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Production and optimization of polyhydroxybutyrate (PHB) from *Bacillus megaterium* as biodegradable plastic

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ABSTRACT: Among biodegradable plastics polyhydroxy alkanate and its polymers have received more attention than other biodegradable polymers because of their complete degradability, flexibility, water resistance and also the ease of production process. Polyhydroxybutyrate is one of the types of polyhydroxy alkanates that is seen as a storage granule in many microorganisms. In this study, *Bacillus megaterium* was prepared from Iranian microbial collection. Glucose and yeast extract were used as the main components of the medium in seed media 9 and 2.5 g/l and in fermentation medium 30 and 7.5 g/l respectively. GC-MASS and FTIR were used to identify the PHB produced. The results showed that the highest amount of biomass (0.221 g/l) and PHB (0.080 g/l) were obtained with glucose at 37°C and shaker speed of 150 rpm for 72 h incubation. The results of GC MASS and FTIR showed the production of PHB by *Bacillus* under investigation. Based on the mean of data on total cell growth conditions, the rate of cell biomass and PHB production in *B. megaterium* were 0.0869 and 0.0171 respectively. According to the results of the experiments, temperature had the greatest effect on biomass production and PHB production. The bioplastics produced by microbes are also highly degradable in the environment, and due to their specific chemical structure, they have been widely used in various fields of the food, pharmaceutical and chemical industries and are likely to replace today's plastics in the near future.

Keywords: Polyhydroxybutyrate; Soudan black; *Bacillus megaterium*; FTIR; GC-MASS.

1. INTRODUCTION

Plastics are used today in the production of all kinds of industrial products, from the automotive industry to the medical world. And in the United States alone, nearly 50 million tons of plastic are produced annually. But these materials, as resistant to microbial degradation, have posed complex environmental challenges [1]. One of the important solutions to solve the problem of polymer waste accumulation in nature is the use of biodegradable polymers including polyhydroxy alkanates (PHAs) and copolymers [2]. But industrial-scale production is constrained by the relatively high cost of substrate, low polymer production, and the high cost of isolation and maintenance of microorganisms [3]. In another report, the cost of producing

degradable polymers in pure culture was estimated to be about 20 times the price of synthetic plastics [4]. Polyhydroxy alkanates are hydroxy alkanates polyesters and their molecular weights range from 2×10^5 to 3×10^6 daltons depending on the type of microorganism and growth conditions [5].

One of the types of polyhydroxy alkanates is polybeta hydroxybutyrate (PHB), a storage granule that is found in many microorganisms. These bacterial polymers are produced in these cells under adverse nutritional conditions and, if they persist, for the survival of the bacteria, they decompose and provide the carbon and energy resources they need [6, 7]. Any adverse conditions do not lead to the production of these compounds, and in fact only if the carbon sources are abundant and more than the bacterial need and on the other hand are the restriction of elements such as nitrogen, phosphorus and magnesium, then the cell's central metabolism towards the production of this polymer precedes. The bacterium can make the most of its available capacity and optimally store available resources [8]. These polymers are in many cases similar to synthetic (petrochemical) plastics, and in fact can be said to be their natural type, but one major difference with synthetic plastics is their degradability, which has made these biopolymers green and harmless plastics [9, 10]. Polyhydroxybutyrate is a non-toxic, environmentally friendly, and biodegradable material that can be produced from renewable sources [11]. Polyhydroxybutyrate was first used as packing layers for bags and containers, then used as a paper gloss. Other uses include plastic, kitchen utensils, fabrics, cosmetics, plastic bottles, and so on [12, 13]. They can also be used as biodegradable carriers for long-term delivery of drugs to specific parts of the body for medical reasons [2, 11]. The main purpose of this study was to investigate the production of polyhydroxybutyrate (PHB) and the production of cellular biomass in various growth factors including temperature, carbon source, time and Shaker Round to produce this polymer.

2. MATERIALS AND METHODS

2.1. Microorganism preparation

In this study, *Bacillus megaterium* PTCC 1656 was prepared from Iranian Microbial Collection Center to produce polyhydroxybutyrate and was used after confirmation as *Bacillus megaterium*by biochemical tests.

2.2. Soudan blackstaining method

Sudan staining was used for staining PHB grains. A drop of bacterial suspension was placed on the slide and fixed on the flame after drying by rapid passage of the slide. The smear surface was then impregnated with black Sudan solution (0.3 g of black Sudan and 100 ml of 70% ethanol). After 10 minutes, the slide was rinsed with distilled water and washed with xylene after drying. After this the smear was impregnated with safranine solution for 15 seconds. After washing with distilled water and drying the slide with polyhydroxy alkanate filter paper, black stains were observed inside the bacteria with a lens of 100 light microscopes [14].

