

Isolation, characterization and Molecular identification of Polyhydroxybutyrate producing bacterium grown on cooking oil wastes

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Abstract: Disposal of petroleum based plastic is a threat to our environment and health since it is non degradable. Polyhydroxybutyrate (PHB) is the best type of biodegradable polymer that accumulated by genera of bacteria. These bacteria used it as a carbon and energy sources. The properties of PHB are identical to the petroleum based plastics. Twenty two bacterial isolates were obtained on nutrient agar and modified C2M agar media from different soil samples, collected from Jeddah, Kingdom of Saudi Arabia. All bacterial isolates used oil as carbon source. They were screened for PHB production using Sudan Black B stain. Three bacterial isolates accumulated PHB and the quantity of PHB was determined by UV-VIS spectrophotometer. The bacterial isolate NM17 is the best PHB producer which accumulates 50.10 % of the cell dry weight. The bacterial isolate NM17 was identified based on morphological, physiological, biochemical tests and 16s rRNA gene sequence as *Bacillus funiculus*. The best condition of PHB production were obtained using medium C2M at pH 6.5, incubation temperature at 37°C and incubation period was 2 days.

Keywords: petroleum based plastic, polyhydroxybutyrate, production, waste cooking oil, *Bacillus*

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I. Introduction:

Disposal of petroleum based plastic is a threat to our environment and health. Chemically, petroleum based plastic synthetic polymers made of a long chains of molecules by petroleum and other fossil fuels (Tuominen *et al.*, 2002). Unfortunately, these petroleum based plastics stick to the environment for a long time in a hard condition and remain protected from the damage of microorganisms and chemicals (Marjadi and Dharaiya, 2011). For this reason, the need to produce bioplastics for available and alternative technology has become important (Zargounet *et al.*, 2015). Biological material has a special type called bioplastics, their chemical nature is degradable and eco-friendly (Kamilah *et al.*, 2011; Jain and Tiwari, 2015). Among different biodegradable plastic, polyhydroxylalkanoate, a biopolymer found as inclusion, used as both carbon and energy sources in bacteria, is the most famous (Zhang *et al.*, 2003; Scheel *et al.*, 2016).

There are more than 155 polyhydroxylalkanoate (PHA) monomers had been identified (Agnew and Pflieger, 2013; Nielsen *et al.*, 2017). Polyhydroxybutyrate that accumulated by numerous microorganisms is considered to be the best characterized and the most studied of polyhydroxylalkanoate (Echo and Antimafia, 2010; Phanseet *et al.*, 2011).

The high cost production of carbon source is the main reason for high cost of polyhydroxylalkanoate. Therefore, the need to investigate inexpensive substrates to make the polyhydroxylalkanoate production economically more attractive than petroleum based plastic (Povolovet *et al.*, 2010; Budde *et al.*, 2011).

Plant oils found as a possible substrate for polyhydroxylalkanoate production (Kaharet *et al.*, 2004; Alias and Tan 2005). Plant oils compared with sugar such as glucose or sucrose can increase the production of PHA and decrease the cost of the production (Daniel, 2006; Chenyu *et al.*, 2012).

Waste cooking oil is a cheaper sustainable source of plant oils that can be used in the production of polyhydroxylalkanoate (PHA) which can be easily collected from several of domestic communities (Zhang *et al.*, 2003; Jiang *et al.*, 2016).

There are about 250 of different bacterial strains that can produce polyhydroxylalkanoate at different levels of quality, yield and efficiency under several conditions (Pollet and Averous, 2011). For example, *Azotobacter*, *Bacillus*, *Archaeobacteria*, *Methylobacteria* and *Pseudomonas* (Lee, 1996). Moreover, *Ralstonia eutropha* (formerly *Alcaligenes eutrophus*) and *Cupriavidus necator* which are the most studied bacteria that can accumulate polyhydroxylalkanoate up to 80 per cent of the dry weight of PHA (Verlinden *et al.*, 2007; ShzeMok *et al.*, 2016). The aim of this study was isolation and characterization of the best polyhydroxybutyrate producers that used waste cooking oil as carbon source.

II. Materials And Methods

Samples collection:

Different soil samples were collecting from gardens in different region in Jeddah, Kingdom of Saudi Arabia. The soil samples were collected by a hand shovel at 10 cm deep and kept it in sterile plastic containers at 4°C until use.

