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Research Article

MOLECULAR CHARACTERIZATION OF A POLY- β -HYDROXYBUTYRATE-PRODUCING *MICROBACTERIUM* ISOLATE

Yehia A. Osman^{1*}, Ahmed Abd Elrazak¹, Wesam Khater¹, EL-Shahat Nashy², Attiya Mohamedin¹

¹ Botany Department, Faculty of Science, Mansoura University, 35516, Egypt

² National Research Center, Dokki, Giza, Egypt

Corresponding author's email: yaolazeik@mans.edu.eg

Abstract

Bacterial poly- β -hydroxybutyrate (PHB) is a natural, biodegradable polymer, which is accumulated in the cells as an energy reserve material due to depletion of nitrogen or phosphorous in the presence of excess carbon source. This polymer is foreseen to possess high industrial potentiality and excellent alternative to the non-degradable petroleum-based plastics. In this study, we isolated and characterized a local bacterial strain WA81 which accumulated 18mg/L PHB after 72 h growth in mineral salt medium under nitrogen deficiency. The PHB granules were detected in the cells using TEM and the genes encode for this polymer were detected by oligonucleotide primers using PCR technology. The *16S rRNA* gene nucleotide sequence for this isolate was used to construct a phylogentic tree against all available sequences in the GenBank. The phylogenetic tree data suggested that the closest type strain to the local bacterium is the *Microbacterium paraoxydans* CF36^T and hence we named it *Microbacterium* sp. strain WA81. Moreover, the set of enzymes responsible for the PHB biosynthetic pathway and their controlling elements were detected in this local isolate using PCR. The genes encode for the biosynthesis enzymes are *phbA* (β -ketothiolase), *phbB* (acetoacetyl CoA reductase), *phbC* (PHB polymerase), while the genes encode for the controlling elements are *phbP* (phasin), *phbZ* (PHB depolymerase). The novelty of this local bacterium lies in its ability to accumulate huge amounts of PHB in its cytoplasm and the presence of a whole set of genes encode for the PHB biosynthetic and catabolic pathways of this polymer.

Keywords: Polyhydroxybutyrate; *Microbacterium*; *16S rRNA*.

Introduction

Plastics have multipurpose characters of strength, lightness, durability and resistance to degradation. They become an important product in the daily life and have replaced glass and paper in packaging. However, its accumulation become one of the greatest problem facing modern civilization due to its degradation resistance (Khanna and Srivastava, 2005a). The alternative solution was the development of eco-friendly and biodegradable biopolymer materials (Khardenavis *et al.*, 2007). One of these biopolymers is polyhydroxyalkanoates (PHAs) which comprise a class of polyesters that are synthesized by most prokaryotes as an insoluble inclusions in their cytoplasm to levels as high as 90% of the cell dry weight (Madison and Huisman, 1999; Pötter and Steinbüchel, 2006). They have mechanical properties similar to polypropylene or polyethylene and can be extruded, molded, spun into fibers, made into films and used to make heteropolymers with other synthetic polymers. They are also completely degraded to water and carbon dioxide under aerobic conditions and to methane under anaerobic conditions by micro-organisms in soil, sea, lake water and sewage (Khanna and Srivastava, 2005a).

PHB is immensely used in medicine due their nontoxic nature, also applied in pharmacy, agriculture, food industry even as raw material for enantiomerically pure chemicals and paint industry (Anderson and Dawes, 1990).

Several prokaryotes synthesize poly (3-hydroxybutyric acid; PHB), from variety of renewable resources under unfavorable growth conditions; i.e. imbalance in the nutrient supply. Its accumulation was favored by adequate availability of a suitable carbon source and a limiting supply of nitrogen, phosphate and dissolved oxygen or microcomponents such as magnesium, sulphate, iron, potassium, manganese, copper, sodium, cobalt, tin and calcium (Koller *et al.*, 2010). Depending on the culture conditions, PHB-producing bacteria were classified into two groups: 1) group one (*Cupriavidus necator*, *Protomonas extorquens* and *Protomonas oleovorans*) requires limitation of essential nutrients such as nitrogen, oxygen and presence of excess carbon source for the efficient synthesis of PHB, and 2) the second group of bacteria does not require nutrient limitation and can accumulate PHB during exponential growth phase. It includes *Alcaligenes latus*, a mutant strain of *Azotobacter*

vinelandii and recombinant *E. coli* harboring the PHB biosynthetic operon of *C. necator* (Khanna and Srivastava, 2005a; Lee, 1996).

