JETIR.ORG ISSN: 2349-5162 | ESTD Year : 2014 | Monthly Issue

JOURNAL OF EMERGING TECHNOLOGIES AND INNOVATIVE RESEARCH (JETIR)
An International Scholarly Open Access, Peer-reviewed, Refereed Journal

MICROBIAL DEGRADATION OF POLYHYDROXYALKANOATES (PHAS) BY AN INDIGENOUS SOIL ISOLATE OF *STREPTOMYCES* SP.ND-3

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Abstract: Microbial polymers such as Polyhydroxybutyrate (PHB) and Polyhydroxyalkanoates (PHAs) can be catabolized by a wide range of microorganisms by intracellular (i-*phaZ*) or extracellular Polyhydroxyalkanoate depolymerases (e-*phaZ*). In this study, various environmental samples (soil & effluent) were collected in and around Madurai dt., and analyzed for e-*phaZ* activity by clear zone method using 0.1% PHB. Among screened isolates, ND-3 was selected on the basis of zone diameter on media enriched with polymer, indicating the presence of an extracellular PHA depolymerase. The degradative ability of ND-3 was examined using other substrates like Poly (3-hydroxybutyric acid-co-3 hydroxyvaleric acid, Poly (*L*-lactide), Poly (1, 4-butylene succinate) and esterase specific substrates such as tributyrin and *p*-nitrophenyl *(pNP)*-alkanoates. The maximum activity (165.8 U/mg) was observed with *p*-nitrophenyl-butyrate at pH 7.5. The strain ND-3 could degrade 0.1% PHB in 4th day at 30°C and the degradation was further confirmed by Scanning Electron Microscopy.16S rRNA phylogeny results revealed that ND-3 has close relatedness to *Streptomyces* sp. (93%). The molecular mass of e-*Pha*Z was determined to be ~48kDa by SDS-PAGE and Zymogram. The results of this study suggest that this distinct enzyme might represent a new subgroup of scl PHA depolymerase.

Index terms: Biodegradation, PHB, PHA depolymerase, *Streptomyces* sp. SEM

1. INTRODUCTION

Biodegradable polymers are receiving much attention from researchers as a solution to problems concerning the build-up of non-degradable polymers in an environment. As of now, there are numerous polyesters which exhibit properties comparable to conventional synthetic polymers. One among them is PHB is the most well-known PHAs, which are naturally occurring energy reserve polyesters synthesized by a number of bacteria and fungi, under unbalanced growth conditions. According to the number of carbon atoms in the monomer units (1), PHAs are divided into three major classes as short-chain- length (3 to 5 carbon atoms), medium-chain-length (6 to 14 carbon atoms) and long chain length (>14 carbon atoms) PHAs. PHB and PHBV are the most important PHAs among 150 different constituents, identified as homo-polymers or as copolymers (2).

PHAs are biodegraded into carbon dioxide and water by lipolytic i-*phaZ* and e-*phaZ* secreted by both bacteria and fungi. In general, lipolytic enzymes are classified into two major classes of hydrolases *viz* lipase (EC 3.1.1.1, triacylglycerol hydrolases) and esterase (EC 3.1.1.3, carboxyl ester hydrolase). Among which, PHB depolymerase was discovered and grouped into new family (family IX) of bacterial esterases (3). *phaZ* are carboxylesterases which belong to the α/β-hydrolase fold family (4,5). The enzymes first adsorb on the polymer surface of the binding domain then through the catalytic domain which hydrolyze the polymeric chain (6). The ability to degrade extracellular PHA in the environment and to use degradation products as sources of carbon and energy depends on

the secretion of e-*phaZ* that could be specific for either short-chain-length (scl)-PHA or medium-chain-length (mcl)-PHA. Also, depending on the depolymerase activity, the end products of PHA degradation are only monomers, both monomers and dimers, or a mixture of oligomers as a result of the enzymatic PHA degradation (7). These water soluble products are further absorbed by the microbial cells. Aerobic metabolism results in carbon dioxide and water (8), whereas anaerobic metabolism results in carbon dioxide, water and methane as the end products (9). The rate of degradation depends upon microbial population and its distribution along with the degradation ability (10).

