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Screening of Microorganisms Producing Polymer (PHB) from Dump Sites Soil in Ilorin Metropolis, Kwara State, Nigeria

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ABSTRACT

This study was aimed at screening microorganisms isolated from dump sites for polymer production. Five samples of dump site soil were gathered from each of 10 distinct sites, totaling fifty samples, throughout the collection process. Microorganisms were isolated using the pour plate method. Bacteria isolates were identified using biochemical tests and molecular analysis using 16S rRNA gene primer while morphological characteristics are 18SrRNA gene primer were used to identify fungi isolate. Screening of the organisms for polymer production was done using Sudan Black Band Nile Blue A. The polymer extracted were analyzed using Fourier Transform Infrared Spectroscopy (FTIR) and Scanning Electron Microscope (SEM). Results revealed the presence of five fungi and seven bacterial species identified to be *Aspergillus niger*, *Penicillium chrysogenum*, *Penicillium marneffei*, *Cladosporium tenuissimum* and *Rhizopus* sp., while bacterial isolates were identified as *Bacillus licheniformis*, *Bacillus cereus*, *Paenibacillus polymyxa*, *Bacillus megaterium, Bacillus mycoides, Bacillus subtillis* and *Enterobacter* species. Only four bacterial isolates were positive for polymer production and molecularly identified as *Bacillus subtilis* strain AGB1 (OM273871), *Bacillus megaterium* strain *AGB2 (*OM216844*), Paenibacillus polymyxa* strain AGB3 (OM273889) and *Bacillus licheniformis* strain AGB4 (OP703543).The polymer has rough surfaces and crystalline in nature, while FTIR result indicated that the polymer had C=O carbonyl functional group and identified as polyhydrxybutyrate (PHB) polymer while SEM showed that it has crystalline surface. It can be concluded that bacterial isolates which were polymer producers can be utilized for large-scale production of polyhydroxybutyrate for industrial purposes.

INTRODUCTION

The increased use of plastic is one of the factors contributing to the worsening state of environmental degradation. Microplastic and other toxins that are based on plastic are being released into the environment as a result of the plastic material that is making its way into our food chain. Since this time, research into bio-based plastics has focused on creating a sustainable, environmentally friendly environment with a less carbon impact [1]. Conventional plastic polymers derived from petroleum have an inextricable role in all aspects of human life. However, these refractory plastics are a source of concern since they are nondegradable, making disposal difficult. As a result, they persist in the environment for an extended length of time, causing serious ecological damage by disrupting natural conditions [2]. Indiscriminate disposal of Petroleum-based polymers on land and open-air burning can lead to the release of toxic chemicals into the environment posing health issues to humans, and animals, and entirely collapsing the biological systems and cycles [1]. Finding a substitute for the petroleum-based polymer is an urgent requirement to avoid and lessen the environmental impacts produced by existing1 polymers owing to excessive consumption and inappropriate disposal. Therefore, searching for novel biodegradable plastics is of increasing interest.

Among the solutions to these difficulties is the discovery of biodegradable polymers. Some bacteria produce biodegradable polymers that their enzymes are capable of degrading[3]. Biodegradable plastics provide a promising strategy for managing trash in the environment, since they may substitute for traditional polymers in cases where recycling or incinerator recovery is impractical or too expensive. Researchers

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prefer to utilize and produce biodegradable polymers from microbes, according to [3], since they are simpler to manage, manufacture, purify, and have more options for genetic modifications with quick biodegradability without harming ecology and the environment. The industrial bioprocess of polyhydroxybutyrate (PHB) is still developing, and microbial resources are still being explored for extensive production and commercialize issues [3].

