

Isolation, Screening and Identification of Low Density Polyethylene (LDPE) degrading bacteria from contaminated soil with plastic wastes

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Abstract

One hundred and sixty nine bacterial isolates were isolated from 64 soil and plastic waste samples from different municipal landfill regions in Baghdad city. Primary screening has been done for the bacterial isolates depending on bacterial growth (OD_{600nm}) in low density polyethylene solid and liquid medium. Forty two isolates show high efficient to degrade LDPE powder, then secondary screening has been done depending on bacterial growth, color change in solid medium, and change in pH medium. Bacterial isolates Mw43, Tw53 and Shw51 were given high ability to degrade LDPE strips. These three isolates have been identified as *Pseudomonas fluorescens*, *Pseudomonas aeruginosa* and *Acinetobacter ursingii*, respectively.

Keywords; Biodegradation, LDPE, Plastic waste, *Pseudomonas*

Introduction

During the past 3-decades, plastic materials have been increasingly used in food clothing, shelter, transportation, construction, medical, and recreation industries. Plastics are advantageous as they are strong, light-weighted, and durable. However, they are disadvantageous as they are resistant to biodegradation, leading to pollution, harmful to the natural environment. Increasing environmental

pollution and waste that cannot be renewed and degrade it encourages research and studies in the field of biosynthetic and biodegradation material. One of the wastes that cannot be destroyed is plastic waste, which is a type of polyethylene plastic.

Low density polyethylene is one of the major sources of environmental pollution. Polyethylene is a polymer made of long chains of ethylene monomers. The use of polyethylene growing worldwide at a rate of 12% per year and about 140 million tons of synthetic polymers are produced worldwide each year. With such a large amount of polyethylene gets accumulated in the environment, generating plastic waste ecological problems are needed thousands of years to efficiently degradation [1].

Microorganisms can degrade plastic over 90 genera, from bacteria and fungi, among them; *Bacillus megaterium*, *Pseudomonas* sp., *Azotobacter*, *Ralstonia eutropha*, *Halomonas* sp., etc. [2] Plastic degradation by microbes due to the activity of certain enzymes that cause cleavage of the polymer chains into monomers and oligomers. Plastic that has been enzymatically broken down further absorbed by the microbial cells to be metabolized. Aerobic metabolism produces carbon dioxide and water. Instead of anaerobic metabolism produces carbon dioxide, water, and methane as end products [1].

This study aims to isolate, screening and identification the bacteria from contaminated soil with plastic waste that can degrade low density polyethylene (LDPE).

Materials and methods

Polyethylene (PE)

Low density polyethylene powder (LDPE) was obtained from BDH (England). Low density polyethylene strips (plastic bags) used in this study were obtained from local markets. LDPE were cut into small pieces of about (5cm ×1cm), washed with 70% ethanol for 30 min, then washed with distilled water, and air dried for 15 minutes in laminar air flow chamber and was added to the medium.

Sample collection

Sixty four of soil and plastic waste samples were collected from four waste disposal sites dumped with polyethylene bags and plastic waste located at Baghdad, two sites located in Al-Kharkh and another sites located in Al-Rousafaat different periods. The samples were collected randomly from the superficial layer of soil (5-10cm) in depth, using pre-sterilized spatula and were transferred into sterile plastic bags and stored at 4 °C till use.

Isolation of low density polyethylene degrading bacteria.

One gram of soil and plastic waste sample was suspended in 50 ml of sterile distilled water and the suspension was incubated in shaker incubator at 37°C for 30 min at 150 rpm, then 0.1ml from different suspensions were spread directly on the surface of nutrient agar plates, then the plates were incubated at 37°C for 24h.[3,4].

Screening of LDPE degrading bacteria.

Primary screening

A) In liquid medium

To screen the LDPE degrading bacteria, loop full from each bacterial isolate was cultured for 2 days in liquid MSM supplemented with glucose 0.1% as carbon source and incubated in shaker incubator 150 rpm at 30°C.

Twenty- five ml of liquid MSM, was dispensed in 100 ml Erlenmeyer flasks and supplemented with 0.1% LDPE powder as a substrate and sole source of carbon .Flasks were sterilized by autoclave, then 1 ml from each liquid MSM supplemented with glucose flask was used as inoculums for each Erlenmeyer flasks with control (flasks with 1ml of sterile distill water)[5]. All flasks were incubated in shaker incubator 150 rpm at 30°C for 7 days. The bacterial growth of isolates was determinate at 600nm by measuring the OD using U.V-visible spectrophotometer.

