

**Treatment of Port Harcourt refinery effluent by a bacterial consortium immobilized on agro-based biocarriers**

**Abstract**

Discharge of poorly treated refinery wastewater has always been a major environmental challenge. Bacterial immobilization is key to maintenance of biomass on contaminated site. In this study, a mixed culture of three bacterial isolates from oil polluted water: *Pseudomonas aeruginosa* (MN294989), *Bacillus tequilensis* (MN294990) and *Micrococcus* sp. immobilized on Groundnut Shell (GS), Melon Husk (MH) and Sugarcane Bagasse (SB) were employed in the bioremediation of Port Harcourt refinery wastewater. Surface area and pore size distribution of the agro-based biocarriers were suitable for bacteria adhesion. The bacterial isolates were screened for phenol, naphthalene and hydrocarbon utilization. Scanning Electron Microscopy (SEM) was used to ascertain the immobilization of the consortium on the agro-base carriers. A 15-days laboratory scale treatment of refinery raw wastewater was compared in the unimmobilized and immobilized consortium. The agro-based residue immobilized consortium enhanced reduction in BOD<sub>5</sub>, COD, oil and grease, phenol by 7%, 9%, 30% and 5% respectively compared to the free form of the consortium. This study underscores the role of immobilization in maintaining high bacterial biomass on contaminated site and possible improvement in bioremediation of refinery wastewater.

**Key words:** immobilized consortium, bioremediation, refinery raw wastewater, biocarriers, phenol utilization

**Introduction**

Nigeria has four operational crude oil refineries with an estimated refining capacity of 445 000 barrels per day (Kadafa, 2012). The Federal Environmental Protection Agency (FEPA) and the Department of Petroleum Resources (DPR) are responsible for setting the minimum standards for industrial effluent discharge. Recent reports indicate that effluents discharge from refinery industries do not meet minimum set standards (Osin, Yu, & Lin, 2017; Yu & Han, 2017). In a review of the current refinery effluent treatment practices of refineries in the Niger Delta region. Osin et al. (2017) reported that receiving water bodies in the Niger Delta region of Nigeria contain most of the examined pollution parameters in concentrations above the set regulatory limits.

Pollutants in refinery effluents pose serious environmental hazards. Due to ineffectiveness of purification systems, wastewaters may become hazardous leading to the accumulation of toxic products in receiving water bodies (Deka, Devi, & G.Bhattacharyya, 2013). The composition of these effluents depends on the

36 type of oil being processed, the plant configuration and operational procedures (Hasan, Mohd, Wan, &  
37 Aziz, 2010; Ishak, Malakahmad, & Isa, 2012). Ishak et al., (2012) reported that the major constituents of  
38 refinery wastewater in general are dissolved and dispersed oil (mixture of hydrocarbons – benzene, toluene,  
39 ethylbenzene, xylenes, polyaromatic hydrocarbons and phenols) and dissolved formation minerals (anions  
40 and cations including heavy metals). Phenol contaminants are relatively soluble in water and their severe  
41 toxicity even in low concentration have been reported worldwide (Mahiuddin, Fakhrudin and Al-Mahin,  
42 2012). The removal of phenol from effluents has been quite challenging and expensive. Of the different  
43 technologies being applied to remove phenolic compounds from polluted areas, biodegradation process is  
44 relatively low cost, no chemicals used, and high public acceptance tends to completely destroy the  
45 pollutants if possible or at least to transform them to less harmful forms (Mahiuddin et al 2012, Poi,  
46 Aburto-Medina, Mok, Ball, & Shabsavari, 2017).

