Chromium Biosorption Potential of *Rummeliibacillus stabekisii* (MN250294.1) and *Alcaligenes faecalis* (MN250293.1) using Fermented Palm Oil Mill Effluent as Carbon Source

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Abstract BACKGROUND AND OBJECTIVE: Heavy metal contaminated water is said to negatively impact environment and it keeps increasing. Treating such water is a challenge since the conventional methods are costly. There is continuous search globally for biological methods which are cost effective and environmentally friendly. Thus, this study aim at evaluating the ability of *Rummeliibacillus stabekisii*(MN250294.1) and *Alcaligenes faecalis* (MN250293.1) to remove chromium in a metal-ladened mine drainage using fermented palm oil mill effluent as carbon source.

METHODS: Metal tolerant *Rummeliibacillus stabekisii* (MN250294.1) and *Alcaligenes faecalis* (MN250293.1) were screened for volatile fatty acids utilization and pH of growth medium using standard methods. Fresh palm oil mill effluent (POME) was fermented in a digester. Biotreatment of the mine drainage samples using fermented POME at 10 and 20% as carbon source was done. Chromium concentration were determined by atomic absorption spectrometry (Model 210 VGP) in triplicate. Data were analysed using independent T-test at $P < 0.05$.

FINDINGS: The volatile fatty acids were utilized at varied level. Both isolates least utilized butyric acid. Optimum growth value was recorded at pH 7.0 for both isolates. After the 21 days of fermentation, propionic acid had the highest concentration of 1.15%. At 20% fermented POME, 100% removal of Cr was achieved by both isolates singly and in combination. The mean percentage reduction of chromium by both isolates in combination ($\overline{X}$ = 100.00) is significantly higher than when the isolates were used singly ($\overline{X}$ = 95.31).

CONCLUSION: Total chromium remediation was achieved by both isolates *Rummeliibacillus stabekisii* and *Alcaligenes feacalis.* Fermented POME is efficient carbon source.

Keywords: Atomic Absorption Spectrometry; Biotreatment; Metal Tolerant; Mine Drainage; Volatile Fatty Acids.

INTRODUCTION

Heavy metals such as chromium, nickel and copper are natural elements having atomic number greater than twenty with relatively high density (at least 5gcm-3) and toxic even at low concentration (1,2). They exist as components found in the environment and have been harnessed for use as importance part of different components especially in the manufacturing companies by human activities [3].

Heavy metals are generated from both anthropogenic sources such as mining and natural sources such as volcanic eruption and weathering of metal-bearing rocks and are eventually discharged into the environment [2,4,]. These has caused a wide spread disruption of the normal biogeochemical cycles of metals leading to a huge deposition of heavy metals in the environment (4,5).

Mainly, chromium, lead, cadmium, arsenic, copper and mercury are of major concern due to their toxic impact on human heath Such impact in human include; fatique, and irritability (Chromium), renal dysfunction and lung cancer (Cd), bronchitis and dermatitis (As), mental retardation in children and congenital paralysis (Pb), central nervous system damage (Mg), tremors and spontaneous abortion (Mercury), nervous membrane damage (Zn), liver and kidney damage (Cu) among many others (6). Rai et al. (7)

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reported that excessive amount of most metals in the environment can degrade soil qualities, reduce crop production and diminish product value of agriculture. The presence of heavy metals in the environment has been a source of concern over the past few decades due to their persistence, potential harm and toxicological hazards (2). The increase in industrialization and urbanization offer ascend to environmental heavy metal pollution which might have resulted from the discharge of effluents containing metals (4,8). Among the various industrial sectors such as iron and steel, textiles and leather, mining and mineral processing, pulp and paper varies, mining and mineral processing facilities produce higher quantity of hazardous and toxic waste than any other industrial sector (9).

In order to alleviate the environmental impacts of heavy metal, several efforts are currently being adopted. Such methods includes thermal, precipitation, chelating, adsorption, ion exchange, membrane technologies and biosorption strategies. However, biosorption has several advantages over other conventional methods of heavy metal remediation due to its accessibility and efficiency (8). One economic importance of biosorption technology is that the biomass used for metal decontamination is natural, easily available and affordable, and it also provides a better performance compared to conventional methods of decontamination. (10,11).

