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Effect of Micronutrients and Macronutrients on the Biodegradation of Phenol in Biological Treatment of Refinery Effluent

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Authors' contributions

This work was a collaborative effort of all the authors. Author IVA designed the study under the guidance of author GCO, performed the statistical analysis, wrote the protocol, wrote the first draft of the manuscript and managed the literature searches. Authors IVA, AAI and GCO managed the analyses of the study and literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Background/Aim: Regulatory agencies in Nigeria and all over the world demand that refinery wastewater (RWW) meet stipulated regulatory limits before discharge into the environment. Biodegradation of toxic hydrocarbon constituents of these effluents, such as phenol, has remained a challenge with regards to compliance with regulatory requirements. This study investigated the effect of micronutrients and macronutrients on the biodegradation of phenol in RWW.
Methods: The micronutrients used in the study were $CoSO_4$, $MnSO_4$, $ZnSO_4$ and $CuSO_4$ while the macronutrients comprised urea and NPK. Range-finding and optimum concentration tests were performed for each of the nutrients. The experiment was carried out in a 3L Erlenmeyer's flask incubated in a rotary shaker under experimentally determined optimum cultural conditions, using a

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fractional factorial design. Phenol concentration (mg/ml) was monitored daily throughout the experiment using spectrophotometric method.

Results: The result obtained from the study revealed that a combination of $CoSO_4$, $MnSO_4$ and NPK was most efficient in enhancing the degradation of phenol in the RWW. After three days of incubation, phenol concentration of 141.99 mg/ml was reduced to 0.1 mg/ml. This value is lower than the phenol concentration of 0.5 mg/ml recommended for discharge of RWW into the environment. The degradation model derived from the study can be represented with the equation, $y = 8.4998e^{-2.302x}$ and $R^2 = 0.961$.

Conclusion: This study has revealed that the combination of $CoSO_4$, $MnSO_4$ and NPK can efficiently enhance phenol degradation in RWW for effectual compliance with the regulatory discharge limit.

Keywords: Refinery wastewater (RWW); biodegradation; phenol; micronutrients and macronutrients; NPK.

1. INTRODUCTION

Petroleum refinery effluents (PREs) are wastewater originating from industries primarily engaged in refining crude oil and manufacturing fuels, lubricants and petrochemical intermediates [1]. The effluents are a major source of aquatic environmental pollution and their impact is deleterious to aquatic organisms [2]. PREs usually contain undesirable environmental pollutants such as cyanides, phenol, hydrocarbons, sulphides, etc. [3]. These pollutants are toxic to several biochemical reactions [4]. If large quantities of pollutants are discharged from a refinery without compliance to the regulatory standards, a major local environmental hazard will be imminent. Some of these hazards include: off odours, pollution of the water body and mortality of flora and fauna of the ecosystem. Fish and shellfish harvested from polluted waters may be unsafe for consumption. It is therefore imperative to treat wastewater to an environmentally acceptable limit before discharge into receiving ecosystems.

In order to reduce the concentration of toxic and undesirable components in the effluent to tolerable limits; effluents are usually treated in Wastewater Treatment (WWT) Plants before discharge into receiving ecosystems. This treatment process is very essential to ensuring discharges that will not be deleterious to the environment. Sometimes, due to technical and/or operational faults, the effluent water quality after treatment may fall short of design specifications or standards set by regulatory bodies. Different treatment options exist for petroleum refinery wastewater. Typical refinery wastewater treatment plants consist of primary and secondary oil/water separation, followed by biological treatment, and tertiary treatment (if necessary). Meanwhile, biological treatment offers the best option for wastewater treatment technology and results in the removal of dissolved organic compounds in the oil refining industry [5].

