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FACTORIAL ANALYSIS ON *BACILLUS* SP. REMOVAL USING GARLIC SOLUTION

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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. Many different microbes can be found in biofilm such as *Bacillus* sp., *Listeria monocytogenes*, *Staphylococcus* sp., *Escherichia coli* and many more. *Bacillus* sp. was used as biofilm model in this study. The purpose of this study is to determine the most affecting factors in *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. *Bacillus* sp. was mixed with nutrient broth before it was incubated. Then, 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. The selected factors were ratio of water to garlic (W/G) (1:1 and 1:5), ratio of garlic solution to *Bacillus* sp. (GS/B) (1:1 and 1:3), agitation speed (50 – 100 rpm) and reaction time between *Bacillus* sp. and garlic (12 – 24 hours). Design Expert software (Version 7) was used to construct experimental table where all the factors were randomized. The most contributing factor and interaction between the factors was analyzed via two level factorial analysis by using the same software. The results showed that the most significant factors were GS/B and agitation speed, while for the interaction effect was between W/G and GS/B. The *Bacillus* sp. removal best condition was determined at W/G at 1:1, GS/B at 1:3, agitation speed at 50 rpm and reaction time at 12 hours. The result of the best condition was 88.46% of *Bacillus* sp. removal.

Keywords: Garlic; *Bacillus* sp.; Two Level Factorial Analysis (TLFA); antimicrobial

1.0 INTRODUCTION

Costerton *et al.*, (2005) said that 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. Biofilm is removed because it may affect the substances in food and pharmaceutical industry by changing the reaction in the substances and can cause disease directly to human by ingestion (Mortensen, 2014; Swichtenberg, 2007). Biofilms may cause infectious disease and device-related infections. Biofilm can grow on medical devices, living tissues, piping, or natural aquatic system. *Bacillus* sp. was used because they have been long used as biofilm model to investigate biofilm (Vlamakis *et al.,* 2013).

Because of safety issue towards chemical usage, at present, people prefer products that have less chemicals and minimum amount of process (Kerekes *et al.,* 2015). In food industry, the number of usable chemical that can be used is limited and their usage can cause unpleasant smell (Kerekes *et al.,* 2015). Wilson and Adams, (2007) found that there is evidence that garlic has properties of antioxidant, anti-inflammatory and antibacterial. Garlic solution can inhibit much more strongly towards bacterial strains compared to antibiotics (Bakri and Douglas, 2004; Lai and Roy, 2004). Bakri and Douglas (2004) found that the garlic extract inhibited the growth and killed most of the organisms tested. For the Gram-negative strains, in term of the minimal inhibitory and minimum bactericidal concentrations, it was lower than those for the Gram-positive strains tested. Time-kill curves for *Streptococcus mutans* and *Porphyromonas ginigvalis* showed that killing of the latter began was faster, whereas there was a delay before *Streptococcus mutans* was killed. Besides that, the trypsin-like and total protease activity of *Porphyromonas gingivali also can be inhibit by the* garlic extract (Bakri and Douglas, 2004). Lai and Roy, (2004) believed that garlic can inhibit *Bacillus subtilis*, *Escherichia coli*, and *Saccharomyces cerevisiae*. Therefore, garlic is used to remove *Bacillus* sp. 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 (Anderson and Whitcomb, 2000).

The objectives are to remove biofilm by using garlic solution and to analyze the factor that affects the biofilm removal process. The scopes of study are included the four factors which were ratio of water to garlic (W/G) (1:1 and 1:5), ratio of garlic solution to *Bacillus* sp. (GS/B) (1:1 and 1:3), agitation speed (50 – 100 rpm) and reaction time between *Bacillus* sp. and garlic (12 – 24 hours).

2.0 MATERIALS AND METHODS

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). 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.

Bacillus sp. Preparation

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^{o}C for 24 hours (Peterson *et al.,* 2011).

