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Biosurfactant Screening and Antibiotic Analysis of *Bacillus salmalaya*

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ABSTRACT

Introduction: Biosurfactants are made up of naturally occurring molecules such as lipopeptide, glycoprotein, lipoprotein, and fatty acids. Using biosurfactants rather than chemical and synthetic surfactants is safer.

Objective: The aim of this study was to investigate biosurfactant properties and antibiotic resistance of soil bacteria, *Bacillus salmalaya* strain 139SI. *Bacillus salmalaya* produces biosurfactant, which capable of reducing surface tension of certain media, as they are surface- active molecules.

Methods: In current study Parafilm M Test, Drop Collapse, Oil spreading methods forBiosurfactants screening were used and antibiotic analysiswas done using using an aseptic technique.

Results: 600µl biosurfactant (460 mg/ml) was the most effective volume to produce large diameter of oil spreading zone when 700 µl of oil was used. 0.23g freeze-dried supernatant powder mixed with 0.5 ml distilled water (460 mg/ml) produced larger clearing zone than powder mixed with 1 ml (230 mg/ml) and 1.5 ml distilled water (153 mg/ml). In cleaning activity of crude oil container, biosurfactant was able to clean oil in the container and this can be applied in cleaning oil waste from crude oil tank and can also be used to clean oil spillage. In antibiotic analysis study, ten soil bacteria isolates included *Bacillus salmalaya* were selected.

Conclusion: All isolates had shown different sensitivity towards different antibiotics. *Bacillus salmalaya* showed sensitivity to almost all selected antibiotic compared to other isolates. This indicated that this strain is safe to be used as it was sensitive to many antibiotics.

Key Words: *Bacillus salmalaya* strain 139SI, Biosurfactant, Oil, Synthetic, Antibiotic resistance, Drop Collapse

INTRODUCTION

Nowadays, crude oil is used widely in industry and these results in huge amount of crude oil waste released into environment. Oil pollution, such as oil spills, has become a serious problem. To overcome this problem, biosurfactants produced by some bacteria can play a role in enhancing oil recovery and oil spill clean-up.[1] Biosurfactant or surface-active agent are amphiphilic compound, which means they can attract both lipid, water molecules, and reduce the surface tension between the two substances. Biosurfactant able to reduce surface tension and interfacial tension between two different substances. This surface reduction property is very essential in degrading oil activity such as in oil waste cleaning activity.[1]

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Biosurfactant consist of naturally occurring molecule such as lipopeptide, glycoprotein, lipoprotein and fatty acid. Thus, it is safer to use biosurfactant instead of chemical and synthetic surfactant.[2] Chemical and synthetic surfactant are expensive, and they cause environmental and toxicological problem. For example, if they are used in oil spill clean up, they may clean the oil spill, but they also may cause harmful effects towards aquatic ecosystem.[3] Thus, biosurfactant is a better option to be used in industry compared to chemical and synthetic surfactants.[4]

The sensitivity of *Bacillus salmalaya* and other selected soil bacteria was performed via the disk diffusion method, which represents a simple procedure for screening the antibiotic activity. It is essential to know the bacterial safety in handling it, and to determine the most appropriate treatment for any infection.Dadrasnia and Ismail (2015) has reported that the biosurfactant produced by cultured strain 139SI showed high physicochemical properties and surface activity in the selected medium.[5]

If there is any infection occur caused by the bacteria, we can know which antibiotic to use to combat the infection. This could be a huge help in preventing further problem such as sickness or death.[6] Thus, it is very important to analyse antibiotic susceptibility in order to determine the safety in handling and using the bacteria and to determine the best treatment or antibiotic for any infection. The aim of this study was to study biosurfactant properties of *Bacillus salmalaya*. Besides, we want to investigate the potentiality of *Bacillus salmalaya* strain 139SI biosurfactant in remediating petroleum crude oil waste, and to determine the sensitivity of soil bacteria including *Bacillus salmalaya* towards different commercial antibiotics.

