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SCREENING FOR HIGH LIPASE PRODUCING MICROORGANISMS

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Abstract

The study of lipolytic enzymes has shown great scientific interest in modern biotechnology researches, due to the discovery of applications such as transesterification and ester stereospecific hydrolysis, used in oil industry, detergents and pharmaceutics. Enzyme catalysis using lipases has been intensely researched since the reaction takes place at regular pressure and 30-40^{o}C, achieving the necessary energy, and reducing at the same time the emergence of unwanted secondary compounds. Lipases used in biotechnology processes are usually microbial in origin and are commercially available, immobilized on different supports. They present the advantage of the possibility of both recycling the biocatalyst and also developing continuous processes. In biotechnological processes, the most exploited lipase is lipase B from Candida antarctica. Given the price of such enzymes, there is a continuous need for novel and better enzymes to be used in biocatalysis reactions. To this purpose, a series of industrial importance strains pertaining to the Microorganisms Collection of the National Institute for Chemical Pharmaceutical Research and Development ICCF Bucharest have been tested in order to determine potential lipase production. The screening was performed on 3 bacterial strains, 16 yeast strains and 14 fungi strains. The microorganisms were grown on several specific lipase inductor media. The enzymes produced by the strains which developed lipolytic activity are to be used later for biodiesel obtainment through biocatalysis.

Keywords: *enzyme, lipase, screening, transesterification.*

INTRODUCTION

The ever increasing and developing industries all around the world have brought us to the challenging situation of needing to discover novel and better biocatalysts, to enhance the production of widely used products, such as food produce, cleaning agents, pharmaceuticals and many more. Also highly developing is the use of microbial populations for the treatment of agricultural, industrial and household wastes.

One of the most versatile groups of enzymes with biotechnological applications is represented by lipases (EC 3.1.1.3.), which carry out hydrolytic reactions in both aqueous and non-aqueous media, by performing interfacial catalysis. Lipases are carboxylesterases which catalyze the hydrolysis of long-chain triglycerides to mono-and di-glycerides, free fatty acids and glycerol (Aehle, 2007). Their catalytic activity covers esterification, trans-esterification, interesterification, acidolysis, alcoholysis and aminolysis (Joseph et al., 2008). Due to their wide area of activity, lipases are used in food and feed processing, fats and oils industry, detergents and various degreasing products, synthesis and production of fine chemicals, cosmetics and pharmaceuticals, bakery and brewing, biofuels and waste treatment (Kazlauskas et al., 1998; Sharma et al., 2001; Babu and Rao, 2007; Hasan et al., 2009; Balakrishnan et al., 2011; Kishore et al., 2011).

The main and most common source of lipases is represented by microorganisms (Gupta et al., 2004), although lipases are part of the physiology of living organisms and can be also found in plants and animals, as well as microbes (Rahnman et al., 2006). However, they are more abundantly found in bacteria, yeasts and fungi (Haki et al., 2003), which constitute nowadays the principal lipase generators for almost all processes that require them.

Given the augmentation of the demand for superior biocatalysts in industries requiring

lipases, the need of finding novel sources of lipolytic enzymes has been increasing as well.
The purpose of this study was to determine the possibility of using and enhancing microbial strains pertaining to an industrial microorganism collection for lipase production, with various applications in biocatalytic processes.

MATERIALS AND METHODS

The strains that we have taken under consideration for our study were chosen from the Industrial Importance Microorganism Collection (IIMC) pertaining to the National Institute for Chemical Pharmaceutical Research and Development ICCF Bucharest, registered in the World Federation of Culture Collections Directory, no. 232, from the conservation vegetative stock. For our study, we have selected for the screening 3 bacterial strains, 16 yeast strains and 14 fungi strains, which we are describing below (Table 1).

