# *In Vitro* and *Ex Situ* Biodegradation of Low-Density Polyethylene by a *Rhizopus* sp. Strain Isolated from a Local Dumpsite in North-East Algeria

**Randa Harrat[1*], Ghania Bourzama[1], Houria Ouled-Haddar[2], and Boudjema Soumati[1]**

*[1]Laboratory of Biochemistry and Environmental Toxicology, Department of Biochemistry, Faculty of Sciences, University of Badji Mokhtar, Annaba 23000, Algeria*
*[2]Laboratory of Molecular Toxicology, University of Jijel, Jijel, Ouled Aïssa 18000, Algeria*

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*** Corresponding author:**
E-mail: harratranda@gmail.com

## ABSTRACT

Low density polyethylene (LDPE) is the most abundant non-degradable plastic waste. Widely used in packaging material, it represents a serious threat to all ecosystems. In the present study, a *Rhizopus* sp. fungal strain was isolated from soil of a landfill located in north-east Algeria and cultured on potato dextrose agar. The *in vitro* biodegradability of pieces of the same plastic bag (0.2, 0.4, and 0.6 g) was estimated in minimal liquid medium and on minimal solid medium. Furthermore, biodegradation of plastic bag pieces was examined in seawater, tap water and soil. The isolated *Rhizopus* sp. strain could degrade the plastic bag waste. The highest *in vitro* rate occurred in the minimal liquid medium for both the 0.4-g and 0.6-g pieces (a 20% decrease in weight). In natural media, the highest weight decrease was different depending on the substrate: 5% in seawater for the 0.2-g piece, 10% in tap water for the 0.4-g piece and 8% in soil for the 0.4-g piece. This strain could also form a biofilm in Malt Extract Broth (MEB). These results revealed that the isolated *Rhizopus* sp. strain has considerable biodegradative ability based on different measures.

## 1. INTRODUCTION

Owing to their chemical stability, good mechanical properties, low production costs and simple processability, plastic materials are the main components in many manufacturing sectors such as packaging, clothing, construction, and automotive (Akhbarizadeh et al., 2020; Thiounn and Smith, 2020). Every year, over 320 million tons of plastic are produced, and this amount is predicted to double in 12 years (Ritchie and Roser, 2018).

Plastic is a polymer of long hydrocarbon chains with a high molecular weight, derived mainly from petrochemicals that are then synthetically arranged by certain chemical processes to produce the long polymer chains (Shimao, 2001). Polyethylene is a thermoplastic polymer produced by monomers of ethylene. There are numerous categories such as high-density polyethylene (HDPE), linear low-density polyethylene (LLDPE) and low-density polyethylene (LDPE), which is a thermoplastic made from petroleum. LDPE materials are generally used for manufacturing various containers, dispensing bottles, plastic bags, and various melded laboratory wares because of their light weight, strength, and durability (Pramila and Ramesh, 2011).

Plastics generate huge problems related to the environment, health, and the management of residual materials. In addition, some materials have generated considerable controversy regarding their toxicological risks due to the presence of additives such as phthalates or bisphenol A (Lewis, 2012). In fact, many countries have restricted the use of certain plastic products. Disposable plastic bags as well as polyethylene containers have been subjected to such measures (Lewis, 2012). Several processes are used to deal with this situation, namely landfills, incineration, and storage, without thinking about the consequences and possible negative effects of these techniques on the environment in the short or long term. On the other hand, recent studies have shown that the degradation

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of plastic bags by microorganisms, particularly fungi, is the main process of decontamination in sediments and deep soils (Bonanni et al., 2012). For example, Sowmya et al. (2014) affirmed the ability of *Penicillium simplicissimum* to use polyethylene as a carbon source and its ability to degrade it, while El Sayed et al. (2021) isolated and identified six fungal strains: *Aspergillus carbonarius*, *Aspergillus* sp., *Aspergillus flavus*, *Aspergillus ochraceus*, *Penicillium* sp., *Fusarium* sp. and proved their ability to use LDPE as a unique source of carbon and energy.

The aim of this work was to isolate and identify a fungal strain involved in plastic biodegradation from a landfill located in north-east Algeria and to evaluate its capacity to degrade plastic bag pieces and to form biofilm. The obtained fungal strain could be used as a tool to depollute contaminated soil.

## 2. METHODOLOGY

### 2.1 Study area and sample collection

A quantity of soil was collected from a public landfill area in north-east Algeria after elimination of the first 5 cm of the soil surface. The sample was transported in a sterile black plastic bag to the laboratory and kept at 4°C and used within 24 h (according to Boughachiche et al. (2005), with modification).

