PRODUCTION OF POLYHYDROXY ALKANOATES BY NEW STRAIN *Bacillus subtilis* MANA18

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ABSTRACT

Anew strain of *Bacillus* isolated from soil of Basrah, Iraq, was identified by 16S rRNA sequencing as *Bacillus subtilis* MANA18. Polyhydroxyalkanoates (PHA) production by this strain was investigated. The extracted PHA was characterized by FTIR spectroscopy. It was found that this strain is PHA producer.

INTRODUCTION

Increased growth of the Earths human population has led to a drastic accretion of non-degradable waste materials; of these, plastic waste is most harmful to the environment (1). Degradation of conventional waste is very slow, and produces very harmful toxins during this process (2). Consumption of petrochemical based plastics is linked directly with diminishing oil resources. The worlds population consumes almost 150t plastic every year each, because of the increasing environmental problems associated with discarded plastics many studies have been directed towards the development of a suitable eco-friendly material that can replace at least some of the commodity plastics , (3) .

Polyhydroxyalkanoates (PHAs) are a group of polyesters synthesized by bacteria, archaea as well as some fungi (4; 5; 6). These polyesters are biodegradable and biocompatible. When the carbon substrate is in excess to other nutrients such as nitrogen, sulfur, phosphorus or oxygen (7; 5; 8), many micro-organisms can accumulate PHAs as intracellular energy and carbon storage inclusions.

MATERIALS AND METHODS

During the study of soil bacteria in Al-Seeba area of Basrah city- Iraq, a New strain of *Bacillus* was isolated. The present study was conducted to investigate the ability of this isolate to produce PHA.

Collection of soil sample:

Soil samples collected from 3.0-5.0 cm deep from surface was used for isolation of the bacteria., placed in a disposable plastic vial and brought to the laboratory.

Isolation and identification of bacterial isolate:
Bacterial isolates were obtained by dilution-pour plate technique. Luria Bertani (LB) agar plates were used. sample of different dilutions (10^{-6}, 10^{-7}) was pour plated onto LB agar plates in triplicates. After incubation of 24h at room temperature (35°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 bacterial isolate for PHA production:
Sudan Black staining method:
The bacterial isolate was qualitatively tested for PHA production following the viable colony method of screening using Sudan Black B dye (9),.For rapid screening of PHAproducers Luria berttine medium consist of was sterilized by autoclaving at 121°C for 20 minutes and cooled to 45°C. The medium was poured into sterile Petri plates and allowed for solidification. The plates were inculcated with bacterial sample . Then were incubated at 30°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 washed with ethanol (96%) to remove the excess stain from the colonies. The dark blue colored colonies were taken as positive for PHA production.

. Staining for PHA Granules
Intracellular PHA granules were observed by staining with Sudan black-B as reported earlier (10). 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 solution in dimethylsulfoxide (DMSO; 0.25 mg/mL) was added to the sterile HM medium. Each isolated strain was incubated at 35 °C for 48 h. The agar plates were exposed to UV light (312 nm) after cultivation to detect PHA accumulation in the grown colonies. (11), (12)

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 (13)

Morphological characterization.
sample was cultured on nutrient agar , Plates was incubated in an incubator at 37 C for 72 hours. Then, the colonies were studied and identified morphologically by recognizing the shape , color, motility and Gram stains .

. **Genetic identification.**

Genomic DNA extraction

One ml of an overnight culture (Luria-Bertani broth) was added to 1.5 ml eppendorff tube, centrifuged at 14000 rpm for 2 min to pellet the cells and decanted the supernatant, washed with distilled water at the same aspreparing to the procedure of the genomic DNA mini kit (Geneaid, Taiwan) as possible, with some changes in concentrations and time for the addition materials.

Detection of genomic DNA by gel electrophoresis

Method of electrophoresis by using 2%agarose has been done according to (14) as possible due to facilities.

Identification by Polymerase Chain Reaction (PCR).

Primers for 16SrDNA ,F(5'-AGAGTTTGATCCTGGC-3') and R (5-GGTTACCTTG TTACGACTT-3) are used (15), along with PCR program selected from (16), adapted by (17) table (1). The amplification steps abstracted from with little changes.

Table (1): PCR amplification program.

Steps	Temperature (°C)	Time	No. 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**

sequensing of 16S rDNA.

The obtained products of PCR were purified and sequenced at Macrogen company laboratories\ Korea *https://www.**macrogen**usa.com* for complete identification of bacterial isolate.