B) In solid medium

Solid MSM supplemented with 0.1% (wt/v) of LDPE powder was used to detect the ability of LDPE degrading isolates.

The plates were inoculated with loop full from each bacterial isolate in the middle of the agar plate; all plates were incubated at 30°C for 10 days. After period of incubation the diameter of bacterial growth for each isolate was determined [5, 1].

Secondary screening

The most active degrading isolate sobtained from primary screening were further screened for LDPE degradation rates by sequence of events which were assessed by two methods.

A) Clear zone assay

LDPE-emulsified agar plates inoculated with loop full from each pure bacterial isolate and spreading over a 1 cm area on the middle of the agar plates; all plates were incubated at 30°C for 3 days, the diameter of color zone change around colonies growth were determined[6]. Bacterial isolates which gave maximum diameter were selected for further screening of biodegradation rates.

B) Determination of bacterial growth

Twenty- five ml of liquid MSM was dispensed in 100 ml Erlenmeyer flasks and supplemented with LDPE strips (5cm×1cm) as a substrate and sole source of carbon .Flasks were sterilized by autoclave, then inoculated with 1ml from 24h. older bacterial culture of isolates in liquid MSM with 0.1% glucose with control (medium inoculated with 1ml distilled water. The flasks were incubated for 7 days at 30°C, 150 rpm. The OD at 600 nm was recorded using UV-VIS spectrophotometer [7]

C) Determination of pH change

Study of pH change was adopted to make sure any metabolic activity of bacterial isolates in supplemented medium, as metabolism shown by microbial cells may greatly support the evidence of degradation. The pH of the medium inoculated with bacterial isolates was measured daily during the study.

Twenty- five ml of liquid MSM was dispensed in 100ml Erlenmeyer flasks and supplemented with LDPE strips as a substrate and sole source of carbon. Flasks were sterilized by autoclave, then inoculated with 1ml from 24h. older bacterial culture of isolates in liquid MSM with 0.1% glucose with control (medium inoculated with 1ml distilled water). The flasks were incubated for 7 days at 30°C, 150 rpm.

The pH probe was inserted in the medium to measure the pH. Initial pH of the medium was ensured to be 7.0 ± 0.2 . The medium uninoculated with bacteria served as negative control.

D) Identification of LDPE degrading isolates.

The most active bacterial isolates were identified according to the cell morphology, arrangement and Gram stain reaction as described in Bergey's Manual of Determinative Bacteriology [8].

Biochemical properties include, indole, methyl red test, Voges Proskauer test, citrate utilization test, starch utilization test, catalase, oxidase, urease producing, gelatin utilization test , sugars fermentation tests , motility test and pyocyanin pigment production[5,8].

Identification with VITIK 2 system was performed with ID-GNB cards, according to the manufacturer's instructions [9].

Results and Discussion

Isolation of LDPE degrading bacteria

As domestic and industrial waste containing huge amount of low density polyethylene, in this study municipal landfill solid waste samples have been collected in order to isolate the bacterial isolates which show potent biodegradation.

One - hundred and sixty nine (169) bacterial isolates were obtained from 64 soil and plastic waste samples from different municipal landfill regions (Al- Shaab, Al-Sader, Al-Karkh and Al-Mansour) in Baghdad city at different periods from March 2013 to May 2013, as shown in table (1).

The results in table (1) were shown variation in isolates number which were obtained from each sample and this was depending on the place and period of samples collection. These results also show that the highest number of isolates was collected from Al-Shaab region (22 bacterial isolates). It is found that the best bacterial isolation was occurred on April (74 isolates), which is might be due to suitable environmental conditions.

Different bacterial isolates were isolated from soil and plastic waste samples. According to the results of this study, the plastic waste samples were considered as a good source for bacterial isolates capable to utilize LDPE in soil contaminated sites.

Screening of LDPE degrading bacteria

A) Primary screening

1- On solid medium

Biodegradation is a favorable solution of plastic disposal which is accumulation problem in nature. All the 169 isolates were screened for their ability to degrade LDPE according to grow on solid MSM with 0.1 % (wt/v) LDPE powder. The efficiency to degrade has been recorded depending on the diameter growth of bacterial colonies as shown in table (2).

Results in table (2) show that 41 isolates give strong growth with a diameter from 2.5 - 3.0 cm as illustrated in figure (1). Growth was started within 4-5 days at respective incubation temperatures. Initially an opaque zone was observed around the colony, however slowly growth zone formed within 10 days at 30°C. Augusta *et al.* [10], have reported that the zone of clearance around the colony is due to extracellular hydrolyzing enzymes secreted by the target organism into suspended polyesters agar medium. All microbes involved in forming a growth zone were selected for further studied. All minerals were supplied along with polyethylene powder as carbon source for the growth of the microorganism.

