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Evaluation of Factors Affecting Microbial Growth Inhibition and Optimization Using Pineapple Leaves Juice

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ABSTRACT

This study optimized microbial growth inhibition conditions using pineapple leaf juice (PLJ). The sugarcane press machine was used to press the PLJ. The study considered four factors to be analyzed by Two-level factorial design (TLFD), which are microbial inhibition time (0.5–5 h), the concentration of total phenolic content (TPC) (0.2563–0.5127 mg GAE/mL), temperature (26–37 °C), and the ratio of PLJ to microbe (PLJ/M) (v/v) (1:1 and 1:3). Colony-forming unit (CFU) method was employed to measure microbial growth inhibition. The microbial growth inhibition was expressed as a percent in terms of CFU/mL. A central composite design (CCD) experimental design created using response surface methodology (RSM) determined the optimum temperature (35–39 °C) and microbial inhibition time (10–50 min) of microbial growth inhibition. The best conditions were 0.5 h of microbial inhibition time, 0.5127 mg GAE/mL of TPC, 1:1 PLJ/M, and a temperature of 37 °C. The analysis of variance (ANOVA) showed that temperature (Factor C) has the greatest contribution (1.56%) to inhibiting microbial growth, accompanied by TPC concentration in PLJ (Factor B) with 1.27%, microbial inhibition time (Factor A) with 1.07% and PLJ/M (Factor D) 0.29%. Optimization studies show that at an optimum temperature of 37 °C and an inhibition time of 34.25 min, maximum microbial growth inhibition of 94.73% with a minimum value of 9.12×10^4 CFU/mL was achieved. This research

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suggests that PLJ can be utilized as a value-added natural product for application in the agricultural sector.

Keywords: Central composite design (CCD), microbial growth inhibition, phenolic compounds, pineapple leaf juice (PLJ), two-level factorial design (TLFD)

INTRODUCTION

Most synthetic microbial growth inhibitor (MGI) agents can cause severe toxicity. Using synthetic MGI to combat disease and infection is impactful, especially for humans and the environment. Therefore, finding a new alternative MGI agent from natural plant sources will be favorable. Nowadays, natural MGI from different sources has been used to inhibit microbial growth and pathogenic microorganisms. More than 30,000 antimicrobial components and 1,350 plants with antimicrobial activities have been extracted (Arshad & Batool, 2017). Pineapple (*Ananas comosus*) is a commercial fruit with MGI properties due to its high phenolic compounds (Domínguez et al., 2018). Pineapple leaves contain seven significant phenolic compounds, including Methyl-5-O-caffeoyl-quinate, octahydrocurcumin, meliadanoside A, stilbostemin D, feralolide, agrimol C and kukoamine A (Ya'acob et al., 2021). Phenolic compounds are important to provide a defensive mechanism against infection. Therefore, using pineapple leaf juice (PLJ) as a natural product will benefit the communities since they are abundantly available waste materials in Malaysia. However, at the current time, it has not been studied yet as it is required (Asim et al., 2015).

Because these factors can influence the process, analyzing the microbial growth inhibition process can consume much energy, money, and time. Therefore, it is decided to use a two-level factorial design (TLFD), a screening experiment to analyze the factors affecting the microbial growth inhibition process by using PLJ. It explains the correlations among various responses resulting from one or more factors (Shane, 2017). Screening designs offer an efficient approach for assessing many factors in a minimal number of experimental runs for further investigation. Thus, the use of TLFD is vital in analyzing the influence of several factors that contributed to the application of PLJ as MGI by evaluating all the interactions involved.

In order to utilize the PLJ as an effective MGI, it is needed to evaluate the optimum condition of inhibition of microbial growth through response surface methodology (RSM). The RSM method can also determine the interaction between the independent variables by decreasing the number of trials (Aydar, 2018). According to Noormohamadi et al. (2018), central composite design (CCD) is advantageous for second-order (quadratic) polynomial fitting, which is beneficial for the study of the optimization process. Ammer et al. (2016) employed RSM under CCD to investigate the antimicrobial potential of

Eucalyptus tereticornis leaf extracts against *Escherichia coli.* On the other hand, the research on microbial inhibition through factorial analysis and optimization with PLJ, on the other hand, has never been published. Thus, factorial analysis and optimization in determining microbial growth inhibition were beneficial in this study. This study aimed to analyze the factor affecting microbial growth inhibition and optimize the conditioning process using PLJ.

MATERIAL AND METHODS

Materials

Potato dextrose agar (PDA) powder (99%), gallic acid (99%), Folin-Ciocalteu reagent (99%), sodium carbonate (Na_2CO_3, 99%), and methanol (99.8%).

