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# Optimization of Spray Drying Process Conditions for Recombinant Stem Bromelain

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ABSTRACT

Bromelain is a plant protease with numerous therapeutic, industrial and analytical applications. Spray drying of enzyme often leads to loss of enzymatic activity arising from thermal denaturation. Hence, the design of a suitable drying process should provide a great level of active enzyme. The present study examined the effect of operating parameters of a laboratory spray dryer on powder characteristics, so as to optimize the production of recombinant bromelain expressed in E. coli BL 21-AI. The recombinant enzyme was spray dried from maltodextrin (10 %w/v), CaCl2 (0.2 % w/v) and sodium metabisulphite (2.5 % w/v) solutions using a laboratory-scale Büchi Mini Spray dryer B-290. The process parameters investigated were: drying air inlet temperature (100-120 ºC), drying air volumetric flow rate – given as % of the maximum aspiration rate (80-100 %), feed volumetric flow rate– expressed as % of the maximum pump rate (10-15 %). On the other hand, the activity of bromelain (U/ml) served as the response of the design. Outlet temperature was maintained at 50 oC. All the studied process parameters had significantly affected the characteristics of the powdered bromelain, at a 95% confidence interval. The higher values of coefficient of determination (R2 = 99.95% and adjusted R2 = 99.84%) attained, showed that there is good compliance between the experimental and the theoretical values predicted by the model. Moreover, the graphical representations of the regression equation generated suggested that the examined independent variables interacted significantly. Thus, the corroboration of reality of the optimal conditions and the validity of the model had been ascertained. Consequently, under optimized process conditions, the study had produced powdered bromelain with greater quality in terms of moisture contents, residual activity and product recovery.

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## INTRODUCTION

Bromelain refers to proteolytic enzyme derived from pineapple plant (*Ananas comosus*). Stem bromelain (EC 3.4.22.32) is the major protease present in extracts of pineapple stem while fruit bromelain (EC 3.4.22.33) is the main protease fraction present in pineapple fruit [1]. Stem bromelain is widely used in food industry; for baking processes, meat tenderization, protein hydrolysate production, as food supplement and in prevention of browning of apple juice [2].

The spray drying technique has been considered as one of the simplest, fastest and economical means of converting solutions into powdered forms. Solid compounds in form of powders with dry matter content greater than 90% are more easily handled and preserved as compared to liquid preparations [3]. The prospective of spray drying technique to rapidly produce dry powder as well as the capability to control particle size distributions have been established [4].

The application of spray drying technique in many enzyme preparations is well documented [3]. In contrast to freeze-drying, spray drying is generally regarded as a promising technique for large-scale processing. This is because the technique is quite cheap and simple [5]. Nevertheless, loss of enzymatic activity is encountered as a

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result of thermal denaturation of the enzyme during spray drying. The presence of additives like polysaccharides and salt in the enzyme preparations reduces enzyme inactivation by spray drying [6]. Consequently, the design of a proper drying process should guarantee a high level of active enzyme. In general, the residual activity of spray dried enzyme depends on the process parameters, physical and chemical properties of the enzyme as well as composition of the original enzyme solution [7]. Thus, drying of each enzyme product should be considered on an individual basis. Moreover, modification of the process parameters may provide a way of altering and controlling the spray dried enzymes properties.

The main limitation for the industrial, therapeutic and analytical applications of enzymes is their liability to various forms of inactivation [8]. Many enzymes are subjected to spray drying process because the enzyme storage stability is better in that form rather than in a liquid formulation. However, enzymes may lose their structure and thus, their catalytic activity when exposed to the high temperatures inside a spray dryer. Hence, both the final product properties and the enzyme activity loss greatly depend on the selection of process conditions [9]. This stimulated research towards the development of strategies for the enhancement of enzymes stability. As a result of this, several studies have been carried out on the stability of bromelain. For instance, freeze drying and spray drying of bromelain had been reported [10]. Similarly, investigations were carried out on the effects of outlet temperature and spray pressure of spray-drying on the activity yield and water content of the bromelain juice [11]. Correspondingly, [12] investigated the effects of drying parameters on the retention of the enzymatic activity and on the physical properties of spray-dried pineapple stem extract. The authors concluded that high processing temperatures yielded a bromelain product with a smaller moisture content, particle size, and lower agglomerating tendency.

