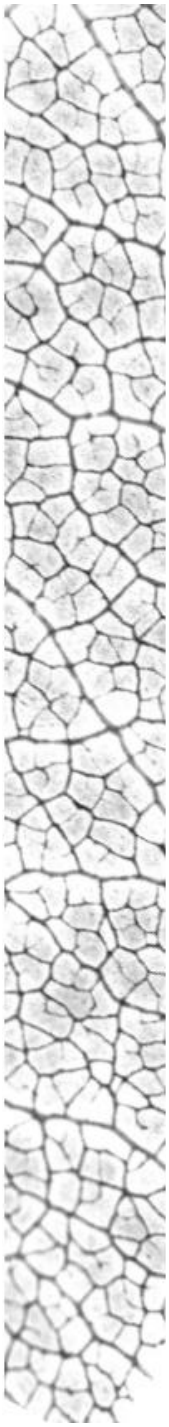

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Study of gentamicin combined with polyhydroxyalkanoates extracted from lactobacillus plantarum

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Abstract

Background: Polyhydroxyalkanoates (PHAs) are natural bio-polymers, which are formed by many micro-organisms in the context of a carbon and energy reservoir. PHAs are mainly dependent on the number of carbons and their molecular weight in the monomer unit. PHAs are biomedical products due to their biocompatible, biodegradable or non-toxic effects. **Methodology:** Nine bacterial isolates were isolated from oil-contaminated soil samples in different locations of Babylon province were diagnosed to Pseudomonas spp via traditional methods. In addition to three isolates of Lactobacillus bacteria obtained from the Advanced Microbiology Laboratory/ College of Science/ Babylon University, they were isolated from dairy. **Results:** all isolates were screened for PHA production by Sudan black B and Nile blue A. six isolates of Pseudomonas spp. and three of Lactobacillus spp. were shown positive for the production of PHA. After primary screening the polymer was extracted by sodium hypochlorite and chloroform. The best producing polymer in 3.4% from cell dry weight was found to be Lactobacillus spp1 was identified and characterized by VITEK 2 compact device; the result showed 87% identical to Lactobacillus plantarum. Fourier transform infrared spectroscopy FTIR was used to show the functional group of extracted polymer from L. plantarum, FT-IR was used to show the functional groups of PHA samples were identified as C-O, C-H and C=O. The FT-IR spectra of PHA containing carbonyl group (C=O) occurred in a strong band at 1739.85cm^{-1}. The solubility of PHA was also measured in several solvents. The results showed that the polymer was well dissolved by chloroform and Dimethyl sulfoxide. The polymer produced from L. plantarum was combined with Gentamicin was added to obtain a 5:1 or 10:1 (w/w) Gentamicin content to improve the performance improvement of Gentamicin against bacterial biofilm. Diagnosed pathogenic bacteria were then obtained from hospitals in Babylon province for later use in Gentamicin and PHA loaded Gentamicin running tests. The minimum inhibitory concentration MIC of the Gentamicin concentration was 0.2 mg/ml. The antibacterial activity of Gentamicin, PHA loaded Gentamicin (5:1 or 10:1) and PHA was studied against two types of gram-positive (Enterococcus spp) and gram-negative (K. pneumoniae) bacteria; the results show the effect of PHA loaded Gentamicin (5:1 or 10:1) stronger than Gentamicin against pathogenic bacteria, while there is not any effect for the alone polymer.The biofilm formation was studied on a wavelength of 630 nm via the ELISA reader and it was found that both isolates can form biofilms. **Conclusion:** The study concluded that Polyhydroxyalkanoate loaded Gentamicin (1:5) was stronger than Gentamicin and Polyhydroxyalkanoate loaded Gentamicin (1:10) against the biofilm formation.

