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Isolation of Photosynthetic Bacteria from Coal Mining Site Having Potential for Nitrate Removal

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ABSTRACT

Wastewater is untreated water that has high amount of nutrients such as nitrate, phosphate, ammonium and chemical oxygen demand (COD). When it is discharged into watercourse, it affects human and aquatic biota. The application of photosynthetic bacteria is considered bio-friendly system than the conventional one. Hence, the present study investigates the effectiveness of robust strain of photosynthetic bacteria for nitrate removal under different concentrations of 85, 135, 190, 235 and 320 mg/L. Serial dilution techniques was used for the isolation of the bacteria. The results showed that three bacterial isolate were obtained and were both screened for nitrate reduction ability. The isolate was able to remove 91, 90, 71, 67 and 55% of nitrate at 85, 135, 190, 235 and 320 mg/L respectively. The bacteriochlorophyll of the isolate was detected at peak range between 689-710 nm. The morphological, physiological and biochemical characterization showed that the isolate was identified as *Rhodopseudomonas* sp. The nutrient removal yield of the nitrate under different concentrations was found to be at range of 0.01- 0.033 g^{-1} DCW g^{-1} NO_3^- This study suggested that the strain could be used as an efficient bacterial candidate for the treatment of wastewater containing high amount of nitrate.

1. INTRODUCTION

The increase in human activities such as agriculture, industrialization, urbanization and population growth produces vast volume of pollutants that are unhealthy to the environment. Several sources of such effluent are municipal and industrial set up. Majority of such set up are constantly faced with dilemma on how to efficiently deal with their discharges in an efficient process before being released into the environment [1]. This effluent may include toxic pollutant such as heavy metals, xenobiotic compounds, phenolic compounds, chemical oxygen demand, nitrate and phosphorus [2]. When these pollutants get integrated into the environment, they confer adverse effect on both human and aquatic biota [3]. For instance, high amount of nitrogen in water was reported to cause several diseases in infant [4].

Chemical method of treatment is the main techniques employed in removing such pollutants from the environment. Moreover, it was reported that chemical treatment produces secondary pollutant, high cost of operation and couple together with promoting the growth of filamentous algae that may clog water treatment plant filters [5]. Biological method is therefore considered the best due to its low cost of operation. Biological method can be done on site, and does not produce secondary pollutant [6]. Hence can also be incorporated with other treatment methods, thereby treating

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complex and mixed waste [7]. In addition, [8] suggested that the efficiency of this method can be improved by the choice of microorganism with the desirable potential of removing complex contaminants.

Photosynthetic bacteria are considered group of bacteria capable of removing nitrogen and phosphorus. They often used these nutrients for assimilation, growth and reproduction. They also produce oxygen for chemotrophic bacteria to degrade organic compound efficiently [9]. Because these bacteria require carbon dioxide for growth, they then purify the polluted atmosphere as well as treat wastewater [7]. Several research studies on the application of bacteria for nitrogen removal are available in the literature. However these research could not get a robust and well adapted isolate that can clean nitrogen efficiently from environment [10]. Therefore this research was conducted in order to assess the potential of bacterial isolate obtained from Maiganga coal mining site for nitrogen removal at different concentrations. Biokinetic analysis will also be conducted with a view to determine the biomass accumulation by the bacteria.

2. MATERIALS AND METHODS

2.1 Samples collection

The source of bacterial isolation was the Maiganga coal mine wastewater site in Kumo, Akko local government area of Gombe State, Nigeria. It is located 8 km off Gombe–Yola road. The area lies between Latitude 09°18' and 11°59'E (Figure 1). The justification of selecting Maiganga coal mining site was due to the adverse activities taking place there which could produce robust bacterial strain. This strain will have capability of stripping recalcitrant pollutants efficiently from environment. Wastewater samples was collected at a depth of approximately 0.5 m aseptically and kept in an air-tight container. This is to reduce oxygen contamination, thus providing semi anaerobic condition. The sample was then transported back to the lab for further analysis.

