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Detection and Analysis of the Random Mutagenesis Site(s) of Xylanase Gene from Mutants of *Bacillus subtilis* subsp. *spizizenii* ATCC 6633

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Authors' contributions

This work was carried out in collaboration between both authors. Author HLH designed and supervised the study, edited and approved the manuscript. Author AMC performed the experiments and managed the analysis. Both authors read and approved the final manuscript.

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ABSTRACT

Aims: A total of five mutant strains of *Bacillus subtilis* subsp. *spizizenii* ATCC 6633 designated as the MXB 1, MXB 2, MXB 3, MXB 4 and MXB 5 were developed using random mutagenesis of ethyl methane sulfonate (EMS) and acridine orange (AO) in our previous study. Based on our present investigation, we identified, verified and sequenced xylanase gene of mutant strains of *B. subtilis* ATCC 6633 as the potent bacterial xylanase producers under submerged fermentation. Furthermore, amino acid analysis and comparison between the xylanases of the mutants and other xylanolytic bacteria were also elucidated. Overall, this study would provide gene and protein molecular information correlating nucleotide and amino acid structure related to the increased xylanase production by random mutagenesis. In respect to the objectives of this study, we compared the endoxylanase sequence of wild type *B. subtilis* ATCC 6633 with its mutants in order to determine their possible site(s) of mutagenesis and to analyse amino acid xylanase sequence of the mutants of *B. subtilis* ATCC 6633.
Methodology: After the verification of xylanase production by all mutants of *B. subtilis* ATCC 6633

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on the xylan agar using Congo-red staining in the previous study, xylanase gene of the mutants was amplified from the genomic DNA to detect the mutagenesis site(s) by synthesizing primers directed against the sequence of xylanase gene obtained from the wild type of *Bacillus subtilis* subsp. s*pizizenii* ATCC 6633.

Results: The comparison of xylanase genes from different mutants of *B. subtilis* and the wild type revealed the site(s) of mutagenesis. Interestingly, the mutations of the mutants of *B. subtilis* ATCC 6633 in this study were significantly reflected at the 5' end of the mutants xylanase genes. The open reading frames (ORF) of the mutant xylanase genes ranged from 644 bp to 684 bp with translated encoding protein between 214 and 228 amino acid residues were obtained. On the other hand, predicted molecular mass from 23.94 kDa to 25.40 kDa and theoretical pI which ranged from 8.63 to 9.16 were attained from all of the mutant strains in this study. Based on the characteristics obtained, the mutant xylanases were suggested to belong to Glycosyl Hydrolase (GH) Family 11 with 98% homology to endo-1,4-beta-xylanase of *B. subtilis* subsp. *spizizenii* of W23. In fact, conserved regions, signal peptide, a cleavage site between the Ala28 and Ala29 residues and four Tyr residues specific to GH Family 11 xylanase were also observed and detected in all mutants. Indeed, two conserved glutamate residues of E94 and E183 that directly involved in the enzyme catalytic mechanism were also detected in the amino acid sequences of the mutants. The analysis of the deduced amino acid sequences revealed that the mutations in the signal peptide regions fostered increased hydrophobic core of xylanase residue. We suggested that these changes would probably be responsible for the increased extracellular xylanase yield in the mutants of *B. subtilis*. On the other hand, all the mutants of *B. subtilis* ATCC 6633 exhibited the tendency to be thermostable based on the Val, Ser and Thr frequency ratios which were almost identical to those of thermophiles. Furthermore, the increase of Thr to Ser ratio and presence of Arg residue found in the mutant strain of MXB 5 would enhance the polar interactions and hence improve the secondary structure stabilization that was usually one of the determining factors in the thermophilic proteins.

Conclusion: In a nutshell, the properties of *B. subtilis* mutant xylanases particularly mutant MXB 5 revealed its relevant potential in the biotechnology applications in bio-bleaching, textile, paper and pulp industries that commonly require high temperature usage in xylanase applications.

Keywords: Xylanase gene; random mutagenesis; thermophiles; mutants; Bacillus subtilis.

1. INTRODUCTION

Xylan is the substrate of xylanase. It is the second most abundant and inexhaustible polysaccharide in nature that responsible for one-third of the Earth's renewable organic carbon [1]. Xylan is present in the secondary cell wall in synergy with lignin which is a complex polyphenolic compound and cellulose, a 1,4-β-glucan forming the polymeric complex of plant cell walls [2]. Due to the heterogeneity and complex chemical nature of plant xylan, it requires the action of a complex hydrolytic enzyme system with diverse specificities and modes of action for its complete breakdown [3]. The most important enzymes responsible for arbitrary cleavage of the xylan backbone consisted of endo-l,4-β-xylanases or also known as 1,4-β-D-xylan xylanohydrolases which hydrolyse β-1,4-glycosidic linkages between xylopyranose unit by reducing xylan to xylooligosaccharides. As a result, the resulting low molecular mass fragments of xylan hydrolysis perform integral roles in the regulation of xylanase biosynthesis. These fragments include xylooligosaccharides, xylose, xylobiose, heterodisaccharides of xylose and glucose [2]. The classification of xylanases is based on the primary structure and comparisons of the catalytic domains that have been established, thus grouping enzymes in families of related sequences are conducted by analysing both the structural and mechanistic features [1]. The coherence of microbial xylanase could be deduced via researches conducted on xylanase structure and function, comprising different combinations of functional elements such as repeated sequences of amino acids, cellulose binding domains, linker regions and catalytic domains [4]. Thus far, xylanases have been classified based on their physico-chemical properties of molecular weight and isoelectric point (pI). Indeed, all characterised xylanases are categorised into two groups where the first group is consisted of xylanases which possesses a lower molecular weight of lesser than 30 kDa with higher pI value whereas another group of xylanases which possesses a higher molecular weight of greater than 30 kDa with lower pI value [5]. Besides that, xylanases have also been

classified based on the kinetic properties, crystal structure, substrate specificity and product profile [6]. Some elaborate genomic studies on *B. subtilis* have revealed the presence of genes encoding enzymes which participate in the degradation of plant cell wall polysaccharides. Updated and revised information deposited in the Carbohydrate-Active Enzyme (CAZy) database on the characteristics and classification of enzymes discovered that xylanases are related to Glycosyl Hydrolase (GH) Families 5, 7, 8, 9, 10, 11, 12, 16, 26, 30, 43, 44, 51 and 62 [7]. Notably, xylanase genes from *B. subtilis* B10 and *B. subtilis* M015 that were cloned, sequenced and expressed in *E. coli* had reported to show similarities with GH Family 11 as conducted by Huang et al. [8] and Banka et al. [9], respectively.

Xylanases are majorly considered to belong to GH Family 10 and 11 based on the hydrophobic cluster analysis of their catalytic domains and amino acid sequence similarities even though, they have further been reported in Families 5, 7, 8, 16, 26, 43, 52 and 62 of GH [10]. On the other hand, in a research conducted by Roncero [11], eight mutants of *B. subtilis* which were deficient in the utilization of xylan were isolated and characterized biochemically and genetically. In fact, there are two genes with the ability of controlling xylan utilization coded for two xylan-degrading enzymes present in the wild type strains. These genes are consisted of an extracellular β-xylanase designated as xylanase A (xyn A) and a cell-associated β-xylosidase designated as xylanase B (xyn B). Notably, xyn A gene in *B. subtilis* 168 demonstrated to encode a GH Family 11 xylanase and members of this family have been investigated and suggested to generate xylobiose and xylotriose along with the aldopentauronate 4-O-methylglucuronosyl-1,2-xylotetraose [12]. On the other hand, John et al. [13] studied the expression and characterisation of xylanase C (xyn C) from *B. subtilis* 168 which happened to be the first complete characterisation of an endoxylanase classified as a GH Family of 5 from a gram-positive bacterium. Consequently, all xylanases produced by *Bacillus* spp have been reported to belong to either GH Family 10 or 11 based on amino acid similarity [14]. Members of these families have exhibited striking similarities based on their primary and tertiary structures comparison [15]. Interestingly, the optimum pH of GH Family xylanases exhibit a wide variation ranged between acidic values as low as pH 2 to alkaline values as high as pH 11 [16]. pH modification or substrate binding has been observed to influence conformational changes in xylanase catalytic sites [17]. In alkaline xylanases, an asparagine acid is found to hydrogen bonded to the acid/base catalyst in the catalytic site, however, it is substituted by an aspartic acid in acidophilic endo-l,4-β-xylanases [17].

Xylanases which defined as the glycosidase enzymes catalyse the hydrolysis of β-1,4 xylan by cleaving β-1,4-glycosidic linkages to xylose and xylooligosaccharides in fact have established their significances through applications in many industries [18,19]. Xylanase has proven to be cost effective in paper manufacturing by reducing lignin in kraft pulp in biobleaching process, as a result, it increases the brightness and quality of paper pulp. Besides that, it improves digestibility of animal feeds, eases modification of cereal food stuffs, enhances textural and staling properties of breads, aids in production of industrial based chemicals including biofuel, ethanol and xylitol [20,21,22,23,24]. In respect to the importance of the xylanase applications in various industries, many investigations on the improved strains of microorganism particularly *B. subtilis* in xylanase production have been conducted. Recently, mutant strains of *Bacillus subtilis* subsp. *spizizenii* ATCC 6633 that designated as MXB 1, MXB 2, MXB 3, MXB 4 and MXB 5 created in our lab have demonstrated higher xylanase enzyme activity at their optimum pH of 6.0 to 6.47. Therefore, in this study, xylanase genes of these mutant strains were sequenced and compared with their parental wild type strain to determine the possible site(s) of mutagenesis and thus to elucidate the xylanase amino acid sequences for the production of the enzyme.

