Production and Characterization of Xylanases from Fungal Isolate Aspergillus Terreus and Development of Low Cost Fermentation Media

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Abstract—**Fungal isolate, *Aspergillus terreus*, was utilized for the production of hydrolytic enzymes through solid state fermentation of wheat bran. After 5 days of incubation, 116 U/g xylanase, 1.53 fpu/g cellulase and 16.99 cmc/g carboxymethyl cellulase was produced. The optimum pH and temperature for the activity of xylanase enzyme was found to be pH 5 and 60 ℃. The zymogram analysis of the native enzyme shows the presence of single xylanase band with the molecular weight of 91.67 KDa. Low cost nutrient supplements such as the corn cob as the carbon substrate and biogas slurry as the nitrogen supplement can offer a low cost medium for the production of xylanase enzyme by *A. terreus*.**

Index Terms—**agricultural residue, biogas slurry, characterization, fungi, xylanase**

I. Introduction

Fungus has proved to be a suitable organism for bioremediation process. Due to an increased cell-to-surface ratio, fungi have a greater physical and enzymatic contact with the environment. The fungal mycelia have an additive advantage over single cell organisms in solubilizing the insoluble substrates through production of extracellular enzymes. The extra-cellular nature of the fungal enzymes is also advantageous in tolerating high concentrations of the toxicants. Further, certain fungal strains can also produce value-added by products such as industrially important enzymes [1]. Recently utilization of microbial enzyme xylanase for pulp-bleaching has been intensely researched and adopted commercially as well. This result in cheaper and cleaner production process by avoiding the use of chlorine and significantly reduces the discharge of the pollutants. Chlorine being the cheapest of all bleaching agents is used excessively for the process. However, it destroys the stratospheric ozone layer, and produces dioxin and organic chlorides. Its low consistency produces a large volume of acidic effluent, which has to be neutralized before waste treatment. For commercial applications, xylanases should ideally be produced quickly and in large quantities from simple and inexpensive substrates. Abundantly available agro-residues are an obvious source of substrate. Enzyme synthesis in solid-substrate fermentation has attracted significant attention. This technique offers distinct advantages over submerged fermentations, including economy of space, greater product yield and provision of a more natural growth environment for microbes. Furthermore, it is easier to control contamination due to low moisture level in the system and has no requirement for complex machinery, equipment or control systems [2]. Also, the possibility of producing xylanolytic systems free of cellulase should be ascertained in strains of *Aspergillus*, since they belong to the best xylanase producers [3].

In the present study, a fungal isolate, *A. terreus,* was utilized for the production of various hydrolytic enzymes through solid state fermentation and resultant crude xylanase enzyme was characterized for its optimum pH and temperature. Low cost alternate media for commercial production of this enzyme was also attempted.

II. Materials and Methods

A. Test Organism

The fungal isolate *A. terreus*, previously isolated from dump sites located at Indian Institute of Technology Delhi (India) was utilized for the production of hydrolytic enzymes through solid state fermentation. The isolate was maintained on the Potato Dextrose Agar slants kept at 4 ℃. Freshly revived slants were utilized for each experiment.

B. Solid State Fermentation

Wheat bran was taken as the substrate for solid state fermentation by A. terreus. The sterilized substrate (5g) was taken in an Erlenmeyer flask and supplemented with a suitable nitrogen rich media containing, gL^{-1}, NH_4NO_3, 0.5; $MgSO_4.7H_2O$, 0.1; K_2HPO_4, 0.5 and Yeast extract, 2.5, pH: 6.5 (1:3 w/v). The flasks were inoculated with 5% spore suspension of A. terreus and kept in an incubator at 30 ℃ for 4 days. After the end of incubation period, 100 ml 0.05 M citrate buffer (pH 5.3) and Tween 80 (0.1 %) were added to the flasks. The flasks were kept in an orbital shaker at 150 rpm for 1 h. The contents of

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the flasks were centrifuged at 10,000 rpm and 4 ℃ for 10 min and supernatant was collected and tested for crude xylanase, cellulase and carboxymethyl cellulase activity as well as for total protein content.

Crude xylanase enzyme activity was determined spectrophotometrically at 540 nm using di-nitrosalicylic (DNS) acid method based on the release of reducing sugars from oat spelt xylan [4] at pH 5.3 and temperature 50 ℃ utilizing 1 % oat spelt xylan as the substrate. 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 [5] utilizing 50 mg Whatman No.1 filter paper as the substrate. One unit of filter paper unit assay is defined as the amount of enzyme required to release 1μmol of glucose equivalents under standard assay conditions (50 ℃ and pH 4.8). Carboxymethyl cellulase activity was estimated using the assay for endo-β-1,4-glucanase described by [5] utilizing 2% carboxymethyl cellulose as the substrate. Total protein was estimated by Lowry's method using Bovine Serum Albumin as standard [6].

