

The Potency of Local Bacterial Isolates Encapsulated Within Sodium Alginate in Carbofuran Degradation

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Abstract. Research on the viability of bacteria encapsulated within sodium alginate and their potential in carbofuran degradation has been done. A total of 8 bacterial isolates have been isolated from slaughter house waste. A 100 ml of *Bushnell-Hass Broth* (BHB) medium containing 146.982 ppm of carbofuran was used as a medium. As much as 2 gr of beads which equal to 10^8 cells.ml⁻¹ was inoculated into each medium culture and incubated for 15 days at ambient temperature and was shaken at 100 rpm. Analysis of carbofuran residues using *High Performance Liquid Chromatography* (HPLC) showed that the best 2 isolates, DN 1 and OR 2, were able to decrease carbofuran phenol concentration up to 30.37 % and 32.09% respectively compared to control. These results suggested that no significant different from the ability of free cell which decreased carbofuran phenol concentration up to 32.54% and 28.29%.

Keywords: biodegradation, carbofuran-phenol, immobilization, local isolate

1. Introduction

Indonesia is an agricultural country with high potency in agricultural and plantation industries. Many efforts such as the use of various types of pesticides, irrigation and fertilizer have been done to improve the quality and quantity of agricultural production. On the other hand, unwise application of pesticides results in accumulation of pesticides residue in the environment and causes a serious environmental problem. The main active compounds of pesticides such as demeton-S-methyl sulfonate, phthalimide, 1,2,3,6-tetrahydrophthalimide and others are carcinogens. Therefore their concentration must be strictly controlled [1].

Pesticides may undergo degradation naturally through photo-oxidation, as well as chemical and biological process. Biodegradation of pesticides by microorganism including bacteria, as well as fungi offers many advantages compared to chemical and physical degradation. Various species of bacteria such as *Pseudomonas*, *Flavobacterium*, *Arthobacter*, *Rhodococcus* and *Stenorophomonas* have been reported to degrade pesticides.

Lipolytic bacteria isolated from slaughter house waste such as *Bacillus* sp. *Pseudomonas* sp. [2] and *Halobacillus trueperi* and *Rhodobacteraceae* bacterium [3] breakdown lipid hydrocarbon compounds by producing surfactants. The effectivity of microorganism in degrading hydrocarbon



compounds such as pesticides is influenced by various environmental factors, and therefore these microorganism has to have good endurance. In order to maintain bacterial capabilities from environmental influences, it is necessary to protect the bacteria, one of which is by encapsulation [4]. Research on the encapsulation of biosurfactant producing bacteria in degrading carbofuran has not been widely studied before. Bacterial encapsulation can sustain and even increase the potential and viability of bacteria in decomposing pesticides in the environment. Therefore, it is necessary to study the viability of local bacteria from slaughter house waste encapsulated in sodium alginate in carbofuran degradation in vitro.

2. Methods

2.1 Isolation and Characterisation of Biosurfactant-producing Bacteria from Slaughter House Waste

As much as 1 g of waste was diluted into 9 ml of distilled water, and homogenized using vortex. 0.1 ml of the suspension was inoculated on the surface of Nutrient Agar medium, incubated at ambient temperature for 24 hours. The grown colonies were observed, characterized and purified.

The purified bacteria were stored in Tryptic Soy Agar (TSA) for further use. Characterization of bacteria including macroscopic observation such as colony morphology (shape, color, edge, elevation) and microscopic observation such as cell shape, arrangement and Gram staining. The observed biochemical properties including starch hydrolysis test, citrate test, hydrogen sulfide test, motility test and catalase test. Bacterial isolates obtained were then grown and stored on TSA medium before used for further observation.

2.2 Measurement of Cell Growth

Two ml of bacterial suspension with the cell density of 10^9 cells.ml⁻¹ was inoculated into 98 ml of BHB medium containing 146.982 ppm of carbofuran as the sole carbon source. The culture were incubated at orbital shaker at 100 rpm at room temperature. Cell growth were observed at day 5, 10 and 15 using Standard Plate Count (SPC) method.