### 2.2. Isolation of fungal strains

Ten grams of soil was added to 90 mL of sterile physiological water. Then, $10^{-2}$, $10^{-3}$ and $10^{-4}$ serial dilutions were prepared, and 0.1 mL of each dilution was inoculated on the surface of a Petri dish containing solid potato dextrose agar (PDA) supplemented with gentamicin. The plates were incubated at 27°C for one week (Davet and Rouxel, 1997). The isolated strains were further purified on solid PDA.

### 2.3. Identification and storage of fungi

Fungal strains were identified based on macroscopic and microscopic characteristics (Pardo-Rodríguez and Zorro-Mateus, 2021). Isolated strains were stored in slant PDA agar tubes (Botton et al., 1990).

### 2.4. Preparation of LDPE pieces

The LDPE films used in this study were plastic white bags of 50 µm thickness, flexible and solid. For the biodegradation studies, LDPE films were cut into small pieces (0.2, 0.4, and 0.6g) and were disinfected with 70% ethanol.

### 2.5. *In vitro* biodegradation test

*2.5.1. Preparation of the minimal medium*

Liquid minimal medium (MM) was prepared by adding 16 g $K_2HPO_4$, 2 g $KH_2PO_4$, 1 g $(NH_4)_2SO$, 0.475 g $MgSO_4$, 0.2 g NaCl, 0.01 g $CuSO_4$, 1 g $CaCO_3$ and 1 g $ZnSO_4$ in 1,000 mL of distilled water. Solid MM was obtained by adding 20 g agar to 1 L liquid MM (according to Nakajima-Kambe et al. (1999), with modifications). The initial pH of the medium was adjusted to 7.0.

*2.5.2. Culture in liquid MM*

The tested fungal strain was screened for its ability to degrade LDPE films. The biodegradation test was performed in liquid MM containing LDPE film as the sole carbon source. A piece of the same plastic bag (0.2, 0.4, and 0.6 g) was added to 250 mL Erlenmeyer flasks containing 150 mL of liquid MM. An agar disc of the fungal strain that had been cultured for seven days was inoculated in each Erlenmeyer flask (Bourzama et al., 2021). The Erlenmeyer flasks were incubated in a shaker incubator for one month at 27±2°C with rotation at 150 rpm. Every 10 days, the pieces were aseptically collected and weighed. The fungus *Aspergillus niger* was used as the control to confirm the degradation of LDPE films.

*2.5.3. Culture on solid MM*

A piece of plastic bag (0.2, 0.4, and 0.6 g) was added to Petri dishes containing solid MM. The fungal strain was added by touch. The plates were then incubated for 30 days at 27±2°C (Bourzama et al., 2021). A Petri dish containing solid MM without the piece of plastic bag served as the control.

### 2.6. *Ex situ* biodegradation test

The fungal strain was tested for its ability to degrade LDPE films in the presence of other carbon sources than LDPE. A piece of plastic bag piece was added to a flask containing 150 mL of sterile seawater, 150 mL of sterile tap water and 200 g of sterile soil. The fungal strain was added by the disc method. The weight of each piece was checked every 10 days during 1-month incubation. Flasks containing a plastic piece with 150 mL of sterile water, 150 mL of sterile tap water and 200 g of sterile soil, without addition of the fungal strain, served as the controls. All tests were carried out in triplicate.

### 2.7. Formation of biofilm by the fungal strain

Malt extract broth (MEB) was used to test the ability of the isolated fungal strain to form biofilm. MEB was prepared as follows: 5 g peptone, 10 g malt extract and 1,000 mL of distilled water. After autoclaving, MEB was poured into three Petri dishes. The tested fungal strain was inoculated by the touch technique and incubated for 3 days. Each day, one plate was examined using a binocular microscope (Siqueira and Lima (2013), with modifications).

### 2.8. Estimation of the biodegradation rate

The percentage weight loss of each plastic bag piece was calculated by the following equation (DSouza et al., 2021):

$$\%\ \text{weight loss} = \frac{\text{Initial Weight} - \text{Final Weight}}{\text{Initial Weight}} \times 100$$

The values obtained were then compared with the controls.

### 2.9. Carbon dioxide production

To estimate carbon dioxide ($CO_2$) production by the tested fungal strain during biodegradation, the cultures were observed by naked eye (according to Khruengsai et al. (2021), with modifications). Positive cultures were recognised by the presence of gas bubbles.

## 3. RESULTS

### 3.1 Screening and identification of LDPE-degrading fungal isolates

Growing the landfill soil samples on solid PDA for seven days yielded a single fungal strain. Based on its macroscopic and microscopic characteristics, the fungal isolate was identified as *Rhizopus* sp. (Figure 1).

