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Contributed Paper

Optimization, Purification and Characterization of β-xylanase by a Novel Thermotolerant Strain of *Microbispora siamensis*, DMKUA 245^T

Antika Boondaeng [a], Sukhumaporn Krajangsang [b], Chanaporn Trakunjae [a], Thanasak Lomthong [a,c], Shinji Tokuyama [d] and Vichien Kitpreechavanich* [a]

[a] Department of Microbiology, Faculty of Science, Kasetsart University, Bangkok 10900, Thailand.
[b] Department of Microbiology, Faculty of Science, Srinakharinwirot University, Bangkok 10110, Thailand.
[c] Division of Biology, Faculty of Science and Technology, Rajamangala University of Technology Thanyaburi, Pathumthani 12110, Thailand.
[d] Department of Applied Biological Chemistry, Faculty of Agriculture, Shizuoka University Shizuoka 422-8529, Japan.
* Author for correspondence; e-mail: fsciwck@ku.ac.th

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ABSTRACT

Investigation of factors affecting β-xylanase production by a newly thermotolerant strain of *Microbispora siamensis*, DMKUA 245^T, using a sequence of statistical methods, indicated that the optimized medium was composed of 10 g/L of xylan, 0.7 g/L of casein, 1 g/L of K_2HPO_4 and 0.15 g/L of $MgSO_4 \cdot 7H_2O$, yielding 44 U/mL of β-xylanase activity in a shaking flask at a temperature of 35 °C for 120 h. This was a 4.4-fold increase compared to when using unoptimized medium. β-Xylanase production of 250 U/mL was obtained in a 3 L stirrer fermenter with an aeration rate of 0.5 vvm, agitation speed of 150 rpm and initial pH of 7.0 for 72 h cultivation. The β-xylanase enzyme from the strain DMKUA 245^T was purified, with a specific activity of 219.4 U/mg protein obtained. SDS-PAGE and gel filtration indicated that the molecular weight of the purified β-xylanase as a monomer was estimated to be 65.8 kDa. The optimum pH and temperature for β-xylanase activity were 5.5 and 60 °C, respectively. The activity of Co^{2+}-, K^+- and Mg^{2+}-treated purified enzymes was more thermostable than the untreated purified enzyme, particularly the Co^{2+}-treated purified enzyme, which was still stable at temperatures up to 90 °C. The hydrolysis products were a series of short-chain xylooligosaccharides, indicating that the purified enzyme was an endo-β-1, 4-xylanase.

Keywords: β-xylanase, purification, characterization, *Microbispora siamensis*, thermotolerant

1. INTRODUCTION

Endo-β-1, 4-xylanase, one the main constituents of the xylanolytic enzyme system, randomly hydrolyzes xylans into short oligomers [1-3]. β-Xylanases are useful in several applications such as clarifying fruit juices and wine [4, 5], food processing in combination with cellulases [6, 7], pre-treatment of forage crops and other lignocellulosic biomass, improving the nutritional properties of swine and poultry cereal-based diets and agricultural silage [8], flour modification for bakery products and saccharification of agricultural, industrial and municipal waste [9]. The degradation of xylan, together with the cellulose found in various lignocellulosic materials, has application in bioethanol production through fermentation of xylose and glucose formed as by-products in the hydrolysis of xylan and cellulose, respectively [10]. Attention on the applications of β-xylanase has led to the discovery of many new enzymes with novel characteristics from various microorganisms [11]. Driven by industrial demand for enzymes that can operate under process conditions, a number of thermostable β-xylanases have been isolated, but the search for new producers with higher potential remains necessary.

A novel thermotolerant actinomycete, *Microbispora siamensis* DMKUA 245^T, isolated from Thai forest soil [12], was characterized as a potent strain for β-xylanase production. This thermotolerant strain showed the highest β-xylanase activity when grown at 40 °C. The strain is of interest for industrial fermentation due to its ability to reduce the cost of cooling systems and potentially by enhancing the thermostability.

Statistically based experimental designs provide an efficient approach for optimization. The Plackett-Burman design is a well-established and widely used statistical technique for screening and selection of critical culture variables [13, 14] Response surface design, including central composite design (CCD), has been increasingly used for optimization of various phases of fermentation [15].

This study aimed to optimize the medium for β-xylanase production by the new thermotolerant *M. siamensis* DMKUA 245^T using a sequence of statistical methods: Plackett-Burman and CCD were employed. In addition, to the best of our knowledge, there have been no reports on the purification and characterization of β-xylanase produced by the genus *Microbispora*. Therefore, the purification and characterization of a β-xylanase from *M. siamensis* DMKUA 245^T were also investigated for further application.

2. MATERIALS AND METHODS

2.1 Microorganism, Inoculum Preparation and Cultivation

The thermotolerant actinomycete *M. siamensis* DMKUA 245^T [12] was maintained on yeast extract-malt extract agar (ISP-2) at 4 °C. The inoculum was prepared by inoculation of a loop of the culture on ISP-2 slant agar in 50 mL of medium with the pH adjusted to 7.0 consisting of (g/L): xylan (Sigma Chemical Co., USA), 10; peptone, 1; K_2HPO_4, 1 and $MgSO_4 \cdot 7H_2O$, 0.2. The inoculum was incubated at 45 °C on a rotary shaker at 180 rpm for 48 h. The cells were harvested by centrifugation at 8000 *g* at 4 °C for 10 min. The obtained cell pellet was then washed with sterile 0.85% (w/v) NaCl. The optical density of the cell suspension was adjusted with 0.85% (w/v) NaCl to an optical density of 0.7 at a wavelength at 600 nm. A 10% (v/v) cell suspension was used as the inoculum.

