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Isolation And Characterization Of Bacteria Associated With Corrosion Of Pipelines In Soils Of Delta State Of Nigeria.

Odesiri-Eruteyan Eunice Asiboroko [1*] and Omonigho S. E.[2]
[1]*Department of Environmental Management and Toxicology, Federal University of Petroleum Resources, Effurun, Nigeria*
[2]*Department of Microbiology, University of Benin, Benin, Nigeria*

Abstract
Isolation and characterization of bacteria associated with corrosion of pipelines was investigated using soil samples collected along oil pipelines and at 500m away in oil producing communities of Delta State. Bacteria enumeration was carried out using nutrient agar, Postgate's medium B (solid), modified Starkey medium, Iron reducing medium (FWA-FE (III) medium), Iron and manganese bacteria agar, Manganese agar no 2, Sulfur amended Mackintosh medium, Thiobacillus medium and Pseudomonas medium A. The isolates were screened for their potentials to corrode metals. The isolates with the best ability to corrode were subsequently identified using standard bacteriological test protocol (Gram reaction and Analytical profile index (API) rapid test kit).The highest bacterial count of soil along pipelines was from Okpai while the lowest was from Otu-Jeremi, with values of 4.65 × 10^7 and 3.30 × 10^7cfu/g respectively. Bacterial counts were higher along pipelines than arable farmland. The screened isolates with corrosion capacity were identified as Pseudomonas putida, P. aeruginosa, P. stutzeri, Shewanella putrefaciens, Desulfovibrio vulgaris and Vibrio vulnificus. The selected identified bacteria were analysed for the presence of these genes (AlgR gene, phz coding gene and rus gene) using their specific primers. These corrosive genes were detected in these test bacteria. The corrosive gene alginate was found to be present in all the organisms while rus gene was found in the three bacteria that were found to influence corrosion more. Detection of these bacteria in the soils of Delta State is indicative of corrosive environment and is of great public health implication.

Keywords: *Corrosion, Microbial Induced Corrosion, Corrosive Genes, Biofilms.*

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I. Introduction

In Nigeria, oil exploration and exploitation are mainly in the Niger Delta Region [1, 2]. Corrosion of oil field equipment is a frequent characteristic of the region. Underground utilities such as pipelines, storage tanks and vessels are constantly under attack from corrosion with consequences of structural damages, structural failures, environmental pollution, risk of public safety and financial losses. Corrosion is generally the deterioration of materials as a result of reactions of the environment. Microbiologically induced corrosion also known as microbial induced corrosion is the inclusion of microbes in the degradation of metals or non-metallic substances [3] [4]. Corrosion occurs in diverse industries and public services [5, 6].

Microbial involvement in corrosion of materials is as a result of adhesion and metabolism on surfaces [7]. Metal surfaces are swiftly adhered by microorganisms in both natural and industrial aquatic environments, leading to a complicated and completely adhering microbial community known as biofilm [8. 9, 10]. The biofilm build up protects a microbial cell from the external force and also it is harmful to the underlying substances thereby resulting into physical degeneration of the metal surface [10]. It is evaluated all over the world that microbial induced corrosion is accountable for losses worth billions of dollars to pipes and industrial machinery. In spite of the use of precautionary measures to attack microbial induced corrosion (MIC), corrosion persists in most cases [11].

Many bacteria associated with corrosion process are acid forming bacteria, exopolysaccharide or extracellular polymeric substances (EPS) or slime forming bacteria, manganese-oxidizing bacteria, iron-oxidizing/reducing bacteria, sulphur-oxidizing bacteria and sulphate-reducing bacteria (SRB) and were all found to participate actively in corrosion processes [12, 13, 14]. These microorganisms are widely spread in nature [15]. They live together in nature in biofilms or communities and are able to influence corrosion reactions through mutual activities which a single organism has difficulty to facilitate [4]. Soils, like any environment can be corrosive. Pipes buried underground are exposed to a lot of factors and conditions that can affect its longevity

[16]. The amount of microbes in the soil and other soil parameters are usually used for assessing soil corrosion potential [17, 18].

Based on operating challenges, gas and oil industries fuse various procedures for the protection and prevention of corrosion and to suppress bacterial growth in gas and oil industries. Nevertheless, it was found that microbes especially extremophiles could adjust to extreme situations created by these applications and withstand tough situations in the surroundings, thus still posing hazard to the substances [19]. It is therefore imperative to reduce corrosion of such important engineering facilities to enhance our global economy and reduce huge economic losses, wastages and damages.

The main objective of this study was to isolate, and identify bacteria associated with corrosion from soil samples along oil pipelines and the environs in selected locations in Delta state in Niger Delta Region.

