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Full Length Research Paper

Potential of *Laceyella sacchari* strain B42 crude xylanase in biobleaching of kraft pulp

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Xylanase producing thermophilic actinomycetes strain B42 was isolated from bagasse. This strain was enriched on oat spelt xylan agar medium and screened onto xylan-congo red agar plate by the xylanolysis method. The phylogenetic analysis using 16S rDNA sequence data confirmed that strain B42 showed highest homology (99.0%) with *Laceyella sacchari* and was identified as *Laceyella sacchari* strain B42. Maximal xylanase production was achieved at the incubation period of 48 h with the xylanase and cellulase activities as 24.5 and 0.08 U/ml, respectively. The optimum pH and optimum temperature of *L. sacchari* strain B42 xylanase was found to be 9.0 and 60°C, respectively. Xylanase was thermostable at 60°C for 1 h and retained 90% of its activity up to 6 h at this temperature, and subsequently enzyme retained 75 and 60% activity at 70 to 80°C, respectively after 6 h. At biobleaching of kraft pulp, enzyme released sufficient amount of phenolic and hydrophobic compounds. The ultraviolet (UV absorption spectrum of the compounds released by enzyme treatment indicated the presence of lignin in the released coloring matter. The enzymatic biobleaching of kraft pulp caused ~12% reduction of kappa number, 6.67 fold releases of reducing sugars and 10% decrease of lignin content at xylanase optimum dose (60 U/g) and time (4 h).

Key words: Biobleaching, kappa number, pulp and paper industry, thermostable, xylanase, 16S rDNA.

INTRODUCTION

The pulp and paper industry is one of the fastest growing industry of India. This industry requires the need of chemicals for pulp processing and bleaching. The major problem associated with this conventional bleaching is the use of large amount of chemicals that leads to environmental pollution (Hongzhang et al., 2002). The present scenario of pulp and paper industry is focused on the modification of pulping, bleaching and effluent treatment technologies to reduce the environmental impact of mill effluents. In order to reduce the impacts of chemical bleaching technology to environment, one environment friendly technology alternative of conventional bleaching (chemical bleaching) strategy has been developed and named as biobleaching technology (Khristova et al., 2006).

Biobleaching needs the application of microbes or enzymes for pulp debarking, pulp refining and pulp dissolving. The concept of using enzymes to enhance the delignification of pulp was first presented by Viikari et al. (1986). Pulp biobleaching is employed by the use of xylanolytic microorganism or enzymes as it is the major industrial enzyme with great biotechnological application. Many workers have studied the applicability of xylanase for biobleaching (Adachi and Chen, 2007). Xylanases based biobleaching decreases the consequence of the use of chemicals in conventional papermaking processes as it releases hazardous effluents (Ayyachamy and Vatsala, 2007). Xylanase treated pulp require less chlorine during subsequent bleaching operation and this treatment results in low amount of chlorinated organic compound released in bleaching effluent, thus, minimizing the risk of environment pollution (Senior and Hamilton, 1992).

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The xylanolytic procedure in paper industry requires the enzyme which exhibited high thermostability and activity in broader pH range (Jacques et al., 2000). Treatment with xylanase at elevated temperature disrupts cell wall structure leading to removal of lignin in various stages of bleaching that ultimately reduces the use of chemicals for bleaching, to achieve comparable levels of paper brightness and paper strength. Xylanase used for paper industry must lack cellulolytic activity to avoid hydrolysis of cellulose fibre. Cellulase free xylanase which shows activity at high temperature and alkaline pH are gaining importance in pulp and paper technology as alternative to the use of toxic chlorinated compounds (Sudan and Bajaj, 2007). Most of the xylanases known to date are optimally active at or below 50°C and at acidic or neutral pH. On the other hand, in the process of enzyme associated pulp bleaching, the incoming pulp has high temperature and alkaline pH (Nanmori et al., 1992). Therefore, attempt was taken for the use of new xylanolytic microorganism to find its suitability for biobleaching.

In order to minimize the risk of environmental pollution in pulp and paper industry, we undertook a study to isolate the strain which produces thermostable, cellulase free xylanase active at alkaline pH. Further, the potential of crude xylanase in biobleaching of kraft pulp was studied.

MATERIALS AND METHODS

Isolation and screening of xylanase producing bacteria-

Thermophilic bacteria were isolated from sugarcane field soil, compost and bagasse collected from Pantnagar, India. Diluted samples were applied on nutrient agar plate and incubated at 60°C for two to three days for the appearance of thermophilic bacterial colonies. Appeared colonies were transferred into xylan containing media for the screening of xylanase producing bacteria. Further, the xylanase producing potential for these isolates was determined using xylanolysis method by incubating these isolates into xylan-congo red agar media consisting: (% w/v): Congo red, 0.02%; oat spelt xylan, 0.18%; agar, 0.5%; gelatin, 0.2%; K_2HPO_4, 0.05%; $MgSO_4$, 0.025%; pH 7.0. Positive isolates produced clear zone of lysis around the bacterial colony due to degradation of xylan-congo red agar. The zone of lysis varies according to the quantity of xylanase produced; more xylanase produced is depicted by the large zone of lysis.

Bacterial identification

Promising isolate B42 was identified by 16S rDNA analysis. The identification of bacteria was done by the alignment of 16S rDNA sequence with already available sequence at European Microbiological Laboratory (EMBL), GenBank (gb, Germany) using the BLAST algorithm available in NCBI (National Centre for Biotechnology information).

