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RESEARCH ARTICLE OPEN ACCESS

# Production of Polyhydroxybutyrate (PHB) by Bacteria Isolated from Soil of Saudi Arabia

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## Abstract

**One of the greatest problems in environment is accumulating of non-biodegradable polymers. Due to the hardness of these polymers, the biodegradation process is very slow. Several microorganisms have the potential for production of biodegradable polymers such as PHAs. Polyhydroxyalkanoates (PHAs) found in the cytoplasm of the bacterial cell as sources of carbon and energy. In the current study, 20 microbial strains related to bacteria were isolated from different areas in Makkah. Collected isolates were screened to examine their ability in production of PHB. *Bacillus* sp (F15) were selected based on their high production of PHB. *Bacillus* was maintained on (Luria Bertani broth medium) which showed PHB maximum production after 48 hours at 30 °C and the optimum pH is 7 under shaking conditions. Carbon and nitrogen sources were used to study their affect on production of PHB. Glucose is the best carbon source for PHB production, while $(NH_4)_2SO_4$ is the best nitrogen source. Strain (F15) was characterized by morphological, biochemical test and identified as *Bacillus* sp.**

**Keywords:** *Bacillus,* Polyhydroxyalkanoates, polyhydroxybutyrate (PHB), optimization.

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## INTRODUCTION

Synthetic polymers waste considered to be the most important problem in developing countries. Discovering biodegradable polymers is one of solutions to solve these problems. Biodegradable polymer is synthesized by some microorganisms and their enzymes are capable to degrade them (Chandra and Rustgi, 1998).

Biodegradable polymers are formed in cells during the growth of some microorganisms; therefore, they are also considered to be natural polymers. They are known as PHAs. These polymers produced biologically by maintaining microorganisms on renewable wastes. In lab, it can be prepared from 4-hydroxybutyric acid, propionic acid and $CO_2$ (Bhalla *et al*., 2007).

Polyhydroxyalkanoates are used in different medical applications such as bone fixation devices, surgical sutures, drug delivery systems, and in agriculture applications as well. (Alistair and Edwin, 1990).

PHAs are found in form of granules inside the cytoplasm of the bacterial cell. The size of granules diameter is arranging from 0.2 to 0.7 mm, and they surrounded by a membrane layer consisting of lipids and proteins. These inclusions can take up to 90% of the cell dry biomass each containing a minimum of 1000 polymeric molecules. The granules of PHAs are stored materials in the cell as a source of carbon and energy. (Braunegg *et al*., 1998, Yu *et al*., 2002).

PHA is insoluble polymer and containing hydroxyalkanoic acids (HA) as monomer units with only R configuration. (Verlinden *et al*., 2007).

PHAs molecular weight are different in microorganism depending on species, carbon source, and the growth conditions. it ranges from (200,000 to 3,000,000) Dalton. (Sudesh and Doi, 2000).

Furthermore, the PHA inclusions appear clearly by staining them with common dyes such as Sudan Black or the Nile Red to detect their presence by using a phase contrast light microscope (Sudesh and Doi 2000, Lee, 1996)

When a bacterial cell is under starvation conditions such as decreasing of an essential nutrient or lack of oxygen, the stored material can be utilized to promote survival of the cell (Koller *et al*., 2010).

Several bacterial species can produce PHA such as *Bacillus sp, methylotrophs, pseudomonads,* and *Escherichia coli, Alcaligenes eutrophus, Alcaligenes latus, Azotobacter vinelandii, Rhizobium sp*, (Mikkili *et al*., 2014). In (Merugu, 2012) article, bacteria producing – Polyhydroxyalkanoates are classified into two groups: (1) bacteria produce short chain length of PHA with monomer units ranged from $C_3$ to $C_5$ such as *Ralstonia eutropha*. (2) bacteria produce moderate chain length of PHB with monomer units range from $C_6$ to $C_{14}$ such as *Pseudomonas oleovoran*s, (Safak *et al*., 2002) studied also the ability of eukaryotic microorganisms to produce poly- beta- hydroxybutyrate. Many species of yeasts were isolated and PHB was found accumulated in their cells.

On another hand, microorganisms can degrade the polymer into simple components and utilize the final products in reasonable time. In particular, PHAs are fully biodegradable with the final products being water and carbon dioxide. (Koller *et al*., 2010).

*Bacillus* is used in many applications such as bioremediation, generation of bioenergy, production of secondary metabolites, and, but a few studies have not paid attention to it as PHA producer. (Singh *et al*., 2009)

The aim of this study is to investigate the potential of some bacteria that isolated from Makkah region to produce the PHB and to study the factors affecting the production of PHB.