Polyhydroxyalkanoates (PHAs) are found to accumulate in varieties of microorganisms as reserve food material for example, *Alcaligenes latus, Ralstonia eutropha, Bacillus megaterium* etc. Polyhydroxybutyrate, or PHB for short, is the most common biodegradable polymer and a viable alternative to manufactured non-degradable plastics. It is a member of the PHA family of biodegradable polymers. In circumstances of nutritional stress, these polymers aggregate as an intracellular membrane-enclosed inclusion that may account for up to 90 percent of the cell's dry weight. These polymers function as an energy reserve material. It is possible to shape it, convert it into films, spin it into monofilaments, and use it to construct heteropolymers with other synthetic polymers. Moreover, it has various uses in agriculture, packaging, and the medical industry due to the fact that it is biodegradable and immunologically compatible [3].

Plant-based polysaccharides (e.g., starch, cellulose, chitosan/chitin), proteins (e.g., casein, gluten), and other carbon sources may be used to create bioplastics. Wheat gluten, like other proteins, can also be turned into useful bioplastics[4]. Sugarcane is exploitable for bioplastic production by bacterial sugar assimilation [5]. Finally, oil is a good carbon source for the production of bioplastic. Cottonseed oil [6], soybean oil [7], crude palm kernel oil, jatropha oil, crude palm oil, palm olein, corn oil, and coconut oil had been previously reported to be a good substrate for biopolymer production [8]. The aim of this investigation is to study the ability of some microorganisms isolated from dump site soils to produce polymer (PHB) and analyzed it using FTIR and SEM techniques.

MATERIALS AND METHODS

Isolation and identification of polymer (PHB) producing microorganisms and media preparation

The collection of dump site soils took place in various locations (10 locations) in the Ilorin metropolis, Kwara State. Samples were taken at 6cm deep and kept in sterile containers and were also immediately transferred to the laboratory where serial dilution was carried out. One (1) gram of sample was mixed with 9ml of distilled water homogenized to dissolve and allowed to settle. From the suspension, 1ml from each sample was transferred to nutrient agar for bacteria and PDA for fungi, which were incubated at room temperature (30 $^{\circ}$C) for 24-72 h and NA plates at 37 $^{\circ}$C for 24-48 h. Identification of isolates was carried out based on morphological, physiological and biochemical characteristics [9] cited by [10] for only viable colonies.

Identification of fungal isolates

The pure culture of fungal isolates were identified on the basis of morphological biochemical and by using lactophenol cotton blue as employed by [11].

DNA Extraction of Bacterial Isolates (16S rRNA)

The DNA extraction procedures for bacterial isolates were carried out. The method of [12] was directly employed which involved the use of the phenol or chloroform method.

The DNA extracted was tested by electrophoresis in agarose gel and spectrometer (Eppendorf, Germany). A series of centrifugation processes were carried out before the application of an equal volume of phenol ratio and chloroform ratio.

Identification of PHB bacteria isolates by (16S rRNA)

Amplifications of DNA were carried out with general methods used by [13] and [12]. The 16S rRNA genes of total bacteria were amplified by PCR employing the universal primer. The sequencing was done by denaturation at 96 $^{\circ}$C; annealing at 50 $^{\circ}$C and elongation at 60 $^{\circ}$C followed by precipitation of amplicons. The contigs were subjected to BLAST for nucleotide similarity search. All the bacterial strains were assigned to a genius based on their 16S rRNA gene sequence percentage similarities.

Media and Growth Conditions for Polymer Extraction

Bacterial and fungal Isolates were screened for detection of polymer granules which was done by staining the colonies with Sudan Black B and Nile Blue-A staining dyes. Polymer-producing cells were sub-cultured in nutrient agar and nutrient broth (pH 7.0) to confirm their viabilities and later transferred and cultured in yeast malt agar and on yeast malt broth to prepare the inoculums for fermentation on an orbital Shaker at 120 rpm for 72 h [14,15, 10].

