Iran. J. Biotechnol. July 2021;19(3): e2849 DOI:10.30498/ijb.2021.244756.2849

Research Article

Production of Bioplastic (Polyhydroxybutyrate) with Local *Bacillus megaterium* Isolated from Petrochemical Wastewater

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Background: Polyhydroxybutyrate is a biodegradable plastic produced by some bacteria and can completely be replaced with petroleum based non-degradable plastics.

Objectives: This study was done to isolate and identify one local strain with a high-production ability for industrial purposes.

Material and Methods: The sampling from petrochemical wastewater was done. The existence of polyhydroxybutyrate in isolates was studied with Sudan Black staining. Using the Sudan Black B plate assay method and estimating produced PHB amount, the most potent isolate was chosen. This isolate was distinguished by morphological and biochemical methods and determining 16S rRNA gene sequencing. The final confirmation of polyhydroxybutyrate synthesis was done by FTIR and ^{1}H NMR. To increase more production of polyhydroxybutyrate, the effect of different factors including carbon, nitrogen, pH, and temperature were assessed.

Results: Six bacterial isolates producing polyhydroxybutyrate were separated, which among them, one new strain of *Bacillus megaterium* named saba.zh was selected as better isolation. 16S rRNA nucleotide sequence of bacterium was assigned accession number: MN519999 in the NCBI database. The optimal conditions to increase the production of polyhydroxybutyrate, are using glucose as a carbon source, ammonium sulfate as the nitrogen source, in the condition with having pH 7 and temperature 30 °C. After optimizing, the production of PHB increased from 56.51% to 85.41%.

Conclusions: This research indicated that *Bacillus megaterium* saba.zh, due to better polymer yield, is a potent PHB producer which can be used for PHB industrial production.

Keywords: Bacillus megaterium, Bioplastic, Petrochemical Wastewater, Polyhydroxybutyrate.

1. Background

Nowadays, among all contaminations, plastic is considered one of the important contaminations which result from polluting environment worldwide. The most important factor for pollution is due to the repletion of synthetic and petroleum-based plastics in the environment (1). Petroleum-based plastics are considered the main part of modern society. Moreover, due to their unique characteristics, they are widely used in daily life (2). The widely use of plastics due to their non-biodegradability causes serious environmental problems. Therefore, alternative and more environmentally friendly plastics are needed (3). Each year about 140 million tons of plastics are produced and used throughout the world. The production of plastics needs approximately 150 million tons of fossil fuels. As a result, a lot of rubbish is produced that their depolymerization can last for thousands of years (4). The amount of plastic gathering in the environment is daily increased, and produced plastic rubbishes are controlled through burying or burning, these processes are expensive and time-consuming (5). Furthermore, plastics and their additives cause making serious problems concerning human health

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(6). Long-lasting gathering of non-biodegradable polymers in soil, in addition to ecological and hygienic problems, leads to reducing soil fertility (7). Bioplastics are the best solution-way for the security of the environment against dangers originated from petroleum-based plastics, because they are eco-friendly. There are different kinds of biodegradable plastics with various degrees of biodegradability, PHB is only biodegradable 100% (8). Due to this reason, PHB can be used as a substitution for petrochemical plastics (9). The accumulation of PHB granules begins in the answer to unpleasant conditions such as nutrient deficiency, including nitrogen, phosphate, oxygen, potassium, sulfate, iron, magnesium, sodium, calcium, copper, manganese, tin, and cobalt in the presence of excess carbon source. These granules are produced by a wide range of bacteria as carbon and energy reserves and prevent bacterial cell death in starvation conditions (10, 11). PHB has wide application in medical, pharmaceutical, tissue engineering materials, veterinary practice, agriculture, and food packaging (12).

2. Objectives

The present study was done to isolate and identify one local PHB producing strain from petrochemical wastewater, with a high production potential of this biopolymer for industrial purposes.

3. Materials and Methods

3.1. Sample Collection and Isolation of Bacteria

The sewage of a petrochemical company naming *JAM* (Assaluyeh, Iran) was sampled to isolate PHB-producing bacteria. The sewage sample was transferred to the lab under sterile and standard conditions. Then, serial dilutions from 10^{-1} to 10^{-6} were provided from the sewage sample. The amount of 0.1 mL of each dilution was plated on a nutrient agar medium containing (g.L^{-1}) peptone, 5.0; sodium chloride, 5.0; yeast extract, 1.5; beef extract, 1.5 and agar 15 (pH 7.4 ±0.2) by spread plate method. The plates were incubated at 37 °C for 24 h. Colonies that had different characteristic features were kept as pure cultures on nutrient agar slants, and then they were stored at 4 °C.

