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SCREENING OF THE POLYHYDROXYALKANOATES PRODUCING BACTERIA ISOLATE FROM DIFFERENT SOURCES IN IRAQ

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Abstract

Soil, sediment and sewage samples collected randomly from different location in Basrah city screened for Polyhydroxyalkanaotes (PHAs) using Sudan Black (B) and Nile blue staining plate assay, the isolates showed positive results. The bacterial isolates were identified by 16S rDNA sequencing to the level of strain, there are twenty one isolates had an identity of 100 % with different strains of *Bacillus* sp., *Enterococcus* sp. and *Clostridium* sp., the bacterial strains were screened for the presence of PHA synthase gene and the accumulation of PHA, the functional groups of extracted PHA granules were identified by Fourier Transform Infrared (FTIR) spectroscopy analysis.

Key words: Polyhydroxyalkanoate producing bacteria, *Bacillus* sp., *Enterococcus* sp., *Clostridium* sp. and FTIR.

1. Introduction

Environmental pollution by the disposal of synthetic polymers (i.e. conventional plastics) is a growing problem. Since, the 1940s plastics have been replacing glass, wood, metal and other constructional materials in numerous applications, Poirier *et al*., 1995. The use of plastics is widespread within our society, this is primarily due to the favourable thermal and mechanical properties of plastics making it a stable and durable material Poirier *et al*., 1995. The extensive global use of plastics has contributed heavily to environmental pollution as plastics are not always properly discarded or recycled and consequently persist within theenvironment. The manufacturing processes required to produce plastic also creates large quantities of chemical pollutants. In recent years, there has been a shift in public opinion with people becoming more ecologically aware. The shift in public opinion has driven industries to investigate biodegradable alternatives to plastic which are not manufactured using petrochemical methods (Braunegg et al., 1998 and Salehizadeh and Van Loosdrecht, 2004). Poly hydroxyl alkanoates (PHAs) are among the top group of biopolymers that have been intensively investigated and commercialized. PHAs are a family of biodegradable polymers produced by a broad range of microorganism. PHA can be synthesized by over 30 % of soil inhabiting bacteria (Wu *et al*., 2000). Many bacteria in activated sludge, in high seas and in extreme environments are also capable of making PHA. In the last 10 years, PHA have been developed rapidly to find applications in various fields (Chen, 2009). PHA have rich properties depending on the structures. Homopolymers, random copolymers, and block copolymers of PHA can be produced depending on the bacterial species and growth conditions. With over 150 different PHA monomers being reported, PHA with flexible thermal and mechanical properties has been

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developed (He *et al*., 1999). Such diversity has allowed the development of various applications, including environmentally friendly biodegradable plastics for packaging purposes, fibers, biodegradable and biocompatible implants, and controlled drug (Kemavongse *et al*., 2008) PHA monomers can also be used to develop biofuels,drugs or chiral intermediates. Oligomers of PHA were reported to be nutrients for animals.

2. Materials and methods

Sample collection

A total of 100 samples were collected from soil, reveres sediment, sewage in Basrah city. Soil samples 3.0 - 5.0 cm deep from surface was used for isolation of the bacteria. About 15 - 20 g of soil samples scraped with sterile spatula was placed in sterile plastic bags. Around 1.0 g of soil sample was serially diluted in sterile saline solution and plated onto Nutrient agar plates, and incubated at 37 °C for 48 hours. After incubation period, the plates were observed for development of different macroscopic colonies. Various colonies of different morphologies were individually picked and inoculated into Nutrient broth and allowed to grow. The bacteria from broth were streaked on to Nutrient agar slants, incubated at 37 °C for 24 - 48 hours, and then stored at 4 °C for further use.

Isolation of bacterial strains

Bacterial isolates were obtained by Serial dilution - Pour plate technique. Luria Bertani (LB) agar plates were used. Each sample of different dilutions (10^{-6} and 10^{-7}) was pour plated onto LB agar plates intriplicates. After incubation of 24 hrs at room temperature (28 °C), pure and isolated single colonies were randomly picked from each of these 3 plates (two dilutions) with sterile loop and were transferred onto fresh second plates.

Screening of native bacterial isolates for PHA production

Sudan Black staining method

All the bacterial isolates were qualitatively tested for PHA production following the viable colony method of screening using Sudan Black B dye (Kemavongse *et al*., 2008 and Kumari and Dhingra, 2013). For rapid screening of PHA producers Luria Bertani medium consist of 10 gm tryptone, 5 gm yeast extract and 10 gm sodium chloride in 950 ml of D.W., after that the pH was adjusted to 7, and the volume was completed to 1000 ml by D.W., was sterilized by autoclaving at 121°C for 20 minutes and cooled to 45 °C. The medium waspoured into sterile petriplates and allowed for solidification. The plates were inculated with bacterial sampels. Then were incubated at 35 °C for 24 hours. Ethanolic solution of 0.02 % Sudan Black B was spread over the colonies and the plates were kept undisturbed for 30 minutes. They were washedwith ethanol (96 %) to remove the excess stain from the colonies. The dark blue coloured colonies were taken aspositive for PHA production. All the positive isolates were assigned the code numbers based on their source ofisolation.

