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LIPOLYTIC AND CUTINOLYTIC ACTIVITY OF MICROORGANISMS ISOLATED FROM POLYETHYLENE SURFACE

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Abstract

Among the most known enzymes used for plastic degradation are esterase, cutinase, and lipase. These enzymes are synthesized by a wide range of microorganisms from different species. The research aimed to estimate lipolytic and cutinolytic activity of microorganisms isolated from the surface of low-density polyethylene (LDPE) films, using three rapid screening methods. The rapid tests used in this research are based on the addition to the culture medium of tributyrin, triacetin and flaxseed oil as inducers, and dyes as indicators of enzymes synthesis. The microorganisms included filamentous fungi and yeasts, pseudomonads and actinobacteria. Among 21 isolated strains of fungi, 90% strains possessed lipase activity, 29% exhibited high lipolytic activity. Only 57% of fungal isolates showed cutinase activity. Both extracellular enzymes were recorded for 43% of yeasts isolates. Lipolytic activity exhibited 83% of the tested strains of Pseudomonas spp., and 50% of Streptomyces spp., while cutinolytic activity only 33% of bacterial isolates. Both lipase and cutinase were produced by 41% of the total number of tested microorganisms. The ability to produce these extracellular hydrolytic enzymes indicates the possibility to metabolize polymers, such as polyethylene.

Key words*: fungi, yeasts, bacteria, lipase activity, cutinase activity, polyethylene, LDPE.*

INTRODUCTION

Plastic pollution is a worldwide issue that has an impact on the environment and demands an eco-friendly solution. The use of microorganisms for bioremediation has proved to be the safest method for the environment over time, as it does not involve the use of hazardous chemicals or expensive machinery, and is more energy-efficient than methods of mechanical and chemical degradation (Soong et al., 2022; Raoufi et al., 2023).

The enzymes secreted by microorganisms can reduce the number of carbonyl groups, break them into carboxylic acids, and hydrolyze the polymer carbon chains into fragments, or bio-fragmentation, including long-chain aliphatic compounds such as alkanes and alkenes (Zhang et al., 2022; Raoufi et al., 2023). Among the most known enzymes used for plastic degradation are cutinase, lipase, polyethylene terephthalate hydrolase (PETase) and esterase. These enzymes are synthesized by a wide range of microorganisms from different species, used in the degradation of PE (polyethylene) and PET (polyethylene terephthalate) polymers in different natural environments (Monahan et al., 2020; Kaushal et al., 2021; Andler et al., 2022).

Lipases (triacylglycerol acylhydrolase, EC 3.1.1.3) are members of the α/β hydrolase fold superfamily. They are a biotechnologically important group of enzymes that act on the carboxyl ester bonds present in triacylglycerols and liberate fatty acids and glycerol. Lipases are reported to be produced by many species of animals, plants, bacteria, yeasts, and fungi (Bornscheuer, 2002; Sreelatha et al., 2017). Lipases of microbial origin are widely used in various industries due to the rapid growth of microorganisms and the possibility of obtaining high yields (Rigoldi et al., 2018). For example, representatives of the genus *Streptomyces* and *Pseudomonas* are recognized for their high exogenous lipolytic activity (Madan & Mishra, 2010; Aly et al., 2012). In addition, microbial lipases are characterized by broad substrate specificity, versatility of molecular structure and stability in organic solvents (Aly et al., 2012).

Cutinases (E.C. 3.1.1.74) are esterases belonging to the *α/β* hydrolase superfamily, usually quite stable, do not require cofactors, are active in organic solvents and can be used both in hydrolysis reactions and in synthesis reactions. Cutinases are mostly produced by phytopathogenic microorganisms, which use

these extracellular enzymes to break down cutin which is the major component of the cell wall in plants. Cutinases of fungal origin are capable of breaking down not only cutin, but also soluble esters and insoluble triglycerides (Macedo & Pio, 2005; Furukawa et al., 2019; Ueda et al., 2021). Many polyester hydrolases related to cutin-hydrolyzing esterases are active against PET (Wei & Zimmermann, 2017; Zhu et al., 2022). Cutinases of bacterial origin, according to the literature, are most frequently synthesized by the genera *Thermobifida*, *Streptomyces and Pseudomonas* (Furukawa et al., 2019; Xu et al., 2020).

