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Production of Bioplastic by Local Strains of *Bacillus subtilis* using Watermelon Peels as Substrate

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Abstract

The amount of environmental contamination brought on by the careless disposal of plastic garbage has increased to 400 million tons every year on a global scale. These synthetically generated traditional polymers are not easily biodegradable. This work was therefore undertaken to isolate Bacillus subtilis from soil with potential to produce bioplastic: Poly-ß-hydroxybutyrate (PHB). Samples of soil were collected from various locations (BG = Botanical Garden, FARD = Fine Art Refuse Dumpsite, SHRD = Suleiman Hall Refuse Dumpsite, FVM = Faculty of Veterinary Medicine animal paddock) within Ahmadu Bello University, Zaria, Nigeria. Spread plate technique was used to isolate B. subtilis on nutrient agar, and the isolates' cultural, morphological, and biochemical properties were identified. The isolates of B. subtilis were screened for PHB production using two different methods: the plate assay method and slide technique using Sudan black B dye. The PHB was then produced using submerged fermentation with watermelon peel as sole source of carbon in the production medium. The PHB was extracted using Sodium-hypochlorite method and the quality of the PHB was determined using FT-IR analysis. Four isolates of Bacillus subtilis were obtained from the soil samples (50 %), one each out of the two samples (50 %) per unit location (BG2, FARD3, SHRD2, and FVM4). The screening revealed that all the isolates were PHB producers. The B. subtilis isolate SHRD2 from the students' dormitory was found to produce the highest PHB yield of 0.98 g/L from the watermelon substrate, whereas isolate from the animal paddock (FVM4) yielded the lowest quantity of the PHB (0.12 g /L). The biopolymer's (bioplastic) identity was confirmed to be PHB based on the peaks in the FT-IR spectra, which displayed wave numbers for a variety of functional groups, including -O-H, C-H, C-O, and C=O. It was concluded that the local isolates of B. subtilis have potentials for PHB production using watermelon peels as source of carbon and energy.

Key words: *Bacillus subtilis, watermelon peels, submerged fermentation, bioplastic, Poly-ß-hydroxybutyrate*

INTRODUCTION

One of the main sources of pollution in the world today is the build-up of non-biodegradable plastic products in the environment (Bhagowati, 2013). Global dimensions have been reached in the environmental damage brought on by the careless disposal of plastic garbage. These typical plastics, which are made synthetically from petroleum and are not easily biodegradable, are therefore seen as dangerous wastes to the environment. Different biodegradable plastics have been created in the hunt for an environmentally benign material to replace the usage of traditional plastics, either by adding natural polymers into formulas for conventional plastics, by chemical synthesis, or by microbial fermentations (El-kadi, 2010).

A family of more than 40 poly-hydroxyalkanoates (PHAs) and their co-polymeric derivatives has emerged among the variety of biodegradable plastics as a very attractive material for bioplastics due to their complete biodegradability. Several bacteria accumulate these polymers or co-polymers as an intracellular carbon reserve when unfavourable environmental and dietary conditions are encountered.

A microbial polyester known as poly-hydroxybutyrate (PHB) is generated by numerous bacteria and kept in cells as granules (El-Kadi, 2010). The PHAs are a group of 3, 4, 5, and 6-hydroxyacid linear polyesters with a range of mechanical, biocompatible, and biodegradable characteristics. They have thermoplastic and elastomeric characteristics and are insoluble in water. Numerous bacteria, including Bacillus sp., produce and store them intracellularly. Additionally, the carbon: nitrogen (C:N) ratio of the media in which the organism is developed has a significant impact on the composition and concentration of the polymer in the cell. The C:N ratio of the medium used to produce PHA must therefore be properly monitored and adjusted.

The composition of the polymer's monomer is influenced by the type of microorganism, the contents of the media, the conditions under which it ferments, as well as the modes of fermentation and techniques of recovery. This influences the polymer's physical and chemical properties (Sukan *et al.*, 2014). Polysaccharides, proteins, and lipids are examples of the natural components used as raw ingredients in the creation of bioplastics. Watermelon peels and other organic waste can be utilized to make starch-based bioplastics, which can help reduce environmental pollution from watermelon peels and conventional plastics (Maulida *et al.*, 2016).

