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# OPTIMIZATION OF CULTURE CONDITIONS FOR THE PRODUCTION OF POLY (3-HYDROXYBUTYRATE) DEPOLYMERASE FROM NEWLY ISOLATED *ASPERGILLUS FUMIGATUS* FROM SOIL

**ADIL FAROOQ LODHI, FARIHA HASAN, ZIAULLAH SHAH, ABDUL HAMEED, SHAH FAISAL AND AAMER ALI SHAH***

*Department of Microbiology*
*Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad, Pakistan.*

**Abstract**

The present research work was aimed for the isolation and identification of poly (3-hydroxybutyrate) (PHB) degrading *Aspergillus fumigatus* and optimization of culture conditions for the maximum production of PHB depolymerase. This strain was isolated from the soil samples, collected from waste disposal sites, Islamabad, Pakistan, through enrichment and plate assay techniques, then culture conditions were optimized using enzyme assay, protein estimation and calculation of specific activity of the enzyme. The optimization experiments were done to determine the optimal combination of parameters, such as temperature, pH, substrate concentration, extra carbon sources and different inoculum sizes for maximum PHB depolymerase production by *Aspergillus fumigatus.* All the experiments were performed for 120hrs and samples were taken after every 24 hours and enzyme activities and protein concentrations were monitored. It was observed that maximum PHB depolymerase was produced at 45°C, pH 7 and 0.2% substrate concentration with specific activity 7.756U/mg, 6.120U/mg and 6.506U/mg respectively, after 24 hours of incubation. Lactose was found to be the most optimum source giving maximum specific activity 6.776U/mg as compared to fructose, sucrose and glucose. An inoculum size of 9% was found optimum for PHB depolymerase production with a maximum specific activity of 7.206U/mg after 24 hrs of incubation using lactose as an extra carbon source, pH 7, substrate concentration 0.2% and 45°C temperature. From the present study, it has concluded that the soil is a rich source of potential microorganisms, both bacteria and fungi, capable of degrading natural polymers.

## Introduction

Organic and inorganic raw materials, such as carbon, silicon, hydrogen, nitrogen, oxygen and chloride are used for the manufacture of plastics that are in use today. The basic materials used for making plastics are extracted from oil, coal and natural gas (Seymour, 1989). The reason behind this widespread use of plastics is stability and durability of plastics in harsh conditions and there resistance to microbial as well as chemical decomposition. Synthetic plastics are therefore so much in use that it is impossible to imagine everyday life without synthetic plastics (Sudesh & Iwata, 2008).

The development of an ecologically useful alternative of synthetic plastics, biopolymer (biodegradable polymer) materials have remained under study for the past two decades so that it may solve the existing problem of plastic waste. These are made from the renewable resources and developed countries are producing biodegradable plastic proactively as an alternative and different kinds such as poly (3-hydroxybutyrate) (PHB), poly (3-hydroxybutyrate-co-3-hydroxyvalerate (PHBV), polycaprolactone (PCL), polylactic acid (PLA), polyethylene succinate (PES), polybutylene succinate-co-adipate (PBSA) and the copolymer or blends of these are produced (Tseng *et al.,* 2007).

*Corresponding author E-mail: alishah_75@yahoo.com,
Tel No. +92-51-90643185, Fax: +92-51-9219888.

Polyhydroxyalkanoic acids (PHAs), among the different types of biodegrable plastics have been extensively studied because of their similarity to conventional plastics characteristically and complete biodegradability and current market domination (Verlinden *et al.,* 2007). Both PHAs and synthetic plastics possess similar properties and can be used in a wide range of applications from packaging to coatings. Polyhydroxyalkanoic acids (PHA) are synthesized and deposited intracellularly in the form of inclusion bodies (``granules'') in bacteria and might amount up to 90% of their cellular dry weight. At the same time, PHAs are renewable by nature; they could be produced from renewable resources such as plant oils (Loo *et al.,* 2005) sugars (Ramachander *et al.,* 2004) and carbon dioxide (Volova *et al.,* 2004)

Among PHAs, poly (3-hydroxybutyrate) PHB and poly (3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) are most commonly used. PHB is a homopolymer of 3-hydroxybutyric acid, which is accumulated in some bacteria during unbalanced growth. PHB is comparable to polypropylene (PP) mechanically and PHBV copolymer can be used to increase the toughness of PHB polymer (Velde & Kiekens, 2002). However the production of these bioplastics is cost effective as compared to synthetic plastics (Khanna & Srivastava, 2005).