Generally, there exist two main categories of biological treatment viz., suspended growth processes and attached growth processes. Ishak et al. [6] however, reviewed different biological treatment methods of refinery wastewater, identifying three categories of biological treatment techniques namely: suspended-growth, attached-growth, and hybrid processes. Suspended-growth processes involve maintaining microorganisms in suspension mode within the liquid in batch reactor. The batch reactor is allowed to operate with mixing under either aerobic or anaerobic conditions. Conversely, in attached-growth process, microorganisms are attached to an inert material (rocks, slag or plastic), which enables the generation of biofilms containing extracellular polymeric substances produced by microorganisms [7]. The hybrid processes is a combination of suspended and attached-growth process in the same reactor; for example, the combination of activated sludge and submerged biofilters (fixed bed biofilters). In a typical hybrid process, a carrier material in a reactor is maintained in suspension by aeration or mechanical mixing (moving bed reactor). Tyagi et al. [8] studied the performance of RBC-polyurethane foam (PUF) to biodegrade petroleum refinery wastewater; they achieved chemical oxygen demand (COD) removal efficiency of 87%, and found PUF advantageous as a structure for microorganism to attach, grow and be protected from high external shear.

In all biological treatment systems, it is important that the process conditions be kept at optimum as to provide the environment necessary for the

microorganisms to carry out their metabolic activities. Maintaining conducive environment entails optimizing the nutritional and environmental parameters that directly or indirectly influence microbial metabolism. Researchers have recognized many factors, including physical, chemical and biological, that may ultimately determine the effectiveness of bioremediation of organic pollutants in refinery wastewater [9]. These factors include: pH [9], temperature [10], nutrients [9], oxygen [9,11-13], salinity [14], biosurfactants [15,16], and water activity/moisture contents [17]. Among these factors mentioned above, nutrient availability is critical to the ability of microorganisms in decontaminating organic pollution. This is particularly true as the growth of heterotrophic bacteria and fungi depend on a number of nutrient elements, an electron acceptor and organic compound that serve as source of carbon and energy.

Regulatory agencies in Nigeria and all over the world demand that RWW meet stipulated regulatory limits before discharge into the environment. Biodegradation of toxic hydrocarbon constituents of effluents, such as phenol, has remained a challenge with regards to compliance with the regulatory requirements. Mohammed et al. [18] reported that the compositions of treated Nigerian crude oil refinery effluent contain high concentrations of Phenol. This study evaluated the effects of micronutrients and macronutrients on the biodegradation of phenol in biological treatment of refinery effluent.

2. MATERIALS AND METHODS

2.1 Sample Collection and Physico-chemical Analysis of RWW

Sample for Microbiological examination was collected in a non-reactive borosilicate glass bottle that had been cleansed and sterilized (in a thermostatically regulated oven at 160^{o}C for 1hr). Representative portions of the effluent sample were collected from the biodisk (inlet point of raw wastewater) with the sterile container. In collecting the effluent sample, air space was left in the bottle to facilitate mixing by shaking and aseptic techniques were adopted to avoid sample contamination. The sample was labelled for proper identification and transported to the laboratory in an ice pack for laboratory analyses.

The physicochemical analyses of the RWW were performed using standard methods. The parameters analysed included pH, temperature, COD, BOD, DO, nitrate, phosphate, sulphate, ammonia, sulphide, chloride, TSS, odour, phenol, PAHs and total petroleum hydrocarbon (TPH).

2.2 Range-finding Test for Micronutrient and Macronutrient Concentration

Different concentrations (0.000001 - 0.1 g/L) of the following micronutrients: $MnSO_4$, $ZnSO_4$, $CuSO_4$, and $CoSO_4$, were examined to find out the most suitable concentration range that can enhance the biodegradation of phenol in RWW (Table 1). For macronutrients, a concentration range of 0.01 – 20 g/L and 1 – 80 g/L were examined for Urea and NPK respectively, to determine the most appropriate concentration (Table 2). In each set-up, appropriate concentration of the micronutrient or macronutrient was added to the RWW in a 250 ml Erlenmeyer's flask and incubated in a rotary shaker incubator at a temperature of 35^{o}C for 7 days. Phenol concentrations in the set-ups were monitored daily throughout the experiment.

2.3 Micronutrient and Macronutrient Combination Set-up

The best concentration that enhanced phenol biodegradation in RWW was used to set-up this phase of the experiment. The different micronutrients ($MnSO_4$, $ZnSO_4$, $CuSO_4$, and $CoSO_4$) were run singly, and in combinations of 2s, 3s, and then all together (Table 3) to determine the best combination that most significantly enhanced the biodegradation of phenol in RWW. The same procedure was applied to the macronutrients: urea and NPK (Table 4).

