RESEARCH ARTICLE

Experimental Design of the Interaction Effect of Independent Variables on Pineapple (*Ananas comosus*) Fruit Bromelain Activity

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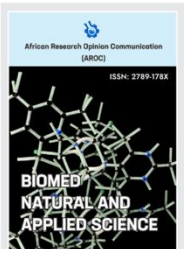

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ABSTRACT

Background: Certain factors are known to influence the rate of enzyme activity. It is paramount to study such independent factors to obtain the ideal conditions required to maximize enzyme activity. The time-consuming traditional one-factor-at-a-time (OFAT) approach of evaluating the effect of independent variables on enzyme activity does not consider the interaction effects of the independent variables on dependent responses. **Methods:** The impact of four independent variables on fruit bromelain activity was evaluated using the complete factorial design of the experiment approach. **Results:** The highest actual fruit bromelain activity (76.83 U/ml) and predicted activity (74.81 U/ml) were recorded at an optimum pH of 7.5, temperature of 50°C, and incubation period of 10 minutes using azocasein substrate. All tested variables except temperature; the interaction between substrate type and incubation time significantly affected fruit bromelain activity ($p < 0.05$). **Conclusion:** The design model analysed the main and interaction effect of the variables on bromelain activity. Therefore, complete factorial design is a better approach than OFAT to determine the effect of independent variables on fruit bromelain activity.

Keywords: Bromelain, Pineapple, Enzyme, One-factor-at-a-time (OFAT), Design of Experiment (DOE).

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1.0 Introduction.

Bromelain is a sulfhydryl protease extracted from the pineapple plant (*Ananas comosus*). Proteases catalyse the hydrolysis of peptide bonds within the polypeptide chains. Proteases are ubiquitous and broadly distributed in plants, animals and microorganisms, but plant proteases account for 43.85% of the total distribution [1]. The most abundant plant proteases with industrial and medical values are the cysteine proteases such as papain from pawpaw (*Carica papaya*), ficin from the latex of fig (Ficus spp.) and bromelain from the *Ananas comosus* [2].

Pineapple (*Ananas comosus*) belongs to the *Bromeliaceae* family. It is a popular edible fruit grown in tropical and subtropical countries, including Costa Rica, Brazil, the Philippines, India, Thailand, Nigeria, China mainland, Indonesia, Mexico, and Colombia [3]. The pineapple fruit is a global diet because of its distinctive sweet taste. It contains nutrients like fibre, vitamins, manganese, and copper. Only about one-third of the pineapple plant was considered valuable; the remaining two-thirds, comprising leaves, crown, stem, and peel, are treated as agricultural waste [4]. Numerous phytochemical studies have shown that pineapple wastes contain alkaloids, flavonoids, saponins, tannins, and bromelain, as present in the fruit extract [5].

Bromelain has been the most attractive extract of the pineapple plant since its discovery. It is grouped into four: the stem (EC 3.4.22.32), fruit bromelain (EC 3.4.22.33), ananain (EC 3.4.22.31) and comosain bromelain, but the most prominent are the stem and fruit bromelain [6]. Fruit bromelain differs from stem bromelain because it has higher proteolytic activity and a broader specificity for substrates than bromelain [7,8].

In plant physiology, bromelain plays a specific defensive role in protecting the pineapple plant throughout the developmental, maturation and ripening stages [9]. Bromelain is widely used as a meat tenderiser, solubilising and clarifying agent in the food and cosmetics industries because of its efficacy and lack of toxic side effects [10,11]. Its pharmaceutical and clinical usage includes reversible inhibition of platelet aggregation, modulation of cytokines and immunity, skin debridement, enhanced antibiotic absorption, wound healing, and fibrinolytic activity [10-11].

Like any other enzyme, the activity of bromelain is affected by substrate concentration, pH and temperature. Studies have reported the effect of some of these parameters on the activity of fruit bromelain using the traditional one-factor-at-a-time (OFAT) approach [12-16]. The OFAT approach involves fixing all parameters while varying the variable under consideration. It is a straightforward approach. However, it is time-consuming and does not consider the interaction effect of variables. It is expensive to conduct with many experimental factors. Thus, this study aimed to evaluate the main and interaction effects of four independent variables on the activity of fruit bromelain using the statistical design of experiments method (DOE).

2.0 Materials and Methods

2.1 Materials

Mature smooth cayenne pineapple fruit was collected from the National Horticultural Research Institute (NIHORT) in Ibadan-Oyo state and transported to the Biotechnology Advanced Research Centre (BARC) Laboratory, Sheda Science and Technology Complex (SHESTCO), Abuja, Nigeria.

