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Production of Cellulase-Free Xylanase from a Novel Yeast Strain Used for Biobleaching in Paper Industry

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Abstract: An investigation was conducted to explore the microbial xylanases that are thermostable and cellulase-free are generally preferred for biobleaching of paper pulp. A novel yeast strain, *Pichia pinus* isolated from a decaying wood produced up to 545 IU mL^{-1} of xylanolytic enzymes when grown on a medium containing 4% oat spelt xylan. The crude xylanase preparation showed no activity towards cellulolytic substrates but low levels of β-xylosidase (0. 1 IU mL^{-1}) and α-L-arabinofuranoside (0.05 IU mL^{-1}) were detected. The temperature and pH optima for the crude xylanase preparation were 55°C and 4.5, respectively. The crude xylanase produced mainly xylose from xylan within 10 min. Prolonged hydrolysis of xylan produced xylobiose and arabinose, in addition to xylose, as the end products. The culture filtrates of this isolate grown on oat spelt xylan or sugarcane bagasse containing 600 UL^{-1} enzyme could bleach sugarcane bagasse pulp in 60 min treatment at 55°C resulting in a 40% reduction in chlorine consumption and a ten point reduction in kappa number. The potential industrial applications of such cellulase-free xylanase, especially from yeast strains having activity at neutral pH, will be attractive in the paper and pulp industries to improve paper pulp quality as well as to minimize environmental pollution which occurs due to the use of hazardous chemicals by these industries.

Key words: Black liquor, biobleaching, Dharwad, lignin, yeast

INTRODUCTION

Hemicelluloses, the second most common polysaccharides in nature, represent about 20-35% of lignocellulosic biomass. Xylans are the most abundant hemicelluloses (Saha, 2003). Xylan consists of β-1, 4-linked xylose with substituents such as acetyl, arabinosyl and glucuronyl residues (Bastwade *et al*., 1994; Thompson, 1983). Xylan is hydrolyzed by β-xylanases attacking internal xylosidic linkages on the xylan backbone while β-xylosidases release xylose residues by endwise attack on xylooligosaccharides (Wong *et al*., 1988). The paper and pulp industry is a potential source of major pollution, generating large volumes of intensively colored effluent for each metric ton of paper produced. In the production of paper, residual lignin from wood pulp is chemically liberated by using chlorine bleaching. Elemental chlorine reacts with lignin and other organic matter in the pulp, forming chlorinated compounds that are extracted with alkali (Elisa *et al*., 1991; Ganjidoust *et al*., 1997; Thompson *et al*., 2001; Raghukumar *et al*., 2004; Wu *et al*., 2005). Only about 40-45% of the original weight of the wood goes into paper production and therefore, the effluent produces is rich in organic matter (Ali and Sreekrishnan, 2001). The effluents (black liquor) have high Biological Oxygen Demand (BOD) and Chemical Oxygen Demand (COD), chlorinated compounds measured as adsorbable organic

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halides, chlorinated lignin derivatives such as chlorolignols, dioxins and sulfur compounds. Some of these compounds are carcinogenic, toxic and recalcitrant to degradation and are known as persistent organic pollutants (Raghukumar *et al*., 2004).

Enzymes that degrade, or help to degrade, hemicelluloses are of great interest to the paper and pulp industry due to their bleach-boosting properties (biobleaching of pulp), which reduces environmentally unfriendly chlorine consumption. Biobleaching is carried out using white-rot fungi to degrade residual lignin in the pulp by using ligninolytic enzymes such as manganese peroxidase and laccase, or by using hemicellulolytic enzymes such as xylanases (Casimir-Schenkel *et al*., 1995; Malabadi and Raghavendra, 1998). The cellulose fibers are protected by lignin and hemicellulose. The bond between lignin and hemicellulose is primarily between lignin and xylan, which can be removed by xylanase. Once this layer of hemicellulose is removed, the lignin layer is easily available for the degradative action of lignolytic enzymes (Eriksson, 1993). In addition, during chlorine bleaching, the exposed lignin layer thus requires less chlorine to achieve the required brightness (Raghukumar *et al*., 2004; Wu *et al*., 2005).

