Characterization of Polyhydroxyalkanoate (PHA) Produced by *Bacillus* species Isolated from Garden Soil

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Abstract: Plastic materials which have made entry in every sphere of human life are now causing serious environmental problems due to their non- biodegradability. This study, therefore, examined the production of biodegradable s polyhydroxybutyrate by *Bacillus* species isolated from environmental samples; the isolates were screened for the presence of polyhydroxybutyrate (PHB) inclusions using Sudan Black B stain. The PHB produced were extracted with chloroform and analyzed using Gas Chromatography Mass Spectroscopy (GCMS). The bacterial isolates identified as *B. mycoies* had 6 different compounds with 1, 2-Benzenedicarboxylic acid, diisooctyl ester as the major compound and *B. subtilis* recorded 4 compounds with 1, 2-Benzenedicarboxylic acid, diisooctyl ester as the major compound. The GCMS analysis confirmed the presence of biodegradable polymers making them interesting candidate in biodegradable polymer production for application in environmental areas.
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1. Introduction

Plastic materials which have made entry in every sphere of human life are now causing serious environmental problems due to their non-biodegradability. Synthetic polymers also known as plastics have become significant since the 1940's when they started replacing glass, wood and other constructional materials, and even metals in many industrial, domestic and environmental applications (Poirier *et al*., 1995; Cain, 1992; Lee, 1996). These widespread applications are not only due to their favorable mechanical and thermal properties but mainly due to the stability and durability. The intrinsic qualities of durability and resistance to degradation, over the last two decades, have been increasingly regarded as a source of environmental and waste management problem emanating from plastic materials (Porier *et al.,* 1995). Because of their persistence in our environment, several communities are now more sensitive to the impact of discarded plastic on the environment, including deleterious effects on wildlife and on the aesthetic qualities of cities and forest. The increased cost of solid waste disposal as well as the potential hazards from waste incineration such as dioxin emission from PVC makes synthetic plastic a waste management problem. The exponential growth of the human population has led to the accumulation of huge amounts of non-degradable waste materials across our planet. Living conditions in the biosphere are therefore changing dramatically, in such a way that the presence of nonbiodegradable residues is affecting the potential survival of many species. (Hema *et al.,* 2010) For this reason, many countries have promoted special programmes directed towards the discovery of new commonly used materials that can be readily eliminated from the biosphere and have designed novel strategies aimed at facilitating the transformation of contaminants. The continuous exhaustion of fossil fuels led to the research for the production of biodegradable plastics from renewable sources. These plastics have high molecular weight and are tightly bonded together which makes them non degradable and their disposal difficult and inversely leads to negative impact on the environment. Furthermore, the synthetic plastics cause deleterious effects to wild life and pose threat to environment and other serene habitats. One option is to produce truly biodegradable polymers, which may be used in the same applications as the existing synthetic polymers. These materials, however, must be processible, impervious to water and retain their integrity during normal use but readily degradable in a biologically rich environment.

The production of biodegradable polymers from renewable resources is the need of the hour, in the face of these ecological facts. Among the candidates for biodegradable plastics, PHA's have been drawing much attention because of their similar material properties to conventional plastics and complete biodegradability (Steinbüchel and Fuchtenbusch, 1998). Also the fact that it can be produced from renewable resources and its efficient processing on equipment just like some polyolefins and other synthetic plastics make PHAs suitable for applications in several areas as a substitute for non biodegradable synthetic polymers. (Steinbüchel and Fuchtenbusch, 1998). Plastics produced from PHA's

have been reported to be truly biodegradable in both aerobic and anaerobic environments (Page, 1995). PHA's are composed mainly of poly-beta hydroxybutyric acid (PHB) and poly-beta hydroxyvaleric acid (PHV), although other forms are possible. Interestingly more than 80 different forms of PHA's have been detected in bacteria (Lee, 1996), Lafferty *et al*. (1988) stated that the accumulation of PHA by microorganisms can be stimulated under unbalanced growth conditions when nutrients such as nitrogen, phosphorus or sulfate become limiting, when oxygen concentration is low, or when the C: N ratio of the feed substrate is higher. PHB is accumulated by numerous microorganisms and is the best characterized PHA (Madison and Huisman, 1999). Polyhydroxybutyrate (PHB) is a biopolymer that can be used as a biodegradable thermoplastic material for waste management strategies and biocompatibility in the medical devices (Steinbüchel, 1995).To achieve a cost effective PHA production, the availability of an efficient bacterial strain is a prerequisite and is a focus of interest for many investigations. Highlighting the importance of supporting researches on PHA will bring the wealth for all humanity.

This present study is aimed at characterizing biodegradable poly (3-hydroxybutyrate) (PHB) inclusions from *Bacillus* species isolated from the environment using the gas chromatography- mass spectrophotometer with the objective to determine their biodegradability.

