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RESEARCH ARTICLE

PARAMETRIC FACTORIAL ANALYSIS OF FERMENTATION CONDITIONS FOR PROTEASE PRODUCTION FROM PINEAPPLE WASTE

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ABSTRACT - Pineapple waste is abundant in cellulose, hemicellulose, lignin, complex carbohydrates, and protein. One simple way to use pineapple waste as a substrate is to utilize it with the probiotic beverage to produce enzymes through submerged fermentation. Studies on fermentation using pineapple waste have mainly focused on the production of cellulase, xylanase, and pectinase, with little information on protease fermentation. Future studies on pineapple waste-based protease fermentation should be conducted. The present work aims to investigate the significant process parameters affecting protease fermentation from pineapple waste. The parametric factorial analysis was performed using the two-level fractional factorial design by Design Expert 7.1.6. In this work, four process parameters were manipulated for protease fermentation which is incubation time from 24 hours to 72 hours, temperature from 20.0 °C to 40.0 °C, substrate concentration from 10.0 % to 30.0 % and pH value from 4.0 to 8.0. The result showed that the most significant process parameters affecting protease fermentation were temperature, pH value and incubation time. This study investigated that the highest protease activity of 0.118 U/mL can be obtained with 48 hours of incubation time, 30.0 °C of temperature, 20.0 (v/v) % of substrate concentration and pH 6.0.

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1.0 INTRODUCTION

Pineapple is an edible fruit that is the third most important tropical fruit after banana and citrus. It contributes more than 20 % of the world's production of tropical fruits [1]. Pineapple is widely grown in Malaysian states of Johor and Selangor in Peninsular Malaysia and Sarawak in East Malaysia [2]. Malaysia exports approximately 20,000 tonnes of fresh pineapples every year [3]. Pineapple is mainly consumed fresh in developing countries and processed as canned fruits, dried fruits, drinks or juices in developed countries [4]. Pineapple (*Ananas comosus Merr.*) is the third most popular tropical fruit, and it is a crucial ingredient in fruit and juice-based products such as juice concentrates, jams, squash, jellies, essence, and pickles. The pineapple pulp contributes to about 30 % of the whole weight of the fruit, 70 % of pineapple tissue is thrown as waste which contains the crown, peel, bottom, stem and trimmings [5]. Stem alone contributes to 20 % of the total waste produced by pineapple processing industry [6]. The main attention of the pineapple industry in Malaysia is fruits and related foodstuffs. Consequently, the other parts of the plant such as its stems, roots and leaves are considered biomass waste, potentially yielding 122,176 metric tonnes [7]. The large quantities of waste from the fruit processing industry are mainly constituted of fibrous residues from epicarp or inedible parts. Valorisation of these wastes not only could add commercial value to the products but also alleviates the detrimental effect on the environment caused by the disposal in municipal landfills [8].

In the bioprocessing industry, there is a new interest in the use of residual materials due to their easy accessibility and the C and N content, necessary elements in fermentation processes [9]; however, the greatest interest is the possibility of reducing the costs associated with the substrate, since this influences between 60 and 70 % of the total cost of the process [10]. Numerous academic studies are being carried out to utilize the pineapple wastes such as protein enrichment, vinegar manufacturing, enzyme production, preparation of activated carbon nanosheets, production of cellulose nanocrystals and others [11]. Waste from pineapple also can be a source of proteolytic enzyme, which is widely used as a therapeutic and meat-tenderiser [12]. Several enzyme fermentation researches have been done by using pineapple waste [11]; [13]; [14]. However, most of the research focused on enzymes such as cellulase, xylanase and pectinase. Studies on bromelain are also popular but the method of extraction varies from one to another. Currently, proteases from microbial origin have been widely utilized for the production of protein hydrolysates with bioactivities because of their low cost, high stability and specificity. However, despite of the currently available commercial proteases, more novel microbial proteases are still needed from natural resources [15]. Proteases are catalytic proteins that could be utilised in the food industry, which may improve the nutritional, bioactive and functional properties of the food proteins, the digestibility, sensory quality (texture, taste or flavor) andantioxidant properties of food [16]. Proteases, peptidases or proteinases, are widely used

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because they hydrolyse protein molecules into peptides and amino acids [17]. Proteases can be produced by animals, plants and microorganisms while microbial proteases are favoured for industrial applications as the production rate is high [18]. The microbial production of proteases usually depends on some factors, such as medium composition, temperature, pH, incubation time and others [19]. The use of lactic acid bacteria (LAB) in the agri-food industry has increased tremendously over the years as they possess technological properties that could be exploited at the industrial level. These include proteolytic, flavoring, cellulolytic, texturizing, amylolytic, and their ability to produce lactic acid [20]. Similar researches indicated that microbial proteases originate from a wide range of sources, such as lactic acid bacteria (LAB), bacilli and yeast [17].