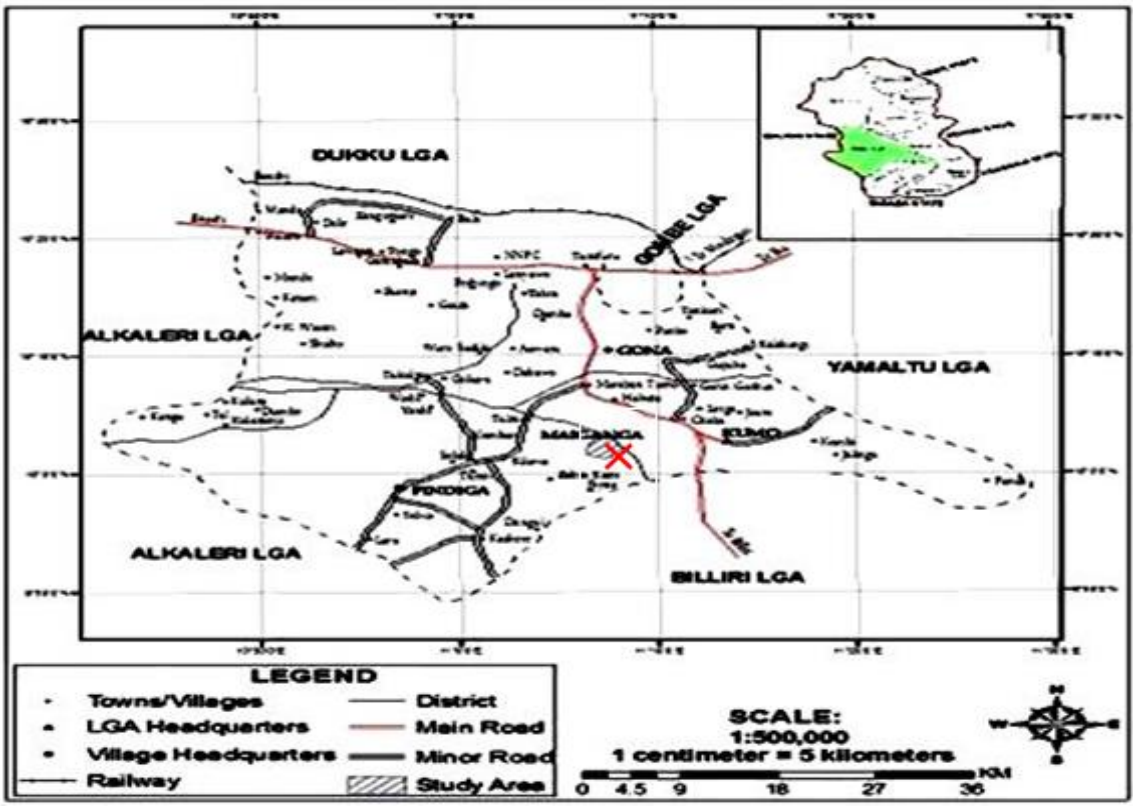

Fig. 1. Map of Maiganga coal mining site, Akko LGA, Gombe State.

Table 1. Chemical composition of PNSBEM medium

Chemical component	Composition (g/L)
NH_4Cl	1.0
$Na2HPO_4$	0.5
NaCl	2.0
MgCl2	0.2
Yeast extract	2.0
Sodium Lactate 80%	6 ml

The entire chemicals were measured and added into a 1 L conical flask containing 1 L of distilled water. The medium was mixed thoroughly and pH adjusted to 7.2 with 1.0 M NaOH before autoclaving. For solid medium, 15 g of agar was added before the pH is adjusted [8]. A 30mL of medium was transferred into appropriate sterile screw capped universal bottles, sealed and autoclaved at 121°C for 15 min. It was then left to cool and stored.

2.2 *Isolation of bacteria*

A serial dilution technique was carried out using 10^{-5} dilution factor. The justification of using dilution is to reduce the bacteria mass growing in the culture in order to obtain a pure isolate. The lowest dilution fold was inoculated into broth medium contained in universal bottle using 10% (v/v). It was then incubated in an anaerobic condition using anaerobic jar with light intensity between 2000 and 3000 lux at room temperature Figure 2. Anaerobic condition was provided using anaerobic jar instrument. Harvesting of the bacterium was done with repeated spread plating technique after the appearance of bacterial bloom. The isolate was then streak on fresh agar plate followed by repeated streaking to ensure purification.

