Original Research Article

Isolation and Identification of biopolymer polyhydroxybutyrate (PHB) by bacterial strains isolated from Soil of Al-Kharj, Saudi Arabia

ABSTRACT

Polyhydroxybutyrate (PHB) is one of Polyhydroxyalkonate (PHAs) group which are biopolymers that have many medical applications such as heart valves, scaffold, suture, and drug delivery. Samples isolated from different natural environments in Al Kharj south of Riyadh, Kingdom of Saudi Arabia were examined for microorganisms that are able to produce poly (3-hydroxy butyrate-ku-4 hydroxypoterate).

Among 50 isolates. After staining with Nile red stain, the only lighted bacterial isolates were selected. Further identification and characterization for the strongest florescent strain using biochemical tests. One of these two strain (G-4) was identified as *Bacillus* sp. For further confirmation, PHB was extracted from the G-4 isolate and analyzed by IR. The production conditions were improved with various sources of carbon and nitrogen, the highest production of PHB with peptone and ammonium chloride was observed. It was found that despite of the type of the polymer-containing media used, the optimum incubation temperature for the tested biopolymer-degrading microorganism was 35°C, while pH value of 7.2 resulted in the best growth pattern in date palm syrup at concentration of 8 % containing media.

***Keywords**: Isolation _ Polyhydroxyalkanoate _ Nile red _ PHA producer _ Microorganisms*

1. INTRODUCTION

Plastics are very useful materials. A special feature of plastics is being as synthetic polymer which allows for any chemical manipulation resulting in a wide range of products with different strength and shape [1-2].

Recently, there has been a lot of concerns about the impact of petrochemical plastics on the environment. The bioplastics are used now as a product friend to the environment and help in solving the pollution problems caused by the synthetic plastics [3, 4]. Among a variety of biodegradable polymer materials, polyhydroxy alkanoates (PHAs), representing a possible replacement for conventional petrochemical plastics due to some reasons [5]**.** The PHAs have similar properties to numerous thermoplastics and elastomers, which further justify their use as substitutes for current plastics. A major advantage of these materials is their capability to completely degrade upon disposal under various environments [6]. A possible mechanism is that they accumulate in numerous microorganisms at specific conditions as a mechanism of storage excess carbon and energy [7]. When PHAs are exposed to soil, compost or marine sediment, they undergo a process called biodegradation [1]. Factors that might interfere with the biodegradation of PHAs are the size of the exposed surface area, pH, moisture, temperature, and molecular weight [8]. There are a long range of Gram +ve and Gram -ve bacteria from at least 75 different genera can synthesize polyesters (PHAs) in various forms [9, 10]. These materials supposed to accumulated intracellularly to reach a level as high as 90% of the total cell dry weight at specific conditions [11]. This study aimed to isolation and characterization of bacteria producing PHB as a nature and nontoxic polymer. Moreover, utilizing the inexpensive

resources and waste materials available in the local environment screen for production valuable products,

2. MATERIAL AND METHODS

2.1. Isolation and selection of PHB-producing strain

Soil samples were collected from different localities in Al-Kharj, kingdom of Saudi Arabia The isolation procedure of all isolates was described by Bormann et al., [12] as follows: 100 µl from each samples were used to inoculate 15 ml test tubes contained 5 ml of (MSM) 0.05% (w/v) NH_4Cl, 0.74%(w/v) KH_2PO_4, NaCl, 2.46g $MgSO_4$ $7H_2O$, 82 mg EDTA, 1.25 mg $ZnCl_2$, 0.75 mg Mn Cl_2, 7.5mg H_3BO_3, 5 mg $CoSO_4$ 7 H_2O, 0.25 mg $CuCl_2$ $7H_2O$, 0.75 mg Na_2MoO_4. $2H_2O$, 0.5 mg $NiCl_2$. 6 H_2O and 7.0 mg $FeCl_3$. 6 H_2O supplemented with 1 mM from the appropriate carbon source. The culture was incubated at 30C for 24 hr, then 100 µl was transferred to newly prepared medium. 1 ml culture was transferred to 100 ml of MSM and 5 mM of the carbon source. After inoculating many time on MSM agar plates pure cultures of bacteria were isolated. Screening test for the production of the PHAs by different types of microorganisms using isolated strains was performed using Nile-Red [9-diethylamino-5H-benzo α- phenoxazine-5-one. (Sigma)] staining approach [13]. 20 µl of Nile red stock solution was spread onto sterilized LB agar plates to reach a final concentration of 0.5 µg Nile Red/ml medium. The plates were incubated after inoculation overnight at 35°C. The plates were then exposed to ultraviolet light at 300 nm to detect the accumulation of the PHB. The lighted plates were recorded positives PHB production and these isolates were selected for the subsequent identification [14].

