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Synthesis of biodegradable polymer (PHB) by Bacillus subtilis isolated from rhizosphere soil sample and Optimization of growth conditions

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Abstract: *The natural biopolymers polyhydroxyalkanote (s) (PHAs) are produced by many prokaryotic organisms as reserve material, when excess carbon source and nutrient limitation are available in the medium. Polyhydroxybutyrate (PHB) is the most famous class from PHAs. Bacteria (53 isolates) from different soil samples, collected from agriculture fields in 5 cities were obtained and screened for PHB production on modified nutrient agar medium (sublimated with 1% glucose as carbon source). All isolates were grown in modified nutrient broth medium for 2 days at 30°C. Growth and % of PHB were calculated. The quantity of PHB was determined by UV-VIS spectrophotometer. Out of 53 isolates, 17 (31%) produced PHB with accumulation percentage ranged from 6-48 % of the cell dry weight. Excellent growth and high percentage of PHB was obtained by isolate SM36. Using morphological and physiological characters in addition to 16S rRNA, the isolate SM36 was identified as Bacillus subtilis SM36 with 95% similarity with B. subtilis strain X-01, Bacillus sp.K7SC-9A, B. velezensis UUS-1. Bacillus Subtilis SM36 was the most active isolate in PHB production and accumulation. Maximum PHB accumulation was obtained in modified nutrient broth medium containing 2% glucose. For the maximum PHB accumulation by the isolate B. Subtilis SM36, the best incubation temperature was recorded to be 30°C and the best medium pH was pH 6.5. Addition ay yeast extract to the medium decreased PHB accumulation. In conclusion, Bacillus subtilis SM36 obtained from soil, showed excellent PHB producer and improving growth conditions enhanced accumulation percentage.*

Keywords: ***Bacillus, Biopolymer, optimization, PHA, Sudan black B, production, 16s rRNA***

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I. Introduction

Polymers are made up from non-renewable as well as renewable feedstock. These polymers are well known for their diverse applications in industries, domestic appliances, transportation, construction, shelters, storage and packaging practices. Such polymers are differentiated according to their chemical nature, structural arrangement, physical properties and applications (Shah *et al*. 2008). Over the past few years, owing to rising petroleum prices and many environmental concerns related to non-degradable material pollutions which have been developed rapidly (Thompson *et al*., 2009). Increasingly reduction of carbon dioxide emissions has become another reason for promoting bio-based plastics amid the worldwide financial tsunami (Chee, *et al*., 2010). It is major challenge faced countries these days is the ways to dispose the non-degradable waste safely because of recycling alone not sufficient (Leonard 2018). The daily general non degradable wastes in the main cities in Saudi Arabia are around 7,000 tons, and 30% of the general wastes are non-biodegradable materials. In fact, non-degradable wastes in general takes up to 1,000 years to degrade in environment. Thus, another alterative, materials must be used to replace plastic.

Polyhydroxyalkonates is biodegradable organic polymers with high molecular weight which can be molded under heat and pressure (Fridovich-Keil, 2016). Thus, in order to create a sustainable environment and prevent the possible disposal of recalcitrant non-biodegradable materials & wastes in the environment, production of biodegradable materials gained a lot of attention due to their biodegradability, meaning that the material returns to its natural state when buried in the ground. Generally, bio-based materials include starch-based materials, protein based materials, and cellulose –blended materials. They can also be blended with conventional plastic such as polyethylene, polypropylene and poly (vinyl alcohol) to get better properties but they are only partially biodegradable .The residual petroleum-based plastic remain as broken pieces creating additional pollution. To produce bio-based materials completely look like plastic based petroleum bacteria are employed to make the building block for polymers from renewable sources, including starch, cellulose, fatty acids and bio waste (Chee, *et al*. 2010). Actually, the word bio- materials can refer either to bio-based plastics synthesized from biomass and renewable resources such as Poly(lactic acid) (PLA) and Polyhydroxyalkanoate

(PHA) or plastics produced from fossil fuel including aliphatic plastics like Polybutylene succinate (PBS), which can also be utilized as a substrate by microorganisms (Mekonnen *et al*., 2013).

The word 'bio- materials' is used mainly for biodegradable polymers which can be degraded by microorganisms. Polyhydroxyalkanoates are the best studied polymers containing at least one monomer synthesized by bacteria and are well known for their biodegradability (Chee *et al*.,2010). In past years, the bio-material properties, whether physical or chemical, and their applications have been studied and developed to reduce their cost or improve their properties (Tokiwa, *et al*., 2009). PHA polymers are produced by a range of microorganisms under various growth conditions (Basnett and Roy 2010). They produced as storage molecules under stress conditions such as excess carbon and limiting nitrogen (Verma *et al*., 2002). Production of these biopolymers in an industrial scale is already well established. They have been produced using cheap carbon sources to achieve economical production (Sodergard and Stolt, 2002.)

