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OPTIMIZATION OF XYLANASE ACTIVITY IN THE PRESENCE OF SALTS OF DIFFERENT METALS FOR THE PULPING OF INDIGENOUS NON-WOODY RAW MATERIAL (*GOSSYPIUM ARBORIUM*)

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ABSTRACT

This study was conducted to optimize the process of degradation of agricultural wastes using xylanase by pulping. The cotton stalk was treated with enzyme and sugar with different concentration was obtained. The substrate concentration, incubation time, cofactors and activators on xylanase were investigated in the pulping and bleaching process. The study presents results that at pH 6.0 with time span (3, 6 and 9 hours) of incubation produced 56 mg/ml, 75 mg/ml and 112.5 mg/ml sugar respectively. While at pH 7.0 with time duration (3, 6 and 9 hours) of incubation 64 mg/ml, 75 mg/ml and 150 mg/ml of sugar was produced and at pH 8.0 with time duration (3, 6 and 9 hours) of incubation produced 64 mg/ml, 220 mg/ml and 225 mg/ml sugar. There was no significant change in production of sugar at pH 9.0, where during the time period (3, 6 and 9 hours) of incubation 30 mg/ml, 37 mg/ml and 45 mg/ml of sugar was produced respectively. While metal salts are good activators, for the time period of 3, 6 and 9 hours of incubation 56 mg/ml, 64 mg/ml and 90 mg/ml of sugar was produced. This work provides an efficient method for organic waste disposal using an environment-friendly approach with a wide industrial application for welfare of society.

Key Words: Optimization, xylanase, hydrolysis, lignocelluloses, saccharification

INTRODUCTION

All lignocellulosic materials contain hemicellulose which is abundantly available in nature. Hemicellulose from cotton stalk gives D-xylose after enzymatic hydrolysis (Saddler, 1993). The bleaching of soft wood kraft pulp by xylanase is a completely chlorine-free, enzyme aided process. xylanase treatment increased the bleaching ability of kraft pulp depending upon the cooking method used in pulp production (Saddler, 1993). Xylanase increases the brightness of kraft pulp and pulp produced by extended cooking process up to the highest degree. Large amount of lignin with high molecular mass can also be extracted from this process after xylanase treatment. It

has previously been reported that xylanase was especially effective in improving the bleaching ability of oxygen delignified pulps (Cloud, 1994). A lot of studies have been conducted on xylanase activity (Suleman et al., 2012a; 2012b; 2016a; 2016b). However, there is a lack of studies on treatment of cotton stalk by xylanase. This study was designed with the objective to treat lignocellulosic waste with xylanase to obtain products that are used in the pulping industry while ensuring environment-friendly dumping of waste.

MATERIALS AND METHODS

Buffer of pH 6.0 (Citrate phosphate)

A solution was made by adding 0.96 g of citric acid (0.2M) in 50 ml of distilled water and another solution was prepared using 1.78 gram of Na_2HPO_4 (0.1M) in 50 ml of distilled water. From these solutions, 17.9 ml of citric acid solution was taken and mixed in 32.1 ml of Na_2HPO_4 solution to obtain a 50 ml solution. The pH was adjusted with pH meter and volume was made 100 ml by distilled water.

Buffer of pH 7.0 (Phosphate)

Na_3PO_4 (0.05M solution) was taken in a 500 ml beaker and 0.05M $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ was added continuously with constant stirring until the pH reached 7.0.

Buffer of pH 8.0 (Phosphate)

Na_3PO_4 (0.05M solution) was taken in a 500 ml beaker and noted its pH by pH meter. $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ was added to this solution and was constantly stirred until its pH reached 8.0.

Buffer of pH 9.0 (Phosphate)

Na_3PO_4 (0.05M solution) was taken in a 500 ml beaker and its pH was noted using pH meter. $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ was added to this solution with constant stirring until its pH reached 9.0.

DNS (Dinitro salicylic acid) solution preparation

Na-K titrate (Rochelle salt) (182 g/L), DNS (10g/L), NaOH (10g/L), Phenol (0.2g/L) and Na_2SO_3 (0.5g/L) were used. The ingredients were dissolved and further dissolution was done by magnetic stirrer.

Substrate for xylanase activity

Xylan solution (1 %: 1gram of xylan (oat spelt) in 100 ml of distilled water) was mixed on a hot stirring plate. Volume was

made 100 ml by adding water after stirring. 1 unit of xylanase activity was considered as the amount of xylanase that can make 1 micro mole of xylose per minute (Kamble and Jadhav, 2012).

