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Poly-*β*-hydroxybutyrate degradation by *Aspergillus fumigates* isolated from soil samples collected from Jeddah, Saudi arabia

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Abstract: *Poly-β-hydroxybutyrate acid (PHB) is thermoplastic biopolymers, synthesized by some bacterial genera under stress conditions. The degradation of PHB was detected in Petri dish by formation of a clear zone around the fungal colonies due to production of depolymerase enzyme which has interesting role in PHB degradation process. The most active PHB degrader fungi was selected and identified as Aspergillus fumigates using morphological characters. The highest PHB degradation in Petri dish by A. fumigates was at pH 5, 30°C and 7days. In liquid medium, degradation by A. fumigates was studied using enzyme assay method (U/ml). All the experiments were performed enzyme activities were monitored. After 3 days of incubation, maximum PHB depolymerase production was at pH 5 and 30°C. In conclusion, PHB can be degraded in solid and liquid medium using fungal depolymerase enzyme.*

Keywords: *depolymerase, Aspergillus fumigates, PHB, degradation, bioplastics,*

I. Introduction

Now in our life, plastics are used in almost all things and it is impossible to function without them although they destroy the environment. Biodegradable plastic or Poly ß-hydroxybutyrate (PHB) are intracellular granules, produced by many bacteria under unfavorable growth conditions (Anderson and Dawes, 1990, Verlinden *et al*., 2007) and these material must be used to replace the dangerous used plastic (Aly *et al*., 2013a). Under excess carbon source and limitation of nitrogen and phosphorous, these granules are formed as energy reserve materials (Senior and Dawes, 1971). Bacteria can produce PHB while in natural environments bacteria and fungi can degraded it to CO_2, H_2O and energy (Jendrossek *et al*., 1996). Biodegradable PHB is largely used in many medical and industrial proposes and degradation of this polymer by depolymerase enzyme was recorded in many fungal and bacterial isolates (Aly *et al*., 2015). The bacterial genera *Bacillus, Pseudomonas, Comamonas, Alcaligenes* and *Streptomyces* have a role in degradation of native and denatured PHB granules due to the presence of extracellular and intracellular depolymerase enzyme (Jendrossek *et al*., 1996, Tokiwa *et al*., 2009, Hsu *et al*., 2012). The PHB polymer was hydrolyzed to water soluble units of PHB which pass through the cell wall (Gilmore *et al*., 1990) and metabolized to either CO_2 or water (Scott, 1990) or methane (Luzier, 1992). The aim of this study is to isolate PHB degrading actinomycete and to optimize the culture conditions for maximum PHB degradation.

II. Material and methods

All experiments were performed using PHB powder with molecular weight of 470,000 g/mol and were obtained from Biomer Inc., Germany. Other substrates were purchased from Aldrich Chemical Co. or Sigma (St. Louis, USA).

Collection of samples

The samples used in this study were soils which were collected from the five different regions (Ubhur, King Abdulaziz University, AL- Sharafiya, AL- Marwa and Bahra) in Jeddah, KSA. Soil samples were collected 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.

Isolation and purification of PHB degrading microbial isolates

About one gram of the soil sample was suspended in 9.0 ml of sterile distilled water. The suspension was shaked well and serially diluted from 10^{-1} to 10^{-3} about 0.1 ml of this suspension was spread on plates of Sabouraud agar medium for isolation of fungi and all plates were incubated at 25°C for 7 days. The colonies were transferred to new plates until pure colonies were obtained. All fungi isolated were maintained on Sabouraud agar slopes held at 4°C. Each 4 month intervals, fresh cultures were prepared by streaking out from stock slopes onto Sabouraud agar plates to check for purity and then sub-culturing onto fresh Sabouraud agar slopes, both plates and lopes were incubated at 25°C.

Screening test to selection the best PHB degrader isolates

All the tested isolates were grown on Mineral PHB degradation medium (Han and Kim, 2002) at pH7 which was composed of (g/l): PHB, 0.0015; KH_2PO_4, 0.7; K_2HPO_4, 0.7; $MgSO_4$, 0.7; $NaNO_3$,1; NH_4Cl, 1; NaCl, 0.005; $FeSO_4$, 0.002 and $ZnSO_4$, 0.007. In case of preparing solid medium, 15 g/l Agar was added. Each Petri dish was divided into 5 equal parts and a disc (5 mm in diameter) of the tested fungal growth (7 days old culture) was put in the center in each part. After incubation for 7 days, all plates were incubated at 30°C, the clear zone was measured. The tested isolate showed the biggest clear zone was selected for more studies (Augusta *et al*., 1993).