METHODOLOGY

Bacterial Cultures Collection

Bacteriology preparations were based on the guidelines of Laboratory Manual in Molecular Biology.[5] Bacterial colonies were all isolated from local agricultural soil and slant stocks were kept at 4°C in the Molecular Biology Laboratory of Institute of Science Biology, Faculty of Science, University of Malaya, Kuala Lumpur.

Screening Methods for Biosurfactant Activity

Haemolytic Activity

Bacterial colonies that grew on blood agar plate were streaked onto fresh blood agar plate using inoculating loops. The plate was sealed with Parafilm M and was incubated at 37° for 24 hours. The bacterial colonies growth was observed, and haemolytic activity was inspected by observing zones of clearing around the colonies, which indicates biosurfactant production. Colonies that showed clear haemolytic activity would be picked to undergo further biosurfactant screening. These methods were done based on methods described by-Bielawski, (1990).[7]

Drop Collapse

10µl of oil was applied to a glass slide. Then, 20µl of bacterial culture supernatant was dropped onto crude oil drop and the shape of the droplet was observed. Distilled water and SDS solution were used as negative and positive control respectively. The shape of droplets observed were compared. These methods were done based on methods described by Jain et al. (1991).[8]

Oil Spreading

50 ml of distilled water was added to a large petri dish followed by addition of 20 µl of crude oil to the surface of the water. 10µl of bacterial culture supernatant was added to the surface of oil. Clear zone formed was observed and measured. Distilled water was used as negative control. This method was repeated using different volume of supernatant, which are 200µl, 300µl, 400µl, 500µl and 600µl.

Emulsification activity

10µl of oil was mixed with 20µl of bacterial culture supernatant in a test tube. The presence of bacterial biosurfactant was shown by production of milky colour, which indicated positive emulsification activity. Distilled water was used as negative control and the colour changes was observed.

Parafilm M Test

25µl of supernatant was added on hydrophobic surface of parafilm M strip. The shape of the drop was observed after 1 minute and the diameter of the drop was measured. The presence of the surface-active compounds in the supernatant was detected by the flat shape of the drop, while the semi spherical shape indicated the absence of biosurfactant. Medium without microorganism such as distilled water was used as control. This method was done based on method described by Larrañeta et al., (2014).[9]

Cleaning Activity of Crude Oil Tank

Container containing crude oil was sprayed with bacteria culture broth using a sprayer. The wall of the container was well sprayed until clean. After a few minutes, wall of the container was observed whether clean or not. Commercial detergent and distilled water were used as positive and negative control respectively.

Antibiotic Analysis of Bacteria

Using a sterile swab was placed into the broth culture of bacteria and then the excess liquid was removed by gently pressing or rotating the swab against the inside of the tube.

The Mueller-Hinton plate was streaked with bacteria by using the swab to form a bacterial lawn. The plate was dried for approximately 1-2 minutes. Antibiotic discs used in this test were Ampicillin, Gentamycin, Tetracycline, Sulfamethoxazole, Chloramphenicol and Erythromycin. Each disc was pressed to the agar gently using flame-sterilized forceps to ensure that the disc is attached to the agar properly. Plate was incubated overnight at an incubation temperature of 37°C. Zone of inhibition was observed and measured.

To measure the diameter, a metric ruler was placed across the zone of inhibition, at the widest diameter, and measured from one edge of the zone to the other edge. After measuring the zone inhibition diameter, the results were interpreted and categorized into sensitive, intermediate or resistant towards antibiotics used(Table 1).

RESULTS

Screening Method of Biosurfactant

Haemolytic Activity

From the haemolytic test observation, there was clear, transparent layer surrounding the colonies. The shown haemolytic activity indicated the presence of biosurfactant as biosurfactant could cause lysis of red blood cell. It was beta type haemolysis. The haemolytic activity proved that *Bacillus salmalaya* strain 139SI contained biosurfactant. The colonies shown a clear haemolytic activity. The colonies from the plate were isolated and used for further screening method of biosurfactant.There were two types of layer of haemolysis which are single and double layer (Figure 1a,b).Haemolytic test using cell free broth and blood in test tube had shown positive result of blood lysis. This further proved that strain 139SI inhibited biosurfactant property as it caused lysis of blood cell (Figure 1c).

