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Characterization and optimization of xylanase and endoglucanase production by *Trichoderma viride* HG 623 using response surface methodology (RSM)

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Xylanase and endoglucanase production from *Trichoderma viride* HG 623 and their properties were investigated in this research. By using response surface methodology, the optimal concentrations for xylanase and endoglucanase production were carbon (rice straw: corn straw=6:1), 26.91 g/L; NH₄Cl, 3.77 g/L; KH₂PO₄, 5.31 g/L and carbon (rice straw:corn straw=6:1), 26.99 g/L; NH₄Cl, 3.80 g/L, and KH₂PO₄, 5.23 g/L, respectively. Under these optimal conditions, the xylanase and endoglucanase activity from *T. viride* HG 623 reached 135.51 and 40.89 IU/g, respectively. Verification of the optimization showed that xylanase and endoglucanase activity were 139.57 and 41.46 IU/g, respectively. The optimal pH of xylanase and endoglucanase activity from *T. viride* HG 623 was 5.0 and the optimal temperature were 60 and 55°C, respectively. The activity of xylanase and endoglucanase were stable when incubated from 35 to 55°C for 1 h. The xylanase and endoglucanase activity of *T. viride* HG 623 were stable from pH 3.0 to 7.5 at 50°C. Xylanase activity showed the highest level (150.36 IU/g) when activated by 75 mM of Co²⁺, and endoglucanase activity reached the highest level (36.99 IU/g) when activated by 75 mM of Mg²⁺. The wheat bran was the optimal natural substrate for enzyme production of *T. viride* HG 623. The results of this study would instruct the cellulase and hemicellulase production on industrial scale.

Key words: *Trichoderma viride* HG 623, xylanase, endoglucanase, response surface methodology, characterization.

INTRODUCTION

It is well known that energy consumption has increased progressively as the result of growing world population and industrialization. Owing to the realization of diminishing natural oil and gas resources, interests in the bioconversion of abundant and renewable cellulosic biomass into fuel ethanol as an alternative to petroleum is rising around the world (Cardona and Sánchez, 2007). Huge quantities of agro-industrial biomass are produced worldwide annually, that is including about 900 million tons of

rice straw (RS) which more than 90% are produced in Asia (Jahromi et al., 2011). The RS mainly consists of cellulose, hemicellulose and lignin. Cellulose and hemicelluloses could be degraded into hexose and pentose which are able to be fermented into ethanol by *Saccharomyces cerevisiae* and *Pichia stipitis*, respectively (Agbogbo and Coward-Kelly, 2008). However, the degradation of cellulose and hemicellulose is the limiting step during the conversion of biomass into bioenergy.

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Xylanases (1,4- β -D-xylan xylanohydrolase; EC 3.2.1.8) are the main constituents of the xylanolytic enzyme system, which hydrolyze xylan into Xylo-oligosaccharide mostly (Geetha and Gunasekaran, 2010; Zhang et al., 2011; Shin et al., 2009). Endoglucanases are parts of the cellulase complex involved in cellulose degradation. They attack cellulose fibres at random and cleave them at more amorphous regions which, in turn, create sites for other enzymes like exoglucanases and β -glucosidases (Yoon et al., 2008). The synergistic action of endoglucanase (EG), cellobiohydrolase (CBH) and β -glucosidase with the help of hemicellulases (mainly xylanases) results in the complete degradation of cellulosic materials (Quiroz et al., 2011; García et al., 2007).

The traditional 'one-factor at a time' technique used for optimizing a multivariable system is not only time-consuming but also often easily misses the alternative effects between components. Conventional methods for optimal culture conditions based on the classical method of 'one-variable-at-a-time' bioprocess design in which, one independent variable is studied while fixing all others at a specific level, may be effective in some situations, but may fail to consider the combined effects of all involved factors and lead to misleading results and inaccurate conclusions (Silva and Roberto, 2001). Recently, many statistical experimental design methods have been employed in bioprocess optimization. Among them, response surface methodology (RSM) is the one suitable for identifying the effect of individual variables and for seeking the optimum conditions for a multivariable system efficiently (Li et al., 2007a; Maria et al., 2009).

