# Production of lipase by a strain of the non-conventional yeast *Yarrowia lipolytica* and isolation of crude enzyme

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## Abstract

*Fermentation experiments were performed at laboratory level, in shake flasks and batch culture system. Lipase production by a wild strain of Yarrowia lipolytica was investigated in cultivation media containing: glucose as carbon and energy source, peptone bacto and yeast extract as the most suitable nitrogen sources, mineral salts ($MgSO_4$, $KH_2PO_4$) and olive oil (0,05 - 2%). The study was focused on the dependence of lipase biosynthesis on aeration and olive oil concentration. Four levels of aeration and five variants of the above mentioned cultivation medium were compared with respect to lipase production and the optimum values of these parameters were established.*

*Precipitation with ethanol was used to isolate crude lipase from the fermentation liquid, after removing the biomass by centrifugation. Aiming to recover the native enzyme as much as possible, our experiments pointed out the optimal conditions for lipase precipitation with ethanol, taking into account the influence of the following parameters: enzymatic solution/ethanol ratio, solvent-enzyme contact duration, precipitation temperature, $Ca^{2+}$ concentration as stabilizer-activator.*

Keywords: fermentation, biosynthesis, C sources, isolation, crude enzyme

## Introduction

Lipases (triacylglycerol acylhydrolases - E.C. 3.1.1.3) are enzymes which catalyses the hydrolysis of esters of glycerol and other alcohols with fatty acids. The hydrolysis of ester bonds takes place at the interface between a lipid substrate phase and the aqueous phase in which the enzyme is dissolved. Lipases show both regional and stereo specificity with respect to the alcohol moiety of their substrates [1] and can be divided into two groups on the basis of the regional specificity exhibited with respect to the position of the ester group form the triacylglycerol substrates [2]. Usual industrial lipases act on fats and oils and hydrolyze them gradually into di- and monoacylglycerols and finally into glycerol and fatty acids. In the absence of water, they are capable of reversing the reaction, leading to esterification. Due to their ability to utilize a wide spectrum of substrates, and also to their chemo-, regio- and enantioselectivity, lipases stand amongst the most important biocatalysts used in organic synthesis for several reactions, such as hydrolysis, esterification and transesterification. [3,4]. Based on these reactions, lipases have a great potential for industrial applications in food, chemical, pharmaceutical or detergent industry [5,6]. Promising fields include the biodegradation of plastics [7], such as polyhydroxyalcanoate and polycaprolactone.

Due to their biotechnological interest, many of these enzymes have been identified cloned and characterized [8]. Nevertheless, the demand for the production of highly active preparations of lipolytic enzymes has led to research on lipase producing micro-organisms and culture strategies [9,10]. Among these micro-organisms, *Yarrowia lipolytica* is one of a great interest, being able to naturally secrete several enzymes, including lipase, depending on the growth conditions [11].

*Yarrowia lipolytica* is the most extensively studied “non-conventional yeast”, being currently used as a model for the study of protein secretion, cell dimorphism, degradation of hydrophobic substrate, including triglycerides etc [12 – 14]. Being strictly aerobic yeast, its growth and lipase secretion are affected by the amount of oxygen available in the culture medium; therefore one of the purpose of our study was to point out the influence of aeration on the biosynthesis of lipase. Extra cellular lipase production by this micro-organism depends also on the composition of the medium, so that another purpose of our study was to establish the optimum concentration of olive oil as an inductor of the enzyme.

Multi-step procedures [15] are applied to isolate and purify the microbial lipases, usually based on non-specific techniques, beginning with ammonium sulphate precipitation, followed by gel permeation and ion exchange chromatography. Most of the commercial lipases available today are crude preparations, which are why our study aimed to investigate the precipitation of crude lipase from *Yarrowia lipolytica* with ethanol, as a first step for further purification.

## Materials and methods

**Materials:** Natural components of nutritive media were purchased from Merck and Oxoid, organic solvents, analytical reagents and mineral salts, from Merck.

