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Cloning, sequencing and expression of the xylanase gene from *Bacillus pumilus* GH in *Escherichia coli*

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Abstract : The thermostable endo-1,4-beta-xylanase gene of *Bacillus pumilus* GH strain was isolated from chromosomal DNA using specific primers designed from *Bacillus pumilus* xylanase gene given in gene bank database then cloned into pET29a (+) vector and transformed into *E. coli* DH5α.The positive clone was selected, sequenced and submitted to gene bank with the accession number KT757524.1. The open reading frame of the xylanase gene was 687 bp encoding a protein of 228 amino acids with a molecular mass of 23 kDa. The sequence of *Bacillus pumilus* GH xylanase gene showed 99 % similarity with other xylanase genes of different *Bacillus pumilus* strains, differ only in two nucleotide bases at positions 579 and 600. The recombinant plasmid was subcloned into the expression host *E.coli* BL21 (DE3) and successfully expressed. The total activity of xylanase was 9 U/ml, 52% (4.7U/ml) of the activity was extracellular and 48 % (4.3 U/ml) intracellular.
Key words: *Bacillus pumilus*; *E. coli*; xylanase; gene cloning and expression; sequence analysis.

Introduction

The plant cell wall is composed of cellulose, hemicellulose (mainly xylan) and lignin[1]. Xylan is a heteropolymer composed of a backbone of D- xylose unites linked by β-1,4 glycosyl bonds branched with arabinofructisyl, glucuronic acid and acetyl side chains [2]. Complete degradation of xylan requires a complex of xylanolytic enzymes including endo-xylanase, xylosidases, glucuronidase, acetylesterase and arabino furanosidase. Endo-xylanase cleavage the long chain xylan into xylose and short xylo-oligosaccharid [3]. Endo-xylanase used in many industrial applications such as paper pulp bleaching, textile, animal feeding, beverage and food industries [4,5]. Xylanase is produces from various microorganisms including bacteria, fungi, yeast and actinomycetes [6,7]. Bacteria have advantage than other microorganisms because it needs less time for growth, also bacterial xylanase show more activity at high temperature and alkaline conditions which more suitable for most applications[8].Many xylanase genes have been cloned and expressed in *E.coli* [9,10,11,12,13,14,15].

In this study, xylanase gene from *Bacillus pumilus* GH strain was isolated, sequenced and expressed in *E.coli*.

Materials and Methods

Bacterial strains, vector and culture medium

Bacillus pumilus GH strain was isolated from soil and identified by 16s RNA in a previous study[16].Bacterial expression vector pET 29a+ (Novagen, Germany) used for cloning of xylanase gene. *E. coli* DH5α used for transformation of recombinant plasmid and *E.coli* BL21(DE3) used as a host for xylanase gene expression.All bacterial strains were cultivated in Luria–Bertani medium (LB) at 37^{o} C. *E.coli* strains carrying the plasmid vector were grown in LB supplemented with (50 µg/ml) Kanamycin.

DNA extraction and xylanase gene isolation

Genomic DNA from *Bacillus pumilus* GH was extracted by lysozyme-sodium dodecyl sulfate method[17]. Xylanase gene was isolated by PCR reaction using forward (xyl-F) and reverse (xyl-R) primers (Table 1). The primers designed from Gene bank (Accession No: X00660, GQ377132, AY526092). The recognition sites for restriction enzymes *Bgl* II and *Xho*I were added to the 5′end of the primers. The xylanase gene band cut from the agarose gel and purified using PCR clean up system (Promiga).

Table (1): primer sequences for xylanase gene isolation and sequencing.

Primer	Sequence
Xyl-F	GGAAGATCTATGAATTTGAGAAAATTAAGACTGTTG
Xyl-R	CCGCTCGAGTTAGTTGCCAATAAACAGCTGA
pET-F	CGTCCGGCGTAGAGGATC
pET-R	ATCCGGATATAGTTCCTCCTTTC

The underlined sequences indicate the recognition sites of restriction enzymes *Bgl*II and *Xho*I for xyl-F (xylanase forwarded) and xyl-R (xylanase reverse) primers, respectively. pET-F and pET-R foreword and reverse primers for pET plasmid vector.

