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ORIGINAL PAPER

Polycarbonate biodegradation by isolated molds using clear-zone and atomic force microscopic methods

M. Arefian · M. Zia · A. Tahmourespour · M. Bayat

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Abstract The accumulation of dry waste containing synthetic polymers due to their resistance to microorganisms and other environmental factors has posed some serious problems to the environment in recent years. On the other hand, plastics constitute the foundations of economy as they are widely used in agriculture, constructions, packaging, health care and also medicine. The aim of this research was to investigate the role of different isolated fungi in the degradation of polycarbonate polymers. For this purpose, sampling was done using the garden soil and waste leachate from Isfahan Waste Management Organization. Samples were enriched in the liquid mineral salt medium supplemented with polycarbonate and then were transferred to the same medium solidified with agar to isolate and identify different fungi. Finally, their biodegradation activity was investigated with the help of clear-zone and atomic force microscopic (AFM) techniques, and also lipase and amylase production was tested. Among 15 isolated genera of mold fungi, *Fusarium, Ulocladium, Chrysosporium* and *Penicillium* showed biodegradation activity. According to the diameter of clear zone around the fungal colonies and also AFM results, the highest rate of degradation was related to *Fusarium*. Lipase activity of all isolated fungi was positive, but amylase activity of *Ulocladium* was negative. It can be concluded that some fungal strains such as *Fusarium* can be used for the biodegradation of plastic materials as it leads to a very eco-friendly biodegradation process.

Keywords Atomic force microscopy (AFM) · Biodegradation · Clear-zone method · Plastic polymers · Polycarbonate (PC)

M. Arefian
Department of Microbiology, Fars Science and Research Branch, Islamic Azad University, Fars, Iran

M. Zia · A. Tahmourespour (✉)
Khorasgan (Isfahan) Branch, Department of Basic Medical Sciences, Islamic Azad University, Isfahan, Iran
e-mail: a.tahmoures.p@gmail.com

M. Bayat
Department of Medical and Veterinary Mycology, Faculty of Veterinary Specialized Sciences, Science and Research Branch, Islamic Azad University, Tehran, Iran

Introduction

The increasing growth of the world population and also technological development has added pollutants such as heavy metals (Gupta et al. 2010, 2011a, b), synthetic dyes (Jain et al. 2003; Mittal et al. 2008, 2010; Gupta et al. 2009, 2011a, b) and synthetic polymers (Gilan et al. 2004; Kumaravel et al. 2010; Boyandin et al. 2012) to the environment. Synthetic polymers are a group of materials that surround the whole environment (Gilan et al. 2004). In recent years, the excessive consumption of synthetic plastics has had an impact on the environment as the majority of them cannot be degraded in the environment, which increases the environmental pollution (Maeda et al. 2005). Plastics are disadvantageous as they are resistant to biodegradation, leading to pollution (Kathirsan 2003). The growth of population has led to the accumulation of huge amounts of non-degradable waste materials; for this reason, many countries have conducted special programs for the discovery of new materials that can be readily eliminated from the environment and have designed novel strategies to facilitate the transformation of contaminants (Kumaravel et al. 2010). Approximately 140 million tones/year of synthetic polymers are introduced as industrial waste products (Friedrich et al. 2007; Shah et al. 2008). The presence of these substances in the environment causes serious problems, including challenges to wastewater treatment plants and pollution of groundwater and surface water. Synthetic polymers are recognized as major solid waste environmental pollutants, and many of them are resistant to chemical and physical degradation; thereby, the biodegradation of synthetic polymers by microorganisms is favorable (Kim and Rhee 2003; Leja and Lewandowicz 2009). These polymers are extremely stable, and their degradation processes are limited in the environment (Bentham et al. 1987).