47

#### 48 **Microbial immobilization**

49 Microbial immobilization occurs naturally around the world (Bayat, Hassanshahian, & Cappello, 2015). It  
50 offers a lot of advantages over the free form of microorganisms in bioremediation, some of which are:  
51 provision of high biomass, high metabolic rate, improving genetic stability, resistance to toxic chemicals,  
52 elimination of cell washout problems etc. (Bayat et al., 2015; Cláudia et al., 2013; Liu, Guo, Liao, &  
53 Wang, 2012)

#### 54 **Materials and Methods**

##### 55 **Bacteria source and consortium**

56 The bacterial consortium used in this study were isolated from crude oil polluted creeks of Bodo, in  
57 Gokana local government area of Rivers State. The water samples were enriched in 98ml Bushnell Haas  
58 media (BHM), prepared according to manufacturer's specification and supplemented with 1% Bonny Light  
59 crude oil (BLCO). One percent crude polluted pond water samples was added to the sterile set up and  
60 incubated in an orbital shaker incubator (Stuart, Germany S150) at 170 r.p.m at 37°C for seven days

61 (Ekwuabu, Chikere, & Akaranta, 2016; Xue et al., 2017). Further enrichments were conducted to obtain the  
62 selected strains, which were sub-cultured separately in nutrient broth medium for 24 hours. After  
63 incubation, the cells were harvested by centrifugation, washed with normal saline (0.85% NaCl) and re-  
64 suspended in fresh normal saline. Equal volumes of the suspension containing the different bacterial strains  
65 were mixed to form the consortium used in the study.

66  
67 The selected strains were identified as 100% *Pseudomonas aeruginosa* (MN294989), 99% *Bacillus*  
68 *tequilensis* (MN294990) and 63% *Micrococcus spp.* using the 16S rRNA approach.

#### 69 **Screening for phenol and naphthalene degrading organism**

70  
71 The method of screening adopted was similar to Velmurugan & Arunachalam, (2009). One ml of water  
72 sample (one gram for soil sample) was serially diluted up to  $10^{-6}$  dilution, and spread on Bushnell Haas  
73 agar medium containing 10 mg/ 100 ml phenol and naphthalene which was added after sterilization, and  
74 the plates were kept for incubation at 28°C for 5 days.

#### 75 76 **Enrichment of the isolated phenol and naphthalene degrading strains**

77 The 24 hours selected cultures were prepared in nutrient broth. One ml of this turbid broth was taken and  
78 added into 99 ml of liquid mineral medium supplemented with 10 mg of phenol and naphthalene in a sterile  
79 250 ml conical flask and kept in a rotary shaker at 120 rpm for 5 days.

#### 80 81 **Selection of potential phenol and naphthalene degrading strains (Plate method)**

82 Minimal agar medium was prepared with increasing concentration of selected phenol and naphthalene as  
83 follows 10 mg/100 ml, 20 mg/100 ml, 30 mg/100 ml, 40 mg/100 ml, 50 mg/100 ml and 100 mg/100 ml of  
84 enriched cultures were inoculated on minimal agar media. Plates were incubated at room temperature for  
85 48-72 hrs.

86 **Biocarriers**

87 Raw materials used as biocarriers (sugarcane bagasse, groundnut shell and melon husk) were all obtained  
88 from Nchia Market in Eleme local government area of Rivers State. These agro-based residues were first  
89 air-dried, grind mechanically and sieved to obtain homogenous particle size of 0.3-0.5mm. They were  
90 washed sequentially with ethanol and distilled water several times to prevent the impurities from affecting  
91 the growth and immobilization of the bacteria. The powder were dispensed in vials and sterilized by  
92 autoclaving at 120°C, 1 atm for 15 min and kept at room temperature until use.

93 **Surface area and pore distribution of biocarriers**

94 The surface area and pore size distribution of the biocarriers were analyzed based on the Brunauer-Emmet-  
95 Teller (BET) theory. This is based on the adsorption of gas molecules on solid surfaces. Prior to analysis,  
96 the samples were left in a desiccator at low temperature to ensure that they have as little remaining water  
97 vapor as possible. The analysis was performed by the BET analysis instrument, according to  
98 manufacturer's specifications. The out-gassed samples are immersed in a liquid nitrogen bath while the  
99 instrument performs the nitrogen adsorption tests.

