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High level expression of a recombinant xylanase by *Pichia pastoris* NC38 in a 5 L fermenter and its efficiency in biobleaching of bagasse pulp

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ABSTRACT

A genetically modified XynA gene from *Thermomyces lanuginosus* was expressed in *Pichia pastoris* under the control of GAP promoter. *P. pastoris* expressed greater levels of xylanase (160 IU ml^{-1}) on BMGY medium without zeocin after 56 h. The xylanase production by recombinant *P. pastoris* was scaled up in a 5 L fermenter containing 1% glycerol and the highest xylanase production of 139 IU ml^{-1} was observed after 72 h. Further studies carried out in fermenter under controlled pH (5.5) yielded a maximum xylanase production of 177 IU ml^{-1} after 72 h. The biobleaching efficacy of crude xylanase was also evaluated on bagasse pulp and a brightness of 47.4% was observed with 50 IU of crude xylanase used per gram of pulp, which was 2.1 points higher in brightness than the untreated samples. Reducing sugars (24.8 mg g^{-1}) and UV absorbing lignin-derived compounds values were considerably higher with xylanase treated samples.

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1. Introduction

An increasing awareness on environmental pollution has enforced the pulp and paper industries to strive for an alternate greener technology which will replace the use of harsh chemicals in their processes with microbial enzymes. The application of biocatalysts not only makes the process less toxic but also decreases costs associated with the production and consumption of resources (water, electricity, fuels). Pulp bleaching with microbial xylanases has already been demonstrated as an environmentally friendly technology for the pulp and paper industries (Shirkolaee et al., 2008). Xylanases aid in enhancing the brightness of pulp, diminish impurities, and retention period of pulp (Viikari et al., 1994; Bajpai, 1999; Singh et al., 2003). Cellulase-free, alkali and thermo-stable microbial xylanases are mostly ideal for biopulping and bleaching processes.

Xylanases are produced by numerous microorganisms, *viz.*, bacteria, actinomycetes and fungi. Xylanases hydrolyse xylan, which is one of the major components of hemicellulose found in the plant cell wall (Jeffries, 1996). Xylan is considered to be the second most abundant polysaccharide in nature; it is composed of a linear backbone of 1,4-β-linked D-xylose units and often contains side chains of other sugar residues such as arabinose and glucuronic acid (Biely, 1985). Due to its heterogeneity and complexity, the complete hydrolysis of xylan requires a repertoire of hydrolytic enzymes: β-1,4-endoxylanase, β-xylosidase, α-L-arabinofuranosidase, α-glucuronidase, acetyl xylan esterase, ferulic and ρ-coumaric acid esterases. All these enzymes act co-operatively to convert xylan into its constituent sugars (Collins et al., 2005).

Potential application of xylanase in pulp and paper industries has urged considerable research efforts towards producing more thermophilic and alkalophilic xylanases by screening for naturally-occurring xylanases. Alternatively, xylanases from extremophiles have been genetically modified for enhanced stability at higher temperature and pH conditions (Stephens et al., 2009). We previously reported on the expression of an alkalo-tolerant fungal recombinant xylanase (able to retain 84% activity at pH 10 for 90 min at 60 °C) developed by directed evolution in *Pichia pastoris* GS511 and *Escherichia coli* BL21. *P. pastoris* exhibited 545-fold higher levels of recombinant xylanase expression (extracellular) than the levels observed intracellularly with *E. coli* (Mchunu et al., 2009). However, the usage of multifaceted undefined medium and addition of antibiotics for the growth and xylanase production by *P. pastoris* will not be economically viable for any industrial applications. Thus the present study was focused on optimizing the growth conditions of *P. pastoris* using buffered medium containing zeocin or devoid of zeocin for obtaining the maximum expression of recombinant xylanase in shake flasks and then

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scaling up further in a lab scale fermentor. The biobleaching efficiency of crude recombinant xylanase was also assessed on bagasse pulp. To the best of our knowledge, this is the first report on biobleaching of bagasse pulp using a recombinant xylanase expressed in *P. pastoris*.

2. Methods

2.1. Strain maintenance

P. pastoris NC38 was used in this study (Mchunu et al., 2009). Yeast cultures were maintained on YPD medium (g l^{-1}: 10 yeast extract, 20 peptone and 20 dextrose) containing zeocin (100 μg ml^{-1}) for plasmid maintenance.

