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Growth Pattern of *Pseudomonas aeruginosa* in different wastewater media

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***Abstract*—** *Restaurant wastewater are a major cause of environmental pollution with the indiscriminate release of the effluent to the environment resulting in blocking of drainages and eutrophication thereby causing serious threat to aquatic life. The growth pattern of a dietary rich oily wastewater degrading bacteria strain, Pseudomonas aeruginosa in different wastewater media composed to mimic the possible constituents of restaurant wastewater was investigated in this study. There was noticeable microbial growth in the synthetic and domestic wastewaters after 48h while the detergent wastewater and heated oil-detergent wastewater did not support the strain's growth. The decrease in the fat content with a corresponding increase in the ash content after 120h was due to the test strain's metabolic activity, which is slightly higher in domestic wastewater than the heated oil-detergent solution. Also, the potassium (K^+), Mg^{2+}, Fe^{2+} and Ca^{2+} contents increased within the same period in both media except in the domestic wastewater where the Ca^{2+} content reduced. Protease enzyme activity (46.440mM/min) was considerably higher in the domestic wastewater than lipase (3.322mM/min) and amylase activity (14.244mM/min) after 72 hours of incubation. The pristine genetic properties of Pseudomonas aeruginosa altered when cultured in various wastewaters probably due to variation in the composition of the substrates.*

***Keywords*— restaurant, domestic wastewater, Pseudomonas aeruginosa, synthetic wastewater, lipase, 16s rRNA sequencing.**

I. INTRODUCTION

Restaurant wastewater usually results from water that has been used for cleaning food products such as meats and vegetables, washing dishes and cooking utensils, or cleaning the floor of restaurants (Zulaikha et al., 2014). The effluent comprises leftovers of food, soup, detergents, fats, oil and grease which generates unpleasant odor when released into drains without proper pre-treatment processes (Xue et al., 2016).

The domestic wastewater is composed of proteins and carbohydrates, smaller amounts of lipids (Odeyemi et al., 2011), anthropogenic organic chemicals and some microbial pathogens (Tchobanoglous et al., 1991). The lipids (oils) that are released into the environment have been reported to be responsible for the clogging of sewer networks and unsettling the balance of water in the treatment plants and implicated as environmental pollutants (Saifudin et al., 2006; Xue et al., 2016).

Several authors have reported different wastewater contaminants in soil and aquatic environments in different parts of Nigeria (Nwachukwu et al., 2001; Adeyemo, 2003; Akpan, 2004; Efe, 2005; Zulaikha et al., 2014). In Nigeria, domestic wastewater especially from bukateria undergoes little treatment (Adeyemo, 2003). It is usually disposed on open lands or drainages where it empties into water bodies causing eutrophication (Akpan, 2004). Studies have shown that the chemical oxygen demand (COD) concentration, animal and vegetable oils and suspended solids are 16 times higher in restaurants wastewaters than domestic wastewater (Fadile et al., 2011; Kshirsagar, 2013; Xue et al., 2016) Leaching into groundwater is also a major part of environmental concern, especially due to the recalcitrant nature of some contaminants (Lapygina et al., 2002).

Biodegradation of fats and oils in wastewater has a potential role in pollution control employing the metabolic capacity and diversity of microorganisms to breakdown these complex substrates and reduce its toxicity (Nelson, 2009). Bioremediation offers a clean and cheaper alternative to conventional clean-up methods (Zhu et al., 2001; Xia et al. 2006; Calvo et al., 2008).

Lipase producing bacteria have been isolated from oil contaminated sites and exploited for their ability to remediate domestic wastewater polluted environments (Odeyemi and Aderiye, 2011). The bioremediation process is typically enzymatic, where amylases, oxygenases and lipases are secreted into the medium which facilitate the breakdown. Predominant among degraders of complex organic substrates including lipids and oils is the genus Pseudomonas which has been investigated for its ability for bioremediation (O'Mahony, 2006; Odeyemi and Aderiye, 2011). With domestic oil waste constituting a big threat to clean and hygienic environment in Nigeria, there is need to further exploit the potential of Pseudomonas aeruginosa previously

associated with the degradation of oil rich waste water (Odeyemi et al., 2014).

