Application of two level factorial design to study the microbe growth inhibition by pineapple leaves juice

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Abstract. The yield of Ananas comosus (pineapple) is susceptible to microbial infection and the usage of chemical pesticides to control it has been often associated with negative impacts to the environment and human health. As pineapple leaves is one of the lavish organic materials with antimicrobial properties due to its total phenolic content (TPC), its potential as a microbial inhibitor is explored in this study. The objective of this research is to study the factors that affect microbe growth inhibition using pineapple leaves juice (PLJ). The factors evaluated were; reaction time between mixtures of PLJ and microbe (0.5 – 5 hours), concentration of TPC in PLJ (0.2563 – 0.5127 mg GAE/ml), reaction temperature (26 – 37°C), and ratio of microbe to PLJ (M/PLJ) (1:1 and 1:3). A two level factorial design was adopted to assess the effect of the above mentioned factors on the microbial inhibition by PLJ. The results show that the most contributing factor of 1.55 % was reaction temperature, meanwhile the highest contribution factor for interaction effect was between concentration of TPC in PLJ and ratio of microbe to PLJ at 5.17%. The best condition for microbe growth inhibition of 20.90% was found to be at reaction time of 0.5 hour, TPC in PLJ of 0.5127 mg GAE/ml, reaction temperature of 37°C, and M/PLJ at 1:1. This study demonstrates that pineapple leaves could be exploited as valuable sources of natural products that could be used as microbial growth inhibitor and thus become one of the cheap and green alternatives for more expensive chemical pesticides.

1. Introduction
The pineapple fruit, scientifically called Ananas comosus is a cone-shaped juicy fruit with the crown at the top and is the leading edible member of the family Bromeliaceae [1]. According to the Asian Pineapple Market (2018), pineapple is extensively grown and export in Asia for example in Malaysia, Thailand, Philippines, China, India, and South Central America [2]. However, microbe-causing diseases have led to low quality pineapple fruits which are left wasted on farms [3]. International Tropical Fruits Network (2016) reported various microbe-causing diseases disturb the handling and storing process which leads to pineapple yield losses. Therefore, it is needed to come up with new substances and methods to overcome these losses [2].

A wide range of formulations that converts a chemical with anti-pathogenic properties have been used to manage the existing crop diseases [4]. However, it may offer hazardous problems to other living things due to its toxicity content. Pineapple leaves are believed to contain antimicrobial properties due to its total phenolic content (TPC). Pineapple leaves fibers has shown antimicrobial activity towards Escherichia coli and Staphylococcus aureus [5,6]. In other study, the pineapple leaves
extract showed nonspecific proteolytic, gelatinolytic, collagenase, fibrinolytic, acid and alkaline phosphatase, nuclease, peroxidase along with considerable anti-bacterial and anti-fungal activities [7]. The significant phenolic content found in pineapple leaves are phytosterol, beta-sitosterol, stigmasterola and campestrol which relates to high anti-oxidant activity [3]. These phenolic compounds are important to plant growth and development to provide a defensive mechanism against infection and injury [8]. Ribera & Zuniga, (2012) also reported that aromatic plants with phenolic compounds have gained interest in the field of plant disease control [9]. Thus, pineapple leaves are seen as potential valuable products since they are abundantly available after pineapple harvest, giving safer impact to the environment [10].

While one-factor-at-a-time (OFAT) approach involves only one variant at a time by making the other variables fixed, Two Level Factorial Analysis (TLFA) help investigators to study the effect of all factors by varying them simultaneously [11]. Bozkir and Saka (2005) also states that TLFA aids in studying the effects caused by independent variables and interactions between those variables [2]. Besides that, another advantage of TLFA over OFAT is that the estimates of the effects of each factor are more precise due to more observations used compared to OFAT which typically only use two of the observations[12]. Therefore, in the present study, TLFA approach is used.

Given the above, the aims of this research were to study the microbe growth inhibition using pineapple leaves juice (PLJ) and to analyse the factor that affects the microbe growth inhibition using TLFA.