**Micro-organism**

A laboratory strain (wild type) obtained from Genetic Laboratory of the University of Bucharest was used. Stock culture were grown at $28^0$C and maintained by periodic transfer on YPD slant agar slants, with the following composition (% g/v): glucose-2, peptone bacto-2, yeast extract-1, agar-1,5, pH 6 and conserved under mineral oil, at 4℃.

**Inoculum growth medium and cultivation conditions**

A microbial suspension in sterile distilled water was prepared from the stock culture. 10 mL of a medium containing (% g/v): yeast extract – 1, peptone – 2, glucose – 2, $MgSO_4 \cdot 7H_2O$ – 0,04, $KH_2PO_4$ – 0,2 (medium A), previously sterilized at $120^0$C, for 20 minutes (glucose and mineral salt solutions were autoclaved separately) was inoculated with 1 mL of the above-mentioned microbial suspension, containing $10^7$ cfu/ml. The inoculum (preculture) was prepared in 50-mL Erlenmeyer flasks, by incubation at $28^0$C, for 20 – 24 hours, on a laboratory rotary shaker (Heidolph – Germany) at 240 rpm.

**Fermentation conditions**

Fermentations were carried out batch wise in 500 mL Erlenmeyer flasks, containing 50, 75, 100, 125 mL of a medium composed of the same nutrients as the inoculum growth medium (A) and, in some experiments, 0,05 – 2% v/v olive oil. The sterilized fermentation medium (as mentioned above) was inoculated with 10 % (v/v) preculture and incubated at $28^0$C, for 48 – 72 hours, on a rotary shaker, at 240 rpm. Aerobic yeast growth and lipase biosynthesis was determined by measuring the OD and lipase activity at different time intervals.

**Biomass separation**

At the end of fermentation process, the cultivation medium with lipolytic activity was centrifuged during 10 minutes at 4000 rpm, in a centrifuge (Hettich – Germany). The sediment was washed with 2 volumes of physiological saline and the obtained suspension was centrifuged again, in the same conditions. The two supernatants were mixed together.

**Supernatant concentration**

The total supernatant was concentrated in a rotary evaporator (Heidolph – Germany) under reduced pressure, to 1/3 from initial volume, at maximum $35^0$C. At the end of concentration, the lipase activity of the solution was determined.

**Lipase precipitation with ethanol**

Different volumes of ethanol were added to the concentrated enzyme solution, at different temperatures, under stirring and the hydro alcoholic solutions were maintained in the same conditions many hours.

The precipitates were filtered under reduced pressure, washed with hydro-alcoholic solutions of the same concentrations as those of the precipitation media and dried under reduced pressure, at room temperature.

**Lipolytic activity determination**

Lipase activity was measured by a titrimetric assay with NaOH 0,1 N [16], using emulsified olive oil as the substrate. 1- 5 mL of enzyme solution, 5 ml 10 mM citrate buffer pH = 7 and 2 mL $CaCl_2$ 0,6% in the above mentioned buffer solution were added to 10 ml emulsion containing 25% (vol./vol.) olive oil and 75% (vol./vol.) gum arabic. The assay was carried out at 37°C during a 60-minute incubation. After this time interval, the reaction was stopped by adding 20 ml acetone-ethanol 1:1 (vol. /vol.) and the amount of fatty acids was then titrated.

One unit of lipase activity was defined as the amount of enzyme that released 1 μ equivalent of carboxyl groups of fatty acid under analysis conditions (temperature = $37^0$C, pH = 7, reaction time = 60 minutes).

**Other analytical methods**

Biomass concentration was determined measuring the optical density (OD) of the fermentation medium by visible spectrophotometry (Helios γ – Thermo Electron Corporation-USA) at 570 nm.

Protein concentration was determined according to the method of Lowry et al. [17].

## Results and discussions

**Influence of aeration on cell growth and lipase biosynthesis**

The study of this parameter was performed in fermentation experiments carried out in 500-mL Erlenmeyer flasks, using 50, 75, 100 and 125 mL nutrient medium A with 2% olive oil, under the following conditions: inoculum concentration = 10% v/v, temperature = $28^0$C, incubation on rotary shaker for 48 hours, at 240 rpm.