Fungi are a preferable source of lipase and cutinase, because in most cases these are extracellular enzymes, which facilitate their extraction from the culture liquid (Gopinath et al., 2005; Rai et al., 2014; Raoufi et al., 2023). Apart from this, due to the fact that these enzymes are extracellular, various rapid screening tests of microorganisms with the capacity to synthesize lipase and cutinase have been developed. However, they differ in sensitivity, cost and ease of preparation (Ramnath et al., 2017).

Three primary screening methods of the ability to synthesize lipase and cutinase enzymes by fungi and bacteria were tested and evaluated in this study. The premise was our previous research, which revealed microbial consortia of mixed composition, isolated from the surface of low-density polyethylene (LDPE). These consortia included filamentous fungi, yeast, and bacteria (Rastimesina et al., 2022). Research has shown that the LDPE films, populated by microorganisms, have undergone processes of mechanical degradation and chemical changes (Rastimesina et al., 2023).

Thus, the research aimed to estimate the lipolytic and cutinolytic activity of microorganisms isolated from the surface of low-density polyethylene films.

MATERIALS AND METHODS

Biological material

The soil was collected from the landfill, mostly of plastic waste, located near the village of Slobozia-Duşca, the Criuleni district, the Republic of Moldova. The sampling of the complex soil sample collected from 20-30 subsamples from determined sampling unit (area of the sampling with relative similar conditions) was performed according to the protocol (GOST 17.4.4.02-2017). The total soil sample volume was near 10 kg. The complex soil sample was taken from the depth 0-10 cm. LDPE films (35 μm) used in this work were produced by Kraus Folie Sp.J. (Poland). After six months of incubation in the soil LDPE films were extracted and placed in the flasks with liquid mineral salt media MSM 2 (g/L: K_2HPO_4 - 1.0, KH_2PO_4 - 1.0, NH_4NO_3 - 1.0, $(NH_4)_2HPO_4$ - 1.0, $MgSO_4 \cdot 7H_2O$ - 0.2, $FeCl_3$ - 0.05, $CaCl_2$ - 0.02) and MSM 4 (g/L: $FeCl_3$ - 10.0, $NaNO_3$ - 2.0, $MgSO_4 \cdot 7H_2O$ - 0.5, $CaCl_2$ - 0.5, KCl - 0.5, $BaCl_2$ - 0.2) (Postolachi et al., 2021) and cultivated under continuous stirring conditions at 28°C. All mineral salts were purchased from Sigma-Aldrich, Millipore, Germany.

Through enrichment techniques cultivation was continued for 270 days for microbial consortia formation. Microorganisms were isolated by serial dilution technique, purified, and identified to the genus. All bacterial isolates were studied for their colony morphology, cell morphology (Gram reaction), pigmentation and spore production as per Bergey's Manual of Determinative Bacteriology (Holt et al., 1994). Differentiation of pseudomonads was performed by culturing on King B Agar medium (Millipore, Germany) (King et al., 1954). The identification of fungi isolates was performed according to the colonial aspects and microscopic morphology (Garybova & Lekomtseva, 2005). Morphological peculiarities of the microorganisms were studied under the optical microscope Optica® Microscopes B-510 PH, Italy. The isolated fungi were maintained on slants of potato dextrose agar medium (Millipore, Germany), bacteria and yeasts on nutrient agar medium (Oxoid, United Kingdom).

Screening for lipase production by the isolated microorganisms

All the previous isolated microscopic fungi and bacteria were inoculated on solid tributyrin agar medium (NutriSelect® Basic, Switzerland) containing (g/L), special peptone 2.5; yeast extract 3.0; agar-agar 12.0 and 10.0 mL tributyrin (glycerol tributyrate) (Sigma-Aldrich,

Switzerland). Final pH was 7.5. The plates were inoculated at 28°C for a period of 10 days. The presence of clear zone around the colonies indicated lipase production. The diameter of the clear zone was measured at regular intervals of 24-h incubation, started from the 3rd day (Gopinath et al., 2005; Aly et al., 2012). Statistical analysis was performed using MS Excel. The results were expressed as mean of two experiments in three individual replicates ± CI (confidence intervals). All differences were considered significant at $P<0.05$. Lipase activity was qualitative evaluated based on the diameter of the clear zone: slight activity - zones 1-2 mm, medium activity - zones 2-5 mm, large activity - zones > 5 mm, absent activity - no zones (Ramnath et al., 2017).

