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Optimisation and Stabilisation of Cellulase and Xylanase Production by *Beauveria bassiana*

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Abstract

This research was conducted to optimise and stabilise cellulase and xylanase production by *Beauveria bassiana* B14532. Optimisation for pH (4.0, 6.5 and 9.0) and temperature (30, 55 and 80°C) was attained by a response surface methodology (RSM) model to fit the data using central composite design (CCD). Results indicated that *B. bassiana* B14532 recorded cellulase and xylanase activity at 3.59 UmL^{-1} (144h) and 39 UmL^{-1} (120h) respectively. Optimum pH and temperature which produced the highest cellulase (3.89 UmL^{-1}) were 6.5 and 80°C respectively while highest xylanase (41.01 UmL^{-1}) was obtained from optimum pH 6.5 and temperature 55°C. Coefficients of determination R^2 were 0.93 (cellulase activities) and 0.98 (xylanase activities). Stability of the cellulase and xylanase enzymes was investigated at the optimised pH values and temperatures for 120 min. Results showed that both enzymes decreased as reaction time increased by every 10 min. Cellulase retained 88, 80, 74, 66 and 55% of its activity at pH 6.5, 80°C post incubation for 50 min, while xylanase retained higher enzyme values at 93, 91, 83, 70, 65, 61 and 50% of its activity at pH 6.5, 55°C post incubation for 70 min.

Keywords: *Beauveria bassiana*; Cellulase; Optimisation; Stabilty; Xylanase

1. Introduction

Applications of entomopathogenic fungi (EPF) are widespread and significant in organic agriculture which is a production system that sustains the health of soils, ecosystems and people through ecological processes and biodiversity. The broad goals of sustainable agriculture include economic profitability, environmental stewardship and community vitality (Goldberger, 2011). *Beauveria bassiana* and Metarhizium anisopliae are well-known EPFs regarding pest management because they can act as parasites to kill or seriously disable crop-destroying insects. They infect by growing through the body of the insect by releasing extracellular cuticle-hydrolysing enzymes (protease, chitinase and lipase) and their induction into cultures containing cuticle as substrates (St. Leger *et al.*, 1986). Moreover, *B. bassiana* produces beauvericin and bassianolide which are correlated with their entomopathogenesis. Promotion of EPF applications is guided by the mass production of *B. bassiana* with cost reduction. Nowadays, the concept of organic waste utilisation has become the primary focus of many industries from an economic and sustainability perspective. Thailand is an agriculturally orientated country with a rich organic agricultural input that can be used

in many lignocelluloses. These are the most abundant biopolymers degraded by cellulase and xylanase enzymes and can be utilised as low cost materials. The main constituents of organic wastes are cellulose and xylan comprising approximately 35-50% of plant dry weight (Lynd *et al*., 2002). Hydrolysis of xylan and cellulose are essential steps towards the efficient utilisation of lignocellulosic materials in nature. Cellulase is an enzyme complex capable of hydrolysing cellulose into glucose molecules (Pérez *et al*., 2002) and xylanases degrade xylan, the main carbohydrate present in some hemicelluloses, into xylose (Kheng and Omar, 2005; Xiong *et al*., 2005). These enzymes can be found in saprophytic fungi that have the ability to attack cellulose and thus lead to the formation and induction activity of cellulose biosynthesis.

B. bassiana is a fungus that grows naturally in soil. It is produced by solid-state fermentation of low-cost materials such as lignocelluloses and used as a mycoinsecticide in organic agriculture. *B. bassiana* has been identified as a saprophytic fungus that can be utilised to produce cellulase and xylanase enzymes. Thus, this research aimed to investigate the ability *B. bassiana* to produce cellulase and xylanase enzymes in order to support organic waste utilisation for cost reduction of EPF production and promote the development and spread of biological control methods to minimise waste. In addition, the application of response surface methodology (RSM) was applied to deduce the relationships between parameters and also the optimum pH and temperature for the production process. Conventional methods are very time consuming for optimisation of a multivariable system and they do not determine interaction effects between the variables. This interaction can result in a synergistic effect, that is, a pH and a temperature condition in which the enzymes have a higher activity value (Vieira *et al*., 2007; Kalogeris *et al*., 2009; Saqib *et al*., 2010).

