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CLONING, CHARACTERIZATION AND ITS POTENTIAL IN PULP BIO BLEACHING BY ALKALI THERMOSTABLE ß-MANNANASE FROM *BACILLUS* SP. 22

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ABSTRACT

The mannanase gene of *Bacillus* sp. 22 was cloned and expressed in an *Escherichia coli* expression system. Recombinant β-mannanase activity was detected on the basis of the clearing of zone around *Escherichia coli* colonies grown on a congo red dye. Mannanase gene was cloned into the vector pSmart LCKan and expressed in DH10β *E. coli*. β-Mannanase activities in the culture supernatant were 7Uml^{-1}. β-Mannanase was partially purified using ammonium sulphate precipitation. The molecular mass of the purified mannanase was 38 kDa as estimated by SDS-PAGE. The optimum temperature and pH of recombinant β-mannanase activity was 70°C and 8.8 respectively. Alkali thermostable β-mannanase was applied on kraft pulp to estimate its bio-bleaching potential in industry. β-mannanase from *Bacillus* sp. 22 shown 11.27% reduction in kappa number.

KEYWORD: *Bacillus*, bio-bleaching, β-mannanase, Kraft pulp, Kappa number.

1 INTRODUCTION

The hemicelluloses are the second most abundant heteropolymers present in nature (Viikari *et al.*, 1992). Depending on their sugar backbone composition, they are classified as either xylans, mannans, arabinogalactans or arabinans. The two most important components of hemicelluloses are the hetero-1,4,-β-D-mannans and hetero-1,4,-β-D-xylans. Endo-1,4,-β-D-Mannanase (EC 3.2.1.78, mannan endo-1,4-β-D-mannosidase) cleave randomly within the 1,4-β-D-mannan main chain of galactomannan, glucomannan, galactoglucomannan and mannan (McCleary, 1988).

Mannan degrading enzymes from various sources has increased in the past years, especially because of their applications in various biotechnological Industries (Singh *et al.*, 2003). Mannanase has been successfully used in pulp bleaching (Lahtinen *et al.*, 1995; Cuevas *et al.*, 1996, Bhoria *et al*., 2009, Sondhi *et al*., 2014), in clarification of fruit juices (Christgau *et al.*, 1994), in manufacturing of instant coffee, chocolate and cocoa liquor (Francoise *et al.*, 1996; Van Zyl *et al*., 2010). Other than these applications, mannanase also applied in the pharmaceutical industry for the production of active oligosaccharides (Lin and Chen, 2004, Fu *et al*., 2006).

Mannanases are well-known to be produced by a variety of actinomycetes, fungi, bacteria, plants and animals. Bacterial mannanases have been reported from various strains in different genera and purified from *Paenibacillus cookie* (Li *et al.*, 2012), *Paenibacillus sp.* MSL 9 (Manjula *et al*., 2010), *Bacillus circulans* (Yoshida *et al.*, 1997), *Bacillus subtilis* (Jiang *et al*., 2006), *B. subtilis* B 36 (Li *et al.*, 2006), *Bacillus* sp.MG-33 (Meenakshi *et al*., 2010), *Bacillus. stearothermophilus* (Talbot and Sygusch, 1990), *Caldibacillus cellulovorans* (Sunna *et al.*, 1999), *Cellulomonas fimi* (Stoll *et al.*, 2000), *C.tertium* (Kataoka *et al.*, 1998), *Dictyoglomus thermophilum* (Gibbs *et al.*, 1999), *Bacillus nealsonii* PN-11 (Chauhan *et al*., 2014a) and many more.

Various studies have been reported on cloning and manipulation of microbial mannanase genes from different organisms with the aim of to enhance the production of enzyme (Chauhan *et al*., 2015). In industrial processes, it is important to understand the structure of protein and protein engineering for the alteration of enzyme properties for its commercial applications. Therefore, the present study was aimed to clone mannanase encoding gene from *Bacillus* sp. 22, characterize the cloned enzyme produced recombinant gene and having application in Pulp Biobleaching.

