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DNA Shuffling을 이용한 *Paenibacillus polymyxa* GS01의 다기능 *β*-Glycosyl Hydrolase (Cel44C-Man26A$_{P558}$) 효소 활성 증가

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Enhancing the Enzymatic Activity of the Multifunctional *β*-Glycosyl Hydrolase (Cel44C-Man26A$_{P558}$) from *Paenibacillus polymyxa* GS01 Using DNA Shuffling

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We previously reported that the truncated Cel44C-Man26A$_{P558}$ *β*-glycosyl hydrolase protein exhibits multifunctional activities, including cellulase, xylanase, and lichenase. DNA shuffling of the truncated Cel44C-Man26A$_{P558}$ enzyme was performed to enhance the enzymatic activity of the multifunctional *β*-glycosyl hydrolase. Two mutant enzymes, M2Cel44C-Man26A$_{P558}$ that carries one mutation (P438A) and M21Cel44C-Man26A$_{P558}$ that carries two mutations (A273T and P438A) were obtained. The enzymatic activity of the M21Cel44C-Man26A$_{P558}$ double mutant was lower than enzymatic activity of the single mutant (M2Cel44C-Man26A$_{P558}$). However, both mutants displayed the enhancements in their enzyme activities that were ≈1.3- to 2.2-fold higher than the original enzymatic activity in Cel44C-Man26A$_{P558}$. In particular, the mutant M2Cel44C-Man26A$_{P558}$ exhibited an approximate 1.5- to 2.2-fold increase in the cellulase, xylanase, and lichenase activities in comparison with the control (Cel44C-Man26A$_{P558}$). The optimum cellulase, linchenase, and xylanase activities of β-glycosyl hydrolase were observed at pH 7.0, pH 7.0 and pH 6.0, respectively. These results, therefore, suggest that the amino acid residue Ala438 plays important roles in the enhancement of the activity of multifunctional *β*-glycosyl hydrolase.

***Keywords*:** *Paenibacillus polymyxa* GS01, Cel44C-Man26A$_{P558}$, DNA shuffling, multifunctional *β*-glycosyl hydrolase

Cellulosic biomass has great potential as a renewable energy source. The enzymatic hydrolysis to produce monomeric sugars is, however, nonetheless hindered by obstacles caused by the complex and inhomogeneous structure of the cellulosic substrates (Varnai *et al*., 2011). The enzymes involved in the metabolism of plant carbohydrate polymers can be grouped into 104 different families based on sequence homology (Cho *et al*., 2006). The glycosyl hydrolase family 44 (GH44) is mostly composed of endoglucanases (EGs), and GH44 enzymes are produced by both aerobic/anaerobic bacteria. At present, 29 amino acid sequences of GH44 family members have been determined (Warner *et al*., 2010). Several researchers reported that GH44 enzymes exclusively cleave *β*-1, 4 bonds between glycosyl and xylosyl residues and that they have different abilities to attack the bonds of xylan, lichenan, and

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different cellulose forms, such as Avicel, acid-swollen cellulose, and carboxymethylcellulose (CMC) (Crameri *et al*., 1998; Cho *et al*., 2006, 2007, 2008a, 2008b; Warner *et al*., 2010). The development of directed-evolution techniques was stimulated by the advent of DNA shuffling (Crameri *et al*., 1998; Kataeva *et al*., 2002), a PCR-like process that introduces and recombines random point mutations within a particular gene (Ash *et al*., 1993). Occasionally, DNA shuffling experiments showed that amino acid changes distant from the active site can affect substrate specificity (Ajay and Kiyoshi, 1995; Hida *et al*., 2007). DNA shuffling consists of four steps: (i) preparation of the gene to be shuffled, (ii) fragmentation with DNase I, (iii) reassembly by thermocycling in the presence of a DNA polymerase, and (iv) amplification of the reassembled products by conventional PCR. Point mutations may be generated during each of these steps (Ash *et al*., 1993; Binay *et al*., 2009; Hu *et al*., 2009; Eisenbesis and Hocker, 2010).