The principal enzyme for the degradation of PHB and oligomers derived from the polymer is PHB depolymerase. Extracellular PHB depolymerase has been isolated from different bacteria as *Alcaligenes faecalis*, *Rhodospirillum rubrum*, *B. megaterium*, *A. beijerinckii* and *Pseudomonas lemoignei*. Polyhydroxyalkanoic acids-degrading fungi have been isolated from various environments, though, very few were recovered from fresh water, sea water and sludge samples (Mergaert *et al.,* 1996). It has been established, that many fungal taxa belonging to Basidiomycotina, Deuteromycotina and Ascomycotina are the predominant degraders of PHAs in the environment, while members of Zygomycotina degrading PHAs are less prevalent (Neumier, 1994). Analysis of PHA-degrading fungi has led to the consideration of *Aspergillus* as one of the most predominant genera (Matavulj & 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). In addition to mesophiles many thermotolerant *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). Therefore, the current study is performed for the isolation of PHB degrading fungi, its identification and optimization of different culture conditions for the maximal production of PHB depolymerase from the isolated strain.

## Materials and Methods

**Isolation of PHB degrading fungi:** Soil samples were collected from waste disposal sites Islamabad, Pakistan and were used to screen for PHB utilizing fungi. Each soil sample (1g) was added to Erlenmeyer flask containing 100 ml of the mineral salt medium and PHB as the sole carbon source and incubated at 30$^{o}$C with shaking (150 rpm min$^{-1}$). The composition of mineral salt medium was as follows: (g/l: $K_2HPO_4$ 8, $KH_2PO_4$ 1.0, $(NH_4)_2SO_4$ 0.5, $MgSO_4$ 0.2, NaCl 0.1, $FeSO_4.7H_2O$ 0.02, $Na_2MoO_4$ 0.0005, $MnSO_4$ 0.0005, $CuSO_4$ 0.0005, $ZnSO_4$, $CaCO_3$ 0.5, Agar 15), and pH 7 (Nishida & Tokiwa, 1993). After a week, 1 ml of culture broth was transferred into another flask containing

99ml of fresh mineral salt medium. This procedure was repeated five times. The culture broth obtained through enrichment technique was tested for the presence of PHB degrading microorganisms by clear zone test using PHB emulsified mineral salt agar plates. Isolates which showed zones of hydrolysis were selected and identified (Klich, 2002).

**Identification of PHB degrading fungus:** The fungal strain observed to form clear zone of hydrolysis on PHB emulsified 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 colour, colony diameter, mycelial colour, exudates, reverse colour, soluble pigment, sclerotia and cleistothecia. Features seen through a compound light microscope were, seriation, vesicle, conidia, stipe, hull cells, cleistothecial wall and colour, size, ornamentation of ascospores (Klich, 2002).

**Preparation of spore suspension:** Spore suspension was prepared by adding autoclaved 0.9% saline suspension directly on to sporulating fungal colonies grown on Petri plates and then spores were counted in the suspension collected under aseptic conditions in screw caped tubes through Haemocytometer. The following formula was used for spore count:

Spores per ml= Average count per square × dilution factor ×volume factor

**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 250ml Erlenmeyer flasks using Mineral salt medium supplemented with PHB as a sole carbon source. All flasks were incubated at 150 rpm for 120 hours. Samples were drawn aseptically after every 24 hours, centrifuged at 10,000 rpm for 10min and then supernatant was taken as a crude enzyme extract to determine the enzyme assay, protein estimation and then specific activity of PHB depolymerase was then calculated.

**Effect of temperature:** To study the effect of temperature fermentations were carried out at 30, 37, 45, 50 and 55°C for 120 hours. Mineral salt medium containing 0.2% of PHB at pH 7 was inoculated with 5ml spore suspension for this experiment.

**Effect of pH:** Flasks containing mineral salt medium, 0.2% of PHB, with different pH ranges (3, 4, 5, 6, 7, 8 and 9) were prepared, inoculated with 5ml spore suspension and incubated at 45°C for 120 hours. pH was adjusted using 1N NaOH and 1N HCl.

**Effect of carbon sources:** PHB emulsified MSM flasks mixed with different carbon sources such as glucose, fructose, sucrose and lactose (1% w/v) at pH 7 were prepared, inoculated with 5ml spore suspension and then incubated at 45°C for 120 hours.

**Effect of substrate concentration:** Flasks containing mineral salt medium and different concentrations of PHB (0.1, 0.2, 0.3, 0.4, 0.5%) were prepared at pH 7 and inoculated with 5ml spore suspension then incubated at 45°C for 120 hours to study the effect of substrate concentration.

**Effect of inoculum size:** The effect of inoculum size on the maximum production of PHB depolymerase by the fungus was studied by inoculating the flasks containing mineral salt medium with 0.2% PHB and lactose 1% w/v with increasing concentrations of inoculum from spore suspension as 1, 3, 5, 7, 9, and 11 % v/v at pH 7 aseptically and incubating at 45°C for 120 hours.

**PHB depolymerase assay:** PHB depolymerase assay was performed according to the method described by Kobayash *et al.,* (1999). About 0.3% Poly (3-hydroxybutyrate) was suspended in 50mM Tris-HCl buffer, pH 7.5 and the suspension was sonicated for 20min in a 300ml flask immersed in as ultrasonic water bath (35KHz, 285W) prior to the dilution to 0.03% in the same buffer. Culture supernatant 0.1ml was added to 0.9ml of the substrate suspension and incubated for 24 hours at 30°C. Activity was measured as the decrease in OD, as measured at 650nm through spectrophotometer, against substrate buffer blanks.