Experimental Setup for *Bacillus sp.* Removal

Bacillus sp. was mixed with nutrient broth and agitated in incubator shaker for 1 hour at 37^{o}C and 100 rpm. 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. The mixture was incubated in the incubator shaker at 37^{o}C for 12 hours. The steps were repeated according to the run in Design Expert (Table 1). The *Bacillus* removal was calculated in Equation 1. Control was referred to the weight of *Bacillus* only without any garlic solution added. BGS was referred to the weight of *Bacillus* after garlic solution was added.

Table 1: Experimental setup that has been constructed by using Two-level factorial analysis by Design Expert software (version 7)

Run	Factor 1 A: Ratio of water to garlic	Factor 2 B: Ratio of garlic solution to *Bacillus* sp.	Factor 3 C: Agitation speed (rpm)	Factor 4 D: Reaction time between Garlic and *Bacillus* sp. (Hours)
1	1:1	1:3	50	12
2	1:5	1:1	100	12
3	1:5	1:3	50	12
4	1:1	1:3	100	24
5	1:1	1:3	100	12
6	1:5	1:1	50	24
7	1:5	1:3	50	24
8	1:1	1:1	100	12
9	1:1	1:1	50	12
10	1:1	1:1	50	24
11	1:5	1:3	100	12
12	1:5	1:1	50	12
13	1:5	1:3	100	24
14	1:1	1:3	50	24
15	1:1	1:1	100	24
16	1:5	1:1	100	24

$$Bacillus\ removal\ (\%) = \frac{Control-(BGS)}{Control} \times 100\% \qquad (1)$$

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, 1 μL 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 into the incubator overnight at 100 °C. The dry centrifuge tube was weighed to determine the weight of *Bacillus* sp.

Experimental Design Setup

In this study, 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.

Table 2: The levels of selected factors

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

3.0 RESULTS AND DISCUSSION

Screening of Factors Affecting on *Bacillus sp.* Removal

Two Level Factorial method in Design Expert was used to study the effective effect on *Bacillus* sp. removal. Table 3 shows 16 runs of experiments were done for this study and the results of *Bacillus* sp. removal. The response was analyzed using ANOVA by using the Design Expert Software V7, at more than 95% of confidence level to identify the most contributing factor and interaction between the factors that has effect on *Bacillus* sp. removal. The range for the response of *Bacillus* sp. removal from 42.13% to 99.59%. Table 4 shows the percentage contribution for each factor towards *Bacillus* sp. removal which is GS/B (Factor B) has the highest percentage with value 16.8%, followed by agitation speed (Factor C), W/G (Factor A) and lastly reaction time between *Bacillus* sp. and garlic (Factor D) has the least percentage with value 0.02%. The lowest value of 42.13% *Bacillus* sp. removal was obtained at 1:1 W/G, 1:3 G/B, 50 rpm of agitation speed for 24 hours; the highest value of 99.59% *Bacillus* sp. removal was obtained at 1:5 W/G, 1:1 G/B, 100 rpm of agitation speed for 12 hours.

Analysis of Variance (ANOVA) for *Bacillus sp.* Removal

ANOVA summary was shown in Table 5 for *Bacillus* sp. removal to estimate the coefficient of the model, check the significance of each parameter, and indicate the interaction strength of each parameter. From ANOVA analysis, it was observed that model terms are significant when p-value less than 0.05. In this case, for main factors, GS/B (Factor B) and agitation speed (Factor C) are significant model terms. For interactions between factors, W/G and GS/B (Factor AB), is significant model terms. Meanwhile when p-value greater than 0.1 indicate the model terms are not significant which are involving reaction time between *Bacillus* and garlic (Factor D), W/G and reaction time between *Bacillus* and garlic (Factor AD), GS/B and agitation speed (Factor BC), GS/B and reaction time between *Bacillus* and garlic (Factor BD), and, agitation speed and reaction time between *Bacillus* and garlic (Factor CD). The coefficient of determination (R^2) and adjusted coefficient of determination (R^2 adj) were 0.95 and 0.82 respectively. Olmez (2009) suggest that a good fit of a model R^2 should be at least 0.80. Since the R^2 for these response variables are higher than 0.8, it specified that the regression models explained the mechanism well. To express the *Bacillus* sp. removal (Y) as a function of independent variables, the final empirical model in terms of actual factors were determined as follows:

% removal of *Bacillus* sp. = 86.74+4.31A–6.32B+5.64C–0.21D+5.90AB+ 2.66BC+3.43CD–6.46ACD+6.12BCD (2)

where A is W/G, B is GS/B, C is agitation speed and D is reaction time between *Bacillus* sp. and garlic.