Biosorption is a biological remediation technology that involves the removal of metal species from a solution by inexpensive biomaterials. Most biomaterials has been reportedly said to be useful biosorbents for heavy metals sequestration and serve as an important passive procedure in organisms except mobile alkali metal cations like K+ and Na+ (12). Most biosorbent materials have excellent biosorption capacities towards all types of metal ions, therefore many affordable and available biosorbents used for heavy metal removal in the environment are majorly derived from bacteria, fungi, algae, plants, and some polysaccharide materials.

Biosorbents of biological origin particularly various microorganisms have received growing interest for heavy metal removal and recovery owing to their greater performance (4, 13). Although biosorption is affected by diverse factors such as temperature, pH, contact time (14), the use of microorganisms as biosorbent materials offers a selective removal of heavy metals under varied physicochemical properties, adsorption and desorption, and high surface area to volume ratio (4,10)

In mine drainage polluted water, sulfate reduction through sulfate reducing bacteria activities is the major technique used in metal removal and recovery. Nevertheless, because concentration of some metals may be highly toxic to sulfate reducing bacterial activity in mine drainage whereas certain aerobic bacteria are capable of resistance to these metal concentrations, a synergic combination of a treatment system for the removal of metals by other biological processes capable of removing metals (such as biosorption) to reduce the inhibiting concentration of metals in mine drainage before biological sulfate reduction has been suggested (15).

Usually in mine drainage, total organic carbon is limited especially in acidic waters (16). Therefore, in microbial treatment of mine drainage, an external supply of carbon source is considered necessary for efficient and effective remediation.

In mine drainage biological treatment, monocarboxylic acids especially the volatile fatty acids and hydrogen are considered to be the preferred carbon source because of their low molecular weight and are effectively utilized by sulfate reducing bacteria in the sulfate reduction process which is considered as the prime biological treatment process for effective simultaneous removal of both metals and sulfate ion (16, 17).

One of the major sources of VFAs is palm oil mill effluent (POME), an effluent from the final oil stages of palm oil production in the mill (18).

Nigeria is one of the largest producers of palm oil in West Africa and there are about 20 oil palm growing states in Nigeria including Osun State (19). Palm oil mill effluent (POME) is a general phrase referring to the effluent from the final oil stages of palm oil production in the mill. POME in its untreated form is a very high strength waste, depending on the operation of the process, the biochemical oxygen demand (BOD) of these wastes ranges from 25000 to 35000 mg/L. It contains about 94% water (20). It is considered as one of the most polluting agro-industrial effluent due to its high values of Chemical Oxygen Demand (COD) and BOD (20), however it is an ideal substrate for bioprocessing because it contains high level of degradable organic material such as lipids, proteins and carbohydrates which results in a net positive energy (20). POME is acidic (pH 4–5), has discharge temperature of 50–60°C and is non-toxic (20).

In the anaerobic digestion of POME, water, carbon dioxide and methane are produced and the order of reactions involved are; hydrolysis, acidogenesis containing acetogenesis and methanogenesis. Volatile fatty acids are produced at acidogenesis stage as the intermediate, and its rapid production is attributed to pH decreased during

acidification which stressed and inhibited the activity of methanogenic bacteria. Therefore, methanogenesis is a rate limiting step (21). This has become advantageous for volatile fatty acids production from POME which then serves as substrate for various bioprocesses such as production of bioplastics (21). However, information on the use of fermented POME to treat mine drainage is very scarce. Therefore, this study is aim at determining the biosorption rate of chromium from mine drainage water by the previously isolated indigenous aerobic bacteria, *Rummeliibacillus stabekisii* (MN250294.1) and *Alcaligenes faecalis* (MN250293.1) (22) using Fermented Palm Oil Mill Effluent as Carbon Source.

MATERIALS AND METHODS

Collection of Bacterial Isolates

Freshly isolated and identified bacterial isolates (*Rummeliibacillus stabekisii* (MN250294.1) and *Alcaligenes faecalis* (MN250293.1)) were collected from Environmental and Biotechnology Laboratory, Microbiology Department, University of Ibadan, Ibadan, Nigeria.

