

*Full Length Research Paper*

# Production and characterization of thermostable xylanase from *Bacillus subtilis* XP10 isolated from marine water

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Accepted 22 June, 2012

**This study aims is to characterize an efficient bacterium, capable of producing thermostable xylanases. Different bacterial isolates were obtained on medium containing xylan as the sole carbon source from samples collected from Jeddah, Saudi Arabia. Out of 15 xylanase producing isolates, the best xylanase producer was XP10 which was selected for the enzyme production. It was identified based on morphological and some biochemical characters as a species belonging to the genus *Bacillus* and identified as *Bacillus subtilis* XP10. Identification was confirmed by 16S rDNA studies and restriction fragment length polymorphism. Factors affecting enzymes production were studied. Maximum xylanase production was 2.82 U/ml obtained at pH 8 for 4 days at 40°C and 120 rpm. The molecular weight of the purified enzyme was 23 kDa. Fivefold increasing in xylanase production was obtained by construction of recombinant *Escherichia coli* 107 harboring recombinant vector pJET 1.2/blunt/*xynA*. The purified thermostable alkalotolerant xylanase can be used in the treatment of agricultural wastes as well as in the bioremediation of xylan-containing materials.**

**Key words:** Xylanase, polymerase chain reaction based restriction fragment length polymorphism (PCR-based RFLP), 16S rDNA, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE), *xynA* gene.

## INTRODUCTION

Hemicellulose is composed mainly of xylan that constitutes about 20 to 40% of total plant biomass (Sa-Pereira, et al., 2003; Ninawe et al., 2008). It is a linear polymer of  $\beta$ -D-xylopyranosyl unit linked by (1 to 4) glycosidic bonds (Zhang et al., 2007). Xylanase enzyme received great attention for the degradation of plant cell wall materials (Omar et al., 2008), the manufacture of bread, food and drinks, textiles, bleaching of cellulose pulp, ethanol and xylitol production as well as in biomass

process for example, biofuel production and future biorefineries (Subramaniyan and Prema, 2002; Keshwani and Cheng, 2009).

Many xylanases in addition to their producing genes have been identified (Ogasawara et al., 2006) and the alkali-tolerant xylanase producers have mainly been found in the genera *Aspergillus*, *Penicillium*, *Streptomyces*, *Thermoactinomyces* and *Clostridium* (Duarte et al., 2003; Techapun et al., 2003). Furthermore, the commercial xylanase used in many industrial and biotechnological applications has been produced from *Trichoderma* (Huitron et al., 2008). Only a few xylanases exhibit stability under high temperature and alkaline pH conditions including xylanases produced from *Bacillus* which are comparatively thermostable, and can tolerate a wide pH range (Haki et al., 2003) but the cost of production and the low yield of these enzymes are

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**Abbreviations:** XB, Xylan broth; PCR, polymerase chain reaction; SDS-PAGE, sodium dodecylsulfate–polyacrylamide gel electrophoresis.

the two major problems for industrial applications (Kulkarni et al., 2003; Techapun et al., 2003). Our study aim is to isolate xylanase - producing bacterium, studying factors affecting enzyme production, characterizing the purified enzyme and over-production of xylanase by recombinant DNA technology. Degradation of some agroresidues and plant wastes using the pure enzyme was also studied.

## MATERIALS AND METHODS

### Isolation of xylanolytic bacterial strains

Different samples (soil, sand, marine water, waste water, decaying wood and plant wastes) were collected from Jeddah. Enrichment method described by Khasin et al. (1993) and Xylan Broth (XB) medium at pH 8 were used for bacterial isolation. XB medium was composed of (g/l): Yeast extract 1 g, Birchwood xylan 5 g,  $(\text{NH}_4)_2\text{SO}_4$  0.2 g,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  0.5 g,  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  0.25 g,  $\text{KH}_2\text{PO}_4$  0.6 g. Incubations were performed at 40°C and 120 rpm for 4 days. 500 µl of the culture broth was spread on XB plates at pH 8 for 3 to 4 days at 40°C and colonies with different morphologies were picked up and maintained in 40% glycerol at -80°C (Sambrook and Russell, 2001). After incubation, the plates were stained with Congo Red solution (0.5% w/v in 50% ethanol) for 15 min and destained with 1 M NaCl. Xylanase producing bacteria, formed yellow zones around their colonies against a red background, were selected and grown in XB medium for 4 days at 40°C and 120 rpm and the bacterial growth (turbidity at 540 nm) and xylanase activity were recorded. The most active xylanase producing bacterial isolate was selected for enzyme production and characterization.

### Identification of the most active isolate in xylanase production

Bacterium XP10 was characterized using light and electron microscopes. Gram, endospore and flagella staining of the selected bacterium were carried out in addition to some physiological testes and carbohydrate utilization.

### Phylogenetic analysis of 16S rDNA sequence

Genomic DNA from the selected isolate XP10 was obtained (Tork et al., 2010; Weisberg et al., 1991) and the 16S rDNA gene was amplified by polymerase chain reaction (PCR) technique, as described by Tork et al. (2010). For restriction endonuclease digestion, a 20 µl reaction mixture was prepared by mixing 10 µl of unpurified PCR amplicons of 16S rDNA gene, with 10 U of the restriction endonuclease *Aat II* (gacgt/c) or *Hpa I* (gtt/aac). The mixture was incubated overnight at 37°C and was analyzed electrophoretically with a 1.5% agarose gel in 0.5× Tris-borate-EDTA. The obtained bands were stained in ethidium bromide and were visualized by UV transilluminator.

### Amplification of xylanase gene *xyn A*

*XynA* gene was amplified by PCR using forward primer 5'-TCAAGGAACGATCAGCAGCCCGTT-3' and reverse primer 5'-TGTATACGAGTGCTACCTCAAAGT-3'. Primers were designed based on sequence published in Genbank database (accession NC\_000964). Initial denaturation was preformed for 2 min at 95°C. After that, the cycle profile was programmed as follows: 1 min at 55°C, 2 min at 72°C, and 30 s at 95°C. This profile was repeated for

30 cycles, and the final 72°C extension step was increased to 7 min. The reaction mixture was subjected to 1.0% agarose gel electrophoresis and visualized by UV transilluminator. A blunt end PCR fragment was purified and was ligated in cloning vector pJET 1.2/blunt (Fermentas). Recombinant pJET 1.2/blunt/*xynA* was transformed in competent *E. coli* JM107 (Sambrook and Russell, 2001) and the recombinant enzyme was assayed.