Polycarbonates (bisphenol-A-carbonate) (PCs) are the most widely used group of engineering plastics because of their superior physical, chemical and mechanical properties as well as their extensive applications. About 2.7 million tones of PCs are produced annually. Biodegradation of these polymers is considered as an important concern in waste management. In the past decades, a great deal of attention has been given to evaluate the various methods for the biodegradation of polymers in the controlled laboratory conditions (Trishual and Mukesh 2008, 2009). Generally, biodegradation is affected by different factors such as polymer characteristics, consistency and organism type (Trishual and Mukesh 2008).

Microorganisms are responsible for the majority of plastic degradations. Microorganism's exoenzymes first break down the complex polymers, giving short chains that are small enough to permeate through the cell walls to be used as carbon and energy sources (Premarj and Mukesh 2005). Many approaches have been employed for solving the international problem of plastic waste, such as recycling and using biodegradable plastic materials. In biodegradation process of plastic materials, microorganisms, especially bacteria and fungi, can play important roles (Bentham et al. 1987). Fungi may be involved in the primary degradation of polymers due to their dominant ability to compose organic matter (Lee et al. 2005) and also can generally exceed the bacterial biomass (Kim and Rhee 2003). There are many reports on bacterial degradation of polymers; however, the reports on fungal degradation are relatively rare (Cosgrove et al. 2007). The present study aimed to investigate the biodegradation activity of molds isolated from soil and waste leachate on polycarbonate polymers in an in vitro condition. So, this research was carried out throughout winter1391–autumn1392 in the laboratory of Isfahan Waste Management Organization.

Materials and methods

Sample collection

Sampling was done from the garden soil, buried polymers in soil (for 9 months) and garbage leachate of Isfahan Waste Management Organization. The samples were accordingly transferred on ice to the laboratory of Isfahan Waste Management Organization.

Isolation of polycarbonate-degrading fungi

Polycarbonate-degrading fungi were isolated from samples by enrichment technique. One hundred microliters of sample in enrichment medium was added to the mineral salt media supplemented with 0.6 % PCs and incubated at 35 °C, with shaking at 150 rpm for 2 weeks (Table 1). The identification of fungus isolates was done based on morphologic and microscopic features. Pure colonies were used in the biodegradation experiment.

Biodegradation experiments

The biodegradation activity of isolated fungi was tested by the following three methods:

Clear-zone test method

Pure colonies were transferred onto the plates with minimal salt media supplemented with PCs that were solidified with agar at pH 7. After incubation period, polycarbonate-degrading activity of the isolated fungi was screened by the formation of clear zones around the colonies (Shah et al. 2007). The diameter of clear zone was measured and recorded after 1 week.

Enzyme production

Amylase and lipase production in fungi was determined as follows:

Amylase test was performed in nutrient agar plate containing 1.0 % soluble starch. The strain was streaked in the center and incubated overnight at 40 °C and flooded with Lugol's iodine. The clear zone indicated the presence of amylase (Behal et al. 2006).

Lipase test was performed in the yeast extract agar supplemented with 5 % butter (pH 7.8). Culture plates

Table 1 Mineral salt media components (Shah et al. 2007)

g/liter	Compositions
1	NH_4NO_3
0.7	KH_2PO_4
0.7	K_2HPO_4
0.005	NaCl
0.7	$MgSO_4 \cdot 7H_2O$
0.002	$FeSO_4 \cdot 7H_2O$
0.002	$ZnSO_4 \cdot 7H_2O$
0.001	$MnSO_4$
6	Polymer

were incubated at 37 °C. The formation of green-blue zones around colonies, after adding 8–10 ml of Cu $(SO_4)_2$ for 10–15 min and washing with tap water, was investigated. The appearance of green-blue zone indicated the presence of lipase.

Atomic force microscopy (AFM)

Polycarbonate films are prepared and placed in a medium containing PCs as the sole carbon and energy source. The media are then inoculated with each fungus. After 15 days of fungal growth, films of PCs were removed and washed, and the changes in surface were studied by AFM. AFM was done to confirm the fungal biodegradation of PCs.

