Degradation of poly (3-hydroxybuthyrate) using *Aspergillus oryzae* obtained from uncultivated soil

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Abstract: The thermoplastic polyester, PHB (poly-3-hydroxybutyric acid) is a synthesized by some bacterial genera including *Azotobacter* and *Bacillus* as a form of intracellular storage material for carbon and energy. PHB accumulated as inclusion granules in the cytoplasm of the previous bacterial genera. PHB degradation by soil fungi collected from various sites was studied. PHB depolymerization was tested in vials containing a PHB-containing medium which were inoculated with isolates from the tested microbes. The degradation activity was detected by the formation of a clear zone below and around the fungal colony. Out of 20 fungal isolates, 11 showed PHB degradation. Most of these fungi were belonging to the deuteromycetes including species of the genera *Penicillium, Aspergillus, Fusarium, Alterneria* and*, Trichoderma. Aspergillus fumigates* and *A. Oryzae* were the most active PHB degrader isolates. For degradation of PHB using *A. Oryzae*, the best temperature was 30^{o}C at pH 6.5 after 5 days of growth in minimal medium containing 1% PHB. In conclusion, PHB or bioplastic can produced mainly from bacteria but fungi can be used successfully in PHB degradation.
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1. Introduction

Polyhydroxyalkanotes (PHAs) are insoluble polyesters of hydroxyalkanoic acids and among the various PHA polymers, poly-(β)-hydroxybutyrate (PHB) is the well studied and characterized polymer. Polyhydroxy butrate, a homopolymer of β-hydroxybutyrate and is insoluble polyesters of hydroxybutric acids which is synthesized inside the cells of many bacterial genera as inclusion bodies and serve as carbon and energy reserves. PHB are thermostable, biodegradable, non toxic and biocompatible, thus they can be used as in medicine and food (Lenz and Marchessault, 2005). Other aliphatic polyesters such as poly (propiolactone) (PPL), poly (ε-caprolactone) (PCL), poly(L-lactide) (PLA), poly(ethylene succinate) (PES), poly(butylene succinate) (PBS), and poly(ester carbonate) (PEC) also have properties of conventional plastics and are being developed for use as biodegradable substitutes for non-degradable plastics (Tokiwa and Calabia, 2004). Degradation of PHAs can take place in soil, compost, water and other natural environments by bacteria, fungi and protozoa (Jendrossek *et al.*, 1996). Many kinds of PHA-degrading bacteria belonging to *genera Bacillus, Pseudomonas, Comamonas, Alcaligenes,* and *Streptomyces* have been isolated (Tokiwa *et al.*, 2009). Many PHA-degrading fungi belonging to more than 95 fungal genera were recorded (Jendrossek *et al.*, 1996). Microorganisms with the ability to be PHB-degrading are present in either mesophilic or thermophylic environments (Tokiwa *et al.*, 2009). Isolation and identification of thermophilic PHB-degrading microbes is essential, thus Tseng *et al.* (2007) isolated many PHAs degrading thermophilic actinomycetes from different environments in Taiwan. Some of these isolates especially *Streptomyces bangladeshensis* degrade three types of polyesters like PHB while the other isolated microorganisms can degrade at least two types. The aim of the present study was isolation and characterization PHB degrading fungi from soil collected from Jeddah. Selection and identification of the most active PHB degrading isolate was carried out.

2. Material and Methods

The commercial grade powder of PHB (MW, 279,000) was supplied by Aldrich Chemical Company.

Test fungi

Twenty different fungal isolates, from the culture collection of Botany Dep., Faculty of Science, Assuit University, Egypt, were obtained on potato dextrose agar (PDA) and all fungal isolates were preserved on the same medium at 4^{o}C until used. All the previous fungal isolates were from soil origin and identification was confirmed according to their macroscopic and microscopic features. After determination of their genera (Domsch et al., 1980, von Arx, 1981, Hanlin 1990, Kiffer and Morelet, 1997), they were transferred to the media recommended by the authors of the selected genus for species identification.

Screening for PHB Degradation

The fungal isolates were screened for PHB

degradation using plate-clearing technique on medium containing PHB as carbon source. Mineral medium was composted of 0.01% peptone, 0.01% yeast extract, and 0.1% PHB. The medium pH was 7 and 15 g/l Agar was added to prepare solid medium. About 0.1 ml of spore suspension of the each fungal isolate was used to inoculate the agar plate. The inoculated plates were incubated for 7 days at 25°C until PHB hydrolysis (presence of a clear zone) as described by Klich, (2002).