II. Materials And Methods

Study Site and Samples Collection: Soil samples were obtained from the forest land basically for oil exploration and exploitation in three oil producing communities and within oil flow stations. Soil samples were collected from a depth of 5 to 10cm and from a distance of 1cm from pipelines and flow stations, and from farmland soil (100meters far from pipeline location) for comparison. Soil samples were collected from oil-producing communities in Kokori (Erhioke Town), Otujeremi and Okpai. Soil samples were obtained from the locations in the months of January, July and December which included the dry and wet seasons. Soils were collected from the respective locations using a soil auger, air-dried and sieved to remove gravel, debris and chunks. The soils were thoroughly mixed to make them more homogenous and placed in labeled sterile polyethylene bags and transported to a Microbiology laboratory for further analysis.

Table 1 Global Positioning System (GPS) Reading of the Sampling Locations

LOCATION	TEST	CONTROL
Kokori (Erhioke), Ethiope East Local Govt Area, Delta State.	$05^0$38’58.2’’N, $006^0$04’10.1’’E	$05^0$34’45.4”N, $005^0$59’05.7”E
Otu-Jeremi , Ughelli-South Local Govt Area, Delta State.	$05^0$26’07.3”N, $005^0$52’56.8”E	$05^0$30’12”N, $005^0$49’18”E
Okpai, Ndokwa East Local Govt Area, Delta State.	$05^0$40’03.7”N, $006^0$30’39.2”E	$05^0$40’06.4”N, $006^0$30’45.5”E

Determination of Total Bacterial Count

Soil samples were analysed for total bacterial count (TBC). This was evaluated by standard pour plate method using nutrient agar. A 1g soil sample was introduced into 9ml of normal saline and subsequently serially diluted. The nutrient agar was prepared from commercially available dehydrated powder according to manufacturer’s instructions. The medium was cooled to 45^{o}C and then dispensed aseptically into sterile petri dishes containing 1ml of the inoculum and swirled to effect even distribution. The plates were incubated at 37^{o}C for 24-48hrs [20, 21]. Discrete bacteria colonies were counted and colony forming unit was determined.

Isolation and Detection of Bacteria associated with corrosion

Different selective media were used for the enumeration of the bacteria. Six classes of bacteria were targeted based on literature sources [12, 13, and 14].

Sulphate Reducing Bacteria: Sulphate reducing bacteria were enumerated with Postgate’s medium B [22, 23, 3]. The pH of medium was adjusted to 7.6 with 1 M NaOH solution and antifungal was added to inhibit fungal growth, the medium was sterilized before used. The medium was then sparged with nitrogen gas for approximately one hour to remove oxygen from it prior to autoclave. The incubation was performed anaerobically in anaerobic jars for seven days at 30^{o}C. Sulphate degradation was a proof as the medium changed from clear to black due to SRB metabolism which degrades the sulphate and as a result, there was FeS production.

Acid Producing Bacteria: Acid producing bacteria were enumerated with sulphur amended Mackintosh medium by [24]. 10g of NaCl was added to the medium. It was sterilized. The evaluation of bacterial growth was done by measuring the pH. It was a positive growth when the pH dropped from 7 to 3.

Sulphur Oxidizing Bacteria: Sulphur oxidizing bacteria were enumerated with modified Starkey medium by [25] and as adopted by [26].

Iron Reducing Bacteria: Iron reducing bacteria were enumerated by using iron reducing medium (FWA-FE (III) medium) by [27]. pH was adjusted to 7.0 and the medium was sterilized.

Iron Oxidizing Bacteria: Iron oxidizing bacteria were isolated by using lron and Manganese Bacteria Agar by [28]. The medium was supplemented with 10g/l of NaCl. Incubation was done aerobically. The evaluation of bacterial growth was by taking the pH. It was a positive growth when the pH dropped from 7 to 3.

Manganese Oxidizing Bacteria: Manganese oxidizing bacteria were enumerated using Manganese Agar No 2 by [28]. pH of the medium was adjusted to 7.5, sterilized and incubation was done aerobically.

Other media used are as follows.

Thiobacillus species: Thiobacillus Medium by [28] was also used for isolation of Thiobacillus species. pH of the medium was adjusted to 4.4±0.2 at 25^0C and sterilized.

Pseudomonas species: Pseudomonas Medium A as described by [28] was used for isolation of Pseudomonas species.

