

# Determination of Yeast Strains Characteristics as Lipase Providers for Enzymatic Transesterification to Biodiesel

ANA AURELIA CHIRVASE<sup>1</sup>, CAMELIA UNGUREANU<sup>2\*</sup>, LUMINITA TCACENCO<sup>3</sup>, NICOLETA RADU<sup>1</sup>

<sup>1</sup>National Research & Development Institute for Chemistry and Petroleum Chemistry, ICECHIM, 202 Splaiul Independenței, 060021, Bucharest, Romania

<sup>2</sup>University Politehnica of Bucharest, 313 Splaiul Independenței, 060042, Bucharest, Romania

<sup>3</sup> National Research & Development Institute of Biological Sciences, Bucharest, Romania

Recently, enzymatic transesterification using lipase has become more attractive for biodiesel fuel production, since the glycerol produced as a by-product can be easily recovered and the purification of the fatty methyl esters is simple to accomplish. Preliminary research work done on testing several yeasts strains as lipase producers demonstrated the most promising were the yeasts *Yarrowia lipolytica* ATCC 8661 and *Candida rugosa* DSM 70761. The paper presents the results of their complete testing to investigate the conditions of lipase formation and the obtained enzymes capacity to catalyze the vegetable oils transesterification to biodiesel. The research methodology comprises: design of cultivation medium and bioprocessing conditions; determination of yeast specific growth rate and lipase activity; isolation of extracellular lipase by centrifugation and ammonium sulfate precipitation; crude enzyme testing in transesterification process.

Keywords: biodiesel, *Candida rugosa*, *Yarrowia lipolytica*, transesterification

Biodiesel is composed of the mono alkyl esters from the long chain fatty acids derived from vegetable oils or animal fats, for use in compression-ignition (diesel) engines [1,2]. Enzymatic technology seems to be starting its application on industrial scale. Recently, it has been claimed that this technology has been applied in a first plant with a capacity of 20,000 t/year in China, and this is the first industrial scale application with lipase as the catalyst in the world to date [3,4]. 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) from the already described microbial producers *Candida rugosa*, *Candida utilis*, *Candida antarctica* and *Pseudomonas cepacia*; (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 major technical disadvantages of the enzymatic process are the slower reaction rate by comparison with the alkaline catalysis and the risk of enzyme inactivation.

In a recent review with focus on process design and economy, the researchers calculate the productivity (kg biodiesel/kg enzyme) based on information from six different studies and consider a range of enzyme prices from 12 to 185 USD/kg as acceptable, depending on the productivity in the application, i.e. per each kg of biodiesel a biocatalyst cost of USD 0.025 could be of economic interest [5,6]

Moreover enzymatic transesterification has become more attractive for biodiesel fuel production [7], since the glycerol produced as a by-product can be easily recovered and the purification of the fatty methyl esters is simple to accomplish [8,9].

## Experimental part

### Materials and methods

The yeasts cultivation conditions were: temperature of 30°C; Erlenmeyer flasks of 300 mL with 100 mL medium; rotary shaker New Brunswick Innova 40 at 300 rpm.

### Experimental variants: Microorganisms and cultivation media

- 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 lipolytica)* ATCC 8661 on M2, 24 h
- D2: *Candida sp.DG 8* on M3, 24 h

### Cultivation media compositions:

	M1		M2		M3
Malt extract	3.78 g/L	NH <sub>4</sub> Cl	5.0 g/L	KH <sub>2</sub> PO <sub>4</sub>	5.00 g/L
Peptone	5.00 g/L	Yeast extract	10.0 g/L	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	1.00 g/L
Tween 80	4.33 g/L	Peptone	10.0 g/L	MgSO <sub>4</sub> ·7H <sub>2</sub> O	0.50 g/L
Rapeseed oil	33.70 g/L	Glucose	10.0 g/L	Yeast extract	10.00 g/L
		Rapeseed oil	5.0 g/L	Rapeseed oil	20.00 g/L

