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SCREENING AND CHARACTERIZATION OF BIOPLASTICS (POLYHYDROXYLALKANOATES) PRODUCING BACTERIA ISOLATED FROM SAWDUST FROM PARTS OF ANAMBRA STATE

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Abstract
Screening and characterization of bioplastics (Polyhydroxyalkanoates PHA) produced by bacteria isolated from sawdust was undertaken. 15 samples of sawdust were randomly collected from different locations in Anambra State of which 20g each sample was suspended in saline water for 1 hr then cultured on nutrient agar, incubated at 37^0c for 48hrs with pH 7.2 ± 0.2. Sudan B Black stain was used to identify positive isolates while Nile Red strain was used to confirm PHA producing bacteria. Morphological and biochemical characterization of positive isolates identified *Bacillus spp.* FTIR confirmed the bond as CH_2 while the molecular and PCR identified bacteria as potent PHA producers. Hence, industrial production and commercialization of bioplastics should be encouraged as means of increasing national GDP and conserving the environment from non-biodegradable petroleum based synthetic plastics.

INTRODUCTION

Biopolymers (PHAs) are plastic materials produced from renewable biomass sources, such as vegetables, fats and oils, corn starch, straw, woodchips, sawdust, recycled food wastes as well as other biodegradable agricultural wastes (Siracusa & Blanco, 2020). Petrochemical based synthetic plastics and pollution have an intense correlation. These non-biodegradable polymers are introduced to the environment daily thereby causing numerous health and environmental hazards especially greenhouse effects (Atiwesh *et al.,* 2021).

Accordingly, the annual production of plastics is continuously increasing worldwide. In 2021, about 390.7 million tons were produced which is 25.5 million tons more than in 2018 and 15.2 million tons more than that in 2020 (Plastics – The Facts, 2022). This extensive production and used plastics generate large amount of plastics wastes thereby constituting nuisance within the environment. But highly non-biodegradable requiring recycling as the only method of proper disposal (Alabi *et al.,* 2019).

A wide range of applications of plastics are indisputable due to its low cost, stability, durability and high mechanical and thermal properties.

Microplastic (MP) are smaller plastic particles that could be found in numerous species including humans by exposure through eating and inhalation. A 2019 U.S study estimated that an adult human ingests 50,000 particles of microplastics each year through food and drink and another 55,000 particles through inhalation (Cox *et al.,* 2019). Commercially available plastics usually contain numerous additives such as UV stabilizers, antioxidants, dyes, plasticizers and flame retardants which can be easily released from MP particles because they are rarely chemically bonded to the plastic polymer (Zolotora *et al.,* 2022, Barrick *et al.,* 2021, Miri *et al.,* 2022).

Polyhydroxyalkanoates (PHA) has become a substitute for petrochemical derived plastic due to similar mechanical properties, PHA is eco-friendly, biodegradable, biocompatible and microbial thermoplastic (Cruz *et al.,* 2018). PHA is a homopolymer straight chain of D-β-hydroxyalkanoates, is produced as granules in different bacteria during the exponential growth phase and transferred into important energy reserves (Charen *et al.,* 2021).

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Bacterial species such as Pseudomonas, Bacillus and Rhodococcus naturally produce PHA (Santo *et al.,* 2020). Among bacteria, the general Pseudomonas and Bacillus are most commonly used for the biodegradation of plastics.

Several attempts have been made to produce bioplastics from renewable biomass sources and agricultural wastes, with premium placed on the effectiveness of carbon sources, supplementation of the medium and utilization by the bacterial strain being screened.

The techniques for tracing PHA producing bacterial granules together with PHA PHA production potential of some isolated bacterial strains under different cultural conditions such as phylogenic analysis, extraction of genomic DNA, sequencing and bioinformatics analysis, DNA quantification, 16SrRNA amplification, PCR, TLC, FTIR and UV-vis spectroscopy have been developed for easy identification, characterization of the bacterial species and the functional group present in the polymer. (Koller & Rodriguez, 2019; Mira *et al.,* 2021).