100 **Scanning electron microscopy**

101 The scanning electron microscopy (SEM) was performed to examine the physical structure of the samples  
102 as well as the adsorption of the microbial cells on them. This was done using SEM model Phenom ProX,  
103 by Phenom World Eindhoven, The Netherlands. Sample which was sputter coated by quorum technologies  
104 model Q150R 5nm of gold was placed on double adhesive which was on a sample stub. Thereafter it was  
105 taken to the chamber of SEM machine where it was viewed via NaVCaM for focusing and little  
106 adjustment. This was then transferred to SEM mode where focusing was automatically adjusted. The  
107 morphologies of different magnification were recorded.

108

109 **Immobilization of bacterial consortium**

110 About 0.5 g of the sterilized agro-based biocarriers were aseptically transferred into 500 mL of Bushnell  
111 Hass broth in separate one litre Erlenmeyer flasks. Five milliliter of the bacterial consortium was  
112 inoculated into the separate flasks containing the different biocarriers. After three days of incubation, the  
113 powder was harvested, washed by sterile 75% normal saline, and air-dried.

114

115 To determine the population of the bacteria consortium that immobilized on the biocarriers. They were  
116 washed with saline solution by centrifugation at 4000 rpm for 10 minutes to remove microorganisms that  
117 were not immobilized on the biocarriers. The microorganisms immobilized on the biocarriers were

118 harvested by centrifugation at 8000 rpm for 10 min. The number of bacteria was approximately  $5 \times 10^7$   
119 CFU/ml in the experiment and was determined using the dilution plate method.

120

### 121 **Wastewater sample collection**

122 The wastewater samples were collected from the raw wastewater and treated wastewater reservoirs in Port  
123 Harcourt Refinery Eleme, Nigeria, and transferred to the laboratory immediately for analysis. All the  
124 collected samples were preserved and processed in accordance with standard guidelines. The samples were  
125 analysed for pH, Biological Oxygen Demand (BOD<sub>5</sub>), Chemical Oxygen Demand (COD), total dissolved  
126 solids, oil and grease, phenol, sulphide and some selected heavy metals.

127

### 128 **Bioremediation using immobilized mixed culture**

129 The efficacy of the immobilized consortium in bioremediation of refinery wastewater was determined in  
130 microcosm trials. Batch cultures were performed using one litre conical flasks containing 500ml refinery  
131 wastewater autoclaved at 121°C for 15 minutes at 15 psi. On cooling, 0.5g of the immobilized consortium  
132 is introduced separately in each of the conical flasks. The control had the sterilized raw wastewater only.  
133 The different treatment options and the content of each representative flask is shown in table 1. The  
134 samples were analyzed for pH, Biological Oxygen Demand (BOD<sub>5</sub>), Chemical Oxygen Demand (COD),  
135 total dissolved solids, oil and grease, phenol, sulphide and some selected heavy metals on every five days  
136 intervals

137

138 Table 1: compositions of the different treatment options

S/N	Treatments	Code	Description
1	Immobilized consortium on Sugarcane bagasse	SB	Immobilized consortium + sterile raw wastewater
2	Immobilized consortium on Groundnut shell	GS	Immobilized consortium + sterile raw wastewater
3	Immobilized consortium on Melon husk	MH	Immobilized consortium + sterile raw wastewater
4	Free form of bacterial consortium	FB	Free form of consortium + sterile raw wastewater
5	Control	NB	Sterile raw wastewater

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143 Table 2: Result for screening of potential phenol and naphthalene degrading strains

S. No	Isolates	Phenol concentrations (mg/100ml)				Naphthalene concentrations (mg/100ml)			
		10	30	50	100	10	30	50	100
1	RWA	+	-	-	-	++	+	-	-
2	RW3	+++	+++	++	+	++	+	-	-
3	RW10	++	+	-	-	++	-	-	-
4	JW1	-	-	-	-	+	-	-	-
5	JW2	+	-	-	-	+	-	-	-
6	JW4	++	+	+	-	+++	++	+	+
7	JW5	+	-	-	-	+	-	-	-
8	GW3	++	-	-	-	++	+	-	-
9	GW4	++	-	-	-	++	-	-	-
10	GW5	+++	++	++	-	+++	++	+	+

