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Immobilization of *Neocallimastix patriciarum* xylanase on artificial oil bodies and statistical optimization of enzyme activity

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ABSTRACT

A thermally stable and alkalophilic xylanase, XynCDBFV, from *Neocallimastix patriciarum* was overexpressed in *Escherichia coli* as a recombinant protein fused to the N-terminus of oleosin, a unique structural protein of seed oil bodies. As a result of the reconstitution of the artificial oil bodies (AOBs), the immobilization of active xylanase was accomplished. Response surface methodology (RSM) was employed for the optimization of the immobilized xylanase activity. The central composite design (CCD) and regression analysis methods were effective for determination of optimized temperature and pH conditions for the AOB-immobilized XynCDBFV. The optimal condition for the highest immobilized xylanase activity (3.93 IU/mg of total protein) was observed at 59 °C and pH 6.0. Further, AOB-immobilized XynCDBFV retained 50% of its maximal activity after 120 min at 60 °C, and it could be easily and simply recovered from the surface of the solution by brief centrifugation, and could be reused eight times while retaining more than 60% of its activity. These results proved it is a simple and effective method for direct immobilization of xylanases.

1. Introduction

Xylan, a heteropolysaccharide containing substituent groups of acetyl, 4-*O*-methyl-D-glucuronosyl and α-arabinofuranosyl residues linked to a backbone of β-1,4,-linked xylopyranose units, is the second-most-abundant renewable resource, with a high potential for degradation to useful end products (Subramaniyan and Prema, 2002). As xylan varies in structure between different plant species, complete hydrolysis requires a large variety of cooperatively acting enzymes such as xylanases, xylosidases, arabinofuranosidases, glucuronidases, acetylxylan esterases, ferulic acid esterases, and *p*-coumaric acid esterases (Kulkarni et al., 1999; Subramaniyan and Prema, 2002; Collins et al., 2005). All of these enzymes act cooperatively to convert xylan into its constituent sugars. Of these, xylanase is of particular significance because it can catalyze the random endohydrolysis of β-1,4-xylosidic linkages in xylan to produce xylooligosaccharides and xylose (Wu et al., 2006).

Xylanases are a major group of industrial enzymes due to their biotechnological utility and potential application 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 in the consistency of beer and the digestibility of animal feed stock (Subramaniyan and Prema, 2002). In the past few years, the potential applications of xylanases for bioconversion of lignocellulosic feedstocks to fuel-grade ethanol have been of particular interest to researchers. Current commercial preparations in lignocellulose hydrolysis have primarily been based on dilute-acid pretreatment where hemicellulose is removed before saccharification. With the development of non-acid pretreatment methods where the hemicellulose fraction remains intact, however, xylanases are required (Gray et al., 2006). Although the cost of commercial xylanase preparations has been reduced significantly in recent years, enzyme costs are still an obstacle to full-scale process commercialization (Lynd et al., 2005). Immobilization on an inert carrier offers the prospect of significant cost savings by facilitating enzyme recycling through multiple cycles of batch-wise hydrolysis (Hudson et al., 2005). Also, enzyme immobilization frequently results in improved thermal stability or resistance to shear inactivation (Tu et al., 2006). The immobilized xylanases are usually packed in a column for convenient application; however, the disadvantage of such an immobilized xylanase column is that the solid property of the sub-

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strate xylan restricts passage through the column (Liu et al., 1997). In addition, the efficiency of the xylanase is decreased when immobilized on solid supports as the bound enzyme is not accessible to bulky insoluble substrate (Tan et al., 2008).

A recently established expression/purification system based on artificial oil bodies (AOBs) provides a novel method of enzyme immobilization (Peng et al., 2004; Chiang et al., 2006). In this system, oleosin, a unique structural protein of seed oil bodies, is used as the carriers. Oleosins possess a lipophilic segment embedded into the triacylglycerol core, with two amphipathic arms protruding on the surface of the oil bodies. Thus, the target protein can be expressed in *Escherichia coli* as an insoluble recombinant protein fused to the N- and/or C-terminus of oleosin. Stable artificial oil bodies are then constituted with triacylglycerol, phospholipid and the insoluble oleosin fusion protein. As illustrated previously, the insoluble recombinant proteins fused to oleosin tend to self-refold spontaneously when displayed on AOBs (Chiang et al., 2005). Thus, this may offer an easy and efficient, single-step method for achieving immobilization of the recombinant proteins (Chiang et al., 2006).

