

Acta Scientiarum. Technology  
ISSN: 1806-2563  
ISSN: 1807-8664  
actatech@uem.br  
Universidade Estadual de Maringá  
Brasil

## Evaluation of the biodegradation of phthalic contaminants originated from polyvinyl chloride commercial films

**Luzia, Márcia Regina Figueiredo; Sarrouh, Boutros; Lofrano, Renata Carolina Zanetti**

Evaluation of the biodegradation of phthalic contaminants originated from polyvinyl chloride commercial films

Acta Scientiarum. Technology, vol. 42, e47010, 2020

Universidade Estadual de Maringá, Brasil

**Disponível em:** <https://www.redalyc.org/articulo.oa?id=303265671039>

**DOI:** <https://doi.org/10.4025/actascitechnol.v42i1.47010>



Esta obra está bajo una Licencia Creative Commons Atribución 4.0 Internacional.

# Evaluation of the biodegradation of phthalic contaminants originated from polyvinyl chloride commercial films

Márcia Regina Figueiredo Luzia  
Universidade Federal de São João Del-Rei, Brasil

DOI: <https://doi.org/10.4025/actascitechnol.v42i1.47010>  
Redalyc: <https://www.redalyc.org/articulo.oa?id=303265671039>

Boutros Sarrouh  
Universidade Federal de São João Del-Rei, Brasil

Renata Carolina Zanetti Lofrano  
Universidade Federal de São João Del-Rei, Brasil  
[renataczlofrano@ufsj.edu.br](mailto:renataczlofrano@ufsj.edu.br)

Recepción: 15 Marzo 2019  
Aprobación: 01 Mayo 2019

## ABSTRACT:

The excessive use of phthalates (PHEs) plasticizers has promoted a serious environmental problem. This study evaluated the biodegradation of PHEs from polyvinyl chloride (PVC) film, aiming to mitigate damage to the environment. Therefore, 23 microorganisms were prospected and isolated from the Serra do Ouro Branco State Park (Minas Gerais, Brazil), where screening was carried out by growth in enriched microbial medium and esterase-activity was monitored by diffusion in solid agar. Three microorganisms were selected. Bacterium (I) presented the highest esterase activity, as evidenced in the diffusion tests in solid medium. Further, evidence of this is given by the biodegradation of PHEs into phthalic acid, because changes the acid concentration from 0 to  $3.10 \pm 0.07 \text{ mol L}^{-1}$  and 28 % mass reduction of PVC film after 24 days. Furthermore, after six days of biodegradation the number of viable cells and ammonia concentration showed no significant change. This indicates that the survival of the Bacterium (I) cells is independent of ammonia consumption, but rather depends on PHEs consumption. The detection of the organic functions of -COOH and -COOR proved the biodegradation of PHEs by Bacterium (I), thus demonstrating its potential to be used in bioremediation of soils and rivers from PHEs contaminants.

**KEYWORDS:** plasticizer, organic contaminant, bioremediation.

## INTRODUCTION

Polymeric materials are present in the everyday world of humanity in various appliances and devices. These include toys, automotive vehicles, packaging materials, various paints -and varnishes, fibers for fabrics and prostheses -and implants. This vast use is the result of the durability, ease of processing and the suitability of these materials for their desired usages. In general, such adaptation requires the use of plasticizers such as the di-n-octyl phthalate (PDO), diethyl phthalate (DEP), dimethyl phthalate (DMP), di-(2-ethylhexil phthalate) (DEHP) and dibutyl phthalate (DBP). These compounds are known as Phthalates (PHEs) and improve the flexibility and malleability of polymeric materials. They are organic compounds, many stable due to their aromatic structure. Due to the high usage of these plasticizers, humans are often exposed to PHEs. This can be through the air, the skin and by ingestion of contaminated food. Once absorbed, these compounds act as endocrine disruptors and become responsible for various health problems. Examples of these are mental retardation, immunodeficiencies and various forms of cancer (Vilela, Bassin, & Peixoto, 2018).

## NOTAS DE AUTOR

[renataczlofrano@ufsj.edu.br](mailto:renataczlofrano@ufsj.edu.br)

High production, use and inappropriate disposal of polymeric materials containing PHEs, have promoted the omnipresence in the environment in measureable levels worldwide (air, water, soil, sediment and mud). The reason behind this is the high water solubility of PHEs (Gani, Tyagi, & Kazmi, 2017). Such characteristics and environmental conditions justify the interest of the scientific community into the research of presence, effects, control, treatment, characterization and identification of PHEs, in order to seek ways to reduce the impact of these compounds, to both naturally occurring and man-made materials (Zhao et al., 2016).

Several surveys report that the aerobic biodegradability is the primary means of mineralization of PHEs in the environment and that the combination of different technologies of wastewater treatment has demonstrated efficiency in removing PHEs in individual treatments (Net, Sempéré, Delmont, Paluselli, & Ouddane, 2015). Xu, Li, and Wang (2008) analyzed the types of pollution and degradation of the compounds DBP and DEHP in soils, whose experiments have confirmed that the two compounds are most degraded through microbial processes (Kong et al., 2019). In addition, the analysis of the degradation products has shown that the metabolism of PHEs studied, begins by hydrolysis of DBP. This hydrolysis forms products ranging as di-esters, mono-esters and alcohols. The DBP was firstly degraded into phthalic acid (PA) by microbial hydrolyzation, then to protocatechuate followed by ring cleavage to obtain beta-ketoadipate and 4-oxalocitratemalate, which was transformed in oxalacetate and pyruvate, and finally was mineralized to carbon dioxide and water accordingly. Similar results were obtained by the biodegradation of DEHP by *Pseudomonas fluorescences*, isolated from activated sludge from petrochemical industries, which produced the PA under aerobic conditions. Other research has indicated that biodegradation appears to be more effective than abiotic degradation in surface water and that PHEs at low molar mass and simple side chains (DMP and DEP) are more easily biodegraded than those with higher molecular masses (Ren, Lin, Liu, & Hu, 2018).

