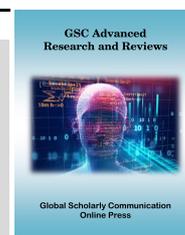


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(RESEARCH ARTICLE)



## Production of poly- $\beta$ -hydroxybutyric acid (PHB) by *Bacillus cereus* on pineapple peels

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### Abstract

Global population increase with the public demands could exceed the limit of natural resources soon and the current world problem on effective means of disposing used plastics which has posed great threat on the environment, therefore, this research was aim at producing biodegradable plastic using pineapple peels inoculated with *Bacillus cereus*. Sudan Black test was done to confirm the *Bacillus cereus* ability to produce polyhydroxybutyrate. Using fed-batch fermentation technology, polyhydroxybutyrate (PHB) pellets were produced, extracted and purified via solvent extraction. Pineapple peels as a substrate for the production of PHB yielded a good result with the cell dry weight of 4.7%. The produced plastic was characterized using Fourier transform Infra-red (FT-IR) and gas chromatography mass spectroscopy (GC-MS). *Bacillus cereus* on pineapple peels can efficiently synthesized PHB which is a cheaper source and an alternative and better way to produced biodegradable plastic.

**Keywords:** Poly- $\beta$ -hydroxybutyrate; Plastics; Biodegradable; *Bacillus cereus*; Pineapple peels.

### 1. Introduction

Plastics, known as synthetic polymer are used in a wide range of household, agricultural, marine and architectural applications as replacement to natural resources, such as metals and stones but its properties of durability have caused serious problems since plastic waste accumulates in the environment. Nature usually cannot handle plastic waste because plastics are not easily degraded by microorganisms [1]. Alternatives to waste disposal such as plastic recycling are not economical and are hazardous such as dioxin emission from PVC incineration [2]. Biodegradable plastics are designed to degrade under environmental conditions or in municipal and industrial biological waste treatment facilities [2]. There are different biodegradable polymer materials which one of them is Polyhydroxyalkanoates (PHAs) [3]. This polymer family is made of two major groups-aliphatic and aromatic. Polyhydroxyalkanoate (PHAs) are aliphatic polymer naturally produced via a microbial process on sugar-based medium, where they act as carbon and energy storage material in bacteria.

Among the PHA family is polyhydroxybutyrate (PHB). PHB is accumulated inside in numerous bacteria under nutrient – limiting conditions with excess carbon. Many researches have shown that number of microorganisms like *Alcaligenes eutrophus*, *Azotobacter beijerinckia*, *Pseudomonas Oleovorans*, *Rhizobium* sp. and *Bacillus* sp. produce PHAs as reserve food material. They can be used in different applications such as packaging film and containers, surgical pins and sutures, and bone replacements. PHB degrade naturally and completely to CO<sub>2</sub> and H<sub>2</sub>O under natural environment by different microorganisms [4, 5, 6]. Commercial production of PHB and its production from cheap and readily available as bio plastic as compared to that of production of synthetic plastics based on petrochemicals is a big challenge. Low cost agro industrial materials can be used to economize the production. Many efforts have been devoted to reducing the production costs by isolating bacterial strains capable of growing and producing PHB from inexpensive raw

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materials and also optimization fermentation conditions for PHB production [7]. Therefore, the objectives of this study was to isolate PHB- producing bacteria and characterize PHB produce on pineapple peels.

## 2. Material and methods

Fresh peels of pineapple were collected from Federal University of Technology, Akure. Pure strain of *Bacillus cereus* was obtained from research laboratory, Microbiology Departmental of Federal University of Technology, Akure. The Pineapple peels were washed with clean water and sun dried for 14 days. The dried peels were ground with electrical blender (VTcl 750 watts) and sieved with 32 mesh size sieve to have fine powders. The fine powders were carefully collected and kept in an air tight container for the production of polyhydroxybutrate.

### 2.1. Sub-culturing of *Bacillus cereus*

In order to preserve and to have enough of the organism, 2g of nutrient agar (NA) was measured and 100 mL of distilled water was added in a flask and carefully shaken to allow homogenous dissolution of the mixture. The mixture was poured into three bottles (Biju bottle) 5 mL each and autoclave at 110 ° C for 15 min. After sterilization, the bottles were removed from the autoclave and slant in a sterile condition to gel. The pure culture of *Bacillus cereus* was introduced to the prepared media with sterile loop and incubated for 24 hrs at 37 ° C, then kept in the refrigerator for later use.