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°C. Clear supernatant is treated with benzamidine 2 mM and sodium azide 0.02% to prevent proteolysis and microbial attack;

2) the supernatant is precipitated with ammonium sulphate 30% at 0 °C, then left to stand for 24 h for achieving precipitation and centrifuged at 10 000 rpm for 30 min at

\*email: ungureanucamelia@gmail.com; Tel.: 0723239120

40°C. The supernatant is precipitated again with 75% ammonium sulphate. After 24 h one centrifuges the sample again and the resulting product is resuspended in 8 mL TRIS buffer, pH 6.8. This crude enzyme is preserved in the freezer, and then one determines the lipase activity by using the volumetric method.

The transesterification to biodiesel was studied in a mixture consisting of 1.22 g ammonium sulfate sample, 2 mM oleic acid 0.564 g, 0.5929 mL butanol and 2.6 mL hexane; this mixture was incubated at 37°C for 5 h in a horizontal shaker at 150 rpm. The reaction was stopped by using 20 mL of stop mixture consisting of acetone-ethanol (1:1) (v/v).

## Results and discussions

Previous research work [11] done on testing several yeast strains as lipase producers demonstrated the most promising were the yeasts *Yarrowia lipolytica* ATCC 8661 and *Candida rugosa* DSM 70761.

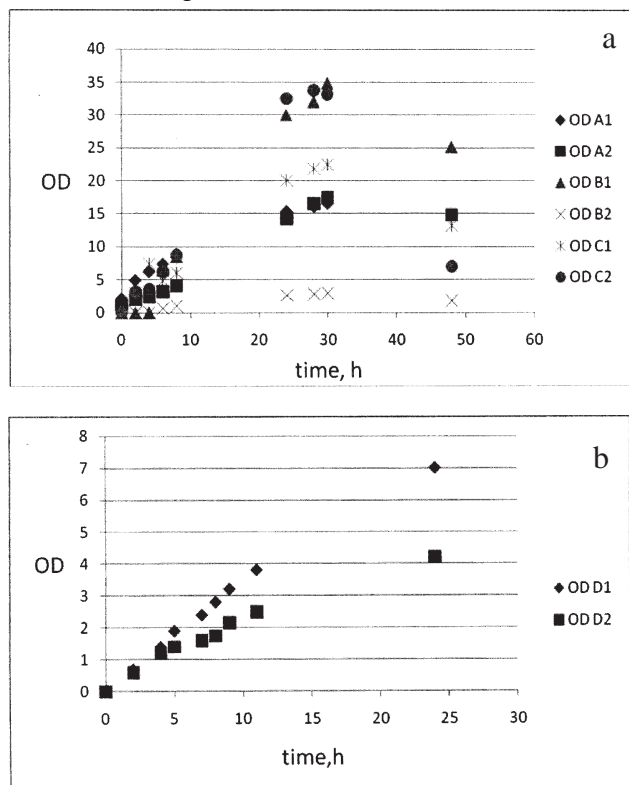


Fig. 1. Optical density (OD) versus time – a) Ai, Bi, Ci, variants b) Di variant (i=1,2)

The paper presents the results of their complete testing together with other interesting strains to investigate the conditions of lipase formation and the enzymes capacity to catalyze the rapeseed oils transesterification to biodiesel. The research methodology comprised: design of cultivation medium and bioprocessing conditions; determination of yeast specific growth rate and lipase activity.

