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Isolation and Identification of Polyethylene Terephthalate Degrading Bacteria from Shatt Al-Arab and Sewage Water of Basrah City

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Abstract:

Biodegradation is utilizing microorganisms to degrade materials into products that are safe for the environment, such as carbon dioxide, water, and biomass. The current study aims to isolate and characterize bacteria with polyethylene terephthalate (PET) degradation ability isolated from Shatt al-Arab water and sewage from Basra, the bacteria were identified as *Klebsiella pneumoniae*. According to the findings, the isolates showed a highly significant difference in degradation of PET (24% during 7 days) and the percent of degradation increased to 46% at 4 weeks compared to the control. The study also involved determining the optimum temperature of *K. pneumoniae* growth, which was 37°C, while the preferred pH was 7-8. The research revealed that PET biodegradation by *K. pneumoniae* can be used as a suitable and environmentally friendly tool.

Keywords: Biodegradation, Degradation Bacteria, *Klebsiella Pneumoniae*, Plastic, Polyethylene Terephthalate(PET).

عزل وتشخيص البكتيريا المحللة للبولي ايثيلين تيريفثاليت من مياه شط العرب ومياه الصرف الصحي في مدينة البصرة

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الخلاصة:

التحلل الحيوي للمواد البلاستيكية هو استخدام الكائنات الحية الدقيقة لتحطيم هذه المواد إلى منتجات آمنة للبيئة ، مثل ثاني أكسيد الكربون والماء والكتلة الحيوية. نهدف في الدراسة الحالية إلى عزل وتشخيص بكتيريا ذات قدرة على تحلل البولي ايثيلين تيريفثاليت PET من مياه شط العرب ومياه الصرف الصحي في مدينة البصرة . وفقا للنتائج فان العزلات أظهرت فرقاً معنوياً عالياً في تحلل PET بنسبة ٢٤٪ خلال ٧ أيام وزادت نسبة التحلل الى ٤٦٪ في ٤ أسابيع مقارنة بالضابطة. كما اشتملت الدراسة على تحديد درجة الحرارة المثلى لنمو البكتيريا وهي ٣٧ درجة مئوية، بينما الرقم الهيدروجيني الامثل للنمو ٧-٨. أظهر البحث أن التحلل البيولوجي لبوليمر PET يمكن ان يتم باستخدام بكتيريا *K. Pneumoniae* كطريقة مناسبة وصديقة للبيئة.

الكلمات المفتاحية: التحلل الحيوي، البكتيريا المحللة، بكتيريا الكلبسيلا الرئوية، المواد البلاستيكية، البولي ايثيلين تيريفثاليت .

Introduction:

