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Factors Affection Degradation of Poly-3-Hydroxybutyrate (Bioplastic) By A Filamentous Bacterium of The Genus Streptomyces

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Abstract: *Actinomycetes are filamentous Gram-positive bacteria mostly found in soil and are known as a best producer of antibiotics and useful enzymes. They have important role in the breaking dawn of organic materials and polymers. About 40 actinomycete isolates were obtained from saline soil collected from Jeddah on starch nitrate agar using serial dilution method. All isolates were screened for poly-3-hydroxybutyrate (PHB) degradation on agar and broth media containing PHB as carbon source. The isolate GHM20 was the most active isolate in PHB degradation and using different characteristic, it was identified as species belong to genus Streptomyces. Identification was confirmed and the isolate was identified as Streptomyces sp. GHM20. The best degradation was recorded at pH 6.5, 25°C after 3 days of incubation in minimal medium containing 2 g/l glucose and 0.2 g/l yeast extract as additional carbon and nitrogen sources, respectively. The used medium contained 1.5% PHB enhanced degradation of PHB and depolymerase production. In conclusion, the species belonging to the genus Streptomyces showed excellent activities in PHB degradation and improving growth conditions enhanced the degradation process and depolymerase production.*

Keywords: *Streptomyces, degradation, poly-3-hydroxybutyrate, depolymerase, biopolymer, bioplastic*

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I. Introduction

Plastics of petroleum are of great importance in today's life and poses wide uses in all things around us which improved the quality of human life. Everywhere plastics materials were found, which are used in packaging foods, instrumentals, cars, and other items. Plastics make progress possible, making electrical goods safer, lighter, more attractive, quieter, and more durable (Andrady, 2003; Andrady and Neal, 2009). Plastic accumulate in the environment at a rate of 25 million tons per year and the main effects of land pollution caused by plastics is the blockage of rain water from getting underground, thus reducing ground water level. Accumulation of plastic wastes in the environment was dangerous and cause ecological threat and diseases. Disposing of plastics by burning pollute the environmental and cause many health problems. Developing more environmentally friendly biodegradable plastic as an alternative is very argent in maintaining the ecosystems peace. Actinomycetes, the branching filaments bacteria, dominate the microbial life in soil and play a major role in decomposition of different polymers and recycling of organic complex polymers (Madigan and Martinko, 2005).

The biopolymer poly-3-hydroxybutyrate (PHB) is polyester produced by certain bacteria processing glucose, corn starch or wastewater. Its characteristics are similar to those of the petroplastic polypropylene. PHB production by bacteria is increasing every year (Gouda et al., 2001). The South American sugar industry, for example, has decided to expand PHB production to an industrial scale (Biobased-busines, 2017). PHB is distinguished primarily by its physical characteristics. It can be processed into a transparent film with a melting point higher than 130 degrees Celsius, and is biodegradable without residue. Most of the plastic degradation microorganism was screened for their potential do degrade them in vitro (Delafield et al.1965, Fields and Finn 197, Oda et al., 1995, Mergaert and swings 1996, Aly et al., 2015). The study aimed to isolate, identify ad study the most active bacterial isolate in PHB degradation.

II. Material Ad Methods

Sample collection and bacterial isolation:

Four different soil samples were used to isolate some actinomycetes on starch nitrate agar at 30°C. Soil samples were collected from the different places of Jeddah, Saudi Arabia. Soil suspensions were prepared ad used to isolate actinomycetes. About 0.1 ml of each sample dilution was spread on starch nitrate agar (Shirling and Gotlieb, 1966). All plates were incubated at 30°C for 5 days and the purified isolates were maintained on