Extraction and Quantification of PHA production

Bacterial strain was subjected to quantification of PHA production (18 , 19) . 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 35ºC for 72 h . After 72 h of cultivation, cells were harvested by centrifugation at 10,000

rpm at 4ºC 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 Petri dish at room temperature. The weight of the residue obtained after evaporation of chloroform was recorded (20) (21).

Fourier Transform Infrared (FTIR) analysis

The chemical structure of the extracted polyhydroxyalkanoates was analyzed by Fourier transform infrared spectroscopy.. The infrared spectra of the sample was recorded in the wave number range from 400 to 4000 cm−1 using a Perkin Elmer Fourier transform infrared (FTIR) spectrophotometer using KBr disc (22).

RESULTS

Isolation and identification of bacterial isolate:

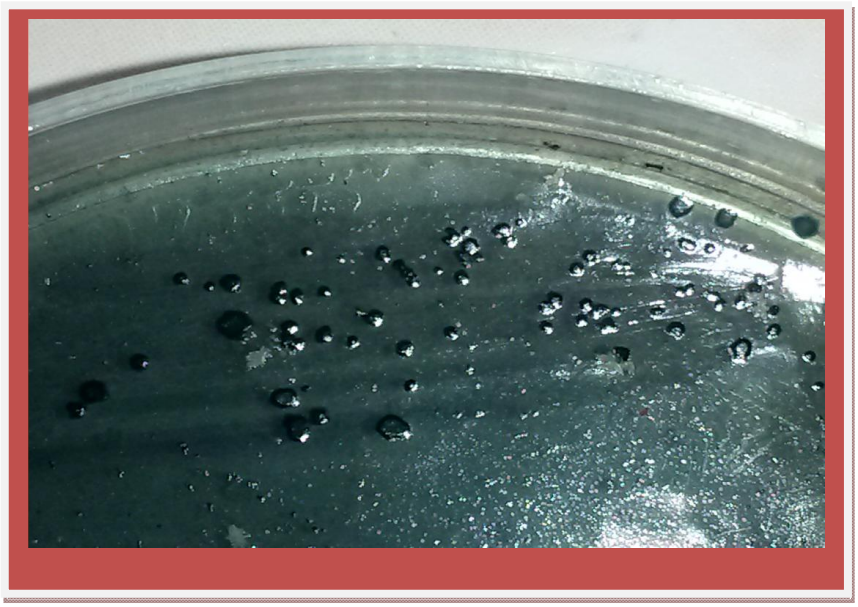
Suspected isolate has been selected from soil samples , and has cod number (18) .

Screening of bacterial isolatefor 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 isolate was exhibited astrong dark blue colar (Fig1).

Residual biomass (g/L) = DCW (g\ml) – Dry weight of extracted PHA (g/ml).
PHA accumulation (%)= Dry weight of extracted PHA (g/L)× 100%\DCW (g/L) .



