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Effect of garlic solution to *Bacillus* sp. removal

N Zainol and S R Rahim

Faculty of Chemical and Natural Resources Engineering, Universiti Malaysia Pahang, Lebuhraya Tun Razak, 26300 Kuantan, Pahang, Malaysia

E-mail: azwina@ump.edu.my

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. Bacilus 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 concentation 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

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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 then was 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 it 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.

Table 2. The levels of selected factors.

Factors	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 37ºC 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 100ºC. 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.

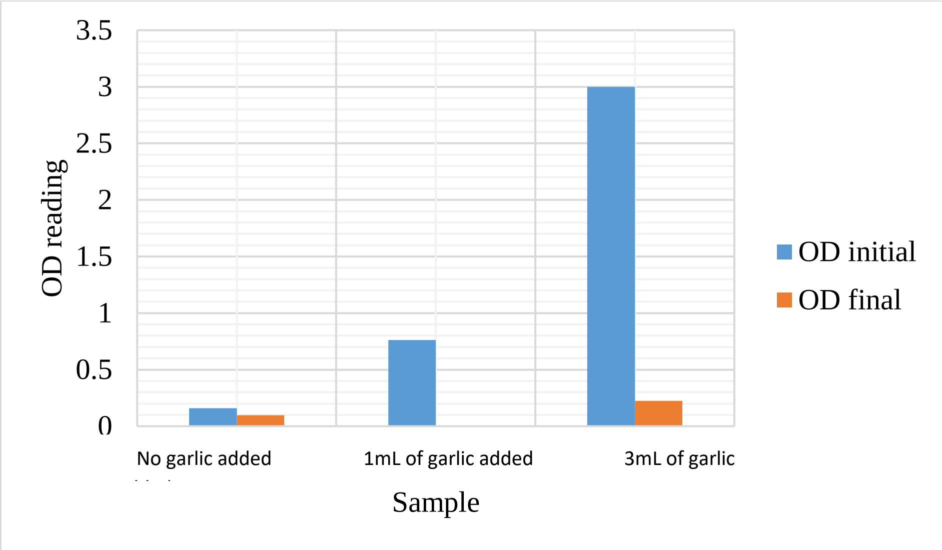

Figure 1. Data analysis of OD on *Bacillus* sp.

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. growth 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.

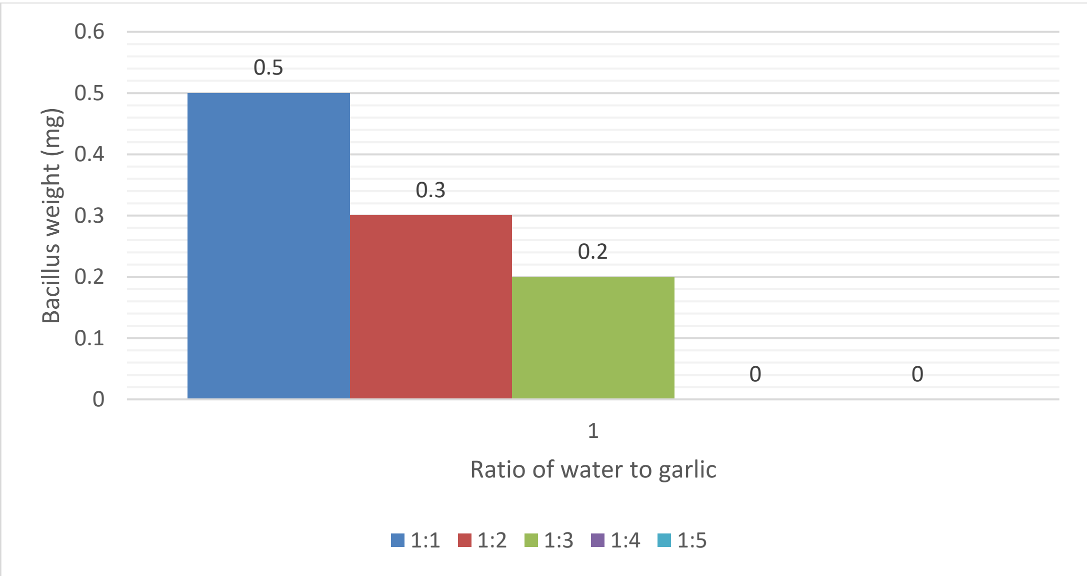

Figure 2. The effect of W/G on weight of *Bacillus* sp.

