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Research Article

Isolation and Immobilization of Biosurfactant-Producing Bacteria Capable of Degrading Carbofuran Pesticide

Nunuk PRIYANI[1], Dwi SURYANTO[2], Edison PURBA[3], Erman MUNIR*[4]

[1,2,4]Ph. Ph.D. student, Graduate Program of Biology, Faculty of Mathematics and Natural Sciences, Universitas Sumatera Utara. Jl. Bioteknologi no. 1, Padang Bulan, Medan Baru, Medan, Indonesia. Post Code 20155
[3]Study Program of Agroekotechnology, Faculty of Agriculture, ¬Universitas Sumatera Utara, Jl. Dr. A. Sofyan No. 3, Padang Bulan, Medan Baru, Medan, Indonesia. Post Code 20155

[1]https://orcid.org/0009-0008-1488-7382, [2]https://orcid.org/0000-0003-0010-2958, [3]https://orcid.org/0000-0002-9366-2489
[4]http://orcid.org/0000-0003-2815-4856

*Corresponding author e-mail: erman@usu.ac.id

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Abstract: Pesticide residue has been detected not only on agricultural lands but also in bodies of water such as rivers, lakes, and the sea. This study was aimed at exploring the potency of local bacterial isolates to degrade carbofuran, an active pesticide compound. Two biosurfactant-producing bacteria were isolated from hydrocarbon-compound-contaminated seas (NF9) and agricultural land with a long-term history of pesticide application (AB2). Bacteria were selected according to their ability to grow on a mineral medium, Bushnell Haas Agar, with the addition of 41.86 ppm of carbofuran pesticide as the sole carbon source. Their growth was characterized morphologically, biochemically, and molecularly based on their 16S rRNA genes. All isolates were Gram+ and indicated as *Bacillus thuringiensis* KD168 for isolate NF9 and *Bacillus paranthracis* C9 for isolate AB2. Both of the isolates were immobilized in sodium alginate and polyurethane matrixes. Both *B. thuringiensis* NF9 and *B. paranthracis* AB2 were able to degrade carbofuran, as indicated by the presence of carbofuran residue that ranged from 1.03 to 1.89 ppm; however, the residue was undetected after 15 days of incubation. We also confirmed that bacterial cells were immobilized and retained in polyurethane as well as in the sodium alginate matrix. The immobilization of the bacterial cells showed the abilities of the cells to degrade pesticides and their potential to be developed as bioremediation agents in polluted areas.

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1. Introduction

In modern agriculture, the increasing use of various forms of pesticides is inevitable. Pesticides are used to limit the reproduction and development of pests and diseases; however, the uncontrolled application of pesticides has caused severe problems on agricultural land and in bodies of water. The accumulation of pesticides can threaten human existence, other organisms, and the environment. In the US, the usage of pesticides has increased more than twofold since 1962, and now it endangers groundwater quality in most parts of the country (Lima et al., 2009). Meanwhile, in developing countries such as African countries, people rely on pesticide use as the best way to protect their crops against

pests, as if pesticides can guarantee their crop yield. The annual global agriculture utilization of pesticides has been estimated to be in the order of five million tons, of which approximately seventy percent is utilized in farming and the rest by public health agencies and government departments for vector control and household goals (Sharip et al., 2017; Nabhan et al., 2018; Sarkar, 2021). Carbofuran, a carbamate pesticide, is a potent inhibitor of acetylcholine esterase (AChE) and butyrylcholinesterase, enzymes vital for the functioning of the central nervous system. In humans, carbofuran is associated with endocrine-disrupting activity and reproductive disorders by mimicking or inhibiting estrogen receptors. It is also believed to be responsible for a decrease in sperm count and a rise in testicular cancer in humans, as well as abnormal sexual development in some wildlife species and cytotoxic and genotoxic abnormalities (Islam et al., 2018; Mishra et al., 2020; Pathak et al., 2021). According to the World Health Organization (WHO), over half a million people are poisoned each year by pesticides, and five thousand of the victims die (Bertolote et al., 2006). It is clear that the amount of carbofuran residue in the environment should be controlled. Bioremediation is an emerging technology that has the potential to reduce environmental toxicity.