Tokiwa *et al.*, [11] and Usha *et al.*, (2011) mentioned that the ability of bacterial isolates to utilize LDPE was estimated by growth ability on solid MSM supplemented with 0.1% of LDPE powder.

2- In liquid medium

Forty-one isolates from 169 LDPE degrading bacteria which shown the highest ability for LDPE biodegradation were examined their ability to grown in liquid MSM with 0.1% LDPE powder, the growth density at 600nm along seven days of incubation was determined. Table (3). The growth of microorganisms and fungi various nutritional factors are required, which includes carbon, nitrogen, phosphorous and other mineral sources. Carbon source plays important role for the growth of the microorganisms and fungi. The growth of the microorganisms and fungi are ensured by the increase in optical density of the culture.

B) Secondary screening

1- Color zone assay

The efficiency of LDPE degradation was determined by the color zone formed on solid LDPE-emulsified plates. Bromocresol purple indicator was used to detect any change in the pH of the medium. The control plates were purple in color, however, LDPE- emulsified agar plates turned to sea-green after plating probably as a result of slight increase in acidity of bacterial isolates on carbon free media with polyethylene as a sole source of carbon and production of coloring zone around the microbial colonies on solid medium were regarded as evidence of ability to utilize LDPE.

2- Bacterial growth in liquid medium

In an attempt to investigate the ability of isolates to degrade LDPE strips, the efficient forty two isolates that showed potential ability to grow on LDPE powder chose to grow on LDPE strip as a sole source of carbon. Table (5) shown the bacterial ability to grow on LDPE strip.

Results in table (5) show that SHw43, Tw53 and Mw51 have high growth. The OD at 600nm was 0.219 for Shw43 and 0.205, 0.195 for Tw53 and Mw51 respectively after 7 days, 150rpm at 30°C. These isolates have been showed respectable density growth compare with other isolates throughout the incubation period, which may be attributed to physiological and genetic properties of these isolates.

Microorganisms utilize PE strip as a sole source of carbon resulting in partial degradation of PE and plastics. They colonize the surface of the PE strip or plastics forming biofilm [12]

3- pH change

Study of pH changes is adopted to make sure any metabolic activity of the bacterial isolates in supplemented medium, as metabolism shown by microbial cells may greatly support the evidence of

degradation. Table (6) shows the variation in pH of the medium during this biodegradation study. From ten efficient LDPE strips degrading bacteria, three isolates SHw43, Tw53 and Mw51 were appeared to decrease in pH medium of value of 6.41, 6.45 and 6.52 respectively. It is found that pH value of medium inoculated with SHw43 was lowest than other media which inoculated with the other isolates.

Microorganisms secrete a variety of enzymes into the media, which begin the breakdown of the polymers. Two types of enzymes are involved in the process, namely intracellular and extracellular depolymerases. Exoenzymes from the microorganisms first breakdown the complex polymers giving short chains or monomers that are small enough to permeate through the cell walls to be utilized as carbon and energy sources by a process of depolymerization [13]. The similar results on *Bacillus amyloliquefaciens* have been also reported by [14].

Identification of bacterial isolates

Three isolates were selected according to their ability to degrade LDPE strip as mentioned above. These isolates were identified depending on morphological, cultural and biochemical characteristics. Bacterial isolates revealed different colonial appearance depending on the selective or differential used media.

Morphological and microscopic observations appeared that the three bacterial isolates were Gram negative, non-spore former, non-lactose-fermenter, one of them was coccobacilli shape and usually arrangement with pairs, sometimes it could be single. It may also take short chain with different lengths, non-motile, while others isolates were rod shape bacilli, motile. Morphological results are shown in table (7), while biochemical tests are shown in table (8).

Results show that these isolates SHw43, Tw53 and Mw51 are identified as a *Pseudomonas fluorescens*; *Pseudomonas aeruginosa* and *Acinetobacter ursingii*, respectively, all of them are mesophilic strains. The morphological and biochemical results for these isolates are in good agreement with that reported by Holt *et al.* (1994).

Burd [15] reported that most of the PE degrading isolates were identified as Gram negative and belong to genus *Pseudomonas* and *Sphingomonas* on the basis of their phenotypic characteristics, while [16], identified *Pseudomonas* sp. which would efficiently degrade LDPE and able to utilize it as a sole carbon source for its survival. *Pseudomonas* isolated from sewage sludge dump was found to degrade both natural and synthetic polyethylene very efficiently [17].