Therefore, this study was aimed at observing the growth pattern of P. aeruginosa in different wastewater media and monitoring the effect of some growth conditions on the genetic makeup of the microbe.

II. HEADINGS

Collection of domestic oil wastewater

Fresh wastewater samples containing a mixture of waste food debris, detergents and domestic oils were collected into sterile 2.5L sampling bottles from Falegan restaurant situated along Ekiti State Secretariat road, Ado-Ekiti, Ekiti State of Nigeria. The restaurant opens between 8am and 4pm daily and samples were collected at 2½h intervals (10:30am and 1pm and 3:30pm). The samples were transported in cold storage to the Microbiology laboratory of the Ekiti State University, Ado-Ekiti for further analyses.

Source of *Pseudomonas aeruginosa*

P. aeruginosa was obtained from the work of Odeyemi *et al*. (2013), previously isolated from domestic oil-rich wastewater sample collected from the same source and purified by sub-culturing on *Pseudomonas* agar (HiMedia, India).

Preparation and analyses of wastewater media

The growth culture media for *P. aeruginosa* include restaurant wastewater, palm oil/detergent solution, detergent solution and synthetic wastewater. Two hundred millilitre (200mL) of fresh restaurant wastewater was homogenized and sieved before autoclaving to prepare sterile wastewater media. For preparation of palm oil/detergent solution, 20mL of palm oil was heated for 15minutes in a ventilated oven at 100ºC, allowed to cool and 20g of detergent (OMO Unilever PLC.) was mixed with the heated palm oil and then dissolved in 200mL of distilled water in 1000mL Erlenmeyer flask prior to autoclaving. Detergent solution was prepared by dissolving 20g of the detergent in 100mL of distilled water, made up to 200mL in 250mL Erlenmeyer flask before autoclaving. Synthetic wastewater was formulated using a modification of the method of Foglar (2004) by dissolving 1g of sodium acetate, 0.1g $MgSO_4.7H_2O$, 0.1 g KNO_3, 0.1g KH_2PO_4, 0.2g meat extract and 0.1g NaCl in 200ml of distilled water prior to autoclaving. The media were then inoculated with the test strain.

Twenty millilitre (20mL) of standard inoculum was transferred into each of the four media, incubated at 30ºC and monitored spectrophotometrically for 5days at 24h intervals. Also, the microbial load, proximate components (moisture, ash, crude protein, crude fibre, fat and total carbohydrate contents) (AOAC, 2005) and mineral analyses (Fe^{2+}, K^+, Ca^{2+}, Mg^{2+}) (AOAC, 2005) were carried out during the same period.

Analysis and Production of Enzymes:

The wastewater media was prepared for enzyme production, with 100mL of each wastewater inoculated into five different 250mL flasks, incubated in an orbital shaker (Stuart shaker) at 150r.p.m and 30ºC. Samples were drawn from each of the flasks at 6h intervals for a period of 48h and each sample was centrifuged at 5000 rpm for 30 min at 4ºC. Cell free supernatant corresponding to each growth phase was used for the assay of crude enzyme (An *et al*., 1994). The activity of protease and amylase was determined according to the methods of An *et al.* (1994) and Berfield *et al*. (1995) respectively while lipolytic activity was determined by colorimetric method of Lotrakul and Dharmsthiti (1997).

Molecular characterization of *P. aeruginosa* (CP004061.1) grown in different wastewater media

Genomic DNA was isolated according to the method of Sambrook *et al*. (1989). The appropriate primer used for the work was designed by Inqaba Biotechnical Industries (South Africa). DNA isolation, PCR and sequencing were carried out at the International Institute for Tropical Agriculture (IITA, Ibadan, Nigeria). The sequences were analyzed using the BLAST (Basic Local Alignment Search Tool) bioinformatics program on the NCBI (National Center for Biotechnology Information) website.

