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Progress in Vegetable Oils Enzymatic Transesterification to Biodiesel - Case Study

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1. Introduction

These days the interest of fuels preparing from sustainable natural resources is continuously increasing due to the rising prices of the fossil fuels and the political instability in the oil producing countries. The fuels manufacturing from local vegetal resources can sustain the every country' prosperity, including rural, agricultural, economically disadvantaged regions. Nowadays only the bioethanol and the biodiesel are already produced at industrial level from sustainable raw materials.

The biodiesel is manufactured by the chemically catalysed transesterification of the triglycerides from the vegetable oils, rapeseed oil in Europe and soya oil in USA. As the methanol is often used as alcohol reagent, the reaction is consequently named methanolysis. The most applied catalysts are alkalines (especially NaOH) or mineral acids. So the biodiesel represents the methyl esters of the fatty acids from the vegetable oils. The present diesel engines can normally use a mixture of diesel with 5% v/v biodiesel.

Biodiesel contains virtually no sulfur or aromatics, and use of biodiesel in a conventional diesel engine results in substantial reduction of unburned hydrocarbons, carbon monoxide and particulate matter. The production and use of biodiesel, compared to petroleum diesel, resulted in a 78.5% reduction in carbon dioxide emissions. Moreover, biodiesel has a positive energy balance.

The chemical transesterification applied at industrial level has important advantages, but also limitations: in spite of the high conversion yields and the short reaction duration, the global transformation is energetically intensive, the glycerol recovery is difficult, the alkaline catalyst must be separated, the wastewaters are to be treated by a rather complex procedure, and both the free fatty acids and water can badly influence the reaction.

These unfavourable situations can be diminished by performing the enzymatic transesterification on conditions that: (a) the immobilised lipase used as biocatalyst must be as cheap as possible; (b) one can obtain the economic efficiency of the whole biotransformation process similar to that characteristic to the chemical process, these objectives being presented function of the research methodology and results. The comparison between the chemical way and the enzymatic way is presented in the Table 1.

Criterion	Alkaline catalysis process	Enzymatic proces
• Reaction temperature	60-70°C	30-40°C
• Free fatty acids from the vegetable oils	Saponification products	Methylic esters
• Water from the raw material	Reaction interference	No influence
• Methylic esters yield	Normal	Higher
• Glycerol recovery	Difficult	Easy
• Methylic esters purification	Repeated washing	No need
• Catalyst preparation price	Cheap	Relatively high

Table 1. Comparison between the alkaline catalysis and the enzymatic method for biodiesel preparation (Bajaj *et all*, 2010)

The now-a-day technological progress regarding the enzymatic transesterification is demonstrated by the realisation of 2 industrial pilots in China (Moore 2008a, 2008b; Uthoff *et all*, 2009) to apply this advanced methodology, though the biodiesel manufacture price still remains higher than the diesel price no matter the transesterification route, due to the raw materials high prices (Bisen *et all*, 2010). Developments to meet the economical framework are needed, including: (a) the introduction of the enzymatic transesterification of plant oils as a part from a comprehensive technology of complete valorisation of the vegetable oil, meaning the application of the bio refinery concept; (b) the increase of the available vegetable oil quantity with limited interference with the vegetable oils' food use; (c) the possible preparation of methanol from natural resources.

2. State of the art in the domain of biodiesel preparation by enzymatic transesterification of vegetable oils

Other advantages of using lipases in biodiesel production are: (a) ability to work in very different media which include biphasic system, and monophasic system, (b) they are robust and versatile enzymes that can be produced in bulk because of their extracellular nature in most manufacturing system, (c) when the lipase is used in a packed bed reactor, no separation is necessary after transesterification, and (d) higher thermo stability and short-chain alcohol-tolerant capabilities of lipase make it very convenient for use in biodiesel production (Ghaly *et all*, 2010). Until now the biodiesel manufactured by chemical catalysis is cheaper than the same product obtained by enzymatic catalysis, but in case of considering the pollution suppressing costs needed after the chemical process performing, the costs of both reaction' types could be comparable.

Enzymatic transesterification can be done with crude or purified vegetable oils, free fatty acids, residual grease from food industry or of animal origin, and residual vegetable oils from fry cooking. Beside methanol and ethanol one can also use as acyl acceptors the propanol, iso-propanol, butanol and iso-butanol. Many microorganisms, bacteria, yeasts or fungi can produce useful lipases for transesterification. Of these microorganisms, *Candida antarctica*, *Candida rugosa*, *Pseudomonas cepacia*, *Pseudomonas fluorescens*, *Rhizomucor miehei*, *Rhizopus chinensis*, *Rhizopus oryzae* and *Thermomyces lanuginosa* have produced the most effective lipases, able to perform the biotransformation with high yields. The combination of two or more lipases can increase the conversion in order to lower the cost. A combination of *Candida antarctica* and *Thermomyces lanuginosa* lipases was used to obtain a 95% conversion in methanolysis using a tert-butanol solvent. From the many lipases it is recommended to use those with reduced region specificity, but with higher substrate specificity.