Commercial films of PVC are expressively consumed today and, once they contain PHEs in their composition, generate waste that contaminates waters and soils with these harmful organic compounds (Gao & Danwen, 2016). Thus, studies on biodegradation of PHEs are allied to bioprospecting of new microorganisms from regions with important biodiversity for the development of a process to mitigate these contaminants from PVC commercial films.

The research, described here, aimed to monitor and evaluate the biodegradation of PHEs found in commercial PVC film by using microorganisms isolated from the Serra do Ouro Branco State Park (Minas Gerais, Brazil), for the construction of a biological treatment process to remove PHEs.

## MATERIAL AND METHODS

### Sample collection

The microorganism bioprospecting was held in the Serra do Ouro Branco State Park (Minas Gerais, Brazil) and established using a tracking device in the Global Positioning System (GPS), at five different collection points. At these points, ten samples were collected from various natural occurring conditions and parts of plants. These were spring water, bryophytes, soil covered by dense vegetation, mounds made of earth, leaves of small trees, bromeliads and the stems of large trees. The samples were packaged in polypropylene beakers of 1 L, which were previously sterilized, due to the ease of handling in these collection areas. The samples were transported in adequate racks and processed over a period of up to 5 hours after collection.

## Microorganism cultivation from the collected samples

For the isolation of the population of microorganisms, present in the bryophytes, leaves, stems of the trees and bromeliads, the collected samples were separated into small fragments and placed in direct contact with the culture medium 'Yeast Malt Agar' (YMA) ( $\text{g L}^{-1}$ ): malt extract (99.5%, Merck), 3; yeast extract (98%, Synth), 3; peptone (95.5%, Synth), 5; glucose (97.5%, Synth), 10; agar (93.8%, Synth), 17. Soon after, the plates were incubated at 30°C until the colonies began to grow (Fuentefria & Valente, 2005). For the water samples, 1.0 mL sample was taken and placed in direct contact with the solid media. Samples of the soil and earth mounds were inoculated by placing a small amount of the samples at the center of the plates. All plates were incubated at 30°C (Silva, Bicas, Sarrouh, & Lofrano, 2016).

## Selection of microorganisms

After the incubation period, the microorganisms were isolated according to the macro morphological characteristics (color and appearance) of the colonies. Subsequently, the colonies were purified by the creation of grooves into the solid YMA media. This procedure was repeated as many times as required, in order to obtain pure colonies. Isolated colonies were then incubated in solid YMA media during 30 days at 4°C. The identification of the isolated microorganisms was performed by Gram staining techniques for bacteria and by microculture test for fungi (Larone, 2011). The isolated microorganisms were inoculated onto solid and liquid media. The colonies were added onto solid mineral medium ( $1.0 \text{ g L}^{-1} \text{ NH}_4\text{Cl}$  (98%, Synth),  $0.5 \text{ g L}^{-1} \text{ K}_2\text{PO}_4$  (99.5%, Synth),  $20 \text{ mg L}^{-1} \text{ MgSO}_4 \cdot 7 \text{ H}_2\text{O}$  (98%, Synth), adding  $17 \text{ g L}^{-1}$  agar (93.8%, Synth) and containing, as the sole source of carbon and energy, approximately 1.0 g PVC film (BOREDA-plot nº 32280) with 11.0  $\mu\text{m}$  thickness (caliper Kingtools 500,150) (Niazi, Prasad, & Karegoudar, 2001).

## Esterase detection and characterization

Esterases were detected by agar diffusion method. The ability to hydrolyze esters was tested in Petri dishes containing 10.0 g peptone (95.5%, Synth), 5.0 g of NaCl (99%, Synth), 0.1 g  $\text{CaCl}_2$  (98.9%, Synth), 15.0 g agar (93.8%, Synth), 10.0 g Tween 80 (99%, Merck), and 1.0 L distilled water. The prepared plates were then inoculated and incubated at 30°C. The plates were examined, in these incubation conditions, for ten days. Esterase activity was identified by the presence of a visible precipitate (opacity) around each colony. The diameters of the halos were measured with a pachymeter (Kingtools 500, 150). From the values obtained, the enzymatic index (EI) was calculated considering the ratio of the average diameters of degradation of the substrates to the average diameter of the respective colonies (Carissimi, Stopiglia, Souza, Corbellini, & Scroferneker, 2007).

## Biodegradation of PVC films

For the biodegradation assay, 25.0 mL inoculum aliquots were distributed in twelve 250 mL Erlenmeyer flasks, containing 125 mL mineral medium ( $1.0 \text{ g L}^{-1} \text{ NH}_4\text{Cl}$  [98%, Synth],  $0.5 \text{ g L}^{-1} \text{ K}_2\text{PO}_4$  (99.5%, Synth),  $20.0 \text{ mg L}^{-1} \text{ MgSO}_4 \cdot 7 \text{ H}_2\text{O}$  (98%, Synth) supplemented with approximately 1.0 g PVC film (as the only carbon source) that was previously split in pieces and sterilized. This experiment was done in duplicate and two control tubes were prepared, with and without the PVC film (as positive and negative controls, respectively). Erlenmeyer flasks containing inoculum and the PVC film and the controls, were capped and

inserted into an orbital incubator (Multitec 430) at 180 rpm and 30°C. These tests were conducted for 24 days and monitored every 48 hours (Chatterjee & Dutra, 2003).