Keywords: Lactobacillus plantarum, Polyhydroxyalkanoate (PHA), gentamicin, chloroform, biofilm

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INTRODUCTION

Polyhydroxyalkanoates (PHAs) are natural bio-polymers, which are formed by many micro-organisms in the context of a carbon and energy reservoir. PHA synthesis generally occurs during fermentation under nutrient limiting conditions with excess carbon. There are two main types of PHAs, short-chain length PHAs (scl-PHAs) and medium-chain length PHAs (mcl-PHAs). The mechanical and thermal properties of PHAs depend mainly on the number of carbons in the monomer unit and its molecular weight. PHAs are promising materials for biomedical applications because they are biodegradable, non-toxic and biocompatible (Nigmatullin, et al. 2015, Poltronieri, & Kumar, 2017) Extensive usage of the synthetic polymers (plastics) and their non-biodegradable nature has led to their accumulation in quantities, which are difficult to manage

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Table 1. Bacterial Isolates Used for PHA Production

Bacteria	Isolation site	Gram stain
Lactobacillus spp1	Dairy products	G+ve rod
Lactobacillus spp2	Dairy products	G+ve rod
Lactobacillus spp3	Dairy products	G+ve rod
Pseudomonas spp.1	Soil	G-ve rod
Pseudomonas spp.2	Soil	G-ve rod
Pseudomonas spp.3	Soil	G-ve rod
Pseudomonas spp.4	Soil	G-ve rod
Pseudomonas spp.5	Soil	G-ve rod
Pseudomonas spp.6	Soil	G-ve rod
Pseudomonas spp.7	Soil	G-ve rod
Pseudomonas spp.8	Soil	G-ve rod
Pseudomonas spp.9	Soil	G-ve rod

and a major cause of environmental pollution. Bacteria have the ability to accumulate Carbon (C) as biopolymers especially under stress conditions. The biopolymers polyhydroxyalkanotes (PHAs) are biodegradable and have properties quite close to those possessed by plastics (Kalia, et al. 2019). The use of PHAs as biodegradable polymers has gained attention because of their biological (microbial) origin and non-toxic nature compared to synthetic plastics, which may be toxic (Koller, et al. 2017). The resistance to antibiotics is defined as the ability of bacteria causing the disease to resist the therapeutic effects of antibacterial drugs. The danger of antibiotic resistance comes from; it resulted in enormous human and economic losses. About 700,000 people have died each year worldwide thanks to the inappropriate antibiotic usage that develops resistance to conventional therapy (Koller, et al. 2017). Bacterial biofilms are serious global health concern due to their abilities to tolerate antibiotics, host defence systems and other external stresses; therefore it contributes to persistent chronic infections (De la Fuente-Núñez, et al. 2013). To enhance the efficacy and action of gentamicin against pathogenic bacteria and the formation of biofilms, Polyhydroxyalkanoate was combined with gentamicin.

MATERIAL AND METHODS

Collection of Samples

Isolation of Bacteria from soil

To obtain isolates of bacteria from soil samples, 1 gm of soil sample was added to 99 ml of sterile distilled water and stirred. After a serial dilution of each sample (10^{-1}, 10^{-2}, 10^{-6}) transfer 0.1ml of 10^{-5} and 10^{-6} dilutions to nutrient agar plates, spread then incubate at 37^0C for 24 hours. The pure colonies of each sample were picked up and sub-cultured to obtained pure culture and kept for the screening of PHA production (Sao, 2017).

Isolates of bacteria

Nine bacterial isolates were isolated from oil-contaminated soils in different location of Babylon province/Iraq were diagnosed to *Pseudomonas spp* via traditional methods. In addition to three isolates of *Lactobacillus* bacteria obtained from the Advanced Microbiology Laboratory/ College of Science/ Babylon University isolated from dairy in order to isolate PHA-producing bacteria as shown in **Table 1**.

Table 2. Bacterial Isolates Used in Extracted PHA Tests

Bacteria	Infection site	Isolation site	Gram stain
Klebsiella pneumoniae	Urinary tract	Al-Sadiq Hospital	G-ve
Enterococcus spp	Urinary tract	Marjan Hospital	G+ve

Isolates of pathogenic bacteria

During the research period, diagnosed bacterial isolates were obtained from the hospitals that were later used in PHA experiments as in **Table 2**.

Primary Screening of Polyhydroxyalkanoates

Screening by Sudan black B Stain

Several drops of microbial broth were fixed on a glass slide by applying heat and then stained with a 3% Sudan Black B (w/v in 70% ethanol) solution for 10 minutes. The slide was then immersed in xylene until completely decolonized. The sample was counterstained with safranin (5% w/v in distilled water) for 10 seconds, washed again with distilled water, and dried. After adding several drops of immersion oil directly to the completely dry slide, the cells were examined by optical microscopy (Dhingra, & Priya, 2013).