Screening for cutinase production by the isolated microorganisms

Basic indicator method

The screening of the cutinase activity of the microorganisms was performed on modified Czapeck-Dox medium (3 g $NaNO_3$, 1 g K_2HPO_4, 0.5 g KCl, 0.01 g $FeSO_4 \cdot 7H_2O$ and 17 g agar-agar in 1 L of distilled water) with the addition of flaxseed oil (10 g/L), as the sole source of carbon, and phenol red (Sigma-Aldrich, India) (5 g/L) as an indicator at pH 9, was used. All mineral salts were purchased from Sigma-Aldrich, Millipore, Germany. Cutinase activity was qualitative evaluated based on the color change of the medium from red to yellow: ++ = growth of microorganisms and detectable color change on selective medium, + = growth of microorganisms but no detectable color change on selective medium, − = no growth (Dickman & Patil, 1986; Rueda-Rueda et al., 2005).

Rhodamine B method

The method involves the cultivation of fungal and bacterial strains on triacetin (TRI) agar medium (NutriSelect® Basic, Switzerland) (g/L: special peptone 2.5; yeast extract 3.0; agar-agar 12.0) with the addition of triacetin (Sigma-Aldrich, United Kingdom) and the colorant Rhodamine B (Sigma-Aldrich, India). The production of cutinase enzyme is determined by the formation of a fluorescent halo around the colony during growth. Cutinase activity was qualitative evaluated based on the appearance of a fluorescent halo: ++ = growth of microorganisms and detectable fluorescent halo on selective medium, + = growth of microorganisms but no detectable fluorescent halo on selective medium, − = no growth of microorganisms.

The cutinase activity of the strains, inoculated on modified Czapek and TRI media with Rhodamine B, was evaluated starting with the 6th day of growth, until the 14th day (Bornscheuer, 2002; Macedo & Pio, 2005). The results represent the data from two experiments in three repetitions.

RESULTS AND DISCUSSIONS

The use of microbial enzymes in the biodegradation of plastic has been demonstrated in a lot of researches. Thus, the cutinase produced by *Thermomonospora* spp. (Raoufi et al., 2023), yeasts *Cryptococcus magnus* (Ueda et al., 2021), filamentous fungi *Fusarium solani pisi*, *Fusarium oxysporum*, *Humicola insolens*, *Aspergillus fumigatus* (Zhu et al., 2022; Raoufi et al., 2023), was effective in degradation of PE, PET, polybutylene succinate (PBS), and polybutylene terephthalate (PBT). Some species of fungi, known as lipase producers, also are involved in the degradation of LDPE (*Phanerochaete chrysosporium*) (Janapaty, 2021), PBS (*Cryptococcus* sp., *Rhizopus delemer, Candida antarctica, Thermomyces lanuginosus, Candida rugosa*) (Srikanth et al., 2022), PET and PE (*Aspergillus, Acremonium, Alternaria, Beauveria, Candida, Eremothecium, Fusarium, Geotrichum, Humicola, Mucor, Ophiostoma, Penicillium, Rhizomucor, Rhizopus, Trichoderma*) (Raoufi et al., 2023).

Although there is more information about cutinases of fungal origin, a number of bacteria capable of actively producing cutinase have been identified. The most studied source of bacterial cutinase is the actinobacteria *Thermobifida fusca*, followed by other representatives of this genus (*T. vulgaris, T. cellulosilytica, T. alba,* and *T. halotolerans*) (Furukawa et al., 2019; Xu, 2020; Amobonye, et al., 2023), as well as representatives of the genus *Streptomyces* (*Streptomyces scabies*)

(Jabloune et al., 2020), which have demonstrated PET-degrading potentials. It was reported about PET degrading ability of the marine bacterium *Pseudomonas aestusnigri* (Bollinger et al., 2020).

In our research from representatives of the order Hypocreales, five strains of the genus *Trichoderma* and one strain of the genus *Fusarium* were isolated according to the colonial aspects and microscopic morphology (Garybova & Lekomtseva, 2005). The representatives of this order mostly showed a weak lipolytic activity, the exceptions being strains *Trichoderma* sp. PE1 and *Trichoderma* sp. PE4, which had a medium tributyrin lysis activity (Table 1). It should be noted that lipolytic activity was not observed in all the strains from the 3rd day of growth and lasted throughout the testing period. Thus, zones of tributyrin lysis appeared from the 4th day of growth of the *Trichoderma* sp. PE4, and the strain *Trichoderma* sp. PE3 on the 10th day of growth no longer presented lipolytic activity. Tests for the determination of cutinolytic activity revealed only *Trichoderma* sp. PE2 as a cutinase-producing strain.