2 MATERIAL AND METHODS

2.1 Strains and vector

The bacterial strain *Bacillus* sp. No 22 which produce an alkali-thermostable β-mannanase was isolated earlier in our laboratory. Electrocompetent *E. coli* DH10β was

used as the host for gene cloning and expression linearized (blunt ends) and dephosphorylated pSMART LC-Kan plasmid (Lucigen Corporation, USA) was used as the cloning vector.

2.2 Media

Luria-Bertani medium (LB) containing 1% (w/v) bactotryptone, 0.5% (w/v) yeast extract and 0.5% (w/v) NaCl, at a pH of 7.0, was used for cultivation of *E. coli* and *Bacillus* sp. No 22. LB agar contained additionally 2% (w/v) agar. One hundred micrograms of Kanamycin per milliliter was added for growing the transformants.

2.3 Chemicals and reagents

Locust bean gum (LBG), guar gum (GG), bovine serum albumin (BSA), were purchased from Sigma-Aldrich Co. (St. Louis, USA). HaeIII and HindIII enzymes were purchased from New England Biolabs (USA). Kanamycin and dinitrosalicylic acid (DNSA) were purchased from Sigma-Aldrich Co. (St. Louis, USA), HiMedia, India. All other chemicals and reagents used in this study were of analytical grade.

2.4 Generation of genomic library

Genomic DNA was prepared as described by Ausubel et al. (2006). The DNA was partially digested by blunt-end tetra cutter enzyme HaeIII and run on a preparative agarose gel along with λ/HindIII marker. The agarose gel piece containing desired fragments (2–6 kb) was excised, and DNA was extracted using the QIAquick Gel Extraction Kit (QIAGEN). Ligation was done using pSMART vector at 22 °C for 4 h. The electrocompetent *E. coli* cells suspension was mixed with ligation mix and electroporated. Then, 100µl of transformed cells were plated on Luria agar plates containing kanamycin (100 μgml^{-1}). The plates were incubated at 37°C for 72 h. Transformants were screened on Luria agar+kanamycin plate containing (locust bean gum 0.5%) by replica plate method. The transformants showing zone of clearance after staining with congo red were selected. Plasmid was isolated and gene inserts were confirmed by restriction analysis.

2.5 Enzyme assay

Mannanase activity was assayed by measuring the amount of reducing sugars released by the enzyme using locust bean gum as a mannan substrate (Chauhan et al. 2014b).

One unit of mannanase activity was defined as the micromoles of a mannose release by 1 ml of enzyme in 1 min under standard assay conditions.

2.6 Purification and molecular weight determination

E. coli DH10β cells harboring mannanase gene from *Bacillus* sp. No 22 were grown in Luria broth containing $100\mu gml^{-1}$ kanamycin. Cell-free supernatant was used as enzyme and mannanase was partially purified by using ammonium sulfate precipitation (60–80%). Mannanase activity was determined by using activity staining (zymography). All the purification steps and zymographic analysis were carried out according to the method of Chauhan et al. (2014d).

2.7 Characterization of mannanases

Recombinant mannanase was produced under optimum conditions. This enzyme was characterized.

2.8 Temperature optima/stability of mannanase enzyme

Effect of temperature on purified mannanase for was determined by incubating the assay mixture in the temperature range of 40-85°C for 5 min. The thermal stability of the enzyme was measured at different temperatures range of 60-75°C and incubating the enzyme for a time period of 0-24 h. At different time intervals, aliquots were withdrawn and residual activity was determined under standard assay conditions.

