Journal of Bioresource Management

Volume 3 | Issue 1 Article 2

2016

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J. Bioresource Manage. (2016) 3(1): .

PRODUCTION AND CHARACTERIZATION OF XYLANASE FROM *ASPERGILLUS NIGER* USING WHEAT BRAN, CORN COBS AND SUGAR CANE BAGASSE AS CARBON SOURCES WITH DIFFERENT CONCENTRATIONS

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ABSTRACT

Xylanases are enzymes that degrade Xylan, a hemicellulose found in plant cell walls, into Xylose. They are a very important class of enzymes to be used in paper and pulp industry. Removal of lignin from paper and pulp by Chlorine and its compounds have caused a serious problem in the environment. Delignification of lignin by Xylanase is an alternative approach that is environmentally friendly. The present research was conducted to produce and characterize Xylanase from the fungus *Aspergillus niger* using agricultural wastes/byproducts like corn cobs, wheat bran and sugar cane bagasse with different concentrations. Submerged fermentation was carried out in 250ml Erlenmeyer flasks using Vogel's medium at 37^{o}C. Culture conditions like pH, temperature, incubation time and concentration of carbon sources were optimized to achieve maximum Xylanase production. Molecular weight was determined by SDS-PAGE to be 27.2KDa. It was revealed that the pH and thermal stability of Xylanase is very important for it to be used in industry.

Keywords: *Aspergillus niger*, Submerged fermentation, Xylanase production, Wheat bran, SDS-PAGE

Introduction

In the present age, the major success of biotechnology lies in the production of various enzymes by using strains of fungi and bacteria in solid state and submerged fermentation methods. For the industrial and commercial production of Xylanase, filamentous fungi are mostly used because enzymes produced by this strain are more efficient than produced by bacteria (Bakri *et al.* 2003).

The balance between nature and the government economy can be maintained in part by the use of agricultural waste as raw material. According to this study, Xylanase can be produced by using bacterial and fungal sources through solid state fermentation methods using agricultural wastes such as wheat bran, corn cobs and sugarcane bagasse. Xylanase was stable for twenty-four hours over a wide range of pH from 3.0 to 9.0. Xylanase was stable at 60^{o}C for one hour. Optimum pH and temperature for Xylanase was as follows. 5.0 to 5.5 and 75^{o}C. (Micheal *et al.* 1992)

Production of thermo stable and cellulase free Xylanase from

streptomyces sp.QG-11-3 was conducted. Solid state fermentation was applied using wheat bran as the prime solid substrate. Maximum Xylanase yield using wheat bran was 2360U/g. When Xylanase treated pulp was further treated with 4.5% chlorine, it resulted in the reduction of kappa numbers up to 25%. This process enhanced the brightness by 20% (% ISO). This treatment improved the pulp properties, such as burst factor and tensile strength, by 8% and 63% respectively. This study found that Xylanase was stable over a wide pH range, between 5.4 and 9.4. Ca^{+2} increases the activity of the Xylanase, whereas Cd^{+2}, Co^{+2}, and Cr^{+3} inhibited Xylanase activity. We also found that Hg^{+2} inhibited Xylanase completely. (Beg *et al.* 2000)

MATERIALS AND METHODS

Citrate Phosphate Buffer of pH 5.6

0.96 g of citric acid was added in 50 ml of distilled water and 1.78 g of Na_2HPO_4 was added in 50 ml of distilled water. From these solutions, 17.9 ml of citric acid was taken and mixed in 32.1ml of Na_2HPO_4 to obtain 50 ml. After adjusting the pH, the volume was filled to 100 ml with distilled water.

Preparation of DNS (Dinitro salicylic acid) solution

Different ingredients were used for the preparation of DNS. These are as follows: K titrate (Rochelle salt) 182 g/l, DNS 10g/l, NaOH 10g/l, Phenol 02g/l, Na_2SO_3, and 0.5g/l. The above ingredients were dissolved and further dissolution was done with a magnetic stirrer. After dissolving all the above ingredients, the solution was filtered through a filter (sintered glass). This solution was stored at room temperature in an amber colored bottle to avoid photo oxidation. It was stable for 6 months.