Pineapple Leaf Juice (PLJ) Preparation

The pineapple leaf and tested microbe, which are mixed culture, were provided by a pineapple plantation in Pekan Pina, Pahang. An electrical press machine prepared the pineapple leaf juice (PLJ) extract and autoclaved it for 15 min at 121 °C.

Total Phenolic Content (TPC) Analysis

Total phenolic content (TPC) was determined using a Folin-Ciocalteu assay with Gallic acid as a standard. First, 10 mL of PLJ was centrifuged at 5000 rpm for 15 min. Next, 2.5 mL of 10-fold diluted Folin-Ciocalteu and 0.5 mL of its supernatant were combined. The mixture was kept at room temperature for 5 min. After that, 2 mL of Na_2CO_3 (7.5%) was added to the mixture and kept for 1 h. Then, the mixture was measured using a UV-Vis spectrophotometer at 450 nm. Gallic acid was prepared in an 80% methanol solution with a 0.1–1.0 mg/mL concentration as a standard curve. The solution was also subjected to a similar treatment, which included the addition of Folin-Ciocalteu reagent and 7.5% Na_2CO_3. Mg of gallic acid equivalent per gram of PLJ extract (mg GAE/mL) was presented (Siddiqui et al., 2017).

Culture Medium

Thirty-nine grams of Potato dextrose agar (PDA) were completely dissolved in 1000 mL of distilled water before autoclaving for 15 min at 121 °C. Approximately 10 mL of the solution was poured into Petri plates.

The Cultivation of Microbe

In this study, a pineapple leaf infected with microbes obtained from a pineapple plantation was used as a microbe for testing. The agar was streaked with the microbe on its plate

from quadrant one to four before incubating at 37 °C for 24 h using a sterile loop (Zainol & Rahim, 2017). The microbe used in this study was mixed culture.

Microbial Growth Inhibition Experiment Set-up

The experiment began with re-culturing the microbe. Next, microbe broth (MB) was prepared by scraping and mixing the re-cultured microbes into the nutrient broth. Approximately one PDA plate of microbe was scraped and mixed with nutrient broth. In an incubator shaker, the MB was agitated at 100 rpm of 37 °C for 1 h. Then, the MB and PLJ was mixed at selected ratio (1:1 and 1:3) and agitated in the incubator shaker at 100 rpm at selected inhibition times (0.5–5h) for factorial design and (10–50 min) for optimization and temperature (26–37 °C) for factorial design and (35–39 °C) for optimization. The experiment was conducted according to factorial and optimization design tables. The colony-forming unit (CFU) count was then performed on all samples.

Analysis of Colony Forming Units (CFU)

One hundred microlitres (100 µL) of microbe and PLJ mixture from section 2.6 was evenly spread on a PDA plate with a triangular cell spreader and incubated for 24 h at 37 °C (Jayaratne & Dayarathna, 2015). After 24 h, the colony count was determined. Microbes were counted at a constant range between 30 and 300 colonies on the Petri plate (O'Toole, 2016). The total CFU/mL obtained was used to calculate the microbial growth inhibition (%) using Equations 1 and 2.

$$\mathrm{CFU/mL} = \frac{\text{No. of colonies} \times \text{dilution factor}}{\text{Volume of culture in plate}} \quad (1)$$

$$\text{Microbial growth inhibition (\%)} = \frac{\left(\frac{\mathrm{CFU}}{\mathrm{mL}}\text{ of control} - \frac{\mathrm{CFU}}{\mathrm{mL}}\text{ of mixture}\right)}{\frac{\mathrm{CFU}}{\mathrm{mL}}\text{ of control}} \times 100 \quad (2)$$

Factorial Analysis Study on Microbial Growth Inhibition

The experimental design of two-level factorial design (TLFD) with some factors at different levels was constructed as shown in Table 1. The factorial design table was designed using Design-Expert software (v7) (Table 2). There are four selected factors for factorial analysis: microbial inhibition time (0.5–5 h), the concentration of TPC (0.2563–0.5127 mg GAE/mL), the ratio of PLJ to microbe (PLJ/M) (1:1 and 1:3) and temperature (26–37 °C). For 1:1 PLJ/M, the ratio was 20 mL PLJ: 20 mL MB, while for 1:3 PLJ/M, the ratio was 10 mL PLJ: 30 mL MB. The experiment began with re-culturing the microbe. Then, the experimental setup for microbial growth inhibition and CFU analysis was carried

out. Finally, the experiment for microbial growth inhibition was conducted based on the factorial design table (Table 2).