Screening by Nile blue A Stain

The bacterial cells cultured in MRS media were smeared, fixed by heating on a glass slide. At first, flooded by the Nile Blue A solution 1%. Incubated in a water bath at 55 °C for 10 minutes and rinsed with tap water. Then rinsed slide with 8% of the acetic acid solution for 1 minute to remove the unbound stain, and rinsed with tap water. A few drops of immersion oil were added directly on the completely dry slide, and the cells were examined by fluorescence microscope and observed with blue excitation wavelength (Legat, et al. 2010).

Extraction of Polyhydroxyalkanoates

The positive isolates from the Sudan Black B and Nile Blue A staining were further quantified for PHA accumulation. The isolates were grown in 250 ml flask containing 50 ml sterile modified MRS medium. The flasks were inoculated with 2.5% (v/v) (McFarland = 0.5) overnight grown culture and were incubated at 37ºC for 48 hr. The biopolymer produced was then extracted by the modified Hypochlorite method (Rawte, & Mavinkurve, 2002). The grown culture was centrifuged at 6,000 RPM for 20 min. The supernatant was discarded and the pellet was treated with 10 ml sodium hypochlorite and the mixture was incubated at 30 °C for 1 h. The mixture was centrifuged at 6000 RPM for 20 min and then rinsed with distilled water, acetone, and methanol respectively. The pellet was dissolved in 5 ml boiling chloroform and evaporated by pouring the solution on a sterile glass tray kept at 4 °C and weighed (Getachew, & Woldesenbet, 2016). The relative PHA accumulation by the different isolates was compared to help in the identification of the best producer. The

percentage of PHA accumulation was estimated as the percentage composition of PHA present in the dry cell weight, which was calculated using the following formula:

PHA accumulation (%) = [Dry weight of extracted PHA (g/L) / cell dry weight (g/L)] ×100

Measurement of cell dry weight (CDW)

The sample (10 mL of culture suspension) was centrifuged in pre-dried and pre-weighted tubes at 10000 rpm for 5 min. The pellet (wet cells) was resuspended in distilled water and centrifuged. The washed cells were dried in hot air at 95°C for at least 24 hours and then a CDW measurement was taken (Altaee, et al. 2016).

Fourier Transform Infrared Spectroscopy (FTIR)

The chloroform extract of PHA 4 mg was mixed thoroughly with potassium bromide KBr (Spectroscopic grade) and dried at 100 ^{o}C for 4 h. Infrared spectra of the PHA sample was recorded and analyzed on a single beam Perkin Elmer device with the following scan parameters: Scan in the range of 4000–400 cm^{-1} number of scans, 16 and resolution, 4.0 cm^{-1} (Rawte, & Mavinkurve, 2002, Daly, 2018).

Determination the Solubility of PHA

Dissolved 0.05 gram of PHA in 10 ml of chloroform, Dimethylsulfoxide, acetone, methanol, ethanol and water, and observing the solubility of the polymer (Terada, & Marchessault, 1999).

Polyhydroxyalkanoate (PHA) / Gentamicin Combination

A gram of PHA that thawed by chloroform. A suitable amount of gentamicin was added to obtain a 5:1 or 10:1 (w/w) gentamicin content. The mixture was incubated at 55◦C in a shaking water bath and vortexed until fully homogeneous. The PHA/gentamicin mixture was then poured into a metal mould. The discs were coated and dried for 24h. (Rossi, et al. 2004; Nurfitriani, & Hindersah, 2015).

Biofilm Formation

Microtiter-plate test was used to detect the biofilm formation for all bacterial isolates as following (Christensen, et al. 1985, Diriba, et al. 2020).

