Production of poly β hydroxybuthyrate from *Pseudomonas putida* MAO12 isolated from wastewater sample

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Abstract: The biodegradable polymers of hydroxyl butyric acid, poly β-hydroxybutyrate (PHB) is a biodegradable material produced mainly from bacteria and has wide range of application. In this study, 20 bacterial isolated was obtained on Nutrient agar medium from soil and waste water samples collected from either date palm farm or Waste water treatment station. Out of 20 isolates, 6 (30%) showed the highest PHB production, 8 (40%) showed moderate production and 6 (30%) were non producer. The isolate MAO12, which was isolated from waste water sample was the most active isolate in PHB production. It was identified as *Pseudomonas putida* MAO12 using morphological and physiological characters. Maximum production was found in Nutrient broth containing 10% glucose, at pH 7.5, 35°C after 2 days of growth at 120 rpm. The selected bacterium were grown in Nutrient broth containing 10% of different carbon sources and maximum percentage of PHB was found using glucose> fructose> sucrose> maltose> cellulose >starch. Moreover, Whey, chitin, molasses, corn steep and starch were used as cheap carbon sources in minimal medium containing 10% glucose. At the end of growth period, growth and PHB production was determined. Maximum PHB was obtained by Corn steep > Molasses > Chitin > Whey > Starch. In conclusion, PHB can be produced by *Pseudomonas putida* using different cheap carbon sources.
[Aburas MMA. **Production of poly β hydroxybuthyrate from *Pseudomonas putida* MAO12 isolated from wastewater sample.** *J Am Sci* 2016;12(5):107-112]. ISSN 1545-1003 (print); ISSN 2375-7264 (online). http://www.jofamericanscience.org. 14. doi:10.7537/marsjas12051614.

Keywords: PHB, Degradation, Production, Bioplastic, Wastes, Bacteria, *Pseudomonas putida*

1. Introduction

The biodegradable polyhydroxy butyrate (PHB) or bioplastic is a biocompatible thermoplastic material of the class of bacterial polyesters which mainly called polyhydroxyalkanoates (PHAs). In harsh environmental conditions, PHB was accumulated intracellular in many bacterial genera like *Azotobacter* and *Bacillus* as distinct white food storage granules (Hanzlikova *et al*., 1985, Amara, 2008). PHB produced from regenerable carbon sources and can accumulate inside the cells during unbalanced growth, in the presence of excess carbon and low nitrogen (Verlinden *et al*., 2007, Belal, 2013). The most active genera in PHB production were *Azotobacter, Ralstonia, Bacillus, Pseudomonas* and *Alicaligens eutrophs* (Singh and Parmar, 2011). Some bacteria have been accumulated using different carbon sources, including sucrose media (Quagliano *et al*., 1994). PHB was first isolated and characterized since 1925 as a primarily a product of carbon assimilation and can be used as a source of energy by bacteria and fungi (Lemoigne, 1926).

The biodegradable plastics PHB was as other plastic, can be used in many application but it had unique properties like UV resistant, highly resistant to hydrolytic degradation, insoluble in water, oxygen permeability, soluble in chloroform, and poorly resistance to acids and bases, and other chlorinated organic materials. PHB is used in many medical applications and the granules are clearly visible as bright granules in the cytoplasm of the cell or after staining and examination using light microscope.

Biodegradable polyesters have a special interest in substituting common plastics due to complete degradation by the environmental microbes. Many bacterial and fungal isolates of soil have the ability to breakdown PHB due to the presence of some enzymes specially depolymerase (Aly *et al.,* 2015) and during the adverse conditions PHB is used by the cell as an internal reserve of carbon and energy. Therefore, the resent study aimed to study the effect of various environmental conditions on PHB production by the isolate, obtained from cultivated soil.