PCR reaction

The PCR started with a 5 minutes denaturation at 94^{o}C, followed by 35 cycles of 30 second at 94^{o}C, 40 second at 52^{o}C, and 1 minute at 72^{o}C. A final extension of 5 minutes at 72^{o}C was performed. Theamplified PCR products were analyzed by agarose gel electrophoresis.

Construction of recombinant plasmid

Both plasmid vector pET 29a+ and PCR product of xylanase gene were double digested by *Bgl* II and *Xho*I restriction enzymes (Thermo scientific) at 37^{o}C for 30 minutes then ligated by T4 ligase at 22^{o}C for 1 hour.

Competent cells preparation

One ml of overnight culture used for inoculating 50 ml LB medium. The culture incubated at 37^{o}C until the O.D $_{600}$ reach to 0.4, the cells chilled on ice for 15 minutes, then collected by centrifugation at 4000 rpm for 5 minutes. Cells resuspended in 50 ml 100 mM $MgCl_2$and put in ice for 10 minutes, centrifuge at 4^{o}C and resuspended in 50 ml mM $CaCl_2$ for 60 minutes. Cells were centrifuged and resuspended in 1 ml 100 mM $CaCl_2$ plus 15%glycerol. Aliquots of 50 µl competent cells per eppendorf were stored at - 80^{o}C[18].

Transformation

The recombinant plasmid with xylanase gene was added to *E.coli* competent cells and incubated for 30 minutes on ice then placed on water path at 42^{o}C for 90 second then transferred immediately to ice for 5 minutes. Pre warmed LB medium (500 µl) was added to the mixture and incubated for 2 hours at 37^{o}C then 100 µl was transferred to LB plates with 50µg/mL Kanamycin and incubated at 37^{o}C overnight[19].

Selection of positive transformed colonies

The transformed colonies that containing the plasmid carrying the xylanase gene was selected by colony PCR using xylanase gene primer (Table 1) with the above PCR conditions. The plasmid was isolated from the positive colony of *E. coli* DH5α by plasmid isolation kits (Qiagene) for xylanase gene sequencing and subcloning of *E.coli* BL21.

DNA sequencing

Xylanase gene was sequenced by the dideoxy-chain termination method[20]. The sequence was analyzed using http://www.ncbi.nlm.nih.gov/BLAST. Multiple sequence alignment was done using clustal X2 program.

Expression of xylanase gene in *E.coli* BL21and cell lysis

Overnight culture of transformed *E.coli* BL21 used to inoculate 50 ml LB medium containing Kanamycin (50µg/mL), the flask incubated at 37^{o}C. Induction of xylanase expression was started by the addition of 1mM IPTG when the $O.D_{600}$ reaches 0.6 and overnight incubated. Cells collected by centrifugation at 6000 rpm for 5 minutes, washed and suspended with (0.1 M) sodium phosphate buffer pH7. Cell disrupted by sonication, cell debris and unbroken cell removed by centrifugation at 13000 rpm for 5 minutes. The supernatant used for measuring the enzyme activity[21,22].

Enzyme assay

Xylanase activity was measured according to the method of Bailey[23] at pH 7and 60°C using 0.5% beechwood xylan (Roth) in 0.1 M sodium phosphate buffer. Xylanase activity was measured by the increase in reducing sugars, as given by the dinitrosalicylic acid (DNS) method[24] using xylose as a standard. One unit of xylanase activity was defined as the amount of enzyme producing l µmol of xylose per minute.

SDS-PAGE

Xylanase expression for induced and un-induced cultures was performed by SDS-PAGE (15%) according to Laemmli method [25].

Results and discussion

Xylanase gene isolation and transformation

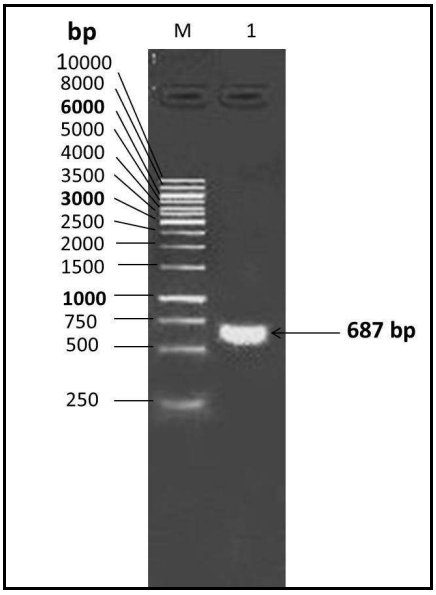

Fig.1. Agarose gel electrophoresis for PCR product of xylanase gene from *Bacillus pumilus* GH. (M) Marker and (1) PCR product of xylanase gene 687 bp.

