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Production and Analysis of Polyhydroxyalkanoate (PHA) by *Bacillus megaterium* Using Pure Carbon Substrates

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Abstract: This study examined the production of polyhydroxybutyrate by *Bacillus megaterium* isolated from garden soil. The isolates were screened for the presence of polyhydroxybutyrate (PHB) inclusions using Sudan Black B stain and subsequently examined on different sugars to find out the best carbon source for PHB production. The PHB produced were extracted with chloroform and analyzed using Gas Chromatography Mass Spectroscopy (GCMS). *Bacillus megaterium* accumulated PHB with varied pure substrates. The results of the GCMS recorded 21 different biodegradable compounds that were produced by the organisms with 9-Octadecanoic acid (Stearic acid), n-Hexadecanoic acid and 9-Octadecanoic acid (Oleic acid) as the major compounds.

Key words: Polymer · Polyhydroxybutyrate · Inclusions · GCMS · Carbon · Bacteria

INTRODUCTION

Synthetic plastics are one of the greatest inventions of mankind and have been developed into a major industry and indispensable commodity in human's life [1]. They are designed in a way to suit the constant performance and trustable qualities that are used for long life span, therefore causing them to be inert to natural and chemical breakdown. 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 [2-4]. Bio-based materials such as polynucleotides, polyamides, polysaccharides, polyoxoesters, polythioesters, polyanhydrides, polyisoprenoids and polyphenols are potential candidates for substitution of synthetic plastics [2, 5]. An important family of biomaterials is bioplastics which are usually built from hydroxy-acyl–CoA derivatives via different metabolic pathways. The term biopolymers include chemically unrelated products that are synthesized by microorganisms under different environmental conditions [1,2,6]. Depending on their microbial origin, bioplastics differ in their monomer composition, macromolecular structure and physical properties. Most of them are biodegradable and biocompatible, which makes them extremely interesting from the biotechnological point of view. Bioplastics are a special type of biomaterial; they are polyesters, produced by a range of microbes, cultured under different nutrient and environmental conditions (Madison and Huisman, 1999). Among these, polyhydroxyalkanoate (PHA), which belongs to the group of polyoxoesters has received intensive attention because it possesses biodegradable thermoplastic properties [7]. They can be assimilated by many species (biodegradable) and do not cause toxic effects in the host conferring upon them a considerable advantage with respect to other conventional synthetic products [8-10]. Owing to their thermoplastic properties and biodegradability, PHAs have attracted industrial interest and have been extensively studied over the last two decades. PHA is synthesized by bacteria under unbalanced growth conditions with a remarkable increase in the production of PHA under nutrient depleted environments [4, 11]. The number and size of the granules, the monomer composition, macromolecular structure and physicochemical properties vary, depending on the producer organism [12, 13]. Highlighting the importance of supporting researches on PHA will bring the wealth for all humanity. This study examined the production of polyhydroxybutyrate by bacillus species isolated from soil samples; their diversity in soil makes them a suitable area for exploit as potential producers of PHB.

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In view of this, this present study is aimed at revealing biodegradable poly (3-hydroxybutyrate) (PHB) inclusions from bacillus species isolated from soil with the objective to determine the carbon source that will give the highest yield of the PHB.

MATERIALS AND METHODS

Collection of Samples: 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.

Isolation Procedures: Samples was 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°C for 15 minutes. The plates were incubated at 37°C for 24 hours. Representative colonies were obtained and purified by repeated streaking on nutrient agar.

Screening Methods for PHB Accumulation in Bacteria
Staining Method for PHB Detection: The pure isolates were grown on nutrient agar plates and incubated at 37°C 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 [14]. 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.

Characterization of PHB Producing Bacterial 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. Identification of isolates was done by comparing the results of these tests with the standard descriptions of the Bergey's Manuals of Determinative Bacteriology [15].

Optimization of Carbon Sources for PHB Production: The screened PHB producing bacterial isolates were grown in 250 ml conical flasks containing 150 ml modified mineral salt medium with different carbon sources. The substrates considered were glucose, lactose, fructose and sucrose.

PHB Production: PHB production in shake flasks was studied using the modified basal mineral salt medium [16] 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 and lactose. Medium (150 ml) was sterilized at 121°C 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 and 150 rpm for 48 hrs.

Identification of PHB
Extraction 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°C to a constant weight in a hot air oven (Memmert).

Cell Dry Weight Measurement: Total cell concentration was determined by weighing the dry cell mass which was dried at 105ºC to a constant weight in a hot air oven then cooled down at room temperature. The drying was repeated until constant weight was obtained [17].

PHB Recovery and Extraction: PHB content from each dried cell was determined by incubating the cells at 37°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°C. The weight of the dried chloroform extract was thus determined [18]

Gas Chromatography Mass Spectroscopy (GCMS) Analysis: 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°C at the rate of 32.7mL/min., injection port temperature 250°C, 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 [19].