Extracellular PHA-depolymerase of aerobic and anaerobic microorganisms are widely distributed in various environments (11, 12). Degradation of PHB and its copolymer has been studied in different natural environments such as soils (13, 14) and natural waters (15, 16) by both Gram-negative and Gram-positive bacteria. But members of this *Streptomyces* genus have since received very little attention for the degradation of PHB. Therefore, this present study was aimed to evaluate and characterize the degradative potential of PHB, co-polymer (PHB/12%HV) and PBS by Gram positive *Streptomyces* sp. isolated from landfill soil. Results of this study clearly demonstrated that the isolate ND-3 has potential biotechnological application in bio-plastic composting systems in future.

2. MATERIALS AND METHODS

2.1. Polymeric Materials and chemicals

The granules of PHB/12%HV, PLA, PBS and powder of PHB and 4-methylumbelliferyl butyrate (MUB) were purchased from Sigma Chemical (St. Louis, MO). Zeba^{TM} Spin Desalting Columns were purchased from thermo scientific. Other chemicals used in this study were reagent grade and purchased from Hi-media (www.himedialabs.com).

2.2. Preparation of biopolymer suspensions and films

PHB emulsified media was prepared by suspending PHB (0.1g) in 100 ml of distilled water and sonicated for 5 min (Sonics Vibra Cell, Amp 20% & Pulse 30sec on 30sec off). P-(3HB) and its copolymer P (3HB-co-12%-3HV) and PBS films were prepared by solvent casting technique. In brief, 100 mg of the polymer was dissolved in 20 ml of chloroform and subjected to mild heating and vigorous stirring for 5 min. The homogenous solution was poured on glass Petri dishes and the solvent was then evaporated at room temperature (17).

2.3. Microorganism, media, growth and maintenance conditions

To investigate growth requirements of the selected six PHA degrading bacterial strains, the cells were grown in Kuster (Glycerol-Casein-KNO_3) medium containing(g/l): Glycerol, 10ml; KNO_3, 2.0; Casein,0.3; NaCl,2.0; K_2HPO_4, 2.0; $MgSO_4.7H_2O$, 0.05; $CaCO_3$, 0.02; $FeSO_4.7H_2O$, 0.01;Agar,(1.5% (wt/vol), pH 7.8 (18). For enzyme production, media was supplemented with 0.1g of PHB as the sole carbon and energy source. The strain was maintained as frozen spore (3 days grown culture mixed with 30% (vol/vol) glycerol) at -80°C. Genomic DNA was isolated from 4 days grown culture (30°C in Glycerol-Casein-KNO_3medium) (19). The culture supernatant was filtered using Whatman filter paper no. 1 and used in enzyme assay (20).

2.4. Taxonomical identification of PHA-degrading microorganisms

The PHB (0.1%) emulsified plates were incubated with serially diluted samples for 3 days at 30ºC and the strain which showed clear zone was selected for biochemical and taxonomical identification as per Bergey's manual of Systematic Bacteriology (21). The selected strain was further examined for its ability to utilize various carbon sources using API HI-Carbo analysis Kit (Hi-media). The taxonomic position of the selected isolate was identified by 16S rRNA gene sequence analysis (22). The 16S rRNA nucleotide sequence of ND-3 was checked for chimera using Decipher (23). Phylogenetic tree was constructed using MEGA6 (24). The analyzed partial 16S rRNA sequence was submitted to NCBI.

2.5. Purification of the PHB depolymerase

The supernatant was collected from 4 days grown ND-3 culture and the extracellular enzyme was purified using ammonium sulphate precipitation. Ammonium sulphate was added (80% saturation) to the supernatant with gentle stirring until the solution reached to particular saturation level. The solution was centrifuged at 10,000 X g for 20 min at 4ºC and the supernatant was collected (25). Dialysis was performed with 2ml sample to remove the excess of ammonium sulphate salts using 50mM Tris buffer pH 7.5. The whole process was performed under cold conditions for overnight. The dialysed protein was subjected to Zeba spin desalting column with size exclusion membrane of 40 kDa to remove the lower proteins and to concentrate the desired protein. The collected samples after desalting spin column were analysed for esterase activity and protein concentration using Lowry's method (26).