2.2. Infra-Red (IR) spectroscopic analysis of PHB

The extracted polymer was qualitative analyzed by Fourier transform infrared spectroscopy (FTIR) (FT-IR - 4100, Jasco, Europe) to determine its functional groups contents. According to the method of Gopi et al., [15], 1mg of each of the PHB standard and the extracted were dissolved in 5 ml of chloroform, then chloroform was evaporated and KBr pellet. IR spectra were recorded in 4000 cm-1 to 400 cm- 1 range.

2.3. Optimization of cultural conditions for maximum PHB production

The production of PHB between the positive isolates was affected by the culture conditions. Therefore, different media: YT medium: 16g trypton, 10g Yeast extract and 10g NaCl [16], SG medium: 25%, (w/v): NaCl, 2%(w/v): $MgSO_4.7$ H_2O, 0.2%, (w/v): KCl, 3%, (w/v): Tri Sodium citrate, 1% (w/v): Yeast extract and 0.75%, w/v): Casmino acids [16] and LB medium: 10 g NaCl, 10 g trypton and 5g yeast extract [17] were used with different cultivation conditions and the composition of each medium was changed throughout the duration of each experiment [18]. In this study, incubation temperature, pH, various carbon sources, different concentrations of phosphate (KH_2PO_4) and nitrogen (NH_4Cl) were tested, with respect to their PHB accumulation, in flasks that incubated on a shaker (200 rpm).

2.4. Extraction and estimation of PHB production efficiency

PHB was extracted from the *Bacillus* by three methods, the first is the extraction by chloroform according the methods of Hahn et al., [19] as follows, cells were collected by centrifugation at 4000 xg for 20 min at 25 ºC, washed with acetone for 20 min. The dried cells were mixed with 50 volumes of chloroform for 48 h at 30ºC. Centrifugation was used for recovery of PHB Finally, pure PHB was obtained by precipitation with a mixture of methanol and water (3:7 v/v) followed by filtration. Second method was the extraction using NaOH as strong base according to the method of Kunasundari and Sudesh, [20]. Cells were collected by

centrifugation at 4000 xg for 20 min at 25ºC and washed with water. The pelleted cells were dissolved in 0.2 N NaOH at 30ºC for 1 h. After one hour the cells were collected by centrifugation at 4000 xg and the pellet was washed by ethanol and acetone. The PHB was leaved in oven at 40ºC until it completely dried. Third method was extraction using sodium hybochlorite according to methods of Daniel et al., [21]. To about 0.2 g of lyophilized cell biomass, 5 ml NaOCl (12 %) was added and the mixture was leaved at 40ºC for 1 h. Subsequently, the PHB granules were collected by centrifugation (2000 xg). The pellet was then washed by water then by ethanol and acetone The insoluble residue was discarded and the pellet was dissolved in chloroform. the PHB granules were collected and weighed after evaporation of chloroform.

