

Bacteria Screening with Sufficient Nitrate Extraction from Wastewater

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Search for effective biological strain for use to remedy water pollution has been limited. This study was performed to search for effective bacteria capable of extracting nitrate from wastewater. Samples from abattoir inside (Abi) and abattoir outside (Abt), fishpond of kwalkwalawa (Fpk) and mabera fishpond (Fpm) wastewater were used. Using conventional standard plate technique as 1.96×10^7 cfu/ml (Abi) had highest bacteria count while 1.05×10^7 cfu/ml (Fpk) had minimal. The obtained pure colonies were morphologically and biochemically characterized including two *Enterobacter* specie, *Proteus* and *Pseudomonas* specie. The isolated organisms were used as a test organism for the removal of nitrate present in high nitrate containing medium. Prior to the extraction, the wastewater samples were physicochemically analyzed using parameters like color, odor, pH, alkalinity, hardness, temperature and nitrate. For nitrate extraction, bacteria were inoculated in a separate tubes containing nitrate broth, incubated in a rotary shaker 150rpm at 37°C for 16hrs. The supernatant from 10minutes centrifuged culture were used for nitrate removal evaluation after a series of reaction and stop using spectrophotometer at 420nm. The absorbance 0.732 *proteus* specie after estimation of nitrate in a medium was maximal while *Enterobacter* specie 0.574 was minimal. Meanwhile, two of the *Enterobacter* specie has similar absorbance 0.012 while

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Proteus and *Pseudomonas* species had 0.016 and 0.010 respectively in the nitrate concentration removed within the media. The nitrate present in the medium was compared with a known standard curve prepared with NaNO_3 at 100-1000 ppm. The result indicated that bacteria from fish pond wastewaters had highest potential for extracting nitrate and that inherent bacteria are capable of removing pollutant nitrate and the bacteria may be able to remove nitrate efficiently if genetically engineered.

Keywords: Nitrate; wastewater; removal; spectrophotometer and remediation.

1. INTRODUCTION

Wastewater may contain high level of nutrients, which when excessively released to the environment can lead to the growth of undesirable microorganism and hence eutrophication may result. One of the major eutrophic nutrient present in wastewater is nitrate [1]. The presence of these nutrient in wastewater, causes ecological impact and public health, thus the control of their emission into receiving water bodies is therefore of essential [2]. Although biological nutrient removal has been attributed mostly to bacteria where there is now increasing evidence that microbes play an important role in nutrient recycling in aquatic ecosystem [3].

Nitrate is recognized as one of the major nutrients which are used by living organisms for their physiological processes. It is commonly added as fertilizer to enhance the quality of soil. However it has emerged as one of the most abundant pollutants in the environment due to it excess usage. The traditional agricultural practices like dry farming with marginal irrigation, flood plain farming and random application of fertilizers are considered as diffused sources of nitrate in soil and aquifers. Besides this, the irregular rainfall during different seasons and the stream flow pattern causes seepage of these contaminants from soil to surface and ground water [4]. The cultivation patterns like terrace farming results in nitrate leaching into aquifers [5].

Increased levels of nitrate up to 400 ppm have been detected in groundwater [6]. Possible sources of nitrate pollution include manure, agricultural fertilizer, industrial effluent, domestic wastewater, septic systems, human waste lagoons, animal feedlots and native soil organic matter, as well as geologic sources [7]. Other point sources of nitrate are municipal sewage canals, septic tanks, sewage dumping grounds [8]. The mining tailings, industrial effluent from nuclear reactors, radioactive waste processing

units mainly those dealing with compounds like plutonium or thorium nitrate [9]. Nitrate contamination is a global problem and stands as second most dangerous pollutant after the pesticides [7]. Environmental protection agency (EPA) has stated a clear begins and ends of the limited level of contaminant concentration 45 ppm for NO_3 . A similar guideline of 50 ppm as NO_3 has been set by the WHO and the European Community (EC). Several conventional technologies adopted for nitrate removal are ion exchange resins, electro dialysis, reverse osmosis and distillation which substantially increase the cost of operation. Therefore the cost-effective alternative may lies in the biological denitrification process [10].

Rapid population growth continually increase rate of pollution affecting the quality of water and ultimately converting the water into toxic storage channel. The nitrate water contamination is now-a-days a matter of concern because of water scarcity in some part of the world [7].

