ENHANCED PRODUCTION OF CELLULASE-FREE XYLANASE BY ALKALOPHILIC *BACILLUS SUBTILIS* ASH AND ITS APPLICATION IN BIOBLEACHING OF KRAFT PULP

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This paper reports high level production of a cellulase-free xylanase using wheat bran, a cost-effective substrate, under submerged fermentation by alkalophilic *Bacillus subtilis* ASH. Production of xylanase was observed even at alkaline pH up to 11.0 and temperature 60 °C, although the highest enzyme titer was recorded at neutral pH and 37 °C. The enzyme production under optimized fermentation was 1.5-fold greater than under unoptimized conditions. Pre-treatment of unbleached pulp of 10% consistency with crude xylanase (6 IU/g o.d. pulp) at 60 ºC for 2 h increased the final brightness by 4.9%. The enzyme treatment reduced the chlorine consumption by 28.6% with the same brightness as in the control. A reduction in kappa number and increase in viscosity was observed after enzyme pre-treatment. Scanning electron microscopy revealed loosening and swelling of pulp fibers. The strength properties viz. grammage, fiber thickness, beating degree, tensile index, breaking length, tear index and double fold of the treated pulp were improved as compared to the control pulp. This study reveals the potential of *B. subtilis* ASH xylanase as a biobleaching agent for the paper and pulp industry.

Keywords: Pulp bleaching; Submerged fermentation; Wheat bran; Xylanase

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INTRODUCTION

Xylanase (endo-1, 4-β-D-xylanohydrolase; EC 3.2.1.8), a hydrolytic enzyme involved in depolymerization of xylan, finds application in many industrial processes such as bleaching of kraft pulp, improving the digestibility of animal feed, softening of fruits, clarification of juices, bioconversion of agricultural wastes, extraction of plant oils, degumming of plant fibers, etc. (Eriksson 1990; Dhiman et al. 2008). It has gained considerable attention in the past few years due to its potential application in the paper and pulp industry. In the transformation of wood to paper, the lignin fraction is removed in two stages: the kraft process and bleaching. The kraft process involves cooking the wood chips at high temperature and at alkaline pH. In this process, part of the xylan is dissolved, and short-chain xylan precipitates in a more or less crystalline form on the surface of cellulose microfibrils. Approximately 95% of the lignin is removed during this process, but the remaining 5% lignin imparts a brown color to the cellulosic fibres

(Damiano et al. 2003). The residual lignin is removed during subsequent chemical bleaching process to produce white pulp. Most industries are using a bleaching sequence based on chlorine, chlorine dioxide, and extraction by sodium hydroxide. The bleaching of pulp with elemental chlorine results in discharge of large amounts of organochlorine compounds in the effluent. Some of these compounds are found to be toxic, mutagenic, persistent, bioaccumulating, and harmful to biological systems (Bajpai and Bajpai 1996). As these compounds cause environmental pollution, paper industry is now facing many restrictions. Thus, there is an urgent need of either replacing or supplementing the existing bleaching process of chlorination by an eco-friendly and cost-effective biological process so as to reduce organic pollutant load on the environment (Kulkarni and Rao 1996; Dhillon et al. 2000).

Biobleaching has proved effective in reducing environmental pollution caused by chemical bleaching processes. Biobleaching of pulp with xylanase has been reported to reduce chlorine requirement during the subsequent bleaching operation, causing release of lower amounts of chlorinated organic compounds in bleach effluents, thus minimizing environmental pollution (Bajpai and Bajpai 1992; Senior et al. 1992; Bajpai et al. 2006). Pre-treatment of pulp with xylanases helps in partial disruption of lignin-carbohydrate bonds, thereby enhancing the accessibility of the subsequent bleaching chemicals to the pulp (Lei et al. 2008). In addition, the reprecipitated and reabsorbed alkali-resistant xylan, which forms a physical barrier against the extraction of residual lignin molecules from the fibers, may be removed from the fiber surface by xylanase treatment (Daneault et al. 1994). The xylanase pretreatment was reported to facilitate access to cellulose fibres, thereby boosting the effect of laccase-mediator system in reducing the content of residual lignin and releasing more hexenuronic acids (Valls and Roncero 2009). Despite the fact that the use of xylanase has become well established technology, its use on a large scale in the paper industry worldwide still faces problems mainly due to its cost of production (Dhillon et al. 2000). To utilize xylanases in the paper and pulp industry, an efficient microbial strain producing cellulase-free xylanase is needed, since the presence of cellulase may destroy the structure of cellulose and diminish the pulp quality. In addition, xylanases active at high temperature and alkaline pH are desirable for pulp bleaching (Viikari et al. 1994).

Xylanase is produced by a variety of microorganisms, including bacteria, fungi, and actinomycetes (Biely 1985; Ball and McCarthy 1989; Sunna and Antranikian 1997). Among the bacterial sources, *Bacillus* is an industrially important source of xylanases (Bataillon et al. 1998; Shah et al. 1999). Although fungi secrete high levels of extra-cellular xylanases, yet the presence of considerable amount of cellulase activity and their lower pH optima render them less suitable for pulp and paper industry (Srinivasan and Rele 1999). Further, fungi need much longer incubation periods for enzyme production. However, bacteria can grow and produce xylanase at high pH and temperature with minimal or no cellulase production. In our laboratory, a novel moderately thermophilic and alkalophilic strain of *Bacillus subtilis* ASH (MTCC 7414) was isolated, which produced high levels of cellulase-free xylanase in solid state fermentation (Sanghi et al. 2007). The present investigation was aimed at optimizing the production of cellulase-free xylanase by this bacterial strain, using cost-effective agro-residues in submerged fermentation, and evaluation of its potential in bleaching of kraft pulp.

EXPERIMENTAL

Microbial Strain

Bacillus subtilis ASH [Microbial Type Culture Collection (MTCC), Chandigarh, India, accession number MTCC 7414], isolated in our laboratory from soil collected locally from Kurukshetra, Haryana (India) was used in this study. It was periodically subcultured on nutrient agar medium (0.5% peptone, 0.5% beef extract and 2% agar) and maintained at 4 °C.

Optimization of Xylanase Production under Submerged Fermentation

Xylanase was produced under submerged fermentation in 250 ml Erlenmeyer flasks, each containing 50 ml of basal medium (g/L: peptone, 5.0; yeast extract, 5.0; K_2HPO_4, 1.0; $MgSO_4.7H_2O$, 0.1; wheat bran, 1.0; pH 7.0). The flasks were inoculated with 0.5 ml of 24 h inoculum (approximately 10^7 CFU/ml) and incubated at 37 ^oC in an incubator shaker at 150 rpm for 48 h. The contents of the flasks were centrifuged at 10,000 x g for 20 min in a refrigerated centrifuge at 4 °C, and the resulting supernatant was used for the assay of xylanase and cellulase activities. Various physical and nutritional parameters for enzyme production were optimized by varying one parameter while keeping all other factors constant. All the experiments were carried out independently in triplicate and the results presented are mean of the three values. Xylanase production was optimized with respect to the following parameters:

Incubation time

The time course of xylanase production by *B. subtilis* ASH was studied by carrying out submerged fermentation in the basal medium for 72h and harvesting samples at 6-hourly intervals for estimation of xylanase activity.

pH

The effect of pH on enzyme production was assessed by cultivating the strain in the basal media of pH ranging from 3.0 to 11.0.

Temperature

The influence of temperature was studied by performing the fermentation at different temperatures i.e. 30, 37, 40, 45, 50, 55, and 60°C.

Age and size of inoculum

The effect of inoculum age on xylanase production was studied by inoculating the production medium with an inoculum of different age i.e. 6 to 42 h old culture of *B. subtilis* ASH. The inoculum size was optimized by varying it from 1 to 20 %.

Agitation rate

The flasks were shaken at different agitation rate i.e. 50,100, 150, and 200 rpm for evaluating the effect of agitation on xylanase production.