Fungal growth in liquid Medium

Maximum PHB degrading fungal isolate was cultivated in 48 ml of PHB mineral broth medium (Lee et al, 2005) in 250 ml Erlenmeyer flask which was inoculated with a 2 ml (2.6×10^4CFU/mL) of the fungal suspension, previously grown Sabouraud broth medium for 2 days at 25°C and 120 rpm. The inoculated flasks were incubated at 25°C and120 rpm for 4 days. Finally, fungal growth were collected by centrifugation at 10,000 rpm for 15 min and the cell-free supernatant was used for PHB depolymerase assay.

PHB Depolymerase Assay

After centrifugation at 4°C for 15 minutes at 12,000 rpm, culture supernatant was used for PHB depolymerase assay using PHB as a substrate (Kobayash *et al.,* 1999). One unit of the enzyme is the decrease in OD_{650}nm per 24 h (Kobayashi *et al.*, 1999). Protein concentration estimation was determined using method suggested by Lowry *et al.* (1951).

Factors affecting PHB degradation

Effect of temperature (10 - 45ºC), pH values (4 - 8), incubation period (1 - 9 days) and concentration of PHB in the growth medium (1 - 4%) on the enzymes production was determined in the culture supernatant. The selected fungal isolate was grown in 250mL Erlenmeyer flasks using minimal medium with 0.1% PHB as a sole carbon source. All flasks were inoculated with 2 ml of the spore suspension then incubated for 4 days at 120 rpm. Samples were drawn, centrifuged at 10,000 rpm for 10 min and then supernatant was taken as a crude enzyme extract to determine the enzyme activity.

Statistical Analysis

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

3. Results

On sold agar medium, PHB degradation was recorded by the clear zone diameter (cm) but in liquid medium, PHB degradation was measured by the activity of depolymerase enzyme (U/ml). Based on the screening data, out of 20 fungal isolates, 11 isolates were PHB degraders and *Aspergillus fumigatus* and *A. oryae* were the most active isolates (Table 1). *A. oryzae* was selected for more detail experiments (Fig 1). In minimal medium PHB degradation varied with incubation temperature, medium initial pH, PHB concentration and presence of carbon source in the medium. The most suitable temperature for growth and depolymerase production was 30°C using minimal medium after 4 days of incubation (Fig. 2). The difference was significant compared to control (25°C). The effect of initial pH of the medium on depolymerase production was shown in Fig. 4. The best pH value for enzyme production was pH 6.5 and maximum enzyme production was after 5 days in minimal medium containing 1% PHB (Fig. 4, Fig. 5). The difference was significant compared to control (pH 7 and days).

Table 1. PHB degradation in solid and liquid medium using different fungal isolate

Tested fungus	Growth on* solid medium	PHB degradation		% of degradation
		Presence of clear zone (cm) after 7 days	Enzyme activity (U/ml)	
Alterneria alternata	+	1.4±0.02	0.71±0.043	14
Aspergillus flavus	++	2.3±0.07	0.91±0.012	23
Aspergillus fumigatus	++	2.4±0.42	0.44±0.002	24
Aspergillus nidulans	+++	4.7±0.22	1.33±0.02	47
Aspergillus niger	++	2.4±0.02	0.67±0.08	24
Aspergillus ochraceus	++	2.4±0.12	0.73±0.015	24
Aspergillus oryzae	+++	4.1±0.82	1.11±0.06	41
Aspergillus parasiticus	++	2.5±0.12	0.17±0.08	25
Aspergillus terreus	++	2.9±0.12	0.47±0.12	29
Fusarium	++	3.4±0.08	0.60±0.002	34
Penicillium	++	3.4±0.034	0.45±0.002	34
Trichoderma	+	1.4±0.042	0.37±0.044	14

*Fungal growth on minimal medium with 0.1% PHB, +: Weak growth, ++: Moderate growth, +++: High growth

Similarly, maximum PHB degradation was in medium using 1% PHB at pH 6.5 and at incubation temperature 30°C.

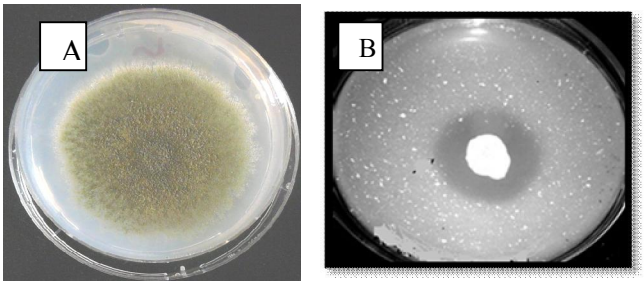

Fig 1. *Aspergillus* isolate on PDA at 25C after 7 days (A), clear zone after fungal growth and PHB degradation (B).