2.3 *Detection of photosynthetic pigments*

Bacteriochlorophyll content of the bacteria was detected using method described by [13]. The method involves inoculating single colony of pure culture into the broth medium. It was then incubated under light, after the medium turned cloudy. About 10 % (v/v) of the culture was further inoculated into fresh medium and incubated for 72 h. The culture was then diluted with bovine serum albumin (BSA). Analysis of the bacterial cell spectrum was done at the range of 300-1100 nm with spectrophotometer (Buck Scientific, Inc).

2.4 *Potentials of nitrate removal*

Nitrate removal ability of the isolate was done using synthetic wastewater. $NaNO_3$ was used as the only nitrogen source for bacterial growth. The composition of the wastewater described in Table 2. The chemical constituents' were accurately measured and added into a 1 L conical flask containing 1 L of distilled water. The mixture was mixed thoroughly. pH of the mixture was adjustment to 7.0 using 1.0 M NaOH before autoclaving at 121°C for 15 minutes

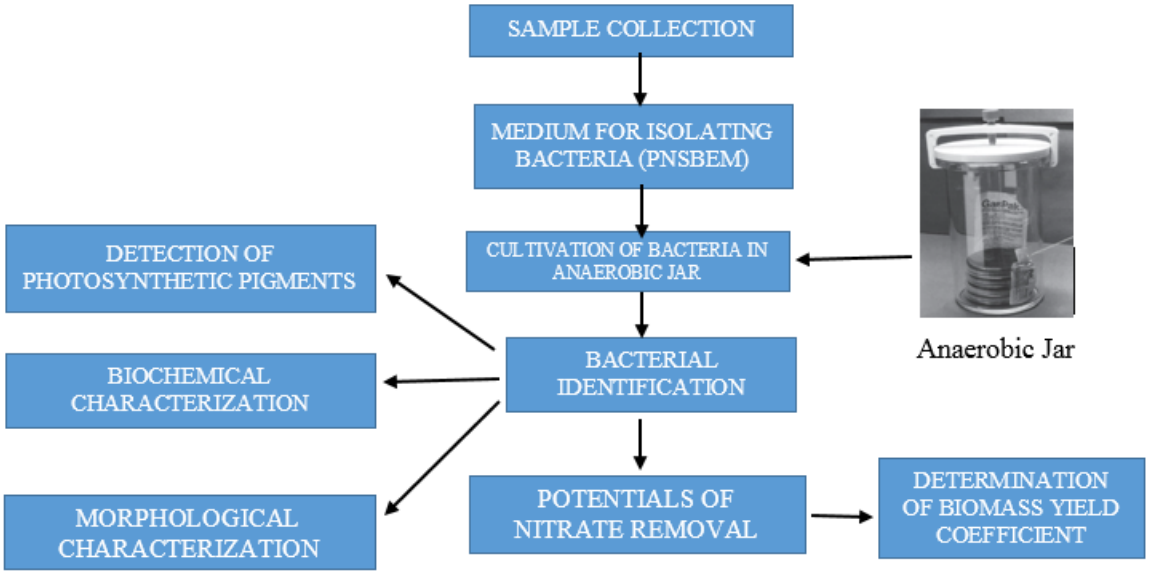

Fig 2: Experimental set up used for isolation and treatment.

Table 2. Chemical composition of synthetic wastewater

Chemical component	Composition (g/L)
KH_2PO_4	0.33
$MgSO_4.7H_2O$	0.33
NaCL	0.33
$CaCL_2.2H_2O$	0.05
Yeast extract	1.0
Sodium succinate	1.0

For the determination of nitrate kinetic coefficient of the bacterium, several concentrations of 85, 135, 190, 235 and 320 mg l^{-1} of nitrate were employed. pH of the wastewater was also adjusted and maintain at 7 using NaOH. In order to

ensure active process performance, the bacterium was left to adapt in 0.5 g l^{-1} $NaNO_3$. At exactly lag phase, about 2% (V/V) was transferred into 50 ml screw cap bottles containing different nitrate concentrated wastewaters. The empirical was done under facultative anaerobic condition without shaking at room temperature with light intensity of 2000-3000 lux. Samples were removed at an interval of every 24 h, the centrifuged; secure the supernatant for the determination of amount of nitrate.