Screening of bacteria Isolates for Volatile Fatty Acids Utilization

The selected aerobic bacterial isolates were screened after purification for their ability to utilize volatile fatty acids such as acetic acid, propionic acid and butyric acid as sole carbon sources using modified method of Van and Chang (23). Growth medium containing (g/l): Sodium Chloride, 1.0; Potassium dihydrogen phosphate, 0.5; Ammonium chloride, 1.0; Magnesium sulfate, 0.05 and each 0.10% C (v/v) sodium acetic acid, propionic acid or butyric acid, the pH was adjusted to 7.0±2 with 1 M NaOH and dispensed in test-tubes. The test-tubes were corked, sterilized and then allowed to cool. Inoculation of the tubes done with loopful of 18-24 hour old culture of each selected isolate and then incubated at 28±2oC. Growth was monitored at 24 hours intervals by measuring the Optical Density (OD) of each isolate at a wavelength of 620 nm using photoelectric colorimeter (model T11D, Techmel and Techmel, USA).

Growth of Bacterial Isolates at different pH.

Growth of the selected aerobic bacterial isolates in peptone water at defferent pH was tested. The pH of the peptone water used was adjusted to pH 3, 5, 7, 9, and 11 with 1 M NaOH or HCl. The medium was dispensed in test-tubes, corked, sterilized and allowed to cool. Tubes were inoculated with loopful of selected bacterial culture (18-24 hour old) and incubated at 28±2oC. Growth was monitored at 24 hours intervals by measuring the Optical Density (OD) of each isolate at a wavelength of 620 nm using photoelectric colorimeter (model T11D, Techmel and Techmel, USA)

Acidogenic Fermentation of Fresh Palm Oil Mill Effluent (POME) to Produce Volatile Fatty Acids (VFAs) as carbon sources

Inoculum Sludge Preparation

Palm oil mill effluent sludge and fresh palm oil mill effluent were collected from a conventional POME pond and POME factory respectively at Igun town, Osun State. The fresh Palm Oil Mill Effluent (POME) was diluted with tap water in ratio 1:4 (v/v) respectively. Equal volume of the POME sludge and the diluted fresh POME were mixed thoroughly. 0.01% thioglycolic acid as a reducing agent and 0.01% Ascorbic acid were added to the mixture and then poured in a clean, air-tight 8 litres Fermenter for up to 10 days at 28±2oC for proper microbial adaptation.

Acidogenic Fermentation of Fresh Palm Oil Mill Effluent (POME)

The acidogenic fermentation of fresh palm oil mill effluent was carried out using the modified method of Maaroff et al. (24). The fermentation experiment was carried out in a clean, air-tight 8 litre Fermenter. The fresh POME was inoculated with 10% (v/v) of the prepared inoculum sludge. 0.01% thioglycolic acid as a reducing agent and 0.01% Ascorbic acid were added to the mixture in the Fermenter. The contents were well mixed and the fermenter tightly covered. Methylene blue solution was used as redox indicator which turns colourless as soon as anaerobic condition was attained within the Fermenter. Sample was taken at 2 days interval through the sampling outlet for analyses

Determination of the Volatile Fatty Acids Produced in the Fermented Palm Oil Mill Effluent (POME).

Volatile fatty acids content, mainly: acetic acid, propionic acid and butyric acid of the fermented POME were determined using the method of APHA (25). A 50ml volume of POME sample was measured into a 200ml Erlenmeyer flask; 20ml benzene was added and shaken thoroughly to extract the fatty acids. The mixture was transferred into a 250ml separation funnel to separate the benzene extract from the aqueous extract. A 5ml aliquot of the benzene extract was pipetted into a 15ml test-tube and 2ml of 10% Copper acetate was added to develop a blue colour. Standard solutions of each volatile fatty acid were prepared in the range 0-10ppm from 100ppm stock solution of each fatty acid. Optical density of sample extract as well as standard solutions of different concentrations was read on a spectrophotometer at a wavelength of 585nm, 515nm and 570nm for Acetic acid, Propionic acid and Butyric acid respectively. The % of each Fatty Acid is obtained using the formula:

$$\% \text{ Fatty Acid} = \frac{\text{Absorbance of sample X GF of a Specific Fatty Acid X DF}}{\text{Volume of Sample X 10,000}}$$

Where DF = Dilution Factor
GF = Gradient Factor

Bioremediation of Abandoned Mine Drainage

The bioremediation of the mine drainage water sample with the use of the bacterial isolates was carried out using the modified method of Murthy et al. (26). To 100 mL drainage water sample, salts containing (g/l): Sodium Chloride, 1.0; Potassium dihydrogen phosphate, 0.5; Ammonium chloride, 1.0; Magnesium chloride, 0.05 and 10% or 20% fermented POME as carbon source were added. The flasks were corked, sterilized and then allowed to cool. Each flask was inoculated with aliquots (10%) of 1.0 McFarland standard (approximately 3 x 108cells) of the suspension of each bacterial isolate of 18-24 hour old. All the set up was incubated at 28±2oC in an orbital shaker at 150 rpm for 3 days. Samples were taken after 3 days and centrifuged at 10000 rpm for 10 minutes at 4oC in a cold centrifuge [Hitachi High Speed Refrigerated Centrifuge (HIMAC CR21GII)] to remove the bacterial cells. The chromium concentration in the supernatant were determined by atomic absorption spectrometry (Model 210 VGP) with air- acetylene gas mixture as oxidant.