Bacterial isolation from the collected soil samples:

A serial dilution method was used to isolate the bacteria from soil samples. 1g of soil samples was added into 9ml of serial distilled water and homogenized by a blender for 1 min. the soil suspension was diluted from 10^{-1} to 10^{-6} . Then, spreading and streaking plate's method were used to isolate the bacteria from the diluted soil suspension. About 1ml of each diluted soil suspension was spread on two solid medium which were Nutrient Agar plates, composed of g/l: 3 g beef extract, 5 g pepton, 8 gNaCl, 15 g bacteriological agar per liter, pH, 7, (Piwpan et al., 2016) and modified C2M Agar plates. This medium composed of 50 ml cooking oil, 3g meat extract, 5g polypeptone, 2g yeast extract, 2gNaCl and 20g bacteriological agar per liter, pH, 7 (Piwpan and Jaturapiree, 2016). All plates were incubated at 37°C for 24 h. After that, the bacterial growth was picked with a needle loop and streaked on each plates until a pure colony appears. Finally, the pure bacterial isolate kept on Nutrient Agar Slant at 4°C for tests (Mikkili *et al.*, 2014) and preserved in 10 % glycerol at -20°C until used (Wu *et al.*, 2016).

Screening for polyhydroxybutyrate producers on solid agar method:

The bacterial colonies were screened by using Sudan Black B stain to detect the polyhydroxybutyrate (PHB) granules. 3 % of Sudan Black B powder dissolved in 70 % of ethanol. Two solid media (Nutrient Agar plates and modified C2M Agar plates) were used to culture the bacterial colonies which were incubated at 37°C for 24 h. Then, the Sudan Black B solution spread over the plates and left it for 30 min. After that, the plates washed with 96 % of ethanol to remove the excess stain. The bacterial colonies that produce polyhydroxybutyrate appears dark blue colored (Liu *et al.*, 1998; Zargounet *al.*, 2015).

Screening for polyhydroxybutyrate (PHB) producers under light microscope method:

The bacterial colonies were spread on a clean slide and heat fixed by passing it with a flame. Then, a few drops of Sudan Black B solution (3 % of Sudan Black B in 70 % of ethanol) added over the slide for 10 min. After that, the slide immersed in xylene to completely decolorized. The slide was stained with safranin solution (5 % in water) for 10 seconds and washed with distilled water. After the slide dried, a drop of immersion oil was added and examined under the oil immersion lens on the light microscope (Burdon, 1946; Zargounet *al.*, 2015).

Morphological, Physiological, biochemical and Molecular characterization of the selected bacterial isolate (NM17):

The identification of the best polyhydroxybutyrate (PHB) producer (NM17) was carried out according to morphological, physiological, biochemical characters(Cappucino and Sherman,1998; Mikkili *et al.*, 2014). The selected bacterial isolate (NM17) was analyzed by 16S rRNA analysis .

Cultivation method:

The pre culture were prepared in 250 ml conical flasks containing 50 ml of nutrient broth media. Each conical flask was inoculated with the selected bacterial isolates (NM17) and incubated in a rotary shaker at 150 rpm rate for 24 h at 37°C (Piwpan and Jaturapiree, 2016).The 250 ml conical flasks contain 48 ml of the production medium which were a modified C2M broth media composed of (50mL cooking oil, 3g meat extract, 5g polypeptone, 2g yeast extract and 2gNaCl) and the pH adjusted to 7 (Piwpan and Jaturapiree, 2016). Each conical flask was inoculated with 2 ml of the pre cultures of the selected bacterial isolates (NM17) and incubated in a rotary shaker at 150 rpm rate for 48 h at 37°C.

The effects of different factors e.g. pH values (5.5, 6, 6.5, 7, 7.5, 8.0 and 8.5), incubation temperature (20, 25, 30, 37 and 40) and incubation period (1, 2, 3, 4 and 5 days) on PHB production were studied. The bacterial growth, dry cell weight and PHB quantity were measured.

