Romanian Biotechnological Letters *Vol. 15, No.5, 2010*

ORIGINAL PAPER

The preparation and immobilization of some yeast lipases for rapeseed oil transesterification to biodiesel

Received for publication, May 12, 2010
Accepted, September 16, 2010

LUMINIȚA TCACENCO[1], ANA AURELIA CHIRVASE[2], CAMELIA UNGUREANU[2], ELENA BERTEANU[1]
[1]*National Institute of Research and Development for Biological Sciences, Bucharest, Romania phone/Fax+4021.220.79.09.e-mail:tcacenco_lumi@yahoo.com*
[2]*National Institute of Research and Development for Chemistry and Petrochemistry, Bucharest, Romania*

Abstract

Biodiesel prepared from vegetable oils, biodegradable and less toxic, represents a promising alternative fuel for use in compression-ignition engines.

Much research work is in progress now-a-days to elaborate an enzymatic technology as effective as the chemical catalysis, oriented to solve, besides the preparation of a cheap catalyst, other important issues: (a) the biosynthesis of lipases with high specificity for the transesterification of plant oil triglycerides; (b) the design of an enzymatic transesterification process with global economic efficiency similar to the chemical methods.

The paper presents the research done to select yeast strains producing lipases with important transesterification activity and, at the same time, the experimental results for the immobilization of the crude enzymes of interest, based on the very large number of techniques described in the dedicated literature.

Keywords: biodiesel, rapeseed oil, transesterification, yeast lipases

Introduction

Now-a-days the preparation and use of biofuels is one of the most important research topics, as the world shifts away from a dependency on petroleum resources. Biodiesel (a mixture of fatty acids esters) prepared from vegetable oils, biodegradable and less toxic, represents a promising alternative fuel for use in compression-ignition engines [1, 2].

Enzymatic production of biodiesel has been proposed to overcome the drawbacks of the conventional chemically catalyzed processes. The main obstacle facing full exploitation of the lipase potential is its cost. Therefore, reuse of lipase is essential from the economic point of view, which can be achieved by using the lipase in immobilized form. In addition, immobilized lipase displays improved stability and activity [3, 4, 5, 6].

The actual research work aimed to elaborate an enzymatic technology as effective as the chemical catalysis is oriented to solve, besides the preparation of a cheap catalyst, other important issues: (a) the biosynthesis of lipases with high specificity for the transesterification of plant oil triglycerides; (b) the design of an enzymatic transesterification process with global economic efficiency [7, 8, 9, 10, 11], similar to the chemical methods.

There is a great number of bacteria, yeasts and fungi strains with interesting lipase production. The bacteria and yeasts strains can be considered as recommended lipase producers based on the cultivation criteria, namely easy to apply and reproducible aerobic bioprocess conditions. Meanwhile these microorganism types can probably form growth associated lipases, in a first stage, linked to the cellular membranes, then released into the

cultivation medium as extracellular enzymes. The crude lipases, after specific separation – purification operations, can be used as enzymatic catalysts for the tranesterification of triglycerides to biodiesel when immobilized on appropriate supports, ready to be used in several biotransformation cycles [12, 13, 14, 15, 16].

The paper presents the research work done to select yeast strains producing lipases with important transesterification activity and at the same time the experimental results for immobilization of the crude enzymes of interest, based on the very large number of techniques described in the dedicated literature [17, 18, 19, 20, 21, 22].

Materials and methods

1. Lipases formation

Several bacteria and yeasts from own / international collections were tested for cell growth and enzyme formation, the cultivation conditions being: temperature of 30°C; Erlenmeyer flasks of 300 mL with 100 mL medium; rotary shaker New Brunswick Innova 40 at 300 rpm.

