

## Full Length Research Paper

# Thermoactive cellulase-free xylanase production from alkaliphilic *Bacillus* strains using various agro-residues and their potential in biobleaching of kraft pulp

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The four bacterial strains were isolated on media containing xylan and screened for xylanase activity. The bacterial strains (Ag12, Ag13, Ag20 and Ag32) were characterized based on morphological, biochemical and physiological characters and identified as belonging to the genus *Bacillus*. The effects of different factors such as pH (7.0 – 10.0), temperature (25.0 – 50.0°C) and inexpensive agro-residues (wheat straw, wheat bran and corncob) on xylanase production of strains were studied under shake flask conditions. Maximal enzyme activities were obtained by cultivation in birch-wood xylan, but high enzyme production was also obtained on wheat straw and corncob when cultivated at pH 8.5. Under optimized fermentation conditions, no cellulolytic activity were detected on the crude extracts. The effects of temperature (40.0 – 80.0°C), pH (6.0 – 10.0) and salt concentration (1.0, 5.0 and 10.0%) on the xylanases activity were determined. The maximum activity was obtained temperature 60.0°C and pH at 9.0. The enzyme was stable at 60.0°C for more than 60 min, suggesting that the xylanases of *Bacillus* strains are thermoactive and being of interests for biobleaching processes. The effectiveness of crude xylanases from the strains Ag12, Ag20 and Ag32 on kraft pulp were carried out at pH 9.0 at 60.0°C. Biobleaching studies of kraft pulp with xylanases and its subsequent treatment with 1.0% EDTA (30 min at 50.0°C) and peroxide (80 min at 70.0°C), showed that the enzymes reduced the kappa number by 27.4, 61.7 and 75.3% and enhanced the brightness by 1.0, 1.5 and 3.0% from xylanases produced by strains Ag12, Ag20 and Ag32, respectively. These results suggest that the application of this xylanases to the paper and pulp industry may be very promising.

**Key words:** Alkaliphilic *Bacillus*, biobleaching, brightness, cellulase-free xylanase, chromophore release, kappa number, kraft pulp, pretreatment, xylanase applications.

## INTRODUCTION

Production of industrial enzymes has been carried out extensively using microbial isolates, which exhibited higher productivity compared to the plants and animal sources. Enzymes are proteins and are considered as potential biocatalysts for a large number of reactions. In living systems, enzymes function in the transformation of macromolecules to energy and new materials, besides for growth, repair and maintenance of cells. Thus, all living things, particularly animal, plant and microorganisms are sources of enzymes. However, for commercial applications of industrial enzymes, microorganisms

are the most important source of various enzymes (Ibrahim, 2008). Thermostable enzymes are highly specific and thus have considerable potential for many industrial applications. The use of such enzymes those are important for industrial utilization because of the possible economic benefits of being able to degrade plant residues at elevated temperatures (Haki and Rakshit, 2003).

Xylanases are groups of enzymes that depolymerize xylan molecules into xylose units used by microbial populations as a primary carbon source (Nath and Rao, 2001). The enzymatic hydrolysis of xylan, a major hemicellulose component of agro-industrial residues, is advantageous for the recovery of hexose and pentose sugars to be used as raw materials in a wide number of biotechnological

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processes. Microbial xylanases represent one of the largest groups of industrial enzymes and they have attracted a great deal of attention during the past few decades. Their potential biotechnological applications in various industries include the food, feed, fuel, textile, detergents, paper and pulp industries and in waste treatment (Beg et al., 2001; Nath and Rao, 2001; Collins et al., 2005; Dhiman et al., 2008). In the paper and pulp industries, xylanases can be used for bleaching kraft pulp. In conventional papermaking processes, manufacturers use more quantity of chemicals, which has resulted in hazardous effluent disposal problems (Ayyachamy and Vatsala, 2007). Treatment with xylanases facilitates the chemical extraction of lignin from pulp and leads to a significant reduction of chlorine dioxide ( $\text{ClO}_2$ ) that hazardous chemical required to achieve comparable levels of paper brightness while simultaneously preserving the paper strength properties, brightness, fibrillation, drainage, among others. For biobleaching applications, the candidate xylanase should be thermostable, alkalitolerant and stable on kraft pulp and its various properties, such as effective molecular weight, net ionic properties and specific action pattern, must suit the process requirements. Moreover, to avoid damage to cellulose pulp, enzyme preparations should be free from cellulase activity (Subramaniyan and Prema, 2002; Damiano et al., 2003; Ayyachamy and Vatsala, 2007; Sudan and Bajaj, 2007).

Although many microorganisms including bacteria (Nakamura et al., 1994; Yang et al., 1995; Gupta et al., 2000; Balakrishnan et al., 2002), actinomycetes (Ball and Mccarthy, 1989; Techapun et al., 2001; Tuncer et al., 2004) and filamentous fungi (Taneja et al., 2002; Angayarkanni et al., 2006; Sudan and Bajaj, 2007) have been reported for xylanase production only a few of them are alkaliphilic or thermophilic. Kraft pulping is carried out under strong alkaline conditions and even after multiple washings, alkali continues to leach from the fiber. Therefore, activity under alkaline conditions is an important characteristic for an enzyme to function in enzymatic prebleaching (Yang et al., 1995). Wild type of *Bacillus* strains capable of secreting xylanases that free of cellulase activity would be attractive for such applications.

Each organism or strain has its own special conditions for maximum enzyme production. Hence, optimization of medium composition has to be carried out in order to maintain a balance among various medium components, thus minimizing the amount of non-utilized components at the end of fermentation. An important factor to be monitored while developing a production medium is the cost of the medium. The use of abundantly available and cost-effective agricultural residues such as wheat bran, corncob and wheat straw provide suitable methods to achieve higher xylanase yields (Gupta et al., 2000; Kapoor et al., 2008).

The present study aimed to report the characteristics of alkaliphilic *Bacillus* strains that we isolated from Soda Lake for xylanase production in alkaline conditions. In

addition, their enzyme productions on various agro-residues and biobleaching potential of crude xylanases on kraft pulp have been described.

## MATERIALS AND METHODS

### Bacterial strains and culture conditions

The alkaliphilic xylanase producing *Bacillus* strains (Ag12, Ag13, Ag20 and Ag32) were isolated by enrichment culture technique from Acı-Göl Lake (Denizli/Turkey) previously (Azeri, 2009). They were maintained in nutrient agar (Sigma) and in modified Horikoshi agar (glucose replaced by 1.0% w/v Birch-Wood Xylan (BW-X, Fluka) (Ikura and Horikoshi, 1987; Horikoshi, 1999) slants supplemented with 2.5% NaCl, at pH 9.0. These bacteria were identified using physiological and biochemical tests according to Bergey's Manual of Determinative Bacteriology (Holt et al., 1994) and other related literatures (Sneath, 1986; Priest and Alexander, 1988; Fritze et al., 1990). For identification of unknown strains, a probability matrix constructed and analyzed using Minitab-pc software package (version 13.20, Minitab Inc., 2000) with the simple matching coefficient ( $S_{SM}$ ). A dendrogram obtained by comparison of the unknown strains with known *Bacillus* species using the single linkage method where scores of 0.85 and above indicated a positive identification. *Bacillus* strains deposited in our culture collection of Microbiology Laboratory, Biology Department, Celal Bayar University, Manisa Turkey.