2.3 Biosurfactant Activity Screening

Screening of biosurfactant activity was carried out using modified Drop Collapsing Test method [5]. Bacterial isolates were grown in BHB medium with 2% of dextrose. Two ml of bacterial suspension with 10^9 cells.ml⁻¹ was inoculated into 98 ml BHB medium containing 2% dextrose. The cultures were then incubated in an orbital shaker at 100 rpm at room temperature for 15 days. At the end of incubation, each culture in incubation medium was centrifuged at 6,000 rpm for 10 min, in which then 4 ml of the supernatant from each isolate was added with 4 ml of N-hexan and 2 ml of distilled water in test tube. The mixture was shaken vigorously using vortex for one minute. The stable emulsion formed in this process were then measured afterwards.

2.4 Production of Biosurfactants

To encourage bacteria to produce biosurfactant, isolates were grown in BHB medium containing 146.982 ppm of carbofuran. Two ml of bacterial suspension with 10^9 cells.ml⁻¹ was inoculated into 98 ml of BHB medium containing 146.982 ppm carbofuran [6]. The culture was then incubated at orbital shaker at 100 rpm at room temperature for 15 days and then centrifuged at 6,000 rpm for 10 minutes to separate the supernatant from free cells. Four ml of supernatant was extracted with 2 ml dietilether for 5 min. The ether layer was aspirated using a pipette, dried and then redissolved in 2 ml of sodium bicarbonate (Na.HCO₃) 0.05 M solution. The solution was then mixed well and carefully added with 3.6 ml of orsinol, heated to boil, cooled at room temperature for 15 minutes, and measured using spectrophotometer with a wavelength of 421 nm. Biosurfactant content was measured based on Rhamnose standart curve.

2.5 Bacterial Encapsulation

Two bacterial isolates were selected for further encapsulation based on cell growth, biosurfactant production, and biosurfactant activity. Bacterial encapsulation using sodium alginate was done using extrusion method. Three gram of sodium alginate was dissolved in 100 ml of distilled water and boiled. The solution was sterilized at 121 °C with 2 atm pressure for 15 minutes and cooled to 30 °C. Bacterial cells suspension was added to the solution and mixed well afterwards. The mixture was loaded into 3 ml of syringe and was carefully dropped the mixture into CaCl₂ solution to form alginate beads in which the bacterial cells have been trapped.

2.6 Effectiveness of Encapsulation

To evaluate the effectiveness of encapsulation, a modified viability test [4] was conducted. The viability test was performed after the beads have been stored for 5, 10, and 15 days at room temperature. One gram beads was dissolved in water and 0.1 ml of suspension was plated on the surface of NA medium to observe the colony growth.

2.7 Culture Condition For Carbofuran Degradation

To determine the potential of bacteria to degrade carbofuran, both isolate as free cells (2 ml bacterial suspension) as well as encapsulated cells (2 g of beads), was inoculated into 98 ml BHB medium containing 148.982 ppm of carbofuran as the sole carbon source. The culture were incubated for 15 days at room temperature on orbital shaker at 100 rpm [7]. The bacterial growth and the carbofuran residue were observed on day 5, 10, and 15 after inoculation. Bacteria released from beads were measured using TPC method, while the carbofuran residue was analyzed using High Performance Liquid Chromatography (HPLC). A BHB medium containing 148.982 ppm carbofuran without the addition of neither free cells nor beads was used as a control.

3. Results and Discussion

3.1 Bacterial Characteristic

Eight isolates were successfully isolated from slaughter house waste. Each isolate showed different characteristic based on their colony and cell morphology as presented in Table 1 below.

