

Effects of culture conditions on growth and poly- β -hydroxybutyric acid production by *Bacillus cereus* MM7 isolated from soil samples from Saudi Arabia.

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Abstract: Poly- β - hydroxybutyrate (PHB) is the most known degradable biopolymers, produced by genera of bacteria under unfavorable conditions. It is generally accepted that PHB can be used instead of plastic to solve one of the greatest problems facing the environment. In this study, 39 different bacterial isolates were obtained on nutrient agar from heated soil samples, collected from Jeddah, Saudi Arabia. All bacterial isolates were screened for PHB production either on solid medium and stained using Sudan black B or in broth medium and assayed for PHB quantity. The quantity of PHB was ranged from 10-43%. The most active bacterium that accumulated high amount of PHB was selected and identified as *Bacillus cereus* MM7, based on morphological and physiological characters in addition to 16S rRNA gene sequences. The effect of different conditions on PHB production by *Bacillus cereus* MM7, including carbon source, nitrogen source, incubation temperature, pH and incubation period were studied. The highest amount of PHB was obtained from the isolate MM7 using medium containing 30 g/l glucose, 0.2 % yeast extract and 0.05 % K₂PO₄, pH 7 and incubation at 37°C and after 2 days. In conclusion, after the optimization of the conditions of PHB synthesis, *Bacillus cereus* MM15 accumulate up to 55 % of its dry weight and can be employed for industrial production.

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1. Introduction

Because conventional plastics are not degradable by microorganisms, many companies have attempted to develop biodegradable alternatives (Wei *et al*, 2011). Bioplastics are naturally made from polyhydroxyalkanoates (PHA) of which poly 3-hydroxy butyric acid (PHB) is the most common (Singh and Parmar, 2011) and was discovered in bacterial cells since 1920 (DeMarco, 2005).

PHB synthesized by a large number of bacteria and have received great attention as sources for biodegradable, biocompatible, and thermoprocessible plastic materials (Ueda *et al.*, 1996, Hyakutake *et al.*, 2011; Cao and Zhang, 2013). Therefore, using biodegradable plastic can reduce the current problems with decreasing fossil resources and environmental impact caused by plastic garbage.

A wide variety of microorganisms are able to naturally accumulate PHB as intracellular energy storage materials under an excess of carbon source and conditions of limiting nutrients such as oxygen, nitrogen and phosphate (Wang *et al* 2009; Hyakutake *et al*, 2011; Rodriguez-Contreras, *et al.*, 2013). *Bacillus* (Halami, 2008) *Alcaligenes eutrophus* (Ueda *et al.*, 1996), *Pseudomonas* (Cai *et al* 2009, Tajima *et al.*, 2012), *Azotobacter vinelandii* (Galehdari *et al* , 2009), *Ralstonia eutropha* (DeMarco, 2005),

Sinorhizobium meliloti and recombinant *Escherichia coli* (Galehdari *et al.*, 2009; Chen, *et al.*, 2011) are producer of PHB.

PHB has many promising applications in medicine, material science, food industries and agriculture. However, the important factor preventing the industrial and commercial production of PHB is its high price of production compared to synthetic plastic (Sangkharak and Prasertsan, 2008).

The aim of this study was to isolate and identify a local isolate producing PHB and enhancing production process by optimizing growth conditions.

2. Material and Methods

Samples collection:

The bacterial isolates were isolated from five different samples, collected from Sewage collector station in south Jeddah, Sea water from North Corniche, sea water from south Corniche, soil from south of Jeddah, soil from king Abdulaziz University. Soil samples were collected from 10-15 cm depth in sterile plastic bags, transported to the laboratory and spread on sterile paper sheet until air dried. Water samples were collected in sterile plastic bottle and preserved at 4°C until used.

