Optimisation of growth conditions for maximum degradation of poly β-hydroxyl butrate by two soil fungi

Magda M. Aly*1, Fardos M. Bokhari1, Mona O. Albureikan1, Nuha M. Alhazmi2

1Department of Biology, Faculty of Science, King Abdulaziz University, Jeddah, Saudi Arabia
2Department of Biology, University of Jeddah, College of Science, Jeddah, Saudi Arabia

ABSTRACT

Poly β- hydroxyl butrate (PHB) is a polymer produced by bacteria and can be used safely in different modern application to replace biobased plastic which has adverse effects on the environments. This study is aimed to isolate some fungi for PHB degradation and optimise the growth conditions for maximum degradation. Contaminated soil samples were collected from the industrial area of Jeddah and used for fungal isolations. All fungal isolates were screened for PHB biodegradation on solid agar medium. Out of 20 isolates, 2 isolates were the most active in PHB degradation. They were identified as Trichoderma asperellum NM 6, and Aspergillus fumigates NM10 using morphological and molecular methods. The effects of some growth factors on growth and PHB degradation by the two isolated fungi were determined. Growth was measured by either CPV (ml) or dry weight (g/l) while PHB degradation was detected by depolymerase assay (U/ml). Presence of yeast extract (2.5 g/l) and glucose (1 g/l) enhanced both growth and PHB degradation by the two isolates. Similarly, adjusting medium pH at 7-7.5 and incubation at 25 °C after five days led to maximum growth and PHB degradation. The presence of PHB in growth medium enhanced both growth and PHB degradation and the maximum growth (CPV ml or dry weight (g/l) were in medium containing 0.5 g/l of PHB for both tested fungi. The maximum growth, measured by either CPV (ml) or dry weight (g/l), was in the medium that was inoculated with 10x10⁶ spore/ml for the two tested fungi. In conclusion, PHB degradation by the two tested fungi was similar. It was affected by the same physical and biochemical factors and optimisation of these conditions enhanced both growth and degradation process by the two tested fungi.

*Corresponding Author

Name: Magda M. Aly
Phone:
Email: mmmohammad@kau.edu.sa

INTRODUCTION

Recently in modern life, many used materials are from petro-based plastics which have various adverse severe effects on aquatic life and human environment and heath due to CO₂ releases, bis-phenol accumulation and ingestion of plastic (Gervet, 2007; Halden, 2010). The need of the time is to use biobased biodegradable plastics to maintain the health of the environment. Biodegradable plastic or environmentally-friendly alternative polymer from renewable resources is urged, and many microorganisms convert plastic polymers to monomers by an effective eco-friendly method. Poly β- hydroxyl butrate (PHB) are polymer produced...
by many genera of bacteria as reserved food, up to 75% of the cell dry weight. These polymers were extracted, characterised and used in many countries as Biobased plastic which is safe and eco-friendly materials. Biodegradation of these polymers has been investigated in soils, composts and natural waters by bacteria and fungi which contained PHB depolymerase (Mergaert et al., 1995, 1994) and rate of degradation depend on the properties of the polymers Oda et al. (1995); Sharma (2019). PHB depolymerase is responsible for extracellular PHB degradation by Aspergillus fumigatus and Penicillium simplicissimum (Jendrossek and Handrick, 2002). Recently, Stenotrophomonas sp. RZS7 was obtained from a dumping yard rich in plastic waste for PHB depolymerase production during growth. This enzyme was extracted and purified by ammonium salt precipitation, column chromatography and solvent purification (Sayyed et al., 2020). The aims of this study were isolation and identification of fungal species for the degradation of PHB and optimised the degradation conditions for maximum activities.

MATERIALS AND METHODS

Sample collection and fungal isolation and selection

Contaminated soil samples (20) from the industrial zone, Jeddah, Saudi Arabia were collected in sterile plastic bags, air-dried and sieved. Different fungal isolates were obtained on Sabouraud dextrose agar after growing for five days at 25°C, and the pure isolates were maintained on the same medium in the refrigerator at 4°C until used. All isolates were screened on mineral salt agar medium containing 50 ml of the basal Mineral salt PHB medium (pH, 7) which contained (g/l): PHB (0.5), KH2PO4 (0.7), K2HP04 (0.7), MgSO4 (0.7), Yeast extract (2.5), Glucose (1), NaCl (0.005), FeSO4 (0.002) and ZnSO4 (0.007). Each flask was inoculated with size 2 ml of freshly prepared spore suspension containing about 6x10⁷ spores/ml, and all flasks were incubated in shaking incubator at 25°C and 100 rpm for five days.

At the end of the growth period, the media was filtered through Whitman filter paper No. 1 and fungal mats were dried at 50°C for two days in an oven. The dry weight was determined as g/l. The growth was also measured after centrifugation at 4000 rpm for 20 minutes, and Cell packed volume (CPV/100 ml) was determined using special tubes (Agwa et al., 2000). PHB degradation by depolymerase enzyme was measured quantitatively in the culture filtrate by the decrease in the absorbance using the spectrophotometric method, according to the process of (Kobayashi et al., 1999). All experiments were made in triplicate, and averages were calculated.

