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Isolation and Identification of Phenol Degrading Bacteria in Refuse Dumping Site

Muhammad Bashir Muhammad[1*], Abubakar Aisami[2], Maianguwa Abdulrashid Dauda[3], Abdullahi Sa'adu Adeseye[4], Isaac John Umaru[5], Helen Oluwabunmi[6], Bilyaminu Habibu[7], Gloria Omonefe Oladele[8]

[1,2,3,4]Gombe State University, Gome State, Nigeria
[5,6,7]Federal university Wukari, Taraba State, Nigeria
[8]Federal Medical Centre Bida Niger State, Nigeria
muhammadbashirmuhammad4@gmail.com

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Abstract

Phenol is a harmful compound found in soil, and its removal is crucial for human health. Phenol or hydroxybenzene, is both a synthetically and naturally produced aromatic compound. Microorganisms capable of degrading phenol are common and include both aerobes and anaerobes. In this study, researchers collected soil samples from INEX refuse dumping site along Bajoga road of kwami, Gombe state, and determined the abundance of heterotrophic and degradation bacteria using serial dilution and most probable number (MPN) methods. This experimental research study was carried out in order to isolate and identify phenol-degrading bacteria in polluted soil, the effect of concentration and pH was analysed during the procedure. Bacteria was isolated from contaminated soil and sub-cultured in a Mineral-salt media

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which was prepared with phenol added as the only carbon source needed for the bacterial growth, The bacterial isolate was identified as Escherichia coli a gram-negative bacteria. The favourable concentration and pH required for the growth of the bacteria was determined as 200mg/L and pH 7 respectively. These findings have significance in bioremediation for employing suitable bacteria in suitable condition for solving environmental pollution crises.

Keywords: Phenol degradation, Hydroxybenzene, Soil contamination, Bioremediation, Heterotrophic bacteria

INTRODUCTION

Environmental pollution is the contamination of the physical and biological components of the Earth's ecosystems to the extent that normal ecological processes are disrupted (Iyyanki *et al.,* 2017). Pollutants like oil, food waste, rubber, and plastic find their way into the environment through the air, soil, and water. The combustion of fuels, industrial activities, and power generation are major sources of air pollution, leading to the release of volatile hydrocarbons into the atmosphere (DOE, 2009). Air pollutants can contribute to various respiratory, cardiovascular, and liver-related health issues (Creek *et al.,* 2004; Ko & Hui, 2010). The release of untreated harmful chemical mixtures and heavy metals are the primary sources of water and soil contamination. Additionally, oil spills, deck overflow from vessels, pipeline and storage tank leaks, and offshore waste disposal are also significant contributors to soil and water pollution (Agahalino *et al.,* 2009; Matkin *et al.,* 2008).

Phenol and its derivatives are widely used in various industrial processes, such as the production of plastics, pesticides, and pharmaceuticals (Shetty *et al.,* 2014). However, the improper disposal or uncontrolled release of these compounds can lead to significant environmental pollution and pose a threat to human health and aquatic ecosystems (Doble & Kumar, 2005). Exposure to phenol can cause a range of adverse effects, including skin and eye irritation, respiratory problems, and even liver and kidney damage (ATSDR, 2008). Phenol has diverse applications, including use as a general disinfectant, a reagent in chemical analysis, and in the production of various organic compounds, such as soaps, pharmaceuticals, and dyes. It is also utilized in the manufacture of fertilizers, explosives,

paints and paint removers, and textiles. Phenol and its derivatives can contaminate the environment through the discharge of municipal or industrial wastewater into surface water bodies and soil. The presence of phenolic pollutants in aquatic environments can adversely impact the biodiversity of these ecosystems due to their toxicity (Lika & Papadakis, 2009; Pradeep *et al.,* 2015). Phenolic compounds can be absorbed through small areas of the skin and mucous membranes and can potentially be harmful to the nervous system, heart, kidneys, and liver in animals. Studies have found that phenol can inhibit the synthesis and replication of DNA cells (Wang *et al.,* 2011). While plants are generally tolerant towards phenol, phenolic toxicity must still be considered. Phenolic industrial wastes are typically treated using physicochemical methods. However, these physicochemical techniques alone are not entirely efficient due to their high cost and the generation of secondary pollutants (Gonzalez *et al.,* 2006; Suhaila *et al.,* 2013). The presence of certain microorganisms can be leveraged for the biodegradation of phenol and other phenolic compounds.