agar slants of the same medium at 4°C until used. All isolates were screened for PHB degradation using plate-clearing technique on Turbid medium containing PHB as carbon source (Augusta et al., 1993). Turbid medium was composted of (g/L): PHB 1, KH_2PO_4, 0.7, $MgSO_4$, 0.7, NH_4NO_3, 1, Agar 15, pH 7. About 0.1 ml of PHB degrading bacterium was used to inoculate the agar plate and all plates were incubated at 30°C for 5 days until the presence of a clear zone which indicated PHB hydrolysis (Klich, 2002). The bacterial isolate with maximum PHB degradation on agar plate was cultivated in 50 ml of PHB emulsified Mineral Turbid broth medium in 250 ml Erlenmeyer flasks. Each flask was inoculated with a 2 ml (5×10^6CFU⁄mL) of the bacterial suspension, previously grown in starch nitrate medium for 2 days at 30°C and 120 rpm. The inoculated flasks were incubated at 30°C and120 rpm for 5 days. Finally, bacterial cells were collected by centrifugation at 5,000 rpm for 10 min and the cell-free supernatant was used for PHB depolymerase assay.

Identification of PHB degrading actinomycete

The isolate with the highest clear zone on PHB emulsified agar plates was identified on the basis of colony morphology, microscopic examination using light and electron microscopy, sensitivity to different antibiotics and biochemical and physiological tests (Aly et al., 2011, 2012).

Optimization of Conditions for PHB Depolymerase Production

Different cultures conditions for the maximum production of PHB depolymerase were optimized. All the experiments were performed in 250 ml Erlenmeyer flasks using Mineral Turbid medium supplemented with 0.1% PHB as a sole carbon source. All flasks were incubated at 120 rpm for 5 days. Samples were drawn aseptically after every 24 h, centrifuged at 5,000 rpm for 10 min and then supernatant was taken as a crude enzyme extract to determine the enzyme activity. To study the effect of temperature, inoculated flasks were incubated at different temperatures ranging from 25- 55°C for 5 day. Flasks containing PHB emulsified Turbid medium with different pH values (pH 5-10) were prepared and inoculated with 2 ml of preculture. After incubation, PHB depolymerase production was measured after 24 hr as described earlier. Moreover, different concentrations of Glucose 0.0-3.0 g/l, yeast extract 0.0-1.0 g/l and PHB 0.25-2.0 g/l were added to PHB emulsified Turbid medium at pH 6.5 and inoculation was carried out after inoculation of each flask with 2 ml bacterial suspension. All flasks were incubated at 25°C for 5 days depolymerase production was measured. Effect of different incubation period on enzyme production was determined.

PHB Depolymerase Assay

After centrifugation at 10,000 rpm at 4°C for 15 minutes, culture supernatant was used for measuring the PHB depolymerase activity using PHB as a substrate according to the method described by Kobayash et al. (1999). About 0.3% Poly (3-hydroxybutyrate) in 50mMTris-HCl buffer, pH 7.5 was sonicated for 20 min in ultrasonic water bath (35KHz285W) prior to the dilution to 0.03% in the same buffer. To 0.1mL of culture supernatant, 0.9 ml of the Poly-β-hydroxybutyrate substrate suspension was added and the mixture was incubated for 24 h at 30°C. The decrease in OD_{650}nm was measured using spectrophotometer, against substrate buffer blanks. One unit of the enzyme is the decrease in OD650nm per 24 h (Kobayashi et al., 1999).

III. Statistical Analysis

Mean of three replicates ±standard deviation were recorded and difference between mean values was determined using Student's t-test. Differences were considered significant when probability was less than 0.05.