2.2 Effect of Carbon and Nitrogen Sources on Enzyme Production

The effect of carbon sources on xylanase production by thermotolerant *M. siamensis* DMKUA 245^T was studied. The strain DMKUA 245^T was cultured in basal medium, with an initial pH of 7.0, consisting of (g/L): peptone, 1.0: K_2HPO_4, 1.0 and $MgSO_4 \cdot 7H_2O$, 0.2 on a rotary shaker at 180 rpm at 45 °C for 120 h. Each of 13 carbon sources (1% w/v): xylan, xylooligosaccharides (Sigma Chemical Co., USA), xylobiose

(Sigma Chemical Co., USA), cellobiose (Sigma Chemical Co., USA), lactose (Sigma Chemical Co., USA), maltose (Wako, Japan), glucose (Ajax Finechem, Australia), fructose (Sigma, USA), xylose (Sigma, USA), galactose (Difco™, USA), mannitol (Sigma, USA), sorbitol (Ajax Finechem, Australia), and xylitol (Sigma Chemical Co., USA) were used. Carbon sources which gave high xylanase production were selected for further study. The effect of various organic nitrogen sources (10 g/L), namely, casamino acid (Wako, Japan), casein (Wako, Japan), gelatin (Fisher Scientific, UK), peptone (HiMedia, India) and skim milk (Sigma Chemical Co., USA), on β-xylanase production by the novel thermotolerant strain *M. siamensis* DMKUA 245^T was evaluated using the “one factor at a time” method in basal medium containing (g/L): xylan, 10; K_2HPO_4, 1.0 and $MgSO_4{\cdot}7H_2O$, 0.2 on a rotary shaker at 180 rpm at 45 °C for 168 h.

To the culture grown on the glucose basal medium at 45 °C on rotary shaking of 180 rpm for 3 days, each of the selected carbon sources was added to make a final concentration of 1.0 % w/v. All cultures were further incubated for up to 5 days. The obtained supernatants were used for analysis of xylanase activity.

2.3 Optimization of β-Xylanase Production by Response Surface Methodology (RSM)

2.3.1 Plackett-Burman design

A Plackett-Burman experimental design [17] consisting of a set of eight combinations was used to determine the relative influence of five factors (X_1 to X_5, representing xylan, casein, K_2HPO_4, $MgSO_4{\cdot}7H_2O$ and temperature, respectively) with three replicates on β-xylanase production by the strain *M. siamensis* DMKUA 245^T. All these variables are quantitative factors and were investigated at two spaced levels coded as “1 (low level) and +1 (high level) (Table 1). The experimental design and statistical analysis of the data were done using SPSS software version 11.5. A significance level of 90% ($P < 0.1$) was chosen for selection of the influential experimental factors.

Table 1. Experimental variables at different levels used for β-xylanase production by *M. siamensis* DMKUA 245^T using a Plackett-Burman design.

Variable code	Variables	Levels	
		Low (-1)	High (+1)
X_1	Xylan (g/L)	10	30
X_2	Casein (g/L)	0.5	2.5
X_3	K_2HPO_4 (g/L)	0.1	1.9
X_4	$MgSO_4{\cdot}7H_2O$ (g/L)	0.1	0.3
X_5	Temperature (°C)	35	45
X_6-X_8	Dummy	0	0

2.3.2 Central composite design (CCD)

RSM was used to determine the optima of the selected factors for maximal β-xylanase production by the strain *M. siamensis* DMKUA 245^T. The best condition from Plackett and Burman design was selected for further optimization. Three factors (casein, $MgSO_4{\cdot}7H_2O$ and temperature) were evaluated each at five levels in a CCD based on a factorial design at two levels, with the addition of star points outside the experimental space coded -1 and + 1 at a distance depending on the number of factors and the resolution (coded -α and + α) and a center point (coded 0) in three replicates. A quadratic model was established as Eq. (1) by using the method of least squares as follows:

$$Y = a_0 + a_2X_2 + a_4X_4 + a_5X_5 + a_{24}X_2X_4 + a_{25}X_2X_5 + a_{45}X_4X_5 + a_{22}X_2^2 + a_{44}X_4^2 + a_{55}X_5^2 \quad (1)$$

where Y is the predicted response (β-xylanase activity, U/mL); X_2, X_4 and X_5 the coded forms of the input variables (casein, $MgSO_4{\cdot}7H_2O$ and temperature, respectively); a_0 a constant (general average); a_2, a_4 and a_5 the linear coefficients; a_{24}, a_{25} and a_{45} the interaction coefficients; and a_{22}, a_{44} and a_{55} the quadratic coefficients. The relation between the coded forms of the input variables and the actual values of casein, $MgSO_4{\cdot}7H_2O$ and temperature are described by Eq. (2):

$$x_i = \frac{X_i - X_0}{\delta X} \quad (2)$$

where x_i is the dimensionless coded value of the variable X_i, X_0 is the value of X_i at the center point and δX is the step change. The values at the center were chosen as a function of the results of the Plackett-Burman experiment.

