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Effects of Operating Conditions in Spray Drying of Recombinant Bromelain

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ABSTRACT

Background: The aim of the present study is to investigate the effect of operating parameter of a Buchi B290 mini spray dryer towards specific activity of recBromelain. Using Response Surface Methodology (RSM), face centred central composite design (FCCCD) was tabulated for optimization. Optimization process was conducted by varying the inlet air temperature (°C), gas flow height (mm) and pump settings (%) of the laboratory Buchi B290 Mini Spray Dryer to obtained optimal spray drying operating processes recovering high recBromelain specific activity as response. Results showed that the optimized process parameter having 126 °C air inlet temperature, 42 mm gas flow height and 12 % feed pump setting resulted in 0.119 ± 0.003 U/mg-protein of spray dried recBromelain with R^2 of 90.12% thus this model was found to be significance and reliable.

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INTRODUCTION

Bromelain is one of the protease enzymes found in the pineapple plant (*Ananas comosus*) and given a lot of attention recently (Arshad *et al*., 2014; Bala *et al*., 2012; Maurer, 2001). Stem bromelain (EC 3.4.22.32) is the major protease present in extracts of pineapple stem while fruit bromelain (EC 3.4.22.3) is the major enzyme fraction present in the juice of the pineapple fruit (Kelly, 1996). Some other minor cysteine endopeptidases (ananain, comosain) are also present in the protease mixture extracted from pineapple stem. Although the fruit bromelain was discovered much earlier than stem bromelain, the biochemical characterization of the latter enzyme has been described in more detail (Harrach *et al*., 1998). Stem bromelain is widely used in industry and medicine, but fruit bromelain is not commercially available, even if it could be easily obtained from pineapple juice by simple ultrafiltration (Larocca, Rossano, Santamaria, & Riccio, 2010). Stem bromelain preparation contains a complex mixture of different thiol-endopeptidases and other partially characterized components such as phosphates, glucosidases, peroxidases, cellulases, glycoproteins and carbohydrates, among others (Maurer, 2001). The entire extracts of stem bromelain exhibit its activity over a wide pH range of 5.5 to 8.0 (Yoshioka, Izutsu, Aso, & Takeda, 1991). Bromelain are widely attributed from pineapple and accepted as therapeutic qualities as a traditions in South America, China and Southeast Asia (Chobotova, Vernallis, & Majid, 2010). The use of bromelain in medicine is gaining more recognition owing to the fact that several clinical studies indicates its applications in oncology, inflammatory conditions, skin debridement, immune regulation, etc. Consequently, there is growing demand for the proteases of both eukaryotic and prokaryotic microorganisms. Bromelain acts systemically, affecting directly several cellular and molecular targets and the relevant therapeutic applications. Recently, recombinant bromelain was produced by recombinant DNA method (Amid, Ismail, Yusof, & Salleh, 2011), characterized (Bala, Mel, Jami, Amid, & Salleh, 2013; Fouz, Amid, & Hashim, 2014), the fermentation condition and downstream processing were also optimized (Bala, Salleh, Amid, Mel, & Jami, 2011; Jamaluddin, Amid, Azmi, & Othman, 2014; Othman, Amid, Jimat, & Jamaluddin, 2014). One of the important downstream processing for recombinant enzyme is the powder preparation where the production cost will be affected mush at this stage, thus spray drying technique may be chosen to obtain lowest production cost. Spray drying efficiently removes initial moisture resulting in a large throughput and low to medium operating cost (Mujumdar, 2001), 30 to 50 times less than the freeze drying (Desobry, Netto, & Labuza, 1997). Spray drying offers the direct formation of droplets undergoing chemical reaction during drying which makes

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this technique widely used for drying heat sensitive foods, pharmaceuticals active ingredients and many under sensitive substance. In addition, spray dried product can be transported at low cost and stored in a stable form. However, dehydration process itself may cause protein inactivation. Removal of the aqueous medium surrounding the enzyme may also lead to breakage of the hydrogen bonds. Further, the interactions between the hydrophilic groups may change (Millqvist-Fureby, Malmsten, & Bergenståhl, 1999). These factors lead to enzyme denaturation and inactivation as mentioned above. Thus, in order to obtain the desired quality of recombinant bromelain powder, optimization of operational parameters for spray drying is needed. Therefore, in this study, recombinant bromelain powder will be produced by using the spray drying technique.