Extraction and assay of polyhydroxybutyrate:

The PHB was extracted by using the hypochlorite method which described by Slepecky and Low (1960). About 1 ml of the polyhydroxybutyrate on a liquid media placed in 10 ml centrifuged tube and centrifuge at 4000 rpm for 10 min. The supernatant was discarded and a 9 ml of the sodium hypochlorite was added to the pellet and incubated for 24 h at 37°C. After that, the mixture was centrifuged for 10 min at 4000 rpm and discarded the supernatants. The pellet washed with distilled water, acetone and ethanol and centrifuged

to discard the supernatants. Finally, the polyhydroxybutyrate was dissolved in a hot chloroform and evaporated. Then, the polyhydroxybutyrate treated with 10 ml of concentrated sulfuric acid (H₂SO₄) and heated at 100°C in water bath for 10 min. The sulfuric acid (H₂SO₄) cause the polyhydroxybutyrate to convert into a crotonic acid. A serial dilution 10⁻¹- 10⁻⁷ of the sulfuric acid were prepared and measured at the absorbance 235 nm with UV_VIS spectrophotometer (Tekinet *al.*, 2011; Salah Elsayed *et al.*, 2013). The crotonic acid concentration were calculated by using a standard curve equation: $y = 0.017x + 0.0419$ where y is the optical density 235 nm while x is the crotonic acid concentration (µg/ml).

The polyhydroxybutyrate (PHB) percentage was calculated by the following formula:

$$\text{PHB\%} = \frac{\text{PHB quantity } (\mu\text{g/ml})}{\text{DCW (g/L)}} \times 100$$

Measurement of bacterial growth and dry cell weight:

The bacterial growth was measured by spectrophotometers with the optical density at 550 nm. Measurement of the dry cell weight, by centrifuge the bacterial media and discarded the supernatant. Then, the dry cell weight measured after the pellet washed with distilled water and dried for three days at 60°C (Shivakumar, 2012).

III. Results:

Twenty two bacterial isolates were obtained from soil gardens in different region in Jeddah city, Kingdom of Saudi Arabia. All bacterial isolates were screened by using Sudan Black B stain on nutrient agar media (Figure 1) and modified C2M agar (Figure 2). The positive polyhydroxybutyrate (PHB) producers (NM7, NM16 and NM17) appears dark blue colored. All positive polyhydroxybutyrate (PHB) producers (NM7, NM16 and NM17) were stained by Sudan Black B stain and examined under light microscope. It showed a black color which refers to the polyhydroxybutyrate (PHB) granules inside red cell.

The positive polyhydroxybutyrate bacterial isolates (NM7, NM16 and NM17) were grown, the bacterial growth, dry cell weight and polyhydroxybutyrate (PHB) quantity were measured. Among the three bacterial isolates (NM7, NM16, NM17), NM17 produce the highest polyhydroxybutyrate (PHB) quantity (Table 1).

Table 2 showed the results of the bacterial isolate NM17 morphological characterization. NM17 was bacilli, motile, gram positive, nonacid fast, spore forming bacteria and cysts were formed. NM17 colonies are white, circular and entire edge with flat elevation. The results of physiological and biochemical characterization of the bacterial isolate NM17 were showed in Table 2,3 and 4. Production of catalase, urease, indole, methyle red, voges-Proskauer, nitrate, citrate, cellulase, H₂S and Hydrolysis of starch were positive. Negative results in the following tests: Hydrolysis of esculin, Hydrolysis of Tween 80, Egg yolk lecithinase, Hemolysis, Utilization of casein, and Gelatin liquefaction. The Optimum temperature was ranged between 30 and 37°C and the Optimum pH was ranged from 5.5 to 7. The NaCl tolerance was up to 5%. The sensitivity to Chlorophenicol, Tetracycline, Ampicillin and Penicillin G were positive while, the sensitivity to Novobiocin, Rythromycin, Rythromycin and Sutamide were negative (Table 4). The selected bacterial isolate (NM17) was analyzed by 16S rRNA analysis and identified as *Bacillus funiculus* (Figure 4).

