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SHORT COMMUNICATION

Production and Characterization of Esterase in *Lantinus tigrinus* for Degradation of Polystyrene

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Abstract

Polystyrene is considered stable to biological degradation. *Lantinus tigrinus* isolated from wood sample produced esterase in growth medium under normal conditions. However, acidic medium, 37°C temperature, presence of tween 80; and urea and yeast extract in mineral salt medium enhance the production of esterase and specific activity. Purified esterase was active at broad pH range and 45°C. FTIR analysis confirmed that esterase produced by *Lantinus tigrinus* effectively degraded polystyrene film and broke macromolecules down to non-toxic molecules. This study concludes that the presence of *Lantinus tigrinus* at dumping sites can be exploited for waste management containing high molecular weight synthetic polymers.

Key words: Lantinus tigrinus, degradation, esterase, inducer, polystyrene

Polystyrene is a synthetic polymer widely used as loose fill packaging foam. The chemical makeup of polystyrene is a long chain hydrocarbon with every other carbon connected to a Phenyl group. Each of the chiral backbone carbon lies at the centre of a tetrahedron, with its four bonds pointing toward the vertices. The presence of chiral backbone carbon makes it highly stable and less susceptible for biodegradation. However, few reports demonstrate that some species of basidiomycetes, *Alcaligenes, Pseudomonas* and *Azotobacter* efficiently degrade polystyrene into low molecular weight compounds (Higashimura *et al.*, 1983; Milstein *et al.*, 1992; Nakamiya *et al.*, 1997; Jang *et al.*, 2006).

Esterases play an important role in maintaining normal physiology and metabolism, detoxifying various drugs and environmental toxicants in living systems (Huang and Ottea, 2004). The hydrolytic mechanism of most of the known esterases resembles the mechanism of lipases and serine proteases (Ollis *et al.*, 1992). Esterases can hydrolyze substrates having regio-, enantio-, or stereo properties (Krishna *et al.*, 2002).

The white-rot fungi genus *Lentinus* is well known to degrade environmental pollutants such as textile dyes (Niebisch *et al.*, 2010); oil pollutants (Adenipekun and

Fasidi, 2005); agro-industry waste and de-colorization of synthetic dyes (Sarnthima et al., 2009); and also other pollutants (Aust and Benson, 1993; Bumpus et al., 1985). This fungus normally possesses an extracellular oxidative enzymatic system; ligninolytic enzymes, for degradation of organic pollutants (Mester and Tien, 2004). Recently, Covino et al. (2010 a, b) reported the presence of cytochrome P-450 epoxide hydrolase system in Lantinus tigrinus involved in degradation of PAHs (chrysene and bezopyrene) and polycyclic hydrocarbons. This strain also produces lignocellulosic enzymes such as cellulose, endoglucanase, laccase and ligninases that degrade aromatic compounds from lignin (Lechner and Papinutti, 2006). The present work demonstrates that production of esterase by Lantinus tigrinus efficiently degrades polystyrene and esterase production is influenced by pH, temperature, inducer and nitrogen source supplied in the medium.

Lentinus tigrinus strain was isolated from wood sample and tested for production of hydrolytic enzymes on mineral salt (MS) medium. The MS media contained 0.25 g/l citric acid; 0.02 g/L CaCl₂; 5 g/L K₂HPO₄; 0.50 g/L MgSO₄·7H₂O; 5 g/L (NH₄)₂SO₄; 2% Tween 80; 10 g/L yeast extract; and 15 g/L agar. *Lentinus tigrinus*

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showed clear zone of hydrolysis on plates. Furthermore, production of esterase was carried out in PYD broth medium containing 5 g/L peptone; 3 g/L beef extract; 5 g/L sodium chloride; 5 g/L yeast extract, and 1 g/L Dextrose. The PYD media was inoculated with 10% fungal homogenate and fermented at 37°C, 150 rpm in shaker incubator for 96 hr. Samples were collected after every 24 hr and esterase activity was performed in cell free extract. To access esterase activity, crude enzyme was treated with 1 M p-nitrophenyl acetate, following the procedure described by Maeda et al. (2005). The optical density was measured in spectrophotometer at 380 nm. Standard curve was drawn by reacting crude enzyme with varying concentration of ρ -nitophenyl (0.01–0.1 mg/ml). One unit of esterase activity is defined as the amount of enzyme which releases 10 µg of p-nitrophenol/ml under the standard assay conditions.