## Titration and molar concentration calculations

A volume of 40.0 mL was collected from the mixtures used in the biodegradation assays and 2 drops of alcoholic solution of bromothymol blue (indicator) were added. The alcoholic solution was created by mixing 50.0 mg bromothymol blue (95.9%, Merck), 4.0 mL aqueous solution of NaOH (97%, Merck), 0.02 mol L<sup>-1</sup>, 20.0 mL absolute ethanol (95%, Sigma Aldrich), and 100.0 mL distilled water. Using a standard solution of 0.0075 mol L<sup>-1</sup> NaOH a standard series was prepared. From the values of volume of NaOH standard solutions consumed in titrations, the Acidity Index (mol L<sup>-1</sup>) for each of the studied samples was calculated according to Equation 1. The same experiment was carried out for the volumes collected in the test mix negative control (absence of microorganism) (Perez, Anjos, Ebeling, & Pereira, 2009).

$$Ma = Mb.Vb / Va \quad (1)$$

where:

Ma: molar concentration of acid (mol L<sup>-1</sup>); Va: standard sample volume (L); Mb: molar concentration of the standard solution of NaOH (mol L<sup>-1</sup>); Vb: volume of NaOH standard solution consumed (L).

## Viable cell counts

Viable cells were monitored by counting colonies on plates for colony forming units (CFU). To this end, serial decimal dilutions of the suspensions of cells were performed using sterile peptone 0.1% (1.0 g L<sup>-1</sup> peptone [99%, Synth] and 8.5 g L<sup>-1</sup> NaCl [99%, Synth]). The counting was made by inoculating the diluted cell suspensions in Petri dishes, containing Agar medium prepared by blending 10.0 g peptone 5.0 g NaCl, 0.1 g CaCl<sub>2</sub> (98.9%, Synth) and 15.0 g agar (98%, Synth) in 1.0 L of distilled water. Inoculation was performed by spreading 1.0 mL of each dilution onto the surface of the media. The Petri dishes were incubated in an incubator at 30°C for 24 hours before counting the colonies. The mean value of colonies obtained (from 130 to 280 units) was used for dilution in the order of 1x10<sup>-8</sup> (Tortora, Funke, & Case, 2017).

## Evaluation of cell death by ammonia concentrations

Cell death was evaluated by ammonia concentrations in the samples (Sharpe & Woodrow, 1971). This was done by removing 50.0 µL aliquots of the mixtures in which the biodegradation occurred and adding 3.0 mL phenol reagent (11.68 mL phenol and 0.0625 g sodium nitroprusside (97.5%, Merck), 3.0 mL sodium hypochlorite reagent (6.25 g NaOH [97%, Merck]) and 3.67 mL sodium hypochlorite (98%, Merck). This was done every 24 hours, after which samples were incubated at 39°C for 20 min. in a water bath (Novatecnica NT 268). The samples were then analyzed in a UV/Vis spectrophotometer at 640 nm (Biospectro SP-220 Spectrophotometer) using a glass cuvette. The concentration of ammonia produced by bacteria was determined through a standard curve of ammonia, previously constructed (Sharpe & Woodrow, 1971).

## PVC mass loss

For determination of the mass loss of the PVC, samples of PVC films were taken during testing half reaction biodegradation over a period of 48 hours. These were washed with water Milli-Q, dried with anhydrous calcium chloride in an incubator (101/150 SOLAB) at 45°C for 48 hours and using an analytical balance (Mars AY220) at room temperature ( $\sim 25^{\circ}\text{C}$ ).

## Ferric hydroxamate test

The test of ferric hydroxamate reagent was prepared using a mixture of 1.0 mL hydroxylamine hydrochloride (99%, Sigma Aldrich)  $0.50 \text{ mol L}^{-1}$  in ethanol (95%, Sigma Aldrich) and 0.4 mL sodium hydroxide (97%, Merck) ( $6.0 \text{ mol L}^{-1}$ ). Where, 300.0  $\mu\text{L}$  from the mixture of biodegradation and 1.0 mL of the mixture of hydroxylamine hydrochloride (hydroxylamine reagent) were mixed in a test tube. It was then heated for 30 s in a water bath (Novatecnica NT 268) to  $40^{\circ}\text{C}$  and then cooled in an ice bath. 2.0 mL of an aqueous solution of hydrochloric acid  $1.0 \text{ mol L}^{-1}$  was then added along with a drop of ferric chloride aqueous solution (96%, Sigma Aldrich) 5% ( $\text{v v}^{-1}$ ). The test tube was slowly stirred afterwards. The appearance of wine/reddish brown color was indicative of the presence of a carboxylic acid functional group. The identical procedure was adopted in the control test without the presence of microorganisms. The ferric chloride test was done in a 50 mL beaker, containing 1.0 mL distilled water. Five drops from the reaction mixture used in biodegradation test and seven drops of aqueous solution ferric chloride (96%, Sigma Aldrich) were added and evaluated. The appearance of a violet, blue or green intense color is indicative of the presence of the phenols in the specimens tested. This procedure was used in the control test without the presence of microorganisms (Engel, Kriz, Lampman, & Pavi, 2012).

## RESULTS AND DISCUSSION

The aim of this study was the conduction of studies of biodegradation of PHEs from PVC commercial films. Emphasizing the screening and use of microorganisms isolated from the region of Alto Paraopeba, State of Minas Gerais/Brazil, known for its regional biodiversity. Thus, aiming to minimize the impacts arising from PHEs in this same location. Due to this, the Serra do Ouro Branco State Park located in the city of Ouro Branco, was selected as the area of bioprospecting because of the peculiar environmental characteristics and a great diversity of plant species with high biotechnological potential in relation to their microorganisms available. The collection of the samples was the subject of licensing by the Directory of Research and Biodiversity Protection, which belongs to the State Secretary for Environment and Sustainable Development of Minas Gerais (SEMAD). Five different georeferenced points for microorganism collection were established, making up a large area of the Park, aiming to isolate a greater diversity of species. To this end, the chosen regions showed a high diversity of habitats and fairly dense vegetation and due to this, ten different samples (water, soil, bryophytes, leaves and stems of trees and bromeliads) were collected. From these samples, 23 species of microorganisms were isolated.