Table 1 the cell growth, cell dry weight and polyhydroxybutyrate (PHB) quantity

Bacterial Isolates	Growth	Cell dry weight (g/l)	PHB quantity (µg/ml)	% PHB
NM7	1.17	0.30	0.4251	34.16
NM16	1.18	0.31	0.1189	33.83
NM17	1.08	1.25	1.0828	44.06

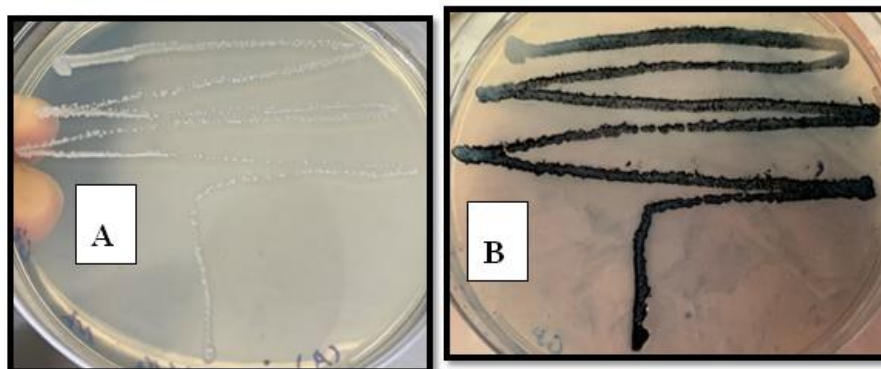


Figure 1. The bacterial isolate NM17 on nutrient agar medium, (A): Before staining, (B): stained by Sudan Black

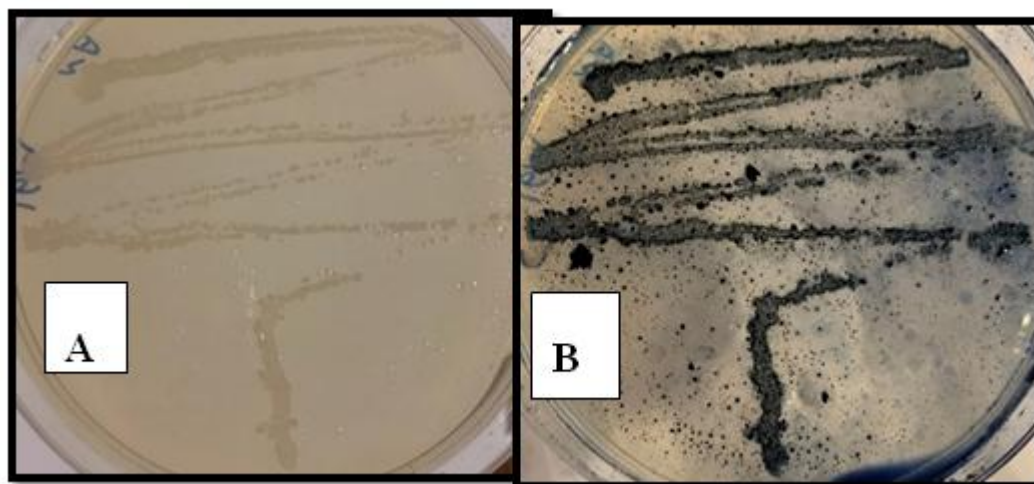


Figure 2. The bacterial isolate NM17 on modified C2M agar medium, (A): Before staining, (B): stained by Sudan Black

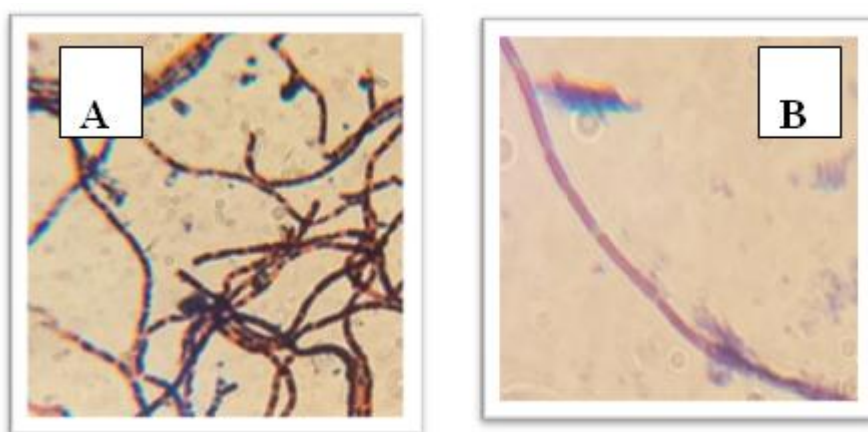


Figure 3 The bacterial isolate NM17 stained by Sudan Black (A), stained by Gram stain under light microscope