1. Methodology:

Bacterial Strain:

Recombinant bromelain clone (patent file no. PI 20095434) was obtained with the courtesy of Assoc. Prof. Dr. Azura Amid.

Cultivation of Recombinant Bromelain:

Five colonies of recombinant *E. coli* BL21-AI harbouring bromelain gene from fresh MDAG plate were inoculated into a 10ml non-inducing media for preparation of the starter culture. The starter culture was grown overnight for 12 hours at 37°C with 300 rpm in an incubator shaker (Stuart, Germany). Then the overnight starter culture was inoculated to 1L fresh medium ZYM-5052 auto-induction media (Studier, 2005)in a 2L working volume bioreactor (Infors, Germany) grown at the same fermentation condition of the starter culture. This ZYM-5052 auto-induction media contains complex nitrogen source, ZY with 1% (w/v) of tryptone and 0.5% (w/v) of yeast extract, 20 ml of 50 X buffering salt, 20 ml of 50 X 5052 carbon source, 2 mM MgSO 4.H_2O, 0.2 ml of 1000 X trace element, 1ml of 100 mg/ml ampicillin and 0.02% of L-arabinose. Cells were then harvested by centrifugation at 5000 rpm for 30 minutes at 4°C using XIR centrifuge (Thermo Fisher, USA). Subsequently, the cell pellet was stored in -20°C

Cell Disruption by Ultrasonication (Othman, et al., 2014):

The harvested cell pellet, 5g was re-suspended in extraction buffer (Ketnawa, Chaiwut, & Rawdkuen, 2011) consisting of 100mM sodium phosphate buffer, 15mM of L-Cysteine and 2mM of EDTA and was chilled on ice before the sonication process. Disruption of cells was performed using lab scale ultrasonic homogenizer (Sartorius, Germany) operated at 30 kHz frequency with variation of 20 – 100% amplitude equipped with a 10mm diameter titanium needle probe. The disruption period was varied from 1 to 5 minutes with 60s intervals for three times with bursting cycle (pulse operation) from 0.2s to 60s on ice. Samples were kept in ice during the sonication process to prevent overheating and denaturation. Lysed cells were centrifuged at 12,000×g for 30 minutes at 4°C to remove cell debris

Partial Purification by Ammonium Precipitation:

Partial purification by ammonium sulfate precipitation was carried out using ammonium salt powder based on (Doonan, 2004) with a slight modification. Fractional precipitation was carried out by adding ammonium salts into the concentrated samples until it completely dissolves and the temperature of the solution was maintained at 4ºC. Sample solution was stirred at slow rate to avoid bubble formation. After the addition is completed, sample solution was stirred until reached equilibrium. Precipitated proteins will be collected by centrifugation at 10,000×g for 30 minutes at 4ºC. Supernatant of the centrifuge sample was decanted for the next fraction. Precipitated protein was dissolved in minimal phosphate buffer saline (PBS) for the next process and kept chilled at 4°C. The amount of $(NH_4)_2SO_4$ required (g) will be calculated using the equation below:

$$\frac{519.1\ (S_2 - S_1)}{100 - 0.3S_2} \times volume\ of\ solution\ (liter)$$

where S_1 initial saturation of solution (%) and S_2 required solution (%). Since the precipitation was done in cold condition (4°C), ammonium sulphate has the molecular weight of 131.14 g/mol thus having solubility at 4°C is 519.1 g/L. the equation was related by the assuming that 100% saturation corresponds to 3.93M of ammonium salt solution.

Feed Mixture Preparation:

Crude bromelain which has been precipitated using ammonium salt was further suspended in phosphate buffer saline (PBS) with ratio 1:20 (w/v) and kept chilled on ice. Homogenous suspension was achieved by mixing for the precipitate crude recombinant bromelain to completely dissolve in the PBS solution

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by gently mixing in a beaker using magnetic stirring bar. Lastly, maltodextrin was added as excipient to prevent of rapid thermal effect towards recombinant bromelain during spray drying.

Spray Drying:

The bromelain solution were spray dried using a laboratory scale spray dryer model Buchi Mini Spray Dryer B-290 having evaporation capacity 1 L/hr. The bromelain solution feed was introduced into the spray drying system by peristaltic pump into 7mm inner standard diameter of two-fluid nozzle atomizer. The system also equipped with compressed air maintaining at 6 bar. Spray dried products are separated by cyclone and collected inside the collection vessel.

