Prediction of optimum reaction conditions for the thermo-tolerant acetylxylan esterase from *Neocallimastix patriciarum* using the response surface methodology

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Abstract

BACKGROUND: Xylan is the second most abundant renewable polysaccharide in nature and also represents an important industrial substrate. The complete degradation of xylan requires the combination of several types of xylanolytic enzymes, including endo-β-1,4-xylanases, β-xylosidases, and acetylxylan esterases. As a biocatalyst, xylanolytic enzymes with good thermal stability are of great interest, therefore, a thermo-tolerant acetylxylan esterase, AxeS20E, was investigated.

RESULTS: The cDNA encoding the carbohydrate esterase (CE) domain of AxeS20E from *Neocallimastix patriciarum* was expressed in *Escherichia coli* as a recombinant His6 fusion protein. The recombinant AxeS20E protein was obtained after purification by immobilized metal ion-affinity chromatography. Response surface modeling (RSM) combined with central composite design (CCD) and regression analysis were then employed for the planned statistical optimization of the acetylxylan esterase activities of AxeS20E. The optimal conditions for the highest activity of AxeS20E were observed at 54.6 °C and pH 7.8. Furthermore, AxeS20E retained more than 85% of its initial activity after 120 min of heating at 80 °C.

CONCLUSIONS: The results suggested that RSM combined with CCD and regression analysis were effective in determining optimized temperature and pH conditions for the enzyme activity of AxeS20E. The results also proved AxeS20E was thermo-tolerant and might be a good candidate for various biotechnological applications.

Keywords: thermo-tolerant; acetylxylan esterase; response surface methodology; optimization

INTRODUCTION

The plant cell wall represents the most abundant reservoir of organic carbon in the biosphere and also represents an important industrial substrate. Xylan, the second most abundant renewable polysaccharide in nature, is one of the building blocks of the plant cell wall and constitutes the major component of hemicelluloses. Xylan is a heteropolysaccharide containing substituent groups of acetyl, 4-O-methyl-D-glucuronyl, and α-arabinofuranosyl residues linked to the backbone of β-1,4-xylopyranosyl units and has binding properties mediated by covalent and noncovalent interactions with cellulose, lignin, and other polymers. As xylan varies in structure between different plant species, the complete degradation of xylan requires not only endo-β-1,4-xylanases and β-xylosidases but also some other accessory enzymes such as α-L-arabinofuranosidases, α-glucuronidases, acetylxylan esterases, ferulic acid esterases, and p-coumaric acid esterases to remove the side chain substituents from the xylan backbone.

Among the accessory enzymes, acetylxylan esterase hydrolyzes specifically the ester linkages of the acetyl groups in positions 2 and/or 3 of the xylose moieties of natural xylan and plays a role in enhancing the accessibility of xylanase to the xylan backbone and subsequent hydrolysis of xylan. Therefore, several studies have demonstrated that xylanase and acetylxylan esterase synergistically enhanced xylan degradation.

Several genes encoding for acetylxylan esterases have been isolated from bacteria, such as *Clostridium cellulovorans*, *Fibrobacter succinogenes*, *Pseudobutyribrio xylanivorans*, *Ruminococcus flavefaciens*, and *Streptomyces lividans*, as well as fungi, such as *Aspergillus niger*, *Penicillium purpurogenum*, *Schizopyllum commune*, and *Trichoderma reesei*. According to the CAZy database (http://www.cazy.org/), carbohydrate esterases...
are classified into 16 families based on their sequence similarities; acetylxylan esterases are found in families 1–7, and 12. To date, only a few studies have focused on the rumen fungal carbohydrate esterases.7,18,19 These carbohydrate esterases belong to families 1, 2, 3, and 6, and most of them have been shown to act synergistically with xylanase.7,18,19