The biochemical characterization occurred through Gram staining of the isolated bacteria. Six of the isolated bacteria showed the shape of rods, where two isolates were characterized as Gram positive and four as Gram negative. These bacteria were isolated from samples of water, leaves, stems of trees and soil. The colonies presented different colors (purple, yellow, orange, beige and white) as well as shiny appearances. Four different species of fungi of the genus *Aspergillus* sp, were morphologically identified. These fungi



originated exclusively from leaves and presented matte and yellow or beige looks. Another genus of fungi, also originating from leaves and identified as *Geotrichum* sp, had a matte appearance.

From the isolated microorganisms, three pure colonies were selected (I, II and III), which had higher growth in solid mineral medium supplemented with commercial PVC film as the sole source of carbon. The bacterium (I), from the water sample, showed brilliant purple colonies and cellular morphology as Gram-negative, rod-shaped bacteria. While both fungi *Aspergillus* sp (II) and (III) were obtained from the leaves and presented frosted, beige colored colonies.

Figure 1 presents an illustrative photo of the Petri dish prepared for selection in solid media. At this stage of the study, eight microorganisms were selected due to their ability to grow in solid mineral medium supplemented with PVC film as the sole source of carbon. It was observed that these microorganisms were able to degrade the PHEs present in PVC film present on solid medium. Analyzing Figure 1, it is observed that the microorganisms were able to colonize and grow in the presence of PVC film. This growth indicates the consumption of PVC film as the sole source of carbon and, because it is a solid medium, the possibility of migration of phthalates by dissolution in water is discarded.

A similar study was developed by Nakamiya et al. (2005), in which the use of microorganisms to degrade and remove DEHP from PVC was investigated. The possible microorganisms, able to degrade the DEHP, were acquired from samples of soil and sewage sludge. These were tested in media containing DEHP (0.3 %) as the only carbon source. From more than 100 samples tested, four were isolated microorganisms, of which only one was able to completely degrade DEHP. Similar results were demonstrated in this study, by having microorganisms selected in solid medium and showing microorganisms able to metabolize the PHEs present in PVC film. As in the previously cited study, the carbon source was a plasticizer and was present in the PVC polymer structure.

According to the obtained results, it was found that microorganisms (I), (II), (III) demonstrated high growth after ten days of biodegradation and in liquid medium reaching an optical density greater than 1.0. In the qualitative detection of enzyme activity in solid media, microorganisms (I), (II), (III) showed the best kinetic growth profile. The results obtained showed that the microorganism (I) presented the largest hydrolysis halo with an enzymatic activity index (EI) exceeding 2.0. This could possibly be due to increased production and capacity of esterase enzymes (Lealem & Gashe, 1994).

In Figure 1, the halos produced are indicative of esterase enzymes, due to the clear zone around the colonies as assessed by Gopinath, Anbu, and Hilda (2005). It is observed that microorganism I generated the largest halo zone. Extracellular enzymatic activity was evaluated by the ratio of the average diameter of the halo of degradation to the average diameter of the colony and expressed as IE. Thus, higher indices indicate elevated enzymatic activity (Hubbell, Morales, & Umali-Garcia, 1978). Esterases are enzymes that cleave ester linkages and therefore microorganisms that synthesize the same can degrade PHEs. The study of Gavala, Yenal, and Ahring (2004) investigated the degradation of three PHEs, DEP, DBP and DEHP, present in sludge generated during wastewater treatment. The esterase enzyme was added to the sludge containing PHEs and subjected to an incubator under agitation, at a temperature of 30°C for 20 days. On the results, the research concluded that the treatment was effective in enzymatic degradation of plasticizers. In addition, it was observed that the enzymatic treatment resulted in the shortest half-life of DEHP in sludge reported so far. Due to the results of this research, it can be considered that microorganisms with the potential to synthesize esterases are probably more apt to degrade PHEs present in PVC film. Studies on fungal colonization and biodegradation by Webb et al. (2000) suggest that fungi produce extracellular esterases that degrade the plasticizers in PVC. Secondly, in Grisa, Cardoso, Zoppas, Brandalise, and Zeni (2011), microorganisms isolated from landfill secrete enzymes which are responsible for metabolism, transformation and breakdown of one substance into another. Therefore, the microbial cells possess enzymatic arsenals that are capable of acting on synthetic plasticizers, through this study it was observed that bacteria and fungi produce the enzyme esterase.

In addition to metabolism, it is important to note that, when they are used as plasticizers, PHEs can migrate or desorb from the polymer structure (PVC) thus promoting possible contamination. In this case, the PHEs act as compounds embedded between the polymer chains, significantly spacing and modifying the physical properties of the material, thereby making it more flexible. Thus, PHEs are adsorbed to certain polar polymer chain sites linked through weak polar bonds, which are induced by their presence on polymer mesh. Eventually, when this material is placed in liquid medium, the PHEs molecules are desorbed. Therefore, facilitating the breakage of their weaker links and consequent migration to the medium. In addition, PHEs present high solubility in water, which facilitates this process of desorption in an aqueous medium. This migration and transfer explain the contamination of different aquatic and terrestrial environments (Erythropel et al., 2016).

The titration method was used for detection of possible variations in acidity of the medium, due to the metabolic breakdown of PHEs into phthalic acid (PA) by microorganisms (biotic) or by transfer or spontaneous migration of PHEs. Another reason this method was chosen, was because it allows for the verification of the variations in the concentration of  $H^+$  ions in samples taken from the spontaneous migration and biodegradation tests. The results, obtained in aerobic biodegradation, demonstrated that there had been an increase in the concentration of  $H^+$  ions over the course of 24 days of testing. This indicates the presence of acidic substances produced during the tests. It was observed that PHEs desorption was due to ester bonds hydrolysis in aqueous medium and also by the oxidative behavior presented by the microorganisms' metabolic route of the biodegradation, as shown in Figure 2.

It was also verified that the greatest release of  $H^+$  ions was due to the bacteria metabolism since biodegradation medium with the presence of microorganisms showed greater concentrations of acids than in the negative control test (medium free of microorganisms). This is illustrated in Figure 3.