(Fig 1) show PHA producing bacteria with Sudan black dye

Staining for PHA Granules

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

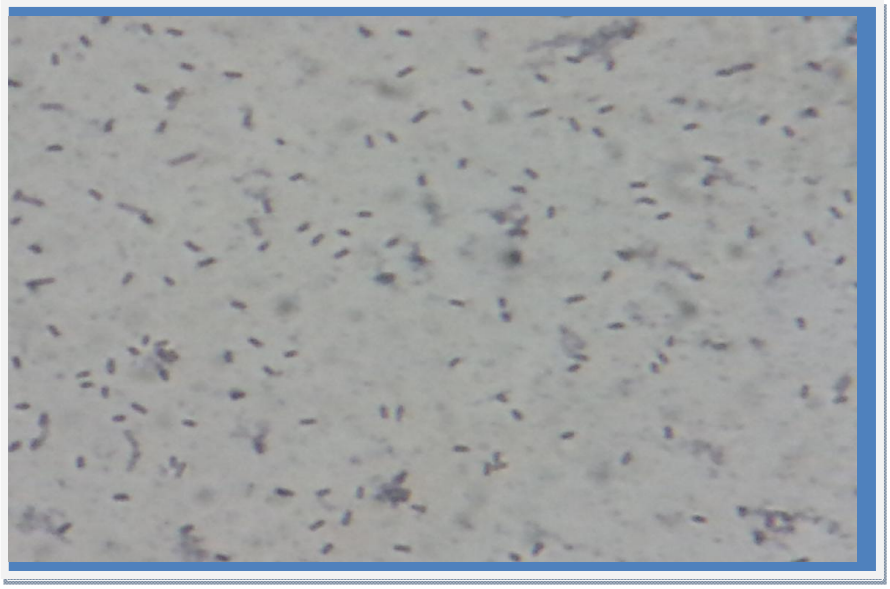
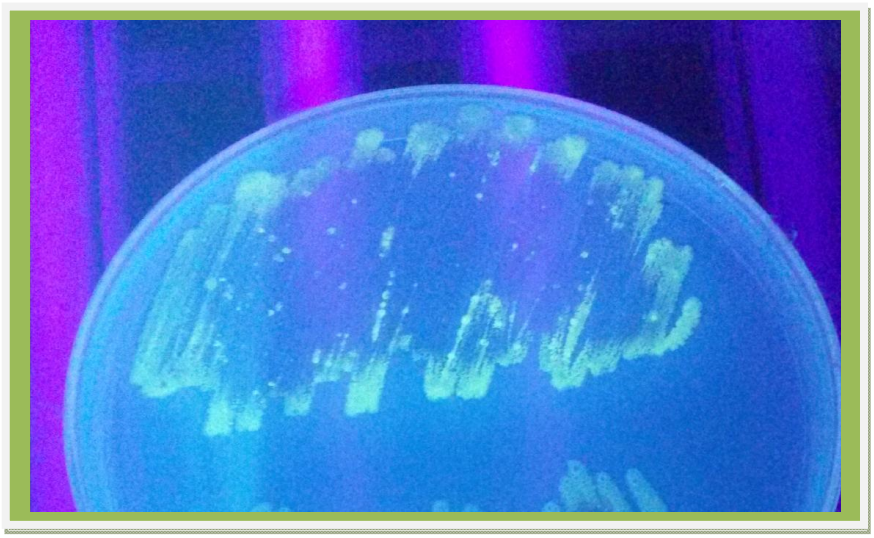


Fig2;show smear of PHA producing bacteria with Sudan black

Detection of polymer-producing in agar plates :

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



Fig(3) showed PHA producing bacteria with Nile blue under UV

Nile Blue A smear Staining

Furthermore microscopic investigation of the strains with Nile blue , showed that the stain imparted fluorescence to the PHA granules which were observed at wave length (460nm) on fluorescence microscope (Fig 4)

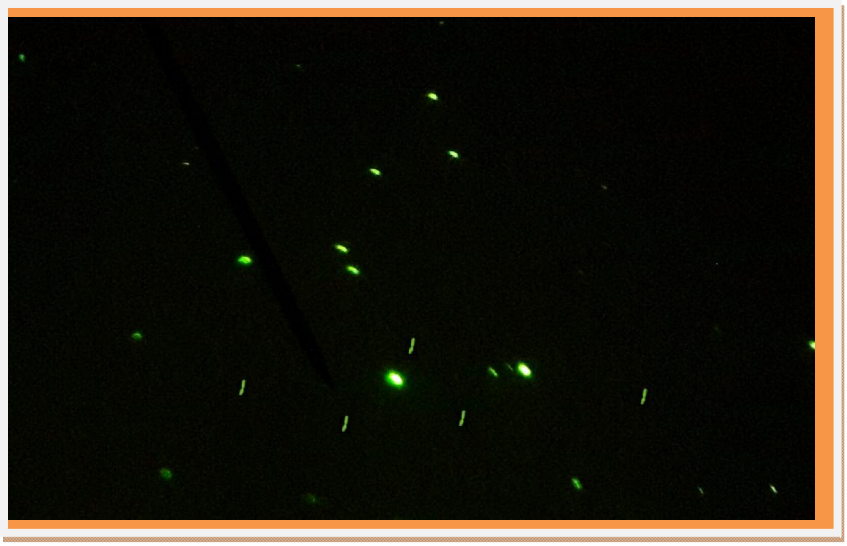


Fig (4) show florecence of PHA produsing bacteria under florescent microscope with Nile blue dye

Morphological characterization.

The suspected isolate was creamish in color on nutrient agar , motile gram positive bacilli .

Genetic identification.

Genomic DNA extraction and detection.

Electrophoresis technique has revealed clear isolated DNA for the isolate

Amplifying of the 16S rDNA gene by (PCR) Technique.