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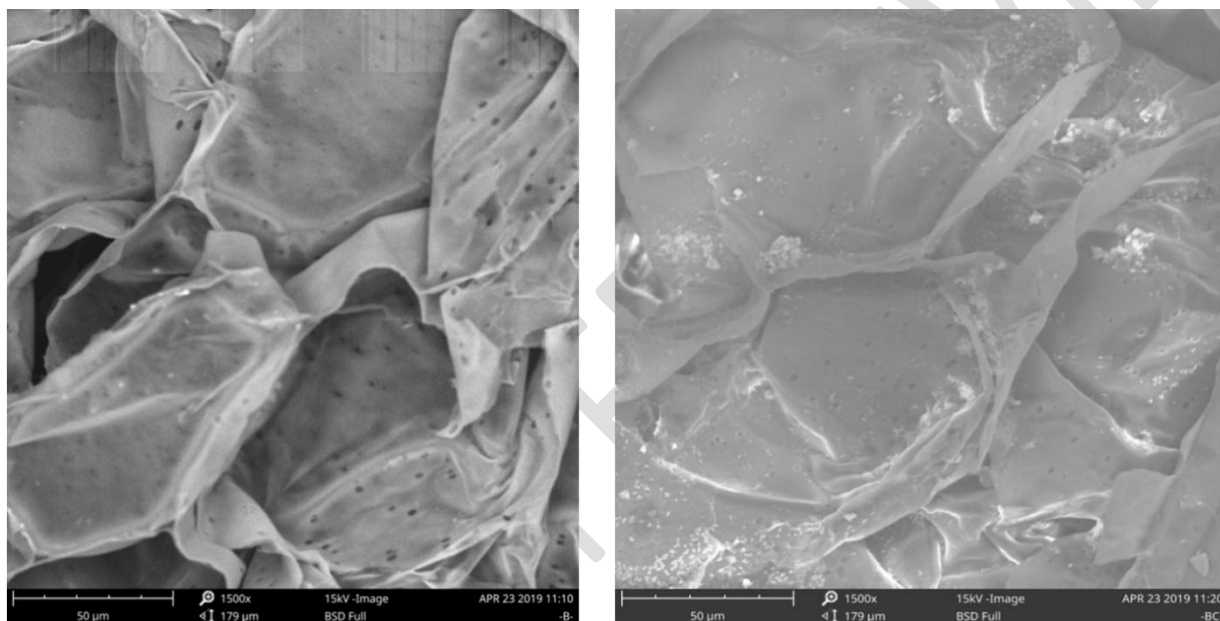
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146 **Table 3: Surface properties and proximate analysis of the agro-based biocarriers**

Biocarriers	Parameters							
	BET surface area (m <sup>2</sup> /g)	Micropore volume (cc/g)	Ash	Crude fat	Crude lipid	Moisture	Crude protein	Carbohydrate
Melon husk	782.1	0.121	6.2±0.21	48.1±0.85	12.2±0.42	8.7±0.21	4.7±0.07	19.6±0.64
Groundnut shell	424.8	0.164	2.8±0.35	58.5±0.70	0.6±0.07	7.9±0.14	4.6±0.28	25.7±0.14
Sugarcane bagasse	532.5	0.155	2.0±0.07	30.8±0.57	1.9±0.14	9.8±0.35	1.9±0.21	52.8±0.56