Table 1
Factors at different levels

Factors	Level	
	Low	High
Microbial inhibition time (h)	0.5	5
TPC concentration in PLJ (mg GAE/mL)	0.2563	0.5127
Temperature (°C)	26	37
Ratio of PLJ to microbe (PLJ/M) (v/v)	1:1	1:3

Table 2
Table of factorial analysis experimental design

Runs	Factor			
	A: Microbial inhibition time (h)	B: TPC concentration (mg GAE/mL)	C: Temperature (°C)	D: Ratio of PLJ to microbe (PLJ/M) (v/v)
1	0.5	0.2563	26	1:1
2	5	0.2563	26	1:1
3	0.5	0.5127	26	1:1
4	5	0.5127	26	1:1
5	0.5	0.2563	37	1:1
6	5	0.2563	37	1:1
7	0.5	0.5127	37	1:1
8	5	0.5127	37	1:1
9	0.5	0.2563	26	1:3
10	5	0.2563	26	1:3
11	0.5	0.5127	26	1:3
12	5	0.5127	26	1:3
13	0.5	0.2563	37	1:3
14	5	0.2563	37	1:3
15	0.5	0.5127	37	1:3
16	5	0.5127	37	1:3

Optimization Study

Microbial inhibition time and temperature factors were chosen to investigate their inhibition of microbial growth effect. The optimization studies were conducted through response surface methodology (RSM) under central composite design (CCD). The selected factors and their level are shown in Table 3, and the experimental design comprised 13 runs. The experiment was carried out based on the experimental design table (Table 4).

Table 3
Factors and level of CCD

Factors	−α	−1 level	0	+1 level	+α
A: Microbial inhibition time (min)	10	20	30	40	50
B: Temperature (°C)	35	36	37	38	39

Table 4
Experimental design table of CCD

Runs	Factor A: Microbial inhibition time (min)	Factor B: Temperature (°C)
1	20	36
2	40	36
3	20	38
4	40	38
5	10	37
6	50	37
7	30	35
8	30	39
9	30	37
10	30	37
11	30	37
12	30	37
13	30	37

Data Analysis

Design-Expert software analyzed the best condition and optimum conditions for inhibition of microbial growth.

Validation Studies

The optimum points suggested by Design-Expert software were further validated to verify the model. Finally, the errors between the experimental values and predicted values were calculated. Equation 3 was used to calculate the error.

$$\text{Error (\%)} = \frac{\text{Experimental} - \text{Predicted}}{\text{Predicted}} \times 100 \quad (3)$$

RESULTS AND DISCUSSIONS

Screening and Analysis by Two-Level Factorial Design (TLFD)

The screening of the four factors was analyzed using TLFD, and 16 experimental runs were carried out, as seen in Table 5. Analysis of variance (ANOVA) Table 6 reveals that temperature (Factor C) has the greatest contribution (1.56%) to inhibiting microbial growth,

accompanied by a concentration of TPC (Factor B) with 1.27%, microbial inhibition time (Factor A) with 1.07% and PLJ/M (Factor D) 0.29%. The highest value of 3.58×10^5 CFU/mL, which indicates the minimum inhibition of microbial growth, was achieved at 1:3 of PLJ/M, 0.5127 mg GAE/mL at 26 °C, and 0.5 h. On the other hand, the lowest value of 1.69×10^5 CFU/mL indicates the maximum inhibition of microbial growth was achieved at 1:1 of PLJ/M, 0.5127 mg GAE/mL at 37 °C, and 0.5 h. Design-Expert software's interpretation of the data analysis indicated that PLJ could only inhibit microbial growth without killing them. It might be explained by the variation of phenolic compounds found in PLJ, which have a certain efficiency in inhibiting microbial growth (Maqsood et al., 2014).

Table 5
Experimental data of factorial study

Std	Factor				Response 1
	A: Microbial inhibition time (h)	B: TPC concentration in PLJ (mg GAE/mL)	C: Temperature (°C)	D: Ratio of PLJ to microbe (PLJ/M) (v/v)	CFU/mL
1	0.5	0.2563	26	1:1	3.57×10^5
2	5	0.2563	26	1:1	2.12×10^5
3	0.5	0.5127	26	1:1	1.94×10^5
4	5	0.5127	26	1:1	3.58×10^5
5	0.5	0.2563	37	1:1	3.47×10^5
6	5	0.2563	37	1:1	2.58×10^5
7	0.5	0.5127	37	1:1	1.69×10^5
8	5	0.5127	37	1:1	2.89×10^5
9	0.5	0.2563	26	1:3	2.30×10^5
10	5	0.2563	26	1:3	2.91×10^5
11	0.5	0.5127	26	1:3	3.58×10^5
12	5	0.5127	26	1:3	2.19×10^5
13	0.5	0.2563	37	1:3	2.50×10^5
14	5	0.2563	37	1:3	2.68×10^5
15	0.5	0.5127	37	1:3	3.03×10^5
16	5	0.5127	37	1:3	2.14×10^5

Factorial Study Analysis of Variance (ANOVA)

