

Biodegradable Plastic Production by *Bacillus spp.* Isolated from Agricultural Wastes and Genetic Determination of PHA Synthesis

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Article Info	Abstract
Received 31/may/2017 Accepted 5/Dec./2017	<p>Polyhydroxyalkanoates (PHAs) are resources that hoard by a variety of microorganisms as a nutrient source. It is an environmentally recyclable biopolymer, and totally corrupted biologically to simple molecules. A total of 38 isolates from agricultural wastes, 9 isolates exhibited optimistic outcome (dark blue particles) for PHA accumulation by Sudan black B staining method. Maximum bacterial growth was achieved at temperature 35°C, PH= 7, for 48 hr. incubation and 0.25% concentration of NaCl. Chosen the isolate (NE1MOK) recognized as <i>Bacillus subtilis</i> when diagnosed by traditional and molecular methods by amplification 16s rRNA gene. <i>Bacillus subtilis</i> had an ability to accumulate 0.26 g/l (40.625 %) of PHA, and characterized by Fourier transforms infrared (FTIR) analysis, which exhibited functional groups of PHA as (C-O, C=O, CH₂, CH₃, C-O-C, C-H and terminal OH). The responsible gene was detected by PCR, and the results of amplification were shown in all isolates with a length of amplicons 505 bp. The aim of this study was to detect more efficient bacterial isolate that produce PHAs from agricultural wastes in optimal conditions, and determining the genes responsible for it.</p> <p>Keywords: <i>Bacillus spp.</i>, PHA production, FTIR, Characterization, synthase gene.</p> <p>الخلاصة</p> <p>بولي هيدروكسي الكانواتس (PHA) من المواد التي تستخدم من قبل مجموعة متنوعة من الكائنات الحية الدقيقة كمصدر للمغذيات. وهو بوليمر قابل لإعادة التدوير بيئياً، ويتحلل تماماً بيولوجياً لجزيئات بسيطة. من مجموع 38 عزله من المخلفات الزراعية، 9 عزلات اظهرت نتيجة واضحة (جزيئات زرقاء غامقة) من تراكم PHA عند صبغها بطريقة التصبغ بالسودان السوداء. ظهر اعلى نمو للبكتريا عند درجة حرارة 35م، اس هيدروجيني 7، حضن لمدة 48 ساعة و 0.25٪ من تركيز كلوريد الصوديوم. عرفت العزلة المختاره والمسماة (NE1MOK) على انها عضية <i>Bacillus subtilis</i> عندما تم تشخيصها بواسطة الطرائق التقليدية والجزيئية عن طريق التضخيم لجينات S16 الريبوسومي. وكانت <i>Bacillus subtilis</i> قادرة على تراكم 0.26 غرام / لتر (40.625٪) من PHA ، والذي شخص بجهاز مطياف الأشعة تحت الحمراء واطهر مجاميع وظيفية للـ PHA مثل (C-O ، C=O ، CH₂ ، CH₃ ، C-O-C ، C-H و OH). تم تعيين الجين المسؤول بالـ PCR وكانت النتائج ايجابية لجميع العزلات وكان طول الامبليكونس 505 زوج قاعدي. ان الهدف من هذه الدراسة هو تحديد العزلة البكتيرية الكفوه لانتاج الـ PHA من النفايات الزراعية في ظروف مثلى، وتحديد الجينات المسؤولة عنه.</p>

Introduction

Today, synthetic polymer has become indispensable part of our life. Petrochemical materials used to manufacture synthetic polymer and plastic, they are defiant to biodegradation, which poses a grave menace to the surroundings [1]. Environment issue due to pollution increases in the price of petroleum and depletion of fossil fuels makes us to

develop bioplastic as a substitute for petrochemical derived plastic [2].

Polyhydroxyalkanoates (PHA) has become a substitute for petrochemical derived plastic due to similar mechanical properties, PHA is ecofriendly, biodegradable, biocompatible and microbial thermoplastic [3]. PHA is a homopolymer straight chain of D-β-hydroxyalkanoates, is produced as granules in different bacteria [4]. PHA synthesis occurs

during the exponential growth phase and transferred into important energy reserves [5]. *Pseudomonas*, *Bacillus*, *Rhodococcus* bacterial species, which naturally produce PHA [7]. Gram-negative bacteria accumulated PHA and used broadly in a range of products. PHA from *Bacillus* species are extensively used in biopharmaceutical applications after lack Lipopolysaccharides from its membrane [8]. Also PHA considered as eco-friendly bioplastics when used in industrial application [9]. PHA genes coded for proteins in biosynthetic pathways, like *phaA*, *phaB*, and *phaZ* that coded to ketothiolase, reductase, and depolymerase, respectively, have revealed to take place in *pha* gene clusters [10]. Recent methods have developed to make PHAs from industrial, agricultural, food, municipal wastes and sludge of paper and pulp mills. Therefore, substitute of non-biodegradable with biodegradable plastic from organic waste can supply many profit to the environment and add to sustainable development [11]. Therefore, our aim was to determine the optimal conditions of bacterial growth and produce PHA from bacteria isolated from agriculture wastes, then amplification of synthase genes by PCR technique.