Bioremediation, which involves the use of microorganisms to degrade or transform environmental contaminants, has emerged as a promising approach for treating sites contaminated with phenol (Vidali, 2001). Certain microorganisms, particularly bacteria, have the ability to utilize phenol as a carbon and energy source, thereby reducing its concentration in the environment (Leahy & Colwell, 1990). The identification and characterization of these phenol-degrading bacteria are crucial for the development of effective bioremediation strategies. The biodegradation of phenol and its derivatives by microbial communities has been a focus of research for over three decades (Chung *et al.,* 2003). Microorganisms are well-suited for the task of pollutant destruction because they possess enzymes that allow them to use organic contaminants as a food source, and they are small enough to easily access and interact with pollutants.

The current bioremediation systems in use rely on microorganisms native to the contaminated sites, promoting their growth by providing optimal levels of nutrients and other chemicals essential for their metabolism. Researchers are now exploring ways to supplement affected sites with non-native organisms, including genetically engineered microbes specifically adapted to degrade phenol in particular locations (Kerr, 1994). Phenol-degrading microorganisms that are locally isolated are the best choice for use in bioremediation, as they are better able to adapt to the local environmental conditions compared to commercially imported organisms. Using non-native microbes could potentially lead to an ecological disaster. Therefore, there is a need to increase the

availability of phenol-degrading microorganisms to prepare for future phenol remediation efforts (Watanabe, 2001).

The findings from this study will contribute to the understanding of the microbial community involved in the biodegradation of phenol and provide valuable insights for the development of efficient bioremediation strategies.

MATERIALS AND METHODS

Chemicals/ Reagents

The chemicals and reagent used include : 4-amminoantipyrine, Acetone, Agar, Ammonium sulphate, Crystal violet, di-Potassium hydrogen phosphate, EDTA, Ethanol, Ferric sulphate, Hydrochloric acid, Lugol's iodine, Magnesium sulphate, Manganese sulphate, Methyl red, Nutrient agar, Nutrient broth, Oxidase reagent, Phenol, Potassium ferric cyanide, Potassium di hydrogen phosphate, Potassium Hydrogen phosphate, Safranin, Saline solution, Simmons citrate agar, Sodium chloride, Sodium hydroxide,Sodium molybdate and Urease broth.

Equipment's

The equipment used include the following: Autoclave, Spectrophotometer, Beaker, Bunsen flame, Centrifuge, Conical flask, Filter paper, Glass slide, Measuring cylinder, Media plates, Microscope, Orbital shaker, Oven, pH meter, Refrigerator, Syringe, Test tubes, Vertex mixer, Weighing Balance, Candle, Matches, Spirit lamp.

Soil Sample Collection

Three (3) specimen collection polythene bag where use to collect soil sample from INEX refuse dumping site along Bajoga road of kwami, Gombe state. During the sample collection, the soil was collected from 3 different locations to represent the sample size.

Soil Sample Preparation

One gram of the soil sample was weight using electronic balance, and dissolved in 9ml distilled water in a test-tube. Five different test-tube containing 9ml distilled water each where use for serial dilution. One of the major reason or purpose of serial dilution is to reduce the number of colonies of bacteria or viruses of unknown sample.

Analytical Method

4-aminoantypyrine was used to determine Phenol degradation, a colorimetric assay based on the reaction of the reagent with phenol in the presence of potassium ferric cyanide under alkaline pH in the presence of potassium ferric cyanide. The absorbance was read at 510 nm (Arif *et al.,* 2013).

Preparation Of Analytical Reagents

Analytical reagents and solutions were prepared according to the supplier's instructions and under sterile conditions.

Preparation Of 4-Aminoantipyrine Solution

2g 4-AAP was dissolved in 100ml distilled water; the mixture was shaken to ensure homogeneity. This solution is prepared every day.

Preparation Of Ferric Cyanide Solution

8g $K_3Fe\ (CN)_6$ dissolved in 100ml distilled water. The solution was shaken thoroughly to ensure homogeneity.This solution can be used for one week.