The effects of various factors on microbial growth were studied by analysis of variance (ANOVA) (Table 6). The statistical test revealed that the significant factors of A, B, C, D, AB, AD, BC, BD, ABD, BCD, and ABCD are based on their prob>F (less than 0.05). The model was accepted as the statistical test. The model was accepted as the linear regression coefficient R^2 of 0.9995. The adjusted R^2 of 0.9980 shows a good data fit (Saunders et al., 2012). The relationship of CFU/mL with the factors was shown through the codified linear regression shown in Equation 4.

$$Y = 2.703E + 005 - 6228.21A - 6789.28B - 7507.49C - 3243.17D + 13320.50AB - 12400.30AD - 116314.76B + 13657.16BD - 51475.21ABD + 4612.22BCD + 12018.73ABCD \quad (4)$$

Y was the predicted response (CFU/mL), A was the microbial inhibition time (h), B was the TPC concentration in PLJ (mg GAE/mL), C was the temperature (°C), and D was the PLJ/M (v/v).

Table 6
ANOVA of factorial study

	Sum of Square	df	Mean Square	*F*-Value	*P*-Value Prob > F	(%) Contribution	
Models	5.792E+010	11	5.265E+009	692.20	< 0.0001		significant
A: Microbial inhibition time	6.206E+008	1	6.206E+008	81.60	0.0008	1.07	
B: TPC concentration	7.375E+008	1	7.375E+008	96.96	0.0006	1.27	
C: Temperature	9.018E+008	1	9.018E+008	118.56	0.0004	1.56	
D: Ratio	1.683E+008	1	1.683E+008	22.12	0.0093	0.29	
AB	2.839E+009	1	2.839E+009	373.23	< 0.0001	4.90	
AD	2.83E+009	1	2.83E+009	323.45	< 0.0001	4.25	
BC	2.158E+009	1	2.158E+009	283.77	< 0.0001	3.72	
BD	2.984E+009	1	2.984E+009	392.34	< 0.0001	5.15	
ABD	4.240E+010`	1	4.240E+010`	5573.58	< 0.0001	73.16	
BCD	3.404E+008	1	3.404E+008	44.75	0.0026	0.59	
ABCD	2.311E+009	1	2.311E+009	303.85	< 0.0001	3.99	
Residual	3.043E+007	4	7.606E+006				
Cor. Total	5.795E+010	15					
R^2	0.9995						
Adjusted R^2	0.9980						

Factors Influencing Microbial Growth Inhibition

Table 7 shows the suggested best conditions obtained for microbial growth inhibition. The suggested best conditions, PLJ/M of 1:1, 0.5127 mg GAE/mL of concentration of TPC, and temperature of 37 °C for 0.5 h of microbial inhibition time, achieved 21.25% of microbial inhibition time with 2.81×10^5 CFU/mL. The main and interaction effects between factors on microbial growth inhibition were illustrated in the Pareto chart shown in Figure 1. The factors with the blue color represent the negative effect, while the orange color represents the positive effect. The negative effect of increasing the factor value lowered the microbial growth factor CFU/mL response value. From Figure 1, the factors A, B, and

C together affect the interaction between the PLJ/M and temperature and reduce the CFU/mL value. It contributes to the greater inhibition of microbial growth. The factors AD (microbial inhibition time and PLJ/M) and BC (concentration of TPC and temperature) interact negatively. When both interaction factors were increased, the CFU/mL value decreased. Figure 2 illustrates the effect of the two most significant factors.

Table 7
Suggested best conditions

Factors	Conditions
A: Microbial inhibition time	0.5 h
B: TPC concentration	0.5127 mg GAE/mL
C: Temperature	37 °C
D: Ratio	1:1
CFU/mL	3.49×10^5 CFU/mL
Microbial growth inhibition	21.25%