2. MATERIALS AND METHODS

2.1 Mutants and Wild Type of *Bacillus subtilis* subsp. *spizizenii* ATCC 6633

The wild type of *Bacillus subtilis* subsp. *spizizenii* ATCC 6633 was obtained from American Type Culture Collection (ATCC), Manassas, USA. Subsequently, the bacteria were subcultured on nutrient agar and incubated at 37℃ before being subjected to random mutagenesis. In our previous study, five mutants of *B. subtilis* ATCC 6633 designated as MXB 1, MXB 2, MXB 3, MXB 4 and MXB 5 were produced after random mutagenesis using ethyl methane sulfonate (EMS) and acridine orange (AO) as the chemical mutagens to induce the random mutation in the

wild type of *B. subtilis* subsp. *spizizenii* ATCC 6633 for overproduction of xylanase [25]. Notably, the mutant strain of *B. subtilis* MXB 1 was created using 100 µg/mL of EMS after exposure of the wild type for 15 min. On the other hand, MXB 2 and MXB 3 were produced after the exposure of 50 µg/mL of AO for 15 min and 30 min, respectively. Besides that, MXB 4 and MXB 5 were attained after 10 µg/mL of AO exposed to wild type for 15 min and 30 min, respectively. Subsequently, qualitative confirmation of xylanase activity produced by five mutant strains of *B. subtilis* ATCC 6633 was conducted using plate hydrolysis assay [26]. At the end of the experiments, the clear ring zone of discoloration on the xylan agar appeared due to the presence of xylanase activity where the xylan substrate was hydrolyzed by xylanase secreted by the mutants of *B. subtilis* ATCC 6633. Consequently, the xylan plate hydrolysis assay was obviously proved all mutants of *B. subtilis* ATCC 6633 were able to produce extracellular xylanase activity that hydrolyzed its beechwood xylan substrate as a result of xylan hydrolysis [26]. Subsequently, the xylanase genes of the mutants of *B. subtilis* ATCC 6633 were sequenced and analysed for the study of the random mutagenesis in the production of the xylanase enzyme.

2.2 Genomic DNA Extraction from Wild Type and Mutants of *B. subtilis* subsp. *spizizenii* ATCC 6633

Genomic DNA from the wild type and mutant strains of *B. subtilis* ATCC 6633 was extracted using Invitrogen PureLink™ genomic DNA mini kit. To begin, 1 mL of wild type bacteria culture was harvested during the exponential growth phase. Subsequently, the bacteria culture was centrifuged at 13,000 g for 2 min before resuspending the cell pellet in PureLink™ lysozyme digestion buffer. Then, the bacteria cells were subjected to freeze-thawing in liquid nitrogen followed by incubation at 37℃ for 30 min with 20 µL of 20 mg/mL Proteinase K. After that, 200 µL of PureLink™ genomic lysis buffer was mixed gently to yield a homogenous solution. Thereafter, 200 µL of 100% ethanol was sequentially added to precipitate the lysate. The precipitated DNA was then washed twice with equal volume of wash buffer before harvested by centrifugation at 10,000 g for 1 min at room temperature. In order to recover more DNA, the pellet was resuspended in PureLink™ genomic elution buffer in a collection tube at room temperature. Subsequently, the DNA suspension was centrifuged at the maximum speed for 1 min. The DNA pellet was then stored in the PureLink™ genomic elution buffer at -20℃ before it was being used as the DNA template of polymerase chain reaction (PCR). The same procedures were also applied to all mutant strains of *B. subtilis* ATCC 6633 to isolate their genomic DNA for PCR as the DNA template to identify the mutagenesis site(s) of each mutant.

2.3 Primers Design and PCR Conditions for the Amplification of Xylanase Gene

Based on the sequence of endoxylanase gene from *B. subtilis* subsp. *spizizenii* ATCC 6633 published in the DDBJ/EMBL/GenBank nucleotide sequence databases (accession number of ADGS01000013), two oligonucleotide primers of s-xyl F as the forward primer and s-xyl R as the reverse primer were designed and synthesized for PCR amplification of the xylanase gene using genomic DNA of wild type of *B. subtilis* ATCC 6633 as the DNA template. In this study, the forward and reverse primers that consisted of 5'-GGAGGGTAACCAAATGAAACA-3' and 5'-CTTTTCCCTCCAATAGTCAG-3' were used for the xylanase gene amplification, respectively. To amplify the xylanase gene, the PCR reaction mixture was comprised of 1 × Taq reaction buffer (New England Biolabs), 0.2 mM deoxynucleoside triphosphates (New England Biolabs), 0.2 µM forward and reverse primers, 100 ng of DNA template consisted of genomic DNA from wild type of *B. subtilis* ATCC 6633 and 1 U of DNA *Taq* DNA polymerase (New England Biolabs) before top up with sterile distilled water to a total volume reaction of 50 µL.

The PCR of xylanase gene was optimised at the initial denaturation temperature of 95℃ for 5 min followed by 35 seconds at 95℃, 35 seconds of annealing at 57℃ and lastly 1 min of extension at 72℃. Thereafter, the PCR reaction was repeated for 35 cycles with the final 72℃ extension step run for 5 min. The expected size of 699 bp of PCR product was anticipated from this amplification reaction.

To identify the mutagenesis site(s) of the mutants, the genomic DNA of the mutant strains consisted of MXB 1, MXB 2, MXB 3, MXB 4 and MXB 5 of *B. subtilis* ATCC 6633 was used as the DNA template for PCR amplification as the comparison with the type wild of *B. subtilis* ATCC 6633, respectively.

2.4 DNA Electrophoresis Analysis

The PCR products of xylanase gene from wild type and mutant strains of *B. subtilis* ATCC 6633 were loaded into 1.5% agarose gel electrophoresis. Then, the DNA band on the agarose gel was stained with 0.2 µg/mL ethidium bromide and visualized using UV illumination. After confirmation of the correct size of PCR products on the agarose gel, the PCR products were recovered and purified from the agarose gel before being subjected to sequencing to identify and detect the mutagenesis site(s) of the mutants of *B. subtilis* ATCC 6633.

2.5 Analysis of Nucleotide and Protein Sequences of Xylanase

After sequencing, DNA sequence chromatogram were intergraded in the contig assembly program (CAP) using Bioedit software before analysed using NCBI database and UniProtKB/TrEMBL database. Sequence analysis tools of EMBL computational services (http://www.ebi.ac.uk/Tools/emboss/) were employed for the comparison and analysis of nucleotide sequences while corresponding amino acid analysis were deduced using various tools in EXPASY Resource portal (http://www.expasy.org/genomics). Using CAZY database, GH Family 11 xylanase sequences with a UniProt code assigned and Xyl-11 sequences of known structure (http://www.cazy.org/GH11_all.html) were opted for amino acid sequences comparison using CLUSTAL W2 tool (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

3. RESULTS AND DISCUSSION

3.1 Amplification of Xylanase Gene from Wild Type and Mutants of *B. subtilis* subsp. *spizizenii* ATCC 6633

The PCR products of xylanase gene were obtained using the genomic DNA of wild type and mutants of *B. subtilis* subsp. *spizizenii* ATCC 6633 as the DNA template, respectively. Based on the agarose gel electrophoresis in Fig. 1, Lane 1 to 5 indicates the single band of PCR products attained from the mutants of *B. subtilis* designated as MXB 1, MXB 2, MXB 3, MXB 4 and MXB 5 whereas Lane 6 reveals the PCR product obtained from the wild type.

Notably, all the sizes of the PCR products of 699 bp were obtained as shown on the agarose gel in Fig. 1. Indeed, there were absences of non-specific DNA fragments on the agarose gel indicates that the optimised amplification of PCR conditions was successfully attained for the amplification of xylanase gene from wild type and mutants of *B. subtilis*. Thereafter, the PCR products of the wild type and mutants of *B. subtilis* were recovered and purified from the agarose gel electrophoresis before sequencing.

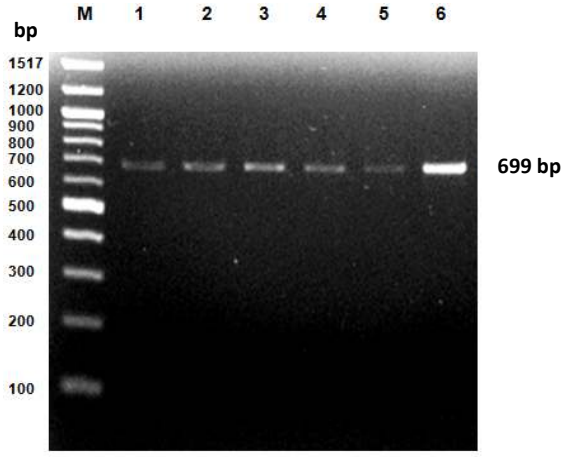

Fig. 1. Agarose gel electrophoresis of xylanase PCR products of wild type and mutants of *B. subtilis* ATCC 6633 on 1.5% agarose gel

Lane M indicates the DNA marker of 100 bp. The xylanase PCR products of mutants MXB 1 is shown in lane 1; MXB 2 in lane 2; MXB 3 in lane 3; MXB 4 in lane 4 and MXB 5 in lane 5. Lane 6 shows the xylanase PCR product of wild type of B. subtilis ATCC 6633

3.2 Identification and Detection of Random Mutagenesis Site(s) of Mutants of *B. subtilis* subsp. *spizizenii* ATCC 6633

After sequencing, the complete nucleotide sequences of xylanase gene from the wild type and mutant strains of *B. subtilis* ATCC 6633 were displayed as the forward and reverse nucleotide chromatograms that had been intergraded in the contig assembly program (CAP) using Bioedit software. Subsequently, identification and detection were performed via pairwise alignment between each mutant and wild type of *B. subtilis* ATCC 6633 to reveal their relationships, to identify regions of similarity and differences and lastly to determine the mutagenesis site(s) of each mutants.