In the present study, a xylanase gene, *xynCDBFV*, from the ruminal fungus *Neocallimastix patriciarum* was cloned and expressed as an oleosin-fused protein in *E. coli*. As a result of reconstitution of the AOBs, the immobilization of active xylanase was successfully accomplished. Response surface methodology (RSM) was then employed for the planned statistical optimization of the immobilized xylanase activity.

2. Methods

2.1. Bacterial culture and DNA manipulation

E. 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) to broth. Plasmid DNA was isolated from *E. coli* using the alkali lysis method (Birnboim and Doly, 1979). 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).

2.2. Construction of xylanase expression plasmids

The uninterrupted DNA sequence encoding xylanase XynCDBFV (Chen et al., 2001), a thermally stable and alkalophilic mutant of the catalytic domain of *N. patriciarum* xylanase XynC (GenBank accession number AF123252), was amplified by PCR from pNZJ021 (Liu et al., 2005) using the oligonucleotide forward primer, 21F (5′ GGTGGTCATATGCAAAGTTTCTGTAGTTC 3′), and the reverse primer, 21R (5′ GGTGGTTGCTCTTCTGCAATCACCAATG 3′). These two primers were designed to place an *Nde*I site at the 5′ end and an *Sap*I site at the 3′ end of the PCR product, respectively. The PCR fragments encoding *xynCDBFV* were digested with *Nde*I and *Sap*I, and then ligated with *Nde*I-*Sap*I-digested pOSP2 (Chiang et al., 2005) to generate pOSP2-xyn, which was then sequenced to ensure that no errors were introduced by PCR. The resultant plasmids were used to transform *E. coli* BL21 (DE3) (Novagene, Madison, WI) by standard techniques (Sambrook and Russell, 2001). Transformants were selected on LB agar plates containing ampicillin (100 μg/mL) (Sigma Chemical Co., St. Louis, MO).

2.3. Expression of recombinant proteins

E. coli BL21 transformant cells were cultured in LB broth, and cell growth was then measured turbidimetrically at 600 nm (OD600). To produce the recombinant protein, the overnight culture was prepared and subsequently seeded into 5 ml of LB broth. The cell cultures were maintained at 37 °C and induced with 100 μM of isopropyl-L-D-thiogalactopyranoside (IPTG; Sigma) for protein production upon reaching OD600 of 0.5. After 4 h of induction, the cells were harvested by centrifugation at 5000*g* for 20 min at 4 °C.

2.4. Immobilization of xylanase on AOBs

AOBs were prepared according to the method described by Chiang et al. (2005). The cell pellet was resuspended in 1 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. Then, 15 mg of olive oil (Sigma) and 150 μg of phospholipid (Sigma) were added into the pellet fraction of the *E. coli* cell lysate, and the mixture was subjected to sonication. Subsequently, the reconstituted AOBs were collected after centrifugation and washed in the sodium phosphate buffer solution (0.1 M, pH 7.4). The protein production in each step was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) (Laemmli, 1970).

2.5. Xylanase activity assays

Xylanase activity on the AOBs was determined using oat spelt xylan (Sigma) as the substrate. Reducing sugar released from the substrate was estimated using the DNS reagent method (Konig et al., 2002). One unit of enzyme activity was defined as that releasing 1 μmol of reducing sugar equivalents per minute from the substrate under the assay conditions, with specific activity expressed as IU/mg protein. Protein concentration was determined using Lowry assay (Lowry et al., 1951) against a standard curve of bovine serum albumin, fraction V (Sigma).