Xylanase production and activity

Xylanase producing ability for positive isolates was tested in production media that contained: oat spelt xylan (OSX), 1%; yeast extract, 0.2%; peptone, 0.5%; $MgSO_4$, 0.05%; NaCl, 0.05%; $CaCl_2$, 0.015%; pH 7.0. Media was inoculated with 10% v/v of overnight culture and incubated at 60°C with aeration in shaker at 200 rpm for 96 h. The enzyme activity and growth (at 600 nm) were determined periodically after time intervals of 12 h. Before assay, the cells were separated by centrifugation at 10,000 *g* for 20 min and clear supernatant was used as crude enzyme.

Enzyme assay

Xylanase activity was assayed by measuring the release of reducing sugar from oat spelt xylan. Reaction mixture consisted of 1% xylan in 0.1 M Tris-HCl buffer (pH, 7.0) and enzyme to give a final volume of 1.0 ml. After incubating for 10 min at 60°C, the release of reducing sugar was determined by Nelson-Somogyi method (Nelson, 1944; Somogyi, 1952). The cellulase activity was measured under the same condition as described above using carboxymethyl cellulose as a substrate. One unit of xylanase and cellulase is defined as the amount of enzyme required to release 1 µmol of reducing sugar, xylose or glucose per min, under above assay condition. The protein concentration was determined by the Lowry method using bovine serum albumin as standard (Lowry et al., 1951).

Effect of pH and temperature on xylanase activity

The activity of xylanase at various pH values were measured by using buffers having pH ranges from 5.0 to 10.0. The buffers used were 0.1 M citrate-phosphate buffer (pH, 5.0 to 6.0), 0.1 M Tris-HCl buffers (pH, 7.0 to 8.0) and 0.1 M Glycine-NaOH buffer (pH, 9.0 to 10.0). The effect of temperature on the enzyme activity was determined by assaying xylanase within temperature range of 40 to 80°C at their optimum pH, 9.0 (0.1 M Glycine-NaOH buffer). Heat stability studies was done by incubating crude xylanase at temperatures ranging from 40 to 80°C for 1 to 6 h. The residual activity was quantified at optimum temperature and pH that is, 60°C and 9.0, respectively using Nelson-Somogyi method.

Biobleaching of kraft pulp

Biobleaching efficiency of crude xylanase was studied by the treatment of pulp sample with the enzyme. Enzyme dose and reaction time for biobleaching were optimized. Various parameters such as, release in hydrophobic compound (λ_{465nm}), release in phenolic compound (λ_{237nm}), release in reducing sugars, decrease in kappa number and percentage of residual lignin were also studied.

Colour removal from the kraft pulp

Unbleached kraft pulp used in the present study was kindly provided by Shivangi paper mill (Kashipur, India). Pulp samples were washed properly before use and after each treatment step with water. All the biobleaching studies were performed at optimum pH and optimum temperature of the enzyme. The kraft pulp was treated with the enzyme doses of 0 to 100 U/g at optimum pH (9.0) and temperature (60°C) for incubation time of 1 to 5 h. The pulp treated with the buffer in a similar manner was used as control. Following incubation period, the pulp samples were washed with distilled water and the absorbance of the filtrate was determined spectrophotometrically from λ_{200} nm to λ_{400} nm.

Optimization of enzyme dose and reaction time for biobleaching

The enzyme dose was optimized by the treatment of pulp with different doses of xylanase, which ranged between 0 to 100 U/g moisture free pulp for the incubation time of 3 h. Similarly, optimum incubation time for biobleaching was achieved by giving the pulp treatment at optimum enzyme dose, that is 60 U/g for 1 to 5 h. Following incubation, the pulp was dewatered on a separating funnel using Whatman No.1 filter paper and filtrate were collected for the study of chromophore content and reducing sugar estimation. Chromophore content in the filtrate was measured with spectrophotometer by optical density at 465 and 237 nm for hydrophobic compounds and phenolic compounds, respectively. Released reducing sugar was estimated by the Nelson-Somogyi method.

Determination of kappa number and residual lignin

After treatment of pulp sample with enzyme, the pulp suspension was filtered through a Whatman No.1 filter paper and air-dried. The delignification was measured as change in kappa number which is indicative of the extent of delignification and bleachability of the pulp. The pulp parameters that is, kappa number was determined according to the standard methods of the Technical Association of the Pulp and Paper Industry (TAPPI, Atlanta, GA, USA) (T236-cm-85) (Tappi test method, 1996). The kappa number is defined as the amount (ml) of a 0.1 N $KMnO_4$ solution consumed by 0.5 to 1.0 g of dried pulp under standard conditions. The kappa number × 0.15 gives the percentage of residual lignin.

RESULTS AND DISCUSSION

In total, 95 thermophilic isolates were screened from soil, compost and bagasse, among which only 24 isolates showed good hydrolysis zone around xylan-congo red promising isolates, *Laceyella sacchari* agar media and were selected for further study.

The moststrain B42 produced the largest zone of hydrolysis on xylan-congo red agar media and selected as best xylanase producer.

Strain B42 identification

Phylogenetic analysis by 16S rDNA validated that isolate B42 was one strain of *L. sacchari* (99% identity with *L. sacchari* strain VTT E-062990) and identified as *L. sacchari* strain B42.

