

comitant increase of the lipase activity (Fig. 4). It is plausible that the presence of the surfactant led to more effective oxygen transport in the medium, as well as better dispersion of the oleic acid in the growth substrate, which made it readily accessible to the microbial cells.

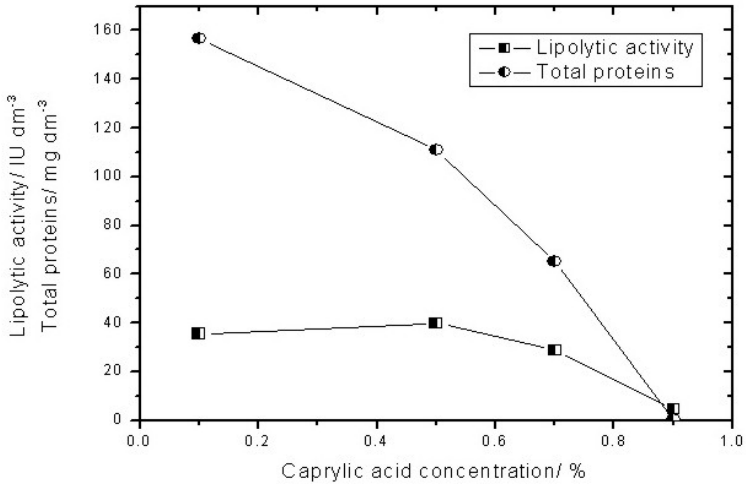

Fig. 3. Effect of caprylic acid concentration on lipase production and total proteins. Fermentation conditions: 2 % malt extract medium, 28 ºC, 120 h.

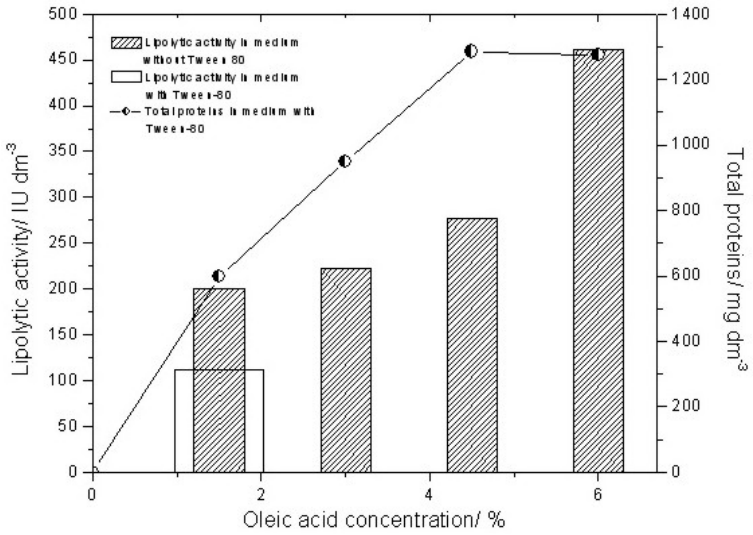

Fig. 4. Combined effect of oleic acid concentration and Tween 80® on lipase production and total proteins. Fermentation conditions: yeast extract 3 % (w/v); KH_2PO_4 1 % (w/v); $MgSO_4 \cdot 7H_2O$ 0.01 % (w/v); maltose 0.5 % (w/v), 28 ºC, Tween 80® 0.1 % (v/v), 120 h.

Effects of nitrogen sources. In previous experiments, yeast extract was used as a nitrogen source. Literature data shows that the production of lipases can be

improved by a suitable selection of a nitrogen source depending on the studied microorganism. For example, yeast extract stimulated lipase production by *Cryptococcus* sp., while urea was the best nitrogen source for lipase production by *Y. lipolytica*.[1,10] In terms of medium optimization, different organic nitrogen sources at a concentration of 3 % (w/v) were investigated instead of yeast extract. The influence of the source of organic nitrogen is shown in Fig. 5.

