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Simultaneous fermentative chitinase and β-1,3 glucanase production from *Streptomyces philanthi* RM-1-1-38 and their antifungal activity against rice sheath blight disease

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Abstract

Chitinase and β-1,3 glucanase are enzymes that play important roles in the biocontrol of fungal plant pathogens. The effects of environmental conditions and culture medium composition on simultaneous chitinase and β-1,3 glucanase production from *Streptomyces philanthi* RM-1-138 were investigated using a conventional (one-factor-at-a-time) method and a response surface methodology (RSM), respectively. The optimum cultivation conditions were at pH 7.5 and a temperature of 30°C. The optimized medium (4.88 $g \cdot l^{-1}$ chitin, 6.27 $g \cdot l^{-1}$ glucose, and 17.05 $g \cdot l^{-1}$ malt extract) exhibited 53- and 80-fold increase in the activity of chitinase (0.53 $U \cdot ml^{-1}$) and β-1,3 glucanase (8.79 $U \cdot ml^{-1}$), respectively, compared to the original medium. The culture filtrate from the original and the optimized medium were partially purified and tested (by agar-well diffusion assay) for their antifungal activities against *Rhizoctonia solani* PTRRC-9 compared to the chemical fungicides carbendazim® and propiconazole® (100 $\mu g \cdot ml^{-1}$). The partially purified enzymes from the optimized medium exhibited 4.0-fold stronger antifungal activities against *R. solani* PTRRC-9 compared to that from the original medium and equal to that of the chemical fungicide propiconazole® but slightly lower than that of carbendazim®.

Keywords: *Streptomyces philanthi*, chitinase, β-1,3 glucanases, response surface methodology, rice sheath blight

Introduction

Fungal diseases pose serious threats worldwide during the cultivation of economically important plants (Brimner and Boland, 2003). Plants are endowed with several defense mechanisms that protect them from fungal infections. A major mechanism involved in the biological control of plant pathogens is parasitism that may involve the production of specific bacteria with extracellular cell wall degrading enzymes, for example, chitinase and β-1,3 glucanase that can lyse pathogen cell walls (Whipps, 1997). The major components of the fungal cell walls are chitin and β-1,3-glucan, hence, they could be hydrolyzed by chitinase and β-1,3 glucanase, respectively (Webster, 1986).

Rice sheath blight disease caused by *Rhizoctonia solani* Kuhn is economically costly and causes diseases in most of the agricultural crops (Kanjanamaneesathian et al., 1998; Pengnoo et al., 2000). Controlling this pathogen is difficult because it has a wide host range, attacks plants from the soil, and survives in the soil for extended periods of time. Disease control methods used currently are primarily based on chemicals; however, chemical control is not an effective means to suppress the aforementioned pathogens (Raaijmakers et al., 2002). Microbial antagonists are widely used for the biocontrol of fungal plant diseases. Many *Streptomyces* sp. including *S. hygroscopicus* and *S. viridodiasticus* are reported to produce chitinase and β-1,3 glucanase (El-Tarabily et al., 2000; Prapagdee et al., 2008). The production of these enzymes could be increased by optimization of the medium composition and environmental growth conditions (Nawani and Kapadnis, 2005; Rahulan et al., 2009; Singh et al., 2009; Rahulan et al., 2011; Mishra et al., 2012). The conventional method or the traditional "one-factor-at-a-time" technique is used for optimization of the culture medium by varying one factor

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(parameter) at a time in order to select the important component, although it requires a considerable amount of time and work. The concept of response surface methodology (RSM) has eased the process of determining the optimum culture conditions in biochemical and biotechnological processes (Bas and Boyaci, 2007). The RSM comprises statistically designed experimental techniques for estimating the coefficients in a mathematical model that can predict the response followed by checking the applicability of the model (Techapun et al., 2002). This study aimed at optimization of the environmental growth conditions and culture medium composition affecting simultaneous production of chitinase and β-1,3 glucanase by *S. philanthi* RM-1-138 using both the conventional method and the response surface methodology (RSM), followed by partial purification of the two enzymes and testing for their antifungal activities against the rice sheath blight disease caused by *R. solani* PTRRC-9.

Materials and methods

Microorganisms and basal medium

The *Streptomyces philanthi* RM-1-138 used in this study was isolated from the rhizosphere soil of chili pepper in southern Thailand (Boukaew et al., 2011). The strain was cultivated in a basal medium containing 0.05% (w/v) $MgSO_4$, 0.02% (w/v) K_2HPO_4, 0.03% (w/v) KH_2PO_4, and 0.001% (w/v) each of $FeSO_4$, $ZnSO_4$, and $MnCl_2$ (Nawani and Kapadnis, 2005). The pH was adjusted to 7.5 with 5 M NaOH before autoclaving.

Rhizoctonia solani PTRRC-9 (AG-1IA), isolated from naturally infected rice plants in the field, was obtained from the Phatthalung Rice Research Center of Thailand. The fungal strains were grown on potato dextrose agar (PDA) slants at room temperature (28 ± 2 °C) for 3 days and kept at 4 °C. They were sub-cultured freshly before use in each experiment.

Enzyme assays

Chitinase activity assay

The chitinase activity was assessed using colloidal chitin (Zhengzhou Sigma Chemical Co., Ltd.) as a substrate. Colloidal chitin 1% (w/v) was prepared and modified according to the procedure given by Roberts and Selitrennikoff (Hsu and Lockwood, 1975); 1 g of chitin powder was added slowly to 20 ml of concentrated HCl and left at 4 °C overnight with vigorous stirring. The mixture was then added to 200 ml of ice-cold 95% ethanol with rapid stirring and kept overnight at 4 °C. The precipitate was collected by centrifugation at 5000 × *g* for 15 min at 4 °C, then it was washed with sterile distilled water until the colloidal chitin became neutral (pH 7.0). Subsequently, the final volume was increased to 100 ml by adding 25 mM sodium acetate buffer (pH 5.0). The assay mixture consisted of 100 µl of enzyme solution and 100 µl of 1% colloidal chitin. After incubation at 40 °C for 30 min, the reaction was stopped by heating the content in a boiling water bath for 3 min and then centrifuged at 10 000 × *g* for 5 min. The supernatant was used for enzyme analysis using the dinitrosalicylic acid (DNS) method (Miller, 1959). One unit (U) of activity was defined as the amount of enzyme that liberated 1 µmol of N-acetylglucosamine from the reaction mixture per min, while N-acetyl-D-glucosamine (GlcNAc) was used as the standard. All experiments were performed twice, with three replicates for each treatment.

β-1,3 glucanase activity assay

The β-1,3 glucanase activity was determined by a colorimetric method (Ghose, 1987). The amount of reducing sugar released from laminarin (S.M. Sigma Chemical Supplies Co., Ltd.) was measured. The standard assay (0.5 ml) contained 10 µl of the crude enzyme solution and 90 µl of 5 mg · ml^{-1} laminarin in 100 mM sodium acetate buffer at pH 5.5. After incubation at 40 °C with gentle shaking for 10 min, the reaction was stopped by boiling the content for 5 min and then 200 µl of 1.0% DNS and 200 µl of sodium acetate buffer was added into the reaction mixture and boiled for another 5 min, then it was placed in an ice bath. The optical absorption was measured at 540 nm. The reducing sugar release was calculated from a standard curve prepared with glucose. One unit of β-1,3 glucanase activity was defined as the amount of enzyme that released 1 µmol of glucose equivalent per min under the standard assay conditions. All experiments were performed twice, with three replicates for each treatment.