Statistical analysis of the data was performed using SPSS software version 11.5 and STATISTICA 10 for Windows™. Response surfaces were drawn for the experimental results obtained from the effect of different variables on β-xylanase production to determine the individual and cumulative effects of these variables and the mutual interactions between them.

2.3.3 Validation of the experimental model

To validate the obtained experimental model (optimization of the conditions), three experiments were carried out using the optimized conditions, to confirm the results from the RSM analysis.

2.4 Batch Fermentation in an Stirrer Fermenter

The batch fermentation was performed in a 3 L stirrer fermenter containing a 2 L working volume of CCD-optimized medium. An inoculum of the strain *M. siamensis* DMKUA 245^T in flasks was transferred (10% v/v) to the medium and the fermentation was operated at 35 °C with an aeration rate of 0.5 vvm, agitation speed of 150 rpm and initial pH of 7.0. The culture was sampled at intervals of 24 h for 120 h and the enzyme activity was determined.

2.5 Analysis

β-Xylanase activity was assayed at 50 °C for 10 min, by measuring the quantity of reducing sugars liberated from 1% beech wood xylan (Sigma Chemical Co., USA) in 100 mM acetate buffer, pH 5.5, by the DNS method [18]. One unit of β-xylanase was defined as the amount of enzyme that liberated 1 µmol of xylose equivalent per min.

Proteolytic activity was assayed by using casein as substrate. The reaction was incubated at 50 °C for 30 min. The protease activity was determined by measuring the released of tyrosine from the reaction using Folin-Ciocalteu reagent (Merck,USA). The absorbance was read at 750 nm. One unit of proteolytic activity was defined as the amount of enzyme that liberated 1 mg of tyrosine per min. [19].

Cell dry weight was determined by centrifuged at 10,000 *g* at 4 °C for 10 min. The cell pellets were washed twice with distilled water, centrifuged and the washed cells were dried in an oven at 70 °C for 24 h and used thereafter for cell dry weight determination.

2.6 Purification of β-xylanase

A 10% inoculum was inoculated into the optimized medium and then the fermentation was operated with a temperature of 45 °C and aeration rate of 0.5 vvm for 48 h. The culture supernatant obtained after centrifugation at 10,000 *g* at 4 °C for 10 min was used for β-xylanase analysis and subjected to purification. Firstly, the crude enzyme was purified by batch method using DEAE-Sepharose (GE Healthcare Bio-Science AB, Sweden) by adding the crude enzyme to 100 mL of DEAE-Sepharose which was previously equilibrated with 50 mM Tris-HCl buffer (pH 8.0) at 4 °C and left overnight. The unbound protein solution which had no β-xylanase activity was discarded. Then, 100 mL of 0.5 M NaCl was added to the remaining DEAE-Sepharose and slowly mixed. The eluted proteins previously bound to DEAE-Sepharose were then subjected to dialysis with 50 mM Tris-HCl buffer (pH 8.0) overnight. The dialyzed enzyme solution was applied to a 100 mL DEAE-Sepharose column (10 × 45 cm) previously equilibrated with 50 mM Tris-HCl buffer (pH 8.0) and further eluted with a linear (0-0.5 M) NaCl gradient. The eluted solution was collected by a fraction collector. Each fraction was used to determine protein and β-xylanase activity. Active fractions were combined and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to check the purity and to determine protein molecular weight. The protein concentration in each step of the purification process was determined by reading the absorbance at a UV wavelength of 280 nm (A_{280})

2.7 Characterization of β-xylanase

2.7.1 Effect of pH and temperature on β-xylanase activity and stability

The effect of pH on purified enzyme was studied under standard conditions as described above. Buffer solutions (50 mM) with varying pH were: acetate buffer (pH 3.0-5.5), phosphate buffer (pH 5.5-7.0), Tris-HCl buffer (pH 8.0-9.0) and glycine-NaOH buffer (pH 9.0-12.0). The effect of temperature on purified RSDE activity was investigated at optimum pH and at various temperatures between 30-80 °C.

The pH stability of purified enzyme was tested by incubating the enzyme solution at 4 °C for 24 h in buffer solutions with pH values in the range of 3.512.0. The thermal stability of the enzyme was checked by incubating the enzyme for 1 h at different temperatures between 30100 °C. The residual activity of the purified enzyme was assayed under optimal conditions.

2.7.2 Substrate specificity and mode of hydrolysis

The activity towards beechwood xylan, oat spelt xylan (Sigma Chemical Co., USA), wood xylan (Daio Seishi Co.Ltd., Japan) and carboxymethyl cellulose (Sigma Chemical Co., USA) at 1% was assayed in 100 mM acetate buffer, pH 5.5. After incubation at 60 °C for 10 min, the reaction was terminated by adding 3.0 mL of 3, 5-dinitrosalicylic acid reagent (Sigma Chemical Co., USA) [18]. The activity of the purified enzyme on 4 mM *p*NP-β-D-xylopyranoside (Sigma, USA) and *p*NP-β-D-glucopyranoside (Sigma Chemical Co., USA) was examined in 100 mM acetate buffer (pH 5.5) at 60 °C for 10 min and detected the released *p*-nitrophenol

(Sigma Chemical Co. USA) at A_{405} [20]. Each of 1% substrate; beechwood xylan, wood xylan and xylobiose was hydrolyzed by the purified enzyme for 24 h at 60 °C. The hydrolysis products were determined by thin-layer chromatography on 10 × 10 cm cellulose HPTLC plates (Merck, USA) with a mixture of n-butanol, acetic acid and water (2:1:1) as a solvent system [17]. The sugar spots were detected by heating the plates to 100 °C after spraying with a solution consisting of 4 g diphenylamine (Merck, USA), 4 mL of aniline, 200 mL of acetone (Merck, USA) and 30 mL of 80% phosphoric acid [21].