Identification of the selected isolate

The fungal strain that form the biggest clear zone of PHB hydrolysis on agar plates was subjected to identification on the basis of macroscopic (colony morphology) and microscopic examination i.e. the features seen through a compound light microscope. Features observed with naked eye were, conidial color, colony diameter, mycelial color, exudates, reverse color, soluble pigment, sclerotia and cleistothecia. Features seen through a compound light microscope were seriation, vesicle, conidia, stipe, hull cells, cleistothecial wall and color, size, ornamentation of ascospores (klich, 2002).

Growth of selected isolate *Aspergillus fumigatus* on solid medium:

The selected isolate was used as test organism for PHB degradation on Mineral PHB degradation agar medium containing PHB as carbon source. Inoculated plates with a disk of fungal growth (5 mm in diameter) were prepared and the clear zone diameters in mm on agar plates due to PHB degradation were measured (Delafield *et al.,* 1965, Manna *et al.,* 1999). The weight loss of polymer with time was calculated of according to the equation of Foster *et al*. (1995) under different growth conditions.

$$\text{Polymer weight loss} = \frac{\text{Clear zone area X Total weight of polymer top layer}}{\text{Total area of Petri dish}}$$

Effect of different pH on PHB degradation:

The agar plates, containing Mineral PHB degradation medium with PHB as carbon source at different pH values (pH 5.0 , 5.4 6.0, 6.5, 7.0, and 7.5), were inoculated in the center with a fungal disk (5mm in diameter) of selected isolate and all plates were at 30°C for 7 days. Clear zone (mm) in plates was measured after 7days and weight loss of polymer was calculated.

Effect of incubation temperature on PHB degradation:

Incubation of plates containing Mineral PHB degradation medium with PHB as carbon source (pH 5) with a disk of 5 mm in diameter of the selected isolate was carried out. All plates were incubated at different incubation temperatures, 25, 30, 37, 40 and 45°C for 7 days. Clear zone (mm) in plates was measured after 7days and weight loss of polymer was calculated.

Effect of different incubation periods on PHB degradation:

A disc (5mm in diameter) of the selected isolate was inoculated on Mineral PHB degradation agar medium with PHB as carbon source (pH 5). The plates were incubated 30°C (the best incubation temperature determined from the previous experiment) for different times, 1, 2, 3, 4, 5, 6 and 7 days). Clear zone (mm) in plates was measured and weight loss of polymer was calculated.

Optimization of different parameters for maximum production of PHB depolymerase by the selected isolate *Aspergillus fumigates*:

Mineral PHB degradation broth medium was used to study the effects of some factors on PHB depolymerase production. In Erlenmeyer Flasks (250 ml capacity), about 2 ml of the spore suspension ($4x10^5$ spore/ml) of selected isolate was transferred to 50 ml of Mineral PHB degradation broth medium and all flasks were incubated in shaker incubation (150 rpm). After growth, cells were harvested by centrifugation at 10000 rpm for10 min and the supernatant was taken as a crude enzyme extract to determine the enzyme assay (Lodhi *et al*., 2011).

PHB depolymerase assay

The activity of PHB depolymerase was assayed according to the method described by Kobayashi *et at*. (1999). Stable suspension of PHB granules was prepared by suspended 0.3% of PHB in 50 mM Tris-HCl buffer, pH 7.5 and used a sonic oscillator (20 kHz, 250 W) for 20 min. About 0.1 ml of culture supernatant was added to 0.9 ml of substrate suspension and incubated at 30°C for 24 hr. The activity of PHB depolymerase was

measured at 650 nm as the decrease in OD and one unit is defined as the activity resulting in a decrease in OD_{650} per 24 hr (Kobayashi *et at*., 1999). Units were calculated using the equation:

$$\text{Uint/ml} = \frac{\text{dil x 2 x OD x Factor}}{\text{Mw x time}}$$

Dil (Dilution) = 10, OD = Observed Density using 650 wavelength, Factor = 208.33, MW of PHB = 59800, Time = 24hr.

