Bacillus Siamensis KCTC 13613(T) Cultured in Plantain Peels Flour for the Production of Polyhydroxyalkanoate

Babaniyi Babafemi Raphael[1*], Adebomi Joshua Ibukun[2], Adejoro Femi[3], Babaniyi Ebunoluwa Elizabeth[4]

[1]*National Biotechnology Development Agency, Lugbe, Abuja, Nigeria*

[2,3]*Federal University of Technology, Akure, Nigeria*

[4]*Adeyemi College of Education, Ondo, Nigeria*

***Abstract*: Wide use of synthetic plastics increases the environmental concerns of non-biodegradable wastes which home pathogenic organisms and contamination with particles of toxic compounds leached into ground water and other organic pollutants associated with plastics wastes. This research aimed at developing a biodegradable polymer using plantain peels flour with *Bacillius siamensis kcTcT 13613(T)*.**

During fermentation, optical density, pH and incubation time were monitored. After 48 hours of incubation, the polymer were extracted using chloroform and 63% dried cell weight were gotten. FT-IR analysis peak at wave numbers 1656 cm^{-1} revealed ester carbonyl (C=O) stretching groups, the GC-MS Chromatogram apex at (7.957) which correspond to polyhydroxybutyrate spectral.

This study provides economic insight use of plantain peels cultured with *Bacillus Siamensis KcTc 13613(T)* is capable of producing biodegradable polymer (PHB) that is environmental friendly.

***Keywords*:**
Bacillus Siamensis KCTC 13613(T)
Polyhydroxybutyrate
Plantain peels
Plastics

1. Introduction

As the population of the world is increasing, the persistent usage of petroleum derived plastics also increased as a mean of domestic and industrial packages. This poses great threat to environmental well-being due to bio accumulation of plastic waste in the environment also, inefficient recycling of plastic wastes causes continuous environmental degradation [1]. Non-biodegradable and expensive cost of recycling plastics coupled with the adverse effects of crude waste on the environment lead to search of natural and environmental friendly materials as alternative materials for domestic and industrials uses [2].

Environmental pollution dues to petroleum derived plastic materials are seriously affecting both terrestrial and aquatic lives. The littering of the environment promotes dirtiness. It also leads to blockage of drainage, gutter and carnal which causes erosion and later aggregated into flood. Aquatic animals can be trapped by plastic wastes, or poisoned through exposure to chemical contents of plastics that could cause mal-functioning of major organs. Humans can also be affected through the disruption of the thyroid hormone or fluctuation in sex hormone levels [3], [4]. Increasing harm of plastic accumulation in recent years highlights the importance of biodegradable plastics with the production of polymer from raw materials that are eco-friendly.

Previous studies showed that the chemicals used in the production of plastics are harmful in nature. Bio monitoring data revealed that these harmful chemicals accumulate within the environment and human body thereby, posing threat to human health. Biodegradable polymers emerged a potential substitute to mitigate environmental pollution posed by traditional plastics waste i.e. bio plastics are degraded by variety of microorganisms which are capable of eliminating any form of threats to the environment and human [5], [6]. Polyhydroxyalkanoates (PHAs) known as microbial polyesters, are metabolic products of some bacteria where essential nutrients like nitrogen, phosphorus or potassium is deficient. PHAs are accumulated as intracellular storage compounds within the bacteria [7], [8]. They are biodegradable, biocompatible, and hydrophobic also have thermoplastic properties. They can be produced readily from renewable carbon sources. Among the PHAs is [poly(3-hydroxybutyrate) PHB] that studies revealed to be the best PHA that can be synthesized by various bacteria, including gram positive bacteria such as *Bacillus substilis*, *B. thuringiensis*, and gram-negative like *Cupriavidus necator* and *Pseudomonas mendocina* [9]-[11,]. The PHB polymer has very similar properties to petroleum polymer, but this polymer degrades completely into carbon dioxide and water under aerobic conditions [12], [13]. PHB has been used in the packaging industry, agriculture, the food industry, and recently in the medical and pharmaceutical fields [8]. Production of PHB can be done industrially by adopting gram negative bacteria with outer membrane lipopolysaccharide (LPS) endotoxins [14].

*Corresponding author: babafemiraphael@gmail.com

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The LPS endotoxins are pyrogenic and co-purify with PHB, and this is, therefore, not too good for medical applications of PHB [15, 16]. On the other hand, gram-positive bacteria lack LPS endotoxins; which are more preferred for PHB production use in biomedical materials [17]. This study aimed at developing a biodegradable polymer using plantain peels flour with *Bacillius siamensis kcTcT 13613(T).*

2. Materials and Methods

Fresh peels of plantain peels (an alkaloid) were collected from cassava processing industry Oda, Akure, Ondo State, Nigeria. Pure strain of *Bacillus Siamensis KcTc 13613(T)* (gram-positive, endospore-forming rod-shaped bacteria) was collected from Ph.D. Research Laboratory, Microbiology Department of Federal University of Technology, Akure. Chemicals used are analytical grade product of Sigma-Aldrich Company Ltd.