Optimization of chitinase and β-1,3 glucanase production from *S. philanthi* RM-1-138 using conventional methodology

Effect of chitin concentration

The seed culture (5 ml, 10^7 spore ml^{-1}) of *S. philanthi* RM-1-138 was transferred into the basal medium (100 ml) containing various chitin concentrations (0, 1,

2, 3, 4, 5, and 6 g·l^{-1}) and incubated at 30 °C on a rotary shaker (at 150 rpm). Samples were taken at 24 and 48 h, then centrifuged at 8,880 × *g* for 20 min. The chitinase and β-1,3 glucanase activities in the culture filtrate were assayed (Prapagdee et al., 2008). The chitin concentration that exhibited the highest enzyme activities was chosen for further analyzes.

Effect of temperature and initial pH

The effect of temperature (25, 30, 35, and 40 °C) and initial pH (5.0, 5.5, 6.0, 6.5, 7.0, 7.5, and 8.0) on the production of chitinase and β-1,3 glucanase from *S. philanthi* RM-1-138 were studied. The initial pH was adjusted to various values using either 1 N NaOH or 0.1 N HCl.

Effect of carbon and nitrogen source and concentrations

The effect of carbon sources (glucose, sucrose, starch, maltose, and fructose each at 1.0 g·l^{-1}) on chitinase and β-1,3 glucanase production from *S. philanthi* RM-1-138 was studied using the basal medium supplemented with the optimum concentration of chitin. The optimum carbon source was selected to find the optimum concentration (0, 1, 2, 4, 6, and 8 g·l^{-1}). Then, the effect of nitrogen sources (yeast extract, malt extract, peptone, $(NH_4)_2SO_4$, and NH_4Cl at 10 g·l^{-1}) was studied. The selected nitrogen sources were tested at various concentrations (at 0, 1, 5, 10, 15, and 20 g·l^{-1}) to find its optimum value.

Time course of chitinase and β-1,3 glucanase production under the optimum conditions from conventional method

Streptomyces philanthi RM-1-138 (10^7 spore ml^{-1}) was cultivated on a basal medium with optimum nutrients and environmental conditions based on the conventional optimization studies. The initial pH was adjusted to the optimum value before autoclaving and incubated at the optimum temperature on a rotary shaker (150 rpm) for 72 h. The samples were collected every 12 h to measure the chitinase and β-1,3 glucanase activities.

Medium optimization on the simultaneous chitinase and β-1,3 glucanase production from *S. philanthi* RM-1-138 using response surface methodology (RSM)

The selected nutrients from the conventional experiments were optimized for chitinase and β-1,3 glucanase production from *S. philanthi* RM-1-138 using RSM based on the central composite design (CCD). A 2^3 factorial CCD with six axial points (with one variable set at an extreme ±2 level and the other variable at a central point level) and six replications around the center points (n_0 = 6) with a total number of 20 experiments were carried out for this study. For statistical calculation, the relation between the coded values and actual values were described by Equation (1) as follows:

$$X_i = \frac{(X_i - X_0)}{\Delta X_i} \quad (1)$$

where X_i is the coded value of the variable, X_i the actual value of the variable, X_0 the actual value of X_i at the center point, while ΔX_i corresponds to the step change of variable and represents the scale of variation in this fact.

To predict the optimal point, second-order polynomial model was fitted to correlate relationship between the variables and responses (Box et al., 1976). Quadratic equation for the variables is shown in Equation (2):

$$Y_i = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i=1}^{k} \sum_{j=1}^{k} \beta_{ij} X_i X_j \quad (2)$$

where Y_i is the predicted response; β_0 the interception coefficient; β_i, β_{ii}, and β_{ij} the linear, quadratic, and interactive coefficients, respectively. X_i is the independent variables. The statistical software package Design-Expert 7.0.0 (trial version), Stat-Ease. Inc., Minneapolis, MN, USA, was used for the regression and graphical analysis of the experimental data obtained.

Antifungal activities of partially purified enzymes of *S. philanthi* RM-1-138 and chemical fungicides against rice sheath blight disease

The crude enzyme from *S. philanthi* RM-1-138 (10^7 spore ml^{-1}) was prepared by cultivating in the basal (both original and optimized) medium under optimum environmental conditions. For partial purification of the two enzymes, the salting-out technique was used. The extracellular enzymes were precipitated by slowly adding ammonium sulfate salt (40-80% saturation) in the cell-free supernatant and left at 4 °C overnight (Park et al., 2012). A second centrifugation (at 9000 × *g* at 4 °C for 15 min) was conducted. The pellet was dissolved in 50 mM Tris–HCl buffer, pH 7.5. The ammonium sulfate salt in the enzyme solution was removed by dialysis using a dialysis bag (molecular weight cut-off 8,000 Da)

and dialyzed at 4 °C for 24 h in the same buffer (300-500 times the volume of the sample). Dialysis buffer was changed every 2 h for the first three times to remove the high amount of ammonium sulfate salt and left overnight to remove residual ammonium sulfate salt. The activities of partially purified enzymes were determined, chitinase and β-1,3 glucanase, using the crude enzyme as control.

The antifungal activity of the partially purified enzymes of *S. philanthi* RM-1-38 against *R. solani* PTRRC-9 that caused sheath blight disease of rice was determined by agar-well diffusion assay (Kavitha et al., 2010). Each 5 mm agar with mycelial growth of the pathogenic fungi was transferred to the center of each PDA plate. About 50 µl of the partially purified enzyme of *S. philanthi* RM-1-138 was placed in the wells at the concentration of 5 mg · ml^{-1}. The inoculated plates were incubated at room temperature (28 °C ± 2 °C) for 3 days and checked for the inhibition zones. The chemical fungicides carbendazem® and propiconazole® (100 µg · ml^{-1}) were used as positive controls and 50 mM Tris–HCl buffer, pH 7.5, was used as a negative control.

Statistical analysis

The data were subjected to analyzes of variance (ANOVA) using SPSS software version 15 for Windows. Statistical significance was evaluated using DMRT (Duncan's Multiple Range Test) and a $P < 0.05$ value was considered to demonstrate a significant difference.

Results

Optimization of chitinase and β-1,3 glucanase production from *S. philanthi* RM-1-138 using conventional methodology

Effect of chitin concentration

The effect of chitin concentrations (0-6 g · l^{-1}, w/v) on the induction of chitinase and β-1,3 glucanase in the presence of glucose as main carbon energy source was examined. The maximum chitinase and β-1,3 glucanase activity (0.21 and 3.73 U · ml^{-1}, respectively) were achieved at the chitin concentration of 4.0 g · l^{-1} (Fig. 1). They were 21.0- and 33.9-fold higher than the controls (without chitin) (0.01 and 0.11 U · ml^{-1}), respectively.