Among more than 250 different natural PHB-producers, only few bacteria have been employed for the biosynthesis of PHB at large-scale. These include *Alcaligenes latus*, *Bacillus megaterium*, *C. necator* and *P. oleovorans*, which are capable of utilizing various carbon sources including plant oils or wastes to produce PHB. In *Ralstonia eutropha*, the PHB is synthesized in a three-step pathway where β -ketothiolase first condenses two molecules of acetyl-CoA to aceto-acetyl-CoA. Then an NADPH-dependent aceto-acetyl-CoA reductase converts it to 3-hydroxybutyryl-CoA. The third and the final step is the polymerization reaction of the 3-hydroxybutyryl-CoA catalyzed by PHB polymerase to PHB (Khanna and Srivastava, 2005a). This biosynthetic pathway is prevalent in the majority of PHB-producing bacteria (Pötter and Steinbüchel, 2006) and their coded genes are *phbA* (β -ketothiolase), *phbB* (acetoacetyl-CoA reductase), and *phbC* (P(3HB) polymerase). The three enzymes are encoded by a single operon *phbCAB* under the control of an upstream promoter of *phbC* which transcribes the complete operon (Madison and Huisman, 1999).

During our screening for a PHB-producing bacterium, a local isolate of *Microbacterium* was isolated in pure culture and identified at morphological and molecular levels. Moreover, it produces significant amount of PHB at the shake-flask level which was evident in the Sudan Black B stained smears and TEM photos. To our surprise, this is the second report ever in the literature of PHB-producing *Microbacterium* isolate.

Materials and Methods

Microorganism isolation and growth conditions

The PHB-producing bacterial isolate was obtained from rhizosphere soil sample collected from Mansoura University Plantation. One gram of rhizosphere soil was serially diluted in sterile distilled water and plated on nutrient agar plates and incubated at 30°C for 24 hours. Various colonies were individually picked and sub-cultured 3-4 times on nutrient agar plates to obtain pure culture (Mohapatra et al., 2014). The purified bacterial isolates were maintained on nutrient agar slants, stabs and stored at 4°C for daily usage, while, long term storage was under 20% glycerol and kept at -20 °C. The culture maintained on nutrient agar slants at 4°C, was sub cultured monthly. The isolate was grown in the mineral salt medium consisted of: 2.0 g/L (NH₄)₂SO₄, 2.0 g/L KH₂PO₄, 0.6 g/L Na₂HPO₄, 0.2 g/L MgSO₄.7H₂O, 20 mg/L CaCl₂, 10 mL/L trace metal solution, 0.1 g/L yeast extract was used. The trace metal solution consisted of: 1.3 mg/L ZnSO₄.7H₂O, 0.2 mg/L FeSO₄.7H₂O, 0.6 mg/L (NH₄)₆Mo₇O₂₄.4H₂O and 0.6 mg/L H₃BO₃. Fructose was used as a carbon source in a concentration of 40g/L for PHB production media and 10

g/L for inoculum development. Fructose and mineral salt solutions were sterilized separately and aseptically mixed together at room temperature prior to inoculation. The pH of the resulting broth was adjusted to 7.0 with 2N NaOH/2N HCl ((Khanna and Srivastava, 2005b).

Screening for PHB-production

Staining

Sudan Black B stain was used for detecting the presence of PHB in the cytoplasm of the isolated bacterial cells (Moreno et al., 2007) grown for 48 h in PHB production broth containing 40g/L fructose (Khanna and Srivastava, 2005b). Moreover, transmission electron micrography (TEM) was used to observe PHB granules in the selected isolate bacterial cells.