A. Sample Preparation

The Plantain peels were washed with slow running water then sun dried for two weeks. The dried peels were ball milled (PM100) in the Technical Workshop Federal University Technology, Akure. The flour was carefully collected and enclosed in a container for polyhydroxyalkanoate production.

B. PHA Production with Plantain Peels using Bacillus Siamensis KcTc 13613(T)

Revamped Kannan and Rehacek medium was used to produce PHA [19]. pH of the media was modified to 6.8 prior to sterilization. The media contained: plantain peels flour 10g/L, yeast extract 2g/L, potassium chloride 3g/L, and ammonium sulphate 2.5g/L.

Plantain peels flour (10g) was soaked in 500 mL distilled water for 24 h at 4˚C. The inoculum was prepared in 250 mL flasks containing 20 mL sterile nutrient broth, media were incubated at 37ºC for 24 h at 80 rpm. The cells were harvested by centrifugation then washed with distilled water, 20 mL of the purified cells was poured into 200 mL Kannan and Rehacek medium in a litre flasks and incubated for 48 h [20].

C. Extraction of PHA

At the completion of 48 h incubation of the production media, the pellets were extracted by centrifugation. Aliquot of the media 50 mL was centrifuged at 4000 rpm for 15 min, the supernatants were removed, and 10 mL sodium hypochlorite solution was added to the slurry in the tube and incubated for another 2 h at 37ºC with 80 rpm. The tubes were centrifuged again at 4000 rpm for another 15 min. this resulted into three layers fragmentations, first layer was the supernatants which contain sodium hypochlorite, that was discarded, the second layer was the layer having the PHA, the third layer contained the biomass resides which are heavier, the slurry were gently cleaned with distilled water 10 mL, follow by addition of acetone 10 mL, the tubes were left for 10 min then decanted, 10 mL ice cold methanol was added to precipitates the dissolved PHA. 10 mL diethyl ether was added to further separate PHB from the biomass. Finally, the pellets were purified with chloroform and harvested with Whatman's (2.5µm) filter paper. During fermentation period, 50 mL aliquot of the broth were collected, extracted and quantified at intervals of 8, 16, 24, 32, 40 and 48 hours [21]. The pH and optical density were checked. PHA production was quantified by measuring the absorbance at 600 nm using 10S UV-Vis (Thermo Scientific,).

D. Dry Cell Weight Determination

After the extraction and purification of the cell pellet, Cell dried weight of the harvested polymer were calculated using gravimetric method, initial reading was taken by weighing then dried to constant weight at 45°C for a week. The weight was taken as dry cell weight (DCW) in gram per litre (g/L).

E. Characterization of the Produced Polymer

1) Fourier transform infrared (FTIR) spectroscopy

Dried sample (2mg) of polymer extracted was used to prepare KBr discs, by using a Spectrum with scanning as a spectrogram between 4000 and 400 cm^{-1} the functional group of the produced polymer was confirmed [21].

2) Gas chromatography-mass spectrometry (GC-MS)

Identification of PHA extracted was done by modifying by the method described by Huijberts 1994 [23]. A portion 2 mg mL^{-1} of the extracted PHA was ground and kept in a flask, 2 mg mL^{-1} ethyl benzoate in chloroform was poured into 3 mL 15% sulphuric acid with methanol (ratio 1: 1) at 100˚C for 4 h in a reflux, then left to cool for an hour, 2.0 mL distilled water was introduced and the tube was vortexed for 2 min, stand for 5 min, organic phase was collected and dried over anhydrous magnesium sulphate. The organic phase collected was filtered and sent for GC-MS analysis at central laboratory. Federal University of Technology Akure (FUTA) Ondo State, Nigeria. The analysis was done using Agilent 1909IS-933H-1MS. The sample in chloroform (1ul) was injected with helium (1mL min^{-1}). The injector temperature was 290˚C and the column temperature rose from 30˚C to 350˚C at 60˚C min^{-1} and kept at the final temperature for 10 min, run time 27.333 min.

F. Water Uptake

The sample was weighed (4g), Mo dry as the initial weight, then immersed in water at 37°C, for a period of one months. The sample was irrigated with fresh water at interval of three days. At the end of the testing period, the sample was withdrawn from the water, weight loss (%WL) and water uptake (%WA) was measured [24].

Water uptake (% WA) = [(Mw wet-Mt dry)/Mt dry] 100

Weight loss (% WL) = [(Mo dry-Mt dry)/Mo dry] 100

Where,

Mo is the initial weight of the sample.

