Article

Optimization of Medium Components for the Production of Antagonistic Lytic Enzymes Against Phytopathogenic Fungi and Their Biocontrol Potential

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In this paper, fractional factorial screening design (FFSD) and central composition design (CCD) were used to optimize the medium components for producing chitinase and gelatinase by *Lysobacter capsici* YS1215. Crab shell powder, nutrient broth and gelatin were proved to have significant effects on chitinase and gelatinase activity by FFSD first. An optimal medium was obtained by using a three factor CCD, which consisted of nutrient broth of 2.0 g L^{-1}, crab shell powder of 2.0 g L^{-1} and gelatin of 1.0 g L^{-1}, respectively with the highest chitinase activity (3.34 U mL^{-1}) and gelatinase activity (14.15 U mL^{-1}). This value was 3.76 and 1.11 fold of the chitinase and gelatinase activity, respectively, compared to the lowest productive medium in the design matrix. In investigating potential of these enzymes partially purified from *L. capsici* YS1215 for biotechnological use, the crude enzymes was found to be inhibition against pathogenic fungal mycelia: *Colletotrichum gleosporioides*, *Phytophthora capsici*, and *Rhizoctonia solani*. In this study, we demonstrated the optimal medium for producing the chitinolytic and gelatinolytic enzymes by the strain YS1215 and the role of their enzymes that may be useful for further development of a biotechnological use and agricultural use for biological control of phytopathogenic fungi.

Key words: *Lysobacter capsici* YS1215, Chitinase, Gelatinase, Response surface methodology, Optimization

Summary of fit, analysis of variance and lack of fit for predictive equation for chitinase and gelatinase production.

		Chitinase	Gelatinase
Summary of Fit	RSquare	0.9008	0.8235
	RSquareAdj	0.7520	0.5587
Analysis of Variance			
Model	DF	9	9
	Sum of Squares	6.7405	2.1128
	Mean Square	0.7489	0.2347
	F Ratio	6.0560	3.1109
	Prob> F	0.0200*	0.0903
Lack Of Fit	DF	5	5
	Sum of Squares	0.5365	0.3596
	Mean Square	0.1073	0.0719
	F Ratio	0.5224	0.7723
	Prob> F	0.7749	0.6933

Square = Coefficient of correlation; RSquareAdj = Adjusted correlation coefficient; DF = Degree of freedom. Data are statistically significant ($p < 0.05$).

Introduction

Crustacean shell wastes containing crab shells generally consist of proteins (30-40%), calcium carbonate (30-50%), and chitin (20-30%) (Crini *et al*., 2009; Kurita, 2006). Thus, crab shell waste is a good source of production of enzymes. Chitin is composed of linear chains of β-1,4-linked N-acetylg-lucosamine residues that are highly cross-linked by hydrogen bonds. Chitinase is a group of enzymes capable of catalyzing the hydrolysis of chitin directly to its monomer N-acetyl-D-glucosamine from variety of sources, such as bacteria, fungi, yeast and plants (Patil *et al*., 2000). Chitinases have been applied to a broad range: (1) cytochemical localization of chitin/chitosan, (2) fungal protoplast technology, (3) preparation of chitooligosaccharides and (4) as biocontrol agents of fungal pathogens and plant parasitic nematodes (Vaidya *et al*., 2003). For example, chitinases from microorganisms can degrade fungal cell walls and the nematode eggshells which composed of chitin (Goodrich and Winter, 2007; Bird and McClure, 1976; Ordentlich *et al*., 1988; Regev *et al*., 1996).

Gelatin is an important hydrocolloid which has been used for production of protease (Rajkumar *et al*., 2010). It is a high molecular weight and water-soluble protein. Gelatin contains all types of amino acids except tryptophan and is, however, low in methionine, cystine and tyrosine (Jamilah and Harvinder, 2002; Chapman and Hall, 1997). The enzymes that degrade gelatin are known as proteases, gelatinases, peptidyl-peptide hydrolases or sometimes, simply gelatinolytic proteins (Rattray *et al*., 1997). Because of the characteristics of these hydrolytic enzymes, it may have an antagonistic role against nematodes (Siddiqui *et al*., 2005). The genus *Lysobacter* are typically found in soil and water habitats and are qualified by gliding motility and the ability to lyse Gram-negative and Gram-positive bacteria, including fungi and nematodes (Lee *et al*., 2013). Several new species of *Lysobacter* have recently been reported with high promise of biocontrol activity against fungi and nematode (Chen *et al*., 2006; Park *et al*., 2008; Postma *et al*., 2009), ascribed to the production of antifungal compounds and nematicidal substances including enzymes such as chitinases, β-1, 3-glucanases and proteases (Islam, 2010). Although chitinase and gelatinase production have been reported in *Lysobacter* species, there is no information on statistical experimental optimization of nutritious conditions for their production.