C. Characterization of Xylanase Produced from A. Terreus and Zymogram Analysis

The crude xylanase enzyme thus collected was characterized for its optimum pH and temperature. 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 ℃ for 5 min after which the reaction was stopped by adding DNS reagent. The optimum pH of crude xylanase was determined at 50 ℃ 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 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 ℃ overnight. The saturated solution was later centrifuged and dissolved in citrate buffer (0.05M). 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 100V 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 ℃. The gel was then submerged in 0.1 % Congo Red solution for 10 min and later washed with 1M 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 Rf value of the markers. Rf value is calculated as the ratio of distance migrated by the marker or sample to that migrated by the marker dye-front [7].

D. Low Cost Nutrient Supplements for Solid State Fermentation

The crude xylanase enzyme thus collected was characterized for its optimum pH and temperature. The Effect of low cost nutrient media (carbon and nitrogen) on xylanase production by *A. terreus* was estimated. Two other low cost carbon substrates: corn cob and waste paper pulp were utilized as the substrate for solid state fermentation by A. terreus. Alternatively, two low cost nitrogen sources; combination of urea and ammonium chloride and biogas slurry were also tested as a replacement for yeast extract in the fermentation media. Solid state fermentation using these sources was conducted as described in Section 2.2.

III. Results and Discussion

A. Solid State Fermentation and Production of Hydrolytic Enzymes

Solid state fermentation of wheat bran by *A. terreus* yielded in the production of various hydrolytic enzymes such as xylanase, cellulase and carboxymethyl cellulase. After 5 days of incubation, the crude enzyme filtrate exhibited 116 U/g xylanase, 1.53 fpu/g cellulase and 16.99 cmc/g carboxymethyl cellulase enzyme activity.

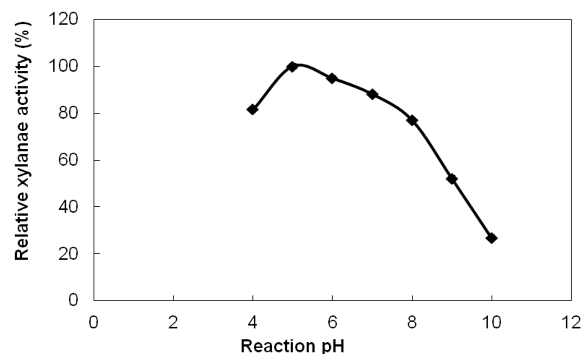

Figure 1. Relative xylanase activity obtained at different reaction pH under standard assay conditions.

B. Characterization of Xylanase Produced from A. Terreus and Zymogram Analysis

The crude xylanase enzyme produced from *A. terreus* was characterized for its optimum pH and temperature value. Fig. 1 shows the pH profile of crude xylanase at different pH values ranging from pH 4-10. The plot gives a bell shaped curve where maximum xylanase activity was observed at pH 5. Lower activities were reported on increasing or decreasing the assay pH. Similar trend was observed for temperature profile (Fig. 2) as well where highest xylanase activity was found at 60 ℃. The xylanase activity decreased when the assay temperature was either decreased to 50 ℃ or increased to 70 ℃. Thus pH 5 and 60 ℃ were found to be optimum for the activity of crude xylanase produced from *A. terreus*. The native PAGE analysis shows a single band with a molecular weight of 91.67 KDa corresponding to partially purified xylanase enzyme from *A. terreus* (Fig. 3).

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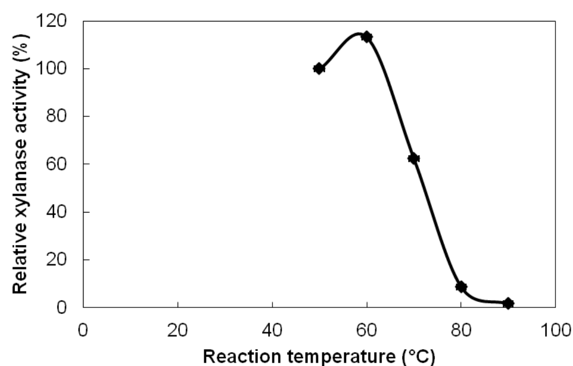

Figure 2. Relative xylanase activity obtained at different reaction temperatures under standard assay conditions.