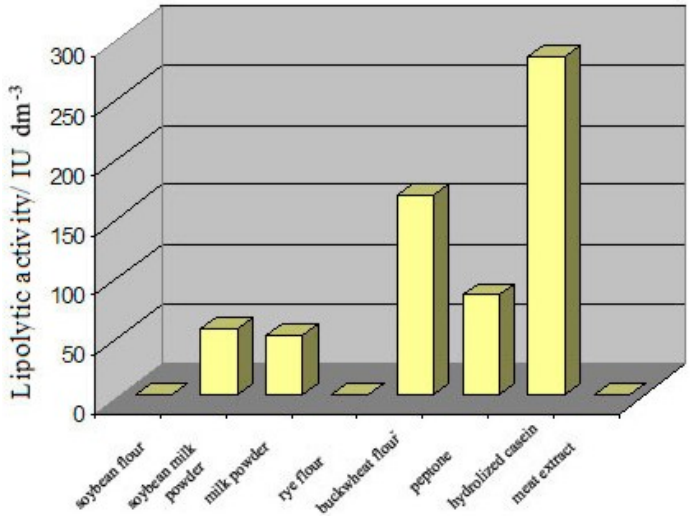

Fig. 5. Effect of nitrogen sources on lipase production. Fermentation conditions: oleic acid 6 % (w/v); KH_2PO_4 1 % (w/v); $MgSO_4 \cdot 7H_2O$ 0.01 % (w/v); maltose 0.5 % (w/v) and Tween 80® 0.1 % (v/v), 28 ºC, 120 h.

The highest yield of lipolytic activity of 284 IU dm^{-3} was achieved in a growth medium with hydrolyzed casein as the nitrogen source. A satisfactory yield of lipase activity was achieved in a medium containing buckwheat flour (166 IU dm^{-3}), while other nitrogen sources did not stimulate lipase production. The favorable effect of hydrolyzed casein on lipase production was previously reported in a study focused on mutant *Candida* sp.[9]

Fermentation of lipase

According to the obtained results, the fermentation was performed in a medium with hydrolyzed casein as the nitrogen source enriched with Tween 80® and oleic acid (6 % w/v). This medium was used to study the kinetics of the production of lipase and the relationship between enzyme secretion, protein production and cell growth.

The kinetics of lipase production, total produced proteins and cell growth during 7 days of fermentation are shown in Fig. 6. The exponential phase of cell growth ceased after 4 days and subsequently the cell concentration decreased rapidly. On the other hand, the increase of lipase activity and total produced pro-

teins was prolonged. Therefore, the maximum lipase activity appeared during the late logarithmic phase, after 5 days of fermentation, which corresponded with the maximum of the total produced proteins. A similar behavior was observed in the production of lipase with *Antrodia cinnamomea*[18] and *C. rugosa*.[19,20]

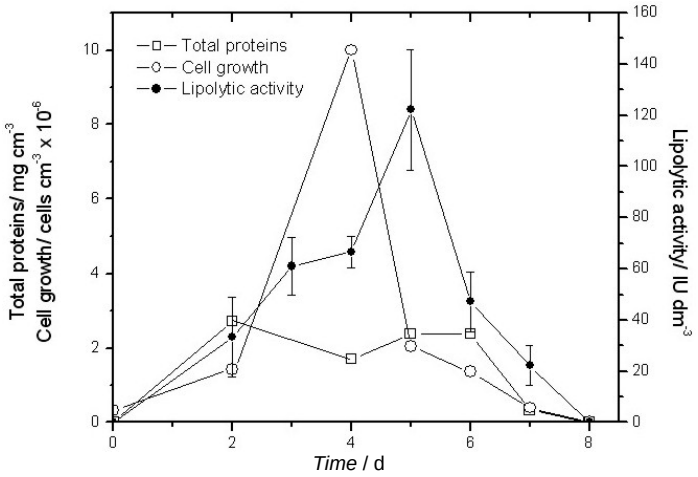

Fig. 6. The time course of lipase production, total proteins and cell growth in the optimized medium. Fermentation conditions: oleic acid 6 % (w/v); hydrolyzed casein 3 % (w/v); KH_2PO_4 1 % (w/v); $MgSO_4 \cdot 7H_2O$ 0.01 % (w/v); maltose 0.5 % (w/v) and Tween 80® 0.1 % (v/v), 28 ºC.

After reaching the lipase production peak, the lipase activity decreased more rapidly than the total produced proteins. It is plausible that the rapid decrease in the lipolytic activity is a consequence of the detrimental effect of proteolytic enzymes released from the yeast cells after cell death.

CONCLUSIONS

In this study, the effects of different factors, such as composition of fermentation medium and addition of inducers, on lipase production by the yeast *Candida utilis* were investigated. Among the examined inducers, the highest lipolytic activity was achieved with oleic and caprylic acids. However, caprylic acid was toxic to yeast cells in concentrations above 0.5 %, while oleic acid exhibited favorable effect on lipase production in concentrations up to 6%.

Among different nitrogen sources, good results were achieved with pepton and buckwheat flour, but the highest lipolytic activity was achieved in a medium containing hydrolyzed casein.

The kinetics of lipase production was studied in the optimized medium and it was observed that the maximum lipase production was achieved after an exponential phase of cell growth, after five days of fermentation. It can be concluded that significant extracellular lipase activity can be achieved using *Candida utilis* and that the obtained lipase activity can be notably enhanced by optimization of the composition of the growth medium.

