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# Nitrogen Bacteria Associated with Bioremediation Soil and their Ability to Degrade Hydrocarbon

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**A B S T R A C T**

The environmental degradation caused globally by crude oil and its by-products have become a menace that must be addressed. The pollution of soil has reduced the land use and the ecosystem functions of the soil have been adversely affected. It has become pertinent that ecologically friendly remediation approaches be adopted to restore the fertility of the soil and in essence its ecosystem functions. Nitrogen is a growth-limiting nutrient that cannot be assimilated without microorganisms, an increase in nitrogen-fixing bacteria after bioremediation is an indication of restoration. The soil samples were analyzed for physicochemical characteristics such as pH, total organic nitrogen (TON), electrical conductivity (Ec), and phosphorus. The microbiological characterization was done for total heterotrophic bacteria (THB) using plate count agar (PCA), the hydrocarbon utilizing bacteria was done using Mineral salt agar (MSA). The THB obtained in the study was2.4 x $10^4$, 1.1 x $10^4$, and 3.4 x $10^4$, the HUB was0.1 x $10^3$, 1.8 x $10^3$, and 2.4 x $10^3$. The bacterial isolates obtained in the study are *Arthrobacter sp, Pseudomonas sp, Nitrosomonas sp, Bacillus sp, Micrococcus sp, Staphylococcus sp, and Escherichia sp*. The degradative potential of these isolates was analyzed, the absorbency of the bacterial isolates was done for two different wavelengths (370 and 570). The degradative potential of the isolates was higher at the wavelength of 570. These organisms will be effective as a consortium for further hydrocarbon degradative study.

## Introduction

The growing usage of crude oil and petroleum products around the world contributes to increased pollution of the natural environment (Chris, 2000; Asif and Muneer, 2007). Technical, industrial, and economic progress is one of the fundamental causes. Every year, around 0.10 to 0.25 percent of petroleum products penetrate the natural environment (Agarry *et al.,* 2010). Because the quality of life is inextricably linked to the condition of the environment, there is a global need to improve and sustain environmental quality. Some development in remediation approaches has been demonstrated in terms of cost, eco-friendliness, and efficacy in restoring the damaged environment (Fanni *et al.,* 2010).

Complete degradation of a pollutant requires the action of several microbes therefore sometimes

potential microbes can be added to the contaminated site for the effective degradation process and this process is called bio-augmentation (Chapin *et al.,* 2002). The reduction of the concentration and toxicity of various chemical pollutants such as pesticides polyaromatic hydrocarbon, halogenated petroleum, hydrocarbon, nitroaromatic compounds, metal, and industrial solvents is bioremediation (Fanni *et al.,* 2010). Bioremediation utilizes living organisms mainly microorganisms, green plants, and their enzymes to remove, degrade, mineralize, transform, and detoxify the environmental pollutants and hazardous components of the environmental waste into innocuous or less toxic forms during the treatment of contaminated sites to return them to their original conditions (Fanni *et al.,* 2010).

Nitrogen is an important element that is required for the survival of all forms of life. It is the most plentiful gas in the Earth's atmosphere (Frank *et al.,* 2003). It can also be found in amino acids and proteins, and it is the source of many other organic molecules formed from the nitrogen fixation process (Egamberdieva *et al.,* 2008). Only prokaryotes, which can be symbiotic or free-living, fix nitrogen biologically. These prokaryotes include aquatic species like Cyanobacteria and free-living soil bacteria like Azotobacter bacteria that create associative relationships with plants (Zahran, 2001). Nitrogen fixation is mediated by the enzyme nitrogenase in soil, but its activity depends on ecological conditions in association with the specific nitrogen fixation capabilities of certain micro-organisms under various climatic conditions (moisture conditions, oxygen concentration, and a supply of organic substrates) Matthew *et al.,* 2008). However, the nitrogen-fixing activity of free-living non-photosynthetic aerobic bacteria is strongly dependent on favorable moisture conditions, oxygen concentration, and a supply of organic substrates (Matthew *et al.,* 2008). The enzyme nitrogenase consists of two conserved proteins**:** an iron (Fe) containing dinitrogenase reductase encoded by the nifH gene and a molybdenum iron (MoFe) dinitrogenase that is encoded by the nifDk genes; this enzyme is irreversibly inhibited by molecular oxygen and reactive oxygen species oxygen stress on diazotrophic (nitrogen-fixing) organism triggers a wide range of protective responses aimed at deterring the inhibitory effects of oxygen on nitrogenous substrates (Zahran, 2001; Matthew *et al.,* 2008).