2.5. Quantification of PHA in cell suspensions

At the present work, a microtiter plates/Nile-Red PHAs quantification method was developed [22]. For each well in a 96 well microtiter plate (Biomaster), 270 µl from the optimized growth medium (MM) 4g $NaNO_3$, 1.5g KH_2PO_4, 0.5g Na_2HPO_4, 0.001g $FeSO_4.7H_2O$, 0.2g $MgSO_4.7H_2O$, and 0.01g $CaCl_2$ and pH was adjusted to 7.0 were added. Subsequently, 20 µl from the overnight growth culture for each isolate were supplied. The plate was then covered with sterilized sticky paper and incubated at 35°C for 24 hours. After the incubation period, 10 µl of Nile Red stock solution was added to each well leaved 10 minutes and read on the fluroscan apparatus (FluroScan, Labosystem, Finland) at wavelengths 960 and 450 nm. A standard curve by adding a serial dilution of a pure PHB instead of the growth culture was constructed. At least six replicates for each dilution were ruined at the same time. Recovery of pure PHB concentration gave excellent results.

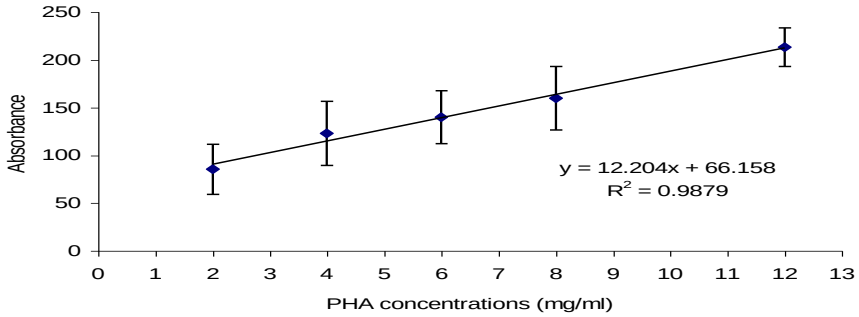

Fig (1): Standard curve showing the low and high PHAs detection concentration limits by using the developed PHB quantification technique.

2.6. Identification of PHB producing isolate

2.6.1. Morphological and biochemical test

The characteristics of colony as its pigment, ability to hydrolyze starch, gelatin, casein and cellulose was detected. Also, the biochemical tests as indole production, methyl red, Vogues Proskauer, citrate utilization, motility, catalase were determined. The fermentation with sugar was also studied by spreading the isolate on medium containing different types of sugars (sucrose, galactose, glucose, fructose, sorbitol, xylose, mannose, rhaffinose, lactose, and ribose. The characteristics of the isolates were compared with the data from Bergey's Manual of Determinative Bacteriology [23].

3. RESULTS AND DISCUSSION

This study has aimed to look for a novel bacterial strain isolated from the soil of Al-Kharj, KSA, able to accumulate in high amount the biodegradable biopolymer poly (3-hydroxybutyric acid) [PHB]. This target was achieved by screening a culture collection consists of 50 bacterial isolates for presence/absence of PHB using a Nile-red staining approach. To quantify the PHB directly inside the bacterial cells without prior extraction of the polymer, an approach based on measuring the PHB content of cell suspensions in microtiter plates was developed. Analysis of the extracted biopolymer using IR spectra was performed. In addition, the amount of accumulated PHB was optimized using different growth conditions. Due to the high production cost of the PHB based on the use of peptone as a carbon source, alternative inexpensive substrates such as date palm syrup was examined.

3.1. Screening of culture collection for PHB producers

By using a Nile-red staining assay, screening of the culture collection for presence/ absence of PHB was performed. Two out of 50 bacterial isolates yielded positive results (Fig 2). The results show that the isolate G4 exhibited a very strong fluorescence in comparing to the negative controls, therefore, they recorded as PHB positives. The morphological and physiological characteristics of G-4 were summarized as shown in Table 1.