И З В О Д

УТИЦАЈ УСЛОВА ФЕРМЕНТАЦИЈЕ НА ПРОДУКЦИЈУ ЛИПАЗЕ ИЗ *Candida utilis*

САЊА ГРБАВЧИЋ, СУЗАНА ДИМИТРИЈЕВИЋ–БРАНКОВИЋ, ДЕЈАН БЕЗБРАДИЦА, СЛАВИЦА ШИЛЕР–МАРИНКОВИЋ и ЗОРИЦА КНЕЖЕВИЋ

Технолошко–металуршки факултет, Карнегијева 4, 11000 Београд

У раду је испитана могућност производње липазе помоћу дивљег соја квасца изолованог из поквареног сојиног уља и идентификованог као *Candida utilis*. Овај микроорганизам је показао малу липолитичку активност када је узгајан у 2 % сладном бујону као универзалном квашчевом медијуму (око 4 IU dm^{-3}). Испитани су утицаји различитих фактора као што су састав хранљиве подлоге и додатак одређених индуктора и стимулатора продукције липаза на повећање липолитичке активности. Олеинска и каприлна киселина су се показале као најефикаснији индуктори продукције липазе. У малим концентрацијама (до 0,5 %) каприлна киселина је показала већи утицај на производњу липазе од олеинске киселине, да би при већим концентрацијама имала токсичан утицај на ћелијски раст. Процес је оптимизован и са аспекта извора азота. Највећа липолитичка активност од 284 IU dm^{-3} остварена је након 5 дана ферментације у оптимизованој подлози која је садржала олеинску киселину и хидролизат казеина као изворе угљеника и азота и Tween 80® као стимулатор продукције ензима. Показано је да се пажљивим избором састава хранљиве подлоге може значајно повећати продукција липаза (више од 70 пута у поређењу са почетном вредношћу добијеном у неоптимизованој подлози).

(Примљено 5. октобра 2006, ревидирано 6. марта 2007)

REFERENCES

1. N. R. Kamini, T. Fuijii, T. Kurosu, H. Iefuji, *Process Biochem*. **36** (2000) 317
2. Z. Knežević, S. Šiler–Marinković, L. Mojović, *Appl. Microbiol. Biotechnol.* **49** (1998) 267
3. D. Bezbradica, D. Mijin, S. Šiler–Marinković, Z. Knežević**,** *J. Mol. Catal. B: Enzym.* **38** (2006) 11
4. H. Abbas, A. Hiol, V. Deyris, L. Comeau, *Enzyme Microb. Technol*. **31** (2002) 968
5. W. O. B. Silva, S. Mitidieri, A. Schrank, M. H. Vainstein, *Process Biochem.* **40** (2005) 321
6. J. Vakhlu, A. Kour, *Electron J. Biotechnol.* **9** (2006) 68
7. A. Makhzoum, J. Z. Knapp, R. K. Owusu, *Food Microbiol.* **12** (1995) 277
8. A. Hiol, M. D. Jonzo, N. Rugani, D. Druet, L. Sarda, L. Comeau, *Enzyme Microb. Technol*. **26** (2000) 421
9. T. Tan, M. Zhang, B. Wang, C. Ying, L. Deng, *Process Biochem*. **39** (2003) 459
10. S. Benjamin, A. Pandey, *Bioresour. Technol*. **55** (1996) 167
11. M. A. Gordillo, J. L. Montesinos, C. Casas, *Chem. Phys. Lipids* **93** (1998) 131
12. E. Dalmau, J. L. Montesinos, M. Lotti, C. Casas, *Enzyme Microb. Technol*. **26** (2000) 657
13. N. Obradors, J. L. Montesinos, F. Valero, F. J. Lafuente, C. Sola, *Biotechnol. Lett.* **15** (1993) 357
14. B.–K. Lee, J. K. Kim, *Aquacult. Eng*. **25** (2001) 111
15. J. Lodder, *The yeast – a taxonomic study*, American Elsevier Publishing Company, New York, 1974
16. O. H. Lowry, N. J. Rosebrough, A. L. Farr, R. J. Randall, *J. Biol. Chem*. **193** (1951) 265
17. G. Corzo, S. Revah, *Bioresour. Technol*. **70** (1999) 173
18. E.–S. Lin, H.–C. Ko, *Enzyme Microb. Technol*. **37** (2005) 261
19. R. V. Muralidhar, R. R. Chirumamila, R. Marchant, P. Nigam, *Biochem. Bioeng. J*. **9** (2001) 17
20. R. K. Saxena, P. K. Gosh, R. Gupata, W. S. Davidson, S. Bradoo, R. Gulati, *Curr. Sci.* **77** (1999) 101.