Extraction of Polyhydroxybutyrates from *Bacillius cereus* isolated from Engine Oil-polluted Soils

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Abstract

In the present study a trial was carried out to isolate bacteria species from engine oil–polluted soils, the soil samples were diluted and spread on solid media at 37 ^{o}C incubation. The most species were then picked up from plates for identification, characterization, and storage for further use. According to the physical morphology and the shapes using gram stain the bacteria were belonged to *Bacillus cerues*, molecular technique using 16S rRNA gene were also carried out to confirm the types of species. The Sudan Black B was used to optimize the PHA granules in bacteria. The bacterium isolate were then grown in appropriate media, and date molasses, which was as carbon source was added to media to promote the growing cells and to accumulate PHB granules as intercellular product at different, range of temperature, pH, agitation speed, and carbon source concentration. The high level of PHB production was

accumulated at $35\ ^{o}$ with natural pH, and 150 rpm when date molasses was added at 15% (v/v). The PHB productions were characterized using biological techniques as FITR to confirm the functional group of PHA at range $4000-400\ cm^{-1}$.

Key words: PHB, FITR, date molasses, *Bacillus cerues*

1. INTRODUCTION

production plastics have become important in all aspects of life, due to the high demand from industries and domestics. Petroleum-based plastics have become attractive due to low production cost. However, these types of plastics are non-degradable and have caused the damage to the natural environment (1). The petroleum-based plastics usually remain in a landfill for a long period of time. The efforts to produce eco-friendly plastics become inevitable to overcome these problems. Polyhydroxyalkonates (PHAs) are bioplastics which have attracted considerable interest in recent years because of its easy possessing, biodegradability and biocompatible properties. They accumulated as carbon or energy reserves (2). The polyhydroxyalkonates are synthesized using natural organisms such as bacteria with limitation in growth conditions like oxygen, nitrogen, or phosphorus and excess amount of

carbon source (3). The vast majority of PHA production based on the type of bacteria strain and carbon sources used as substrate (4). This biopolymer can be divided into three main types according to the number of repeating units in the polymer, shorter chain length (SCL), medium-chain-length (MCL), and large chain length (LCL). SCL composes of three to five carbon atoms while for MCL the polymer contains six to sixteen carbon atoms. As for LCL, it composes of fourteen to more carbon atom (5, 6). Polyhydroxybutyrate P (3HB) polymers have been recognized as common type of PHAs and first described by Lemiogne 1926 in *Bacillus megaterium*, to continue with other Gram- negative bacterial strains to be clear as intracellular reserve accumulates in cytoplasm of the cell (7). The main benefits of PHB are that; the composition of PHB are biodegraded to water, carbon dioxide and methane by anaerobic microorganisms in different environments, as sea water, soil, and lake water to decrease the pollution resulting from the consumption of high amounts of plastic

(8). On the other hand, PHB has other advantages; it is insoluble in water and resistant to ultraviolet radiation and is

impermeable to oxygen making it good in food packaging industry (9).

In this study, PHB producing bacteria were isolated from engine oil-polluted soils. The identification of isolated bacteria was done using morphological and molecular analysis. The use of inexpensive carbon source such as date molasses was assessed for PHB production. PHB production was confirmed using FITR spectrophotometer.

2. Materials and Methods

2.1 Collection of Samples

Engine oil-polluted soils were collected from various workshops, Tripoli and all samples were collected using glass sterile containers and transferred to lab for isolation PHB producing bacteria.

2.2 Isolation PHB producing Bacteria

The bacteria was isolated by dilution of inoculated soils and then were spread onto enrichment nutrient agar plates by sterile glass spreader and were incubated at 37 ^{o}C for 24 hour, the enrichment media supplemented with data molasses and used

to enhance the growth rapidly, in addition it has been a commonly used to grow heterotrophic bacteria.. The colony was individually picked and sub cultured many times on nutrient agar to get pure colony. The pure bacterium was stored in 15% glycerol at −20 or− 80 °C for long term, and some colonies were maintained at 4 °C and refreshed on nutrient agar slant every 30 days for using (10).