Table 1. Strains that have been studied for lipase producing potential from the ICCF collection

Strain name	Registration
Pseudomonas aeruginosa F– ATCC* 9027	ICCF 90
Pseudomonas sp. no. 2	ICCF 399
Pseudomonas sp. no. 3	ICCF 400
Candida arborea – CBS** 64	ICCF 193
Candida glabrata	ICCF 182
Candida famata	ICCF 181
Candida robusta	ICCF 194
Yarrowia lipolytica– ATCC 16618	ICCF 214
Yarrowia lipolytica	ICCF 215
Candida boidinii-CMBG*** 221	ICCF 26
Candida paraffinica	ICCF 190
Candida paraffinica	ICCF 184-1
Candida paraffinica	ICCF 184-2
Candida albicans– ATCC 10231	ICCF 91
Candida utilis-CMGB 237	ICCF 191
Candida guillermondii– CMGB 229	ICCF 183
Pichia pastoris – CMGB 267	ICCF 189
Candida utilis– CMGB 237	ICCF 263
Candida sp.	ICCF 315
Aspergillus niger	ICCF 250
Aspergillus awamory	ICCF 171
Aspergillus niger	ICCF 21
Aspergillus awamory	ICCF 173
Aspergillus niger– ATCC 16404	ICCF 92
Aspergillus niger	ICCF 164
Aspergillus awamory	ICCF 167
Aspergillus awamory	ICCF 165
Aspergillus oryzae	ICCF 24
Aspergillus flavus	ICCF 233
Aspergillus ochraceus	ICCF 401
Aspergillus nigricans	ICCF 402
Rhizopus stolonifer	ICCF 223
Aspergillus versicolor	ICCF 232

American Type Culture Collection
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** Center for Research, Forming and Consultancy in Microbiology, Genetics and Biotechnology – MICROGEN, University of Bucharest

In order to determine lipase production by the microorganisms taken into study, various screening media have been used.
For the selection of lipase producing bacteria nutrient agar with added 1.0%g/V Tween 80 and 0.1%g/V $CaCl_2$ was used.
For the yeast strains a selective YPG medium containing calcium chloride and Tween 80 as lipase production inductor has been used (%g/V) : Glucose 2.0; Peptone 0.5; Yeast extract 0.5; Tween 80 1.0; $CaCl_2$ 0.1; Agar 2.0; pH 6.0-6.5, 120^{o}C for 15 min sterilization.
For the lipase producing fungi the media (YPG, malt and Czapeck-Dox) supplemented with Tween 80, $CaCl_2$ and sodium deoxycholate were used. Sodium deoxycholate was added to diminish colony diameter and facilitate lipase extraction through hyphae permeabilization.
YPG (%g/V) : Glucose 2.0; Peptone 0.5; Yeast extract 0.5; Tween 80 1.0; $CaCl_2$ 0.1; Sodium deoxycholate 0.05; Agar 2.0; pH 6.0-6.5, 120^{o}C for 15 min sterilization.
Malt extract medium (%g/V): Malt extract 4.0; Tween 80 1.0; $CaCl_2$ 0.1; Sodium deoxycholate 0.05; Agar 2.0; pH 6.0-6.5, 120^{o}C for 15 min sterilization.
Czapeck-Dox (%g/V): Sucrose 3.0; $NaNO_3$ 0.33; K_2HPO_4 0.1; $MgSO_4 \cdot 7H_2O$ 0.05; KCl 0.5; $FeSO_4 \cdot 7H_2O$ 0.004; $ZnSO_4 \cdot 7H_2O$ 0.008; $CaCl_2$ 0.1; Tween 80 1.0; Sodium deoxycholate 0.05; Agar 2.0; pH 6.0-6.5; 120^{o}C for 15 min sterilization.
All of the microorganisms were grown in Petri dishes, 10 cm in diameter, 24-48 hours at 26-28^{o}C.
The method of determination of lipase activity for the strains grown on agar media with inducers consists in the presence or absence of opacity areas around the colonies.
The opacity areas are a proof that the saponification reaction has taken place, being composed out of calcium soap crystals, as a

result of extracellular lipolytic enzymes on the Tween, in the presence of inducers in the medium.

Lipolytic activity of microorganisms was expressed through the ratio between the diameter of the opacity area added to the diameter of the colony and the diameter of the colony (Ionita et al., 1997), as follows:

$$Vr = \frac{R + r}{r}$$

Where:

R = opacity area diameter;

r = colony diameter;

V_r = size of the opacity area.

