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Isolation and Characterization of Alkalophilic Denitrifying Bacteria from Lake Chitu

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Abbreviations

AOB	Ammonia Oxidizing Bacteria
AUR	Amonia Utilization Rate
BNR	Biological nitrogen removal
DO	Dissolved Oxygen
MA	methylamines
mg	milli gram
ml	milli liter
NTN	Total Nitrogen
NUR	Nitrate Utilization Rate
pH	Potential Hydrogenation
SO-NR	Sulfur Oxidizing- Nitrate Reducing
VFA	volatile fatty acids and alcohols,

.

Abstract

The objectives of the study were to isolate and characterize alkaliphilic denitrifying organisms from Ethiopia soda lake, Lake Chitu. Eighty seven potential denitrifying organisms were isolated. Two isolates were selected (designated as BACC280 and BACC118) as efficient denitrifiers for further characterization. These were Gram-negative, oxidase and catalase positive cocci, mesophilic and facultative anaerobe. Isolate BACC118 can optimally grow from 0 to 5% NaCl and 0.25 to 1% Na_2CO_3 concentration but optimum salt concentration for BACC280 is relatively higher. Salt requirement for denitrification activity of the isolates were found to be similar with growth concentration of NaCl but Na_2CO_3 optimum was at 0.25% to 2% for BACC280 and 0.5 to 3% for BACC118. The pH optimum of both buffered and un buffered were 12 for BACC280 and BACC118. Both isolates were able to denitrify aerobically and anoxically. These isolates may play great role in recycling of nitrogen compounds in to gaseous end product in their natural habitat. They have also potential to treat wastes that contain high amount of nitrogen, high salt concentration and high pH values.

Key words: Denitrifying bacteria, Soda Lake, Alkalophiles, Halophilis

1. Introduction

1.1 Soda Lake as a microbial habitat

Soda Lakes represents the most stable, naturally occurring alkaline and haline environment. These lakes contain sodium carbonate/ bicarbonate as the dominant soluble salts that provide the buffering capacity to lake (Baumagrite, 2003; Sorokin and Kuenen, 2005a; Ulukanli, 2002).

Soda lakes are located in areas with a dry climate that facilitate salt accumulation in depressions (Sorokin and Kuenen, 2005a). The main mechanism for the formation of the lakes is leaching of the sodium-rich rocks by high CO_2 containing ground water under condition of low Ca and Mg content (Jones, *et al.,* 1998; Baumagrite, 2003; Sorokin and Kuenen, 2005a).

Through evaporation such waters rapidly achieve saturation with respect to alkaline earth cations which precipitate as insoluble carbonates leaving Na^+, Cl^- and HCO_3^-/CO_3^{2-} as the major ion in the solution that leads to the development of alkaline and saline environment (Baumagrite, 2003; Grant, 2006). The presence of sodium carbonate that has high buffering capacity creates a unique and stable alkaline habitat. The pH of these environments ranges between 9.5-10.5 (Sorokin and Kuenen 2005a; Jones *et al*., 1998).

The well- known soda lakes are located in the East African Rift Valley, in the western mountain shadowed desert of the USA and in Central Asia (Table.1) (Baumagrite, 2003). The lakes are extensively studied by geologists because of the potential for the deposition of soda minerals and to some extent by microbiologists because of their extreme nature (Sorokin and Kuenen 2005a; Baumagrite, 2003).

Table 1. Worldwide distribution of soda lakes and soda deserts (Baumagrite, 2003)

Continent	Country	Location
Africa	Libya	Lake Fezzan
	Egypt	Wadi Natrun
	Ethiopia	Lake Aranguadi, Lake Kilotes, Lake Abiata, Lake Shala, Lake Chitu, Lake Hertale, Lake Metahara
	Sudan	Dariba Lakes
	Kenya	Lake Bogoria, Lake Nakuru, Lake Elmenteita, Lake Magadi,, Lake Simbi Crater Lake (Lake Sonachi), Lake Oloidien
	Tanzania	Lake Natron, Lake Eyasi, Lake Magad, Lake Manyara, Lake Balangida, Bosotu Crater Lake, Lake Kusare, Lake Tulusia, El Kekhooito, Momela Lakes Lake Lekandiro, Lake Reshitani, Lake Lgarya, Lake Ndutu
	Uganda	Lake Rukwa North Lake Katwe, Lake Mahenga, Lake Kikorongo, Lake Nyamunuka
	Chad	Lake Munyanyange, Lake Murumuli, Lake Nunyampaka Lake Bodu, Lake Rombou, Lake Dijikare, Lake Monboio Lake Yoan
Asia	Siberia	Kulunda Steppe, Tanatar Lakes, Karakul, Chita, Barnaul Slavgerod,Lake Baikal region, Lake Khatyn
	Armenia	Araxes Plain Lakes
	Turkey	Lake Van, Lake Salda
	India	Lake Looner, Lake Sambhar
	China	Outer Mongolia, various “nors”; Sui-Yuan, Cha-Han-Nor and Na-Lin-Nor;Heilungkiang, Hailar and Tsitsihar; Kirin, Fu-U- Hsein and Taboos-Nor; Liao-Ning, Tao-Nan Hsein; Jehol, various soda lakes; Tibet, alkaline deserts; Chahar, Lang-Chai; Shansi, U-Tsu-Hsein; Shensi, Shen-Hsia-Hsein; Kansu, Ning- Hsia-Hsein, Qinhgai Hu
Australia		Lake Corangamite, Red Rock Lake, Lake Werowrap, Lake Chidnup
Central America	Mexico Lake	Texcoco
Europe	Hungary Former Yugoslavia	Lake Feher, Pecena Slatina

Despite the apparently inhospitable conditions of Soda Lakes by high alkalinity and high salinity, they contain alkaliphilic representative of prokaryotes. They are also extremely productive environment because of high ambient temperature, high light intensity and effectively unlimited supplies of CO_2 via the $HCO_3/CO_3^{-2}/CO_2$ equilibrium (Baumagrite, 2003; Ulukanli, 2002).

They are the natural habitat for alkaliphilic anoxygenic phototrophic bacteria, which provide substantial contribution to the primary production. The primary producers support large numbers of chemo-organotrophic bacteria and the vast flocks of the lesser flamingo (*Phoeniconaias minor*) that graze on these lakes (Baumagrite, 2003; Grant, 2006)

Attempts to cultivate alkaline organisms' resulted in remarkably diverse organisms. This has permitted the identification of most of the major trophic groups responsible for the cycling of carbon, sulfur and nitrogen in the lakes (Fig.1) (Sorokin and Kuenen, 2005b).