Before their cultivation for enzyme formation the microorganisms were grown on liquid media to develop preinoculum and inoculum stages of 24 hours duration, using an inoculation volume of 5-10 % V/V. The most representative results were obtained with the microorganisms:

Bacteria: *Pseudomonas putida* (*P. sp. 1)* and *Pseudomonas aeruginosa* (*P. sp. 3)*
Yeasts*: Yarrowia sp./ Candida lipolytica* ATCC 8661 and *Candida rugosa* DSM 70761

Cultivation media:

M1 for bacteria: (variant a-without rapeseed oil; variant b-with rapeseed oil 10 mL/L)	**M2 for yeasts:**
Glucose: 4 g/L	Glucose: 10 g/L
Peptone: 0.5 g/L	Peptone: 10 g/L
Yeast extract: 5 g/L	Yeast extract: 10 g/L
Na_2SO_4: 2 g/L	Rapeseed oil: 5 g/L
KH_2PO_4: 1 g/L	
K_2HPO_4: 3 g/L	
$MgSO_4.7H_2O$: 0.1 g/L	

M1 is a rich medium, with 2 organic N sources (peptone and yeast extract), and glucose as C source.

M2 is also rich, with 2 organic N sources, 1 mineral N source, 1 C source for growth - glucose and rapeseed oil for enzymatic induction (adaptive lipase formation).

The growth characteristics were evaluated by measuring OD_{500}; the lipase activity was determined by using the volumetric method [14], considering one unit of lipase activity as corresponding to 1 μmol of fatty acid obtained by the hydrolysis of the triglycerides from the rapeseed / olive oil, the reaction conditions being: temperature of 37ºC, pH=7, duration 60 minutes.

Isolation of extracellular lipase was made by centrifugation (1) and ammonium sulfate precipitation (2).

1) biosynthesis medium was centrifuged at 10 000 rpm for 30 min. at 4 ^{0}C. Clear supernatant was treated with benzamidine 2 mM and sodium azide 0.02% to prevent proteolysis and microbial attack and 2) the supernatant is precipitated with ammonium sulfate

30% at 0 ^{0}C, then left to stand for 24 hours for achieving precipitation and centrifuged at 10 000 rpm for 30 minutes at 4^{0}C. The supernatant is precipitated again with 75% ammonium sulfate. After 24h, the sample is centrifuged again and the resulting product is dissolved in 8 ml TRIS buffer, pH 6.8. This crude enzyme is preserved in freezer.

2. Lipases immobilization

a) Lipase immobilization by adsorption on silicagel or celite support

For immobilization by adsorption of crude lipase obtained from *Yarrowia lipolytica* and *Candida rugosa* yeasts, the precipitate obtained with 70% ammonium sulfate was dissolved in 0.05 M phosphate buffer, pH 7. Then the adsorbent was added until the limit activity in the supernatant is reached, respectively: for *Yarrowia lipolytica* 2.5 g silicagel G at 800 mL extract, 22 g of celite in the same volume of extract and for *Candida rugosa* 11 g celite at 800 ml extract. Adsorption duration was approx. 2 hours at ambient temperature and mechanical stirring.

b) Lipase immobilization by adsorption on chitosan support

1. Cross-linking with glutaraldehyde:

30 mL chitosan 1% solution was prepared by adding 2mL CH_3COOH p.a. acetic acid , 19.8 mL 0.5 N NaOH by heating to 50 ^{0}C and stirring for 10 minutes to complete dissolution of chitosan. 0.5 mL 25% of glutaraldehyde was added dropwise under high stirring. Microspheres thus obtained were filtered and thoroughly washed with distilled water and 0.05 M phosphate buffer, pH 7.

1g wet chitosan microspheres were used for immobilization; they were suspended in 2 mL 0.05 M phosphate buffer, pH 7 and mixed with 2mL solution of lipase (*Candida rugosa*) obtained by solving the crude enzyme precipitated with ammonium sulfate into 0.05 M phosphate buffer, pH 7, 1:5 (w / v) ratio. The mixture was stirred for 1 hour at 37 ^{0}C.

2. Cross-linking with carbodiimide:

1g wet chitosan particles was obtained by injecting 25 mL solution of 3% chitosan into 250 mL solution of NaOH 1N and alcohol ethylic 26%. The chitosan particles were suspended into 3 mL 0.75% carbodiimide solution, prepared in 0.05 M phosphate buffer, pH 6, 25 ^{0}C. After 10 minutes of activation, the particles were washed with distilled water and transferred to 10 mL 1% lipase solution immersed in 0.05 M phosphate buffer, pH 6. The adsorption duration was 60 minutes; then the immobilized enzyme was washed 3 times with distilled water.

