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Optimization and Analysis of Polyhydroxyalkanoate (PHA) by *Bacillus sp*. Strain CL33 and *Bacillus flexus* Strain S5a from Palm Oil Mill Waste

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Abstract*—Polyhydroxyalkanoate (PHA) is a biodegradable polymer that microorganisms can synthesize amidst non-optimal growth conditions with excess carbon sources. Palm oil, rich in fatty acids, serves as a carbon source for PHA synthesis. The bacterial PHA production can be influenced by carbon concentration in the growth medium. Therefore, determining the optimal concentration of palm oil as a carbon source is crucial for PHA production. Additionally, it is possible to determine the type of PHA generated by bacteria, which can then be utilized as information when processing utilizing the PHA. The experiment employed palm oil concentrations of 0.5%, 1%, and 2% and was carried out for periods of 48, 72, 96 hours. It was discovered that *Bacillus sp.* strain CL33 and *Bacillus flexus* strain S5a produced the most effective PHA at a concentration of 25 with an incubation period of 96 hours. The PHA generated by these bacteria was quantitatively analyzed through measurements of total bacterial growth, cell dry weight, and the levels of crotonic acid. PHA types were also analyzed using GC-MS, with monomers including 2-hydroxybutyrate(-2HB), 2-hydroxy-3-phenylpropionate (2H3PhP), 3-Hydroxyhexanoate (3HHx), 3-hydroxyoctanoate (3H2O), and 3-hydroxydecanoate (3HD). The *Bacillus sp.* strain CL33 yielded a PHA level of 92.23%. Meanwhile, *Bacillus flexus* strain S5a synthesized a polyhydroxyalkanoate comprising mostly 3-hydroxyhexanoate (3HHx) and polydimethylsiloxane (PDMS). The monomers used were decamethyltetrasiloxane, dodecamethylpentasiloxane, hexamethylcyclotrisiloxane, octamethylpentasiloxane, and dodecamethylcyclohexasiloxane. The type of PHA produced accounted for 85.93% of the total.

***Keywords*— *Bacillus flexus* S5a; *Bacillus sp.* CL33; polyhydroxyalkanoate; palm oil.**

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I. INTRODUCTION

Plastic is a highly utilized product and has become an essential component of human life. Over time, global plastic production increased tremendously from about 2 million metric tons in 1950 to 8.3 billion in 2015. This trend will persist as the world population grows. However, 6.3 billion metric tons (or 76%) of this production takes the form of waste that is challenging to decompose, harming the environment, ecosystems, and human health. The fragility of petroleum-based and non-biodegradable plastics serves as the foundation for the creation of biodegradable plastics (known as bioplastics) [1], [2], [3].

One of the most commonly utilized forms of bioplastics is polyhydroxyalkanoate (PHA) [4]. PHA is a biodegradable plastic with material attributes comparable to conventional plastics but can be completely degraded in nature to carbon dioxide, air, and organic compounds [5], [6]. PHA is a polymer that microbes can consume and accumulate], [8]. PHA is synthesized by bacteria as granules in their cells under unfavorable growth conditions with an abundant carbon source but a lack of nitrogen, magnesium, or oxygen [9], [10], [11].

O250 types of bacteria have been identified as capable of producing approximately 150 PHA structures, including both gram-positive and gram-negative species [12], [13]. Among the gram-negative bacteria that produce PHA are *Burkholderia*, *Azotobacter*, *Pseudomonas*, and the well-known *Cupriavidus necator* (also known as *Ralstonia eutropha*) [14], [15]. *Ralstonia eutropha* H16 has even been utilized as a model in studies [16], especially research on PHA synthesis enzymes [17]. However, gram-negative bacteria

have a disadvantage in PHA production compared to gram-positive bacteria due to lipopolysaccharide (LPS) endotoxins, necessitating a purification step [18]. *Bacillus* is a gram-positive bacterium that produces more PHA than gram-negative bacteria [19]. Among the confirmed PHA-producing *Bacillus* strain are *Bacillus aquimaris*, *B. bataviensis*, *B. flexus*, *B. vallismortis*, *B. vietnamensis* [20], *B. megaterium* [21], and *B. endophyticus* [22] *Bacillus sp.* strains CL33 and *Bacillus flexus* strain S5a, found in waste from palm oil mills, are also known as bacteria capable of producing PHA [23].

