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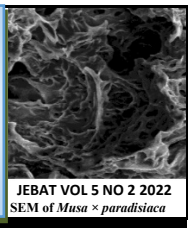

Isolation and Characterization of Poly-3-hydroxybutyrate (PHB)-producing Bacteria from some Municipal Waste within Maiduguri Borno State Nigeria

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ABSTRACT

The present work was conducted to isolate, screen and identify polyhydroxybutyrate (PHB)-producing bacteria from municipal wastes. Samples were collected from dumping sites of Maiduguri Borno State and were checked for their bacterial population using nutrient agar and PHB Detection agar (PDA). A total of 20 PHB-producing bacteria were isolated and identified. Of the total, four of the isolates were found to be from *Paenibacillus* spp. while the remaining 16 were *Bacillus* spp. Isolates from Ngomari Custin denoted as NC 5, 2; NC 7, and NC 5A were suspected to be *Paenibacillus favisporus, Bacillus lentus,* and *Bacillus firmus* respectively while others of NC 7A X, NC 6A and NC 7A Y of the same site were all found to be *Bacillus smithii*. Isolates from Old Maiduguri Graveyard denoted as OMG 5X, OMG 7, OMG 6 and OMG 5A were all found to be *Bacillus smithii.* The isolates from Flour Mill Area denoted as FMA 5A, FMA 6A, FMA Y, and FMA7B were found to be *Bacillus smithii, Paenibacillus nanensis, Bacillus acidiceler and Paenibacillus septentrionalis* respectively. On the other hand, isolates from Ramat Square site denoted as RS 6,2; RS 6A, RS 5, RS 7,2 were registered as *Bacillus nealsonii, Paenibacillus cookie, Bacillus firmus and Bacillus megaterium* respectively. Those of RS 5B and RS 7A of the same sample site were both *Bacillus acidiceler* and their granules were stained by Nile Blue A staining and observed under the fluorescence microscope and were classified for the extent of their PHB production based on the intensity of fluorescence emitted under the microscopy. NC 7 produced the highest PHB yield compared to all other isolates. Hence, municipal wastes are a rich reservoir of PHB-producing bacteria and can readily produce (PHB), which has enormous advantages over petroleum-based polymers by means of cost effectiveness and eco-friendliness

INTRODUCTION

Presently, Poly-3-hydroxybutyrate (PHB) is getting more attention as bioplastic, it has properties similar to that of petroleum-based thermoplastics such as polypropylene [1], making them useful in wide-ranging applications for industries and commercial requirements. It is also useful in large-scale production as it can replace petroleum-based plastics currently in use [2]. However, a serious problem for wide-scale production of PHBs is the high cost of production as compared with the currently in-use petroleum-based plastics. Several reports regarding PHB production pointed out that, the high cost of production of PHB is due to the carbon substrate cost [3]. Studies were committed to reducing the cost of PHB production through efficient bacterial strain development and optimizing fermentation and recovery processes [4,5]. Moreover, the larger solid waste produced has created much concern for the production of biodegradable polymers. Apart from solving environmental issues, PHB can also be effectively and efficiently be used in the field of medicine and medical sciences, chiefly to

its biodegradability characteristics, as PHB is present in 1.3 mol/L concentration in the human blood [6]. One undisputable significance of PHB in its biodegradability is that its fibres get thinner and thinner over time when used to suture wounds in mouse [7]. In the same line, PHB can readily be converted into nanoparticles, facilitating drug delivery to all body parts even to the smallest capillaries of 5-6mm diameter [8]. PHBs are a group of natural polyesters that are ready stored as intracellular inclusions by a large variety of bacteria [9] such as A*zotobacter, Nocardia, Pseudomonas, Rhizobium* [10], *Staphylococcus, Alkaligens, Bacillus, Micrococcus and Rhodococcus* [11] and possess similar characteristics to the synthetic plastics in use [12].