In a few steps, fresh agar-isolated bacteria were inoculated in tubes filled with a 1 % glucose sterile triyptone soy broth (TSB) and incubated for 24 hours at 37 ° C. This culture was diluted into the fresh media at 1:100. Then, a sterile 96-wells flat-bottom microtiter with a suspension of 200µl was applied to it and incubated for 48 hours at 37 ° C. The phosphate buffer saline (pH7.2) washed the bacterial suspension of the well and washed gently 3 times. Plates with absolute methanol have been fixed and then stained with 220µL crystal violet (CV, 0.1% w/v) for 15min at room temperature. Each well was washed with PBS three times to eliminate unbound CV tint. After drying, 220µL of ethanol (95%) was added to each well. Lastly, the solubilized CV was transferred to a new microtiter plate. The optical density (OD) of the biofilm was measured by a microplate ELISA reader at a wavelength of 630nm.

The cut-off value (ODc) was established, it was defined as three standard deviations (SD) above the OD mean of the negative control. The OD values of tested isolates were expressed as the average OD value of the isolate reduced by ODc value. OD = average OD of isolate – ODc). Finally, the biofilm results of isolates were divided into the following categories (Stepanović, et al. 2000).

- Non-biofilm producer (OD ≤ ODc)
- Weak biofilm producer (ODc < OD ≤ 2 x ODc)
- Moderate biofilm producer (2 x ODc < OD ≤ 4 x ODc)
- Strong biofilm producer (4 x ODc < OD).

RESULTS

Isolation of PHA-producing bacteria

At first, nine bacterial isolates were isolated from oil-contaminated soils in different locations of Babylon province were diagnosed to Pseudomonas spp via traditional methods. In addition to three isolates of Lactobacillus bacteria obtained from the Advanced Microbiology Laboratory/ College of Science/ Babylon University isolated from dairy and All isolates were screened through a primary screening to ensure that polyhydroxyalkanoate was produced. Six soil isolates belonging to Pseudomonas spp and three isolates of Lactobacillus bacteria were positive results for Black Sudan B showing the presence of black granules within cells and was also confirmed by the Blue Nile A more selective stain for the presence of PHA granules that appear in orange fluorescent. After primary screening the polymer was extracted by sodium hypochlorite solution and chloroform according to **Fig. 1** Lactobacillus spp1 was selected to be the best PHA-producing bacteria in 3.4% percent from cell dry weight, which is identified and characterized later by VITEK 2 compact device in **Fig. 6**; the result showed 87% identical to Lactobacillus plantarum.

Primary Screening of Lactobacillus Plantarum

L. plantarum was examined by black Sudan B staining, and the results showed the presence of black granules inside the cells indicating that they are positive for the production of Polyhdroxyalkanoate (PHA) as **Fig. 2.a** and this result is similar to the previous study of (Mascarenhas, & Aruna, 2017). which was confirmed by the coloration of the Blue Nile in **Fig. 2.b**, the results shown in fluorescent orange colour indicate the accumulation of intracellular PHA granules according to a study (Hassan, et al. 2016). and thus L. plantarum was used in the production of PHA.