Preparation Of Mineral Salt Media

K_2HPO_4 (400 mg/L), KH_2PO_4 (200 mg/L), $MgSO_4$(100 mg/L), $Fe_2(SO_4)$.h20(10 mg/L), NaCl (100 mg/L), $NaMoO_4.2H_2O$(10 mg/L), $MnSO_4.2H_2O$(10 mg/L) and $(NH_4)2SO_4$ were dissolved in 1 L of distilled water, and the Ph adjusted to 7.2 mg/L before autoclaving at 121^OC for 15 min. Phenol to the final concentration of 500 mg/L was introduced as the only source of carbon before use.

Procedure For 4-Aminoantipyrine Assay

Sample (1.5ml) was first centrifuged for 15 minutes at 10,000 × g and the supernatant was collected. One hundred µL each of 4-amino antipyrine and potassium ferric cyanide solutions were added to the 1 ml of the supernatant, and the pH was adjusted to 10 using 1 M NaOH. The mixture was incubated at room temperature for 15 minutes, and the absorbance was read against the blank at 510 nm using a spectrophotometer (Soltan & Rashed, 2003).

Identification Of Bacteria

The isolates were identified based on biochemical characterization. Many tests have been known over years for the classification of microorganisms into families, genera, species and even sub specie. Some of these tests are simple which can be performed in department laboratory and some require equipment and chemicals which are not present in the

department laboratory. The tests done in the laboratory involves Gram Staining test, catalase test, coagulase test, oxidase test

Gram Staining

This test is done to differentiate between gram positive and gram negative organisms. Gram positive organisms contain a thick layer of protein-sugar complex called peptidoglycans in their cell wall whereas for gram negative, it only comprises of 10-20% of the cell wall (Capuccino & Sherman, 2017).

The procedure is as follows:

The isolate was fixed on a smear slide, the slide was then flooded with crystal violet stain as primary stain, It was then rinsed with water after 1 minute and then the slide was flooded with iodine solution, It was again rinsed with water after another 1 minute, It was then flooded with acetone alcohol as decolorizer and rinsed with water after 15 seconds. Finally it was rinsed with safranin as secondary stain and again rinsed with water after 1 minute. The slide was blot dry and observed under microscope. Gram positive bacteria will stain purple while Gram negative bacteria will stain red/pink.

Catalase Test

This is used to test for the presence of the enzyme catalase.

A loopful of colony of pure culture was picked up and kept on a clean glass slide. 2 drops of 3% H_2O_2 was added and waited for 15 seconds.

Oxidase Test

Principle: The enzyme oxidase oxidizes a redox dye such as tetramethyl paraphenylene diamine dihydrochloride (TMPPDH) to deep purple color. This enzyme is produced by some aerobic bacteria as part of their respiratory oxidation mechanism.

Procedure: Two drops of the oxidase reagent on the sample and the color change was observed.

Coagulase Test

Principle: The enzyme, coagulase causes the plasma to clot by converting fibrinogen to fibrin. Coagulase is produced by *s. Aureus*. It is considered a pathogenicity test for *S. aureus*.

Procedure: A drop of saline was dropped on a slide, the organism was emulsified in the drop to make a thick suspension and mixed with a wire and the mixture was observed for clumping.

Citrate Utilisation Test

Principle: It is based on the ability of an organism to utilize citrate as its only source of carbon, and ammonium as its only source of nitrogen. The citrate is metabolized to acetoin and CO_2.

Procedure: The organism was suspended lightly in saline and inoculated in Simmon's citrate agar.

RESULTS AND DISCUSSION

Soil Sample Collection

sterile polyethene bags where use to collect soil sample from three different location to represent the whole sample collection location

Gram Staining Of The Isolate

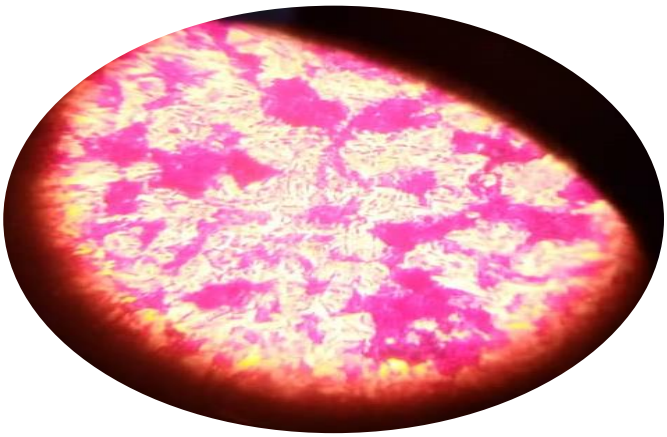


Fig 1; Microscopic view of the isolate

The isolate was seeing to be gram-negative bacteria because it possesses the pink colour, rod shape and motile, as shown in fig 4.2 aboved.

