USE OF AN EXTREMELY HIGH SPECIFIC ACTIVITY XYLANASE IN ECF AND TCF PULP BLEACHING

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ABSTRACT

A 25 kDa catalytic domain of Xylanase A from the anaerobic fungus *Orpinomyces* sp. *strain PC – 2* was produced in *Escherichia coli*. The enzyme was stable for at least 120 minutes at pH 5.0 to 10.0 and 40^{o}C. During the incubation of oxygen bleached hardwood kraft pulp for 120 min at 40^{o}C, the xylanase released both lignin and reducing sugars at pH 6.0, 7.0 and 8.0. The enzyme reduced the kappa number and increased the brightness of the pulp at all the pH values studied. The benefits obtained with the enzymatic treatment of the pulp were most obvious at pH 8.0. The xylanase treatment reduced the kappa number of the pulp by approximately 25% and increased the brightness by 2.7% ISO at this pH. A higher brightness ceiling could be achieved when the pulp was treated with the xylanase and bleached using either an ECF bleaching (OXDP) or a TCF bleaching sequence (OXZP). With the xylanase treatment at pH 8.0, pulp was bleached with both the ECF and the TCF sequences to a higher than 90% ISO brightness, which was unattainable without xylanase treatment under the same conditions. There was no change in the strength properties of the paper at the end of both bleaching sequences.

KEYWORDS - enzyme, xylanase, high specific activity, kraft pulp, elemental chlorine free, totally chlorine free, bleaching.

INTRODUCTION

Traditionally and even today bleaching of wood pulp is carried out using molecular chlorine and chlorine based chemicals. Use of chlorine based chemicals results in formation of AOX (1). AOX compounds are acutely toxic and may persist in the environment for a long time. Reducing the AOX levels in the bleach plant effluents has therefore been a major concern for the pulp and paper industry.

AOX formation can be reduced by minimizing the amount of lignin to be removed and by employing ECF bleaching sequences. ECF bleaching is usually carried out in multiple stages using chlorine dioxide. Alkaline extraction is done in between the stages. Part of the chlorine dioxide can be replaced by hydrogen peroxide to further reduce AOX formation.

AOX can be avoided in the effluents by using TCF bleaching sequences. Ozone has so far shown a high potential as a alternative bleach chemical for use in TCF bleaching sequences. However ozone can damage the cellulose component, since reaction of ozone with phenolic lignin structures produces hydroxyl radicals which attack the cellulose and decrease paper strength (2, 3). Phenolic lignins can be removed from the pulp in a prebleaching, oxygen delignification stage prior to ozonation. Ozone can also react with cellulose directly. However, ozone has more affinity for lignin than for cellulose and lignin therefore protects cellulose during ozonation of the pulp (4). Ozone slowly reacts with cellulose when in excess. By carefully monitoring the contact time and concentration of ozone with the pulp, paper of high brightness and acceptable strength can be obtained. Oxygen delignification and alkaline hydrogen peroxide extraction are usually incorporated to fortify ozone bleaching.

Enzymes have been used to improve the bleachability of pulps (5). Hemicellulases and ligninases have been tried both at laboratory and pilot plant scales to treat pulp. Only xylanases have so far shown promise for use in mills and a number of mills are currently using xylanases to treat pulp at commercial levels. A xylanase treatment of the pulp before bleaching saves chemicals in subsequent bleaching stages (6). Xylanases have also been implicated in direct brightening of the pulp and achieving high brightness ceilings for fully bleached pulps. Xylanases increase the efficiency of bleaching by making lignin more accessible to the bleach chemicals (7). The benefits achieved with xylanase treatment of the pulp vary with the sources of xylanases (8). Conditions like pH and temperature of the pulp differ for individual enzymes. Also the amount of lignin and chromophores released from the pulp by different enzymes varies (9). A xylanase capable of releasing high amounts of chromophores and lignin from the pulp under alkaline conditions is desirable for mill scale application. The pulp industry is not much concerned about the temperature and is generally able to provide the required temperatures for the xylanase treatment.

The benefits obtained from xylanase treatment of pulp so far have been limited and have attracted relatively little attention. The pulp and paper industry desires greater benefits. In this paper we report a 2.7% ISO brightness gain with the *Orpinomyces* xylanase step at pH 8 alone and an overall 4.3% ISO brightness gain over the control (xylanase untreated) pulps at the end of an ECF (OXDP) or a TCF (OXZP) bleaching sequence. The benefits reported are higher than for any other xylanase treatment of pulp at pH 8 using less than 1 g of enzyme protein to treat 1 ton of pulp.