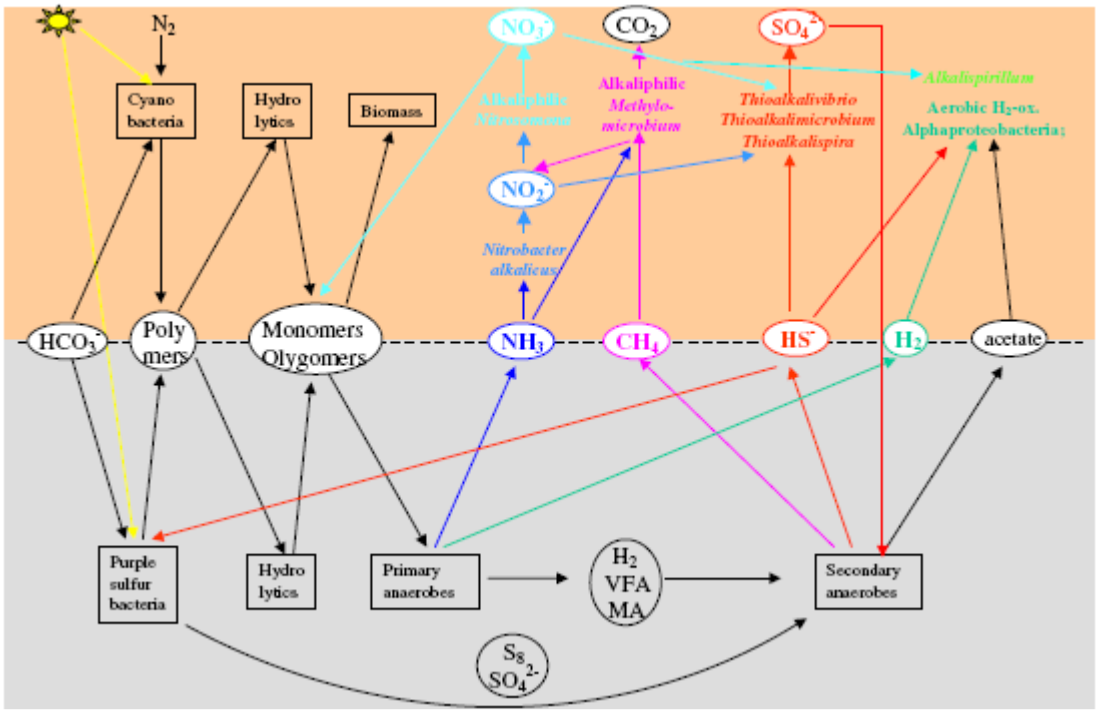


Figure 1. Role of microorganisms in the nutrient cycling in soda lakes (Sorokin and Kuenen, 2005b)

VFA – volatile fatty acids and alcohols,
MA – methylamines.

Organisms that are isolated from Soda Lake include aerobic and anaerobic carbon utilizing organisms. Such as methanogenes and methylotrophic bacteria that utilize a variety of different one-carbon compounds including methane, methanol and methylated amines. (Joye, 1999; Grant, 2006; Trotsenko and Khmelenina, 2002).

There is active sulfur cycle in this environment by sulfur reducing (anaerobic) and sulfur oxidizing bacteria (aerobic/ anoxic). An example of sulfur reducing bacteria (SRB) is *Desulfonatronovibrio hydrogenovorans* that utilize H_2 and reduce sulfate (Zhilina, *et al.*, 1997; Grant, 2006). The sulphur cycle also has an aerobic component and aerobic sulphur-oxidizing bacteria occupy a narrow zone where H_2S and O_2 coexist. Three genera of alkaliphilic chemo-lithoautotrophic sulphur-oxidizing bacteria have been isolated (Sorokin and

Kuenen 2005a; Sorokin, *et al*., 2001b; Sorokin, *et al.*, 2004a: Sorokin, *et al.*, 2004b).

There is also undoubtedly an active nitrogen cycle in Soda Lakes. Several strains of lithotrophic and alkaliphilic ammonia and nitrite oxidizers are known from Siberian and Kenyan soda lakes and there is also active ammonia oxidation in Mono Lake, California (Sorokin and Kuenen 2005a; Sorokin, *et al.*, 2004; Baumagrite, 2003; Joye, 1999). Nitrate reduction and denitrification occurs widely, which is carried out by both lithotrophic bacteria like *Thioalkalivibrio denitrifcans* and the widely distributed chemoorganotrophic halomonads (Sorokin and Kuenen 2005a; Baumagrite, 2003; Grant, 2006).

1. 2 Ecophysiology of soda lake inhabitants

The pH and salt concentration of soda lakes are the most significant difference from that of the optimum for intracellular enzymes (that is found to be around neutrality in most alkaliphlic and halophilic organisms (Horkoshi, 1999; DasSarma and Arora, 2001). Therefore, organisms that survive in these environments need some mechanisms that allow them to adapt to highly saline and alkaline habitats. To overcome the problems some use their cell wall and others use cell membrane (Horkoshi, 1999; Banciu, *et al.*, 2005; Sorokin and Kuenen, 2005a). There is also accumulation of organic osmoregulator, and ions resulting in modification of the chemical composition of their membrane (Banciu, *et al.*, 2005, Detkova and Boltyanskya, 2006; Trotsenko and Khmelenina, 2002).

In alkaliphilic organisms one of the key features that discriminate and maintain the intracellular neutrality is the cell surface. The cell wall contains certain acidic polymers in addition to peptidoglycan such as galacturonic acid, aspartic acid and phosphoric acids (Horkoshi, 1999). The negative charge on the acidic component may give the cell surface the potential to adsorb sodium and hydronium ion and repulse hydroxide ion. Plasma membrane also

maintain pH homeostasis by using the Na^+/H^+ antiporter system ($\triangle\Psi$ dependant and $\triangle$pH dependant) the K^+/H^+ antiporter and ATPase - driven H^+ expulsion (Horkoshi,1999). Some strains have substantial amounts of Squalene (a polar lipid, C30 isoprenoid) which can serve as a barrier decreasing the membrane permeability for ions in haloalkaliphiles (Sorokin and Kuenen, 2005a; Banciu, *et al.*, 2005).

To prevent loss of cellular water under saline conditions, halophiles employ two fundamentally different strategies of adaptation (Oren, 1999; Detkova and Boltyanskya, 2006). Most halophiles generally accumulate high solute with in the cytoplasm. The compatible solutes or osmolytes that accumulate in halophiles are usually amino acids and polyols, e.g. glycine, betaine, ectoine, sucrose, trehalose and glycerol, which do not disrupt metabolic processes and have no net charge at physiological pH (Roberts, 2005; Aston and Peyton, 2007; DasSarma and Arora, 2001; Detkova and Boltyanskya, 2006).

Some others use salt in mechanism (the "salt-in" strategy) by maintaining a high intracellular concentration of salts that are osmotically equivalent to the extra cellular concentration as a consequence, modify the intracellular proteins and other macromolecules so that they are able to function in solutions with a high ionic strength (Boltyanskya, *et al.*, 2005; Detkova and Boltyanskya, 2006; Detkova and Boltyanskya, 2007).

1.3 Microbial Transformation of Nitrogen

Microorganisms play a major role in nitrogen cycling in an environment. Transformation of nitrogen has different mechanisms and there exist very complex relationship among different nitrogen species (such as ammonium, nitrite and nitrate) (Paredes *et al.*, 2007). Organic nitrogen is made up of different compounds such as amino acids, amino sugars, urea and uric acid and purines and pyrimidines. These organic compounds are converted to ammonium through hydrolysis and deamination/ decomposition.

Ammonia can be oxidized by microorganisms to nitrite and nitrate, under aerobic condition. Two different groups of bacteria play a role in nitrification. These are ammonia oxidizers and nitrite oxidizers (Paredes *et al.*, 2007). The Denitrifires on the other hand reduce nitrite and nitrate to dinitrogen gas or other gaseous nitrogen compounds. Finally microbial nitrogen fixation converts gaseous dinitrogen to ammonia for assimilation and connects the nitrogen cycle (Fig. 2).

Though 79% of the earth's atmosphere is composed of molecular nitrogen (N_2), this type of nitrogen is unavailable to be used directly by most organisms. Biological nitrogen fixation is restricted to specialized groups of prokaryotes which possess the enzyme nitrogenase (Herber, 1999). Since the nitrogen nitrogen triple bond is extremely stable, the biological reduction of dinitrogen to ammonia is an energy demanding process estimated to be equivalent to a minimum of 16 ATP per molecule of N_2 fixed (Herber, 1999). The fixed nitrogen is used for assimilation purposes.