PHB production is most widely carried out with *Alkaligens eutrophus* for it is easy to grow, stores large amount of PHB (about 80% of dry cell weight (DCW) using a simple medium, as a result of their more comprehensive physiology and biochemistry involved [13]. Nearly 40-48% of cost of PHB production comes from the raw materials where about 70-80% of the total cost goes to carbon source [13]. Therefore, the selection and choosing of suitable and efficient carbon substrate is of paramount importance. And as such, a proactive approach is to select a renewable, most readily available and economically feasible carbon substrates for the growth of the microorganisms and PHB production respectively [13]. Hence, this study shall isolate and characterize an indigenous bacterium that can produce bioplastic (PHB), which have enormous advantages over petroleum-based polymers by means of cost effectiveness, eco-friendliness, and user-friendly.

MATERIALS AND METHODS

Study area

Maiduguri is the capital and the largest city of Borno State, Nigeria which has an East-West extension of about 280 km. From North to the south it is approximately 320 km in North-Eastern Nigeria. The city of Maiduguri with the Latitude and longitude coordinates 11.8311° N, 13.1510° E sits along the seasonal Ngadda River which disappears into the *Firki* swamps in the areas around Lake Chad [14].

The city was established more than 100 years ago and used to be called Yerwa by the local population. In the middle of the 20th century, it became the center of the region and the capital city of the state. Today, it is home to slightly more than half a million people. The current population of Maiduguri metropolis in 2021 is 803,000, a 2.16% increase from 2020 (786,000), 1.81% increase from 2019 (772,000) and 1.58% increase from 2018 [14]. The city is mainly agricultural and commercial, with a number of large farmer's markets and the so-called "Monday Market" being especially popular. It is one of the warmest regions in Nigeria with an average daily high temperature of 37 ºC. With a yearly average of 37 ºC. The climate is very warm but has only a very few tropical and humid months. It is yearlong warm or hot. Sometimes humidity isn't unpleasantly high from June to September [14].

Sampling sites

The soils were sampled at 4 different sites in Maiduguri, in the central part of Borno State and the quota sampling method was employed in sampling at four (4) municipal waste areas viz Flour Mill Area Dumping Site, Old Maiduguri Graveyard Dumping Site, Ramat Square Dumping Site and Ngomari Custing Dumping Site respectively, all in Maiduguri Metropolis, Borno State, Nigeria.

Sample collection

A sample was collected from the subsoil of the four (4) municipal waste areas as described above. The samples were collected at a depth of 1m into sterile glass bottles and labelled accordingly. They were transported on ice to the laboratory and processed within two hours of their collection.

Isolation and enumeration of PHB-producing bacteria

Isolation of bacteria was performed by serial dilution technique for all four different samples respectively. Soil samples were sieved to a uniform size. The samples were taken in a test tube and seven test tubes, each with 9 ml of sterile distilled water were taken. Sterile pipettes were used during the serial dilutions. Aliquots (I ml) of serially diluted soil samples (10^{-5}, 10^{-6} and 10^{-7} respectively) [14] were poured in duplicates onto nutrient agar plates and PHA Detection Agar (PDA) [(g/L: Glucose 20, $(NH_4)_2SO_4$ 0.2, KH_2PO_4 13.3, $MgSO_4$ 1.3, Citric Acid 1.7, Trace element solution 10 mL, [(g/L): $FeSO_4.7H_2O$ 10, $ZnSO_4.7H_2O$ 2.25, $CuSO_4.5H_2O$ 1, $MnSO_4.5H_2O$ 0.5, $CaCl_2.2H_2O$ 2.0, $Na_2B_4O_7.10H_2O$ 0.23, $(NH_4)_6Mo_7O_{24}$ 0.1, 35% HCl 10 mL]; Agar, 20.0, pH 6.8 - 7.0) [14]. The nutrient agar plates were incubated at 29 ± 1 ºC for 24 h while the PDA plates were incubated at 29 ± 1 ºC for 72 h [15].

