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Degradation of poly(butylene succinate) and poly(butylene succinate-co-butylene adipate) by a lipase from yeast *Cryptococcus* sp. grown on agro-industrial residues

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ABSTRACT

The yeast, *Cryptococcus* sp. MTCC 5455 was grown on various agro-industrial residues for the production of lipase. A maximum lipase activity of 753 ± 19 U g dry substrate^{-1} (U gds^{-1}) and a biomass of 103 ± 5 mg gds^{-1} was obtained at 25 °C and 120 h using cottonseed oil cake with 71% moisture content and 30% (v/w) inoculum. The recovery of the enzyme was increased by 14.1%, when the fermented substrate was extracted in 2 mM $CaCl_2.2H_2O$ solution. The crude enzyme partially purified by $(NH_4)_2SO_4$ precipitation showed a major 22 kDa protein on SDS-PAGE. The enzyme has good potential for hydrolysis of poly(butylene succinate) (PBS) and poly(butylene succinate-co-butylene adipate) (PBSA) and complete degradation of the polymeric films were observed at 72 h and 16 h respectively. The degradation was evaluated by Fourier transform infrared (FTIR) and ^{1}H NMR spectroscopy. The complete hydrolysis of polymers by *Cryptococcus* sp. lipase makes the process ideal and also serves as a baseline for its exploitation in polymer degradation.

1. Introduction

In the past decade, persistence of synthetic polymers in the environment poses a major threat to natural ecological systems (Hidayat and Tachibana, 2012). These plastics are not incorporated into the carbon cycle and have diminished landfill capacity owing to their low biodegradability. Degradable polyesters, as green materials are considered as an alternative to the current traditional thermoplastics from the perspective of ecological security and waste management (Li et al., 2011; Novotny et al., 2015; Shah et al., 2015). They are designed to degrade completely under environmental conditions or by microbial attack into water soluble compounds. Accordingly, various biodegradable aliphatic polyesters including poly(β-hydroxybutyrate) (PHB), poly(ε-caprolactone) (PCL), poly(butylene succinate) (PBS), poly(butylene succinate-co-butylene adipate) (PBSA) and poly(lactic acid) (PLA) have been developed. Among them, PBS and PBSA are synthesized from di-acids and diols with relatively low production cost and act as substitute for routine plastics due to their equivalent mechanical properties to that of polyethylene and polypropylene (Zhao et al., 2005). There are reports on microbial degradation of PBSA and PBS using *Leptothrix* sp. TB-7 (Nakajima-Kambe et al., 2009), *Azospirillum brasilense* (Wu, 2012), *Aspergillus versicolor* (Zhao et al., 2005) and *Bacillus pumilus* 1-A (Hayase et al., 2004), *Aspergillus fumigates* (Ishii et al., 2008) respectively. However, enzymatic degradation of these plastics is very much limited (Maeda et al., 2005; Li et al., 2011).

Lipases are currently receiving much attention due to their multifaceted applications in various industrial processes such as hydrolysis of oils and fats, alcoholysis, aminolysis, peroxidations, interesterifications, in synthesis of food ingredients and as additives in detergents (Rodrigues and Fernandez-Lafuente, 2010). In

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particular, microbial lipases are more suitable because of their low cost, simple production, susceptibility to expression in host microorganisms, diverse specificity, wide range of pH and thermal operational optima (Sharma et al., 2001). Extensive studies have been carried out with bacterial and fungal lipases and reports are available on lipase producing yeasts such as *Candida rugosa* (Rao et al., 1993; Benjamin and Pandey, 1997), *Yarrowia lipolytica* (Dominguez et al., 2003), *Aureobasidium pullulans* HN2.3 (Liu et al., 2008) and *Pichia lynferdii* (Kim et al., 2010). However, confined investigation has been directed towards the lipase of *Cryptococcus* (Chen et al., 1997). The yeast, *Cryptococcus* sp. MTCC 5455 (earlier cited as *Cryptococcus* sp. S-2) produces α -amylase, lipase, polygalacturonase and xylanase (Kamini et al., 2000) and could be utilized for wastewater treatments. The efficacy of *Cryptococcus* sp. lipase has been shown in transesterification of rice bran oil (Kamini and Iefuji, 2001), removal of triglyceride soil from fabrics (Thirunavukarasu et al., 2008) and degradation of polymers, polylactic acid (Masaki et al., 2005) and polyurethane (Thirunavukarasu et al., 2015).

The material cost of the medium is one of the important parameters for commercialization of any fermentation processes to make it viable. An intriguing approach to produce industrial enzymes is solid state fermentation (SSF) as it involves low cost substrates, less energy and space prerequisite, simpler equipment, higher productivity and easier downstream processing (Pandey, 2003; Demir and Tari, 2016). Moreover, there is an increasing trend towards effective use of agro-industrial residues for production of value-added products using SSF (Nidheesh et al., 2015). Most of the studies on lipase production by SSF have been carried out with fungi and bacteria, while only few reports are available on synthesis of lipase by yeasts (Rao et al., 1993; Benjamin and Pandey, 1997; Dominguez et al., 2003). This paper describes the production of an extracellular *Cryptococcus* sp. MTCC 5455 lipase, using cottonseed oil cake, for the hydrolysis of PBS and PBSA, since enzymatic degradation is considered to be an ideal plastic waste treatment strategy for polymer recycling.