2.9 pH optima/stability of mannanase enzyme

Mannanase activity was determined for Locust Bean Gum (0.5%) as substrate by performing the enzyme assay at different pH values ranging from 7.2 (0.1 M phosphate buffer), 8.0 and 8.8 (0.1 M Tris-HCl buffer) and 9.2 (0.1 M carbonate-bicarbonate buffer). The assay was done at 70°C. The pH stability of mannanase was determined by suitably diluting the enzyme in the buffer of pH 8.0 and then incubating the enzyme at room temperature for 4 hours. At various time intervals, aliquots of enzyme were withdrawn and the residual activity was examined as per standard assay conditions. Tris buffer of pH 8.0 was used for determining pH stability.

2.11 Application of Mannanase in Biobleaching of softwood pulp

Softwood pulp was washed with distilled water and dried at 55ºC in an oven overnight. Pulp with 5% consistency was treated with 40 Ug-1 oven-dried pulp of cloned mannananse enzyme at a temperature of 70ºC and pH 8.8 for 4 h (conditions standardized in our laboratory). Pulp treated under the same conditions but without enzyme was taken as control. Both the control and enzyme-treated pulp samples were filtered through muslin cloth, washed with distilled water and then dried in oven at 55–60ºC overnight. Kappa number of pulp was determined according to the TAPPI method (Technical Association of pulp and paper industry, Atlanta, USA) T 236 and T 452, respectively.

3 RESULTS AND DISCUSSION

3.1 Cloning analysis

Bacillus sp. No. 22 produces an important alkalithermostable mannanase which has application in pulp and other industries (Chauhan et al. 2014b, c, d, e). To study the enzyme at molecular level, which can be helpful for further improvement of enzyme properties, β-mannanase gene from *Bacillus* sp. No. 22 was cloned by functional screening of *E. coli* cells transformed with pSMART/HaeIII genomic library. Out of the 2.6×10^3

transformants, a mannanase positive transformant was selected; on sequencing, it was found to have an insert size of 3174 bp.

3.2 Screening

A clone in which mannanase gene is expressed would be able to utilize LBG (Locust Bean Gum) as substrate therefore Luria Agar containing 0.5% LBG and 100ng ml^{-1} of kanamycin drug was used for screening of transformants. The replica plating of the transformants was done by pick poking method in duplicates. One set of plates was stained with 0.2% Congo Red solution followed by destaining with 1N NaCl. About 3000 transformants were screened and transformant number 1628 was found to give a zone of clearance. (Figure 1).

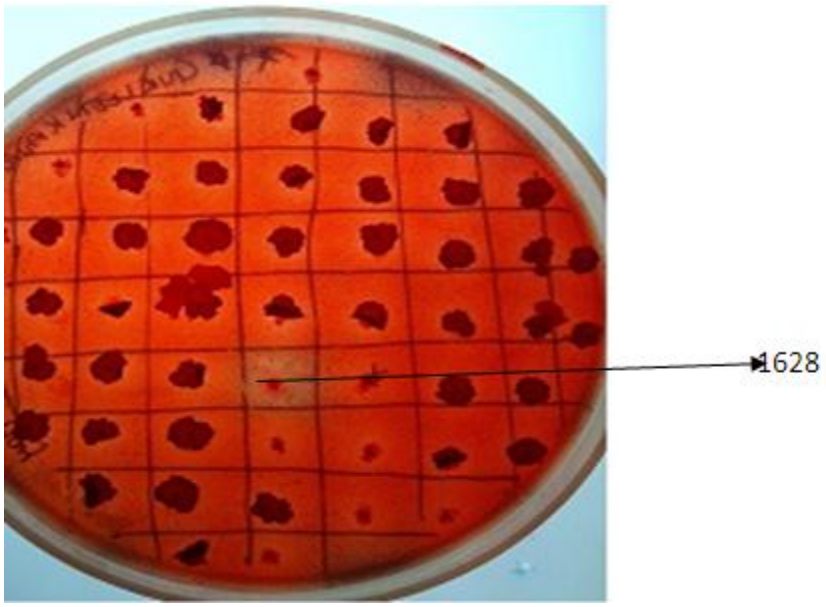


Figure 1 LA plate containing 0.5% LBG showing zone of clearance around transformant no. 1628.