MATERIALS AND METHODS

Enzyme - The *xynA-2* gene of the anaerobic fungus *Orpinomyces* sp. strain PC – 2 was cloned into *E coli* (10). The gene and its recombinant proteins were patented as *xynA-2* and XynA-2 respectively (11). The encoded XynA is a 362 amino acid polypeptide containing a signal peptide, followed by the xylanase catalytic domain, a linker region and a non-catalytic repeated peptide domain (NCRPD). The NCRPD facilitates binding of the xylanase to other polypeptides for the formation of cellulase/hemicellulase complexes. The Xyn A-2 enzyme used in this study is a truncated protein containing only the xylanase catalytic domain and differs from the patented protein (11). To produce just the catalytic domain, two oligonucleotides were designed and

synthesized to amplify with polymerase chain reactions the region coding for amino acid residues from 12 to 235 (10). Two restriction sites, *Bam*HI and *Eco*RI, were added to the ends of the two oligonucleotides. The DNA fragments and plasmid pRSET B (Invitrogene) were digested with *Bam*HI and *Eco*RI, purified and ligated. *E. coli* strain BL21 (DE3) (Stratagene) was transformed using the plasmid. The recombinant xylanase was produced in batch fermentation (20 lit) with *E. coli* growing at 37^{o}C in Luria Bertani medium, pH 6.5, consisting of 1% tryptone, 0.5% yeast extract, 0.5% NaCl, and 50 mg/l ampicillin. When the optical density at 600 nm reached 1.0, ITPG (1 mM) was added. The culture was continued for additional 4 hr. Cells were harvested by centrifugation, resuspended in 20 mM sodium citrate buffer, pH 6.0, and broken in a French Press. Cell debris were removed by centrifugation at 10,000 x g for 30 min at 4^{o}C. The supernatant was used as enzyme for this study.

Xylanase assay - The reaction mixture, 250 µl, consisted of 200 µl of 1% birchwood xylan in 0.05 M buffer and 50 µl of the xylanase solution. Incubation was done at 40^{o}C for 15 minutes. The reducing sugars liberated were estimated by the dinitrosalicylic (DNS) method (12) as xylose equivalents.

One unit of enzyme activity is defined as the amount of enzyme required to liberate one micromole of xylose min^{-1} under the assay conditions.

Buffers - The buffers used in the study were : citrate phosphate (pH 5 , 6), sodium phosphate (pH 7, 8) and glycine NaOH (pH 9, 10). There was no change in the pH of the pulp at the end of 120 minutes of enzymatic treatment.

Pulp - Oxygen bleached hardwood kraft (HWKO) pulp obtained from Champion Mill in South Carolina was used in this study. The pulp was thoroughly washed with water before use.

Enzymatic treatment of the pulp

Xylanase treatment of the pulp was carried out according the conditions described in **Table I**. Pulp at 5% consistency in 0.05 M buffer was incubated with enzyme for different time intervals (0 to 120 min) at different pH values (pH 6, 7 , and 8). A control pulp treated with boiled enzyme was run every time along with the enzyme treated pulp under similar conditions. At the end of the enzymatic incubation the pulps were filtered through Whatman filter paper no. 1 using a Buchner funnel. The filtrate was retained for further analysis. Handsheets made from the enzyme treated and control pulps were tested for brightness and the lignin content was determined by estimating the kappa number.

Table I. Conditions for the bleaching of HWKO pulp

Symbol	Chemical Charge %	Reaction time, min	Temperature ^{o}C	pH	Consistency %
X	2 , 2.4, 3 U xylanase per o. d. pulp	0 – 120	40	6,7,8	5
D	As active chlorine : 1.5	120	70	2.5-3.0	5
E	NaOH : 1.0	10	60	> 10	5
P	H_2O_2 : 2	180	90	> 10	5
Z	O_3 : 0.75 g / o. d. pulp	2	amb.	2.5-3.0	5

Lignin release - Release of lignin from the pulp was monitored by reading the absorbance of the filtrate at 280 nm (13)

Sugar release - Reducing sugars released from the pulp were estimated by the DNS method (12) as xylose equivalents.

