HUMIC SUBSTANCE UTILIZING POTENTIAL OF BACTERIA ISOLATED FROM THE "BLACKWATER" ECOSYSTEM OF ENIONG RIVER, OKOPEDI-ITU, NIGERIA

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ABSTRACT

The effect of humic substance (HS) on the growth profile of bacteria isolated from the "blackwater" or humic freshwater system of Eniong River in the Niger Delta Region of Nigeria was investigated using standard analytical procedures to determine their degradability in nature. The study revealed that Eniong River harbors diverse species of bacteria whose occurrence and survivability in humic substance (HS) simulated water medium varied with the isolates. Of the 15 bacterial species isolated from the ecosystem, *Pseudomonas aeruginosa, Bacillus subtilis* and *Micrococcus* sp were the most abundant bacterial species in both water and sediment samples and best survivors. Based on the analysis, *P. aeruginosa* exhibited the best survival potentials in the humic water within 24 hours as it had the highest number of generations (n = 1.26316) within the shortest time (Gt = 18.99996 hours) followed by *Micrococcus* sp (n = 1.0001; Gt = 23.99761 hours). The isolates also exhibited varying potentials to utilize various concentrations of HS extracted from the sediment as a sole source of carbon by their increase in cell density overtime. Our findings have shown that *Bacillus subtilis, Pseudomonas aeruginosa* and *Micrococcus* isolated from the blackwater system of Eniong River in Nigeria do not only survive the inhibitory stress of humic substance, they do so with high levels of viable cells generate-ability and within short periods. These are attributes which can be exploited for the degradation of recalcitrant humic wastes and for the bioconversion of lignolytic wastes into useful products

INTRODUCTION

Humic substances are groups of organic compounds occurring in the soil, marine and fresh waters and their sediments as well as in peat-bogs and in ground water (Donderski and Burkowska, 2000; Hou *et al.,* 2014). They are composed of polyelectrolytes-spherocolloids that are formed as a result of very complex processes of biochemical condensation and polymerization of decomposition products of plant, animal and microorganism metabolites (Gonet, 1993). In freshwater ecosystems, humic substances (HS) are complex organic molecules that make up most (50-80%) of the dissolved organic matter (DOM) (Wetzel, 2001; Hou *et al*., 2014). Similarly, Moran and Hodson (1990) reported that humic substances constitute 25-26% of dissolved organic carbon in surface waters, and up to 90% in swamps and peat-bogs. In these environments they have their own properties which result from the way they are formed, their chemical structure, and the role they play. Humic substances increase the growth rate of various forms of beneficial microorganisms by stimulating enzymes activities (Pouneva, 2005, Burkowska and Donderski, 2007), acting as electron shuttle for anaerobes (Finneran *et al.* 2002) and complexing and delivering trace elements like iron to microbial cell surfaces (Chen and Wang, 2008).

In the present study, humic substances are not viewed as pollutants *sensu stricto*, rather as a chemical species whose presence in the environment may antagonise or potentiate effects of other toxic substances, since they are naturally present in virtually every soil type and sediment that contains organic matter. Hydrophobic Organic Contaminant (HOCs) tends to associate to

dissolved macro-molecules (such as humic acid) present in the environmental matrix. This association will in some exposure scenarios lead to a decrease in the freely dissolved concentration and then likely to lower biodegradation rates, because degrading bacterial cells usually take up dissolve HOC by diffusion from the surrounding aqueous phase (Wick *et al.,* 2001 and Yang *et al.,* 2009). However, in other situations, the presence of humic substance can lead to an enhancement in the biodegradation rate of these HOCs (Ortega-Calvo and Saiz-Jimenez, 1998; Haftka *et al.,* 2008 and Smith *et al.,* 2009).

Indeed, the addition of humic acid is currently considered as a valid biostimulation strategy to speed up bioremediation (Plaza *et al.,* 2009; Yang *et al.,* 2011). Various forms of humic substances can accelerate the biodegradation of PAHs, increasing the apparent solubility and enhancing the diffusive mass transfer of these contaminants, thus promoting their availability to microorganisms (Smith *et al,* 2009).The molecular form of humic acid has been described as a micellar microstructure similar to surfactants, which can incorporate dissolved PAHs (Engebreston and Vonwandruszka, 1994)

However, humic substances are known to be recalcitrant in nature due to its stability which is based on two mechanisms: its resistance to microbial attack due to its aromatic cores and the reaction of the soil humic substance to soil minerals surfaces, which reduces microbial degradation. Their stability is responsible for the intense coloration due to humic substances and possibly soluble iron that results to it "blackwater" status (Essien *et al*. 2015; Abraham and Essien 2016). Despite the fact that humic substances have been focused upon in many research studies and investigations, knowledge of the role played by humic substance on the growth and survival of benthic bacterial species and their degradability in "blackwater" ecosystem is still insufficient. This work is therefore designed to isolate bacteria that can survive and grow under HS with high potential to utilize the substance as the sole carbon and nitrogen source for growth.