3.3 Isolation of recombinant plasmid

Plasmid of the mannanase positive tranformant number 1628 was isolated by the Alkali Lysis method**.** Gel Electrophoresis of the isolated plasmid showed the presence of plasmid DNA. (Figure 2).

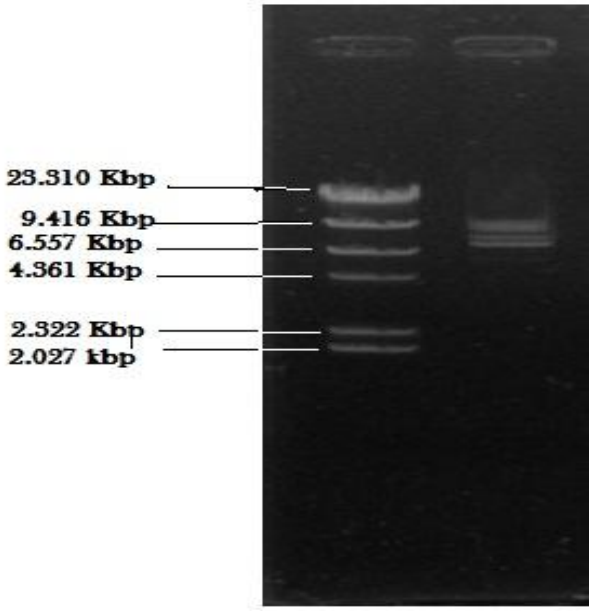


Figure 2Gel Electrophoresis of the plasmid isolated from recombinant No 1628, Lane 1: λ/Hind III marker, Lane 2: Isolated recombinant plasmid.

3.4 Digestion of recombinant plasmid

The recombinant plasmid from transformant number 1628 was digested with restriction enzymes EcoR1 and EcoRV both having unique cutting sites in the cloned vector on both sides of cloning site. With EcoR1 two bands of 9 Kb and 2 Kb were visible. As the vector is of size 2 Kb therefore the digestion pattern indicates that the insert is of 9 Kb. However with EcoRV 3 bands of size 6 Kb, 3 Kb and 2 Kb were seen which means that there is an EcoRV site within the inserted sequence. (Figure 3).