Optimization of fermentation can increase the yield and productivity of polyhydroxyalkanoate (PHA) produced by bacteria. Various factors, such as carbon ratio and incubation time [24], [25], affect PHA optimization. Among the carbon sources, palm oil waste produces higher levels of PHA because it has more carbon content per gram than sugar. This is because the waste from palm oil extraction contains cellulose and fatty acids that serve as a source of reducing sugars [26]. This study aims to identify the optimal carbon concentration for PHA production and the type of PHA produced by *Bacillus sp.* strain CL33 and *Bacillus flexus* strain S5a, which were isolated from palm oil mill waste.

II. Materials and Methods

A. Regeneration of PHA Bacterial Isolates

Bacillus sp. strain CL33, and *Bacillus flexus* strain S5a were each inoculated with one dose of pure isolate in nutrient agar (NA) media supplemented with 1% palm oil and 1% glucose in a petri dish. The inoculated mixture was then incubated for 24 hours at 37°C.

B. Variations in Carbon Source Concentration in PHA Production

A total of 5 ml of inoculum isolate, with a standardized OD value (25%T), was cultured in 100 ml of minimal medium [27] containing 0.5%, 1%, and 2% palm oil as carbon sources. The culture was incubated at room temperature for 48, 72, and 96 hours, utilizing a shaker at 150 rpm. Total bacterial growth was calculated by standard plate count (SPC) at each time period. Bacterial cultures were incubated for 48, 72, and 96 hours. The cultures were then centrifuged at 400 rpm for 15 minutes. The resultant cell mass was then washed with deionized water and centrifuged again for 15 minutes. The cell mass was rewashed and suspended in 5 ml of distilled water. Subsequently, the dry weight of the cell mass was determined using 1 ml of cell suspension, and PHA levels were analyzed via UV-Vis spectrophotometry using another 1 ml of the suspension.

C. Calculation of Total Microbial Growth

A total of 1 ml of bacterial culture was collected from the designated time intervals (48, 72, and 96 hours) and diluted with sterile distilled water before being cultured in Petri dishes on PCA media. The resulting samples were then incubated at 37 °C for 24 hours.

D. Measurement of Cell Mass Dry Weight

Containers with a known dry weight were filled with 1 ml of the cell suspension and dried in an oven at 70 °C (140 °F). The weight of the dried cell suspension was measured repeatedly to determine its weight.

E. PHA Analysis Using a UV-Vis Spectrophotometer

One milliliter of cell suspension, 3 milliliters of PH 7.0 phosphate buffer, and 1 milliliter of 5% NaOCl (sodium hypochlorite) were added, followed by incubation for 24 hours at room temperature and 180 rpm. The leftover pellets were collected through centrifugation at 4000 rpm for 15 minutes. Then, the supernatant was discarded. After adding the cell pellet and 5 ml of distilled water, the centrifuge was run for another 15 minutes at 4000 rpm. After discarding the supernatant, the cell pellet and 3 ml of acetone were combined and centrifuged at 4000 rpm for 15 minutes. The resulting pellet was rinsed with 3 ml of diethyl ether and allowed to stand for 5 minutes. The ether was then discarded along with the supernatant. Next, 3 ml of concentrated H_2SO_4 was added to the dry pellets, and the mixture was heated in a water bath at 100 °C for 10 minutes. Using a UV-Vis spectrophotometer at a wavelength of 235 nm and H_2SO_4 as a control, the optical density value of the crotonic acid produced was determined [28].