Several xylan-degrading enzymes have been isolated and characterized from filamentous fungi and bacteria (Bastwade, 1992; Wong *et al*., 1988). Among the yeasts *Crytococcus albidus* (Biely *et al*., 1978) *Aureobasidium pullulans* (Leathers, 1986) and *Rhodotorula mucilginosa* (Lee *et al*., 1987) are known to produce xylan-degrading enzymes. Certain naturally occurring isolates of *Aureobasidium pullulans*, previously described as color variants, overproduce extracellular xylanase with exceptionally high specific activity. Yeasts are quantitatively and economically important group of microorganisms commercially exploited my man (Malabadi, 1994; Malabadi and Raghavendra, 1994, 1995, 1998). Yeasts are widely distributed in nature and very limited information is available on the production of hemicellulolytic enzymes from yeasts, fungi and bacterial cultures liberating both xylose and arabinose (Dekkar, 1985; Boyle *et al*., 1992). The present work describes the isolation of yeast strain producing high levels of hemicellulose-degrading enzymes. Our objective was therefore, to explore the potential use of this yeast strain as a source of xylanases suitable for use in the biobleaching of the paper pulp.

MATERIALS AND METHODS

Isolation, Maintenance and Identification of Yeasts

The yeast strains were isolated from the decaying wood of *Dalbergia sisoo, Bombax malbaricum* (Bombaceae), *Psidium gujava, Spathodea companulata* (Bignoniaceae), *Tectona grandis, Pinus roxburghii*, *Santalum album* (Sandal wood) and *Mangifera indica* (Anacardiaceae) (Table 1). The small pieces of decaying wood were collected in sterilized vials and surface washed with sterile distilled water (Malabadi, 1994; Malabadi and Raghvendra, 1994, 1995). The samples were diluted to 5 mL with sterilized distilled water and one ml of diluted sample was plated by pour plate method on YM agar medium (Wickerham, 1955). YM agar medium contained 3 g L^{-1} yeast extract, 3 g L^{-1} malt extract, 5 g L^{-1} peptone, 10 g L^{-1} glucose, 50 μg mL^{-1} penicillin, 25 μg mL^{-1} chloramphenicol and 20 g L^{-1} agar. The pH of the medium was adjusted to 4.5 with dilute HCl before the medium was autoclaved. The petridishes were incubated at 30°C for 72 h. Cells from yellowish-brown-colored colonies thus obtained were initially examined under the microscope and screened for xylan-degrading activity by growing them on basal medium containing 1% oat spelt xylan (Sigma, St Louis Mo) at 30°C. The basal medium contained 2 g L^{-1} KH_2PO_4, 1.5 g L^{-1} $MgSO_4$. $7H_2O$, 0.132 g L^{-1} $CaCl_2$. $2H_2O$, 1 m g L^{-1} thiamine-HCl, 28 mL of 1.2 mM ammonium tartrate and 18 g L^{-1} of agar. The medium was prepared with distilled water and pH was adjusted to 4.5 with dilute HCl. After 72 h of incubation at 30°C, the clearance zones produced around the yeast colonies in these media identified the isolates as xylanase positive (Haack, 1992; Mackenzie and Williams, 1984). Xylan hydrolysis was visualized using Congo red (Mackenzie and Williams, 1984). The yeast strain was identified using the

methods explained by Lodder (1970) and Barnett *et al*. (1983) using conventional identification keys (morphological, cultural, sexual and physiological characteristics including DBB test) (Malabadi, 1994; Malabadi and Raghvendra, 1994, 1995).