Biochemical Test Result

Catalase test, coagulase test, oxidase test and citrate test were among the biochemical tests done and result were presented in table 4.1 below

Table 1. BIOCHEMICAL TEST RESULT

Test	Result
Catalase	**Positive**
Coagulase	**Negative**
Oxidase	**Negative**
Citrate	**Negative**

Oxidase test is an enzyme produced by some aerobic bacteria as part of their respiratory oxidation mechanism, this test is employed to aid in the identification of *Pseudomonas, Neisseria, Vibrio* and other groups, the enzyme oxidase will oxidize a reedox dye such as tetramethyl paraphenylalanine diamene dihyrochloride (TMPPDH) to deep purple colour.

Therefore, based on the result observed for this test, there was no any purpled cloured form this indicate that the result read was negative.

Coagulase an enzyme that causes plasma to clot by converting fibrinogen to fibrin was found to be negative as the plasma did not clot; this enzyme is produced by *Staphlycoccus aureaus*.

Citrate is employed by organisms as its only source of carbon, and ammonium as its only source of nitrogen, the citrate is metabolized to acetoin and CO_2. A positive result is indicated by a growth of blue colour on the media; meanwhile there was no change of colour which refers to a negative result

Effect Of Phenol Concentration On The Phenol Degradation

To study the effect of high concentration on the Phenol degrading bacteria, the experiment was carried out using phenol of200mg/L, 500mg/L and 800mg/L.

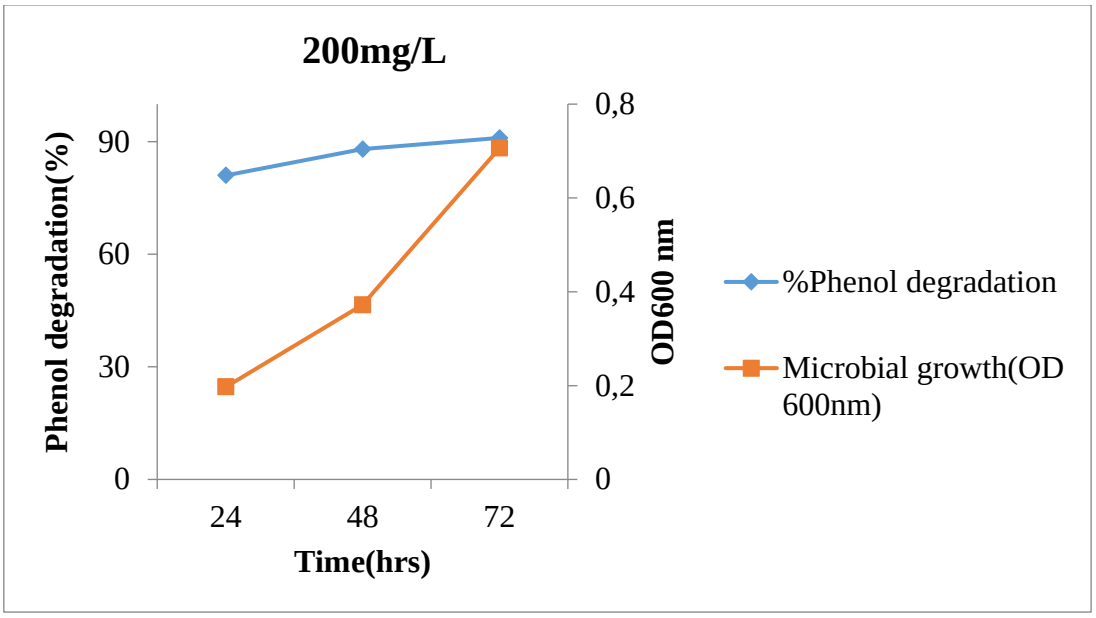


Figure 2: Effect of 200mg/L of phenol concentration on the Bacteria

The above result shows the growth of the bacteria at different time interval ranges from 24hrs, 48hrs, and 72hrs, where the bacteria degrade most of the phenol at 72hrs.