2.5 Biochemical test for identification of the bacterium

Urease Test

The chemicals used for the preparation of Christensen urease agar was described (Table 3). The media was prepared by dissolving 38.71 grams of urease agar in a flask containing 1000 ml distilled water. The mixture was swirled squarely to dissolve the particle before being autoclave at 121 ^{0}C for 15 minutes. It was then distributed in sterile tubes. Production of orange color at the surface of slant indicated that the bacteria produce urease enzymes.

Table 3. Composition of Christensen urease agar

Chemicals	Composition (g/L)
Peptone	1.0
Dextrose	1.0
Sodium Chloride	5.0
Potassium phosphate	2.0
Urea	20.0
Phenol red	0.012
Agar	15.0

Citrate Test

The composition of Simmons Citrate Agar was shown in Table 4. Accurately, about 24 g of Simmons Citrate Agar was transferred into a 1000 ml bottle filled with distilled water. The mixture was reflux in order to dissolve the particles. It was then shaken to ensure uniform distribution in test tubes. It was then sterilize in autoclaving machine at 121°C for 15 minutes. The appearance of growth at slant and intense blue coloration indicate positive results **for** Citrate **[13].**

Table 4. Components of Citrate Agar

Composition	Quantity (g/L)
Magnesium Sulfate	0.20
Ammonium Dihydrogen Phosphate	1.00
Dipotassium Phosphate	1.00
Sodium Citrate	2.00
Sodium Chloride	5.00
Thymol Blue	0.08
Agar	15.00

pH : 6.8

Catalase Test

An inoculum of bacterial colony was transferred from an active growth culture into a clean, dried glass slide using sterile loop. It was followed by the addition of drop of 3% H_2O_2 on the slide. Observation of instant oxygen evolvement/bubbling from the prepared slide indicate positive for catalase.

Nitrate reduction test

Aseptically, bacterial isolates was inoculated into a nitrate broth. It was then incubated at 37^{o}C for 48 hours. The procedure was completed by the addition of one drop of sulfanilic acid. Followed by another one drop of a α-naphthylamine to the broth. Appearance of red color indicates that the organism is a nitrate reducer.

Oxidase Test

In this method, a clean filter paper was soaked in a glass containing tetramethyl-p-phenylenediamine dihydrochloride. After 5 minutes, the paper was then moistened with distilled water. A wire loop was used to pick a colony and smear it on the paper, the appearance of deep blue or purple color within 10-30 second indicate a positive results

Motility Test

A dry and clean needle was used for this test. The needle was used to swab the surface of active bacterial growth on agar medium. It was stabbed once to a depth of only 1/3 to ½ inch in the middle of the tube. Incubation of the culture was done at 37°C. Positive growth was defined by observing hazy growths that spread throughout the medium rendering it slightly opaque.

D-glucose Test

A wire loop was used to pick a bacterial colony and placed it in test medium in a test tube. It was then covered by thin layer of liquid paraffin. It was then incubated at 37°C for 48 hours. The appearance of Acid production characterized by yellow color indicates positive results.

D-glucose Test

A wire loop was used to pick a bacterial colony and placed it in test medium in a test tube. It was then covered by thin layer of liquid paraffin. It was then incubated at 37°C for 48 hours. The appearance of Acid production characterized by yellow color indicates positive results.

3. RESULTS AND DISCUSSIONS

3.1 Isolation and screening of Bacteria

A total of three different strains of bacteria were isolated in this study. Isolation of the pure colony of the potential bacteria was carried out using serial dilution technique and streaking techniques. The major distinctive feature of this bacterium is their ability to produce reddish color. The reddish color was as results of photosynthetic pigment commonly known as bacteriochlorophyll. The photosynthetic pigment analysis using UV-Vis spectroscopy showed whole cell absorption reading at 300 nm to 1100 nm. Characteristics bacteriochlorophyll signals were found at 689 nm and 710 nm (characteristics of purple non-sulfur bacteria). The isolated photosynthetic bacteria were characterized on the basis of colony, morphology, colour, growth characteristics, Gram staining, mobility, catalase, oxidase, urease, nitrate reduction and carbohydrate utilization tests (Table 5 and 6). The use of morphological, physiological and biochemical test for the identification of the isolate were adopted from the research of [14]. In his research he confirmed that all the isolates produced yellow-brown to yellow-green cultures under anaerobic phototrophic growth conditions. The color of the cultures turned to red when they were exposed to air. This was the case with members of the genera ***Rhodobacter*** and ***Rhodovulum*** which was also observed in the course of this research. This further gives credence to the identification of the isolates (Figure 3).