Pulping of non woody raw material (Gossypium Arborium)

Cotton stalk (*G. arborium*) was taken from cotton fields of Faisalabad in the form of sticks. These sticks were dried by direct sunlight. These cotton sticks were then ground to semi powdered form. Application of Xylanase against *G. arborium* has a potential for the availability of Xylose to be used for the production of alcohol and paper pulp.

Pre-treatment of lignocellulosic substrate

The lignocellulosic substrate (cotton stalk) was ground into small particles and pretreated with 2 % NaOH and H_3PO_4 in the ratio of 1:20 (W/V) for 2 hours at room temperature.

Xylanase Assay

Each 0.2 ml of xylanase was taken in 10 different Eppendorf tubes. These Eppendorf tubes were incubated at 55°C, 65°C and 75°C in shaking incubator. After every 5 minutes, one tube was taken out. All the tubes were then cooled in ice for 30 minutes. 0.5 ml of appropriately diluted xylanase was taken in 10 test tubes. 1ml of citrate phosphate buffer was added in each test tube. 0.5 ml of xylan was added in each test tube except the control. These test tubes were incubated for 5 minutes in shaking water bath at 50 °C.

DNS (1 mL) was added in each test tube. These test tubes were boiled for 5 minutes in boiling water. After boiling, test tubes were cooled in cold water. These solutions were centrifuged for 5 minutes in micro-centrifuge. Absorbance (Optical Density) was taken by UV-visible

spectrophotometer (Labomed, USA) and reducing sugars were calculated (Miller, 1959).

Enzyme activity

Xylanase enzyme hydrolyses xylan into xylose. The free xylose units thus produced react with DNS to form color complex. This color was detected by Spectrophotometer (Labomed) at 550 nm. If large amount of xylose is formed, then color of the solution was dark, and more light will be absorbed. The optical density was measured using UV spectrophotometer.

Xylanase activity at pH 6.0 (acidic) with change in substrate concentration and time

Different substrate concentrations (1 g, 2 g, 3 g and 4g) were taken into four different flasks. Xylanase enzyme (2 cm³) was added to each flask. Buffer of pH 6.0 was prepared and 25 cm³ of phosphate buffer was added one by one in each flask. Then these flasks were placed in an incubator at 50°C for 3 hours. After 3 hours, aliquots of 0.5 ml were taken and assayed for reducing sugar. In the same way, reducing sugars were calculated after 6 hours and 9 hours.

Xylanase activity at pH 7.0 (neutral) with change in substrate concentration and time

Different substrate concentrations (1 g, 2 g, 3 g and 4 g) were taken into four different flasks. Xylanase enzyme (2 cm³) was added in each flask. Buffer of pH 7.0 was prepared with Na₂HPO₄.2H₂O and Na₃PO₄, 25 cm³ of this was added in each flask respectively. Then these flasks were placed in oven at 50°C for 3 hours. After 3 hours, amount of reducing sugar produced was calculated. In the same way reducing sugar was calculated after 6 hours and 9 hours.

Xylanase activity at pH 8.0 (basic) with change in substrate concentration and time

Different substrate concentrations (1 g, 2 g, 3 g and 4 g) were taken into four different flasks. Xylanase enzyme (2 cm³) was added in each flask. Buffer of pH 8.0 was prepared with Na₂HPO₄.2H₂O and Na₃PO₄, 25 cm³ of this was added in each flask. Then these flasks were placed in oven at 50°C for 3 hours. After 3 hours reducing sugar was calculated. In the same way, quantity of reducing sugar produced was calculated after 6 hours and 9 hours.

Xylanase activity at pH 9.0 (Basic) with change in substrate concentration and time

Different substrate concentrations (1 g, 2 g, 3 g and 4 g) were taken into four different flasks. Xylanase enzyme (2 cm³) was added in each flask. Buffer of pH 9.0 was added in each flask and it was also prepared with Na₂HPO₄.2H₂O and Na₃PO₄. Then these flasks were placed in oven at 50°C for 3 hours. After 3 hours reducing sugar was calculated. In the same way reducing sugar was calculated after 6 hours and 9 hours.