Following the success of Bacillus subtilis in the manufacture of metabolites, bioremediation, and the production of bioenergy, it has attracted attention as a possible producer of PHB. It has previously been acknowledged in the industrial scale manufacture of amino acids, recombinant proteins, and fine compounds but has never been tested for its potentials to produce biopolymers. Gram-positive bacteria *Bacillus subtilis*, sometimes referred to as grass bacilli, are a well-known species that can thrive in a variety of settings. They appear to be broadly suited to grow in a variety of environmental conditions due to their capacity to be separated from different environments. Like other bacillus species members, *Bacillus subtilis* may develop a very hardy dormant endospore in response to food restriction and other environmental challenges. Many prokaryotes can produce intracellular storage compounds when cultivated in situations where development is restricted due to the exhaustion of a crucial nutrient like nitrogen or phosphorus and carbon substrate is still available (El-kadir., 2014).

Therefore, the objective of this research was to identify local *Bacillus subtilis* strains from soil and evaluate their capacity to create the bioplastic Poly-ß-hydroxybutyrate (PHB).

MATERIALS AND METHODS

Collection of Soil Samples and Watermelon Peels

A total of eight (8) soil samples were collected in sterile polythene bags from various locations (BG = Botanical Garden, FARD = Fine Art Refuse Dumpsite, SHRD = Suleiman Hall / Students' dormitory Refuse Dumpsite, FVM = Faculty of Veterinary Medicine animal paddock) within Ahmadu Bello University, Zaria main campus. The samples were labelled appropriately and transported to the laboratory, Department of Microbiology, Ahmadu Bello University Zaria for analysis.

A total of 1000 g of fresh watermelon peels were collected in clean polythene bags from watermelon vendors at Samaru Market in Samaru Village, Zaria - Nigeria. The watermelon peels were then washed to remove soil particles and dirt before they were sliced into small pieces and dried under shade at room temperature. The dried watermelon peels were later used as substrate for PHB production by the *B. subtilis* via submerged fermentation.

Isolation and Identification of *Bacillus subtilis*

Twenty-five (25) grams of each sample were separately suspended into 225 mL of sterile distilled water and mixed thoroughly. The stock soil suspension was heat-shocked at 80 °C for 20 min. to activate the bacterial endospores. Serial dilution (10^{-1} to 10^{-5}) of each sample was carried out and 0.1mL aliquot from each of the last three dilutions (10^{-3}, 10^{-4}, 10^{-5}) were separately inoculated onto already prepared nutrient agar plate by spread plate method. The plates were then incubated at 37 °C for 24 h (El-Kadi, 2014). The isolates obtained after overnight incubation were characterized by cultural morphology, Gram's staining, endospore staining and various biochemical tests (catalase test, starch hydrolysis, methyl red test (MR), Voges–Proskauer (VP) and citrate utilization tests) according to standard procedures described in Cowan and Steel (1993) for the identification of presumptive isolates of *Bacillus subtilis*.

Screening of the *Bacillus subtilis* Isolates for PHB Production

The *Bacillus subtilis* isolates were later screened for Polyhydroxybutyrate granules using Sudan black B staining technique (Gayathiri *et al.*, 2017). Smears of the positive *Bacillus subtilis* isolates were prepared on glass slide, allowed to air-dry, and stained with Sudan black B stain, which was being added continually for 30 min without allowing the stain to dry. The slides were then washed and counter-stained with safranin for two minutes and rinsed with water.

The slides were air-dried and observed under a phase contrast microscope (Indira *et al.*, 2014). Additionally, Sudan Black plate assay methodwas also used to detect the presence of PHB in the isolates. Plates of nutrient agar were inoculated with the isolate, incubated at 37 °C for 24 h before being flooded with Sudan black dye solution, which was left to stand for 30 min (for the dye to diffuse). The plates were then rinsed with ethanol to remove excess dye and the whole plates were observed for the presence of coloured complex formation (Mascarenhas *et al.*, 2017) considered to be Polyhydroxybutyrate granules.