Fungal growth in liquid medium

Spores suspension of the tested fungi was prepared by collecting the growth of 7 days old culture slants with 10 ml sterilised saline solution (0.9% NaCl) and shake vigorously for 1 min. The number of spores in ml was counted by a hemocytometer to adjust the count approximately to 6x10⁷ spores/ml. Two ml of the previous spore suspension was used to inoculate 50 ml of Sabouraud dextrose broth medium (preculture) and all flasks were incubated at 25°C for four days at 100 rpm. Every 2 ml of the preculture (6x10⁷ spores/ml) was made in triplicate, and averages were calculated.
spores/ml) was used to inoculate every 50 ml of Mineral salt broth medium containing PHB (0.5 g/l) as carbon source. All flasks were incubated in shaking incubator (100 rpm) at 25°C for five days. After growth and PHB degradation, the fungal growth was determined, and the fermentation medium was centrifuged at 7000 rpm for 15 min in a cooling centrifuge. The supernatant was used to measure the depolymerase activity.

Optimisation of conditions for PHB depolymerase production

Different cultures conditions for the maximum production of PHB depolymerase were optimised. All the experiments were performed in 250 ml Erlenmeyer flasks containing 50 ml of Mineral salt medium supplemented with 0.5 % PHB as a sole carbon source. All flasks were incubated at 100 rpm for five days.

Mineral salt medium with PHB as carbon source (pH 7) was used to determine the effect of different concentrations of yeast extract (0.5, 1.0, 1.5, 2.0) and 2.5 g/l or glucose (0.5, 1.0, 1.5, 2.0 and 2.5 g/l) on fungal growth and depolymerase activity. Similarly, the effects of different pH values (6, 6.5, 7.0, 7.5 and 8.0), incubation temperature (20, 25, 30, 35, 40 and 45°C), incubation period (3, 4, 5, 6, 7, 8, 9 and 10 days) and different concentrations of PHB (0.25, 0.5, 1.0, 1.5, 2.0 and 2.5 g/l) were determined at the end of growth period, growth and depolymerase activity were determined. Finally, the effect of different inoculum size on fungal growth and depolymerase production was studied by inoculating the flasks containing 50 ml of the mineral salt medium at pH 7.5 with 0.5% of PHB with increasing concentrations of fungal inoculum (4, 6, 8, 10 and 12 x10⁶ spore/ml). After incubation at 25°C for seven days, fungal growth and depolymerase production was detected as described before.

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 the mean ± standard deviation (Mean ± SD). The significance of the difference between samples was determined using t-test. The difference was regarded as significant when P<0.05 and non-significant when P>0.05. Additionally, the two-way ANOVA test was applied when necessary and significant differences were recorded at P<0.05.

RESULTS

Soil samples were collected from four contaminated areas around the plastic factories in the industrial zone in Jeddah, Saudi Arabia and different fungi were isolated on Sabouraud dextrose agar (SDA) using the dilution plate method. All plates were incubated at 25°C for seven days.

The frequency of each isolate was calculated. Taxonomic identification by the morphology of fungal isolates was mainly based on many identification keys. The percentage of isolation was ranged from 3.3 to 33.3 %. The highest frequencies were 10 and 8 times, respectively for the isolates NM1 and NM4. The lowest rate one time for the isolates NM6 and NM10, whereas the percentage of occurrence for the two fungal isolates were 3.3% only Table 1. All the isolates were screened on MSA medium containing 0.5% PHB with increasing concentrations of fungal inoculum (4, 6, 8, 10 and 12 x10⁶ spore/ml). After incubation at 25°C for seven days, fungal growth and depolymerase production was detected as described before.
Table 1: The isolated fungi from contaminated soil samples, frequency and percentage of isolation and Identification of the isolated fungal species and their growth on MSA medium

| Fungal isolate | Color                  | F  | Percentage (%) | Growth     | Clear zone diameter (mm) |
|----------------|------------------------|----|----------------|------------|--------------------------|
| NM 1           | Green                  | 10 | 33.3           | +          | 15                       |
| NM 2           | Brown Cinnamon         | 3  | 10.0           | ++         | 22                       |
| NM 3           | Light green            | 3  | 10.0           | ++         | 29                       |
| NM 4           | Black                  | 8  | 26.7           | ++         | 26                       |
| NM 5           | Dark green             | 1  | 3.3            | ++         | 40                       |
| NM 6           | Pale green             | 1  | 3.3            | +++        | 48                       |
| NM 7           | Green gray             | 1  | 3.3            | ++         | 20                       |
| NM 8           | Green                  | 1  | 3.3            | +          | 12                       |
| NM 9           | Brown                  | 1  | 3.3            | +          | 7                        |
| NM 10          | Bluish Green           | 1  | 3.3            | +++        | 42                       |

F: Frequency (no. of occurrence), +++: high growth, ++: moderate growth, +: little growth

Table 2: The growth and PHB degradation by the ten identified fungal isolates

| Fungal isolate | Fungi species                  | Growth (Dry weight, g/l) | PHB Degradation (U/ml) |
|----------------|--------------------------------|--------------------------|------------------------|
| NM 1           | Aspergillus versicolor         | 0.0267±0.013             | 0.069±0.006            |
| NM 2           | Aspergillus terrues            | 0.0311±0.021             | 0.067±0.008            |
| NM 3           | Aspergillus flavus             | 0.0344±0.010             | 0.025±0.007            |
| NM 4           | Aspergillus niger              | 0.0227±0.012             | 0.013±0.009            |
| NM 5           | Trichoderma koningii           | 0.0193±0.003             | 0.004±0.000            |
| NM 6           | Trichoderma asperellum         | 0.0558±0.004             | 0.1672±0.005           |
| NM 7           | Talaromycesbysschlamydoides    | 0.0309±0.009             | 0.033±0.005            |
| NM 8           | Trichoderma pseudokoningii     | 0.0387±0.009             | 0.038±0.004            |
| NM 9           | Rhizopus stolonifer            | 0.0221±0.005             | 0.035±0.004            |
| NM 10          | Aspergillus fumigates          | 0.0586±0.005             | 0.1995±0.001           |

p-value p-value
0.03* 0.0013*

LSD ANOVA

|                  | DF | SS    | MS    | F    | p-value |
|------------------|----|-------|-------|------|---------|
| Between groups   | 3  | 419.2 | 182.21| 6.45 | 0.0013  |
| Within groups (Error) | 36 | 959.58| 26.65 |      |         |
| Total            | 39 | 1378.78|      |      |         |

*:Significant Results

Figures 1 and 2. The lowest growth and the smallest clear zone were recorded for NM8 and NM9.