The reaction can be realised either in organic solvents, or in solvent-free media (where there are only the substrates' mixture). Normally in organic solvents' systems the lipases can catalyse the biotransformation when the alcohol is added stepwise at the beginning (a „batch" system), by comparison with the free-solvent media, where the alcohol is added several times for maintaining a certain molar ratio with the oil concentration.
The **key factors affecting the enzymatic transesterification** are presented in the Figure 1.

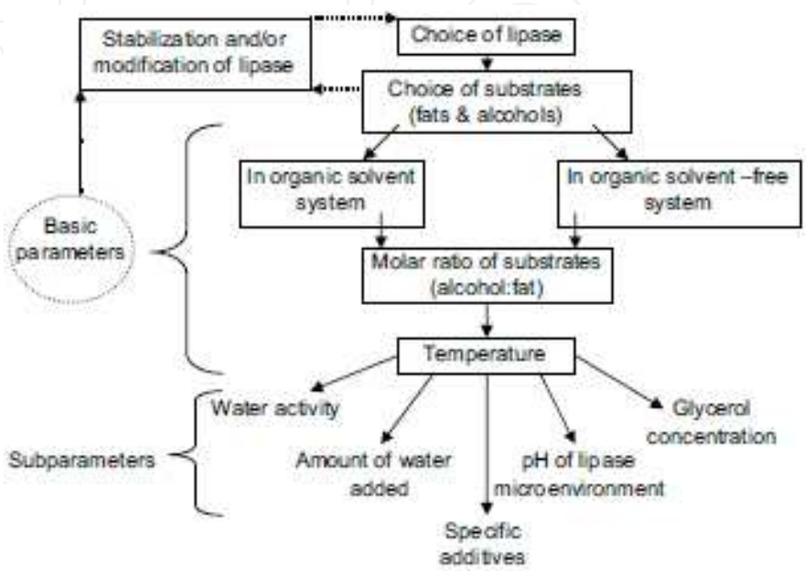


Fig. 1. Key factors of influence on the enzymatic transesterification (Antczak *et all*, 2009)

There are two categories of enzymatic biocatalysts: (1) extracellular lipases (i.e. the enzyme has previously been recovered from the cultivation broth and then purified) especially from the microbial producers *Candida rugosa*, *Candida utilis*, *Candida antarctica* and *Pseudomonas cepacia*, generally bacteria and yeasts; (2) intracellular lipases which still remain either inside or attached to the cellular wall; in both cases the enzymes are immobilized directly or together with the whole cell and this use can eliminate downstream operations and assure the enzyme recycling.
The **extracellular lipases** are mostly produced by bacteria and yeasts and the large scale production of these lipases should be economical, fast, easy and efficient. Unfortunately, the cost of specific separation and purification operations is high enough. Still the majority of immobilized lipases that are commercially available are extracellular. The most commonly used is: Novozym 435 which is the lipase from *Candida antarctica*. Meanwhile the bacteria and yeasts can probably form growth associated lipases, in a first stage, linked to the cellular membranes, then released into the cultivation medium as extracellular enzymes.
When preparing the **intracellular lipases** the costly step of purification can be eliminated and this has led to using whole cells as biocatalysts. After the intracellular production of lipases the direct use of fungal cells immobilized within porous biomass support particles as a whole biocatalyst represents an attractive process for bulk production of biodiesel (Fjerbaek *et all*, 2009)
The main criteria to choose between the two lipase types can be: (a) the bacteria and yeasts strains which biosynthesise extracellular lipases, can be considered as recommended producers based on the cultivation conditions, namely easy to apply and reproducible

aerobic bioprocesses; (b) using intracellular lipases slows down the transesterification process due to mass transfer limitations.

Immobilization of an enzyme must solve both mass transfer limitations types-internal or external (last case due to formation of an external film layer). Choice of the appropriate lipase immobilization technology is determined by the following objectives: (a) long term enzyme reuse; (b) easy enzyme recovery from the reaction medium; (c) improved activity and thermal, chemical and mechanical stability of the enzyme; (d) potential to run continuous processes. The immobilization support is to be as low cost as possible, condition which is difficult to be observed when the other ones should be fulfilled at the same time (Ghaly *et all*, 2010). Among the great number of immobilization techniques, they can be classified under four general categories: (a) adsorption, (b) cross linking, (c) entrapment and (d) encapsulation. Adsorption seems to be the most attractive, as it is simplest and cheap, retaining high enzyme activity and allowing a good mass transfer, combined or not with the cross linking. The carriers used in adsorption via weak forces include: celite, cellulose, acrylic, silica gel, textile membranes, spherosil, sepharose, sephadex and siliconized glass. The major drawback of the adsorption is the low stability of the enzyme when adsorbed, which determines only limited reuse.

The **stability of the lipase** with low loss of the catalytic activity is the most important characteristic, when used in biodiesel preparation in connection with the enzyme r**ecovery and reuse.**

The most commonly used reactor type for the biodiesel enzymatic preparation is a batch-stirred tank reactor, though this biofuel must be considered as a commodity product and therefore produced in continuously operated installations. Possible alternative solutions could be packed bed reactors, fluid beds, expanding bed, recirculation membrane reactors. A wide range of configurations are applicable to perform the transesterification.