## MATERIALS AND METHODS

### Isolation and screening of bacteria producing PHB

Soil samples were collected from different regions in Makkah province, Saudi Arabia. Soil samples were taken at 10 cm deep and kept in sterilize bag to transfer it to the lab. Serial dilution method was used to isolate the bacteria by suspending 1 g of soil in 99 ml distal water. Serial dilution was done up to $1:10^{-6}$. 1 ml of suspension transferred to nutrient ager and incubated at 30°C for three days.( Panigrahi and Badveli,2013) and (Mercan *et al*., 2002), (Singh *et al*., 2011). After incubation for 72 hours at 30°C, detection of PHB granules was done by staining bacterial colonies with Sudan black. The isolates which show maximum PHB granules were selected for more studies.

**PHB production and extraction from bacteria**

PHB production was carried out in Luria Bertani medium contained (g $L^{-1}$) Trypton, 1.0; NaCl, 1.0; yeast extract 0.5; yeast extract 0.5. (Sambrook and Russell, 2001).The inoculum was added to 100ml of sterilize medium in 250ml conical flask at 37°C for three days. The PHB assayed by using method of (Law and Splepecky method, 1961). Centrifugating at 10000 rpm for 20 minutes for (1-2ml) containing cellular growth was placed in centrifuge tube, and 9ml of alkaline hypochlorite reagent was added, after that the pellet was lyophilized. The residue centrifuged again at 8000 rpm for 20 minutes, then washed it twice with 10 ml of different solvents such as water, ethanol, and acetone. Purified material was dissolved in chloroform and make sure solution evaporates completely. 5 ml of concentered $H_2SO_4$ was added and heated for 40 minutes at 100°C in water bath. Optical density was read at 235 nm using Ultraviolet–visible spectrophotometer. PHB concentration was calculated by comparing the optical density value with a standard. the isolates which show high production ability were selected for further studies and maintaining them on suitable medium.

**Identification of the selected bacterial isolate *Bacillus* sp.**

Identification of *Bacillus* sp (PHB producing bacteria) was done based on morphological and physiological characteristics as described by ("Bergey's Manual of Determinative Bacteriology" 8th ed., 1993).

**Optimization of culture conditions for PHB production**

The selected isolate which showed maximum heights PHB production were tested. Physicochemical factors were studied such as different medium, different carbon and nitrogen sources, pH, temperature, incubation periods, aeration, and adding cheap carbon sources.

**Study the effective of different media on PHB production**

Three different medium were used to maintain the bacteria: The first medium was used for the study is: Luria Bertani broth medium (Sambrook and Russell, 2001), the second medium is : nutrient broth medium (Atlas, 1997), and the third medium is: Synthetic medium (Burdman *et al*., 1998). 100 ml of each previous media was sterilized at 121°C for 20 minutes and inoculated with 1ml of inoculum containing ($4x10^4$ CFU / ml) bacteria. Flasks were incubated at 30°C for 48 hours.

**Effect of aeration**

1ml of inoculum containing ($4x10^4$ CFU / ml) of bacteria inoculated in a production medium and incubated at agitation conditions (150rpm) at 30°C for two days. Flasks were also incubated at static conditions at 30°C for two days.

**Study the effective of different incubation periods on PHB production**

100 ml of a production medium was prepared and inoculated with 1ml of inoculum containing ($4x10^4$ CFU / ml) of bacteria. Flasks were kept at 30°C for different incubation periods (24,48,72,96,120) hours.

**Study the effective of different temperature degrees on production of PHB**

1ml of inoculum containing ($4x10^4$ CFU / ml) of bacteria inoculated in a production medium and incubated at different temperature degrees such as (20°C, 25°C, 30°C, 35°C, and 40°C, 45°C) for two days.

**Study the effective of different pH values on production of PHB**

pH degrees were tested such as (6, 6.5, 7, 7.5, 8) using 0.1N NaOH and 0.1N HCl. Flasks containing a production medium was inoculated with 1ml of inoculum containing ($4x10^4$ CFU / ml) of bacteria and incubated at 30°C for two days.

**Study the effective of different of carbon sources production of PHB**

Different carbon sources such as (Mannitol, Sucrose, Glucose and Starch) were added with 1% to the production medium separately. Flasks were inoculated with 1ml of inoculum containing ($4x10^4$ CFU / ml) of bacteria and incubated at 30°C for two days.