2.6. Enzyme Assays

2.6.1. Tributyrin plate assay

The qualitative lipase/esterase activity was measured by a halo in tributyrin plate. Esterase producing microorganisms showed hydrolysis as a zone of clearance, when the appropriate dilutions were spread on the TBA medium containing per liter of Tryptone, 10 g; Sodium Chloride, 10g; Yeast Extract, 5 g; Tributyrin, 10ml and Agar, 20g. The clear zone was measured in 4th day of incubation at 30ºC (27). The experiment included negative controls to identify spontaneous hydrolysis which is not due to enzymatic activity.

2.6.2. Esterase activity assay

Quantitative esterase activity was determined spectrophotometrically using *p*NP-alkanoates substrate at 410 nm. In brief, for esterase activity assay, reaction mixture in a total volume of 1 ml contained 50 mM Tris–HCl buffer (pH 7.5), 1 mM *p*-nitrophenyl ester and 50 µg of the crude protein (esterase) extract to the reaction mixture (28). The reaction was started by addition of the partially purified (enzyme) extract at 37ºC for 10 min. One unit of esterase activity was the amount of enzyme that released 1 µmol of *p*-nitrophenol per min under standard conditions. Blanks without enzyme were also performed.

2.6.3. PHB Depolymerase Assay

Extracellular PHB depolymerase activity was assessed by spot test assay (28) as well as with turbidimetric method with slight modifications. In order to test the *e-phaZ* activity through spot assay, PHB enriched agar plates were prepared by mixing sonicated homogeneous 0.1% PHB suspension with 1.5% (w/v) agar in 50 mM Tris–HCl buffer (pH 7.5). Partially purified sample (100 µg) was loaded in 5mm in diameter hole in the PHB–agar plate and incubated at 30°C for 20 h. On the other hand, in turbidimetric method, a total of 200µL of standard reaction mixture containing 50mM Tris–HCl buffer (pH 7.5) and 0.1% of sonicated homogenous suspension of PHB in deionized water was used. The reaction was started by the addition of 100µl enzyme solution (100 µg) and after 24 hr incubation; the turbidity decreased at 650 nm was measured against substrate buffer blanks. One unit (U) of enzyme activity was defined as the amount of enzyme which catalyzes the decrease of 0.01 absorbance units per minute at 650 nm in the assay conditions (30).

2.7. Biodegradation studies on other polymers

Qualitative estimation of the hydrolytic activity of PHA depolymerase from *S.*sp.ND-3 toward different polymers was performed by a drop test on indicator plates. Briefly, 5 ml of 1% (wt/vol) polymer emulsion was mixed with 5 ml of 1.5% (wt/vol) agar in 50mM Tris-HCl buffer, pH 7.5 and poured on a glass plate. The bacterial cells were grown on the gel at 30°C. The diameters of the resulting clearing zones were observed after a period and it is correlated with the enzyme activity.

2.8. SDS–PAGE and Zymogram analysis for butyrate esterase

The molecular mass of the protein was determined using SDS-PAGE (31) and Esterase hydrolytic activity was identified by zymogram. The proteins were resolved on native gel (10 %) at a constant voltage (50 V) at 4ºC. After electrophoresis, gel was washed with 50 mM Tris-HCl (pH 7.5) twice for 10 min each. The gel was soaked in the 0.1 % (w/v) methyl umbelliferyl butyrate (dissolved in Dimethylformamide) prepared by the same 50mM Tris-HCl (pH 7.5) buffer and incubated at 30ºC for 30 min. After visualizing the gel under UV transilluminator, the region of flurogenic hydrolysis was noted by the appearance of fluorescing zone.

2.9. Effect of pH and temperature on the enzyme activity

The activity of butyrate esterase was evaluated at different pH values and temperature. Optimum pH for butyrate esterase activity was determined over a pH range of 4 to 13 and also the optimum temperature range for enzyme activity was determined by carrying out the enzyme assay at different temperatures ranging from 20 to 100 ºC.

2.10. SEM analysis of degraded polymeric film

For SEM examination, polymer pieces, which had been incubated in Glycerol-Casein-KNO_3 with and without bacterial inoculum, were immersed in PBS buffer. The samples were mounted on aluminium stumps, coated with gold in a sputtering device for 3-10 min at 15 mA and examined under a Scanning Electron Microscope (TESCAN VegaTc, CIe, Central Instrumentation facility, Madurai Kamaraj University).