3.2. Screening of PHB Producing Isolates

3.2.1. Sudan Black B Plate Assay Method

Isolates were grown as a single colony on a plate containing nutrient agar. The plates were incubated at 37 °C for 48 h. After incubation, an ethanolic solution of (0.3% w/v) Sudan Black B was spread over the colonies, and the plates were kept undisturbed for 30 min. Then, the plates were washed with ethanol (96%) to remove the excess stain from the colonies. At last, dark blue colored colonies were taken as positive for PHB production (13).

3.2.2. Sudan Black B Under Light Microscope

For microscopic studies, a thin smear of isolated bacteria was prepared on glass slides and fixed by heat. Then smear surface was stained by Sudan Black B solution (0.3% Sudan Black B in 70% ethanol) for l0 min, washed with xylene, and stained with 0.5% safranin for 5 min. After washing with tap water and drying with absorbent paper, the smear was observed under a light microscope at 1000x magnification (14).

3.3. Identification of the Most Potent Isolate

The morphological and biochemical properties of isolates were investigated due to Bergey's Manual of Determinative Bacteriology (15). For 16S rRNA analysis, the genomic DNA was extracted using GeneAll Bacterial Genomic DNA Extraction Kit (Korea). The primers fD1 (5′-AGAGTTTGATCCTGGCTCAG-3′) and rP2 (5′-ACGGCTACCTTGTTACGACTT-3′) were used for amplification of 16S rRNA (16). PCR was performed in a thermal cycler (Bio-Rad, USA) with conditions as follows: initial denaturation at 94 °C for 5 min, 40 cycles of denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min and extension at 72 °C for 1.5 min and a final extension at 72 °C for 10 min. The PCR products were checked by 1.5% agarose gel electrophoresis and observed with a UV transilluminator. Due to the manufacturer's instruction, the PCR product was purified using a PCR purification kit (Qiagen, Germany) and sent to Macrogen Company in Korea for sequencing. Sequencing results using the blast program were analyzed in NCBI (National Center for Biotechnology Information, http://www.ncbi.nml.nih.gov) site. Phylogenetic tree was delineated MEGA X software with neighbor-joining method.

3.4. PHB Production and Extraction

The pre-culture medium was prepared and incubated at 37 °C for 48 h at 150 rpm by transferring one loopful of selected isolate which was inoculated from slant nutrient agar to 10 mL nutrient broth medium containing (g.L^{-1}): peptone, 5.0; sodium chloride, 5.0; beef extract, 1.5; yeast extract, 1.5. Then, 1 mL of seed culture was inoculated into 100 mL PHB producing media contains mineral salt medium (MSM) with

following composition (g.L^{-1}): $MgSO_4.7H_2O$ (0.2), $Na_2HPO_4.12H_2O$ (9.0), $CaCl_2.2H_2O$ (0.02), Fe^{+3} NH_4 citrate (0.0012), KH_2PO_4 (1.5), NH_4Cl (1.0). One mL trace element solution contained (g.L^{-1}): $ZnCl_2$ (0.84), $CoCl_2.6H_2O$ (0.1), H_3BO_4 (0.1), EDTA (50), $FeCl_3$ (8.3), $CuCl_2.2H_2O$ (0.13), $MnCl_2.6H_2O$ (0.016), were added to production medium after preparation and sterilization. Then, Erlenmeyer flask was incubated at 37 °C for 48 h on a rotary shaker at 150 rpm (17, 18). After incubation, the culture medium was collected and centrifuged at 4500 × g for 10 min at 4 °C. The supernatant was discarded, and the cell pellet was washed with phosphate-buffered saline (pH 7.5). The cell pellet was dried and its weight was determined. Then, the cell pellet was suspended in 10 mL sodium hypochlorite (4% w/v chlorine) and incubated at 37 °C for 90 min. The mixture was centrifuged at 4500 × g for 10 min at 4 °C, and the pellet was washed with distilled water, acetone, and methanol. The mixture was again centrifuged at 4500 × g for 10 min at 4 °C. The cell pellet was re-suspended in 10 mL of hot chloroform and allowed to evaporate at 40 °C to obtain PHB crystals (19, 20). At last, PHB accumulation was calculated as follows:
PHB%= Dry weight of extracted PHB (g.L^{-1}) / Dry cell weight (g.L^{-1}) × 100

3.5. Optimization of Culture Medium Conditions for PHB Production
The fermentation medium was supplemented with various carbon sources such as maltose, glucose, sucrose, fructose, and starch at 1% concentration separately for carbon source optimization. To optimize the nitrogen source, the medium was separately supplemented with different nitrogen sources (ammonium nitrate, ammonium sulfate, ammonium chloride, peptone, and yeast extract) at 1% concentration. To analyze the effect of pH, various fermentation medium was prepared at different pH values (5, 6, 7, 8 and 9). To analyze the effect of temperature, the incubation temperature was maintained at 25 °C, 30 °C, 37 °C, 40 °C, and 45 °C.