3.2. Identification of the efficient of PHB production isolate

Preliminary identification indicated that G-4 isolate was Gram positive-bacilli, spore forming bacteria, catalase positive, urease positive, indole formation negative, methyl red test positive, and Voges-Proskauer test negative, citrate test positive and formation of glucose, lactose, mannitol positive but without gasses in glucose and grow at 10, 30, 37°C and NaCl 7%. The results clearly showed that G4 most probably is species of Bacillus sp. According to Bergey's Manual of Systematic Bacteriology [23]. The result obtained by Hassan et al., [24] was the most results similar to our results, who interest in the identification of *Bacillus* sp. strains. Other studies cleared that the production of PHB can be isolated from microorganisms belonging to the genera *Azotobacter, Alcaligenes, Pseudomonas*, and *Bacillus* [25]. Also other studies reported, PHB can be isolated from *Bacillus* spp. isolated from natural sources [26, 27, 28].

3.3. Extraction of PHB from bacterial cells

To extract the PHB, various extraction methods either by chloroform, sodium hypochlorite and sodium hydroxide were performed. The results presented in Fig. (3) clearly show that the use of chloroform extraction method was time consuming and yielded low purity. On the other hand, the use of NaOH gave the best results than the chloroform and sodium hypochlorite.

3.4. IR spectroscopic for analysis of the extracted PHB

As shown in Fig (4) the absorption bands appeared in the spectrum are associated with the side chains from the ester C=O stretching vibration at 1727 cm^{-1}, the CH_3-deformation peak at 1286 cm^{-1} and the ester C-O-C at 1072 cm^{-1} which clearly put the extracted polymer sample obtained from isolate G-4 in the class of polyhydroxybutyric acid. Gurubasappa et al., [29] reported that, FTIR spectra of the extracted polymer show peaks at 1731.92 cm-1 and 1215.47 cm-1 corresponds to specific rotations around carbon atoms specific to certain functional groups. Another study carried out by Oliveira et al., [30] show the peak at 1731.92 cm-1 corresponds to C=O stretch of the ester group present in the molecular chain of highly ordered crystalline structure. While the study of Rohini et al., [31] reported, the peak at 1215.47 cm-1 corresponds to –CH group. These peaks are corresponding to the peaks obtained for the standard PHB, at 1730 cm-1 and 1216 cm-1 exactly confirming that the extracted polymer is PHB and the Fourier-transform infrared (FTIR) absorption band at about 1,730 cm-1 is a characteristic of

the carbonyl group and that a band at about 1,280–1,053 cm-1 characterizes valance vibration of the carboxyl group.

3.5. Optimization of the growth conditions

3.5.1. Effect of different fermentation media on Cell growth

To achieve enough biomass from isolate G-4 required as inoculum for PHB production, various growth media (YT, LB and SG) and incubation times (24, 48 and 72 hr) at 30ºC were changed. As shown in Fig. (5), YT medium yielded the highest cell biomass, which therefore used in the further studies and incubation time for 72 hours (Fig. 6).

3.5.2. The effect of different (pH) on the cell growth of the bacterial isolate G-4

In this experiment, the optimized (MSM) was prepared as previously described and the medium was initially adjusted at different pH values ranged from 5.5 to 8.5. Under the optimized growth conditions (incubation at 37°C for 48 hours in YT medium, the effect of pH on the growth rate of isolate G-4 was examined. As shown in Fig. (7), the results revealed that the highest growth rate (O.D 600 =0.85) was observed at pH 7.2. Rukman et al., [2] investigated the *Halomonas elongate* can used as bioplastic producer. The indication as bioplastics Poly-hydroxybutyrate (PHB) producer was evaluated by growing in Nile red-containing medium and bacterial colonies displayed bright orange fluorescent under ultraviolet light. The effect of different carbon sources, nitrogen, and pH values on PHB production by strains of *Rhizobium meliloti* was investigated by Tavernler et al., [32], these strains showed higher PHB content at pH 7.0. These findings are in agreement our previous study [33, 34]. These results supported by Sangkharak and Prasertsan, [35]. Pozo et al., [36] studied effects of culture conditions on PHB production by *Azotobacter* sp. and showed that growth conditions including pH, temperature plays an important role in the production rate of PHB.