Design of Experiment:

To verify the influence of drying process consisting inlet air temperature (X1), gas flow height (X2) and pump settings (X3) towards the specific activity as the response, a face centered central composite(FCCD) design with three replicates at the centre point were employed to optimize these parameters. The variation of the parameters was derived according to the design formulated by the statistical software,Stat-Ease DesignExpert® v8.0 (Minneapolis, USA). A total number of 17 experimental runs were summarized in Table 1 for obtaining optimal conditions for recbromelain specific activity (Y).

Table 1: Summary for the parameters involved to obtain high recBromelain specific activity. Three operating parameters encoded with inlet air temperature (X1), gas flow height (mm) and pump settings (X3) with three levels (-1, 0, +1).

Code	Parameter	Unit	Range		
			-1	0	+1
X1	Inlet Air Temperature	°C	100	125	150
X2	Gas Flow Height	mm	40	45	50
X3	Pump Settings	%	10	12.5	15

Total Protein Quantification:

The total protein content of the homogenized samples will be determined using the Quick Start™ Bradford Assay Kit (Bio-Rad, USA) with 1X Bradford dye reagent and bovine serum albumin (BSA) as standard. BSA Standard curve was conduct by varying concentrations of the BSA ranging from 0.125mg/mL to 2mg/mL. 20µL of test sample and blank in triplicates was added into 1000µL of the protein assay reagent in 1.5mL micro centrifuge tube and mixed by vortexing. The mixed samples were incubated at room temperature (25˚C) for 5 minutes, transferred into half-micro cuvette (3mL) and absorbance was read at 595nm using spectrophotometer MultiskanGo (Thermo Fisher, USA). Sample reading was done in triplicates.

Bromelain Enzymatic Assay:

Recombinant bromelain activity in the crude extract was determined by using protease assay as outlined by Sigma-Aldrich protocol (St. Louis, USA). Here, the activity was measured by the rate of substrate reaction towards bromelain using spectrophotometric measurement at 340 nm. About 100µl of enzyme solution was mixed with buffer A (30mM sodium acetate buffer with 100mM potassium chloride and 1mM L-Cysteine). The mixture was inverted few times in a cuvette and equilibrated at room temperature before adding NáCBZ-L-Lysine ester (LNPE) as the substrate. Absorbance measurement of enzymatic reaction was measured spectrophotometrically for 5 minutes. One unit of bromelain activity releases 1.0 µmoles p-nitrophenol per minute at pH 4.6 at 25°C.

Statistical Analysis:

The recBromelain specific activity is the dependent variable in this study. The quadratic model for obtaining the optimal recBromelain activity was expressed according to the equation below:
Equation

$$Y = \beta_0 + \beta X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + X_1^2 + X_2^2 + X_3^2 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2$$

where Y is the recBromelain specific activity (U/mg-protein), while X1, X2 and X3 are coded level of independent variables corresponded to inlet air temperature (°C), gas flow height (mm) and pump settings (%). β_0 is the intercept term, β_1, β_2 and β_3 are linear coefficients, β_{11}, β_{22} and β_{33} are quadratic coefficients, β_{12}, β_{13} and β_{23} are the interactive coefficient. The statistical significance of the second-order model equation was determined by F-value and the proportion of variance explained by the model obtained was given by the multiple coefficient of determination, R^2.

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RESULTS AND DISCUSSIONS

Effect of Operating Parameters:

The tabulated optimization results (Table 2) of operating parameters for spray drying of recBromelain shows three factors involved (inlet air temperature, gas flow height and pump settings) with recBromelain specific activity (U/mg-protein) as response. FCCCD of three variable of parameters value with three center points (in bold and italic) shows the actual coded values along with the experimental and predicted responses. The effect of inlet temperature and gas can be observed in the red region of the 3D surface in figure 1.A. Highest point in the red region clearly shows the optimal range of response. It clearly shows that the inlet temperature increase and gas flow height decrease these reduced the specific activity of the spray dried recombinant bromelain. At the inlet temperature region of 110°C to 130°C, with gas flow height from 40mm to 50mm, the specific activity is at optimal value. Devakate and co-workers reported that high activity of bromelain was achieved when sample was spray dried at low temperature (Devakate, Patil, Waje, & Thorat, 2009). Denaturation of protein might occur at high inlet temperature thus reduces the activity (Devakate, *et al*., 2009). However, enzyme inactivation for bromelain reported by Devakate and co-workers was at 65-70°C, interestingly with the of addition of 10% (w/v) maltodextrin, the recombinant bromelain activity for this study managed to sustain high temperature more than 120°C with 50% activity recovery(Devakate, *et al*., 2009).

