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Isolation and Identification of Bacterial Strains Able to Biopolymer Polyhydroxybutyrate (Phb) Production from Soil of Al-Kharj Probes, Saudi Arabia

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Author's contribution

The sole author designed, analyzed and interpreted and prepared the manuscript.

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ABSTRACT

Polyhydroxyalkanoates (PHAs), which is produced by several bacteria, is a biodegradable polymer that has many industrial and medical applications such as heart valves, scaffold, suture and drug delivery. The aim of the present study is to isolate and identify the bacteria producing polyhydroxybutrate (PHB) from the soil sample of Al-Kharj, south of Riyadh, Kingdom of Saudi Arabia. After staining the bacterial isolates with Nile red stain, the only lighted isolates were selected for further identification. The strongest florescent strain (G-4) was identified by morphological and biochemical tests as *Bacillus* sp. For further confirmation, PHB was extracted from the G-4 isolate by three different methods of extraction and analyzed by IR. The effect of different conditions on PHB produced by bacterial isolate (G-4) including carbon sources, nitrogen sources, incubation temperatures, pH and incubation periods were studied. The highest production of PHB was observed with cultural media containing 8% of date palm syrup (Khalas) at the pH of

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7.2. These results show that *Bacillus* species identified in soil sample of Al-Kharj produced highest concentration of PHB by using date palm syrup as a carbon source that can substantially reduce substrate and production costs of PHB.

Keywords: Polyhydroxybutrate; date palm syrup; nile red; PHA producer; Bacillus sp.

1. INTRODUCTION

Plastics are synthetic polymers which have wide applications ranging from packaging, domestic, aquatic and architectural industries. The synthetic plastics are characterized by light weight, and durability. Due to their non-biodegradable nature, they accumulate in the environment causing global environmental pollution [1]. Now need arises to develop biodegradable plastics that will opened a way for new strategies for waste management. Polyhydroxybutyrate (PHB) is one of the most biodegradable and biocompatible thermoplastic synthesized by many microorganisms, collectively called polyhydroxyalkanoates (PHAs) [2,3]. These biodegradable plastics are considered the best solution for solving the environmental pollution problems by replacing conventional plastics industries [4]. Because of their biological nature, PHAs completely hydrolyzes into water and carbon dioxide by different microorganisms found in soil [5,6]. Because of its biodegradable characteristics, these compounds can be used as biodegradable carriers for long-term dosage of drugs, medicines, hormones, insecticides and herbicides [7]. They are also used as osteosclerotic stimulants owing to their piezoelectric properties, in bone plates, surgical sutures and blood vessel replacements [8]. PHB might be synthesized by wide range of Gram positive and negative bacteria from different genera [9]. These PHB producing bacteria should have optimal cultural conditions including high concentration of carbon source and limited concentration of nitrogen, phosphorus, sulphur and/or trace elements [10]. In fact, to commercialize PHAs, substantial effort has been devoted to reducing the production cost through the development of bacterial strains and more efficient fermentation/recovery processes because the price of the substrate has the largest influence on the production cost of PHA [11]. The flora of microorganisms in Al-Kharj area are not utilized, therefore, this study will utilize these natural resources for production of useful biodegradable biopolymer and using inexpensive resources available in Saudi Arabia such as date palm syrup as alternate carbon source for production of PHB from the isolate (G-4).

2. MATERIALS AND METHODS

2.1 Isolation and Selection of PHB-Producing Bacteria

Soil samples collected from different localities in Al-Kharj were used for isolation of the PHB producing bacteria according to the method of Bormann et al. [12] as follows. 1.0 g of sample was serially diluted in sterile distilled water and plated onto nutrient agar plates and incubated at 30ºC for 24 hours. Various colonies of different morphologies were individually picked and subcultured 3-4 times on nutrient agar plates. The bacterial colonies were streaked on nutrient agar slants, incubated at 30ºC overnight and then stored at 4ºC for further use. Screening test for the PHB production of the 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 Extraction of Date Syrup with Water

Date pulp (100 gm) of date fruits (*Phoenix dactylifera* L.) of Khalas variety at the tamr stage of maturity put in an erlenmeyer flask (1 L) and water were added at 1:5 ratios. The date pulp/water sample was blending using a hand-held blender (Phillips, Holland). The pH was adjusted to 6.0 ± 0.2. The sample, in triplicate, was placed in water bath at 70ºC for 2 h. After heating the slurry was filtered through a cheese cloth with a hand press to remove large impurities and insoluble matters then centrifuged at 8000 g for 10 min, and the supernatant was decanted.

