International Journal of Agricultural Technology 2019 Vol. 15(6):913-924
Available online http://www.ijat-aatsea.com
ISSN 2630-0192 (Online)

Optimization of microbial collagenolytic enzyme production by *Bacillus subtilis* subsp. *Subtilis* S13 using Plackett-Burman and response surface methodology

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Khamson, A., Sumpavapol, P., Tangwatcharin, P. and Sorapukdee, S. (2019). Optimization of microbial collagenolytic enzyme production by *Bacillus subtilis* subsp. *Subtilis* S13 using Plackett-Burmanand response surface methodology. International Journal of Agricultural Technology 15(6):913-924.

Abstract *Bacillus subtilis* subsp. *subtilis* S13, a new isolate from soil is found to be the collagenase producing bacteria. The results showed that gelatin concentration, initial pH, and incubation time were accounted for significant factors from PB design and then applied for a central composite design (CCD) under response surface methodology (RSM) for optimization of significant factors were performed. The optimum parameters for the enhancing gelatinase production through CCD and response surface methodology were 19 g/l of pork gelatin, initial pH 6.16 for culture medium, and 59 h of incubation time, which provided the predicted maximum collatinolytic activity of 65.36 U/ml. This condition allowed approximately increasing 4-folds as compared to un-optimized condition (15 U/ml). The obtained optimal activity of this enzyme might be expressed the potential collagenase for several applications including meat tenderizing enzyme.

Keywords: Protease production, Collagenolytic protease, Central composite design

Introduction

Nowaday important enzymes, accounting for more than 65% of the total ecumenical enzyme sales, are proteases (Oskouie *et al*., 2008). Among these proteases, predominant market shares of commercial enzymes are originated from microbial proteases secreted from *Bacillus* species (Gupta *et al*., 2002a, b). *B. subtilis* is considered generally recognized as safe (GRAS) and extensively utilized in biotechnological application (Huang *et al*., 2018). This microorganism provides adequate yields and is able to secrete large amounts of extracellular enzymes, but not produce toxins or any other undesired products in culture medium (Priest, 1977).

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Generally, the protease enzyme from microorganisms is greatly impacted by the composition of medium, particularly carbon and nitrogen sources. In addition, other factors such as temperature, pH, incubation time, agitation, and density of inoculum also play an important role in protease production (Johnvesly and Naik, 2001). Because about 30–40 % of the production cost for an industrial enzyme is accounted from the media composition, various statistical methods involving Plackett–Burman designs (PBD) and response surface methodology (RSM) that was used for the optimization of enzyme production (Reddy *et al*., 2008). To rapidly screening the main significant effects from large numbers of variables, the PBD is carried out (Yang *et al*., 2014). The information obtained from the PBD can set as the method for primary screening of medium components, which is necessary for further optimization (Priya *et al*., 2011; Yang *et al*., 2014). Thereafter, RSM is performed for optimizing a small number of variables, and it usually involes an experimental design such as Central composite design (CCD) to fit a second-order polymomial by a least squres technique (Dean and Voss, 2000).

Recently, *B. subtilis* subsp. *Subtilis S13* was isolated from soil and exhibited some interesting characteristics for meat tenderization (Sorapukdee and Tangwatcharin, 2019). It can hydrolyze connective tissue proteins (collagen and elastin) that contribute meat toughness, providing softer meat texture (Sorapukdee and Tangwatcharin, 2019). There are numerous studies of collagenase producing bacterium, such as *B. subtilis* FS-2, *B. subtilis* CN, *B. subtilis* AS1.398, *B. pumilus* CoI-J and *B.licheniformis* N22 (Akiyama *et al*., 1999; Nagano, 1999; Tran and Nagano, 2002; Rui *et al*., 2009; Wu *et al*., 2010). However these researches are focused on different screening purposes depending on target enzyme application. Therefore, it was necessary to formulate the medium as well as cultural condition which provides the higher production of collagenase for meat tenderizing enzyme. The objective of this study was to optimize the collagenase production by *B. subtilis* subsp. *Subtilis* S13 using statistical methods of PBD and CCD.