An increase in the cell mass and lipase activity was observed (figure 1) as a result of a stronger aeration (lipase activity = 3 U/ml and OD = 1,96 – 1,36, in the experiments with 50 and 75 mL cultivation medium/500-ml Erlenmeyer flasks, towards 2,4 U/ml and OD = 1,11 – 1, in the experiments with 100 and 125 mL cultivation medium/500-ml Erlenmeyer flasks). Therefore, an aeration coresponding to a ratio of 1:10 fermentation medium volume: flask volume seemd to be the best for enzyme biosynthesis.

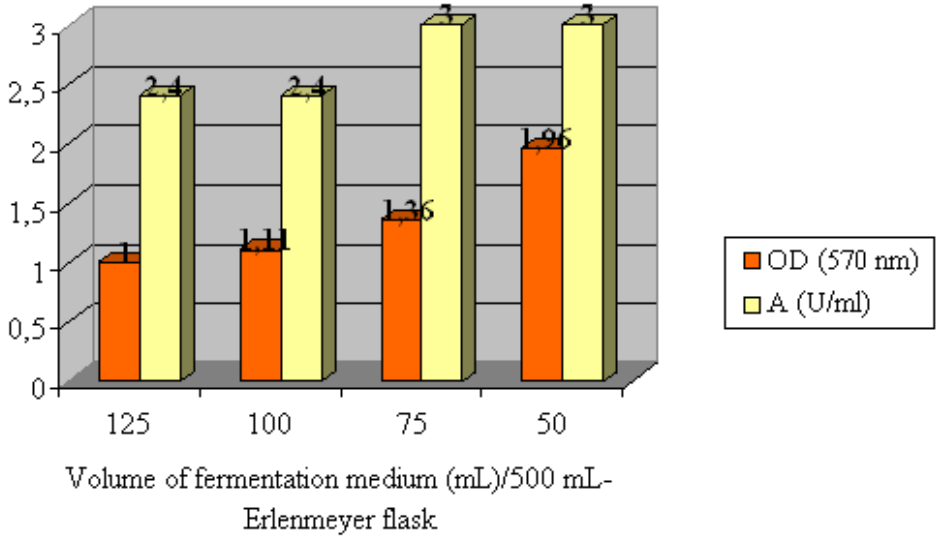


**Figure 1.** Influence of aeration on cell growth and lipase activity in fermentation medium

**Influence of olive oil concentration on cell growth and lipase biosynthesis**

Four variants of fermentation medium containing glucose as carbon source and different concentration of olive oil as well as a fermentation medium without glucose but with 2% (v/v) olive oil (table 1) were tested for the cultivation of the *Yarrowia lipolytica* strain in the following conditions: 50 mL fermentation medium/500-mL Erlenmeyer flask, inoculum concentration = 10% v/v, temperature = $28^0$C, incubation on rotary shaker for 48 hours, at 240 rpm.

**Table 1.** Variants of fermentation media for lipase biosynthesis by *Yarrowia lipolytica* strain

| No. of variant | Glucose 2% (g/v) | Olive oil conc. % (v/v) |
|---|---|---|
| 1. | + | 0.05 |
| 2. | | 0.10 |
| 3. | | 0.50 |
| 4. | | 2.00 |
| 5. | - | 2.00 |

The results obtained in these experiments (figure 2) pointed out that lipolytic activity in the above mentioned cultivation conditions decreases with the increase of olive oil concentration from 0,05% to 2% (v/v). A maximum enzyme concentration of 12 U/mL was obtained in the fermentation medium with 2% glucose and 0,05% olive oil.

