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**RESEARCH ARTICLE**

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

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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 activity was induced more strongly by PBSA than by tributyrin which are typical substrate of lipase.These observation strongly suggest that this lipase was involved in the PBSA biodegradation in strain S-32.From taxonomical studies, the strain was tentatively described to belong to the genus *Pseudomonasaeruginosa*.



## Introduction

Plastics have been widely used as basic materials in various industries because of their excellent characteristics and low cost. On the other hand, unfortunately, plastics have caused severe environmental problems with increased waste quantity. For this reason, biodegradable plastics which can be degraded into water and carbondioxide by microorganisms have attracted attention recently. Biodegradable plastics lend themselves to microbial degradation, however, studies on the degrading microorganisms in particular to biochemical and enzymologic research on such degrading microorganisms, have been releatively sparse[1-3]

Poly (butylene succinate co adipate) (PBSA) is one of the synthetic polyester based biodegradable plastic which possess many good characteristics and its use is expected to become widespread as an alternative material to ordinary plastics. However, information about PBSA degrading microorganisms is limited [1-5]. In this study, we have isolated and identified PBSA degrading bacteria from various environmental sources & enzyme activity was observed using PBSA as a substrate.

## Material and Methods

### Preparation of emulsified PBSA

PBSA (Bionolle # 3020:showa Highpolymer co Ltd) 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 microorganisms

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 $l^{-1}$): $KH_2PO_4$, 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 g l -1 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 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 method with Bovine Serum Albumin as a standard.

**Enzyme purification (Lipase)**
The cell-free supernatant was prepared by centrifugation (10 000rpm, 15 min) of culture broth and it was passed through a 0.45-Wm pore size membrane filter at 4˚C. To the filtered supernatant, ammonium sulfate was slowly added under stirring to achieve saturation. The suspension was centrifuged (10 000rpm, 20 min) and then, ammonium sulfate was added to the supernatant to reach 20 – 100 % saturation at 4˚C. The final supernatant was collected by centrifugation at 10 000rpm for 20 min, 4˚C. The dissolved enzyme solution was dialyzed against the same buffer for 16 hrs at 4˚C to remove the residual ammonium sulfate.

**Enzyme assay**
The enzyme assay was done both in crude enzyme and partially purified enzyme.

**A] Lipase assay in crude enzyme**
The fermented culture was harvested and filtered and centrifuged. The cell free supernatant was collected and used as a crude enzyme. The lipase activity in the crude enzyme was determined titrimetrically on the basis of olive oil hydrolysis [14].

**B] Lipase assay in partially purified enzyme**
The lipase activity in the partially purified enzyme was determined titrimetrically on the basis of olive oil hydrolysis[14] at different saturation levels 20 - 100%

**Enzyme characterization**

**A] Effect of carbon source**
The utilization of various carbon sources like solid and emulsified PBSA, succinic acid, 1,4 butanediol andAdipic acid were used for lipase production at different periods of incubation as 24, 48, 72 and 96 hrs.

**B] Substrate specificity in solid media**
The hydrolytic abilities of the PBSA degrading isolate was examined by the clear zone method using substrate (PBSA) with different concentration of0.1 ,0.2 , 0.3 ,0.4 , 0.5 , 0.6 , 0.7 ,0.8 , 0.9 and 1ml/gm in basal medium plates.

**C] Substrate specificity in liquid media**
The strain was inoculated with different concentration of emulsified PBSA from 0.1 to 1 ml and the lipase activity was determined titrimetrically on the basis of olive oil hydrolysis.

D] **Effect of Temperature on Lipase**
For the optimum temperature determination, lipase activity was determined by titration at different temperature in the range of 27, 37, 45, 55, 65 and 75°C at pH 7.0 at 100mM phosphate buffer for crude as well as partially purified enzyme.

**E] Assay for Thermostable Lipase**
Partially purified lipase was incubated at 60, 65, 70, and 75°C for upto 24hrs in 100 mM phosphate buffer at pH 7.0 and residual activity was determined.

**F] Effect of pH on Lipase**
The crude and partially purified lipase was incubated at 60°C with different pHof 6, 7, 8, 9 and 10 in 100mM phosphate buffer. The activity was determined by titrimetrically.