As the actual major technical limits of the enzymatic process are still the slower reaction rate by comparison with the alkaline catalysis and the risk of enzyme inactivation, with focus on process design and economy, the researchers calculate the productivity (kg biodiesel/kg enzyme) based on information from different studies and considering a range of enzyme prices from 12 to 185 USD/kg as acceptable, depending on the application characteristics, i.e. per each kg of biodiesel a biocatalyst cost of USD 0.025 could be of economic interest. An increased enzyme life of around 6 years would make enzymes competitive based on productivity again. To this must be added increased reactor costs as enzymes lead to longer space times than alkaline catalysts, but reduced separation costs and low waste water treatment costs will be the benefits.

3. Case study: Enzymatic transesterification of the rapeseed oil with yeast lipases

The chapter presents the research activity done by the authors regarding the rapeseed oil transesterification with yeast lipase, and is structured in three parts: lipase formation in aerobic bioprocessing; lipase recovery and immobilization; enzymatic transesterification with immobilized lipase produced by the yeast *Candida rugosa* DSM 70761.

3.1 Lipase formation

3.1.1 Materials and methods

Several bacteria and yeasts from own / international collections were tested for cell growth and enzyme formation, the cultivation conditions being: rotary shaker New Brunswick

Innova 40 at 300 rpm; temperature of 30°C; Erlenmeyer flasks of 500 mL with 150 mL medium. 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. Several cultivation media, specific for the studied strains, were tested and both the cellular growth and enzymatic activity were measured.

Microorganisms and cultivation media:

Bacteria: *Pseudomonas putida (P. sp. 1) and Pseudomonas aeruginosa (P. sp. 3)*
Yeasts: *Yarrowia sp. / Candida lipolytica ATCC 8661, Candida sp. DG 8, Pseudozyma aphidis DSM 70725, and Candida rugosa DSM 70761.*

M1 for bacteria: (variant a: no rapeseed oil; variant b: with 10 mL/L rapeseed oil)	M2 for yeasts	M3 for yeasts	M4 for yeasts
Glucose: 4 g/L	Glucose: 10 g/L	KH_2PO_4: 5 g/L	Malt extract: 3.78 g/L
Peptone: 0.5 g/L	Peptone : 10 g/L	$(NH_4)_2SO_4$: 1 g/L	Peptone: 5 g/L
Yeast extract: 5 g/L	Yeast extract: 10 g/L	Yeast extract: 10 g/L	Tween 80: 4.33 g/L
Na_2SO_4: 2 g/L	NH_4Cl : 5 g/L	$MgSO_4.7H_2O$: 0.5 g/L	
KH_2PO_4: 1 g/L	Rapeseed oil: 5 g/L	Rapeseed oil: 20 g/L	Rapeseed oil: 33.7 g/L
K_2HPO_4: 3 g/L			
$MgSO_4.7H_2O$: 0.1 g/L			

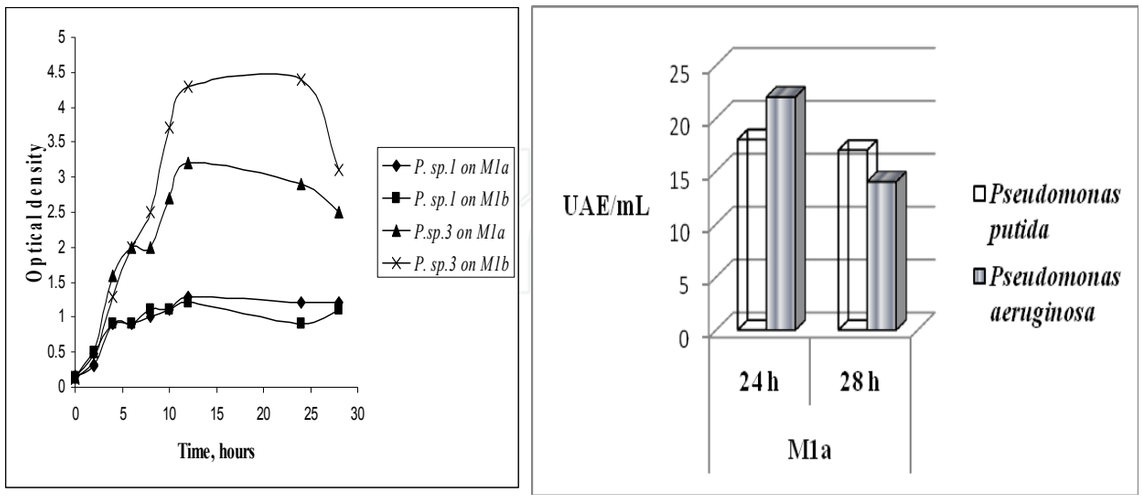
Table 2. Cultivation media composition

The growth characteristics were evaluated by measuring OD_{500}; the lipase activity was determined by using the volumetric method (Tcacenco *et all*, 2010), 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 37°C, pH=7, duration of 60 minutes.
Isolation of extracellular lipase was made by centrifugation (1) and ammonium sulphate precipitation (2): (1) biosynthesis medium was centrifuged at 10 000 rpm for 30 min. at 4 °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 sulphate 30% at 0 °C, then left to stand for 24 hours for achieving precipitation and centrifuged at 10 000 rpm for 30 minutes at 4°C. The supernatant is precipitated again with 75% ammonium sulphate. 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 the freezer.