Incubation time on xylanase production and activity

L. sacchari strain B42 produced low level of xylanase in early stages of incubation. The enzyme production steadily reached a maximum level (24.5 U/ml) after 48 h of incubation (Figure 1) and decreased with further increase in incubation period. The reduction in xylanase yield after optimum period was probably due to the depletion of nutrient available to microorganism or due to proteolysis. Similar finding was reported for *Streptomyces* sp. by Godden et al. (1989). Comparable to our results, the optimum incubation time reported by other workers were 72 h for *Streptomyces* sp.CD-3, 24 h for isolate AS1, AS2 and CS1, 48 h for isolate CD-4 and CS1 (Sharma and Bajaj, 2005). Georis et al. (2000) reported that *Micromonospora* strain LL23 and *Amycolata autotrophica* strains (DSM 43089; DSM 43099) produced maximum xylanase after 6 days of incubation.

The xylanase activity for *L. sacchari* strain B42 was found to be 24.5 U/ml, which may be compared with previously reported *Bacillus substilis* strain (3.2 U/ml) (Khanongnuch et al., 1998), *B. subtilis* (12 U/ml) (SaPereria et al., 2002), *Streptomyces* sp. EC22 (9 U/ml), *Streptomyces* sp. EC1 (12 U/ml) and *Thermomonospora fusca* MT100 (8 U/ml) (Ball and McCarthy, 1989). Cellulase activity of crude enzyme preparations was estimated at their optimum pH and temperature. *L. sacchari* strain B42 showed 0.08 U/ml of cellulase activity; comparable to finding of Yang et al. (1995) with cellulase activity of 0.1 U/ml for alkaline active xylanase from *Bacillus* sp. Similarly, Grabski and Jeffries (1991) observed cellulase activity of 0.12 U/ml for *S. sclerotialus* NRRL B-2317 and 0.1 U/ml for *S. flavisclerotic*us NRRL B-11018, respectively. Xylanase for industrial uses must be cellulase-free.

The presence of cellulase in commercial xylanase preparations can result in a loss of fiber strength, leading to degradation in pulp quality and increase effluent treatment cost (Paice, 1992). The presence of 0.08 U/ml of cellulase activity in *L. sacchari* strain B42 meets these requirements.

Effect of pH and temperature on xylanase activity

Optimum pH of *L. sacchari* strain B42 xylanase was 9.0, making it suitable for bio-bleaching applications. Earlier workers also reported the pH optima of xylanase from several *actinomycetes* strains ranging from pH 6.0 to 9.0 (Georis et al., 2000). Xylanases produced by most alkaliphiles reported to date have their optimum pH around neutrality, therefore alkaline xylanases are considered to have good potential for application in the pulp and paper industry. The use of these enzymes is expected to greatly reduce the need for pH readjustments before enzyme addition. The xylanase from *L. sacchari* strain B42 having pH optima around 9.0 makes it suitable for bio-bleaching applications.

Temperature optima for *L. sacchari* strain B42 xylanase was 60°C; additionally it also showed good activity at 70 and 80°C. The optimum temperature value for *L. sacchari* strain B42 xylanase was similar or somewhat higher than the optimal temperature reported for *Streptomyces* sp. CH-M-1035 (Flores et al., 1997), *Streptomyces* sp. QG-11-3 (Beg et al., 2000c), *Streptomyces* sp. strain AMT-3 (Nascimento et al., 2002) and *S. roseiscleroticus* (Grabski and Jeffries, 1991).

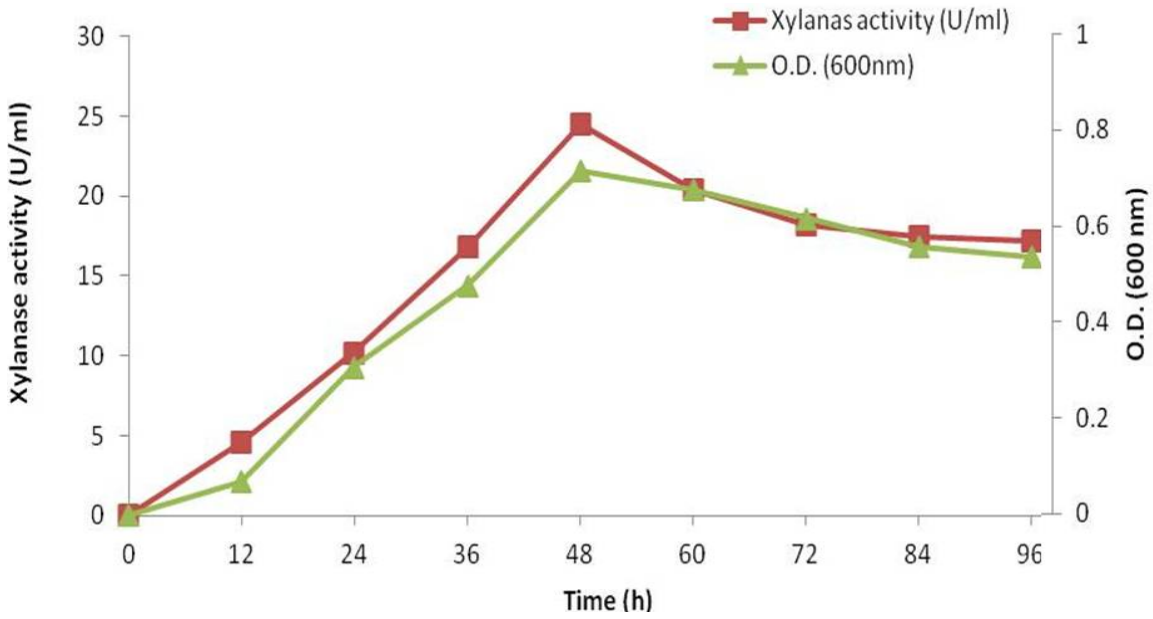

Figure 1. Effect of incubation time on xylanase production.