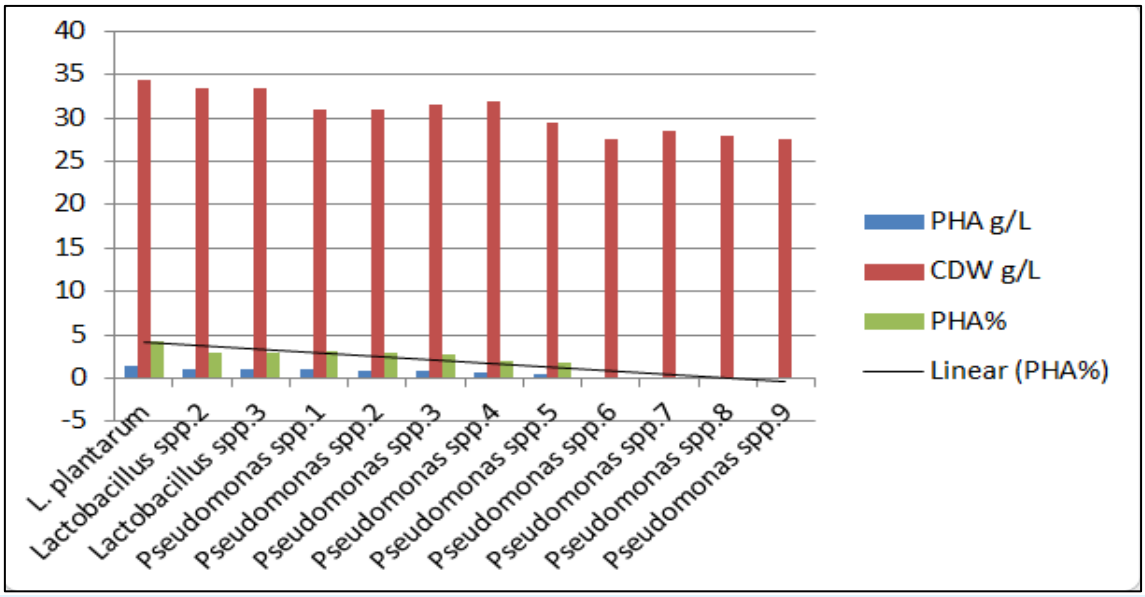


Fig. 1. CDW, PHA, PHA% for each isolate

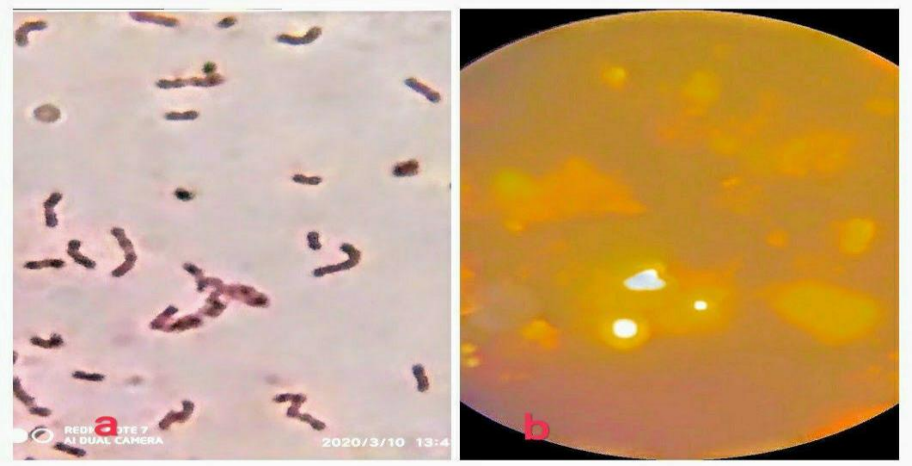


Fig. 2. (a) Nile blue A staining of L. plantarum under the fluorescent microscope. (b) Sudan black B staining of L.plantarum under microscopic with 100X magnification

Table 3. PHAs production by some Pseudomonas species and Lactobacillus species

Bacteria	PHA g/L	CDW g/L	PHA%
L. plantarum	1.5	34.5	4.3
Lactobacillus spp2	1	33.5	3.0
Lactobacillus spp3	1	33.5	3.0
Pseudomonas spp.1	0.95	31	3.1
Pseudomonas spp.2	0.9	31	2.9
Pseudomonas spp.3	0.85	31.5	2.7
Pseudomonas spp.4	0.65	32	2.0
Pseudomonas spp.5	0.55	29.5	1.9
Pseudomonas spp.6	0	27.5	-
Pseudomonas spp.7	0	28.5	-
Pseudomonas spp.8	0	28	-
Pseudomonas spp.9	0	27.5	-

Extract of Polyhyroxyalkanoates (PHAs)

Polyhydroxyalkanoates (PHAs) were determined in Pseudomonas species and Lactobacillus species. They were grown on an appropriate broth for 24h. Cell biomass was obtained by centrifugation. The cell walls were washed with sodium hypochlorite and washed with water, acetone and methanol and then dissolved with hot chloroform. The yields of PHA (%) accumulated in the cells according to dry weight were different according to in **Table 3** show three types of Lactobacillus spp. and six types of Pseudomonas spp. able to produce PHA.

The yields of PHA (%) accumulated within cells according to dry weight were 4.3% for L.Plantarium and appears as white powder as in **Fig. 3.b**.