3.6. Characterization of PHB

3.6.1. Fourier Transform Infrared Spectroscopy (FTIR)
The presence of different functional groups in PHB extracted from the isolate S3 was analyzed by fourier transform infrared spectroscopy (WQF- 510A, China). The sample of PHB was mixed with potassium bromide pellet, and FTIR absorption spectra were recorded in 400- 4000 cm^{-1} range.

3.6.2. Nuclear Magnetic Resonance (NMR)
Sample of PHB was dissolved in deuterochloroform ($CDCL_3$) and ^1H NMR spectra were recorded on a Bruker Avance II 500 spectrometer (21).

3.7. Statistical Analysis
Data analysis was done using SPSS software version 22. Tests were performed by 3 times repetition. Moreover, to analyze the influence of sources i.e., carbon, nitrogen, pH, and temperature in PHB production, the researcher used one-way ANOVA (one-way variance statistical test) and then the Duncan method in post hoc test. P-values ≤ 0.05 were considered as statistically significant.

4. Results

4.1. Isolation and Screening of PHB Producing Bacteria
15 bacterial isolates were separated from petrochemical wastewater. Six isolates out of 15 isolates were positive for Sudan Black B staining. One *Bacillus* isolate was selected among positive isolates due to the content of polymer inside the bacterial cytoplasm. **Figure 1A** showed the black granules of PHB inside the pink-colored cytoplasm. Sudan Black B plate assay method also indicated the presence of lipophilic granules inside the isolates, and PHB positive isolates were separated due to the intensity of black stain (**Table 1**). In this technique, isolate S3 indicated the highest intensity of black stain comparing with other isolates (**Fig. 1B**). As shown in **Table 1**, among PHB positive isolates, isolate S3 produces the highest amount of PHB (2.43 g.L^{-1} PHB per 4.30 g.L^{-1} DCW (56.51% of the DCW)). Due to the results, isolate S3 was selected for further studies.

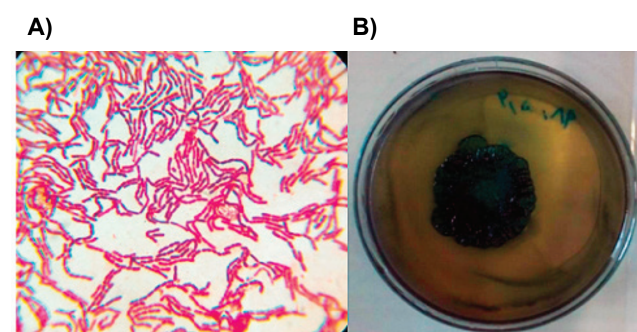

Figure 1. Sudan Black B staining for identification of PHB producing selected isolate. **A)** Black PHB granules observed under light microscope; **B)** Blue black colored colony after Sudan Black B staining

Table 1. Screening of PHB producing bacteria

NO.	Isolate code	Gram Reaction	Shape	Plate assay method*	DCW (g.L^{-1})	PHB (g.L^{-1})	PHB Content (%)
1	S1	Gram Positive	bacilli	+++	3.17 ± 0.088	1.63 ± 0.088	51.41
2	S2	Gram Positive	bacilli	++	1.80 ± 0.200	0.57 ± 0.098	31.66
3	S3	Gram Positive	bacilli	++++	4.30 ± 0.152	2.43 ± 0.348	56.51
4	S4	Gram negative	bacilli	++	2.33 ± 0.037	0.87 ± 0.033	37.33
5	S5	Gram Positive	bacilli	+	1.47 ± 0.115	0.23 ± 0.075	15.64
6	S6	Gram Positive	bacilli	++	1.60 ± 0.200	0.45 ± 0.072	28.12

* Intensity of Sudan Black B in a plate assay method: (+) Poor stained colonies; (++) Medium; (+++) Good and (++++) Excellent

4.2. Characterization of the Most Potent Isolate

Table 2 summarizes the morphological and biochemical characteristics of *Bacillus megaterium* saba.zh. The electrophoresis of the PCR product for 16S rRNA gene on 1.5% agarose gel approved the presence of approximately 1500 bp fragment (**Fig. 2**). The percentage of 16S rRNA sequence homology of selected bacterial isolate with other bacteria was performed using BLAST program in NCBI site. BLAST result showed that the isolate belongs to genus *Bacillus* and species *megaterium* which has 23% homology with *Bacillus megaterium* DSM32 (NR118962). Therefore, the bacterium was identified as *Bacillus megaterium* saba.zh. The nucleotide sequence of selected isolate was deposited in the NCBI database under accession number MN519999. The result of the phylogenetic analysis of 16S rRNA gene sequence of *Bacillus megaterium* saba.zh and related strains was shown in the **Figure 3**.

