

## Characterization of Poly and Depolymerase From *Amycolatopsis* sp.



### Botany

**KEYWORDS :** *Amycolatopsis*, Poly(3-hydroxy butyrate) (P-3HB), PHB depolymerases, SDS-PAGE, butyric acid

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### ABSTRACT

*Poly (3-hydroxy butyrate)(P-3HB) and its copolymers are accumulated as an intracellular storage compound within the cells of a wide variety of bacteria. P (3HB) and its copolymers are a biodegradable material that serves as a exogenous carbon source for many microorganism in the environment. The microorganism secrete extracellular PHB depolymerases to degrade environmental P(3HB) and utilize the decomposed compounds as nutrients. A study was conducted to isolate *Amycolatopsis* strain from the rhizosphere soil and degradation of emulsified PHB by the strain. The polymerases was characterized. The single PHB de polymerases degrade high molecular weight PHB to butyric acid. The concentration of >90% was significantly decreased within 8 days by bacteria. The molecular mass of PHB depolymerases was 24kDa was determined by SDS-PAGE. The optimum conditions for the enzyme activity were pH 5.0 and 45°C. The enzyme was stable for 30minutes at a temperature lower than 50°C and stable at pH higher than 2.0 but it was unstable at pH 1.0.*

### INTRODUCTION

Bacterial polyesters like (P-3HB) have attracted industrial attention as environmentally degradable thermoplastic used for a wide range of agricultural, marine and medical applications (Holmes,1985). A number of (P-3HB) degrading microorganism have been isolated from various environments such as soil (Jendrossek et al.,1993, Kausya et al.,1994, Mergaert et al.,1993) laboratory atmosphere, sea water (Mukai et al.,1993 and Kita et al.,1995), lake water (Mukai et al.,1994), activated sludge, and anaerobic sludge (Janssen and Harfoot,1990). Analysis of structural genes of several PHB depolymerases has shown that the enzymes are comprised of an N-terminal catalytic domain comprising lipase box as an active site, C-terminal putative substrate binding domain (SBD), and a linear domain connecting the two domains. (Briese et al., 1997).

Extracellular PHB depolymerases can be classified into three types based on the location of the active site (lipase box) in the catalytic domain and on the sequence of the linker domain. Recently (Ikura and Kudo,1999) also isolated: *Amycolatopsis* sp. 3118 and identified it as PLA degrading bacterium. With the development of petrochemical industry, about one hundred million tons of chemically synthesized plastics are produced per year, are nor biodegradable cause rising environmental pollution. (Doi, 1990). Poly (3-hydroxy butyrate) (P-3HB) has been synthesized by more than 75 microbial species. The microorganisms accumulate PHB as a polymeric material for preserving energy in their cells (Anderson and Dawes, 1990).

Some PHB depolymerases have serine, histidine and aspartate residues in their active sites and most PHB depolymerases are deactivated by diisopropyl fluorophosphate Phenylmethylsulfonyl fluoride and dithiothreitol, indicating the importance of serine residues and disulfide bonds in the activation of the enzymes (Sadocco et al., 1997). The used PHB is normally buried the ground and can be decomposed by the extracellular PHB depolymerases secreted by the soil microbes. In this study, the extracellular PHB depolymerases from *Amycolatopsis* was isolated from soil, was purified in order to examine the capability for PHB decomposition, biochemical characteristics and the active sites of the enzyme.

### MATERIALS AND METHODS

Rhizosphere soil sample were plated on a screening agar media to isolate *Amycolatopsis* sp. The organism was further confirmed using conventional microbiological methods such as Gram staining, culture on selective media and biochemical methods based on standard protocols described by Gordon *et al.*,1974.

### Morphological and biochemical characteristics of the PHB degrading strains

PHB degrading colonies on the agar plate containing ISP medium 2 were fixed with 2% (v/v) glutaraldehyde, degraded in 50 to 100% (v/v) ethanol lyophilized in 2, 2-dimethylpropanol by the critical point method. The lyophilized colonies coated with platinum-vanadium and were observed under a Hitachi S-4200 scanning electron microscope. Menaquinones and phospholipids were extracted and analysed by Minnikin *et al.*,1984.

### Degradation and assimilation of emulsified PHB in the cultures of *Amycolatopsis* sp.

PHB degrading strains were cultured in ISP medium 1 at 37°C for 2 days and collected by centrifugation. The collected bacteria (wet weight, 1g) were inoculated into the PHB emulsified liquied mineral medium (150ml) and cultivated at 37°C with shaking at 125 strokes per minute. Two milliliters of the cultures were taken every 24hours were lyophilized. The lyophilized samples were hydrolysed in 1M NaOH at 100°C for 1hr. The hydrolysates were neutralized with 1M HCl. Butyrate was eluted with the same buffer and determined spectrophotometrically at 210nm. Lithium butyric acid was used as a standard.