Substrate for enzyme activity

1% substrate was prepared by dissolving 1g of oat spelt Xylan in 100 ml of distilled water. It was sterilized by passing through filter paper.

EXPERIMENTAL METHOD

Substrate

For biosynthesis of the Xylanase enzyme, indigenous carbon sources like wheat bran, corn cobs and sugarcane bagasse were dried, ground through 40mm mesh and treated with 2.0% NaOH. These substrates were separately stored in air tight containers for their further utilization in the Xylanase synthesis.

Fermentative Organism

Pre-isolated and purified cultures of the fungus *Aspergillus niger* were taken from the biotechnology laboratory of Government College University Lahore and stored in a refrigerator at a suitable temperature for further use.

Germination of Fungus (*Aspergillus niger)* on agar for sporulation

100 ml of the sporulation medium was prepared in a 250ml Erlenmeyer flask by adding 4g of potato dextrose agar in 100ml distilled water. The Erlenmeyer flasks were cotton plugged and placed in an autoclave at 115^{o}C for 20 minutes. After sterilization, the medium in the Erlenmeyer flasks was cooled and transferred into sterile test tubes. Test tubes were placed in a stand to allow the agar medium to solidify. These test tubes were inoculated from the mother culture of *Aspergillus niger* with a pre-sterilized inoculation loop. These test tubes were

cotton plugged and inoculated for 72 hours at 37^{o}C for germination (Ahmad *et.al.* 2009).

Preparation of inoculums

Inoculation medium was prepared at pH 5.6 and temperature was maintained at 37^{o}C. This medium was sterilized by autoclaving. Sporulation medium and inoculums medium was transferred aseptically into 500 ml flasks. This medium was incubated at 37^{o}C in an orbital shaker at 130 revolutions per minute for three days. Composition is as follows: (NH4)2SO4 1.40 g/100 ml, MgSO4 0.30 g/100 ml, Urea 0.30 g/100 ml, Proteose peptone 0.75 g/100 ml, $CaCl_2$ 0.30 g/100 ml, KH2PO4 2.00 g/100ml, Glucose 10.00 g/100 ml, and Yeast extract 0.25 g/100 ml.

Enzyme production

Carbon sources like wheat bran, corn cobs and sugar cane bagasse were used for the biosynthesis of the Xylanase enzyme, using different temperature pH and incubation times. In each fermentation flask, 3% inoculums medium was added containing the above-mentioned substrate. After a suitable interval of time, the biomass from these flasks was filtered through Whatman™ filter paper No.1 and centrifuged at 4000 rpm for 10 minutes at 10^{o}C. All these prepared samples were refrigerated in sterilized glass bottles.

Optimization

Carbon sources

Different indigenous carbon sources like corn cobs, wheat bran and sugar can bagasse were used with different concentrations separately (2.5, 3.0 and 3.5%). Fermentation was carried out for a period of 72 hrs for the optimization of the substrate.

pH

To find out the optimum pH for Xylanase production, biosynthesis of enzymes was carried out at different pH values, such as 5.0, 5.6, 6.0, 6.6, 7.0, etc.

Temperature

To optimize the temperature, Xylanase production was carried out at different temperatures (20, 25.30 and 35^{o}C).

Incubation Time

To find out the optimum time required for maximum Xylanase activity, samples were harvested for a period of 12, 24, 48, 72 and 96 hrs.

Harvesting of sample

After incubation of the medium at different time intervals, the medium from the flasks was filtered by Whatman™ filter paper of suitable grade. The filtrate was centrifuged at 11000 rpm at 15^{o}C for 15 minutes. Centrifugation was done to remove mycelia and spores of the organism. Supernatant was carefully refrigerated in sterilized glass bottles.

Standard curve for Xylose

Different concentrations (0.2, 0.4, 0.6, 0.8, 1.0, 1.2, and 1.4 mg/ml) of Xylose were prepared in distilled water. 0.5 ml of each concentration was added in test tubes along with 0.5ml of DNS solutions. These test tubes were boiled for 5 minutes in boiling water and then cooled. A blank was also prepared by dissolving 1ml each of DNS and distilled water. Absorbance of these solutions was determined by using a Labomed spectro photometer at 550 nm. A graph was drawn by plotting concentration against absorbance.

