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# Nucleotide Sequence and Analysis of a Xylanase gene (*xynS*) from Alkali-tolerant *Bacillus* sp. YA-14 and Comparison with Other Xylanases

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**The nucleotide sequence of the xylanase gene (*xynS*) from alkali-tolerant *Bacillus* sp. YA-14 was determined and analyzed. A 639 base pairs open reading frame for *xynS* gene was observed and encoded for a protein of 213 amino acids with a molecular weight of 23,339. S1 nuclease mapping showed that the transcription initiation site of the *xynS* gene did not exist in the cloned DNA. Ribosome binding site sequence with the free energy of −18.8 Kcal/mol was observed 8 base pairs upstream from the initiation codon, ATG. The proposed signal sequence consisted of 28 amino acids, of which 3 were basic amino acid residues and 21 were hydrophobic amino acid residues. When the amino acid sequences of xylanases were compared, *Bacillus* sp. YA-14 xylanase showed 48% homology with *Bacillus* sp. YC-335 xylanase and 96% homology with xylanases from *B. subtilis* and *B. circulans*.**

Xylan is an important enzyme for the utilization of hemicellulose which has a large xylan content. Several xylanase genes have been cloned and sequenced (1, 7, 9, 10, 15, 20, 26, 39), and the relationship between their protein structures and functions have been investigated.

In our laboratory, several alkalophilic *Bacillus* strains were isolated from soil (13, 32, 33). These strains produced various useful enzymes (3, 4, 30, 31) and the properties and the expression of genes coding for these enzymes were investigated (14, 19, 21, 28, 29, 34, 35, 37). Recently, xylanase gene from *Bacillus* sp. YC-335 (3) was cloned (38) and sequenced (36). In order to compare this xylanase with others, we had cloned another xylanase gene from alkalophilic *Bacillus* sp. YA-14 (28).

In this study, we subcloned the xylanase gene from *Bacillus* sp. YA-14 into M13 vector, and determined the nucleotide sequence of the functional domain in the cloned DNA fragment, and analyzed it.

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Key words: Xylanase gene, amino acid hmology, alkali-tolerant *Bacillus* sp.

## MATERIALS AND METHODS

### Bacterial Strains and Plasmids

Alkali-tolerant *Bacillus* sp. YA-14 (33) was the donor strain of the xylanase gene and *Escherichia coli* HB101 [*supE44 hsdS20 recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1*] (2) was used as the host for the expression of the cloned gene. *E. coli* JM109 ($r_k^-$, $m_k^-$, *rec*A1, *end*A1, *gyr*A96, *thi*, *hsd*R17, *sup*E44, *rel*A1, $\lambda^-$, Δ (*lac-pro*AB)/F', *tra*D36, *pro*AB, *lacI*$^q$ ZΔM15) (27) was used as a recipient for bacteriophage M13mp vectors. Plasmid pYDC21 (28) was the recombinant plasmid harboring xylanase gene.

### Media

The media compositions for the cultivation of *E. coli* cells were described earlier (36).

### Enzymes and Reagents

All restriction endonucleases and T4 DNA ligase were purchased from Promega and New England Biolabs and were used as recommended by the suppliers. Cyclone I Biosystem was obtained from International Biotechnologies, Inc. Isopropyl-β-D-thiogalactopyranoside (IPTG), 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-

Gal) and ribonucleoside-vanadyl complex were from Sigma Chemical Co., St. Louis, Mo.

**DNA Isolation**

Rapid isolation of plasmid DNA from *E. coli* was done by the miniscreen method (22). Bacteriophage RF DNA was isolated from *E. coli* by the method of Messing *et al.* (18). A large-scale plasmid purification was obtained by banding in CsCl-ethidium bromide density gradients (12). DNAs were resolved by doing horizontal gel eletrophoresis in acetate or borate buffer (16).