3.3 Mutations of Xylanase Gene in Mutant MXB 1

Interestingly, mutations were significantly reflected at the 5' end of the xylanase gene of mutant MXB 1. There were nucleotide substitutions at the positions 17 and 24 of T/G and T/C. Additionally, insertions of nucleotides T, C and A at the positions 21, 22 and 657 of the mutant MXB 1 were also detected. Further deletions of nucleotides A and C at the positions 638 and 649 (of the wild type) also occurred in mutant MXB 1 as demonstrated in Fig. 2.

```
MXB1         1 ATGAAACAAAAAAGAATGAATCGTTTTTGTTAGCAGTTTTATGTAGTCTA     50
               ||||||||||||||||.|||  |.||||||||||||||||||||||||||
W.T          1 ATGAAACAAAAAAGAAGGAA--GCTTTTGTTAGCAGTTTTATGTAGTCTA     48

MXB1        51 TTGCTGTCATTACCTGCATGGTCCGCTCAGGCGGCAACTACAATCACCTC    100
               ||||||||||||||||||||||||||||||||||||||||||||||||||
W.T         49 TTGCTGTCATTACCTGCATGGTCCGCTCAGGCGGCAACTACAATCACCTC     98

MXB1       101 AAATCAAACCGGCTCCCAAGATGGCTACGACTATGAATTGTGGAAGGATT    150
               ||||||||||||||||||||||||||||||||||||||||||||||||||
W.T         99 AAATCAAACCGGCTCCCAAGATGGCTACGACTATGAATTGTGGAAGGATT    148

MXB1       151 ATGGGAACACCAGTATGACGCTCAATAGCGGCGGCGCGTTTAGTGCCCAG    200
               ||||||||||||||||||||||||||||||||||||||||||||||||||
W.T        149 ATGGGAACACCAGTATGACGCTCAATAGCGGCGGCGCGTTTAGTGCCCAG    198

MXB1       201 TGGAGTAACATTGGGAATGCTTTATTCCGAAAAGGCAAGAAATTCGATAC    250
               ||||||||||||||||||||||||||||||||||||||||||||||||||
W.T        199 TGGAGTAACATTGGGAATGCTTTATTCCGAAAAGGCAAGAAATTCGATAC    248

MXB1       251 AACCAAGACTCACTCAGAGCTTGGAAACATATCAATTAATTACAACGCAA    300
               ||||||||||||||||||||||||||||||||||||||||||||||||||
W.T        249 AACCAAGACTCACTCAGAGCTTGGAAACATATCAATTAATTACAACGCAA    298

MXB1       301 CCTTCAATCCGAGAGGAAATTCCTATCTATGTGTTTATGGATGGACGAAG    350
               ||||||||||||||||||||||||||||||||||||||||||||||||||
W.T        299 CCTTCAATCCGAGAGGAAATTCCTATCTATGTGTTTATGGATGGACGAAG    348

MXB1       351 GATCCGCTCGTAGAATATTATATCGTCGATAATTGGGGCACTTATAGACC    400
               ||||||||||||||||||||||||||||||||||||||||||||||||||
W.T        349 GATCCGCTCGTAGAATATTATATCGTCGATAATTGGGGCACTTATAGACC    398

MXB1       401 GACCGGTACGCCAAAGGGTACTATTACTGTTGATGGGGGCACATATGACA    450
               ||||||||||||||||||||||||||||||||||||||||||||||||||
W.T        399 GACCGGTACGCCAAAGGGTACTATTACTGTTGATGGGGGCACATATGACA    448

MXB1       451 TTTATGAGACCACTCGGATCAACCAACCTTCTATTATAGGTATCGCAACC    500
               ||||||||||||||||||||||||||||||||||||||||||||||||||
W.T        449 TTTATGAGACCACTCGGATCAACCAACCTTCTATTATAGGTATCGCAACC    498

MXB1       501 TTTAAACAGTATTGGAGTGTCCGGCAGACAAAACGAACGAGCGGAACCGT    550
               ||||||||||||||||||||||||||||||||||||||||||||||||||
W.T        499 TTTAAACAGTATTGGAGTGTCCGGCAGACAAAACGAACGAGCGGAACCGT    548

MXB1       551 ATCTGTCAGCGAACACTTCAAGAAGTGGGAAAGCTTAGGAATGCCAATGG    600
               ||||||||||||||||||||||||||||||||||||||||||||||||||
W.T        549 ATCTGTCAGCGAACACTTCAAGAAGTGGGAAAGCTTAGGAATGCCAATGG    598

MXB1       601 GTAAAATGTATGAAACCGCGCTTACGGTAGAGGGATATC-AAAGTAATGG    649
               ||||||||||||||||||||||||||||||||||||||| ||||||||||
W.T        599 GTAAAATGTATGAAACCGCGCTTACGGTAGAGGGATATCAAAAGTAATGG    648

MXB1       650 -CAGTGCTAAATGTATCTACTAATGTACTGACTATT    684
                ||||||| ||||||||||||||||||||||||||||
W.T        649 CCAGTGCT-AATGTATCTACTAATGTACTGACTATT    683
```

Fig. 2. Pairwise alignment comparing the xylanase gene of wild type *B. subtilis* ATCC 6633 with MXB 1 mutant sequence via EMBOSS Needle

Mutagenesis sites are substitutions of nucleotides T/G and T/C as shown in yellow whereas the insertions of nucleotides T, C and A and deletions of A and C are highlighted in green

3.4 Mutations of Xylanase Gene in Mutant MXB 2

Mutations were also found at the 5' end of the xylanase gene in mutant MXB 2. The xylanase gene of MXB 2 also disclosed mutagenesis sites at the nucleotide positions of 20, 25 and 662 corresponding to single nucleotide substitutions of C/G, T/C and A/T, respectively. Furthermore, mutation was also generated by insertions of nucleotides T at the positions 17, 18 and 19 whereas deletions of nucleotides A and C at the positions 638 and 649 (of the wild type) were indeed observed as revealed in Fig. 3.

```
MXB2         1 ATGAAACAAAAAAGAATTTCGAAGTTTTTGTTAGCAGTTTTATGTAGTCT     50
               |||||||||||||||||   .||||.|||||||||||||||||||||||||
W.T          1 ATGAAACAAAAAAGAA---GGAAGCTTTTGTTAGCAGTTTTATGTAGTCT     47

MXB2        51 ATTGCTGTCATTACCTGCATGGTCCGCTCAGGCGGCAACTACAATCACCT    100
               ||||||||||||||||||||||||||||||||||||||||||||||||||||
W.T          48 ATTGCTGTCATTACCTGCATGGTCCGCTCAGGCGGCAACTACAATCACCT     97

MXB2       101 CAAATCAAACCGGCTCCCAAGATGGCTACGACTATGAATTGTGGAAGGAT    150
               ||||||||||||||||||||||||||||||||||||||||||||||||||||
W.T          98 CAAATCAAACCGGCTCCCAAGATGGCTACGACTATGAATTGTGGAAGGAT    147

MXB2       151 TATGGGAACACCAGTATGACGCTCAATAGCGGCGGCGCGTTTAGTGCCCA    200
               ||||||||||||||||||||||||||||||||||||||||||||||||||||
W.T         148 TATGGGAACACCAGTATGACGCTCAATAGCGGCGGCGCGTTTAGTGCCCA    197

MXB2       201 GTGGAGTAACATTGGGAATGCTTTATTCCGAAAAGGCAAGAAATTCGATA    250
               ||||||||||||||||||||||||||||||||||||||||||||||||||||
W.T         198 GTGGAGTAACATTGGGAATGCTTTATTCCGAAAAGGCAAGAAATTCGATA    247

MXB2       251 CAACCAAGACTCACTCAGAGCTTGGAAACATATCAATTAATTACAACGCA    300
               ||||||||||||||||||||||||||||||||||||||||||||||||||||
W.T         248 CAACCAAGACTCACTCAGAGCTTGGAAACATATCAATTAATTACAACGCA    297

MXB2       301 ACCTTCAATCCGAGAGGAAATTCCTATCTATGTGTTTATGGATGGACGAA    350
               ||||||||||||||||||||||||||||||||||||||||||||||||||||
W.T         298 ACCTTCAATCCGAGAGGAAATTCCTATCTATGTGTTTATGGATGGACGAA    347

MXB2       351 GGATCCGCTCGTAGAATATTATATCGTCGATAATTGGGGCACTTATAGAC    400
               ||||||||||||||||||||||||||||||||||||||||||||||||||||
W.T         348 GGATCCGCTCGTAGAATATTATATCGTCGATAATTGGGGCACTTATAGAC    397

MXB2       401 CGACCGGTACGCCAAAGGGTACTATTACTGTTGATGGGGGCACATATGAC    450
               ||||||||||||||||||||||||||||||||||||||||||||||||||||
W.T         398 CGACCGGTACGCCAAAGGGTACTATTACTGTTGATGGGGGCACATATGAC    447

MXB2       451 ATTTATGAGACCACTCGGATCAACCAACCTTCTATTATAGGTATCGCAAC    500
               ||||||||||||||||||||||||||||||||||||||||||||||||||||
W.T         448 ATTTATGAGACCACTCGGATCAACCAACCTTCTATTATAGGTATCGCAAC    497

MXB2       501 CTTTAAACAGTATTGGAGTGTCCGGCAGACAAAACGAACGAGCGGAACCG    550
               ||||||||||||||||||||||||||||||||||||||||||||||||||||
W.T         498 CTTTAAACAGTATTGGAGTGTCCGGCAGACAAAACGAACGAGCGGAACCG    547

MXB2       551 TATCTGTCAGCGAACACTTCAAGAAGTGGGAAAGCTTAGGAATGCCAATG    600
               ||||||||||||||||||||||||||||||||||||||||||||||||||||
W.T         548 TATCTGTCAGCGAACACTTCAAGAAGTGGGAAAGCTTAGGAATGCCAATG    597

MXB2       601 GGTAAAATGTATGAAACCGCGCTTACGGTAGAGGGATATC-AAAGTAATG    649
               |||||||||||||||||||||||||||||||||||||||| |||||||||
W.T         598 GGTAAAATGTATGAAACCGCGCTTACGGTAGAGGGATATCAAAAGTAATG    647

MXB2       650 G-CAGTGCTAATGAATCTACTAATGTACTGACTATT    684
               | ||||||||||.|||||||||||||||||||||||
W.T         648 GCCAGTGCTAATGTATCTACTAATGTACTGACTATT    683
```