Effect of temperature and initial pH

The effect of the cultivation temperature (25 °C-40 °C) and the initial pH (5-8) on the production of chitinase and β-1,3 glucanase from *S. philanthi* RM-1-138 was studied in a basal medium supplemented with 4.0 g · l^{-1} chitin. The highest chitinase and β-1,3 glucanase activities were obtained at 30 °C (0.27 and 3.67 U · ml^{-1}, respectively) (Fig. 2) and an initial pH of 7.5 (0.26 and 3.69 U · ml^{-1}, respectively) (Fig. 3). Therefore, the en-

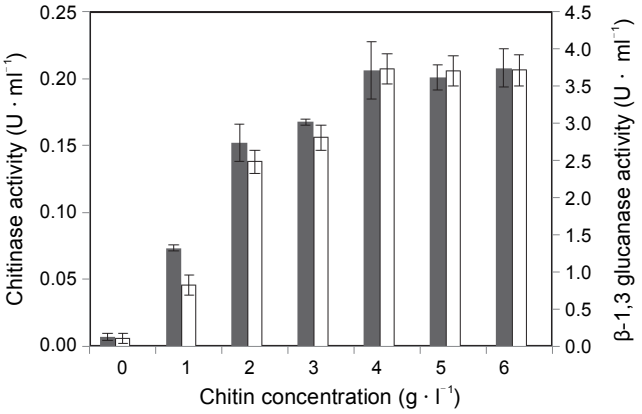

Fig. 1. Effect of chitin concentrations on the production of chitinase (24 h) (*black bar*) and β-1,3 glucanase (48 h) (*white bar*) from *S. philanthi* RM-1-138 in a basal medium

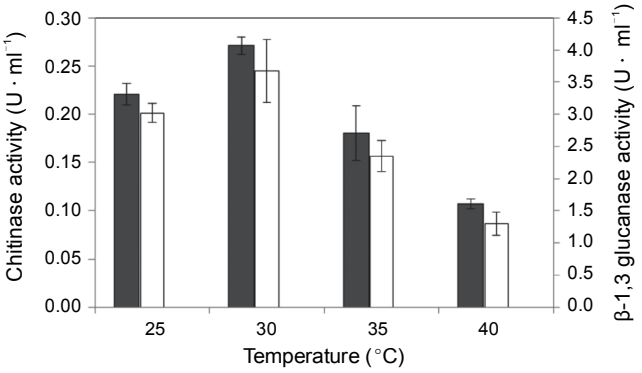

Fig. 2. Effect of temperature on the production of chitinase (24 h) (*black bar*) and β-1,3 glucanase (48 h) (*white bar*) from *S. philanthi* RM-1-138 in a basal medium with 4.0 g · l^{-1} chitin

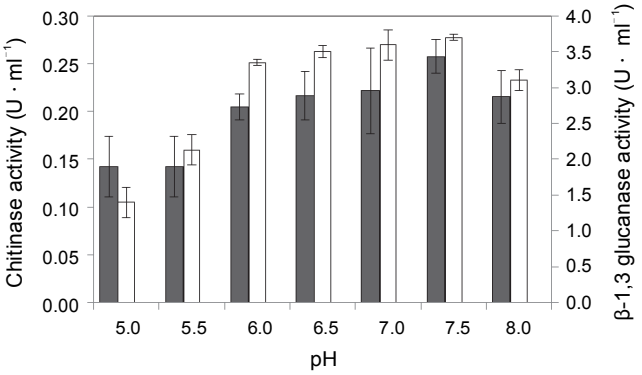

Fig. 3. Effect of initial pH on the production of chitinase (24 h) (*black bar*) and β-1,3 glucanase (48 h) (*white bar*) from *S. philanthi* RM-1-138 in a basal medium with 4.0 g · l^{-1} chitin

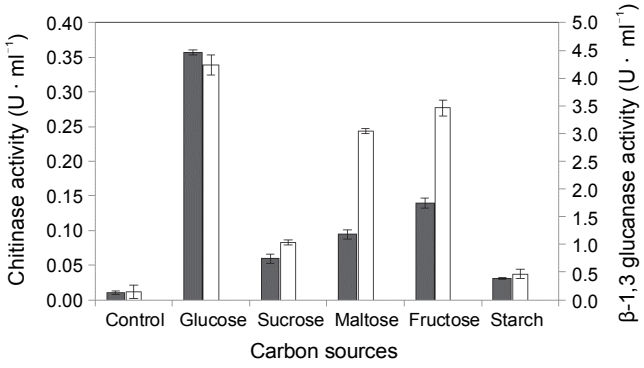

Fig. 4. Effect of carbon source (1.0 $g \cdot l^{-1}$) on the production of chitinase (24 h) (*black bar*) and β-1,3 glucanase for 48 h (*white bar*) from *S. philanthi* RM-1-138 in a basal medium with 4.0 $g \cdot l^{-1}$ chitin

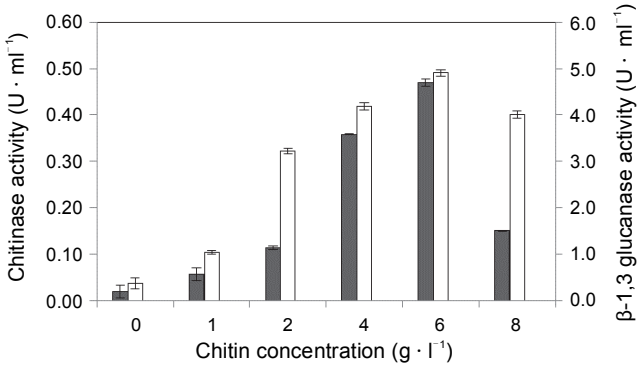

Fig. 5. Effect of glucose concentrations (0-8 $g \cdot l^{-1}$) on the production of chitinase (24 h) (*black bar*) and β-1,3 glucanase (48 h) (*white bar*) from *S. philanthi* RM-1-138 in a basal medium with 4.0 $g \cdot l^{-1}$ (w/v) chitin

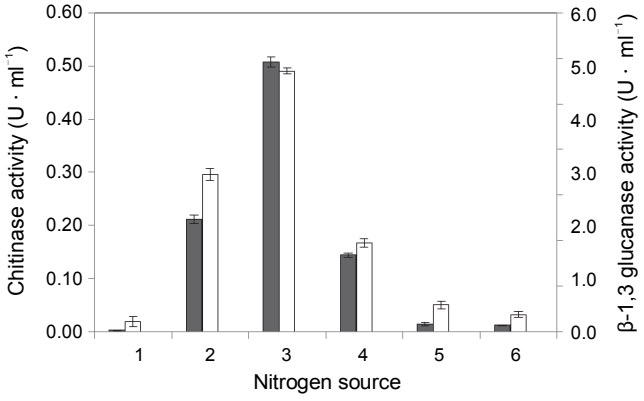

Fig. 6. Effect of nitrogen sources (10 $g \cdot l^{-1}$) on the production of chitinase (24 h) (*black bar*) and β-1,3 glucanase (48 h) (*white bar*) from *S. philanthi* RM-1-138 in a basal medium with 4 $g \cdot l^{-1}$ chitin and 6 $g \cdot l^{-1}$ glucose

vironmental conditions were the same similar to the previous experiments.

