Egyptian Journal of Aquatic Biology and Fisheries
Zoology Department, Faculty of Science,
Ain Shams University, Cairo, Egypt.
ISSN 1110 – 6131
Vol. 28(6): 589 – 601 (2024)
www.ejabf.journals.ekb.eg

Molecular Identification and Polymer Determination of Poly(3-hydroxybutyrate) Producing Bacteria Isolated from the Fish *Lutjanus* sp.

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ARTICLE INFO

Article History:
Received: Oct. 29, 2023
Accepted: Nov. 16, 2024
Online: Nov. 18, 2024

Keywords:
Bioplastic,
GC-MS,
Isolation,
Fish bacteria,
Nile blue A,
Screening P(3HB)

ABSTRACT

Poly(3-hydroxybutyrate) is a biodegradable bio-plastic that can be produced by bacteria and used as a substitute for petrochemical plastics. Various sources of Poly(3-hydroxybutyrate) producing bacteria have been successfully studied. However, few bacteria from fish samples have been identified. More sources of Poly(3-hydroxybutyrate) producing bacteria need to be found to obtain potential bacteria. Therefore, it is necessary to carry out molecular identification of poly(3-hydroxybutyrate) producing bacteria isolated from *Lutjanus* sp. and determine the polymer content. The methods of this research include the isolation and screening of isolates of Poly(3-hydroxybutyrate) producing bacteria, molecular identification of Poly(3-hydroxybutyrate) producing bacteria, and production and determination of Poly(3-hydroxybutyrate) content using gas chromatography-mass spectrometry (GM-MS). The results indicate that during the bacterial screening stage, two isolates capable of producing Poly(3-hydroxybutyrate) were isolated from the intestine (UKA-2) and gill (IKA-4) of *Lutjanus* species. Molecular identification of UKA-2 and IKA-4 bacteria revealed their similarity to *Enterobacter cloacae* and *Bacillus* species. The Poly(3-hydroxybutyrate) content testing by GC-MS showed that the content value of UKA-2 was 1.39%, while IKA-4 had a content of 78%. This demonstrates the significant potential of the IKA-4 isolate, *Bacillus* sp. isolated from the gill of *Lutjanus* sp. to produce Poly(3-hydroxybutyrate).

INTRODUCTION

Plastic from which products are made represents an important material nowadays. The huge levels of plastic usage can cause environmental problems. Plastic can cause a buildup of plastic waste since it is difficult to decompose. Petroleum-based plastics also have bad effects on the environment and health **(Proshad *et al*., 2017)**. Some dangerous plastic contents are bisphenol A and phthalate **(Halden, 2010)**. Since this issue causes global environmental problems, researchers are trying to find alternatives. One alternative

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that can be done is by using bioplastics **(Prasteen *et al*., 2018)**. Bioplastics have the property of being easily decomposed by decomposing microorganisms. One of the bioplastics that is being widely researched is Poly(3-hydroxybutyrate).

Poly(3-hydroxybutyrate) P(3HB) is a polymer produced by bacteria. P(3HB) is a short-chain polymer form of Polyhydroxyalkanoate **(Tan *et al*., 2014)**. P(3HB) can be accumulated by bacteria in conditions of high carbon sources and limited nitrogen sources **(Ali & Jamil, 2014)**. There has been a lot of research on bacteria that can accumulate P(3HB) in their cells. Screening for PHB-producing bacteria can be done using the Nile blue A and Sudan black staining **(Ostle & Holt, 1982; Musa *et al*., 2016)**. The Nile blue dye is superior to Sudan black dye in screening PHB-producing bacteria **(Ostle & Holt, 1982)**. In addition, P(3HB) has a similar material and can replace polypropylene (PP) and polyethylene (PE). P(3HB) is easily decomposed in the marine environment, hence it can reduce marine debris and microplastics; therefore, P(3HB) is very environmentally friendly **(Markl *et al*., 2018)**. P(3HB) can decompose within a certain time if it comes into contact with an environment containing decomposing microorganisms **(McAdam *et al*., 2020)**.