Polymer Assay

At the end of fermentation, the method of [15], used by [10] was directly employed. This method involved centrifuging fermented materials at 10000 rpm for 20 min after 72 h of incubation. The cellular growth was placed in a centrifuge tube and 9 mL of alkaline hypochlorite reagent was added and incubated at 50 $^{\circ}$C for 1 hour to allow lysis of cells.
It was mixed with ethanol (70%) and refrigerated for 12 h. The mixture was centrifuged at 8,000 rpm for 15 min and washed with 10 ml of distilled water, acetone and absolute ethanol. The residue was dissolved in 10ml of chloroform after washing and incubated overnight at 40 ^{0}C it was then evaporated at room temperature and was finally dried at 40 $^{\circ}$C for 24 h and weighed (g/L) [10,16-18].

FTIR Analysis

Fourier Transform Infrared Spectroscopy (FTIR) was carried out by using a modified method of [18]. The FTIR analysis of the polymer sample in this study was done on the Shimadzu 48005 FTIR machine. The machine was turned on and allowed to warm for 30 min. The machine was programmed to scan between 400 and 4000 cm^{-1} at a resolution of 4 cm^{-1}. After that, the background spectrum was scanned and recorded. The pre-prepared KBr windows were each coated with a separate application of the polymer samples, resulting in the formation of a thin layer of the sample liquid between the two KBr windows. The polymer samples from the KBr windows were put into the sample compartment and affixed to the sample holder. After scanning each of the samples, the spectrum was formed and shown on the screen of the computer. The noise signals were removed from the spectrum with the use of software called "IR solution" that was placed into the system, and the spectrum was then printed after it had been smoothed down. All the other polymer samples went through the same process.

Scanning Electron Microscopic (SEM) Analysis of Polymer Samples

The sample that had the highest percentage of the polymer was taken for SEM analysis. The SEM revealed information about the morphological characteristics of the material making up the samples which include: texture, chemical composition,

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crystalline structure e.t.c. (SEM, Phenotype model: pro x). SEM is a surface analytical technique used to generate magnified topographical images of the material surface 20 × to cover 100, 000 ×.

In this study, the analysis was done at a magnification 750 ×. The polymer sample studied was first electrically conductive by ion sputtering with platinum. The examination was typically done along the sample axis. A focused stream of high-energy electrons is what the scanning electron microscope (SEM) utilizes to create a variety of signals at the surface of solid objects. Using the field emission cathode in the SEM's electron gun, narrower probing is possible at low and high electron energy, leading to better spatial resolution and less sample charge and damage. Signals resulting from the interaction of electrons with the sample gave information on the morphology of the sample's constituent material.

RESULT AND DISCUSSION

Identification of Isolated Organisms

The fungi species identified in this study were *Aspergillus niger, Cladosporium* sp., *Penicillium marneffi, Rhizopus*s and *Penicillium chrysogenum;* while bacterial species include *Bacillus subtilis, Bacillus megaterium, Bacillus cereus, Bacillus polymyxa, Bacillus licheniformis, Bacillus mycoides* and *Enterobacter* sp (**Table 1**). Based on molecular analysis, the fungal isolates were identified as *Cladosporium tenuissimum* strain AGF1 (OM273981), *Penicillium chrysogenum*strainAGF2 (OM273982) and *Aspergillus niger* strainAGF3 (OM273994) and were confirmed with sequences amplified homologies. The four bacterial isolates that were positive for polymer production were molecularly identified as *Bacillus subtilis* strain AGB1 (OM273871), *Bacillus megaterium* strainAGB2 (OM216844), *Paenibacillus polymyxa* strain AGB3 (OM273889) and *Bacillus licheniformis* strain AGB4 (OP703543).

Some of the bacteria species showed Black- Blue colouration which was positive for the polymer production while the fungal species screened negative. However, only four (4) Bacilli groups were polymer producers especially polyhydrxybutyrate (PHB) and they were Gram positive rod-shaped bacteria. They showed bright orange florescence under UV light bypolyhydroxybutyrate (PHB).

