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Cloning and Characterization of Xylanase Gene from *Bacillus licheniformis* NBL420

Bacillus licheniformis NBL420 유래의 Xylanase 유전자의 클로닝과 특성 검토

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Abstract

The gene encoding endoxylanase (xylS) was isolated from a genomic library of *Bacillus licheniformis* NBL420. Two positive clones, which harbor 1.5 kb and 0.8 kb inserts respectively, were screened on RBB dyed-xylan plates and the recombinant plasmids were named as pBX3 and pBX5. The nucleotide sequencings of two inserts revealed the existence of common 639 bp of open reading frame which encode 232 amino acids. The xylS gene was successfully subcloned into pET22b(+) vector and overexpressed. Enzymatic properties including optimum pH, optimum temp, thermostability and pH stability were investigated. Activity staining of XylS was identical with that of original *Bacillus licheniformis* NBL420.

키워드 : *Bacillus licheniformis* NBL420, *xylanase-cellulase 융합단백질*
Keywords : *Bacillus licheniformis* NBL420, *xylanase-cellulase fusion protein*

1. INTRODUCTION

Xylan, a major component of plant hemicellulose, is second only to cellulose in nature abundance and is now regarded as a usable biomass that is convertible to biofuels, chemicals and many other valuable compounds [3].

Xylanase catalyze the hydrolysis of xylan. Xylan can be degraded to xylose by the sequential reaction of xylanase (1,4-β-D-xylan xylanohydrolase; EC 3.2.1.8) and β-xylosidase (1,4-β-D-xylohydrolase; EC 3.2.1.37) [7, 17]. β-Xylosidase degrades not only xylobiose but also xyloligosaccharides. The various applications of xylanase have stimulated research on the biochemical and molecular aspects of this important enzyme of the family of glycosyl hydrolases. Microbial xylanase are the preferred catalysts for xylan hydrolysis due to their high specificity, mild reaction conditions, negligible substrate loss, and side product generation [3, 11 and 18]. However, the cost of enzymatic hydrolysis of biomass is one of the factors limiting the economic feasibility of the process. Xylanase are produced by a plethora of organisms like bacteria, algae, fungi, protozoa, gastropods, and arthropods [5, 10]. Most of the bacteria and fungi secrete extracellular xylanases which act on the hemicellulosic material to liberate xylose as a directly assimilable end product allowing the organisms to grow heterotrophically on xylan. Unlike cellulose,

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xylan is a complex polymer consisting of a β-D-1,4-linked xylopyranoside backbone substituted with acetyl, arabinosyl and glucuronosyl side chains. Therefore, the xylotic enzyme system carring out the xylan hydrolysis is usually composed of a repertoire of hydrolytic enzymes: β-1,4-endoxylanase, β-xylosidase, α-L-arabinofuranosidase, α-glucuronidase, acetyl xylan esterase, and phenolic acid esterase (Fig. 1.). [10]

It was reported that a polymer-producing *Bacillus licheniformis* NBL420 secrete various hydrolases containing xylanase, proteases as well as cellulase [14]. The genetic system of xylanse in this new *Bacillus* was unknown so far. For this reason, we cloned a gene (xylS) encoding a new endoxylanase of *Bacillus licheniformis* NBL420 and overexpressed in *E. coli*. The recombinant XylS protein was partially purified by Ni-NTA (Ni^{2+}-nitrilotriacetic acid) column chromatography. The enzymatic characteristics of recombinant xylanases were further characterized.

2. MATERIALS AND METHODS

2.1 Bacterial strains, plasmids, and growth conditions

E. coli DH5α and DH10B were used as the host strain for the plasmid maintenance and gene cloning. Ampicillin (Sigma) was added to the media at a final concentration of 50 ㎍/㎖. Plasmid pUC18 (GibcoBRL) and pET15b(+) (Novagen) were used for the cloning, subcloning, and expression.

2.2 Preparation of soluble xylan

The soluble fraction of commercial xylan (Oat spelt xylan, Sigma) was used as the substrate for all assays. The commercial xylan (2g) was shaken in 100 ㎖ of double distilled water for 20 hr at room temperature. Insoluble and soluble fractions were separated by centrifugation. They were then lyophilized to complete dryness [6, 11].

