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Optimization of culture conditions for the biodegradation of low-density polyethylene films by plastic waste dumpsite bacteria

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Accumulation of low-density polyethylene films used in consumer packaging in the environment has generated a lot of problems due to its recalcitrance. The ability of bacteria recovered from three different plastic waste dumpsite soil located within Ekiti State University and the Ado-Ekiti metropolis to degrade this pollutant was evaluated and the optimal conditions for biodegradation was determined for the screened degrading species. The average count from the three sites ranged from 2.0 x 10^7 CFU/g to 4.38x 10^7 CFU/g in the waste polyethylene samples and 2.78 x 10^7 CFU/g to 5.53 x 10^7 CFU/g in the soil samples. Six isolates were able to utilize effectively the polyethylene amongst the 38 isolates recovered and they were identified via 16S rRNA sequencing as *Lysinibacillus xylanilyticus* strain BN-13 16S, *Pseudomonas aeruginosa* RI-1, *Pseudomonas aeruginosa* strain JAY2N, *Stenotrophomonas maltophilia* strain T7D7, *Pseudomonas aeruginosa* strain SMVIT-1 and *Achromobacter xylosoxidans* strain YEB RH5. The optimization of culture conditions for polyethylene degradation revealed that *L. xylanilyticus* strain BN-13, *A. xylosoxidans* strain YEB RH5 and *S. maltophilia* strain T7D7 grew optimally at 37 °C while *P. aeruginosa* RI-1, *P. aeruginosa* strain V1 and *P. aeruginosa* strain PW5a grew optimally at 45 °C after 24 h of incubation. Also, the optimal pH was between pH 6 and 7 in all the isolates while they were also able to utilize up to 5% w/v of polyethylene although at lower rates with most favouring 1% w/v concentration of the substrate. This study confirms the potential of these isolates for polyethylene degradation.

Keywords: optimization, polyethylene, bacteria, pH, temperature.

INTRODUCTION

Plastics are synthetic organic heteroatomic polymers that originate from oil, coal and natural gas (Azeko *et al*., 2015). Their extensive application is mainly due to their lightweight, ready availability, cheapness of raw materials, inertness and durability (Hemashenpagam *et al*., 2013). It also has widespread use in consumer packaging; water sachets, food packaging etc (Temitope *et al*., 2015). Low-density polyethylene (LDPE) is the most widely produced polymer, accounting for 21% of global production (Harrison *et al.,* 2014). Pure water" sachets are LDPE products widely in packaging of potable water (Temitope *et al*., 2015). Its desirable properties like extreme chemical stability, durability and cheapness results in its accumulation in the environment resulting in severe environmental pollution (Mukherjee *et al*., 2017; Kulkarni and Dasari, 2018). Indiscriminate sachet disposal is a common feature in Nigeria with empty sachets littering the nook and corners of the Nigerian landscape, highways, homes, schools etc

(Temitope *et al*., 2015). It is also responsible for clogging drains with consequent flooding issues (Kalilu, 2013). Conventional methods of remediation are either expensive, are limited in application and have resultant health detriments Hence, microbial approach through biodegradation offers a better option in the current situation (Mohan *et al*., 2016).

This study investigates the degradation ability of bacteria species recovered from LDPE dumpsite and also determine the optimum pH, temperature and polyethylene concentration required by isolates for maximal degradation of LDPE films.

MATERIALS AND METHODS

Sampling Sites and Sample Collection

Soil samples and waste sachet water bags popularly called 'pure-water' were collected from three sampling spots which were identified based on the extent of plastic contaminations around Ado-Ekiti. The first spot was a restaurant located within the Ekiti State University community (Coordinates 7.7141° N, 5.2601° E), the second spot was a student hostel dumpsite just outside the school vicinity while the third spot was a dumpsite located in Adehun area of Ado Ekiti metropolis (Coordinates 7.6602° N, 5.2376° E). These sampling spots have had polyethylene waste disposed extensively for an extended period. The waste water sachet and the soil samples were collected at a depth of 5-10cm, in a sterile container transported to the Microbiology laboratory of the Ekiti State University for microbiological analysis

Collection and processing of LDPE sheets

LDPE used for sachet water packaging were obtained from a water processing and packaging factory located within Ekiti State University (EKSU), Ado-Ekiti campus and was used for the degradation studies. Polyethylene powder (PEP) was prepared using the method described by Odusanya *et al*. (2013) and kept for further studies.

Isolation of LDPE utilizing bacteria species

One gram of soil sample attached to waste sachets and surrounding soil was added in 99 mL of sterile distilled water. Each suspension was vortexed for 15 minutes and serially diluted. One milliliter aliquot was transferred to sterile Petri dishes prior to pouring of Nutrient agar. The plates were incubated for 24 hours at 35 ^{o}C, sub-culture of distinct colonies was done to get pure isolates before being kept in slants for further studies (Gupta *et al*., 2010)

Primary screening of potential bacterial isolates.

Primary screening of the recovered isolates was done. Polyethylene powder was added to Mineral Salt medium at a concentration of 0.1% (w/v) and sonicated for 1hr at 120rpm. After sonication, agar was added and autoclaved at 121^{0}C, 15lbs pressure for 15 min. Sterilized media was cooled and poured into sterile Petri plates. Once solidified, the isolated colonies were inoculated and then incubated at 30-35^{0}C for 2 weeks. The organisms producing zone of clearance were selected for further analysis (Usha *et al*., 2011).

Identification of Polyethylene Degrading Bacteria

The molecular characterization of the bacterial isolates was done using 16s rDNA sequencing. The genomic DNA of the bacterial strains was isolated according to Sambrook and Russell (2001). Polymerase chain reaction (PCR) amplification of partial 16s rRNA gene was carried out with the bacterial primer set forward 16SF: GTGCCAGCAGCCGCGCTAA. PCR was be performed in an automated gene amplification PCR system 9700 thermal cycler (Institute for International Tropical Agriculture, Ibadan). The ladder used was hyper ladder 1 from Bioline. The expected base pair of the amplicon is around 850bp. The PCR product was purified and was loaded on the ABI 3500 genetic analyzer from Applied Biosystems USA. The sequences were then analyzed by basic local alignment search tool (BLAST) at NCBI database. The isolates were identified based on the similarity scores and the sequences were submitted to NCBI Gen Bank to obtain accession number (Soni, *et al*., 2009).

