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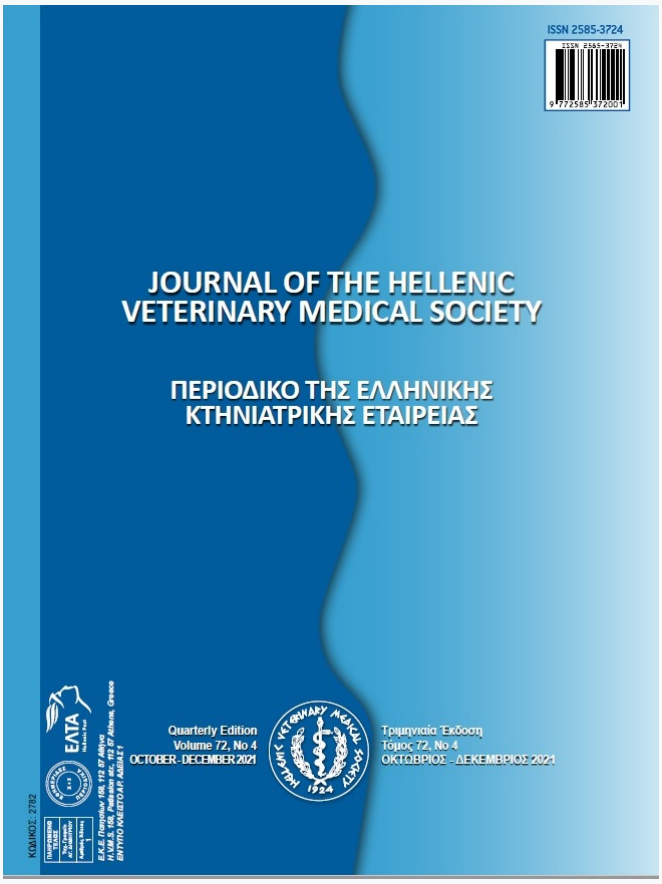

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Research article
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Cloning of the xylanase gene from soil *Streptomyces* into *Escherichia coli* for the poultry industry application

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ABSTRACT: *Streptomyces* are gram-positive aerobic strains that are isolated from soil, water, sediments, and various sources. The bacteria are capable of producing secondary metabolites, such as enzymes that sometimes play unique functional roles in industry, and are one of the important bio-control agents. This study aimed to isolate and clone the*xylanase* gene from soil *Streptomyces*. Soil samples were collected from Markazi Province, Iranafter specific biochemical examinations, isolation of bacteria, and DNA extraction. PCR was then performed to identify the strains containing the *xylanase* gene. The gene from the positive strains was cloned into an *E.coli* host-vector by TA cloning technique and finally, the expression of genes in *E. coli origami* was measured by Real-Time PCR technique. ClustalX and Mega5 software were used to draw the phylogenetic tree. A total of twelve *Streptomyces* isolates were identified from the soil samples. Among all the isolates, threeones had the *xylanase* gene. After cloning the *xylanase* genes, the cloned strains were isolated. To confirm the DNA cloning, Real-Time PCR was performed, and finally, the PCR product where sequenced. In this study, *Streptomyces* was identified as a native strain for the expression of xylanase after generating recombinant plasmid and TA cloning. It can be stated that cloning of the xylanase gene from soil *Streptomyces* in *E. coli* can be used in the poultry industry.

Keywords: *Streptomyces, Xylanase*, TA-Cloning

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INTRODUCTION

Cellulose and hemicellulose arethe most abundant renewable polymers in plant cell walls. Xylan is the most crucial compound in lignocellulosic agro-residues (Verma et al., 2013).It is composed of β-1,4-linked xylosyl residues and different groups (arabinosyl, acetyl,and glucuronosyl) in its side chains. Xylan is a significant compound in hemicellulose that comprises 20-40% of total plant biomass. This compound has an essential role in maintaining the cell wall integrity through the creation of covalent and non covalent bonds with cellulosic fibers and lignin (Li et al., 2008).Xylanasesare glycosidases that catalyze the endohydrolysis of 1, 4-β-D-xylosidic linkages in xylan to shorten xylooligosaccharides (Li et al., 2008). Xylan needs a complex of xylanolytic enzymes for degradation, such as xylanase (Emam et al., 2016).Regarding the amino acid sequence homologies and hydrophobic cluster analysis, xylanases are divided into two families of glycosylic hydrolyses (I) family for GH 10 and (II) family G or GH 11 (Kishishita et al., 2014).Xylanase gene is commonly isolated from different microorganisms and expressed in *Escherichia coli*. It was reported that xylanase is not only produced at lower activity levels in bacteria compared to fungi that are restricted to the intracellular or periplasmic fractions (Ahmed et al., 2009).