2.3 Preparation of dyed xylan

Xylan was dyed with Remazol brilliant blue R (RBB), following the procedure of Biely, Mislovicova and Toman [4] with simple modifications. The RBB dye (0.5 g) was dissolved in a solution of xylan.(1 g in 30 ㎖ of DDW). A solution of Na2SO4 (10 ㎎ in 10 ㎖ of DDW) was added dropwise with stirring for 5min. The mixture was then alkalized with NaOH (0.5~1 g in 10 ㎖ of DDW) and stirred at room temperature for 90min. The dye product was precipitated with 2 vol of ethanol (96%), collected by filtration, and washed with a mixture ethanol-0.05 M sodium acetate in DDW 2:1(v/v) until the filtrate was colorless. The product was successively washed with ethanol-DDW 4:1(v/v), ethanol, and acetone, and dried at room temperature.

2.4 Genomic library construction and screening

Electroporation was performed by the procedure of Dower et al., (1988). Electroporation of bacteria was performed in a MicroPulser electroporator system (Bio-Rad, USA) with an amplitude setting program of EC2 for *E. coli*. Transformants were spreaded on LB agar plates containing 0.1% of RBB-dyed xylan and 50 ㎍ of ampicillin per ㎖. The plates were incubated at 37℃ overnight. Xylanase positive colonies were detected by the appearance of a clearing zone around active colonies.

2.5 Xylanase subcloning

A 558 bp of fragment which encode a mature protein excluding the tentative signal sequence, was amplified with specific primers (Xyl22F and Xyl22R) by PCR (Table 1). The PCR products were digested with Xho I and BamH I restriction enzymes and ligated into similarly cleaved pET-22b(+)(Novagen, Germany) plasmid. The construct, referred to as pxylS, was transformed into *E. coli* BL21(DE3) cells and plated on LB agar plates containing 50 ㎍/㎖ ampicillin.

2.6 Over-expression and crude purification of the XylS

A single colony containing pET-22b-xylS was grown in 50 ml LB containing ampicillin until the cell absorbance reached to approximately 0.6 at 600 nm. The culture was then adjusted to 0.4 mM IPTG, and further incubated at 30℃ for 8 hour. Cells were harvested by centrifugation at 15,000 rpm for 5 min., resuspended and incubated in binding

Table 1. PCR primers and PCR program for xylS amplification

Primer	Sequence
Xyl22F(*BamH* I)	5'-CGGGATCCGGCTAGTCCAGACTACT - 3'
Xyl22R(*Xho* I)	5'-CCGCTCGAGCCACACTGTTACGT - 3'
PCR conditions	28 cycle
Pre-denaturation	94℃, 1 min
Denaturation	94℃, 15 sec
Annealing	56℃, 30 sec
Extension	72℃, 1 min
Last-extension	72℃, 7 min

buffer (Novagen, Germany). It was followed by sonication with a Artec Sys. Co-150 ultrasonicator to release intracellular proteins. The cell-free extract was centrifuged at 15,000 rpm for 10 min. to remove cell debris, and the supernatant was used to purify the protein. Purification procedure was performed by the manufacturer's method with His • Bind® purification kit (Novagen, Germany).

2.7 Gel electrophoresis and activity staining

The SDS-PAGE was performed in a 15% SDS polyacrylamide gel containing 0.1% soluble Xylan [9, 11]. The samples were heat-treated for 5 min at 80℃ before being loaded and run at 40 mA for 40 min using a Bio-Rad Mini-Protein III Gel Kit. After the electrophoresis, the gels were washed for 30 min. at 4℃ with shaking in a 10 mM sodium phosphate buffer (pH 7.0) and further incubated for 90 min. at 50℃. The protein gel was stained with Comassie brilliant blue G (Sigma) while the activity gels were stained with a 0.1% Congo red (Sigma) solution for 10 min and destained with a 1 M NaCl solution.