**Construction of Overlapping Deletion Subclones**

Overlapping deletions were generated with Cyclone I Biosystem as described by Dale *et al.* (5). In order to subclone into M13mp19 DNA, plasmid pYDC21 (28) was digested with *Hin*dIII, and the 3.0 kb DNA fragment was ligated with *Hin*dIII-digested M13mp19 to give mp 19-DCA0 and mp19-DCB0. The 3.0 kb insert DNA fragments were deleted with the exonuclease activity of T4 DNA polymerase from the 3'-end of the inserted DNA, tailed with poly A, ligated , and transformed into *E. coli* JM109. Single strand DNAs were isolated from the transformed white plague, electrophoresed and fractionated.

**Xylanase Assay of M13 Subclones**

Pellets of *E. coli* JM109 subclones produced above were suspended in 1 ml of 10 mM Tris-HCl buffer at pH 8.0 and sonicated at 20 MHz for 1 min. The supernatant obtained by centrifugation at 10,000×g for 10 min was assayed for xylanase activity as described earlier (36).

**Nucleotide Sequence Analysis**

Fragments of appropriate size in the M13 deletion subclones were sequenced by the dideoxy chain termination method of Sanger *et al.* (23). DNA chain termination sequencing with sequenase enzyme (United States Biochemicals Corp.) and [α-$^{35}$S]dATP (Amersham Corp.) were performed according to the protocols given by U.S. Biochemicals. Electrophoresis was carried out on a 6% polyacrylamide/8 M urea gel. The sequence were read from both strands, and each sequence was read an average of 4 times in each directions. Sequence data were analyzed by the Pustell DNA Analysis Program (International Biotechnologies, Inc., New Haven, CT).

**Isolation of RNA**

Total cellular RNA was extracted from *E. coli* HB101 harboring plasmid pYDC21 cells as described earlier (36).

**S1 Nuclease Mapping**

To prepare a probe, plasmid pYDC21 was digested with *Hin*dIII-*Pst*I and dephosphorylated with calf intestinal alkaline phosphatase. The fragment (162 bp) was electroeluted from a 5% polyacrylamide gel and the 5'-ends were $^{32}$P labeled by using [γ-$^{32}$P]dATP (Amersham Corp.) and T4 polynucleotide kinase (Boeringer Mannheim Biochemicals) as described by Maniatis *et al.* (16). S1 nuclease mapping was performed by the method of Gilman and Chamberlin (8). The single strand probe was annealed to total RNA (50 μg) isolated from *E. coli* HB101 harboring plasmid pYDC21 during late logarithmic phase and digested with S1 nuclease. The S1 nuclease resistant DNA fragments were resolved by 6% polyacrylamide gel electrophoresis.

**Nucleotide Sequence Acession Number**

The nucleotide sequence of the xylanase gene has been submitted to the EMBL Data Library under Accession Number X59058.

## RESULTS

**Construction of Deletion Subclones**

The size of the DNA fragment cloned into pBR322 was 3.0 kb. This size was larger than those of other most characterized xylanases (6, 7, 11). In order to determine which part of the DNA is essential for xylanase activity, inserted DNA fragment was sequentially deleted from the 3' end by using exonuclease activity of T4