2.3 Screening the PHB Producing Bacteria by Sudan Black B stain

The screening of the isolated bacteria is necessary distinguisng between PHB producing and non producing bacteria. Two types of stains used which are Sudan Black B. The colonies were screened for detect PHB producing bacteria using Sudan Black B. 0.2% Sudan Black B solution was spread over the colonies of isolated bacterium and the plates kept undisturbed for 30 minutes. After that, the plate was washed with ethanol (96%) to remove the excess stain from the colonies (11). The detection also achieved by fixation the colony on clean slide by heating in oven for few minutes and then stained with a 3% Sudan black B (w/v in 70% ethanol, Sigma) solution for 10 min. After that, the

slides were immersed in xylene for decolorizing the cells. The safranin 5%, w/v in distilled water was used for 10 second. Finally, the slides washed with distilled water, and dried to be ready for examination under optical microscope (12). The test was done using control plates and slides of negative PHB producing bacteria isolated from soil.

2.4 Identification of Isolated Bacteria

The colonies shape, size, and color were observed in culture plates with nutrient agar. Gram staining was also used to confirm the morphological of bacteria. The bacteria were smeared on clean slides and treated with three types of stains which were crystal violet, gram's iodine and Safranin that used as a counter. The slides were dried and observed under light microscope at 60 x magnification. In addition, the biochemical tests were carried out to confirm the spices.

2.5 Endospore Staining

The method utilizes malachite green to stain the endospore and safranin to stain the vegetative portion of the cell. The smear of heat –fixed bacteria was covered with small piece of filter paper and saturated with malachite green. The smear was steamed

over boiling water for 5 minutes. Additional stain was added when it boiled off. After the slide cooled down, the paper was removed and the slide was rinsed with water before counterstained with safranin for 20 seconds. Excess stain was washed off and air-dried smear was examined under oil immersion. (13).

2.6 Bacteria Identification by 16S r RNA Gene

For more reliable result , 16s rRNA was carried out to indicate the bacteria identity. The genomic DNA of bacteria was isolated using promega kit. The 16s rRNA gene amplification was achieved using two universal primers which are forward 5' – AGA GTT TGA TCC TGG CTC AG-3' and reverse primer 5'- ACGGTC ATA CCT TGT TAC GAC TT-3'. PCR amplification was carried out at 95 °C/1min, 55 °C/1 min, 72 °C/2 min with final extension at 72 °C/5 min. the PCR products were purified using Qiagen PCR purification kit (14). The purified PCR were send to First BASE Laboratories Sdn Bhd, Malaysia to obtain DNA sequence. The received sequences were analyzed using NCBI-BLAST (National Center for Biotechnology Informationhtt://www.ncbi.nml.nih.gov)program.

2.7 PHB Production

Liquid culture were performed by pure colonies from solid medium to 250 ml Erlenmeyer flasks containing 100 ml of nutrient media and 50 ml of date molasses was added to media as carbon source at different concentration range 10, 15, and 20% (v/v). The pH was adjusted to 7.0 with a solution of 1 M NaOH. Bacteria were incubated at 37 °C for 48 hours under continuous shaking at 150 rpm. The optical density of bacteria (OD) was measured using spectrophotometer at 600 nm interval. PHB production was studied under different ranges of temperature at 30, 35, 40, 45 °C, pH was 6, 7, and 8. The PHB biosynthesis conducted triplicate.

2.8 PHB Extraction

The samples were subjected to centrifugation at 10000 rpm for 15 minutes after 4 days of fermentation. The pellets were suspended in distilled water with EDTA pH 5 at 5°C for 24 h. This condition ensures the lysis of cells. These lysed suspensions were centrifuged at 10000 rpm for 15 minutes. The pellets were then washed repeatedly with distilled water to remove carbon source. The final pellets were dried in the oven

at 80°C until a constant weight. The white dusts resulting from this treatment were dissolved in chloroform for 24 hours. Finally, the chloroform was evaporated at room temperature and thin films of polyhydroxybutyrates were obtained (15). The films were characterized by FITR spectrophotometer.

2.9 Total Cell Dry Weight

The cell dry weight was carried out after centrifugation the volume of the fermentation broth at 10000 for 15 minutes. The bacteria pellet was dried in oven at 80 °C until constant the weight.