The strains for which $V_r > 2$ are considered to be good lipase producers.

Throughout the experiments, the value of the opaque area represents an average of 5 estimations for each of the strains chosen for the screening.

RESULTS AND DISCUSSIONS

Once the incubation period was finished, viability and presence or absence of certain opacity areas around the colonies was determined.

Table 2 shows the results obtained from the yeast strains taken under study, the type of halo formed, as well as the value of the ratio indicating lipase activity. The Petri dishes were studied after 5, 10 and 20 days of inoculation, and the table presents the general aspect at the end of the observation period, as well as the initial and final Vr values (Vr_1 after 5 days and Vr_2 after 20 days observation), if modified.

Table 2. The aspect of yeast strains on Petri dishes with agar medium, containing lipase inducers

Crt.	Yeast name and registration no.	Aspect	$V_{r\,1}$	$V_{r\,2}$
1	*Candida arborea* ICCF 193	Clarifying halo	3	4.75
2	*Candida glabrata* ICCF 182	Semi-compact clarifying halo	2.25	3.5
3	*Candida famata* ICCF 181	Compact wavy halo	2.66	3.33
4	*Candida robusta* ICCF 194	Absent halo	0	0
5	*Yarrowia lipolytica* ICCF 214	Compact matt halo	2.6	2.6
6	*Yarrowia lipolytica* ICCF 215	Compact matt halo	2.5	3
7	*Candida boidinii* ICCF 26	Semi-compact halo	3.35	3.35
8	*Candida paraffinica* ICCF 190	Semi-compact halo	3.4	3.4
9	*Candida paraffinica* ICCF 184-1	Compact matt halo	3.8	3.8
10	*Candida paraffinica* ICCF 184-2	Compact matt halo	2.05	2.05
11	*Candida albicans* ICCF 91	Compact diffuse halo	2.95	3
12	*Candida utilis* ICCF 191	Clarifying halo	0.2	0.2
13	*Candida guillermondii* ICCF 183	Diffuse crystal halo	3.75	5
14	*Pichia pastoris* ICCF 189	Absent halo	0	0
15	*Candida utilis* ICCF 263	Clarifying halo	0.2	0.2
16	*Candida sp.* ICCF 315	Diffuse matt crystal halo	2.08	2.08

The test aimed at distinguishing lipolytic activity for the yeast strains on YPG medium with Tween 80 and calcium chloride as inducers.

The halos of interest which proved lipase production on the agar media are compact matt halos.

The best results were obtained for the *Candida paraffinica*– ICCF 184-1 strain, which presented a well formed halo, 3.8 cm in diameter after 5 days.

The yeast strain *Candida famata*– ICCF 181 presented a slightly different halo, which continued its development during the observation period.

Yarrowia lipolytica– ICCF 215, a cell line modified for lipase production, and *Candida albicans*– ICCF 91 developed also a significantly large halo (Vr = 3 cm), which modified itself in size over the 20 days observation interval.

The strains *Yarrowia lipolytica*– ICCF 214, *Candida sp.* – ICCF 315 and *Candida paraffinica*– ICCF 184-2 also presented lipolytic activity, with halos over the 2 cm threshold value: Vr = 2.6, 2.08, and 2.05, respectively.

The clarifying halos which appear around the yeast colonies indicate the presence of

amylolytic activity (*Candida arborea* – ICCF 193, *Candida utilis*– ICCF 191, *Candida utilis*– ICCF 263)

The results observed are also presented in figure 1 (colony aspect) and figure 2 (halos highlighting lipase secretion in the agar medium).