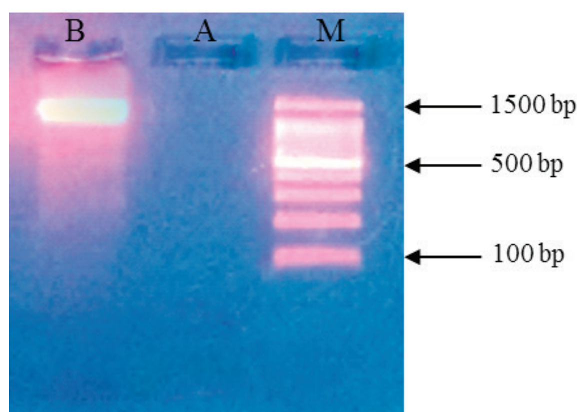

Figure 2. Gel electrophoresis of 16S rRNA gene PCR product of *Bacillus megaterium* saba.zh. M: Ladder (SMOBIO, DM2100), A: Negative control, B: PCR Product (1500 bp)

Table 2. Morphological and biochemical characteristics of *Bacillus megaterium* saba.zh

Characterization	Results
Shape	Rode
Gram Staining	G+
Sporulation	+
Motility	+
Catalase	+
Oxidase	-
Methyl Red	+
Voges-Proskauer	-
Citrate utilization	+
Nitrate reduction	+
Starch hydrolysis	+
Casein hydrolysis	+
Gelatin hydrolysis	+
Urea hydrolysis	-
Indole production	-
H2S production	-
Growth in 3% NaCl	+
Growth in 5% NaCl	+
Growth in 10% NaCl	+
Growth in 15% NaCl	+
Carbohydrates fermentation:	
Glucose	+
Sucrose	+
Lactose	+
Galactose	+
Fructose	+
Maltose	+
Sorbitol	-
Adonitol	-
Raffinose	-
Mannose	+
Xylose	+
Mannitol	+

