Utilization of Central Composite Design in Optimization of Microbial Growth Inhibition using *Ananas Comosus* Leaves Juice

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Abstract. Microbial growth inhibitors have been very effective for the prevention of disease transmission and infection. For this study, pineapple (*Ananas comosus*) leaves juice (PLJ) was use as microbial growth inhibitor (MGI) because of its higher phenolic content. Microbe from contaminated pineapple leaves was use as tested microbe. The selected operating conditions in this study were temperature and microbial inhibition time with the range between 35 to 39 °C and 10 to 50 min respectively. Microbial growth inhibition experimental design set-up was constructed using Design Expert software (Version 7.1.6) to analyze the condition required. The experiments was conducted according to the central composite design (CCD) in two variables of the surface response methodology (RSM) to determine the optimum conditions for inhibiting microbial growth. Quadratic polynomial model was constructed to predict the microbial growth inhibition. A quadratic polynomial model was generated to predict microbial growth inhibition. Microbial growth inhibition was measured using the method of colony formation unit (CFU) counting. The RSM result showed that microbial inhibition time had a major impact on inhibition of microbial development. The optimum conditions were 37 °C and 34.25 min with a 94.73% microbial growth inhibition. In this condition, microbe growth was predicted to be at a minimum rate due to inhibition of the phenolic compound in PLJ. The optimum state of this study will be useful for the use of PLJ in pineapple plantation.

Keywords: pineapple leaves juice; phenolic compounds; microbial growth inhibitor; optimization; response surface methodology

1. Introduction

Microbial growth inhibitor is an agent that inhibits cell growth, or can be known as a static agent [1]. Nowadays, microbial growth control was required in many practical situations such as in agriculture, medicine and food science. Microbial growth inhibitors have been very effective in preventing the spread of infection and contamination by undesirable microorganism growth. Although some chemical agents have been commercially available, these chemicals can cause bad effect to humans, plants and animals. There was therefore an urgent need to look for natural agent as a microbial growth inhibitor (MGI). Pineapple leaves was selected consider that pineapple has a high phenolic content relative to other fruits [2, 3]. A study by Ti et al. [4] found the total phenolic content (TPC) of pineapple peels was 7.98 mg Gallic Acid Equivalent/g dry weight which also 148.91 mg Gallic Acid Equivalent /100 g of

fresh weight. Phenolic compounds and their antioxidant biomolecules have been subjects to research for many years that may inhibit the transmission of a pathogen. These antioxidants primarily derived from plants of phenolic compounds such as flavonoids, phenolic acids, ascorbic acids, isoflavones, flavonoses, quinones, stilbenes, carotenoids and tannins. In recent years, plant phenolics have developed significant interest in their possible effects on microorganisms. Seven phenolic compounds were identified from pineapple leaves, namely ananasate, 1-O-caffeoylglycerol, 1-O-p-coumaroylglycerol, caffeic acid, p-coumaric acid, β-sitosterol and daucosterol [6]. Caffeic acid has been significantly affected the growth of *Listeria monocytogenes*. Shimbe et al. [7] discovered the antimicrobial activity of β-sitosterol isolated from ethyl acetate root extract of *Hymenocardia acida.* Therefore, the use of pineapple leaves juice (PLJ) as a natural MGI may considered as a suitable alternative. In addition, pineapple leaves have been one of the abundant waste materials available in Malaysia and have not yet been used [8].

Analyzing the process may consume much time, cost, and energy since these factors may affect the process. It is therefore desirable to use the response surface methodology (RSM) for the design of experiments, the design of models, the evaluation of effect factors and optimal conditions for desirable responses [9]. According to Gao and Ju [10], the number of experimental points in the central composite design (CCD) was sufficient to assess the statistical viability of the models and the lack of fitness of the models. RSM can define the various interactions between different parameters with a minimum number of experiments. It was widely used for medium optimization [11]. RSM was a mixture of statistical and mathematical approaches to select the reliable experimental conditions in order to achieve the required results [12]. A study conducted by Ammer et al [13] successfully optimized the process variable using RSM in the CCD design of the antimicrobial activity of *Eucalyptus tereticornis* leaf extracts against *E. coli*. However, the study on microbial inhibition by process optimization using PLJ extract was never been reported. Thus, the use of PLJ optimization in the inhibition of microbial growth has become useful. Therefore, the present study aimed to determine the optimum conditions for the microbial growth inhibition.