Primary screening for PHB production

All the PHA Detection Agar (PDA) culture plates were qualitatively tested for PHB production using Sudan Black B dye. Ethanolic solution of Sudan Black B (0.05%) was poured over the colonies and the plates were kept undisturbed for 30 min. They were then washed with ethanol (98%) to remove the excess stain from the colonies. Smears of stained samples were made and observed under oil immersion at 100 × magnification with direct bright-field illumination using an Olympus CX31 microscope with a Lumenera infinity 2 CCD camera [15]. The dark blue-coloured colonies were positive for PHB production and were individually picked and subcultured 3-4 times on nutrient agar plates for purification. Well-isolated, morphologically distinct colonies were stored at 4 ˚C by streaking onto nutrient agar slants till further use [16].

Identification of the PHB-producing bacteria

The PHB-positive isolates were identified based on morphological and biochemical characteristics using Bergey's manual of determinative bacteriology [17].

RESULTS

Population of total heterotrophic bacteria on nutrient agar medium

The total heterotrophic bacterial counts from the sampling sites show OMG (50 x10^7cfu/g), FMA (52 x10^7cfu/g), RS (61 x10^7cfu/g) and NC (48 x10^7cfu/g).

The highest population count was at RS whereas the lowest count was at NC as shown in **Fig**. **1**. The total PHB-producing bacterial counts from the sampling sites show OMG (45 x10^7cfu/g), FMA (48 x10^7cfu/g), RS (46 x10^7cfu/g) and NC (38 x10^7cfu/g), the highest population count was at FMA. Whereas, the lowest count was at NC as shown in **Fig**. **2**. Gram staining and biochemical tests were conducted for the identification of the PHB-positive isolates. From the samples of the four (4) sampling sites, 20 PHB-producing bacteria were isolated. The Gram reaction and the biochemical tests conducted were used in the identification of the isolates using Bergey's Manual of Determinative Bacteriology and ABIS Online-Bacterial Identification Software. The result is shown in **Table 1**.

The growths of PHB-positive isolates on plates were subjected to Fluorescence Microscopy and the intensity of fluorescence light emitted by the PHB granules of the isolate was used to estimate different amounts of PHB inside the cells. *Bacillus lentus* was observed to have emitted the brightest light intensity and is therefore recorded as the highest PHB-producing bacteria among the tested isolates. The result is shown in **Table 2.**

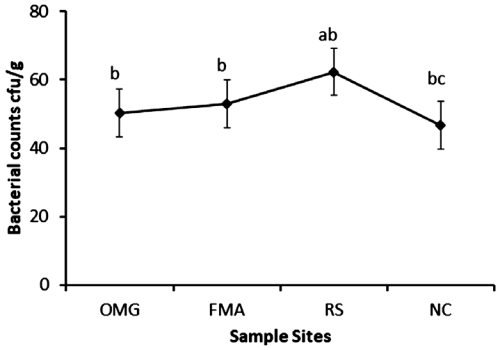

Fig. 1. Population of total heterotrophic bacteria on nutrient agar medium bars (means + SD, n=3) with different letters within treatments are significantly different based on LSD ($p < 0.05$). CFU= Colony Forming Unit OMG= Old Maiduguri Grave Yard Dumping Site (Sample location)**,** FMA= Flour Mill Area Dumping Site (Sample location), RS= Ramat Square Dumping Site (Sample location) NC= Ngomari Custin Dumping Site (Sample location).

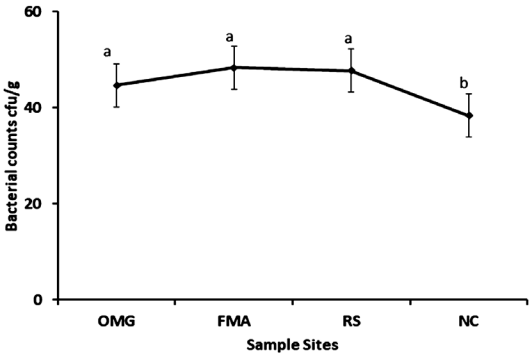

Fig. 2. Population of PHB producing bacteria on PHA detection agar (PDA) bars (means + SD, n=3) with different letters within treatments are significantly different based on LSD ($p < 0.05$). CFU= Colony Forming Unit OMG= Old Maiduguri Grave Yard Dumping Site (Sample location)**,** FMA= Flour Mill Area Dumping Site (Sample location), RS= Ramat Square Dumping Site (Sample location) NC= Ngomari Custin Dumping Site (Sample location).

