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Optimization of *Aspergillus terreus* Mp1 for Xylanase Production and Application in Eco-friendly Bleaching Technology

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This study aims to characterize and optimize the xylanases production using marine sponge - derived *Aspergillus terreus* MP1. *A.terreus* showed optimal enzyme activity on day 6 while sucrose and urea were used as carbon source and nitrogen source respectively. The enzyme was purified by dialysis and the purified enzyme exhibited optimal activity at pH 5.6 in ambient temperature of 55ºC . Zymogram analysis displayed four activity bands corresponding to xynalolytic activity and molecular weight was determined to be 66, 45, 35 and 19 kDa. The optimized Xylanase was also utilized effectively as an environmental friendly technique of dye degradation process.

Key words: Microbial Xylanase, *Aspergillus terreus,* LSF, SSF, Deinking, Dye degradation.

Xylan, the major hemicellulose component in a plant cellwall, is easily found in solid agricultural and agroindustrial residues, as well as in effluents released during wood processing (Collins *et al*., 2005; Anthony *et al*., 2005). Frequent inappropriate discarding of xylan caused great damage to the ecosystem (Prade, 1995). Xylanases are the key enzymes, which play important role in the hydrolysis of xylan (Vardakou *et al*., 2008; Collins *et al*., 2002) and therefore it is important in biopulping wood, bleaching pulp, treating animal feed, and bioconversion of lignocellulose materials to fermentative products. Further, the xylanases are especially used in enzymatic treatment of kraft pulp before bleaching in the paper industry, which has a positive impact on the environment. In recent years, many kinds of xylanases have been isolated from various microorganisms (Stricker *et al.*, 2008; Canakci *et al.*, 2007). Filamentous fungi such as *Aspergillus* spp. and *Trichoderma* spp. are of particular interest, and many microbial xylanases from other fungi and bacteria have been purified and characterized (Manimaran and Vatsala, 2007). Various thermostable xylanases have been isolated from cultures of *Schizophyllum commune* (Katarina *et al.*, 2005) and *Bacillus* spp. (Okazaki *et al.*, 1985). Microbial enzyme production is obtained using various substrates both in submerged and solid state fermentation processes. Though number of xylanases productions was done using submerged systems, solid state fermentation is found to be more economical mainly due to the cheap and abundant availability of agricultural wastes which can be used as substrates (Pandey, 1992). It has been proven in numerous published studies that xylanase as pre-bleaching agent is a clean, eco-friendly, economically feasible technology that can decrease the amount of bleaching chemicals required to attain a given brightness in subsequent chemical bleaching stage (Zhao *et al.* 2006). The continuously growing paper manufacturing industry imposes a severe demand on green plants

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that forms the basic raw material. Thus, it is not an environment-friendly option. Recycling of used paper is an alternative that can alleviate this stress exercised on the environment.

MATERIALS AND METHODS

Sample collection

Sponge samples were collected by scuba during scientific expedition in a rocky slope at water deeper than 20 m from the coastal water of Kovalam Coast which is situated on the west coast of Kerala about 14 km to the south of Thiruvananthapuram at 8° 23' N latitude and 76° 57' E longitude in India. Specimens were washed and stored at -20°C until used for extraction. The sponge sample was washed with sterile distilled water: sea water (1:1) and ground in a mortar and pestle under aseptic conditions. Serial dilution was performed and spread plating was done on Sabourauds dextrose agar for each dilution. The plates were then incubated at 27°C for 5 days. After 5 days, the plates were examined and the pure culture obtained were transferred to another sterile SDA plate. The culture were transferred to potato dextrose broth and incubated at room temperature in dark for 48 to 72 hours.

Identification of fungi

The genomic DNA was isolated and the ITS region of 5.8sRNA was amplified using primer ITS1 TO 5' TCCGTAGGTGAACCTGCGG 3' and primer ITS5 5' TCCTCCGCTTATTGATATGC 3' [7] and sequenced using automated sequencer.

Production and optimization of xylanase production

The fungal filtrate was subjected to primary xylanases enzyme screening using modified assay of Kheng *et al*., 2005. Agro waste such as Wheat bran, Rice bran, Bean hull, Black gram hull, Sago waste etc., were used in enzyme production medium. These substrates were inoculated with 10% inoculum of 16 hrs. old culture of *A.terreus* and incubated at room temperature for 48-72 hrs and extracted with 100ml of buffer(1mM). The extract was centrifuged at 10000 rpm and supernatant was used as the enzyme source. Optimization of Carbon and Nitrogen source was performed by following the protocol of Lemos *et al.*(2001). Effect of pH and temperature on Xylanase production was analysed by Lenartovicz *et al.*(2003) assay.