### Factors affecting xylanase production by the selected bacterium

Erlenmeyer flasks (in triplicate) containing 48 ml of XB medium (pH 8) supplemented with 1% xylan (w/v) were inoculated with 2 ml of preculture ( $4 \times 10^6$  CFU/ml) and incubated for 4 days at temperatures of 25, 35, 40, 45 and 50°C. Similarly, XB medium was prepared at different pH (3 to 9) in 250 Erlenmeyer flasks which were inoculated as before and incubated at 40°C for 4 days at 120 rpm. The growth and xylanase production was determined. The effect of shaking rate was determined by preparing Erlenmeyer flasks as described before and incubation was carried out at 40°C at different shaking rate (80 to 180 rpm). After each experiment, growth and xylanase production were measured.

### Xylanase assays

Xylanase was determined according to Bailey et al. (1992), using 1.0% (w/v) xylan in 0.5 M Tris buffer (pH 9.0) after 5 min of reaction time. The assay mixture (2.0 ml substrate solution and 0.5 ml of culture filtrate) was incubated at 40°C for 5 min and the reaction was stopped by the addition of 3.0 ml dinitrosalicylic acid reagent, followed by keeping at 90°C for 5 min to stop enzyme reaction and measuring the absorbance at 540 nm. The amount of reducing sugar liberated was determined (Miller, 1959) using xylose (Sigma) as a standard. One unit (U) of xylanase activity was defined as the amount of enzyme required to liberate 1 µmol of xylose /minute under the assay conditions. The substrate and the inactivated enzyme (at 100°C for 30 min) were used as control.

### Protein determination

Protein concentration was measured using Lowry method (Lowry et al., 1951) and bovine serum albumin (Sigma, USA) was used as a standard. Protein content of the fractions obtained from the chromatographic column was measured by monitoring the optical density at 280 nm.

### Production and purification xylanase

The selected bacterium XP10 was grown at 40°C in 2 L of XB medium supplemented with 1% xylan at pH 8 and distributed in 500 ml Erlenmeyer flasks. Each flask was inoculated with 2 ml of the preculture ( $4 \times 10^6$  CFU/ml). After growth, the cultural filtrate was collected and precipitated with ammonium sulfate (80%) at 4°C and the precipitate was collected. The obtained precipitate was dissolved in a 0.05 M Na-phosphate buffer, pH 9 and dialyzed against the same buffer at 4°C for two days. Purification was carried out using Sephadex G-200 (Sigma-Aldrich Co., USA) and anion exchange DEAE Sepharose FF (Sigma-Aldrich Co., USA) column chromatography.

### Characterization of the purified xylanase

The effect of different pH on xylanase activity was measured in a

**Table 1.** Morphological characteristics of the isolate XP10.

Morphological characteristic	Result
Source	Marine water
Shape	Monobacillus
Motility	Motile
Presence of capsule	No
Arrangement of Flagella	Polar
Gram reaction	positive
Spore formation	Spore formed
Diameter	(0.4-0.7, 1.4-5 $\mu\text{m}$ )
<b>Cultural Characteristic</b>	
Color	white
Form	Circular
Margin	lobate

reaction mixture containing 0.8 ml of oat spelt xylan solution and 0.2 ml of the purified enzyme within pH range of 5 to 11. 50 mM of the following buffers were used acetate; buffer for pH 4.0, 5.0, sodium phosphate buffer for pH 6.0, 7.0, Tris-HCl buffer for pH 8.0 to 10.0 and enzyme mixture was incubated at 40°C for 15 min. The effect of different temperature on enzyme activity was measured using 50 mM Tris-HCl buffer (pH 8) and was incubated at temperature range of 30 to 95°C for 15 min. The enzyme activity was estimated and the relative activity was calculated. Thermostability of xylanase was studied at different temperatures (50 to 90°C) for different time intervals up to 30 min using 50 mM Tris buffer pH 8.

#### Xylanase production using different plant materials

Wheat straw, wheat bran, oat bran, barley bran and corncob were ground to particles of 0.5 to 1 cm. After delignification (Pham et al., 1998), each obtained material was added to XB medium at 5% (w/v) instead of xylan. Growth and xylanase production were measured as described before.

#### Molecular weight determination

Molecular weight of xylanase was detected according to Laemmli (1970). Low molecular weight protein standard (Amersham Pharmacia) ranged from 14 to 97 kDa was used to determine the enzyme molecular weight.

#### Statistical analysis

Each experiment was carried out in three replicates. Means of variable and standard deviation were recorded and *t*-student test was carried out to detect any significant differences between the results of control and the treated samples

## RESULTS

Fifteen bacterial isolates were screened on XB medium. Xylanase production was detected using Congo Red

solution. The diameter of the yellow zones around the colonies was ranged from 13 to 22 mm. Xylanase production in liquid medium ranged from 0.11 to 0.21 U/ml (data not shown). The most active isolate in xylanase production (2.82 U/ml) was the isolate XP10, which was isolated from marine water (pH 7.9 and salt concentration 9.75%), collected from Jeddah.

#### Taxonomical studies

Bacterial isolate XP10 was characterized according to a great variety of morphological and physiological features (Table 1). XP10 was capable of growing over a wide range of pH (from 5.0 to 10.0) and at temperatures ranging from 25.0 to 50°C. The optimal temperature and pH was 40°C and 8.5, respectively. It had the ability to hydrolyze starch and gelatin (Table 2).