2.2. Growth and recombinant xylanase production by P. pastoris on buffered minimal glycerol yeast (BMGY) medium incorporated with or without zeocin

Two hundred millilitres of BMGY containing glycerol (1%), yeast nitrogen base with ammonium sulphate (13.4%), biotin (0.02%), 1 M potassium phosphate buffer (20 ml, pH 6.0) and zeocin (100 μg ml^{-1}) was dispensed into 500 ml Erlenmeyer flasks. An additional set of experiment was conducted using BMGY medium deprived of zeocin. A 10% (v/v) of 16-h-old *P. pastoris* cultures grown on BMGY medium was used as inoculum. Culture flasks were incubated at 30 °C with shaking at 200 rpm for 72 h. Five millilitres of sample was withdrawn every 8 h to determine the optical density (600 nm) and pH. For xylanase activity, the culture broths were withdrawn every 8 h and centrifuged at 10,000*g* for 10 min at 4 °C and the supernatant was used for the assay.

2.3. Effect of glycerol concentration on the growth and xylanase production by P. pastoris

Two hundred millilitres of BMGY medium devoid of carbon sources was dispensed into 500 ml Erlenmeyer flasks. Glycerol concentrations of 0.1%, 1%, 2.5% or 5% were added. A 10% (v/v) of 16-h-old *P. pastoris* culture grown on the BMGY medium was used as an inoculum. Culture flasks were incubated at 30 °C with shaking at 200 rpm for 72 h. Samples (5 ml) were withdrawn every 8 h to determine optical density (600 nm), pH and xylanase activity.

2.4. Laboratory scale production of recombinant xylanase by P. pastoris

This study was carried out in a 5-L glass fermenter (Minifors, Infors HT, Switzerland) with a working volume of 3 L BMGY medium containing 1% glycerol as a carbon and 13.4% yeast nitrogen base with ammonium sulphate as a nitrogen source. A seed culture of *P. pastoris* (16-h-old) was prepared in BMGY broth and inoculated at 10% (v/v). The agitation, aeration rate and temperature were maintained at 200 rpm, 1.5 vvm (gas volume per liquid reactor volume per minute) using the cascade mode and 30 °C, respectively, for 72 h. Using the similar growth conditions, a separate batch run was carried out by maintaining the pH at 5.5. Fifty millilitre samples were withdrawn through the sampling port and centrifuged (10,000*g* for 10 min) and the clear supernatant was used to determine the xylanase activity.

2.5. Determination of β-xylanase activity

The xylanase activity was determined according to the method described by Bailey et al. (1992). The reaction mixture consisted of 1.8 ml of 1% birchwood xylan (Roth, Karlsruhe, Germany) solution in sodium citrate buffer (50 mM, pH 6.5) and 0.2 ml of appropriately diluted enzyme. After 5 min incubation at 50 °C, the liberated reducing sugars were determined as xylose equivalents. One unit (IU) of enzyme activity was defined as the amount of enzyme that released 1 μmol reducing sugars (xylose) per minute.

2.6. Biobleaching of bagasse pulp with a recombinant xylanase

Unbleached sugarcane bagasse pulp was obtained from Sappi Fine Paper, Stanger, South Africa. The pulp was carefully washed using tap water to remove the fine and water soluble contaminants. For biobleaching, bagasse pulp sample (10 g) was pre-treated with 500 IU of crude xylanase obtained from *P. pastoris* for 3 h at 10% pulp consistency (Manimaran et al., 2009). The reaction was terminated by heating the enzyme pre-treated pulp samples at 100 °C for 10 min. Ten millilitres of biobleached test and control samples were centrifuged at 10,000*g* for 15 min and the supernatant was analysed for reducing sugars (Bailey et al., 1992). Lignin-derived compounds in the supernatant were also observed by measuring the absorption spectrum between 200 and 465 nm using UV–Vis Spectrophotometer (Varian Cary 100, USA) against the control supernatant from sample pre-treated with denatured enzyme. The pulp was subjected to standard handsheet making processes and the final paper product was analysed (TAPPI, 1996). Biobleaching efficiency of crude xylanase (50 IU g^{-1}pulp) on bagasse pulp was also examined at various temperatures (30–70 °C) and pH (5–9) conditions for 3 h.