### Physical treatment of agro-residues

Wheat straw, wheat bran and corncob were ground to particles of 0.5-1 cm. For delignification, 100 g of agro-residues in 500 ml Erlenmeyer flasks were soaked in 1.0% NaOH and autoclaved at 121°C for 20 min. After the alkali treatment, the materials were washed with tap water until neutral and oven-dried. Treated substrates were passed through 0.5 mm screens (Pham et al., 1998).

### Optimization of xylanase production and fermentation

Various fermentation variables were studied to monitor their effect on xylanase production from *Bacillus* strains. These were incubation time in hours (estimating the amount of xylanase produced at different time, up to 48 h at a regular interval of 6 h), initial pH (7.0-10.0) of the medium and temperature (25.0-50.0°C). Several agricultural residues as a carbon sources (BW-X replaced with wheat straw, wheat bran and corncob) were studied by adding them separately at a final concentration of 1.5% (w/v) in the optimized medium.

For xylanase production, initially the strains were inoculated in NA (supplemented with 2.5% NaCl and 0.5% BW-X, pH 9.0) medium that incubated at 35.0°C for 24 h and was used for development of inoculums. The seed culture was prepared in 250 ml Erlenmeyer flasks containing 25 ml of medium by inoculating 2.0 (5.0%, v/v) ml of bacterial suspension and cultivated under agitation (150 rpm) at 35.0°C for 24 h. After optimization of the fermentation parameters, 2.0 ml (5.0%, v/v) of bacterial suspension was transferred to 500 ml Erlenmeyer flasks containing 200 ml of the fermentation medium. Fermentation was carried out in modified DX medium (Nakamura et al., 1993; Yang et al., 1995) containing: BW-X 10.0 g or pretreated agro-residues 15.0 g,  $\text{K}_2\text{HPO}_4$  3.0 g,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  0.5 g, NaCl 20.0 g, trace element solution (Shirling and Gottlieb, 1966) 1.0 ml and distilled water 1000 ml. The pH was adjusted to 8.5 with 1.0 N NaOH prior to sterilization. After required incubation, the filtrate was centrifuged at 14,000 rpm for 10 min to

obtain cell-free supernatant for using enzyme assays.

### Enzyme assays

Xylanase was determined according to Bailey et al. (1992), using 1.0% (w/v) BW-X in 0.5 M tris buffer (pH 9.0) after 5 min reaction time. The assay mixture containing 2.0 ml substrate solution and 0.5 ml suitably diluted enzyme solution in the buffer was incubated at 60.0°C for 5 min and reaction was stopped by addition of 3.0 ml dinitrosalicylic acid (DNS) reagent followed by keeping at 90.0°C for 5 min and absorbance read at 540 nm (Spectrophotometer model: Optima Phototech 301-D). The amount of reducing sugar liberated was determined by the DNS method (Miller, 1959) using xylose (Sigma) as a standard. One unit (IU) of xylanase activity was defined as the amount of enzyme required to liberate one  $\mu\text{mol}$  of xylose per minute under the assay conditions. The substrate alone and the inactivated enzyme (at 100°C for 20 min) were used as controls.

A similar method was used to assay carboxymethyl cellulose activity (CMCase), using 1.0% (w/v) carboxymethyl cellulose (Sigma, low viscosity) as a substrate and D-glucose as the standard.

### Effect of temperature, pH and salt concentration on xylanase activity

The optimum temperature of xylanase was determined by incubating the enzyme for different time at different temperatures ranging from 35.0 to 80.0°C, with soluble BW-X (1.0%) in 50 mM glycine-NaOH buffer at pH 9.0. The pH profile of xylanase was evaluated by incubating the enzyme for 10 min, at 60.0°C, in the presence of BW-X solubilized in appropriate buffers: 50 mM sodium phosphate (pH 6.0-8.0) and 50 mM Glycine-NaOH buffer (pH 9.0 - 10.0) (Taneja et al., 2002). Residual enzyme activity was measured by the standard assay. The effect of the presence of different concentrations of NaCl (1.0, 5.0 and 10.0%) on xylanase activity was also measured under optimized conditions.

### Thermal stability of the crude enzyme

The temperature stability of enzyme extract was determined by incubation at 60.0°C for intervals of 5, 25, 45 and 60 min at pH 9.0 and the residual enzyme activity measured (Subramaniyan and Prema, 2000; Beg et al., 2001).

### Biobleaching

Unbleached kraft pulp was obtained from SEKA Çaycuma, Cellulose and Paper Industry (Zonguldak/Turkey) Limited. The pulp was subjected to the following treatments.

### Enzyme treatment and prebleaching

The pulp was subjected to the enzyme treatment in plastic bags. For this, two lots of pulp were taken. One lot was added with enzyme and the other lot as a control was subjected to the similar conditions adding distilled water. The pulp consistency was 5.0% and the enzyme treatment of pulp has been carried out at pH 9.0 at 60.0°C. Enzyme dosage was 10.0 IU/g oven-dried (OD) pulp. Samples were occasionally mixed by kneading the bags during the 2-h incubation. After enzyme treatment, the pulp was washed with distilled water until pH decreased to 7.8. The enzyme filtrates collected were then analyzed for the color and reducing sugar (as

xylose) to find out the efficacy of the enzyme. When not used immediately it was stored in a plastic bag and frozen for later chemical bleaching (Modified from Chauhan et al., 2006).

Chromophore release was carried out on pulps at 5.0% consistency. Methods were the same as described for the enzyme treatment. After incubation for 2-h at 60.0°C, the pulp was dewatered on a separating funnel using Whatman No. 1 filter paper. Chromophore content in the filtrate was measured with Elrepho 3300 spectrophotometer (DataColor-Switzerland) by optical density at 465 nm (Yang et al., 1995) and 237 nm for hydrophobic compounds and phenolic compounds, respectively (Gupta et al., 2000).

### Chemical bleaching

Both the treated and control pulps were subjected to the multistage bleaching process after enzyme treatment according to a modified method by Atik et al. (2006). The EDTA treatment was carried out at 1.0% consistency using 1.0% EDTA for 30 min at 50.0°C. To improve the final brightness of the pulps, all the pulps were given an additional peroxide treatment. Peroxide bleaching was carried out at a consistency of 5.0%, NaOH 1.0%, H<sub>2</sub>O<sub>2</sub> 2.5% at 70.0°C for 80 min. After each bleaching step, pulps were thoroughly washed with distilled water and pulp properties were determined.