Some factor effecting on PHB depolymerase production:

Effect of different pH on PHB depolymerase production, incubation temperature and incubation periods was studied in Mineral PHB broth medium with PHB as carbon source. Fifty ml of Mineral PHB degradation medium were dispensed in 250 ml flasks with different pH values and each flask was inoculated with 2 ml of fungal suspension (4×10^5 spore/ml). The flasks incubated using shaking incubator (150 rpm and 30^oC for 7 days). Similarly, mineral PHB degradation medium (pH 5) was inoculated with 2 ml of fungal spore suspension and the flasks were incubated at different incubation temperature and 120 rpm. The effect of incubation periods (1-8 days) on depolymerase production was also determined in Mineral PHB degradation medium. At the end of incubation period, culture filtrate was centrifuged and the supernatant was used as a crude enzyme extract to determine the enzyme activity (U/ml)

Statistical analysis:

Three replicates of each treatment were prepared and mean value ±standard deviation was determined. Student's t-test was applied to detect the difference between means which considered significant if p value was less than 0.05.

III. Results

In this research many fungal isolates (Figure 1), obtained from soil were screened for PHB degradation in Mineral PHB degradation agar and broth medium. In this connection, among 16 fungal isolates, 8 (50%) gave clear zone on solid medium containing PHB as carbon source. The eight fungal isolates were identified according to morphological and physiological characters. Six isolates were belonging to genus *Aspergillus*, one to the genus *Penicillium* and one to the genus *Alterneria* (Table 1). The degree of fungal growth was determined for each isolate and was very high (+++) for isolate TF 4 and TF 5, moderate (++) for isolate TF6 and low (+) for isolates TF1, TF2, TF 9, TF11 and TF14 (Table 1, Figure 2). PHB degradation (clear zone diameter, mm) was very high (50 mm) for isolate TF5, moderate (14-33 mm) for isolates TF4 and TF6 and low for isolates TF9,TF11 and TF14. Table 1 showed that the best microbe for degradation of PHB on solid medium was TF5. Thus, the fungal isolate TF5 was selected for more studies. The fungal isolate TF5 was identified as *Aspergillus fumigatus* on the basis of macroscopic (colony morphology) and microscopic examination i.e. the features seen through a compound light microscope (Figure 2). In macroscopic examination, grayish turquoise or dull green conidial color, white mycelia color, yellowish to brown reverse color and uncoloured exudates were observed. Colony diameter was 40-70 mm after 4-7 days and no soluble pigment, sclerotic and cleistothecia was recorded. In microscopic morphology, uniseraite seriation, 15-30 µm diameter of vesicle, globosely to broadly ellipsoidal conidia with 2-3mm diameter, smooth walled, 4.5-6 µm ascospores and absence of hull cells and cleistothecial wall confirmed the strain to be *Aspergillus fumigatus*.

Optimization of culture conditions for the selected isolates *A. fumigatus* for PHB degradation

The optimization of culture conditions for growth and PHB degradation by the selected isolates was studied by applied different factors affecting PHB degradation like; different incubation temperature, incubation period and initial pH values.

Effect of different initial pH values on PHB degradation in Petri dish

The selected isolate *A. fumigatus* was grown for 7 days at 30^oC in Mineral PHB degradation agar medium at different pH values, 5.0, 5.4, 6.0, 6.5, 7.0 and 7.5. The activity was measured of clear zone (mm) in Petri dish after 7 days and calculated the weight lost according to the equation of Foster *et al* (1995). Similarly, Mineral PHB degradation medium broth medium at different pH values was prepared and inoculated with the selected isolate *A. fumigatus* and after 5 days of incubation, depolymerase (U/ml) in the supernatant was determined. Table 2 and Figure 4 and Figure 5 showed that maximum PHB degradation was at pH 5 while the minimum amount of PHB degradation was observed at pH 7.5. Thus, pH5 was selected for maximum PHB degradation.

Effect of different incubation temperature on PHB degradation

The selected isolate of A. fumigatus was grown for 7 days in the Mineral PHB degradation agar medium at pH 5 in Petri dishes, incubated at different temperature (25, 30, 35, 40 and 45°C). The activity was measured by measuring the clear zone (mm) after 7 days and calculated the weight loss in the PHB (Table 3). The results showed that the maximum degradation of PHB for the selected isolate was at 30oC, while the minimum PHB degradation was observed at 45oC (Figure 6 and 7). Similarly, in liquid Mineral PHB degradation medium, maximum depolymerase production was at 30°C (Table 3, Figure 6), so, 30°C was selected for maximum PHB degradation. The results in Table 4, Figure 8 and Figure 9 showed that the degradation of PHB in either Mineral PHB degradation agar medium or broth medium was after 7 days and 3 days, respectively. From the previous results, rate of PHB degradation measured using clear zone diameter on solid medium or using depolymerase production in liquid medium varied with incubation temperature, incubation period and initial pH of the medium