3. Cross-linking with glutaraldehyde and reduction with sodium borohydride

A mixture was prepared from 0.5 g chitosan, 1.041 mL 2M acetic acid, 25 mL distilled water and 1.041 mL of 1M sodium acetate, maintained on water bath at 50^{0}C with stirring. For the immobilization of *Aspergillus niger* lyophilized lipase (Fluka), 0.1 g of lipase immersed in 0.5 M phosphate buffer, pH 5.6 was added to this mixture. Then 2.5 mL 50% glutaraldehyde dissolved in 25 mL double distilled water was added. To accomplish the gel formation, the mixture rested for 30 minutes at 4 ^{0}C. 0.25 g sodium borohydride was added in portions, during 15 minutes, by using ice pieces to low the temperature, and finally a vacuum filtration of the mixture was done. The immobilized product thus obtained was washed with double distilled water and 0.5 M phosphate buffer, pH 5.6.

Lipase activity and immobilization yield were evaluated for each application.

Results and discussion

1. Growth and enzyme formation Bacteria

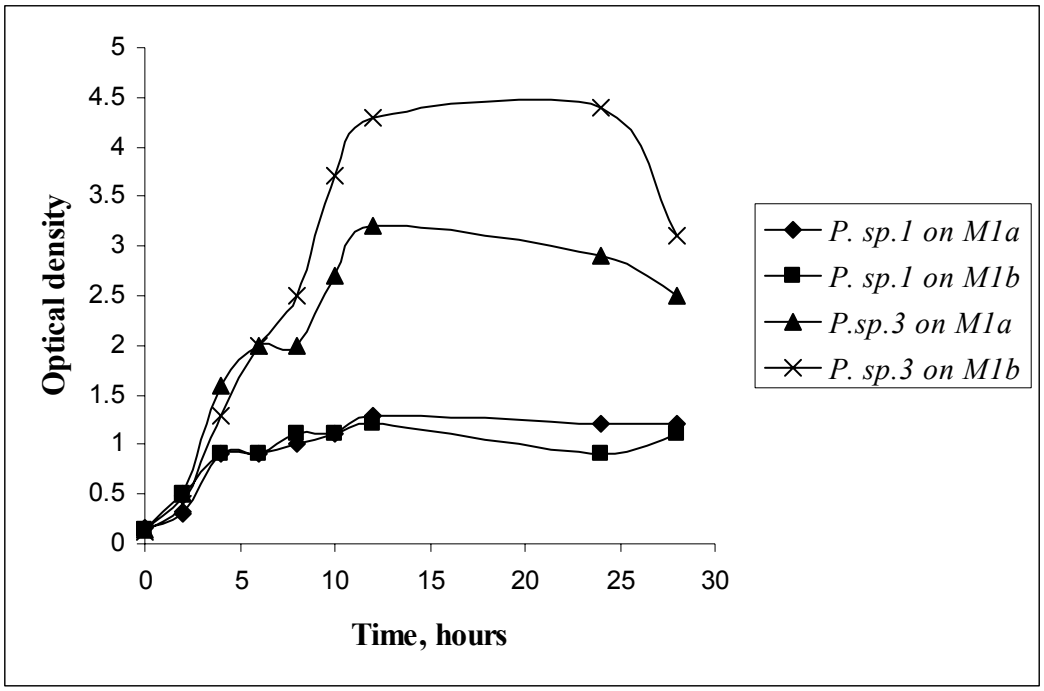
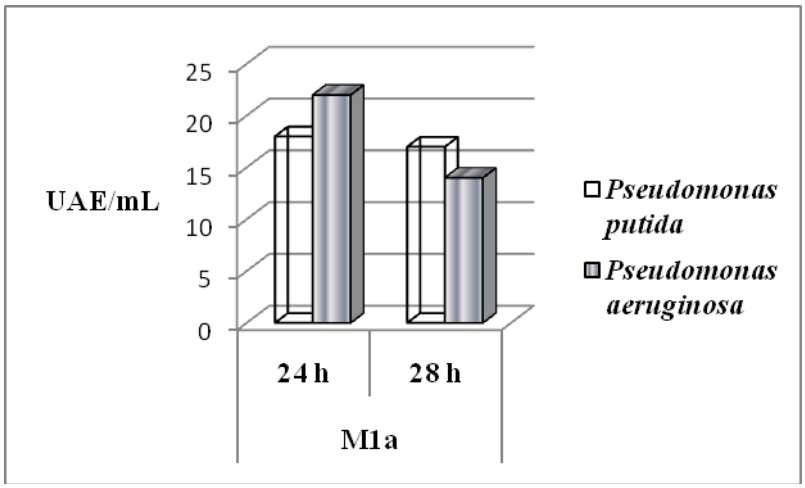