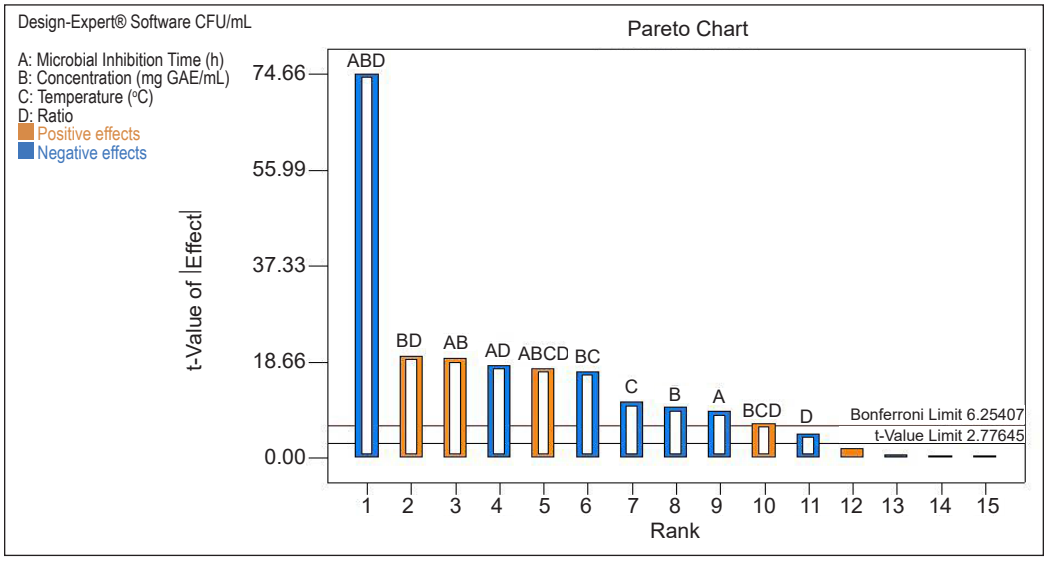
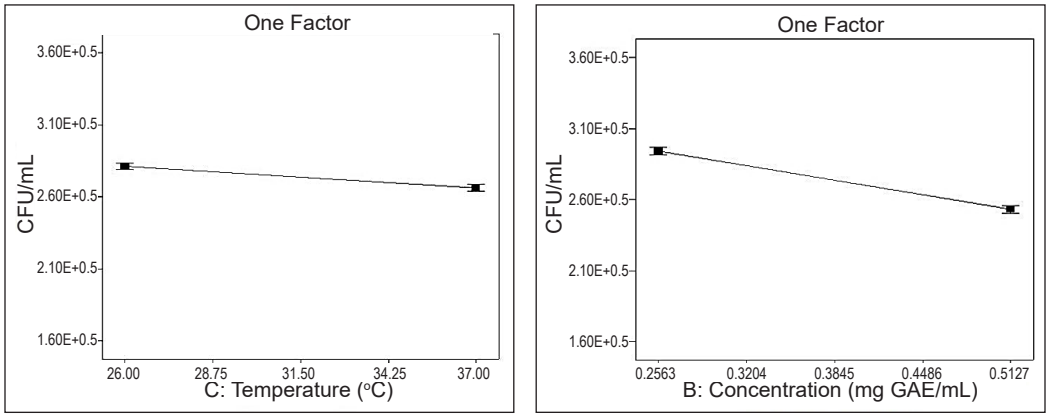


Figure 1. The factorial study's Pareto chart



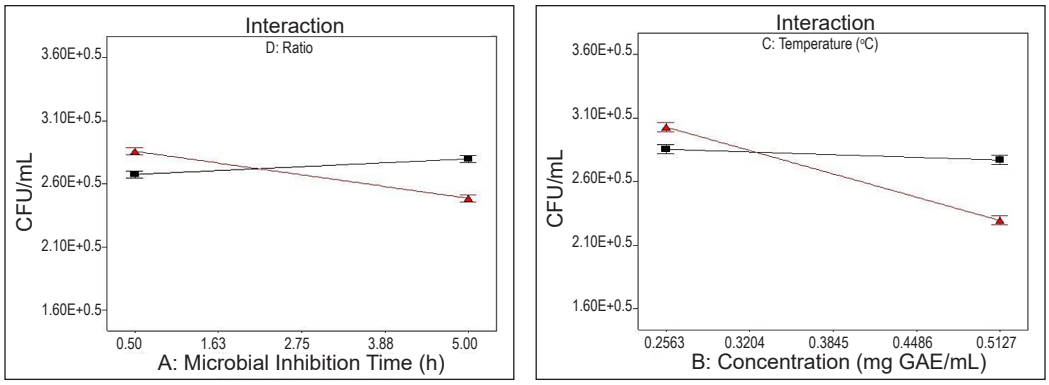
(a) (b)

Figure 2. Factors on CFU/mL (a) C (temperature) and (b) B (TPC concentration)

From Figure 2(a), CFU/mL was slightly decreased with the increasing temperature from 26 to 37 °C. Higher temperatures cause the biofilm's thickness and no longer protect the microbe (Reichhardt et al., 2014). Figure 2(b) shows that CFU/mL decreased when the concentration of TPC increased from 0.256 to 0.513 mg GAE/mL. A higher concentration of TPC could increase the number of antioxidants, resulting in a higher inhibitory effect (Lobo et al., 2010).