2.7.3 Effect of various reagents on enzyme activity

The influence of various reagents on the purified enzyme was investigated by incubating purified enzyme with 1 mM of each reagent: Ca^{2+}, Co^{2+}, Fe^{3+}, K^{+}, Mg^{2+}, Mn^{2+}, Na^{+}, Zn^{2+} and SDS. Residual activity was measured using a standard assay procedure. An activity assay without metal ions was considered to be the reference value (100%).

3. RESULTS AND DISCUSSION

3.1 Effect of Carbon and Nitrogen Sources

Among the 13 carbon sources, *M. siamensis* DMKUA 245^{T} produced high β-xylanase activity of 9.4 and 3.5 U/mL on the basal medium using xylan and mannitol, respectively (Table 2). The induction study on β-xylanase production by *M. siamensis* DMKUA 245^{T} using xylan and mannitol as inducers in the basal medium that contained only glucose as a sole carbon indicated that the addition of xylan and mannitol to the 3rd day of glucose-grown culture increased the enzyme activity compared to when using glucose basal medium, as shown in supplementary Figure 1. This result was similar to the finding that production of thermo-stable xylanase from *Bacillus tequilensis* strain ARMATI which used birchwood xylan (1% w/v) as a carbon source in the basal medium, gave the maximum enzyme production as compared to the other carbon sources [22], while *Geobacillus stearothermophilus* KIBGE-IB29 gave the highest β-xylanase production using 0.5% (w/n\v) xylan (beechwood) as a carbon source [23]. This may be due to xylan being metabolized more slowly by the bacteria as a complex carbon and energy source by xylanase, which produced and liberated xylooligosaccharides which acted as good inducers to yield the maximum amount of enzyme in the fermentation medium. [22]. Carbon source provides energy and carbon skeletons for cell growth and enzyme production, but some simple utilizable sugars caused catabolic repression of enzyme production [24].

Among the organic nitrogen sources, the maximum activities of β-xylanase (12 U/mL) were obtained at 72 h in the medium using casein as a nitrogen source (Figure 1). Casein is a proteinaceous substance which supports microbial growth and enzyme production [25]. Xylan and casein were therefore selected as parameters for further study regarding optimization of enzyme production using a Plackett-Burman experimental design.

Table 2. Effect of various carbon sources on dry weight and β-xylanase activity produced by thermotolerant *M. siamensis* DMKUA 245^T after cultivation for 72 h.

Carbon source (1% w/v)	Dry weight (mg/mL)	β-xylanase activity (U/mL)
None	1.0 ± 0.9	0.3 ± 1.4
Xylan	ND	9.4 ± 0.6
Xylooligosaccharides	3.3 ± 0.03	0.5 ± 1.6
Xylobiose	3.9 ± 0.05	0.6 ± 1.1
Cellobiose	3.7 ± 0.04	0.31 ± 0.7
Lactose	3.3 ± 0.03	0.5 ± 1.2
Maltose	3.2 ± 0.08	0.4 ± 0.7
Glucose	1.3 ± 0.03	0.4 ± 0.6
Fructose	3.6 ± 0.12	0.7 ± 0.2
Xylose	3.1 ± 0.06	0.2 ± 1.0
Galactose	4.2 ± 0.02	1.3 ± 0.7
Mannitol	5.9 ± 0.04	3.54 ± 0.8
Sorbitol	3.5 ± 0.05	0.8 ± 0.4
Xylitol	3.2 ± 0.07	0.5 ± 1.2

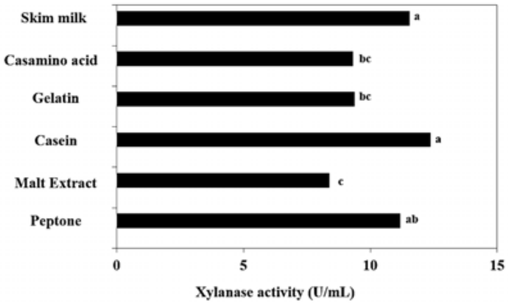

Figure 1. Effect of various nitrogen sources on β-xylanase activity produced by thermotolerant *M. siamensis* DMKUA 245^T after cultivation for 72 h.

3.2 Optimization of Culture Conditions by RSM

The Plackett-Burman design was applied to select those parameters affecting enzyme production. The Plackett-Burman design matrix and response values are listed in Table 3. Among the five variables which were expected to play a critical role in enhancing xylanase production, three factors (casein, $MgSO_4 \cdot 7H_2O$ and temperature) significantly affected enzyme production. The regression analysis data for the Plackett-Burman design are shown in supplementary Table 1. The model had a coefficient of determination (R^2) of 0.990, which could explain 99.0% of the variability of the data. The Plackett-Burman design, a powerful technique for screening important variables, has successfully been used in various reports [26-28]. Model terms having values of '*p*' less than 0.1 were considered significant and hence casein, $MgSO_4 \cdot 7H_2O$ and temperature having the lowest values were found to be statistically significant in affecting β-xylanase production (Supplementary Table 1). Therefore, casein, $MgSO_4 \cdot 7H_2O$ and temperature were selected as parameters for further investigation with respect to optimization of enzyme production.