Oil Spreading

Oil spread test had shown positive result as there was clear zone formed when biosurfactant was placed on oil layer in a large petri dish. The formation of the clear zone indicated that biosurfactant was present in the supernatant of *Bacillus salmalaya*. The diameter of the clear zone was 5.2 cm. Oil spread test was a qualitative test, which can be used for relatively small amount of biosurfactant. The larger the clear zone, the more concentrates the biosurfactant. From the observation, we can see that *Bacillus salmalaya* had moderate amount of biosurfactant (Figure 2a).

Oil spreading activity depended on the concentration and the volume of biosurfactant. The more concentrated the biosurfactant, the larger the diameter of clearing zone. Besides, the volume of cell free broth used also plays important role. The greater the volume of cell free broth, the larger the clearing zone. The size of clearing zone of oil depended on the concentration of biosurfactant. Figures below recorded the size of clearing zone with different concentration of biosurfactant (Figure 2b,c,d).

The size of clearing zone of oil can also be determined by the volume of supernatant containing biosurfactant used. The higher the volume, the larger the diameter of clearing zone. The volume of oil used also plays crucial factor in the diameter of clear zone formed. The higher the volume of oil, the larger the size of clearing zone (table 2).

Parafilm M Testand Drop Collapse Test

Droplet of *Bacillus salmalaya* strain 139SI had flat shape compared to negative control, distilled water that had semi-spherical shape. The flat shape of the supernatant droplet indicated that biosurfactant is present (Figure 3a,b,c).

Emulsification Activity

Emulsification activity were done by mixing crude oil with biosurfactant and distilled water in test tubes. After few minutes, the changes in colour were observed (Figure 3d).The mixture of *Bacillus salmalaya* cell free broth with crude oil produces milky colour, which indicated the activity of emulsification. The emulsification activity indicated the presence of biosurfactant in the cell free broth of *Bacillus salmalaya*. This was because biosurfactant caused emulsification of oil. Meanwhile, the mixture of distilled water with crude oil showed no changes in colour of the mixture. The oil layer was totally separated from the water layer. This indicated the absence of biosurfactant in the mixture (Figure 3d).

Cleaning Activity of Crude Oil Tank

Container containing crude oil were sprayed with biosurfactant, detergent and distilled water in order to clean the oil container. The results obtained can be observed (Figure 4a).

In the beginning, the container was fully coated with crude oil. After been sprayed with bacteria culture broth, the oil layer on the container surface was cleared. The surface of the container was clean from oil. The oil layer flew down to the base of the container. This activity shows that the biosurfactant in the bacteria culture broth has the ability to clean or remove the oil layer from the surface of the container. Three container filled with crude oil were tested using bacteria culture broth, detergent and distilled water. The three container were coated with the same amount of crude oil and then sprayed with the same amount of fluid. The results can be observed in figure 4b&c. From the observation, container sprayed with broth containing biosurfactant was the cleanest one compared to positive and negative controls. This showed that biosurfactant had better ability to clean crude oil tank compared to other controls.

Antibiotic Analysis

Antibiotic test of *Bacillus salmalaya* strain 139SI using Kirby-Bauer Test. Antibiotics used were Erythromycin, Chloramphenicol, Sulfamethoxazole, Gentamicin, Ampicillin and Tetracycline. As observed, we can see that *Bacillus salmalaya* bacteria was sensitive to antibiotic. This strain had large zone of inhibition surrounding Erythromycin, Chloramphenicol, Tetracycline and Gentamicin,small zone of inhibition aroundAmpicillin and no zone of inhibition around Sulfamethoxazole (Figure 5). All isolates showed different sensitivity towards different antibiotics, whichwere Erythromycin, Chloramphenicol, Sulfamethoxazole, Gentamicin, Ampicillin and Tetracycline (Table 3 and 4).