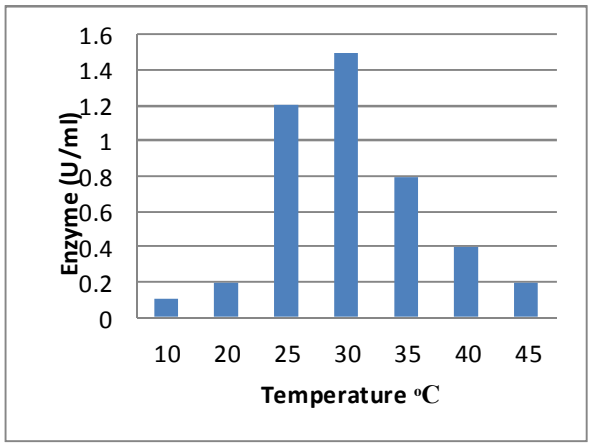

Fig. 2. Effect of different temperature on PHB degradation (enzyme production, U/ml) by the selected fungal isolate

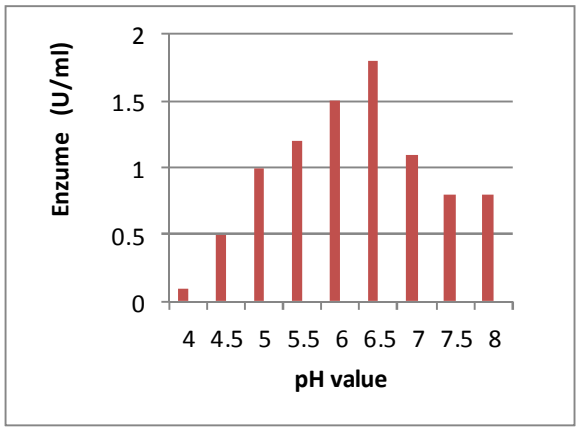

Fig. 3. Effect of different pH values on PHB degradation (enzyme production, U/ml) by the selected fungal isolate.

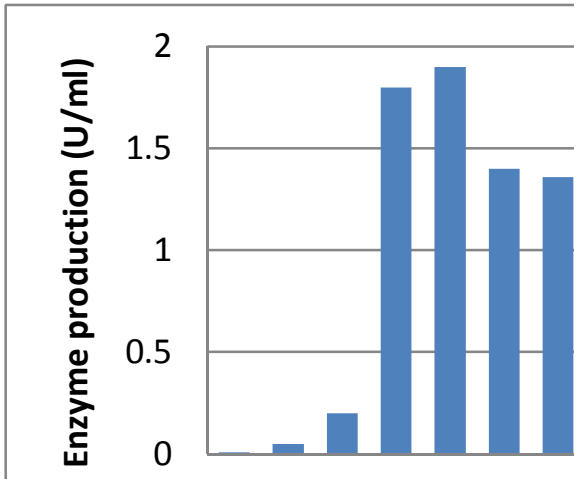

Fig. 4. Effect of different incubation period on PHB degradation (enzyme production, U/ml) by the selected fungal isolate.

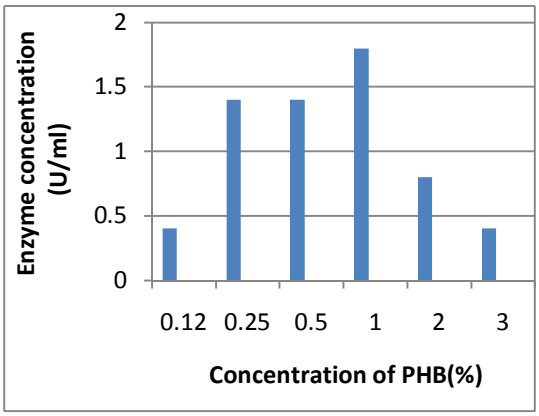

Fig. 5. Effect of different concentration of PHB on PHB degradation (enzyme production, U/ml) by the selected fungal isolate.

4. Discussions

The accumulation of plastics in the environment becomes a matter of great concern leading to long-term environment, economic and waste management.

Problems in poor and developed countries. In the present world, bioplastic materials of PHB become ones of the most popular materials and indispensable because their flexibility, toughness, excellent barrier and physical properties.

In order to overcome these problems, significant attention has been placed on biodegradable polymers, and also, on the identification of microorganisms with degradation potential upon polymeric materials. In present paper, the fungal degradation of polymeric material PHB was carried out in solid and liquid medium and the percentage of degradation was