After isolation, thirty fungal isolates were obtained, and they belonged to ten species. They were identified as Aspergillus Versicolor, Aspergillus terrues, Aspergillus flavus, Aspergillus niger, Trichoderma koningii, Trichoderma asperellum, Talaromyces bysschlamydoides, Trichoderma pseudokoningii, Rhizopus stolonifer and Aspergillus fumigates. The ten fungal isolates were grown in MSB medium containing 0.5% PHB as carbon source. All isolates grow well in the previous medium but with different degrees. It was found that the isolates T. asperellum and A. fumigates grow strongly after hydrolysing PHB by producing depolymerase enzyme.

The enzyme concentrations for the two fungal isolates T. asperellum and A. fumigates were 0.1672 and 0.1995 U/ml, respectively. The lowest growth and PHB degradation were recorded for Trichoderma koningii. The results of the growth and PHB degra-
Figure 3: *Trichoderma asperellum* (A) and *Aspergillus fumigates* (B) morphology under scanning electron microscope.

Figure 4: The phylogenetic tree of *Trichoderma asperellum* NM6 based on ITS region.
Figure 5: The phylogenetic tree of *Aspergillus fumigates* NM10 based on ITS region.

dation by the ten fungal isolates in Mineral salt broth medium were shown in Table 2. In broth medium, *T. asperellum* and *A. fumigates* showed the best growth and depolymerase production. Thus, the two isolates were selected for details studies and examined under the scanning electron microscope Figure 3.

After examination of the selected fungus, *Trichoderma asperellum* under the scanning electron microscope, the hyphae and spores were characterised. It belongs to the phylogenetically *Trichoderma* section and similar to *T. asperellum* which frequently isolated from soil. *Trichoderma* growth was high on agar and broth medium. Morphological observations showed that after five days of culture, the front of the novel isolate’s colony was dark green (spore colour), and the back was white (mycelium colour).

The morphology of the mycelium was coarse, and dark green spores began to form in the centre of the colony at high rates. Based on preliminary morphological observations, the strain was confirmed to be *Trichoderma*. Cultures are typically fast-growing at 25°C, colonies are white at first on medium and change to dark green. Mycelium and conidia usually form within one week in compact or loose tufts in shades of green or yellow or less frequently white. A yellow pigment may be secreted into the growth medium. Conidiophores are highly branched and thus difficult to define or measure, loosely or compactly tufted, often formed in distinct concentric rings or borne along the scant aerial hyphae. Typically, the conidiophore terminates in one or a few phialides. Phialides generally are enlarged in the middle but may be cylindrical or nearly subglobose. Phialides may be held in whorls, at an angle of 90° concerning other members of the whorl, or they may be variously penicillate. Conidia typically appear dry but, in some species, they may be held in drops of transparent green or yellow liquid. Conidia are smooth and ellipsoidal, 3-5x 2-4 μm Figure 3A

After examination of the selected fungus, *Aspergillus fumigates* under the scanning electron microscope, the hyphae and spores were characterised. *A. fumigatus*, a saprotroph widespread in nature, is typically found in soil. The fungus produces from conidiophores which carry thousands of minute grey-green conidia (2-3 μm) that readily become airborne. The fungus is capable of growth at 25°C and can grow at temperatures up to 50°C, with conidia surviving at 70°C. Its spores are ubiquitous in the atmosphere, and it is estimated that everybody inhales several hundred spores each day. Colonies granular to cottony, velvety, and powdery, usually white at first then darkening to green, green-grey, or green-brown with a white apron at the margin. The
Table 3: Effect of different concentrations of yeast extract on *Trichoderma asperellum* and *Aspergillus fumigates* growth and PHB degradation

| Yeast extract Conc. (g/l) | *Trichoderma asperellum* (mean ±SD) | *Aspergillus fumigates* (mean ±SD) |
|--------------------------|------------------------------------|------------------------------------|
|                          | Trichoderma asperellum | Aspergillus fumigates |
|                          | Growth (Dry weight, g/l) | PHB degradation | Growth (Dry weight, g/l) | PHB degradation |
| 0.0 (Control)            | 2.50 ±0.01 | 0.07 ±0.055 | 2.50 ±0.02 | 0.07 ±0.001 |
| 0.5                      | 3.75 ±0.006 | 0.08 ±0.005 | 3.50 ±0.03 | 0.08 ±0.010 |
| 1.0                      | 5.00 ±0.009 | 0.07 ±0.003 | 4.50 ±0.02 | 0.09 ±0.005 |
| 1.5                      | 5.25 ±0.004 | 0.09 ±0.004 | 4.00 ±0.046 | 0.07 ±0.025 |
| 2.0                      | 6.25 ±0.004 | 0.10 ±0.020 | 4.00 ±0.05 | 0.11 ±0.005 |
| 2.5                      | 6.75 ±0.005 | 0.10 ±0.090 | 5.00 ±0.005 | 0.15 ±0.005 |