Carbon source

Various lignocellulosic substrates (wheat straw, wheat bran, rice husk, ground nut shells, sugarcane bagasse, and saw dust) and carbohydrates (glucose, xylose, sucrose, lactose, maltose, cellulose, CM-cellulose, starch, birch wood xylan, and oat spelt xylan) at 2% were tested as carbon source for enzyme production. The control was devoid of any carbon source.

Nitrogen source

Various organic (peptone, yeast extract, beef extract, tryptone, and casein hydrolysate) and inorganic ($NaNO_3$, KNO_3, NH_4NO_3, and $(NH_4)_2H_2PO_4$) nitrogen sources at 0.5% concentration were incorporated into the production medium (devoid of nitrogen source) to select the best nitrogen source for xylanase production. The control was devoid of nitrogen source.

Effect of additives

The xylanase production by *B. subtilis* ASH was studied by supplementing the production medium with various additives (at 0.1% w/v) like metal salts (NaCl, KCl, $CaCl_2$, $MgSO_4$, $ZnSO_4$, $CuSO_4$), EDTA, and SDS.

Enzyme Assays

The xylanase activity was assayed according to the method of Bailey et al. (1992) by measuring the amount of reducing sugars (xylose equivalent) liberated from xylan using 3, 5-dinitrosalicylic acid (Miller 1959). The reaction mixture contained 0.5 ml of 1% birch wood xylan (Sigma) as substrate, 0.02 ml of appropriately diluted enzyme extract, and phosphate buffer (0.05 M, pH 7.0) in a total volume of 1.0 ml. After 5 min incubation at 55 ºC, the reaction was terminated by adding 3.0 ml of 3,5-dinitrosalicylic acid reagent. A control was run simultaneously that contained all the reagents but the reaction was terminated prior to the addition of enzyme extract. The contents were placed in a boiling water bath for 10 min and then cooled to room temperature. The absorbance of the resulting red color was measured against the control at 540 nm in a spectrophotometer.

Cellulase activity (CMCase and FPase) was determined according to the method of Ghose (1987). The reaction mixture for carboxymethyl cellulase (CMCase) activity containing 0.5 ml of 2% carboxymethyl cellulose (prepared in 0.05 M sodium citrate buffer, pH 4.8) and 0.5 ml of enzyme was incubated at 50 ºC for 30 min. The reaction mixture for Filter paper (FPase) activity contained a Whattman No. 1 filter paper strip (1X6 cm), 1.0 ml of 0.05 M sodium citrate buffer, pH 4.8 and 0.5 ml enzyme extract, followed by incubation at 50 ºC for 60 min. In both cases, the reaction was then terminated by adding 3 ml of 3,5-dinitrosalicylic acid reagent. The mixture was boiled for 5 min in a boiling water bath, cooled and added 20 ml of distilled water to it. The colour intensity was measured at 540 nm in a spectrophotometer. A control was run simultaneously which contained all the reagents but the reaction was terminated prior to the addition of enzyme extract.

One unit (IU) of xylanase or cellulase activity was defined as the amount of enzyme that catalyzes the release of 1 μmol of reducing sugar as xylose or glucose equivalent per min under the specified assay conditions.

Biobleaching of Kraft Pulp (XC/DED_1D_2)

The unbleached mixed pulp prepared from six types of wood namely poplar, eucalyptus, eucalyptus rulla, small vaneer, bamboo, and debarka bamboo hardwood was obtained from Balarpur Industries Limited, Yamuna Nagar, India. It was subjected to enzymatic pre-bleaching followed by conventional chemical bleaching.

Enzymatic pre-bleaching

Unbleached pulp at 10% consistency (w/v) was treated with *B. subtilis* ASH xylanase under varying reaction conditions (Xylanase 0-10 IU/g o.d. pulp; pH 7.0-10.0; temperature 50-70 ºC; time 0-180 min) in plastic bags so as to optimize the reaction conditions for enzyme pre-treatment. After enzyme treatment, the pulp was de-watered on a Büchner funnel using Whatman No.1 filter paper, followed by washing with cold water. A control sample of pulp (not treated with xylanase) was also processed under identical conditions. The pulp was analyzed for kappa number and brightness.

Chemical bleaching

Xylanase-treated and untreated pulp samples were chemically bleached in a multi-stage process using C/DED_1D_2 sequence under the conditions specified in Table 1. After each treatment, the pulp was dewatered and washed with cold water. At C/D stage, both the pulps were treated with chlorine-chlorine dioxide in a ratio of 90:10 keeping total chlorine percentage constant at 3.8%. Alkali extraction was done with 2.25% NaOH. At D_1 and D_2 stages, pulps were treated with 0.6-0.8% and 0.2-0.3% chlorine dioxide, respectively. Thereafter, various experiments were conducted in which the dose of chlorine treatment given to xylanase-treated pulp was reduced progressively to the level so as to achieve the same brightness as in the control, keeping all other parameters constant.

Table 1. Conditions for Bleaching (XC/DED_1D_2) of Kraft Pulp

Treatment	Pulp consistency (%)	Temperature (˚C)	Time (min)	pH
X (enzymatic prebleaching)	10	55	120	7.0
C/D (chlorine-chlorine dioxide)	3	Ambient	45	1.0-2.0
E (alkali extraction)	10	65-70	120	11.0-12.0
D_1 (chlorine dioxide stage 1)	10	70-75	180	3.8-4.0
D_2 (chlorine dioxide stage 2)	10	70-75	180	3.8-4.0

Physical and chemical characterization of kraft pulps

After chemical bleaching, control and xylanase-treated pulps were thoroughly washed, and handsheets were prepared according to Technical Association of Pulp and Paper Industry (TAPPI) standard methods [T-205om-88, TAPPI Test Methods 1996] so as to evaluate their strength properties using the standard procedures. Kappa number, a measure of lignin content, was determined by reaction of pulp samples with acidified

potassium permanganate (TAPPI Method T 236 om-99). Brightness, a measure of diffused reflectance at 457 nm was measured as % ISO (International Organization of Standardization) with ISO Colourtech, USA (TAPPI method T 452 om-87). P. no. (permanganate number) was estimated by reaction of pretreated pulp with acidified permanganate solution using starch and potassium iodide as indicator. Viscosity, a measure of cellulose chain length, was determined by dissolving delignified pulp in cupriethylenediamine (CED) and measuring the viscosity of a 0.5% solution with an Ostwald viscometer (TAPPI Method T 230 om-94). Such a test gives a relative indication of the degradation (decrease in cellulose molecular weight) resulting from the pulping and/or bleaching process.

Strength properties of handsheets viz. grammage (TAPPI T 410), fiber thickness (TAPPI T 411), beating degree, bulk (TAPPI T 500), tensile index (TAPPI T 404), breaking length, burst index (TAPPI T 403), tear index (TAPPI T 414), and double fold (TAPPI T 511) were tested according to the standard methods of TAPPI (TAPPI Test Methods 1996). Scanning electron microscopy (SEM) of hand-made paper sheets was performed at the Sophisticated Analytical Instrumentation Centre, Punjab University, Chandigarh, India to examine the loosening and swelling of pulp fibers at magnification of 200X and 500X.

RESULTS AND DISCUSSION

Optimization of Xylanase Production under SmF

Incubation period

B. subtilis ASH was grown in production medium (pH 7.0) at 37 °C under shaking conditions at 150 rpm in an orbital shaker incubator, and xylanase activity was determined in its cell free extract at 6-hourly intervals up to 72 h. The production of xylanase could be detected after 18 h of incubation with maximum (298 IU/ml) at 48 h i.e. during stationary growth phase of *Bacillus* (Fig. 1).

