

Isolation and Degradation Study on Carbosulfan Containing Pesticide of Biosurfactant-Producing Bacteria From Belawan Sumatera

Frans Grovy Naibaho¹, Erman Munir² and Nunuk Priyanti²

¹Undergraduate student, Department of Biology, Faculty of MIPA, Universitas Sumatera Utara, Jln. Bioteknologi No.1 Kampus USU, Padang Bulan, Medan 20155

²Department of Biology, Faculty of MIPA, Universitas Sumatera Utara, Jln. Bioteknologi No.1, Kampus USU, Padang Bulan, Medan 20155.

Abstract. Uncontrol use of synthetic pesticide causes environmental pollution and leaves its residue in soil and water. The objective of this research was to obtain bacterial isolates capable of producing biosurfactant from Belawan North Sumatera and to observe their biosurfactant activities as well as their potential in degrading of carbosulfan pesticide. Bacteria were isolated using selective medium, Bushnell-Hass Agar (BHA) containing 2% of pesticide with carbosulfan as the active compound. The result showed that all bacterial isolates were able to grow on the medium and consumed carbosulfan as the carbon source. The highest cell population was shown by isolate Sp.1 with total count was 7.6×10^9 CFU/ml. The highest biosurfactant activity was shown by isolate Sp.6 with the volume of emulsion was 5.627 cm³, meanwhile the highest production of biosurfactant shown by Sp.7 with concentration was 54.6 ppm. Two isolates, Sp.1 and Sp.6 were selected for further test to study their abilities in carbosulfan degradation. Both isolates were able to degrade carbosulfan completely after 21 days of incubation.

Keyword: Carbosulfan, biodegradation of pesticide, bioremediation, biosurfactant

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1 Introduction

The use of pesticide to control pest and plant diseases still plays an important role in agriculture. It is required to improve both quality and quantity of the crop [1]. Sutamihardja [2] reported that pesticide, mainly insecticide application is an important part of plant protection. Yet, unwise application of pesticide including insecticide may cause serious problem since its residue polluted soil and both surface and ground water [3].

Pesticide residual pollution has various negative effects on life. Its accumulation result in agricultural land pollution and if it enters the food chains its toxic compound may cause severe

*Corresponding author at: Jl. Bioteknologi No. 1 Kampus Univ. Sumatera Utara, Padang Bulan, Medan, Indonesia

E-mail address: nunuk@usu.ac.id

diseases such as mutation, cancer as well as *Chemically Acquired Deficiency Syndrome* (CAIDS) [4].

Pesticide residue that pollutes the environment consists of hydrocarbon compounds. These compounds are quite stable in the environment and are toxic to organisms [5]. Carbosulfan included in carbamat group and is an active compound of pesticide which is widely used mainly in Sumatera Utara. It has an aromatic ring structure, and is a potent inhibitor of cholinesterase, an enzyme vital for functioning of the central nervous system. As a result, it is highly toxic to mammals with 50% lethal dose ranging from 90 – 250 mg/kg in rat. Therefore its concentration should be strictly controlled.

An effective and safe method to heal the polluted area is bioremediation technique. It is a technique to remedy, to remove pollution from the environment using microorganism. The microbe functions as the agent of remediation through adsorption, and oxidation and reduction reactions. To metabolism those compounds, microorganism secretes biosurfactant to increase their solubility which in turn will be attacked by hydrolytic enzymes. Bertrand [6] reported that microorganism with high production of biosurfactant capable of degrading aromatic hydrocarbon effectively.

Bacteria isolated from the sea abled to produce biosurfactant and to degrade an aromatic hydrocarbon naphthalene [7]. Furthermore some bacteria isolated from Belawan sea, Sumatera Utara were reported to degrade glifosate [8]. Parra [9] said that biosurfactant-producing bacteria could be readily found in area contaminated by aromatic hydrocarbon compounds. This study reports the potency of bacterial isolates from Belawan sea in degrading carbosulfan.

2 Materials and Methods

Chemicals

All chemicals were from sigma except the pesticide was bought from a local market. Biosurfactan concentration was determined by analyzing rhamnose concentration using spectrophotometer while carbosulfan residue was detected using Gas Chromatography ZB-1 (30m x 0.25mm x 0.25 μ m) at 300°C.

Isolation of microorganism and characterization

The Biosurfactant-producing bacteria were isolated from Belawan sea, a big public sea port in Medan Sumatera Utara. The water sample was inoculated on Bushnel Haas Agar medium containing pesticide as the carbon source. The growing colonies were purified and characterized morphologically and biochemically. All colonies were maintained at slant agar media.

All colonies were characterized based on their shape, edge, color, elevation of colonies, cell's shape and arrangement as well as Gram staining. Simple biochemical test including ability to metabolize citrate, starch, gelatin. hydrogen sulfide and catalase were performed to obtained isolates which were different each other.