Transmission Electron Microscopy (TEM)

Cells with age 18h were harvested and fixed in Millonig's phosphate buffer supplemented with 3% glutaraldehyde and incubated for several days at 4°C. The samples were extra fixed in 1% osmium tetroxide for 1 h at room temperature. The samples were then rinsed with Millonig's phosphate buffer and regularly dehydrated with ethanol. For TEM, samples of the diaphragm and housing were infiltrated with resin and ethanol, embedded in the resin overnight, cut with a diamond knife to a thickness of 60-80 nm, pulse-stained in uranyl acetate and lead citrate, and in the last stage examined under a transmission electron microscope (Sangkharak and Prasertsan, 2008).

Time course of PHB production by the local bacterium

The time course of bacterial growth and PHB production was studied by growing the bacterium in mineral salt medium. The seed culture (a 150 ml containing 10g/L fructose) was grown shaking (150 rpm) at 30°C for 24 h. Fifteen flasks (100 ml medium/each) containing 40 g/L fructose were inoculated with 10 mL from the seed flask and kept under shaking conditions for 72 h at 30°C. Samples were withdrawn at 0, 2, 4, 6, 9, 12, 15, 24, 30, 36, 48, 60 and 72 h and analyzed for growth (OD₆₀₀), and PHB concentrations (Sangkharak and Prasertsan, 2008).

Extraction and quantification of PHB

The bacterial cell pellet collected by centrifugation was suspended in sodium hypochlorite and incubated at room temperature for 1h, then centrifuged again and the supernatant was discarded. The pellet containing PHB was subjected to two consequent washes with acetone and ethanol. Finally, the polymer granules were dissolved in hot chloroform, filtered and to the filtrate, concentrated 10 ml hot sulfuric acid (H₂SO₄) was added. The addition of H₂SO₄ converts the polymer into a brown colored crotonic acid. The solution was cooled and the absorbance read at 235 nm against a sulfuric acid blank and PHB concentration was extrapolated from the standard curve (Law and Slepecky, 1961).

DNA extraction and 16S rRNA gene amplification

The Insta-Gene Matrix Genomic kit (Bio-Rad, USA) was used to extract the total genomic DNA from the isolate under investigation and used as a template for amplification of the 16S rRNA gene. PCR reaction was prepared as followed: 5 µl master mix, 20 µl each of primers 518F (5'-CCA gCagCCgCggTA ATA Cg -3') and 800R (5'-TAC CAgggT ATC TAA TCC -3'), 3 µl of 50 mM MgCl₂, 0.5 µl of AmpliTaq DNA polymerase, and 1 µl of genomic DNA and the total volume was made up with distilled water to 50 µl. The PCR products were checked by agarose gel electrophoresis (1 % w/v; 30 min at 100 V, 0.59 TBE). The amplified fragments were compared with 100 bp molecular size marker (MBI Fermentas, Lithuania). The PCR product was stored at -20° C for subsequent analysis.

Purification and sequencing of 16S rRNA genes

The PCR product was purified using Montage PCR Clean up kit (Millipore) before being sequenced. Sequencing was performed by using ABI PRISM®BigDye™ Terminator Cycle Sequencing Kits. A 2µl of the kit was mixed with 5 µl of PCR mixture in a 1.5 ml sterile screw tube, incubated for 15 min at 37°C, followed by a second incubation for 15 min at 80°C. The purified PCR product was then sequenced using an Applied Biosystem model 3730XL automated DNA sequencing system (Applied BioSystems, USA). The quality and quantity of the sequence obtained was checked with Finch TV version 1.4.0. While, DNA baser (version 3.55.0.199) software was used to assemble the gene. The sequence was identified by BLAST and SeqMatch against Genbank database.

Detection of the gene encodes for PHB

Polymerase chain reaction (PCR) and specific oligonucleotide primers (Table 1) were used to detect the genes encoding and regulating PHB synthesis (Mullis, 1990). The PCR was performed in 25µl reaction volume containing 1x buffer (10mM Tris- HCl PH 8.3, 50mM KCl, 2m M MgCl₂), 250µM each of dGTP, dATP, dCTP and dTTP, 2.5 units of Taq DNA polymerase, 100 p mol of each primer reported in Table 1 and DNA template.

Table 1: primers sequences used in PCR reaction for detecting PHB production genes.