The present work concerns the Cel44C-Man26A of the GH44 family from *Paenibacillus polymyxa* GS01, a Gram-positive, mesophilic, spore-forming, endophytic bacterium. This strain was shown to produce a wide variety of secondary metabolites, such as plant growth-regulating substances, antibiotic compounds, and plant cell wall-degrading enzymes (Cho *et al*., 2006, 2007, 2008a, 2008b). In addition, we previously reported that the truncated Cel44C-Man26A$_{P558}$ protein was a trifunctional β-glycosyl hydrolase, demonstrating cellulase, xylanase, and lichenase activities (Cho *et al*., 2006). In the present study, we increased the activities of the trifunctional β-glycosyl hydrolase (Cel44C-Man26A$_{P558}$) using the DNA shuffling method.

Materials and Methods

Bacterial strains, vectors, and materials

Escherichia coli DH5α, BL21 (DE3), and recombinant *E. coli* were cultured in Luria-Bertani (LB) containing 50 μg/ml ampicillin at 37℃. Restriction enzymes and DNA-modifying enzymes were purchased from Promega (USA). Other chemicals were purchased from Sigma-Aldrich Chemical Co. (USA).

Antibiotics were purchased from Sigma-Aldrich Chemical Co., and ampicillin was used at 50 μg/ml. The pGEM-T easy vector (Promega) was used for cloning and sequencing. The LB media were purchased from Difco (Becton Dickinson Co., USA).

Recombinant DNA techniques

Standard procedures for restriction endonuclease digestion, agarose gel electrophoresis, DNA ligation, and other cloning-related techniques were followed (Sambrook and Russell, 2001). Nucleotide sequences were determined using the dideoxy-chain termination method with the PRISM Ready Reaction Dye Terminator/Primer cycle Sequencing kit (PerkinElmer Corp., USA). Assembly of the nucleotide sequences and the amino acids sequence analysis were performed using the DNAMAN analysis system (Lynon Biosoft, Canada). The experimental three-dimensional (3D) structure data were obtained from the Protein Data Bank (PDB).

DNA shuffling

DNA shuffling was performed using a slight modification of the Stemmer method (Stemmer, 1994). The target for the shuffling procedure was a 1.8 kb PCR product obtained by the amplification of 0.3 μg template DNA (Cel44C-Man26A$_{P558}$/ pBluescript II SK+). The PCR product was purified using the PCR purification kit (INtRON Biotechnology, Korea). Approximately 2 to 4 μg of the purified PCR product was digested with 0.15 units of DNase I at 37℃ for 15 min in a total volume of 30 μl in 10 mM Tris-HCl buffer (pH 7.4). Fragments with sizes of 100 to 300 bp were purified from the corresponding region of 2% agarose gels using the INtRON gel extraction kit (INtRON Biotechnology, Korea). These fragments were then amplified by PCR (1 min at 94℃, 40 cycles of 30 sec at 94℃, 30 sec at 52℃, 2 min at 72℃, and a final step of 10 min at 72℃). The full-length gene was isolated using a second PCR amplification (initial denaturation 95℃ for 5 min, 25 cycles of 94℃ for 30 sec, 55℃ for 30 sec, 72℃ for 5 min, and a final step of 72℃ for 10 min). This step was performed using the correct primers for the 5′ *Bam*HI (N-terminus) and 3′ *Sal*I (C-terminus) sites. All of the reactions were performed in the presence of 0.2 mM each dNTPs, 2 mM $MgCl_2$, 1× Taq buffer, and 2.5 U *Taq/Pfu* enzyme mixture. The ≈1.8 kb product was digested with *Bam*HI and *Sal*I prior to cloning into a similarly cut pBluescript II SK+ vector and the plasmids were used to transformation *E. coli* DH5α competent cells.

Screening

E. coli cells harboring the recombinant plasmids were grown and selected on LAXI [LB medium containing 50 μg/ml ampicillin, 6 μg/ml X-gal, 5 μg/ml IPTG, and 1.5% agar (w/v)] agar plates. The positive subclones were obtained by the detection of the bacterial colonies on cellulase, xylanase, and lichenase indicator media [LB medium containing 50 μg/ml ampicillin and 1.0% carboxylmethylcellulose (CMC, w/v), 1.0% oat spelt xylan (OSX, w/v), or 1.0% lichenan (w/v), respectively, and 1.5% agar (w/v)]. After growth at 37℃ for 24 h, the plate for cellulose was stained with 0.5% (w/v) Congo Red solution for 30 min, rinsed with water, washed twice with 1 M NaCl and then stained with 0.1 N HCl for cellulase activity. Positive clones exhibiting extracellular cellulase activity were