Optimization of the parameters by statistical method can remove limitations of time consuming and expensive approaches. Response surface methodology (RSM) is a statistical method for exploring optimum condition in the biochemical process. This program has effectively been used to analyze the influence of several independent variables altogether on the desirable responses in a wide range of applications (Myers and Montgomery, 2002). The objectives of using RSM are to improve quantity as well as quality, including reduction of variability, having an improved process and product performance. The main advantage of this method is the reduced number of experimental trials needed to evaluate multiple parameters and their interactions (Gunaraj and Murugan, 1999; Kincl *et al*., 2005).

Lysobacter capsici YS1215 was isolated from the rhizosphere soil from fields near Suncheon, Jeolanamdo, Korea and produced several enzymes such as gelatinase and chitinase. Numerous studies on optimum media composition (Akolkar *et al*., 2009; Singh *et al*., 2009; Ghorbel-Bellaaj *et al*., 2012) have been investigated but no such research has been reported with *Lysobacter capsici* YS1215 on the production of chitinase and gelatinase in a systematic way. Therefore, the present investigation was carried out to optimize the medium composition for chitinase and gelatinase production using RSM. In addition, antifungal activities against pathogenic fungi were studied with a partially purified enzyme (80% ammonium sulfate precipitate) in *in vitro* experiments.

Materials and Methods

Preparation of media composition and bacterial culture
Lysobacter capsici YS1215 was grown in 250 mL Erlenmeyer flasks in laboratory conditions cultured at 30ºC with shaking. The compositions of the medium used for growth of *L. capsici* YS1215 were as follows (g L^{-1}): L-monosodiumglutamate (MSG) 1.0; sucrose (Beksul, Korea) 3.0; KH$_2$PO$_4$ 1.4; (NH$_4$)$_2$SO$_4$

Table 1. CCD pattern and responses.

Variables			Chitinase (U mL^{-1})		Gelatinase (U mL^{-1})	
X_1	X_2	X_3	Observed	Predicted	Observed	Predicted
0.66	1.5	0.75	2.15	1.88	13.10	12.81
1.0	1.0	0.5	1.85	2.19	12.77	12.90
1.0	1.0	1.0	0.89	0.76	12.74	12.93
1.0	2.0	0.5	1.59	1.68	12.77	13.05
1.0	2.0	1.0	1.37	1.59	13.36	13.33
1.5	0.66	0.75	1.61	1.62	13.28	13.16
1.5	1.5	0.33	1.93	1.68	13.15	12.93
1.5	1.5	0.75	1.87	2.20	13.06	13.29
1.5	1.5	0.75	2.52	2.20	13.49	13.29
1.5	1.5	1.17	1.43	1.59	13.38	13.45
1.5	2.34	0.75	2.11	2.01	13.87	13.85
2.0	1.0	0.5	2.55	2.39	13.01	13.14
2.0	1.0	1.0	2.41	2.37	13.65	13.47
2.0	2.0	0.5	1.83	2.02	13.67	13.58
2.0	2.0	1.0	3.63	3.35	14.19	14.16
2.34	1.5	0.75	3.36	3.53	13.56	13.71

X_1= Nutrient broth (g L^{-1}), X_2= Crab shell powder (g L^{-1}), X_3= Gelatin (g L^{-1})

1.0; $CaCO_3$ 1.0; $MgSO_4 \cdot 7H_2O$ 0.3; $CuSO_4 \cdot 5H_2O$ 0.02; $ZnSO_4 \cdot 7H_2O$ 0.02; $FeCl_3 \cdot 6H_2O$ 0.03 at pH 7.0 and other nutrients (Table 1). After 5 days of incubation, the bacterial culture was used to measure chitinase and gelatinase activities in *in vitro* experiments.

Assay of antifungal activity A crude enzyme was tested for antifungal potentials against several fungal pathogens namely: *Phytophthora capsici* KACC 40483, *Fusarium oxysporum* f. sp*lycopesici* (Saccardo) Snyder & HansenKACC 40032, *Colletotrichum gleosporioides* KACC40003, and *Rhizoctonia solani* AG-2-2 (IV) KACC 40132 obtained from KACC (Korea Agriculture Culture Collection, 225 Seodun-dong, Suwon, Gyunggi Province, Korea). The plugs (6 mm) of six old potato dextrose agar (PDA) culture of the fungal pathogens were inoculated on PDA plates. Discs containing the partially purified enzyme (10 μL or 20 μL) by 80% ammonium sulfate saturation were placed on the plates and incubated at 26°C for 5 days.

