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Polycarbonate biodegradation by newly isolated *Bacillus* strains

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Abstract: As polycarbonate is frequently used in many products, its accumulation in landfills is absolutely harmful to the environment. The aims of this study were the screening and isolation of polycarbonate-degrading bacteria (PDB) and the assessment of their ability in the degradation of polycarbonate (PC) polymers. Nine-month buried--PC films were used for PDB isolation and identification. The biodegradation ability of the isolates was determined by growth curve, clear zone formation, lipase and amylase production, AFM and FTIR.

Bacillus cereus and *Bacillus megaterium* were identified and considered as PDB. The degradation ability of *B. megaterium* was significantly higher than that of *B. cereus.* Both were lipase and amylase positive. AFM and FTIR results showed the initiation of bacterial attachment.

The PC biodegradation ability of isolates can be very efficient. Finding such efficient isolates (which was less studied before) will promise a decrease in plastic contamination in the future.

Introduction

With the increase of world populace and the technology development, the plastic supplies have found broad applications in each life aspect and industries, and, consequently, many plastics cause waste management problems (Tokiwa et al. 2009). From an ecological point of view, the global accumulation of plastic waste which contributes to 10–13% of the total municipal solid waste is an environmentally growing concern as the rate of plastics manufacture goes over 25 million tons per year (Moharir and Kumar 2018).

The development and use of synthetic plastic have changed the nature of waste in the last 3–4 decades. The plastic durability is one of its most advantageous qualities, but, this same property leads to a main crisis of our environment (Harshvardhan and Jha 2013). Synthetic polymers are very stable and their degradation processes are limited in the environment. Approximately140 million tons of these plastic polymers are produced worldwide every year and become an environmental nightmare. Living conditions in the ecosphere are therefore changing notably, so that the presence of non-biodegradable residues can affect the potential survival of many organisms (Cosgrove et al. 2007).

Polycarbonate (PC) is a commonly used polymer because of its greater mechanical, physical, and chemical properties. Burying in a landfill, recycling and incineration are the major methods for management of plastic waste with their own inherent limitations. Many other approaches also have been projected for solving the problems of plastic waste, such as biodegradation or using biodegradable plastic materials (Kale et al. 2015). Biodegradation is a process in which bacteria or other microbes secrete polymer-degrading enzymes that lead to polymer decomposition (Boll et al. 2019). Bacterial exoenzymes play a fundamental role in polymers depolymerization in which breaking down the complex polymers and changing them into smaller chains occur to penetrate throughout the membrane and used as carbon and energy sources (Kale et al. 2015; Moharir and Kumar 2018). The enzymes responsible for degradation include esterases, serine hydrolases, amylases, and lipases. Also, it was observed that lipases can increase the polymer degradation (Banerjee et al. 2014).

Recently, much attention has been attracted toward the improvement of technologies which utilize microbial activity for various plastic polymer degradation such as polyethylene (Auta et al. 2017; Ren et al. 2019; Guzik et al. 2014), polyethylene terephthalate and polystyrene (Auta et al. 2017), and high density polyethylene (Devi et al. 2016) because this process is cheap and much more efficient without producing secondary pollutants (Moharir and Kumar 2018). But till to date (Artham and Doble 2009; Artham and Doble 2012; Sivalingam and Madras 2004) there has been a small number of reports concerning the biodegradation of PC. Biodegradation of PC are recognized to be performed either throughout the action of bacterial whole cells or pure enzymes. This study aimed to isolate polycarbonate degrading bacteria (PDB) from PC films buried in landfill soil and to assess their biodegradation ability by different methods.

Materials and methods

Sampling

Soil sampling was done from 10 cm depth of the landfill with a long history of contamination, transferred to the lab, passed through a sieve (2-mm). Soil pH and electrical conductivity (EC) were determined. Also, total heterotrophic bacteria (THB) were enumerated through a standard plate count method on Nutrient agar. The soil was then put into pot. Five PC films (70 · 40 · 1 mm) were weighted and buried in the soil for 9 months at room temperature. The soil was amended with a mineral salt solution (same composition as MSM medium) to keep moisture and minerals constant (~ 0.35 w/w). This step was performed in order to let the soil PDB attach to the PC films and their isolation become possible. After 9 months, the PC films were recovered and washed in phosphate buffered saline (PBS) solution in order to remove unattached cells. Then, the PC films with remaining attached bacteria were placed in a sterile tube containing PBS (5 ml) and vortexed well for detachment of all attached cells. The films were washed again, left to dry at room temperature and the weight loss percentages of them were measured. The remaining solution was used as the source of PC degrading bacteria.