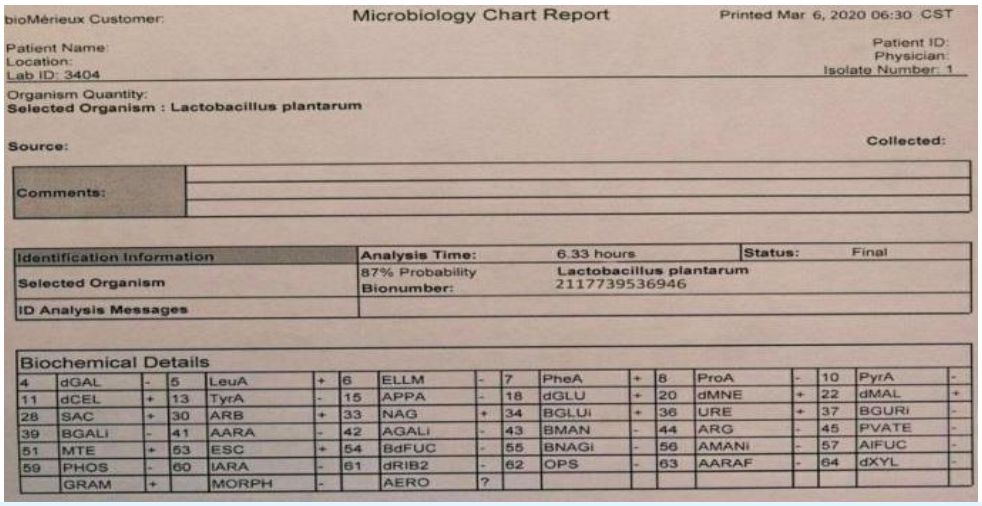
Identification of Best PHA Producing Lactobacillus

In addition to traditional methods, it was also diagnosed by VITEK 2compact device as 87% Lactobacillus plantarum demonstrated in **Fig. 4**.

Characterization of Polyhydroxyalkanoates

Fourier Transform Infrared Spectroscopy (FTIR)

Fig. 3. PHA extraction from L. plantarum, (a) pouring the solution on a sterile class tray as a film in the oven at 60 ^{0}C for drying. (b) After collection as powder and kept in an opaque tube



Microbiology Chart Report

Printed Mar 6, 2020 06:30 CST

bioMérieux Customer:

Patient Name:
Location:
Lab ID: 3404

Patient ID:
Physician:
Isolate Number: 1

Organism Quantity:
Selected Organism : Lactobacillus plantarum

Source:

Collected:

Comments:

Identification Information	Analysis Time: 6.33 hours	Status: Final
Selected Organism	87% Probability Bionumber:	Lactobacillus plantarum 2117739536946
ID Analysis Messages		

Biochemical Details

4	dGAL	-	5	LeuA	+	6	ELLM	-	7	PheA	+	8	ProA	-	10	PyrA	-		
11	dCEL	+	13	TyrA	-	15	APPA	-	18	dGLU	+	20	dMNE	+	22	dMAL	+		
28	SAC	+	30	ARB	+	33	NAG	+	34	BGLUi	+	36	URE	+	37	BGURi	-		
39	BGALi	-	41	AARA	-	42	AGALi	-	43	BMAN	-	44	ARG	-	45	PVATE	-		
51	MTE	+	53	ESC	+	54	BdFUC	-	55	BNAGi	-	56	AMANi	-	57	AIFUC	-		
59	PHOS	-	60	IARA	-	61	dRIB2	-	62	OPS	-	63	AARAF	-	64	dXYL	-		
	GRAM	+		MORPH	-		AERO	?											