Thermostability profile of *L. sacchari* strain B42 xylanase showed desirable activity at 60ºC up to 6 h and retained 99% of its activity after 1 h and 90% activity after 6 h of incubation at this temperature. Enzyme also retained 75 and 60% of its activity at 70 and 80°C, respectively, at 6 h incubation (Figure 2). The observed stability of xylanase was much better as reported by Duarte et al. (2000) for *B. pumilus* strain 5_2, 5_{14},13_a and 4_a, Sharma and Bajaj (2005) for *Streptomyces* sp. CD-3, Sa Pereira et al. (2002) for *B. substilis* and Kohli et al. (2001) for *T. thalophilus*. The process of enzyme assisted kraft pulp bleaching require high temperature, hence a thermostable xylanase from B42 would fulfill these requirement.

Biobleaching of kraft pulp

Colour removal from the kraft pulp

The amount of colour release was increased with the increase in enzyme dose. The peak at 280 nm in the UV spectrum indicated the presence of lignin in the released coloured compounds and showed the effectiveness of xylanase on pulp. The *L. sacchari* strain B42 crude xylanase treatment gave the absorption value of 0.828 (at $\lambda_{280\ nm}$) with the treatment of 100 U/g xylanase at their optimum temperature and pH, for 3 h (Figure 3).

At the optimum enzyme dose (60 U/g oven dry pulp), the enzyme gave absorption of 0.875 (at $\lambda_{280\ nm}$) with the optimum incubation time of 4 h (Figure 4). Khandeparkar and Bhosle (2007) demonstrated that *Arthrobacter* sp. xylanase gave the absorption of 1.5 when treated with 20 U/g of oven dried pulp, while Kulkarni and Rao (1996) showed absorption of 0.8 at 20 U/g of oven dried pulp. Nissen et al. (1992) concluded that increase in absorbance at 280 nm emphasize the release of lignin.

Enzyme dose and reaction time optimization

The biobleaching efficiency of crude xylanase from *L. sacchari* strain B42 was studied by giving the treatment to pulp with the enzyme dose ranging from 0 to 100 U/g of pulp. With the increase in enzyme dose-kappa number, release of phenolic compounds, hydrophobic compounds and reducing sugars were increased. Increases in the release of phenolic compounds (at $\lambda_{237\ nm}$) were observed from 0.016 to 1.44, while increases in hydrophobic compound (at $\lambda_{465\ nm}$) were observed from 0.10 to 0.41 (Figure 5) at 100 U/g enzyme dose for 3 h. The optimum enzyme dose for biobleaching of pulp was found to be 60 U/g as it released the maximum reducing sugars form pulp sample. At this optimized enzyme dose, ~14.7% reduction of kappa number with the 4 fold release of reducing sugars was observed (Figure 7).

With the treatment of pulp for different incubation time which ranged from 1 to 5 h, the release in phenolic compounds (at $\lambda_{237\ nm}$) were observed from 0.68 to 1.25 and the release of hydrophobic compounds (at $\lambda_{465\ nm}$) were observed from 0.17 to 0.41 (Figure 6). At optimum reaction time (4 h) and enzyme dose (60 U/g), crude xylanase produced ~12% reduction in kappa number and 6.67 fold increase in release of reducing sugars (Figure 8). The efficacy of the enzyme on biobleaching is well correlated with the release of chromophore and total sugar. Khandeparkar and Bhosle (2007) observed maximum biobleaching efficiency at 2 h and 70°C

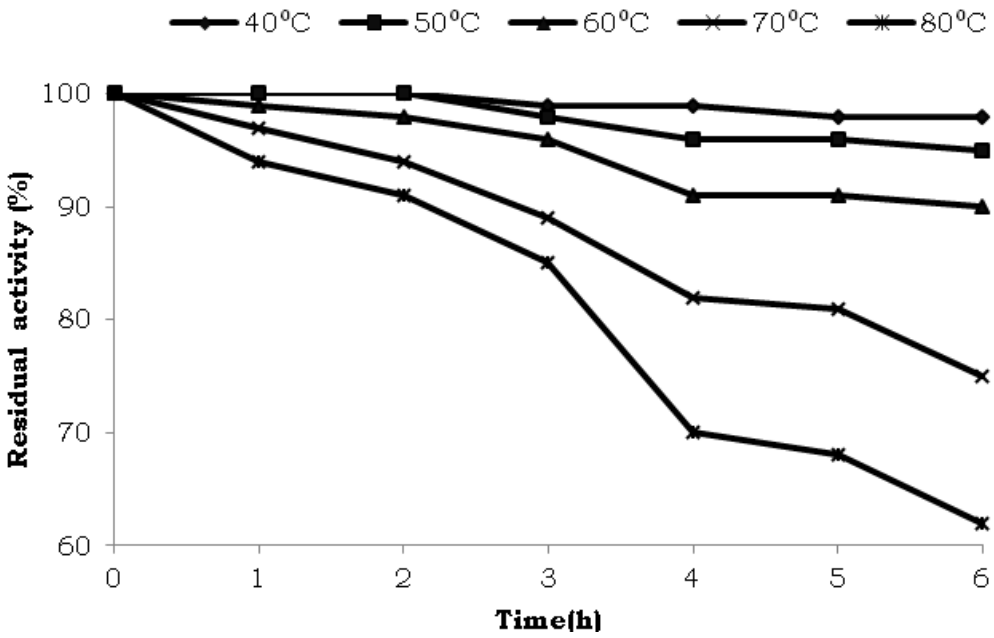

Figure 2. Thermostability profile of xylanase. Crude xylanase were preincubated at various temperatures (40 to 80°C) for 1 to 6 h prior to xylanase determination at pH, 9.0 assay condition. Residual activity is presented as a percentage of the original without heat treatment.

