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# Bioremediation of *Spent Bleaching Earth* (SBE) Wastes Using Lipolytic Bacteria (*Bacillus cereus*) with Variation of Inoculum Volume

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## ABSTRACT

*Spent Bleaching Earth* (SBE) is a solid waste was generated from the CPO refining step into cooking oil. SBE that was discharged directly into the environment has the potential to pollute the environment because in the SBE waste contained oil and acid residues, which are easy to be oxidized and flammable. Therefore, SBE must be processed first before being discharged into the environment. One way to manage SBE waste is by bioremediation. Bioremediation is a method of cleaning the environment from contaminants by using biological agents, such as bacteria, fungi, etc. The bacterial isolates used in this study were *Bacillus cereus*. This study aims to obtain the best inoculum and to know the ability of *Bacillus cereus* bacteria in degrading the oil content in Spent Bleaching Earth (SBE) waste. This study used a Completely Randomized Design with the volume of *Bacillus cereus* bacteria inoculum as a treatment, consisting of 6 treatment levels of 0 mL $kg^{-1}$, 25 mL $kg^{-1}$, 50 mL $kg^{-1}$, 75 ml $kg^{-1}$, 100 mL $kg^{-1}$ and 125 mL $kg^{-1}$. Each treatment level was repeated 3 times so that 18 experimental units were obtained. The observation was done once a week, in a month. Parameters observed were bacterial population, the percentage of oil degradation and oil content after degradation. The best treatment result for the bacterial population was obtained at the treatment of 100 mL $kg^{-1}$, at week 4, which was 7.4 x 108 Cfu $g^{-1}$, and for the oil, degradation was obtained at 50 ml $kg^{-1}$ on the treatment at week 4 as big as 90.43%.

*Keywords: Spent Bleaching Earth (SBE), Bacillus cereus, bioremediation, degradation*

## 1. INTRODUCTION

Indonesia is the second largest of palm oil producer in the world. CPO that was produced from palm oil can be used for various industries including cooking oil industry. Based on a survey conducted by CDMI (2014), Indonesia's cooking oil production in the last five years (2009-2013) increased by an average of 16.5% per year from 7.13 million tons per year in 2009 to 13.0 million tons per year in 2013.

The processing of CPO into cooking oil is carried out through various stages. One of the stages in the refining stage, which consists of degumming, bleaching, filtration, deodorization, and deaeration. Each stage is producing residues (wastes). The most massive wastes were contained in the bleaching process (purification). The waste generated from the bleaching process is a solid waste known as Spent Bleaching Earth (SBE). According to Prokopov & Mechenov (2013), SBE is generated from the bleaching process that is disposed of as waste in landfills. The disposal of SBE on landfill can lead to environmental pollution. SBE contains oil residues that are easy to be oxidized and flammable. Kheang *et al.* (2006), states that each SBE particle contains an average of absorbed oil derived from the bleaching process of CPO, which is contained of 20-30% from the weight of SBE. Regarding the vast amount of oil were still left behind, the management of SBE requires particular attention in order to reduce the impact on the environment.

One of the ways to process the SBE waste is by bioremediation method, by utilizing biological agents such as bacteria. Bacteria that can be used in reducing oils residue are classified as lipolytic bacteria. Lipolytic bacteria produce and excrete lipase enzymes that can hydrolyze fat to fatty acids and glycerol. Based on previous research conducted by Widjajanti and Munawar (2015), one of the bacteria that can reduce oil in SBE waste is *Bacillus cereus* bacteria. This bacterium is able to reduce the oil in SBE waste by 49.06%. Biodegradation by *Bacillus cereus* bacteria is influenced by biotic and abiotic factors, including bacteria, substrates, nutrients, acceptor and environmental factors, such as pH, temperature and salt content (Notodarmojo, 2005). Nugroho (2006), adding that

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the degradation process of a compound is caused by the enzyme product produced by the bacteria, so the degradation process depends on the number of bacteria sufficient to degrade the oil through its metabolic pathway. As one of the factors that influence the bioremediation process, it is necessary to research about the right concentration of inoculum so that the bioremediation process is going well. This study aims to obtain the best inoculum volume and to know the ability of Bacillus cereus in SBE waste bioremediation.