Various groups of microorganisms like algae, fungi and bacteria are capable to convert the nitrate ions into organic matter through assimilatory nitrate reduction process. This involves utilization of several enzymes including nitrate and nitrite reductases. The assimilatory nitrate reductase enzyme is repressed in the presence of ammonia or reduced nitrogenous organic metabolites. This enzyme is not inhibited in the presence of atmospheric oxygen. The formation of ammonia due to assimilatory nitrate reductase rapidly incorporates into organic nitrogen. However the formation of excess ammonia acts as a feedback inhibitor to shut off nitrate reduction [11].

The contamination by nitrate has emerged as a global problem and its potential threat is marked on the environmental sustenance as well as on the public health. The aim of the present study was to determine an array of bacteria which can reduce nitrate load in wastewater and that can significantly be use for bioremediation.

2. MATERIALS AND METHODS

2.1 Collection of Samples

Samples were collected based on the procedure described by Zahoor and Rehman [12]. Waste water were collected from main abattoir of kasuwar Daji, fish poultry farm of Kwalkwalawa and Maberu area sokoto using sterile sample bottles. The samples were properly labeled with the date, time and location of sample taken. These were transported in an ice box to the laboratory aseptically.

2.2 Isolation Media

The media used were Nutrient agar, Nitrate agar (1% of potassium nitrate added to a nutrient agar), Nitrate broth, and biochemical test media. All preparations were based on manufacturer's instructions as described by [13].

2.3 Isolation and Screening of Bacteria

Waste water samples each were serially diluted up to 10^{-6} . The samples diluted 0.1 ml was spread plated on sterilized plates containing nitrate media. These were incubated for 1 day at 37°C. The colonies developed on NA plates were counted and expressed as colony forming units per ml (CFU/ml). Colonies were repeatedly subcultured to obtain pure colonies. Isolated colonies were characterized based on their cultural, morphological and biochemical characteristics [14]. The bacterial colonies each were inoculated again into NB (nitrate broth) and incubated at 37°C for 2 days for extraction of nitrate. Medium without nitrate was the control. These were monitored at subsequent intervals.

2.4 Biochemical Characterization

Biochemical specifications were based on the method described by [15]. Among the parameters include, catalase, citrate, Methyl red-Voges Proskauer (MR-VP), Gas, H₂ S, Glucose and Lactose utilization/production by bacteria.

2.5 Physicochemical Determination of Wastewater

Sub-part of the wastewater samples (Abattoir; inside and outside, Kwalkwalawa and Maberu fish pond) were physically observed and chemically analysed adopting the procedure described by [16].

2.5.1 pH determination

The pH was determined using pH meter, the electrode probe was inserted into a glass beaker containing 20 ml of the water sample and the beaker was gently swirled until the pH reading stabilized and the result was read from the screen and recorded.

2.5.2 Temperature determination

The temperature of the sample was determined using a temperature thermometer. The thermometer was inserted into the beaker containing 20 ml of the water sample and the result was read after the thermometer was stabilized.

2.5.3 Alkalinity determination

This test is used for the determination of carbonate and bicarbonate using titrimetric method. The carbonate was determined using Phenolphthalein as the indicator for carbonate, 50 ml of the water sample was poured inside a beaker followed by the addition of the Indicator, there was no colour change, few drops of methyl orange was added to the 50 ml of the sample which was used as the indicator for the bicarbonate the sample was then titrated against 0.05 mole of H₂SO₄ contained in a burette till the colour changed to yellow.

2.5.4 Nitrate determination

Kjeldhal method was adopted, as described by [17]. About 10 ml of boric acid was poured into a conical flask and it was placed under a condenser, 50 ml of each water sample was taken into a distillation flask, 0.2 g of magnesium oxide and 0.4 g of Devard's alloy was added into each waste water sample and it was mounted in a distillation apparatus and it was heated up to a concentration. A quantity of 20 ml of distillate was collected into 10 ml of the boric acid indicator under the condenser. The distillate was then titrated against 0.01 mole of H₂SO₄, until the colour changed to a pink.