Primers	Nucleotide sequence (5' → 3')	Product Size(bp)
<i>phbA</i> F <i>phbA</i> R	GTTCCCATCCTGATTCCGCA GGTCAGACCCAGTTCCTTGG	216
<i>phbB</i> F <i>phbB</i> R	TTCAACGTGACCAAGCAGGT GGTGGAGTAGTTGGTCTGGC	117
<i>phbC</i> F <i>phbC</i> R	AACGGCGATTCCACCAATCT TCCAGGGGACGATGTGATCT	181
<i>phbP</i> F <i>phbP</i> R	CCAATTGGAAGCTTGCTGG TTCATCAGACTCACCACCGC	363
<i>phbZ</i> F <i>phbZ</i> R	ACCTTACCAATCCGCTGAG TACTCGTCGTAGAAGCGCAC	827

Components were overlaid with a drop of mineral and DNA amplification started with denaturing the template DNA at 94°C for 5 min followed by 35cycles. Each cycle consisted of: denaturation at 94°C for 1 min, annealing at 50°C for 45 sec and extension at 72°C for 3 min, and a final extension step at 72°C for 7 min. The PCR products were analyzed by agarose gel electrophoresis.

Results and Discussion**Morphological and molecular identification of the local bacterium**

A group of bright yellow, smooth and sticky colonies were prevalent in our study. They were Gram-positive, small, coryneform rod-shaped bacteria. Sudan black-B smears of all morphological types of bacteria isolated from rhizosphere soil of a potato plantation showed the accumulation of bluish black, ovoid or spherical granules of PHB, in their cytoplasm. From amongst all isolates a particular bacterium with a dense PHB granule was identified as *Microbacterium* sp.WA81 depending on morphological and 16S rRNA gene sequencing analysis. The electron microscope photographs showed the enlarged PHB granules inside the local bacterial isolate of *Microbacterium* strain WA81. The size of the PHB granules ranged from 0.17 to 0.3 µm (Fig.1). This finding is supported by the single published report which provided an evidence for the ability of *Microbacterium* to produce PHB (Shivakumar, 2012) . This emphasized the novelty of this local isolate in terms of its dependence on PHB as reserve energy source under nutrient limitation conditions.

The 16S rRNA sequencing (Fig.2) followed by basic local alignment search tool (BLAST) and SeqMatch analysis. The 16S rRNA sequence was submitted to the GenBank and assigned an accession number KM191355. The 16S rRNA gene sequence (Fig. 2) was used to build a phylogenetic tree (Fig. 3), by performing automated BLAST searches, to determine the closest type strain to the isolate under investigation. The phylogenetic tree suggested that the local isolated strain is definitely a member of the genus *Microbacterium* and formed a common phyletic lineage that could be equated with a novel local species. Low 16S rRNA gene sequence similarity values (<99%) were obtained between the novel strain WA81 and all species with valid published names from the genus *Microbacterium*. Close reading of the 16S rRNA gene sequences analysis revealed that the local PHB-producing bacterial isolate had the highest matching similarities, arranged in ascending order, to the *Microbacterium paraoxydans* CF36^T strain (99.5%), followed by *M. oxydans* (99.2%), *M. lequificiens* (98.7%), *M. luteolum* (98.9%) and *M. saperdae* (98.3%) as indicated from the phylogenetic tree (Fig. 3). (Janda and Abbott, 2007; Keller et al., 2010).

