

**INDUSTRIAL APPLICATION OF XYLANASE IN THE CRUDE ENZYME EXTRACT FROM *TRICHODERMA sp.*MS 2010**M. SATHIYAVATHI<sup>1\*</sup> and R. PARVATHAM<sup>2</sup>

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**ABSTRACT**

In this study, *Trichoderma sp.*MS 2010 (GenBank accession number, HM 124450) was used as the source for xylanase. Sugarcane bagasse, an inexpensive agro-industrial waste rich in lignocellulosic materials exhibited maximum production of xylanase. It was found that initial pH 5.0, cultivation temperature of 27°C, incubation period of 10-12 days and 80% moisture content as the optimum growth conditions required for the highest production of xylanase by *Trichoderma sp.* MS 2010. Among the nitrogen supplements, both organic and inorganic nitrogen sources increased the production of xylanase. Inorganic metal salts like ZnSO<sub>4</sub>, MnSO<sub>4</sub>, MgSO<sub>4</sub>, FeSO<sub>4</sub>, NaCl, and CaCl<sub>2</sub> were found to support xylanase production. Prebleaching of pulp by xylanase in the crude enzyme extract followed by multistage chemical treatment resulted in enhancement of the brightness, which was attained with 50% reduction in chemical consumption.

**Keywords:** *Trichoderma sp.* MS 2010, Xylanase, sugarcane bagasse, Prebleaching

**INTRODUCTION**

The environment is endlessly polluted by a huge array of hazardous chemicals with different structures and toxicity levels which are released mainly from industrial activities. The pollutants are the major risk factors for human, animals and environment<sup>1</sup>. Hence, the utilization of microbial enzymes in the industrial processes is considered to be a clean strategy, entirely free from the use of harmful chemicals and capable of producing similar results as those obtained during the course of conventional methods<sup>2</sup>. Biobleaching and bioprocessing of pulps using xylanases is one of the most appropriate biological means used in the pulp and paper industry<sup>3</sup> to reduce and/or completely eliminate the use of chlorine and chlorine dioxide<sup>4</sup>.

Large quantities of lignocellulosic materials are produced as agro-industrial wastes and most of these wastes are disposed by burning, which results in the loss of valuable resources and is regarded as the major factor in global warming<sup>5</sup>. The exploitation of agro-industrial wastes as substrates for solid state fermentation (SSF) is a beneficial process in the aspects of efficient utilization of substrates and reduction in the production cost<sup>6</sup>. Therefore, it is obligatory to search for a novel microorganism with the potential to formulate commercial plus green industrial processes. Hence, the present work "Industrial Application of Xylanase in the crude enzyme filtrate from *Trichoderma sp.*MS 2010, is designed to study the effect of crude enzyme filtrate in bleaching of Kraft wood pulp, which is produced through SSF.

**MATERIALS AND METHODS****Source organism**

The fungal organism used in this study was *Trichoderma sp.*MS 2010 (GenBank accession number, HM 124450) isolated from paper industry effluent.

**Screening of agro-industrial waste material as SSF substrate for maximum enzyme production**

Various agro-industrial waste materials such as wheat bran, sugarcane bagasse, rice bran, corncob and banana peel were selected as substrates and screened for the maximum production of xylanase through SSF. The substrates were dried in an oven at 80°C for 2 hours, ground with blender, sieved into finer particles and then used. Erlenmeyer flasks (250ml) containing 10g each of the five substrates were added with 30ml of Mandels and Sternburg's basal medium as moistening agent<sup>7</sup>. The composition of the medium was as follows: Peptone 1 g/l; (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> 1.41 g/l; KH<sub>2</sub>PO<sub>4</sub> 2.0 g/l; Urea

0.3 g/l; CaCl<sub>2</sub> 0.3 g/l; MgSO<sub>4</sub>.7H<sub>2</sub>O 0.3 g/l; Trace Elements FeSO<sub>4</sub>.7H<sub>2</sub>O 5.0 mg/l; MnSO<sub>4</sub>.H<sub>2</sub>O 1.6 mg/l; ZnSO<sub>4</sub>.7H<sub>2</sub>O 1.4 mg/l; CoCl<sub>2</sub> 20.0 mg/l; Tween 80 0.1 % (v/v); pH 5.0. The flasks were

sterilized at 121°C for 30 minutes and allowed to cool. After cooling, the flasks were inoculated with 2 agar discs (2mm) prepared from 10 days old PDA slants of *Trichoderma sp.*MS 2010 grown at 28°C. The flasks were incubated at 28°C under static condition for 10 days. At the end of the tenth day, the crude enzyme extract was prepared and was used as the source of crude enzyme<sup>8</sup>. The same procedure was used for fungal cultivation for enzyme production for all experiments that are followed in the present investigation.

**Assessment of xylanase activity<sup>9,10</sup>**

One unit of xylanase activity is expressed as the amount of enzyme that releases 1μmol of xylose (ml<sup>-1</sup>min<sup>-1</sup>) at pH 5.0 and at 30°C.

**Optimization of culture conditions for enzyme production**

Culture conditions for maximum production of xylanase by *Trichoderma sp.*MS 2010, were optimized with respect to pH, temperature, moisture content, incubation period, organic and inorganic nitrogen sources and inorganic metal ions. The effect of initial pH of the medium was observed by adjusting the initial pH to 4.0, 5.0, 6.0, 7.0 and 8.0 with 50mM citrate (4-6) and phosphate buffer (7-8). The effect of cultivation temperature was studied at different temperatures starting from 17°C to 57°C with an interval of 10°C at pH 5.0. The fungal organism was cultivated for a period of 20 days at pH 5.0 and 27°C and the enzyme production was determined with 48 hours interval to examine the effect of incubation period. The effect of moisture content of the medium on the enzyme production was tested in different moisture level (50%, 60%, 70% and 80%). The effect of the supplementing nitrogen sources was studied by adding either one of the organic nitrogen sources (peptone and urea in a concentration of 1%) or one of the inorganic nitrogen sources (NaNO<sub>3</sub>, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, NH<sub>4</sub>NO<sub>3</sub>, NH<sub>4</sub>Cl in a concentration of 10mM) to the modified Mandels and Sternburg's basal medium (without any nitrogen source). A Control was maintained simultaneously without any of the nitrogen sources. The effect of metal ions was studied by adding 10mM each of CuSO<sub>4</sub>, FeSO<sub>4</sub>, MnSO<sub>4</sub>, MgSO<sub>4</sub>, NaCl, ZnSO<sub>4</sub>, CaCl<sub>2</sub>, CoCl<sub>2</sub> to the modified Mandels and Sternburg's basal medium (without any microelements). The control assay was also conducted simultaneously without the addition of metal salts.