Optimization studies

Optimization of culture conditions for *in vitro* LDPE degradation was monitored adopting the method described by Al-Jailawi *et al*. (2015). The effects of temperature, pH and substrate concentration was monitored. The effect of temperatures (30- 50 ^{o}C) different pH values (6.0-9.0) and substrate concentrations (0.1- 5 %) on the ability of screened bacteria isolates to degrade polyethylene was evaluated. Mineral salt medium was supplemented with final concentration of 0.1% w/v of Polyethylene film and inoculated with the test isolates prior to incubation in shaker incubator (180 rpm) at for three days. The pH was adjusted to 6.5 for both temperature and concentration measurement while temperature was adjusted to

35°C for pH and substrate concentration measurement. The optimal conditions were then determined and subsequently employed for the degradation studies, depending on the optical growth density measurement (OD_{600}).

RESULTS

The average total bacterial colony count from the soil of polyethylene dump sites and polyethylene samples after 24h of incubation on nutrient agar is presented in Table 1. The soil samples from Ekiti State University (EKSU) Ado-Ekiti Satellite Phase 1 hostel dump site averaged 3.25 x 10^7 CFU/g of soil while the polyethylene samples taken from the same dump site averaged 2.0 x 10^7 CFU/g of sample. Also, the average bacteria count from Ekiti State Waste Management Authority (EKSWMA) dump site was 2.75 x 10^7 CFU/g for soil samples and 2.0 x 10^7 CFU/g for used sachet water samples (Table 1). The restaurant's average count for soil was 5.53 x10^7 CFU/g of soil while the used sachet water averaged 4.38 x10^7 CFU/g. Deepika and Jaya (2015) reported bacteria count ranging from 9.8 x 10^7 CFU/g to 4.0 x10^9 CFU/g which was significantly higher than what was reported in this study. Also, Vijaya & Reddy (2008) reported the average number of heterotrophic bacteria found in association with polythene film and plastic cups were 3.7 x 10^5 cfu/g and 3.84 x 10^5 cfu/g respectively while Poonam *et al*. (2013) enumerated the presence of bacteria from various plastic wastes associated soil samples and the highest total bacteria count were in range of 2.4 × 10^4 to 3.2 × 10^4 CFU/g in the soil sample collected from municipal landfills.

Thirty-eight morphologically distinct colonies were recovered after purification from the soil and PE samples collected from the three polluted sites. Of the 38 isolates, 21 were obtained from the polyethylene waste dumpsite located in Ado-Ekiti metropolis, seven from the restaurant dumpsite while ten were recovered from the University hostel dumpsite

Ten out of the thirty-eight isolates recovered from these sites were able to grow on mineral salt agar plates after 7 days. The isolates were codenamed B14, B15, B20, S1, S6, W5, W6, W11, PE2, PE4 and they were further grown in mineral salts broth with 2% w/v LPDE powder for 28 days. The ability of the ten isolates to grow on the agar medium with LDPE as sole carbon source is indicative of their potentials to degrade polyethylene as reported by Priyanka and Archana (2011). Six isolates PE4, PE2, W5, W11, S1, S6 were selected for degradation studies based on the weight loss recorded after 28 days. According to Gajendiran *et al*. (2016), weight loss is the simplest and quickest way to assess biodegradation of polymers. After the incubation period, LDPE films were treated with SDS as surfactant that denatures the microbial cells and washes them off from the surface.

The six isolates were identified using molecular characterization as *Lysinibacillus xylanilyticus* strain BN-13 16S (PE2), PE4 as *Pseudomonas aeruginosa* RI-1, Isolate S1 as *Pseudomonas aeruginosa* strain JAY2N, Isolate S6 as *Stenotrophomonas maltophilia* strain T7D7, Isolate W5 as *Pseudomonas aeruginosa* strain SMVIT-1, Isolate w11 as *Achromobacter xylosoxidans* strain YEB RH5 (Table 4). These species have been associated with biodegradation of polymers including LDPE wastes. *Pseudomonas* sp. are ubiquitous, metabolically versatile organisms associated and have been implicated in biodegradation of wate LDPE (Yoon *et al*., 2012; Tribedi and Sil 2013b; Bhatia *et al*., 2014; Hussein *et al*., 2015; Wilkes *et al*., 2017). Esmaeili *et al.* (2013) isolated *Lysinibacillus xylanilyticus* with remarkable abilities to degrade non-UV irradiated LDPE (15.8%), Deepika and Jaya (2015);

Stenotrophomonas sp. has been found to play important role in biodegradation of keratin (Yamamura *et al*., 2002), RDX (Binks *et al*., 1995), geosmin (Zhou *et al*., 2011), atrazine (Rousseaux *et al*., 2001), p-nitrophenol (Liu *et al*., 2007) and monocyclic hydrocarbons (Urszula *et al*., 2009), phenanthrene (Gao *et al*., 2013) and styrene. Skariyachan *et al*. (2017) and Montazer *et al*. 2018 in their investigation observed that *Stentrophomonas maltophilia* possessed LDPE degradation properties. Also, Kowalczyk *et al.*, (2016) isolated a new bacteria strain *Achromobacter xylosoxidans* with degradation effect on the structure of high-density polyethylene (HDPE), a polymer resistant to degradation in environment.