Xylanolytic enzymes are used in a range of industrial processes, such as biobleaching in the paper and pulp industry, bioconversion of lignocellulosic material and agro-wastes to fermentative products, clarification of juices, and improvement of the digestibility of animal feed stocks.3 To satisfy the industrial need, xylanolytic enzymes with better thermal stability are of great interest because they could be used in applications where a cooling step would be uneconomical or where high temperatures are required to increase the bioavailability and/or solubility of substrates, to reduce viscosity and/or to reduce the risk of contamination.2,20 Therefore, there has been considerable interest in the basic properties and industrial applications of thermally stable xylanolytic enzymes from thermosto philes and mesophiles. A number of thermal stable xylanases have been isolated from bacterial resources, such as Bacillus firmus,21 Bacillus thermop hilus,22 Thermotoga sp.,23 Thermoascus aurantius,24 and Clostridium thermocellum.24 Less frequent, thermally stable acetylxylan esterases have also been isolated from Streptomyces lividans8 and Thermobifida fusca.6 In a previous study, a cDNA encoding a bifunctional acetylxylan esterase/xylanase, XynS20E, was cloned from the ruminal fungus Neocallimastix patriciarum.25 Translation of the open reading frame of xynS20E revealed a protein of 671 amino acids with a predicted molecular weight of 72.4 kDa. A putative conserved domain of carbohydrate esterase (CE) family 1 was observed at the N-terminus and a putative conserved domain of glycosyl hydrolase (GH) family 11 was detected at the C-terminus of XynS20E. Interestingly, the optimal conditions for the highest xylanase activity of the recombinant XynS20E were observed at a temperature of 49 °C and a pH of 5.8 while those for the highest carbohydrate esterase activity were observed at a temperature of 58 °C and a pH of 8.2. Under the optimal conditions for the enzyme activity, the xylanase and acetylxylan esterase specific activities of the recombinant XynS20E toward birchwood xylan were 128.7 and 167.4 U mg⁻¹, respectively. In addition, the acetylxylan esterase activity exhibited greater thermal stability than the xylanase activity of XynS20E.25

In this study, the cDNA encoding the carbohydrate esterase domain of XynS20E was expressed in Escherichia coli as a recombinant His₆ fusion protein (AxeS20E). A purified AxeS20E protein was obtained after purification by immobilized metal ion-affinity chromatography. Response surface modeling (RSM), combined with central composite design (CCD), and regression analysis were then employed for the planned statistical optimization of the acetylxylan esterase activities of AxeS20E. The thermostability of AxeS20E was also studied.

MATERIALS AND METHODS

Expression and purification of the recombinant AxeS20E

The uninterrupted cDNA sequence encoding acetylxylan esterase AxeS20E, the CE family 1 catalytic domain of N. patriciarum bifunctional acetylxylan esterase/xylanase XynS20E (GenBank accession number FJ529209), was subcloned into the E. coli expression vector pET-29a (Novagen, Madison, WI) to generate pET-xynS20E-CE1.25 The resultant plasmids were used to transform E. coli BL21 (DE3) (Novagen) using standard techniques.26 Transformants were selected on Luria-Bertani (LB) agar plates containing kanamycin (30 μg mL⁻¹) (Sigma Chemical Co., St Louis, MO). To produce the recombinant protein, E. coli BL21 transformant cells were cultured in LB broth and cell growth was then measured turbidimetrically at 600 nm (OD₆₀₀). The overnight culture was prepared and subsequently seeded at a 1 : 100 dilution into 5 mL of fresh LB broth. The cell cultures were maintained at 37 °C and induced with 100 μmol L⁻¹ of isopropyl-L-D-thiogalactopyranoside (IPTG; Sigma) for protein production reaching an OD₆₀₀ of 0.5. After 4 h of induction, the cells were harvested by centrifugation at 5000g for 20 min at 4 °C.

The cell pellet was resuspended in 1 mL of 0.1 mol L⁻¹ sodium phosphate buffer (pH 7.4), sonicated for 10 min with an ultrasonicator (Model XL, Misonix, Farmingdale, NY), and fractioned into supernatant and pellet fractions by subsequent centrifugation. The recombinant proteins were present mainly in the pellet and so were treated with 8 mol L⁻¹ urea to induce protein unfolding. The proteins were then purified by immobilized metal ion-affinity chromatography using a prepacked HiTrap Ni-Sepharose column (GE Healthcare, Piscataway, NJ). Finally, the soluble recombinant proteins were obtained by on-column refolding using a Hitrap desalting column (GE Healthcare). The purified AxeS20E was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)27 and then the enzyme activities were determined. 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).