## 2. EXPERIMENTAL SECTION

### 2.1 Materials and Instrumentation

The tools were being used in this research are stationery, aluminum foil, autoclave, bunsen burner, petri dish, tissue, Erlenmeyer, beaker, measuring cylinder, hot plate, incubator, ose needle, digital camera, cotton, filter paper, label paper, refrigerator, pH meter, stirrer, magnetic stirrer, paper disk, micron pipette, serological pipette, basin, plastic, rubber, reaction tube rack, rotary shaker, reaction tube, analytical scales, vortex, microscope, counting chamber, and soil tester. The materials were being used are aquadest, the bacterial isolates of Bacillus cereus, mineral medium, NA medium, NB medium, 70% alcohol, and SBE waste.

### 2.2 Sampling Method

This study used a Completely Randomized Design (RAL) with the treatment of bacterial inoculum of Bacillus cereus, which consisted of: 0 ml $kg^{-1}$ (control), 25 ml $kg^{-1}$, 50 mL $kg^{-1}$, 75 ml $kg^{-1}$, 100 mL $kg^{-1}$ and 125 mL $kg^{-1}$. Each treatment was repeated 3 times.

### 2.3. Procedure

#### *2.3.1 Sampling*

Sampling was done at PT. Tunas Baru Lampung, South Sumatera. SBE waste samples were taken directly from the drainage stack using randomizes sampling, as much as 50 kg. Sample of wastes were solid wastes, grayish black, smells sharp and flammable.

#### *2.3.2. Sterilization Tools and Media*

The tools and media that being used were sterilized by autoclaving at 1 atm pressure at 121°C for 15 minutes and the non-heat resistant equipments were sterilized using 70% alcohol.

#### *2.3.3. Medium Creation*

*2.3.3.1 NA Medium (Nutrient Agar)*

The NA medium (Nutrient Agar) was weighed 1.2 g and then dissolved in 60 mL of aquades in Erlenmeyer. Erlenmeyer which contain the media was clogged with cotton and aluminum foil, and then was heated over the hot plate until the solution becomes light yellow, next was sterilized in the autoclave at a temperature of $121^0$C pressure 1 atm for about 15 minutes. As much as 10 ml of nutrient agar was inserted to the reaction tube amounting of 6 pieces, settled at room temperature until the media freezes in a tilted position, and then the tilted nutrient agar is ready for bacterial rejuvenation.

2.3.3.2. Making NB Medium

NB Medium (Nutrient Broth) was 13 g and dissolved in 1000 mL of aquades, then being heated until dissolved, and cooled first, and the tube was covered with cotton and aluminum foil. The nutrient broth solution was being heated in the autoclave to $121^0$C at 1 atm pressure for 15 minutes and then stored in the refrigerator.

2.3.3.3. Making of Mineral Medium

Making mineral medium by dissolving $K_2HPO_4$ 4.5g; $(NH_4)2SO_4$ 1 g; $7H_2O$ 0.2 g; NaCl 0.1g; $CaCl_2$ 0.1 g; $FeCl_3$ 0.02 g and CPO 44.3 ml into 1000 mL of aquades on erlenmeyer. The solution was homogenized by using a magnetic stirrer over the hot plate. After homogeneous, the media was sterilized using an autoclave at a temperature of 121°C with a pressure of 1 atm for 15 minutes (Modifikasi Atlas, 2010).

#### *2.3.4. Rejuvenation of Lipolytic Bacteria*

Lipolytic bacterial isolates were inoculated into a prepared Nutrient agar (NA) medium, i.e. as many as 6 reaction tubes. The bacterial isolate was taken by using an ose needle aseptically, and taken as much as 1 ose, and then the cultured ose needle was placed at the base of the slope of agar and withdrawn with zigzag movement (Modifikasi Lay, 1994), then being incubated in the incubator for ± 48 hours at room temperature.

#### *2.3.5 Growth Curve of Lipolithic Bacterial Growth*

The growth curve of bacterial isolate was made by inoculating as much as 4% (v/v) bacterial inoculum on 100 ml of mineral medium, which was added by the CPO. The number of bacterial cells was calculated from the time of initial inoculation until it showed the decreased of cell density with observation intervals every 3 hours, whereas the diagitation cultures were at 120 rpm at room temperature, and the calculations were performed direct microscopically by counting chamber. The number of isolate cell data for each observation was presented in graphical form, so that the growth phases are being known. The obtained isolate growth curve was determined as the shortest generation time in its exponential growth phase. The shortest generation time was used as the basis for making and giving inoculum on testing the ability of bacteria to degrade the SBE waste.