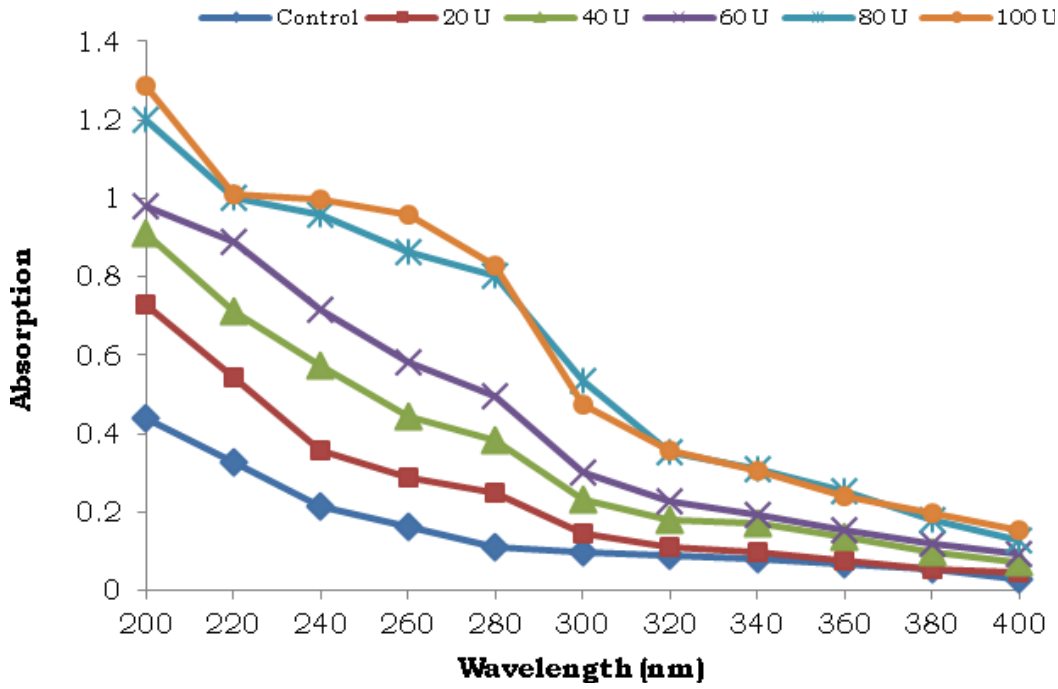

Figure 3. UV spectrum of the compounds released during crude xylanase treatment at different enzyme dose (U/g) oven dry pulp.

treatment for *Arthrobacter* sp. by 20 U/g (9.0 pH) which produced 20% reduction in kappa number and 6 to 7 fold increase in release of reducing sugars. The optimum condition for biobleaching was also reported by other worker as *B. licheniformis* 77-2 xylanase optimized dose was 14 U/g at pH 6.0 and 60°C for 4 h (Damiano et al., 2003); enzyme dose of 5 to 10 U/g and incubation time 2 to 3 h was used for biobleaching (Sindhu et al., 2006;

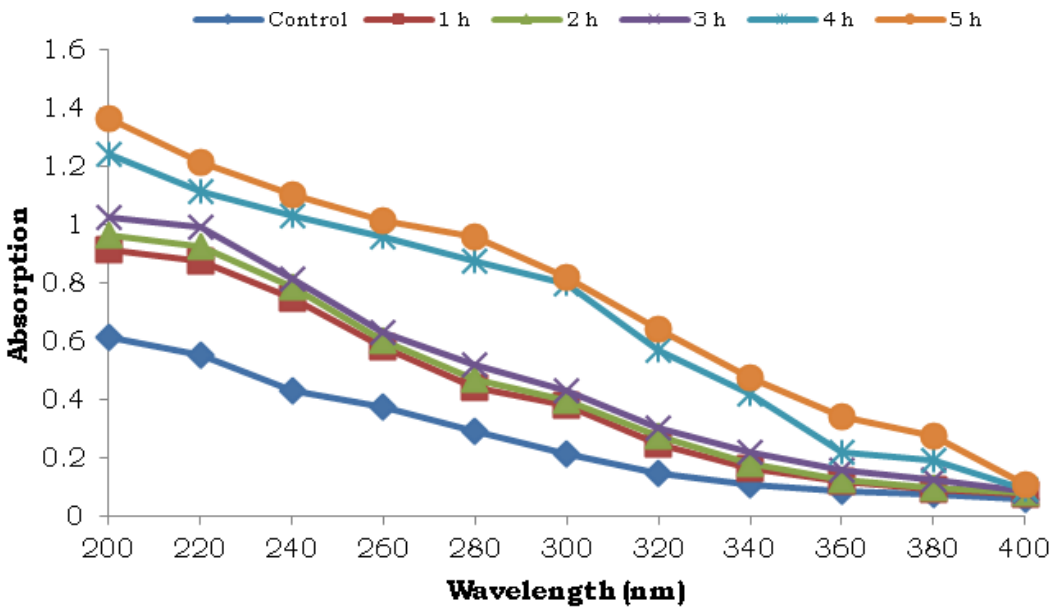

Figure 4. UV spectrum of the compounds released during crude xylanase treatment at different time interval.