Table 1. Lipolytic and cutinolytic enzymes in representatives of the order Hypocreales

Strains	Lypolitic activity, diameter of the lysis zone (mm)	Rating Phenol red	Rating Rhodamine B
Trichoderma sp. PE1	2.88 ± 0.67	+	-
Trichoderma sp. PE2	1.08 ± 0.25	++	+
Trichoderma sp. PE3	1.33 ± 0.51	+	-
Trichoderma sp. PE4	4.83 ± 0.32	-	-
Trichoderma sp. PE5	1.96 ± 0.13	+	+
Fusarium sp. PE1	1.42 ± 0.25	-	-

Penicillium strains were found to be more active producers of extracellular cutinolytic and lipolytic enzymes. Thus, out of 13 representatives of the genus *Penicillium*, 4 had increased activity of lipase production, 6 strains - average activity, 1 strain - reduced synthesis, and 2 strains were lipase-negative, but produced cutinase (Table 2). In some strains, the synthesis of the lipase enzyme started later, for example at *Penicillium* sp. PE3 from day 4, at *Penicillium* sp. PE5 from day 5, and *Penicillium* sp. PE11 from day 6. There were strains at which lipase synthesis was not fixed throughout the experiment. So, at *Penicillium* sp. PE1 the tributyrin lysis activity disappeared in the 7th day, and at the strains *Penicillium* sp. PE6 and *Penicillium* sp. PE10 - on the 10th day.

Tests to determine the synthesis of cutinase enzyme by *Penicillium* strains revealed the discoloration of phenol red or the appearance of fluorescent halos in 10 isolates.

It was established that 8 from 13 strains of genus *Penicillium* show both lipolytic and cutinolytic activity. *Penicillium* sp. PE8, *Penicillium* sp. PE11 and *Penicillium* sp. PE13 showed only lipolytic activity, and *Penicillium* sp. PE1 and *Penicillium* sp. PE4 showed only cutinolytic activity.

The strain *Aspergillus* sp. PE1 proved to be an active producer of the lipase enzyme, but does not produce cutinase, while the representative of the genus *Alternaria* sp. PE1 active produces both enzymes.

Table 2. Lipolytic and cutinolytic enzymes in representatives of the order Eurotiales and Pleosporales

Strains	Lypolitic activity, diameter of the lysis zone (mm)	Rating Phenol red	Rating Rhodamine B
Penicillium sp. PE1	0.17 ± 0.32	++	-
Penicillium sp. PE2	3.08 ± 0.91	++	-
Penicillium sp. PE3	1.54 ± 0.31	++	-
Penicillium sp. PE4	0.00	++	++
Penicillium sp. PE5	3.17 ± 0.51	++	+
Penicillium sp. PE6	3.25 ± 0.34	++	++
Penicillium sp. PE7	5.79 ± 0.31	++	++
Penicillium sp. PE8	17.00 ± 1.76	+	+
Penicillium sp. PE9	2.92 ± 0.25	++	-
Penicillium sp. PE10	6.83 ± 0.51	-	-
Penicillium sp. PE11	2.75 ± 0.34	++	++
Penicillium sp. PE12	6.67 ± 0.75	++	++
Penicillium sp. PE13	4.00 ± 0.56	+	-
Aspergillus sp. PE1	8.25 ± 0.86	+	+
Alternaria sp. PE1	10.88 ± 0.26	+	++

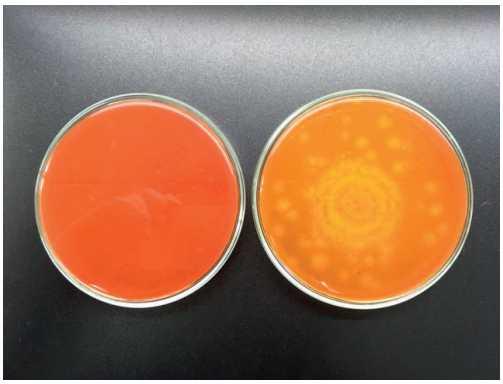

Figure 1. Phenol red method: orange plate - *Ascomycota* sp. PE1, without cutinolytic activity, yellow plate - *Penicillium* sp. PE6, demonstrates cutinolytic activity

Among the representatives of Ascomycota filum were three strains of yeasts (PE3, PE4, PE5), which had the property of synthesis of both enzymes, lipase and cutinase, and the strain *Ascomycota* sp. PE1, which actively produces lipase (Table 3). It should be mentioned that the activity of tributyrin lysis in the Yeast PE5 strain was reported starting with the 5th day of growth.