DISCUSSION

In this research, *Bacillus salmalaya* had shown positive result of beta haemolysis where colourless, clear zone area formed surrounding the colonies after 24 hours incubation at 37 °C. This indicated the presence of biosurfactant in the bacteria. From the observation through this research, clear zone formed surrounding the colonies varies. In this case, *Bacillus salmalaya* can produce two types of clear zones layer, which are single and double layer. The single layer is when only one layer of clear zone formed and double layer when two layers of clear zone formed. Based on Bielawski(1990), haemolytic test is used to determine the presence of biosurfactant producing bacteria.[7] Biosurfactant producing bacteria will cause the lysis of red blood cell. This can be seen in the haemolytic test where red blood cell will lyse and cause the formation of clear zone surrounding the colonies of bacteria. This test is used as preliminary test to detect the presence of biosurfactant producing bacteria.[10]

Drop collapse test is an easy and simple test to determine the presence of biosurfactant producing bacteria.[11] Loganathan et al. (2010) reported that the results of the present study also suggested that the drop collapse method suits well as a primary screening method for biosurfactant production.[12]

In the experiment, we can observe that the droplet of supernatant containing biosurfactant had flat shape compared to negative control, distilled water. The diameter of the droplet of supernatant also larger than distilled water, which was caused by the collapsed activity of the droplet. This was because biosurfactant can reduce the surface tension between the droplet and oil surface, which caused the droplet to collapse. Bodour et al., (2003), prove this where it was reported that the semi-sphere shape droplet forms because the polar water molecules are repelled from the hydrophobic surface.[11]

GOTO (1985) first demonstrated oil-spreading method where biosurfactant was dropped onto thin oil layer.[13] This method measures the diameter of clear zones formed when a drop of a biosurfactant-containing solution is placed on an oil–water surface.[14] It is an easy and rapid method, which only required small amount of biosurfactant. Oil spreading technique is a reliable method to detect biosurfactant production by diverse microorganisms as demonstrated by Youssef et al. (2004).[15]

In this study, technique used for oil spreading method was slightly modified from what have been demonstrated by Morikawa et al. (2000).[14] Hence, we tried this method with different volume and concentration of biosurfactant. Different volume of biosurfactant were used which are 200, 300, 400, 500 and 600µl. The diameter of clear zone increased with the increase of the biosurfactant volume. Besides, different concentration of biosurfactant were used in this method. 0.23 g of freeze-dried supernatant powder was diluted with different amount of distilled water, which were 0.5ml, 1.0 ml and 1.5 ml.

In Parafilm Mtest, 20µl of biosurfactant was dropped onto hydrophobic surface of parafilm m strip. The result showed that *Bacillus salamalya* droplet had flat shape compared to semi-sphere shape of distilled water, which is the negative control. *B. salmalaya* had larger diameter of droplet compared to distilled water, which is 0.6 cm and 0.5 cm respectively.

From the observation, we can say that the presence of biosurfactant in *B. salmalaya* droplet influenced the flat shape and large diameter of droplet. Besides, we can say that biosurfactant reduced the surface tension between the droplet and hydrophobic surface of parafilm m strip causing the flat droplet shape. Meanwhile, in distilled water, there was no biosurfactant presence, thus the semi-sphere shape and smaller diameter of droplet.

Loganathan et al. (2010) reported that biosurfactant production might be also detected by using emulsification activity. Emulsification activity is a test where biosurfactant is tested whether it can emulsify oil or not. The emulsification of oil will produce cloudy layer when oil is mixed with biosurfactant. The emulsification occurs because oil is emulsified with oil.[12]

It was reported that biosurfactant produced from P. *aeruginosa* RB 28 emulsified hydrocarbons, hydrocarbon mixtures and vegetable oils and formed stable emulsions.[15] The ability of the produced biosurfactants to emulsify kerosene and crude oil is an important feature for bioremediation applications.[16]

In this study, emulsification formed when crude oil was mixed with biosurfactant. The emulsification layer occurred after test tube containing oil and biosurfactant mixture was mixed for few minutes. We can clearly see the distinct layer of emulsified oil and non-emulsified oil in the test tube. This test was repeated using positive control, Sodium Dodecyl Sulphate (SDS) and the result showed completely emulsified

oil layer was formed. SDS is a synthetic surfactant, which is commonly used as emulsifier. Thus, the test result in emulsification test showed positive result. Meanwhile, emulsification test done with negative control, which is distilled water, showed negative result of emulsification.

