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Cloning, Expression and Characterization of Xylanase (*xyn-akky1*) from *Bacillus subtilis* in *Escherichia coli*

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ABSTRACT

In this study, *Bacillus subtilis* akky1 strain was isolated from the soil of beech forest in Akkuş City, Ordu Province, Turkey. akky1 strain was identified by 16S rRNA analysis. The full-length 16S rRNA sequence of akky1 strain showed the 100% similarity with *Bacillus subtilis* strain B7 (KC310823.1) . A 642 bp DNA fragment was obtained from genomic DNA using primers designed based on the gene sequence of *Bacillus subtilis* xylanase given in GenBank. The gene encoding xylanase was cloned into pET28b (+) plasmid vector, sequenced and expressed in *Escherichia coli* BL21 (DE3). The hexahistidine (6xHis) tagged fusion protein was purified using nickel affinity chromatography and the xylanase activity was measured. The molecular mass of the purified xylanase was approximately 26 kDa as estimated by SDS-PAGE. The xylanase had optimal activity at pH 6.0 and 60°C. The K_m values of the recombinant enzyme towards beechwood was 3.33 mg/ml.

Keywords: *Bacillus subtilis,* Xylanase, Recombinant Protein, Industrial Enzymes, *Escherichia coli*

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1. INTRODUCTION

Hemicellulose is a heterogeneous polymer composed of pentose (such as xylose, arabinose) and hexose sugars (such as mannose, glucose, galactose) and sugar acids. Hemicelluloses collectively are classified into three groups as xylan, glucomannan, arabinogalactan (1). An essential component of the xylan is 5-carbon sugar, D-xylose, which can be converted into chemical fuel by microbial cells (3). Complete degradation of plant xylanes requires the collaboration of several hydrolytic enzymes because of the complex chemical structure and heterogeneity of the xylan. Therefore, it is not surprising that producing a multitude number of polymer disintegrate enzymes by the xylan digesting microbial cells. The xylanolytic enzyme system which is performed xylan hydrolysis usually consists of several hydrolytic enzymes: β-1,4-endoxylanase, β-xylosidase, α-L-arabinofuranosidase, α-glucuronidase, acetyl xylan esterase and phenolic acid esterase (ferulic acid and p-coumaric acid). Among them, endo-1,4- β-xylanase (1,4-β-D-xylan-xylan hydrolase E.C. 3.2.1.8) is the key enzyme. This enzyme breaks down the glycosidic bonds in xylan structure. Initially, the product of hydrolysis is β-D-xylopyranosyl oligomers and at the later stages small molecules such as mono-, di- and trisaccharides of β-D-xylopyranosyl (16). Endo-1,4- β-xylanase is produced by various microorganisms such as funguses (3; 2), actinomycetes (6) and bacteria (4).

Xylanases derived from microorganisms caught major attention due to their expended industrial applications including textile industry (5), production of xylo-oligosaccharides (14), clarification of juices (3), waste-water treatment (17), bioconversion of lignocellulosic wastes into useful economical products (ethanol, sugar syrups, gaseous fuels etc.) (5), biobleaching of pulp (11, 18).

In this study, xylanase-producing *Bacillus subtilis* strain akky1 was isolated from the soil of beech forest in Akkuş City, Ordu Province, Turkey. The identification of the strain akky1 was performed with PCR amplification of 16S rRNA. Xylanase gene was amplified from the genomic DNA of akky1 strain by polymerase chain reaction using two oligonucleotides. After *Xyn-akky1* gene had been sequenced, it was cloned into the pET28b vector and expressed in *Escherichia coli*. The recombinant xylanase was characterized by biochemical methods.

2. MATERIALS AND METHODS

2.1. Microorganism Isolation and Screening Xylanase Activity

The soil of beech forest was collected from Akkuş City, Ordu Province, Turkey. The growth medium contained 0.25% yeast extract, 0.5% peptone, 0.1% glucose and adjusted to pH 4.8 using HCl. Culter was incubated at 37°C and 250 rpm for 30 h. The diluted cultures were spread on agar plates containing 0.5% peptone, 0.25% yeast extract, 1.0% beechwood xylan and 2.0% agar (pH 4.8). Congo red method has been used to screen xylanase-producing strains (1) The strains identified as xylanase producers were inoculated into 5 ml of PCA medium pH 5.5 and incubated overnight at 37°C at 250 rpm agitation. The isolation of genomic DNA from overnight culture after incubation was carried out in accordance with the manufacturer's recommendation using the kit ‘Fermantes. The isolated genomic DNA and two oligonucleotides were utilized in order to amplify the 16S rRNA (Table 1). Sequence analysis was performed by Refgen Company. So that choosen xylanase-producing strains was identified by 16S rRNA analysis.