Estimation of cell population

Bacterial cell population was determined by Standard Plate Count method. 0.1 ml of each culture was spread on plate count agar (PCA) and was incubated for 24 hrs. The bacterial growth was observed on day 7, 14 and 21th of incubation. The number of colonies was counted using colony counter based on following formula:

$$\text{Number of cells} = \text{Numbers of colony} \times \frac{1}{\text{dilution factor}} \text{ (CFU/ml)}$$

Biosurfactant activity

The activity of biosurfactant secreted by each isolate was measured using *Drop Collapsing Test* method [10]. For this purpose, the supernatant and n-hexan (1:1 v/v) were taken into a tube vortexed for 2 min and left for 24 hrs. In this case we used 2 ml of supernatant and 2 ml of n-hexan. The volume of emulsion which is formed was measured.

Quantification of Biosurfactant Concentration

To analyze the concentration of biosurfactant which is produced during incubation time, a standard curve of rhamnose was used. A serial of rhamnose dilution in sodium bicarbonate (NaHCO_3) 0.05M, 2 ml each (10, 50, 100 dan 200 ppm), was added with 3.6 ml orcinol solution, heat to boil, let them cool at room temperature for about 15 min. Measure their absorbance at λ 421 nm. The regression line equation of rhamnose standard curve was determined using Least Square method.

Bacterial growth condition for biosurfactant production

A 24 hrs old culture was used as inoculum. Two ml of inoculum of each isolate, which is equal to Mc Farland standard was inoculated into 98 ml of BHB medium containing 2% of carbosulfan pesticide. The cultures were incubated in water bath shaker at 150 rpm, 30°C, and in dark condition for 21 days. The cultures were centrifuged at 6,000 rpm for 10 min. four ml of supernatant was added with 2 ml of diethylether and shake vigorously to extract the biosurfactant. The ether layer was separated carefully. The extraction was repeated three times. The ether layer was dried and dissolved into 2 ml of NaHCO_3 0,05 M solution, vortexed and added with 3.6 ml of orcinol solution. The solution is heated it, let it cool for 15 min and measure its absorbance at λ 421 nm. The quantification of biosurfactant concentration was done on 7, 14, and 21 days of incubation.

Biodegradation of carbosulfan by local isolates

Bacterial degradation of carbosulfan was studied in mineral BHB medium containing 2% of carbosulfan pesticide. Twenty ml of inoculum of each isolate which is equal to Mc Farland standard was added aseptically into 980 ml of BHB medium. The cultures were grown in waterbath shaker at 150 rpm, temperature of 30°C in the dark condition for 21 days. Cultures were centrifugated at 6,000 rpm for 10 minutes, the supernatant was used for analysing the residue of carbosulfan.

Culture supernatant (300 ml) was taken into separated funnel, added with 100 ml of dichloromethane and shake vigorously. Two layers were formed, the dichloromethane layer was separated. The extraction was repeated twice by addition of 50 ml of dichloromethane and shake well. Dichloromethane was evaporated, added with a mixture of isooctane : toluene (9:1) to get 3 ml volume. The carbosulfan concentration was analyzed using Gas Chromatography Varian CP-3800 column ZB-1 (30m x 0,25mm x 0,25 µm) with detector ECD, column temperature 200-270°C, detector temperature 300°C, injector temperature 280°C

3 Result and Discussion

Characters of obtained isolates

Nine isolates were able to grow on BHA medium indicating that those bacteria could metabolize carbosulfan and use the compound as the carbon source. They varied in colony morphology such as: colony shape were circular and irregular; edge of colony are entire, undulate and lobate; elevation were flat and convex. Base on microscopic observation it was shown that among 9 isolates, 7 isolates were Gram negative while the rest are Gram positive. Five isolates are cocci while 4 others are bacilli. The result of biochemical test gave evident that those isolates are different.

Bacterial Cell Population

The bacterial growth during 21 days of incubation was shown in Figure 1 below. It was known that all bacterial isolates increased in cell numbers at different rate. The initial population was 2.0×10^6 cells/ml.

The highest cell growth on day 7th was shown by isolate Sp. 7 which was 46×10^8 CFU/ml while isolate Sp. 9 showed the lowest one which was 22×10^8 CFU/ml. It was assumed that isolate Sp. 7 was able to adapt more quickly to metabolize carbosulfan and used the compound as the carbon and energy sources. As the result, Sp. 7 has the shortest lag phase compare to others. On the day 14th all isolates decreased in cell numbers, except isolate Sp. 1 and Sp. 6 which showed their best growth with the cell numbers were 19×10^8 CFU/ml and 12×10^8 CFU/ml. Meanwhile

isolate Sp. 7 showed the lowest population, 5.9×10^8 CFU/ml. Decreasing in cell numbers could be caused by limited available carbon source.