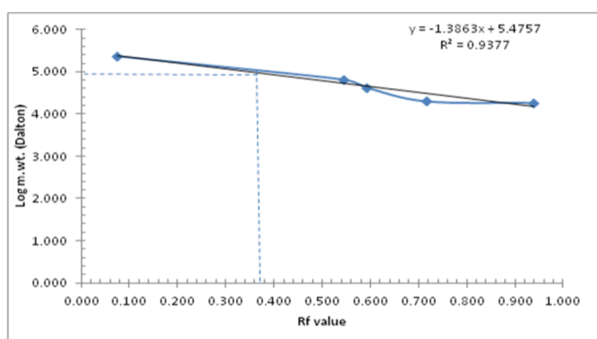

Figure 3. Plot of Rf value against molecular mass of standard protein marker and partially purified enzyme.

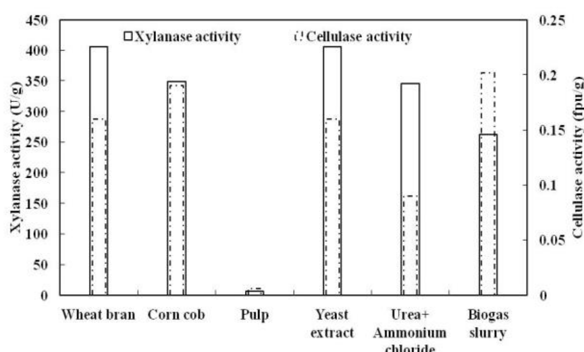

Figure 4. Crude xylanase and cellulase activity obtained with different carbon and nitrogen supplements.

C. *Production of Low Cost Alternate Media for Xylanase Production*

Low cost media supplements including carbon substrate and nitrogen media were tested as the source of nutrient for solid state fermentation. For carbon substrates, corncob and waste paper pulp were utilized for solid state fermentation by *A. terreus*. A significant decrease in xylanase activity (348.57 U/g) was observed when corncob was used as the substrate for solid state fermentation (Fig. 4). Very less amount of xylanase (6.57 U/g) was produced when waste pulp from handmade paper units was utilized as the substrate for solid state fermentation. Similar observation was made for cellulase enzyme where 1.9 fpu/g was produced when corncob was used as the substrate as compared to 0.06 fpu/g obtained with waste pulp. The specific xylanase activity obtained with corn cob was 43.81 U/mg (47.78 U/mg obtained with wheat bran). The results show that corn cob which is a waste agricultural residue can utilized as the substrate for producing xylanase enzyme and can replace wheat bran which finds use as a food supplement for humans owing to its nutritional value.

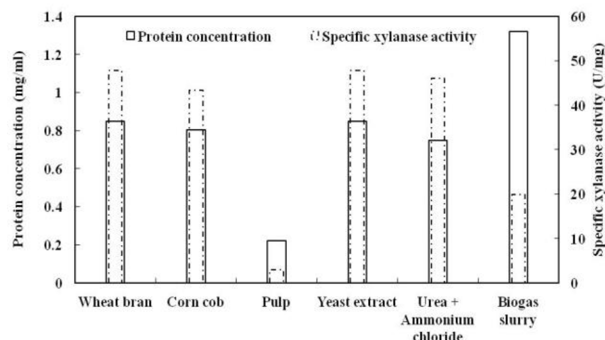

Figure 5. Total protein and specific xylanase activity obtained with different carbon and nitrogen supplements.

Two other low cost nitrogen sources, biogas slurry and a combination of urea and ammonium chloride, were tested as the replacement for yeast extract in fermentation media. In the presence of urea and ammonium chloride, 345.89 U/g crude xylanase activity was obtained (Fig. 5) which is less than that obtained with yeast extract (405.29 U/g). Further reduced xylanase activity was obtained when biogas slurry was used as the nitrogen source (261.91). Nevertheless, the obtained crude xylanase activities are good enough and can further be increased through optimization of process parameters such as incubation time, substrate concentration, incubation temperature and pH. The results thus provide an efficient fungal strain, *A. terreus*, capable of producing xylanase enzyme along with trace amounts of cellulase enzyme through solid state fermentation of waste agricultural residues such as wheat bran and corn cob.

IV. Conclusions

Thus from the above studies it can be concluded that fungal isolate *A. terreus* produces a thermophilic and cellulase-free xylanase enzyme which can be produced on a low cost media comprising waste residues through solid state fermentation.

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