Fig. 3. Pairwise alignment comparing the xylanase gene of wild type *B. subtilis* ATCC 6633 with MXB 2 mutant sequence via EMBOSS Needle

Mutagenesis sites are substitutions of C/G, T/C and A/T as highlighted in yellow while insertions of nucleotides T, and deletions of nucleotides A and C are shown in green

3.5 Mutations of Xylanase Gene in Mutant MXB 3

Xylanase gene of mutant strain MXB 3 reflected most of its mutation at the 5' end. Substitutions nucleotides of T/A, C/G and T/C at the positions 19, 20 and 25 were present whereas insertions of nucleotides T, A and T at the positions 16, 17 and 18 were also observed. In fact, the deletions of nucleotides A and C at the positions 638 and 649 (of the wild type) were also seen to have materialized in mutant MXB 3 as shown in Fig. 4.

```
MXB3         1 ATGAAACAAAAAAGATATTCGAAGTTTTTGTTAGCAGTTTTATGTAGTCT     50
               ||||||||||||||||   ..|||||.|||||||||||||||||||||||||
W.T          1 ATGAAACAAAAAAGA---AGGAAGCTTTTGTTAGCAGTTTTATGTAGTCT     47

MXB3        51 ATTGCTGTCATTACCTGCATGGTCCGCTCAGGCGGCAACTACAATCACCT    100
               ||||||||||||||||||||||||||||||||||||||||||||||||||||
W.T          48 ATTGCTGTCATTACCTGCATGGTCCGCTCAGGCGGCAACTACAATCACCT     97

MXB3       101 CAAATCAAACCGGCTCCCAAGATGGCTACGACTATGAATTGTGGAAGGAT    150
               ||||||||||||||||||||||||||||||||||||||||||||||||||||
W.T          98 CAAATCAAACCGGCTCCCAAGATGGCTACGACTATGAATTGTGGAAGGAT    147

MXB3       151 TATGGGAACACCAGTATGACGCTCAATAGCGGCGGCGCGTTTAGTGCCCA    200
               ||||||||||||||||||||||||||||||||||||||||||||||||||||
W.T         148 TATGGGAACACCAGTATGACGCTCAATAGCGGCGGCGCGTTTAGTGCCCA    197

MXB3       201 GTGGAGTAACATTGGGAATGCTTTATTCCGAAAAGGCAAGAAATTCGATA    250
               ||||||||||||||||||||||||||||||||||||||||||||||||||||
W.T         198 GTGGAGTAACATTGGGAATGCTTTATTCCGAAAAGGCAAGAAATTCGATA    247

MXB3       251 CAACCAAGACTCACTCAGAGCTTGGAAACATATCAATTAATTACAACGCA    300
               ||||||||||||||||||||||||||||||||||||||||||||||||||||
W.T         248 CAACCAAGACTCACTCAGAGCTTGGAAACATATCAATTAATTACAACGCA    297

MXB3       301 ACCTTCAATCCGAGAGGAAATTCCTATCTATGTGTTTATGGATGGACGAA    350
               ||||||||||||||||||||||||||||||||||||||||||||||||||||
W.T         298 ACCTTCAATCCGAGAGGAAATTCCTATCTATGTGTTTATGGATGGACGAA    347

MXB3       351 GGATCCGCTCGTAGAATATTATATCGTCGATAATTGGGGCACTTATAGAC    400
               ||||||||||||||||||||||||||||||||||||||||||||||||||||
W.T         348 GGATCCGCTCGTAGAATATTATATCGTCGATAATTGGGGCACTTATAGAC    397

MXB3       401 CGACCGGTACGCCAAAGGGTACTATTACTGTTGATGGGGGCACATATGAC    450
               ||||||||||||||||||||||||||||||||||||||||||||||||||||
W.T         398 CGACCGGTACGCCAAAGGGTACTATTACTGTTGATGGGGGCACATATGAC    447

MXB3       451 ATTTATGAGACCACTCGGATCAACCAACCTTCTATTATAGGTATCGCAAC    500
               ||||||||||||||||||||||||||||||||||||||||||||||||||||
W.T         448 ATTTATGAGACCACTCGGATCAACCAACCTTCTATTATAGGTATCGCAAC    497

MXB3       501 CTTTAAACAGTATTGGAGTGTCCGGCAGACAAAACGAACGAGCGGAACCG    550
               ||||||||||||||||||||||||||||||||||||||||||||||||||||
W.T         498 CTTTAAACAGTATTGGAGTGTCCGGCAGACAAAACGAACGAGCGGAACCG    547

MXB3       551 TATCTGTCAGCGAACACTTCAAGAAGTGGGAAAGCTTAGGAATGCCAATG    600
               ||||||||||||||||||||||||||||||||||||||||||||||||||||
W.T         548 TATCTGTCAGCGAACACTTCAAGAAGTGGGAAAGCTTAGGAATGCCAATG    597

MXB3       601 GGTAAAATGTATGAAACCGCGCTTACGGTAGAGGGATATC-AAAGTAATG    649
               |||||||||||||||||||||||||||||||||||||||| |||||||||
W.T         598 GGTAAAATGTATGAAACCGCGCTTACGGTAGAGGGATATCAAAAGTAATG    647

MXB3       650 G-CAGTGCTAATGTATCTACTAATGTACTGACTATT    684
               | ||||||||||||||||||||||||||||||||
W.T         648 GCCAGTGCTAATGTATCTACTAATGTACTGACTATT    683
```

Fig. 4. Pairwise alignment comparing the xylanase gene of wild type *B. subtilis* ATCC 6633 with MXB 3 mutant sequence via EMBOSS Needle

Mutations are substitutions of T/A, C/G and T/C as highlighted in yellow while insertions of nucleotides T, A and T and deletions of nucleotides A and C are indicated in green

3.6 Mutations of Xylanase Gene in Mutant MXB 4

Similar to the mutations present in other mutants of *B. subtilis*, the xylanase gene of mutant MXB 4 showed mutations at the positions 17 and 25 indicating single nucleotide substitutions of T/G and G/C. Addition mutations were also detected by insertions of nucleotides T, G and C at the positions 20, 23 and 24 whereas the deletions of nucleotides A and C at the positions 638 and 649 (of the wild type) were observed as displayed in Fig. 5.

```
MXB4         1 ATGAAACAAAAAAGAATGATAGGCGTTTTGTTAGCAGTTTTATGTAGTCT     50
               |||||||||||||||||.|| ||  .|||||||||||||||||||||||
W.T          1 ATGAAACAAAAAAGAAGGA-AG--CTTTTGTTAGCAGTTTTATGTAGTCT     47

MXB4        51 ATTGCTGTCATTACCTGCATGGTCCGCTCAGGCGGCAACTACAATCACCT    100
               ||||||||||||||||||||||||||||||||||||||||||||||||||
W.T          48 ATTGCTGTCATTACCTGCATGGTCCGCTCAGGCGGCAACTACAATCACCT     97

MXB4       101 CAAATCAAACCGGCTCCCAAGATGGCTACGACTATGAATTGTGGAAGGAT    150
               ||||||||||||||||||||||||||||||||||||||||||||||||||
W.T          98 CAAATCAAACCGGCTCCCAAGATGGCTACGACTATGAATTGTGGAAGGAT    147

MXB4       151 TATGGGAACACCAGTATGACGCTCAATAGCGGCGGCGCGTTTAGTGCCCA    200
               ||||||||||||||||||||||||||||||||||||||||||||||||||
W.T         148 TATGGGAACACCAGTATGACGCTCAATAGCGGCGGCGCGTTTAGTGCCCA    197

MXB4       201 GTGGAGTAACATTGGGAATGCTTTATTCCGAAAAGGCAAGAAATTCGATA    250
               ||||||||||||||||||||||||||||||||||||||||||||||||||
W.T         198 GTGGAGTAACATTGGGAATGCTTTATTCCGAAAAGGCAAGAAATTCGATA    247

MXB4       251 CAACCAAGACTCACTCAGAGCTTGGAAACATATCAATTAATTACAACGCA    300
               ||||||||||||||||||||||||||||||||||||||||||||||||||
W.T         248 CAACCAAGACTCACTCAGAGCTTGGAAACATATCAATTAATTACAACGCA    297

MXB4       301 ACCTTCAATCCGAGAGGAAATTCCTATCTATGTGTTTATGGATGGACGAA    350
               ||||||||||||||||||||||||||||||||||||||||||||||||||
W.T         298 ACCTTCAATCCGAGAGGAAATTCCTATCTATGTGTTTATGGATGGACGAA    347

MXB4       351 GGATCCGCTCGTAGAATATTATATCGTCGATAATTGGGGCACTTATAGAC    400
               ||||||||||||||||||||||||||||||||||||||||||||||||||
W.T         348 GGATCCGCTCGTAGAATATTATATCGTCGATAATTGGGGCACTTATAGAC    397

MXB4       401 CGACCGGTACGCCAAAGGGTACTATTACTGTTGATGGGGGCACATATGAC    450
               ||||||||||||||||||||||||||||||||||||||||||||||||||
W.T         398 CGACCGGTACGCCAAAGGGTACTATTACTGTTGATGGGGGCACATATGAC    447

MXB4       451 ATTTATGAGACCACTCGGATCAACCAACCTTCTATTATAGGTATCGCAAC    500
               ||||||||||||||||||||||||||||||||||||||||||||||||||
W.T         448 ATTTATGAGACCACTCGGATCAACCAACCTTCTATTATAGGTATCGCAAC    497

MXB4       501 CTTTAAACAGTATTGGAGTGTCCGGCAGACAAAACGAACGAGCGGAACCG    550
               ||||||||||||||||||||||||||||||||||||||||||||||||||
W.T         498 CTTTAAACAGTATTGGAGTGTCCGGCAGACAAAACGAACGAGCGGAACCG    547

MXB4       551 TATCTGTCAGCGAACACTTCAAGAAGTGGGAAAGCTTAGGAATGCCAATG    600
               ||||||||||||||||||||||||||||||||||||||||||||||||||
W.T         548 TATCTGTCAGCGAACACTTCAAGAAGTGGGAAAGCTTAGGAATGCCAATG    597

MXB4       601 GGTAAAATGTATGAAACCGCGCTTACGGTAGAGGGATATC-AAAGTAATG    649
               ||||||||||||||||||||||||||||||||||||||||| |||||||||
W.T         598 GGTAAAATGTATGAAACCGCGCTTACGGTAGAGGGATATCAAAAGTAATG    647

MXB4       650 G-CAGTGCTAATGTATCTACTAATGTACTGACTATT    684
               | ||||||||||||||||||||||||||||||||
W.T         648 GCCAGTGCTAATGTATCTACTAATGTACTGACTATT    683
```

