Bima Journal of Science and Technology, Vol. 8(3B) Oct, 2024 ISSN: 2536-6041

DOI: 10.56892/bima.v8i3B.920

Nitrate Removal Potential of *Rhodobacter sphaeroides* Isolated from Abattoir Wastewater of Gombe Township, North-Eastern Nigeria

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ABSTRACT

Nitrogen is one of the essential nutrients in ecosystem but its accumulation could results into the generation of wastewater and also affects nitrogen cycle. This means that, employment of denitrifying bacteria for treatment is needed to protect our environment. In this study, a denitrifying photosynthetic bacterium capable of nitrate removal was isolated using serial dilution and streak plating method and was characterized using morphological, biochemical and physiological methods. The isolate was found to show spherical patches of red coloration detected at 300 and 1,100 nm respectively using ultraviolet spectrophometer. The isolate was also found to respond positively to oxidase, urease, catalase, citrate, malate, ethanol and glucose test with a strong physiological potential of growing under anaerobic-light and dark condition but could not thrive under aerobic light and dark condition. The active growth of the isolate was detected between days 0-1, followed by stationary phase and then decline phase. The highest biomass concentration of 4.1 mg/L was produced at day 7 culture under 85 mg/L whereas the lowest was detected at 320 mg/L of 1.3 mg/L at day 6. The effect of various initial concentrations of the nitrates (85, 135, 190, 235, 290 and 320 mg/L) were measured on the rate of nitrate removal. At 85 mg/L, highest nitrate removal of 88% without accumulation was achieved, followed by 81, 77, 73, 67, and 60 % at 135, 190, 235, 290 and 320 mg/L respectively. The results showed that increase in concentration resulted into the decrease in nitrate removal. Thus, this strain can serves as good for use in wastewater treatment plan.

Keywords: Nitrogen, Accumulation, Denitrifying Bacteria, Biomass.

INTRODUCTION

Gombe State, located in northern Nigeria is currently faced with problem of water pollution due to high level of nitrogen discharged from agricultural activities, for example fertilizer application, aquaculture and livestock farming (Mustapha *et al*., 2011). High level of nitrate in water often causes digestive tract cancer in human (Idi *et al*., 2015). In infants it causes methemoglobinemia, a disease that resulted into inability of red blood cell to contained enough oxygen for proper cell functioning (Fewtrell, 2004; Johnson, 2019). High amount of nitrate in water bodies also causes water aging and death of aquatic microorganism (Qadri and Faiq, 2020). In natural environment, anthropogenic activities have a negative effect on nitrogen cycle which also elevates the nitrogen level in water body thereby causing a serious problem to organisms leaving in water as well as the chemical status of the water body (Saidu *et al*., 2022).

The government of Nigeria have been setting and reviewing laws regulating the discharge of water containing high amount of nitrate into the water bodies (Umar, 2020). Despite that, water management and regulatory boards reported the continue consumption of

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ground water by millions of people having nitrate content exceeding the permissible limit of 10 mg/L (Mustapha *et al*., 2011).

Physicochemical methods that are considered effective for the removal of this pollutant from wastewater were found to be less efficient (Saravanan *et al*., 2021; Saidu *et al*., 2017). These methods are expensive and inappropriate to be applied for *in-situ* process (Choudhary *et al*., 2022). To overcome such challenges, biological nitrate removal should be considered due to its simplicity and complete removal of nitrate from the water body in order to save both humans and aquatic organisms. Microorganism have evolved to contained several cellular structures, this gave them an ability to tolerate different types and concentrations of nutrient or pollutant. For instance, Aslan and Kopdan, (2006) reported that, Chlorella spp. can tolerate high amount of nitrogen and phosphorus in synthetic wastewater.