RESULTS

Volatile Fatty Acids (VFAs) Utilization by the Bacteria Isolated

The result of the volatile fatty acid (VFAs) utilisation test for each bacterial isolates is shown in Figure 1. The two bacteria utilised the acetic acid, propionic acid and butyric acid as carbon source for their growth however at varied level of utilization.

Among the three tested volatile fatty acids, propionic acid supported the growth of *Rummeliibacillus stabekisii*(MN250294.1) and *Alcaligenes faecalis* (MN250293.1) most followed by acetic acid. Butyric acid was the least utilized by the bacterial isolates.

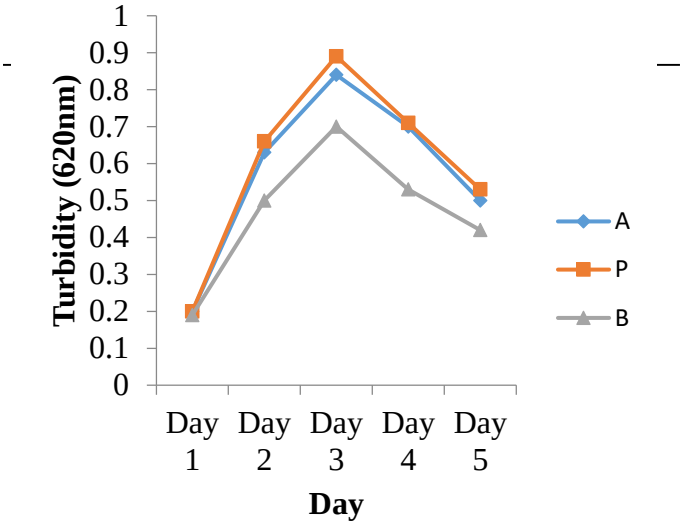

Fig 1a: Volatile Fatty Acids Utilization by *Rummeliibacillus stabekisii* (MN250294.1)
KEY: A=Acetic acid, P=Propionic acid, B=Butyric acid

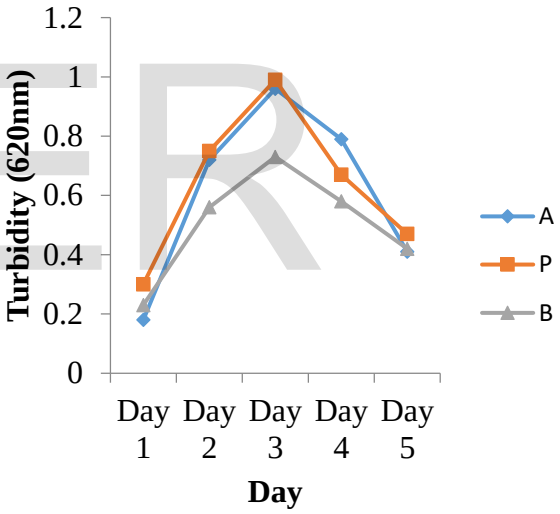

Fig 1b: Volatile Fatty Acids Utilization by *Alcaligenes faecalis* (MN250293.1)
KEY: A=Acetic acid, P=Propionic acid, B=Butyric acid

Effect of Different pH on the Growth of Selected Bacteria Isolated

The result of the effect of different pH values on the growth of the aerobic bacterial isolates is shown in Figure 2. The bacterial isolates which were *Rummeliibacillus stabekisii* (MN250294.1) and *Alcaligenes faecalis* (MN250293.1) had a significant exponential growth at pH 5 and 7. However, pH 3 and 11 did not support the bacterial growth.