Lytic enzyme assays Gelatinase activity was determined by modification of the method of Moore and Stein (1948) and Mandl *et al.* (1953). Gelatin (Sigma) was used as a substrate to assess the gelatinase activity. Enzyme solution (0.05 mL) was added to 1.0 mL of 10 mM Tris-HCl buffer (pH 8.0) containing 0.5% gelatin in eppendorf tubes. The reaction mixtures were incubated at 37°C for 60 min followed by addition of 0.06 mL of 100% trichloroacetic acid to terminate the reaction and then keeping them in 4°C for 10 min. The mixtures were then centrifuged at 10,000 *g* for 10 min and after centrifugation, 0.05 mL of supernatant was mixed with 0.7 mL of 2% ninhydrin solution and boiled for 15 min. After boiling, the reaction mixtures were diluted with 0.7 mL of 50% n-propanol and then absorbance was read at 570 nm in UV-spectrophotometer (UV-1650PC, Shimadzu, Japan). One unit liberates peptides from gelatin equivalent in ninhydrin color to 1.0 μM of leucine for 1 h at pH 8.0 at 37°C in the presence of calcium ions.

To assess the chitinase activity colloidal chitin was used as a substrate. Enzyme solution (0.05 mL) was added to 0.45 mL of 50 mM acetate buffer (pH 5.0) and then mixed with 0.5 mL of 0.5% colloidal chitin. The reaction mixtures were incubated at 37°C for 60 min and then mixed with 0.2 mL of 1.0 M NaOH to terminate the reaction. The mixtures were then centrifuged at 10,000 *g* for 10 min and supernatants were collected. Supernatant (0.75 mL) was then mixed with 1 mL of Schales' reagent (0.5 M sodium carbonate and 1.5 mM potassium ferricyanide) and boiled for 15 min. The amount of reducing sugars produced in the supernatant was determined by the method of Lingappa and Lockwood (1962). One unit (U) of chitinase activity was defined as the amount of enzyme that liberated 1.0 μM of N-acetyl-glucosamine per hour.

Statistical design of experiments The experiment was carried out using fractional factorial screening design (FFSD) of Design of Experiment (DOE) which is a software developed by JMP, SAS (trail version) to determine the optimum medium conditions for the production of enzymes. Four independent variables were assayed at three codes levels (-1, 0, +1) in the following concentrations (X_1-X_4 in g L^{-1}): X_1, nutrient broth (0.0, 0.5, 1.0); X_2, yeast extract (0.0, 0.5, 1.0); X_3, crab shell powder (0.2, 0.5, 0.8); and X_4, gelatin (0.2, 0.5, 0.8). The design matrix consists of 18 trials with one number of center points and one number of replicates. The behavior of the system was explained by following equation:

$$Y = \beta_0 + \Sigma_{i=1}^{n} \beta_i x_i + \Sigma_{i<j}^{n} \beta_{ij} x_i x_j \qquad (1)$$

Where Y is the predicted response (the production of chitinase and gelatinase), β_0 is a constant, β_i is linear coefficient, and β_{ij} is the second-order interaction. As four variables were involved in the present study, therefore, the above equation may be written as:

$$Y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_4 x_4 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{14} x_1 x_4 \qquad (2)$$

Where x_1, x_2, x_3 and x_4 are the 4 input factors (independent variables), β_0 is a constant and β_1, β_2, β_3 and β_4 are linear coefficients. β_{12}, β_{13}, and β_{14} are cross-product coefficients. After yeast extract was screened out as non-important variable in FFSD, central composite design (CCD), employed with five levels of each of nutrient broth, crab shell powder and gelatin, were run to evaluate the second-order polynomial effects and interactions between variables. In the CCD with $\alpha = 1.682$, three variables were assayed at five coded levels (-α, -1, 0, +1, +α) in the following concentrations (X_1-X_3, in g L^{-1}): X_1, nutrient broth (0.66, 1.0, 1.5, 2.0, 2.34); X_2, crab shell powder (0.66, 1.0, 1.5, 2.0, 2.34); and X_3, gelatin (0.32, 0.5, 0.75, 1.0, 1.17). The design matrix consisted of 16 trials with two numbers of center points and zero number of replicates. The behavior of the system was explained by following equation:

$$Y = \beta_0 + \Sigma_{i=1}^{n} \beta_i x_i + \Sigma_{i<j}^{n} \beta_{ij} x_i x_j + \Sigma_{t=1}^{n} \beta_{ij} x_i^2 \qquad (3)$$

Where Y is the predicted response, x_i is the input variables that influence the response variable Y, β_0 is the intercept, β_i is the *i*th linear coefficient, β_{ii} is the *i*th quadratic coefficient and β_{ij} is the *ij*th interaction coefficient, respectively.