Photosynthetic bacteria are prokaryotes that can be found in habitats like soil, lakes, paddy fields, oceans, rivers, and activated sludge having high potential for nitrate removal (Lu et al., 2019). They are can be either oxygenic utilizing water as an electron donor during photosynthesis as in the case of cyanobacteria and prochlorophytes or can be anoxygenic, those that that assimilate nitrogen, carbon dioxide (CO_2) and other organic or inorganic compounds as in case of *Rhodobacter* and *Rhodopseudomonas* (Idi *et al*., 2014). Therefore this research intend to isolate robust strain of photosynthetic bacteria that could assimilate nitrate dissimilatorily by providing useful kinetic data that could assist in designing bioreactor for pilot scale water treatment.

MATERIALS AND METHODS

Collection of Abattoir Wastewater

Sample of wastewater was collected from Gombe township abattoir in a clean plastic container and was transported back to the laboratory for analysis.

Bacterial Culture

Purple Non-Sulfur Bacteria culture medium (PNSBEN) was used for the cultivation. The medium contsist of NH4Cl 1.0 Na2HPO4 0.5 NaCl 2.0 MgCl2 0.2 Yeast extract 2.0 Sodium Lactate 80% 6 ml. Each of the chemicals were accurately weigh and placed in 1 litre conical flask, filed with distilled water, mixed thought and the pH was adjusted to 7. The medium was then autoclave at 121^{o}C for 15 minute. For solid medium, 10g of agar was used for solidification purpose.

Isolation of Bacteria

Pure isolate of the bacteria was obtained using serial dilution method. The method consists of four bottles containing 9 mL of distilled water; 1 mL of original sample was transfer into each and every one of the bottles and lowest dilution factor was used for the experiment via transferring into a nutrient broth. The media was then incubated in an anaerobic jar with light intensity of 2000 lux at room temperature. After the medium turns cloudy, inoculum loop was used to streak on agar plate and that was repeated until a separated, clear and visible colony observed

Morphological Examination of the Isolate

The morphological features of the isolate were detected using light microscope according to the method described by Sanders, (2012). The method involve the use of a wire loop which was initially sterilized using ethanol and flame, the loop was used to scrap the surface of bacterial colony in an agar plate and was transferred to clean microscope slide. The slide was covered with cover and transferred to light microscope for an observation under (10× magnification).

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Biochemical and Physiological Test for Identification of the Bacterium

Biochemical test such as Urease, catalase, oxidase, citrate and nitrate reductase were conducted according to the standard method as described by Saidu et al., (2021). Specifically, urease agar was used to test for urease, hydrogen peroxide for testing catalase, tetramethyl-p-phenylenediamine dihydrochloride reagent for testing oxidase; Simon citrate agar for testing citrate and nitrate reductase using sulfanilic acid and α-naphthylamine.

Citrate test

Citrate test was conducted using simoom's citrate agar culture medium. The medium was prepared by dissolving 20g of citrate in 1 liter of distilled water and sterilized in autoclave for 121°C for 15 minutes. An inoculum of the bacterial isolated was picked and streaked in a slang agar medium, it was then incubated in an anaerobic condition at room temperature for 3 days; if microbes are present, the condition will turn alkaline thereby causing a change in colour from green to blue (Hemraj *et al*., 2013).

Urease test

Urease test was conducted using ureases agar which was prepared by dissolving 30 grams of agar in 1 liter distilled water, followed by sterilization as described in the earlier section. A bacterial inoculum was placed on the surface of slant agar and incubated at 35°C for 5 days. A change in colour of the medium might occur from light orange to red/pink if the bacteria possessed urease enzymes.

Catalase test

A wire loop was used to transfer small portion of bacterial growth into microscope slide, then three drop of 3% hydrogen peroxide was added and then observed under microscope. Appearance of immediate oxygen bubbled confirmed a positive result.

Oxidase test

This method involves the use of a disk which was prepared by soaking a paper (preferably filter paper) in a glass containing tetramethyl-p-phenylenediamine dihydrochloride for about 5 minutes. A wire loop was used to transfer bacterial colony from agar and spread on disk. After three minute, if the area of the inoculum indicates the appearance of deep blue, maroon or purple color, it is positive results (Gilani et al., 2015).