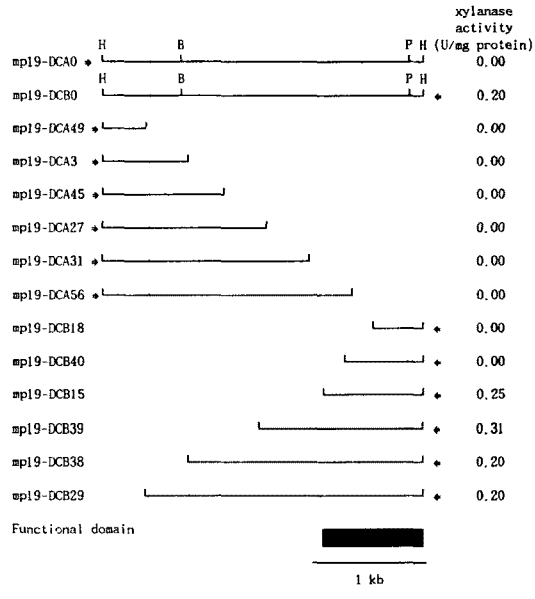


**Fig. 1. Map of the functional domain of *Bacillus* sp. YA-14 xylanase gene cloned in mp19-DCA0 and mp 19-DCB0.**

The 3.0 kb *Bacillus* sp. YA-14 DNA insert was subcloned by Cyclone I Biosystem. Xylanase activities of various subclones are given on the right. The approximate location and extent of the xylanase gene functional domain are indicated by the filled box. The arrow indicates the direction and location of the *lacZ'* gene promoter exited in the M13mp19 vector. H, *Hin*dIII; P, *Pst*I; B, *Bam*HI.

DNA polymerase. The remaining part of the DNA was religated, yielding deletion subclones mp19-DC3 through mp19-DC56 (Fig. 1). When the xylanase activities of these subclones were assayed and compared with their inserted size, mp19-DCB15 had the smallest inserted size and showed xylanase activity. So we proceeded to further experiment to determine its nucleotide sequence.

**Sequencing Strategy**

The structure of plasmid pYDC21 is shown in Fig. 2 and the nucleotide sequence of deleted insert DNA was determined by the strategy outlined in Fig. 2.

All of the sequenced region was read from at least two subclones as shown by the arrows in the lower part of the Fig. 2, and the results were analyzed by computer matching.

**Nucleotide Sequence of the Xylanase Gene**

The nucleotide sequence of 975 bp covering the entire xylanase gene and its flanking regions is shown in Fig. 3. The nucleotide sequence of xylanase gene has an open reading frame of 639 bp which translates to the amino acids shown below the nucleotide sequence. Since the other two reading frames contained numerous stop codons, we concluded that the open reading frame translated in Fig. 3 encodes pre-xylanase, and named it *xynS* (EMBL accession No: X59058).

The encoded 213-amino-acid polypeptide had a calculated molecular weight of 23,339, and its pI was 10.7.

**S1 Nuclease Mapping**

In order to identify the promoter region of the xylanase gene, transcription initiation site was first determined by S1 nuclease mapping. Total RNA was isolated from *E. coli* HB101 harboring plasmid pYDC21 and hybridized to the 162 bp *Hin*dIII-*Pst*I fragment labeled on the 5'-terminus. As shown in Fig. 4, The 162 nucleotides were protected, which corresponded to the size

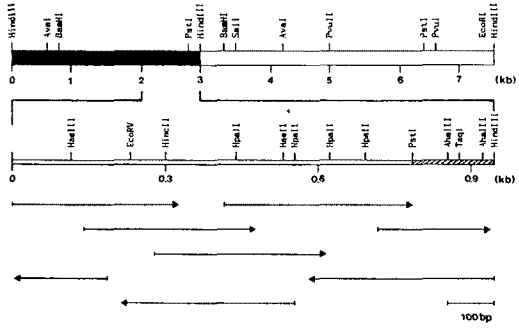


**Fig. 2. Restriction map of plasmid pYDC21 and strategy of nucleotide sequencing. pYDC21 is a hybrid plasmid consisting of the 3.0 kb *Hin*dIII fragment of *Bacillus* sp. YA-14 DNA (filled bar) and pBR322 (open bar).** Fine mapping of the region containing the xylanase gene and the sequencing strategy are enlarged. The arrows show the direction of reading from inserts in M13mp19. The hatched region is the 162-nucleotede *Hin*dIII-*Pst*I probe used for S1 nuclease mapping.