Results and discussion

Fractional factorial screening design (FFSD) FFSD was employed to determine the influence of independent variables on the production of chitinase and gelatinase by *L.*

capsici YS1215. The data obtained from the experiment will be analyzed in linear model. From the model generated by JMP, the predicted value was obtained from Eq. (4), Eq. (5), for the production of chitinase and gelatinase, respectively (see Materials and methods). All equations, explaining the behavior of the system, were derived from the parameter estimates data analyzed in linear model.

$$Y = 3.15 + 0.61x_1 + 0.74x_2 + 0.31x_3 - 0.36x_4 - 0.25x_1x_2 - 0.69x_1x_3 - 0.53x_1x_4 \quad (4)$$

$$Y = 11.88 + 0.08x_1 - 0.11x_2 + 0.06x_3 - 0.03x_4 - 0.01x_1x_2 + 0.01x_1x_3 - 0.08x_1x_4 \quad (5)$$

[*The *P* values for regression coefficients in bold characters were significant at *P*<0.05]

For both chitinase and gelatinase, the *P* values of the F tests were highly significant and *P* values of lack of fit of the regression model were not significant. The goodness of fit of the models was checked by the determinations of the coefficient of 0.97 and 0.72 for chitinase and gelatinase, respectively. The fit of the model based on the experimental design can be checked by coefficient of correlation (R^2), which provides a measure of how much variability in the observed response values can be explained by the experimental factors and their interactions (Kaushik *et al.*, 2006). A highly significant model was obtained from R^2 and adjusted correlation coefficient (R^2adj) for chitinase production.

Maximized desirability of chitinase and gelatinase can be obtained from a medium containing high concentration of nutrient broth (1.0 g L^{-1}), low concentration of yeast extract (0.0 g L^{-1}), high concentration of crab shell powder (0.8g L^{-1}) and low concentration of gelatin (0.2g L^{-1}). The medium yielded 1.15 and 1.03 fold of chitinase activity, and gelatinase activity, respectively, higher than that of the less productive medium in the design. This suggested that yeast extract would not be necessary in RSM.

Optimization of screened medium components Three medium components were selected and further optimized using CCD. Table 1 shows the results of experimental and predicted values from design of experiment. From the model generated by JMP, the predicted value was obtained from Eq. (6), and Eq. (7) for the production of chitinase and gelatinase, respectively.

$$Y = 2.2 + 0.49x_1 + 0.11x_2 - 0.02x_3 + 0.04x_1x_2 + 0.35x_1x_3 + 0.34x_2x_3 + 0.18x_1^2 - 0.13x_2^2 - 0.19x_3^2 \quad (6)$$

$$Y = 13.28 + 0.26x_1 + 0.2x_2 + 0.15x_3 + 0.07x_1x_2 + 0.06x_2x_3 - 0.01x_1^2 + 0.07x_2^2 - 0.03x_3^2 \quad (7)$$

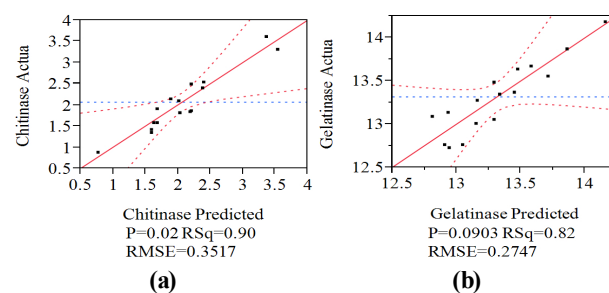[*The *P* values for regression coefficients in bold characters were significant at *P*<0.05]

Base on the data from the regression coefficients and *t* values (Table 2), it can be concluded that the production of chitinase was determined primarily by nutrient broth, and the production of gelatinase was determined by nutrient broth and crab shell powder. Interactions between two of the three factors (nutrient broth, crab shell powder and gelatin) were also found significant for the production of chitinase (nutrient broth and gelatin; crab shell powder and gelatin), but not significant for the production of gelatinase.