### 2.2. Confirmatory test (sudan black staining)

Sudan black solution was prepared by dissolving 0.3g of the powder in 100 mL of 70% ethanol. 2g of nutrient agar was measured into a conical flask, 100 mL of distilled water was added and gently mixed, corked and autoclaved at 110 ° C for 15 min. The media was poured into petri dish and allow to gel; then streaked with sterile loop, inoculated in a sterile condition and incubated for 24 hrs at 37 ° C. The organism was smeared on grease free slide using sterile loop. The slide was allowed to dry, heat fixed, then stained for 10 min. Excess sudan black dye was drained from the heat fixed slide: clarified with xylene blotted solution for 5s, rinsed in slow running tap water, air dried and viewed using X100 oil immersion lenses of celestron digital microscope [8].

### 2.3. Seed culture preparation in nutrient broth for the production of polyhydroxybutyrate (PHB)

The culture was prepared by dissolving 0.5g of nutrient broth in 50 mL of distilled water, then heat with frequent agitation for one minute until complete dissolution. 7 mL was dispensed into test tube, corked and autoclaved at 110 ° C for 15min. After cooling, the broth was inoculated with *Bacillus cereus* in a sterile condition and incubated for 24 hrs at 37 ° C with 120rpm. The broth was centrifuge for 20 mins at 4000rpm. The supernatant was discarded and the organism (*Bacillus cereus*) settled at the base of the tube which was used for the production of polyhydroxybutyrate [9].

### 2.4. Production of polyhydroxybutyrate (PHB)

PHB was produced by preparing Kannan and Rehacek medium (Composition of media shown in table 1.0) [10]. With slight modification on pineapple peels as substrate. The substrate was soaked in 100 mL distilled water and kept at 4 ° C for 24 hrs. The substrate was sterilized separately at 110 ° C for 10 min while the rest of the chemicals were sterilized at 121 ° C for 15 min. after cooling, they were all mixed together in erlenmeyer flask in a sterile condition and the pH adjusted to 6.8 using 1 N HCL and 1 M NaOH prior to the inoculation of the carbon source, the inoculum used was the fresh nutrient broth prepared in 2.3 above. The mixture was incubated for 48 hrs at 37 ° C with 120 rpm the optical density, pH and rate of production of PHB were monitored 8 hourly by taken 50 mL aliquots and centrifuge at 4000rpm for the optical density, the frequency was set at 600nm and curvet was properly cleaned for each of the reading.

**Table 1** Composition of media

Composition	Yeast Extract	Potassium Chloride	Ammonium Sulphate	Pineapple (Powder)	peels
g/l	2.0	2.5	3.0	10.0	

### 2.5. Extraction of PHA

Extraction was done according to the methods described by Pooja 2014 and valappil et al., 2007 [9, 10] with a bit modification. After 48 hrs of incubation, the cells were harvested. The slurry was poured into 50 mL centrifuging tube and centrifuged at 4000rpm for 20 min, the supernatants were discarded, and the sediments were treated with 10 mL

sodium hypochlorite solution and incubated again for 2 hrs at 37 °C with 120rpm. The tubes were centrifuged again at 4000rpm for another 15 min. this resulted into formation of three layers, first layer was the supernatants which contain sodium hypochlorite, that was discarded, the second layer was the layer having the PHB, the third layer contained the biomass residues which are heavier, the slurry were gently washed with 10 mL distilled water, acetone was added to further wash the remains of the PHB locked within the biomass, the tubes were stand for 10 min then decanted and 10 mL ice cold methanol was added to precipitates the dissolved PHB. 10 mL diethyl ether was added to further separate PHB from the biomass. Finally, 10 mL chloroform was added and the PHB float on the surface which were harvested with 44 (2.5µm) Whatman's filter paper. Weighed and oven dried at 28 °C for one week. Cell dried weight of the composite were calculated using gravimetric method.

## 2.6. Cell dry weight (CDW)

This was done by gravimetric method by weighing the inertial weight of the harvested PHB and the final weight of the PHB. The PHB harvested was been weighed daily as it dried until constant weight was attained.

$$\text{Cell dry weight} = W_o - W_f$$

Where:  $W_o$  is the inertial weight of PHB when it was produced

$W_f$  is the final weight of the PHB when it dried.

## 2.7. Characterization of the polymer produced and standard (PHB)

### 2.7.1. Fourier transform infrared spectroscopy (FTIR)

The dried sample of the polymer produced (PHB) 2mg was used to prepare KBr discs. An FT-IR Spectrum 1720 X Spectrometer. The analysis was done with window material, csl, 10 scan, resolution 4cm<sup>-1</sup>, and spectral range 4400-350 cm<sup>-1</sup>. The detector was a temperature- stabilized coated DTGs detector. This analysis was done at central laboratory, university of Ibadan Nigeria.