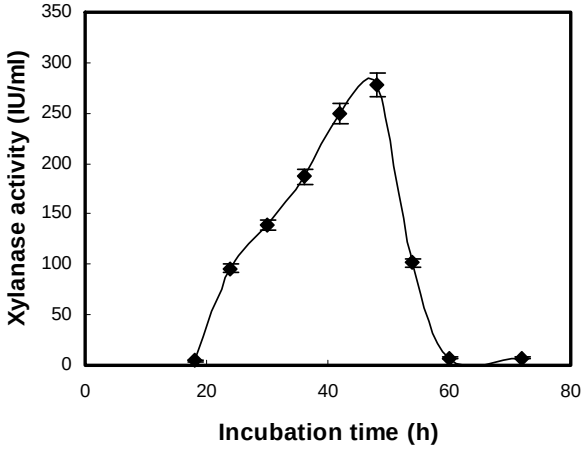

Fig. 1. Time course of xylanase production by *B. subtilis* ASH in submerged fermentation at 37°C, pH 7.0 under shaking at 150 rpm with 2% wheat bran as carbon source and 0.5% peptone+yeast extract as nitrogen source. The inoculum was 24 h old and used at 1%.

On further incubation, there was a decline in enzyme production. Similarly, other workers have reported maximum xylanase production by *Bacillus* sp. during the incubation period of 24-48 h (Ratto et al. 1992; Breccia et al. 1998; Qureshy et al. 2002). However, Damaso et al. (2000) observed maximum production of xylanase by *Thermomyces lanuginosus* after 96 h of cultivation.

pH

The pH of the production medium was varied from 3.0 to 11.0, and enzyme activity was determined in the cell free extract. *B. subtilis* ASH, being alkalophilic, was capable of producing xylanase even up to pH 11.0; however, appreciable xylanase activity was observed between pH 5 and 9, with the highest at pH 7.0 (Fig. 2).

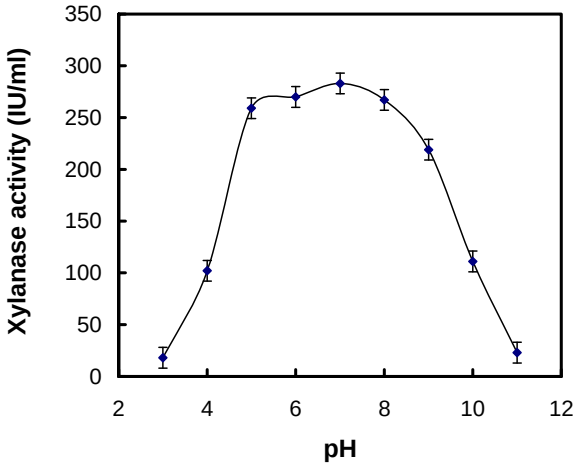

Fig. 2. Effect of pH on xylanase production by *B. subtilis* ASH in submerged fermentation after 48 h of incubation at 37 °C under shaking at 150 rpm with 2% wheat bran as carbon source and 0.5% peptone+yeast extract as nitrogen source. The inoculum was 24 h old and used at 1%.

When compared with other *Bacillus* sp., the enzyme titre at pH 7.0 (283 IU/ml) in this study was much higher than *B. circulans* AB-16, which produced 55 IU/ml (Dhillon et al. 2000). Moreover, xylanase production at pH 9.0 (219 IU/ml) was greater than 12.95, 56.9, and 90 IU/ml reported for *Geobacillus thermoleovorans*, *B. thermoalkalophilus* and *Bacillus* sp., respectively (Rajaram and Varma 1990; Anuradha et al. 2007; Sharma et al. 2007).

Temperature

Xylanase production by *B. subtilis* ASH, a moderate thermophile, was monitored at temperatures ranging from 30 to 60 °C. The enzyme production was observed over the temperature range under study with a maximum of 289 IU/ml at 37 °C, which was markedly higher as compared to 1.1 IU/ml for *B. circulans* Teri-42 (Qureshy et al. 2002). At temperatures as high as 55 °C and 60 °C, the residual enzyme activity was 38.4% and 31.1%, respectively (Fig. 3).

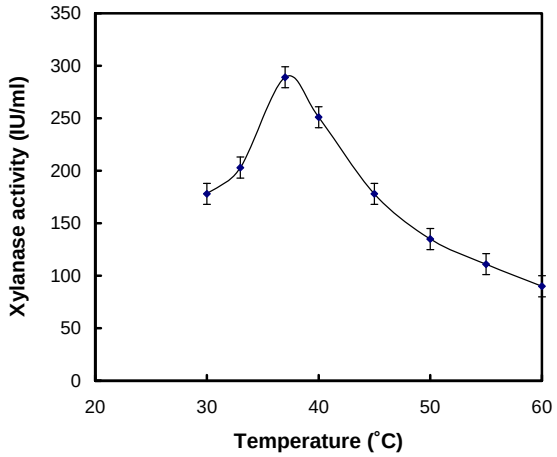

Fig. 3. Effect of temperature on xylanase production by *B. subtilis* ASH in submerged fermentation after 48 h of incubation, pH 7.0 under shaking at 150 rpm with 2% wheat bran as carbon source and 0.5% peptone+yeast extract as nitrogen source. The inoculum was 24 h old and used at 1%.

The enzyme production at higher temperatures was better than the earlier reports from thermotolerant *Bacillus* sp. (Rajaram and Varma 1990; Dhillon et al. 2000; Anuradha et al. 2007). However, *B. licheniformis* was found to produce 756 IU/ml at 50 °C (Archana and Satyanarayan 1998).

Size and age of inoculum

On varying the size of inoculum from 1-20%, it was found that 2% inoculum resulted in maximum xylanase production (Fig. 4).

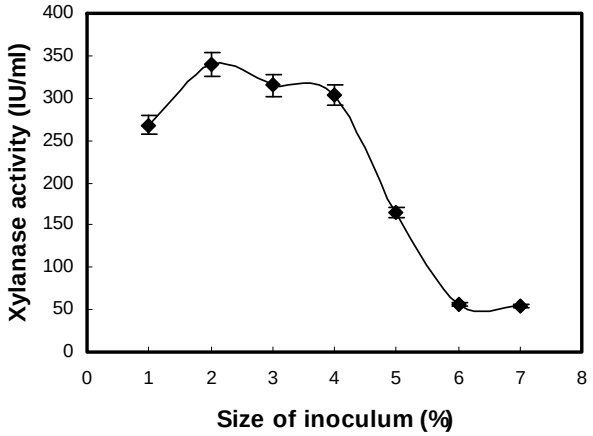

Fig. 4. Effect of inoculum size on xylanase production by *B. subtilis* ASH in submerged fermentation after 48 h of incubation, pH 7.0 at 37 °C under shaking at 150 rpm with 2% wheat bran as carbon source and 0.5% peptone+yeast extract as nitrogen source. The inoculum used was 24 h old.

The effect of inoculum age was studied by inoculating 50 ml of the production medium with 1.0 ml of 6 to 42 h old culture of *B. subtilis* ASH. The xylanase activity was highest when 18 h old culture was used as inoculum (Fig. 5).

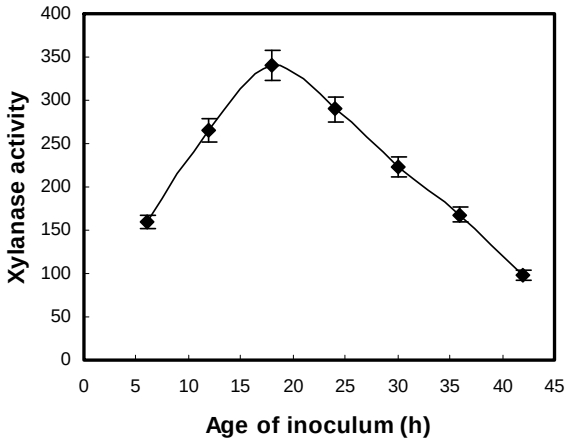

Fig. 5. Effect of age of inoculum on xylanase production by *B. subtilis* ASH in submerged fermentation after 48 h of incubation, pH 7.0 at 37 °C under shaking at 150 rpm with 2% wheat bran as carbon source and 0.5% peptone+yeast extract as nitrogen source. The inoculum was used at 2%.