Materials and Methods

Collection of Samples and bacterial isolation

Samples collected from agricultural wastes (leaf wastes) in gardens of Baghdad University under a septic condition using standard practices [12]. Isolation of bacteria from wastes done by serialized dilution (10⁻¹ - 10⁻⁶), then bacterial isolation done by using pour plate technique on nutrient agar (Hi media, India). Plates incubated for 24 h at 37 °C.

Screening for PHA-producing by bacteria

PHA producing bacteria detected by Sudan black B staining method [13]. Briefly Sudan black stain was prepared as a 0.3% solution (w/v) in 60% ethanol. Smears of bacteria were prepared on glass slides and heat fixed, then stained for 10 min with Sudan black solution, rinsed with water and counter-stained with 0.5% safranin for 5 sec. Stained samples were observed under oil immersion at 1000x

magnification by using an Olympus microscope.

Effect of various growth parameters on Bacterial growth

Several cultural parameters like salt concentration (NaCl=0.25, 0.5, 1, 2.5, 5%), incubation time (24-120 h), pH (4-9), and temperature degree (25, 30, 35, 40, 45 and 50°C) were evaluated to establish their effect on bacterial growth [14]. Briefly, tubes with Nutrient broth were accustomed with each parameter, and inoculated with bacterial culture, then incubated with shaking incubator (150 rpm). After that, growth of culture in each tube measured and recorded the O.D at 600 nm.

Bacterial PHA production

Bacillus spp. inoculated in producing media, containing of: yeast extract (2.5 g/l), tryptone (4.0 g/l), glucose (1% w/v), and sodium chloride (0.25 g/l). 2% (v/v) inoculum added to the production medium (100 ml) then incubated in a shaker (150 rpm) at 35° C for 48 h [15].

Cell dry weight:

After incubation, bacterial culture centrifuged (10,000 rpm) for 15min. Cell pellets, dried to determine dry cell weight (DCW) as a g/l unit [16].

PHA extraction

Extraction of PHA was performed following sodium hypochlorite-chloroform method [12]. About 5 ml of culture was centrifuged at 10,000 g for 10 min. and supernatant was discarded. The pellet was suspended in 2.5 ml of 4 % sodium hypochlorite for digestion and 2.5 ml of hot chloroform and was incubated at 37°C for 1 hour. The suspension was centrifuged at 1500 g for 10 minutes. The bottom phase containing PHA with chloroform was collected and further was followed by extraction with hot chloroform and precipitated with ethanol and acetone (1:1). The precipitate was allowed to evaporate to dryness at 30⁰ C to obtain PHA crystals.

Quantification of PHA

PHA was liquefied in chloroform and evaporated completely, and determined as dry weight in unit of g/l [17]. Percentage of producing PHA was calculated according to the equation:

$$\text{Accumulation of PHA (\%)} = \frac{\text{Dry weight of PHA (g/l)}}{\text{Dry cell weight (g/l)}} \times 100.$$

Characterization of polymer by FTIR

FTIR spectrum was taken using Fourier Transform IR Spectrophotometer (Schimadzu-8400s). The spectra were recorded in range of 4000-600 cm⁻¹ (according to many searches).

Identification of PHA –producing by bacteria

Biochemical tests: Many biochemical tests achieved to detect PHA producing bacteria [18].

Molecular identification

DNA extraction: It's done by using the Genomic DNA purification Kit (Wizard®) according to the manufacturing of the company. The purity of DNA checked by nanodrop (Promega), and the range was between (50-100 np) [11].

Selection of Primers:

PCR amplification was done by using the primers (Alpha DNA, USA) [11], which specific for 16sRNA for *Bacillus spp.* according to the manufacture companies' information (Advanced Scientific Bureau), Primer sequence listed in Table 1.

Table 1: Primer sequence of 16sRNA for Bacillus spp.