Treatment of pulp in subsequent bleaching stages

Treatment of pulp in subsequent bleaching stages was done according to Table I. The enzyme treated and control pulps were bleached using an ECF bleaching sequence OXDP and a TCF bleaching sequence OXZP known as the EnZone process developed by Eriksson *et al.* (14) at the University of Georgia in USA.

Pulp properties - The following standard TAPPI test methods were used to analyze various properties of pulps – formation of handsheets for physical tests of pulp (T205 om – 88), Kappa number of pulp (T 236 cm – 85), Brightness of pulp (T 525 om – 86), Tensile strength (T 494 om – 88) and Tear index (T 414 om – 88).

RESULTS

Properties of the xylanase - The truncated XynA-2 of *Orpinomyces sp. strain PC-2* is a polypeptide of 224 amino acid residues from 12 – 235 of XynA-2 (10, 11). The calculated mass and pI are 25 kDa and pH 8.4 respectively. The recombinant protein has been purified and the purified protein had a specific activity of 3,500 U / mg.

Effect of pH on the activity and stability of the xylanase - The pH activity profile of the enzyme was determined by assaying the enzyme at 40^{o} C at different pH values (pH 5 to 10) using 1% birchwood xylan as substrate, **Fig. 1**. The optimum for enzyme activity was at pH 6. The enzyme had 83% and 65% of optimum activity at pH 7 and pH 8, respectively. At pH 9 and pH 10 the enzyme showed only 25% and 10% of optimum activity, respectively. The enzymatic

treatment of the pulp was therefore carried out at pH 6, 7 and 8 only. The amount of enzyme required to release reducing sugars from birchwood xylan equivalent to that released by 2 U at pH 6 was calculated for pH values pH 7 and 8 and found that to be 2.4 U and 3 U respectively (**Table II**). This was done to have same effective amount of activity present at each pH.

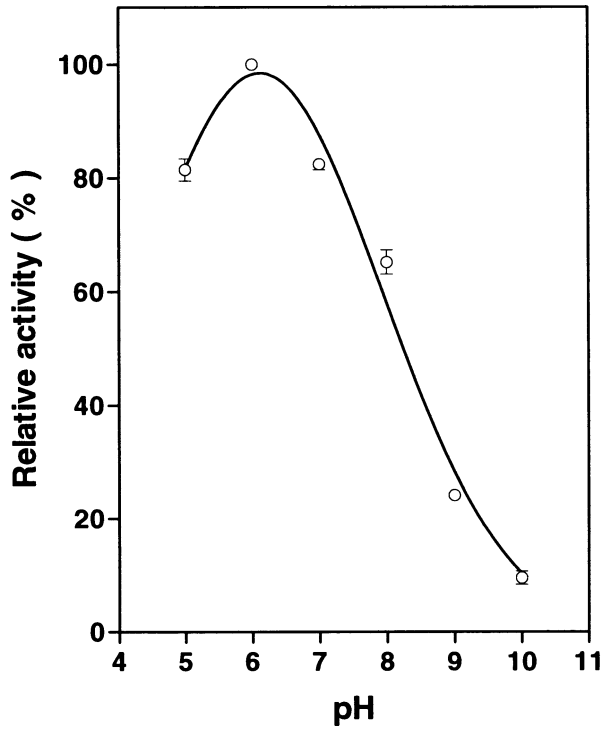

Fig. 1 pH activity profile of *Orpinomyces* xylanase
Each data point represents a mean of 3 independent experiments carried out in duplicates under identical conditions. Error bars represent standard deviation.

Table II. Enzymatic treatment of pulp

pH of pulp	Xylanase added U / g o. d. pulp
6	2
7	2.4
8	3

To determine the stability of the enzyme at different pH values (pH 5 to 10) at 40^{o}C, the enzyme was incubated in different buffers at pH 5 to 10. Samples were withdrawn at 30 minute intervals to estimate the residual enzyme activity. There was no loss in enzyme activity at the end of 120 minutes of incubation for any of the buffers and pH - levels studied.

Lignin and sugar release - The release of lignin and reducing sugars by the enzyme at pH 6, 7 and 8 are presented in **Fig. 2 and 3**. The enzyme could release lignin and reducing sugars from the pulp at all pH values studied. However, maximum release of both lignin and sugars was at pH 8. The amount of lignin released from pulp by the enzyme after 120 minutes of incubation at pH 8 was almost twice than that released at pH 6. The sugars released at pH 8 were more than 3 times than that released at pH 6.