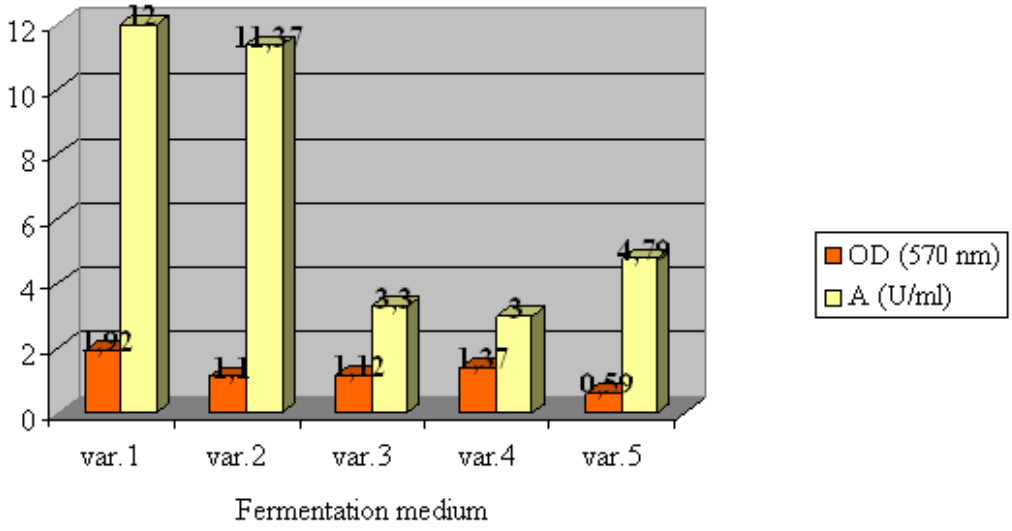


**Figure 2.** Influence of medium composition on cell growth and lipase activity

Comparing figures 1 and 2, it can be seen that the replacement of glucose with olive oil, in the same concentration of 2% (v/v), had a favourable effect on lipase biosynthesis, which increased from 3 U/mL to 4,79 U/mL, even if cell growth was lesser.

**Post-biosynthesis processing for isolation of crude lipase**

In order to establish reproducible conditions for a first step isolation of extra cellular lipase from the cultivation medium, we applied the method of enzyme precipitation with ethanol from a concentrated aqueous solution of 36 U/ml lipase activity. To recover the native enzyme as much as possible, we studied some factors that influence this process, taking into account that enzyme activity may be significantly affected by the organic solvent concentration and, on the other hand, activated by divalent cations, such as $Ca^{2+}$ [4]. Therefore, to point out the optimal conditions for lipase precipitation with ethanol, our experiments aimed to study the influence of the following parameters:

- enzymatic solution/ethanol ratio;
- solvent-enzyme contact duration;
- temperature;
- $Ca^{2+}$ concentration

Concentrated solution with 36 U/ml lipolytic activity was treated with ethanol under the following conditions:

- continuous agitation;
- aqueous solution / solvent ratio: 1:0,5; 1:; 1:2; 1:3; 1:4; 1:5;
- contact duration with solvent: 1 hour, 2 hours, 3 hours;
- temperature during precipitation: $+4^0C$, $+20^0C$, $+30^0C$
- $Ca^{2+}$ concentration (as $CaCl_2$ solution): 0,01%; 0,05%; 0,10%; 0,15%; 0,20%; 0,25% (with respect to the volume of reaction medium).

**The influence of ethanol concentration**

Precipitation was conducted at $+4^0C$, during 4 hours, under stirring. As it can be seen in figure 3, the highest lipolytic activity of the dry enzymatic powder (2400 U/g) was obtained when a ratio of 1/ 3 enzyme solution /solvent was used.