Nitrogen-fixing organisms are generally active in plant root zone soil; plants that can produce exudates have higher nitrogen fixation activity in soil (Egamberdieva *et al.,* 2008). Nitrates, according to Matthew *et al.,* (2008), are transformed into plant and animal proteins, DNA, and RNA. Bacteria and fungi decompose the dead remnants of plants or animals or their waste products to ammonia (NH3), which nitrifying bacteria convert to nitrites and ultimately to nitrates. Plants absorb and digest some of the nitrate generated in the soil. Finally, nitrates are converted to nitrogen gas by denitrifying bacteria in the soil.

The emergence of adaptive systems that protect nitrogen from molecular oxygen and reactive oxygen. Physico-ecological patterns in microbial morphology, biochemistry, physiology, and community structure can be used to identify species along a gradient from anaerobic to totally aerobic environments. (Rashid *et al.,* 2008) At the ecosystem level, the patterns and balance of nitrogen inputs and outputs constrain nitrogen fixation rates, whereas regional and global patterns of nitrogen fixation are controlled by patterns of land cover and use, bioredistribution, global climatic patterns, and nitrogen deposition patterns (Shridhar, 2012). Nitrogen fixers are exposed to physiological controls that determine their ability to accumulate molybdenum at the organismal level. Furthermore, the capacity of nitrogen-fixing organisms to colonize or persist in each ecosystem is determined by the competitive interaction, predation pressure, and availability of limiting nutrients. This collection of ecological controls is found at the third hierarchical level (Shridhar, 2012). At the ecosystem level, the patterns and balance of nitrogen inputs and outputs constrain nitrogen fixation rates, whereas at the highest level, regional and global patterns of

nitrogen fixation are controlled by patterns of land cover and use, bio-redistribution, global climatic patterns, and nitrogen deposition patterns (Shridhar, 2012).

Plant and bacterium can live separately but the associations are very beneficial for them. It is reported that in plants, up to 25% of total nitrogen came from nitrogen fixation (Al Abboud, *et al.,* 2014). The activity of nitrogen-fixing microorganisms depends greatly upon an excessive amount of carbon compounds and an adequately low level of combined nitrogen (Shridhar, 2012). The plant canopy hosts a wide array of microorganisms having beneficial, harmful, and neutralizing effects. It is well established that many soil and plant-associated bacteria groups can synthesize phytohormones. (Bashan and De-Bashan, 2010).The genera under this list are steadily growing and presently include Gram-negative and Gram-positive symbiotic and Nitrogen-fixing bacteria (Sharma *et al.,* 2020). Nitrogen-fixing bacteria such as *Azotobacter*, *Azospirillum*, *Rhizobium*, *Mesorhizobium*, and *SinoRhizobium* are well known for their ability to improve plant development (Singh *et al.*, 2019).

This work is aimed at isolating nitrogen bacterial species from stimulated soil, characterizing them and their degradative ability with time.

## Materials and Methods

The research was carried out on a farm in Eliozu, Obi Akpor, Port Harcourt. Nigeria.

## Sample collection

Soil samples were collected from the site after bioremediation using cow dung at a depth of 10 – 30cm. A sample of unpolluted soil was also taken using a sterile auger, placed in black polyethylene bags, sealed, and transported to the laboratory for further analysis. All materials, apparatus, and media were used and prepared according to the manufacturer's instructions.

## Enumeration of culture-dependent microbes

The microbiological analyses done were total heterotrophic bacteria (THB) and hydrocarbon utilizing bacteria (HUB). A serial dilution was carried out using 1 g of soil to 9 ml of normal saline to make a ten-fold dilution, 0.1 ml diluent was placed on a plate count agar and incubated for 24 hours at 37$^{o}$C.

The colonies were counted and recorded. The visible colonies were sub-cultured and characterized biochemically. The hydrocarbon utilizing bacteria was analyzed using 0.1 ml diluent on Mineral Salt Agar.

## Physicochemical characterization of samples

The total petroleum hydrocarbon(TPH) and polyaromatic hydrocarbon (PAH) contents of soil samples were determined unpolluted soil (A), polluted soil (B), and bioremediation soil (C) using methods adapted from USEPA 8015C and USEPA 8270D respectively.

Other physicochemical parameters monitored include pH (EPA 9045D), electrical conductivity (ASTM D1125), total organic nitrogen (ASTME258), temperature (APHA 2005), total organic matter (routine colorimetric method), and phosphorus (Flame Photometer).