D-glucose test

Glucose utilization by the isolate was measured by using wire loop sterilized under flame. The loop was used to transfer a small amount of colony and was placed in 500 mL test tube containing medium. A two layer of paraffin was used to cover the test tube and incubated at room temperature for 2 days, if the color of the medium turns yellow is a signed of positive results.

Nitrate reduction test

In this method, a sterilized wire loop was used to transfer an inoculum of bacteria into broth, incubated at room temperature for 48 h. To a small portion of growth, one drop of reagents called sulfanilic acid and α-naphthylamine was added. If the bacterium is a nitrate reducer, a reddish coloration will appear.

Physiological test

The growth of the bacteria under anaerobic-dark condition was done by transferring small amount of bacterial inoculum in medium containing 20 mM malate, 0.05% yeast extract and 20mM potassium nitrate. The culture was incubated in the absence of air at 30°C in schott bottle and the bacterial growth was observed (Ohki et al., 2012).

Detection of Photosynthetic Pigments

The photosynthetic pigment of bacteria was detected following the method of Idi et al., (2015). In this method, single colony of bacteria was placed in broth and incubated

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for 72h in light until active growth. About 10 mL of culture was diluted with 2 mL bovine serum albumin (BSA) and was analyzed for bacterial cell spectrum at 300-1100 nm using spectrophotometer.

Potentials of Nitrate Removal

The potential of nitrate removal was investigated using synthetic growth medium because it is easier to operate and study. The composition of the synthetic medium is same as PNSBEM medium described above but using $NaNO_3$ as the sole source of nitrate for the growth of the bacteria. The initial concentration of the nitrate were varied as 85, 135, 190, 235 and 320 mg/L and 2% (v/v) of bacteria was inoculated in each of these concentrations and was monitored at interval of 24h for period of 7 days. In each of these cases, after centrifugation, it is the supernatant that was used for measuring the concentration of nitrate using spectrophometer. The percentage nitrate removal was computed by subtracting initial concentration (Ci) from final concentration (Cf) divide by initial multiply by 100.

Determination of Cell Dry Weight

The cell dry weight of the bacterial biomass was determined using furnace. Five mL of sample was removed; centrifuge for 15 minute to obtain the pellets. The palate was place on foil paper and was heated in further furnace at 120^{o}C until dry. The weight of the biomass was then taken using weighing balance.

RESULTS AND DISCUSSION

Isolation and Screening of Bacteria

The results of the isolation revealed a successful isolation of pure colony of photosynthetic bacterial isolate from abattoir wastewater. This was obtained after subjecting the colony obtained from 10^{-4} serial dilution for growth in agar medium containing nitrate. The colony that has better growth was selected and was characterized using biochemical (catalase, urease, oxidase, gram staining) and morphological features (color, shape, mobility) Table 1. Based on the results obtained from Table 1, the isolate was found to respond positively to oxidase, urease, catalase, citrate, malate, ethanol and glucose test. It was reported that the isolate has physiological potential of growing under anaerobic-light and dark condition. Moreover, the isolate could not thrive under aerobic light and dark condition. This could be due to the fact that photosynthetic bacteria can utilize light for photosynthesis but in the absence of light, they switch the photosynthetic system by utilizing organics compounds such as ammonium and sulfide as a source of carbon as literature clarified (Lu *et al*., 2019). Furthermore, it was reported that photosynthetic bacteria can thrive in wide range of temperature variation 20-30^{o}C and pH of 6-7. These features were found to be more related to *Rhodobacter sphaeroides.*