### PHB Degradation by the concentrated culture supernatant

The PHB emulsion (0.9ml) was mixed with 100 fold concentrated culture supernatant (0.1ml) and the mixture was incubated at 37°C for 24hours. A portion (0.1ml) of the culture was withdrawn, lyophilized and solubilised in 10µl of 1M HCl. The solubilised sample was spotted onto a thin layer plate. The thin layer plate was developed with mixture of ethyl acetate, toluene, water and formic acid (2.3/1.2/0.9, v/v) was sprayed with 5% phosphomolybdate.

### Assay and purification of PHB depolymerases activity

The (0.1%Wt/vol) was emulsified with plysurf A210G (0.01%, WT/VOL) in 10Mm potassium phosphate buffer (pH-7.0) and was used as a substrate. Mixtures of enzymes solutions (5ul) and the PHB emulsion (45µl) were put into the wells of a 96 well multiple and were kept at 37°C for 30 min with continuous shaking at 500rpm. The emulsion was measured at a wavelength of 630nm using a multiplate reader. The concentrated culture supernatant was dialyzed against 20mM potassium phosphate buffer (pH6.0) and applied onto a TSK gel CM-Toyopearl 650m column equilibrated with the same buffer. Adsorbed proteins were eluted with a descending linear gradient of ammonium sulfate (1 to 0M), and active fractions were combined and dialyzed against 10mM potassium phosphate buffer (pH7.0). The purified PLA depolymerases thus obtained was divided into small portions and was kept at 80°C until use.

### PHB depolymerase assay

The culture solution from mineral PHB media was centrifuged for 15min at 8000rpm and the supernatant was collected. Dry

cell weight was determined according to (Odo *et al.*, 1995) after extraction of the PHB in the cells with chloroform/water (1:1) mixture followed by drying at 105°C for 18 hrs. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was done (Laemmli,1970). The protein bands of the PHB depolymerases in an SDS-polyacrylamide gel was blotted onto a polyvinylidene difluoride sheet by (Matsudaira, 1987). The gel was washed 30 min in a 12.5% trifluoroacetic acid solution in demineralized water and incubated for 50 min in a butyric acid 3% HAC buffer. The butyric acid was washed away carefully with dematerialized water after which the gel was stained with Coomassie brilliant blue R-250. The protein concentration was measured with Bio-Rad protein assay using Bovine serum as the standard protein.

**Effect of PH and temperature on the PHB degrading activity of the purified enzyme**

PHB degrading activity of the purified (0.1µg) was assayed under standard conditions expect for pH (pH 3.5 to 10) and temperature (30 to 100°C). The purified enzyme (0.13µg) was kept at pH 3.5 to 10 at 40c for 24 hrs and residual activity was assayed under standard conditions. The purified enzyme (0.13µg) was kept at 30 to 100°C for 1hrs, and residual activity was assayed under standard conditions. The PHB degrading activity obtained under standard conditions was considered 100% activity.

**RESULT AND DISCUSSION**

Isolation of PHB degradation *Amycolatopsis sp* from Rhizosphere soil were studied for their morphological and biochemical properties (Fig-1). Degradation of the emulsified PHB by the strains, residual PHB was quantified as the concentration of butyric acid after alkaline hydrolysis of the culture fluid. The concentration of butyric acid significantly decreased after the cultivation for 2 to 3 days and >90% of PHB was consumed by the bacteria within 8 days. Degradation products from PHB were analysed using thin layer chromatography (Fig-2). The spots to monomeric and oligomeric butyric acid were visible (Nakamura *et al.*,2007).

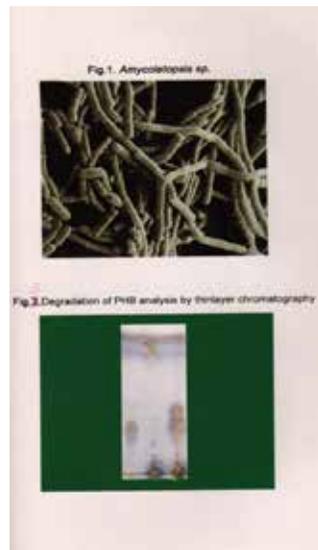
Purification of an extracellular PHB depolymerases from K104-2 and molecular weight and N-terminal amino acid sequence of the purified protein (Table-1). PHB degrading activity was assayed as the ability to decrease turbidity of the PHB emulsion enzyme fractions were incubated with 0.1% (w/v). PHB emulsion in 10mM Potassium phosphate buffer (pH-7.1) at 37°C for 0 to 30min and turbidity of the PHB emulsion was monitored spectrophotometrically at 630nm (Fig-4). The purification of the enzyme by (Nakajima *et al.*,1993).