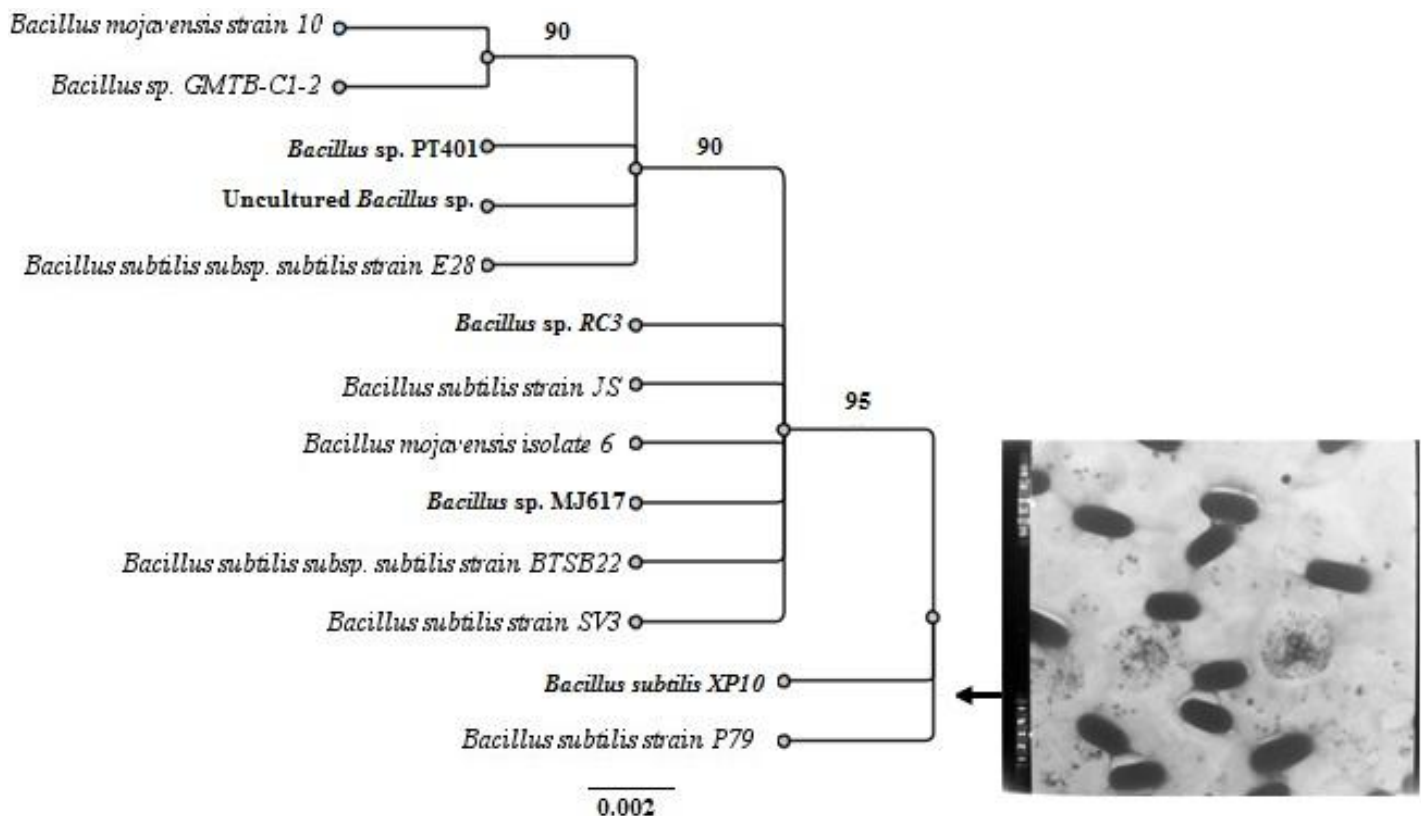
Molecular techniques were used to confirm the identification of the isolate XP10 to the species level. The 16S rDNA sequence was determined and compared to the Genbank database (<http://www.ncbi.nlm.nih.gov>) using the BLASTN 2.2.6 program. The 16S rDNA sequence of XP10 isolate showed high levels of sequence similarity with members of the genus *Bacillus* such as *Bacillus subtilis* (99%) (Figure 1). The partial nucleotide sequence of the 16S rDNA gene was deposited in the Genbank database (the accession number JF807058).

The restriction pattern derived from the distribution of restriction sites within 16S rDNA gene of the isolate XP10 was determined using web cutter database ([www.webcutter.com](http://www.webcutter.com)) (Figure 3). It was demonstrated that endonuclease *HpaI* did not restrict 16S rDNA gene of the isolated strain, whereas, the restriction enzyme *Aat II* digested it. The obtained fragments were 1015, 333 and 169 bp (Figure 2A).

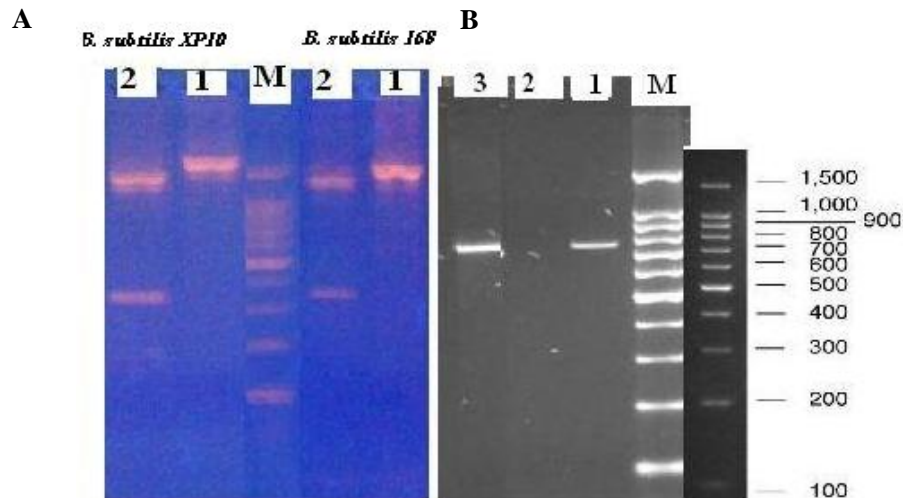
Maximum xylanase production by the bacterium XP10