Chromosomal DNA from *Bacillus pumilus* GH used as a template for isolation of xylanase gene by PCR amplification. PCR product (687 bp) (Fig. 1) was purified from the gel and cloned to pET 29a (+) plasmid vector then transformed to *E.coli* DH5α host cell. The positive colonies that grow on LB plate containing Kanamycin were subjected to colony PCR using xylanase gene primers and the previous PCR conditions that described in materials and methods section for further selection of colonies harboring plasmid with xylanase gene. Eight colonies were selected as shown in Fig. (2).

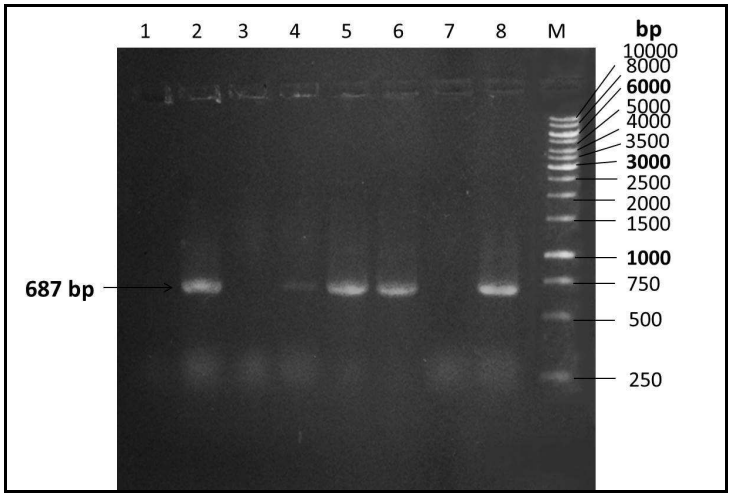

Fig.2. Agarose gel electrophoresis for colony PCR products of transformed *E.coli* DH5α using xylanase gene primers. (M) Marker, (1:8) selected colonies.

Plasmid isolation

One of the positive colonies was selected for isolation of recombinant plasmid. To analyze whether the plasmid contained the xylanase fragment or not, both recombinant and non-recombinant plasmids were double digested by *Bgl* II and *Xho*I restriction enzymes. The results presented in Fig. (3) revealed that, the non-recombinant plasmid gives one fragment at about 5000 bp while the recombinant plasmid gives two fragments, one at 5000 bp for the plasmid and the other one at 687 bp for xylanase gene.

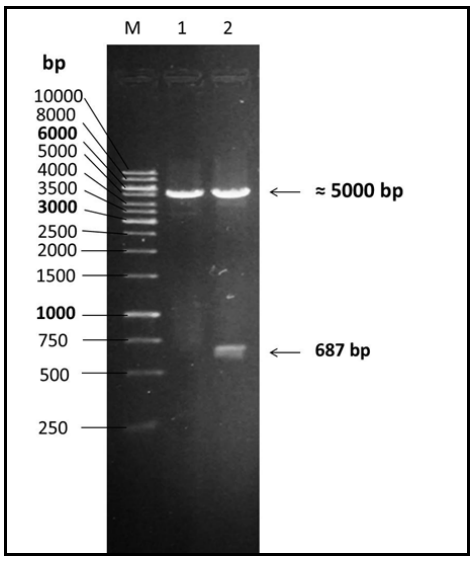

Fig.3. Agarose gel electrophoresis of isolated plasmids digested by *Bgl* II and *Xho* I restriction enzymes. (M) DNA marker, (1) non recombinant plasmid and (2) recombinant plasmid with xylanase gene.