Most of the workers have reported optimum inoculum size between 1 and 5%. However, 10% inoculum was suggested as optimum for xylanase production by *Bacillus* sp NCIM 59 (Kulkarni and Rao 1996). A 2% inoculum of 18 h old culture of *B. subtilis* resulted in the highest xylanase production. The decline in enzyme yield at larger inoculum size might be due to formation of thick suspensions and improper mixing of substrates in shake flasks.

Agitation

Production medium was inoculated with 2% inoculum and incubated at 37 °C for 48 h under both stationary and shaking conditions viz. 50, 100, 150, 200, and 250 rpm. The xylanase production was higher under shaking as compared to stationary conditions and increased with agitation rate, reaching a maximum at 200 rpm (Fig. 6).

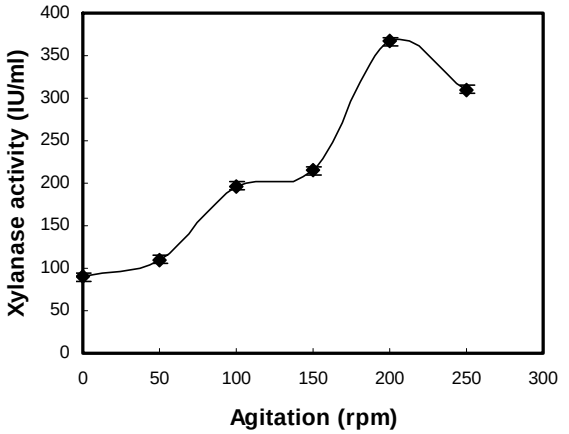

Fig. 6. Effect of agitation on xylanase production by *B. subtilis* ASH in submerged fermentation after 48 h of incubation, pH 7.0 at 37 °C with 2% wheat bran as carbon source and 0.5% peptone+yeast extract as nitrogen source. The inoculum was 18 h old and used at 2%.

Enhancement in xylanase production, after agitation might be due to effective mixing of the medium contents, uniform air distribution and prevention of cell clumping.

Carbon source

Various carbohydrates and agricultural residues/byproducts were tested as sole carbon sources for xylanase production. Among the various carbon sources used, wheat bran favored maximum xylanase production, followed by wheat straw, oat spelt xylan, and birch wood xylan. The enzyme titre was low in the presence of other natural lignocellulosic materials such as sugarcane bagasse, rice husk, saw dust, and groundnut hulls as well as carbohydrates other than xylan (Fig. 7).

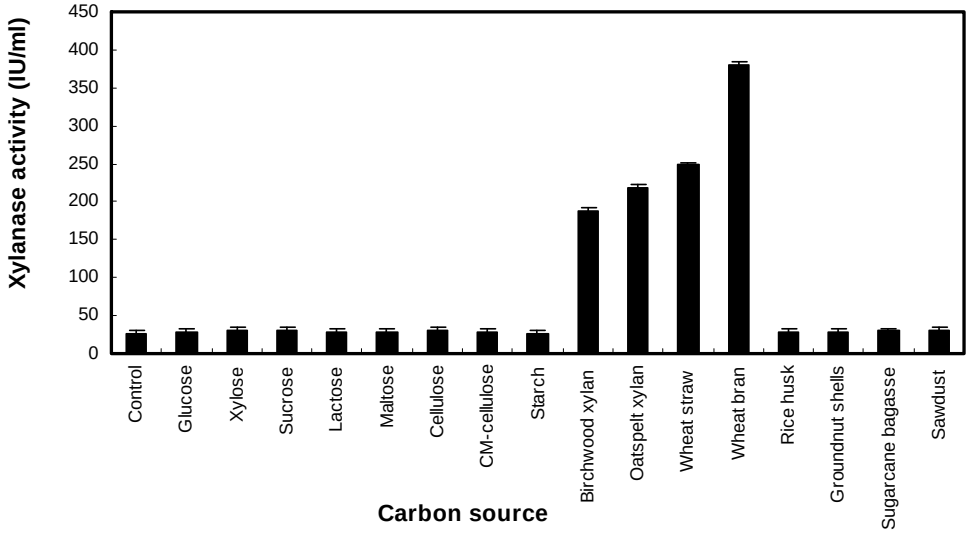

Fig. 7. Effect of carbon source on xylanase production by *B. subtilis* ASH in submerged fermentation after 48 h of incubation, pH 7.0 at 37 °C under shaking at 200 rpm with 0.5% peptone+yeast extract as nitrogen source. The inoculum was 18 h old and used at 2%.

In the present investigation, 2% wheat bran resulted in maximum xylanase production. Enhanced xylanase production by wheat bran might be due to the fact that it contained sufficient nutrients and was able to remain loose under moist conditions, thereby providing large surface area. Besides, the cell wall polysaccharides of wheat bran are rich in xylan (40%), the substrate for xylanase (Thiago and Kellaway 1982). Also, wheat bran has low lignin content and 28% protein, which is the source of carbon and nitrogen for growth of the *Bacillus*. Thus, wheat bran, a cost-effective agricultural residue, offers an excellent substrate for xylanase production by *B. subtilis* ASH. Goyal et al. (2008) also found higher xylanase production with lignocellulosics as compared to commercial xylan as carbon source. In contrast, some workers have reported higher xylanase production from pure xylan as compared to lignocellulosic materials (Rizzati et al. 2001; Battan et al. 2007). However, the use of pure xylan as a substrate is uneconomical for commercial production.

Nitrogen source

The production medium devoid of any nitrogen source was supplemented with different inorganic and organic nitrogen sources to evaluate their effects on xylanase titre. Organic nitrogen sources resulted in higher enzyme production than inorganic sources. This could be due to the fact that complex nitrogen sources like peptone released NH_4^+ ions, which stimulated growth. Among the organic nitrogen sources tested, peptone+ yeast extract favored highest xylanase production. Tryptone, beef extract, and yeast extract were slightly less effective in supporting enzyme production (Fig. 8).

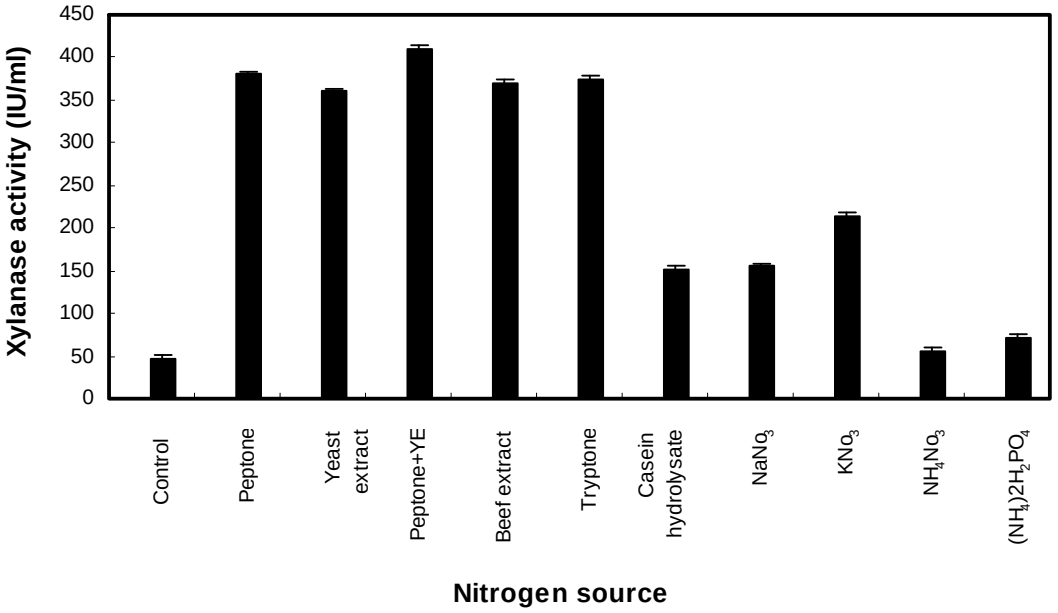

Fig. 8. Effect of nitrogen source on xylanase production by *B. subtilis* ASH in submerged fermentation after 48 h of incubation, pH 7.0 at 37 °C under shaking at 200 rpm with 2% wheat bran as carbon source. The inoculum was 18 h old and used at 2%.