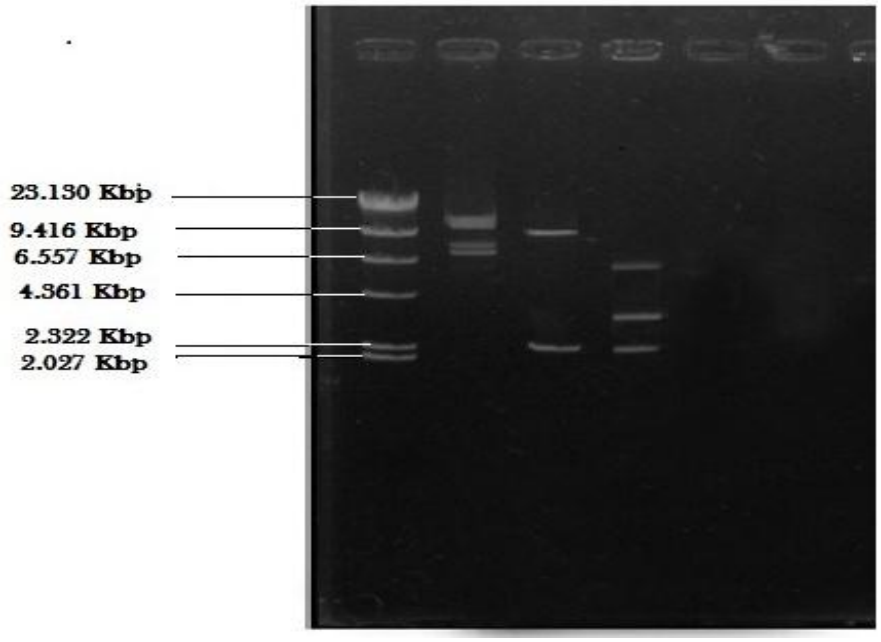


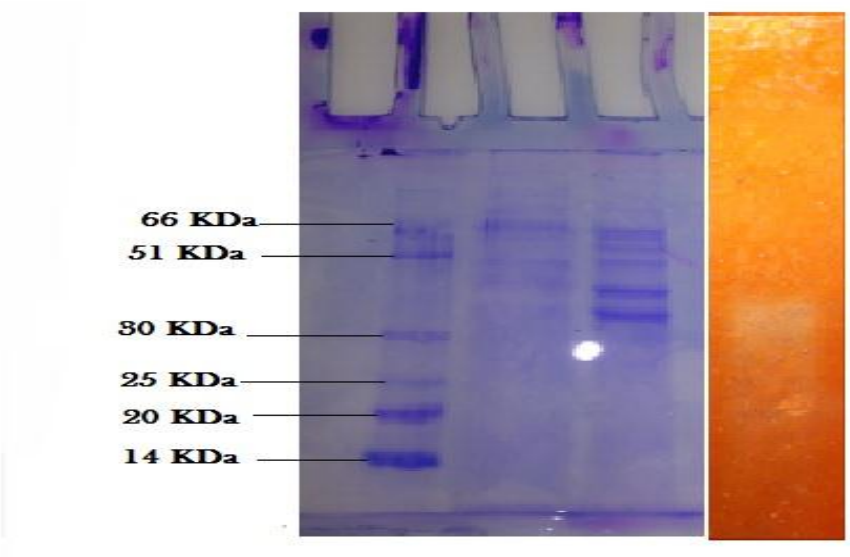
Figure 3 Digestion of recombinant plasmid Lane 1: λ/Hind III marker, Lane 2: Uncut plasmid, Lane 3: Plasmid digested with restriction enzyme EcoR1, Lane 4: plasmid digested with EcoRV.

3.5 Enzyme preparation from the recombinant strain in liquid medium

Mannanase enzyme was produced in liquid media by inoculating 20 ml of Luria Broth containing 0.5% LBG and kanamycin Drug (100µg ml^{-1}) with a single colony of the mannanase positive transformant of *E.coli* and incubated in a shaker (150 rpm) at 37°C for 24 hours. Next day 40ml of LB was inoculated with 1% inoculum of the overnight grown culture and the mannanase assay was done with cell free supernatant after 24h. The enzyme activity was analyzed in cell free supernatant and was observed to be 7 U ml^{-1} which was higher than the wild type. The wild type *Bacillus* sp.22 gave enzyme activity of 3 U ml^{-1}. This indicates that enzyme activity of *Bacillus* sp.22 increased by 2.3 folds on heterologous expression which can be due to increase in the mannanase gene's copy number. Similarly β-Mannanase production has been increased through heterologous expression in a number of cases such as by cloning mannnanase from *Biospora* sp. MEY-1 in *P. pastoris* (Luo *et al*. 2009) and Bacillus sp. N16-5 in *K.cicerisporous* (Pan *et al*. 2011). *Bacillus nealsonii* PN-11 (Chauhan *et al*., 2015).

3.6 Partial Purification of Recombinant mannanase

Cloned mannanase was concentrated by precipitation with ammonium sulphate (100%) and 2 lanes of the concentrated sample were run on SDS –PAGE gel. One lane of the gel was stained and destained to determine the Molecular Weight of the protein and the other lane was cut and zymogharphy was performed. On comparing with the protein marker a major protein band of about 35-38 KDa was visible on the gel which corresponded to the zone of clearance on zymograph. (Figure 4).



(A) **(B)**

Figure 4 SDS-PAGE of recombinant mannanase. Lane 1: SDS PAGE protein marker, Lane 2: Crude enzyme, Lane 3: 100% Ammonium sulphate concentrated enzyme (B) Zymograph.