SDS-Page and zymogram analysis:

The proteins were separated using SDS-Polyacrylamide gel electrophoresis with separating and stacking gel [10% and 5% (w/v)] . Xylan zymogram was performed by the slightly modified method of Morag *et.al.*, (1990) in polyacrylamide slab gels containing SDS and Xylan (0.5%) as a copolymerized substrate.

Application of xylanase

Environment friendly approach for de inking of paper

10g of paper pulp were boiled at 50°C for 2 hour and reacted with 0.2M sodium acetate buffer, pH 5.8 for 10 mins. The reaction with the pulp occurred for 48 hours with continuous slow mixing and control assays were made in parallel.

Dye degradation

White cotton cloth was cut into small squares and immersed in different dyes viz., Orange ME2RL, Red CLBL, Yellow mg4G, Black B, Yellow F_3R , Brown I, Red F_3B, T Blue G for 2-3 hrs. The cloth pieces were then rinsed with distilled water and dried for 2 hrs. After drying the cloth pieces were subjected to 0.2 M sodium acetate buffer maintained at pH 5.8 and crude enzyme solution and left for 24 hours.

RESULTS AND DISCUSSION

Marine sponge associated fungi were isolated and identified based on it's ITS sequence (Genbank Accession number HQ 449678). Primary screening confirmed the synthesis of extracellular enzymes by *Aspergillus terreus* MP1 by producing 21mm zone of clearance on xylan substrate (Fig.1). Qualitative and quantitative results indicate that enzyme grown on 0.05% of birchwood xylan gave maximum activity on day 6 (Fig.2) which corresponds to the reults of Duarte *et al*., (1999). The addition of sucrose resulted in increased xylanase production compared to the cultivation on other carbon sources (Fig. 3) which corresponds to earlier work of Montarroyos *et al*., (2007) performed on *M.musicola* and Seyis and Aksoz (2003) work on *T.harzianum*. The addition of urea resulted in increased xylanases production compared to the cultivation on other nitrogen sources (Fig. 4).

The purified enzyme after dialysis was subjected to bioassay produced maximum enzyme

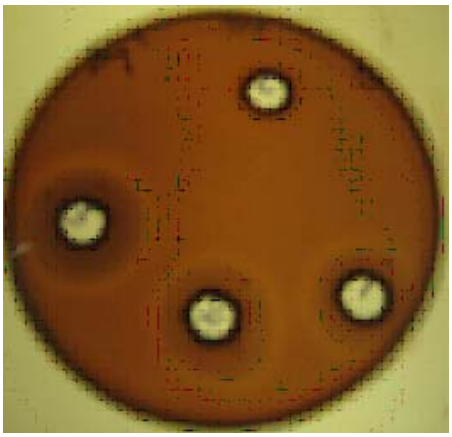

Fig 1 : Zone of clearance on Xylan substrate

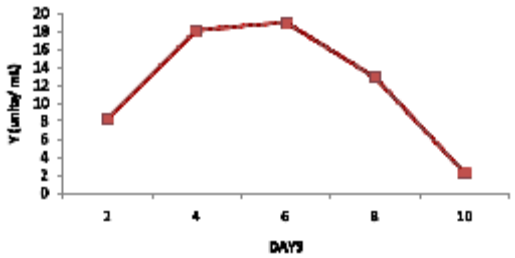

Fig. 2. Day optimization of optimim Xylanase enzyme production

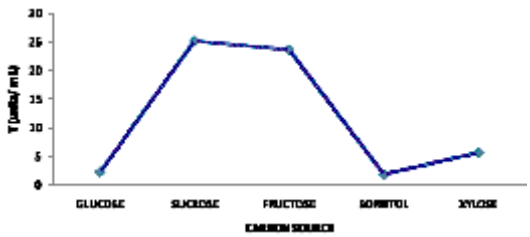

Fig. 3. Carbon source optimization of optimim Xylanase enzyme production

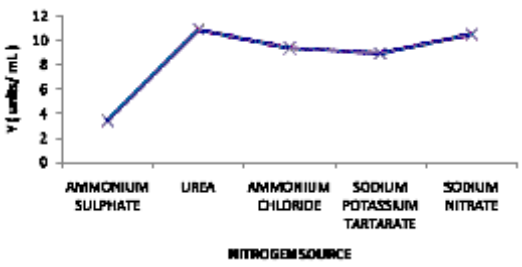

Fig. 4. Nitrogen source optimization of optimim Xylanase enzyme production

concentration of 20.17 mg/mL compared to 3.78 mg/mL before dialysis . Results indicate that the xylanases of *Aspergillus terreus* MP1 exhibited the activity within a acidic to neutral pH range, optimum being 5.6 (Fig.5), This result suggest that the enzyme would be useful in process of wide range of pH change from slightly acidic to alkaline and vice versa . Yang *et al*., (2006) showed xylanases from *A.niger* 3486 to be active at 5.5 which is very close to the result obtained in this study. Another study by Kormelink *et al*., (1993) on Endoxylanase I and II from *Aspergillus awamori* showed optimum activity of xylananses at pH 5.5 – 6.0 and 5.0, respectively . The effect of

