Screening and selection of microorganisms for oil-based plastics biodegradation

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Abstract

Oil-based plastics have raised severe environmental and health concerns because they accumulate due to their refractoriness to biological degradation. Consequently, the search for an efficient biological technique for their treatment is a current challenge. Several authors have reported microorganisms and specific enzymes capable of breaking down these polymers, but their number is quite limited and inefficient in most cases. Therefore, there is an urgent need to enlarge the resources available for that purpose. Twenty-seven microorganisms (bacteria and fungi) isolated from composting and olive mill wastewater sludges (OMWs) were used for this study. Microorganisms were tested for the expression of lignin-degrading enzymes (laccase, oxidase, and polyphenoloxidase) as well as esterases (lipase, cutinases, and polyurethanase). The microorganisms isolated from composting and OMWs covered the full spectrum of enzymatic activities tested, however, the highest proportion of microorganisms bearing most enzymatic activities came from composting. The most frequently expressed enzymatic activities in composting isolates were related to lignin metabolism while isolates from OMWs mainly expressed esterases. Thus, microorganisms isolated from composting are considered the most suitable candidates for the biodegradation of oil-based plastics due to the wide range of enzymes produced. Among them, two fungi *Rhodotorula* RHM1, *Aspergillus* RHM15 and the bacteria *Bacillus* RBM2 were chosen because they showed the greatest range of enzymatic activities, including lipases, cutinases and ligninases. Therefore, composting could be considered as a source of functionally efficient microorganisms to use them as biotechnological tools for biodegradation of recalcitrant xenobiotics compounds, such as oil-based plastics.

Keywords: Composting, olive oil mill wastewater, enzymatic profile, ligninases, esterases.

1. Introduction

Oil-based plastics are a set of diverse synthetic long chain polymeric materials, mainly formed by carbon bonds which tend to accumulate in the environment due to their poor biodegradability [1]. Currently, an estimated 8.5 billion tons of plastic have been produced, of which 6.3 billion tons (76%) have become waste. Approximately, 9% of plastic waste have been recycled, 79% stored in landfills or dumped into the environment and 12% has been incinerated [20].

Due to the recent incorporation into the ecosystems, microorganisms have not coevolved to develop the enzymatic mechanisms necessary for the mineralization of these recalcitrant materials, which makes the accumulation of plastic waste a serious threat to the sustainability of our planet sustainability [3]. The scientific community has focused on the research of biological systems as an efficient alternative to the depolymerization of oil-based plastic wastes by enzymes, increasing the accessibility for cellular assimilation [22].

Most oil-based plastics exhibit high resistance to biodegradation, generally caused by their stable C-C bonds, being heteroatomic polymers with hydrolyzable bonds in their backbone the most susceptible to biodegradation [19]. The vast majority of enzymes described so far and employed to attack plastics are ligninases and lipases [2]. Lignin is the most recalcitrant compound found in nature due to its structural complexity and its wide variety of bonds, existing a strong relationship between enzymes involved in lignin metabolism and their success in degrading recalcitrant xenobiotic compounds [9]. Some of these enzymes are laccases, specific phenol-oxidases described oxidizers lignin-associated compounds and polyphenoloxidases, responsibles for the degradation of structurally complex compounds by oxidation of aromatic rings with molecular oxygen [6]. Fatty acids contain ester bonds similar to those of some plastics, and some authors have described a structural relationship between some plastic degrading enzymes and lipases, cutinases and polyurethanases. Lipase, or triacylglycerol esterase is capable to produce monoglycerides and free fatty acids in presence of water from short-chain fatty acid triglyceride, used as pre-screening for cutinases, serine esterases capable of hydrolyzing acid esters with higher affinity for short-chain esters. Polyurethanases are a group of esterases with hydrophobic polyurethane-surface-binding domain essential for polyurethane degradation, which degradation mechanism is the result of

synergistic activity between endopolyurethanases and exopolyurethanases [8]. Thus, this enzymes group has been the focus of attention in recent research on bioremediation of plastic waste [13].