Isolation and identification of PDB (polycarbonate degrading bacteria)

PDB were isolated from the above solution by an enrichment culture technique using MSM (mineral salt medium) in which PC was used as the sole carbon and energy source. The MSM contained (per 1000 mL; Merck, Germany): NH_4NO_3 (1.0 g); KH_2PO_4 (0.7 g); K_2HPO_4 (0.7 g); $MgSO_4$–$7H_2O$ (0.7 g); NaCl (0.005 g); $FeSO_4$–$7H_2O$ (0.002 g); $ZnSO_4$–$7H_2O$ (0.002 g) and $MnSO_4$–H_2O (0.001 g). PC was used as granules (Petrochimi Co. Iran).

The amount of one milliliter of washing solution was added to MSM supplemented with 0.6% PC (6 gr/L) and incubated in a shaker incubator at 150 rpm, and 35ºC for 2 weeks. The streak plate method was then used to isolate colonies in their pure form. Pure colonies were used for identification and biodegradation experiment.

The bacterial strains were identified according to macroscopic, microscopic examinations and biochemical tests. Also 16S rDNA sequence analysis of isolates was done with the universal primers (27F and 1492R) for identification confirmation (Isfahani et al. 2018).

Biodegradation studies

Clear-zone test method: The MSM agar plates were supplemented with PC powder at a final concentration of 0.2% (pH 7), the mixture was sonicated for 1 hour at 90 duty cycles and autoclaved. Then, the pure colonies obtained from the previous step were transferred to these media. After the incubation period of one week, the polycarbonate degrading activity of bacterial isolates was confirmed by clear-zone formation around the colonies and the clear-zone or halo diameter was measured (Arefian et al. 2013).

Enzyme production: Amylase and lipase production of PDB was determined as previously described method (Arefian et al. 2013), briefly:

- Nutrient agar plates containing 1.0% soluble starch were prepared and inoculated with each isolates for determining amylase production. The plates were then incubated overnight at 40°C and flooded with Lugol's iodine. The halo zone indicates starch degradation.
- The yeast extract agar plates supplemented with 5% butter (pH 7.8) were inoculated with each isolate to determine lipase production. The plates were kept at 37ºC. Green-blue zones around colonies were measured after adding 8–10 ml of Cu $(SO4)_2$ for 10–15 min and washed with tap water.

Bacterial growth rate: The cell pellets of overnight bacterial cultures were resuspended in PBS buffer to be sure that there is not any culture medium brought into the MSM medium. Then, the amount of 5 ml cell suspension adjusted to 0.5 Mc Farland standard (equal to $1.5 \cdot 10^8$ CFU/ml) was inoculated to flasks containing MSM (250 ml) supplemented with one piece of PC film and incubated in a shaker incubator at 150 rpm for 15 days. In this medium, PC utilization as the only carbon and energy source leads to bacterial growth and the bacterial growth is the sign of PC degradation or decomposition which is monitored regularly by reading the broth optical density at the wavelength of 600 nm (OD600) at time intervals by UV-Visible spectrophotometer (Chatterjee et al. 2010; Devi et al. 2016 and Nzila et al. 2016).

Atomic Force Microscopy (AFM) and FTIR spectroscopy

After fifteen days of bacterial growth in the MSM supplemented with PC, the PC films were recovered from the culture media, washed, dried and studied by AFM without any preparation or staining. The AFM was performed to confirm the modifications caused by bacterial degradation on the surface topography of the PC films (Tribedi and Sil 2013).