By using a universal primer, results have obtained the required band of 16S rDNA for the isolate along with electrophoresed ladder in the region of 1500bp, as in the figure (5)

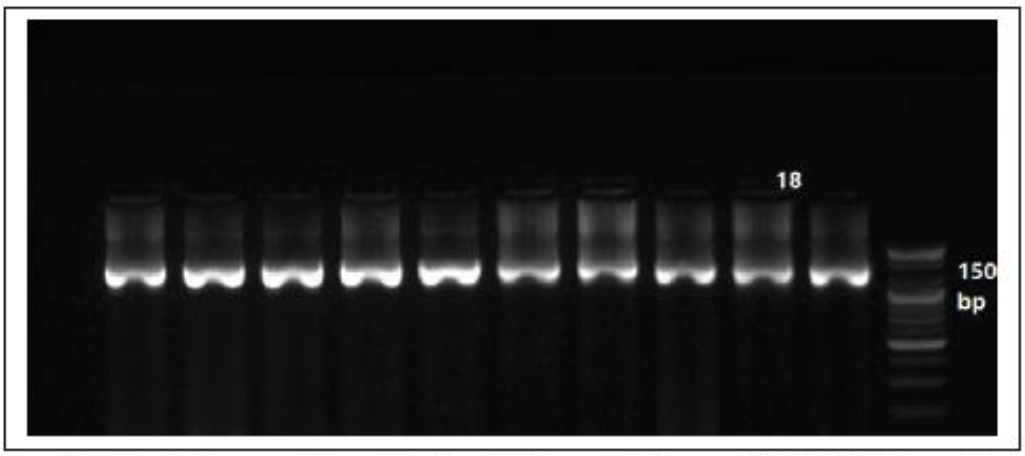


Fig (5) Gel electrophoresis for PCR technique;,DNA ladder of 1Kb, :16S rDNA gene 1500bp.

.Sequencing of 16S rDNA.

Bacterial isolate with number 18 which is different from its reference strain in one position of Nucleotide sequence .The isolate was published by the European Nucleotide Archive (ENA) and National Center for Biotechnology Information (NCBI) which is a part of the United States National Library of Medicine , the databases of these strain was recorded in gen Bank for DNA sequences . The new isolate ***Bacillus subtilis*** strain MANA18 (Genbank : LN999780.1) was closely related (99%) to *Bacillus subtilis* strain H171 , but with a gene or point mutation transversion (T instead G) at position (1104), (Fig 6), (7)

GTTGTGTATTTTCTTGACCTATACATGCAAGTCGAGCGGACAGATGGGAGCTTGCTCCC
TGATGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGAT
AACTCCGGGAAACCGGGGCTAATACCGGATGCTTGTTTGAACCGCATGGTTCAAACAT
AAAAGGTGGCTTCGGCTACCACTTACAGATGGACCCGCGGCGCATTAGCTAGTTGGTG
AGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCAC
ACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGC
AATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTA
AAGCTCTGTTGTTAGGGAAGAACAAGTACCGTTCGAATAGGGCGGTACCTTGACGGTA
CCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGC
AAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCTCGCAGGCGGTTTCTTAAGTCTGAT
GTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCA
GAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAAC
ACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGAGCGAAAGCGTGG
GGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTG
TTAGGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAG
TACGGTCGCAAGACTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAG
CATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGACA
ATCCTAGAGATAGGACGTCCCCTTCGGGGGCAGAGTGACAGGTGGTGCATGGTTGTCG
TCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTA
TTTGCCAGCATTCAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGG
TGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATG
GACAGAACAAAGGGCAGCGAAACCGCGAGGTTAAGCCAATCCCACAAATCTGTTCTCA
GTTCGGATCGCAGTCTGCAACTCGACTGCGTGAAGCTGGAATCGCTAGTAATCGCGGA
TCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGA
GAGTTTGTAACACCCGAAGTCGGTGAGGTAACCTTTTAGGAGCCAGCCGCCGAAGGTG
GAACGGTTTGA

(Fig 6) Gene sequencing of *Bacillus subtilis* MANA18 isolated in the present study