Bacterial isolation from the collected water and soil samples:

About 1 g of each soil sample was suspended in 10 ml of sterile distilled water and the suspension was shaken vigorously. Water samples and soil suspensions were heated at 80°C in a water bath for 15 min. About 100 µl of each heated sample were spread directly on the surface of nutrient agar plates which were incubated at 45°C for two days. Bacterial colonies were selected and transferred to new plates until pure colonies were obtained. All pure bacterial isolates were preserved on nutrient agar slants at 4°C.

Morphological, physiological and biochemical characterization of the isolate MM7:

After isolation of bacteria from different sources and sites, the best producer of PHB was selected and identified. The tested bacterial isolate was grown on nutrient agar plates and the cellular morphology was examined with light and scanning electron microscope. Identification was carried out according to morphological, physiological and biochemical characters. These characters were studied following the standard microbiological methods described by Williams *et al.* (1994).

Molecular analysis

The sequencing of 16S rDNA and the taxonomic studies of strain *Bacillus cereus* were performed at Special Infectious Agents Unit, King Fahd Medical Research Center KAU in Jeddah, KSA. 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.

Screening for PHB on solid agar

The bacterial colonies were examined for PHB accumulation by staining with Sudan black (0.3% in 70% ethanol) using rapid screening method. The bacteria isolates were grown on nutrient agar medium supplemented with 1 % glucose. The plate was divided into 6 equal parts and in each part, a bacterial isolate was spotted. The plates were incubated at 30°C for 24 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 (Mohamed *et al.*, 2012).

Screening for PHB production using light microscope

Smears of 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 (Legat *et al.*, 2010).

Cultivation method:

The preculture was prepared in 250 ml Erlenmeyer flasks containing 50 ml of nutrient broth (Khanna and Srivastava, 2006). Each flask was inoculated with a loop of the tested cultures and incubated in a rotary shaker at 130 rpm at 30°C for 24 h. The Erlenmeyer flask containing 50 ml of the production medium which composed of (g/l): glucose, 10; MgSO₄, 0.2; NaCl, 0.1; KH₂PO₄, 0.5; and yeast extract, 2.5, were inoculated with 2 ml of the preculture of the tested bacterium. The inoculated flasks were incubated at 37°C on a rotary shaker at 130 rpm for 48 h. (Shivakumar, 2012).

In the production medium, the carbon sources (glucose) were replaced with sucrose, Lactose and Maltose. The medium containing no carbon source was used as negative control. Different concentrations of glucose ranging from 10-50g/l were used as carbon source and after 2 days, growth and PHB were determined. In the production medium, the nitrogen source (yeast extract) was variously replaced by peptone, tryptone, casein, NH₄Cl or No nitrogen source. After two days of growth at 37°C on a rotary shaker at 120 rpm, the growth and PHB production were determined. Moreover, the effect of different incubation temperatures (20- 45°C) and different initial pH (6- 8), and different incubation period (24h, 48h, 72h, 96h, 120h) were studied.

Extraction and assay of PHB

The best Sudan Black B positive bacterial isolates were subjected to quantification of PHB production by the method of John 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 (Adwitiya *et al.*, 2009) and 10 ml 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 (Mohamed *et al.*, 2012).

Quantifications of bacterial growth and dry weight

Cell growth was monitored by measuring the turbidity at an optical density at 520 nm (Legat *et al.*, 2010; Tekin *et al.*, 2012). After centrifugation of the culture medium, the supernatant was discarded and the cell pellet was washed with distilled water. Cell dry weights were measuring as described by Berekaa and Thawadi (2011) after growth drying at 60°C for 2 successive days until constant dry weight.

Statistical analysis:

All measurements were performed in triplicate, and all values reported are the mean of three replicates. P-values were calculated for all possible variable pairs and were performed using SPSS 16.0. The asterisks * indicate significance at $p < 0.05$.

3. Results

Different bacterial isolates (39) were obtained from heated soil suspensions and waste water collected from Jeddah on nutrient agar at 45°C. All the bacterial isolates were screened on solid agar medium using Sudan black (Figure 1) On ager medium, out of 39 isolates, 12 were PHB producers as detected by Sudan black. They were grown in liquid medium for 48 hr. and PHB was extracted and quantified (Table 1).