Vitek 2 which is the powerful technique to identify the bacterial isolates has been used to confirm the conventional diagnosis. The results of present study were in good agreement with that obtained using biochemical technique. The three isolates SHw43, Tw53 and Mw51 were identified as *Pseudomonas fluorescens* (93% probability), *Pseudomonas aeruginosa* (99%) and *Acinetobacter ursingii* (99%), respectively. To solve any discrepancies between the Vitek-2 direct and standard method, phenotypic assays were performed. The Vitek-2 system integrates several advantages that may be of clinical interest for routine testing of Gram negative bacilli isolated from the samples: Rapid

identification (three hrs.), a high level of automation, a simple methodology and taxonomically updated databases [18]. Vitek-2 system correctly identified 85.3 to 100% of enterobacteriaceae. Dina and Rania, (2014) have used the Vitek-2 system, where 165 strains out of total 170 strains have been identified correctly (with percentage 97.05%). One strain (0.6%) remained unidentified while 4 strains (2.35%) were misidentified.

Table (1): LDPE degrading bacterial isolates which isolated from plastic waste and contaminated soil with plastic waste.

No.	Sample sites	Samples collecting date	No. of bacterial isolates	Symbol
1	Al- Shaab	19/3/2013	8	Shs3
			10	Shw3
2	Al-Sader	19/3/2013	6	Ts3
			8	Tw3
3	Al-Karkh	31/3/2013	5	Ks3
			8	Kw3
4	Al-Mansour	31/3/2013	5	Ms4
			7	Mw4
5	Al-Shaab	14/4/2013	10	Shs4
			12	Shw4
6	Al-Sader	14/4/2013	6	Ts4
			12	Tw4
7	Al- Karkh	28/4/2013	7	Ks4
			9	Kw4
8	Al-Mansour	28/4/2013	8	Ms5
			10	Mw5
9	Al-Shaab	12/5/2013	5	Shs5
			6	Shw5
10	Al-Sader	12/5/2013	4	Ts5
			6	Tw5
11	Al- Karkh	26/5/2013	3	Ks5
			5	Kw5
12	Al-Mansour	26/5/2013	4	Ms5
			5	Mw5
			Σ=169 isolates	

Table (2) :Primary screening of LDPE degrading bacterial isolates, using solid mineral salt media with 0.1% (wt/v)LDPE powder, pH7 at 30°C for 10 days.

No.	No. of bacterial isolates	Growth ability of bacterial isolates*			ND
		Strong	Moderate	Weak	
1	41	+++			
2	30		++		
3	45			+	
4	53				-

*The ability of bacterial isolates were measured depending on the diameter of bacterial colonies developing as the following:

Strong = diameter of bacterial colonies ≥ 2.5

Moderate = diameter of bacterial colonies between 1.0 –2.5

Weak = diameter of bacterial colonies ≤ 1.0

ND = Growth not detect

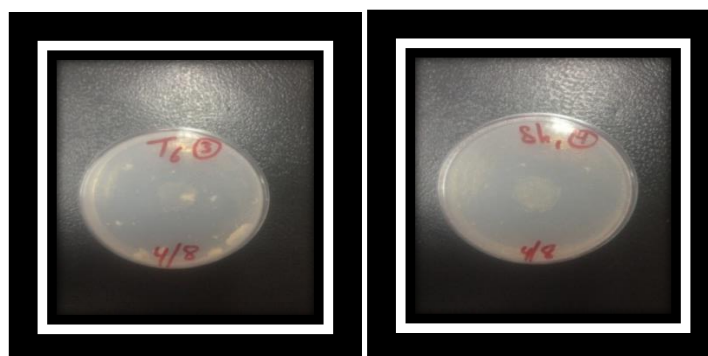


Figure 1:Bacterial growth of some isolates on solid MSMwith 0.1% (wt/v) LDPE, pH7 after 10 day of incubation at 30°C.

Table 3:Primary screening of LDPE degrading bacterial isolates, using liquid mineral salt mediumwith 0.1% (wt/v) LDPE powder, pH7, 150 rpm at 30°C for 7 days.