PHB is a polymer produced intracellularly by bacteria as an energy reserve. PHB can be applied for industrial purposes in agriculture, cosmetic containers, packaging, and medical to replace petrochemical plastics **(Musa *et al*., 2016)**. P(3HB) could be used as a matrix for urea slow-release fertilizer **(Djamaan *et al*., 2015)**. Several studies have been carried out isolating P(3HB) producing bacteria from several samples such as sludge and sea **(Kavitha *et al*., 2018; Narayanan *et al*., 2021)**. Some PHB-producing bacteria that have been identified are *Acinetobacter nosocomialis* and *Erythrobacter aquimaris* **(Reddy *et al*., 2019; Mostafa *et al*., 2020)**.

There have been many studies regarding the isolation and potential of bacteria in producing P(3HB) from various sources. However, little research on the potential of P(3HB) from fish samples has been carried out. Bacteria in fish that live in environments contaminated with plastic or microplastics are thought to be able to accumulate P(3HB) in their cells. Fish can be contaminated with plastic or microplastics obtained from the aquatic environment. One of the fish contaminated with microplastics is *Lutjanus* sp. **(Murugan *et al*., 2021; Susanti *et al*., 2022)**. Therefore, it was necessary to carry out molecular identification of bacteria isolated from *Lutjanus* species to determine the P(3HB) content that can be produced by bacteria.

MATERIALS AND METHODS

Isolation of bacteria from *Lutjanus* sp.

The fish used in this research were those of *Lutjanus* sp. obtained from the fish market in the Padang Beach area, Indonesia. The parts of the fish that were isolated were the intestines and gills. Isolation of bacteria from fish was performed using the multilevel dilution method up to 10^{-5}. The specific isolation medium used was bacto agar, glucose,

and $(NH_4)_2.2H_2O$. The samples were incubated at 28ºC for 24 hours to 48 hours **(Djamaan & Dewi, 2014)**.

Screening for P(3HB) producing bacteria

Bacteria were isolated from the gills and intestines of *Lutjanus* sp. Screening for P(3HB)-producing bacteria was conducted using the Nile blue A **(Ostle & Holt, 1982)**. It was was applied to the bacterial colonies and left for 30 minutes. Bacteria capable of producing P(3HB) exhibited an orange fluorescent color when observed under UV light with a wavelength of 365nm.

Molecular identification of P(3HB) producing bacteria

Amplification of the 16s rRNA gene used the primer pair 16SrRNA_27F (5' AGA GTT TGA TCM TGG CTC AG 3') and 16SrRNA_1525R (5' AAG GAG GTG WTC CAR CC 3') **(Weisburg *et al*., 1991)** with an estimated product size of 1,498 bp. DNA amplification begins with making a PCR mix (Table 1) and PCR program (Table 2).

Table 1. PCR mix

Reagent	Volume
KOD one blue master mix (Toyobo, Japan)	25 µl
Primer 16SrRNA_27F (10 ng/µl)	2 µl
Primer 16SrRNA_1525R (10 ng/µl)	2 µl
Bacterial genomic DNA (10 ng/µl)	2 µl
Nuclease free water	19 µl
Total	50 µl

Table 2. PCR reaction program

Stage	Temperature (°C)	Time	Cycle
Pre denaturation	95	2 minutes	
Denaturation	95	45 seconds	35
Annealing	56	45 seconds	
Extensions	72	1 minute	
Final extension	72	5 minutes	
Cooling	8	pause	

The Sanger sequencing method was used to sequence the 16S rRNA gene PCR product of bacterial samples in a bi-directional manner. The sequencing process was completed at 1st base, a sequencing services provider located in Singapore. SeqMan ™ was used to alter and manipulate the sequencing of every sample. The 16S rRNA gene base sequence for every bacterial sample was then found using BLAST on the NCBI website **(Zhang *et al*., 2000)**. Following the BLAST results, 20 bacterial sample sequence data were chosen from the Genebank and utilized for alignment, phylogenetic

tree construction, and genetic distance calculation using the MEGA X program **(Kumar *et al.*, 2018)**. The clustal W algorithm was used to perform alignment. Neighbor-joining **(Saitou & Nei, 1987)** and evolutionary techniques were used to create phylogenetic trees.

Production of P(3HB) producing bacteria

Bacterial isolates UKA-2 and IKA-4, which have the potential to produce P(3HB), were inoculated 1-2 times into 10ml of sterile nutrient broth (NB). The NB media was incubated in a rotary shaker for 24 hours at 200rpm to grow bacteria. NB media growing bacteria were used for the production of P(3HB). The fermentation media used were glucose, mineral sources, and microelement solutions. 3ml of seed culture was transferred into 100ml of fermentation media. The fermentation media were incubated in a rotary shaker for 48 hours at 30°C with a speed of 200rpm **(Djamaan & Dewi, 2014)**. The process of separating biomass and supernatant was carried out by centrifugation at a speed of 3000 rpm for 20 minutes. Biomass was used to see the P(3HB) content produced by bacteria in cells, which is determined using gas chromatography-mass spectrometry (GS-MS).