Optimum pH and temperature of enzyme activity of the recombinant AxeS20E

RSM, its main effects and the interactions between the different factors at each level, were simultaneously investigated. CCD with two variables at five levels and five replicates at the central point, for a total of 13 experiments were conducted. According to preliminary experimental results, pH and temperature were identified as the major factors affecting the enzyme activity of the recombinant AxeS20E and were chosen as factors in the experimental design. In the statistical model, Y denotes units of acetylxylan esterase activity and the scaled values were defined as follows: $X_1 = (pH - 8.5)/1.06$ and $X_2 = (T - 50)/7.07$. The experimental index number, scaled, and real values are shown in Table 1. The experimental design, data analysis 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 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 the 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. Predicted residual sum of the squares (PRESS) was used as a measure of the fit of the model to the points in the design. The smaller the PRESS statistic is, the better the model fits the data.28

After the optimal conditions for enzyme activity were predicted, a series of experiments were repeated three times in order to check the reliability of the predicted values and experimental data. The results were analyzed using Student’s t test, available in Statistical
Optimization of acetylxylan esterase activity by using RSM www.soci.org

Analysis System software (SAS; version 8.1; Statistical Analysis System Institute, Cary, NC).

Acetyl esterase activity assays

Acetyl esterase activity was determined by measuring the amount of 4-methylumbelliferone released from 4-methylumbelliferyl acetate as described by Shao and Wiegel 1995.29 To determine the optimum pH and temperature of AxeS20E activity, 100 µL of 200 mmol L\(^{-1}\) sodium phosphate buffer (pH 7 to 8.5) or glycine sodium hydroxide buffer (pH 9 to 10) was mixed with 280 µL of distilled water. Following this, 10 µL of enzyme solution (1.25 µg mL\(^{-1}\)) was added to the buffer, and the reaction was initiated within 1 min by adding 10 µL of 100 mmol L\(^{-1}\) 4-methylumbelliferyl acetate in dimethyl sulfoxide. After incubation for 10 min at the respective reaction temperature, the reaction was stopped by adding 600 µL of 50 mmol L\(^{-1}\) citric acid, and the absorbance of the solution was measured at a wavelength of 354 nm. One unit of enzyme activity was defined as that releasing 1 µmol of product per minute from the substrate under the assay conditions. Specific activity was expressed as U mg\(^{-1}\) protein.

Kinetic parameters of the recombinant AxeS20E

To determine the kinetic parameters of AxeS20E, 4-methylumbelliferyl acetate at concentrations ranging from 0.25 to 4 mmol L\(^{-1}\) was incubated with 0.5 µg of the recombinant AxeS20E in a final reaction volume of 400 µL. Reactions were conducted at the optimal conditions for the highest acetyl esterase activity of recombinant AxeS20E for 10 min. A typical Lineweaver–Burk plot was obtained when 1 /[\(v\)] was plotted against 1 /[\(S\)]. Kinetic parameters (\(K_m\) and \(V_{max}\)) were estimated by linear regression from the Lineweaver–Burk plot.

Thermal stability of AxeS20E

The thermal stability of AxeS20E was determined by incubating 0.5 µg of the purified recombinant AxeS20E in 50 mmol L\(^{-1}\) sodium phosphate buffer (pH 7.8) containing 2.5 mmol L\(^{-1}\) 4-methylumbelliferyl acetate (Sigma) at 60, 70 and 80 °C. Aliquots were withdrawn at intervals of 0, 30, 60, 90, and 120 min, and the residual enzyme activity was measured.