### Biobleaching of Hardwood kraft pulp by crude xylanase

Unbleached hardwood kraft pulp was obtained from Sesashayee Papers Boards Pvt Ltd, Erode, Tamilnadu, India. The pulp had an initial solid consistency of 20%, kappa number of 28 and brightness of 28.2%. The pulp was stored in sealed plastic bags in 4°C until required. The optimization of treatment conditions are carried out as described by Sadhasivam *et al.*<sup>11</sup> with modifications. The pulp samples with 3 % consistency with citrate buffer (pH 5.0) were taken in polyethylene bags and treated with crude enzyme extract containing xylanase. Enzyme charges of 20, 40, 60 U /g of pulp; incubation periods of 1.0, 2.0, 3.0, 4.0, 5.0 and 6.0 hours; various pH ranging from 4.0, 5.0, 6.0, 7.0 and 8.0; temperatures ranging from 20°C to 60°C with an interval of 10°C were tested to determine the optimum reaction conditions. Simultaneously, a control was maintained without crude enzyme extract for all the treatments.

### Processing and analysis of treated pulp and pulp free filtrate

The pulp samples treated in the above experiments were filtered and washed with distilled water after the incubation period. One part of the pulp was processed to determine kappa number (TAPPI method). The other part of the pulp was dried and hand sheets were prepared by standard TAPPI method to determine the brightness. The pulp free filtrate was analyzed for the release of lignin derived compounds (A<sub>237</sub>, A<sub>254</sub>, and A<sub>280</sub>) and hydrophobic compounds (A<sub>465</sub>)<sup>12</sup>. The release of reducing sugar was analyzed by DNS method<sup>10</sup>.

### Assessment of prebleaching efficiency of xylanase in the crude enzyme extract<sup>13</sup>

For the assessment of prebleaching efficiency of xylanase from *Trichoderma* sp. MS 2010, a comparative study was done. The pulp was made up at 3.0 % consistency and divided into three parts. One part was treated with 40U/g of crude xylanase, mixed thoroughly and incubated at the optimum conditions observed in the above processes. The enzyme treated pulp was treated with 7 % sodium hypochlorite (NaOCl), incubated for 1 hour at 60°C, followed by the addition of 1 % H<sub>2</sub>O<sub>2</sub> and incubated at 60°C for one hour. Second part of the pulp was first treated with 40U/g of crude xylanase, mixed thoroughly and incubated at the optimum conditions observed in the above processes. The enzyme prebleached pulp was then treated with 3.5 % NaOCl, followed by the H<sub>2</sub>O<sub>2</sub> treatment as above. The third part of the pulp was treated with 7 % NaOCl, followed by 1% H<sub>2</sub>O<sub>2</sub> without enzymatic pretreatment as above. Untreated pulp sample was used as control. The effect of the treatments was assessed by calculating the pulp properties such as kappa number and brightness as mentioned above.

### Statistical analysis

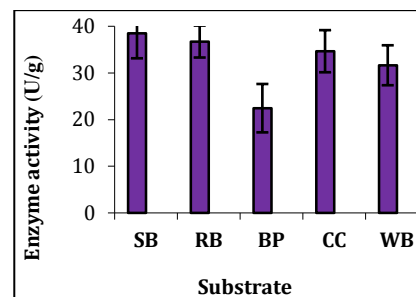
The statistical analysis was carried out using the software Graphpad prism version 5.0 (GraphPad Software, San Diego, California, USA). The difference between more than two groups was calculated using Bonferroni's multiple comparison test (one way ANOVA). The results of obtained were subjected to statistical analysis wherever required. P value less than 0.05 was considered as statistically significant.

## RESULTS AND DISCUSSION

### Screening of low cost agro industrial waste materials for their suitability for the production of xylanase by *Trichoderma* sp. MS 2010 in SSF

It was obvious from the results depicted in **Figure 1** that sugarcane bagasse is a better substrate, when compared to the other selected substrates. Hence, it was selected as an appropriate substrate for xylanase production in SSF to perform further investigation using *Trichoderma* sp. MS 2010. The culture of *Trichoderma* sp. MS 2010 produced higher amount of xylanase in sugarcane bagasse, when compared to rice bran, corn cob and wheat bran. However, banana peel was found to produce significantly ( $p < 0.05$ ) lower amount of xylanase. The findings of the present work are in accordance with Pang *et al.*<sup>14</sup> who reported the highest xylanase production by *Trichoderma* spp. FETL c3-2 with sugarcane bagasse, when compared to rice bran and palm kernel cake. Bakri *et al.*<sup>15</sup> found that

the highest xylanase production by *Cochliobolus sativus* was obtained on wheat straw, whereas, wheat bran and corn hulls showed lower production.



**Figure 1: Effect of substrate on xylanase production by *Trichoderma* sp. MS 2010**

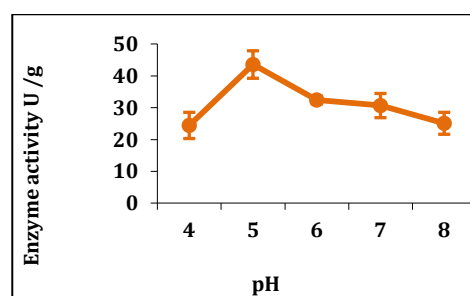
Values are mean  $\pm$  SD of triplicates. Values are expressed as U/g of dry fermented substrate at pH 5.0 and at 28°C.

One unit of xylanase activity is expressed as the amount of enzyme that releases 1 $\mu$ mol of xylose ( $\text{ml}^{-1}\text{min}^{-1}$ ) at pH 5.0 and at 30°C.