2. Materials and methods

2.1. Chemicals and Reagents

All reagents and chemicals used were analytically graded and had high purity which are Potato Dextrose Agar (PDA) powder (<1mm; 99%), gallic acid (66.66 μm; 99%), methanol (99.8%), Folin-Ciocalteu reagent (99%) and sodium carbonate (Na_2CO_3, 99%).

2.2. Material and PLJ Extraction Preparation

Pineapple leaves and the tested microbe was obtained from a pineapple plantation in Pekan Pina, Pahang. The pineapple leaves juice (PLJ) was extracts using an electrical sugarcane pressing machine. PLJ was then autoclaved at 121 °C for 15 min.

2.3. Total Phenolic Content (TPC) Analysis

The total phenolic content (TPC) was analyzed using the Folin-Ciocalteu's reagent by using Gallic acid as the standard [14]. Initially, 10 mL of PLJ was centrifuged at 5000 rpm for 15 min. 0.5 mL of the supernatant was injected into test tubes and mixed with 2.5 mL of 10-fold diluted Folin-Ciocalteu reagent. Then, the prepared mixture was kept at normal temperature in a dark place for 5 min. Following, 2 mL of 7.5% Na_2CO_3 was added and allowed to rest for another 1 h. Lastly, the mixture was measured at 450 nm using UV-Vis Spectrophotometer. The TPC compositions were compared to Gallic acid standard curve. The concentration of gallic acid was prepared at 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1.0 mg/mL in 80% methanol solution and mixed with Folin-Ciocalteu reagent and 7.5% Na_2CO_3. Lastly, the TPC was presented as mg Gallic acid equivalent per gram of PLJ extract (mg GAE/mL).

2.4. Culture Medium

Potato Dextrose Agar (PDA) was pour in distilled water, stirred until the powder was completely dissolved prior to sterilize in the autoclave. Then the solution was left to be cooled and approximately 10 mL was used to pour into the plates. After the agar solidified, the plates were labelled and stored in chiller at 4 °C until further use.

2.5. Cultivation of the Microbe

Microbe on contaminated pineapple leaves was used as a tested microbe. The microbe was streaked on the agar surface in the petri plate using a sterile loop around the quadrant one before quadrant four. The plate was incubated at 37 °C for 24 h [15].

2.6. Experimental Set-up for Microbial Growth Inhibition

The experiment was started by re-cultured the microbe from section 2.5 into the new PDA plate. The re-cultivated microbe was scraped out from potato dextrose agar (PDA) plate and mix with nutrient broth to produce microbe broth (MB). The MB was agitated in the incubator shaker for 1 h at 37 °C and 100 rpm. The MB was mixed with PLJ at a ratio of 1:1 (20 mL of MB and 20 mL of PLJ). The mixture was placed in the incubator shaker at 100 rpm at the selected time and temperature, respectively. The steps were repeated based on the optimization design table (Table 2). Then colony count analysis was conducted to all samples run.

2.7. Colony Forming Unit (CFU) Analysis

The microbe and pineapple leaves juice (PLJ mixture from section 2.6 was spread on PDA evenly using a triangle stick. The plate was incubated at 37 °C for 24 h [16]. After 24 h, colony count was performed. The plate count was linear for microbe over the range of 30 to 300 CFU on a standard sized petri plate [17]. Microbial growth inhibition (%) was estimated in terms of CFU/mL and determined using equations (1) and (2) respectively.

$$\mathrm{CFU/mL} = \frac{\text{number of colonies x dilution factor}}{\text{volume of culture plate}} \quad (1)$$

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

2.8. Optimization Study

The experiment was conducted according to the central composite design (CCD) with two variables under response surface methodology (RSM) to determine the optimum conditions. Design Expert software [18] was used for model development and optimization. The selected factors and their degree were shown in Table 1. Experiments were performed in accordance with the experimental design (Table 2) referred to in Sections 2.6 and 2.7.