2.3 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] 1 mg 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.

2.4 Optimization of Cultural Conditions for Maximum PHB Production

The production of PHB from the positive isolate was affected by the culture conditions. Therefore, different media (LB) medium: 16 g trypton, 10 g Yeast extract and 10 g 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 Mineral Salt Medium (MSM): 0.05% (w/v) NH_4Cl, 0.74%(w/v) KH_2PO_4, NaCl, 2.46 g $MgSO_4\ 7H_2O$, 82 mg EDTA, 1.25 mg $ZnCl_2$, 0.75 mg Mn Cl_2, 7.5 mg 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 $FeCl_3.\ 6\ H_2O$ [17] were used with different cultivation conditions [18]. Minimal salts medium (MM): 4 g $NaNO_3$, 1.5 g KH_2PO_4, 0.5 g Na_2HPO_4, 0.001 g $FeSO_4.7H_2O$, 0.2 g $MgSO_4.7H_2O$, and 0.01 g $CaCl_2$ [17] was used as (control) in all experiments in this work.

For optimization of the best incubation periods to production of PHB, different incubation time (24h, 48h, 72h) were studied. For pH optimization, culture was incubated in carbon rich nutrient medium with different pH range from 6.5 to 8.5. Each culture was taken from different flask and PHA production was determined. Effect of media ingredients like carbon, phosphate and nitrogen sources on PHA production was determined by simply using carbon sources i.e. (starch, yeast, peptone, sucrose, maltose, glucose and date palm syrup), sugars were added at equivalent weights and the date palm syrup was used at different concentration ranged from 4% to 30% v/v. Also the effect of different concentration of potassium phosphate and ammonium chloride in range equivalent to 0.1 to 1% (v/v) were tested.

2.5 Extraction and Estimation of PHB Production Efficiency

PHB was extracted from the *Bacillus* by three methods, the first is the extraction by chloroform according to the methods of Hahn et al. [19] as follows, cells were collected by centrifugation at 4000 x g for 20 min at 25ºC, washed with acetone for 20 min. The dried cells were mixed with 50 volumes of chloroform for 48 h at 30°C. 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 Na OH 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 hypochlorite according to methods of Daniel et al. [21]. To about 0.2 g of lyophilized cell biomass, 5 ml Na OCl (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.

2.6 Quantifications of Bacterial Growth and Dry Weight

Cell growth was monitored by measuring the optical density (O.D) at 600 nm using spectrophotometer Ten milliliter culture medium was centrifuged at 10,000 rpm, 4ºC for 15 min and cell pellet was washed with 10 mL distilled water. Cell pellet was harvested by centrifugation and dried at 105ºC for 48 h [22] Cell mass concentration was determined by the standard calibration curve between OD 600 and cell dry weight.

PHA accumulation (%) = Dry weight of extracted PHA (g/L) / Cell dry weight (CDW) (g/L) × 100%

2.7 Quantification of PHA in Cell Suspensions

Standard PHA sample (0.02-0.1 g) was digested by heating in concentrated H_2SO_4 at 100ºC for 10 min estimated at 235 nm in UV/visible spectrophotometer to determine slope and easily calculate factor = 1/slope. By referring to the standard curve, the quantity of PHA produced was determined [23].

2.8 Identification of PHB Producing Isolate

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 [24].

2.9 Statistical Analysis

Statistical analysis was done using GraphPad InStat software. Results were expressed as mean ± SE. Statistical significance for data was determined using a one-way analysis of variance (ANOVA) with post-Dunnett test. The level of significance was accepted as $P < 0.05$.

3. RESULTS AND DISCUSSION

This research work is aimed to isolate and identify PHB producing bacteria by utilization of inexpensive alternate carbon sources native to Saudi Arabia such as date palm syrup. This target was achieved by screening of the bacterial isolates for their presence/absence of PHB using a Nile-red staining approach. The extracted PHB was analyzed and confirmed using IR spectra. Due to the high production cost of the PHB based on the use of multiple carbon sources, date palm syrup as alternative inexpensive substrates was used. By using a Nile-red staining assay, screening of the culture collection for presence/absence of PHB was performed. The bacterial isolate yielded positive results as indicated in (Fig 2). The results show that the isolate G-4 exhibited a very strong fluorescence in comparison to the negative controls, therefore recorded as PHB positive strains. The morphological and biochemical characteristics of G-4 were summarized as shown in Table 1.