Nwodo *et al.*<sup>16</sup> demonstrated that sugarcane pulp and wheat bran were found to be the best substrates for xylanase production by *Penicillium crysogenum* and *Aspergillus niger* when compared to saw dust. According to Sonia *et al.*<sup>17</sup> the observed difference in xylanase production with different substrate materials may be due to variable nature and degradation of their hemicellulosic material. The presence of suitable nutrients during production might also play a key role for differential production of enzymes.

### Effect of initial pH on xylanase production by *Trichoderma* sp. MS 2010 with sugarcane bagasse as substrate

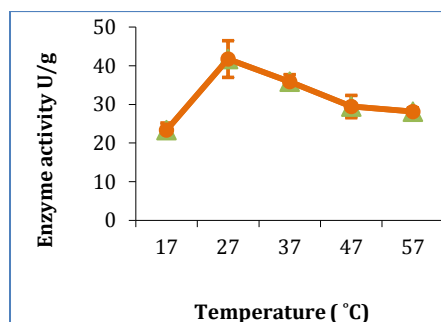
pH is one of the important parameters in fungal cultivation. The results of the effect of pH on enzyme production by *Trichoderma* sp. MS 2010 are depicted in **Figure 2**. The optimum pH for maximum xylanase production was found to be pH 5.0, an increase in xylanase activity was noticed from pH 4.0-5.0, but thereafter enzyme production was decreased with increase in pH. This may be attributed to the fact that change in pH may alter the three dimensional structure of the enzymes. Similar findings were noticed by Isil *et al.*<sup>18</sup> for *Trichoderma harzianum* 1073 D3, which produced highest xylanase activity with wheat bran at pH 5.0. However, Azin *et al.*<sup>19</sup> showed maximum production of xylanase at pH 4.5 with *Trichoderma longibrachiatum* using a combination of wheat straw and wheat bran. Gupta *et al.*<sup>8</sup> found that 5.5 was the optimum pH for xylanase production by *Fusarium solani* F7 in SSF of wheat straw.



**Figure 2: Effect of initial pH on xylanase production by *Trichoderma* sp. MS 2010**

### Effect of temperature on xylanase production by *Trichoderma* sp. MS 2010 with sugarcane bagasse as substrate

The optimum temperature for the production of xylanase was found to be 27°C with sugarcane bagasse. A lower activity was recorded above and below this temperature



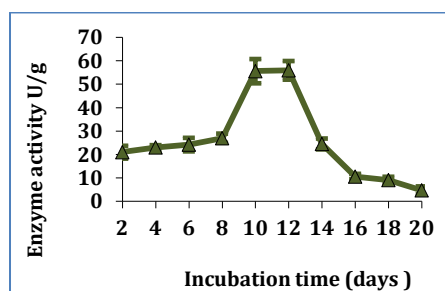
**Figure 3: Effect of temperature on xylanase production by *Trichoderma* sp. MS 2010**

Values are mean  $\pm$  SD of triplicates. Values are expressed as U/g of dry fermented substrate at pH 5.0

The results obtained indicated that the optimum temperature for enzyme production corresponded closely to room temperature, i.e. 28°C. This temperature is similar to the temperature of the natural habitat of the fungus, from where it was initially isolated. The observations of the present study were nearer to the results of Azin *et al.*<sup>19</sup> who reported the optimum temperature as 25°C for xylanase production by *Trichoderma longibrachitum* through SSF in wheat bran and wheat straw. Ahmad *et al.*<sup>20</sup> and Gupta *et al.*<sup>8</sup> reported 30°C as optimum temperature for maximum xylanase production with wheat bran by *Aspergillus niger* and with wheat straw by *Fusarium solani* F7 respectively. The temperature requirement for xylanase production reported in literature differed between 25°C and 30°C with the nature of the substrates and microorganisms used. This might be due to the heterogeneous nature of substrates which is influenced differently by the environmental factors such as temperature and pH<sup>21</sup>.

#### Effect of incubation time on xylanase production by *Trichoderma* sp. MS 2010 with sugarcane bagasse as substrate

The maximum xylanase activity was exhibited on 12<sup>th</sup> day of incubation period at 27°C and pH 5.0 (Figure 4). Findings of the corresponding experiments revealed that the time course of enzyme production varies with the nature of substrate, microorganisms used, cultivation conditions and inducers added in the medium.



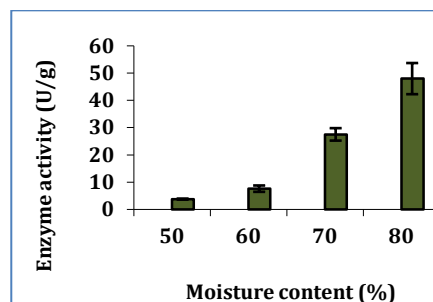
**Figure 4: Effect of incubation time on xylanase production by *Trichoderma* sp. MS 2010**

Values are mean  $\pm$  SD of triplicates. Values are expressed as U/g of dry fermented substrate at pH 5.0 and 27°C.

Reddy *et al.*<sup>22</sup> had reported that the SSF of banana leaf and pseudostem by *Pleurotus ostreatus* produced maximum xylanase with 20 days of time duration, whereas the time course required by *Pleurotus sajor-caju* with the same substrates was 10 days. According to Gupta *et al.*<sup>8</sup> the maximum xylanase production occurred on 6<sup>th</sup> day of incubation during the SSF of wheat straw with the culture of *Fusarium solani* F7. The findings of the present investigation were on par with the previous findings.

#### Effect of moisture content on xylanase production by *Trichoderma* sp. MS 2010 with sugarcane bagasse as substrate

The present investigation clearly indicated that xylanase activity increased with increase in moisture content from 50% to 80% and the maximum activity was obtained at 80% (Figure 5). Ferreira *et al.*<sup>23</sup> had shown that the optimal moisture requirement for the production of xylanase with *Aspergillus tamarii* was found to be 86%, 80% and 75% for wheat bran, corn cob and sugarcane bagasse respectively. Christopher *et al.*<sup>24</sup> reported that 83% moisture condition was the prerequisite to obtain maximum production of xylanase through the SSF of sugarcane bagasse pulp by *Thermomyces lanuginosus*. Pang *et al.*<sup>14</sup> had revealed that 75-80% moisture content in SSF medium with sugarcane bagasse resulted in significant production of xylanase by *Trichoderma* spp. FETL c3-2.