Table 1. Two oligonucleotides were utilized in order to amplify the 16S rRNA

Primer	Sequence (5'→3')	Accession Number
Unv-Bac-27F	agagtttgatcmtggctcag	AB579660–765
Unv-Bac-1525R	aaggaggtgwtccarcc	

2.2. Cloning and Expression of the *xylanase* Gene in *Escherichia coli*

Two different oligonucleotides were used to amplify DNA fragment encoding the xylanase gene for *Bacillus subtilis* akky1 (Table 2).

Table 2. Two oligonucleotides were utilized in order to amplify the xylanase gene

Primer	Sequence (5'→3')
xyn1	ttttggatccgatgtttaagtttaaaaag
xyn2	ttttctcgagttaccacactgttacgtt

Genomic DNA used as template DNA for PCR, isolated from *Bacillus subtilis* akky1 using Fermantes genomic DNA prufication kit. *xylanase* gene was cloned to construct the pET28b-xyn recombinant vector DNA using *Xho*I and *Bam*HI restriction enzymes (Figure 1). The positive clones for recombinant xylanase were identified using the Congo Red.

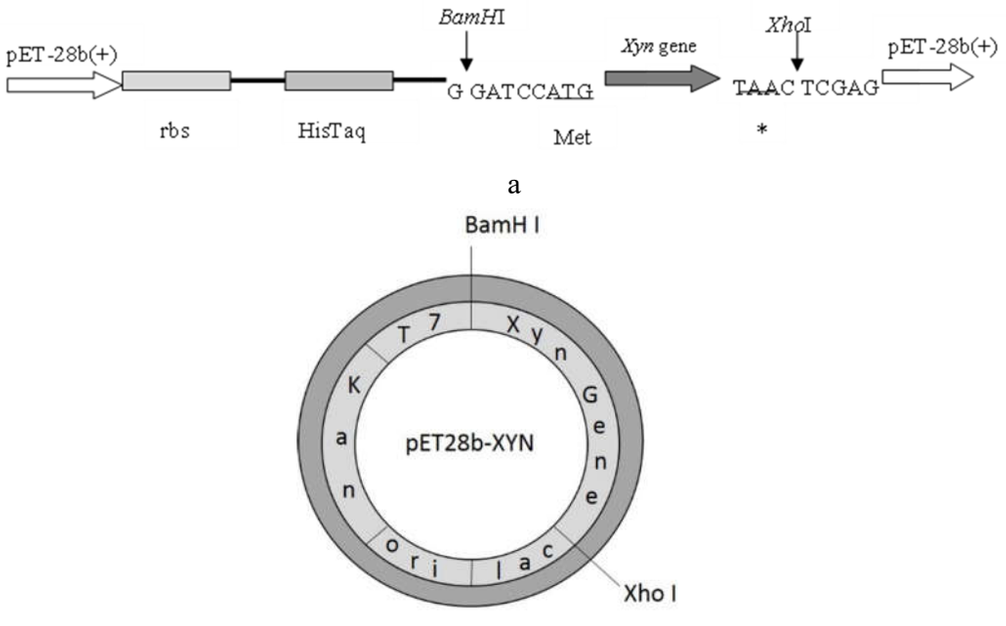

Figure 1. a. Schematic diagram of the gene region where the Xyn DNA sequence transferred to pET-28b (+) vector. b. Circular the pET28b-*xyn* map of construct which used to produce *Bacillus subtilis* xyn-akky1 xylanase.

2.3. Purification of Recombinant Xylanase

The *E. coli* BL21 (DE3) was transformed with the pET28b-xyn construct and growth on Luria Bertani agar containing kanamycin (50 mg/ml). Briefly, the transformant was inoculated into 3 ml culture tube containing LB brorth and incubated overnight at 37 °C and 200 rpm. Then this culture was inoculated into 500 ml of LB containing kanamycin and grown at 37 °C with shaking at 200 rpm. The