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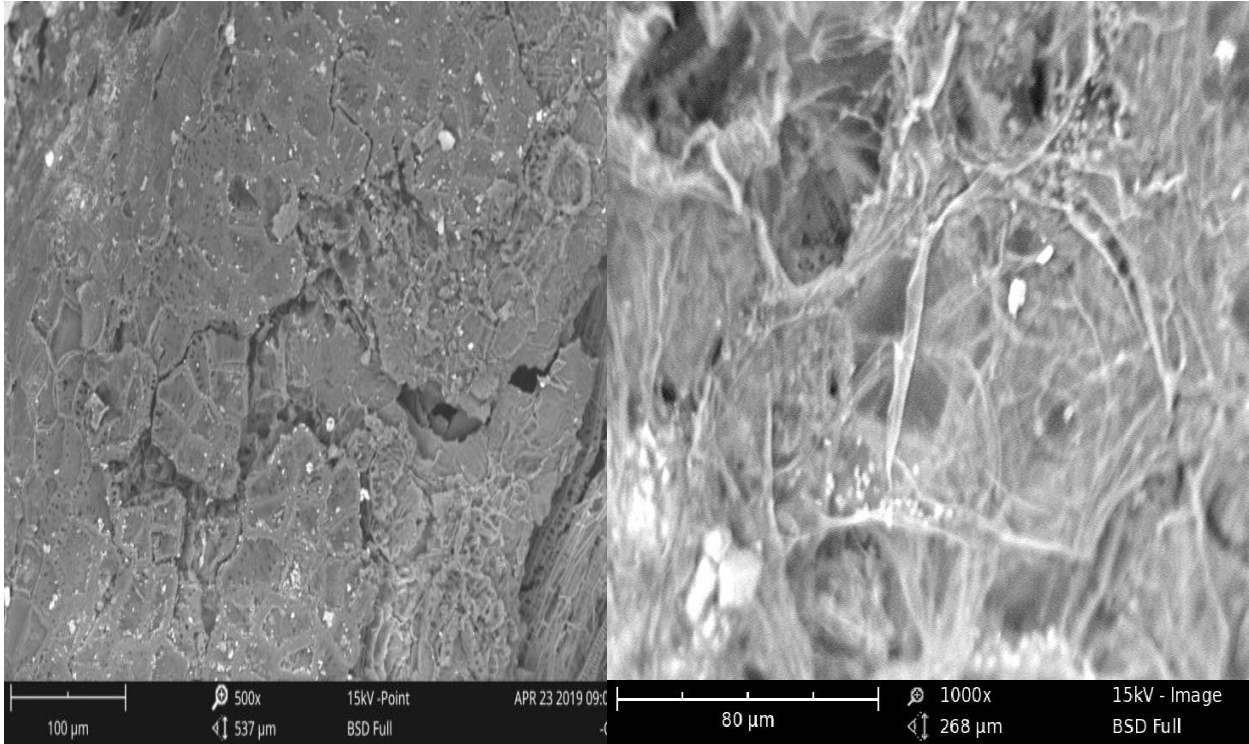
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150 **Figure 1:** Scanning electron microscopic images of sugarcane bagasse without the consortium (A), with the  
 151 consortium (B)

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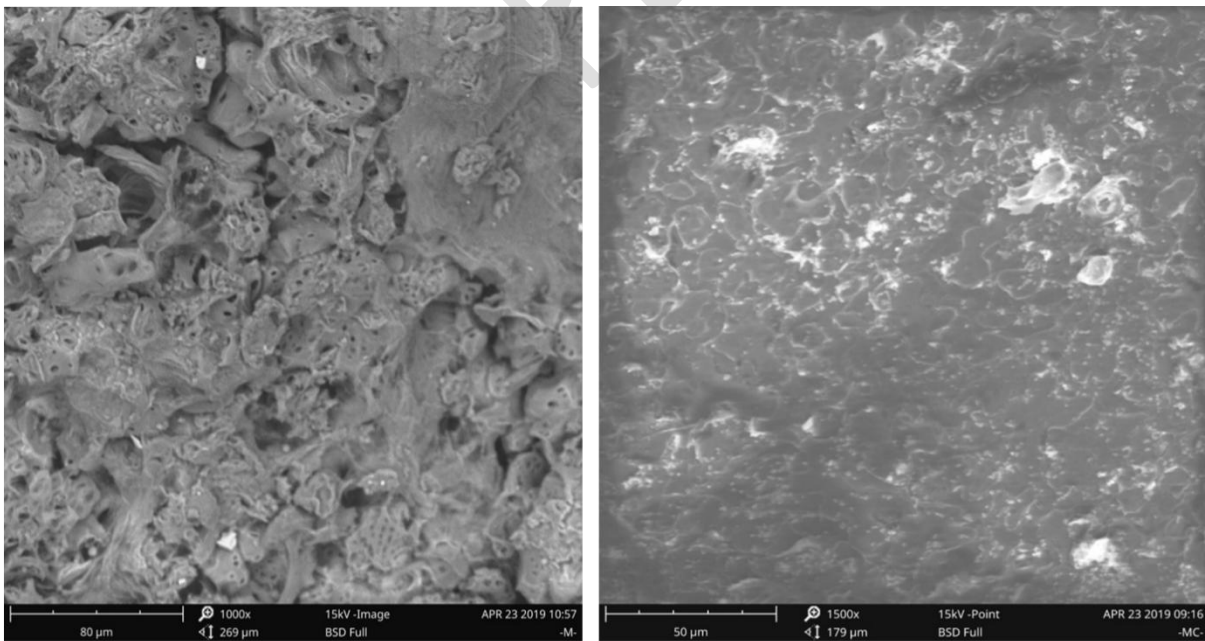