**Unit of activity:** One unit is defined as the activity resulting in a decrease in $OD_{650}$ per 24 hours (Kobayashi *et al*., 1999).

**Protein estimation:** Protein concentration estimation was performed for each enzyme sample using method suggested by Lowry *et al.,* (1951). Color developed during the assay procedure was read at 650nm. Finally concentration of protein in the culture supernatant was determined using standard curve of bovine serum albumin.

**Determination of specific activity of the crude extract:** The specific activity (U/mg) in case of each sample was determined by dividing the enzyme activity (U/ml) of the sample by its protein content (mg/ml).

## Results

**Isolation of PHB degrading fungi:** A fungal strain capable of degrading PHB, was isolated from the soil samples, collected from waste disposal sites, Islamabad, Pakistan, through enrichment and plate assay technique. The zones of hydrolysis on the PHB emulsified mineral salt agar plates indicated that the fungus is utilizing PHB as a sole carbon source. Maximum size of zones was observed after 10 days (240 hours) of incubation. No further increase in zones size was observed after that period (Fig. 1).

**Identification of PHB degrading fungus:** The fungal strain 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 (Holt, 1993). In macroscopic examination grayish turquoise or dull green, conidial colour was observed, white mycelial colour, yellowish to brown reverse colour and uncoloured exudates were observed, 40-70µm colony diameter after 4-7 days, and absence of soluble pigment, sclerotic and cleistothecia showed the strain to be *Aspergillus fumigatus*.

In microscopic morphology, uniseraite seriation, 15-30 µm diameter of vesicle, globosely to broadly ellipsoidal conidia with 2-3mm diameter, smooth walled stipe (grayish near the apices, purple lenticular, 4.5-6 µm, ascospores and absence of hull cells and cleistothecial wall confirmed the strain to be *Aspergillus fumigatus*.

**Optimization of different parameters for maximum production of PHB depolymerase by *Aspergillus Fumigatus***

**Effect of temperature:** It was evident from the results that maximum production of PHB depolymerase was observed at 45°C after 24 hours of incubation, indicated by the maximum value of specific activity being 7.756 U/mg. At 30, 37, 50, and 55°C the specific activity was 6.046, 7.197, 5.756 and 6.003 U/mg respectively (Fig. 2).

**Effect of pH:** *Aspergillus fumigatus* was able to produce PHB depolymerase at wide pH range, but maximum production was observed at pH 7 after 24 hours of incubation, as evident by the maximum value of specific activity 6.120 U/mg. In case of pH 3, 4, 5, 6, 8 and 9, the specific activity was 4.653, 4.817, 5.459, 5.686, 5.851 and 4.381 U/mg respectively (Fig. 3).

**Effect of substrate concentration:** The optimum substrate concentration required for the production of PHB depolymerase production was 0.2% (w/v) after 24 hours of incubation as shown by the maximum value of specific activity that is 6.506 U/mg. In the presence of 0.1, 0.3, 0.4 and 0.5% of substrate, the specific activity was 5.738, 3.499, 3.322 and 3.152 U/mg respectively (Fig. 4).

**Effect of carbon sources:** Maximum degradation of PHB was observed in the presence of lactose, as confirmed by the results of specific activity 6.776 U/mg after 24 hours of incubation while in the presence of other carbon sources, fructose, sucrose and glucose the maximum specific activity was 4.289, 2.379 and 2.774 U/mg respectively (Fig. 5).

**Effect of inoculum size:** Degradation of PHB was affected when different inoculum sizes were used. Inoculum size ranging from 1, 3, 5, 7, 9 and 11 ml were used and a maximum production of PHB depolymerase was observed when 9ml of inoculum was used as evident by the specific activity 7.206 U/mg after 24 hours of incubation. The specific activity of PHB depolymerase was 5.660, 5.675, 5.748, 6.091 and 6.116 U/mg in the presence of 1, 3, 5, 7 and 11 ml of inoculum respectively (Fig. 6).

**Discussion**

A variety of microorganisms such as bacteria and fungi are involved in the degradation of both natural and synthetic plastics (Gu *et al.,* 2000). The biodegradation of plastics proceeds actively under different soil conditions according to their properties, because the microorganisms responsible for the degradation differ from each other and they have their own optimal growth conditions in the soil. This action leads to the recycle of carbon, the mineralisation ($CO_2$, $H_2O$ and salts) of organic compounds and the generation of new biomass however, at present, the complexity of biodegradation is better understood and cannot be easily summarised (Lucas *et al.,* 2008) Therefore, the present study deals with the isolation of natural polymer Poly (3-hydroxybutyrate) (PHB) degrading microorganisms from soil samples. A fungal strain, *Aspergillus fumigatus* was isolated from soil through enrichment and plate assay technique. The polymer-mineral salt agar plates were used to determine the PHB degradation by *Aspergillus fumigatus.* The results showed that *Aspergillus fumigatus* was capable of degrading PHB by producing clear zones of hydrolysis.