culture was induced for 3 hours with a final concentration 1mM IPTG when the OD_{600} reached 06-07.
Next, the cells were harvested by using centrifugation (at +4°C, 8000 rpm 5 min), followed by re-suspended the pellet using RNAse (20 µg/ml) and DNAse (20 µg/ml) with 20 mM phosphate buffer (pH 8.0) and protease inhibitors (0.5 mM Phenyl methyl sulfonyl fluoride (PMSF) and 2 mM Benzamidine). Cells were first lysed using a sonicator (Sonics VCX 130), then high-speed (30,000 rpm) centrifugation was performed for 1 hour. Qiagen Ni-NTA affinity column was used to prufication of soluble recombinant protein carrying N-terminal 6x histidine. The column was washed first with 50 mM phosphate buffer (pH 8.0) and then 50 mM phosphate buffer (pH 8.0) containing 30 mM imidazole. The protein was eluted from column with 300 mM imidazole in 50 mM phosphate buffer (pH 8.0). Purity of this isolated protein was checked by SDS-PAGE. Concentration of protein was determined by UV absorption at 280 nm (19).

2.4. Plate Assay

E. coli strain BL21 (DE3) containing the recombinant plasmid pET28b-xyn was inoculated on LB agar plate containing 1% beechwood xylan, 50 mg/ml kanamycin and 100 mg/ml IPTG. Following overnight incubation at 37ºC, staining of the plates done using 1% Congo-red solution and destained by three washes using 1 M NaCl followed by 0.1 N NaOH. The enzyme activity was examined by a clear zone formation around the colony (Wood et al., 1998).

2.5. Biochemical Characterization

3,5-dinitrosalicylic acid (DNS) method was used to determine the recombinant xylanase activity (10). The optimal pH for purified 6x His tagged enzyme was determined at 37ºC. Beechwood xylane was used as a substrate in wide pH which ranging from 4.0 to 10.0. pH range of substrate was adjusted by Mcllvaine buffer for pH 4-7, Tris-HCl buffer for pH 8, and glycine-NaOH buffer for pH 9-10. To determine the optimal temperature for enzymatic activities the enzyme was incubated between 30ºC to 70ºC in precense of Mcllvaine buffer (pH 6.0). The thermo-stability of the xylanase was tested by pre incubating the enzyme in Mcllvaine buffer (pH 6.0) at 50ºC, 55ºC, 60ºC without substrate. Km and V_{max} values for purified enzyme were calculated in Mcllvaine buffer (pH 6.0 at 60ºC using 1-10 mg/ml beechwood xylan as a substrate). The data were plotted by Lineweaver-Burk method (13).

2.7. Nucleotide sequence accession numbers

Bacillus subtilis strain akky1 16S rRNA nucleotide sequences and xylanase gene were deposited in the GenBank (accession numbers KJ540929.1 and KJ540928.1).

3. RESULTS AND DISCUSSION

3.1. Microorganism Identification Using PCR

Six strains isolated from soil samples collected from Ordu province, Turkey demonstrated xylanolytic activity. New strains were identified using 16S rRNA sequences. The xylanase activity was revealed by strain akky1 which produce highest zone clearance on agar plate containing xylan (Figure 2a,b).

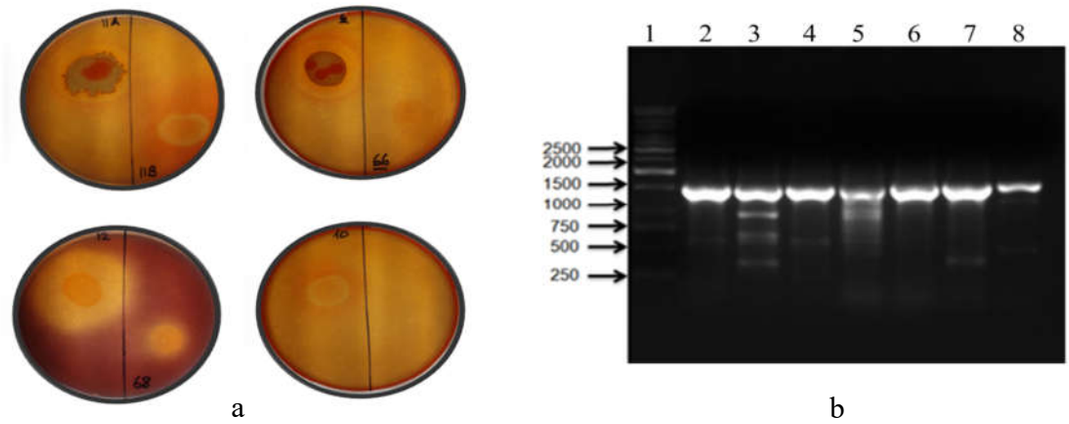

Figure 2. a. Hydrolysis zones of the xylanase-producing microorganisms. b. The result of agarose gel (%1) electrophoresis shows PCR products for the 16S rRNA of the xylanase-producing microorganisms. 1. λ-EcoR I /Hind III DNA marker, 2-8 16S rRNA PCR product of xylanase-producing microorganisms.