Table 1: Average Bacterial Colony Count from sampling sites

Site	Average bacterial colony count (CFU/ml)	Colour/ Soil Type
EKSU Satellite Hostel dump site		
a. Soil	3.03 x 10^7	Dark/loamy soil
b. Polyethylene	2.0 x 10^7	
EKSWMA dump site		
a. Soil	2.78 x 10^7	Dark Brown/clay soil
b. Polyethylene	2.0 x 10^7	
Mama Toyin Restaurant EKSU		
a. Soil	5.53 x10^7	Dark/loamy soil
b. Polyethylene	4.38 x10^7	

Table 2: Distribution of isolates recovered from the sampling sites

SITES	ISOLATES RECOVERED	
	WASTE SACHET BAGS	SOIL
EKSWMB Dumpsite	W6, W7, W8, W9, W10, W11	B1, B2, B3, B4, B5, B6, B7, B8, B9, B10, B11, B12, B13, B14, B15
Satellite Hostel Waste Dumpsite	W1, W2, W3, W4, W5	B11, B19
Restaurant Soil	PE1, PE2, PE3, PE4	S1, S2, S3, S4, S5, S6

Table 3: Percentage weight loss after enrichment initial weight is 2.00g

Isolates	Final Weight	% Weight Loss
B14	2.0g	0
S6	1.940g	3.0
W11	1.866g	6.7
B20	1.948g	2.6
W10	2.0g	0
S1	1.737g	13.15
W5	1.793g	10.35
PE4	1.854g	7.3
B15	2.0g	0
PE2	1.909g	4.55

Table 4: Molecular identification of the selected strains

Isolate code	Names	% Similarity	Accession Number
PE2	*Lysinibacillus xylanilyticus* strain BN-13	99%	MF188191.1
PE4	*Pseudomonas aeruginosa* RI-1	97%	AB900067.1
W5	*Pseudomonas aeruginosa* strain V1	98%	KT860423.1
W11	*Achromobacter xylosoxidans* strain YEB RH5	80%	MK258170.1
S1	*Pseudomonas aeruginosa* strain PW5a	88%	MK026869.1
S6	*Stenotrophomonas maltophilia* strain T7D7	99%	EF552366.1

The effect of different pH ranges, temperature and polyethylene substrate concentrations was observed in the strains. *Pseudomonas aeruginosa* strain PW5a had its highest growth at pH 7 (0.875) after 48hours, optimum temperature of 37^{0}C (0.769) and polyethylene concentration of 0.1 percent (0.829). *P. aeruginosa* strain V1 had its highest growth at pH 7 (0.995) after 36hours, temperature of 37ºC (1.01) and optimum polyethylene concentration of 1 % (0.823). *Achromobacter xylosoxidans* strain YEB RH5 had its highest growth at pH 7 (0.774) at 36hours, temperature of 37^{0}C (0.989) after 60hours, and could utilize LDPE at concentration as high as 5% w/v (0.986) after 48hours.

Stenotrophomonas maltophilia strain T7D7 had its highest growth at pH 7 (0.986) at 48hours, temperature of 37^{0}C (0.912) at 48 hours, and polyethylene concentration of 0.1 percent (0.999) at 36hours. *Lysinibacillus xylanilyticus* strain BN-13 16S (PE2) had its highest growth at pH 7 (1.118) at 48hours, temperature of 37^{0}C (1.001) at 36hours, and polyethylene concentration of 0.5 percent (1.103) at 48hours. *Pseudomonas aeruginosa* RI-1 had its highest growth at pH 7 (0.475) at 48hours, temperature of 45^{0}C (0.943) at 36hours, and polyethylene concentration of 0.1 percent (0.898).

At pH 6, isolate *Pseudomonas aeruginosa* strain PW5a had the highest growth in terms of optical density (OD_{600}) reaching a peak of 1.103 over the period of 72 h of monitoring with peak observed between 36 and 48 h of incubation while PE4 was noticed to have the least growth during the poerid of monitoring (Fig. 1).

At pH 7, all the isolates had similar growth patterns, although *P. aeruginosa* PE4 didn't grow well at that pH compared to the other isolates. Also, it was observed that the isolates' optimal pH was around pH 7 as revealed by the high OD_{600} values observed over the 72 hours of monitoring Fig. 2.