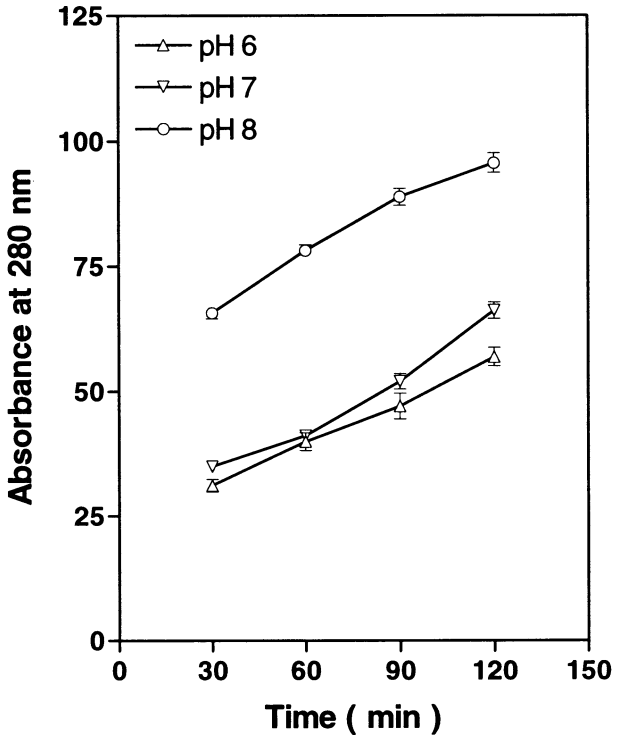

Fig. 2 Lignin release from the pulp during enzymatic treatment
Each data point represents a mean of 3 independent experiments carried out in duplicates under identical conditions. Error bars represent standard deviation.

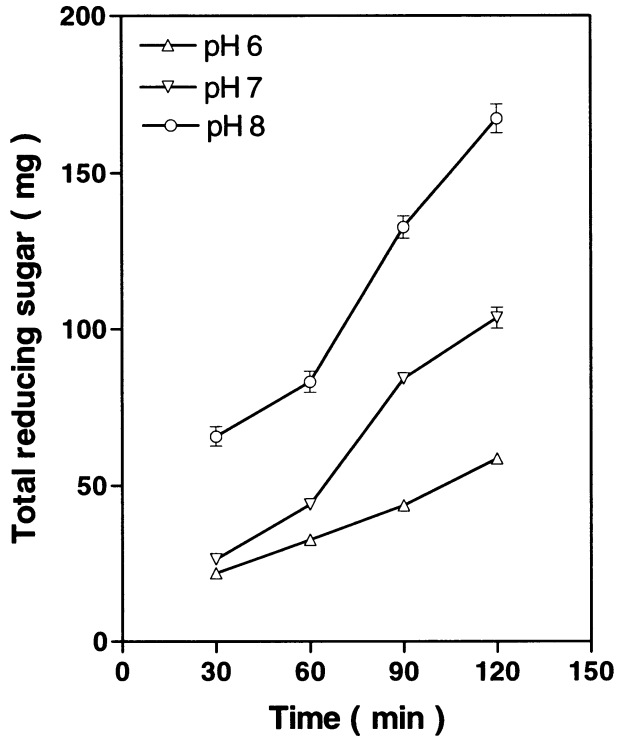

Fig. 3 Sugar release from the pulp during enzymatic treatment
Each data point represents a mean of 3 independent experiments carried out in duplicates under identical conditions. Error bars represent standard deviation.

Kappa number reduction brightness increase - The enzyme could reduce the kappa number of the pulp at all pH values studied (**Fig. 4**). Maximum reduction in kappa number, approximately 25%, was also observed at pH 8. The xylanase treatment of the pulp resulted in an increase in pulp brightness. Maximum brightness gain was obtained when the enzymatic treatment was carried out at pH 8 (**Table III**). A 2.7% ISO brightness gain over the control pulp was observed at this pH.