The % of PHB of cell dry weight was determined. It was ranged from 10-43% of the cell dry weight. The most active isolate MM7 produced 0.52 g/l PHB (43% of cell dry weight). It was

selected for more detailed studies. It was Gram positive bacilli, non acid fast and was spore forming bacterium. Examination with light and scanning electron microscope revealed that it was non motile with the diameter of 0.5-0.7, 5-10 μm and the colony had creamy color on nutrient agar medium (Figure 2). It was resistant to Novobiocin, Rythromycin and Sutamide (Table 2). Production of catalase, urease, indole, lecithinase and cellulose were positive (Table 3). After cell staining using Sudan black and safranin, PHB granules were detected inside the cells as black color against red back ground (Figure 3A). After growth in liquid medium, PHB was extracted, purified and dried (Figure 3A). The isolate MM7 was very similar to *Bacillus cereus* and 16SrDNA analysis revealed that this isolate is belonging to genus *Bacillus* and very closely related to *B. cereus* ATCC14579 (Figure 4).

Maximum PHB production was determined using 3% glucose as carbon source (Figure 5, 6). Furthermore, using production medium with 0.2 % yeast extract as nitrogen source and 0.05 % K_2PO_4 as phosphorus source enhanced both growth and PHB production (Figure 7, 8). In addition, the production medium with initial pH 7 (Figure 9), incubation temperature of 37°C (Figure 10) and shaking rate 120 rpm after 2 days of growth, yielded the maximum PHB production (Figure 11).

Table 1 Growth (g/l) and PHB production by 12 bacterial isolates obtained from different sources, collected from Jeddah

Isolate	Source	PHB detection on agar medium	Cell dry weight (g/l)	Quantity of PHB (g/l)	% PHB/cell dry weight
MM1	Soil	+	2.30 \pm 0.4	0.31 \pm 0.3	13.4
MM 5	Soil	++	2.50 \pm 0.25	0.39 \pm 0.1	15.6
MM7	Soil	+++	1.20 \pm 0.08	0.52 \pm 0.0*	43
MM9	Soil	+	2.40 \pm 0.49	0.28 \pm 0.0	11.6
MM13	Soil	++	1.70 \pm 0.33	0.18 \pm 0.0	10.6
MM14	Soil	+	2.10 \pm 0.69	0.38 \pm 0.0	18
MM15	MW	+++	0.80 \pm 0.15	0.28 \pm 0.1	35.6
MM19	MW	+	1.90 \pm 0.35	0.19 \pm 0.0	10
MM22	MW	++	3.50 \pm 0.65	0.38 \pm 0.0	10.8
MM30	WW	+	1.60 \pm 0.55	0.28 \pm 0.0	17.5
MM33	WW	++	1.30 \pm 0.08	0.48 \pm 0.0*	36
MM39	WW	++	0.98 \pm 0.10	0.20 \pm 0.1	20.4
<i>B. subtilis</i> **		++	0.99 \pm 0.40	0.18 \pm 0.1	18.1

*: significant result, +: Low production, ++: Moderate production, High: production. **: *B. subtilis* ATCC6633 (Control), WW: Waste water, MW: Marine water

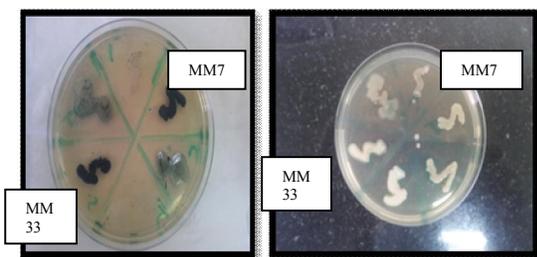


Figure 1. Screening of some bacterial isolates on solid agar medium using Sudan Black, (A): Before staining (B) After staining