2.1 Material and Methods

2.1.1 Isolation procedures

Soil samples were collected from Department of Microbiology; River behind the Department of Microbiology and runoff sewage beside Department of Zoology all in the University of Ibadan, Nigeria respectively in sterile bottles aseptically and used for isolation of the bacteria. Samples were serially diluted in sterile distilled water and plated onto nutrient agar plates using standard pour plate technique. Sterilization of the nutrient agar was carried out by autoclaving at 121^0C for 15 minutes. The plates were incubated at 37^0C for 24 hours. Representative colonies were obtained and purified by repeated streaking on nutrient agar. Each colony of pure culture was individually picked based on distinct morphological characteristic.

2.1.2 Screening Methods for PHB Accumulation in Bacteria

The pure isolates were grown on nutrient agar plates and incubated at 37^0C for 24 hours. A loopful of each culture was smeared on a grease free slide using a sterile inoculating loop. Slides were allowed to dry. The dried slides were heat-fixed and stained with Sudan Black solution according to the method of Smibert and Krieg (1981). Heat fixed slides of the bacterial samples were stained with Sudan Black solution for 10 minutes and clarified with xylene drops, dried with filter paper, counterstained with 0.5% aqueous safranine for 5 seconds and rinsed off with slowly running water. The slides were subsequently air dried and viewed under × 100 oil immersion lenses. The PHA granules appeared as blue-black granules inside pink cells for the cells that stained positive; and only pink cells for those that were negative.

2.1.3 Characterization of PHB Producing *Bacillus* Isolates

The positive PHB producing bacterial isolates were subjected to a set of morphological, physiological and biochemical tests for the purpose of identification. Eighteen to twenty four hour old culture was used for each test viz; gram staining, spore staining, indole, methyl red, catalase, starch hydrolysis, sugar fermentation test (glucose, galactose, fructose, lactose, sucrose, maltose, arabinose, xylose, sorbose, sorbitol and mannitol). Eighteen to twenty four hour old culture was used for each test. Identification of isolates was done by comparing the results of these tests with the standard descriptions of the Bergey's Manuals of Determinative Bacteriology (Buchanam and Gibbons, 1994). PHB production in shake flasks was studied using the modified basal mineral salt medium of Mahmoudi *et al*. (2010) with appropriate carbon source. The selected bacterial isolates were grown in 250 ml conical flasks containing 150 ml MSM broth with different carbon sources *viz.,* glucose, fructose, sucrose, maltose and lactose, Medium (150 ml) was sterilized at 121^0C for 15 minutes in 250 ml capacity Erlenmeyer flasks. The medium was inoculated with culture inoculums grown in nutrient broth and incubated at 37°C for 48 hrs.

2.1.4 Extraction and Identification of PHB

After incubation the cell in broth culture was concentrated by centrifugation at 3,500 rpm for 30mins (Hermle Z 323, Germany) and dried at 105^0C to a constant weight in a hot air oven (Memmert). PHB content was determined using a modified method of Arnold *et al*. (1999). PHB content from each dried cell was determined by incubating the cells at 37^0 C(Stuart, S150 incubator) for one hour with 5ml of 0.4% sodium hypochlorite solution to break the bacterial cell walls. The supernatant was obtained by centrifugation at 13,000rpm for 10mins and was transferred into separating flasks for extraction. Cell lipids and other molecules (PHA present) were extracted by adding 5ml of 96% ethanol and 5ml of 96% acetone. PHB was thereafter extracted by adding 10ml of chloroform to the

mixture in a hot water bath at 60^0C. The weight of the dried chloroform extract was thus determined. The analysis of the Polyhydroxybutyrate polymers extracted from the bacterial isolates were carried out on a gas chromatograph mass spectroscopy instrument (Shimadzu, GCMS QP2010) with capillary column (HP5MS), 30 m x 0.25 mm i.e., coated with DB-5, 0.25 µm film thickness; column oven temperature of 60^0C at the rate of 32.7mL/min., injection port temperature 250^0C, constant pressure of carrier gas (helium)72.8kPa, flow rate 1.20mL/min, acquisition parameters full scan, scan range 30 to 500 amu. Sample was dissolved in chloroform and subsequently evaporated, the dried extract was dissolved in n-hexane and 1µl of the sample was dispensed into the GCMS vial for analysis (Das *et al*., 2005).

3. Results

The isolates were first screened for the production of PHB and further characterized using conventional methods of identification

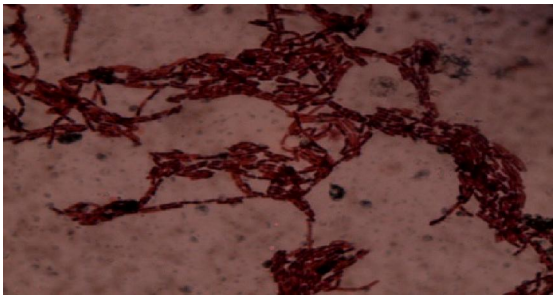

Figure 1: Photomicrograph of isolate *Bacillus subtilis,* showing the PHB granules produced in form of dark granules inside the bacterial cells.