The equations used to determine generation time are:

$$G = \frac{t}{3{,}3 \log (b/B)}$$

Description :
G = generation time
t = time interval between measuring the number of cells in the population at a time in the log phase (B) and then again at a later point (b)
B = initial population
b = population after time t
log = $\log_{10}$
3.3 = conversion factor log2 to $\log_{10}$
(Pelczar and Chan, 2010).

The shortest generation time was obtained according to the bacterial growth curve is 6 hours.

#### *2.3.6 Inoculum Making of Lipolytic Bacteria*

The making of bacterial inoculum by taking the cultured of the rejuvenated bacterial isolates, as much as 15 ose, then inserted

into 2400 mL of NB medium, NB medium containing bacterial isolates were being homogenized by rotary shaker for 6 hours (according to the shortest time in bacterial growth curve), after 6 hours, calculated the density of bacteria by using counting chamber, then calculated until the density of ±108 cells/ml. Once the bacterial density is in accordance with the desired, then, the bacterial inoculum is ready to be inoculated into the bioreactor.

#### *2.3.7. Bioreactor Preparation for Bioremediation*

Bioreactor prepared as many as 18 pieces. Each bioreactor was filled with 2 kg of spent bleaching earth (SBE) waste, with a predetermined waste treatment condition, i.e. for pH 6-7, and 50% moisture. The pH measured at the start of the study was 3.11 (very acidic). The pH was raised by calcification. Lime that being used was calcite chalk type (CaCO3), as much as 1.08 kg for 50 kg of SBE waste. After the lime is applied to the SBE waste, it was homogenized by stirring, after homogenous then covered by plastic for 2 weeks. After 2 weeks, pH was measured again by using pH meter, and obtained pH value of 5.89. Furthermore, moisture was measured using a soil tester. At the time of bioreactor preparation, the moisture has reached at 50%. After the prepared bioreactor was in accordance with the desired conditions, the prepared bacterial inoculum was ready for inoculation into each bioreactor according to the treatment (0 ml $kg^{-1}$, 25 ml $kg^{-1}$, 50 ml $kg^{-1}$, 75 $kg^{-1}$, 100 ml $kg^{-1}$, 125 ml $kg^{-1}$). The bacterial inoculum was inoculated by using spray evenly over the entire portion, and then being homogenized again by stirring so that it was completely mixed. Then each bioreactor was covered by a plastic that has been perforated and finally the bioreactor was placed in a sterile place (to avoid contaminants). Furthermore, the bioreactor was allowed to stand for one month with intervals of observation and controlling the moisture in every week.

#### *2.3.8. Analysis of Gravimetric Oil Levels*

The oil content was analyzed by Gravimetry according to reference of SNI 06-06989-10 which has been modified. The oil content analysis was carried out by inserting 5g of SBE into the erlenmeyer, 100 ml of n-hexane was added and then shaken for ± 5 min using a shaker, then the shaked SBE and n-hexane were filtered with filter paper in a weighted bottle. There are 3 layers of oil, n-hexane and water. The water layer was disposed; the oil layer and n-hexane were inserted into the distillation flask, then disoxhlet until n-hexane runs out. The flask was heated in the oven at 100°C for 1 day until n-hexane runs out, the water runs out and only the oil was left. The flask was lifted and settled in the desiccator for 30 minutes. Make sure the flask is dry and weigh until it gets a fixed weight.

The formula for calculating the oil content is:

$$\% \text{ Oil contents} = \frac{(A-B) \times 100\%}{\text{gram sample}}$$

Description:
A = weight of flask and extract (g)
B = empty flask weight (g)
(SNI 06-06989-10, 2004)

#### *2.3.9. Calculation of Bacterial Population*

The calculation of bacterial colonies used by the Total Plate Count (TPC) method. The SBE was taken from the bioreactor and weighed 5 g, then added 45 mL of aquadest. Then perfomed dilutions of 6 series (10-1, 10-2, 10-3, 10-4, 10-5 10-6). From the 6 series dilutions, only 3 dilution series (10-4, 10-5, 10-6) were taken to be placed on a sterile petri dish for each treatment. Then the NA medium is poured into an existing substrate petri dish, and then incubated for 24 hours at 37 ° C. The result of bacterial population contained in petri dish is calculated by using colony counter.