Sequencing and alignment of xylanase gene

Xylanase gene from positive recombinant plasmid was sequenced by plasmid forward primer pET-F and reverse primer pET-R showed in Table (1). The open reading frame ORF of xylanase gene has 687 bp encoding a protein of 228 amino acids. This result is in accordance with previous studies[26,27,28,29]. The sequence was submitted to gene bank database at ncbi website with the accession number KT757524.1. Complete nucleotide sequence of endo-1,4-beta-xylanase gene from *Bacillus pumilus* GH strain was presented in Fig.(4). Multiple sequence alignment with different *Bacillus pumilus* strains from gene bank was done (Fig.5). Xylanase gene from *Bacillus pumilus* GH strain showed 99 % ident with *Bacillus pumilus* strains of accession numbers AY887130.1, AY526092.1 and X00660.1, it differ only in two nucleotide bases at positions 579 and 600, 97% ident with *Bacillus pumilus* strain HY-20 of accession number GQ377132.1, 88 % ident with AF220528.1 and 76 % ident with *Bacillus pumilus* PJ19 strain of accession number DQ663783.1. This similarity suggested that there are high conservatives of xylanase gene sequence in the same species[30].

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   1 atgaatttgagaaaattaagactgttgtttgtgatgtgtattggactgacgcttatactg

  61 acggctgtaccagcccatgcgagaaccattacgaataatgaaatgggtaaccatagcggg

 121 tacgattatgaattatggaaggattatggaaacacctcgatgacactcaataacggcggg

 181 gcatttagtgcaggctggaacaatatcggaaatgctttatttagaaaagggaaaaagttt

 241 gattccactagaactcaccatcagcttggcaacatctccatcaattacaacgcaagtttt

 301 aacccaggcgggaattcctatctatgtgtctatggctggacacaatctccattagcagaa

 361 tactacattgttgattcatggggcacatatcgtccaacaggagcgtataaaggatcattt

 421 tatgctgatggaggcacatatgacatttatgaaacaacccgtgtcaatcagccttccatt

 481 atcgggatcgcaaccttcaagcaatattggagtgtacgtcaaacgaaacgtacaagcgga

 541 acggtctccgtcagtgcgcattttagaaaatgggaaagtttagggatgccaatggggaag

 601 atgtatgaaacggcatttactgtagaaggctaccaaagcagcggaagtgcaaatgtgatg

 661 accaatcagctgtttattggcaactaa
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Fig.4. Complete nucleotide sequence of endo-1,4-beta-xylanase gene from *Bacillus pumilus* GH strain.

