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SCREENING OF MICROBIAL STRAINS ABLE TO PRODUCE EXTRACELLULAR LIPASES

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Abstract

Lipases have gained attention worldwide, due to their potential for diverse applications and their stability and selectivity. The study's aim was to identify microorganisms with the ability to synthesize extracellular lipase. In order to achieve this, 20 microbial strains were cultivated on selective solid agar media and lipase producing microorganisms were selected based upon their ratio of the diameter of the halo (if present) and the colony's diameter. The bacterial strains were cultivated on three different selective media, the results indicating that the best screening medium was TBA and the strain that was estimated to have the highest lipase activity was Bacillus subtilis ICCF 20. Yeast strains were cultivated on two selective media and M4D was selected as the best screening medium. The highest producer of lipases was considered to be Yarrowia lipolytica ATCC 16618 ICCF 214. The fungal strains were cultivated on two selective media and the best screening medium was determined to be YS. Two fungal strains were selected as having the highest lipase activity: Aspergillus niger (P4 C36) ICCF 24 and Aspergillus awamory (P2 C114) ICCF 259.

Key words*: lipase, screening, bacteria, yeast, fungi.*

INTRODUCTION

Due to their usefullness in various versatile industrial applications, lipases are ranked as the third most used enzymes after proteases and amylases (Javed et al., 2017).

Depending upon the source, lipases can be grouped into: plant lipases, animal lipases (milk, pancreatic lipases, lipoprotein lipases, hormone-sensitive lipases) and microbial lipases (Jurcoane et al., 2009). Among all of the types of lipases, microbial ones are preferable because they have high specificities for their substrates, higher stability, and lower production costs compared to lipases synthesized from plants and animals (Lee et al., 2015).

Various industries require enzymes that can be used as organic catalysts in various commercial-scale processes. The specialized literature consider that microbial enzymes are of particular interest, including lipases and amylases (Tomulescu et al., 2015).

Lipases are glycerol-ester hydrolases and therefore are present in the form of aqueous emulsions, in a heterogeneous system they are carboxyl-esterases, which hydrolyze glycerides (Gerhartz, 1990; Pascoal et al., 2018). Lipase is responsible for the catalysis of the hydrolysis of triglycerides at the oil-water interface with the formation of glycerol and fatty acids, with a high rate of cleavage (Javed et al., 2017; Gopinath et al., 2013; Guldhe et al., 2015). Also, they have the ability to catalyze transesterification and interesterification reactions into organic solvents (Singh and Mukhopadhyay, 2012; Villeneuve et al., 2000). Due to these special properties, lipases are widely used in the detergent industry, food production and processing, pharmaceuticals, paper, cosmetics, and chemical synthesis industry (Pascoal et al., 2018; Guldhe et al., 2015; Jaeger and Reetz, 1998).

According to the data from the specialized literature, some confusions were noted regarding the exact meaning of the terms lipase and esterase, since both hydrolyze the ester bonds of carboxylic acids. Some researchers believe that the essential difference between lipases and esterases lies in the physical state of the substrate on which they act. Thus, esterases

can hydrolyze soluble or totally dispersed substrates, and lipases cannot. That is why it was proposed by Wills and Jensen that lipases are enzymes that hydrolyze esters at an oil-water interface, in a heterogeneous environment (Jurcoane et al., 2009).

Due to their wide applications, low production costs and higher stability, a lot of research work was done in order to find new microbial strains capable of lipase synthesis at superior level. Yeats and fungi belonging to *Candida*, *Yarrowia*, *Aspergillus, Penicillium* and *Trichoderma* genera were investigated for their ability of extracellular lipase production. Also, a lot of strains of *Bacillus, Pseudomonas, Staphylococcus, Serratia* and *Streptomyces* species were used for obtaining this type of hydrolases with thermostability and higher enzymatic activity at neutral and alkaline pH (Chandra et al., 2020).

The aim of the present study was to investigate 6 bacterial strains, 7 yeast strains and 7 fungi strains for their ability to produce high amount of lipase. The microorganisms were cultivated on specific culture media (3 for bacteria, 2 for yeasts and 2 for fungi) and the lipolytic activity was determined according to the area of opacity around the microbial colonies.

MATERIALS AND METHODS

The microbial strains tested in this study are from the Collection of Industrially Important Microorganisms (IIMC) belonging to the National Institute for Chemical Pharmaceutical (Table 1).