IV. Results And Discussion

Poly B- hydroxybutyric acids (PHB), a promising compound for making biodegradable plastics, has been investigated for its degradation in many terrestrial and aquatic environments (Jendrossek and Handrick, 2002, Aly et al., 2015). Among different types of biodegrable plastics, PHB has been extensively studied, because of the similarity to conventional plastics, complete biodegradability and current market domination (Verlinden et al., 2007). In this study, forty actinomycetes isolates were obtained from soil samples on starch nitrate agar. All isolates were preserved on agar slants of the previous medium. All isolates of Actinomycetes which were isolated from various soil samples, collected from Jeddah, were screened for degradation of poly β-hydroxybutyrate) (PHB) on minimal medium with PHB as carbon source by the clear-zone method. Out of 40 actinomycetes, only eight isolates (20%) recorded different growth ranging from high (+++), moderate (++) or low (+) using emulsified PHB as carbon source and maximum diameter of the zones of hydrolysis was observed after 7 days (Table 1). Presence of zone of hydrolysis o agar medium indicated the presence of an extracellular PHB depolymerases (Hsu et al., 2012). Tseng et al. (2007) recorded that out of 341 strains of thermophilic actinomycetes, 105 isolates were PHB-degraders (30.8%) and based on the morphological and chemical characteristics, these isolates belonged to genera *Streptomyces* (48.4%), *Actinomadura* (12.9%), *Microbispora* (25.8%), *Thermoactinomyces* (9.7%) and *Saccharomonospora* (3.22%).

The isolate GHM20, obtained from saline soil sample, collected from cultivated area, Jeddah, was the most active in growth (+++), PHB degradation (diameter of clear zone, 31 mm) and depolymerase production in liquid medium was 0.5 U/ml as in Figure 1. Similarly, Tseng et al. (2007) used the same technique (diameter of clear zone) for determining the PBH degrader actinomycetes. Thus, based on the screening data, the isolate GHM20 was selected for more detail studies. Identification of the selected isolate was carried out. It was characterized by some morphological, physiological and biochemical properties. The isolate GHM20 was belonging to filamentous Gram positive bacteria with pink color colonies and the growth was heavy on starch agar medium. The spores were cylindrical and with smooth surface and found in chains. Table 2 summarized the morphological and physiological characters of the selected isolate. Microscopic observation of the isolate GHM20 showed substrate and aerial mycelia bearing a chain of conidia, which had smooth surface (Figure 2). Comparison between the characteristics of the active actinomycete isolate GHM20 with the former isolates indicated that it belongs to the genus *Streptomyces*. The isolate was similar to genus *Streptomyces* and identified as *Streptomyces* sp. Various *Streptomyces* strains have been used to produce a wide variety of enzymes and other secondary metabolites (Manteca et al., 2008). Many different PHB-degrading bacteria and fungi including members of *Bacillus, Streptomyces, Aspergillus* and *Penicillium* have been isolated from soil (Mergaert et al., 1993). Because of their ability to degrade extracellular PHB, these microorganisms have the potential to become useful for industrial applications. Hsu et al., (2012) isolated a PHB-degrading bacterium *Streptomyces bangladeshensis* which were isolated from soil of Bangladesh and was previously identified by Al-Bari et al. (2005). *Streptomyces lydicus* MM10 produce excellent depolymerase enzyme in the culture supernatant after 2 days of growth on PHB containing medium (Aly et al., 2015). Extracellular PHB depolymerase has been isolated, purified and characterized from different bacteria as *Alcaligenes faecalis, Rhodospirillum rubrum, B. megaterium, A. beijerinckii* and *Pseudomonas lemoigne*i (Mergaert et al., 1996; Panagiotidou et al., 2014). The expression of PHB-degrading enzyme in cells occurred in the presence of PHB in the growth medium, indicating that the synthesis PHB depolymerase is inducible. Depolymerase enzyme is not required for balanced bacterial growth and may be synthesized in response to energy or nutrient limitation (Lodhi et al., 2011). They also reported that PHB depolymerase is inducible enzyme and need the presence of PHB in growth medium. This induction was maximal when 2 g/l % was added to the culture medium.

Hsu et al., (2012) reported that the synthesis of the PHB-degrading enzyme was diminished when higher concentrations of PHB, suggesting that the production of the enzyme is tightly regulated.