Fig. 5. Pairwise alignment comparing the xylanase gene of wild type *B. subtilis* ATCC 6633 with MXB 4 mutant sequence via EMBOSS Needle

Mutations are substitutions of T/G and G/C as highlighted in yellow whereas insertions of nucleotides T, G and C and deletions of nucleotides A and C are displayed in green

3.7 Mutations of Xylanase Gene in Mutant MXB 5

The 5' end of the xylanase gene of MXB 5 was significantly possessed mutations. The single nucleotide substitutions of T/G and C/T were found at the positions 17 and 25. Other mutations including insertion of nucleotides C and A at the positions 20 and 23 and deletion of nucleotide C at position 649 (of the wild type) were also observed and shown in Fig. 6.

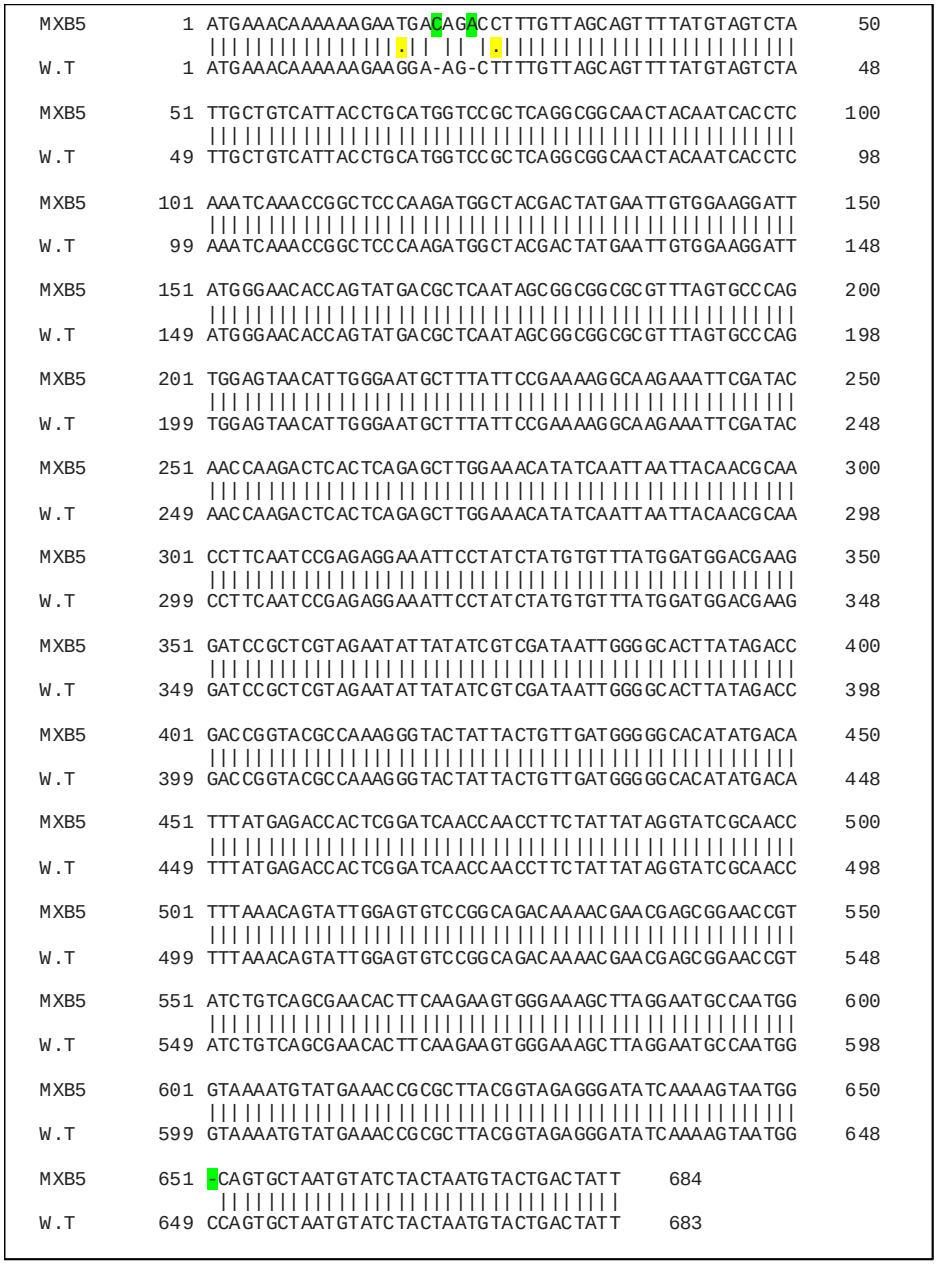

Fig 6. Pairwise alignment comparing the xylanase gene of wild type *B. subtilis* ATCC 6633 with MXB 5 mutant sequence via EMBOSS Needle

Mutations are substitutions of T/G and C/T as highlighted in yellow whereas insertions of nucleotides C and A with deletion of nucleotide C are displayed in green

3.8 Identification and Nucleotide Sequences Analysis of Mutants Xylanase Genes

In an attempt to confirm the identification of the mutant strains xylanase genes, the nucleotide sequences of their amplified xylanase genes were determined. The sequenced results were subjected to nucleotide BLAST analysis conducted in GenBank and EMBL-EBI databases. Based on our results as shown in Table 1, the mutant strains were confirmed to belong to the genus of *Bacillus*. The gene sequence data of the mutants exhibited homology with the same hit organisms with approximately 50 BLAST hits displaying related identities. Notably, the closest sequence matched results indicated 99% analogy with two best hits of endo-1,4-beta-xylanase gene of *B. subtilis* subsp. *spizizenii* strain NRS 231 (also known as *B. subtilis* ATCC 6633) with the GenBank accession number of CP010434.1 and *B. subtilis* subsp. *spizizenii* str. W23 with the GenBank accession number of CP002183.1. On the other hand, 95% similarity of the sequence result revealed endo-1,4-beta-xylanase A of *B. subtilis* subsp. *spizizenii* TU-B-10 with the GenBank accession number of CP002905.1. Furthermore, 92% nucleotide similarity identities the xyn A gene of *Bacillus* sp. JS with the GenBank accession number of CP003492.1, endo-1,4-beta-xylanase gene of *Bacillus amyloliquefaciens* TA208 with the GenBank accession number of CP002627.1 and glycoside hydrolase gene of *Bacillus amyloliquefaciens* XH7 with the GenBank accession number of CP002927.1 in addition to 81.3% homology with beta-xylanase precursor gene of *Bacillus pumilus* with the EMBL accession number of HM536195.

3.9 Amino Acid Sequences of *B. subtilis* ATCC 6633 Mutant Strains

The xylanase nucleotide sequences identified from the mutants resulted in open reading frames (ORF) of 669 bp, 684 bp, 684 bp, 684 bp and 644 bp using EXPASY-PROSITE Translate tool with the initiated nucleotides from 3 to 671, 1 to 684, 1 to 684, 1 to 684 and 3 to 647 for xylanases MXB 1 to MXB 5, respectively. The translated encoding protein which composed of 222, 228, 228, 228 and 214 amino acid residues were also identified. Based on the deduced amino acid sequences, the predicted theoretical molecular mass and pI of the mutant strains xylanase protein were comprised of 24.73 kDa with pI of 8.63, 25.38 kDa with pI of 9.05, 25.40 kDa with pI of 9.16, 25.28 kDa with pI of 9.07 and 23.94 kDa with pI of 8.91 for mutant strains of MXB 1 to MXB 5, respectively. The deduced amino acid sequences of the mutants were subjected to the BLAST search program to decipher the homologous sequences. Observed homology of mutants xylanase amino acid was detected to be endo-1,4-beta-xylanase of *B. subtilis* subsp. *spizizenii* W23 with 98% identity (EMBL_E0TVS7), *B. subtilis* subsp. *spizizenii* TU-B-10 with 94% identity (EMBL_Q5EFR9), *B. subtilis* subsp. *inaquosorum* KCTC 13429 with 92% identity (EMBL_L8Q1B0) and *B. stratosphericus* LAMA 585 with 75% identity (EMBL_M5QXB9), respectively. Furthermore, the mutant xylanases also possessed high amino acid similarity with Xyn A of *B. amyloliquefaciens* with 93% identity (EMBL_E1UUS4) and *B. pumilus* with 75% identity (EMBL_P00694). On the other hand, some reports in the databases revealed that the deduced amino acid sequence demonstrated extensive homology to xylanases belonged to GH Family 11 xylanases as classified by Gilkes et al. [27]. GH Family 11 xylanases are commonly characterized by a basic pI, a low molecular weight of lesser than 30 kDa, two glutamates functioning as the catalytic residues via a double displacement catalytic mechanism and a jelly roll fold structure [1]. GH Family 11 xylanases are solely active on D-xylose containing substrates. Besides that, they also possess a lower catalytic variability than the Family 10 xylanases. In fact, the products resulting from their action can be hydrolysed further by the Family 10 xylanases. As a result, GH Family 11 xylanases are also regarded as the true xylanases [28]. Based on the findings in this study, the sequence analogous to a typical signal peptide were present at the 5' end of the open reading frame of each mutant strain. The reputed signal peptide of the respective mutant strains possessed 28 amino acid residues with copious hydrophobic residues [29]. Therefore, from the results, we suggested that the xylanases from the mutant strains of *B. subtilis* in this study could be anticipated to belong to the GH Family 11 xylanases.

