THE CONSTRUCTION OF BIFUNCTIONAL FUSION XYLANOLYTIC ENZYMES AND THE PREDICTION OF OPTIMUM REACTION CONDITIONS FOR THE ENZYME ACTIVITY

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Four chimeric xylanolytic enzymes were formed by fusion of a thermally stable xylanase XynCDBFV either to the N-terminus or C-terminus of a thermally stable acetylxylan esterase AxeS20E, with or without a Gly-rich flexible linker (S2). The three-dimensional (3D) structures of the chimeric enzymes were predicted using the I-TASSER server, and the results indicated that the structures of Axe-S2-Xyn and Xyn-S2-Axe were more similar to the native structures than were those of Axe-Xyn and Xyn-Axe. Axe-S2-Xyn and Xyn-S2-Axe were expressed in *Escherichia coli* and purified by means of affinity chromatography. Response surface modeling (RSM), combined with central composite design (CCD) and regression analysis, was then employed to optimize the xylanase activities of the chimeric enzymes. Under the optimal conditions, Xyn-S2-Axe had greater hydrolytic activities on natural xylans and rice straw than did the parental enzymes. These results suggested that the chimeric enzyme Xyn-S2-Axe could be effective at hydrolyzing xylan in biomass and that it has potential to be used in a range of biotechnological applications.

Keywords: Chimeric enzyme; Neocallimastix patriciarum; Xylanase; Acetylxylan esterase

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INTRODUCTION

Hemicelluloses are the second most abundant renewable bioresource on the earth and have a high potential to be converted into useful end products (Collins *et al.* 2005). As a major component of hemicelluloses, xylan is a heteropolysaccharide that consists of a backbone of β-1,4-xylopyranosyl units and has substituent groups including acetyl, α-arabinofuranosyl, and 4-*O*-methyl-D-glucuronosyl residues in its side chain (Subramaniyan and Prema 2002). These substituent groups in the xylan structure mediate the binding of the xylan to cellulose, lignin, and other polymers by covalent and noncovalent interactions. They also hinder the contact between xylanolytic enzymes and xylan, thereby reducing the catalytic efficiency of xylanolytic enzymes with regards to the hydrolysis of xylan. Therefore, the complete hydrolysis of xylan requires the cooperation of a large number of enzymes, including acetylxylan esterases, arabinofuranosidases, *p*-coumaric acid esterases, ferulic acid esterases, glucuronidases,

xylanases, and xylosidases (Subramaniyan and Prema 2002; Collins *et al.* 2005). Of these xylanolytic enzymes, endo-1,4-β-xylanase (EC 3.2.1.8) is necessary and of particular importance because it can depolymerize xylan by random hydrolysis of the β-1,4-xylosidic backbone linkages to produce xylooligosaccharides, which are further degraded by other accessory enzymes.

Among the accessory enzymes, acetylxylan esterase (EC 3.1.1.72) specifically hydrolyzes the ester linkages of the acetyl groups in position 2 and/or 3 of the xylose moieties of natural xylan. Acetylxylan esterase used along with xylanase enhances sugar release during the enzymatic saccharification of lignocelluloses (Selig *et al.* 2008a). This may be attributable to acetylxylan esterase cleaving the ester linkages between xylan and lignin and enhancing the accessibility of xylanase to the xylan backbone and the subsequent hydrolysis of xylan (Dupont *et al.* 1996).

Xylanolytic enzymes have potential applications in a wide range of industrial processes, such as biobleaching in the paper and pulp industry, bioconversion of lignocellulosic material and agro-wastes into fermentative products, clarification of juices, and improvement of the digestibility of animal feed stock (Subramaniyan and Prema 2002). Since most of these processes normally operate at high temperatures, thermally stable xylanolytic enzymes are considered economic advantages because they are suitable in bioconversion processes where high temperatures are required to increase substrate solubility, to increase the bioconversion rate, to reduce solution viscosity, or to reduce the risk of contamination (Collins *et al.* 2005; Wu *et al.* 2006).

In a previous study a sample of thermally stable xylanase, XynCDBFV, was obtained from the error-prone polymerase chain reaction (PCR) mutant library of XynC (GenBank accession number AF123252) from the ruminal fungus *Neocallimastix patriciarum* (Chen *et al.* 2001). In another previous study, a cDNA encoding an acetylxylan esterase AxeS20E (GenBank accession number FJ529209) was cloned from *N. patriciarum* (Pai *et al.* 2010a). AxeS20E exhibited great thermal stability and has potential to be used synergistically with other thermally stable xylanases (Pai *et al.* 2010b). Since both XynCDBFV and AxeS20E showed good thermal stability, they could be used effectively in combination with each other to promote the more efficient hydrolysis of xylan. Compared to mixtures of multiple single enzymes, chimeric enzymes possessing two or more enzyme activities are more cost- and time-effective in industrial enzyme production (Fang *et al.* 2009). Therefore, the chimeric enzymes XynCDBFV and AxeS20E might have the potential to be developed as high-efficiency, cost-saving xylanolytic enzymes.

This study discusses the construction of the four chimeric xylanolytic enzymes by means of the fusion of XynCDBFV to either the N-terminus or C-terminus of AxeS20E, with or without a Gly-rich flexible linker. The three-dimensional (3D) structures of the chimeric enzymes were predicted and generated using the I-TASSER server. RSM, combined with CCD and regression analysis, was then employed to optimize the xylanase activities of the chimeric enzymes. The specific activities of the chimeric enzymes on natural xylans and rice straw were determined. The thermal stability and kinetic parameters of the chimeric enzymes were also studied.

EXPERIMENTAL

Bacterial Culture and DNA Manipulation

Escherichia coli were grown at 37 °C in Luria-Bertani (LB) broth (Difco Laboratories, Detroit, MI). Agar plates were prepared by adding agar (1.5% w/v) (Difco Laboratories) to the broth. Plasmid DNA was isolated from *E. coli* using the QIAprep Miniprep Kit (Qiagen Inc., Valencia, CA). Restriction enzymes and T4 DNA ligase (New England BioLabs Inc., Beverly, MA) were used according to the manufacturer's instructions. All other DNA manipulations were performed using established procedures (Sambrook and Russell 2001).

Design of the Chimeric Enzymes

Four chimeric enzyme models were designed based on XynCDBFV and AxeS20E as follows: model 1, AxeS20E at the N terminus and XunCDBFV at the C terminus (Axe-Xyn); model 2, XynCDBFV at the N terminus and AxeS20E at the C terminus (Xyn-Axe); model 3, AxeS20E at the N terminus and XunCDBFV at the C terminus separated by an S2 linker (Axe-S2-Xyn); model 4, XynCDBFV at the N terminus and AxeS20E at the C terminus separated by an S2 linker (Xyn-S2-Axe). S2 represents a Gly-rich flexible linker (GGGGSGGGGS), which can retain the independent folding of the two domains it connects (Lu and Feng 2008). The 3D structures of the parental and chimeric enzymes were predicted by an automated homology modeling method using the I-TASSER server (Zhang 2008). A benchmark scoring system that included the confidence score (C-score), root-mean-square deviation (RMSD), and the estimated template modeling score (TM-score) was used for *in silico* quantitative assessments of the I-TASSER models. The predicted structure with the highest C-score reflected a model of better quality with greater structural similarity between the predicted and native structures (Zhang 2008; Nahar *et al.* 2012). The predicted structures of the chimeric enzymes were then superimposed onto those of the parental enzymes using the structural alignment program TM-align (Zhang and Skolnick 2005).