Biodegradation of plastics proceeds actively under different conditions according to microbe properties and each microbe has its own optimal growth conditions (Lucas et al., 2008). The best conditions for depolymerase production varied with incubation temperature, incubation period, medium initial pH, PHB concentration and presence of carbon source. On Turbid broth medium, the most suitable temperature for growth and depolymerase production were 30 and 25°C, respectively after 24 h of incubation (Figure 3). A number of mesophilic microbes have been found to be responsible for degrading PHB in soil and aquatic environments (Mergaert et al., 1994; Kim et al., 2000). The effect of initial pH of the medium on depolymerase production was shown in Figure 4. It was found that pH 6.5 was the best for PHB degradation while pH 7.0 was the best for bacterial growth. It was also found that minimal medium supplemented with 2 g/l glucose, 0.2 g/l yeast extract and 1.5% PHB enhanced PHB depolymerase production (Table 3). Moreover, growth and PHB depolymerase production were the best after 3 day of incubation. Papaneophytou et al. (2009) reported maximum enzyme production by *Thermus thermophilus* HB8 after 24 hr of incubation. The gradual decrease in the production of enzyme after 24 h was as a result of utilization of substrate and other nutrients (Shivam et al., 2009). On contrast, *Aspergillus fumigates* M2A needed 150 hr of incubation in liquid medium to degrade PHB by extracellular PHB depolymerase (Scherer et al., 1999).Moreover, maximum enzyme production by S. lydicus MM10 was using Turbid medium at pH 7. Increasing pH more than 7 affected the charges on the amino acids within the enzyme active site. Similarly, maximum production of PHB depolymerase by *Aspergillus fumigates* was observed at pH 7 after 24 h of incubation in liquid medium (Lodhi et al., 2011).

In the current study, presence of glucose in the growth medium along with PHB enhanced activity of PHB depolymerases. On contrast, lactose enhanced PHB degradation by *A. fumigates* (Lodhi et al., 2011). According to Manna and Paul (2000), degradation of PHB by bacterial strains was affected significantly when the PHB containing medium was supplemented with easily consumable carbon sources. Glucose, fructose and arabinose supplementation lowered the extent of degradation (Manna and Paul, 2000). In conclusion; *Streptomyces* from soil can be used in PHB degradation. Optimization of culture conditions enhanced the degradation process.

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Table 1. Growth and degradation of PHB by different bacterial isolates obtained from soil on solid medium containing PHB as carbon source

Bacterial isolate	Color of the bacterial isolate	Shape	Degree of growth	Clear zone (mm)	Depolymerase activity (U/ml)
GHM 1	White	Filament	+	21±2.1	0.21
GHM 4	Gray	Filament	+	20±1.0	0.26
GHM 7	Creamy	Filament	++	19±0.2	0.31
GHM 13	Gray	Filament	++	15±3.2	0.11
GHM 16	White	Filament	+	20±3.6	0.27
GHM 17	Brown	Filament	+	15±4.2	0.24
GHM 20	Pink	Filament	+++	31±2.1	0.51
GHM 25	Gray	Filament	+	22±1.2	0.33

(+++); High growth, (++); Moderate growth, (+); Low growth

Table 2. Morphological character of the selected isolate GHM20

Tested character	**Results**
Gram stain	Gram positive
Source of isolation	Soil
Motility of spore	Absent
Shape of spore	Cylindrical (3-4 and, 5-7 µm)
Spore chain	Straight chain
Spore Surface	Smooth
Number of spore/ chain	5-33
Aerial and substrate hyphae	Well developed
Zoospore, sporangium, sclerichia and Fragmented mycelia	Absent
Chitinase, gelatinase and pectinase production	Positive

Table 3. Effect of different incubation period on PHB degradation by the selected isolate after growth on solid and in liquid media (*; significant results compared to control)

Glucose concentration (g/l)	Depolymerase activity (U/ml)	Yeast extract concentration (g/l)	Depolymerase activity (U/ml)	PHB (g/l)	Depolymerase activity (U/ml)
0 (control)	0.60	0.0 (control)	0.717	0.25 (control)	0.514
0.5	0.69	0.1	0.731	0.75	0.645
1.0	0.71	0.2	0.888*	1.0	0.88
1.5	0.73*	0.4	0.806*	1.25	0.805
2.0	0.79*	0.8	0.744	1.5	0.874
3.0	0.66*	1.0	0.690	2.0	0.88