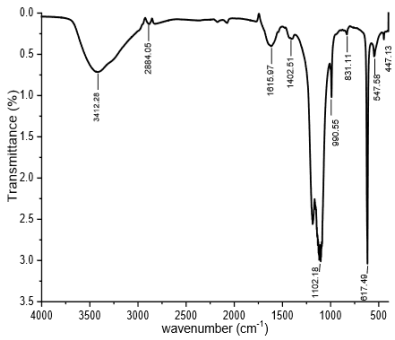


Fig. 1. FT-IR Analysis. FRT- IR spectrum of PHB produced by B. *megaterium* strain AGB2, resolution = 4cm^{-1} , scanning speed= 2mms^{-1} (A) 3412.28 (B) 2884.05 (C) 1615.97 (D) 1402.51 (E) 1102.18 (F) 990.55 (G) 831.11 (H) 617.49 (I) 547.58 (J) 447.13.

The wave number (cm^{-1}) varies for different functional groups. The wave number of polymer (PHB) in this study was between 1615 to 1700 and above which was the wave number of carbonyl functional groups (C=0). Therefore, FTIR analysis (**Fig. 1**) revealed that the functional group of extracted polymers was identified as C=0 group (carbonyl group).

Table 1. Colonial, microscopic and biochemical characteristics of bacterial isolates.

Isolates	Colonial characteristics				Microscopy			Biochemical reactions										Sugar fermentation					Probable Identity
	Colour	Elevation	Edge	Optical characterization	Surface	Gram reaction	Spores staining	Cell Arrangement	Motility	Catalase	Starch Hydrolysis	Methyl Red	Penicillinase	Glucose	protease	lecithinase	Citrate	Glucose	Lactose	Maltose	Mannitol	Sucrose	Galactose	
AGB1	Cream	flat	Entire	translucent	smooth	+	+		Rod	+	+	+	+	+	+	+	+	+	+	+	+	+	+	*Bacillus subtilis*
AGB2	Cream	Flat	Entire	Opaque	shinning	+	+		Rod	+	+	+	+	-	+	+	+	+	+	+	+	+	+	*Bacillus megaterium*
AGB3	Yellowish	Raised	Rhizoid	translucent	smooth	+	+		Rod	+	+	+	+	+	+	+	+	+	+	+	+	+	+	*Bacillus polymyxa*
AGB4	Cream	Raised	Rhizoid	Opaque	smooth	+	+		Rod	+	+	+	+	+	+	+	+	+	+	+	+	+	+	*Bacillus licheniformis*
AGB5	Cream	Raised	Round	translucent	smooth	+	+		Rod	+	+	+	+	+	+	+	+	+	+	+	+	+	+	*Bacillus cereus*
AGB6	White	flat	Round	Opaque	smooth	+	+		Rod	+	+	+	+	+	+	+	+	+	+	+	+	+	+	*Bacillus mycoides*
AGB7	Greenish or	Raised	Rhizoid	Opaque	shinning	-	-		Short rod	-	-	-	-	+	-	-	-	+	-	-	-	-	-	*Enterobacter*sp.

Key: AGB1 – AGB7 (Bacterial Isolates)
+ Positive (fermentation)
- Negative (no fermentation)

Phylogenetic Tree of Bacterial Isolates

Nucleotide sequence analysis of test bacterial isolate (**Fig. 2**) revealed that isolate AGB1 showed maximum homology 98% with *Bacillus subtilis* strain NRRL NRS744 and isolate AGB2 showed homology 100% with *B. megaterium* strain DSM32, isolate AGB3 showed maximum homology 95% with *Paenibacilluspolymyxa* strain B719W while *Bacillus*

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licheniformis strain AGB4 showed maximum homology 100% with B. *licheniformis* strain BCRC11702.

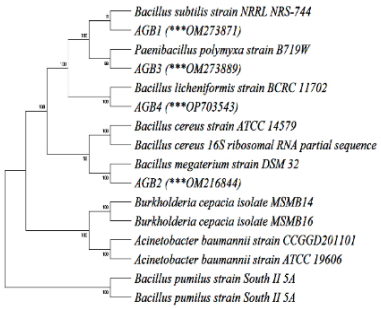


Fig. 2. Phylogenetic tree constructed by Neighbor-Joining method derived from analysis of the 6S rRNA gene sequences of native isolates and related sequences obtained from NCBI.Scale bar, 0.02 substitutions per nucleotide position.