Carbofuran (2,3-dihydro-2,2dimethylbenzene-7-methylcarbamate) is one of the active ingredients in pesticides. Carbofuran is a broad-spectrum insecticide used extensively in agriculture to inhibit the digestion of insects and other pests. Carbofuran residue becomes a global issue due to its solubility in water and high mobility in soil, resulting in significant groundwater contamination and severe toxic effects on mammals due to cholinesterase inhibition. Numerous European nations have already prohibited the use of chlorpyrifos and carbofuran, and some Asian nations will follow suit by 2022 (PAN Asia Pacific, 2022). As an agricultural nation, Indonesia has a massive pesticide market worth approximately US$ 600 million, of which more than 90 percent is imported (ACECHEM, 2022). Utami et al. (2020) report that the pesticide use estimation was an average of 24.6 kg/ha/year. Mostly, pesticides are used in line with the recommended dosage, but about a quarter are used in larger amounts than the prescribed amount (Utami et al., 2020). Carbofuran is the most toxic carbamate pesticide among pesticides and is sold under various brand names, including Furadan, Jordan, Propinep, Methomyl, and others. Carbofuran is extensively used as an insecticide, acaricide, and nematicide in global agriculture, and its residues will remain in soil and water. Several studies have shown that pesticide behavior in soil is influenced by the absorption, motility, and degradation processes. The adsorption of pesticides in soil is very important because it can lead to environmental problems. Pesticides that do not bind to the soil particles will be degraded to produce less toxic metabolites, while in comparison, adsorbed pesticides will continue to be in the surroundings for a number of years and may build up into food chains many years after their use in soil (Cheah et al., 1997). As an example, it is estimated that carbofuran, which is widely utilized in paddy soil, remains in the water at about 54% and in the soil at about 46% (Arifin and Sukirah, 2020). Another way to improve soil fertility is the application of organic fertilizers including vermi compost, cattle and chicken manure (Alp and Sensoy, 2023; Rahimi et al., 2023).

Numerous bacterial species have been isolated through extensive research on the biodegradation of a variety of pesticides under varying conditions. Microorganisms that play major roles in degrading and utilizing carbamates as a sole source of carbon include *Achromobacter* spp. (Karns et al., 1986; Tomasek and Karns, 1989), *Arthrobacter* sp. (Racke and Coats, 1988), *Sphingomonas* spp. (Feng et al., 1997; and Xu et al., 2009), *Pseudomonas* spp. and *Chrysobacterium* spp. (Bano and Musarrat, 2004), *Bacillus brevis* (Kamboj et al., 2005), *Gliocladium* sp. (Slaoui et al., 2007), *Novosphingobium* sp. FND-3 (Yan et al., 2007), *Burkholderia cepacia* PCL3 (Plangklang and Reungsang, 2011), *Rhodococcus* sp., *Sphingobium* sp., *Bosea* sp., and *Microbacterium* sp. (Shin et al., 2012), and *Cupriavidus* sp. ISTL7 (Gupta et al., 2019). The removal of pesticide-active compounds is restricted by their toxicity. Biological degradation was devised to eliminate these toxic environmental pollutants. This cleaning method is regarded as eco-friendly and cost-effective in comparison to other methods (Mishra et al., 2020).

The immobilization of microorganisms is a multidisciplinary subject that bridges the pure and applied sciences. It has become an emerging method for wide applications in the areas of environmental bioremediation, bioprocessing, and biomedical technology (Nemati and Webb, 2019). Compared to conventional suspension systems, cell entrapment technology has numerous advantages, including high biomass, high enzyme activities, strong resistance to toxic compounds, possible reutilization of microorganisms, and being highly efficient in harsh environments (Partovinia and Rasekh, 2018). At 4 ^{o}C, a species of *Pseudomonas* degraded carbofuran substantially more efficiently in agar-immobilized cells than in free cell suspension (Fareed et al., 2019). Therefore, immobilized microbial technology has

been investigated as a potential wastewater treatment instrument in recent decades. The objective of this investigation was to isolate bacteria from hydrocarbon-contaminated sites and to compare the pesticide degradation efficiency of local isolates as immobilized cells versus their free cell suspensions.

2. Material and Methods

2.1. Chemicals

Analytical grade (99.9%) carbofuran and other chemicals (except as otherwise stated) have been purchased from Merck and Sigma Aldrich Singapore. Molecular identification was done using their 16S rRNA genes. All the samples were sequenced in Macrogen, Singapore.

2.2. Sampling and Screening of isolates

Water and soil samples were collected for the isolation of bacteria. A water sample was obtained from a polluted area by gasoline at the beach area at Belawan Port. Meanwhile, 100 g of topsoil was obtained from agricultural land that has been treated with carbofuran pesticide for a long time in Berastagi. Both are located in North Sumatra, Indonesia. One ml of water sample or 1 g of soil sample was diluted in 9 ml of phosphate buffer, repeated twice to obtain a dilution up to 10^{-2} times. As much as 0.1 ml was inoculated on Bushnell Haas Agar (BHA) medium that contained 41.86 ppm of carbofuran as the only carbon source. The composition of BHA medium per liter was: 0.2 g of magnesium sulfate, 0.02 g of calcium chloride, 1 g of monopotassium phosphate, 1 g of dipotassium phosphate, 1 g of ammonium nitrate, 0.05 g of ferric chloride, and 20 g of agar. pH was adjusted to 7.5 by the addition of NaOH. The obtained colonies were isolated and characterized to get the pure isolate.

2.2. Cell immobilization

Two different matrixes, sodium alginate, and polyurethane, were used to immobilize the isolates.