MATERIALS AND METHODS

Study Area and Sample Collection

The study area is a humic freshwater ecosystem of Eniong River which is a tributary of the middle course of the Cross River located in the South-eastern coast of the Niger Delta, Nigeria (Figure 1). Water and sediment samples were collected from three locations (Station A – Lat. $05^0\,15^1\,56.0^{11}$ N and Long. $007^0\,56^1\,59.1^{11}$E, Station B - Lat. $05^0\,13^1\,50.9^{11}$N and Long. $007^0\,57^1\,28.7^{11}$ E and Station C - Lat. $05^0\,12^1\,05.4^{11}$ N and Long. $007^0\,58^1\,48.6^{11}$ E.) in the humic freshwater ecosystem of Itu, Nigeria using standard techniques. The samples were transported to the laboratory in an iced- packed chest for analysis

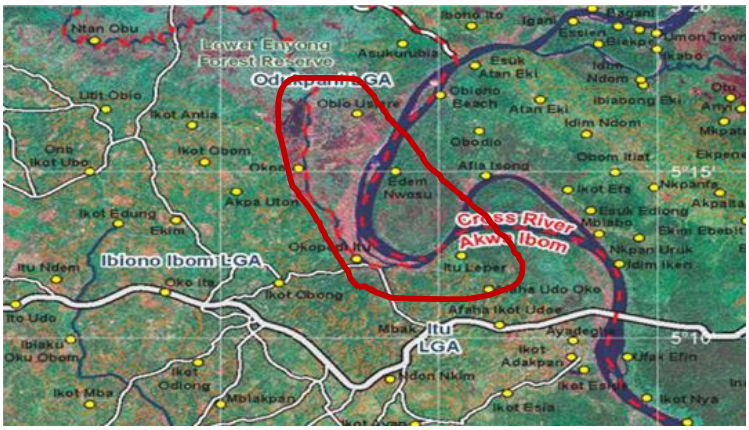

Figure 1: Satellite Image of the Study area – Eniong River showing the sample stations

Isolation and Characterization of Culture-able Bacterial Isolates

Ten-fold serial dilution of the soil and sediment samples was carried out and plated with sterile molten Nutrient agar (NA) using the pour plate technique. The plates were incubated for 24 hours at 28^{O}C after which discrete colonies on the plate were sub- cultured unto freshly prepared NA medium. The purified isolates were characterized using standard procedures as described by Cowan (1985) and Holt *et al.,* (1994); Collins *et al.,* (2004). The most prevalent bacterial species were selected for further study.

Growth and Survival of Selected Bacterial Species in Humic Freshwater Sample

To determine the growth and survivability of the bacterial isolates in humic freshwater, 1 ml of 24-hour old broth culture of the selected bacterial species were reintroduced into a membrane filtered humic freshwater and incubated at 30^{o}C for 24 hours. Prior to this, the number of colonies in 1 ml of the sample was determined using pour plate technique. After incubation, the number of colonies in 1 ml of the culture was determined again using pour plate technique (Collins *et al.,* 2004). The determinations of the number of colonies were carried out in triplicate and the colonies counted averaged and recorded in colony forming units per milliliter (CFU/ml). The number of cells obtained before and after incubation was used to determine the growth rate (Gr), generation time (gt) and number of generations (N) of the isolates as describe by Akinjogunla, (2016) as follows:

(i) Number of generation (n)

Most bacteria reproduce by binary fission, which results in doubling of the number of viable bacterial cells. Hence, under favorable condition, bacterial population will increase geometrically at a specific time interval. The specific time interval between two subsequent binary fissions is known as generation of doubling time.

$$No \rightarrow 2^1 \rightarrow 2^2 \rightarrow 2^3 \rightarrow\rightarrow 2^n \qquad eqn.1$$

Where No = the initial population number

Thus, the number of cells present in a culture after time "t" (hours) of incubation can be related to the initial cell number as

$$Nt = No \times 2^n \qquad eqn.2$$

Where Nt = number of cell at time 't'

No = initial number of cells, n = the number of generation in time t.

