Effect of garlic solution to *Bacillus* sp. removal

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**Abstract.** Biofilm is a microbial derived sessile community characterized by cells that are irreversibly attached to a substratum or interface to each other, embedded in a matrix of extracellular polymeric substances that they have produced. *Bacillus* sp. was used as biofilm model in this study. The purpose of this study is to determine the effect of Garlic solution in term of ratio of water and Garlic solution (W/G) and ratio of Garlic solution to *Bacillus* sp. (GS/B) on *Bacillus* sp removal. Garlic solution was used to remove *Bacillus* sp. In this study, Garlic solution was prepared by crushing the garlic and mixed it with water. The Garlic solution was added into *Bacillus* sp. mixture and mixed well. The mixture then was spread on nutrient agar. The *Bacillus* sp. weight on agar plate was measured by using dry weight measurement method. In this study, initially Garlic solution volume and Garlic solution concentration were studied using one factor at time (OFAT). Later two-level-factorial analysis was done to determine the most contributing factor in *Bacillus* sp. removal. Design Expert software (Version 7) was used to construct experimental table where all the factors were randomized. *Bacillus* sp removal was ranging between 42.13% to 99.6%. The analysis of the results showed that at W/G of 1:1, *Bacillus* sp. removal increased when more Garlic solution was added to *Bacillus* sp. Effect of Garlic solution to *Bacillus* sp. will be understood which in turn may be beneficial for the industrial purpose.

1. **Introduction**

*Bacillus* sp. have been long used as biofilm model to investigate biofilm [1]. Garlic solution can inhibit much more strongly towards bacterial strains compared to antibiotics [2, 3]. [4] found that the garlic extract inhibited the growth and killed most of the organisms tested. [3] believed that garlic can inhibit *Bacillus subtilis*, *Escherichia coli*, and *Saccharomyces cerevisiae*. Therefore, garlic is used to remove *Bacillus* sp. The concentration of garlic (biocide) also can be defined as the ratio of water and garlic to remove microbes. Concentration of biocide is one of important aspects that should be considered when it is used to remove microbes as it affects the efficacy [4] concentration of biocide usually used as they can remove microbes greater and rapidly compare than low concentration of biocide. The activity of inhibitory natural biocides is directly proportional to the concentration of natural biocide [5]. According to [6], as the concentration of the garlic extraction increase, the diameter of inhibition of microbes increase too. As the garlic solution concentration increases, the inhibitory effect increases. In other words, the inhibitory effect of the extract is proportional to its concentration [5]. As the ratio of biocides extract increases to microbes, it can inhibit the microbes higher [7]. [8] reported that with garlic extract, an initial lag phase of 30 to 60 min occurred at concentrations of 0.34 to 2.75 mg/ml. Lag phases may reflect delay in uptake of garlic molecules...
and/or of toxic effect upon metabolic processes. However, at the higher concentrations used, a potential for rapid bactericidal action of garlic extract was indicated by complete loss of viability during the first 2 h. The objectives is to study the effect of garlic solution to Bacillus sp. removal. One factor at time (OFAT) analysis and response surface method (RSM) were used to evaluate the significance of W/G and GS/B values. Factorial analysis via two level factorial is used to allow chemist to study the effect of each factor on response and study the correlation between the factors [9].

2. Materials and Method

2.1. Garlic solution preparation
Garlic was obtained from a grocery shop in Gambang. The garlic sample was peeled, washed and prepared into two samples with different ratio of water to garlic (W/G).

2.2. Bacillus sp. preparation
Bacillus sp. from the previous study has been used in this research [10]. Bacillus sp. was streaked on the surface of the nutrient agar in the petri dish by swabbing it across quadrant number 1 by using sterile inoculation loop. It was repeated until quadrant number 4 before incubating it at 37°C for 24 hours [11].

2.3 Experimental setup for Bacillus sp. removal
Bacillus sp. was mixed with nutrient broth and agitated in incubator shaker for 1 hour at 37°C and 100 rpm. Then the Bacillus sp. broth was mixed with garlic solution. Then the mixture was spread on nutrient agar. The mixture was incubated in the incubator shaker at 37°C for 12 hours. Finally the Bacillus removal was calculated [12].

2.4 Experimental set-up for one factor at time (OFAT)

2.4.1 Effect of garlic solution volume on Bacillus sp.
One colony of Bacillus sp. was mixed with 10 mL of broth and was shaken for 1 hour at 37°C. The Bacillus broth was mixed with 1 mL of garlic solution and slightly shaken before incubate it. The initial OD of mixture was analysed and recorded. The mixture was then incubated at 37°C and the OD of the mixture after 4 hours. The steps were repeated by using 3 mL of garlic solution.

2.4.2 Effect of garlic solution on the growth of Bacillus sp.
Five samples of garlic solution were prepared according to their ratio as shown in Table 1. Half plate of Bacillus sp. was mixed with 50 mL of broth and was shaken in incubator shaker at 37°C for 1 hour. 1 mL of 1:1 of garlic solution sample was spread onto the agar plate by using sterile inoculation loop. Then 1 mL of sample that have been agitate before also was spread on the same plate. The agar plate was incubated at 37°C for 24 hours.