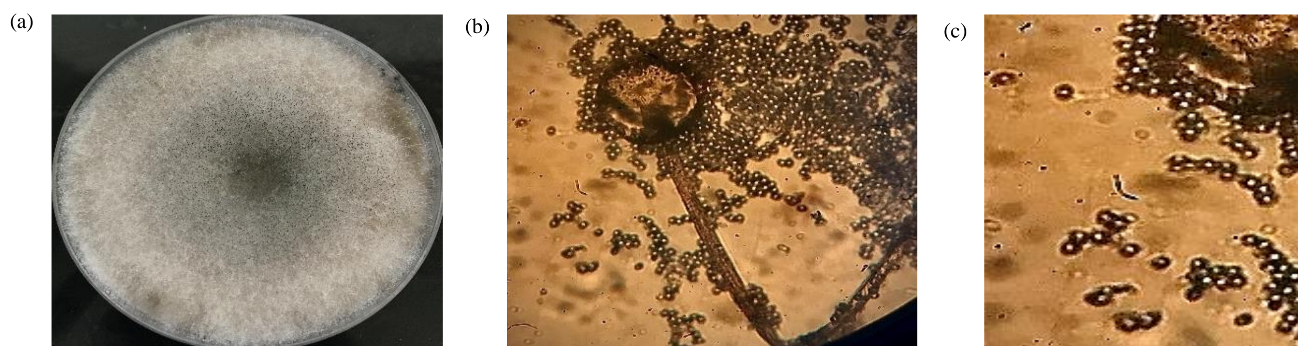


**Figure 1.** Morphology of the isolated *Rhizopus* sp. strain after 7 days of incubation: (a) macroscopic appearance, (b) microscopic appearance (40X magnification), and (c) spores of *Rhizopus* sp. under optical microscopy

### 3.2. *In vitro* biodegradation test

#### *3.2.1. In liquid MM*

The isolated *Rhizopus* sp. strain was tested for its ability to degrade plastic bag pieces in liquid MM. The cultures were incubated with continuous shaking (100 rpm) at 27±2°C at neutral initial pH, the weight change in plastic bag pieces was examined after 10, 20, and 30 days. Compared to the control *Aspergillus niger*, *Rhizopus* sp. showed better results.

The weight change of the 0.2-g plastic bag piece is presented in Figure 2(a). After the first 20 days, there had been no change in weight. After 30 days, the weight had decreased by 5%. Figure 2(b) shows the variation in the weight of the 0.4-g plastic piece. After 10 days, there had been a 16% decrease in weight. The weight continued to decrease, and at 30 days it had decreased by 20%. The 0.6-g plastic piece showed a weight loss similar to the 0.4-g plastic piece (Figure 2(c)).

#### *3.2.2. On solid MM*

On the plates with solid MM containing the plastic bag pieces, *Rhizopus* sp. appeared like a tangle of white filaments and small black and white dots. No growth was observed in the control plate (Table 1).

**Table 1.** Appearance of plates after incubation

| Plate | Macroscopic observation |
|---|---|
| Plate with the 0.2-g piece | Growth of the tested fungal strain above the plastic bag piece |
| Plate with the 0.4-g piece | Growth of the tested fungal strain above the plastic bag piece |
| Plate with the 0.6-g piece | Growth of the tested fungal strain above the plastic bag piece |
| Control plate | Death of the tested fungal strain seeded on the plate |