The maximum specific growth rate  $\mu_m$  [ $h^{-1}$ ] was calculated for 8 representative runs (A<sub>i</sub>÷D<sub>j</sub>) in accordance with an exponential model [10] considering X-cells' concentration:

$$\frac{dX}{dt} = \mu_m X \quad (1)$$

After the integration and the linearization the equation (1) becomes:

$$\ln \frac{X}{X_0} = \mu_m t \quad (2)$$

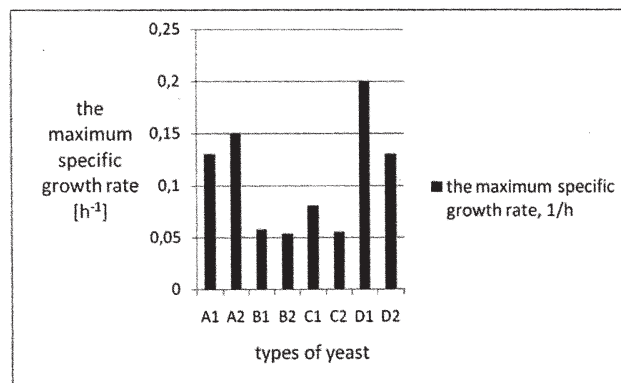


Fig. 2. The maximum specific growth rate of the studied yeasts

For the same balanced cultivation medium the growth rate for two yeasts were close enough (*Yarrowia lipolytica* (D1): specific growth rate of  $0.2 h^{-1}$  and *Candida rugosa* (A2): 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.

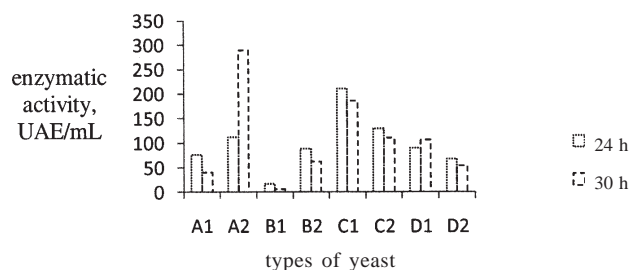


Fig. 3 Enzymatic activity of the studied yeasts

The interesting results are probably due to the M2 medium composition, a nutrient rich formula: both types' sources of N (organic and mineral); two C sources-carbohydrate for microbial growth and rapeseed oil for enzyme induction.

At the same time the transesterification results obtained by thin layer chromatography demonstrated both lipases have high enough catalysis activities.

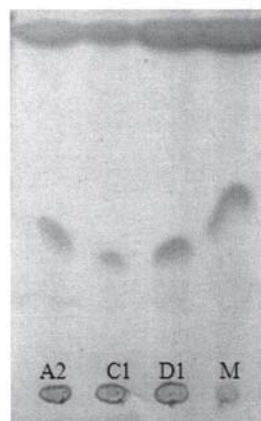


Fig. 4 Thin layer chromatography A2-lipase from *Candida rugosa* DSM 70761/M2; C1-lipase from *Candida rugosa* DSM 70761/M3; D1- lipase from *Yarrowia lipolytica*/M2; M - Control, ester of oleic acid

The UV absorption spectra of the reaction product formed with the mixture n-butanol/hexane as substrate under the action of the lipase crude extract clearly put into evidence the substrate modification: if initially substrate presents two peaks in the range of wavelength 210 - 220 nm (control spectrum), after the catalysis action of the 2 samples of crude lipase extracts obtained from the experimental cultivation variants A2 and D1, the substrate