Fig. 1 shows the actual values and the predicted values determined by the model Eq. (6), (7) for the production of chitinase and gelatinase, respectively. The correlation coefficient values are $R^2 = 0.90$, and $R^2 = 0.82$ for chitinase and gelatinase, respectively, meaning a high significance of the model (Table 3). Furthermore, 98% and 90% of chitinase and gelatinase activities can be explained by the model. Another indicative proof of the high significance of the model is that the *P* values of lake of fit were not significant for both models.

Table 2. Parameter estimates form the data of RSM.

Term	Chitinase		Gelatinase	
	Estimate	Prob>|t|	Estimate	Prob>|t|
Intercept	2.2005	0.0001*	13.2880	<.0001*
N.B (1,2)	0.4904	0.0021*	0.2682	0.0112*
C.S.P (1,2)	0.1150	0.2720	0.2064	0.0321*
Gelatin (0.5,1)	-0.0262	0.7923	0.1528	0.0855
N.B*C.S.P	0.0350	0.7878	0.0722	0.4849
N.B*Gelatin	0.3542	0.0292*	0.0742	0.4738
C.S.P*Gelatin	0.3360	0.0355*	0.0622	0.5456
N.B*N.B	0.1776	0.1751	-0.0097	0.9175
C.S.P *C.S.P	-0.1361	0.2833	0.0768	0.4273
Gelatin*Gelatin	-0.1997	0.1345	-0.0350	0.7114

N.B = Nutrient broth (g L^{-1}). C.S.P = Crab shell powder (g L^{-1}), Gelatin (g L^{-1})

Chitinase Predicted
P=0.02 RSq=0.90
RMSE=0.3517
(a)

Gelatinase Predicted
P=0.0903 RSq=0.82
RMSE=0.2747
(b)

Fig. 1. Actual by predicted plot of chitinase (a) and gelatinase (b). The actual values form a scatter of points around each leaf mean. A diagonal line represents the locus of where predicted and actual values are the same.

According to the program, maximized desirability of chitinase (3.34 U mL^{-1}) and gelatinase (14.15 U mL^{-1}) was obtained from a medium containing nutrient broth (2.0 g L^{-1}), crab shell powder (2.0 g L^{-1}) and gelatin (1.0 g L^{-1}). This optimum medium yielded 3.76, and 1.1 fold of chitinase activity and gelatinase activity respectively, higher than that of the less productive medium in the design matrix.

The three-dimensional response surface plot was developed to explain the interaction of medium components and the optimum concentrations of each component required for the chitinase and gelatinase production (Fig. 2). Each figure indicated the effect of two variables, while the other variable one is held at zero level. It was evident that chitinase and gelatinase activities increased as concentrations of crab shell powder and nutrient broth increased. This relationship was not linear. The elliptical nature of the contour plot indicated that the interaction between crab shell powder and nutrient broth was significant.

There are a few reports on optimizing medium compositions for the production of chitinase and gelatinase by microorganisms. Optimized medium components to improve chitinase activity of *Streptomyces* sp. DA11 associated with South China sea sponge *Craniella austaliensis* were examined by Plackett-Burman design and Box-Behnken RSM (Han *et al*., 2008). The author found that the maximum chitinase activity was 39.2-fold higher than that of the control basic medium. Using similar statistical method, chitinase production was found to increase from 12 to 29 U mL^{-1} by *Alcaligenes xylosoxydans* (Vaidya *et al*., 2003). The optimal medium components were (g L^{-1}): $(NH_4)_2SO_4$, 1.0; KH_2PO_4, 1.36; $MgSO_4 \cdot 7H_2O$, 0.3; Tween 20, 0.12; yeast extract, 0.3 and chitin powder, 1.5. CCD has been used in optimizing chitinase production by *Moniliophthora perniciosa* (Lopes *et al*., 2008), *Streptomyces* sp. NK157, NK528 and NK951 (Nawani *et al*., 2005), and chitin deacetylase production

Table 3. Summary of fit, analysis of variance and lack of fit for predictive equation for chitinase and gelatinase production by *Lysobacter capsici* YS1215.