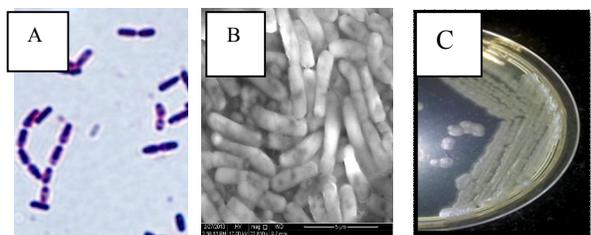


Figure 2. The selected bacterial isolate MM7 after staining with Gram X 1000 (A), under scanning electron microscope (B), On Nutrient agar after 4 days of growth at 45°C

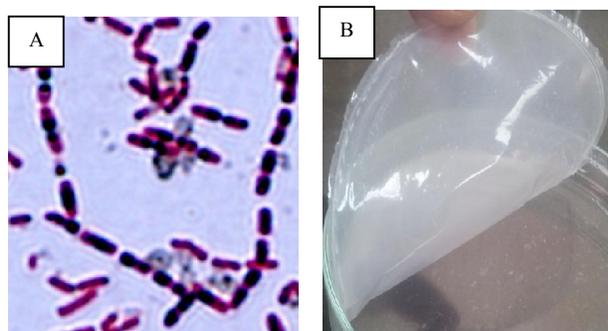


Figure 3. The stained smear with Sudan black and saffranin under light microscope X10000 (A), the produced PHB plastic sheet extracted from the selected isolate MM7 after optimization of growth conditions (B)

After optimization of growth conditions, the isolate MM7 was grown one liter conical flask containing 200 ml of the modified production medium prepared with 3% glucose and 0.25% yeast extract at pH7. After 2 days of incubation at 37°C and 120 rpm, PHB was extracted, purified, dried (Figure 3B) and weighted. After optimization of growth factors, % of PHB was enhanced from 43% to 55%.

Table 2: The morphological characters and sensitivity to some antibiotics of the selected bacterial isolate MM7

Character	Result	Antibiotic	Sensitivity
Gram stain	+ve	Chloromphenicol	+
Acid fast	-ve	Tetracycline	+
Shape-motion	Bacilli, non motile	Novobiocin	-
Color	Creamy	Rythromycin	-
Colonies	Regular	Sutamide	-
Colony edge	Entire	Ampicillin	+
Endospore formation	+ve	Penicillin G	+

+ve: Positive result, -ve: Negative results, -: Resistance, +: Sensitive

Table 3. Physiological and biochemical characters of the selected bacterial isolate MM7

Character	Result	Character	Result
Optimum temperature	30-37	Nitrate production	+
Temperature range	15-50	Gelatin liquefaction	-
Optimum pH	5-8	Hydrolysis of esculin	-
NaCl tolerance	5-15%	Hydrolysis of Tween 80	-
Catalase	+	Egg yolk lecithinase	-
H ₂ S	+	Hydrolysis of starch	+
Voges –proskauer	+	Hemolysis	-
Urease	+	Cellulase	+
Indole	+	Utilization of casein	+