Streptomyces are aerobic, filamentous Gram-positive soil bacteria that produce extracellular enzymes able to degrade natural polymers such as xylan (Sevillano et al., 2016).Their use as hosts for the production of the enzyme can improve problems encountered with other systems. Bacteria have many advantages compared to other microorganisms such as requiring less growth time.Also, bacterial xylanase is more functioning at high temperature and alkaline conditions, which are more appropriate for most applications (Subramaniyan et al., 2001). Some xylanase genes have been cloned and expressed in *E.coli* (Akyol et al., 2009; Guo et al., 2009).

The arabinoxylan present in cell walls of poultry feeds has an anti-nutrient effect on poultry. Xylan in soluble form increases the viscosity of the ingested feed, influencing the mobility and absorption of other components (Mirzaie et al., 2012).Dietary inclusion of xylanase in poultry feeds may improve the digestion of nutrients in the initial part of the digestive tract, and improve energy utilization (Polizeli et al., 2005). Regarding the importance of xylanase in the poultry industry, for the first time, we aimed to isolate the xylanase gene from soil *Streptomyces* into *Escherichia coli* for application in the poultry industry.

MATERIALS AND METHODS

Microorganism isolation and culture

We isolated *Streptomyces* sp. from soil samples obtained in Arak, Iran. To separate and identify proteolytic microorganisms, skim milk agar medium, and biochemical tests were used. To isolate Streptomyces sp., heat and dried treatments were used, and dilution was conducted. To dilute soil samples, 10 g aseptic soil was added to 90 ml sterilized distilled water. The samples were mixed, and then1 ml of sample was transferred into a tube containing 9 ml physiological serum. An amount of 0.2 ml of the mixture was transferred into skim milk agar medium, homogenously distributed by glass spread under aerobic condition, incubated at 30 ˚C for 72 h, and investigated for growth. *Streptomyces* sp. were obtained by biochemical tests and morphological properties in specific cultures. To keep a short time and long time, the isolates were prepared for the microbial bank and screening step. All the strains were transferred into tubes after purification. The samples were cultured, incubated, and observed at 2-5 ˚C. The samples were investigated for survival after 30 days.

Identification of bacterial isolates, and DNA extraction

To determine bacterial isolates based on morphological properties, microscopic and biochemical properties were used, including motility, indole, methyl red, VP, citrate, pigment production, gelatin hydrolysis, nitrate reduction, oxidase, catalase, casein hydrolysis, starch hydrolysis, sucrose, and SH2 production tests were used.

To extract bacterial DNA, a DNA extraction kit (Iran Genetic Reservoir Center) was used. Mini column extraction was conducted by the kit.

PCR reaction

For cloning of the obtained xylanase gene, the gene fragment was amplified by polymerase chain reaction (PCR). The primers were (F, 5′-*CCCGCTAGCATGACAGCGAGTTTGAGGAAGA*-3′; R, 5′-*CCCCTCGAGTTACGGCGTGTTTCCGTAGC*-3′). 10 µl PCR Master Mix 2x, 1 µl primers with concentration of 10 pm/µl, 4 µl template DNA(150 ng), and 4 µl distilled water were added. The PCR was set up as follows, initial denaturation at 95 ˚C for 5 min, 35 cycles of de-

naturation at 95 ˚C for the 30s, annealing at 60 ˚C for 30s, replication at 72 ˚C for 1 min, and final replication at 72 ˚C for 10 min. To conduct DNA electrophoresis, 1.5% agarose gel was prepared. For the post-run staining, Ethidium bromide was used.