### Determination of pulp properties

The treated pulp was thoroughly washed and used to make hand sheets that were prepared under standardized pressure (3.87 kg/cm<sup>2</sup> for 5 min), light, humidity and temperature, following the Technical Association of Pulp and Paper Industry (TAPPI) recommendations (TAPPI T 205 SP-95). The Kappa number was determined by the reaction of pulp samples with acidified potassium permanganate (TAPPI Protocol, T 236 OM-85). The brightness (%ISO) of the paper sheet was measured by reflectance at 457 nm with an Elrepho 3300 DataColor instrument (TAPPI Protocol, T 452 OM-87) (Anon, 1991). The results of reducing sugar, color of filtrate, brightness and kappa number presented here are the average value of two experimental values.

## RESULTS AND DISCUSSION

### Characterization of the *Bacillus* strains

*Bacillus* strains were isolated from a soda lake (pH: 8.2, salt concentration 9.90%). They characterized according to a great variety of morphological, cultural, physiological and biochemical features. The strains are gram-positive, catalase and oxidase-positive, bacil-shaped bacteria, aerobic and grow readily on most of the nutrient media. The organisms are capable of growing over a wide pH range (from 7.0 to 10.0). All strains grow at temperatures ranging from 25.0 to 50.0°C and an optimal temperature was 40.0°C, but they do not grow at 55.0°C. Some differences occurred in hydrolysis of starch and gelatin; only Ag12 reduced nitrate. There were growth detected at higher concentrations of NaCl (up to 10.0%), but Ag32 may be halophilic bacterium due to it does not growth without NaCl. Other physiological and biochemical features of strains are summarized in Table 1. For determination growth curves; strains cultivated in modified Horikoshi medium supplemented with 2.5% NaCl at pH

**Table 1.** Morphological, physiological and biochemical characteristics of *Bacillus* strains.

Characteristics	Isolates			
	Ag12	Ag13	Ag20	Ag32
Gram reaction	+*	+	+	+
Morphology	Bacil	Bacil	Bacil	Bacil
Metabolism	aerobic	aerobic	aerobic	aerobic
Oxidase	+	+	+	+
Catalase	+	+	+	+
Nitrate reduction	+	-	-	-
Acid from				
Glucose	+	+	+/-	+/-
Mannitol	+	+	+	+
Lactose	-	-	-	-
Xylose	+	+	+	+
TSI	+	+/-	-	-
Gas from glucose	-	-	-	-
H <sub>2</sub> S (TSI)	-	-	-	-
Hydrolysis profile				
Starch	-	+/-	+	+
Casein	-	-	-	-
Tyrosine	-	-	-	-
Gelatin	-	+	-	+
Lecithinase	-	-	-	-
Indole formation	-	-	-	-
Citrate utilization	-	-	-	-
Methyl red (MR)	+/-	-	+	-
Voges-Proskauer (VP)	+/-	-	+	-
Salt tolerance (% w/v)				
%0.0	+	+	+	-
%2.5	+	+	+	+
%5.0	+	+	+	+
%10.0	+	+	+	+
Growth at pH 10.0	+	+	+	+
Growth temperature (°C)				
50.0°C	+	+	+	+
55.0°C	-	-	-	-

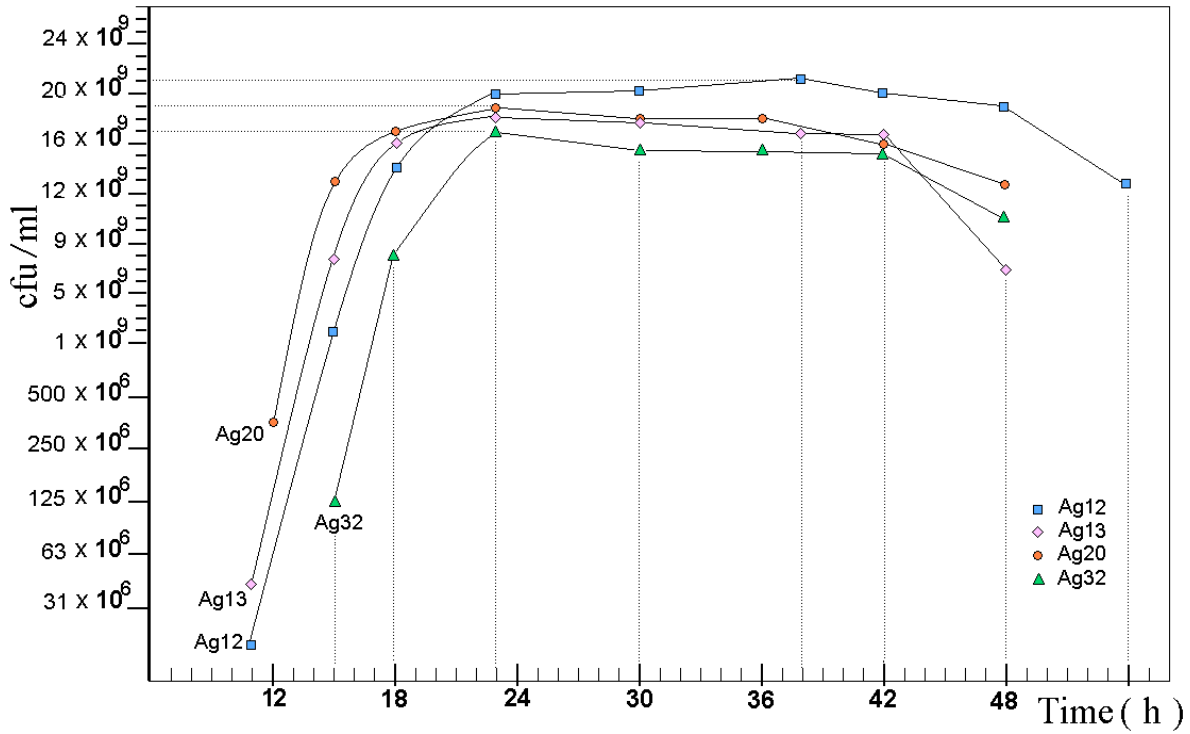
\* Symbols: +, positive; -, negative; +/-, weak positive; TSI, triple sugar iron agar.

8.5 and bacterial cell counted both cultivation in suitable media and microscopy at 6 h intervals. The growth curves were similar to that shown in Figure 1.