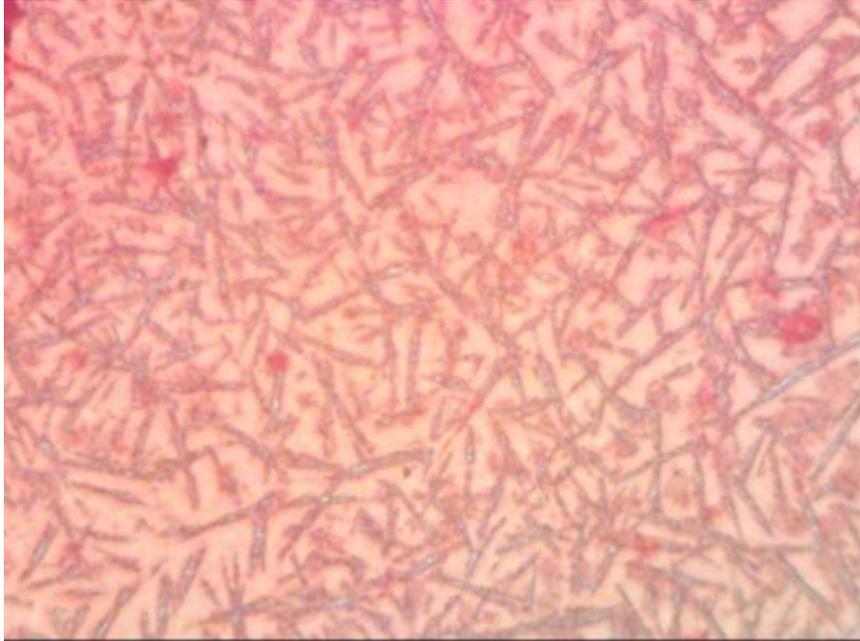
### 2.7.2. Gas chromatography- mass spectroscopy (GC-MS)

Identification of polymer produced was done using GC-MS analysis of the methanolysed polymer. A quantity of 0.5g of sample and 5 mL ethylbenzoate in chloroform 5 mL, was added to a mixture of 3 mL, 15% Sulphuric acid in methanol 5 mL (ratio 1:1) at 100 °C for 4 hrs reflux. The reaction was left over night, 10 mL distilled water was added and the tube was vortexed for 2 min, after phase separation, the organic phase was collected and dried over anhydrous magnesium sulphate. This was filtered and sent for GC-MS analysis at central laboratory. Afe Babalola University (ABUAD) Ado-Ekiti, Ekiti State, Nigeria. The analysis was done using Agilent 1909IS-933H-1MS. The sample in chloroform (1ul) was injected with helium (1ml min<sup>-1</sup>) as the carrier gas. The injector temperature was 290 °C and the column temperature was increased from 30 °C to 350 °C at 60 °C min<sup>-1</sup> and held at the final temperature for 10 min run time 27.333 min.

## 3. Results and discussion

### 3.1. *Bacillus cereus* confirmatory test (sudan back staining)

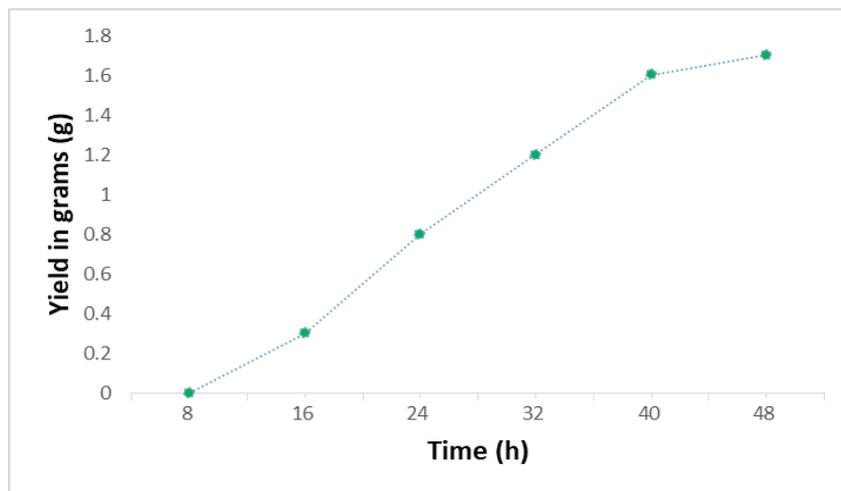
Figure 1 shows the result of the sudan back staining of the *Bacillus cereus*. Sudan black stained *Bacillus cereus* appeared as gram positive rod shaped cells under oil immersion which was similar to the sudan back staining result of Nasir et al. 2016 [11] on production and characterization of Polyhydroxybutyrate (PHB). The positive strains were isolated and inoculated in the broth and prepared in nutrient broth for the production of polyhydroxybutyrate (PHB) in a sterile condition for further studies.