**Figure 5: Effect of moisture content on xylanase production in *Trichoderma* sp. MS 2010**

Values are mean  $\pm$  SD of triplicates. Values are expressed as U/g of dry fermented substrate at pH 5.0 and 27°C.

Yang *et al.*<sup>25</sup> had obtained the highest production of xylanase by *Pacilomyces thermophila* J18 in the SSF of wheat straw with the moisture content of 83%. The outcome of similar experiments demonstrated that the moisture requirement for most of the filamentous fungi in SSF processes for the enzyme production is between 40% and 80%. However, as reported by Raimbault<sup>26</sup> the requirement of moisture content for maximum enzyme production varies with the organism and substrate used for cultivation.

#### Effect of nitrogen source on xylanase production by *Trichoderma* sp. MS 2010 with sugarcane bagasse as substrate

The effect of both organic and inorganic nitrogen sources on xylanase production by *Trichoderma* sp. MS 2010 were assessed and the results are depicted in Table 1. It was observed that NH<sub>4</sub>Cl supported the highest production of xylanase ( $p < 0.05$ ), when compared to control. NH<sub>4</sub>NO<sub>3</sub> and (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> peptone, urea and NaNO<sub>3</sub> also produced considerable levels of xylanase ( $p < 0.05$ ). The results thus obtained did not show any significant difference between organic and inorganic nitrogen sources on xylanase production by *Trichoderma* sp. MS 2010.

**Table 1: Effect of nitrogen source on xylanase production by *Trichoderma* sp. MS 2010**

S.No	Nitrogen source	Xylanase activity (U/g)
1	Control	14.95 $\pm$ 0.56
2	Peptone (1%)	35.48 $\pm$ 4.00*
3	Urea (1%)	33.48 $\pm$ 1.98*#
4	NH <sub>4</sub> Cl (10mM)	43.65 $\pm$ 3.08*
5	NH <sub>4</sub> NO <sub>3</sub> (10mM)	41.77 $\pm$ 2.18*
6	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> (10mM)	39.83 $\pm$ 4.28*
7	NaNO <sub>3</sub> (10mM)	38.47 $\pm$ 2.65*

Values are mean  $\pm$  SD of triplicates. Values are expressed as U/g of dry fermented substrate at pH 5.0 and 27°C.

\*Significant ( $p < 0.05$ ), when compared to the control.

\*#Significant ( $p < 0.05$ ), when compared to the highest value within the test groups

The previous investigations regarding the requirement of nitrogen source for the production of xylanase varied considerably depending on the type of organisms and the method of cultivation. The results

of the present study were in accordance with the findings of Gupta *et al.*<sup>9</sup> who recorded that *Fusarium solani* F7 was found to produce higher xylanase activity both with organic and inorganic nitrogen sources under SSF process with wheat straw. Svarachorn<sup>27</sup> reported that among the inorganic nitrogen sources tested,  $\text{NH}_4\text{NO}_3$ ,  $(\text{NH}_4)_2\text{SO}_4$ , and  $\text{NH}_4\text{Cl}$  were found to be the best for xylanase production by *Aspergillus fumigatus* strain 4-45-1F in SSF with rice straw. Yang *et al.*<sup>25</sup> observed that the xylanase production by *Pacilomyces thermophila* J18 in SSF of wheat straw was best with organic nitrogen source.

#### Effect of metal ions on xylanase production by *Trichoderma* sp. MS 2010 with sugarcane bagasse as substrate

According to the data are summarized in **Table 2**, the presence of inorganic metal salt  $\text{ZnSO}_4$  in the medium induced maximum xylanase production by *Trichoderma* sp. MS 2010 and other metal salts like  $\text{MnSO}_4$ ,  $\text{MgSO}_4$ ,  $\text{FeSO}_4$ ,  $\text{ZnSO}_4$ ,  $\text{NaCl}$  and  $\text{CaCl}_2$  significantly ( $p < 0.05$ ) enhanced the xylanase production by *Trichoderma* sp. MS 2010, when compared to control. Though  $\text{CoCl}_2$  increased the production of xylanase, it was not significant.  $\text{CuSO}_4$  was found to inhibit xylanase production, however it was insignificant.

**Table 2: Effect of metal ions on the production of xylanase by *Trichoderma* sp. MS 2010**

S.No	Metal (10mM)	Xylanase activity (U/g)
1	Control	15.88 ± 0.45
2	$\text{CaCl}_2$	24.35 ± 2.40*#
3	$\text{NaCl}$	25.30 ± 2.90*#
4	$\text{MnSO}_4$	36.58 ± 3.37*
5	$\text{MgSO}_4$	38.52 ± 2.71*
6	$\text{CuSO}_4$	12.70 ± 2.77
7	$\text{ZnSO}_4$	39.28 ± 2.32*
8	$\text{CoCl}_2$	23.07 ± 0.79
9	$\text{FeSO}_4$	38.45 ± 2.68*

Values are mean ± SD of triplicates. Values are expressed as U/g of dry fermented substrate at pH 5.0 and 27°C.

\*Significant ( $p < 0.05$ ), when compared to the control.

\*#Significant ( $p < 0.05$ ), when compared to the highest value within the test groups

Rani and Nand<sup>28</sup> recorded similar observations for *Clostridium absonum* CFR-702, and observed the highest xylanase activity in the presence of  $\text{Zn}^{2+}$  followed by ferrous ion. Copper was found to be inhibitory for growth and enzyme production. Sanghvi *et al.*<sup>29</sup> demonstrated the enhancement of xylanase production in the solid state cultures of *Trichoderma harzianum* using wheat straw as the substrate in the presence of  $\text{Ca}^{2+}$  and  $\text{Zn}^{2+}$ .