After selecting the best micronutrient and the best macronutrient combinations, respectively, both were combined based on the combinations that most effectively enhanced phenol degradation in the RWW.

The optimized nutrient were used to set-up the final experiment in a 3L Erlenmeyer's flask and incubated in a rotary shaker incubator (100 rpm) at a temperature of 35°C for 7 days. Phenol concentrations in the set-ups were monitored and recorded daily throughout the experiment (Tables 5 and 6).

Table 1. Range-finding test design for micronutrient selection

No. of runs	Factors (Micronutrients)	Treatments levels (Concentrations (g/L))
1	$CoSO_4$	0.000001
2	$MnSO_4$	0.000001
3	$ZnSO_4$	0.000001
4	$CuSO_4$	0.000001
5	$CoSO_4$	0.00001
6	$MnSO_4$	0.00001
7	$ZnSO_4$	0.00001
8	$CuSO_4$	0.00001
9	$CoSO_4$	0.0001
10	$MnSO_4$	0.0001
11	$ZnSO_4$	0.0001
12	$CuSO_4$	0.0001
13	$CoSO_4$	0.001
14	$MnSO_4$	0.001
15	$ZnSO_4$	0.001
16	$CuSO_4$	0.001
17	$CoSO_4$	0.01
18	$MnSO_4$	0.01
19	$ZnSO_4$	0.01
20	$CuSO_4$	0.01
21	$ZnSO_4$	0.1
22	$CuSO_4$	0.1
23	Control	-

Table 2. Range-finding test design for macronutrient selection

No. of runs	Factors (Macronutrients)	Treatments levels (Concentrations (g/L))
1	NPK	-
2	Urea	0.01
3	NPK	-
4	Urea	0.1
5	NPK	1
6	Urea	1
7	NPK	10
8	Urea	10
9	NPK	20
10	Urea	20
11	NPK	40
12	Urea	-
13	NPK	80
14	Urea	-
15	Control	-

2.4 Phenol Determination

The concentration of phenol was determined using the direct photometric method described by American Society for Testing and Materials (ASTM D1783-01). Phenol standard curve was prepared and the phenol concentration in the sample determined by comparing the absorbance reading with the standard curve.

2.5 Isolation, Identification, and Characterization of the Phenol-degrading Microorganisms in the Refinery Wastewater Treatment Plant

The mineral salt medium of Hill and Robinson [19] was prepared for the isolation of phenol-degrading bacteria and fungi with the following components in mg/L of deionized water: Phenol,

235; KH_2PO_4, 420; K_2HPO_4, 375; $(NH_4)_2SO_4$, 244; NaCl, 30; $CaCl_2$, 30; $MgSO_4$, 30; and $FeCl_2$, 3. For the isolation of bacteria, 0.1% w/v of chloramphenicol was added while the medium for fungi was amended with 0.01% Nystatin.

Table 3. Micronutrient combination design for RWW biodegradation

No. of runs	Treatments
1	Control
2	$CoSO_4$ + $MnSO_4$
3	$CuSO_4$ + $MnSO_4$
4	$CuSO_4$ + $CoSO_4$
5	$ZnSO_4$ + $MnSO_4$
6	$ZnSO_4$ + $CoSO_4$
7	$CuSO_4$ + $ZnSO_4$
8	$CuSO_4$ + $CoSO_4$ + $MnSO_4$
9	$CuSO_4$ + $ZnSO_4$ + $MnSO_4$
10	$CuSO_4$ + $ZnSO_4$ + $CoSO_4$
11	$ZnSO_4$ + $CoSO_4$ + $MnSO_4$
12	$CuSO_4$ + $ZnSO_4$ + $CoSO_4$ + $MnSO_4$
13	$ZnSO_4$
14	$CuSO_4$
15	$MnSO_4$
16	$CoSO_4$

Table 4. Macronutrient combination design for RWW biodegradation

No. of runs	Treatments
1	Control
2	NPK
3	Urea
4	NPK + Urea

Table 5. Selected micronutrient and macronutrient combination design

No. of runs	Treatments
1	$ZnSO_4$ + $MnSO_4$
2	$CoSO_4$ + $MnSO_4$
3	NPK + $MnSO_4$ + $ZnSO_4$
4	NPK + $MnSO_4$ + $CoSO_4$
5	Control 1
6	Control 2
7	NPK

The phenol-degrading bacteria growing on the mineral salts-phenol agar were purified on nutrient agar and stored in nutrient agar slants at refrigeration temperature. Similarly, the phenol-degrading fungi were purified on PDA and stored in PDA slants.