The molecular mass of the purified PHB depolymerases was 24 kDa determined by SDS-PAGE (Fig.3). The isoelectric acid point of the enzyme was >10 as estimated by using a 5% polyacrylamide gel containing Ampholine at pH-7 to 11. Effect of pH and temperature on the PHB degrading of the emulsified PHB of the purified enzyme was assayed under standard conditions expect for pH (pH 3.5 to 10). In the pH, range of >5, PHB degrading activity of the enzyme increased with increasing pH, reaching the maximal value at pH 9 (Fig.5). In contrast, no enzyme activity was detected under acidic conditions (pH<5). The PHB degrading activity of the enzyme was assayed at temperature of 30 to 100°C. The maximal activity of the enzyme was observed at 55 to 60°C, but no activity was detected at >80°C (Fig.6). Therefore supernatant of K 104-2 using various enzymatic substrates such as synthetic substrates for proteases, esterase and lipase. Further characterization of the PHB depolymerases and molecular cloning of the gene encoding the enzyme is in progress.

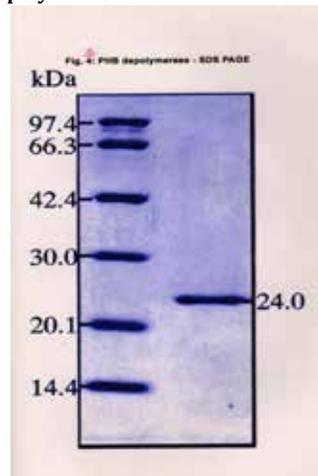
**Fig-1. *Amycolatopsis sp.***



**Fig-2. Degradation of PHB analysis by TLC**



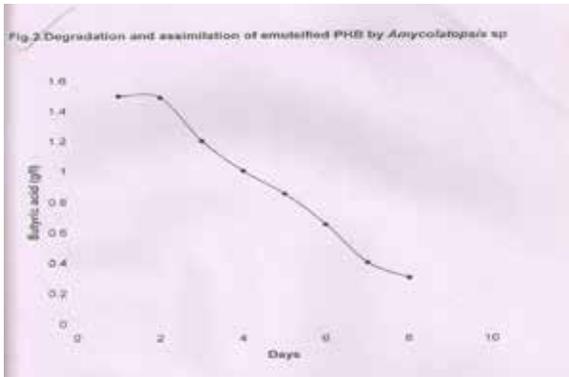
**Fig-3 PHB depolymerase-SDS PAGE**



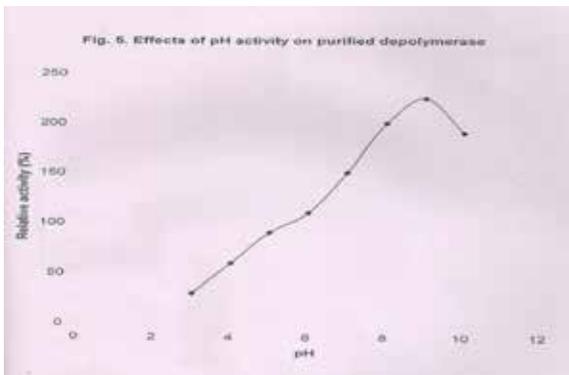
**Table-1. Purification of PHB-degrading enzyme from the culture supernatant of *Amycolatopsis sp.***

S. No	Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg of protein)	Yield (%)	Purification
1.	Culture supernatant	12.1	13.3	1.10	100	1
2.	CM-Toyopeal 650 M	0.881	6.69	7.58	50.1	6.9
3.	CM-5PW	0.188	4.07	21.6	30.5	19.7
4.	Phenyl- 5PW	0.093	2.39	25.7	17.9	23.5

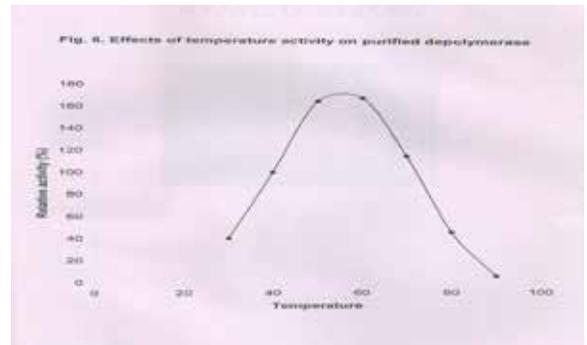
**Fig-4 Degradation and assimilation of emulsified PHB by *Amycolatopsis* sp.**



**Fig-5. Effects of pH activity on purified depolymerase**



**Fig- 6. Effects of temperature activity on purified depolymerase**



**ACKNOWLEDGEMENT**

The authors thank to Dr. A.Panneerselvam, Associate Professor of A.V.V.M Sripushpam college, Poondi and Sri Gowri Biotech Research Academy, Thanjavur (Dt), Tamilnadu for the laboratory facility.