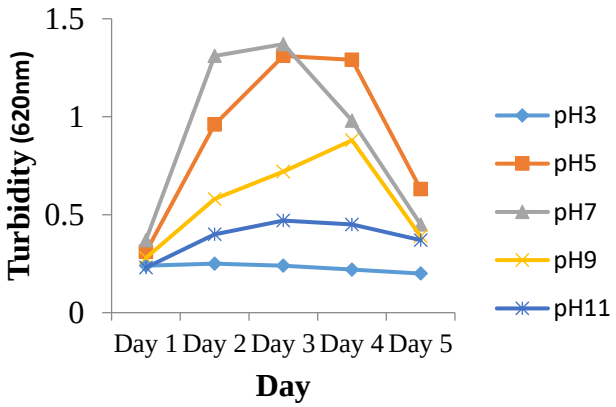

Fig 2a: Effect of Different pH on the Growth of *Rummeliibacillus stabekisii* (MN250294.1)

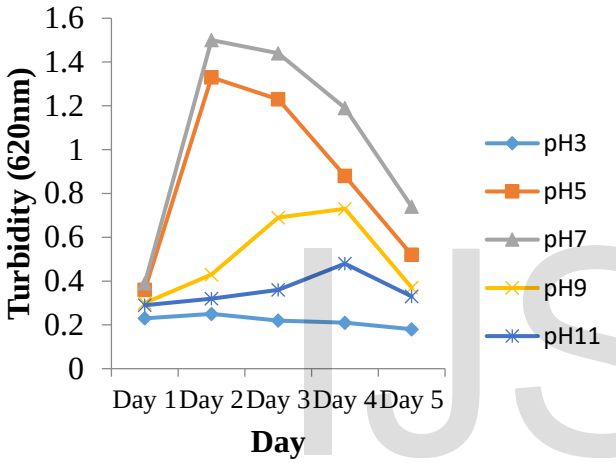

Fig 2b: Effect of Different pH on the Growth of *Alcaligenes faecalis* (MN250293.1)

Volatile Fatty Acids Content of the Fermented Palm Oil Mill Effluent (POME)

Figure 3 is showing the volatile fatty acids content of the fermented palm oil mill effluent (POME) in the anaerobic fermenter. At zero (0) day, propionic acid and butyric acid were detected at 0.012 and 0.015% respectively in the POME while 0.092% of acetic acid was detected in the POME. The concentrations of the acetic acid, propionic acid and butyric acid increased with time. After the 21 days of acidogenic fermentation, propionic acid had the highest concentration of 1.15% while the acetic acid had the least concentration of 0.78%. Butyric acid concentration was 1.05%.

Chromium Removal from the Dry Season Drainage Water by the Bacterial Isolates

Table 1 shows the percentage reduction of chromium in the mine drainage water. At 10% fermented palm oil mill effluent (POME) concentration, the AMD water treated with *Rummeliibacillus stabekisii* (MN250294.1) + *Alcaligenes faecalis* (MN250293.1) had the highest percentage removal of 100% while the AMD water treated with only *Rummeliibacillus stabekisii* (MN250294.1) had the lowest percentage removal of 95.03%. At 20% fermented palm oil mill effluent (POME) concentration, the AMD water treated with *Rummeliibacillus stabekisii* (MN250294.1), *Alcaligenes faecalis* (MN250293.1) and *Rummeliibacillus stabekisii* (MN250294.1) + *Alcaligenes faecalis* (MN250293.1) had 100% percentage removal of chromium.

Table 2 shows the summary of the independent T-test analysis. The result shows that the use of the bacterial isolates (*Rummeliibacillus stabekisii* (MN250294.1) and *Alcaligenes faecalis* (MN250293.1) singly and combination significantly influence the percentage chromium reduction [t (3) = -200.86; P < 0.05]. Further analysis revealed that the mean percentage reduction for *Rummeliibacillus stabekisii* (MN250294.1) and *Alcaligenes feacalis* (MN250293.1) in combination ($\overline{X}$ = 100.00) is significantly higher than the mean percentage reduction when the isolates were used singly ($\overline{X}$ = 95.31).