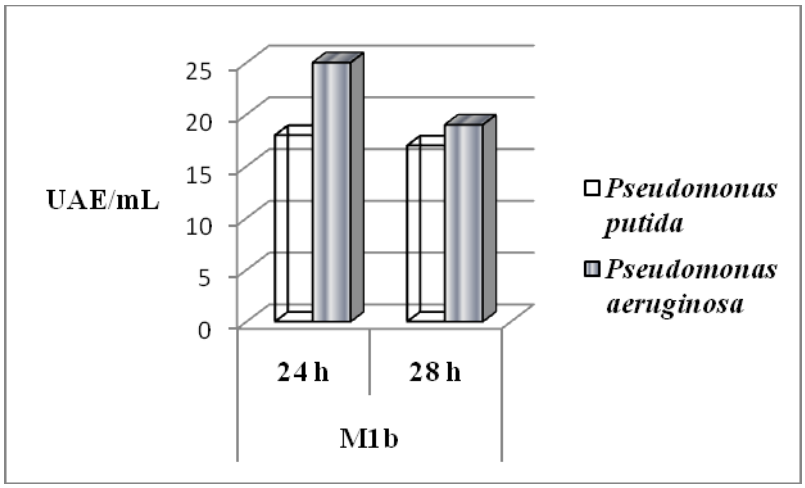
Figure 1. Growth of strains *Pseudomonas putida* (*P. sp.1*) and *Pseudomonas aeruginosa* (*P. sp. 3*) on cultivation medium M1, a and b

a) *Pseudomonas putida* (*P. sp.1*) strain shows exponential growth phase between 2-12 h, then the culture reaches the stationary phase. No differences were observed between growths on the cultivation media variants M1a and M1b, so the vegetable oil does not stimulate the cells multiplication.
b) *Pseudomonas aeruginosa* (*P. sp.3*) strain shows the same growth behavior as the previous bacterium, but the growth is better on the medium variant M1b, so the use of both substrates-glucose and oil seems possible.

Both bacterium strains have similar small lipase activity levels, the cultivation duration of 24 h being enough, and no induction by the rapeseed oil (figure 2).



a)



b)

Figure 2. Lipase activity of *Pseudomonas* strains (UAE/mL medium) on cultivation medium M1, a and b.

Yeasts.

Candida rugosa DSM 70761 is preserved on agar medium with the general formula for yeast:

Yeast extract: 3 g
Malt extract: 3 g
Soya peptone: 5 g
Glucose: 10 g
Agar: 15 g
Distilled water: 1000 mL

This yeast cultivated on Petri dishes is presented in the following (figure 3) microscopic image (Olympus microscop, 500 X)

The cells are cylindrical-ovoid, of different dimensions, the characteristic diameters being in the range 2.5-13 μm, with budding in some cases.

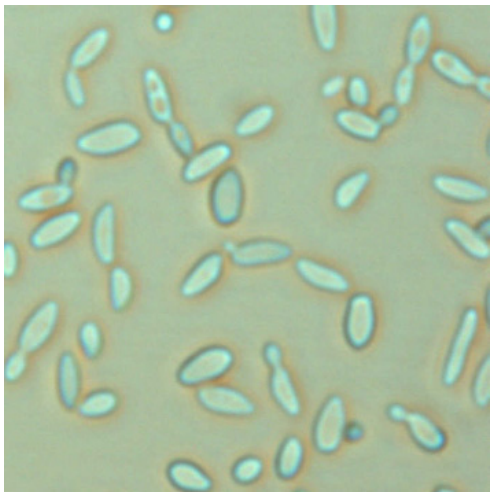


Figure 3. Microscopic view of the yeast *Candida rugosa* DSM 70761

The growth characteristics are presented in the figure 4.