After the above-mentioned biodegradation process, changes in the PC films structure, as the formation or disappearance of functional groups in the presence of bacterial inoculation, were analyzed by FTIR Spectroscopy (Jasco Inc. 6300; Japan) in the frequency range of 4000–650 cm^{-1} (Ren et al. 2019).

Statistical analysis: All of the experiments were performed in three replicates. The ANOVA analysis of variance (SPSS software) was used to determine the statistical significance of data. In order to draw charts and also find regression equation, Excel software was used. Statistical significance was assumed at a P value of 0.05.

Results

The pH, EC and total heterotrophic bacterial count of the tested landfill soil, which was used for the PC films burial, were 8.1, 8.2 ds/m^2 and 43 × 10^7 CFU/gr, respectively. The significant weight changes ($P<0.05$) of 9 month buried PC films were recorded (Fig. 1); the means of the pair (x1–x2: initial and final weight) were significantly different according to ANOVA analysis variance and Tukey HSD (Table 1). The mean weight loss was calculated as 0.218%.

After enrichment culture or 3 successful transfers, colonies were isolated in pure form. Only two bacterial strains, with the ability of growth in enriched cultures containing PCs as the only source of carbon and energy, were isolated and purified. The isolates were considered as degrading bacteria for further studies.

According to macroscopic and microscopic observations, biochemical tests, and 16SrDNA sequence analysis with the percentage similarity of more than 99%, they were recognized as *Bacillus megaterium* and *Bacillus cereus* (Table 2).

In biodegradation studies, the clear-zone diameter around colonies for bacterial strains was measured after one week. As a result, the clear-zone diameters for *B. megaterium* and *B. cereus* were 5 mm and 4 mm, respectively. It is clear that *B. megaterium* has greater ability in PC utilization than *B. cereus*, although the difference is not significant (P>0.05).

Both of the bacterial isolates were lipase and amylase positive. The growth rate of bacterial isolates was shown in the presence of PC as only carbon and energy source (Fig. 2). The results correspond to a non-linear increase of bacterial number which shows the ability of bacteria to utilize PC as the only source of carbon and energy, and grow. The fittest line for the calibration of the bacterial number during the time required a 3rd order polynomial (R2=0.99). Analysis of the growth rate results showed that the decomposition ability of *B. megaterium* was higher than that of *B. cereus.* The images obtained from AFM (Fig. 3) also confirmed the PC decomposition and surface roughness caused by *B. megaterium* in comparison with the control film. Finally, probable changes in the structure of PC films during bacterial growth were analyzed by FTIR--spectroscopy and shown in Figure 4. The FTIR spectra of polycarbonate exposed to *B. megaterium* and control showed tghat major absorbance intensities of 1,770 and 1,505 cm^{-1} correspond to carbonate carbonyl and aromatic C=C stretching vibrations, respectively. The bonds at 2,800–3,200 cm^{-1} correspond to the CH stretching bond. The only difference found between control and the treated film was the appearance of a very negligible vibrational peak at 3,400 cm^{-1} in the presence of *B. megaterium.*

Discussion

Soil bacteria are considered as a key constituent of biodegradation due to their stability and efficiency (Zawierucha et al. 2014). Today, it is known that microorganisms are responsible for the degradation of various plastic polymers but very little is known about PC biodegradation. The tested soil in this study was prepared from the landfill with a long history of plastic disposal wastes which can provide an adopted bacterial population. It was classified as saline-alkaline soil, according to its pH and EC, which is an extreme habitat for the isolation of the specific prokaryotic community with the ability to tolerate high EC and pH. The resident bacteria of such niches acquire unique adaptation mechanisms for survival in the presence of environmental stresses, which biotechnologically and commercially is really considerable (Keshri et al. 2013).