2.6. Optimum pH and temperature of immobilized xylanase

The response surface model, its main effects, and the interaction between the different factors, each at different levels, can be simultaneously investigated. The central composite design (CCD) was used, with two variables, at five levels and five replicates at the central point, for a total of 13 experiments. In this experimental design, pH (X_1) and temperature (X_2) were chosen as factors, with units of xylanase activity were denoted by *Y*. According to our preliminary experimental results, the experimental index number, and scaled and real values are shown in Table 1. The scaled values

Table 1
Process variables used in the CCD, showing the treatment combinations and the mean experimental responses for AOB-immobilized xylanase

Treatment	Coded setting levels (X_1 = pH; X_2 = T)		Actual levels (X_1 = pH; X_2 = T)		Xylanase activity (IU/mg of total protein)
	X_1	X_2	X_1	X_2	
1	−1	−1	4	45	0.78
2	0	−1.41	6	38.8	1.53
3	−1.41	0	3.17	60	0.41
4	0	0	6	60	3.75
5	1	1	8	75	0.39
6	0	0	6	60	3.86
7	0	0	6	60	3.73
8	1.41	0	8.83	60	0.35
9	0	0	6	60	3.67
10	1	−1	8	45	0.94
11	0	1.41	6	81.2	0.10
12	0	0	6	60	3.82
13	−1	1	4	75	0.48

Results represent the mean of three experiments.

were defined as follows: $X_1 = (pH-6)/2$; $X_2 = (T-60)/15$. Statistical Analysis System software (SAS; version 8.1; Statistical Analysis System Institute, 1998) was used for regression analysis of the experimental data. The CCD was used to allow expression of the variables as second-order polynomial models of the form:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{12} X_1 X_2 + \varepsilon$$

where β_0 is constant, β_1 and β_2 are the main effect of each process variable (pH and temperature), β_{12} is the interaction effect between the variables, β_{11} and β_{22} are effect of the square of the variables, Y is xylanase activity, X_1 and X_2 represent the independent variables (pH and temperature), and ε is the random error.

Finally, a series of experiment was conducted in triplicate and repeated three times in order to check the reliability of the response surface model, with the predicted values and experimental data compared. The results were analyzed using the SAS analysis of variance (ANOVA) function. Duncan's multiple range test (Montgomery, 1999) was used to detect differences between the predicted and observed values.

2.7. Thermal stability of immobilized xylanase

The thermal stability of AOB-immobilized xylanase was determined by incubation at 60 °C in phosphate buffer (pH 6.0) containing 0.5% oat spelt xylan as the substrate. Aliquots were withdrawn at intervals of 0, 20, 40, 60, 80, 100, and 120 min, and the residual enzyme activity measured.

2.8. Reusability of immobilized xylanase

The reusability of AOB-immobilized xylanase was determined by incubation at 60 °C for 20 min in phosphate buffer (pH6.0) containing 0.5% oat spelt xylan as the substrate. At the end of the reaction, the immobilized enzyme was recovered by centrifugation and the supernatant was removed for further analysis. The reaction was reinitiated by administration of the recovered enzymes in a fresh substrate solution.

3. Results and discussion

3.1. Immobilization of xylanase XynCDBFV on AOBs

The uninterrupted DNA fragments encoding xylanase XynCDBFV, a thermally stable and alkalophilic mutant of the catalytic domain of *N. patriciarum* xylanase XynC (GenBank accession number AF123252), were amplified by PCR and subcloned into the AOB expression/purification system vector pOSP2. XynCDBFV was overexpressed in *E. coli* as a recombinant protein fused to the N-terminus of oleosin by a linker polypeptide. After induction with IPTG at 37 °C, the induced and non-induced recombinant bacteria containing the xylanase gene were analyzed using SDS-PAGE. A band of about 61 kDa corresponding to the XynCDBFV-oleosin fusion protein was observed in the induced recombinant bacteria (Fig. 1, lane 2). The XynCDBFV-oleosin fusion protein was predominately found in the insoluble fraction of cell lysate after centrifugation (Fig. 1, lanes 3 and 4). After reconstitution into AOBs, the XynCDBFV-oleosin fusion protein was almost entirely present in the oil-body fraction after centrifugation (Fig. 1, lanes 5 and 6). Further examination of the reconstituted AOBs by light microscopy showed that these AOBs were extremely stable and maintained integral for at least 7 days at 4 °C (results not shown). The DNS method was then used to confirm xylanase activity of the reconstituted AOBs. A detectable level of xylanase activity was observed in the reconstituted AOBs from the induced recombinant bacteria