In view of the above, the purpose of this study is to maximize the activity of spray dried recombinant stem bromelain by optimization of some spray drying process operational conditions.

## MATERIALS AND METHODS

### *Chemicals:*

All the chemicals used were pure and of analytical grade.

### *Expression of Recombinant Bromelain:*

Expression of recombinant bromelain was conducted as described in our earlier studies [13,14]. Transformants were grown in shake flasks overnight in LB media containing 100 μg/ml ampicillin. The overnight culture was diluted 50 folds in a fresh LB media and then grown in a 2-liter bioreactor at 27 °C, air flow rate of 1.0 vvm with 350 rpm agitation until a cell density of $OD_{600nm} = 0.6$ was attained. Subsequently, L-arabinose (0.15 % w/v, final concentration) was added and induction was allowed to continue for 8 hours. Cells were harvested from the spent media by centrifugation (6000×*g*) at 4 °C for 20 min.

### *Purification of Enzyme:*

The harvested cells were subjected to sonication on ice using six-10 second burst with 10 seconds interval at high amplitude. This was followed by centrifugation at 4 °C, 12000 rpm for 30 minutes and the supernatant was collected and purified by nickel-NTA affinity chromatography as described elsewhere [14,15](Muntari et al., 2012; 2013).

### *Measurement of Bromelain Activity:*

The bromelain activity was measured on N-α-cbz-L-Gln-*p*-nitrophenyl ester using the Silverstein's method [16] with modification. The assays were conducted at 45 °C in 0.1 M Tris–HCl buffer (pH 8.0), containing 25 mM cysteine and 1 mM of the substrate in the reaction mixture. UV-visible spectrophotometer was used to measure the *p*-nitrophenol released at 405 nm. One unit was defined as the amount of protease that released one micromole of *p*-nitrophenolate per min in the assay conditions. The enzyme residual activity was calculated as percentage of differences of activity before and after the drying process.

### *Bromelain Sample Preparations:*

Prior to the spray drying process, some additives like maltodextrin (10 %w/v), $CaCl_2$ (0.2 % w/v) and sodium metabisulphite (2.5 % w/v) were supplemented to the purified bromelain preparations.

### *Spray Drying Process:*

The recombinant bromelain solutions were dried using a laboratory Büchi Mini Spray-Dryer B-290 (Büchi Labortechnik AG, Flawil, Switzerland) with a 0.7-mm two-fluid nozzle. The solution was sprayed in a co-current flow by means of air as drying medium. The humidity of the inlet drying air was regulated and maintained below 20%. The spray-dried particles were separated from the drying air by a high-performance cyclone [17](Maury et al., 2005). The process parameters studied were: inlet temperature (100-120 °C), aspirator setting (80-100 %) and pump setting (10-15 %) as presented in Table 1. Outlet temperature was maintained at 50 °C. The spray dried powders of bromelain were collected, weighed and stored in capped glass vials for further analysis.

**Table 1:** Operating variables for enhanced recombinant bromelain spray drying.

| Operating variable[a] | Range and level | | Unit |
|---|---|---|---|
| | −1(Low) | +1 (High) | |
| A ( inlet temperature) | 100 | 120 | °C |
| B (aspirator setting) | 80 | 100 | % |
| C (pump setting) | 10 | 15 | % |

*Design of Experiment by Full Factorial Design (FFD):*

In order to evaluate the effect of spray drying process parameters that enhance the quality of powdered bromelain and its activity, a randomized $2^3$ full factorial design (two-level and three-factor with three center points) was devised. The independent variables selected were: (A), drying air inlet temperature, (B), drying air volumetric flow rate – given as % of the maximum aspiration rate and (C), feed volumetric flow rate– expressed as % of the maximum pump rate. On the other hand, the activity of bromelain (U/ml) served as the response of the design. Table 1 summarizes the levels of operating variables. For choosing the three factors and their corresponding ranges, practical considerations and literature reports were applied.

Statistical software (MINITAB release 14) was employed for the designing and analysis of the experimental data so as to measure the effects of those operating variables on bromelain activity. The design generated a sum of 11 experiments comprising of 3 center points (Runs 4, 10 &11). The detailed experimental design containing coded and actual values of the three factors is presented in Table 2.