When compared to the EUCAST database, Dadrasnia and Ismail (2015) discovered that *Bacillus salmalaya* strain 139SI was most resistant to Ciprofloxacin, Amikacin, Doripenem, Ampicillin, and Linezolid, but susceptible to Aztreonam.[5]

Bacillus salmalaya had shown great sensitivity towards most antibiotics which means that it was safe to be used and handled. This analysis was very important to make sure the safety in handling the bacteria and the bacteria is safe to be used.

Based on zone of inhibition size, differences in size of only 2 to 3 mm can mean the difference between describing bacteria or isolates as being sensitive to the antibiotic or resistant, which means the antibiotic would be ineffective. Zone of inhibition that falls between sensitive and resistant is called as intermediate.

In this study, *B. salmalaya* had formed inhibition zone towards Erythromycin 15, Chloramphenicol, Tetracycline, Ampicillin and Gentamicin. From the result interpretation, Bacillus salmalaya was sensitive to Erythromycin, Chloramphenicol and Ampicillin, and had intermediate sensitivity towards Tetracycline. This mean that these antibiotics are effective in stopping or preventing the growth of bacteria.

However, this strain was resistant to Sulfamethoxazole and Gentamicin. There was zone of inhibition formed surrounding Gentamicin antibiotic disc, but it was stated as resistant because the zone of inhibition size fall into resistant category where the size was not large enough to become sensitive towards the antibiotic. There was no zone of inhibition surrounding Sulfamethoxazole 25, which indicates that this antibiotic could not stop the growth of bacteria thus it also indicates that this antibiotic cannot be used to combat any infection caused by *B. salmalaya* as this strain was resistant towards the antibiotic.[17]

CONCLUSION

Bacillus salmalaya had shown positive result for all biosurfactant screening tests. In haemolytic test, *Bacillus salmalaya* showed positive result of beta haemolytic activity where colourless, clear zone area formed surrounding the colonies. In drop collapsed and parafilm M test, *Bacillus salmalaya* had shown good surface reduction property of biosurfactant where flat drop of supernatant containing biosurfactant can be observed. It also gave positive result in oil spreading test where biosurfactant formed clear zone on oil layer. The higher the volume and concentration of biosurfactant, the larger the size of clearance zone. In emulsification test, biosurfactant emulsified crude oil forming cloudy layer, which indicated that oil is emulsified. Positive result in cleaning activity of oil container shows the potential of *Bacillus salmalaya* in industries to be used as biosurfactant. In Kirby-Bauer test, all 15 isolates had shown different sensitivity towards different antibiotics. *Bacillus salmalaya*was sensitive towardsErythromycin, Chloramphenicol and Ampicillin. It had intermediate sensitivity towards Tetracycline but resistant towards Sulfamethoxazole 25 and Gentamicin. If any infections occur, Erythromycin, Chloramphenicol, Ampicillin and Tetracycline can be used to combat the infection.

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Table 1: shows the classification of bacteria sensitivity as sensitive, intermediate or resistant towards antibiotics used based on the zone of inhibition measurement

Antibiotics used	Zone diameter measured (mm)		
	Sensitive	Intermediate	Resistant
Ampicillin	≥ 10	Not applicable	≤ 9
Chloramphenicol	≥ 23	12-22	≤ 11
Erythromycin	≥ 19	16-18	≤ 15
Gentamycin	≥ 23	Not applicable	≤ 22
Sulfamethoxazole	≥ 21	11-15	≤ 10
Tetracycline	≥ 33	16-32	≤ 15