Table 3: Experimental result of *Bacillus* sp. removal on different factors

Run	Factor 1	Factor 2	Factor 3	Factor 4	Response 1	Response 2
	A: Ratio of water to garlic	B: Ratio of garlic solution to *Bacillus* sp.	C: Agitation speed (rpm)	D: Reaction time between Garlic and *Bacillus* sp. (Hours)	Weight of *Bacillus* sp. (mg)	% removal of *Bacillus* sp.
1	1:1	1:3	50	12	17	82.14
2	1:5	1:1	100	12	1	99.59
3	1:5	1:3	50	12	16	83.19
4	1:1	1:3	100	24	41	95.77
5	1:1	1:3	100	12	268	60.77
6	1:5	1:1	50	24	6	96.20
7	1:5	1:3	50	24	84	81.01
8	1:1	1:1	100	12	8	96.72
9	1:1	1:1	50	12	7	97.13
10	1:1	1:1	50	24	15	90.51
11	1:5	1:3	100	12	3	99.56
12	1:5	1:1	50	12	8	76.47
13	1:5	1:3	100	24	12	98.76
14	1:1	1:3	50	24	256	42.13
15	1:1	1:1	100	24	20	94.22
16	1:5	1:1	100	24	22	93.64

Table 4: Percentage contribution for every factor

Term	% contribution
A: Ratio water to garlic	7.83
B: Ratio garlic solution to *Bacillus*	16.80
C: Agitation speed (rpm)	13.37
D: Reaction time between *Bacillus* sp. and garlic (hours)	0.02

Table 5: ANOVA analysis on *Bacillus* sp. removal

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	3621.52	11	329.23	7.12	0.04
A-ratio of water to garlic	297.88	1	297.88	6.44	0.06
B-ratio garlic solution to *bacillus*	639.30	1	639.30	13.83	0.02
C-agitation speed	508.98	1	508.98	11.01	0.03
D-reaction time between *Bacillus* and garlic	0.70	1	0.70	0.02	0.91
AB	556.77	1	556.77	12.05	0.03
AD	38.89	1	38.89	0.84	0.41
BC	112.99	1	112.99	2.44	0.19
BD	10.00	1	10.00	0.22	0.67
CD	187.88	1	187.88	4.06	0.11
ACD	668.57	1	668.57	14.46	0.02
BCD	599.55	1	599.55	12.97	0.02
Residual	184.89	4	46.22		
Cor Total	3806.41	15			
R-Squared	0.95				
Adj R-Squared	0.82				

Analysis of Main Effect and Interaction Effect Between Factors on *Bacillus sp.* Removal

Figure 1 shows the main effects and interaction effects of the factors. For main effect, it showed that there are two main factors contribute to *Bacillus* sp. removal. Factor B (GS/B) has shown the highest effects followed by Factor C (agitation speed). In Table 3, Factor B does give the highest contribution among other factors. Based on Table 1, for Factor 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 1, Factor B gave negative effects towards the respond. Therefore, when the value of Factor B is increasing, the *Bacillus* sp. removal is decreasing. Factor 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 Factor B should be on low level. On the other hand, Factor C gave a positive effect toward the *Bacillus* sp. removal. Positive effect is when the factor is increasing, the responds is increasing as well. To obtain the higher *Bacillus* sp. removal, the agitation speed should be increasing too. For interaction effects, it showed that there was only one interaction that gives positive effect towards *Bacillus* sp. removal which is W/G and GS/B (Factor AB). Positive effects referred to *Bacillus* sp. removal increased when the value of Factor AB increased.