**Table 2** Physiological characteristics of the bacterium XP10.

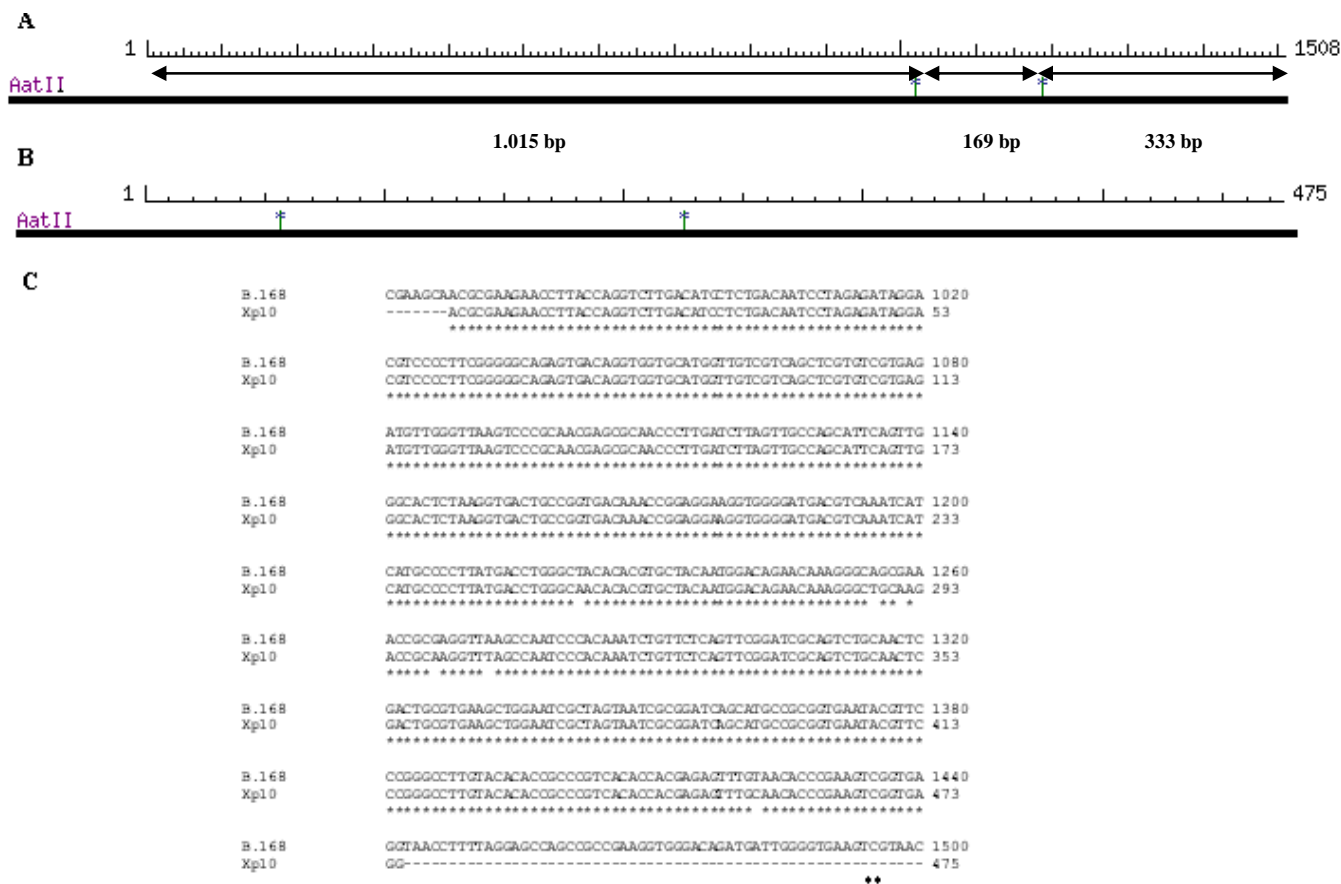
Reaction	Result
Catalase	+
Oxidase	+
Aerobic growth	+
Growth at 10- 45°C	+
50°C	-
Growth at pH 5.0 - 8.0	+
Growth in 3-5 % NaCl	+
Voges-Proskauer	+
DNase	+
Urease	+
Citrate	+
Nitrate reduction	+
Aesculin	+
L-Arginine	+
Dihydroxyacetone	+
Hydrolysis of: Casein, xylan, chitin, cellulose	+
<b>Resistance to antibiotic</b>	
Ampicillin 25 µg	Resistant
Cotrimoxazole 25 µg	Sensitive



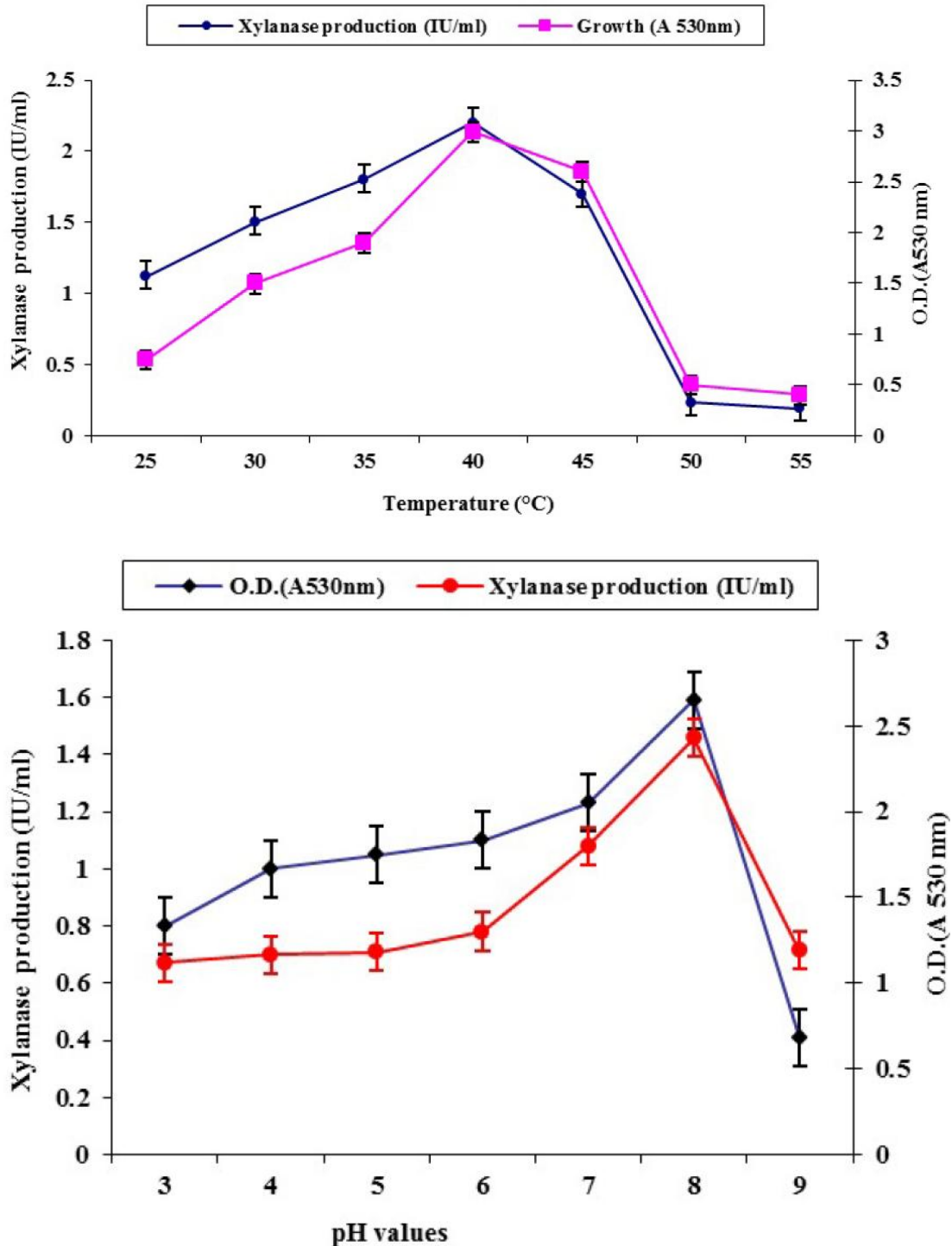
**Figure 1.** Phylogenetic tree of the partial sequence of 16S rDNA of the isolate XP10 with respect to closely related sequences available in Genbank database.