Construction of the Chimeric Enzymes

The DNA sequences encoding AxeS20E and XynCDBFV were amplified by PCR from the plasmids pTriplEx2-S20E (Pai *et al.* 2010a) and pNZJ021 (Liu *et al.* 2005), respectively. The DNA sequences encoding the chimeric enzymes Axe-S2-Xyn and Xyn-S2-Axe were obtained by a modified overlap extension PCR method as described by Wurch *et al.* (1998). The primers for the overlap extension PCR were designed to introduce the DNA fragment encoding the S2 linker between AxeS20E and XynCDBFV. The sequences of overlap extension PCR primers used are listed in Table 1. All of these primers were designed to place an *Nde*I site at the 5′ end and an *Xho*I site at the 3′ end of the PCR product. The PCR fragments encoding AxeS20E, XynCDBFV, Axe-S2-Xyn, and Xyn-S2-Axe were digested with *Nde*I and *Xho*I and ligated with *Nde*I-*Xho*I digested pET-29a (Novagen, Madison, WI) to generate pET-axe, pET-xyn, pET-axe-S2-xyn, and pET-xyn-S2-axe, respectively. The resultant plasmids were sequenced by an automatic sequencing service provided by Genomics BioSci & Tech Inc. (Taipei, Taiwan) in order to ensure that no errors had been introduced by PCR. The plasmids were then used to

transform *E. coli* BL21 (DE3) (Novagen) by standard techniques (Sambrook and Russell 2001). The *E. coli* transformants were selected on LB agar plates containing kanamycin (30 μg/mL) (Sigma-Aldrich Co., St. Louis, MO).

Table 1. The Sequences of Overlap Extension PCR Primers used in this Study

Primers	Sequence (5'-3')	Purpose
AxeF1	CATATGCAAGGAGCTGGTAGAGATATTC	Forward primer for amplifying *axeS20E* to construct *axe-S2-xyn*
AxeR1	CGAGCCACCGCCACCCGAGCCACCGCCACCAGAAACTGGACCATCTAC	Reverse primer for amplifying *axeS20E* to construct *axe-S2-xyn*
XynF1	GGTGGCGGTGGCTCGGGTGGCGGTGGCTCGCAAAGTTTCTGTAGTTC	Forward primer for amplifying *xynCDBFV* to construct *axe-S2-xyn*
XynR1	CTCGAGATCACCAATGTAAACC	Reverse primer for amplifying *xynCDBFV* to construct *axe-S2-xyn*
XynF2	CATATGCAAAGTTTCTGTAGTTCAG	Forward primer for amplifying *xynCDBFV* to construct *xyn-S2-axe*
XynR2	CGAGCCACCGCCACCCGAGCCACCGCCACCATCACCAATGTAAACCTTTG	Reverse primer for amplifying *xynCDBFV* to construct *xyn-S2-axe*
AxeF2	GGTGGCGGTGGCTCGGGTGGCGGTGGCTCGCAAGGAGCTGGTAGAGATATTC	Forward primer for amplifying *axeS20E* to construct *xyn-S2-axe*
AxeR2	CTCGAGAGAAACTGGACCATCTAC	Reverse primer for amplifying *axeS20E* to construct *xyn-S2-axe*
Underlined sequences are the overlap annealing sequences that encode the S2 linker (GGGGSGGGGS).		

Purification of the Recombinant Proteins

The *E. coli* BL21 transformed cells were cultured in LB broth, and cell growth was then measured turbidimetrically at 600 nm (OD_{600}). To produce the recombinant proteins, the overnight culture was prepared and subsequently seeded at a 1:100 dilution into 50 mL of fresh LB broth. The cell cultures were maintained at 37 °C, and upon reaching an OD_{600} of 0.5 they were induced with 100 μM of isopropyl-L-D-thiogalactopyranoside (IPTG; Sigma-Aldrich Co.) for protein production. After 4 hours of induction, the cells were harvested by centrifugation at 5,000 ×*g* for 20 min at 4 °C.

The cell pellet was resuspended in 10 mL of 0.1 M sodium phosphate buffer (pH 7.4), sonicated for 10 min with an ultrasonicator (Model XL, Misonix, Farmingdale, NY), and fractioned into supernatant and pellet parts by subsequent centrifugation. The recombinant proteins were present mainly in the pellet and so were treated with 8 M urea to induce protein unfolding. The proteins were then purified by immobilized metal ion-affinity chromatography using prepackaged HisTrap Ni-Sepharose columns (GE Healthcare, Piscataway, NJ). Finally, the soluble recombinant proteins were obtained by on-column refolding using HiTrap desalting columns (GE Healthcare). The purified recombinant proteins were then analyzed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to the method described by Laemmli (1970), followed by determination of the enzyme activities. The total protein concentration was measured using the Bradford protein assay (Bio-Rad Laboratories, Inc., Hercules, CA) against a standard curve of bovine serum albumin (Sigma-Aldrich Co.).

Optimum pH and Temperature for Xylanase Activity of the Chimeric Enzymes

CCD was conducted with two variables at five levels and with five replicates at the central point, which amounted to a total of 13 experiments. The results of CCD were analyzed using RSM. According to our preliminary experimental results, pH and temperature were identified as the major factors affecting the enzyme activity of the chimeric enzymes and were thus chosen as the factors in the experimental design. In the statistical model, Y_1 and Y_2 denoted the xylanase activities of Axe-S2-Xyn and Xyn-S2-Axe, respectively, and the scaled values were defined as follows: $X_1 = (pH - 5)$; $X_2 = (T - 50)/20$. The experimental design, data analyses, and regression model building were performed using Design Expert software (version 7.13, Stat-Ease Inc., Minneapolis, MN). The responses, as linear, quadratic, and cubic functions of the variables, were tested for adequacy and fitness using analysis of variance (ANOVA). Model analysis and the lack-of-fit test were used for the selection of adequacy models. A model with *P*-values ($P > F$) less than 0.05 was regarded as significant. The highest-order significant polynomial was selected. The lack-of-fit test was used to compare the residual and pure errors at replicated design points. The response predictor was discarded where lack-of-fit was significant, as indicated by a low probability value ($P > F$). The model with no significant lack-of-fit was selected (Segurola *et al.* 1999).

After the optimal conditions for enzyme activity had been predicted, a series of experiments was conducted in triplicate and repeated three times (nine tests in all per condition) in order to check the reliability of the predicted values and the experimental data. The results were analyzed using Student's t test available from the Statistical Analysis System software (SAS; version 8.1; Statistical Analysis System Institute, Cary, NC).

Xylanase Activity Assays

To determine the optimum pH and temperature for the xylanase activities of the chimeric enzymes, 5 µg of purified Axe-S2-Xyn or Xyn-S2-Axe was incubated with 0.5 % w/v birchwood xylan (Sigma-Aldrich Co.) in 100 mM sodium citrate buffer (pH 3 to 5) or sodium phosphate buffer (pH 6 to 7), amounting to a final reaction volume of 300 µL. After incubation for 20 min at the respective optimum reaction temperatures for each enzyme, the xylanase activity was determined by measurement of the amount of reducing sugars released from the birchwood xylan using the dinitrosalicyclic acid (DNS) reagent method as described by Konig *et al.* (2002). One unit of enzyme activity was defined as that which released 1 µmol of product per minute from the substrate under the assay conditions. Specific activity was expressed as U/mg protein.