Table 1. Volume of water and garlic solution needed for each ratio.

| Ratio of water to garlic | Volume of water (mL) | Volume of garlic solution (mL) |
|-------------------------|----------------------|-------------------------------|
| 1:1                     | 25                   | 25                            |
| 1:2                     | 25                   | 50                            |
| 1:3                     | 25                   | 75                            |
| 1:4                     | 25                   | 100                           |
| 1:5                     | 25                   | 125                           |

2.5 Experimental setup for Response Surface Method (RSM) analysis
Design Expert software (Version 7.1.3, Stat-Ease, Inc., Minneapolis, MN) program was used in
RSM analysis. The experimental table was constructed in two level factorial of response surface methodology (RSM). Results from OFAT were utilized to determine the range for RSM analysis. Four selected factors were: ratio of water to garlic (W/G), ratio of garlic solution to Bacillus sp. (GS/B), agitation speed and reaction time between Bacillus sp. and garlic. These factors were studied to determine their effects on the Bacillus sp. removal by using a 2^4 factorial design. Table 2 shows the factors and its levels where low level indicates the lowest range of the factors and high level indicates the highest range of the factors. Experimental design table was constructed by using the Design Expert Software V7 and experimental data was analyzed using the same software. For 1:1 of W/G, the garlic solution was prepared by blending 25 mL of garlic and 25 mL of distilled water. Meanwhile, for 1:5 of W/G, the garlic solution was prepared by blending 125 mL of garlic and 25 mL of water. For 1:1 of GS/B, 10 mL Bacillus broth was mixed with 10 mL of garlic solution. On the other hand, for 1:3 of GS/B, 30 mL Bacillus broth was mixed with 10 mL of garlic solution.

|                | Low level (-1) | High level (+1) |
|----------------|----------------|-----------------|
| Ratio of water and garlic solution | 1:1            | 1:5             |
| Ratio of garlic solution to Bacillus sp. | 1:1    | 1:3             |
| Reaction time between garlic solution and Bacillus sp. (hours) | 12 | 24 |
| Agitation speed (rpm) | 50 | 100 |

2.6. Analysis of Bacillus sp. weight
The weight of Bacillus sp. on the agar plate was measured by using dry weight measurement method to determine the amount of Bacillus sp. for each run. First, by using sterile inoculation loop, 1µL of the sample that has been shaken before was spread onto the agar in petri dish. The petri dish was incubated in the incubator at 37°C for 24 hours. After 24 hours, all the Bacillus sp. growth on the agar was scraped out into the 10 mL broth in the centrifuge tube. It should be noted that all the empty centrifuge tube need to be weighed before proceeding to the next step. Then, the mixture was centrifuged at 5000 rpm for 15 minutes to separate Bacillus sp. and the broth. The broth was discarded from the centrifuge tube and the centrifuge tube was placed at 100°C. The dry centrifuge tube was weighed to determine the weight of Bacillus sp.

3. Results and Discussion

3.1 Effect of garlic solution volume to Bacillus sp.
This study was conducted to determine the effect of different garlic solution (GS) volume towards Bacillus sp. For effect of GS volume to Bacillus sp., the result was analysed by OD analysis for each sample. Figure 1 shows the result of by adding different volume of garlic had effect on Bacillus sp. growth after 4 hours. There was a small reduction of Bacillus sp. growth when there is no garlic added into the sample. However, huge reduction of Bacillus sp. growth can be seen when 3 mL of garlic added into the sample which is from 3.0000 to 0.2257 followed by 1 mL garlic added with value 0.7620 to 0. This is because when the GS volume was increasing, it reacts with Bacillus sp. stronger since it contains more garlic. [13] mentioned that when optical density reduced, this is because the cells had become less motile. OD initial for 3 mL of garlic added is the highest because of the concentration of GS itself is high and cause the OD reading high.
3.2 Effect of garlic solution on the growth of Bacillus sp.

This preliminary study was carried out to study the effect of water to garlic ratio (W/G) on Bacillus sp. growth. The value to study the W/G were 1:1, 1:2, 1:3, 1:4, and 1:5. 1:1 was set as low level of WG, meanwhile 1:5 was set as high level. Figure 2 shows the effect of W/G on Bacillus sp. weight. From the figure, it shows that when W/G increases, the Bacillus sp. weight decreases. As the level of WG increasing, the concentration of GS increased. It means that the Bacillus sp. weight decreases as the GS concentration increases. The data follow the result from [6] in which Bacillus sp. weight decreased as GS concentration increased. Since allicin compound in garlic can inhibit the growth of Bacillus sp., as the GS increased, the growth of Bacillus sp. decreased. Figure 3 and 4 showed the different of Bacillus sp. growth on different W/G.