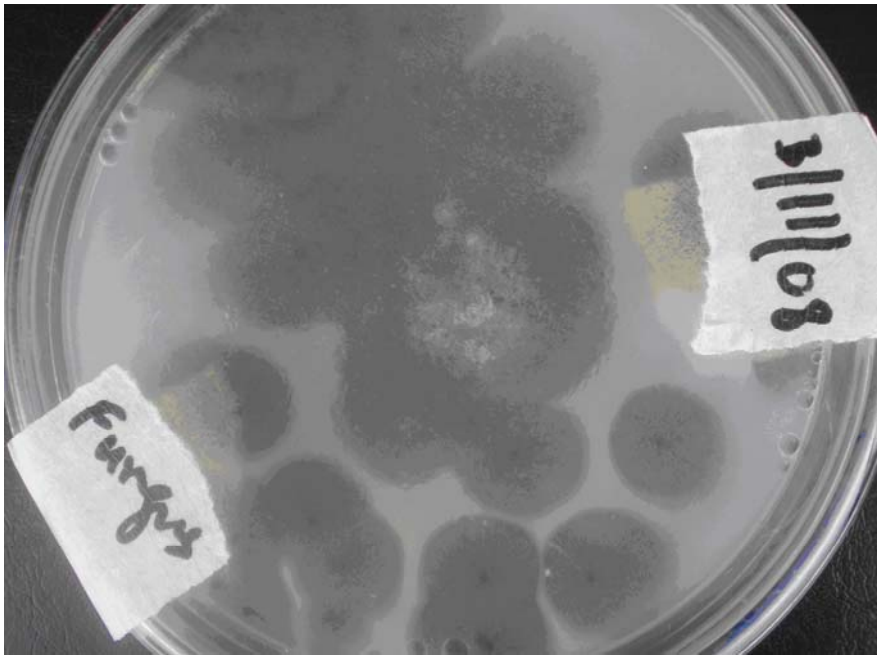


Fig. 1. Degradation of poly (3-hydroxybutyrate) by fungal strain on mineral salt agar, shown by clear zone of hydrolysis after 10 days of incubation.