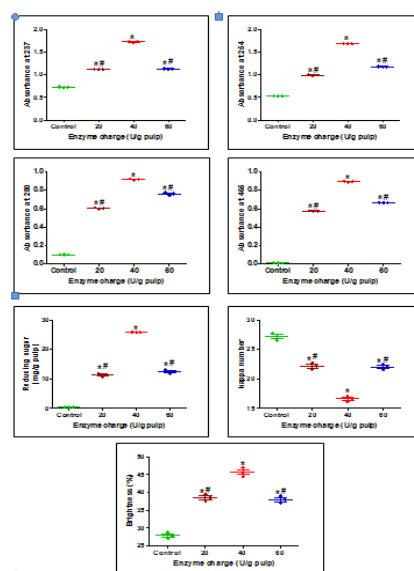
#### Optimization of treatment conditions for biobleaching of hardwood kraft pulp by xylanase in the crude extract of *Trichoderma* sp. MS 2010

##### Effect of enzyme charge

The observations (**Figure 6**) of the present study clearly showed that the brightness was significantly ( $p < 0.05$ ) higher with all enzyme dosages when compared to the control. When the enzyme charges were compared between 20, 40 and 60 U/g, the enzyme dosage of 40 U/g of pulp yielded the maximum brightness. The kappa number was significantly ( $p < 0.05$ ) reduced at the enzyme dosage of 40 U/g pulp, when compared to control and among the three enzyme charges used in the present study. It was also clear that the increase in brightness and decrease in kappa number was significantly correlated with the increase in the amount of reducing sugar and the chromophoric material ( $A_{237}$ ,  $A_{254}$ ,  $A_{280}$ ,  $A_{465}$ ) released in the pulp filtrate. However, further increase in enzyme dosage to 60 U/g did not enhance the biobleaching, but reducing sugar and brightness decreased with increase in kappa number.

Saleem *et al.*<sup>30</sup> treated the wood kraft pulp of 5% consistency with cell free extracts of xylanase of thermophilic *Bacillus* sp. XTR-10 with an enzyme charge of 40 IU/g and reported reduction in kappa number and increase in brightness and further increase in enzyme

dose to 50 IU/g did not result in substantial improvement of the brightness. Kumar *et al.*<sup>31</sup> had obtained maximum brightness when the pulp (10% consistency) samples were treated with 50 U of crude xylanase from *Thermomyces lanuginosus* MC 134 mutant and observed no further increase in the brightness, when the enzyme dosage was increased to 100 U/g pulp. Khandeparkar and Bhosle<sup>13</sup> demonstrated 20% reduction in kappa number using purified xylanase from *Arthrobacter* sp. MTCC5214 in a dosage of 20U/g with 6.0% pulp consistency and reported that higher enzyme dosage had no role in enhancing the biobleaching efficiency. The results of the above experiments indicate that the bleaching ability of the enzyme is dependent on the nature of the enzyme, the source and the pulp consistency.



**Figure 6: Effect of enzyme charge on the physicochemical properties of the enzyme bleached pulp**

\*Significant ( $p < 0.05$ ), when compared to the control.

\*#Significant ( $p < 0.05$ ), when compared to the highest value within the test groups

##### Effect of reaction time for biobleaching of hardwood kraft pulp

The effect of reaction time was observed and the data are shown in **Figure 7**. The brightness of the treated pulp increased significantly ( $p < 0.05$ ) with increase in reaction time up to 3 hours, at which, maximum increase in brightness was observed when compared to control. However, after that, the increase in reaction time did not enhance the brightness. The kappa number was reduced significantly ( $p < 0.05$ ). The discharge of chromophoric materials and reducing sugar significantly increased ( $p < 0.05$ ) with the increase in reaction time up to 3 hours, which are correlated with the increase in brightness and decrease in kappa number. The results of the present study are in agreement with the observations of Kumar *et al.*<sup>31</sup> who obtained maximum improvement in brightness with crude xylanase from *Thermomyces lanuginosus* MC 134 mutant at 3 hours of reaction time and found that extended time played no role for the improvement of the brightness. In contrast, Saleem *et al.*<sup>30</sup> reported 8 hours as optimum reaction time to achieve the maximum brightness of wood kraft pulp with crude xylanase from *Bacillus* sp. XTR-10.

##### Effect of pH for biobleaching of hardwood kraft pulp

The results of enzyme bleaching of hardwood kraft pulp at various pH ranging from 4.0 to 8.0 are summarized in **Figure 8**. When pH was studied as one of the factors affecting biobleaching efficiency of xylanase, maximum brightness was achieved at pH 5.0. The brightness was increased and the increase in brightness was significant ( $p < 0.05$ ), when compared to control. The significant reduction ( $p < 0.05$ ) in kappa number was correlated with the increase in brightness. It was obvious from the results of the present study that the increase in brightness from pH 4.0 to 8.0 was

significant ( $p < 0.05$ ). This indicated that the xylanase could be used to treat the wood kraft pulp in acidic as well as in alkaline conditions. The release of lignin derived compounds and reducing sugar was also significantly higher ( $p < 0.05$ ) at pH 5.0 and substantiated the increase in brightness and decrease in kappa number. Kumar *et al.*<sup>31</sup> demonstrated the pH dependent biobleaching process by crude xylanase from *Thermomyces lanuginosus* MC 134 mutant and revealed that the maximum brightness of bagasse pulp was achieved at pH 6.0. Li *et al.*<sup>32</sup> found that the purified xylanase from *Thermomyces lanuginosus* CBS 288.54 produced maximum brightness of wheat straw pulp at pH 9.0.

