

## التحري الجزئي عن العزلات البكتيرية المنتجة للبولي هايدروكسي بيوتيريت من الترب المختلفة

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### Summary

The different soil samples were screened fir bacterial isolation, only three pure isolates were obtained, these isolates were selected and kept in glycerol for long time. The bacteria for polyhydrinybutyrate production were detected using the very selective and specific stain which named Nile red stain. Only one isolate had the positive result with Nile red stain the DNA of isolate that selected for the current study was used to detecting phac gene which represent the synthetic gene of PHB ١٠ confirmed its ability for PHB production. The PhoC gene encoded the synthase enzyme which was considered as the key enzyme for PHA biosynthesis, G-D and G-١R primers were used for the amplification of Phat gene by PCR with a size of ٥٠٨ bp

### Literature Review:

Plastics have now become the essential ingredient to enhance the comfort and quality of our life, due to their versatile qualities of strength, lightness, durability and low production cost. Plastics material have become an integral part of contemporary life because of their many at a rate of desirable properties including durability and resistance to degradation. These non-degradable plastics accumulate in the millions of ton per year causing several problems. Recently, issues environment concerning the global environment and solid waste management have created much interest in the development of biodegradable plastics (Anderson et al., ١٩٩٠), Poly-lhydroxybutyrate (PHB) (bioplastic), the best-known poly hydroxyl alkanoutes (PHA) is an energy and/or carbon storage Material synthesized and accumulated by numerous microorganisms (Lee et al., ١٩٩٩).

Poly-B-hydroxybutyrate (PHB) is a biodegradable thermoplastic which can be extracted from a wide range of bacteria. PHB is the intracellular granule synthesized by bacteria and acts as an energy storage facility. Under normal growth conditions, the nutrient sources are used for the synthesis of proteins essential for the growth in bacteria. In some *Bacillus* sp, it supplies energy for sporulation (Tanadchangsaeng et al., ٢٠٠٩). The storage granules are synthesized by the microorganisms, when the cell's surroundings contain an unbalanced growth condition such as limited concentration of O, N, P, S or trace elements, e.g., Mg, Ca, Fe and high carbon concentration (Sudesh et al., ٢٠٠٠).

PHB can be extracted from a wide range of bacteria. PHB is an important molecule on cytoplasm and cell walls. *Bacillus* sp. has been shown to accumulate PHB during the sporulation of bacterial growth. The PHB production capacities of bacteria have been investigated for possible application in industry (Verlinden et al., ٢٠٠٧). When the sporulating strain is grown under conditions favorable for polymer production, no spores are formed. When the sporulating strain is grown under conditions unfavorable for polymer production but favorable for sporulation, less polymer is produced and peak production occurs during the log phase of growth. Rapid utilization of the polymer proceeds sporulation. If the PHB production medium is made with addition of glucose and acetate and vigorous aeration is favorable for good polymer production and subsequent utilization.

The high carbon concentration and C/N ratio ranging from ٢٠/١ to ١٠٠/١ has the most beneficial effect on PHB biosynthesis. The cost of PHB production is rather high thus hindering its practical applications. Despite many cost-cutting efforts in the past few years, the price of "Biopol" (a commercial PHB/V copolymer) is still high, \$٤-٨/kg compared to \$٠.٦-٠.٩/kg for conventional synthetic plastics (Schubert et al., ١٩٩١; Li et al., ١٩٩٧). The cost of the carbon source contributes significantly to the overall production costs. For the process with recombinant *Escherichia coli*, the cost of the carbon source is as high as ٣٠.٧% of the total cost (Qi and Rehm, ٢٠٠١). Cheaper substrates were used for the production of PHB (Tokiwa et al., ٢٠٠٩).

### Materials and methods:

#### ١-Collection of soil samples

Samples of soil were collected from a different fertile land in Iraq and Malaysia. Each sample was kept separately in the sterile plastic bags and transferred to the laboratory.

#### **٢-Isolation of bacteria**

To obtain the bacteria from soil samples, 1 gm of soil sample was added in 99 ml of sterile distilled water and stirred. The mixture was filtered by What man filter paper to remove the debris. After a serial dilution of each sample (10<sup>-1</sup> to 10<sup>-6</sup>)- transfer 0.1 ml of each dilution to nutrient agar plates medium and spread then incubate at 30 C for 3 days. The pure colonies of each sample were picked and sub cultured for several times and kept for screening of PHA production.