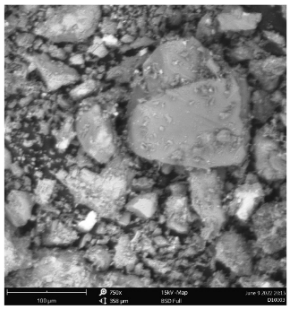


Fig. 3. SEM Analysis (Scanning Electron Microscopic) of polymer (PHB) produced by *Bacillus licheniformis* (rough with the crystalline surface.)

Polyhydroxybutyrate (PHB) producing bacteria have been reported and isolated from various environments, but only a few had been reported from dump soils from where the bacteria used in this investigation were obtained. Potential characterization and identification of the best bacteria in the production of PHB were done by studying their morphological, and cultural characteristics such as Gram staining, motility test, spore test and other physiological and biochemical tests described by [9] cited by [10]. *Bacillus licheniformis* strain AGB4 (OP703543) produced the highest PHB 56 percent followed by *Bacillus megaterium* strain AGB2 (OM216844)50 percent and *Paenibacillus polymyxa* strain AGB3 (OM273889) 40 percent. This observation was similar to the report of [10] though the name of the organism was not mentioned but labelled as "F_{14}" isolate that produced (42.1 percent).

The result of this study was also in line with the report of [13] who produced PHB from bacteria. The special media that was used to culture the bacteria for PHB production (Yeast Malt Agar and Yeast Malt Broth) also aid in the multiplication of the bacteria and the production of biopolymer (PHB). This result was favourably compared with the report of [17];[10] who used special media for culturing PHB bacteria producers. The orbital shaker that was used for incubation during submerged fermentation of bacteria assisted to produce maximum PHB at 29 ^{0}C and this agrees with the observation of [13]. High temperature and high pH range were not favourable to the production of PHB because it was not favoured the survival and activity of the bacteria.

The pH range of 6.5 and 7 favoured the production of PHB by the bacteria isolates. Anything higher or lower than the above pH caused the decrease in the activity of the PHB bacteria producers and this agrees with the record of other researchers [17]; [19]; [15] and [10]. The quantity of PHB produced by *Bacillus subtilis* was also encouraging (25 percent) and it was in line with the report of other authors who had used *Bacillus subtilis* for the production of PHB [20-22]. The observation of FTIR spectroscopy analysis was similar to the report of [13,14]. The analysis was used to confirm the functional groups of the extracted polymer (C=O) which were also related to the report of [23] during their study on the characterization of polyhydroxybutrate (PHB) synthesized by newly isolated *Haloarchaea halolamina*. The PHB production and yields (i.e. the percentage) were similar to the observation of [24] in their study of novel lactate utilizing *Bacillus* spp. YHY22 for PHB production. Scanning electron microscopy analysis (**Fig. 3**) revealed the crystalline nature of the polymer samples and it was in line with other researcher's reports also produced PHB using bacteria such as *Bacillus* species and analysed it with SEM [10,13].

CONCLUSION

It can be concluded that the importance of PHB produced can now become a suitable substrate for the production of bioplastics (bioplastic PHB) and this is biodegradable plastic. Also, *Bacillus licheniformis* strain AGB4 (OP703543) and *Bacillus megaterium* strain AGB2 (OM216844) that produced maximum PHB can be attractive bacteria for the production of biodegradable plastics. Therefore, *Bacillus* species isolated from dump site soils can be employed for the industrial production of PHB. The PHB (polyhydroxybutyrate) extracted from the bacterial isolates have functional groups that were identified as C=O group. This was revealed and confirmed by FTIR spectroscopy.

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