The optimization of culture medium and culture conditions for improvement of the production of CMCase and xylanases have been reported by many scholars (Evangelos et al., 2003; Sonia et al., 2005; Irfan et al., 2012; Romdhane et al., 2010). In this study, response surface methodology (RSM) was used to determine the effects of several variables and to optimize enzyme production conditions. The mathematical models were established and showed the relation of the enzyme activity to independent variables. By this way, the maximum enzyme activity was ensured through the prediction of the optimum values of the independent variables. Furthermore, the characterization of xylanase and endoglucanase from the *T. viride* HG 623 were researched.

MATERIALS AND METHODS

Microorganism and preparation of spore suspension

T. viride HG 623 was selected from a screening from *T. viride* CICC13038 (obtained from China Center of Industrial Culture Collection) mutated by physical and chemical methods. Briefly, spore suspension of *T. viride* CICC13038 (5 mL) was treated with 2% diethyl sulfate for 20 min and then illuminated 2 min with an 30-w ultraviolet lamp. The mutant producing the highest enzyme activity was selected and named *T. viride* HG 623. It was cultured on potato dextrose agar (PDA) for 10 generations, and then maintained on PDA at 4°C.

Spore suspension of *T. viride* HG 623 was washed from the PDA plate with sterile water then inoculated in 100 mL of potato dextrose broth in a 500-mL Erlenmeyer flask, and the spores were grown at 28°C on PDA at 250 rpm for 48 h.

Raw material

Rice straw and corn straw were dried overnight at 70°C and cut into approximate 1 cm size, then sifted by 50 meshes sieve. The fractions passing through sieve were used as the substrate for submerged cultivation.

Medium and culture conditions

The medium used for enzyme production was composed of (g/L): Tween 80, 1.5; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.3; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.3; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.005; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.0014; $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 0.0016 and $\text{CoCl}_2 \cdot 7\text{H}_2\text{O}$, 0.002 (Van Wyk and Mohulatsi, 2003). The concentrations of Carbon, NH_4Cl and KH_2PO_4 were adjusted according to the experimental design. The pH was adjusted to 5.0-6.0. The spore culture (10.0%) was inoculated in 20 mL of individual test media in 50 mL shake-flask. Then, the flasks were incubated on a shaker at 28°C, 250 rpm for 5 days. Crude enzyme preparation was obtained from the enzyme-containing broth by centrifugation at 5,000x g for 10 min.

Enzyme assay

The xylanase activity was determined by measuring the release of reducing sugars from oat spelt xylan (1% w/v) using the dinitrosalicylic acid method (Miller, 1959). The reaction mixture, containing 1 ml of a solution of 1% oat spelt xylan in a citrate buffer 50 mM, pH 5.0 plus 1 ml of the diluted crude enzyme, was incubated for 30 min at 50°C. One unit of xylanase was defined as the amount of enzyme required to release 1 mmol of xylose from xylan per minute under the assay conditions.

The CMCase activity was measured by using Meinke's procedure (Meinke et al., 1995) with some modifications. The reaction mixture, consisting of 1 ml 1% CMC-Na as the substrate and 0.5 ml of cultured supernatant after centrifugation, was incubated at 50°C for 30 min, supplemented with 0.5 ml of 3,5-dinitrosalicylic acid and boiled for 10 min. After cooling, the reduced sugars released in response to CMCase activity were measured at 540 nm. One unit of CMCase activity was defined as the amount of enzyme required for releasing total reduced sugar equivalent to 1 μmol glucose per minute.

Plackett-Burman experimental design

The Plackett-Burman experimental design (Plackett and Burman, 1946) based on the first-order model:

$$Y = \beta_0 + \sum \beta_i X_i \quad (1a)$$

Used to screen the important variables that influence enzyme production. Total number of trials to be carried out according to the Plackett-Burman was k+1, where k is number of variables (medium components). Each variable is represented at two levels (high and low) that are denoted by (+1) and (-1), respectively (Table 1).

Central composite design

According to the central composite design (CCD) (Box and Wilson,

Table 1. Range of variables at different levels for the fractional factorial design.