For the morphological identification, Lantinus tigrinus was grown on malt extract media (Merck). After seven days of culture at 37°C, morphological characters were observed. For molecular identification, total genomic DNA was extracted according to the method of Anderson et al. (1996) following amplification of ITS regions by PCR. Universal primers ITS-1 5'-TCCG-TAGGTGAACCTGCGG-3' and ITS-4 5'-TCCTC-CGCTTATTGATATGC-3' were used as previously described by Webb et al. (2000). Amplified products were purified and sequenced using the ABI BigDye Dideoxy Terminator Cycle sequencing kit (Applied Biosystems Inc., Warrington, United Kingdom) following forward and reverse sequences alignment using ABI Auto-assembler software (Applied Biosystems Inc.). The overlapping consensus sequence was compared with sequences in the NCBI database using FASTA 3 sequence homology searches.

For production of maximum esterase, PYD medium was used as described before in all experiments otherwise mentioned. For pH optimization, the pH of the media was adjusted to 3, 4, 5, 6, 7, 8, 9 and 10. For temperature condition standardization, inoculated media (pH 5) flasks were kept on orbital shaker at 30°C, 37°C and 45°C at 150 rpm. Three different substrates as inducer (Tween 20, Tween 80, and olive oil) at 1% each were also examined for enzyme production. Interaction of application of inducer with rage of temperature (30°C, 37°C and 45°C) was also studies. For optimization of nitrogen source, Mineral medium (0.25 g/L Citric acid; 0.02 g/L CaCl.; 5 g/L K, HPO.; 0.50 g/L MgSO₁·7H₂O) with different nitrogen sources was used. All the experiments were performed in triplicate and samples were collected at 24 hr intervals. Enzyme assay was performed as mentioned before. Total protein was also analyzed in all samples following the method described by Lowry et al. (1951).

For partial purification of esterase, the culture filtrate was concentrated by 70% (NH₄)₂SO₄ in 50 mM Tris HCL buffer (pH 8). Concentrated esterase was dialyzed by ultra-filtration (dialyzed by using 12000 M.W cut off dialyzing bag) and chromatographed using Sephadex G-75, eluted with 50 mM tris HCl buffer, pH 8. A volume of 3 ml fraction was collected and assayed for enzyme activity and amount of protein. The procedure for enzyme assay as defined before was followed for the degradation of plastics except that instead of ρ-nitophenyl acetate 0.1% polystyrene film was incubated at 37°C. After 72 hr of incubation, the films were removed and thoroughly washed with distilled water. Degradation of the film surface was initially examined microscopically following FTIR spectroscopic analysis in an Analect RFX-75 spectrometer through standard protocol.

Growth of Lentinus tigrinus in mineral salt medium produced hydrolytic enzyme. The clear region in agar containing petri plates confirmed release of enzyme in the medium under normal conditions. Colonies of the strain were light colored, smooth and flat on the surface with sporous appearance. The isolate was identified by 5.8S, 18S, 28S rRNA internal transcribed spacer (ITS) region sequencing. Sequences were compared with known sequences and deposited to NCBI with accession number EU543989. Increase in pH from acidic to basic decreased production and specific activity of esterase (Fig. 1). Maximum esterase production and specific activity was observed on 4th day when cultured at acidic medium. However; at pH 7 and 8, maximum activity was observed on 5th day of culture. Lantinus tigrinus also prefered neutral to acidic condition for production of esterase like other esterase producing microbes (Buzzini and Martini, 2002; Kudanga et al., 2007). At 30°C, esterase production gradually increased up to 5th day of culture (Fig. 2). However, 37°C was found optimum for production of esterase. At this temperature maximum esterase production (10 U/ml) and maximum specific activity (0.4 µg/ml protein) was attained on 5th day of culture. At all the temperatures, production and specific activity drastically decreased when culture was incubated for more then five days. Maximum esterase production from Aureobasidium pullulans has been reported at 35°C and its production decreased with increase of culturing temperature (Kudanga et al., 2007).

Tween 80 had a positive role in production of esterase (Fig. 3). In all the cases esterase production and specific activity was maximum at 37°C as compared with 30 and 45°C. Tween 20 was also found better for production of esterase at 30 and 37°C. However, in the presence of tween 80 specific esterase activity was higher. The results also describe that *Lantinus tigrinus* does not prefer the presence of fatty acids



Fig. 1. Effect of different pH inducing esterase production by *Lantinus tigrinus*.
 (A) pH 3.0; (B) pH 4.0; (C) pH 5.0; (D) pH 6.0; (E) pH 7.0; (F) pH 8.0; (G) pH 9.0; (H) pH 10.0, enzyme activity is expressed in U/ml and specific activity in µg/ml protein.



Fig. 2. Effect of different temperature inducing esterase production by *Lantinus tigrinus*.
(A) 30°C; (B) 37°C; (C) 45°C, enzyme activity is expressed in U/ml and specific activity in μg/ml protein.