Effect of type and concentration of carbon source

Production of chitinase and β-1,3 glucanase from *S. philanthi* RM-1-138 in a basal medium containing either glucose, sucrose, starch, maltose, or fructose (1.0 $g \cdot l^{-1}$) as a carbon source supplemented with 4.0 $g \cdot l^{-1}$ chitin were compared. Glucose was the optimum carbon source as it gave the highest chitinase and β-1,3 glucanase activities (0.35 and 4.23 $U \cdot ml^{-1}$, respectively) (Fig. 4). Among various glucose concentrations (0-8 $g \cdot l^{-1}$) tested, the highest chitinase and β-1,3 glucanase activities (0.47 and 4.90 $U \cdot ml^{-1}$, respectively) were produced in the medium with 6.0 $g \cdot l^{-1}$ glucose (Fig. 5). Under the optimum glucose concentration, the activity of chitinase and β-1,3 glucanase increased 1.34 and 0.16 folds, respectively.

Effect of type and concentration of nitrogen source

The effect of nitrogen sources (yeast extract, malt extract, peptone, $(NH_4)_2SO_4$, and NH_4Cl) at 10 $g \cdot l^{-1}$ on chitinase and β-1,3 glucanase production from *S. philanthi* RM-1-138 was studied in a basal medium supplemented with 4.0 $g \cdot l^{-1}$ chitin and 6.0 $g \cdot l^{-1}$ glucose. The highest chitinase and β-1,3 glucanase activities (0.50 and 5.72 $U \cdot ml^{-1}$, respectively) were observed with malt extract as the nitrogen source (Fig. 6). Among the five different malt extract concentrations (0-20 $g \cdot l^{-1}$), the maximum chitinase and β-1,3 glucanase activity of *S. philanthi* RM-1-138 (0.58 and 6.74 $U \cdot ml^{-1}$, respectively) were achieved at 15 $g \cdot l^{-1}$ malt extract (Fig. 7). The activities of the two enzymes increased by 1.23 and 1.38 folds, respectively, compared to the previous experiment on the carbon source (0.47 and 4.90 $U \cdot ml^{-1}$).

Time course of chitinase and β-1,3 glucanase production from S. philanthi RM-1-138

Time course on the production of chitinase and β-1,3 glucanase from *S. philanthi* RM-1-138 was studied using a basal medium with optimized medium composition and conditions. The modified basal medium composed of 4.0 $g \cdot l^{-1}$ chitin, 6.0 $g \cdot l^{-1}$ glucose, and 15 $g \cdot l^{-1}$ malt extract, with an initial pH of 7.5 and cultivated on a shaker (at 150 rpm) at 30°C for 72 h. The levels of chitinase and β-1,3 glucanase activities sharply increased during the exponential phase of growth. The maximum chitinase (0.59 $U \cdot ml^{-1}$) and β-1,3 glucanase (7.57 $U \cdot ml^{-1}$) activities were obtained at 24 and 48 h, respectively (Fig. 8). Therefore, the sampling times chosen for en zyme analysis of chitinase and β-1,3 glucanase in the pre-

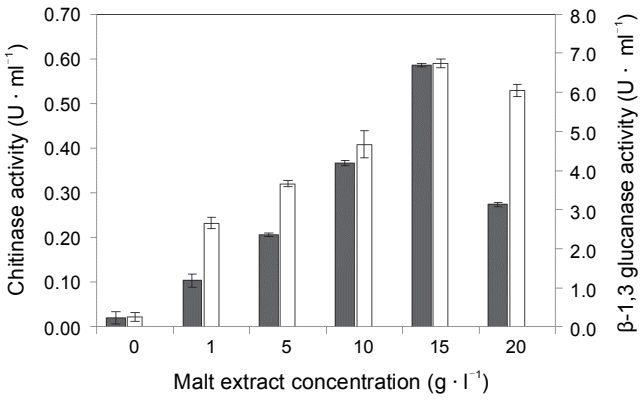

Fig. 7. Effect of malt extract concentrations (0-20 $g \cdot l^{-1}$) on the production of chitinase (24 h) (*black bar*) and β-1,3 glucanase (48 h) (*white bar*) from *S. philanthi* RM-1-138 in a basal medium with 4.0 $g \cdot l^{-1}$ chitin and 6.0 $g \cdot l^{-1}$ glucose

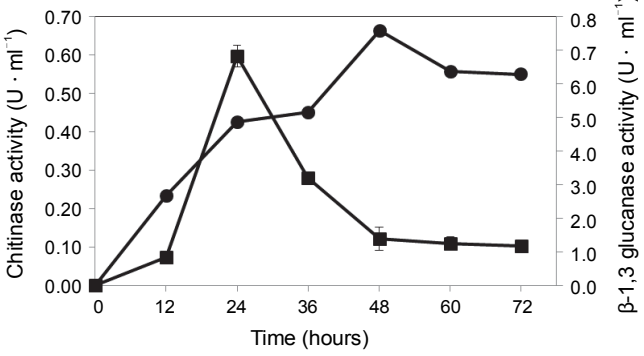

Fig. 8. Time course on the production of chitinase (*square*) and β-1,3 glucanase (*circle*) from *S. philanthi* RM-1-138 in a basal medium with 4.0 $g \cdot l^{-1}$ chitin, 6.0 $g \cdot l^{-1}$ glucose, and 15 $g \cdot l^{-1}$ malt extract

vious experiments were already at the optimal point for each enzyme.

Optimization of chitin, malt extract, and glucose concentration on chitinase production by *S. philanthi* RM-1-138 using RSM

The results from the conventional method revealed that chitin (X_1), malt extract (X_2), and glucose (X_3) are the important factors in the enzyme production, so they were selected as variables to find their optimum concentrations and interactions using CCD. By applying multiple regression analysis on the experimental data, the following second-order polynomial equation was obtained to describe the chitinase production as a function of the significant variables (Eq. 3):

$$Y_1 = 0.51 + 0.056(X_1) + 0.067(X_2) + 0.019(X_3) - 0.078(X_1)^2 - 0.038(X_2)^2 - 0.074(X_3)^2 - 0.041(X_1X_2) + 0.06(X_1X_3) - 0.11(X_2X_3) \quad (3)$$

where Y_1 is the chitinase production ($U \cdot ml^{-1}$) and X_1, X_2, and X_3 are the actual values of chitin ($g \cdot l^{-1}$), malt extract ($g \cdot l^{-1}$), and glucose ($g \cdot l^{-1}$), respectively. ANOVA of the fitting model (Table 1) showed that the model term was significant ($P < 0.01$). The lack of fit was not significant ($P > 0.05$). Non-significant lack of fit indicated that the model equation was sufficient for predicting the chitinase production under various combinations of the variables. The R^2 value of 0.97 indicated the accuracy of the model and also explained how much the response variables and their interactions varied (Acikel et al., 2010). All these values indicated that Equation (3) could illustrate the effect of response variables on the chitinase production as explained in this study.