Effect of cofactors or activators on xylanase activity

Xylanase activity was checked at pH 8.0 with different activators to see extent of enhancement in xylanase activity. Some cofactors were used like Cobalt Chloride (CoCl₂), Zinc Chloride (ZnCl₂), Calcium Chloride (CaCl₂) and Mercuric Chloride (HgCl₂). One gram of treated substrate was taken in four flasks separately. Xylanase enzyme (2 cm³) was added. 25 cm³ of buffer of pH-8 was added in each flask. 1 M solution of Zinc Chloride (ZnCl₂), Cobalt Chloride (CoCl₂), Mercuric Chloride (HgCl₂) and Calcium Chloride (CaCl₂) was prepared. Then, 2 cm³ of ZnCl₂ solution was added in flask 1, 2 cm³ of CoCl₂ solution in flask 2, 2 cm³ of CaCl₂ solution in flask 3, and 2 cm³

of HgCl_2 solution in flask 4. These four flasks were placed in an oven for 3 hours, 6 hours and 9 hours at 50°C for incubation. After particular (3, 6 and 9 hours) time intervals reducing sugar was calculated to see enhancement by different co-factors.

RESULTS AND DISCUSSION

Xylan is a very large and abundantly-found complex structure in wheat, cotton stem, Birchwood and bagasse etc. It is the second most abundant natural polysaccharide (Polizeli et al., 2005). Xylanases are hydrolyzing enzymes produced by various microorganisms (fungi and bacteria). However, xylanase synthesis has been reported to be repressed by Xylose. Significant level of Xylanase was secreted when *Aspergillus niger* was grown in the presence of wheat bran as carbon source at optimum temperature and pH conditions (Ayyachamy and Vatsala, 2007).

Xylanase activity at pH 6.0 (3 hours, 6 hours and 9 hours) of incubation

When the substrate was incubated at 50°C for 3 hours using a buffer solution of pH 6, 56 mg/ml of sugar was produced (Figure 1). After 6 hours, the amount of released sugar increased from 56 mg/ml to 75 mg/ml (Figure 2). After 9 hours, the amount of sugar released increased from 75 mg/ml to 112.5 mg/ml (Figure 3).

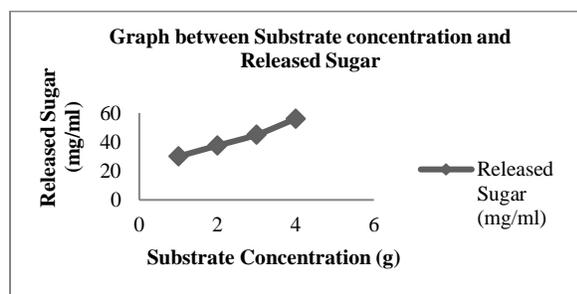


Figure 1: Xylanase activity at pH 6.0 for 3 hours incubation with different concentrations of substrate

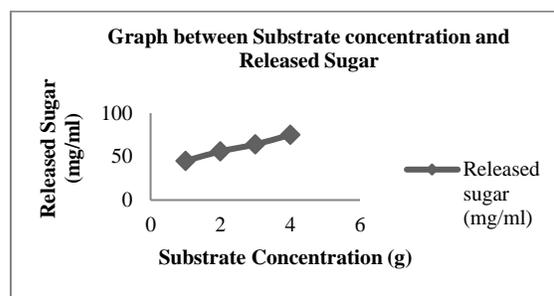


Figure 2: Xylanase activity at pH 6.0 for 6 hours incubation with different concentrations of substrate

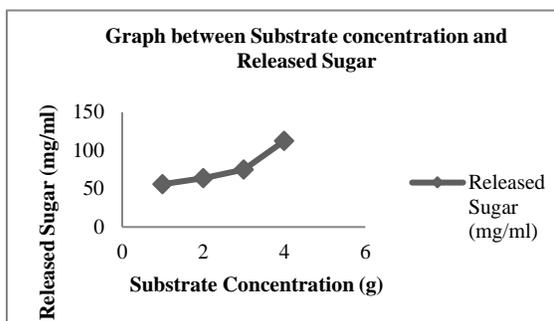


Figure 3: Xylanase activity at pH 6.0 for 9 hours incubation with different concentrations of substrate

Xylanase activity at pH 7.0 (3 hours, 6 hours and 9 hours) of incubation

When the substrate was incubated at 50°C for 3 hours using a buffer solution of pH 7, 64 mg/ml of sugar was produced (Figure 4). After 6 hours, the concentration of released sugar was 75 mg/ml (Figure 5). After 9 hours, the concentration of released sugar was 150 mg/ml sugar (Figure 6).

