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# Screening of Poly(3-Hydroxybutyrate) P(3HB) Producing Bacteria from Mackerel Fish (*Rastrelliger* sp.)

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**Abstract:** Bioplastic P(3HB) is a plastic that can be decomposed by decomposing microorganisms. Bacteria can produce P(3HB) in conditions of low nutrition and high carbon sources accumulated in cells as energy reserves. Isolation of bacteria-producing bioplastic P(3HB) was carried out in mackerel fish's gills and intestines, allowing direct contact with polluted waters. This research aimed to determine the presence of P(3HB) bioplastic-producing bacteria in mackerel fish samples and to screen for P(3HB) bioplastic-producing bacteria. The stages of the research were the isolation of P(3HB) producing bacteria from the gills and intestines of mackerel fish, screening of P(3HB) bioplastic-producing bacteria by using Nile Blue A staining, and molecular identification of bioplastic-producing bacteria using 16S rRNA gene. The results of bacterial isolation in the gills and intestines of mackerel obtained 10 bacterial isolates. P(3HB) screening found 1 bacterium showing orange fluorescence, namely IKE-1 isolate which was isolated from the gills of mackerel fish and included in the Gram-negative group with a monobacilli cell shape. Molecular identification of bacteria by using the 16S rRNA gene that isolate IKE-1 is *Enterobacter roggenkampii*. These results indicate that *Enterobacter roggenkampii* can produce P(3HB) which was confirmed by the screening results.

**Keywords:** *Enterobacter roggenkampii;* Isolation; Nile Blue A; P(3HB); Screening

## Introduction

The high use of plastic causes plastic waste to become a serious problem. Plastic is widely used because the characteristics of plastic are durable, not easily damaged, light and water resistant. High use of plastic can cause a buildup of plastic waste, one of which is in water. Plastic waste that is thrown away floats and then settles in the ocean and is very difficult for bacteria to decompose. Plastic waste such as plastic bags and food wrappers contributes the largest type of waste to fishing activities by fishermen (Selvam et al., 2021). The most common types of waste are plastic (rubber and soft plastic) and lost fishing gear, especially nets and fishing lines (Mortensen & Mortensen, 2018). Efforts that can be made to reduce plastic waste are by producing plastic that is easily biodegradable, namely bioplastic. One of the bioplastics that is being widely researched is Poly(3-hydroxybutyrate).

P(3HB) is a microbial polymer belonging to SCL PHA which can be accumulated by bacteria (Vicente et al., 2023). Nile Blue A and Sudan Black staining is a way to identify bacteria that can accumulate P(3HB) (Ostle & Holt, 1982). P(3HB) can be produced by biosynthesis using carbon sources such as glucose, date seed oil, date molasses, fish oil waste, and glycerol (Meng et al., 2015; Purama et al., 2018; Thuoc et al., 2019). P(3HB) synthesis can be carried out under growth conditions without nutrient limitations, then polymer production under growth conditions with a high carbon source and limited P, Fe, Mg, and C nutrient sources (Alves et al., 2017). P(3HB) can be applied in biomedical, aquaculture, and agriculture (Vigneswari et al., 2019).

The P(3HA) group of compounds, the polymer poly(3-hydroxybutyrate) P(3HB) and their copolymer

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poly (3-hydroxybutyrate-co-3-hydroxyvelerate) P(3HB-ko-2HV) are compounds that have been extensively studied because they have 100% easily decomposed within a certain time when disposed of into the environment (Djamaan, 2015). Many genera of bacteria-producing bioplastic P(3HB) have been identified. Bacteria that can synthesize PHA include groups from the genera *Pseudomonas, Bacillus, Ralstonia, Aeromonas, Rhodobacter,* and several groups of Arkhaea (Ojumu et al., 2004). PHB-producing bacteria have been isolated from aerobic sludge, agricultural waste, soil, dan marine sponge (Aryaraj & Pramitha, 2021; Biradar et al., 2018; Getachew & Woldesenbet, 2016; Zheng et al., 2015).