Materials and methods

Microorganism

The microorganism with collagenolytic activity used in this study was isolated from soil. It was identified as *B. subtilis* subsp. *Subtilis* S13 by 16S rRNA gene sequencing (Sorapukdee and Tangwatcharin, 2019). A bacterium was kept frozen at -20 ℃ in nutrient broth (NB) supplemented with 20 % (v/v) glycerol.

Inoculum preparation and culture condition

The strain was sub-cultured in NA plate. The cultures were grown at 37 ℃ for 24 h. A loopfull of strain in sub-culture medium was transferred to 10 ml of NB in sterile 50-ml centrifuge tubes and incubated at 37 ℃, 180 rpm for 24 h in an incubator shaker (NB-205, N-Biotek, N-Biotek Co., Ltd., Gyeonggi-Do, Korea). To produce collagenase, culture was inoculated with 50 ml of production medium into a 250-ml Erlenmeyer flask. The compositions of production medium and growth conditions were defined as presented in Table 1 and Table 2. After that, the cells were removed by centrifuged at 13,000 ×g, 4 ℃ for 5 min (Sigma 2-16 KL, Sigma, Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany). After that, the cell-free supernatant was used for collagenolytic activity analysis.

Table 1. Statistical analysis of PBD results on collagenolytic enzyme production by *B. subtilis* subsp. *Subtilis* S13 for the eleven variables

No.	Variables	Levels		Estimate	Coefficient	*t*-value	*p*-value
		-1 level	+1 level				
X_1	Gelatin	10	15	11.17	5.58	13.40	0.047[/1]
X_2	Glucose	0	5	-6.83	-3.42	-8.20	0.077
X_3	Initial pH	6.5	7.5	-11.50	-5.75	-13.80	0.046[/1]
X_4	NaCl	0	10	-7.83	-3.92	-9.40	0.067
X_5	Glycerol	0	10	-0.83	-0.42	-1.00	0.500
X_6	Inoculation size	2	7	-1.83	-1.42	-1.00	0.500
X_7	Incubation time	24	48	12.83	6.42	15.40	0.041[/1]
X_8	Speed	100	200	5.83	2.92	7.00	0.090
X_9	$MgSO_4$	0	0.2	1.167	0.58	1.40	0.395
X_{10}	$CaCl_2$	0	0.2	-6.50	-3.25	-7.80	0.081
X_{11}	Yeast extract	0	1	7.5	3.75	9.00	0.070

[1]/ Significant at $P < 0.05$

Table 2. Twelve-trial PBD for eleven variables and observed collagenolytic activity of *B. subtilis* subsp. *Subtilis* S13

Runs	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	X_{11}	U/ml
1	15	0	6.5	0	10	7	48	100	0.2	0.2	0	40
2	15	5	6.5	10	10	2	48	100	0.0	0.0	1	39
3	10	0	7.5	10	10	2	48	200	0.0	0.2	0	15
4	15	5	6.5	10	0	2	24	200	0.2	0.2	0	20
5	10	5	6.5	0	0	7	48	200	0.0	0.2	1	35
6	10	0	6.5	10	10	7	24	200	0.2	0.0	1	28
7	10	5	7.5	0	10	2	24	100	0.2	0.2	1	6
8	15	0	7.5	0	0	2	48	200	0.2	0.0	1	50
9	10	0	6.5	0	0	2	24	100	0.0	0.0	0	23
10	15	5	7.5	0	10	7	24	200	0.0	0.0	0	20
11	15	0	7.5	10	0	7	24	100	0.0	0.2	1	15
12	10	5	7.5	10	0	7	48	100	0.2	0.0	0	10

Assay for collagenolytic activity

Collagenolytic activity was determined by using a gelatin as substrate according to the reported of Tran and Nagano (2000) with a slight modification. The reaction mixture composed of 0.6 mL of 0.2% (w/v) gelatin in water, 0.4 mL of 150 mM Tris-HCl, pH 7.5 containing 12 mM $CaCl_2$ and 0.2 ml of cell-free supernatant. The reaction was incubated at 37 ℃ for 30 min and then stopped by the summation of 1.2 ml of 0.1 N HCl. The ninhydrin method was used to estimate the content of liberated free amino acid. One unit (U) of collagenolytic activity was determined as the amount of enzyme which emancipated 1 μg of glycine per minute.