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	+
Indole formation	-
Methyl red test	-
Voges-Proskauer test	+
Citrate test	+
Growth in NaCl 7%	Grow
Growth at 10, 30 and 37°C	Grow
Hydrolysis of	
Casein	+
Cellulose	+
Starch	-
Carbohydrates fermentation	
Glucose	+
Sucrose	+
Fructose	+
Lactose	-
Xylose	+
Ribose	+

Preliminary identification indicated that G-4 isolate was Gram + 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%. According to Bergey's Manual of Systematic Bacteriology, the isolate G-4 is most probably *Bacillus* sp. [24] which was also confirmed by the results obtained by Hassan et al. [25]. Some other studies reported that PHB can be isolated from microorganisms belonging to the genera *Azotobacter*, *Alcaligenes*, *Pseudomonas*, and *Bacillus* [26,27,28].

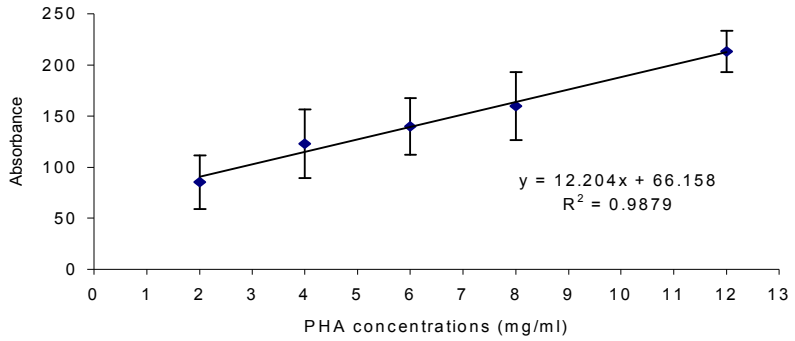

Fig. 1. Standard curve for PHA detection

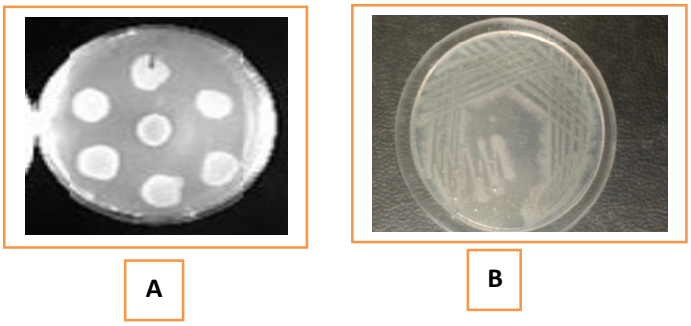

Fig. 2. Nile-red staining assay for presence/absence of PHB, (A) shows positive isolate & (B) negative isolate

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 Na OH gave the best results compared to chloroform and sodium hypochlorite.

As shown in Fig (5) 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.

To achieve enough biomass from isolate G-4 required as inoculum for PHB production, various growth media (LB, MSM and SG) and incubation times (24, 48 and 72 hr) at 30ºC were changed. As shown in Fig. 5, MSM medium yielded the highest cell biomass, which therefore used in the further studies and incubation time for 72 hours (Figs. 6 & 7).

The optimized (MSM) was prepared as previously described [17] and the medium was initially adjusted at different pH values ranged from 6.5 to 8.5. Under the optimized growth conditions (incubation at 37ºC for 48 hours in MSM medium, the effect of pH on the growth rate of isolate G-4 was examined. As shown in Fig. (8), the results revealed that the highest growth rate (O.D 600 =0.85) was observed at pH 7.2.

