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ISOLATION AND CHARACTERIZATION OF *PSEUDOMONAS AERUGINOSA* AND *BREVIBACILLUS* SPECIES AND THEIR POTENTIAL TO BIODEGRADE POLYETHYLENE MATERIAL

A.S. Gumbi[1,2*], M.S. Abdulsalam[2], A.B. Suleiman[3], N. Egbe[2], Z. Umar[2], A.M. Shitu[3], A.S. Gumbi[3] and S.M. Yahuza[4]

[1] Standards Organisation of Nigeria, No 44 Kachia Road, Kaduna, Nigeria
[2] Department of Biological Sciences, Faculty of Sciences, Nigerian Defence Academy, Kaduna, Nigeria
[3]Department of Microbiology, Faculty of Science, Ahmadu Bello University Zaria, Kaduna, Nigeria
[4] Department of Veterinary, Public Health and Preventive Medicine, Ahmadu Bello University Zaria, Kaduna, Nigeria

Corresponding Author's Email Address: nyaleyjo02@yahoo.com Phone: +2347035867336

ABSTRACT
This study was conducted to screen for Pseudomonas aeruginosa and Brevibacillus species from soil and investigate their ability to biodegrade low density polyethylene materials. The organisms were isolated using phenotypic characterization and molecular identification by polymerase chain reaction (PCR). The PCR confirmed the presence of two different strains of Pseudomonas aeruginosa and absence of Brevibacillus specie from the soil sample. The bacteria were inoculated in a nutrient broth to which 2% polyethylene was amended for a period of three weeks in a shaker incubator at 180rpm. Effect of temperature, pH and concentration of polyethylene on the biodegradation process was also studied. The initial and final dry weights of the polyethylene were recorded and the % degraded was calculated. It was found that both strains of Pseudomonas aeruginosa were capable of degrading the polyethylene. Strain C3 produced a maximum degradation of 20% at 37°C and pH 6. Strain B3 achieved a maximum degradation of 15% at 37°C at pH 6 and 7. In addition, it was found that both strains of Pseudomonas aeruginosa were able to survive up to 6% of polyethylene producing a maximum degradation of 55%. Therefore strains B3 and C3 of Pseudomonas aeruginosa can be effective in biodegradation of polyethylene in dump sites if their potentials are well exploited.

Keywords: Gumbi, Pseudomonas, PCR, Biodegradation, Polyethylene

INTRODUCTION

Polyethylene is a polymer made up of long chain monomers of ethylene. It is a thermoplastic commodity mostly used for packaging. The worldwide utility of polyethylene is expanding at a rate of 12% per annum (Shimao, 2001) and approximately 140 million tons of synthetic polymers are produced worldwide each year (Shimao, 2001).

With a huge amount of polyethylene getting accumulated in the environment, their disposal evokes a big ecological issue. It takes a thousand years for their efficient degradation. The durability, light weight and processability of polyethylene cause it to linger in nature for centuries and end up in landfills and/or natural water resources (Jang *et al.,* 2002).

In Nigeria, waste disposal is very poor and polyethylene constitutes most of the waste. Most of the polyethylene waste originates from consumption of products packaged in polyethylene sachets, bags or other containers. Polyethylene are chemically synthesized organic compounds most of which do not occur in nature (Ojo, 2007). They are compounds that are foreign to living organisms. Where these compounds are not easily recognized by existing degradative enzymes, they accumulate in the soil and water (Ojo, 2007). The environmental pollution caused by polyethylene includes soil, water and air contamination, blocking of drains and sewage lines in and around the cities. Since polyethylene is non- degradable, it remains intact in water and soil for many years and are not productive to the soil as reported by Aziegbe (2007) and FEPA (1998); Lugwig (1990); Ibanga (2000); Shanwar and Kanwar (2007).