		Chitinase	Gelatinase
Summary of Fit	RSquare	0.9008	0.8235
	RSquareAdj	0.7520	0.5587
	Root Mean Square Error	0.3516	0.2747
	Mean of Response	2.0654	13.3154
	Observations (or Sum Wgts)	16	16
Analysis of Variance			
Model	DF	9	9
	Sum of Squares	6.7405	2.1128
	Mean Square	0.7489	0.2347
	F Ratio	6.0560	3.1109
	Prob> F	0.0200*	0.0903
Error	DF	6	6
	Sum of Squares	0.7420	0.4527
	Mean Square	0.1236	0.0754
C. Total	DF	15	15
	Sum of Squares	7.4825	2.5656
Lack Of Fit			
Lack Of Fit	DF	5	5
	Sum of Squares	0.5365	0.3596
	Mean Square	0.1073	0.0719
	F Ratio	0.5224	0.7723
	Prob> F	0.7749	0.6933
Pure Error	DF	1	1
	Sum of Squares	0.2054	0.0931
	Mean Square	0.2054	0.0931
Total Error	DF	6	6
	Sum of Squares	0.7420	0.4527

Square = Coefficient of correlation; RSquareAdj = Adjusted correlation coefficient; DF = Degree of freedom. Data are statistically significant ($p < 0.05$).

(a)   **(b)**
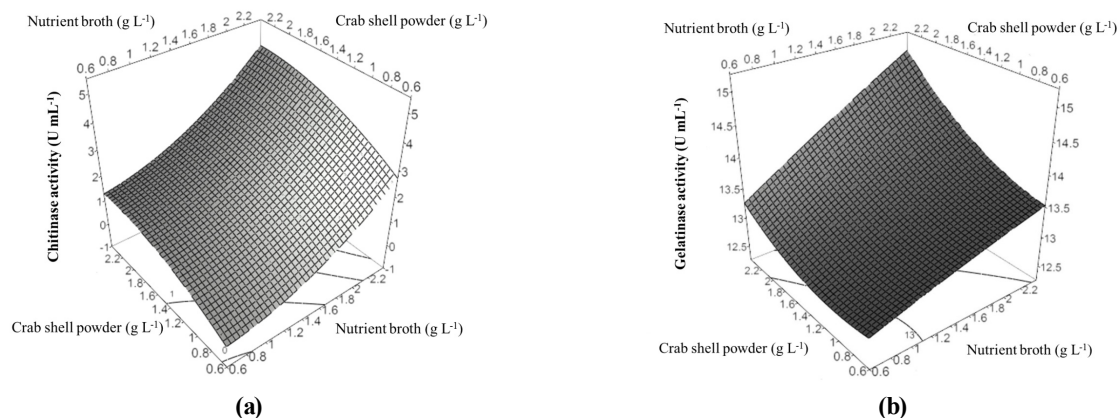
Fig. 2. Response surface plots showing the effect of the most influential variables (nutrient broth and crab shell powder) on the production of chitinase (a) and gelatinase (b).
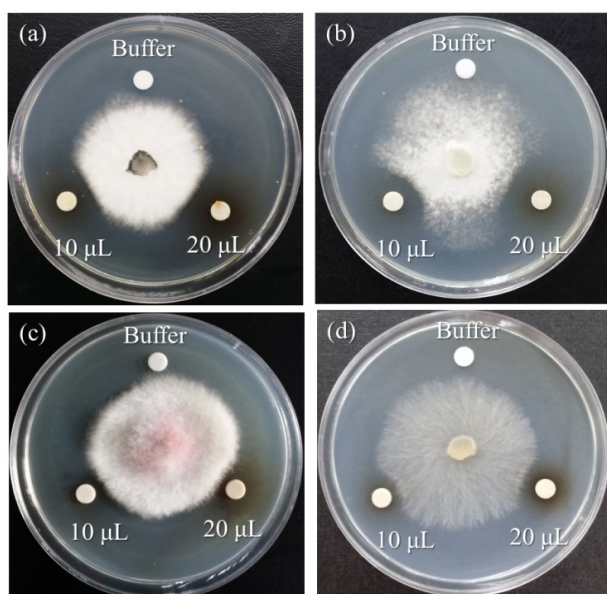
Fig. 3. Inhibition activities of a partially purified enzyme (treatments of 10 μL or 20 μL)against (a) *Colletotrichum gleosporioides*, (b) *Phytophthora capsici*, (c) *Fusarium oxysporum*, and (d) *Rhizoctonia solani* after 5 days. Buffers were treated as a control.

by *Penicillium oxalicum* SAE$_M$-51 (Pareek*et al*., 2011). Plackett-Burman screening design and CCD were employed to optimize simple culture conditions for biomass production of an ochratoxigenic mould biocontrol yeast strain (Pelinski*et al*., 2012). The authors also found that yeast extract would not be necessary for higher productivity. The partially purified enzyme showed significant inhibition of fungal mycelia growth against *Phytophthora capsici*, *Colletotrichum gleosporioides*, and *Rhizoc-tonia solani* AG-2-2 (IV) exception of *Fusarium oxysporum* f. sp *lycopesici*(Saccardo) Snyder & Hansen (Fig. 3). This result indicates that chitinase and gelatinase from *L. capsici* YS1215 may be used in the biological control of phytopathogenic fungi through mass production of these enzymes.