Kinetic Parameters of the Chimeric Enzymes

To determine the kinetic parameters of the chimeric enzymes, birchwood xylan at a concentration ranging from 1 to 6 mg/mL was incubated with 5 µg of Axe-S2-Xyn or Xyn-S2-Axe in a final reaction volume of 300 µL. The reactions were conducted for 10 min at the respective optimal conditions for xylanase activity for the enzymes Axe-S2-Xyn and Xyn-S2-Axe. A typical Lineweaver-Burk plot was obtained by plotting $1/[v]$

against 1/[*S*] (Lineweaver and Burk 1934). The kinetic parameters (K_m and V_{max}) were estimated by linear regression from the Lineweaver-Burk plot.

Substrate Specificity of the Chimeric Enzymes

In order to compare the enzyme activities in equal numbers of molecules, XynCDBFV, Axe-S2-Xyn, Xyn-S2-Axe, and the AxeS20E-XynCDBFV mixture were incubated with 0.5 % w/v beechwood, birchwood, or oat-spelt xylans (Sigma-Aldrich Co.) with the final concentration of each enzyme 0.3 μM. All reactions were conducted at the respective pHs and temperatures found to be optimum for enzyme activity (pH 6.0 and 62.0 °C for XynCDBFV and the AxeS20E-XynCDBFV mixture; pH 5.4 and 62.9 °C for Axe-S2-Xyn; pH 5.47 and 59.3 °C for Xyn-S2-Axe). After incubation for 20 min, the xylanase activities were determined by measurement of the amount of reducing sugars released from the substrates using the DNS reagent method as described above. Specific activity was expressed in U/nmol protein.

Thermal Stability of the Chimeric Enzymes

The thermal stabilities of XynCDBFV, Axe-S2-Xyn, and Xyn-S2-Axe were determined by incubating each purified recombinant enzyme at a final concentration of each enzyme of 0.3 μM at 60, 70, and 80 °C, respectively, and at the optimum pH for enzyme activity established for each respective enzyme. Aliquots were withdrawn at intervals of 0, 30, 60, 90, and 120 min, and the residual enzyme activities were measured using the DNS reagent method as described above.

Hydrolytic Activity toward Rice Straw

Rice (*Oryza sativa* L.) straw was obtained from local farms outside of the city of Taipei. The rice straw was washed with distilled water and then cut into 1 to 2 cm pieces. The chopped rice straw was dried at 70 °C until a constant weight was obtained. The initial composition of the rice straw was determined to be 46.2% cellulose, 11.4% hemicellulose, 11.3% lignin, and 1.4% ash.

The chopped rice straw was pretreated with alkali before enzymatic hydrolysis according to the method described by Saha and Cotta (2008). In brief, the chopped rice straw (15.0% w/v) and calcium hydroxide (1.5% w/v) were slurried in water, mixed, and autoclaved at 121 °C for 1 hour. Then, the solid residue was collected via filtration and washed extensively with distilled water until a neutral pH was achieved. Subsequently, this pretreated rice straw, which was to be used as the substrate for enzymatic hydrolysis, was dried in an oven at 50 °C until it was able to maintain a constant weight. The enzymatic hydrolysis of the pretreated rice straw was performed in accordance with a modified method based on the Laboratory Analytical Procedure of the US Department of Energy's National Renewable Energy Laboratory (Selig *et al.* 2008b). XynCDBFV, Axe-S2-Xyn, Xyn-S2-Axe, and AxeS20E-XynCDBFV mixture were incubated with 5% w/v pretreated rice straw at their respective optimum pHs and temperatures (pH 6.0 and 62.0 °C for XynCDBFV and the parental enzyme mixture; pH 5.4 and 62.9 °C for Axe-S2-Xyn; pH 5.47 and 59.3 °C for Xyn-S2-Axe) in a final enzyme concentration of 0.3 μM. Aliquots were withdrawn at intervals of 0, 12, and 24 hours, and the quantity of

reducing sugars released from the pretreated rice straw was measured using the DNS reagent method as described above.

Statistical Analysis

All results were analyzed using the general linear-model procedure available from the Statistical Analysis System software package version 8.1 (SAS Institute Inc., Cary, NC). Duncan's multiple range test (Montgomery 1996) was used to detect differences between treatment means. Each assay was conducted in triplicate.

RESULTS AND DISCUSSION

Design of the Chimeric Xylanolytic Enzymes

Chimeric enzymes possessing two or more enzyme activities are more cost- and time-effective in industrial enzyme production than are mixtures of multiple single enzymes. Since XynCDBFV and AxeS20E showed good thermal stability, they could be used effectively in combination with each other to promote more efficient hydrolysis of xylan. To take advantage of this feature, we designed chimeric enzymes based on XynCDBFV and AxeS20E. Chimeric enzymes are usually created by an end-to-end fusion technique, in which the N terminus of one catalytic domain is linked to the C terminus of the other catalytic domain (Khandeparker and Numan 2008). The sequential order of catalytic domains in chimeric enzymes may affect the enzyme specific activities (An *et al.* 2005; Hong *et al.* 2006). In addition, proper linker peptides between the individual catalytic domains are able to adopt the original conformation and maintain the functionality of the individual domains (Lu and Feng 2008). Therefore, we constructed four chimeric enzymes by fusion of XynCDBFV to the N-terminus or the C-terminus of AxeS20E, with or without an S2 linker. In order to compare the structures of the parental enzymes with those of the chimeric enzymes, 3D structures of these enzymes were generated using the I-TASSER server. In the I-TASSER server, five top-ranked 3D structures of the individual enzymes were generated. The accuracy of each structure was evaluated by a benchmark scoring system that included C-score, RMSD, and TM-score (Zhang 2008). The top-ranked structure of each enzyme was the one with the highest C-score, and it is shown in Fig. 1.

The predicted structures of the chimeric enzymes were then superimposed onto those of the parental enzymes using the structural alignment program TM-align, which identified the best alignment between the parental and chimeric structures using the heuristic iteration of dynamic programming based on the TM-score rotation matrix (Zhang and Skolnick 2005). The value of the TM-score lies between 0 and 1. In general, a TM-score lower than 0.2 indicates that there is no similarity between the two structures, while a TM-score higher than 0.5 means that the structures share the same fold (Zhang and Skolnick 2005). As shown in Table 2, the TM-scores of Axe-S2-Xyn and Xyn-S2-Axe were higher than those of Axe-Xyn and Xyn-Axe, indicating that the structures of Axe-S2-Xyn and Xyn-S2-Axe were more similar to the native structures.