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156

A

B

**Figure 2:** Scanning electron microscopic images of groundnut shell without the consortium (A), with the consortium (B)



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**Figure 3:** Scanning electron microscopic images of melon husk without the consortium (A), with the consortium (B)

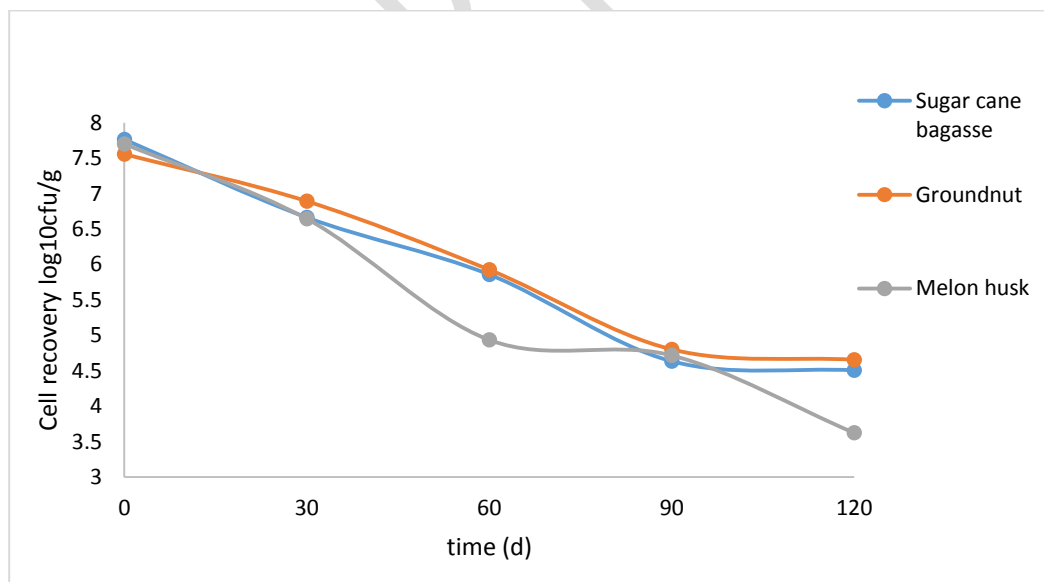


159 **Table 4:** Comparison of refinery wastewater effluents with the different treatment options  
 160 and discharge standard of DPR/EGASPIN