Table 1. Specific and relative activities for the hydrolysis of carboxymethylcellulose, oat spelt xylan, and lichenan by Cel44C-Man26AP558, M2Cel44C-Man26AP558, and M21Cel44C-Man26AP558 enzymes

Proteins	Specific activity[a] (U/mg)/Relative activity (%)		
	Cellulase[b]	Xylanase[c]	Lichenase[d]
Cel44C-Man26A$_{P558}$	334/100	142/100	356/100
M2Cel44C-Man26A$_{P558}$	514/154	310/218	612/172
M21Cel44C-Man26A$_{P558}$	427/128	216/152	484/136

[*] All values were calculated as mean value after determination more than three times.
[a] Specific activity was expressed as μmol glucose and xylose min^{-1} mg^{-1} protein.
[b] Cellulase activity were determined at pH 7.0 and 50℃ for 30 min.
[c] Xylanase activity was determined at pH 6.0 and 50℃ for 30 min.
[d] Lichenase activity was determined at pH 7.0 and 50℃ for 30 min.

surrounded by a yellow halo against a red background (Cho *et al*., 2006).

Expression and purification of enzyme

For high expression, the PCR products generated with primers 5′-AAGGATCCAGAGCGAAAAATAGTAGTAGTAT-TC-3′ (forward, *Bam*HI underlined) and 5′-TTGTCGACTGGAGACGTGTAATTGCC-3′ (reverse, *Sal*I underlined) were cloned into the expression vector pET-29a(+) (Novagen, USA) using the *Bam*HI and *Sal*I sites, resulting in the addition of a C-terminal $(His)_6$ tag, to produce pET-29a(+)/Cel44C-Man26A$_{P558}$, pET-29a(+)/M2Cel44C-Man26A$_{P558}$, and pET-29a(+)/M21Cel44C-Man26A$_{P558}$. Enzyme was purified as described previously (Cho *et al*., 2008a).

Enzyme assay

The substrates for the cellulase, xylanase and lichenase activities, from pH 3 to 11, were prepared. Each enzyme activity assay was conducted using a DNS-based method to determine the optimal pH for each activity. To ascertain the optimal temperature, each activity test was performed at eight different temperatures, ranging from 10℃ to 80℃, with the optimum respective pH. The cellulase, xylanase, and lichenase activities of the recombinant *E. coli* clones were determined by measuring the amount of reducing sugars produced during the incubation with the substrate. One unit of enzyme activities for cellulase, xyalnase and lichenase was defined as described elsewhere (Cho *et al*., 2008a).

Results and Discussion

Using DNA shuffling (Stemmer, 1994; Crameri *et al*., 1998; Binay *et al*., 2009; Eisenbesis and Hocker, 2010) of the Cel44C-Man26A$_{P558}$ gene, we amplified a 1.8 kb DNA fragment (Fig. 1A). The amplicon was randomly cleaved with nonspecific DNase I, and the resulting fragments were amplified again by PCR (Figs. 1B to 1C). A library of full-length mutant genes was generated after several rounds of