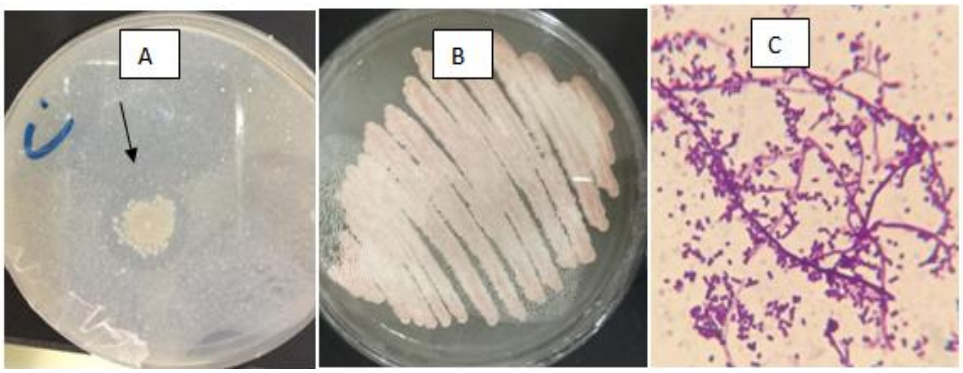

Figure 1. The selected isolate GHM20 on mineral salt medium containing PHB as carbon source (A), on starch nitrate agar (B) and under light microscope after staining with gram stain x 1000 (C).

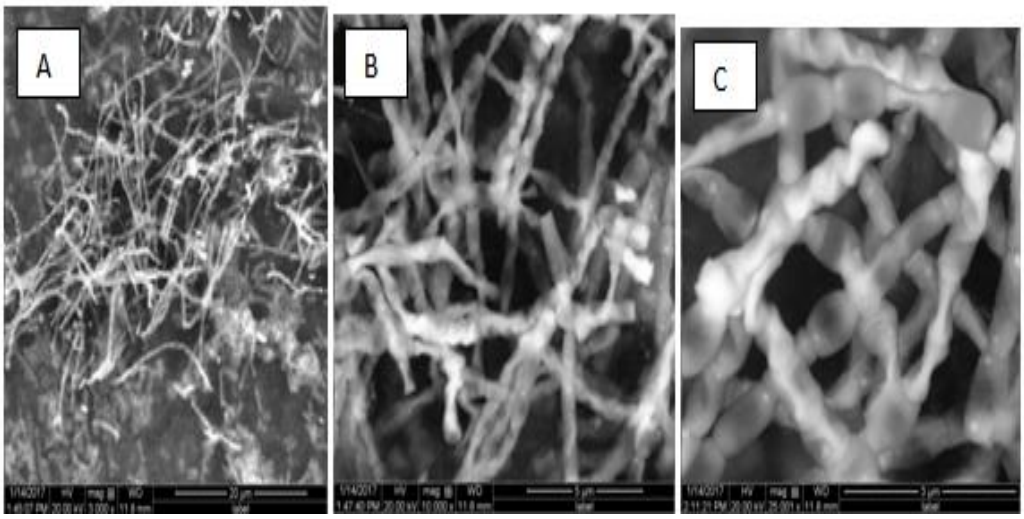

Figure 2. Scanning electron micrograph of Streptomyces sp. GHM20 after 14 days of growth on starch nitrate agar plates using different magnification x 10 000 (A) , x 20 000 (B) and x 30 000 (C).