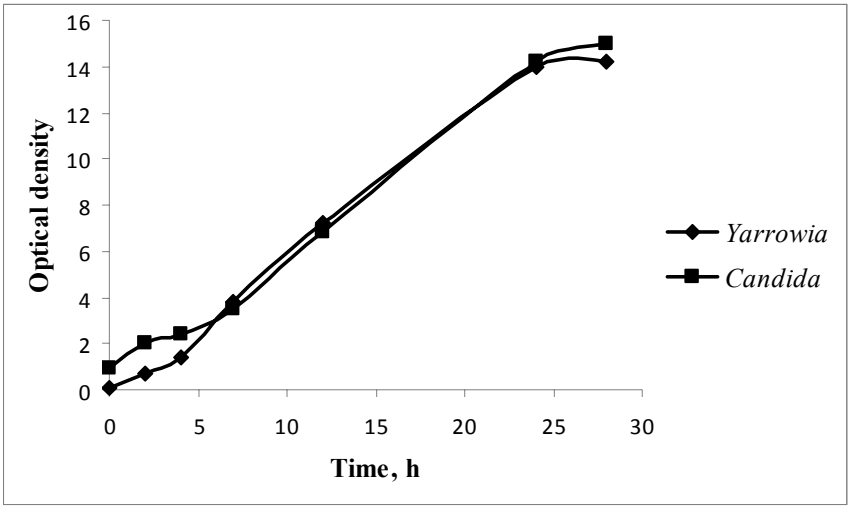


Figure 4. Growth characteristics of the tested yeasts *Yarrowia lipolytica* ATCC 8661 and *Candida rugosa* DSM 70761

Both yeasts grow better than the studied bacteria, with no major differences between them.

Determined lipase activity is presented in figure 5.

For the same balanced cultivation medium, the final enzyme activity was higher for *Candida rugosa*, 362.0 UAE/mL (28 h) by comparison with *Yarrowia lipolytica* enzymatic activity of 106.0 UAE/mL (28 h).

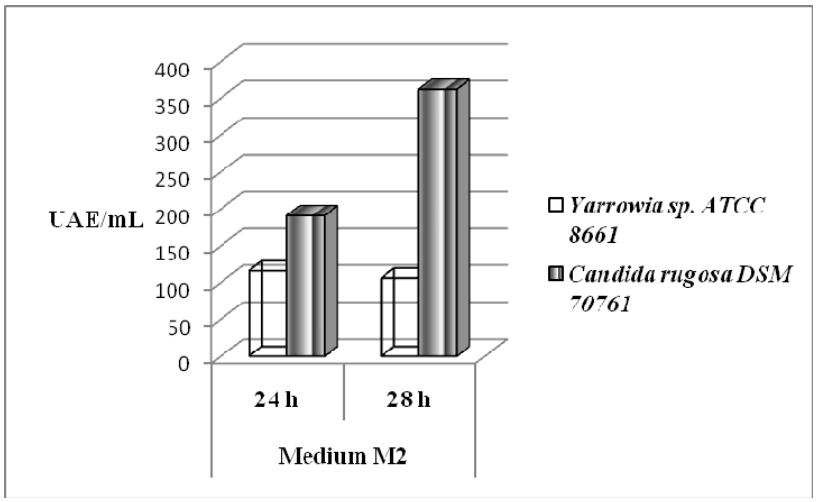


Figure 5. Lipase activity of the yeasts *Yarrowia lipolytica* ATCC 8661 and *Candida rugosa* DSM 70761 (UAE/mL).