Table 1. Selected ranges value for optimization

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

Table 2. Experimental design table Set-up by Design Expert software.

Run	Factor A: Microbial inhibition time (min)	Factor B: Temperature (°C)
1	20.00	36.00
2	40.00	36.00
3	20.00	38.00
4	40.00	38.00
5	10.00	37.00
6	50.00	37.00
7	30.00	35.00
8	30.00	39.00
9	30.00	37.00
10	30.00	37.00
11	30.00	37.00
12	30.00	37.00
13	30.00	37.00

2.9. Data Analysis
Responses have been evaluated using Design Expert tools. Validation experiments were performed based on the optimum conditions.

2.10. Experimental Set-up for Effect of Culture Age on Microbial Growth Inhibition
The effect of culture age of the microbe towards microbial growth inhibition was studied. Culture age can be defined as the age of the microbe growing in culture medium. In this study, the experiment was conducted to observe the influence of the microbial culture age on the microbial growth inhibition process by PLJ. The experiment was started by re-culturing the microbe at two different times (24 h and 48 h). Then the experiment was conducted following the method mentioned in section 2.6 and 2.7 at optimum condition of inhibition time and temperature respectively.

3. Results and discussion

3.1. Optimization of PLJ
13 run experiments with different temperature and microbial inhibition time were conducted based on the CCD (Table 3). With the experimental data, a second-order polynomial equation was generated to fit the microbial growth inhibition. The models used to express microbial inhibition as a function of independent variables was shown in equation (3) and (4) respectively (in terms of coded and actual levels).

$$\frac{\text{CFU}}{\text{mL}}(\text{coded}) = 0.036 - 0.045x_1 - 0.014x_2 - 0.00028x_1x_2 + 0.053x_1^2 + 0.031x_2^2 \quad (3)$$

$$\frac{\text{CFU}}{\text{mL}}(\text{actual}) = 42.8203 - 0.0257x_1 - 2.2741x_2 - 0.000028x_1x_2 + 0.0000528x_1^2 + 0.03065x_2^2 \quad (4)$$

The fitting of data to different models and their subsequent analysis of variance (ANOVA) shows the CFU/mL was better represented by the quadratic polynomial model. The R^2 of the quadratic model (0.9108) was higher than other models. ANOVA was used to measure the fitting of the RSM equation and to check the model significance, each factor and the frequency of the interaction of each factor (Table 4). ANOVA result show the *p*-value of the model was 0.0015 with confidence level greater than

95%. The *p*-value model below 0.05 was important, indicate that the model was optimal for this experiment. The effect of temperature and the interaction of main effects in terms of CFU/mL were insignificant based on the values greater than 0.05, 0.2254 and 0.8822 respectively. Microbial inhibition time effect has an important effect on the inhibition of microbial development, explained by 0.0039 of *p*-value. The coefficient of determination (R^2) and the modified coefficient of determination (R^2adj) were 0.9108 and 0.8471, respectively, suggesting that the calculated model matches the experimental data in a satisfactory manner. Lee and Lemieux [19] proposed that R^2 should be at least 0.80 for a reasonable fit of the model.

Figure 1 shows the correlation of actual and predicted values in terms of CFU/mL. A linear distribution suggesting a well-fitting model was obtained. The predicted values were similar to the CFU values observed. The effect of two independent variables on the CFU/mL was shown in Figures 2a and 2b. The plotted data demonstrates the effects of microbial inhibition time and temperature on CFU/mL. CFU/mL decreased from 20 to 35 min with increasing microbial inhibition time as shown in Figure 2a. This result shows that increasing microbial inhibition time has a massive effect on CFU/mL. The phenol coefficient has potency against certain microbe after 10 min [20]. It could be the minimum time needed for phenolic compounds to be efficient in inhibiting the microbe. Meanwhile, when microbial inhibition time increased from 35 to 40 min, CFU/mL increased again. This was due to the lack of phenol, which made it not able to inhibit the microbe. However, Leite et al [21] stated that 2 to 3 h of microbial inhibition period was between citral and C. Albicans could kill 99.9% of the inoculum as well. The effect of temperatures was shown in Figure 2b. It shows that the optimal temperature of the microbial growth inhibition process is at 37 °C. The temperature had no effect on the CFU/mL, as the variation between the CFU/mL from 35 to 39 °C was smaller. This was confirmed by the *p*-value of 0.2254 derived from ANOVA. The effect of temperature on CFU/mL was insignificant. Thus, only a temperature of 37 °C was required for microbial growth inhibition, since the microbe from plants can be killed at this temperature [22].