Independent variables X_i (g/L)	Level	
	-1	1
X_1 *Carbon	10	40
X_2 NH_4Cl	1	5
X_3 KH_2PO_4	1	5
X_4 $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	0.003	0.007
X_5 $\text{MnSO}_4 \cdot \text{H}_2\text{O}$	0.001	0.002
X_6 $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	0.001	0.002
X_7 $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.1	0.5
X_8 CaCl_2	0.1	0.5
X_9 $\text{CoCl}_2 \cdot 7\text{H}_2\text{O}$	0.001	0.003
X_{10} Tween 80	1	3

*Carbon contained rice and corn stalk (6:1).

1951), a five-level, three-factor factorial central composite design and nine replicates at the center points leading to 23 runs was employed for the optimization of the enzymatic production. The variables were coded according to Equation:

$$x_i = (X_i - X_0) / \Delta X_i \quad (1b)$$

Where, x_i is the coded value, X_i is the real value, X_0 is the real value at the center point, and ΔX_i is the step change value.

A second-order polynomial model for predicting the optimal point was expressed as Equation:

$$Y = A_0 + \sum A_i X_i + \sum A_{ii} X_i^2 + \sum A_{ij} X_i X_j \quad (1c)$$

Where, Y is the predicted response, A_0 is the interception coefficient, A_i is the linear effect, A_{ii} is the squared effect, and A_{ij} the interaction effect. The accuracy and general ability of the above polynomial model could be evaluated by the coefficient of determination R^2 .

The characterization assays of enzymes

The stability of pH was measured by incubating the crude enzyme in different pH using buffer solutions 50 mM sodium citrate (pH 3.0-6.0), sodium phosphate (pH 6.5 - 8.0), and Tris-HCl (pH 8.5-9.0) for 48 h at 4°C, and then the residual activity was measured under standard conditions (pH = 5.0). The optimum pH was explored at 50°C between pH 3 and 9 at intervals of 0.5 pH units.

Thermal stability was assayed by incubating the crude enzyme at different temperature (ranging 30-90°C at intervals of 5 °C) in 50 mM citrate buffer, pH 5.2, for 1 h, and then the residual activity was measured by incubation at 50°C for 30 min. The optimum temperature was studied at pH 5.6 between 30 and 90°C at 5°C intervals for 30 min, using CMC-Na and oat spelt xylan as substrate

To investigate the effect of ions on enzymatic activity, the enzyme activity was assayed in the reaction buffer supplemented with 75 mM of metal ion. Several different buffer solutions were prepared; each contained a different metal salt (MgSO_4 , AgNO_3 , ZnSO_4 , CuSO_4 , BaCl_2 , FeCl_3 , FeSO_4 , CoCl_2 , MnSO_4 , $\text{Al}_2(\text{SO}_4)_3$, CaCl_2). All of the above experiments were completed in triplicate, and average values were calculated based on results from three independent experiments.

To study the effect of various substrates on enzyme activity, 1.5 ml 1% substrates was added into reaction system, containing of 0.5 ml of crude enzyme preparation, and incubated for 30 min at 50°C. Carboxymethyl cellulose sodium (CMC-Na), filter, cotton, rice stalk,

corn stalk, wheat bran, wheat straw, sawdust, soybean straw, coconut shell and CCM were used as different substrates.

RESULTS

Screening of Significant Nutrient Components for Xylanase and Endoglucanase Production by *T. viride* HG 623

Ten factors were chosen to optimize the condition for enzyme production of *T. viride* HG 623 (Table 1). Table 2 shows the Plackett–Burman design for 12 trials, which were two levels of concentrations for ten different nutrient components and corresponding enzyme activity. It was found that the variables X_1 (Carbon), X_2 (NH_4Cl), and X_3 (KH_2PO_4) had significant influence on xylanase and endoglucanase production ($P < 0.05$) (Table 3) in ten variables.