3.1.2 Results and discussion

1. Bacteria growth and enzyme formation

The growth of both bacteria is low, only *Pseudomonas aeruginosa* (*P. sp.3*) grows more on the medium variant M1b, so the use of both substrates-glucose and oil seems useful. Both bacterium strains have similar small lipase activity levels, the cultivation duration of 24 h being enough for the maximum lipase production, and there is no induction by the rapeseed oil (Fig. 2).

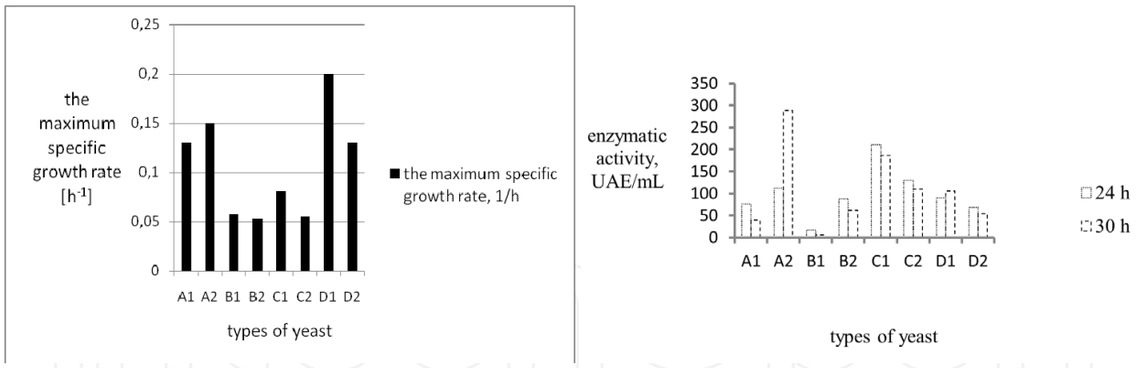


(a) (b)

Fig. 2. Growth and lipase activity of bacteria *Pseudomonas putida* (*P. sp.1*) and *Pseudomonas aeruginosa* (*P. sp. 3*) on cultivation medium M1, a and b; (a) growth (OD); (b) lipase formation

2. Yeasts growth and lipase formation
   Experimental variants:
   A1: *Candida rugosa* DSM 70761 on M1, 48 h
   A2: *Candida rugosa* DSM 70761 on M2, 48 h
   B1: *Pseudozyma aphidis* DSM 70725 on M1, 48 h
   B2: *Pseudozyma aphidis* DSM 70725 on M2, 48 h
   C1: *Candida rugosa* DSM 70761 on M3, 48 h
   C2: *Pseudozyma aphidis* DSM 70725 on M3, 48 h
   D1: *Yarrowia (Candida lypolitica)* ATCC 8661 on M2, 24 h
   D2: *Candida sp. DG 8* on M3, 24 h

For the cultivation medium M2 the growth rate for the yeasts *Candida rugosa* DSM 70761 and *Candida lypolitica* ATCC 8661 were higher and close enough: variant D1 *Yarrowia lipolytica* with the specific growth rate of 0.2 h^{-1} ; variant A2 *Candida rugosa* with the specific growth rate of 0.15 h^{-1}. But the final enzyme activity was higher for the second yeast: *Candida rugosa* final enzymatic activity of 289.0 UAE/mL by comparison with *Yarrowia lipolytica* enzymatic activity of 106.0 UAE/mL. At the same time the growth and lipase activity of both yeasts were much higher than those of the studied bacteria. So the immobilization study was to be performed with these already mentioned yeasts. In a first step, the preliminary transesterification results, obtained by thin layer chromatography, demonstrated that both lipases have high enough catalysis activities. 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.



(a) (b)

Fig. 3. The maximum specific rate and the lipase activity of the yeasts for the experimental variants A_i-D_i (i=1, 2); (a) max specific growth rate (μ^{-1}); (b) lipase activity (UAE/mL) (Chirvase *et all*, 2010)

3.2 Lipase immobilization

3.2.1 Materials and methods

The techniques by physical adsorption were chosen due to the fact they are simple and cheap, so the price of the immobilized biocatalyst is expected to be low.

a. Lipase immobilization by adsorption on silicagel or celite support

The crude lipase obtained from *Yarrowia lipolytica* and *Candida rugosa* yeasts after the precipitation with 70% ammonium sulphate 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 under 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. , 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 washed with H_2O dist. 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 sulphate 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 C_2H_5OH 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. The mixture rested for 30 minutes at 4 ^{0} C. 0.25 g sodium borohydride was added in portions, during 15 minutes, with ice pieces to low the temperature, and finally the mixture was filtrated in vacuum. 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.