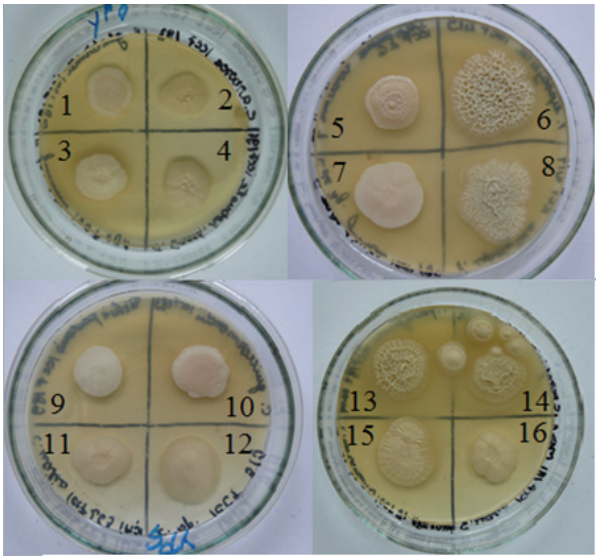

Figure 1. Yeast colonies on Petri dishes

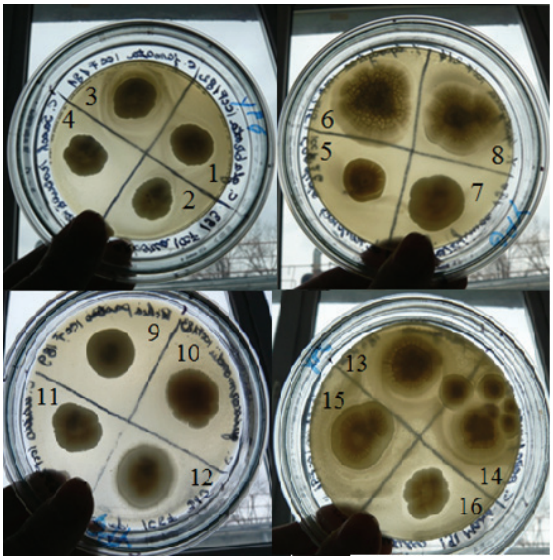

Figure 2. Presence of halos around the yeast colonies, proving lipase activity

Where:

1 = *Candida glabrata* – ICCF 182; 2 = *Candida arborea* – ICCF 193; 3 = *Candida famata* – ICCF 181; 4 = *Candida robusta* – ICCF 194; 5 = *Candida boidinii* – ICCF 26; 6 = *Yarrowia lipolytica* – ICCF 215; 7 = *Candida paraffinica* – ICCF 190; 8 = *Yarrowia lipolytica* – ICCF 214; 9 = *Pichia pastoris* – ICCF 189; 10 = *Candida guillermondii* – ICCF 183; 11 = *Candida utilis* – ICCF 263; 12 = *Candida sp.* – ICCF 315; 13 = *Candida paraffinica* – ICCF 184-1; 14 = *Candida paraffinica* – ICCF 184-2; 15 = *Candida albicans* – ICCF 91; 16 = *Candida utilis* – ICCF 191

As can also be inffered from figure 2, where the opacity halos can be clearly distinguished, the yeast strains which presented lipolytic activity were: no. 3 –*Candida famata*– ICCF 181, no. 6 –*Yarrowia lipolytica*– ICCF 215, no. 8 –*Yarrowia lipolytica*– ICCF 214, no. 12 –*Candida sp*. – ICCF 315, no. 13 –*Candida paraffinica*– ICCF 184-1, no. 14 –*Candida paraffinica*– ICCF 184-2, no. 15 –*Candida albicans*– ICCF 91,

The fungi strains have known rapid development, covering up the surface of the Petri dishes in 48 hours on the specific culture media (YPG and malt extract, respectively) and showed no presence of lipase activity under the form of specific halos (Figure 3).