The isolated bacteria was able to degrade 93% of the phenol at 72hrs time interval.

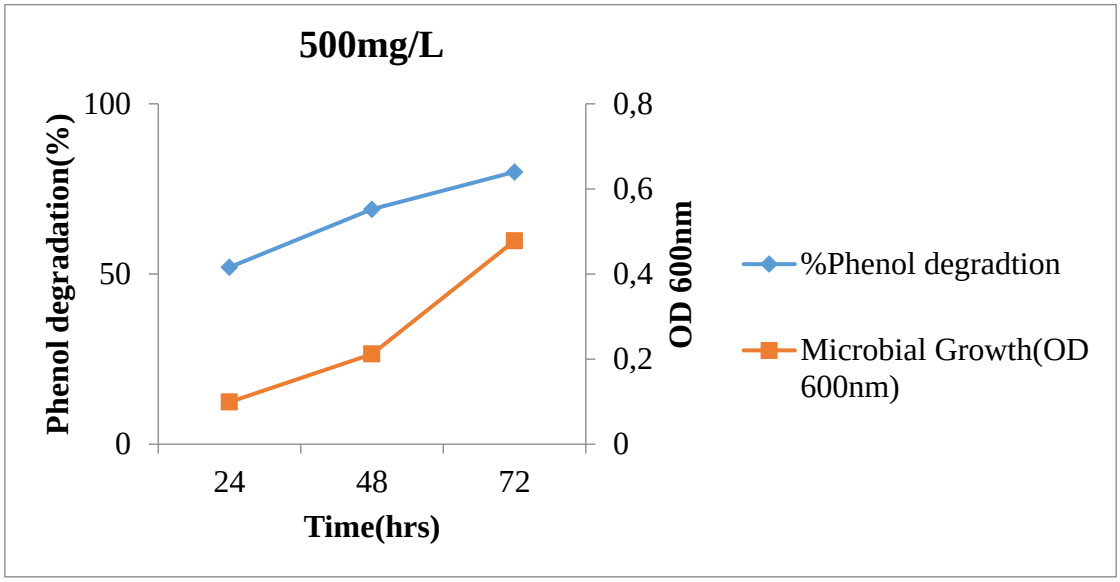


Figure 3: Effect of 500mg/L of phenol concentration on the Bacteria

The above result shows the growth of the bacteria at different time interval ranges from 24hrs, 48hrs, and 72hrs, where the bacteria degrade most of the phenol at 72hrs.

The isolated bacteria was able to degrade 74% of the phenol at 72hrs time interval .