2.1.1. Cell immobilization using alginate

Cell suspension was prepared by measuring the absorbance of the cell culture using a spectrophotometer at 600 nm to achieve optical density (OD) = 1 which is equal to 10^9 cells ml^{-1}. The cell suspension was concentrated into 10^{13} cells ml^{-1} by centrifugation, and the pellet was suspended in a smaller volume of buffer. The bacterial cell suspension was mixed with 3% Na-alginate. Using a 3 ml syringe, the mixture was dropped into a 0.1–0.2 M $CaCl_2$ solution. Alginate polymerized spontaneously, forming alginate beads containing bacterial cells (Fravel et al., 1985). Alginate beads were rinsed with distilled water for 10 minutes to remove the $CaCl_2$. To evaluate the efficiency of cell entrapment by alginate beads, 10 alginate beads were soaked in 10 ml of 0.85% NaCl for 30 minutes, then transferred into a sodium citrate solution (60 g l^{-1}) for 30 minutes and shaken in a rotary shaker until the beads dissolved completely. Total bacteria were observed by standard plate counting (Schoebitz et al., 2013). The experiments were done in triplicate.

2.1.2. Cell entrapment using polyurethane

The equal volume (50 ml each) of polyurethane solutions A and B (obtained from the local supplier) were mixed and the mixture solidified at room temperature (28 ^oC) to form a hard foam of polyurethane. By using a cutter the foam was cut into pieces with a size of 2 cm square and 0.2 cm of thickness. As many as 0.5 g of polyurethane cubes were mixed with bacterial suspension with a population of 10^{13} cells ml^{-1}. The mixture was incubated for 30 minutes with a 125 rpm shaking incubator at 28 ^oC (Moon et al., 2024). Cell attachment efficiency was evaluated by counting the cells from polyurethane foam after the foam had been shaken vigorously using a vortex for 2 minutes. The cell present in the buffer was plated on Nutrient Agar in a Petri dish. The colonies were counted using the colony counter. The experiments were done in triplicate.

2.3. Determination of cell attachment on polyurethane using Scanning Electron Microscope (SEM) and Pesticide residue analysis

The preparation and observation of the sample using SEM were done at the National Research and Innovation Agency in Bogor, Indonesia. The types of SEM are JSM-5000, MAG-X-15,000, and ACVV-20kV. Meanwhile, the residue of the pesticide was analyzed using HPLC. Waters HPLC 2-2695 series with condition Column: C18, 250 mm x 4.6 mm, 5µ; Flow rate: 1.0 mL/min; Wavelength: 282 nm; Column temperature: 30 °C; Injection volume: 20 µL; Run time: 10 minutes; Diluent: Mobile phase; Elution: Isocratic; Needle wash: Water: Acetonitrile 90:10 (v/v).

2.4. Culture condition for determination of carbofuran degradation

One gram of sodium alginate beads or polyurethane cut into pieces was added to 99 ml of Bushnell-Haas Broth (BHB), whose composition is the same as BHA but without agar addition. As much as 41.86 ppm of carbofuran was added to the medium that served as the only carbon source. The isolates were incubated in a shaking incubator (Vison, Model: VS-8480SN) at 125 rpm and 28 °C for 15 days in a dark condition. The growth of cells was determined, and the residual pesticide was analyzed on days 5, 10, and 15. The same cultures with the addition of free cell suspension were conducted as a positive control. By using 2 ml of bacterial suspension, OD600 = 1 (equal to 10^9 cells ml^{-1}) was added to 98 ml of BHB with the same amount of carbofuran. The same medium with no bacteria was used as a negative control. There is no replication for HPLC analysis.

3. Results and Discussion

3.1. Bacterial isolation

From the screening of the ability of local isolates to grow on the medium with pesticide as the sole carbon source, 17 isolates were able to grow. They varied in morphology, colonies, some basic metabolism (biochemical tests), types of Gram staining, shape, and cell arrangement, as shown in Tables 1 and 2. The isolates with the NF code came from Belawan, the water sample, and the HS and AB codes came from agricultural land in Berastagi. Soils, mainly those with a history of pesticide application, are the main source of microorganisms. Pesticides showed shorter half-lives in soil with a history of pesticide application, mostly compared to soil that has no history of pesticide application (Cycon et al., 2017). The long application of pesticides has caused numerous microorganisms to expand their metabolic systems to break down toxic compounds through different mechanisms, approaches, and enzymatic pathways. The first bacterial isolate capable of degrading organophosphate was from a paddy field in the Philippines in 1973. Later on, numerous strains capable of metabolizing pesticides have been isolated by many researchers from different geographical regions (Das et al., 2005; Talwar et al., 2014; Wu et al., 2014).