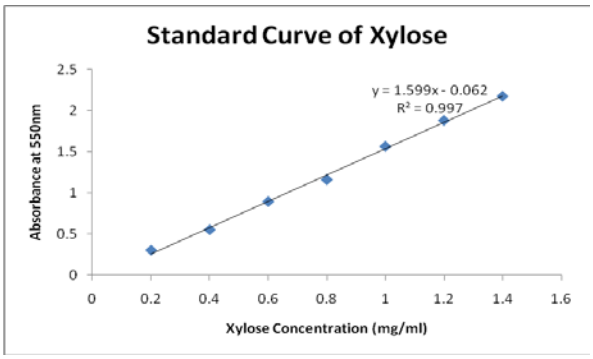

Figure 1: Standard curve for Xylose.

Enzyme Activity

Xylanase enzyme hydrolyses the substrate Xylan in Xylose. The free Xylose units produced react with DNS to form a color complex. This color was detected by a Spectro photometer (Labomed) at 550nm greater the amount of Xylose produced. Darker will be the color and more light will be absorbed. OD's were taken using a UV visible spectrophotometer. These OD's were used to calculate enzyme activities. One unit of enzyme activity may be defined as the amount of Xylanase that produces one micromole of reducing sugar (Xylose) per minute under assay conditions using the DNS method (Miller, 1959).

Activity Assay for Xylanase

Crude Xylanase assays were performed using 0.5ml of 1% oat spelt Xylan using citrate phosphate buffers of pH 5.6. The composition of the reaction mixture was 0.5ml substrate and 0.5ml of crude enzyme. This reaction mixture was incubated in a shaking water bath at 50^{o}C for 15 minutes. After incubation, 1ml of DNS was added. A reddish brown color was developed when the reaction tubes were boiled in boiling water for 5 minutes. After cooling the reaction tubes, OD was measured at 550nm with Xylose as standard material, where one unit of enzyme may be defined as the amount of Xylanase that can produce one micromole of reducing sugar (Xylose) in one minute under standard assay conditions.

Enzyme Purification

Ammonium sulphate precipitation of protein

25 ml of the crude enzyme solution was taken in a 250ml beaker. Different fractions of ammonium sulphate were added into the above solution. These solutions were placed on a constant magnetic bar for constant slow stirring at 4^{o}C. After the addition of each fraction, the solution was centrifuged at 4^{o}C and 4000 rpm for 15 minutes. Supernatants were removed carefully and the pellets obtained were dissolved in 5ml of 0.1 molar phosphate buffers at pH 6.0.

Dialysis

Dialysis tubes were filled with 5ml of the crude enzyme solution and tightened from both ends. A 1000ml beaker was filled with a phosphate buffer at pH 6.0 and 4^{o}C. The apparatus was left with constant slow stirring. Buffer solution was changed after a regular interval of time. This process was carried on for 24 hours (Norazlina *et al.* 2013).

Gel Filtration

The precipitates of the dialyzed enzymes obtained after dialysis was purified by gel filtration. 5ml of enzyme solution was used. The column was equilibrated with a phosphate buffer at pH 6.0. After a regular interval of time, 5ml of the enzyme sample was collected and used for Xylanase activity. The fraction which gives better Xylanase activity was collected while the rest of

the fraction was discarded. (Carmona *et al.*1998)

Estimation of Protein

Protein was determined by using the Bradford method (1976) using a Bovine Serum Albumin (BSA) as a standard by comparison with the standard curve. (Bradford, 1976) In this method, dye binding reagent was prepared by dissolving a suitable amount of commassie brilliant blue in ethanol and orthophosphate. Volume was made to 1000ml with distilled water. For protein estimation, 2.0 ml of dye binding reagent was mixed with 50 micro litres of the protein sample. OD was taken at 550 nm against blank after every five minutes.