Tables 1. Gram Staining and Biochemical tests for the identification of the PHB positive isolates.

	Gram Rxn	BIOCHEMICALS							TSI				Possible identity of bacteria
Isolates		Ind.	MR	Cit.	Ure.	Ox.	Cat.	VP	Butt	Slant	H_2S	Gas	
NC 5,2	+ rod	_	+	_	+	+	+	_	Y	Y	_	_	*Paenibacillus favisporus*
NC 7A X	+ rod	_	+	+	+	+	+	_	Y	R	_	_	*Bacillus smithii*
NC 7	+ rod	_	+	+	+	+	+	_	Y	Y	_	_	*Bacillus lentus*
NC 6A	+ rod	_	+	_	_	+	+	_	Y	R	_	_	*Bacillus smithii*
NC 7A Y	+ rod	_	+	+	+	+	+	_	Y	R	_	_	*Bacillus smithii*
NC 5A	+ rod	_	+	_	_	_	+	_	Y	R	_	_	*Bacillus firmus*
OMG 5X	+ rod	_	+	+	+	+	+	_	Y	R	_	_	*Bacillus smithii*
OMG 7	+ rod	_	+	_	_	+	+	_	Y	R	_	_	*Bacillus smithii*
OMG 6	+ rod	_	+	+	+	+	+	_	Y	R	_	_	*Bacillus smithii*
OMG 5A	+ rod	_	+	_	_	+	+	_	Y	R	_	_	*Bacillus smithii*
FMA 5A	+ rod	_	+	_	_	+	+	_	Y	R	_	_	*Bacillus smithii*
FMA 6A	+ rod	_	+	_	+	+	+	+	Y	R	_	_	*Paenibacillus nanensis*
FMA Y	+ rod	_	+	_	_	+	+	+	Y	R	_	_	*Bacillus acidiceler*
FMA 7B	+ rod	_	_	_	+	_	+	+	Y	R	_	_	*Paenibacillus septentrionalis*
RS 6,2	+ rod	_	+	_	_	+	+	_	Y	Y	_	_	*Bacillus nealsonii*
RS 6A	+ rod	_	+	_	_	+	+	+	Y	Y	_	_	*Paenibacillus cookii*
RS 5B	+ rod	_	+	_	_	+	+	+	Y	R	_	_	*Bacillus acidiceler*
RS 5	+ rod	_	+	_	_	_	+	_	Y	R	_	_	*Bacillus firmus*
RS 7,2	+ rod	_	_	+	_	_	+	_	Y	Y	_	_	*Bacillus megaterium*
RS 7A	+ rod	_	+	_	_	+	+	+	Y	R	_	_	*Bacillus acidiceler*

Keys:
MR = Methyl Red test
VP = Voges-Proskauer test
- = Negative
+ = Positive
Y = Yellow
R = Red

Tables 2. List of PHB positive isolates with the extent of PHB production.

S/N	*Bacterial isolates*	Extent of PHB Production
1	*Bacillus lentus*	+ + +
2	*Paenibacillus septentrionalis*	+ +
3	*Bacillus firmus*	+ +
4	*Bacillus smithii*	+ +
5	*Bacillus acidiceler*	+ +
6	*Bacillus megaterium*	+ +
7	*Paenibacillus nanensis*	+
8	*Paenibacillus favisporus*	+
9	*Bacillus nealsonii*	+
10	*Paenibacillus cookie*	+

Keys:
+ = Low PHB producers
++ = Medium PHB producers
+++ = Highest PHB producer

DISCUSSIONS

Materials of petrochemical origin are one of the major environmental pollutants not only because they persist in the environment, but because they are also disposed of in large quantities [18]. The potential to avoid these problems lies in taking advantage of bacterial metabolic activities, particularly those related to the generation of new eco-friendly and sustainable plastics [18]. Biodegradable polymers have found applications in a lot of areas, e.g., in medicine such as sutures, surgical implants, wound dressings and controlled-release drug delivery systems [19]. In addition to that, there is an excessive demand for biodegradable plastics for use as food and beverage containers, garbage bags, and for mulching.