## Detection of hydrocarbon-degrading bacteria

The degradative potential of isolates was carried out using the calorimetric method, the absorbance over time was measured.

## Results and Discussion

The total heterotrophic bacteria isolated from samples A (Uncontaminated soil), B (Contaminated soil), and C (Bioremediated soil) was 2.4 x $10^4$, 1.1 x $10^4$, and 3.4 x $10^4$(Fig 1) while the hydrocarbon utilizing bacteria was 0.1 x $10^3$, 1.8 x $10^3$, and 2.4 x $10^3$ (Fig 2) respectively. The uncontaminated soil

had the highest heterotrophic count at 2.4 x $10^4$ and the polluted soil was the lowest, this may be because of the adverse effect of the crude oil on the microbes in the polluted soil.

The presence of crude oil in soil reduces the oxygen availability in soil, reduces the nutrient content, and allows only microorganisms with the ability to use hydrocarbon as their source of energy and growth. This agrees with Akande and Obire (2008).

The hydrocarbon utilizing bacteria use hydrocarbon to produce amino acids for growth. The uncontaminated soil had the lowest observed HUB while the soil after bioremediation treatment with cow dung had the highest HUB, this may be attributed to the effect of biostimulation in reducing pollutants concentration and increasing the microbial diversity.

During biostimulation, the nutrient provided by the manure obtained from organically grazed cows was able to effectively increase the microbial community with the ability to degrade hydrocarbon. This agrees with Neethu *et al.,* (2019) and Baranu *et al.,* (2021).

The physicochemical characteristics of the samples showed pH ranging between 6.0-6.98. this pH is tolerable for hydrocarbonoclastic organisms to grow. The phosphorus content reduced with contamination, the total organic carbon increased from the quantity in uncontaminated soil and was highest in the remediated soil. This increase may be due to the nutrient provided by the organic manure during bioremediation.

This suggests that the bioremediation soil may be able to support plant growth. The electrical conductivity of the samples indicates that sample C had the highest observed value for salinity, this may be due to the addition of organic manure during bioremediation. The increase in electrical conductivity is an indication of nutrient availability and its value in C suggests the remediated soil is returning to its original state of nutrient availability as seen in Fig 3.

The morphological and biochemical characterization revealed the organisms isolated in this study to be *Arthrobacter sp, Pseudomonas sp, Nitrosomonas sp, Bacillus sp, Micrococcussp, Staphylococcus sp, and Escherichia sp*.(Tables 1 and 2).

The most frequent amongst the bacterial species isolated was *Arthrobacter sp, Nitrobacter sp, Nitrosomonas sp, and Micrococcus sp.* Most of which are nitrogen-fixing bacteria. The least frequent is *Bacillus sp and Staphylococcus sp.* The organisms isolated in Fig 4 suggest that the soil microbes will be able to support plant growth and restore the ecosystem service function of the soil.

The absorbance of the bacterial isolates decreased with time indicating the growth of organisms and hence possible degradation. However, the degradation at a wavelength of 570 is higher than at the wavelength of 370 with time (Fig 5a and b) (El Hanafy *et al.,* 2017).

The highest degradation was observed at 24 hours for both wavelengths with *Micrococcus sp, Bacillus sp, and Pseudomonas sp* being the highest. This suggests that these bacterial isolates will effectively degrade hydrocarbon in the soil if used as a consortium for the degradation of hydrocarbon. This agrees with Dawoodi *et al.,* (2015) and Mukread *et al.,* (2008).

The most frequent organisms isolated and characterized in this study are *Arthrobacter sp, Pseudomonas sp, Nitrosomonas sp, Bacillus sp, Micrococcus sp, Staphylococcus sp, and Escherichia sp*. While the bacterial species associated with nitrogen fixation is abundant in the soil, the presence of Pseudomonas which converts nitrogen from nitrates to its inorganic $N_2$ form suggests active nitrogen cycling processes in the samples.

This can reduce the fertility of the soil; however, the presence of more nitrogen-fixing bacteria suggests the soil's fertility will not be compromised. This suggests that bioremediation is an effective method for restoring the soil's ecosystem service function.