Screening of Corrosive Isolates

About thirty (30) isolates were subjected to pilot test by screening them for corrosion potential. Each isolate was inoculated into sterile peptone water singly while a sterile nail was dropped into each medium. The initial and final weights of each nail were taken. Weight loss and discoloration from clear to cloudy and intensity of black discoloration were used to determine corrosion potential of each isolate. These were now screened down to seven by visual of colour intensity, turbidity and weight loss. Differences in turbidity and colour intensity were used to select seven isolates based on visual observation.

Identification and Characterization of Isolates

The seven isolates obtained from the pilot test were subjected to further identification and characterization using Gram stain technique, motility and Analytical Profile Index (API).

Analytical Profile Index (API) Test: Analytical Profile Index (API) 20 NE biochemical kit (Biomerieux) was used in this research as used by [29]. Pure bacterial cultures were also used. The bacteria to be identified were isolated using a suitable selective media where the bacteria grew. Pure isolates were tested using the API 20 NE test kit and identified using the API web^{TM}. Test procedure was followed according to the manufacturer's directives.

Confirmation of Genes Responsible for Corrosion

The genetic analysis of the seven identified organisms was carried out. The procedure included DNA extraction, amplification of the various genes using PCR and their respective primers, then gel electrophoresis and photography. These were carried out on all the screened organisms. Three genes were determined: AlgR Gene, *phz* Coding Genes and *rus* Coding Genes based on literature sources of genes associated with corrosion [30, 31].

III. Results And Discussion

Bacteriological Analyses

Bacteriological characteristics of soils collected along pipeline routes and farmland routes are presented in Figure 1. The total bacterial count of soil samples ranged from 1.55±0.53 in Jeremi farmland to 4.65±0.14 in Okpai pipeline route. Bacterial growth was observed more on soils along pipeline than on arable farmland. Figure 3.2 showed bacterial counts on different selective media. Bacterial growth was also observed more on soils along pipeline than on most arable farmland. Sulphur, iron oxidizing bacteria and Thiobacillus were not detected in all the locations but Sulphate reducing bacteria were present in all the locations. *Pseudomonas spp.* was present in all locations, with higher counts along pipelines than in arable farmlands. Manganese oxidizing and Iron reducing bacteria were present in all locations and the counts were generally higher along pipelines. The same trend was repeated with acid producing bacteria.

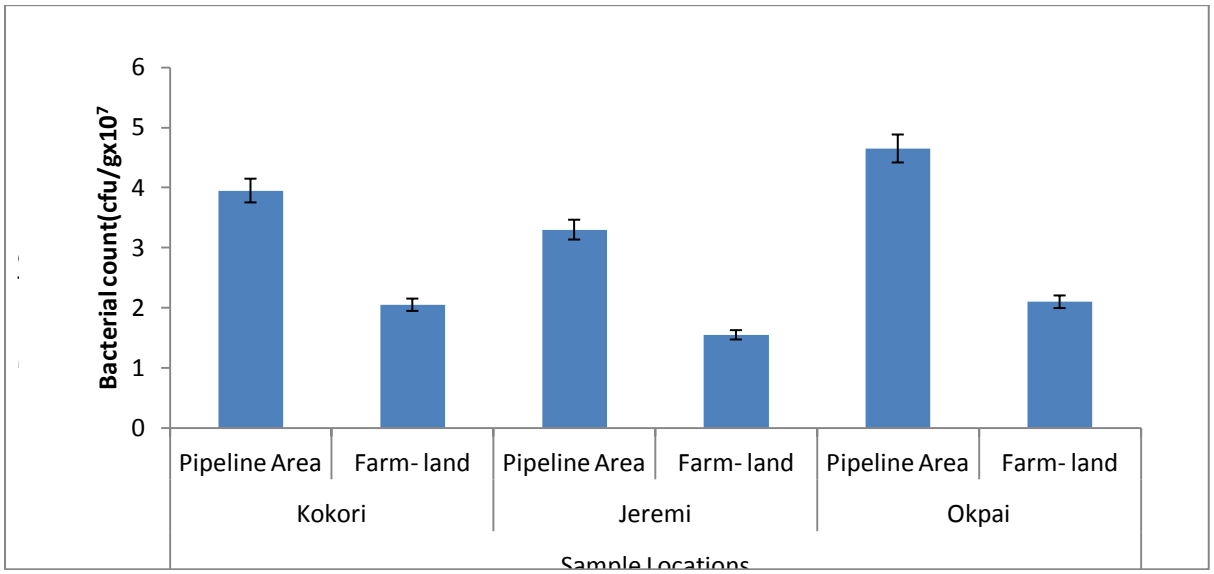

Figure1: Bacterial Counts of various soil samples collected from different Locations on Nutrient Agar

Okpai location was found to be more contaminated than other locations based on the bacterial counts on both nutrient and selective media. The presence of *Pseudomonas species* in all the locations could be attributed to their aerobic metabolism as they are known to be initial colonizers of an environment. They inhabit an environment first and create favourable conditions for other colonizers. There was no growth on Thiobacillus medium, Sulphur oxidising medium and Iron oxidising medium. Their absence in these media does not necessarily mean that they were completely absent from the environment, it could be due to the fact that they were difficult to culture in the laboratory as previously reported [14].