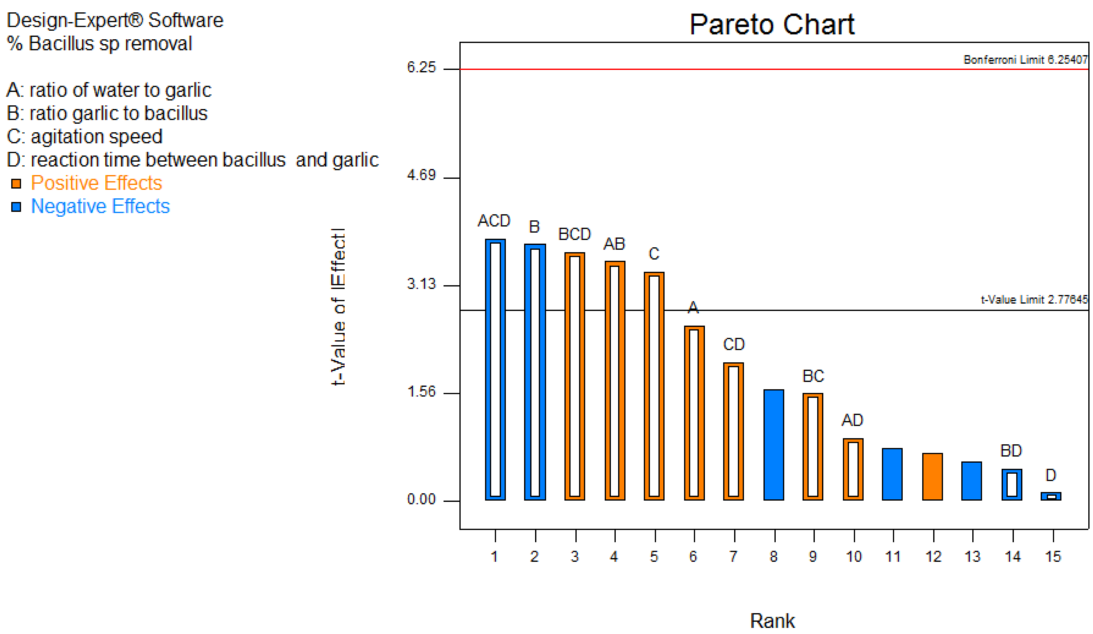
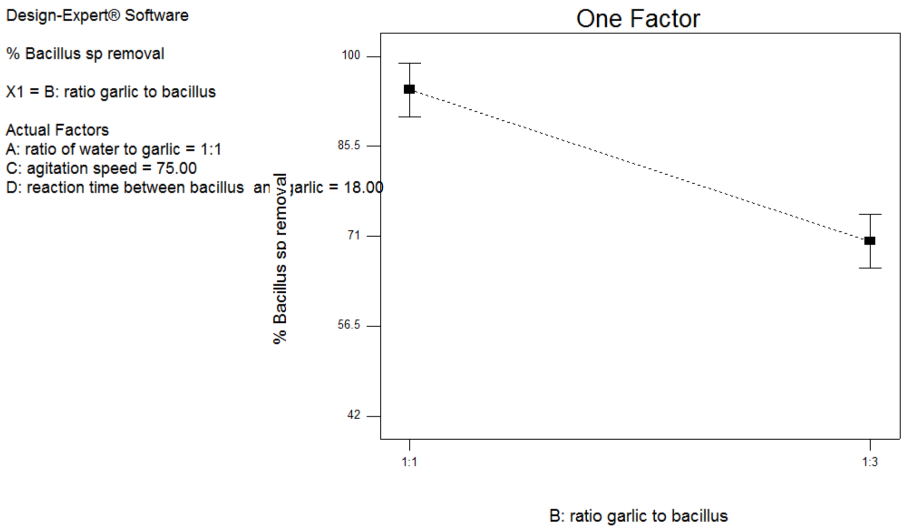