Table 1. Potential lipase-producing microbial strains that were studied from the ICCF collection

Microbial strains analysed		
Bacteria	**Yeast**	**Fungi**
Bacillus megaterium CA1 ICCF 280	*Candida albicans ATCC 10231 ICCF 91*	*Aspergillus awamory (P2 C114) ICCF 259*
Bacillus subtilis ICCF 20	*Candida famata ICCF 181*	*Aspergillus awamory (P2 C287) ICCF 245*
Bacillus subtilis ICCF 77	*Candida paraffinica ICCF 184*	*Aspergillus niger (P4 C36) ICCF 249*
Bacillus subtilis ICCF 84	*Candida tropicalis CMGB 114*	*Aspergillus niger (P6 C317) ICCF 244*
Bacillus subtilis NCIB 8646 ICCF 19	*Rhodotorula glutinis CMGB 169*	*Aspergillus niger ICCF 170*
Bacillus subtilis USAMV 4 ICCF 294	*Yarrowia lipolytica ATCC 16618 ICCF 214*	*Aspergillus niger IP 106 ICCF 222*
-	*Yarrowia lipolytica ICCF 215*	*Beauveria bassiana CA 6 ICCF 335*

ATCC - American Type Cultures Collectionst; Center for Research, CMGB - Forming and Consultancy in Microbiology, Genetics and Biotechnology - MICROGEN, University of Bucharest; NCIB - National Collection of Industrial Bacteria, Aberdeen, Scotland; USAMV -University of Agronomic Sciences and Veterinary Medicine of Bucharest; CA - Antibiotics Research Center Iasi.

Different specific culture media were used for the selection of microbial strains with lipase-producing potential. The three culture media tested for the selection of lipase-producing bacteria are: M4B, TBA and T80.

M4B: tryptone 1% (w/V), calcium chloride 0.1% (w/V), Tween 80.1% (w/V), yeast extract 0.5% (w/V), agar 2% (w/V).

TBA: bacto-peptone 0.5% (w/V), tributyrin 1% (w/V), meat extract 0.3% (w/V), agar 2% (w/V).

T80: bacto-peptone 1% (w/V), Tween 80.1% (w/V), sodium chloride 0.5% (w/V), calcium chloride 0.01% (w/V), agar 2% (g /V).

Two specific culture media were used for screening lipase-producing yeasts: M4D and YS.

M4D: tryptone 1% (w/V), glucose 2% (w/V), calcium chloride 0.1% (w/V), Tween 80.1% (w/V), yeast extract 0.5% (w/V), agar 2% (w/v).

YS: bacto-peptone 0.5% (w/V), glucose 2% (w/V), Tween 80.1% (w/V), calcium chloride 0.1% (w/V), yeast extract 0.5% (w/V), agar 2% (w/V).

All the five culture media described above were sterilized at 121°C for 20 minutes and the pH was adjusted to 6.5.

Two specific culture media were tested for screening of lipase-producing fungi: CDA si YS.

CDA: sodium nitrate ($NaNO_3$) 0.2% (w/V), magnesium sulfate ($MgSO_4*7H_2O$) 0.05% (w/V), potassium chloride (KCl) 0.05% (w/V), iron sulfate ($FeSO_4*7H_2O$) 0.001% (w/V), dipotassium phosphate (K_2HPO_4) 0.01% (w/V), starch 1% (w/V), agar 2% (w/V).

These culture media were sterilized at 115^0C for 30 minutes, adjusting the pH to 6.5.

All microorganisms were cultivated in Petri dishes, with a diameter of 10 cm, for 24-72 hours at 28-30^0C. The method for determining lipase activity for the strains grown on inducer agar

media consists of the presence or absence of areas of opacity (halo) around the colonies.
The lipolytic activity of microorganisms was expressed by the ratio of the diameter of the opacity zone added to the colony diameter and the colony diameter (Ionita et al., 1997), as follows:

$$Vr = (R+r)/r$$

Where:
R = diameter of the opacity zone;
r = colony diameter;
Vr = size of the opacity area.
Strains for which Vr > 2 are considered to be good lipase producers.

RESULTS AND DISCUSSIONS

The six bacterial strains mentioned above were tested on the three specific culture media, the lipase activity being assessed according to the Vr value (Table 2).
After the end of the incubation period, several aspects were analysed: the presence or absence of the opacity area around the colonies and the halo aspect, thus determining the lipolytic activity for the bacterial strains.