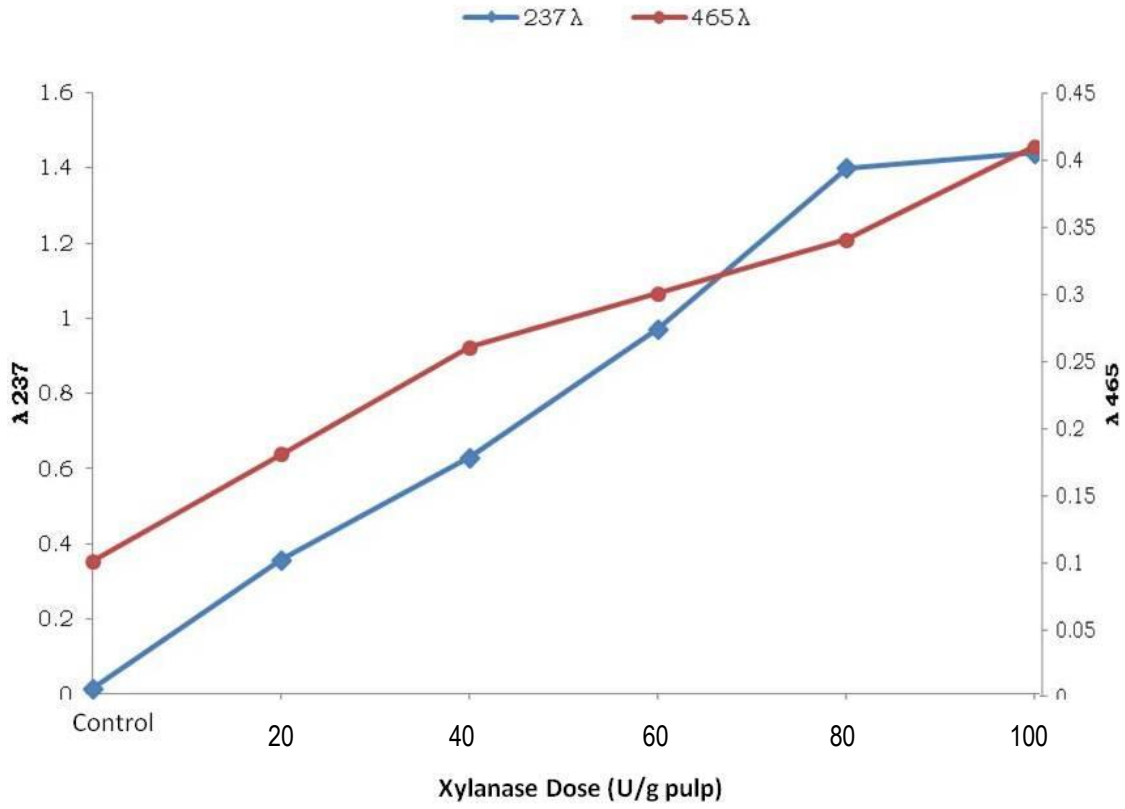

Figure 5. Release of hydrophobic and phenolic compounds by different xylanase dose (U/g pulp) at biobleaching of pulp.

Battan et al., 2007). Maximum bleaching was reported at enzyme dose of 20 U/g (Khanderparker and Bhosle, 2007), 150 U/g (Bissoon et al., 2002), 10 to 40 U/g at pH 7.0 for 4 h (kulkarni and Rao, 1996) and for