Production of PHB by the *B. subtilis* using Watermelon Peels as Substrate

Watermelon peels which are usually considered as biowastes were utilized in this research by the *B. subtilis* isolated from the soil to produce the bioplastic by submerged fermentation. The dried watermelon peels were pulverized, and 50 g was added into a 250 mL Erlenmeyer flask containing 200 mL of distilled water. The mixture was heated and then filtered after cooling. The filtrate was sterilized by autoclaving at 121 °C for 15 min and 15 psi pressure. The medium used for the bioplastic production composed of mineral salts medium to which the filtrate of the watermelon peels was added. This mineral salt medium was prepared by dissolving 2.0 g $(NH_4)_2SO_4$, 1.5 g K_2HPO_4, 0.2 g $MgSO_4.7H_2O$, 0.2 g yeast extract and 0.2 g peptone in 200 mL of distilled water and sterilized (El-kadi., 2014). The fermentation was carried out in a 250 mL Erlenmeyer flask, in which 50mL of the sterile mineral salt medium was mixed with 10 mL of the sterile watermelon peel filtrate. Aliquot of 2.5 mL of the standardized (using 3.0 McFarland standard) *Bacillus subtilis* suspension was added into the production medium and then incubated at 37 °C for 48 h (El-Kadi, 2014).

Extraction, Purification and Quantification of the PHB Produced

Sodium hypochlorite and chloroform were used to extract the PHB that was created; following the fermentation, 10 mL of the fermented broth was centrifuged at 4000 rpm for 30 minutes, with the supernatant being discarded. Following that, the pellet was dissolved in 5 mL of 4 % sodium hypochlorite and 5 mL of chloroform, and it was incubated at 37 °C for 1 hour. The suspension was centrifuged at 3000 rpm for 10 minutes after incubation. The middle phase, which contained chloroform and cell debris, and the upper phase, which contained sodium hypochlorite, were eliminated. To precipitate the granules, 5 mL of an ethanol and acetone combination (1:1) was added to the bottom phase containing the PHB after another 5 mL of chloroform was added. The weight of the PHB recovered was calculated after the precipitate dried out at a temperature of 30 °C (Wala'a *et al.* 2017). The extracted poly-hydroxybutyrate powder was transferred to a clean test tube, 10 mL of concentrated sulphuric acid (H_2SO_4) was added, the tube was sealed, and the PHB was quantified using crotonic acid analysis. This procedure involved heating the test tube for 20 minutes at 100 °C in a water bath. Dehydration transformed the PHB crystals into crotonic acid. The resulting crotonic acid was well blended and allowed to cool. The procedure described by Darshen *et al.* (2014) was adopted, where a sample was placed into a quartz cuvette, and the absorbance was measured at 235 nm in a UV spectrophotometer against a sulphuric acid blank.

Characterization of the PHB Produced

Fourier transformed infrared spectroscopy (FT-IR) was also carried out to analyse the presumed PHB extracted and check for the presence of various functional groups (Shreema, 2014).

RESULTS

Table 1 revealed the cultural, microscopic, and biochemical characteristics of *Bacillus subtilis* isolated from different soil samples. All the four isolates were observed to be greyish white in color, round and dry. They appeared as spore-forming Gram-positive rods capable of utilizing tryptophan (indole positive) and citrate. The isolates were also found to degrade hydrogen peroxide (catalase positive), carry out butanediol/acetoin fermentation (VP positive) but not mixed acid fermentation (MR negative). These isolates were then tentatively confirmed to be *Bacillus subtilis*.

Table 2 revealed the percentage occurrence of *Bacillus subtilis* from the various soil samples analyzed. An overall occurrence of 50 % was obtained since *Bacillus subtilis* was isolated only from four of the eight samples analyzed. Similarly, only one sample out of the two from each of the sampling locations was positive for *Bacillus subtilis*, also giving a percentage occurrence of 50 % in relation to the type of sampling location. Table 3 shows the results of the screening of the *B. subtilis* isolates to produce the bioplastic (polyhydroxy butyrate) by plate and slide techniques. Dark-colored colonies indicating PHB granules were observed during the plate assay, whereas purple, rod-shaped cells with characteristic dark granules observed under the microscope during slide assay indicated the presence of PHB within the Bacilli.