Cloning

For the cloning the PCR product PCR TA-Cloning package of CinnaGen Company (Tehran, Iran) was used. Based on the company's guidelines, a linear vector of PTG19-T was used. Application of linear vector of PTG19-T caused a direct connection between PCR product and cloning vector. T4 DNA ligase enzyme created a covalent bond to the linear vector, and it did not need any enzymatic digestion. PCR TA-Cloning has 5 steps, including 1) Production of PCR product by Taq DNA polymerase, 2) Ligation of the PCR product in the PTG19-T cloning vector, 3) Transformation of vector to *E.coli* XL1-Blue host, 4) Approval of the cloning, and 5) Identification of positive clones. The approval of the cloning, was performed through four steps, 1) Conducting PCR by vector primer, 2) Colony selection by Blue/White screening and growth in presence of ampicillin, 3) Gene expression by Real-Time PCR, and 4) Sequencing junctions. To conduct ligation, a 10 µl mixture was prepared, including 2 µl PTG19-Tcloning vector, 1 µl T4 ligase enzyme, 1 µl buffer, 1.5 µl PCR products, and 4.5 µl water. The mixture was transferred into a vial at 22 ˚C and heated by a hotplate. To transform the vector to *E. coli*, liquid, and solid LB Broth were used.

Preparation of competent cells

E. coli uragami was obtained from Tehran University, and cultured for 16-17 hours at 37 ˚C and centrifuged at 10000 rpm for 10 min.the upper solution was removed and 100 µl buffers was added to the pellet and centrifuged at 12000 rpm for 1 min. Then, 50 µl buffers was added as well and placed on ice for 5 min. It was centrifuged at 12000 rpm for 1 min, and 500 µl buffers were added again. It was placed at 42 ˚C for 90 s and placed on ice for 10 min. finally, 400 µl of culture media was transferred into 1.5 ml vials and transfer materials were added and placed at an incubator for 45-60 minutes. Ampicillin was also added to the medium. The mixture was centrifuged at 650 rpm for 1 min, the upper solution was removed and the bacterial pellet was cultured on an LB plate, incubated at 37 ºC for 2 days, to approve the cloning. The cloning vector of PTG19-T contained the Lac z gene. Since the medium contained ampicillin, colony growth could show resistance to ampicillin as a result of plasmid transfer.

Recombinant bacteria were incubated before PCR and extracted after 15h incubation. RNA was extracted after 15h by RNase micro-kit (Qiagen Co, Germany). The bacterial suspension (Bacteria resistant to xylanase gene) were in the logarithmic phase (OD_{600}=0.4-0.6).

Quantitative and qualitative assessment

The extracted RNA was assessed for qualitative and quantitative evaluations. To evaluate RNA, light absorption was used in 260 and 280 nm. 2 µl extracted RNA was positioned in nano drop and absorption was investigated in 260 and 280 nm.

The cDNA was obtained by Reverse AMV enzyme (Roche Co, Switzerland) in the concentration of 25 µl/unit. The extracted RNA was incubated at 65˚C for 3 mins. Reverse transcription was performed at 42˚C for 60 mins by using 2 µl Random Primer, 8 µlAMV Reverse Transcriptase, 2 µl dNTP (10 mmol), 1 µl RNase inhibitor, and 2 µl 10x buffer of AMV enzyme. AMV enzyme was incubated at 99 ˚C for 5 minutes and inactivated.

Real-Time-PCR was performed in a volume of 20 µl by GeNetBio Cat.No: Q9210 (South Korea). To conduct Real-Time-PCR, 10 µl Prime Qmaster mix (2x) with cyber green, 5 µl Depc water, 1 µl forward primer, 1 µl reverse primer, 1 µl Rox dye, and 2 µl cDNA were used. Fragment replication was conducted in Corbet apparatus by using initial denaturation in 95 ˚C for the 30s, followed by 35 cycles of 59 ˚C for 40s, and 72 ˚C for 60s. 16srRNA housekeeping gene was used as an internal control. To calculate the expression of xylanase, and illustration of figures, specific software was used. The expression analysis was conducted compared to the standard strain.