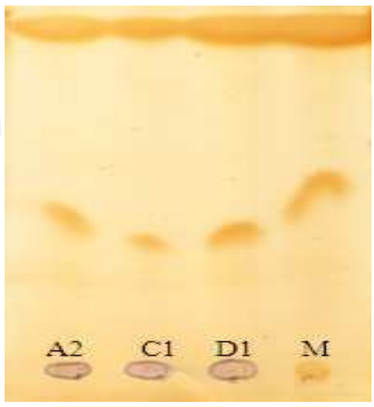
3.2.2 Results and discussion

The final activities and isolation yields obtained when the crude lipases were separated from the cultivation medium by precipitation with ammonium sulphate are presented in the Table 3. High 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 got for both variants (24 hr. and 28 hr.), while when the same procedure was applied for *Candida rugosa* samples the isolation yields were lower: 62% for 24 hr. extract and only 29.6% for 28 hr. extract.

No.	Strain / duration of bioprocessing	Extract volume (mL)	Initial activity (UEA)	Quantity $(NH_4)_2SO_4$ (g)	Final activity (UEA)	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

Table 3. The final activities and isolation yields determined for the crude lipases separated from the cultivation media of the yeasts strains *Candida rugosa* DSM 70761 and *Yarrowia (Candida lypolitica)* ATCC 8661

At a first step the preliminary transesterification results obtained by thin layer chromatography demonstrated both lipases have high enough catalysis activities.



Legend:
A2-lipase from *Candida rugosa DSM 70761*/M2; C1-lipase from *Candida rugosa DSM 70761*/M3; D1-lipase from *Yarrowia lipolytica*/M2; M - Control, ester of oleic acid

Fig. 4. Thin layer chromatography of the products obtained by the transesterification

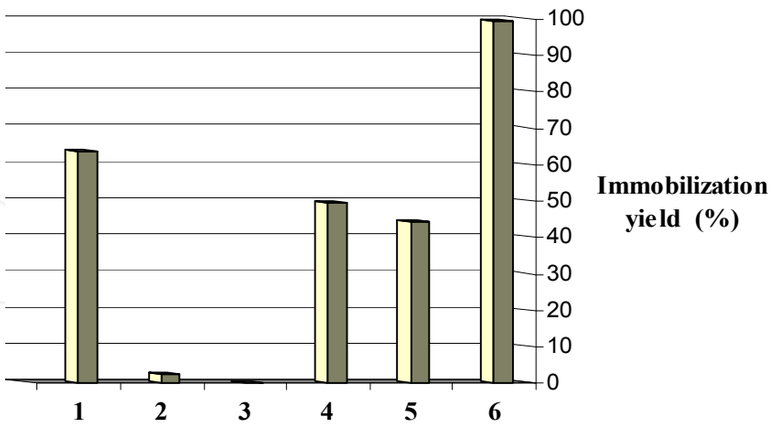


Fig. 5. Immobilization efficiency of the tested lipases (Tcacenco *et all*, 2010)

The experimental results are presented in the Figure 5, obtained with the described immobilization techniques for both crude lipases.
The immobilization techniques, characterized in the following table, were performed in comparison with the immobilization of a lipase from the fungus *Aspergillus niger.*

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

Table 4. Applied immobilization techniques

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 44 - 49% for the same lipase immobilized by adsorption on Celite or Silicagel. On the contrary the immobilization of *Aspergillus niger* lipase gave unsatisfactory results.
In order to improve the immobilization yield of the lipase from the yeast *Candida rugosa* DSM 70761 on Celite support a supplementary treatment with acetone as organic solvent

was done, the obtained results in comparison with the control procedure (without acetone adding) are presented in the following table.

No.	Support	Lipase (mL)	Initial activity		Final activity		Immobilization yield (%)
			UEA/mL	Total activity	UEA/mL	Total activity	
1.	Celite 545	800	362.0	289 600	840.2	142 841.7	49.32
2.	Celite 545 + acetone	1150	182.6	210 000	2537.2	204 246.2	97.26

Table 5. The immobilization yields of the lipase from the yeast *Candida rugosa* DSM 70761 on Celite support with / without acetone treatment

The acetone treatment had as consequence a big improvement of the immobilization yield on Celite from 49% to 97% in case of the lipase from *Candida rugosa* DSM 70761. It seems that the system hydration degree highly increases due to the support treatment with organic solvent, which determines a better adsorption of the enzyme. The improved procedure to get the immobilized biocatalyst was further applied in the research regarding the immobilized enzyme characteristics: static activity, operational activity, and transesterification performance.

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 6.