**Figure 2.** (A) RFLP based on 16S rDNA gene analysis of strain Xp10. 16S rDNA gene digested by *HpaI* (Lane 1), by *AatII* (Lane 2). (B) A 740 bp of *xynA* gene, lane 1 is the PCR product of XP10. Lane 2 represents the negative control (*Bacillus* sp.). Lane 3 is the positive control (*B. subtilis* 168). M is DNA Molecular Weight Marker (100 bp).



**Figure 3.** Restriction pattern for *B. subtilis* 168 (A) and for XP10 (B) flanked by restriction enzyme *AatII*. Expected fragments are indicated with arrows. (C) Alignment of partial DNA sequence of 16S rDNA gene of XP10 and with that from *B. subtilis* 168, conserved and identical nucleotides are indicated with asterisks (\*).



**Figure 4.** Effect of different pH values (A) and different incubation temperatures (B) on growth and xylanase production by bacterium XP10.

was obtained at 40°C, pH 8 and at 120 rpm (Figures 4 and 5). The highest enzyme production (2.82 U/ml) was produced on xylan followed by corncob (1.8 U/ml) and wheat straw (1.7 U/ml) whereas much lower levels of

xylanase activities (1.03, 1.06 U/ml) were obtained with oat and barley bran (Figure 6).

Purification of xylanase was carried out using different column chromatography (Figure 7A). A single protein

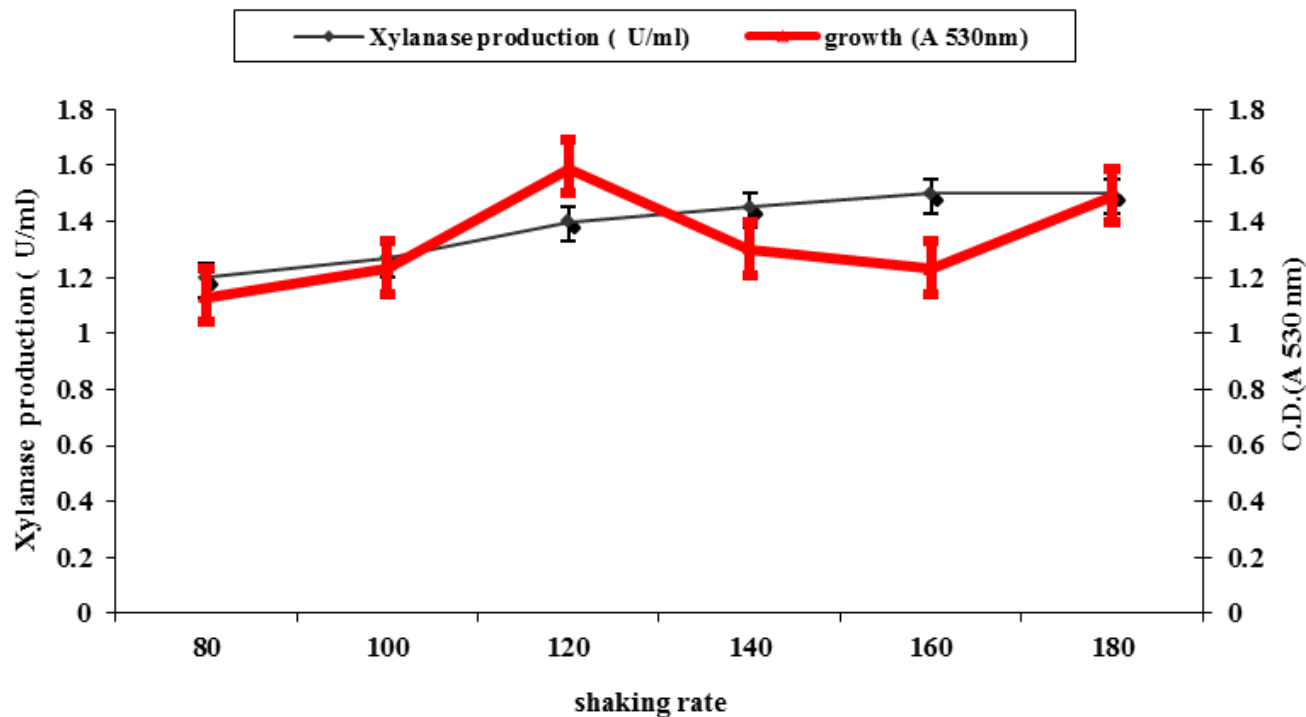


Figure 5. Effect of shaking rate on growth and xylanase production by bacterium XP10.