RESULTS AND DISCUSSION

Expression and purification of AxeS20E

Among the xylanolytic enzymes, thermally stable xylanases have potential application in many industries and have received tremendous attention. In contrast to xylanases, relatively little information on acetylxylan esterases is available. In a previous study, a cDNA encoding a bifunctional acetylxylan esterase/xylanase, XynS20E, was cloned. The optimal reaction temperature and pH values for XynS20E acetylxylan esterase activity were higher than those for its xylanase activity.25 In the present study, the cDNA encoding the carbohydrate esterase domain of XynS20E was cloned and expressed in E. coli as a recombinant His\(_6\) fusion protein AxeS20E, which was then purified and characterized.

After induction with IPTG at 37 °C, the induced and non-induced recombinant bacteria harboring the AxeS20E expression plasmids were analyzed using SDS-PAGE. A band of about 24 kDa corresponding to the AxeS20E-His\(_6\) fusion protein was observed in the sample from the induced recombinant bacteria (Fig. 1, lane 2). After centrifugation, the expressed recombinant proteins were predominately found in the insoluble fraction of cell lysate (Fig. 1, lane 4). After treatment with 8 mol L\(^{-1}\) urea, the recombinant proteins dissolved (Fig. 1, lane 5). The purified AxeS20E-His\(_6\) fusion proteins were obtained after purification by affinity chromatography and desalting (Fig. 1, lanes 6 and 7). The respective specific activity for each purification step was further confirmed by measuring the amount of 4-methylumbelliflourone released from 4-methylumbelliferyl acetate. Only the refolded protein obtained after purification by desalting column showed acetylxylan esterase activity, indicating that the purified AxeS20E was folded into the active structure. The yield of the purified recombinant AxeS20E was 62.18 ± 4.52 µg, starting from 100 mg (wet weight) of E. coli cells.

Figure 1. SDS-PAGE of recombinant AxeS20E. Lane M, molecular weight marker; lane 1, cell lysate of the recombinant E. coli before IPTG induction; lane 2, cell lysate of the recombinant E. coli after IPTG induction; lane 3, supernatant fraction of the cell lysate after centrifugation; lane 4, pellet fraction of the cell lysate after centrifugation; lane 5, soluble fraction after the pellet was treated with 8 M urea; lane 6, urea-soluble AxeS20E after HisTrap chromatography; lane 7, purified AxeS20E after HiTrap chromatography.

### Table 1. Process variables used in the CCD, showing the treatment combinations and the mean experimental responses

<table>
<thead>
<tr>
<th>Treatment</th>
<th>(X_1)</th>
<th>(X_2)</th>
<th>(X_1)</th>
<th>(X_2)</th>
<th>Acetylxylan esterase activity(^a) (U mg(^{-1}) of total protein)</th>
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<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>7.44</td>
</tr>
<tr>
<td>2</td>
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<td>0</td>
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<tr>
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<td>0</td>
<td>0</td>
<td>0</td>
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</tr>
<tr>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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</tr>
<tr>
<td>5</td>
<td>1</td>
<td>1</td>
<td>9.56</td>
<td>57</td>
<td>59.17</td>
</tr>
<tr>
<td>6</td>
<td>0</td>
<td>0</td>
<td>8.50</td>
<td>50</td>
<td>59.17</td>
</tr>
<tr>
<td>7</td>
<td>0</td>
<td>0</td>
<td>8.50</td>
<td>50</td>
<td>59.17</td>
</tr>
<tr>
<td>8</td>
<td>1.41</td>
<td>0</td>
<td>10.00</td>
<td>50</td>
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</tr>
<tr>
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<td>8.50</td>
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<tr>
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<td>13</td>
<td>1</td>
<td>1</td>
<td>7.44</td>
<td>57</td>
<td>59.17</td>
</tr>
</tbody>
</table>

\(^a\) Results represent the mean of three experiments.

Optimization of AxeS20E activity

The classical method of ‘one-variable-at-a-time’ bioprocess design may be effective in some situations, but fails to consider
the combined effects of all factors involved. RSM is an empirical modeling technique used to evaluate the relationships between a set of controllable experimental factors and observed results. CCD, which minimizes the number of experimental runs, was used to determine the effects of independent variables on the dependent variables. According to preliminary experimental results, temperature and pH were identified as the major factors affecting the acetylxylan esterase activity of the recombinant AxeS20E. The process variables used in the RSM experimental design and results for enzyme activities are shown in Table 1. The central points (treatments 4, 6, 7, 9, and 12) and treatment 13 showed the highest levels of acetylxylan esterase activity (range from 759.28 to 867.52 U mg⁻¹ of total protein).