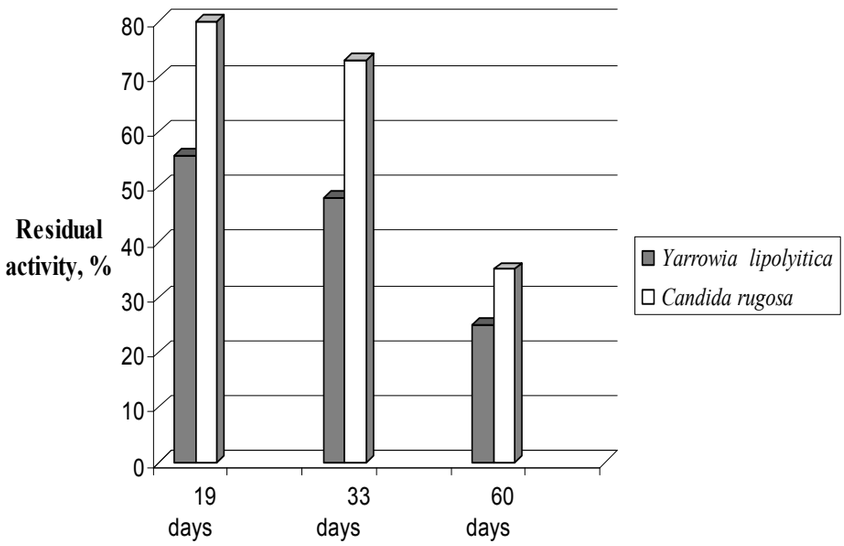


Fig. 6. Static stability determination by yield of the residual activity for the Celite adsorption immobilized lipases from *Yarrowia lipolytica* and *Candida rugosa*

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.

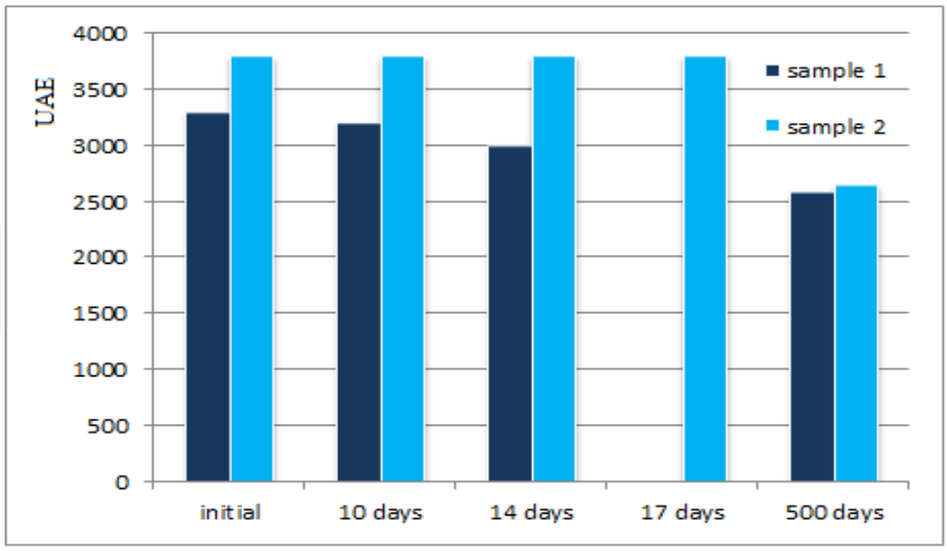


Fig. 7. The effect of the acetone treatment associated to Celite immobilization on the static activity of the immobilized lipase from *Candida rugosa* DSM 70761 (sample 1-no acetone treatment; sample 2-with acetone treatment)

The static stability for the enzyme from *Candida rugosa* DSM 70761 immobilized on Celite with / without acetone treatment was determined for a long period of time, the results being presented in the Figure 7. The biocatalyst obtained with the lipase from the above mentioned yeast immobilized on Celite 545 by physical adsorption with or without organic medium treatment presented a high static stability, when preserved in freezer. The residual activity was as high as 82% after 1 year and half, and after the first 2 weeks the residual activity was practically unchanged in both cases. These findings were considered as a selection criterion between the lipases from the two studied yeasts, so the lipase produced by *Candida rugosa* DSM 70761 with a better static stability was further used to continue the research. Firstly the biocatalyst prepared by the described procedure with the lipase from the yeast *Candida rugosa* DSM 70761 was tested for its operational activity. The test consisted of using the same biocatalyst quantity in several reaction cycles and measuring the enzymatic activity at the beginning and after each reaction phase. The results are presented in the following table.

No. of cycles	Initial activity (UEA/g)	Final activity (UEA/g)	Activity loss (%)
1.	705.00	606.72	13.34
2.	606.72	549.75	9.40
3.	549.75	481.36	12.44
4.	431.36	413.97	14.00

Table 6. Evolution of the operational activity of the lipase from *Candida rugosa* DSM 70761 immobilized on Celite 545

The results from the table indicate a biocatalyst half time of 5-6 reaction cycles, because after 4 cycles the residual activity was 58.71%.

3.3 Lipase transesterification

3.3.1 Materials and methods

The experimental study was done with rapeseed oil of Romanian origin or soya oil and by using the lipase from the yeast *Candida rugosa* DSM 70761 obtained in aerobic bio processing, isolated from the cultivation medium and immobilized by adsorption on Merck Celite support (lipase activity of 4701 UEA / g support).

The transesterification was done in two variants: (a) in anhydrous medium without organic solvents adding; (b) in hexane (Biosolve).