The presence of metal pipes and petroleum containing hydrocarbons in the environment could have led to high total bacterial counts, especially along pipeline routes. The pipes containing hydrocarbons were used as sources of energy for growth and development by the microbes and hence higher growth and bacterial counts along pipeline routes. Higher bacterial count in soil along the pipelines was also an indication of accessibility of abundant nutrients supply, the more hydrocarbons are present, the more microorganisms are present too.

The elements present in the oil and gas transporting media enable the survival of microorganisms [32]. Previous reports showed that petroleum is an ideal food supply for many micro-organisms [19]. Research has also revealed the use of metal for growth and development by microbes [33, 34]. High number of microbes from this region indicates that these organisms are the degraders of the environment. Bacterial counts higher than 10^4cfu/g in soil indicates corrosion tendency. Therefore, microbiological characteristics of the soil samples could enhance microbial corrosion in the environment.

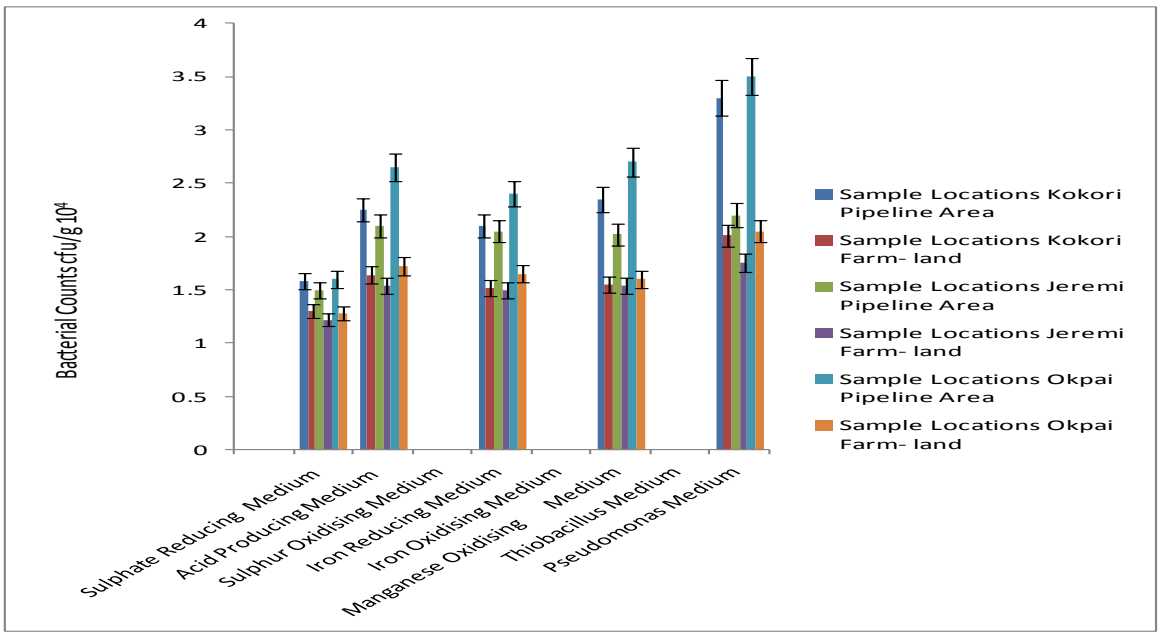

Figure 2: Bacterial Counts of various soil samples collected from different Locations on different selective media

Screening of Isolates for Biocorrosion Potential.