Observing Figure 3, there has been a change in the concentration of  $H^+$  ions from 0.05 to 3.1 mol L<sup>-1</sup> during twenty-four days. Thus, indicating an increase of 47% from the onset of the experiment to the sixth day of biodegradation assays. From the eighth to the twenty-fourth day, a variation from 1.8 to 3.1 mol L<sup>-1</sup> was detected, showed approximately 58% increase in the concentration of  $H^+$  ions in the culture medium used. This presents an approximately 58% increase in the concentration of  $H^+$  ions in the growth medium used. Such increases coincide with the PA initial formation (Figure 2), only when two hydrogens are dissociated, followed by the formation of protococatechuate (a phenolic compound), beta-ketoadipate, 4-oxalocitratemalate, oxaloacetate and pyruvate, respectively. In this way, possibly, the 58% increase in the concentration of  $H^+$  ions, can be assigned to the increase in the number of hydrogens dissociated from phenolic compounds formed. Thus, indicating the same behavior with biodegradation of PHEs present in the commercial film of PVC, according to the literature (Ferreira & Morita, 2012).



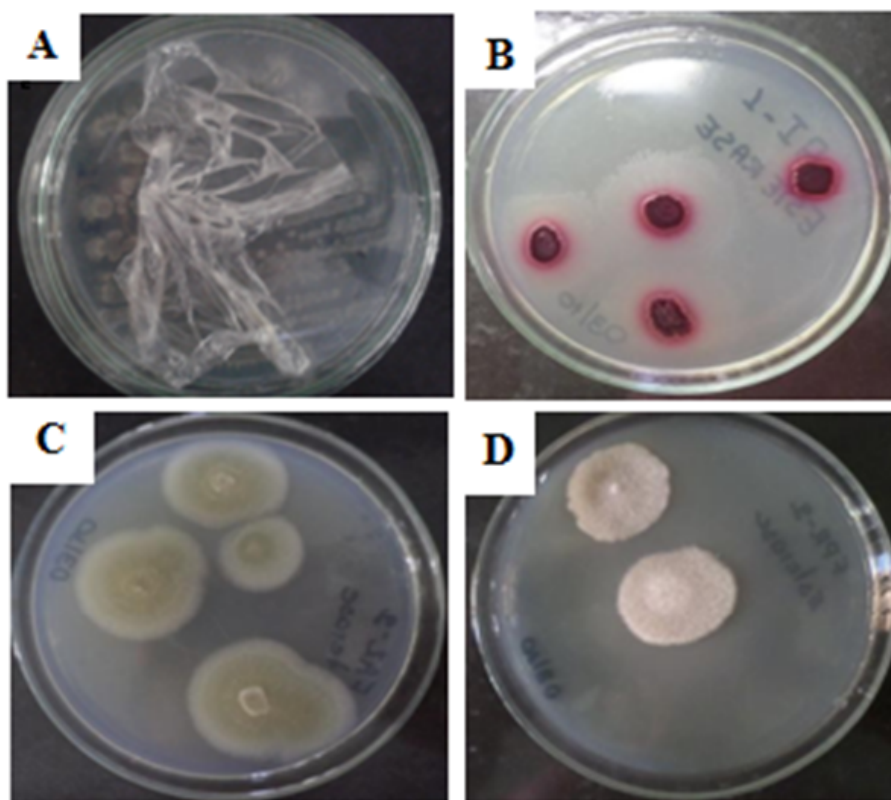


FIGURE 1.

Illustrative photo of *Geotrichum* sp. growing on solid medium, containing PVC film as a substrate (A); Pictures of the halos produced by microorganisms (I) (B) (II) (C) and III (D) after 10 days of growth on solid medium supplemented with Tween 80 (1%).

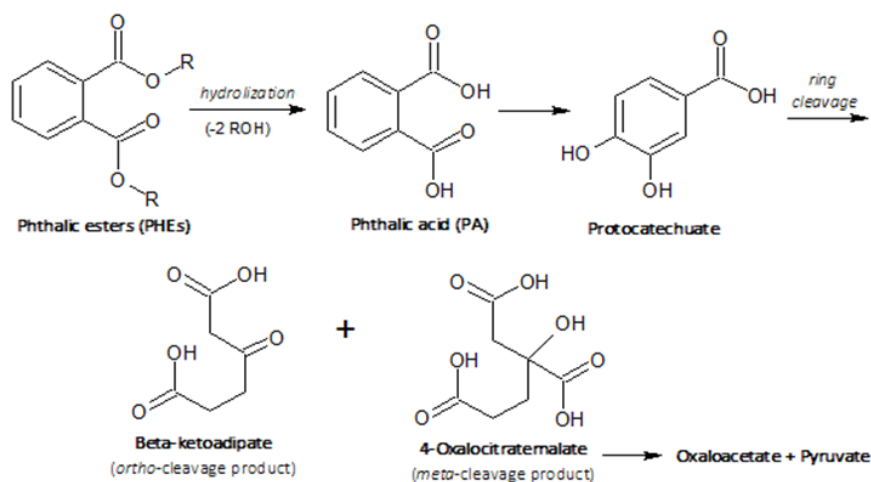


FIGURE 2.

The aerobic metabolic pathways of PHEs and PA (Adapted from Ren et al., 2018).

Similar results were reported by Al-Saleh, Shinwari, and Alsabbaheen (2011), who determined the presence of PHEs in ten different brands of bottled water available in the markets of Saudi Arabia. The researchers concluded that the plasticizers are transferred from the polymeric materials of packaging to the mineral water inside each bottle. The authors concluded that the increase in the concentration of the acid in the degradation test was due to the transfer of the phthalate, as phthalic acids, to the mineral medium.

Similar result was reported by Juneson, Ward, and Singh (2001) that evaluated the biodegradation of DEHP in reactors. This research also noted an increase in the concentration of acids during the thirty days of experiments due to the acidic nature of the biodegradation products of PHEs. This result corroborates the results obtained herein by the titration method, since the concentration of  $H^+$  ions had a considerable increase. This justifies the increase in concentration of PA in the medium (Figure 3).