The results of GCMS analysis obtained for *Bacillus mycoides* as represented in Table 1 showed six compounds. The major compounds were 1, 2-Benzenedicarboxylic acid, diisooctyl ester with a total percentage of 46.14, molecular weight of 390 and corresponding retention time of 15.969minutes; Diclohexyl ester with a percentage of 24.72, molecular weight of 310 and retention time of 19.318minutes. The lowest molecular weight of 248 was recorded for Propanoic acid, 3-chloro, decyl ester with a retention time of 18.860minutes.

The result of GCMS analysis for the polymer extracted from *Bacillus subtilis* is shown in Table 2; four different biodegradable compounds were obtained. The major compounds observed were 1, 2- Benzenedicarboxylic acid, diisooctyl ester with a total percentage of 62.98 and the highest molecular weight of 390 with a corresponding highest retention time of 15.965. The lowest retention time of 7.118minutes was recorded for tert-butyl acrylate with a corresponding lowest total percentage of 5.00 and molecular weight of 128.

Table 1: Chemical Composition of the Biodegradable Polymer of *B. mycoides* as revealed by GCMS analysis (RT- Retention time in minutes, %T- Percentage Total, CN- Compound Name, MW- Molecular weight, MF- Molecular formular)

RT	%T	CN	MW	MF
13.204	6.95	Acetic acid, octadecyl ster	312	$C_{20}H_{40}O_2$
14.470	7.98	Hexanedioic acid, bis(2-ethylhexyl)ester	370	$C_{22}H_{42}O_4$
15.969	46.14	1,2-Benzenedicarboxylic acid, diisooctyl ester	390	$C_{24}H_{38}O_4$
18.860	8.64	Propanoic acid,3-chloro,decyl ester	248	$C_{13}H_{25}CLO_2$
19.318	24.72	Hexanedioic acid,diclohexyl ester	310	$C_{18}H_{30}O_4$
19.413	5.56	Hexanedioic acid,bis(2-ethylhexyl)	370	$C_{22}H_{42}O_4$

Table 2: Chemical Composition of the Biodegradable Polymer of *B. subtilis* as revealed by GCMS analysis (RT- Retention time in minutes, %T- Percentage Total, CN- Compound Name, MW- Molecular weight, MF- Molecular formular)

RT	%T	CN	MW	MF
6.524	15.88	Cycohexane,isothiocyanato	141	$C_7H_{11}NS$
7.118	5.00	Tert-butyl acrylate	128	$C_7H_{12}O_2$
9.392	16.14	1,2-Benzenedicarboxylic acid di-2-propenyl ester	246	$C_{14}H_{14}O_4$
15.965	62.98	1,2-Benzenedicarboxylic acid,diisooctyl ester	390	$C_{24}H_{38}O_4$

4. Discussions

The results of this study confirm that PHB producers can be isolated from soil; Choi and Lee, (1999) have also successfully isolated PHB producing bacteria from the soil. *Bacillus* species isolated from the soil for PHB production have been reported among diverse bacteria as being potential PHB producers (Ramsay *et al*., 1990; Aslim *et al*., 2002; Katircioglu *et al*., 2003 and Yuksekdag *et al*., 2004). The PHB producers isolated have the ability to utilize pure substrates such as glucose, fructose, lactose and sucrose which has also been reported by Labuzek and Radecka(2001); Mercan and Beyalti (2005) and Valappil *et al*.(2007). The production of these polymers occurs at the stationary phase of growth, the physiological state of the bacterial strain as well as the composition of the fermenting medium is a determinant factor in the type of biodegradable polymer produced. It is also necessary to mention that PHA accumulation in this study was carried out

under uncontrolled environmental (pH and dissolved oxygen) conditions of shake flasks incubator. Further, the carbon-nitrogen ratio may not be the optimum condition for the PHA production. The gram positive *Bacillus* species are known to accumulate PHB during growth phase. Hence, in order to achieve a good PHB content, cultivation techniques to improve the biomass should be adopted for *Bacillus* genera.

The result of GCMS analysis of chloroform extract of biodegradable polymers produced by the different bacillus species where *B. mycoides* had 6 different compounds with 1,2-Benzenedicarboxylic acid, diisooctyl ester as the major compound and *B. subtilis* recorded 4 compounds with 1,2-Benzenedicarboxylic acid, diisooctyl ester as the major compound; these aliphatic biodegradable polyester family due to hydrolysable ester bonds was reported by Wallen and Rohwedder (1974); Riis and Mai (1988); Abe *et al*. (1994); Cromwick *et al*. (1996); Jung *et al*. (2000).

PHB producing *Bacillus* species were successfully isolated and characterized from the environmental samples. This study showed that *Bacillus subtilis* and *Bacillus mycoides* showed more preference for C_{14} and C_{24} monomers. PHAs containing unsaturated monomers can be further modified by chemical reactions such as cross-linking, double bond hydration, epoxidation to produce new polymers having different thermal and mechanical properties.

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