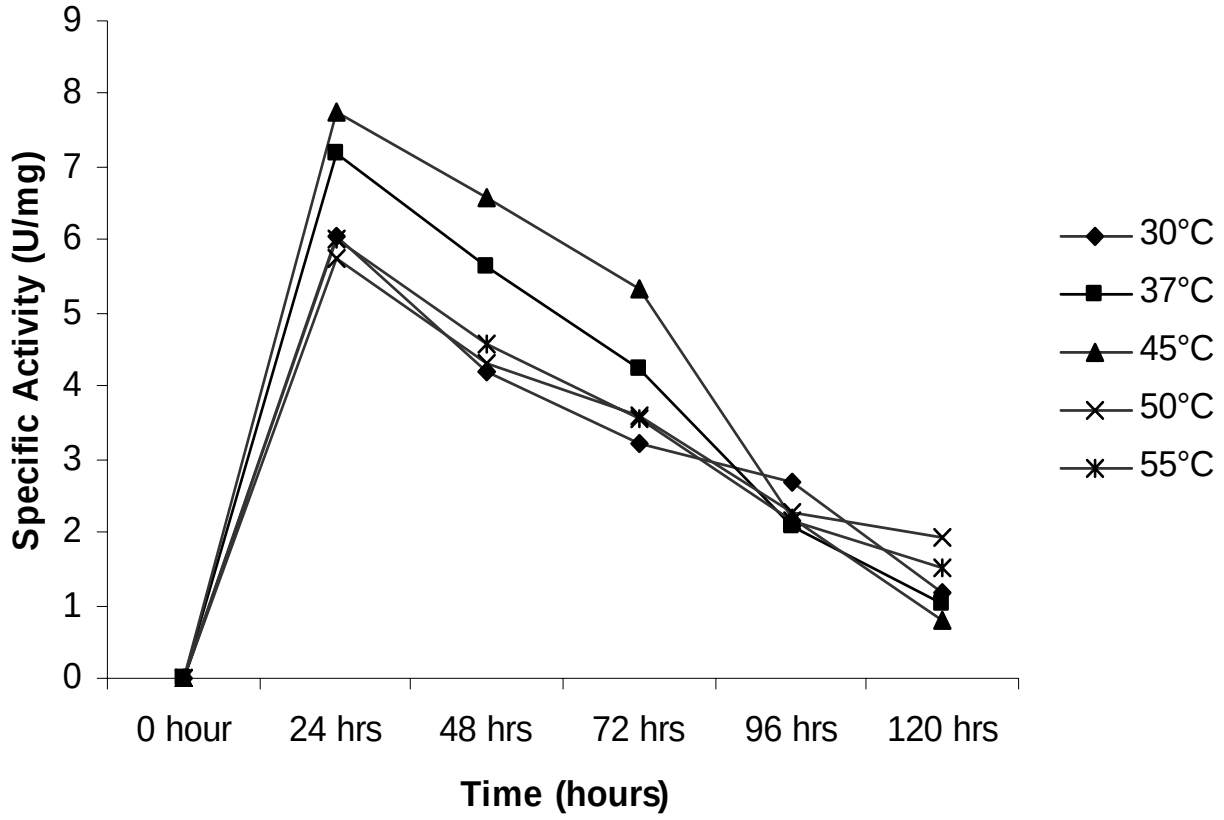


Fig. 2. Effect of temperature on PHB depolymerase production.

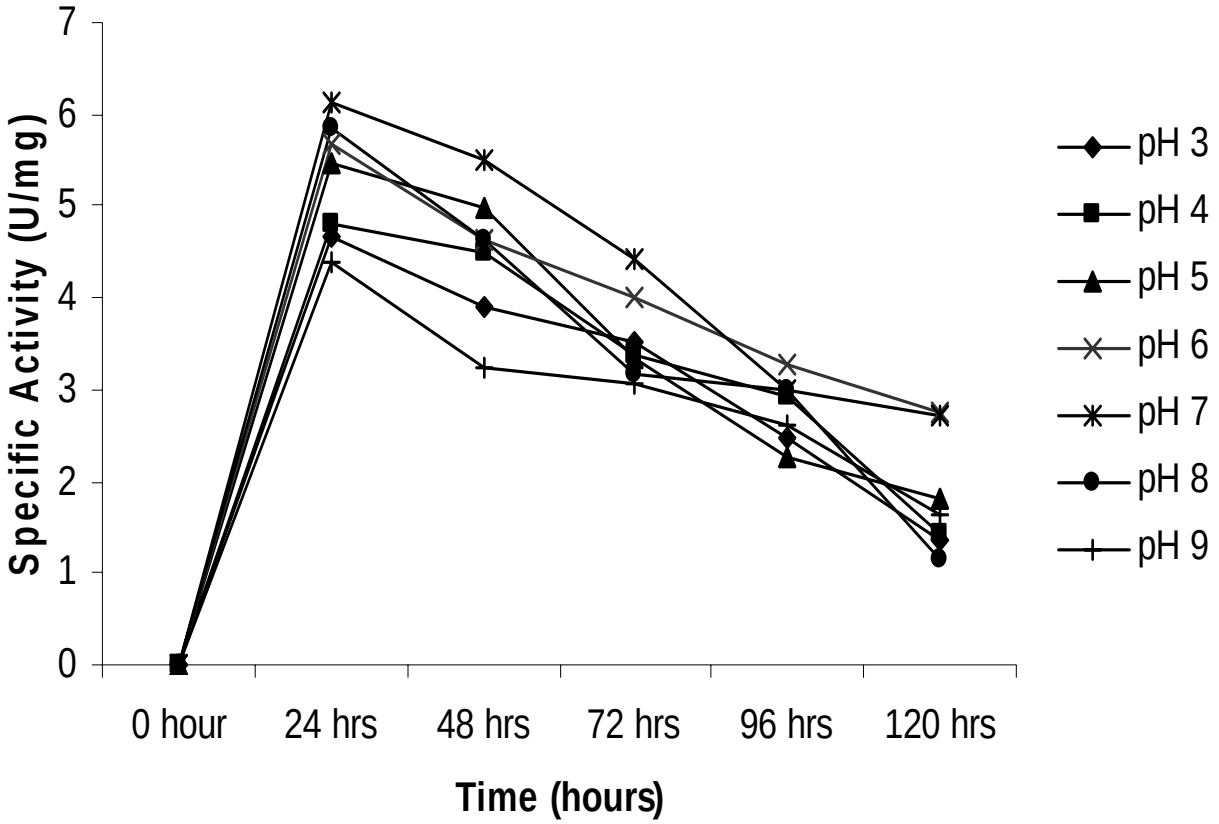


Fig. 3. Effect of pH on PHB depolymerase production.