The aim of this work was the search for plastic-degrading related enzymes within a collection of microorganisms isolated from compost and OMWs. Composting is an aerobic and thermophilic biological process, in which organic matter is biotransformed by a wide variety of microorganisms, resulting in a stable product called compost, which improves soil characteristics and is used as a fertilizer. This process is carried out in different stages, characterized by extensive nutritional changes involving a wide variety of substrates, including lignocellulose. Ligninolytic microorganisms can be isolated from different environments, being composting one of the most appropriate because of the effective biodegradation of lignin that occurs in that organic matter biotransformation process [14]. Another less-known habitat for these microorganisms is concentrated of olive oil mill wastewater sludges (OMWs). OMWs a liquid residue from the olive oil extraction industry, which is rich in lignin-like compounds and a large variety of remaining fatty acids [12]. Consequently, composting and OMWs are excellent environments for the isolation of microorganisms with a potential enzymatic system capable of degrading plastic waste.

2. Material and methods

2.1 Chemicals

Impranil® DLN-SD emulsion was provided by COVESTRO (Leverkusen, Germany). Tributyrin 97% was provided by Acros-Organics. Polycaprolactone, tannic acid, phenol red, α-naftol and guaiacol 99% were provided by Sigma-Aldrich/Merck (St. Louis, Missouri, USA). Glycerol monostearate purified powder was supplied by Alfa Aesar (Ward Hill, Massachusetts, USA).

2.2 Microorganisms collection

Twenty-seven microorganisms (fungi and bacteria) previously isolated from compost (11 strains) and plant waste composting piles (16 strains) [10, 12].

2.3 Analysis of relevant enzymes associated with the degradation of plastics

Microorganisms collection was subjected to qualitative tests for determination of several enzymes related to plastic degradation, including ligninases (polyphenol oxidase, laccase and oxidase) and lipases (cutinases and long-chain fatty acid degraders) on plate assays tests with specific media for each case as explained below [11]. All media were inoculated with a 1 cm^2 plug of a 5 days-old fungal culture on PDA or by streaking the bacteria previously grown on APHA (Panreac) for 24h. After inoculation all media were incubated at 30 ºC for 24h (bacteria) or 5 days (fungi).

Polyphenoloxidase activity (PO) was determined by inoculation on PDA and APHA supplemented with 0.5% (w/v) tannic acid. After incubation, brown color around the microorganism was taken as positive .Laccases, oxidase, and peroxidase activity were detected by adding 2-3 drops of each specific reagent on colonies previously grown in general mediums PDA or APHA for fungi and bacteria respectively, as described in Rayner and Boddy [16]. When incorporating a guaiacol solution (1.24 g in 100 ml of 96% ethanol) the color of the colonies with laccase activity (LAC1) turned purple; extracellular oxidase (LAC2) was determined by the appearance of red color when adding gum guaiac (0.5 g in 30 ml of 96% ethanol) on positive strain.

Lipase (LIP), polycaprolactone-degrading (CUT2), and polyurethanase (PU) activities were tested on opaque solid media as sole carbon sources which positive strains showed a clearance halo after incubation. LIP contains 1% (w/v) of universal substrate tributyrin with a short-chain fatty acid triglyceride. CUT2 were tested through polycaprolactone 10% (v/v) as structural analog from cutine. Impranil® DLN-SD 1% (v/v) was used as an anionic aliphatic polyester polyurethane like inducer for PU screening on mineral basal medium agar. By contrast, culture medium with glycerol monostearate purified powder 2%

(w/v) was used as part of molecular structure from cutine like substrate of cutinase (CUT1). Phenol red has been used as a dye indicator of pH change as a result of the hydrolysis reaction releasing acid compounds turning the color from red to yellow.