3. RESULTS AND DISCUSSION

There is a world-wide research effort to develop biodegradable polymers as an alternative option for non-degradable polymers accumulated in the environment. Biodegradation (i.e. biotic degradation) is a chemical degradation of polymers provoked by the action of microorganisms such as bacteria, fungi and algae (32). There are number of environmental isolates involved in the biodegradation of PHA include, *Bacillus*, *Pseudomonas, Streptomyces, Aspergillus, Penicillium*, *Acidovoraxs* and *Variovorax* (33, 34), on the other hand, to the knowledge, PHB degradation by Gram positive, particularly by *Streptomyces* sp. has not been reported much. Therefore, in this work, a widely employed screening technique such as agar plates containing emulsified polymer was used to screen and assess the degradation potential of different microorganisms, since, a promising biodegradable polymer degradation have been investigated in various environments such as terrestrial and aquatic by many researchers Mergaert et al. (1995) (16) and Mergaert et al. (1993) (13) .Six PHB-degrading extracellular depolymerase producing bacteria were isolated from natural environmental sample such as effluent and landfill soil. Serial dilutions of homogenized samples were spread on PHB-mineral agar plate consisting of 0.1% PHB with M9 medium (35) and their abilities to degrade aliphatic biodegradable polyester PHB was evaluated by clear zone method. Among the screened bacterial isolates, only ND-3 was found to be potential due to its ability to utilise 0.1% PHB at 30ºC (**Fig. 1A**) by producing a clear zone (diameter) around the colony. This happens when the polymer-degrading microorganisms excrete extracellular enzyme which diffuse through the agar and degrade the polymer into water soluble materials. The degradation activity of the strain *S.sp*.ND-3 was detectable by the formation of a clear zone around the colony on 4th day itself by secreting e-PHB depolymerase and degradation rate was increased with increasing period of incubation and complete clarification of the turbid medium was obtained in 4th day of incubation. Previous work demonstrated that the complete degradation of PHB was studied for a period of 8 days (36) and efficient degradation of PHB by marine isolate observed in 4th day of incubation (37). This could be the first report that the soil isolate ND-3 completely degraded PHB in 4 days. The esterase activity of strain ND-3 was examined also by tributyrin supplemented agar plate and a spectrometric assay using p-nitrophenyl esters as substrates. Both methods had been generally employed in the screening of esterase (38).The esterase activity of strain ND-3 was tested preliminarily on 1% tributyrin plate with an enzyme extract of ND-3 or cells of ND-3. The plates were incubated at 30ºC for 96h, resulting in the halo formation depending on the enzyme activity. The tributyrin-hydrolyzing activity was quantified based on the diameter of the clearance (**Fig. 1B**). PHB depolymerase activity of ND-3 was estimated from the time of appearance and the diameter of clearing zones in drop tests on thin agar plates containing 0.1% (w/v) PHB in 50 mMTris-HCl, pH 7.5 at 30°C by using protein sample. Zone diameter was found to be 20 mm (**Fig. 1C**). The seleceted *S*.sp. ND-3 was able to degrade other polymers such as P (3HB-co-12%-3HV), Poly (L-lactide) and Poly (1, 4-butylene succinate), which was further confirmed by the presence of clear zone. Distinct clear zone was observed on PHBV and PBS plates after 14-15 and 28-30 days respectively at 30ºC. The strain did not form clear zone on PLA (**Fig. 1D, E & F**). The clear zone method was confirmed that the population of aliphatic polyester-degrading microorganisms at 30ºC decreased in the order of PHB > PHBV > PBS. Pranamuda et al. (1997) (39) and Tansengco and Tokiwa (1998) (40) reported that the population of aliphatic polyester-degrading microorganisms at 30 and 50°C decreased in the order of PHB = PCL > PBS > PLA. Suyama et al. (1998) (14) reported that 39 bacterial strains of class Firmicutes and Proteobacteria isolated from soil were capable of degrading aliphatic polyesters such as PHB, PCL and PBS, but no PLA-degrading bacteria were found. These results showed that PLA-degrading microorganisms are not widely distributed in the natural environment and thus, PLA is less susceptible to microbial attack in the natural environment than other microbial and synthetic aliphatic polyesters. However, further studies on *S.sp*.ND-3 may be useful for the possible exploitation in industrial applications.