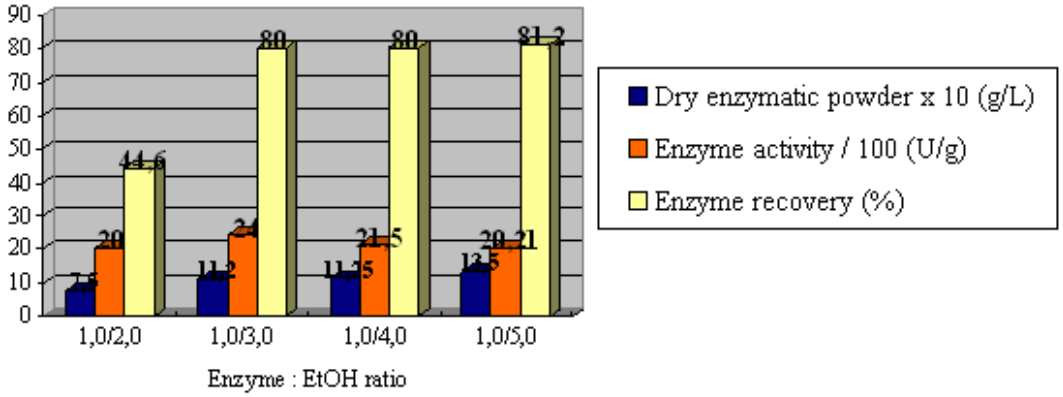


**Figure 3.** The influence of ethanol concentration on lipase isolation.

Although the enzyme recovery yields were similar or even higher (81,2%) when ratios of 1/4 and 1/5 enzyme solution/EtOH were used, the specific lipase activity was lower. This means that increasing of the EtOH volume determined the precipitation of other substances together with lipases.

**Time dependence of lipase precipitation**

Study of this parameter was performed in four experiments in which 3 volumes of EtOH per 1 volume of concentrated enzyme solution were used and the mixtures were kept under stirring at $+4^0$C, for 1 – 3 hours. The obtained results indicate that, after 2 hours, lipase activity and recovery yield are higher than those obtained after 1 hour and equal to those obtained after 3 hours (figure 4). Therefore, we concluded that 2 hours of stirring at $+4^0$C, after EtOH treatment, are enough for complete precipitation of lipase from concentrated aqueous solution.

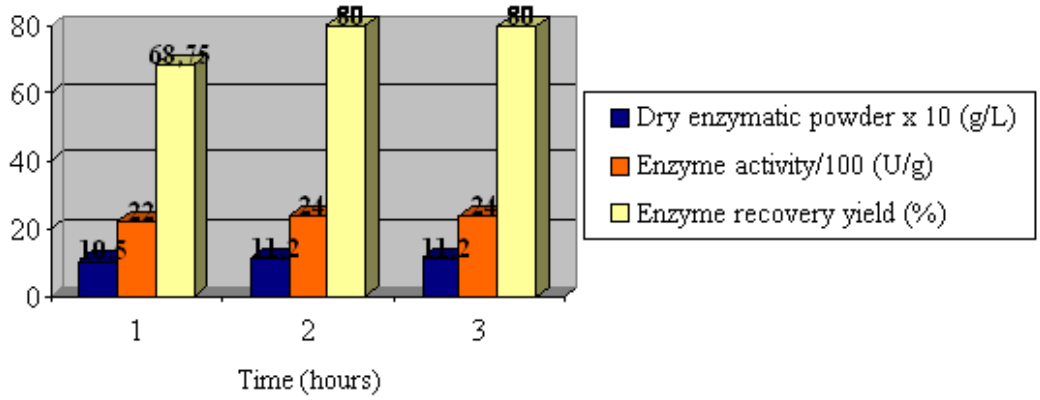


**Figure 4.** Time evolution of lipase precipitation

**The influence of temperature on enzymatic activity and enzyme recovery yield**

In order to establish the dependence of enzymatic activity and enzyme recovery yield on precipitation temperature, 3 volume of ethanol were added to 1 volume of concentrated enzyme solution under stirring at different temperatures, during 2 hours. The results obtained pointed out the positive influence of a lower temperature ($+4^0$C). Thus, lipase activity of the product obtained by precipitation at $+4^0$C was 2400 U/g compared to 2350 U/g obtained at $20^0$C-$30^0$C. The quantity of precipitated enzyme is also important, as it is proved to be higher at $+4^0$C, compared to $20^0$C - $30^0$C, leading to the maximum enzymatic activity recovery yield.