Table 2. Evaluation of lipase activity synthesized by bacterial strains according to the calculated Vr values

	TBA		**M4B**	
Bacillus subtilis NCIB 8646 ICCF 19	Compact, matte, clear halo	2.83	Semi-compact, matte halo	2.66
Bacillus subtilis ICCF 20	Compact, clear, transparent halo	5.33	It developed on this medium, but the halo is missing	-
Bacillus subtilis ICCF 77	It did not develop on this medium	-	Compact, matte halo	2.66
Bacillus subtilis USAMV 4 ICCF294	Compact, transparent, well-defined halo	2.88	It developed on this medium, but the halo is missing	-
Bacillus subtilis ICCF 84	Compact, matte halo	2.33	It developed on this medium, but the halo is missing	-
Bacillus megaterium CA1 ICCF 280	Compact, transparent halo	2.5	It developed on this medium, but the halo is missing	-

On the specific T80 culture medium, in the conditions of the experiment, only the strain *Bacillus subtilis ICCF 84* developed, but it didn't show any halo, while the other tested strains of bacteria did not develop on this medium.
The best lipolytic activity was recorded for the *Bacillus subtilis ICCF 20* strain, with a well-formed and clear halo of 5.33, on TBA medium (Figure 1). In fact, on this culture medium the tested strains of bacteria showed lipolytic activity with halos over 2 cm, except for *Bacillus subtilis ICCF 77* that did not grow on TBA medium.
On M4B specific medium, all the bacterial strains were developed, but only two of them synthesized lipases: *Bacillus subtilis NCIB 8646 ICCF 19* and *Bacillus subtilis ICCF 77* (Figure 2).

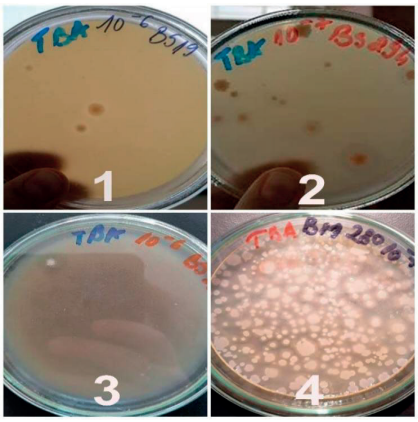

Figure 1. Screening of bacterial strains grown on TBA medium:
1- *Bacillus subtilis NCIB 8646 ICCF 19;*
2- *Bacillus subtilis USAMV 4 ICCF294;*
3- *Bacillus subtilis ICCF 20;*
4- *Bacillus megaterium CA1 ICCF 280*

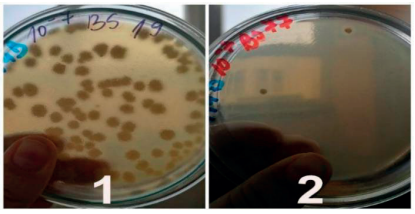

Figure 2. Screening of bacterial strains grown on M4B medium:
1- *Bacillus subtilis NCIB 8646 ICCF 19;*
2- *Bacillus subtilis ICCF 77*

For estimating the lipolytic activity, several aspects were monitored for the yeasts cultivated on the two specific media: the type and the diameter of the obtained halo and the area of opacity (Table 3).

The enzyme activity with the highest value was recorded for the strain *Yarrowia lipolytica ATCC 16618 ICCF 214*, on the M4D medium (Vr = 4.33), and the strains *Candida albicans ATCC 10231 ICCF 91* and *Candida tropicalis CMGB 114 USAMV* presented well-defined halos with a Vr equal to 3, grown on M4D medium (Figure 3).

Table 3. Evaluation of lipase activity synthesized by yeast strains according to the calculated Vr values

	YS		M4D	
Candida albicans ATCC 10231 ICCF 91	It has developed on this medium, but it does not show a halo	-	Compact, transparent halo	3
Candida famata ICCF 181	It has developed on this medium, but it does not show a halo.	-	It has developed on this medium, but it does not show a halo.	-
Candida paraffinica ICCF 184	It has developed on this medium, but it does not show a halo.	-	It has developed on this medium, but it does not show a halo.	-
Yarrowia lipolytica ATCC 16618 ICCF 214	It has developed on this medium, but it does not show a halo.	-	Compact, matte and well-defined halo	4.33
Yarrowia lipolytica ICCF 215	It has developed on this medium, but it does not show a halo.	-	It has developed on this medium, but it does not show a halo.	-
Rhodotorula glutinis CMGB 169 USAMV	Compact, transparent halo	2.5	It did not develop on this medium.	-
Candida tropicalis CMGB 114 USAMV	It has developed on this medium, but it does not show a halo.	-	Compact, transparent halo	3