2.8 Enzyme assay of xylanase

The xylanase activity of recombinant XylS protein was determined by measuring the amount of released reducing sugar using the dinitrosalicylic acid method. The reaction mixtures containing 100㎕ 1%(w/v) of soluble Xylan in 20 mM sodium phosphate buffer, pH 6.0, and 10 ㎕ enzyme solution, were incubated at 50℃ for 20 min. One unit of the enzyme activity was defined as the amount of enzyme that produced 1 μmol of reducing sugar per min.

2.9 DNA sequence accession number

The DNA sequence reported here was deposited in the GenBank under accession No. AF441773 (xylS).

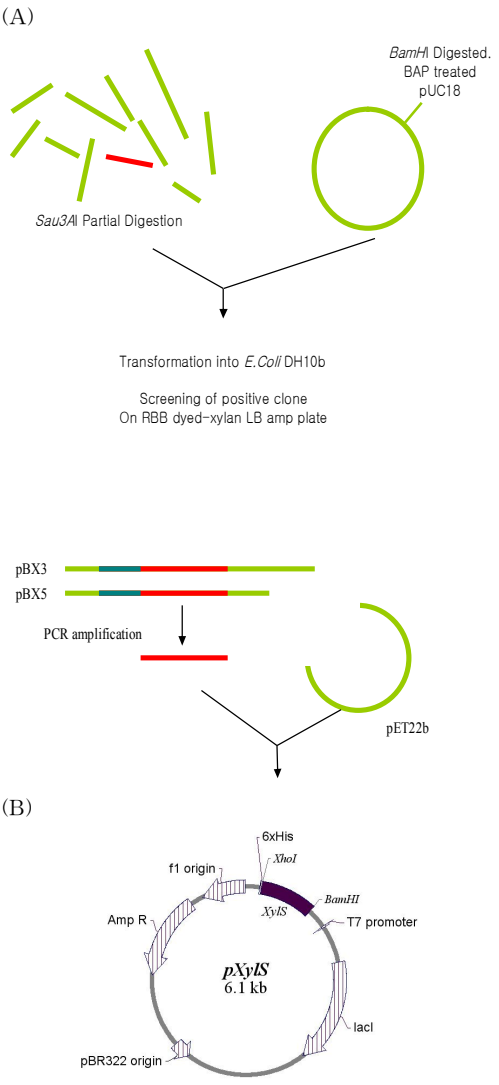

Fig. 2. Strategy for cloning of endoxylanase and subcloning procedure.

3. RESULTS AND DISCUSION

3.1 Cloning and nucleotide sequencing of xylS

The chromosomal DNA of *B. licheniformis* NBL420 was digested with Sau3A I and ligated to BamHI digested and dephosphorylated pUC18 (Takara, Japan). It was then transformed into *E. coli* DH10B. Visual screening of transformants on LB plates containing ampicillin (50㎍/㎖) and RBB-dyed xylan (0.1%, w/v) showed two positive clones with clear zone. Two clones carrying the recombinant plasmids (pBX3, pBX5) were further analyzed by DNA sequencing. It was revealed that two inserts (0.9 and 1.5 kb respectively) in recombinant plasmids contained a common 0.8 kb DNA segment carrying structural gene of xylanase. pBX5, a clone carrying the small insert was selected for further study (Fig. 2A). The xylanase gene, designated xylS, consisted of an open reading frame of 639 bp encoding 213 amino acids with a predicted molecular mass of 23 kDa.