2. Materials and Methods

2.1 Microorganisms

Beauveria bassiana B14532 was purchased from the National Centre for Genetic Engineering and Biotechnology. It has been shown to have capability of cellulase enzyme production (Petlamul *et al*., 2017); therefore, it was selected to investigate the optimisation of pH and temperature for xylanase and cellulase production. *B. bassiana* B14532 was cultured on potato dextrose agar (PDA) as stock culture and stored at 4°C until required for use (Pham *et al*., 2009; Petlamul and Prasertsan, 2012).

2.2 Synthetic Media

Basal salt medium used for enzyme assay contained 1 /gL KH_2PO_4, 0.5 g/L $MgSO_4 \cdot H_2O$, 0.01 g/L $FeSO_4 \cdot H_2O$ and 0.5 g/L KCl. The medium was sterilised at 121°C for 15 min. Yeast extract was used as the nitrogen source. Stock solution of yeast extract (10.0 gL^{-1}) was autoclaved separately and added to the basal salt medium as required (Gao and Lui, 2010).

2.3 Cellulase and Xylanase Production

B. bassiana B14532 in potato dextrose agar (PDB) was cultured at 30±2°C for 7 days. Then it was harvested and the suspensions were mixed thoroughly by vortex mixer and diluted to obtain a standard inoculum of 108 spores mL-1. For enzyme production, 10 mL of spore suspension (108 spores mL^{-1}) was inoculated in 90 mL basal salt medium. This medium was separately supplemented with 1% carboxymethyl cellulose (CMC) for cellulase and 1% xylan for xylanase production. These cultures were incubated on an orbital shaker at 180 rpm and 30±2°C. Cultures were taken at different incubation times 0, 24, 48, 72, 96, 120, 144, 168 and 192h to study cellulase and xylanase production. They were then centrifuged at 10,000 rpm for 10 min and the supernatant was obtained as crude enzyme for cellulase and xylanase assay.

2.4 Cellulase and Xylanase Assay

Cellulase was measured by incubating 0.1 mL of 1% (w/v) CMC in 0.1M Tris-HCl buffer (pH 8.0). After pre-incubation at 60°C for 5 min, enzymatic reaction was initiated by adding 0.2 mL of crude enzyme at 60°C for 10 min. The reaction was terminated by boiling in a water bath for 10 min followed by cooling in ice-cold water. Absorbance of the resulting colour was

measured against the control at 540 nm in a spectrophotometer. Xylanase was assayed as above, using xylan (1.0%) in place of CMC. A control that contained all the reagents was run simultaneously but the reaction was terminated prior to the addition of enzyme. One unit of xylanase or cellulase was defined as the amount of enzyme that catalysed the release of 1.0 µmol of reducing sugar as xylose or cellulose or glucose equivalent per min under the specified assay conditions. Release of reducing sugar was measured as cellulose by the Somogyi-Nelson method. The procedure was modified from the method described by Kumer *et al.* (2011).

2.5 Experiment design for the optimisation of pH and temperature for cellulase and xylanase production

In all the determinations, cellulase and xylanase were measured using CMC and xylan as the substrates. The post-incubation time of the highest enzyme activity was used to explore the optimum pH and temperature. Optimum pH and temperature for cellulase and xylanase were determined by assaying the corresponding activity at different temperatures (from 30, 55 and 80ºC) and pH values (from 4.0, 6.5 and 9.0). The experimental design to evaluate the optimum pH and temperature was applied by response surface methodology (RSM) using central composite design (CCD) comprising eleven runs, corresponding to four cube points, four axial points and three central points with the experiments carried out in a random order (Mutanda *et al.*, 2008). Factors and levels investigated are shown in Table 1. The dependent variables (responses) were cellulase and xylanase activities. Statistica software (StatSoft version 6.0) was used to analyse the experimental data, generation of the ANOVA (analysis of variance) data and plotting of response surfaces. The design was represented by a second-order polynomial regression model as Equation (1) to generate contour plots:

$$Y = b_0 + b_1X_1 + b_2X_2 + b_{12}X_1X_2 + b_{11}X_1^2 + b_{22}X_2^2 \quad \text{eq.1}$$

Where, Yi represents the response variable, b_0 is the intercept, b_1 are the coefficients of linear effects, bii are the coefficients of single factor quadratic effects, and bii are the coefficients of interaction effects. The symbol Xi denotes the coded level of Xii variable in the experimental design.