Table 1: Biochemical and physiological characterization of the isolate

Characterization of the isolate			
Biochemical		**Physiological**	
Oxidase	+	Growth Temp. (^{0}C)	30
Urease	+	Aerobic-light	-
Catalase	+	Aerobic-dark	-
Nitrate reduction	+	Anaerobic-light	+
Citrate	+	Anaerobic-dark	+
Malate	+	Growth at pH	7.2
Ethanol	+	Gram staining	-
D-Glucose	+	Colony Morphology	Orange, large
Motility	+	Mobility	Motile

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For morphological characterization, the isolate was found to show spherical patches of red coloration detected at 300 and 1100nm respectively using ultraviolet spectrophometer (Figure 1). The reddish feature is an indication that the isolate contained bacteriochlorophyl.

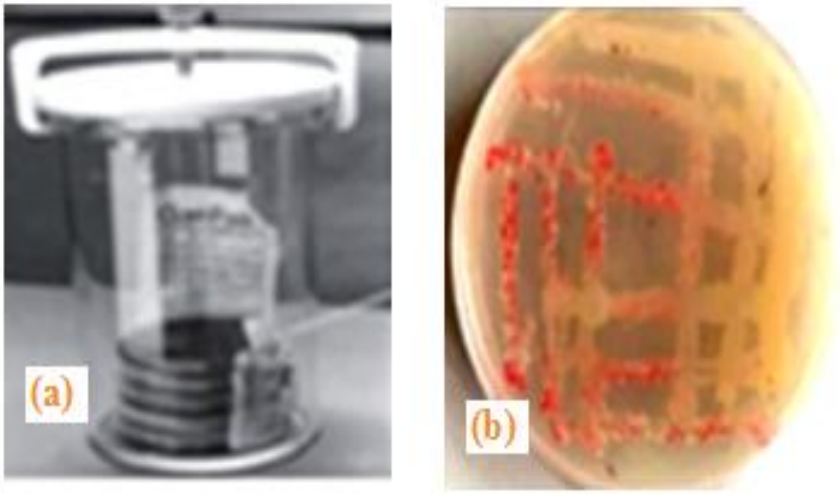

Figure 1: Growth of bacterial isolate; (a) anaerobic jar, (b) agar plate.

Teves, (1998) reported the isolation of eight photosynthetic bacteria where all the cultures showed un-flagellated yellowish-reddish coloration. In addition, research study involving analysis of biochemical and physiological features of bacteria as a tool for identification was reported in literature. For instance, Choi et al., (2002) reported the isolation and identification of photosynthetic bacterium based on its growth rate, gram staining, shape, chemical response and ability to produce value-added substances.

Nitrate Removal Potential of the Isolate

The rate of nitrogen removal was found to be dependent on its initial concentration. This is because, at a certain amount, the enzymes presence in the bacteria cannot work efficiently due to toxicity. This is crucial in determining the tolerant level bacteria to a given nutrient removal. Based on the result presented in Figure 2, the initial nitrate concentration of 85 mg/L gave the highest nitrate removal of 88%. As the concentration increases to 135 mg/L, the percentage of nitrate removal decreases to 81%. Further increase in concentration to 190, 235 and 290 mg/L gave a progressive decrease in the percentage of nitrate been removed of about 77, 73 and 67% respectively. 60% of nitrate removal was recorded as least under maximum initial nitrate level of 320 mg/L (Figure 2). It was observed that an increase in nitrate concentration causes a decrease in the percentage removal.

This result was found to be similar with the finding of Idi et al., (2015) who reported the nitrate removal ability of *Rhodobacter sphaeroides* ADZ101 in synthetic wastewater where an increase in initial concentration of the nitrate causes a decrease in percentage of nitrate removal efficiency. The season for the low percentage of nitrate removal under high nitrate concentration could be due to the toxicity as the amount of the nitrate is high for normal metabolism of the bacteria, thereby affecting its growth.