Discussion

Volatile fatty acids (VFAs) are short-chain fatty acids consisting of six or fewer carbon atoms which can be distilled at atmospheric pressure (27). The microbial utilization of such acids as carbon source for various biological processes has been reported by several authors (21, 28). Munir and Jamil (29) reported that both *Bacillus* sp. CS8 and *Pseudomonas* sp. ST2 were able to utilize volatile fatty acids (acetic and propionic acids) as carbon source for the production of Polyhydroxyalkanoates (PHA). Shoda (30) reported VFAs (acetate) as carbon source in heterotrophic nitrification and aerobic denitrification by *Alcaligenes faecalis* No.4 while using mixed volatile fatty acid as carbon source, Venkateswar et al. (28) reported *Pseudomonas otitidis* as a potential biocatalyst for polyhydroxyalkanoates (PHA) synthesis. These reports are similar to the result of this present study where *Rummeliibacillus stabekisii* and *Alcaligenes faecalis* were able to utilize volatile fatty acids as carbon source for growth.

Acetic acid was mostly utilized followed by propionic acid while butyric acid was least utilized therefore the two aerobic bacterial isolates had the least percentage increase in biomass concentration in the butyric acid medium compared to the acetic acid and propionic acid media. This observation might be as a result of the higher molecular weight of butyric acid compared to propionic and acetic acids which has been similarly reported in the work of Bedaso (31) who stated that bacteria have preference for low molecular weight volatile fatty acid for utilization as carbon source.

Volatile Fatty Acids Content of the Fermented Palm Oil Mill Effluent (POME)

Due to the high level of degradable organic material such as lipids, proteins and carbohydrates of Palm Oil Mill Effluent (POME), it is considered as an ideal substrate for various bioprocesses (28, 29). Therefore, its anaerobic fermentation to break down or hydrolyse its complex organic materials to simpler organic molecules such as VFAs as immediate carbon source for microbial growth in biological processes has been reported by several authors. Such VFAs produced in the anaerobic fermentation of POME are usually numerous however, acetic acid, propionic acid and butyric acid are the major fatty acids produced (32)

Mamimin et al. (33) reported the concentration of volatile fatty acids in the POME hydrogenic effluent collected from a pilot-scale continuous stirred-tank reactor (CSTR) for hydrogen production. Butyric acid (3.95g/L; 0.395%) was

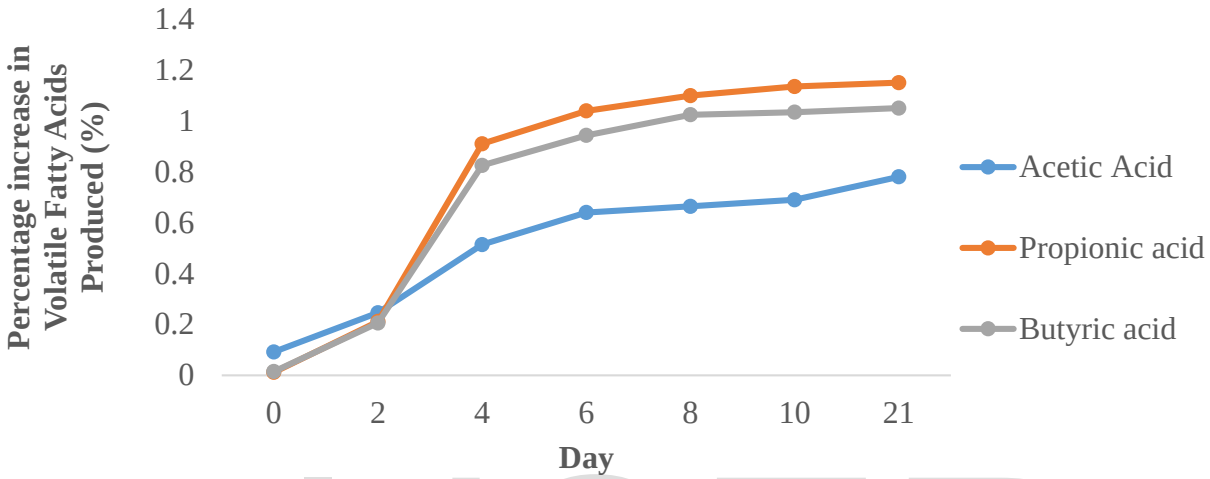

Fig 3: Percentage Increase in Volatile Fatty Acids Produced in the Fermented Palm Oil Mill Effluent

Table 1: Percentage Reduction in the Chromium Concentration Present in the Mine Drainage Water Sample by the Bacterial Strains after the 72hrs Biosorption Experiment

Bacterial Treatment	Initial Concentration (mg/L)	Final Concentration (mg/L)	Percentage Removal (%)
Control1	2.155	2.155	0.00
A10	2.155	0.107	95.03
B10	2.155	0.094	95.63
AB10	2.155	0.000	100.00
Control2	2.240	2.240	0.00
A20	2.240	0.000	100.00
B20	2.240	0.000	100.00
AB20	2.240	0.000	100.00