According to the results in Figure 4, it was observed that from the sixth day of the biodegradation assay the number of viable cells and ammonia concentration showed no significant variation, thus remaining constant. This proves that the survival of the bacterial cells is mainly dependent on the consumption of PHEs present in PVC films, used as the sole carbon source, and not on the consumption of ammonia possibly present in the degradation medium, due to cellular lysis (endogenous consumption). Cellular lysis can be explained by the inability of the microorganism to use PHEs as the only carbon source in the biodegradation assay.

Considering Figure 4, it can be observed that from the tenth day onwards, there were significant variations in the number of microbial colonies. This indicates that bacterium I was able to use the phthalates available in the biodegradation medium, as a source of energy. Since there was no significant cell death, however, a gradual loss of weight of the PVC film was observed, as shown in Figure 5.

In accordance with the obtained results, bacterium I was able to degrade the PHEs, evidenced by the decrease in the mass of PVC films by approximately 28% after 24 days of biodegradation (Figure 5). Similar results were evidenced in Ferreira and Morita (2012).

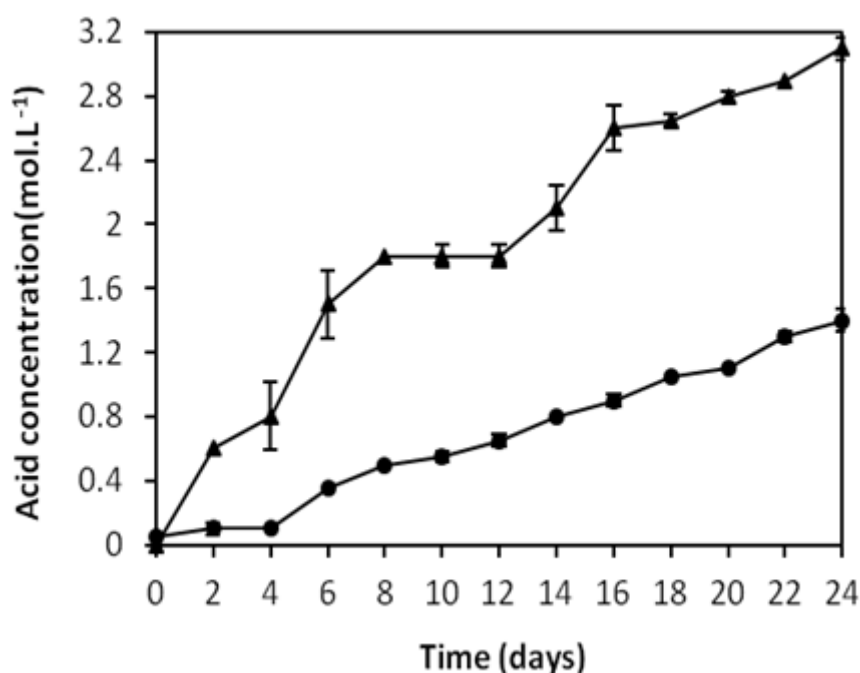


FIGURE 3.

Concentration of the acid ( $\text{mol L}^{-1}$ ) in the biodegradation tests (▲) and control (●) of the PHEs presents in the PVC film. Results are obtained by titration method.

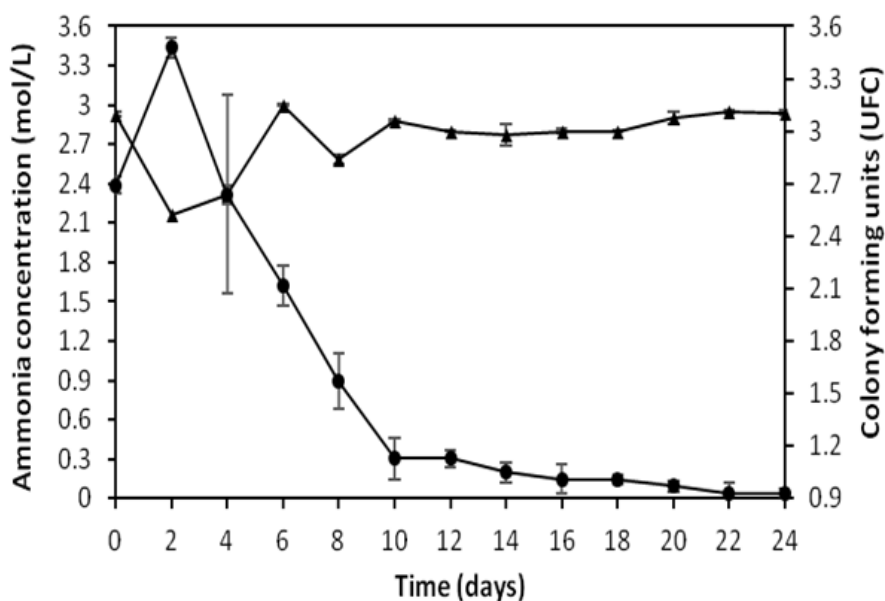


FIGURE 4.

Variation in ammonia concentration (▲) caused by the number of viable cells (●) during the biodegradation tests using bacterium I.

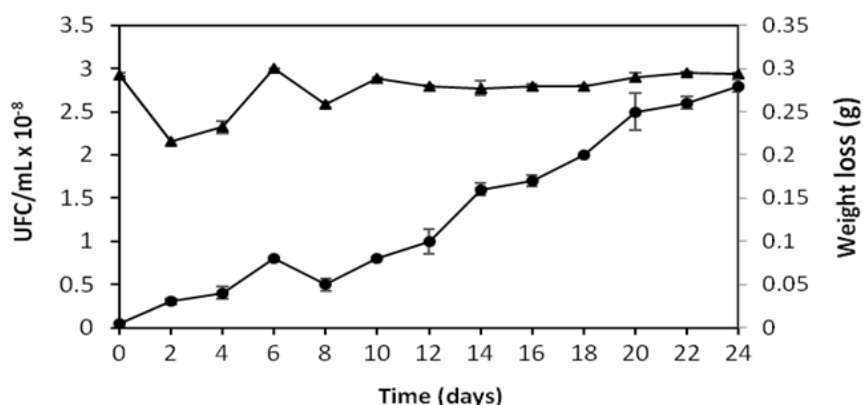


FIGURE 5.