3.5.3. Effect of different carbon sources on the production of PHB

Under the optimized growth conditions, different concentrations of various carbon sources (2-40g/l) were tested. The data presented in Fig. (8) show that the PHB yield was recorded as follows: glucose (10g), mannose (5g/l), maltose (9g/l), lactose (10g/l), sucrose (20g/l), Yeast extract (15g/l). The strain *P. hydrogenovora* DSM 1749 is able to cometabolize glucose and galactose from lactose-hydrolyzed whey permeate to produce PHB [37].

Pseudomonas cepacia ATCC 1775 produce high quantity of PHB at supplementation of culture media with lactose and xylose [38]. While Gerhart, [39] reported that *Pseudomonas hydrogenovora* produce PHA when the media supplemented with whey permeate. Nath et al., [40] reported PHB production achieve from lactose and sucrose supplementation by *Methylobacterium* sp. ZP24 and from bagasse as well as from food wastes [41].

3.5.4. PHB production from date palm syrup khalas as inexpensive Carbone source

The effect of different concentrations of date palm syrup (1-30%; v/v) as inexpensive carbon source in (MSM) with incubation time for 48 h at 37°C, and date palm syrup as a carbon source with different concentrations ranged from zero to 30% (v/v) were tested. The data revealed that the highest amount of PHB (5mg/ml) produced by the isolate G-4 was achieved with date palm syrup (Khalas) concentration of 8% (Fig 9). Page, [42] reported that, molasses, the byproduct in sugarcane industry has been used as a carbon source in PHB production by *Azotobacter vinelandii* instead of glucose. While, Page, [43] reported that the *Rhizobium* produced less PHB in yeast extract mannitol (YEM) broth media with different carbon such as sucrose, arabinose and glucose.

3.5.5. The effect of ammonium and phosphate on PHB production

The effect of phosphate (KH_2PO_4) and ammonium (NH_4Cl) on the PHB production was examined. Isolate G-4 was grown under the above-optimized growth conditions in (MSM) containing various concentrations of ammonium and phosphate ranged from 0.1-1% (w/v). As shown in (Fig 10 & 11), it was observed that the concentrations of phosphate (0.04%) and ammonium (0.2%) gave the highest PHB yield. The results of this study were agreement with

those obtained by Shaaban et al., [44]; Khanna and Srivastav, [45], who reported, the high content of PHB production at using MSM medium supplemented with ammonium sulphate by *R. eutropha*, *Stenotrophomonas* sp. and *Pseudomonas* sp. As well as Raje and Srivastav, [46] reported PHB accumulated by *A. eutrophus* with different supplementation of the culture media with ammonium salts.

Table 1: Morphological and biochemical characterization of the isolate G-4

Test	Reaction
Staining	Gram positive Bacilli
Spore staining	Spore forming bacteria
Biochemical properties	
Catalase test	Production of gas bubbles
Urease test	+ve
Indole formation	- ve
Methyl red test	- ve
Voges-Proskauer test	+ve
Citrate test	+ve
Growth in NaCl 7%	Grow
Growth at 10, 30 and 37°C	Grow
Hydrolysis of:	
Casein	+ve
Cellulose	+ve
Starch	- ve
Carbohydrates fermentation	
Glucose	+ve
Sucrose	+ve
Fructose	+ve
Lactose	- ve
Xylose	+ve
Ribose	+ve

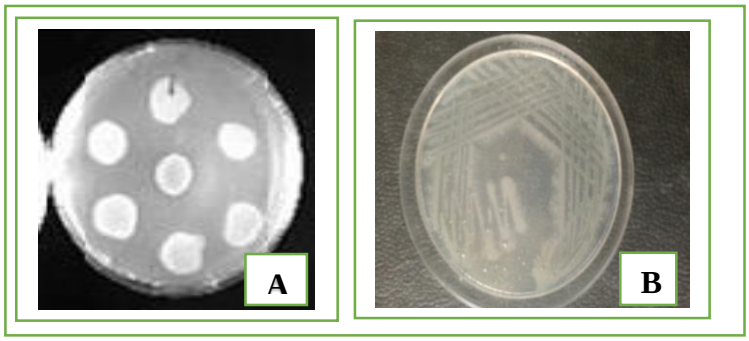

Fig 2: Nile-red staining assay for presence/absence of PHB, (A) is the positive isolate & (B) showing negative isolate