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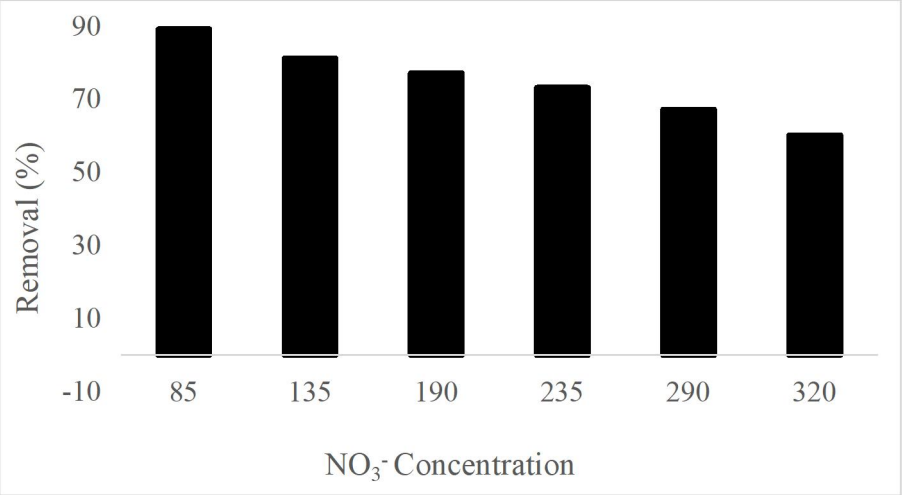

Figure 2: Relationship between initial concentrations of nitrate and its removal efficiency.

Growth of the Isolate Under Different Concentrations of Nitrate

In order to understand pattern of nutrient utilization, the isolate was cultured under different concentrations of nitrogen and the growth was monitored for over 7 days. Cell dry weight method was used to measure an increase in the level of biomass while concomitantly removing the nitrate as shown in Figure 3.

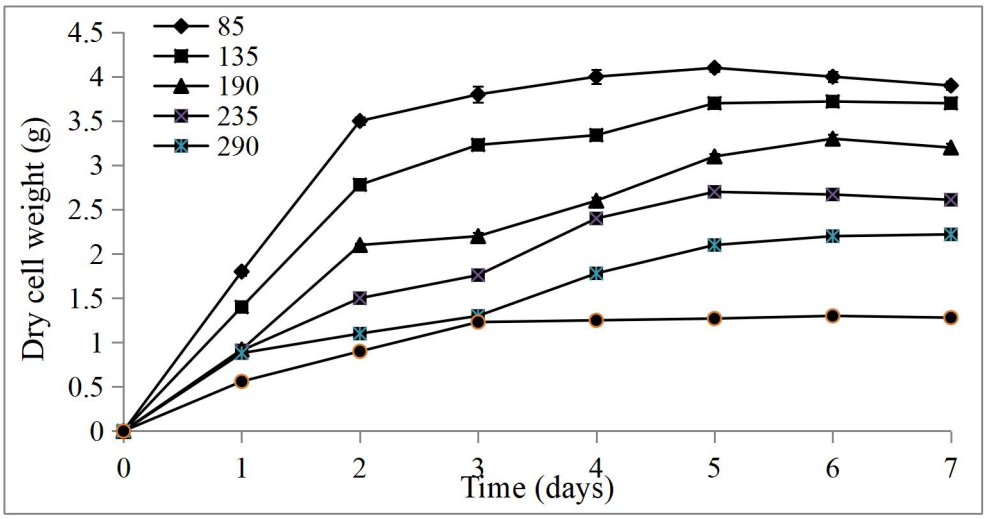

Figure 3: Growth of bacterial strain in a culture containing various initial concentrations of nitrate.