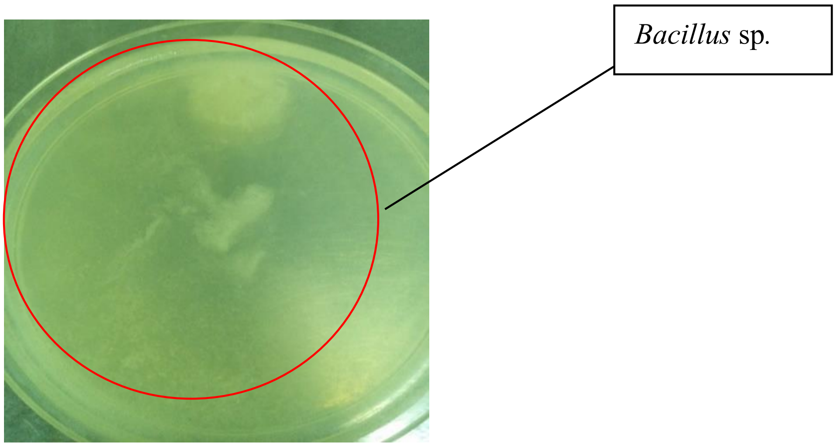

Figure 3. *Bacillus* sp. growth for W/G at 1:1

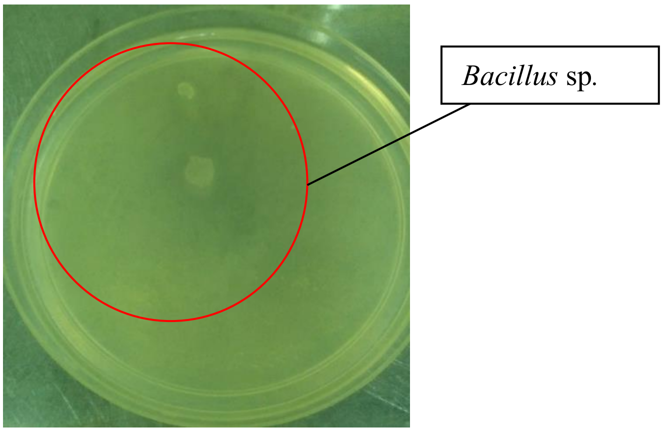

Figure 4. *Bacillus* sp. growth for W/G at 1:2

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.

% *Bacillus* sp removal = 86.74+4.31*A - 6.32 * B +5.64 * C - 0.21 * D +5.90 * AB +1.19 *AC+1.56* AD +2.66 * BC + 3.43 * CD - 6.46 * ACD + 6.12 * 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 A: ratio water to garlic	Factor 2 B: ratio garlic solution to *bacillus*	Factor 3 C: agitation speed (rpm)	Factor 4 D: reaction time (hours)	Predicted *Bacillus* sp. removal (%)	Experiment *Bacillus* sp. removal (%)
1:1	1:3	50	12	80.65	88.46

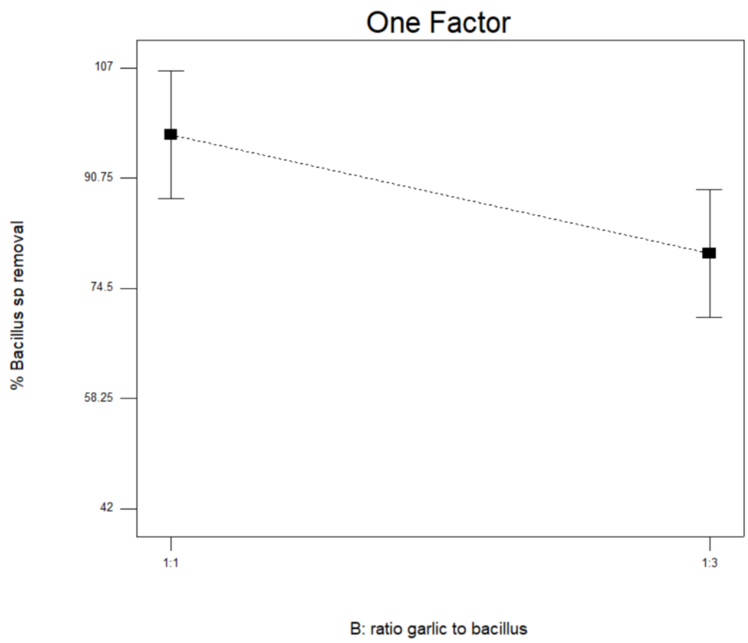

Figure 5. One factor plot of the interaction between GS/B on *Bacillus* sp removal.