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GATCAAAAACTTTGGCATTAGTAATTAATAATGTTTTAAATCTATACGAGTGCTACCTCA   60
TGTCAAAGTCAGAATAATTATTATAGGAGGTAACATATGTTTAAGTTTAAAAGGAATTTC  120
                                    M   F   K   F   K   R   N   F
                                    1   2   3   4   5   6   7   8
TTAGTTGGATTAACGGCAGCTTTAATGAGTATTAGCTTGTTTTCGGCAACCGCCTCTGCA  180
 L   V   G   L   T   A   A   L   M   S   I   S   L   F   S   A   T   A   S   A
 9  10  11  12  13  14  15  16  17  18  19  20  21  22  23  24  25  26  27  28
GCTAGTCCAGACTACTGGCAAAATTGGACTGATGGCGGCGGAACAGTAAACGCTGTTAAT  240
 A   S   P   D   Y   W   Q   N   W   T   D   G   G   G   T   V   N   A   V   N
29  30  31  32  33  34  35  36  37  38  39  40  41  42  43  44  45  46  47  48
GGGCCTGGCGGGAATTACAGCGTTAATTGGTCTAATACCGGAAATTTCGTTGTTGGTAAA  300
 G   P   G   G   N   Y   S   V   N   W   S   N   T   G   N   F   V   V   G   K
49  50  51  52  53  54  55  56  57  58  59  60  61  62  63  64  65  66  67  68
GGTTGGACTACAGGTTCGCCATCTAGGACAATAAACTATAATGCCGGAGTTTGGGCGCCG  360
 G   W   T   T   G   S   P   S   R   T   I   N   Y   N   A   G   V   W   A   P
69  70  71  72  73  74  75  76  77  78  79  80  81  82  83  84  85  86  87  88
AATGGCAATGGAATATTGCCTTTATATGGTTGGACGGAGGCATCTCTCATAGAATATTAT  420
 N   G   N   G   Y   L   A   L   Y   G   W   T   R   A   P   L   I   E   Y   Y
89  90  91  92  93  94  95  96  97  98  99 100 101 102 103 104 105 106 107 108
GTAGTGGATTCATGGGGTACTTATAGACCTACTGCAACGTATAAAGGTACTGTAAAAAGT  480
 V   V   D   S   W   G   T   Y   R   P   T   G   T   Y   K   G   T   V   K   S
109 110 111 112 113 114 115 116 117 118 119 120 121 122 123 124 125 126 127 128
GATGGGGGCACATATGACATATATACAACTACACGTTATAATGCACCTTCCATTGATGGC  540
 D   G   G   T   Y   D   I   Y   T   T   R   Y   N   A   P   S   I   D   G
129 130 131 132 133 134 135 136 137 138 139 140 141 142 143 144 145 146 147 148
GAAAAAACTACTTTCAGCAGTACTGGAGTGTTCGCCAGACGAAGACACCAACTGGAAGC  600
 E   K   T   T   F   T   Q   Y   W   S   V   R   Q   T   K   R   P   T   G   S
149 150 151 152 153 154 155 156 157 158 159 160 161 162 163 164 165 166 167 168
AACGCTAAAATCACTTTCAGCAATCATGTTAGAGCATGGAAGACTCATGGAATCAATCTG  660
 N   A   K   I   T   F   S   N   H   V   R   A   W   K   S   H   G   M   N   L
169 170 171 172 173 174 175 176 177 178 179 180 181 182 183 184 185 186 187 188
GGTAGTATTTGGTCTTATCAAGTCTTACGCACAGAGGGATATCAAAGTAGTGGAAGTTCT  720
 G   S   I   W   S   Y   Q   V   L   A   T   E   G   Y   Q   S   S   G   S   S
189 190 191 192 193 194 195 196 197 198 199 200 201 202 203 204 205 206 207 208
AACGTAACAGTGTGGTAACAGATCATCTTTAATCAGGCGTAGCGGCTGTTGATCCATGTC  780
 N   V   T   V   W   ·
209 210 211 212 213   ·
CTCCAGCACGATATCAAAACGGACGTCACCGATTTTTCCGACAATGCGCGGATC        834
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Fig. 3. Nucleotide sequence of *B. licheniformis* NBL420 xylanase (*xylS*) in pBX5.

The N-terminus of the deduced amino acid sequence includes a bacterial signal peptide with suitable cleavage sites for a signal peptidase (Ala28-Ala29). The calculated size of mature xylS sequence was almost identical to 20 kDa of recombinant protein (XylS) resulted from activity staining result. A putative ribosome binding site (Shine-Dalgarno sequence) was identified from upstream of the initiation codon (Fig. 3.). The DH10B host cell harboring pUC18 itself did not show any xylanase activity.

The *xylS* gene showed a high similarity (up to 93.4%) in amino acid sequence with other previously reported xylanases (Fig. 4.). The sequences shown are CAA84276 from *Bacillus subtilis*, NP389765 from *Bacillus subtilis*, P09850 from *Bacillus circulans*, CAA41783 from *Bacillus* sp. YA-14 and AAD10834 from *Bacillus* sp.