The species that have a similarity of 16S rRNA sequences was analysed by BLAST server at the NCBI public database. A taxonomy report was established by using the Taxonomy Report tool within BLAST.

According to taxonomy report, 16S rRNA sequence of *Bacillus subtilis* strain akky1 (KJ540929) exhibited 100% nucleotide identity with *Bacillus subtilis* strain therefore new species were classified under the genus *Bacillus subtilis* (Figure 3).

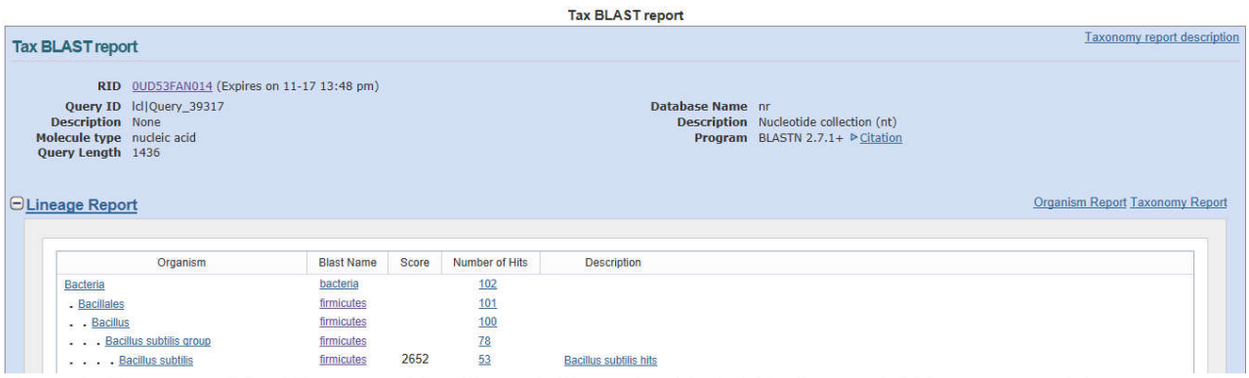

Figure 3. Taxonomy BLAST report of Bacillus subtilis strain akky1 16S ribosomal RNA gene, partial sequence (KJ540929)

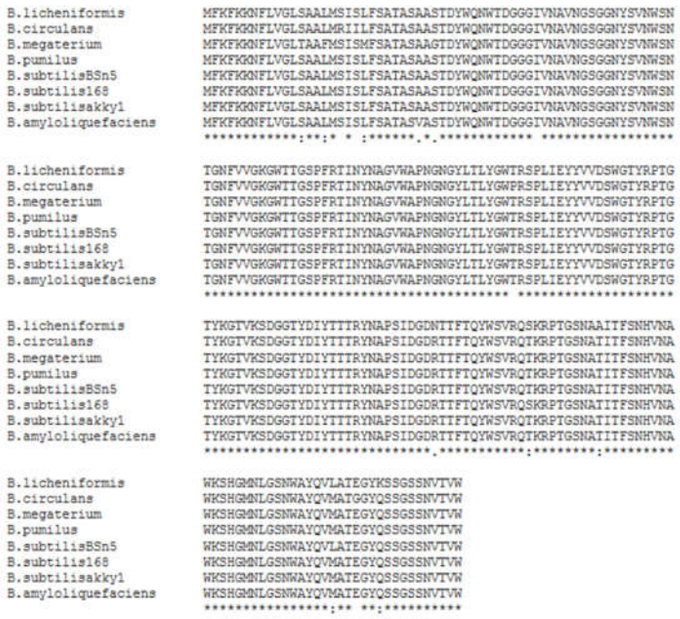

Figure 4. The multiple alignment of amino acid sequence of the xylanase enzymes of different species of *Bacillus* with the amino acid sequence isolated from *Bacillus* and used for cloning and expression studies of xylanase enzyme, by using ClustalW2 program. The accession numbers are: *B. subtilis* strain akky1, KJ540928.1; *Bacillus pumilus*, AAZ17390.1; *Bacillus subtilis* subsp. subtilis str. 168, NP_389765.1; *Bacillus subtilis* BSn5, YP_004203820.1; *Bacillus amyloliquefaciens*, AAZ17388.1; *Bacillus megaterium*, ACT21830.1; *Bacillus licheniformis*, AAZ17387.1; *Bacillus circulans*,AAM08360.1