**Study the effective of different of nitrogen sources production of PHB**

Different nitrogen sources such as (Ammonium sulfate, Malt extract, Pepton and Yeast extract) were added with 1% to the production medium separately. Flasks were inoculated with 1ml of inoculum containing ($4x10^4$ CFU / ml) of bacteria and incubated at 30°C for two days.

**Effect of cellulose and fruit peel on production of PHB**

Cellulose and fruits peel were used instead of carbon sources and added to the medium of production separately. Flasks were inoculated with 1ml of inoculum containing ($4x10^4$ CFU / ml) of bacteria and incubated at 30°C for two days.

**Statistical Analysis**

Standard Deviation was used in this study to compare mean data.

## RESULTS AND DISCUSSION

**Isolation and Screening of bacteria producing PHB**

Twenty bacterial strains were isolated from soil of western region (Makkah province). Thirteen isolates are gram positive (Seven of them are baciili and the others are cocci). Seven isolates are gram negative bacilli. Screening of these isolates was carried out to test their ability in production of PHB table (1). All obtained isolates were screening for PHB production by staining with Sudan black and examine them by using fluorescent staining methods fig (1). Potential isolates which showed PHB production were identified by staining them with Sudan black and measuring the fluorescence intensity to detect them. Isolate (F15) was the best in production of PHB. This isolate was grown in a production medium for further examination.

**Table 1.** Growth and PHB production of twenty bacterial isolates maintained on Luria Bertani medium

| Isolates | Cell dry weight (g / L) | PHB (g / L) | % PHB yield |
|---|---|---|---|
| F1 | 2.1 ±0.06 | 0.3±0.05 | 14.2 |
| F2 | 1.3±0.03 | 0.2±0.04 | 15.3 |
| F3 | 0.9±0.01 | 0.1±0.02 | 11.1 |
| F4 | 1.6±0.90 | 0.6±0.1 | 37.5 |
| F5 | 2.4±0.1 | 0.7±0.90 | 29.17 |
| F6 | 3.1±0.07 | 0.4±0.50 | 12.90 |
| F7 | 3.4±0.99 | 1.4±0.03 | 41.17 |
| F8 | 2.7±0.02 | 0.9±0.2 | 33.4 |
| F9 | 1.8±0.01 | 0.5±0.3 | 27.8 |
| F 10 | 2.7±0.3 | 1±0.05 | 37.03 |
| F 11 | 4.1±0.03 | 1.7±0.09 | 41.4 |
| F 12 | 1.30±0.09 | 0.5±0.01 | 38.46 |
| F 13 | 0.5±0.07 | 0.007±0.6 | 1.4 |
| F 14 | 1.9±0.04 | 0.8±0.06 | 42.1 |
| F15 | 4.3±0.09 | 2.7±0.05 | 62.79 |
| F 16 | 3.8±0.1 | 0.5±0.08 | 13.15 |
| F 17 | 2.9±0.01 | 1.1±0.2 | 37.93 |
| F 18 | 2.6±0.04 | 0.78±0.01 | 30 |
| F 19 | 4.5±0.01 | 1±0.06 | 22.3 |
| F 20 | 0.7±0.2 | 0.08±0.3 | 11.4 |

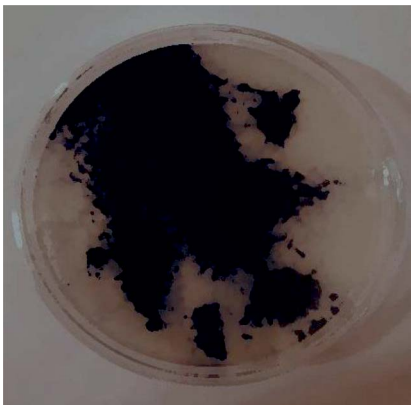

**Fig. 1.** Colored gunnels staining by Sudan black

**Identification of bacteria**

Identification of the best isolate in production of PHB was done by studying it's morphological, cultural characterizations table 2, Fig. 2. The Identification was based on Gram stain, spore test, motility, and several physiological and biochemical tests described by ("Bergey's Manual of Determinative Bacteriology" 9th ed., 1994).