ANOVA of the model (Table 2) exhibited that the linear effect of chitin concentration and malt extract and the quadratic effect of chitin concentration, malt extract, and glucose ($P < 0.01$) were significant, which indicated that these terms had an impact on the chitinase production. In contrast, the linear effect of glucose was not significant ($P > 0.05$). Subsequently, the maximum production of 0.56 $U \cdot ml^{-1}$ was derived from Equation (3) at the chitin, glucose, and malt extract concentrations of 5.76, 7.42, and 15.58 $g \cdot l^{-1}$, respectively. The response surface plots and corresponding contour plots are shown in Figure 9, where two variables were left within the experimental range and one was kept constant at its optimum level. The 3D response surface showed the effect of chitin and malt extract (Fig. 9A), chitin and glucose (Fig. 9B), and malt extract and glucose (Fig. 9C) combinations on the chitinase production. It showed that all factors significantly and interactively influenced the chitinase production.

Optimization of chitin, malt extract, and glucose concentration on β-1,3 glucanase production by *S. philanthi* RM-1-138 using RSM

The influences of the key factors from conventional method including chitin (X_1), malt extract (X_2), and glucose (X_3) on β-1,3 glucanase production were investigated using a CCD. Regression analysis of the experimental data from Table 1 resulted in the second-order polynomial equation (Equation 4) as follows:

$$Y_2 = 8.32 + 1.29(X_1) + 0.67(X_2) + 0.14(X_3) - 1.10(X_1)^2 - 0.83(X_2)^2 - 1.32(X_3)^2 - 0.09(X_1X_2) + 1.36(X_1X_3) - 0.82(X_2X_3) \quad (4)$$

Table 1. The Central Composite experimental design with three independent variables for simultaneous production of chitinase and β-1,3 glucanase from *S. Philanthi* RM-1-138 grown in a basal medium

Run	Chitin [g·l^{-1}]		Malt extract [g·l^{-1}]		Glucose [g·l^{-1}]		Chitinase activity [U·ml^{-1}]	β-1,3 glucanase activity [U·ml^{-1}]
	X_1	Code X_1	X_2	Code X_2	X_3	Code X_3		
1	2	−1	10	−1	3	−1	0.07	3.91
2	6	1	10	1	3	−1	0.16	3.50
3	2	−1	20	1	3	−1	0.52	6.56
4	6	+1	20	1	3	−1	0.38	5.98
5	2	−1	10	−1	9	1	0.23	2.69
6	6	1	10	−1	9	1	0.50	7.95
7	2	−1	20	1	9	1	0.19	2.27
8	6	1	20	1	9	1	0.36	6.94
9	0	−2	15	0	6	0	0.10	1.11
10	8	2	15	0	6	0	0.35	6.96
11	4	0	5	−2	6	0	0.24	3.36
12	4	0	25	2	6	0	0.53	6.83
13	4	0	15	0	0	−2	0.20	2.54
14	4	0	15	0	12	2	0.28	3.73
15	4	0	15	0	6	0	0.56	8.51
16	4	0	15	0	6	0	0.53	8.42
17	4	0	15	0	6	0	0.50	7.25
18	4	0	15	0	6	0	0.53	8.96
19	4	0	15	0	6	0	0.51	8.62
20	4	0	15	0	6	0	0.50	8.34

where Y_2 is the β-1,3 glucanase production (U·ml^{-1}) and X_1, X_2, and X_3 are the actual values of chitin (g·l^{-1}), malt extract (g·l^{-1}), and glucose (g·l^{-1}) respectively. ANOVA of the fitting model (2) showed that the model term was significant ($P < 0.01$). The lack of fit was not significant ($P > 0.05$). The model demonstrated a high determination coefficient ($R^2 = 0.97$), which proves it as a statistically significant model. The results of an ANOVA test (Table 2) exhibited that the linear effects of chitin and malt extract concentration and the quadratic effect of chitin, malt extract, and glucose concentration on the β-1,3 glucanase production were significant ($P < 0.01$). Hence, the *P*-values of the linear effect of glucose ($P = 0.3489$) and interactive effect between chitin and malt extract ($P = 0.6531$) were not significant ($P < 0.05$). The maximum β-1,3 glucanase production of 8.97 U·ml^{-1} was obtained from Equation (4), where the concentrations of chitin, glucose, and malt extract were 3.63, 4.61, and 19.53 g·l^{-1}, respectively.

The response surface plots and corresponding contour plots are shown in Figure 10, where two variables were left within the experimental range and one was kept constant at its optimum level. The 3D response surface showed the effect of chitin and malt extract (Fig. 10A), chitin and glucose (Fig. 10B), and malt extract and glucose (Fig. 10C) combinations on the chitinase production. Similar to previous studies, it showed that the interactive effect between chitin and glucose and malt extract and glucose significantly and interdependently influenced the β-1,3 glucanase production.

Validation of the model for chitinase and β-1,3 glucanase

To validate the predicted optimal conditions of the models, verification tests were done by batch experiments. The maximum chitinase production (run 1) and β-1,3 glucanase (run 2) were obtained (Table 3) under the predicted optimal concentrations of chitin, malt

Table 2. Analysis of variance (ANOVA) for the model regression represents chitinase and β-1,3 glucanase production

Source	Sum of squares	df	Mean square	F value	Prob > F
Model of chitinase production [U · ml^{-1}]	0.50	9	0.056	41.08	< 0.0001
X_1	0.056	1	0.050	36.29	0.0001
X_2	0.067	1	0.072	52.46	< 0.0001
X_3	0.019	1	0.006	4.40	0.0622
X_1X_2	−0.041	1	0.014	9.98	0.0102
X_1X_3	0.061	1	0.030	22.00	0.0009
X_2X_3	−0.106	1	0.090	66.21	< 0.0001
X_1^2	−0.078	1	0.013	67.46	< 0.0001
X_2^2	−0.038	1	0.092	9.40	0.0119
X_3^2	−0.074	1	0.007	101.50	< 0.0001
Residual	0.014	10	0.001	–	–
Lack of fit	0.011	5	0.002	4.08	0.074
Pure error	0.003	5	0.001	–	–
Cor total	0.518	19	–	–	–
CV = 10.20					
Coefficient of determination (R^2) = 0.97					
Model of β-1,3 glucanase production	120.01	9	13.33	39.64	< 0.0001
X_1	26.626	1	26.63	79.16	< 0.0001
X_2	7.076	1	7.08	21.04	0.0010
X_3	0.325	1	0.32	0.97	0.3489
X_1X_2	0.072	1	0.07	0.21	0.6531
X_1X_3	14.906	1	14.91	44.31	< 0.0001
X_2X_3	5.379	1	5.38	15.99	0.0025
X_1^2	13.928	1	13.93	41.41	< 0.0001
X_2^2	7.889	1	7.89	23.45	0.0007
X_3^2	43.809	1	43.81	130.24	< 0.0001
Residual	3.364	10	0.34	–	–
Lack of fit	1.678	5	0.34	0.99	0.50
Pure error	1.686	5	0.34	–	–
Cor total	123.373	19	–	–	–
CV = 10.14					
Coefficient of determination (R^2) = 0.97					

extract, and glucose. Under the optimal conditions, the model predicted the chitinase production as 0.56 U · ml^{-1} and β-1,3 glucanase as 8.97 U · ml^{-1}. These predicted values were close to the actual values obtained with the chitinase production as 0.53 U · ml^{-1} and β-1,3 glucanase as 8.86 U · ml^{-1} (Table 3).