Staining for PHA Granules

Intracellular PHA granules were observed by staining with Sudan black - B as reported earlier (Sanathanam and Sasidharan, 2010). After incubation, heat fixed smears were prepared and dipped in 0.3 % Sudan Black - B prepared in ethylene glycol for 20 minutes. Slides were then decolorized in xylol and after drying, counter - stained with safranin solution for 30 seconds. Slides were washed and observed under the microscope.

Detection of polymer-producing bacteria

For the detection of polymer producing bacteria, Nile Blue A (Sigma-Aldrich, St. Louis, MO, USA) solution in dimethyl sulfoxide (DMSO; 0.25 mg/mL) was added tothe sterile HM medium which consist of (g/L): NaCl, 30; $MgSO_4.7H_2O$, 0.25; $CaCl_2$, 0.09; KCl, 0.5; NaBr, 0.06; Peptone, 5; Yeast extract, 10; Glucose, 1; and Granulated agar, 20; and pH was adjusted to 7

(Quillaguaman *et al*., 2004). Each isolated strain was incubatedat 35 °C for 48 hrs. The agar plates were exposed to UV light (312 nm) after cultivation to detect PHB accumulationin the grown colonies. The cells were observed under fluorescent microscope (Labophot Microscope, Nikon Instruments).

Nile Blue A smeer staining

Heat fixed bacterial smears were stained with 1 % (w/v) aqueous Nile Blue A Stain and heated in a water bath at 55 °C for 10 minutes. The stain was washed off with 8 % (v/v) aqueous solution of acetic acid for 1 minute. The slide was allowed to dry and then viewed under fluorescence microscope at 460 nm excitation wavelength (Ostle *et al*., 1982).

Molecular detection of the isolated bacteria

Extraction of bacterial DNA

DNA extraction by kit was used as recommended by manual Kit instruction (According to Gene aid Kit).

Agarose gel preparation

DNA Loading and running - Bromophenol blue (3 ml) was added to the DNA solution. The bands of DNA were examined under UV.

Bacterial identification by (16S rDNA)

The bacteria specimens were diagnosed and detected by amplifying universal 16S rDNA by primers as shown Table - 1 and PCR amplification regents are summarized in Table - 2 and PCR condition was described in Table – 3.

Electrophoresis running of 16S rDNA

DNA ladder (1 kb) as 8 ml and 10 ml of 16S rDNA (PCR product) were run for on 2 % agarose gel for 60 min (70 V). The bands were photographed under UV light by digital camera.

Purification, sequencing and manipulation of data for PCR product

The obtained products of PCR were purified and sequenced at Macrogen company laboratories, Korea for complete identification of bacterial isolates.

Detection of phac1and phac2 genes

Amplification of phac1and phac2 genes

The of *phac1*and *phac2* genes which responsible of PHA production were detected according to protocol of (Solaiman *et al*., 2000; Jamil *et al*., 2007 and Abdul *et al*., 2013) by using I-179 L and I-179 R primers, the primer sequence was listed in Table – 4. Reagents of PCR amplification *phac1*and *phac2* genes described in Table – 5.

Electrophoresis running

According to Sambrook and Russell, 2001, the steps in 3.8.1.2 and 3.8.2.2 were followed in order to detect the *phac1*and *phac2* genes with some differences required according to Abdul Basit Khan *et al*., 2013 as follows: 1) using 1 % agar and 2) using DNA ladder 100 bp (100- 1500). To analyse the PCR products, gels were exposed to UV light andphotograph was taken after electrophoresis.

Extraction and Quantification of PHA production

Twenty two bacterial strains were subjected to quantification of PHA production (Kalaivani and Sukumaran, 2013 and Raghul *et al*., 2013). A simplified media PYM in 250 ml flasks was inculcated with 0.1 from 10^{-6} bacterial suspension. The inoculated flasks were incubated on rotary shaker (150 rpm) at 35 ºC for 72 hrs. After 72 hrs of cultivation, cells were harvested by centrifugation at 10,000 rpm at 4 ºC for 15 min, washed aseptically with sterile distilled water. Cell pellet was dried at 60 ºC oven till dry. To estimate the dry cell weight (DCW) in units, residual biomass was estimated as the difference between dry cell weight and weight of extracted PHA. For PHA extraction, 10 ml of sodium hypochlorite was added to the culture pellet and centrifuged at 5,000 rpm for 20 minutes. The

pellet was washed sequentially with distilled water, acetone and methanol, respectively. The pellet obtained after washing was suspended in 5 ml of chloroform, and evaporated ina glass petridish at room temperature. The weight of the residue obtained after evaporation of chloroform was recorded (Kannahi and Rajalakshmi, 2012).

Residual biomass (g/L) = DCW (g\ml) – Dry weight of extracted PHA (g/ml)

PHAaccumulation (%)= Dryweight of extracted PHA (g/L)× 100%\DCW (g/L)

Fourier Transform Infrared (FTIR) analysis

The chemical structure of the extracted Polyhydroxyalkanoates was analyzed by Fourier transform infrared spectroscopy. The infrared spectra of the samples were recorded in the wave number range from 400 to 4000 cm^{-1} using a Perkin Elmer Fourier transform infrared (FTIR) spectrophotometer using KBr disc (Shamala *et al*., 2003).