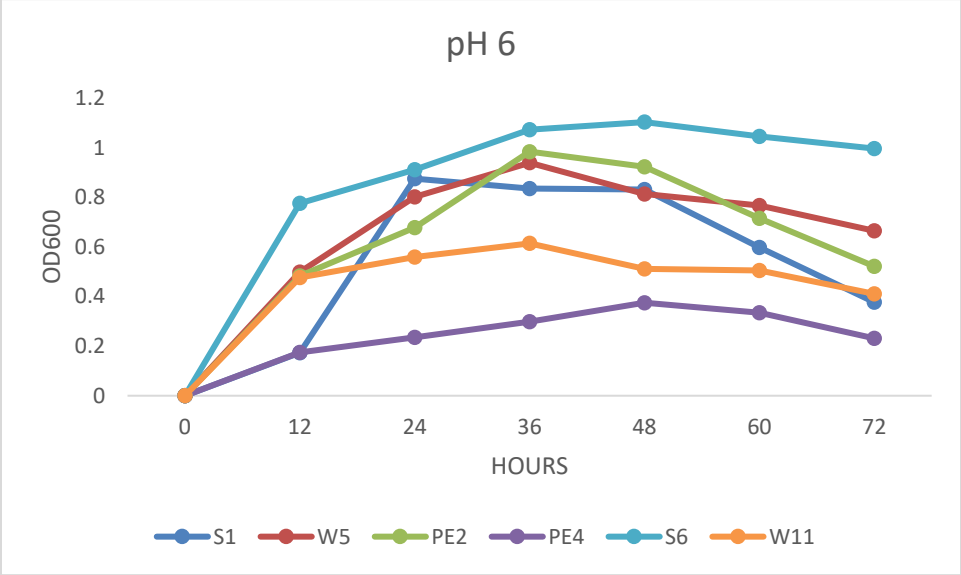

Figure 1: Growth profile of the test isolates at pH 6 within 72 hours

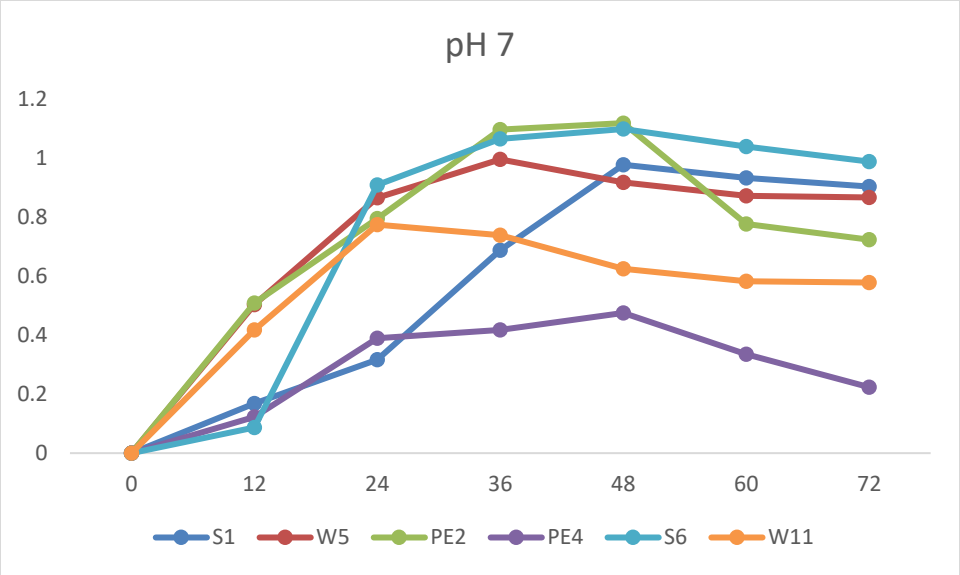

Figure. 2: Growth profile of the test isolates at pH 7 within 72 hours

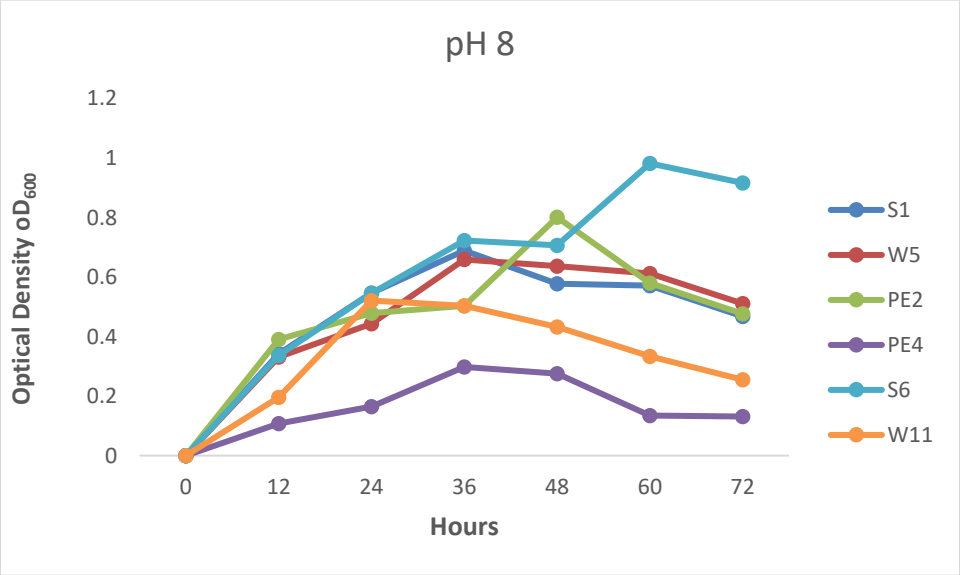

Figure. 3: Growth profile of the test isolates at pH 8 within 72 hours

At pH 8, lower optical density values were observed in all the isolates thus suggesting that the pH isn't optimal for the isolates. *P. aeruginosa* PE4 also had the least optical density value while *P. aeruginosa* strain W5, *P. aeruginosa* strain S1 and *S. maltophilia* strain S6 grew best at that pH as compared to the others (Fig. 3). At pH of 9, the growth of the organisms was markedly reduced as shown by the optical density readings taken within 72 h (Fig. 4).

The effect of temperature on the growth of the test organisms was monitored for three days. At 30 °C, *L. xylanilyticus* PE2 and *A. xylosoxidans* W11 had the highrest optical density reading while the growth of the others was average. *P. aeruginosa* PE4 however had the lowest growth pattern over

the 72 h of incubation (Fig. 5).

At 35 °C, the isolates *L. xylanilyticus*, *A. xylosoxidans*, *S. maltophilia* grew best with high OD readings which peaked at the 36 h of incubation for all thew isolates. *P. aeruginosa* W5 however peaked after 60 h while the growth of *P. aeruginosa* PE4 was least amongst the isolates (Fig. 6).