Inorganic nitrogen sources resulted in very low enzyme production. Subramaniyan and Prema (1998) also reported enhanced xylanase production by *Bacillus* sp. SSP-34 using peptone+yeast extract. On the other hand, xylanase production was reported to be highest with peptone (Subramaniyan et al. 2001), tryptone (Dhillon et al. 2000) and a combination of peptone, yeast extract and KNO_3 (Battan et al. 2007).

Effect of additives

Xylanase production was monitored in the presence of additives (1 %) viz. NaCl, KCl, $CaCl_2$, $MgSO_4$, $ZnSO_4$, $CuSO_4$, SDS, and EDTA under optimized conditions. $ZnSO_4$, $CuSO_4$, SDS, and EDTA reduced the xylanase production to a great extent, whereas other additives had no effect (Fig. 9).

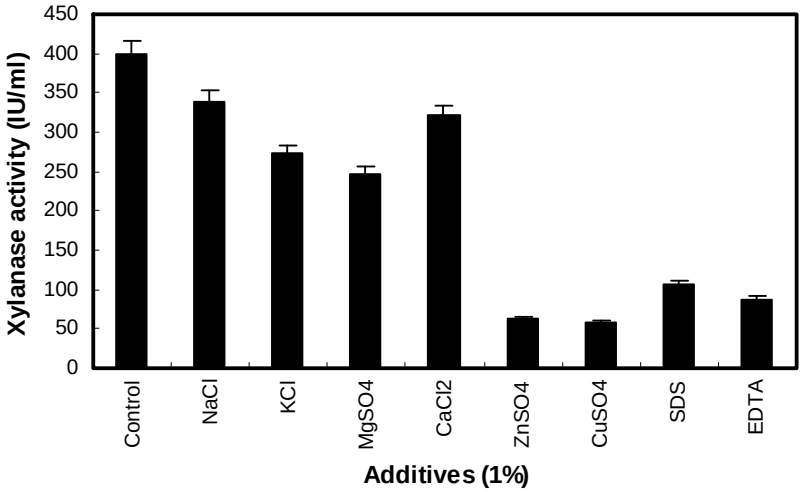

Fig. 9. Effect of additives on xylanase production by *B. subtilis* ASH in submerged fermentation after 48 h of incubation, pH 7.0 at 37 °C under shaking at 200 rpm with 2% wheat bran as carbon source and peptone+yeast extract as nitrogen source. The inoculum was 18 h old and used at 2%.

A control without any additive showed maximum enzyme activity. As none of these additives had a stimulatory effect, they were not used for enzyme production.

Xylanase Production under Optimum Conditions

Under optimized submerged fermentation conditions, (pH 7.0, temperature 37 ºC, 48 h incubation period**,** shaking at 200 rpm, 2% inoculum, 2% wheat bran, and 0.5% peptone + yeast extract), *B. subtilis* ASH produced 410 IU/ml of xylanase, which was 1.5-fold higher than under unoptimized conditions (time of incubation, 48 h; pH, 7.0; temperature, 37 °C; amount of inoculum, 1%; inoculum age, 24 h; agitation, 150 rpm; carbon source, 2 % wheat bran; nitrogen source, peptone+yeast extract). It was higher than the production of 180 IU/ml xylanase by *Bacillus* sp. GRE7 under submerged fermentation using oat spelt xylan as substrate (Kiddinamoorthy et al. 2008).

Biobleaching of Kraft Pulp with Xylanase

Cellulase-free xylanase produced by *B. subtilis* ASH under optimized submerged fermentation conditions was used for pre-bleaching of kraft pulp so as to evaluate its potential use as biobleaching agent**.**

Enzymatic Pre-Bleaching (E)

The conditions for biobleaching must be optimized to obtain effective dispersion of enzyme (Bajpai 1999). The pulp was pre-treated with xylanase under varying reaction conditions (enzyme dose, reaction time, incubation temperature, and pH), and the effectiveness of treatment was evaluated by reduction in kappa number and increase in brightness. The results on the optimization of enzymatic pre-bleaching (Tables 2-5) revealed that the bleaching was most effective at an enzyme dose 6 IU/g upon incubation for 2 h at 55 °C and pH 7.0.

Table 2 . Effect of Enzyme Dose on Pulp Biobleaching

Xylanase dose (U/g)	Brightness (% ISO)	Kappa no.
0	29.0±1.22	17.3±0.45
2	29.3±1.23	17.1±0.43
4	31.1±1.27	16.8±0.41
6	32.2±1.28	16.5±0.40
8	32.2±1.29	16.5±0.40
10	32.1±1.27	16.5±0.40

Table 3 . Effect of Retention Time on Pulp Biobleaching

Retention time (min)	Brightness (% ISO)	Kappa no.
0	28.9±1.25	17.3±0.47
30	29.1±1.27	17.2±0.46
60	29.4±1.28	16.9±0.44
90	31.0±1.28	16.7±0.43
120	32.2±1.29	16.5±0.40
150	32.1±1.27	16.5±0.41
180	31.9±1.27	16.5±0.40

Table 4 . Effect of Temperature on Pulp Biobleaching

Temperature (ºC)	Brightness (% ISO)	Kappa no.
50	28.9±1.26	17.3±0.48
55	32.2±1.29	16.4±0.46
60	32.1±1.28	16.5±0.45
65	31.9±1.27	16.5±0.45
70	31.8±1.28	16.6±0.47

Table 5. Effect of pH on Pulp Biobleaching

pH	Brightness (% ISO)	Kappa no.
7	32.2±1.29	16.5±0.45
8	31.1±1.28	16.7±0.47
9	29.6±1.26	17.0±0.49
10	29.0±1.25	17.1±0.48

These results are in agreement with those reported earlier by several researchers, where an enzyme dose of 5-10 U/g, incubation time 2-3 h and temperature 50°-60 °C was used for pulp bleaching (Dhillon and Khanna 2000; Sindhu et al. 2006; Battan et al. 2007). In contrast, maximum bleaching was reported at an enzyme dose of 20 U/g (Khandeparker and Bhosle 2007) and 150 U/g (Bissoon et al. 2002). Kulkarni and Rao (1996) performed bleaching with 10-40 IU of xylanase at pH 7 and temperature 50 °C for 4 h.

In the present study, pre-bleaching of kraft pulp with xylanase under optimized conditions reduced the kappa number from 17.3 to 16.5 and increased the brightness from 28.9 to 32.2% ISO. The effectiveness of xylanase treatment might be due to partial disruption of lignin-carbohydrate bonds thereby enhancing the accessibility of the subsequent bleaching chemicals to the pulp (Lei et al. 2008). In addition, the reprecipitated and reabsorbed alkali-resistant xylan, which forms a physical barrier

against the extraction of residual lignin molecules from the fibers, may be removed from the fiber surface by xylanase treatment (Daneault et al. 1994). So, xylanase treatment may be employed as the first step to boost pulp bleaching.

Chemical Bleaching (C/DED_1D_2)

Both control and xylanase-treated pulps were subjected to the chemical bleaching sequence (C/DED_1D_2), and the results are given in Table 6.