Two bacterial strains, namely *B. megaterium* and *B. cereus* could survive and grow on MSM broth containing PC better than others which, confirmed their PC decomposition ability and the existence of some PC degrading mechanisms to access its carbon content. Such ability is the result of their adaptability to plastic disposal environment because, under the stress condition, bacteria express new and necessary enzymes or other metabolites that help them to overcome the stress (Artham and Doble 2012). Muthukumar and Veerappapillai (2015) in their brief review of biodegradation of plastics concluded that there were many different bacterial strains with the ability of plastics degradation of which the *Bacillus* genus was the most frequent one. It is in agreement with our results. The growth curve of the isolates during the in vitro degradation study revealed that both isolates showed a significant growth phase from 1st to 3rd day of incubation and the steady stationary phase thereafter. The growth profiles correspond to a non-linear increase of bacterial

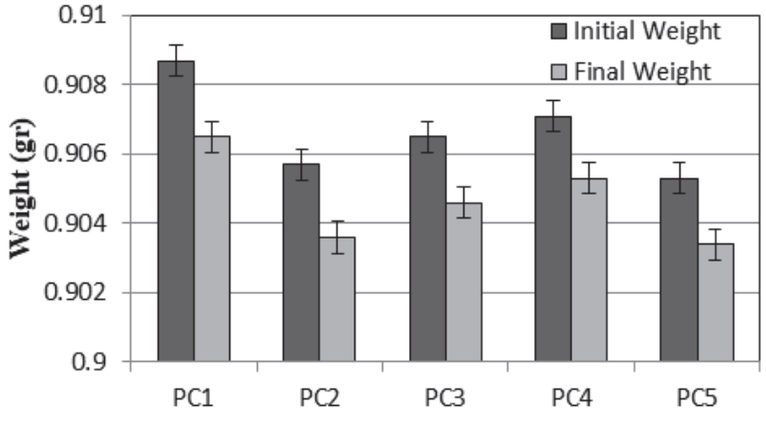

Fig. 1. The initial and final weight of PC films before and after 9 months burial (The error bars show the significant difference between initial and final weight of each PC films)

Table 1. ANOVA analysis variance of the means of soil buried-PC films' initial and final weight

Source	DF	Sum of Square	Mean Square	F Statistic	P-value
Between groups	1	0.00000980100	0.00000980100	5.739972	0.0434590
Within groups	8	0.0000136600	0.00000170750		
Total	9	0.0000234610	0.00000260678		

Table 2. Tannin derading bacterial isolates identification and charachterization

Test	Isolate 1	Isolate 2
Morphology	Rod	Rod
Gram strain	+	+
Spore formation	+	+
Gloucose	+	+
Xylose	–	–
Arabinose	–	+
Manitol	–	–
Urease	–	V
Indole	–	–
Citrate	+	+
Nitrate reduction	+	+
Voges-proskauer	–	+
Blood agar hemolysise	–	+
Motility	+	+
Growth in 6.5% Na Cl	+	+
Casein hydrolysis	+	+
Lesithinase	–	+
Catalase	+	+
Gelatinase	+	+
Amylase	+	+
Lipase	+	+
Microorganisms	***Bacillus (megaterium)***	***Bacillus (cereus)***
Similarity percentage with GenBank strains	***>99%***	***>99%***
Clear zone diameter (mm)	***5***	***4***

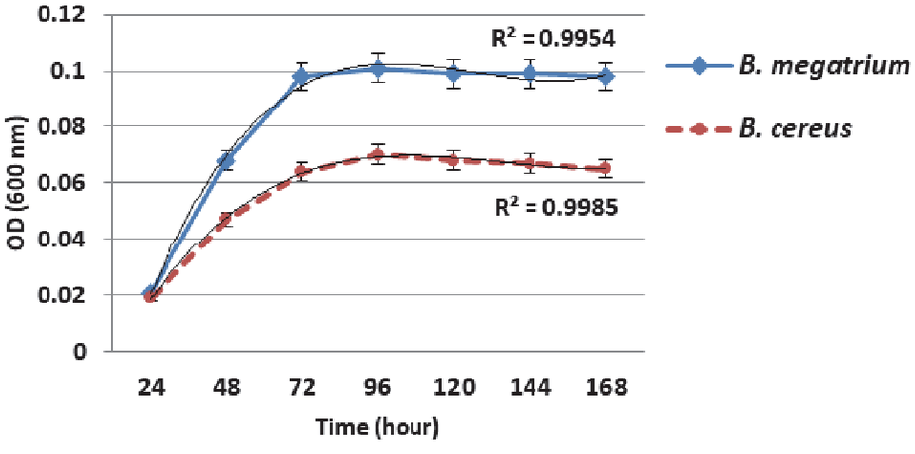

Fig. 2. The growth rate of bacterial isolates in the presence of PC as the sole source of carbon and energy