Significant results at p< 0.5 and CPV: Cell packed volume

Figure 6: Effect of different concentrations of yeast extract on *T. asperellum* and *A. fumigates* growth (dry wt., g/l) and PHB degradation (U/ml)

Table 4: Effect of different concentrations of glucose on *Trichoderma asperellum* and *Aspergillus fumigates* growth and PHB degradation

| Glucose concentrations (g/l) | *Trichoderma asperellum* (mean ±SD) | *Aspergillus fumigates* (mean ±SD) |
|-----------------------------|------------------------------------|------------------------------------|
|                            | Trichoderma asperellum | Aspergillus fumigates |
|                            | Growth (Dry weight) | PHB degradation | Growth (Dry weight) | PHB degradation |
| 0.0 (Control)              | 1.75 ±0.009 | 0.07 ±0.000 | 2.75 ±0.002 | 0.07 ±0.004 |
| 0.5                        | 2.75 ±0.008* | 0.10 ±0.003* | 3.50 ±0.01 | 0.06 ±0.004 |
| 1.0                        | 13.00 ±0.006* | 0.20 ±0.008* | 8.00 ±0.19 | 0.13 ±0.005* |
| 1.5                        | 4.50 ±0.007* | 0.12 ±0.006* | 7.00 ±0.02 | 0.02 ±0.004* |
| 2.0                        | 2.00 ±0.002* | 0.12 ±0.006* | 7.00 ±0.04 | 0.01 ±0.001* |
| 2.5                        | 1.50 ±0.007* | 0.13 ±0.008* | 5.00 ±0.04 | 0.03 ±0.007* |

Significant results at p< 0.5, CPV: Cell packed volume
Figure 7: Effect of different concentrations of glucose on *T. asperellum* and *A. fumigates* growth (dry wt., g/l) and PHB degradation (U/ml)

Reverse is generally white to tan. Conidiophores are long (300-500 µm) and have club-shaped vesicles that are 30-50 µm in diameter. Vesicles are uniseri-ate and are covered by phialides-conidia on only the distal half. Conidia arise in chains and tend to sweep toward the central axis Figure 3B.

The phylogenetic tree of *Trichoderma asperellum* and *Aspergillus fumigates* based on ITS region was showed in Figures 4 and 5.

*Trichoderma asperellum* and *A. fumigates* were the most active isolates for PHB degradation. Thus they were selected for more detail studied. Each fungal isolate was grown in medium containing PHB as a carbon source, and at the end of the incubation period, growth and PHB degradation were measured.

*Trichoderma asperellum* and *A. fumigates* were grown in medium containing PHB as a carbon source and different concentration of yeast extracts, 0.5 to 2.5 g/l. After five days, the growth and PHB degradation were measured. From the results, it was clear that the presence of yeast extract in the growth medium enhanced both growth and PHB degradation. Maximum growth measured by either CPV (ml) or dry weight (g/l) were in medium containing 2.5 g/l of yeast extract for both tested fungi. Similarly, the highest PHB degradation, measured by enzyme activity (U/ml) for the two tested fungal isolates, was obtained in MSB medium containing 1.0 g/l of glucose. The lowest growth and PHB degradation were observed at control (0.0% yeast extract) for both *T. asperellum*, and *A. fumigates* (Table 3 and Figure 6).

Both *T. asperellum* and *A. fumigates* were grown in medium containing PHB as a carbon source in addition to different concentration of glucose, 0.5 to 2.5 g/l to enhance the growth. After five days, the growth and PHB degradation were measured for each fungal isolate. From the results, it was clear that presence of glucose in growth medium enhanced both growth and PHB degradation and the maximum growth measured by either CPV (ml) or dry weight (g/l) were in medium containing 1.0 g/l glucose for both tested fungi. Similarly, the highest PHB degradation, measured by enzyme activity (U/ml) for the two tested fungal isolates, was obtained in MSB medium containing 1.0 g/l of glucose. The lowest growth and PHB degradation were observed at control (0.0% glucose) for both *T. asperellum*, and *A. fumigates* (Table 4 and Figure 7).

Similarly, *T. asperellum* and *A. fumigates* were grown in medium containing PHB as a carbon source in addition to 2.5 g/l yeast extracts and 1.0 g/l glucose. The medium was prepared at different pH values, pH 6.0- pH 8.0. The inoculated flasks were incubated at 25°C for five days, and growth and PHB degradation were measured. From the results, it was clear that adjusting pH of the medium at 7.5 enhanced growth, measured by either CPV (ml) or dry weight (g/l) and PHB degradation (U/ml). Similarly, the highest PHB degradation, measured by enzyme activity for the two tested fungal isolates were at pH 7.5. The lowest growth and PHB degradation were observed at pH 6.0 for *T. asperellum*, and pH 8.0 for *A. fumigates* (Table 5 and Figure 8).