Table 6. Final set-up design with optimized nutrients

No. of runs	Treatments
1	NPK + $MnSO_4$ + $CoSO_4$
2	Control 1
3	Control 2

Mineral salt medium was prepared for isolating phenol-degrading bacteria and fungi. The phenol-degrading bacteria were identified by morphological and biochemical techniques using Bergey's Manual of Determinative Bacteriology [20]. Whereas, Colour Atlas [21,22] as well as wet mount were employed for fungal identification.

2.6 Statistical Analysis and Modelling

The results were compared by one-way analysis of variance (one-way ANOVA) and multiple range tests to find the differences between the measurement means at 5% (0.05) significance level. The analyses were performed using IBM® SPSS® Statistics Version 20.0 (Jean-Loup Gailly and Mark Adler, US).

3. RESULTS

3.1 Physicochemical Analyses of RWW

The physicochemical results of the RWW are presented in Table 7. The conductivity of the sample was 373.3±0.58 mg/L; the pH was close to neutral, phenol concentration was 79.9+0.75, and TPH level was 43.18±0 mg/L.

3.2 Concentration Range-finding Test for Micronutrient and Macronutrient Selection

Fig. 1A to 1D show the results of the concentration-range-finding test for micronutrient selection. From the results obtained, the preferred concentration for $CuSO_4$, $ZnSO_4$, and $CoSO_4$ was 0.00001 g/L; while for $MnSO_4$ the concentration was 0.001 g/L. Below or above these chosen concentrations, there was corresponding decline in the ability of the micronutrients to enhance phenol degradation in the RWW.

The results of the concentration range-finding test for macronutrient selection are presented in

Fig. 2A and 2B. From the results obtained the best concentration for both urea and NPK was 1 g/L.

Table 7. The physicochemical analysis of the RWW

Parameter	Value
pH	6.63±0.09
Conductivity (NS/cm)	373.3±0.58
TDS (mg/L)	176.3±0.17
Temperature (°C)	26±0.06
Turbidity (FRU)	37.7±1.53
TSS (mg/L)	21.7±1.53
Odour	Objectionable
COD (mg/L)	351.7±0.58
Nitrate (mg/L)	6.5±0.1
Sulphate (mg/L)	26±2
Phosphate (mg/L)	2.38±0.29
Ammonia (mg/L)	0.45±0.04
Sulphide (mg/L)	0.05±0
Chloride (mg/L)	58±3
TPH (mg/L)	43.18±0
BOD-5 (mg/L)	35±2
Phenol	79.9+0.75

3.3 Effect of Micronutrient and Macronutrient Combinations on Phenol Degradation

The results of the micronutrient and macronutrient combinations are presented in Fig. 3A, and 3B, respectively. The best micronutrient combinations that showed optimal performance were ZnMn ($ZnSO_4$ + $MnSO_4$) and CoMn ($CoSO_4$ + $MnSO_4$), whereas, only NPK yielded the best result when compared with a combination of NPK and Urea.

The result of the combination of the best micronutrients and NPK is presented in Fig. 3C. The combination, $CoSO_4$, $MnSO_4$ and NPK showed optimum phenol degradation after 3 days of incubation. When this best combination was used to set-up the biodegradation of RWW experiment, it showed 97% degradation of phenol after 24 hours (Fig. 4). A percentage degradation of 98.9% was obtained after 3 days and a greater than 99.5% after four days.

3.4 Identification of Phenol-degrading Bacteria and Fungi Involved in the Bioremediation of the Refinery Wastewater

Twelve (12) bacteria belonging to nine different genera were identified based on their phenotypic and biochemical characteristics. The phenol-degrading bacteria identified included: *Acinetobacter junii*, *Pseudomonas putida*, *Pseudomonas fluorescens*, *Burkholderia* sp., *Xanthomonas* sp., *Azotobacter chroococcum*, *Enterobacter cloacae*, *Streptomyces* sp. *Serratia marcescens*, *Xanthomonas sacchari*, *Campylobacter jejuni*, and *Campylobacter lari*.