Production of Xylan-Degrading Enzyme

The xylan-clearing cultures were grown at 30°C for up to 7 days in basal medium (pH 4.5) containing 1% oat spelt xylan (Sigma) suspended in liquid YM broth medium (Wickerham, 1955). Production of xylanase in different xylan sources such as breakfast oats (commercial preparation), birchwood xylan (Sigma) and sugar cane bagasse powder (supplied by Dandeli paper mills and Athani sugar mills) suspended in half strength liquid YM medium with some of the isolates. The cultures were filtered through Whatman GF/F filters, the filtrates centrifuged at 10, 000 g for 15 min at 2°C and the clear supernatants used for enzyme assay. All the assays were carried out in triplicate and repeated at least thrice for confirmation. Xylanase from the selected yeast colonies was partially purified and characterized. For this purpose, the culture was grown in the above-mentioned half-strength basal medium with distilled water containing oat spelt xylan (Sigma).

Enzyme Assays

Xylanase activity was assayed using 3, 5-Dinitrosalicyclic Acid (DNSA) to measure the amount of xylose-equivalent reducing sugars liberated from oat spelt xylan (Sigma) solution in 50 mM sodium acetate buffer of pH 4.5 incubated at 30°C for 30 min. Endoglucanase activity was assayed under similar conditions to those described above except that 1% (w/v) carboxymethylcellulose (Sigma) was used as substrate instead of xylan. One unit of activity (U) is defined as the amount of enzyme capable of releasing reducing sugars equivalent to 1 μmol xylose, glucose per minute per ml of crude filtrates from the appropriate substrate under conditions described (Nakamura *et al*., 1993). All the experiments were repeated for three times. Protein concentrations were measured by the method of Lowry *et al*. (1951) using bovine serum albumin as the standard. Cellulase activity was measured according to Ghosh, (1987). β-D-xylosidase and α-arabinofuranoside activities in the crude cultures were assayed at 50°C at pH 4.5 in acetate buffer using p-nitrophenol-β-D-xylopyranoside and p-nitrophenol-α-L-arabinofuranoside respectively. The reaction was terminated by the addition of 2 mL of 2% (w/v) sodium-carbonate solution. The p-nitrophenol liberated was measured at 430 nm (Smith and Wood, 1991). One unit of activity (U) is defined as the amount of enzyme capable of releasing 1 μmol nitro phenol per minute under conditions described.

Effect of Black Liquor on Xylanase Activity

Black liquor (obtained from Local Paper mills, Karnataka, India) at final concentrations of 0.1 and 0.2% was added to an enzyme reaction mixture containing buffer and oat spelt xylan. Xylanase activity was measured after incubation for 30 min as described earlier.

Enzyme Characterization

To study the properties of xylanase, the culture filtrates from the cultures grown in oat spelt xylan medium prepared in half-strength YM medium were used. The organism was grown at different initial pH (3.0-7.0) at 30°C and enzyme production was monitored up to 24 h of incubation. The reaction of DNSA with known amount of xylose at different pH was also carried out to check the sensitivity of the DNSA assay to pH. The effect of temperature of the reaction was assessed by incubating the reaction mixtures (pH 4.5) at different temperatures in the range of 12, 20, 25, 30, 35 and 40°C. Thermostability was monitored by incubating the enzyme at pH 4.5 for the specified period at different temperatures followed by rapid cooling in ice and carrying out the routine assay at 30°C and pH 4.5. Enzyme blanks and substrate blanks were similarly treated at corresponding pH values and temperatures for the same lengths of time and their absorbance's substracted from those of reaction mixtures to arrive at actual enzyme activities.

Enzyme Purification

The crude culture filtrates was brought to 60-80% saturation with ammonium sulphate. The pellet obtained after centrifugation was suspended in minimum volume of 50 mM acetate buffer (pH 4.5) and loaded on a 140 mL volume Sephadex G-100 column at a flow rate of 20 mL h^{-1}. The protein was eluted with acetate buffer and 3 mL fractions were collected. The fractions were monitored at 280 nm for protein and assayed as above for xylanase activity. The active fractions were pooled and concentrated by saturating to 80% with ammonium sulphate. All the above operations were carried out at 4°C in a cold room.