2.3. Preparation of inoculum (seed culture medium) and microorganism production and culture medium

The desired microorganism was inoculated by sterile loop from the plate into the seed media with the following composition: glucose, fructose and maltose (individually) 9 g/l, yeast extract 2.5 g/l, KH_2PO_4 2.5 g/l, K_2HPO_4 2.5 g/l, NH_4NO_3 0.5 g/l, $(NH_4)_2HPO_4$ 1 g/l, $MnSO_4\cdot7H_2O$ 0.007 g/l, $MgSO_4\cdot7H_2O$ 0.2 g/l, $FeSO_4\cdot7H_2O$ 0.01 g/l. Three types of media were seeded with different carbon sources and poured into ML10 tubes and incubated at 37°C after bacterial culture. After 48 hours of incubation, 1 ml of seed medium was inoculated and 10 ml of production medium was inoculated and warmed. The composition of the production

medium was similar to that of the seed medium but the concentration of glucose and yeast extract in the production medium were 30 and 7.5 g/l, respectively. It should be noted that they were separately sterilized when preparing the medium to prevent adverse reactions to glucose and yeast extract [15].

2.4. Determination of dry cell biomass

To optimize and evaluate PHB growth and production from carbon (glucose, fructose and maltose), temperature (25, 30 and 37°C), aeration (0, 150 and 200 rpm) and time (24, 48 and 72 hours) was used. The fermentation medium was sampled at specified 24-hour intervals. 10 ml of the samples were discarded for 15 minutes at 5000 rpm centrifuge and the supernatant was discarded. To remove the additives, the precipitated biomass was suspended in 5 ml distilled water and discarded again for 15 minutes at 5000 rpm centrifuge and the supernatant was discarded. The obtained biomass was kept for 24 hours at 90°C until complete evaporation and cellular weight stabilization and then cell dry weight was measured [14].

2.5. PHB percent spectrophotometer

For quantitative analysis, the dried cells containing intracellular poly-beta-hydroxybutyrate were hydrolyzed using concentrated sulfuric acid. For this purpose, 10 ml of concentrated sulfuric acid was added to the dried biomass in the bolted tubes and stored at 100°C for one hour in Ben Murrayand the maximum optical absorption (λmax) at 235 nm was calculated and calculated using the following formula [12].

$$PHB\% = \text{Absorbance in 235 nm} / CDW \times 10 \text{ ml} \times 100$$

$$\text{Biomass in mg} = CDW$$

$$\text{Volume of sulfuric acid in dilution} = 10 \text{ ml}$$

2.6. Analysis of PHB beads by Mass Spectrometry Chromatography (GC MASS) and Infrared Spectroscopy (FTIR)

The standard material of 3-hydroxybutyrate from Sigma USA was used to confirm the production of PHB beads. The FTIR device was used to identify the PHB carbon groups produced. The quantitative analysis of PHB grains produced by the device (GC MASS) was used. 2 ml of chloroform and 1 ml of acidic methanol (Includes 85% volumetric volume methanol, 15% volumetric sulfuric acid and 45 g/l benzoic acid as internal standard) were obtained and the standard polyhydroxybutyrate sample was added. The samples and standard were kept at 100°C for 2 h until the esterification reaction was complete. Then, 1 ml of water was distilled twice in each sample and shaken vigorously for one minute [14-16]. With intense shaking and sedation, three distinct phases were formed (upper phase containing sulfuric acid, middle phase containing microbial residues and lower phase containing methyl ester of hydroxy alkanate), the upper two phases being discarded by pipette and phase pipette. The clean test was transferred to a refrigerator before being injected into the gas chromatograph. Two microliter volumes of samples and standard samples were injected separately into GC MASS and FTIR. Specifications of the GC MASS are as follows: Model GC 6890N, NS S973N and HPMSM, 0.25 µm fixed phase particle size, 30 m column length and 0.25 mm column diameter, manufactured by US Agile Company, 250°C injection temperature and representative temperature 280°C was used. Helium was used as carrier gas at a volume flow rate of 2 ml/min. The apparatus was heated at 90°C for 1 minute, then at 5°C/min at 150°C and finally at 35°C/min at 220°C.