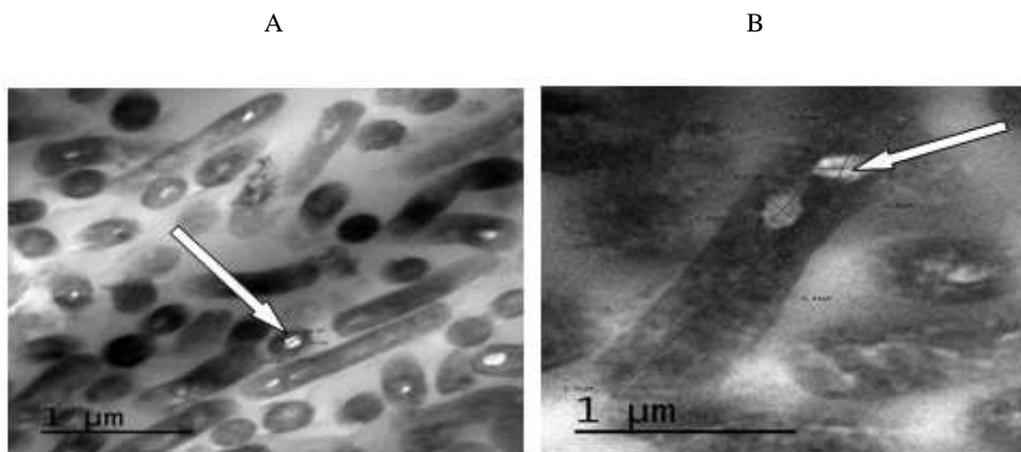


Fig. 1: TEM of the local PHB-producing bacterium. Panel A): *Microbacterium* WA81 showing accumulation of PHB granules inside the bacterial cells. Panel B): Dimensions of the individual PHB granule after 18 h.

5'-
 TCAGGATGAACGCTGGCGGCGTGCTAACACATGCAAGTCGAACGGGAACACGGAGCTTGCTCTGTGGGATCAGT
 GGCGAACGGGTGAGTAACACGTGAGCAACCTGCCCTGACTCTGGGATAAGCGCTGGAAACGGCGTCTAAACT
 GGATATGTGACGTGCCGCATGGTCTGCGTTGGAAAGATTTTTTCGGTTGGGGATGGGCTCGCGCCTATCAGCTTG
 TTGGTGAGGTAATGGCTACCAAGGCGTCGACGGGTAGCCGGCCTGAGAGGGTGACCGGCCACACTGGGACTG
 AGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCAA
 CGCCGCGTGAGGGATGACGGCCTTCGGGTTGTA AACCTCTTTTAGCAGGGAAGAAGCGAAAGTGACGGTACCTG
 CAGAAAAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGTAATACGTAGGGCGCAAGCGTTATCCGGAATTATT
 GGGCGTAAAGAGCTCGTAGGCGGTTTGTGCGCTCTGCTGTGAAATCCCCGAGGCTCAACCTCGGGCCTGCAGTGG
 GTACGGGCAGACTAGAGTGCAGTGGGGAGATTGGAATTCCTGGTGTAGCGGTGGAATGCGCAGATATCAGGA
 GGAACACCGATGGCGAAGGCAGATCTCTGGGCCGTA ACTGACGCTGAGGAGCGAAAGGGTGGGGAGCAAACAG
 GCTTAGATAACCCTGGTAGTCCACCCCGTAAACGTTGGGAACTAGTTGTGGGGTCCATTCCACGGATTCCGTGACG
 CAGCTAACGCATTAAGTTCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAA ACTCAAAGGAATTGACGGGGACC
 CGCACAAGCGGCGGAGCATGCGGATTAATTCGATGCAACGCGAAGAACCTTACCAAGGCTTGACATATACGAG
 AACGGGCCAGAAATGGTCAACTCTTTGGACTCTGTAACAGGTGGTGCATGGTTGTCGTACGCTCGTGTCTGTG
 AGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGTTCTATGTTGCCAGCACGTAATGGTGGGAACTCATG
 GGATACTGCCGGGGTCAACTCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCTTATGTCTTGGGCTTC
 ACGCATGCTACAATGGCCGGTACAAAGGGCTGCAATACCGTGAGGTGGAGCGAATCCCCAAAAGCCGGTCCCA
 GTTCGGATTGAGGTCTGCAACTCGACCTCATGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCAACGCTGCGGT
 GAATACGTTCCCGGGTCTTGACACACCCGCGTCAAGTCATGAAAGTCGGTAACACCTGAAGCCGGTGGCCTA
 ACCCTTGTGGAGGGAGCCGTCG-3'

Fig. 2: The *16S rRNA* gene nucleotide sequence of the local *Microbacterium* sp. strain WA81 arranged from the 5` end to the 3` end.