Regression models of response

The variables X_1 (Carbon), X_2 (NH_4Cl) and X_3 (KH_2PO_4) were confirmed as important factors through the factorial analysis of Plackett–Burman experiment. The coded values of variables are shown in Table 4. The optimal concentrations of these three variables needed to be further measured by CCD design. The experimental responses for the 23 runs are presented in Table 5. The multiple regression equations (Equation 2) for xylanase and endoglucanase production were performed on the experimental data.

$$Y_1 = 126.195 + 9.3169X_1 + 8.3884X_2 + 8.7119X_3 + 0.5727X_1X_2 + 0.6790X_2X_3 - 0.2320X_1X_3 - 6.9511X_1^2 - 5.9909X_2^2 - 6.9047X_3^2 \quad (2a)$$

$$Y_2 = 36.3283 + 4.4854X_1 + 4.8224X_2 + 3.4610X_3 + 0.3781X_1X_2 + 0.7968X_2X_3 + 0.6994X_1X_3 - 3.7323X_1^2 - 3.4738X_2^2 - 3.7455X_3^2 \quad (2b)$$

Where, Y was the predicted response [xylanase (Y_1) and

Table 2. Plackett-Burman design matrix for ten variables with the experimental values of xylanase and endoglucanase activity by *T. viride* HG 623.

Run	X ₁	X ₂	X ₃	X ₄	X ₅	X ₆	X ₇	X ₈	X ₉	X ₁₀	Xylanase activity (IU/g)	Endoglucanase activity (IU/g)
1	1	-1	1	-1	-1	-1	1	1	1	-1	74.78	25.22
2	1	1	-1	1	-1	-1	-1	1	1	1	80.36	24.96
3	-1	1	1	-1	1	-1	-1	-1	1	1	74.08	23.01
4	1	-1	1	1	-1	1	-1	-1	-1	1	76.40	23.73
5	1	1	-1	1	1	-1	1	-1	-1	-1	81.19	25.21
6	1	1	1	-1	1	1	-1	1	-1	-1	98.57	30.61
7	-1	1	1	1	-1	1	1	-1	1	-1	70.12	21.78
8	-1	-1	1	1	1	-1	1	1	-1	1	64.58	20.06
9	-1	-1	-1	1	1	1	-1	1	1	-1	33.11	10.28
10	1	-1	-1	-1	1	1	1	-1	1	1	71.75	22.28
11	-1	1	-1	-1	-1	1	1	1	-1	1	71.72	22.27
12	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	31.45	9.78

Table 3. Effect estimates for xylanase and endoglucanase production from the results of the Plackett-Burman design.

Factor	MC	Xylanase				Endoglucanase			
		Es.	SE	T ratio	P value	Es.	SE	T ratio	P value
X ₁	Carbon#	22.999	0.925	24.85	0.0256*	7.476	0.0459	162.80	0.0039*
X ₂	NH ₄ Cl	20.659	0.925	22.32	0.0285*	6.082	0.0459	132.46	0.0048*
X ₃	KH ₂ PO ₄	14.823	0.925	16.02	0.0397*	4.937	0.0459	107.51	0.0059*
X ₄	FeSO ₄	-2.766	0.925	-2.99	0.2055	-1.193	0.0459	-25.97	0.0245
X ₅	MnSO ₄	3.074	0.925	3.32	0.1861	0.621	0.0459	13.53	0.0470
X ₆	ZnSO ₄	2.540	0.925	2.74	0.2224	0.456	0.0459	9.92	0.0639
X ₇	MgSO ₄	6.695	0.925	7.23	0.0875	2.412	0.0459	52.53	0.0121
X ₈	CaCl ₂	3.022	0.925	3.27	0.1892	1.272	0.0459	27.70	0.0230
X ₉	CoCl ₂	-3.285	0.925	-3.55	0.1748	-0.687	0.0459	-14.96	0.0425
X ₁₀	Tween 80	8.276	0.925	8.94	0.0709	2.237	0.0459	48.72	0.0131

MC, Medium components; Es., estimate; SE, standard error. *Significant at 5% level ($P < 0.05$); #Carbon contained rice and corn stalk (6:1)

Table 4. Coded values of variables used in central composite design.