2.7. Analytical methods

Standard paper sheets were made from enzyme pre-treated and untreated pulps using the handsheet former (PTI Lab equipment, Austria) and air dried according to TAPPI methods (TAPPI, 1996). The brightness and opacity of the final paper product was measured using a reflectance meter (Technidyne, USA). The ceramic reference (TAPPI) with a brightness of 47.5% was used as a standard. The paper was also tested for tensile index, burst factor and tear index. For scanning electron microscopy, xylanase pre-treated and control pulp samples were mounted on stubs coated with gold palladium and examined under a scanning electron microscope (Philips, USA) at 10 kV.

3. Results and discussion

3.1. Xylanase production in shake flask cultures

Xylanases are secreted by a number of microorganisms and their productivity diverges significantly with respect to cultivation conditions (Collins et al., 2005). In this study, the thermo and alkalo-tolerant recombinant fungal xylanase was expressed in *P. pastoris* and its growth conditions were optimized for maximum xylanase production. *P. pastoris* expressed a high level of xylanase (165 IU ml^{-1}) on BMGY medium incorporated with zeocin after 56 h and there was no considerable improvement in xylanase production after 48 h. Comparatively, the xylanase production level was slightly lower (160 IU ml^{-1}) in the yeast cells grown on BMGY medium devoid of zeocin. Although, there was a negligible difference in enzyme production levels, the xylanase expression trend was comparable on both the media.

The growth pattern of yeast cells was relatively similar between the cells grown on these two media. The cell OD was steadily increased and maximum growth was observed after 48 h in both the yeast cultures. The shake flask experiments indicated that there was no difference in yeast cell growth pattern. However, a marginal diminution in xylanase expression level was noticed in