Correlation between growth and PHB production

The time course analysis of samples withdrawn from cultures (mineral salt medium) under nitrogen-limiting growth conditions indicated that PHB was a growth-associated product and its accumulation significantly increased when the culture reached the exponential growth phase as PHB production was noticeable after 35 h and showed a sharp increase from 50 h upwards until it reached maximum PHB value of 18 mg/l after 72 h (Fig.4). This is a fascinating phenomenon, since previous physiological

studies of PHB production by *A. eutrophus* and *Azotobacter* spp. produced very little PHB during the exponential phase of batch growth, although *Alcaligenes latus* and a respiratory-impaired mutant of *Azotobacter vinelandii* (UWD) were exceptions (Henderson and Jones, 1997). The growth-associated production of PHB by the local isolate agreed well with *Rhodobacter sphaeroides* ES16, *Methylobacterium* sp. ZP24 and *Alcaligenes latus* and substantially different from *Ralstonia eutropha* which accumulated PHB at the stationary phase (Nath et al., 2008; Sangkharak and Prasertsan, 2008).

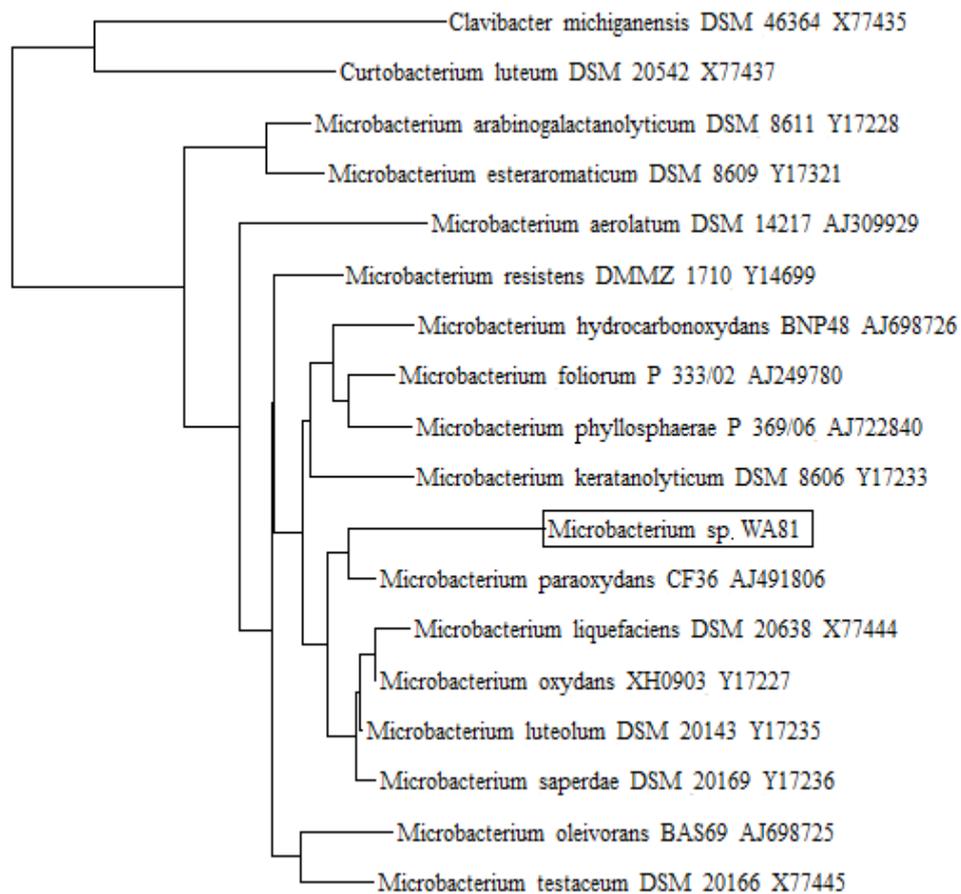


Fig.3: Phylogenetic dendrogram based on *16S rRNA* gene sequence analysis showing the phylogenetic position of *Microbacterium* sp. WA81 compared to closely related species of the genus *Microbacterium*. The sequences of *Curtobacterium luteum* DSM 20542^T and *Clavibacter michiganensis* subsp. *michiganensis* DSM 46364^T are used as external references.