Plastic waste scattered in marine ecosystems is very high. Aquatic microorganisms consisting of heterotrophic, autotrophic, predatory, and symbiont bacteria live together and live on the hydrophobic plastic surface which is rich in nutrients (Zettler et al., 2013). Fish whose habitat is in water can come into direct contact with waters contaminated with rubbish. Mackerel (*Rastreliger* sp.), which is a pelagic fish, has a habit of eating filter feeders, namely filtering its food so that there is a possibility that aquatic microbes can be eaten by the fish (Tobing et al., 2020).

Seeing a large amount of plastic waste pollution in marine ecosystems, it is suspected that bioplastic P(3HB)-producing bacteria are found in fish that live in waters polluted by plastic waste. Based on this, this study will isolate and screen bacteria-producing poly(3-hydroxybutyrate) bioplastics from the intestines of mackerel (*Rastrelliger* sp.).

## Method

*Sample Preparation, Tool, and Media Sterilization*

The sample used in this research is mackerel fish purchased from fish traders in the Padang Beach area, Padang City. The fish were immediately brought to the Sumatran Biota Laboratory using a cool box filled with bulk ice. The fish was cut, weeded and then the gills and intestines were separated for further research. The tools used are washed and dried. Then the tools and media were sterilized using an autoclave at 121$^{o}$C with a pressure of 15 lbs for 15 minutes.

*Isolation of Bacteria from Mackerel*

Isolation of fish bacteria using bacto agar bacterial isolation media with glucose and $(NH_4)_2.2H_2O$ added. A total of 7.5 grams of bacto agar, 0.55 grams of $(NH_4)_2.2H_2O$, and 7.31 grams of glucose were dissolved in 500 ml of distilled water. The solution is sterilized by autoclaving. Then pour into petri dishes 15 ml each. The gills and intestines of the fish were weighed as much as 1 gram each pulverized with a sterile mortar and then put into 9 ml of physiological saline. Multilevel dilutions were carried out up to $10^{-5}$. The results of the $10^{-3}$-$10^{-5}$ dilution were poured into the isolation medium and incubated for 24 – 48 hours at 28-30$^{o}$C in the incubator.

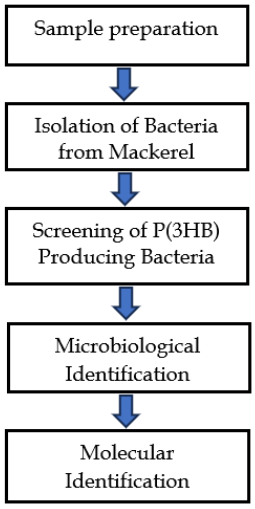


**Figure 1**. Research procedure

*Screening of P(3HB) Bioplastic Producing Bacteria Isolates*

The dye used to screen P(3HB) bioplastic-producing bacteria was 1% Nile Blue A powder. As much as 1 gram of Nile Blue A was dissolved in absolute ethanol to a volume of 100 ml. The media that has been overgrown with bacterial colonies is dripped with 1% Nile Blue A solution and allowed to stand for 30 minutes at room temperature. Then viewed under ultraviolet light at a wavelength of 365 nm. The result is positive if the bacterial colonies produce orange fluorescence.

*Microbiological Identification*

Microbiological identification is carried out by observing macroscopically and microscopically. Macroscopic observations can be carried out directly by observing the characteristics of isolated bacterial colonies including shape, edges, elevation, color, and size of bacterial colonies. Microscopic observations can be made by observing the shape of the bacterial cells and Gram staining. Observations were observed with a light microscope with 100x magnification dripped with immersion oil. Gram-positive bacteria are marked in purple and Gram-negative bacteria are marked in pink.

*Molecular Identification*

Identification of P(3HB) bioplastic producing bacteria using 16S rRNA gene. The primers used were 16SrRNA_27F (5' AGA GTT TGA TCM TGG CTC AG 3') and 16SrRNA_1525R (5' AAG GAG GTG WTC CAR CC 3'). The PCR program used was pre-denaturation for 2 minutes at 95°C, denaturation for 45 seconds at 95°C, annealing for 45 seconds at 56°C, extension for 1 minute at 72°C, final extension for 5 minutes at a temperature of

72°C, and cooling at a temperature of 8°C. The base sequence of the 16S rRNA gene of the bacterial sample was then BLAST on the NCBI website and a genetic phylogenetic tree was constructed using the MEGA X program.