The maximum simultaneous chitinase and β-1,3 glucanase production was obtained at the optimal conditions of 4.88 g · l^{-1} chitin, 6.27 g · l^{-1} glucose, and 17.05 g · l^{-1} malt extract (Table 3 run 3). Under the optimal conditions, the predicted chitinase and β-1,3 glucanase productions were 0.54 U · ml^{-1} and 8.82 U · ml^{-1}, respectively. The actual results obtained were 0.53 and 8.79 U · ml^{-1}, respectively, which were close to the predicted values (Table 3). This confirmed that CCD is an efficient tool for predicting optimal conditions for

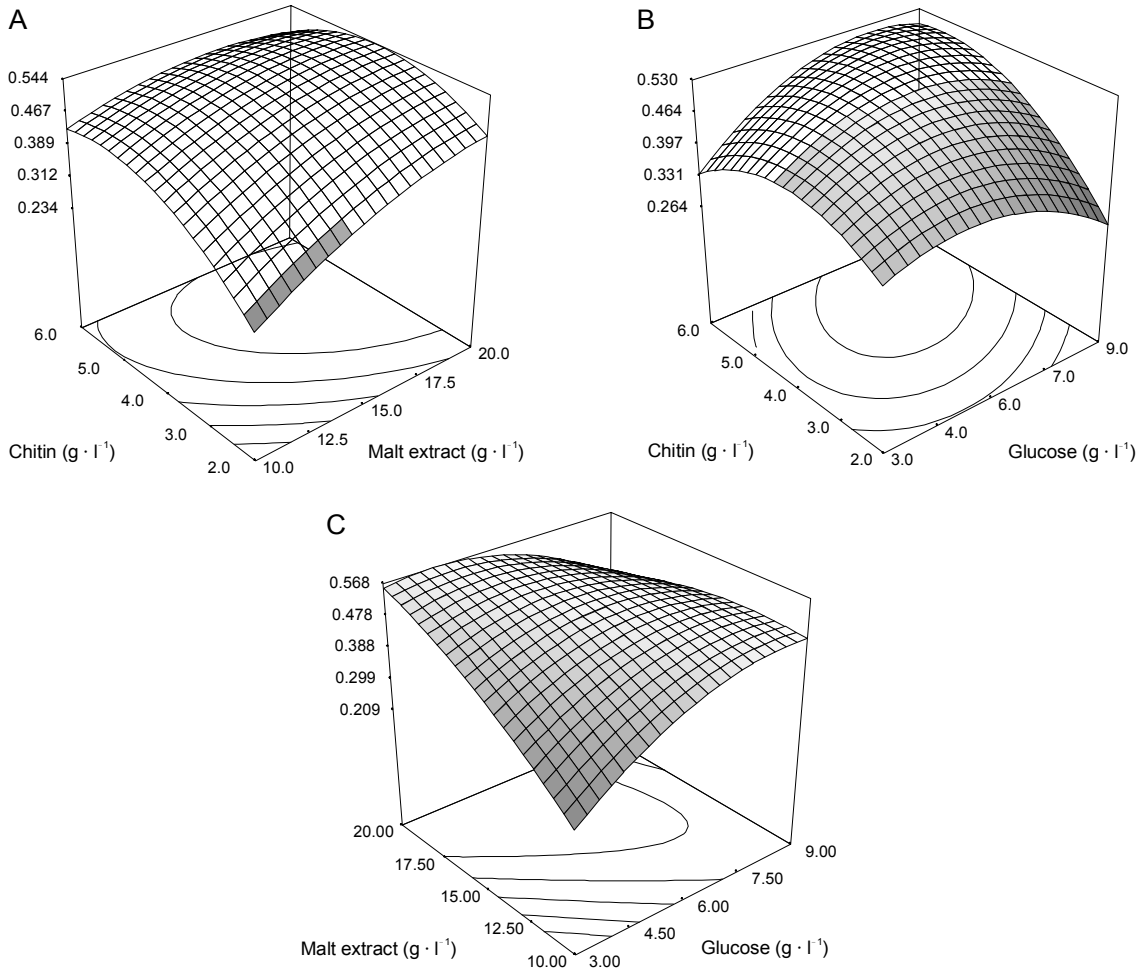

Fig. 9. 3D surface plots of the chitinase activity of *S. philanthi* RM-1-138 as a function of A – chitin and malt extract; B – chitin and glucose; and C – malt extract and glucose (in coded values)

a combination of predicted chitinase and β-1,3 glucanase production from *S. philanthi* RM-1-138.

Antifungal activity of partially purified enzymes of *S. philanthi* RM-1-138 and chemical fungicides against rice sheath blight disease

The crude and partially purified enzymes solutions (chitinase and β-1,3 glucanase) from the cultivation of *S. philanthi* RM-1-138 in the original and optimized basal medium were tested to compare their antifungal activities. It exhibited a clear zone of inhibition against *R. solani* PTRRC-9. The antifungal activity from chitinase and β-1,3 glucanase reached the maximum values in the optimized medium (15.0 and 22.8 mm, respectively) compared to the original medium (5.30 mm). The partially purified enzymes from the optimized medium for β-1,3 glucanase production (22.8 mm) exhibited slightly stronger antifungal activities against *R. solani* PTRRC-9 than the propiconazole® (21.8), but lower than the carbendazim® (28.2 mm) (Table 4). In each optimized medium, partial purification increased the antifungal activities of chitinase and β-1,3 glucanase by 1.56 and 1.48 folds, respectively, or approximately 1.5 folds for both the enzymes.