**Figure 1** *Bacillus cereus* stained with Sudan black

### 3.2. PHB production from the substrate

The PHB production yield result of the pineapple peels is shown in Figure 2. Although, been moderately deferent to many observations recorded by different researchers, product yield increased as the media pH changed from neutral conditions to acidic condition. Figure 3 revealed changes in optical density (OD) and pH of the substrate during 48 hrs of incubation. Higher product was obtained from the pineapple (0.37 g) at pH value of 4.8, optical density of 1.902 and 48 hrs. And the PHB settles at the top of the tube which was also different from observations of previous researchers like pooja [9, 12]. They reported that the PHB settles at the base of the tube. These observations may be due to the difference in carbon sources or the method used. The total dry cell at the end of the fermentation was 4.7 which was a good yield also showed that the *Bacillus cereus* used up the substrate effectively.



**Figure 2** Substrate yield during incubation

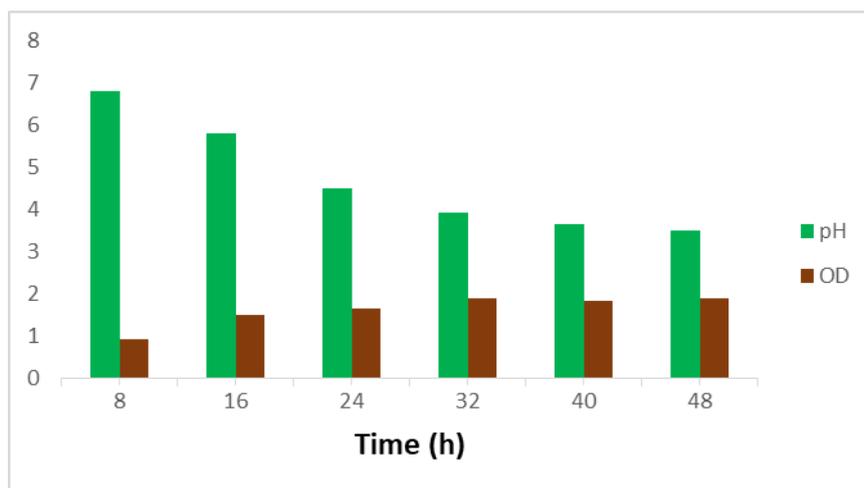


Figure 3 OD and pH of the substrate during incubation period

### 3.3. Characterization of the produced polymer

#### 3.3.1. Fourier transform infrared spectroscopy (FT-IR) analysis

The FT-IR characterization of the polymers is shown in Figure 4. The polymers extracted for pineapple peels showed the intense absorption at  $3405\text{ cm}^{-1}$  were attributed to the terminal O-H bonding or water adsorption on the PHB. Adsorption bands at  $2949\text{ cm}^{-1}$  for C-H stretching group were observed in the produced polymer corresponds to the stretching and deformation vibrations of the O-H groups. The presence of the peak above  $2900\text{ cm}^{-1}$  may be due to the C-H hydrogen bond [13, 14]. IR spectra of the polymers revealed the presence of marked peaks at wave numbers  $1643\text{ cm}^{-1}$  representing the presence ester carbonyl (C=O) stretching groups, band recorded at  $1022\text{ cm}^{-1}$  is generally known to be typical characteristics of sugar derivatives such as guluronic acid, manuronic acid and uronic acid.