Table 1. The coded levels using central composite designs for xylanase and cellulase production

Independent variable	Symbol	Coded levels		
		-1	0	1
pH	X1	4	6.5	9
Temperature (ºC)	X2	30	55	80

2.6 Enzyme Stability

Determination of cellulase and xylanase stability was explored under the optimised pH and temperature (as previous condition). Cellulase and xylanase enzymes were mixed with substrate in Tris buffer (0.08M Tris, 0.1M HCl, pH 6.5) and then incubated for 120 min at 80ºC for cellulase and 55ºC for xylanase. Reaction tubes were placed in ice (Bhella and Altosaar, 1985). Enzyme was then assayed. Incubation time was recorded as the half-life ($t_{0.5}$) that showed the loss of 50% activity (Farinas *et al.*, 2010).

3. Results and Discussions

3.1 Optimisation of pH and temperature for cellulase and xylanase production

Cellulase and xylanase enzymes produced from *B. bassiana* B14532 were obtained at the highest enzyme at different incubation times. The highest cellulase was achieved at 3.59 U/mL from post-incubation for 144h and the highest xylanase was achieved at 39.00 U/mL from post-incubation for 120 h. Thus, investigation on optimisation for pH and temperature of cellulase and xylanase was explored at 144h and 120 h respectively. Since pH and temperature are significant factors, the optimum pH (4.0, 6.5 and 9.0) and temperature (30, 55 and 80ºC) for cellulase and xylanase activities were investigated by RSM using CCD. *B. bassiana* B14532 gave cellulase and xylanase in all 11 experiments (Table 2). *B. bassiana* B14532 produced higher quantities of cellulase and xylanase enzyme at the optimised pH and temperature. Highest cellulase of 3.89 U/mL, increased from 3.59

U/mL, was obtained from optimised pH 6.5 and temperature 80ºC. Highest xylanase of 41.01 U/mL, increased from 39 U/mL, was obtained from optimised pH 6.5 and temperature 55ºC. Table 2 shows the coefficients of the mathematical model and statistical parameters for these results that were fitted with quadratic equations by regression analysis. The mathematical models representing the cellulase and xylanase (Y) in the experimental region studied were expressed by Equation (2) and Equation (3) respectively giving the following response surface models:

$$Y_{cellulase} = 1.71 - 0.057X_1 - 0.14X_2 + 0.062X_1X2 - 0.28X_1^2 + 0.17X_2^2 \quad \text{eq. 2}$$

$$Y_{xylanase} = 6.21 - 1.00X_1 - 0.25X_2 + 0.084X_1X_2 - 0.88X_1^2 + 0.20X_2^2 \quad \text{eq. 3}$$

where, Y cellulase and Y xylanase are the response values of enzyme, and X_1 and X_2 are the code levels of pH and temperature respectively.

ANOVA was employed for the determination of significant parameters and to estimate the specific cellulase and xylanase. The lack of fit was not significant ($p>0.05$). Non-significant lack of fit indicated that the model equation was sufficient for predicting cellulase and xylanase under combinations of the variables. ANOVA of model (Table 3) exhibited the linear effect of temperature for both cellulase and xylanase but the linear effect of pH for only xylanase. The quadratic effect of pH was significant ($p<0.05$) for cellulase production. This indicated that these terms had impact on cellulase and xylanase production from *B. bassiana* B14532. A summary of ANOVA for the model fit is given in Table 3.

Table 2. Experimental design and results of central composite design

Run	Code levels		Cellulase (UmL^{-1})		Xylanase ($U\ mL^{-1}$)	
	X1	X2	Actual value	Predicted value	Actual value	Predicted value
1	9	80	3.19	3.1	15.31	13.75
2	6	55	2.79	2.95	39.21	38.65
3	9	55	1.66	1.88	17.97	19.19
4	4	55	2.17	2.19	37.45	39.9
5	6	30	2.89	3.04	39.05	38.98
6	6.5	80	3.89	3.98	29.98	33.72
7	4	30	2.49	2.47	39.99	39.72
8	6.5	55	3.37	2.95	39.41	38.65
9	6.5	55	2.93	2.95	41.01	38.65
10	4	80	3.03	3.03	37.66	35.48
11	9	30	1.89	1.77	19.69	20.03

Table 3. Conditions anxd results of the statistical experimental design for cellulase and xylanase production

	Cellulase			Xylanase		
	Co-efficient	p-value	F-value	Co-efficient	p-value	F-value
Model		0.0079**	12.21		0.0005**	39.78
Intercept	1.71	0.0066**		6.21	0.0012**	
pH	-0.057	0.138			0.0001**	
T	0.14	0.0073**		-0.25	0.0350*	
pH.T	0.062	0.178		-0.084	0.461	
pH^2	-0.28	0.0023**		-0.88	0.0011**	
T^2	0.17	0.0182*		-0.2	0.189	
Lack of Fit	0.616			0.080		
CV	4.81			3.76		
R^2	0.93			0.98		

*Note. * Statistically significant at 95% of confidence level. ** Statistically significant at 99% of confidence level.*

Data analysis allowed the definition of an optimum range of pH and temperature for higher enzyme as well as the degree of significance of each variable and their interaction. Coefficients of pH and temperature (both linear and quadratic effects) variables showed a negative effect on xylanase, while the pH and temperature in linear effect variables showed a negative effect on cellulase.