Independent variable Xi (g/L)	Level				
	-1.68	-1	0	1	1.68
X ₁ #Carbon	3.18	10	20	30	36.82
X ₂ NH ₄ Cl	1.32	2	3	4	4.68
X ₃ KH ₂ PO ₄	0.64	2	4	6	7.36

#Carbon contained rice straw and corn stalk (6:1).

endoglucanase (Y_2) production]; X_1 , X_2 , X_3 were coded values of carbon concentration, NH₄Cl concentration and KH₂PO₄ concentration, respectively. The statistical significance of Equation 2 was done by the analysis of variance (ANOVA) in Table 6. The coefficients of determination (R^2) were 0.9496 and 0.9607 for xylanase and endoglucanase production, illustrating that the sample variation of more than 94.96% and 96.07% were explained by the fitted models.

The significant coefficients of the full second-order polynomial model of xylanase and endoglucanase production were shown through the Student t -distribution and the corresponding P -value (Table 7). The P -values of less than 0.05 indicated the more significant correlation of coefficients. Table 7 suggests that the independent variables X_1 (Carbon concentration), X_2 (NH₄Cl concentration), X_3 (KH₂PO₄ concentration) and the quadric term of these three variables had a significant effect on xylanase pro-

Table 5. Central composite experiment design matrix with experimental values of xylanase and endoglucanase production by *T. viride* HG 623.

Trial number	Variable			Response	
	X ₁ (Carbon)	X ₂ (NH ₄ Cl)	X ₃ (KH ₂ PO ₄)	Xylanase activity (IU/g)	CMCase activity (IU/g)
1	-1	-1	-1	89.36	16.32
2	-1	-1	1	99.50	19.26
3	-1	1	-1	95.67	21.99
4	-1	1	1	114.65	27.13
5	1	-1	-1	100.47	22.03
6	1	-1	1	115.81	26.78
7	1	1	-1	115.20	28.22
8	1	1	1	127.13	37.15
9	-1.68179	0	0	85.14	17.06
10	1.68179	0	0	125.46	35.95
11	0	-1.68179	0	88.08	16.60
12	0	1.68179	0	127.94	37.87
13	0	0	-1.68179	86.82	18.88
14	0	0	1.68179	124.04	34.05
15	0	0	0	126.55	36.75
16	0	0	0	126.33	36.85
17	0	0	0	125.08	35.24
18	0	0	0	125.38	35.23
19	0	0	0	126.94	36.40
20	0	0	0	127.00	36.92
21	0	0	0	125.07	35.28
22	0	0	0	127.71	37.79
23	0	0	0	126.13	36.24

Table 6. Analysis of variance for the response of xylanase and endoglucanase production.

Source	DF	Xylanase ^a				Endoglucanase ^b			
		SS	MS	F-value	P > F	SS	MS	F-value	P > F
Linear	3	3182.95	—	148.25	<0.0001*	755.94	—	172.40	<0.0001*
Quadratic	3	2095.55	—	97.6	<0.0001*	635.99	—	145.05	<0.0001*
Cross product	3	6.74	—	0.31	2.3070	10.14	—	2.31	1.2815
Total model	9	5256.85	584.09	27.20	<0.0001*	1393.40	154.82	35.31	<0.0001*
Total error	13	279.12	21.47	—	—	57.00	4.38	—	—

DF, Degree of freedom; SS: Sum of squares; MS, Mean square. ^acoefficient of variation (CV) = 4.05; coefficient determination (R^2) = 0.9496; correlation coefficient (R) = 0.9745; ^bcoefficient of variation (CV) = 7.0206; coefficient determination (R^2) = 0.9607; correlation coefficient (R) = 0.9802. *Significant at 5% level ($P < 0.05$).

duction and endoglucanase production. Interactions between the three variables had no significance ($P > 0.05$).