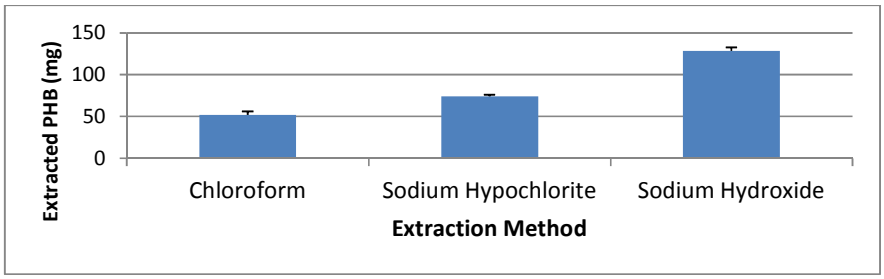

Fig. 3. PHB yields with using different extraction methods

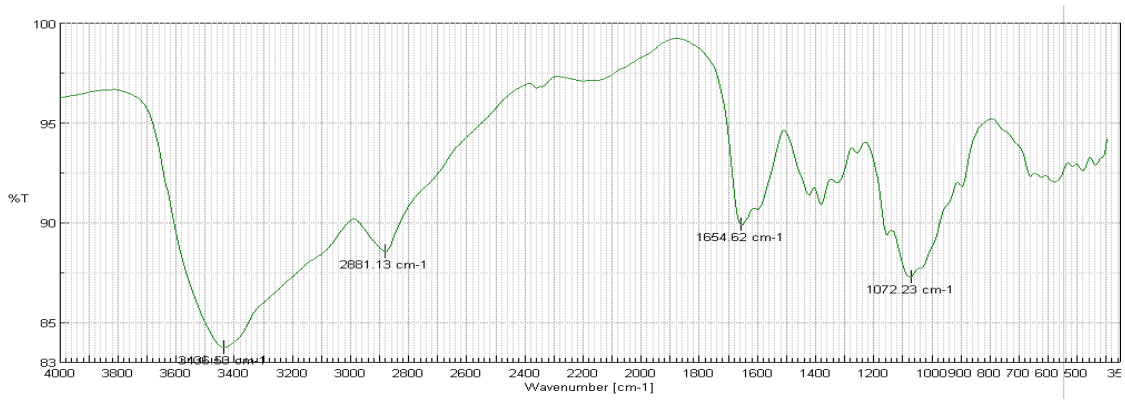

Fig. 4. IR spectrum of the standard PHB

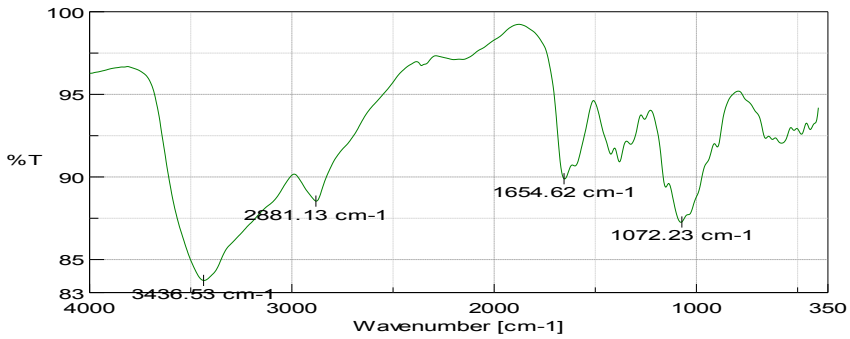

Fig. 5. IR spectrum of the extracted biopolymer from isolate G-4

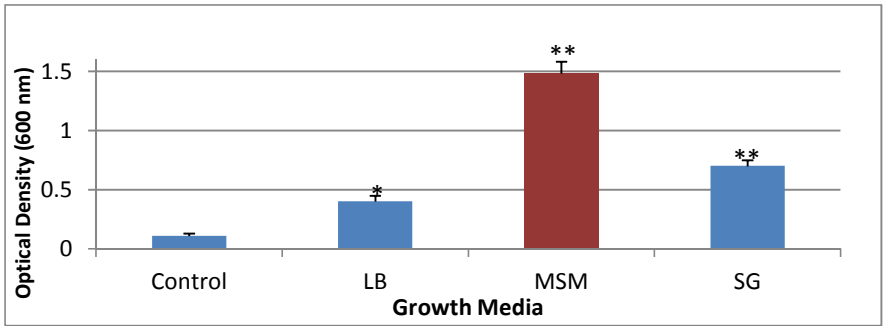

Fig. 6. Growth optimization of isolate G-4 on different nutrient media. Experiments were done in triplicate and data represented as an average ± standard deviation.