Table 1. Characteristic of isolates

No.	Isolate	Colony Morphologies				Gram Staining	Shape and cell arrangement
		Shape	Edge	Elevation	Color		
1.	NF 1	Circular	Entire	Raised	White	-	Basil
2.	NF 3	Circular	Entire	Flat	Krem	+	Streptobasil
3.	NF 4	Irregular	Irregular	Flat	Milky white	+	Basil
4.	NF 5	Circular	Entire	Flat	Beige	+	Streptobasil
5.	NF 6	Circular	Entire	Flat	Beige	+	Streptobasil
6.	NF 7	Circular	Irregular	Flat	Beige	-	Basil
7.	NF 8	Irregular	Irregular	Flat	Beige	-	Basil
8.	NF 9	Circular	Entire	Raised	Yellow	+	Streptobacil
9.	AB 2	Circular	Undulate	Flat	Light yellow	+	Mono, diplobacil
10.	HS 1	Circular	Entire	Flat	Light brown	-	Mono, diplococcus
11.	HS 2	Circular	Undulate	Convex	Milky white	-	Mono, diplococcus
12.	HS 3	Irregular	Entire	Flat	Light brown	-	Mono, diplococcus
13.	HS 4	Irregular	Entire	Flat	Milky white	-	Mono, diplococcus
14.	HS 5	Circular	Entire	Flat	Milky white	-	Mono, diplococcus
15.	HS 6	Circular	Entire	Flat	Light yellow	-	Mono, diplococcus
16.	HS 7	Irregular	Entire	Flat	Light brown	+	Mono, diplococcus
17	HS 8	Circular	Undulate	Convex	Light orange	-	Mono, diplococcus

Soil ecosystems comprised of microorganisms in soil are able to metabolize carbamate pesticides and easily adapt themselves to various forms of that pesticide. Nevertheless, pesticides and their metabolism products play an important role in the soil's microflora and productivity (Gupta et al., 2016). Parekh et al. (1994) have gathered samples from five field locations with varying carbofuran treatment histories. The chemical was hydrolyzed more quickly in all soils treated with carbofuran earlier than in samples of identical soils that had not been treated. Sixty-eight bacteria, capable of degrading carbofuran as the sole source of carbon and nitrogen, were isolated from liquid cultures of treated soils. All carbofuran-degrading isolates were gram-negative aerobic rods that broke down the carbofuran to carbofuran phenol.

Table 2. Biochemical characteristics of isolates

No.	Isolate	Types of Biochemical test					
		Starch	Gelatine	Citric	Sulfide	Motility	Catalase
1.	NF 1	-	-	+	+	-	+
2.	NF 3	-	-	-	+	+	+
3.	NF 4	+	-	-	+	+	+
4.	NF 5	+	-	+	+	+	+
5.	NF 6	-	-	-	+	+	+
6.	NF 7	-	-	-	+	-	-
7.	NF 8	-	-	-	-	+	+
8.	NF 9	-	-	-	-	+	+
9.	AB 2	-	+	+	+	+	+
10.	HS 1	-	+	-	+	+	+
11.	HS 2	-	+	-	+	+	+
12.	HS 3	-	-	-	-	+	-
13.	HS 4	-	-	-	+	+	-
14.	HS 5	-	+	-	+	-	+
15.	HS 6	-	+	+	+	+	-
16.	HS 7	-	+	-	+	+	+
17.	HS 8	-	-	-	+	+	+

All isolates that were grown in BHB medium with carbofuran as the sole carbon source grew well, as shown in Table 3. Among all isolates, NF9 showed the best growth, steadily increasing weekly. In addition to the ability to break down carbofuran as their carbon source, the other important characteristic of hydrocarbon-degrading bacteria is their capability to secrete biosurfactants. Microbial biosurfactants are low-molecular-weight surface-active compounds that are stable under several environmental conditions. Biosurfactant functions to reduce the surface tension, thus facilitating the emulsification and solubilization of highly hydrophobic pollutants (Eras-Muñoz et al., 2022). It is assumed that bacteria secrete the biosurfactant into the medium. The biosurfactant was obtained by centrifugation of the culture to separate the cell pellet from the medium. The medium is considered a biosurfactant. Biosurfactant activity could be evaluated by measuring the emulsion volume produced by the mixture of biosurfactant and hydrophobic compound, n-hexane. Data in Table 3 showed that NF9 and AB2 had the highest and second highest biosurfactant activity, with an Emulsion Index (EI24) value of 43% and 38%, respectively.

Two isolates, NF9 and AB2, were selected for further testing based on their abilities to grow best among others and their highest biosurfactant activities. These isolates showed distinct colony characteristics, in which the NF9 isolate has a flat elevation and a white color, while the AB2 isolate has a raised elevation and a yellow color. They share a common form, margin, Gram+ staining, and streptobacillus arrangement. Based on molecular identification of 16S rRNA analysis, NF9 and AB2 isolates were closely related to *Bacillus thuringiensis* strain KH 168 with a percent identity of 99.73% and *Bacillus paranthracis* strain C9 with a percent identity of 99.54%, respectively. The phylogenetic structure of these strains is shown in the following figure. Subsequently, *Bacillus thuringiensis* NF9 and *Bacillus paranthracis* AB2, with accession numbers SUB14158312 NF9 PP152281 and SUB14158312 AB2 PP152282, respectively, are used as the strains of our isolates.