Localization of optimum condition

The optimal values of the independent variables for enzyme production could be observed from the 3D response surface plots and the corresponding contour plots (Figures 1 to 3). Figure 1A and B show the effect of Carbon and NH₄Cl on the endoglucanase and xylanase production by *T. viride* HG 623, while KH₂PO₄ was fixed

at its middle level (4 g/L). The endoglucanase and xylanase production reached a maximum point when concentrations of carbon and NH₄Cl were used between 24-30 and 3.6-4.2 g/L, respectively. Figure 2A and B present the effects of carbon and KH₂PO₄ on the endoglucanase and xylanase production, while NH₄Cl concentration was fixed at its middle level (3 g/L). The maximum endoglucanase and xylanase activity predicted from the model when concentrations of Carbon and KH₂PO₄ were used between 24-30 and 5.0-6.0 g/L, respectively. Figure 3A and B show the effects of NH₄Cl and KH₂PO₄ on the

Table 7. The least-square fit and parameters (significant of regression coefficient) for the response of xylanase and endoglucanase production.

Model term	DF	Xylanase				Endoglucanase			
		Estimate	SE	t-value	P> t	Estimate	SE	t-value	P> t
Intercept	1	126.195	1.544	81.76	<0.0001	36.328	0.698	52.08	<0.0001*
X ₁	1	9.317	1.254	7.43	<0.0001*	4.485	0.567	7.92	<0.0001*
X ₂	1	8.388	1.254	6.69	<0.0001*	4.822	0.567	8.51	<0.0001*
X ₃	1	8.712	1.254	6.95	<0.0001*	3.461	0.567	6.11	<0.0001*
X ₁ X ₂	1	0.573	1.638	0.35	0.7322	0.378	0.740	0.51	0.6181
X ₂ X ₃	1	0.679	1.638	0.41	0.6853	0.797	0.740	1.08	0.3014
X ₁ X ₃	1	-0.232	1.638	-0.14	0.8895	0.699	0.740	0.94	0.3620
X ₁ ²	1	-6.951	1.162	-5.98	<0.0001*	-3.732	0.525	-7.10	<0.0001*
X ₂ ²	1	-5.991	1.162	-5.15	0.0002*	-3.474	0.525	-6.61	<0.0001*
X ₃ ²	1	-6.905	1.162	-5.94	<0.0001*	-3.746	0.525	-7.13	<0.0001*

DF, Degree of freedom; SE, standard error.*Significant at 5% level (P<0.05).

Figure 1A. Response surface plot and contour plot of the combined effects of Carbon and NH₄Cl on the CMCASE production by *T. viride* HG 623 with constant cultivation time (120 h).

endoglucanase and xylanase production, while Carbon concentration was fixed at its middle level (20 g/L). The optimum pairs of NH₄Cl and KH₂PO₄ concentration for maximum endoglucanase and xylanase activity were between 3.6-4.2 and 5.0-6.0 g/L, respectively. Based on these results, the optimal concentrations of Carbon, NH₄Cl and KH₂PO₄ for xylanase production were calculated as 26.91, 3.77 and 5.31 g/L, respectively. When these concentrations of carbon, NH₄Cl and KH₂PO₄ were used, the maximum xylanase activity predicted from the

model was 135.51 IU/g. The optimum concentrations for endoglucanase production were 40.89 IU/g when concentrations of Carbon, NH₄Cl and KH₂PO₄ were 26.99, 3.80 and 5.23 g/L, respectively.

The evaluated experiments were carried out under optimal condition. The xylanase and endoglucanase activity were 139.57 IU/g and 41.46 IU/g when the optimal concentrations of carbon, NH₄Cl and KH₂PO₄ were used. The mean values of xylanase and endoglucanase were 3.0% and 1.4% more than the predicted value, respectively.

Figure 1B. Response surface plot and contour plot of the combined effects of Carbon and NH_4Cl on the xylanase production by *T. viride* HG 623 with constant cultivation time (120 h).

Figure 2A. Response surface plot and contour plot of the combined effects of Carbon and KH_2PO_4 on the CMCase production by *T. viride* HG 623 with constant cultivation time (120 h).