This paper aims to study the effect of different incubation times (24 to 72 hours), temperatures (20 to 40 °C), substrate concentration (10 % to 30 %v/v) and pH value (4.0 to 8.0) during fermentation on the protease activity. The objective of this research is to investigate the significant process parameters affecting the production of protease enzymes by using pineapple waste and probiotic drinks consisting of lactic acid bacteria using factorial design.

2.0 MATERIALS AND METHOD

2.1 Materials

Pineapple waste from fresh pineapple and probiotic drinks were purchased at the local market at Gambang, Pahang Malaysia. Monopotassium phosphate, dipotassium phosphate, sodium acetate and acetic acid were used to prepare the buffer solution. The casein powder, sodium carbonate, trichloroacetic acid, Folin – Ciocalteu's phenol reagent and commercial L-tyrosine were used for protease assay. All the other used chemicals were of analytical grade and purchased from Sigma Chemical-Aldrich, Malaysia.

2.2 Substrate Preparation

Pineapple waste is a product made from the peels, crown leaves and core, of the pineapple fruit. Substrate preparation was carried out as described earlier by Selvanathan & Masngut [21].

2.3 Experimental Design and Analysis

Design-Expert® software (Version 7.1.6, Stat-Ease, Inc., Minneapolis, MN) was used to analyse and construct the screening experimental design of the fermentation process parameters in the enzyme synthesis from the pineapple waste juice fermentation [14]. Incubation period, temperature, pH of the medium and substrate concentration were chosen as four parameter components in a 2^4 partial factorial design. The coded and actual levels of the parameters for protease production are shown in Table 1. The Design Expert 7.1.6 software, was used to screen the most significant factors affecting the fermentation of protease. Half factorial design was employed, and ANOVA analysis was used to estimate the statistical parameter.

Table 1. Factors, their codes and their actual level used in the 2^4 full factorial designs for protease production

No.	Factors	Code	Type of Factor	Actual values of coded levels			Units
				-1	0	+1	
1	Fermentation Time	A	Numerical	24	48	72	hours
2	Temperature	B	Numerical	20	30	40	°C
3	Substrate Concentration	C	Numerical	10	20	30	%
4	pH of Medium	D	Numerical	4	6	8	-

2.4 Fermentation of Protease

The fermentation samples were prepared by mixing the media of fermentation (probiotic drink), pineapple waste substrate earlier and buffer solution in 250 mL conical flask. The fermentation of protease was carried out in an incubator shaker with a constant rotational speed of 200 rpm. The conditions of the fermentation were adjusted using different screening parameters, which were incubation time (24, 48 and 72 hours), temperature (20.0, 30.0, 40.0°C), substrate concentration (10, 20 and 30 % v/v) and pH value (pH 4, 6 and 8). This method was modified from a few studies [22-24].

2.5 Protease Assay

Lactobacillus Casei, a type of lactic acid bacteria are versatile extracellular protease producers [25]. The protease assay was conducted by following the protocol by Sigma Aldrich, using casein as the substrate [26].

3.0 RESULTS AND DISCUSSION

3.1 Analysis of Protease Production from Pineapple Waste

The 2^4 partial factorial designs of the experiment are shown in Table 2. From Table 2, the 3rd run achieved the highest protease activity with the value of 0.118 U/mL while the 4th and 10th runs had the lowest protease activities which are 0

U/mL. The operating parameters for the 3rd run are fermentation time of 48 hours, incubation temperature of 30 °C, 20 % substrate concentration and pH of the medium is 6.

Table 2. Factors, their codes and their actual level used in the 2^4 full factorial designs for protease production

Run	Coded values of factors				Protease Activity (U/mL)
	A	B	C	D	
1	+1	-1	-1	+1	0.024
2	-1	-1	+1	+1	0.006
3	0	0	0	0	0.118
4	-1	-1	-1	-1	0.000
5	0	0	0	0	0.112
6	0	0	0	0	0.107
7	+1	+1	+1	+1	0.082
8	0	0	0	0	0.111
9	+1	+1	-1	-1	0.049
10	+1	-1	+1	-1	0.000
11	-1	+1	+1	-1	0.032
12	-1	+1	-1	+1	0.067

3.2 Model of the FFD

The regression model, in coded parameters is shown in equation 1 for protease production. This equation 1 could be used for predicting responses under various operating situations. The coded parameters for protease production:

$$Y = 0.033 + 6.4 \times 10 - 3A + 0.025B - 2.425 \times 10 - 3C + 0.012D + 1.825 \times 10 - 3AB + 4.725 \times 10 - 3AC + 2.150 \times 10 - 3AD \quad (1)$$

where Y represents the enzyme activity. The fermentation time, incubation temperature, substrate concentration and pH of the medium are represented as A, B, C, and D, respectively. AB, AC and AD are the terms for the interaction effects between the parameters.