3. RESULTS

3.1. Investigation of production of PHB beads by Sudan staining

After Sudan black staining and observation by light microscopy 100X, PHB grains within the bacterium was visible as dark blue (Fig. 1).

Figure 1. Light microscopy with 100X after Sudan black staining.

3.2. Effect of various physical and chemical factors such as temperature, shaker speed, time and carbon source on PHB production

Temperature: Temperatures of 25, 30 and 37°C were used to evaluate biomass production and PHB production. The highest PHB production at 37°C was 0.025 g/; and the lowest production at 25°C was 0.01 g/l (Fig. 2).

Carbon Source: Carbon can be used as a substrate for the production of polyhydroxy alkanates to produce PHB. Glucose, fructose and maltose were used to optimize and evaluate different carbon sources and biomass production and PHB production in each carbon source were investigated. Was and the lowest PHB production in the fructose source medium was 0.011 g/l (Fig. 3).

Time: 24, 48 and 72 hours were used to evaluate the highest biomass production and PHB production. The highest PHB production occurred at 0.021 g/l in 72 hours and the lowest PHB production was 0.012 g/l at 24 h (Fig. 4).

Shaker rotation: Due to the different growth of bacteria at different aeration rates, shaker rotation of 0, 150 and 200 rpm was used to evaluate biomass and PHB production. The highest PHB production occurred at shaker speed 150 (0.021 g/l) and the lowest production at zero rpm (0.012 g/l) (Fig. 5).

Optimum Conditions for Cell Biomass and PHB Production: The Bacillus strain had the highest biomass and PHB production at 37°C, shaker speed of 150 rpm, 72 h time, and consumption of glucose carbon source. In this condition, the bacterium produced the highest biomass (0.211 g/l) and the highest PHB (0.08 g/l) and Based on the mean data, the total cell growth conditions of *Bacillus* was 0.0869 g/l biomass and 0.0171 g/l PHB (Table 1).

According to the statistical data, the effect of temperature, shaker speed, incubation time and carbon source on the biomass and PHB of the bacteria were significant at 1% probability level ($p \leq 0.01$).

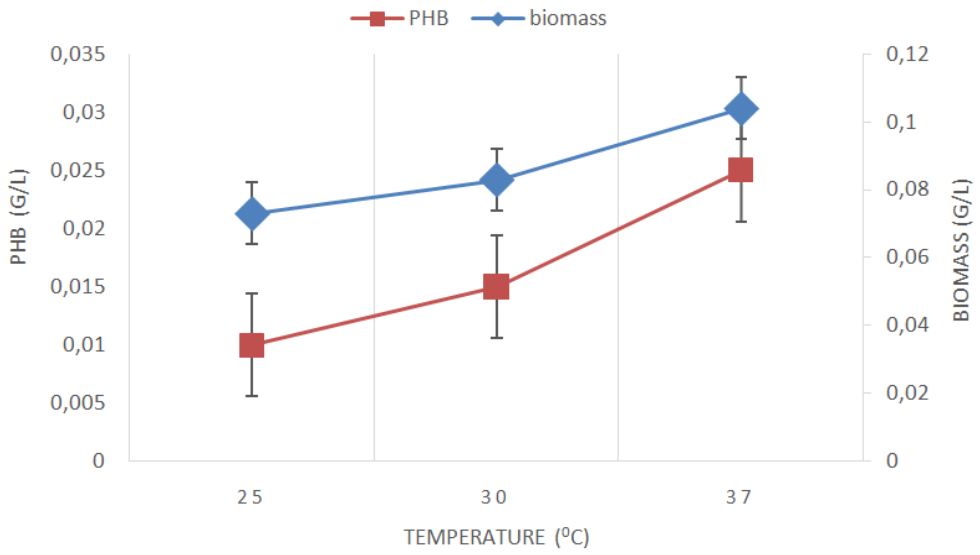

Figure 2. Optimization of biomass and PHB production at different temperature levels.