The annual global production of synthetic polymers is about 140 million tonnes and, due to their exceptional stability, these polymers do not easily enter into the degradation cycles of the biosphere. The environmental pollution of synthetic polymers, such as that caused by waste plastics and water-soluble synthetic polymers in wastewater, has been acknowledged as a significant problem^{1,2}. One of the most commonly used plastics is polyethylene terephthalate (PET), which contains ethylene glycol and terephthalic acid as repeating units. PET is used in the manufacture of fibers, containers, films, and bottles because of its remarkable properties such as durability, ability to be molded, and light weight³. The waste of PET keeps on in terrestrial and marine environments, which frequently causes harm or death to some of the organisms⁴. PET contamination is controlled via chemical, thermal, and mechanical methods. However, these techniques either produce extra pollutants or cost a lot of money; therefore, alternative methods must be found⁵. The process of a polymer's chemical structure changing from a more complex to a simpler one under the influence of several biological agents, such as bacteria, fungi, and various atmospheric microorganisms, is known as biodegradation⁶. More than 90 genera of microorganisms that break down polymers such as: *Actinomyces*, *Thermoactinomyces*, *Azotobacter*, *Alcaligenes*, *Streptomyces*, *Mycobacterium*, *Micromonospora*, *Flavobacterium*, *Escherichia*, *Rhodococcus*, *Streptococcus*, *Klebsiella*, *Nocardia*, *Pseudomonas*, *Comamonas*, and *Staphylococcus*^{7,8}. The plastic biodegradation mechanism involves many levels. Firstly, the microbial attachment changes the physical and chemical characteristics of the plastic surface, followed by enzymatic cleavage, which breaks down the large polymers into smaller molecules of oligomers and monomers (biofragmentation). Numerous hydrolysing enzymes, including esterases, ureases, or proteases, catalyze the dissolution of various polymer linkages. The broken polymer is embraced by bacteria through assimilation, where it is mineralized into CH₄, CO₂, and H₂O, etc^{9,10}. There are several aspects that can be investigated for improved polymer plastic biodegradation, including: utilizing surface-active substances or stimulating microorganisms to make a surfactant that will improve microbe adhesion to the surface of the polymer; mixing polymers with biodegradable materials; also using bio-stimulation that means the addition of nutrients that could be scarce in the environment¹¹⁻¹³. Many researchers have reported numerous organisms that destruct many types of polymers. Montazer et al.¹⁴ have been reported the degradation of untreated PE by *Micrococcus luteus* IRN20, *Acinetobacter pittii* IRN19 and *Pseudomonas putida* IRN22. Ren et al.¹⁵ isolated *Enterobacter* sp. D1 from the *G. mellonella* gut. After treatment, they observed physical changes in the PE-film, which they concluded were produced by oxidation reactions brought on by the bacteria. Giacomucci et al.¹⁶ found that *Bacillus flexus* and *Pseudomonas citronellolis* have the ability to biodegrade polyvinylchloride(PVC) film. These strains have been demonstrated to produce a thick biofilm on the surface of the plastic film and reduce the PVC mean molecular weight. Yin et al.¹⁷ isolated *Bacillus* sp. strain NyZ451 and *Acinetobacter* sp. strain NyZ450 from the gut of *T. molitor* larvae and evaluated their ability to degrade LDPE. Both strains' cells have the ability to depolymerize LDPE, but neither could thrive on it. Over the course of 30 days, their co-culture consumed LDPE and eliminated 18% of the LDPE films. This implies that several types of bacteria are needed for LDPE biodegradation.

Although microbial degradation is a slow process, it is a non-cost and environmentally friendly assay, so the aim of this project involves the investigation of the PET degradation ability of *Klebsiella pneumoniae* and determining the optimum pH and temperature that are necessary for bacterial growth in the presence of the PET.

Materials and Methods:

Materials

The films of PET (drinking water bottles) were prepared by cutting the bottles into equal pieces with (0.6 gm), Nutrient agar, MacConkey ager, Eosin methylene blue, Mineral salt medium (MSM) for PET biodegradable consisting of: NaNO₃ 2.5g, K₂HPO₄ 1.0g, MgSO₄ 0.5g, KCl 0.1g, KH₂PO₄ 0.5g, FeSO₄ 0.01g, CaCl₂ 0.01g, NH₄NO₃ 0.39g, Na₂HPO₄ 5.6g, the components supplemented with Glucose 30g and Distilled water 1000 ml.

Isolation of Bacteria from Shatt al-Arab and Sewage Water

Samples were collected from three locations at a depth of 5-15cm and brought to the laboratory. For the isolation of degradable bacteria, 1 ml of sample (water and sewage) was mixed with 10 ml of distilled water, which was then rotated at 150 rpm for 30 minutes at 37 °C. After that, 1 ml of the solution was pipetted into 9 ml of distilled water. The goal was to make 10⁻¹ decimal and supply inocula of the dilutions (10⁻², 10⁻³, 10⁻⁴) by sterilized pipette and 1 ml placed into a petri dish and poured into nutrient agar, MacConkey, and eosin methylene blue yeast tryptophan. It was then incubated for 24 hours at 37°C¹⁸.

Identification of Isolates

The isolates were identified using biochemical and morphological assays as described in Bergey's Manual of Determinative Bacteriology¹⁹.

Maintenance of Isolates:

Isolates were kept in nutrient agar screw-capped tubes that were 20% glycerol coated²⁰.

Biodegradation of PET

Bacterial isolates were injected into a mineral medium and a thin film of PET plastic was then aseptically implanted and incubated for 1–4 weeks at 37°C. After that, the thin plastic film was cleaned with 70% ethyl alcohol and sterile distilled water. The film was placed into the oven at 80°C till the weight was consistent, which represented the final weight that was recorded²¹. The control consists of PET with the mineral medium. The reduction in weight calculated from the Eq.:

$$\% \text{ Weight reduction of plastic} = (R_1 - R_2) \times 100 / R_1 \dots \dots \dots (1)$$

whereas: R₁ = Initial plastic film weight.

R₂ = Final plastic film weight.

