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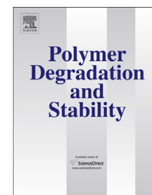
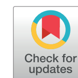


# Growth associated degradation of aliphatic-aromatic copolyesters by *Cryptococcus* sp. MTCC 5455



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A B S T R A C T

The degradation of aliphatic-aromatic copolyesters, poly[(butylene succinate/terephthalate/isophthalate)-*co*-(lactate)] (PBSTIL) and poly(butylene adipate *co*-terephthalate) (PBAT) under mesophilic conditions was investigated using the yeast, *Cryptococcus* sp. MTCC 5455. Complete degradation of PBSTIL and PBAT films was observed at 25 °C within 96 and 216 h, respectively. Lipase was produced during the course of degradation and the encoding gene was expressed in *Escherichia coli* BL21 (DE3). The production of lipase was enhanced by cumulative induction with 0.1 mM IPTG and 1.5 mM lactose for 24 h at 25 °C, which resulted in a maximum lipase activity of 27.75 ± 1.0 U/mL. Spectral studies of the degraded polymeric films confirmed the hydrolysis of ester bonds by the lipase. Scanning electron microscopy revealed the formation of cracks and holes on the surface of the polymeric films during degradation. The results indicated the feasibility of the process towards degradation of polymeric waste at ambient temperatures within a short period of time.

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## 1. Introduction

The use of synthetic polymers in various industries has caused serious environmental problems due to its lack of degradability and accumulation in landfill sites [1,2]. It has been estimated that around 8 million tons of global plastic waste reaches the oceans every year polluting the marine ecosystem [3]. Degradable polymers are considered as viable alternative to combat the adverse effects of conventional plastic waste due to the presence of heteroatoms in their main chain that are susceptible to hydrolytic cleavage [4]. The developed aliphatic biodegradable polyesters like poly(L-lactide) and poly(butylene succinate) have limited market coverage owing to their weak cohesive energies, poor mechanical and thermal properties. These properties were improved by addition of aromatic units into the main chain of aliphatic polyesters [5]. The commercially produced aliphatic-aromatic copolyesters combine the biodegradability of aliphatic polyesters with the excellent material properties of aromatic polymers [6]. These polymers are used in the manufacture of compost bags, agricultural mulch film, packaging materials, transparent films for wrapping food, polyester fabrics and other biodegradable resins [7].

The reports available on microbial degradation of aliphatic-aromatic copolyesters are limited. Among them, the thermophilic actinomycetes, *Thermobifida fusca* [8] and *T. alba* [9] are known to degrade aliphatic-aromatic copolyesters at high temperatures. Partial degradation of these polymers was reported using the mesophilic bacterium *Leptothrix* sp. TB-71 [10] and *Roseateles depolymerans* TB-87 [11]. Recently, the degradation of such polymers have been reported under anaerobic conditions using *Clostridium hathewayi* [12], *C. botulinum* [13] and *Pelosinus fermentans* [14] and their esterase genes expressed in *Escherichia coli*. It is expected that the production and disposal of these bioplastics in

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natural environment might increase to 7.8 million tons by 2019 [15] and this led researchers to screen for potent microorganisms, which could hydrolyse the polymers at ambient temperatures. The yeast, *Cryptococcus* sp. MTCC 5455 produces an extracellular lipase that could be used in the methanolysis of vegetable oil [16], removal of triglyceride soil from fabrics [17], production of omega 3-polyunsaturated fatty acid concentrates [18] and degradation of various aliphatic polymers [19–21]. In order to broaden the degradation activity of *Cryptococcus* sp. towards aliphatic-aromatic copolyesters in mesophilic environments, attempts were made to hydrolyse poly[(butylene succinate/terephthalate/isophthalate)-*co*-(lactate)] (PBSTIL) and poly(butylene adipate *co*-terephthalate) (PBAT). The lipase produced during the process was expressed in *E. coli* BL21 (DE3) and the parameters influencing its production were optimized and the enzyme purified to homogeneity. The purified lipase was also evaluated for degradation of polymers, since enzymatic degradation is considered to be an effective strategy for polymer recycling.

## 2. Materials and methods

### 2.1. Materials

The yeast strain, *Cryptococcus* sp. MTCC 5455 used in this study was maintained on potato dextrose agar (PDA) slants at 4 °C [22]. The chemicals used were of analytical or molecular biology grade and acquired from Hi-Media Limited, Sigma-Aldrich and S.D. Fine Chemicals Limited, Mumbai, India. The expression host *E. coli* BL21 (DE3) and plasmid pET-22b(+) were procured from Novagen, USA. The aliphatic-aromatic copolyester, PBAT (Ecoflex™) was obtained as a gift from BASF AG, Germany and the polymer, PBSTIL was synthesized in the molar proportion described by Nakajima-Kambe et al. [10]. The chemical structures of PBSTIL and PBAT is given in Supplementary data, Fig. S1.