## Result and Discussion

*Isolation of Bacteria from Mackerel*

Isolation of bacteria-producing bioplastic P(3HB) was carried out in the intestines and gills of mackerel fish. Intestines and gills. Bacterial isolation was carried out using the pour plate method dilutions of $10^{-3}$ to $10^{-5}$ using specific media. The media used is a medium high in glucose and few nutrients that can grow bioplastic-producing bacteria. Total bacterial colonies that grew were counted after 24 hours of incubation. The results of bacterial TPC calculations can be seen in Table 1.

**Table 1.** Isolation of bacteria from mackerel

| Sample type | Total Colonies (cfu/ml) | Total isolates |
|---|---|---|
| Mackerel intestines | $1.50 \times 10^{4}$ * | 6 |
| Mackerel gills | $1.10 \times 10^{5}$ | 4 |

Information:
*: Outside the range of the number of colonies 25-250

The results of isolating bacteria from the gills and intestines of mackerel obtained four isolates from the gills of mackerel and six isolates of bacteria from the intestines of mackerel with a total bacterial colony count of 1.5x104 *CFU/ml in the gills of mackerel and 1.1x105 in the gills of mackerel. CFU/ml (Table 1). Bacterial isolates were more commonly found in the intestines than gills of mackerel. The intestine is where the digestive process takes place, so many normal bacteria are found in the intestine. One of the factors that influences the abundance of bacteria is the total organic amount in the aquatic environment. Marwan et al., (2015) said the relationship between total bacteria and total organic matter in aquatic environments correlated 92% where the higher the amount of organic matter, the higher the number of bacteria.

The presence of bacteria in the gills and intestines of fish can be influenced by the fish's living environment. Fish that live in polluted environments have the potential to be contaminated by microplastics that carry bacteria. Amadi et al., (2020) examined the assimilation of microplastics and bacteria found in the gills, intestines, and tissues of fish. Microplastics were detected in the internal organs of the fish and the bacteria were identified as Staphylococcus epidermis, Pseudomonas xiamenensis, Bacillus licheniformis, Klebsiella pneumoniae, Bacillus lentus, Escherichia coli and Vibrio alginolyticus.

*Screening of P(3HB) Bioplastic Producing Bacteria Isolates*

Screening for P(3HB) bioplastic-producing bacteria was carried out on 17 isolates. Screening uses Nile Blue A solution which is dropped on isolation media that has bacteria growing. After 30 minutes, the bacteria were viewed under 365 nm UV light. Isolates with orange fluorescence indicate that the isolate has the potential to produce P(3HB) bioplastic.

Screening results showed that only IKE-1 bacterial isolates showed orange fluorescence (Table 2; Figure 2). Bacteria that can produce P(3HB) are characterized by the presence of orange fluorescence in bacterial colonies seen under 365 nm UV light. Ostle & Holt, (1982) report that P(3HB) granules found in bacteria will show orange fluorescence if dropped with a 1% Nile Blue A. This is in accordance with research by Yanti et al., (2021) that bacterial colonies that accumulate PHB in their cells, after Nile blue A staining, show bright orange fluorescence when irradiated with UV light. The discovery of bacteria producing bioplastic P(3HB). Awe et al., (2023) have carried out PHB screening with Nile blue A and Sudan black staining to identify isolates isolated from dumpsite soil.

**Table 2.** Screening results for P(3HB) producing bacteria

| Mackerel gill isolate | P(3HB) screening | Mackerel intestine isolate | P(3HB) screening |
|---|---|---|---|
| IKE-1 | + | UKE-1 | - |
| IKE-2 | - | UKE-2 | - |
| IKE-3 | - | UKE-3 | - |
| IKE-4 | - | UKE-4 | - |
| | | UKE-5 | - |
| | | UKE-6 | - |

Information:
+ : Shows orange fluorescence
- : Does not show orange fluorescence

**Figure 2**. Screening results of bioplastic-producing bacteria isolate P(3HB) isolate IKE-1

There are three staining methods to detect PHB granules in cells, namely Nile Blue, Nile Red, and Sudan Black which have high affinity for PHB (Ostle & Holt, 1982; Spiekermann et al., 1999). So these three stains can be used to detect PHB. The discovery of bacteria producing bioplastic P(3HB) in the intestines of mackerel has never been reported before, so it is interesting to study further. Bacterial isolates that show orange fluorescence are then subjected to microbiological identification.