Screening of significant variable by the PBD

To evaluate significant variables for collagenolytic enzyme production, varieties of ingredients in culture medium and cultivation parameters were tested and identified by the PBD method. A total of eleven parameters were implicated including (1) gelatin, (2) glucose, (3) glycerol, (4) sodium chloride, (5) magnesium sulfate, (6) calcium chloride, (7) yeast extract, (8) initial pH, (9) inoculation size, (10) speed, and (11) incubation time (Table 1). Each parameter was tested with two levels include high (+1) and low (-1). The set of 12 experiments was conducted (Table 2). The significant effect of each variable on collagenolytic enzyme production was assigned by Student's t-test with 95% confidence levels.

Optimization by CCD under RSM approch

In the present study, RSM with CCD was conducted for further optimization of collagenolytic enzyme production. The significant variables obtained from the PBD, including gelatin, initial pH, and incubation time, was estimated at five coded levels (Table 3). The zero-code was used for the central values of all variables. The minimum and maximum values of the variables were examined and the full experimental plan is shown in Table 4. The response values (*Y*) in each probation were the equalize of the triplicates. A second-order polynomial model was used for the demonstration data and the regression coefficients were obtained by multiple regression as the following quadratic equation.

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{i=1}^{3} \sum_{j=2}^{3} \beta_{ij} X_i X_j \quad (1)$$

Where *Y* is the predicted response (collagenolytic activity), β_0 is the center point of the system, β_i is the linear coefficient, β_{ii} is the quadratic coefficient, and β_{ij} is the interaction coefficient, while X_i, X_i^2 and X_j are the

linear, quadratic and interaction terms of the independent variables, respectively.

Table 3. Independent variables, symbol, code levels for the RSM experiment for optimization of collagenolytic enzyme production by *B. subtilis* subsp. *Subtilis* S13

Independent variables	Units	Symbol	Code levels				
			-1.682	-1	0	+1	+1.682
Gelatin	g/l	X_1	12.64	14.00	16.00	18.00	19.36
Initial pH	-	X_3	6.16	6.50	7.00	7.50	7.84
Incubation time	h	X_7	15.11	25.00	39.50	54.00	63.89

Statistical analysis

The Minitab v.17 statistical was used for the experimental design. All of the optimization experiments were proceeded in triplicate, and the average data of collagenolytic enzyme yields were further analyzed with the Minitab v.17.

Table 4. Experimental designs according to CCD matrix used in the RSM study and values of observed collagenolytic activity (U/mL)

Run	Variable			Collagenolytic activity (U/ml)	
	Gelatin (X_1)	Initial pH (X_3)	Incubattion time (X_7)	Mean Observed[/1]	Predicted
1	0	0	+1.682	41.80 ± 3.62	41.77
2	0	0	0	40.04 ± 3.11	44.53
3	-1	-1	-1	24.39 ± 4.14	25.51
4	0	0	-1.682	18.46 ± 1.34	17.92
5	1.682	0	0	47.57 ± 4.45	50.89
6	+1	+1	+1	54.31 ± 1.30	50.25
7	0	0	0	52.22 ± 0.00	44.53
8	0	-1.682	0	42.77 ± 1.62	43.46
9	-1.682	0	0	33.14 ± 5.32	29.27
10	+1	-1	+1	59.82 ± 0.59	57.20
11	0	0	0	54.50 ± 3.31	44.53
12	-1	+1	-1	28.15 ± 3.15	27.85
13	+1	-1	-1	39.43 ± 7.29	35.07
14	0	0	0	40.80 ± 0.95	44.53
15	0	0	0	48.68 ± 2.32	44.53
16	0	+1.682	0	40.82 ± 4.45	39.59
17	+1	+1	-1	31.41 ± 1.22	30.01
18	0	0	0	37.04 ± 1.14	44.53
19	-1	-1	+1	35.14 ± 3.27	33.62
20	-1	+1	+1	32.64 ± 1.30	34.07

[1]/ Values are mean ± SD (n = 2).