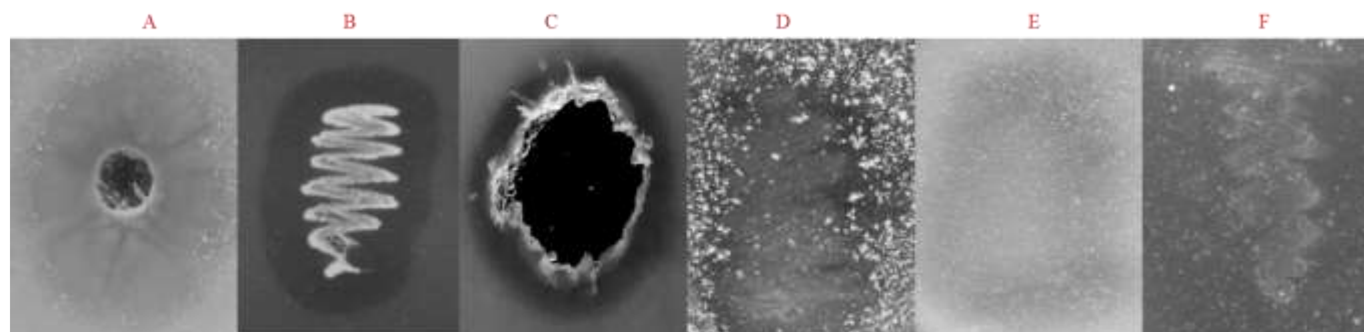

Fig. 1 Clear zone formation of *Streptomyces* sp.ND-3 in Nutrient Agar and M9 Solid medium. A- Zone of clearance by *Streptomyces* sp.ND-3 on 0.1% PHB emulsified M9 media (30mm in diameter). B- Zone of clearance by *Streptomyces* sp.ND-3 on 1% Tributyrin containing M9 media.C- Spot test assay of PHB depolymerase on Tris-Agar media emulsified with 0.1% PHB. The diameter of the zone was measured (20mm in diameter). Degradative ability of *Streptomyces* sp.ND-3 on other aliphatic polymers D-PHBV, E-PBS, F-PLA.

The selected isolate was further characterized by morphological, physiological and biochemical properties. Strain ND-3 showed 93% identity with *Streptomyces* species (**Fig. 2**) and the phylogenetic tree was constructed. The sequence was analysed using BLASTN (41) and deposited in NCBI GenBank (Accession No: KM823664) database.