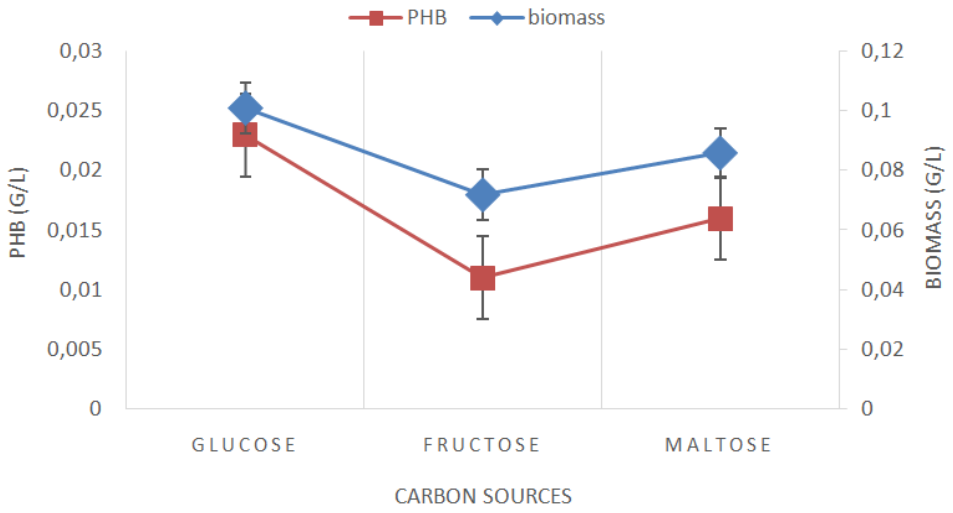

Figure 3. Optimization of biomass and PHB production rates using different carbon sources.

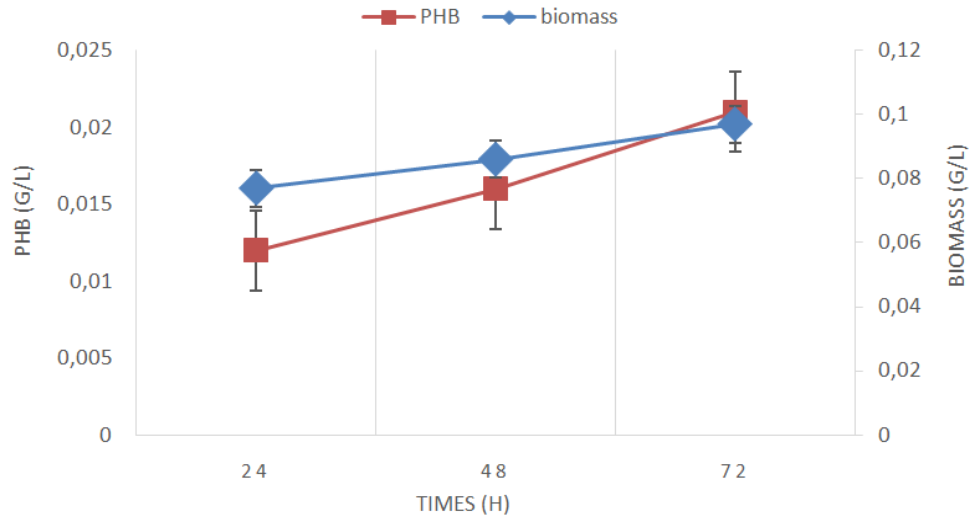

Figure 4. Optimization of biomass and PHB production at different times.

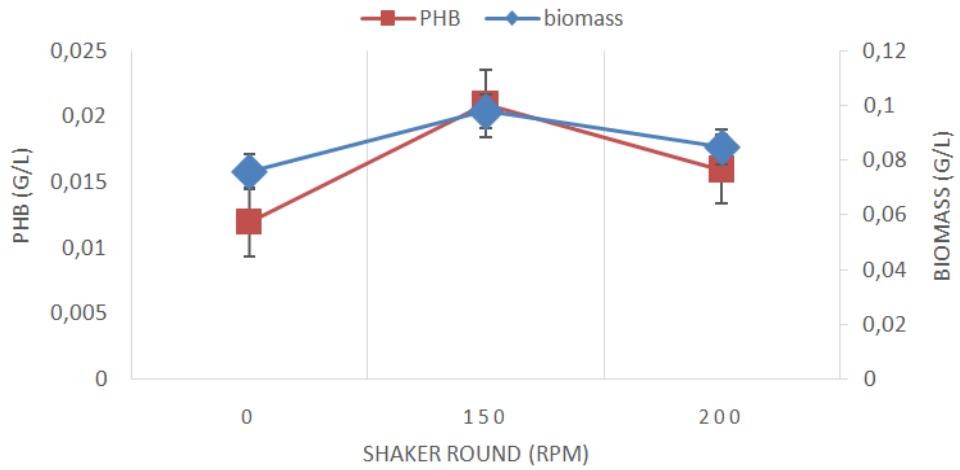

Figure 5. Optimization of biomass and PHB production at different shaker round.