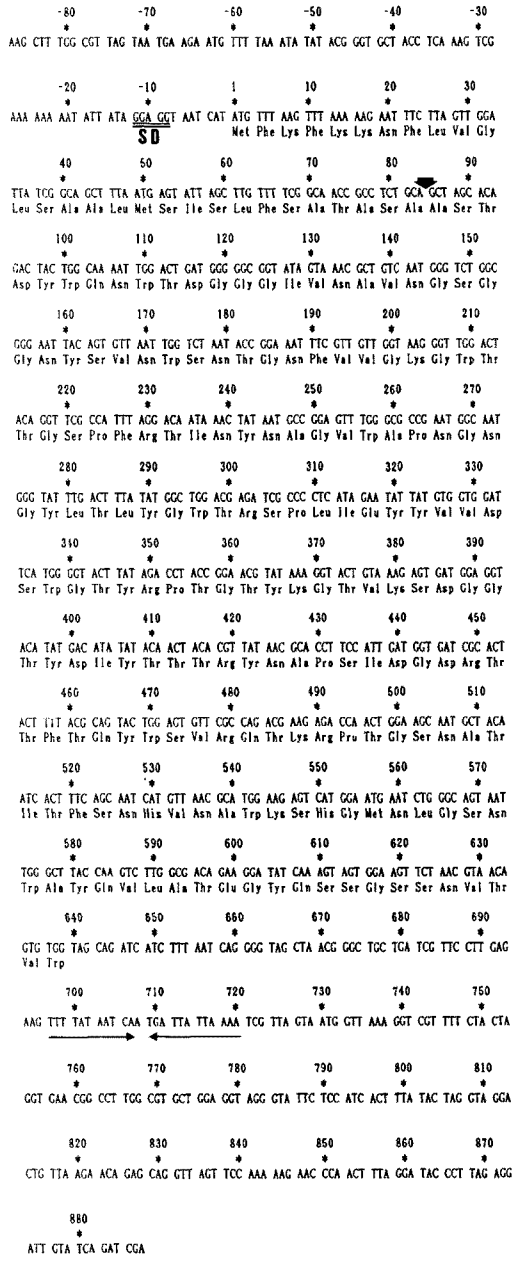


**Fig. 3. Nucleotide sequence and deduced amino acid sequence of the xylanase gene of *Bacillus* sp. YA-14.** Numbering of both nucleotides and amino acids starts with the begining of the coding sequence. The putative Shine-Dalgarno sequence (SD) are double-underlined. The inverted repeat structure for the transcription terminator is indicated by inverted arrows. The putative position of processing of the signal sequence is shown by an arrowhead.

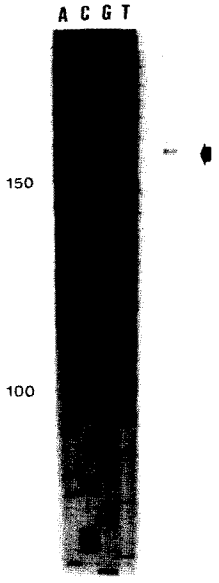


**Fig. 4. Transcriptional start site mapping of the *xynS* gene by S1 nuclease analysis. RNA was isolated from stationary phase cultures of *E. coli* HB101 containing plasmid pYDC21. The protected band is shown with the arrow.**
The sequence ladder of M13mp18 DNA serves as the size marker for the protected band.

of probe DNA used in experiment. This indicates that transcrption initiation site was not at the cloned DNA.

**Ribosome Binding Site**

The ribosome binding sites of Gram-positive mRNAs exhibit extensitive complementarity to the 3′ region of *B. subtilis* 16S rRNA (17). The putative ribosome binding site of the xylanase gene complementary to the 3′ region of *B. subtilis* 16S rRNA was located 8 base upstream from the translation start codon, ATG (Fig. 3).

**Signal Sequence**

Xylanase is an enzyme secreted in *Bacillus* sp. YA-14 and more than 50% of enzyme synthesized in *E. coli* HB101 harboring plasmid pYDC21 is secreted into culture broth and periplasmic space (28). A hydropathy profile of xylanase shows a hydrophobic region near the $NH_2$-terminus and this region represents a signal peptide (Fig. 5) which is good conformity with the typical signal peptide structure (25).