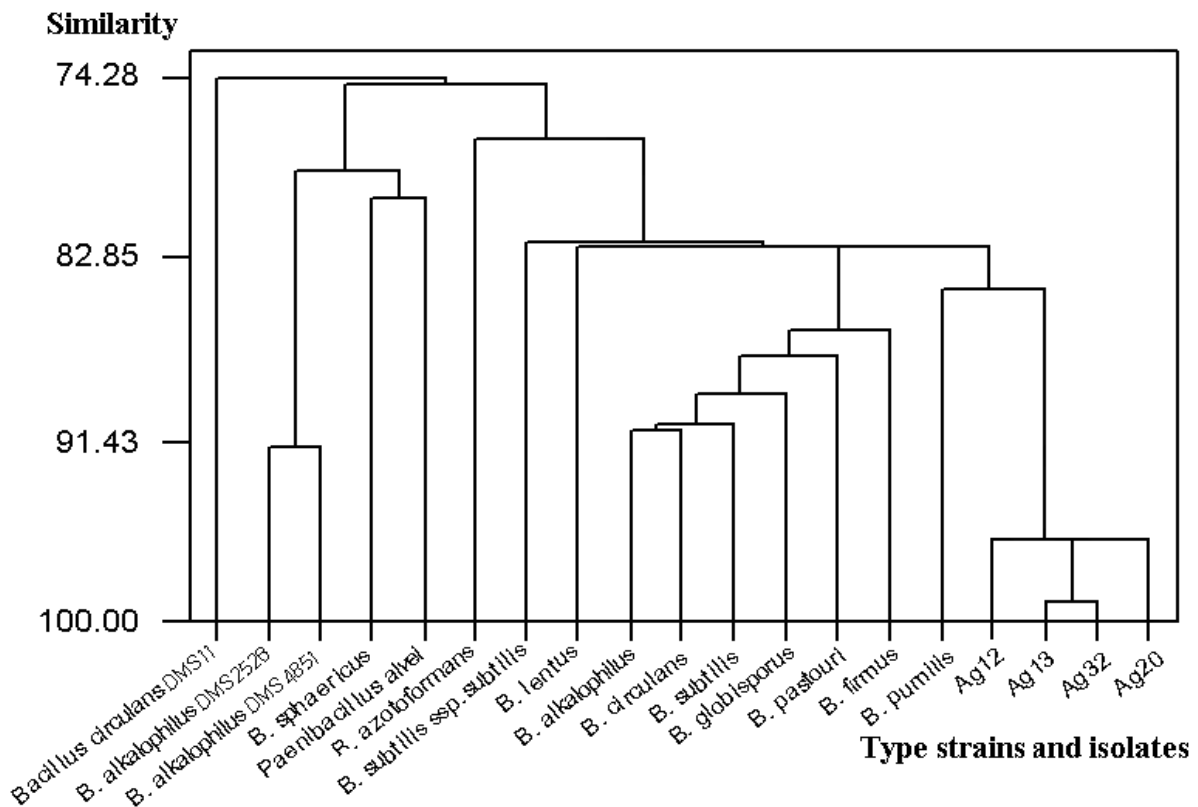
Based on the morphology and physiological characteristics indicated that the organisms belonging to the genus *Bacillus* and appeared closely related to *B. pumilis* using standard techniques and protocols mentioned in Bergey's Manual of Systematic Bacteriology (Figure 2) (Sneath, 1986). However, molecular analyses based on 16S rDNA and DNA-DNA hybridization with related species has to be determined to find out their exact taxonomic position.

#### Effect of various agricultural residues on xylanase production and optimization of fermentation parameters

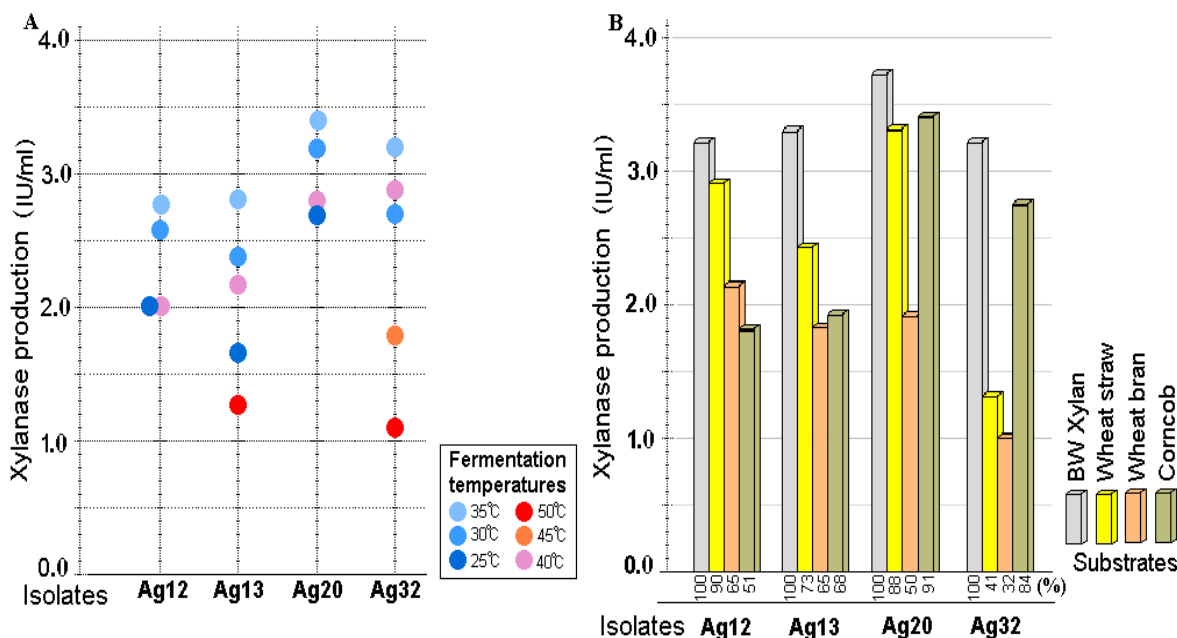
Preliminary studies were conducted in an alkaline medium containing birchwood xylan as the primary carbon source to production of xylanase of *Bacillus* strains at pH 8.5 at different temperatures (25.0-50.0°C) under shaking conditions (150 rpm). For xylanase activity, the dinitrosiacylic acid (DNS) method (Miller, 1959) is used under standard conditions. Maximum xylanase values of strains were similar, ranging from 2.4 to 3.7 IU/ml, obtained



**Figure 1.** Growth curve of *Bacillus* strains (values was obtained by microscopy counting and confirmed with culturing on respective culture media, cfu; colony forming unit).



**Figure 2.** Dendrogram showing relationships based on phenotypic characters of selected *Bacillus* isolates and type strains.