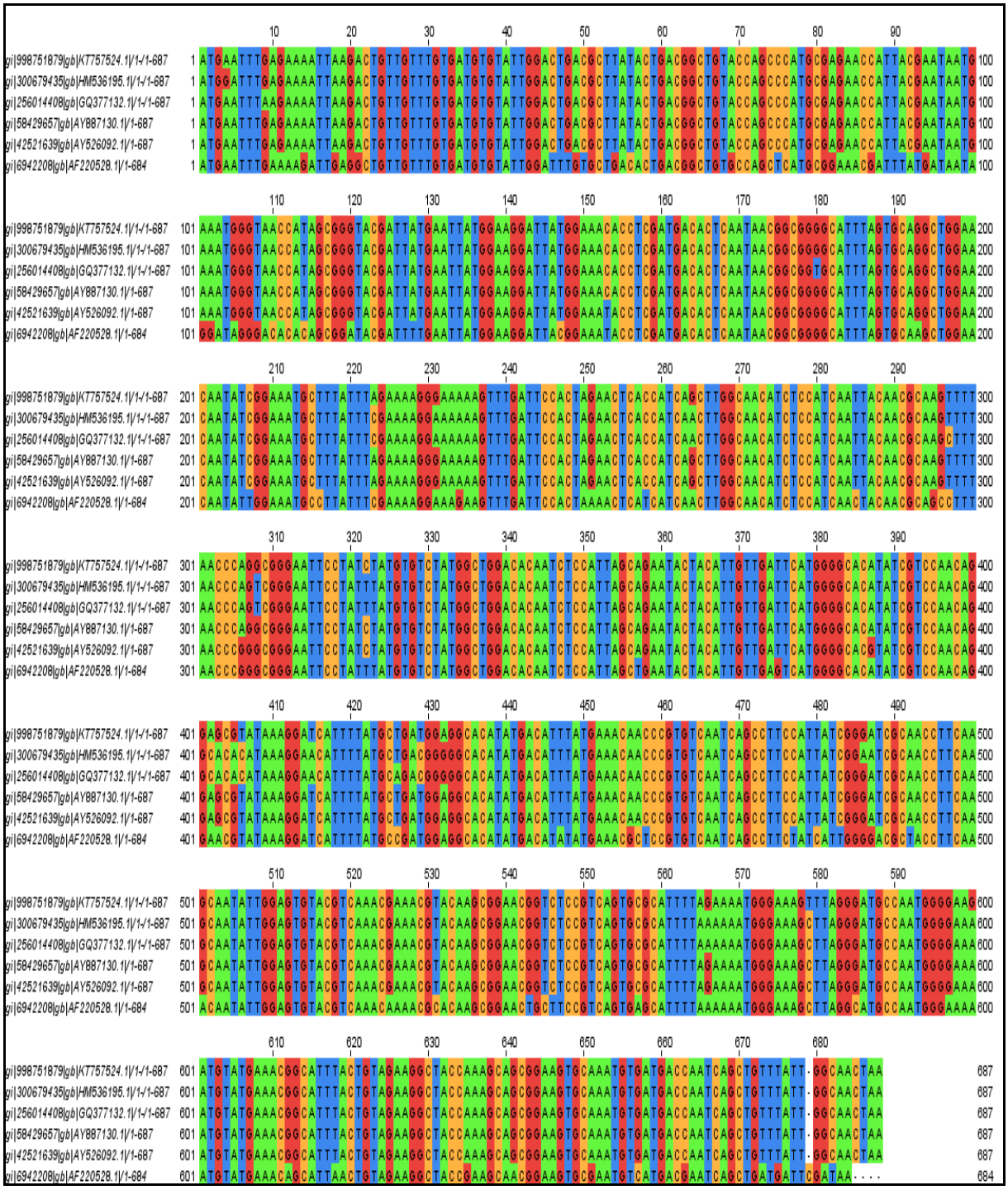

Fig.5. Multiple sequence alignment of xylanase gene from different *Bacillus pumilus* strains based on nucleotide sequences.

Cloning and expression of xylanase gene in *E.coli* BL21

The recombinant plasmid containing xylanase gene was transformed to expression host *E.coli* BL21.The positive colonies growing on kanamycin agar plate were further tested by colony PCR using xylanase gene primers (Fig. 6) to make sure that the recombinant plasmid is inserted to *E.coli* cells. The expression of xylanase gene in *E.coli* cells was analyzed by SDS-PAGE of total protein from cell lysate. Results in Figure (7) showed that the profile pattern of IPTG induced sample and un-induced sample were the same except one band for xylanase at about 23 KDa appeared in induced sample, indicating that the recombinant xylanase gene was successfully expressed in *E.coli* BL21cells.This results is in accordance with previously reported results[28,2931,32].

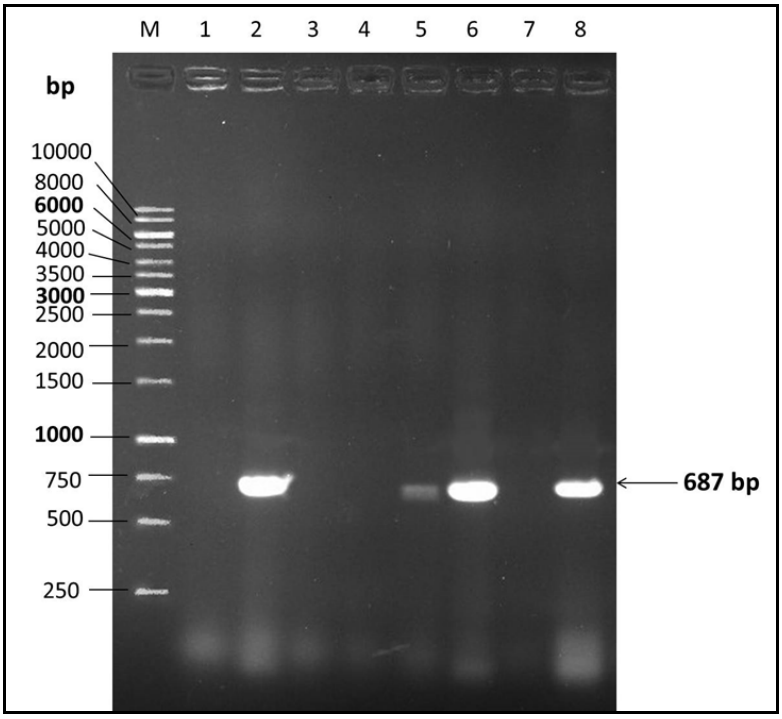

Fig.6. Agarose gel electrophoresis for colony PCR products of transformed *E.coli* BL21using xylanase gene primers. (M) Marker, (1:8) selected colonies.