Discussion

In this study, an optimization process was employed to determine the best combination of parameters such as chitin, pH, temperature, carbon, and nitrogen source for extracellular chitinase and β-1,3 glucanase production by *S. philanthi* RM-1-138 prior to the testing for antifungal activity against *R. solani* PTRRC-9 (a pathogen causing the rice sheath blight disease). The microbial chitinases production can be induced by chitin, even in the presence of a readily utilizable carbon source such

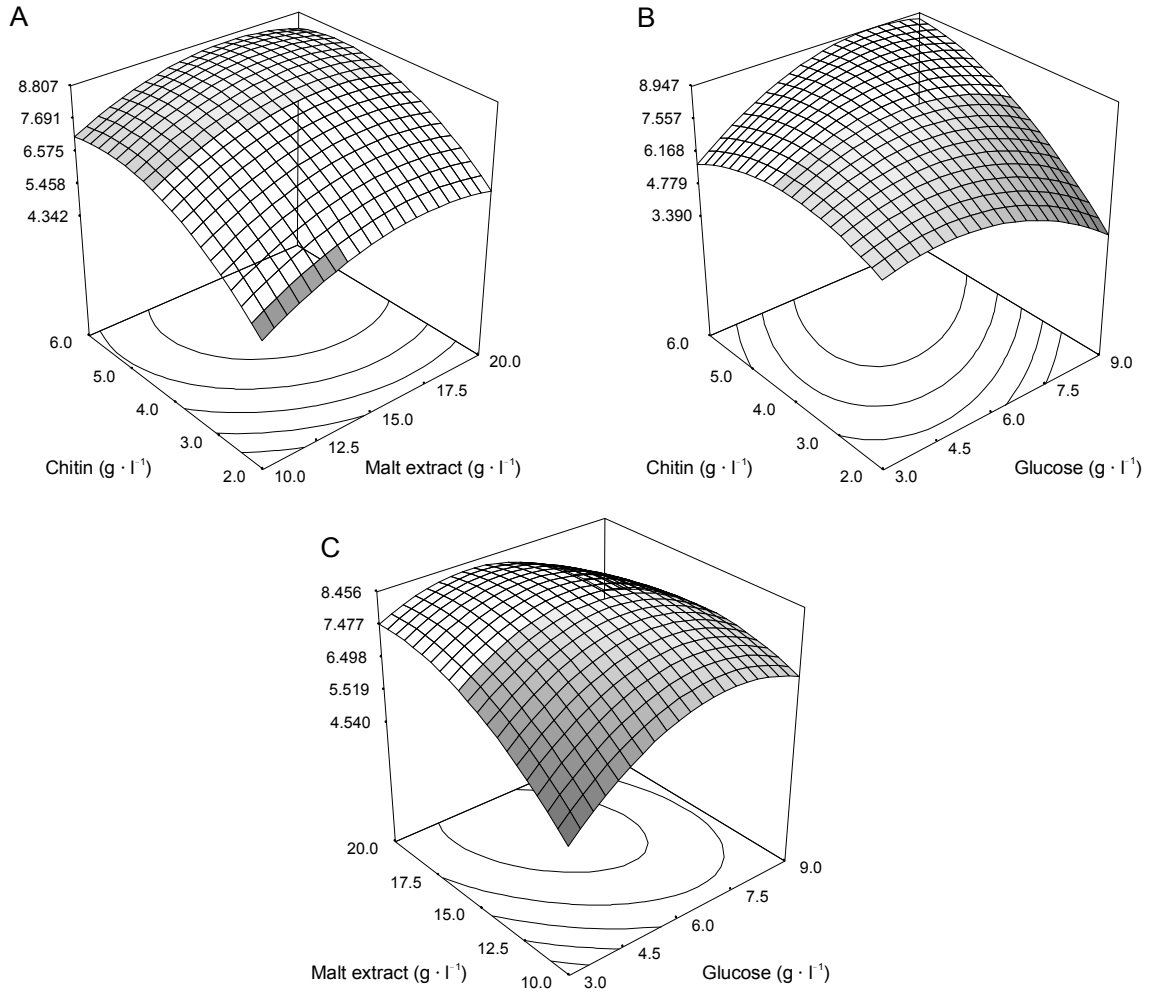

Fig. 10. 3D surface plots of the β-1,3 glucanase activity of *S. philanthi* RM-1-138 as a function of A – chitin and malt extract; B – chitin and glucose; and C – malt extract and glucose (in coded values)

as glucose (Miyashita et al., 2000) and also stimulated by the oligosaccharides, chitobiose, and/or N-acetyl-D-glucosamine (Patil et al., 2010). The culture medium supplied with chitin at 4 $g \cdot l^{-1}$ could increase the chitinase and β-1,3 glucanase activities (0.21 and 3.73 $U \cdot ml^{-1}$, respectively) of *S. philanthi* RM-1-138 by 21.0 and 33.9 folds, respectively, compared to the control. This optimum chitin concentration for *S. philanthi* RM-1-138 was higher than those obtained for *S. halstedii* (2 $g \cdot l^{-1}$ of chitin) (Joo, 2005) and of 1 $g \cdot l^{-1}$ of chitin (Taechowisan et al., 2003; Faramarzi et al., 2009; Narayana et al., 2008).

Environmental factors such as temperature and initial pH are known to have a profound influence on the growth and enzymatic activities (chitinase and β-1,3 glucanase) in *Streptomyces* species (Taechowisan et al., 2003; Nawani and Kapadnis, 2005). The optimal temperature of 30 °C for *S. philanthi* RM-1-138 (with chitinase and β-1,3 glucanase of 0.27 and 3.67 $U \cdot ml^{-1}$, respectively) was within the range of 30 °C-35 °C for *S. aureofaciens* CMUAc130 (0.0833 $U \cdot ml^{-1}$ chitinase) (Taechowisan et al., 2003), but lower than that obtained for *Streptomyces* sp. (40 °C) (Carrillo and Gomez, 1998, Gomes et al., 2001). Therefore, the difference in the optimum temperature for chitinase production clearly depends on the species of *Streptomyces*.

It is also known that pH influences the proton-donating or proton-accepting groups (ionization) in the catalytic sites of enzymes (Faramarzi et al., 2009). Changes in the external pH of the growth medium affects many cellular processes such as regulation and biosynthesis of secondary metabolites (Chang et al., 1991; Datta and Kothary, 1993; Sole et al., 1997). A majority of bacteria have been reported to produce the maximum amount of

Table 3. Experimental evaluation of the optimal conditions predicted by RSM for the production of chitinase and β-1,3 glucanase

Run	Conditions	Response variable					
		Chitinase [U · ml^{-1}]			β-1,3 glucanase [U · ml^{-1}]		
		predicted	experimental	error (%)	predicted	experimental	error (%)
1. Chitinase production	5.76 g · l^{-1} chitin, 7.42 g · l^{-1} glucose, 15.58 g · l^{-1} malt extract	0.56	0.53	5.35	–	–	–
2. β-1,3 glucanase production	3.63 g · l^{-1} chitin, 4.61 g · l^{-1} glucose 19.53 g · l^{-1} malt extract	–	–	–	8.97	8.86	1.23
3. Simultaneous chitinase and β-1,3 glucanase production	4.88 g · l^{-1} chitin, 6.27 g · l^{-1} glucose, 17.05 g · l^{-1} malt extract	0.54	0.53	1.85	8.82	8.79	0.34

Table 4. Comparison of the influence of crude and partially purified enzymes of *S. philanthi* RM-1-138 and chemical fungicides (100 μg · ml^{-1}) on growth of *R. solani* PTRRC-9 using agar-well diffusion assay