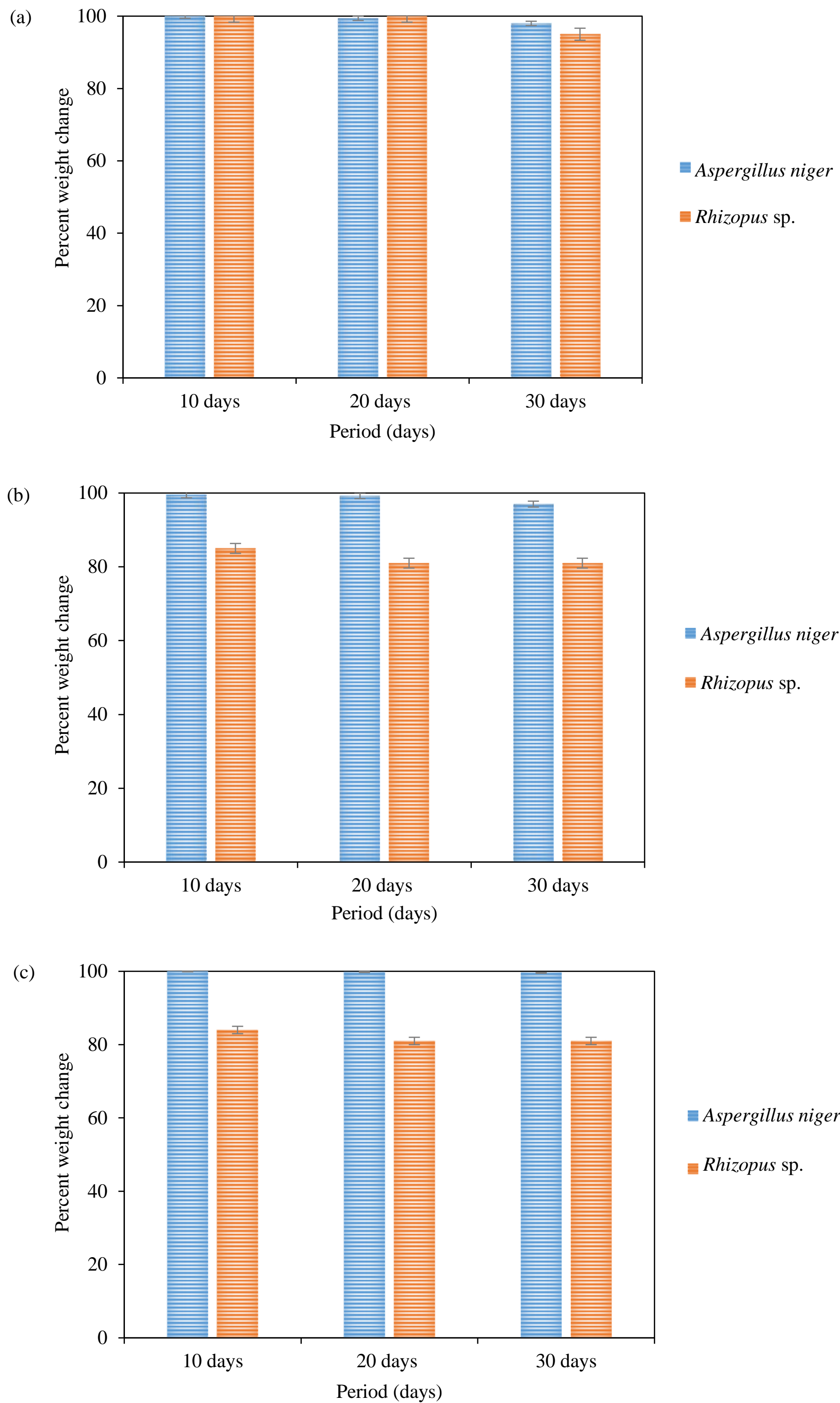


**Figure 2.** Percent weight change of (a) 0.2-g, (b) 0.4-g, and (c) 0.6-g plastic bag pieces incubated with the isolated *Rhizopus* sp. strain at 27±2°C for 30 days in liquid minimal medium compared with *Aspergillus niger*

### 3.3. *Ex situ* biodegradation test

*3.3.1. In seawater*

There was a slow weight loss of the 0.2-g and 0.4-g plastic bag pieces during the entire 30-days test period, 5% and 2.5%, respectively (Figure 3(a) and 3(b)). There was a slight variation in weight for the 0.6-g plastic bag piece (Figure 3(c)). After the first 10 days, there had been no change in weight. After 20 and 30 days, there had been a stable decrease in weight (1.5%).