Optimum Conditions of PET Biodegradable

Effect of Temperature on PET Biodegradable

A five ml inoculum of *Klebsiella pneumoniae* was added to a 250 ml Erlenmeyer conical flask containing 100 ml of MS medium (PH = 7) and 0.5% PET film, which was inoculated and incubated for 7 days in a shaker incubator (180 rpm) at various temperatures (15,20, 25,30, 37,40°C). The growth was recorded in terms of optical density (OD) at 600 nm by using a spectrophotometer²¹.

Effect of pH on PET Biodegradable

A five ml inoculum of *Klebsiella pneumoniae* was added to a 250 ml Erlenmeyer conical flask containing 100 ml of MS medium and 0.5 % of PET film, which was inoculated and incubated in a shaker incubator 180 rpm at diverse pH (4,5,6,7,8,9,10) by adding HCl or NaOH for 7 days at 37°C, the growth was recorded in terms of OD at 600 nm by using a spectrophotometer²¹.

The methods were performed in triplicate and the statistical analysis was carried out using one-way ANOVA to detect significant differences.

Results and Discussion

Isolation and Characterization of Bacteria:

The separation of microorganisms from mixed cultures can be accomplished using a variety of methods. However, spread plating on a solid agar medium is the simplest and most basic method of isolation. Fig. 1 shows the isolated bacteria from Shatt Al-Arab and sewage water using spread plating. Table 1 illustrates the identification of isolates according to morphological, physiological, and biochemical features, which was done according to Bergey's Manual of Determinative Bacteriology. According to the obtained outcomes, the bacterial isolates were identified as *Klebsiella pneumoniae*.

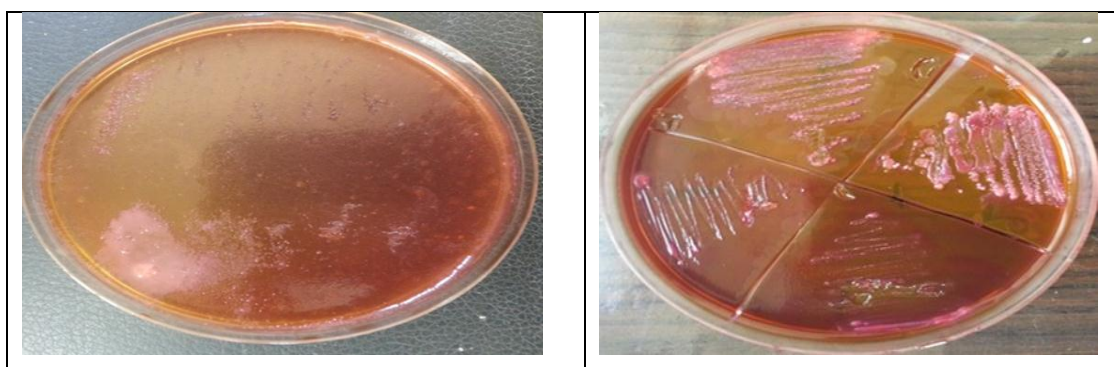


Figure 1: *K. pneumoniae* isolation on macConkey agar magnification 4.1X

Table 1: Morphological, Physiological, and Biochemical Tests of Isolate

Character	Result	Character	Result	Character	Result
Capsule	+	Growth In KCN	+	Pigment	-

Catalase	+	H ₂ S	-	Shape	Rod
Citrate	+	Indole	-	Spore	-
Flagella	-	Motility	-	Urease	+
Gas	+	MR(Methyl Red)	-	VP(Voges Proskauer)	+
Gelatin Hydrolysis	-	Nitrate Reduction	+	Dnase	-
Gram Staining	-	Oxidase	-	Glucose	+
Glycerol	+	Glycerol	+	Maltose	+
Mannitol	+				