**G] Determination of molecular weight**
The molecular mass of the partially purified lipase was determined by SDS – PAGE as described by[15] using 12.5% acrylamide gel. Standard protein marker was used.

## Result and Discussion

**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 (3), Cochin(14), Chennai(9) andTrichy (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).

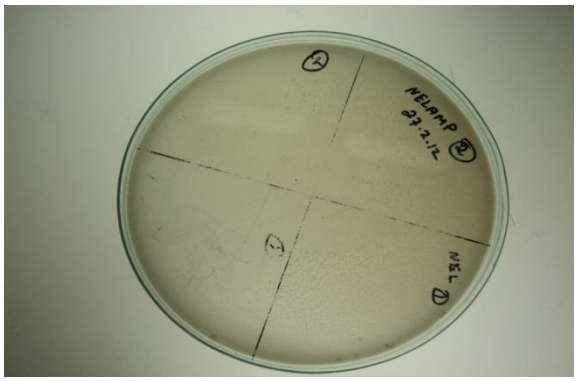

**Fig 1. PBSA degrading organism in solid agar**

| 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** |

**Table 1. Degradation of PBSA pellets by different strains**

**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 *Psuedomonasaeruoginosa***

CCCTCTTTTCGCCCCTCAGTGTCAGTATCAGT
CCAGGTGGTCGCCTTCGC

CACTGGTGTTCCTTCCTATATCTACGCATTTC
ACCGCTACACAGGAAATT

CCACCACCCTCTACCGTACTCTAGCTCAGTAG
TTTTGGATGCAGTTCCCA

GGTTGAGCCCGGGGATTTCACATCCAACTTG
CTGAACCACCTACGCGCGC

TTTACGCCCAGTAATTCCGATTAACGCTTGCA
CCCTTCGTATTACCGCGG

CTGCTGGCACGAAGTTAGCCGGTGCTTATTCT
GTTGGTAACGTCAAAACA

GCAAGGTATTAACTTACTGCCCTTCCTCCCAA
CTTAAAGTGCTTTACAAT

CCGAAGACCTTCTTCACACACGCGGCATGGC
TGGATCAGGCTTTCGCCCA

TTGTCCAATATTCCCCACTGCTGCCTCCCGTA
GGAGTCTGGACCGTGTCT

CAGTTCCAGTGTGACTGATCATCCTCTCAGAC
CAGTTACGGATCGTCGCC

TTGGTAGGCCTTTACCCCACCAACTAGCTAAT
CCGACCTAGGCTCATCTG

ATAGCGTGAGGTCCGAAGATCCCCCACTTTC
TCCCTCAGGACGTATGCGG

TATTAGCGCCCGTTTCCGGACGTTATCCCCCA
CTACCAGGCAGATTCCTA

GGCATTACTCACCCGTCCGCCGCTGAATCCA
GGAGCAAGCTCCCTTCATC

CGCTCGACTTGCATGTGTTAGGCCTGCCGCC
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AATTCAAACTCTAAAATTTCTAAGTTTCCTA

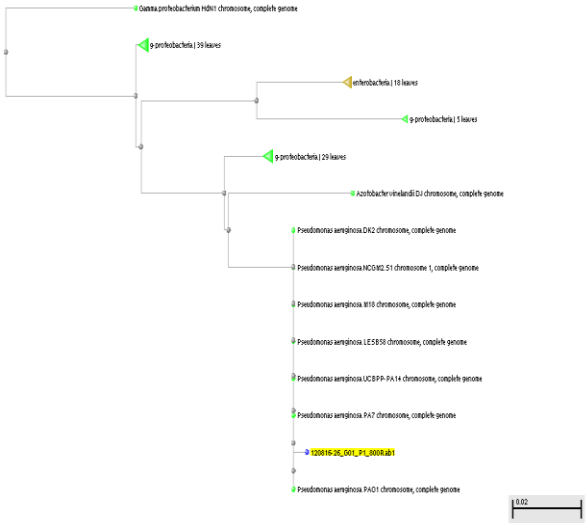


Fig:2 Phylogenetic Tree of *P.aeruginosa* showing 99% sequence similarity.