Isolates were screened for biocorrosion potential as presented in Table 2. Isolates were selected based on high weight loss, colouration and turbidity of the cultures by using clear and cloudy. Isolates causing the higher weight losses and very dark discolouration were selected for further tests. The identities of the selected isolates based on morphological, microscopic (Gram-reaction) and Analytical Profile Index tests are presented in Table 3. *Pseudomonas spp.* was the dominant isolates. Seven bacterial species were identified. These are aerobic, anaerobic and falcultative anaerobic bacteria. These species are; ***Pseudomonas putida(1), Vibrio vulnificus, Pseudomonas stutzeri, Shewanella putrefaciens, Pseudomonas aeruginosa, Desulfovibrio vulgaris, Pseudomonas putida(2).***

These species have been observed and reported in biocorrosion studies. *Pseudomonas spp* such as *Pseudomonas putida* and *P. aeruginosa* were isolated from petroleum products transporting pipelines and water samples from a cooling tower [35]. There was a report on the involvement of *Desulfovibrio vulgaris* at corrosion sites [36]. *Pseudomonas* sp. and *Shewanella* sp. are the common iron reducing bacteria involved in corrosion [37]. *Shewanella* strain was isolated from black powder of gas pipelines [38]. *Vibrio sp* was found at corrosion site and its involvement was as a result of biofilm formation [39]. Although, *Vibrio spp* is generally isolated from marine system, they produce potent toxins and known to be virulent and this could be why it was implicated in corrosion.

Many microorganisms can produce corrosives but have less affinity for metals. This could also be why *Vibrio spp* was found in this environment. *Pseudomonas spp* are always found at corrosive environments and corrosion sites. They are the initial colonizers of metal surfaces, they utilizing the oxygen over time and thereby build favourable and ideal conditions for adhesion of other microorganisms such as *Desulfovibrio spp, Shewanella spp* and others that are anaerobic and falcutative anaerobes. It was also reported in previous studies that bacteria producing exopolysaccharide substances (EPS) are the most significant contributors to microbial induced corrosion of metals. *Pseudomonas* is the main genus of EPS producer.

EPS forming bacteria are initial inhabitants, with the capability of EPS production. They influence electrochemical properties of metal surface producing appropriate environment for colonization of other microorganisms, hence accelerating biofilm production and favourable microbial corrosion [4, 40]. This explains why *Pseudomonas spp*, (aerobes), falcutative aerobe such as *Shewanella sp* and obligate anaerobe such *Desulfovibrio vulgaris* were found together at these sites. Their presence is indicative of corrosive environments.

Table 2: Biocorrosion Potential of Isolates

Isolate	Weight loss (%)	Colour change	Turbidity
1 (APBM, Acid Producing BacteriaMedium)	2.4% (5g-4.88g)	--	+
2(MOBM Manganese Oxidising Bacteria Medium)	3% (5g-4.85g)	+	+
3(SRBM, Sulphate Reducing Bacteria Medium)	3.6% (5g-4.82g)	+	+
4*(APBM)	5.6% (5g-4.72g)	++	+
5(PM, *Pseudomonas* Medium)	3.4% (5g-4.83g)	+	+
6(IRBM,Iron Reducing Bacteria Medium)	3.2% (5g-4.84g)	+	+
7(MOBM)	2.6% (5g-4.87g)	--	+
8* (IRBM)	5.2% (5g-4.74g)	++	+
9(IRBM)	3% (5g-4.85g)	+	+
11*(SRBM)	5.6% (5g-4.72g)	++	+
12(PM)	2.8% (5g-4.86g)	--	+
13(MOBM)	3.4% (5g-4.83g)	+	+
14(APBM)	2.2% (5g-4.89g)	--	+
15*(PM)	5.6% (5g-4.72g)	++	+
16(IRBM)	2.4% (5g-4.88g)	--	+
17(IRBM)	2.8% (5g-4.86g)	--	+
18(MOBM)	3.2% (5g-4.84g)	+	+
19*(MOBM)	4.8% (5g-4.76g)	++	+
20(IRBM)	2.8% (5g-4.86g)	--	+
21*(APBM)	5.4% (5g-4.73g)	++	+
22 (SRBM)	3.4% (5g-4.83g)	+	+
23(MOBM)	3.4% (5g-4.83g)	+	+
24*(MOBM)	5.2% (5g-4.74g)	++	+
25(IRBM)	3% (5g-4.85g)	+	+
26(IRBM)	2.2% (5g-4.89g)	--	+
27(SRBM)	3.2% (5g-4.84g)	+	+
28(PM)	3.8% (5g-4.81g)	+	+
29(APBM)	2.6% (5g-4.87g)	--	+
30(APBM)	3% (5g-4.85g)	+	+
Control	0.4% (5g-4.98g)	--	--

NB: Colour change (--, no change; + black; ++ very black), Turbidity(-- clear; + turbid)
*Selected isolates (isolates 4, 8, 11, 15, 19, 21 and 24).