(G+) Gram positive, (+) positive reaction, (-) negative reaction

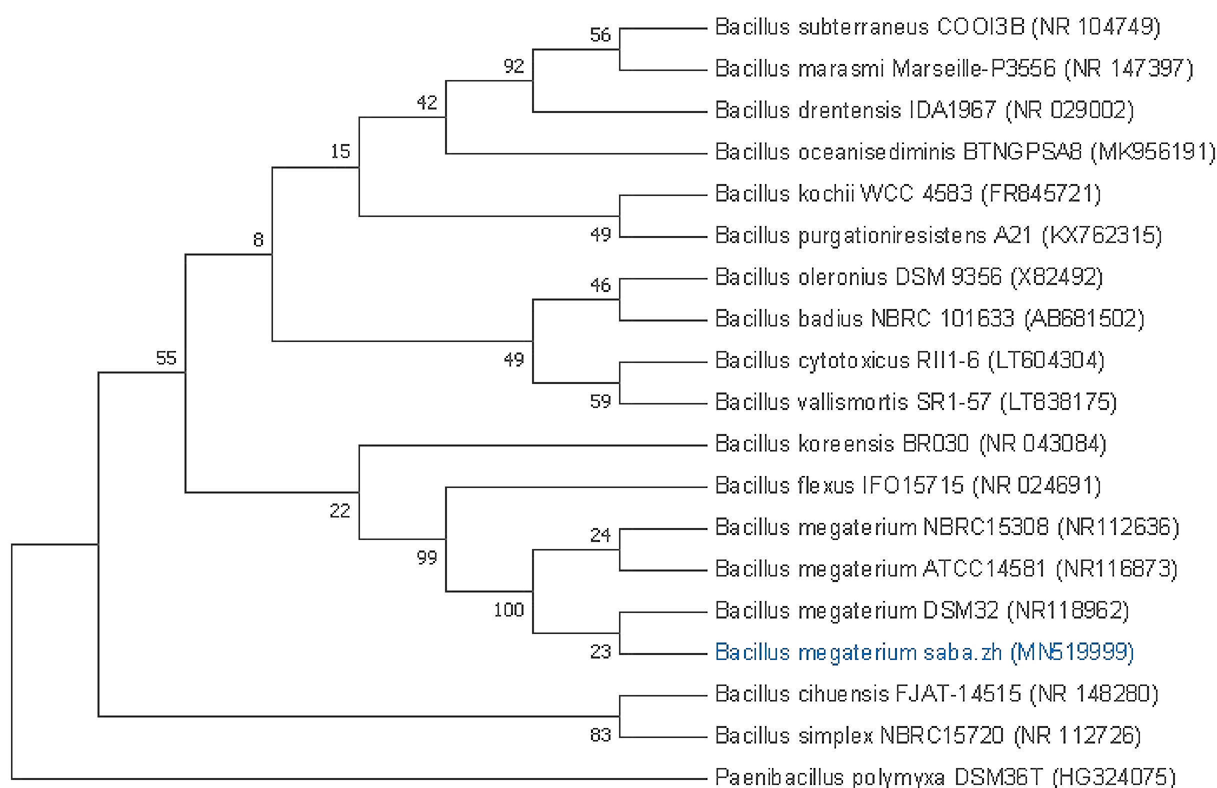

Figure 3. Phylogenetic analysis of 16S rRNA gene sequence of *Bacillus megaterium* saba.zh and related strains. The tree was constructed by neighbor- joining method using MEGA X: Molecular Evolutionary Genetics Analysis (MEGA) software version X. The accession number of each strain is indicated in parenthesis.

4.3. Characterization of PHB

Due to **Figure 4A**, the FT-IR spectrum of PHB compound extracted from *Bacillus megaterium* saba.zh showed six main peaks at 2959, 2924, 1725, 1530, 1442, and 1382. The observed peak spots at the 2924-2959 and 1382-1442 related to methine groups, followed by a peak at 1725, which displays the presence of –C=O group. The observation of these known peaks in FT-IR spectrum showed the presence of PHB. The ^{1}H NMR spectrum of PHB extracted from *Bacillus megaterium* saba.zh has been shown in **Figure 4B**. The peaks observed in the spectra correspond to all of the carbon atoms present in PHB structure. The peak observed at 1.29 ppm represents the methyl protons. The peak observed at 2.46–2.65 ppm represents the diastereotopic protons. Furthermore, the peak observed at 5.28 ppm represents the proton bonded to oxygen.

4.4. Optimization of Culture Medium Conditions for Maximum PHB Production

The effects of carbon sources on PHB production yield are indicated in **Figure 5A**. This study accurately showed that glucose was the best source of carbon for the maximum PHB production 4.37 g.L^{-1} using an isolate. The least amount of PHB production belonged to the starch as carbon source 0.89 g.L^{-1}. **Figure 5B** showed that ammonium sulfate is the most suitable nitrogen source among different nitrogen sources with PHB production yield 4.47 g.L^{-1} PHB, 5.5 g.L^{-1} DCW. Ammonium chloride is the second suitable nitrogen source for PHB production. The lowest level of PHB accumulation 1.43 g.L^{-1} was observed in the medium with yeast extract as nitrogen source (**Fig. 5B**). **Figure 5C** indicates that the accumulation maximum of PHB is 4.67 g.L^{-1} in PHB 7. The minimum level of PHB production yield 0.23 g.L^{-1} was gained at pH 5. As indicated in **Figure 5D**, the maximum PHB production was found at 30 °C. At this temperature, the isolate was able to produce 5.27 g.L^{-1} PHB. The temperature of 45 °C with an average production of 1.17 g.L^{-1} devoted itself to the least amount of PHB synthesis.