parameters	DPR specification	Raw wastewater	Treated wastewater	Sugarcane bagasse	Groundnut shell	Melon husk	Unimmobilized consortium
pH	-	7.9±0.20	6.4±0.18	7.0±0.06	7.1±0.058	6.9±0.13	6.9±0.10
Conductivity	1400	140.2±6.20	168.2±5.4	159.7±1.80	168.3±2.8	167.2±1.13	158.1±4.25
Salinity	NA	28.1±0.30	18.4±1.2	18.5±0.14	16.3±0.57	15.1±0.14	14.9±0.16
TDS	<2000	464.6±18.5	248.4±14.2	226.4±26.8	245.0±8.6	268.3±11.4	250.0±12.8
Phenol	0.5	96.8±6.54	1.2±0.60	0.7±0.10	0.29±0.04	2.5±0.28	5.62±1.20
BOD <sub>5</sub>	10	146.8±8.24	18.6±0.51	4.6±0.49	6.4±0.49	14.5±0.21	14.8±0.46
COD	40	269.4±12.50	38.4±1.2	24.5±0.21	28.4±0.28	46.6±0.00	48.6±2.51
Oil and Grease	10	48.5±0.5	8.6±0.21	2.7±0.14	6.3±0.64	12.5±0.07	17.6±1.32
Ammonia-nitrogen	0.2	0.91±0.21	0.62±0.11	0.19±0.01	0.31±0.01	0.38±0.03	0.21±0.10
Phosphate	0.2	1.8±0.21	2.20±0.42	0.42±0.00	0.81±0.01	0.60±0.01	0.26±0.00
Iron	1.0	0.52±0.20	0.25±0.01	0.18±0.01	0.21±0.01	0.19±0.00	0.22±0.01
Zinc	1.0	0.06±0.00	0.02±0.01	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00

161 *Data presented as Mean ± Standard deviation; Similar superscripts in a column imply there was no*  
 162 *significant difference, those with different superscripts are significant at p-value <0.05 (all results*  
 163 *are in mg/l, conductivity μS/cm )*  
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166

167 Figure 4: Variation in total recoverable heterotrophic bacteria counts during storage period

168 **DISCUSSION**

169 This work aims at mitigating the challenges of maintaining high bacterial biomass on contaminated sites by  
170 bacterial immobilization on organic biocarriers. Figures 1 to 3 shows the scanning electron micrographs of  
171 the adsorbed consortium on the agro-based biocarriers. Surfaces properties affects bacterial adsorption on  
172 them. Organic biocarriers have many functional groups and this affects the degree of colonization by  
173 microorganisms (Dzionic, Wojcieszynska, & Guzik, 2016). The micrographs shows strong attachment of  
174 the consortium on the pores and surfaces of the agro-wastes. Cell immobilization through adsorption brings  
175 about a direct contact between nutrients and immobilized cells (Lin, Liu, Chen, Wang, & Hu, 2014).  
176 Udawatte & Sotheeswaran, (2015) suggested that nutrient absorbed from carrier substrates could be an  
177 advantage to efficient colonization.

178 The results for screening of potential phenol degrading strains as shown in table 3 indicated the ability of  
179 the isolates to thrive in concentrations of 50mg/100ml. All the isolates produced visible colonies after five  
180 days of incubation at 170 r.p.m at 37°C. At concentration of 100mg/ml, only the *Bacillus tequilensis*  
181 produced three visible colonies after 72 hours of incubation.

182 Results of the 15 days laboratory scale treatment of refinery raw wastewater is presented in table 4. The  
183 initial concentration of phenol in both the raw wastewater and the refinery treated wastewater were higher  
184 than the DPR effluent discharge standard. After the treatment period, 99.7% removal of phenol was  
185 recorded in GS-treatment option while the least removal was in the FB-treatment. However, only the GS-  
186 treatment reduced phenol concentration below the DPR limit.

187 Phenol degradation by microorganisms can involve the use of enzymes such as hydroxylase,  
188 monooxygenase and dioxygenase (Rehman and Ilyas, 2008). Aerobically, phenol is first converted (through  
189 oxygenation) to catechol, and subsequently degraded via the ortho or meta fission to intermediates of  
190 central metabolism. This ring fission process is catalyzed by either an ortho cleaving enzyme, catechol 1,

191 2- dioxygenase or by a meta cleaving 2, 3- dioxygenase enzyme (Sridevi, Lakshmi, Manasa, & Sravani,  
192 2012) Compared to physical and chemical methods, biological treatment are preferable as it is relatively  
193 cheaper and reduces the challenges of by-products production.