In our study, the fractional factorial was used for the first time in screening design, and followed by CCD to utilize optimum medium component for the production of chitinase and gelatinase by *Lysobacter capsici* YS1215. We used crab shell powder and gelatin to optimize the production of chitinase and gelatinase by *L. capsici* YS1215 because they are obviously simpler and cheaper than the chitin powder and other expensive nutrients (Wang *et al*., 2006; Liang *et al*., 2007). In conclusion, the use of the statistically based designs has aided a lot in finding out the medium components, which are effective on the production of these enzymes that can be applied as biological control agents on agricultural fungal pathogens. Moreover, the utilization of chitin-containing waste such as crab shell powder not only solves environmental problems but also reduces the production costs of microbial enzymes.

References

Akolkar, A., N. Bharambe, S. Trivedi, and A, Desai. 2009. Statistical optimization of medium components for extracellular protease production by an extreme haloarchaeon, *Halobacterium* sp. SP1(1). Lett. Appl. Microbiol. 48:77-83.

Bird, A.F.and M.A. McClure. 1976. The tylenchid (nematode) egg shell: structure, composition and permeability. Parasitology. 72:19-28.

Chen, J., W.H. Moore, G.Y. Yuen, D. Kobayashi, and E.P. Caswell-Chen. 2006. Influence of *Lysobacter enzymogenes* Strain C3 on Nematodes. J. Nematol. 38(2):233-239.

Crini, G., E. Guibal, M. Morcellet, G. Torr, and P.M. Badot. 2009. Chitine et chitosane. Préparation, propriétés et principales applications. In: *chitine et chitosane. Du biopolymère à l'application*, 1st Ed., Presses universitaires de Franche-Comté, France. p. 19-54.

Ghorbel-Bellaaj, O., L. Manni, K. Jellouli, N. Hmidet, and M. Nasri. 2012. Optimization of protease and chitinase production by *Bacillus cereus* SV1 on shrimp shell waste using statistical experimental design. Biochemical and molecular characterization of the chitinase. Ann. Microbiol. 62:1255-1268.

Goodrich, J.D. and T.W. Winter. 2007. β-Chitin Nanocrystals Prepared from shrimp shells and their specific surface area measurement. Biomacromolecules. 8:252-257.

Gunaraj, V. and N. Murugan. 1999. Application of response surface methodologies for predicting weld base quality in submerged arc welding of pipes. J. Mater. Process Technol. 88:266-275.

Han, Y., Z. Li, X. Miao, and F. Zhang. 2008. Statistical optimization of medium components to improve the chitinase activity of Streptomyces sp. Da11 associated with the South China Sea sponge Craniella australiensis. Process Biochem. 43(10):1088-1093.

Islam, M.T. 2010. Mode of antagonism of a biocontrol bacterium Lysobacter sp. SB-K88 toward a damping-off pathogen Aphanomyces cochlioides. World J. Microbiol. Biotechnol. 26:629-637.

Jamilah, B., and K.G. Harvinder. 2002. Properties of gelatins from skins of fish-black tilapia (Oreochromis mossambicus) and red tilapia (Oreochromis nilotica). Food Chem. 77:81-84.

Kaushik, R., S. Saran, J. Isar, and R.K. Saxena. 2006. Statistical optimization of medium components and growth conditions by response surface methodology to enhance lipase production by Aspergillus carneus. J. Mol.Catal. B.Enzym. 40:121-126.

Kincl, M., S. Turk, and F. Vrecer. 2005. Application of response surface methodology in development and optimization of drug release method. Int. J. Pharm. 291:39-49.

Kurita, K. 2006. Chitin and Chitosan: Functional biopolymers from marine crustaceans. Marine.Biotechnol. 8:203-226.

Lee, Y.S., M. Anees, H.N. Hyun, and K.Y. Kim. 2013. Biocontrol potential of Lysobacter antibioticus HS124 against the root-knot nematode, Meloidogyne incognita, causing disease in tomato. Nematology. 15(5):545-555.