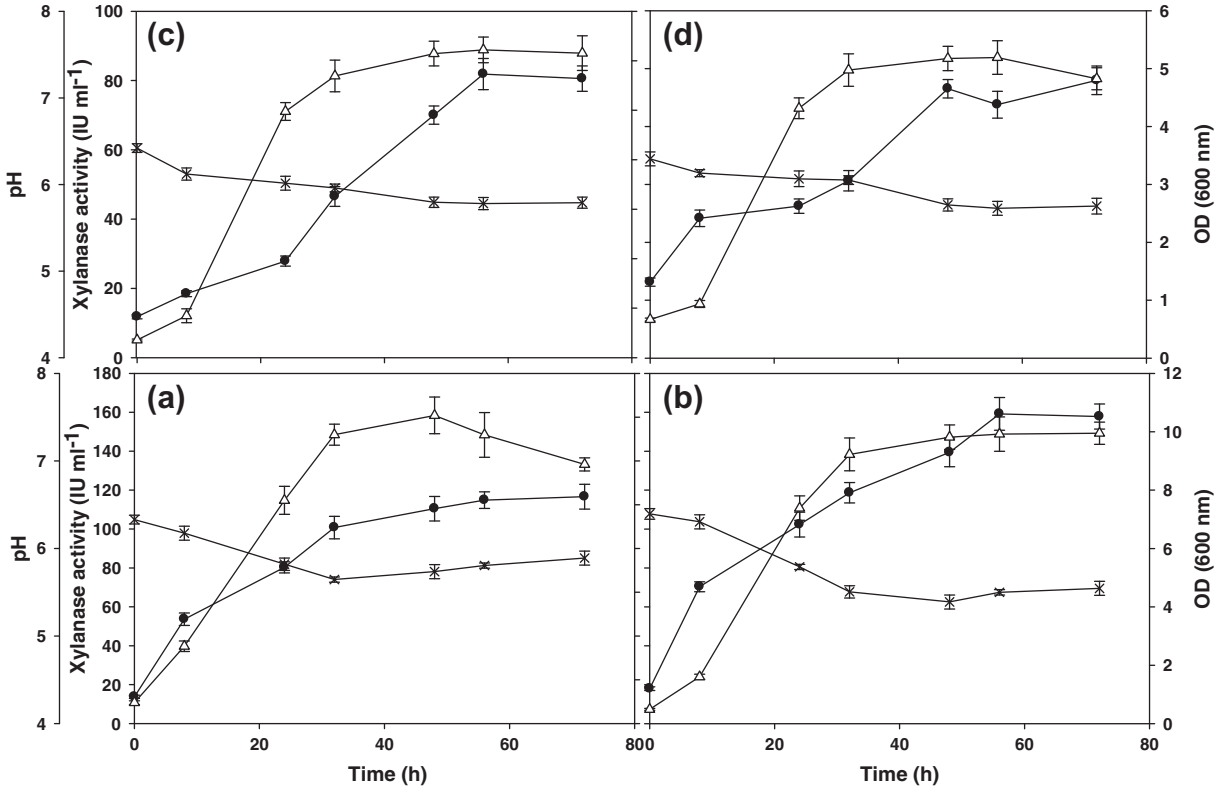

Fig. 1. Effect of glycerol concentration on growth (△), medium pH (×) and recombinant xylanase (●) production by *P. pastoris*: (a) 0.1%, (b) 1%, (c) 2.5%, (d) 5%. Each value represents the mean of triplicate determinations ± standard deviation.

P. pastoris grown on medium lacking zeocin. Owing to the exorbitant cost of zeocin, its use in the culture medium is not economically viable for large scale xylanase production. Therefore, further studies on *P. pastoris* were conducted using the medium devoid of zeocin.

E. coli has been widely used for the expression of a number of recombinant proteins. However, proteins are expressed at relatively low levels (Potvin et al., 2010). On the other hand, *P. pastoris* has been successfully utilized for the overexpression of recombinant proteins with greater success (Li et al., 2010; Fan et al., 2011). Moreover, *P. pastoris* being a eukaryotic organism has an efficient growth rate at high cell densities in economical culture media, and the recombinant protein produced by this strain is secreted to the culture medium, facilitating the protein recovery process (Cereghino and Cregg, 2000). We have also reported that the recombinant xylanase expression in *P. pastoris* was significantly higher than *E. coli* due to the protein being secreted extracellularly while in *E. coli,* the enzyme was cell bound and the need for further purification steps decreased the overall protein yield (Mchunu et al., 2009). *P. pastoris* provides a safe and convenient system for competitive and reproducible processes for producing industrially important bio-products at laboratory as well as industrial scale (Hollenberg and Gellissen, 1997; Cereghino et al., 2002).

3.2. Effect of glycerol concentration on growth and recombinant xylanase production by P. pastoris

Glyceraldehyde 3-phosphate dehydrogenase constitutive promoter (pGAP) has been widely used to express numerous heterologous proteins without any process control strategies and safety concerns related with methanol induced promoter (Potvin et al., 2010). Generally, glucose or glycerol is used as carbon source for pGAP directed expression of recombinant genes in *P. pastoris*. Glucose is commonly used in shake flask cultures whereas glycerol is used for large scale recombinant protein production (Zhang et al., 2009).

The effect of varying concentrations of glycerol on growth and recombinant xylanase production by *P. pastoris* was studied by growing the yeast cells on BMGY medium containing different concentrations of glycerol. The highest xylanase activity of 159 IU ml^{-1} with a cell OD of 9.9 was noticed at 1% glycerol after 72 h, whereas, the xylanase activity dropped to 117 IU ml^{-1} in cells grown on medium containing lower levels (0.1%) of glycerol (Fig. 1). The optimal cell growth was also observed on BMGY incorporated with 1% glycerol. Xylanase expression by *P. pastoris* is associated with cellular growth. In contrast to AOX promoter, the biomass and protein synthesis occur at the same time in GAP systems (Vassileva et al., 2001).

Higher concentrations of glycerol inhibited the yeast cell growth and also xylanase production (Fig. 1). In contrast, Chang and Ching (2008) have reported that xylanase production by *P. pastoris* was found to be higher (5400 IU ml^{-1}) on BMGY supplemented with 4% glycerol after 10 days through three stages of fermentation, viz., glycerol batch, glycerol fed batch and methanol induction. An increased concentration of glycerol tends to inhibit the production of heterologous proteins in *P. pastoris* (Chen et al., 1996). Similarly, Ghosalkar et al. (2008) have reported that a chemically defined medium should contain 20 g l^{-1} of glycerol for an efficient biomass and protein synthesis in *P. pastoris*. Several heterologous proteins have effectively been expressed in pGAP based *P. pastoris* and the expression levels varied extensively depending on the properties of proteins (Hollenberg and Gellissen, 1997; Damaso et al., 2003; Delroisse et al., 2005).