Table – 1: Universal 16S rDNA primer used in PCR amplification

Gene	Sequence	Size of product (bp)
Universal bacterial 16S rDNA	F- 5'-AGAGTTTG ATCCTGGC-3'	1500
	R- 5'-GGTTACCT TGTTACGACTT-3'	

Table – 2: Reagents of PCR amplification (50 µl) for 16S rDNA

S.No	Reagent	Amount
1	DNA template	10 µl (30 ng)
2	Forward primer	1.5 µl
3	Reverse primer	1.5 µl
4	Taq Green Master Mix.2x	5 µl
5	Nuclease-free Water	8 µl
Total volumes		**25 µl**

Table – 3: Program was used in PCR amplification

Steps	Temperature (°C)	Time	Number of cycle
Initial Denaturation	92 °C	2 min	1
Denaturation	94 °C	30 sec	30
Annealing	51.8 °C	45 sec	
Extension	72 °C	1.5 min	
Final Extension	72 °C	5 min	1

Table - 4: Sequence of *phac1*and *phac2* genes

Gene	Sequence	Size of product (bp)
I-179 F	5'-ACAGATCAACAAGT TCTACATCTTCGAC-3	540
I-179R	5'-GGTGTTGTCGTTGT TCCAGTAGAGGATGTC-3	

Table - 5: Reagents of PCR amplification *phac1*and *phac2* genes

S. No	Reagent	Amount
1	DNA template	10 µl
2	Forward primer	0.5 µl
3	Reverse primer	0.5 µl
4	Taq Green Master Mix.2x	6.5µl
5	Nuclease-free Water	7.5 µl
Total volumes		**25 µl**

Table - 6: Program was used in PCR amplification for *phac1* and *phac2* genes

Steps	Temperature (°C)	Time	Number of cycle
Initial Denaturation	94 °C	5 min	1
Denaturation	94 °C	1 min	30
Annealing	50 °C	2 min	
Extension	72 °C	2 min	
Final Extension	72 °C	5 min	1

3. Results

Samples collection

Seventy eight bacterial isolates were isolated from 100 samples collected from different locations (Soil, Sewage and Sediment) in Basrah. The isolates were storage for the further experiments.

Screening of native bacterial isolates for PHA production

Sudan Black staining method

The bacterial colonies were initially screened using Sudan Black (B) staining of PHA granules to detect the production of PHA. The isolates have shown different degrees of staining with sudan black and only 35 isolates were exhibited astrong dark blue colar (Fig - 1) and numbers of isolates not stained with sudan black.

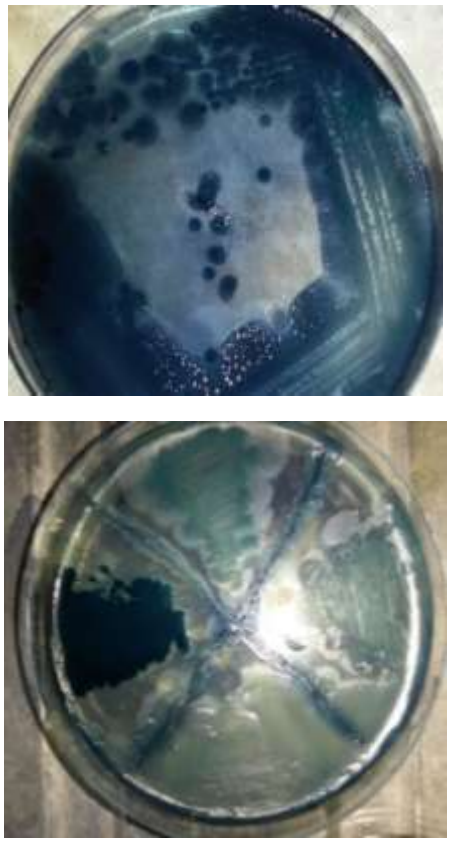


Fig - 1: PHA producing bacteria with sudan black dye

Staining for PHA Granules

The intercellular PHA granules were observed under microscope by staining smeers with 0.02 % sudan black prepared in ethylene glycol. The granules within the cells were stains with blue violet to black color (Fig - 2).

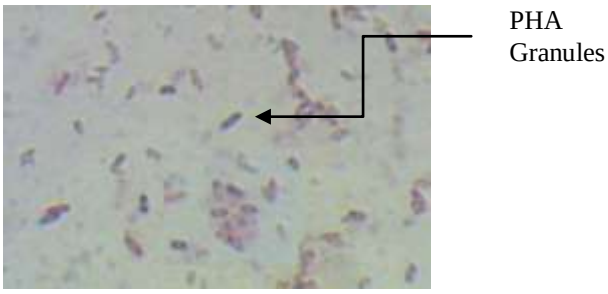


Fig - 2: Smear of PHA producing bacteria with sudan black

Detection of polymer-producing bacteria

The posetive to sudan black isolated strains were re cultivated on agar H.M-1 medium containing Nile blue for 2 - 3 days and then exposed to UV light (312 nm). The day produced fluorescence on binding to PHA granules in the cells (Fig - 3).