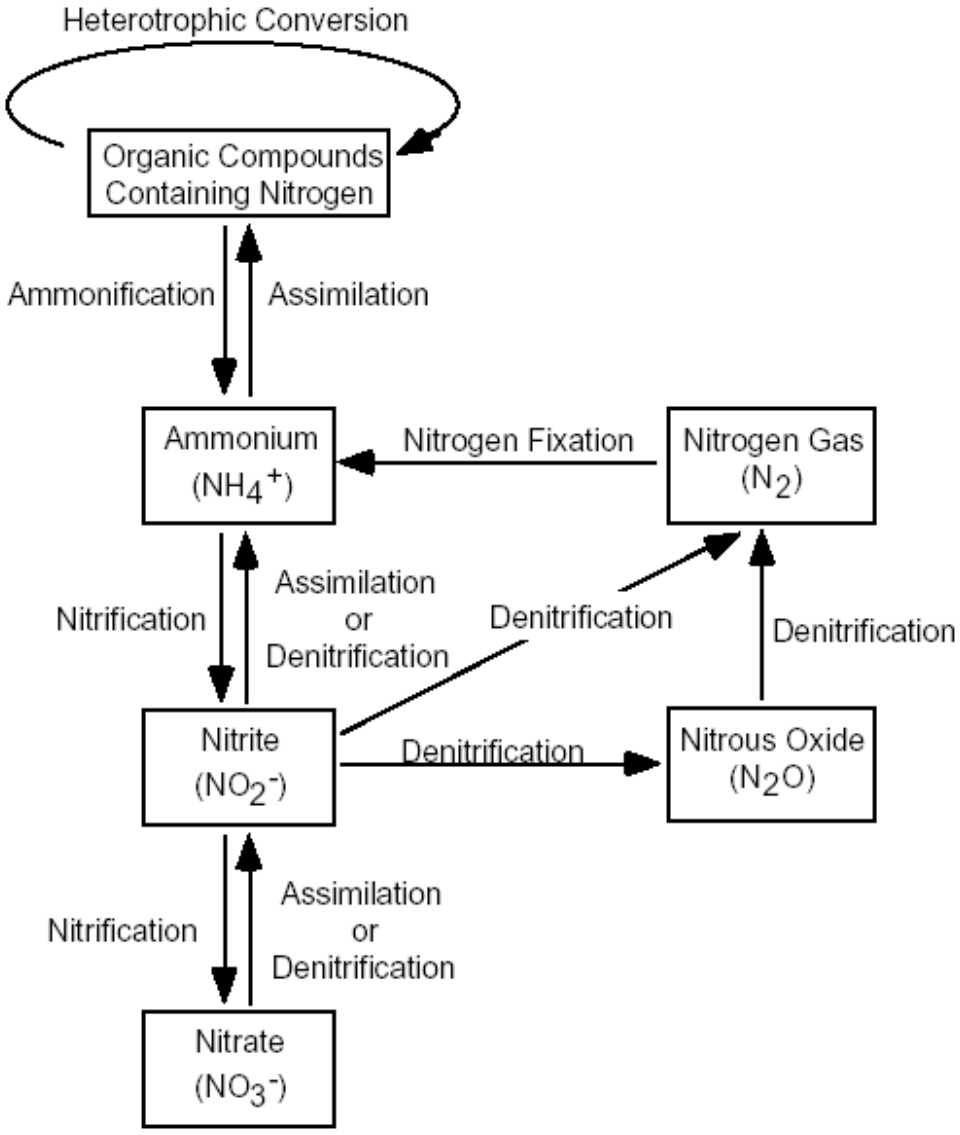


Figure 2. Simplified Biological Nitrogen Cycle (Madison and Brunett, 1985)

1.3.1 Ammonification

Irrespective of its origin, all living matter contains nitrogen containing macromolecules, such as nucleic acids, proteins and polyamino-sugars. These compounds become available up on death of the cells to decomposer organisms. Organic nitrogen compounds are converted to ammonia through decomposition and hydrolysis. The release of ammonium from this nitrogenous matter is termed as ammonification. Depending on the structural

complexity of the organic matter ammonification can be simple deamination or complex hydrolysis reaction (Herber, 1999). Ammonia can be assimilated to new cellular material or oxidized to nitrate/ nitrite by different organisms.

1.3.2 Nitrification

Nitirification is an important part of the biological nitrogen cycle. It is regarded as a two step process. The first step is the aerobic oxidation of ammonium to nitrite (eqn.1) by ammonia oxidizing bacteria and then followed by oxidation of the produced nitrite to nitrate (eqn.2) by nitrite oxidizing bacteria (Gerardi, 2002; Ishizuka, *et al*., 1995).

$$NH_4^+ + 1.5\ O_2 \xrightarrow{\text{ammonia oxidizing bacteria}} NO_2^- + 2\ H^+ + H_2O \qquad (1)$$

$$NO_2^- + 0.5\ O_2 \xrightarrow{\text{nitrite oxidizing bacteria}} NO_3^- \qquad (2)$$

Based on 16S RNA sequence data ammonia oxidizing bacteria (AOB) can be subdivided in to two distinct groups. These are δ & β-*Proteobacteria* that contains *Nitrosococcus oceanus* and *Nitrosomonas* spp. respectively (Herber, 1999; Schmidt *et al.,* 2003). Most organisms that are associated with nitrification are lithotrophics. Among the lithotrphic ammonia oxidizers are *Nitrosomonas, Nitrosococcus, Nitrosospira* and *Nitrosovibrio*. Among the nitrite oxidizers are *Nitrbacter, Nitrococcus*, *Nitrospira* and *Nitrospina* are included. There are also heterotrophic nitrifires including fungi, actinomycets and bacteria (Sabalowsky, 1999; Gerardi, 2002; Prosser, 1989).

A number of physcochemical and biological factors are important in regulating nitrifying activity both in natural ecosystem and wastewater treatment plants. These include dissolved oxygen (OD), pH, temperature, presence of inhibitor and microbial population (Herber, 1999; Schmidt *et al.,* 2003). Since nitrifying bacteria are obligate aerobes and dissolved oxygen

(DO) concentration of more than 1 mg/l is necessary for nitrification to occur. Depth distribution of nitrifying bacteria is constrained by the limits of downward oxygen diffusion (Herber, 1999). Oxygen diffusion in turn is dependant on organic matter content, temperature and degree of mixing. In general, nitrite oxidizers appear to be more sensitive to low DO concentrations than ammonia oxidizers. Two mg/l DO is typically considered as the cut of value to fully ensure nitrification is not oxygen limited (Prosser, 1989; Sabalowsky, 1999).

Nitrification is also affected by pH. The pH affects several different factors including growth and activity of organisms involved. Nitrification in general occurs in the pH range of 6.5 to 8.0. However, there are some nitrifires that are adapted to low pH values of about 4 and there are also nitrifiers such as *Nitrobacter alkalicus* that are adapted to a very high pH values of about 10 such as Soda lake inhabitants (Schmidt 2002; Sorokin *et al.,* 2001c).

There is an apparent optimal temperature for any organism and the optimal temperature for growth will not necessarily be the same as the optimal temperature for substrate oxidation or reduction (Sabalowsky, 1999; Gerardi, 2002). The optimum growth temperature for nitrifying bacteria can range from 4^0c to 45^0c. The optimal temperature for nitrifying activity has been reported to be between 15^0c and 30^0c (Sabalowsky, 1999). Nitrifying organisms are also sensitive to a wide range of organic and inorganic inhibitors. The oxidation of ammonia is considered to be more sensitive for inhibitor than nitrite oxidation.