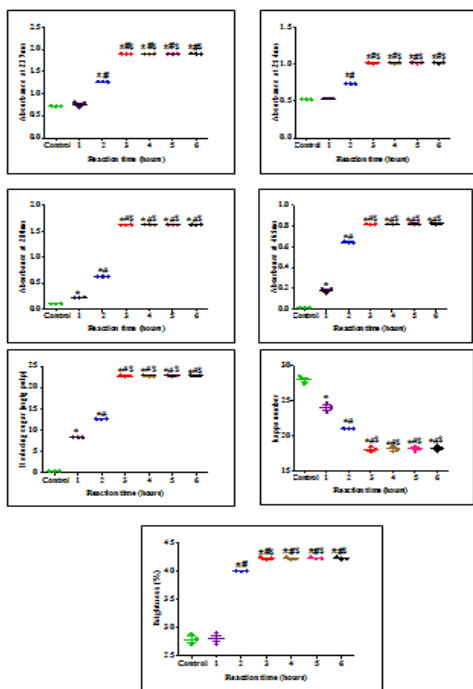


Figure 7: Effect of reaction time on the physicochemical properties of the enzyme bleached pulp

\*Significant ( $p < 0.05$ ), when compared to the control  
 \*# Significant ( $p < 0.05$ ), when compared to the value at 1 hour  
 \*## Significant ( $p < 0.05$ ), when compared to the value at 2 hour

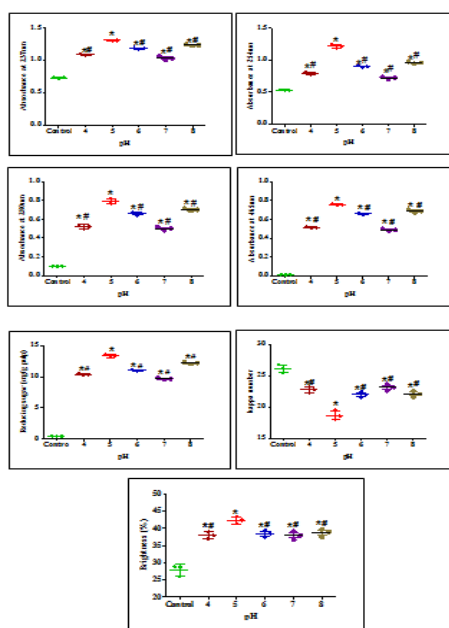


Figure 8: Effect of pH on the physicochemical properties of the enzyme bleached pulp

\*Significant ( $p < 0.05$ ) when compared to the control  
 \*## Significant ( $p < 0.05$ ) when compared to highest value within the test groups

Effect of temperature for biobleaching of hardwood kraft pulp

From the results presented in Figure 9, it was clearly indicated that the maximum brightness was achieved at 60°C. The kappa number was reduced significantly ( $p < 0.05$ ). The increase in brightness and decrease in kappa number is correlated with the release of higher amounts of reducing sugar and chromophores. A significant ( $p < 0.05$ ) increase of brightness was noticed at temperatures between 30°C and 70°C, when compared to control. This clearly revealed the fact that the xylanase from *Trichoderma* sp. MS 2010 is a thermotolerant enzyme and could be exploited in a wide range of temperatures. The findings of the present experiment was on par with Savitha *et al.*<sup>33</sup> who reported that 60°C was the optimum temperature for attaining maximum brightness of waste paper pulp with the purified xylanase from *Graphium putredinis* and *Trichoderma harzianum*. The release of reducing sugar ( $\lambda_{550}$ ), chromophores ( $\lambda_{237}$ ,  $\lambda_{254}$ ,  $\lambda_{280}$ ) and hydrophobic compounds ( $\lambda_{465}$ ) is due the substantial dissociation of lignin carbohydrate complex (LCC) from the cellulose fibers. In wood, the lignin is closely adhered to cellulose network with the support of hemicellulose. Xylan is sandwiched between lignin and hemicellulose. When the wood kraft pulp is pretreated with xylanase, the xylan is degraded by the xylanase and the xylose is released. Lignin and phenolic compounds are also released along with xylose, which are eventually responsible for the enhancement of absorbance of pulp free samples at  $\lambda_{237}$ ,  $\lambda_{254}$ ,  $\lambda_{280}$ , and  $\lambda_{465}$  compared to the control<sup>13</sup>.

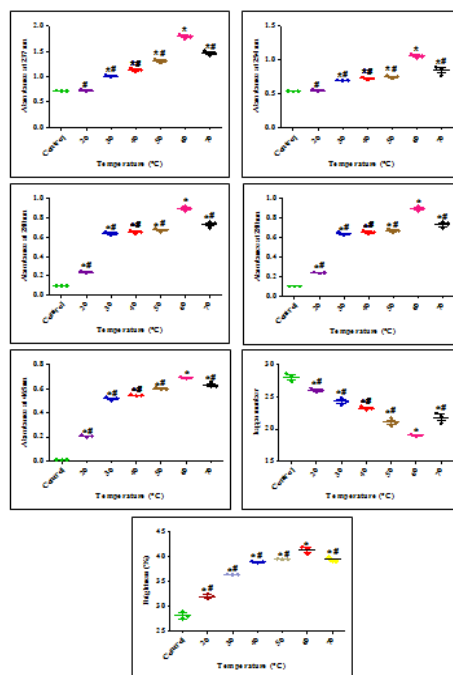


Figure 9: Effect of temperature on the physicochemical properties of the enzyme bleached pulp

\*Significant ( $p < 0.05$ ), when compared to the control  
 \*## Significant ( $p < 0.05$ ), when compared to highest value within the test groups.

Biobleaching of Hardwood Kraft Pulp by Xylanase in the Crude Enzyme Extract from *Trichoderma* sp. MS 2010

The criteria for the assessment of the prebleaching efficiency of enzyme are to achieve higher final brightness of pulp with the lesser use of toxic chlorinated compounds<sup>30</sup>. The present study also intended to reduce the amount of chlorinated compounds for pulp bleaching to a larger extent, in order to achieve better result. The

results of relative multistage bleaching sequence with and without

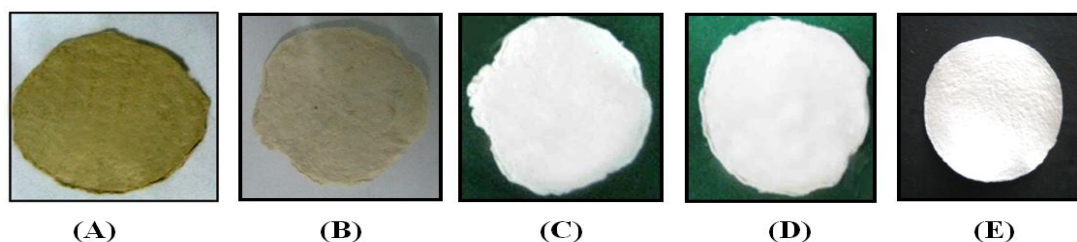
the xylanase treatment is presented in Table 3 and Plate 1.