Nile Blue A smear Staining

Further more microscopic investigation of the strains with Nile blue showed that the stain imparted fluorescence to the PHA granules which were observed at wavelength (460 nm) on fluorescence microscope (Fig - 4).

Fig - 3: PHA producing bacteria with nile blue under UV light

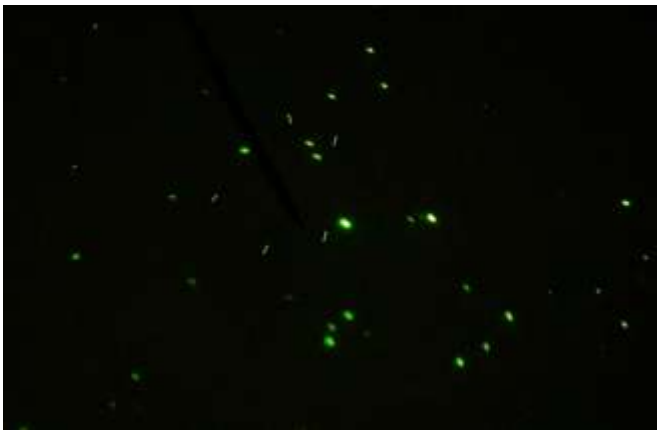


Fig - 4: Florecence of PHA produsing bacteria under florescent microscope with nile blue dye

Molecular detection of the isolated bacteria

Extraction of bacterial DNA

The DNA of 34 isolates was extracted by genomic DNA purification kit. The result was detected by electrophoresis on 0.8 % Agarose and showed under UV light in which the DNA appears as bands Fig – 5.

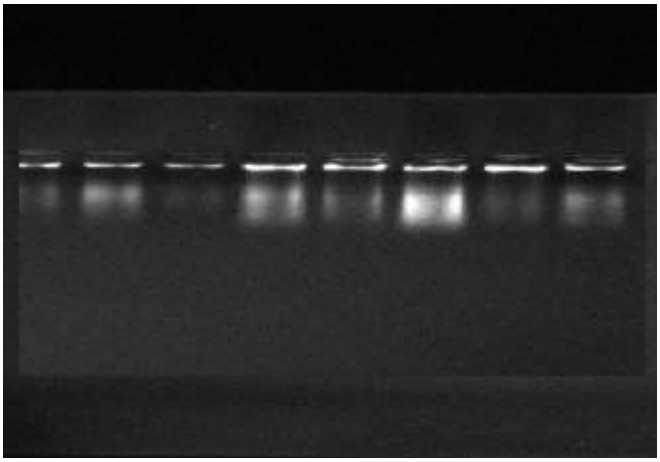


Fig – 5: Total genomic DNA

Bacterial identification by 16S rDNA

The extracted DNA (Fig - 5) from each isolate (n = 35) was subjected to PCR for amplifying 16S rDNA (Fig - 6). The individual band of the gene was characterized by 1500 bp by comparison with the standard molecular DNA Ladder (1Kb).

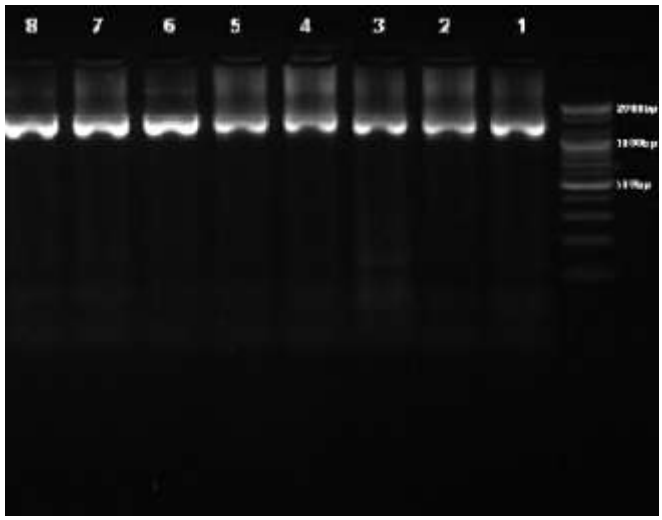


Fig - 6: Amplification of 16S rDNA

Sequencing for 16S rDNA and identification of bacterial species

The results of 16S rDNA nucleotides sequencing for 34 isolates were presented in Table - 7. The isolates were identified to the level of strain, there are twenty one isolates had an identity of 100 % with different strains of *Bacillus* sp., *Enterococcus* sp. and *Clostridium* sp. The unidentified bacteria were twelve isolates.

Detection of *phac1and phac2* genes

Phac1 and *phac2* genes which responsible for the production of PHA were detected by Polymerase Chain Reaction (PCR) from 23 diagnosed isolates used primer 1-179 L and 1-179 R . The PCR product were obtained in 540 bp (Fig - 7).