No.	Bacterial growth at 600nm	No. of bacterial isolates
1	Good*	41
2	Moderate**	30
3	Weak***	45
	ND****	53

*Optical density of bacteria growth ≥ 0.1

** Optical density for bacteria growth between 0.05 – 0.1

*** Optical density for bacteria growth < 0.05

****Growth no detected

Table(4) : Secondary screening for selected bacterial isolates using bromocresol purple indicator in solid mineral salt medium with LDPE, plates were incubated at 30°C for 3 days.

No.	Bacterial isolates	Diameter of color zone(cm)	No.	Bacterial isolates	Diameter of color zone(cm)
1	Shs31	2.1	22	Shw41	2.3
2	Ts31	2.1	23	Shw42	2.1
3	Ks31	2.2	24	Shw43	2.4
4	Ms31	2.2	25	Shw44	2.5
5	Shw31	2.7	26	Tw41	2.3
6	Shw32	2.3	27	Tw42	2.4
7	Shw33	2.2	28	Tw43	2.7
8	Tw31	1.5	29	Tw44	2.7
9	Kw31	2.3	30	Kw41	2.1
10	Kw33	2.2	31	Kw42	2.7
11	Mw31	2.3	32	Kw43	2.0
12	Mw32	2.5	33	Kw44	2.7
13	Mw33	2.5	34	Mw41	2.4
14	Shs41	2.3	35	Mw42	1.5
15	Shs42	2.5	36	Shs51	2.4
16	Ts41	2.5	37	Ks52	1.5
17	Ts42	2.7	38	Mw51	2.9
18	Ks41	2.7	39	Tw51	1.6
19	Ks42	2.0	40	Tw53	3.0
20	Shw43	3.2	41	Kw52	2.0
21	Ms42	2.7			

Table 5: Secondary screening of selected bacterial isolates using optical density as a bacterial growth (O.D) in liquid mineral salt medium, 150 rpm at 30°C for 7 days.

No.	Bacterial isolates	Optical density of bacterial growth	No.	Bacterial isolates	Optical density of bacterial growth
1	Shs31	0.122	22	Shw41	0.143
2	Ts31	0.124	23	Shw42	0.139
3	Ks31	0.130	24	Shw43	0.155
4	Ms31	0.129	25	Shw44	0.162
5	Shw31	0.181	26	Tw41	0.151
6	Shw32	0.156	27	Tw42	0.154
7	Shw33	0.144	28	Tw43	0.175
8	Tw31	0.139	29	Tw44	0.178
9	Kw31	0.142	30	Kw41	0.167
10	Kw33	0.129	31	Kw42	0.179
11	Mw31	0.149	32	Kw43	0.143
12	Mw32	0.163	33	Kw44	0.176
13	Mw33	0.136	34	Mw41	0.165
14	Shs41	0.139	35	Mw42	0.122
15	Shs42	0.148	36	Shs51	0.156
16	Ts41	0.165	37	Ks52	0.123
17	Ts42	0.172	38	Mw51	0.195
18	Ks41	0.167	39	Tw51	0.120
19	Ks42	0.119	40	Tw53	0.205
20	Shw43	0.219	41	Kw52	0.119
21	Ms42	0.177			

Table 6 :Changes in pH values of liquid MSM

No.	Bacterial isolates	Values of pH medium
	Control	7.0
1	Shw43	6.41
2	Tw53	6.45
3	Mw51	6.52
4	Kw42	6.78
5	Shw31	6.74
6	Kw44	6.76
7	Tw43	6.89
8	Tw44	6.67
9	Ms42	6.72
10	Ts42	6.92

Table (7):Morphological characteristics of bacterial isolates.

Morphological test name	Code number of bacterial isolates		
	Shw43	Tw53	Mw51
Colony configuration	Circular	Circular	Circular
Colony margin	Entire	Wavy	Entire
Colony elevation	Raised	Flat	Raised
Colony surface	Smooth	Smooth	Convex
Spore location	-	-	-
Motility test	Motile	Motile	Non motile
Pigment production	Blue green color	Green pigment color	Cream
Gram reaction	-	-	-
Cell shape	Rod	Rod	Coccobacilli

+ positive results , - negative results

Table (8):Biochemical characteristics of bacterial isolates.

Biochemical test name	Code number of bacterial isolates		
	Shw43	Tw53	Mw51
Catalase test	+	+	+
Oxidase test	+	+	-
Methyl red	-	-	-
Voges – Proskauer test	-	-	-
Indol test	-	-	-
Gelatin hydrolysis	+	+	-
Starch hydrolysis	-	-	-
Urease	+	-	-
Citrate utilization	+	+	+
Glucose fermentation	+	+	+
Sucrose	+	-	-
Lactose	-	-	-

+ positive results , - negative results

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