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Effect of Lipase and esterase in Biodegrading Poly (butylene succinate co adipate) (PBSA)

KEYWORDS: Microorganism isolation, enzymatic degradation of PBSA, PBSA Poly(butylene succinate co adipate), Thermostable lipase and esterase

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ABSTRACT *Various microorganisms were screened for their ability to degrade poly (butylene succinate co adipate) (PBSA). Strain S-32, which was newly isolated from a soil sample, was selected as the best strain S-32 could degrade both solid and emulsified PBSA. During the degradation, a lipase activity was observed in the culture broth. This lipase and esterase activity was induced more strongly by PBSA than by tributyrin which are typical substrate of lipase and esterase. These observation strongly suggest that this lipase and esterase was involved in the PBSA biodegradation in strain S-32. From taxonomical studies, the strain was tentatively described to belong to the genus Pseudomonas aeruginosa.*

Introduction

Plastic wastes is the one of the factors in causing environmental pollution, because of their semi- permanent stability in the environmental. According to a National Academy of science report, approximately 4.5 × 10^4 metric tons of plastic are discarded to the ocean annually (Edward *et al*., 1975). There is growing interest in developing biodegradable polymers for at least two reasons: one is the serious environmental problem of plastic wastes: the other is the effective medical application of biodegradable polymers. Biodegradable polymers are degraded to low molecular weight compounds because of water or the action of the enzymes excreted by microorganisms. Aliphatic polyesters, such as Poly (butylene succinate co adipate) (PBSA), poly (butylene succinate)(PBS) poly(^{4}-hydroxybutrate)(PHB), and poly(lactic acid), are known be the biodegradable polymers that can be easily decomposed by the cleavage of ester bond linkage in an acidic and basic condition (Chandra *et al*., 1998; Hocking *et al* 1992). It is known that factors affecting biodegradation behaviour are polymer structures such as stereochemistry, hydrophilicity, flexibility of chains, and polymer morphology such as the crystallinity, size, form, and number of crystallites.(Abe *et al*., 1995; Koyama *et al*.,1997)

Poly (butylene succinate co adipate) (PBSA)and its copolymers have been developed for use as biodegradable plastics. Their biodegradability has been confirmed by the filed tests in soil and sea water (Kumagai *et al*., 1992). However, the degradation mechanism has not yet been well understood because few enzymes have been isolated from the microorganisms that degrade PBS. Considering the responsibility of the enzymes that are liberated from microorganisms for the biodegradation, the enzymatic degradation of various biodegradable polymers has been a subject to study. For example, the enzymatic degradability of poly (butylene succinate co adipate) (PBSA), Poly(butylene succinate) (PBS) has been studied various type of lipases that are either conventionally available or newly isolated from the specific bacteria.(Tokiwa *et al*., 1986; Doi *et al*., 1990). Their degradation products have also been isolated and identified to clarify the mechanism of enzymatic degradation. For instance, it has been reported that PBSA is degraded by commercially available hydrolytic enzymes, such as lipase (Ando *et al*., 1998), but enzymes which are actually secreted by PBSA degrading microorganisms in PBSA still unknown.

Assessment of the distribution of the plastic degrading microorganisms at waste sites and the effects of disposal of biodegradable plastics on the microbial ecosystem is important since these plastic should be used as environmentally releasable materials, For this purpose, studies about plastic degrading microorganisms are essential for the synthesis and evaluation of new biodegradable plastics.

In this paper, we reported on the physiological characteristic and degradation activity of a novel PBSA degrading bacterium isolated from soil. Studied about PBSA degrading enzymes and their inductivity are also shown.

Methodology

Preparation of emulsified PBSA

Cylindrical-shaped Poly (butylene succinate co adipate) (PBSA Bionolle # 3020) and Poly(butylene succinate) (PBS Bionolle #1020: showa Highpolymer co Ltd). The sizes of PBS and PBSA were 2.5 mm diameter × 4 mm. The average molecular weight of PBSA was 58,000 g mol and 60,000 g mol, respectively. Emulsions were prepared by using the detergent Plysurf A210G (Daiichi Kogyo Seiyaku Co., Kyoto, Japan). 2 g of PBSA was dissolved in 40 ml of dichloromethane and transferred into 250 ml of distilled water containing 40 mg of Plysurf A210G and blended in a homogenizer (10,000 rpm for 4 min). The emulsified solution was then incubated at 80°C for 3 hrs under a lab hood to remove dichloromethane.