III. RESULTS AND DISCUSSION

The growth and the degrading potential of Pseudomonas aeruginosa (CP004061.1) was monitored in different wastewater media for about 120h. This bacterial strain recovered from restaurant wastewater, after dish washing (with soap solution, 5g/100ml detergent in water), first and second rinsing of the dishes, and run-off into open sewers along the drainage was capable of growing well in dietary oil (Odeyemi et al., 2014).

The synthetic wastewater and detergent solution were used as experimental controls. It was observed that the synthetic media was best suited for the growth of the organism after 144h when compared to other media. There was about 553% increase in microbial growth than when cultured in the domestic wastewater. Meanwhile, there was no visible growth in either the detergent wastewater or the heated detergent wastewater (Figure 1). However, Odeyemi et al. (2013) reported that this Pseudomonas strain was capable of growing in detergent concentration as high as 5g/100ml which is lower compared to the 20g/100ml used in this study. Ambilly et al. (2014) reported the growth of Pseudomonas

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aeruginosa (MTCC 10311) in 96% of Sodium dodecyl sulfate after 48h incubation in detergent contaminated soil.

The various mineral salts present in the synthetic wastewater might have influenced the high count of the Pseudomonas sp. (Usharani et al., 2011). Odeyemi et al. (2013) also reported an appreciable increase in the weight of Pseudomonas aeruginosa with 66.7% weight gain after 5days incubation in domestic oil wastewater. The increase in the weight may be attributed to the ability of the strain to produce lipase which is responsible for dietary oil degradation.

The nutritive quality of any medium is better evaluated by assessing its proximate composition which provides information on the basic chemical components of the medium and the type of growth that takes place within such medium (Adeolu and Enesi, 2013). The nutritive qualities of the fresh heated oil/ detergent wastewater and domestic wastewater were examined. Similarly, these qualities were investigated after 144h incubation.

The proximate components of the culture media revealed that the ash content increased in both the synthetic and domestic waste water after 120h (0.16% to 0.24% and 0.21% to 0.26% respectively). However, the crude protein was found to decrease in the synthetic wastewater (0.45-0.37%) but increased in the domestic wastewater (0.38-0.40%). Also, there was reduction in the fat content of both media (0.11 to 0.07% and 0.10 to 0.06% respectively). There was however no significant difference in the moisture content in the synthetic wastewater and the domestic wastewater (0.02%) (**Table 1**). This report is similar to that of Odeyemi *et al*. (2014) where a low value of 3.2% crude protein, 3.1% carbohydrate and 1.2% fat was reported in the domestic waste water. The low organic matter observed might be due to variation in the organic components in the wastewater.

Ash content provides an estimate of the inorganic quality of a substrate (Adebowale and Bayer, 2002; Adeolu and Enesi, 2013). There was notable increase in the ash content of the two wastewater media after 120h incubation as shown in Table 1. The various activities of *Pseudomonas aeruginosa* on the organic component of the wastewater are likely responsible for the level of ash detected in the wastewater medium. Tchobanoglous *et al*. (1991) indicated that the organic matter in wastewater is mostly composed of proteins and carbohydrates and smaller amounts of lipids. The rate of microbial activity (fermentation) on the fibre content of the wastewater sample may have contributed to the slight increase in the carbohydrate and ash contents. This study also revealed the low crude protein content of the domestic wastewater and the heated oil/detergent wastewater during incubation. This observation is contrary to the findings of Effiong *et al*. (2009), who reported high values (between 26.2% and 36.8%) for ash in wastewater after treatment. According to Hanif *et al*. (2006), these amounts may be attributed to high nitrogen contents in those wastewaters with vegetables.