Table 3. Plackett-Burman experimental design matrix with β-xylanase production by *M. siamensis* DMKUA 245^T.

Run	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	Y (U/mL)
1	1	1	1	-1	1	-1	-1	1	25.47 ± 0.8
2	1	1	-1	1	-1	-1	1	1	13.60 ± 2.3
3	1	-1	1	-1	-1	1	1	1	7.20 ± 3.2
4	-1	1	-1	-1	1	1	1	-1	12.00 ± 2.1
5	1	-1	-1	1	1	1	-1	1	24.39 ± 4.8
6	-1	-1	1	1	1	-1	1	-1	28.95 ± 5.5
7	-1	1	1	1	-1	1	-1	-1	29.56 ± 2.2
8	-1	-1	-1	-1	-1	-1	-1	-1	7.68 ± 1.0

CCD is a very useful tool for determining the optimal level of medium constituents and their interaction. Based on the results from the Plackett-Burman design, casein, $MgSO_4 \cdot 7H_2O$ and temperature were selected for the further evaluation of their effects on xylanase production by CCD. The basal medium based on run number 7 from Plackett-Burman design was selected for further optimization. The results obtained by CCD were analyzed by standard analysis of variance (ANOVA); the predicted and observed responses are presented in Table 4. The second-order regression equation provided the levels of β-xylanase production as a function of initial values of casein, $MgSO_4 \cdot 7H_2O$ and temperature, which can be predicted by the following equation:

$$Y = 30.823 - 11.507X_5 - 3.162X_2^2 - 4.790X_5^2 \qquad (3)$$

where Y is the predicted response (β-xylanase activity, U/mL) and X_2 and X_5 are coded values of casein and temperature, respectively.

Table 4. Experimental design used in response surface methodology of three independent variables, casein (X_2), $MgSO_4 \cdot 7H_2O$ (X_4) and temperature (X_5), with three center points, and the observed and predicted β-xylanase activity.

Treatment Number	Level			Actual level			Xylanase activity (U/mL)	
	X_2	X_4	X_5	X_2	X_4	X_5	Observed	Predicted
1	-1	-1	-1	0.2	0.1	35	33.155 ± 2.8	31.30
2	-1	-1	1	0.2	0.1	45	2.135 ± 3.2	5.95
3	-1	1	-1	0.2	0.5	35	33.925 ± 3.5	30.45
4	-1	1	1	0.2	0.5	45	3.475 ± 1	5.91
5	1	-1	-1	0.8	0.1	35	33.33 ± 0.8	33.48
6	1	-1	1	0.8	0.1	45	5.2 ± 5.1	11.26
7	1	1	-1	0.8	0.5	35	31.54 ± 0.7	30.30
8	1	1	1	0.8	0.5	45	4.455 ± 2.2	8.89
9	-1.68	0	0	0.004	0.3	40	21.03 ± 1.1	19.38
10	1.68	0	0	1.004	0.3	40	30.915 ± 5.1	23.72
11	0	-1.68	0	0.5	0.036	40	33.535 ± 4.8	22.90
12	0	1.68	0	0.5	0.036	40	27.25 ± 5.4	20.19

Table 4. Continued.

Treatment Number	Level			Actual level			Xylanase activity (U/mL)	
	X_2	X_4	X_5	X_2	X_4	X_5	Observed	Predicted
13	0	0	-1.68	0.5	0.3	31.6	33.385 ± 3.3	37.59
14	0	0	1.68	0.5	0.3	48.4	9.37 ± 4.2	-1.69
15	0	0	0	0.5	0.3	40	30.465 ± 0.5	30.84
16	0	0	0	0.5	0.3	40	30.4 ± 0.4	30.84
17	0	0	0	0.5	0.3	40	30.16 ± 0.7	30.84

The results of the second-order response surface model fitting in the form of ANOVA are given in supplementary Table 2. The model presented a determination coefficient ($R^2 = 0.847$) explaining 84.7% of the variability in the response. Model coefficients estimated by regression analysis for each variable are shown in supplementary Table 3. The significance of each coefficient was determined by *t*-values and *P*-values. The larger the magnitude of *t*-test value and smaller the *P*-value indicates the higher significance of the corresponding coefficient. The results revealed that temperature (X_5) and quadratic term of X_2 and X_5 had a significant effect on β-xylanase production. However, no interactions between the two variables were found to contribute to the response at a significant level. The observed xylanase activity (the response) and those from the empirical model Eq. (3) are shown in supplementary Figure 2. The dots represented the actual residuals which lie approximately along a straight line, suggesting a normal distribution of residuals, therefore, the model was adequate.