2.4 Identification of isolates

The identity of selected isolates were determined by molecular methods according to [10]. Amplification was carried out using MyCycler thermal cycler (Biorad). Fungal DNA extraction was performed by plantDNAzol kit (Invitrogen) in accordance with manufacturer's protocol. Universal primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') were used for PCR amplification of 5.8 ITS ribosomal region for fungi. In case of bacterial genomic DNA extraction, pure colonies were suspended in 100 µL of sterile distilled water subjected to a thermal shock (97 ºC) for five minutes and instant ice bath for five minutes. The identities of specific isolates were determined based on partial or nearly full length 16S rRNA gene using universal primers: 27F (5'-AGAGTTTGATCATGGCTCAG-3') and 907R (5′-CCGTCAATTCMTTTRAGTTT-3′). Forward and reverse sequences were edited, assembled and aligned using the programs Sequence Scanner v1.0 (Applied Biosystem), Reverse Complement (www.bioinformatics.org/sms/rev_comp.html), Clustal X v2.0.11, and MEGA 5 v5.2. The partial or nearly full-length sequences were compared for similar nucleotide sequences with the BLAST search of the National Center of Biotechnology Information (NCBI, http://blast.ncbi.nlm.nih.gov/Blast.cgi). The following thermal profile was used for the PCR for bacteria and fungi, respectively: 95 ºC for 2 min, 34 cycles (94 ºC for 1 min, 55 ºC for 1 min and 72 ºC for 1.5 min), 72 ºC for 10 min; 94 ºC for 10 min, 30 cycles (94 ºC for 1 min, 51 ºC for 1 min, and 72 ºC for 3 min), 72 ºC for 10 min.

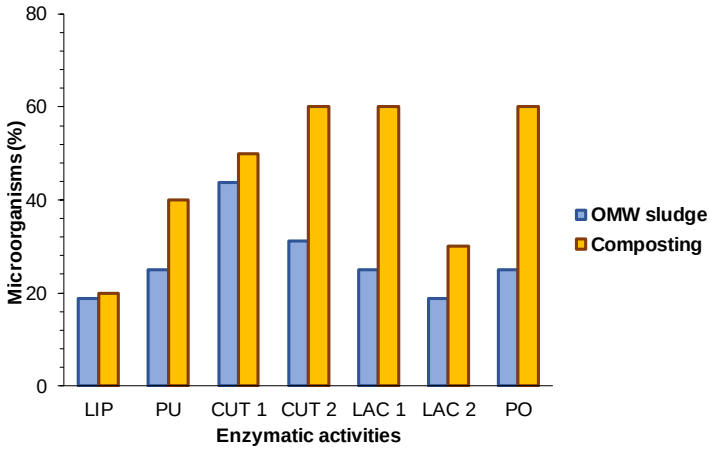
3. Results and discussion

3.1 Comparison of the activities of the collection according to environment of origin.

Comparisons were made between the enzymatic activities of microorganisms isolated from the different compost and OMW environments. The results are intended to clarify the range of enzymatic activities present in each environment, as well as the percentage of microorganisms from each environment with the capability to express plastic-degrading enzymatic activities.

Microorganisms isolated from composting and OMW sludges covered the full spectrum of enzymatic activities tested. However, the highest proportion of microorganisms bearing most enzymatic activities came from composting (Figure 1).

a)



b)

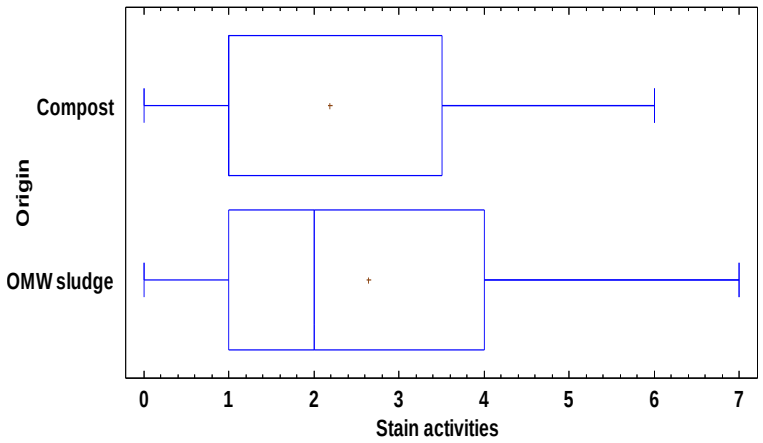


Fig. 1 a): Proportion of microorganisms isolated from OMW sludge (blue) and composting (orange), with the capability to express plastic-degrading enzymatic activities such as lipase (LIP), polyurethanase (PU), cutinases (CUT1 and CUT2), laccases (LAC1 and LAC 2) and polyphenoloxidase (PO). b): Number of enzymatic activities per strain from compost (up) and OMWs (below).