2. Materials and Methods

2.1 Materials

Pineapple leaves (average length of 60 cm) and the studied microbe were collected from a pineapple plantation located at Pekan Pina, Pahang. Potato Dextrose Agar (PDA) powder (average particle size, <1mm; purity, 99%) was purchased from Sigma Aldrich Co. (Malaysia) to prepare the culture media. Gallic acid (average particle size, 66.66µm; purity, 99%), methanol (purity, 99.8%), Folin-Ciocalteu reagent (purity, 99%) and sodium carbonate (Na₂CO₃, 99%) used to prepare a Gallic Acid standard curve, were purchased from Sigma Aldrich Co. (Malaysia).

2.2 Mechanical extraction of PLJ

The uncut pineapple leaves were extracted 5 times using an electrical sugarcane press machine (Himitzu, Malaysia) to obtain the pineapple leaves juice (PLJ). The PLJ was then autoclaved in an autoclave machine (Hirayama, USA) at 121°C for 15 minutes. Approximately 60 mL of PLJ was obtained when 0.1 kg of pineapples leaves juice extracted.

2.3 Cultivation of the microbe

Microbe growing on the infected pineapple leaves at Pekan Pina pineapple plantation was collected as the tested microorganism. The microbe was streaked on the agar surface in the petri dish by swabbing it using a sterile loop across quadrant 1 until quadrant 4 before it was incubated at 37°C for 24 hours [14].

2.4 Total Phenolic Content (TPC) determination

The total phenolic content (TPC) determination was performed using the Folin-ciocalteu reagent, with gallic acid as the standard. 10mL of sample extracted was centrifuged at 5000 rpm for 15 minutes. 0.5 mL of the supernatant was then inserted into test tubes and mixed with 2.5 mL of 10-fold diluted Folin-ciocalteu reagent and allowed to react for 5 min. Two mL of 7.5% Na₂CO₃ was added and the mixture left to react for 1 hour at room temperature. The absorbance was measured at 450 nm using UV Visible Spectrophotometer (Varian Cary 50, Germany). The composition of TPC were compared to a gallic acid standard curve and expressed as mg GAE/mL [13].
2.5 Experimental setup for microbe growth inhibition
In order to produce a microbe broth, the cultured microbe was scraped from the PDA using an inoculation loop and mixed with nutrient broth before it was agitated in incubator shaker (INFORS HT, Ecotron, UK) for 1 hour at 37°C and 100 rpm. For 1:1 of M/PLJ, 20mL microbe broth was mixed with 20 mL of autoclaved PLJ. As for 1:3 of M/PLJ, 10 mL microbe broth was mixed with 30 mL of autoclaved PLJ. Meanwhile, for 0.5127 mg GAE/mL of TPC in PLJ, zero dilution factor is used, whereas for 0.2563 mg GAE/mL of TPC in PLJ, two-dilution factor is used from the original juice concentration. The steps were repeated based on the factorial design table provided by the Design Expert.

2.6 Cell dry weight analysis
The cell growth on the agar plate for each run was measured using dry weight measurement method. 200 µL mixture of autoclaved PLJ and microbe broth was spread onto the surface of new PDA and swabbed evenly by using triangle stick. The petri dish was incubated at 37°C for 24 hours. After 24 hours, the microbe grown on the agar was scraped out into a 10 mL nutrient broth in the centrifuge tubes. Prior to that, all empty centrifuge tubes need to be weighed. The mixture was centrifuged at 5000 rpm for 15 minutes to separate the microbe and the broth. The broth was discarded from the centrifuge tube and the centrifuge tube was kept in the oven overnight at 60°C. The dry centrifuge tube filled with microbe was weighed to determine the cell dry weight of microbe.

2.7 Factorial design
Design Expert software is used in the design of experiment where all factors will be randomized. By using 24 fractional factorial design, a total of 16 experimental runs were performed. Input factor levels were −1 (low level) and +1 (high level), which indicates the lowest range of factors and highest range of factors respectively. The analysis of factors was performed using Minitab 14 statistical software to identify the design factors that contribute significant effects and the statistical parameters in the experiment [14]. Experiment was conducted according to set-up in Design Expert software and the output was analyzed using ANOVA with p-value of 95% confidence level. Table 1 shows the designed factors and levels to be employed for the experiments.

| Table 1. Selected factors and their input level |
|-----------------------------------------------|
| Factors                                      | Level               |
| ---------------------------------------------|---------------------|
| Reaction time between mixtures of PLJ with microbe (hours) | 0.5 5               |
| Concentration of TPC in PLJ (mg GAE/mL)       | 0.5127 0.2563       |
| Reaction temperature (°C)                     | 26 37               |
| Ratio of microbe to PLJ (v/v)                 | 1:1 1:3             |