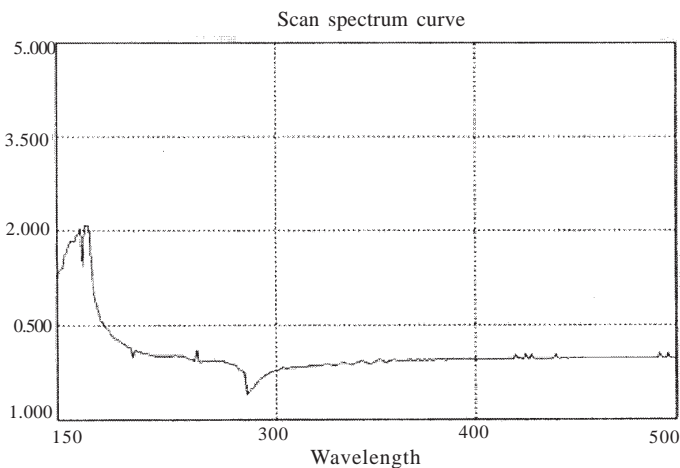
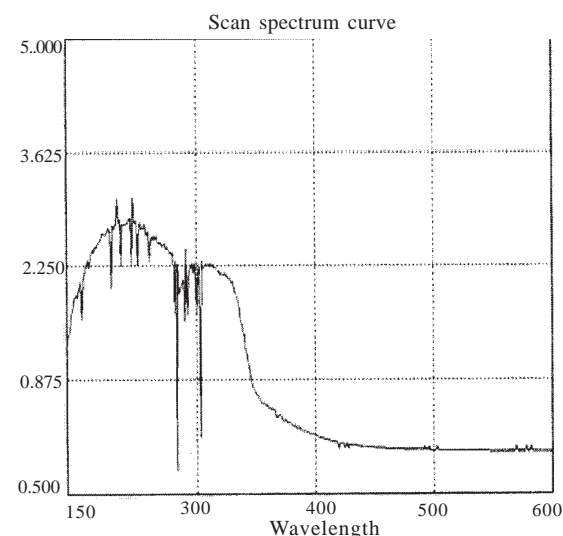
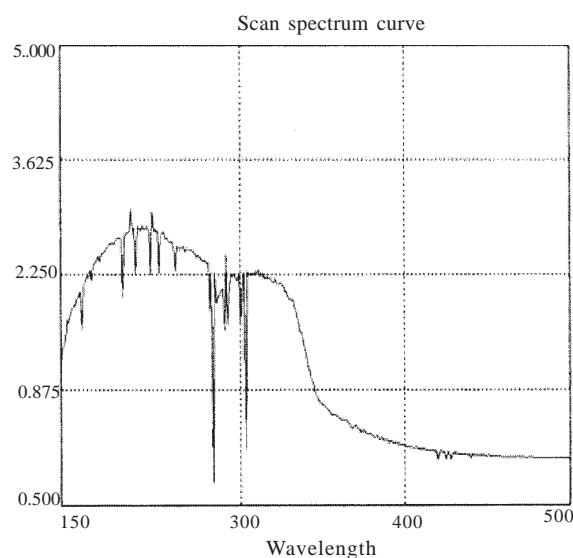


Fig. 5. Adsorption UV spectrum for blank (control)



a) substrate after catalysis with lipase prepared in cultivation variant A2



b) substrate after catalysis with lipase prepared in cultivation variant D1

Fig.6. Modification of substrate adsorption UV spectra after the lipase catalysis

spectra are moving to the range of 250-300 nm, which means that the substrate was modified. During a next phase of research the obtained spectra will be compared with the spectrum of a witness methyl ester.

### Conclusions

The research work performed to test several yeasts strains as lipase producers to be used to catalyze the rapeseed oils transesterification to biodiesel demonstrated the most promising were the yeasts *Yarrowia lipolytica* ATCC 8661 and *Candida rugosa* DSM 70761. For the same rich cultivation medium with mineral and organic N and glucose and rapeseed oil as C substrates the growth rate of both yeasts were close enough (*Yarrowia lipolytica* specific growth rate of  $0.2 \text{ h}^{-1}$  and *Candida rugosa* specific growth rate of  $0.15 \text{ h}^{-1}$ ), 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 preliminary transesterification results obtained by thin layer chromatography demonstrated both lipases have high enough catalysis activities.

### Notation

X - cell concentration, [g/L]  
 $X_0$  - initial cell concentration, [g/L]  
 t - time, h

OD - optical density

### Greeks

$\mu_m$  - maximum specific growth rate,  $\text{h}^{-1}$   
 $\lambda$  - wavelength, nm

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