1. 3. 3 Denitrification

Denitrification is reductive processes where by some prokaryotic organisms utilize nitrate or nitrite as a terminal electron acceptor, instead of oxygen, in respiration and reduce it to dinitrogen gas or other gaseous nitrogen products (Ishizuka, *et al*., 1995). Denitrification is a key process in the global nitrogen

cycle, since it removes excess nitrogen available to the primary producers, as the gaseous end product (Herber, 1999). Denitrification involves four successive steps of nitrogen nitrogen bond formation in the transformation of its intermediates nitric oxide (NO) and nitrous oxide (N_2O) to the next lower oxidation state (eqn. 3). The reduction of nitrite to nitric oxide is the important step for denitrification because it differentiates denitrifires from other nitrate respirers (Zumft, 1997).

$$NO^-_3 \dashrightarrow NO^-_2 \dashrightarrow NO \dashrightarrow N_2O \dashrightarrow N_2 \qquad (3)$$

The ability to denitrify is widely distributed amongst different taxonomic group of bacteria. It is more common among alpha and beta classes of the proteobacteria (Zumft, 1997). Denitrifies are also metabolically divers organisms. Some are Phototrophic bacteria such as *Rhodobacter, Rhodospsedomonas* and *Rhodoplanes* (Choi *et al*., 2005) and others are hetrotrophic organisms including *Pseudomonas*, *Hyphomicobium* and *Altrosomonas*. Denitrification could also take place aerobically, for example, *Pseudomonas aeruginosa* is aerobic denitrifies (Ka *et al.,* 1997). Some chemolithotrophs that oxidize inorganic compounds to generate their energy also have the ability to denitrify.

These include sulfur oxidizing- nitrate reducing (SO-NR) organisms such as *Beggiatoa, Thiobacillus, Thiospheara, Thioplacca* and *Thialkalivibrio* species (Dereje Teshome 2007; Gupta,1997, Sorokin *et al.*, 2004b). Among chemolithotrophic denitrifires some are hydrogen oxidizer and ammonia oxidizer include *Ralstonia* and *Nitrobacter* respectively (Zumft, 1997, Gerardi, 2002). Respiration of nitrate /nitrite to produce dinitrogen or other gaseous nitrogen compounds can also be an activity of some archea, actinomycetes and fungi (Kumon *et al*., 2002; Guest and Smith, 2002; Zumft, 1997).

Denitrification can be affected by different biochemical and environmental factors including pH, temperature, organic loadings and DO. The optimal pH for denitrification is in the range of 7 to 8 (Gerardi, 2002). The process of denitrification itself produces alkalinity by the reduction of nitrate to nitrogen gas which eventually inhibits the process (Sabalowsky, 1999). However, there are denitrifying organisms whose pH optimum is around 10. These organisms are isolated from soda lakes and include *Thioalkalivibrio denitrificance*, *Thioalkalivibrio nitratireducens, Halomonas mongoliensis, Thioalkalispira microaerophila* and *Halomonas kenyensis* (Boltyanskaya *et al.,* 2007; Sorokin *et al*., 2001b; Sorokin *et al.,* 2002; Sorokin *et al*., 2003a).

Denitrification may occur in a wide range of temperature from 5 to 50 ^{0}c with its optimum reaction rates occurring at 35 to 50 ^{0}c (Gerardi, 2002). In general denitrifying organisms are less sensitive than nitrifying organisms for inhibitory substances (Juliastuti *et al,* 2003; Seyoum Leta, *et al*., 2004a; Seyoum Leta, *et al.*, 2004b).

Since nitrate/ nitrite serves as an electron acceptor during denitrification, low DO level is required for most organisms to denitrify. And conventional thought long has held that nitrate reduction, or denitrification, does not take place when oxygen is present. However, these days data are showing that some organisms have the ability to denitrify in the presence of molecular oxygen (both O_2 and NO_3 are reduced simultaneously) (Chen, *et al*., 2003; Matsuzaka; *et al*., 2002 and Elen, 2004).

1. 4 Importance of Nitrogen removal

Nitrogen compounds are very important components of all living things. Primary producers are in need of available nitrogen (NO^-_2/ NH_3) and it plays a critical role as a limiting nutrient in many ecosystems on earth including many forests, wet lands and some lakes (Davidson and Seitzinger, 2006). Despite the fact that nitrate plays an important role as an essential nutrient, it has

become a pollutant of ground and surface water, causing a major problem for the supply of drinking water. High concentration of nitrate in drinking water causes the disease called methemoglobinemia in small children. After ingestion nitrate is reduced to nitrite in the gut and nitrite reacts with hemoglobin in the blood, forming methemoglobin that reduce the oxygen carrying capacity of the blood. As a result the body become bluish and so called blue baby syndrome. Due to this and other diseases linked to NO_3^- the national drinking water set standard at maximum of 10 mg/l contaminant level (Schnobrich *et al.,* 2007).

Introduction of reactive nitrogen compounds in to the biosphere by human activity is becoming very high. This reactive nitrogen is primarily due to nitrogen fertilizer production and fossil fuel combustion used to support the food and energy demand of a rapidly expanding human population. Increased inputs of N_2O and NO to the atmosphere increases tropospheric ozone depletion, N_2O is next to CO_2 and CH_4 in its importance as potent green house gases. Increased nitrogen oxide compounds can acidify soils, streams and lakes (Davidson and Seitzinger, 2006).

In addition, discharge of high nitrogen containing wastes to water bodies by different industries result in, eutrophication of aquatic ecosystems. Among many nutrients in water, phosphorus and nitrogen compounds are the major cause of eutriphication (Howarth and Marino, 2006; Jeon and Park, 2000). Excess nitrogen, normally in the form of nitrates, due to its soluble nature and mobility through soil, can cause the stimulation of growth, resulting in algal blooms of overgrowth of aquatic plants. This can have serious consequences for the receiving water such as odors, accumulation of unsightly biomass, biological decomposition of carbonaceous wastewater. This exerts an oxygen demand that greatly depletes the dissolved oxygen, and causes mortality of fish and other aquatic organisms (Makaya et al., 2007).

Therefore, industries such as tannery and slaughterhouses, fertilizer industries etc. that have large amount of nitrogen containing wastes should be treated at least to maximum standard level set before disposal (Dereje Teshome, 2007; Tesfaye Minuta, 2006; Seyoum Leta, *et al.*, 2003; Kabdaslı, *et al.*, 2003). There are several treatment methods capable of reducing nitrogen compounds. These processes involve exploiting the physical and biochemical interactions that occur naturally in aquatic systems to remove nitrogen. From the transformation mechanism it can be seen that nitrogen reduction can be achieved using bacteria. In theory, the nitrogen in wastewater will be converted to nitrogen gas and lost to the atmosphere (Ishizuka, *et al*., 1995; Olanrewaju, 2007).

1.4.1 Nitrogen Removal Technologies

Nitrogen removal technologies can be categorized in to three: Physical, Chemical processes and biological processes. Physical treatments include screening, sedimentation, filtration and flotation. Chemical treatments include disinfection, adsorption, and precipitation (Ishizuka *et al.,* 1995; Bigornia-Vitale Wu, 2007). The biological nitrogen removal (BNR) technologies usually form the secondary or tertiary treatment step of wastewater processing and mimic the natural treatment processes that occur in the environment, but allowed to take place under controlled conditions in a constructed environment (Olanrewaju, 2007; Bigornia-Vitale Wu, 2007).

Out of the three technologies biological nitrogen removal technologies are the most used, cost effective and environmentally friendly for the removal of nitrogen (Ishizuka *et al.,* 1995). These works based on the general principle of biological reduction using microorganisms such as bacteria and involve two processes in sequence that is nitrification and denitrification. The Biological treatment processes can be classified in to; attached growth system and suspended growth system (Olanrewaju, 2007 and Bigornia-Vitale Wu, 2007).

Attached growth system is based on flowing wastewater across some media and material. It works on the principle that organic matter is removed from wastewater by microorganisms that grow on a filter media and recycling the dissolved organic material into a film that develops on the media. The technology is also known as fixed film processes and there are two basic designs of attached growth systems; one that hold media in place, allowing the waste to flow over the bed (like trickling filter) and in other the media is in a motion relative to the wastewater (such as rotating biological contactor) (Ishizuka *et al.*, 1995; Teixeira and Oliveira, 2001; Olanrewaju, 2007).