### 2.4 Variable observations

Observation variables observed in this study are as follows:

#### *2.4.1. Bacterial Population*

The bacterial population at each treatment was calculated in CfU g-1 every one week from once for 4 weeks.

#### *2.4.2 Percentage of Oil Content*

The calculation of the oil content of the sample in units of% (%) is done once a week for a month to find out how much oil content is left in the SBE waste.

#### *2.4.3 Percentage of Oil Degradation*

The calculation of oil degradation was done once a week in a month to determine the residual oil lost during the bioremediation process. Calculation of the degradation percentage of oil content by using equation as follows:

$$\text{Percentage of Oil Degradation} = \frac{A - B}{A} \times 100\%$$

Description:
A = Initial oil content (%)
B = Final oil content (%)

### 2.5 Data Analysis

The data which was obtained include bacterial population data, percentage of oil degradation, composition and compounds contained in SBE waste. Bacterial population data and percentage of oil degradation were being analyzed by using variance analysis (ANAVA) and if it was significantly different, then continued with Duncan New Multiple Range Test (DNMRT) test with α 5%. The data was being analyzed using Software Statistic 8.0.

## 3. RESULT AND DISCUSSION

The result of Varian Analysis (ANAVA) showed that the volume of bacterial inoculum of Bacillus cereus had a significant effect on bacterial population, oil content and oil degradation. Further test results of DNMRT α5% on the variations of inoculum volume on bacterial population, oil content and oil degradation are presented in Table 3.1, 3.2 and 3.3.

### 3.1 Bacterial Population

The best inoculum volume treatment for bacterial population was 100 mL $kg^{-1}$ at week 4, i.e, 7.74 Cfu $g^{-1}$. These results indicate that at these concentrations support for optimal bacterial growth. Furthermore, in Table 3.1 there was an increase in the number of bacterial cells along with the increase of inoculum volume, although the increasing result did not affect the significant differences between treatments. This explains that the treatment of large amounts of

Table 1. The average (log) population of Bacillus cereus lipolytic bacteria on each treatment

| volume of inoculum | Population lipolytic bacteria on Week of (Cfu g-1) | | | |
|---|---|---|---|---|
| (mL/Kg$^{-1}$) | 1 | 2 | 3 | 4 |
| 0 | 1,00 a | 1,00 a | 1,00 a | 1,00 a |
| 25 | 3,90 ab | 4,90 ab | 7,40b | 7,43 b |
| 50 | 4,13 ab | 4,99 ab | 7,63bc | 7,52 b |
| 75 | 5,96 b | 5,46 ab | 7,88 bc | 7,52 b |
| 100 | 6,33 b | 7,89 b | 7,92 bc | 7,74 b |
| 125 | 6,38 b | 7,92 b | 8,03 c | 7,87 b |

Note: letter indexed after values are Duncan Multiple Rang Test category

Table 2. The average of oil content after degradation in each week of observations

| volume of inoculum | Oil Level on the Week of (%) | | | |
|---|---|---|---|---|
| (mL/Kg$^{-1}$) | 1 | 2 | 3 | 4 |
| 0 | 3,52 a | 3,07 a | 2,67 a | 1,82 a |
| 25 | 2,32 b | 2,26 b | 1,22 b | 1,11 ab |
| 50 | 1, 95 bc | 1,25 c | 1,15 b | 0,67b |
| 75 | 1,78 bcd | 1,39 c | 0,76 b | 0,35 b |
| 100 | 1,52 cd | 1.13 c | 0,70 b | 0,39 b |
| 125 | 1,13 c | 0,83 c | 0,76 b | 0,72b |

Note: letter indexed after values are Duncan Multiple Rang Test category

inoculum did not guarantee the number of bacterial cells more than the treatment of fewer inoculums. The results of Zam (2010) showed that there was no correlation between giving a large amount of inoculum to the growth of microorganisms. According to Doelle (1994), sufficient inoculum concentration is one of the requirements that the fermentation process can take place optimally.