The selection of an adequate model (Table 2) was determined using model analysis, lack-of-fit, and R-squared analysis, as outlined by Chen et al., Hung et al., and Zeng et al. Table 2(a) compares the validities of the linear, quadratic and cubic models for the responses according to their F-values. A model with F-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 (Table 2(b)). The response predictor was discarded where lack-of-fit was significant, as indicated by a low P-value (\( P > F \)). The model with no significant lack-of-fit was selected. The quadratic model appeared to be the most accurate, with a statistically significant model analysis (\( P < 0.05 \)), insignificant lack-of-fit (\( P > 0.05 \)), and the smallest PRESS value (Table 2(c)). In addition, the goodness of fit of the quadratic model was checked using the coefficient of determination (Table 2(c); \( R^2 = 0.9839 \)), indicating that 98.39% of the total variation could be explained by the model. This confirms that the accuracy and general ability of the quadratic model was good, and analysis of the associated response trends was reasonable.

The quadratic model generated by the design is:

\[
Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{12} X_1 X_2 + \epsilon
\]  

where \( \beta_0 \) is constant, \( \beta_1 \) and \( \beta_2 \) are the main effects of each process variable (pH and temperature, respectively), \( \beta_{11} \) and \( \beta_{22} \) are effects of the squares of the variables, \( \beta_{12} \) is the interaction effect between the variables, \( Y \) is acetylxylan esterase activity, \( X_1 \) and \( X_2 \) represent the independent variables (pH and temperature, respectively), and \( \epsilon \) is the random error.

The RSM yielded the following regression equation:

\[
Y = 791.75 - 233.10 \times \text{pH} + 164.85 \times T - 254.90 \times \text{pH}^2 - 209.54 \times T^2 - 162.76 \times \text{pH} \times T
\]  

where \( Y \) is the predicted response for acetylxylan esterase activity (U mg⁻¹ of total protein), and pH and T are the coded values for pH and temperature (Table 1).

The significance of the coefficients determined by Student’s t-test and the related P-values were checked. The P-values were used to check the significance of each coefficient, and also indicated the strength of the interaction between each independent variable (i.e. the smaller the P value, the more significant the corresponding coefficient). In this study, the P-values of pH, T, second-order pH and T, and the interaction coefficient of pH and T were highly significant (\( P < 0.01 \)). The high significance of the pH and T second-order model indicates that it can act as a limiting factor, with even small variations substantially altering acetylxylan esterase activity. The model also clearly reveals significant interactions between pH and T (\( P < 0.01 \)), therefore, treating them separately may not reflect their real influence on the acetylxylan esterase activity (e.g. optimum pH activity changes along with T).
as reported by Huang et al. Fibrobacter succinogenes of xylanase XynC from Marrone the enzyme specific activity more than two-fold, according to were 1 purified recombinant AxeS20E for 4-methylumbelliferyl acetate often functioned independently of each other. Several studies and xylan-binding domains. The domains of the modular enzymes posed of catalytic domains linked to one or more noncatalytic AxeS20E had a higher level of affinity for the substrate. An RSM for enzyme activity as a function of pH and temperature of AxeS20E is depicted in Fig. 2. The results indicate that the optimal conditions for AxeS20E acetylxylan esterase activity occur at 54.6 °C and pH 7.8. To confirm the applicability of the model, acetylxylan esterase activity at the suggested optimum condition was determined. In this condition, the model predicted enzymatic activity of 923.01 U mg⁻¹ (range from 855.49 to 990.53 U mg⁻¹) at the 95% confidence level. The experimental enzymatic activity of 881.84 ± 69.78 U mg⁻¹ confirmed the accuracy of the model. Under the optimal conditions, the Kₘ and Vₘₐₓ of the purified recombinant AxeS20E for 4-methylumbelliferyl acetate were 1.41 ± 0.27 mg ml⁻¹ and 110.83 ± 12.89 μmol min⁻¹ mg⁻¹, respectively.