In suspended growth systems, the organism grows in liquid phase and has to be separated from the effluent in clarifiers. The biomass is well mixed with the wastewater and can be operated in a smaller space than the fixed film system that treats the same amount of waste. The most common example of suspended growth system is the activated sludge systems that consists two parts; aeration tank and a settling tank of clarifier (Olanrewaju, 2007).

Now a day more elaborate and perhaps more technologically advanced modifications to the systems are being introduced, which can be referred as hybrid systems. In one of the systems, fixed film materials are added to the aeration tank of suspended growth systems. While in another case, there might just be slight variation to the original system. Hybrid systems include moving bed biofilm reactor and membrane bioreactor (Olanrewaju, 2007; Bigornia-Vitale Wu, 2007).

Therefore, it is important to use (BNR) technologies for removing nitrogen compounds from wastewaters that involves two processes in sequence that is nitrification and dentrification. The role of denitrification is utmost importance in this context, as it is the only mechanism by which reactive forms of nitrogen are transformed back into dinitrogen (N_2) gas, which is the dominant component of the earth's atmosphere (Boyer, *et al*., 2006; Herber, 1999).

However, some industries result in alkaline and haline nitrogenous waste or byproduct. For this reason they need organisms that are tolerant to the extreme environment to be treated. Soda lakes are one of the habitats that represent the most stable, naturally occurring alkaline and haline environment. They contain organisms that are adapted to these double extreme conditions (Baumagrite, 2003).

Lake Chitu is one of the productive alkaline soda lakes in the Ethiopian Rift Valley system. The lake is known to be highly productive, where the main primary producer is *Spirullina platensis*, forming almost a monoculture. Spirullina is very well known for its high protein content. To support such high algal cell density with high protein content the lake must contain sufficient nitrogen in the form of nitrate. Since there is no inflow or outflow of water into or out of the lake, one assumption has been that the lake might contain a certain amount of nitrogen which is absorbed by Spirullina. When the algal cells die the protein and other nitrogen containing organic compounds are biologically degraded and the nitrogen converted to nitrate. Since most biological denitrification reactions take place at around neutral pH, in this alkaline environment, after nitrate is formed, no further nitrogen removal was expected. However, recent study showed that sample taken from Lake Chitu contains high biological denitrification activity (Tesfaye, 2006). If denitrifying bacteria exist in Lake Chitu, it raises a serious question on the nitrogen economy of the lake. However, in his study Tesfaye's (2006) was a sample from Lake Chitu as inocullum for biological nitrogen removal from tannery wastewater at high pH. However, no denitrifying bacteria were isolated and characterized. The objective of this study was therefore:

1. To isolate alkaliphilic denitrifying bacteria from Lake Chitu and characterize the organisms.
2. To evaluate the growth and denitrification ability of the isolates under different culture and aeration condition.

2. Material and Methods

2.1 Samples and sampling sites

Samples of water and mud sediments were collected from Lake Chitu. Which is located around 250 Km south of Addis Ababa in the rift valley at an altitude of 1,600m with maximum depth of 21m (Elizabeth kebede, 1996).

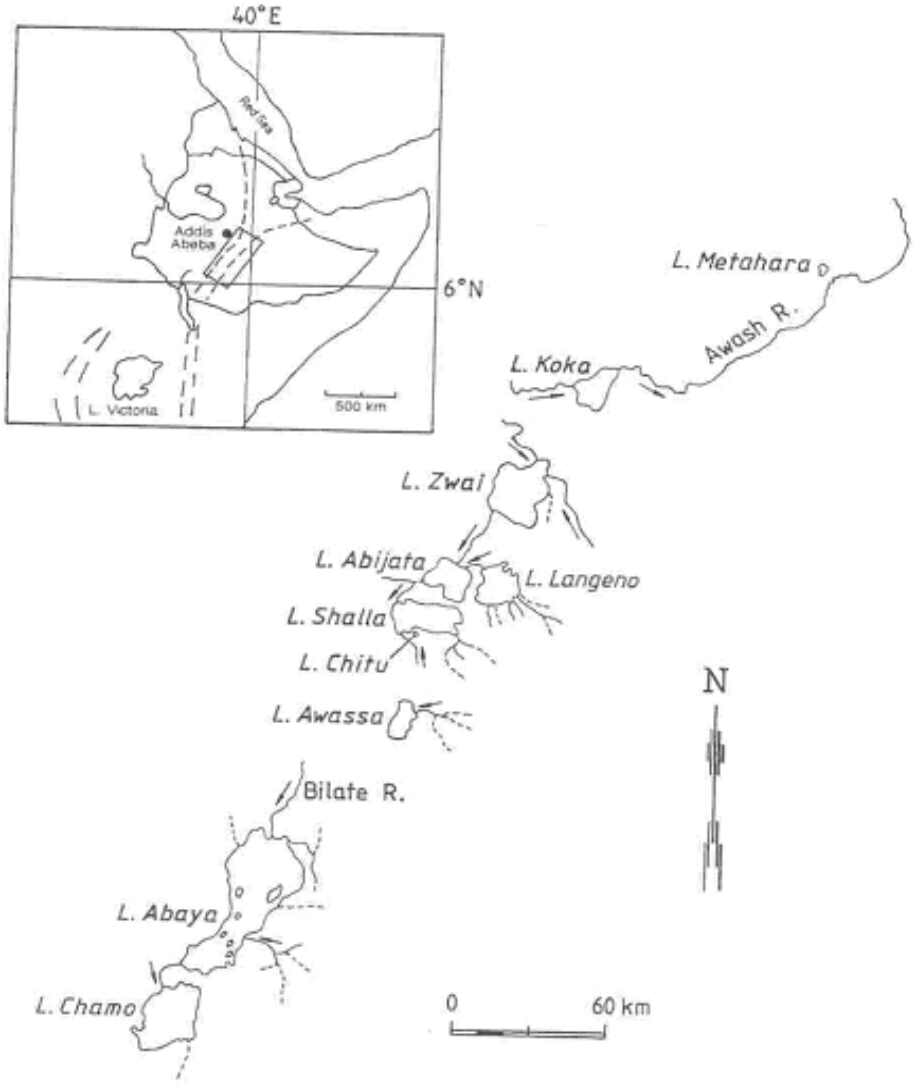


Figure 3 Location of the study area

The water samples were determined for total N, NH^{+4}–N, NO_3^-–N, and S^{-2} in the laboratory by colorimetric methods using spectrophotometer (DR/2010 HACH, Loveland, USA), according to HACH instructions (HACH, 1996-1999). Conductivity, pH and dissolved oxygen level were measured on site.

Lake Chitu is one of the most saline and alkaline lake among Ethiopian Rift Valley lakes with average pH of around (10.4 and conductivity of 64.4 mS). The lake has average temperature of around 22 ^{0}C. It has 10.0, 23.1, 2.2 and 0.021 mg/l concentration of total nitrogen, nitrate, ammonium, and sulfide respectively (Table 2).

Table 2 Different parameters of Lake Chitu

Parameters	**values**
Temperature	22^0c
pH	10.4
Conductivity	64.4 mS
DO	9.72mg/l
Total nitrogen	10.0mg/l
NO_3^-–N (Nitrate)	23.1mg/l
NH^{+4}–N (Ammonium)	2.2mg/l
S^{-2} (Sulfide)	0.021mg/l

2.2 Isolation and Purification of Isolates

The water samples were serially diluted in 0.85% sterilized saline solution from 10^{-1} to 10^{-7} in test tubes. Then 100µl of the suspensions were spread plate on sterile newly constructed enrichment medium (media composition in Table 3 & 4) by using alcohol flamed glass rod to observe the formation of single colonies. The plates were incubated at 30^0c from 3 to 7 days.