3.0 Results and Discussion

3.1 Isolation and Identification PHB-Producing Bacteria

After the spreading the engine oil-polluted soil samples on agar media, the bacteria colony were observed after 24 hrs to recultured again on enrichment agar, the growth of bacteria achieved, all the color of colonies were white-creamy dots shape, smooth and somewhat elevated.

The result of gram staining for bacteria was observed under light microscope at 60X, magnification using immersion oil. The colonies of bacteria were appared with blue colour under light microscope. So that, bacteria identified as gram positive bacteria. The isolated bacteria strain was expected to be from the genus *Bacillus* result of their rod in chains shape and gram positive characteristics. the endospore staining was carried out as next step to confirm the positive result of *Bacillus* strain, the results of bacteria were negative stain even after repeating the experiment many times, this could occurred by many reasons which were the bacteria strain has not reached unfavorable condition yet for producing spore. In addition, the characterististic of spore is considered very difficult to stain.

3.2 Screening of PHB Producing Bacteria

The colonies were screened for PHB production using Sudan Black B for 30 minutes, the slides completely immersed with Sudan Black B and the cells decolorized with xylene to washed with water with be ready for drying. The microscopy results showed the positive PHB producing bacteria colored with black while the negative was pink color. The figure 1 illustrated that.

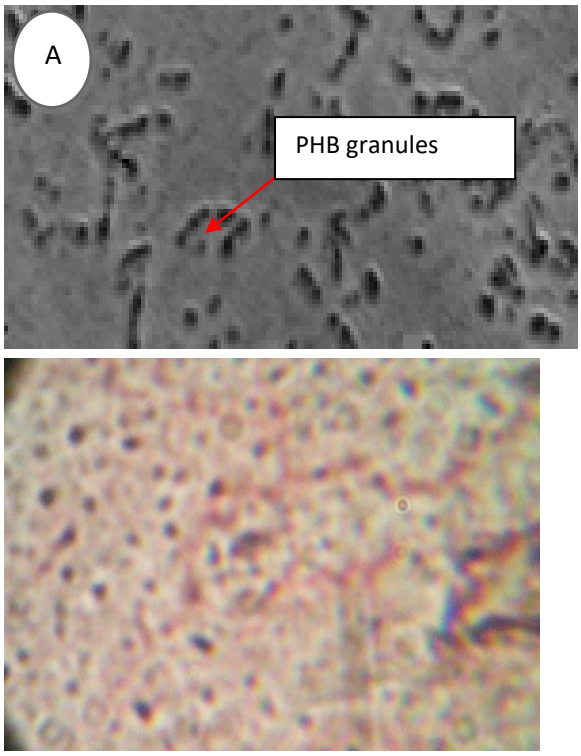

Figure 1. (A) showed the PHB producing Bacteria under optical microscope stained with black color. (B) showed with pink color of bacteria that is negative PHB producing bacteria.

3.3 16 SrRNA Gene Sequence Analysis

For more reliable results 16S rRNA gene sequencing also used to identity isolated bacteria accurately. DNA template for 16S rRNA was successfully extracted and amplified for purification to

obtain high purity of PCR product to get accurate result for DNA sequencing. Beside the purity of PCR product was checked out by running gel electrophoresis as shown in the figure 2.