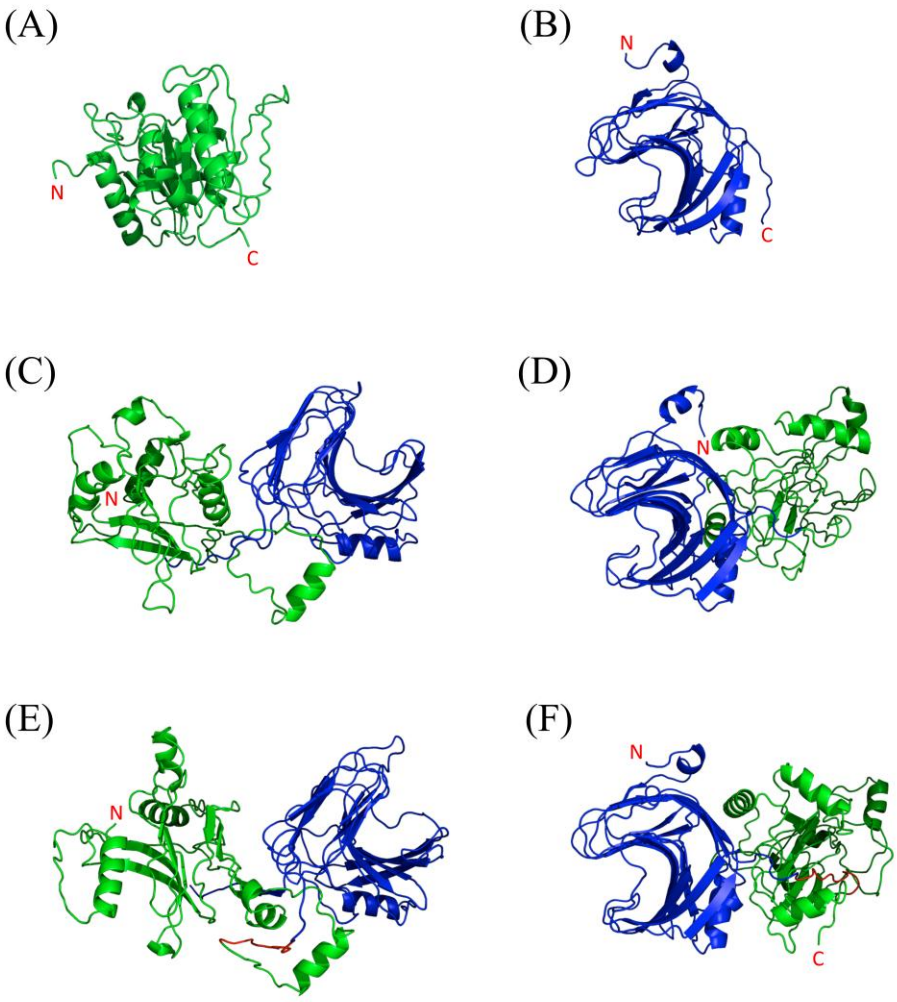

Fig. 1. The predicted structures of the chimeric enzymes. (A) AxeS20E, (B) XynCDBFV, (C) Axe-Xyn, (D) Xyn-Axe, (E) Axe-S2-Xyn, and (F) Xyn-S2-Axe. AexS20E, XynCDBFV, and S2 structures are shown in green, blue, and red, respectively.

Expression and Purification of the Chimeric Enzymes

In accordance with the results of the structural prediction, Axe-S2-Xyn and Xyn-S2-Axe were further expressed, purified, and characterized. The PCR fragments encoding AxeS20E, XynCDBFV, Axe-S2-Xyn, and Xyn-S2-Axe were each subcloned into a pET-29a expression vector. The recombinant enzymes were then expressed in the *E. coli* as His_6 fusion proteins. After induction with IPTG at 37 °C, the expressed recombinant proteins were mainly found in the insoluble fraction of the cell lysate. The recombinant proteins were subsequently dissolved into 8 M urea. After purification by affinity chromatography followed by desalting procedures, the purified recombinant proteins were obtained. As shown in Fig. 2, the purified recombinants AxeS20E, XynCDBFV, Axe-S2-Xyn, and Xyn-S2-Axe revealed bands of about 25.1, 24.5, 49.6, and 49.6 kDa, respectively.

Table 2. TM-align-generated Scores Obtained by Superimposition of the Structures of the Parental and Chimeric Enzymes

Protein	TM-score	RMSD (Å)
Axe	ND [a]	ND
Axe-Xyn	0.308	6.14
Axe-S2-Xyn	0.339	6.09
Xyn-Axe	0.335	5.83
Xyn-S2-Axe	0.345	5.71
Xyn	ND	ND
Axe-Xyn	0.844	2.51
Axe-S2-Xyn	0.847	2.46
Xyn-Axe	0.842	2.51
Xyn-S2-Axe	0.883	2.03

[a] ND: not done.

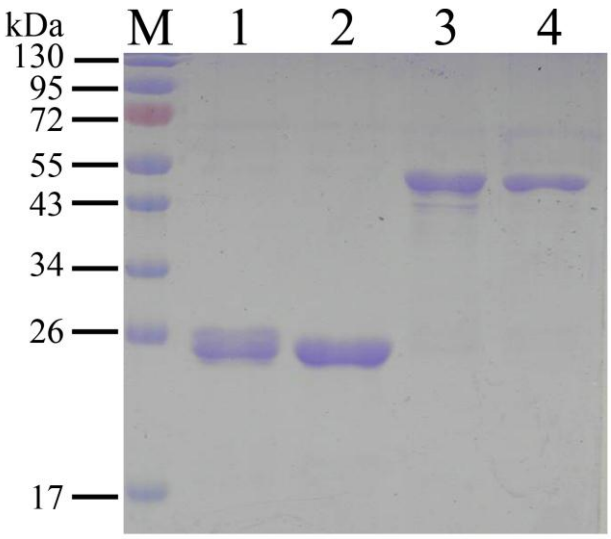

Fig. 2. SDS-PAGE analysis of purified recombinant proteins. M: molecular weight marker; 1: AxeS20E; 2: XynCDBFV; 3: Axe-S2-Xyn; 4: Xyn-S2-Axe (2 µg of protein in each lane).

Optimization of Enzyme Activity of the Chimeric Enzymes

In previous studies, the optimal reaction conditions for enzyme activities were usually investigated by the use of the conventional method, the 'one-variable-at-a-time' approach (Chen *et al.* 2001; Wu *et al.* 2006). However, it is hard to consider the combined effects of all factors involved. In this study, we combined CCD with RSM for the planned statistical optimization of the xylanase activities of the chimeric enzymes. The CCD experimental design uses statistically significant regression equation modeling to determine the effects of independent variables on the dependent variables and thus minimize the number of experimental runs (Heck *et al.* 2006). RSM is a statistical-mathematical method that evaluates the relationships between a set of controllable experimental factors and the observed results. RSM could reduce the number of experimental runs and simplify the calculation of the model parameters (Montgomery

1996). Therefore, we used RSM to analyze the CCD results in this study. According to our preliminary experimental results, temperature and pH were identified as the major factors affecting the xylanase activity of the chimeric enzymes. Table 3 shows the process variables used in the experimental design and the results for the enzyme activities. Both Axe-S2-Xyn and Xyn-S2-Axe had high levels of xylanase activity (4078.6 and 4334.5 U/mg of total protein, respectively) with treatment 7 (pH 6.0, 70 °C) but showed no xylanase activity with treatment 6 (pH 4.0, 30 °C).

Table 3. Variables Used in the CCD, Treatment Combinations, and the Mean Experimental Responses

Treatment	Coded setting levels (X_1 = pH; X_2 = T)		Actual levels (X_1 = pH; X_2 = T)		Xylanase activity[a] (U/mg of total protein)	
	X_1	X_2	X_1	X_2	Axe-S2-Xyn	Xyn-S2-Axe
1	0	1.41	5.0	78.28	2864.3	2304.8
2	0	0	5.0	50	3650.0	4114.3
3	-1	1	4.0	70	1554.7	1560.7
4	-1.41	0	3.59	50	60.7	78.6
5	0	0	5.0	50	3459.5	3209.5
6	-1	-1	4.0	30	0	0
7	1	1	6.0	70	4078.6	4334.5
8	1.41	0	6.41	50	2286.9	1917.9
9	1	-1	6.0	30	1138.1	2608.4
10	0	0	5.0	50	4048.8	3578.6
11	0	0	5.0	50	3334.5	3025.0
12	0	-1.41	5.0	21.72	846.4	1757.1
13	0	0	5.0	50	3667.9	3453.6

[a] Results represent the mean of three experiments.