Illustration of the phylogenetic tree

The analyses for sequencing were investigated by BioEdit software and ordered by DNA Baser. The obtained sequences were compared with registered sequences in NCBI. Each sequence was searched by Blast and sequences were set by W Cluster. Phylogenetic analysis was illustrated by W Clustral by using 5.10 MEGA software in the Neighbor-Joining method.

RESULTS

Screening and identification of *Streptomyces* sp.

Twelve isolates of *Streptomyces* sp. were isolated

based on morphological, microscopical, and biochemical tests. They absorbed theGram staining (Gram positive). Also, the other results were as follows: citrate, casein, oxidase, and catalase tests were positive, but nitrate reduction and VP tests were negative.

PCR

PCR was performed for the xylanase gene by the mentioned primers. The results showed that three strains had the xylanase gene. (Figure 1).

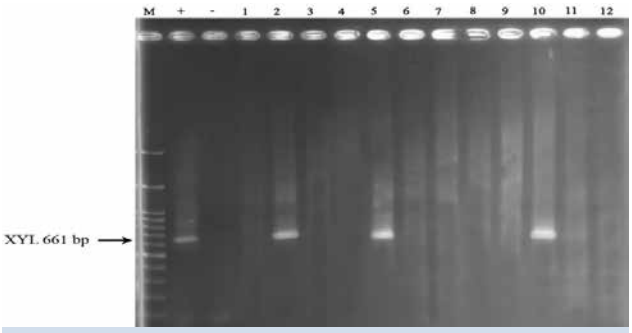

Figure 1. Xylanase gene PCR result

Xylanase gene cloning

After cloning of xylanase genes (blue/white), cloned strains were isolated (Figure 2). The results for RNA extraction confirmed RNA extraction as well (Figure 3).

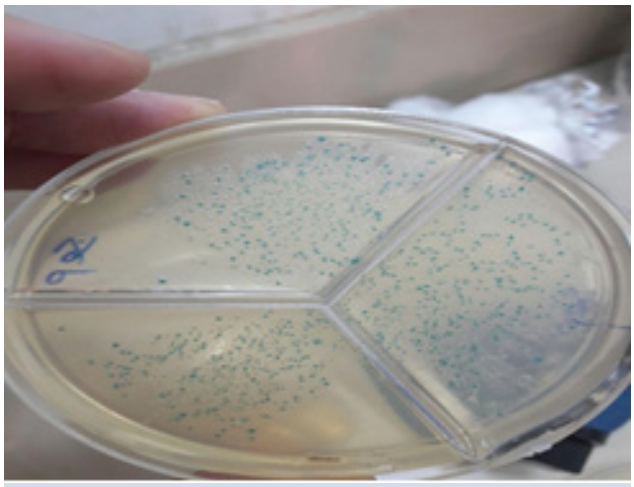

Figure 2. Colony selection(blue/white colony) of the xylanase enzyme

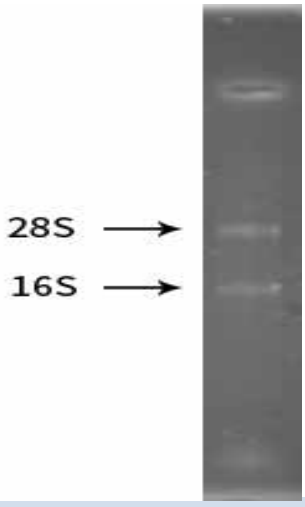

Figure 3. RNA extraction confirmation

Expression of the cloned gene by Real-Time PCR

To approve RNA cloning, cDNA was produced and investigated by Real-Time PCR for the expression of the xylanase gene (Figure 4). The results for the melting curve of PCR are shown in Figure 5.