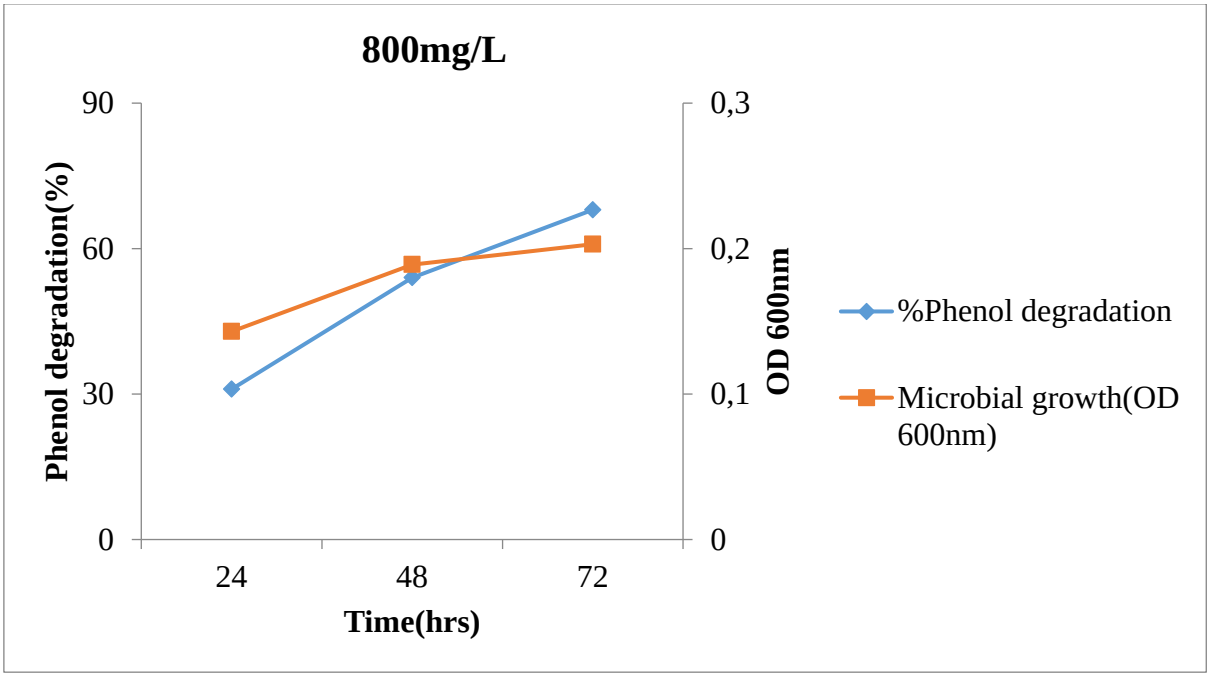


Figure 4: Effect of 800mg/L of phenol concentration on the Bacteria

The above result shows the growth of the bacteria at different time interval ranges from 24hrs, 48hrs, and 72hrs, where the bacteria degrade most of the phenol at 72hrs.

The isolated bacteria were able to degrade 65% of the phenol at 72hrs time interval .

In bioremediation, high concentration of the toxic substances impact the limit of the microorganisms to create and degrade phenol and other biological poisons. High concentration of phenol have been represented to quell biodegradation (Jiang *et al.,* 2013; Liu *et al.,* 2016).

Effect Of pH Of The Medium On Phenol Degradation By Escherichia Coli

The previous writing on biodegradation contemplates detailed that Bacteria have an inclination the pH scopes of 6.5 to 7.5 for both the bacterial development and the biodegradation rates (Demoling and Baath, 2008). pH going from 5 to 8 was chosen for this exploration.

The preceding literature on biodegradation studies reported that bacteria have a preference the pH ranges of 6.5 to 7.5 for both the bacterial growth and the biodegradation rates (Demoling and Baath, 2008). pH ranging from 5 to 8 was selected for this research.