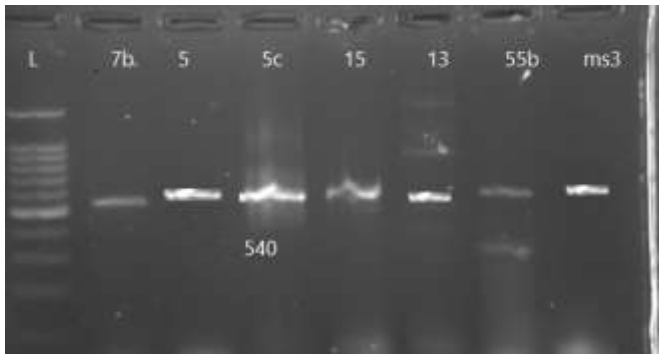


Fig - 7: Detection of phac1and phac2 genes

Table – 7: Identified bacterial strains by gene sequencing

Isolates code	Bacterial species	Identical to strain (100 %)	Length (bp)
7B	*8-F-Bacillus axarquiensis*	DSG5100	674
3B	*2-B- Bacillus thuringiensis*	W64100	647
5	*14-B- Bacillus cereus*	VKR01100	780
5C	*20-F- Bacillus subtilis*	SCODB99100	436
12	*e21-F+R- Bacillus subtilis*	NG4-12 100	1422
6	*25-F- Enterococcus faecium*	KSG2100	329
14	*4-B- Bacillus cereus*	S2 16S 100	1048
15	*8-B- Bacillus subtilis*	6,HB100	1117
21	*6-F- Bacillus methylotrophicus*	Se08100	861
13	*23-B- Bacillus cereus*	ZL-1,100	760
19	*35-B- Bacillus cereus*	S2-8100	1160
26	*9-F- Bacillus cereus*	ZZ62100	598
36B	*3-B- Bacillus cereus*	HYM88100	1028
M21	*11- B- Bacillus oceanisediminis*	DD174100	285
M22	*1-F- Enterococcus durans*	qz1182100	722
Ms3	*7-B-Bacillus myloliquefaciens*	BCRh101100	818
3	*12-B- Bacillus thuringiensis*	FS213P 100	403
55B	*7-B- Bacillus thuringiensis*	FS213P100	915
9B	*2-F- Clostridium bifermentans*	0910-06083 100	569
G3	*3-F- Bacillus cereus*	AZ-7100	296
Sweg1	*5-R- Clostridium bifermentans*	0912-02001100	826

Extraction and Quantification of PHA production

The PHA produced by the bacterial strains was extracted by lysing the cells with Sodium hypochlorite and chloroform as a solvent for the extraction (Fig - 8). The weight of extracted PHA obtained in (mg) were tabulated in Table - 8 and residual biomaswas estimated as difference between the dry cell weight and dry extract of PHA. The percentage of intracellular PHA accumulation was estimated as the percentage composition of PHA present in the dry cell weight. From these results strain *Bacillus amyloliquefacience* BCRh showing high PHA production 66.18 %, the strain *Bacillus methylotrophicus* Se08 63.95 %, *Bacillus cereus* HYM88 48. *Enterococcus faecium* KSG2 47.6 % while the strain *Bacillus oceanisediminis* DD174 showing less production 5.57 % compared with the other strains which ranges between the high and less values (Table – 8; Fig - 9).