**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.

**Enzyme purification – Lipase**

The culture filtrate was purified by ammonium sulfate precipitation method using the range of 20, 40, 60, 80 and 100%. Lipase activity has been evaluated on each range. Among the ranges, 60 - 80 % of ammonium sulfate precipitation alone showed highest activity of lipase. (5100 U/ml) in (Table 2).

Units/mlenzyme=NaOH×MolarityofNaOH×1000×2/ 1.

NaOH=Volume of NaOH used for test-Volume of NaOH used for blank

1000=Conversion factor from milliequivalent to microequivalent

2=time conversion factor from 30min to 1hr (Unit definition)

1=Volume of enzyme used.

| S.No | Percentage (%) | U/ml |
|---|---|---|
| 1 | 0 – 20 | 2600 |
| 2 | 20 – 40 | 3600 |
| 3 | 40 – 60 | 4500 |
| 4 | 60 – 80 | 5100 |
| 5 | 80 – 100 | 3000 |

**Table 2. Lipase activity in Ammonium sulfate precipitation**

**Enzyme characterization**

**Effect of carbon source**

The utilization of various carbon sources and lipase production by strain S32 were examined (Table3). The strain could grew on glucose ,tributyrin, succinic acid,adipic acid and PBSA but the lipase activity was more induced by emulsified PBSA.

| S. No | Medium | 24hr (U/ml) | 48hrs (U/ml) | 72hrs (U/ml) | 96hrs (U/ml) |
|---|---|---|---|---|---|
| 1 | BM+ Glucose (1%) | 200 | 200 | 200 | 200 |
| 2 | BM+ Maltose (1%) | 300 | 200 | 300 | 200 |
| 3 | BM+ Sucrose (1%) | 200 | 200 | 300 | 200 |
| 4 | BM+ Starch (1%) | 300 | 300 | 600 | 300 |
| 5 | BM+ Lactose (1%) | 300 | 200 | 600 | 300 |
| 6 | BM+1,4-Butanediol | 600 | 400 | 300 | 200 |
| 7 | BM+Tributyrin | 400 | 400 | 400 | 300 |
| 8 | BM+ Succinic acid | 200 | 300 | 300 | 200 |
| 9 | BM+Adipic acid | 200 | 300 | 300 | 200 |
| 10 | BM+ PBSA | 600 | 900 | 1200 | 900 |

Table:3 Effect of carbon source (crude enzyme)

**Substrate specificity in solid media**

The hydrolytic abilities of the PBSA degrading isolate was examined by the clear zone method using substrate (PBSA) with different concentration of 0.1 ,0.2 , 0.3 ,0.4 , 0.5 , 0.6 , 0.7 ,0.8 , 0.9 and 1ml/mg in basal medium plates. Halo zone formation was observed in all the plates but highest halo zone was seen in 1 ml concentration.