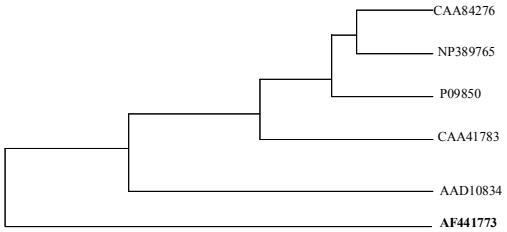


Fig. 4. Phylogenic tree of *xylS*(AF441773) from *Bacillus licheniformis* NBL420.

The open reading frame of *xylS* without its putative signal sequence was amplified by PCR, and ligated into plasmid pET22b(+) (Novagen, Germany). The resulting plasmid was designated as pxylS, and introduced into *E. coli* BL21(DE3) (Fig. 2B). The cloned xylanase activity was further conformed by RBB-dyed xylan plate and activity staining (Fig. 5, 6).

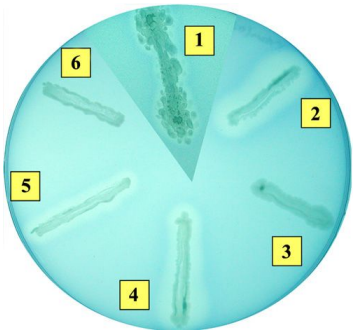


Fig. 5. Detection of xylanase activity of NBL420 and recombinant *E. coli* clones on LB and LB amp plate containing RBB dyed xylan (1) *B. licheniformis* NBL420 on LB plate; (2) *E.coli* BL21(DE3) containing pxylS; (3) *E.coli* BL21(DE3) containing pET22b as a negative control; (4) *E.coli* DH5α containing pBX5; (5) *E.coli* DH5α containing pBX3 and (6) *E.coli* DH5α containing pUC18 as a negative control.

3.2 Southern Hybridization

To demonstrate that the cloned XylS was originated from *B. licheniformis* NBL420, Southern hybridization analysis was performed using chromosomal DNAs from *E. coli* and *B. licheniformis* NBL420 (Fig. 7)

A 558bp of PCR product (xylS) encoding the mature XylS was used as a probe. The DIG-labelled probe was hybridized to a positive control as well as *HindⅢ*, *EcoR I* double-digested *B. licheniformis* NBL420 chromosomal DNA. On the contrary, any hybridization band was not detected in *E. coli* DH5α chromosomal DNA. These results confirmed that xylS was originated from *B. licheniformis* NBL420.

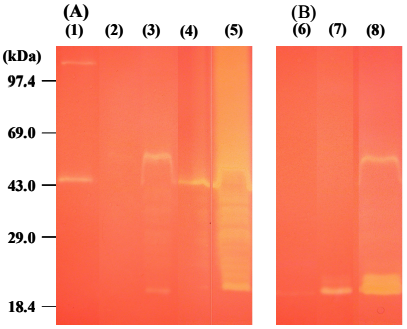


Fig. 6. Activity staining on 0.1% soluble xylan + 12% SDS-PAGE gel. (A), (1) *B. licheniformis* NBL420 culture broth; (2) *E.coli* DH5α containing pUC18 as a negative control; (3) *E.coli* DH5α containing pBX5; (4) *E.coli* BL21(DE3) containing pxylS (IPTG -); (5) *E.coli* BL21(DE3) containing pxylS (IPTG +) (B) 80℃ 5min heat treated sample, (6) *B. licheniformis* NBL420 culture broth; (7) *E.coli* DH5α containing pBX5; (8) *E.coli* BL21(DE3) containing pxylS (IPTG +)

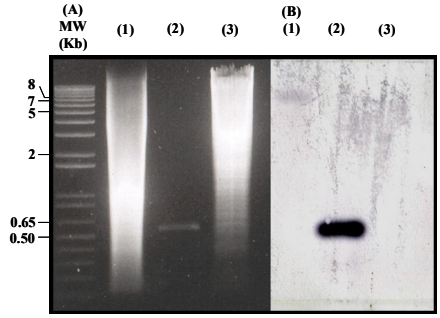


Fig. 7. Southern hybridization of *Bacillus licheniformis* NBL420 chromosomal DNA. (A): Agarose gel pattern with Et-Br, (1) *B.licheniformis* NBL420 chromosomal DNA; (2) PCR product of xylS as a positive control; (3) *E.coli* DH5α chromosomal DNA as a negative control. (B): DNA pattern of southern blot.