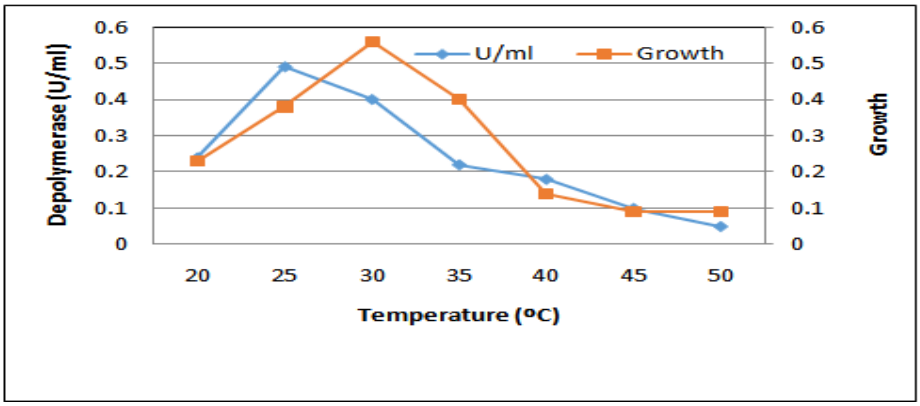

Figure 3. Effect of different incubation temperature on growth and depolymerase production by the selected isolate

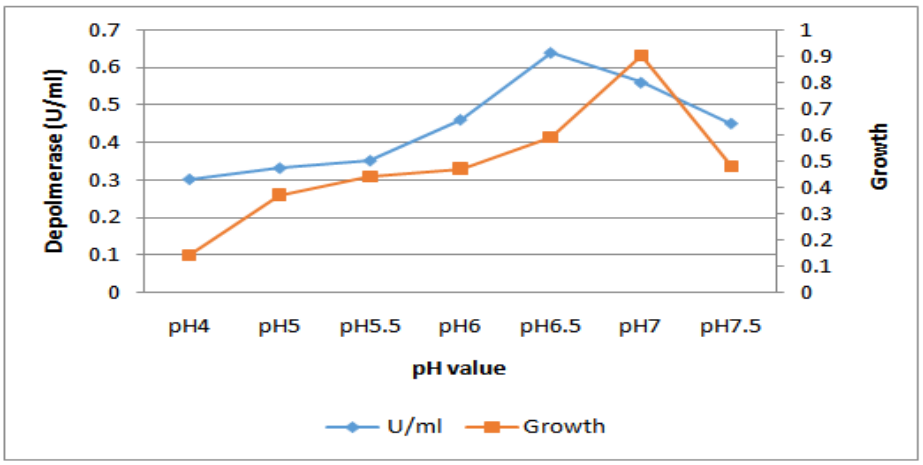

Figure 4. Effect of different initial pH values on growth and depolymerase production by the selected isolate

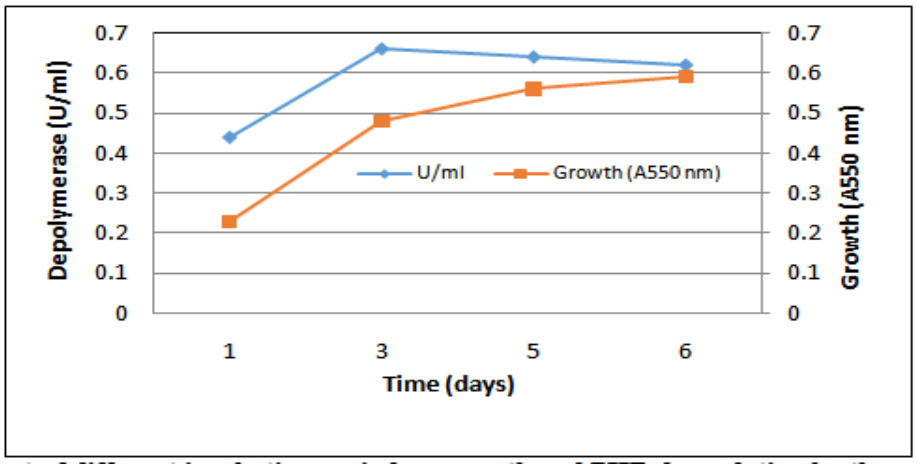

Figure 5. Effect of different incubation period on growth and phb degradation by the selected isolate

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