Chromatographic Analysis of Xylan Hydrolysis Products

End product analysis was carried out by hydrolyzing xylan with crude enzyme preparation (100 IU mL^{-1}). The hydrolysis was carried out at 30°C for 5, 10, 30 and 60 min and the hydrolyzed samples were boiled for 10 min to stop the reaction. The end products were identified using ascending paper chromatography with a solvent system of n-butanol: Pyridine: Water (46:36:19 v/v) by the method of Trevelyen *et al*. (1950). After 20 h the chromatic paper was air dried and sprayed with 0.1% (w/v) silver nitrate in aqueous acetone. Subsequently the paper was sprayed with 0.5% (w/v) NaOH in aqueous ethanol to intensify the reducing sugar spots. Finally, the chromatograph was washed with 5% (w/v) sodium thiosulfate followed by thorough washing with distilled water and then air dried.

Gel Electrophoresis

Polyacrylamide gel electrophoresis on native gels (10% separating gels) was carried out in Tris-glycine buffer as described by Sambrook *et al*. (1989) and the gels subjected to silver staining to visualize the protein bands. For *in situ* detection of the enzyme, electrophoresis was carried out at 8°C on native composite gels of 10% polyacrylamide containing 1% xylan. The gels were then incubated in 50 mM acetate buffer (pH 4.5) for 30 min at 30°C. After a further incubation for 30 min (at room temperature) in a 0.1% Congo red solution, the gels were destained in 1 M NaCl until clearance bands of xylanase activity were obtained (Morales *et al*., 1993). We found that the bands were more prominent when the gels were finally transferred into 50 mM acetate buffer (pH 4.5) for 10 min.

Estimation of Molecular Weight

Gel filteration of the protein standards alcohol dehydrogenase (150 kDa), bovine serum albumin (67 kDa), carbonic anhydrase (29 kDa), ribonuclease A (13.7 kDa) and cytochrome C (12.4 kDa) was carried out on a Sephadex G-100 column as described above. The void volume was calculated using blue dextran as marker and a calibration curve prepared.

Pulp Treatment and Determination of Kappa Number

The pulp from sugarcane after extraction of sugar is called sugarcane baggasse and is used alone or mixed with wood chips for making paper. The screened, unbleached pulp from sugarcane baggasse (10% consistency) was incubated with crude culture filtrates at a ratio of 10 g pulp to 100 mL enzyme (containing 58 U xylanase, 0.32 U α-L-arabinofuranosidase and 26 U β-D-xylosidase activity) at 30°C for 1 h. After incubation, 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 kappa number is the volume of 0.1 N potassium permanganate solution consumed by 1 g moisture-free pulp under the conditions described in standard procedure of Technical Association of Paper and Pulp Industries (TAPPI) test method T236-cm-85 (Anonymous, 1988). The kappa number ×0.15 gives the percentage of residual lignin.

RESULTS AND DISCUSSION

In the present study yeasts were successfully isolated from the decaying wood from the environs of Dharwad. The isolation techniques were slightly modified according to the needs with that of Lodder (1970). Techniques adopted were useful in getting the number of yeast isolates from the decaying wood (Table 1). All the isolated yeasts were maintained on YM agar medium (Wickerham, 1955) as mother cultures before subjecting for the identification. All the yeast strains were identified by the methods of Lodder (1970) and Barnett *et al.* (1983) using the conventional identification keys as mentioned in materials and methods (Malabadi, 1994; Malabadi and Raghavendra, 1994, 1995). The identified yeast strains from the corresponding decaying wood were documented in Table 1. Out of eight samples, only five samples yielded yeast strains and were successfully identified as *Candida utilis*, *Torulopsis versatilis*, *Pichia pinus*, *Candida rhagii* and *Kluyeromyces polysporus*. All these identified five yeast strains were tested for the production of xylanase in the liquid culture. On the basis of this preliminary screening, one of the yeast strain *Pichia pinus* with yellowish-brown colonies (Table 2) producing high levels of xylanase was selected for the further studies and the rest of the yeast strains were failed to produce xylanase in the liquid culture.