The experimental working procedure was: the transesterification reaction was performed in Erlenmayer flasks of 100 mL, containing the tested vegetable oil in a concentration to determine a final triglycerides content of 0.08 mol / L and methanol (this last reagent in molar ratios between 3:1 and 8:1 with the triglycerides substrate). The immobilized enzyme was added in a chosen concentration after a period of 30 minutes at 37^0C. The reaction was done with continuous mixing of 250 rpm. Each 4 hours' sample from the liquid was analysed by thin layer chromatography and gas chromatography to determine the reaction advancement.

a. Thin layer chromatography was done by using the Silicagel G on Al support as stationary phase and the migration solvent was a mixture of petroleum ether: ethylic ether: acetic acid = 80:30:1; the spots were put into evidence in a iodine vapour atmosphere.
b. The fatty acids content in methylic esters was analysed by gas chromatography (GC) using a capillary column with a stationary phase composed from 5% phenyl – 95% methylpolysilane.

The apparatus was a gas chromatograph 6890N – AGILENT with FID detector and autosampler 7683B; column HP 5, L=30m; φ=0.32mm.

Reagents: N-hexane; the methylic esters of several fatty acids mostly presented in the vegetable oils (rapeseed oil or soya oil).

Working conditions:

- column temperature: initial temperature of 160°C, during 2min.; final temperature of 240°C, during 5min.; heating rate of 5°/min.
- injection temperature of 280°C.
- detector temperature of 300 ^{0}C.
- nitrogen flowrate of 2.0 mL / min.
- hydrogen flowrate of 40 mL / min.
- air flowrate of 370mL / min.
- nitrogen flowrate (make-up) of 25 mL / min.
- sample volume of 1µl.
- analysis duration of 23 min.

The evaluation is done by the determination of the content in palmitic, oleic, arachidonic and erucic acids. The external standard method is applied.

Three experimental models were studied:

a. Batch enzymatic transesterification with methanol and without organic solvent, characterised by : vegetable oil concentration of 0.09 M; methanol concentration of 0.54 M (ratio of 8:1 methanol: triglycerides substrate); biocatalyst concentration of 5000 UEA / 100 mL reaction medium; reaction temperature of 37^0C; mixing of 250 rpm; reaction total duration of 24 hr.

b. "Semi-batch" enzymatic transesterification with methanol and without organic solvent, characterised by the same reaction conditions, except the fact that the methanol is added two times, each addition realizing a ratio between alcohol and the triglycerides substrate of 4:1.
c. Batch enzymatic transformation in hexane characterized by: vegetable oil concentration 0.09M; methanol concentration 0.09 M; biocatalyst concentration 5000 UEA / 100 mL reaction medium; reaction temperature of 37^0C; mixing of 250 rpm; reaction total duration of 24 hr.

3.3.2 Results and discussion

The most important transesterification results are presented in the Figure 8.

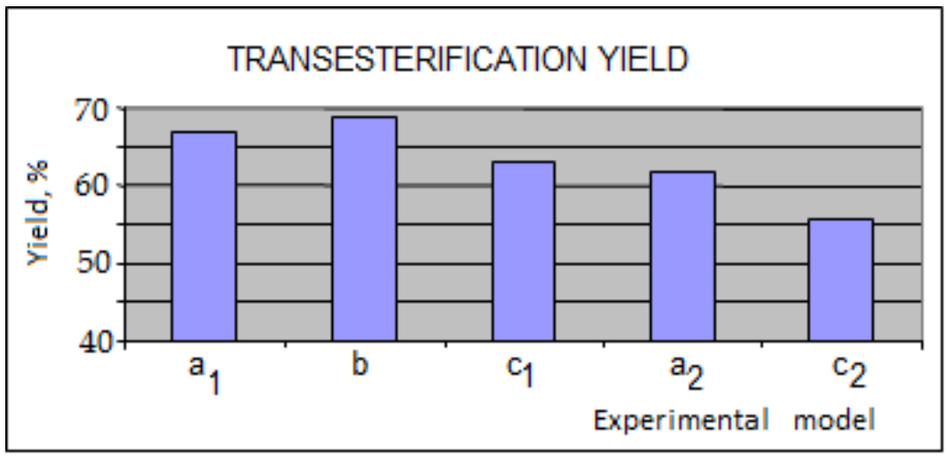


Fig. 8. The vegetable oils transesterification yield for the 3 experimental models: a_1-batch model without solvent, with soya oil; a_2-batch model without solvent, with rapeseed oil; b-semibatch model without solvent, with soya oil; c_1-batch model in hexane with soya oil; c_2-batch model in hexane with rapeseed oil

The transesterification was realised with the following biotransformation yields: 56-67 % for the experimental variant a, 69% for the variant b, and 56-63% for the variant c; these results can be improved by adequate optimization procedures to be applied for each technological phase, comprising enzyme obtaining in aerobic bioprocess, lipase immobilization and transesterification performing.

4. Conclusions

1. The main objectives of the research to replace the actual chemical transesterification with the enzymatic process are: (a) the preparation of cheap and stable immobilized lipases; (b) the realization of biotransformation systems characterized by the biocatalyst long use in many reaction cycles. One of the raisons to choose between extracellular or intracellular lipases is the immobilization of extracellular enzymes by physical adsorption, a low price technology, but imposing to improve the shorter duration. Moreover these lipases are normally biosynthesized by bacteria or yeasts, easier to cultivate in aerobic bioprocess than the intracellular lipases producing' fungi.