Fig. 4. Biochemical characterization of Lactobacillus plantarum

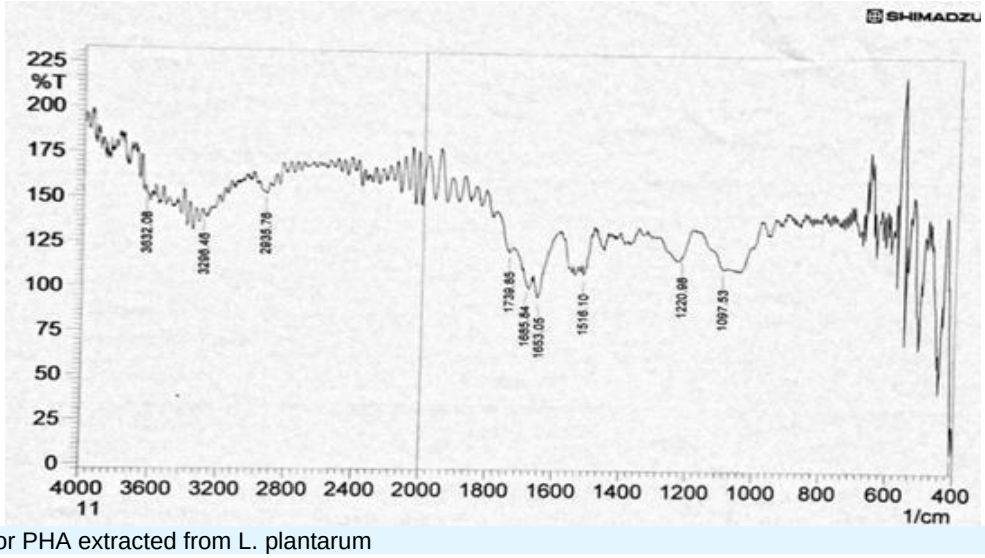


Fig. 5. FTIR for PHA extracted from L. plantarum

The Polyhydroxyalkanoate extracted from L plantarum that was positive for both Nile blue A and Sudan black stain was scanned between a wavenumber of (400 and 4,000 cm $^{-1}$). In **Fig. 5**, the results showed that the functional groups for PHA samples were identified as C-O, C-H and C=O (Hong, et al. 1999). The FT-IR spectra of PHA containing carbonyl group (C=O) occurred in a strong band at 1739.85cm^{-1} (Zhang, et al. 2005, Sharmaet al. 2017). In addition, the medium-strong of (CH_3) bond happened at 2935.76 cm-1 and a medium-weak (CH_2) stretching bond appeared at 1653.05 cm^{-1} (Gumelet al. 2012).

Table 4. Solubility of PHA

Solvents	Solubility of PHA
Chloroform	+
Dimethyl sulfoxide (DMSO)	+
Methanol	-
Ethanol	-
Water	-
Acetone	-

The solubility of Polyhroxyalkanoates (PHAs)

The polymer extracted from L. plantarum in this study was dissolved in chloroform and Dimethylsulfoxide (DMSO) but does not dissolve in acetone, methanol, ethanol and water **Table 4**. The previous study of (Aramvash, et al. 2016). use of two non-halogenated solvents (Ethanol and Acetone) as solvents to PHA at 70°C, while these solvents have been shown to dissolves less than about 1% (w/v) of the PHA at room temperature (PHA-poor solvents).

Minimum Inhibitory Concentration MIC for Gentamicin

Klebsiella pneumoniae and Enterococcus spp strains used in this study are clinical isolates from urinary tract infections in Al-Sadiq Hospital and Marjan Hospital, Babylon, Iraq according to **Table 2**. In **Fig. 6** a serial dilution of gentamicin powder was started with 0.4 and the final volume in the tube is 1.0 ml. The bacterial strains were tested for gentamicin susceptibility by a broth dilution method. MIC was defined as the lowest concentration of gentamicin that inhibited visible growth of the test bacteria. The MIC was the first gentamicin concentration of 0.2 mg/ml.

Biofilm

Bacterial isolates (K. pneumoniae and Enterococcus spp) screened for biofilm formation such as **Fig. 7**. They were measured by a microplate ELIZA reader at a wavelength of 630 nm. The results showed the ability to produce weak biofilm (ODc<OD<2×ODc) compared to (Branda, et al. 2005). Gentamicin and Polyhydroxyalkanoate loaded Gentamicin by (1:5, 1:10) ratio were used to study their effect on the biofilm formation of bacteria. The results showed that the inhibition of biofilm formation with Polyhydroxyalkanoate loaded Gentamicin (1:5) stronger than Gentamicin and