Table 2: Shows the diameter of clearance zone decrease with the increase the amount of distilled water added to 0.23 g of freeze-dried supernatant powder.While diameter of clearing zone increases with the increases in volume of supernatant used when 700 µl of crude oil was used

Volume of distilled water added to freeze-dried supernatant (ml)	Diameter of clearing zone (cm)
0.5	5.4
1.0	4.0
1.5	2.2
Volume of supernatant (µl)	Diameter of clearing zone (cm)
200	0.2
300	1.0
400	3.2
500	4.6
600	5.2

Table 3: Shows the zone inhibition measurement for 14 bacterial isolates towards Gentamycin, Sulfamethoxazole, Tetracycline,Ampicillin, Chloramphenicol and Erythromycin antibiotics in disc diffusion test.

Isolates No.	Antibiotic Disc (ug) / Zone Inhibition measurement (mm)					
	Gentamycin (Gm 30)	Trimethoprim/ Sulfamethoxazole (Sxt 25)	Tetracycline (Te 30)	Ampicillin (Am 10)	Chloramphenicol (Cm 30)	Erythromycin (E 15)
B. salmalaya	19	6	21	10	25	24
HC1	22	6	21	9	25	22
HC2	18	6	12	8	19	20
HC3	19	6	24	8	25	25

Table 3: *(Continued)*

Isolates No.	Antibiotic Disc (ug) / Zone Inhibition measurement (mm)					
	Gentamycin (Gm 30)	Trimethoprim/ Sulfamethoxazole (Sxt 25)	Tetracycline (Te 30)	Ampicillin (Am 10)	Chloramphenicol (Cm 30)	Erythromycin (E 15)
HC4	24	6	19	8	29	28
HC5	20	6	21	12	23.3	24.5
HC6	24	6	19	9	28	27
HC7	19.5	6	21	9	24	24
HC8	23	6	22	8	27	28
HC9	25	6	6	25	30	6
HC10	23.5	6	19	7	24	26.5
HC11	19	6	21	10	25	24
HC12	19	6	20	8	23	23
HC13	19	6	15	7	24	25
HC14	19	6	14	7	24	23

Table 4: Shows the interpretation of antibiotic test of the bacterial isolates in term of sensitive, intermediate or resistant towards Ampicillin, Chloramphenicol, Erythromycin Gentamycin, Sulfamethoxazole and Tetracycline antibiotics.

Isolates No.	Antibiotic Disc (ug) / Sensitive, intermediate or resistant					
	Ampicillin (Am 10)	Chloramphenicol (Cm 30)	Erythromycin (E 15)	Gentamycin (Gm 30)	Trimethoprim/ Sulfamethoxazole (Sxt 25)	Tetracycline (Te 30)
B. salmalaya	Sensitive	Sensitive	Sensitive	Resistant	Resistant	Intermediate
HC1	Resistant	Sensitive	Sensitive	Resistant	Resistant	Intermediate
HC2	Resistant	Intermediate	Sensitive	Resistant	Resistant	Resistant
HC3	Resistant	Sensitive	Sensitive	Resistant	Resistant	Intermediate
HC4	Resistant	Sensitive	Sensitive	Sensitive	Resistant	Intermediate
HC5	Sensitive	Sensitive	Sensitive	Resistant	Resistant	Intermediate
HC6	Resistant	Sensitive	Sensitive	Sensitive	Resistant	Intermediate
HC7	Sensitive	Sensitive	Sensitive	Resistant	Resistant	Intermediate
HC8	Resistant	Sensitive	Sensitive	Sensitive	Resistant	Intermediate
HC9	Sensitive	Sensitive	Resistant	Sensitive	Resistant	Resistant
HC10	Resistant	Sensitive	Sensitive	Sensitive	Resistant	Intermediate
HC11	Sensitive	Sensitive	Sensitive	Resistant	Resistant	Intermediate
HC12	Resistant	Sensitive	Sensitive	Resistant	Resistant	Intermediate
HC13	Resistant	Sensitive	Sensitive	Resistant	Resistant	Resistant
HC14	Resistant	Sensitive	Sensitive	Resistant	Resistant	Resistant