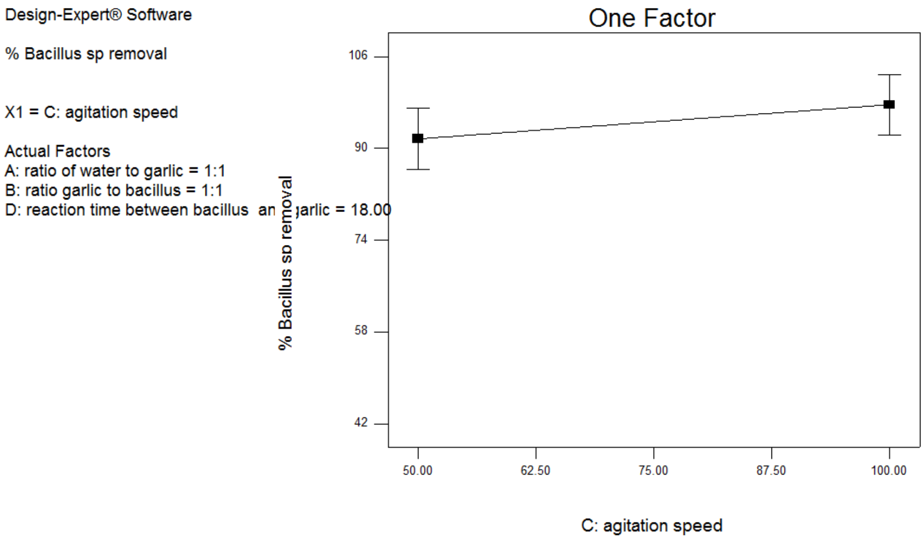
Figure 1: Pareto Chart for factorial analysis on *Bacillus* removal

Effect of Independent Processing Factors on *Bacillus sp.* Removal

The effect of two independent variables on the *Bacillus* sp. removal is shown in Figure 2a and 2b. Factor B gave the highest contribution in *Bacillus* sp. removal. From Figure 2a, the *Bacillus* sp. removal is increasing when GS/B is decreasing. *Bacillus* sp. removal was higher when GS/B at 1:1 with value 94.64%, meanwhile, it reduced to 70.20% 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 (Soliman & Badeaa, 2002). From Figure 2b, the *Bacillus* sp. removal was the lowest when agitation speed at 50 rpm with value 91.66%, and the highest when agitation speed at 100 rpm with value 97.63%. Since Factor C gave the positive effects on the respond, the *Bacillus* sp. removal increased proportionally with the agitation speed. This is due to the break of cell wall of bacteria, change in the morphology of filamentous microorganisms, and mechanical inactivation of the bacteria (Porcel *et al.,* 2005). However, if the agitation speed was exceeding 130 rpm, it will affect the growth of bacteria due to shear stress on bacteria cell (Purwanto *et al.,* 2009)



a) B



b) C

Figure 2: Analysis of most effective independent factors in *Bacillus* sp. removal

Interaction Effects between Factors on *Bacillus sp.* Removal

Figure 3 shows the interaction between Factor A (W/G) and B (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 94.64%. The *Bacillus* sp. removal was slightly reduced when the W/G changed to 1:5 while GS/B remain constant. It was supposed that when W/G increases, the *Bacillus* sp. removal increases. However, it reacted reversely. This was due to the reduction of removal activity that was affected by mass transfer limitation. Uckun Kiran *et al.* (2013) stated that by increasing the solute and solid contents in the medium can cause the mass transfer limitation which could decrease the activity. Based on that statement, the solid content represents garlic and it could decrease the activity of *Bacillus* sp. removal. Lebrero *et al.* (2010) stated that when mass transfer is limited, the metabolic rate of the microorganisms decreases and the microorganisms may respond adversely to the resulting stress. On the other hand, the *Bacillus* sp. removal was lowest when GS/B and W/G at 1:3 and 1:1 respectively with value 70.20%. However, the *Bacillus* sp. removal increased till 90.63% as W/G increasing to 1:5. The concentration of garlic was increasing when W/G was increasing. The *Bacillus* sp. removal was proportional to the concentration of antimicrobial agent. The activity of inhibitory natural biocides is directly proportional to the concentration of natural biocide (Soliman and Badeaa, 2002).