IV. Discussion

PHB, a promising compound for making biodegradable plastics, has been investigated for its degradation in many terrestrial and aquatic environments (Jendrossek and Handrick, 2002). Many different PHB-degrading bacteria and fungi including members of *Bacillus*, *Streptomyces*, *Aspergillus*, *Penicillium*, *Acidovoraxs*, and *Variovorax* 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. Degradation of PHB was also followed in Petri plate and the clear zone formed proved the hydrolysis of the polymer. These results are in accordance with those previously published (Augusta *et al*.,1993; Doi *et al*.,1992) which suggested that the polymer hydrolysis depends on excretion of exoenzymes, diffusion through the surrounding medium and interaction between the enzymes and the polymer. Many factors influencing on PHB degradation including pH, incubation temperature and incubation period by *Aspergillus fumigates* in plates were studied. Ghanem *et al*. (2005) used the Mineral PHB degradation medium with plates to determine the degradation activity by *Nocardiopsis aegyptia* which isolated from marine sediment. Lee et al. (2005) used 0.1 to 0.02% PHB concentrations with agar plates to more easily visualize clear zones.

Our result showed that in plates, pH5 is the optimum pH for PHB degradation (clear zone diameter of 50 mm which equal to weight loss of 11.7mg) by *Aspergillus fumigates.* Belal (2013) reported that the optimum pH for PHB hydrolase was at pH7, a second optimum pH appeared at pH 6 on agar plates. It is known also that the optimum pH for growth of *Thermobifida fusca* or *T. bispora* on mineral salt medium with glucose was pH 7. Our results showed that, the best temperature for PHB degradation in agar plates was observed at 30°C for *Aspergillus fumigates.* Hsu *et al*. (2012) observed the maximum clear zone at 45°C but very tiny clear zones were produced at 28°C, and no clear zones were seen at 55°C. Previous results suggest that pH and temperature affect PHB degradation in agar plates. For *Aspergillus fumigates*, the effect of time course on PHB degradation was studied. Growth for 7 days was the optimum incubation period for PHB degradation in agar medium containing 2% PHB as carbon source. This result differed with that obtained by Hsu *et al*. (2012) who isolated *Streptomyces bangladeshensis* 77T-4 from soil and observed maximum PHB degradation on plates after 4 days while Ghanem *et al* (2005) found that the maximum degradation activity was observed in test tubes after 21 days but the maximum activity in plates after 25 days. PHB degradation in plates was faster than test tubes. Lodhi *et al.* (2011) used *Aspergillus fumigates* to produced PHB depolymerase under optimum condition using Mineral salt broth medium. Analysis of PHA-degrading fungi has led to the consideration of *Aspergillus* as one of the most predominant genera (Matavulj and Molitoris, 1992). A number of mesophilic fungi belonging to the genera *Aspergillus*, *Penicillium* and *Paecilomyces* have been found to be responsible for degrading PHAs in soil and aquatic environments (Kim *et al.*, 2000) while *A. fumigatus* and *A. penicilloides* is responsible for the degradation of PHB in the soil (Mergaert *et al*., 1994). Takaku *et al*. (2006) observed the maximum activity of extracellular PHB depolymerase produced by *Bacillus megaterium* was at pH 9.0 at 65ºC. Briese *et al*., 1994 used sewage sludge as inoculums and observed the highest degradation rate was at pH 7.5 and pH 8.0. The optimum activity of the PHB depolymerase produced by *Penicillium* sp. was obtained by ultraviolet (UV) light mutagenesis at pH 8.6 (Qin *et al*., 2006). In our study, the best incubation period of PHB depolymerase production was observed in liquid media after 3 days by *Aspergillus fumigates* followed by a gradual decrease in production of enzyme. Scherer *et al*. (1999) found that *Aspergillus fumigatus* M2A was able to degrade PHB after 150 hours of incubation in liquid media by the production of extracellular PHB while **L**odhi *et al.* (2011) reported that 24 hr of incubation was optimum incubation period of PHB depolymerase production in Mineral Salt liquid medium. Christos *et al*., (2009) reported the production of extracellular PHB depolymerase by *Thermus thermophilus* HB8 after 24 hours of incubation. Most of the PHB degrading microorganisms consist of a wide range of different microorganisms at ambient or mesophilic temperatures while only a few species like *Bacillus* strain TT96 (Tansengco and Tokiwa, 1998) and *Streptomyces* strain MG (Tokiwa and Calabia, 2004) were reported capable of degradation at higher temperatures. Still there is little information on microbial