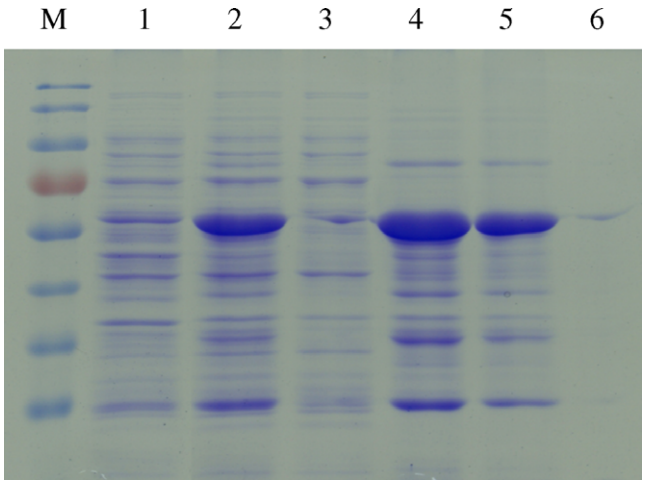

Fig. 1. SDS–PAGE analysis of XynCDBFV immobilized on AOBs. Lane M, molecular weight marker; lane 1, cell lysate of the recombinant *E. coli* before IPTG induction; lane 2, cell lysate of the recombinant *E. coli* after IPTG induction; lane 3, soluble fraction of the cell lysate after centrifugation; lane 4, insoluble fraction of the cell lysate after centrifugation; lane 5, AOBs; lane 6, remaining supernatant after AOBs recovery.

(data not shown), indicating that XynCDBFV might be immobilized on the AOB surface and fold into a correct structure. Many intrinsic soluble proteins overexpressed in *E. coli* are inclined to form insoluble aggregates confined in inclusion bodies. Nevertheless, the AOB system used in this study provides excellent machinery to functionally recover intrinsic soluble proteins that are overexpressed as insoluble aggregates in *E. coli*. Our results suggest that the folding of XynCDBFV was first tangled with the hydrophobic fusion oleosin. Constitution of AOB attracts the hydrophobic oleosin portions into the TAG matrix, thus leaving XynCDBFV alone for self-refolding right after the physical segregation of oleosin and XynCDBFV. As XynCDBFV self-folds into its correct conformation on the surface of an AOB, its functional activity may be recovered concurrently.

Many reports on the immobilization of xylanase have been published (Roy et al., 2003; Hudson et al., 2005; Tan et al., 2008). The immobilized xylanases are usually packed in a column for convenient application. However, the main disadvantage for an immobilized xylanase column is that the solid property of the substrate xylan makes it hard to move through the column (Liu et al., 1997). In addition, efficiency of xylanase conversion is decreased when immobilized on solid supports as the bound enzyme is not accessible to the bulky insoluble substrate (Tan et al., 2008). Compared to conventional immobilization methods, the use of AOBs as immobilization matrices reduces the requirement for xylanase purification and eliminates the cost of immobilization. Particularly, xylanases immobilized on AOBs can be easily recovered from the surface of the solution simply by a brief centrifugation. These results prove that AOBs immobilization is a comparatively simple and effective method of direct immobilization of xylanases.

3.2. Optimization of immobilized xylanase activity

Base on the previous study, temperature and pH were identified as the major factors affecting the activity of xylanase XynCDBFV (Chen et al., 2001). RSM is an empirical modeling technique used to evaluate the relationship between a set of controllable experimental factors and observed results. The CCD experimental design, which minimizes the number of experimental runs, was used to determine the effects of independent variables on the dependent variables. The process variables used in the experimental design

and results for enzyme activities are shown in Table 1. Treatments 4, 6, 7, 9 and 12 (central points) showed the highest levels of xylanase activity (3.75, 3.86, 3.73, 3.67, and 3.82 IU/mg of total protein, respectively). These results suggest that the immobilized xylanase has greater enzyme activity at higher temperatures and moderate acidic pH.

Analysis of variance (ANOVA) of immobilized xylanase activity as a function of pH and temperature is presented in Table 2. The computed *F*-value (456.57) was higher than the analogous value in the statistical tables (<0.0001), indicating that the model was highly significant (Box and Wilson, 1951). The goodness of fit of the model was checked using the coefficient of determination ($R^2 = 0.9969$), indicating that just 0.31% of the total variation was not explained by the model. This confirms that the accuracy and general ability of the quadratic model was good, with the analysis of the associated response trends considered to be reasonable. In addition, the lack-of-fit test was insignificant, suggesting that the model accurately represents the data in the experimental region.