Biodegradation and Optimum Conditions

The investigation of the biodegradation ability of *K. pneumoniae* was carried out by using PET as a pollution source. The loss of mass is used frequently in degradation testing. The results in Fig. 2 and Table 2 showed a highly significant difference in PET degradable with $p < 0.01$ and the loss of weight increased with increasing the incubation time. The reduction in weight increased from 24% in the first week to 46% after 4 weeks. This result is evidence of the use of polymer as a source of carbon by bacteria, which might not be possible without a strong bonding between bacterial cells and the substrate surface. Many researchers have reported on similar investigations into PET biodegradation utilizing various microorganisms. Gao et al.²² used transcriptomic methods to investigate the PET and PE degradation processes by marine bacterial strains. The study found that *Exiguobacterium sp.*, *Halomonas sp.*, and *Ochrobactrum sp.* collectively degraded PET and PE films faster than single isolates. Janczak et al.²³ examine the biodegradation of two types of polymer PET and polylactide(PLA) using different microorganisms. They concluded the biodegradation of the analyzed sheets was most rapid in the presence of the *S. plymuthica* and *L. laccata* strains. Taniguchi et al.²⁴ reported PET degradation by a microbial consortium and its bacterial resident, *Ideonella sakaiensis* and elucidated the mechanism of PET degradation into simple monomers by PET hydrolase and mono(2-hydroxyethyl) terephthalic acid (MHET) hydrolase from *I. sakaiensis*.

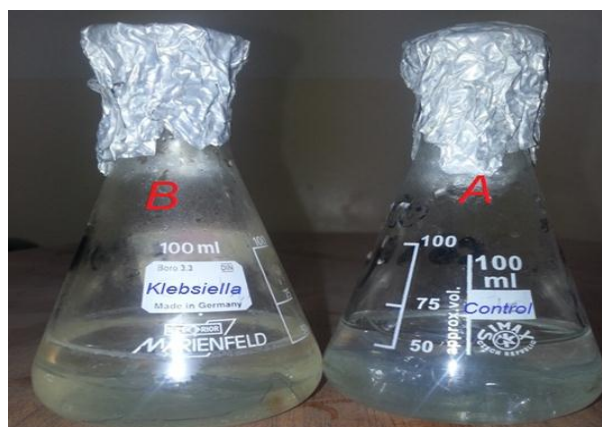


Figure 2: The different in degradation between, A: control, B : *K. pneumoniae*

Table 2: PET Degradation by *K. Pneumoniae* ($p < 0.01$ and $LSD = 0.28000$)

Time (days)	Weight(gm) of PET before degradation	Weight (gm)of PET after degradation with <i>K. pneumoniae</i>	%PET degradation	Weight(gm) of PET in control sample
7	0.600	0.456	24	0.581
14	0.600	0.401	33	0.580
21	0.600	0.387	35	0.578
28	0.600	0.321	46	0.577

Polymers are implicit substrates on which microorganisms can grow. The degradation of polymers by microbes is influenced by many factors that involve: the pretreatment nature, characteristics of polymer (tacticity, molecular weight, mobility, substituents type), and presence of particular bacteria on the surface of

materials^{25,26}. The biodegradation of the polymer chain into oligomers and monomers is aided by microbial specific enzymes and the process depends on the existence of polymer sites for enzyme attack. After the enzyme binds to the polymer surface, a hydrolytic cleavage is initiated in the polymer chain, resulting in water-soluble compounds that are absorbed by microbial cells and used in their metabolism.

The degradation via creating grooves is the main process for *Klebsiella pneumoniae* enzymes, which include: peroxidase, laccase, tyrosinase, and lipase. Also, the produces small chain polymers act as surfactants that facilitate the entry of organisms into the polymer²⁷⁻²⁸. The appropriate compatibility between the enzyme structure and polymer chain is necessary for PET hydrolysis to be successful²⁹. A crucial step in the hydrolysis of PET and any polymer is enzyme adsorption, in which the enzyme clings to the polymer surface. This binding happens when any hydrophobic amino acid moieties come into contact with hydrophobic PET fibers³⁰. According to numerous reports, PET hydrolase enzymes preferentially degrade PET's amorphous regions and what facilitates the process is the presence of easy-to-break ester bonds in PET that can be cleaved by a variety of hydrolytic enzymes³¹⁻³⁴. The enzymatic mechanism of PET biodegradation includes its conversion to mono(2-hydroxyethyl) terephthalic acid (MHET), with small amounts of bis(2-hydroxyethyl)-terephthalic acid and terephthalic acid (TPA) at the first step. In the second step, the MHET breaks down into the two monomers, terephthalic acid and ethylene glycol (EG). Many organisms use EG as a source of carbon and energy^{35,36}.