Results and discussion

In this study, five fungal strains with the ability of growth in enriched cultures containing PCs as sole carbon and energy source were isolated. The isolated fungi were considered as biodegrading fungi for further studies.

With the growth of global population and advances in technology, the worldwide use of plastic materials has increased, causing many waste management problems (Cosgrove et al. 2007; Tokiwa et al. 2009). A considerable attention has been devoted to the biodegradability of polymeric materials mainly because of the environmental pollution by waste polymers. The used materials should be degraded after being discarded in order to cause no environmental problem (Shrivastva and Tripathi 2011). Many efforts, such as production of bioplastics, have been made toward plastic polymer recycling; however, they proved to be very expensive. Therefore, the best method for the degradation of plastics seems to be biodegradation using different microorganisms (Tokiwa et al. 2009). Notably, several types of plastics undergo biodegradation process in the environment, and an understanding of how this process occurs may help the development of strategies to improve it for waste management purposes. Biodegradation of polymers is seen as one of the solutions for the current plastic waste management problems. In fact, biodegradable polymers are the materials that can be degraded into carbon dioxide, water and biomass as a result of the action of living organisms or enzymes. Degradation of polymers may proceed by one or more mechanisms, including microbial degradation in which microorganisms such as fungi and bacteria consume the material (Chonde Sonal et al. 2012). It is known that fungi can be responsible for the degradation of polymers, and different studies have affirmed the role of fungi in degradation of plastics (Gilan et al. 2004; Reddy et al. 2008; Vijaya and Malikarjuna 2008).

Macroscopic and microscopic investigations of isolated fungi of this study identified them as *Fusarium*, *Penicillium, Ulocladium* and *Chrysosporium* (Fig. 1). More details of macroscopic and microscopic features are seen in Table 2.

Fusarium, Penicillium, Ulocladium and *Chrysosporium* were tested for their ability of PC degrading by clear-zone formation around the fungus colonies in mineral salt media containing PCs (Fig. 2). The above-mentioned tests were in fact a rapid and sensitive screening assay for PC degrading, which revealed that *Fusarium* had the best activity in degrading polycarbonate polymers, as the diameter of clear zone was significantly bigger than that formed by other fungi