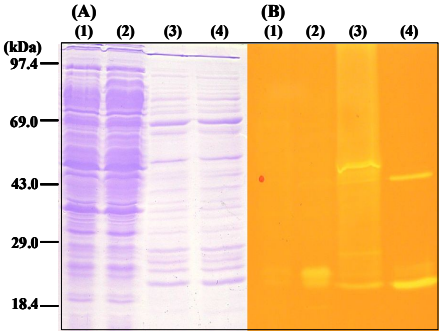


Fig. 8. Partial purification of XylS using Ni-NTA column. (A) protein band profile , (1) Crude extract of *E.coli* BL21(DE3) containing pxylS (2) 80℃ 5min treated crude extract of *E.coli* BL21(DE3) containing pxylS; (3) Partially purified XylS; (4) 80℃ 5min treated, Partially purified XylS; (B) Activity staining result.

3.3 Enzymatic characterization

Activity staining analysis of XylS expressed from pxylS revealed a single activity band approximately 20 kDa in size, which corresponds to the predicted size of XylS based on the xylS gene sequencing (Fig. 8).

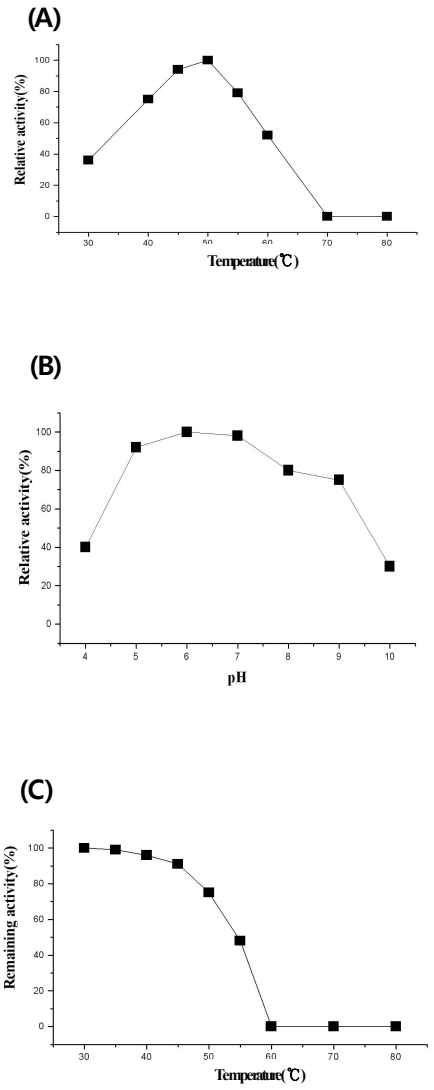
Enzyme characteristics of recombinant XylS were determined using crude XylS proteins purified from Ni-NTA column.

The optimum activity of XylS was showed at 50℃ (Fig. 9A) while the half of XylS activity was disappeared at 55℃ (Fig. 9C). The optimum pH was found near 6.0 (Fig. 9B). However, XylS enzyme showed a substantial high activity across a broad pH range, with over 50% of maximum activity from pH 5.0 to over pH 10.0 (Fig. 9D).

3.4 Computer model generation of XylS

A secondary structure prediction of XylS was done by SOPMA, tool for protein secondary structure prediction by consensus prediction from multiple alignments. (Institut de Biologie et de Chimie des Proteines, UPR 412-CNRS, Lyon, France) (Fig. 10) and possible three-dimensional structure of XylS was predicted by Swiss-Model, a knowledge-based protein modeling tool (Fig. 11).

The XylS consists of mainly a typical beta-sheet domain motif which was found in other *Bacillus* sp. xylanases.