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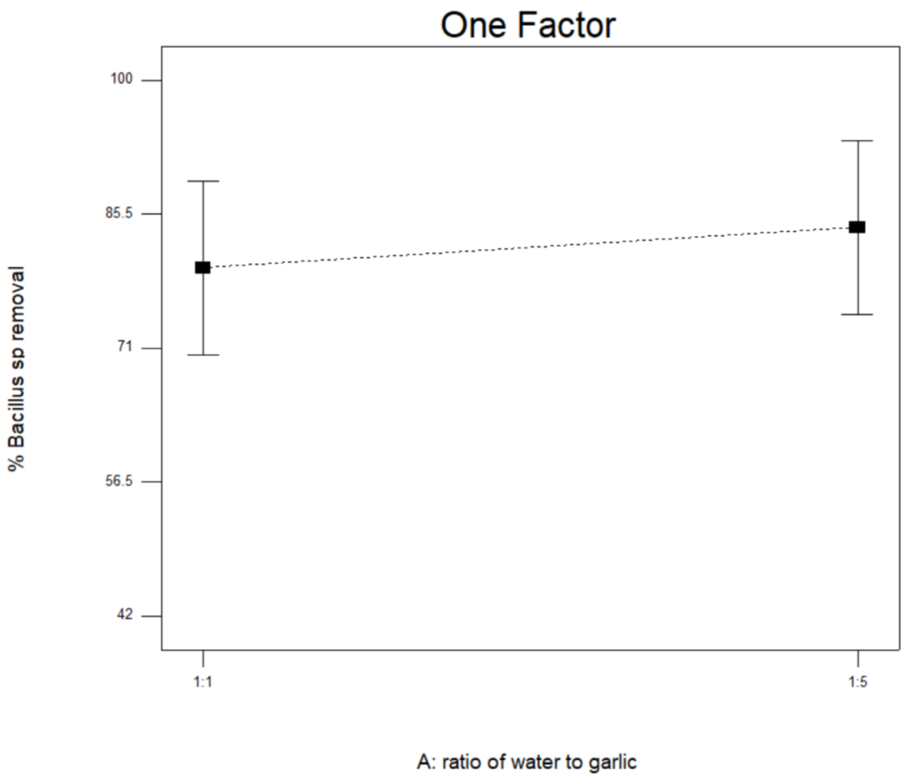

Figure 6. One factor plot of the interaction between W/G on Bacillus sp removal.

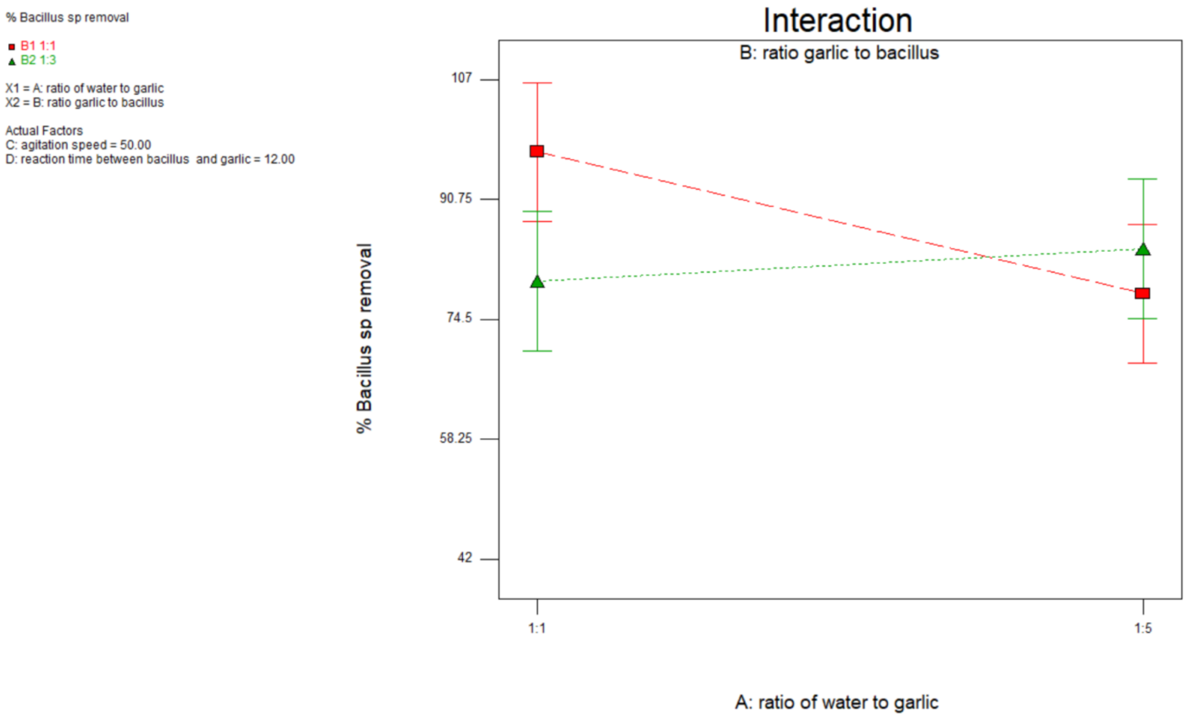

Figure 7. Analysis of interaction effects (Factor AB) on *Bacillus* sp. removal.

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.

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