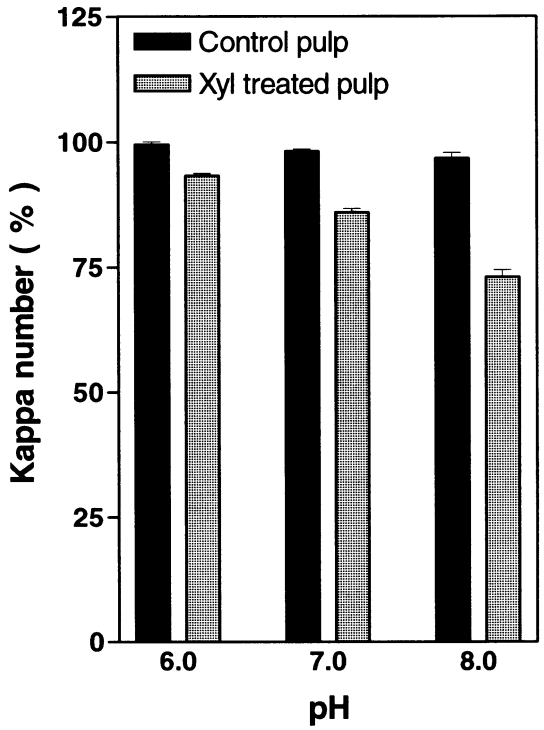

Fig. 4 Kappa number reduction
100% represents a kappa number of 8.4 which was the kappa number of the original pulp used in the study.
Each data point represents a mean of 3 independent experiments carried out in duplicates under identical conditions. Error bars represent standard deviation.

Table III. Brightness values of xylanase treated and control pulps

Symbol	pH 6	pH 7	pH 8
O	46.5	46.5	46.6
OX	47.3	47.7	49.3
ODP	87.3	87.4	87.4
OXDP	8.6	89.4	91.8
OZP	86.1	86.1	86.1
OXZP	86.7	87.8	90.3

Each value represents a mean of 3 independent experiments carried out in duplicates. The variations were less than 0.5% ISO brightness between the 3 independent experiments.

Treatment of the pulp in subsequent bleaching stages - Brightness greater than 85% ISO was obtained for both enzyme treated and control (xylanase untreated) pulps when bleached with either the ECF or the TCF bleaching sequences. However it was observed that the xylanase treatment of the pulp increased the brightness ceiling of the pulps. In all cases the xylanase treated pulp showed higher brightness values than the control pulp when bleached under the same conditions. With a xylanase treatment at pH 8, brightness as greater than 90% ISO were achieved at the end of both bleaching sequences which was not achievable for the control pulps (without the xylanase stage). When the pulp was treated with xylanase at pH 8 and bleached with either the ECF or the TCF bleach sequences, a maximum of 4.3% ISO brightness increase was obtained over the control pulp.

DISCUSSION

The *xynA-2* gene product of *Orpinomyces sp. strain PC – 2* used in this study is a truncated protein containing only the catalytic domain of the enzyme (11). It has a small mass of 25 kDa and an alkaline pI of 8.4. In addition, the specific activity of this enzyme, 3500 U/mg on birch wood xylan is much higher than any of those reported in the literature (**Table IV**). Since the specific activity is so high, less than 1 g of enzyme protein might be required to treat 1 ton of pulp at pH 8 according to the conditions in Table I. The enzymatic treatment of the pulp was carried out at pH 6, 7 and 8. The pH of the pulp was below the pI of the enzyme. The enzyme therefore carried a net positive charge. The pulp was negatively charged because of its content of carboxylic acid groups. This increases the physical interaction of the enzyme with the pulp and thereby its effectiveness. Viikari *et. al.* (15) have reported that the superior ability of the pI 9.0 xylanase from *Trichoderma* to enhance bleaching of pulp under neutral conditions was due to the reason that the xylanase remained bound to the pulp when the pH was below its pI.