(olive oil) for production of esterase. The presence of surfactants increases cell membrane permeability, increasing the adsorption of nutrients and release of enzyme (Okagbue *et al.*, 2001), substrate mobility (Marcozzi *et al.*, 1998); and enhance better dispersion of the lipodal substrate which facilitates better utiliza-







Fig. 3



Esterase production - Esterase specific activity

Fig. 3. Effect of different inducers inducing esterase production by Lantinus tigrinus.

(A) Tween20 at 30°C; (B) Tween20 at 37°C; (C) Tween20 at 45°C;
(D) Tween80 at 30°C; (E) Tween80 at 37°C; (F) Tween80 at 45°C;
(G) olive oil at 30°C; (H) olive oil at 37°C; (I) olive oil at 45°C, enzyme activity is expressed in U/ml and specific activity in µg/ml protein.

tion by the enzyme (Arthur *et al.*, 1984). Kudanga *et al.* (2007) and Okagbue *et al.* (2001) also reported positive influence of Tween 80 for production of esterase and α -glucosidase, respectively. The maximum esterase production (38.62 U/ml) and specific activity (0.85 µg/ml protein) was achieved when cultured in the presence of urea and yeast extract (Fig. 4). Replacing the urea with ammonium sulfate decreased both production and specific activity of esterase. Other nitrogen sources did not positively influence the production of esterase. It can be stated that maximum esterase production through *Lantinus tigrinus* can be achieved by providing organic nitrogen sources. However, the production of enzyme in the presence of different nitrogen source also depends upon fungal strain (Kudanga *et al.*, 2007).

Lentinus tigrinus purified esterase was optimally active at 45°C and pH9. At 25, 30 and 35°C, minute esterase activity (<1.0 µg/ml protein) was observed. The esterase activity reached at maximum (4.1 µg/ml protein) at 45°C and gradually decreased when temperature was increased up to 50 and 55°C. Blum et al. reported optima temperature 30°C for production of maximum acetyl xylan esterase from rumen fungus Orpinomyces sp. strain PC-2 (Blum et al., 1999). At acidic pH (4, 5) the activity was $<0.4 \mu g/ml$ protein and it increased up to maximum (4.8 µg/ml protein) at pH9. Loss of enzymatic activities at different pH and temperature have been reported and found that varying the assay conditions the enzyme activity may be sudden lost or minute activity may be observed (Topakas et al., 2007). The half life of the enzyme also varies by varying the temperature and pH condition either during production or during assay (Ewis et al., 2004). However, many scientists reported that esterases



Fig. 4. Effect of different nitrogen source inducing esterase production by *Lantinus tigrinus*.
 (A) yeast extract; (B) urea and yeast extract; (C) meat extract; (D) yeast extract and (NH₄)₂SO₄; (E) peptone; (F) KNO₃; (G) (NH₄)₂SO₄; (H) yeast extract and KNO₃, enzyme activity is expressed in U/ml and specific activity in µg/ml protein.



Fig. 5. FTIR analysis of polystyrene film. (A) before treatment; (B) after treatment of esterase isolated from *Lantinus tigrinus*.

produced by different bacterial and fungal isolates have optimum temperature 37°C and basic pH conditions (Suzuki et al., 2004). The pH of the medium in which the enzyme is exposed also affects the ionization state of its amino acids which dictate the primary and secondary structure of the enzyme, thus controlling its activity. The influence of pH of the cultivation medium may be directly related with the stability of enzymes (Ueda et al., 1982). The polystyrene film treated with purified esterase confirmed degradation of polystyrene surface (Fig. 5). FTIR spectroscopy analysis (Fig. 5A) revealed that aromatic peaks (1449, 1493 and 1601 cm-1), alkane (1026-1369 cm-1) and C-H stretch (2850-3060 cm-1) are reduced in Fig. 5(B). This shows that esterase breaks down the bonds between small units of styrene and also degrades alkanes and aromatic rings. Generally, polystyrene is considered a stable macromolecule (Zheng et al., 2005); however, long but simple chemical structure and small molecular weight polymer made it easy to be degraded by enzymatic hydrolysis. Many studies reveal that polystyrene and other stable polymers can be

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degraded by different enzymes (Takamoto *et al.*, 2001; Chattopadhyay and Madras, 2003).

This study concludes that *Lantinus tigrinus* releases esterase enzyme in the culture medium. Production and release of esterase was influenced by temperature, pH, nitrogen source and inducer. The enzyme was active at 45°C and basic medium was optimum for its activity. FTIR analysis confirms degradation of polystyrene by reduction in peak wavelength. The results presented in this study can be further exploited for environmental waste management especially when containing synthetic polymers such as polystyrene.

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