Liang, T.W., Y.Z. Chen, Y.H. Yen, and S.L. Wang. 2007. The antitumor activity of the hydrolysates of chitinous materials hydrolyzed by crude enzyme from Bacillus amyloliquefaciens V656. Process Biochem. 42:527-534.

Lingappa, Y., and J.L. Lockwood. 1962. Chitin media for selected isolation and culture of actinomycetes. Phytopathology. 52:317-323.

Lopes, M.A., D.S. Gomes, M.G. Koblitz, C.P. Pirovani, J.C. Cascardo, A. Góes-Neto, and F. Micheli. 2008. Use of response surface methodology to examine chitinase regulation in the basidiomycete Moniliophthora perniciosa. Mycol. Res. 112(3): 399-406.

Mandl, I., J.D. MacLennan, E.L. Howes, R.H. DeBellis, and A. Sohler. 1953.Isolation and characterization of proteinase and collagenase from Cl. Histolyticum. J. Clin. Investig. 32:1323-1329.

Moore, S., and W.H. Stein. 1948. Photometric ninhydrin method for use in the chromatography of amino acids. J. Biol. Chem. 176:367-388.

Myers, H.M., and D.C. Montgomery. 2002.Response surface methodology: process and product optimization using designed experiments. 2nd Ed. New York: A Wiley inter-science publication.

Nawani, N.N., and B.P. Kapadnis. 2005. Optimization of chitinase production using statistics based experimental designs. Process Biochem. 40(2):651-660.

Ordentlich, A., Y. Elad, and I. Chet.1988.The role of chitinase of Serratia marcescens in biocontrol of Sclerotium rolfsii. Phyto-pathology.78:84-88.

Pareek, N., R.P. Singh, and S. Ghosh. 2011. Optimization of medium composition for enhanced chitin deacetylase production by mutant Penicillium oxalicum SAEM-51 using response surface methodology under submerged fermentation. Process Biochem. 46(8):1693-1697.

Park, J.H., R. Kim, Z. Aslam, C.O. Joen, and Y.R. Chung. 2008. Lysobacter capsici sp. nov., with antimicrobial activity, isolated from the rhizosphere of pepper, and emended description of the genus Lysobacter. Int. J. Syst. Evol. Microbiol. 58:387-392.

Patil, R.S., V. Ghomade, and M.V. Deshpande. 2000. Chitinolytic enzymes: an exploration. Enzym. Microb. Tech. 26:473-483.

Pelinski, R.P. Cerrutti, M.L. Ponsone, S. Chulze, and M. Galvagno. 2012. Statistical ptimization of simple culture conditions to produce biomass of an ochratoxigenic mould biocontrol yeast strain. Lett. Appl. Microbiol. 54(5):377-382.

Postma, J., L.H. Stevens, G.L. Wiegers, E. Davelaar, E.H. Nijhuis. 2009. Biological control of Pythium aphanidermarum in cucumber with a combined application of Lysobacter enzymogenes strain 3.1T8 and chitosan. Biol. Contr. 48:301-309.

Rattray, F.P., P.F. Fox, and A. Healy.1997.Specificity of an extracellular proteinase from Brevibacterium linens ATCC 9174 on bovine b-casein. Appl. Environmental. Microbiol. 63:2468-2471.

Regev, A., M. Keller, N. Strizhov, B. Sheh, E. Prudovsky, I. Chet, I. Ginzberg, Z. Koncz-Kalman, C. Koncz, J. Schell, and A. Zilberstein. 1996. Synergistic activity of a Bacillus thuringiensis δ-endotoxin and a bacterial endochitinase against Spodoptera littoralis larvae. Appl. Environ. Microbiol. 62:3581-3586.

Siddiqui, I.A., D. Haas, and S. Heeb. 2005. Extracellular protease of Pseudomonas fluorescens CHA0, a biocontrol factor with activity against the root-knot nematode, Meloidogyne incognita. Appl. Environ. Microbiol. 71:5646-5649.

Singh, A.K., G. Mehta, and H.S. Chhatpar. 2009. Optimization of medium constituents for improved chitinase production by Paenibacillus sp. D1 using statistical approach. Lett. Appl. Microbiol. 49(6):708-14.

Vaidya, R., P. Vyas, and H.S. Chhatpar. 2003. Statistical optimization of medium components for the production of chitinase by Alcaligenes xylosoxydans. Enzym. Microb. Tech. 33(1):92-96.

Wang, S.L., P.Y. Yeh. 2006. Production of a surfactant- and solvent-stable alkaliphilic protease by bioconversion of shrimp shell wastes fermented by Bacillus subtilis TKU007. Process Biochem. 41:1545-1552.