KEY:
Control1: AMD Water + 10% Fermented Palm Oil Mill Effluent
Control2: AMD Water + 20% Fermented Palm Oil Mill Effluent
A: *Rummeliibacillus stabekisii* (MN250294.1)
B: *Alcaligenes faecalis* (MN250293.1)
AB: *Rummeliibacillus stabekisii* (MN250294.1) + *Alcaligenes faecalis* (MN250293.1)
10: Addition of 10% Fermented Palm Oil Mill Effluent
20: Addition of 20%Fermented Palm Oil Mill Effluent

Table 2: Summary of Independent t-Test Showing Difference Between the Mean Percentage Chromium Reduction for *Rummeliibacillus stabekisii* (MN250294.1) and *Alcaligenes faecalis* (MN250293.1) in Singly and Combination.

Chromium	N	$\overline{X}$	df	t	p
A and B Singly	3	95.31			
			4	-200.86	<0 .05
A and B Combination	3	100.00			

said to be highest, followed by acetic acid (2.13g/L; 0.213%) while the propionic acid (0.25g/L; 0.025%) was the least. This result is not in agreement with this present study where propionic acid was most produced. Also, the percentage concentration of the volatile fatty acid in the POME hydrogenic effluent was comparatively lower than the percentage increase for acetic acid (0.78%), propionic acid (1.15%), and butyric acid (1.05%) in this present study. Differences in the initial organic carbon content of the POME used might have caused the variation in the volatile fatty acids content.

In the work of Aznury et al. (32), POME was anaerobically fermented in continuous bioreactor. The author reported that butyric acid had highest concentration of 3.04g/L in their fermented POME. These results are not also in accordance with this present study where propionic acid was observed to have the highest concentration (1.15%) in the fermented POME. Such variation in the composition and concentration of VFAs in fermented POME has been attributed to different reaction pathways that may be involved during acetogenesis. The possible accumulation of propionic acid and butyric acid in the anaerobic fermenter as observed in this present study can be resulted from the instability in the interaction between the acetogens (propionic and butyric degrading bacteria to produce acetic acid) and methanogens (hydrogen-utilizing bacteria) in what is called syntrophic association to create a thermodynamic balance environment because accumulation of hydrogen ion greatly affect acetogens (34).

Removal of Chromium Composition of the Mine Drainage Water Sample

The two bacterial isolates employed for chromium removal set up in this present study showed a marked ability to immobilize/remove heavy metal components of the AMD water sample as shown in the percentage removal of chromium in the batch culture. In the batch culture, the percentage removal of the chromium component of the AMD water sample by the two bacterial isolates ranged from 95.03-100%, where both *Rummeliibacillus stabekisii* and *Alcaligenes faecalis* (with 20% fermented POME), and the combination of *Rummeliibacillus stabekisii* and *Alcaligenes faecalis* were able to remove 100% of the chromium. This percentage chromium removed is higher than what was obtained in the study of Sodhi *et al*. (35) and Sukumar *et al*. (36) where the latter reported biosorption of chromium from wastewater by *Bacillus subtilis* SS-1isolated from soil sample of electroplating industry in Coimbatore, India. In the study, 98.7% of the total chromium in the wastewater was removed. The former reported that the biosorption capacity of the *Alcaligenes* sp. MMA isolated from River Yamuna (India) was 48.93% for chromium. The difference in the percentage chromium removal could be as a result of the possible variation in the initial chromium concentration in the effluent and the different bacterial species used in the biosorption experiment.

The independent T-test analysis shows that the use of the isolates singly and combination significantly influence the percentage chromium reduction [t (3) = -200.86; P < 0.05]. Further analysis revealed that the mean percentage reduction for *Rummeliibacillus stabekisii* and *Alcaligenes feacalis* in combination (= 100.00) is significantly higher than the mean percentage reduction when the isolates were used singly ($\overline{X}$ = 95.31). This could be as a result of synergy effect of the two bacterial isolates when they were used in combination for the chromium removal.

CONCLUSION

The result of this present studies shows that total remediation of chromium was achieved by both *Rummeliibacillus stabekisii* and *Alcaligenes feacalis*. Fermented POME is efficient carbon source for the bacterial isolates in the bioremediation process.

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