Open burning of refuse dumps in developing countries including Nigeria, is a common practice (Izuagbara and Umoh, 2004). In a bid to minimize the pollution effects, burning of polyethylene waste materials has been adopted as a management strategy. However, it has been realized that if burnt, polyethylene products produce harmful toxins which can threaten the air quality (UNEP, 2002). Other thermal/oxidative degradation products of polyethylene in controlled condition have been studied and found to consist of chemicals that are harmful to human health and areas of environmental concern (Sojaka *et al*., 2006). This method in effect, constitutes a greater part of environmental pollution with many greenhouse effects. Gases such as carbon dioxide, carbon monoxide and subsequently sulphur and nitrogen oxides are released into the atmosphere giving rise to acid rains, ozone depletion and global warming (Yang *et al*., 2004). There has always been search for the efficient disposal of polyethylene yet the biological means holds certain concern other than the chemical modes. Some possible measures employed for the purpose are biodegradation and bio-recycling (Yang *et al*., 2004). However, biodegradation serves a tangible alternative. Synthetic fibers like polyethylene and polypropylene are practically non-degradable (Esteve-Nenez *et al*., 2001). Although, more organisms are being described as being able to degrade these anthropogenic molecules, some xenobiotics have been shown to be unusually recalcitrant (Esteve-Nenez *et al*., 2001). The ability of *Pseudomonas* species to degrade organic pollutants has been reported in earlier studies (Nwachukwu *et al*., 1999; 2000). *Pseudomonas* species are known to have strong ability to resist toxic substances, including heavy metals such as mercuric compounds and including heavy metals such as mercuric compounds and disinfectants (Baron *et al*., 1994). *Pseudomonas*

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species are equally highly amenable to genetic manipulation, a desired attribute of microorganisms favoured in bioremediation.
Brevibaccillus borstelensis strain 707 is a thermophilic strain also capable of degrading and utilizing polyethylene as its sole source of carbon (Hadad *et al.*, 2005). Hadad demonstrated that non-degradable plastics like polyethylene can be degraded under appropriate conditions (Hadad *et al.*, 2005).
Polyethylene constitutes a very important part of the waste and causes a lot of pollution problem. However all available management methods have not been effective and this necessitates search for alternative methods including biodegradation by microorganisms
The aim of this research is to isolate and evaluate the capability of *Pseudomonas aeruginosa* and *Brevibacillus* bacteria to degrade polyethylene.

MATERIALS AND METHODS

Sample Collection
The samples were collected from Unguwan Shanu area of Kaduna State located on Latitude 10.5448 north and Longitude 7.43007 east on the map with an average elevation of 620metres (2034 feet). The soil samples were collected from a waste disposal site dumped with polyethylene bags in the above mentioned areas of the state, at a depth of 3cm using a sterile stainless steel spatula. The collected samples were transferred to pre-sterilized, labeled, self-sealed plastic bags and transported to the Bacteria and Zoonosis laboratory in the Department of Public Health and Veterinary Medicine ABU, Zaria. Nine samples were available for the study viz A1, A2, A3, B1, B2, B3, C1, C2, and C3.

Bacterial Enumeration and isolation
Selective isolation of *Pseudomonas using* PAB-CFC and isolation of bacillus using nutrient agar containing amphotericin B was achieved. The isolates obtained were subcultured severally to obtain pure culture.

Identification of isolates
The isolates obtained in pure culture were identified using morphological characterization, biochemical identification and molecular characterization using PCR.

Preparation Of Polyethylene
The general grade polyethylene employed for commercial grocery carriage purpose which was obtained from the waste disposal site, was washed with 70% ethanol until all the visible particles adhered to it were washed off completely. It was then rinsed with distilled water for an hour and dried in a hot air oven for 1 h 30minutes and until dry. The large pieces were crushed by grinding using mortar and pestle along with sufficient amount of crystalline NaCl to make the grinding easier (Sonil and Smiti, 2010). It was ground till they got minced in form of fine ruptured threads. Rupturing the polyethylene with the salt solution assisted in washing away all impurities and organic matter adhering to it. The mixture was transferred into a conical flask with distilled water and was mixed well in a shaker for 1 hour. The solution was then filtered using Whatman no. 41 filter paper. Polyethylene particles recovered from over the filter paper were dried in the oven for an hour (Sonil and Smiti, 2010).