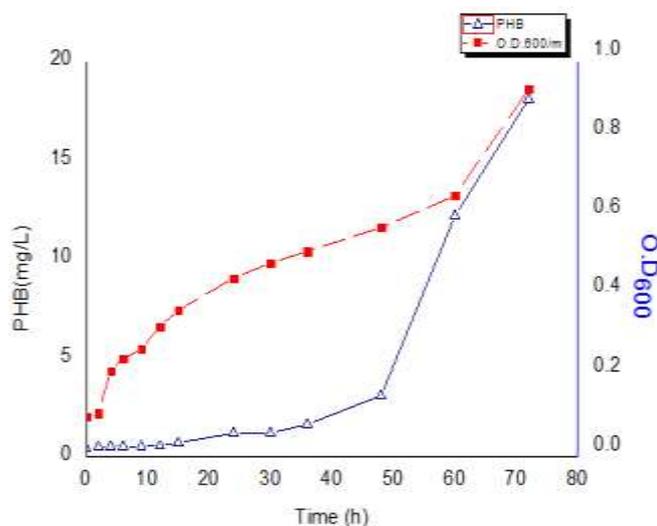


Fig.4: Bacterial growth and PHB accumulation in relation to incubation period (h).

Detection of the genes encode for PHB biosynthetic pathway

PCR was very useful tool to detect the genes encode for PHB biosynthetic pathway in the isolate under

investigation. In these reactions PCR amplicons of molecular masses of 216 bp, 117 bp, 181 bp, 363 bp and 827 bp produced (Fig.5). They are characteristic of genes encodes for a PHB β -ketothiolase (*phbA*), acetoacetyl CoA reductase (*phbB*), PHB polymerase (*phbC*), phasin (*phbP*), PHB depolymerase (*phbZ*), respectively. Of all the PHAs, PHB is the most extensively characterized polymer, The PHB biosynthetic pathway depends on three major enzymatic reactions catalyzed by three distinct enzymes similar to those reported in the two of the natural PHB producers: *Zoogloea ramigera* and *Ralstonia eutropha*. The first reaction is accomplished by β -ketothiolase enzyme encoded by *phbA* gene by condensation of two acetyl coenzyme A (acetyl-CoA) molecules into acetoacetyl-CoA. The second reaction is catalyzed by acetoacetyl-CoA reductase enzyme encoded by *phbB* gene and it is carried out by the reduction of acetoacetyl-CoA to (R)-3-hydroxybutyryl-CoA. Lastly, PHB polymerase enzyme, encoded by *phbC* gene, polymerizes the (R)-3-hydroxybutyryl-CoA monomers into poly(3-hydroxybutyrate) (Madison and Huisman, 1999). The detection of these genes in *Microbacterium* sp. strain WA81 is in accordance with the same genes of several other bacteria, however, their location on the chromosome and/or

plasmid was not established. In *R. eutropha* the genes encoding β -ketothiolase (*phbA*), acetoacetyl-CoA reductase (*phbB*), and PHB polymerase (*phbC*) are closely linked on the chromosome (Korotkova and Lidstrom, 2001; Peoples and Sinskey, 1989; Schubert *et al.*, 1991). In *Rhizobium meliloti*, *Paracoccus denitrificans*, and *Z. ramigera* the two genes, *phbA* and *phbB*, are organized in an operon (Korotkova and Lidstrom, 2001). In *P. denitrificans* *phbC* is located on the chromosome together with genes encoding PHB depolymerase (*orf1*), PHB granule associated protein (*phbP*), and a regulator for PHB granule associated protein (*phbR*) (Maehara *et al.*, 1999). The PHB polymerase gene (*phbC*) was isolated and characterized from *M. extorquens* strain IBT no.6. In this strain, it has been shown that *phbC* is located separately from other genes encoding enzymes for PHB biosynthesis (Valentin and Steinbüchel, 1993).