Poly(3-hydroxybutyrate) [P(3HB)] is the most common PHA and was first described by Lemoigne, a French scientist in year 1925 (Chee *et al*., 2010). Since then, various bacterial strains among archaebacteria ,Gram positive and Gram negative bacteria and photosynthetic bacteria including cyanobacteria have been identified to accumulate P(3HB) both aerobically and anaerobically (Chaitanya *et al*., 2014). The recognition of the role of P(3HB) as a bacterial storage polymer that possesses a function almost similar to starch and glycogen. Macrae and Wilkinson (1973) noticed that *Bacillus megaterium* initiated the accumulation of PHB homopolymer when the ratio of glucose to nitrogen in the culture medium was high and the subsequent intracellular degradation PHB occurred in the absence of carbon and energy sources. The hydroxyl-butaric acid monomer is the only constituent of this polymer which changed after a year of its acceptance as the only bacterial storage material when other types of monomers were discovered.

This research aim to isolate and molecular identify PHB producing soil bacterium. Optimization of cultural parameters for maximum growth and PHA production were recorded.

II. Materials and Methods

All substrates used in these experiments were purchased from Sigma-Aldrich Chemical Co. (St. Louis, USA). Sudan Black B and other substrates were purchased from Aldirch Chemical Co. or Sigma (St. Louis, USA).

Collection of Sample:

The samples used in this study were soils which were collected from the five different agricultural soils in Saudi Arabia and were used for Bacteria isolation. Soil samples were collected from 10-15 cm depth in sterile plastic bags and spread on paper sheet at room temperature until air dry followed by grinding and sieving using 0.2 mm sieves. Each sample was collected in sterile test tube and stored at 4°C until used.

Isolation of PHB Producing Bacteria From Soil Collected Samples:

About one gram of the soil sample was suspended in 9.0 ml of sterile distilled water. The suspension was shaken well and serially diluted from 10^{-1} to 10^{-3}, then about 0.1 ml of this suspension was spread on plates of nutrient agar medium for isolation of bacteria and all plates were incubated at 30°C for two days. The colonies were transferred to new plates until pure colonies were obtained. All bacteria were preserved on nutrient agar slants at -4°C until used.

Screening test for PHB Using Sudan Black B On Solid Agar

The bacterial colonies were examined for PHB accumulation by staining with Sudan Black B (0.3% in 70% ethanol) using rapid screening method. The bacterial isolates were grown on nutrient agar medium supplemented with 10 gram glucose (Aly *et al*., 2011, 2013). The plate was divided into 4 equal parts and in each part, a bacterial isolate was spotted. The plates were incubated at 30°C for 48 hours. Ethanolic solution of 3% (w/v in 70% ethanol) Sudan Black B was spread over the colonies and the plates were kept undisturbed for 30 minutes. Then the plates were washed with ethanol (96%) to remove the excess stain from the colonies. The PHB producing colonies which give dark blue colored were taken as positive. All the positive isolates were assigned the code numbers based on their source of isolation (Aly *et al*., 2013).

Screening test for PHB Using Sudan Black B under light microscope

Smears cells deposited on a glass slide were heat fixed and stained with a 3% (w/v in 70 % ethanol) solution of Sudan Black B for 10 min, then immersion of the slide in xylene until it completely was decolorized. The sample was counterstained with safranin (5% w/v in deionized water) for 10 s, washed with water and dried. A few drops of immersion oil were added directly on the completely dry slide, and the cells were examined by phase contrast microscopy and take photos (Aly *et al*., 2013).

Identification of the selected bacterial isolates SM23 and SM36:

Morphological, physiological and biochemical characterization of the bacterial isolates

After isolation of bacteria from different sources and sites, the best producer of PHB was selected and identified. Strains were preliminarily identified according to traditional morphological criteria, including morphology and growth on nutrient agar. Characteristics of the bacterial colonies on the agar plate, the morphology of spores, color of the produced pigment were carried out. The cellular morphology of the bacterial isolates SM36 was examined under light microscope after staining with Gram and scanning electron microscope. Moreover, biochemical characterization of the isolates was carried out as described before. Tests such as catalase, citrate oxidase, indold production were also carried out.

Molecular Identification

The sequencing of 16S rDNA and the taxonomic studies of strain were performed at Special Infectious Agents Unit, King Fahd Medical Research Center, Jeddah, KAU. A partial 16S rDNA fragment of approximately 1.5 kb was amplified using high-fidelity polymerase chain reaction (PCR) polymerase. The PCR product was sequenced bidirectional using the forward, reverse, and internal primers. The sequence data were then aligned and analyzed to identify the bacterium and find the most closely related strains. The DNA sequence was compared to the GenBank database at the National Center for Biotechnology Information (NCBI) using the BLAST program

Cultivation method:

The pre culture was prepared in 250 ml Erlenmeyer flasks containing 50 ml of the fresh sterile nutrient broth (Khanna and Srivastava, 2006). Each flask was inoculated with a loop of the selected bacterium and the flasks were incubated at 30°C and 200 rpm on the orbital shaker incubator (PEMED 3525) for 24 h. The Erlenmeyer flasks containing 50 ml of the production medium were inoculated with 5 ml of the pre culture of the selected bacterium. The inoculated flasks were incubated at 37 °C and 200 rpm on the orbital shaker incubator for 48 h. (Shivakumar, 2012).

Factors affecting PHB production by the selected bacteria

Different factors affecting on PHB production by the best selected bacterium was optimized for maximum PHB production. Effect of different concentration of glucose, yeast extract, incubation temperature and, pH values were studied in broth medium. Each 50 ml in Erlenmeyer flask was inoculated with 2 ml of the preculture ($4x10^6$ CFU/ml).