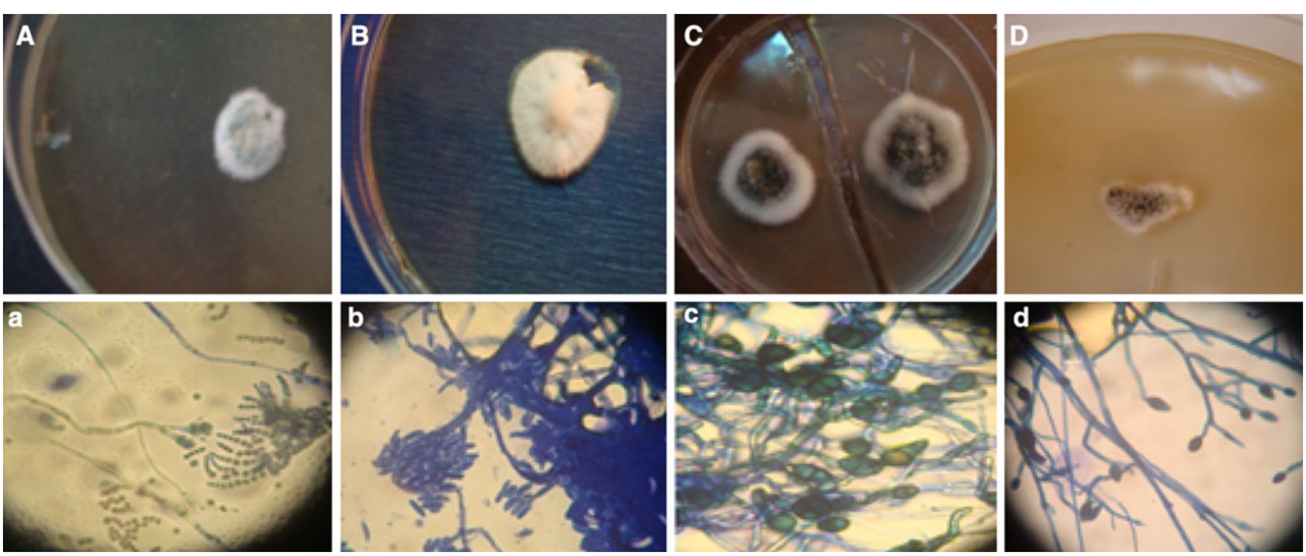

Fig. 1 Macroscopic and microscopic images of *Penicillium* (Aa), *Fusarium* (Bb), *Ulocladium* (Cc) and *Chrysosporium* (Dd)

Table 2 The basic identification features of *Fusarium*, *Penicillium*, *Ulocladium* and *Chrysosporium*

Isolate Number	Size of conidia (μm)	Shape of conidia	Arrangement of conidia	Shape of conidiophore	Arrangement of conidiophore	Colony morphology	Growth rate	Other helpful features	Result
1	3 × 6 & 5 × 50	Cylindrical microconidia, fusiform macroconidia	Solitary microconidia, whorls of macroconidia	Tapering phialides	Solitary, occasional whorls	Cottony pastel (typically pink or purple)	Rapid	Colony pigment is water soluble	*Fusarium*
2	2–5	Round	Chains	Penicillus	Solitary and erect	Velvety or powdery pastel	Rapid	Phialides have relatively short necks with blunt tips	*Penicillium*
3	11–18	Globose or elliptical	Individual	Erect and geniculate, with branching	Individual	Velvety black, with whit to gray over growth and black reverse	Rapid	Conidia are muriform, conidiophores are septate and zigzag simpodially	*Ulocladium*
4	6–8	One-celled ovoid to clavate conidia	Within and along the hyphae	Poorly differentiated from the hyphae	Individual	Granular or cottony, glabrous or velvety, white to tan to yellow and white to light brown reverse	Rapid	Numerous arthroconidia, unicellular conidia on conidiophores or directly on the hyphae	*Chrysosporium*

followed by *Ulocladium, Chrysosporium* and *Penicillium* (45 mm for highest and 3 mm for the lowest activity).

A study by Boyandin et al. (2012) showed that *Paecilomyces*, *Penicillium* and *Trichoderma* genera exhibited PHA-degrading activity in Vietnamese soils, and *Paecilomyces*, *Penicillium*, *Acremonium* and *Verticillium* in Siberian soils (Boyandin et al. 2012). Suyama et al. (1998) and Pranamuda et al. (1999) isolated poly(hexamethylene carbonate)- and poly(butylene carbonate)-degrading microorganisms that were phylogenetically diverse (Pranamuda et al. 1999; Suyama et al. 1998). Cosgrove et al. (2007) investigated the fungal communities associated with degradation of polyester polyurethane (PU) in soil and reported that different fungi can degrade PU in different soils (Cosgrove et al. 2007). Also, the degradation of poly-3-hydroxy-butyric acid (PHB) by fungi isolated from various environments was detected by clear-zone method, and fungi such as *Aspergillus* and *Penicillium* showed PHB degradation activity (Lee et al. 2005).

Lipase activity of all isolated fungi was positive, but amylase activity of *Ulocladium* was negative. It has been shown that the degradation of synthetic polymers may also occur by enzymatic hydrolysis. The enzymes that are responsible for biodegradation are serine hydrolases, esterases, amylases and lipases. Five fungal isolates used in this study were lipase positive. Lipase is an enzyme with the ability to catalyze the hydrolysis of ester bonds in polyesters. It was found that certain lipases enhanced the degradation of polycaprolactone when compared with incubation only in buffer (Azevedo and Reis 2005). Suyama and Tokiwa (1997) reported that cholesterol esterase, lipoprotein lipase and lipase of microorganisms were involved in biodegradation of polymer processes. They also declared that lipase and lipoprotein lipase from *Pseudomonas* sp. could also degrade high molecular weight poly(butylene carbonate) (Suyama and Tokiwa 1997). Tokiwa et al. (2009) also reported that lipase from *rhisopus delmar* could also degrade poly-ethylene terephthalate, poly-butylene terephthalate and poly-ethylene isophthalate (Tokiwa and Suzuki 1981).