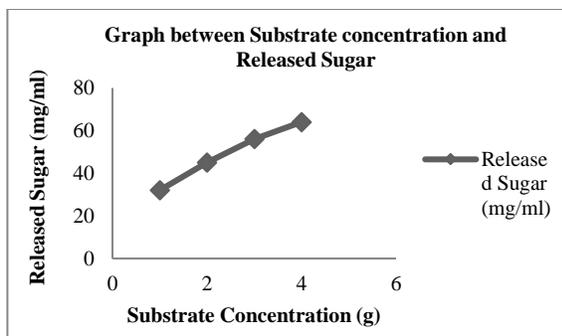


Figure 4: Xylanase activity at pH 7.0 for 3 hours incubation with different concentrations of substrate

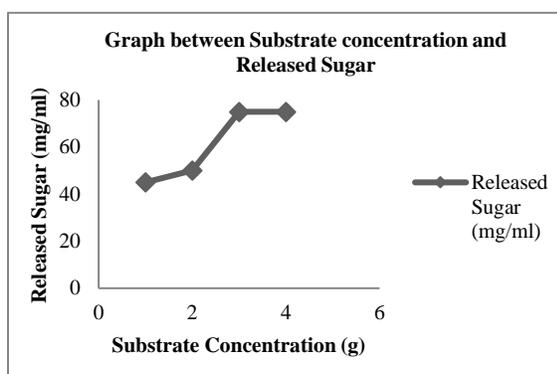


Figure 5: Xylanase activity at pH 7.0 for 6 hours incubation with different concentrations of substrate

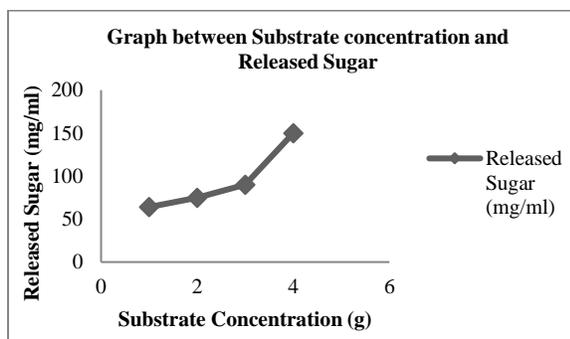


Figure 6: Xylanase activity at pH 7.0 for 9 hours incubation with different concentrations of substrate

Xylanase activity at pH 8.0 for (3 hours, 6 hours and 9 hours) of incubation

When Xylanase was incubated for 3 hours at 50°C using a buffer solution of pH 8, 64 mg/ml of sugar was produced (Figure 7). After 6 hours, the concentration of released sugar was 200 mg/ml (Figure 8). After 9 hours, the concentration of released sugar was 225 mg/ml (Figure 9).

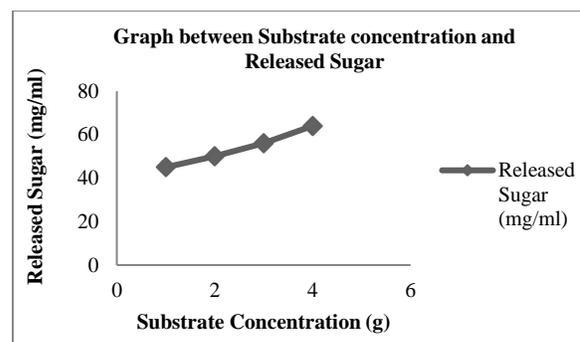


Figure 7: Xylanase activity at pH 8.0 for 3 hours incubation with different concentrations of substrate

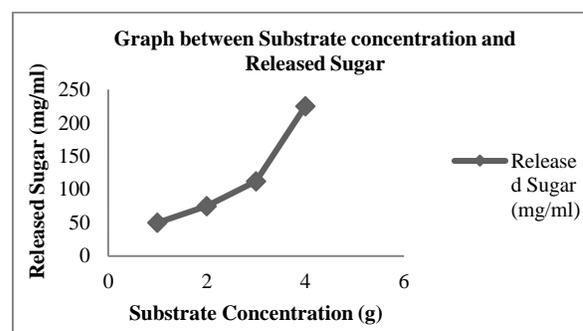


Figure 8: Xylanase activity at pH 8.0 for 6 hours incubation with different concentrations of substrate