Due to the above presented results, the following studies were performed with the yeast lipases. In a first step, the preliminary transesterification results, obtained by thin layer chromatography, demonstrated that both lipases have high enough catalysis activities [23].
After the confirmation of the transesterification capacity, it was of interest to develop appropriate immobilization techniques for these lipases, so to be able to use the immobilized enzymes in several cycles of biotransformation.

2. Enzyme immobilization

The final activities and isolation yields obtained when the crude lipases were separated from the cultivation medium by precipitation with ammonium sulfate are presented in the following table:

Table 1: The final activities and isolation yields determined for the crude lipases separated from the cultivation media of the yeasts strains

No.	Strain / duration of bioprocessing	Extract volume (mL)	Initial activity (UT)	Quantity $(NH_4)_2SO_4$ (g)	Final activity (UT)	Isolation yield (%)
1.	*Candida rugosa* 24 hr	20	3 820	14	2 368	62.0
2.	*Candida rugosa* 28 hr	800	289 600	560	85 721	29.6
3.	*Yarrowia lipolytica* 24 hr	20	2 320	14	2 204	95.0
4.	*Yarrowia lipolytica* 28 hr	800	85 200	560	80 940	95.0

Highly efficient lipases isolation was done by precipitation of the cultivation medium of *Yarrowia lipolytica* yeast with $(NH_4)_2SO_4$; a yield of 95% was recorded for both variants (24 h and 28 h), while when the same procedure was applied for *Candida rugosa* samples, the isolation yields were lower: 62% for 24 h extract and only 29.6% for 28 h extract. The experimental results obtained with the applied immobilization techniques (table 2), tested for both crude and immobilized lipases in comparison with a lipase from *Aspergillus niger* are presented in the figure 6.

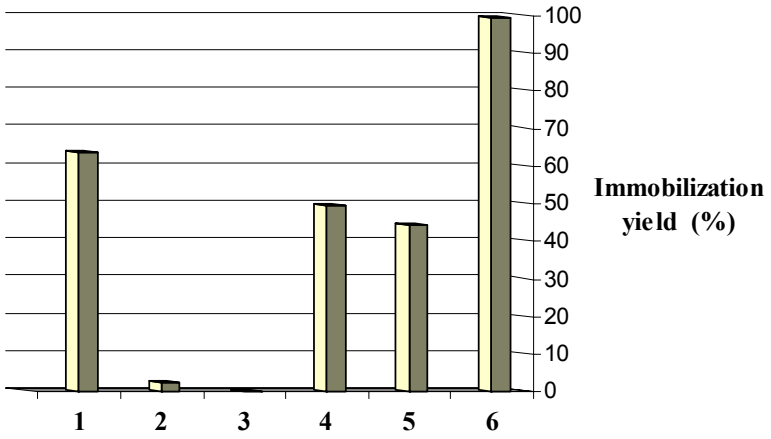


Figure 6. Immobilization efficiency of the tested lipases

Table 2: Applied immobilization techniques

No.	Lipase source	Immobilization technique
1	*Candida rugosa,* DSM 70761	Chitosan adsorption and cross-linking with glutaraldehyde
2	*Aspergillus niger* (Fluka)	Chitosan adsorption and cross-linking with carbodiimide
3	*Aspergillus niger* lyophilized lipase *(*Fluka)	Chitosan adsorption, cross-linking with glutaraldehyde and granulation with sodium borohydride
4	*Candida rugosa,* DSM 70761	Adsorption on celite 545
5	*Candida rugosa,* DSM 70761	Adsorption on Silicagel G
6	*Yarrowia lipolytica* ATCC 8661	Adsorption on celite 545

The experimental study regarding the immobilization of lipases gave interesting results: high yield of 99% obtained for the immobilization of *Yarrowia lipolytica* lipase by adsorption on celite support, good yields of 63.26% for the immobilization of *Candida rugosa* lipase by adsorption on chitosan cross linked with glutaraldehyde and respectively 49.32 - 44% for the same lipase immobilized by adsorption on celite or silicagel. On the contrary, the immobilization of *Aspergillus niger* lipase gave unsatisfactory results.

The immobilized lipases from both yeasts *Yarrowia lipolytica* and *Candida rugosa* prepared by celite adsorption were preserved in a freezer at -18°C and tested for static stability at different time duration. Results are presented in the Figure 7.