Results

Screening of significant variable by the PBD

A total of eleven variables analyzed for deteriming significant impact on collagenolytic enzyme production through the PBD is presented in Table 1. These eleven parametes could generate the design matrix of 12-run expermients as shown in Table 2. Morevoer, the accuracy of the model was computed and variables that exhibit statistically significant effects were computed for analysis of variance with Student's *t*-test (Table 3). Factors with *p*-value lower than 0.05 were regarded to have significant effects on collagenolytic enzyme production. The result from PBD showed that incubation time (*p*-value of 0.041) was consulted as the greatest significant factor for production of collagenolytic enzyme from *B. subtilis* subsp. *Subtilis* S13, followed by initial pH (*p*-value of 0.046), and gelatin concentration (*p*-value of 0.047), respectively. Therefore, these three factors were chosen for further optimization using a CCD under RSM methodology.

Optimization by CCD under RSM approach

The results of collagenolytic enzyme production of 20 trials design matrix of CCD from three independent variables (incubation time, initial pH, and gelatin concentration) is shown in Table 4. Thereafter, the analysis of variance (ANOVA) was analyzed the data from CCD, and collagenolytic enzyme production was predicted with quadratic regression (Table 5). The *p*-value of the model was 0.004, which indicated that the model was statistically significant. The regression coeffieient (R^2) pointed out that the 84.92% of variation in the response expermiment could be described by this model, indicating the fitness of the model. In addition, the significant of each independent variable of the model was determined by *p*-value (Table 5). It was evident that gelatin concentration (X_1), incubation time (X_7), and interaction between initial pH and incubation time (X_3X_7) were significant variables ($p < 0.05$). Therefore, the regression equation (2) shows the response of collagenolytic enzyme production (Y) by *B. subtilis* subsp. *Subtilis* S13.

$$Y = -507 + 24.0\,X_1 + 89.5\,X_3 + 0.96\,X_7 - 1.85\,X_1X_3 + 0.1209\,X_1X_7 - 0.065\,X_3X_7 - 0.394\,X_1^2 - 4.26\,X_3^2 - 0.02469\,X_7^2 \quad (2)$$

Where Y is the predicted collagenolytic enzyme production (U/ml) and X_1, X_3, and X_7 are the values of gelatin concentration (g/l), initial pH, and incubation time (h), respectively.

The three-dimensional response surface plots and two-dimensional contour plots based on the model equations were plotted to investigate the

interaction among variables as shown in Figure 1. Additionally, these plots were used to assign the optimum concentration of each factor from maximum collagenase production by *B. subtilis* subsp. *Subtilis* S13. An increase in gelation concentration of 19 g/l and initial pH of culture medium in the range of pH 6.2-6.4 cased an increase in the enzyme production (Figure 1a). Regarding gelatin and incubation time, the increasing of gelation concentration in the range of 16.5-19 g/l with 40-60 h of incubation time had a positive influence on enzyme production (Figure 1b). The response of the interactive factors of initial pH and incubation time is shown in Figure 1c. It was found that the production of enzyme was maximized by the initial pH in the range of pH 6.2-7.5 and incubates time for 40-60 h.