In the production medium, different concentrations of glucose as source of carbon ranging from 5-40 g/l was tested to find best concentration for PHB production. Ea and after 2 days at 30°C growth and PHB was determined. In the production medium, different concentrations of yeast extract as source of nitrogen ranging from 2-5.5 g/L was tested to find best concentration for PHB production and after 2 days at 30°C growth and PHB were determined. Moreover, the effect of different incubation temperatures (25-50 °C) and different initial pH (5.5- 8.5) were studied.

Quantifications of Cell Growth and Cell dry weight

Cell growth was monitored by measuring turbidity at an optical density 520 nm. Cell dry weight was measured as method described by Aly *et al*., (2013). After centrifugation of the culture medium, the supernatant was discarded and the cell pellet was washed with distilled water, and dried at 60°C for three days to constant weight.

Extraction and essay of PHB

The best Sudan Black B positive bacterial isolates were subjected to quantification of the PHB production by the method of (Johan and Ralph, 1961). Bacterial cells containing the polymer were collected after centrifugation at 4000 rpm for 10 min. The Pellet was resuspended in equal volume of 4% sodium hypochlorite and incubated at 37 °C for 24 hr. Then the pellet was washed with acetone, ethanol and water to remove the unwanted materials. The whole mixture was centrifuged again and the supernatant was discarded. Finally, the polymer granules were dissolved in hot chloroform, allowed evaporating (Aly *al*., 2013) and 10 ml concentrated and hot H2SO4 was added to the polymer granules. The addition of sulfuric acid converts the polymer into crotonic acid which has brown colored. The solution was cooled and the absorbance at 235 nm was determined against a sulfuric acid blank by referring to the standard curve, the quantity of PHB produced was determined (Aly *et al*., 2013) and percentages of PHB/cell dry weight was calculated.

Statistical Analysis

Statistical analyses were performed using the statistical Package for Social Science (SPSS for windows, version 16) (SPSS Inc., Chicago, IL, USA). The variability degree of the result is expressed as mean ± standard deviation (Mean ± SD).The significance of the difference between samples was determined using t-test . The difference was regarded significant when $P< 0.05$ and non-significant when $P>0.05$.

III. Results

In this study, from agricultural soil samples, 53 bacterial isolates were purified and maintained on nutrient agar medium. All isolates were screened with Sudan black B (Figure 1A, B) on solid agar medium using rapid screening method. Out of 53 isolates, 17 were PHB producers as detected for by Sudan black B. They were grown in liquid medium for 48 hr. and PHB was extracted and quantified (Table 1). The isolate SM36 showed dark black color after staining with Sudan black B on plates. After smear staining using Sudan black and safranin, PHB granules were detected inside the cells as black color against red back ground or o a slide (Figure 1 C).

The percentages of PHB of the cell dry weight were determined. They were ranged from 6 - 46 % of the cell dry weight. The most active isolate was isolate SM36 which produced 60 mg/l PHB (43% of the cell dry weight). It was selected for more detailed studies.

Table 1: Detection of PHB in some positive PHB producing bacteria on modified nutrient agar medium and % of PHB in cells grown in broth medium.

Bacterial Isolate	City of isolation	PHB detection on solid medium	PHB extraction from cell grown in liquid medium			
			Cell dry weight (g/l)	Absorbance	Quantity of PHB (mg)	%PHB/cell dry weight
SM1	Al-Bahah	++	0.112	0.140±0.1	**7.11**	**6.25**
SM2	Al-Bahah	++	0.234	0. 809±0.12	**40.8**	**17.0**
SM4	Jeddah	++	0.231	1.91±0.23*	**95.5**	**41.1***
SM7	Jeddah	+++	0.457	1.224±0.114	**61.3**	**13.5**
SM10	Jeddah	+++	0.438	1.44±0.22	**72.4**	**16.7**
SM12	Jeddah	+++	0.658	1.35±0.141	**67.5**	**10.3**
SM15	Jeddah	++	0.555	1.733±1.09	**85.2**	**15.0**
SM16	Makkah	+++	0.512	0.272±0.28	**13.5**	**13.5**
SM17	Makkah	++	0.144	0.223±0.306	**11.0**	**7.60**
SM21	Al-Taif	+++	0.333	1.61±1.38	**53.0**	**15.9**
SM23	Al-Taif	+++	0.229	1.9943±0.79*	**99.5**	**45.0***
SM27	Al-Taif	++	0.202	1.541±1.701	**77.0**	**38.1***
SM30	Al-Taif	++	0.201	0.297±0.331	**14.5**	**7.20**
SM36	Al-Madina	+++	0.123	1.21±0.496	**60.1**	**48.0***
SM37	Al-Madina	++	0.388	1.57±0.813	**78.4**	**20.1***
SM39	Al-Madina	+++	0.451	1.156±0.13	**57.5**	**12.6**
SM41	Al-Madonna	+++	0.321	1.06±0.77	**53.1**	**16.5**

***:** Significant result compared to *Bacillus subtilis* (control), +++: darkest blue colored, ++ : moderate blue colored +: light blue colored.