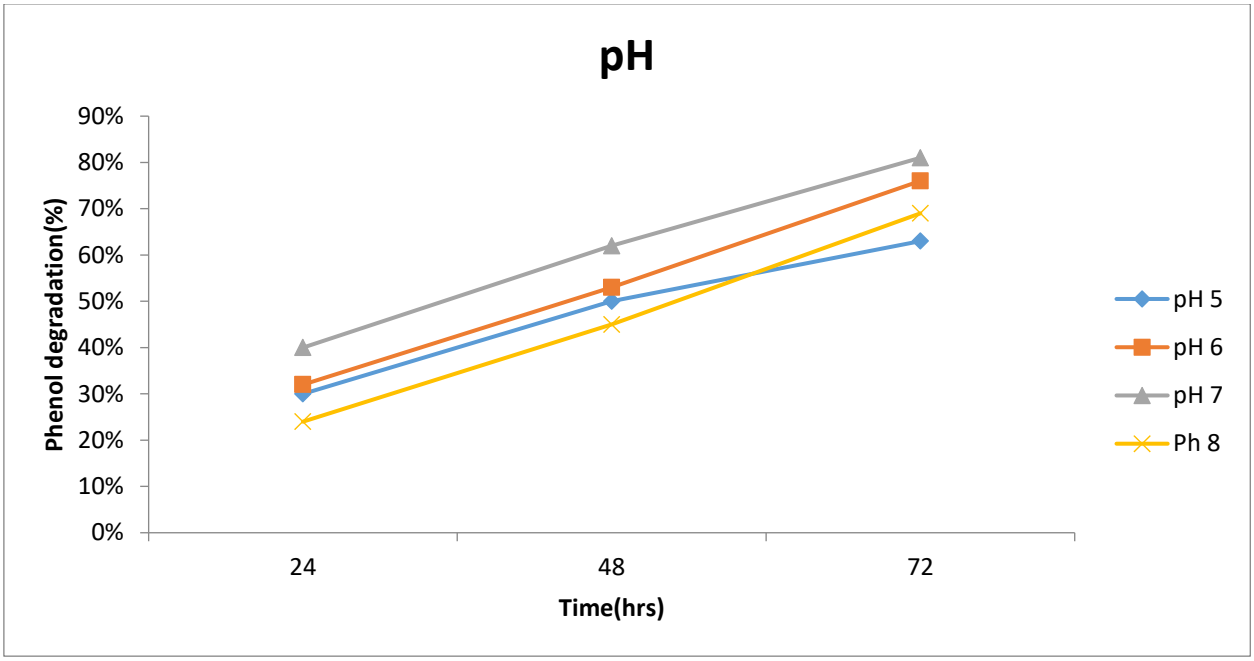


Figure 5: Effect of pH on phenol degradation by *Escherichia coli*

Four pH values from 5 to 8 were investigated, at pH 5, phenol was degraded up to 30% at 24hrs, 50% at 48hrs and 63% at 72hrs. At pH 6, 32% of phenol was degraded at 24hrs, 53% at 48hrs and 76% at 72hrs. At pH 7, 40% degradation at 24hrs, 62% at 48hrs and 81% at 72hrs. At pH 8, 24% was degraded at 24hrs, 45% at 48hrs and 69% at 72hrs.

Phenol was debased quickly at pH 7. At this pH, phenol debasement was high when contrasted with other pH esteems. In any case, the phenol corruption at pH 5, 6 and 8 were slower. These outcomes showed that Escherichia coli debased more phenol each day at pH 7 than some other pH esteem.

DISCUSSION

In evaluating the growth of isolated species during one week, the capacity of bacteria in phenol elimination was evaluated. most phenol degrading bacteria belong to Pseuodomonas and the least to Providensia spp. The growth curve of isolated species show almost the same pattern of growth for all isolated species in 0.2 g of phenol. According to Figure 5 which is same for all species, 0.2 and 0.05 g/l (remains) of phenol reached their maximum growth after 24 h, and after 48 h, phenol completely disappeared. Different

methods have been used for the elimination of phenol, but the use of bacteria can be one of the cheap (Afr *et al.,* 2001).

By comparising, the growth of isolated bacteria at different concentration was evaluated. E.coli growth in mineral salt media isolate obtained from inex dumping site along Bajoga road gombe, ware grown in different media such as nutrient agar and nutrient broth. Phenol degradation was also examined by measuring the optical density at 600nm and estimation of phenol was checked at 510nm.

Different concentration of phenols where use which include 200mg/L, 500mg/L, and 800mg/L at different time interval of 24hrs, 48hrs and 72hrs. Finally, at 200mg/L the bacteria degrade 93% of the phenol at 72hrs which is also similar to a research carried out by (farshid *et al.,* 2010) 90% was degraded at 72hrs, at 500mg/L 74% of the phenol was degraded by the bacteria at 72hrs which is also similar to a reaserch carried out by (Bhavna *et al,*. 2010) where 76% was degraded, lastly at 800mg/L the bacteria degrade 65% of the phenol at 72hrs which also nearly to a research by (farshid *et al.,* 2010) where 68% was degraded. The most favorable pH for the growth of the isolate was found to be P^H 7 as reported by many write-ups which include (farshid *et al.,* 2010) which report it to be around pH 6.5 to 7.0.

CONCLUSION

A phenol-degrading bacterium was identified as *Escherichia coli* Using the Gram-staining technique and other biochemical Tests carried out. The data obtained in this study shows that the 200mg/L concentration had the highest percentage of phenol degradation by the bacterial isolates compared to the other concentrations used for the Test. *Escherichia coli* was degraded more at pH 7 than any other of the pH values determined in this study.

The ability of these isolates to grow on phenol as the sole carbon source, coupled with their high degradation efficiencies, makes them promising candidates for implementation in biological treatment systems for the removal of phenolic contaminants from industrial effluents and contaminated environments. Further optimization of culture conditions and evaluation of their performance in real-world applications will be necessary to fully harness the potential of these phenol-degrading bacteria for environmental bioremediation.

Overall, this study contributes to the growing body of knowledge on the diversity of microorganisms capable of degrading phenolic pollutants, which is essential for developing

effective, sustainable, and eco-friendly strategies for the treatment of phenol-containing waste streams.

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