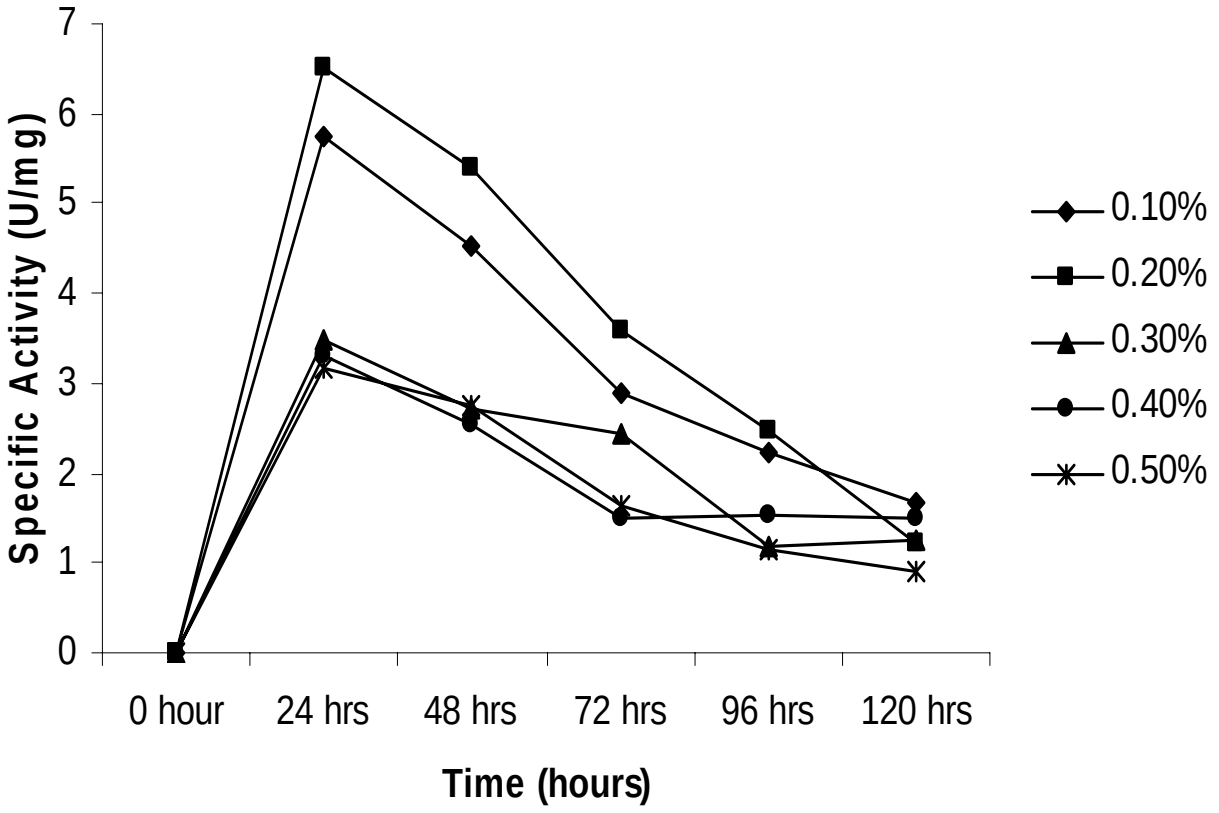


Fig. 4. Effect of substrate concenteration (%) on PHB depolymerase production.