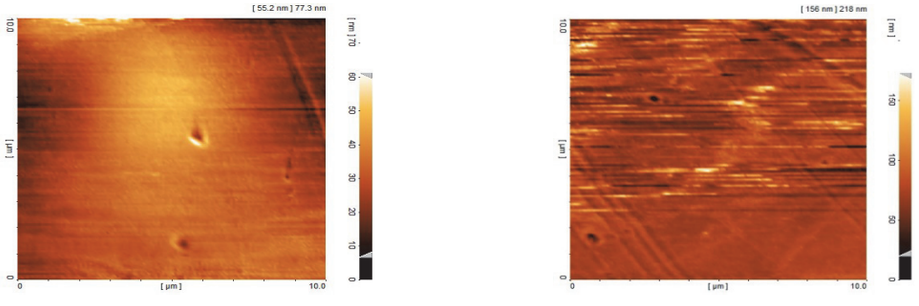

Fig. 3. AFM figures of surface erosion of polycarbonate films; A: Control; B: treated with *B. megaterium*; cavities and bacterial biofilm are shown as dark and light areas on PC plastic surfaces, respectively

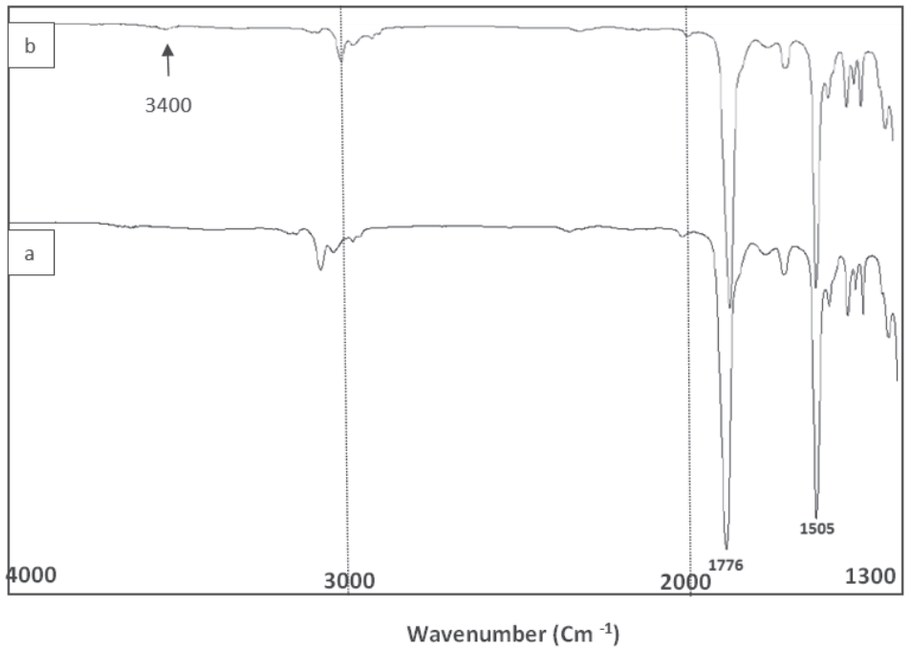

Fig. 4. The FTIR spectrum of polycarbonate film (A: control; B: treated with *B. megatrium*)

count during the process. The best-fitted equation model for the experimental data was a 3rd-order polynomial (R^2>0.99) showing that all the variables in the experiment could be explained by the model. As shown in Fig. 2, the turbidity was increased slowly, which is probably due to bacterial biofilm formation during the process. This phenomenon was previously described (Chatterjee et al. 2010; Devi et al. 2016).

According to the results, *B. megatrium* had better growth and degradation activity than *B. cereus* with significant differences (P<0.05) due to the higher OD600 of the bacterial solution and the greater clear-zones diameter. The obtained results also showed that the clear-zone method is a suitable method for the investigation of the ability of microorganisms in the degradation of polymers. The halos formed are circular, distinct, and reproducible demonstrating the degradation of polymer particles around the colonies (Tokiwa et al. 2009).