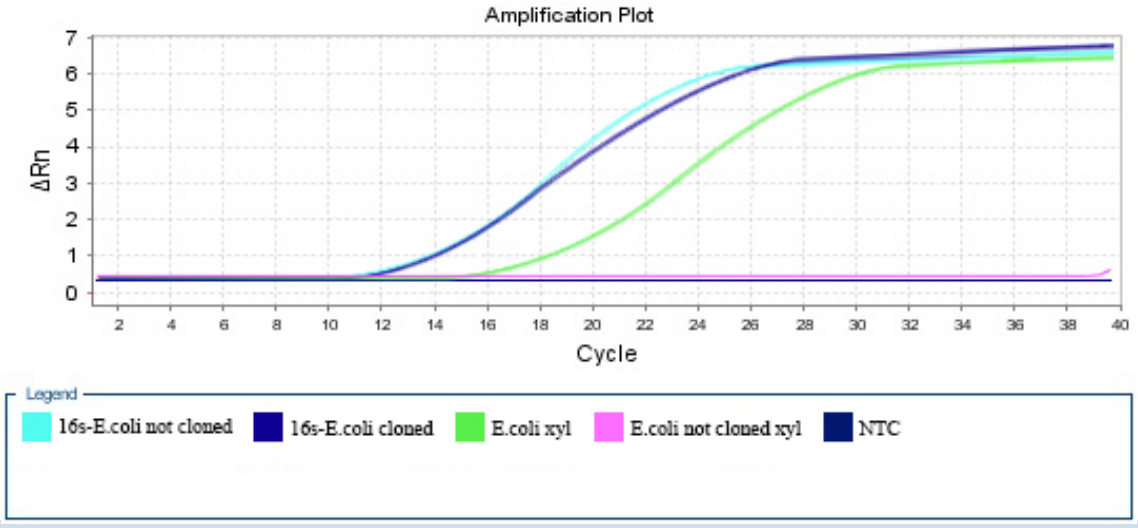

Figure 4. Real-Time PCR results

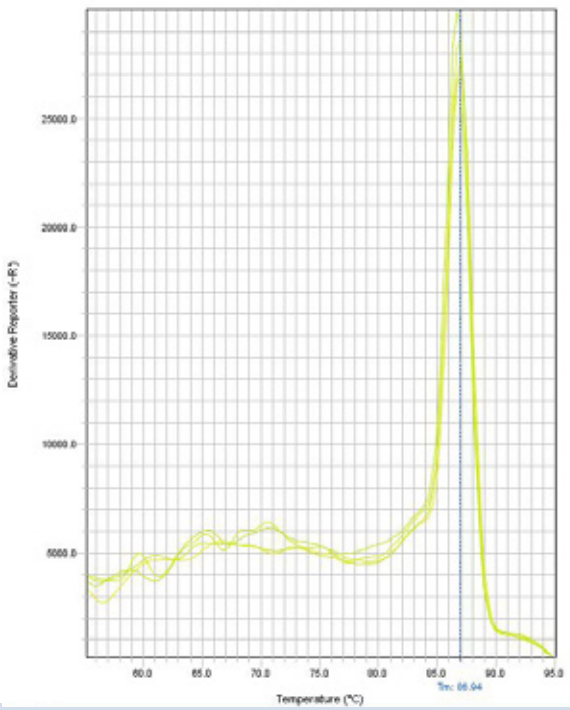

Figure 5. Melting curve of Real-Time PCR

Identification of molecular characteristics of *Streptomyces* sp.

To characterize *Streptomyces sp.,* 16SrRNA was used (Figure 6), and the PCR product was sent to Bioneer Company, South Korea for investigation of BLAST (Figure 7).

The results for the phylogenic tree by neighbor-joining method showed that BPSEAC7 *Streptomyces sp.* was in the same cluster with MI02-7b *Streptomyces* that shows a closed relation between them.

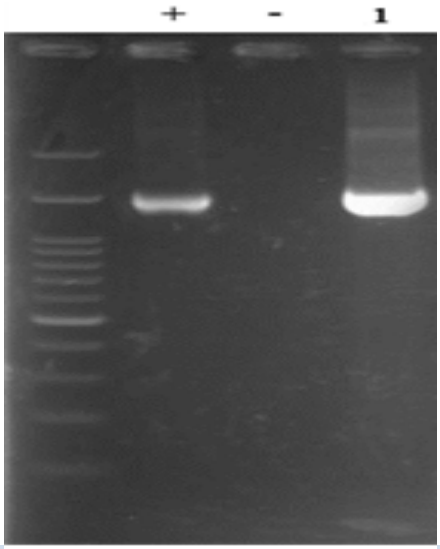

Figure 6. PCR test by 16S rRNAto identify molecular characteristics of *Streptomyces sp.*