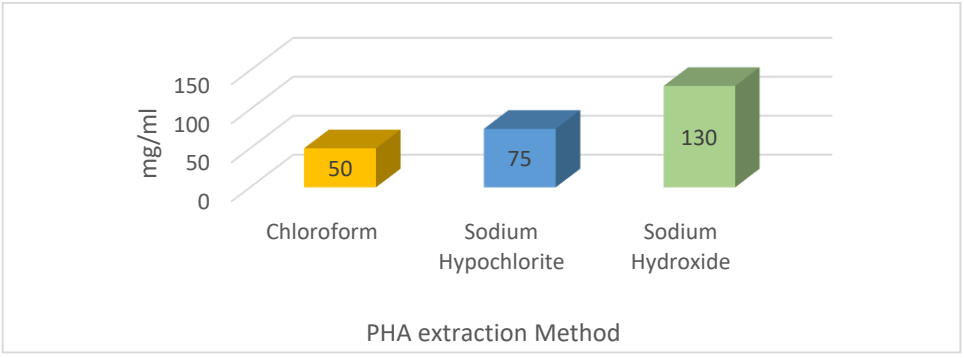

Fig. (3): PHA yields extracted from 100 ml bacterial growth culture with different extraction methods.

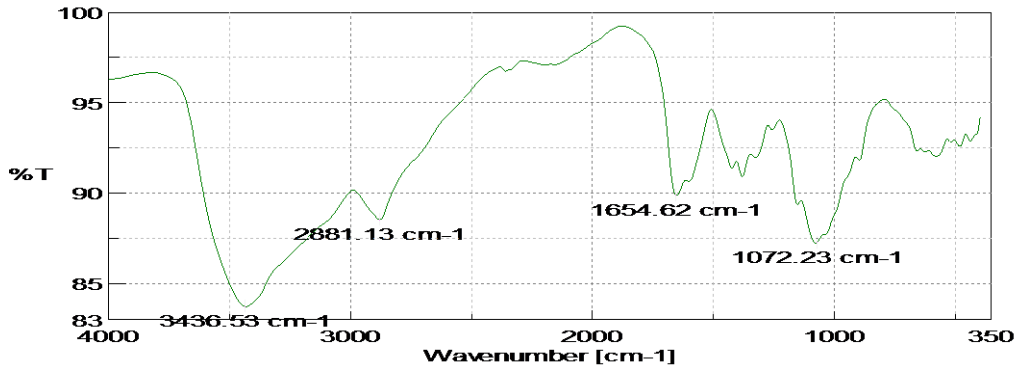

Fig. 4: IR spectrum of the extracted biopolymer from isolate G-4

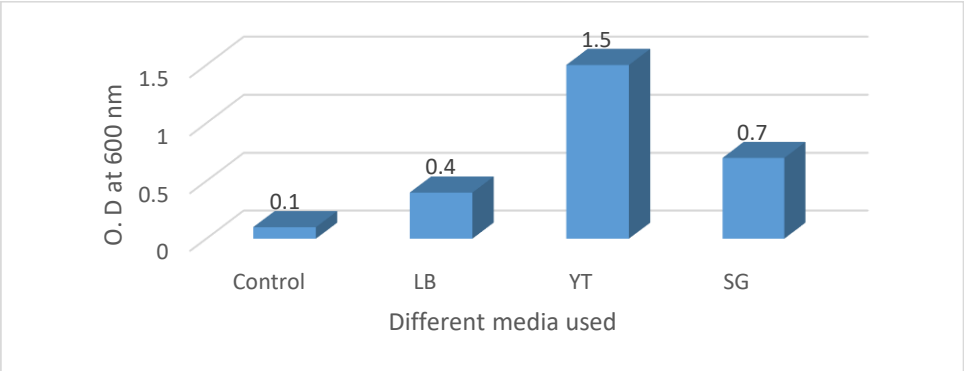