The ANOVA analysis for cellulase and xylanase indicated that the coefficients of correlation were 0.93 and 0.98 respectively. The interactive effect between pH and temperature was not significant ($p<0.05$) for both cellulase and xylanase. The individual effect of pH was not significant for cellulase but the effect of temperature was significant ($p<0.001$). In case of xylanase, both effects of pH and temperature were significant at $p<0.001$ and $p<0.05$ respectively. The model of cellulase and xylanase was significant ($p<0.001$) with F-value at 12.21 and 39.78 respectively. The lack of fit was not significant ($p>0.05$) which implied that the proposed model fitted the experimental data.

The prediction model was used to describe the response surface plots of cellulase and xylanase. A plot of the three-dimensional response surface was employed to assess the interaction factor, which showed a significant effect on cellulase (Fig. (a)) and xylanase (Fig. (b)) from *B. bassiana* B14532. Cellulase tended to decrease with increase in pH and tended to decrease with decrease in temperature. The contour parallel between the two axes suggested that the two parameters were quite independent of each other (Fig. 1).

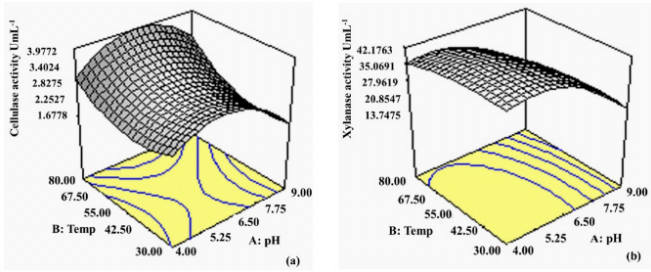

Figure. 1 Response surface showing the pH and temperature effect on cellulase (a) and xylanase (b) enzyme produced by *Beauveria bassiana* B14532.

Other studies on the characterisation of cellulase and xylanase reported optimum pH and temperature at specific values. Farinas *et al.* (2010) found optimum pH of 4.5 and temperature 55ºC for xylanase. Xiong *et al.* (2004) found the maximum xylanase at 70ºC and the optimum pH of 6.5 for xylanase produced from *Thermomyces lanuginosus*. Mmango-Kaseke *et al.* (2016) achieved cellulase production in the presence of CMC (1% w/v) under an incubation temperature of 25ºC (198 U/mL), pH 5 (173 U/mL) and incubation period of 96h (102 U/mL). Highest activity of cellulase from *B. bassiana* was found in 4-to-5-day-old mycelia (Leopold and Samšiňáková, 1970). Xylanase was produced maximally in xylan (1% w/v) at a temperature of 25ºC (1,007 U/mL), pH 10 (2,487 U/mL) and under an incubation period of 84h (1,296 U/mL). As for xylanase, the significance of the interaction effects between pH and temperature revealed the synergistic effect of these variables. For estimation of the optimum temperature of the enzyme, the activity was determined by carrying out the assay at several temperatures between 30 and 80ºC and pH 4-9. It was observed that the enzyme activity had a broad pH range. Several reported studies indicated varying temperature optimum values for the production of both cellulase and xylanase by different microorganisms. Different strains of *Bacillus sp.* gave a maximum yield of xylanase production from incubation temperature at 45ºC and 55ºC (Simphiwe *et al.*, 2011). The effect of pH strongly influenced many enzymatic reactions by affecting the transport of a number of chemical products and enzymes. For example, *Bacillus subtilis* and *B. circulans* gave the maximum cellulase at pH 7-7.5 (Ghatora *et al.*, 2007). Alteration in pH tolerance observed during xylanase production might be due to different enzyme mixtures secreted and/or the post-translational alterations in the xylanase secretion procedure (Park *et al.*, 2002).