The effect of inlet temperature and feed flow settings can be observed in Figure 1.B. At temperature between 120°C to 130°C, with feed pump settings from 10% to 20%, optimal specific activity is achieved, shown in red color. The bulk density of the dried powder is varied with variation of feed pump settings (Chegini & Ghobadian, 2007). Increased the particle size and moisture content of the dried powder were relative to the increase of the feed pump setting (Phisut, 2012). Increased in feed pump setting would increase the moisture content of the final dried powders and reduced the hygroscopic effect (Phisut, 2012)

The effect of gas flow height and feed flow setting shows a strong interactions, as the red region covers majority the surface structure in Figure 1.C. Optimal region predicted by the software with low gas flow height and low feed pump setting, also at low gas flow height and high feed pump setting produces optimal specific activity of spray dried recombinant bromelain. The movement of air is determined by the amount of air being permitted in the system. High volume of air introduced more moisture inside the system. Residence time of drying was decreased with the increased of gas flow (Phisut, 2012) which increased the moisture content in the system. Larger bulk volume of dried powder can be achieved with high moisture content with high gas flow (Phisut, 2012). Devakate and co-workers spray dried bromelain by varying gas flow, keeping pump setting constant and maintaining the outlet temperature low than 50°C to achieve high moisture content in the dried bromelain powder to recover the high bromelain activity (Devakate, *et al*., 2009). The outlet temperature is also determined by varying the pump setting. The increase of feed flow rate relatively increased the amount of water in the sample (Maltesen, Bjerregaard, Hovgaard, Havelund, & van de Weert, 2008). At lower pump setting, low amount of feed flow rate was introduced thus reduced the water content and decreased the chance of condensation dispersion in the drying system resulting in better yields. In this study, the maximum specific activity (U/mg-protein) of spray dried recombinant bromelain was obtained inlet temperature is 125 °C, gas flow height 45 mm and feed flow rate is 12.5 % (Run 8, Table 2) with 0.1120 ± 0.001 U/mg-protein.

Table 2: Recombinant bromelain specific activity affected by the spray drying processes.

Run	A		B		C		Specific Activity (U/mg-protein)	
	Inlet Temperature		Gas Flow Height		Feed Flow Rate			
	°C	Coded	mm	Coded	%	Coded	Exp	Std. Dev
1	150	+1	40	-1	10	-1	0.020	0.002
2	125	0	45	0	15	+1	0.033	0.002
3	100	-1	50	+1	15	+1	0.060	0.001
4	150	+1	50	+1	10	-1	0.029	0.001
5	100	-1	50	+1	10	-1	0.008	0.001
6	150	+1	50	+1	15	+1	0.059	0.002
7	100	-1	45	0	12.5	0	0.066	0.002
8	***125***	***0***	***45***	***0***	***12.5***	***0***	***0.112***	***0.006***
9	100	-1	40	-1	10	-1	0.065	0.001
10	***125***	***0***	***45***	***0***	***12.5***	***0***	***0.111***	***0.002***
11	125	0	50	+1	12.5	0	0.083	0.001
12	100	-1	40	-1	15	+1	0.074	0.002
13	***125***	***0***	***45***	***0***	***12.5***	***0***	***0.109***	***0.003***
14	125	0	40	-1	12.5	0	0.070	0.002
15	150	+1	40	-1	15	+1	0.073	0.002
16	125	0	45	0	10	-1	0.095	0.003
17	150	+1	45	0	12.5	0	0.068	0.007

Table 3: Analysis of variance (ANOVA) for the selected quadratic model for specific activity of spray dried recombinant bromelain.