**Table.1** Biochemical characterization of isolates

| | GRAM REACTION | SHAPE | CAT | OXI | CIT | MOT | IND | MR | VP | STH | GLU | LAC | SUC | MAN | Suspected Organism |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | Positive | Rod | + | - | + | - | - | + | + | - | - | A | A | A | *Arthrobacter* |
| 2 | Positive | Rod | + | - | + | + | - | + | + | + | A | - | A | - | *Bacillus sp* |
| 3 | Positive | Rod | + | - | - | - | - | - | - | - | A | A | A | A | *Micrococcus sp* |
| 4 | Negative | Rod | + | - | + | -→ | + | + | - | + | AG | AG | AG | AG | *Escherichia coli* |
| 5 | Negative | Rod | + | - | - | - | - | - | - | - | A | - | - | - | *Nitrosomonas* |
| 6 | Positive | Cocci | + | - | + | - | + | + | + | + | A | A | A | A | *Staphylococcus* |
| 7 | Negative | Rod | + | - | + | - | - | + | - | - | - | A | - | - | *Nitrobacter sp* |
| 8 | Negative | Rod | + | + | + | + | - | - | - | - | - | - | - | - | *Pseudomas sp* |

**Table.2** Morphological characterization of bacterial isolates

| Isolates code | Color | Elevation | Texture | Shape | Opacity | Surface | Size |
|---|---|---|---|---|---|---|---|
| ***Arthrobacter*** | Creamy | Raised | Moist | Round | Opaque | Smooth | Large |
| ***Bacillus sp*** | Creamy | Flat | Moist | Round | Opaque | Rough | Large |
| ***Micrococcus sp*** | Creamy | Flat | Moist | Round | Opaque | Rough | Small |
| ***Escherichia coli*** | Grayish | Flat | Dry | Round | Opaque | Smooth | Small |
| ***Nitrosomonas*** | Yellow | Raised | Moist | Round | Opaque | Rough | Large |
| ***Staphylococcus*** | Yellow | Raised | Moist | Round | Opaque | Smooth | Small |
| ***Nitrobacter sp*** | Grayish | Raised | Dry | Round | Opaque | Rough | Large |
| ***Pseudomonas sp*** | Creamy | Raised | Moist | Round | Opaque | Smooth | Small |