The results of interaction between temperature and microbial inhibition time on CFU/mL were displayed by the contour plot and the three-dimensional response surface (Figures 3a and 3b). The exploration of interactions between factors by contour plots has helped to select variable ranges to meet the optimization objective [23]. Decreasing of CFU/mL shows the higher microbial growth inhibition. The microbial growth inhibition of 94.73% at 9.12×10^4 CFU/mL was obtained at optimum conditions of 37 °C and 34.25 min. At this stage, the minimum value of the CFU/mL implies the maximum microbial inhibition. A similar study by Segundo [24] found the use of bromelain extracted from pineapple stems inhibited 90% growth of *F. verticilioides*. It shows that these results reported were similar to the results of present study.

Table 3. Experimental data of RSM.

Run	Factor A: Microbial inhibition time (min)	Factor B: Temperature (°C)	Response 1: CFU/mL	Response 2: Microbial growth inhibition (%)
1	20.00	36.00	2.38E+05	58.26
2	40.00	36.00	2.07E+05	63.70
3	20.00	38.00	1.89E+05	66.88
4	40.00	38.00	1.37E+05	75.87
5	10.00	37.00	6.76E+05	18.43
6	50.00	37.00	2.33E+05	59.01
7	30.00	35.00	3.42E+05	40.08
8	30.00	39.00	2.49E+05	56.31

9	30.00	37.00	3.37E+04	94.09
10	30.00	37.00	3.39E+04	94.05
11	30.00	37.00	8.70E+04	84.74
12	30.00	37.00	8.78E+04	84.62
13	30.00	37.00	1.24E+05	78.20

Table 4. Response surface quadratic model of ANOVA

Source of variation	Sum of Squares	df	Mean Square	F Value	P-value Prob > F	
Model	0.097	5	0.019325	14.30	0.0015	significant
A-Microbial Inhibition Time	0.024	1	0.024147	17.87	0.0039	
B-Temperature	0.002389	1	0.002389	1.77	0.2254	
AB	3.19E-05	1	3.19E-05	0.024	0.8822	
A^2	0.064	1	0.06395	47.31	0.0002	
B^2	0.024	1	0.021532	15.93	0.0052	
Residual	0.009461	7	0.001352			
Lack of Fit	0.007561	3	0.00252	5.30	0.0704	not significant
Pure Error	0.001901	4	0.000475			
Cor Total	0.11	12				
R^2	0.9108					
Adjusted R^2	0.8471					