2. Material and Methods

Bacterial isolation and screening for PHB accumulation

In this study, the used bacteria was collected from soil and waste water samples collected from either date palm farm or Waste water treatment station. About 200 g of each soil samples were collected in sterile plastic bags or 200 ml of water samples were collected in sterile glasses. All samples were directly transferred to the Microbiological Lab., under cooling. All soil samples were spread above clean papers and allowed to air to dry. The dried soil samples grinded, sieved and various dilutions were prepared. About 0.5 ml of each water sample were spread directly on the agar plates. Nutrient agar medium containing g/l: peptone 2 g, Yeast extracts 2

g, NaCl 1 g and Agar 20 g. All the bacterial isolates were screened qualitatively using Sudan Black Dye (Panigrahi and Badveli, 2013) on Nutrient agar medium with 1% of glucose and all plates were incubated for 24 hours. The growth was covered with 0.02% Sudan Black Dye in ethanol for 30 min, followed with washing with 96% ethanol to remove excess stain from the examined colonies.

Quantification of PHB production

All the Sudan Black positives isolates were grown in 250 ml Erlenmeyer flasks, each containing 50 ml of the nutrient broth medium, containing 10% glucose and each flask was inoculated with 2 ml of the preculture medium containing, $4x10^6$ CFU/ml. After growth at 37°C for 2 hr., cells were collected by centrifugation at 10,000 rpm for 10 min. The obtained pellet was washed with acetone and ethanol (1:1 V/V), re-suspended into 4% of sodium hypochlorite solution and incubated for 30 min at room temperature and centrifuged. The supernatant was discarded, the obtained cell pellet was washed with equal volume of acetone and ethanol and the washed pellet was dissolved in hot chloroform. After filtration, 10 ml of concentrated sulphuric acid was added to each tube and the obtained crotonic acid was measured at 235 nm against sulfuric acid as blank (Bonartseva and Myshkina, 1985, Aly *et al*., 2011).

Factors affecting PHB production:

Different growth factors were optimized for maximum PHB production by the selected bacterial isolate. Effect of different incubation temperature (25, 30, 35, 37, 40 and 45 °C, initial pH 5.0, 6.0, 7.0, 7.5, 8.0 and 8.5 and incubation period (1, 2, 3 and 4 days) on growth and PHB production was determined in 500 ml conical flasks containing 100 ml of the Nutrient broth medium with 10% glucose. After incubation at 120 rpm, growth (dry weight, g/l) and % of PHB were determined. Similarly, the effect of different carbon sources at concentration of 10 % like glucose, sucrose, maltose, fructose, and cellulose was determined. The selected bacterium was grown in Nutrient broth with the tested carbon sources. All flasks were inoculated with 2 ml of the preculture ($4x10^6$) incubated at 35ºC and 120 rpm for 2 days. PHB was quantified spectrophotometerically (Law and Slepecky, 1961). Furthermore, the effect of whey, chitin, molasses, corn steep and starch at concentration of 10 g /l, as a cheap carbon sources, were determined in minimal medium (Himedia) containing 10% glucose at pH 6.8±0.2. The minimal medium contained (g/l) Disodium phosphate 7.9, Potassium phosphate 3.0, Sodium chloride 0.5 and Ammonium chloride 1.0.

3. Results

In this study, 20 bacterial isolated was obtained on Nutrient agar medium from soil and waste water samples collected from either date palm farm or Waste water treatment station. All the isolates were screened on the previous medium for PHB production using Suddan black. Out of 20 isolates, 6 (30%) showed the highest PHB production which was detected by very dark black color of the bacterial colonies after washing. Moreover, 8 (40%) showed moderate production which appear as pale black color of the colonies and 6 (30%) showed no production, have gray color after whishing (data not shown).

The six isolates with maximum PHB production were grown in liquid medium (Nutrient broth) with 10% glucose at 30ºC and 120 rpm for 2 days. The growth and PHB production were determined (Table 1). The isolate MAO12 was the most active isolate in PHB production. The production was 0.05 g/l and the accumulation percentage was 37% of the cell dry weight. The isolate was Gram negative bacterium, isolated from waste water. On solid Nutrient agar medium with 10% glucose, it showed the highest growth and PHB production. It gave positive result as very dark color with Sudan black on agar medium.