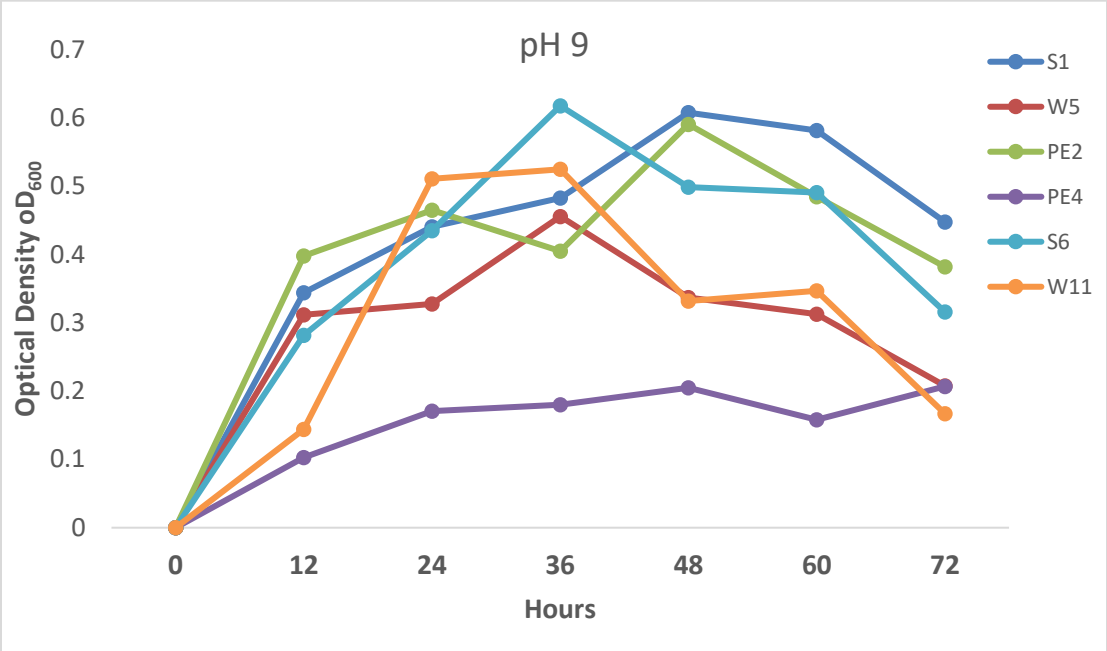

Figure. 4: Growth profile of the test isolates at pH 9 within 72 hours

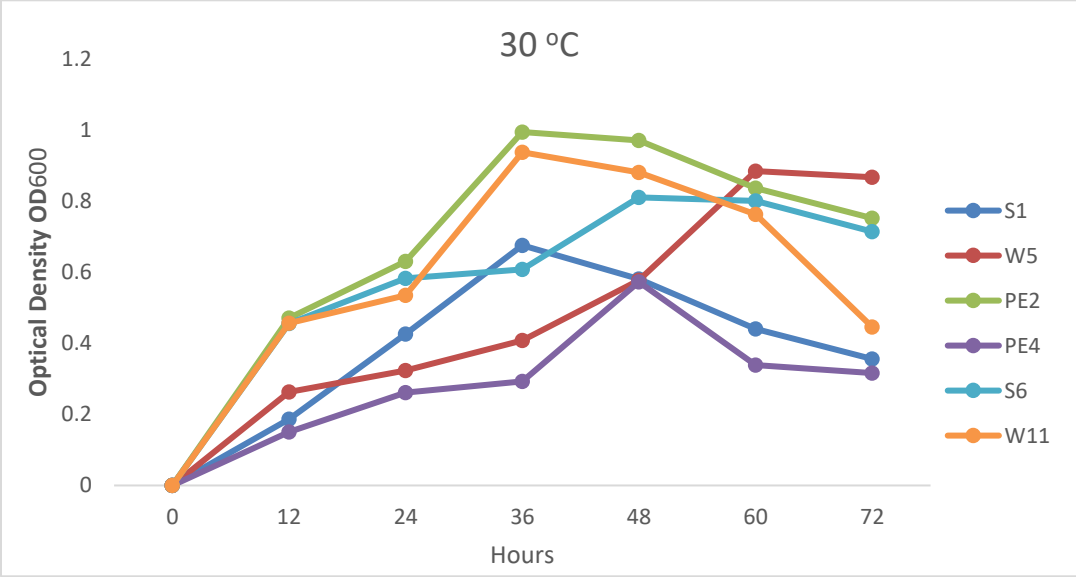

Figure. 5: Growth profile of the test isolates at 30 °C within 72 hours

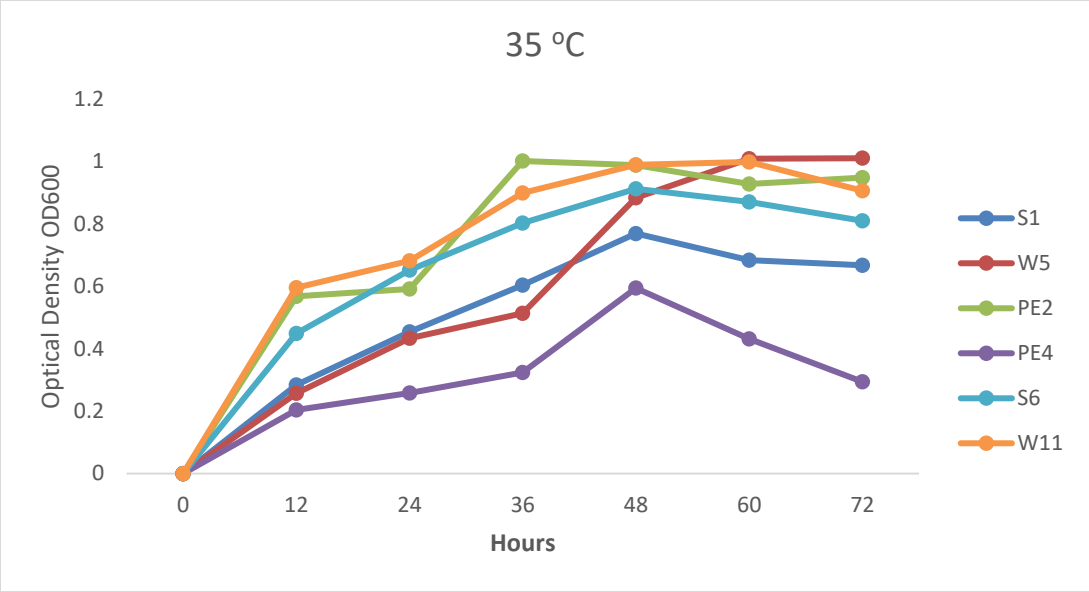

Figure. 6: Growth profile of the test isolates at 35 °C within 72 hours

P. aeruginosa PE4 had the highest optical density reading and best growth pattern amongst the isolates at 45°C although the optical density was not as high as what was observed at 35 °C and 30 °C thus suggesting that although some of the isolates could grow at 45 °C, they would prefer the lower temperatures for degradation (Fig. 7).

All the isolates were able to grow at this temperature though not optimally as compared to the lower temperature *A. xylosoxidans* w11, had the highest OD600 value after 24 h, *P. aeruginosa* w5 after 36 h while *P. aeruginosa* PE4 had the highest OD value after 48 h. Most of the isolates could grow optimally between 30 and 40 °C however *P. aeruginosa* PE4 had its highest growth observed at 45 °C. The effect of increasing concentration of LDPE on the growth patterns of the test isolates was studied and it was observed that all the isolates were able to utilize the polyethylene as carbon source as shown in Figures 9-12 respectively. Isolate *S. maltophilia* S6 grew optimally at this concentration (0.999) at 36 h folowed by *P. aeruginosa* W5 (0.893). most of the isolates peaked at 36 h except *P. aeruginosa* PE4 which peaked at 60 h. Most of the isolates peaked in density between 36 and 48 h of incubation. They all utilized the substrate at this concentration thus indicating they can grow on LDPE as sole carbon source with *L. xylanilyticus* PE2 growing best at this concentration of LDPE.