This can be able to be accomplished by ascertaining new organisms having a wide range of substances and high growth rates [19]. Previous research has presented that a large number of bacterial species of both Gram-positive and Gram-negative, produce PHBs [20,11] and also degrade them [20,11].

At the same time, the huge potential to screen for novel bacterial species capable of producing high levels of PHB remains untapped [21]. However, some reports have investigated the presence of PHB-producing bacteria in different environments such as marine microbial mats existing in cannery waste streams [21], oil-contaminated soils in oil fields [22,11] activated sludges from pesticide and oil refineries effluent treatment plants [23] and salty waters [23] In this study, we have isolated and identified PHB-producing bacteria (**Table 1**) present in consistently used municipal wastes areas.

Altogether, 20 distinct colonies were chosen based on their colour after staining with Sudan Black B which confirms the presence of PHB granules. The successful isolation of these PHB-producing bacteria is a direct achievement of our first objective. They exhibited many morphological features on the culture plate, ranging from having creamy and milky colour; surfaces of raised, rough, smooth and flat surfaces and elevation; entire and undulate margins; mucoid and dry texture. The results of the morphological and biochemical identification of the PHB-positive isolates confirmed by Sudan Black B and Nile Blue A staining were shown in **Table 1**. The biochemical tests conducted in conjunction with the gram staining were used to identify the isolates by using Bergey's manual of determinative bacteriology [24]. The identification scheme is based on the comparison between the morphological and biochemical character values of the isolated strain and standard values of taxa contained in the database [24] (**Table 1**).

The organisms isolated from the sample of Ngomari Custin Dumping Site, denoted by NC 5,2; NC 7, and NC 5A were suspected to be *Paenibacillus favisporus, Bacillus lentus, and Bacillus firmus* respectively while others of NC 7A X, NC 6A and NC 7A Y of the same sample site were all *Bacillus smithii*. Those isolates from "Old Maiduguri Graveyard Dumping Site" denoted by OMG 5X, OMG 7, OMG 6 and OMG5A were all found to be *Bacillus smithii.* The isolates from "Flour Mill Area Dumping Site", denoted by FMA 5A, FMA 6A, FMA Y, and FMA7B were found to be *Bacillus smithii, Paenibacillus nanensis, Bacillus acidiceler and Paenibacillus septentrionalis* respectively. And on the other hand, isolates from Ramat Square Dumping Site denoted by RS 6,2; RS 6A, RS 5, RS 7,2 were also registered as *Bacillus nealsonii, Paenibacillus cookie, Bacillus firmus and Bacillus megaterium* respectively. Those of RS 5B and RS 7A of same sample site are both *Bacillus acidiceler.*

This screening and identification result conforms to our objective two. The intensity of fluorescence was used to estimate different amounts of PHB inside the cells of these organisms as reported by [25,22]. Positive isolates for PHB production were classified for the extent of their PHB production based on the intensity of fluorescence emitted on fluorescence microscopy (**Table 2**). Our study is the first to report *Paenibacillus favisporus, Paenibacillus septentrionalis, Bacillus smithii, Bacillus acidiceler, Bacillus lentus, Bacillus nealsonii, Paenibacillus cookie* and *Paenibacillus nanensis* as PHB producers. Other isolates obtained are *Bacillus firmus* as reported by [25] and *Bacillus megaterium* also reported by [27,28]. Interestingly, amongst the PHB producers detected in this study, *Bacillus lentus* is shown to be the highest PHB producer (**Table 2**) determined by the intensity of fluorescence.

CONCLUSION

This study was able to identify PHB-producing bacteria in municipal waste dump sites in Maiduguri Borno State. Ten species of PHB-producing bacteria belonging to two genera (*Paenibacillus* ***and*** *Bacillus)* were isolated and characterized. *Bacillus smithii* was found to be the commonest as it appeared in three sites while *Bacillus lentus* only appeared on one site and it has the highest potential for PHB production.

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