Table 3. Lipolytic and cutinolytic enzymes in representatives of the phylum Ascomycota

Strains	Lypolitic activity, diameter of the lysis zone (mm)	Rating Phenol red	Rating Rhodamine B
Yeast PE1	0.00	+	+
Yeast PE2	0.00	+	+
Yeast PE3	3.00 ± 0.39	+	++
Yeast PE4	5.00 ± 0.79	+	++
Yeast PE5	6.83 ± 0.32	+	++
Yeast PE6	0.00	+	+
Yeast PE7	0.00	+	+
Ascomycota sp. PE1	7.17 ± 0.51	+	+

Representatives of the genus *Pseudomonas*, for the most part, possess lipolytic activity except for the strain *Pseudomonas* sp. PE1 (Table 4). Testing the cutinolytic activity showed that all strains of pseudomonads grew on the media with the addition of Phenol red and Rhodamine B, but only 2 strains, Pseudomonas sp. PE2 and Pseudomonas sp. PE5, reacted positively by forming fluorescent halos. It should be noted that these two strains also have good lipolytic activity.

Table 4. Lipolytic and cutinolytic enzymes in representatives of the genus *Pseudomonas*

Strains	Lypolitic activity, diameter of the lysis zone (mm)	Rating Phenol red	Rating Rhodamine B
Pseudomonas sp. PE1	0.00	+	-
Pseudomonas sp. PE2	3.00 ± 0.56	+	++
Pseudomonas sp. PE3	1.67 ± 0.51	+	+
Pseudomonas sp. PE4	2.67 ± 0.32	+	+
Pseudomonas sp. PE5	3.29 ± 0.41	+	++
Pseudomonas sp. PE6	3.25 ± 0.34	+	+

Among the representatives of actinobacteria, the genus *Streptomyces*, of 6 strains, 3 formed zones of tributyrin lysis, denoting good (*Streptomyces* sp. PE3 and *Streptomyces* sp. PE6) and very good (*Streptomyces* sp. PE5) lipolytic activity (Table 5). Cutinolytic activity was observed only in two strains, *Streptomyces* sp. PE3 and *Streptomyces* sp. PE6.

The research has revealed that 19 out of the 21 isolated filamentous fungi strains produce exocellular enzymes with lipolytic activity (90%), including 6 isolates (29%) showed high lipolytic activity. The number of strains exhibiting cutinase activity was significantly smaller - 12, which is 57% of the total micromycetes. Both extracellular enzymes were recorded for 43% of yeasts isolates (three of 7 cultures).

Table 5. Lipolytic and cutinolytic enzymes in representatives of the genus *Streptomyces*

Strains	Lypolitic activity, diameter of the lysis zone (mm)	Rating Phenol red	Rating Rhodamine B
Streptomyces sp. PE1	0.00	+	-
Streptomyces sp. PE2	0.00	-	-
Streptomyces sp. PE3	3.17 ± 0.32	+	++
Streptomyces sp. PE4	0.00	-	-
Streptomyces sp. PE5	5.58 ± 0.47	+	-
Streptomyces sp. PE6	4.17 ± 0.75	+	++

Of the 6 tested strains of pseudomonads, 83% (5 strains) exhibited lipolytic activity, 2 of which also had cutinolytic activity (33%). Of the tested streptomycetes, 2 strains produce

lipase and cutinase (33%), and one strain has high lipolytic activity.

The data obtained in our research are in accordance with the multiple researches presented in the specialized literature (Dickman & Patil, 1986; Rueda-Rueda et al., 2005; Gopinath et al., 2005; Aly et al., 2012; Ramnath et al., 2017).

CONCLUSIONS

The screening study of the production of extracellular enzymes by the microorganisms populating the LDPE surface demonstrates the effectiveness of rapid testing. Namely, it has been shown that not all strains of microorganisms produce lipases or cutinases, that the production of extracellular enzymes is an individual physiological process, which in different strains begins at different growth phases.

Of the total number of tested microorganisms, including fungal and bacterial strains, 75% possess lipolytic activity and 46% cutinolitic activity, 41% can produce both lipase and cutinase. It can be highlighted, that from 17 isolates which possesses both activities, 59% are mycelial fungi, including representatives of the genus *Penicillium*, making up 47% of active strains.

The ability to produce these extracellular hydrolytic enzymes indicates the possibility to metabolize polymers, such as polyethylene.

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