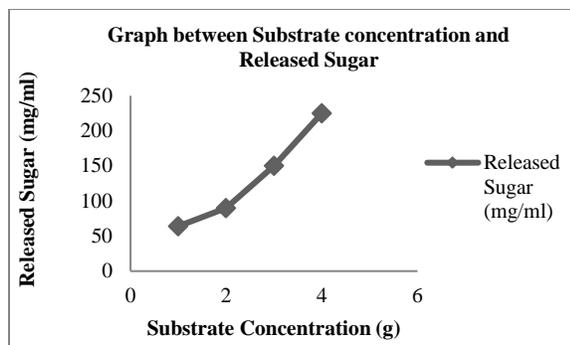


Figure 9: Xylanase activity at pH 8.0 for 9 hours incubation with different concentrations of substrate

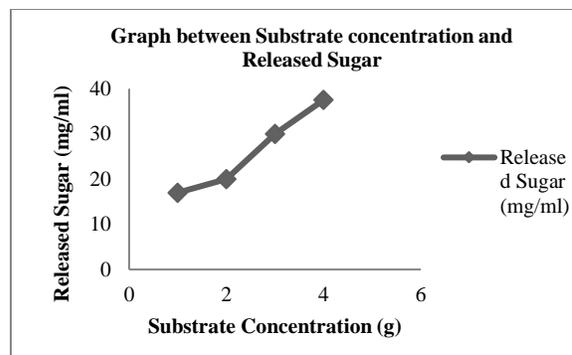


Figure 11: Xylanase activity at pH 9.0 for 6 hours incubation with different concentrations of substrate

Xylanase activity at pH 9.0 for (3 hours, 6 hours and 9 hours) of incubation

When Xylanase was incubated for 3 hours at 50°C using a buffer solution of pH 9, 30 mg/ml sugar was released (Figure 10). After 6 hours, the concentration of released sugar was 37.5 mg/ml (Figure 11). After 9 hours, the concentration of released sugar was 45 mg/ml (Figure 12). It was revealed from the data that amount of released sugar decreased with the increasing pH from 6.0 to 9.0 hours. The amount of released sugar decreased by changing the medium of the reaction mixture from slightly alkaline to more alkaline.

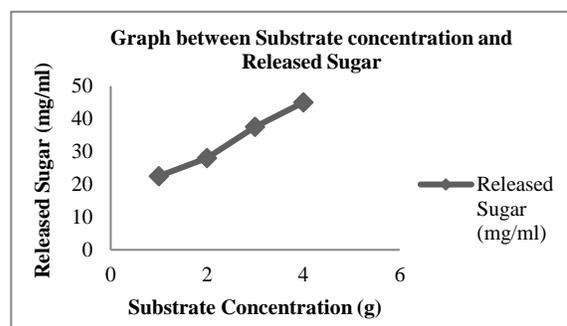


Figure 12: Xylanase activity at pH 9.0 for 9 hours incubation with different concentrations of substrate

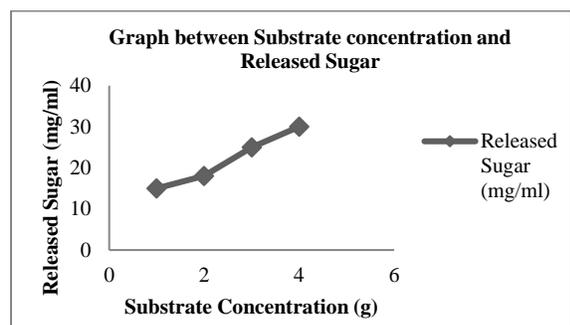


Figure 10: Xylanase activity at pH 9.0 for 3 hours incubation with different concentrations of substrate

Xylanase activity for 3 hours, 6 hours and 9 hours of incubation (Effect of Metal Salts)

Graphical representation of the data showed that 56 mg/ml sugar was released when xylanase was incubated at 50°C for 3 h in the presence of CaCl₂. Maximum released sugar was shown for CaCl₂ (56 mg/ml) and minimum 0 mg/ml for HgCl₂ (Figure 13).