**Fig. 2.** Isolate (F 15)

**Optimization of culture conditions of the selected bacteria for PHB production**

**Effect of different media on growth and production of PHB**

Table 3 shows that the maximum production of PHB was found in Luria Bertani broth medium. The selected bacteria produced

**Table 2.** Physiological and biochemical characteristics of *Bacillus sp.*

| Morphological and Culture characterization | |
|---|---|
| Shape and arraignment | Short , Chain |
| Capsule | Positive |
| Gram stain | Gram Positive |
| Spore staining | Positive, rounded, terminal |
| Motility | Motile |
| Acid fast stain | Non–fast acid |
| Colonies | White- abundant mucilage |
| Temperature range | Optimum 30-37°C |
| Growth | abundant |
| Form | Irregular |
| Margins | Serrate |
| Elevation | Flat |
| Density | Translucent |
| **Physiological and Biochemical characters** | |
| Catalase | Positive |
| Oxidase | Negative |
| Urease | Positive |
| Nitrate reduction | Positive |
| Methyl red | Negative |
| $H_2S$ production | Negative |
| Vogues- Proskauer | Positive |
| Indole | Negative |
| Utilization of Citrate | Negative |
| Mannitol fermentation | Positive |
| Sucrose fermentation | Positive |
| Glucose fermentation | Positive |
| Maltose fermentation | Positive |
| Lactose fermentation | Negative |
| hydrolysis of gelatin | Positive |
| Hydrolysis of lipid | Positive |
| hydrolysis of starch | Positive |

PHB with percentage (69.8%) of cell dry weight. Nutrient broth medium showed also a good result of production PHB (57.1%) while synthetic medium showed a lowest production of PHB with (33.3%) of cell dry weight. Therefore, LB broth medium was chosen as a production medium in all experiments.

**Effect of (Shaking and Static) conditions on growth and production of PHB**

Table 4 shows that aeration conditions are better than static conditions for production of PHB. The selected bacteria showed the maximum production of PHB in shaking conditions with (65.1%) of the cell dry weight.

**Table 3.** Effect of LBM, NBM, and Synthetic medium on production of PHB by *Bacillus* sp.

| Medium | Cell dry weight (g / L) | PHB (g / L) | % PHB yield |
|---|---|---|---|
| Luria Bertani broth medium | 5.3± 0.2 | 3.7± 0.21 | 69.8 |
| Nutrient Broth medium | 4.2± 0.23 | 2.4± 0.3 | 57.1 |
| Synthetic medium | 2.7± 0.13 | 0.9 ± 0.21 | 33.3 |

**Table 4.** Effect of different conditions on production of PHB by *Bacillus* sp.

| State | Cell dry weight (g / L) | PHB (g / L) | % PHB yield |
|---|---|---|---|
| Shaking | 4.3± 0.3 | 2.8± 0.12 | 65.1 |
| Static | 3.1± 0.23 | 1.5± 0.3 | 48.3 |

**Effect of incubation periods on production of PHB**

The selected bacterial was tested at different incubation periods for production of PHB. The results in table 5 show that the maximum production of PHB was after 48 hours of incubation. After two days the production of PHB was decreasing with the increasing of incubation period. This result agrees with (Irsath *et al*., 2015) study, they found *B.subtilis* recorded the highest production of PHB after 48 hours. (Sangkharak, and Prasertsan, 2008) reported that PHB production and its accumulation significantly increased when the growth reached the logarithmic phase after 18 hours until stationary phase.

**Table 5.** Production of PHB by *Bacillus* during different incubation periods

| Incubation time (hour) | Cell dry weight (g / L) | PHB (g / L) | % PHB yield |
|---|---|---|---|
| 24 | 4.1± 0.31 | 1.9± 0.23 | 46.34 |
| 48 | 5.6± 0.22 | 3.7± 0.11 | 66.07 |
| 72 | 4.7± 0.21 | 2.1± 0.31 | 44.68 |
| 96 | 2.9± 0.1 | 1.1 ± 0.2 | 37.93 |
| 120 | 2.1 ±0.03 | 0.6 ±0.01 | 0.28 |

**Effect of temperature on production of PHB**

Different temperature degrees in table 6 were tested to study their effect on production of PHB. Selected bacterial isolate showed a maximum

**Table 6.** Effect of temperature on production of PHB by *Bacillus* sp.

| Temperature (°C ) | Cell dry weight (g / L) | PHB (g / L) | % PHB yield |
|---|---|---|---|
| 20 | 1.1± 0.19 | 0.34± 0.15 | 30.9 |
| 25 | 1.2± 0.3 | 0.4± 0.31 | 33.3 |
| 30 | 5.4± 0.12 | 3.6± 0.13 | 66.7 |
| 35 | 4.1±0.2 | 1.5± 0.1 | 36.58 |
| 40 | 1.8±0.4 | 0.6± 0.11 | 33.33 |
| 45 | 0.9±0.11 | 0.2±0.13 | 22.22 |

production of PHB at 30°C, while increasing in temperature lead to decrease the bacterial activity in production of PHB.