Isolate SM36 was Gram positive bacilli, non-acid fast and was spore forming bacterium (Table 2, 3). Examination with light and scanning electron microscope revealed that it was motility with the diameter of 0.5-0.7, 4-7 µm and the colony had creamy color on nutrient agar medium (Figure 2). Antibiotic sensitivities were determined by the disk diffusion method depending on the criteria published by the Clinical and Laboratory Standards Institute (CLSI). *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 were used as controls It was resistant to Cephalothin (30 µg/ml), Cefoxitin (30 µg/ml) Ampicillin (10 µg/ml), Ceftazidime (30 µg/ml) and Aztreonam (30 µg/ml). as in (Table 2). Production of catalase, urease, indole, lecithinase and cellulase were positive (Table 3). The isolate SM36 was very similar to *Bacillus Subtilis* and 16SrDNA analysis revealed that this isolate is belonging to genus *Bacillus* and is very closely related to *B. Subtilis* ATCC14579, *B. subtilis* strain X-01, *Bacillus* sp.K7SC-9A, *B. velezensis* UUS-1 (Figure 3).

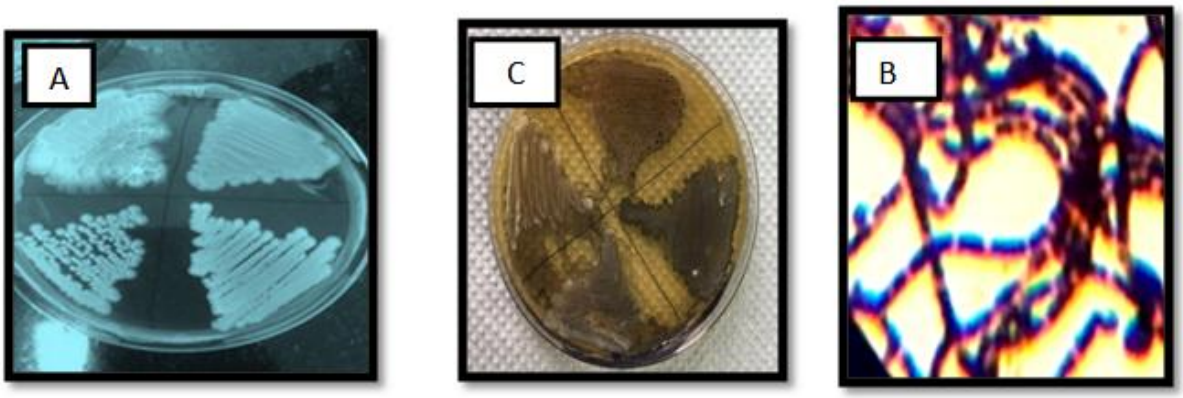

Figure 1: Screening of some bacterial isolates on solid agar medium using Sudan Black B (A) Before staining (B) After staining, stained smear with Sudan black and Safranin under light microscope X1000 (C).

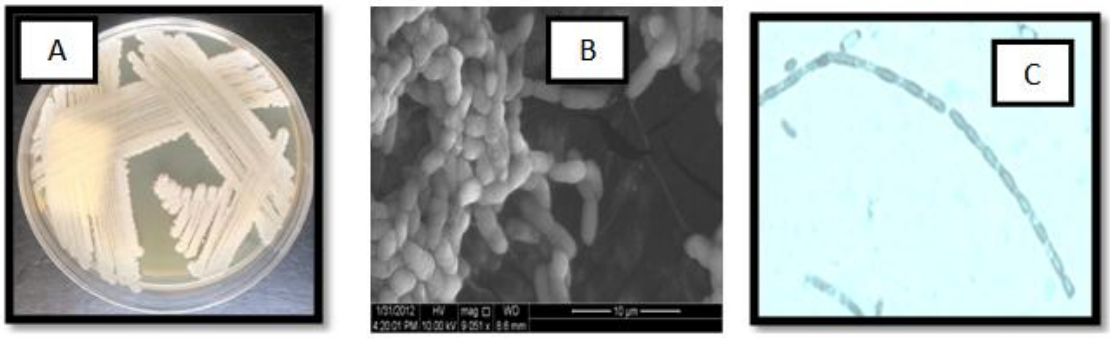

Figure 2 The selected bacteria isolate SM36, A: grown on nutrient agar medium, B: under Scanning electron microscope and C: under light microscope X1000 after Gram stain

Table 2 The morphological characters and sensitivity to some antibiotics of the selected bacterial isolates (SM36).

Characteristics	**Isolate SM36**	**Antibiotic used**	**Sensitivity (mm)**
Gram stain	Gram –positive	PRL	S (32.3)
Colony size	Large	IMI	S (35)
Shape	Rod-shaped	CIP	S (29.5)
Acid fast stain	Negative	ATM	R
Endospore formation	Positive	CAZ	R
Motility	Positive	GM	R

Imipenem (IMI; 10 µg), ciprofloxacin (CIP; 5 µg), ceftazidime (CAZ; 30 µg), aztreonam (ATM; 30 µg), piperacillin (PRL; 100 µg) and gentamicin (GM; 10 µg).