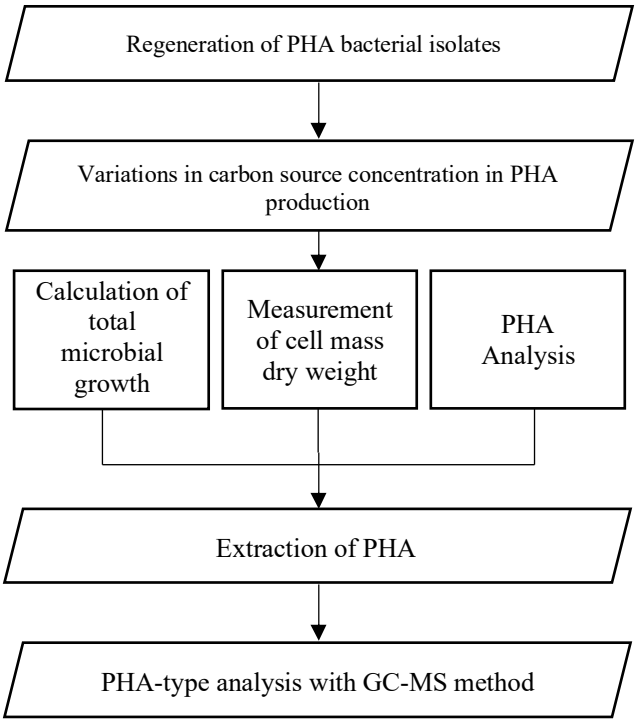

Fig. 1 Flow chart of this research

F. Extraction of PHA

PHA was obtained by adding 1 gram of dry cells to 50 ml of chloroform diluted with sodium hypochlorite. Prior to adding the dry cells, 5 ml of bacterial cell culture was centrifuged at 5000 rpm for 10 minutes and dried at 70 °C to achieve a constant weight. PHA was obtained by adding 1 gram of dry cells to 50 ml of chloroform diluted with sodium hypochlorite. The solution was shaken for 2 hours at 150 rpm and sonicated for 20 minutes, after which the cell cultures were incubated for 1 hour at 38 °C. The cell cultures were centrifuged at 5000 rpm for 10 minutes, resulting in three phases. The bottom phase containing chloroform and PHA was evaporated and mixed with cold methanol (9:1 v/v). The

PHA polymer granules were filtered using filter paper, and the constant dry weight was determined [29]. PHA concentration is calculated by the formula [30]:

$$\text{PHA Accumulation (\%)} = \frac{\text{Dry weight of PHA extract (g/L)}}{\text{Dry weight of cell (g/L)}} \times 100 \quad (1)$$

G. Analysis of the Type of PHA

The PHA samples extracted earlier were metabolized in a mixture of 1 ml of methanol and 1 ml of chloroform, both containing 2.8 M H_2SO_4, without any subjective evaluations. After incubating the samples at 100 °C for two hours, they were left to cool before adding 0.5 ml of distilled water. Abbreviations were explained the first time they occurred. The resulting organic phase containing methyl ester was analyzed utilizing GCMS-QP2010 Ultra [31]. The GC-MS apparatus was set up using the split less method, 76.9 kPa of pressure, 14 ml/min of flow rate, and a ratio of 1:10, with the injector temperature set at 250 °C. The ion source and interface are maintained at temperatures of 200 °C and 280 °C, respectively. The solvent cut duration is fixed at 3 minutes while m/z varies within a range of 400-700. Chromatography employs an SH-Rxi-5Sil MS column with a length of 30 m and an inner diameter of 0.25 mm. The column took a gradual temperature increase from 70 °C to 200 °C, at a rate of 10 °C per minute, and then to a final temperature of 280 °C at a rate of 5 °C per minute. The NIST and Wiley 9 libraries are used to process the chromatogram data.

III. Result and Discussion

A. Calculation of Total Bacteria Using the Standard Plate Count (SPC) Method

Bacillus sp. strain CL33 and *Bacillus flexus* strain S5a showed different growth patterns on Ramsay medium supplemented with 1% glucose and varying concentrations of palm oil. Glucose and palm oil were included in the medium to provide supplementary carbon sources for PHA production during bacterial growth. This is because the formation of PHAs is usually induced by nutrient limitation, such as oxygen, and excess carbon sources [32].

The growth of *Bacillus sp.* strains CL33 and *Bacillus flexus* S5a was measured using the SPC method. Analysis revealed overall bacterial growth increased from 48 to 96 hours. This increase can be attributed to the metabolic processes that occur when bacteria utilize carbon sources in the media, providing them with a means to thrive and reproduce. Carbon sources are a fundamental requirement for ell growth and metabolism [33].

Bacterial growth was the lowest at a 0.5% oil concentration and the highest at a 2% oil concentration, indicating that higher concentrations of palm oil increase bacterial growth. The media's palm oil concentration impacted the growth of the bacteria. Palm oil contains numerous fatty acids that can serve as a carbon source, yielding more carbon sources for bacterial growth when palm oil is more concentrated. Palm oil is composed of 48% saturated fatty acids, with palmitic acid (85%) being the largest component and 52% unsaturated fatty acids, with oleic acid (88%) being the largest component [34].