3.10 Amino Acids Characteristics of Wild Type and Mutant Strains of *B. subtilis* ATCC 6633

In retrospect of our previous study, we endeavored to fathom the relationship between the enzyme activities of the wild type and mutant xylanases via amino acid sequence comparison and analysis. Our studies revealed mutant strains MXB 3 and MXB 5 of *B. subtilis* ATCC 6633 possessed the most and the least related

identity to the wild type of *B. subtilis* ATCC 6633, respectively. As a result of the random mutagenesis, it could be observed that the amino acids M, K, Q and R of the wild type *B. subtilis* subsp. *spizizenii* ATCC 6633 were replaced at the positions 5, 1, 2 and 4 by N, E, T and K in mutants of MXB 1 and MXB 5 as shown in Table 2, respectively. Amino acid K was substituted by E at the position 6 and the insertion of S at the position 7 was seen in mutant MXB 1. Furthermore, amino acids N, V, S and T were observed to be replaced by K, C, I and Y at the positions 219, 220, 221 and 222 correspondingly in MXB 1 mutant. MXB 5 exhibited K substitution to D, F substitution to P, S substitution to K and insertion of R at the positions 6, 8, 214 and 7, respectively. M was deleted in both mutants of MXB 1 and MXB 5 at the position 1 of the wild type. On the other hand, in the mutant MXB 2, amino acids M, K and V were observed to be replaced by I, S and E at the positions 6, 7 and 221 while the insertion of K was seen at the position 8. Similar to mutant MXB 2, mutant MXB 3 exhibited varying mutations at the same sites. In mutant MXB 3, amino acids M and K were noticed to be replaced by Y and S at the positions 6 and 7 with the insertion of K at the position 8, respectively. However, mutant MXB 4 was observed to possess amino acids mutation of K and F which were substituted by I and V at the positions 7 and 9 correspondingly. Insertion mutation was also seen by G at the position 8 in mutant MXB 4 as shown in Table 2.

3.11 Amino Acid Sequences Analogy between *B. subtilis* Mutant Xylanases and GH Family 11 Xylanases

The comparison between the deduced amino acid sequences of xylanases of the mutant strains of *B. subtilis* with those of known GH Family 11 xylanases of *B. subtilis* subsp. *spizizenii* is shown in Fig. 7 while those with other *Bacillus* origins are evaluated in Fig. 8. A high level of sequence homology was seen to be *B. subtilis* W23 with the Genbank accession number of ADM37853.1 and *B. subtilis* NRS 231 (ATCC 6633), respectively. Indeed, the structural features of the mutant strain xylanases including the active site were found to be highly conserved. According to Henrissat [30], a direct relationship between sequence and folding similarities based on the comparison of the primary sequences of Glycosyl Hydrolases (GH) are better reflected the structural similarity and thus proven to be useful especially with the increasing number of GH (xylanase) genes that are being sequenced. The sequence alignment of the mutant xylanases with other known GH 11 xylanases of other *Bacillus* species origin obtained from the CAZy database were also analysed. Notably, *B. subtilis* cho40 (HM159999.1), *B. subtilis* B230, *B. stratus-phericus* LAMA 585 (M5QXB9), *B. altitudinis* (HQ51), *B. amyloliquefaciens* DSM 7 (CBI42956.1), *B. pumilus* and *B. akibai* ATCC 43226 (W4QUM4) revealed similarities to a lesser extent with major differences in the amino acids at n-terminal regions [31,32]. However, most structural features of the xylanases including the active site are highly conserved [33]. Structurally and catalytically vital residues of xylanase from divergent organisms are usually considered to be conserved in the course of evolution [32]. Amino acids in the n-terminal region were present in all other xylanases with the exception of *B. subtilis* B230 and *B. subtilis* cho40. These amino acids are thought to bury hydrophobic residues in the core of the protein which could contribute to thermostability in the xylanases that carry them [31]. Several mutant xylanases from bacteria by random mutagenesis have been evaluated. However, more xylanase analysis considerably focused on site directed mutagenesis. The xylanase n-terminal amino acid sequences of the mutants and *B subtilis* subsp. *spizizenii* confirmed a characteristic signal peptide with a cleavage site between the Ala28 and Ala29 residues and also an intergenic region of the mature polypeptide with potential xylanase regions [29,31]. Amino acid homology with GH Family 11 xylanases of *B. subtilis* subsp. *spizizenii* and *Bacillus* of other origins disclosed two highly conserved residues of Glu94 and Glu183 which are considered to be the key catalytic residues of our mutant *B. subtilis* xylanases and are suggested to be of GH Family 11 xylanases origin as shown in Fig. 7 [32]. Xylanase catalytic residues of these mutants were found in two conserved regions [30] which are homogenous to xyn 11X in *B. subtilis* 230 based on sequence comparison [31]. The authentication of xyn 11X in *B. subtilis* 230 was based on comparison with the three-dimensional structure and site-directed mutagenesis of *B. pumilus* xylanase [32]. Further confirmation based on comparison with site-directed mutagenesis of Glu78 and Glu172 (corresponding to Glu94 and Glu183 of *B. subtilis* 230) in *B. circulans* conducted by Wakarchuk et al. [34] signified the Glu residues played significant catalytic roles in which one glutamate functions as an acid/base catalyst whereas the

other acts as a nucleophile and stabilizes the reaction intermediate [15]. The two conserved catalytic glutamate residues are located opposite to each other in an open active site cleft [35]. Besides that, the results obtained by Esteves et al. [36] also revealed that Tyr 16, Tyr 83, Tyr 95, and Tyr 185 of the mutant *B. subtilis* xylanases are also conserved in all GH Family 11 xylanases as shown in Fig. 8.

3.12 Functional Roles of Deduced Signal Peptides of Mutant Xylanases

Using chemicals in random mutagenesis, we have created several mutant strains of *B. subtilis* subsp. *spizizenii* ATCC 6633 that demonstrated xylanase activity on plate hydrolysis assay. The mutations materialized at the signal peptides of the mutant xylanases. According to Page *et al.* [37], the deletion or modification of the xylanase A signal peptide shows variations in xylanase production based on the nature of the signal sequences. Bearing this in mind, the increase in charged residues found in the n-domain of the signal peptide of the mutants xylanase seemed to be of significant benefit for the mutants xylanase export into the culture medium. The xylanase (xyn) of MXB 1 displayed substitution mutations (K1E) and xyn of MXB 5 exhibited (K1E, K6D and insertion of R) amino acids mutations which potentially resulted in increased xylanase yield. Comparably, the signal peptide mutations on xylanase C secretion in *Streptomyces lividans* using random mutagenesis obtained from the replacement of a positive charge by a negative charge of K38E in the n-domain of the signal peptide caused a higher xylanase yield according to Li et al. [38]. In fact in their study, the replacement of a negative charge of D41N as well as removal of a positive charge of K38I in the signal peptide by site directed mutagenesis resulted a double increase in the xylanase yield. Interestingly, the related mutations also occurred in xyn of MXB 1(M5N), xyn of MXB 2 (M6I), xyn of MXB 4 (K7I) and xyn of MXB 5 (M5N) which could be suggested as the factors for the increased in xylanase production although, their distribution in the n-domain is of more importance than the number of positively or negatively charged residues [38,39]. The effect of random mutagenesis fostered the increased hydophobic core residue of xylanases in MXB 2, MXB 4 and MXB 5. Mutations (M6I) in xyn of MXB 2

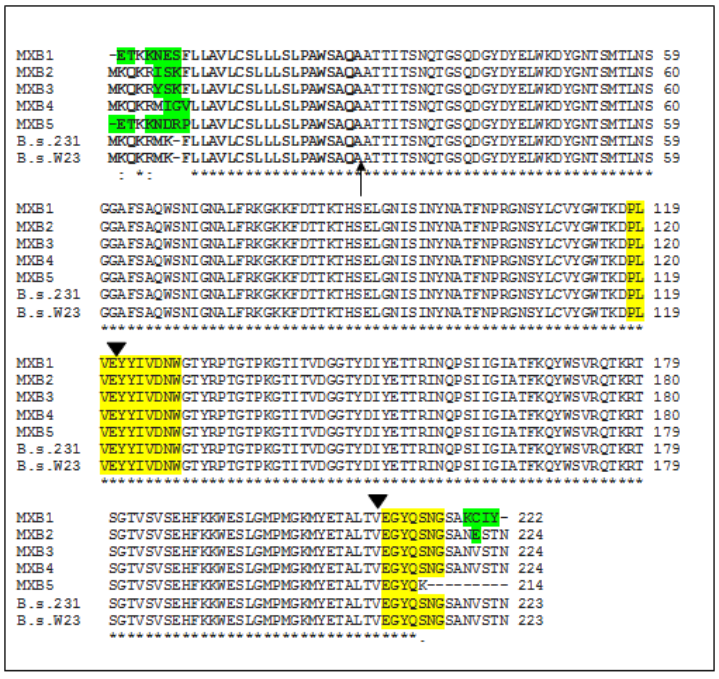

Fig. 7. Xylanase amino acid sequences of mutant strains of *B. subtilis* ATCC 6633

The amino acids conserved in all the sequences are shown by asterisks (*). The signal peptide of 28 amino acids is shown in bold with the mutation sites highlighted in green. Numbering of the protein starts with Ala 1 of the mature protein. The vertical arrow indicates the cleavage site. The sequence equivalent to the GH Family 11 'EGYQSNG' and 'PLVEYYIVDNW' are highlighted in yellow and the amino acids Glu94 and Glu183 that are essential to the catalytic activity are marked by (▼)

(15 residues), (K7I, F9V and insertion of G) in xyn of MXB 4 (16 residues) and (F8P) in xyn of MXB 5 (14 residues) in comparison with the xylanase of the wild type with hydrophobic core length of 14 residues emanated an invariably increase in xylanase production into the medium over the production level of the wild type. A similar, correlation between the length of the hydrophobic core of the signal peptide and the production level in xyn A conducted via the replacement of signal peptide to cellulase A (27 amino acids) and Lam B (25 amino acids long) demonstrated 75% and 44% increase in the production of xyn A compared to the wild type.