#### **٣-Maintenance of the pure isolates**

The pure isolates were maintained on a nutrient agar plate and in glycerol stocks. These isolates were sub-cultured monthly to maintain the cell viability. Glycerol stocks were used for a long term storage. The pure culture of bacteria was inoculated into 50 ml of a nutrient broth in 250 ml C for 24 hours. Then, 12.5 ml of at 30 shaker flask and incubated sterilized glycerol 99% was added into the culture and stirred for 10 the bacterial cells. Finally, pipette aseptically 1 ml resuspend minutes to C tubes and kept at -20 Eppendorf of the suspension into producing bacteria by Nile polyhydroxybutyrate Screening of -4 (1999, et al Spiekermann) Red

The isolates were qualitatively tested for PHB production by culturing the colonies on MM plate supplemented with Nile Red at concentration 0.5 µg/ml. The agar plates were examined by UV light (312 nm) after a suitable period of incubation at 30 C.

#### **-<sup>o</sup>Detection and sequencing PHA synthase gene (PhaC)**

The DNA of the selected bacteria was extracted by following the instruments for DNeasy kit and used as a template to detect PhaC gene using the universal primers G-D primer (GCXCT) as forward (5'-GTGOCOCC

)AG(GC)ATCAACAAGT-3') and G-IR primer as reverse (5. GTTCCAG (AT)ACAG(GC)A(GT)(AG)TCGAA-3') (Romo et al., 2007). The PCR components and setting were listed in Table (1) and Table (2) using Veriti thermocycler. The detection of PCR products was carried out by electrophoresis as described previously (2.2.8.4.), part of PCR product was

sent for sequencing at first base laboratory and analyzed by blasting the result in NCBI (National Center for Biotechnology Information) to confirm the result.

**Table (١): PCR components.**

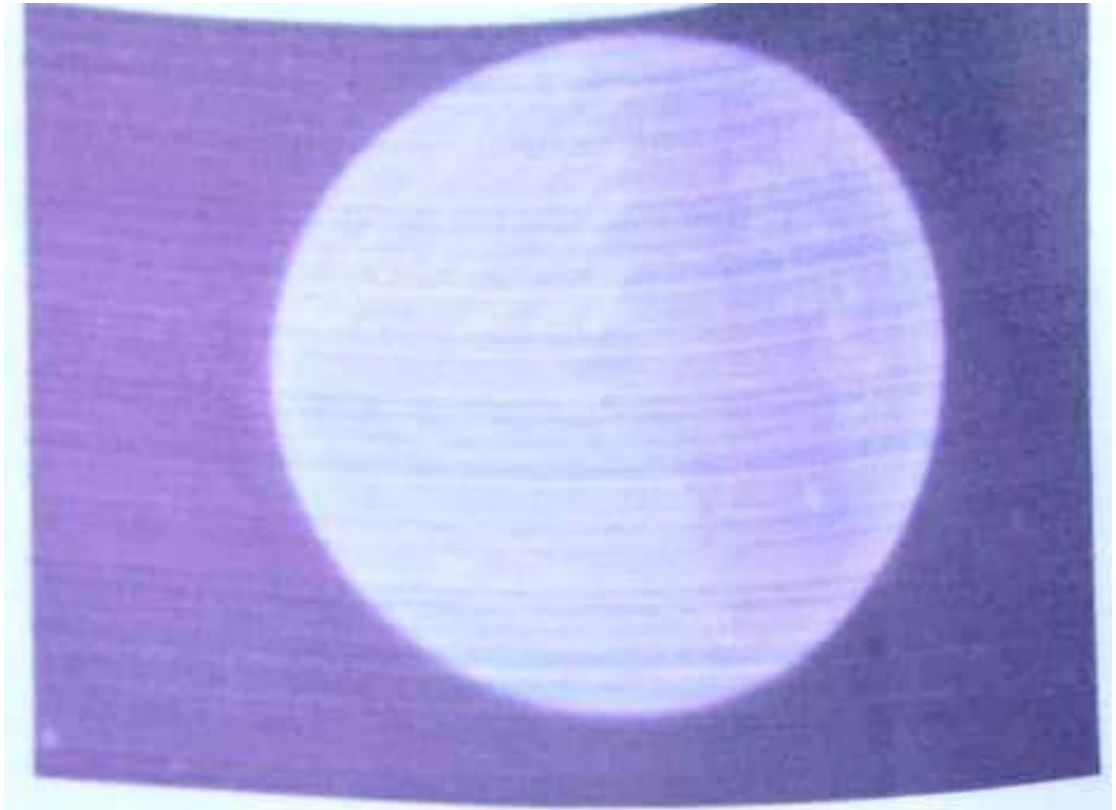
Components	Amount (ul)
Water nuclease free	١٤.٥
٥X PCR buffer	٥
dNTP mix. ١٠mM each	٠.٥
Forward primer, ١٠ μM	١.٢٥
Reverse primer, ١٠ μM	١.٢٥
DNA template	١
Thermostable DNA polymerase (٢ U/ul)	٠.٢٥
Additive	١.٢٥
TOTAL	٢٥