3.3 Analysis of variance table (ANOVA)

Analysis of the variance summary of protease activity produced from fermented pineapple waste juice was tabulated to estimate the coefficient of the model, to determine the significance of each parameter and to check the interaction between the parameters in Table 3.

Table 3. ANOVA Summary of Protease Activity

Source	Sum of Squares	Mean Square	F Value	p-Value	
Model	6.807×10^{-3}	9.725×10^{-4}	47.60	0.0045	significant
A-Fermentation Time	3.277×10^{-4}	3.277×10^{-4}	16.04	0.0279	
B-Temperature	4.990×10^{-3}	4.990×10^{-3}	244.26	0.0006	
C-Substrate concentration	4.705×10^{-5}	4.705×10^{-5}	2.30	0.2264	
D-pH of Medium	1.786×10^{-4}	1.786×10^{-4}	8.74	0.0597	
R^2				0.9911	
Adjusted R^2				0.9703	

P value with higher than 95% confidence level, which means the value less than 0.05. The factors that are below the value of 0.05 where factor A (0.0045), factor B (0.0279), factor D (0.0006). A good fit model's coefficient of determination (R^2) should be close to 100 % or 1 and at least 80 % or 0.80 [27]. This model showed the coefficient of determination, R^2 is 0.9911, and the adjusted R-squared is 0.9703. Hence, it can be interpreted as the model fits the data and can be accepted.

3.4 Contribution Percentage of Factors for Protease Activity

The Pareto chart of the effects of different factors on protease fermentation is shown in Figure 1. The factor B and the factor D had the values of effect on protease fermentation, around 15.00 and 7.00, respectively, which is located above Bonferroni limit, 6.58. The factor A lay between the Bonferroni limit line and the t-value limit line (3.18), which had the value of around 3.80. Variables above the Bonferroni limit can be deemed as factors that are greatly significant, undoubtedly whereas coefficients below t-value limit line are described as parameters that are insignificant, and any value between these two levels is considered as a significant coefficient [23]. In this work, the most significant factor affecting protease production is temperature, followed by the pH value, while the substrate concentration is the least significant.

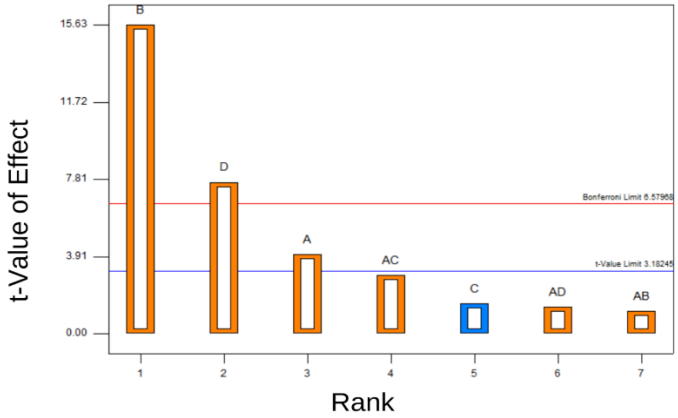

Figure 1. Pareto Chart on Protease Fermentation

Table 4 shows the percentage of the contribution for each factor towards the protease activity produced by fermenting pineapple waste. The temperature has the highest percentage of contribution with the value of 21.01% and followed by pH value with the 5.05% of contribution. Then, it was followed by the incubation time with the percentage of contribution, 1.38%, and the least percentage of contribution factors is substrate concentration with the value of 0.20 %. The interaction factor between incubation time and substrate concentration (AC) also contributed effectively to protease activities which have 0.75%. The significantly high contribution percentage of factor B (temperature) on protease activity was justified as the protease activity showed an increasing trend when the operating temperature getting close to the optimal temperature, 60°C [28].