In addition to oil content, the incubation period also affected the growth of the two bacterial isolates. The growth of both bacterial isolates increased at every oil concentration after 48, 72, and 96 hours. The maximum bacterial growth for each oil concentration was noted after 96 hours of incubation, matching the findings of a previous study [35], in which *Bacillus tropicus* showed continuous growth even after 96 hours of incubation.

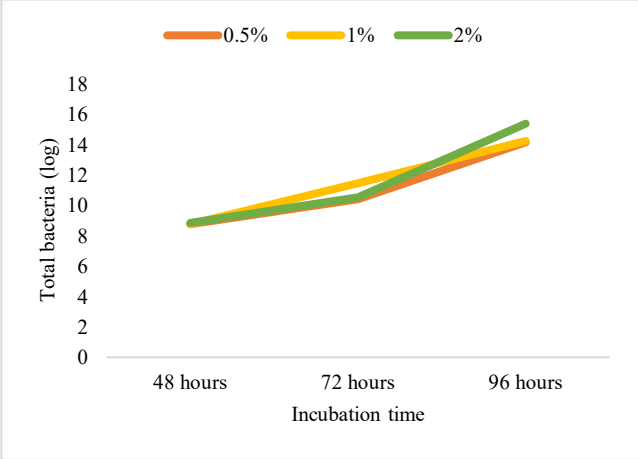

Fig. 2 The graph of total bacterial calculation results for *Bacillus sp.* strain CL33

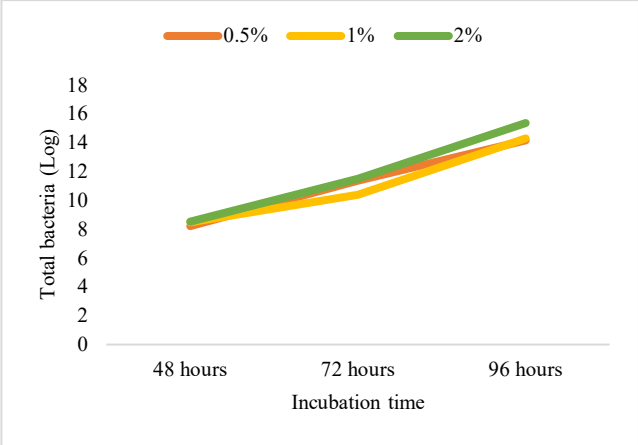

Fig. 3 The graph of total bacterial calculation results for *Bacillus flexus* strain S5a

B. Measurement of Dry Weight of Cell Mass

PHA was analyzed quantitatively through cell mass measurements. The amount of PHA is directly related to the dry weight value of the cell due to the bacterial cells' accumulation of PHA granules. The dry weight calculation of the two bacterial isolates demonstrated that the lowest cell dry weight occurred at a 0.5% oil concentration, while the highest cell dry weight was observed at a 2% oil concentration during each time interval. These cell dry weight values align with the results of total bacterial growth, which indicate that as the concentration of palm oil in the media increases, so does the dry weight of bacterial cells. The identical outcomes were observed in *Burkholderia cepacia* JC-1 bacteria. The maximum PHA production of 36.07 ± 1.42% was accomplished at 2% palm oil concentration, which yielded the highest cell dry weight of 2.87 ± 0.10 g/L [36].

The incubation time influenced the amount of cell dry weight produced by bacteria. Bacillus sp. strain CL33 and Bacillus flexus strain S5a produced the highest cell dry weight

after 96 hours of incubation. Similar results were found in the research on *Paraburkholderia sp.* PFN 29, where the highest cell dry weight produced after 96 hours of incubation, was 5.14 ± 0.17 g/L. It should be noted that cell dry weight decreases rapidly beyond this incubation period [37].