Source of antifungal substance	Zone inhibition [mm] ± SE
Original medium (basal medium)	5.30 ± 0.18^{e}
Culture filtrate from the optimized medium for chitinase production	9.6 ± 0.12^{d}
Partially purified enzymes optimized medium for chitinase production	15.0 ± 0.24^{c}
Culture filtrate from the optimized medium for β-1,3 glucanase production	15.4 ± 0.04^{c}
Partially purified enzymes of optimization medium for β-1,3 glucanase production	22.8 ± 0.15^{b}
Carbendazim®	28.2 ± 0.29^{a}
Propiconazole®	21.8 ± 0.19^{b}
50 mM Tris–HCl buffer, pH 7.5	0 ± 0.00^{f}

Data are the mean of six replicates ± standard errors (SE). Data followed by same letter within each column show no significant differences using ANOVA after DMRT at $P < 0.05$

chitinase at a neutral or slightly acidic pH, whereas fungi mostly prefer acidic conditions (Gomaa, 2012). *S. philanthi* RM-1-138 produced the maximum chitinase and β-1,3 glucanase at a pH of 7.5 (0.26 and 3.69 U · ml^{-1}, respectively). This was within the optimum pH range for chitinase activity of other species of *Streptomyces* (3.3-7.5) (Carrillo and Gomez 1998; Gomes et al., 2001) and *Alcaligenes xylosoxydans* (7.0-9.0) (Vaidya et al., 2001).

A number of carbon and nitrogen sources were investigated for their effect on the growth of *S. philanthi* RM-1-138 and on its chitinase and β-1,3 glucanase production. Glucose proved to be an excellent carbon source, although sucrose was also a good source for production of antibiotics (Juan and Arnold, 1980; Lebrihi et al., 1992; Juan, 2004; Ripa et al., 2009). In this study, *S. philanthi* RM-1-138 produced high levels of chitinase and β-1,3 glucanase in the medium supplemented with glucose at 6 g · l^{-1}. This was higher than that obtained for *S. halstedii* (4 g · l^{-1} glucose) for chitinase production (Joo, 2005). The type of carbon source and/or strain variations have a great influence on the cell growth and secondary metabolite production of *Streptomyces* species (Jonsbu et al., 2002). Higher glucose concentration (more than 8 g · l^{-1}) inhibited chitinase and β-1,3 glucanase production by *S. philanthi* RM-1-1-38. It also inhibits the biosynthesis of antibiotics (Juan and Arnold, 1980; Chu and Li, 2002; Cao, 2003) or acts as a repressor of secondary metabolisms (Demain, 1999).

The nitrogen source often has a profound influence on enzyme production as it is an essential precursor for

protein biosynthesis. The nitrogen source can also affect the pH of the medium, which in turn, may influence the activity and stability of the enzymes (Nizamudeen and Bajaj, 2009). Malt extract (15 $g \cdot l^{-1}$) was favorable for higher chitinase and β-1,3 glucanase production in *S. philanthi* RM-1-138 than the inorganic nitrogen sources. This was expected as organic nitrogen sources contain higher amounts of amino acids and short peptides which could better support enzyme production. This result is in agreement with the endoglucanase production (7.86 and 6.93 $U \cdot ml^{-1}$, respectively) by *Streptomyces* sp. BRC1 and BRC2 in a medium containing malt extract (10 $g \cdot l^{-1}$ w/v) (Chellapandi and Jani, 2008).

Till date, there has been no report on the simultaneous chitinase and β-1,3 glucanase production by *Streptomyces* sp. using RSM. Only one study was carried out using RSM to optimize the production of chitinase by three strains of *Streptomyces* sp. (NK1057, NK 528, and NK 951) (Nawani and Kapadnis, 2005). In this study, it has been determined that *S. philanthi* RM-1-138 produced simultaneous chitinase (0.53 $U \cdot ml^{-1}$) and β-1,3 glucanase (8.79 $U \cdot ml^{-1}$) under the optimum medium containing 4.88 $g \cdot l^{-1}$ chitin, 6.27 $g \cdot l^{-1}$ glucose, and 17.05 $g \cdot l^{-1}$ malt extract (Table 3). Besides these two enzymes, *S. philanthi* RM-1-138 also produces other highly valuable products such as antifungal metabolites that inhibited a range of plant pathogens by both volatile and non-volatile compounds (Boukaew et al., 2013a; Boukaew et al., 2013b; Boukaew et al., 2014c; Boukaew et al., 2014d).

A large number of microorganisms, including fungi, bacteria, actinomycetes, and plant species, possess the ability to excrete cell-wall hydrolases, such as proteases, chitinase, cellulase, and β-1,3-glucanase. These hydrolases play an important role in the reactions between biocontrol agents and pathogens by strongly inhibiting the spore germination, tube elongation, and mycelial growth (Chernin and Chet, 2002). Extracellular enzymes produced by *S. philanthi* RM-1-138 showed a potential antifungal activity against *R. solani* PTRRC-9. However, the exact mechanism of this process has not yet been clarified, although the activity of chitinase and β-1,3 glucanase against the fungal cell wall is evident (Shapira et al., 1989; Lim et al., 1991). In addition, other carbohydrases or other hydrolytic enzymes such as proteases could also be involved in this process. Chitinases and β-1,3 glucanases have been known to lyse fungal cell walls (Webster, 1986), where chitin and β-1,3 glucan are two of the major components. The same mechanism may also apply to the antifungal activity of chitinase from *S. venezuelae* P10 against *Aspergillus niger* (Mukherjee and Sen, 2006), and from *S. halstedii* against *Fusarium oxysporum* (Joo, 2005), and from *Streptomyces* sp. 385 against *F. oxysporum* (Singh et al., 1999). Mycelial growth of *Aspergillus niger*, *Helminthosporium sativum*, and *Alternaria alternata* was inhibited by the application of 10 μg of purified chitinase from *S. venezuelae* P10 (Mukherjee and Sen, 2006). Furthermore, the potent antifungal activity of *Streptomyces* sp. by other or unknown mechanisms has been reported elsewhere (El-Abyad et al., 1993; Errakhi et al., 2007; Patil et al., 2010; Li et al., 2011). Prominent applications of chitinase as a biocontrol agent was reported earlier but this is the first report on β-1,3 glucanase and its antifungal activity.

Conclusions

The optimum medium composition for maximizing the simultaneous production of chitinase and β-1,3 glucanase contained 4.88 $g \cdot l^{-1}$ chitin, 6.27 $g \cdot l^{-1}$ glucose, and 17.05 $g \cdot l^{-1}$ malt extract. The maximum chitinase production of 0.53 $U \cdot ml^{-1}$ and β-1,3 glucanase of 8.79 $U \cdot ml^{-1}$ were achieved. The three major components (chitin, glucose, and malt extract) of this optimum medium for simultaneous production of the two enzymes were lower than those in the optimum medium for each enzyme separately. Hence, the production cost could be reduced while synthesizing both enzymes at the same time. The partially purified enzymes from the optimized medium exhibited 4.0-fold stronger antifungal activities against *R. solani* PTRRC-9 compared to the original medium. Their antifungal activity was equal to that of the chemical fungicide propiconazole® but slightly lower than carbendazim®.

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