Table 3. Physiological and biochemical characters of the selected bacterial isolate SM36

Character	**Result**	**Character**	**Result**
Optimum growth temperature (^oC)	37	Citrate	Positive
Temperature range (^oC)	20-55	Methyl Red	Positive
Optimum pH range	7- 8	Oxidase	Positive
NaCl tolerance	3-5%	Nitrate production	Positive
Catalase	Positive	Gelatin liquefaction	Positive
H_2S	Negative	Hydrolysis of Tween 80	Positive
Voges-proskauer	Positive	Hydrolysis of starch	Positive

Urease	Negative	Hemolysis	Po Negative
Indole	Negative	Cellulase	Positive

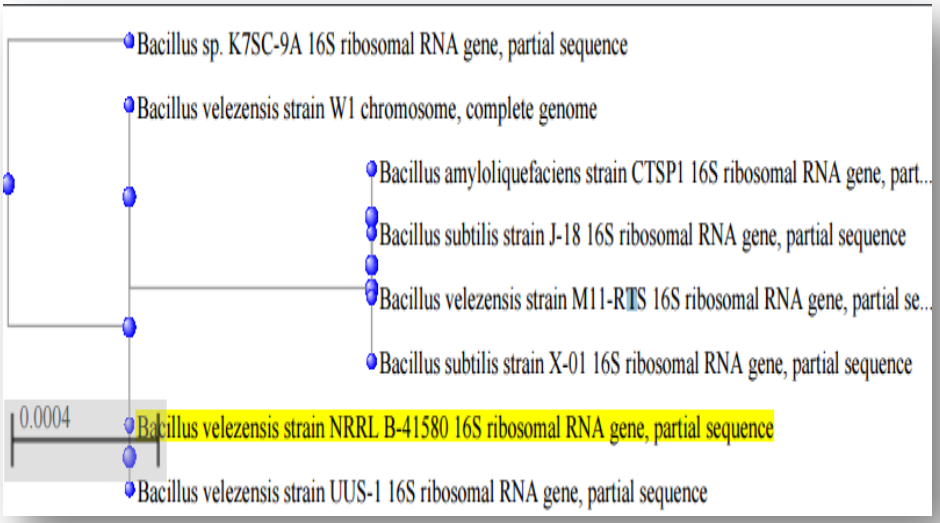

Figure 3. Phylogenetic tree based on 16S rRNA for *Bacillus subtilis* SM36

Bacterial growth were determined at different incubation coditios SM36 showed the maximum production of PHB after shaking at 120 rpm for 2 days and in medium containing 20 g glucose as carbon source (Table 4, Figure 4). Moreover, SM36 showed the maximum production of PHB and bacterial growth in medium containing no yeast extract as nitrogen source (Table 5, Figure 5). In addition, maximum PHB production was determined afer incubation 30°C for two days (Table 6, Figure 6). Moreover, the production medium with initial pH 6.5 give maximum PHB production (Table 7, Figure 7) yielded the maximum PHB production. After optimization of growth conditions, the isolate SM36 was grown in one liter conical flask containing 200 ml of the modified production medium prepared with 20g glucose at pH7. After 2 days of incubation at 30°C and 120 rpm, PHB was extracted, purified, dried (Figure 8) and weighted. After optimization of growth factors, % of PHB was enhanced by 9.3% from 48% to 53%.

Table 4 Effect of different glucose concentration on growth and PHB production by selected bacterium SM36

Concentration of Glucose (g)	Growth (mean SD)	Cell dry weight (g/l)	**Absorbance**	**Quantity of PHB (mg)**	**%PHB/cell dry weight**
5	0.69±0.040	0.15± 0.03	1.32±0.57	65	43*
10 (control)	0.90±0.02	0.18±0.06	1.631±0.57	80	44*
20	1.183±0.51	0.17±0.04	1.78±0.76	85	50
30	1.03±0.05	0.18±0.04	1.66±0.76	83	45*
40	0.90 ±0.04	0.12±0.01	1.69±0.76	84	46*

***:** Significant result compared to control

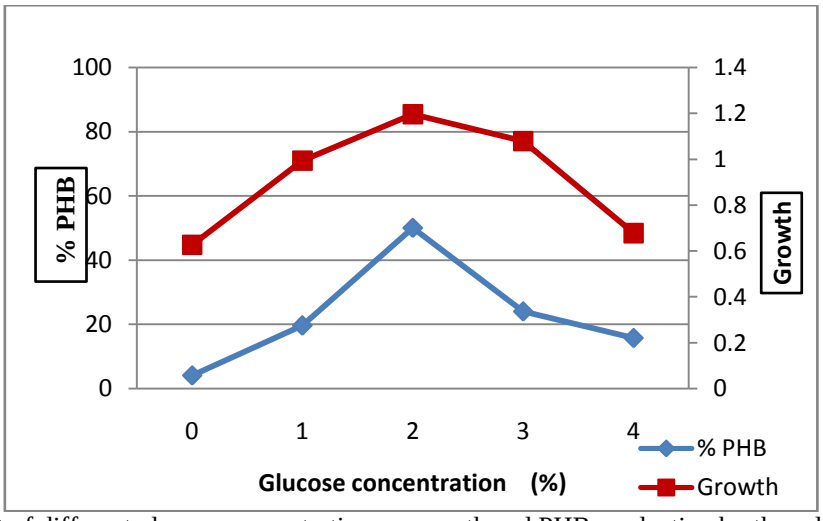

Figure 4. Effect of different glucose concentration on growth and PHB production by the selected bacterium SM36

Table 5. Effect of different yeast concentration on growth and PHB production by selected bacterium SM36
***:** Significant result compared to control