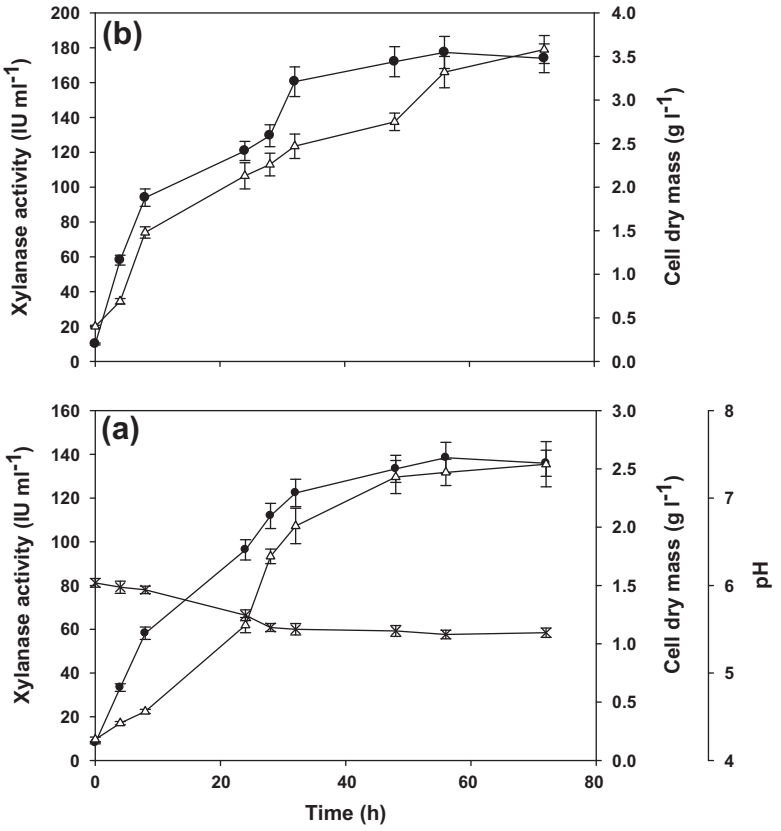

Fig. 2. Laboratory-scale production of recombinant xylanase by *P. pastoris* in a 5 L fermenter. The cultures were grown on BMGY at 30 °C for 72 h. (a) With controlled pH, (b) uncontrolled pH. Agitation and aeration rates were maintained at 200 rpm and 1.5 vvm, respectively, with the evaluation of the following parameters: xylanase activity (△) and pH (×) and biomass (●). Each value represents the mean of triplicate determinations.

3.3. Laboratory-scale production of recombinant xylanase by P. pastoris in a 5-L fermenter

The main objective of this study was scaling up the recombinant xylanase production by *P. pastoris* in a lab scale fermenter using the optimized BMGY medium containing 1% glycerol. The xylanase production was increased linearly up to 56 h and did not show further increase thereafter (Fig. 2a). Highest xylanase production of 139 IU ml^{-1} was observed after 56 h. However, the xylanase production level in a fermenter was 13% lower than shake flask cultures. The pH of the culture medium reached 5.5 at the end of yeast cell cultivation (Fig. 2a). Similar observations were also made in shake flask experiments.

Thermophilic fungi have been reported as a versatile source for hyper xylanase production. The highest xylanase production of 2009 IU ml^{-1} in a 5 L fermenter was observed in case of *Thermomyces lanuginosus* MC 134 mutant after 6 days (Santhosh Kumar et al., 2009). Xylanase production by filamentous fungi in a bioreactor does not succeed in the presence of solid materials which burden the agitation, lowers O_2 availability and absorbs the produced enzyme. Furthermore the enzyme synthesis depends on the hydrolysis rate of xylan substrates and typically takes several days to attain the peak enzyme production. In the case of *P. pastoris*, a substantial production of xylanase could be achieved within 72 h.