The following phenol-degrading fungi namely: *Aspergillus flavus*, *Aspergillus sydowii*, *Cladosporium tenuissimum*, *Aspergillus japonicas*, *Trichosporon montevideense*, *Phanerochaete sordida*, and *Monacrosporium eudermatum* were isolated and identified from the refinery wastewater. The fungi belonged to five (5) different genera with genus *Aspergillus* dominating.

3.5 Modelling of Phenol Degradation in RWW Using the Optimized Medium

Phenol degradation in RWW was monitored for a period of seven (7) days using the optimized medium. Mathematical modelling of phenol degradation through non-linear regression method revealed that phenol content degradation followed an exponential pattern (Fig. 5) The derived phenol degradation model is given by the equation $y = 84.998e^{-2.302x}$. The coefficient of determination R^2 (also known as the Goodness of Fit), a measure of how well the derived model fits the experimental data, had a value of 0.961 (96 %). The R^2 value can be interpreted as the proportion of the variance in phenol content attributable to the variance in time. This shows that the derived model could be satisfactorily applied in predicting further degradation of phenol content as time progresses.

4. DISCUSSION

This project investigated the effect of micronutrients and macronutrients on the biodegradation of phenol in biological treatment of refinery effluent. Physicochemical analyses of the RWW revealed that the sample was contaminated with phenol. The regulatory discharge limit for RWW as stipulated by EGASPIN is 0.5 mg/L. The results obtained during system monitoring revealed that the treated RWW did not comply with the regulatory limit. Non-compliance of treated RWW with regulatory standards has been reported by Anyadiegwu and Ohia [23]. Such non-compliance to regulatory limits can result in

deleterious consequences on the receiving environment. According to Hou et al. [24] and Poulopoulos et al. [25] the discharge of wastewater from refinery into the environment without compliance to regulatory limits can: affect drinking water and groundwater resources, endanger aquatic lives and the health of human beings; cause pollution of the atmosphere; limit optimal crop production; and lead to general land degradation.

The micronutrients employed in this study, included: $CoSO_4$, $CuSO_4$, $MnSO_4$ and $ZnSO_4$. Sa´ and Boaventura [26] formulated a medium for growing phenol-degrading *Pseudomonas putida* with $CoSO_4$ in hydrated form as a component. This shows how important the mineral is for the growth of polycyclic aromatic-degrading microbes. Although microorganisms need trace metal salts in small amount, these salts are however necessary for their metabolism. Cobalt is an essential trace element for many living organisms; it plays a key biological role as the centrally coordinated ion in cyclic tetrapyrroles known as corrin rings [27,28]. When used singly and in combination with $MnSO_4$, $CoSO_4$ demonstrated the ability to stimulate phenol degradation in the RWW.