Concerning the effect of incubation temperature, *T. asperellum* and *A. fumigates* were grown in medium containing PHB as a carbon source in addition to 2.5 g/l of yeast extracts and 1 g/l glucose. After five days of incubation at different temperatures, 20 to 45°C, the growth and PHB degradation were measured. From the results, it was clear that increasing temperature enhanced both growth and PHB degra-
Table 5: Effect of different pH on *Trichoderma asperellum* and *Aspergillus fumigates* growth and PHB degradation

| pH value | *Trichoderma asperellum* (mean ±SD) | *Aspergillus fumigates* (mean ±SD) |
|----------|-----------------------------------|-----------------------------------|
|          | CPV Growth (Dry weight) PHB degradation | CPV Growth (Dry weight) PHB degradation |
| 6.0      | 7.00 0.08 ±0.002* 0.05 ±0.001* | 9.50 0.06±0.001* 0.23 ±0.003 |
| 6.5      | 7.00 0.09 ±0.007* 0.09 ±0.009 | 6.50 0.05±0.001* 0.24 ±0.003 |
| 7.0      | 6.50 0.11 ±0.009 0.12±0.004 | 6.00 0.08±0.003 0.22 ±0.005 |
| (control)| | 7.50 0.15±0.005* 0.24±0.008 |
| 7.5      | 8.50 0.04 ±0.00* 0.14 ±0.001* | 7.50 0.05±0.001* 0.14 ±0.002* |
| 8.0      | 8.00 0.07 ±0.005* 0.14 ±0.001* | | |

Significant results at p < 0.5, CPV: Cell packed volume

Figure 8: Effect of different pH value on *T. asperellum* and *A. fumigates* growth and PHB degradation (U/ml)

Table 6: Effect of different incubation temperature on *Trichoderma asperellum* and *Aspergillus fumigates* growth and PHB degradation

| Incubation temperature OC | *Trichoderma asperellum* (mean ±SD) | *Aspergillus fumigates* (mean ±SD) |
|---------------------------|-----------------------------------|-----------------------------------|
|                           | CPV Growth (Dry weight) PHB degradation | CPV Growth (Dry weight) PHB degradation |
| 20                        | 8.50 0.09±0.009* 0.144±0.009* | 10.00 0.08±0.005* 0.15 ±0.005* |
| 25(control)               | 12.00 0.19 ±0.05 0.30 ±0.05 | 10.00 0.12±0.00 0.41±0.008 |
| 30                        | 10.00 0.12 ±0.08* 0.29 ±0.05 | 7.00 0.12±0.09 0.34 ±0.007* |
| 35                        | 7.50 0.11 ±0.08* 0.25 ±0.07 | 7.50 0.08±0.065* 0.35 ±0.001* |
| 40                        | 7.00 0.08 ±0.05* 0.16 ±0.09* | 6.00 0.07±0.009* 0.20 ±0.000* |
| 45                        | 5.00 0.08 ±0.01* 0.13 ±0.01* | 5.50 0.07±0.009* 0.19 ±0.003* |

Significant results at p < 0.5, CPV: Cell packed volume
Figure 9: Effect of different incubation temperature on *T. asperellum* and *A. fumigates* growth and PHB degradation (U/ml)

Table 7: Effect of incubation period on *Trichoderma asperellum* and *Aspergillus fumigates* growth and PHB degradation

| Incubation periods (days) | *Trichoderma asperellum* (mean ±SD) | *Aspergillus fumigates* (mean ±SD) |
|--------------------------|--------------------------------------|-----------------------------------|
|                          | CPV Growth (Dry weight, g/l) | PHB degradation     | CPV Growth (Dry weight, g/l) | PHB degradation     |
| 3                        | 10.00 ±0.11| 0.15±0.01 | 10.00 ±0.10| 0.10±0.09 | 0.22 ±0.55* |
| 4                        | 8.50 ±0.11| 0.15±0.02 | 10.50 ±0.10| 0.10±0.01 | 0.22 ±0.04* |
| 5 (control)              | 11.00 ±0.11| 0.15±0.03 | 11.00 ±0.11| 0.11±0.00 | 0.27 ±0.04 |
| 6                        | 8.50 ±0.12| 0.37±0.03* | 7.50 ±0.11| 0.11±0.03 | 0.40 ±0.04* |
| 7                        | 12.50 ±0.15| 0.47±0.04* | 13.00 ±0.16| 0.09±0.09* | 0.55 ±0.01 |
| 8                        | 8.00 ±0.10| 0.21±0.01* | 9.50 ±0.12| 0.01±0.01* | 0.55 ±0.00* |
| 9                        | 10.00 ±0.10| 0.17±0.00* | 10.00 ±0.08| 0.05±0.05* | 0.23 ±0.05 |
| 10                       | 7.00 ±0.08| 0.08±0.05* | 9.00 ±0.07| 0.05±0.05* | 0.19 ±0.00* |

Significant results at p < 0.05 and CPV: Cell packed volume

Figure 10: Effect of incubation periods on *T. asperellum* and *A. fumigates* growth and PHB degradation (U/ml)
Table 8: Effect of different concentrations PHB on *Trichoderma asperellum* and *Aspergillus fumigates* growth and PHB degradation

| PHB concentration (g/l) | *Trichoderma asperellum* (mean ±SD) | *Aspergillus fumigates* (mean ±SD) |
|-------------------------|-------------------------------------|------------------------------------|
|                         | CPV Growth (Dry weight, g/l)        | CPV Growth (Dry weight, g/l)       |
|                         | PHB degradation                      | PHB degradation                     |
| 0.25                    | 11.50 ± 0.11 ± 0.005                | 0.31 ± 0.55                         | 6.50 ± 0.9 ± 0.05 | 0.37 ± 0.55 |
| 0.5 (control)           | 12.00 ± 0.11 ± 0.005                | 0.33 ± 0.07                         | 9.50 ± 0.11 ± 0.00 | 0.40 ± 0.008 |
| 1.0                     | 8.50 ± 0.10 ± 0.067                 | 0.31 ± 0.01                         | 6.50 ± 0.10 ± 0.07 | 0.38 ± 0.005 |
| 1.5                     | 8.00 ± 0.09 ± 0.014                 | 0.30 ± 0.08                         | 9.00 ± 0.10 ± 0.04 | 0.39 ± 0.006 |
| 2.0                     | 8.00 ± 0.10 ± 0.007                 | 0.32 ± 0.05                         | 7.00 ± 0.08 ± 0.007 | 0.39 ± 0.007 |

