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Dual application of agricultural residues for xylanase production and dye removal through solid state fermentation

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ABSTRACT

The present study is a novel attempt to utilize agricultural residues for addressing the dual challenge faced by pulp and paper industries to control pollution caused due to pulp bleaching and release of colored waste water. Fungal isolate, *Aspergillus lentulus*, was utilized for the production of xylanase through solid state fermentation utilizing various low-cost agro-residues as substrate. Maximum xylanase production was obtained on the 4th day of incubation using wheat bran as the substrate (158.4 U/g) followed by corn cob (153.0 U/g), sugarcane bagasse (129.9 U/g) and wheat straw (49.4 U/g). These activities were accompanied by very low cellulase activities. The enzyme exhibited good stability at high pH and temperature (>75% activity retained at pH 9 and 70 °C). Later, the left over spent fermented slurry was utilized to remove anionic (>85.0% removal) and cationic (>96.0% removal) dyes. Results indicate cellulase-free; pH and thermo stable nature of the xylanase enzyme which is required during biobleaching process. Moreover, successful utilization of spent residues from fermentation in dye removal process signify that the proposed technology can be utilized to meet the requirements of pulp and bleaching industries through an effective and sustainable approach.

Introduction

Pulp and paper industries have been considered as major polluting units across the world (Sumathi and Hung, 2006; Ratia et al., 2012). Different processes such as the pulp bleaching, deinking, production of colored paper etc. contribute to the pollution caused by these industries. Last decade has seen an increase in the number of studies focusing on the possible pollution reduction, alternative waste treatment technologies, and waste management (Monte et al., 2009).

Recently, utilization of microbial enzyme xylanase for pulp-bleaching has been intensely researched and adopted commercially (Polizeli et al., 2005). This results in cheaper and cleaner production process by avoiding the use of chlorine and significantly reducing the discharge of the pollutants. To make the enzyme application in industries more cost effective, its production using negative value substrates like agro-wastes has been recommended by many workers (Dhillon et al., 2011). Utilization of these agro-residues in bioprocesses has dual advantage of providing alternative substrates as well as solving their disposal problems. Researchers have been utilizing sugarcane bagasse (Song and Wei, 2010), wheat bran (Garai and Kumar, 2013), coba husk (Fang et al., 2010), jatropha cake (Chaturvedi et al., 2010) etc. as the substrates for the production of enzymes through fermentation.

Kraft pulping requires high temperatures and high pH and as most of the available xylanases are produced from mesophilic organisms, they rapidly lose activity at temperatures above 50 °C and at pH above 7. Currently efforts are being made to produce cellulase-free xylanases from thermophilic/thermotolerant microorganisms which can retain their activity at alkaline pH and high temperatures (Collins et al., 2005; Nigam, 2013). Also, during xylanase production through solid state fermentation, after the extraction of enzyme, a lot of slurry comprising of degraded substrate and fungal biomass is produced which again has to be disposed off. If this spent slurry can be utilized for the dye removal process (another pollutant from paper industries) followed by composting of the dye laden slurry, a zero waste objective could be accomplished. Vermicomposting of such dye laden fermented slurry by Kaushik et al. (2013) have been successfully demonstrated where the end product could be used as a soil conditioner. Although economically and ecologically favourable, such integrations have not been tested practically at a larger scale.

The fungal isolate *A. lentulus* is an alkali, thermo and halo tolerant fungus (Kaushik and Malik, 2010) and has already been

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implicated for dye (Kaushik and Malik, 2013) and metal (Mishra and Malik, 2012) remediation processes. The ability of this strain for treatment of dye bearing wastewaters from textile and pulp and paper industries has also been established. Thus in the present study, the fungal isolate was tested for the production of xylanase enzyme utilizing various low cost substrates and nitrogen source through solid state fermentation. Further the potential for application of this enzyme in bleaching process in terms of cellulase-free nature, pH and temperature optima and pH and thermo stability profiles was evaluated. Simultaneously, utilization of spent fermented slurry (slurry left over after the enzyme elution) for the removal of anionic (Acid Navy Blue) and cationic (Methylene Blue) dye was also attempted.

Materials and methods

Test organism

The experiments on xylanase production were performed with a strain of *A. lentulus* FJ172995 previously isolated (Sharma, 2009) from the textile effluent procured from Baddi (Himachal Pradesh, India). The fungal isolate was maintained on slants of Potato Dextrose Agar. Freshly revived cultures were used for all the experiments.

Screening of A. lentulus for xylanase production

The fungal isolate *A. lentulus* was initially screened for xylanase production on xylan-agar plates containing 1% xylan and 0.1% Congo Red (Bajaj and Singh, 2010). Plates were spot inoculated with the fungal spores and incubated at 30 °C for 5 days. The diameter of the clear zone formed around the fungal colony was measured on daily basis.