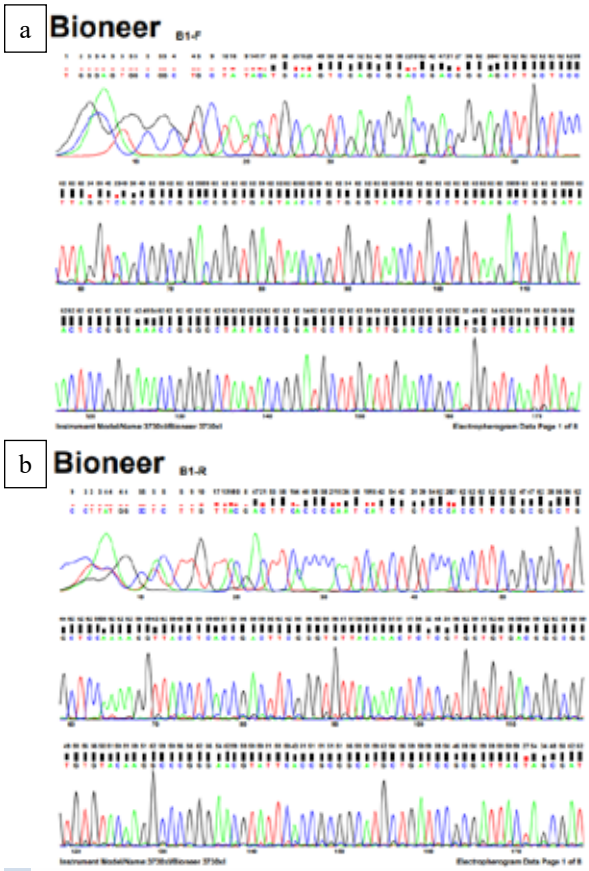

Figure 7. Sequencing and BLAST of forward (a) and reverse (b) of xylanase

DISCUSSION

This study was conducted for cloning the xylanase gene from soil *Streptomyces* into *E. coli* for application in the poultry industry. Xylanase gene was cloned by blue/white screening, and all the strains were isolated, and the PCR product sequence of xylanase gene expression in the bacteria *E. coli origami* was endorsed. Several xylanase genes were previously isolated from various microbial organisms and expressed in *E. coli* (Goswami et al., 2014). It was reported that cloning and expression of a GH11 xylanase gene from *Aspergillus fumigatus* MKU1 in *Pichia pastoris* (Jeyaet al., 2009). Hwang et al. (2010) performed the cloning of xylanase, KRICT PX1 from the strain *Paenibacillus sp*. HPL-001. Wang et al. (2011) showed direct cloning, expression, and enzyme characterization of a novel cold-activexylanase gene (*Xyn*GR40). In another study, Lin et al. (2013) showed cloning and expression of a thermostable *xylanase* from *Bacillus halodurans* C-125 (C-125 *xylanase* A).Kishishita et al. (2014) showed cloning and expression of cellulose inducible endo-β-1, 4-*xylanase* (*Xyl*10A) from the mesophilic fungus *Acremonium cellulolyticus*. The

isolates of this study showed similarity and closeness to *Streptomyces* species.In this work, we isolated a novel indigenous *Streptomyces* strain with the capability to express the xylanase enzyme. The novelty of our study is about transferring the enzyme gene to *E.coli origami* through the recombinant plasmid of PTG19. As the plasmid carried an ampicillin resistant gene so it was a perfect match for transferring xylanase gene by as the *E.coli* is sensitive to ampicillin; growing in presence of ampicillin proves the successful transfer of the xylanase gene. Also, as the plasmid carried *lac* gene, the blue-white screen was another way to ensure the success in cloning. The next step we are taking is to characterize the enzyme and trying to improve the expression through genetic engineering. from soilfor the first time, the xylanase gene was isolated from *Streptomyces sp.*and cloned in *E. coli* origamiby recombinant plasmid of PTG19and TA cloning.The expression of cloned xylanase gene in the extracellular medium of *E. coli*not only approved the successful cloning process but also draws a promising image for future commercial applications in the poultry industry.