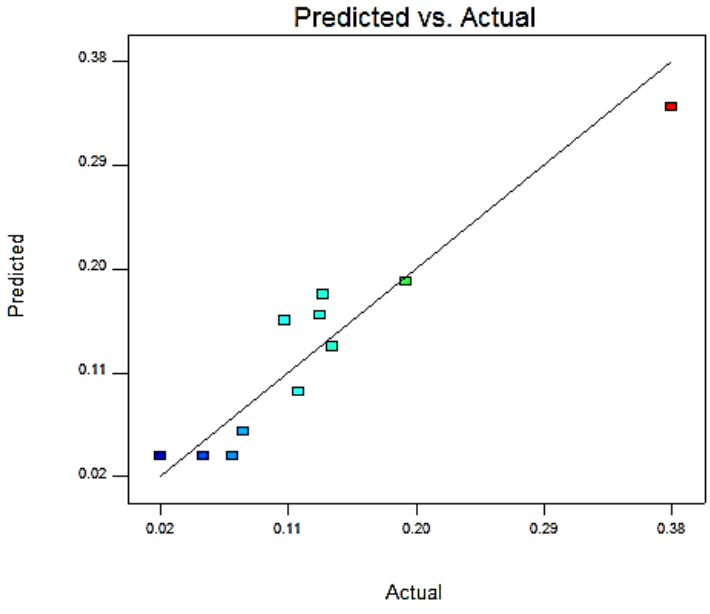
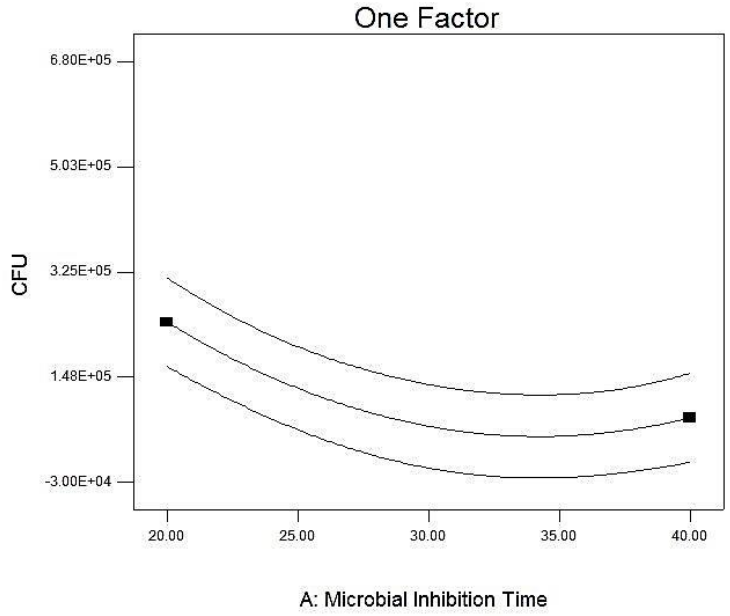
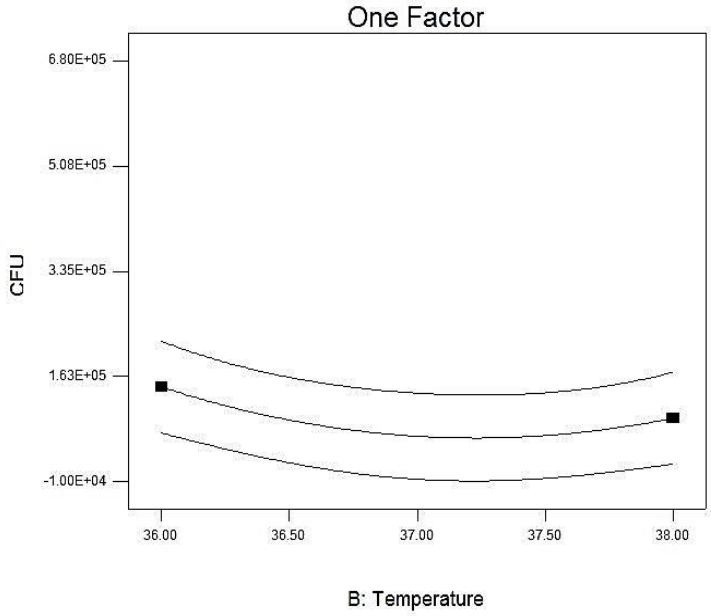