Determination of P(3HB) content using GS-MS

Determination of the P(3HB) content was carried out using GS-MS method using cooled and dried bacterial biomass **(Brandl *et al.*, 1988)**. The gas chromatography program was set with a detector temperature of 250°C and an injector temperature of 260°C. The initial column temperature was 50°C, held for 5 minutes, followed by a temperature program of 10°C per minute, reaching a final column temperature of 220°C, which was maintained for 2 minutes. The P(3HB) content was analyzed by measuring the area under the curve formed on the chromatogram.

RESULTS

Screening for P(3HB) producing bacteria

Isolation of bacteria from *Lutjanus* sp. obtained two isolates originating from the intestine (UKA-2) and gill (IKA-4). The two bacterial isolates were screened for producing Poly(3-hydroxybutyrate) by dripping the Nile blue A dye onto the bacterial colony. The screening results showed that isolate UKA-2 and isolate IKA-4 could produce Poly(3-hydroxybutyrate) in their cells as indicated by the presence of orange fluorescence seen under UV light 365nm (Fig. 1).

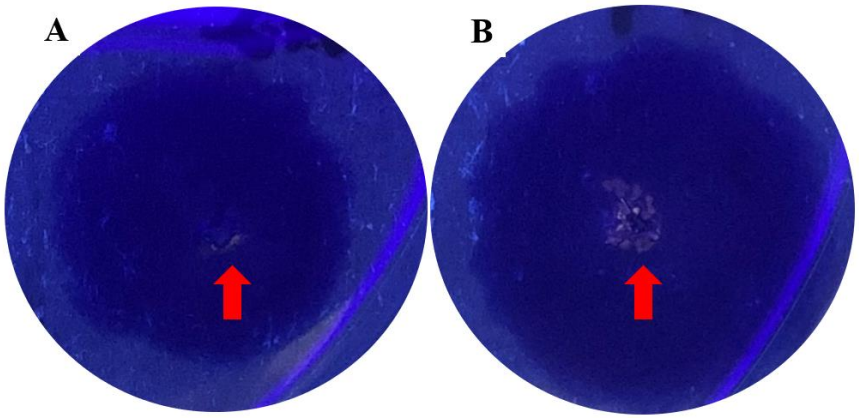

Fig. 1. Screening results for P(3HB) producing bacteria with the Nile blue *A.* A = Isolate UKA-2 B = Isolate IKA-4

Molecular identification of P(3HB) producing bacteria

The PCR visualization results of the 16S rRNA gene obtained a target band of ± 1498bp. In both bacterial samples, the target band was successfully obtained according to the estimated size indicated by the red arrow (Fig. 2). Apart from the target band, other thin bands of various sizes also appeared in each bacterial sample. These thin bands appear to be caused by the presence of a multicopy 16S rRNA gene in the genome of the bacterial sample, resulting in the attachment of the primer to another region in the genome.

The 16S rRNA gene sequences of the bacterial isolates UKA-2 and IKA-4 (Fig. 3) were analyzed using BLAST (http://blast.ncbi.nlm.nih.gov/Blast). BLAST results show information based on the taxonomy of 100 selected sequence data. Taxonomically, it was found that the UKA-2 isolate bacteria belong to the genus *Enterobacter*. 58 of 106 hits is *Enterobacter cloacae.* The phylogenetic tree shows the relationship between the UKA-2 isolate and 20 comparison bacteria from the Genebank based on the 16S rRNA gene fragment sequence (Fig. 4a).

Taxonomically, it was found that the IKA-4 isolate bacteria belong to the genus *Bacillus.* 62 out of 100 hits is *Bacillus cereus.* This was followed by 15 hits of *Bacillus thuringiensis.* Alignment results with Clustal W for the IKA-4 bacterial isolate sequence data with 20 other bacterial data did not show base variations. This is ascribed to the analyzed sequences of the 16S rRNA gene having 100% similarity. The phylogenetic tree shows the relationship between the IKA-4 isolate and 20 comparison bacteria from the Genebank based on the 16S rRNA gene fragment sequence (Fig. 4b).