Both assays therefore revealed the presence of the bioplastic in all the four isolates screened.

The result of the PHB production from the watermelon peels by submerged fermentation is presented on Table 4 in g/L. The *B. subtilis* isolate SHRD2 from the students' dormitory was found to produce the highest PHB yield of 0.98 g/L, whereas isolate from the animal paddock (FVM) yielded the lowest quantity of the PHB (0.12 g /L). The PHB yield was recorded based on the absorbance of the suspension which was determined at a wavelength of 235 nm.

Figure 1 shows the FT-IR spectra of the extracted PHB indicating the wave numbers which are characteristics of the functional groups associated with PHB. Table 5 shows the specific wave numbers of each of the functional group detected in the extracted PHB. Some of the peaks revealed the presence of -O-H, C-H, C-O and C=O.

Table 1: Cultural, Microscopic and Biochemical Characteristics of Bacterial Isolates from Soil

Isolate's code	Growth on NA	GRM	End	SH	C	I	MR	VP	CU	Probable Bacterium
BG2	greyish white, round dry	G +ve rod	+	+	+	-	-	+	+	*B. subtilis*
FARD3	greyish white, round dry	G +ve rod	+	+	+	-	-	+	+	*B. subtilis*
SHRD2	greyish white, round dry	G +ve rod	+	+	+	-	-	+	+	*B. subtilis*
FVM4	greyish white, round dry	G +ve rod	+	+	+	-	-	+	+	*B. subtilis*

Key: MR = Methyl red, **VP** = Voges Proskauer, **CU** = Citrate Utilisation, **C** = catalase, **I** = Indole, **SH** =Starch Hydrolysis, **GRM** = Gram Reaction and Morphology, **End** = Endospores, **BG** =Botanical Garden, **FARD =** Fine Art Refuse Dumpsite, **SHRD =** Suleiman Hall Refuse Dumpsite, **FVM =** Faculty of Veterinary Medicine animal paddock

Table 2: Percentage Occurrence of *Bacillus subtilis* Isolated from Soil

Sample location	Number of samples analyzed	NumberPositive (%)
BG	2	1 (50)
FARD	2	1 (50)
SHRD	2	1 (50)
FVM	2	1(50)

Key: BG = Botanical Garden, **FARD** = Fine art Refuse dumpsite, **SHRD** = Suleiman Hall Refuse dumpsite,
FVM = Faculty of Veterinary Medicine animal paddock

Table 3: Polyhydroxy butyrate Production by the Screened *B. subtilis* Isolates

Isolate's code	Sudan black B plate assay	Slide technique	Inference
BG2	Dark colored colony	purple rod with dark granule	positive
FARD3	Dark colored colony	purple rod with dark granule	positive
SHRD2	Dark colored colony	purple rod with dark granule	positive
FVM4	Dark colored colony	purple rod with dark granule	positive

Key: BG = Botanical Garden, **FARD** = Fine art Refuse dumpsite, **SHRD** = Suleiman Hall Refuse dumpsite,
FVM = Faculty of Veterinary Medicine animal paddock

Table 4: PHB Production by *B. subtilis* isolates using Watermelon peel as substrate

Sample	Absorbance at 235 nm	PHB (g/L)
BG2	0.0982	0.275
FARD3	0.0984	0.143
SHRD2	0.2045	0.980
FVM4	0.0392	0.120

Key: BG = Botanical Garden, **FARD** = Fine art Refuse dumpsite, **SHRD** = Suleiman Hall Refuse dumpsite, **FVM** = Faculty of Veterinary Medicine animal paddock