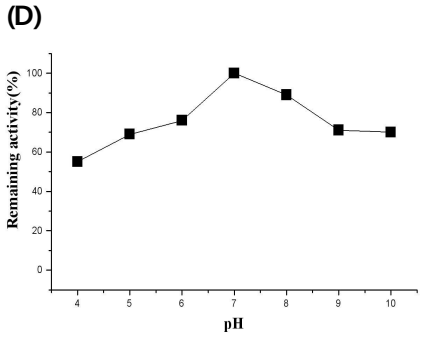


Fig. 9. Enzymatic properties of XylS. (A) Optimum temperature (B) Optimum pH (C) Thermostability (D) pH stability.

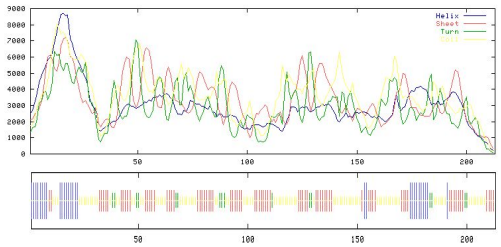


Fig. 10. Secondary structure prediction of XylS. Total 213 amino acids sequence were analized by SOPMA, tool for protein secondary structure prediction by consensus prediction from multiple alignments. (Institut de Biologie et de Chimie des Proteines, UPR 412-CNRS, Lyon, France)

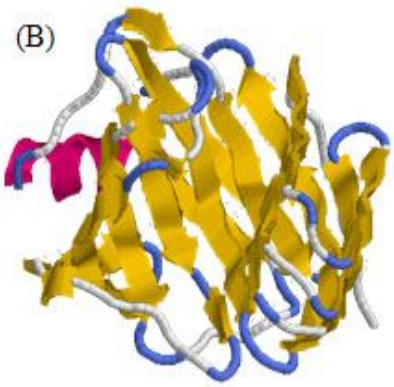


Fig. 11. 3-D model protein of XylS based on previously identified Engineered *Bacillus* Bifunctional Enzyme Gluxyn-1 A fragment (PDB Number :1AXKA). The homology between the amino acid sequence of CelA and the model protein was about 91.5 percent. (A) A model protein 1AXK. (B) Predicted 3-D model of XylS

참 고 문 헌

[1] Alberto R. A., L. Garda, and I. S. Ramon, "Overproduction, purification, and biolchemical characterization of a xylanase (Xyl1) from *Streptomyces halstedii* JM8", *Appl. Environ. Microbiol.,* 61: pp.2414-2419, 1995.

[2] Araki T., S. Hashikawa, and T. Morishita, "Cloning, sequencing, and expression in *Escherichia coli* of the new gene encoding β-1,3-xylanase from a Marine bacterium, *Vibrio* sp. Srtrain XY-214", *Appl. Environ. Microbiol.,* 66(4): pp.1741-1743, 2000.

[3] Beg Q.K., M. Kapoor, and L. Mahajan, "Microbial xylanases and their industrial applications: a review", *Appl. Microbiol. Biotechnol.,* 56: pp.326-338, 2001.

[4] Biely P., D. Mislovicova, and R. Toman, "Soluble chromogenic substrates for the assay of endo-1, 4-β-xylanases and endo-1, 4-β-glucanases", *Anal. Biochem.,* 144: pp.142-146, 1985.

[5] Blanco A., P. Diaz, P. parascandola, and F. I. J. Pastor, "A multidomain xylanase from a *Bacillus* sp. with a region homologous to thermostabilizing domains of thermophilic enzymes", *Microbiology*, 145: pp.2163-2170, 1999.

[6] Cazemier A. E., J. C. Verdoes, and H. J. M. OP den camp, "Molecular and biochemical characterization of two xylanase encoding genes from *Cellulomonas pachnodae*", *Appl. Environ. Microbiol.,* 65: pp.4099-4107, 1999.

[7] Cho S. G., and Y. J. Choi, "Nucleotide sequence analysis of an endo-xylanase gene (xynA) from *Bacillus stearothermophilus*", *J. Microbiol. Biotech.,* 5(3): pp.117-124, 1995.