Table 1. Morphology colony and cells and Gram staining

Bacterial Isolates	Colony morphology				Cell Morphology		Gram
	Form	Edge	Elevation	Color	Form	Setup	
DB 1	Irregular	Lobate	Flat	Beige	Basil	Diplobasil	+
DB 2	Rhizoid	Curled	Flat	White	Basil	Monobasil	+
DN 1	Irregular	Circular	Flat	Beige	Basil	Diplobasil	-
DN 2	Irregular	Lobate	Flat	White	Focus	Streptococcus	-
EM 1	Irregular	Filamentous	Raised	White	Basil	Monobasil	-
EM 2	Irregular	Filamentous	Flat	White	Focus	Monococcus	+
OR 1	Irregular	Entire	Flat	Beige	Basil	Streptobacil	+
OR 2	Rhizoid	Filamentous	Raised	White	Focus	Diplococcus	+

Biosurfactant-producing isolates from areas contaminated with petroleum showed a large amount of gram-negative bacterium that is as much as 155 isolates and a small portion is gram-positive bacteria as much as 17 isolates [8].

To ensure that they are different isolates, some simple and common biochemical tests have been done. The result are shown in the Table 2 below

Table 2. Biochemical Test of Eight Isolates

Bacterial Isolates	Biochemical Test						
	Starch Hydrolysis	Gelatin Hydrolysis	Citrate Test	Hydrogen Sulfide		Motility	Catalase
				<i>Slant</i>	<i>Butt</i>		
DB 1	+	-	+	Yellow	Yellow	+	-
DB 2	+	-	-	Yellow	Yellow	+	-
DN 1	+	-	+	Yellow	Yellow	+	-
DN 2	+	-	+	Red	Yellow	+	-
EM 1	-	-	+	Red	Yellow	+	-
EM 2	+	-	-	Yellow	Yellow	+	-
OR 1	-	-	+	Red	Yellow	+	-
OR 2	-	-	+	Red	Yellow	+	-

Note:(+) = Positive Test;(-) = Negative Test

Some isolates such as DB1, DB2, DN1, EM1, EM2, and OR1 shared common characteristics of biochemical test, but showed different results on colony and cell morphologies test.

The main objective of morphological characterization, biochemical test and gram staining is to distinguish isolates one from another. Each of the isolate should show different characteristics in each test performed. Bacterial identification can be based on morphology, staining properties, colony growth patterns and some growth reactions in carbohydrates and the use of amino acids [9].

3.2 Bacterial Growth

The bacterial growth on day 5, 10, and 15 was observed from the isolates, including control to ensure that there was no contamination during the treatment. All isolates shared the same pattern as shown in Figure 1 below.

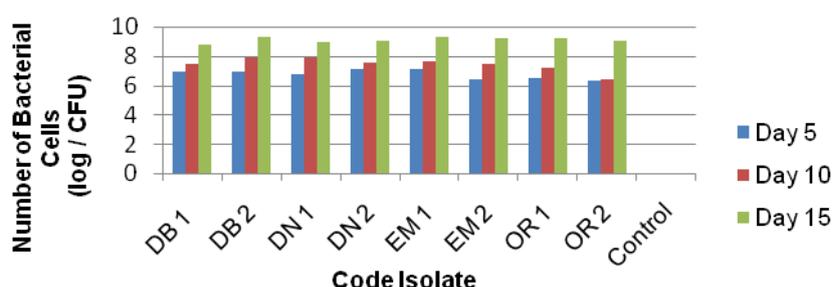


Figure 1. Bacterial Growth

According to Figure 1 above, all isolates grew from day 5 to day 15, but the growth of isolate OR2 on day 5 and 10 was not significant. The growth patterns of each isolate reached its peak on day 15. These results is similar with the results of research conducted by some isolates originating from agricultural land in Berastagi, North Sumatera, showing that bacterial cells continued to grow up to day 21. Increased cell growth that occurred in each isolate showed that each isolate has a high adaptability in media containing propineb which is toxic [6].