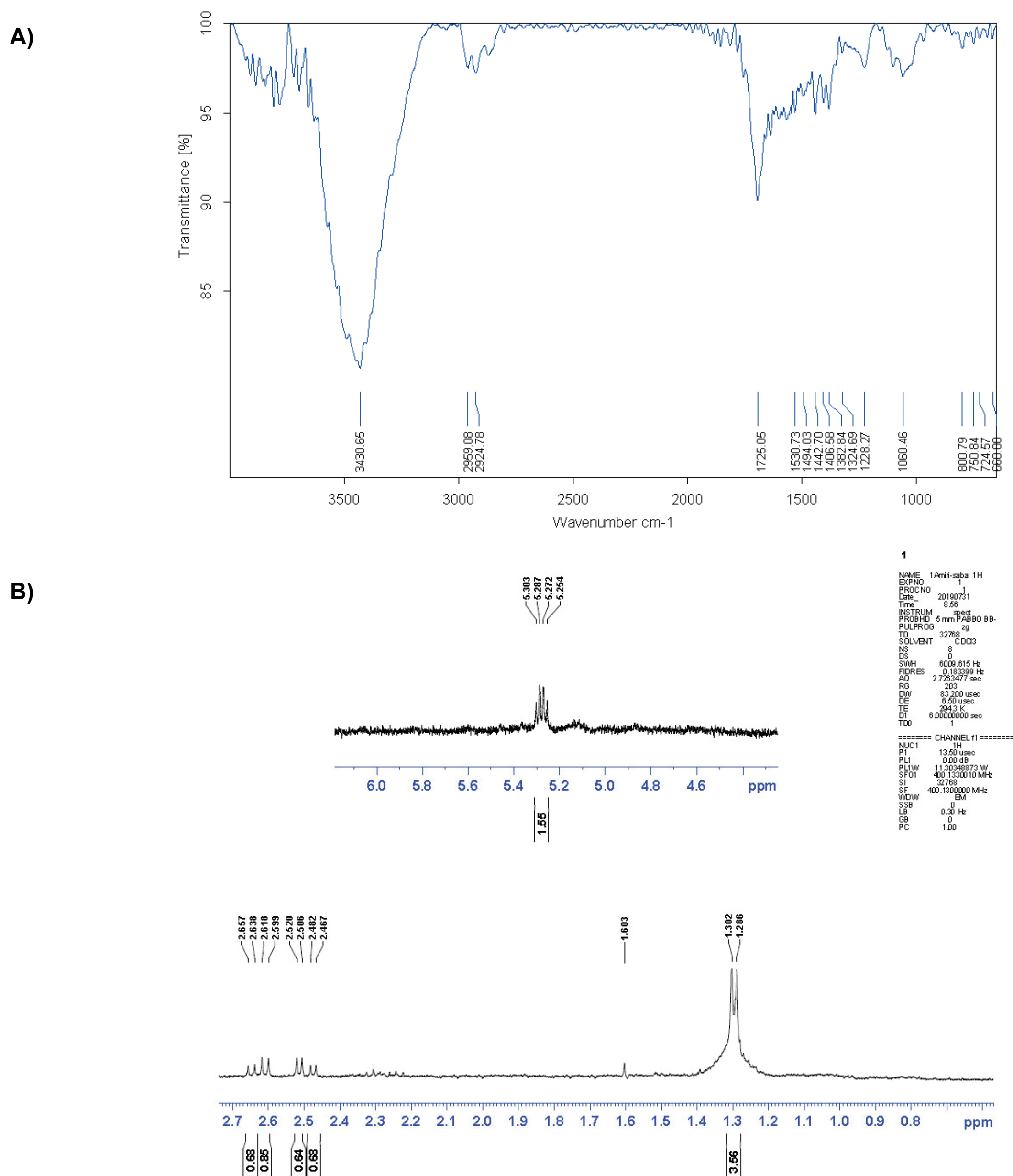

Figure 4. A) FTIR spectrum of PHB extracted from *Bacillus megaterium* saba.zh **B**) ^{1}H NMR spectrum of PHB extracted from *Bacillus megaterium* saba.zh

5. Discussion

The isolate was obtained from petrochemical wastewater of Assaluyeh in Iran. This study is the first report of separating bacteria producing PHB from this area. The PHB production in *Bacillus axaraqunsis* BIPC01 isolated from Bandar Imam petrochemical wastewater (Mahshahr, Iran) was analyzed by Mayeli *et al.* 2015 and the highest yield of polymer was reported 66% (22). Petrochemical industry wastewater exposed in hydrocarbon and petroleum contaminations. Therefore, it can be said that in this ecosystem, bacteria use hydrocarbons as carbon and energy source and preserve it in the form of PHB. In the current study, one *Bacillus* strain was chosen among other bacteria with the ability of PHB production, as the best isolate due to Sudan Black B plate assay and microscopic analysis. Phanse *et al.* 2011 (23) and Desouky *et al.* 2014 utilized this method to screen out PHA-producing bacteria (24). Regarding the production of bioplastic is so much expensive and it is considered the main obstacle in PHB production, a lot of techniques were selected for production on large scale. The selection of suitable strains out of bacteria which are capable of producing or accumulating