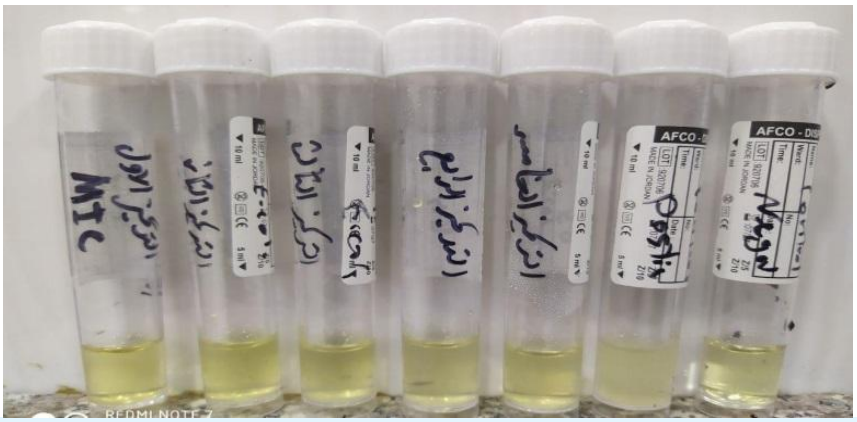
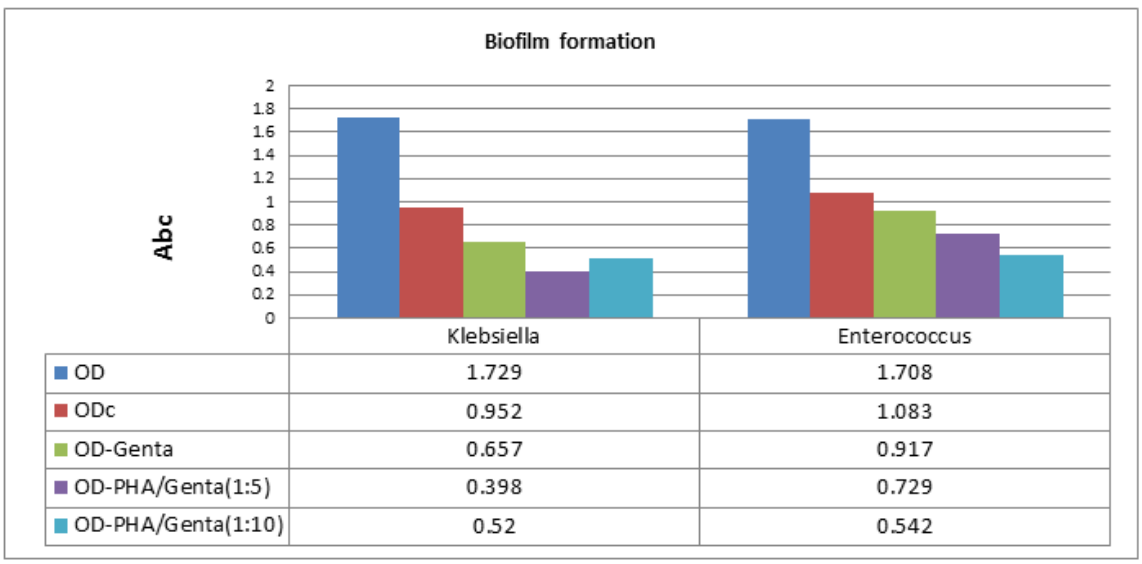


Fig. 6. MIC for gentamicin is 0.2 mg/ml



	Klebsiella	Enterococcus
OD	1.729	1.708
ODc	0.952	1.083
OD-Genta	0.657	0.917
OD-PHA/Genta(1:5)	0.398	0.729
OD-PHA/Genta(1:10)	0.52	0.542

Fig. 7. The effect of gentamicin, the ratio of gentamicin-loaded polyhydroxyalkanoates (1: 5, 1:10) against the formation of biofilms

Polyhydroxyalkanoate loaded Gentamicin (1:10) for K.pneumoniae. However, using Polyhydroxyalkanoate loaded Gentamicin (1:10) effect on biofilm formation stronger than Gentamicin and Polyhydroxyalkanoate loaded Gentamicin (1:5) for Enterococcus spp.

CONCLUSION

Polyhydroxyalkanoates extracted from *L.plantarum* are more efficient in the Drug delivery system for Gentamicin against bacterial biofilm formation.

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