CONFLICT OF INTEREST

There is no conflict of interest to be declared.

REFERENCES

Ahmed S, Riaz S, Jamil A (2009) Molecular cloning of fungal xylanases: an overview. Appl MicrobiolBiotechnol 84: 19-35.

Akyol I, Comlekcioglu U, Kar B, Ekinci MS, Ozkose E (2009) Cloning of a xylanase gene xyn2A from rumen fungus *Neocallimastix sp*. GMLF2 in *Escherichia coli* and its partial characterization. Biologia 64: 664-670.

Emam MTH, Mohamed KA, Badawy FMI, and Ibrahim SA (2016) Cloning, sequencing and expression of the xylanase genefrom *Bacillus pumilus* GH in *Escherichia coli*. Int J ChemTech Res9(12): 114-124.

Guo B, Chen XL, Sun CY, Zhou BC, Zhang YZ (2009) Gene cloning, expression and characterization of a new old-active and salt-tolerant endo-β-1,4-xylanase from marine *Glaciecola mesophila* KMM 241. Appl Microbiol Biotechnol 84: 1107-1115.

Goswami GK, Krishnamohan M, Nain V, Aggarwal C, Ramesh B (2014) Cloning and heterologous expression of cellulose free thermostable xylanase from *Bacillus brevis*. Springer Plus 3:20-26.

Hwang IT, Lim HK, Song HY, Cho SJ, Chang JS, Park NJ (2010) Cloning and characterization of a xylanase, KRICT PX1 from the strain *Paenibacillus* sp. HPL-001. Biotech Adv 28: 594-601.

Jeya M, Thiagarajan S, Lee JK, Gunasekaran P (2009) Cloning sequencing and expresstion of GHxylanase geneAspergillus fumigatus MKU1 in pichia pastoris. J Biosci Bioeng 108: 24-29.

Kishishita S, Yoshimi M, Fujii T, Taylor LE, Decker SR, Ishikawa K, Inoue H (2014) Celluloseinducible xylanase Xyl10A from Acremonium cellulolyticus: Purification, cloning and homologous expression. Protein Expr Purif 94: 40-45.

Li N, Meng K, Wang Y, Shi P, Luo H, Bai Y, Yang P, Yao B (2008) Cloning, expression, and characterization of a new xylanase with broad temperature adaptability from Streptomyces sp. S9. Appl Microbiol Biotechnol 80:231-240.

Mirzaie S, Zaghari M, Aminzadeh S, Shivazad M (2012)The effects of non-starch polysaccharidescontent of wheat and xylanase supplementation onthe intestinal amylase, aminopeptidase and lipaseactivities, ileal viscosity and fat digestibility inlayer diet. Iran J Biotech 10: 208-214.

Polizeli MLTM, Rizzatti ACS, Monti R, Terenzi HF,Jorge JA, Amorim DS (2005) Xylanases fromfungi: properties and industrial applications. ApplMicrobiol Biotechnol 67: 577-591.

Verma D, KawarabayasiY, Miyazaki K, Satyanarayana T (2013) Cloning, Expression and Characteristics of a Novel Alkalistable and Thermostable Xylanase Encoding Gene (Mxyl) Retrieved from Compost-Soil Metagenome. PLoS ONE 8(1): e52459. doi:10.1371/journal.pone.0052459.

Sevillano L, Vijgenboom E, van Wezel GP, Santamara RI (2016) New approaches to achieve high level enzyme production in *Streptomyces lividans.* Microb Cell Fact 15:28. Doi: 10.1186/s12934-016-0425-7.

Subramaniyan S, Sandhia GS, Prema P (2001) Control of xylanase production without protease activity in *Bacillus* sp. by selection of nitrogen source. Biotechnol Lett 23**:** 369-371.

Wang G, Luo H, Wang Y, Huang H, Shi P, Yang P,Meng K, Bai Y, Yao B (2011) A novel cold-activexylanase gene from the environmental DNA ofgoat rumen contents: direct cloning, expression andenzyme characterization. Bioresour Technol 102:3330-3336.