3. Results and Discussion

3.1 Factorial design screening on microbe growth inhibition
Design Expert software was used to design the experimental setup where all the factors were randomized. The degree of effect that PLJ has on the microbes was determined using the response of all the factors. The interactions between independent factors were determined using analysis of variance (ANOVA) and the main effects of microbe cell dry weight were identified based on the P-
value with confidence level greater than 95%. The microbe growth inhibition (%) was ranged from −66.33% to 21.25%. Table 2 shows that the reaction temperature between PLJ and microbe (Factor C) has the most contribution of 1.55% towards microbe growth inhibition, followed by concentration of TPC in PLJ (Factor B), reaction time between PLJ with microbe (Factor A) and M/PLJ (Factor D) with 1.26%, 1.08% and 0.29% contribution respectively. The lowest value of −66.33% of microbe growth inhibition was obtained at 1:3 of M/PLJ, 0.5127mg GAE/mL at 26°C for 0.5 hour; the highest value of 21.25% of microbe growth inhibition was obtained at 1:1 of M/PLJ, 0.5127mg GAE/mL at 37°C for 0.5 hour. Based on the results revealed by the Design Expert software, it was observed that the PLJ was unable to kill but can only inhibit the microbes’ growth. This is probably due to different phenolic compounds which have different efficiencies in retarding or inhibiting the growth of the tested microbe [15].

Table 2. ANOVA analysis on microbe growth inhibition

| Source  | Squares | DF  | Square | F Value | P-value Prob > F | % Contribution |
|---------|---------|-----|--------|---------|-----------------|----------------|
| Model   | 0.018   | 11  | 0.001633| 677.66  | <0.0001         | significant    |
| A-Reaction time | 0.000194 | 1   | 0.000194| 80.48   | 0.0009          | 1.08           |
| B-Concentration | 0.000227 | 1   | 0.000227| 94.32   | 0.0006          | 1.26           |
| C-Temperature | 0.000278 | 1   | 0.000278| 115.41  | 0.0004          | 1.55           |
| D-Ratio  | 5.29E-05| 1   | 5.29E-05| 21.97   | 0.0094          | 0.29           |
| AB       | 0.000884| 1   | 0.000884| 366.72  | <0.0001         | 4.92           |
| AD       | 0.000766| 1   | 0.000766| 317.89  | <0.0001         | 4.26           |
| BC       | 0.000672| 1   | 0.000672| 278.95  | <0.0001         | 3.74           |
| BD       | 0.000929| 1   | 0.000929| 385.46  | <0.0001         | 5.17           |
| ABD      | 0.013139| 1   | 0.013000| 5453.24 | <0.0001         | 73.12          |
| BCD      | 0.000105| 1   | 0.000105| 43.39   | 0.0028          | 0.58           |
| ABCD     | 0.000714| 1   | 0.000714| 296.44  | <0.0001         | 3.97           |
| Residual | 9.64E-06| 4   | 2.41E-06|         |                 |                |
| Cor Total| 0.01797 | 15  |        |         | 0.9995          |                |
| R²       | 0.9995  |     |        |         | 0.9980          |                |

3.2 Analysis of variance (ANOVA) for microbe growth inhibition

The interactions between independent factors were determined using analysis of variance (ANOVA) with confidence level greater than 95% as shown in Table 2. From ANOVA analysis, it was known that Factor A, B, C, D, AB, AD, BC, BD, ABD, BCD, and ABCD are significant model terms due to their “Prob>F” of less than 0.05. This model is accepted since the coefficient of determination (R²) obtained is 0.9995 and according to Saunders, Russell and Crab (2012), an R² of above 0.9 represents a perfect data fit [16]. The modified linear regression equation in (1) shown was used to develop the relationship of cell dry weight with the four factors.

\[
Y = 0.15 - 0.0034814 A - 0.003769 B - 0.004169 C - 0.001819 D + 0.007431 AB - 0.006919 AD
- 0.006481 BCD + 0.007619 BD - 0.029 ABD + 0.002556 BCD + 0.00681 ABCD
\] (1)
Where $Y$ is the predicted response (cell dry weight of microbe), $A$ represents reaction time between mixtures of PLJ with microbe (hour), $B$ represents concentration of TPC in PLJ (mg GAE/mL), $C$ represents reaction temperature ($^\circ$C), and $D$ represents M/PLJ (v/v). The effects included in the equation could not be ignored from the model since they are significant at a 5% of probability level.