**REFERENCE**

Anderson, A.J. and Dawes, E.A., 1990. Occurrence, metabolism, metabolic rate and industrial uses of bacterial polyhydroxyalkanoates. Microbiol. Rev. 54:450-472. | Briese,B.H., Jendrossek,D. and Schlegel H.G., 1997. Degradation of poly (3- hydroxybutyrate-co-3-hydroxyvalerate) by aerobic sewage sludge. FEMS Microbiol.Lett.117:107-112. | Doi.Y.1990 Microbial polyester, VCH, Newyork. | Gordon,R.E., Barnett,D.A., Handernan,J.E and Pang C.H.1974. Nocardia coeliaca,Nocardia autotrophica and the Nocardian strain. Int.J.Syst. Bacteriol.:54- 63. | Holmes,PA.1985. Application of PHB:A microbially produced biodegradable thermoplastic. Phys.Technol.16:32-36. | Jendrossek,D., Knoke,I., Habibian,R.H., Steinbu chel, A. and Schlegel, H.G., 1993. Degradation of poly(3-hydroxybutyrate),PHB, by bacteria and purification of a novel PHB depolymerases of Comamonas sp. J.Environ.Polym.Degrad.1:53-63. | Kasuya,K., Y. Doi and Yao. T., 1994. Enzymatic degradation of poly(R)-3- hydroxybutyrate) by Comamonas testosterone ATSU of soil bacterium. Polym.Deg.Stab.45:379-386. | Kita,K., K.Ishimaru, M.Teraoka, H.Yanase and N.Kato.1995. Properties of poly (3- hydroxybutyrate) depolymerases from a marine bacterium, Alcaligenes faecalis AE122. Appl.Environ.Microbiol.61:1727-1730. | Laemmli,U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685. | Matsudaira, P., 1987. Sequence from Picomole Quantities of Proteins Electroblooded onto Polyvinylidene Difluoride Membrane. The Jorl. Biol. Chem., (262): 10035-10038. | Mergaert, J., Webb, A., Anderson, C., Wouters, A. and Swings, J., 1993. Microbial degradation of poly (3-hydroxybutyrate) and poly (3-hydroxybutyrate co-3- hydroxyvalerate ) in soils. Appl.Environ.Microbiol., 3233-3238. | Minnikin,D.E., A.G.O'Donnell, M.Goodfellow, G.Alderson, M.Athalye, A.Schaal and J.H.Parlett.1984. An integrated procedure for the extraction of bacterial isoprenoid quinines and polar lipids. J.Microbiol.Methods, 2:233-241. | Mukai,K., Yamada, K. and Doi. Y., 1993. Kinetics and mechanism of heterogenous hydrolysis of poly (R)-3-hydroxybutyrate) film by PHA depolymerases. Int.J.Biol.Macromol.15:361-366. | Mukai,K., Yamada, K. and Doi. Y., 1994. Efficient hydrolysis of polyhydroxyalkanoates by Pseudomonas stutzeri YM1414 isolated from lake water. Polym.Deg.Stab.43: 319 - 327. | Nakajima,N., Mihara, H. and Sumi. H., 1993.Characterization of potent fibrinolytic enzymes in earthworm, Lumbricus rubellus. Biosci. Biotechnol.Biochem.57:1726-1730. | Nakamura, Y., Tanaka, F., Haraguchi, N., Mimori, K., Matsumoto, T., Inoue, H., Yanage, K., Mori, M., 2007.Clinicopathological and biological significance of mitotic centromere associated kinesin over expression in human gastric cancer. Br.J.Cancer 97(4):543-549. | Oda,Y., Asare, H., Urakami, T. and Tonomura, K., 1995. Microbial degradation of poly(3- hydroxybutyrate) and polycaprolactone by filamentous fungi. J.Ferment.Bioeng.80:265-269. | Sadacco,P., Nocerino, S., Dubini-Paglia, E., Seres, A. and Elegir, G., 1997. Characterization of a poly (3-hydroxybutyrate) depolymerase from Aureobacterium saperdae: Active site and kinetics of hydrolysis studies. J.Environ.Polym.Deg rad.5: 57-65.