**Table (٢-٢): Thermal cycling protocol.**

Cycle steps	Temp. (C)	Time	Number of cycles
Initial denaturation	٩٨	٢ min	١
Denaturation	٩٨	١٠ s	٣٠
Annealing	٦٠	٣٠ s	
Extension	٧٢	٣٠ s	
Final extension	٧٢	١٠ min	١

### **Results and Discussion:**

The Nile Red staining of viable colonies was used as a direct and a rapid tool for direct detection of PHB accumulation within the bacteria cells Amirul et al. (٢٠٠٩, One isolate of three isolates had a positive result with visible and more fluorescent with Nile Red stain isolated from fertile soil sampled and used as a reference positive strain in the present study. A pink fluorescent under UV illumination was obtained as a result of the binding between stain and polymeric granules) Berlanga et al. (٢٠٠٦ The production of pink fluorescent can discriminate the isolate that produced PHB as shown in Figure (١).

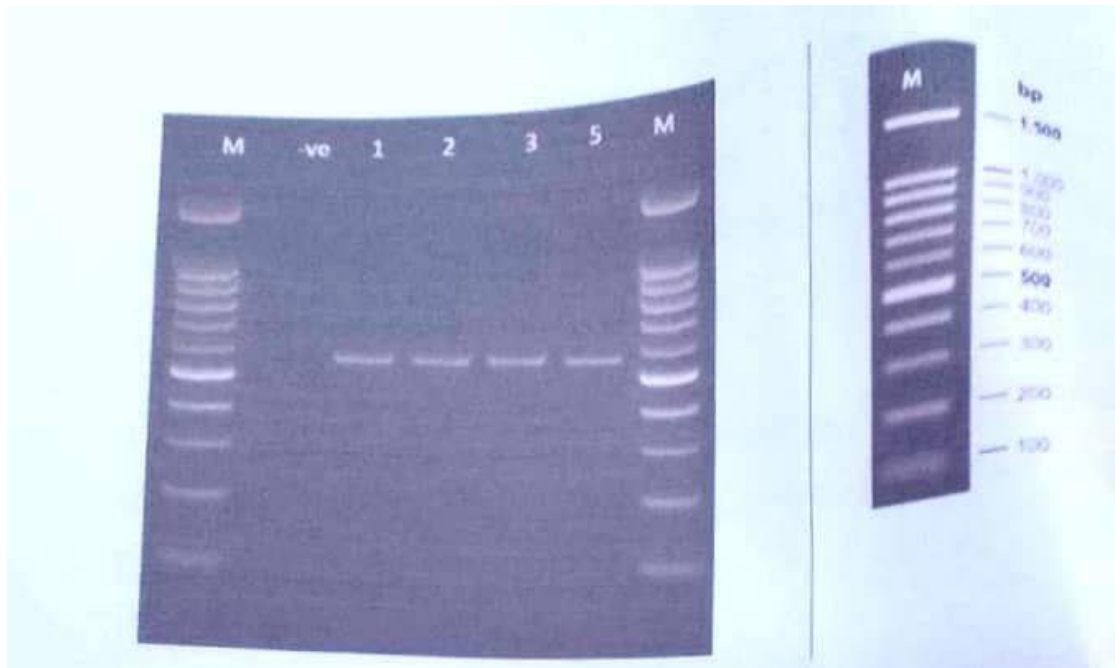


**(red Nile) bacteria stained with selective stain: (١) Figure**

**gene phaC Amplification and sequencing of**

**The PhaC gene was encoded to the PHA synthase enzyme which is the key enzyme of PHAs biosynthesis. The detection of PhaC gene reported the capacity to express the PHA synthase enzyme and the ability to accumulate and produce of PHA.**

**The electrophoresis of PCR product for PhaC gene as in Figure (٣- ١٦) demonstrated that the primers could be specifically used for the amplification of DNA fragment from PhaC gene. The duplicated bands in Figure (٢) showed the PhaC gene with size approximately ٥٠٨ bp.**



**Figure (٢):** The phac gene of bacteria that had positive result with Nile red stain. (all bands were duplicates of selected isolate).

#### **Conclusions and Recommendations:**

The aim of this study was to screen and select suitable organisms for PHB production. Among three isolates it was found only one bacterial isolate has the ability for PHB production and the phaC gene was detected for the isolate that gives pink fluorescence under UV-light. The focus of future research would be to sequence the synthetic gene and production of biopolymer by using cheap substrates to reduce the pollution of the environment that resulted from using petroleum plastic. In addition, we recommended to isolate the bacteria from different sources.

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