Bioplastic production by *Bacillus spp* isolated from agricultural wastes using molecular methods by amplification of 16SrRNA gene, as well as the production of bacterial polymer from low cost sustainable sources such as rice bran and soy molasses, sugarcane molasses, dairy and paper mill effluents, brewer's spent grain hydrolysed with various composition and pre-treatment methods with high bacterial efficiency have been developed (Zaki, 2019; Aljuraifani *et al.,* 2019; El-kadi, 2021 and Rathika *et al.,* 2019).

Sawdust (or wood dust) is a bye product or waste product of wood working operations such as sawing, sanding, milling and routing (Tan *et al.,* 2019). It is composed of very small chips of wood. These operations can be performed by machinery but can be a significant fire hazard in some manufacturing industries.

The objectives of this study include screening and characterization of prospective PHA-producing bacteria from sawdust obtained from parts of Anambra State, Nigeria.

MATERIALS AND METHODS

Collection and Processing of Sawdust Samples

A total of 15 samples of sawdust (depths 0cm, 5cm, 10cm) were collected from various locations in Ekwulobia, Igboukwu, Awka Etiti and Nnewi, Anambra State of Nigeria. The samples were collected inside clean polyethylene bags then transferred immediately to the laboratory of the Department of Microbiology, Chukwuemeke Odumegwu Ojukwu, University, Uli, Anambra State for analysis. All the samples were kept at 4^0C until required for use 20g each of the sawdust samples was suspended in sterilized saline solution (0.85% w/v) for 1 hour and was streaked on nutrient agar medium using sterile cotton wool swab.

Primary screening for PHA producers

Primary screening of all the isolates was carried out using Sudan B Black dye assay method (Porras *et al.,* 2018). The colonies were covered with an ethanolic solution of (0.05%). Sudan B Black and the plates were left undisturbed for 30 minutes. Excess stain from the colonies was washed with 96% ethanol first. The colonies that retain dark blue colour were considered positive for producing PHA (Aljuraifani *et al.,* 2019).

Confirmatory staining for PHA producers

Nile Red was added to the agar plates. All positive 10 isolates on Sudan B Black were streaked on the Nile Red containing plates and reincubated at 37^0C for 24 hours. Plates were observed under UV light for confirmation of PHA accumulation. Colonies showing bright pink or orange fluorescence are considered positive for PHA accumulation (Rogala *et al.,* 2020).

Morphological and biochemical characterization of potent PHA producers

After the isolation of bacteria, ten potent PHA producers were selected and cultured on Luria Bertani (LB) agar using a spread plate method using sterile cotton swab according to Bhuwal *et al.,* (2013). Triplicate plates were inoculated and incubated at 37^0C for 48 hrs with sterile control. Cellular morphology was examined with a light microscope. Biochemical tests were carried out.

Bacterial isolation

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The isolation method was accomplished using carbon enrichment medium via Mineral Salt Medium (MSM) according to Ratnaningrum *et al.,* ((2019). The MSM contains in gram per litre of K_2HPO_4 (5.8), KH_2PO_4 (3.7), $(NH_4)_2$ SO_4 (1.1), $MgSO_4.7H_2O$ (0.2), $FeSO_4.7H_2O$ (2.78), $C_aCl_2.2H_2O$ (1.67) with microelements including $MnCl_2.4H_2o$ (0.17) and $ZnSO_4.2H_2O$ (0.29).

10g ml of the bacterial culture growth was centrifuged at 8000 rpm for 10 mins then washed with mixture of alcohol and acetone (1:1 v/v).

The pellet was resuspended in 15ml of boiling chloroform. The chloroform was allowed to evaporate according to Mannima *et al.,* (2019). The final product was obtained as PHA pellets.