Only a few carbohydrate esterases from rumen fungi have been purified and characterized to date. These carbohydrate esterases belong to families 1, 2, 3, and 6 on the basis of the CAZy classification system.7,18,19 The optimal pH for AxeS20E activity was found to be pH 7.8, which is in the range reported for the other rumen fungal carbohydrate esterases (pH 5.5 to 9.0) and is higher than those for the other fungal carbohydrate esterases (pH 5.5 to 7.0).7,8,37 Under the optimal conditions, the Kₘ of AxeS20E (1.41 ± 0.27 mg mL⁻¹) was significantly lower than that of XynS20E (16.7 ± 2.3 mg mL⁻¹) as described by Pai et al.,25 indicating that AxeS20E had a higher level of affinity for the substrate. Xylanolytic enzymes often exhibit a modular structure composed of catalytic domains linked to one or more noncatalytic domains such as dockerin domains, cellulose-binding domains, and xylan-binding domains. The domains of the modular enzymes often function independently of each other. Several studies indicated that deletion of noncatalytic domains from full-length enzymes could increase the specific activities of the catalytic domain. Deletion of dockerin domains significantly increased the specific activity of xylanases Xyn11A and Xyn11B of N. frontalis, as reported by Huang et al.37 Deletion of the dockerin domain of xylanase XynC from Fibrobacter succinogenes S85 increased the enzyme specific activity more than two-fold, according to Marrone et al.38 It is possible that the dockerin domain sterically hinders the release of products from the catalytic domain, thus removal of the dockerin domain would expose the active site cleft and facilitate the more rapid release of hydrolysis products, and hence increase the specific activity. In the present study, the specific activity of AxeS20E under the optimal reaction conditions on 4-methylumbelliferyl acetate was more than 1.5 times that of XynS20E as described by Pai et al.25 The synergistic effect of xylanase and acetylxylan esterase combinations was demonstrated in previous studies.7,18,19 Interestingly, AxeS20E did not display a lower, but instead, showed a higher level of specific acetylxylan esterase activity than XynS20E. This could be because, under the optimal reaction conditions for the acetylxylan esterase, the secondary structure of XynS20E was less stable than that of AxeS20E. Therefore, the GH domain of XynS20E could not act synergistically with the CE domain. Further investigation is required to verify whether the deletion of the GH and dockerin domains alters the conformation of the CE domain of AxeS20E and subsequently increases its acetylxylan esterase activity.

Thermal stability of AxeS20E
Thermal stability is a very important issue when considering the industrial applications of enzymes. In terms of the thermal stability of the recombinant AxeS20E in our study, more than 85% of the original activity was retained after 120 min of heating at 80 °C (Fig. 3). The thermal stability profiles of AxeS20E were similar to those of XynS20E as described by Pai et al.,25 indicating that removal of GH and dockerin domains from the full-length enzyme did not affect the thermal stability of the CE domain. Xylanolytic enzymes have potential applications in a wide range of industrial processes, such as food, feed, and paper and pulp industries. Most of these processes are carried out at high temperatures, so that thermally stable enzymes would give an advantage. Since AxeS20E retained more than 85% of its initial activity after 120 min of heating at 80 °C, it could be used synergistically with other thermally stable xylanases, thus promoting more efficient hydrolysis of xylan. This thermal stability makes AxeS20E a good candidate for various biotechnological applications.
CONCLUSION
RSM was employed for the planned statistical optimization of the acetylxylan esterase activity of the recombinant AxeS20E. CCD and regression analysis were used to determine optimized temperature and pH conditions for AxeS20E enzyme activity. The optimal conditions for AxeS20E activity were 54.6 °C and pH 7.8. In addition, AxeS20E retained more than 85% of its initial activity after 120 min of heating at 80 °C. These results show the potential of AxeS20E for various biotechnological applications.

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