**Effect of pH values on PHB production**

Table 7 shows the maximum production of PHB observed at pH 7 with (63.6 %). Increasing of pH degrees lead to decrease PHB production. This result agrees with the result of (Wei *et al*., 2011) they found the highest PHB production observed at pH 7 with (43.04%). (Palleroni and Palleroni, 1978) also recommended the pH in range (6.0 - 7.5) is good for PHB production and microbial growth.

**Table 7.** Effect of pH values on PHB production by *Bacillus* sp.

| pH | Cell dry weight (g / L) | PHB (g / L) | % PHB yield |
|---|---|---|---|
| 6 | 1.66 ± 0.24 | 0.5± 0.16 | 30.12 |
| 6.5 | 2.4± 0.13 | 1.1± 0.13 | 45.8 |
| 7 | 5.11± 0.24 | 3.25± 0.11 | 63.6 |
| 7.5 | 4.80 ± 0.13 | 2.41 ± 0.13 | 50.20 |
| 8 | 1.66± 0.18 | 0.7 ± 0.21 | 42.16 |

**Effect of carbon sources on PHB production**

Different carbon sources were added to medium to test the activity of bacteria in production of PHB. Table 8 shows that the glucose is the best carbon source of production PHB, while starch and fructose recorded the lowest percentage at (22.2%) and (16.7%) respectively. This result agrees with (Joraleerut *et al*.,2014) and (Shah, 2014). (Singh *et al*., 2009) mentioned that *Bacillus* sp can produce PHB when glucose was used as a sole source of carbon in the medium.

**Table 8.** Effect of different carbon source on production of PHB by *Bacillus* sp.

| Carbon sources | Cell dry weight (g/L) | PHB (g/L) | %PHB yield |
|---|---|---|---|
| Mannitol | 1.9± 0.12 | 0.8 ± 0.1 | 42.10 |
| Glucose | 3.1± 0.03 | 1.5± 0.03 | 48.38 |
| Sucrose | 1.3± 0.11 | 0.5± 0.01 | 38.5 |
| Fructose | 1.2± 0.2 | 0.2± 0.01 | 16.7 |
| Starch | 1.8 ± 0.03 | 0.4 ± 0.11 | 22.2 |

**Effect of different nitrogen sources on PHB production**

Based on table 9 the maximum production of PHB was (48.38%) observed in medium containing ammonium sulfate. Minimum PHB production noticed in media containing Peptone which recorded (16.7 %) PHB of cell dry weight. In (Lakhawat *et al*., 2012) study, ammonium acetate was the best nitrogen source and enhances the growth of bacteria and PHB production while PHB accumulation was slow when ammonium sulphate was used as a nitrogen source.

**Table 9.** Effect of different nitrogen source on PHB production by *Bacillus* sp.

| Nitrogen source | Cell dry weight (g/L) | PHB (g/L) | %PHB yield |
|---|---|---|---|
| Ammonium nitrate | 2.8± 0.03 | 1.2± 0.03 | 42.85 |
| Malt extract | 1.8 ± 0.01 | 0.7 ± 0.2 | 38.88 |
| Peptone | 1.2± 0.2 | 0.2± 0.01 | 16.7 |
| Yeast extract | 1.3± 0.11 | 0.4± 0.01 | 30.76 |
| Ammonium sulphate | 3.1± 0.12 | 1.5 ± 0.1 | 48.38 |

**Effect of cheap carbon sources on production of PHB**

The waste carbon sources were used to test their effective on PHB production. The highest percentage of PHB production was observed in the medium containing cellulose with (53.39%) table 10. In (Israth *et al*., 2015) study, *B.subtilis* BP1 showed maximum production at (60%) of sugar industry waste water and 50% of fruit peel extract respectively. (Joraleerut *et al*., 2014), (Gurubasappa *et al*., 2015) and (Singh *et al*.,2013) demonstrated that *B. cereus* strain can utilize

cheap carbon sources such as sugar industry waste water, molasses and crude glycerol for production of PHB.

**Table 10.** Influence of cheap carbon sources on production of PHB by *Bacillus* sp.

| Cheap carbon | Cell dry weight (g/L) | PHB (g/L) | %PHB yield |
|---|---|---|---|
| Cellulose | 2.06±0.41 | 1.1 ± 0.2 | 53.39 |
| Fruits peels (banana peels) | 1.68 ± 0.13 | 0.8 ± 0.42 | 47.61 |

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**DATA AVAILABILITY**

All datasets generated or analyzed during this study are included in the manuscript.

**ETHICS STATEMENT**

This article does not contain any studies with human participants or animals performed by any of the authors.

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