Microorganisms grow optimally at certain ranges of various conditions which may vary for different microbes (Lodhi *et al*., 2011). In this study, the conditions that were investigated were temperature, pH and LDPE concentration. Microbial activities are controlled by enzymes which work optimally at various conditions (Nigam, 2013). Like other proteins, enzymes in bacteria are affected by extreme pH such as strong acid or alkali. Determination of bacterial growth rate was studied using the spectrophotometric optical density readings at 600nm. optimal growth parameters evaluated for the study bacteria were: temperature range 30-50˚C, pH range 6-9 and LDPE concentration range 1-5%. *Lysinibacillus xylanilyticus* strain BN-13, *Achromobacter xylosoxidans* strain YEB RH5 and *Stenotrophomonas maltophilia* strain T7D7 grew optimally at temperature 37 °C while *Pseudomonas aeruginosa* RI-1, *Pseudomonas aeruginosa* strain V1 and *Pseudomonas aeruginosa* strain PW5a grew optimally at 45 °C. The optimal condition was reached at 24 h. Also the optimal pH was between pH 6 and pH 7 in all the isolates. Esmaeili *et al.* (2013) in their study noted that the suitable pH for LDPE film degradation in soil by *Lysinibacillus xylanilyticus* was 7.5. This was also similar to the results of Veethahavya *et al*. (2016) who found out that the degrading species they recovered including some *Pseudomonas* species were able to biodegrade LDPE well at pH 7.5. Kashimozhi and Perinbam

(2010) however observed that the optimum pH and temperature range for esterase production by *Pseudomonas* sp. to be between 8.0-8.5 and 37-42 °C, respectively, a finding similar to what was observed in this study.