degradation of PHB at high temperatures. Moreover, degradation in liquid media takes a much lesser time than that observed in solid media. In current study, the maximum activity of PHB depolymerase by *Aspergillus fumigates* was at 30°C. *A. fumigatus* strains that are capable of degrading PHB and Poly (3HB-co-3HV) at high temperatures of above 40°C have been isolated from soil samples (Kim *et al.*, 2000) and compost (Mergaert *et al.*, 1994). Lodhi *et al.* (2011) was observed that the *Aspergillus fumigatus* was able to degrade PHB better at 45°C after 24 hours of incubation in liquid media while Takaku *et al*., 2006 reported that 65ºC was the best incubation temperature of extracellular PHB depolymerase produced by *Bacillus megaterium.* In consolation, fungi can used PHB as carbon and energy sources and many factors affecting degradation process including pH, temperature, PHB concentration and incubation time.

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

Fungal isolate	Color of the isolate	Identification	Growth	Clear zone (mm)
TB 1	Black	*Alterneria* sp.	+	11±2.1
TB 2	Yellow	*Aspergillus nidulans*	+	19±1.0
TB 4	Black	*Aspergillus niger*	+++	33±0.2
TB 5	Bright green	*Aspergillus fumigatus*	+++	50±3.2
TB 6	Yellowish green	*Aspergillus* sp.	++	14±2.1
TB9	Pale yellow	*Aspergillus oryzae*	+	0.0
TB 11	Dark yellow	*Aspergillus* sp.	+	0.0
TB 14	Blue	*Penicillium* sp.	+	0.0

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

Table 2. Effect of different initial pH values on PHB degradation by the selected isolate *A. fumigatus* after growth in solid and liquid

	Solid medium		Liquid medium
pH **value**	**Clear zone (mm)**	**Weight loss (g)**	Depolymerase activity (U/ml)
4.5	40±2.0*	0.0094	0.61
5	50±3.0*	0.0117	0.81
5.4	19±2.0	0.0044	0.74
6	17±0.2	0.004	0.51
6.5	18±0.2	0.0042	0.52
7 (control)	15±2.3	0.0035	0.41
7.5	12±1.1	0.0028	0.42

*: significant results at $p < 0.05$ compared to control

Table 3 Effect of different incubation temperature on PHB degradation by the selected isolate *A. fumigatus* after growth in solid and liquid

	Solid medium		Liquid medium
Temperature (°C)	**Clear zone (mm)**	**Weight loss (g)**	Depolymerase activity (U/ml)
25	13±3.4*	00.003	0.42
30 (control)	50±2.1	0.0117	0.90
35	15±2.4*	0.0035	0.72
40	14±0.2*	0.0032	0.59
45	12±2.2*	0.0028	0.50

*: significant results at $p < 0.05$ compared to control

Table 4. Effect of different incubation period on PHB degradation by the selected isolate *A. fumigatus* after growth in solid and liquid

	Solid medium		Liquid medium
Incubation period (days)	Clear zone (mm)	Weight loss (g)	Depolymerase activity (U/ml)
1	17±0.7*	0.0040	0.22
3	20±1.9*	0.0047	1.17
4	43±1.6	0.0094	1.11
5	45±2.6	0.0102	0. 91
7 (control)	50±0.2	0.0117	0.89
8	50±1.2	0.0117	0.72

*: significant results at $p < 0.05$ compared to control

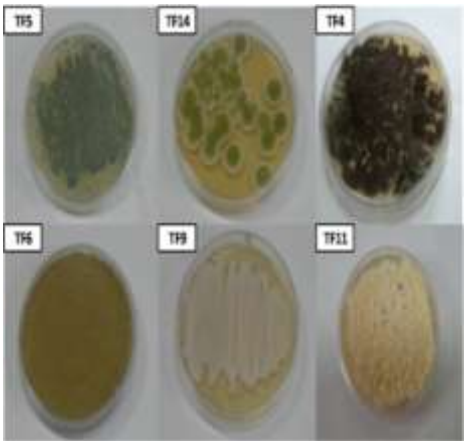

Figure 1. Growth and Color of PHB degrading fungal isolates on Sabouraud agar medium