Table 3 Compositions of the enrichment medium

Component	Amount (g/l)
K_2HPO_4	1
$MgSO_4.7H_2O$	0.1
$CaCl_2$	0.15
$FeCl_3.6H_2O$	0.02
$MnSO_4.4H_2O$	0.01
$C_2H_3NaO_2$	2
$NaNO_3$	2
Nutrient broth	5
Agar	15
Trace Metal Solution	10ml
25% Na_2CO_3	4ml

Table 4 Composition of the Trace Metal Solution

Component	Amount(g/100ml)
EDTA	0.05
$ZnSO_4.7H_2O$	0.02
$MnCl_2.2H_2O$	0.05
$(NH_4)_6Mo7O_{24}.4H_20$	0.01
$CuSO_4.5H_2O$	0.015
$CoCl_2.6H_2O$	0.02

The plates were examined for single colonies. Single colonies which appear to be different in color, size, and shape were picked by using flame sterilized loop and streak transferred to sterilized plate media. Transferring single colonies were continued until purity and uniformity was achieved. The pH of the media were adjusted to around 10.3 by using 1% Na_2CO_3.

2.3 Selection of Potential Denitrifying Isolates

A total of 87 isolates were inoculated in to nutrient broth supplemented with 0.15 $NaNO_2$ in the presence of 0.1% agar in order to create anoxic environment (Gerhardt *et al.*, 1981). They were incubated at 30^0c and bubble formation was observed in screw capped tubes. Finally isolates with gas emergence were selected as positive for further studies.

2.4 Bacterial Designation and Preservation

The pure isolates were transferred to agar slant and incubated until well growth were observed then the slants were kept at 4^0c. The isolates were also preserved by using broth media and 15% glycerol in straws. The straw were foiled with aluminum foil and kept for 24 hours at 4^0c and stored at -70^0c. The isolates were designated as BACC followed by specific number to indicate, Biological Alkaliphils Culture Collection and the isolate number.

2.5 Measurement of Denitrifying Activity

To identify isolates showing relatively better rate of denitrification, the eight isolates that have shown qualitatively superior denitrification after observing the gas production were measured for nitrite utilization rate. From an over night culture 10 mg of the isolates were suspended in to 1ml nutrient broth and mixed with $NaNO_2$ in microtiter plates (Gumaelius *et al*., 1996). The bacterial suspensions was transferred in to nitrite detection reagent, every 20 minute until the nitrite is consumed. Absorbance was measured at 540 nm. The rates of substrate utilization of the selected strains were conducted on triplicate and nitrite concentration was plotted versus time.

2.6 Characterization

2.6.1 Morphological Characterization

An over night culture of each isolate was streaked in to sterile plate media and incubated at 30^0c until single colonies observed. Then the isolates were examined for morphology, gram reaction and biochemical properties. The gram staining experiment was done by following Pacarynuk (2005). The shape and texture of the colonies of the isolates were also determined. In addition, all these properties were compared with the standard characteristics described in the ninth edition of Bergey's Manual of Systematic Bacteriology (Holt *et al.*, 1994).

2.6.2 Physiological Characterization

2.6.2.1 Growth rate determination for the isolates

Growth rate of the isolates was measured following Pacarynuk (2005) with some modifications. One ml of an over night culture of the test isolate was inoculated in to 100 ml liquid media in 250 ml Erlenmeyer flask. The culture was incubated at ambient temperature in a rotary shaker at 120rev./min. Samples were taken from the culture for OD reading every 6 hours starting from zero hour until constant reading achieved at 600nm.

2.6.2.2 Growth at different NaCl concentrations

The ability of isolates to grow at different salt concentration was tested by inoculating 1ml of the test organisms in 100ml liquid media containing 0, 0.5, 1, 2, 5, 10, 15 and 20 % w/v NaCl in triplicates. Cultures were incubated at ambient temperature in a rotary shaker at 120rev. /min. Growth was measured by taking samples of the culture for OD reading every 6 hours starting from zero hour at 600nm.

2.6.2.3 Growth at different Na_2CO_3 concentrations

The isolates were tested for growth at different Na_2CO_3 concentration by inoculating 1ml of overnight culture into 100ml of sterile liquid media that contain 0.25, 0.5, 1.5, 2.5, 3, 5, 7, and 10% w/v Na_2CO_3. The cultures were incubated at ambient temperature in a rotary shaker at 120rev. /min. OD was measured ever 6 hours starting from zero hour at 600nm.

2.6.2.4 Growth at different temperature

An over night culture of the isolates were streaked on to sterile plate media in triplicate. The plates were kept at various temperatures (i.e., 5, 10, 15, 20, 30, 35, 40, 45 and 50) and growth of isolates were evaluated qualitatively as (+) for growth and (-) for no growth. Streaked plates of both isolates were also incubated at 30 ^{0}c as control.

2.6.2.5 Measurement of denitrifying activity at different NaCl concentrations

Selected isolates were measured for nitrite utilization at various NaCl concentrations. Over night culture of test isolates were inoculated into broth media containing 0, 1, 2, 5, 7, 10 and 15% w/v NaCl. The culture was transferred to microtitre plates with nitrite detection reagent in 20min. time interval and the result was measured at 540nm absorbance.

2.6.2.6 Measurement of denitrifying activity at different Na_2CO_3 concentrations

Denitrification activity of isolates was measured at different Na_2CO_3 concentration. Isolates were made to suspend in to a liquid culture media containing different concentration of Na_2CO_3 (0.25, 0.5, 1.5, 2.5, 3, 5 and 7). They were transferred to a microtiter plate with nitrite detection reagent at 20 min. time interval and nitrite consumption was measured at 540 nm absorbance using spectrophtometer.

2.6.2.7 Measurement of denitrifying activity at different pH

To measure denitrification activity of isolates at different pH values (not buffered), liquid media were adjusted to different pH (i.e., 8, 8.5, 9, 10, 11, 12 and 12.5) by using 1N NaOH and HCl. While measuring denitrification activity of isolates in different buffered pH, sterile liquid media were adjusted to different pH (i.e., 7, 8, 8.5, 9, 10, 11, 12 and 12.5) by using buffers such as phosphate (pH 7-8), glycine-NaOH (pH 9.0-11.0) and potassium KCl/NaOH (12-12.5). These media with sodium nitrite were inoculated with an over night culture of the selected isolates. The suspension was transferred to nitrite detection reagent every 20 minutes and results were read at 540 nm absorbance and nitrite concentration was plotted verses time.

2.6.2.7 Measurement of denitrification aerobically and anoxically

To determine the denitrification (aerobically and anoxically) ability of the isolates, denitrification activity measurement were combined with modified AUR and NUR experiment (Gumaelius *et al*., 1996; Seyoum Leta, 2004a). The two isolates were grown overnight aerobically in the absence of sodium nitrate in a liquid media. The culture was transferred in two 100 ml containers for aerobic denitrification. One percent of 0.75%w/v $NaNO_2$ was added to the

culture immediately before zero hour sample was taken. The containers had two openings for oxygen inlet and sampling port. They were aerated continuously throughout the experiment. Samples were taken every thirty minute starting from zero minute to evaluate nitrite reduction. The samples were transferred into microtiter plate and absorbance was measured at 540nm.

The over night culture each isolate was transferred in two 100 ml containers for anoxic denitrification. The containers had two opening. In the first opening nitrogen gas was flushed to replace oxygen and immediately inserted into 3N KOH so that nitrogen gas (which is produced during denitrification process) could be removed from the container. The other opening was used as a sampling port. Then 1% of 0.75% w/v $NaNO_2$ was added to the culture and samples were taken every thirty minute starting from zero minute to evaluate nitrite reduction.

3. Results

3.1 Selection of potential Denitrifying Isolates

An organism to be a denitrifier, it should consume nitrate or nitrite and evolve N_2 gas as a by-product. In this study a total of eighty seven bacterial isolates were tested for potential denitrification and eight isolates which showed relatively fast and active denitrifying activity were selected for further study.