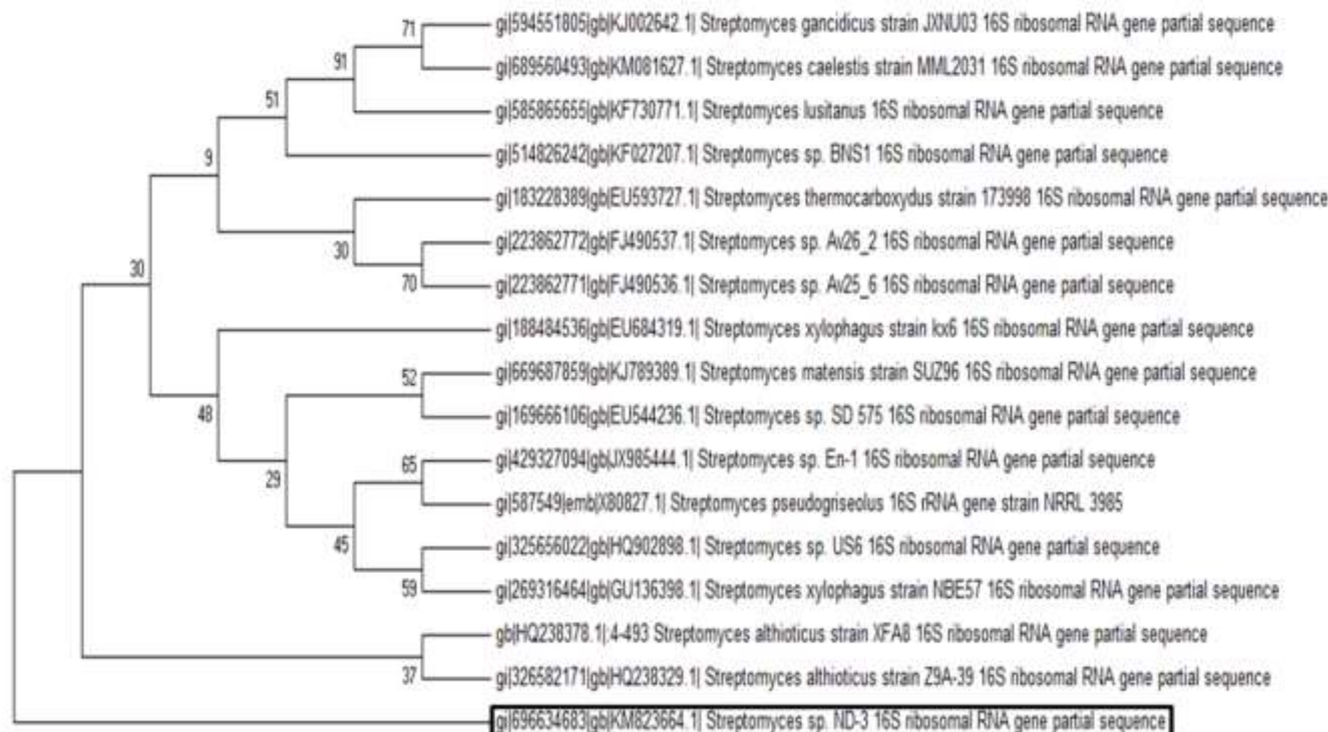

Fig. 2 Phylogenetic neighbor-joining tree construction using partial 16S rRNA gene sequences of *Streptomyces* sp.ND-3 by MEGA6

Specific substrate preference of esterase was determined by comparing the specific activity towards various *p*-NP ester substrates such as p- *p*-NP butyrate (C4),p- *p*-NP hexanoate (C6), *p*-NP octanoate (C8), *p*-NP decanoate (C10), *p*-NP dodecanoate(C12) and *p*-NP palmitate (C16) under standard assay condition (28). The esterase activity of ND3 was showed the highest with *p*NPB (165.8U/mg protein) compared to other substrates (*p*NP-hexanoate, *p*NP-octanoate, *p*NP-decanoate, *p*NP-dodecanoate & *p*NP-palmitate 121.6, 40.8, 23.83, 14.28 & 6.12 U/mg respectively) (**Fig. 3**). The quantification of esterase activity revealed that the strain *S*.sp.ND-3 showed substrate preference with *p*-nitrophenlybutyrate than other substrates. This may be due to the substrate specificity of ND-3. On the other hand, it activity with mcl *p*NP-alkanoate was significantly lower. When compared with mcl PHA, scl shows more esterase activity. The colonial growth of the *Streptomyces sp.* over the entire plates interfered with the observation of clear zone formation. Therefore, an alternative liquid medium was used to assay the degradation activity.

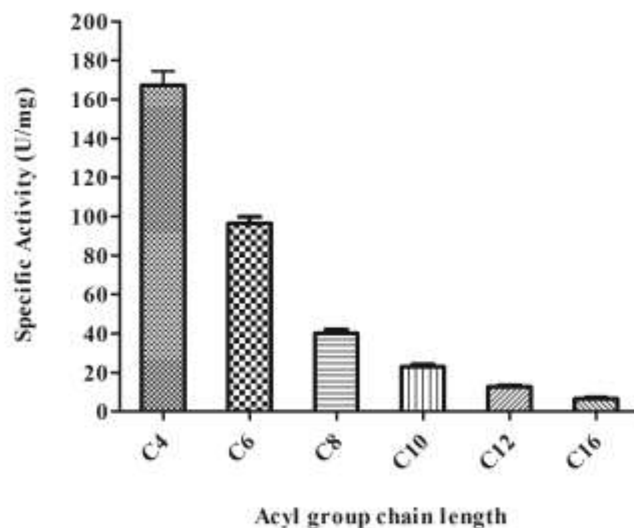

Fig. 3 Specific activity of esterase. The substrate specificity of esterase was measured by hydrolysis of *p*-nitrophenyl carboxylic acid esters using different *pNP*-alkanoates as substrates. This graph summarises three independent experiments.C4- Butyrate, C6- Hexanoat, C8- Octanoate , C10- Decanoate ,C12- Dodecanoate and C16- Palmitate . Graph was plotted by Graph pad Prism.