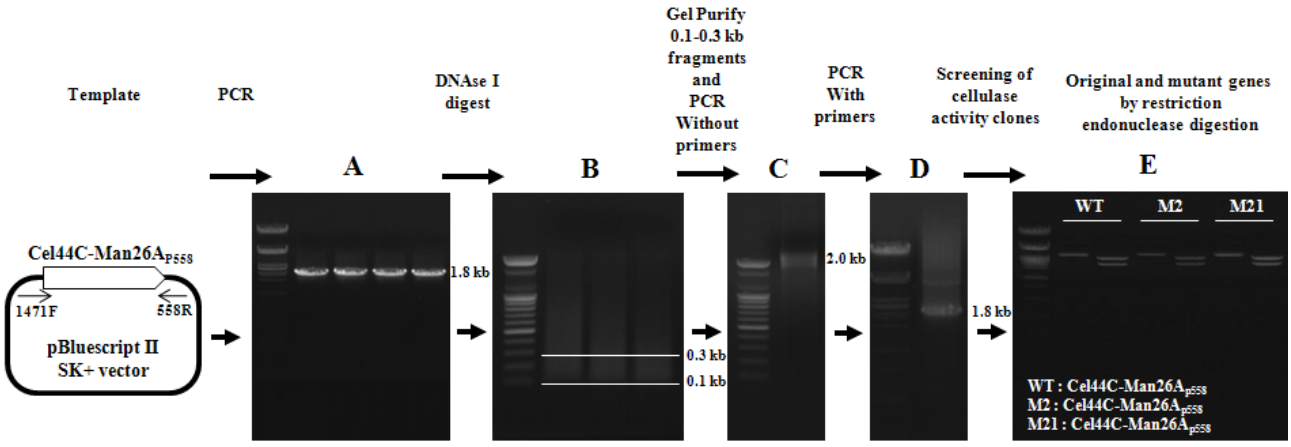

Fig. 1. Reassembling of 1.8 kb gene from 0.1 to 0.3 kb random fragments. (A) 1.8 kb DNA fragment encoding *cel*44C-*man*26A$_{P558}$ was amplified by PCR. (B) DNase I digest of gene: DNA fragments (0.1 to 0.3 kb) purified from an agarose gel. (C) Assembling of purified fragments into a full-length gene at high fragment concentration (50 ng/μg) in the absence of primers. (D) PCR yields, a single product. Cloning of this into plasmid generated 60% clones displaying cellulase activity, reflecting that mutation occurred during the reassembly process. (E) Detection of cellulase-positive clones harboring *cel*44C-*man*26A$_{P558}$ and other mutant genes by restriction endonuclease (*Bam*HI and *Sal*I) digestion.

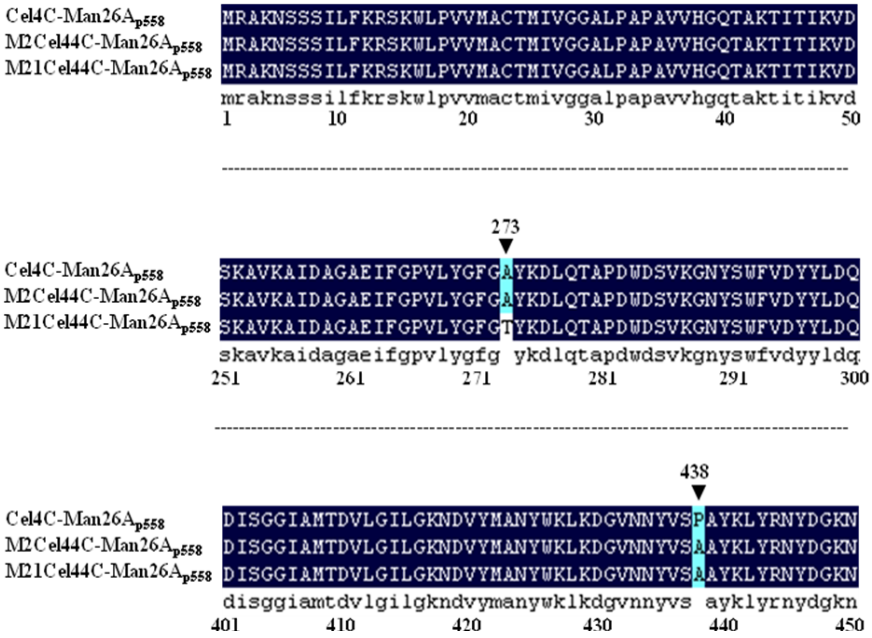

Fig. 2. Homology alignment of the deuced amino acid sequence of Cel44C-Man26A$_{P558}$, M2Cel44C-Man26A$_{P558}$, and M21Cel44C-Man26A$_{P558}$. The amino acids at positions 273 and 438 see replaced with T and A respectively.

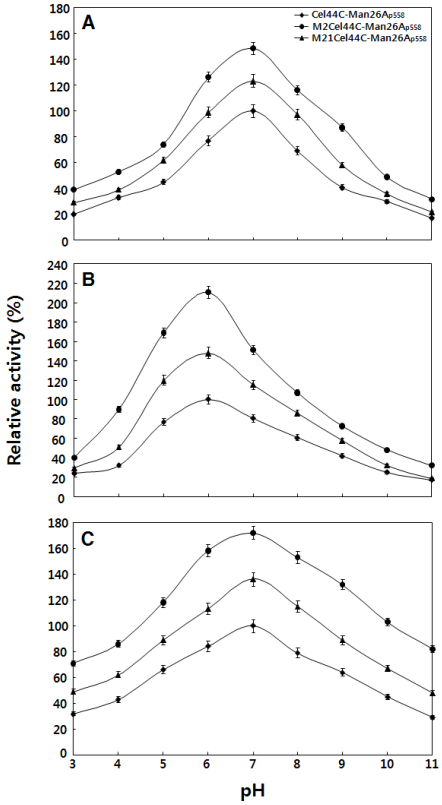

Fig. 3. The Cel44C-Man26A$_{P558}$, M2Cel44C-Man26A$_{P558}$, and M21Cel44C-Man26A$_{P558}$ enzymes were assayed for the β-glycosyl hydrolase activity carboxymethylcellulose, oat spelt xylan, and lichenan as substrate at different pH values at 50℃ for 30 min. (A) cellulase activity, (B) xylanase activity, (C) lichenase activity