Significant results at p < 0.5, CPV: Cell packed volume

Figure 11: Effect of different concentrations PHB on *T. asperellum* and *A. fumigates* growth and PHB degradation (U/ml)

dation up 25°C, then the growth and PHB degradation were decreased by increasing incubation temperatures. From the results, maximum growth and PHB degradation for both tested fungi, *T. asperellum* and *A. fumigate* were recorded at 25°C after five days of incubation. In contrast, the lowest growth and PHB degradations were at incubation temperature 20 and 45°C for both fungal isolates (Table 6 and Figure 9).

Also, *T. asperellum* and *A. fumigates* were grown in medium containing PHB as a carbon source, yeast extracts and glucose. All flasks were incubated at 25°C for 3, 4, 5, 6, 7, 8, 9 and 10 days. At the end of the growth period, growth and PHB degradation were measured. From the results, it was clear that incubation at seven days enhanced both growth and PHB degradation and the maximum growth measured by either CPV (ml) or dry weight (g/l) were in broth medium after seven days of growth at 25°C for both tested fungi, *T. asperellum* and *A. fumigates*. Similarly, the highest PHB degradation, measured by enzyme activity (U/ml) for the two tested fungal isolates, was obtained in MSB medium after seven days of incubations. The lowest growth and PHB degradation were observed after ten days for both *T. asperellum*, and *A. fumigates* (Table 7 and Figure 10).

The growth and PHB degradation of *T. asperellum* and *A. fumigates* were measured after grown in medium containing different concentrations of PHB as a carbon source in addition to 2.5 g/l yeast extracts and 1 g/l glucose. After five days.

From the results, it was clear that presence of PHB in growth medium enhanced both growth and PHB degradation and the maximum growth measured by either CPV (ml) or dry weight (g/l) were in medium containing 0.5 g/l of PHB for both tested
Table 9: Effect of different inoculum size on *Trichoderma asperellum* and *Aspergillus fumigates* growth and PHB degradation

| Inoculum size (x10^6 spore/ml) | *Trichoderma asperellum* (mean ±SD) | *Aspergillus fumigates* (mean ±SD) |
|-------------------------------|-------------------------------------|-----------------------------------|
|                              | CPV | Growth (Dry weight, g/l) | PHB degradation | CPV | Growth (Dry weight, g/l) | PHB degradation |
| 2                             | 8.00 | 0.10 | 0.32 | 11.00 | 0.06 | 0.39 |
| 4                             | 11.50 | 0.10 | 0.32 | 12.00 | 0.06 | 0.39 |
| 6                             | 11.50 | 0.11 | 0.35 | 14.50 | 0.05 | 0.40 |
| 8                             | 14.00 | 0.11 | 0.35 | 17.50 | 0.05 | 0.43 |
| 10                            | 8.50 | 0.16 | 0.34 | 16.00 | 0.08 | 0.46 |

Significant results at p < 0.5

Figure 12: Effect of different inoculum size on *T. asperellum* and *A. fumigates* growth and PHB degradation (U/ml)

fungi, *T. asperellum* and *A. fumigates*. Similarly, the highest PHB degradation, measured by enzyme activity (U/ml) for the two tested fungal isolates, was obtained in MSB medium containing 0.5 g/l of PHB. Increasing the PHB concentrations showed no apparent effect on both growth and PHB degradation. The lowest growth and PHB degradation were observed at 2.0 g/l of PHB as a carbon source for *T. asperellum,* and *A. fumigates* (Table 8 and Figure 11).

Finally, *T. asperellum* and *A. fumigates* were grown in medium containing PHB as a carbon source. Different inoculum size was used, (4-12) x10^6 spore/ml. After five days, the growth and PHB degradation were measured. Increasing inoculum size, increased growth and PHB degradation and the maximum growth measured by either CPV (ml) or dry weight (g/l) were in medium inoculated with 10x10^6 spore/ml for both tested fungi, *T. asperellum* and *A. fumigates*. Similarly, the highest PHB degradation, measured by enzyme activity (U/ml) for the two tested fungal isolates, was obtained in MSB medium inoculated with 10x10^6 spore/ml (Table 9 and Figure 12).

Independent variables are yeast extract concentrations, Glucose concentrations, pH, Incubation temperature, Incubation periods, PHB concentrations and inoculum size. The dependent variable is PHP degradation. To investigate the effect of different factors on PHB degradation, two-way ANOVA test was applied. The result shown in Table 10 illustrated the significant values of the tested element and PHB degradation by the two established fungal genera.