The full factorial experimental design employed is derived from the first order model:

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_{12} AB + \beta_{13} AC + \beta_{23} BC \quad (1)$$

Where Y is the dependent variable (bromelain activity); $\beta_0$ is an intercept term; $\beta_1$, $\beta_2$ and $\beta_3$ are linear coefficients; $\beta_{12}$, $\beta_{13}$ and $\beta_{23}$ are the interaction coefficients; and A, B and C are the independent variables standing for inlet temperature, aspirator setting and pump setting, respectively.

In order to investigate and assess the model and the best operating conditions for the bromelain spray drying, the combination of the coefficient of determination ($R^2$), coefficients of all effects, ANOVA, two-dimensional (2D) as well as three-dimensional (3D) plots were all used.

**Table 2:** Full factorial design of three independent variables with three center points showing the actual and coded values

| Run order | Inlet temperature (°C) Actual & coded values | Aspirator setting (%) Actual & coded Values | Pump setting (%) Actual & coded values |
|---|---|---|---|
| 1 | 120 (+) | 100 (+) | 10.00 (−) |
| 2 | 100 (−) | 80 (−) | 10.00 (−) |
| 3 | 120 (+) | 80 (−) | 10.00 (−) |
| 4 | 110 (0) | 90 (0) | 12.50 (0) |
| 5 | 120 (+) | 100 (+) | 15.00 (+) |
| 6 | 120 (+) | 80 (−) | 15.00 (+) |
| 7 | 100 (−) | 80 (−) | 15.00 (+) |
| 8 | 100 (−) | 100 (+) | 10.00 (−) |
| 9 | 100 (−) | 100 (+) | 15.00 (+) |
| 10 | 110 (0) | 90 (0) | 12.50 (0) |
| 11 | 110 (0) | 90 (0) | 12.50 (0) |

Some sets of experiments within the design space were used for the establishment of the precision of the predicted model with respect to all the three operating variables as presented in Table 3.

**Table 3:** Validation of the experimental model.

| Experiment | Inlet temperature (°C) | Aspirator setting (%) | Pump setting (%) |
|---|---|---|---|
| 1 | 110 | 100 | 15.00 |
| 2 | 110 | 90 | 12.50 |
| 3 | 100 | 90 | 10.00 |
| 4 | 120 | 80 | 12.50 |

The experimental run which exhibited highest bromelain activity was subjected to analysis for determination of moisture content, residual protein content and product recovery yield.

*Results:*

In order to produce formulated recombinant bromelain of greater activity, this research work was geared towards investigating the effects of some operating parameters on bromelain spray drying. This technique was applied to quantify the influence of aspirator setting, air inlet temperature and pump setting (feed flow rate) on the activity of the enzyme. The selected variables were used to identify the optimum conditions which enhance the spray drying of recombinant bromelain via FFD. The entire results obtained for bromelain activity from the 11 experiments (comprising of the predicted and experimental values) are shown in Table 4.

**Table 4:** Full factorial design of three independent variables with three centre points showing the actual and coded values along with the experimental and predicted responses

| Run order | Inlet temperature ($^{o}$C) | Aspirator setting (%) | Pump setting (%) | Bromelain activity (U/ml) | |
|---|---|---|---|---|---|
| | | | | Experimental | Predicted |
| 1 | 120 (+) | 100 (+) | 10.00 (−) | 62.78±0.04 | 62.64 |
| 2 | 100 (−) | 80 (−) | 10.00 (−) | 55.87±0.06 | 55.73 |
| 3 | 120 (+) | 80 (−) | 10.00 (−) | 54.87±0.04 | 55.00 |
| 4 | 110 (0) | 90 (0) | 12.50 (0) | 76.82±0.08 | 76.73 |
| 5 | 120 (+) | 100 (+) | 15.00 (+) | 73.89±0.06 | 74.02 |
| 6 | 120 (+) | 80 (−) | 15.00 (+) | 62.26±0.07 | 62.12 |
| 7 | 100 (−) | 80 (−) | 15.00 (+) | 60.98±0.05 | 61.12 |
| 8 | 100 (−) | 100 (+) | 10.00 (−) | 55.32±0.04 | 55.46 |
| 9 | 100 (−) | 100 (+) | 15.00 (+) | 65.24±0.08 | 65.10 |
| 10 | 110 (0) | 90 (0) | 12.50 (0) | 77.02±0.09 | 76.73 |
| 11 | 110 (0) | 90 (0) | 12.50 (0) | 76.34±0.08 | 76.73 |