Concentration of yeast (g)	Growth (mean SD)	Cell dry weight (g/l)	**Absorbance**	**Quantity of PHB (mg)**	**%PHB/cell dry weight**
0 (control)	0.490±0.11	0.11±0.11	1.88±0.56	85.1	50.5
2	0.460±0.11	0.13±0.11	1.78±0.76	85.1	51.0
2.5	0.595±0.123	0.19±0.10	1.49±0.57	71.9	46.6*
3.5	0.551±0.43	0.31±0.40	1.43±0.34	70.4	22.2*
4.5	0.752±0.053	0.54±0.45	1.44±0.23	70.7	12.9*
5.5	0.790±0.133	0.54±0.26	1.18±0.18	55.1	10.4*

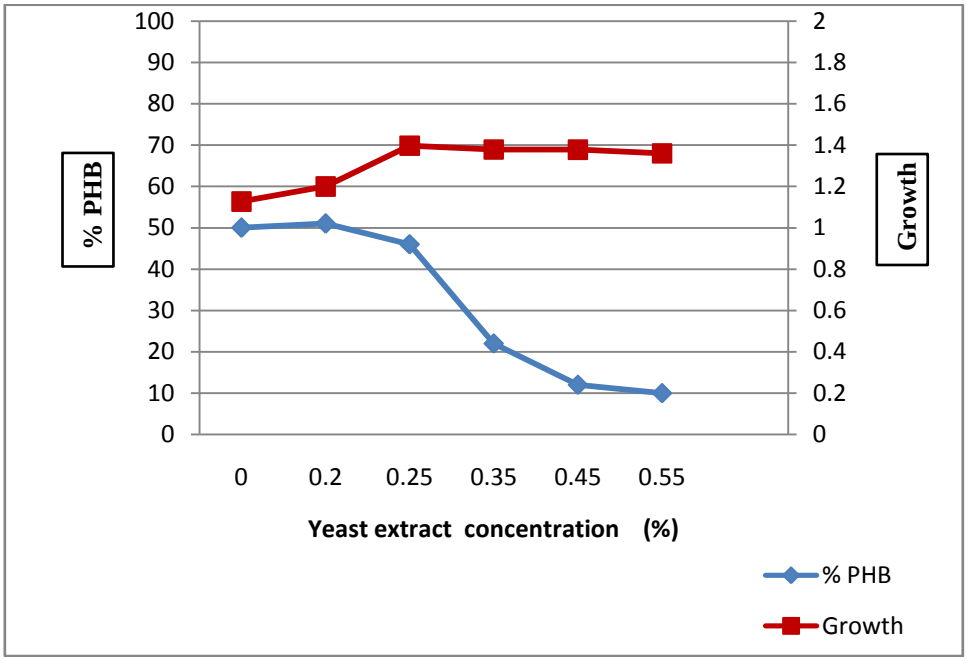

Figure 5. Effect of different yeast concentration on growth and PHB production by the selected bacterium SM36

Table 6. The effect of different temperature on growth and PHB production by the isolate SM36

Different incubation of temperature	Growth (mean SD)	Cell dry weight (g/l)	Absorbance	Quantity of PHB (mg)	%PHB/cell dry weight
25°C	0.96±0.075	0.12±0.201	0.86±0.86	42	35*
30°C (control)	1.34±0.11	0.13±0.04	1.78±0.26	85.1	50
37°C	1.287±0.144	0. 14±0.16	1.39±0.3942	69.6	49*
45°C	1.149±1.541	0.12±0.119	0.96±0.24	48	40*
50°C	1.001±0.198	0.1458±1.31	1.07±1.315	50.1	35*