Mw is the weight of the sample after the immersion in the water

Mt is the dry weight of the samples after the study.

3. Result and Discussion

A. Effect of Incubation Time, OD and pH on the Substrate Yield of PHA

Plantain peels flour fed cultures of *Bacillus Siamensis KcTc*

13613(T) produced significantly higher 6.3 DCW of 10.00 g/L with total pH accumulation of 63%. pH ranged from 6.8 to 4.3 Production of PHA progressed as the media pH shifted from neutral conditions to acidic. Optimum production was attained at pH 4.3. Meanwhile, at this pH there was no noticeable difference in DCW produced from the substrates, these findings correspond to Valappil's [25] finding on Polyhydroxyalkanoate biosynthesis in *Bacillus cereus* SPV under varied limiting conditions and an insight into the biosynthetic genes involved.

Incubation period ranged from 8–48 h. Product accumulation increased as the incubation time shifted up to 40 h and stable at 48 h then reduced as production period further increased. Highest production was 0.96g at 48 h and lowest yield at 8 h. this result was in line with Khadeejah [20] on Production and Characterization of Polyhydroxyalkanoate (PHA) Using Mango Seed Kernel as an Alternative to Glucose also agreed with the finding made by thammasittirous [22] that polyhydroxybutyrate (PHB) accumulation increased and reached maximum of 2.7g/L at 48 hours. The pH of the culture medium reduced during the fermentation, from 6.8 to 4.3. Cessation of logarithmic growth coincided with the approach of the pH minimum and rapid consumption of the carbon sources (plantain peels flour). The P (3HB) accumulation rose rapidly during the stationary phase and reached a maximum concentration of 63% of dry cell weight at 48 h of growth. Absorption of UV-Vis of the media increases from 0.593-1.588 while the transmitter shifted from 3.1-1.5 leading to more yield. Once P(3HD) optimum concentration was achieved, the P(3HD) concentration remained almost constant. Hence, Low pH inhibit production of polymer [23].

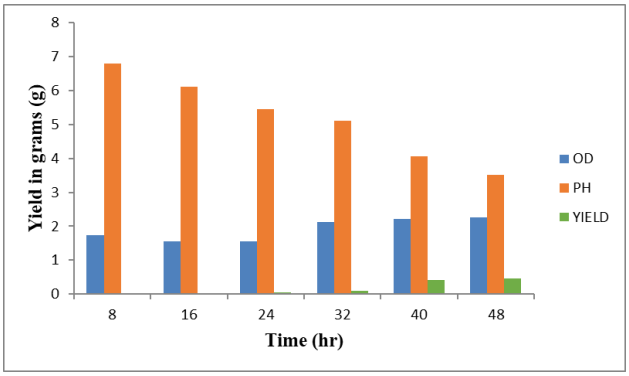

Fig. 1. Effect of Incubation Time, OD and pH on the Substrate Yield of PHA

B. Fourier Transform Infrared Spectroscopy (FT-IR) Analysis

FT-IR analysis of polymer extracted is presented in Figure 2. The peak at 3309 cm^{-1} corresponds to the terminal O-H bonding. Adsorption bands at 2526 cm^{-1} for C-H stretching group seen correspond to vibrations of the O-H groups. The peak above 2900 cm^{-1} may be due to the C-H hydrogen bond. IR spectra of the polymers showed wave numbers 1656 cm^{-1} represent ester carbonyl (C=O) stretching groups [17].

C. Gas Chromatography-Mass Spectroscopy (GC-MS) Analysis

Figure 3.3 showed GC-MS of the PHA produced. Methyl-3-hydroxybutyrate showed peak at (7.957), which was similar to the standard mass spectra [17]. The internal standard 2-ethyl-2-hydroxybutyric acid showed peak at 4.61 min. Fragmentation at 6.64 min correspond to the mass spectrum of the methyl ester of 3-hydroxyoctanoate, indicating that the polymer produced contained monomer units of the 3-hydroxyoctanoate. 3HB was the prominent monomer unit in the produced polymer [21].