The recombinant bromelain activity (U/ml) varied broadly from 54.83 U/ml to 77.02 U/ml. It can be observed from the results (Table 4) that center points (runs 4, 10 and 11) yielded the highest activity. Equally, run 3 gave the lowest enzyme activity. In this situation, the inlet temperature was at its peak (120 $^{o}$C) while the remaining two factors were at their minimum heights (10 % & 80 %).

The experimental results obtained were then evaluated by the model comprising of the effects of linear and interaction that generated the following equation with bromelain activity (U/ml) as a function of inlet temperature (A), aspirator setting (B) and pump setting (C).

$$\text{Bromelain activity (U/ml)} = 259.486 - 1.793A - 2.419B - 4.071C + 0.0198AB + 0.0174AC + 0.043BC \quad (2)$$

According to the t-test, the main variable effects plus their interactions were assessed as shown in Table 5. It can be deduced that within the examined experimental range, all the operation variables have exerted significant effects on spray drying of bromelain. The statistical model was confirmed by F-test and the analysis of variance (ANOVA) presented in Table 6 verified the model F-value of 615.31.

**Table 5:** Estimated effects and coefficients for bromelain activity (U/ml).

| Term | Net effect | Coefficient | Standardized effect (T) | *p*-value |
|---|---|---|---|---|
| Constant | | 61.40 | 479.96 | 0.000 |
| Inlet temperature (A) | 4.10 | 2.05 | 16.01 | 0.001 |
| Aspirator setting (B) | 5.81 | 2.91 | 22.72 | 0.000 |
| Pump setting (C) | 8.38 | 4.19 | 32.76 | 0.000 |
| AB | 3.96 | 1.98 | 15.47 | 0.001 |
| AC | 0.87 | 0.43 | 3.39 | 0.043 |
| BC | 2.13 | 1.07 | 8.33 | 0.004 |

*Note*. Standard error coefficient for all cases= 0.128, $p< 0.05$ were considered to be significant.

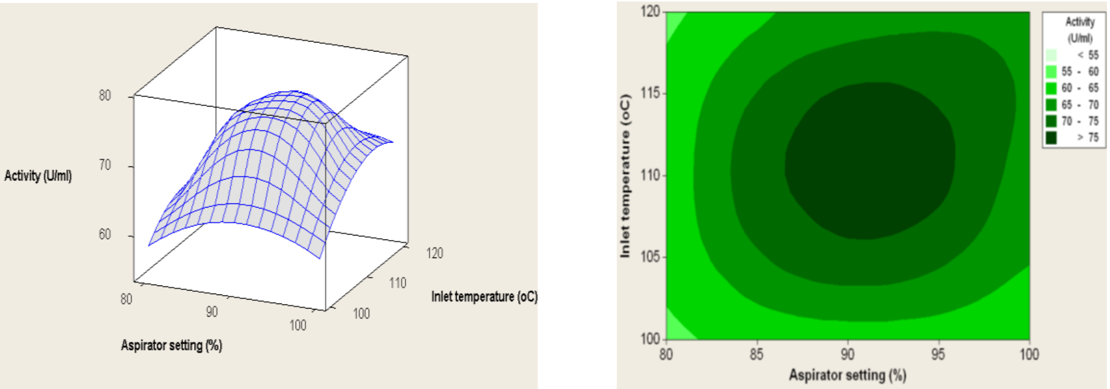
**Table 6:** Analysis of variance for bromelain activity (U/ml)

| Source | Degree of freedom | Sum of squares | Mean squares | F-value | *p*-value |
|---|---|---|---|---|---|
| Main effects | 3 | 241.68 | 80.56 | 615.31 | 0.000 |
| 2-way interaction | 3 | 41.92 | 13.98 | 106.74 | 0.002 |
| Curvature | 1 | 512.44 | 512.44 | 3913.96 | 0.000 |
| Residual error | 3 | 0.39 | 0.131 | | |
| Total | 10 | 796.43 | | | |

*Note*. $R^2$=99.95, $R^2$ (adj) = 99.84% and Lack of fit (F-value=1.22 and *p*-value=0.385).

