Original Research Article

Treatment of Port Harcourt refinery effluent by a bacterial consortium immobilized on agro-based bio carriers

Abstract

Discharge of poorly treated refinery wastewater has always been a major environmental challenge. Bacterial immobilization is key to the maintenance of biomass on a 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 bio carriers 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 immobilised and immobilized consortium. The agro-based residue immobilized consortium enhanced the reduction in BOD_5, 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.

Keywords: immobilized consortium, bioremediation, refinery raw wastewater, bio carriers, 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 discharged from refinery industries do not meet minimum set standards (Osin et al, 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 contains most of the examined pollution parameters in concentrations above the set regulatory limits.

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

being processed, the plant configuration and operational procedures (Hasan et al, 2010; Ishak et al, 2012). Ishak et al., (2012) reported that the major constituents of refinery wastewater, in general, are dissolved and dispersed oil (a mixture of hydrocarbons – benzene, toluene, ethylbenzene, xylenes, polyaromatic hydrocarbons and phenols) and dissolved formation minerals (anions and cations including heavy metals). Phenol contaminants are relatively soluble in water and their severe toxicity even in low concentration have been reported worldwide (Mahiuddin et al, 2012). The removal of phenol from effluents has been quite challenging and expensive. Of the different technologies being applied to remove phenolic compounds from polluted areas, biodegradation process is relatively low cost, no chemicals used, and high public acceptance tends to destroy the pollutants if possible or at least to transform them to less harmful forms (Mahiuddin et al 2012, Poi et al, 2017).

Microbial immobilization

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

Materials and Method

Bacteria source and consortium

The bacterial consortium used in this study were isolated from crude oil polluted creeks of Bodo, in Gokana local government area of Rivers State. The water samples were enriched in 98ml Bushnell Haas media (BHM), prepared according to manufacturer's specification and supplemented with 1% Bonny Light crude oil (BLCO). One per cent crude polluted pond water samples were added to the sterile setup and incubated in an orbital shaker incubator (Stuart, Germany S150) at 170 r.p.m at 37^{o}C for seven days (Ekwuabu et al, 2016; Xue et al., 2017). Further enrichments were conducted to obtain the selected strains, which were sub-cultured separately in nutrient broth medium for 24 hours. After incubation, the cells were

harvested by centrifugation, washed with normal saline (0.85% NaCl) and re-suspended in fresh normal saline. Equal volumes of the suspension containing the different bacterial strains were mixed to form the consortium used in the study.

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

Screening for phenol and naphthalene degrading organism

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

Enrichment of the isolated phenol and naphthalene degrading strains

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

Selection of potential phenol and naphthalene degrading strains (Plate method)

Minimal agar medium was prepared with increasing concentration of selected phenol and naphthalene as 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 enriched cultures were inoculated on minimal agar media. Plates were incubated at room temperature for 48-72 hrs.

Biocarriers

Raw materials used as bio carriers (sugarcane bagasse, groundnut shell and melon husk) were all obtained from Nchia Market in Eleme local government area of Rivers State. These agro-based residues were first

air-dried, grind mechanically and sieved to obtain a homogenous particle size of 0.3-0.5mm. They were washed sequentially with ethanol and distilled water several times to prevent the impurities from affecting the growth and immobilization of the bacteria. The powder was dispensed in vials and sterilized by autoclaving at 120˚C, 1 atm for 15 min and kept at room temperature until use.

Surface area and pore distribution of carriers

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

Scanning electron microscopy

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

Immobilization of bacterial consortium

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

To determine the population of the bacteria consortium that immobilized on the bio carriers. They were washed with saline solution by centrifugation at 4000 rpm for 10 minutes to remove microorganisms that were not immobilized on the carriers. The microorganisms immobilized on the bio carriers were harvested by centrifugation at 8000 rpm for 10 min. The number of bacteria was approximately 5×10^7 CFU/ml in the experiment and was determined using the dilution plate method.

Wastewater sample collection

The wastewater samples were collected from the raw wastewater and treated wastewater reservoirs in Port Harcourt Refinery Eleme, Nigeria, and transferred to the laboratory immediately for analysis. All the collected samples were preserved and processed following standard guidelines. The samples were analysed for pH, Biological Oxygen Demand (BOD_5), Chemical Oxygen Demand **(**COD), total dissolved solids, oil and grease, phenol, sulphide and some selected heavy metals.