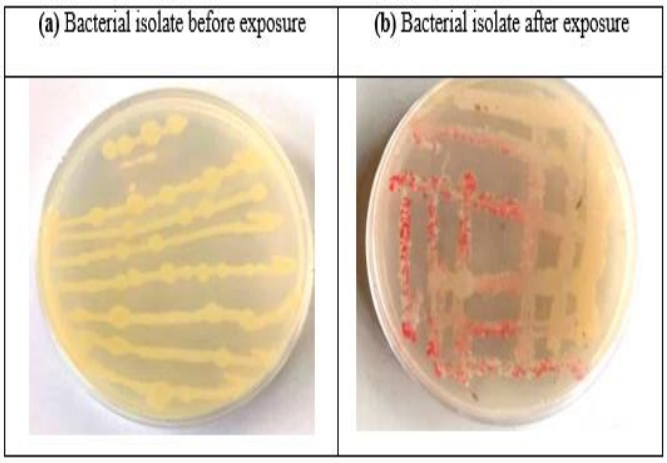

Fig. 3. Colour changes of the bacterial isolate when exposed to air.

3.2 Nitrate removal rate

The isolate with the highest growth in the $NaNO_3$ was selected for the research. However, this study focused on nitrate concentration because it is easier to control compare to pH and dissolve oxygen. The effect of various initial concentrations of nitrate on nitrate removal was given in Figure 4.

The figure explained how each initial concentration of nitrate affect the efficiency of the isolate to remove nitrate.

Table 5. Physiological test for the confirmation of the isolates

Characteristics	Bacterial isolates		
	RI	R2	R3
Growth Temp. (^{0}C)	30	30	30
Aerobic-light	+	+	+
Aerobic-dark	+	+	+
Anaerobic-light	+	+	+
Anaerobic-dark	-	+	+
Growth at pH	7.2	7.2	7.2
Gram staining	-	-	-
Colony Morphology	Orange, large	Red ,tiny	Red, small
Mobility	Motile	Non-Motile	Motile

Table 6. Biochemical test for the confirmation of the isolates

Characteristics	R1	R2	R3
Citrate	+	-	+
Urease	+	+	+
Catalase	+	+	+
Nitrate reduction	+	-	-
Oxidase	+	+	+
Succinate	+	+	+
Ethanol	+	+	-
D-Glucose	+	+	+
Motility	+	-	+
Malate	+	+	+
Identified genus	*Rhodo pseudo monas* sp.	*Rhodomicrobium* sp.	*Rhodobacter* sp.

Positive/Utilization: (+) ; Negative/Non Utilization : (-)

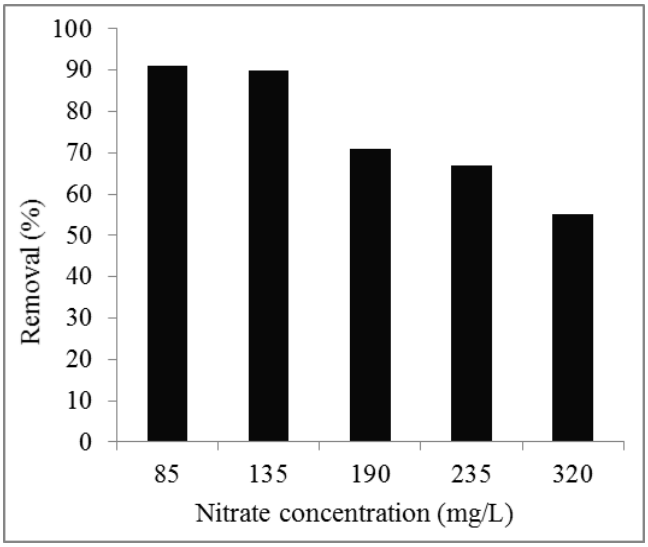

Fig. 4: Effect of various initial concentrations on nitrate removal

This is because certain concentration of nitrate might increase the bacterial growth thereby increasing the nitrate removal capacity of the isolate. Whereas in some concentration are going to be toxic which might affect the growth of the bacterium.

