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Biodeinking of old newspaper pulp using a cellulase-free xylanase preparation of *Aspergillus niger* DX-23

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ABSTRACT

Old newspapers (ONP) are a significant source of raw materials for recycled paper industry. To obtain bright pulp for recycling, the ink from the ONP pulp is removed by chemical deinking process which is highly polluting and expensive. Due to high xylan content in ONP, the ink particles adhering on ONP pulp surface can be removed through detachment of ink/fiber bond by action of xylanases. In the present study, cellulase-free xylanases from fungi were *specifically screened* for deinking of ONP pulp. Among 16 cellulase-free xylanase producing isolates, strain DX-23 (identified as *Aspergillus niger*) produced maximum xylanase (48.9 ± 0.02 U ml^{-1}). The xylanase of *A. niger* DX-23 (50 U g^{-1} pulp), efficiently deinked the ONP pulp which exhibited 34.5% ISO brightness (22% higher than the untreated pulp). Improvement up to 78.8% ISO brightness in ONP pulp compared to untreated pulp was achieved after optimization of conditions for deinking by response surface methodology and subsequent mild H_2O_2 treatment of deinked pulp. ATR-FTIR spectra, X-ray diffraction analysis and scanning electron micrographs of pulps confirmed the removal of surface ink particles from ONP pulp. The xylanase of *A. niger* DX-23 possessed molecular weight of 15.0 kDa, showed maximum activity at 50 °C and pH 5.0. Additives such as Mn^{+2}, Fe^{+2}, and sodium dodecyl sulfate enhanced the activity of xylanase, whereas, Zn^{+2}, Mg^{+2}, Ca^{+2} and Hg^{+2} and EDTA completely inhibited the activity. The K_m and V_{max} for the xylanase of *A. niger* DX-23 for hydrolysis of Birchwood xylan was found to be 2.38 mg/ml and be 230.8 μM/min/mg, respectively.

1. Introduction

Globally the paper industry is facing problem of raw material i. e cellulosic pulp due to shortage of forest based raw materials. Old newsprint (ONP) is primary raw material for recycled newsprints (Chen et al., 2015; Chutani and Sharma, 2015). To obtain bright pulp for recycling, the ink from the ONP pulp is removed by chemical deinking process using large amounts of chemicals such as NaOH, Na_2SiO_3, chelating agents and surfactants which makes deinking process expensive and environmentally damaging (Ibarra et al., 2012; Kumar and Satyanarayana, 2014; Virk et al., 2013). Enzymatic peeling-off of ink particles from fiber surface using cellulases, hemicellulases and laccases is eco-friendly and efficient alternative for chemical deinking. Among these, cellulase decreases the pulp strength due to its action on cellulose fibers (Maity et al., 2012), while laccases reduce brightness and cause yellowing of pulp (Hager et al., 2002). Since newspapers contain 25–40% hemicelluloses (Howard et al., 2003), xylanases offer promise as non-damaging deinking agent. The pH of the writing and printing papers varies from 5.0 to 7.5 and hence xylanases working in acidic pH are more effective for deinking of ONPs (Dutt et al., 2013). Although compared to bacteria and yeast, filamentous fungi produce acidic xylanases in high concentrations and extracellularly (Bajaj and Abbas, 2011; Polizeli et al., 2005) the reports on deinking of ONP pulp using fungal xylanases is very scarce. Therefore, in the present study, we report specific screening, evaluation and characterization of a xylanase of *Aspergillus niger* DX-23 for efficient deinking of ONP pulp. To the best of our knowledge present study is the first report on specific screening and evaluation of fungal xylanases for effective deinking of ONP pulp.

2. Materials and methods

2.1. Screening of xylanase producing fungal cultures

A 0.1 ml aliquote of the homogenate of xylan-rich decomposed samples (e.g. leaves, corn cobs) prepared in sterile normal saline was spread onto Martin Rose Bengal Agar and incubated at 25 ± 1 °C for 48 h. The fungal colonies obtained were spot inoculated individually onto Mandel's media (pH 5.5) (Mandels and

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Weber, 1969) containing 0.5% (w/v) birchwood xylan/carboxymethyl cellulose (CMC) and Tween 20 (1.0 ml L^{-1}). The plates after incubation at 25 ± 1 °C for 48 h were flooded with 0.5% (w/v) congo red solution, kept for 10 minutes, and washed with 1.0 M NaCl solution to visualize pale yellow zone surrounding the xylanase/cellulase producing colonies.

2.2. Xylanase and cellulase production under submerged fermentation (SmF) condition

Mandel's medium (50 ml in 250 ml Erlenemeyer flask) supplemented with either birchwood xylan or CMC at a concentration of 0.5% (w/v) and 1% (w/v), respectively was inoculated at 5.0% (v/v) level with spores (10^6 ml^{-1}) of respective fungal cultures and incubated on an orbital shaker (150 rpm) at 30 ± 1 °C for 48 h. The culture broth after growth was centrifuged at 10,000*g* for 10 min at 4 °C to remove fungal biomass and subsequently used as source of xylanase (hereinafter referred to as cellulase-free xylanase preparation). Xylanase, CMCase and cellulase were assayed as described earlier (Torres et al., 2013) by determining reducing sugars liberated after hydrolysis of birch wood xylan/CMC/Whatman filter paper 1 in 100 mM sodium citrate buffer (pH 5.5) at 50 °C after 10 min of hydrolysis using dinitrosalicylic acid (DNS) reagent. One unit (U) of enzyme activity was defined as the amount of enzyme which released 1 µmol of reducing sugar as xylose/glucose per min.