Table 6. Effect of *Bacillus subtilis* ASH Xylanase Treatment on Chlorine Consumption and Physical Properties of Pulp

Particulars	Control * (C/DED_1D_2)	Xylanase treated XC/DED_1D_2)				
		I	II	III	IV	V
Kappa no.	17.3	16.5*	16.5	16.5	16.5	16.5
Cl_2 added at CD stage (%)	3.8	3.8	3.4	3.0	2.7	2.4
NaOH added (%)	2.25	2.25	2.25	2.25	2.25	2.25
P. no.	2.7	2.2*	2.1	2.8	3.3	3.4
Initial Brightness (% ISO)	43.7	46.8*	45.3	43.3	41.1	40.5
ClO_2 added at D_1 stage (%)	0.8	0.6	0.6	0.7	0.8	0.8
ClO_2 added at D_2 stage (%)	0.3	0.2	0.3	0.3	0.3	0.3
Final Brightness (% ISO)	81	85*	84	83	82	81
Viscosity	9.78	14.34*	14.30	14.25	14.23	14.20
Total Chlorine used (%)	4.9	4.6	4.3	4.0	3.8	3.5
Reduction in Chlorine (%)	100.0	6.1	12.2	18.4	22.4	28.6

- Control pulp was not treated with xylanase; it was subjected to only chemical bleaching according to C/DED_1D_2
- Xylanase treated pulps I, II, III, IV and V were bleached with successively reduced concentrations of chlorine with respect to control
- *P<0.05, paired t test

In the enzyme pre-treated pulp, the final brightness increased from 81 to 85 (4.9%). This indicated that xylanase pre-treatment enhanced the process of lignin removal from the pulp. The P. no. was reduced by 18.5% after enzyme treatment in comparison to the chemical bleaching. To evaluate the potential of *B. subtilis* ASH cellulase-free xylanase in reducing the chlorine consumption, bleaching of enzyme pre-treated pulp was carried out by successively reducing the amount of chlorine to get the same brightness as in the control. The results shown in Table 6 (I-V) revealed that enzyme pre-treatment resulted in 28.6% reduction in chlorine consumption with the same brightness as in the control. This makes the biobleaching process not only economical but also eco-friendly.

In concurrence with our results, xylanase treatment has earlier been documented to cause reduction in kappa number and chlorine consumption but increase in brightness. Jiang et al. (2006) reported the reduction in kappa number by 1.1 points and enhancement in pulp brightness by 5.5% after treatment of wheat straw pulp with recombinant XynB at 10 U/g. Treatment of eucalyptus kraft pulp with commercial xylanases such as Novozyme 473, VAI xylanase, and Cartazyme HS-10 reduced chlorine consumption by 31% at chlorination stage and increased the final brightness by 2.1- 4.9 points (Bajpai et al. 1994). Madlala et al. (2001) reported that xylanase P (a commercial enzyme)

improved the brightness of kraft pulp by 5.6 points when used at 10 U/g of moisture-free pulp and caused about 10% reduction in chlorine dioxide consumption. Damiano et al. (2002) reported that the application of crude xylanase from *Bacillus licheniformis* for bleaching of kraft pulp resulted in a 5% increase in brightness and 30% reduction in the requirement of chlorine dioxide in comparison to the enzymatically untreated samples in order to obtain same value of brightness. In the present study, saving in chlorine charge with the use of *B. subtilis* ASH xylanase was either greater or comparable to that reported earlier (Bim and Franco 2000; Dhillon and Khanna 2000; Beg et al. 2001; Bissoon et al. 2002; Li et al. 2005; Sindhu et al. 2006; Zhao et al. 2006; Battan et al. 2007; Kiddinamoorthy et al. 2008). However, Garg et al. (1998) found a reduction in chlorine consumption in the range of 30-35% using cellulase-free xylanase from *Streptomyces thermoviolaceus*.

The enzyme treatment resulted in an increase in the viscosity of pulp from 9.78 to 14.34 (46.6%), as shown in Table 6. An increase in viscosity has also been reported in *B. pumilus* (Battan et al. 2007). Viscosity is a measure of cellulose chain length, and treatment of pulp with xylanase preparations containing cellulase results in a reduction in the degree of polymerization of the cellulose fibers and drop in product quality (Beg et al. 2001). The increase in viscosity after xylanase treatment might be due to degradation of hemicelluloses with shorter degree of polymerization (DP), leaving longer DP cellulose. The use of cellulase-free xylanases may help in selective removal of hemicellulose components with minimal damage to cellulose (Srinivasan and Rele 1995). As the xylanase produced by *B. subtilis* ASH was free of cellulase activity, it was unlikely to affect cellulose fibres. Hence, it was suitable for use in pulp bleaching.

Effect of xylanase treatment on paper quality

In order to test the effect of *B. subtilis* ASH xylanase pre-treatment on paper quality, the physical strength properties of handsheets made from enzyme treated pulp were compared with those of the control. The strength properties viz. grammage, fiber thickness, beating degree, tensile index, breaking length, tear index, and double fold of handsheets made of enzyme treated pulp showed an improvement compared to the untreated pulp (Table 7). Although, the values for bulk and burst index were statistically not significant at $P<0.05$ yet these were preserved in comparison to control.

Bajpai et al. (1994) reported that the physical strength properties of eucalyptus kraft pulp treated with three commercial xylanases, Novozyme 473, VAI xylanase, and Cartazyme HS-10 were preserved in comparison to a control. An enhancement in the strength properties of the enzyme-treated pulp compared to the control has also been reported by Battan et al. (2007). Maximo et al. (1998) observed that the enzyme-treated handsheets retained most of the required strength properties like bulk, burst index, tensile index, tear index, although there was an appreciable drop in the number of double folds. In the present studies, the improvement in various physical properties together with reduction in chlorine consumption after enzyme treatment implied that *B. subtilis* ASH xylanase could be used as a biobleaching agent.

Table 7. Strength Testing on Handsheets made from Pulp Pre-treated with *Bacillus subtilis* ASH Xylanase

Parameters	Control (C/DED_1D_2)	Xylanase-treated (XC/DED_1D_2)
No. of revolutions (PFI)	2700	2700
Grammage (g/m^2)	60	63*
Fiber thickness (µm)	82	87*
Beating degree (°SR)	31	42*
Bulk (cm^3g^{-1})	1.33	1.40
Tensile index (Nm/g)	70.02	79.43*
Breaking length(m)	7140	8095*
Burst index ($kPa.m^2/g$)	4.70	5.00
Tear index ($mN.m^2/g$)	8.03	9.53*
Double fold (No.)	267	448*
Pulp of 10% consistency was pre-treated with xylanase (6 IU/g o.d. pulp) at 55 ˚C, pH 7.0 for 2 h and then subjected to conventional chemical bleaching. *P<0.05, paired t test		

Scanning electron microscopy of handsheets

The handsheets made from untreated and enzyme-treated pulps were analyzed by scanning electron microscopy (SEM) to observe changes in pulp fibers after xylanase pre-treatment. SEM studies clearly showed that the application of crude xylanase caused morphological changes in the pulp fibers (Fig. 10). As compared to untreated pulp (Fig. 10 a & c), scanning electron micrographs of xylanase-treated pulp revealed loosening and swelling of fibers (Fig.10 b & d).

The fiber separation resulting from enzyme treatment might increase accessibility of pulp to bleaching chemicals. Similar observations on wood pulp fibers after xylanase treatment have been reported (Poorna and Prema 2007; Lei et al. 2008). Manimaran et al. (2009) also observed morphological changes in the bagasse pulp fibers after treatment with xylanase (50 IU/g pulp) at 50°C for 3 h.

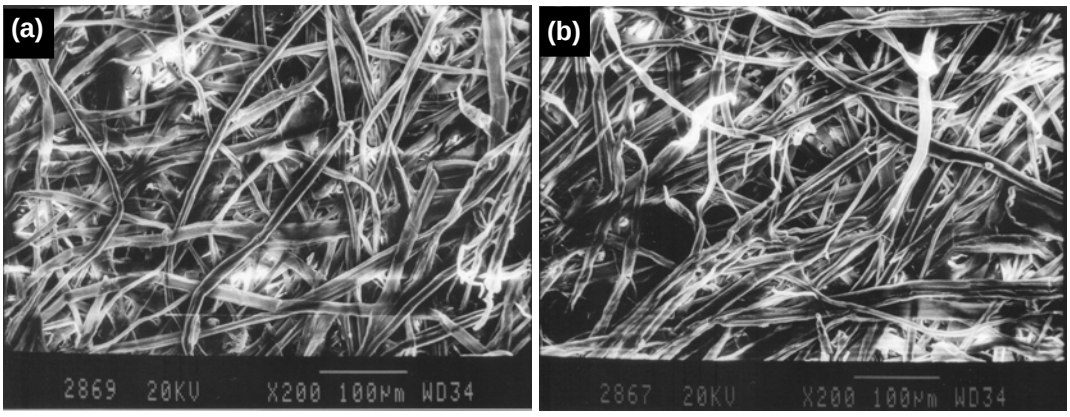

Fig. 10 (a and b). Scanning Electron Micrographs of handsheets made from untreated (a & c) and xylanase-treated (b & d) pulp. The pulp was pre-treated with xylanase (6 IU/g) at pH 7.0and 55 ºC for 2h followed by chemical bleaching. After washing, handsheets were prepared and analysed by SEM.