Maximum xylanase activity of 177 IU ml^{-1} was observed when *P. pastoris* was grown on BMGY under controlled pH conditions (Fig. 2b). The xylanase production level at constant pH (5.5) was higher than uncontrolled pH conditions (Fig. 2). Similarly, cell biomass was also increased to 1.2 g l^{-1} after 56 h (Fig. 2b). Xylanase production by several recombinant *Pichia* strains has been reported. Cheng et al. (2005) have reported the xylanase activity of 342 IU ml^{-1} in *P. pastoris* (expressed with *Thermobifida fusca* xylanase under the control of AOX promoter) after 144 h through methanol induction. Xylanase production of 3476 IU ml^{-1} was observed in *P. pastoris* under the control of AOX1 promoter using the high cell density cultivation at 3% (v/v) methanol induction (Ruanglek et al., 2007).

Recombinant protein production under pGAP is affected by culture conditions, notably pH and temperature. The optimum conditions vary depending on the products. Zhao et al. (2008) have reported that the recombinant lipase production by *P. pastoris* was optimal at pH 6.5 (26 °C) whereas, the optimal hGM-CSF expression in *P. pastoris* was observed at pH 5 and 30 °C (Pal et al., 2006). Based on the reports, the optimal operating pH for recombinant protein production in *P. pastoris* ranged from 5.5 to 8, which helps in minimizing the pH dependent proteolytic activity and also maintains the recombinant protein stability (Idiris et al., 2010).

E. coli has been considered for a long time as the vector of choice for the production of foreign proteins, but the drawbacks are that these proteins are secreted intracellularly. In comparison to *E. coli*, *P. pastoris* (a methylotrophic yeast) is able to express foreign proteins extracellularly and is capable of performing post-translational modifications, such as glycosylation, and requires an inexpensive and undefined medium for growth.

One of the major factors determining the large scale utilization of xylanases is the cost involved in xylanolytic enzyme preparations. Major development has been made in the last decade to identify the process parameters which have a vital role in the production of high enzyme titre and that consequently impact the

Table 1
Lignin derived compounds and reducing sugars released from bagasse pulp treated with xylanase (50 U g^{-1} pulp) in 50 mM sodium citrate buffer (pH 5–6), sodium phosphate buffer (pH 7–8) and glycine–NaOH buffer (pH 9) at 50 °C for 3 h.

pH	Lignin derived compounds			Reducing sugar released (mg g^{-1} pulp)
	Ab_{237}	Ab_{280}	Ab_{465}	
5	1.48 ± 0.01	2.037 ± 0.01	0.012 ± 0	25.2 ± 0.15
6	1.49 ± 0.02	1.909 ± 0.04	0.047 ± 0	25.1 ± 0.20
7	2.219 ± 0.05	2.132 ± 0.03	0.138 ± 0	25.4 ± 0.17
8	2.029 ± 0.07	1.969 ± 0.03	0.099 ± 0	24.6 ± 0.15
9	2.230 ± 0.02	2.275 ± 0.01	0.080 ± 0	23.2 ± 0.15

Ab: Absorbance at wavelength (nm).

Table 2
Lignin derived compounds and reducing sugars released from bagasse pulp treated with xylanase (50 U g^{-1} pulp) in 50 mM sodium citrate buffer (pH 8) at 37 °C, 60 °C, 70 °C or 80 °C for 3 h.

Temperature	Lignin derived compounds			Reducing sugar released (mg g^{-1} pulp)
	Ab_{237}	Ab_{280}	Ab_{465}	
37	2.86 ± 0.01	2.61 ± 0.01	0.27 ± 0	22.3 ± 0.15
50	2.23 ± 0.05	2.28 ± 0.02	0.31 ± 0	24.8 ± 0.20
60	1.83 ± 0.02	2.29 ± 0.04	0.15 ± 0	23.8 ± 0.15
70	1.57 ± 0.05	1.95 ± 0.03	0.11 ± 0	23.5 ± 0.20
80	1.45 ± 0.07	2.07 ± 0.03	0.13 ± 0	23.4 ± 0.15

Ab: Absorbance at wavelength (nm).

process economics. The cost of the carbon source as well as additional nutrients in the production medium plays a critical role in the process commercialization. Pure xylan has been frequently used in laboratory scale studies on xylanase production by filamentous fungi, but for use on a larger scale it is not economically feasible. On the other hand, *P. pastoris* can be cultivated on non-xylan carbon sources and the produced enzyme is pure enough for any bioprocesses/industrial applications. Moreover, there was no reduction in the enzyme production by *P. pastoris* observed while scaling up the xylanase production as compared to 40% reduction in the filamentous fungi (Santhosh Kumar et al., 2009).