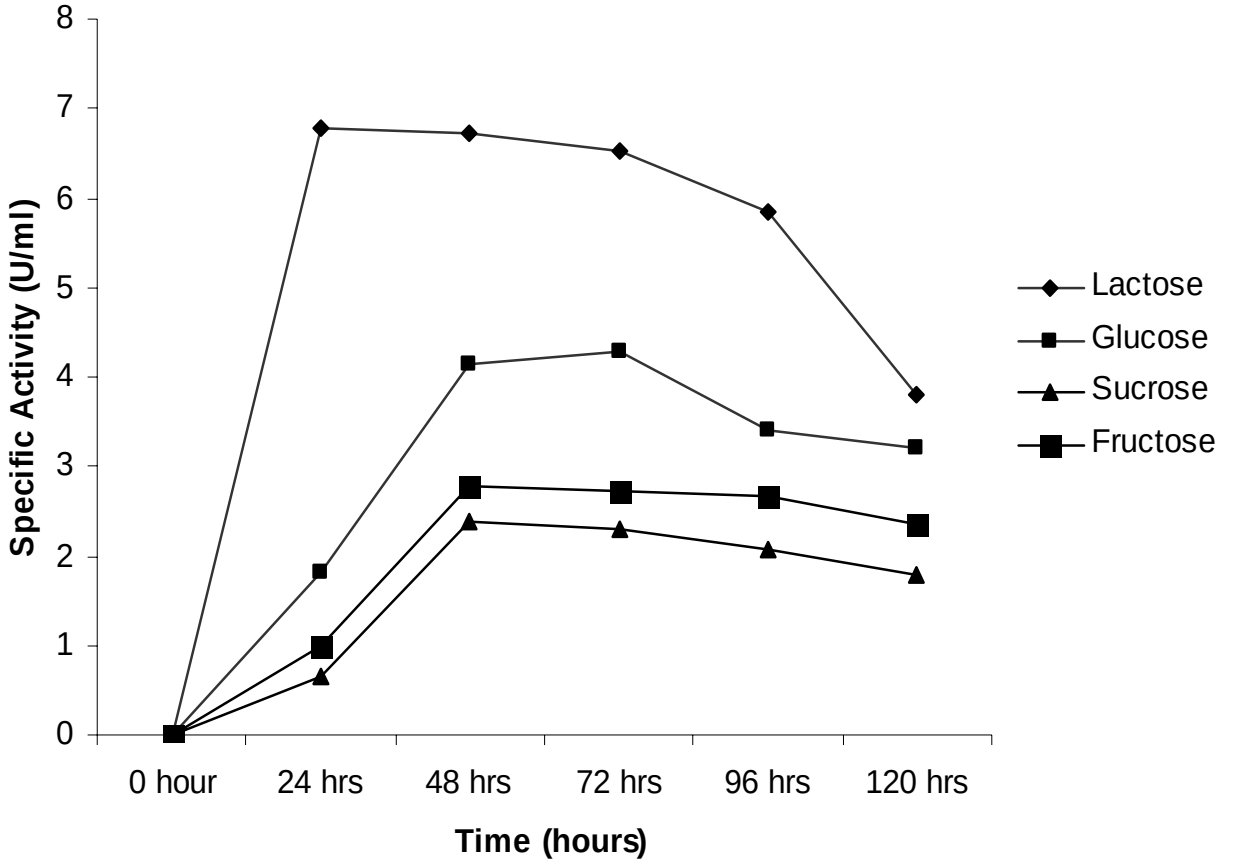


Fig. 5. Effect of extra-carbon sources on PHB depolymerase production.

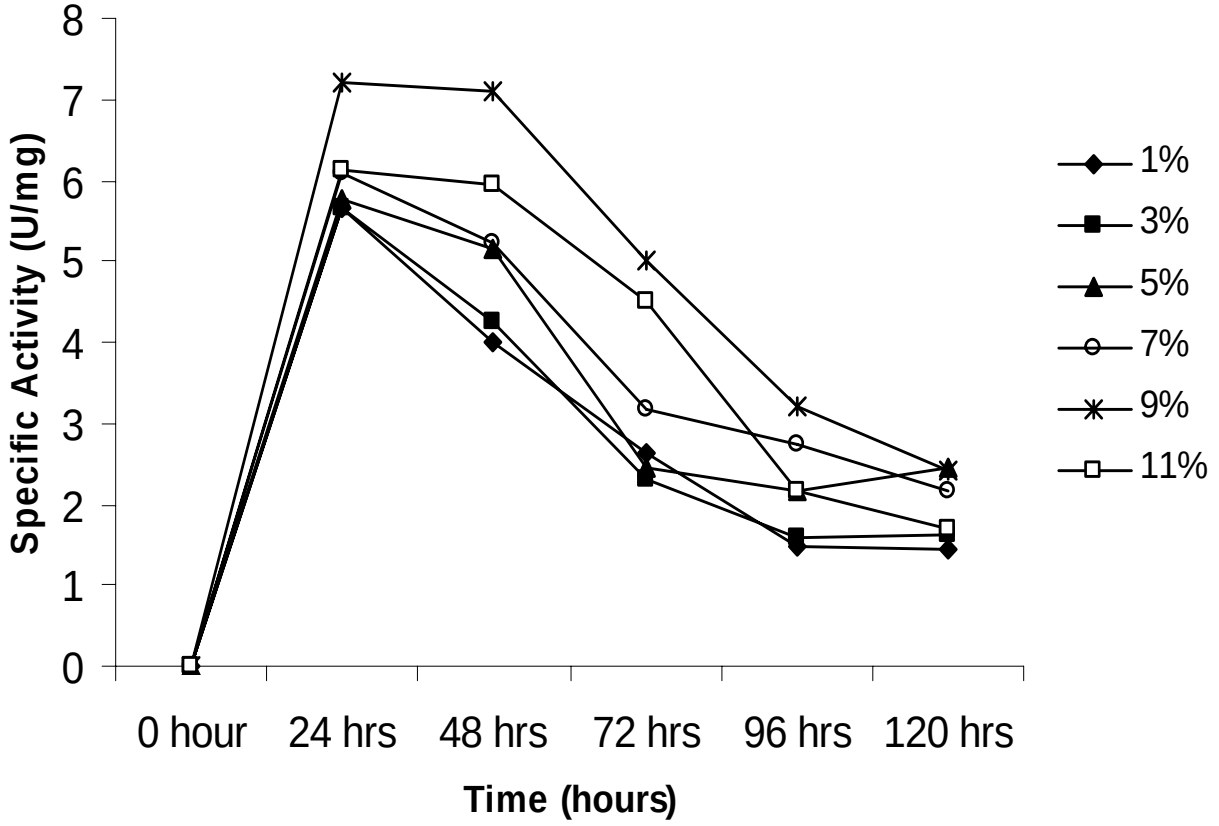


Fig. 6. Effect of different inoculum sizes (%) on PHB depolymerase production.