Screening of PBSA degrading microorganism

Soil samples were collected from various sites from South India (Tamil Nadu & Kerala) for screening of PBSA-utilizing bacteria. 0.2 g of each soil samples were transferred to different test tubes which contained 10 ml of the basal medium composition with PBSA as the sole carbon source (mg l^{-1}):

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KH_2PO_4, 200: K_2HPO_4, 1600: $(NH_4)_2SO_4$, 1000: $MgSO_4.7H_2O$, 200: $FeSO_4.7H_2O$, 10: $MnSO_4.4H_2O$: 0.5; $ZnSO_4.7H_2O$, 1: $CuSO_4$., 0.1 and $CaCO_3$, 5000. The final pH was 7.0 For the solid medium, emulsified PBSA was added and 15 mg/L agar was added.) The medium was incubated at 30°C with shaking condition. After a week, 0.5 ml of culture broths was transferred into the tubes containing fresh basal medium. This procedure was repeated for five times. Single-colony isolation was done using PBSA-emulsified solid medium. Isolates which showed a halo zones were stored for further work.

Identification of bacterial strain
Isolated soil bacteria were characterized and identified using standard biochemical methods according to Bergey's (Holt *et al.*, 1994) manual of systematic bacteriology. Moreover 16S rRNA gene sequencing was carried out to find the species level.

Protein Estimation
The protein concentration was determined by (Lowry's *et al.*, 1951) method with Bovine Serum Albumin as a standard.

Partial purification by ammonium sulphate fractionation
The intracellular lipase was partially purified by ammonium sulphate fractionation for use in esterification. The cell free extract was subjected to protein fractionation by addition of small increments of solid ammonium sulphate at 4°C with constant stirring to obtain three fractions i.e 0-10%, 10-20%, 20-30%, 30-40%, 40-50%, 50-60%, 60-70%, 70-80%, 80-90%, and 90-100%. When all the ammonium sulphate was dissolved at the end of each fractionation range, the mixture was allowed to stand for overnight at 4°C followed by its centrifugation at 10,000 x g for 30 minutes at 4°C. The pellet was collected and the supernatant was used as starting material for next fractionation. The collected precipitate of each fractionation range was re-suspended in small volume of 0.05M phosphate buffer (pH 7.0) and checked for the enzyme activity and protein content.

Enzyme assay
Assay of Lipase
The activity of free lipase was determined spectrophotometrically using p-nitrophenyl palmitate (p-NPP) as substrate according to the method of (Nawani *et al.*, 2006) with some modifications. The reaction mixture containing 0.3 mL of 0.05M phosphate buffer (pH 8.0), 0.1 mL of 0.8 mM p-NPP and 0.1 mL of lipase was incubated at 37 °C for 10 min. The reaction was then terminated by adding 1 mL ethanol. A control was run simultaneously, which contained the same contents but the reaction was terminated prior to the addition of enzyme. Absorbance of the resulting yellow colored product was measured at 410 nm in a spectrophotometer.

Esterase activity assay
The esterase activity was determined by using *p*-nitrophenyl butyrate (*p*-NPB) as substrate. Briefly, 0.4 ml of substrate solution (10 mM dissolved in 2-propanol) was mixed with 3.6 ml of a solution containing 50 mM Tris-HCl buffer (pH 10.0) and 11.7% NaCl. After pre-incubation at 30°C for 10 min, the enzymatic assay was initialled by adding 0.2 ml of the purified enzyme solution to the reaction mixture and incubated at 50°C for 10 min. Following the addition of 2.0 ml of Na2CO3 solution (0.25 M) to stop the reaction, the amount of *p*-nitrophenol (*p*-NP) released was measured at 410 nm against a blank. One unit (U) was defined as the amount of enzyme liberating 1 µmol of *p*-NP per minute. The specific activity was expressed in the units of enzyme activity per milligram of protein.

Determination of molecular weight
The molecular mass of the partially purified lipase was determined by SDS – PAGE as described by (Laemmli *et al.*,1970) using 12.5% acrylamide gel. Standard protein marker was used.