2. Materials and methods

2.1. Materials

In the present study, all the chemicals used were of AR grade and acquired from Hi-Media Limited, Sigma–Aldrich and S.D. Fine Chemicals Limited, Mumbai, India. The industrial residues, coconut oil cake (COC), cottonseed oil cake (CtOC), gingelly oil cake (GOC), wheat bran (WB) and rice bran (RB) and the inducers were purchased from local market, Chennai, India. Crude protein content of the substrates were analysed according to the ISO 5983–1979 and the oil content was determined in a Soxhlet apparatus according to the ISO 659–1988. The poly(butylene succinate)-co-(butylene adipate) (PBSA- 58,000) and poly(butylene succinate) (PBS-60,000) used in this study were provided by Showa Highpolymer Co. Ltd., Tokyo, Japan.

2.2. Inoculum and fermentation conditions

The yeast strain, *Cryptococcus* sp. MTCC 5455 grown in the YM medium (3 g l^{-1} yeast extract, 5 g l^{-1} malt extract, 5 g l^{-1} peptone and 10 g l^{-1} dextrose, pH 6.2 ± 0.2) for 40 h at 25 °C (5.4–5.8 × 10^8 cells ml^{-1}) was used as the inoculum. Lipase production was carried out in 250 ml Erlenmeyer flasks, each containing 10 g of various agro-residues with an initial moisture content altered to 60% with distilled water. The flasks were inoculated with 30% (v/w) of the inoculum and the contents were well-mixed and incubated at 25 °C for 5 days. Samples were taken at 24 h intervals and assayed for enzyme activity from 48 h, since lipase activity was low at 24 h. Results are expressed as the mean ± SD from repetition of all the experiments in triplicates.

2.3. Lipase production by SSF

Consecutive optimization studies were carried out by varying the initial moisture (50%–75%) and inoculum concentration (15–60%, v/w) for 10 g of substrate. The effect of addition of various organic nitrogen sources (beef extract, meat extract, peptone, tryptone and yeast extract, 5%, w/w), inorganic nitrogen sources (NH_4NO_3, $(NH_4)_2SO_4$, $NH_4H_2PO_4$ and $NaNO_3$, 5%, w/w), carbon sources (fructose, glucose, lactose, maltose, sucrose and starch, 5%, w/w) and inducers (olive oil, sardine oil, sesame oil, soybean oil and sunflower oil, 5%, w/w) to the substrate was studied for optimal lipase production. Time course studies were carried out using optimized parameters to estimate biomass and lipase production in 2.8 L Fernbach flasks with 100 g substrate. For this, 10 g of substrate inoculum grown for 48 h was transferred into Fernbach flask containing 90 g of substrate and incubated for a period of 120 h and assayed for lipase activity.

2.4. Enzyme assay and biomass estimation

At the end of fermentation period, 1 g of the fermented substrate was homogenized with 10 ml of phosphate buffer (0.1 M, pH 7.0) using a pestle and mortar and centrifuged at 8000 rpm for 5 min. The resultant supernatant was used as the enzyme source. The lipase activity was estimated by the spectrophotometric method using *p*-nitrophenyl laurate (*p*-NPL) as substrate at 410 nm and a molar extinction coefficient of 1.68 (Isobe et al., 1988). 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 and the lipase activity was expressed as U g of dry substrate^{-1} (U gds^{-1}). Enzyme from the fermented substrate was extracted with tap water, distilled water, phosphate buffer (0.1 M, pH 7.0), salts (NaCl, $(NH_4)_2SO_4$, $CaCl_2.2H_2O$) and surfactants (Tween 80 and Triton X-100), respectively. The specific activity was determined by estimating the protein content of the enzyme as per the method Lowry et al. (1951). The biomass estimation was carried out by adding 10 ml of distilled water to 1 g of fermented substrate, vortexed for 5 min and centrifuged at 1000 rpm for 5 min to separate the substrate particles. The supernatant thus obtained was again centrifuged at 8000 rpm for 5 min and the biomass was dried at 70 °C until constant weight was achieved.

2.5. Partial purification of Cryptococcus sp. lipase

The crude enzyme produced under the optimum conditions was partially purified by ammonium sulphate precipitation (80% saturation). The precipitate collected by centrifugation was dissolved in 0.1 M phosphate buffer (pH 7.0), dialysed against the same buffer and lyophilized. The molecular weight of the protein was analysed using SDS-PAGE and the proteins were stained with coomassie brilliant blue R-250.

2.6. Scanning electron microscopic (SEM) studies

Fermented substrate samples taken at different time intervals were fixed in 4% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4 for 1 h at 30 °C and washed three times with the same buffer. The samples were then impregnated with 1.25% OsO_4 in 0.1 M phosphate buffer (pH 7.4) for 1 h and dehydrated through a series of 10–100% ascending series of ethanol for 15 min at each stage and finally dried in liquid nitrogen. The dried samples were coated with