Bioremediation using immobilized mixed culture

The efficacy of the immobilized consortium in bioremediation of refinery wastewater was determined in microcosm trials. Batch cultures were performed using one-litre conical flasks containing 500ml refinery wastewater autoclaved at 121^{o}C for 15 minutes at 15 psi. On cooling, 0.5g of the immobilized consortium is introduced separately in each of the conical flasks. The control had the sterilized raw wastewater only. The different treatment options and the content of each representative flask are shown in table 1. The samples were analyzed for pH, Biological Oxygen Demand (BOD_5), Chemical Oxygen Demand **(**COD), total dissolved solids, oil and grease, phenol, sulphide and some selected heavy metals on every five days intervals

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 the Melon husk	MH	Immobilized consortium + sterile raw wastewater
4	The free form of bacterial consortium	FB	The free form of consortium + sterile raw wastewater
5	Control	NB	Sterile raw wastewater

Table 2: Result for the 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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145 **Table 3: Surface properties and proximate analysis of the agro-based bio carriers**

Biocarriers	Parameters							
	BET surface area (m^2/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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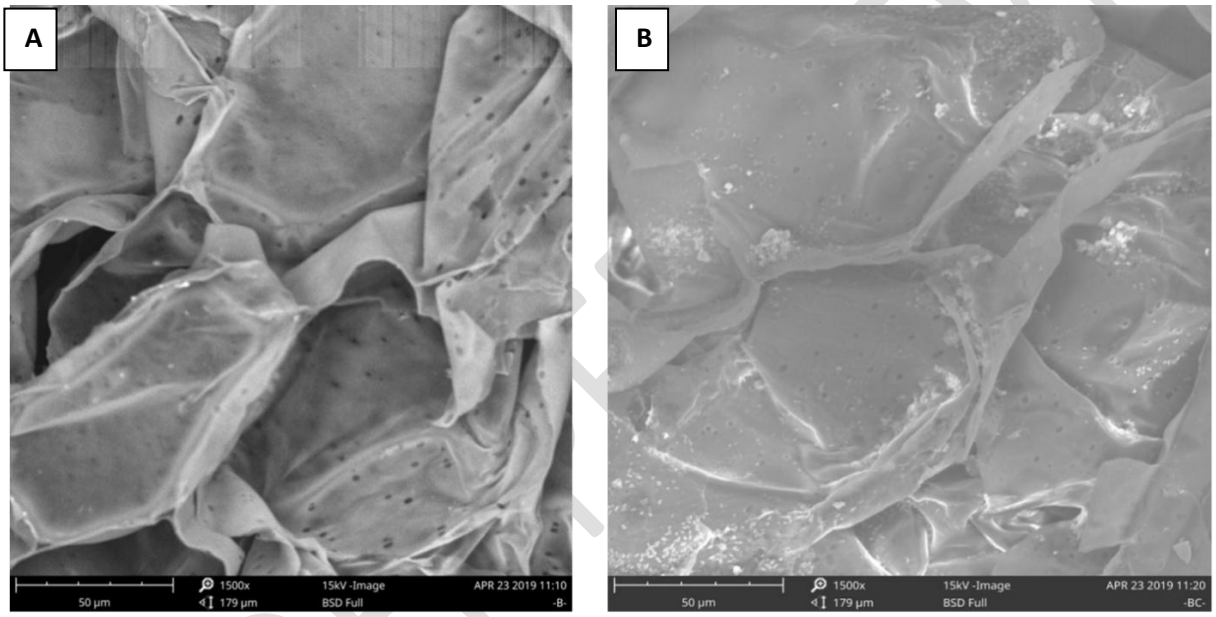

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149 **Figure 1**: Scanning electron microscopic images of sugarcane bagasse without the consortium (A), with the
150 consortium (B)