All enzyme activities were expressed by microorganisms isolated from both environments, being in higher proportion those isolated from compost. On the other hand, there is greater variability in the number of enzyme activities expressed in the OMWs environment than composting, being the mean 2.67 versus 2.16 activities per strain, respectively. This highlights the great suitability of the two environments as a source of microorganisms with enzyme profiles associated with oil-based plastic degradation, where a large percentage of the target enzyme activities are expressed in by adequate amounts of strains.

The most frequently expressed enzymatic activities in composting isolates were related to lignin metabolism (LAC 1 and PO). Robledo-Mahón et al. [18] tested a total of 40 fungal and 128 bacterial strains were tested from composting who 42% expressed PO and 15% of fungus expressed LAC, postulating microorganisms from composting as ligninase producers. This could be explained because of the selective pressure of that environment, in which extensive production of different enzymes for the biodegradation of lignocellulose are required. Isolates from OMW sludge mainly expressed esterases such as CUT, albeit lignin-degrading activity was also detected in some isolates. Castro-Ochoa et al. [5] purified and identified for the first time a cutinase produced by an strain of *Aspergillus* induced by olive oil due its large different lipidic and carbon sources, present compounds in OMWs.

3.2 Comparison of the activities of the collection according to genus

In order to classify by genre the enzymatic activities with the greatest potential for the degradation of oil-based plastics, the percentage of enzymatic activities most frequent by the different genera of the collection of microorganisms was studied (Figure 2). All genres studied expressed some of the activities related to the biodegradation of oil-based plastics, being the most outstanding *Bacillus*, *Aspergillus* and *Rhodotorula* genres. Employment of these genera for plastic biodegradation has been successfully demonstrated in numerous studies.