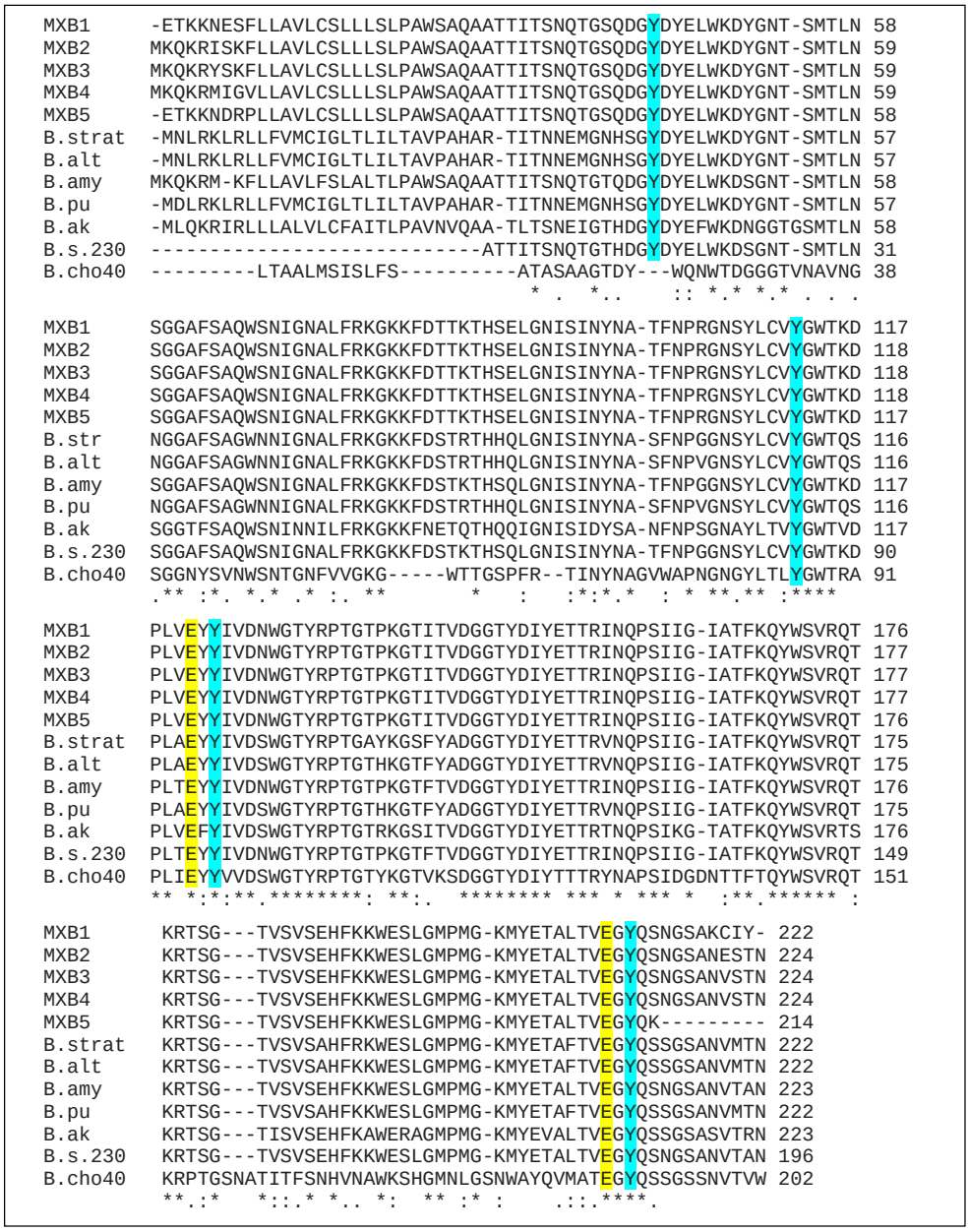

Fig. 8. Amino acid sequences of the mutant strains of *B. subtilis* ATCC 6633 compared with those of GH Family 11 xylanases of *Bacillus* origins

Conserved amino acids among the xylanases are marked by asterisks (*). The corresponding catalytic Glu94 and Glu183 and four Tyr residues of the mutant xylanases of MXB 1 to MXB 5 are highlighted in yellow and blue. Abbreviations: B. strat: B. stratosphericus LAMA 585; B. alt: B. altitudinis HQ51; B. amy: B. amyloliquefaciens DSM 7; B. pu: B. pumilus; B. ak: B. akibai ATCC 43226; B. s.230: B. subtilis B230; B. cho40: B. subtilis cho40

The difference was based on the hydrophobic core length, (19 residues) for the cellulase A signal peptide and (14 residues) for the Lam B signal peptide [40].

3.13 Amino acid Composition and Predicted Thermal Stability Characteristic of Mutant Xylanases

Increased thermal stability of GH Family 11 xylanases was seem to emanate from various minor modifications in the amino acid which include increase of the Thr to Ser ratio and increase number of charged residues particularly arginine (Arg). These modifications prompt enhanced polar interactions and improved secondary structure stabilization via insertions at some regions and thereby resulting the enhanced interactions or increased number of aromatic residues on protein surface [41]. Thus, it could be suggested that the xyn of MXB 5 with increased Arg percentage composition could probably be thermophilic. Reported sequence comparisons have demonstrated that thermophilic proteins contain more Arg on the protein surface in comparison to mesophilic proteins [42]. Furthermore, Noorbatcha et al. [43] demonstrated that the lysine (K) mutation with Arg formed additional hydrogen bond throughout the structure and was thought to give an impact to the thermostability of xylanase. Ser to Thr mutations have also been reported as one of the stabilizing mutations by Turunen et al. [44] and Zhou et al. [45]. Kumar et al. [42] observed the decrease in the frequency of Ser but not the increase of Thr. The present study reports the increase in Thr and decrease in Ser in mutants of *B. subtilis.* MXB 1, MXB 2, MXB 3 and MXB 4 possessed approximately similar Ser to Thr percentage composition to the wild type. Nonetheless, MXB 5 showed a reduced percentage of Ser. The composition of all 20 amino acids for the mutant *B. subtilis* xylanases is compiled in Table 3. The wild type and mutant xylanases possessed relatively high abundance of threonine (11.7% to 12.1%), serine (8.4% to 9.6%) and glycine (9.2% to 9.6%) highlighted in dark red accent whereas methionine (1.8% to 2.6%), cysteine (0.9% to 1.4%) and histidine (0.9%) contents were the lowest highlighted in bright red accent. The amounts of the hydroxyl amino acids, threonine and serine (especially the former) were speculated to impart flexibility in the structure of the xylanases (polysaccharide hydrolases) [46]. The amino acid residues of the wild type and mutant xylanases exhibited the common characteristic of low methionine content for microbial xylanases according to Nakamura et al. [47]. Notably, the very low cysteine content present in the mutant xylanases in this study is almost identical to *B. subtilis* cho40 xylanase with no cysteine detected [33]. Indeed, a similar histidine content of approximately 1.0% in this study with *B. subtilis* cho40 was also observed [33].

Based on the availability of three-dimensional structures for thermophiles and mesophiles of GH Family 11 xylanases, Hakulinen et al. [41] compared the amino acid composition of these xylanases and summarized in Table 3. According to Hakulinen et al. [41], Val is considered to have a good β-forming tendency and its lower frequency in the β-strands of the thermophilic xylanases is not of primary importance in xylanases, in fact, some other characteristics are more vital for thermal stability [41]. Besides the low frequency of Val in the xylanase gene, the significant differences between Val, Ser and Thr frequency ratios in the xylanase gene are also another vital characteristic for the determination of thermal stability of the xylanase protein. The Val, Ser and Thr frequency ratios in the xylanase gene of mesophiles are expressed as 8.1%, 13.0% and 10.4% compared to thermophiles of 5.8%, 9.5% and 12.4% as emphasized by Hakulinen et al. [41], respectively. Notably, a closely related pattern to the thermophiles was observed in xyn of MXB 5 with 4.2%, 8.4% and 12.1% in comparison to xyn of MXB 1 with 4.1%, 9.5% and 11.7%, xyn of MXB 2 with 4.4%, 9.6% and 11.8%, xyn of MXB 3 with 4.8%, 9.6% and 11.8% and xyn of MXB 4 with 5.3%, 9.2% and 11.8%. Thus, by considering the thermal stable characteristics of the wild type and mutant xylanases, all the mutants of *B. subtilis* ATCC 6633 were in fact exhibited the tendency to be thermostable based on the Val, Ser and Thr frequency ratios which were almost identical to those of thermophiles.