Molecular Weight Determination by SDS-PAGE

The Molecular weight of the Xylanase enzyme was determined by using Sodium Dodecyl Sulphate poly acryl amide gel electrophoresis (SDS-PAGE). This method was carried out according to Carmona, *et al.* (1998). In this method, caster and casting plates were set. Resolving gel was prepared. Run of Gel was done at 60 V for 20 minutes and brought to 100 V for about one hour. Gel was stained in commassie brilliant blue R250. Gel was destained in destaining solution for about 24 hours and an image was drawn through gel doc.

Optimization of pH and Temperature

Optimum pH and temperature of the enzyme was obtained (Coral *et al.* 2002) by caring out enzyme activity at different pH and temperature using Citrate Phosphate buffers.

RESULTS AND DISCUSSION

Effect of Carbon Sources for Xylanase Production

Xylanase production by *Aspergillus niger* was studied by testing the Xylanase production in the culture medium using different carbon sources at 20, 25, 30, 35 ^{O}C for different pH levels like 5.0, 5.6, 6.0, 6.6 and 7.0 over a period of 4 days. Results indicate that at lower temperature like 20 ^{O}C, *Aspergillus niger* yielded little Xylanase for all carbon sources, that is, 28.2, 18.4 and 14.3 IU/ml for wheat bran, corn cobs and sugarcane bagasse respectively. When the experiments were performed at higher temperatures, production of Xylanase was effected positively for all carbon sources. At 35 ^{O}C, activities of fungus, wheat bran, corn cobs and sugarcane bagasse were 80.04, 63.02 and 45.01 IU/ml respectively.

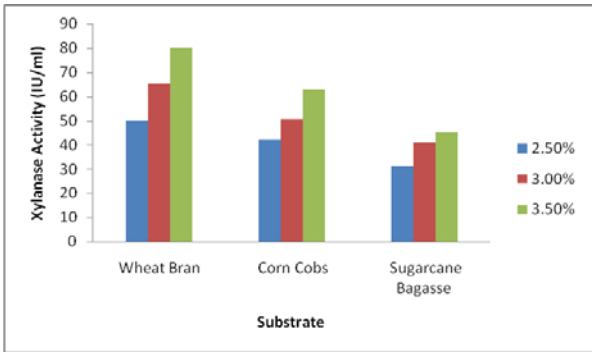

Figure 2: Xylanase Production at different concentrations of wheat bran, corn cobs and sugarcane bagasse by *Aspergillus niger*.

Effect of Incubation time for Xylanase Production

It is apparent from the results that the *Aspergillus niger* synthesized small activities during the 24 hours of incubation. With the increase of incubation time, the organism produced the maximum Xylanase production for 3 days incubation (72 hours), that is, 46.7, 35.6, 27.0 IU/ml of enzyme activities for wheat bran, corn cobs and sugarcane bagasse respectively. With a 96 hour

incubation, enzyme activities decreased for all three substrates due to nutrient depletion by the action of the organism in the growth medium. These results are closely related to the results of Haq *et al.* (2002), who reported maximum Xylanase recovery for 72 hours of incubation.

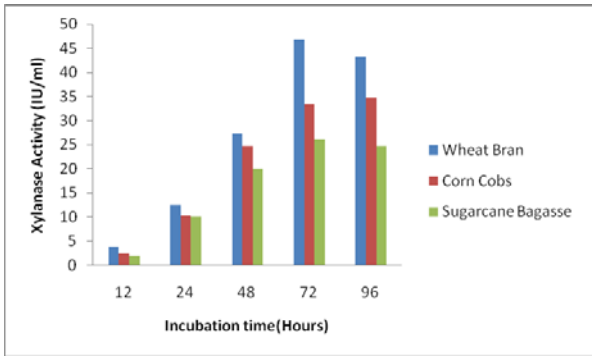

Figure 3: Xylanase Production at Different Incubation and Different Substrate by *Aspergillus niger*

Effect of pH for Xylanase Production

The effect of different pH, such as 5.0, 5.6, 6.0, 6.6 and 7.0, was studied on the enzyme production, when A. niger was grown on wheat bran, corn cobs and sugarcane bagasse. It is obvious from the results that at pH 5.6 of the culture medium, A. niger showed the highest activities of Xylanase enzymes for all carbon sources. Activities for wheat bran, corn cobs and sugarcane bagasse are 39.2, 33.7 and 23.0. It was concluded that 5.6 pH was the most suitable to produce maximum Xylanase activity by using the above carbon sources.