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 & Tokiwa, 1998) and *Streptomyces* strain MG (Tokiwa & Calabia, 2004) were reported capable of degradation at higher temperatures. Still there is little information on microbial degradation of PHB at high temperatures. In the present study, degradation of PHBV by *Aspergillus fumigatus* was checked at different temperatures (30, 37, 45, 50, 55°C). It was observed that the *Aspergillus fumigatus* was able to degrade PHB better at 45°C after 24 hours of incubation in liquid media. There was a gradual decrease in production of enzyme after 24 hours. This has been observed in various other reports as well that degradation in liquid media takes a much lesser time than observed in solid media as for example Scherer *et al*., (1999) found that the strain of *Aspergillus fumigatus* M2A was able to degrade PHB after 150 hours of incubation in liquid media by the production of extracellular PHB depolymerase while Christos *et al*., (2009) reported the production of extracellular PHB depolymerase by *Thermus thermophilus* HB8 after 24 hours of incubation. *Aspergillus parasiticus* MTCC-2796 produced maximum amount of α-galactosidase after 24 hours of incubation in liquid media. There was gradual decrease in the production of enzyme after 24 hours, as a result of utilization of substrate and other nutrients (Sivam *et al*., 2009).

In the present study, maximum production of PHB Depolymerase by *Aspergillus fumigatus* was observed at pH 7 after 24 hours of incubation in liquid medium, indicated by the maximum specific activity. Many fungi show broad pH optima from 5.5 to 7.5, but the enzymes needed in many natural environments often have specific pH optima and function over a quite narrow pH range (Ellaiah *et al*., 2004). 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). The highest degradation rates were reported at pH 7.5 and pH 8.0 when sewage sludge was used as inoculum (Briese *et al*., 1994). The optimum activity of the PHB depolymerase produced by *Penicillium* sp., DS9713a-01 obtained by ultraviolet (UV) light mutagenesis was observed at pH 8.6 (Qin *et al*., 2006).

In the present study, the maximum production of PHB depolymerase was found at 0.2% of substrate concentration as indicated by the maximum value of specific activity 6.506U/mg, while it decreased with further increase in polymer concentration. The decrease in enzyme activity at higher concentrations of polymer in growth medium might be due to substrate level inhibition. Substrate concentration above the optimum level as a rule suppresses the activity of the enzyme (Manna & Paul, 2000). In case of *Arthrobacter* sp., Strain W6 the optimal concentration of PHB was 0.1% reported by Asano & Watanabi (2001). Most recently the production of extracellular PHB depolymerase by *Thermus thermophilus* HB8 was reported after 24hours of incubation when 0.1% PHB was used. The specific activity obtained in this case was 2.0U/mg (Christos *et al.,* 2009).

In the current study presence of lactose in the solid medium along with PHB showed increased activity of PHB depolymerases by *Aspergillus fumigatus*. The specific activity in this case was observed as 6.776U/mg. This has been reported in other cases as well as for example Shah *et al.,* (2007) reported a maximum specific activity of PHBV depolymerase as 0.2U/mg by *Bacillus* sp., AF3. It is important to mention, that carbon catabolite repression (CCR) by lactose was observed for the organisms degrading polyhydroxyalkanoates i.e., cells sense the presence of a favorable carbon source, in this case lactose, and transmit the information to the relevant control units. Consequently, PHB as a C-source is not depolymerized to act as a catabolite. According to Manna &

Paul (2000) degradation of PHB by bacterial strains isolated from soil and sewage sludge 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 while for most of the isolates it was almost not affected when lactose was supplemented in the medium (Manna & Paul, 2000). PHB depolymerase expression is repressed in the presence of a soluble carbon source that permits high growth rates. However, after exhaustion of the nutrients, the synthesis of PHB depolymerase is derepressed. This regulation has been reported in bacteria as well (Jendrossek *et al*., 1996)

The effect of inoculum size showed an increase in the production of PHB depolymerase and when an inoculum of 9% (9ml spore suspension) was used the maximum production of PHB depolymerase was obtained after a 24hours of incubation with a specific activity value of 7.206U/mg, however the effect of increasing the inoculum further had no such effect. It has been reported in other findings also as Ikram-ul-haq *et al*., (2003) reported that in case of *Rhizopus Oligosporous* HIS13 an inoculum size of 10 % was optimum for the production of proteases. Abusham *et al*., (2009) reported that an increase in the production of enzyme protease by different bacterial strains can be achieved by increasing the inoculum size uptil 5%. A small amount of inoculum can lead to insufficient number of microbial cells and a reduced amount of the secreted enzyme while a much higher inoculum could lead to or cause a lack of oxygen and depletion of nutrients in the culture media (Abusham *et al*., 2009). In the present case it was found that an increase in inoculum size of upto 9% is best for the production of PHB depolymerase after 24 hours, along with a temperature 45˚C, pH 7, substrate concentration of 0.2%, and using lactose as extra carbon source.

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