Xylanase production through solid state fermentation

Xylanase production by *A. lentulus* through solid state fermentation of wheat bran was recorded at various time intervals. Six 250 mL flasks were taken and 5 g of wheat bran was added to each of them. The substrate was moistened with 15 mL of composite media (CM) comprising, Yeast extract 2.5 g/L, $MgSO_4$ 0.1 g/L, K_2HPO_4 0.5 g/L, NH_4NO_3 0.5 g/L and NaCl 1 g/L. The flasks were autoclaved at 121 °C and 15 psi for 20 min. Sterile flasks containing substrate were then inoculated with 5% spore suspension and incubated at 30 °C. One flask was removed after every 24 h for six days and 100 mL of 0.05 M citrate buffer (pH 5.3) and Tween 80 (0.1%) were added to it and it was agitated at 180 rpm for 1 h in an orbital shaker. The contents of the flasks were filtered through Whatman No. 1 filter paper and the filtrate was centrifuged at 10,000 rpm and 4 °C for 10 min and supernatant was collected and tested for crude endo-β-1,4-xylanase activity. Crude endo-β-1,4-xylanase enzyme activities were determined spectrophotometrically at 540 nm using di-nitrosalicylic (DNS) acid method based on the release of reducing sugars from oat spelt xylan (Bailey et al., 1992) at pH 5.3 and temperature 50 °C utilizing 1% xylan as the substrate. For the sake of convenience and as per the literature, this activity has been termed as xylanase activity throughout the text. One unit of xylanase activity is defined as the amount of enzyme required to produce 1 μmol of xylose per minute under standard assay conditions. Crude cellulase activity was determined through filter paper unit assay (Ghosh, 1987). One unit of filter paper unit assay is defined as the amount of enzyme required to release 1 μmol of glucose equivalents under standard assay conditions (50 °C and pH 4.8). Total protein was estimated by Lowry's method using Bovine Serum Albumin as standard (Lowry et al., 1951).

Zymogram analysis

The crude enzyme extract obtained on the 4th day of solid state fermentation was partially purified through ammonium sulphate precipitation. Ammonium sulphate was added to the crude enzyme extract to 40 and 75% saturation and stirred at 4 °C overnight. The saturated solution was later centrifuged and dissolved in citrate buffer (0.05 M). The dialyzed sample was then analyzed by native PAGE utilizing 10% separating gel using BIORAD electrophoresis apparatus. The native protein markers and the partially purified enzyme extract were loaded. Electrophoresis was performed at a constant voltage of 100 V for 3 h. After the run was completed, the gel containing the sample band was cut and rinsed with citrate buffer twice and incubated in 1% oat spent xylan for 5 min at 60 °C. The gel was then submerged in 0.1% Congo Red solution for 10 min and later washed with 1 M NaCl till the enzyme band appeared visually. The rest of the gel containing protein markers was visualized through Coomassie Brilliant Blue R-250 staining. The molecular weight of the active enzyme was estimated using the plot of log value of weight of the markers versus the R_f value of the markers. R_f value is calculated as the ratio of distance migrated by the marker or sample to that migrated by the marker dye-front.

Effect of different substrates and nitrogen sources

Different agro-residues like wheat bran, wheat straw, corn cob and sugarcane bagasse were taken as the substrates for production of xylanase enzyme through solid state fermentation. Wheat bran was procured from the Yusuf Sarai Market (New Delhi, India) whereas wheat straw, corncob and sugarcane bagasse were procured from agricultural fields of Bulandshahar, Uttar Pradesh (India). The agricultural residues were characterized for water soluble, hemicellulose, cellulose and lignin content using the sequential acid fractionation and gravimetric method described by Datta (1981). For solid state fermentation, 5 g of the substrate was taken in 250 mL flask and 15 mL CM was added to the flask and mixed well. The flasks were autoclaved at 121 °C and 15 psi for 20 min. Sterile flasks containing 5 g substrate were then inoculated with 5% spore suspension and incubated at 30 °C for 4 days. After 4 days, 100 ml 0.05 M citrate buffer (pH 5.3) and Tween 80 (0.1%) were added to the flasks and the flasks were agitated at 180 rpm for 1 h in an orbital shaker. The contents of the flasks were filtered through Whatman No. 1 filter paper and the filtrate was centrifuged at 10,000 rpm and 4 °C for 10 min and supernatant was collected and tested for crude xylanase and cellulase activity. Total protein in the supernatant was also estimated. Effect of nitrogen source on xylanase production was tested by replacing the CM by the modified media (MM) containing urea and ammonium chloride (Urea 0.3 g/L, NH_4Cl 0.2 g/L, $MgSO_4$ 0.1 g/L and K_2HPO_4 0.5 g/L).

Characterization of crude xylanase

The temperature optima of the crude xylanase was determined by carrying out the enzyme reaction with substrate (1% xylan) at different temperatures ranging from 50 to 90 °C for 5 min after which the reaction was stopped by adding DNS reagent. The optimum pH of crude xylanase was determined at 50 °C by carrying out the enzyme reaction at different pH values using the following buffers: 0.05 M citrate buffer (pH 4–6); 0.05 M phosphate buffer (pH 7–10).

The thermo-stability of the xylanase was studied at pH 5.3 by exposing the crude enzyme to a given temperature (ranging from 50 to 80 °C) for varying time intervals up to 3 h and assaying the residual xylanase activity under standard reaction conditions. The pH stability of crude enzyme was studied by diluting the crude