-: Negative results, +: positive results

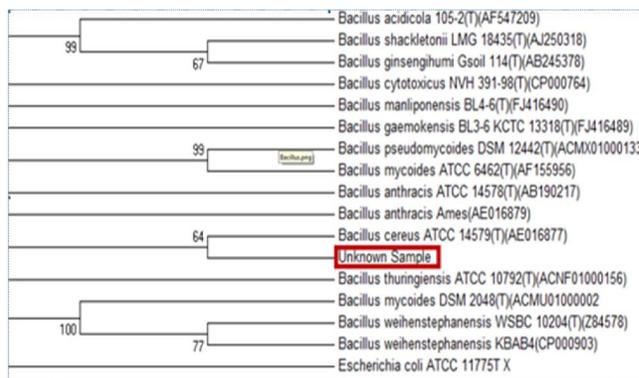


Fig 4. Phylogenetic tree of the isolate MM7 and the most related isolates

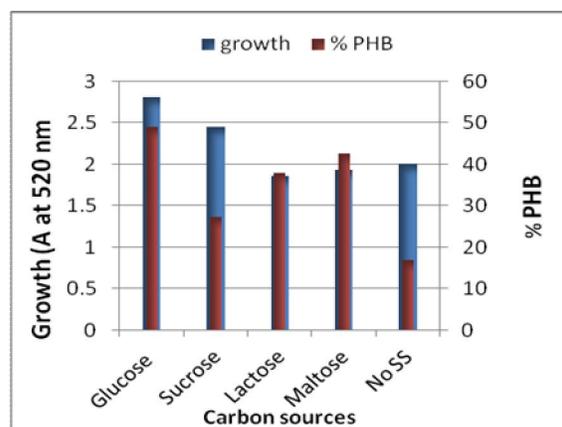


Fig. 5: Effect of different carbon sources on growth and PHB production by the selected bacterium MM7

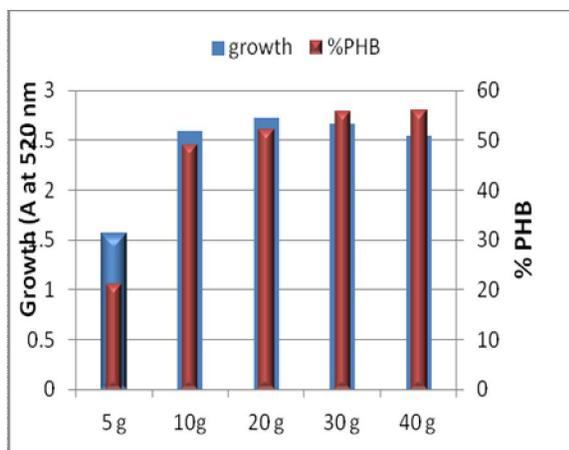


Fig.6: Effect of different glucose concentrations on growth and PHB production by the selected bacterium MM7

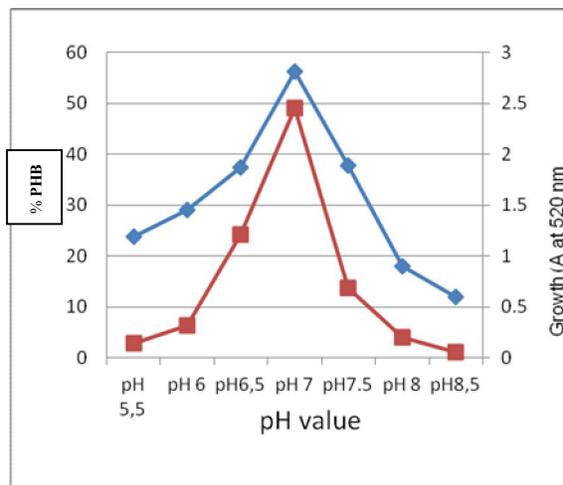


Fig. 9: Effect of different initial pH values on growth and PHB production by the selected bacterium MM7

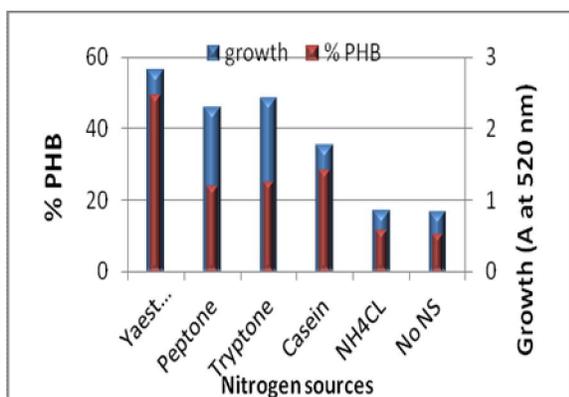


Fig. 7: Effect of different nitrogen sources on growth and PHB production by the selected bacterium MM7