**= p<0.05; **= p<0.01*

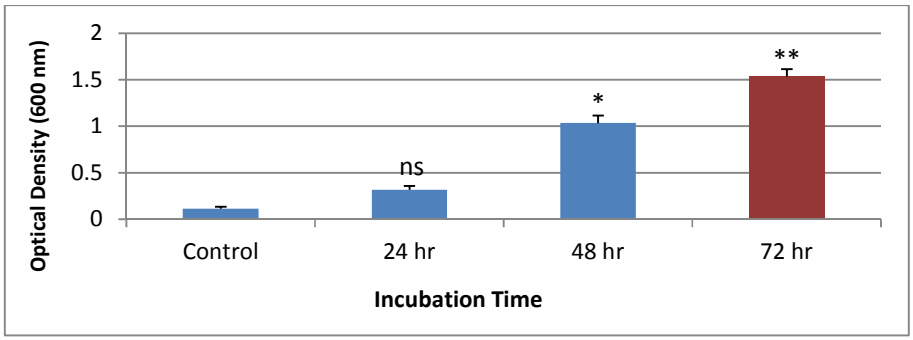

Fig. 7. Growth optimization of isolate G-4 on different incubation times. Experiments were done in triplicate and data represented as an average ± standard deviation.
*ns= Non significant; *= p<0.05; **= p<0.01*

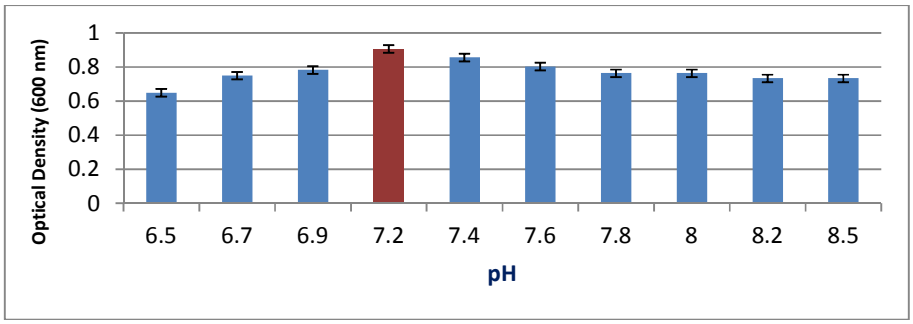

Fig. 8. The effect of different pH values on the growth of bacterial isolate G-4

Rukman et al. [32] investigated the Halomonas elongate can used as bioplastic producer. The indication as bioplastics PHB producer was evaluated by growing in Nile red-containing medium and bacterial colonies displayed bright orange fluorescent under ultraviolet light [33]. The effect of different carbon sources, nitrogen, and pH values on PHB production by strains of Rhizobium meliloti was investigated by Tavernler et al. [34] these strains showed higher PHB content at pH 7.0. These findings are in agreement previously reported studies [35]. These results supported by Sangkharak and Prasertsan [36]. Pozo et al. [11] 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.

Under the optimized growth conditions, different concentrations of various carbon sources (2-40 g/l) were tested. The data presented in Fig. (9) show that the PHB yield was recorded as follows: glucose (10 g), mannose (5 g/l), maltose (9 g/l), lactose (10 g/l), sucrose (20 g/l), Yeast extract (15 g/l).

The strain P. hydrogenovora DSM 1749 has been reported to co-metabolize glucose and galactose from lactose-hydrolyzed whey permeate to produce PHB [37,38]. *Pseudomonas* cepacia ATCC 1775 produce high quantity of PHB at supplementation of culture media with lactose and xylose [39]. Nath et al. [40] reported the PHB production from lactose and sucrose supplementation by Methylobacterium sp. ZP24 and from bagasse as well as from food wastes [41].

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 37ºC, were tested. The data revealed that the highest amount of PHB (5 mg/ml) produced by the isolate G-4 was achieved with date palm syrup at concentration of 8% (Fig. 10).

Page, [42] reported that instead of glucose, molasses, the byproduct in sugarcane industry can be used as a carbon source for PHB production by *Azotobacter vinelandii.*

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 (Figs. 11 & 12), 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. [43] Khanna and Srivastav [44], 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, [45] reported PHB accumulated by A. *eutrophus* with different supplementation of the culture media with ammonium salts.