Table IV. Strength properties of bleached pulps

Sequence	pH of xylanase treatment	Tensile strength mNm^2 / g	Tear index Nm / g
ODP	-	19.18 ! 0.67	9.43 ! 0.51
$OXDP^1$	6	19.00 ! 0.43	9.32 ! 0.30
$OXDP^2$	7	18.87 ! 0.71	9.38 ! 0.21
$OXDP^3$	8	19.38 ! 0.32	9.52 ! 0.54
OZP		18.76 ! 0.68	8.89 ! 0.12
$OXZP^1$	6	17.96 ! 0.93	8.84 ! 0.32
$OXZP^2$	7	18.60 ! 0.64	9.12 ! 0.53
$OXZP^3$	8	18.88 ! 0.22	9.03 ! 0.39

Each value represents mean ! sd , n = 6

Although pH 6 was the optimum for the enzyme to hydrolyze birchwood xylan, the enzyme released more lignin and sugar from the pulp at pH 8 (Fig. 2 and 3). This could be because of a greater swelling of the pulp fibers under alkaline conditions. The optimum pH for enzymatic treatment of pulp is not always the same as the pH optimum of the enzyme towards a soluble substrate such as low MW birchwood xylan (16).

The enzyme reduced the lignin content (kappa number) of the pulp at all pH values studied (Fig. 4). At pH 8 the enzyme reduced the kappa number of the pulp from an initial value of 8.4 to a final value of 6.4 after 120 minutes of enzymatic treatment. The xylanase treatment of the pulp also increased its brightness . The gain in brightness could be due to the removal of chromophores and part of the residual lignin from the pulp by the enzyme.

A major concern for using ozone to bleach pulp has been the decrease in strength of the bleached paper. One way of minimizing damage to the cellulose would be to limit the contact time and concentration of ozone. However this would necessitate incorporation of additional steps like oxygen delignification and alkaline peroxide extraction in order to bleach the pulp to a brightness greater than 85% ISO. In the present study hardwood kraft pulp could be bleached to a brightness greater than 90% ISO using the EnZone Process (OXZP) without any loss in the strength properties of the pulp (see **Table** III and **V**).

Table V. Specific activities of xylanases from various sources

Organism	Specific activity μmol / min / mg	Reference
Orpinomyces sp. strain PC-2	3,500	This study
Aureobasidium pullulans	*2,500*	Li *et al*., 1994 (18)
Clostridium acetobutylicum	22.4	Lee, Forsberg and Rattry, 1987 (19)
Streptomyces roseiscleroticus	305	Grabski and Jeffries, 1991 (20)
Trichoderma longibrachiatum	130	Roger and Nakas, 1991 (21)
Trichoderma viride	152	Ujiie and Yaguchi, 1991 (22)
Fibrobacter succinogenes S85	33.6	Matte and Forsberg, 1992 (23)
Bacillus sp. strain T-6	288	Khasin *et al*., 1993 (24)
Aspergillus syndowii	204	Ghosh and Nanda, 1994 (25)
Aspergillus fischeri	588	Raj and Chandra, 1996 (26)

Two beneficial effects have been observed with xylanase treatment of the pulp before bleaching – direct brightening and bleach boosting (17). Both of these benefits were observed in this study (Table III). A brightness gain was observed for pulp treated with xylanase at all pH values. When the pH of the xylanase treatment was pH 8, brightness increased from 46.6 to 49.3% ISO following the xylanase treatment step. A bleach boosting effect of the xylanase treatment was

observed when the xylanase treated and control pulps were bleached with both the ECF and TCF bleaching sequences (Table III). The xylanase treated pulps had higher brightness ceilings than control pulps when bleached with either an ECF or a TCF sequence under the same conditions. An increase of approximately 4.3% ISO in the final brightness was achieved when the xylanase treatment was carried out at pH 8 (Table III). Pilot scale studies are underway to evaluate the *Orpinomyces* sp. strain PC – 2 xylanase in a TCF sequence (OXZP) for bleaching of both hardwood and softwood pulps. The cost of the treatment, yield loss studies, as well as environmental impact of the waste waters will be evaluated.

CONCLUSIONS - An *Orpinomyces sp strain PC – 2* xylanase pretreatment of oxygen bleached hardwood kraft pulp results in direct brightening and bleach boosting of the pulp.

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List of abbreviations

AOX	-	Adsorbable organic halogens
D	-	Chlorine dioxide treatment
ECF	-	Elemental chlorine free
HWKO	-	Oxygen bleached hardwood kraft pulp
IPTG	-	Isopropyl - β - D - thiogalactoside
kDa	-	Kilo dalton
MW	-	Molecular weight
NCRPD	-	Non-catalytic repeated peptide domain
O	-	Oxygen delignification
P	-	Peroxide treatment
TCF	-	Totally chlorine free
X	-	Xylanase treatment
xynA	-	Gene coding for Xylanase A
Z	-	Ozone treatment

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