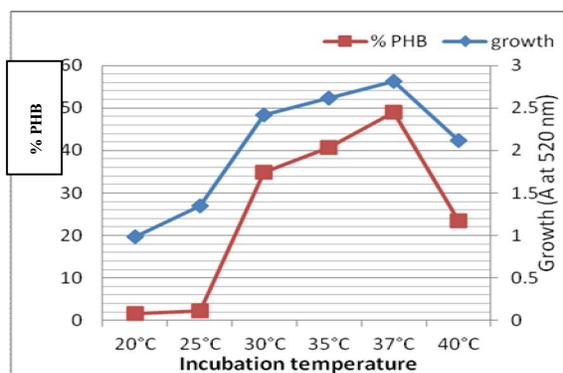


Fig. 10: Effect of different temperature on growth and PHB production by the selected bacterium MM7

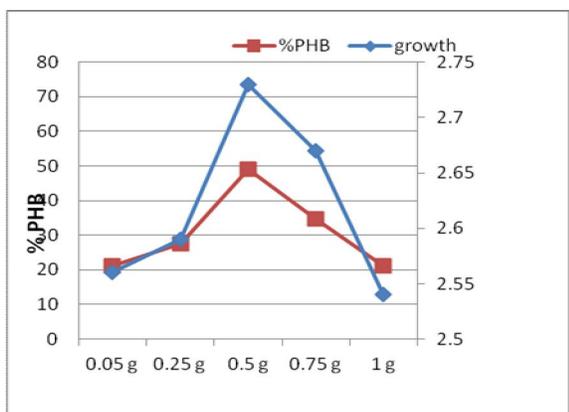


Fig. 8: Effect of different initial concentration of K₂PO₄ on growth and PHB production by the selected bacterium MM7

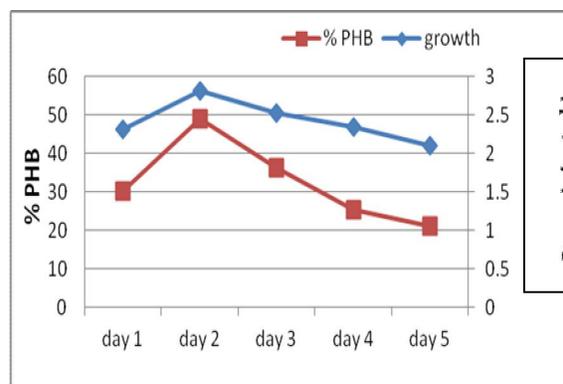


Fig. 11: Effect of different incubation period on growth and PHB production by the selected bacterium MM7

4. Discussions

Many environmental problems affect our entire world and one of the largest problems affecting the world is solid plastic waste management. Therefore, biodegradable plastic, may be a solution for the environmental problem concerning plastic products.

PHB, a polyester compound, is gaining interest among the group of biodegradable plastics. PHB biodegradable, biocompatible and naturally accumulated by several bacteria such as *Alcaligenes*, *Pseudomonas*, *Bacillus*, *Rhodococcus*, *Cupriavidus* and some species of photosynthetic bacteria under conditions of nutrient stress.

It was interesting to note that bacteria belonging to *Bacillus* accumulated a high concentration of PHAs (Sangkharak and Prasertsan, 2012; 2013). *Bacillus* sp. have ability to syntheses both short chain length with C3–C5 hydroxyacids as monomers and medium chain length with C6–C16 hydroxyacids as monomers (Tajima *et al.*, 2003). Reports suggesting the use of *Bacillus* for the production of a range of different PHAs by utilizing different carbon sources are known (Halami, 2008).