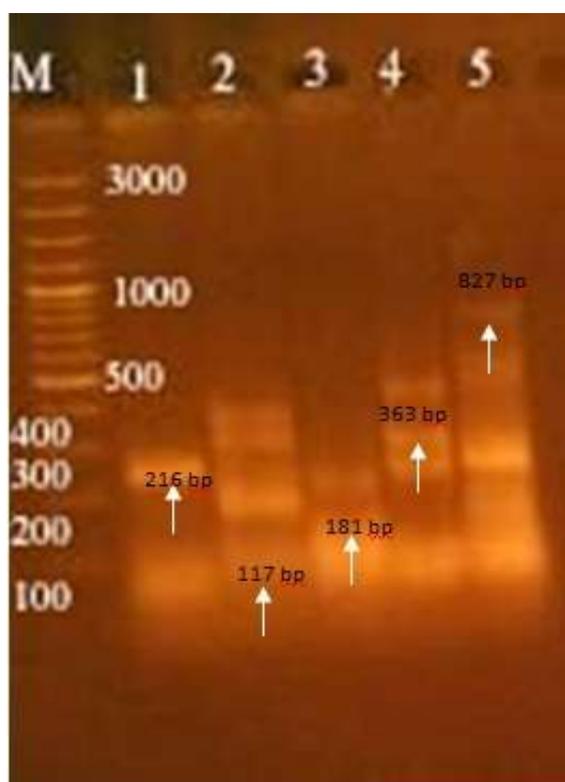


Fig.5: PCR product showing genes responsible for PHB production : 1)*phbA*, 2)*phbB*, 3)*phbC*,4) *phbP* and 5)*phbZ*

More evidences comes from immunochemical analysis of some PHB-producing strains, Phasin (PhbP) is a natural granule-binding protein that determines the size of PHA granules and it was not found to be in a free state in the cytoplasm of the wild type cells. The overexpression of PhbP resulted in the formation of many small PHB granules while a *phbP* mutant contained only a single PHB granule. These results indicating the importance of the concentration of PhbP which is inversely related to the size of the granule (Madison and Huisman, 1999). Depolymerases are highly specific for the polymers consisting of monomers in the (R) configuration. PHAs depolymerase are carboxyesterases that hydrolyze the water insoluble polymer to water-soluble

monomers and /or oligomers and lastly to water and carbon dioxide or methane. Early PHB degrading bacteria were isolated by selection for microorganisms able to utilize PHB as the sole source of carbon and energy. PHAs degrading bacteria differ from each other depending on the type of PHAs they degrade, however some bacteria revealed a rather broad polyester specificity and are able to utilize a wide range of PHAs (Amara and Moawad, 2011).

Detection of all these genes, in the local bacterium, was not only to understand PHB synthesis and degradation, but more importantly, to understand carbon (fructose) flow and accumulation of PHB during the growth of the *Microbacterium* sp. WA81. Knowing that two major pathways for PHB synthesis are documented in bacteria, our data suggest that the *Microbacterium* strain WA81 can be grouped with *Ralstonia eutropha*, *Methylobacterium extorquens*, *Zoogloea ramigera*, and *Azotobacter beijerinckii*. Where PHB is synthesized from acetyl coenzyme A (acetyl-CoA) as a result of sequential action of three enzymes: β -ketothiolase, NADPH-dependent acetoacetylCoA reductase, and PHB polymerase (Belova *et al.*, 1997; Fukui *et al.*, 1987; Haywood *et al.*, 1988; Korotkova and Lidstrom, 2001; Peoples and Sinskey, 1989) However, PHB synthesis is catalyzed by five enzymes: β -ketothiolase, NADH-dependent acetoacetyl-CoA reductase, L-(1) and D-(2)-specific crotonyl-CoA hydratases (crotonases), and PHB polymerase in *Rhodospirillum rubrum* and *Methylobacterium rhodezianum* (Moskowitz and Merrick, 1969; Mothes and Babel, 1994, 1995). Moreover, the local *Microbacterium* sp. strain WA81 contained genes *phbP* (phasin), *phbZ* (PHB depolymerase) which encode for the PHB degradation. The PHB degradation in most bacteria is catalyzed by PHB depolymerase, β -hydroxybutyrate dehydrogenase, acetoacetate-succinate-CoA transferase and β -ketothiolase. (Anderson and Dawes, 1990).

In conclusion, different techniques were used to identify bacteria, especially the *16S rRNA* sequence analysis. This systematic process narrows down the types of bacteria to the genus or may reach the species level. The dependence upon bacteriological, biochemical and recently, molecular methods such as the *16S rRNA* gene, beside the qualitative observations and comparison to the data banks are much more accurate and time saving in reaching the reliable identity of novel bacterial strains such as the *Microbacterium* sp. WA81.

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