Number of viable cells variation (●) and the loss of mass of the PVC (▲) during the biodegradation tests using bacterium I.

In order to detect and identify the resulting organic functional groups obtained either by liquid migration (negative control, without the presence of microorganisms) or by the biodegradation assays of PHEs found in PVC film, the Feigl method was used (Engel et al., 2012). These tests are of chromogenic nature, meaning they are specific tests indicated by color change. The presence of PHEs is due to hydrolysis of the ester into carboxylic acid functional groups. In the case of biodegradation, it is expected to obtain the monoester (initially), their alcohol and subsequently the PA. This is because these are the primary degradation products, as described previously (Ren et al., 2018).

The carboxylic acid functional group (-COOH) was assessed by ferric hydroxamate test. In this test, carboxylic acid enters in contact with hydroxylamine. This reaction promotes the formation of hydroxamic acids, which react with salts of iron, to produce a reddish-purple intense color. This was detected through tests conducted in the analytes produced during the biodegradation assays by the appearance of reddish-brown color, indicating the presence of PA from the eighth to the twenty-fourth day of reaction. The obtained

results can be confirmed by the increased concentration of  $H^+$  ions, with consequent drop in pH of the medium during PHEs biodegradation. The absence of PA in the first six days of assays, even with the increased concentration of  $H^+$  ions (Figure 3), may not have been detected, because it could be below the detection limit of the respective probe, which is in the order of micrograms. In the case of analysis done on the control test in analytes, we also observed the presence of PA, due to the positive results obtained for the carboxylic acid function from the second to twenty-fourth day of repeated experimentation. This result is confirmed by the gradual decrease of pH, especially from the fourth day of the test onwards (Figure 3). The negative result previously obtained from the second day was evaluated and again may be related to the concentration of PA below the detection limit of the test performed. Samples containing phenol, when subjected to the test of ferric chloride, form a complex of phenols with the Fe (III) ion. Results show that dark blue color can indicate the effectiveness of the process of biodegradation, in producing phenolic compounds, such as protocatechuate, beta-ketoadipate, 4-oxalocitratemalate, oxalacetate or pyruvate, as exclusive products of aerobic biodegradation. The results were positive for the presence of phenolic compounds, starting from the twelfth day of biodegradation, thus indicating that these products depend on the formation of PA, and thus can only be detected days after their production .

## CONCLUSION

The obtained results demonstrated that the bioprospection of microorganisms carried out in Serra do Ouro Branco State Park, Minas Gerais/Brazil, was able to isolate an efficient microorganism, identified as bacterium (I), for biodegradation of PHEs found in PVC films. This bacterium was able to promote, over a time period of 24 days, a 28% reduction in the mass of the commercial PVC film studied. From our findings, it can be concluded that isolated microorganisms, from regional biodiversity, has the potential to be used for bioremediation and biodegradation of PHEs found in commercialized PVC.

## ACKNOWLEDGEMENTS

The authors would like to thank the State Institute of Forestry, Environment and Sustainable Development from the State of Minas Gerais for the permission to gather and transport botanical material for scientific purposes in the State of Minas Gerais. Authorization COL: 006/13 and Process SIGED – IEF/DPBIO/GPROP: 0000003821012013-15.

## REFERENCES

- Al-Saleh, I., Shinwari, N., & Alsabbaheen, A. (2011). Phthalates residues in plastic bottled Waters. *Journal of Toxicological Sciences*, 36(4), 469-478. doi: 10.2131/jts.36.469
- Carissimi, M., Stopiglia, C. D. O., Souza, T. F., Corbellini, V. A., & Scroferneker, M. L. (2007). Comparison of lipolytic activity of *Sporothrix schenckii* strains utilizing olive oil-Rhodamine B and Tween 80. *Tecno-lógica*, 11(1), 33-36.
- Chatterjee, S., & Dutta, T. K. (2003). Metabolismo f butyl-benzyl phthalate by *Gordonia* sp strain MTCC 4818. *Biochemical and Biophysical Research Communications*, 309(1), 36-43. doi: 10.1016/S0006-291X(03) 01513-4
- Engel, R. G., Kriz, G. S., Lampman, M. G., & Pavi, D. F. (2012). *Química orgânica experimental: técnicas em escala pequena*. São Paulo, SP: Cengage Learning.
- Erythropel, H. C., Shipley, S., Cormann, A. B., Nicell, J. A., Maric, M., & Eask, R. L. (2016). Designing green plasticizers: Influence of molecule geometry and alkyl chain length on the plasticizing effectiveness of diester plasticizers in PVC blends. *Polymer*, 89, 18-27. doi: 10.1016/j.chemosphere.2015.04.014