194 Phenol removal in refinery effluents has been a very challenging process in wastewater treatment. The  
195 chemical treatment with hydrogen peroxide prior to loading in the rotary bio disc is a very expensive  
196 procedure. The result shows that the selected consortium were able to degrade the phenol in the  
197 wastewater. The bacteria employed as a consortium in this study have previously been associated in  
198 hydrocarbon degradation. *Pseudomonas aeruginosa* and *Bacillus* sp. had been used as a consortium in  
199 phenol degradation (Poi et al., 2017; Velmurugan & Arunachalam, 2009) while *Micrococcus luteus* had  
200 previously been mentioned as hydrocarbon degraders (Obuekwe & Al-Muttawa, 2001; Rehman and Ilyas  
201 2008).

202 Results of the physicochemical parameters of the Port Harcourt refinery raw wastewater effluents before  
203 and after treatment with the agro-waste immobilized bacterial consortium are presented in table 4. The  
204 major source of concern in effluent discharge into the environment include the presence of polycyclic and  
205 aromatic hydrocarbons especially phenol, metal derivatives, high COD and BOD<sub>5</sub>.

206 BOD<sub>5</sub> and COD values are often used as an indicator of water quality and their removal efficiencies are  
207 used to comparatively analyze a variety of wastewater treatment systems (Baharvand, Reza, & Daneshvar,  
208 2019). The efficiency of treatment plants can be assessed based on the chemical parameters of BOD and  
209 COD (Carducci & Verani, 2013). The BOD<sub>5</sub> and COD values in the raw wastewater were 146.8±8.24 and  
210 269.4±12.50. After a 15-day treatment with the different carrier-immobilized consortia, BOD<sub>5</sub> values of  
211 4.6±0.49, 6.4±0.47, 14.5±0.21 and COD values of 24.5±0.21, 28.4±0.28 and 46.6±0.00 were obtained for  
212 SB, GS and MH respectively while the unimmobilized consortium had values of 14.8±0.46 and  
213 48.6±2.51 for BOD<sub>5</sub> and COD respectively. The results indicated that the immobilized consortium were

214 more effective in the treatment process than the free form of the consortium, with SB and GS options  
215 showing higher efficiencies in BOD<sub>5</sub> and COD removal.

216 When oil containing hydrocarbons are discharged into water body, they can cause depletion of  
217 dissolved oxygen due to transformation of organic components into inorganic compounds (Ajao, Yakubu,  
218 Umoh, & Ameh, 2013), and this has potential damaging effects on aquatic organisms. Oil and grease  
219 values were reduced by 94%, 87%, 74% and 64% in the SB, GS, MH and FB treatment options. Oil and  
220 grease removal was highest in SB treatment option and least in the unimmobilized consortium.

221 Bacterial recovery during storage is shown in figure 4. After storage for 120 days, the highest cell recovery  
222 was recorded in the GS-immobilized consortium and the least was in the MH-immobilized consortium.  
223 Viable counts of up to 10<sup>4</sup> for GS and SB, and 10<sup>3</sup> for MH were recorded after 120 days from initial count  
224 of 10<sup>7</sup>. Nuñal et al., (2014) suggested that the obtained bacterial counts may be lower than the actual counts  
225 as strongly adsorbed bacteria may be difficult to dislodge. Nuñal et al., (2014) had reported cell viability  
226 after storage of rice hull and cocopeat-immobilized bacterial consortia at temperatures of -30°C, 0°C, and  
227 room temperatures after six months. This result suggest that immobilized cells can be stored without losing  
228 their metabolic activities.

## 229 **Conclusion**

230 This work underscores the potential of agro-waste immobilized consortium of three hydrocarbon utilizing  
231 bacteria isolated from oil polluted environment in Bodo creeks, of Gokana local government area of Rivers  
232 state to effectively treat refinery raw wastewater. Bacteria immobilization on agro-waste materials could be  
233 introduced into refinery wastewater treatment protocols as a means of enhancing the treatment process.

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