Increased cell growth might be caused by the amount of nutrient content in the media was still abundant that isolates entered the logarithmic phase on day 14. It could also be due to the fact that each isolate is capable of producing high amounts of biosurfactant to utilize carbofuran as a source of carbon and nitrogen [10].

3.3 Biosurfactant Assay

The biosurfactant activity from each bacterial isolate can be observed by measuring the volume of emulsion produced upon the addition of N-Hexan to the supernatant. The result of emulsification test obtained from 8 isolates of bacteria are presented in Figure 2.

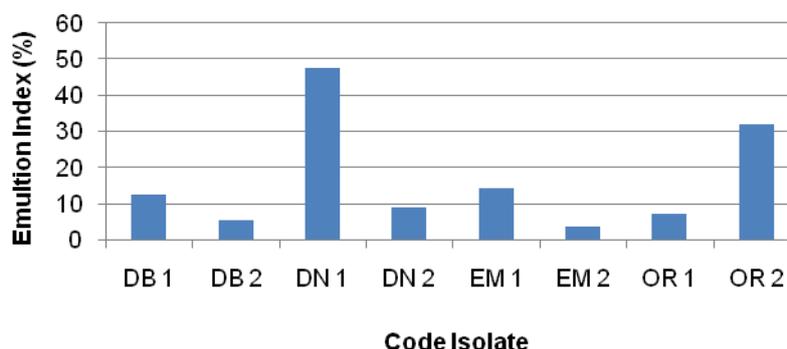


Figure 2. Bacterial Biosurfactant Activity

It can be seen from Figure 2 above that all isolates have different biosurfactant activities. Isolates with the high emulsion index is expected to represent high biosurfactant activity as well. The highest emulsion index was obtained from isolate DN1 which was 47.68%, followed by OR2 with 31.79%, while the lowest index was shown by EM2 with 3.53%. Differences in the emulsion index values produced by each isolate is due to the specific metabolic system from each isolate to survive under certain substrate conditions. The biosurfactants produced by each species may have different quantities and qualities. The higher the emulsion index produced by the isolates, the better ability it has to reduce the surface tension to helps the bacteria to utilize carbon compounds on the media for its growth.

The ability of bacteria to produce biosurfactant will determine the ability of these bacteria to use carbon from the growth substrate [10]. According to Kurniati [11], the type of biosurfactants produced, as well as its quality and quantity, are affected by the nature of the carbon substrate, the concentration of nitrogen, phosphorus, magnesium, iron and manganese ions in media, and culture conditions such as pH, temperature and agitation.

3.4 Biosurfactant Production Assay

Biosurfactant production assay was performed in BHB medium with 146.982 ppm of carbofuran as the sole carbon source. Results of biosurfactant production by each isolate is shown in Figure 3.

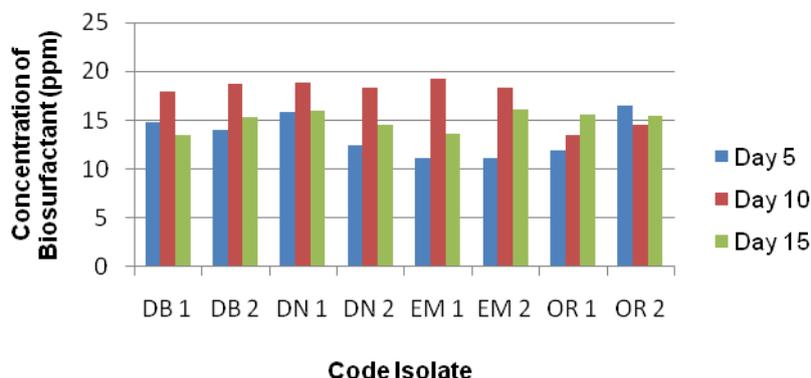


Figure 3. Production of Biosurfactant

The amount of biosurfactant produced by each isolate during the 15 days of incubation was varied one from another. Most isolates showed the same pattern, in which the maximum production occurred 10 days after incubation. OR1 isolate was the only isolate that produced biosurfactant at highest volume on day 15.