Thus, at the concentrations of 85 mg l^{-1} and 135mg l^{-1} about 91% and 90% of nitrate was removed. As the concentration increased to 190 mg l^{-1} and 235 mg l^{-1}, 71% and 67% were removed respectively. The percentage dropped down to 55% when 320 mg l^{-1} was used. This clearly indicates that lower concentrations have higher nitrate removal.

The pattern of nitrate removal was also found to be related to bacterial growth in Figure 5. Figure 5 explained the growth behaviour of the bacterial isolate under different nitrate concentrations for a period of five (5) days. The amount of growth was observed to be higher at lower concentration and decreased steadily as the nitrate concentration increased. Growth was not observed when the concentration was further increased to 550 mg l^{-1}.

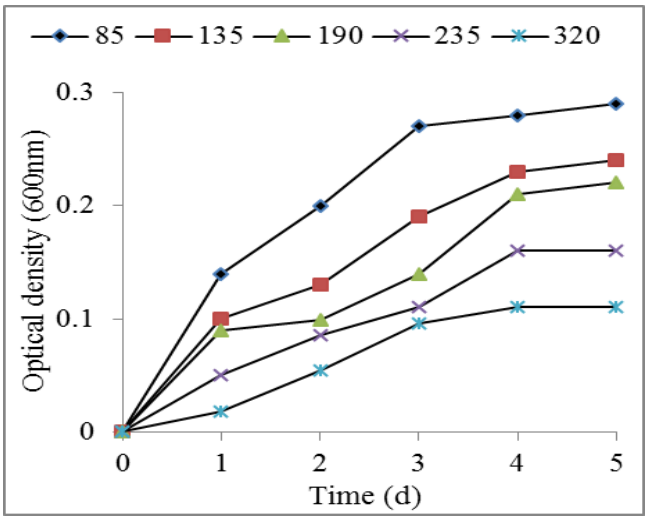

Fig. 5: Growth pattern of isolates on different initial concentrations of nitrate. (**a**) 85 mg-l, (**b**) 190 mg-l, (**c**) 135 mg-l (**d**) 320 mg-l.

The inhibition of growth at this concentration may be attributed to toxicity of nitrate to the bacteria at that particular concentration [16]. The growth curve shows a shift in the lag phase of the bacterial growth. At the concentration of 85 mg l^{-1}, 135 mg l^{-1} and 190 mg l^{-1}, no lag phases were observed within 24h while at the concentration of 235 mg l^{-1} and 320 mg l^{-1}, the lag phase was extended to the end of 48 h (Figure 5).

This is because the bacteria need more time to acclimatize in higher concentrations which is more toxic than lower concentrations.

Nitrate reduction curve was plotted in Figure 6. The figure explained the nitrate reduction pattern of the isolate over 5 days cultured. The aim was to analyse the nitrate removal behaviour of the isolate between days.

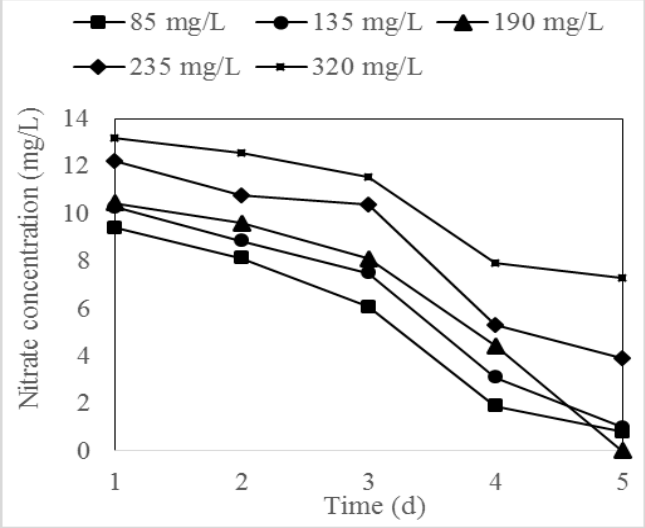

Fig. 6: Nitrate removal curve of Isolate in different concentrations of nitrate over 5 day's culture.