***:** Significant result compared to control

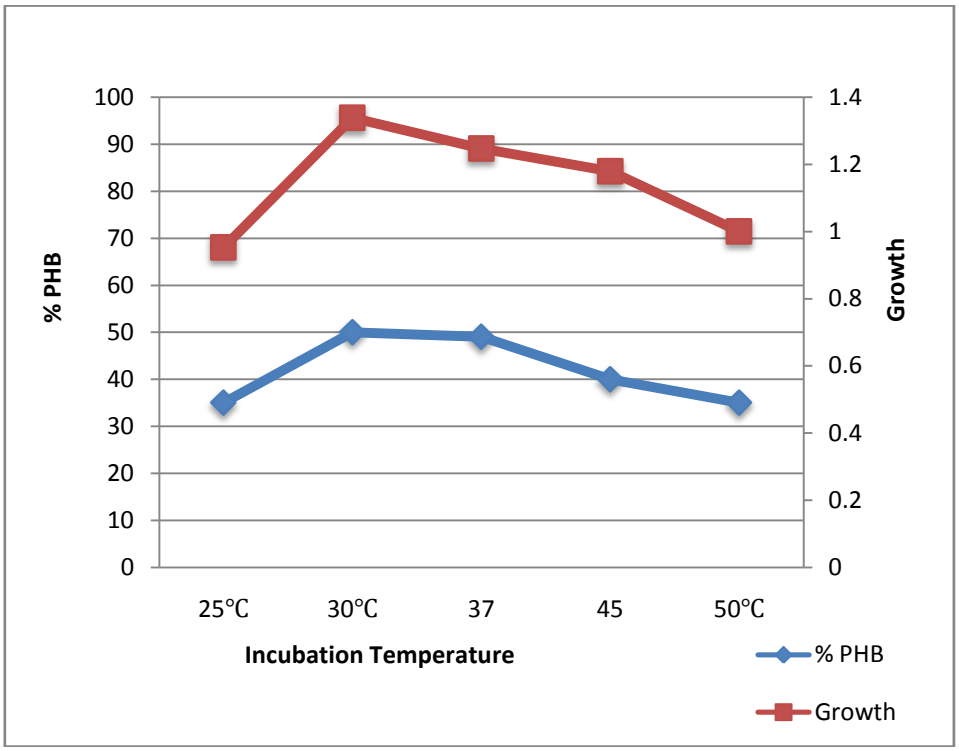

Figure 6. The effect of different temperature on growth and PHB production by the isolate SM36

Table 7 Effect of different pH values on growth and PHB production by selected bacterium SM36

Different pH values	Absorbance (540 nm)	Cell dry weight (g/l)	Absorbance	Quantity of PHB (mg)	%PHB/cell dry weight
5.5	1.023±0.064	0.104±0.232	1.00 ±0.24	50.0	48.1
6.5	1.373±0.38	0.16±0.01	1.88±0.16	85.1	53.1
7 (control)	1.1±0.005	0.14 ±1.1489	1.29±0.05	64.1	45.4
7.5	1.036±0.068	0.193±0.0835	1.34±0.04	67.0	35.3
8.5	0.92±0.045	0.19468±0.0168	1.39±0.04	65.3	34.2

***:** Significant result compared to control

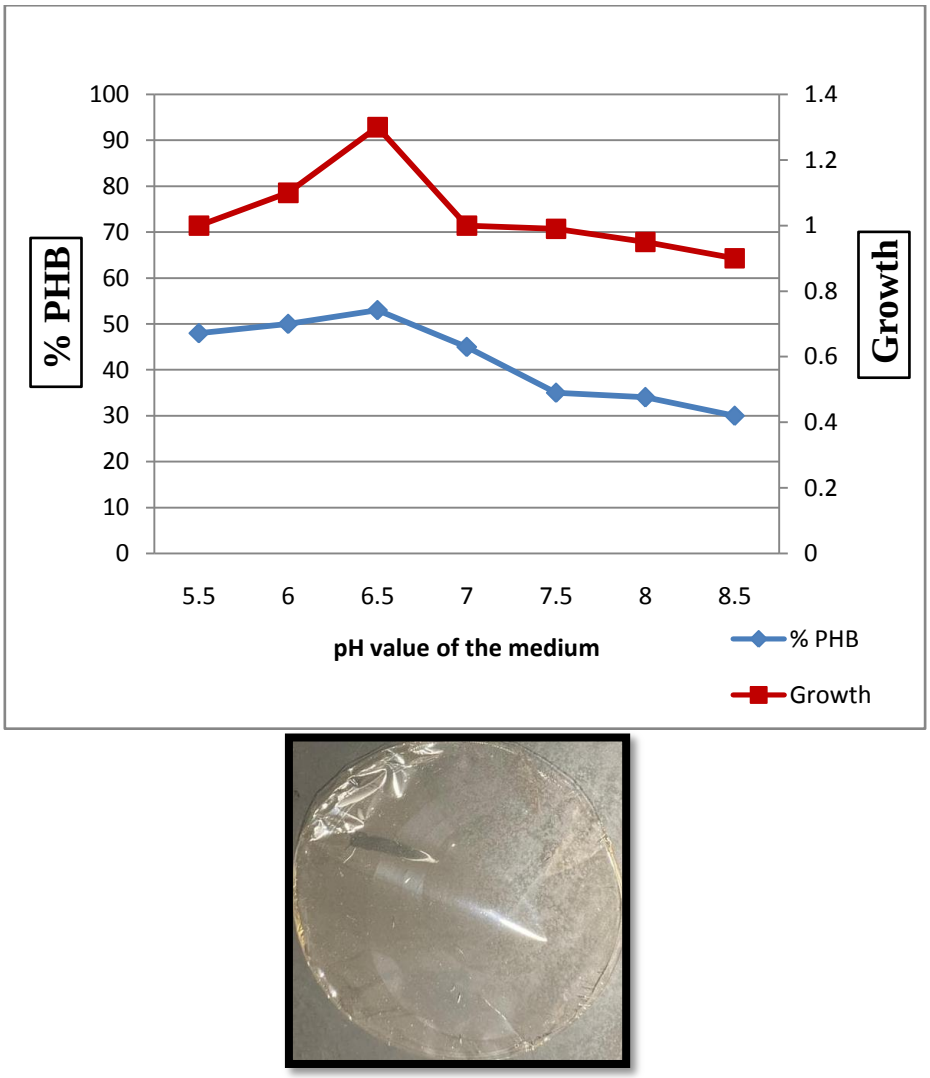

Figure 8. Polyhydroxybutyrate (PHB) sheet from the bacterial isolates NM17

IV. Discussions

Petroleum based plasticis disposal is a threat to environment and health because they are non degradable and le stayed for long time in soil and water. These materials are protected from damage by microorganisms and chemicals (Marjadi and Dharaiya, 2011). Bioplastics are available as degradable and eco-friendly alternative materials (Kamilah *et al*., 2013, Jain and Tiwari, 2015, Zargoun*et al*., 2015).

