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Biochemical evaluation of xylanases from various filamentous fungi and their application for the deinking of ozone treated newspaper pulp

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D-Xylose (Pub Chem CID: 644160)
Potassium dihydrogen phosphate (Pub Chem CID: 516951)
Magnesium sulfate heptahydrate (Pub Chem CID: 24843)
Ammonium sulfate (Pub Chem CID: 6097028)
Glacial acetic acid (Pub Chem CID: 176)
Acetone (Pub Chem CID: 180)
Tween 80 (Pub Chem CID: 5281955)
Sodium hydroxide (Pub Chem CID: 14798)
Sodium chloride (Pub Chem CID: 5234)

ABSTRACT

Filamentous fungi, *Aspergillus oryzae* MDU-4 was biochemically selected among different species of *Aspergillus* and *Trichoderma*, for xylanase production. The enzyme activity and specific activity of partially purified xylanase from *A. oryzae* MDU-4 was 7452 IU/ml and 13,549 IU/g, respectively. Temperature and pH optima for xylanase were found to be 60 °C and 6.0, respectively. The reaction kinetics of xylanase was found to be K_m (3.33 mg/ml) and V_{max} (18,182 μmol/mg). The implementation of ozone treatment in the deinking of newspaper pulp resulted in high crystallinity index (72.1%) and more fibrillar surface. Furthermore, the xylanase treated pulp showed significant improvement in optical properties such as brightness (57.9% ISO) and effective residual ink concentration (211 ppm). Scanning electron microscopy analysis suggests perforations in xylanase treated pulp samples. Here we report biochemical evaluation of xylanases and a combination of ozone treatment followed by catalytically efficient fungal xylanase selected for the cost competitive deinking of newspaper pulp.

1. Introduction

The pulp and paper industries are mainly dependent on the wood logs, which are the basic raw material for paper production but their continuous use may cause habitat loss and also create an environmental imbalance (Shrinath, Szewczak, & Bowen, 1991). To overcome this problem, paper industries are now inclined toward the recycling of paper, which contributes to approximately 35% of total solid municipal waste. There is enormous pressure on forests for pulp and paper production. Therefore, recycling of paper is an effective and eco-friendly way to preserve our forest resources, which eventually saves the natural diversity and conserve energy. In the early 1970s, 7% of the waste paper was used as raw material in the paper industry, whereas nowadays, the contribution has reached to 47%. Furthermore, recovery rate of paper by paper mills in India is 27%, which is very low when compared with developed countries like Germany—73%, Sweden—69%, Japan—60%, Western Europe—56%, USA—49% and Italy—45%, according to Indian Agro and Recycled Paper Mills Association (IARPMA) estimates (Maity et al., 2012).

In the recycling process, the major problem is to remove impact ink from newspapers (Pala, Mota, & Gama, 2004). In the traditional deinking process, a large number of chemicals such as sodium hydroxide, sodium carbonate, sodium silicate, diethylenetriaminepentacetic acid, hydrogen peroxide, chlorine based derivatives,

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surfactants and chelating agents are used, which increase the process cost as well as generates hazardous byproducts (Shrinath et al., 1991; Virk, Puri, Gupta, Capalash, & Sharma, 2013). On the contrary, the use of enzymes makes the deinking process eco-friendly, very efficient and significantly improves the optical properties of treated newspaper (Kuhad, Mehta, Gupta, & Sharma, 2010; Virk et al., 2013). Therefore, in recent years the use of microbial enzymes has increased tremendously at the industrial scale. A large number of enzymes such as xylanases, cellulases, amylases, lipases, esterases and laccases have been reported for the deinking purposes (Kuhad et al., 2010; Virk et al., 2013). Among all these enzymes, xylanases have been recognized to have great potential in the deinking processes (Viikari, Kantelinen, Sundqvist, & Linko, 2001; Virk et al., 2013). It degrades xylan, which is the chief constituent of hemicelluloses and the second most abundant polysaccharide in nature after cellulose. Xylan is a complex heterogeneous polysaccharide, therefore several enzymes, i.e., β-1,4-endoxylanase, β-xylosidase, α-L-arabinofuranosidase, α-glucuronidase, acetyl xylan esterase and phenolic acid esterase are involved in its complete hydrolysis (Singh, Madlala, & Prior, 2003). However, the key enzyme of the microbial xylanolytic system is endo-1,4-β-xylanase (EC 3.2.1.8) (Bailey & Viikari, 1993), that depolymerizes xylan by hydrolyzing β-1,4-xylosidic linkages at random or specific positions between xylopyranose units and produce short chain xylo-oligosaccharides of varying lengths (Wong, Tan, & Saddler, 1988).