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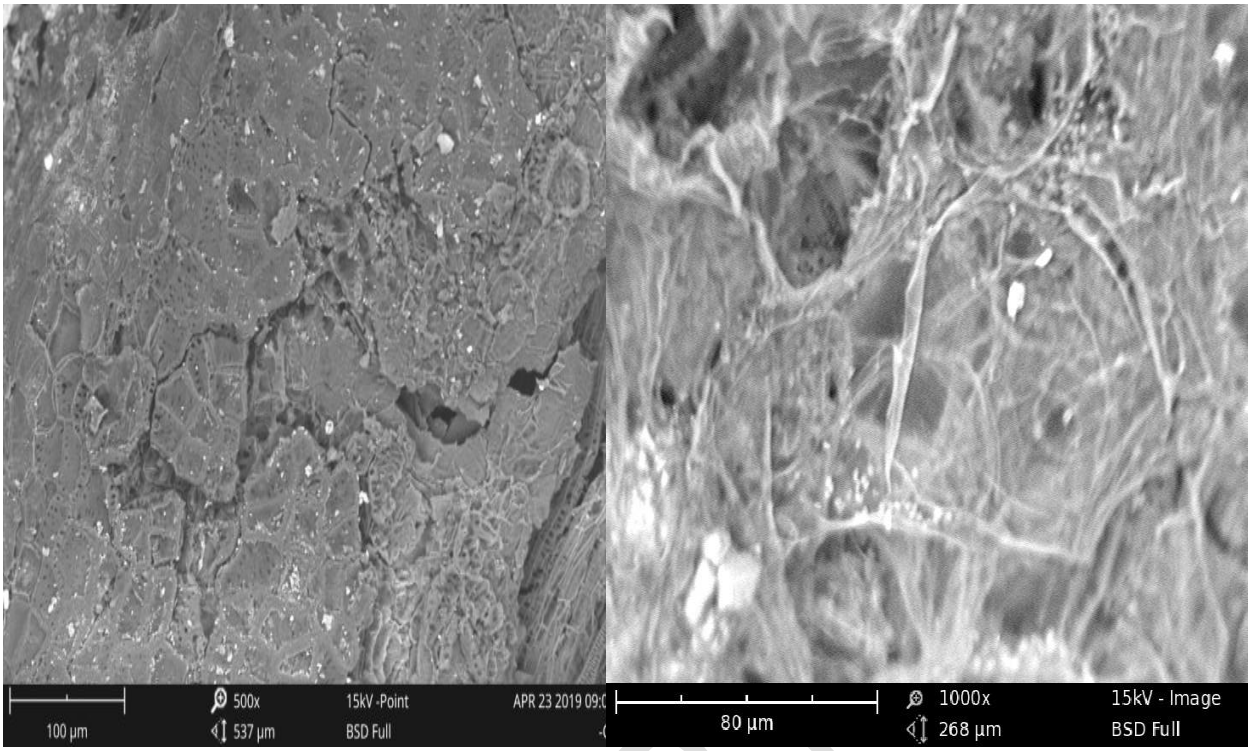

A B

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

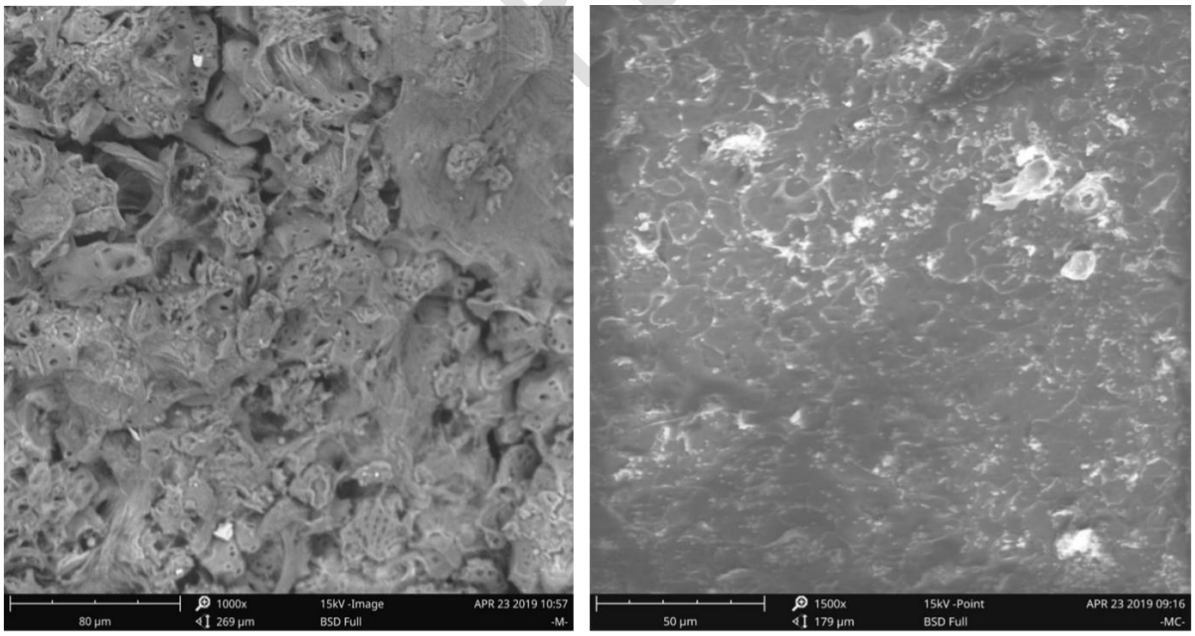

Figure 3: Scanning electron microscopic images of melon husk without the consortium (A), with the consortium (B)

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

parameters	DPR specification	Raw wastewater	Treated wastewater	Sugarcane bagasse	Groundnut shell	Melon husk	An immobilized consortium
pH	-	7.9±0.20	6.4±0.18	7.0±.06	7.1±.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_5	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