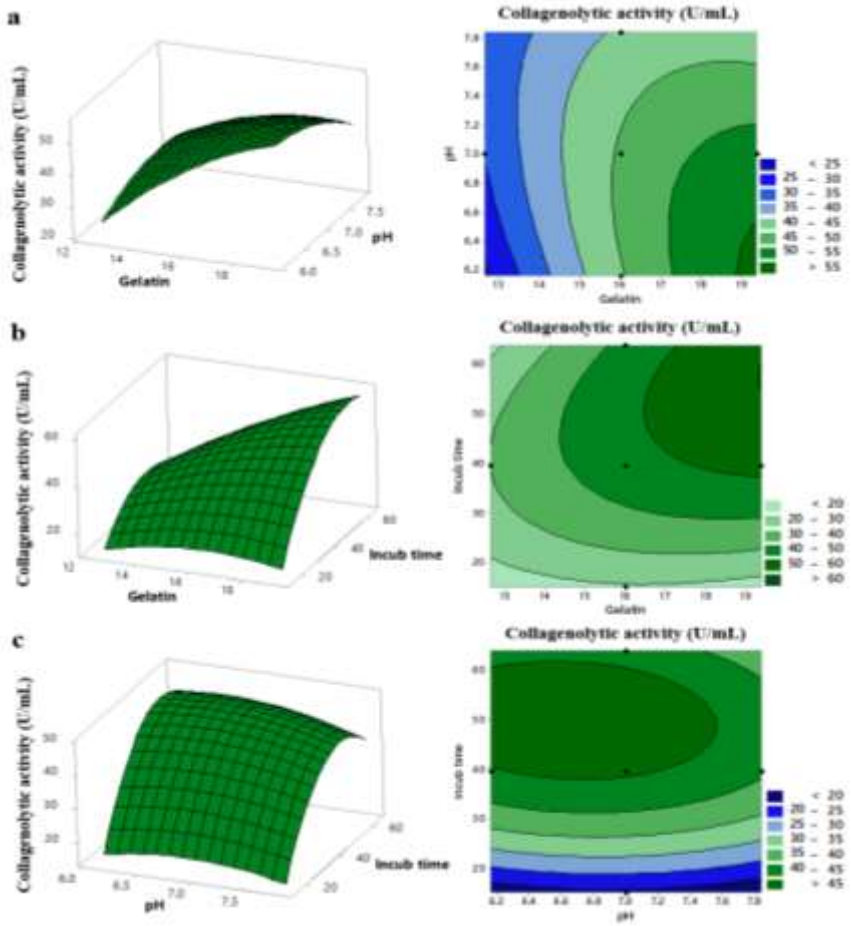

Figure 1. Response surface plots (left) and 2D conture plots (right) of effects of (a) gelatin vs. pH, (b) gelatin vs. incubation time and (c) pH vs. incubation time on collagenolytic activity

Table 5. ANOVA for the response surface methodology (RSM)

Source	*DF*	*SS*	*MS*	*F*-value	*p*-value
Model	9	1,832.18	203.575	6.26	0.004[/1]
X_1	1	578.94	578.938	17.80	0.002[/1]
X_3	1	17.74	17.736	0.55	0.477
X_7	1	700.10	700.099	21.52	0.001[/1]
$X_1 X_3$	1	35.87	35.873	1.10	0.318
$X_1 X_7$	1	16.36	16.365	0.50	0.494
$X_3 X_7$	1	388.49	388.491	11.94	0.006[/1]
X_1^2	1	27.35	27.347	0.84	0.381
X_3^2	1	98.35	98.351	3.02	0.113
X_7^2	1	1.77	1.768	0.05	0.820
Residual error	10	325.33	32.533		
Lack of fit	5	65.57	13.114	0.25	0.921
Pure error	5	259.76	51.953		
Correlation of total	19	2,157.51			

R^2, 0.8492; Adj-R^2, 0.7135; Pred-R^2, 0.5890
DF degree of freedom, *SS* sum of squares, *MS* mean square
[1]/ Significant at $P < 0.05$

When the independent variables were fited to equation (2), the optimum condition for collagenolytic enzyme production were proposed as follows: gelation concentration of 19 g/l, initial pH of culture medium as pH 6.16, and incubation time of 59 h. The predicted maximum production of collagenolytic enzyme from *B. subtilis* subsp. *Subtilis* S13 was 65.36 U/ml. For the validation of the experimental model, the cultivation of this bacteria strain under this optimized condition was performed in triplicate. The observed experiment value was 65 ± 4.20 U/ml (data not shown), which close relationship with the predicted value (65.36 U/ml).