Table 4 validates the reliability of the linear, quadratic, and cubic models for the responses according to their *F*-values. In the cases of Axe-S2-Xyn and Xyn-S2-Axe, the quadratic model had insignificant lack-of-fit ($P > 0.05$) and significant statistical model analysis ($P < 0.05$), indicating that the quadratic model accurately represented the experimental data. Next, the coefficient of determination was calculated to check the goodness of fit of the quadratic model. The *R*-squared values were 0.9730 and 0.8594 for Axe-S2-Xyn and Xyn-S2-Axe, respectively, indicating that only 2.70% and 14.06%, respectively, of the total variation could not be explained by the model. These results confirmed that the quadratic model was accurate and reliable, and that analysis of the associated response trends was reasonable.

The quadratic models generated by the design were:

$$Y_1 = -33479.17 + 11831.18*\text{pH} + 170.27*T - 1184.60*\text{pH}^2 - 2.11*T^2 + 17.32*\text{pH}*T$$

$$Y_2 = -32980.70 + 11756.05*\text{pH} + 157.50*T - 1086.15*\text{pH}^2 - 1.42*T^2 + 2.07*\text{pH}*T$$

where Y_1 and Y_2 are the predicted responses for the xylanase activity of Axe-S2-Xyn and Xyn-S2-Axe, respectively, and pH and T are the actual values for pH and temperature.

Table 4. Model Analysis (a), Lack of Fit Tests (b), and *R*-squared Analysis (c) for Measured Responses

Source of variation	Axe-S2-Xyn		Xyn-S2-Axe	
	Sum of squares	*P* > *F*	Sum of squares	*P* > *F*
(a) Model analysis[a]				
Mean	73,880,000		78,490,000	
Linear	12,550,000	0.0431*	10,030,000	0.0545
Quadratic	13,120,000	<0.0001**	9,504,000	0.0080**
Cubic	369,800	0.1692	1,756,000	0.1363
Residual	357,200		1,440,000	
Total	100,800,000		101,200,000	
(b) Lack of fit[b]				
Linear	14,040,000	0.0024**	12,010,000	0.0166*
Quadratic	433,300	0.2609	2,503,000	0.0815
Cubic	63,563	0.4047	747,400	0.1064
Pure error	293,600		692,900	
(c) *R*-square analysis[c]	*R*-square	PRESS	*R*-square	PRESS
Linear	0.4668	23,280,000	0.4411	21,960,000
Quadratic	0.9730	3,540,000	0.8594	18,880,000
Cubic	0.9867	4,527,000	0.9367	48,910,000

* Statistically significant at 95% of confidence level.
** Statistically significant at 99% of confidence level.
[a] Model analysis: select the highest order polynomial where the additional terms are significant.
[b] Lack of fit: want the selected model to have insignificant lack of fit.
[c] *R*-squared analysis: focus on the model minimizing the "PRESS".

Figure 3 depicts the RSMs for xylanase activity as functions of the pH and temperature of the chimeric enzymes. The results indicated that the optimal conditions for the xylanase activity of Axe-S2-Xyn occurred at pH 5.4 and 62.9 °C (Fig. 3A), while those for Xyn-S2-Axe occurred at pH 5.47 and 59.3 °C (Fig. 3B). To confirm the accuracy of the model, the xylanase activities of Axe-S2-Xyn and Xyn-S2-Axe at the suggested optimum conditions were determined. At their respective optimum conditions, the model predicted that Axe-S2-Xyn would have a xylanase activity of 4114.61 U/mg (range, 3769.47 to 4458.85 U/mg) and that Xyn-S2-Axe would have a xylanase activity of 3827.2 U/mg (range, 3122.53 to 4531.86 U/mg) at a confidence level of 95%. The experimental xylanase activities of Axe-S2-Xyn and Xyn-S2-Axe were 4288.29 ± 71.33 and 4370.64 ± 47.09 U/mg, respectively, confirming the accuracy of the models. Under the optimal conditions, the K_m and V_{max} of Axe-S2-Xyn were 15.14 ± 1.77 mg/mL and 62.13 ± 5.09 mM/min, respectively, whereas those of Xyn-S2-Axe were 9.22 ± 3.27 mg/mL and 23.23 ± 3.96 mM/min, respectively.

Most commercial xylanases are produced by filamentous fungi such as *Aspergillus* and *Trichoderma*. The specific activities of the commercial xylanase XynIV from *Trichoderma reesei* and xylanase XynII from *Aspergillus aculeatus* were 283 and 1020 U/mg (Berlin *et al*. 2006; Kofod *et al*. 2001), respectively, which were lower than those of the chimeric enzymes Axe-S2-Xyn and Xyn-S2-Axe. Thus we suggested that these chimeric enzymes have potential for used in industrial applications. The general utility of xylanases in industry has spurred considerable research into the production of more thermally stable and alkaline-tolerant variants.

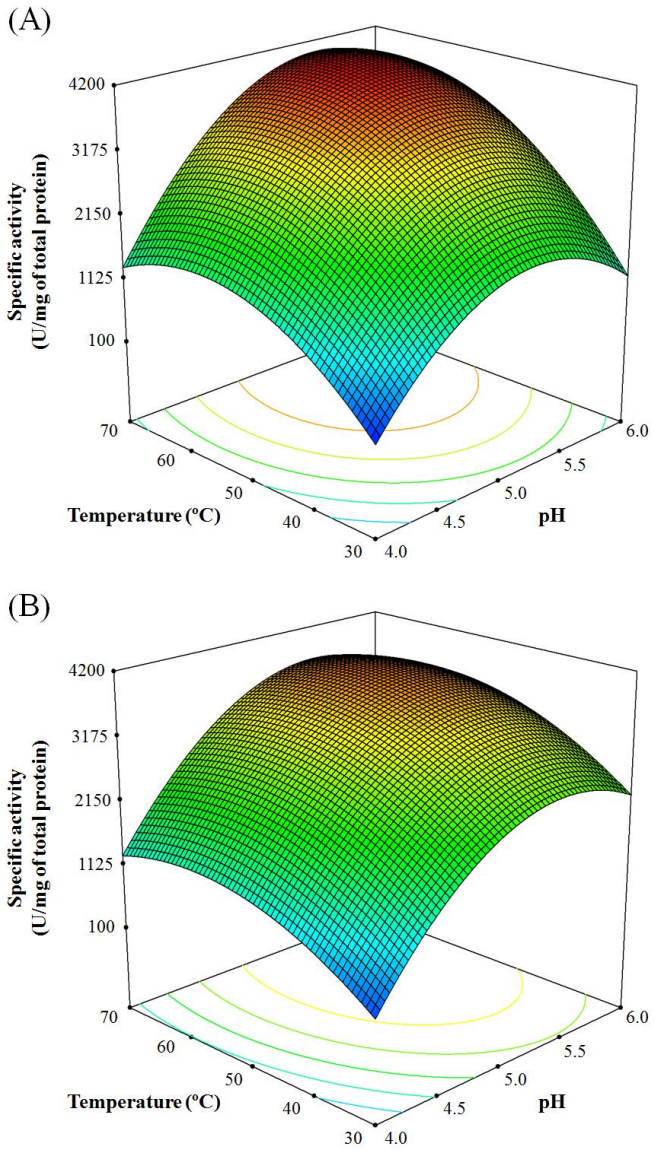

Fig. 3. Response surface plot of the effects of pH and temperature on the xylanase activities of Axe-S2-Xyn (A) and Xyn-S2-Axe (B)