In comparison with other microorganisms, filamentous fungi are more promising for large scale production of extracellular enzymes on a commercial scale (Haltrich, Nidetzky, Kulbe, Steiner, & Zupancic, 1996). Among filamentous fungi, the genus *Aspergillus* and *Trichoderma* are more widely used for the production of efficient hydrolytic enzymes (Haltrich et al., 1996). Several xylanases have been reported from these fungal strains for various industrial and biotechnological applications, i.e., enzymatic deinking (Pathak, Bhardwaj, & Singh, 2014), pulp bleaching (Viikari et al., 2001), to improve the nutritional properties of animal feed, in pharmaceutical industries (Wong et al., 1988), for the production of xylo-oligosaccharides (Polizeli et al., 2005) and saccharification of lignocellulosic substances into xylose sugars (Kuhad et al., 2010). In order to fulfill specific industrial needs, enzymes must possess pH and thermostability, high specific activity and most importantly high affinity for the substrate (Dobrev et al., 2009). To the best of our knowledge, this is the first exhaustive report on physical and biochemical evaluation of xylanases from different species of *Aspergillus* and *Trichoderma*, which includes *Aspergillus oryzae* MDU-4, *Aspergillus flavus* MDU-5, *Trichoderma citrinoviride* MDU-1, *Trichoderma harzianum* MDU-2 and *Trichoderma longibrachiatum* MDU-6. This is also the first report on combinatorial approach of using ozone with biochemically suitable xylanase produced from *A. oryzae* MDU-4 using wheat bran as a substrate for the effective and cost competitive deinking of newspaper pulp.

2. Materials and methods

2.1. Materials

All the chemicals used were of analytical grade. Potato dextrose agar (PDA) was obtained from Hi Media Laboratory (Mumbai, India), while xylose, oat spelt xylan and beechwood xylan were purchased from Sigma Chemicals (St. Louis, USA). Wheat bran and newspapers were procured locally.

2.2. Microorganisms

The fungal cultures used in this study include *A. oryzae* MDU-4 (accession no. KC914095), *A. flavus* MDU-5 (Accession no. KC914096), *T. citrinoviride* MDU-1 (accession no. KC807226), *T. harzianum* MDU-2 (accession no. KC807227), and *T. longibrachiatum* MDU-6 (accession no. KC914097) taken from the laboratory culture collection and maintained on PDA slants at 4 °C.

2.3. Inoculum preparation

A homogeneous spore suspension was harvested from 72 h old PDA slants of all the fungal cultures by using sterile saline containing 0.1% (v/v) Tween 80 to obtain 1×10^8 spores/ml and used as inoculum.

2.4. Xylanase production under submerged fermentation (SmF) condition

Different species of *Aspergillus* (two) and *Trichoderma* (three) were screened for their ability to produce extracellular xylanases under SmF condition during their growth on modified Horikoshi medium containing (g/l): beechwood xylan, 2.5; peptone, 5.0; yeast extract, 5.0; KH_2PO_4, 1.0; $MgSO_4 \cdot 7H_2O$, 0.1 (Ikura & Horikoshi, 1987). The flasks were inoculated with 1×10^8 spores of respective cultures and incubated at 30 °C on an orbital shaker (125 rpm). Culture supernatant was withdrawn at regular interval of 24 h, centrifuged at 13,201 × *g* for 10 min at 4 °C and the cell free culture filtrate was used for estimating xylanase activity.

2.5. Effect of temperature and pH on xylanase production

The effect of incubation temperature ranging from 28 to 37 °C on xylanase production was studied. The xylanase production was also studied by pre-adjusting the medium pH from a range of 3 to 9 with 1 N NaOH/HCl.

2.6. Analytical procedures

Xylanase activity was assayed by measuring the release of reducing sugar by dinitrosalicylic acid (DNS) method (Miller, 1959) at 50 °C, using 0.6% (w/v) beechwood xylan. One unit of xylanase is defined as the amount of enzyme required to release 1 μmol of xylose per minute under standard assay conditions. All the assays were carried out in triplicate and the results are represented as mean values with standard deviations (SD). Protein content was estimated by Lowry's method using bovine serum albumin (BSA) as standard (Lowry, Rosebrough, Farr, & Randall, 1951).

2.7. Partial purification of xylanases

Xylanases were partially purified by adding ammonium sulfate salt to the culture supernatant with constant stirring at low temperature to achieve 15–60% saturation. The precipitates were collected by centrifugation at 9168 × *g* for 15 min at 4 °C and dissolved in minimum volume of 50 mM citrate buffer, pH 6.2 and dialyzed overnight at 4 °C against the same buffer.

2.7.1. Effect of pH and temperature on xylanase activity and stability

The xylanase activity was estimated in the pH range of 3.0–10.0 using buffers of varying pH, citrate buffer (pH 3.0–6.0), phosphate buffer (pH 7.0–8.0), Tris–HCl buffer (pH 9.0) and carbonate–bicarbonate buffer (pH 10.0). The effect of temperature on xylanase activity was also determined by incubating the reaction mixture at various temperatures ranging from 30 to 100 °C.

The pH and thermostability of xylanase was determined by incubating the enzyme sample at a particular pH for up to 4 h and at various temperatures from 40 to 70 °C up to 8 h. At regular time