Figure 3 illustrates the most significant interaction effect between microbial inhibition time and PLJ/M (v/v) (Factor AD) and also the concentration of TPC and temperature (Factor BC). As can be seen in Figure 3(a), the interaction effect of Factor AD indicates that the CFU/mL value was lower for 1:3 (PLJ/M) (v/v) when microbial inhibition time was 5 h and lower for 1:1 (PLJ/M) (v/v) when microbial inhibition time was 0.5 h. As for the interaction effect between Factor BC [Figure 3(b)], the temperature of 37 °C contributed to reducing CFU/mL when the concentration of TPC was 0.5127 mg GAE/mL. However, a temperature of 26 °C does not affect the CFU/mL at low and high TPC concentrations in PLJ. This claim was supported by Hajdu et al. (2010), in which an increase in the temperature of antimicrobial agents in certain plant-related infection treatments led to a higher decrease in microbe growth. It could be due to a decrease in the thickness of the microbe biofilm caused by the high temperature that triggers the release of the cells from the biofilm. The biofilm appears as a host defense for the microbes and acts as a protective barrier against antimicrobial agents (Reichhardt et al., 2014).

Additional research needs to be done to understand better how PLJ can act as an effective MGI agent and thus enhance maximum microbial growth inhibition. In order to construct substantially improved models, the CCD enables further assistance in optimizing the conditions of the variables identified in a factorial study. It also reduces the number of experimental runs required while giving the most powerful effect on the inhibition of microbial growth.



(a) (b)

Figure 3. Interaction effects on CFU/mL (a) Factor AD (microbial inhibition time–ratio) and (b) Factor BC (concentration–temperature)

Optimization Study

The RSM method optimized the process parameters of the best conditions obtained from TLFD screening. The best conditions obtained were 0.5 h of microbial inhibition time, 0.5127 mg GAE/mL of concentration of TPC in PLJ, the temperature of 37 °C, and a 1:1 ratio of PLJ to microbe (PLJ/M) (v/v). For factorial analysis, the range and values used were higher than optimization, which used a smaller range and values. In order to assess the optimum conditions, the range and values chosen for optimization were smaller with temperature (35–39 °C) and microbial inhibition time (10–50 min). Temperature and microbial inhibition time are two major factors governing microbial growth inhibition. These factors can be systematically optimized with CCD. Thirteen experimental runs using the design shown in Table 8 with varying temperatures and microbial inhibition times were carried out. A model was developed to describe the microbial growth inhibition caused by independent variable levels (coded) and actual levels, as shown in Equations 5 and 6. X_1 was the temperature (°C), and x_2 was the microbial inhibition time (h).

$$\text{CFU (coded)} = 64761.63 - 80544.01x_1 - 25333.17x_2 - 5071.98x_1x_2 + 94855.91x_1^2 + 55041.41x_2^2 \quad (5)$$

$$\text{CFU (actual)} = 7.69E{+}007 - 46201.62x_1 - 4.083E{+}006x_2 - 507.20x_1x_2 + 948.56x_1^2 + 55041.41x_2^2 \quad (6)$$

From the analysis of variance (ANOVA) in Table 9, the regression analysis revealed a good fit of the experimental results to the polynomial model with a regression coefficient

Table 8
Experimental data of optimization study

Run	Factor A: Microbial inhibition time (min)	Factor B: Temperature (°C)	Response 1: CFU (CFU/mL)	Response 2: Microbial growth inhibition (%)
1	20.00	36.00	2.38×10^5	58.26
2	40.00	36.00	2.07×10^5	63.70
3	20.00	38.00	1.89×10^5	66.88
4	40.00	38.00	1.37×10^5	75.87
5	10.00	37.00	6.76×10^5	18.43
6	50.00	37.00	2.33×10^5	59.01
7	30.00	35.00	3.42×10^5	40.08
8	30.00	39.00	2.49×10^5	56.31
9	30.00	37.00	3.37×10^4	94.09
10	30.00	37.00	3.39×10^4	94.05
11	30.00	37.00	8.70×10^4	84.74
12	30.00	37.00	8.78×10^4	84.62
13	30.00	37.00	1.24×10^5	78.20

Table 9
ANOVA of optimization study

	Sum of Squares	df	Mean Square	*F*-Values	*P*-Values Prob > F	
Model	3.115E+011	5	6.230E+010	14.30	0.0015	significant
A: Microbial Inhibition Time	7.785E+010	1	7.785E+010	17.87	0.0039	
B: Temperature	7.701E+009	1	7.701E+009	1.77	0.2254	
AB	1.029E+008	1	1.029E+008	0.024	0.8822	
A^2	2.062E+011	1	2.062E+011	47.31	0.0002	
B^2	6.942E+010	1	6.942E+010	15.93	0.0052	
Residual	3.050E+010	7	4.358E+009			
Lack of Fit	2.438E+010	3	8.125E+009	5.30	0.0704	not significant
Pure Error	6.128E+009	4	1.532E+009			
Cor. Total	3.420E+010`	12				
R^2	0.9108					
Adjusted R^2	0.8471					

(R^2) value of 0.9108 and model *F*-value of 14.30. At the same time, the adjusted R^2 was 0.8471. Lee and Lemieux (2010) suggested that R^2 should be at least 0.80 to get a good fit. The ANOVA model significantly affects microbial inhibition with a *p*-value of 0.0015 (< 0.05) and a confidence level greater than 90%. With *p*-values of 0.2254 and 0.8822, respectively, the interactions between temperature and microbial growth inhibition in CFU/mL were insignificant. The *p*-value of 0.0039 shows that the microbial inhibition time was a significant factor in the microbial growth inhibition.