Gene name	Primer sequence
16sRNA	F 5- GGTGGAGCATGTGGTTTA-3
	R 5- CCATTGTAGCACGTGTGT-3

Amplification Reaction

In the present study, 30 cycles of amplification used, for all genes, and the cycling conditions were applied according to [11]. The PCR amplification of 16S rRNA was done at 94°C for 4 min, 94°C for 20 s, 58°C for 30 s, 72°C for 2 min, and 72°C for 9 min withhold at 4°C.

Electrophoresis by agarose gel

PCR products were evaluated by electrophoresis as a method of [19]. DNA ladder (1000 bp) was the marker of the procedure, and power supply established at 7 V/cm for 2 hours. Gel exhibited by UV-Transilluminator then it's photographed.

16S rRNA gene Sequencing

PCR products were purified by extraction kit (GenElute™ gel-Sigma). Sanger sequencing method was used in sequencing PCR products according to [20]. The obtained primers for gene sequence were subjected to search at National Centre for Biotechnology Information (NCBI), and analyzed by sequencing Ready Reaction Kit (Applied Biosystems, USA). The chromatogram was prepared by Chimeras 5.2 software at (NCBI).

Molecular analysis for synthase genes of PHA

Template Preparation

Phenol- chloroform extraction method used as the first stage to extract template according to [12]. Then supernatant removed to another tube. In the following stage, Isopropanol (0.6 volume) was added and mixed lightly until a tough white DNA precipitate made. Latest, the suspension centrifuged at 10000 rpm for 5 min and the pellet dried until ethanol completely evaporated, then TE buffer (30 µl) added, and DNA stored at -20°C.

Primers descriptions

Two primers used for amplification of synthase genes in PHA producing bacteria [12]. Complete characteristics showed in Table 2.

Table 2: Descriptions of synthase genes .

Gene name	Sequence of nucleotides
Class 1-F	5'-ATGAAAGAGGTTGTAATCGTCGCT-3'
Class 1-R	5'-TCAACGCTCCACTGCGAG-3'
Class 2-F	5'-ATGAGCAATCAACGAATTGCA-3'
Class 2-R	5'-TCATTGCATGTTTCAGACCGC-3'

PCR conditions

A reaction mixture of PCR had 5 μ l of each (primer, template DNA, PCR buffer, MgCl₂), 2 μ l of DNA polymerase and 1.2 μ l of dNTPs. Cyclic conditions were (95^o C for 5 min used as initial denaturation, 35 cycles of denaturation at 95^o C for 2 min, 60^o C for 30 sec. used to anneal, 72^o C for 2 min and for 10 min used for extension and a final extension respectively), then embrace at 4^o C [21].

Results and discussion

Isolation of PHA-producing bacteria

From 38 samples of agricultural wastes, nine isolates (23.6%) were exhibited dark blue particles after staining by Sudan Black B. [12] demonstrated that marine and organic-wastes can produce bioplastic from 32 isolates, while [11] showed that 42 isolates of PHA producers when stained by Sudan black B exhibited black-blue particles. [22] showed that agricultural wastes have organic materials with good values of biological materials, which effect on growing of bacteria, take nutrients of the wastes and change it to expensive products.

Effect of different parameters on bacterial growth

1-Effect of Temperature on Bacterial growth

Maximum bacterial growth was achieved at temperature 35 °C as shown in Figure 1. High or low temperature effect on cell biomass and PHA synthesis by Bacillus subtilis may lead to decrease on PHA production probably due to the low enzyme activity at these temperatures [23].

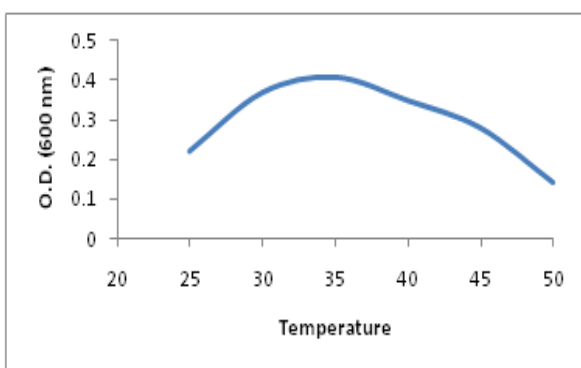


Figure 1: Temperature effect on growth of bacteria.

2-Effect of incubation time on Bacterial growth

The incubation time was showing optimum bacterial growth in 48 hr. for all strains. The incubation time was exhibited in Figure 2.