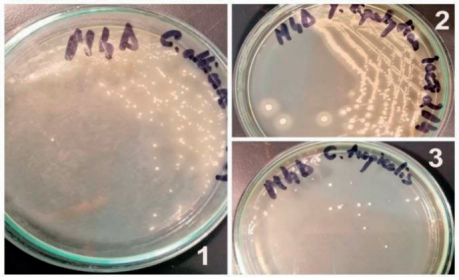

Figure 3. Screening of yeast strains grown on M4D medium:
1- *Candida albicans ATCC 10231* ICCF 91;
2- *Yarrowia lipolytica ATCC 16618 ICCF 214;*
3- *Candida tropicalis CMGB 114* USAMV

On the specific YS medium, all seven yeast strains used in this screening experiment developed, but the strain *Rhodotorula glutinis CMGB 169 USAMV* was the only one that showed enzymatic activity and a well-defined halo (Figure 4).

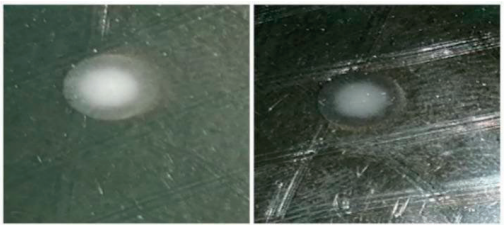

Figure 4. Screening of the yeast strain *Rhodotorula glutinis CMGB 169 USAMV* grown on YS medium

The 7 strains of fungi, analysed in this study, were cultivated on the two specific culture media YS and CDA, and the lipase activity was evaluated according to the diameter of the halo (Table 4).

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Table 4. Evaluation of lipase activity synthesized by fungal strains according to the calculated Vr values

	YS		CDA	
Aspergillus niger ICCF 170	It has developed on this medium, but it does not show a halo.	-	Compact and transparent halo	2.4
Aspergillus niger (P6 C317) ICCF 244	It has developed on this medium, but it does not show a halo.	-	It has developed on this medium, but it does not show a halo.	-
Aspergillus niger (P4 C36) ICCF 249	Compact and transparent halo	3.75	It did not develop on this medium.	-
Aspergillus niger IP 106 ICCF 222	Compact halo, matte	3	It has developed on this medium, but it does not show a halo.	-
Aspergillus awamory (P2 C287) ICCF 245	It did not develop on this medium.	-	It did not develop on this medium.	-
Aspergillus awamory (P2 C114) ICCF 259	Semi-compact and matte halo	4	It did not develop on this medium.	-
Beauveria bassiana CA 6 ICCF 335	Compact halo, matte	3	It has developed on this medium, but it does not show a halo.	-

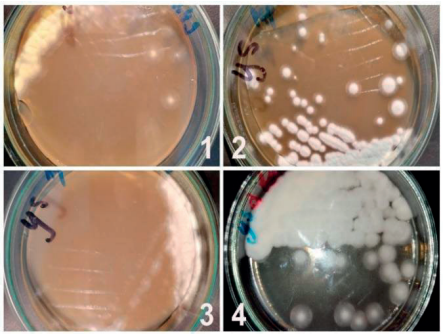

Figure 5. Screening of fungal strains grown on YS medium:
1- *Aspergillus niger (P4 C36)* ICCF 249;
2- *Aspergillus niger IP 106* ICCF 222;
3- *Aspergillus awamory(P2 C114)*ICCF 259;
4- *Beauveria bassiana CA 6* ICCF 335

The strain *Aspergillus awamory (P2 C114) ICCF 259* recorded the highest lipolytic activity when cultivated on the specific culture medium YS, with a Vr value of 4. The strain *Aspergillus niger IP 106 ICCF 222* and *Beauveria bassiana CA 6 ICCF 335* registered an equal lipase activity, Vr = 3, on the same culture medium, YS.

On the specific culture medium for fungi, CDA, enzymatic activity was observed only for the strain *Aspergillus niger ICCF 170*, with a compact and transparent halo. The other six strains of fungi tested in this experiment did not show any lipolytic activity.