PCR in the presence of primers for the 5′-*Bam*HI and 3′-*Sal*I sites (Fig. 1D). This library was ligated back into pBluescript SK II(+), transformed into *E. coli* DH5α cells and plated on cellulase activity indicator media.

The PCR procedure adopted here employed smaller DNA fragments than the DNA templates used in normal PCR. The rate of point mutation may depend upon the size of the DNA fragments that are used in the reassembly. In contrast to PCR, the DNA reassembly is an inverse reaction: in PCR, the number of polymerase start sites and the number of DNA molecules grow exponentially, whereas the number of start sites and the number (but not the size) of the molecules decrease over time during DNA reassembly. Such a reaction may be used for the reassembly of genes from highly fragmented fossil DNA. By conducting two rounds of DNA shuffling and recombination, two types of mutants of the multi-functional glycosyl hydrolases from *P. polymyxa* GS01 were obtained in this study. The specific activity of one type of mutant increased relative to the wild-type enzyme, and the other type of mutant showed unaltered catalytic capacity.

The first round of DNA shuffling yielded 64 clones on the basis of the production of a yellow halo on selective plates. Among the different clones, three showed larger halos than the parents (Cel44C-Man26A$_{P558}$ protein). The enzyme activity of the M32Cel44C-Man26A$_{P558}$ gene product was similar to the control. The sequencing of the two shuffled genes (M2Cel44C-Man26A$_{P558}$ and M21Cel44C-Man26A$_{P558}$) revealed

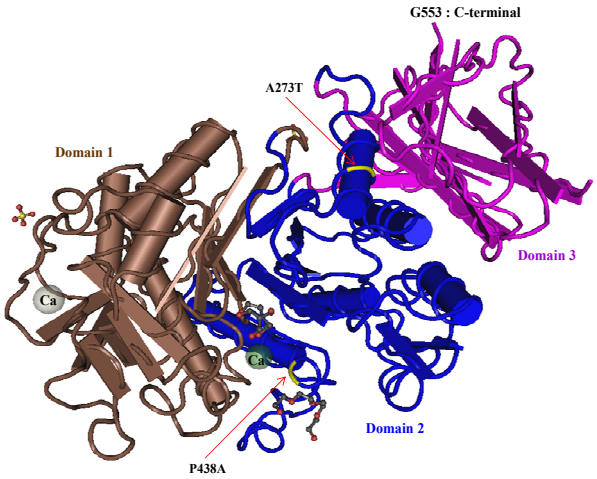

Fig. 4. The structure of Cel44C-Man26A$_{P558}$ from *P. polymyxa*. The Cel44C-Man26A$_{P558}$ has three domains as depicted in brown (Domain 1), blue (Domain 2), and pink colors (Domain 3). A273T and P438A mutation enhanced the glucosyl hydrolase activity (PDB 2YKK_A; http://www.ncbi.nlm.nih.gov/Structure/index.shtml).

that a single amino acid residue (Pro438 to Ala438) was substituted in the M2Cel44C-Man26A$_{P558}$ gene. Pro438 was substituted in both shuffled genes with Ala (Fig. 2). The β-endoglucanase activities of the parent (Cel44C-Man26AP558 protein) and the two mutant enzymes (M2Cel44C-Man26A$_{P558}$ and M21Cel44C-Man26A$_{P558}$ genes) using carboxymethylcellulase, oat spelt xylan, and lichenan were determined at 50℃ and pH values of 7.0, 6.0, and 7.0, respectively. The shuffled M2Cel44C-Man26A$_{P558}$ and M21Cel44C-Man26A$_{P558}$ enzymes showed higher (≈1.3- to 2.2-fold) enzymatic activity than that of the original Cel44C-Man26A$_{P558}$. In particular, the M2Cel44C-Man26A$_{P558}$ mutant showed the highest cellulase, xylanase, and lichenase activities (Table 2). The optimal cellulase, linchenase and xylanase activities of these proteins were found at pH 7.0, pH 7.0 and pH 6.0, respectively (Fig. 3). The interest in cellulose hydrolysis is focused on animal nutrition and on the potential development of environmentally benign processes for the microbial conversion of fuel ethanol and other compounds (Zaldivar *et al*., 2001; Chen and Jin, 2006).