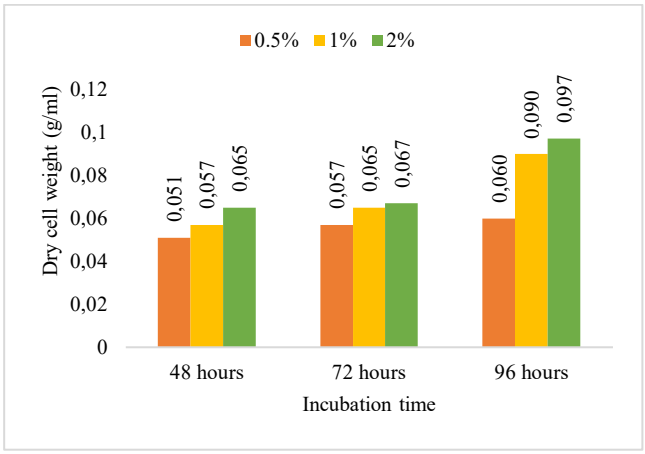

Fig. 4 Histogram of cell dry weight ratio of *Bacillus sp.* strain CL33 at each interval of palm oil concentration and incubation time

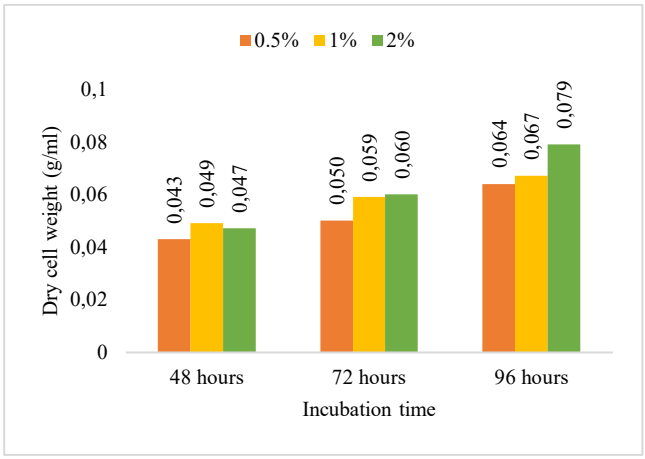

Fig. 5 Histogram of cell dry weight ratio of *Bacillus flexus* strain S5a at each 3-interval of palm oil concentration and incubation time

C. PHA Analysis Using UV-Vis Spectrophotometer

PHA analysis was conducted by measuring the absorbance of crotonic acid generated after adding H_2SO_4 with a UV-Vis spectrophotometer. Technical term abbreviations were explained upon first use. The formation of crotonic acid resulting from the addition of H_2SO_4 confirmed the PHA analysis, while the dehydration and conversion of PHA granules in bacterial cells via a heating process produced crotonic acid [35].

According to the absorbance values of the two isolates, it was determined that a concentration of 2% palm oil yielded the maximum absorbance value at each time interval. The elevated absorbance value at 2% concentration can be attributed to the abundant presence of fatty acids in palm oil for PHA synthesis. As per [38], fatty acids undergo oxidation before producing a monomer by acetyl Co-A, which will subsequently undergo polymerization via PHA synthase in a reaction that generates PHA. The absorbance values of both isolates peaked after 96 hours of incubation. A study conducted on *Bacillus megaterium* JHA demonstrated the production of maximum PHA at 54.51% after 96 hours of incubation [19]. The prolonged incubation time is vital for the bacteria to convert fatty acids from palm oil into PHA polymers.

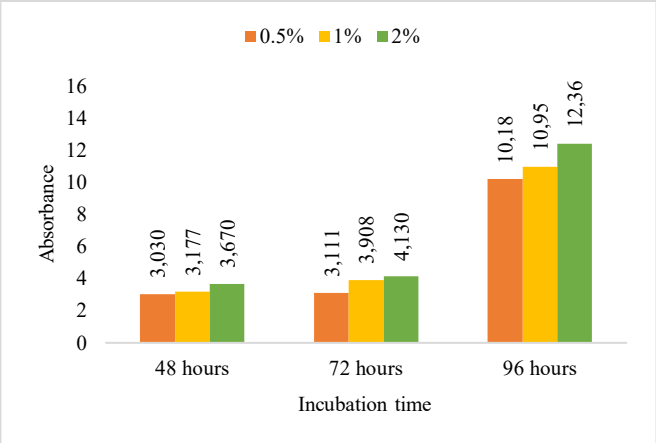

Fig. 6 Histogram of the comparison of *Bacillus sp.* strain CL33 absorbance values at each interval of palm oil concentration and incubation time