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	6.71	7	0.96	11.73	0.0007	*significant*
A-Inlet Air Temp	0.3	1	0.3	3.62	0.0894	
B-Gas Flow Height	0.056	1	0.056	0.69	0.4285	
C-Feed Flow Settings	5.51E-07	1	5.51E-07	6.74E-06	0.998	
AB	1.85	1	1.85	22.61	0.001*	
AC	0.74	1	0.74	9.02	0.0149	
BC	1.07	1	1.07	13.05	0.0056	
A^2	2.4	1	2.4	29.37	0.0004*	
Residual	0.74	9	0.082			
Lack of Fit	0.58	7	0.083	1.08	0.5594	*not significant*

Note: The (*) symbol indicates the significant terms

3D Surface Plots:

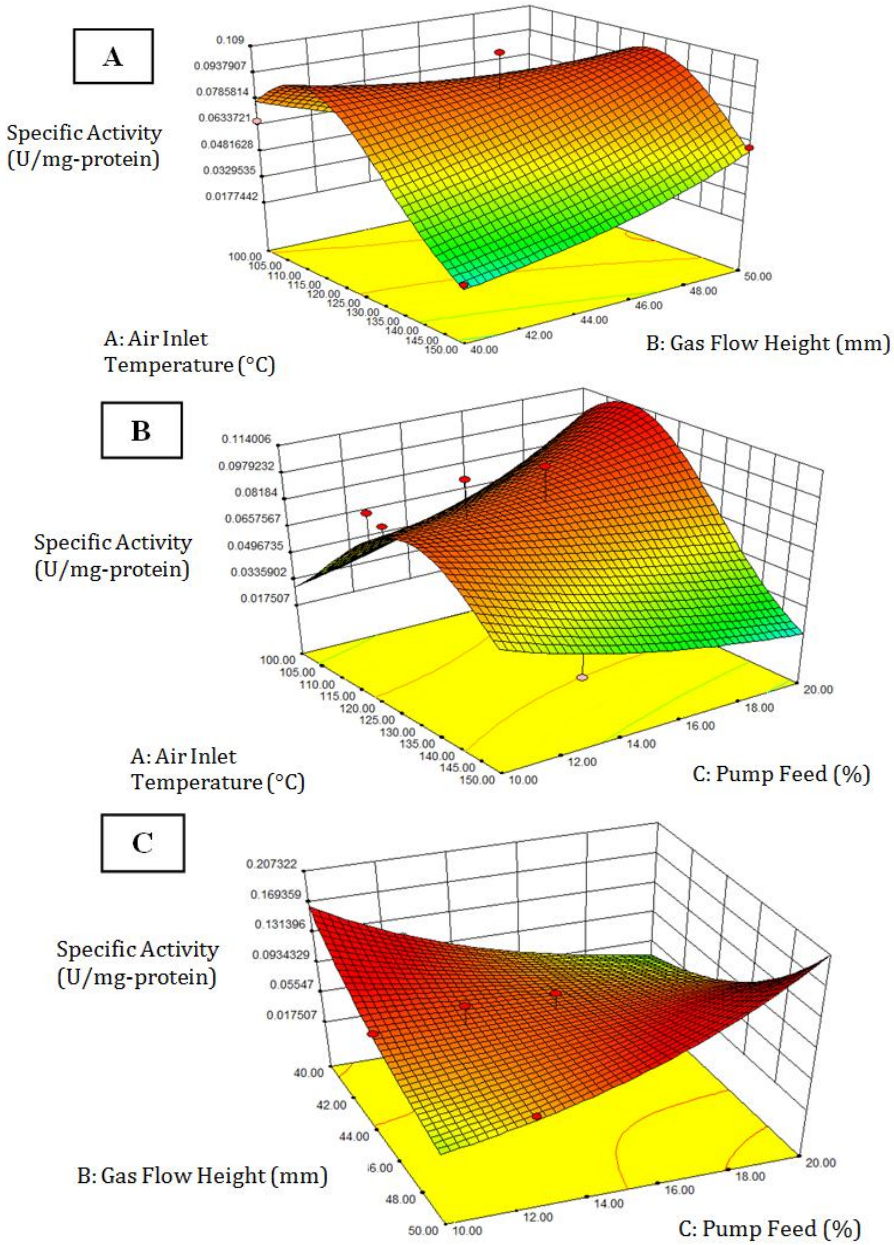

Fig. 1: The 3D surface plot . A, the effect of inlet air temperature, A (°C) and gas flow height on recombinant bromelain specific activity; B, the effect of inlet air temperature, A (°C) and feed pump settings, C (%) and; C, effect of the gas flow height, B (mm) and feed flow settings, C (%) towards specific activity (U/mg-protein) of spray dried recombinant bromelain.