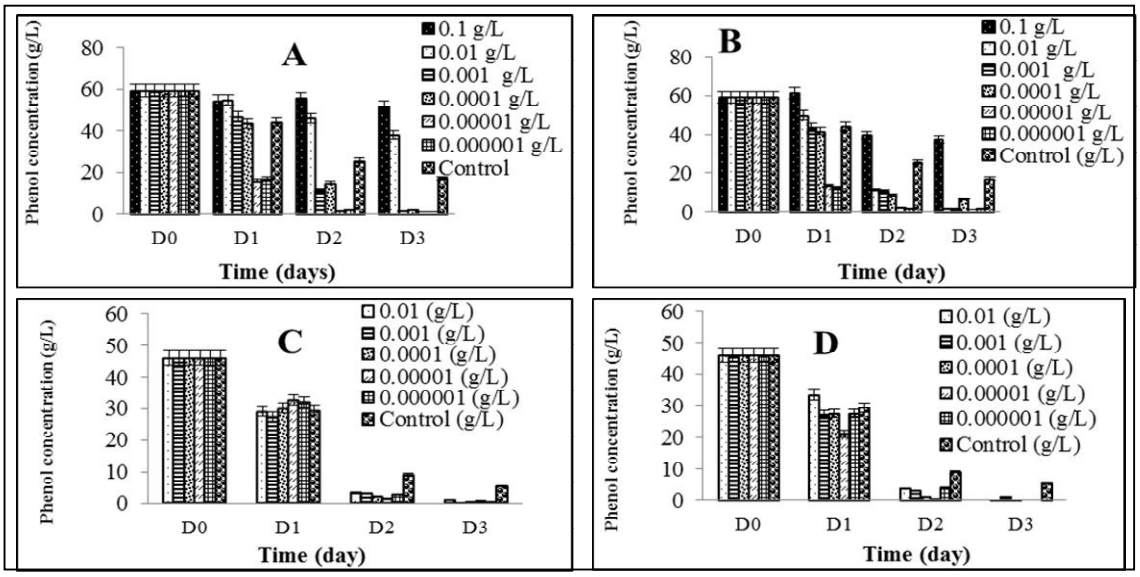

Fig. 1. Concentration range finding test for micronutrients
(A: $CuSO_4$ and B: $ZnSO_4$, C: $MnSO_4$; D: $CoSO_4$)

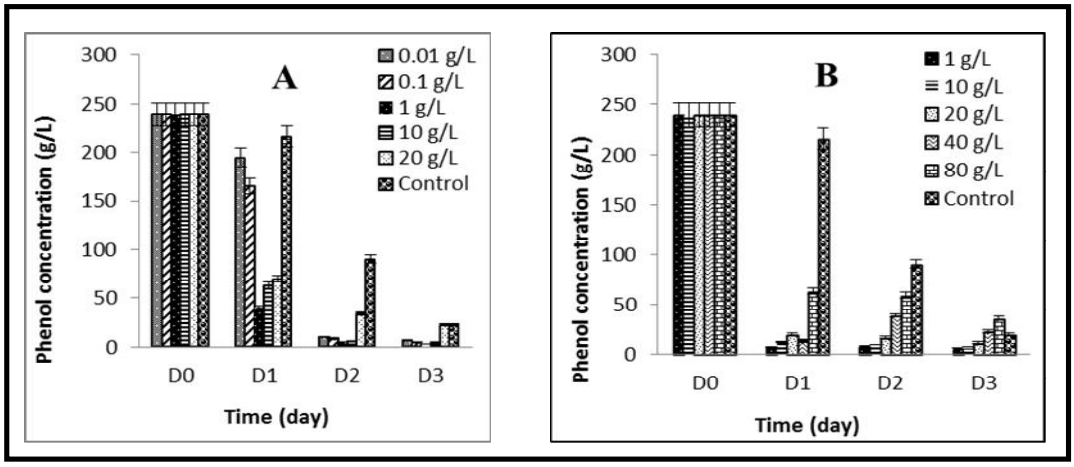

Fig. 2. Concentration range finding test for macronutrients
(A: Urea and B: NPK)