The liquid portion containing PHA was dried at 60^0c to form pellets. The pellets were washed twice with distilled water and was dried in an oven at 100^0C for 10 minutes. 10ml Sulphuric acid (H_2SO_4) was added to the biomass in the tube for the conversion of PHA to Crotonic acid and the samples were placed in water bath at 100^0C for 1hr. The volume of remaining sample in the water bath was kept and stored at -4^0C. However, 0.2ml of the residual sample was picked up and placed in Spectrophotometer to determine the amount of polymer produced on the basis of observed absorption in the presence of produced Crotonic acid. The maximum absorbance of each sample at 235nm which is the maximum absorbance for Crotonic acid. Also PHA was noted in that Crotonic acid being absorbed in the range 100 – 1000 μg/ ml. UV irradiation at 235nm which appeared as a sharp peak in the spectrum. The extent of UV irradiation absorbed by Crotonic acid was directly correspond to the amount of PHA present in the sample since PHA is converted to Crotonic acid.

The following equation was used to determine the amount of produced PHA in samples (as described by Gnanasekhar, 2012).

$$y = 0.0226 x - 0.0945$$

Where y is the absorbance, and x is the amount of crotonic acid corresponding to PHA content in μg.

$$PHA\ \% = \frac{X}{CDW} x\ 100$$

CDW is the cell dry weight obtained by weighing method.

FTIR analysis of PHA for characterization

The structural groups of the powder PHA was identified using Fourier Transform Infarred Spectroscopy (FTIR spect). FTIR has been established as the most effective method for analysing components since it identifies the kinds of chemical bonds and functional groups that are present in the sample (Stanley *et al.,* 2020). The PHAs extracted (2mg was dissolved in 500μl of chloroform and layered o $NaCl_2$) crystal form the PHA was subjected to FTIR analysis by spectra recording in the range of 400-4000cm^{-1} at a resolution of 4cm^{-1} with procedures described by Liu *et al.,* (2018).

Molecular identification (DNA extraction)

Genomic DNA purification kit (Wizard) was used according to the manufacturer. The purity of DNA was checked by nanodrop (Promega) and the range between 50-100np (Bhuwal *et al.,* 2013).

RESULTS AND DISCUSSION

The isolation of *Bacillus spp* from sawdust obtained from parts of Anambra State, Nigeria, revealed the presence of diverse bacterial strains capable of utilizing sawdust as carbon source. The abundance and diversity of *Bacillus spp* isolated in this research could be linked to the availability of essential nutrients that support bacterial life in those environment. Many microbes that accumulate PHA as storage materials could be found in natural environments such as soil, seawater, sewage sludge or compost. Hence, the isolation of *Bacillus spp* from sawdust was not coincidental (Gmbh, 2018).

The results characterization (Table 1) revealed that ten isolates out of the eighteen were positive to Nile Red fluorescence microscopy. These findings signified that 55.6% of all the isolated bacteria are potential PHA producers. Similar observations have been reported by Poras *et al.,* (2018), Cruz *et al.,* (2022). This method

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remains a powerful means of confirming PHA producers with high degree of certainty. Hence, the results of screening demonstrated the presence of promising PHA producer with similar approach to Aljuraifani *et al.,* (2019) and Rogala *et al.,* (2020).

Table 1 Qualitative features of the selected Polyhydroxylbutyrate (PHA) bacterial strains.

Strain	Sudan B black	Nile Red
PHA 2	+	++
PHA 5	++	++
PHA 6	++	+
PHA 8	++	+
PHA 9	++	+

This table shows that PHA strains have varying reactions to Sudan B Black and Nile Red stains.

The results of bacterial identification (Table 2), showed that *Bacillus spp* were the most frequently isolated bacteria. The identity of these isolates was inferred based on their 16SrRNA sequences which were found similar to reports of Bhuwal *et al.,* (2013), Rogala *et al.,* (2020).

Table 2: Biochemical characteristics of the isolated and selected bacterial strains.