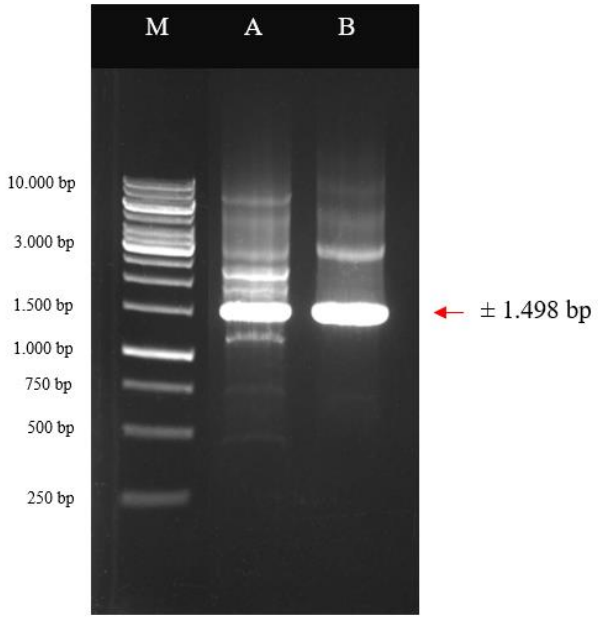

Fig. 2. Visualization of Bacterial 16SrRNA Gene Amplification Results. M= Marker 1kb gene ruler. A= PCR product of the *16SrRNA gene* of UKA-2 isolate bacteria. B=PCR product of the 16SrRNA gene of IKA-4 isolate bacteria

A

CGGTAGCACAGAGAGCTTGCTCTCGGGTGACGAGTGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGGGATAA
CTACTGGAAACGGTAGCTAATACCGCATAACGTCGCAAGACCAAAGAGGGGGACCTTCGGGCCTCTTGCCATCAGATGTGCCCAG
ATGGGATTAGCTAGTAGGTGGGGTAACGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAAC
TGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTG
TATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGGGAGGAAGGTGTTGTGGTTAATAACCGCAGCAATTGACGTTACCCG
CAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAG
CGCACGCAGGCGGTCTGTCAAGTCGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCGAAACTGGCAGGCTAGAGTCTT
GTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGAC
AAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGATTT
GGAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAGCTAACGCGTTAAATCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCA
AATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTGGTCTTGACATC
CACAGAACTTAGCAGAGATGCTTTGGTGCCTTCGGGAACTGTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAAT
GTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCGGTTCGGCCGGGAACTCAAAGGAGACTGCCAGTGAT
AAACTGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGACCAGGGCTACACACGTGCTACAATGGCGCATACAAA
GAGAAGCGACCTCGCGAGAGCAAGCGGACCTCATAAAGTGCGTCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTC
GGAATCGCTAGTAATCGTAGATCAGAATGCTACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTG
GGTTGCAAAAGAAGTAGGTAGCTTAACCTTCGGGAGGGCGCTTACCACTTTGTGATTCATGACTGGG

B

TGCAAGTCGAGCGAATGGATTAAGAGCTTGCTCTTATGAAGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCCATAA
GACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATAACATTTTGAACCGCATGGTTCGAAATTGAAAGGCGGCTTCGGCTG
TCACTTATGGATGGACCCGCGTCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGG
GTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTC
TGACGGAGCAACGCCGCGTGAGTGATGAAGGCTTTCGGGTCGTAAAACTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAG
CTGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATC
CGGAATTATTGGGCGTAAAGCGCGCGCAGGTGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGA
AACTGGGAGACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGAGATATGGAGGAACACCAGTG
GCGAAGGCGACTTTCTGGTCTGTAACTGACACTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCAC
GCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGAAGTTAACGCATTAAGCACTCCGCCTGGGGAGTA
CGGCCGCAAGGCTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAG
AACCTTACCAGGTCTTGACATCCTCTGAAAACCCTAGAGATAGGGCTTCTCCTTCGGGAGCAGAGTGACAGGTGGTGCATGGTTG
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CTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTG
CTACAATGGACGGTACAAAGAGCTGCAAGACCGCGAGGTGGAGCTAATCTCATAAAACCGTTCTCAGTTCGGATTGTAGGCTGCA
ACTCGCCTACATGAAGCTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCC
CGTCACACCACGAGAGTTTGTAACACCCGAAGTCGGTGGGGTAACCTTTTTGGAGCCAGCCGCCTAAGGTGGGACAGATGATTGG
GG