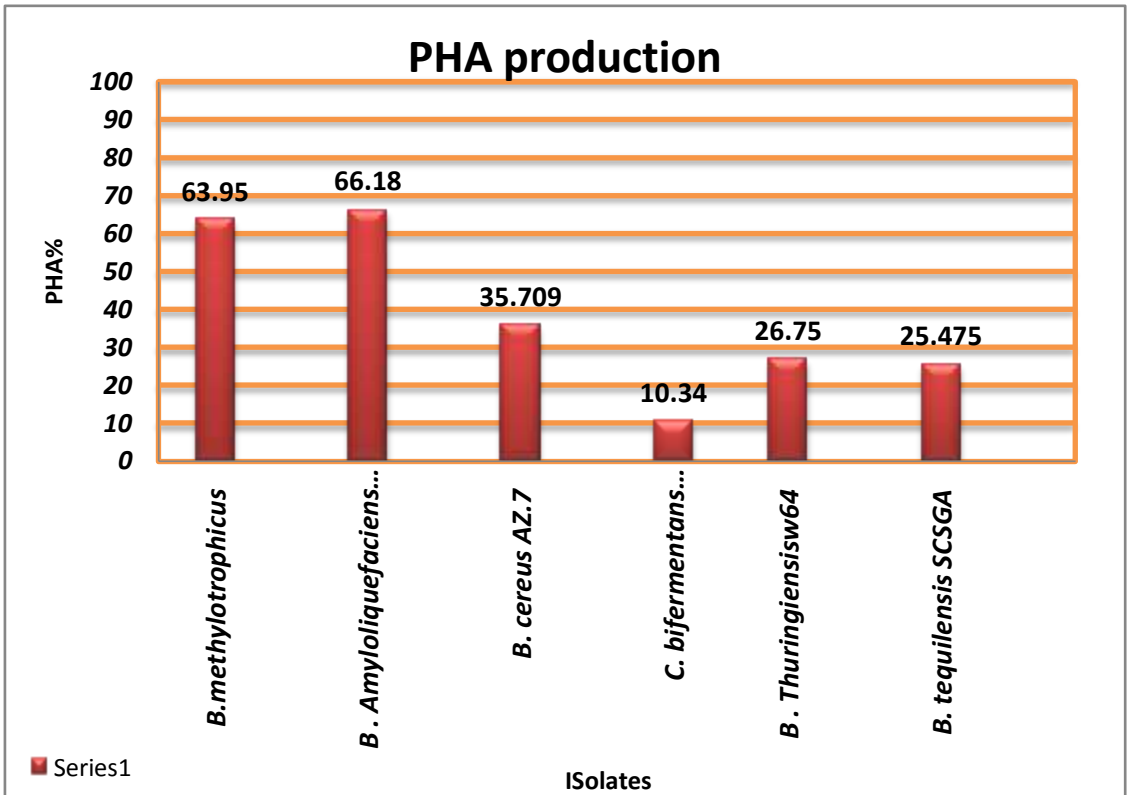
Fig – 8: Extracted PHA

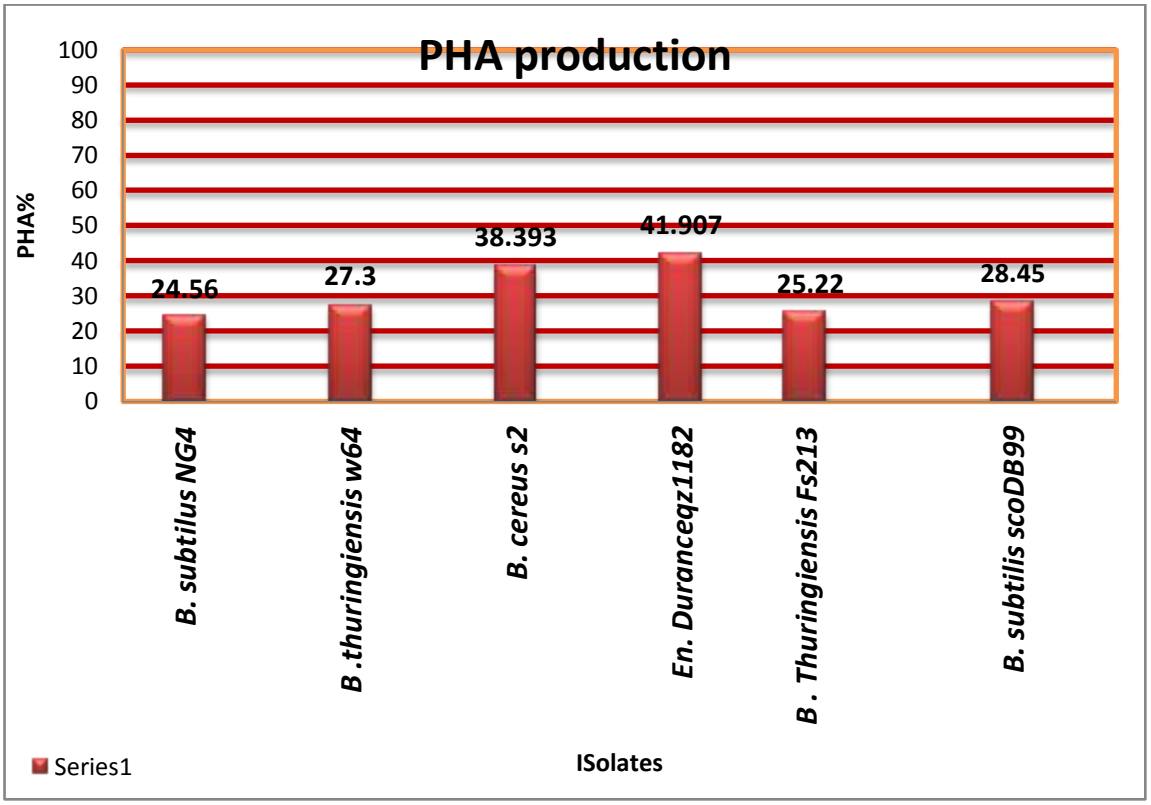
Fourier Transform Infra Red Analysis (FTIR)