The increasing of bacterial population at 25 mL kg$^{-1}$, 50 mL kg$^{-1}$, 75 mL kg$^{-1}$, 100 mL kg$^{-1}$ and 125 mL kg$^{-1}$ acts every week during the bioremediation process. This suggested that bacteria have been able to utilize the nutrients present in the substrate and be able to adapt to the substrate environment. In the first weeks, bacteria were still in the adaptation stage, i.e. bacteria still in the preparation stage and adjustment to environmental growth conditions, such as nutrient source conditions and substrate environmental conditions. Susanti (2003), stated that in the adaptation phase to the environment the bacteria will hydrolyze the required medium components for metabolism and cell growth. According to Kusumaningati *et al.* (2013), in the slow phase (lag phase) bacteria perform metabolism in preparing and adjusting to the growth conditions in new environments so that there is no significant increase in the number of cells. In the adaptation stage, the increasing of cell activity will continue, along with the improvement of the absorption of nutrients as a source of energy. Waluyo (2007), stated that the better the bacteria absorb the nutrients contained in the substrate, then the growth of bacterial numbers is getting faster. After the adaptation stage, bacterial growth will enter the exponential phase. In this phase the bacterial cell will reach the highest number (climax), and then it will decrease or stagnate. Kusumaningati *et al.* (2013), stated that the exponential phase is the phase of cell number multiplication, cell activity increases. The bacterial population at week 3 were continues to increase, showed that the 25 mL kg$^{-1}$ treatment has entered the exponential phase. Bacterial population at 4th week, seen began to decrease or becoming stagnated. Bacterial growth at 4th week was in the stationary phase. In this phase, the source of nutrients that were available carbon is getting more and more exhausted because it has been exploited by bacteria as a food source. Susanti (2003), stated that in the phase of death, the nutrients was being needed by bacteria are reduced and the results of bacterial excretion have been buried in the medium, thus disrupting the breeding and the next bacterial growth. Anggriani *et al.* (2012), stated that the factors that cause the death of microbial population are the nutrients in the medium are exhausted and no reserve energy in the cell. Therefore the bacterial cell growth will decrease or has a tendency to be stagnant.

### 3.2 Oil content

The results of this study indicate that there was a decrease in oil levels for all treatments along with the increasing of the volume numbers of inoculum in each week of observation, although the difference in oil levels among treatments were significant and insignificant. Preliminary data of oil content in SBE waste before inoculation was 7%. Presumably this is caused by a certain amount of inoculum, the bacteria have been able to decompose the oil optimally. Januar *et al.* (2013), stated that the decrease in lipid levels is an indication of the role of bacteria in oil remodel. Mishra *et al.* (2001), stated that the suitability between the inoculum ratio and the substrate composition can affect the petroleum degradation process. The degradation of oil content came along with the length of time of bioremediation (Table 3.2). Presumably this is caused by the lack of nutritional sources along with the length of bioremediation, thus causing a competition in getting food. This competition leads to the death of bacteria, so the number of bacteria is reduced, eventually the degradation process also decreased. According to Chorom *et al.* (2010), that time has a significant effect on the degradation process. Schaefer and Juliane (2007) suggested that the longer the bioremediation time, the growth of bacteria and the rate of degradation of oil were being decreased.

Furthermore, if we refer to the Decree of Kepmen LH No. 128 (2003), it can be seen that the 125 mL kg$^{-1}$ treatment with the value level of oil as much as 0.83% at week 2, has fulfilled the requirements set by the government that is at a maximum of 1%. And if the bioremediation process was continued for up to three weeks, the 75 mL kg$^{-1}$ treatment is a treatment that can be applied with a 0.76% oil content, and if the bioremediation process were being continued until week 4, then 50 mL kg$^{-1}$ treatment with the oil content of 0.67 is a usable treatment.

### 3.3. Percentage of oil degradation

The result of the percentage of oil degradation showed that the greater of the inoculum volume, the higher the percentage of degradation obtained, although, there were significant and insignificant differences among treatments. The percentage of degradation will increase along with the decrease of oil content. According to Zulaika *et al.* (2012), the higher of the number of cells were being used; the resistance and the reduction power of Bacillus will be higher. Nugroho (2006), stated that the degradation process of a compound is caused by the enzyme product produced by bacteria, so the degradation process depends on the adequate amount of bacteria to degrade the oil through its metabolic pathway. Atlas and Bertha (1992) suggested that the remodel activity is caused by enzyme oxygenase released by bacteria to increase direct contact between oil and bacteria, so bacteria can use the oil as a carbon source.