In a nutshell, with the increase ratio of Thr to Ser, presence of Arg residue and identical Val, Ser and Thr frequency ratios with thermorphiles found in the mutant of MXB 5, we anticipated MXB 5 to be potentially thermophilic useful at the elevated temperature in xylanase applications.

3.14 Summary of the Characteristics of the Mutants of *B. subtilis* ATCC 6633

A summary of the various characteristics in the mutants of *B. subtilis* ATCC 6633 is detailed in Table 4.

Table 1. BLAST result for the mutants of *B. subtilis* ATCC 6633

Hit organism	Feature (gene)	Identity (%)	Accession number	E-value
Bacillus subtilis subsp. *spizizenii* strain NRS 231 (ATCC 6633)	1,4-beta-xylanase	99	CP010434.1	0.0
Bacillus subtilis subsp. *spizizenii* str. W23	endo-1,4-beta-xylanase	99	CP002183.1	0.0
Bacillus subtilis subsp. *spizizenii* TU-B-10	endo-1,4-beta-xylanase A	95	CP002905.1	0.0
Bacillus sp. JS	xyn A	92	CP003492.1	0.0
Bacillus amyloliquefaciens TA208	endo-1,4-beta-xylanase	92	CP002627.1	0.0
Bacillus amyloliquefaciens XH7	glycoside hydrolase	92	CP002927.1	0.0
Bacillus pumilus	beta-xylanase precursor	81.3	HM536195	2.1E-21

Table 2. Amino acids characteristics of *B. subtilis* ATCC 6633 mutant strains after random mutagenesis

Wild type	Types of mutation			Mutants				
	Substitution	Insertion	Deletion	MXB 1 (position number)	MXB 2 (position number)	MXB 3 (position number)	MXB 4 (position number)	MXB 5 (position number)
M- Met	→			N-Asn (5)	I-Ile (6)	Y-Tyr (6)		N-Asn (5)
			→	-				-
K- Lys	→			E-Glu (1)	S-Ser (7)	S-Ser (7)	I-Ile (7)	E-Glu (1)
	→			E-Glu (6)				D-Asp (6)
Q- Gln	→			T-Thr (2)				T-Thr (2)
R- Arg	→			K-Lys (4)				K-Lys (4)
F- Phe	→						V-Val (9)	P-Pro (8)
S- Ser	→			I-Ile (221)				K-Lys (214)
N-Asn	→			K-Lys (219)				
V-Val	→			C-Cys (220)	E-Glu (221)			
T-Thr	→			Y-Tyr (222)				
---		→		S-Ser (7)	K-Lys (8)	K-Lys (8)	G-Gly (8)	R-Arg (7)

Table 3. Percentages composition of xylanase amino acid sequences of mutants, wild type, *B. subtilis* cho40, mesophiles and thermophiles

Amino acid	MXB 1 (%)	MXB 2 (%)	MXB 3 (%)	MXB 4 (%)	MXB 5 (%)	Wild type (%)	*B. subtilis* cho40 (%)	Mesophiles (%)	Thermophiles (%)
Ala (A)	5.40	5.30	5.30	5.30	5.10	5.40	7.40	5.70	5.10
Arg (R)	2.70	3.10	3.10	3.10	3.30	3.10	3.00	2.50	4.50
Asn (N)	6.30	6.60	6.60	6.60	6.10	6.70	9.40	8.90	7.20
Asp (D)	3.60	3.50	3.50	3.50	4.20	3.60	3.50	3.40	4.30
Cys (C)	1.40	0.90	0.90	0.90	0.90	0.90	0.00	0.40	0.70
Gln (Q)	3.60	3.90	3.90	3.90	3.70	4.0	2.50	3.90	4.30
Glu (E)	4.50	3.90	3.50	3.50	4.20	3.60	1.00	2.80	2.90
Gly (G)	9.50	9.20	9.20	9.60	9.30	9.40	12.90	12.50	13.60
His (H)	0.90	0.90	0.90	0.90	0.90	0.90	1.00	1.50	1.60
Ile (I)	5.40	5.70	5.30	5.70	5.10	4.90	3.00	3.70	3.90
Leu (L)	6.80	7.00	7.00	7.00	7.00	6.70	3.50	2.50	3.70
Lys (K)	6.80	6.60	6.60	6.10	7.00	6.70	2.50	1.20	1.50
Met (M)	1.80	2.20	2.20	2.60	1.90	2.70	1.50	0.80	0.60
Phe (F)	3.20	3.10	3.10	2.60	2.80	3.10	2.50	3.90	3.00
Pro (P)	3.20	3.10	3.10	3.10	3.70	3.10	3.00	2.80	2.80
Ser (S)	9.50	9.60	9.60	9.20	8.40	9.40	9.90	13.00	9.50
Thr (T)	11.70	11.80	11.80	11.80	12.10	11.70	13.90	10.4	12.40
Trp (W)	3.20	3.10	3.10	3.10	3.30	3.10	5.40	3.50	4.00
Tyr (Y)	6.80	6.10	6.60	6.10	6.50	6.30	7.40	8.40	8.50
Val (V)	4.10	4.40	4.80	5.30	4.20	4.50	6.90	8.10	5.80

Table 4. Summary of the various characteristics in the mutants of *B. subtilis* ATCC 6633

Mutants of *B. subtilis*	DNA mutagenetic site(s) (insertion) [deletion]	Predicted theoretical molecular mass and pI	Amino acid mutagenetic site(s) (insertion) [deletion]	Intrinsic relationship of GH Family 11	Amino acid composition *M.C.H/ *G.S.T	Predicted thermo-stability
MXB 1	T/G and T/C, (T,C,A), [C,A]	24.73 and 8.63	M/N, K/E, Q/T, R/K. S/I, N/K, V/C, T/Y, (S) [M]	Glu94, Glu183 and 4 Tyr residues	1.80, 1.40, 0.90/ 9.50, 9.50, 11.70	+
MXB 2	C/G, T/C and A/T, (T,T,T), [A,C]	25.38 and 9.05	M/1, K/S, V/E, (K)	Glu94, Glu183 and 4 Tyr residues	2.20, 0.9, 0.9/ 9.20, 9.60, 11.80	+
MXB 3	C/G, T/C and A/T, (T,A,T), [A,C]	25.40 and 9.16	M/Y, K/S, (K)	Glu94, Glu183 and 4 Tyr residues	2.20, 0.9, 0.9/ 9.20, 9.60, 11.80	+
MXB 4	T/G and G/C, (T.C,G), [A,C]	25.28 and 9.07	K/I, F/V, (G)	Glu94, Glu183 and 4 Tyr residues	2.60, 0.9, 0.9/ 9.60, 9.20, 11.80	+
MXB 5	T/G and C/T, (C,A), [C]	23.94 and 8.91	M/N, K/E, K/D, Q/T, R/K, F/P, S/K, (R) [M]	Glu94, Glu183 and 4 Tyr residues	1.90, 0.9, 0.9/ 9.30, 8.40, 12.10	++

**M: Methionine, C: Cysteine, H: Histidine; *G: Glycine, S: Serine, T: Threonine*

4. CONCLUSION

This work presents an attempt to verify, determine and analyse the improved xylanase secretion capability of *B. subtilis* subsp. *spizizenii* ATCC 6633 mutant strains after random chemicals of AO and EMS mutagenesis. Identification of the observed effects on the genes coding for xylanase production in the respective mutants of *B. subtilis* subsp. *spizizenii* ATCC 6633 prompted the DNA sequence and analysis of the mutant xylanase genes. Comparison of xylanase genes obtained from the different mutants of *B. subtilis* with the wild type revealed the site(s) of mutagenesis. Interestingly, the mutations are significantly reflected at the 5' end of the mutant xylanase genes. Corresponding amino acids displayed high levels of amino acid similarity on BLAST analysis showing extensive homology with GH Family 11 xylanases and a reduced similarity with xylanase A enzyme. Furthermore, the amino acids of the mutants had predicted molecular mass ranging from 23.94 kDa to 25.40 kDa at the predicted pI ranged between 8.63 and 9.61. Amino acid composition of the mutants showed high abundance of threonine (11.7% to 12.1%), serine (8.4% to 9.6%) and glycine (9.2% to 9.6%). In fact, the mutant xylanase proteins in this study possess low methionine (1.8% to 2.6%), cysteine (0.9% to 1.4%) and histidine (0.9%) which are the characteristics for microbial xylanases. Based on the mutations in the mutant xylanases genes, the corresponding amino acids also showed substitution and insertion mutations. The comparison of the xylanase proteins with known xylanase protein sequences of *B. subtilis* subsp. *spizizenii* strains featured two catalytic residues in the intergenic region, four Tyr residues and signal peptides at the n-terminal linked by a cleavage site to the mature proteins. Xyn of MXB 1 displayed substitution mutations (K1E) whereas the xyn of MXB 5 exhibited (K1E, K6D and insertion of R) amino acids mutations. Furthermore, mutations were observed in xyn of MXB 1(M5N), xyn of MXB 2 (M6I), xyn of MXB 4 (K7I) and xyn of MXB 5 (M5N) which potentially resulted in increase of xylanase yield. The mutants xylanase of MXB 1 to MXB 5 showed approximate Arg compositions to the wild type. On the other hand, MXB 1, MXB 2, MXB 3 and MXB 4 possessed approximately similar Ser to Thr percentage composition to the wild type. Indeed, MXB 5 showed reduced percentage of Ser. Overall, mutants of MXB 1, MXB 2, MXB 3, MXB 4 and MXB 5 were seemed to possess close related pattern of Val, Ser and Thr which are identical to the thermophilic xylanases. Consequently, we suggested that these mutants would find biotechnological importance in textile and bio-bleaching industries that require high temperature usage in xylanase applications particularly mutant strain of MXB 5. Indeed, mutant strain of MXB 5 would probably be anticipated as being thermophilic based on the related amino acids pattern to the thermophiles.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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