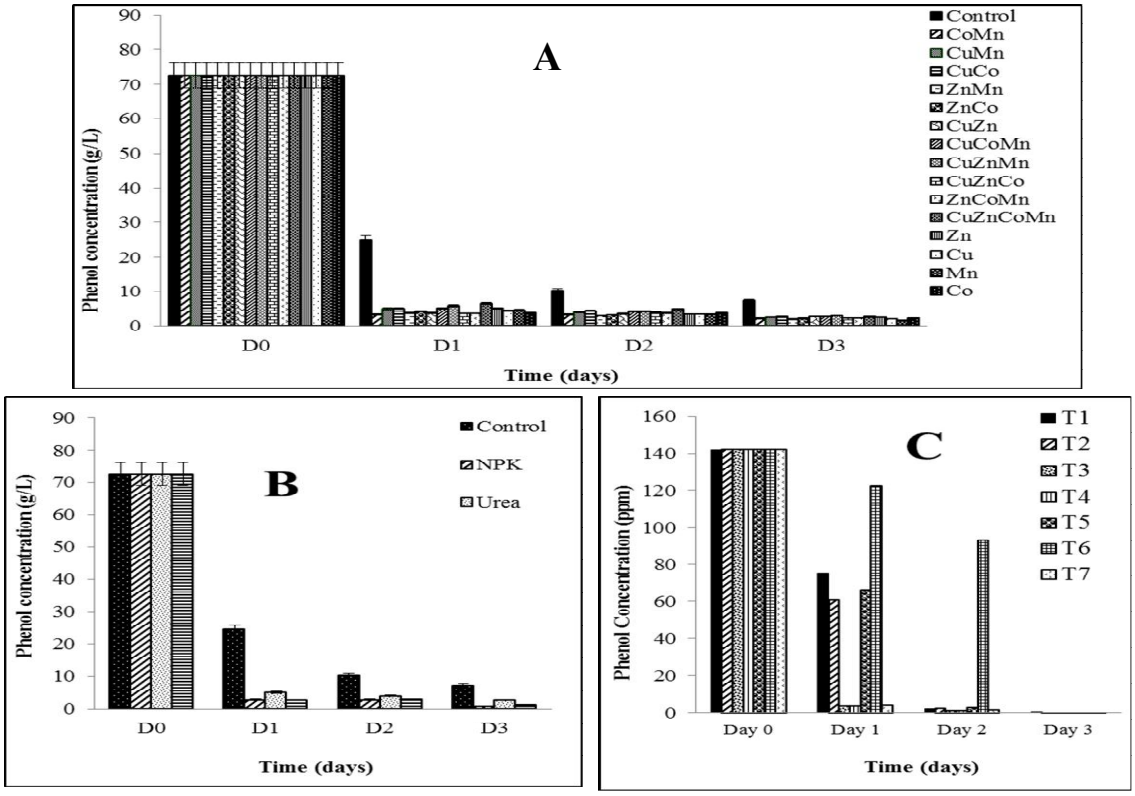

Fig. 3. Effect of micronutrient and macronutrient combinations on degradation of phenol in RWW

(A: Micronutrient combinations; (Co: CoSO4; Mn: $MnSO_4$; Zn: $ZnSO_4$; and Cu: $CuSO_4$); B: Macronutrient combinations; C: Micronutrient and Macronutrient final combinations screening (T1: $ZnSO_4$+$MnSO_4$; T2: $CoSO_4$+$MnSO_4$; T3: NPK+$MnSO_4$+$ZnSO_4$; T4: NPK+$MnSO_4$+$CoSO_4$; T5: Control 1; T6: Control 2; T7: NPK)

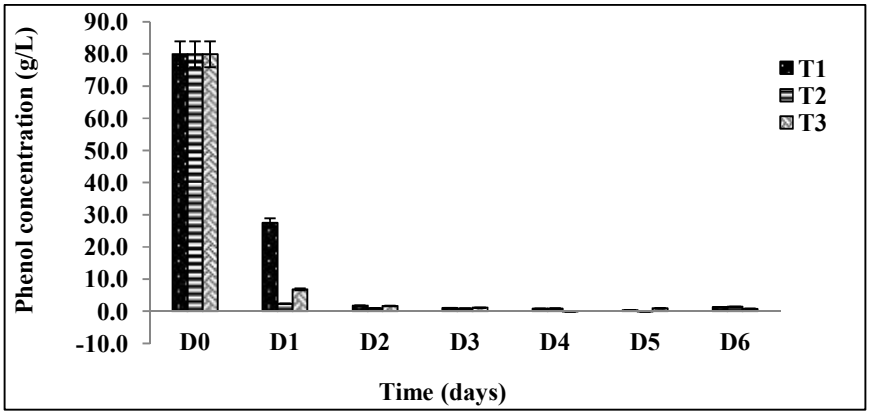

Fig. 4. Effect of the optimized nutrient combination on phenol degradation

(T1: Control; T2: Optimized nutrients and RWW; T3: Optimized nutrients, RWW and inoculum)

The result obtained in this study regarding the efficiency of $MnSO_4$ in improving biodegradation of phenol in the RWW is consistent with the findings of other studies. Gallego [29] and Pandimadevi et al. [30] reported the stimulation of bioremediation of polycyclic aromatic hydrocarbons with $MnSO_4$ and $ZnSO_4$ as components of the medium that supported the microbial consortium for the bioremediation process.