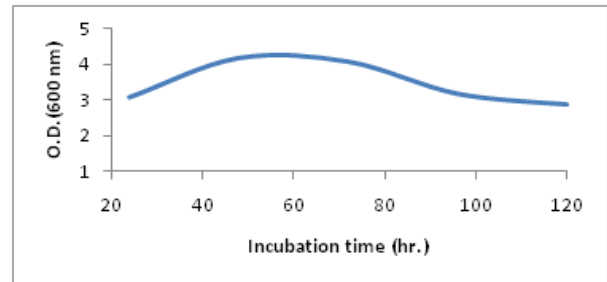


Figure 2: Incubation time effect on bacterial growth.

After 72 h micronutrients were decreased, and there was an increase in metabolites, this showed the depressing result of bacterial growth and lead to decrease in PHA production [24].

3-pH effect on growth of bacteria

Low and high pH decreased the bacterial growth, while pH 7 was optimized for growth as in Figure 3.

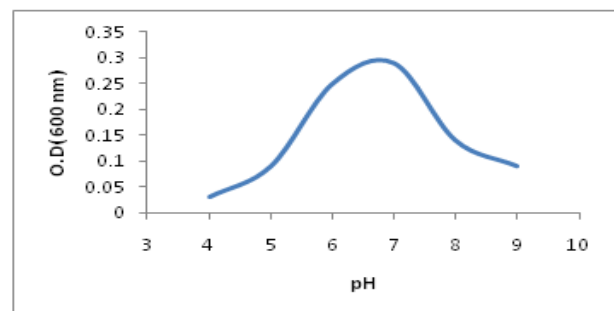


Figure 3: pH effect on growth of bacteria.

In general, slight changes in pH affected on metabolic process of bacteria. (25) Exhibited that pH range from 6.5–7.5 caused greatest PHA production by *B. sphaericus*, while higher pH value decreased the production and this owing to its effect on the enzymes which degraded bioplastic.

4-NaCl effect on Bacterial growth

The 0.25% NaCl concentration was suitable for growth of all the PHA isolates, and increasing concentration of NaCl resulted in the decrease of the growth Figure 4.

Salt concentration causes changes in bacterial growth depends on the osmotic balance required for such growth [14]. [17] Exhibited that concentration of 0.1% NaCl was appropriate for PHA bacterial producers.

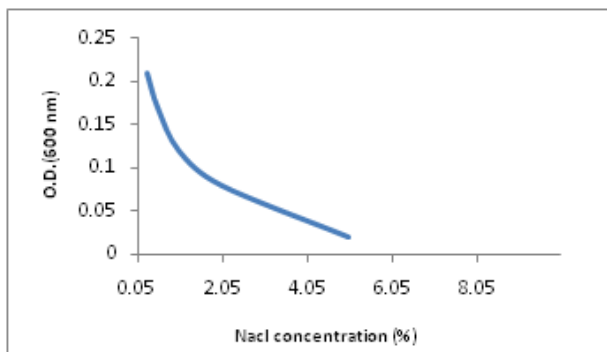


Figure 4: NaCl Concentration effect on Bacterial growth.

PHA Quantification

Bacillus subtilis was able to produce 0.26 g/l (40.625 %) of PHA Figure 5. [26] Showed that highest PHA production from the bacteria *Bacillus brevis* M6 was (41.67% w/v), while (27) exhibited that PHA product from the bacteria *P. corrugata* A.1 was 1.93 g/L (47.3 %).



Figure 5: PHA polymer after extraction and drying
Polymer analysis by FTIR.

Polymer analysis by FTIR

The functional groups present in the structure of PHA. It exhibited bands at 1286.56 and 1730.21 cm^{-1} which related to C-O and C=O stretch of the ester group present in the molecular chain of highly ordered crystalline structure, and corresponding to specific rotations around carbon atoms specific to certain functional groups. These bands exactly confirming that the extracted polymer is PHA.

Bands for $-\text{CH}_2$, CH_3 , and C-O-C groups occurred at 1411.94 cm^{-1} , 1357.58 cm^{-1} , and 1178.55 cm^{-1} . The absorption bands at 1566.25 and 3308.03 cm^{-1} related to C-H and terminal OH groups. These results related to (28) who showed the characteristic peaks at (1226.77, 1454.38 and 1726.35) cm^{-1} corresponds to the (C-O, C-H and C=O) respectively, and refer to the functional groups present in the structure of pure PHA.

Biochemical and molecular characterization of PHA-producing bacteria

The selected isolates were identified as *Bacillus sp.* according to morphological and biochemical characterization Table 3.

Table 3: Morphological and Biochemical characteristics of *Bacillus* isolate.