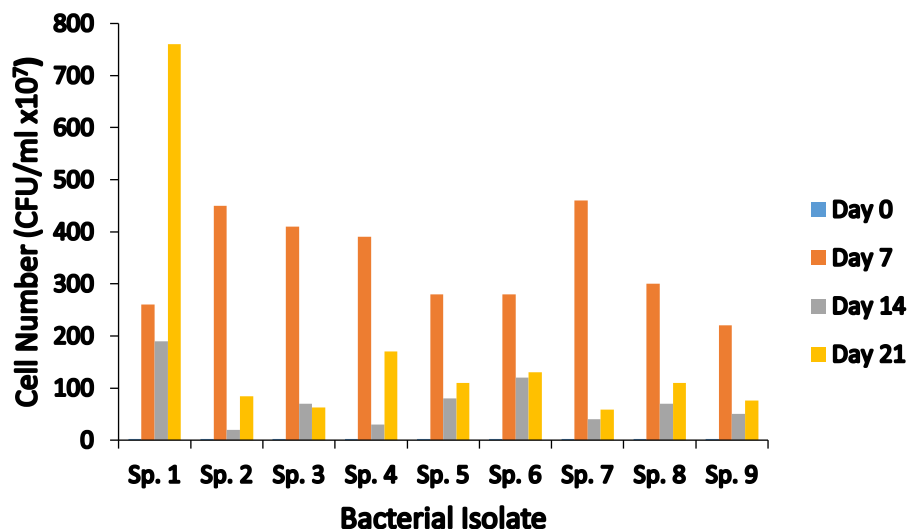


Figure 1. Growth of isolates in BHB medium during 21 days of incubation

Some isolates still able to increase their viable cells on day 21th, mainly isolates Sp. 4 which increased significantly. For these isolates, it was assumed that they were able to use carbon source other than carbonsulfan. In addition to active compound, pesticide also contains surfactant that function to increase solubility of active compound in water. This surfactant could also serve as carbon source. Banat [11] said that biosurfactant-producing bacteria increase their metabolism since the biosurfactant secreted on the surface of the cells enhances nutrition transport into the cells and promote their growth rate.

Biosurfactant Activity

The activity of biosurfactant could be examined from its ability to lower surface and interfacial tensions, CMC (critical micelle concentration) and formation of stable emulsion.

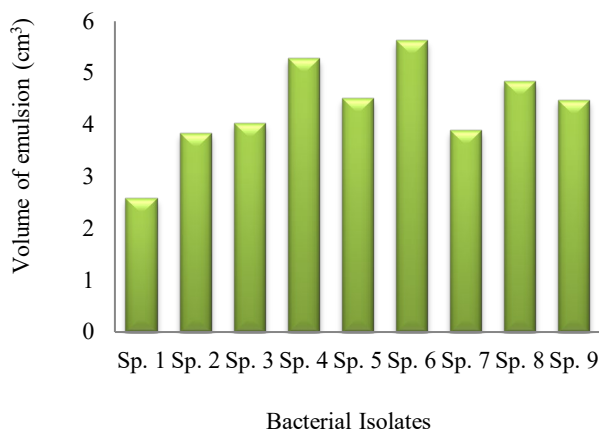


Figure 2. Biosurfactant activities of 9 isolates tested.

In this study we observed the formation of stable emulsion. Different biosurfactant activities were shown by all isolates as shown by Figure 2. The highest biosurfactant activity was shown by isolate Sp. 6 with the emulsion volume was 5.627 cm³ followed by isolate Sp. 4 which was 5.273 cm³. Meanwhile isolate Sp. 1 had the lowest activity with emulsion volume 2.582 cm³.

Prak [12] reported that the activity of biosurfactant depend on several factors such as: the structure and composition of biosurfactant, ion strength, the presence and types of additive compounds, and temperature. Isolate Sp. 1 showed the lowest biosurfactant activity despite its highest cell growth (76x10⁸CFU/ml). On the contrary, isolate Sp. 6 which had much less cell number (12x10⁸ CFU/ml) showed the highest biosurfactant activity.

Biosurfactan Production

All isolates have shown to produce biosurfactant with different yield as well as duration of incubation. In general isolates secretes more biosurfactant as the incubation time is longer, except isolate Sp. 2, Sp. 6 and Sp. 8. The later isolates reached the highest production of biosurfactant on day 7th and decreased significantly afterward. Isolate Sp. 7 showed the highest biosurfactant concentration on day 14th which was 54.6 ppm and decreased sharply on the day 21th. Meanwhile isolate Sp. 1 showed the lowest yield of biosurfactant which was only 10.4 ppm.