Table 2 The Morphological characterization of the bacterial isolate NM17

Characters	Results
Gram stain	positive
Acid fast	-
Shape-Motion	Bacilli,
Color	white
Colonies	Circular
Colony edge	Entire
Elevation	Flat
Endospore	+
Cyst	-

+: Positive result, -: Negative result

Table 3 The physiological and biochemical characterization of the bacterial isolates NM17

Characters	Results	Characters	Results
Temperature range	15-45	H ₂ S	+
Optimum pH	8-9	Hydrolysis of esculin	-
NaCl tolerance	3-5	Hydrolysis of Tween 80	-
Catalase	+	Hydrolysis of starch	+
Urease	+	Egg yolk lecithinase	-
Methyl red	+	Hemolysis	-
Indole	+	Cellulase	+
Voges-proskauer	+	Utilization of casein	-
Nitrate	+	Gelatin liquefaction	-

+: Positive result, -: Negative result

Table 4 The sensitivity of some antibiotics of the bacterial isolates NM17

Antibiotic	Sensitivity
Chlorophenicol	+
Tetracycline	+
Ampicillin	+
Penicillin G	+

+: Sensitive, -: Resistance

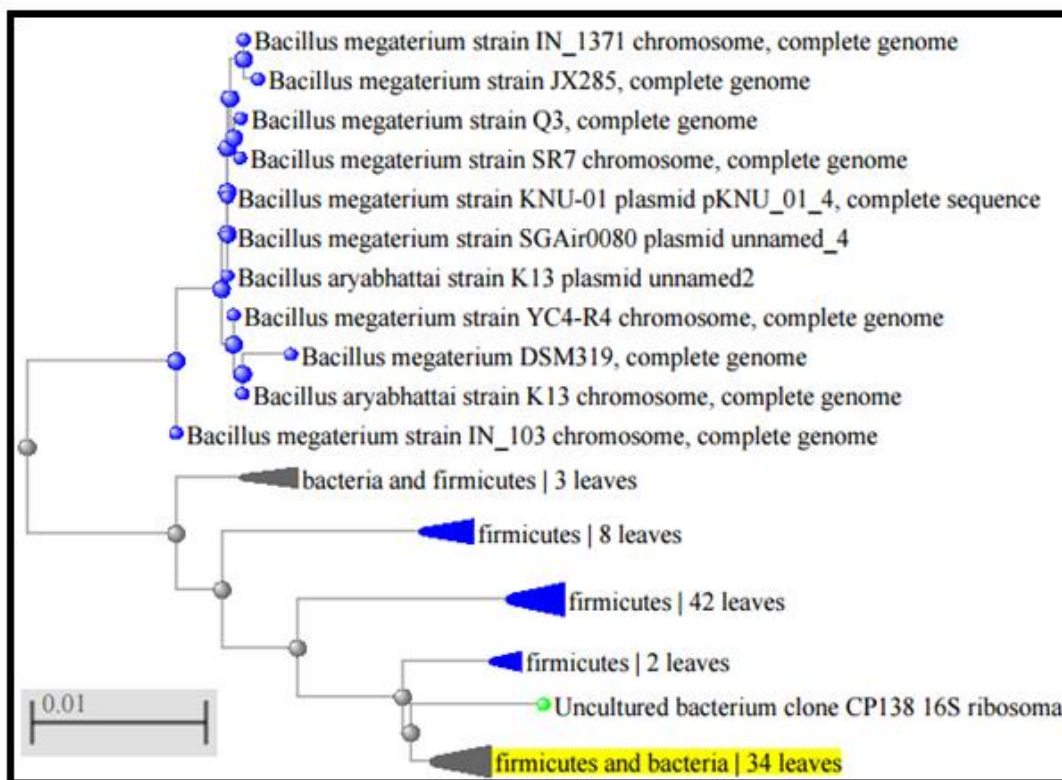


Figure 4. Phylogenetic tree of the bacterial isolates NM17

The maximum growth and polyhydroxybutyrate production were determined after incubation for different periods. Maximum growth was after 2 days while maximum PHB were after 3 days. The lowest % of PHB was recorded after 5 days of growth (Figure 5). The effect of temperature on growth and % of PHB were recorded at different temperatures. It is clear that maximum growth and % of PHB were recorded at 37°C (Figure 6). Similarly, the effect of different pH values on growth and % of PHB were recorded. It was found that maximum growth and % of PHB were recorded at pH 6.5 (Figure 7). Polyhydroxybutyrate was extracted and dried as a sheet as recorded in Figure 8.