**DISCUSSION**

The degradation of biopolymers helps to overcome some of the pollution problems associated with the use of petroleum polymers. PHB, a promising compound for making biodegradable plastics, has been investigated for its degradation in many terrestrial
Table 10: Statistical analysis of the different tested factors on PHB degradation using two-way ANOVA test

| Source                        | Sum of Squares | Df (n-1) | Mean Square | F      | P value | Sig. |
|-------------------------------|----------------|----------|-------------|--------|---------|------|
| Yeast extract                 | 344574.11      | 5        | 544574.511  | 23.733 | 0.017   | Sig. |
| Glucose                       | 44414.656      | 5        | 74804.885   | 11.020 | 0.049   | Sig. |
| pH                            | 43556.506      | 4        | 64518.835   | 43.700 | 0.017   | Sig. |
| Incubation temp.              | 797929.935     | 5        | 893246.93   | 51.011 | 0.034   | Sig. |
| Incubation periods            | 2226774.51     | 7        | 319633.66   | 37.571 | 0.011   | Sig. |
| PHB concentrations            | 34714.65       | 4        | 726845.44   | 6.044  | 0.05    | Non sig. |
| Inoculum size                 | 556.506        | 5        | 14518.835   | 3.733  | 0.517   | Non sig. |

Aspergillus fumigates

| Source                        | Sum of Squares | Df (n-1) | Mean Square | F      | P value | Sig. |
|-------------------------------|----------------|----------|-------------|--------|---------|------|
| Yeast extract                 | 57346.98       | 5        | 599999.919  | 21.444 | 0.022   | Sig. |
| Glucose                       | 56891.56       | 5        | 977591.885  | 12.066 | 0.040   | Sig. |
| pH                            | 14559.20       | 4        | 766156.835  | 13.144 | 0.034   | Sig. |
| Incubation temp.              | 79777.90       | 5        | 876999.93   | 31.088 | 0.031   | Sig. |
| Incubation periods            | 67594.59       | 7        | 983021.66   | 37.333 | 0.029   | Sig. |
| PHB concentrations            | 34714.65       | 4        | 726845.44   | 3.224  | 0.048   | Non sig. |
| Inoculum size                 | 891.506        | 5        | 334679.135  | 6.733  | 0.0917  | Non sig. |

and aquatic environments (Jendrossek and Handrick, 2002). In this study, different fungi were obtained from soil samples on SDA medium. The identification was carried out according to Raper (1950); Pitt (1979) for Penicillium, Guchi et al. (2014) for Aspergillus, Ellis (1971, 1976) for dematiaceous hyphomycetes, Booth (1971) for Fusarium, Cannon (1986) for Chaetomium. Mycologists have traditionally used morphology as a sole means of identifying fungal species (Hyde et al., 2011), and even nowadays it is still the potential means of species identification within the mycological community (Lutzoni et al., 2004; Wang et al., 2016) and many problems of using morphology alone in the identification of fungi at the species level exist. Thus, the newly emerged DNA sequence-based methods have shown higher potentiality for identifying species within the megadiverse fungi (Hibbett and Taylor, 2013; Hibbett et al., 2016, 2011). Therefore, molecular techniques were used to confirm the classification. Many different PHB biodegrading bacteria and fungi, including members of Bacillus, Streptomyces, Aspergillus, Penicillium, Acidovorax, and Variovorax have been isolated from soil (Mergaert et al., 1994). Because of their ability to biodegrade extracellular PHB, these microorganisms have the potential to become useful for industrial applications. Polyhydroxy alkanoic acids or bioplastic, among different types of biodegradable plastics, have been extensively studied, because of their similarity to conventional plastics, complete biodegradability and current market domination (Verlinden et al., 2007).

The biodegradation of plastics proceeds actively under different conditions according to microbe properties, and each microbe has its optimal growth conditions (Lucas et al., 2008). All fungal isolates were screened for depolymerase production on solid Turbid medium containing 0.5% PHB as inducer. As it is well known, the depolymerase enzyme was inducible, and the presence of PHB as an inducer in the medium is necessary for enzyme formation (Aly et al., 2015). Induction and expression are subjected to a complex regulation and depolymerase enzyme is not required for balanced fungal growth and may be synthesised in response to energy or nutrient limitation. In this work, all the tested fungi (100% of the tested fungi) grow on medium containing PHB as carbon source and are producer for depolymerase. At the same time, Aly et al. (2015) reported that 50% of the screened actinobacteria were depolymerase producing, and this activity was detected as clear zones accompanying the growth in solid agar medium.

Similarly, 31(48%) bacterial strains out of 67 showed PHB degradation, detected as a clear zone on assay medium (Lee et al., 2005) found that among 16 fungal isolates, 8 (50%) gave clear area 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 family Penicillium and one to the genus Alternaria.

Depolymerase enzyme detection was obtained using plate-clearing technique and/or measuring the enzyme activity in a liquid medium. Plate-clearing technique was a straightforward method to detect polymer degradations in the agar medium and used to identify the PHB degradation by A. fumigates as a clear halo zone around the fungal colonies. This method is usually applied to screen microorganisms that can biodegrade a specific polymer on solid medium (Augusta et al., 1993). For fungi, growth on agar medium is generally more comfortable than in broth medium (Aly et al., 2017). Thus, no further assessment was conducted for isolates that showed weakly activity on solid agar medium containing PHB as a carbon source. The weak or lack of action in some isolates may be due to a loss of trait in the isolate, or the used culture conditions was not effective in inducing the depolymerase enzyme. Out of 30 fungal isolates, two new isolates were proven to biodegrade poly-β-hydroxybutyrate (PHB).

Enzyme assay for these isolates indicated the optimal environmental conditions required for depolymerase enzyme to induce the highest level of biopolymer degradation. The depolymerase enzymes obtained by fungi are extracellular, whereas the enzymes from bacteria are either extracellular or intracellular. The extracellular depolymerase production of the isolate NM6 and NM10 were selected for more detail studies.