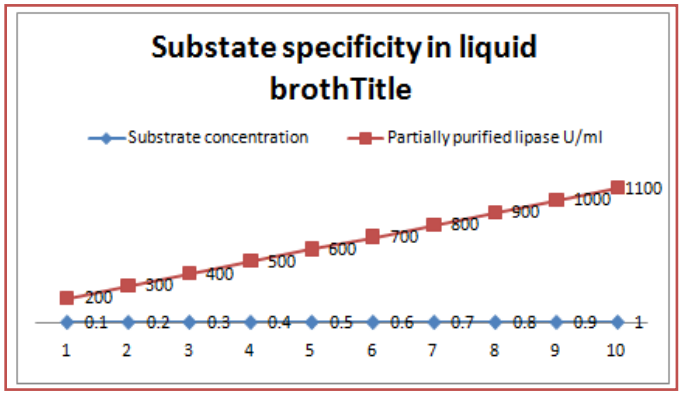


**Fig3.Substate specificity in liquid broth**

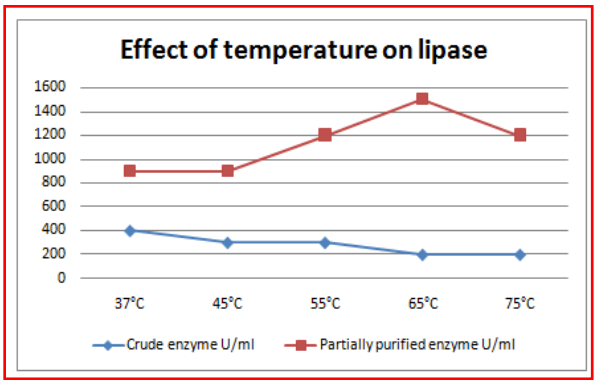


**Fig4. Effect of temperature on lipase**

Table shows the effect of temperature on lipase activity. It was detected in temperature range of 37 - 75°C at pH 7.0. The optimum temperature for crude enzyme and partially purified enzyme was 65°C and by 55°C respectively. The optimum temperature with highest activity were chosen for thermostability study.

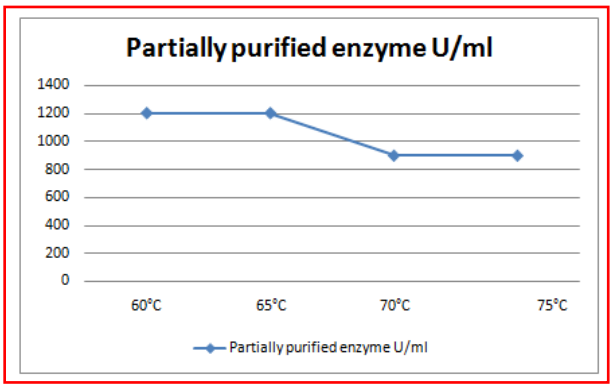


**Fig5. Assay for Thermostable Lipase**

Table shows the results of thermostability lipase. Partially purified lipase was incubated at 60, 65, 70, and 75°C for up to 24 hrs in 100 mM phosphate buffer at pH 7.0 and residual activity was determined.

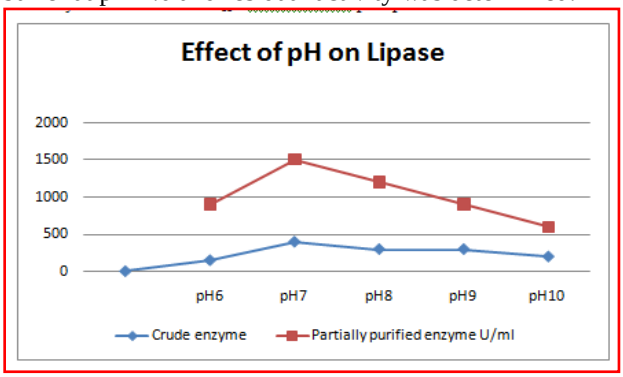


**Fig6. Effect of pH on Lipase**

The crude and partially purified lipase was incubated at 60°C at different pH of 6, 7, 8, 9 and 10 in 100 mM phosphate buffer. The activity was determined by titrimetrically. Maximal activity was observed at $P_H$7 in Potassium phosphate buffer.

**Determination of molecular weight**

SDS –PAGE analysis of lipase exhibited a single band with molecular mass estimated to be 30KDa. Lipases with lower molecular weight have advantage as smaller enzymes and are more stable due to smaller changes in tertiary structure [16].

## Discussion

Although some mesophilic and thermophilic microorganisms have been reported to be PBSA degraders(1-5),*Pseudomonas* 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 activity was detected in the culture broth of *Pseudomonas* which grew on PBSA. This lipase activity was induced more strongly by PBSA than by tributyrin which are typical substrate of lipase. These observationsstrongly Suggests that this lipase is a PBSA degrading enzyme. It is a well known that PBSA are hydrolyzed by lipase[17,18],Unfortunately, most of the enzymatic analysis reported in the literature have been performed using commercially available enzymes and not enzymes isolated from PBSA degrading microorganisms.

There are only a few reports on enzymes degrading synthetic polyester type biodegradable plastics. The diversity of plastic degrading enzymes will be clarified if the characteristics of many other plastic degrading enzymes from microorganisms are also studied in future.

## 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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