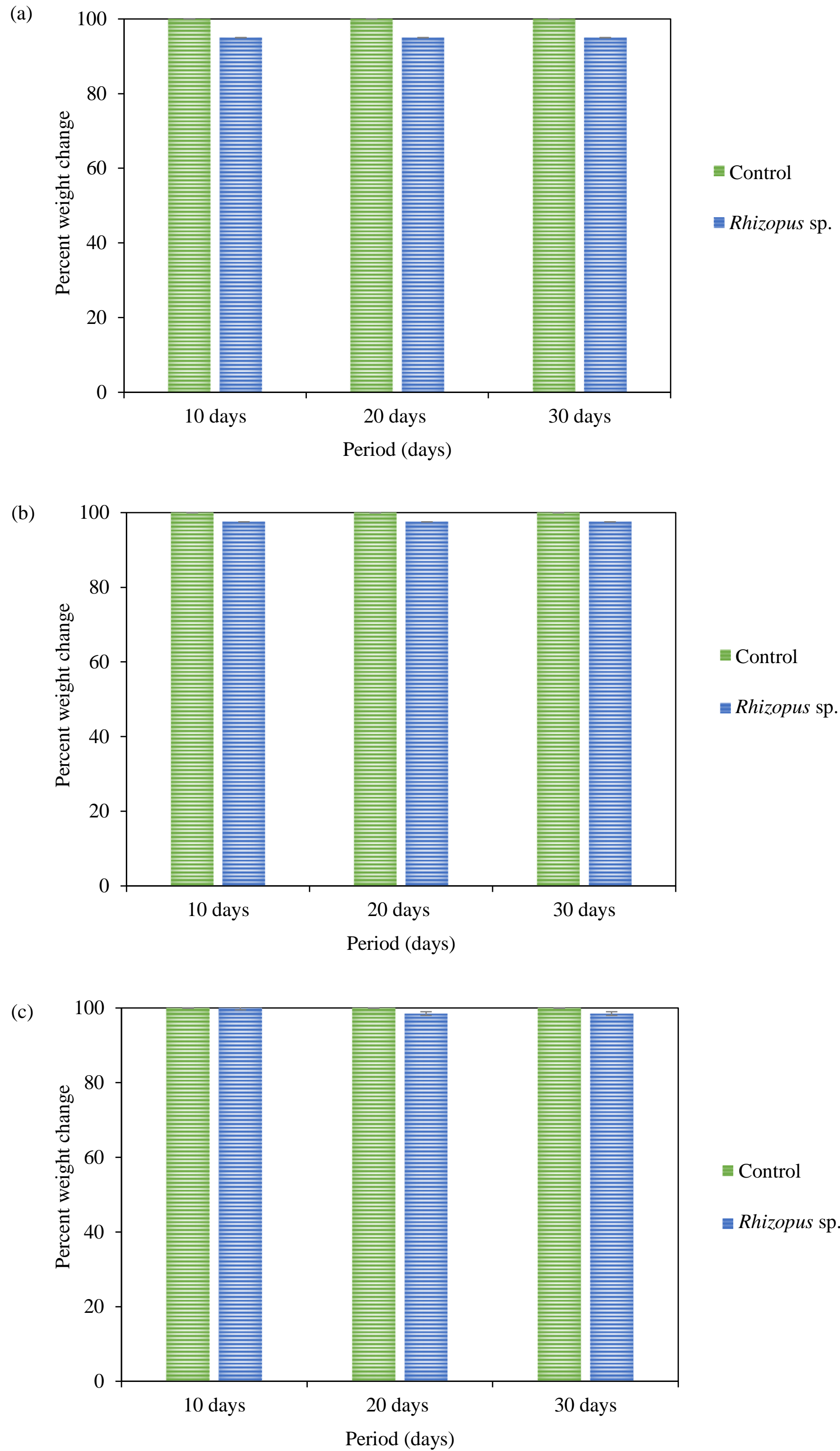


**Figure 3.** Percent weight change of (a) 0.2-g, (b) 0.4-g, and (c) 0.6-g plastic bag pieces incubated with the isolated *Rhizopus* sp. strain at 27±2°C for 30 days in seawater compared with the control

*3.3.2. In tap water*

The weight of the 0.2-g plastic bag piece had decreased by 5% after 30 days (Figure 4 (a)). The weight of the 0.4-g plastic bag piece also changed (Figure 4(b)). After 10 days, there had been no change. After 20 days, there had been a slight weight loss and it continued until day 30, when it had decreased by 10%. There had been no change in weight for the 0.6-g plastic bag piece after 20 days, but after 30 days the weight had decreased by 1.5% (Figure 4(c)).