Properties of xylanase and endoglucanase from *T. viride* HG 623

The xylanase activity rose slowly from 30 to 60°C, reached its summit at 60°C, and reduced beyond 60°C. The endoglucanase activity increased slowly from 30 to 55°C, reached its peaked at 55°C, and decreased beyond 55°C. Conclu-

sively, the optimal temperature for the xylanase and endoglucanase activity was 60°C and 55°C, respectively (Figure 4A). The xylanase and endoglucanase activities were stable after incubation for 1 h from 35 to 55°C and decreased rapidly when temperature was beyond 60°C (Figure 4B). The activities of xylanase and endoglucanase were the highest at pH 5.0 (Figure 4C). The xylanase

Figure 2B. Response surface plot and contour plot of the combined effects of Carbon and KH_2PO_4 on the xylanase production by *T. viride* HG 623 with constant cultivation time (120 h).

Figure 3A. Response surface plot and contour plot of the combined effects of NH_4Cl and KH_2PO_4 on the CMCase production by *T. viride* HG 623 with constant cultivation time (120 h).

and endoglucanase activities were stable at 50°C when pH was between 3.0 to 7.5. The xylanase and endoglucanase activities were reduced dramatically beyond pH 8.0 (Figure 4D). The xylanase and endoglucanase from *T. viride* HG 623 were stable over a wide pH range (pH 3.0-7.5) (Figure 4D) and the optimum enzyme activity of xylanase and endoglucanase was at 60 and 55°C , respectively (Figure 4A).

To investigate the effect of metal ions on enzymatic activity, the crude enzyme was dissolved in the reaction buffer, which was added with a metal ion at a concentration of 75 mM. Several different buffer solutions were prepared, each spiked with a different metal. The variance analysis showed that xylanase ($F = 8.45$, $df = 11$, $P < 0.05$) and endoglucanase ($F = 835.55$, $df = 11$, $P < 0.01$) activities were significantly different in the presence of

Figure 3B. Response surface plot and contour plot of the combined effects of NH_4Cl and KH_2PO_4 on the xylanase production by *A. T. viride* HG 623 with constant cultivation time (120 h).

Figure 4. Activities and properties of endoglucanase and xylanase from *T. viride* HG 623. **A.** Effects of temperature on endoglucanase and xylanase activities of *T. viride* HG 623. The enzyme activity was measured at 30 to 90°C for 30 min at 5°C intervals (pH 5.6). **B.** Effects of temperature on endoglucanase and xylanase stability of *T. viride* HG 623. The dialyzed fraction (pH 5.2) was incubated at 30 to 90°C for 1 h at 5°C intervals, and then the residual activity was measured by incubation at 50°C for 30 min. **C.** Effects of pH on endoglucanase and xylanase activities of *T. viride* HG 623. The enzyme activity was measured in the reaction buffer at different pH values between 3 and 9 at intervals of 0.5 pH units. **D.** Effects of pH on endoglucanase and xylanase stability of *T. viride* HG 623. The dialyzed fraction was incubated in different pH buffers (pH 3.0–9.0) at 4°C for 48 h, and then the residual activity was measured under standard conditions (pH=5.0). All the experiments were performed three times.

Figure 5. Effects of exposure to different metal ions on the endoglucanase and xylanase activities of *T. viride* HG 623. **A.** Effect of different metal ions on the endoglucanase activity. **B.** Effect of different metal ions on the xylanase activity. Several different reaction buffers were prepared, each spiked with 75 mM of a metal ion. One unit of endoglucanase and xylanase activity were defined as the amount of enzyme required for releasing total reduced sugar equivalent to 1 mmol glucose or xylose per minute, respectively. Con: control; the enzyme activity of *T. viride* HG 623 was measured under the normal reaction condition without any additional ions. The experiments were performed three times. Different letters above the columns indicate a significant difference determined by Duncan's multiple comparisons test (A: $P < 0.01$; B: $P < 0.05$).

different metal ions. The activity of endoglucanase in *T. viride* HG 623 was stimulated slightly by Mg²⁺, Co²⁺, Fe³⁺, Ca²⁺ and Zn²⁺, and strongly by Mg²⁺ and Co²⁺, but was inhibited by Ag⁺. When Co²⁺ was present, the activity of xylanase was stimulated strongly. However, xylanase activity was inhibited by Al³⁺ (Figure 5).