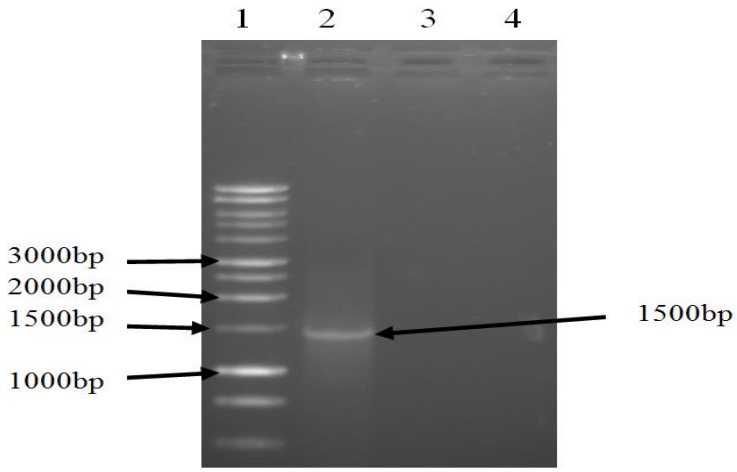

Figure 2. The band of bacteria at 15kbp using gel electrophoresis

The partial 16S rRNA gene sequencing also used on isolated bacteria using NCBI BLAST (National centre for Biotechnology Information http://www.ncbi.nml.nih.gov). The complete sequences were aligned to the homologous sequence available for *Bacillus*. The sequences of the 16S rRNA gene of the isolated strains were deposited in the GenBank sequence database and found closely to *Bacillus cereus* with 99% similarity. This work supports the finding by Laila et al. (2015)

who isolate *Bacillus* bacteria from waste cooking oil (16). The growth PHB producing bacteria in engine oil-polluted soil may due to the mechanism of utilizing the oil by bacteria that can be described in terms of lipolytic activity (17).

3.4 Phylogenetic Tree Analysis

Phylogenetic tree was established using BLAST-Webpage and MEGA5 Software. different species from BLAST analysis related to the bacterium *Bacillus* were chosen together with sequence of *Bacillus* to perform alignment using Neighbor-joining method and MEGA5 software. Figure 3 showed bacterium *Bacillus* was clustered with a bootstrap value of 1000 and scale bar 0.5 substitutions per site with a clad consisting *Bacillus cereus*. This suggested that isolated bacterium was closely related to the respective *Bacillus Cereus strain P17.* The isolated bacteria could be divided into two major groups based on the phylogenetic analysis *Bacillus cereus* and *Bacillus sp* formed a group with a bootstrap value of 100%. Similarities within each morphotype were relatively high, within the range of 96% to 100%.

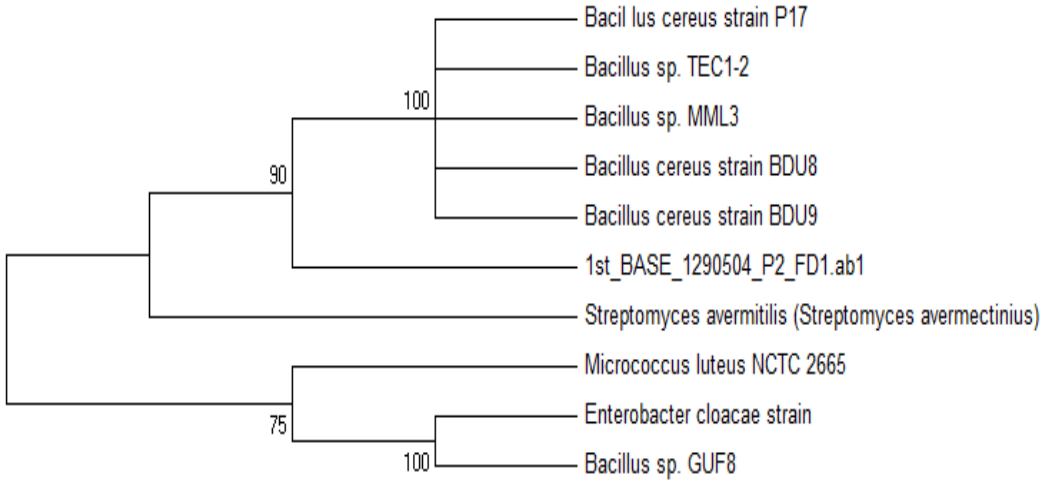

Figure 3. phylogenetic tree for *Bacillus cereus strain P17*

3.5 PHB Production

Bacterial isolates selected for PHB production was studied under different growth condition by varying the temperature, pH, and carbon source concentration. The most important factor effecting PHB production was carbon source, which was date molasses. The maximum CDW was 0.8 g/l and accumulation of PHB was 33.3% when date molasses used 10% v/v, and the temperature was 35 °C with natural pH. The low and high carbon source concentration reduced PHB production.