The yeast isolate *Pichia pinus* when grown at 30°C in half-strength liquid YM medium with a variety of xylan-containing substrates (Table 3) such as 1% oat spelt xylan, breakfast oats (commercial

Table 1: Isolation and identification of yeast strains from the decaying wood

Name of the plant	Source of isolation	Name of identified yeast strain	Xylanase activity
Dalbergia sisoo	Decaying wood	Nill	Nill
Bombax malbaricum (Bombaceae)	Decaying wood	*Candida utilis*	Nill
Psidium gujava	Decaying wood	*Torulopsis versatilis*	Nill
Santalum album (Sandal wood)	Decaying wood	*Pichia pinus*	Very high
Tectona grandis	Decaying wood	*Candida rhagii*	Nill
Pinus roxburghii,	Decaying wood	*Kluyeromyces polysporus*	Very low
Spathodia companulata (Bignoniaceae)	Decaying wood	Nill	Nill
Mangifera indica (Anacardiaceae)	Decaying wood	Nill	Nill

Table 2: Important characteristics of yeast strain showing high xylanase activity isolated from the decaying wood of *Spathodia comanulata* (Bignoniaceae)

Name of the yeast strain	Important characteristics of *Pichia pinus* (Test)	Results
Pichia pinus	1) Nature of cells	1) Cells are spherical and larger measuring (7.32×7.32 μ) colonies are yellowish brown in color, mucoid,flat and shining, Pseudomycelium absent.
	2) Fermentation activity	
	a) Glucose +++	Positive
	b) Mannitol – or +	Negative
	c) Starch ++	Positive
	d) Lactose –	Negative
	3) DBB test	Negative
	4) Growth in vitamin free medium	Negative or weekly positive
	5) Cycloheximide resistance	Absent
	6) Xylanase activity	Very high

Table 3: Effect of substrate on the production of xylanase (UL^{-1})*

Substrate	Yeast isolate	YM medium prepared with distilled water and growth was assayed at pH 4.5
Sugarcane bagasse	*Pichia pinus*	457±35
Commercial breakfast oats	*Pichia pinus*	340±32
Oat spelt xylan	*Pichia pinus*	545±36
Birchwood xylan	*Pichia pinus*	460±27

*Table represents only optimum results. Experiments were repeated for three times and ±SE represent standard error

Table 4: The effect of different xylan concentrations on xylanase production

	12 h*	12 h	24 h*	24 h	48 h*	48 h	72 h*	72 h
Xylan (%)	IU	pH	IU	pH	IU	pH	IU	pH
0.5	25	6.3	180	7.3	230	7.2	280	7.4
1.0	24	5.4	192	6.4	215	6.7	300	7.2
2.0	20	5.3	180	6.5	210	6.4	285	7.1
3.0	16	4.7	148	6.0	180	6.3	268	6.4
4.0	14	4.2	134	5.5	215	6.0	545	6.2
5.0	10	4.0	112	4.9	199	5.6	500	6.0
6.0	7	3.8	75	4.2	168	4.8	480	5.4

*Xylanase activity (UL^{-1}) expressed as µmol xylanase equivalents released per minute