Bacteria are considered as the source of enzymes and able to break down different kinds of substrates (Guzik et al. 2013). In this study, the production of enzymes, including lipase and amylase by *B. megaterium* and *B. cereus* was confirmed. Tokiwa et al. (2009) also showed that Pseudomonas-secreted serine hydrolases, esterases, and lipases are enzymes responsible for polymer biodegradation. Russell et al. (2011) stated that the active enzymes involved in polymer degradation are grouped as proteases, esterases, lipases, and ureases. Sivalingam et al. (2004) have reported the bisphenol-A, PC biodegradation by lipases which are stable and active enzymes. The α-amylase is the enzyme belonging to the endo-amylases family, which can catalyze the starch initial hydrolysis into smaller oligosaccharides throughout the cleavage of α- D-(1-4) glycosidic bonds (Souza 2010). There is some evidence to indicate the role of α-amylase in PC biodegradation (Artham and Doble 2009; Shah et al. 2008). However, there is still a lack of information on the complete metabolic pathways involved in the process, in the structure, and identity of all the enzymes involved. The enzymatic degradation of polycarbonate has also been shown (Premraj and Doble 2005).

The technique of AFM has been evolved to visualize the evolution of surface changes. In this study, the images of AFM showed the cavity formation (dark areas) and bacterial biofilm (light areas) on PC plastic surfaces, but the control sample surface remained smooth and no attached cells of bacteria were observed. The bacterial attachment to the polymer surface enables them to efficiently use the substrate (non-soluble) by the secreted extracellularly products that might break the complex molecular structure of polymers (Phukon et al. 2012; Shah et al. 2008). Ren et al. (2019) reported Enterobacter attachment and biofilm formation to the surfaces of polyethylene by AFM technique and concluded that biofilm formation can facilitate the contact of polymer with microbial enzymes. Tribedi and Sil (2013) also showed the biofilm formation of Pseudomonas sp. during degradation. However, it is useful to monitor the bacterial growth and attachment, also, the polymer structure during the degradation process (Da Costa et al. 2018; Lwanga et al. 2018). The appearance of a very negligible vibrational peak at 3,400 cm^{-1} in the presence of *B. megaterium* in FTIR, might be due to the hydroxyl group formation which suggests the carbonate hydrolysis initiation. In this study, the slight changes in the location of peaks around 3000 cm^{-1} were also observed between the FTIR spectra bonds of PC films in control and treatment due to a very short exposure time of 15 days. Artham and Doble (2012) also found a broad peak at 3,400 cm^{-1} in the presence of microbial consortium in an in vitro study which was due to the formation of hydroxyl groups suggesting the hydrolysis of carbonate bonds in a longer period of 12 months. Das and Kumar (2015) also observed the formation of new and disappearance of functional groups in their low-density polyethylene degradation experiments by *B. amyloliquefaciens*. So, each change in the peak location

and values of almost all functional groups can support the conformational change on the polymer sample.

Conclusion

Although plastic wastes cause negative effects on ecosystems, there is not enough accurate knowledge and protocol to cope with them. However, significant researches are being conducted in the polymer biodegradation field because it is very cost-effective, eco-friendly, and an attractive professional option of plastic waste disposal. Hence, the isolation and identification of polymer-degrading microbes are the first steps. In this study, *B. megateriun and B. cereus* strains were isolated from buried PC in the presence of PC as the only source of carbon and energy (which has been less studied to date). Further biodegradation experiments also confirmed the decomposition and enzymes production ability of isolates, the properties which offer them biotechnological and commercial advantages. So, optimization of PC biodegradation of such isolates and the study of interactions between plastics and bacteria need urgent investigation because the ecosystem is in a serious danger and the natural adaptability of microbes needs too much time. Furthermore, these bacterial isolates are not only considered as the suitable PC degrading bacteria, but also as good sources of enzymes such as lipase and amylase with many biotechnological applications.

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