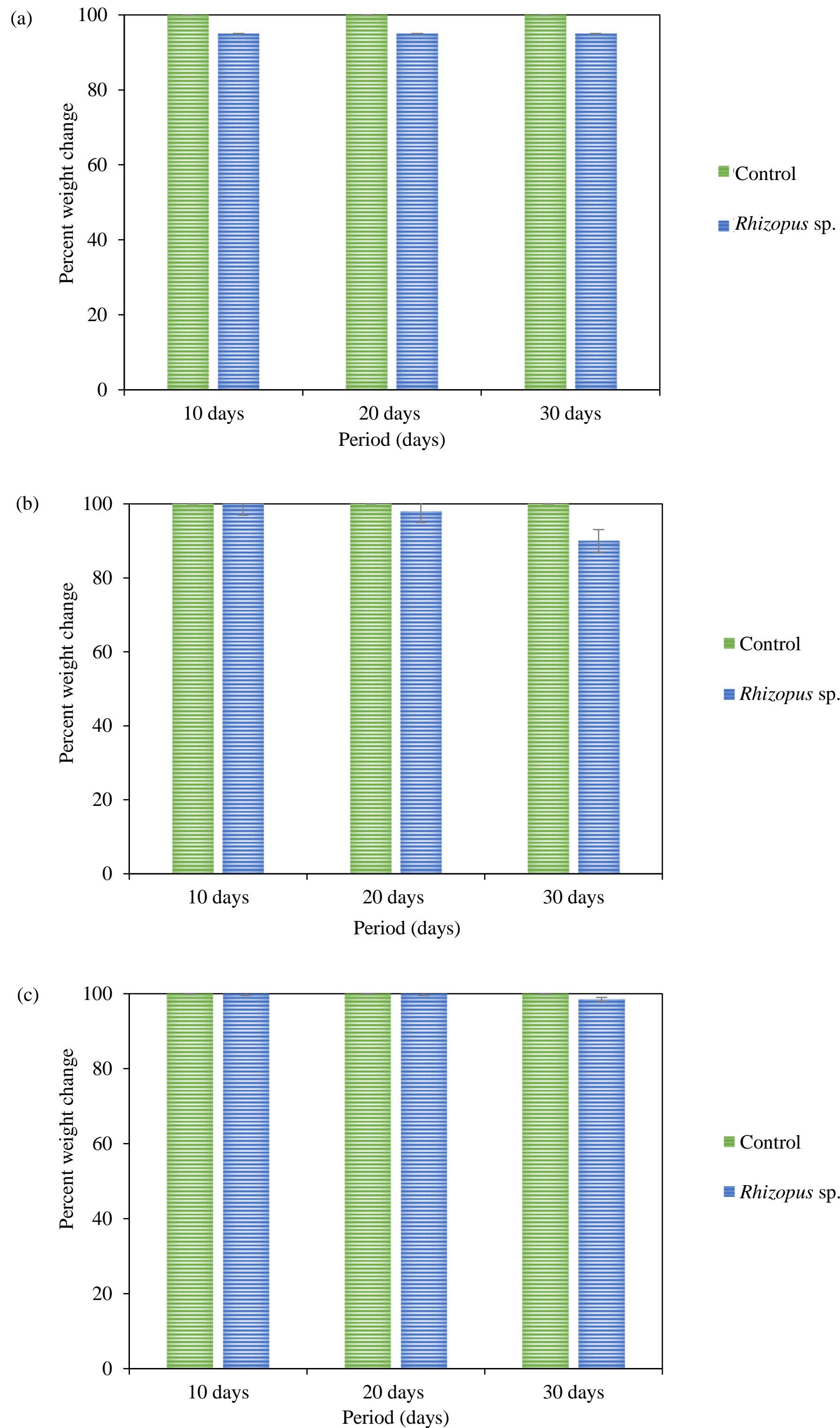


**Figure 4.** Percent weight change of (a) 0.2-g, (b) 0.4-g, and (c) 0.6-g plastic bag pieces incubated with the isolated *Rhizopus* sp. strain at 27±2°C for 30 days in tap water compared with the control

*3.3.3. In soil*

The weight of the 0.2-g plastic bag piece had not changed after 20 days of incubation. After 30 days, however, the weight had decreased by 5% (Figure 5(a)). The weight of the 0.4-g plastic bag piece varied during the experiment (Figure 5(b)). After 10 days, there had been no change in weight. After 20 days, there had been a slight weight loss that continued until 30 days, when it had reached 8%. Moreover, the 0.6-g plastic bag piece exhibited weight loss throughout the entire test period, reaching its maximum (6.5%) after 30 days (Figure 5(c)).