2.5.5 Determination of nitrate extracted by bacteria

Nitrate extraction was based on the methods used by Neha and Aditi [13]. The isolates were inoculated in nitrate broth medium, and incubated in a rotary shaker 150 rpm for 16 hrs at 37°C. The cell free supernatant was taken for

evaluation of nitrate removal after centrifuging each culture at 10,000 rpm for 10 mins. About 200 μ l of Salicylic acid (5% H_2SO_4 with Salicylic acid) measured were used to each separate tube, into which 40 μ l of cell free supernatant were added and vortexed well. Dark space was used for incubation of tubes for 600seconds. About 2 ml of 4N NaOH was used to stop the reaction mixture of each individual separate tube. Spectrophotometer (JENWAY 6305, UK) was used and determined optical density (OD) of the solutions at 420 nm following 20 mins. Known concentrations of $NaNO_3$ (1000 ppm) standard curve prepared was compared to resulted optical density and determined the extra nitrate in the medium.

2.6 Data Analysis

Data generated in this study were analyzed using descriptive statistic in form of frequency and percentage. A simple table was used to present result where there is no need for test of difference.

3. RESULTS AND DISCUSSION

The results of the study were presented in Tables 1 to 5. Table 1 shows bacterial enumeration of wastewater samples. The colony count of bacteria ranges between 9.3×10^6 to 2.46×10^7 cfu/ml. The wastewater sampled from outside the Abattoir (Abt) showed maximum growth of bacteria 9.3×10^6 cfu/ml while wastewater sampled from inside the abattoir (Abi) exhibited minimal growth 1.96×10^7 cfu/ml. Wastewater 1.05×10^7 cfu/ml for kwalkwalawa fish pond (Fpk) exhibited minimal growth and 2.64×10^7 cfu/ml for mabera fish pond wastewater (Fpm) exhibited maximum growth. Abattoir had greater population of bacteria with all the samples almost bear similar pH level. Their greater counts may depicts bacteria from wastewater of abattoir can be utilize to bring back to normal level any nitrogen-inhibited environment. Their growth may be favored by maximal content of protein in the abattoir wastewater as described by [18]. Presence of maximal bacteria in the abattoir wastewater conformed to the report by [19].

Table 2 presented observed biochemical characterization of the bacteria isolated from pond and abattoir wastewater from the study area. All the bacteria from wastewater were observed to bring out bubbles in the presence of hydrogen peroxide as catalase positive. Similarly, they all utilize citrate, changing the

color of citrate agar after 2-days of incubation to bright blue. All bacteria from abattoir wastewater samples produces gas while unable to ferment lactose. Bacteria from fish pond wastewater are methyl red (MR) negative and voges proskaur (VP) positive while none of the bacteria can ferment glucose. The observed bacteria include *Pseudomonas* sp, *Enterobacter* sp and *Proteus* sp. The *Enterobacter* sp was isolated twice from both Abt and Fpm.

Table 1. Total bacterial colony count of the wastewater

Samples	Bacterial load (CFU/ml)
Abt	9.3×10^6
Abi	1.96×10^7
Fpk	1.05×10^7
Fpm	2.46×10^7

Keys: CFU/ml- colony forming unit per milliliter, Abt- Abattoir waste water from outside, Abi- Abattoir waste water from inside, FPk- fish pond waste water of kwalkwalawa area, Fpm- Fish pond waste water of Mabera area

The physiochemical analyses of wastewater sampled were presented in Table 3. The parameters include pH, temperature, colour, total hardness, alkalinity and nitrate. The result shows temperature (25°C, 27°C, 27°C and 26°C), pH (7.21, 7.12, 7.22, 7.24), Alkalinity (1.6, 1.7, 1.5, 1.6), Hardness (1.7, 1.9, 2.9, 1.5) and Nitrate (4.8, 4.5, 5.6, 5.8) for Abt, Abi, Fpk and Fpm respectively.