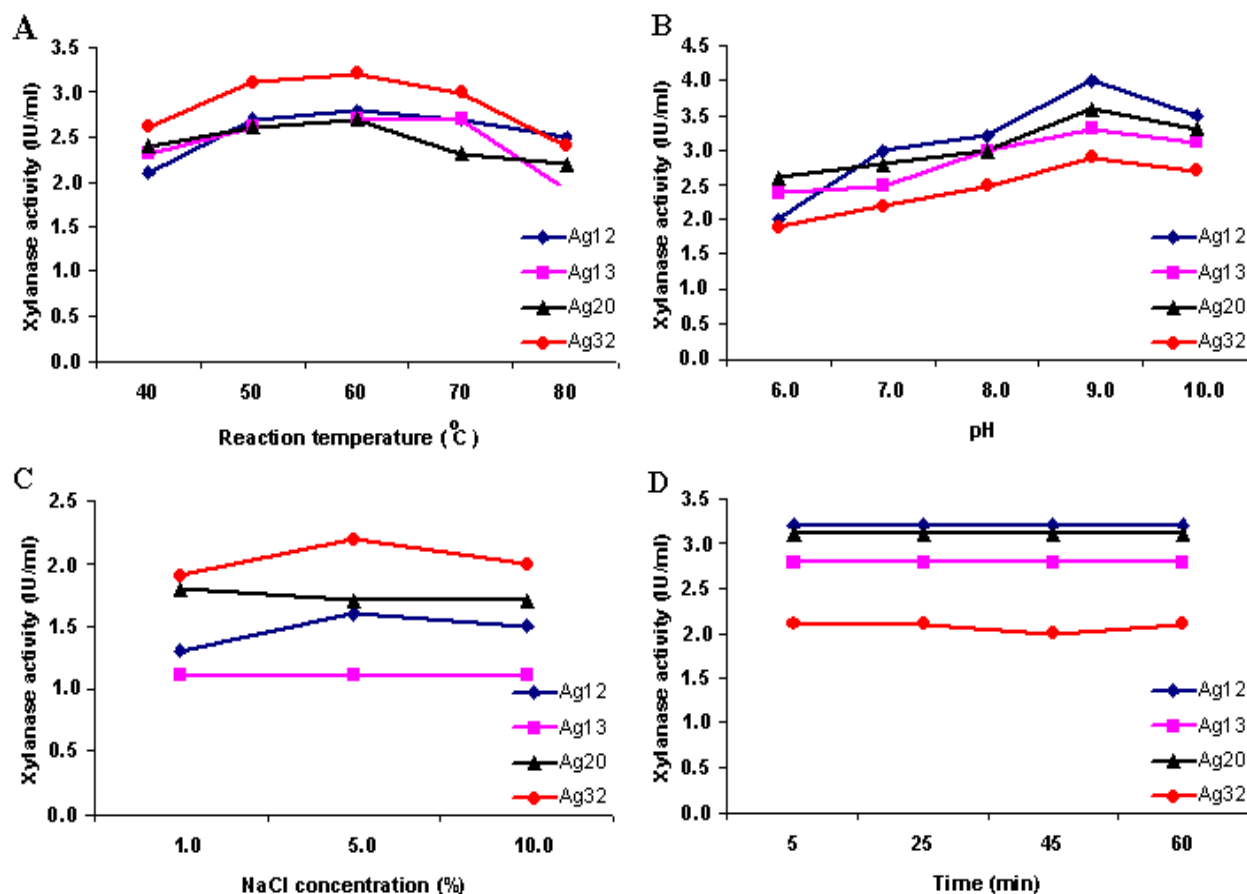
**Figure 3.** Effect of cultivation temperature (A) and various agro-residues (B) on xylanase production by *Bacillus* strains. The cultures were cultivated in modified Horikoshi broth including 1.0% BW-X or 1.5% agro-residues under shaking conditions (150 rpm), at pH: 8.5, enzyme activity expressed as IU/ml  $\pm$  0.1. The activity for birchwood xylan was defined as 100%.

at 42-48 h incubation, at 35.0°C (Figure 3A). The most xylanase production strain was Ag20. On the other hand, our results show that the xylanase production of strains were maximum during the stationary phase. In parallel, experiments other enzyme activities such as cellulase (CMCase) was examined, but no cellulase activity was detected for all strains.

The influence of different agro-residues on extracellular xylanase production was investigated. In these experiments, lignocellulosic carbon sources were wheat bran, wheat straw and corncob at a concentration of 1.5%. In the present study, agricultural substrat dosage has been chosen according to the literature reported value and further optimization will have to be carried out. Enzyme activities were measured after 48 h of incubation at 35.0°C at pH 8.5. The highest (3.7 IU/ml by Ag20) of xylanase was produced on birchwood xylan (the activity for birchwood xylan was defined as 100%) following treated (with alkaline conditions at 121°C for 20 min) corncob (91%) and treated wheat straw (88%) whereas much lower levels of xylanase activities (1.1 IU/ml by Ag32; activity 32%) were produced with wheat bran. Similar results of xylanase production were obtained on these substrates for all strains (Figure 3B). The xylanase synthesis level on xylan or xylan containing substrates such as corncob, wheat straw and wheat bran suggests that xylan is necessary for the effective induction of xylanase by *Bacillus* strains. This data may be explained not only because xylan is the main carbon source, but probably also because its hydrolysis products act as

inducers. Our results clearly indicate that alkaline treatment was an effective method for improving the lingo-cellulosic materials as substrate for higher yields of xylanase. The increased value of xylanase in delignified materials could be due to easier access of the hemicelluloses to the organism. However, it should be noted that the differences and lower xylanase production by *Bacillus* strains grown on agricultural residues might be attributed to less accessible sites in the substrate for xylanase attack due to the presence of lignin (Pham et al., 1998).

Among the xylanases from *Bacillus* species, similar results have been reported for *Bacillus polymyxa* (Pham et al., 1998) and *Bacillus circulans* (Bocchini et al., 2003). Yang et al. (1995) reported that an alkaliphilic *Bacillus* sp isolated from kraft pulp produced 49.0 IU/ml of xylanase when cultivated in alkaline medium at pH 9.0. Maximal enzyme activity was obtained by cultivation with 2.0% birchwood xylan and 1.0% corn steep liquor at pH 9.0. Nevertheless, high enzyme production was also obtained on wheat bran. However, in their study, the xylanase temperature optimum (at pH 7.0) was 55.0°C that was higher than our results. Kapoor et al. (2008) have optimized the cultural conditions (pH 9.0, agitation 200 rpm, inoculum size 1.25% and inoculum age 2 h) for maximum xylanase production by an alkaliphilic *Bacillus pumilus* strain MK001. The bacterium secretes high levels of xylanase on agricultural residues (wheat bran 1220 IU/ml; wheat straw 900 IU/ml) as well as on synthetic xyans (birch wood xylan 1190 IU/ml; oat spelt xylan 1150 IU/ml). How-



**Figure 4.** Influence of the temperature (A), pH (B), salt concentration (C) on the activity of xylanases and thermal stability profile of them (D) produced by *Bacillus* strains (Buffers used: phosphate buffer (pH 6.0 – 8.0), glycine–NaOH buffer (pH 9.0 – 10.0). Thermal stability was determined by preincubating the xylanase at pH 9.0; at 60.0°C for 1 h. Influence of the temperature on enzyme activity was determined by DNS assay, at pH 9.0 and 60°C, for 5 min, by DNS assay, and was expressed as the IU/ml  $\pm$  0.1.

ever, in the present study, xylanases production using wheat bran and wheat straw were very less. In another study, a *Bacillus subtilis* strain isolated from a hot spring was shown to produce xylanolytic enzymes using oat spelts xylan as xylanase inducer. Optimal xylanase production was achieved 12.0 UI/ml at pH 6.0 and 50.0°C, within 18 h fermentation (Sá-Pereira et al., 2002).