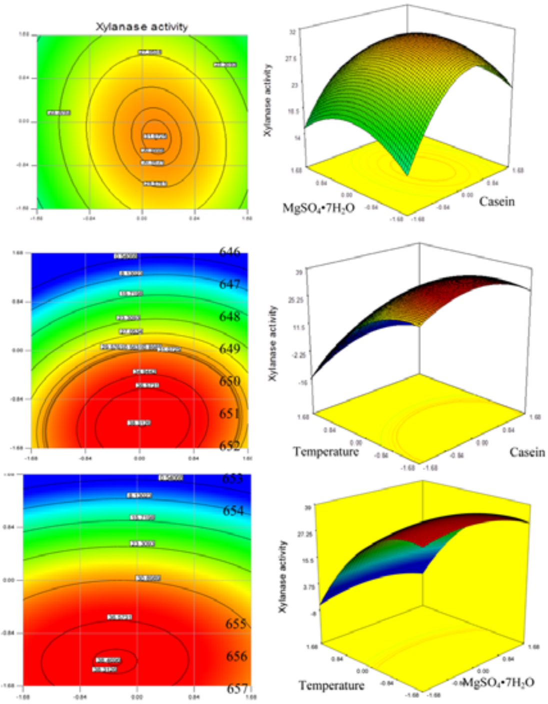

Figure 2. Response plot of the combined effects between casein and temperature (A) and between $MgSO_4 \cdot 7H_2O$ and temperature (B) on β-xylanase production by *M. siamensis* DMKUA 245^T.

A response plot between casein (X_2), $MgSO_4 \cdot 7H_2O$ (X_4) and temperature (X_5) is shown in Figure 1. Decreasing the concentration of casein, $MgSO_4 \cdot 7H_2O$ and temperature resulted in increased xylanase activity. A high casein and $MgSO_4 \cdot 7H_2O$ concentration may result in high production of proteolytic enzymes that can degrade β-xylanase protein during fermentation. This result was supported by the work of Thangamani et al. [29], who reported that a low level of β-xylanase was produced by an alkali-tolerant *Aspergillus fumigatus* AR1 due to the action of high proteolytic degradation on xylanase protein during fermentation. From the second-order polynomial equation (Eq. 3), the maximum predicted value of β-xylanase production was obtained. The maximum β-xylanase production (37 U/mL) was predicted when using X_2, X_4 and X_5 at 0.7 g/L casein, 0.15 g/L $MgSO_4 \cdot 7H_2O$ and a temperature of 35 °C, respectively.

3.3 Validation of the Experimental Model

To verify the optimization results, the experiment was performed under the predicted optimum conditions. The verified condition of the test variables was done at 0.7 g/L casein, 0.15 g/L $MgSO_4 \cdot 7H_2O$ and a temperature of 35 °C. The validity of the experimental model and regression equation was tested by carrying out β-xylanase production in the medium. The predicted response and actual response for β-xylanase production were 37 and 44 U/mL, respectively, which was acceptable values since the model had a determination coefficient (R^2) = 0.847 explaining 84.7% of the variability in the response and about 15.3% of total variation affected by other variables.

The β-xylanase production by the strain *M. siamensis* DMKUA 245^T in the optimum medium as determined by the result of CCD experiments was carried out in a 3 L stirrer fermenter at 35 °C with an aeration rate of 0.5 vvm, agitation speed of 150 rpm and initial pH of 7.0. The maximum xylanase production at 72 h cultivation was 250 U/mL with the productivity of 3.47 U/mL/h. From these studies, an increase of 5.7 and 9.38 folds in enzyme production and productivity, respectively as compared to the shaking flask (Supplementary Figure 3). The increased enzyme activity produced in the stirrer fermenter indicates that aeration and agitation may be required for the growth and enzyme production of β-xylanase by this strain, which may be a key factor in enhancing the enzyme production. The result was similar to the findings of Sukkhum et al. [30] who showed that increasing the aeration rate in the airlift fermenter up to 0.43 vvm increased the production PLA-degrading enzyme by *Actinomadura keratinilytica* strain T16-1. In addition, Techapun et al. [31] reported that the aeration rate was important factor in the stirrer fermenter which yielded the highest xylanase production by *Streptomyces* sp. Ab 106 at aeration rate of 1.0 vvm

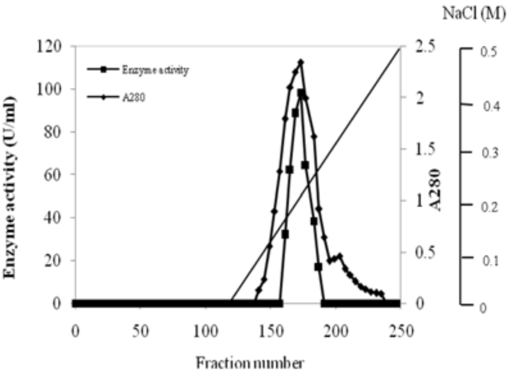

Figure 3. Elution profile of protein and β-xylanase activity on DEAE-Sepharose column chromatography.

3.4 Purification and Characterization of β-xylanase

The enzyme was purified 88-fold with a recovery of 34% and a specific activity of 219.4 U/mg protein (Table 5). The protein profile at a UV wavelength of 280 nm and β-xylanase activity are shown in Figure 3. SDS-PAGE and zymogram analysis, as shown in Figure 4A and 4B, indicated that the molecular weight of purified β-xylanase from the strain DMKUA 245^T was estimated to be 65.8 kDa. The zymogram revealed the presence of a zone of hydrolysis that corresponded with the Coomassie-stained band of purified β-xylanase on SDS-PAGE, confirming the purified protein as β-xylanase (Figure 4B).

Table 5. Purification of β-xylanase from the culture supernatant of thermotolerant *M. siamensis* DMKUA 245^T.