Furthermore, Table 4.3 also showed that although the percentage of degradation increased along with the increasing of in-

Table 3 Average percentage of oil degradation (log) at each treatment at week 1,2,3,4 after application

| volume of inoculum (mL/Kg$^{-1}$) | Percentage of Oil Degradation (%) on Week of | | | |
|---|---|---|---|---|
| | 1 | 2 | 3 | 4 |
| 0 | 50,43 a | 56,04 a | 61,86 a | 70,05 a |
| 25 | 66,76 b | 67,71 b | 82,57 b | 84,14 ab |
| 50 | 72,13 bc | 80,09 c | 83,53 b | 90,43 b |
| 75 | 74,57 bcd | 80,43 c | 89,19 b | 94,95 b |
| 100 | 78,33 cd | 83,86 c | 90,00 b | 94,38 b |
| 125 | 83,81 d | 88,14 c | 89,19 b | 89,72 b |

Note: letter indexed after values are Duncan Multiple Rang Test category

oculum volume in every week of observations, the statistic showed that the increase was not significantly different among treatments. Presumably, this is because the concentrations of bacteria are causing inadequate medium for the bacterial growth, effected to the competition between the bacteria, and eventually the growth and degradation process becomes low. Astuti (2003), stated that the competition in the use of substrate effected to the growth of culture becomes poor, because the increasing of cells number or biomass become low. According to Gibbson and Westby (1986), too high inoculum concentrations can lead to a reduction in cell viability. Anggriani *et al.* (2012), adding that too high bacterial populations can lead to the rivalry of the similar types of bacteria (*Bacillus sp*) and indigenous microorganisms in nutrient or substrate intake that eventually the bacterial activity become inhibited. Inoculum volume of 50 ml kg$^{-1}$, is the appropriate and economical inoculum volume treatment in SBE waste bioremediation. The treatment supports for optimal bacterial growth when being compared with other treatments.

The correlation analysis between the bacterial population and the percentage of degradation indicates the bacterial population affects the percentage of degradation. The correlation analysis between the bacterial population and the degradation percentage yielded a correlation coefficient (r) of 0.6006. The correlation coefficient value between the bacterial population and the percentage of degradation is positive; it means the relationship between the bacterial populations (Cfu g$^{-1}$) was in line with the percentage of degradation. If the population of bacteria (Cfu g$^{-1}$) (x) is high then the percentage of degradation produced is also high and the oil content (%) will decrease. According to Ichor *et al.* (2014), stated that the number of bacterial cells responsible to the oil degradation.

The results of simple linear regression analysis showed that the association of the bacterial population and the percentage of degradation can be seen in the following equation:

$$y = 63.072 + 2.828x$$

Description: y: percentage of oil degradation
x: bacterial population

The simple linear regression equation showed that the bacterial population (Cfu g$^{-1}$) was influenced by the constant 63,072 and the regression coefficient + 2,828. The regression coefficient is positive value, then the regression model is positive or in line with the direction of the percentage of oil degradation (%). Based on linear regression equation above can be concluded that bacterial population (x) have the positive effect on degradation percentage with total influence of 35,9%. This positive influence means the higher of the bacterial population; the higher of the percentage of degradation. This relationship is also seen in the linear graph between the bacterial population and the percentage of degradation (Figure 1).

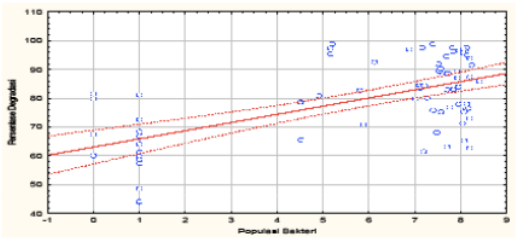


Figure 1. Simple linear regression graph between the bacterial population and the percentage of degradation

## 4. CONCLUSION

The volume of inoculum affected to the bacterial growth and oil degradation. The best treatment of bacterial inoculum volume of Bacillus cereus to the bacterial population is 100 mL kg$^{-1}$ at week 4 of 7.4 x 108 Cfu g$^{-1}$ and the most efficient treatment in degrading oil in SBE waste during the bioremediation process, was the inoculum volume of 50 mL kg$^{-1}$ treatment at week 4 as much as 90.43%.

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