3.2. Cloning of the Xylanase gene in *Escherichia coli*

DNA fragment encoding-xylanase from *Bacillus subtilis* strain akky1 xylanase was amplified and this fragment was cloned to pET 28b (+) vector using *Xho*I and *Bam*HI restriction enzymes. Final plasmid was named as a pET28b-xyn. The results obtained from colony PCR (template: the *E.coli* DH5α strains harboring pET28b-xyn; primers: xyn1-xyn2) (Figure 5 a), restriction fragment analysis (Figure 5 b), confirm the success of cloning. Furthermore DNA sequencing was done to verify correct insertion of xylanase-encoding DNA fragment. *E. coli* DH5α cells transformed by constructed pET28b-xyn were selected with kanamycin selection. Consecutive plasmid preparation method was utilized for sequencing DNA samples and transformation of competent *E. coli* BL21 (DE3) cells. Figure 1b. demonstrates the circular plasmid map of the construct.

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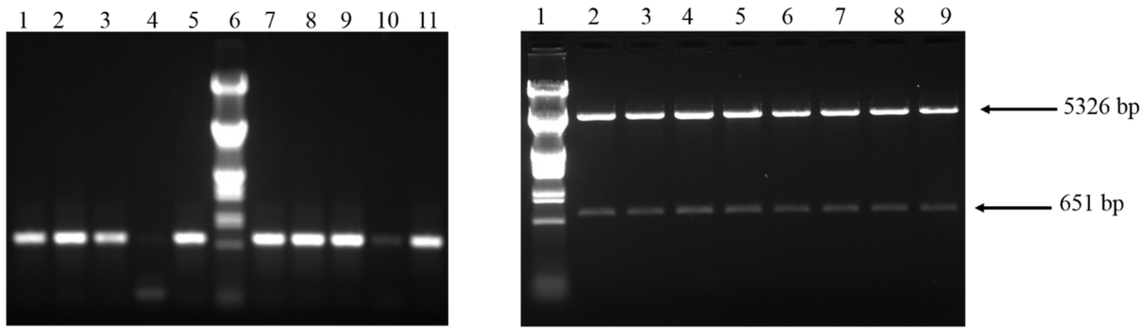
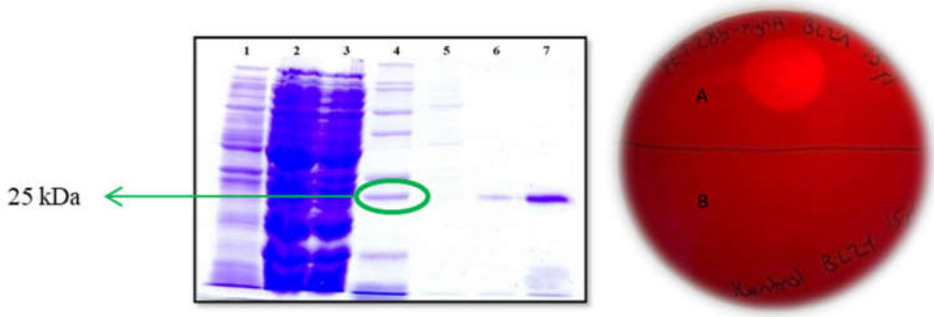


a b

Figure 5. a. Agarose gel (1%) electrophoresis result showing PCR verification of recombinant plasmid pET28b-xyn after the cloning. Columns 1-5 and 7-11 indicates PCR products from the colonies and column 6 indicate, λ-EcoR I /Hind III DNA marker. b. 1% Agarose gel demonstrating digesting of recombinant plasmid pET28b-xyn with *Nco*I restriction enzyme after the cloning. 1, λ-EcoR I /Hind III DNA marker 2-9, DNA fragments obtained after the digestion.

3.3. Qualitative Analysis of the Purified Protein

The 6xHis tagged recombinant xylanase was purified from *E. coli* cell lysate by Ni-NTA chromatography as described above. Eluted samples were analysed on SDS-PAGE and the purified enzyme migrated on the gel as a single band with a molecular mass of around 26.0 kDa (Figure 6 a). The calculated molecular mass of protein using the "ExPASy ProtParam Tool" was 26970.6 Da which is very close to the experimental molecular mass.