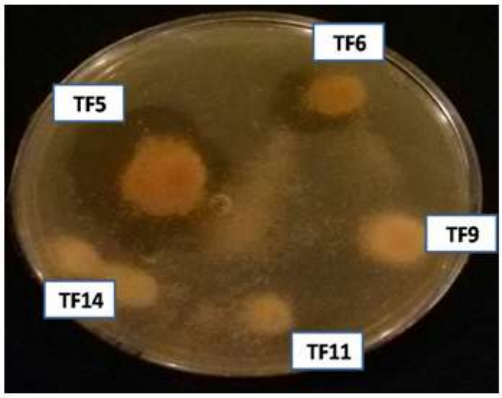

Figure 2. Growth and degradation of BHP by some fungal isolates on Mineral PHB degradation agar medium

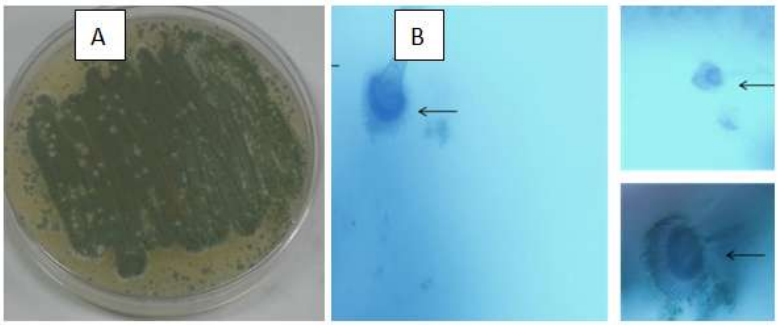

Figure 3. The selected fungal isolate TF5 on solid medium (A) and under light microscope (B).

Figure 4. Effect of different initial pH values on PHB degradation and depolymerase production by the selected isolate *A. fumigatus*.

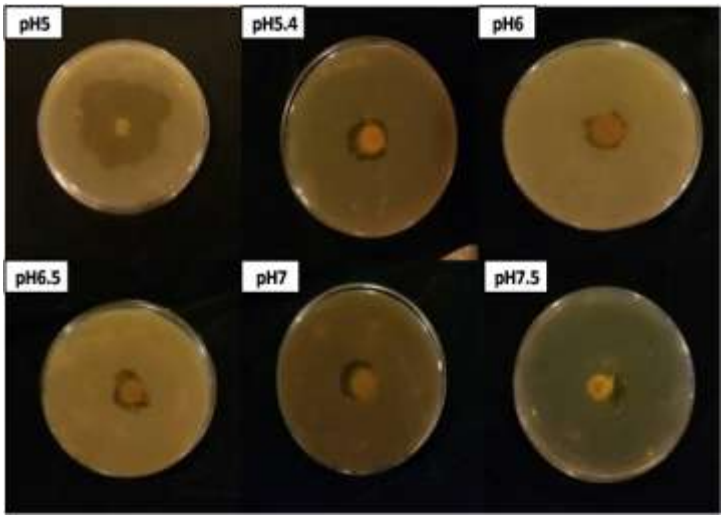

Figure 5. Effect of different initial pH values on PHB degradation by the selected isolate *A. fumigates* after week of growth in Petri dishes

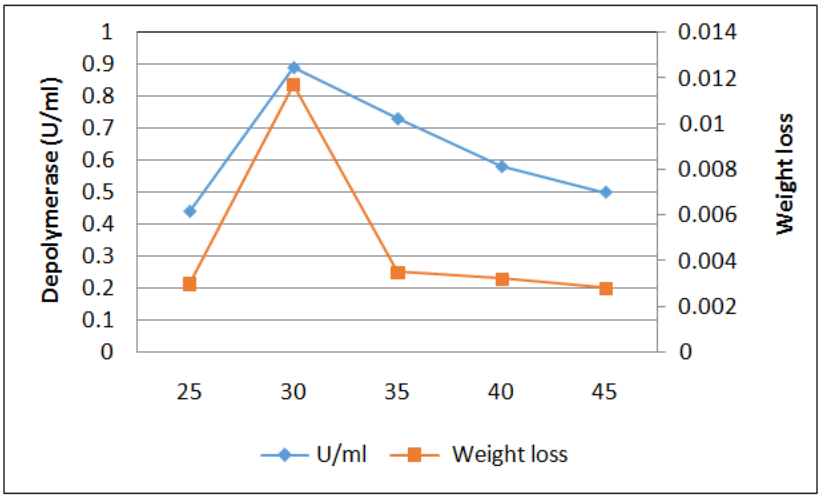

Figure 6. Effect of different incubation temperature on PHB degradation and depolymerase production by the selected isolate *A. fumigates*