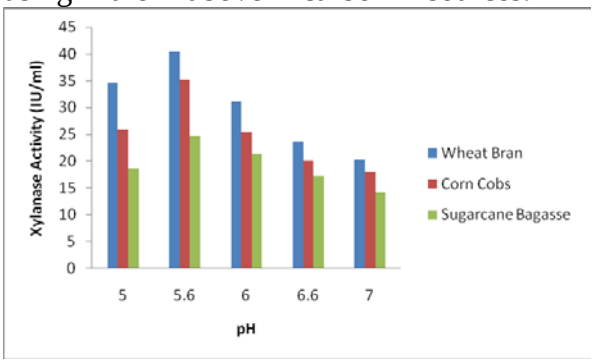

Figure 4: Effect of pH for Xylanase Production.

Effect of temperature for Xylanase Production

Xylanase biosynthesis was carried out at different temperatures, 20, 25, 30, 35, and 40 ^{O}C, for the different carbon sources: wheat bran, corn cobs and sugarcane bagasse. It was revealed that maximum Xylanase production was observed at 35^{O}C for wheat bran.

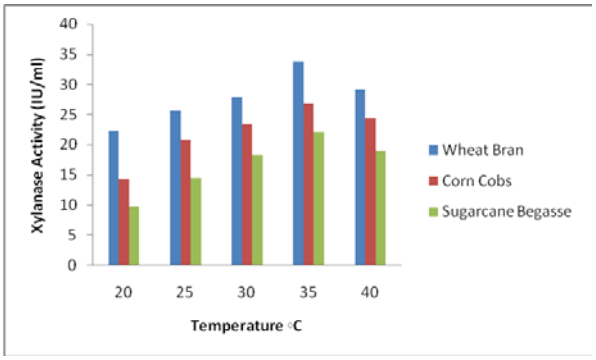

Figure 5: Effect of Temprature for Xylanase Production

Purification and characterization

After the purification, characterization of the Xylanase was done to find the optimum pH, temperature, heat stability and molecular mass. Data obtained after characterization of Xylanase will help us find its application in the paper and pulp industries.

Optimization of temperature for Xylanase activity

It is revealed that when enzyme assay was performed at different temperatures, from 20 ^{o}C to 100 ^{o}C, Xylanase activities increase with rises in temperature up to 50 ^{o}C, with maximum activity at 50 ^{o}C. When temperature was further increased, there was a decline in activity. Minimum activity was found at 100^{O}C. This result shows a close resemblance with the results of Carmona *et al.* (1998), who calculated optimum activity at 55^{o}C.

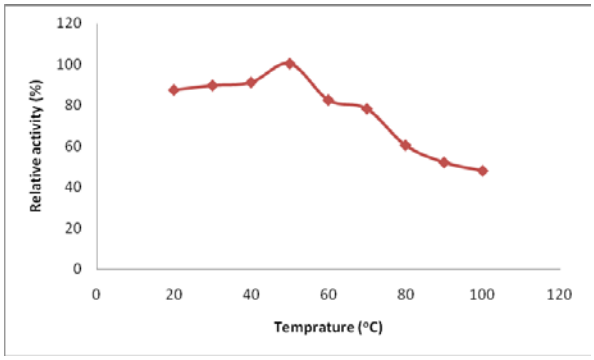

Figure 6: Relative activity of Xylanase at pH 5.5 under different temperatures.

Effect of pH on Xylanase activity

It is obvious from the results that when Xyalanse assay was carried out at 50^{O}C, the enzyme showed minimum activity at pH 2. The graphs showed that activity decreased with the increase of pH up to 5.0. When the pH was raised, there was a gradual increase in the enzyme activity. Maximum activity was found at pH 8.0. Further increases in pH showed a gradual decline in Xylanase activity.