Properties of the Particles:

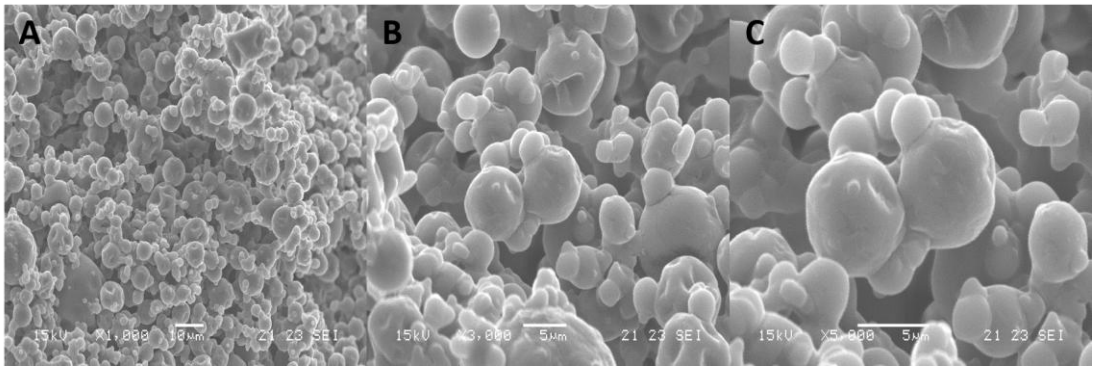

Fig. 2: SEM image shows the particles of spray dried recbromelain. There stages of magnification at (A)1000X, (B) 3000X and (C) 5000X.

Analysis of Variance (ANOVA):

Table 3 shows the significance of the data is evaluated by its p-value (0.0007) that is closer to zero. For any effect to be statistically significant at 95% confidence level, the p-value should be less than or equal to 0.05. Table 3 summarizes the analysis of variance (ANOVA) for specific activity of the spray dried recombinant bromelain. The model F-value 11.73 implies the model is significant and there is a 0.07% chance that this model could occur due to noise. Values of "Prob-F" is less than 0.05 indicated that the model terms are significant. The model also indicated that the inlet air temperature (A), gas flow height (B) and feed flow rate (C) and A2 were highly significant model terms since all values of "Prob-F" are lower than 0.1000. Therefore, all terms has significances and major effect towards the specific activity of spray dried recombinant bromelain. The "Lack of Fit F-value" of 1.08 implies the Lack of Fit is not significant relative to the pure error. There is a 55.94% chances that a "Lack of Fit F-value this large could occur due to noise. The software mentioned that a non-significant lack of fit is good where the model should be fit.

Validation of the model:

In order to validate the statistical experimental strategies and expand the understanding of spray dried recombinant bromelain and the spray drying condition, with 126 °C inlet air temperature, 42 mm gas flow height and 12% pump setting was the chosen parameter for the validation process. The dried recombinant bromelain was subjected to scanning electron micrograph (SEM) for particle morphology observation. Figure 2 shows the images captured at different magnification. It can be seen that with the optimized conditions and excipient added the particle is in spherical form compared to the one that was not optimized. Rapid dehydration process and thermal changes caused low specific activity of recombinant bromelain when it was dried without excipients and not in the optimized drying conditions. This is due to the liquid evaporation from the enzyme mixture, shrinkage of the droplets due to the water escaping during the transition of heat in the spray drying (Maa *et al*., 1998; Saluja *et al*., 2010) Moisture content is relatively low, due to the rapid water removal during the drying process thus reduced the surface area (Phisut, 2012; Saluja, *et al*., 2010)

4. Conclusion:

In conclusion, optimization of spray drying recombinant bromelain involves three parameters which are inlet temperature (T_i), gas flow height (mm) and feed pump setting (%) were investigated using RSM FCCD. On statistical analysis, the specified optimum conditions was established having inlet temperature of 126°C, 42mm gas flow height and 12% feed pump settings resulted in 0.119 ± 0.0025 U/mg-protein of spray dried recombinant bromelain with the value of 90.12% coefficient of determination, R^2.

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