Figure 1. Correlation of actual and predicted values

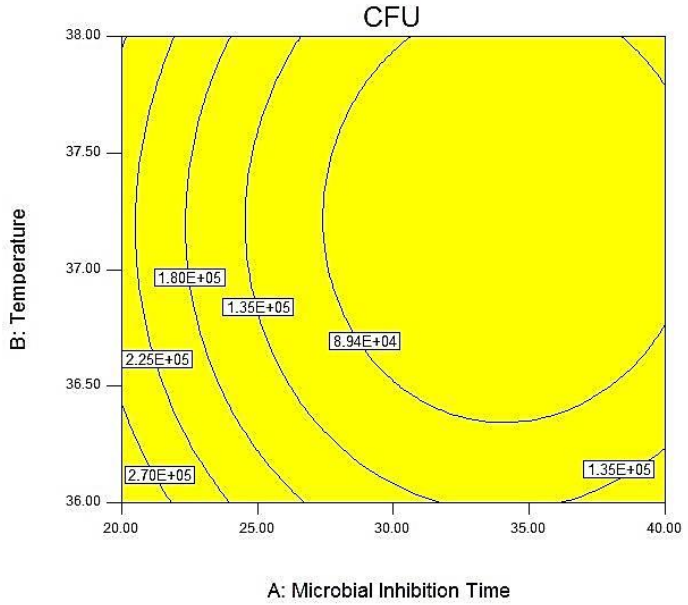


(a)

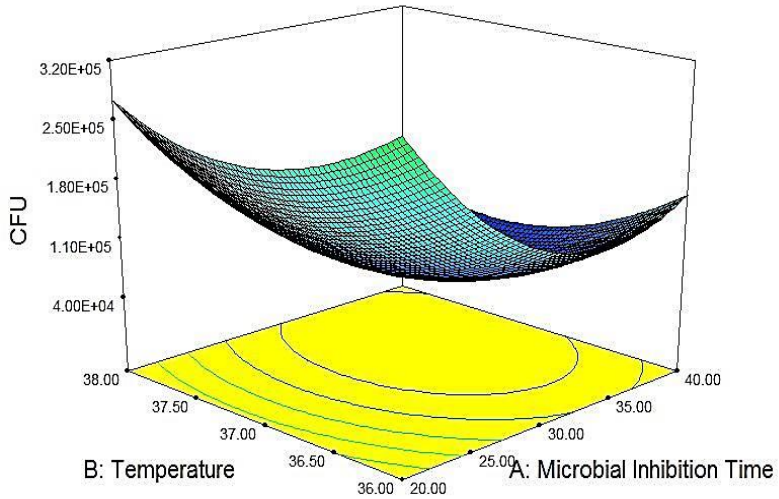


(b)

Figure 2. (a) Effect of microbial inhibition time, (b) Temperature on CFU/mL.



(a)



(b)

Figure 3. (a) Contour plot, (b) Three dimensional response surface plot of CFU/mL

3.2. Optimum Conditions

Table 5 shows the suggested optimum conditions generated from response surface methodology (RSM) analysis developed by the Design Expert software. It was reported that 91.65% microbial growth inhibition achieved was at 37 °C and 34.25 min microbial inhibition time. Table 6 shows the predicted and validation value of validation experiment. With three duplicates, the validation experiments carried out showed that the experimental values were relatively similar to the expected value. The highest experimental inhibition of microbial growth achieved was 94.73 %. The error was estimated using equation (5). The error reported ranged from 1.73% to 8.03%.

$$\text{Error (\%)} = \frac{\text{Experimental}-\text{Predicted}}{\text{Predicted}} \text{ x } 100 \quad (5)$$

Table 5. Suggested optimum conditions.

Factors	Value
A: Microbial inhibition time	34.25 min
B: Temperature	37°C
CFU	4.77E+04 CFU/mL
Microbial growth inhibition	91.65 %

Table 6. Predicted and validation experimental of microbial growth inhibition at optimum conditions.

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

3.3. Effect of Culture Age on Microbial Growth Inhibition

The effect of culture age of microbe was shown in Table 7 and Figure 4. It can be define as a method of multiplying microbial organisms by letting them reproduce in culture medium. The effect of culture age of microbe towards microbial growth was studied in order to achieve optimized operating conditions and maximum microbial growth inhibition by pineapple leaves juice (PLJ). Culture age is important to determine the production of cells as older culture cells reproduce at a higher rate than younger culture. Therefore, makes it less resilience and lack of inhibition. As the culture age of microbe increased, the microbial growth inhibition decreased. This trend shows that the younger culture age was better compared to the older culture age which indicates the higher microbial inhibition. Lee et al [26] studied the impact of fungal culture age on plant growth. The plant growth depends on VOCs produced by the filamentous fungi. The fungal culture age affects the production of the VOCs. Aging results in a thicker cell wall that makes the cells more resilient and vulnerable. The old cells lead to high treatment failure rate and loss of inhibition, indicating that treatment preferentially inhibits younger cells [27]. Older culture cells reproduce at a higher rate than younger culture. Therefore when the CFU increased which caused the phenolic compounds to be ineffective at a certain point. Hence, the microbial inhibition process of the older culture to take place was slower compared to the younger culture.