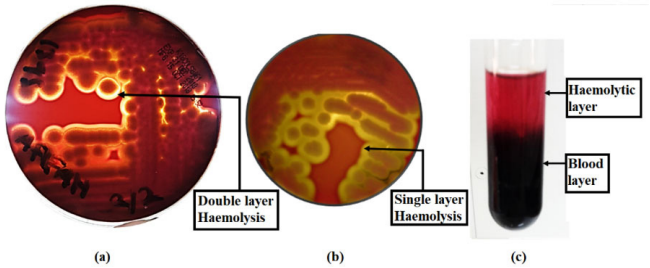

Figure 1: (a) Arrow shows the haemolytic layer which is the clear zone formed around the colonies of 139SI after 24 hours of incubation at 37°C. Strain 139SI had shown strong haemolytic activityThe arrow shows double layer haemolytic zone. There were two distinct layers of clear zone observed around the colonies. (b)The arrow shows one layer haemolysis formed around the colonies. (c)Arrows show haemolytic layer and blood layer in a test tube. This showed that *Bacillus salmalaya* had strong haemolytic activity.

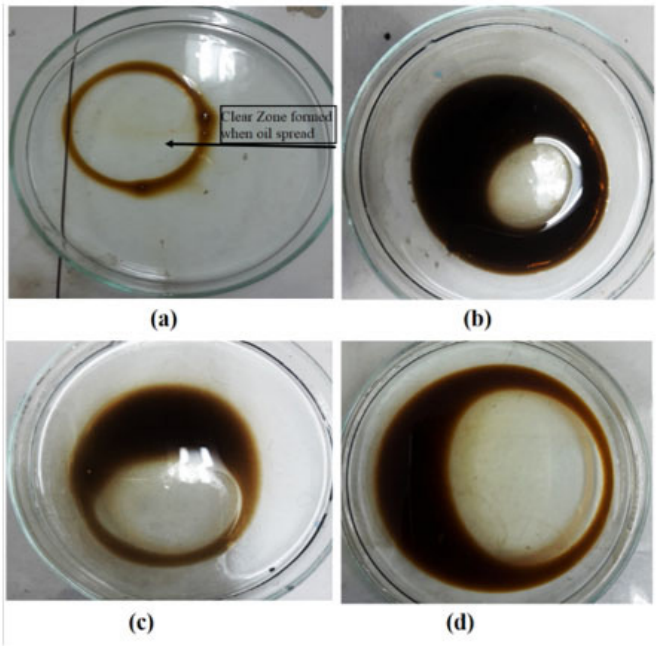

Figure 2: (a)The arrow shows clear zone or displacement zone of oil after biosurfactant was dropped onto the oil layer in the petri dish. *Bacillus salmalaya*showed positive result to oil displacement test. (b)Small displacement zone which was 2.2 cm formed when low concentration biosurfactant was added onto the oil layer in the petri dish.(c)Medium size of clearing zone which was 4.0 cm formed when medium concentration of biosurfactant was added onto oil layer in the petri dish. (d) Large clearing zone which was 5.4 cm of oil formed when high concentration biosurfactant was added onto oil layer in petri dish.