**Fig.1** Total heterotrophic bacteria (THB)

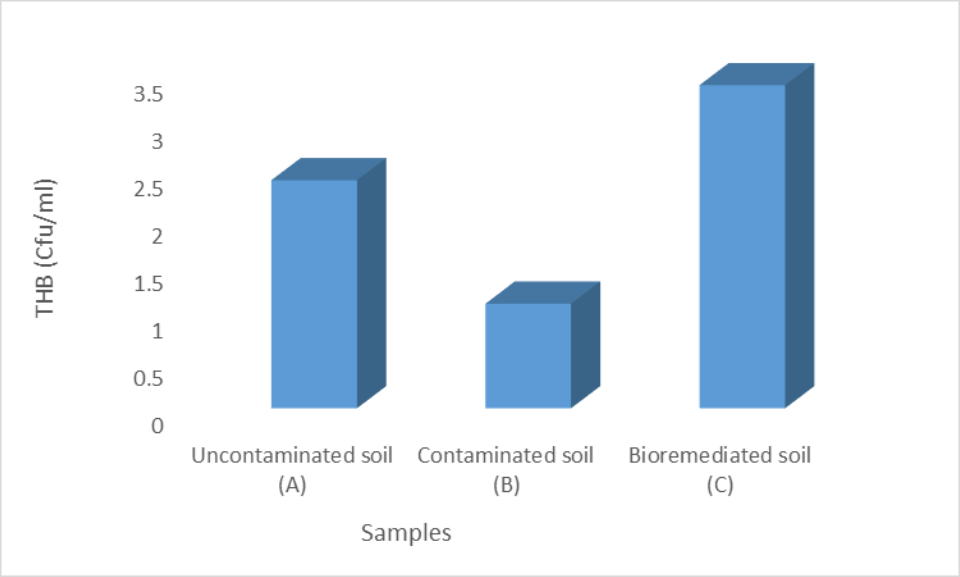



**Fig.2** Hydrocarbon utilizing bacteria (HUB)

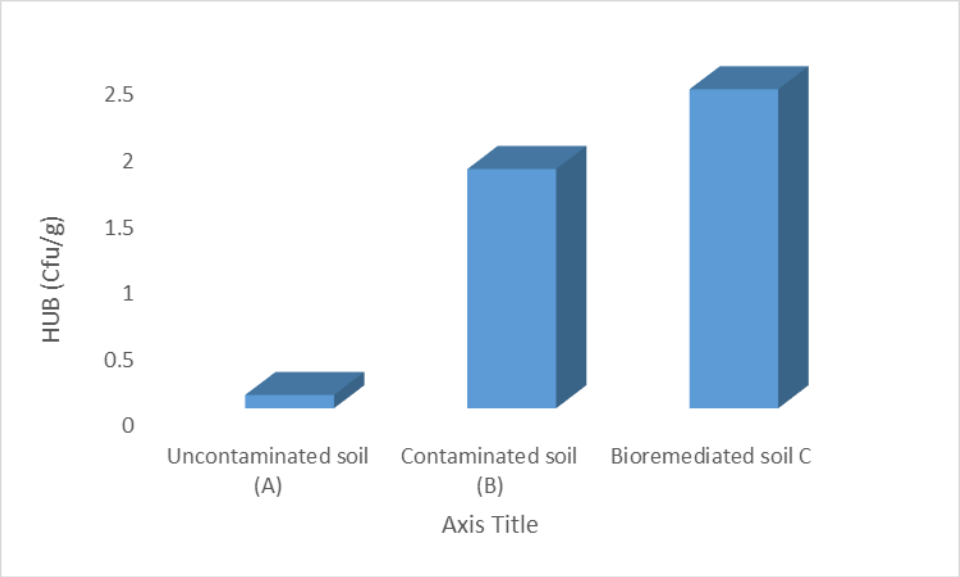


**Fig.3** Physicochemical characteristics of samples

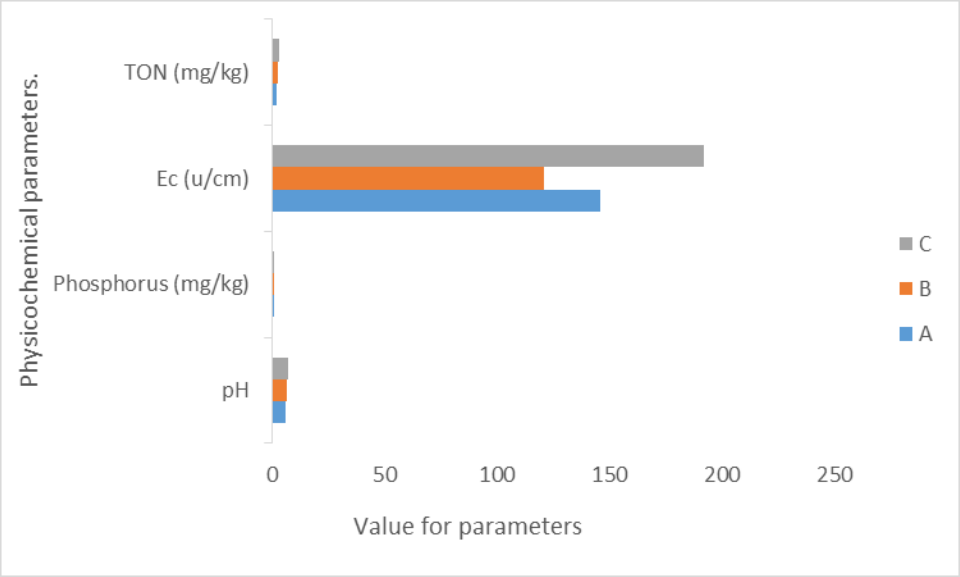



**Fig.4** The frequency of microbial isolates

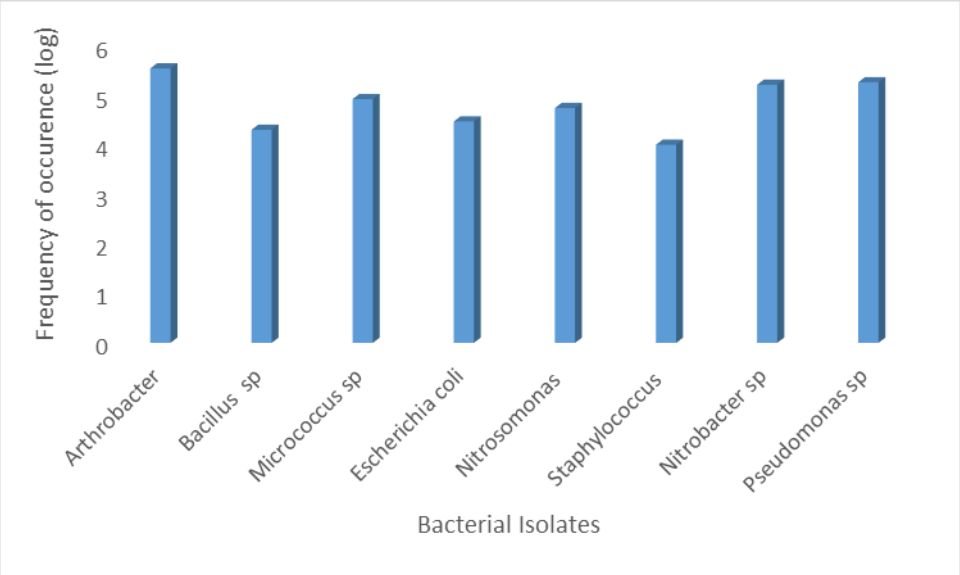