All the isolate showed a rapid growth at log phase of growth between days 0-1, immediately after day 1, the growth enters stationary phase for 290 and 320 mg/L concentrations whereas for other concentrations, the growth continued. Isolate growing under 320 mg/L produces the lowest biomass concentration of 1.23 g at day 3 and then remain at stationary until day 7. This has corresponded to the results presented in Figure 1 where nitrate utilization reduces at 320 mg/L. Highest biomass of 4.1 mg/L was produce when growing under 85 mg/L nitrate concentration at day 5. At concentration of 135, 190, 235, 290 mg/L, the following biomass concentrations were produced 3.72 g and 3.33g at day 6, and 2.7 g at day 5 and 2.2 g

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at day 7 respectively (Figure 3). The pattern of biomass production was also found to be related to nitrate concentration, higher amount of biomass are produce at lower concentration and decrease steady as the concentration increased, and this is because the bacteria need more time to acclimatize in higher concentration which is more toxic than lower concentration.

Pattern of Nitrate Removal

The result showing the pattern of nitrate removal was plotted in form of a curved where the values at vertical axis represent nitrate concentrations and horizontal axis represent time of the culture (Figure 4).

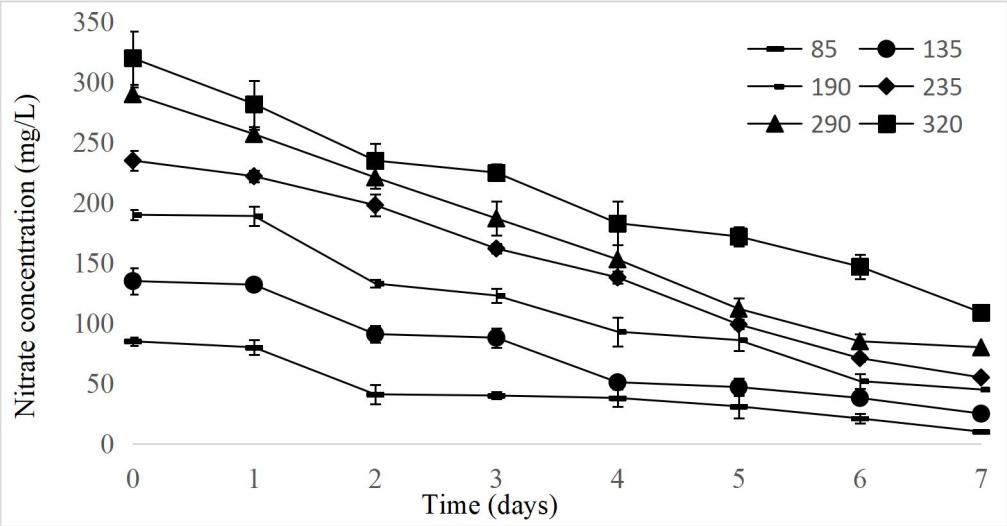

Figure 4: Nitrate removing curve of the isolate over seven days culture.

The nitrate removing curve under 85, 135 and 190 mg/L were found to be parallel between days 1 to 4. For 135 mg/L, it experiences a brief stationary decline between day 1 to 3 and then fall progressively. Nitrate reduction under 320 and 290 mg/L decline continuous at a parallel trend until day 7. All the nitrate reduction length brought to halt at exactly day 7.

CONCLUSION

Conclusively, the study reported the successful isolation of *Rhodobacter sphaeroides* from abattoir wastewater and confirmed its potential for nitrate removal. The isolate can remove nitrate under different initial concentration of nitrate. 85 mg/L was found to support active growth of bacteria whereas 320 mg/L was toxic. The bacteria produce maximum biomass of 4.1 mg/L at day 7. The nitrate removal curve indicated that, increase in initial concentration of nitrate lowers its efficiency for nitrogen removal. The highest nitrate removal was achieved under 85 mg/L. Therefore, *R. sphaeroides* can be a good candidate for wastewater treatment containing high amount of nitrate.

Acknowledgement

The author would like to acknowledge Tertiary Education Fund (TETFund) Nigeria for funding this research via 2024 cycle of Institutional Based Research (IBR). The first author also thanks Gombe State University for providing an enabling environment for the conduct of the research.

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