The signal peptide of pre-xylanase consists of 28 amino acids, of which 3 are basic amino acid residues, Lys, in the region near the N-terminus, and 21 are hydrophobic amino acid residues. The processing site recognized by signal peptidase could be after Ala-28. The signal peptides of various xylanases are compared in Table 1.

**Homology Comparison of Xylanase Primary Structure**

**Table 1. Comparison of signal peptides of xylanases**

| Organism | Amino acid sequence* |
|---|---|
| *Bacillus* sp. YA-14 | Met Phe Lys Phe Lys Lys Asn Phe Leu Val Gly Leu Ser Ala Ala Leu Met Ser Ile Ser Leu Phe Ser Ala Thr Ala Ser Ala ↓ Ala |
| *Bacillus* sp. YC-335 | Leu Lys Gln Val Lys Ile Met Phe Leu Met Thr Met Val Leu Gly Ile Gly Leu Phe Phe Ser Glu Asn Ala Glu Ala ↓ Ala |
| *B. pumilus* | Met Asn Leu Arg Lys Leu Arg Leu Leu Phe Val Met Cys Ile Gly Leu Thr Leu Ile Leu Thr Ala Val Pro Ala His Ala ↓ Arg |
| *B. subtilis* | Met Phe Lys Phe Lys Lys Asn Phe Leu Val Gly Leu Ser Ala Ala Leu Met Ser Ile Ser Leu Phe Ser Ala Thr Ala Ser Ala ↓ Ala |
| *B. circulans* | Met Phe Lys Phe Lys Lys Asn Phe Leu Val Gly Leu Ser Ala Ala Leu Met Ser Ile Ser Leu Phe Ser Ala Thr Ala Ser Ala ↓ Ala |
| *Bacillus* sp. C-125 | Met Ile Thr Leu Phe Arg Lys Pro Phe Val Ala Gly Leu Ala Ile Ser Leu Leu Val Gly Gly Gly Ile Gly Asn Val Ala ↓ Ala Ala |
| *C. thermocellum* | Met Ser Arg Lys Leu Phe Ser Val Leu Leu Val Gly Leu Met Leu Met Thr Ser Leu Leu Val Thr Ile Ser Ser Thr Ser Ala ↓ Ala |
| *C. saccharolyticum* | Met Arg Cys Leu Ile Val Cys Glu Asn Leu Glu Met Leu Asn Leu Ser Leu Ala Lys Thr Tyr Lys Asp Tyr Phe Lys Ile Gly Ala Ala ↓ Val Thr ↓ Ala ↓ Lys |

* Positively charged amino acids are underlined and the possible processing sites are shown by the ↓

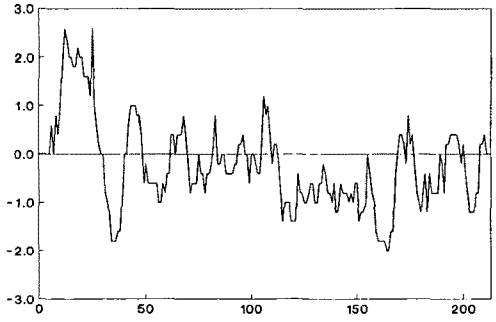


**Fig. 5. Hydropathy profile of the xylanase from *Bacillus* sp. YA-14.**
The abscissa of the panel shows amino acid number. The ordinate shows the average hydrophobicity (positive ordinate) or hydrophilicity (negative ordinate) of 1 amino acid residue.