### 2.2. Inoculum

*Cryptococcus* sp. was grown in the yeast medium (3.0 g/L yeast extract, 5.0 g/L meat extract, 5.0 g/L peptone and 10.0 g/L dextrose) for 40 h at 25 °C and used as the inoculum ($5.4–5.8 \times 10^8$ cells/mL). The degradation of aliphatic-aromatic copolyesters by *Cryptococcus* sp. was investigated on PDA plates, which were overlaid with PBSTIL and PBAT emulsion, respectively and incubated at 25 °C for 96 h. The emulsions (2 g/L final concentration) were prepared by dissolving the individual polymers in dichloromethane and homogenized using the detergent, Plysurf A210G as per the protocol described by Uchida et al. [23].

### 2.3. Degradation of aliphatic-aromatic copolyesters by *Cryptococcus sp.*

Degradation studies were carried out in 250 mL Erlenmeyer flasks in the medium of Kamini et al. [22] with slight modifications. The composition of the degradation medium was as follows (g/L): yeast extract, 10; lactose, 5; $KH_2PO_4$, 10.0; $MgSO_4.7H_2O$, 1.0 and polymeric film, 1.0. The films (about 0.2 mm thickness) were prepared by heat pressing the polymeric pellets, sterilized in 70% ethanol for 10 min under UV light and used. The flasks were then inoculated with 1% (v/v) of the inoculum and incubated at 25 °C in a shaker at 120 rpm and observed for complete degradation of the polymeric films. Polymer degradation was monitored at 24 h intervals by measuring the weight of film before and after incubation. Films were separated from the medium by filtration using a muslin cloth, washed twice with distilled water, air dried and weighed. Biomass was determined by centrifuging 5 mL of the filtrate at $9000 \times g$ for 10 min and drying the pellet at 105 °C to a constant weight. The lipase activity was estimated from the filtrate by spectrophotometric method using *p*-nitrophenyl laurate (*p*-NPL) as substrate [24]. One unit of lipase activity was defined as the amount of enzyme that liberates 1 μmol of *p*-nitrophenol per minute under the standard assay conditions. The degradation products were extracted with chloroform and evaporated to dryness using a rotary evaporator for further analysis.

### 2.4. Expression of lipase in *E. coli*

The DNA fragment encoding the *Cryptococcus* sp. lipase (NCBI accession number - AB671329) was cloned into *MscI*- and *NotI*-digested pET-22b(+) vector in order to express the lipase gene fused to the *pelB* leader sequence at the N-terminus [25]. The constructed plasmid (Fig. 1) was transformed into *E. coli* BL21 (DE3) for expression studies. The cells were grown in LB broth containing 100 μg/mL ampicillin to mid log phase (0.5–0.8 OD) at 37 °C and induced with 0.1 mM isopropyl β-D-thiogalactopyranoside (IPTG) for 18 h at 25 °C. Consecutive optimization for enhanced production of recombinant lipase was carried out by varying the concentrations of IPTG (0.1–3.0 mM), lactose (0.1–3.0 mM), induction temperature (25–37 °C) and time (6–30 h). The cumulative induction effect of IPTG and lactose on the lipase activity was also determined. The culture was centrifuged at $9000 \times g$ for 10 min to separate the supernatant and cell pellet. The cell pellet was resuspended in lysis buffer composed of 10 mM Tris, 1 mM EDTA, 0.1% SDS and 0.2 mg/mL lysozyme (one fourth of the original culture volume), disrupted by sonication and the supernatant containing the soluble intracellular proteins were recovered. The extracellular (culture supernatant) and intracellular (soluble proteins) fractions were analyzed for lipase activity.

The extracellular supernatant was concentrated by ammonium sulfate precipitation (80% saturation) and subjected to hydrophobic interaction chromatography (HIC) using an Octyl Sepharose CL-4B column packed in a AKTA FPLC system (Wipro GE Healthcare, India) at 25 °C. Gradient elution was carried out using $(NH_4)_2SO_4$ and methanol as described by Nakajima-Kambe et al. [26]. The protein concentration was determined by Lowry et al. [27] and the molecular mass of the purified lipase was estimated by SDS-PAGE using 15% polyacrylamide gel [28].

The purified recombinant lipase was assessed for the degradation of PBSTIL and PBAT films (40–45 mg) in 100 mL screw capped Erlenmeyer flasks containing 10 mL of 0.1 M phosphate buffer (pH 7.0) and 500 U of enzyme at 25 °C and 120 rpm.

### 2.5. Mechanical properties

An Instron universal test system 3369 (Norwood, USA) was used to study the changes in the tensile strength and percent elongation of PBSTIL and PBAT films during the course of degradation.

### 2.6. Fourier transform infrared (FTIR) spectroscopy

FTIR spectra of the control polymer film and degraded products were measured in the transmission mode between the spectral region of 4000 and 500 $cm^{-1}$ using a FTIR-4200 spectrometer (Jasco, Japan) equipped with an Attenuated Total Reflectance (ATR) accessory.

### 2.7. $^1H$ Nuclear magnetic resonance (NMR) spectroscopy

$^1H$ NMR spectra of the polymeric films in deuterated chloroform (7.2 ppm) and degraded products in deuterated water (4.7 ppm) was obtained using an Avance III Bruker spectrometer

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