Isolate/Parameter	PHA2	PHA5	PHA6	PHA8	PHA9
Gram reaction	+	+	+	+	+
Cell shape	Rod	Rod	Rod	Rod	Rod
Oxidase	+	+	+	+	-
Hydrogen sulphide	+	_	+	+	+
Glucose	+	+	+	+	+
Sucrose	_	+	_	+	_
Lactose	_	+	_	+	_
Citrate	+	_	+	+	+
Indole	_	_	_	_	_
Nitrate reduction	+	+	+	+	+
Voges Proskauer	+	+	+	+	+
Xylose	+	+	+	+	+
Arabinose	+	+	+	+	+
Galactose	+	+	+	+	+
Methyl red	_	_	_	_	_
Catalase	+	+	+	+	+
Identity	*Bacillus sp.*	*Bacillus sp.*	*Bacillus sp.*	*Bacillus sp.*	*Bacillus sp.*

Table 2 shows the different biochemical reactions used to identify *Bacillus spp.*

The result pf the PHA quantification showed that the maximum absorbance was 245nm that appeared as a sharp peak which is equivalent to the absorbance by the Crotonic acid amounting to the PHA present (Gnansekhar, 2012).

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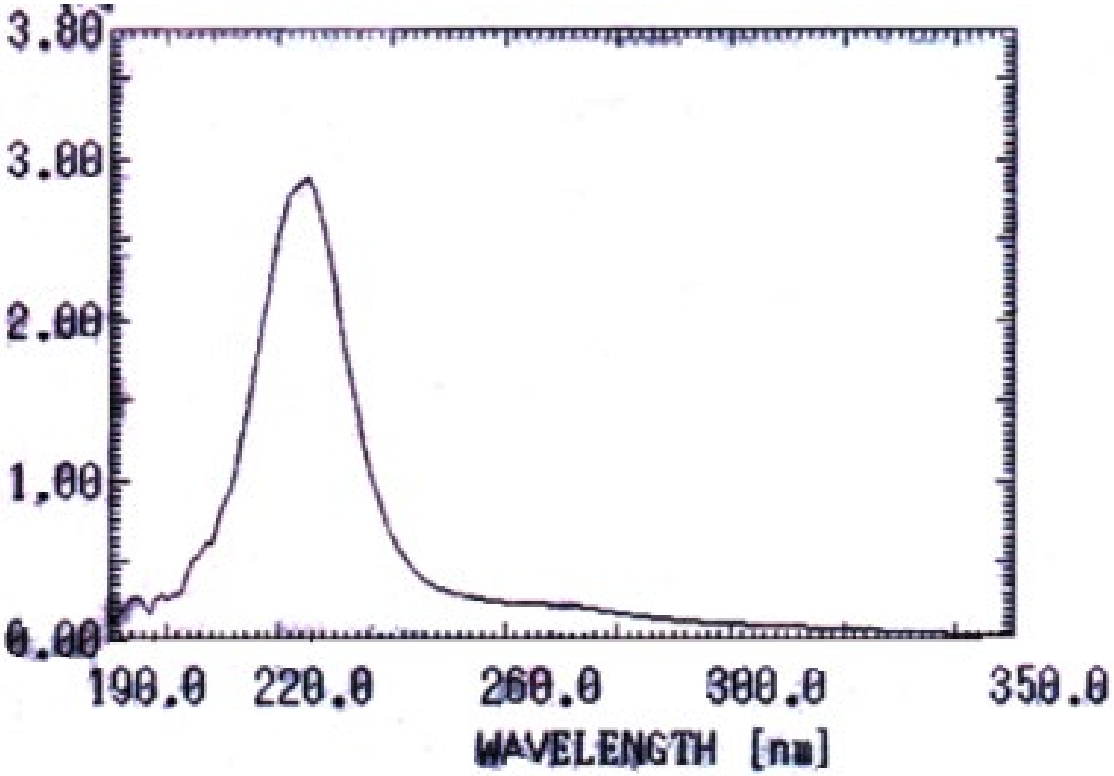


Figure 1: UV–Vis spectra profile of PHB extract of the consortium bacterial strains (PHA2 and PHA5)

The results of the FTIR analysis was presented in fig 2 showing the pattern of spectra profile. The was found at 1455.36cm^{-1} band and 1453.67cm^{-1} which corresponds to the asymmetrical deformation of the C-H bond in CH_2 group (Nark & Ranade, 2020). These bands represents C-H stretching and also characteristics of PHA. These prominent absorption bands provide evidence that polyhydroxyalkanoate was the polymer isolated from sawdust.