3.4. Bio-bleaching of bagasse pulp using a recombinant xylanase expressed in P. pastoris

Kraft pulping process using wood chips is not attractive with regard to economic and environmental assessments (Khristova et al., 2006; Shirkolaee et al., 2008). Henceforth, the pulp and paper industries have already started to utilize plant secondary fibres and non-woody pulps as alternate materials. Sugar cane bagasse is one of the most important non-woody components for paper production in developing countries. In recent years, attention has been paid for effective utilization of bagasse in pulp and paper industries owing to its high yield and superior mechanical properties.

Biobleaching efficacy of the recombinant xylanase on bagasse pulp was evaluated at different reaction temperatures and pH. Of the various pH ranges tested, a maximum brightness of 48.6 ± 0.07%, 2.9 points higher than control (untreated) sample was observed at pH 5. Reducing sugars and lignin derived compounds released from pulp further substantiated the brightness increase in enzymatic pre-treated pulp samples (Tables 1 and 3). However, the recombinant xylanase expressed in *P. pastoris* was stable at alkaline pH and furthermore there was only a slight difference in the pulp brightness at pH 8 as compared to pH 5, therefore biobleaching studies at different temperatures were conducted at pH 8.

Among the biobleaching experiments carried out at various temperatures, maximum release of lignin derived compounds, reducing sugars and brightness (47.4 ± 0.04%) was observed at 50 °C (Tables 2 and 4). However, the brightness achieved at high temperatures was comparable with pulp bleaching performed at 50 °C. The biobleaching results indicate that the recombinant xylanase remained active at high pH and temperature and these attributes are highly preferred in enzymatic pulp bleaching industries. Data on lignin derived compounds and reducing sugar release from bagasse pulp further substantiated that the enzyme remained active at high temperature and alkaline conditions.

The crude recombinant xylanase pre-treated bagasse pulp under optimized conditions showed 2.1 points increase in the brightness and 1.2 points decrease in kappa numbers as compared to control (untreated) pulp samples. Several studies have focused on pulp bleaching with different bacterial and fungal xylanases. To the best of our knowledge, this is the first report on pulp bleaching with a recombinant xylanase produced by *P. pastoris*.

However, insignificant difference in tensile strength, breaking length and burst factor of the paper product are promising (Tables 3 and 4). SEM results showed that bagasse pulp pre-treated with recombinant xylanase affected the fibre morphology (Supplementary Fig. S1) and the pulp became uneven with the enlargement of extended peeled fibres on the surface, whereas, control bagasse pulp fibres were smooth with uniform surface. These results indicated that enzyme did not alter the quality of the pulp but aided in brightness enhancement. Similar observations on xylanase pre-treated bagasse pulp have been reported (Bissoon et al., 2002; Manimaran et al., 2009; Santhosh Kumar et al., 2009).

The biochemical properties of several commercial hemicellulases are not disclosed by the companies and therefore it is difficult to employ these enzymes into the existing pulp bleaching process. Industries require enzymes that should function efficiently on pulp materials without affecting the quality of cellulosic fibres. Recombinant xylanase used for biobleaching of bagasse pulp is cellulase-free (which can act only on xylan deposited on lignocellulosic fibres) and therefore it did not affect the quality of the final product.

Table 3
Properties of paper made after bagasse pulp treated with xylanase (50 U g^{-1} pulp) in 50 mM sodium citrate buffer (pH 5–6), sodium phosphate buffer (pH 7–8) and glycine–NaOH buffer (pH 9) at 50 °C for 3 h.