Taking log of both sides of eqn. 2

$$\log Nt = \log No \times \log 2^n \text{ (applying law of logarithm)}$$

$$\log Nt = \log No + n \log 2$$

$$logNt - \log No = n \log 2$$

Making 'n' the subject of the formula, divide both side by log 2

$$n = (\log Nt - \frac{\log No)}{\log 2}$$

Therefore, number of generation (n)

$$n = \frac{\log Nt - \log No}{0.3010}$$

(ii) Growth Rate Constant (k):

During the exponential (or logarithmic growth phase), a bacterial culture mimics a first order chemical reaction i.e. the rate of increase of cells at any particular time is proportional to the number of bacteria present at that time. The constant of proportionality is an index of the rate of growth and is called the exponential growth rate constant 'K' - defined as number of doublings in unit time, and is usually expressed as the number of doubling in an hour.

It is calculated from the following equation:

$$Nt = No \times 2^{Kt} \qquad eqn.3$$

Where Nt = Population at time t

No = Population at time 0, t = time

Taking the logarithms $\log Nt = \log No + Kt \log 2$

Solving the equation for K

$$K = \frac{logNt - \log No}{t \log 2} \qquad eqn.4$$

(iii) Generation time (gt) Gt = 1/k

Where k = growth rate constant

Extraction of Humic Substance (HS) from Sediment Samples

Humic substance was extracted from the sediment's samples using the method described by Martins *et al.,* (2014). Two (2) liters of 0.1 M HCl mixed with 400 g of the sediment sample, shaken for 4 h at room temperature and decanted overnight. The supernatant was then eliminated and the residue neutralized with 2 liters of 0.1 M NaOH under an oxygen free atmosphere. Here the mixture was shaken for 8 hours at room temperature then allowed to settle overnight. The mixture was then decanted and the supernatant acidified with 6 M HCl to pH of1 and re-suspended for 24 h. The mixture was centrifuged at 7500 rpm for 10 min, the solid phase was dissolved by adding 4.4 g KCl and 200 ml 0.1 M KOH. The solution was shaken for a few minutes under an O_2-free atmosphere and centrifuged at high speed to remove suspended solids. Thereafter, the HA in the supernatant was precipitated by addition of 6.0 M HCl and the suspension was stored at -20^{o}C for 24 h. After centrifugation, the precipitated HS was filtered and washed several times with sterile distilled water and kept in a stove at 36^{o}C for 5 days to allow elimination of water.

Humic Substance Utilizing Potentials of Prevalent Bacterial Species

The most abundant and fast-growing bacterial species will be selected to determine their ability to utilize the extracted HS as sole source of carbon and nitrogen for growth. The modified enrichment technique was employed. 1ml broth culture of the selected bacterial species was introduced to liquid medium containing per liter, 6.0g K_2HPO_4, 12g NaCl, 6.0g KH_2PO_4, 2.6g $MgSO_4.7H_2O$, 0.16g $CaCl_2.2H_2O$ and a different concentration (0.25%, 0.125% and 0.05%) of the extracted humic substance as the sole Carbon and Nitrogen source (Fu *et al.,* 2017). The inoculated tubes were incubated at room temperature in an aerobic shaker at 110m/s revolution for a period of 14 days. Inoculated tubes containing un-supplemented MSM were also prepared to serve as controls. The utilization of the humic substance by the various bacterial isolates as a substrate for growth was determined by their ability to grow in the supplemented medium. This was accessed by determining the total viable cell (TVC) counts of the inocula in the various growth medium after every 48 hours using the pour plate technique (Collins *et al.,* 2004).

RESULTS

Diversity of Bacteria in Humic Ecosystem

A total of 15 different bacterial species were isolated and characterized from the samples (Table 1). Of this, the most encountered bacterial species in the water sample were *Enterobacter* sp., *S. aureus, B. subtilis, Pseudomonas aeruginosa* and *Micrococcus* sp while the least encountered isolates were *V. cholerae, Shigella* sp and *B. pseudomalle*i. Similarly, *B. subtilis, P. aeruginosa* and *Micrococcus* sp exhibited the highest occurrence rate in the sediment samples, while *V. cholarae, V. parahaemolyticus, Desulfovibrio* sp., *Desulfuromonas* sp and *B. pseudomallei* were the least encountered bacterial species in the humic sediment.