*S.*sp.ND-3 cell-free culture supernatant were precipitated with increasing amounts of ammonium sulphate (0-100%) and maximal precipitation was achieved with 80% $(NH_4)_2SO_4$. The molecular mass of the ammonium sulphate purified enzyme by SDS-PAGE was approximately 48kDa (**Fig. 4A**). The zymogram analysis using 10% native PAGE of PHB depolymerase with flurogenic substrate 4-methylumbelliferyl butyrate (MUB), which is a substrate for butyrate esterase revealed the zone of fluorescence at ~48kDa (**Fig. 4B**). SDS-PAGE analysis of depolymerase gave protein band around 48kDa which was confirmed by the flurogenic substrate 4-methylumbelliferyl butyrate (MUB). The 4-methylumbelliferone moiety, released from the hydrolysed substrate, is fluorescent when viewed at 366 nm with an intensive UV source (42). Partially purified butyrate esterase from *S.*sp.ND-3 was active over wide range of pH of 6-9 with maximum activity at pH 7.5 (**Fig. 5A**). It is evident from **Fig. 5B** that the enzyme was active at the temperature range of 30–50 ^{o} C and showed a steep decent in activity above 50 ^{o} C.

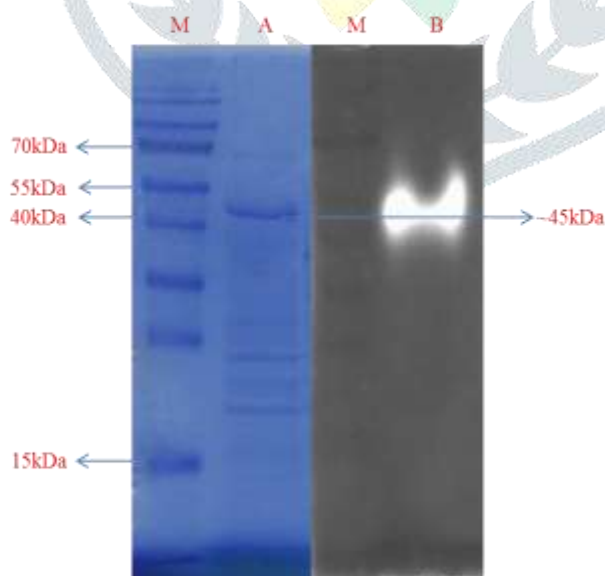

Fig. 4 SDS PAGE (A) and Zymogram (B) analysis of partially purifed PHB depolymerase(btyrate esterase) protein (80% saturated and dialysed) from *S.*sp. ND-3. Lane M - Protein marker (fermentas), lane A & B- PHB depolymerase from *Streptomyces* sp.ND-3.

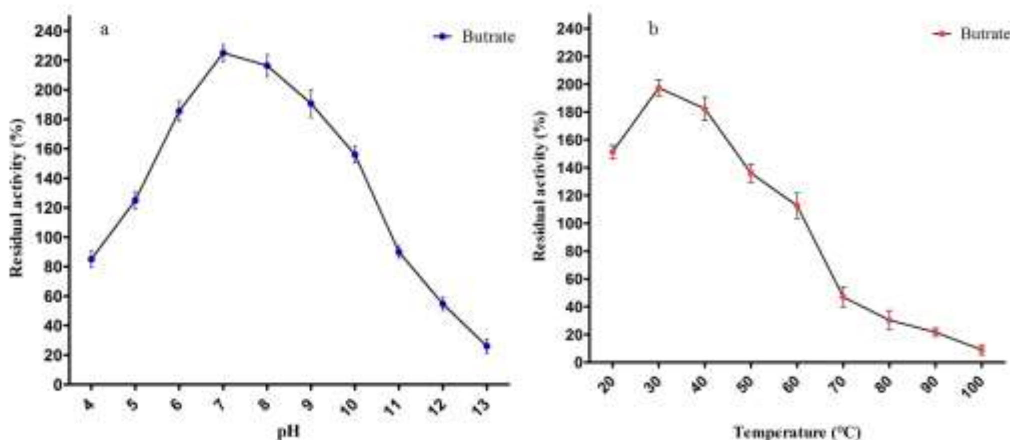

Fig. 5 Effect of physical parameters on butyrate esterase.A- Effect of pH on enzyme activity; B- Effect of temperature on enzyme activity.