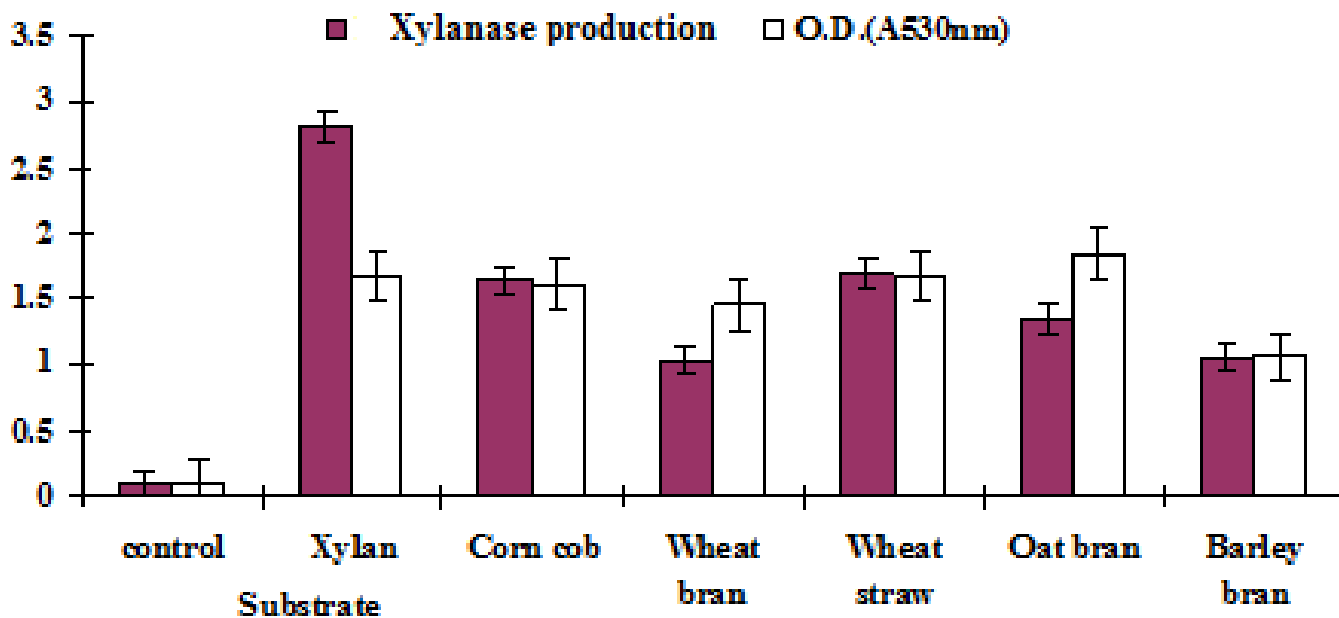
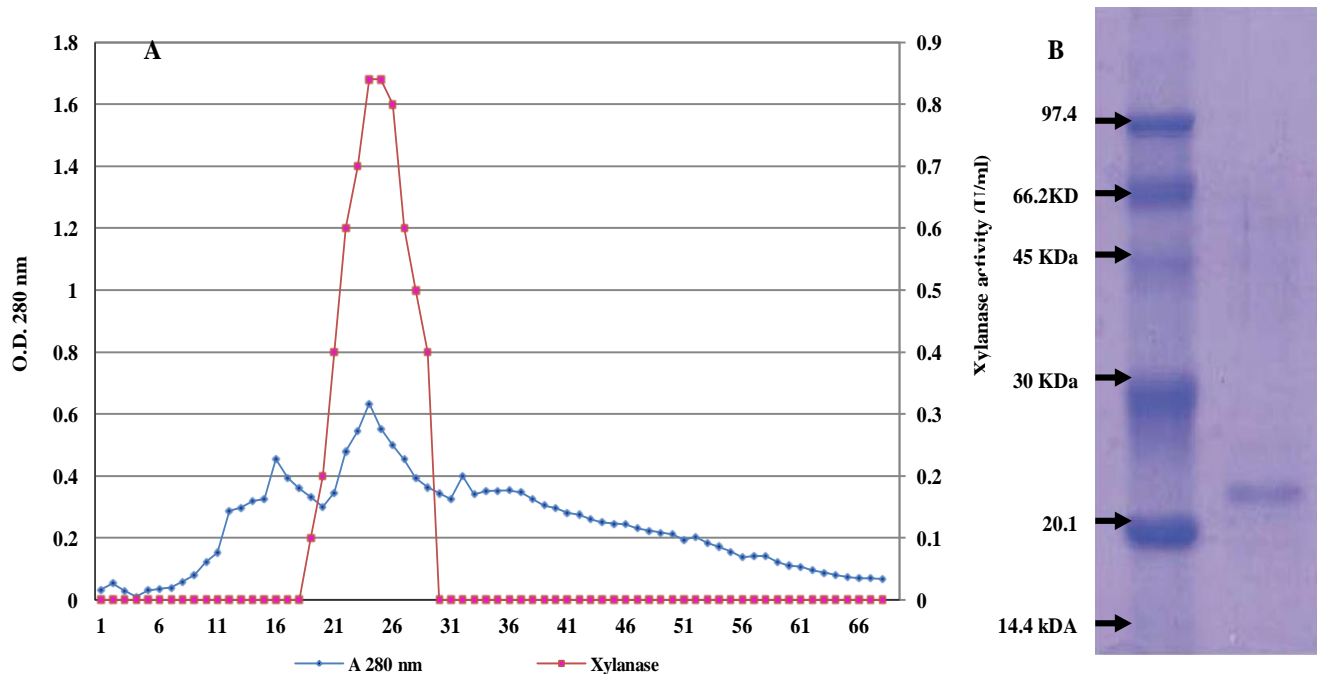