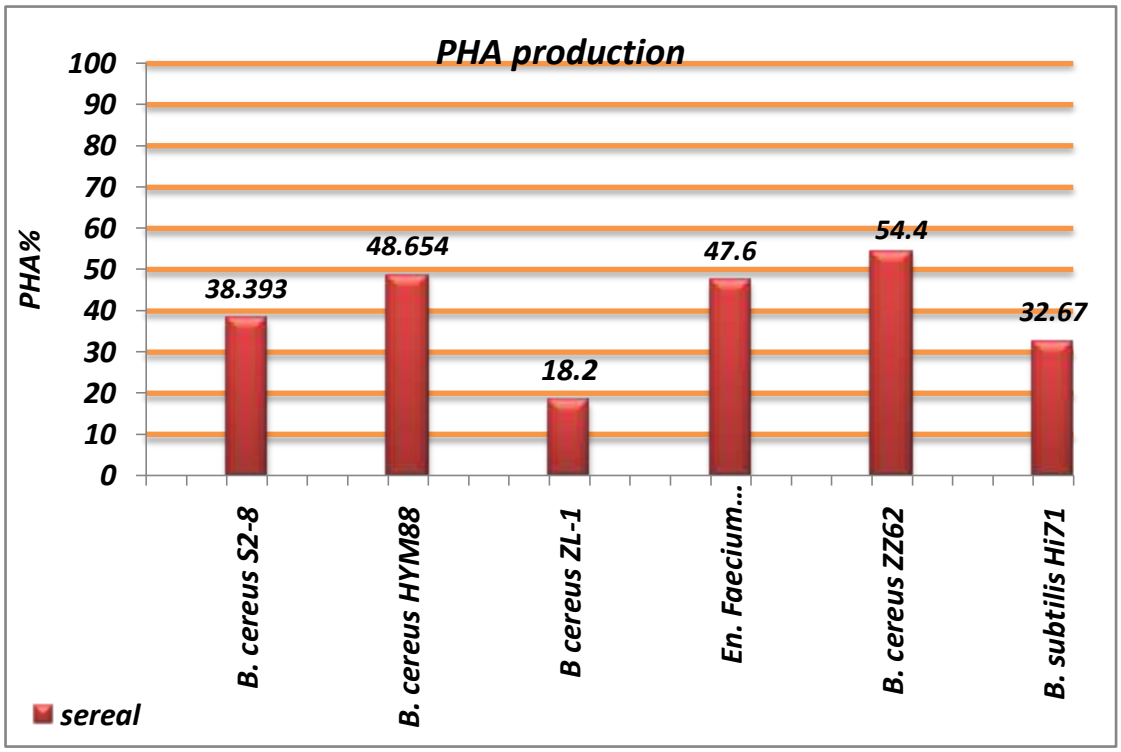
To further confirm the production of PHA by the bacterial strains, powdered PHA were analysed by FTIR to determine the functional groups of PHA. In the present study, FTIR

Table - 8: PHA production values and residual biomass

No	Bacterial isolates	Dry weight of extracted PHA		Cell dry weight CDW (g\ml)	Residual biomass (g\ml)
		g\100ml	g\ml		
1	*Bacillus subtilis NG4*	2.155	0.0215	8.77	0.0151
2	*Bacillus cereus S2*	0.9798	0.009798	2.4898	0.0662
3	*Enterococcus durans-qz1182*	2.1905	0.02191	0.05227	0.0304
4	*Bacillus thuringiensis FS213P*	0.715	0.0072	0.0283	0.0211
5	*Bacillus thuringiensis FS213P-915*	0.74	0.0074	0.0271	0.0197
6	*Bacillus subtilis SCODB99*	2.467	0.0246	0.087	0.0624
7	*Bacillus cereus strain AZ-7*	0.92988	0.009298	0.02604	0.01674
8	*Bacillus amyloliquefaciens BCRh*	4.50725	0.0451	0.0681	0.022997
9	*Bacillus thuringiensis W64*	0.7375	0.007375	0.0276	0.02023
10	*Clostridium bifermentans-0910-06083*	3.634	0.036	0.3523	0.316
11	*Bacillus tequilensis -SCSGAB0139*	0.7375	0.00738	0.02895	0.0216
12	*Bacillus cereus HYM88*	0.9615	0.009615	0.051475	0.04186
13	*Enterococcus faecium KSG2*	2.09	0.0209	0.0439	0.023
14	*Bacillus methylotrophicus -Se08*	1.84375	0.01844	0.028825	0.010387
15	*Bacillus cereus S2-8*	3.3988	0.03398	0.08853	0.05451
16	*Bacillus oceanisediminis DD174*	0.4525	0.00452	0.08123	0.877
17	*Clostridium bifermentans -0912-02001*	0.7725	0.0082	0.03177	0.02357
18	*Bacillus subtilis HB*	2.6	0.026	0.09235	0.0664
19	*Bacillus cereus VKR01*	0.73	0.0073	0.0293	0.022
20	*Bacillus axarquiensis -DSG5*	3.0875	0.030875	0.06583	0.03495
21	*Bacillus cereus ZL-1*	5.272	0.0527	0.0481	0.00464
22	*Bacillus cereus -ZZ62*	3.436	0.03436	0.0631	0.02874