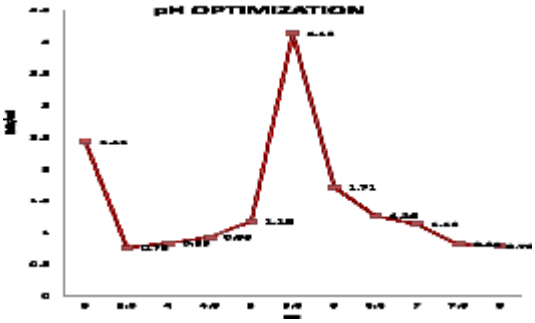

Fig. 5. Effect of pH for optimim Xylanase enzyme production

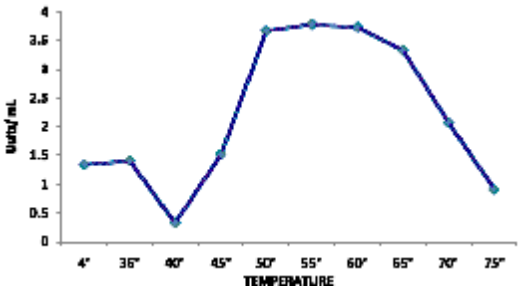

Fig. 6. Effect of Temperature for optimim Xylanase enzyme production

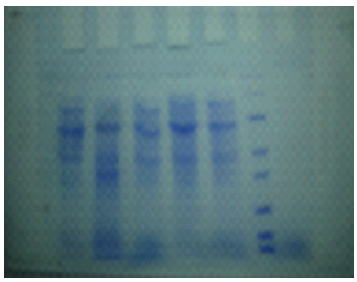

Fig. 7. Zymogram analysis of Xylanase enzyme production

temperature on xylanase production by *Aspergillus terreus* MP1 was examined to have maximum activity at ambient temperature (55ºC) (Fig.6) which is identical to the work of Yang *et al*., (2006). Temperature stability studies indicate general increase in the stability of enzyme with time 5 to 60 minutes. Zymogram analysis of xylanase from *A. Terreus* MP1 fungi (Fig.7) showed four activity bands corresponding to xynalolytic activity and molecular weight was determined by SDS-PAGE with apparent molecular masses of 66, 45, 35 and 19 kDa which is almost closer to the results obtained by Yang *et al*., (2006).

Results of the present study clearly indicates that xylanase from A.terreus MP1 shows tremendous potential for eco-friendly technique of biological deinking. Subsequent decolorization of dislodged ink particles is perhaps brought about by low molecular weight compounds that generate free radical by enzyme system. Enzyme treatments showed substantial improvements in residual ink-removal relative to the control assay. Angayarkanni (2006) reported that Aspergillus spp. xylanase induced the release of pentosans from the pulp, which indicates the removal of hemicelluloses from the paper pulp resulting in an eco friendly method for paper recycling. Another significant finding reports the dye decolourization ability of xylanases from *Aspergillus terreus* MP1 on 9 dyes. Six dyes Black B, Yellow mg 4 , Brown I, T Blue G, Red CLBL, Orange ME2RL were decolorized completely. The other Three dyes Yellow F_3R, Red F_3B,Reactive Orange M3R were partially degraded. However, the extent of colour removal was not consistent with all the dyes. Decolourization depends upon on the enzyme production, media and nature of dyes. The similar observation regarding the dye degradation by the white rot fungus *P.chrysosporium* has been observed by Cripps *et al*. *(*1990) and Spardaro *et al.* (1992). The efficacy of microbial xylanases in bleaching process has been studied for *A. oryzae* (Christov *et al*., 1999), *A. niger* (Zhao *et al*. 2002) and *A. nidulans* (Taneja *et al*. 2002).

The only drawback with scaling up and commercialization of microbial by-products is the expensive experimental setup and low product yield which can be overcome by developing mutant strains to enhance enzyme production by genetic engineering methods. This method can be practically applied in industrial sector by introducing mutant strains with increased xylanase production with more efficacy. This study is first of its kind to elucidate an environment- friendly approach for de-inking of paper and dye degradation by marine sponge associated fungi from Gulf of mannar origin. This would pave way for a scientific breakthrough to minimize chemical intervention in conventional paper recycling and dye removal technologies.

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