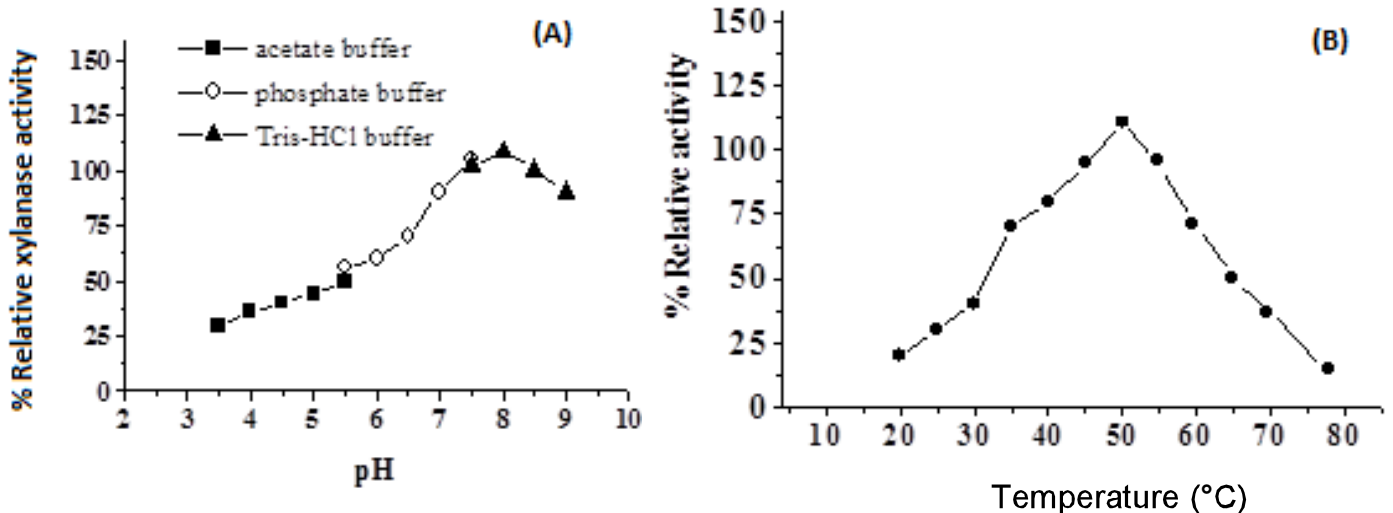
Figure 6. Growth and xylanase production by bacterium XP10 with different plant wastes.

band was shown on sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gel electrophoresis at 23 kDa (Figure 7B). The purified

xylanase exhibited maximum activity at pH 8 and 50°C (Figure 8 A, B). The purified XP10 xylanase retained high activity (100%) at 50°C. Only 33% loss of enzyme activity



**Figure 7.** (A) Elution profile of XP10 xylanase on CM-Sepharose column chromatography. (B) Molecular weight of XP10 xylanase by SDS-PAGE, lane 1: protein standard (97- 14.4 kDa), lane 2: Purified xylanase.



**Figure 8.** (A) Optimum pH of XP10 xylanase. (B) Optimum temperature of XP10 xylanase.

was recorded at 60°C, 20% of activity still remained at 70°C and thermally inactivated at 90°C after incubation for 30 min (Figure 9).

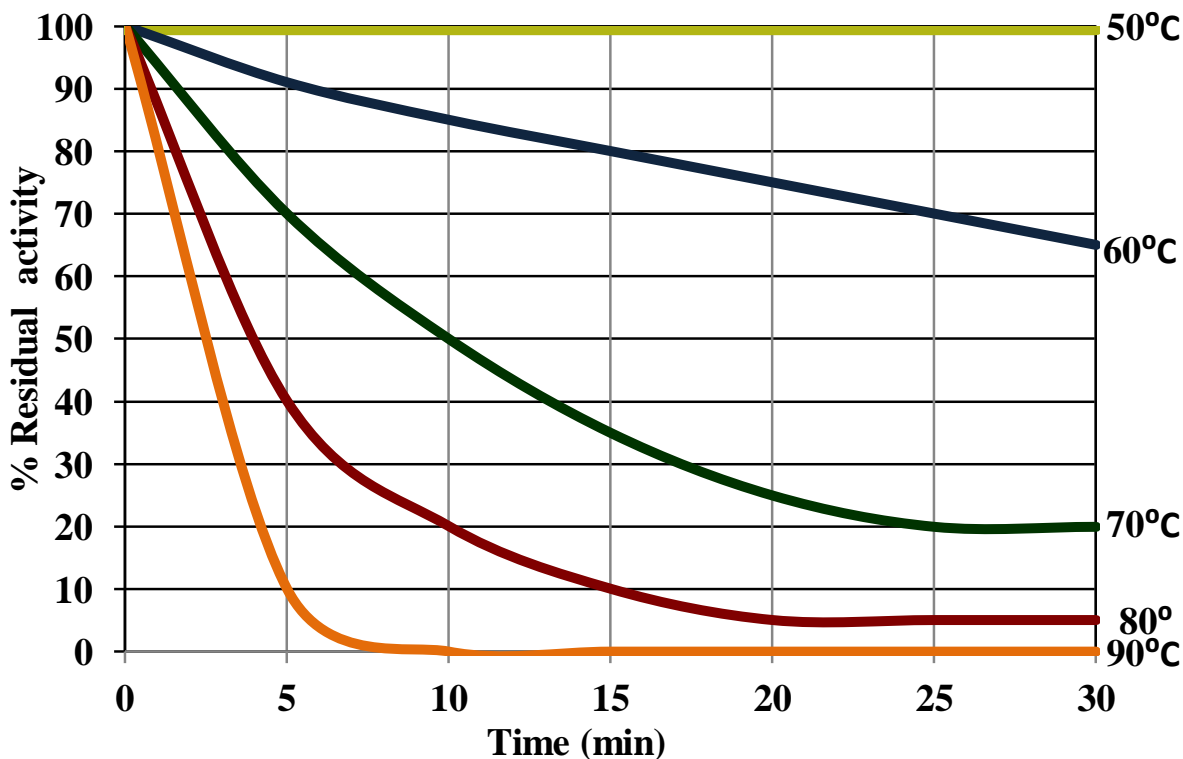
The gene *xynA* without its promoter and terminator was amplified and a 740 bp PCR product (Figure 2B) was cloned and transformed into *E. coli* JM107 (Figure 10). The recombinant xylanase was purified and its activity

increased five times (data not shown) compared to that obtained from the bacterium XP10.

## DISCUSSION

A new local xylanase - producing bacterium XP10 was





**Figure 9.** A schematic diagram showing the effect of the temperature on the stability of an enzyme reaction.

isolated from marine water. A few xylanases from marine bacteria encompassing some unique characters, such as good salt-tolerance have been reported (Guo et al., 2009; Raghukumar et al., 2004; Subramaniyan and Prema, 2002; Wu et al., 2006).

The bacterium XP10 was belonging to genus *Bacillus* and was similar to *B. subtilis* by 99%. (Fritze, 2004; Tork et al., 2010). Restriction pattern confirmed that the isolate XP10 related to *B. subtilis* strains. The previous technique was used by Figueras et al. (2008) to differentiate many species of *Helicobacter*.