The parental xylanase, XynCDBFV, used in this study is a thermally stable mutant of the catalytic domain of *N. patriciarum* xylanase XynC. It was previously determined that the optimum pH and temperature for XynCDBFV were pH 6.0 and 62 °C (Chen *et al.* 2001). In this study, the maximum activity of Axe-S2-Xyn was found at pH 5.4 and 62.9 °C, whereas that of Xyn-S2-Axe was found at pH 5.47 and 59.3 °C. Therefore, the optimum pH for the xylanase activity of the chimeric enzymes had decreased slightly. Instances of chimeric enzymes having shifted pH optima and activity profiles exist; for example, a xylanase/arabinofuranosidase chimeric enzyme shifted its optimum pH for xylanase activity from pH 8 to pH 6 (Fang *et al.* 2009). This shift in

optimum pH was suggested to be attributable to either the change in the microenvironmental pH generated by the chimera or to alteration of the tertiary structure (Bulow 1987).

Specific Activities of the Parental and Chimeric Enzymes on Natural Xylans

The possibility of synergy between AxeS20E and XynCDBFV was studied using natural xylans such as beechwood xylan, birchwood xylan, and oat spelt xylan as substrates. The results showed that the xylanase activity of the parental enzyme mixture (a combination of equal numbers of molecules of AxeS20E and XynCDBFV) was significantly higher than that of XynCDBFV (Table 5), indicating that AxeS20E acted in synergy with XynCDBFV to facilitate the hydrolysis of xylan. This could be attributed to a release of acetic acid from xylan by AxeS20E, which enhanced the accessibility of XynCDBFV to the xylan backbone and the subsequent hydrolysis of xylan. Both chimeric enzymes Axe-S2-Xyn and Xyn-S2-Axe were also found to have greater xylanase activities, compared to the parental enzyme XynCDBFV, toward natural xylans (Table 5). It is noteworthy that the xylanase activities of Xyn-S2-Axe on beechwood and birchwood xylans were significantly greater than the activities of XynCDBFV, the parental enzyme mixture, and Axe-S2-Xyn.

As the complete hydrolysis of xylan requires a large variety of cooperative enzymes, engineering a bi- or multi-functional xylanolytic enzyme has the potential to facilitate successively catalytic reactions via the positive proximity effects between the two neighboring catalytic sites (Mesta *et al.* 2001). In addition, a chimeric enzyme possessing two or more enzyme activities has the potential to be more cost-effective in industrial enzyme production than do mixtures of multiple single enzymes (Fan *et al.* 2009). In this study, it was found that the xylanase activities of Xyn-S2-Axe on beechwood and birchwood xylans were significantly greater than the activities of XynCDBFV or the parental enzyme mixture. Therefore, it is suggested that Xyn-S2-Axe has the potential to be used in a range of applications due to its efficient synergy in the degradation of xylan and its potential to save production costs.

Table 5. The Specific Activities of XynCDBFV, Axe-S2-Xyn, Xyn-S2-Axe, and the AxeS20E-XynCDBFV Mixture towards Natural Xylans

Substrate	Xylanase activity (U/nmol protein)			
	XynCDBFV	Axe-S2-Xyn	Xyn-S2-Axe	XynCDBFV+AxeS20E
Beechwood xylan	136.3 ± 6.0^{c}	246.1 ± 1.0^{b}	261.9 ± 1.8^{a}	247.0 ± 6.4^{b}
Birchwood xylan	157.1 ± 9.1^{c}	231.3 ± 2.0^{b}	263.5 ± 11.4^{a}	225.9 ± 12.7^{b}
Oat spelt xylan	165.0 ± 2.6^{c}	223.3 ± 2.4^{b}	219.1 ± 3.6^{b}	316.1 ± 12.1^{a}
$^{a\text{-}c}$ Means in a row with different superscripts are significantly different ($p < 0.05$).				

Fusion protein techniques are commonly used for purifying recombinant proteins, monitoring protein expression, displaying proteins on the cell surface, biological screening, targeting proteins, and so on (Hong *et al.* 2006). Fusion proteins are usually created by an end-to-end fusion technique, in which the N terminus of one domain is linked to the C terminus of the other domain (Khandeparker and Numan 2008). Several

studies have indicated that selection of proper linkers with a certain degree of flexibility and hydrophilicity is very important (Shan *et al.* 1999; Xue *et al.* 2004; Lu and Feng 2008). Only when proper linker peptides were inserted between the individual domains were the fusion proteins able to adopt the original conformation and maintain the functionality of the individual domains (Lu and Feng 2008). In this study, we introduced the S2 linker between the AxeS20E and XynCDBFV domains in the chimeric enzymes. Both chimeric enzymes Axe-S2-Xyn and Xyn-S2-Axe showed higher xylanase activity than the parental enzyme, indicating that both the CE and GH domains in the chimeric enzymes were able to adopt their original conformation and maintain the functionality of the individual domains, therey displaying synergy in the degradation of xylan.

Thermal Stability of the Chimeric Enzymes

Xylanolytic enzymes are used in a range of industrial processes. Most of these processes are carried out at high temperatures, so thermally stable xylanolytic enzymes are of great interest (Collins *et al.* 2005). In terms of the thermal stability of XynCDBFV, more than 80% of the original activity was retained after 120 min of heating at 60 or 70 °C (Figures 4A and 4B). The thermal stability profiles of Xyn-S2-Axe were similar to those of XynCDBFV. However, the thermal stability profile of Axe-S2-Xyn was lower than that of XynCDBFV and Xyn-S2-Axe. Only 64% of the original activity of Axe-S2-Xyn was retained after 120 min of heating at 60 or 70 °C. When incubated at 80 °C for 120 min, XynCDBFV retained more than 50% of its original activity, whereas Xyn-S2-Axe retained about 36% of its original activity. Axe-S2-Xyn retained only 11% of its original activity (Fig. 4C). These results demonstrated that the sequential order of catalytic domains in the chimeric enzymes could affect the enzyme′s thermal stability.

Hydrolytic Activity toward Rice Straw

The hydrolytic activities of the chimeric enzymes toward lignocelluloses were evaluated using rice straw as the substrate. After incubation for 12 hours, the parental enzyme and the chimeric enzymes had released similar amounts of reducing sugars from the rice straw, which was significantly less than that released by the parental enzyme mixture (Fig. 5). After incubation for 24 hours, Xyn-S2-Axe had released a significantly greater quantity of reducing sugar from rice straw than had XynCDBFV and Axe-S2-Xyn, but these amounts did not differ significantly from those of the parental enzyme mixture.