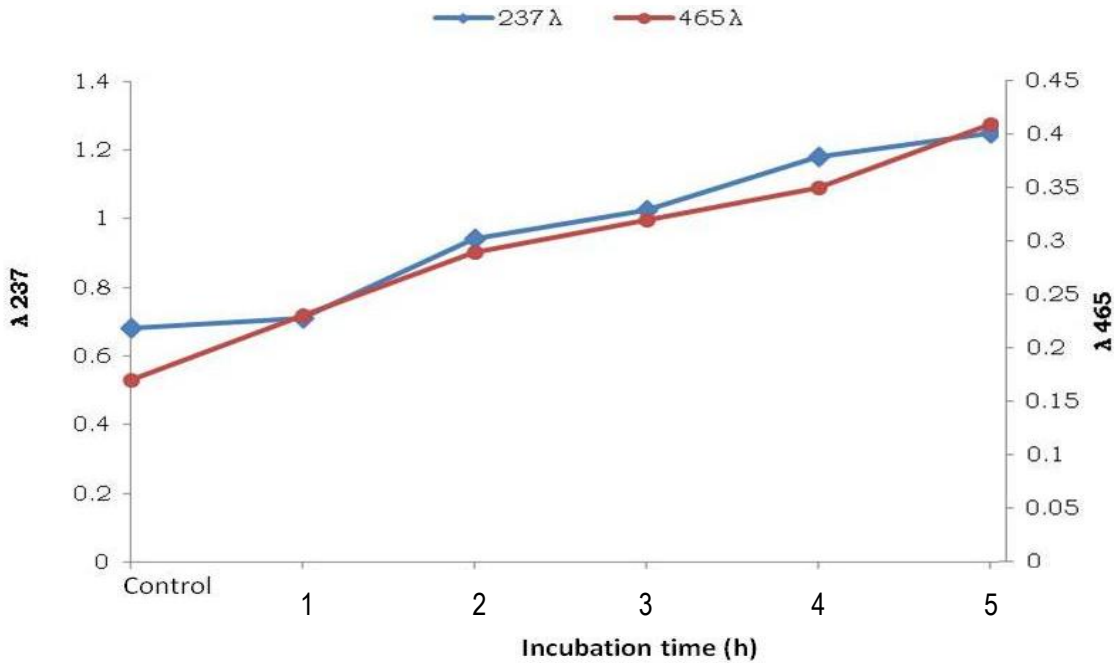

Figure 6. Release of hydrophobic and phenolic compounds after different time intervals with 60 U/g crude xylanase dose at 60°C.

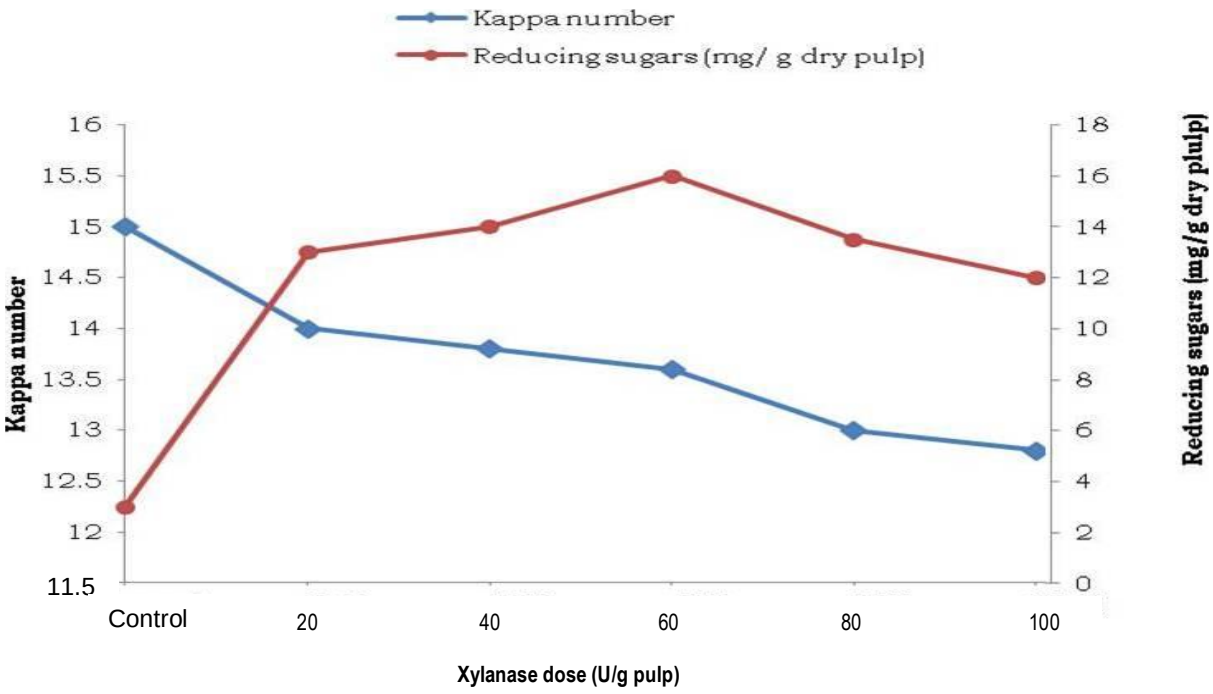

Figure 7. Kappa number and reducing sugars estimation by crude xylanase dose (U/g pulp) for biobleaching of pulp.

Cellulosimicrobium sp. MTCC10645, at 2 U/g (Kamble and Jadhav, 2012).

The correlation between the release of chromophores ($\lambda_{237\ nm}$) and hydrophobic compounds ($\lambda_{465\ nm}$) and the reduction in kappa number coupled to the release of reducing sugars suggested the dissociation of lignin-carbohydrate complex (LCC) from the pulp fibers. The enzyme doses that produces maximum reducing sugar generation is regarded as optimum dose. At biobleaching of kraft pulp, the xylan sandwiched between lignin and