### 3.3 Analysis of main effect and interaction effect between factors on microbe growth inhibition

From Figure 1, there are three main factors that affect the microbes’ cell dry weight which measures the microbe growth inhibition. Negative effect is desired to show that the factor contributes to microbe growth inhibition as increasing the factor value will lower the response value. In this case, the response value is microbes’ cell dry weight. Factor $C$ (reaction temperature) shows the highest negative effect, followed by Factor $B$ (concentration of TPC in PLJ) and Factor $A$ (reaction time between PLJ with microbe). It simply means that increasing the temperature, concentration and reaction time will decrease the cell microbes’ cell dry weight which means higher inhibition of microbe growth.

![Pareto Chart](image)

**Figure 1.** Pareto Chart for factorial analysis on cell dry weight of microbe.

Meanwhile, for the interaction effects, Factor $AD$ (reaction time and M/PLJ) followed by Factor $BC$ (concentration of TPC in PLJ and reaction temperature) give high negative effects. Increasing both of each interaction factor will contribute to higher microbe growth inhibition. The effect of two most significant independent variables are shown in Figure 2. From Figure 2(a), when the reaction temperature increased from 26°C to 37°C, the cell dry weight of microbe is slightly minimized, as higher temperature reduces the biofilm thickness which no longer protects the microbe [17]. Meanwhile, Figure 2(b) shows that the cell dry weight of microbe decreases when TPC concentration increases from 0.256 mg GAE/mL to 0.513 mg GAE/mL. This might be due to the fact that higher TPC concentration contains higher antioxidants which scavenges free radicals and results in higher inhibitory effect [18].
3.4 Validation experiment

The validation experiment was done by determining the error of predicted results and actual microbe growth inhibition based on the suggested best condition: 0.5 hour reaction time (A), 0.5127 mg GAE/mL concentration of TPC in PLJ (B), 37°C reaction temperature (C), and 1:1 ratio of M/PLJ (D). The best condition provided by Design Expert Software is obtained by adjusting the setting of optimization criteria. The conditions were selected out of many possible solutions for 2 combinations of categorical factor levels under numerical optimization [16]. By using these suggested best conditions, 3 runs of experiment were done. The experimental error calculated were less than 10% as shown in Table 3.
Table 3. Validation experiment data

| Run | Microbe growth inhibition (%) | Error (%) |
|-----|-------------------------------|-----------|
|     | Predicted | Actual |               |
| 1   |           | 19.29  | 7.70         |
| 2   |           | 20.90  | 4.02         |
| 3   |           | 19.19  | 8.18         |

4. Conclusion

In conclusion, Design Expert Software is an efficient tool in analysing the most contributing factors for microbial inhibition by PLJ. The present study uses Full Factorial Design (FFD) which aids in studying the main and interaction effects between the factors. The results revealed that the main effects were concentration of TPC in PLJ and reaction temperature. On the other hand, the significant interaction effects were reaction time of PLJ and microbe with M/PLJ (Factor AD), and concentration of TPC in PLJ with reaction temperature (Factor BC). The best condition for microbe inhibition of 20.90% was achieved at M/PLJ of 1:1, 0.5127mg GAE/mL of TPC content, at 37°C for 0.5 hour of reaction time. Based on these results, it shows that PLJ has high potential as microbe growth inhibitor due to the presence of phenolic compound as its defending mechanism. However, the limitation of this pineapple leaves juice is that it must be applied to the plant surface repeatedly as new tissues are growing when the plant grows. Further study needs to be done to produce PLJ that can inhibit the microbe growth in a long period of time or killing them at once without repeated application. In order to create significantly better models, Central Composite Design will further help to provide in-depth information about a few variables identified during this FFD process with a smaller number of experiments, as having the largest effect on the microbe growth inhibition. The results of this study highlight the potential of PLJ as a raw material for development of value-added products with antimicrobial properties for application in agricultural sector.

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