Fig 5: Growth optimization of isolate G-4 on different nutrient media

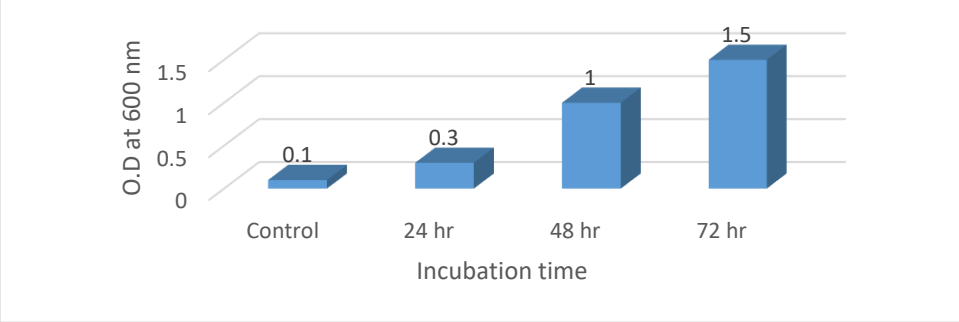

Fig 6: Growth optimization of isolate G-4 on different incubation times.

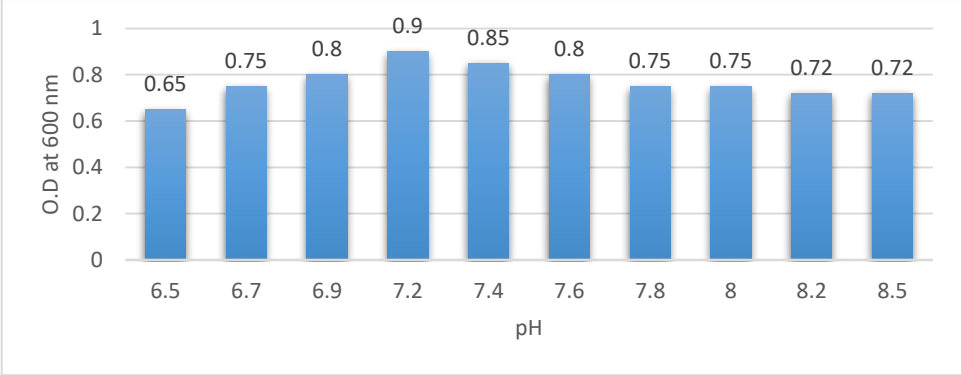

Fig 7**:** The effect of different pH values on the growth of bacterial isolate G-4.