Rice straw is one of the most abundant lignocellulosic agricultural residues in the world (Zhang and Cai 2008). It consists of cellulose (46.2% on a dry basis) and hemicelluloses (11.4% on a dry basis) in close association with lignin (11.3% on a dry basis). Rice straw is considered a waste material because of its low value as an animal feed due to low digestibility and high ash contents. Nonetheless, rice straw can be hydrolyzed by enzymes, converted to reducing sugars, and subsequently fermented by suitable microorganisms to produce other target products (Zhang and Cai 2008). In this study, Xyn-S2-Axe exhibited highly xylanolytic activity. Therefore, it is reasonable to believe that Xyn-S2-Axe could be used synergistically with other cellulolytic enzymes to promote the more efficient hydrolysis of rice straw.

Xyn-S2-Axe had both greater thermal stability and greater xylanolytic activity on the rice straw than did Axe-S2-Xyn (Figs. 4 and 5).

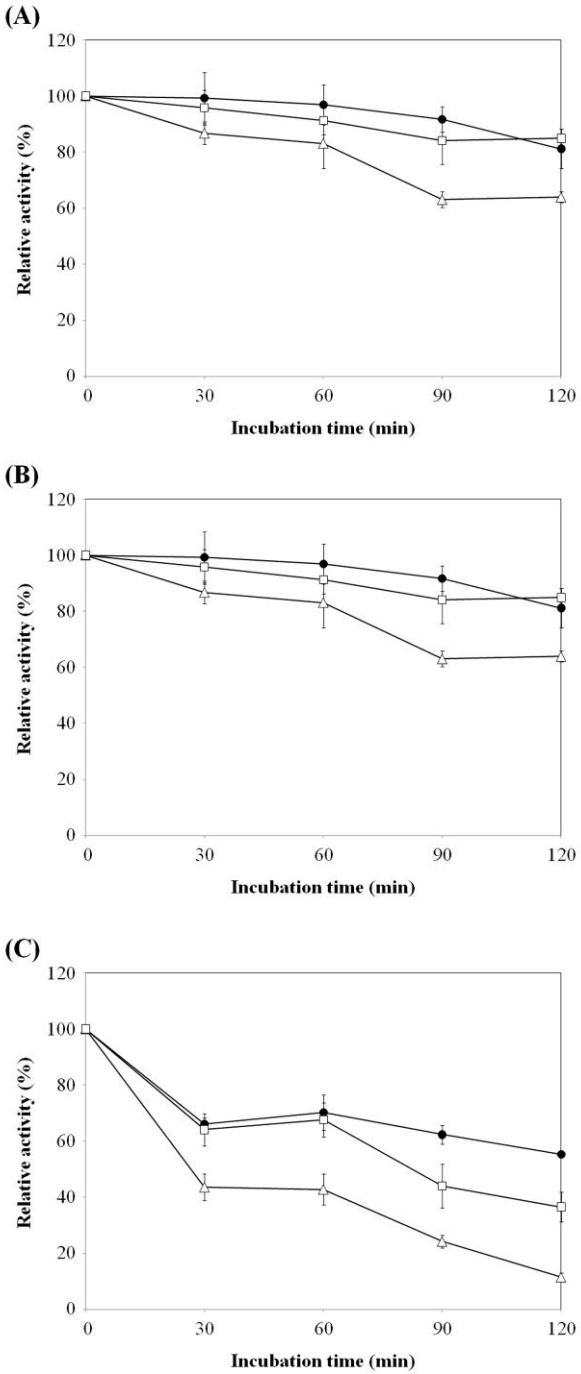

Fig. 4. Thermal stability of XynCDBFV (●), Axe-S2-Xyn (△), and Xyn-S2-Axe (□) incubated at 60 (A), 70 (B), and 80°C (C) for 120 min at the respective optimum pHs for enzyme activity. The bars represent standard errors of the means of triplicate measurements.

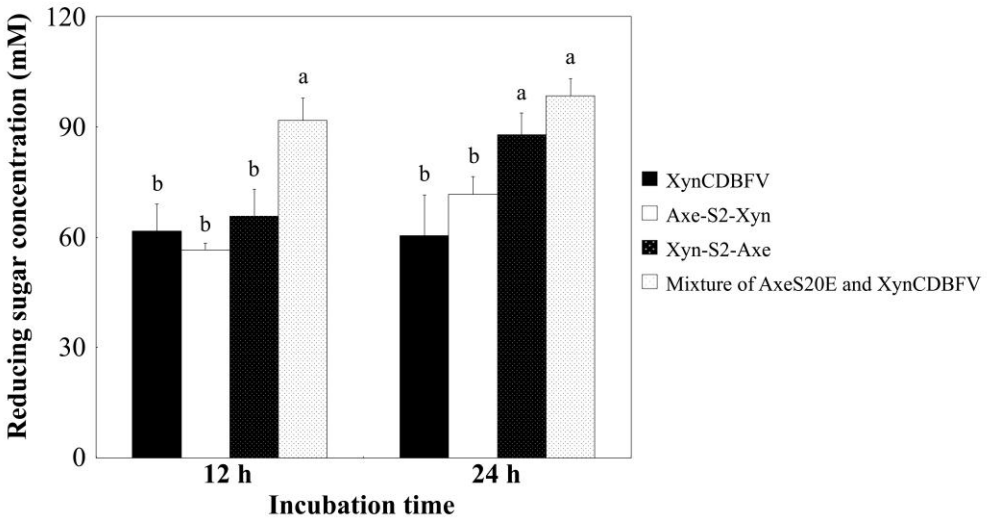

Fig. 5. Amounts of reducing sugars released from the alkaline-pretreated rice straw by XynCDBFV, Axe-S2-Xyn, Xyn-S2-Axe, and the AxeS20E-XynCDBFV mixture. The bars represent standard errors of the means of triplicate measurements. Locations marked by different letter are significantly different ($p < 0.05$).

The mechanism behind this may be that the sequential order of the catalytic domains affected the protein folding and led to the change in enzyme properties. An *et al.* (2005) constructed chimeric enzymes containing two different catalytic domains: XynX and Cel5Z. They found that the chimeric enzyme exhibited both cellulase and xylanase activity when Cel5Z was fused downstream of XynX, but no activities were observed when Cel5Z was fused upstream of XynX. A similar result was reported by Hong *et al.* (2006). However, the chimeric enzymes constructed in these two previous studies did not contain linker sequences between the catalytic domains. Therefore, it is suggested that the proper sequential order of catalytic domains and the proper length and composition of the linker are important for providing proper intra-peptide chain interactions in chimeric enzymes.

CONCLUSIONS

1. The chimeric enzyme Xyn-S2-Axe was found to have greater xylanase activities on beechwood xylan and birchwood xylan than did the parental enzyme or the parental enzyme mixture. In addition, Xyn-S2-Axe had a thermal stability profile and xylanolytic activity on rice straw similar to those of the parental enzyme mixture.
2. Since chimeric enzymes are more cost- and time-effective in industrial enzyme production than are mixtures of multiple single enzymes, Xyn-S2-Axe could be a good candidate for various biotechnological applications requiring thermostable xylanase activity.

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REFERENCES CITED

An, J. M., Kim, Y. K., Lim, W. J., Hong, S. Y., An, C. L., Shin, E. C., Cho, K. M., Choi, B. R., Kang, J. M., Lee, S. M., Kim, H., and Yun, H. D. (2005). "Evaluation of a novel bifunctional xylanase-cellulase constructed by gene fusion," *Enzyme Microb. Technol.* 36, 989-995.

Berlin, A., Maximenko, V., Gilkes, N., and Saddler, J. (2006). "Optimization of enzyme complexes for lignocellulose hydrolysis," *Biotechnol. Bioeng* 97, 287-296.