Except *Ulocladium*, other fungi were amylase positive. α-Amylase is an endo-specific enzyme that catalyzes the hydrolysis of α-1,4-glycosidic linkages of starch to maltose and dextrins, reducing the molecular size of starch. The combination of the two enzymes contributes to significant differences in the bands of both starch and polycaprolactone (PCL), indicating degradation of both components of the polymeric blend (Azevedo and Reis 2005). Amylase from *Aspergillus niger* could also degrade starch-based plastic polymers (Suyama and Tokiwa 1997).

Results of AFM analyses of this research indicated that isolated fungi can form biofilm and cavity on PC films (Fig. 3). Figure 3d shows more biofilm formation of

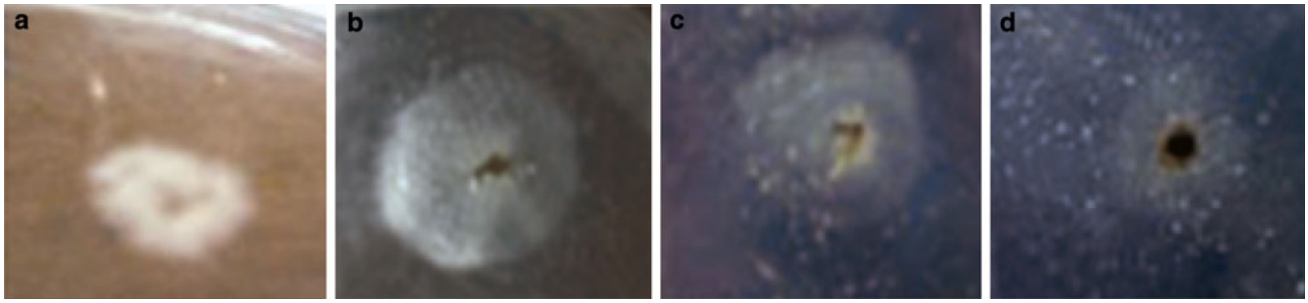

Fig. 2 Clear zones of *Penicillium* (**a**), *Fusarium* (**b**), *Ulocladium* (**c**) and *Chrysosporium* (**d**)