**Fig.5a** The degradation of bacterial isolates at a wavelength of 370.

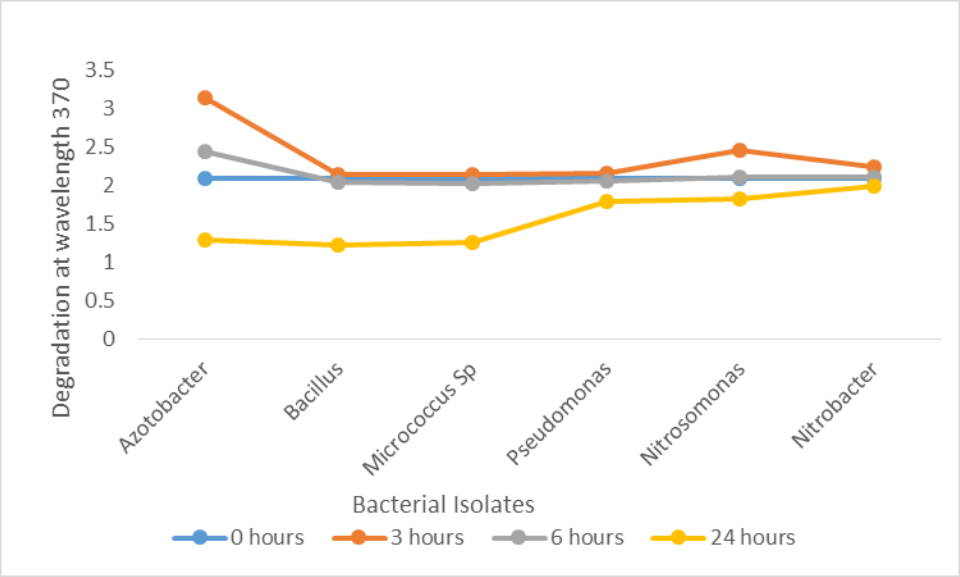
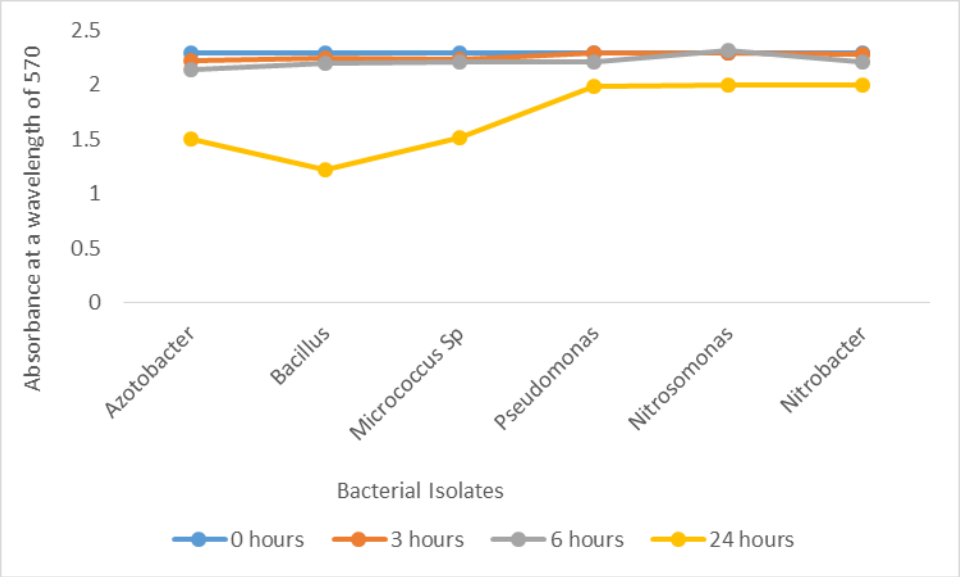



**Fig.5b** The degradation of bacterial isolates at a wavelength of 570.

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