Growth and survival of bacterial species isolated from humic ecosystem

The growth and survival of the most occurring bacterial isolates (occurrence rate $\geq$ 66.7% in both water and sediment samples) in humic water within 24-hours incubation is presented on Table 3. From the analysis, *P. aeruginosa* exhibited the best survival potentials in the humic water within the study time as it had the highest number of generations (1.26316) within the shortest time (18.99996 hours). On the other hand, *Salmonella* sp had the longest generation time of 85.67272 hours with 0.280136 generations.

Utilization of Humic Substance (HS) by Bacteria Isolated from Humic Sediment

Three bacterial species (*B.* subtilis, *P. aerogenosa* and *Micrococcus* sp.) were further selected for HA utilization test based on their high abundance (100%) in the water and sediment samples and there relatively short generation time.

Table 1: Biochemical Characteristics of the Bacterial Isolates Associated with the Samples

Morphology	Gram Stain	Catalase	Citrate	Coagulase	Indole	Motility	Oxidase	Methyl Red	Voges	Urease	Glucose	Sucrose	Lactose	Mannitol	Maltose	Probable Organism
Comma	-	+	+	-	+	+	+	-	+	-	A	A	-	A	A	*Vibrio cholera*
Comma	-	-	+	-	+	+	+	+	-	-	A	-	-	A	A	*V. parahaemolyticus*
Rod	-	+	-	-	+	+	-	+	-	-	AG	A	AG	AG	AG	*Escherichia coli*
Rod	-	+	+	-	-	+	+	+	-	-	AG	A	A	A	AG	*Salmonella* sp
Rod	-	+	-	-	-	-	-	+	-	-	A	A	A	-	A	*Shigella* sp
Rod	-	+	+	-	-	+	-	-	+	-	AG	AG	AG	AG	AG	*Enterobacter* sp
Cocci	+	+	+	+	-	-	-	-	+	-	AG	A	A	AG	AG	*Staphylococcus aureus*
Rod	-	+	-	-	+	+	-	+	-	+	AG	-	-	-	-	*Proteus vulgaris*
Rod	-	+	+	-	-	+	-	-	+	-	AG	A	A	AG	-	*Bacillus subtilis*
Rod	+	+	+	-	-	+	+	-	+	-	AG	AG	AG	A	-	*Pseudomonas aeruginosa*
Rod	+	+	-	-	-	+	+	-	+	+	AG	A	A	AG	-	*Desulfovibrio* sp
Rod	-	+	+	-	-	+	+	-	+	-	A	A	A	A	A	*Desulfuromonas* sp
Rod	-	-	+	-	-	-	-	-	+	-	AG	A	A	A	AG	*Desulfobacter* sp
Cocci	+	+	+	-	-	-	-	+	+	-	A	A	AG	AG	A	*Micrococcus* sp
Rod	-	+	+	+	+	-	-	+	+	-	AG	A	A	AG	A	*Burkholderia pseudomallei*

The rate of occurrence of the various bacterial isolates in the samples are presented in Table 2. *B. subtilis, Pseudomonas aeruginosa* and *Micrococcus* sp were selected for further study as they were the only bacterial group that were most abundant (100%) in the humic water and sediment samples.

Table 2: Occurrence Rate of the Various Bacterial Species in the Humic Water and Sediment Samples

Isolates	Water Sample (n = 3)	Occurrence Rate (%)	Sediment Sample (n = 3)	Occurrence Rate (%)
Vibrio cholera	+ (1)	33.3	+ (1)	33.3
V. parahaemolyticus	+ (2)	66.7	+(1)	33.3
Escherichia coli	+ (2)	66.7	+(2)	66.7
Salmonella sp	+ (2)	66.7	+ (2)	66.7
Shigella sp	+ (1)	33.3	+(2)	66.7
Enterobacter sp	+ (3)	100	+ (2)	66.7
S. aureus	+ (3)	100	+ (2)	66.7
Proteus vulgaris	+(2)	66.7	+ (2)	66.7
Bacillus subtilis	+(3)	100	+ (3)	100
P. aeruginosa	+(3)	100	+ (3)	100
Desulfovibrio sp	- (0)	0	+ (1)	33.3
Desulfuromonas sp	- (0)	0	+(1)	33.3
Desulfobacter sp	- (0)	0	+ (2)	66.7
Micrococcus sp	+(3)	100	+ (3)	100
B. pseudomallei	+(1)	33.3	+(1)	33.3

Key: + = present; Values in parenthesis are the number of samples

The analysis revealed remarkable variation in species capabilities to utilize various concentration of HA as their sole carbon and nitrogen source for growth. This was reflected in the ability of the selected bacterial species to increase in cell density over time