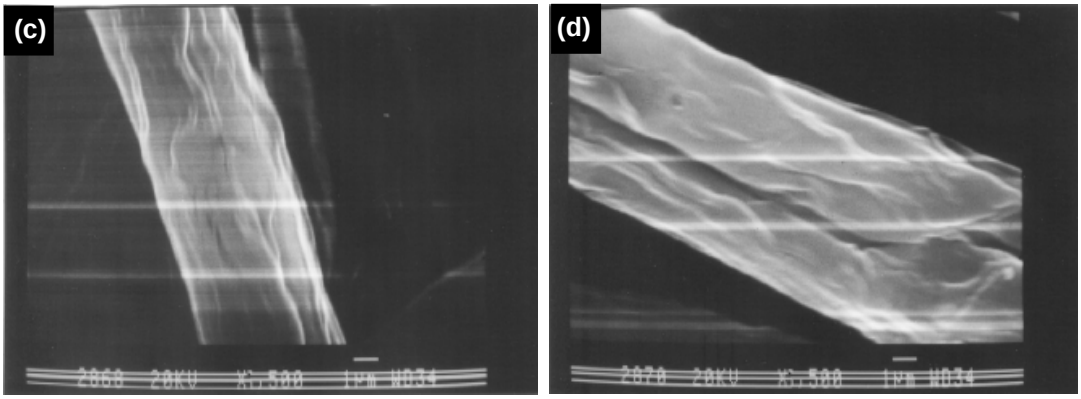

Fig. 10 (c and d). Scanning Electron Micrographs of handsheets made from untreated (a & c) and xylanase-treated (b & d) pulp. The pulp was pre-treated with xylanase (6 IU/g) at pH 7.0and 55 ºC for 2h followed by chemical bleaching. After washing, handsheets were prepared and analysed by SEM.

CONCLUSIONS

1. The bacterial strain used in this study produced xylanase in high titer (410 IU/ml) under submerged fermentation using wheat bran, a cost-effective substrate.
2. The cellulase-free nature of the enzyme and its production at alkaline pH and temperature as high as 60°C would make it potentially effective for pulp biobleaching.
3. Optimization of fermentation conditions enhanced xylanase production by 1.5-fold as compared to unoptimized conditions.
4. Xylanase produced by *B. subtilis* ASH has been shown to reduce chlorine consumption by 28.6%, thereby making the bleaching process economical and environmentally friendly. Further, the strength properties of pulp compared very favourably with that of control at constant brightness. It may be implied from the chlorine saving with concomitant improvement in various physical properties after enzyme treatment that *B. subtilis* ASH xylanase could be used as a biobleaching agent in the paper and pulp industry.

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REFERENCES CITED

Anuradha, P., Vijayalakshmi, K., Prasanna, N. D. and Sridevi, K. (2007). " Production and properties of alkaline xylanases from *Bacillus* sp. isolated from sugarcane fields," *Curr. Sci.* 92(9), 1283-1286.

Archana, A. and Satyanarayan T. (1998). "Cellulase-free xylanase production by thermophilic *Bacillus licheniformis* A-99," *Indian J. Microbiol.* 38, 135-139.

Bailey, M. J., Biely, P. and Poutanen, K. (1992). "Laboratory testing of methods for assay of xylanase activity," *J. Biotechnol.* 23, 257-70.

Bajpai, P., Anand, A., Sharma, N., Mishra, S. P., Bajpai, P. K., and Lachenal, D. (2006). "Enzymes improve ECF bleaching of pulp," *BioResources* 1(1), 34-44.

Bajpai, P. (1999). "Application of enzymes in the pulp and paper industry," *Biotechnol. Prog.* 15, 147-157.

Bajpai, P. and Bajpai, P. K. (1992). "Biobleaching of kraft pulp," *Process Biochem.* 28, 319-324.

Bajpai, P. and Bajpai, P. K. (1996). "Realities and trends in enzymatic prebleaching of kraft pulp," *Adv. Biochem. Eng. Biotechnol*., T. Schepper, ed., Springer-Verlag, Berlin, 56, pp. 1-31.

Bajpai, P., Bhardwaj, N. K., Bajpai, P. K. and Jauhari, M. B. (1994). "The impact of xylanases on bleaching of eucalyptus kraft pulp," *J. Biotechnol.* 38, 1-6.

Ball, A. S. and McCarthy, A. J. (1989). "Saccharification of straw by actinomycete enzymes," *J. Appl. Bacteriol.* 66, 439-444.

Bataillon, M., Nunes-Cardinali, A. P., and Duchiron, F. (1998). "Production of xylanase from newly isolated alkalophilic thermophilic *Bacillus* sp.," *Biotechnol. Lett.* 20, 1067-1071.

Battan, B., Sharma, J., Dhiman, S. S., and Kuhad R. C. (2007). "Enhanced production of cellulose-free thermostable xylanase by *Bacillus pumilus* ASH and its potential application in paper industry," *Enzyme Microbiol. Technol.* 41, 733-739.

Beg, Q. K., Kapoor, M., Mahajan, L. and Hoondal, G. S. (2001). "Microbial xylanases and their industrial applications: a review," *Appl. Microbiol. Biotechnol.* 56, 326-338.

Biely, P. (1985). "Microbial xylanolytic systems," *Trends Biotechnol.* 3, 286-290.

Bim, A. and Franco, T. (2000). "Extraction in aqueous two phase systems of alkaline xylanase produced by *Bacillus pumilus* and its application in kraft pulp bleaching," *J. Chrom. B* 743, 349–356.

Bissoon, S., Christov, L., and Singh, S. (2002). "Bleach-enhancing effects of purified xylanase from *Thermomyces lanuginosus* SSBP on bagasse pulp," *Process Biochem.* 38, 571–577.

Breccia, J. D., Sineriz, F., Baigori, M. D., Castro, G. R., and Hatti-Kaul, R. (1998). "Purification and characterization of a thermostable xylanase from *Bacillus amyloliquefaciens*," *Enzyme Microb. Technol.* 22, 42-49.

Damaso, M. C. T., Andrade, C. M. M. C., and Pereira, N. Jr. (2000). "Use of corncob for endoxylanase production by thermophilic fungus *Thermomyces lanuginosus* IOC-4145," *Appl. Biochem. Biotechnol.* 84–86, 821-834.

Damiano, V. B., Bocchini, D. A., Gomes, E., and Da Silva, R. (2003). "Application of crude xylanase from *Bacillus licheniformis* 77-2 to the bleaching of eucalyptus Kraft pulp," *World J. Microbiol Biotechnol* 19, 139-144.

Daneault, C., Leduc, C., and Valade, J. L. (1994). "The use of xylanases in kraft pulp bleaching: A review," *Tappi Journal* 77(6), 125-131.

Dhillon, A. and Khanna, S. (2000). "Production of a thermostable alkali-tolerant xylanase from *Bacillus circulans* AB16 grown on wheat straw," *World J. Microbiol. Biotechnol.* 27, 325-327.

Dhillon, A., Gupta, J. K., Jauhari, B. M. and Khanna, S. (2000). "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," *Bioresour. Technol.* 73, 273-277.