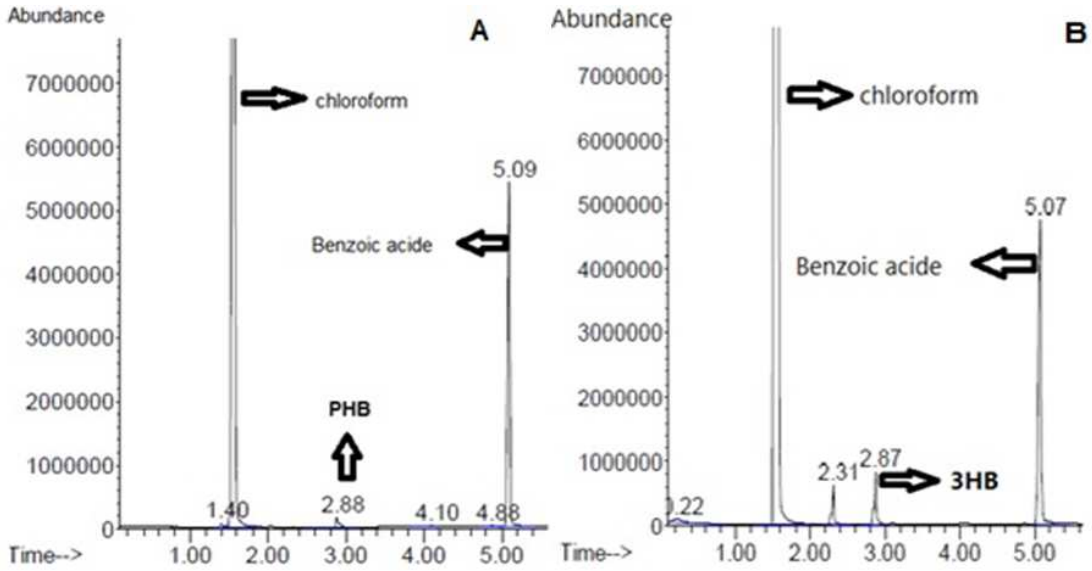

Figure 6. (A) GC MASS chart for PHB produced by *Bacillus megatrium*, (B) Standard PHB chart.

Table 1. Effect of different growth factors on cell biomass and PHB production.

Bacillus megaterium		**Different growth factors**	
PHB (g/l)	Biomass (g/l)		
0.01	0.073	25	Incubator temperature (^{0}C)
0.015	0.083	30	
0.025	0.104	37	
0.023	0.101	glucose	Carbon sources
0.011	0.072	fructose	
0.016	0.086	maltose	
0.012	0.076	0	Shaker Round (rpm)
0.021	0.095	150	
0.016	0.085	200	
0.012	0.077	24	Times (hours)
0.016	0.086	48	
0.021	0.097	72	