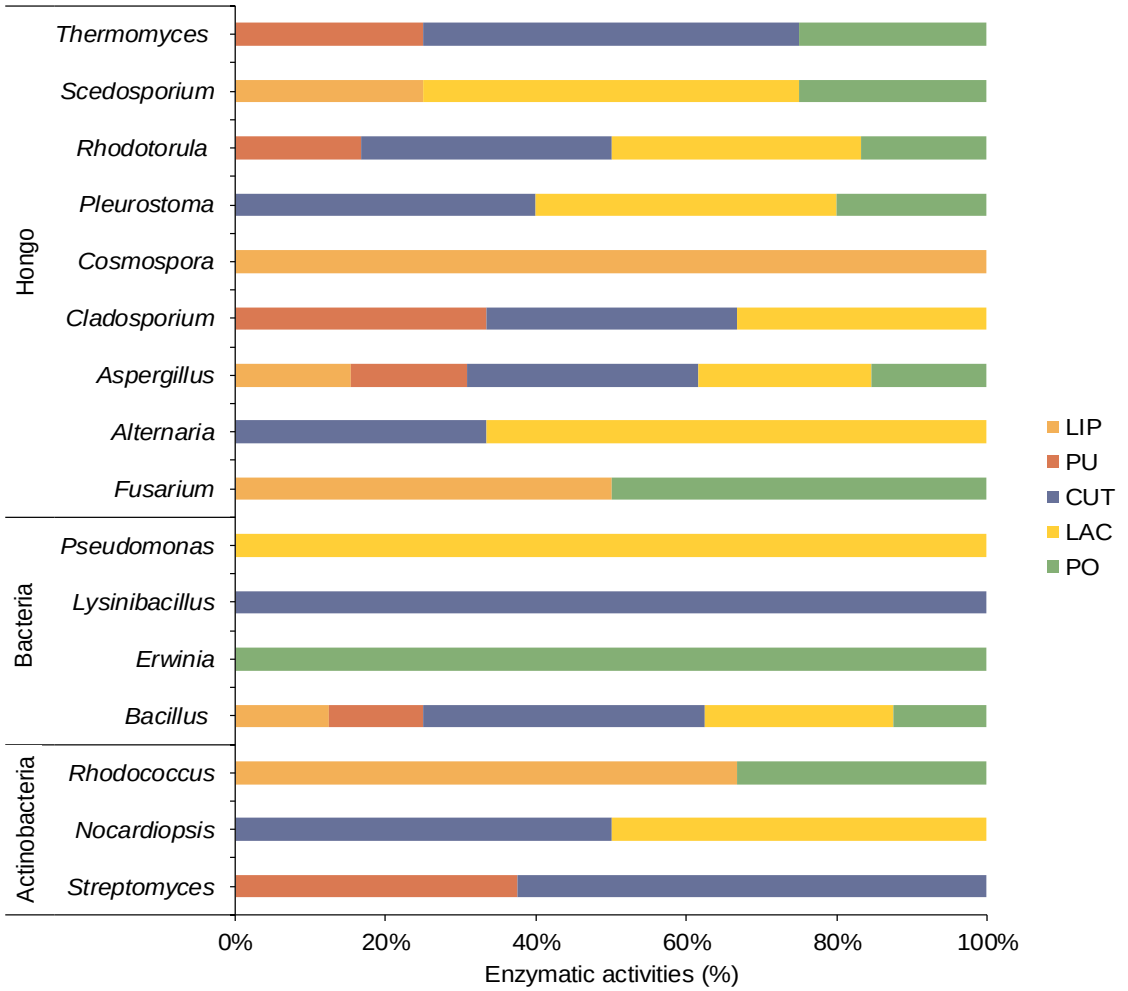


Fig. 2 Most common enzymatic activities distributed per genres.

As shown in Figure 2, *Aspergillus*, *Rhodotorula* and *Bacillus* genres covered the full spectrum of enzymatic activities proportionally about 10 - 20% of each one, being *Bacillus*' case the most interesant due to his bacteria condition. Raaman et al. [15] isolated two *Aspergillus* strains from polyethylene polluted region and confirmed by means of a microbial degradation test of plastic with low density polyethylene (LDPE) the weight loss about 11% in a month. Further the surface of degraded LDPE was analyzed by SEM, showing a clear alteration with hole formation. Das and Kumar [7] studied low-density polyethylene weight loss, SEM surface alteration and assessment of mineralization level from two *Bacillus* strains with positive results, reaching about 14%. *Rhodotorula* performs an optimus enzymatic profile for plastic biodegradation, showing four different activities expressed about proportionally, while *Bacillus* showed a major proportion of cutinases. Cutinases production are strongly related with oil-based plastic degradation. Yoshida et al. [21] discovered an enzyme capable of degrading PET produced by a microorganism isolated from a plastic polluted site, which by crystallographic studies was determined to have a structure similar to cutinase. Roberts et al. [17] employed a microbial consortium of *Pseudomonas* and *Bacillus* where cutinase activity is used for the PET degradation, reporting this genus and enzyme as a key for biotechnological treatment to solve the plastic challenge. Moreover, Barratt et al. [4] studied the colonization sequence on plastics, occupying *Rhodotorula* genre the second place in mineralization action after filamentous fungus.

Among them, *Rhodotorula* RHM1, *Aspergillus* RHM15 and *Bacillus* RBM2 were chosen. These strains showed the greatest range of enzymatic activities, including lipases, cutinases and ligninases, which are considered valuable for the biodegradation of plastics.

Conclusions

Both environments are potential candidates as a source of microorganisms with enzymatic profiles associated with plastic degradation, being microorganisms isolated from composting considered the most suitable candidates for the biodegradation of oil-based plastics due to the wide quantity and range of enzymes lipases and ligninases produced.

Acknowledgments

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