Biosurfactant produced by microbes varied depending on the types of microbes and nutrients consumed. Similarly, for the same type of microbe, the amount of surfactant produced is different according to the nutrients it consumed [12]. Nutrient played an important role for the growth of microbes, including biosurfactant-producing bacteria. Several studies showed that macro element that played an important role in supporting the growth of biosurfactant-producing bacteria are carbon and nitrogen [13].

3.5 Encapsulation Effectiveness

Two out of eight isolates were chosen for further encapsulation. The isolates were selected based on bacterial growth, volume of biosurfactant produced, and biosurfactant activity. The effectiveness of encapsulation was examined by conducting bacterial viability test after the beads has been stored for four weeks at room temperature. The result is shown in Table 3 below.

Table 3. Effectiveness of Encapsulation After Four Weeks Storage

Code of Isolate	Initial Cell population	Cell Population at the end of storage
DN1	7.2×10^{10}	2.9×10^7
OR2	4.1×10^{10}	1.8×10^7

Result showed that bacterial cells could survive after being kept at room temperature for four weeks. The advantage of bacterial immobilization is to protect the cells from harsh environment.

The bacterial viability during application in medium BHB containing 146.982 ppm of carbofuran was also observed on day 5, 10, and 15 as the cells degrade carbofuran. The bacterial viability during 15 days of incubation is shown in Figure 4.

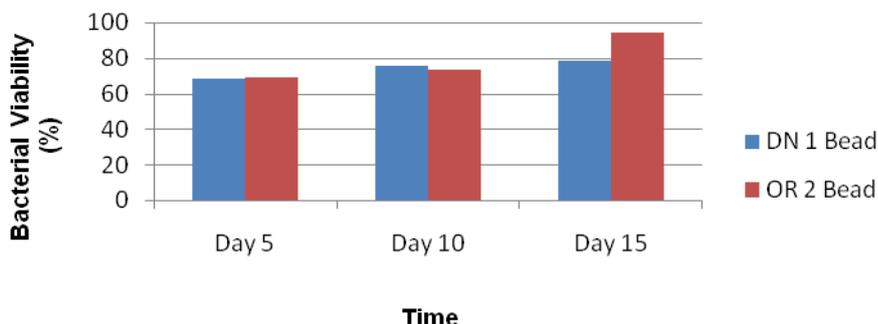


Figure 4. Viability of Bacteria in BHB medium containing 146.982 ppm Carbofuran

The initial population of isolate DN1 in the bead was 7.2×10^{10} cell.g⁻¹ while the initial population of isolate OR2 was 4.1×10^{10} cells. g⁻¹. Both isolates decreased in population after five days of incubation. This means that some bacteria were unable to adapt to utilize carbofuran as the carbon source. After 10 days of incubation, the bacterial population started to increase and at the end of the incubation, the bacterial population was almost the same as its initial population with the highest viability of 96.4% by isolate OR2. This information showed that alginate encapsulation was an excellent matrix that the bacteria showed high survivability and were able to utilize carbofuran as their carbon and energy sources.

Cell encapsulation is a technique used to maintain microorganisms in the matrix, to increase the efficiency of microorganisms to work as well as to maintain or protect microorganisms from environmental influences that are unfavorable to the cell [13]. Report on *Bacillus* encapsulated in alginate showed survivability in medium containing mercury and could reduce mercury up to 10 mg / L within 11 hours [14].

3.6 The Potency of Encapsulated Bacteria and Free Cell Bacteria in Degrading Carbofuran *In-vitro*

The ability of both isolate DN1 and OR2 as encapsulated and free cell to degrade carbofuran was examined in BHB medium containing carbofuran which was incubated for 15 days. The carbofuran residue was analyzed using HPLC on day 5, 10, and 15. The result is shown in Table 4 below.