Inoculation of isolates with polyethylene.
0.5 McFarland standard concentrations of the different isolated samples suspected *Pseudomonas* species were individually inoculated in to 100 ml of nutrient broth in respective conical flasks except the control. 0.2 g of polyethylene was amended to each flask except the blank.
All the treatments were incubated in an incubator shaker at 180 rpm for 3 weeks. PC3 and PB3 were incubated at 37°C, 45^{0}C, 50^{0}C, pH 6, 7, pH 8 and at different concentrations of the polyethylene. Negative controls and Blanks were used to examine the viability of the bacteria. The weight loss in the reaction mixture and in the polyethylene weight was calculated.

RESULTS

Morphological Identification

Table 1: morphological characterization of *pseudomonas aeruginosa on* PABCFC plate and *Brevibacillus species* nutrient agar plate

PAB-CFC PLATE	NUTRIENT AGAR PLATE
COLOUR a. Green Coloured Colonies b. White Colonies	COLOUR: Milky White Colonies
SIZE : Tiny	Tiny, Small and Medium Colonies
APPEARANCE	APPEARANCE
Rough, swollen and moist	Moist
ELEVATION : Flat and Raised	ELEVATION: Flat

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Table 2: Gram Stain

Sample Response to Gram stain	
Sample: NA plate	**PAB-CFC plate**
A1 +short rods	- short rods
A2 + short rods	- short rods
A3 + long rods	- short rods
B1 + short rods	- long rods
B2 *	+ short rods
B3 *	- short rods
C1 *	- short rods
C2 +short rods	- short rods
C3 + short rods	- short rods

Biochemical Identification

Table 3: Biochemical Identification

	Suspected *P.aeruginosa*					Suspected Brevibacillus					
Test	**A 3**	**B 1**	**A 3**	**C 2**	**C 3**	**A 1**	**A 2**	**A 3**	**B 1**	**C 2**	**C 3**
Oxidase	+	+	+	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+	+	+	+
Citrate	+	+	+	+	-	-	-	-	-	-	-
Urease	-	±	-	±	-	-	-	-	-	-	-
Methyl red	+	+	-	+	-	-	-	-	-	-	-
VP	-	-	+	-	+	+	+	+	+	+	+
Indole	+	-	-	-	-	-	-	-	-	-	-
Motility	+	+	+	+	+	-	-	-	+	+	+
Mannitol	-	-	-	-	-	-	±	-	-	-	±
Maltose	+	+	+	+	-	-	-	+	-	-	-
Sucrose	+	+	+	+	-	±	-	±	±	+	+
Alkaline test	+	+	+	+	+	-	-	-	-	-	-
H_2S formation	-	-	-	±	-	-	-	-	-	-	-

Molecular Identification

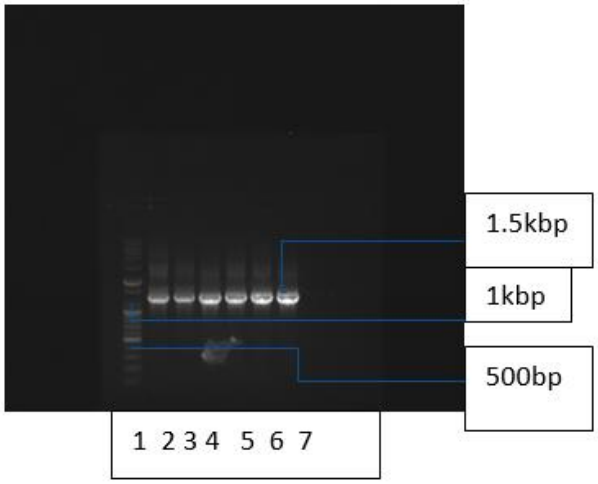

Plate: 1 Image of Bands of Molecular Weight 1.5kbp Formed for suspected *Brevibacillus* samples. Where lane 1 = molecular weight ladder of varying length, Lane 2= sample A1, Lane 3= sample A2, Lane 4= A3, Lane 5= B1, Lane 6= C2, Lane 7= C3 and lane 7= negative