The association between the actual values of CFU/mL and predicted values of CFU/mL as microbial inhibition response was illustrated in Figure 4. Figure 5 shows the influence of two main factors on CFU/mL. These plots illustrate the influence of temperature and microbial inhibition time on CFU/mL. By increasing the timing of microbial inhibition from 20–30 min, CFU/mL was also decreased [Figure 5(a)]. This result shows that the increase influenced the CFU/mL value at microbial inhibition. Pineapple leaves contain seven significant phenolic compounds, including methyl-5-O-caffeoyl-quinate, octahydrocurcumin, meliadanoside A, stilbostemin D, feralolide, agrimol C and kukoamine A (Ya'acob et al., 2021). According to Hoskeri et al. (2012), phenolic compounds have potency as an agent against some microbes after 10 min.

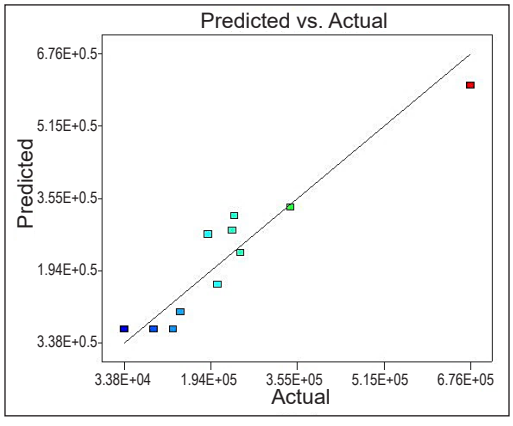
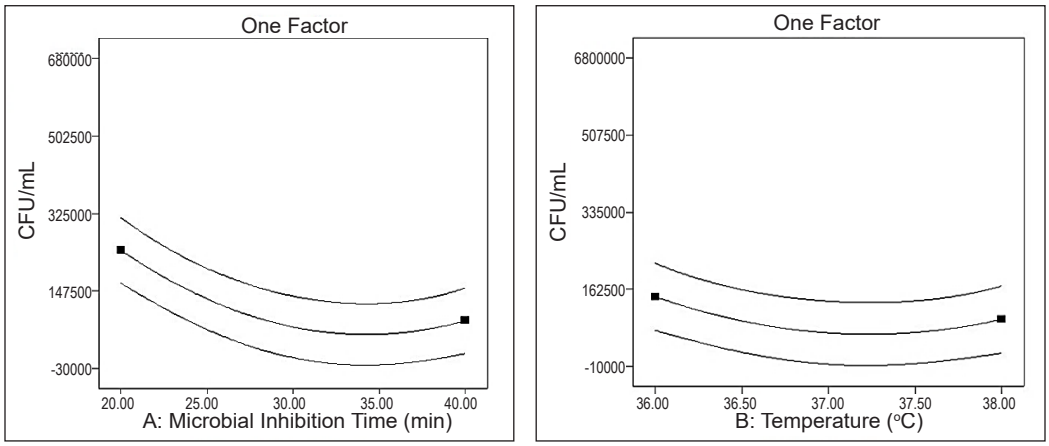


Figure 4. Actual and predicted values

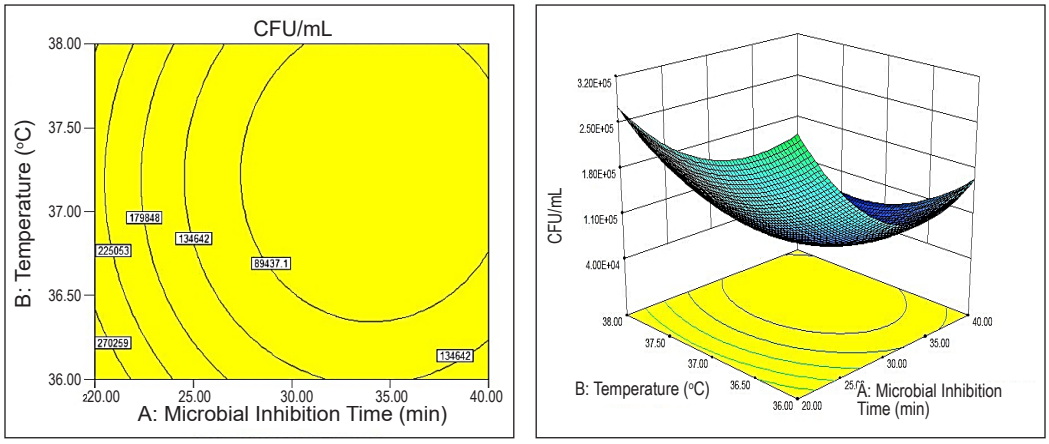


(a) (b)