Table 1. Growth and PHB detection and production on either Nutrient agar or in Nutrient Broth containing 10% glucose by six bacterial isolates

Isolate	Source	Gram Stain	Solid medium		Liquid medium	
			Growthon solid medium	PHB detection on solid medium	PHB (g/l)	% PHB
M AO3	Soil	Gram positive, Bacilli	+++	+	0.031±0.006	25.3
MAO5	Soil	Gram positive, Bacilli	+++	+	0.024±0.002	14.2
MAO6	Soil	Gram positive, Bacilli	+++	+	0.029±0.018	11.0
MAO9	Soil	Gram positive, cocci	+++	+	0.03±0.004	21.4
MAO12	Waste water	Gram negative, Bacilli	+++	+	0.05 ± 0.001	37.0
MAO15	Waste water	Gram negative, Bacilli	+++	+	0.03± 0.003	22.0

The isolate MAO12 has bacillus shape with no capsule or spore. It belongs to Gram negative bacteria and give positive results with catalase test and negative results with H_2S and indole production. Urea, gelatin and starch

hydrolysis were negative. Fermentation of sugars to acids (Methyl Red Test) was negative (Table 2). It was identified as *Pseudomonas putida* MAO12 using morphological and physiological characters.

Table 2. Morphological and physiological characters of the selected bacterial isolate MAO 12

Test	Result	Test	Result
Source	Contaminated water	Catalase test	Positive
Gram stain	Positive	H_2S production	Negative
Acid fast	Negative	Urea test	Negative
Shape	Bacilli	Indole	Negative
Motion	Motile	Methyl red	Negative
Capsule	No capsule	Starch hydrolysis	Negative
Spore	No spore	Gelatin liquefaction	Negative

Maximum production was found in Nutrient broth containing 10% glucose at incubation temperature of 35°C and initial pH 7.5 (Figure 1 and 2). Maximum PHB production was found after 2 days of growth at 120 rpm (Figure 3). The selected bacterium was grown in Nutrient broth containing 10% of different carbon sources including starch, maltose, fructose, sucrose and cellulose. Glucose was used as control. Maximum % of PHB was found using glucose followed by fructose, sucrose, maltose, cellulose and finally starch (Figure 4). Moreover, Whey, chitin, molasses, corn steep and starch were used as cheap carbon sources in minimal medium containing 10% glucose. At the end of growth period, growth and PHB production was determined. Maximum PHB was obtained by Corn steep > Molasses > Chitin > Whey > Starch (Figure 5). In conclusion, PHB can be produced by *Pseudomonas putida* using different cheap carbon sources.

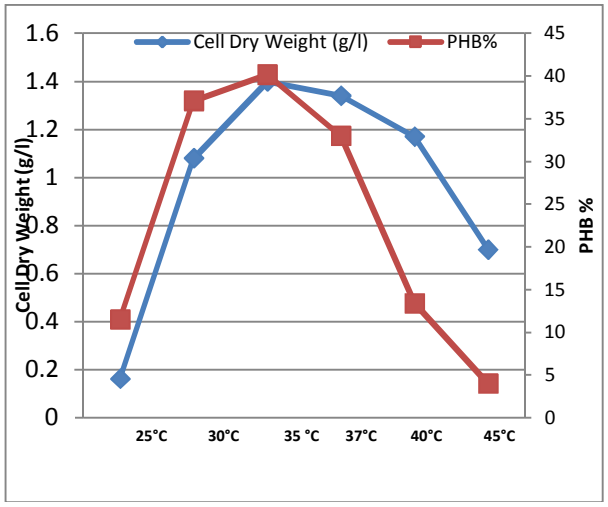

Figure 1. Growth and production of PHB at different incubation temperatures by the selected bacterium