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

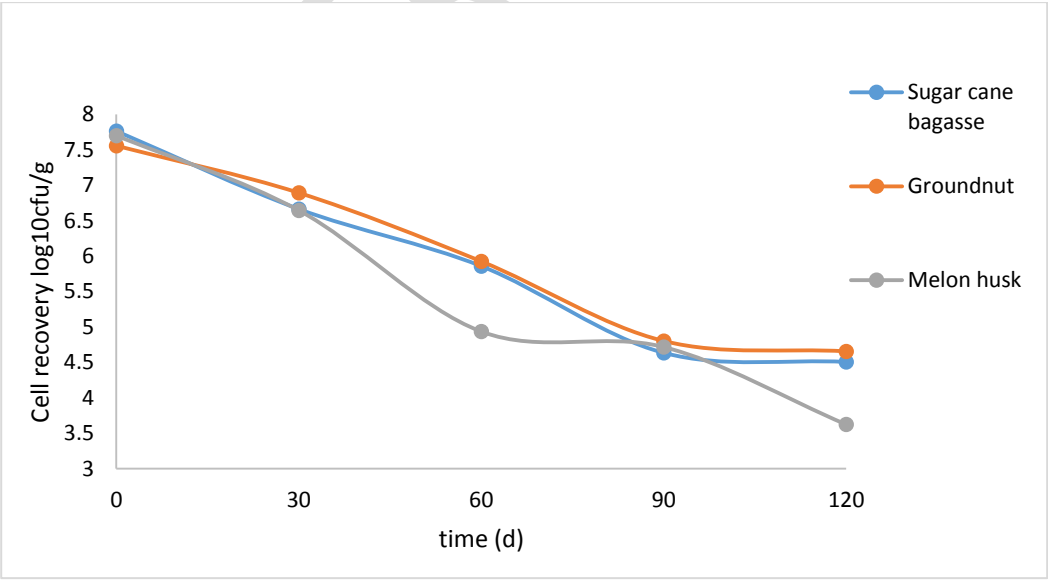

Figure 4: Variation in total recoverable heterotrophic bacteria counts during the storage period

DISCUSSION

This work aims at mitigating the challenges of maintaining high bacterial biomass on contaminated sites by bacterial immobilization on organic bio carriers. Figures 1 to 3 shows the scanning electron micrographs of the adsorbed consortium on the agro-based bio carriers. Surfaces properties affect bacterial adsorption on them. Organic bio carriers have many functional groups and this affects the degree of colonization by microorganisms (Dzionek et al, 2016). The micrographs show the strong attachment of the consortium on the pores and surfaces of the agro-wastes. Cell immobilization through adsorption brings about direct contact between nutrients and immobilized cells (Lin et al, 2014). Udawatte & Sotheeswaran, (2015) suggested that nutrient absorbed from carrier substrates could be an advantage to efficient colonization.

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

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

Phenol degradation by microorganisms can involve the use of enzymes such as hydroxylase, monooxygenase and dioxygenase (Rehman and Ilyas, 2008). Aerobically, phenol is first converted (through oxygenation) to catechol and subsequently degraded via the ortho or metha fission to intermediates of central metabolism. This ring fission process is catalyzed by either an ortho cleaving enzyme, catechol 1, 2- dioxygenase or by a meta cleaving 2, 3- dioxygenase enzyme (Sridevi et al, 2012)

Compared to physical and chemical methods, biological treatment is preferable as it is relatively cheaper and reduces the challenges of by-products production.

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

Results of the physicochemical parameters of the Port Harcourt refinery raw wastewater effluents before and after treatment with the agro-waste immobilized bacterial consortium are presented in table 4. The major source of concern in an effluent discharge into the environment includes the presence of polycyclic and aromatic hydrocarbons especially phenol, metal derivatives, high COD and BOD_5.

BOD_5 and COD values are often used as an indicator of water quality and their removal efficiencies are used to comparatively analyze a variety of wastewater treatment systems (Baharvand, Reza, & Daneshvar, 2019). The efficiency of treatment plants can be assessed based on the chemical parameters of BOD and COD (Carducci & Verani, 2013). The BOD_5 and COD values in the raw wastewater were 146.8±8.24 and 269.4±12.50. After a 15-day treatment with the different carrier-immobilized consortia, BOD_5 values of 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 SB, GS and MH respectively while the immobilised consortium had values of 14.8±0.46 and 48.6±2.51for BOD_5 and COD respectively. The results indicated that the immobilized consortium was more effective in the treatment process than the free form of the consortium, with SB and GS options showing higher efficiencies in BOD_5 and COD removal.

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

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

Conclusion

This work underscores the potential of an agro-waste immobilized consortium of three hydrocarbon utilizing bacteria isolated from the oil-polluted environment in Bodo creeks, of Gokana local government area of Rivers state to effectively treat refinery raw wastewater. Bacteria immobilization on agro-waste materials could be introduced into refinery wastewater treatment protocols as a means of enhancing the treatment process. The agro-based residue immobilized consortium enhanced the reduction in BOD_5, 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.

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