3.2 Measurement of denitrifying activity

The eight isolates that showed better denitrifying activities on qualitative test were measured quantitatively (substrate /nitrite consumption over time). From these isolates two organisms show relatively faster consumption of the substrate (Fig.4). These are BACC118 and BACC280, which were used for further characterization and evaluation.

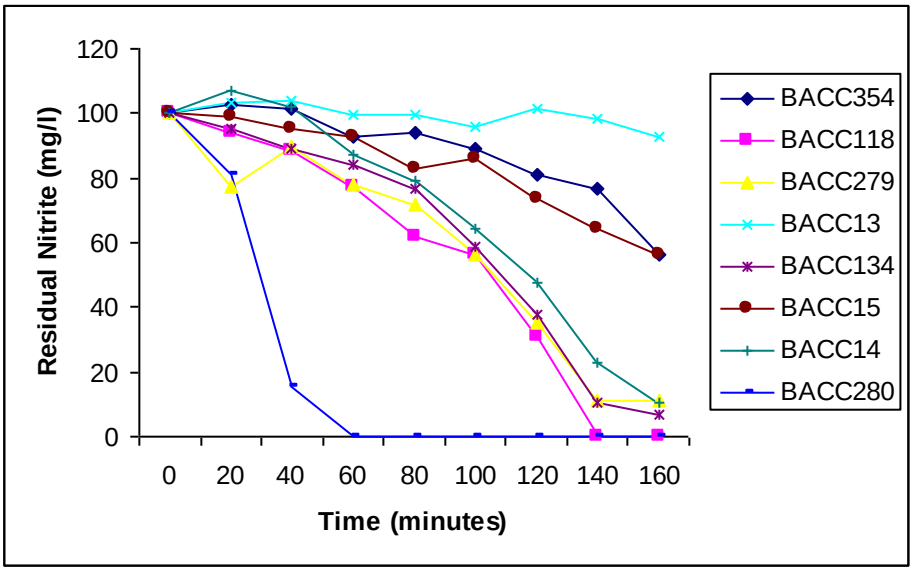


Figure 4. Denitrification Curve using NO_2^- as a sole substrate

3.3 Morphological and physiological Characterization

3.3.1 Morphological appearances of isolates

The result of morphological characterization indicates that both the selected isolates are Gram reaction and oxidase negative. This shows that the organisms don't contain cytochrom c oxidase. Both isolates were catalase positive, and they were able to grow in both anoxic and aerobic conditions suggesting that the organisms are facultative anaerobes. Isolates colony appears to be very small and circular, BACC280 of them have creamy color and BACC118 was light brown (Table 5). Both isolates can not grow at 5 ^{0}c and 50 ^{0}c. Moreover, isolate BACC118 is unable to resist temperature above 45 ^{0}c whereas isolate BACC280 was able to grow till 45 ^{0}c. Optimum growth temperature for the isolates was 25 to 30 ^{0}c. It was difficult to clearly put the two isolates in to Genus of the Bergey's Manual of Systematic Bacteriology because the organisms resemble Genus *Paracoccus* and *Halomonas* except for its being motile and for the color of the colony, respectively.

Table 5. Morphological and Biochemical test result for the selected isolates

Testes		Bacterial isolates	
		BACC280	BACC118
Gram reaction		-	-
Oxidase		+	+
Catalase		+	+
Sucrose		+	+
Mannitol		+	+
Lactose		+	+
Nitrate reduction		+	+
Temperature (^{0}c) tolerance	5	-	-
	10	+	+
	40	+	+
	45	-	+
	50	-	-
Colony color		Creamy	Light brown
Colony shape		Circular	Circular
Colony appearance		Translucent	Translucent
Colony size		Very small	Very small
Colony texture		Buttery	Buttery
Cell shape		Cocci	Cocci
Cell arrangement		Streptococcus	Diplocccus
Motility		Motile	Motile

3.3.2 **Physiological Characterization of the isolates**

3.3.2.1 Growth rate determination for the isolates

Quantitative study was carried out to evaluate the growth rate of the two isolates that show relatively better denitrifying ability (isolates BACC280 and BACC118). The adaptation period of BACC280 was until 12 h while BACC118's was until 6 hours. Both organisms are fast growing isolates. Their logarithmic phase is until 24th hours, and then stationary phase started (Fig. 5).

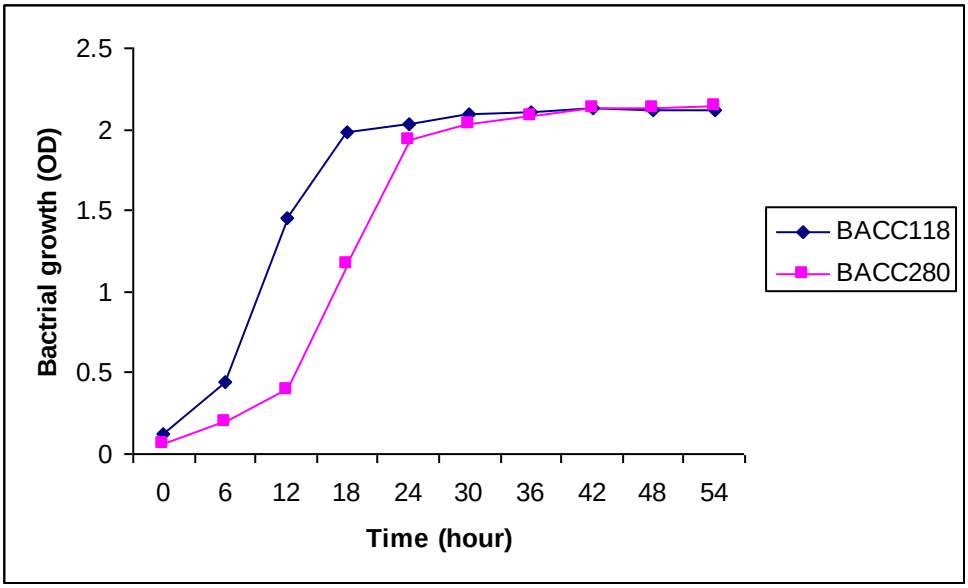


Figure 5. Growth rate of isolates

3.3.2.2 Salt (NaCl) tolerance

The effect of NaCl on the growth of the isolates was tested by varying the salt concentration on growth media. BACC118 can optimally grow from 0 to 2% NaCl concentration afterwards the growth starts to decline. However, optimum concentration for the growth of BACC280 is between1 to 5% NaCl (Fig. 6).

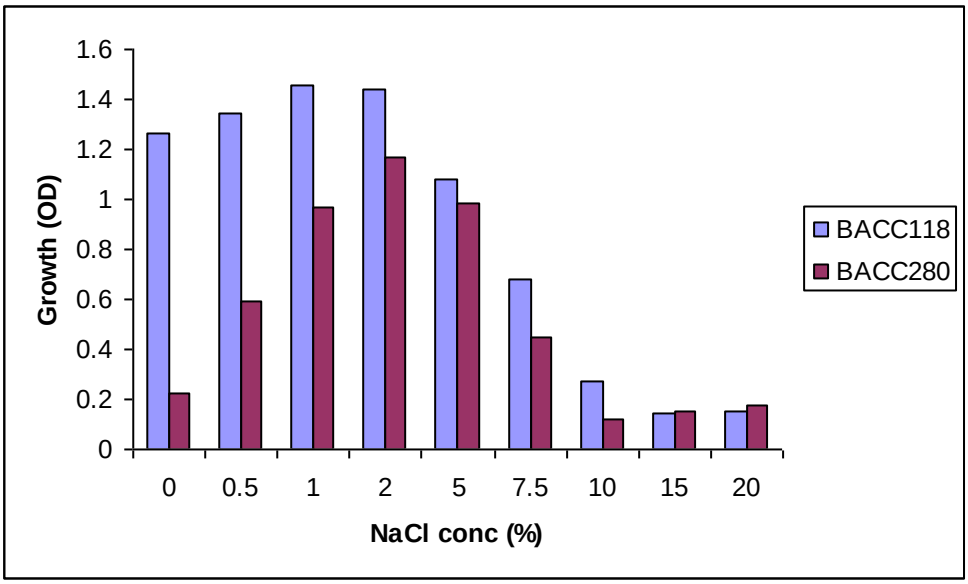


Figure 6. NaCl tolerance for the isolates growth.