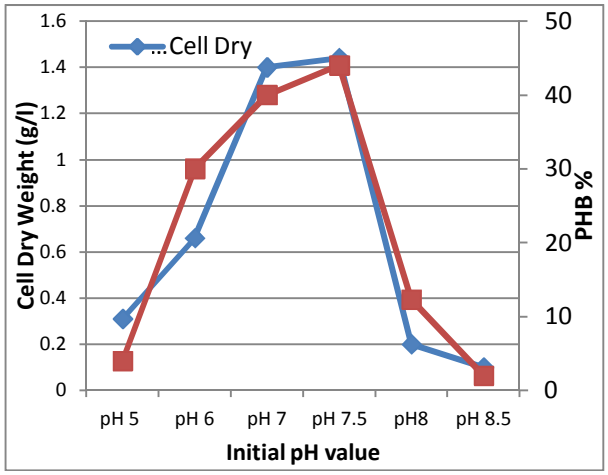

Figure 2. Effect of different pH values on growth and PHB production by the selected bacterial isolate

4. Discussions

Bioplastic which chemically named polyhydroxyalkanoate (PHA) can be used to replace plastic in many applications (Eggersdorfer *et al.*, 1992). Many fat polymer materials are belonging to PHA polymers and poly-β- hydroxybutyrate (PHB) is one of the most famous and known PHA. In this study, we tried to isolate PHB producing bacteria from poor habitats either soil or waste water. It is well known that bacteria in natural environment (soil or water) which is poor in nutrient sources exhibit higher survival rate and growth ability than those living in rich nutrient area e.g. alimentary tract of higher animal

The observation in the present study showed that the oak forest was typically moister than the pine forest which is consistent with the study of Saxena survival rate and growth ability than those living in rich nutrient area e.g. alimentary tract of higher organisms (Anderson and Dawes, 1990, Hahn *et al.*, 1994).

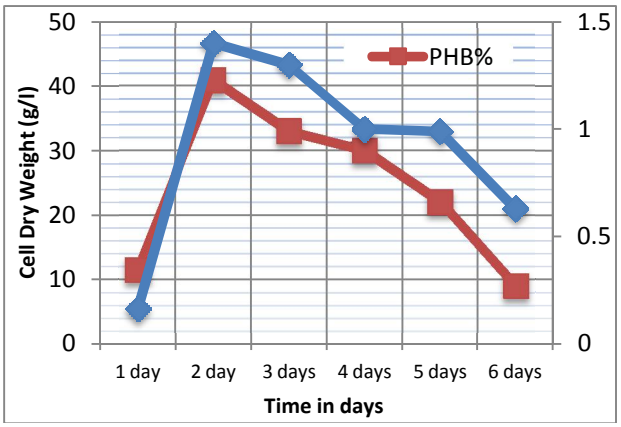

Figure 3. Growth and production of PHB by the selected bacterium at different incubation period