Enzymatic degradation of biopolyesters including PHB, PHBV, PBS and PLA were studied using a polyhydroxyalkanoate (PHA) depolymerase produced by *S*.sp ND-3 by SEM analysis. The depolymerase cleaves the ester bonds and disintegrates the polymer. The PHA degradation activity observed was in the descending order as PHB>PHBV>PBS. PHBV and PBS degradation was very slow compared to PHB homopolyester. At the same time, PLA could not be degraded by the strain *S*.sp.ND-3. This explains that the distribution of PLA-degrading microorganisms in the environment is limited which inferring that the degrading agent excreted by the strain shows a wide range of substrate specificity. This is in agreement with the earlier reports of (43, 37).

The degradation patterns of PHB and P (HB/12% HV) were observed by Scanning Electron Microscopy. The polymer samples incubated for seven days at 30°C with ND-3 and without were removed and processed for SEM analysis. Examination of polymers showed that the complete degradation of PHB and irregular pits and lesions in case of PHBV, when the polymer was incubated with ND-3 (**Fig. 6C**). The SEM examination of control film and polymer incubated in sterile culture media were smooth, continuous, without any holes but possessed well-defined shallow pits of variable size (**Fig. 6A &B**). The SEM observation indicated that the degradation starts as soon as the microorganisms begin to colonize the film and secretes enzymes that breakdown the polymer into monomer. This suggested that surface morphology might have a relationship with polymer degradation, i.e., when degradation starts, holes on the surface allowed enzymes and water molecules/microbes to get in contact with the surface, thus around the holes faster degradation starts, thereby holes became bigger and bigger more enzymes and water molecules leading to maximum degradation. It is assumed that the mycelial growth of the bacterium penetrates into the lesion and secretes depolymerase to initiate the hydrolysis of polymer (44).

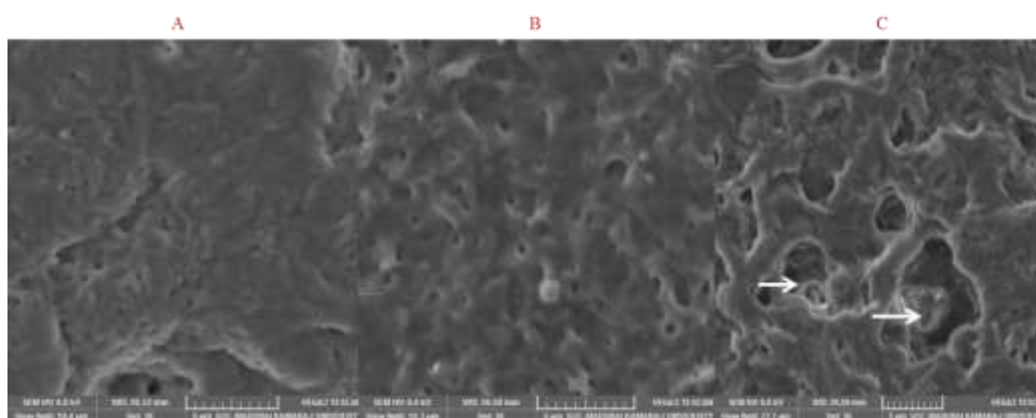

Fig. 6 Scanning Electron Microscopic examinations of PHBV films A- Control PHBV, B- PHBV without microbe, C- PHBV Incubated with *Streptomyces* sp.ND-3. Arrow indicates the PHBV degradation by *Streptomyces* sp.ND-3.

4. ACKNOWLEDGEMENTS

The authors sincerely thank University Grants Commission, New Delhi for financial support (UGC-MRP No.F.No.39-206/2010 (SR)**.** The authors are thankful to UGC-NRCBS, DBT-IPLS, SBS, for state-of-art facility and central instrumentation facility, MKU. The authors sincerely thank Department of Zoology and Biotechnology, Lady Doak College for academic support.

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