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification fold (fold)	Recovery (%)
Crude enzyme	18000	7215	2.5	1	100
Batch-method of DEAE-Sepharose	7730	201.5	38.4	15	43
DEAE-Sepharose column	6098	27.8	219.4	88	34

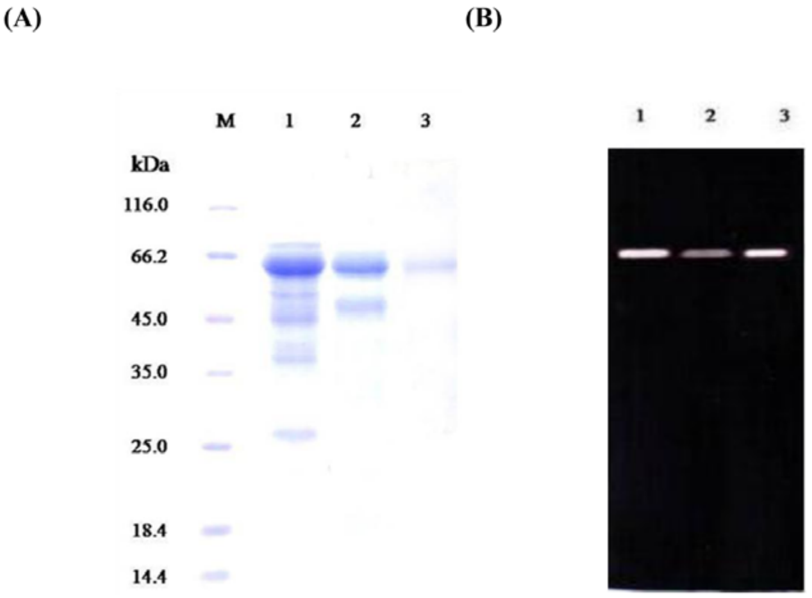

Figure 4. SDS-PAGE (A) and zymogram (B) of the β-xylanase produced by thermotolerant *M. siamensis* DMKUA 245^T. Lane M: LMW protein marker; lane 1: crude enzyme; lane 2: batch-process of DEAE-Sepharose; lane 3: purified β-xylanase. The enzyme was electrophoresed by SDS-PAGE (12%, w/v) and protein bands were stained with Coomassie Brilliant Blue R-205.

3.4.1 Effect of pH and temperature on enzyme activity and stability

The purified β-xylanase was assayed at various pHs and temperatures, as described above. The optimal pH and temperature for β-xylanase activity were 5.5 (Figure 5A) and 60 °C, respectively (Figure 5B). The β-xylanase was active in a pH range of 4-11 (Figure 5C). Moreover, the enzyme was also active in a temperature range of 30-60 °C (Figure 5D); however, it was completely inactivated when pre-incubated for 30 min at over 70 °C. This was similar to the results obtained for xylanase produced from *Streptomyces* sp. CS624 which revealed its highest activity at a temperature of 60 °C and pH 6.0 but its stability was in the pH range 4.5-10.0 and at temperatures ≤ 60 °C [32]. While the purified xylanase from *Bacillus* sp. SN5 had maximum activity at 40 °C and pH 7.0 [33].

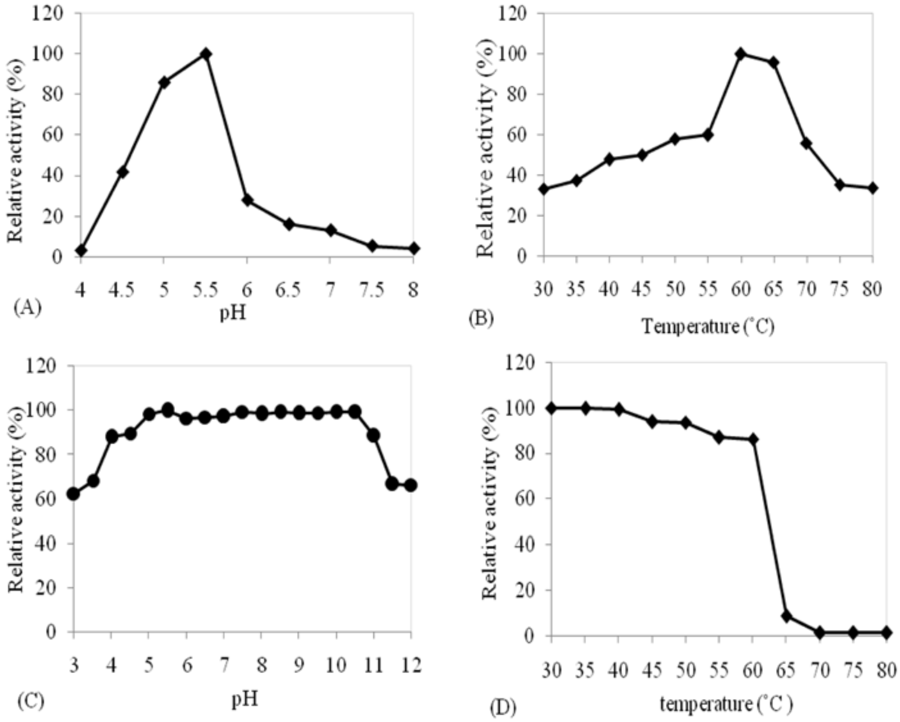

Figure 5. Effect of pH (A) and temperature (B), pH stability (C) and thermostability (D) on the purified β-xylanase produced by thermotolerant *M. siamensis* DMKUA 245^T.

These findings demonstrate the tolerance and resistance characteristics of the new thermotolerant *M. siamensis* DMKUA 245^T β-xylanase against changes in pH and temperature, which are desirable for biotechnological applications such as simultaneous saccharification and fermentation of xylan in agricultural waste for lactic or ethanol production at acidic pH.