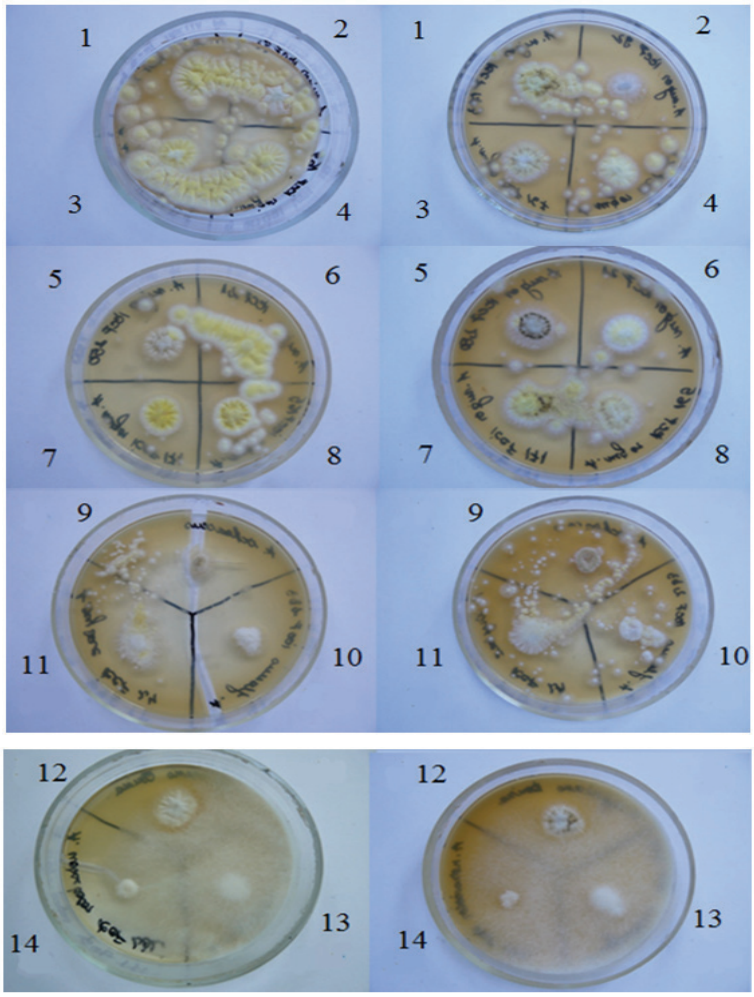

Figure 3. Aspect of fungi strains on Petri dishes containing agar media: YPG (left column) and Malt extract (right column)

Where:

1 = *Aspergillus awamory*– ICCF 173
2 = *Aspergillus niger*– ICCF 92
3 = *Aspergillus awamory*– ICCF 167
4 = *Aspergillus niger*– ICCF 164
5 = *Aspergillus niger*– ICCF 250
6 = *Aspergillus niger*– ICCF 21
7 = *Aspergillus awamory*– ICCF 171
8 = *Aspergillus awamory*– ICCF 165
9 = *Aspergillus ochraceus* ICCF 401
10 = *Aspergillus flavus*– ICCF 233
11 = *Aspergillus oryzae*– ICCF 24
12 = *Aspergillus nigricans* ICCF 402
13 = *Rhizopus stolonifer*-ICCF 223
14 = *Aspergillus versicolor*-ICCF 232

The *Pseudomonas* strains formed no specific opaque halos on the agar medium (Figure 4).

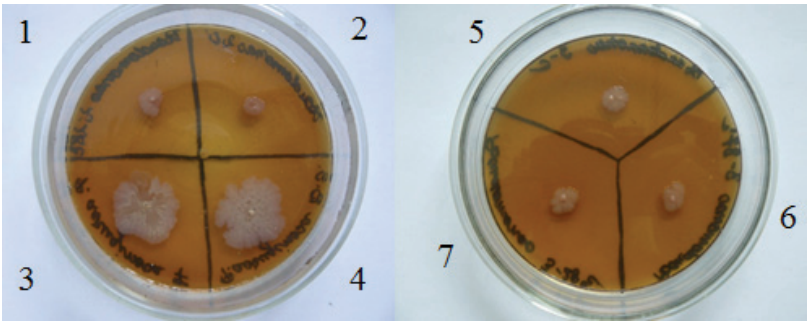

Figure 4. Appearance on Petri dishes of the Pseudomonas strains

Where:
1 = *Pseudomonas sp.* nr. 2 – ICCF 399 – grown on test tube at 28°C
2 = *Pseudomonas sp.* nr. 2 – ICCF 399 – vegetative stock strain
3 = *Pseudomonas aeruginosa* F – ICCF 85
4 = *Pseudomonas aeruginosa* F – ICCF 90
5 = *Pseudomonas sp.* nr. 3 – ICCF 400 – vegetative stock strain
6 = *Pseudomonas sp.* nr. 3 – ICCF 400 – grown on test tube at 37°C
7 = *Pseudomonas sp.* nr. 3 – ICCF 400 – grown on test tube at 28°C

CONCLUSIONS

The aim of the study on industrial strains pertaining to the Industrial Importance Microorganism Collection (IIMC) registered to the National Institute for Chemical Pharmaceutical Research and Development ICCF Bucharest was the determination of lipase producing microorganisms, selected from several bacterial, yeast and fungi strains.