XB medium with 1% xylan at optimum temperature 40°C, 120 rpm and initial pH 8 were suitable for high xylanase production after 4 days. Similar results were obtained for *B. subtilis* where highest enzyme activity was achieved at 60°C (Raghukumar et al., 2004) and for *Streptomyces* sp. that showed maximum xylanase production at 50°C (Techapun et al., 2003). This strikingly high temperature contrasts strongly with results obtained by Heck et al., (2005) for *B. subtilis*, where highest enzyme activity was achieved at low temperature (25°C). The time for highest xylanase activity (3 days) corresponds to mid-stationary growth phase which is in agreement with findings obtained by Heck et al. (2005). On contrast, Bocchini et al. (2002) reported that the optimum cultivation time for xylanase production by *Bacillus circulans* D1 is of 48 hr and the pH did not

influence xylanase production. It can be concluded that optimization of culture conditions for xylanase production by *B. subtilis* XP10 increased its production from 2.82 to 3.85 U/ml.

The highest xylanase production (2.82 U/ml) was obtained using xylan which acts as inducer, followed by corncob and wheat straw whereas much lower levels of xylanase activities were obtained with oat and barley bran. Similar results of xylanase production using waste products were obtained (Azeri et al., 2000). Xylanase synthesis on xylan or xylan containing substrates 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.

The molecular weight of the purified enzyme was 23 kDa which was lower or higher than some others reported (Helianti et al., 2010). Xylanase from *B. subtilis* XP10 was thermostable at 50°C, but thermally inactivated at 90°C. Subramaniyan and Prema (2002) previously reported similar results.

The xylanase from *B. subtilis* XP10 was successfully cloned and introduced in *E. coli* JM107. Similar results were found by Helianti et al. (2010). High production of recombinant enzyme (5 fold) may be useful in pulp pre-bleaching process to remove the hemicelluloses (Helianti

GTATTATACTGAAGGGGACG

ATCAAAGCTTTGGCGTTAGTAATTAAGAATGTTTTAAATG**TGATA**

**CGAGTGCTACCTCAAAGT**CGGAAAAAATATTAT**AGGAGG**TAAACAT

742 atgtttaagtttaaaaagaatttcttagttggattatcggcagct  
M F K F K K N F L V G L S A A

697 ttaatgagtattagcttgttttcggcaaccgcctctgcagctagc  
L M S I S L F S A T A S A A S

652 acagactactggcaaaattggactgatggggcggtatagtaaac  
T D Y W Q N W T D G G G I V N

607 gctgtcaatgggtctggcgggaattacagtgttaattggtcta  
A V N G S G G N Y S V N W S N

562 accggaaatttgttgggttaaagggttgactacaggttcgcca  
T G N F V V G K G W T T G S P

517 tttaggacgataaactataatgccggagtttgggcgccgaatggc  
F R T I N Y N A G V W A P N G

472 aatggatatttaactttatagttggacgagatcacctctcata  
N G Y L T L Y G W T R S P L I

427 gaatattatgtagtggattcatggggacttatagacctactgga  
E Y Y V V D S W G T Y R P T G

382 acgtataaaggactgtaaaaagtgatgggggtacatatgacata  
T Y K G T V K S D G G T Y D I

337 tatacaactacacggtataacgcaccttccattgatggcgatcgc  
Y T T T R Y N A P S I D G D R

292 actacttttacgcagtagtggagtggttcgacagtcgaagagacca  
T T F T Q Y W S V R Q S K R P

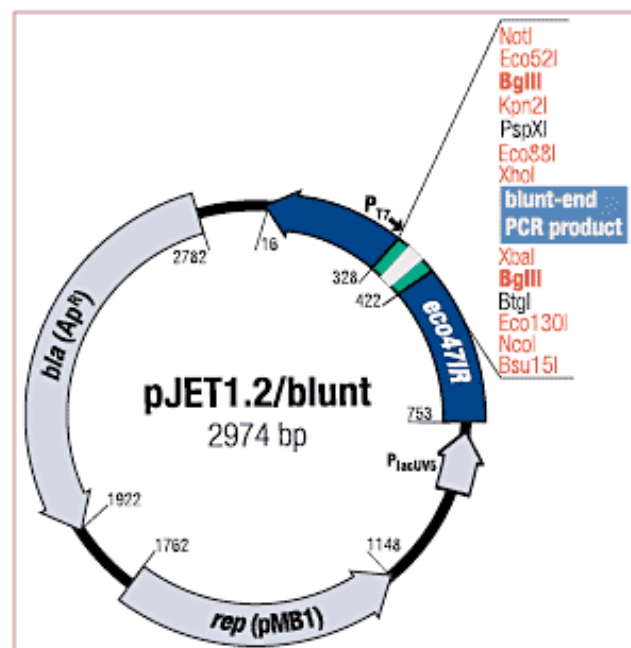
247 accggaagcaacgctacaatcactttcagcaatcatgtgaacgca  
T G S N A T I T F S N H V N A

202 tggaagagccatggaatgaatctgggcagtaattgggcttaccaa  
W K S H G M N L G S N W A Y Q

157 gtcattggcgacagaaggatatcaaagtagtgaagttctaacgta  
V M A T E G Y Q S S G S S N V

112 acagtgtggttaaTATTTTTTCCG**ACTTTGAGGTACGGGCTGCTGA**  
T V W \*101  
**TCGTTCTTGA**GAAAGTTTTATAATCAATGATTATTAATAATCGTTAG

TAATGGTTAAAGGTT



**Figure 10. A:** Nucleotide sequence of *Xyn A* gene and its flanking regions and deduced amino acids sequence. The putative Shine-Dalgrano-type ribosome binding site is indicated in bold capital letters and is underlined. The forward and reverse primers were indicated in bold capital letters and is undelined red colored. the transitional stop codon is marked by asterisk(\*). **B:** pJET1.2/blunt vector map.

et al., 2010). Characterization of the recombinant enzyme will be studied further.

## ACKNOWLEDGMENT

This study was funded by Deanship of Scientific Research (DSR), King Abdulaziz University, Jeddah, Saudi Arabia, under grant No. 17-014/ 430.

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