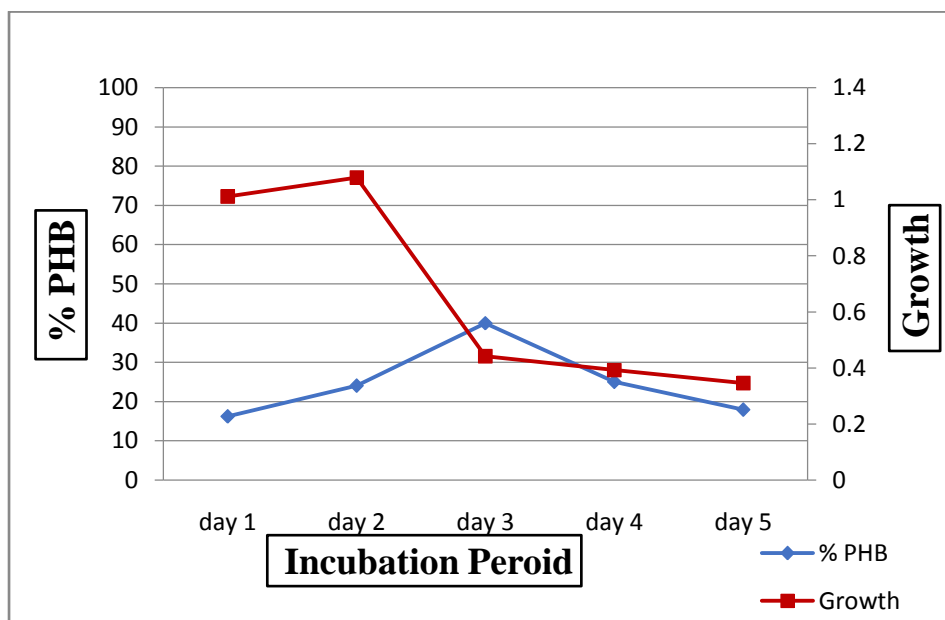


Figure 5 Effect of different incubation period on growth and polyhydroxybutyrate production by the selected bacterial isolates NM17

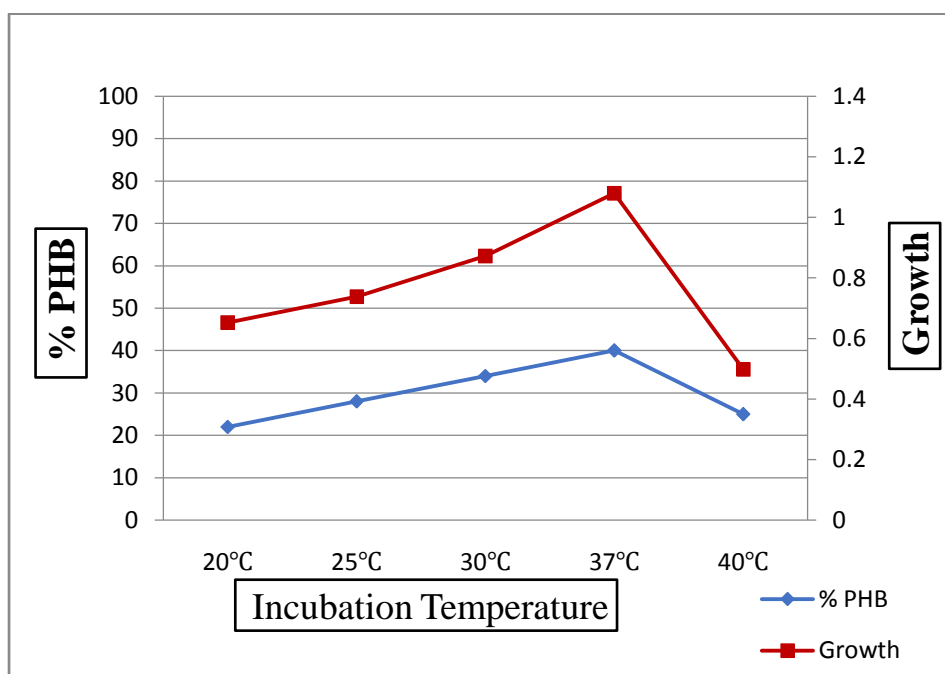


Figure 6. Effect of different incubation temperature on growth and polyhydroxybutyrate production by the selected bacterial isolates NM17