Additionally, to validate the model, the goodness of fit was evaluated by the coefficients of determination ($R^2$). The higher coefficient of determination ($R^2$ = 99.95% and adjusted $R^2$ = 99.84%) explained that there is good conformity between the experimental and the theoretical values predicted by the model and that approximately all the variations could be accounted for by the model equation.

The graphical illustrations of the regression equation (2D contour plots and 3D response surface) were generated using MINITAB release 14. The results revealed that the interactions among the tested independent variables were significant. In all cases, the maximum bromelain activity was positioned at the center of the experimental regions. The activity of spray dried bromelain varied significantly with the changing of aspirator setting and inlet temperature as presented in Fig. 1. Furthermore, the observed interaction between inlet temperature and pump setting was found to be significant ($p < 0.05$) as shown in Fig. 2.



(a) (b)

**Fig. 1:** Response surface and contour plots showing the effect of aspirator setting (%) and inlet temperature ($^{o}$C) on the activity (U/ml) of spray dried bromelain (a) 3D response surface (b) 2D contour plot.

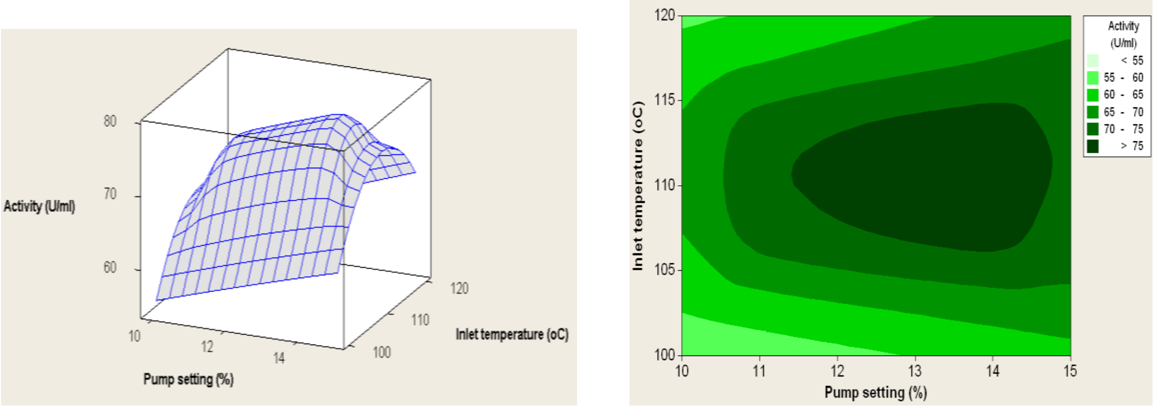


**Fig. 2:** Response surface and contour plots showing the effect inlet temperature ($^{o}$C) and pump setting (%) on the activity (U/ml) of spray dried bromelain (a) 3D response surface (b) 2D contour plot.