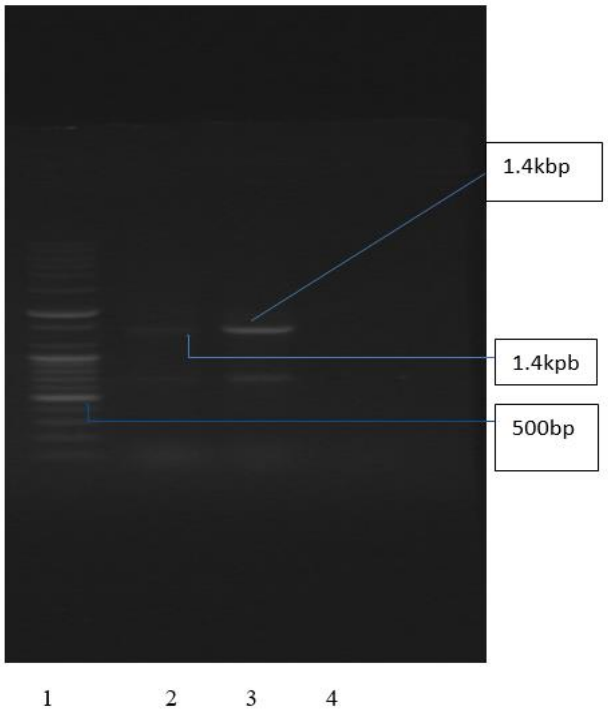

Plate 1: Bands produced by *Pseudomonas.*Where lane 1 = molecular weight ladder, Lane 2= sample B3, Lane 3= sample C3, Lane 4= Negative

DISCUSSION

Bacterial genomic extraction was achieved using phenol/chloroform extraction followed by the PCR reaction which was conducted in DNA thermal cycler PTC-100 with a total of 2µl

of DNA solution and a master mix Accupower hot start PCR premix by Bioneer and 1µl of the reconstituted primers for each of the organism.

Out of all the samples isolated from the soil, none of the suspected *Brevibacillus species* yielded the desired bandwidth of 1.078 kbp for any of the *Brevibacillus specie* when amplified using 16S rDNA amplification of the two universal primers; 63F (5'-CAGGCCTAACACATGCAAGTC – 3') and 1389R (5' – ACGGGCGGTGTGTACAAG – 3') (Bioneer Inc. CA, San Francisco USA). This confirms that *Brevibacillus species* were not isolated from the soil sample collected. However, two of the samples C3 and B3 for the suspected *Pseudomonas aeruginosa* yielded two different bandwidth of the same base pair and were suspected to be two different strains of the bacteria as it produced the expected bands of 1400 base pairs after the 16S rRNA gene region was amplified using the universal primer; forward primer 27F

(5' AGAGTTTGATCCTGGCTCAG3')

reverse primer 1492R

(5' TACCTTGTTACGACTT 3') (Bioneer Inc. CA, San Francisco USA)