2.2 Methods

2.2.1 Sample Preparation

The crown was detached from the fruit before washing thoroughly with tap and distilled water. The fruit was peeled, and the core was separated from the fleshy part.

2.2.2 Bromelain Extraction

Bromelain was extracted according to the methods of [10,17]. Briefly, the fleshy part of the pineapple fruit was chopped into tiny bits and blended with a 10 mM Sodium phosphate buffer solution (Ph, 7). The homogenised solution was filtered through a triple-folded Muslin cloth. To free extracts of insoluble debris, filtrates were centrifuged at 10,000 rpm for 10 min in a refrigerated temperature of 4 ^{o}C. The collected supernatant (crude extract) was frozen at –20 ^{o}C until further use.

2.2.3 Enzymatic Assay of Fruit Bromelain Extract

The activity of the bromelain extract was assayed via the product formation method using a 1 % casein solution. Absorbance was read at 275 nm in a UV-VIS spectrophotometer, and bromelain activity was extrapolated from the tyrosine standard curve prepared with 0.1 mg/ml tyrosine stock solution and diluted to a working solution of 20 -120 µg/ml [10,12]. Azocasein (2 %) was used for the substrate utilisation assay approach, and absorbance was read at 440 nm. A molecular mass of 23.6 kDa and a percentage extinction coefficient ($E_{1\%}$) of 35 were used to

determine the concentration of azocasein utilised [18,19]. Bromelain activity (U/ml) was calculated based on the expression:

$$\frac{\text{Conc of A (µmol)} \times \text{B}}{C \times D}$$

Where A = Tyrosine or Azocasein, B = total volume (ml) of the reaction assay, C = volume (ml) of the bromelain extract used and D = time (min) of the reaction incubation.

2.2.4 Statistical Design of the Experiment

The effect of pH, substrate type, temperature and incubation time on the activity of fruit bromelain was evaluated based on a complete factorial design with 22 runs comprising 6 central points generated using the Design Expert software version 13 (Stat-Ease Inc., Minneapolis, MN, USA). Parameter values were chosen based on optimum conditions reported in literature [20-22].

3.0 Results and Discussions

3.1 Effect of pH, Substrate type, Temperature and Incubation time on the Activity of Fruit Bromelain.

Enzymes have maximum activity at optimum pH and temperature. A reduction in optimum conditions of these parameters slows down the rate of enzyme activity. An increase above the optimum range also decreases enzymatic activity due to the enzyme's denaturation and reduction in substrate binding specificity [23]. This study recorded maximum bromelain activity of 76.83 U/ml in run number 2 (Table 1) at an optimum pH of 7.5, temperature of 50 ^{o}C, and incubation time of 10 min with Azocasein substrate type. These corroborated the optimum conditions previously reported in literature [20-22].

However, these optimum conditions also produced lower bromelain activity when visualised separately without considering their interaction effects. Run numbers 3, 6, 10, 12, 17, 18, and 20 produced varied and lower bromelain activity within the 56.23-70.47 U/ml despite being assayed at an optimum pH of 7.5. Run numbers 3, 9, 11, 12, 14, and 19 produced varied and lower bromelain activity within the 53.17-69.40 U/ml despite being assayed at an optimum temperature of 50 C. A similar trend was observed with type of substrate and incubation time at run number (1, 6, 7, 10, 13, 14, 15, 16, 19, and 20) and (4, 6, 7, 1, 12, 14, and 18) respectively. This implies that the correct combination of optimum conditions is essential for optimum enzymatic activity, which is not possible with the OFAT approach. The optimum conditions were obtained simultaneously within a run (number 2) of all the 22 experimental runs, thus saving time and resources that would have been utilised with the OFAT method, which requires separate runs for each of the four parameters.

Table 1: Effect of independent variables on the activity of fruit bromelain

Run	pH	Substrate	Temp (°C)	Incubation time (min)	Actual Bromelain activity (U/ml)	Predicted Bromelain activity (U/ml)
1	6.5	Azocasein	30	30	58.23	56.31
2	7.5	Azocasein	50	10	76.83	74.81
3	7.5	Casein	50	30	56.23	55.09
4	6.5	Casein	30	10	53.00	53.11
5	7.0	Casein	40	20	54.20	56.41
6	7.5	Azocasein	30	10	70.47	70.71
7	6.5	Azocasein	30	10	65.33	64.21
8	7.0	Casein	40	20	60.13	56.41
9	6.5	Casein	50	30	53.17	52.58
10	7.5	Azocasein	30	30	60.63	58.82
11	6.5	Casein	50	10	57.10	57.21
12	7.5	Casein	50	10	62.70	63.71
13	7.0	Azocasein	40	20	60.90	63.24
14	6.5	Azocasein	50	10	69.40	68.31
15	7.0	Azocasein	40	20	60.05	63.24
16	7.0	Azocasein	40	20	60.77	63.24
17	7.5	Casein	30	30	55.61	56.26
18	7.5	Casein	30	10	60.83	59.61
19	6.5	Azocasein	50	30	55.70	55.14
20	7.5	Azocasein	50	30	57.37	57.66
21	7.0	Casein	40	20	54.90	56.41
22	6.5	Casein	30	30	52.67	53.74