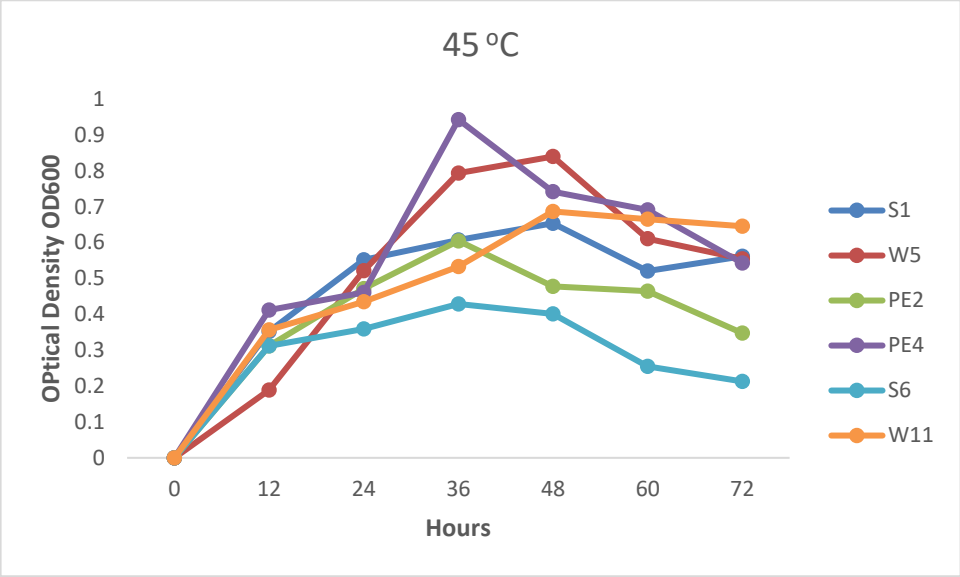

Figure 7: Growth profile of the test isolates at 45 °C within 72 hours

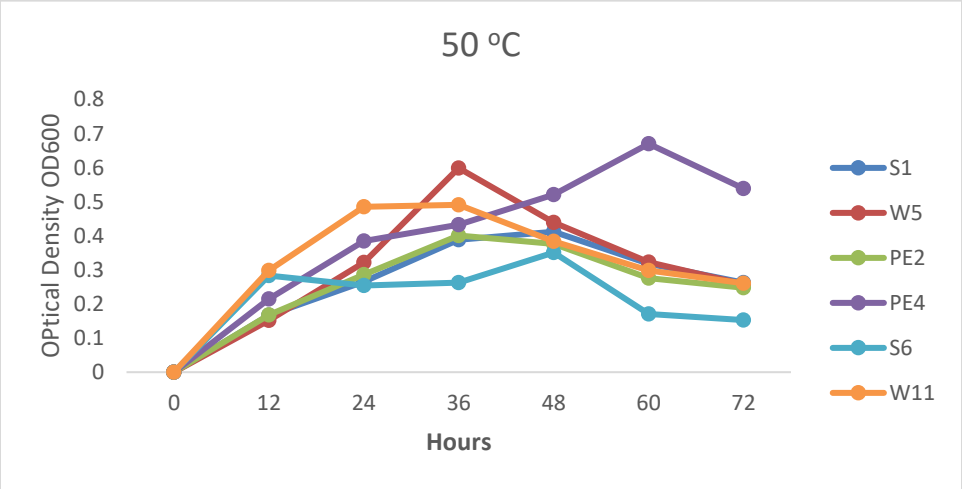

Figure 8: Growth profile of the test isolates at 50 °C within 72 hours

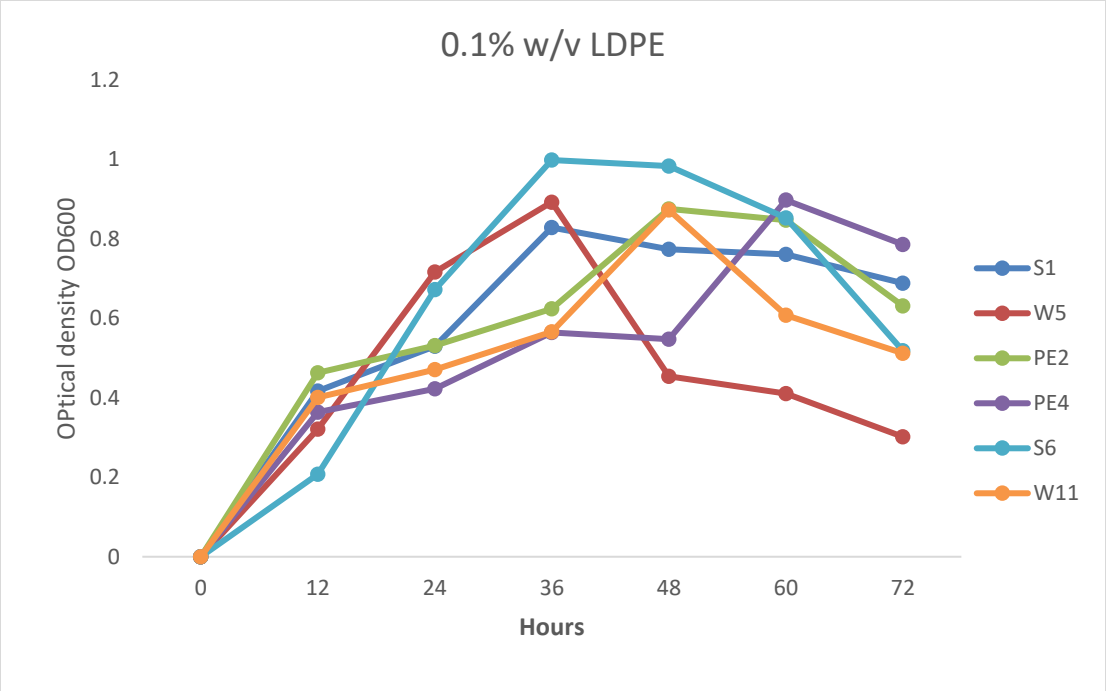

Figure 9: Growth profile of the test isolates at 0.1% (w/v) LDPE within 72 hours

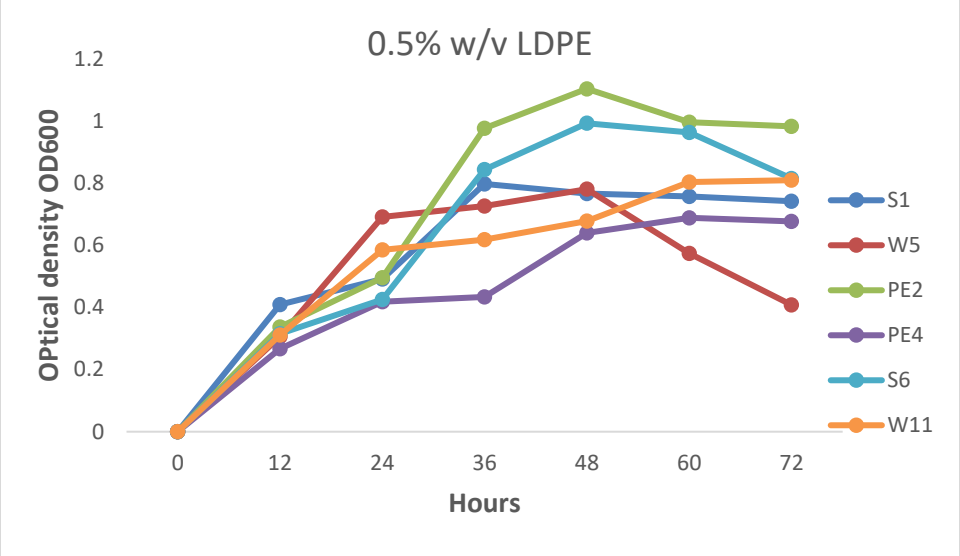

Figure. 10: Growth profile of the test isolates at 0.5 % (w/v) LDPE within 72 hours