Fig. 3. 16S rRNA gene sequence of the bacterial. A= Isolate UKA-2, B= Isolat IKA-4

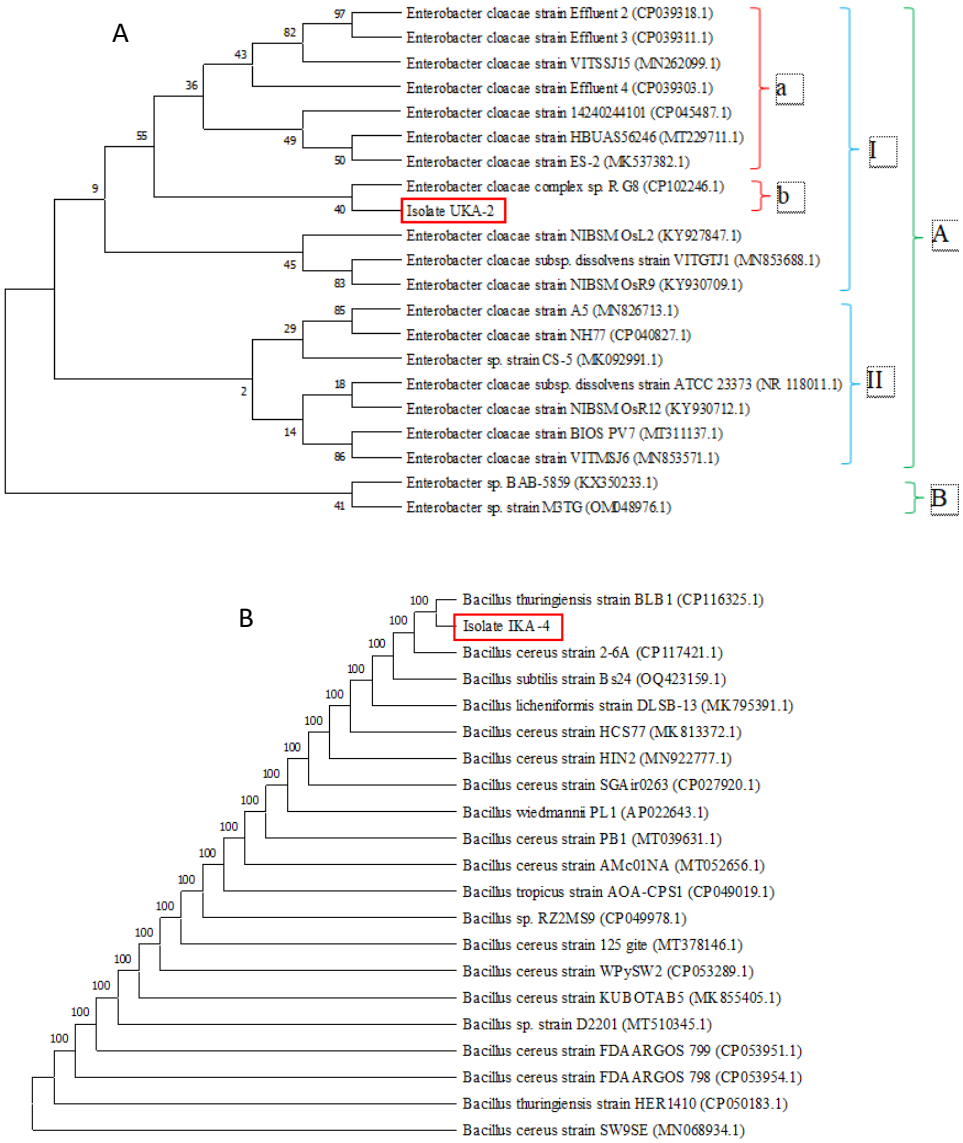

Fig. 4. Phylogenetic tree. A=Isolate UKA-2, B=Isolate IKA-4

Production and determination of P(3HB) content

P(3HB) production was carried out by fermentation using a liquid medium. The fermentation medium contains a high carbon source and limited nutrient sources. The production results and P(3HB) content in bacteria are presented in Table (3). The chromatograms of isolates UKA-2 and IKA-4 are displayed in Fig. (5).