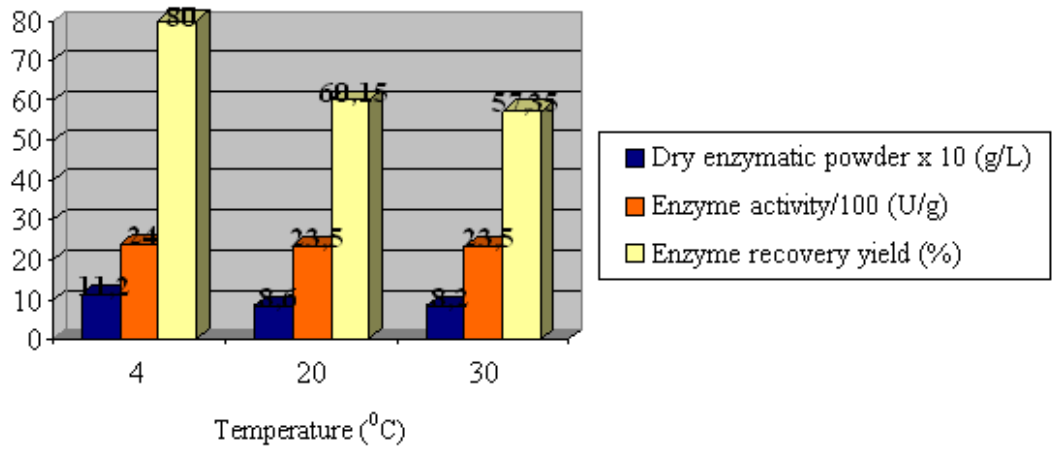


**Figure 5.** Influence of temperature on lipase precipitation

**The influence of $Ca^{2+}$ on enzymatic activity and enzyme recovery yield of lipase**

Precipitation of enzymes from hydro-alcoholic solutions is influenced by metal ions as: $Ca^{2+}$, $Mg^{2+}$. Besides a stimulating effect on enzymatic activity, metal ions have a positive influence on precipitation duration and precipitation yield [18].

In this research we studied the influence of $Ca^{2+}$ which was introduced as $CaCl_2$, in concentration of 0,01-0,25% in the enzyme solution. Precipitation was performed with 3 volume of ethanol, at $+4^0$C, under stirring, for 2 hours.

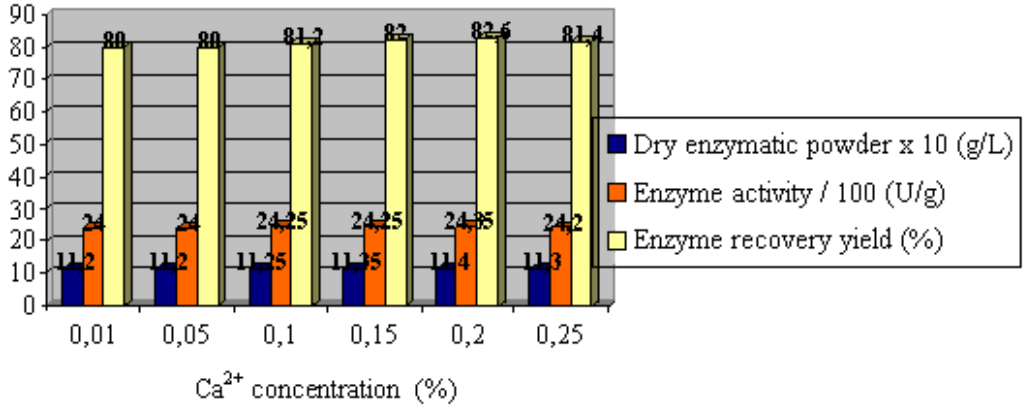


**Figure 6.** Influence of $Ca^{2+}$ concentration on lipase precipitation

Correlating the best results obtained in the above mentioned studies with those presented in figure 6, we can conclude that lipase recovery yield after precipitation with ethanol can be improved in the presence of 0,2% $CaCl_2$.

## Conclusions

Experiments performed in order to establish the influence of the olive oil concentration as an inductor for the biosynthesis of lipase by *Yarowia lipolytica* pointed out that maximum enzyme production was accomplished in the presence of 0,1% (v/v) olive oil and 2% (g/v) glucose as carbon and energy source; comparing with glucose, olive oil used as sole carbon source, led to better results, at the concentration of 2%.

Isolation of crude lipase by ethanol precipitation from de fermentation medium, as a first step of post-biosynthesis processing, was influenced by the following parameters: aqueous solution/solvent ratio, contact duration with solvent, temperature during precipitation, $Ca^{2+}$ concentration, for which the optimum values were determined.

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