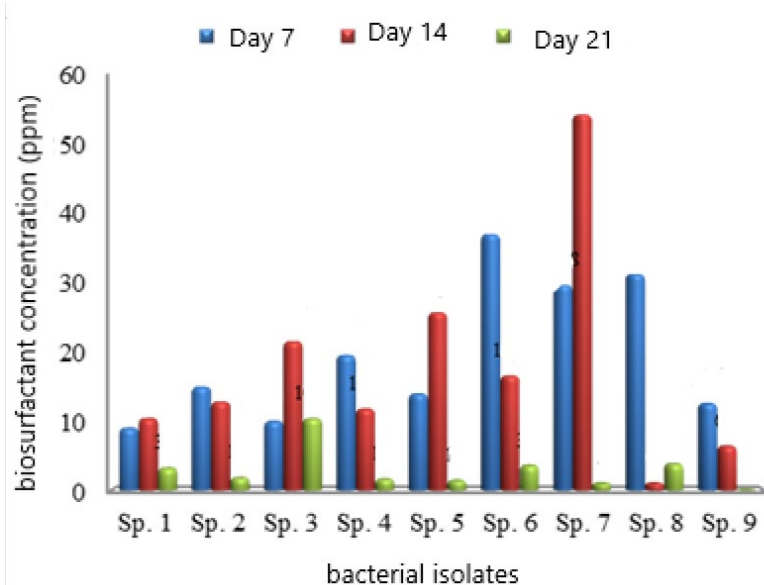


Figure 3. Concentration of biosurfactant produced by isolates.

Compare to the data of bacterial growth which showed that the isolate Sp. 7 grew steadily up to day 7th of incubation and decreased sharply on day 14th and 21th, the result seemed to be not correlated. It is likely that the bacteria secreted more biosurfactant up to day 14th to metabolize limited source of substrate. The high population of isolate Sp. 1 produced very less biosurfactant

compare to others. There are two types of biosurfactant produced by microorganism. First, biosurfactant is secreted into the medium so it could be detected when the supernatant is analyzed. The second type is biosurfactant which is secreted but it still attached to the cell wall, is not released into the medium. The presence of this biosurfactant can't be detected when the supernatant was analyzed. Rufino [13] reported that 8 g/L of crude biosurfactant could be extracted from *Candida lipolitica* after 72 hours of incubation and showed a growth-associated production. Another report from Sobrinho [14] said that *Candida sphaerica* had a biosurfactant yield of 4.5 g/L after 144 hours of incubation.

Other factor that affect biosurfactant production is environmental condition such as temperature, pH and salinity. It was assumed that isolate Sp. 7 grew best at pH 7 and secreted more biosurfactant. Other *Pseudomonas* increased the biosurfactant production as increasing the pH from 6 to 7. The the medium pH higher than 7 the biosurfactant production decreased [15].

Carbosulfan degradation

Base on above results, growth rate, biosurfactant activity, and the production of biosurfactant, both isolate S.1 and Sp.6 were chosen to be tested on their abilities in degrading carbosulfan. The result showed that the bacteria were able to degrade completely carbosulfan present in medium so no more carbosulfan could be detected on day 21th. Meanwhile the concentration of carbosulfan in the control reduced 16% as shown by the following figure.

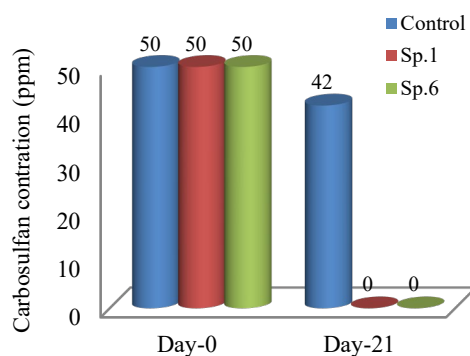


Figure 4. Carbosulfan residue after 21 days of incubation.

Decreasing carbosulfan concentration in control indicated that carbosulfan could be degraded naturally without participation of microorganism. The presence of light, photooxidation and temperature affect the rate of degradation. In this study, effect of light is excluded since the bacterial cultures were incubated in the dark. It was assumed that naturally, carbosulfan could be degraded very slowly at room temperature.

The ability of both isolate Sp.1 and Sp.6 to degrade carbosulfane completely confirmed with the data about their ability to grow in medium containing carbosulfan as the sole source of carbon and energy. Those isolates showed high activity of biosurfactant and produced enough

biosurfactant to metabolize carbosulfan completely. Warsito [16] reported that bacterial isolate that produced high concentration of biosurfactant were able to reduce naphthalene concentration up to 94%.

4 Conclusion

This investigation clearly indicated that bacterial species isolated from Belawan sea were able to grow and metabolize carbosulfan. The ability of isolates to break down carbosulfan was closely related to the ability of isolates to produce biosurfactant. The biosurfactant was well function to emulsify carbosulfan and promote carbosulfan degradation.

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