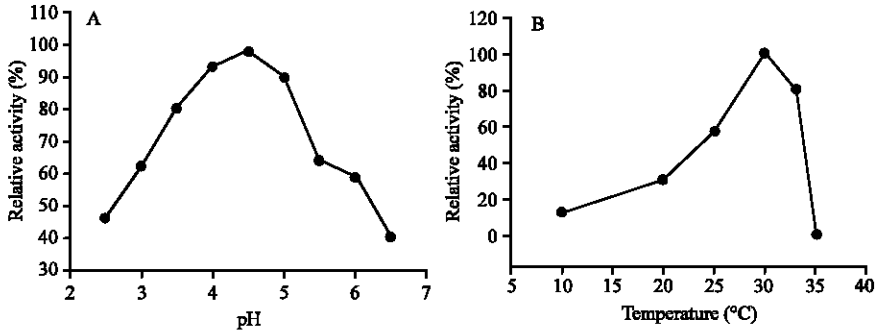

Fig. 1: Effect of pH (A) and temperature (B) on xylanase production by yeast

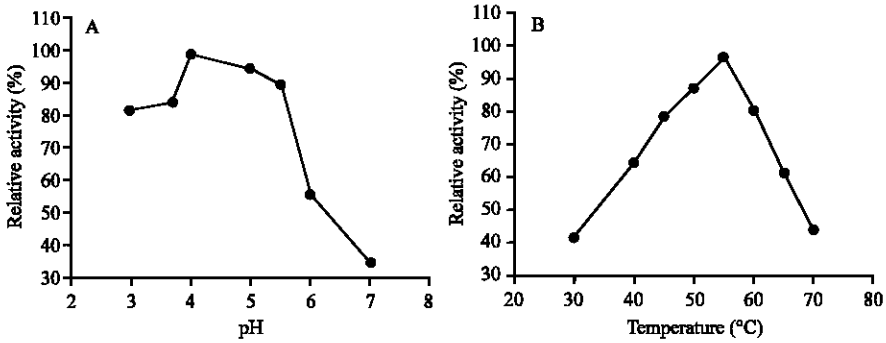

Fig. 2: Effect of pH (A) and temperature (B) on xylanase activity

preparation), birchwood xylan (Sigma) and sugar cane bagasse powder), the culture produced a good amount of (300 I U mL^{-1}) of xylanase but low levels of β-D-xylosidase (0.2 IU mL^{-1}) and α-arabinofuranoside (0.08 IU mL^{-1}). The xylanase preparation did not show any activity towards Carboxymethylcellulose (CMC) (Table 4). The effect of initial pH on xylanase production is shown in Fig. 1A. Growth at pH values above 5.5 decreased xylanase production. Maximum xylanase production was observed at pH values between 3.5 and 5.5 (Fig. 1A). The optimum temperature for xylanase production was 30°C, whereas no enzyme production was observed at 35°C (Fig. 1B). The organism grew well between temperatures 25-30°C but no growth was obtained at 35°C (data not shown). The pH and temperature optima were determined for the crude xylanase preparation. The optimum pH for enzyme activity was 4.5 (Fig. 2A), while the optimum temperature for enzyme activity was 55°C (Fig. 2B). The main breakdown product of xylan hydrolysis was xylose with traces

Table 5: Ammonium sulphate fractionation of culture filtrate and further purification of xylanase from the yeast isolate

Fraction	Total activity (U)	Specific activity (U mg protein^{-1})
Crude culture filtrate	44.5	4.2
0-35%	14.0	1.6
35-50%	2.0	5.0
50-80%	24.6	16.3
80-90%	2.4	5.0
Sephadex G-100 Fraction (Xy I A)	4.6	400.8

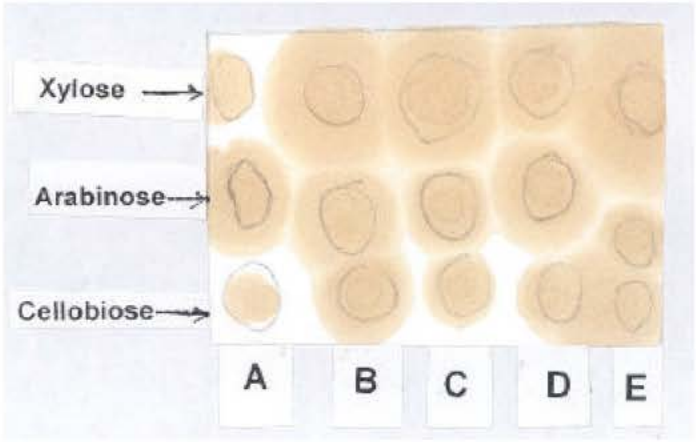

Fig. 3: Paper chromatography of the products of xylan hydrolysis by crude xylanase preparation after different time intervals: A) Standards, B) 5 min, C) 10 min, D) 30 min and E) 60 min