The nitrate reduction curves in Figure 6 have parallel trend line across the different concentrations of nitrate except at 235 mg/L. This indicated that there is no much difference in the pattern of nitrate removal by *Rhodobacter* spp over different concentrations [17]. Nitrate removal curves at 320 mg/L and 325 mg/L almost immediately reached stationary phase between days 1 to 3. In this system, insufficient nitrate was the limiting nutrients for growth. In the case of 85 mg/L a negligible removal was observed between days 4 to 5. This coincides with steady growth of the bacteria when growing between days 4 to 5 as initially presented in Figure 5. This was further supported by the assertion availability of nutrient constitute an important factor in stimulating growth of bacteria [7].

3.3 Determination of the biomass yield coefficient of the isolate

The Yield coefficient for Nitrate removal was calculated by using Eqs. (1);

(Biomass) f − (Biomass) i = YN [NO_3^-)0 − (NO_3^-)f] ……..(1)

- where (Biomass)f is the final biomass concentration (mg l−1),
- (Biomass)i is the initial biomass concentration (mg l−1) at the beginning of the experiments,
- (NO_3^-)0 and (NO_3^-)f are the initial and the final Nitrate concentrations (mg l−1), respectively

Based on the slope of the graph, the yield coefficient of nitrate removal (Y) at different concentrations were computed as shown in Figure 7. The slope of the biomass ((Biomass)f-(Biomass)i) versus $(NO_3^-)_i - (NO_3^-)_f$ give yield coefficient values of NO_3^- at 85, 135, 190, 235 and 320 mg/L concentrations to be 0.01, 0.022, 0.21, 0.22 and 0.33 g^{-1} DCW g^{-1} NO_3^- respectively. The data represent the biomass production strength of the isolate when cultivated in different concentration of nitrate. Moreover, these data were summarized and compared with other research as shown in Table 7.