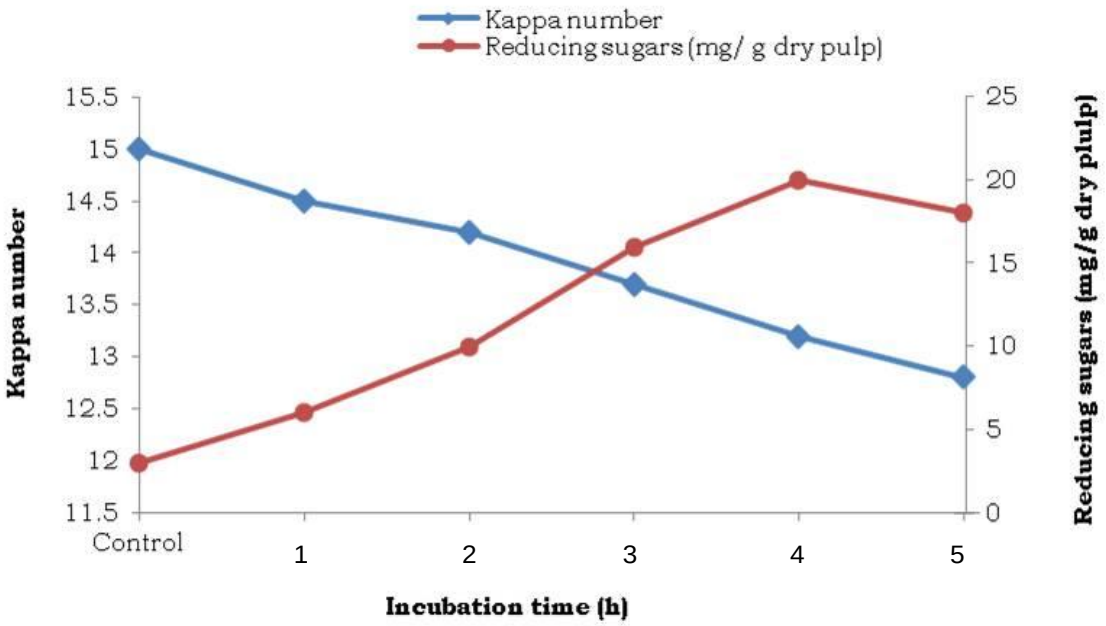

Figure 8. Kappa number and reducing sugars estimation by crude xylanase with 60 U/g pulp at different incubation time for biobleaching of pulp.

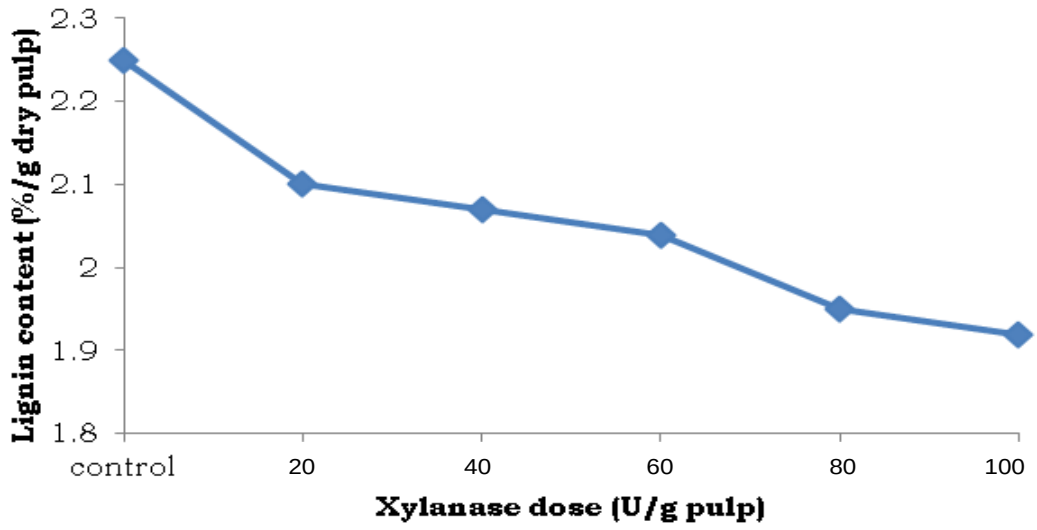

Figure 9. Lignin content of pulp at different enzyme dose.

cellulose layer is degraded by xylanase and releases xylose, reducing sugars and free sugars from the hemicellulose layer that ultimately resulted in an increase in the free sugar content in the pulp sample. It also resulted in the release of lignin and phenolic compounds from the pulp fiber that ultimately leads to the enhancement in absorbance ($\lambda_{237\ nm}$) of pulp free samples compared to the control.

Kappa number and lignin content

Kappa number is the measure of the amount of lignin present in the pulp. The optimum enzyme dose (60 U/g) and incubation time (4 h) reduced the kappa number by ~12%. Lignin content in the pulp was decreased with the enzyme treatment, at the optimized enzyme dose and incubation time; lignin content was decreased by 10% (Figure 9). The reduction in kappa number reported for other xylanase are as follows: *B. plumillus* by 14% (Bim and Franco, 2000), *Staphylococcus* sp. SG-13 by 30% (Gupta et al., 2000), *Bacillus* sp. NCIM59 by 21% (Kulkarni and Rao, 1996), xylanase of three fungi *A. indicus, A. flavus* and *A. niveus* reduced kappa number from 18.60 to 5.0 to 6.8 (Angayarkanni et al., 2006) and

B. subtilis decreased from 70.0 to 56.0 (Saleem and Akhtar, 2002).

Conclusion

The pulp and paper technology is one of the fastest growing industries and the use of thermostable xylanases seems attractive, since they provide global environmental benefits. This study explored the isolation and identification of thermostable, alkaliphilic, cellulase free xylanase producing actinomycetes *L. sacchari* strain B42. Xylanase from this source was active at alkaline pH (9.0) with broad range of thermostability at 60 to 70°C for a period of 6 h. Good thermal stability with minimal cellulase activity of isolated xylanase makes it important in pulp and paper industry; as the biobleaching process needs xylanases that are active at high temperature and pH. The *L. sacchari* B42 xylanase with high temperature and pH optima thereby suggest its potential in biobleaching processes. The biobleaching study also gave satisfactory results with the release of chromophore (hydrophobic and phenolic) compounds and reducing sugars with the reduction in kappa number and lignin content. All of these properties suggested the applicability of *L. sacchari* strain B42 xylanase for biobleaching and this enzyme could fulfill the demand of pulp and paper industry.

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