RSM yielded the following regression equation:

$$Y = -32.56 + 5.34^{*}\mathrm{pH} + 0.69^{*}T - 0.43^{*}\mathrm{pH}^2 - 0.005761^{*}T^2 - 0.002125^{*}\mathrm{pH}^{*}T$$

where Y is the predicted response for xylanase activity (IU/mg of total protein), and pH and T are the coded values for pH and temperature (see Table 1).

The significance of the coefficients determined by Student's *t*-test and the related *P*-values are presented in Table 3. The latter 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) (Akhnazarova and Kafarov, 1982). In this study, the *P*-value of pH, second-order T, and the interaction coefficient of pH and T were highly significant ($P < 0.005$). The high significance of the T second-order model indicates that it can act as a limiting factor, with even small variations substantially altering xylanase activity (Heck et al., 2006). The model also clearly reveals significant interactions between pH and T ($P < 0.0001$), therefore, treating them separately may not reflect their real influence on the xylanase activity (e.g., optimum T activity changes along with pH).

Table 2
Analysis of variance (ANOVA) for model regression

Source of variation	Sum of squares	Degree of freedom	Mean square	*F*-value	*F*-value in statistic table
Model	29.410	5	5.880	456.57	<0.0001
Residual	0.090	7	0.013		
Lack of fit	0.067	3	0.022	3.96	0.1086
Pure error	0.023	4	0.00569		
Total	29.50	12			

Model regression is $Y = -32.56 + 5.34^{*}\mathrm{pH} + 0.69^{*}T - 0.43^{*}\mathrm{pH}^2 - 0.005761^{*}T^2 - 0.002125^{*}\mathrm{pH}^{*}T$.
$R^2 = 0.9969$; standard error of estimate = 0.0005; significance level = 95%.

Table 3
Coefficient estimates by the regression model

Factor	Coefficient estimate	Standard error	*P*-value
Intercept	3.770	0.051	
T	−0.002	0.040	0.9674
pH*	−0.200	0.040	0.0016
$T \cdot T$*	−1.740	0.043	<0.0001
$T \cdot$ pH*	−1.300	0.043	<0.0001
pH · pH	−0.064	0.057	0.2984

* Statistically significant at 99% of confidence level.

The RSM for enzyme activity as a function of pH and temperature of the immobilized xylanase is depicted in Fig. 2. The results indicate that the optimal conditions for the immobilized xylanase activity occur at 59 °C and pH 6.0. To confirm the applicability of the model, xylanase activity at the suggested optimum condition was determined. In this condition, the model predicted enzymatic activity of 3.93 IU/mg at a confidence level of 95%. The experimental enzymatic activity of 3.90 IU/mg confirmed the accuracy of the model.

The general utility of xylanases in industry has spurred considerable research into the production of more thermally stable and alkalophilic variants. The xylanase, XynCDBFV, used in this study is a thermally stable and alkalophilic mutant of the catalytic domain of *N. patriciarum* xylanase XynC. It was previously determined that the optimum temperature and pH of free XynCDBFV was 62 °C and pH 6.0, respectively (Chen et al., 2001). In this study, the maximum activity of immobilized XynCDBFV was determined at 59 °C and pH 6.0. Therefore, immobilization of the XynCDBFV on AOBs did not significantly change the optimum pH of the xylanase activity, and only slightly decreased the optimum temperature. At pH 8.5, 47 °C, and at pH 6.0, 77 °C, the free XynCDBFV maintained approximately 45% and 10% of maximal activity, respectively (Chen et al., 2001), while the analogous maxima for the immobilized form were 16% and 51% in this study. Thus, it appear reasonable to suggested that immobilization on AOBs may improve the thermal stability of XynCDBFV but not its alkalinity tolerance.