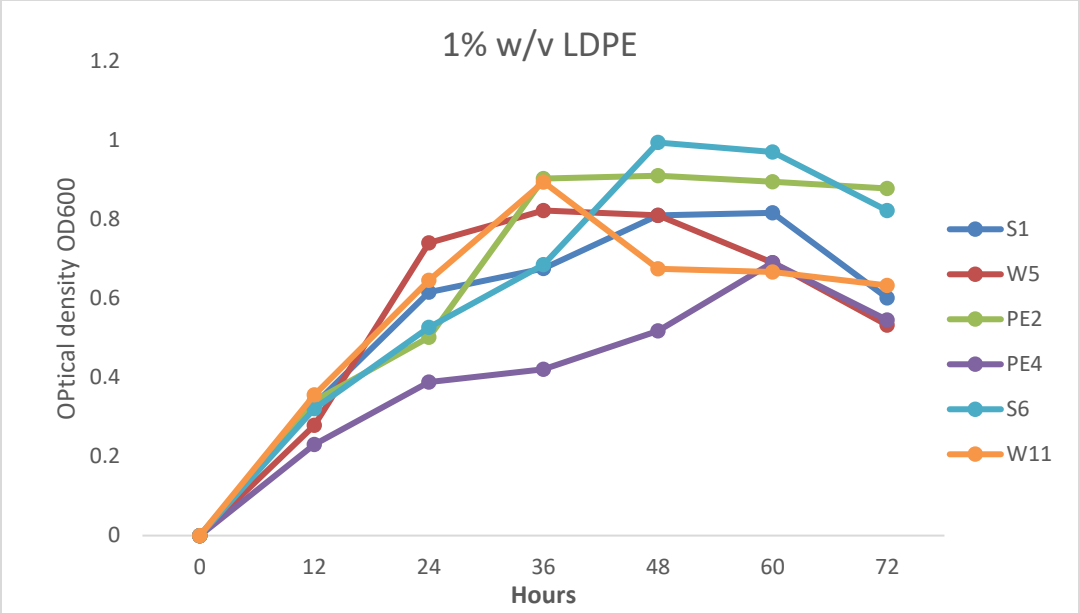

Figure 11: Growth profile of the test isolates at 1 % (w/v) LDPE within 72 hours

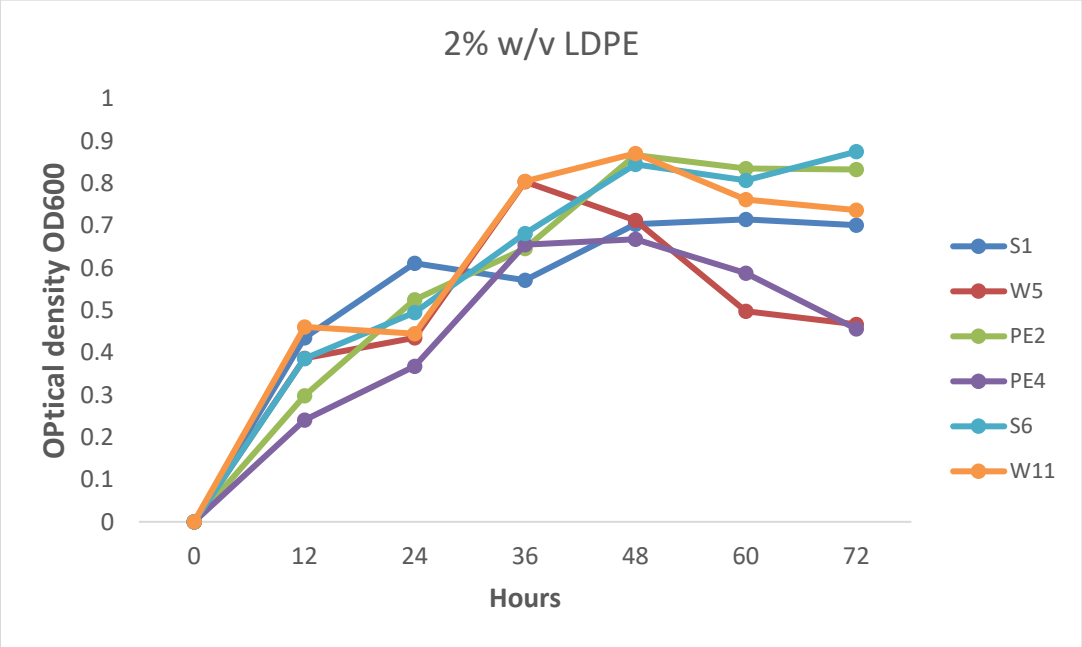

Figure. 12: Growth profile of the test isolates at 2 % (w/v) LDPE within 72 hours

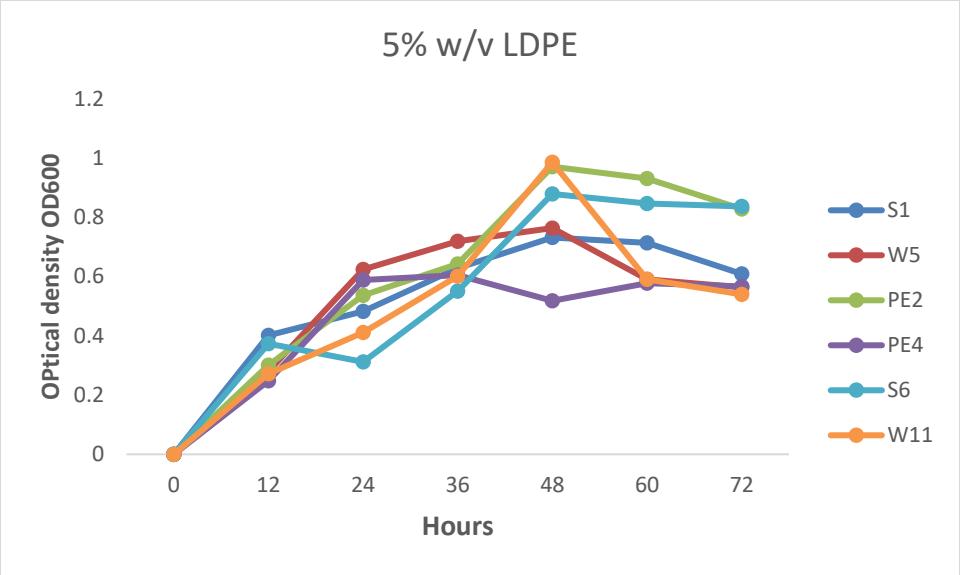

Figure. 13: Growth profile of the test isolates at 5 % (w/v) LDPE within 72 hours

Also, Skariyachan *et al*., (2015) revealed that *Pseudomonas* sp. was able to utilize plastic as a sole source of carbon and showed 20–50% weight reduction over 120 days and this rate was optimally achieved at 37 °C in pH 9.0 while Al-Jailawi *et al*., (2015) also pointed out that the optimum temperature for growth of *Pseudomonas putida* S3A, a poly-ethene degrader was 37 °C which was a quite lower than what was observed in this study. Kumari *et al.* (2009) however noted that high temperature (40 °C) was found to enhance degradation rate of PE more effectively by 24-28% compared to temperature of 30 °C (18-21%).

In general enzymes as catalysts have certain temperature that it withstands and normally at optimum temperature will catalyze reaction most rapidly. Microbial enzymes likewise function best at optimum temperature which related to an organism's normal environment. The temperature range where optimum growth was observed in the isolates recovered and screened still fall within the optimum range for mesophilic organisms (20-40 °C) (Nzila, 2018).

The isolates in this study were able to utilize up to 5% w/v of polyethylene although at lower rates as measured by spectrophotometric readings. The optimal LDPE powder concentrations for *Pseudomonas aeruginosa* strain PW5a was between 1% and 2 % after 24 h, 1% for *Pseudomonas aeruginosa* strain V1 and *Achromobacter xylosoxidans* strain YEB RH5 while *Stenotrophomonas maltophilia* strain T7D7 optimally utilized LDPE at 0.1 %. Several investigations have reported degradation at low concentrations of LDPE. Yoon *et al*. (2012) studied LDPE degradation by *Pseudomonas* at 0.5% w/v while Al-Saraf and Al-Jailawi (2013) also pointed out that *P. putida* S3A was able to degrade nylon 6 at an optimized concentration of 0.1 %.

CONCLUSION

This preliminary study revealed that the bacteria isolates recovered from several dumpsites were found to be capable of utilizing Low-density polyethylene and as such are available for further investigation into the mitigation of pollution caused by indiscriminate disposal of water sachet bags into the environment, a common feature in most Nigerian cities. The isolates were also found to have varying optimal conditions for growth but could tolerate high concentrations of the polymer hence they should be suitable for on-site remediation. Further investigation into the mechanism of degradation, the roles of enzymes and plasmids in the degradation of LDPE should be elucidated as well as strain improvement methods that will enhance the ability of these pristine isolates.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

ACKNOWLEGEMENT

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AUTHOR CONTRIBUTIONS

OOA and OJO designed the research work and proofread the manuscript, OOA wrote the manuscript too. OTO assisted with the editing of the manuscript and proofreading. DA, AA and AO carried out the experiments. All authors read and approved the final version.

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