The mineral concentrations of both the heated oil/domestic and domestic oil wastes also increased after 120h. There was increase in the potassium (K^+) 7.20mg/L - 7.50mg/L (4.2%), Mg^{2+} (25.00mg/L - 28.10mg/L) (12.4%), Ca^{2+} (11.30mg/L - 11.35mg/L) (0.4%) and Fe^{2+} (0.01mg/L - 0.03mg/L) (200%) in the heated oil/detergent wastewater after 120h. Similarly, there was slight increase in the potassium content (6.50mg/L-7.00mg/L) (7.7%), Mg^{2+} (27.01mg/L-29.30mg/L) (8.5%), Fe^{2+} (0.01mg/L-0.03mg/L) (200%) in the domestic oil wastewater. Meanwhile, calcium reduced significantly (99.75%) from (12.00mg/L-0.03mg/L). Phosphorus was not detected in both media. The increase in the mineral contents of the waste media may be responsible for the high ash content observed after 120h in the waste media (**Table 2**).

Similar report was obtained by Odeyemi *et al.* (2013) where the mineral content of the domestic wastewater rose on the ninth day of degradation of the samples. Despite the low mineral contents, the media were still rich enough to support microbial growth due to low nutrient demand of microorganisms.

The pH and temperature of the wastewater culture media were also monitored. There was no significant change in the temperature of the media, which averaged 27.25ºC. However, there was a significant drop in the pH of the media except the synthetic media. Synthetic wastewater showed a pH range of 8.80-10.24, while the pH values in the domestic wastewater varied between 9.06 and 10.5; detergent wastewater varied between 9.85 and 11.31 and the heated oil wastewater between 8.75 and 11.15 (**Fig. 2)**.

The various biochemical reactions that took place within the medium depend on the enzymes which facilitate the processes (Kirk *et al*., 2005). The activity of the enzymes (amylase, lipase and protease) secreted by *Pseudomonas aeruginosa* into the growth media during incubation was examined at different time intervals. The activities of these enzymes increased during the incubation of domestic oil waste water. It was however observed that lipase activity (0.333, 2.778 and 3.322 mM/min) in the domestic wastewater was low when compared to amylase and protease activities. The results obtained for lipase activity were similar to those of Orapin *et al*. (2002) who reported higher fat (73%) and oil (88%) degradation in the wastewater treated with a pure culture of *Pseudomonas aeruginosa* after 7days.

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Amylase activity in the domestic oil waste water was 378.4% greater (14.24mM/min) after 120h storage than its initial value with protease activity exhibiting a tremendous influence on biodegradation of the waste water (46.44mM/min after 120h). Meanwhile, Laura *et al*. (2013) reported some toxicity impacts of sewage effluent on the amylase activity of pigeon pea (*Cajanus cajan* L.) in 50% and 100% sewage effluent for 8days. Interestingly, a corresponding increase in the protein concentration in the waste water was recorded after 120h (41.82mg/ml). The protease activity in the domestic oil wastewater was similar to the report of Irina and Yana (2010) who observed an accelerated increase in protein hydrolysis and the rate of protein removal from 10% to an average of 74% within 72h in kitchen wastewater treated with proteolytic bacteria which without the organism would have taken 135h. The high production of protease can be attributed to the meat, fish and other proteinaceous foods present in the domestic wastewater (**Table 3**).

The recovered genomic DNA extracted from the strain grown at optimal conditions in the different wastewater samples was studied. The entire 1.5 kb 16S rRNA gene was amplified (Figure 2) and sequenced. A search of the Genbank using the BLAST tool showed that the pristine strain cultured in the heated oil wastewater and in the domestic wastewater, at 98% identity value and 0.0 E-value was similar to *Pseudomonas* sp. (R3. 1B) and *Pseudomonas* sp. strain (PO150) respectively. Also, it showed 98% identity value and 0.0 E-value similarity and 97% identity value and 0.0 E-value similarity with *Pseudomonas* sp. strain (YR20) and *Pseudomonas* sp. strain (PO150) respectively, after BLAST.

Sequences reported here are available at the Genbank Nucleotide Sequence Database under the accession numbers KM058081.1, HG93439.1, HM224401.1 and KC433649.1 respectively.

The 16s rRNA sequencing analysis revealed that the *Pseudomonas* sp. strain (PO150) was present in both the domestic wastewater and synthetic wastewater and these can be named *Pseudomonas* sp. strain (PO150a) and *Pseudomonas* sp. strain (PO150b) respectively to distinguish them. *Pseudomonas* sp. (PO150b) had the highest degrading activity and this might be attributed to the composition of the synthetic wastewater (Figure 3). This is in agreement with high cell concentration observed in the synthetic medium after 120hours incubation.