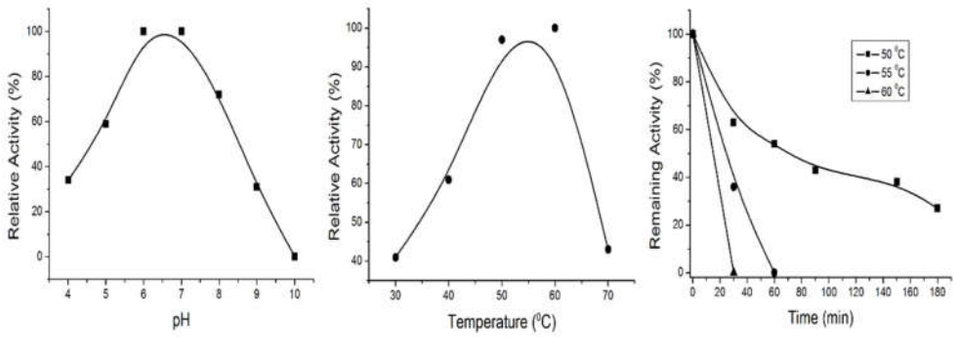


a b

Figure 6.a. Purification of xylanase enzyme was confirmed with SDS-PAGE (%12). Samples from induced *E. coli* BL21 pLysE cell lysate carrying pET28b plasmid (1). Samples from induced *E. coli* BL21 pLysE cell lysate carrying pET28b-xyn plasmid (2). Collected supernatant after centrifugation of the lysate (3). BioRad dual colour precision plus protein marker (4). The eluate collected from Ni-NTA agarose affinity column (imidazole concentrations are respectively 10, 25, 300 mM) (5-7). b. The image of zone formation at the periphery of the recombinant colony by Congo-red plate containing beechwood xylan. A. Recombinant colony B. *E. coli* BL21 without plasmid

3.4.Biochemical Characterization

Activity of recombinant xylanase was observed for various pH values. The optimum condition for activity of recombinant xylanase predicted as follows: pH 6.0 (Figure 7a) and at 60°C (Figure 7b). 35% of the enzyme activity was able to maintain stability for 200 minutes at 55°C (Figure 7c). Km value for xylanase was 3.33 mg/ml when beechwood xylan was used as substrate(Figure 8).



a b c

Figure 7. Characterization of recombinant xylanase. a. Effect of pH. b. Effect of temperature. c.Thermostability of recombinant xylanase

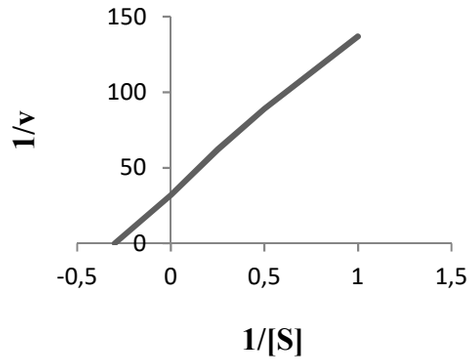


Figure 8. Lineweaver-Burk curve for the purified xylanase enzyme

Several xylanases from microorganisms, such as fungi, bacteria and yeast, were isolated, cloned and expressed in *E. coli* (9, 10, 21, 22) . In this study, xylanolytic *Bacillus subtilis* strain akky1 was isolated from the soil of beech forest in Akkuş City, Ordu Province, Turkey. *Bacillus* species are soil bacteria with gram-positive cell membrane. These species can utilize complex carbohydrates in their native environment by expressing and secreting a variety hydrolytic enzymes (9).

In the current study, we first identified the xylanase gene sequence of xylanase from public database (NCBI/GenBank accession no NC000964) and further designed the primers to amplify of 642-bp DNA fragment by PCR using genomic DNA as a template. DNA sequence of xylanase gene from akky1 and the xylanase gene (accession no NC000964) showed 99.7% similarity with only two bases differences (in positions 484 and 498). These two bases cause mutation of serine at position 160, becomes threonine (S160T) (Figure 4). The observed phenomenon could be due to similarity in the some species caused by high conservation of xylanase gene sequence in the microorganism.

Xyn-akky1 derivatives exhibit substantial thermal and pH stability which make possible for their use in industries. Xyn-akky1 retains its activity at pH 6 to 8 range therefore it can be useful in the paper industry (8).

Xyn-akky1 enzyme is more useful for pulp bleaching than non-enzymatic multistage process because of its cost effectiveness and eco-friendly characteristics. Additionally, the combination of our xylanase with other xylan-degrading enzymes may be applicable in processing food products, manufacturing vegetable and juice products and optimization of animal feedstock digestibility.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interests.

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