Table 3. Growth of isolates on carbofuran and the activity of their biosurfactant

No.	Isolate Code	Total Bacterial Cells (10^7 cells/ml)			Biosurfactant Activity (% Emulsion Index/EI_{24})
		Day 7th	Day 14th	Day 21st	
1.	NF 1	2	58	78	-
2.	NF 2	8	38	58	38
3.	NF 4	4	25	17	18
4.	NF 5	9	25	25	36
5.	NF 6	48	49	50	39
6.	NF 7	2	12	12	-
7.	NF 8	4	118	43	24
8.	NF 9	8	48	86	43
9.	AB 2	4.9	22	18	38
10.	HS 1	2	12	6	27
11.	HS 2	2.5	18.6	10.4	29
12.	HS 3	5	16	10.9	29
13.	HS 4	1.6	8.2	9.6	26
14.	HS 5	1.5	4	3.2	-
15.	HS 6	2.8	16	18.8	38
16.	HS 7	7.8	11.5	6.5	24
17.	HS 8	5.2	14.5	16	39

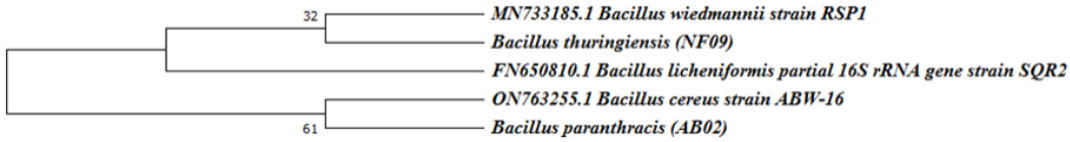

Figure 1. Phylogenetic tree of NF9 and AB2 isolates. The neighbor-joining tree is based on the 16S rRNA sequence, demonstrating the phylogenetic position of each strain.

Pesticide biodegradation by microorganisms is not new. Microorganisms simply supply all the required energy sources for simple chemical reactions to take place (Pandey et al., 2010). Many groups of microorganisms are characterized by the growth and degradation abilities of pesticides (Ishag et al., 2016). Isolation and characterization of microbes for pesticide degradation bring about new tools to restore environments polluted with pesticides. Several microbial species capable of degrading pesticides, such as *Pseudomonas*, *Flavobacterium*, *Achromobacterium* sp., *Sphingomonas* sp., *Arthrobacter*, and *Bacillus* species, have been isolated and characterized in an effort to know their mechanisms for removing pesticides. Various strains of *Bacillus* have been isolated from different types of contaminated soil as well as contaminated water. *B. amyloliquefaciens* FZB42, *Bacillus* sp. NH 217, and *B. subtilis* NH-100 showed high biosurfactant activities, and *B. atrophaeus* 176s lipopeptide biosurfactant, in addition to its very good activity, also showed antifungal activity (Sarwar et al., 2018). To improve further application of those isolates, the bacteria were encapsulated in sodium alginate and polyurethane.

3.2. The effectiveness of cell immobilization

The effectiveness of cell immobilization in alginate and polyurethane is shown in Table 4.

Table 4. The average number of cells immobilized in alginate and polyurethane

Bacterial Species	Number of cells (CFU) ml^{-1}		
	ml^{-1} cell suspension	g^{-1} alginate	g^{-1} Polyurethane
***B. thuringiensis* NF9**	2.08×10^{13}	6.60×10^{13}	1.25×10^{12}
***B. paranthracis* AB2**	4.07×10^{13}	1.94×10^{13}	6.60×10^{12}

The number of cells entrapped in alginate was statistically higher than that in the cell suspension. It is assumed that the bacterial cells were entrapped more easily in sodium alginate beads compared to the attachment of the cells to the polyurethane surface. Similarly, *B. thuringiensis* NF9 cells, which were

entrapped in alginate, were also significantly higher than *B. paranthracis* AB2. Conversely, there was no significant difference in the number of cells entrapped in polyurethane between the two strains. For almost thirty years, there has been interest in the immobilization of entire microbial cells and their applications to bioprocessing. In order to produce extracellular enzymes, whole cells can be immobilized. This has a number of advantages, including the ability to easily extract the cell mass from the bulk liquid for potential reuse, the ability to operate continuously for extended periods of time, increased reactor productivity, and increased catalysis efficiency (Kar and Ray, 2008).

A recent study reported by Jeon et al. (2019) stated that immobilization using a polyvinyl alcohol-sodium alginate matrix bead resulted in outstanding porosity, chemical stability, and mechanical strength. Moreover, based on the topology of the beads in the SEM image, Jeon et al. (2019) reported that the pores' coarse and irregular appearance will improve their specific surface area and interaction potential.

The number of bacterial cells that were released into the medium during incubation was observed and compared to the number of cells that remained in the matrix after incubation to evaluate the stabilization of cell immobilization. The result showed that the number of cells that were released into the medium remained stable (Table 5).