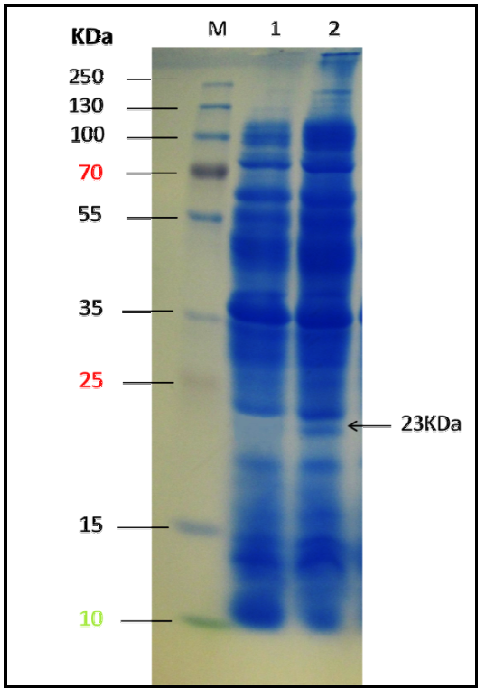

Fig.7. SDS–PAGE analysis of total cell protein from transformed *E.coli* BL21, (M) protein marker, (1) un-induced culture and (2) induced culture.

Xylanase activity

Enzyme activity of xylanase gene from induced and un-induced transformed *E.coli* BL21 was evaluated. This activity was measured by DNS method for both intracellular and extracellular crude extracts as indicated in table (2). The total xylanase activity is 9 U/ml, 4.7 U/ml of the activity was extracellular (52 %) and 4.3 of the activity was intracellular (48%). This activity is lower than *Bacillus pumilus* GH wild type strain (80 U/ml). However, many xylanase genes from different microorganisms including *Bacillus* species have been

cloned and expressed in *E.coli* to improve xylanase activity[33]. Some researchers reported that the activity of recombinant xylanase was lower than their parent organism. Xylanase activity of *Bacillus subtilis* lowering from 2.0 I.U. to 0.5 I.U. when transferred to *E.coli* WA802[34]. The activity of xylanase gene of *Bacillus pumilus* decreased from 600mU/ml to 36 mU/ml when transformed to *E.coli* but the activity increased to 1800mU/ml when the xylanase gene transformed to *Bacillus subtilis*[35]. In addition, *Paenibacillus* xylanase activity decreased from 1.7 U/ml to 1.5 U/ml when transformed to *E.coli*[36].When xylanase gene was transferred from *Bacillus Lyticus* to both *Escherichia coli* and *Bacillus subtilis*, the results indicated that the transformed *Bacillus subtilis* gives activity higher than transformed *E.coli*[37]. On the other hand, the xylanase gene from both *Bacillus subtilis* and *Bacillus circulans* were overexpressed in *E.coli*[38]. Xylanase activity from *Bacillus brevis* increased about two times when xylanase gene transformed to *E. coli* BL21[39]. However, lowering of protein expression in heterologous host due to several reasons including protein toxicity, formation of inclusion bodies and post translation modification[40].

Table (2): Enzyme activity and specific activity of xylanase gene from transformed *E.coli BL21*.

Transformed *E.coli BL21*	Enzyme source	Enzyme activity (U/ml)	Proteinconc. (mg/ml)	Specific activity (U/mg)
Transformed Bl21(Un-induced)	extracellular	0.09	0.11	0.9
	intracellular	0.11	0.043	2.55
Transformed Bl21(Induced)	extracellular	4.7	0.39	12.05
	intracellular	4.3	0.15	28.67

One unit of xylanase activity was defined as the amount of enzyme producing l µmol of xylose per minute.

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