- Ferreira, I. D., & Morita, D. M. (2012). Biorremediação de solo contaminado por isobutanol, bis-2-etil-hexilftalato e di-isodeciltalato. *Revista Brasileira Ciência do Solo*, 36(2), 643-652. doi: 10.1590/S0100-06832012000200033
- Fuentefria, A. M., & Valente, P. (2005). Screening of enzyme-producing yeast and yeast like fungi from the phylloplane of Hibiscus rosa-sinensis in Brazil. *Revista Tecno-Lógica*, 9(1), 9-24.
- Gani, K. M., Tyagi, V. K., & Kazmi, A. A. (2017). Occurrence of phthalates in aquatic environment and their removal during wastewater treatment processes: a review. *Environmental Science and Pollution Research*, 24(21), 17267-17284. doi: 10.1007/s11356-017-9182-3
- Gao, D. W., & Danwen, Z. (2016). Phthalate esters in the environment: A critical review of their occurrence, biodegradation, and removal during wastewater treatment processes. *Science of the Total Environment*, 541, 986-1001. doi: 10.1016/j.scitotenv.2015.09.148
- Gavala, H. N., Yenal, U., & Ahning, B. K. (2004). Thermal and enzymatic pretreatment of sludge containing phthalate esters prior to mesophilic anaerobic digestion. *Biotechnology and Bioengineering*, 85(5), 561-567. doi: 10.1002/bit.20003
- Gopinath, S. C. B., Anbu, P., & Hilda, A. (2005). Extracellular enzymatic activity profiles in fungi isolated from oil-rich environments. *Mycoscience*, 46(2), 119-126. doi: 10.1007/S10267-004-0221-9
- Grisa, A. M. C., Cardoso, V., Zoppas, B. C. D. A., Brandalise, R. N., & Zeni, M. (2011). Degradação biológica do PVC em aterro sanitário e avaliação microbiológica. *Polímeros*, 21(3), 210-216. doi: 10.1590/S0104-14282011005000046
- Hubbell, D. H., Morales, V. M., & Umali-Garcia, M. (1978). Pectolytic enzymes in Rhizobium. *Applied and Environmental Microbiology*, 35(1), 210-213.
- Juneson, C., Ward, O. P., & Singh, A. (2001). Biodegradation of bis(2-ethylhexyl)phthalate in a soil slurry-sequencing batch reactor. *Process Biochemistry*, 37(3), 305-313. doi: 10.1016/S0032-9592(01)00196-0
- Kong, X., Jin, D., Ta, X., Yu, H., Duan, G., Yan, X., ... Deng, Y. (2019). Bioremediation of dibutyl phthalate in a simulated agricultural ecosystem by *Gordonia* sp. strain QH-11 and the microbial ecological effects in soil. *Science of The Total Environment*, 667, 691-700. doi: 10.1016/j.scitotenv.2019.02.385
- Larone, D. H. (2011). *Medically important fungi: a guide to identification*. Washington, DC: American Society for Microbiology Press.
- Lealem, F., & Gashe, B. A. (1994). Amylase production by a gram-positive bacterium isolated from fermenting tef (*Eraglostis tef*). *Journal of Applied Bacteriology*, 77(3), 348-352. doi: 10.1111/j.1365-2672.1994.tb03084.x
- Nakamiya, K., Hashimoto, S., Ito, H., Edmonds, J. S., Yasuhara, U., & Morita, M. (2005). Microbial treatment of bis-(2-ethylhexyl) phthalate in polyvinyl chloride with isolated bacteria. *Journal of Bioscience and Bioengineering*, 99(2), 115-119. doi: 10.1263/jbb.99.115
- Net, S., Sempéré, R., Delmont, A., Paluselli, A., & Ouddane, B. (2015). Occurrence, fate, behavior and ecotoxicological state of phthalates in different environmental matrices. *Environmental. Science. Technology*, 49(7), 4019-4403. doi: 10.1021/es505233b
- Niazi, H. J., Prasad, T. D., & Karegoudar, T. B. (2001). Initial degradation of dimethylphthalate by esterases from *Bacilo* species. *FEMS Microbiology Lett*, 196(2), 201-205. doi: 10.1111 / j.1574-6968.2001.tb10565.x
- Perez, D. V., Anjos, L. H. C., Ebeling, A. G., & Pereira, M. G. (2009). Comparison of H/AL stoichiometry of mineral and organic soils in Brazil. *Revista Brasileira de Ciência do Solo*, 33(4), 1071-1076. doi: 10.1590/S0100-06832009000400031
- Ren, L., Lin, Z., Liu, H., & Hu, H. (2018). Bacteria-mediated phthalic acid esters degradation and related molecular mechanisms. *Applied Microbiology and Biotechnology*, 102(3), 1085-1096. doi: 10.1007/s00253-017-8687-5
- Sharpe, A. N., & Woodrow, M. N. (1971). A rapid test for biodegradability of PVC film by *pseudomonads*. *Journal of Applied Bacteriology*, 34(2), 485-489. doi: 10.1111/j.1365-2672.1971.tb02308.x
- Silva, B. D., Bicas, J. L., Sarrouh, B., & Lofrano, R. C. Z. (2016). Bioprospecção de microrganismos produtores de enzimas de interesse industrial realizada no Parque Estadual Serra do Ouro Branco, Brasil. *Interbio*, 10(1), 13-24.
- Tortora, G. J., Funke, B. R., & Case, C. L. (2017). *Microbiologia*. Porto Alegre, RS: Artmed.



- Vilela, C. L. S., Bassin, J. P., & Peixoto, R. S. (2018). Water contamination by endocrine disruptors: Impacts, microbiological aspects and trends for environmental protection. *Environmental Pollution*, 235, 546-559. doi: 10.1016/j.envpol.2017.12.098
- Webb, J. S., Nixon, M., Eastwood, I., Greenhalgh, M., Robson, G. D., & Handley, P. S. (2000). Fungal colonization and biodeterioration of plasticized polyvinyl chloride. *Applied and Environmental Microbiology*, 66(8), 3194-3200. doi: 10.1128/aem.66.8.3194-3200.2000
- Xu, G., Li, F. S., & Wang, Q. H. (2008). Occurrence and degradation characteristics of dibutyl phthalate (DBP) and di-(2-ethylhexyl) phthalate (DEHP) in typical agricultural soils of Chin. *Science of The Total Environment*, 393(2-3), 333-340. doi: 10.1016/j.scitotenv.2008.01.001
- Zhao, H. M., Du, H., Feng, N. X., Xiang, L., Li, Y. W., Li, H., ... Mo, C. H. (2016). Biodegradation of di-n-butylphthalate and phthalic acid by a novel *providencia* sp. 2D and its stimulation in a compost-amended soil. *Biology and Fertility of Soils*, 52(1), 65-76. doi: 10.1007/s00374-015-1054-8