More than 50 isolates from different ecological soil were obtained on nutrient agar medium. The simplest first line screening program for PHB producing bacteria is the use of Sudan black B staining. The isolates which were found positive for PHB granules after Staining with Sudan black B (i.e., showing dark spot inside the pink coloured cells) were further confirmed for their PHB producing potential in broth medium. There are about 250 of different bacterial strains that can produce different types and yields of PHA under several conditions (Pollet and Averous, 2011). Different bacteria produced PHB inside cells as inclusion bodies. Genus *Bacillus* produced biodegradable plastic, and used it as both carbon and energy sources (Zhang *et al*., 2003; Scheel *et al*., 2016). Other bacteria for example, *Azotobacter*, *Bacillus*, *Archaebacteria*, *Methylobacteria* and *Pseudomonas* produced PHB (Lee, 1996). Gómez Cardozo *et al*. (2016) found a low amount of polyhydroxybutyrate (PHB) production by *Bacillus megaterium*. Moreover, *Ralstonia eutropha* (formerly *Alcaligenes eitrophus*) and *Cupriavidus necator* are the most studied bacteria, accumulate PHB up to 80 per cent of the dry weight of PHA (Verlinden *et al*., 2007). There are more than 155 PHA products that accumulated by numerous bacteria had been identified (Agnew and Pfleger, 2013; Nielsen *et al*., 2017). The high cost production of carbon source is the main reason for high cost of PHB production. Therefore, there is need to investigate inexpensive substrates to make the PHB production economically more attractive than petroleum based plastic (Povolo *et al*., 2010; Budde *et al*., 2011).

The isolate SM36 used in the present study was morphologically and biochemically characterized. The isolate on the basis of their morphological and biochemical characteristics identified as *Bacillus sp.* (Bergey's Manual 9th ed.) when further characterized with 16S r-RNA genes for sequence homology (using BLAST and distance matrix based nucleotide sequence homology. The maximum growth and PHB production were noted in medium after 48 h of incubation at 30°C. The percentage of PHB was 48% and become 53% after optimization of growth factors. Tufail *et al*. (2017) report that the maximum PHB production was 38.4% of the dry cell weight by *Bacillus subtilis* and the maximum production *Bacillus cereus* was 18.1% of the dry cell weight using waste cooking oil as a carbon source.

Glucose and yeast extract not only affected the microbial growth but also PHB accumulation. This may be due to the fact that carbon and other nutrient support microbial growth. The highest level of PHB accumulation was observed in the media with protease peptone as nitrogen sources by Aslim et al. (2002) in *B. subtilis* and *B. megaterium*. Under unoptimized conditions *Bacillus* was observed to accumulate about 60 mg/L (48% of cell dry weight) of PHB after 2 days. The decrease of PHB production after 24 h might indicate that the bacteria used PHB as nutrient source. Matavulj and Molitoris (1992) reported that the highest PHB level in *Agrobacterium radiobacter* was achieved during stationary growth phase after 96 h. The observation was supported by Yüksekdağ et al. (2004). The maximum PHB production was recorded at 30°C after 2 days. The increase of temperature beyond 40°C has negative impact on PHB production. The decrease in PHB production at high temperature could be due to low PHB polymerase enzyme activity (Yüksekdağ et al., 2004).

The effect of pH variations on PHB production is shown. From analysis it is clear that pH 7 was favourable for PHB production by *Bacillus subtilis* NG220 in sugar industry waste water. The current observation was in agreement with Aslim et al. (2002) who observed that the maximum PHB was produced (at pH 7 by *Rhizobium* strain grown on yeast extract mannitol broth while studying the effect of different pH on exo-polysaccharide and PHB production in two strains of *Rhizobium meliloti*. Tavernier et al. (1997) reported that these two strains showed higher PHB content at pH 7.0. Shivakumar (2012) reported optimum pH between 6.8 and 8.0 for PHB production by *Alcaligenes eutrophus*. To find the best available carbon source and its optimum concentration for maximum PHB production, commercial carbohydrates dextrose, xylose, sucrose, rhamnose, mannitol, maltose, lactose monohydrate, mannose, galactose, starch, and raffinose were tested as a sole carbon and maltose supplementation to production media gave higher PHB yield (Singh et al., 2012). Hori et al. (2002) reported that PHB content in *B. megaterium* reached maximum level in a medium containing glucose as carbon source. The study conducted by Wu et al. (2001) reported that *Bacillus sp.* JMa5 accumulated 25%–35%, (w/w) PHB during sucrose fermentation (Singh et al., 1999). Working with different carbon sources in MSM broth, Khanna and Srivastava (2005) observed higher PHB yield on fructose by *A. Eutrophus*. Out of various organic and inorganic nitrogen sources ammonium sulphate (1% w/v) supported the maximum (5.297 g/L) PHB production followed by peptone and tryptone (Singh et al., 2012). In the literature, ammonium sulphate is reported to be the best nitrogen source for PHB production in different microorganism such as *Alcaligenes eutrophus*, *Methylobacterium sp.*, and *Sinorhizobium fredii* (Koutinas et al., 2007, Kim et al., 2006, Liangqi et al., 2006, Liangqi et al., 2006).

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