The PCR reveals that there has been genetic modification in the pristine *Pseudomonas* strain when grown in domestic wastewaster and heated oil/ detergent media (Fig. 3). The molecular weight of the pristine isolate and synthetic media DNA sequence was around 0.75kbp compared to that from the same isolate grown in domestic wastewater and heated oil/ detergent media which was about 1.5kbp thus suggesting that the conditions present in the culture media of the domestic wastewater and heated oil/ detergent media may have affected the genome sequence. The nucleotide sequence tree relates the similarity of all the isolates; *Pseudomonas* sp. strain (Heated), *Pseudomonas* sp. strain (Domestic), *Pseudomonas* sp. strain (Pristine) *Pseudomonas* sp. strain (Synthetic) grown under the same condition in wastewater media.

IV. FIGURES AND TABLES

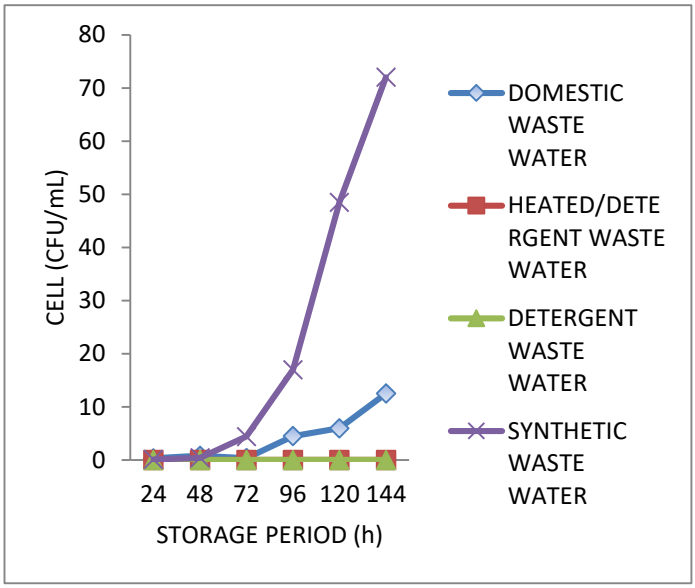
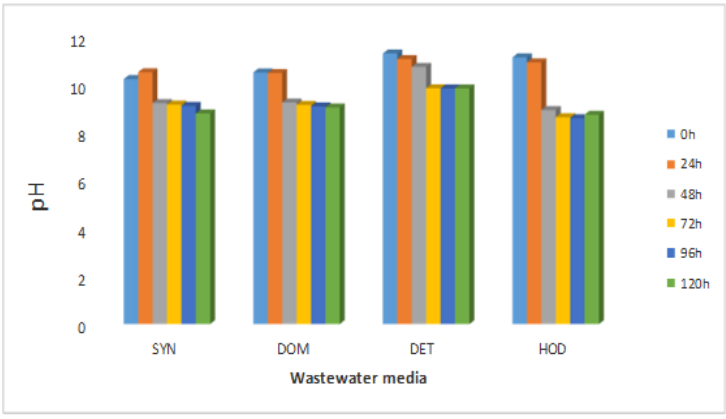


Figure 1: Growth of *Pseudomonas aeruginosa* in the different media



Legend: SYN (synthetic media), DOM (domestic oil wastewater media), DET (detergent wastewater media), HOD (heated oil detergent wastewater media)