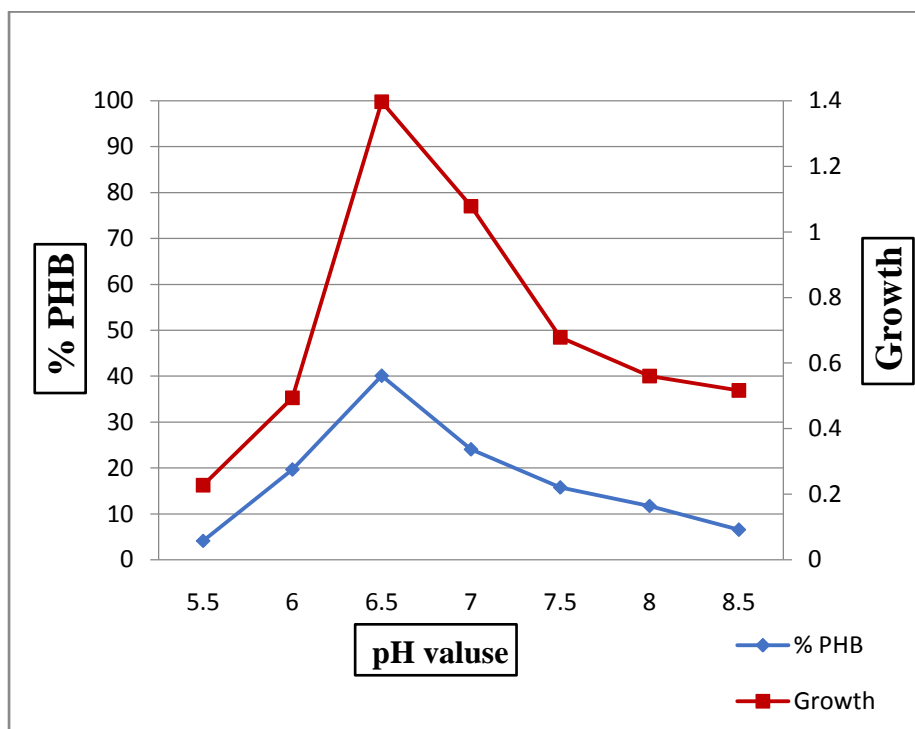


Figure 7. Effect of different pH values on growth and polyhydroxybutyrate production by the selected bacterial isolates NM17



Figure 8. Polyhydroxybutyrate (PHB) sheet from the bacterial isolates NM17

IV. Discussion:

Polyhydroxybutyrate (PHB) is a macromolecule synthesized by bacteria. They are inclusion bodies accumulated as reserve materials when the bacteria grow under different stress conditions. Because of their fast degradability under natural environmental conditions, PHBs are selected as alternatives for production of biodegradable plastics. This study showed the bacterial isolates NM17 which identified as *Bacillus funiculus* able to grow and accumulate polyhydroxybutyrate using waste cooking oil as a carbon source. *Bacillus funiculus* showed the maximum polyhydroxybutyrate quantity and percentage at 6.5 pH when incubate in a rotary shaker at 150 rpm, 37°C for 48 h. Tufail *et al.* (2017) report that the maximum polyhydroxybutyrate production was 38.4% of the dry cell weight by *Bacillus subtilis* and the maximum production of polyhydroxybutyrate by *Bacillus cereus* was 18.1% of the dry cell weight using waste cooking oil as a carbon source while Cardozo *et al.* (2016) found a low amount of polyhydroxybutyrate production by *Bacillus megaterium*. Results of these studies confirmed that cheaply available oil can be used for the production of PHB and reducing the cost of biodegradable plastics, reducing environmental pollution problems caused by conventional plastics and solving disposal problem of the oil wastes.

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