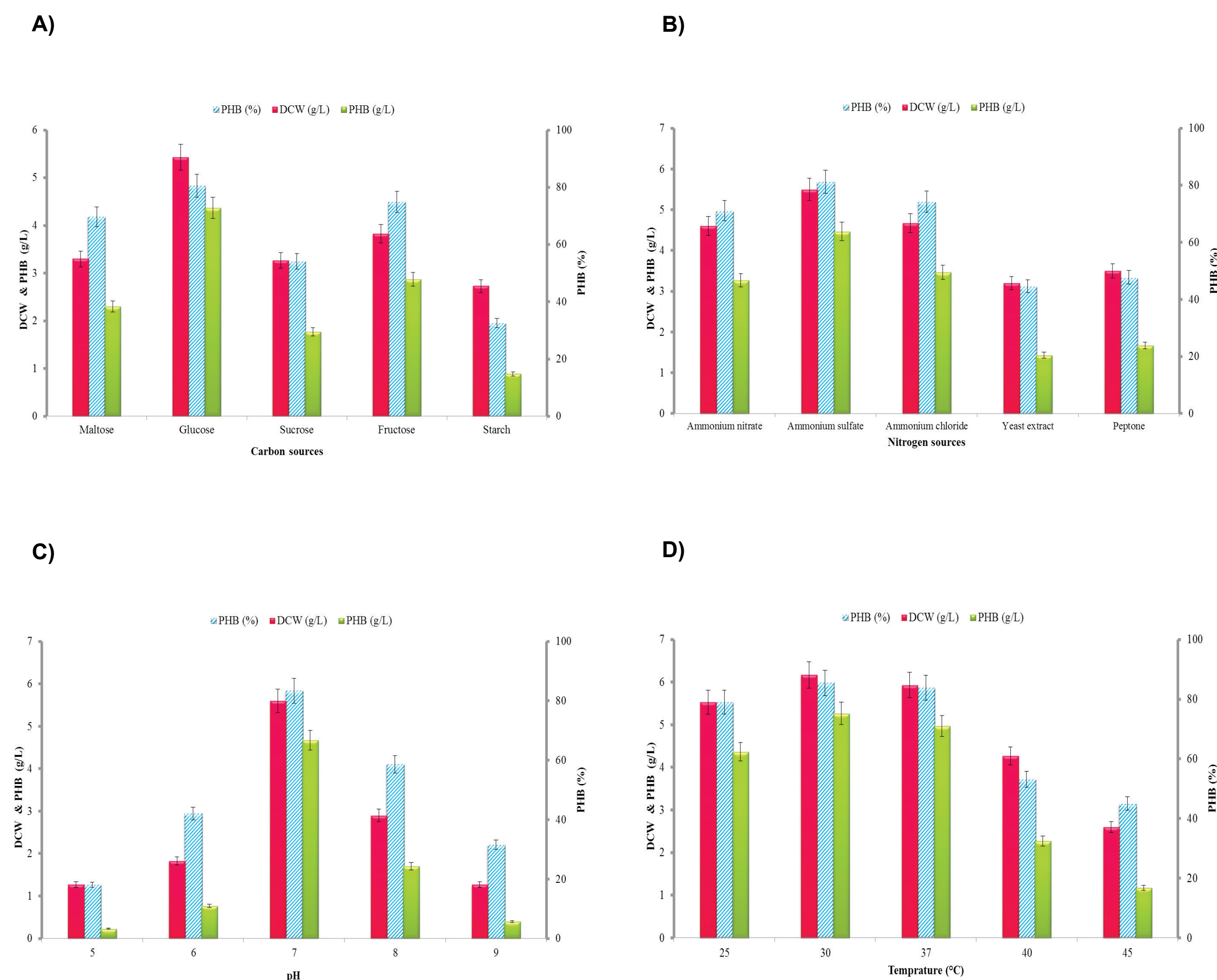

Figure 5. Optimization of culture medium conditions for maximum PHB production. (**A**) Effect of different carbon sources; (**B**) Effect of different nitrogen sources; (**C**) Effect of different pH; (**D**) Effect of different temperature