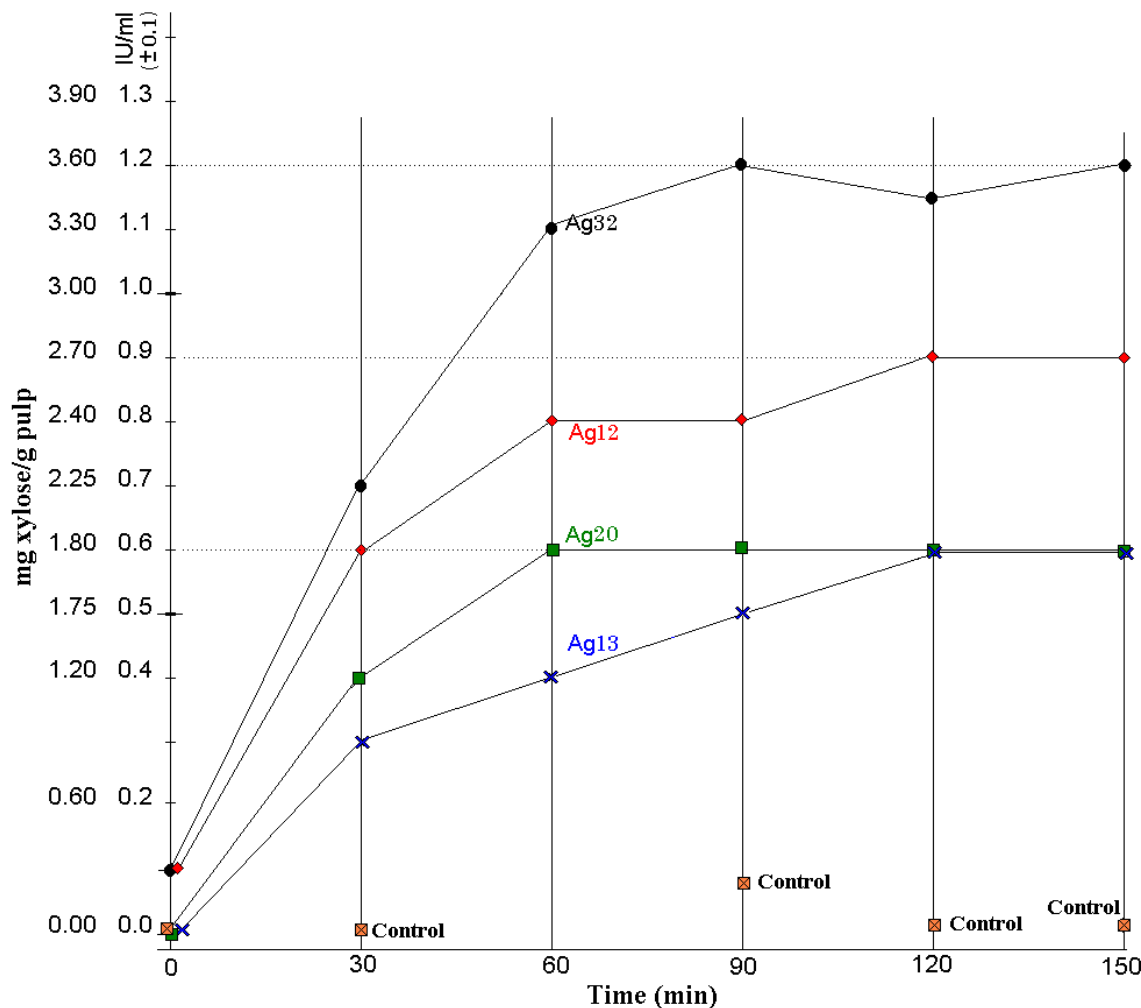
#### Effect of temperature, pH and salt concentration on xylanase activity and thermal stability

In biobleaching process using xylanase, major important parameters are enzyme stability at higher pH and temperature. For the effect of temperature on enzyme activities, the reaction mixtures were determined in the temperature range of 40.0–80.0°C at a pH of 9.0 using the methods as described in material and method section. The xylanases remained their activity high at 80.0°C and they reached the maximum activity at 60.0°C (Figure 4A).

The optimum reaction pH was investigated with crude

culture supernatant within pH 6.0–10.0 at a temperature of 60.0°C. It has been observed that the xylanase activity of the *Bacillus* strains detected over a wide pH range from 6.0 to 10.0 and reached the maximum at pH 9.0 (Figure 4B). When the effect of NaCl concentrations was studied, maximum xylanase activity observed at 5.0% NaCl and was stable up to 10.0% NaCl (Figure 4C). The long-term temperature stability of crude xylanases from bacteria grown at pH 8.5 were stable at temperatures up to 60.0°C even after 60 min (Figure 4D). As indicated above, the four *Bacillus* strains produced xylanases possessing similar activity of pH, temperature, NaCl concentration and thermal stability. This may be attributed to the strains showing high relationships with each other based on phenotypic characters and their extracellular xylanases may be have same chemical structure.

Sá-Pereira et al. (2002) reported that optimal xylanase activity of a *B. subtilis* strain was at 60.0°C on phosphate buffer, at pH 6.0. The xylanase was thermostable, presenting full stability at 60.0°C during 3 h. They also reported that further increase in the temperature caused



**Figure 5.** Released sugars values of xylanases on kraft pulp produced by *Bacillus* strains (released sugar was expressed as the mg xylose/g OD pulp).

a correspondent decrease in the residual activity. At 90.0°C, 20% relative activity remains after 14 min.

### Evaluation of enzyme as a biobleaching agent

In the present study, the effect of the treatments on paper quality was analyzed by determining the amount of chromophores and released sugars, kappa number and brightness. The crude xylanase dose of three strains (Ag12, Ag20 and Ag32) of *Bacillus* for biobleaching of kraft pulp has been evaluated at 60.0°C at pH 9.0, pulp consistency of 5.0% and optimized enzyme as 10.0 IU/g OD. The cell-free crude enzyme efficiency on kraft pulp was maximum after 2 h of incubation. The color released from the pulp (chromophores) (A237 nm) and hydrophobic compounds (A465 nm) was also observed to be maximum after 2 h of reaction time. The effect of enzyme treatment on pulps reduced the kappa number and increased the release of sugars. Highest released sugars

obtained xylanase by the strain Ag32 (3.6 mg/g OD pulp) followed by Ag12 (2.7 mg/g OD pulp) (Figure 5). Maximum kappa number reduction was observed at higher pH of 9.0. The effect of higher enzyme dose and prolonged incubation was not enhanced the biobleaching significantly. After the enzymatic treatment of pulp, subsequent EDTA treatment and bleaching with hydrogen peroxide has been carried out. To determination of the quality of the paper, the treated and untreated pulps were used to make hand sheets for analyzing optical properties. The chemical treatment process itself reduced the kappa number from 19.93 to 9.71 (48.7%) and increased the brightness from 51 to 79 (54.9%) ISO units. However when the enzyme of pretreatment process applied (prior to EDTA and peroxide treatment) by Ag12, Ag20 and Ag32 reduced the kappa number by 7.04 (27.4%), 3.72 (61.7%) and 2.39 (75.3%), increased the brightness by 80 (1.0%), 80.5 (1.5%) and 82 (3.0%) ISO units, respectively (Table 2).