Bulow, L. (1987). "Characterization of an artificial bifunctional enzyme, betagalactosidase/galactokinase, prepared by gene fusion," *Eur. J. Biochem.* 163, 443-448.

Chen, Y. L., Tang, T. Y., and Cheng, K. J. (2001). "Directed evolution to produce an alkalophilic variant from a *Neocallimastix patriciarum* xylanase," *Can. J. Microbiol.* 47, 1088-1094.

Collins, T., Gerday, C., and Feller, G. (2005). "Xylanases, xylanase families and extremophilic xylanases," *FEMS Microbiol. Rev.* 29, 3-23.

Dupont, C., Daigneault, N., Shareck, F., Morosoli, R., and Kluepfel, D. (1996). "Purification and characterization of an acetyl xylan esterase produced by *Streptomyces lividans*," *Biochem. J.* 319, 881-886.

Fang, Z., Werkman, J. R., and Yuan, L. (2009). "Engineering of a multifunctional hemicellulase," *Biotechnol. Lett.* 31, 751-757.

Heck, J. X., Flores, S. H., Hertz, P. F., and Ayub, M. A. Z. (2006). "Statistical optimization of thermo-tolerant xylanase activity from Amazon isolated *Bacillus circulans* on solid-state cultivation," *Biores. Technol.* 97, 1902-1906.

Hong, S. Y., Lee, J. S., Cho, K. M., Math, R. K., Kim, Y. H., Hong, S. J., Cho, Y. U., Kim, H., and Yun, H. D. (2006). "Assembling a novel bifunctional cellulose-xylanase from *Thermotoga maritime* by end-to-end fusion," *Biotechnol. Lett.* 28, 1857-1862.

Khandeparker, R., and Numan, M. T. (2008). "Bifunctional xylanases and their potential use in biotechnology," *J. Ind. Microbiol. Biotechnol.* 35, 635-644.

Kofod, L. V., Kauppinen, M. S., Christgau, S., Heldt-Hansen, H. P., Dalboge, H., Andersen, L. N., Si, J. Q., Jacobsen, T. S., Munk, N., and Mullertz, A. (2001). "Enzymes with xylanases activity from *Aspergillus aculeatus*," US patent 6,228,630.

Konig, J., Grasser, R., Pikor, H., and Vogel, K. (2002). "Determination of xylanase, β-glucanase, and cellulase activity," *Anal. Bioanal. Chem.* 374, 80-87.

Laemmli, U. K. (1970). "Cleavage of structural proteins during the assembly of the head of bacteriophage T4," *Nature* 227, 680-685.

Lineweaver, H., and Burk, D. (1934). "The determination of enzyme dissociation constants," *J. Am. Chem. Soc.* 57, 685.

Liu, J. R., Yu, B., Liu, F. H., Cheng, K. J., and Zhao, X. (2005). "Expression of rumen microbial fibrolytic enzyme genes in probiotic *Lactobacillus reuteri*," *Appl. Environ. Microbiol.* 71, 6769-6775.

Lu, P., and Feng, M. G. (2008). "Bifunctional enhancement of a β-glucanase-xylanase fusion enzyme by optimization of peptide linkers," *Appl. Microbiol. Biotechnol.* 79, 579-587.

Mesta, L., Rascle, C., Durand, R., and Fevre, M. (2001). "Construction of a chimeric xylanase using multidomain enzymes from *Neocallimastix frontalis*," *Enzyme Microb. Technol.* 29, 456-463.

Montgomery, D. C. (1996). *Design and Analysis of Experiments*, John Wiley and Sons, New York, NY.

Nahar, N., Rahman, A., Mos, M., Warzecha, T., Algerin, M., Ghosh, S., Johnson-Brousseau, S., and Mandal, A. (2012). "*In silico* and *in vivo* studies of an *Arabidopsis thaliana* gene, *ACR2*, putatively involved in arsenic accumulation in plants," *J. Mol. Model.* DOI 10.1007/s00894-012-1419-y.

Pai, C. K., Wu, Z. Y., Chen, M. J., Zeng, Y. F., Chen, J. W., Duan, C. H., Li, M. L., and Liu, J. R. (2010a). "Molecular cloning and characterization of a bifunctional xylanolytic enzyme from *Neocallimastix patriciarum*," *Appl. Microbiol. Biotechnol.* 85, 1451-1462.

Pai, C. K., Zeng, Y. F., Yueh, P. Y., Chen, M. J., Tung, L. C., and Liu, J. R. (2010b). "Prediction of optimum reaction conditions for the thermo-tolerant acetylxylan esterase from *Neocallimastix patriciarum* using the response surface methodology," *J. Chem. Technol. Biotechnol.* 85, 628-633.

Saha, B. C., and Cotta, M. A. (2008). "Lime pretreatment, enzymatic saccharification and fermentation of rice hulls to ethanol," *Biomass Bioenergy* 32, 971-977.

Sambrook, J., and Russell, D. W. (2001). *Molecular Cloning: A Laboratory Manual*, 3rd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Shan, D., Press, O. W., Tsu, T. T., Hayden, M. S., and Ledbetter, J. A. (1999). "Characterization of scFv-Ig constructs generated from the Anti-CD20 mAb 1F5 using linker peptides of varying lengths," *J. Immunol.* 162, 6589-6595.

Segurola, J., Allen, N. S., Edge, M., and McMahon, A. (1999). "Design of eutectic photoinitiator blends for UV/visible curable acrylated printing inks and coatings," *Prog. Org. Coat.* 37, 23-37.

Selig, M. J., Knoshaug, E. P., Adney, W. S., Himmel, M. E., and Decker, S. R. (2008a). "Synergistic enhancement of cellobiohydrolase performance on pretreated corn stover by addition of xylanase and esterase activities," *Bioresour. Technol.* 99, 4997-5005.

Selig, M., Weiss, N., and Ji, Y. (2008b). *Enzymatic saccharification of lignocellulosic biomass*, The US Department of Energy's National Renewable Energy Laboratory, Golden, Colorado.

Subramaniyan, S., and Prema, P. (2002). "Biotechnology of microbial xylanases: Enzymology, molecular biology, and application," *Crit. Rev. Biotechnol.* 22, 33-64.

Wu, S., Liu, B., and Zhang, X. (2006). "Characterization of a recombinant thermostable xylanase from deep-sea thermophilic *Geobacillus* sp. MT-1 in East Pacific," *Appl. Microbiol. Biotechnol*. 72, 1210-1216.

Wurch, T., Lestienne, F., and Pauwels, P. J. (1998). "A modified overlap extension PCR method to create chimeric genes in the absence of restriction enzymes," *Biotech. Tech*. 12, 653-657.

Xue, F., Gu, Z., and Feng, J. (2004). "LINKER: A web server to generate peptide sequences with extended conformation," *Nucleic Acids Res*. 32, 562-565.

Zhang, Y., and Skolnick, J. (2005). "TM-align: A protein structure alignment algorithm based on the TM-score," *Nucleic Acids Res.* 33, 2302-2309.

Zhang, Y. (2008). "I-TASSEr server for protein 3D structure prediction," *BMC Bioinformatics* 9, 40.

Zhang, Q. Z., and Cai, W. M. (2008). "Enzymatic hydrolysis of alkali-pretreated rice straw by *Trichoderma reesei* ZM4-F3," *Biomass Bioenergy* 32, 1130-1135.

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