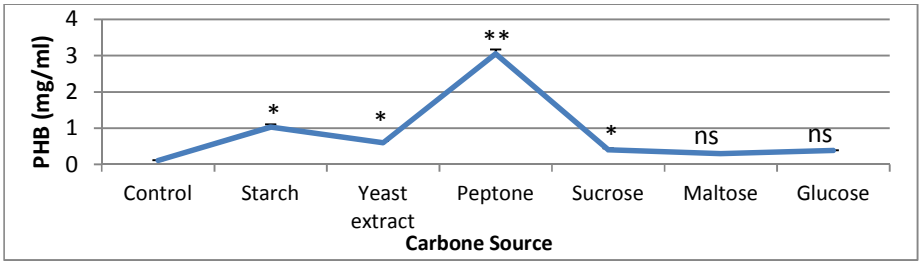

Fig. 9. The effect of different carbon sources on PHB content. Experiments were done in triplicate and data represented as an average ± standard deviation.
*ns=Non significant; *= p<0.05; **= p<0.01*

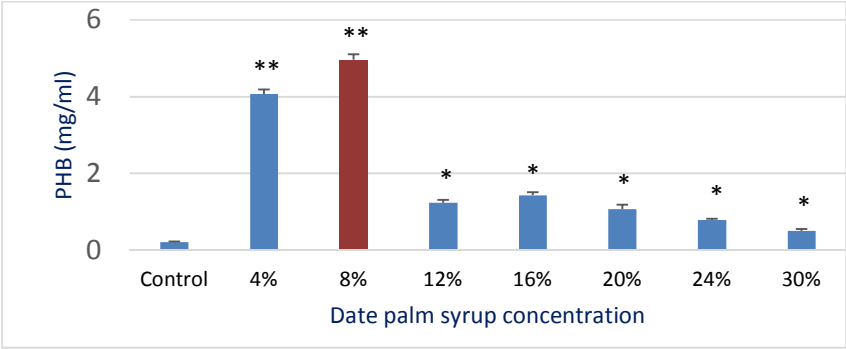

Fig. 10. The effect of different date palm syrup concentrations on the production of PHB. Experiments were done in triplicate and data represented as an average ± standard deviation.
**= p<0.05; **= p<0.01*

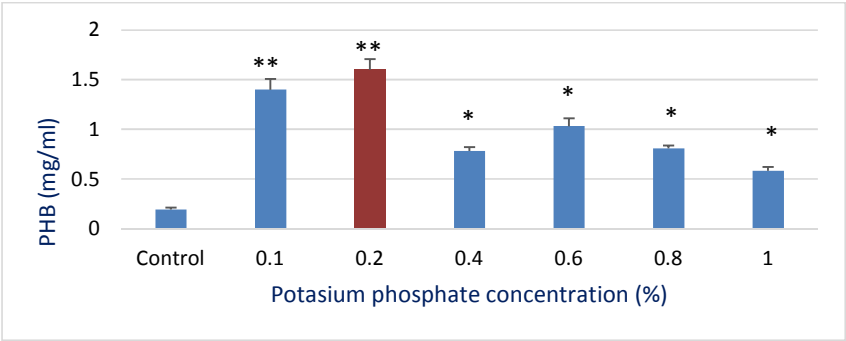

Fig. 11. The effect of different Phosphate concentrations on the production of PHB. Experiments were done in triplicate and data represented as an average ± standard deviation.
**= p<0.05; **= p<0.01*

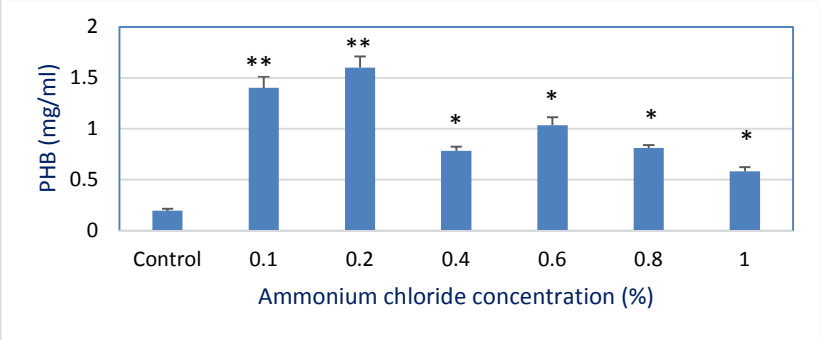

Fig. 12. The effect of different Ammonium concentrations on the production of PHB. Experiments were done in triplicate and data represented as an average ± standard deviation.
**= p<0.05; **= p<0.01*

4. CONCLUSION

These results show that *Bacillus* species identified in soil sample of Al-Kharj produced highest concentration of PHB by using date palm syrup as a carbon source that can substantially reduce substrate and production costs of PHB.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Author has declared that no competing interests exist.

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