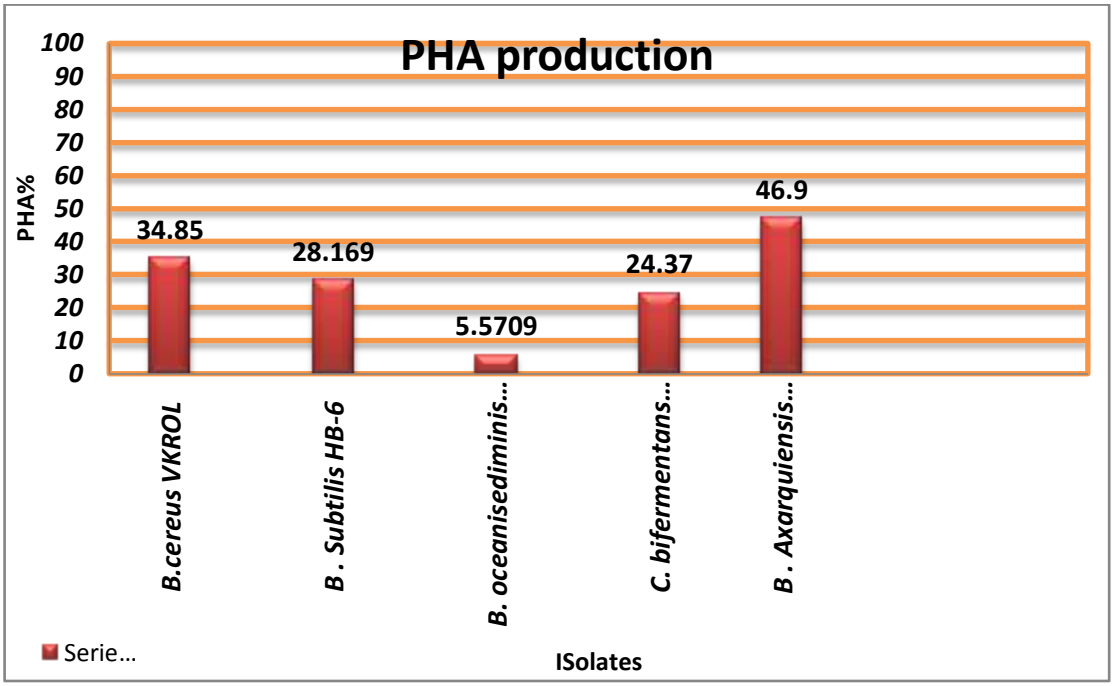


Fig – 9: Percentage of PHA

spectroscopy was performed between frequency ranges of 4000 - 400 cm^{-1}. The polymers extracted showed the intense absorption at 3325 and 3293.34 cm^{-1} for (O-H) bending group, other absorption bands at 2960, 68 and 2934 cm^{-1} for (C-H) stretching group. IR spectra of the polymers revealed the presence of marked peaks at wave numbers 1634 and 1648.84 cm^{-1} representing the presence thioester carbonyl (C=O) stretching groups, the marked peaks at wave numbers of 1734 due to the presence of (C=O) ester stretching groups. Other absorption bands at 1443, 1400, 1453 cm^{-1} were observed for (CH_3) group (Fig - 10).

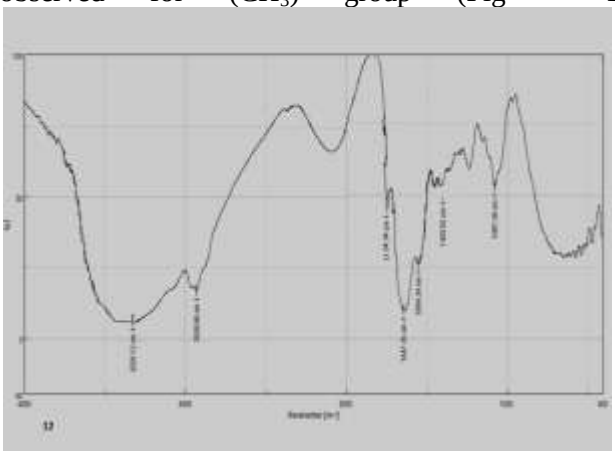


Fig - 10: FTIR for the isolated bacteria

4. Discussion

Petroleum derivative plastic are widely used in our daily lives, but they cause environmental pollution because they are persistent for hundreds of years. Because of this biodegradable polymer producing (microbial thermoplastics) has gained importance. Polyhydroxy alkanoates (PHA) is an important raw material for microbial plastics. Today, most research efforts in this field concentrate on the isolation of PHA producing microorganisms from different sources and improvement of PHA producing abilities of microorganisms (Muthazhagan and Thangaraj, 2014). In the present study, different bacterial isolates were obtained from soil, sediment and sewage in Basrah city. For the rapid detection and isolation of PHA producing bacteria Sudan black B and Nile blue A staining viable colony methods were used. A total of 34 isolates showed black - blue coloration when stained with Sudan black B, preliminary screening agent for lipophilic compounds and these isolates showed positive results with Nile blue A staining specific dye for the PHA granules. Teeka *et al*.

(2010), used these methods to screen the potential PHA producing bacteria from soil and Ramachandran and Abdullah, 2010, also observed the colonies formed on rich medium under ultraviolet light (UV) to screen for the fluorescence which indicated the presence of PHA producers. By applying PCR technique an amplified region was shown in 1500 bp approximately which was in agreement with the result of Hussein, 2013, because the 16S rDNA appears in this region in all bacteria.

Bacterial PHA are chromosomally encoded by the *phc1* and *phc2* genes of the expected length of 540 bp were amplified from bacterial strains, Kung *et al*., 2006, succeeded in amplification of the *phc* gene from *Pseudomonas* and *Escherichia coli* by using PCR technique. The bacterial strains showed different values in PHA accumulation, percentage of PHA accumulation in bacterial cells dependent on many physiological and regulatory factors (Willian *et al*., 2013). The FT-IR technique was used in this study with spectra range of 4000 - 400 cm^{-1} several methods have been reported for the qualitative analysis of PHA, including GC, NMR etc. but FT-IR was reported to be the most rapid and simple method (Hong *et al*., 1999), it does not require complicated sample preparation and can be used to detect the extracted PHA as well as intracellular PHA in the dried cells.

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