Table 5. The average number of bacterial cells encapsulated within sodium alginate which is released into the medium during incubation

Bacterial Species	**Number of cells which is released into medium (CFU) ml^{-1}**		
	Day 5th	Day 10th	Day 15th
***B. thuringiensis* NF9**	2.08×10^{13}	6.45×10^{12}	9.77×10^{12}
***B. paranthracis* AB2**	7.94×10^{12}	3.89×10^{12}	3.80×10^{11}

During 15 days of incubation, the number of cells that were released from the beads varied between the two isolates. *B. paranthracis* AB2 showed a continued decrease in the number of cells, while *B. thuringiensis* NF9 showed a slightly increasing cell population at the end of incubation. However, both strains showed that there was no statistically significant difference in cell numbers released into the medium on days 5, 10, and 15. As the incubation time proceeded, the beads absorbed some water and appeared to be a little bit swollen (*B. thuringiensis* NF9). This caused more bacteria to be released into the medium, but other beads of *B. paranthracis* AB2 remained intact. The stability of the interaction between bacterial cells and alginate beads is affected by the chemical characteristics and composition of the alginate beads and the bacterial cells. It was reported that the combination of alginate and other compounds such as active carbon, biochar, and cornstalk cubes improves the surface area and pore distribution of alginate beads (Fareed et al., 2019; Fravel et al., 1985; Li et al., 2020). The pores protect bacteria from harsh environments that could harm the cells. Furthermore, Jeon et al. (2019) noted that keeping an eye on the beads' structural integrity was necessary to monitor their ability to function properly and provide a sign of any deterioration. Other studies reported that after 102 days of use, alginate beads became transparent and their shape began to alter from spherical to round (Damayanti et al., 2021). It is thought that the bead's damage was caused by calcium ions, which dissolved over time and left the bead translucent, mushy, and partially fractured. The beads eventually break and become more brittle (Hu and Chen, 2007).

Table 6. The average number of bacterial cells entrapped in polyurethane which is released into the medium during incubation

Isolate	**Number of cells which is released into medium (CFU ml^{-1})**		
	Day 5th	Day 10th	Day 15th
***B. thuringiensis* NF9**	3.63×10^{13}	1.99×10^{12}	6.02×10^{9}
***B. paranthracis* AB2**	5.12×10^{12}	1.34×10^{13}	1.34×10^{13}

Unlike cell release from alginate beads, in polyurethane, cell release of *B. thuringiensis* NF9 decreased steadily, while *B. paranthracis* AB2 remained stable. Statistically, there was no significant difference in the number of released cells during incubation time, except for strain NF9 on day 5. During five days, the average number of cells showed the highest, 3.63×10^{13} CFU/ml.

Alginates are the polymers of choice in most systems of immobilization because they are easy to handle, nontoxic to humans, the environment, and entrapped microorganisms, legally safe for human use, available in large quantities, and inexpensive. From a physiological perspective, a major advantage of alginate is that immobilized cells do not suffer extreme changes in physicochemical conditions during the procedure of immobilization, and the gel is transparent and permeable (Buque et al., 2002). Organic carriers are such as modified celluloses, dextran, and chitosan agarose (Lu and Toy, 2009).

Despite providing high mechanical strength, cell immobilization on polyurethane showed some disadvantages, such as cell leakage and releasing cells into the medium. Moon et al. (2020) said that bacterial cell entrapment in polyurethane foam was not effective for thermal stabilization presumed to be due to the poor direct covalent linkage of the enzyme to the polyurethane matrix.

At the end of incubation, the number of cells retained in the beads was still high. Those results demonstrated that alginate beads served as an excellent matrix for cell immobilization. One advantage of applying cell immobilization is the possibility of using the beads several times without reducing the ability of cells to undergo pesticide degradation. A study by Soo et al. (2017) showed that increasing concentrations of alginate and $CaCl_2$ enhanced the stability of beads for up to 15 days in a vigorous shaking incubator. Furthermore, combining sodium alginate with chitosan extends the reusability of the beads up to 10 times in oil waste treatment (Jeon et al., 2019).

Table 7. The average number of bacterial cells retained in the sodium alginate beads and polyurethane after incubation

Isolates	**Number of cells (CFU ml^{-1})**			
	At the initial incubation		At the end of incubation	
	Alginate	Polyurethane	Alginate	Polyurethane
B. thuringiensis NF9	6.60 x 10^{13}	1.25 x 10^{12}	1.64 x 10^{11}	7.58 x 10^{10}
B. paranthracis AB2	1.94 x 10^{13}	6.60 x 10^{12}	9.50 x 10^{10}	2.88 x 10^{11}

At the end of the incubation, the number of bacterial cells in polyurethane was still relatively high. Statistically, there was no significant difference between the initial number of cells and the number of cells at the end of incubation, except for the number of *B. thuringiensis* NF9 cells in alginate beads. In this case, the average number of cells decreased significantly from 6.60×10^{13} to 1.64×10^{11}. It indicated that polyurethane foam provides excellent support for bacterial cells to attach. Due to its excellent mechanical characteristics, high porosity, and substantial adsorption surface, polyurethane foam has recently gained significant relevance as a carrier. Furthermore, it is cost-effective (De Ori et al., 2020). Compared to *B. thurungiensis* NF9, *B. paranthracis* AB2 showed a higher cell population. It was assumed that *B. paranthracis* AB2 had a better, stronger interaction between the bacterial cell and the polyurethane matrix. It was assumed that *B. parathracis* AB2 had a better, stronger interaction between the bacterial cell and polyurethane. Figure 2. shows how *B. parathracis* AB2 cells attached randomly to the polyurethane matrix.