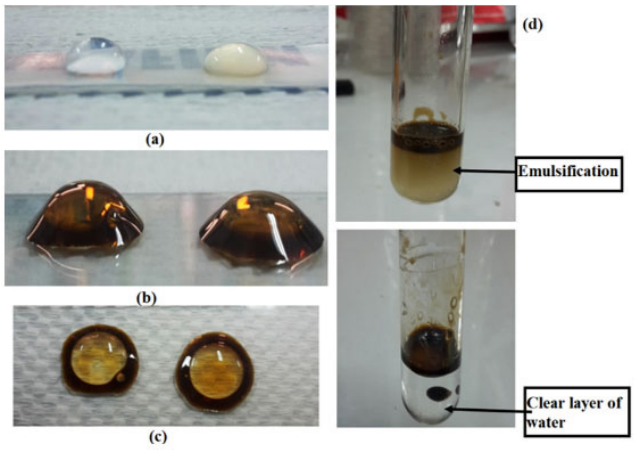

Figure 3: (a) From left was the droplet of distilled water and to the right is *B. salmalaya* cell free broth droplet. Droplet of *B. salmalaya* was flat compared to semi-sphere shape of distilled water droplet. Droplet of *B. salmalaya* had 0.6 cm diameter while distilled water had 0.5 cm diameter. (b) (a) was the droplet of distilled water and (b) was*B. salmalaya* cell free broth droplet. Droplet of *B. salmalaya* was flatter compared to distilled water droplet (c): (a) was the negative control droplet and (b) was*B.salmalaya* droplet on oil layer. *B. salmalaya* droplet had bigger diameter size compared to distilled water diameter, which were 0.9 cm and 0.7 cm respectively.(d)Arrow in (a) showed emulsification layer formed in the mixture of B. salmalaya cell free broth and crude oil mixture while in (b) arrow showed clear layer in distilled water and oil mixture.

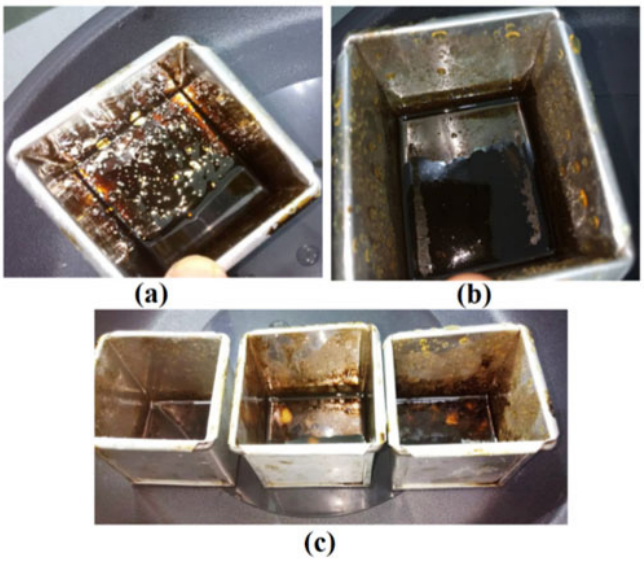

Figure 4: (a) The condition of oil container after being sprayed with bacteria broth containing biosurfactant. (a) was before cleaning activity and (b) was after cleaning activity.(b) shows the container containing oil after cleaning activity by bacteria culture, detergent and distilled water. (c)From left was container cleaned with *B.salmalaya* bacterial culture followed by detergent and lastly distilled water.

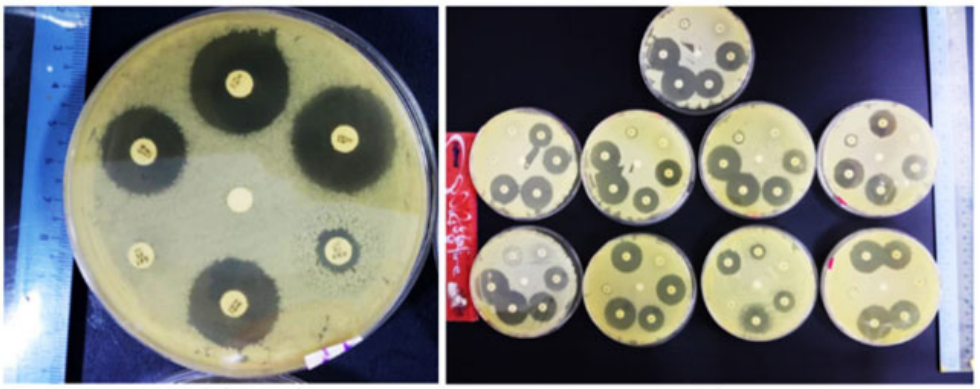

Figure 5: (a) showing*Bacillus salmalaya* antibiotic test plate(b)shows plates of other bacteria isolates tested with Kirby-Bauer test.