Fig.2: The pH values of the wastewater media

http://dx.doi.org/10.22161/ijeab/2.5.44 *ISSN: 2456-1878*

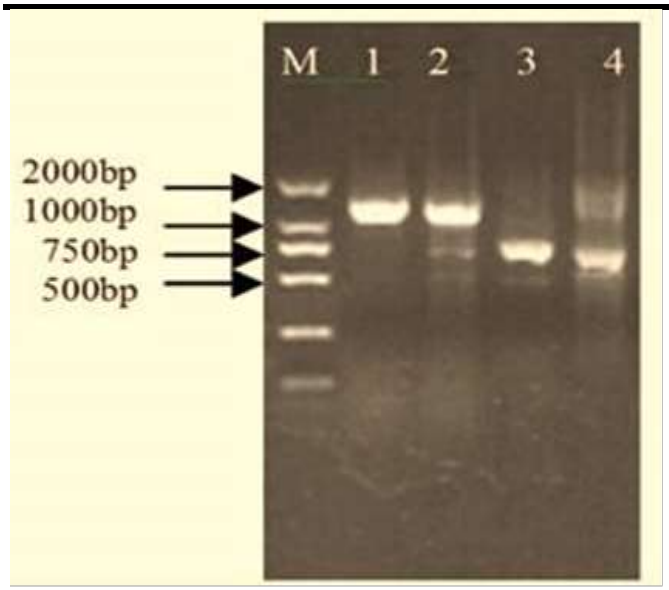


Fig.3: PCR results of the amplified genomic DNA of Pseudomonas aeruginosa.

Keys: M-DNA ladder: 2000bp, 1- *Pseudomonas* sp. (Domestic), 2- *Pseudomonas* sp. (Heated), 3- *Pseudomonas* sp. (Pristine) and 4- *Pseudomonas* sp. (Synthetic).

Fig.4: Nucleotide sequence tree of the Pseudomonas aeruginosa

Table 1: Proximate component of wastewater

Wastewater Sample	Proximate component (%)				
	Ash	Crude Protein	Carbohydrate	Fat	Moisture
Fresh Wastewater					
Heated oil and detergent wastewater	0.16±006^{c}	0.45±010^{a}	1.05±010^{a}	0.11±010^{a}	98.24±010^{c}
Domestic oil-rich wastewater	0.21±010^{b}	0.38±010^{bc}	0.12±010^{d}	0.10±010^{a}	99.21±010^{a}
After 120h Incubation					
Heated oil and detergent wastewater	0.24±010^{a}	0.37±010^{c}	0.60±10^{b}	0.07±010^{b}	98.71±010^{b}
Domestic oil-rich wastewater	0.26±010^{a}	0.40±010^{b}	0.40±10^{c}	0.06±010^{b}	98.89±015^{b}

All data were mean ± standard deviation of triplicate determinations

V. CONCLUSION

Microbial communities are prone to adapt to a substrate when it is a regular contaminant, such is the situation for the Pseudomonas sp. inoculated into the wastewater media. The genetic sequence of the original inoculum changed when grown in the different wastewater media which might be due to the variations in the media composition since the organism was exposed to the same environmental conditions. However, the nature and mechanism of the genetic changes of this organism in the different growth media is still open to investigation.

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Table 2: Mineral component of fresh growth media and after 120h of incubation

Sample	Mineral content (mg/L)			
	Potassium	Magnesium	Calcium	Iron
Fresh medium				
Heated oil and detergent wastewater	7.20±0.06^{b}	25.00±0.00^{d}	11.30±0.00^{a}	0.01±0.00^{a}
Domestic oil-rich wastewater	6.50±0.00^{d}	27.01±0.06^{c}	2.00±0.00^{b}	0.01±0.00^{a}
After 120hours Incubation				
Heated oil and detergent wastewater	7.50±0.00^{a}	28.10±0.00^{b}	11.55±0.10^{a}	0.03±0.10^{a}
Domestic oil-rich wastewater	7.00±0.00^{c}	29.30±0.00^{a}	0.03±0.10^{c}	0.03±0.10^{a}

All data were mean ± standard deviation of triplicate determinations

Table 3: Activity of enzymes secreted by *Pseudomonas aeruginosa* in domestic oil rich wastewater

Incubation period	Enzyme activities (mM/min)			Protein concentration (mg/mL)
	Amylase	Lipase	Protease	
0h	3.764	0.333	0.485	4.318
72h	10.258	2.778	24.515	17.500
120h	14.244	3.322	46.440	41.818

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