3.6 Extraction PHB from Bacterial Cells

In this work, the PHB produced by *Bacillus cereus* P17 cells were extracted successfully. The using 50 mM of EDTA pH 8 was for lysing the cell wall of bacteria then, The pellets were centrifuged at 10000 rpm for 10 minutes after that the pellet washed many times to confirm removing the color of carbon source from the product. The releases of PHB were dried in the oven to estimate DWC, and then were dissolved in chloroform for overnight at room temperature. The PHB was appeared as white layer in the bottom of the cylinder. After weight the extracted PHB film, the product was subjected to FITR analysis.

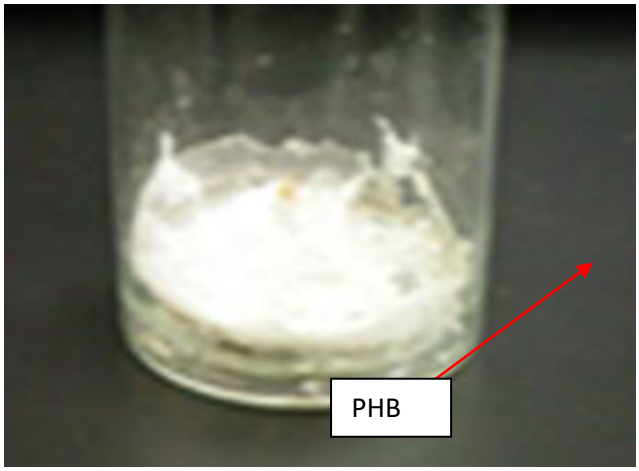

Figure 4. The PHB layer Extracted by Chloroform

3.7 Analysis PHB using FITR

Fourier transform infrared spectroscopy was carried out at range of $4000-400$ cm^{-}FTIR analysis of the isolated polymer shown the presence of different conformational bands in the extracted PHB from mixed culture. The next figure displayed the character of PHB molecule is the presence of carbonyl bands C=O at 1719 cm^{-1} absorption band, the band at about 1381 cm^{-1} is assigned to methyl $(-CH3)$ groups and the bands at 1176 and 1259 cm^{-1} are characteristic of the stretching of the C–O groups, respectively. While the band at 1460 cm^{-1} is assigned to methylene group $(CH2)$. This result was compared with previous study as standard that stated absorption c=o group at 1719 cm^{-1} (18).

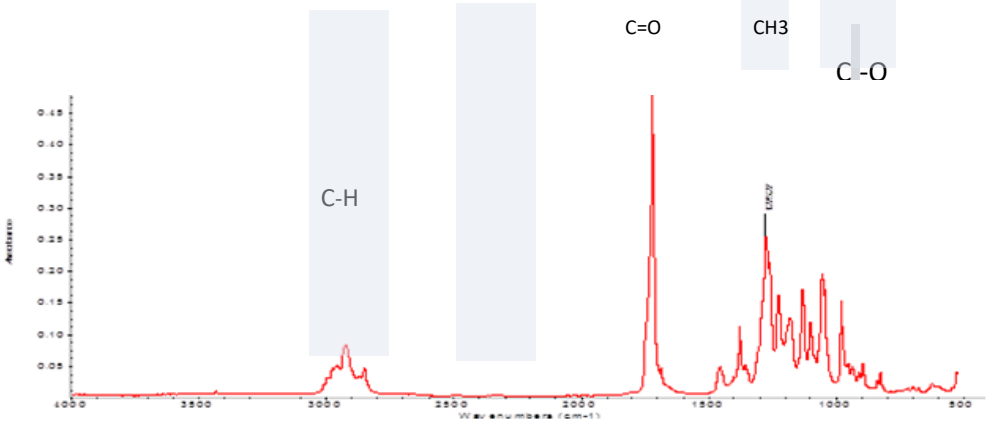

Figure 5. FTIR Spectrum of Extracted PHB Showed Absorption

4.0 CONCLUSION

The colonies isolates from engine oil-polluted were identified using molecular technique and screened for PHB production after that were utilized to produce PHB with presence of date molasses as cheap carbon source to reduce the cost of PHB production.

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