Table 7. Effect of culture age of microbe.

Factor A: Microbial inhibition time (min)	Factor B: Temperature (°C)	Incubation time of microbe (h)	Culture age of microbe (h)	Microbial growth inhibition (%)
				Average
34.25	37	24	24	89.69
34.25	37	24	48	63.08

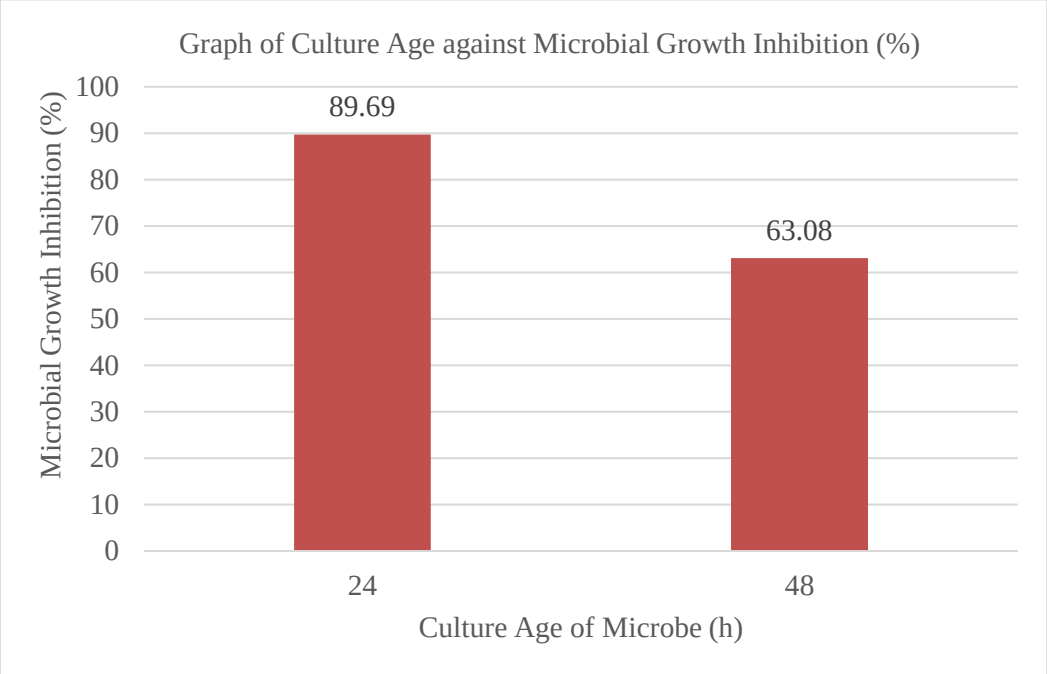


Figure 4. Culture age of microbe against microbial growth inhibition (%).

4. Conclusion

This research aimed to optimize the conditions of microbial growth inhibition by the use of pineapple (*Ananas comosus*) leaves juice. *P*-value from the ANOVA test indicates that the microbial inhibition time has a significant impact on the microbial growth inhibition. Response surface methodology (RSM) analysis was proven to be a useful way to visualize process parameter interaction. The maximum microbial growth inhibition achieved was 94.73% at optimum conditions of 37 °C and 34.25 min. The effect of culture age on microbial growth inhibition was studied. The younger culture age resulted in increasing of microbial growth inhibition. The result shows that pineapple leaves were potentially valuable material for microbial growth inhibitor because they were abundantly available after harvest, resulting in a better and greener effect on the environment.

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