Table 3. Production and determination of P(3HB) content

Isolate code	Dry biomass weight (gr)	Area Bottom of the Curve	Content P(3HB) (mg/20mg)	Percentage P(3HB) (%)
UKA-2	1.80	3215179	0.2787	1.39
IKA-4	1.65	176896331	15.3387	76

P(3HB) content produced by the UKA-2 isolate was 1.99%, while the IKA-4 isolate could produce 76% P(3HB). This shows the ability of the IKA-4 isolate to produce P(3HB). The UKA-2 isolate is *Enterobacter cloacae* bacteria isolated from the intestines of *Lutjanus* sp. UKA-2 *Enterobacter cloacae* can produce P(3HB) of 0.2787mg/ 20mg with a percentage of 1.39%.

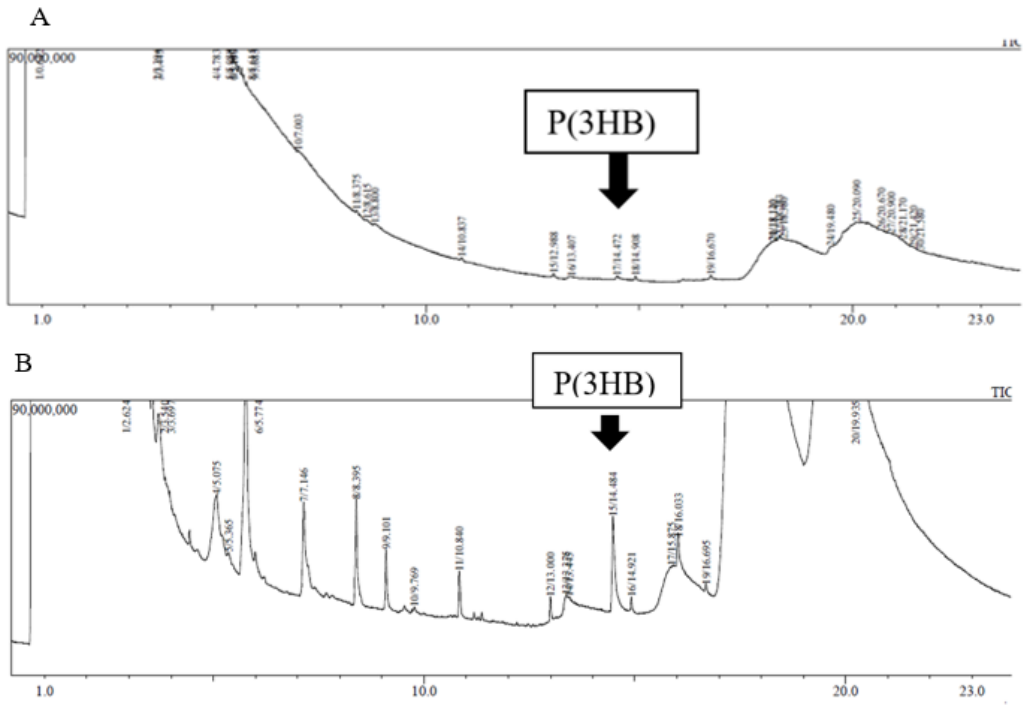

Fig. 5. Chromatogram of GC-MS. A= Isolate UKA-2, B= Isolate IKA

DISCUSSION

The first step in this study was to isolate and screen P(3HB)-producing bacteria from *Lutjanus* species. When bacteria colonies are exposed to 365nm UV radiation, they exhibit orange fluorescence, which is indicative of bacteria that can generate P(3HB). According to **Ostle and Holt (1982)**, bacteria containing P(3HB) granules emit an orange glow when treated with 1% of the Nile blue A. **Gatia *et al*. (2017)** screened P(3HB) producing bacteria with the Nile blue A dye which was detected, upon using a UV trans-illuminator with a wavelength of 312nm, producing bright orange fluorescence. In this

context, **Altaee *et al.* (2017)** used the Nile blue A staining to determine bacteria that can accumulate PHB in their cells, which is shown by a bright orange fluorescent color using a fluorescent microscope.