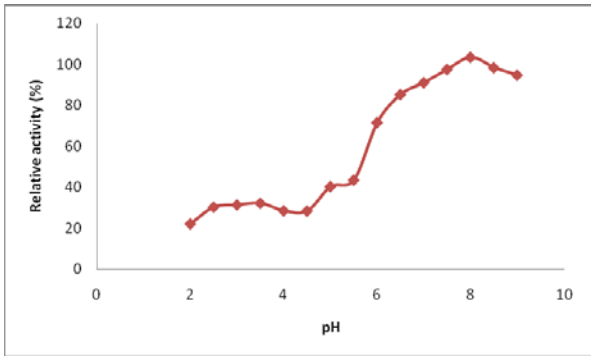

Figure 7: Relative activity of Xylanase at 50 ^{o}C under different pH.

Thermal stability of Xylanase at differnet temperatures

Thermal stability of Xylanase is promising for its application in different industrial processes. When activity of Xylanase was observed at different temperatures, it showed maximum activity at 50^{O}C. When the enzyme was subjected to higher temperatures, there was a negative effect on the enzyme performance. The decrease in the heat stability is due to denaturation of the enzyme at higher temperatures. These results share a close resemblance with the results of Micheal *et al.* (1992). They produced Xylanase that was stable at a temperature of 55-60^{o}C.

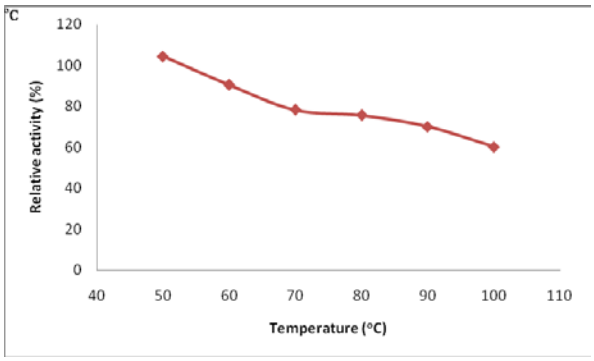

Figure 8: Heat stability of Xylanase at pH 5.5 and different temprature.

Molecular Weight

Molecular weight of the purified Xylanase was found by the subjecting it to SDS-PAGE. The results of SDS-PAGE have revealed that the molecular weight of the protein was 27.2kDa. The results found by the study were similar to Arief Widjaja *et al.* (2009) as 27kDa. Likewise Sardar *et al.* (2000) found the molecular weight of purified Xylanase as 24kDa.

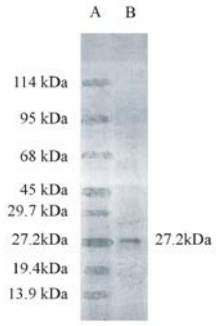

Figure 9: SDS-PAGE analysis for molecular weight determination

CONCLUSION

It was concluded that Xylanase bio synthesis can be conducted using the fungal strain *A. niger*. Agricultural residues/wastes can be used for Xylanase biosynthesis. Local agricultural wastes like sugarcane bagasse, wheat bran and corn cobs with different concentrations were used. It was revealed that maximum Xylanase was produced using wheat bran (3.5%). Results of the fermentation showed that 35^{O}C, 5.6pH, and incubation time of 72 hours with 3.5% carbon source were the best conditions for the biosynthesis of Xylanase. Crude Xylanase have 45.65 IU/mg protein, but purified enzymes showed specific activity of 619.31 IU/mg proteins. Characterization of Xylanase showed that pH 8.0 and 50^{O}C were optimum conditions for maximum activity of Xylanase. SDS PAGE analysis shows purified Xylanase has a molecular weight of 27.2kDa. This Xylanase is thermally stable at 50 ^{O}C, and further increases in temperature decrease Xylanase activity due to the denaturation of protein.

ACKNOWLEDGEMENT

This research work is a part of PhD thesis of Mr. Muhammad Suleman. The Author highly acknowledged the financial support of HEC (Higher

Education Commission of Pakistan) for successful completion of his research work.

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