To determine the optimum pH and temperature requisite for *K. pneumoniae* growth on PET film, MS medium was prepared at different pH levels ranging from 4 to 10 at 37°C, and isolates were incubated at various temperatures ranging from 15 to 40°C, respectively. The results for both cases were significantly different with $p < 0.01$. The optimum temperature of the tested *K. pneumoniae* was 37°C Fig.3, whereas this temperature was necessary for the highest growth of isolates after 7 days, and 15°C recorded the lowest growth for isolates. At low temperatures, there is little growth due to the stiffening of the membrane lipids and that decreases the efficiency of membrane transport and respiratory proteins³⁷. The population in the bacterial culture was influenced by high temperatures. The results showed the bacterial isolates gradually lost their growth at increasing temperatures. This was likely caused by a deficiency in nutrients and oxygen or by an amassing of metabolites created by the bacteria's oxidation processes³⁸. From the results in Fig.4 the highest growth for *K. pneumoniae* in pH 7-8. pH has the most noticeable effects on bacterial enzymes, and bacteria are killed by extreme changes in the pH balance³⁹. Han et al.⁴⁰ obtained approach results, as the optimal temperature was 30°C, whereas the optimum pH was 9.0 for PET film degradation.

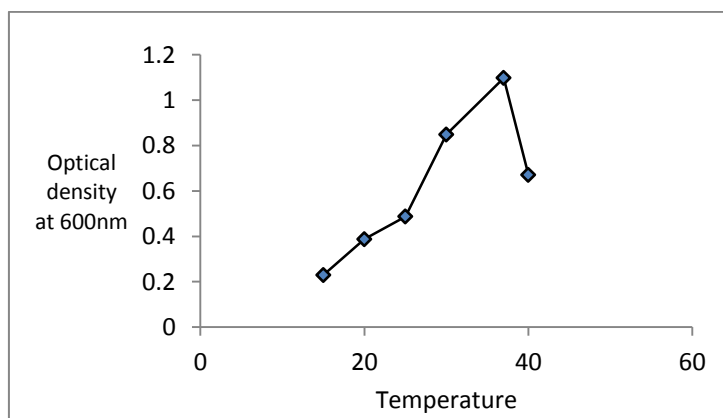


Figure 3: Effect of temperature on PET degradation by *K. pneumoniae* after 7 days

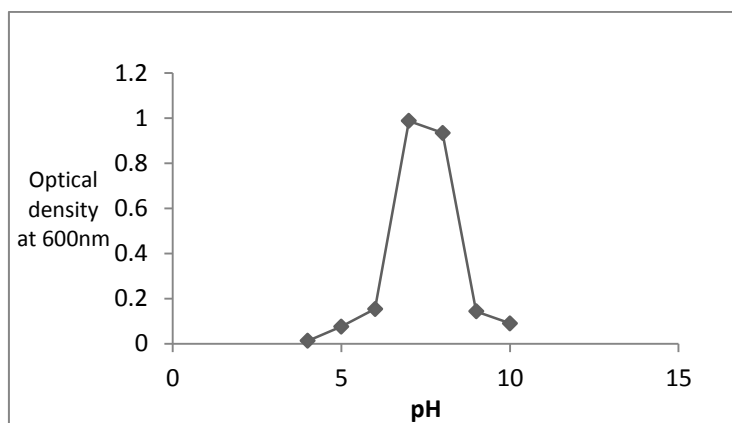


Figure 4: Effect of pH on PET degradation by *K. pneumoniae* after 7days

Conclusion

Biodegradation can be the best strategy to overcome the plastic environmental pollution problem. The goal of this study was to isolate the *Klebsiella pneumoniae* from Shatt Al-Arab and sewage water of Basrah city and test the ability of this bacteria to degrade PET plastic. The biodegradation of the polymer was inferred by the weight difference, as the weight altered from 24% in the first week to 46% in the fourth week. This is in reference to the use of polymer as a source of carbon by isolated bacteria. The research also included studying the optimum temperature and pH, which recorded the highest bacterial growth. The findings showed that 37°C and pH 7-8 were the best conditions for growth. The result inferred that *Klebsiella pneumoniae* can be used for PET degradation as a safe and environmentally friendly assay at optimum conditions.

Authors' declaration:

-Conflicts of Interest: None.

-We hereby confirm that all the Figures and Tables in the manuscript are mine ours. Besides, the Figures and images, which are not mine ours, have been given the permission for re-publication attached with the manuscript.

-Ethical Clearance: The project was approved by the local ethical committee in University of Basrah.

Authors' contributions:

E.M., H.A., and S.A. contributed to the design and implementation of the research, to the analysis of the results and to the writing of the manuscript.

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