**Table 3: Effect of xylanase pretreatment on kappa number and brightness of kraft pulp**

Properties of pulp	Untreated pulp	Xylanase treated	Untreated pulp with		Xylanase pretreated Pulp with	
			7% NaOCl + 1% H <sub>2</sub> O <sub>2</sub>	3.5% NaOCl + 1% H <sub>2</sub> O <sub>2</sub>	7% NaOCl + 1% H <sub>2</sub> O <sub>2</sub>	
Kappa number	27.06±0.83 <sup>a</sup>	18.1±0.61 <sup>b</sup>	6.35±0.34 <sup>c</sup>	6.03±0.12 <sup>c</sup>	3.90±0.14 <sup>d</sup>	
Brightness	28.01±1.02 <sup>a</sup>	42.7±1.62 <sup>b</sup>	77.33±1.31 <sup>c</sup>	77.61±1.51 <sup>c</sup>	82.21±1.19 <sup>d</sup>	

Values are mean ± SD of triplicates. The values with different superscripts are significant (p<0.05) when compared with untreated pulp.

**Plate 1: Effect of xylanase pretreatment on kappa number and brightness of kraft pulp**



**A) Untreated pulp, (B) Xylanase treated pulp, (C) Untreated pulp with 7%NaOCl + 1%H<sub>2</sub>O<sub>2</sub>, (D) Xylanase pretreated pulp with 3.5%NaOCl + 1% H<sub>2</sub>O<sub>2</sub>, (E) Xylanase pretreated Pulp with 7%NaOCl + 1%H<sub>2</sub>O<sub>2</sub>**

From the results of the present investigation, it was observed that xylanase treatment of the pulp resulted in significant (p<0.05) difference in the pulp properties. When the pulp was treated with crude enzyme extract, a significant increase (p<0.05) in brightness and significant (p<0.05) reduction in kappa number was noticed, when compared to untreated pulp. The untreated pulp, when treated with 7% NaOCl followed by 1% H<sub>2</sub>O<sub>2</sub>, the kappa number was significantly (p<0.05) reduced. The reduction in kappa number correlated with the significant (p<0.05) increase in brightness, when compared to untreated pulp.

Enzyme prebleached pulp was treated with 3.5% NaOCl and 1% H<sub>2</sub>O<sub>2</sub>, in order to reduce the usage of chlorinated compounds. In this sequential treatment, reduction in the kappa number was found and an increase in brightness was observed, which were significant (p<0.05), when compared to the untreated pulp. In xylanase prebleached pulp, when subjected to treatment with 7% NaOCl and 1% H<sub>2</sub>O<sub>2</sub>, the kappa number was reduced and the brightness was increased, which were found to be significant (p<0.05). It was obvious from the results of the present work that the same level of brightness of pulp could be attained with 50% of chemical consumption. For example, the enzyme prebleached pulp, when treated with 3.5% NaOCl followed by 1% H<sub>2</sub>O<sub>2</sub> resulted in a similar level of brightness (77.61%), as compared with the brightness (77.33%) of the untreated pulp obtained with 7% NaOCl and 1% H<sub>2</sub>O<sub>2</sub>. In a similar investigation, Khandeparkar and Bhosla (2007) demonstrated 28.5% reduction in chlorine consumption when kraft pulp was treated with purified xylanase from *Arthrobacter* sp. MTCC 5214. Dhillon *et al.*<sup>34</sup> reported that the prebleaching of rice straw with crude xylanase from *Bacillus circulans* AB 16 was accountable for 20% reduction in chlorine usage. Bissoon *et al.*<sup>35</sup> found 18% reduction in chlorine dioxide consumption for pretreated bagasse pulp with the purified xylanase from *Thermomyces lanuginosus* SSBP.

## CONCLUSION

Prebleaching of pulp by xylanase from *Trichoderma* sp. MS 2010 and its following multistage chemical treatment reduced the load of chlorinated compounds. The requirement of chlorine reduction due to the application of crude enzyme in the bleaching process may suitably be considered as a major aspect for the management of cost and risk related to the bleaching process. The present study has demonstrated that the xylanase from *Trichoderma* sp. MS 2010 is significantly active in acid and alkaline pH range and at high temperature (60°C), which is a prerequisite for biobleaching. Thermostability and pH tolerance of the crude xylanase firmly suggested that this enzyme could be of considerable commercial interest.

## REFERENCE

- Rao MA, Scelza R, Scotti and Gianfreda L. Role of enzymes in the remediation of polluted environments. *J. Soil Sci. Plant Nutr.*, 2010; 10:333-353.
- Dhiman SS, Sharma J and Battan B. Pretreatment processing of fabrics by alkalothermophilic xylanase from *Bacillus stearothermophilus* SDX. *Enzyme Microb. Tech.*, 2008; 43:262-269.
- Beg QK, Kapoor M, Mahssajan L and Hoondal GS. Microbial xylanases and their industrial applications: a review. *Appl. Microbiol. Biotechnol.*, 2001; 56:326-338.
- Bae H, Kim HJ and Kim YS. Production of a recombinant xylanase in plants and its potential for pulp biobleaching applications. *Bioresource Technol.*, 2008; 99:3513-3519.
- Waite MJ, Morgan NL, Rockey JS and Higton G. Microbial biomass production. in industrial microbiology: An introduction, Iowa State University Press, U.S.A., 2001.
- Risna RA and Suhirman. Ligninolytic enzyme production by *Polyporaceae* from Lombok, Indonesia. *Fungal Diversity*, 2002; 9:123-134.
- Mandels M and Sternberg D. Recent advances in cellular technology. *J. Ferment. Tech.*, 1976; 54:267-286.
- Gupta VK, Gaur R, Gautam N, Kumar P, Yadav IJ and Darmwal NS. Optimization of xylanase production from *Fusarium solani* F7. *American J. Food Technol.*, 2009; 4:20-29.
- Miller G. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical Chem.*, 1959; 31:426-428.
- Bailey MJ, Biely P and Poutanen K. Interlaboratory testing of methods for assay of xylanase activity. *J. Biotechnol.*, 1992; 23:257-270.
- Sadhasivam S, Sivasubramanian S and Krishnasamy S. Deployment of *Trichoderma harzianum* WL1 laccase in pulp bleaching and paper industry effluent treatment. *J. Cleaner Production*. 2010; 18:799-806.
- Wong KKY, Jong ED, Saddler JN and Allison RW. Mechanism of xylanase aided bleaching of kraft pulp, Part I: Process Parameters. *Appita Journal*, 1997; 50:415-422.
- Khandeparkar R and Bhosle NB. Application of thermoalkalophilic xylanase from *Arthrobacter* sp. MTCC 5214 in biobleaching of kraft pulp. *Bioresource Technology*. 2005; 98:897-903.
- Pang PK, Darah I, Poppe L, Szakacs G and Ibrahim CO. Xylanase production by a local isolate, *Trichoderma* spp. FETL c3-2 via solid state fermentation using agricultural wastes as substrates. *Malaysian Journal of Microbiology*, 2006; 2:7-14.
- Bakri Y, Jawhar M and Eddin MI. Improvement of xylanase production by *Cochliobolus sativus* in solid state fermentation. *Braz. J. Microbiol.*, 2008; 39:602-604.