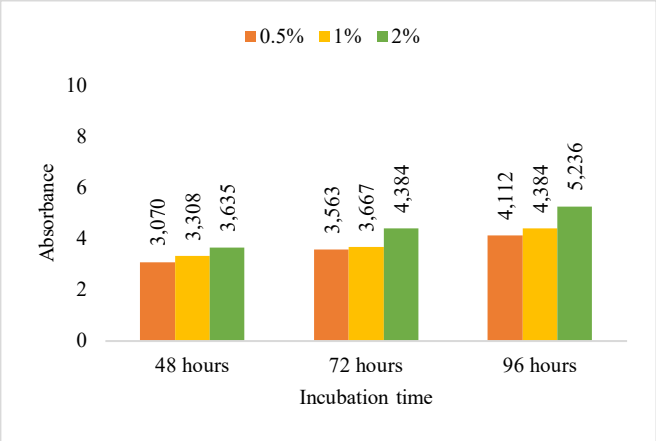

Fig. 7 Histogram of the comparison of *Bacillus flexus* strain S5a absorbance values at each interval of palm oil concentration and incubation time

D. Extraction of PHA

The optimal carbon concentration and incubation time determined served as the foundation for the extraction process of PHA. PHA was extracted by cultivating the isolates of *Bacillus sp.* strain CL33 and *Bacillus flexus* strain S5a in Ramsay media containing a 2% palm oil concentration for 96 hours of incubation time. The values of PHA concentration indicated that *Bacillus sp.* strain CL33 produced a larger amount of PHA compared to *Bacillus flexus* S5a. The variation in PHA accumulation is influenced by the bacterial type's structure [39]. Additionally, it is worth noting that enzyme activity plays a crucial role in PHA production. Enzymatic activity has been shown to contribute significantly to the high levels of PHA production [40]. In addition, the lipase enzyme is considered the dominant enzyme in the PHA production process due to its ability to convert fatty acids from palm oil to PHA [41] by catalyzing the hydrolysis of triacylglycerol (TAG) lipids to diacylglycerol (DAG), monoglycerol (MAG), glycerol, and free fatty acids (FFA) on the surface between lipids and water. Fatty acids are metabolized through the β-oxidation pathway, resulting in the

formation of (R)-3-hydroxyacyl-CoA, which acts as the foundation for PHA monomers [42].

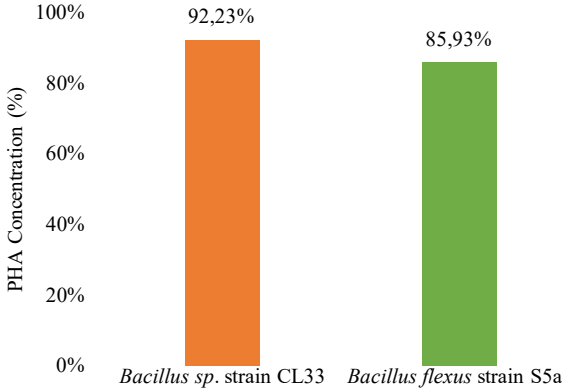

Fig. 8 Histogram of PHA concentration values at an incubation time of 96 hours with 2% palm oil concentration

E. Analysis of the Type of PHA

The analysis of the types of PHA was carried out on the extracted PHA by the technique of gas chromatography-mass spectrometry (GC-MS). The GC-MS report revealed the presence of 100 monomers in the polyhydroxyalkanoate (PHA) obtained from *Bacillus sp.* strain CL33, some of which were PHA monomers. These PHA monomers belong to the group of aromatic PHAs due to the presence of phenyl groups on their side chains and are identified as 2-hydroxybutyrate or 2-hydroxybutanoate (-2HB) and 2-hydroxy-3-phenylpropionate (2H3PhP). The characteristics of aromatic PHAs differ from aliphatic PHAs due to the presence of phenyl groups and exhibit superior physical properties in comparison to aliphatic [43].

Other monomers produced by *Bacillus sp.* strain CL33 are palmitic acid (hexadecanoic acid), stearic acid (octadecanoic acid), and myristic acid (tetradecanoic acid). These monomers can be precursors for the PHA types 3-hydroxyhexanoate (3HHx), 3-hydroxyoctanoate (3H2O), and 3-hydroxyodecanoate (3HD) [44].

GC-MS analysis of *Bacillus flexus* strain S5a PHA revealed 62 monomers, including poly (dimethylsiloxane) monomer (PDMS) which was produced as a PHA monomer. The identified monomers are decamethyltetrasiloxane, dodecamethylpentasiloxane, hexamethylcyclotrisiloxane, octamethylcyclotetrasiloxane, and dodecamethylcyclohexasiloxanes. Another monomer produced by *Bacillus flexus* strain S5a is palmitic acid monomer or hexadecanoic acid, which serves as a precursor for 3-hydroxyhexanoate (3-HHx) [44].