RESULTS AND DISCUSSION

B. megaterium had a cell dry weight of 0.0203g/l on glucose, 0.1125g/l when fructose was employed as carbon source with 0.2501g/l and 0.1360g/l on lactose and sucrose substrates respectively with corresponding PHB yields of 0.0143g/l on glucose, 0.0652g/l on fructose, 0.0791g/l on lactose and 0.0788g/l on sucrose.

GCMS Analysis of PHB Extracts Obtained from *Bacillus megaterium:* The results of GCMS analysis for *Bacillus megaterium* showed that twenty one different biodegradable compounds were obtained from the chloroform extract of the polymer as shown in Table 1. The major compounds obtained were Octadecanoic acid as the highest occurring compound with a total percentage of 24.88, molecular weight of 284; n-Hexadecanoic acid with a percentage of 20.54 and 9 Octadecanoic acid with a percentage of 20.38. The molecular weight seemed to have no effect on the retention time; the highest molecular weight of 402 was recorded for the compound Tributyl acetyl citrate with a retention time of 13.564mins, Formic acid ethenyl ester which had the lowest molecular weight of 72 had a retention time of 7.815mins and 2-Propenoic acid, 2 Methyl-ethenyl ester had the lowest retention time of 4.284mins with a molecular weight of 112.

In this study, *B. megaterium* was able to produce substantial amounts of PHB during growth using the modified mineral salt medium when the carbon substrate was varied (glucose, fructose, lactose and sucrose) in excess of 10g/l with nitrogen source; this is in agreement with previous work carried out [20] who reported *B. megaterium* as accumulating 39.9% polymer weight with glucose substrate. The result of GCMS analysis of chloroform extract of biodegradable polymers produced by *B. megaterium* recorded a high number of compounds

Table 1: Chemical Composition of the Biodegradable Polymer of *B. megaterium* as revealed by GCMS analysis

S/N	RETENTION TIME(minutes)	%TOTAL	COMPOUND NAME	MOLECULAR WEIGHT	MOLECULARFORMULA
1	4.284	4.26	2-Propenoic acid,2-methyl-ethenyl ester	112	$C_6H_8O_2$
2	4.552	2.48	Propane 1,1-diol diacetate	160	$C_7H_{12}O_4$
3	4.735	1.23	Heptanol	116	$C_7H_{16}O$
4	5.234	4.170	Octanoic acid	144	$C_8H_{16}O_2$
5	5.850	0.49	Propanoic acid,2-methylpropyl ester	144	$C_8H_{16}O_2$
6	5.850	7.97	Nonanoic acid	158	$C_9H_{18}O_2$
7	6.218	0.85	1-Propanol,2,2-dimethyl	88	$C_5H_{12}O$
8	6.408	0..72	Napthalene,2-methyl	142	$C_{11}H_{10}$
9	6.519	0.66	Cyclohexanone,3,3,5-trimethyl	140	$C_9H_{16}O$
10	6.699	1.12	Heptanoic acid	130	$C_7H_{14}O_2$
11	7.815	0.15	Formic acid, ethenyl ester	72	$C_3H_4O_2$
12	8.550	0.55	Ethyl citrate	276	$C_{12}H_{20}O_7$
13	9.370	1.12	n-Decanoic acid	172	$C_{10}H_{20}O_4$
14	11.402	20.54	n-Hexadecanoic acid	256	$C_{16}H_{32}O_2$
15	12.188	2.38	1-Heptadecanol	256	$C_{17}H_{36}O$
16	12.699	20.38	9-Octadecenoic acid	282	$C_{18}H_{34}O_2$
17	12.883	24.88	Octadecanoic acid	284	$C_{18}H_{36}O_2$
18	13.376	0.92	Pentadecanoic acid,3-methylbutyl ester	242	$C_{15}H_{30}O_2$
19	13.564	1.56	Tetradecanedioic acid	258	$C_{14}H_{26}O_4$
20	13.564	1.56	Tributyl acetylcitrate	402	$C_{20}H_{34}O_8$
21	14.012	2.60	Benzoic acid,4(methylamino)	165	$C_9H_{11}NO_2$

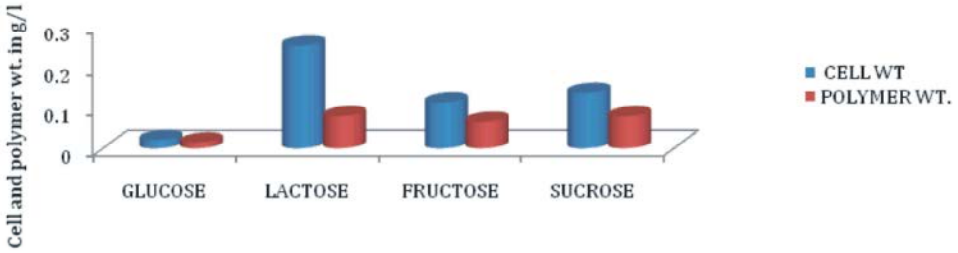

Fig. 1: Effect of different carbon substrates on the dry weight and polymer weight of *B. megaterium*

which makes it an interesting candidate for the production of PHAs. Moreover, 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. Chemical modification of biosynthetic PHAs is a promising approach to expend bacterial polyesters to be used in the medical and environmental areas.

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