Table 3: Growth and survival of bacteria isolated from humic freshwater ecosystem

Organism	Nos. of cells x 10^5CFU/ml [Incubation Periods (h)] 0 hours	24 hours	N	K	Gt (h)
Micrococcus sp.	3.2	6.4	1.0001	0.041671	23.99761
P. aeruginosa	2.0	4.8	1.26316	0.052632	18.99996
Bacillus subtilis	3.0	5.2	0.793628	0.033068	30.24086
Salmonella sp.	2.8	3.4	0.280136	0.011672	85.67272
E. coli	1.6	2.4	0.585021	0.024376	41.02418
Shigella sp.	1.2	1.8	0.415079	0.017295	57.82034
Enterobacter sp	5.6	7.8	0.478095	0.019921	50.19923
Proteus vulgaris	2.1	3.6	0.777685	0.032404	30.86082
S. aureus	1.4	2.2	0.652142	0.027173	36.80182

Key: h = hours; n = Number of generation; k = Growth rate constant; Gt = Generation time

The growth profile of these selected bacterial species on various concentration of HA is as shown on Figure 1.

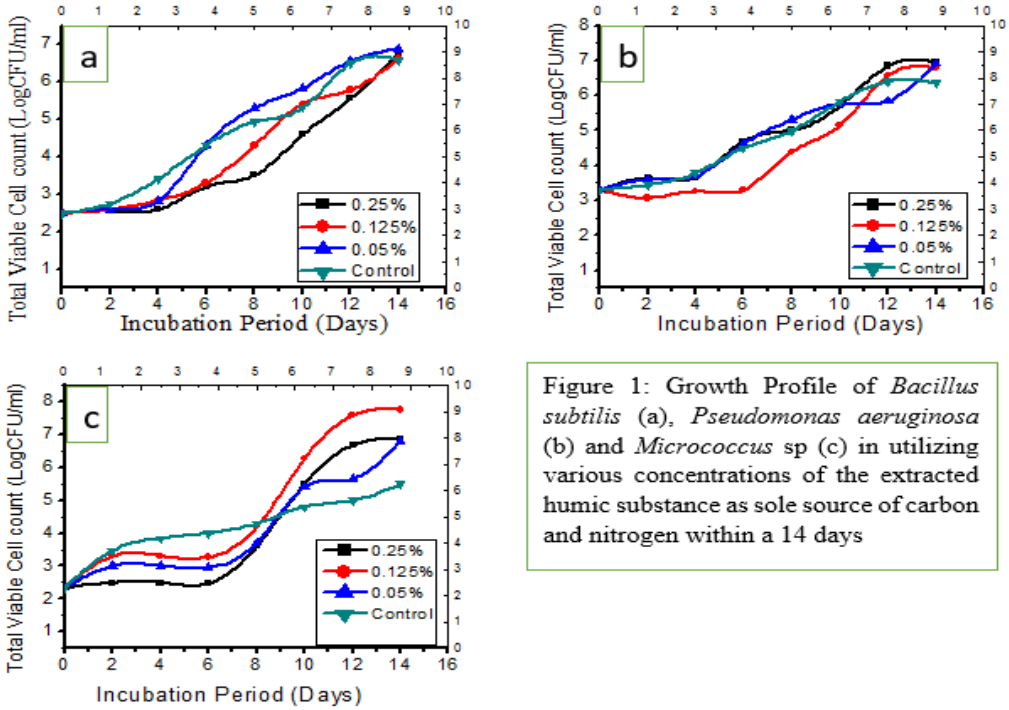

Figure 1: Growth Profile of *Bacillus subtilis* (a), *Pseudomonas aeruginosa* (b) and *Micrococcus* sp (c) in utilizing various concentrations of the extracted humic substance as sole source of carbon and nitrogen within a 14 days

DISCUSSION

Understanding the consequences of biodiversity on ecosystem functioning is becoming increasingly critical in view of the profound interest of human activity on natural ecosystems and goods and services humans receive from them. Humic substances (HS) represent the main carbon reservoir in the biosphere (Fu *et al.*, 2017). Due to their crucial role and control of the biogeochemistry of organic carbon in the global ecosystem, HS are therefore extremely important to environmental processes (Grinhut *et al*., 2007). The role of humic substances in indirectly fueling the heterotrophic component of freshwater ecosystem is also well known. The present study has revealed the rich bacterial assemblage of humic ecosystems in the Niger Delta of Nigeria. Both surface water and sediment of the blackwater ecosystem like other