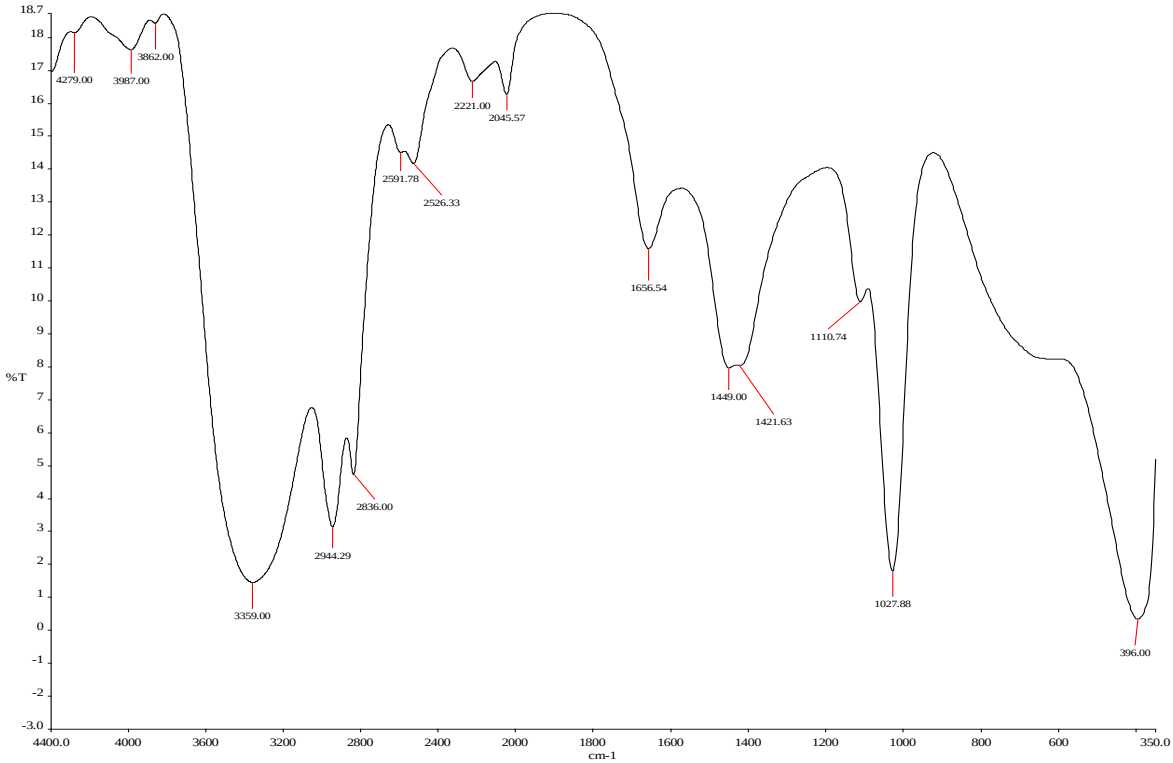

Fig. 2. Fourier transform infrared spectra analysis of Polymer produced from plantain flour using *Bacillus Siamensis KcTc 13613(T)*

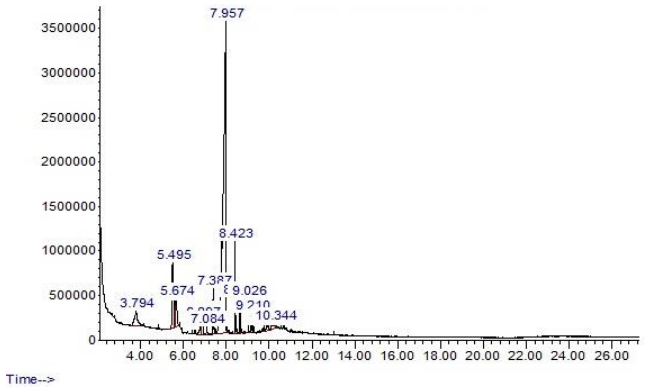

Fig. 3. GC-MS analysis of polymer extracted from plantain peels flour

D. Water Uptake

The polymer absorbs water as the day's progresses from day 6 till day 15. The polymer showed a good resistance to water permeability at the initial stage of the study which is an indication that it could be used for packaging, the polymer bond begins to lose its grip from day 6 due to adhesion of water molecules thereby causing weakening of polymer bond. Constant weight was attained in day 15 (6g). The polymer texture, colour, and firmness begins to change, particles were seen in the pot which account for weight loss (3.4g) at the end of the study, and this observation can be linked to the polymer degradability potentials.

4. Conclusion

This study showed, for the first time, that *Bacillus Siamensis KcTc 13613(T)* with plantain peels has the potential of producing PHBs. The optimised medium and culture gave the highest PHB production of 0.93 at 48 h of incubation and total DCW of 63% was harvested. *Bacillus Siamensis KcTc 13613(T)* showed a unique potential synthesis of 3HB with its monomer content and metabolic capacity to efficiently synthesized these monomers which were seen in the FT-IR spectral and the chromatogram of the GC-MS analysis

This study presents the potentials of *Bacillus Siamensis KcTc 13613(T)* grown in plantain peel flour as capable of producing biodegradable polymer that is environmental friendly. However, study will be done using fresh plantain peels slurry and compare with the dried flour in an effort to increase the economic feasibility of plantain peels as substrate in fermentative production of PHB and 3HD and blend with polyethylene to investigate its compatibility and degradability.

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