Code	Gram Stain	Shape	Aerobic	Motility	H_2S Production	NO_3 (Nitrate Reduction)	TRY (Tryptophane)	GLU (Glucose Acidification)	ADH (Amino acid arginine Decarboxylation)	URE (Urea)	ESC (Esculin Hydrolysis)	GEL (Gelatinen Hydrolysis)	PNG (P-nitrophenyl-beta-d-galactopyranoside)	GLU (Glucose Assimilation)	AR (Arabinose Assimilation)	MNE (Mannose Assimilation)	MA (Mannitol Assimilation)	NAG (N-acetyl-glucosamine assimilation)	MAL (Maltose Assimilation)	GNT (Gluconate Assimilation)	CAP (Caprate Assimilation)	ADI (Adipate Assimilation)	MLT (Malate Assimilation)	CIT (Citrate Assimilation)	PAC (Phenyl-acetate Assimilation)	OX (Oxidase test cytochrome Oxidase)	% of Identification	Identified Isolate
Isolate 4	-	Rod	Obligate Acrobes	+	-	+	+	+	-	+	+	+	+	+	+	-	+	-	+	+	-	-	-	+	+	+	94.8	Pseudomonas putida (1)
Isolate 15	-	Rod	Obligate Acrobes	+	-	+	+	+	-	+	+	+	+	+	+	-	+	-	+	+	-	-	-	-	+	+	92.8	Pseudomonas aeruginosa
Isolate 8	-	Rod	Falcutative Acrobes	+	-	+	-	-	-	-	+	+	-	-	-	-	-	+	-	-	+	-	+	-	-	+	99.9	Shewanella putrefaciens
Isolate 24	-	Rod	Obligate Acrobes	+	-	+	-	-	-	-	-	-	-	+	-	-	+	-	+	+	+	-	+	+	-	+	99.5	Pseudomonas stutzeri
Isolate 11	-	Rod	Obligate Anacrobes	+	+	+	+	+	-	-	-	-	-	+	-	-	+	+	-	-	-	+	+	+	-	+	99.9	Desulfovibrio vulgaris
Isolate 21	-	Rod	Obligate Acrobes	+	-	+	+	+	-	+	+	+	+	+	+	-	+	-	+	+	-	-	-	+	+	+	99.5	Pseudomonas putida (2)
Isolate 19	-	Rod	Falcutative Acrobes	+	-	+	+	+	-	-	+	+	+	+	-	-	-	-	-	+	-	-	+	+	-	+	99.9	Vibrio vulnificus

Table 3: Identification and Isolation of Isolates.

Identification of Genes Coding for Corrosion

Molecular analyses to determine genes coding for corrosion are shown Table 4 and Figure 3, 4 and 5. These show Agarose gel electrophoresis of the PCR products of three genes in the seven selected bacteria isolates. The plates showed DNA bands of the different isolates separated in a gel. The length of the DNA

fragments was compared to a marker containing fragments of known length. The three genes are AlgR gene, phz gene and rus gene. Gel Figure 3 indicates that AlgR gene was present in all the isolates. Figure 4 also indicates that phz gene was absent in five isolates but present in ***Pseudomonas aeruginosa*** **and** ***Pseudomonas putida (1).*** Figure 5 indicates that rus gene was absent in four isolates but present in ***Desulfovibrio vulgaris, P. aeruginosa and P. putida (1).***

Table 4 : Agarose Gel Electrophoresis of the PCR products of three Genes in the Seven Selected Bacteria Isolates

Organism	AlgR Gene	*phz* Gene	*rus* Gene
Pseudomonas putida(1)	+	+	+
Pseudomonas aeruginosa	+	+	+
Shewanella putrefaciens	+	-	-
Pseudomonas Stutzeri	+	-	-
Desulfovibrio vulgaris	+	-	+
Pseudomonas putida(2)	+	-	-
Vibrio vulnificus	+	-	-