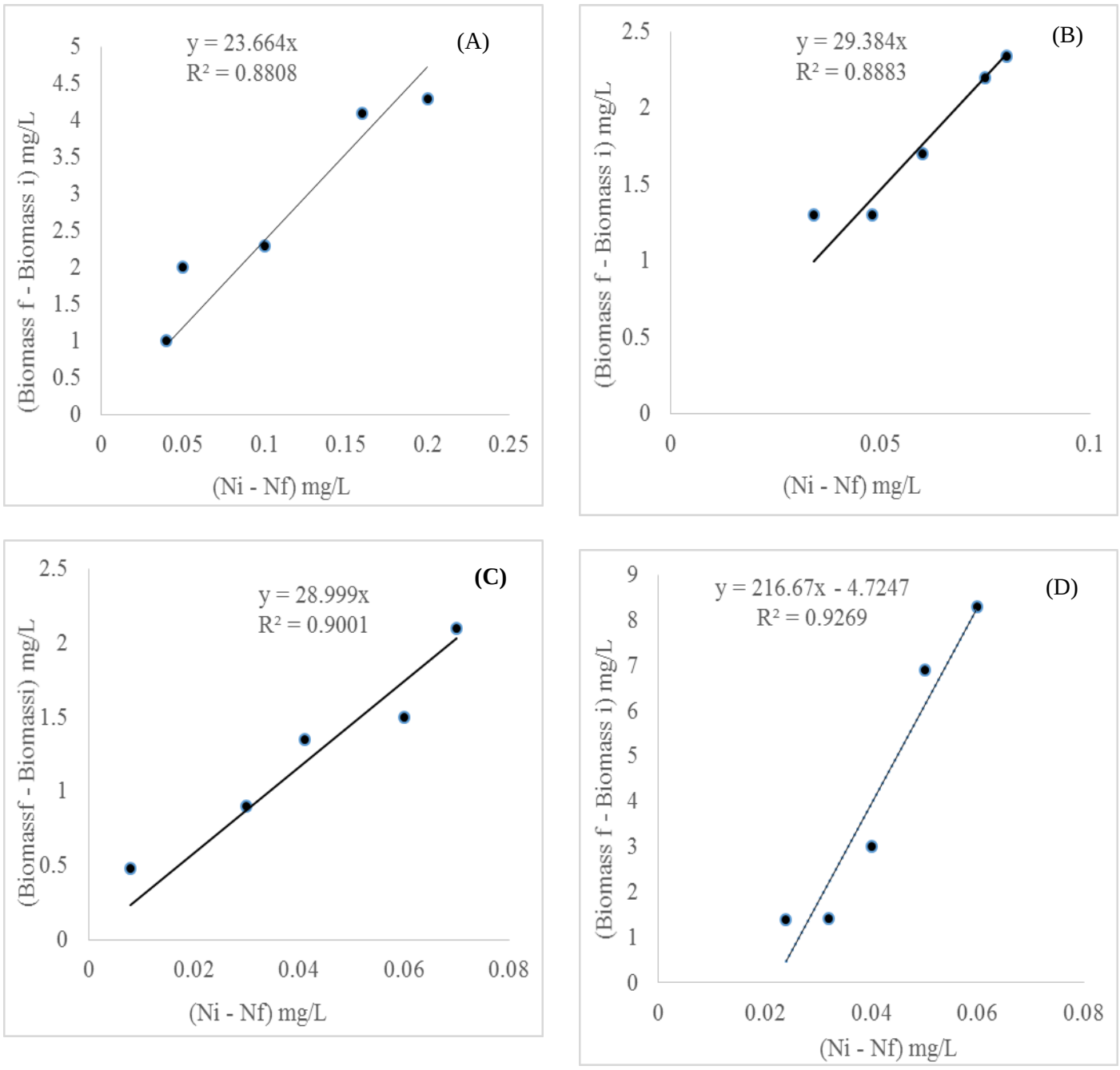

Fig. 7: Determination of yield coefficient for nitrate removal by bacterial isolate (**a**) 85 mg-l, (**b**) 190 mg-l, (**c**) 135 mg-l (**d**) 320 mg-l.

Table 7: Comparison of nutrient removal yield of nitrate from wastewater

Type of bacteria	Substrate	Nutrient	Y	References
R. sphaeroides ADZ101	Synthetic wastewater	NO_3^-	0.15 mg DCW mg/L NO_3^-	[18]
C. sorokiniana	Palm Oil Mill Efffluent	NO_3^-	0.1 g^{-1} DCW g^{-1} NO_3^-	[6]
Rhodopseudomonas sp	Synthetic wastewater	NO_3^-	Range 0.01-0.033 g^{-1} DCW g^{-1} NO_3^-	Present study

The nitrogen removal efficiency reported from this study varies depending on the media composition and environmental condition such as initial nutrient concentration, light intensity and species of bacterial. The nitrogen removal obtain from this study was lower than those reported from other studies. For instance, [18] cultivated photosynthetic bacteria in synthetic wastewater and reported the biomass production yield of 0.15 mg DCW mg/L NO_3^-.

4. CONCLUSION

Photosynthetic bacteria (*Rhodopseudomonas* sp.) were successfully isolated from wastewater obtained from Maiganga coal mining site. The isolate identified using biochemical, physiological and morphological tests. Hence Analysis of bacteriochlorophyll was also conducted in order to validate the identity of the isolate. Nitrogen removing ability of the isolate was tested under different concentration of nitrogen. The isolate removed 91% nitrate at 85 g/L concentration. At 135, 190, 235 and 320 mg/L concentration, the removal efficiencies were found to be 90, 71, 67 and 55% removal respectively. The biomass yield of the isolate at 85, 135, 190, 235 and 320 mg/L were found to be 0.01, 0.022. 0.21, 0.22 and 0.33 g^{-1} DCW g^{-1} NO_3^- respectively. This study confirms that Bacteria *Rhodopseudomonas* sp. has the great ability for nitrogen removal at high amount which could be beneficial in wastewater treatment system. More study should be carried out on the ability of the isolate to remove other recalcitrant pollutants.

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