Table 4. Percentage of Contribution for Factors towards Protease Activity

Factor	**% Contribution**
A – Incubation Time	1.38
B – Temperature	21.01
C – Substrate concentration	0.20
D – pH value	5.05
AB	0.11
AC	0.75
AD	0.16

Temperature is a critical physical element that influences enzyme synthesis [29]. Temperatures of the samples for protease fermentation were adjusted to 20 °C, 30 °C and 40°C. It is observed that the protease activities increased drastically from 20.0 °C to 30.0 °C. The highest protease activity was 0.1180 U/mL at 30.0 °C. The ideal temperature for enzyme synthesis varies depending on the microbes involved. The research of Limkar et al. [30] stated that the maximum protease activity was observed at 30.0 °C. According to Butt et al. [31], temperature is a critical parameter that could affect bio-processing. This backs up a finding that temperature influences extracellular and intracellular enzyme production at the translational and transcriptional levels. As a consequence, it is critical to research the best temperature for enzyme synthesis, which varies depending on the organism [30]. Due to a huge quantity of metabolic heat, the temperature of fermenting feed rises at higher temperatures. The protease activities in this study decreased when the temperature rose to 40 °C. Because of the protein denaturation, it may impede microbial growth and reduce metabolic processes, resulting in fewer enzyme productions [29].

Various pH values were screened through the protease fermentation, which were pH 4.0, 6.0 and 8.0. pH 6.0 achieved the highest protease activity, and the highest protease activity was 0.1180 U/mL. Protease activities for pH 4.0 were the lowest among the three pH values. According to Doan et al. [32], the optimum pH value for protease was determined to be pH 6.0 – 7.0. Butt et al. [31] also reported that the highest protease activity was observed at pH 7.5. [30] investigated the synthesis of protease at various pH medium levels ranging from 6 to 9. It was observed that protease at pH 7.5 has a higher enzyme yield then the other used pH. The external or media pH has an important impact on the transportation and the ionization of the nutritional components across the cell membrane which makes the synthesis of enzyme reliant on the pH too. This affects their availability to the organism and activities of the enzymes, affecting cell development and product creation even more. Similarly, each enzyme has an optimal pH at which it produces the most. Optimum pH value allows both H + and OH - ions to influence the hydrogen and ionic bonds of the enzyme in order to make the active site shape of the enzyme is utmost complimentary to the shape of the substrate. For maximal usage of an organism's ability for enzyme levels of productivity, the pH should be at its optimum. At optimal pH, enzymes are in their most active phase. Extremely basic or acidic conditions may cause denaturation and deactivation of enzymes [30]. Ridzuan et al. [33] reported no protease activity up to 96 hours of fermentation using pineapple waste. The highest protease activity was at 144 hours of fermentation with 0.010 U/mL. The fermentation time is shorter, and the protease activity is higher in this study due to the use of probiotics.

3.5 Validation of the predicted model

A validation study was performed in triplicate to test the models provided by the Design Expert software. The discrepancy between the experimental results and the model's predicted protease activity was assessed by the percentage of error. According to the data provided, the calculated error percentage is between 0.34 % and 1.21 %. The value of error that is tolerable depends on the experiment, but a margin of error of 10% is commonly accepted [34]. As a result, the model's estimated parameter contribution to enzyme production is validated. The temperature of the fermentation and the pH of the medium are important factors for protease synthesis. The most desirable process conditions for the protease activity were 72 hours of fermentation time, 40.0 °C of incubation temperature, 30.0 % of substrate concentration and pH of the medium 8.0, as suggested by the Design Expert software.

Table 5. Validation runs of protease production

Run	Conditions	Protease Activity (U/mL)		Error (%)
		Predicted	Experimental	
1	72 hrs, 40 °C, 30 %, pH 8.0	0.082	0.082	0.34
2	72 hrs, 40 °C, 29.74 %, pH 7.98	0.082	0.083	0.79
3	70.8 hrs, 40°C, 29.95 %, pH 8.0	0.082	0.083	1.21

4.0 CONCLUSION

The purpose of this research was to investigate the effect of different factors during fermentation on protease activity. The most significant process parameter that affects the production of protease enzyme by using pineapple waste was temperature with 21.01 % of contribution, and pH of the medium with 5.05 % of contribution. Best fermentation conditions for the highest protease activity, 0.0784 U/mL were 72 hours of fermentation time, 40.0 °C of incubation temperature, 30.0 % of substrate concentration and pH of medium 8.0, which was suggested by the software. These variables can be utilised to optimise enzyme synthesis in the future. Further researches could also be done on the protease fermentation by using the bacterial strain extracted from the probiotic drink.

5.0 CONFLICT OF INTEREST

The authors declare no conflicts of interest.

6.0 AUTHORS CONTRIBUTION

P. Sivanesan (Methodology; Software; Writing - review & editing)

T. G. Han (Investigation; Formal analysis; Writing - original draft; Validation)

Z. I. M. Arshad (Conceptualisation; Data curation; Visualisation)

R. C. Man (Conceptualisation; Data curation; Visualisation)

S. K. A. Mudalip (Conceptualisation; Data curation; Visualisation)

S. Z. Sulaiman (Conceptualisation; Data curation; Visualisation)

S. M. Sharaani (Conceptualisation; Data curation; Resources; Funding acquisition; Supervision)

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