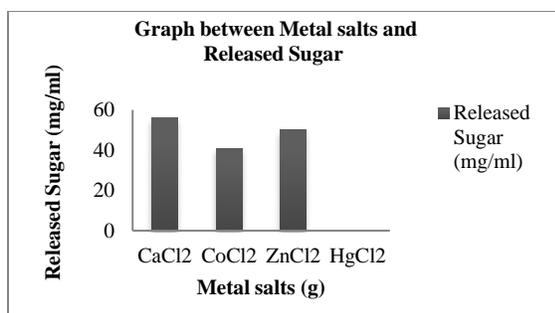


Figure 13: Xylanase activity after 3 hours with different metal salts

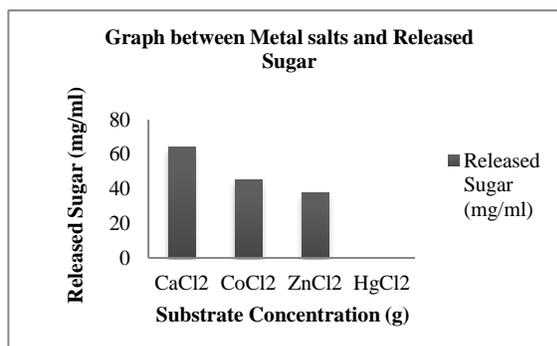


Figure 14: Xylanase activity after 6 Hours with different metal salts

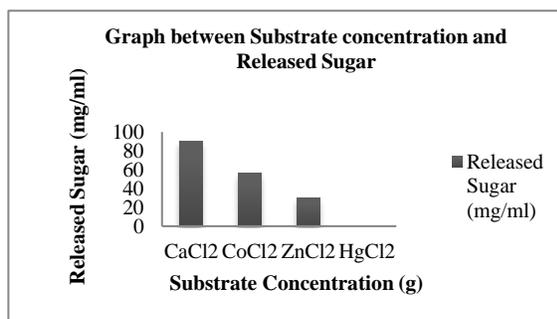


Figure 15: Xylanase activity after 9 Hours with different metal salts

The results of current research showed that xylanase enzyme is active against cotton stem Xylan. Effectiveness of Xylanase was confirmed by transformations of hemicelluloses into simple sugars. Activity of Xylanase was optimized at different levels of pH and temperature and in presence of certain activators (metal ions).

Study of the effect of different substrates concentration on hydrolysis showed that higher saccharification values were obtained with lower substrate concentration. This suggests that lower substrate concentration like 1 g, 2 g, 3 g and 4 g per liter gives high concentration of released sugars. However, increasing the substrate concentration two-fold did not result in the expected exponential. Even at pH 7.0, 8.0 and 9.0 same results were observed, which suggests that by increasing the substrate concentration released sugar was increased with low factor. This finding colludes with the observations by Butt et al. (2008).

When xylanase activity was compared at different pH conditions, it was observed that with 1-gram substrate maximum activity was given at pH 8 and minimum activity was found at pH 9.0. This suggests that xylanase activity declines with increase in pH. Same results were obtained with 2 g, 3 g and 4 g substrate concentrations. When results of different pH levels were observed it was concluded that maximum values of released sugars were found at pH 8.0. When time duration for optimum temperature (50 °C) was changing from 3 hours to 6 hours and 6 hours to 9 hours there was a very little enhancement in released sugar values. Same observations were taken at pH 6.0, 7.0, 8.0 and 9.0 by varying the incubation time at optimum temperature there was a very little change but significant effect of time duration was noted.

Xylanase activity was significantly inhibited by HgCl₂ (mercuric chloride) and ZnCl₂ (zinc chloride). While enhanced by CaCl₂ and CoCl₂. The mechanism by which metal ions enhanced the activity is through their binding to the substrates, so as to orient them properly for enzymatic reaction. Metal ions may facilitate the reaction through the

charge shielding of bound substrate (Mahajabeen, 1998).

CONCLUSION

It may be concluded that xylanase is active against cotton stalk xylan; xylanase converts the xylan into xylose leaving behind disperse cellulose fiber which are starting materials for paper and pulp and the released sugar is fermented to alcohols. It was concluded that xylanase activity is affected by many factors like pH, temperature, time of incubation, activators and inhibitors. Our main purpose was to check enhancement of xylanase activity by changing conditions and different factors. The best enzymatic activity was observed at pH 8 with 9 h time of incubation at 50°C. Results of pH 7 were almost close to that of pH 8. Optimum temperature was 50°C for activity of xylanase against cotton stalk xylan. Effect of some metal ions was observed and CaCl₂ was found to be the best activators for xylanase. HgCl₂ was found to inhibit the xylanase activity very strongly. By increasing the incubation time, good results were observed with 9 h duration.

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