Bacterial Isolates	Carbofuran Residue (ppm)			
	Days After Incubation			
	0	5	10	15
DN 1 Bead	146.982	-	-	-
DN 1	146.982	-	-	-
OR 2 Bead	146.982	-	-	-
OR 2	146.982	-	-	-
Control	146.982	-	-	-

The result showed that no carbofuran can be detected on day 5 after application in all treatments of both isolates as encapsulated and free cells, as well as in control. This information showed that carbofuran can be degraded not only by microorganism but also spontaneously. The same result has been reported by Onunga et al. [15] in which there is a peak emerged right after carbofuran standard. It was thought that the peak was carbofuran phenol. Carbofuran phenol itself is a compound derived from carbofuran which has toxic property in the environment. Bacteria will degrade carbofuran

phenol further into 3-(2-hydroxy-2 methylpropyl) benzene-1,2-diol, in which afterwards it will be hydrolyzed into methylamine.

Pesticide degradation can occur naturally through several ways such as enzymatic degradation, photodegradation and spontaneous degradation. Based on the result of this study, the concentration of carbofuran phenol resulted from spontaneous degradation increased steadily during the incubation period. This means that no carbofuran phenol utilization was shown in the control treatment and therefore it can be assumed that this compound was not degraded further, but instead they are accumulated. On the other hand, different results was observed from the encapsulated and free cells treatment. The concentration of carbofuran phenol on isolate DN1 as encapsulated cells and as free cells showed to decrease during incubation period as shown in Figure 5 below.

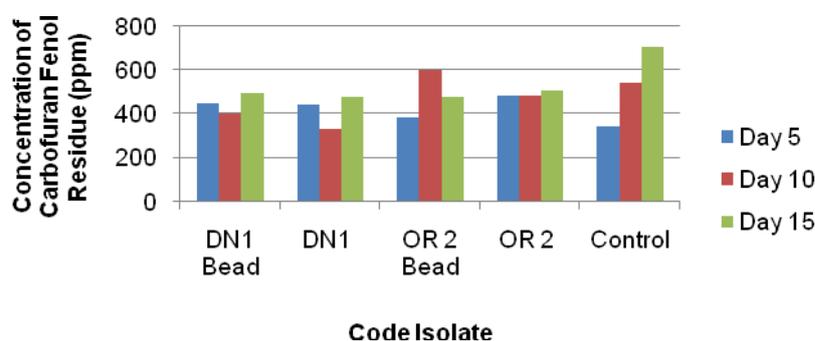


Figure 5. Carbofuran Fenol Residue

It can be assumed that both isolates, DN1 and OR2, were able to degrade carbofuran into carbofuran phenol, and degrade and utilize it further as a source of carbon and energy for their growth and other activities. This result is confirmed with the increase in population of both isolates as discussed earlier. Bacteria can use carbofuran as the sole source of carbon and nitrogen as a nutrient to grow and form carbofuran phenol [5]. The greater amount of carbofuran is degraded and consumed, the higher amount of carbofuran phenol produced [15]. The ability of isolate DN1 on degrading carbofuran phenol was slightly better than that of OR2. Furthermore, the ability to reduce carbofuran phenol of both isolates in encapsulated form was slightly higher compared to free cells. Encapsulated DN1 and OR2 isolates were able to decrease carbofuran phenol concentration up to 30.37% and 32.09% respectively compared to control. Meanwhile, the free cells of DN1 and OR2 decreased carbofuran phenol concentration to 32.54% and 28.29% respectively.

4. Conclusion

Based on the results of the research on Viability Of Bacteria Encapsulated Within Sodium Alginate In Carbofuran Degradation, it could be concluded that isolates DN1 and OR2 were able to degrade carbofuran into carbofuran phenol and decrease carbofuran phenol concentration up to 30.37% and 32.09% respectively. Bacterial encapsulation using alginate showed comparable result as free cells in carbofuran degradation.

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