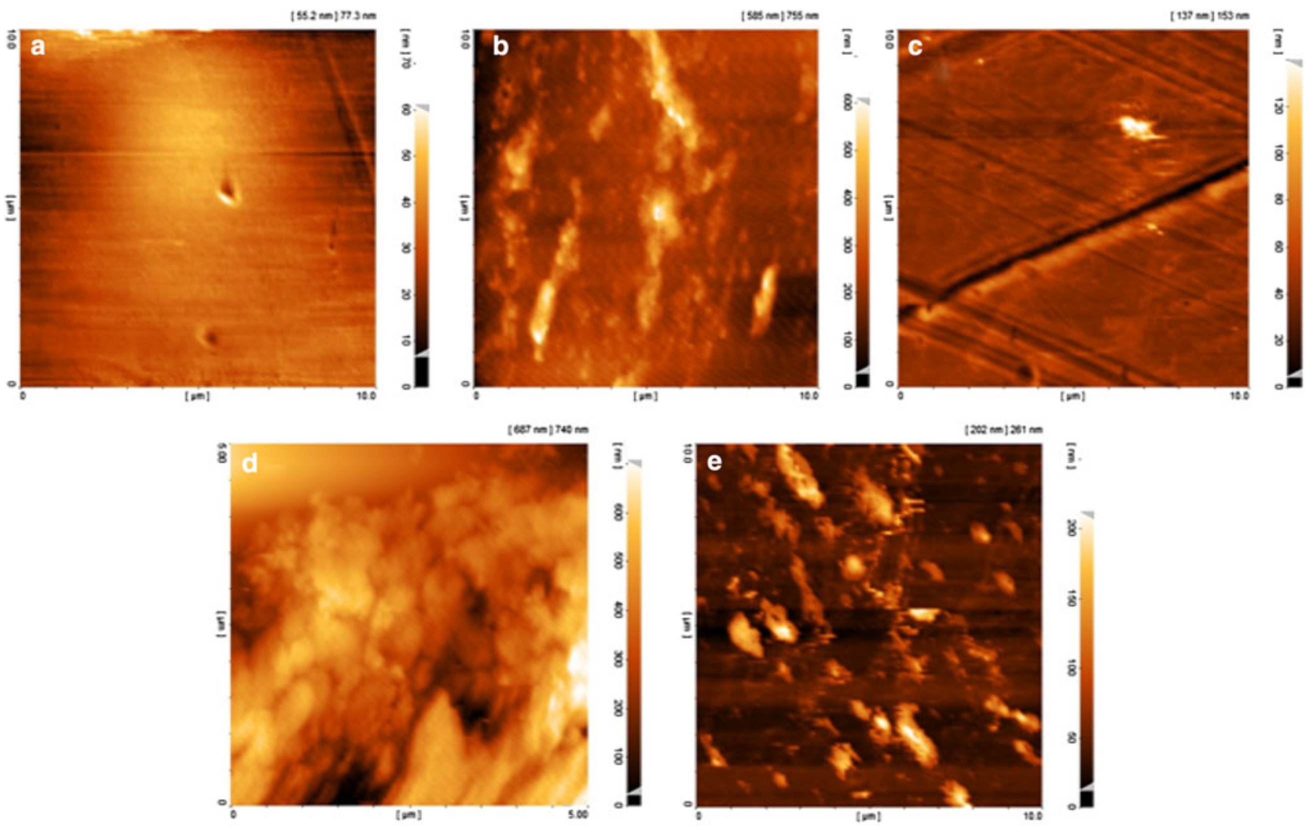

Fig. 3 AFM analyses of standard sample (**a**), *Ulocladium* (**b**), *Chrysosporium* (**c**), *Penicillium* (**d**) *and Fusarium* (**e**)

Penicillium with 600 nm height and both biofilm and cavity formation as a result of fungal growth.

Referring to Fig. 3, bright areas indicate the formed biofilm and black areas show the formed cavity.

The AFM technique has been developed to visualize the evolution of surface changes. Thus, it was employed to investigate the degradation or biodegradation of polymers. In this study, the AFM images showed the formation of cavities (dark areas) and fungal biofilms (light cloud-like areas) on PC plastic surfaces. This might be due to formation of biofilm on the polymer surface to enable the microbes to efficiently utilize the non-soluble substrate by the compounds secreted extracellularly that may break the complex molecular structure of polymers (Priyanka and Archana 2011; Shah et al. 2007).

According to Fig. 3d, *Penicillium* needed more time to degrade PCs because it formed more biofilm (more evident high area of about 600 nm in thickness) on PC surface.

Gilan et al. (2004) also reported the isolation of a strain of *Rhodococcus ruber* that colonized polyethylene surface and formed a massive biofilm on it, which was a process seemed to be a prerequisite for biodegradation (Gilan et al. 2004).

Conclusion

It can be concluded that some isolated fungal species such as *Fusarium, Penicillium*, *Ulocladium* and *Chrysosporium* have the ability to degrade PC polymers. The effect of *Fusarium* on degradation of polycarbonate polymers was estimated to be significant and better than that of other isolated fungi. Hence, it can be suggested to be used for the biodegradation of plastic materials as it leads to a very eco-friendly biodegradation process.

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