Results
Screening of PBSA degrading microorganisms
Total of 100 different soil samples have been collected from states of Tamil nadu and Kerala and stored in sterile air tight bag. The number of sample from different regions were as follows: Coimbatore(12), Tirupur (8), Erode (6), Salem (8), Ooty (5), Metupalayam (5), Madurai (12), Theni (3), Kambam (2), Palakad (9), Cochin(14), Chennai(9) and Trichy (7). 32 strains were primarily isolated from 100 samples by using basal medium containing PBSA as a sole carbon source. By performing subsequent experiments, 5 isolates showed highest ability to form haloes on the emulsified PBSA agar plates and it was confirmed by weight reduction method. (Fig no:1).

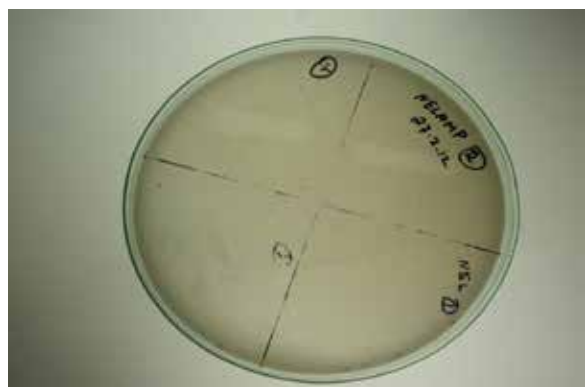

Fig 1. PBSA degrading organism in solid agar

Table 1. Degradation of PBSA pellets by different strains

S.No	Strains No	PBSA (Before) in mg	PBSA (After) in mg
1	1	58	52
2	2	61	57
3	5	67	64
4	9	104	87
5	14	108	105
6	17	88	81
7	21	85	82
8	23	105	101
9	29	107	102
10	32	135	62

Identification of PBSA degrading strain:
Strain S-32 was a rod – shaped aerobic G^{-ve} bacterium, Oxidase – positive, Catalase – positive, nitrate reduction – positive, Indole production – negative, Urease – negative, Gelatin hydrolysis – positive. Based on the biochemical characteristic, the strain was identified as *Pseudomonas* sp. 16S rRNA gene sequencing was performed and submitted to Gen bank (The Gen Bank accession No. is KC522650). The 16S rRNA gene sequence of S_{32} was close to *Pseudomonas aeruginosa.*

Sequence Alignment of *Psuedomonas aeruoginosa*
CCCTCTTTTCGCCCCTCAGTGTCAGTATCAGTCCAGGTGGTCGCCTTCGC