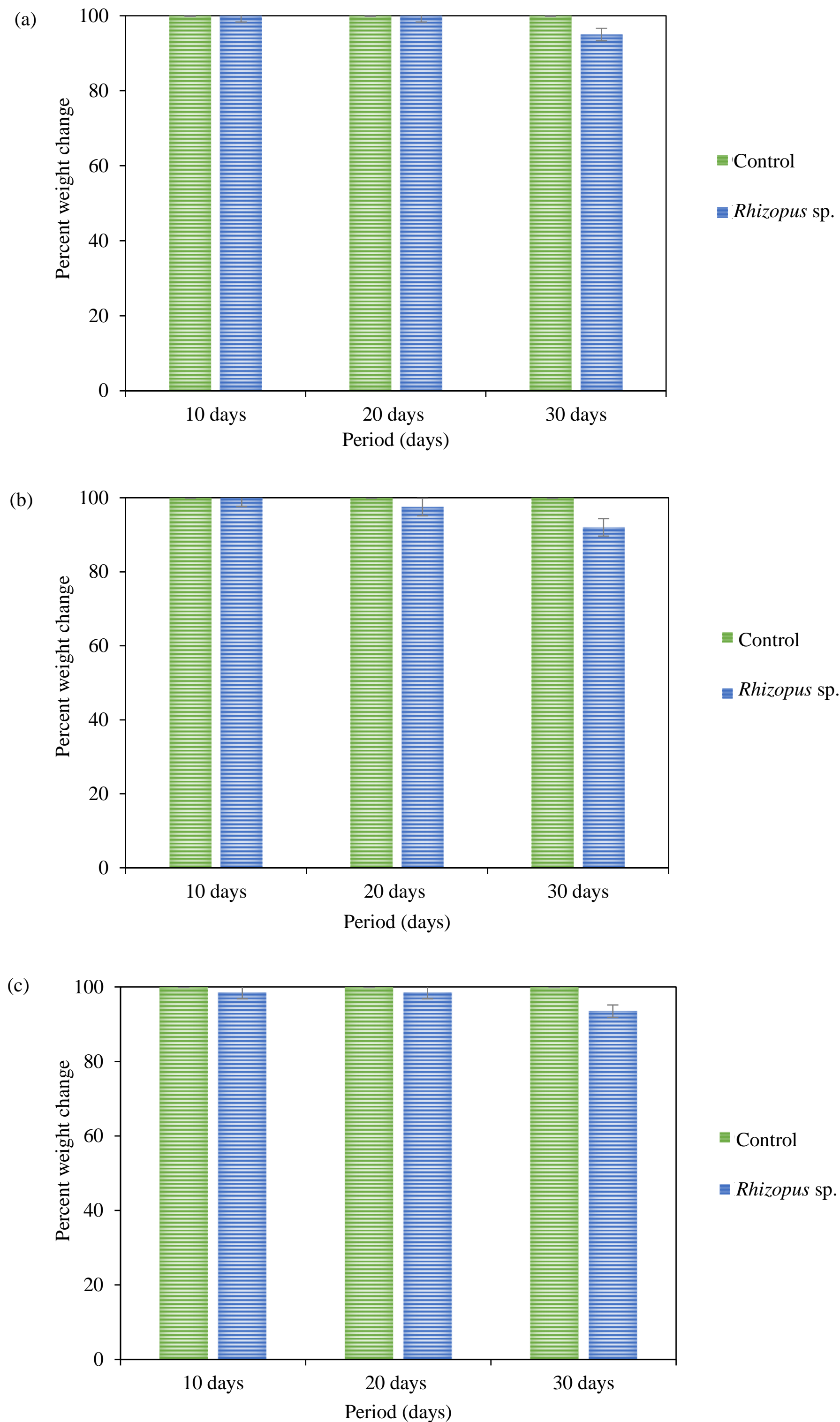


**Figure 5.** Percent weight change of (a) 0.2-g, (b) 0.4-g, and (c) 0.6-g plastic bag pieces incubated with the isolated *Rhizopus* sp. strain at 27±2°C for 30 days in soil compared with the control

### 3.4. $CO_2$ production

*In vitro* and *ex situ* biodegradation of polyethylene by the isolated fungal strain resulted in $CO_2$ production that was observed by naked eye as gas bubbles in all cultures except for the controls (Table 2).

### 3.5. Formation of biofilm

*Rhizopus* sp. was able to quickly form biofilm in MEB (Table 3).

**Table 2.** Production of carbon dioxide ($CO_2$) gas bubbles after 1 month of incubation of *Rhizopus* sp. *in vitro* and *ex situ* cultures

| Test | Culture | Production of gas bubbles |
|---|---|---|
| *In vitro* | On liquid minimal medium | + |
| | On solid minimal medium | + |
| *Ex situ* | In seawater | ++ |
| | In tap water | +++ |

Note: +: low production of $CO_2$; ++: average production of $CO_2$; +++: good production of $CO_2$

**Table 3.** Microscopic observation of biofilm formation

| Time | Biofilm formation |
|---|---|
| After 1 day | + |
| After 2 days | + |
| After 3 days | ++ |

Note: +: beginning of biofilm formation; ++: actual formation of biofilm

## 4. DISCUSSION

There have been numerous studies evaluating the biodegradation of polyethylene by micro-organisms. Although many studies have used bacterial cultures in natural environments, only few studies have used fungal species in controlled environments (DSouza et al., 2021). The aim of this study was to isolate and identify a fungal strain from a landfill in north-east Algeria capable of degrading plastic bag pieces *in vitro* and *ex situ*. The isolated strain was identified as *Rhizopus* sp.

In liquid MM, the weight loss of LDPE pieces during the incubation with the tested fungus *Rhizopus* sp. resulted from the utilization of the polyethylene as the sole carbon source. The results indicated that this fungal strain was capable of degrading LDPE pieces compared to *A. niger*, which has been reported to have good degradation ability (Khruengsai et al., 2021). In fact, the degradation potential of the tested *Rhizopus* sp. strain is explained by the good adaptation of the fungus in liquid MM and its ability to produce the necessary biodegradation enzymes. In addition, the growth of the tested fungal strain on solid MM containing plastic bag pieces and the release of $CO_2$ gas bubbles affirms its capacity to degrade this polymer and to use it as a carbon source.