natural environment harbour diverse bacterial population varying in physiology and nutrition. Heterotrophic bacteria are major components of microbial population in the aquatic sediment (Ramamurthy *et al*., 1990). Fifteen culture-able bacterial species with varying level of occurrence were isolated. The high occurrence of *Staphylococcus aureus, Enterococus faecalis* and *Escherichia coli* may be ascribed to the persistent human activities in the environment. *Staphylococcus, Enterococcus faecalis* and *Escherichia* species are common human skin and intestinal microflora (Jawetz *et al.*, 1989; Farell, 1993). Other dominant bacterial genera encountered included species of *Bacillus subtilis* and *Pseudomonas aeruginosa* which are common soil-borne bacteria and may be autochthonous in the environment under study. The presence of many of the isolates encountered in this study, in aquatic sediments of the Niger Delta has earlier been reported by Itah and Essien (2005).

The isolates exhibited variable potentials to survive in filter - sterilized humic water. The results specifically revealed that *P. aeruginosa* with the lowest generation time (18.99996 hours) exhibited the best growth when cultured on sterilized humic water sample. The isolates with the weakest ability to proliferate in humic water were *Salmonella, Shigella, Enterobacter* and *E. coli* with generation time of 85.67272, 57.82034, 50.19923 and 41.02418 hours respectively. This may be attributed to the fact that they are not autochthonous to the ecosystem and might have gotten into the ecosystem as a result anthropogenic activity. The seeming increase in the growth of these enteric bacteria was unexpected. However, it has been reported that such is possible and may be ascribed to a low level of endogenous metabolism, together with a low maintenance energy requirement (Varnam and Evans, 2000). In all, growth rate analysis has shown that *Bacillus subtilis, Pseudomonas aeruginosa* and *Micrococcus* survived and grew comparatively better in humic-laden water and could be used for the bioconversion of humic substrates.

The question as to whether or not HS are taken up by organisms has been argued intensely in the literature. Currently, empirical evidence has been revealed that HS are indeed taken up. In a cell culture study, Wang *et al*. (1999) showed that HS or at least a fraction thereof, were found inside the cells and even in the DNA. Steinberg (2003) presented evidence that ^{14}C- labeled HS- like substances were taken up and bio-concentrated by freshwater organisms. The growth and proliferation of *Bacillus subtilis, Pseudomonas aeruginosa* and *Micrococcus* sp on humic substance based medium has confirmed that indeed, these microorganisms can actually utilize humic substrate for growth. The isolates increased in biomass with increasing incubation time with varying lag phases in the HS – supplemented MSM medium as well as the non HS – supplemented MSM medium. This plausibly may be ascribed their ability to elaborate the necessary catabolic enzymes.

Due to their large size, HA macromolecules are not likely to be taken up by microbial cells; they are therefore initially degraded by extracellular enzymes (Kastner & Hofrichter 2001; Grinhut *et al.,* 2007). The most efficient lignin degraders thanks to their nonspecific oxidizing enzymes: manganese peroxidase (MnP), lignin peroxidase (LiP) and laccase (Hatakka 1994; Grinhut *et al.,* 2007). These enzymes (making up the so-called ligninolytic system) lead to the formation of unstable compounds (e.g., phenoxy and carboxy radicals), which can then undergo either condensation and polymerization (humification) (Zavarzina *et al.* 2004; Grinhut *et al.,* 2007) or further degradation, and even mineralization (Steffen *et al.* 2002 and Grinhut *et al.,* 2007). The pathway followed by each enzymatic product (degradation or polymerization) is probably dependent not only on the enzymes and substrates involved, but also on reaction conditions, such as pH, humidity, percent oxygen and electrical conductivity, as well as on the presence of other compounds (Grinhut *et al.,* 2007). Previous studies have shown that species of *Bacillus subtilis* (Zhu *et al.,* 2017), *Pseudomonas aeruginosa* (Yang *et al.,* 2018) and *Micrococcus* sp (Taylor *et al.* 2012) isolated in this research can grow elaborate lignolytic enzymes in nature.

CONCLUSION AND RECOMMENDATION

Our findings have shown that *Bacillus subtilis, Pseudomonas aeruginosa* and *Micrococcus* isolated from the blackwater system of Eniong River in Nigeria do not only survive the inhibitory stress of humic substance, they do so with high levels of viable cells generate-ability and within short periods. These are attributes which can be exploited for the degradation of recalcitrant humic wastes and for the bioconversion of lignolytic wastes into useful products

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