3.2 Stabilisation of Cellulase and Xylanase

Cellulase production of *B. bassiana* B14532 retained a value of enzyme activity lower than xylanase production. Cellulase retained 88, 80, 74, 66 and 55% of its activity at pH 6.5, 80ºC post incubation for 50 min while xylanase retained a higher value of enzyme activity at 93, 91, 83, 70, 65, 61 and 50% at pH 6.5, 55ºC post incubation for 70 min. Half-life of the cellulase enzyme at pH 6.5, 80ºC was 50 min (Fig. 2 (a)) while half-life of the xylanase enzyme at pH 6.5, 55ºC was 70 min (Fig. 2 (b)). In this case, this result indicated that the xylanase enzyme produced by *B. bassiana* B14532 was stronger than the cellulase enzyme (Fig. 2).

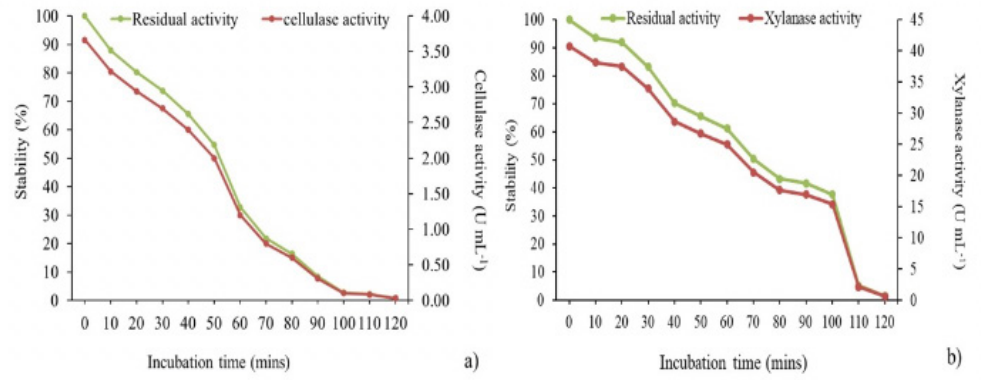

Figure. 2 Stability of cellulase analyzed at pH 6.5, 80ºC (a) and xylanase analyzed at pH 6.5, 55ºC (b) produced from *Beauveria bassiana* B 14532

Stability can be defined as the retention of activity after an enzyme is extracted at a selected temperature for a prolonged period of time. The half-life of an enzyme is the time after which the enzyme activity reduces to 50% of the original activity at a given temperature (Viikari *et al*., 2007). The pH and temperature stability of the enzyme are very important factors when studying the industrial importance of the enzyme. Xylanase stability dropped to 90.1h when incubation was at 50ºC (Farinas *et al*., 2010). Shah and Madamwar (2005) found that xylanase produced by a newly isolated *Aspergillus foetidus* was highly stable at 40ºC but retained only 36% of its activity at 50ºC after 3h. At pH 6, cellulase gradually decreased while xylanase at pH 11 significantly lost activity with only 34% retained (Bano *et al*., 2013). Abo-State *et al*. (2013) reported on the pH stability for crude enzymes produced from *Bacillus* (MAM-29 and MAM-38) at different pH values of 3.0, 4.0, 4.8, 5.0, 5.6, 6.0, 7.0 and 7.6. Their results indicated that both cellulase and xylanase enzymes showed high activity and remained stable at higher pH values. At a temperature of 80ºC (for 60 min) CMCase, FPase, avicelase, xylanase and extracellular protein retained 94, 96, 99, 95 and 95% of their activity respectively.

4. Conclusions

Both pH and temperature are reported to impact on consequent metabolic product formation of *B. bassiana*. In addition, many enzymatic processes and the transport of various components across the cell membrane are strongly affected by the pH and temperature condition. Enzyme production of microorganisms depends on the genetic nature of the organism, the physio-chemical parameters, the fermentation medium components and their concentrations. Thus, optimisation of pH and temperature to achieve maximum yields and produce a compelling bioprocess framework for industrial applications is essential.

Entomopathogenic fungi were found to be capable of degrading substantial lignin mass in lignocellulosic material such as wheat straw and wood (Kluczek, 2007). Amounts of saprophytic fungi as *B. bassiana* which were grown in wheat straw, rice, sorghum and sawdust as carbon sources were able to initiate cellulase production (Kaur *et al*., 2007). In particular, *B. bassiana* was capable of liberating cellulase into the medium. The methodology employed here was very effective in estimating enzyme behaviour under different pH and temperature conditions. By using statistical methodology, it was possible to find an optimum temperature and pH range rather than a specific value, allowing more flexibility during process development.

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