A 3D structure of Cel44C-Man26A$_{P558}$ is shown Fig. 4. The Cel44C-Man26A$_{P558}$ structure was divided by color into three domains: brown color (Domain 1), blue color (Domain 2), and pink color (Domain 3). The mutations of A273T and P438A enhanced the glucosyl hydrolase activity of the area. The atomic coordinates of the native Cel44C-Man26A$_{P558}$ from *P. polymyxa* were obtained from the PDB file 2YKK (Hu *et al*., 2009; Eisenbesis and Hocker, 2010). The 3D structure of the active site of Cel44C-Man26A$_{p558}$ protein may change based on the ionic and hydrophilic/hydrophobic nature of the amino acids (Tang *et al*., 2006; Ryu *et al*., 2008; Binay *et al*., 2009). In many cases, the correlation between hydrophobic interactions and protein stability has been demonstrated. In particular, the side chains of alanine, valine, leucine, and isoleucine tend to cluster together within proteins, stabilizing protein structure by means of hydrophobic interactions (Tang *et al*., 2006; Cho *et al*., 2008a). In the case of the P438A substitution, it is proposed that the increased hydrophobicity of the environment after the substitutions contributed to the enhanced enzyme activity of the trifunctional β-glycosyl hydrolase. However, A273T mutation might affect to decrease the enzymatic activity because single mutant M2 had a lower e nzymatic activity compared to P438A and A273T substitutions in M21 double mutation. In addition, P438A was located near the Ca^{2+} ion in the model. Previously, a study published that LiP pYEX1 (A140G, S190P, P193A, and S196F) mutation may exert its influence on the H_2O_2 stability and thermal stability by affecting Ca^{2+} binding (Ryu *et al*., 2008).

In conclusion, we determined the catalytic capacity of a mutant multi-functional glycosyl hydrolase *cel*44C-*man*26A$_{P558}$ in *E. coli*; the mutant enzymes likely retain the structure and properties of their parental enzymes. Our results may be considered a long-awaited first step toward the elucidation of the function of multi-functional glycosyl hydrolases and the synergism between the host and symbiotic enzymes at the molecular level in *P. polymyxa* GS01. In the future, such microbial glycosyl hydrolases may be utilized in industry and for biomass conversion.

적 요

본 연구자들은 이전에 cellulase, xyalnase 및 lichenase의 다기능 효소활성을 지니는 절단된 Cel44C-Man26A$_{P558}$의 β-glycosyl

hydrolase를 보고하였다. 본 연구에서는 절단된 Cel44C-Man26AP558 효소의 다기능성 β-glycosyl hydrolase 활성을 증가시키기 위해 DNA shuffling을 시도하였다. DNA shuffling에 의해 단일변이(P438A)를 가진 M2Cel44C-Man26A$_{P558}$와 이중변이(A273T 및 P438A)를 가진 M21Cel44C-Man26A$_{P558}$를 얻었다. 이중변이를 가진 M21Cel44C-Man26A$_{P558}$은 단일변이를 가진 M2Cel44C-Man26A$_{P558}$ 보다 효소활성이 낮게 나타났으나, M2Cel44C-Man26A$_{P558}$와 M21Cel44C-Man26A$_{P558}$은 대조구인 Cel44C-Man26A$_{P558}$ 보다 약 1.3에서 2.2배 정도 높은 효소활성을 나타내었다. 특히, 단일변이를 가진 M2Cel44C-Man26A$_{P558}$는 대조구인 Cel44C-Man26AP558보다 cellulase, xylanase 및 lichenase 효소활성이 약 1.5에서 2.2배 정도 높게 나타났다. β-Glycosyl hydrolase의 cellulase, linchenase 및 xylanase 최적 효소활성은 각각 pH 7.0, 7.0 및 6.0에서 이었다. 이러한 결과는, 아미노산 잔기인 Ala438이 다기능성 β-glycosyl hydrolase 활성을 증가시키는 중요한 역할을 한다고 추정할 수 있다.

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