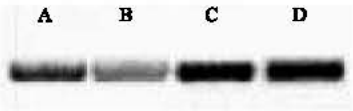

Fig. 4: *In situ* detection of xylanase derived from cultures of yeast *P. pinus* after non-denaturating electrophoresis on a polyacrylamide gel containing oat spelt xylan. Lanes A, B crude extract containing 80 and 165 μg protein, respectively; Lane C, D, 50-80% ammonium sulphate fractions containing 58 and 105 μg protein, respectively. The xylanase activity bands (XyI A) are prominent

of xylobiose within 5 min of hydrolysis (Fig. 3). During further digestion of xylan up to 60 min, increases in xylose and arabinose were observed by the paper chromatography confirming the xylanase to be a exoxylanase type. The presence of xylose also indicated β-xylosidase activity. Ammonium sulphate fractionation of the culture filtrate yielded maximum xylanase activity at 50-80% saturation (Table 5). The bands of xylanase activity in the crude (lanes A and B) and 50-80% ammonium sulphate fraction (lanes C and D) were seen on a polyacrylamide gel containing 1% oat spelt xylan (Fig. 4). The elution profile of the 50-80% ammonium sulphate fraction subjected to column-chromatography on a Sephadex G-100 matrix showed one distinct protein peak, which had xylanase activity. This active fraction in peak was pooled, concentrated by saturation them on 80% with ammonium sulphate and designated as XyIA. This fraction showed specific activities of 400.8 U mg^{-1} protein (Table 5). The xylanase-active peak on Sephadex G-25 matrix corresponded to molecular weights of 16 KDa. These results indicate successful separation of distinct xylanase.

The crude culture filtrate of the yeast isolate was tested for its biobleaching potential. The culture filtrates of this isolate grown on oat spelt xylan or sugarcane bagasse containing 600 UL^{-1} enzyme

could bleach sugarcane bagasse pulp in 60 min treatment at 55°C resulting in a 40% reduction in chlorine consumption and a ten point reduction in kappa number (trials conducted independently by paper mills). The unbleached baggasse showed a kappa number of 52 and the enzyme-treated pulp showed a kappa number of 36. The drop in kappa number after biobleaching indicates reduction in chlorine consumption during further bleaching of the pulp. The stability of the enzyme for nearly 4 h at 55°C suggests that a crude enzyme solution could be used directly for bleaching of cooked paper pulp without requiring any substantial decrease in the temperature of the pulp. About 50 U xylanase from *Thermononospora fusca* reduced the kappa number of softwood Kraft pulp from 18.0 to 13.0 within 2 h at 70°C. In our present study a crude culture filtrates having a xylan-degrading enzyme system (devoid of cellulase activity) that could be used in the biobleaching process would eliminate steps involved in purification of the enzymes, bringing down the costs involved in their production, thus increasing economic viability (Raghukumar *et al*., 2004).

Xylanases are receiving much attention due to their applications in industry as well as in various fields of biochemistry. Xylanases from fungi have been well documented and intensively studied. Among yeasts, *Trichosporon* (Stevens and Payne, 1977), *Cryptococcus albidus* (Biely *et al*., 1980) and *Cryptococcus flavus* (Yasui *et al*., 1984) produce low levels of xylanase. A naturally occurring color variant of *Aureobasidium pullulans* was reported to produce very high levels of xylanase (Leathers, 1986, 1989). The results of this paper reveal that the newly isolated yeast strain from the decaying wood produced xylanase in high quantities (465-545 IU mL^{-1}) when it was grown on spelt oat xylan. This level of xylanase activity is quite high compared to those of other yeast strains including the color variant of *Aureobasidium pullulans* (Leathers, 1986, 1989). High specific activity has also been reported for a xylanase from *Aureobasidium pullulans*. Arnaud, which produces low molecular weight extracellular xylanase with a specific activity of 2, 000 U mg^{-1} protein (Leathers, 1986). Essentially no growth or xylanase production by newly isolated yeast strain was observed on cellulose or caboxymethylcellulose, indicating the absence of cellulases in this yeast strain. Similar observations were reported for *Aureobasidium pullulans* (Leathers, 1986, 1989). The potential industrial applications of such cellulase-free xylanase, especially from yeast strains having activity at neutral pH, will be attractive in the paper and pulp industries to improve paper pulp quality as well as to minimize environmental pollution which occurs due to the use of hazardous chemicals by these industries (Sumathi and Phatak, 1999; Elisa *et al*., 1991).

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