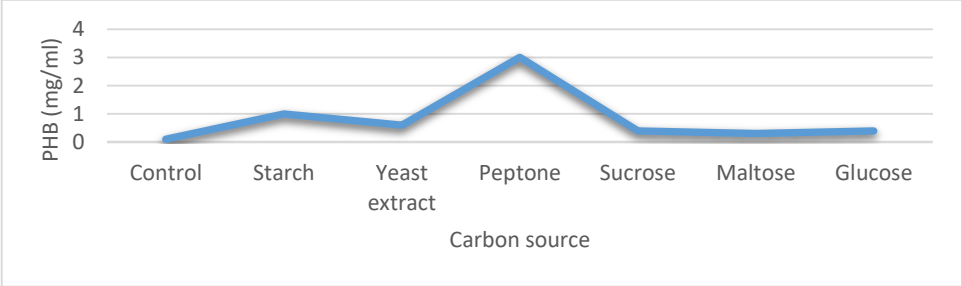

Fig 8: The effect of different carbon sources on PHB content

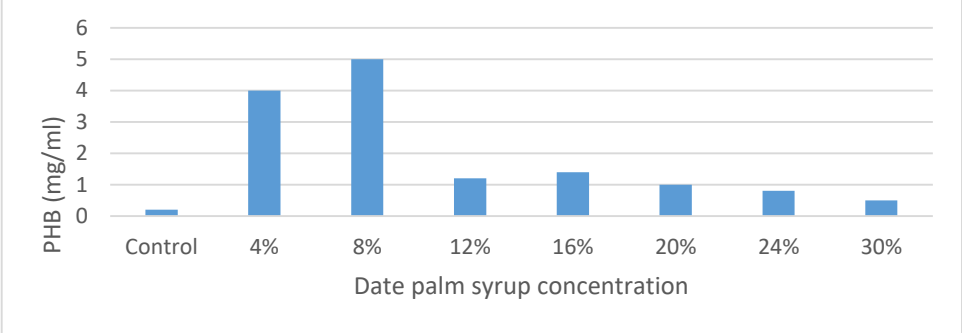

Fig 9: The effect of different Date Palm syrup concentrations on the production of PHB

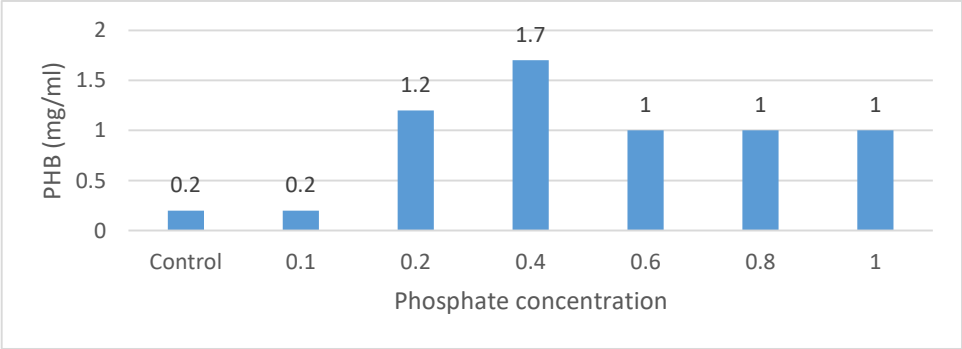

Fig 10: The effect of different Phosphate concentrations on the production of PHB

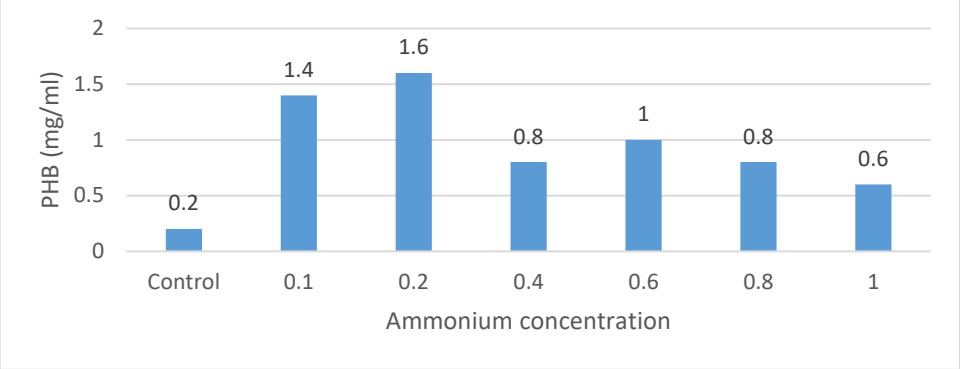

Fig 11: The effect of different Ammonium concentrations on the production of PHB

4. CONCLUSION

In the present study, many of bacterial isolates were obtained from soil in Al-Kharj and screened by Nile red as detector for PHB production and using date palm syrup as inexpensive carbon source. One bacterial isolate G-4 was selected according to its lighted strength with Nile red, and it was successfully characterized and identified using morphological and biochemical characters, it belongs to *Bacillus* sp. Moreover, the using NaOH in the extraction process give 72% of PHB recovery cell dry weight. Moreover, the PHB produced was characterized using FTIR which confirm that the biopolymer produced is highly purified PHB. Also the optimization of culture conditions as pH, incubation time, type of culture media and different types of carbon sources were done on the *Bacillus* Sp. G-4.

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