*Identification of P(3HB)-producing Bacterial Isolates*

Identification of bacterial isolates producing P(3HB) was carried out macroscopically and microscopically. Macroscopic observations look at shape, edges, elevation, pigment, and size. After macroscopic observations were carried out, they were continued with microscopic observations. One of the biochemical tests includes Gram staining of bacteria. Observation of bacterial cells using a microscope at 100x magnification. The results of macroscopic and microscopic observations of P(3HB) producing bacterial isolates are shown in Table 3.

The results of macroscopic observations on the IKE-1 isolate showed that it was white cocci with a small size. The elevation and edges of the bacterial colonies are smooth and convex (Table 3; Figure 3). Alshehrei, (2019) obtained macroscopic results on isolates of PHB-producing bacteria with short shapes, irregular form, serrate margins, flat elevation, and white colors. Susianingsih et al., (2020) also reported the morphological characteristics of five isolates with circular, widened, and rather flat shape. Flat, sandy, and bubbly margin with creamy and white colors. Pure culture is a culture that only contains one type of bacteria. The shape of the colony can vary between species and is characteristic of a particular species (Lay, 1994).

**Table 3.** Results of macroscopic and microscopic test observations on bacterial isolates

| Morphology | IKE-1 |
|---|---|
| Shape | Coccus |
| Margin | Convex |
| Elevation | Smooth |
| Color | White |
| Size | Small |
| Gram | Negative |
| Cell Type | Monobasil |

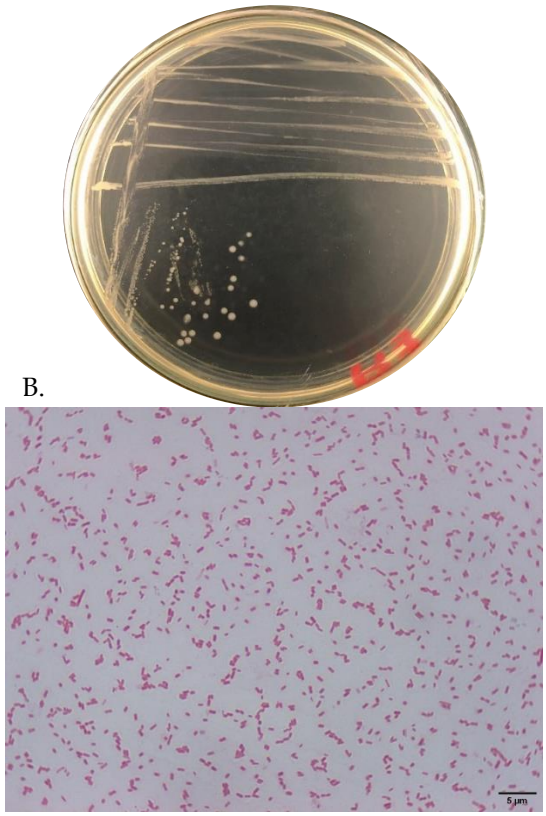


**Figure 3.** Observation results of IKE-1 isolate bacteria. A. Macroscopic, B. Microscopic

The results of Gram staining on IKE-1 isolate were Gram-negative with a monobacilli cell shape (Table 3; Figure 3). Gram staining is used to determine the morphology of bacterial cells and to differentiate Gram-positive and Gram-negative bacteria. Alshehrei, (2019) reported the results of the Gram stain bacterial isolates isolated from soil samples in the Gram-positive group with the short shape. Susianingsih et al., (2020) isolated five isolates from shrimp culture resulting in five isolates from the positive group with rod and chained coccus shape. Gram-positive and Gram-negative bacteria are capable of producing PHB granules within their cells. Bacteria classified as Gram-positive are those that can absorb the deep purple color of crystal violet stain, whereas Gram-negative bacteria are those that can absorb the red color of safranin (Byrom, 1987).