After growth on specific agar media containing lipase inducers on Petri dishes, the colonies have been observed for lipolytic activity in the form of opaque halos surrounding the cultures.

The *Pseudomonas* bacterial strains showed no presence of lipases on the medium, and neither did the fungi strains selected for the screening, the latter of which developed quickly, covering the surface of the Petri dishes in the cases of both specific growth media (YPG and malt extract) used.

Out of the 16 yeast strains considered for the screening, 7 showed lipolytic activity on the specific YPG media containing Tween 80 and calcium chloride as lipase production inducers. The yeast strains that developed opaque halos are: *Candida famata*– ICCF 193, *Yarrowia lipolytica*– ICCF 215, *Yarrowia lipolytica*– ICCF 214, *Candida sp*. – ICCF 315, *Candida paraffinica*– ICCF 184-1, *Candida paraffinica*– ICCF 184-2, *Candida albicans*– ICCF 91.

These yeast strains are going to be subjested to further analysis in order to quantify lipase production and enzymatic activity, for subsequent use in biodiesel obtainment through biocatalytic processes.

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National Institute for Chemical Pharmaceutical Research and Development ICCF Bucharest for setting at our disposal their Industrial Importance Microorganism Collection (IIMC), for the performance of the screening tests.
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REFERENCES

Aehle W., 2007, Industrial enzymes. In: Aehle W. ed. Enzymes in Industry, Production and Applications, Wiley, 99-262.
Babu IS, Rao GH., 2007 Lipase production by *Yarrowia lipolytica* NCIM 3589 in solid state fermentation using mixed substrate. Res J Microbiol 2: 469-474.

Balakrishnan, P., et. al., 2011, Production of lipase enzyme from Lactobacillus spp. And its application in the degradation of meat, World Applied Sciences Journal, 12 (10), 1798-1802

Bhavani M1,3,*, G V Chowdary2, David M1, Archana G1, 2012, Screening, Isolation and Biochemical Characterization of Novel Lipase Producing Bacteria from Soil Samples, International Journal of Biological Engineering, 2 (2) : 18-22

Gupta R., Gupta N., Rathi P., 2004, Bacterial lipases: an overview of production, purification and biochemical properties. Appl Microbiol Biotechnol 64: 763-81.

Haki GD, Rakshit SK., 2003, Developments in industrially important thermostable enzymes: a review. Bioresour Technol, 89: 17-34.

Hasan, F., Shah, A.A., and Hameed A., 2009, Methods for detection and characterization of lipase: A comprehensive review, Biotechnology Advances, 27, 782-798

Ionita, A.; Moscovici, M.; Popa, C.; Vamanu, A.; Popa, O.; Dinu, L., 1997, Screening of yeast and fungal strains for lipolytic potential and determination of some biochemical properties of microbial lipases. *J. Mol. Catal., B Enzym*., 3, 147-151

Joseph B, Ramteke PW, Thomas G., 2008, Cold active microbial lipases: some hot issues and recent developments. Biotechnol Adv 26: 457-470.

Kazlauskas, R.J., and Bornscheuer, U.T., 1998, Biotransformation with lipases, Biotechnology, New York: VCH., 8:37-192

Kishore, J.P., Manoj, K. Z. C., and Raghunath, T. M., 2011, Lipase biodiversity, Indian Journal of Science and Technology, 4 (8), 971-982 Advances, 19, 627-662

Rahman RNZRA, Salleh AB, Basri M., 2006, Lipases: introduction. In: Salleh AB et al. ed. New Lipases and Proteases. Nova Science Publishers; pp. 1-22.

Sharma, R., Christi, Y., and Benerjee, U. C., 2001, Production purification characterization and applications of lipases, Biotechnology