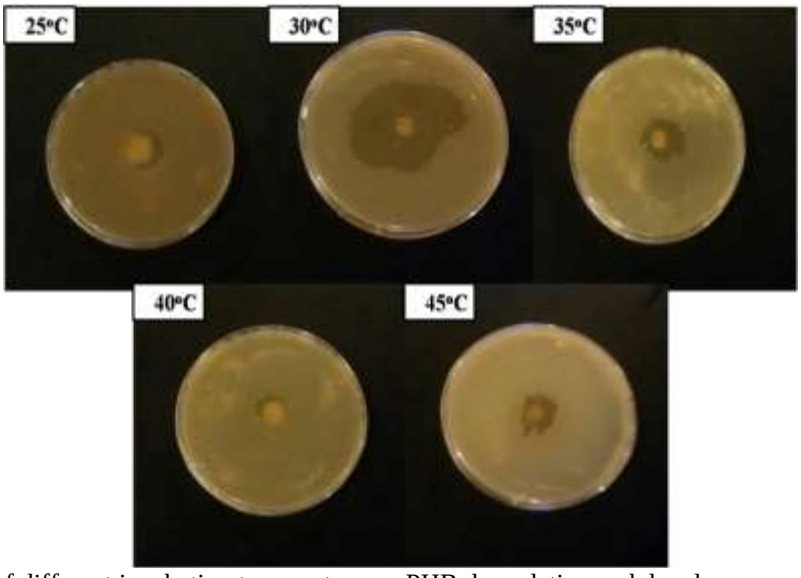

Figure 7. Effect of different incubation temperature on PHB degradation and depolymerase production by the selected isolate *A. fumigatus*

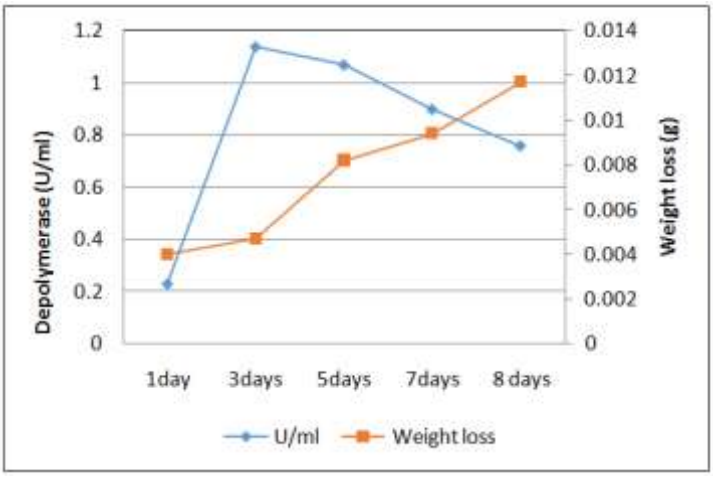

Figure 8. Effect of different incubation period on PHB degradation and by the selected isolate of *A. fumigatus* after different incubation periods

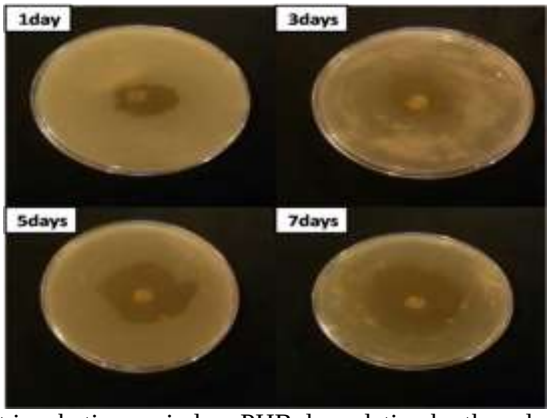

Figure 9. Effect of different incubation period on PHB degradation by the selected isolate of *A. fumigatus*.