Figure 5. Effect of one factor on CFU/mL (a) microbial inhibition time and (b) temperature

It may be the shortest amount of time these seven phenolic compounds need to inhibit the microbe effectively. However, from 35 to 40 min, CFU/mL was increased, probably due to the loss of some phenolic compounds, thus making it ineffective to inhibit. A study by Zhang et al. (2021) stated that change could be explained by the different degradation rates and/or synthesis of each phenolic. Different phenolic have different chemical structures and present different structures in different fractions, such as free and bound phenolic fractions. Structural difference plays an important role in individual reducing capacity. The phenolic compound was sensitive to the presence of oxygen at ambient temperature. The yield of phenolic compounds increased during storage time due to the release of free acids from their bonds (Klimczak et al., 2007). The oxidation reaction is one of the processes that can cause modification of extracted pineapple leaves juice during storage (Zafrilla et al., 2003). In Figure 5(b), 37 °C was found optimum for temperature. The temperature did not significantly affect the CFU/mL value, as there was only a slight difference between the CFU/mL values from 35–39 °C. The result was validated by a 0.2254 *p*-value obtained from ANOVA. Thus, 37 °C was adequate to inhibit the process of microbial growth. At this temperature, plant microbes can be killed (Eddleman, 1998).

The contour and three-dimensional response surface plots of microbial growth inhibition in CFU/mL are shown in Figure 6. The surface plots [Figure 6(a)] show that the variables interacted significantly. The contour plot [Figure 6(b)] showing the interaction between the factors helps in the selection of variable ranges to accomplish the goal of targeted optimization (Zhang et al., 2012). Decreasing CFU/mL shows a higher inhibition of microbial growth. The data obtained show the optimum conditions of 37 °C and microbial inhibition time of 34.25 min resulted in maximum microbial growth inhibition of 94.73% and 9.12×10^4 CFU/mL. López-García et al. (2012) studied that bromelain extract from pineapple stems could inhibit 90% of *F. verticillioides* growth. The experimental response



(a) (b)

Figure 6. Contour and 3D surface plot (a) Contour plot and (b) 3D response surface plot

obtained (94.73%) based on the modeled optimum conditions was reasonably close to the predicted response.

Optimum Conditions

Table 10 presents the optimum conditions suggested by the Design Expert. Microbial growth inhibition by up to 91.65% was obtained at temperature and inhibition time of 37 °C and 34.25 min, respectively. Table 11 indicates the predicted and experimental values of microbial growth inhibition. The optimum conditions were experimentally verified with 94.73% of microbial growth inhibition, corresponding to a percentage error of 1.73% to 8.03%. The error was acceptable as the error percentage was less than 10%. The microbial growth inhibition obtained from the best conditions of factorial analysis was 21.74%. Compared to optimum conditions obtained by optimization, the percentage of microbial inhibition was increased to 94.73%. These results show a better increment of microbial inhibition than the optimization process could achieve.

Table 10
Suggested optimum condition

Factors and responses	Values
A: Microbial Inhibition Time	34.25 min
B: Temperature	37 °C
CFU/mL	4.77×10^4 CFU/mL
Microbial growth inhibition (%)	91.65%

Table 11
Predicted and experimental microbial growth inhibition (%)

Runs	Microbial growth inhibition (%)		Error (%)
	Predicted	Experimental	
Run 1	91.65	90.06	1.73
Run 2	91.65	94.73	3.36
Run 3	91.65	84.29	8.03

CONCLUSION

This study focuses on understanding the effects of several factors involved in microbial inhibition growth by applying the microbial growth inhibitor (MGI). Based on the Full Factorial Design Analysis (FFD), microbial inhibition time, the concentration of TPC, and temperature were found to significantly contribute to the microbial growth inhibition process with contribution percentages of 1.07%, 1.27%, and 1.56%, respectively. The best condition for microbial inhibition of 21.74% was achieved at PLJ/M of 1:1, 0.5127 mg GAE/mL of TPC concentration, and at 37 °C for 0.5 h of microbial inhibition time. The major factors contributing to the inhibition of microbial growth were further optimized through a central composite design (CCD). The quadratic model involving temperature and microbial inhibition time was best fitted to predict the microbial growth inhibition process. The maximum microbial growth inhibition was 94.73%. The microbe was most effectively inhibited at an optimum temperature and inhibition time of 37 °C and 34.25 min. It can be concluded that the maximum microbial growth inhibition was increased from 21.74% to 94.73% using the optimization process. This study shows that PLJ can be an alternative natural MGI from plant sources with microbial growth inhibition properties.

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