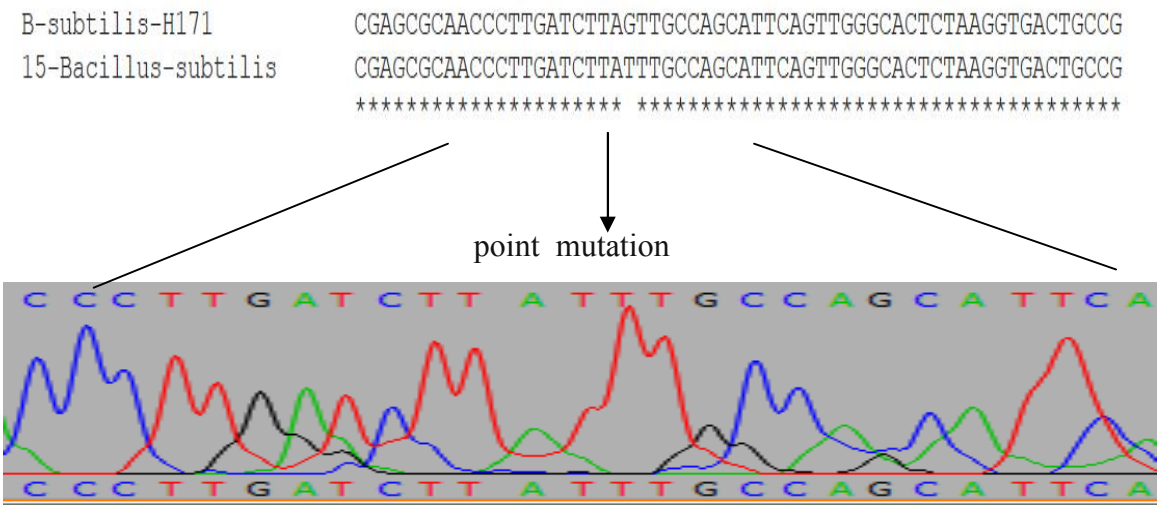


Fig (7): CLUSTALW for comparison of nucleotide sequences alignment for 16SrDNA of new strain to *Bacillus subtilis* strain H171, showing point mutation at the position 1104bp.

Extraction and Quantification of PHA production

The PHA produced by the bacterial strain ***Bacillus subtilis*** strain MANA18 was extracted by lysing the cells with Sodium hypochlorite and chloroform as a solvent for the extraction Fig (8) . The residual biomass was 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 Table (2)

Bacterial isolates	Dry weight of extracted PHA		Cell dry weight CDW g\ml	Residual biomass g\ml	PHA%
	g\100ml	g\ml			
***Bacillus subtilis* MCANA18**	**2.8925**	**0.0289**	**0.08853**	**0.0755**	**32.67%**

Table (2) PHA production values and residual biomass

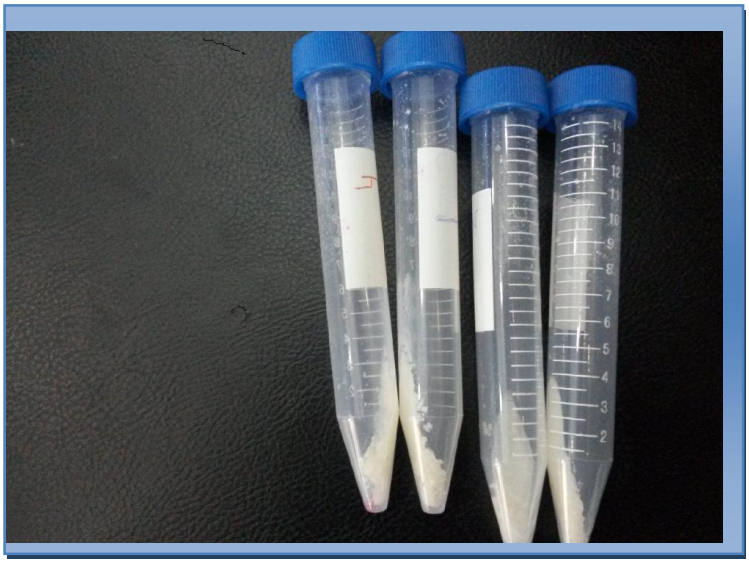
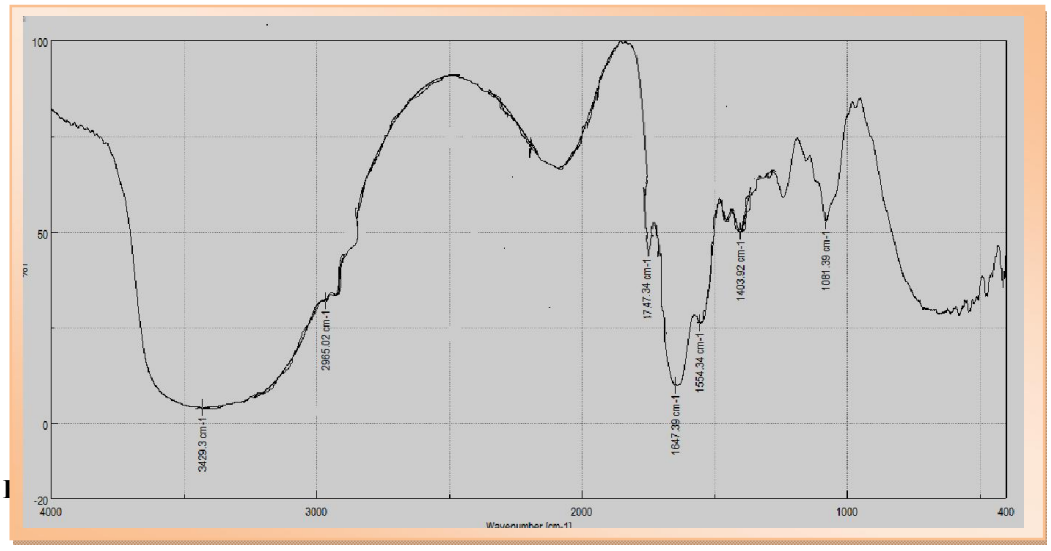