[8] Gibbs M. D., R. A. Reeves, and P. L. Bergquist, "Cloning, sequencing, and expression of a xylanase gene from the extreme thermophile *Dictyoglomus thermophilum* Rt46B.1 and activity of the enzyme on fiber-bound substrate", *Appl. Environ. Microbiol.,* 61: pp.4403-4408, 1995.

[9] Gupta N., V. S. Reddy, and A. ghosh, "Cloning, expression, and sequence analysis of the gene encoding the alkali-stable, thermostable endoxylanase from alkalophilic, mesophilic *Bacillus* sp. Strain NG-27", *Appl. Environ. Microbiol.,* 66: pp.2631-2635, 2000.

[10] Henrik D., "Expression cloning of fungal enzyme genes; a novel approach for efficient isolation of enzyme genes of industrial relevance", *FEMS Microbiology Rev.*, 21: pp.29-42, 1997.

[11] Jeong K. J., P. C. Lee, M. S. Kim, and S. C. Kim, "Molecular cloning and characterization of an endoxylanase gene of *Bacillus* sp. in *Escherichia coli*", *Enzyme Microb. Technol.*, 22: pp.599-605, 1998.

[12] Khowala S., M. Mukherjes, and S. Sengupta, "Carboxymethyl xylan-a specific substrate directly differentiatinf backbone-hydrolysing and side chain-reacting β-D-(1→4)-xylanases of the mushroom *Termitomyces clypeatus*", *Enzyme Microb. Technol.,* 10: pp.563-567, 1988.

[13] Kulkarni N., A. Shendye, and M. Rao, "Molecular and biotechnological aspects of xylanases", *FEMS Microbiology Rev.*, 23: pp.411-456, 1999.

[14] Kulkarni N., M. Lakshmikumaran, and M. Rao, "Xylanase II from an alkaliphilic *Bacillus* with a distinctly different structure from other xylanases:

Evolutionary relationship to alkaliphilic xylanases", *Biochem. Biophys. Res. Comm.,* 263: pp.640-645, 1999.

[15] Lapidot A., A. Mechaly, and Y. Shoham, "Overexpression and single-step purification of a thermostable xylanase from *Bacillus stearothermophilus* T-6", *J. Biotech.,* 51: pp.259-264, 1996.

[16] Na K. H., J. M. Kim, and J. H. Yu, "Recombinant plasmid DNA Containing xylanase and β-xylosidase gene of *Bacillus* sp.YA-14", *Kor. J. Appl. Microbiol. Biotech.,* 18(2) pp.195-198, 1990.

[17] Sung N. K., H. R. Ko, Y., H., Kho, H. K. Chun, and Y. C. Chung, "Subcloning and enhanced expression of the β-xylosidase gene cloned from alkalophilic *Bacillus* sp. k-17", *Kor. J. Appl. Microbiol. Bioeng.,* 17(4) pp.283-288, 1989.

[18] Tabernero C., P. Perez, and R. I. Santamaria, "Cloning and DNA sequencing of xyaA, a gene encoding an endo-β-1,4-xylanase from an alkalophilic *Bacillus* strain (N137)", *Appl. Environ. Microbiol.,* 61: pp.2420-2424, 1995.

[19] Tomme P., S. Mccrae, and M. Claeyssens, Chromatographic separation of cellulolytic enzymes, *Affinity Chromatography of Cellulases* 187-193.

[20] Yoon K. H., H. N. Yun, and K. H. Jung, "Molecular cloning of a *Bacillus* sp. kk-1 xylanase gene and characterization of the gene product", *Biochem. Mol. Biol. International.*, 45: pp.337-347, 1998.

[21] Yu J. H., D. C. Park, Y. J. Chung, and I. S. Kong, "Cloning and expression of a xylanase gene from alkali-tolerant *Bacillus* sp. YA-14 in *Escherichia coli", Kor. J. Appl. Microbiol. Bioeng.,* 17(2): pp.154-159, 1989.

[22] Yu J. H., Y. S. Park, D. Y. Yum, I. S. Kong, and D. H. Bai, "Nucleotide sequence and analysis of a xylanase gene (xynS) from alkali-tolerant *Bacillus* sp. YA-14 and comparison with other xylanases", *J. Microbiol. Biotech.,* 3(3): pp.139-145, 1993.