2. The lipases with advanced specificity are not useful in the transesterification to produce biodiesel; the most recommended are the lipases with reduced region specificity, but more developed specificity for the substrate.
3. The molar ratio of the substrates used in the biotransformation of vegetable oils to biodiesel must be determined for each studied system: alcohol – oil – lipase.
4. The rapeseed oil is of interest as raw material in the transesterification, as it is largely produced by the European agriculture and also in Romania, and at the same time it is used in the alkaline catalysed transformation. But in USA the soya oil is in charge.
5. The aerobic bio processing of several bacteria and yeasts from Romanian research collections or from international collections demonstrated that two yeasts, *Candida rugosa* DSM 70761 and *Yarrowia lipolytica* ATCC 8661 produced lipases characterized by high activity in simple and short duration cultivation. The media composition and the cultivation parameters were optimized for both yeasts' lipases formation.
6. The immobilisation techniques by physical adsorption were studied for the lipases from the above mentioned yeasts. First of all the extracellular lipases from the yeasts *Candida rugosa* DSM 70761 and *Yarrowia lipolytica* ATCC 8661 can be easily separated in the liquid fraction by centrifugation and further on the crude enzymes can be obtained by ammonium sulphate precipitation. 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 44 - 49% for the same lipase immobilized by adsorption on Celite or Silicagel.
7. As the lipase from the yeast *Candida rugosa* DSM 70761 was immobilized on Celite 545 support with yields of 49 – 63%, and higher yields are obtained for the immobilization of the lipase from *Yarrowia lipolytica* ATCC 8661, and the immobilization procedure is easy and low price, the laboratory experimental model was developed on this support.
8. In order to improve the immobilization of the lipase of *Candida rugosa* DSM 70761, a treatment with acetone as organic solvent was introduced and this operation had as consequence a big increase of the immobilization yield on Celite from 49% to 97%.
9. A higher static 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. The residual activity was as high as 82% after 1 year and half, and after the first 2 weeks the residual activity was practically unchanged for the first biocatalyst. These findings were considered as a selection criterion between the lipases from the two studied yeasts, so the lipase produced by *Candida rugosa* DSM 70761 with a better static stability was further used to continue the research.
10. This biocatalyst operational stability was also tested and the immobilized enzyme half time was of about 5-6 reaction cycles, as after 4 reaction cycles the residual activity was still 58.7%.
11. Three experimental models were considered to perform the transesterification: (a) batch enzymatic transesterification with methanol and without organic solvent; (b) "semi-batch" enzymatic transesterification with methanol and without organic solvent; (c) batch enzymatic transformation in hexane. The reaction yields were good enough for all the tested experimental models and for both -soya and rapeseed oils, the results variation being in the range of 56 – 69%. They can be improved by adequate

optimization procedures to be applied for each technological phase, comprising enzyme obtaining in aerobic bioprocess, lipase immobilization and transesterification.

Further research work is to be developed in two directions: (1) the use of the glycerol formed as by product in the transesterification process, especially as C source in several other bioprocesses; (2) as beside this product there are several others, the most important future research direction will be the technical application of the bio refinery concept realised for the vegetable oils extracted from many plants specific to each geographical area. A possible future bio refinery will integrate physical, chemical, and biological procedures for the biodiesel preparation, conversion of solid residue with high carbohydrates or protein content; glycerol use, the whole application being characterised by both high economic efficiency and reduction of solid or liquid residues.

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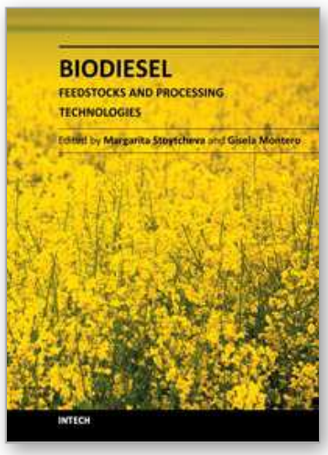
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Biodiesel - Feedstocks and Processing Technologies

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The book "Biodiesel: Feedstocks and Processing Technologies" is intended to provide a professional look on the recent achievements and emerging trends in biodiesel production. It includes 22 chapters, organized in two sections. The first book section: "Feedstocks for Biodiesel Production" covers issues associated with the utilization of cost effective non-edible raw materials and wastes, and the development of biomass feedstock with physical and chemical properties that facilitate it processing to biodiesel. These include Brassicaceae spp., cooking oils, animal fat wastes, oleaginous fungi, and algae. The second book section: "Biodiesel Production Methods" is devoted to the advanced techniques for biodiesel synthesis: supercritical transesterification, microwaves, radio frequency and ultrasound techniques, reactive distillation, and optimized transesterification processes making use of solid catalysts and immobilized enzymes. The adequate and up-to-date information provided in this book should be of interest for research scientist, students, and technologists, involved in biodiesel production.

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