In *in vitro* and *ex situ* tests, the weight of the plastic bag pieces decreased. However, the degradation rate was higher in tests using MM compared with tests using natural media because the latter contain carbon sources other than polyethylene. Indeed, the greatest weight loss occurred in liquid MM (20%), while in natural media, the greatest weight loss occurred in tap water (10%). Moreover, environmental factors and the presence of another carbon sources have an important influence on the degradation of carbonyl polymers which leads to a good production of $CO_2$ in natural media compared to MM, in particular on the biological activity of fungal species (Lefaux, 2005).

The obtained results showed that the tested strain has a good biodegradation activity because different measures confirmed its potential to degrade plastic bag pieces. In fact, DSouza et al. (2021) obtained similar results with a maximum weight loss of LDPE estimated at 26.15% when using a consortium of *Aspergillus* species under controlled conditions.

Shah (2007) buried pieces of LDPE in soil mixed with wastewater sludge for a period of 10 months. Microscopic examination showed the presence of a fungal layer on the surface of the plastic material, indicating its use by microorganisms as a carbon source. On the other hand, DSouza et al. (2021) used a fungal consortium consisting of *Aspergillus niger*, *Aspergillus flavus* and *Aspergillus oryzae* for the biodegradation of LDPE in laboratory conditions. The weight loss of the samples was attributed to the degradation of the carbon polymers by fungal enzymes. The resulting monomers and oligomers were used directly by the fungal species as a unique carbon source. Furthermore, Torres et al. (1996) showed that some fungal strains can also use lactic acid and oligomers released from the abiotic degradation of polylactic acid polymer.

Polyethylene macromolecules are large and cannot be digested by intracellular enzymes because they cannot penetrate the microbial cell barrier (Ammala et al., 2011). Fungi produce extracellular enzymes such as laccases, peroxidases and esterases, which are directly involved in fixation on a polyethylene surface and subsequent biodegradation (Wei and Zimmermann, 2017). So, biodegradation occurs via secreted enzymes that digest polyethylene macromolecules until they are small enough for bioassimilation: the microbial cells metabolise it, assimilate it into their structure and then mineralise it (Wyart, 2007).

Enzyme-mediated degradation of poly-L-lactic acid was first reported by Williams (1981) using proteinase K from *Tritirachium album.* Oxidative degradation is an appropriate pathway for polymers lacking easily hydrolysable groups, such as polyethylene (Yuan et al., 2020). Indeed, microbial

enzymes create functional groups that enhance the hydrophilicity of the polymer and improve its biodegradation (Shah et al., 2008). Moreover, in presence of *Aspergillus nomius* (Abraham et al., 2017) and *Rhizopus oryzae* (Awasthi et al., 2017), there was an increase in the amount of carbonyl groups in LDPE, a sign that was directly associated with peroxidase attack (Sudhakar et al., 2008).

Our results are comparable with the general model that describes the beginning of polymer biodegradation as a surface erosion process (Gajendiran et al., 2016). Microorganisms adhere to and colonise the plastic surface, a phenomenon involving the production and secretion of extracellular enzymes and other molecules that mediate biofilm formation (Koutny et al., 2006; Sen and Raut, 2015; Sheik et al., 2015), a fundamental step to initiate microbial biodegradation (Mathur et al., 2011). In fact, the large size of the enzymes can present an obstacle to penetrate deep into the material (Gajendiran et al., 2016). Besides, the undegraded macromolecules are too large to pass through the cell membranes (Koutny et al., 2006; Sen and Raut, 2015). Finally, biofilm formation can prevent the hydrophobicity of polyethylene and help microorganisms to survive in a nutrient-poor environment by using the solid substrate (Shah et al., 2008; Sen and Raut, 2015; Ojha et al., 2017; Yuan et al., 2020).

## 5. CONCLUSION

Compared to *Aspergillus niger*, the tested *Rhizopus* sp. strain has a good potential for plastic biodegradation as confirmed by several *in vitro* and *ex situ* tests: reducing the weight of plastic bag pieces, formation of visible biofilm on plastic bag pieces and $CO_2$ production. Based on the obtained results, it is evident that nature offers an alternative pathway to solve some environmental issues like plastic pollution. *Rhizopus* sp. could be used for the bioremediation of polluted environments to eliminate plastic waste that is harmful to the environment (flora and fauna) and to human health.

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