Xylanase from alkaliphilic *Bacillus licheniformis* 77-2 at



**Table 2.** Physicochemical properties of chemical treated and xylanases - chemical treated pulps.

Pulp	Kappa number	Brightness (% ISO)	Absorbance at 237 nm	Absorbance at 465 nm	Efficiency of delignification <sup>c</sup>
Control (untreated)	19.93	51	ND	ND	ND
Chemical treated pulp <sup>a</sup>	9.71	79	ND	ND	51
Ag12 <sup>b</sup>	7.04	80	3.6	0.23	64
Ag20	3.72	80.5	2.80	0.17	82
Ag32	2.39	82	4.1	0.22	88

<sup>a</sup> Pulp bleaching by EDTA (1.0%) followed by hydrogen peroxide treatment.

<sup>b</sup> The enzyme treatment of pulp has been carried out at pH 9.0 at 60.0°C. Enzyme dosage was 10.0 IU/g OD pulp. ND, not determined.

<sup>c</sup> Efficiency of delignification was calculated according to Duarte et al. (2003).

a dose of 14.0 IU/g dry of eucalyptus kraft pulp at pH 6.0 and 60.0°C for 4 h, reduced the kappa number and resulted in an increase in brightness of the paper. Thus Kappa number and brightness, respectively 28.5 and 30% less ClO<sub>2</sub> was required in comparison to the enzymatically untreated samples (Damiano et al., 2003). Zheng et al. (2000) have reported that cellulase-free xylanase from an alkaliphilic *Bacillus* sp. was maximally active at pH 10.0 and 60.0°C on biobleaching of ramie fibers and they have reported a brightness increment of 5.2% and boosted the effect of H<sub>2</sub>O<sub>2</sub> bleaching. Biobleaching of kraft pulp with xylanases from *Staphylococcus* sp. SG-13 (Gupta et al., 2000) and its subsequent treatment with 8% hypochlorite, at a dose 1.8 IU/g, reduced the kappa number by 30%, enhanced the brightness by 11% and improved the strength properties of the pulp. The treatment of bagasse pulp with xylanase from *Bacillus* sp. NCIM 59 resulted in 21% reduction in kappa number and 2.5% increased in brightness (Kulkarni and Rao, 1996). Angayarkanni et al. (2006) reported that xylanase enzymes of three fungi, *Aspergillus indicus*, *A. flavus* and *A. niveus*, increased in brightness of 42.0 – 45.0 ISO units from 19.83 ISO units and reduced kappa number of 5.0-6.8 from 18.60 after the enzyme pretreated pulp when subjected to alkali extraction process. Thus, increase of brightness and kappa number reduction observed in this study with xylanases treated of kraft pulp are consistent with other studies.

## Conclusions

The pulp and paper technology is one of the fastest growing industries and the use of thermostable xylanases seems attractive since they provide global environmental benefits. However, scaling up of the enzyme production from the respective microorganisms to the level required by the industry remains to be seen. It is also worth mentioning that, extreme thermophiles that are able to secrete xylanase are few. The search for a thermophile with high yield of enzyme and the desired characteristics is still being pursued (Haki and Rakshit, 2003). On

the other hand, thermostable xylanase were studied from a number of bacterial and fungal origins and in the majority of studies are found to be optimally active at, or near, mesophilic temperatures (approximately 40.0 – 60.0°C). Xylanases are also active at pH from 2.0 to 11.0 (Horikoshi, 1996).

The results obtained in the present study had an optimum pH of 9.0 and temperature of 60.0°C. The xylanases were stable for more than 60 min at 60.0°C. The xylanases produced by *Bacillus* strains isolated in this study were stable over a wide range of temperature (40.0-80.0°C) and also increased the brightness and reduced the kappa number during bleaching processes. In addition, the cost of xylanases production will be greatly reduced with inexpensive agricultural substrates such as wheat bran, wheat straw and corncob. Further optimization process will have to be carried out for maximum xylanases production by other genetic engineering methods.

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## REFERENCES

- Angayarkanni J, Palaniswamy M, Pradeep BV, Swaminathan K (2006). Biochemical substitution of fungal xylanases for prebleaching of hardwood kraft pulp. *J. Biotechnol.* 5: 921-929.
- Anon (1991). TAPPI Test Methods. TAPPI Press. Atlanta, USA.
- Atik C, Imamoğlu S, Bermek H (2006). Impact of xylanase pre-treatment on peroxide bleaching stage of biokraft pulp. *Int. Biodet. Biodeg.* 58: 22-26.
- Ayyachamy M, Vatsala TM (2007). Production and partial characterization of cellulase free xylanase by *Bacillus subtilis* C01 using agriresidues and its application in biobleaching of nonwoody plant pulps. *Lett. Appl. Microbiol.* 45: 467-472.
- Azeri C (2009). Investigation of Aerobic Bacteria That Isolated From Denizli Acıgöl Lake In Terms of Their Productions of Xylanases Useable in Paper Industry. Ph.D. Thesis. Institute of Natural and Applied Sciences, Celal Bayar University, p. 146.
- Bailey MJ, Biely P, Poutanen K (1992). Inter laboratory testing of