3.2 Interaction Effect of Independent Variables on Fruit Bromelain Activity

The main and interaction effects of the independent variables on fruit bromelain activity are illustrated with the Pareto plot (Figure 1). Positive effects corresponded to high activity, and negative effects corresponded to low activity. An increase in the interaction term between incubation time and other factors: pH (AD), substrate type (BD), and temperature (CD) decreased fruit bromelain activity just as an increase in incubation time alone beyond the optimum value reduced enzyme activity. This explains the lower fruit bromelain activity recorded in run numbers 1, 10, 13, 16, 19, and 20 in Table 1.

An increase in pH and temperature increased fruit bromelain affinity for azocasein, thus resulting in higher fruit bromelain activity. This finding corroborated the high thermal stability, better affinity for azocasein and alkaline nature of fruit bromelain reported by [8, 12, 22]. Incubation time, substrate type, pH and the interaction effect of incubation time with substrate type laid above the Bonferroni limit, indicating that these factors significantly affected bromelain activity ($p < 0.05$) [24]. The temperature-incubation time interaction term (CD) between the Bonferroni and t-limit lines was only highly probable to have significantly affected bromelain activity. In contrast, factors that appeared below the t-limit line were insignificant ($p > 0.05$) [24, 25].

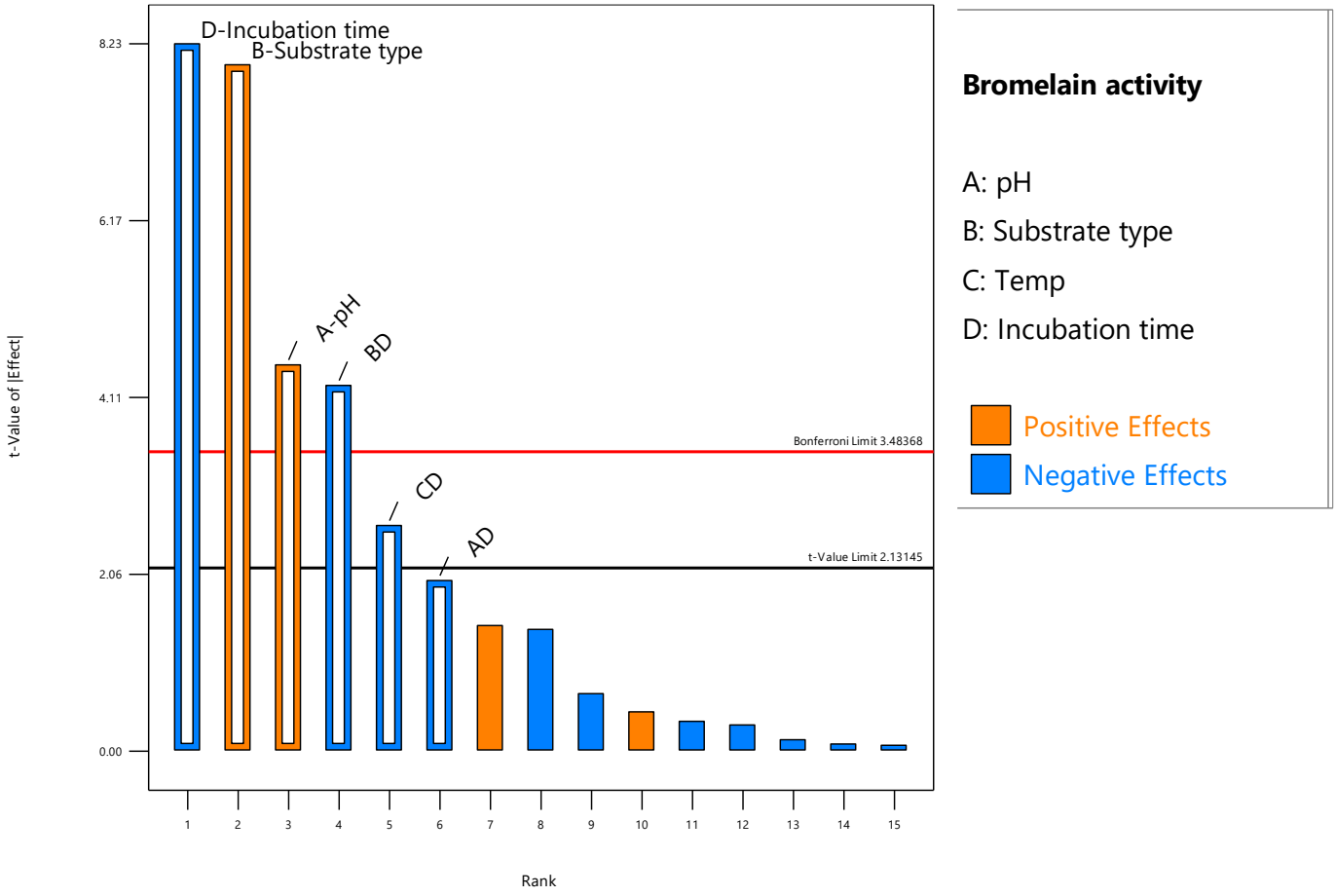

Figure 1: Main and interaction effect of four independent factors on fruit bromelain activity.

3.3 Generation of Regression Equation and Validation of the Model.

Data in Table 1 were fitted automatically by the software to a regression equation (Equation 1) to predict the fruit bromelain activity. There was no significant difference between the actual and predicted experimental values ($p > 0.05$) (Table 1). Therefore, the model equation was suitable for predicting the fruit bromelain activity. ANOVA of the fitted mathematical model (Table 2) showed that the randomised 2-level factorial model with an F-value of 23.53 is significant, and there is < 0.0001 chance that an F-value this large could occur due to noise. The lack of fit F-value of 0.77 implied that the lack of fit is not significant relative to the pure experimental error, and there is a 66.68 % chance that a lack of fit F-value this large could occur due to noise. The non-significant lack of fit is good because the primary objective is for