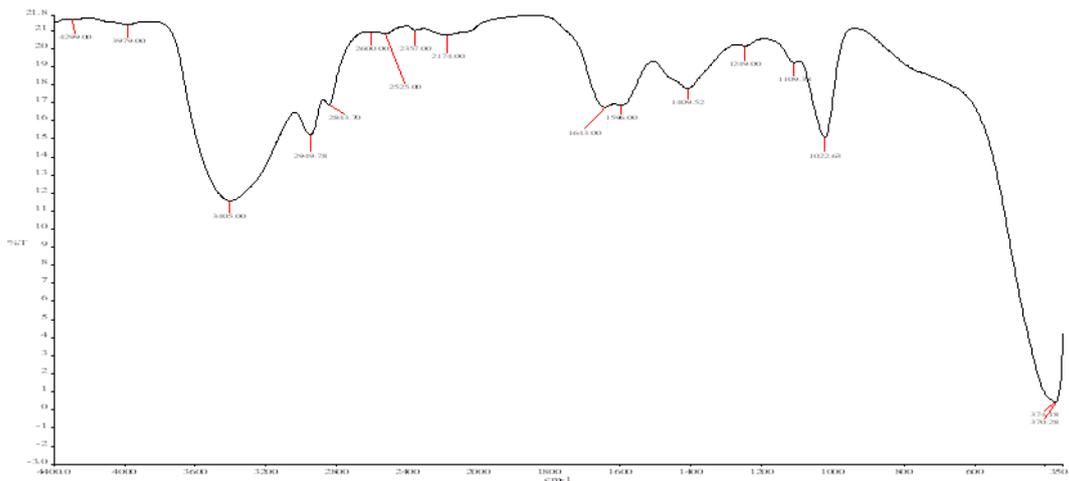
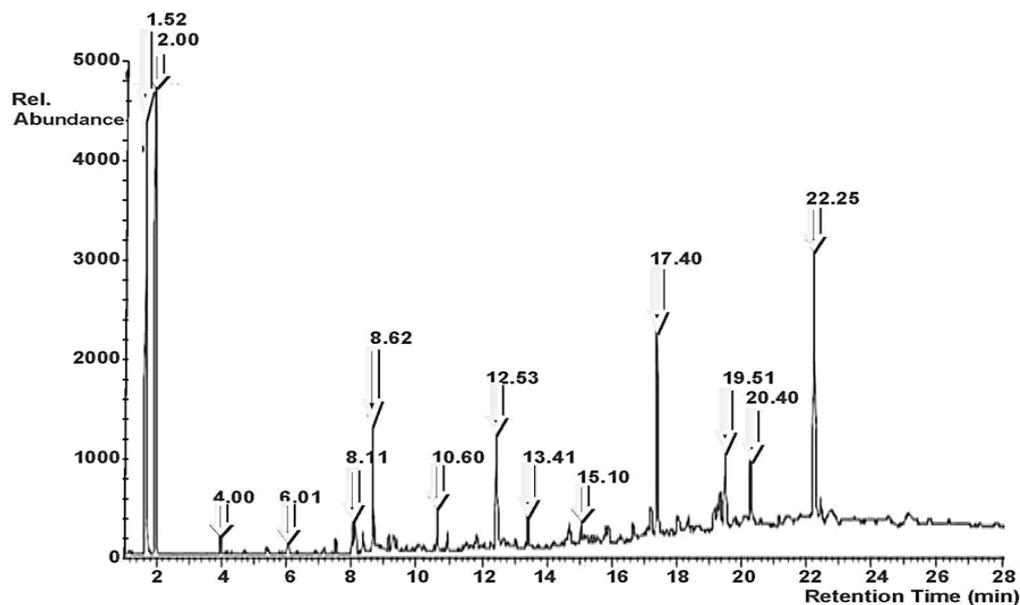


Figure 4 The FT-IR result of the PHB produced from pineapple peels.

#### 3.4. Gas chromatography-mass spectroscopy (GC-MS) analysis

The result of the GC-MS analysis is shown in fig.5 below, the analysis elucidated the characteristic fragmentation patterns, suggesting the presence of PHB. The carbonyl and hydroxyl ends of the corresponding hydroxybutyrate were identified from the specific peaks in the chromatogram. The fragmentation patterns were in concordance with the results given by Mohan et al. (2010). The mass spectra for propyl-esters of 3-hydroxyalkanoates corresponding 3HB peaks with RT values of 10.62 corresponding to derivatized products of Butenoic acid confirming the presence of polyhydroxybutyrate.



**Figure 5** GC-MS analysis of PHB extracted from pineapple peels

#### 4. Conclusion

The research has shown that the substrate (Pineapple peels) that was used for the production of PHB using *Bacillus cereus* are effective with a good yield. It was noted that during the production, there was increase in optical density with yield and decrease in the pH of the media. FT-IR and GC-MS analysis further affirmed that it was PHB that was produced. However, PHB production using pineapple peels with *Bacillus cereus* can be optimized for medical applications in future study.

#### Compliance with ethical standards

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##### Disclosure of conflict of interest

No conflict.

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