calculated. The bioplastics degradation was found under different growth conditions according to microbe properties and growth conditions (Lucas *et al.,* 2008). Some bacterial and fungal isolates were screened for depolymerase production and PHB degradation on solid medium with PHB as inducer. In the presence of PHB, depolymerase enzyme was induced (Lodhi *et al.*, 2011) and the induction process are subjected to a complex regulation and synthesized in energy or nutrient limited conditions (Lodhi *et al.*, 2011). On solid agar, about 60% of the tested fungi were depolymerase enzyme producing which hydrolyze PHB which appear as clear zone around fungal growth. Similarly, 48% of the examined bacterial strains showed PHB degradation (Lee *et al.*, 2005). The most active isolate was *A. fumigates* and *A. oryzae*. *A. oryzae* was selected for detail studies because *A. fumigates* are considered as a pathogen. *A. oryzae* is a fungus widely used in traditional Japanese fermentation industries, including soy sauce, sake, bean curd seasoning and vinegar production. Filamentous fungi generally have the ability to produce various and vast amounts of enzymes in a secretary manner (Christensen *et al.*, 1988). Among filamentous fungi, *A. oryzae* is known to have prominent potential for the secretary production of various enzymes. In addition, developments in genetic engineering technology have led to the application of *A. oryzae* in the production of industrial enzymes in modern biotechnology. *Aspergillus oryzae* was used for the first example of commercial production of heterogonous enzyme, the lipase for laundry detergent (Gomi and Abe, 2007). Fungi are widely used in biodegradation studies due to their robust nature and for their great source of diverse enzymes (Lowe, 1992).

Aspergillus oryzae has the ability to grow and degrade PHB at different temperature and pH ranges. The degree of microbial degradation depends on the culture medium and on composition of polymeric materials (Jecu *et al.*, 2009). *Aspergillus niger* can utilize the biopolymer followed by *A. nidulans, A. flavus* and *A. terreus* while *A. fumigatus* and *A. parasiticus* could not utilize the polymer as carbon source (Merugu, 2012).

Degradation of PHB by measuring clear zone diameter was used for *Aspergillus fumigates* (Lodhi *et al.*, 2011) and this technique is used for solid medium to detect microorganisms that can degrade a certain polymer (Nishida and Tokiwa, 1993; Augusta *et al.*, 1993; Abou-Zeid, 2001) because on agar medium microbial growth is usually easier than in broth medium (Choi *et al.,* 2001). The weak or lack of PHB degradation activity may be due to the used culture conditions or/and the absence of the gene responsible for effective degradation.

It is well known that depolymerase enzymes obtained by fungi are extracellular (Lodhi *et al.*, 2011, Augusta *et al.*, 1993). The roles of fungi in hydrolysis of PHB are not well studied but need to be elucidated in further studies. Thus, this study concerning the selection of fungal isolate that produce excellent depolymerase enzyme in presence of PHB which have a wide distribution in fungi.

Temperature, initial pH, incubation period and polymere concentration are the most critical parameters that controlled in any bioprocess. A number of mesophilic fungi have been found to be responsible for degrading PHB in soil and aquatic environments (Mergaert *et al.*, 1994; Kim *et al.*, 2000). On contrast, many thermotolerant strains of soil and compost are capable of degrading PHB at high temperatures ≥ 40°C (Mergaert *et al.*, 1994; Kim *et al.*, 2000). For *A. oryzae*, pH 6.5 was the best for PHB degradation while pH 7 was the best for the growth of many fungi.

The optimum conditions for PHB depolymerase from *Paecilomyces lilacinus* were pH 6.5 to 7.5 at 50°C (Oda *et al.*, 1995). 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 hr of incubation in liquid medium (Lodhi *et al.*, 2011). The maximum activity of extracellular PHB depolymerase produced by *Bacillus megaterium*N-18-25-9, was observed at pH 9.0 at 65ºC (Takaku *et al.*, 2006) and at pH 7.5 - 8.0 when sewage sludge was used as inoculum (Briese *et al.*, 1994). In liquid medium, *Aspergillus fumigates* M2A degrade PHB by extracellular PHB depolymerase after 150 h of incubation (Scherer *et al.*, 1999). In the present study, the maximum production of PHB depolymerase was found at substrate concentration of 0.1% as indicated by the maximum enzyme activity, while it decreased with further increase in polymer concentration which might be due to substrate level inhibition (Manna and Paul, 2000). Similar results were recorded for *Arthrobacter* sp. W6, the optimal concentration of PHB was 0.1% (Asano and Watanabi, 2001). Production of extracellular PHB depolymerase by *Thermus thermophilus* HB8 was using 0.1% PHB (Christos *et al.,* 2009). In the presence of carbon source in the growth medium with PHB, the activity of PHB depolymerase enzyme was enhanced (Lodhi *et al.*, 2011, Manna and Paul, 2000). PHB depolymerase expression is repressed in the presence of a soluble carbon source that permits high growth rates and after exhaustion of the nutrients, the synthesis of PHB polymerase is decreased (Jendrossek *et al.*, 2002). In conclusion, bioplastic was used instead of plastic from the petroleum origin. Fungi has excellent role in PHB degradation and optimization of culture conditions increased PHB degradation.

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