Note: + = Positive - = Negative

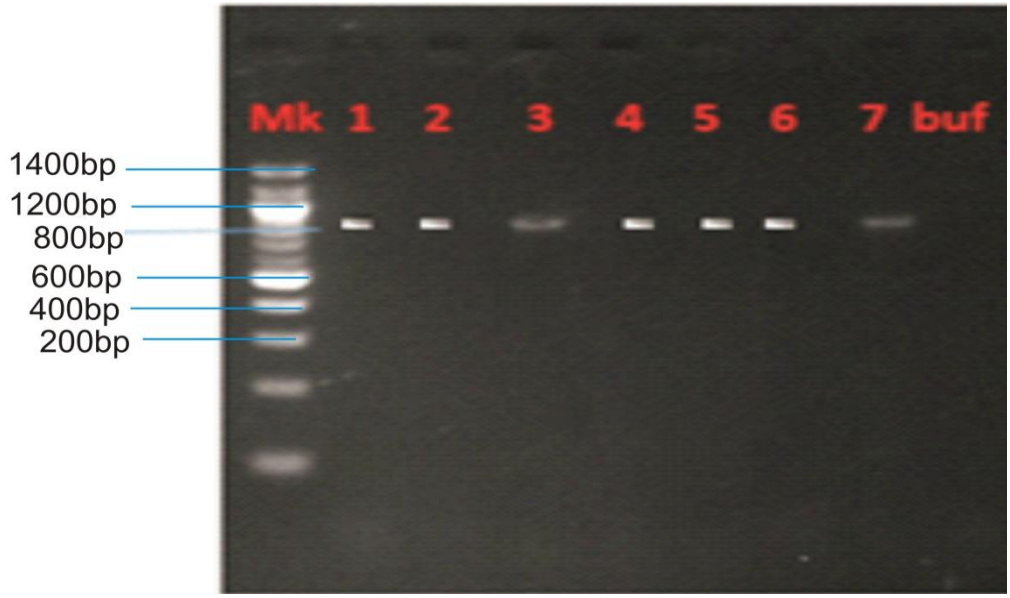

Figure 3: Agarose Gel Electrophoresis of the PCR products of *AlgR* Gene in Selected Tested Bacteria (Band size approximately 842 bp).

Note: Gel image indicates a positive amplification in all samples suggesting the presence of *AlgR gene* in all the seven (7) isolates. Loading arrangement as follows: (1) ***Desulfovibrio vulgaris***, (2) ***Shewanella putrefaciens***, (3) ***Pseudomonas stutzeri***, (4) ***Pseudomonas aeruginosa***,(5) ***Pseudomonas putida****(2)* (6) ***Pseudomonas putida****(1) and* (7) ***Vibrio vulnificus***.

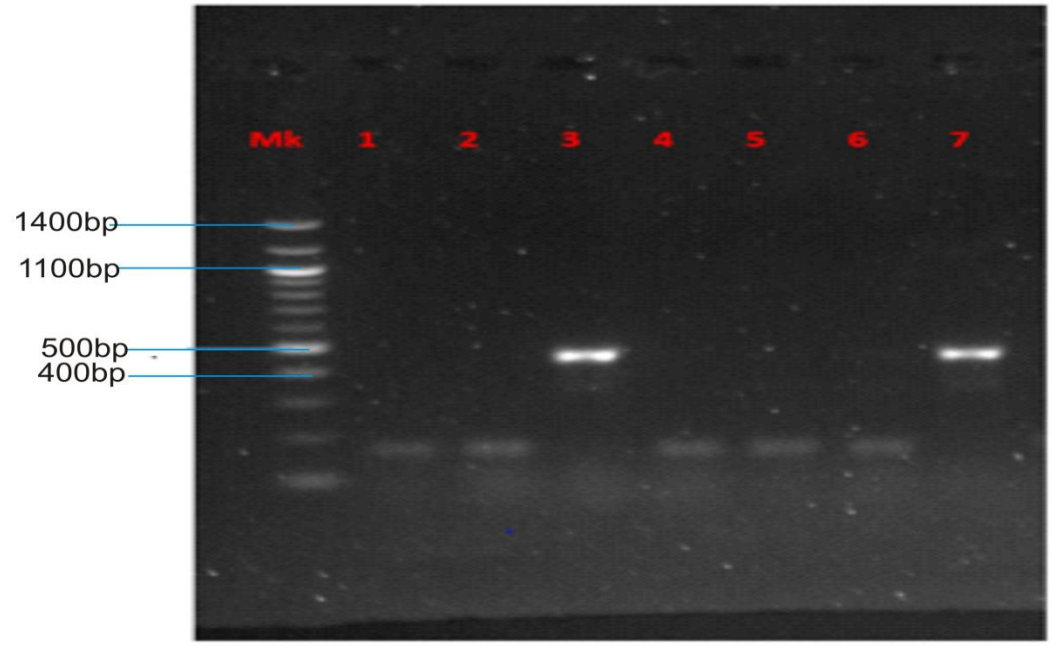

Figure 4: Agarose Gel Electrophoresis of the PCR products of *phz* Gene in Selected Tested Bacteria *(Band size approximately 484 bp).*

Note: Gel image indicates a negative amplification in all samples except ***Pseudomonas aeruginosa*** *and* ***Pseudomonas putida****(1)* indicating the presence of phz gene in only ***Pseudomonas aeruginosa*** and ***Pseudomonas putida****(1).* Loading arrangement as follows:(1) ***Desulfovibrio vulgaris,*** (2) ***Shewanella putrefaciens***,(3) ***Pseudomonas aeruginosa***, (4) ***Pseudomonas stutzeri***, (5) ***Vibrio vulnificus***,(6) ***Pseudomonas putida****(2) and* (7) ***Pseudomonas putida****(1)*