2.3. Evaluation of deinking ability of xylanase of fungal isolates

2.3.1. ONP pulp preparation and deinking

ONP pulp for evaluating the deinking ability of xylanases was prepared as described by Virk et al. (2013). Newspapers (6 month old) were shredded and soaked in water (60 °C) containing Tween 20 (0.1% w/v) for 2 h and then disintegrated in a domestic mixer. The excess water from the pulp was removed by straining and the pulp was oven-dried at 50 °C. The deinking ability of cellulase-free xylanase preparation was evaluated as described by Das et al. (2013). The dry ONP pulp (6.0 g) was soaked in 60.0 ml of 100 mM Na-citrate buffer (pH 5.5) for 30 min to obtain pulp having 10% (w/v) consistency. Cellulase-free xylanase preparation was added to the pulp (50 U/g of pulp) and incubated at 55 ± °C for 20 min on an orbital shaker (150 rpm) for deinking reaction. For comparison, the ONP pulp was also chemically deinked as described in INGEDE Method 11 (International Association of the deinking industry, 2012). Briefly, the pulp was treated with NaOH (0.6% w/v), sodium silicate (1.8% w/v), H_2O_2 (0.7% w/v) and oleic acid (0.8% v/v) for 20 min at 45 °C. Ink from both chemically and enzymatically treated pulp was separated by washing over a wire mesh and dried at 50 °C (Singh et al., 2012). The paper brightness (%) (as per TAPPI T452/ISO 2470), yellowness (as per ASTM D-1925) and CIE whiteness was measured using Premier color scan spectrometer. Untreated ONP pulp was kept as control..

2.4. DNA isolation and identification of fungal culture DX-23

The genomic of DNA fungal isolate DX-23 was isolated and the 5.8 S RNA was amplified and sequenced as described previously (Bakri et al., 2010; Moller et al., 1992). The most homologs sequence to the 5.8 S rRNA gene sequence of isolate DX-23 were identified in the GeneBank database using the BLASTN algorithm, aligned using multiple sequence alignment software and the taxonomic position of the isolate was determined based on the phylogenetic tree constructed by neighbor joining method (Saitou and Nei,1987)

2.5. Optimization of deinking process and characterization of pulps

2.5.1. Optimization of deinking process using response surface methodology (RSM)

In 60.0 ml system, the effect of variation of three factors viz. (A) concentration of xylanase, (B) reaction temperature and (C) reaction time on ISO brightness, whiteness and yellowness of ONP pulp was studied using Box-Behnken experimental design (Box and Behnken, 1960) comprising of 15 randomized experiments (12 unique combinations + 3 center point runs) created using Design Expert 9 software (StatEase Inc Minneapolis, USA). The data obtained were fitted to quadratic polynomial model and the linear, quadratic, and interaction effects of the model were estimated by multiple regression analysis.

2.5.2. H_2O_2 treatment of ONP pulp deinked using xylanase of *A. niger* DX-23

ONP pulp (60.0 ml, 10% w/v) was deinked using cellulase-free xylanase preparation of *A. niger* DX-23 (50 U/g of pulp) in either 0.1 mM Sodium citrate buffer (pH 5.5) or tap water at 30 °C for 100 min and the detached ink was removed from the pulp by washing. The deinked pulp was then resuspended into 60.0 ml of either tap water or 0.1 mM Sodium citrate buffer (pH 5.5) both containing 0.7% (v/v) H_2O_2. After incubation at 30 ± 1 °C for 100 min, the treated pulp was filtered, made into handsheet and analyzed for brightness, whiteness and yellowness.

2.5.3. Fourier transformed infrared spectroscopy

The surface functional groups of the hand sheet samples made from ONP pulp was studied by Fourier transformed infrared (FTIR) spectrometry using the attenuated total reflectance (ATR) measuring cell. FTIR spectra of untreated chemically treated and xylanase treated ONP pulp samples were recorded at wave number range of 4000–375 cm^{-1} in a Bruker Alpha ATR machine.

2.5.4. X-ray diffraction analysis

The X-ray diffraction pattern of handsheets prepared from deinked ONP pulp was analyzed using Bruker D2 Phaser diffractometer at intensities between 10° and 80° of 2θ (scattering angle) and scanning speed of 0.02°/scan. The crystalline index was calculated using equation given by Kim and Holtzapple (2006):

$$\frac{X_C = (I_{002} - I_{am}) x\ 100\ \%}{4}$$

I_{002}

where I_{002} and I_{am} are the peak intensity of crystalline and amorphous phase from the (0 0 2) lattice plane. The average sizes of crystallites obtained from (*hkl*) diffractions were determined by the use of the Scherrer equation as follows:

$$D(hkl) = \frac{k\lambda}{\beta(hkl)cos\theta}$$

where (*hkl*) is the lattice plane, $D(hkl)$ is the size of the crystallite, k is the Scherrer constant (0.84), β is the wavelength of the incident X-ray (0.1542 nm), β (*hkl*) is the full-width at half maximum (FWHM) of the peak angle of the (0 0 2) reflection and θ is the Bragg angle corresponding to the (0 0 2) plane.

2.5.5. Scanning electron microscopy

The hand sheet samples were coated with a thin film of gold and examined under scanning electron microscope (SEM) (Leo Scanning Electron Microscope, 20 kV).

2.6. Purification and characterization of xylanase of *A. niger* DX-23

The xylanase of *A. niger* DX-23 was concentrated from the cell free culture broth by precipitation using ammonium sulfate (0% to 100% saturation, 4 °C). A 2.0 ml aliquot of the fraction that showed highest xylanase activity was loaded onto Sephadex G-100 column