16. Nwodo SC, Uzorna AO, Thompson NE and Victoria IO. Xylanase production of *Aspergillus niger* and *Penicillium crysogenum* from ammonia pretreated cellulosic waste. Research Journal of Microbiology, 2008; 3:246-253.
17. Sonia KG, Chadha BS and Saini HS. Sorghum straw for xylanase hyperproduction by *Thermomyces lanuginosus* (D2W3) under solid state fermentation. Bioresource Technol., 2005; 96:1561-1569.
18. Isil S and Nilufer A. Investigation of Factors Affecting Xylanase Activity from *Trichoderma harzianum* 1073 D3. *Brazilian Archives of Biology and Technology*. 2005; 48:187-193.
19. Azin M, Moraavej R and Zareh D. Production of xylanase by *Trichoderma longibrachiatum* on a mixture of wheat bran and wheat straw: optimization of culture condition by Taguchi method. *Enzyme Microb. Tech.*, 2007; 40:801-805.
20. Ahmad Z, Butt MS, Anjum FM and Asgher M. Effect of wheat bran concentration on xylanase biosynthesis by *Aspergillus niger*. *Int. J. Agri. Biol.*, 2009; 11:571-576.
21. Gomez PI and Gonzalez MA. The effect of temperature and irradiance on the growth and carotenogenic capacity of seven strains of *Dunaliella salina* (Chlorophyta) cultivated under laboratory conditions, *Biol Res.*, 2005; 38:151-162.
22. Reddy GV, Babu PR, Komaraiah P, Roy KRRM and Kothari IL. Utilization of banana waste for the production of lignolytic and cellulolytic enzymes by solid substrate fermentation using two *Pleurotus* species (*P. ostreatus* and *P. sajor-caju*). *Process Biochem.*, 2003; 38:1457-1462.
23. Ferreira G, Boer CG and Peralta RM. Production of xylanolytic enzymes by *Aspergillus tamaritii* in solid state fermentation, *FEMS Microbiol. Lett.*, 1999; 173:335-339.
24. Christopher L, Bissoon S, Singh S, Szendefy J and Szakacs G. Bleach enhancing abilities of *Thermomyces Lanuginosus* xylanase produced by solid state fermentation. *Process Biochemistry*, 2005; 40:3230-3235.
25. Yang SQ, Yan QJ, Jiang ZQ, Li LT, HM and Wang YZ. High level of xylanase production by the thermophilic *Paecilomyces themophila* J18 on wheat straw in solid-state fermentation. *Bioresource Technol.*, 2006; 97:1794-1800.
26. Raimbault M. General and microbiological aspects of solid substrate fermentation, *EJB*, 1998; 1:1-15.
27. Svarachorn A. Production of fungal-xylanase using agricultural waste by solid state fermentation. *J. Sci. Res. Chula. Univ.*, 1999; 24:13-20.
28. Rani DS and Nand K. Production of thermostable cellulase-free xylanase by *Clostridium absonum* CFR-702. *Process Biochem.*, 2000; 36:355-362
29. Sanghvi GV, Koyani RD and Rajput KS. Thermostable xylanase production and partial purification by solid state fermentation using agricultural waste wheat straw. *Mycology*, 2010; 1:106-112.
30. Saleem M, Tabassum MR, Yasmin R and Imran M. Potential of xylanase from thermophilic *Bacillus* sp. XTR-10 in biobleaching of wood kraft pulp. *Int. Biodeter. Biodegr.*, 2009; 63:1119-1124.
31. Kumar K, Manimaran A, Permaul K. and Singh S. Production of  $\beta$ -xylanase by a *Thermomyces lanuginosus* MC 134 mutant on corn cobs and its application in biobleaching of bagasse pulp. *J. Biosci. Bioeng.*, 2009; 107:494-498.
32. Li XT, Jiang ZQ, Li LT, Yang SQ, Feng WY, Fan JY *et al.* Characterization of a cellulase free neutral xylanase from *Thermomyces lanuginosus* CBS 288.54 and its biobleaching effect on wheat straw pulp. *Bioresource Technol.*, 2005; 96:1370-1379.
33. Savitha S, Sadhasivam S and Swaminathan K. Modification of paper properties by the pretreatment of wastepaper pulp with *Graphium putredinis*, *Trichoderma harzianum* and fusant xylanases. *Bioresource Technol.*, 2009; 100:883-889.
34. Dhillon A, Gupta JK, Jauhari BM and Khanna S. A cellulase-poor, thermostable, alkali-tolerant xylanase produced by *Bacillus circulans* AB 16 grown on rice straw and its application in biobleaching of eucalyptus pulp. *Bioresource Technol.*, 2000; 73:273-277.
35. Bissoon S, Christov L and Singh S. Bleach boosting effects of purified xylanase from *Thermomyces lanuginosus* SSBP on bagasse pulp. *Process Biochemistry*, 2002; 37:567-572.