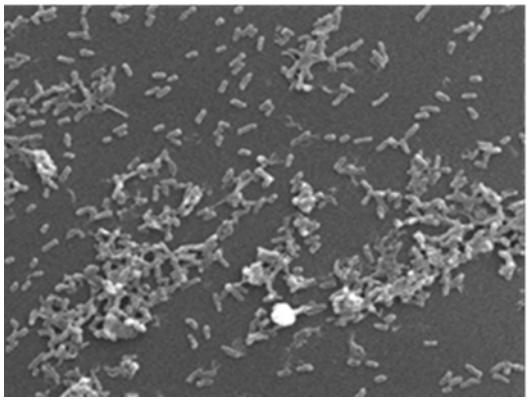

Figure 2. The attachment of *Bacillus parathracis* AB2 on polyurethane foam.

It is assumed that cell wall hydrophobicity enhanced the adhesion of bacterial cells to polyurethane foam. Jeon et al. (2019) reported that SEM imaging of *Acinetobacter* and *Paenibacillus* that were entrapped in sodium alginate showed that the shape of bacteria affected the interaction between bacteria and alginate. It was said that *Paenibacillus*, which is rod-shaped, is more easily entrapped or absorbed during immobilization compared to *Acinetobacter*, which is coccobacillus-shaped. Matshui and Tomohiko (2017) reported that *Brevibacterium ketoglutamicum*, which is immobilized in polyurethane, was able to degrade n-tetradecane in repeated batches up to 300 h with only a slight loss of activity.

3.3. Carbofuran degradation

Figure 3. shows the results of carbofuran degradation by two isolates in the form of immobilized cells as well as free cell suspensions. The result showed that all isolates were capable of degrading carbofuran completely after 15 days of incubation. Meanwhile, there was still 18.71 ppm (55.3%) of carbofuran in the control. No residue of carbofuran could be detected after 5 days of incubation when the carbofuran was treated with *B. parantraxis* AB2 as immobilized cells in alginate beads. Meanwhile, the residue of carbofuran was undetected after 15 days of incubation by those two isolates in all conditions. Based on the data of the number of cells retained within alginate beads, both strains of isolates showed almost the same population, which was $\pm 10^{10}$ cells ml^{-1}. It indicated that the *B. parantracis* AB2 strain had much better activity in carbofuran degradation. The result indicated that immobilized cells within alginate beads degraded carbofuran better than its free cell suspension. A study on carbofuran biodegradation by *Bacillus* sp. strain DT1, a soil bacterium, showed a similar result. When the isolate was immobilized in rice straw, it was capable of decreasing pesticide concentrations up to 97.5%, which was 19.8% higher than its free cell suspension (Duc, 2022).