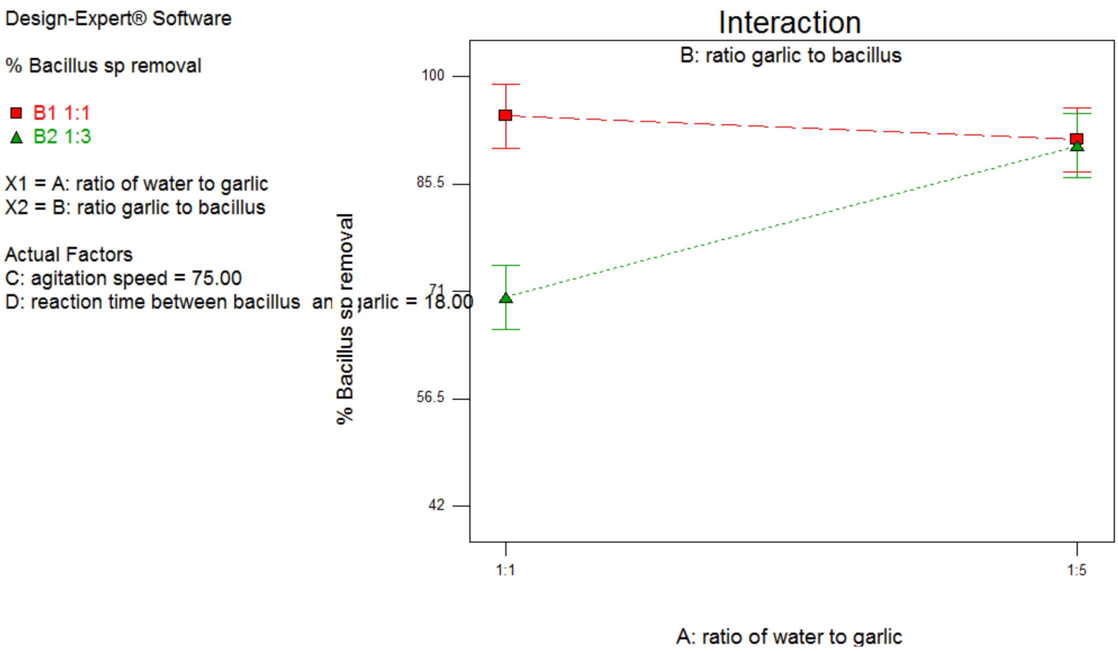


Figure 3: Analysis of interaction effects (Factor AB) on *Bacillus* sp. removal

Validation Experiment

Equation 2 was used to predict the *Bacillus* sp. removal values by using the suggested best condition from Design Expert Software V7. The criteria for determination of best condition is given in Table 6.

Table 6: Criteria for determination of best condition

Criteria	Goal	Value
W/G	Equal to	1:1
GS/B	Equal to	1:3
Agitation speed	In range	50rpm - 100rpm
Reaction time between *Bacillus* sp. and garlic	Minimize	-
Bacillus sp. removal	Maximize	-

The suggested best conditions and their predicted *Bacillus* sp. removal are shown in Table 7. Triplicate experiments were run in order to validate the suggested best condition and the result of the experiment is shown in Table 7. The error from this experiment was calculated by using Equation 3 and the errors were 2.28%, 8.82% and 4.84% for Run 1,2 and 3 respectively.

$$\text{Error (\%)} = 100\% \times \left|\frac{\text{actual} - \text{predicted}}{\text{actual}}\right| \qquad (3)$$

Table 7: 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 (%)		
					Run 1	Run 2	Run 3
1:1	1:3	50	12	80.65	78.85	88.46	76.92
				Error	**2.28%**	**8.82%**	**4.84%**

4.0 CONCLUSIONS

The purpose of this study is to determine the most affecting factors in *Bacillus* sp removal. Design Expert software (Version 7) was used to construct experimental table and to analyze experimental data (via two level factorial analysis) to determine the most contributing factor and interaction factors. 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. The contribution factor rank of *Bacillus* removal were GS/B> agitation speed >W/G> reaction time. This come to the conclusion only GS/B and agitation speed were the significant factors while for the interaction effect was between W/G and GS/B. The *Bacillus* sp. removal best condition was determined at W/G at 1:1, GS/B at 1:3, agitation speed at 50 rpm and reaction time at 12 hours and 88.46% of *Bacillus* sp. removal was achieved at this condition. Results from this study show 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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