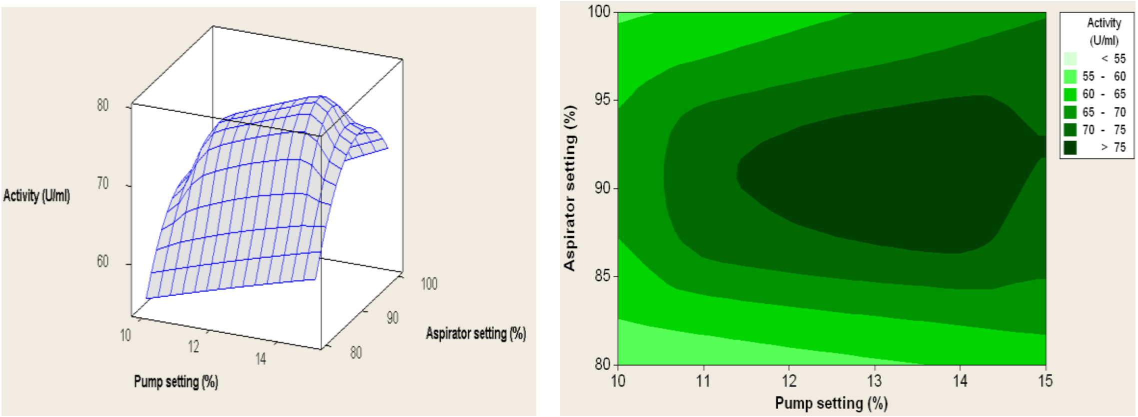


**Fig. 3:** Response surface and contour plots showing the effect of aspirator setting (%) and pump setting (%) on the activity (U/ml) of spray dried bromelain (a) 3D response surface (b) 2D contour plot.

The circular or elliptical shapes of the response surface explain whether interactions between variables are significant or not. The elliptical nature of the contour plots obtained between aspirator setting and pump setting implies that mutual interaction between these set of variables has a profound significant effect on bromelain activity (Fig. 3). In order to verify the validity of the statistical experimental strategies and expand an improved understanding of recombinant bromelain spray drying process, a set of four experiments were carried out (in duplicate) within the design space as presented in Table 7.

The results obtained for the predicted and experimental values (Table 7) revealed the existence of good conformity between them. Hence, there is an establishment of the authentication of the validity of the model and the reality of the optimal conditions. The specified optimum conditions obtained (aspirator setting, 90 (%); inlet temperature, 110 ºC; and pump setting, 12.5 (%) exhibited the maximum activity of 78.08±0.07 U/ml for the spray dried recombinant bromelain.

**Table 7:** Validation of the model

| 1 | Inlet temperature ($^{o}$C) | Aspirator setting (%) | Pump setting (%) | Bromelain activity (U/ml) | |
|---|---|---|---|---|---|
| | | | | Experimental | Predicted |
| 1 | 110 | 100 | 15.00 | 68.96±0.09 | 69.56 |
| 2 | 110 | 90 | 12.50 | 78.08±0.07 | 76.73 |
| 3 | 100 | 90 | 10.00 | 56.38±0.09 | 55.59 |
| 4 | 120 | 80 | 12.50 | 57.78±0.06 | 58.56 |

After the production of spray dried bromelain under the validated optimized conditions, the enzyme powder was subjected to various analyses. Some of the parameters and their values estimated on the enzyme are shown in Table 8. It can be observed that a greater bromelain residual activity of about 92.64±0.09 had been achieved. As stated earlier, one of the most important properties of enzyme which is affected during enzyme spray drying is residual activity. This clearly indicated that the investigated parameters had played significant role in producing powdered bromelain of higher residual activity.

**Table 8:** Effects of some spray drying parameters on recombinant bromelain

| Parameter | Value |
|---|---|
| Moisture content (%) | 9.83±0.04 |
| Residual protein (%) | 88.46±1.2 |
| Residual activity (%) | 92.64±0.09 |
| Yield (%) | 89.28± 0.08 |

## *Discussion:*

Based on the results obtained (Table 4), bromelain spray drying was found to be robustly dependent on all the three operating variables (aspirator setting, air inlet temperature and pump setting). This is a normal trend in spray drying process. The *p*-value is the probability that is applied to verify the impact of each of the coefficients, which in turn may specify the pattern of interactions between the variables. Th*e p*-value that is closer to zero is used to evaluate the significance of the data. Additionally, when the *p*-value is less than or equal to 0.05, then the observed effect is statistically significant at 95% confidence level. Accordingly, from the results obtained in Table 5, both interaction coefficients and linear interactions were established to be statistically significant.

Moreover, the results of ANOVA (Table 6) indicated that the model is very significant with a lower probability value ($p$ model F < 0.001). This implies that there is only about 0.1% chance that the model F-value could occur due to noise. The values obtained with $p > F$ less than 0.05 point out that model terms are significant. In addition, the lack of fit was not significance relative to the pure error owing to its F-value of 1.22 . Similarly, It can be observed from both 2D and 3D plots (Fig. 1) that the raise in inlet temperature and aspirator setting leads to an increase in the bromelain activity to optimal values (77.02 U/ml), while further increase causes the decrease in overall bromelain activity (54.83 U/ml). This is supported by the findings of [10] that an increase in higher inlet temperature leads to decrease in the residual bromelain activity. At higher inlet air temperature, the enzyme is exposed to higher outlet air temperature which leads to the denaturation of the proteins. Both high inlet air temperature and aspirator rates increase the energy available for the drying process and hence increase the outlet air temperature.