Figure 1. Data analysis of OD on Bacillus sp.

Figure 2. The effect of W/G on weight of Bacillus sp.
3.3 RSM analysis
Two level factorial of RSM analysis was used to analyze the process factor by correlating the interaction between input and output variable (equation 1). It also used to provide a clear understanding of the interactions between all selected factors and Bacillus sp. removal. In OFAT analysis, Bacillus sp. removal was increased when garlic solution and WG increased.

\[
\% \text{ Bacillus sp. removal} = 86.74 + 4.31 \times A - 6.32 \times B + 5.64 \times C - 0.21 \times D + 5.90 \times AB + 1.19 \times AC + 1.56 \times AD + 2.66 \times BC + 3.43 \times CD - 6.46 \times ACD + 6.12 \times BCD
\] (1)

where
A-ratio of water to garlic
B-ratio garlic to Bacillus
C-agitation speed
D-reaction time between Bacillus and garlic

Bacillus sp. removal is strongly affected by allicin in garlic due to antimicrobial properties of the allicin. According to the software the suggested best condition for Bacillus sp. removal is as mentioned in Table 3 with the predicted Bacillus sp removal at 80.65%. However, from the experiment, the Bacillus sp. removal was higher than that the predicted (88.46%).

Figure 5 shows the effect of ratio of garlic solution to Bacillus sp (GS/B) on Bacillus sp. removal.
Based on Table 2, for GS/B, 1:1 was set as low level and 1:3 was set as high level. Negative effect is when the factor is not proportional to the response value. From Figure 5, GS/B gave negative effects towards the respond. Therefore, when the value of GS/B is increasing, the Bacillus sp. removal is decreasing. GS/B was increased by increasing the Bacillus sp. concentration. To get higher Bacillus sp. removal, the concentration of Bacillus sp. should be lower in which GS/B should be on low level. Bacillus sp. removal was higher when GS/B at 1:1 with value 97.2%, meanwhile, it reduced to 79.7% when GS/B was at 1:3. This is because when the concentration of Bacillus sp. was higher than the allicin concentration in garlic, the garlic cannot react or inhibit Bacillus sp. since the amount of GS was insufficient to kill Bacillus sp. In order to inhibit Bacillus sp., the allicin concentration in garlic should be same or higher than the Bacillus sp. concentration [5].

Figure 7 shows the interaction between (W/G) and (GS/B). For Factor A and B, 1:1 was considered as low level, and, 1:5 and 1:3 respectively were considered as high level. The Bacillus sp. removal was highest when GS/B at 1:1 and W/G at 1:1 with value 97.2%. On the other hand, the Bacillus sp. removal was lowest when GS/B and W/G at 1:1 and 1:5 respectively with value 78%. However, the Bacillus sp. removal did not really affected by W/G factor when the GS/B was at high value (1:3). The concentration of garlic was increasing when W/G was increasing. The Bacillus sp. removal was proportional to the concentration of antimicrobial agent (Figure 6). The activity of inhibitory natural biocides is directly proportional to the concentration of natural biocide [5].

Table 3. Data from validation experiment.

| Factor 1 | Factor 2 | Factor 3 | Factor 4 | Predicted Bacillus sp. removal (%) | Experiment Bacillus sp. removal (%) |
|----------|----------|----------|----------|-----------------------------------|-------------------------------------|
| A: ratio water to garlic | B: ratio garlic solution to bacillus | C: agitation speed (rpm) | D: reaction time (hours) | | |
| 1:1 | 1:3 | 50 | 12 | 80.65 | 88.46 |

Figure 5. One factor plot of the interaction between GS/B on Bacillus sp removal.
Figure 6. One factor plot of the interaction between W/G on Bacillus sp removal.

Figure 7. Analysis of interaction effects (Factor AB) on Bacillus sp. removal.
4. Conclusion
The purpose of this study is to determine the effect of garlic solution to Bacillus sp removal. The effect of garlic solution was studied in term of ratio of water and garlic solution (W/G) and ratio of garlic solution to Bacillus sp. (GS/B). Garlic content could increased the Bacillus sp. removal. Bacillus sp removal is strongly affected by allicin in garlic due to antimicrobial properties of the allicin. The concentration of garlic was increasing when W/G was increasing. The Bacillus sp. removal was proportional to the concentration of antimicrobial agent. However, GS/B gave negative effects towards the response where the value of GS/B is increasing when the Bacillus sp. removal is decreasing. The maximum FA yield predicted by RSM was 80.65% with best condition at W/G at 1:1, GS/B at 1:3, agitation speed at 50 rpm and reaction time at 12 hours. However, the real value from experimental shows higher yields of 88.46% with the best condition. Results from OFAT and RSM do agreed with one another. Results from this study shows the potential of garlic solution application for Bacillus sp. removal. Garlic solution can replace other commercial biocides especially in food industry.

Acknowledgement
The author wish to acknowledge the Universiti Malaysia Pahang for funding the project under grant RDU170346.

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