3.4.2 Substrate specificity and mode of hydrolysis

The purified β-xylanase showed the highest activity towards beechwood xylan, and slightly decreased activity to insoluble oat spelt xylan. The β-xylanase did not act towards carboxymethyl cellulose, *p*NP-β-D-glucopyranoside, and *p*NP-β-D-xylopyranoside. The hydrolysis products as determined by the TLC of wood xylans by the purified β-xylanase were a series of short-chain oligosaccharides (Supplementary Figure 4). Xylobiose was slightly detected from beech wood xylan. These results may be due to differences in composition and structure of beechwood xylan and wood xylan; structures of xylooligosaccharides were derived from the initial action of the purified enzyme, and which therefore contained different side chains, resulting in a variety of hydrolysate products. In addition, the purified β-xylanase could not hydrolyze xylobiose. This result confirmed that the purified β-xylanase from *M. siamensis* DMKUA 245^T was endo-β-1, 4-xylanase.

3.4.3 Effect of reagent and metal ions on xylanase activity

The effect of reagent and metal ions on β-xylanase activity of purified enzyme were measured under standard assay conditions. It was found that β-xylanase activity of purified enzyme was stimulated by Co^{2+}, K^+ and Mg^{2+} to 182%, 125% and 159%, respectively (Table 6). These results illustrate the ability of metal ions to increase enzyme activity, and are similar to other reports, such as enhancement of activity by Co^{2+} and Mg^{2+} [24] and inhibition by Mn^{2+} [34, 35]. The xylanase produced from *Streptomyces* sp. CS624 enhanced its activity by the addition of Co^{2+} but was strongly inhibited by Mg^{2+} [32]. SDS showed little effect on the activity. This may indicate that the enzyme molecule is the monomeric.

According to metal ion analysis, Co^{2+}, K^+ and Mg^{2+} stimulated the enzyme activity; therefore, the stability of purified β-xylanase with these ions was investigated. To the purified β-xylanase, each of 1 mM Co^{2+}, K^+ and Mg^{2+} was added and kept at 4 °C overnight. It was found that the residual activity of the metal-treated purified enzymes after incubation at temperatures of 40-90 °C was higher than the control, especially with Co^{2+} which was still stable when the temperature was up to 90 °C (Figure 6). This result confirmed that Co^{2+}, K^+ and Mg^{2+} enhance the thermostability of the enzyme. This is the first report to illustrate that these ions enhance the thermostability of β-xylanase activity.

Table 6. Effect of metal ions and reagents on the activity of purified β-xylanase.

Metal ions (1 mM)	Relative activity (%)
Control	100 ± 7
Ca^{2+}	97 ± 2
Co^{2+}	182 ± 5
Fe^{3+}	97 ± 2
K^+	125 ± 3
Mg^{2+}	159 ± 2
Mn^{2+}	56 ± 4
Na^+	108 ± 8
Zn^+	82 ± 7
SDS	93 ± 2

The data are mean ± standard deviations of the means.

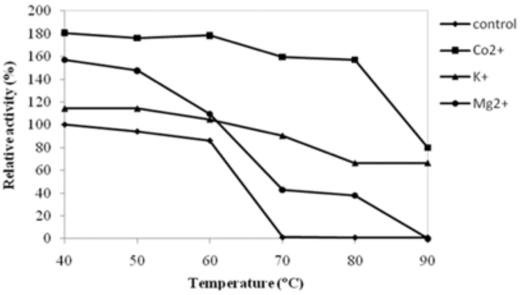

Figure 6. The effect of 1 mM Co^{2+}, K^+ and Mg^{2+} at different temperatures on the thermostability of purified xylanase produced by thermotolerant *M. siamensis* DMKUA 245^T.

According to our findings, Co^{2+} could significantly increase the thermostability of purified β-xylanase activity; therefore, the effect of $CoCl_2 \cdot 6H_2O$ on β-xylanase production was investigated. A concentration of 1 mM (0.238 g/L) $CoCl_2 \cdot 6H_2O$ was added to the optimized medium obtained by CCD. In the presence of $CoCl_2 \cdot 6H_2O$, the β-xylanase activity was higher than in its absence (Supplementary Figure 5). However, proteolytic activity, about 30 U/mL, was found in the medium with or without addition of $CoCl_2 \cdot 6H_2O$, indicating that $CoCl_2 \cdot 6H_2O$ does not inhibit protease production. The increase of β-xylanase production may, therefore, be due to the effect of Co^{2+} on the increase of enzyme thermostability during cultivation. The evolution of β-xylanase production by *M. siamensis* DMKUA 245^T is summarized in Supplementary Table 4.

4. CONCLUSIONS

The optimal conditions for maximum enzyme production by the new thermotolerant *M. siamensis* DMKUA 245^T were successfully determined by a CCD experiment which estimated the optimum values of the factors obtained from a Plackett-Burman design. The optimization resulted in xylanase production of 44 U/mL and 250U/mL observed in a shaking flask and 3 L stirrer fermenter, respectively. Characterization of the purified β-xylanase demonstrated its tolerance and resistance against changes in pH and temperature, which are desirable for biotechnological applications such as simultaneous saccharification and fermentation of xylan in agricultural waste for xylitol, lactic acid or ethanol production at acidic pH or use in the feed industry.

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