In the same vein, from Fig. 2, the smallest ellipse in the contour plot depicted the region of maximum response and in this case, interactions between all the independent variables exhibited elliptical contours. Elliptical contours suggest perfect interactions between the independent variables [18]. Additionally, Pump setting (feed flow rate) also affects the outlet temperature. For instance, [19] evaluated the spray-drying of a budesonide/formoterol solution and reported that outlet temperature was mainly affected by pump setting, aspiration capacity, atomization air flow rate and inlet air temperature. Pump setting increases the amount of solvent available for evaporation and thus decreases the outlet air temperature [20]. For lower pump rates, water loading was almost totally evaporated; decreasing the chance of dispersion condensation on the chamber walls and thus giving better process yields. The moderate pump setting used in this study, contributed in lowering the outlet temperature and hence, higher residual bromelain activity was recorded.

The observed improvement on the process yield (Table 8) is as a result of the moderate aspirations that enhanced separation rate in the cyclone, greater thermal levels in the spray chamber, and elevated drag forces [21]. In a study conducted by [22] on spray drying of insulin, it was observed that aspiration was the main effect with the highest impact on the yield of a spray-dried insulin solution. The authors further reported that low pump setting and low inlet temperature stabilized the protein. Besides, [23] also reported that pump setting and inlet air temperature exerted significant effects on the process yield of spray dried acai fruit (*Euterpe oleraceae)* extract. Both of these studies to some extent lend support to our findings on the observed significant effects of the two parameters on the activity of spray dried bromelain.

The observed effect is largely due to the maintenance of lower oulet temperature (40-50 ºC) during the spray drying process by the intractions of the studied parameters. This is supported by the findings of [10] in which the spray dried bromelain was totally inactivated at outlet temperature of 65 ºC. The bromelain produced by these authors had residual activity and residual protein content of 78 % and 73%, respectively. These reported values are quite lower than what we obtained in our study (Table 4.13).

Moreover, higher product recovery of powdered recombinant bromelain (89.28± 0.08 %) had been accomplished in this study (Table 8). This value is also higher than that obtained by [10] (73 %). This signifies that the study has produced viable and stable spray dried recombinant bromelain. In addition, the moisture content of the product was found to be about 9.83±0.04 %. This value falls within the desirable limit during spray drying. [24] found out that the residual enzymatic activity decreases when the moisture content in the spray-dried enzyme concentrate is reduced. Hence, it is required that the product leaving the spray dryer to have moisture content of not less than 8 to 10%. This results in generating low outlet temperatures from the drying system. It also reduces the hygroscopic effects of the product. Equally, [11] had spray dried bromelain under optimized conditions and obtained product with activity yield and water content of 47.1 % and 5.83 %, respectively. These values are also lower than our reported ones. In the same vein, [12] produced spray dried bromelain with about 90 % residual activity and moisture content of less than 6.5%.

### *Conclusion:*

The effects of some operating variables (aspirator setting, air inlet temperature and pump setting) on bromelain spray drying had been investigated. The results obtained indicated that the interaction and linear coefficients were found to be statistically significant. The higher value of coefficient of determination ($R^2$ = 99.95% and adjusted $R^2$ = 99.84%) obtained showed that there is good compliance between the experimental and the theoretical values predicted by the model. Moreover, the graphical representations of the regression equation generated suggested that the examined independent variables interacted significantly. Thus, the corroboration of reality of the optimal conditions and the validity of the model had been ascertained. The specified optimum conditions obtained (aspirator setting, 90 (%); inlet temperature, 110ºC; and pump setting, 12.5 %) generated the maximum activity of 78.08±0.07 U/ml for the spray dried recombinant bromelain. The study had produced powdered bromelain with greater quality in terms of moisture contents, residual activity and product recovery.

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