3.7 Characterization of Recombinant mannanase

3.7.1 Temperature optima

The optimum temperature at which cloned β-mannanase showed maximum activity at 70°C. Further increase in incubation temperature reduced the enzyme activity. At 75°C, it retained 65% of its activity. At below optimum temperature i.e. at 50°C, it retained 54% of its activity (Figure 5). The optimum temperature of the cloned enzyme is same as that of the native type. Similarly same temperature optima of 70°C has been reported in both native and cloned mannanase of *Bacillus subtilis* N16-5 by Lin *et al*., 2007 and He *et al*., 2008; Pan *et al*., 2011 respectively. Native and cloned mannanase of *Cellulomicrobium* sp.HY-13 also have same temperature optima of 50°C (Kim *et al*., 2011a and 2011b; Aditya *et al.*, 2014; Kumar *et al.*, 2014; Kumar *et al.*, 2016;).

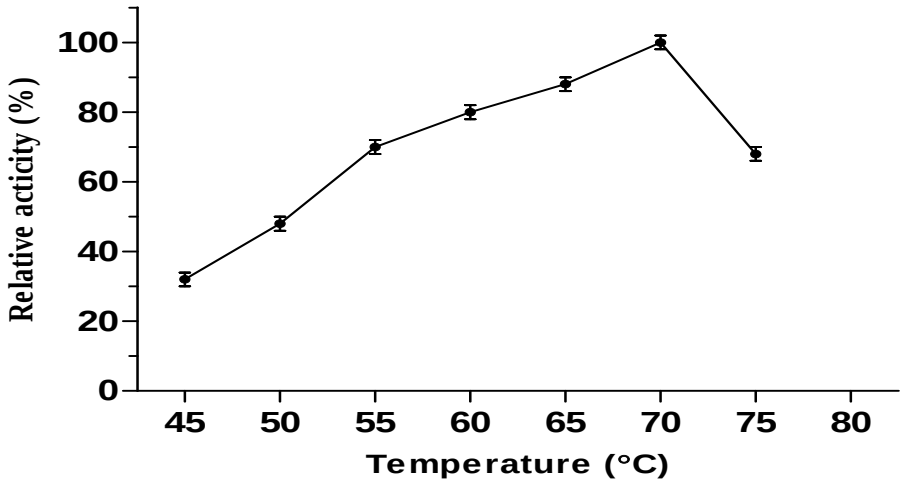


Figure 5 Temperature optima of cloned mannanase of *Bacillus* sp.22.

3.7.2 Temperature stability

The thermostability of mannanase was examined at temperatures 60°C, 65°C and 70°C. The enzyme was incubated at respective temperatures and aliquots were withdrawn sequentially at different time intervals and residual activity was measured under standard assay conditions. The enzyme showed maximum stability at 60°C and retained 80% of its activity even after 2 hours. At 65°C the enzyme retained almost 60% of its original activity after 2hours but the residual activity declined to 30% at 70°C after 2hours (Figure 6). The cloned enzyme has thermal stability slightly higher than the native type as the native mannanase was not stable for more than 10 minutes at 70°C. Similarly an increase in thermal

stability in *Bacillus subtilis* N16-5 on heterologous expression has been reported by He *et al*., 2008. Recombinant *Trichoderma reesei* man1 mannanase is also reported to have higher thermal stability than native type. (Wei *et al*., 2005).