Among 30 fungal isolates recovered, two new isolates were proven to biodegrade PHB. The two isolates were characterised at the morphological level as T. asperellum NM6, and A. fumigates NM 10. Enzyme assay for these isolates indicated the optimal environmental conditions required for depolymerase enzyme to induce the highest level of biopolymer degradation. The two isolates were characterised at the morphological level as T. asperellum NM6, and A. fumigates NM 10 using standard approaches of the molecular level based on the highly diverged rDNA gene. Within this gene, two regions of the large ribosome subunit (LSU) namely internal transcribed spacer (ITS) and 25S-28S were utilised in the analysis. The first region comprises the ITS1/5.8S/ITS2 domains (>500 bp), and Sanger sequencing was conducted for the two isolates using the forward and reverse primers ITS1 and ITS4, respectively, for the ITS region. Multiple sequence alignment indicated 99% identities of the first domain of S1 isolate with T. asperellum isolates XP22. BLAST of the primary domain of NM 10 isolates showed 100% identities with A. fumigatus isolate YNCA0338 (ID: KP068684.1), while 99% of identities with A. fumigatus isolate CCA101 (ID: KT877346.1). BLAST of the second domain of isolate NM 6 indicated 99 and 100% identities with only two strains of T. asperellum namely TR 3 (ID: HM466685.1) and G (ID: KF723005.1), respectively.

The knowledge of the participation and role of fungi in the hydrolysis of PHB are minimal and need to be elucidated in further studies. This study is essential in isolation of some fungi which produce excellent depolymerase enzyme in the culture supernatant after growth on PHB as a carbon source. Although this enzyme has a wide distribution in fungi and some algal genera, bacterial depolymerase has considerable potential in commercial applications due to substrate specificity, resistance to proteolysis and catalytic efficiency (Choi et al., 2001; Shah et al., 2008). Extracellular PHB depolymerase has been isolated from different soil and wastewater in the plastic factories in the industrial zone in Jeddah, Saudi Arabia purified and characterised as T. asperellum NM6, and Aspergillus fumigates NM 10. Temperature is one of the most critical parameters to be controlled in any bioprocess. Several mesophilic microbes are responsible for biodegrading PHB in soil, and aquatic environments, and many the thermotolerant strains are capable of biodegrading PHB at high temperatures ≥ 40°C from soil and compost (Kim, 2000). Therefore, the current study is performed for the isolation of PHB biodegrading fungi which produce thermostable depolymerase enzyme. Maximum depolymerase production by fungi was at 25°C while Bacillus strain TT96 (Tansengco and Tokiwa, 1998) and Streptomyces strain MG (Tokiwa and Galabia, 2004) were capable of degradation at higher temperatures. Aspergillus fumigates was able to biodegrade PHB better at 45°C after 24 h of incubation in a liquid medium and little information on microbial degradation of PHB at high temperatures was available. Maximum degradation was after five days, and there was a gradual decrease in the production of the enzyme after that. As it is well known, degradation in liquid medium was affected with time. On the contrast, (Papaneophytou et al., 2009) reported maximum enzyme production by Thermus thermophilus HB8 after 24 h of incubation. The gradual decrease in the creation of the enzyme after five days was as a result of the utilisation of substrate and other nutrients (Scherer et al., 1999; Mishra et al., 2009). Moreover, maximum enzyme production by fungi was using Turbid medium at pH 7.5 while Aly et al.
Degradation increased with increasing period of incubation (Mabrouk and Sabry, 2001; García-Hidalgo et al., 2013). Previous researchers showed that the percentage for PHB was raised as the incubation time of degradation increases in soil (Altæe et al., 2016). Decreasing the level of glucose or PHB increased the degradation efficiency of PHB. The synthesis of PHB depolymerase seemed to be highly regulated. The presence of a low concentration of soluble carbon sources as glucose in addition to the polymer in the medium, this increased PHB depolymerase specific activity, indicating that PHB depolymerase expression is repressed in the presence of a high level of soluble carbon source (Mabrouk and Sabry, 2001). Our results are supported by other researchers demonstrating that all other known PHB bacterial depolymerase, is repressed in the presence of a soluble carbon source (Jendrossek et al., 1995). Our results are those reported for Pseudomonas lemoignei, (Steinbüchel and Hein, 2001). This result is in good agreement with a previous investigation which demonstrated the importance of carbon sources in the growth medium for enzyme production as the rate of polymer degradation was influenced by the degree and availability of secondary carbon and by the primary carbon source (Shivakumar, 2013; Wang et al., 2016).

A novel poly-b-hydroxybutyrate (PHB) biodegrading bacterium Stenotrophomonas sp. RZS 7 was isolated from soil samples of contaminated plastic sites of the municipal area in Shahada, India and grow well in minimal salt medium containing PHB as the only source of the nutrient. The optimum yield of the enzyme was obtained on the of 5 days of incubation at 37°C and pH 6.0 (Wani et al., 2016).

CONCLUSION

Plastic causes many soil fungi problems and had a terrible impact on human health. Bacteria mainly produce PHB polymers, but excellent degradation was obtained fungi. In this study, two fungal isolates were characterised and identified as T. asperellum, and A. fumigates. Enhancing the degradation process was carried out by optimisation of different growth conditions. Effect of various growth factors, glucose, yeast extract and PHB concentrations, temperature, pH, time and inoculum size were studied. Microbial degradation of PHB was carried out by secretion of depolymerase enzyme. Many considered were needed for detecting the characters of depolymerase enzymes which have the leading roles in PHB degradation.

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Conflict of Interest

The authors have no conflicts of interest to declare.

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