Molecular identification is carried out to identify bacterial species. Based on its position on the phylogenetic tree (Fig. 4a), the UKA-2 isolate has the closest relationship to the *Enterobacter cloacae complex* sp. *R G8.* Given the results of BLAST analysis, alignment, genetic distance calculations, and phylogenetic tree construction, it was concluded that the UKA-2 isolate is a bacterial species of Enterobacter cloacae. This is in accordance with a research by **Shyam *et al.* (2021)** who isolated *Enterobacter cloacae* bacteria from seawater for PHB production. In addition, **Omar and Salim (2020)** reported that *Enterobacter cloacae* isolated from soil samples can produce PHB. **Brahmi *et al.* (2018)** postulated that *Enterobacter cloacae* bacteria were found in the intestine and gill of fish. *Enterobacter cloacae* bacteria can produce polyhydroxyalkanoates isolated from agricultural waste and sugar cane **(Pungsungvorn & Wisetsing, 2021; Kingsly *et al.*, 2022)**.

The phylogenetic tree of IKA-4 constructed resulted in only one main branch and one branch of comparative bacteria consisting of 21 bacteria analyzed (Fig. 4b). This occurs due to the sequence of the 16S rRNA gene fragment analyzed having a similarity percentage of 100%, and this is confirmed by the results of BLAST, alignment, and genetic distance calculation. Identification of IKA-4 bacterial isolates can only be done at the genus level. Based on this, it was concluded that the IKA-4 isolate was *Bacillus* sp. Identification of *Bacillus* bacteria genus using the 16S rRNA gene does not show discrete results representing the species. Therefore, to identify bacteria of the genus *Bacillus*, it is recommended to use genes barcoding using other genes; for example, by using housekeeping genes such as *dnaJ, dnaK, mutL, pheS,* and *yycH,* as described by **Huang *et al.* (2018)**. **Bhagowati *et al.* (2015)** elucidated that the bacteria *Bacillus* sp. isolated from the sea can produce PHB. Furthermore, *Bacillus* sp. bacteria were reported with potential to produce PHB isolated from soil **(Mohapatra *et al.*, 2014; Hassan *et al.*, 2016)**.

Bacterial isolates are fermented to produce P(3HB). The polymer content of P(3HB) can be seen using GC-MS. **Pungsungvorn and Wisetsing (2021)** assessed that *Enterobacter cloacae* bacteria produced as much polyhydroxyalkanoate as 1.30% with sugar cane extract carbon source. Additionally, **Omar and Selim (2020)** produced Poly(3-hydroxybutyrate) using *Enterobacter cloacae* bacteria, with a maximum value of 2186mg/ l, incubated for 48 hours with a sucrose carbon source. In this respect, **Kingsly *et al.* (2022)** determined that *Enterobacter cloacae* can produce polyhydroxyalkanoate as much as 4.13 – 4.98g/ L or 48 - 56%, with molasses as a carbon source. The IKA-4 isolate is a *Bacillus* sp. isolated from *Lutjanus* sp. Isolate IKA-4 has great potential in producing P(3HB) of 76%, using glucose as a carbon source. **Hassan *et al.* (2016)**

recorded that the P(3HB) content of *Bacillus sp.* N- 2 isolated from soil is 20% of the dry weight of cells.

PHB synthesis is carried out by a series of enzymes, namely (1) β-ketothiolase which catalyzes the acid derivative acetyl-CoA to become acetoacetyl-CoA, (2) acetoacetyl-CoA reductase, which catalyzes the hydrogenation of acetoacetyl CoA to become [R]-3-hydroxybutyryl-CoA, which is PHB monomer, and (3) PHB synthase which catalyzes the polymerization of PHB monomer into PHB **(Lenz & Marchessault, 2005)**.

CONCLUSION

P(3HB) producing bacteria from the intestine and gill of *Lutjanus* sp. were successfully isolated and screened with the Nile blue A staining, and molecularly identified using the 16S rRNA gene. The UKA-2 isolate is *Enterobacter cloacae* bacteria and the IKA-4 isolate is *Bacillus* sp. The UKA-2 isolate can produce 1.99% P(3HB), while the IKA-4 isolate can produce 76% P(3HB). This shows the great ability of the IKA-4 isolate, *Bacillus* sp. isolated from the gill of *Lutjanus* sp. to produce P(3HB).

ACKNOWLEDGMENTS

The author would like to thank the Graduate School of Andalas University for funding the research and publication of this article, under the Basic Research Scheme (RD) Research Contract Number: B 56 / UN16.DIR / PT.01.03 / 2023 for the fiscal year 2023. Additionally, gratitude is extended to LPPM Universitas Andalas for their guidance in preparing this article through Scopus Camp Batch 5.

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