the model to fit the experimental data. The R^2 value of 0.9217 shows that the model obtained gave a reasonable estimate of the bromelain activity in the range studied. The Predicted R^2 of 0.8485 is in sufficient agreement with the adjusted R^2 of 0.8825 because the difference is less than 0.2. Adequate precision measures the signal-to-noise ratio. As a rule of thumb, a ratio greater than 4 is desirable [26]. Thus, a ratio of 17.442 in this study indicated an adequate signal that this model can be used for this study.

Fruit bromelain activity (U/ml) = +59.83 + 2.25 pH + 3.42 Substrate + 0.7331 Temp – 4.13 Incubation time – 0.9956 pH * Incubation time – 2.13 Substrate * Incubation time – 1.32 Temp* Incubation time (Equation 1).

Table 2: ANOVA for the effect of four independent variables on fruit bromelain activity

Source	Sum of Squares	Degree of freedom	Mean Square	F-value	P-value	
Model	735.71	7	105.1	23.53	< 0.0001	significant
A-pH	81.32	1	81.32	18.2	0.0008	
B-Substrate type	256.64	1	256.64	57.45	< 0.0001	
C-Temp	8.6	1	8.6	1.93	0.187	
D-Incubation time	272.66	1	272.66	61.04	< 0.0001	
AD	15.86	1	15.86	3.55	0.0805	
BD	72.89	1	72.89	16.32	0.0012	
CD	27.75	1	27.75	6.21	0.0259	
Residual	62.54	14	4.47			
Lack of Fit	41.12	10	4.11	0.7678	0.6668	not significant
Pure Error	21.42	4	5.36			
Cor Total	798.25	21				
Statistical parameters for the model						
Std. dev.	2.11	R-Square	0.9217			
Mean	59.83	Adj. R-Square	0.8825			
C. V %	3.53	Pred. R-Square	0.8485			
PRESS		Adeq. Precision	17.4416			

4.0 Conclusion

Based on the higher fruit bromelain activity recorded when the optimum conditions for the reaction rate were considered simultaneously along with their interaction terms, it can be concluded that the statistical design of the experiment compared to the one-factor-at-a-time method is a better approach to evaluating the effect of independent variables on the fruit bromelain activity. Validation of the model result also showed that a 2-level complete factorial design can be used to assess the impact of independent factors on the bromelain activity.

Authorship contribution: K. O. Nasir-Naeem: Conceptualization, Design, Literature search, software, Formal analysis, Writing - original draft. O. K. Shittu: Supervision, Formal analysis, Resources, Validation, Writing - review & editing. A. Y. Kabiru: Supervision, Writing - review & editing. O. A. Asojo: Supervision, Writing - review & editing. S. A. Akande: Writing - review & editing.

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