Out of all the treatments, C3 and B3 gave the best biodegradability of 55% at 37^0C at a concentration of 0.6% polyethylene. This was followed by C3 which gave 50% at 0.4% concentration of polyethylene and a temperature of 37^0C. At 0.2g, B3 produced the highest degradation at 37^0C and at both pH6 and pH7 while C3 produced 20% degradation at pH 6, respectively. Hence, it might be assumed that the temperature has a greater effect on the biodegradation process than the pH. Probably because the *Pseudomonas* species have a wide range of pH; (6.5-7) and could readily survive its changes. It is also assumed that the basal nutrients from the media were entirely utilized by the bacteria with the polyethylene acting as the readily available source of carbon and energy. This is similar to the findings of Kyaw *et al* (2012). They reported the highest level of polyethylene degradation (weight loss) out of the four bacteria they were working with was found as 20% by *Pseudomonas aeruoginosa* after 120 days. A research by Balasubramamian *et al* (2012) also reported 15% degradation by *Pseudomonas* specie after 30 days of incubation. In a study on the biodegradation of natural and synthetic polyethylene by *Pseudomonas specie,* the highest weight loss percentage of natural polythene was 46.2% and synthetic polythene 29.1% was reported with *Pseudomonas* specie collected from sewage sludge dumping site (Nanda *et al.,*). Sonil in 2012 also reported 40.5% biodegradation of polyethylene by *Pseudomonas* at pH 6.5 and a temperature of 37^0C. This was the best he recorded.

Pseudomonas aeruginosa had a long lag phase in adapting to the changing environment but during the second and the third week a boost in the biodegradation rate was found which would have been possible for the enhanced biofilm formation and enzyme activities on polyethylene particles. The biofilm helped the bacteria to act collectively and produce sufficient metabolites to degrade the polymer and utilize it as the carbon source (Sonil and Smiti, 2010). The biofilm develop structures that perform functions which enable the bacteria to survive the environment (Donlan, 2002). It protects the entire biofilm organisms from physical and chemical attack and against adverse conditions (Abdel-Aziz and Aeron, 2014). The biofilm has been reported to play a key role in supporting bioremediation of hydrocarbon contaminated soils. Bioremediation could therefore be facilitated by enhanced gene transfer among biofilm organisms and by the increased availability of pollutants for degradation as a result of chemotaxis as suggested by Harrison *et al.* (2007). Strategies for improving for bioremediation efficiency could include genetic engineering to improve strains.

Conclusion and Recommendation

The aim of this research was to isolate *Pseudomonas aeruginosa* and *Brevibacillus* species from the soil and evaluate their ability to biodegrade polyethylene. *Pseudomonas aeruginosa* was successfully isolated from the soil as it exists abundantly in the soil of Unguwan Shanu area of Kaduna state where the sample was obtained *Brevibacillus* was not in the sample site. The isolated bacteria were identified at the molecular level by Polymerase Chain Reaction which confirmed the presence of *Pseudomonas* and absence of *Brevibacillus* specie. The ability to degrade polyethylene was investigated. It was observed that *Pseudomonas* can biodegrade polyethylene. The biodegradation is greatly affected by physical factors like pH, temperature and concentration of polyethylene. Certain specification can increase or decrease the rate of biodegradation by the organism.

Pseudomonas seems to be the most efficient organism that biodegrades polyethylene from literatures. The species should be *isolated* from all the sources and *screened to know the efficient isolates.*

Some extracellular enzymes are responsible for the biodegradations of the polyethylene (Aswale, 2010). These enzymes should be characterized and the catabolic genes responsible for those enzymes with polyethylene-utilizing ability should be *identified.* Once the genes responsible for the degradation of polyethylene are identified and the mechanism understood, they could also be manipulated to make the biodegradation more efficient. After field trials, the most efficient polyethylene degrading microbes should *be multiplied* at large scale to decompose the polythene at commercial level and curb the menace of polyethylene pollution.

Authors Contributions

This work was carried out in collaboration between all authors. Author ASG, MSA, ABS designed the study, wrote the protocol and the initial draft of the manuscript. Authors ASG, ABS, SMY, GAS and AS handled the bacterial isolation, morphological characterization, biochemical identification and also monitored experimental system for polyethylene degradation. Authors ASG, ABS and UZ handled the molecular characterization of the bacterial isolates and analysis of the result. Authors ASG, MSA, ABS, NE managed all the literature searches. They all read and approved the final manuscript.

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