*Molecular Identification*

The phylogenetic tree in Figure 4 has two main branches that divide the grouping of bacteria into two clusters. The first cluster is called Cluster A (consisting of 17 bacteria) and Cluster B (consisting of 4 bacteria). Cluster A is divided into sub-clusters A.I and A.II. The IKE-1 bacterial isolate shown in the red box is in sub-cluster A.II. In the nearest branch, the IKE-1 isolate was close to the *Enterobacter roggenkampii* strain KQ-01. Then in the next branch followed by *Enterobacter roggenkampii* strain DSM 16690 and *Enterobacter roggenkampii* strain

WCHER090065. Based on the construction of a phylogenetic tree, it was concluded that the IKE-1 isolate was the *Enterobacter roggenkampii* species.

*Enterobacter roggenkampii* was successfully isolated from the gills of mackerel. This is the first discovery of *Enterobacter roggenkampii* that can accumulate P(3HB). Ji et al., (2021) also reported that *Enterobacter roggenkampii* bacteria were found in wastewater. Gan et al., (2021) isolated *Enterobacter roggenkampii* found in soil as a bacterium that degrades composite films of chitosan. *Enterobacter roggenkampii* can accumulate P(3HB) in its cells based on screening results with Nile Blue A staining. *Enterobacter roggenkampii* has a pathway for PHB synthesis in bacterial cells. P(3HB) is synthesized by 3 types of enzymes: 3-Ketothiolase (PhaA), acetoacetyl-CoA reductase (PhaB), and P(3HB) synthase (PhaC) (Kawaguchi & Doi, 1992; Roohi et al., 2018).

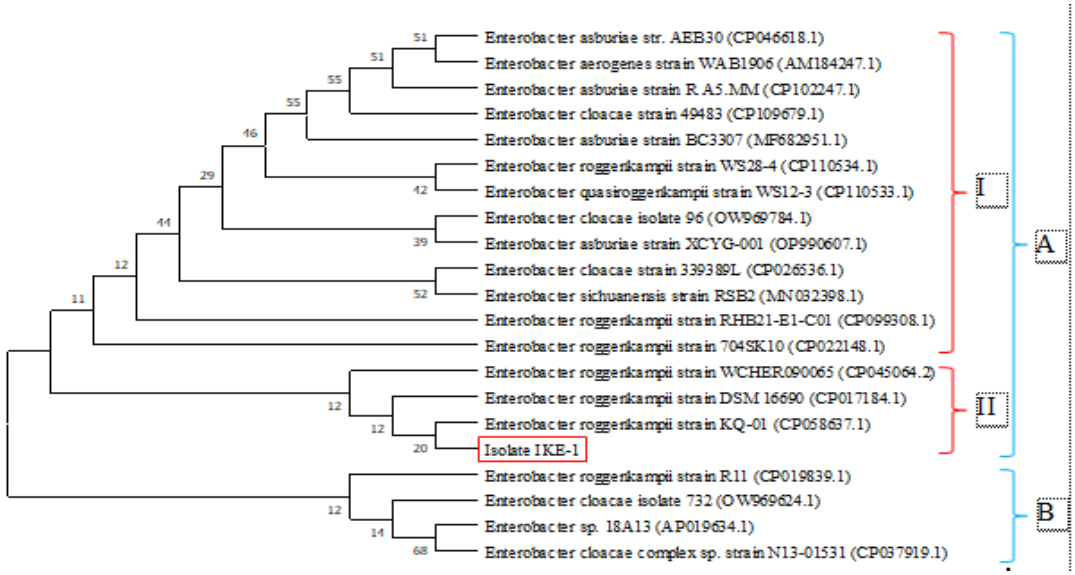


**Figure 4.** Phylogenetic tree of IKE-1 isolate

## Conclusion

Based on the research, it can be concluded that the bacterial isolate isolated from the gills of mackerel (IKE-1) can accumulate P(3HB) in its cells as seen in the screening results with Nile Blue A staining. The bacteria is *Enterobacter roggenkampii.*

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**Author Contributions**

All authors made an equal contribution to this research. all authors reviewed the results and approved the final version of the manuscript.

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**Conflicts of Interest**

The author's interest in publishing this article is to fulfill the research output in the form of publication in a scientific journal as proof of the required performance. No conflict of interest.

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