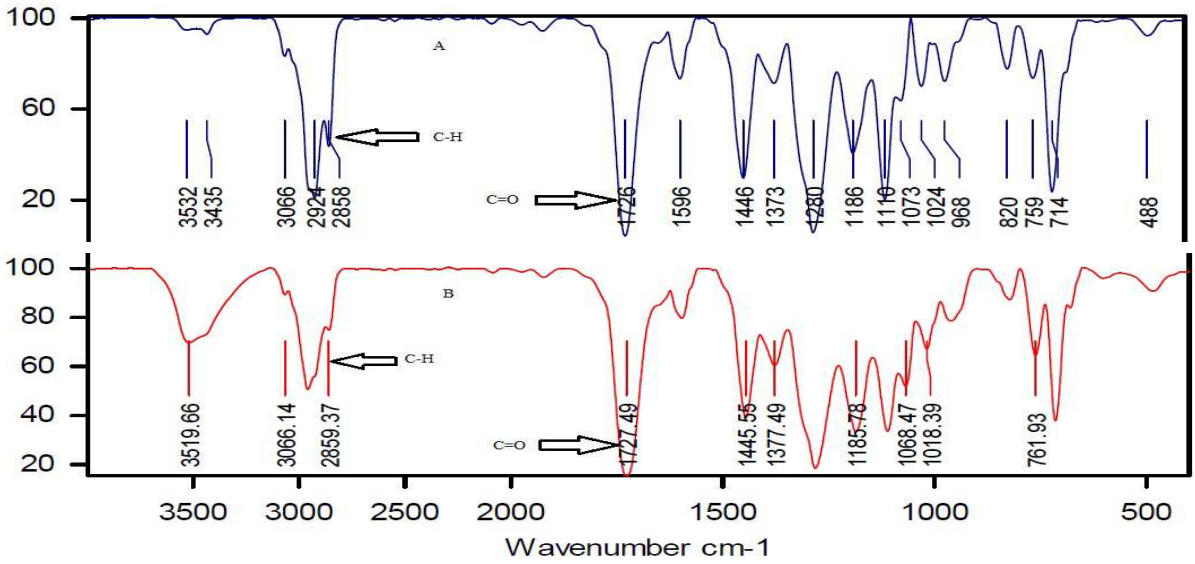

Figure 7. (A) FTIR for PHB produced by *Bacillus megaterium* and (B) FTIR for standard PHB.

3.3. Results of Mass Spectrometry Chromatography (GC MASS) and FTIR

The obtained polymer as well as the poly (3-hydroxybutyrate) prepared as a control were separated from the organic phase (chloroform solution) and injected into the GC MASS. Finally, diagram A was obtained and compared with diagram from control sample (diagram B), indicating and confirming the existence of polyhydroxybutyrate (Fig. 6). The obtained polymer and chloroform solution were also injected into the FTIR apparatus. The graph shows absorption band at 1726 Cm^{-1} for the carbonyl group (C = O) and at 2858 Cm^{-1} for the C-H group reflecting the structure PHB (Fig. 7).

4. DISCUSSION AND CONCLUSION

In this study, *B.megaterium* PTCC 1656 was used to produce polyhydroxybutyrate and the effect of different growth factors on PHB and biomass production was investigated. These bacteria were evaluated for physiological and biochemical properties. The results showed that this bacteriawas heterotrophic, aerobic, gram positive and had characteristics such as spore, catalase reaction, nitrate and citrate. *B. megaterium* at 37°C, shaker speed 150 rpm, 72 h and glucose consumption had the highest biomass production of 0.221 g/l and PHB production of 37% at 0.08 g/l. According to the results of GC MASS, the amount of PHB produced by *B. megaterium* was 41%. Polyhydroxybutyrates (PHBs) have been observed among more than twenty bacterial isolates strains including *Alcaligenes*, *Bacillus*, *Azotobacter*, *Rhodospirillum*, *Rhizobium*, and *Pseudomonas*. Among *Bacillus* strains, the highest PHB content was observed in *Bacillus megatrium* Y6 (48.13%) [17]. During the study on different strains of cyanobacteria in BG11 medium 10.8-65.00% of PHB and in Allen medium 11.89-85.45% of PHB was observed. Another study on PHB production in *Lactobacillus*, *Streptococcus* and *Lactococcus* showed that PHB production in *Lactobacillus* isolates was 0.93-9%, *Lactococcus* 7.09-16% and *Streptococcus* 5.47-21.15%, respectively and most PHB production has been reported in *Streptococcus thermophilus* to 21.15% [18]. Ghatnekar et al. showed that the amount of PHB in *Methylobacterium* sp. V49 can be increased to 98% by changing the culture medium [19]. Also, Khanafari et al. showed that by culturing azotobacter in whey broth at 35°C and 122 rpm shaker, PHB production levels could be as high as 4 ml/l [3].

According to the results of these experiments, temperature had the greatest effect on biomass production and PHB production. Based on the growth of *B. megaterium* in different cell conditions, the production of this biopolymer can be significantly increased by changing bacterial growth conditions including; pH of the medium andinoculation rate. The biodegradable plastic produced by microbes are also highly degradable in the environment, and due to their specific chemical structure, they have been widely used in various fields of the food, pharmaceutical and chemical industries and are likely to replace today's plastics in the future, therefore the purpose of biodegradable plastic production in laboratory scale is Paving the way of production for industrial production.

Authors' Contributions: SA-A: involved in planning and supervised the work, contributed to the interpretation of the results, other contribution. HR: conceived and designed the experiments, carried out the experiment, processed the experimental data, performed the analysis, wrote the manuscript, contributed to the interpretation of the results. MS: edited and review the manuscript, review designed the experiments and processed the experimental data, edited and review the analysis performed, wrote the manuscript, contributed to the interpretation of the results. PZ: edited and wrote the manuscript, drafted the manuscript and designed the figures. DS: drafted the manuscript.

Conflict of Interest: The authors declare no conflict of interest.

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