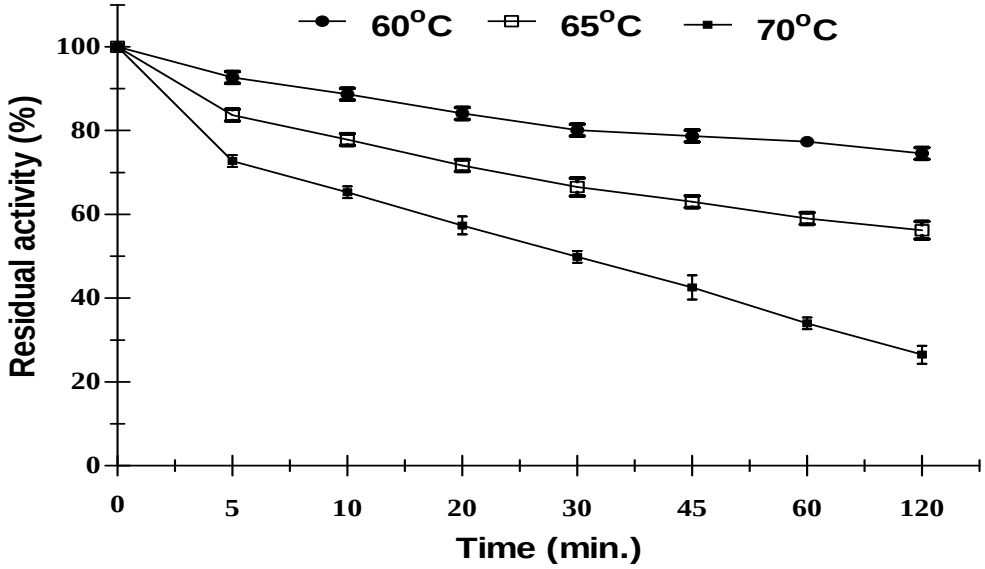


Figure 6 Temperature stability of the cloned mannanase of *Bacillus* sp. 22.

3.7.3 pH Optima

The optimal pH for enzyme activity was determined by preparing the substrate as well as suitable enzyme dilutions in buffers of different pH (7.0, 7.4, 8.0, 8.8 and 9.2) and performing the enzyme assay under standard conditions. The optimum pH of the enzyme was observed to be at pH 8.8. At pH 8.0, the enzyme could retain 85% of its activity and almost 70% of its activity at pH7.4 (Figure 7). The pH optima of the cloned enzyme is same as that of the wild type. Similar to the above observation the cloned enzyme of *Bacillus stearothermophilus* (Ethier *et al*., 1998) has been reported to have same pH optima as the native type. In contrast to this, decrease in pH optima on heterologous expression of mannanase has been reported in *Cellulomicrobium* sp.HY-13. (Kim *et al*., 2011a, Kim *et al*., 2011b) and increase in pH optima has been reported in *Trichoderma reesei* man1 (Stalbrand *et al*, 1993; Wei *et al*., 2005).

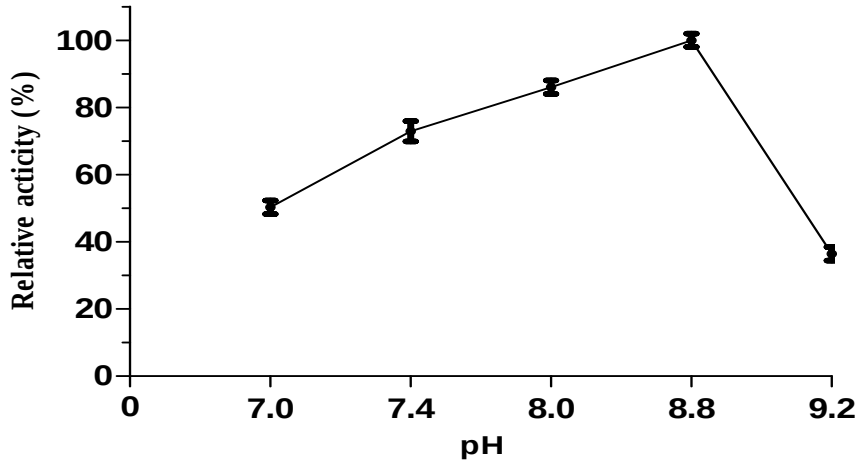


Figure 7 pH optima of the cloned mannanase of *Bacillus* sp.22.