In this study, new polyhydroxybutyric acid producing isolate was selected and it was identified as a *B. cereus* MM7 using sequencing of 16S rDNA as molecular test (Valappil *et al.*, 2007b; Reddy *et al.*, 2009), and microbial tests like Gram stain and other morphological and biochemical properties (Naheed *et al.*, 2011). Bacterial colonies on agar medium were identified by colors, elevation, form and edge appearance according to Sirockin and Cullimore (1969). As it is well known, PHA accumulation in the bacterial cell can be determined quite easily with Sudan Black using rapid screening plate method (Chandrashekharaiah, 2005; Mohamed *et al.*, 2012), or by microscopic screening (Ceyhan and Ozdemir, 2011; Singh and Parmar, 2011; Mohamed *et al.*, 2012; Dhingra and Priya, 2013), and all the two methods were previously used to select PHB producing bacteria.

In general, PHB polymer is synthesized by the bacterial cells under limiting growth conditions, when the carbon source is in excess and nitrogen, phosphorus, magnesium, sulfur or oxygen is present in a limiting concentration (Reddy *et al.*, 2009). The medium we used in this study is the nitrogen-deficient medium; it was the best medium for *B. cereus* in terms of both the cellular growth and PHA accumulation (De Vries *et al.*, 2004; Vallapil *et al.*, 2007a; Reddy *et al.*, 2009). On the other hand, *Bacillus* sp was able to use a large variety of carbon sources like sugars and fatty acids for PHA production (Vallapil *et al.*, 2007a; Vallapil *et al.*, 2007b; Halami, 2008) The carbon source is one of the most factor affecting PHB biopolymer production. In this study, the effect of different carbon sources like (Glucose, sucrose, Lactose, Maltose) on PHB production by *Bacillus* sp. were investigated (Kumar *et al.*, 2009).

The Results showed the maximum PHB production was attained when glucose was used as a sole carbon source. Similar results were obtained by Alvarez *et al.* 2000; Valappil *et al.*, 2007a; Valappil *et al.*, 2007b; Reddy *et al.*, 2009; Kumar *et al.*, 2009; Naheed *et al.*, 2011; Berekaa and Thawadi, 2012. The best amount of growth and PHB accumulation was measured after 48 h incubation. which is agree with Valappil *et al.*, 2007a; Singh *et al.*, 2009; Kumar *et al.*, 2009; Shivakumar, 2012; Berekaa and Thawadi, 2012; Rodriguez-Contreras *et al.*, 2013 but not agree with other researches that found 24 h is the best incubation period (Shamala *et al.*, 2003; Khanafari *et al.*, 2006; Pal *et al.*, 2009; Wang *et al.*, 2009; Omar *et al.*, 2011; Bhubalan *et al.*, 2011) or that indicated that the highest concentration of the polymer was mostly produced by 72hr. (Mizuno *et al.*, 2010; Rodriguez-Contreras *et al.*, 2013). Also, our results showed that the 37°C is the optimum temperature to produce high amount of polymer with *B. cereus* MM7. Similar results were obtained by Chen *et al.*, 2010; Naheed *et al.*, 2011; Shivakumar, 2012; Hamieh *et al.*, 2013; Sangkharak and Prasertsan, 2012; Berekaa and Thawadi, 2012), while other research found that some *Bacillus* sp need to incubated at 30°C to produce maximum amount of PHB (Shamala *et al.*, 2003; Valappil *et al.*, 2007a; Reddy *et al.*, 2009; Mizuno *et al.*, 2010). Moreover, the medium pH affect growth and PHB production and production medium with pH7 resulted in a maximum PHB production which is in agreement with De Vries *et al.*, 2004, Valappil *et al.*, 2007a; Aarthi and Ramana, 2011; Van - Thuoc *et al.*, 2012), and low pH conditions inhibit utilization of the PHB polymer (Valappil *et al.*, 2007a).

In conclusion, soil is a rich source of bacteria producing PHB spinally genus *Bacillus* and optimization of growth conditions enhancing the production

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