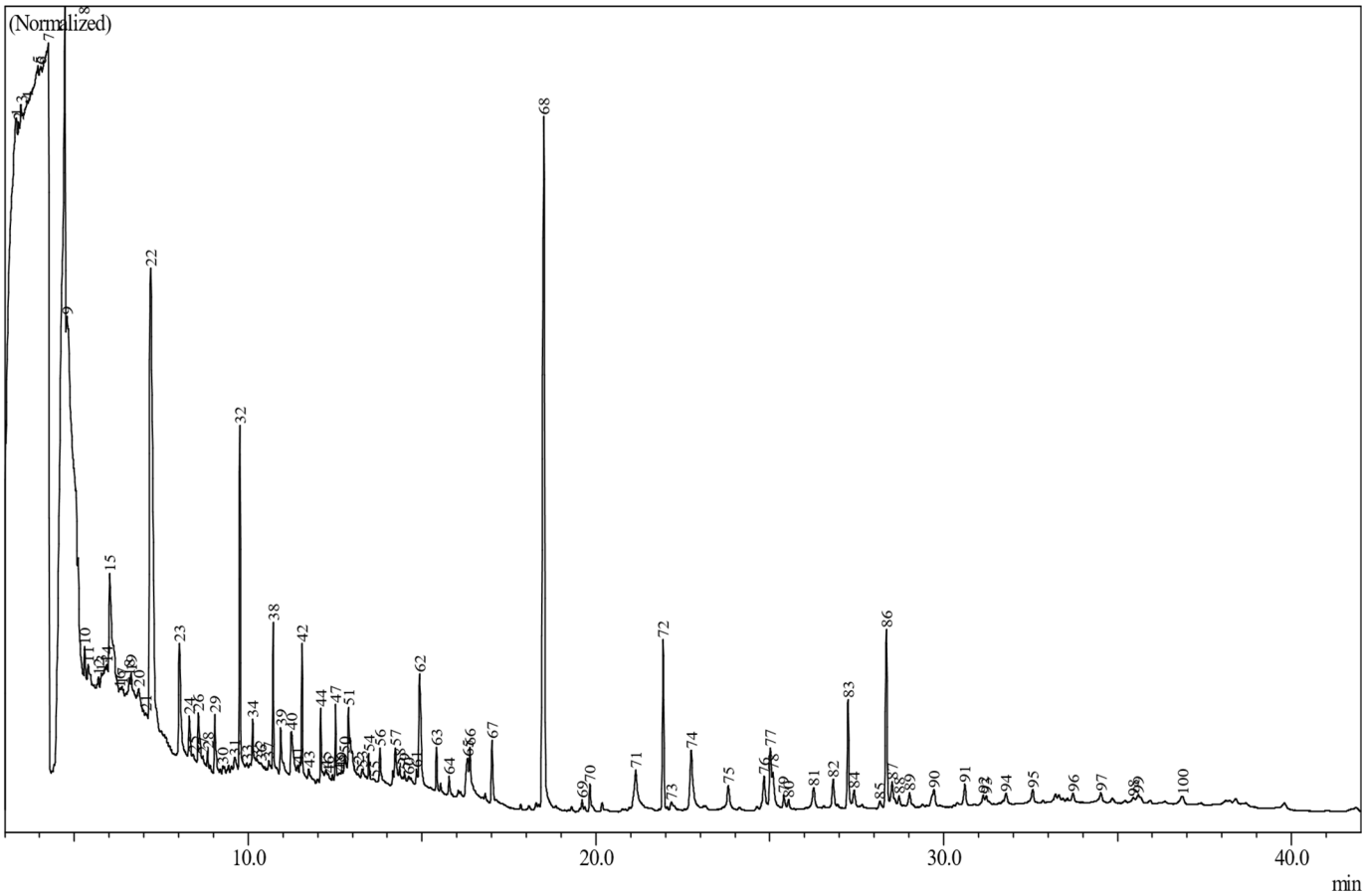

Fig. 9 PHA monomer chromatogram of *Bacillus sp.* strain CL33

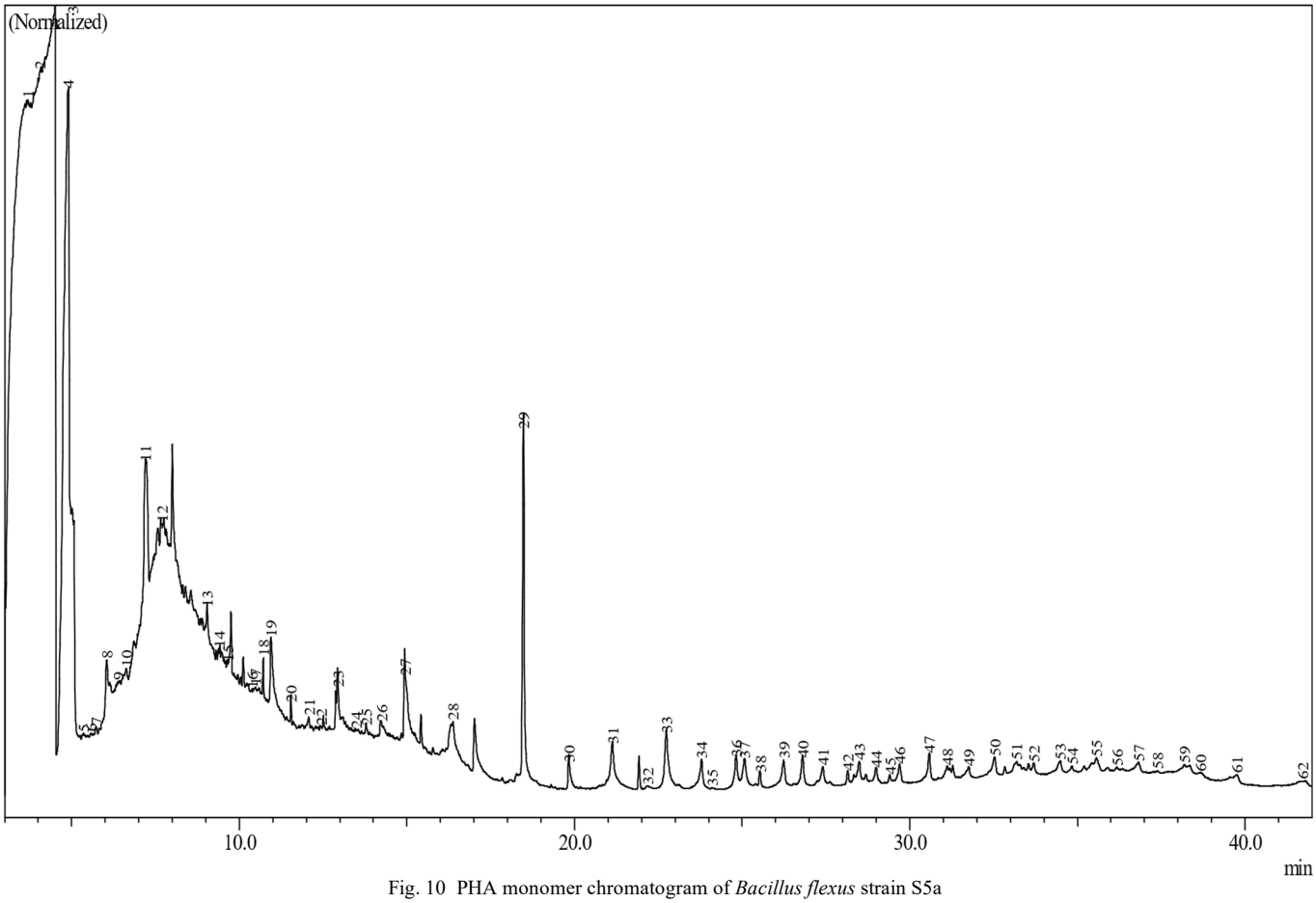

Fig. 10 PHA monomer chromatogram of *Bacillus flexus* strain S5a

IV. Conclusion

This study demonstrates the ability of *Bacillus sp.* strain CL33 and *Bacillus flexus* strain S5a to synthesize PHA using palm mill waste carbon source. Both bacteria achieved PHA production of 92.23% (scl-PHA) and 85.93% (mcl-PHA) after 96 hours of exposure to a 2% concentration of palm oil.

Acknowledgment

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