- methods for assay of xylanase activity. *J. Biotechnol.* 23: 257-270.
- Balakrishnan H, Kamal Kumar B, Dutta-Choudhury M, Rele MV (2002). Characterization of Alkaline Thermoactive Cellulase-free Xylanases from Alkaliphilic *Bacillus* (NCL 87-6-10). *J. Biochem. Mol. Biol. Biophys.* 6: 325-334.
- Ball AS, McCarth AJ (1989). Production and properties of xylanases from actinomycetes. *J. Appl. Bacteriol.* 66: 439-444.
- Beg QK, Kapoor M, Mahajan L, Hoondal GS (2001). Microbial xylanases and their industrial applications: a review. *Appl. Microbiol. Biotechnol.* 56: 326-338.
- Bocchini DA, Damiano VB, Gomes E, Da Silva R (2003). Effect of *Bacillus circulans* D1 Thermostable Xylanase on Biobleaching of Eucalyptus Kraft Pulp. *Appl. Biochem. Biotechnol.* 106: 393-402.
- Chauhan S, Choudhury B, Singh SN, Ghosh P (2006). Application of xylanase enzyme of *Bacillus coagulans* as a prebleaching agent on non-woody pulps. *Process Biochem.* 41: 226-231.
- Collins T, Gerday C, Feller G (2005). Xylanases, xylanase families and extremophilic xylanases. *FEMS Microbiol. Rev.* 29: 3-23.
- Damiano VB, Bocchini DA, Gomes E, Da Silva R (2003). Application of crude xylanase from *Bacillus licheniformis* 77-2 to the bleaching of eucalyptus Kraft pulp. *World J. Microbiol. Biotechnol.* 19: 139-144.
- Dhiman SS, Sharma J, Battan B (2008). Industrial Applications and Future prospects of Microbial Xylanases: a review. *Bioresources* 3: 1377-1402.
- Duarte MCT, Da Silva EC, Gomes IMB, Ponezi AN, Portugal EP, Vicente JR, Davanzo E (2003). Xylan-hydrolyzing enzyme system from *Bacillus pumilus* CBMAI 0008 and its effects on *Eucalyptus grandis* kraft pulp for pulp bleaching improvement. *Bioresour. Technol.* 88: 9-15.
- Fritze D, Flossdorf J, Claus D (1990). Taxonomy of alkaliphilic *Bacillus* strains. *Int. J. Syst. Bacteriol.* 40: 92-97.
- Gupta S, Bhushan B, Hoondal GS (2000). Isolation, purification and characterization of xylanase from *Staphylococcus* sp. SG-13 and its application in biobleaching of kraft pulp. *J. Appl. Microbiol.* 88: 325-334.
- Haki GD, Rakshit SK (2003). Developments in industrially important thermostable enzymes: a review. *Bioresour. Technol.* 89: 17-34.
- Holt J, Krieg N, Sneath P, Staley J, Williams S (1994). Group 18; Endospore-forming Gram-positive rods and cocci. In: *Bergey's Manual of Determinative Bacteriology*, Williams and Wilkins, Baltimore 9: 559-564.
- Horikoshi K (1996) Alkaliphiles-from an industrial point of view. *FEMS Microbiol. Rev.* 18: 259-270.
- Horikoshi K (1999). Alkaliphiles: some applications of their products for biotechnology. *Microbiol. Mol. Biol. Rev.* 63: 735-750.
- Ibrahim CO (2008). Development of applications of industrial enzymes from Malaysian indigenous microbial sources. *Bioresour. Technol.* 99: 4572-4582.
- Ikura Y, Horikoshi K (1987). Stimulatory effect of certain amino acids on xylanase production by alkaliphilic *Bacillus* sp. *Agric. Biol. Chem.* 51: 3143-3145.
- Kapoor M, Nair LM, Chander Kuhad R (2008). Cost-effective xylanase production from free and immobilized *Bacillus pumilus* strain MK001 and its application in saccharification of *Prosopis juliflora*. *Biochem. Eng. J.* 38: 88-97.
- Kulkarni N, Rao M (1996). Application of Xylanase from Alkaliphilic Thermophilic *Bacillus* sp. NCIM 59 in biobleaching of bagasse pulp. *J. Biotechnol.* 5: 167-173.
- Miller GL (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal. Chem.* 31: 426-428.
- Nakamura S, Nakai R, Wakabayashi K, Ishiguro Y, Aono R, Horikoshi K (1994). Thermophilic alkaline xylanase from newly isolated alkaliphilic and thermophilic *Bacillus* sp. strain TAR-1. *Biosci. Biotech. Biochem.* 58: 78-81.
- Nakamura S, Wakabayashi K, Nakai R, Aono R, Horikoshi K (1993). Production of alkaline xylanase by a newly isolated alkaliphilic *Bacillus* sp strain 41M-I. *World J. Microbiol. Biotechnol.* 9: 221-224.
- Nath D, Rao M (2001). pH dependent conformational and structural changes of xylanase from an alkaliphilic thermophilic *Bacillus* sp (NCIM 59). *Enzyme Microb. Technol.* 28: 397-403.
- Pham PL, Taillandier P, Delmas M, Strehaiano P (1998). Production of xylanases by *Bacillus polymyxa* using lignocellulosic wastes. *Ind. Crops Prod.* 7: 195-203.
- Priest FG, Alexander B (1988). A frequency matrix for probabilistic identification of some bacilli. *J. Gen. Microbiol.* 134: 3011-3018.
- Sá-Pereira P, Mesquita A, Duarte JC, Aires Barros MR, Costa-Ferreira M (2002). Rapid production of thermostable cellulase-free xylanase by a strain of *Bacillus subtilis* and its properties. *Enzyme Microb. Technol.* 30: 924-933.
- Shirling EB, Gottlieb D (1966). Methods for characterization of *Streptomyces* species. *Int. J. Syst. Bacteriol.* 16: 313-340.
- Sneath PHA (1986). Endospore-forming Gram-positive rods and cocci. In *Bergey's Manual of Systematic Bacteriology*, Ed: Sneath PHA, Mair NS, Sharpe ME, Holt JG, Williams and Wilkins, Baltimore 2: 1104-1207.
- Subramaniyan S, Prema P (2002). Biotechnology of Microbial Xylanases: Enzymology, Molecular Biology, and Application. *Crit. Rev. Biotechnol.* 22: 33-64.
- Sudan R, Bajaj BK (2007). Production and biochemical characterization of xylanase from an alkalitolerant novel species *Aspergillus niveus* RS2. *World J. Microbiol. Biotechnol.* 23: 491-500.
- TAPPI Test Methods 1996-1997 (1996). T 205 SP-95. TAPPI Press, Atlanta.
- Taneja K, Gupta S, Rao RC (2002). Properties and application of a partially purified alkaline xylanase from an alkaliphilic fungus *Aspergillus nidulans* KK-99. *Bioresour. Technol.* 85: 39-42.
- Techapun C, Sinsuwongwat S, Poosaran N, Watanabe M, Sasaki K (2001). Production of a cellulase-free xylanase from agricultural waste materials by a thermotolerant *Streptomyces* sp. *Biotechnol. Lett.* 23: 1685-1689.
- Tuncer M, Kuru A, Isikli M, Şahin N, Çelenk FG (2004). Optimization of extracellular endoxylanase, endoglucanase and peroxidase production by *Streptomyces* sp. F2621 isolated in Turkey. *J. Appl. Microbiol.* 97: 783-791.
- Yang VW, Zhuang Z, Elegir G, Jeffries TW (1995). Alkaline-active xylanase produced by an alkaliphilic *Bacillus* sp isolated from kraft pulp. *J. Ind. Microbiol.* 15: 434-441.
- Zheng L, Du Y, Zhang J (2000). Biobleaching effect of xylanase preparation from an alkaliphilic *Bacillus* sp. on ramie fibers. *Biotechnol. Lett.* 22: 1363-1367.