Fig (8) Extracted PHA

Fourier Transform Infrared (FTIR) analysis

To further confirm the production of PHA by the bacterial strain , powdered PHA were analyzed by FTIR to determine the functional groups of PHA . In the present study FTIR spectroscopy was performed between frequency ranges of 4000-400 cm^{-1} . The polymers extracted showed the intense absorption at 3425 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 thio ester 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 (9) .



DISCUSSION

In the present study bacterial isolates was obtained from soil in Basrah city, it was rod gram positive motile bacteria . For the rapid detection and isolation of PHA producing bacteria Sudan black B and Nile blue A staining viable colony methods were used The bacterial isolate showed black - blue coloration when stained with Sudan black B , preliminary screening agent for lipophilic compounds , and these isolate showed positive result with Nile blue A staining specific dye for the PHA granules . (23)used these methods to screen the potential PHA producing bacteria from soil , and (24) 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, we have observed that amplified region is found in 1500 bp approximately, which are agree with the result of (17) for the isolate, because the 16S rDNA appears in this region in all bacteria. (25) reported that for many years sequencing of the 16S rDNA gene has served as an important tool for determining phylogenetic relationships between bacteria and it is a powerful mechanism for identifying
new pathogens in patients with suspected bacterial disease, and more recently this technology is being applied in the clinical laboratory for routine identification of bacterial isolates.

Throughout sequencing, we have obtained that the isolate with cod number (18) which had identity of 99%w ith the reference strain ***Bacillus subtilis*** strain H171, which led us to suggest the probability of obtained
new strain.

By using of 16S rDNA sequence analysis, it had been demonstrated by (26) that in the absence of an accepted cutoff value, they retained a 99% similarity as a suitable cutoff for identification at the species level and a 97% similarity as a suitable cutoff for identification at the genus level and while the introduction of these sharp values was necessary to analyze a large collection of unidentified isolates belonging to different genera, further evaluations need to be performed to assess the accuracy of these values, besides; because bacterial genera do not evolve at the same speed, it may be necessary to use different cutoff values depending on the bacterial genus under investigation.

In our study, we have assumed 1% difference (99% identity) with sequence of 16S rDNA from the reference strains in the identification of our suspected new strain. The different of the isolate number 18 has been expressed as a point mutation type transition by the deletion of nucleotide Guanine which probably changed the encoded target amino acid. (27) and (17) adopted 1% difference (99% identity) with sequence of 16S rDNA gen from the reference strains in the identification of their novel strains studied also.

The values in PHA accumulation , percentage of PHA accumulation in bacterial cells dependent on many physiological and regulatory factors (28)

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 is reported to be the most rapid and simple method (29) , 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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انتاج polyhydroxyalkanoites من سلالة جديدة لبكتريا *Bacillus subtilis* MANA 18

ميثم ايوب الحمداني * نورس نوري جابر **

قسم علوم الحياة ، كلية التربية للعلوم الصرفة ، جامعة البصرة ، العراق *

فرع الاحياء المجهرية ، كلية الطب البيطري ، جامعة البصرة ، العراق**

الخلاصة : -

تم عزل سلالة جديدة من بكتريا *Bacillus subtilis* من التربة في محافظة البصرة، العراق وتم وأكد انتاج 16*S rDNA* التشخيص الجيني للعزلة عن طريق دراسة التتابعات للقواعد النيتروجينية لجين البوليمر من العزلة كما تم استخلاص المركب والتعرف على المجاميع الفعالة بأستخدام تقنية ال FTIR.

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