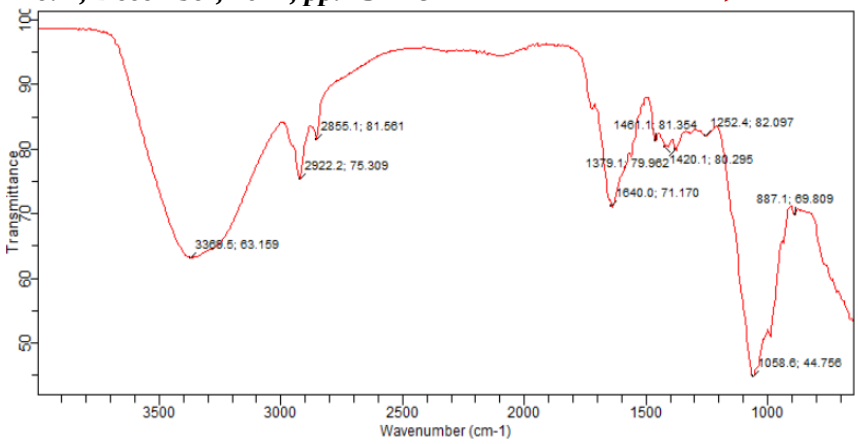

Figure 1: FT-IR Spectra of the Polyhydroxy butyrate Produced from Watermelon Peels

Table 5: Functional Groups of the PHB and their Characteristic Peaks.

Functional group	Peaks (Wave number in cm^{-1})
C=O	1640.0
C-O	1058.8
C-H	2855.1
-O-H	3385.5

DISCUSSION

In this study, out of the total of eight soil samples analysed, 50 % were found to be positive for *Bacillus subtilis* with one sample each (50 %) from the four sampling locations being positive. The occurrence of *Bacillus subtilis* in all the soil samples might be attributed to the environmental adaptability of the organism since it can form a resistant endospore which can be carried by air to various locations. Based on previous studies, PHB producing bacteria have been isolated from different environments such as soil polluted with oil, heavy metal, and effluent from industries (Mascarenhas *et al.*, 2017). Interestingly, all the four isolates screened were potential producers of poly-hydroxybutyrate (PHB). This, therefore, shows that the two techniques are both reliable in detecting the presence of PHB in *Bacillus subtilis*. This variation in PHB production potential from the same bacterium but of various origins might not be unconnected with the nature of the environment from which the organism was originally isolated, which might have affected the physiologic and metabolic performances of the organism (Shreema, 2014). The FT-IR spectra produced from the extracted PHB indicate the functional groups associated with PHB. The most dominant absorption peaks observed around 3385.5 cm^{-1} and 2922.2cm^{-1} could be attributed to the stretching vibrations of –OH group and C-H bond in CH_2 respectively as also reported by Mostafa *et al.* (2015). Also, the characteristic peak at 1640.0cm^{-1} and 1058.8cm^{-1} indicate the absorption peaks for C=O and C-O of carbonyl and ester respectively. The spectra obtain correspond to those extracted from *Bacillus thuringiensis* (Thammasittirong *et al.*, 2015) with exception of the spectrum for C=O which corresponds to the spectrum of the PHB extracted from *Bacillus spharicus* NII 0838 (Shindhu e*t al.*,2011) as well as PHB synthesized by *Staphylococcus epidermidis* as reported by Darshan *et al.* (2014) and Mostafa *et al.* (2015). The characteristics of the PHB produced in this study in comparison with those reported by other researchers shows that, the PHB from *Bacillus subtilis* isolates have almost similar properties and was confirmed to be Polyhydroxybutyrate and of good quality.

CONCLUSION

It was concluded that the soil from all the sampling locations are a rich source of isolating *Bacillus subtilis*. Four isolates of *Bacillus subtilis* were identified and characterized. The isolates screened were found to be capableof polyhydroxybutyrate production. Polyhydroxybutyrate (PHB) was successfully produced from watermelon peels through intracellular biosynthesis in the *Bacillus subtilis* by submerged fermentation with the isolate SHRD2 having the highest production potential (0.98 g/L). The presence and quality of the polyhydroxybutyrate was determined and confirmed using FT-IR analysis.

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