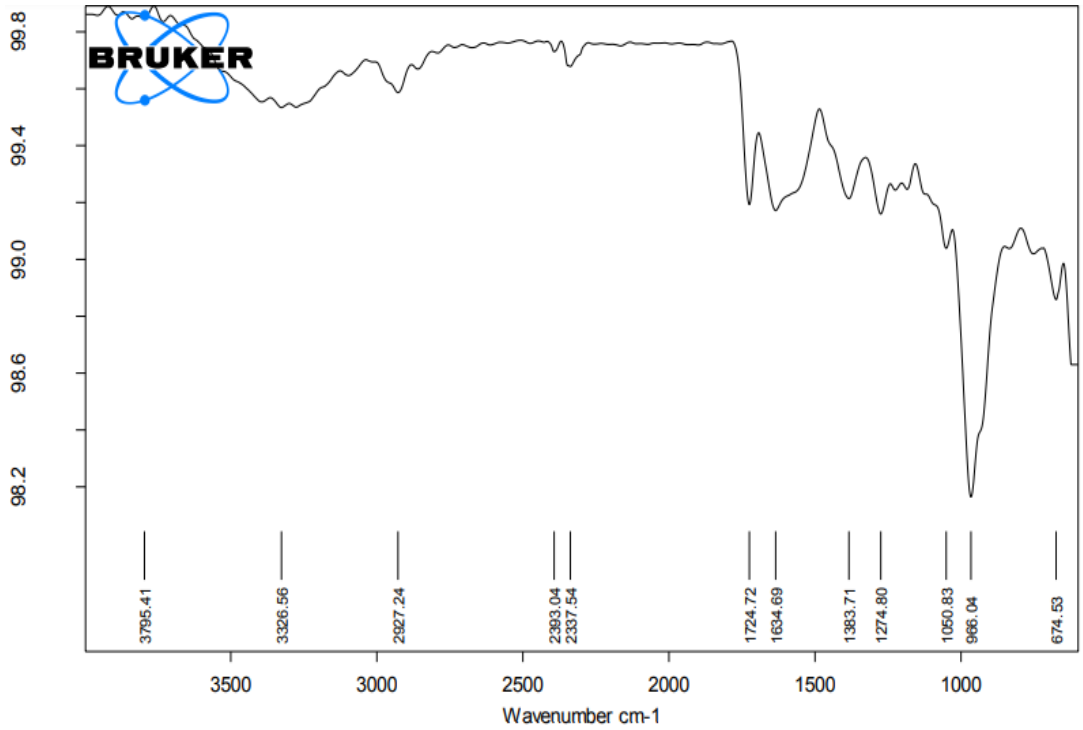


Figure 2: Fourier Spectral Profile of the Polyhydroxybutyrate Produced by the Consortium Strains (PHA 2 and PHA 5)

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It was keenly observed that the optimal temperature for screened *Bacillus spp.* was 35^0C ± 1 with the pH 7.4 ± 0.1. Depth had no significant effects on PHA production from sawdust.

Conclusion

This study revealed the capability of sawdust bacteria for PHA accumulation. Results showed that 56% of the isolated bacteria tested positive for PHA accumulation thus proved that reserve polymers like Polyhydroxyalkanoates promote bacterial survival in dynamic habitats. Furthermore, FTIR analysis showed prominent functional group CH_2 confirming the extract as PHA. Hence, all the ten positive isolates were capable of producing PHA that can be used for industrial production and commercialization of biodegradable plastics.

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