Dhiman, S. S., Sharma, J., and Battan, B. (2008). "Industrial applications and future prospects of microbial xylanase: A review," *BioResources* 3(4), 1377-1402.

Eriksson, K. E. L. (1990). "Biotechnology in the pulp and paper industry," *Wood Sci. Technol.* 24, 79-101.

Garg, A. P., Roberts, J. C. and McCarthy, A. J. (1998). "Bleach boosting effect of cellulase-free xylanase of *Streptomyces thermoviolaceus* and its comparison with two commercial enzyme preparations on birchwood kraft pulp," *Enzyme Microb. Technol.* 22, 594–598.

Ghose, T. K. (1987). "Measurement of cellulase activities," *Pure Appl. Chem.* 59, 257-268.

Goyal, M., Kalra, K. L., Sareen, V. K., and Soni, G. (2008). " Xylanase production with xylan rich lignocellulosic wastes by a local soil isolate of *Trichoderma viride*," *Brazilian J. Microbiol.* 39, 535-541.

Jiang, Z. Q., Li, X. T., Yang, S. Q., Li, L. T., Li,Y. and Feng, W. Y. (2006). "Biobleach boosting effect of recombinant xylanase B from the thermophilic *Thermotoga maritime* on wheat straw pulp," *Appl. Microbiol. Biotechnol.* 70, 65-71.

Khandeparker, R. and Bhosle, N. B. (2007). "Application of thermoalkalophilic xylanase from *Arthrobacter* sp. MTCC 5214 in biobleaching of Kraft pulp," *Bioresour. Technol.* 98, 897-903.

Kiddinamoorthy, J., Anceno, J. A., Haki, D. G., and Rakshit, S. K. (2008). "Production, purification and characterization of *Bacillus* sp. GRE7 xylanase and its application in eucalyptus Kraft pulp biobleaching," *World J. Microbiol. Biotechnol.* 24, 605-612.

Kulkarni, N., and Rao, M. (1996). "Application of xylanase from alkalophilic thermophilic *Bacillus* sp. NCIM 59 in biobleaching of bagasse pulp," *J. Biotechnol.* 51, 167-73.

Lei, X., Lin, L., and Li, K. (2008). "Effect of xylanase pretreatment on wood chips on fiber separation in the CTMB refining process," *BioResources* 3(3), 801-815.

Li, X.T., Jiang, Z. Q., Li, L. T., Yang, S. Q., Feng, W. Y., Fan, J. Y. and Kusakabe, I. (2005). "Characterization of a cellulase-free, natural xylanase from *Thermomyces lanuginosus* CBS 288.54 and its biobleaching effect on wheat straw pulp," *Bioresour. Technol.* 96, 1370-1379.

Madlala, A. M., Bissoon, S., Singh, S. and Christov, L. (2001). "Xylanase induced reduction of chlorine dioxide consumption during elemental chlorine-free bleaching of different pulp types," *Biotechnol. Lett*. 23, 345-351.

Manimaran, A., Kumar, K. S., Permaul, K., and Singh, S. (2009). "Hyper production of cellulose-free xylanase by *Thermomyces lanuginosus* SSBP on bagasse pulp and its application in biobleaching," *Appl. Microbiol. Biotechnol.* 81, 887-893.

Maximo, C., Costa-Ferreira, M., and Duarte, J. (1998). "Some properties of eucalyptus kraft pulp treated with xylanase from *Aspergillus niger*," *World J. Microbiol. Biotechnol.* 14, 365-367.

Miller, G. L. (1959). "Use of dinitrosalicylic acid reagent for determination of reducing sugar," *Anal. Chem.* 31, 426-428.

Peixoto-Nogueira, S. D. C., Michelin, M., Betini, J. H. A., Jorge, J. A., Terenzi, H. F., and Polizeli, M. D. L. T. D. M. (2009). "Production of xylanase by *Aspergillus* using alternative carbon sources: Application of the crude extract on cellulose pulp biobleaching," *J. Ind. Microbiol. Biotechnol.* 36, 149-155.

Poorna, C. A., and Prema, P. (2007). "Production of cellulase-free endoxylanase from novel alkalophilic thermotolerent *Bacillus pumilus* by solid-state fermentation and its application in waste paper recycling," *Bioresour. Technol.* 98, 485-490.

Qureshy, A. F., Khan, L. A., and Khanna, S. (2002). Cloning, regulation and purification of cellulase-free xylanase from *Bacillus circulans* Teri-42," *Indian J. Microbiol.* 42, 35-41.

Rajaram, S. and Varma, A. (1990). "Production and characterisation of xylanase from Bacillus thermoalkalophilus grown on agricultural wastes," *Appl. Microbiol. and Biotechnol.* 34, 141-144.

Ratto, M., Poutanen, K., and Viikari, L. (1992). "Production of xylanolytic enzymes by an alkalitolerant *Bacillus circulans* strain," *Appl. Microbiol. Biotechnol.* 37, 470-473.

Rizzati, A. C. S., Jorge, J. A., Terenzi, H. F., Rechia, C. G. V., and Polizeli, M. L. T. M. (2001). "Purification and properties of a thermostable β-D-xylosidase produced by a thermotolerant *Aspergillus phoenicis*," *J. Ind. Microbiol. Biotechnol.* 25, 156-160.

Sanghi, A., Garg, N., Sharma, J., Kuhar, K., Kuhad, R. C., and Gupta, V. K. (2007). "Optimization of xylanase production using inexpensive agro-residues by alkalophilic *Bacillus subtilis* ASH in solid-state fermentation," *World J. Microbiol. Biotechnol.* 24, 633-640.

Senior, D. J., Hamilton, J., Bernier, R. L., and Manoir, J. R. (1992). "Reduction in chlorine use during biobleaching of kraft pulp following xylanase treatment," *TAPPI J.* 75, 125–130.

Shah, A. K., Sidid, S. S., Ahmed, A., and Rele, M. V. (1999). "Treatment of bagasse pulp with ceIlulase-free xylanases from an alkalophilic *Bacillus* sp. Sam-3," *Bioresour. Technol.* 68, 133-140.

Sharma, A., Adhikari, S., and Satyanarayan T. (2007). "Alkali-thermostable and cellulase-free xylanase production by an extreme thermophile *Geobacillus thermoleovorans*," *World J. Microbiol. Biotechnol.* 23, 483-490.

Sindhu, I., Chhibber, S., Caplash, N. and Sharma, P. (2006). "Production of cellulase-free xylanase from *Bacillus megaterium* by solid state fermentation for biobleaching of pulp," *Curr. Microbiol.* 53, 167-172.

Srinivasan, M. D., and Rele, M. V. (1999). "Microbial xylanases for paper industry," *Curr. Sci.* 77, 137-142.

Subramaniyan, S., Sandhia, G. S., and Prema, P. (2001). "Biotech control of xylanase production without protease activity in *Bacillus* sp. by selection of nitrogen source," *Biotechnol. Lett.* 23, 369-371.

Sunna, A. and Antranikian, G. (1997). "Xylanolytic enzymes from fungi and bacteria," *Crit. Rev. Biotechnol.* 17, 39-67.

TAPPI Test Methods. (1996). *Technical Association of the Pulp and Paper Industry*, TAPPI Press, Atlanta, GA.

Thiago, L. R., and Kellaway, R. C. (1982). "Botanical composition and extent of lignification affecting digestibility of wheat and oat straw and pastalum hay," *Anim. Feed Sci. Technol.* 7, 71–81.

Valls, C., and Roncero, M. B. (2009). "Using both xylanase and laccase enzymes for pulp bleaching," *Bioresour. Technol*. 100, 2032-2039.

Viikari, L., Kantelinen, A., Sundquist, J., and Linko, M. (1994). "Xylanases in bleaching: From an idea to the industry," *FEMS Microbiol. Rev.* 13, 335-350.

Zhao, J., Li, X. and Qu, Y. (2006). "Application of enzymes in producing bleached pulp from wheat straw," *Bioresour. Technol.* 97, 1470-1476.

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