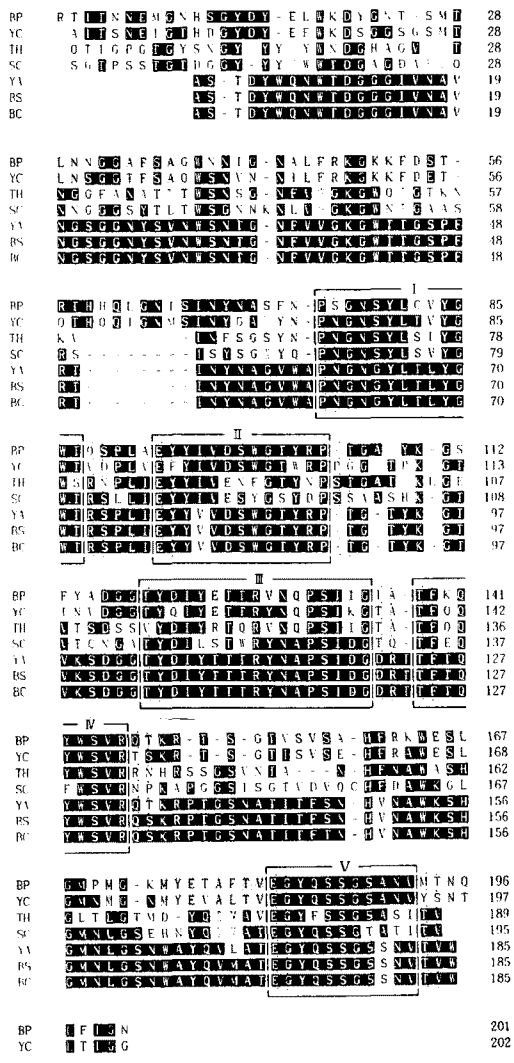

**Fig. 6. Alignment of xylanase amino acid sequences from *B. pumilus* (BP), *Bacillus* sp. YC-335 (YC), *Trichoderma harzianum* (TH), *Schizophyllum commune* (SC), *Bacillus* sp. YA-14 (YA), *B. subtilis* (BS) and *B. circulans* (BC).**

Only the sequences of the processed xylanases are shown and the numbers denote amino acid positions of the proteins. Identical amino acids are in reversed font. If the amino acids are not present in all of the protein sequences compared, they are shown either in stippled boxes or in outlined letters. Dashed lines indicate where it is necessary to introduce gaps for better alignment.

Comparison of the predicted amino acid sequence of the xylanase from *Bacillus* sp. YA-14 with those of other xylanases revealed significant similarities between the xylanase from *Bacillus* sp. YA-14 and the xylanases from the following strains (Fig. 6): *B. pumilus* (BP), *Trichoderma hazianum* (TH), *Schizophyllum commune* (SC), *B. subtilis* (BS), *B. circulans* (BC). In the processed enzymes, *Bacillus* sp. YA-14 xylanase showed 48% homology with *Bacillus* sp. YC-335 xylanase and 96% homology with *B. subtilis* and *B. circulans* xylanases.

### Codon Usage

The usage of codon usage for pre-xylanase is shown in Table 2. There appears to be a bias for codon selection, for example, TAT for Tyr, AAT for Asn, GAA, for Glu. There are no codons for Cys.

## DISCUSSION

The complete nucleotide sequence of the xylanase gene (*xynS*) and its flanking regions was determined and analyzed. Possible sequences of the *Bacillus* promo-