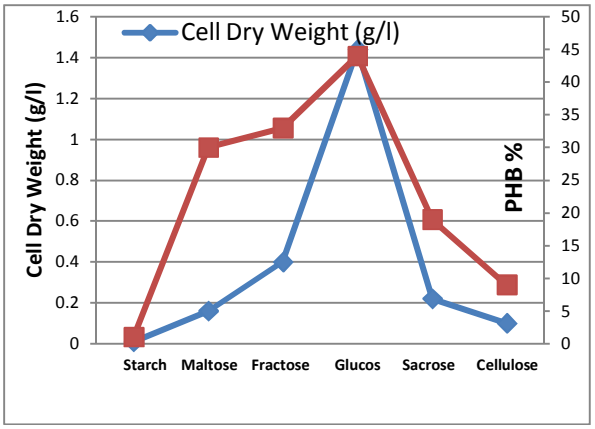

Figure 4. Growth and production of PHB using different carbon sources

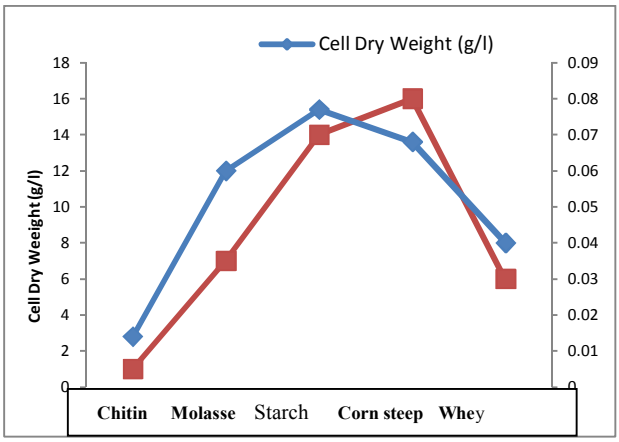

Figure 5. Growth and production of PHB using different cheap waste products as carbon sources

The most active isolate in PHB production was the isolate MAO12 which was identified as *Pseudomonas putida* MAO12 according to gram strain, morphological and Physiological characters described by Williams *et al.* (1994). As we known, inclusion bodied of lipids or PHB are accumulated in many bacteria during growth limitation conditions at stationary phase and later these lipids are used as an internal reserve of carbon granules. In this study, the content of PHB in bacteria differed from bacteria to another and the maximum percentage of PHB in the bacterial cells was 37.0% of dry cell weight of the isolate MAO12 while higher PHB percentage was found in the genus *Azotobacter*, ranging from 35 to 50% per cell dry weight (Tombolini and Nuti, 1989) and the lowest PHB productivity was found in *Rhizobium* spp. 3173 (1.38%) (Mercan, 2002). The occurrence of PHB in both Gram-positive and negative bacteria was approved by many authors (Khanna and Srivastava, 2005, Naranjo *et al*., 2013). Stockdale *et al.* (1968) used 2% glucose as carbon source for PHB production in *Azotobacter macrocytogenes* and soil bacteria was the best for PHB production up 24% of dry weight after 48 h of (Elsayed *et al*., 2013).

Mercan (2002) found that there is a relationship between PHB production and dry cell weight. The content of the PHB polymer in rhizobia ranged from 30 - 55% of the dry cell weight and PHB production was affecting by with different carbon and nitrogen sources (Tombolini and Nuti, 1989, Bonartseva *et al*., 2002). In our study, the production of PHB in *Pseudomonas putida* MAO12, which produced the maximum PHB percentage, was determined in different at different temperature, pH values and carbon sources and the highest PHB accumulation level was observed after 2 days of growth at 35°C, pH7.5 and using 10% glucose. It was reported that when PHB production conditions were optimized, the highest PHB production was observed in Bacillus after 72h at 30°C and pH 7.0, using raffinose and peptone as carbon and nitrogen sources (Singh *et al*., 2011). Javers and Karunanithy (2012) found that *Pseudomonas putida* KT217 produce PHA in medium containing ethanol and biodiesel, while glycerin at 75 g/l enhance PHA content to 30 g/L but 153 g/L of sunflower soap stock enhance PHA content to 17 g/l (17% of the dry weight). The effects of different carbon and nitrogen sources and different pH values on PHB production was studied and at acidic pH, there was a decrease in PHB content in the medium (Mercan, 2002). Moreover, they found that maximum PHB (65% of dry cell weight) was obtained during growth on a medium with sucrose and nitrate and the lowest PHB content was found using organic acids as

carbon source. PHB can be produced ny bacteria using waste products (Arun *et al*., 2006). In this work, *Pseudomonas putida* MAO12 used different waste products to produce PHB. Similarly maximum PHB production by *Lactobacillus acidophilus* and *Bacillus thuringiensis* was after 4 days of growth on date molasses while maximum PHB production was by *Bacillus subtilis* after 6 days of on growth using whey supplemented with glucose, yeast extract, and peptone (Hamieh *et al*., 2013). In conclusion, *Pseudomonas putida* MAO12 from waste water can be used to produce PHB in large scale using different waste products.

Acknowledgements:

Author is grateful to the Department of Biology, Faculty of Science, King Abdulaziz University, Saudi Arabia.

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5/3/2016