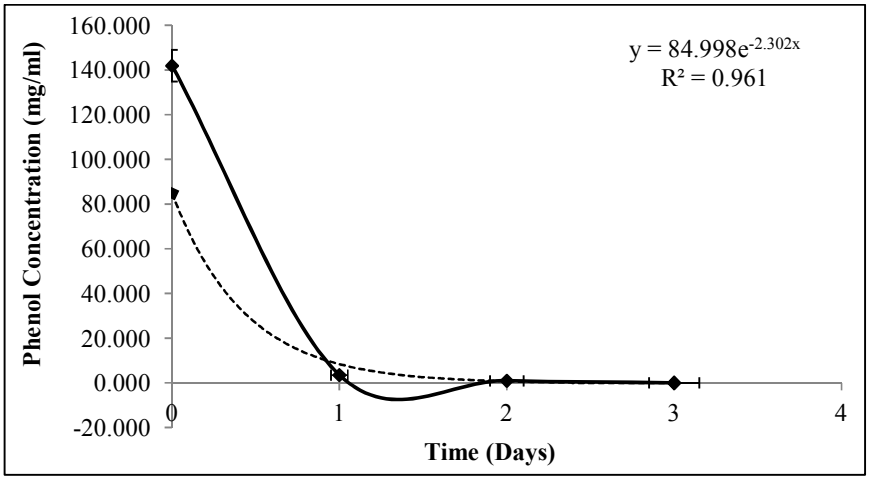

Fig. 5. Model of phenol degradation in refinery effluent using the optimized parameters (T2)

A total of 12 phenol-degrading bacteria and 7 phenol-degrading fungi were identified based on phenotypic, biochemical and microscopic characteristics. Other studies [31-33] have reported the biodegradation of phenol by bacterial strains isolated from wastewater. These bacteria: *Acinetobacter junii*, *Pseudomonas putida*, *Pseudomonas fluorescens*, *Burkholderia* sp., *Xanthomonas* sp., *Azotobacter chroococcum*, *Enterobacter cloacae*, *Streptomyces* sp. *Serratia marcescens*, *Xanthomonas sacchari*, *Campylobacter jejuni*, and *Campylobacter lari* have been implicated in phenol degradation. Liu et al. [34] reported the biodegradation of phenol by *Acinetobacter calcoaceticus* PA isolated from phenolic wastewater. Similarly, Zhang et al. [35] identified amongst cultured bacteria *Pseudomonas* sp., in addition to *Bacillus subtilis* and *Nitrospira* sp. Gu et al. [36] identified the phenol-degrading bacterium *Campylobacter* sp. as well as other bacterial strains different from the ones identified in this study such as *Niastella* sp., *Deinococcus* sp., *Delftia* sp., *Achromobacter* sp., and *Agrobacterium* sp., from drinking water. Krastanov et al. [37] reported that *Pseudomonas* spp. and *Acinetobacter* spp. are some of the most widely implicated phenol-degrading bacteria; a result which is consistent with the findings of this present study.

Juárez et al. [38] reported that *Azotobacter chroococcum* can grow up using polyphenolic compounds as an individual source of carbon and energy supply. They studied the degradation of simple phenolic compounds by this strain using a gas chromatography coupled mass spectrometry method. Other studies have reported the ability of *Serratia marcescens* [39], *Streptomyces* sp. [40], *Burkholderia* sp. [41], *Xanthomonas* sp. [42], and *Enterobacter cloacae* [43] to degrade phenolic wastewater.

This study revealed the presence of *Aspergillus flavus*, *Aspergillus sydowii*, *Cladosporium tenuissimum*, *Aspergillus japonicas*, *Trichosporon montevideense*, *Phanerochaete sordida*, and *Monacrosporium eudermatum* in the refinery wastewater. The capacity of these fungal isolates to degrade phenol is supported by other studies. *Trichosporon* spp. are one the most widely reported phenol-degrading fungi [37]. The identification of *Aspergillus* spp. in the RWW is consistent with another study [44]; which implicated *Aspergillus* spp. in the degradation of phenol in wastewater.

5. CONCLUSION

This study has demonstrated the positive effect of micronutrients and macronutrients on the biodegradation of refinery effluent. Amongst all the nutrients screened, the combination of NPK, $MnSO_4$ and $CoSO_4$ was most efficient in enhancing the biodegradation of phenol in RWW. This could be as a result of preferential assimilation of these specific nutrients by the microbial culture involved in the degradation. Operators of petroleum refineries in Nigeria should employ the synergistic effect of micronutrients and macronutrients in stimulating the microbial culture for optimal biodegradation of phenol in RWW as identified in this research.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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