The obtical density for nitrate content extracted by bacteria *Enterobacter specie*, *Proteus specie*, *Pseudomonas specie* and *Enterobacter specie* as 0.574, 0.587, 0.732 and 0.670 respectively was presented in Table 4. Obtical density for bacteria screened for nitrate removal was presented in Table 5 as such 0.012, 0.010, 0.016 and 0.012 for *Enterobacter* sp., *Pseudomonas* sp., *Proteus* sp. and *Enterobacter* sp. respectively. *Pseudomonas* sp had the highest potential in reducing nitrate followed by *Enterobacter* sp. and many researches informed that, reaction for nitrate reduction does not depend on the reactant concentration that is they are saturated by the reactants, so may not be reduced to very minute contents. Meanwhile, microbes (nutrient reducers) were classified into true, sequential and respirers nitrate reducers [19,13]. Shirey and Sexstone [20] reported *Enterobacter* sp as one population to reduce nitrate. Many bacteria are capable of producing enzymes that catalyzes the reduction and are capable of utilizing nitrate via assimilatory or

Table 2. Biochemical characteristics of isolated bacteria

Sample	Gram	Catalase	Citrate	MR	VP	Gas	H ₂ S	Glucose	Lactose	Sucrose
Abt	- rod	+	+	-	+	+	-	-	-	-
Abi	rod	+	+	-	-	+	-	+	-	-
Fbk	- rod	+	+	-	+	-	+	-	+	+
Fbm	- rod	+	+	-	+	+	-	-	-	+

Table 3. Physicochemical analysis of wastewater

Sample	Temperature (°C)	pH	Alk (mg/l)	Hrd (mg/l)s	NO ₃ (mg/l)	Colour	Odour
Abt	25	7.21	1.6	1.7	4.8	Brown	Pungent smell
Abi	27	7.12	1.7	1.9	4.5	Red	No smell
Fpi	27	7.22	1.5	2.9	5.6	cloudy	Fishy smell
Fpm	26	7.24	1.6	1.8	5.8	cloudy	No smell

Keys°C – Degree Celsius, Mg/l – milligram per liter, Alk – alkalinity, Hrds – hardness, NO₃ – Nitrate, PO₃ – Phosphate

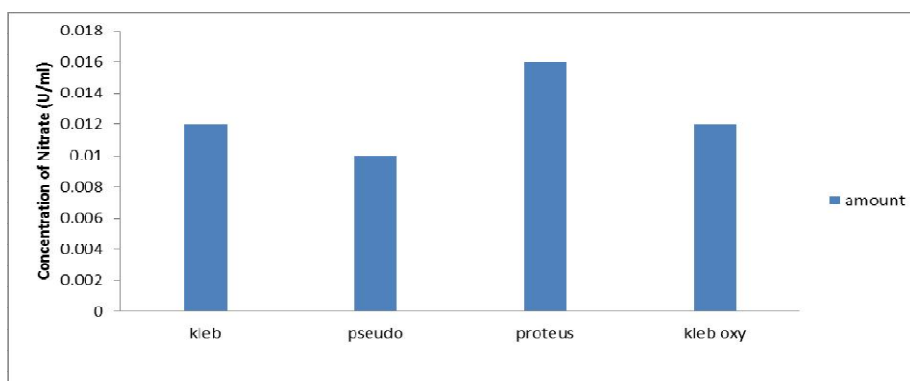


Fig. 1. Remaining nitrate

dissimilatory pathway and some uses nitrate as final electron acceptor also, reduction may be enhanced by organic matter present in a medium containing nitrate [13]. According to literatures, different industrial water contains more than 200 mg/l and their biological denitrification usually takes few days [21]. The study is in line with [22] who reported that *pseudomonas sp* have a strong ability to remove nitrate in waste water.

Table 4. Nitrate Projected from a layer in the nitrate medium

Samples	Absorbance (420 nm)
<i>Enterobacter sp</i>	0.574
<i>Pseudomonas sp</i>	0.587
<i>Proteus sp</i>	0.732
<i>Enterobacter sp</i>	0.670

Key: nm – Nanometer

Table 5. Removed nitrate concentration by bacteria in the nitrate medium

Samples	Absorbance (420 nm)
<i>Enterobacter sp</i>	0.012
<i>Pseudomonas sp</i>	0.010
<i>Proteus sp</i>	0.016
<i>Enterobacter sp</i>	0.012

4. CONCLUSION

The present study is a successful attempt to isolate efficient nitrate removing bacteria from wastewater which can be used for remediation of waste water by reducing their nitrate load. In addition optimization of the waste water treatment parameters by these isolate in future could not only lead to environmental protection

but also sequestration of essential plant growth nutrients from the waste, which in turn could be reused.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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