CACTGGTGTTCCTTCCTATATCTACGCATTTCACCGCTACACAGGAAATT

CCACCACCCTCTACCGTACTCTAGCTCAGTAGTTTTGGATGCAGTTCCCA

GGTTGAGCCCGGGGATTTCACATCCAACTTGCTGAACCACCTACGCGCGC

TTTACGCCCAGTAATTCCGATTAACGCTTGCACCCTTCGTATTACCGCGG

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CTGCTGGCACGAAGTTAGCCGGTGCTTATTCTGTTGGTA-ACGTCAAAACA

GCAAGGTATTAACTTACTGCCCTTCCTCCCAACTTAAAGT-GCTTTACAAT

CCGAAGACCTTCTTCACACACGCGGCATGGCTGGA-TCAGGCTTTCGCCCA

TTGTCCAATATTCCCCACTGCTGCCTCCCGTAGGA-GTCTGGACCGTGTCT

CAGTTCCAGTGTGACTGATCATCCTCTCAGACCAGT-TACGGATCGTCGCC

TTGGTAGGCCTTTACCCCACCAACTAGCTAATCCGAC-CTAGGCTCATCTG

ATAGCGTGAGGTCCGAAGATCCCCCACTTTCTCCCTCAG-GACGTATGCGG

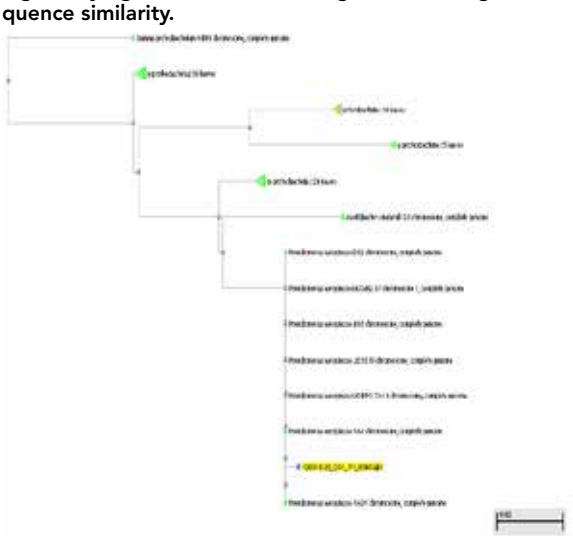
TATTAGCGCCCGTTTCCGGACGTTATCCCCCACTACCAG-GCAGATTCCTA

GGCATTACTCACCCGTCCGCCGCTGAATCCAGGAGCAA-GCTCCCTTCATC

CGCTCGACTTGCATGTGTTAGGCCTGCCGC-CAGCGTTCAATCTGAGCCAG

AATTCAAACTCTAAAATTTCTAAGTTTCCTA

Fig:2 Phylogenetic Tree of P.aeruginosa showing 99% sequence similarity.



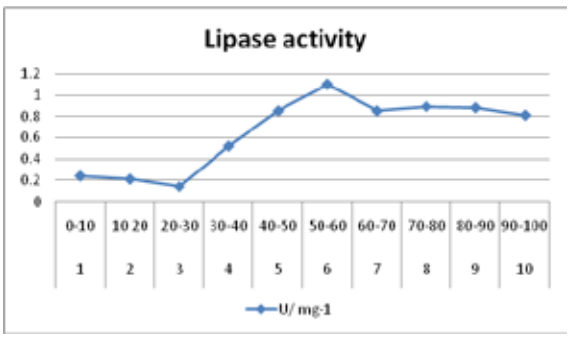
Protein Estimation

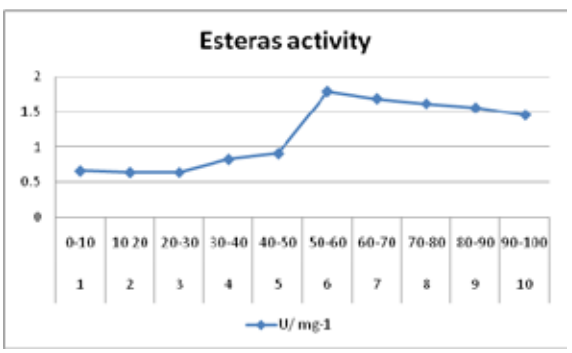
The protein concentration was determined by Lowry *et al* method with Bovine Serum Albumin (BSA) as a standard and S_{32} was used as a test organism. Estimation of protein has been performed on supernatant and pellets of crude culture filtrate.

Partial purification using ammonium sulphate fractionation

The culture filtrate was purified by ammonium sulphate precipitation method using the range of 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100%. Most of the lipase and esteras activity was recovered at 60% saturation. This partially purified lipase and esteras had specific activity of 1.10 IU mg-1 and 1.78 IU mg-1.

Fig:3 Lipase Activity





Determination of molecular weight

SDS –PAGE analysis of lipase exhibited a single band with molecular mass estimated to be 30 KDa.

Lipases with lower molecular weight have advantage as smaller enzymes and are more stable due to smaller changes in tertiary structure (Sharon *et al.*, 1998).

Discussion

Although some mesophilic and thermophilic microorganisms have been reported to be PBSA degraders (Pranamuda *et al.*, 1996; Kleeberg *et al.*,1998), *Pseudomonas aeruginosa* isolated in this study had not been reported previously and is a novel PBSA degrading bacterium. Moreover, no PBSA degrading microorganism has been reported before, so this is the first report of a bacterial isolate degrading PBSA. However, we suppose that most PBS degraders possess PBSA degrading activity.

Since and PBSA have very similar chemical structures and physical properties. A lipase and esterase activity was detected in the culture broth of *Pseudomonas aeruginosa* which grew on PBSA. This lipase activity was induced more strongly by PBSA than by tributyrin which are typical substrate of lipase. These observations strongly Suggests that this lipase is a PBSA degrading enzyme. It is a well known that PBSA are hydrolyzed by thermostable lipase (Ando *et al.*, 1998; Tokiwa *et al.*, 1977).

Conclusion

Thus from the present study, it is concluded that the enzyme thermo stable lipase was proved to be the enzyme responsible for PBSA degradation and was highly active at pH7 and at a temperature of 55°C and thus the same environmental condition was also highly effective in degrading poly (butylene succinate co adipate) (PBSA).

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