Discussion

In this research, the PBD and CCD under RSM approaches were effective statistical method to find the optimized condition for the collagenolytic enzyme production from *B. subtilis* subsp. *Subtilis* S13. Among 11 independent factors, the results from PBD revealed that the incubation time, initial pH, and gelatine concentration were significant factors that impacted on enzyme production. Furthermore, the CCD experiment was carried out to find the best set of factor levels to achieve the optimum condition for collagenolytic enzyme production. It was found that the maximum enzyme production was predicted and validated (65.36 and 65 U/ml, respectivly), when this bacterial strain was cultured at

gelatin concentration (19 g/l) and prepared initial pH 6.16 of culture medium with incubation time for 59 h. The result suggested that hight gelatin concentration could increase the production of collagenolytic enzyme from *B. subtilis* subsp. *Subtilis* S13. These results are accord with Patel *et al*. (2005), who stated that a *Bacillus* sp. is capable of producing protease in a wide range of gelatin concentrations from 0 to 20 g/l. Moreover, Suphatharaprateep *et al*. (2011) also found that gelatin concentration of 15-10 g/l showed a higher collagenase production by *B. cereus* CNA1 than those of 5-10 g/l. Gelatin is one of the various types of collagen supplemented in culture media for the production of extracellular collagenase enzyme (Pal and Suresh, 2016). In the media with gelatin, *B. subtilis* subsp. *Subtilis* S13 could produce collagenase with a higher extent, possibly since their catabolite repression, allowing bacteria to synthesize more collagenolytic enzyme (Deutscher, 2008). Similar results by Lama *et al*. (2005) reported that the production of protease with gradually increased by *Salinivibrio* genus when increasing gelatin concentration from 10 to 20 g/l. They suggested that gelatin was an organic nitrogen source that preformed as an inducer for enzyme production.

Regarding intial pH of culture medium, the predicted value in present study for the optimum condition was pH 6.5. Liu *et al*. (2010) stated that initial pH was the most significant variables impacting protease production by *B. Sphaericus* DS11. Suphatharaprateep *et al*. (2011) found that *B. cereus* CNA1 had the optimum intial pH for collagenase production at pH 7.5. While Sela *et al*. (1998) stated that the optimum of collagenolytic enzyme activity for *B. cereus* was recorded in the pH range of 5.4–8.2. In some study, the production of collagenolytic enzyme by *B. alvei* DC-1 was decreasing at a pH lower than 6.0. (Kawahara *et al*., 1993). The culture pH strongly alters enzymatic processes and fluxes of various nutrients across the cell membrane of bacteria (Moon and Paruleka, 1991). Different microbial cell have an own ability to maintain the variation in the pH of the extracellular medium (Nielsen and Villadsen, 1994). Generally, neutrophilic *Bacillus* species (such as *B. subtilis* and *B. licheniformis*) had the intracellular aqueous in cytoplasm at pH 7.5, while alkalophilic *Bacillus* species (such as *B. firmus*) had this value at pH 8.2-8.5 (Çalık *et al*., 2001). This confirmed that collagenase in present study is obtained from neutrophilic *Bacillus* sp.

Nomally, a broad cultivation time in the range of 24 to 120 h was used to produce a protease enzyme, depending on *Bacillus* strains and target enzyme (Chu *et al*., 1992; Mabrouk *et al*., 1999; Beg and Gupta, 2003). For the report of Tran and Nagano (2002), the maimum collagenase activity produced by *B. Sublilis* CN2 was attained at 14 h of inculation time. Suphatharaprateep *et al*. (2011) showed that collagenase production by *B. cereus* CNA1 exhibited the

maximum enzyme yield at 48 h of incubation. The longer incubation time (54 h) for production of collagenolytic enzyme by *B. subtilis* subsp. *Subtilis* S13 than those studies might relate to higher gelatin concentration in culture medium. The increasing gelatin concentration caused a shift for longer incubation time, but this condition could also increase the gelatinolytic enzyme production by *B. amyloliquefaciens* H11 (Sai-Ut *et al*., 2014).

Acknowledgement

This study was supported by King Mongkut's Institute of Technology Ladkrabang under a grant from the faculty of Agricultural Technology (Scholarship for Graduate Student, Grant No. 2563-02-04-016). The authors would like to sincerely thanks to the Thailand Research Fund (TRF) through Research Grant for New Scholar (MRG6180240) to Supaluk Sorapukdee for the additional financial support.

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(Received: 18 July 2019, accepted: 30 October 2019)