To investigate the effect of different substrates on enzymatic activity, the variance analysis showed that enzyme activity was significantly different ($F = 166.36$, $df = 10$, $P < 0.01$) in the presence of different substrates. CMC synthesized substrates, did not have significant differences with wheat bran (Figure 6). These results indicate that wheat bran was optimal substrate for enzyme production in natural substrate.

DISCUSSION

The nutritional component showed the significant effect on cellulase and hemicellulase production of microorganisms. The enzyme activities of cellulase and hemicellulase could be improved when cultured in mixed carbon resources. It has been reported that culture medium containing rice straw and wheat bran (1:3) as carbon source increased maximal cellulases and xylanases production by *Scytalidium thermophilum* (Jatinder et al., 2006). Sushil Nagar reported a cumulative effect of peptone, yeast extract, and KNO₃ on xylanase production by *Bacillus pumilus* SV-85S (Nagar et al., 2010). Phosphate concentrations had a potential influence on the fungus morphology and

Figure 6. Effects of exposure to different substrates on the enzyme activity *T. viride* HG 623. Con: control, the activity of *T. viride* HG 623 was measured under the normal reaction condition with CMC-Na as carbon sources. The experiments were performed three times. Different letters above the columns indicate a significant difference determined by Duncan's multiple comparisons test ($P < 0.01$).

xylanase activity (Siedenberg et al., 1997). These indicated that various fungi had different ability of utilization of nutrients for enzyme production. In our research, mixed carbon resource, NH_4Cl and KH_2PO_4 showed significant effects on enzymes production at the 5% level. The lower value of coefficient of variation (CV) shows the higher reliability of experiment (Box et al., 1978). In our study, the lower value of CV (4.05 and 7.0206) shows the better accuracy and reliability of the experiments (Table 6).

Different microorganisms have different enzyme characterization. The optimal temperature of xylanase and endoglucanase from *T. viride* HG 623 were at 60°C and 55°C, respectively, and they were stable over a wide pH range (pH 3.0-7.5) (Figure 4A and 4D). In comparison, the optimal temperature of xylanase from *T. viride* HG 623 was higher than that of the *Bacillus* sp JB99 (45°C) (Kumar et al., 2011), and endoglucanase from *T. viride* HG 623 had a wider pH range than that of *Mucor circinelloides* (pH 5.0-9.0) (Saha, 2004). The activity of endoglucanase in *T. viride* HG 623 was stimulated strongly by Mg^{2+} and Co^{2+} , but was inhibited by Ag^+ . When Co^{2+} were present, the activity of xylanase was stimulated strongly. However, xylanase activity was inhibited by Al^{3+} (Figure 5). Quay et al. (2011) reported that endoglucanase from *Aspergillus niger* was inhibited by Mn^{2+} , Co^{2+} , Zn^{2+} , Mg^{2+} , Ba^{2+} , Fe^{2+} , Ca^{2+} and K^+ . The xylanase was strongly inhibited by Hg^{2+} in *Aspergillus niger* RS2. These results show that enzyme from different species may be affected by different ions. Wheat bran was the

optimal substrate for enzyme activity, which could maybe relate to the concentrations and structure of cellulose and hemicelluloses of the wheat bran.

In summary, a maximum endoglucanase activity was 40.89 IU/g following final optimal condition [Carbon (26.99 g/L), NH_4Cl (3.80 g/L), and KH_2PO_4 (5.23 g/L)] and a maximum xylanase activity was 135.51 IU/g following final optimal condition [Carbon (26.91 g/L), NH_4Cl (3.77 g/L), and KH_2PO_4 (5.31 g/L)]. The xylanase and endoglucanase activity of *T. viride* HG 623 showed the highest at 60 and 55°C at pH 5.6. The activity of xylanase was stimulated strongly by 75 mM of Co^{2+} , and the activity of endoglucanase was stimulated strongly by 75 mM of Mg^{2+} and Co^{2+} .

The xylanase and endoglucanase enzymatic activity of *T. viride* HG 623 were stable at pH 3.0 to 7.5 at 50°C or when incubated from 35 to 55°C for 1 h. Wheat bran was the optimal substrate for enzyme activity in natural substrate.

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