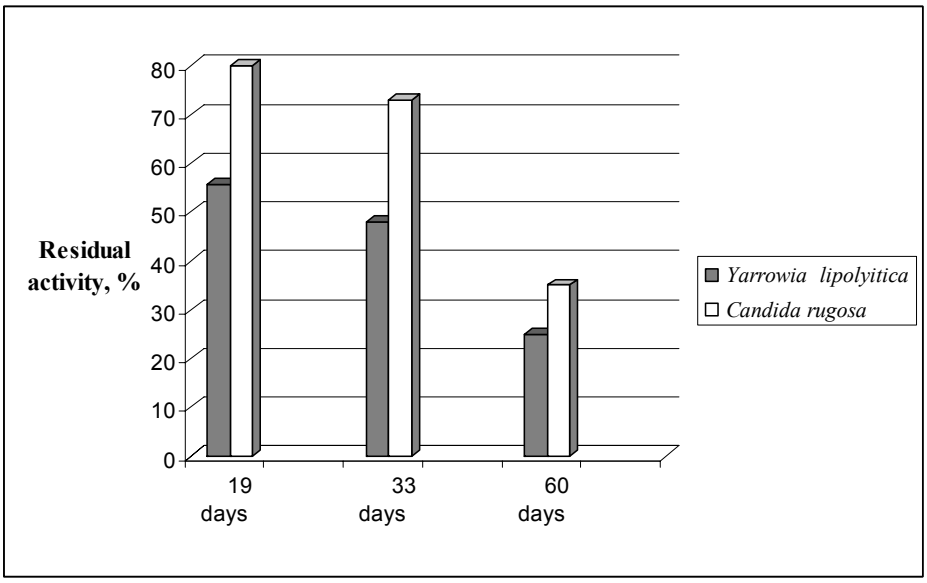


Figure 7. Static stability expressed as residual activities of the celite adsorption immobilized lipases from *Yarrowia lipolytica* and *Candida rugosa*.

The results demonstrated a higher static time stability for the celite adsorption immobilized lipase from the yeast *Candida rugosa* DSM 70761, with 73% residual activity after more than 1 month, by comparison with only 48% residual activity for the immobilized lipase from the yeast *Yarrowia lipolytica* ATCC 8661, although the immobilization yield was higher for this last enzyme.

Conclusions

The paper presents the research work performed to select yeast strains producing lipases with important transesterification activity of rapeseed oil and at the same time the experimental results regarding the immobilization of the crude enzymes of interest.

1. Both the lipase activities were higher for the tested yeasts *Candida rugosa* DSM 70761 and *Yarrowia lipolytica* ATCC 8661 by comparison with the studied bacteria, but in case of the yeast *Candida rugosa* the enzymatic activity was superior (362 UAE/mL) to that determined for the enzyme from *Yarrowia lipolytica* (106 UAE/mL). So the study was continued with the yeasts lipases.
2. The isolation yields obtained by the precipitation with ammonium sulfate were higher (95%) for the enzyme from *Yarrowia lipolytica* yeast than for the enzyme from *Candida rugosa* yeast (30-62%).
3. The immobilization yields for the studied techniques were good for both yeast lipases: 44-63% for the crude lipase from *Candida rugosa* DSM 70761 and 99% for the crude lipase from *Yarrowia lipolytica* ATCC 8661.

4. A higher static time stability was determined for the celite adsorption immobilized lipase from the yeast *Candida rugosa* DSM 70761, with 73% residual activity after more than 1 month, by comparison with only 48% residual activity for the immobilized lipase from the yeast *Yarrowia lipolytica* ATCC 8661.
5. So both yeasts can give lipases with interesting activities for the transesterification of the rapeseed oil; the research work will be continued in order to improve the isolation yield by the precipitation with ammonium sulfate for the crude lipase from *Candida rugosa* DSM 70761 and especially to test the immobilized enzymes as catalysts of the rapeseed oil transesterification.

Acknowledgements

This research is financially supported by the National Agency for Research under the II-end National Program, Project 61-032/2007.

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