3.7.4 pH Stability

The pH stability of enzyme was determined by preparing the substrate as well as suitable enzyme dilution in buffers of pH 8.4 and 8.8 and incubating the enzyme at room temperature. Aliquots were withdrawn at different time intervals and residual activity was measured under

standard assay conditions. The enzyme was highly stable at pH 8.4. It could retain 75% of its activity after 2 hours. The enzyme was fairly stable at ph 8.8 as it retained almost 50% of its activity after 2 hour (Figure 8). The pH stability of the cloned mannanase is in the same range as the native type. Similarly same pH stability of the native and cloned mannanase has been reported in *Cellulomicrobium sp*.HY-13(Kim *et al*., 2011a; Kim *et al.*, 2011b). An increase in pH stability on heterologous expression of mannanase has been reported in *Bacillus subtilis* N16-5 by He *et al.,2008.*

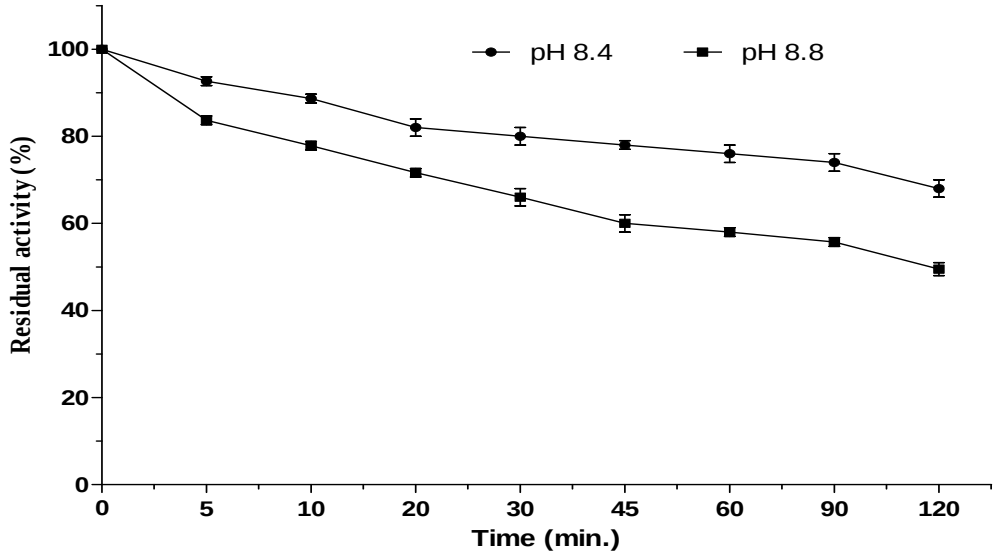


Figure 8 pH stability of cloned mannanase of *Bacillus* sp.22.

3.8 Application of β Mannanase in pulp Biobleaching

β Mannanase from *Bacillus* sp.22 was applied for the biobleaching of kraft pulp under following conditions such as enzyme dose (30 U), temperature (60°C), pH (8.4) and optimum time period(2h). The pre-bleaching with mannanase showed the decrease in kappa (κ) number by 11.27% shown in table 1. Sunna *et al*., 2010 have reported a 13% reduction in Kappa number of Kraft pulp on treatment with cloned mannanase from *Caldi Bacillus cellulovorans*.

Table1: Physiochemical properties of kraft pulp treated with recombinant β Mannanase from *Bacillus* sp.22.

Sample	K= $(p_x f)/w$	%reduction= (C-T)/Tx100
Control	15.00	0
Test	13.48	11.27

CONCLUSION

In conclusion, a mannanase gene, cloned from *Bacillus* sp. 22, was found to encode an alkaline mannanase that may be useful in the pulp and paper industry. β-mannanase was exhibited high thermal and alkli-stability. We are presently attempting to obtain the mannanase gene, which should allow us to study the interaction of recombinant enzymes in the biobleaching of wood pulp.

Moreover, the enzyme is capable of making significant reduction in kappa number thus making the process industrially viable. This knowledge can be helpful for the further improvement of this commercially important mannanase enzyme.

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