PHB in large amount is the most important criterion (1). Regarding the results of the current study and earlier similar studies, it seems that PHB-producing gram-positive bacteria such as *Bacillus* species, are a potential candidate for PHB production due to lacking of LPS and growth on various cost-effective substrates and they produce more pure PHB. While gram-negative bacteria need expensive structurally related substrates for PHB production. Due to this reason, gram-positive producers were highly received attention by researchers and they can reduce the production cost of PHB to a great extent. Moreover, we could identify *Bacillus megaterium* saba.zh by morphological, biochemical, and molecular techniques as Iran local strain with potential ability to produce PHB. In strain *Bacillus megaterium* saba.zh as a promising producer, the culture medium conditions optimization was done to maximize the production of PHB. By optimizing the conditions, the amount of producing PHB increased from 56.51% to 85.41%. Therefore, the production amount of PHB can be developed by fermentation medium optimization. Carbon sources include three different functions: synthesis of biomass, cell maintenance, and carbon source for PHB polymerization in an organism (19). Bora 2013 reported that maximum production of PHB was obtained with *Bacillus megaterium* with 1.38 $g.L^{-1}$ glucose as a carbon source in the fermentation medium (25). In the research of Issazadeh *et al*. 2015, glucose was introduced as the best source of carbon to

produce PHB to the amount of 24.70% by a standard strain of *Bacillus megaterium* PTCC 1656 that it was less compared to the present study. This difference in the results indicates the better potential of local strains compared to standard strains in producing PHB. *Bacillus megaterium* saba.zh as a local strain in medium containing glucose as the best carbon source with 80.47% of PHB production showed considerable metabolic ability (26). A simple sugar like glucose can be used via bacteria easily and this process increases PHB yield. On the contrary, a complex molecule like starch can't is easily used by bacteria (14). Thus, an increase in starch complexity leads to a decrease in PHB yield. Ammonium sulfate with a production amount of 81.27% PHB, among other studied nitrogen sources, was selected as the best nitrogen source. Alshehrei 2019 isolated *Bacillus*.sp from the soil in Saudi Arabia to produce PHB. This bacterium can synthesize 48.38% of PHB as a superior isolator in a medium containing ammonium sulfate as the best nitrogen source (27). Besides, Iman *et al.* 2017 reported that the highest amount of PHB (1.03 $g.L^{-1}$, 39% of DCW) was obtained from *Pseudomonas aeruginosa* Dw7 using medium containing ammonium sulfate (28). Therefore, the type of nitrogen source and used microorganisms are key factors for efficient PHB production (19). Investigating pH effects showed that pH 7 is the optimum pH to produce PHB. Moreover, by considering the low efficiency of production for pH 5 and 9, it can be concluded that pH higher and lower than optimum boundary can probably influence the enzymes involved in PHB synthesis and destroy them. Bharathi *et al.* 2016 also reported that pH 7 is optimum for PHB production by *Bacillus cereus* BB613-A (29). The results of our study showed that the amount of produced PHB is 4.67 $g.L^{-1}$ in pH 7. Also, Chandani *et al.* showed that the maximum PHB production of about 2.77 $g.L^{-1}$ was achieved by *Acinetobacter* sp. K3 in pH 8 (19). This difference in results can be due to a difference in the bacterial strain. Thus, correct controlling of pH has high importance, since slight changes in pH can affect the metabolic process of an organism. Among 5 different temperature conditions, the maximum level of PHB production was observed at 30 °C and the minimum at 45 °C. The decrease in PHB yield at high temperatures could be due to low PHB polymerase enzyme activity (30). Belal and Farid reported that maximum PHB production (3.6 $g.L^{-1}$, 66.7% of DCW) was achieved by *Bacillus cereus* E6 at 30 °C (31). In the present study, identified *Bacillus megaterium* saba.zh in 30 °C as the best temperature showed higher ability compared to reported study results in the PHB production (5.27 $g.L^{-1}$, 85.41% of DCW). Hamieh *et al.*, introduced 37 °C as optimum temperature to produce PHB by *Bacillus thuringiensis* and *Bacillus subtilis* (32). The results gained from the current study and other reported results confirmed the findings of Thirumala *et al.* 2010 which claimed, PHB can be produced by *Bacillus* species in the temperature range of 30-38 °C (33).

6. Conclusions

Regarding the present study, optimum culture conditions to produce maximum PHB by isolated bacteria from petrochemical wastewater include glucose as carbon source and ammonium sulfate as a nitrogen source, in PH 7 and temperature of 30 °C. The local strain *Bacillus megaterium* saba.zh can be used as an appropriate candidate for industrial production of PHB. Moreover, the wastewater of petrochemical industries is valuable as suitable habitat to isolate bacteria producing this biopolymer. To improve the ability for producing biodegradable polymer by microorganisms and decreasing production cost, using inexpensive substrates such as molasses, petrochemical and agro-industrial wastes as carbon sources are suggested. Moreover, genetic modification in responsible genes in biopolymer synthesis is suggested.

Acknowledgments

This study's authors thank dear personnel of the microbiology research laboratory in Islamic Azad University, Tonekabon Branch due to their executive supports.

Conflict of Interest

The authors declare no conflict of interest

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