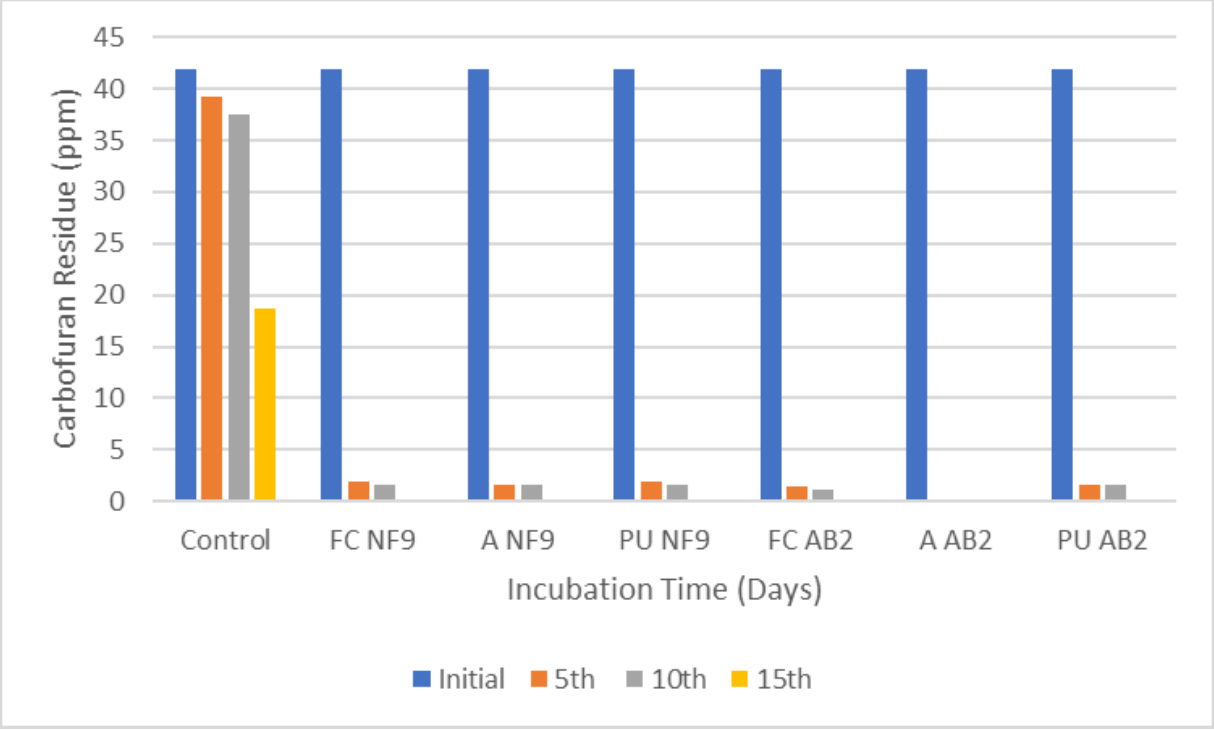

Figure 3. The residual of carbofuran after being incubated with bacterial cells in the form of free cells and immobilized in alginate beads and polyurethane. FC: Free Cells; A NF9, A AB2: Alginate NF9 and Alginate AB2; PU NF9, PU AB2: Polyurethane NF9 and Polyurethane AB2.

At the end of incubation, the residue of carbofuran in the negative control was 18.71 ppm. It meant that carbofuran underwent natural degradation up to 52.4%. When pesticides reach vegetation, soil, aquatic environments, or even the air, they can be degraded spontaneously by photooxidation. Herbicides like 2,4-d bromoxynil will be degraded completely in the presence of sunlight. Chemical

degradation through oxidation-reduction, hydrolysis, and ionization were other examples of natural degradation. Those processes might occur in aquatic environments and are mostly closely related to environmental pH. The strong acid or base environment inhibits the growth of microorganisms or inhibits the enzymes of microorganisms. Such conditions favor the chemical degradation of pesticides (Morel-Cheville et al., 1996).

In general, *B. paranthracis* AB2 degraded carbofuran higher than *B. thuringiensis* NF2 in all conditions, as a free cell suspension immobilized within alginate as well as in polyurethane. Comparing the matrix of immobilization, the result showed that alginate is a better carrier for both isolates and is better than their free cell suspension. On the contrary to immobilization by alginate, *B. paranthracis* AB2 immobilized on polyurethane showed lower degradation ability compared to its free cell suspension. It was assumed that the ability of *B. paranthracis* AB2 to immobilize in polyurethane was not as efficient as in alginate. With a total population of 9.50 x 10^{10} cells per g of alginate, it could degrade carbofuran completely on day 5 of incubation. Meanwhile, the bacterial cells entrapped in polyurethane were 10^{12} cells g^{-1}. Polyurethane at initial was much higher than that in alginate, which was $\pm 10^{10}$ cells g^{-1}. Likewise, the number of cells released into the medium ($\pm$ 10^{13} cells ml^{-1}) was significantly higher than in the alginate medium. Various species of bacteria have been reported to degrade carbofuran. Gupta et al. (2019) reported that *Cupriavidus* sp. ISTL7 degraded carbofuran efficiently, 98% in 96 hours. As in *Pseudomonas*, the bacteria break the chemical compound carbofuran into carbofuran-7 phenol and methyl amine by producing EPS such as glucose, xylose, sorbitol, and fructose.

Conclusion

Two local bacterial isolates capable of degrading carbofuran, *Bacillus thuringiensis* NF9 and *B. paranthracis* AB2, have been isolated from Belawan Port and Berastagi agricultural land. The isolates were immobilized in alginate beads and polyurethane. The immobilization processes were highly efficient, in which the number of cells reached $\pm$ 10^{13} cells g^{-1} alginate and 10^{12} cells g^{-1} polyurethane. The immobilized cells showed very good stability; the number of cells in the polyurethane matrix was about the same between the initial and after 15 days of incubation, allowing them to be applied on a larger scale. Both strains, in the form of free cells as well as immobilized cells, degraded carbofuran within 15 days of incubation. The strain *B. paranthracis* AB2, which was immobilized in alginate, was able to hydrolyze carbofuran completely, even much faster, within 5 days of incubation. This study suggests that both local strains have the potential to be further developed as agents of bioremediation, not only against carbofuran but also other hydrocarbon compounds. Furthermore, this study allows the possibility of using polyurethane as plastic waste to be utilized as a matrix of immobilization.

Ethical Statement

Ethical approval is not require for this study because no animal is used.

Conflict of Interest

There are no known conflicts of interest associated with this publication.

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Author Contributions

Nunuk Priyani: Material and equipment engagement, literature search, monitored search, data analysis and experimental development and wrote manuscript.

Edison Purba: Designed research methodology and data analyses.

Dwi Suryanto: Designed research methodology, conducted field sampling and data interpretation.

Erman Munir: Conceived the original idea, design the study and review and approval of manuscript.

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