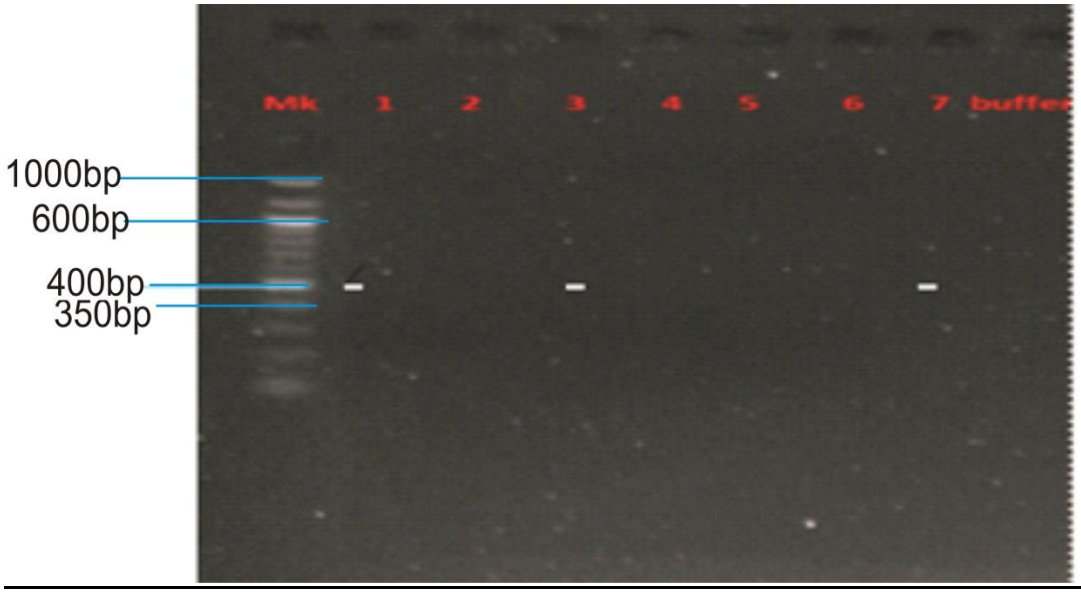

Figure 5: Agarose Gel Electrophoresis of the PCR products of *rus* Gene in Selected Tested Bacteria (Band size approximately 388bp).

Note: Gel image indicates a negative amplification in four isolates (indicating the absence of *rus genes* in the four), and a positive amplification in three isolates (***Desulfovibrio vulgaris***, ***Pseudomonas aeruginosa***, and ***Pseudomonas putida***(1)) indicating the presence of *rus gene* in the three. Loading arrangement as follows: (1)***Desulfovibrio vulgaris***, (2)***Shewanella putrefaciens***, (3) ***Pseudomonas aeruginosa***, (4) ***Pseudomonas stutzeri***, (5)***Vibrio vulnificus***, (6) ***Pseudomonas putida****(2)* and (7) ***Pseudomonas putida****(1).*

The presence of these genes in these microbes is indicative of corrosion and corrosive environments. The presence of Alginate genes in ***Pseudomonas aeruginosa*** isolated from corroded metal surfaces has been

reported [30]. Research also showed the presence of rus gene and other corrosive genes in bacteria from corrosion site [31]. This research revealed that the seven organisms isolated along pipeline in the soils possessed genes that could favour and accelerate corrosion.

IV. Conclusion

Corrosion is a threat to the environment. Researches revealed that many industries have lost several billions of dollars as result of corrosion. Reports showed that many oil industries underground structures ruptured due to corrosion that led to oil spillages and hence environmental pollution. As a result of this, resources are lost in repairing the damages caused by corrosion. Corrosion can also cause adverse effects on health, if there is escape of corrosive products into the environment from the corroded structures. Detection of corrosive genes AlgR, phz and rus gene in these test organisms is of great public health implication. There is a need to prevent and control these metal corrosive bacteria as they may find their ways into domestic pipes in homes. Thus, corrosion should be given much attention and adequate measures should be taken to curb it as lives are being endangered in this serious problem.

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Odesiri-Eruteyan Eunice Asiboroko, et. al. "Isolation And Characterization Of Bacteria Associated With Corrosion Of Pipelines In Soils Of Delta State Of Nigeria." *IOSR Journal of Environmental Science, Toxicology and Food Technology (IOSR-JESTFT)*, 16(06), (2022): pp 41-50.