CONCLUSIONS

The purpose of this research was to identify the microorganisms able to produce lipases, testing bacterial, yeasts and fungi strains from the Culture Collection Of Industrial Importance Microorganisms (CMII), from the National Institute of Chemical and Pharmaceutical Research and Development in Bucharest (ICCF) and two yeast strains (*Rhodotorula glutinis CMGB 169 USAMV* and *Candida tropicalis CMGB 114 USAMV*) from the Faculty of Biotechnology of the USAMV of Bucharest.

The best culture medium for testing bacteria was TBA, and the strain that showed high lipolytic activity was *Bacillus subtilis ICCF 20*.

The best screening medium for testing yeast strains was M4D, and the strain that synthesized lipase with high activity was *Yarrowia lipolytica ATCC 16618 ICCF 214*.

The fungi synthesized lipases especially when cultivated on the specific culture medium, YS, and the strains that were selected as best lipase producers were *Aspergillus niger (P4 C36) ICCF 249* and *Aspergillus awamory (P2 C114) ICCF 259.*

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REFERENCES

Benjamin, S., & Pandey, A. (1996). Optimization of liquid media for lipase production by *Candida rugosa*. *Bioresource Technology*, 55(2), 167-170.

Chandra, P., Enespa, Singh, R. *et al.* (2020) Microbial lipases and their industrial applications: a

comprehensive review. *Microbial Cell Factories* 19, 169

Gerhartz, W. (1990). Enzymes in industry: production and applications. *Vch Verlagsgesellschaft mbH*. Vol. 43. p. 89-90.

Ghiorghiță, A., Campeanu, G., Popescu, M., Israel, F., & Neață, G. (2012). Enzymatic bioprocessing of vegetable oils for the production of biodiesel. *Scientific Bulletin, Series F. Biotechnologies.* Vol. XVI.

Gopinath, S. C., Anbu, P., Lakshmipriya, T., & Hilda, A. (2013). Strategies to characterize fungal lipases for applications in medicine and dairy industry. *BioMed research international*

Guldhe, A., Singh, B., Mutanda, T., Permaul, K., & Bux, F. (2015). Advances in synthesis of biodiesel via enzyme catalysis: Novel and sustainable approaches. *Renewable and Sustainable Energy Reviews*, 41, 1447-1464.

Jaeger, K. E., & Reetz, M. T. (1998). Microbial lipases form versatile tools for biotechnology. *Trends in biotechnology*, 16(9), 396-403.

Javed, S., Azeem, F., Hussain, S., Rasul, I., Siddique, M.H., Riaz, M., Afzal, M., Kouser, A. and Nadeem, H., 2018. Bacterial lipases: a review on purification and characterization. *Progress in biophysics and molecular biology*, 132, pp.23-34.

Jurcoane, Ş., Săsărman, E., Roşu A., Banu A., Lupescu I, Tamba, R. B., Rădoi, F. (2009). *Tratat de biotehnologie*. Vol.1., Ed. Tehnică. Bucureşti.

Lee, L. P., Karbul, H. M., Citartan, M., Gopinath, S. C., Lakshmipriya, T., & Tang, T. H. (2015). Lipase-secreting *Bacillus* species in an oil-contaminated habitat: promising strains to alleviate oil pollution. *BioMed research international*

Pascoal, A., Estevinho, L. M., Martins, I. M., & Choupina, A. B. (2018). Novel sources and functions of microbial lipases and their role in the infection mechanisms. *Physiological and molecular plant pathology*, 104, 119-126.

Priji, P., Sajith, S., Faisal, P. A., & Benjamin, S. (2016). Microbial lipases− properties and applications. *The Journal of Microbiology, Biotechnology and Food Sciences*, 6(2), 799.

Sharma, R., Chisti, Y., & Banerjee, U. C. (2001). Production, purification, characterization, and applications of lipases. *Biotechnology advances*, 19(8), 627-662.

Singh, A. K., & Mukhopadhyay, M. (2012). Overview of fungal lipase: a review. *Applied biochemistry and biotechnology*, 166, 486-520.

Tomulescu, C., Moscovici, M., Ghiorghita, A., Petrescu, M., Vladu, M., Tamaian, R., & Vamanu, A. (2015). Microbial screening for lipase and amylase production using newly isolated strains from various biotopes. *Scientific Bulletin. Series F. Biotechnologies*, 19, 271-278.

Villeneuve, P., Muderhwa, J. M., Graille, J., & Haas, M. J. (2000). Customizing lipases for biocatalysis: a survey of chemical, physical and molecular biological approaches. *Journal of molecular catalysis B: enzymatic*, 9(4-6), 113-148.