	Control	pH 5	pH 6	pH 7	pH 8	pH 9
Brightness (% ISO)	45.7 ± 0.06	48.6 ± 0.07	47.4 ± 0.05	47.6 ± 0.06	47.3 ± 0.05	45.3 ± 0.07
Kappa number	10.5 ± 0.10	9.3 ± 0.10	9.1 ± 0.20	9.3 ± 0.10	9.2 ± 0.10	9.9 ± 0.10
Opacity	95.8 ± 0.20	93.4 ± 0.10	93.0 ± 0.2	93.9 ± 0.10	93.3 ± 0.20	92.9 ± 0.10
Tensile strength (N/15 mm)	16.4 ± 0.09	15.9 ± 0.08	12.1 ± 0.05	14.1 ± 0.06	13.6 ± 0.08	15.9 ± 0.07
TEA (J/M^2)	10.2 ± 0.07	8.9 ± 0.07	8.6 ± 0.14	7.8 ± 0.15	8.2 ± 0.09	7.6 ± 0.15
Break	2.1 ± 0.05	1.7 ± 0.04	1.6 ± 0.04	1.7 ± 0.02	1.7 ± 0.05	1.8 ± 0.04
Stretch (%)	2.1 ± 0.07	1.7 ± 0.03	1.6 ± 0.06	1.8 ± 0.04	1.6 ± 0.03	1.9 ± 0.07
CD-residual	3.01 ± 0.02	2.20 ± 0.02	2.45 ± 0.04	2.85 ± 0.02	2.43 ± 0.05	2.84 ± 0.02
Burst factor ($kPa/m^2/g$)	201 ± 4	191 ± 4	194 ± 5	190 ± 4	189 ± 5	192 ± 4

Table 4
Properties of paper made after bagasse pulp treated with xylanase (50 U g^{-1}) in 50 mM sodium phosphate buffer (pH 8) at 37 °C, 60 °C, 70 °C or 80 °C for 3 h.

	Control	37 °C	50 °C	60 °C	70 °C	80 °C
Brightness (% ISO)	45.7 ± 0.07	46.1 ± 0.05	47.4 ± 0.04	46.8 ± 0.05	46.6 ± 0.08	47.0 ± 0.05
Kappa number	10.7 ± 0.10	9.2 ± 0.10	9.5 ± 0.10	9.3 ± 0.10	9.2 ± 0.10	9.4 ± 0.10
Opacity	95.8 ± 0.10	94.5 ± 0.20	93.2 ± 0.20	93.3 ± 0.20	92.6 ± 0.10	93.0 ± 0.10
Tensile strength (N/15 mm)	16.6 ± 0.07	16.3 ± 0.06	14.6 ± 0.07	14.5 ± 0.06	14.0 ± 0.06	13.9 ± 0.05
TEA (J/M^2)	10.3 ± 0.09	10.0 ± 0.08	9.8 ± 0.09	9.5 ± 0.12	9.2 ± 0.08	8.9 ± 0.15
Break	2.1 ± 0.04	1.7 ± 0.04	1.7 ± 0.05	1.8 ± 0.04	2.1 ± 0.04	2.1 ± 0.05
Stretch (%)	2.2 ± 0.06	1.6 ± 0.02	1.5 ± 0.04	1.7 ± 0.02	2.1 ± 0.01	1.8 ± 0.06
CD-residual	3.0 ± 0.05	2.6 ± 0.01	2.7 ± 0.03	3.0 ± 0.01	3.2 ± 0.03	3.1 ± 0.02
Burst factor ($kPa/m^2/g$)	206 ± 4	193 ± 4	195 ± 5	191 ± 4	190 ± 5	194 ± 4

The brightness improvement by recombinant xylanase derived from *P. pastoris* was slightly lower as compared to biobleaching results of crude fungal xylanases (Bissoon et al., 2002; Manimaran et al., 2009). This could be attributed to the lack of accessory hemicellulolytic enzymes in *P. pastoris*. Accessory enzymes are required for the complete conversion of xylan polymer. Further studies on combination of this recombinant xylanase with other microbial accessory enzymes such as α-arabinofuranosidases, β-xylosidases, and non-chlorinated processes will help to develop an efficient enzyme aided bleaching process.

4. Conclusions

Although many xylanases used in industry are from mesophiles, xylanases from thermophilic sources may be of tremendous use in several biotechnological applications. *T. lanuginosus* is known as a hyper producer of endoxylanase, nevertheless the expression of the genetically modified xylanase in fungal system would require several days of cultivation whereas *P. pastoris* produces good titre of recombinant xylanase within a short period of time. Thus, *P. pastoris* is a suitable alternative candidate for recombinant xylanase production and the biobleaching experiments on bagasse pulp revealed that crude xylanase has a potential application in enzyme aided bleaching of non-woody plant fibres.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.biortech.2011.07.059.

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