**Table 2. Codon usage of pro-xylanase from *Bacillus* sp. YA-14**

| | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| TTT | Phe | 5 | 2.3% | TCT | Ser | 4 | 1.9% | TAT | Tyr | 11 | 5.2% | TGT | Cys | 0 | .0% |
| TTC | Phe | 3 | 1.4% | TCC | Ser | 1 | .5% | TAC | Tyr | 4 | 1.9% | TGC | Cys | 0 | .0% |
| TTA | Leu | 4 | 1.9% | TCA | Ser | 1 | .5% | TAA | – | 0 | – | TGA | – | 0 | – |
| TTG | Leu | 3 | 1.4% | TCG | Ser | 4 | 1.9% | TAG | – | 1 | – | TGG | Trp | 11 | 5.2% |
| CTT | Leu | 0 | .0% | CCT | Pro | 2 | .9% | CAT | His | 2 | .9% | CGT | Arg | 1 | .5% |
| CTC | Leu | 1 | .5% | CCC | Pro | 1 | .5% | CAC | His | 0 | .0% | CGC | Arg | 2 | .9% |
| CTA | Leu | 0 | .0% | CCA | Pro | 2 | .9% | CAA | Gln | 3 | 1.4% | CGA | Arg | 0 | .0% |
| CTG | Leu | 1 | .5% | CCG | Pro | 1 | .5% | CAG | Gln | 2 | .9% | CGG | Arg | 0 | .0% |
| ATT | Ile | 2 | .9% | ACT | Thr | 10 | 4.7% | ATT | Asn | 14 | 6.6% | AGT | Ser | 9 | 4.2% |
| ATC | Ile | 1 | .5% | ACC | Thr | 3 | 1.4% | AAC | Asn | 5 | 2.3% | AGC | Ser | 4 | 1.9% |
| ATA | Ile | 4 | 1.9% | ACA | Thr | 9 | 4.2% | AAA | Lys | 2 | .9% | AGA | Arg | 3 | 1.4% |
| ATG | Met | 3 | 1.4% | ACG | Thr | 4 | 1.9% | AAG | Lys | 6 | 2.8% | AGG | Arg | 1 | .5% |
| GTT | Val | 7 | 3.3% | GCT | Ala | 5 | 2.3% | GAT | Asp | 5 | 2.3% | GGT | Gly | 8 | 3.8% |
| GTC | Val | 2 | .9% | GCC | Ala | 2 | .9% | GAC | Asp | 2 | .9% | GGC | Gly | 5 | 2.3% |
| GTA | Val | 3 | 1.4% | GCA | Ala | 5 | 2.3% | GAA | Glu | 2 | .9% | GGA | Gly | 9 | 4.2% |
| GTG | Val | 3 | 1.4% | GCG | Ala | 2 | .9% | GAG | Glu | 0 | .0% | GGG | Gly | 4 | 1.9% |

ter for the −10 and −35 regions are not found in the upstream of the translation start codon. By S1 nuclease mapping, the promoter and transcription initiation site were found to be deleted during the cloning step. The ribosome binding site is complement to the 3′ end of *B. subtilis* 16 S rRNA at 10 out of 15 bases. The free energy between the 3′ end of *B. subtilis* 16S rRNA and the ribosome binding site is calculated to be −18.8 Kcal/mol by the method of Tinoco (24). This is higher than that of the avarage free energy in Gram positive bacteria, −17.6 Kcal/mol (17). This homology, which includes the proposed ribosome binding sites, is also observed in the 5′ region of *B. pumilus* xylanase (7). The free energy of xylanase gene from *Bacillus* sp. YC-335 was reported to be −17.0 Kcal/mol (36). The distance of the ribosome binding site from the translation start codon is within the range determined for a number of *Bacillus* sequence (17). The inverted repeat sequences are found in the 3′ downstream which may act as an transcription terminator. The free energy of the secondary structure formed by the inverted repeat sequences is calculated as −5.4 Kcal/mol. The secondary structure with this low free energy has difficulty in acting as a transcription terminator, and 3′ end of this structure did not contain poly Us. Therefore, the transcription of the xynS gene could have been be terminated by the rho factor dependent type.

Xylanases can be classified in two groups, Type A and B, by comparing their deduced polypeptides sequences. Type A xylanases include xylanases from *Streptomyces lividans, Clostridium thermocellum, Bacillus* strain C-125, *Cryptococcus albidus, Cellulomonas fimi* and *Caldocellum saccharolyticum.* Type B xylanases include xylanases from *S. lividans, Bacillus pumilus, B. subtilis, Schyzophillum commune, Aureobasidium, Clostridium acetobutylicum,* and *Trichoderma hazianum.*

Our xylanase belongs to type B, and the homologies among *xynS* and type B xylanases were more than 50%. Type B xylanases share five large regions of high homology in their sequences (Fig. 6). Especially, more than 96% homology is found among xylanases from *Bacillus* sp. YA-14, *B. subtilis,* and *B. circulans.* There is no homology between type A and B xylanases.

## Acknowledgement

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