3.3. Thermal stability of immobilized xylanase

Thermal stability is very important in the industrial application of enzymes. In terms of the thermal stability of the immobilized XynCDBFV in our study, 50% of maximal activity was retained after 120 min at 60 °C. However, this result contrasts with that of Chen et al. (2001), who found that the activity of free XynCDBFV was reduced to 30% of maximum at the same time and temperature. Thus, better thermal stability was demonstrated for the immobilized XynCDBFV in our study which compared to the free enzyme. Chiang et al. (2006) immobilized *Agrobacterium radiobacter* D-hydantoinase on AOBs and also demonstrated that the immobilized enzyme exhibited a higher thermal tolerance than the free enzyme. In addition, Li et al. (2007) immobilized cellulase on the outer membrane of liposomes and indicated that the liposome membrane was favorable for maintaining the activity of the cellulase in these cellulase-containing liposomes. It appears reasonable to suggest, therefore, that the interaction between enzyme and oil-

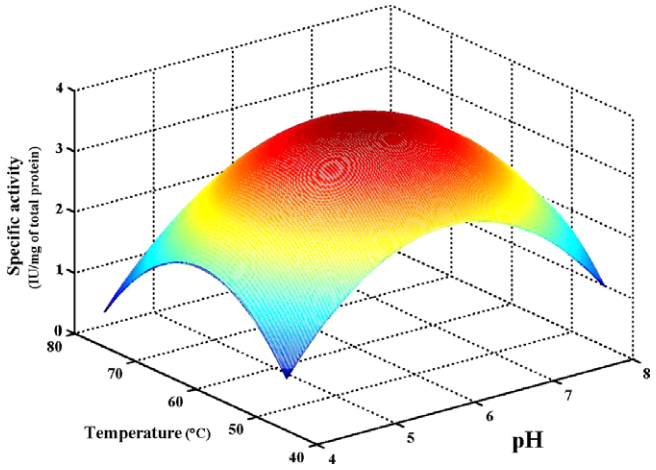

Fig. 2. Response surface plot for the effects of pH and temperature on AOB-immobilized xylanase activity.

body membrane is beneficial in terms of reducing the enzyme deactivation.

3.4. Reusability of immobilized xylanase

Immobilized enzymes are preferred as in this form they can be recycled, thereby reducing production costs. Further, AOBs can be separated from the reaction mix by flotation centrifugation (Chiang et al., 2006), facilitating the recovery and recycling of the xylanase immobilized thereon. The recovery and operational stability of AOB-immobilized XynCDBFV was examined during eight successive rounds of xylan hydrolysis. The AOB-immobilized XynCDBFV could be reused for three cycles at 60 °C without loss in activity. After that, the enzyme activity gradually declined and still retained more than 60% of initial activity after eight-time recycling of the enzyme (data not shown). It is interesting to note that because xylanase immobilized on AOBs floats rather than sinks, it may be much easier to recover in heterogeneous mixtures which contain precipitable solids, which could confound attempts to recover enzymes bound to solid matrices. However, evaluation of the operational stability of enzymes is necessary as this property is one of the most important factors affecting the successful applications of immobilized systems. Liu et al. (1997) fused the *N. patriciarum* xylanase gene *xynC* to an oleosin gene, and then introduced the fusion gene into *Brassica napus*. These workers found that XynC was immobilized on the surface of oil-bodies extracted from the transgenic canola seeds, and that it could be reused several times without impairment of the xylanase activity (Liu et al., 1997). In this study, XynCDBFV was immobilized on AOBs, and it was demonstrated that AOB-immobilized XynCDBFV could be reused eight times and still retain more than 60% of its activity. It appears reasonable to suggest, therefore, that these results prove that xylanase immobilized on AOBs can be reused, thereby reducing the cost of enzymes of industrial processes where the cost of enzyme is significant.

4. Conclusions

The ruminal fungus *N. patriciarum* xylanase gene, *xynCDBFV*, was cloned and expressed as an oleosin-fused protein in *E. coli*. Immobilization of active xylanase was accomplished as a result of reconstituting AOBs, with RSM employed for the planned statistical optimization of the immobilized xylanase activity. It was demonstrated that CCD and regression analysis methods were effective in determining optimized temperature and pH conditions for AOB-immobilized XynCDBFV. In this study, the optimal conditions for the immobilized xylanase activity were 59 °C and pH 6.0. In addition, it was demonstrated that AOB-immobilized XynCDBFV can be easily and simply recovered from the surface of the solution by brief centrifugation, and that it can be reused eight times while retaining more than 60% of its activity. These results prove that immobilization of xylanase on AOBs is a comparatively straightforward and effective means of direct immobilization of xylanases.

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