Morphological character	Result
Color of colony	White
Texture of colony	Smooth
Gram stain	Positive
Shape of cell	Rod
Spore formation	Positive
Biochemical test	Result
Catalase	Positive
Indole test	Positive
MR-test	Negative
VP-Test	Negative
Citrate utilization test	Negative
H ₂ S test	Positive

PCR performed by using 16s rRNA primer for *Bacillus* spp. From the results it found in 100% of testing isolates and the product size was (303 bp) compared to the ladder Figure 6 and the strain NE1MOK was identified as gram positive *Bacillus subtilis*.

(3) Exhibited that 15 isolates of *Bacillus sp.* NA10 accumulated PHA from cardboard industry waste water, while [26] showed that 29 isolates of *Bacillus* from soil in Turkey could accumulate PHB.

After NE1MOK isolates was identified and confirmed as *Bacillus subtilis*, this sequence

matched with bacterial sequences existing in the GenBank database Figure 7. The sequence of this species is 100% similarity with *B.subtilis* according to GenBank: KX840010.1.

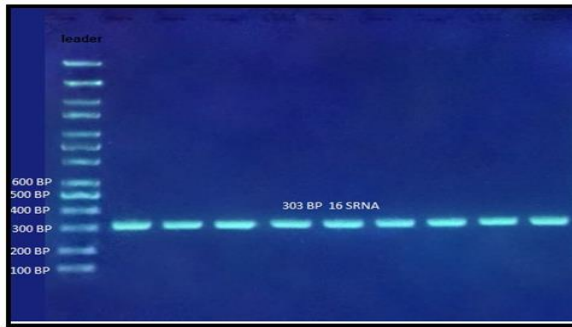


Figure 6: Amplification of 16S rRNA for *Bacillus* spp. (1.5% agarose, 7V/cm, for 120 min).

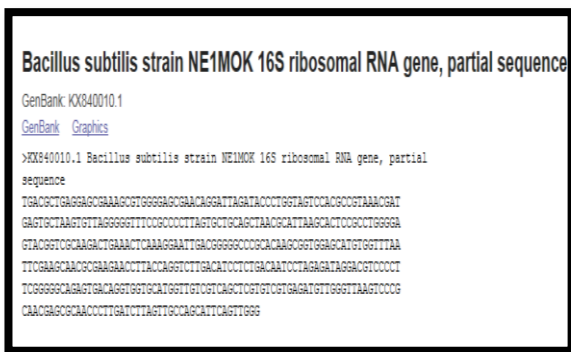


Figure 7: Sequence of 16s rRNA gene of *B.subtilis* NE1MOK.

[29] used S4 isolate to show relation to *Bacillus aryabhatai* (similarity was 99.70%) based on 16S rRNA sequences, which could produce PHA. [30] also used 16S specific primers for samples, and analyzed phylogenetically to detect *Bacillus subtilis*.

Molecular determination for synthase gene

Products of PCR were showed both class I and II PHA synthase gene in all tested isolates, and the length of PCR amplicons expected of 505 bp of PHA synthase Figure 8.

According to related searches, these two genes were accountable for the synthesis of PHA accumulation. [31] Showed that all PCR amplicons of PHA synthase possessed the expected length of 551 bp.

If soil bacteria have the potency to accumulate PHA, then over-expressed by bioplastic genes were increased the production of PHA [12].

Azotobacter spp. could produce PHA, and genes accounts of PHA synthesis were recognized. But the regulation of the genetic expression of these genes was very strong in the bacteria and also little [32].

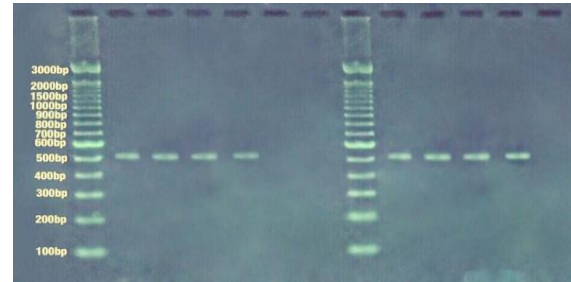


Figure 8: PCR results of PHA synthase genes of *Bacillus subtilis* .

Conclusions

At present, bacteria showed high ability to accumulate biodegradable plastic particles in their cells, so researches focusing on developing methods to isolate producing bacteria from natural sources. Among 9 selected isolates from agricultural wastes, NE1MOK strain belongs to *Bacillus subtilis* has the ability to produce (0.26 g/l (40.625 %) of PHA. Also numerous physical parameters (Temperature, pH, Time incubation and Nacl concentration) affected on bacterial growth. Synthase genes (505 bp) were accountable for PHA synthesis in all isolates of producing bacteria.

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