3.3.2.3 Growth at different Na_2CO_3 concentration

The effect of Na_2CO_3 on the growth of the isolates was tested by varying the salt concentration on growth media. The two selected isolates show similar property against Na_2CO_3 concentration (Fig. 7). They were able to grow optimally from 0.25 to 1.5 % Na_2CO_3 concentration and then the growth started to decline in both of the isolates.

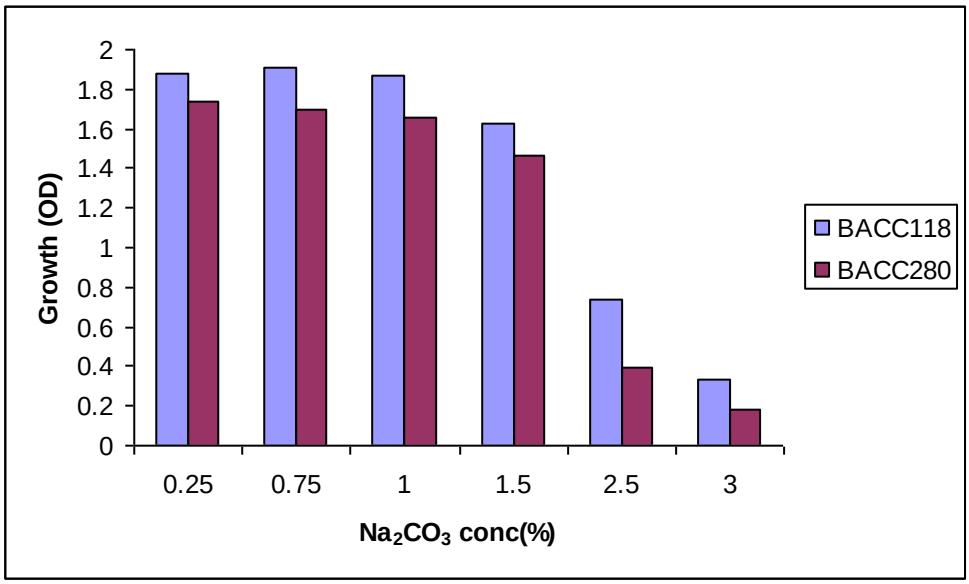


Figure 7. Growth of isolates at different Na_2CO_3 concentration

3.3.2.4 Effect of NaCl on denitrification activity

Both denitrifying isolates displayed similar denitrification activity when the concentration of NaCl is variable. That is, there was high activity in concentrations between 0 to 5% for BACC280 but optimum concentration for BACC118 was from 0 to 2% (Fig. 8).

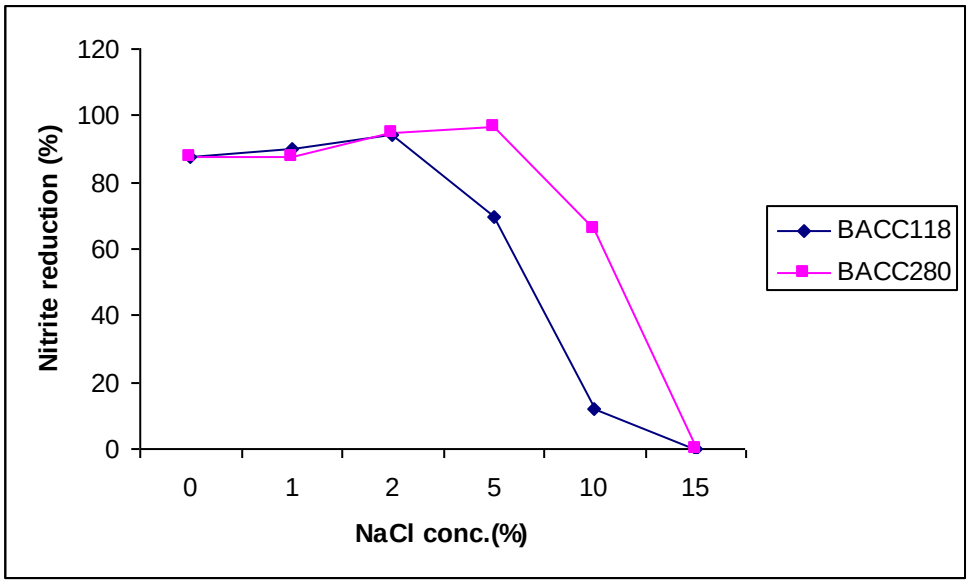


Figure 8 Denitrification activities of isolates at different NaCl concentration

3.3.2.5 Effect of Na_2CO_3 concentration on denitrification activity

The two isolates showed negligible denitrification activity until 0.5% Na_2CO_3 concentration. Isolate BACC280 showed gradual increase in activity until the maximum is reached at 3.5% Na_2CO_3 concentration. However, isolate BACC118's denitrification activity is optimum from 0.5 to 3.5% Na_2CO_3 concentration and start to fall down after wards (Fig. 9).

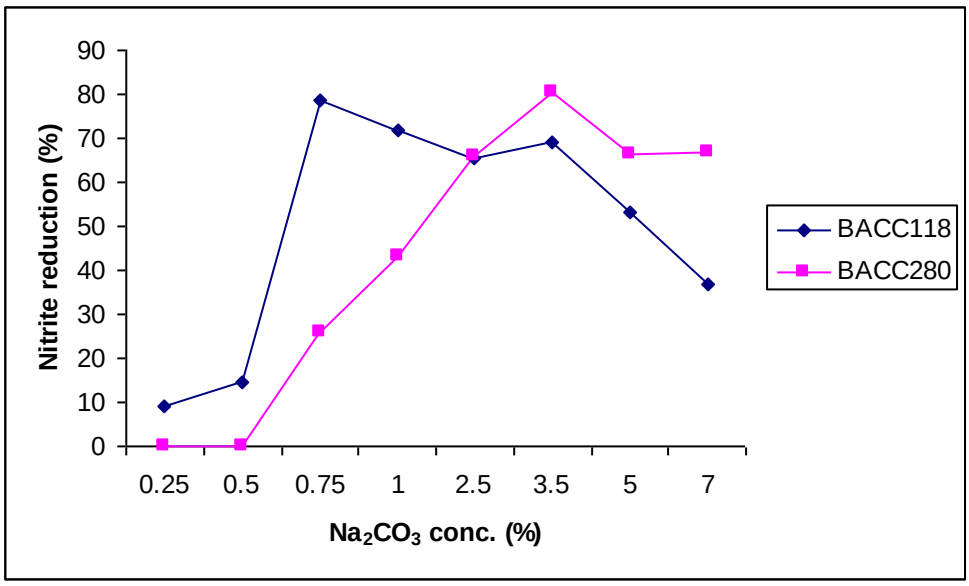


Figure 9. Denitrification activities of isolates at different Na_2CO_3 concentration

3.3.2.6 Denitrification activity at different pH (buffered)

The two isolates are observed to show no denitrification activity from pH 7 to 8.5. BACC118 started its denitrification activity from pH 8.5 and gradually increased until the maximum was achieved at pH 12. While, BACC280's denitrification activity slightly increases from 8.5 to 11 and around 12 it shows the maximum denitrification (Fig. 10).

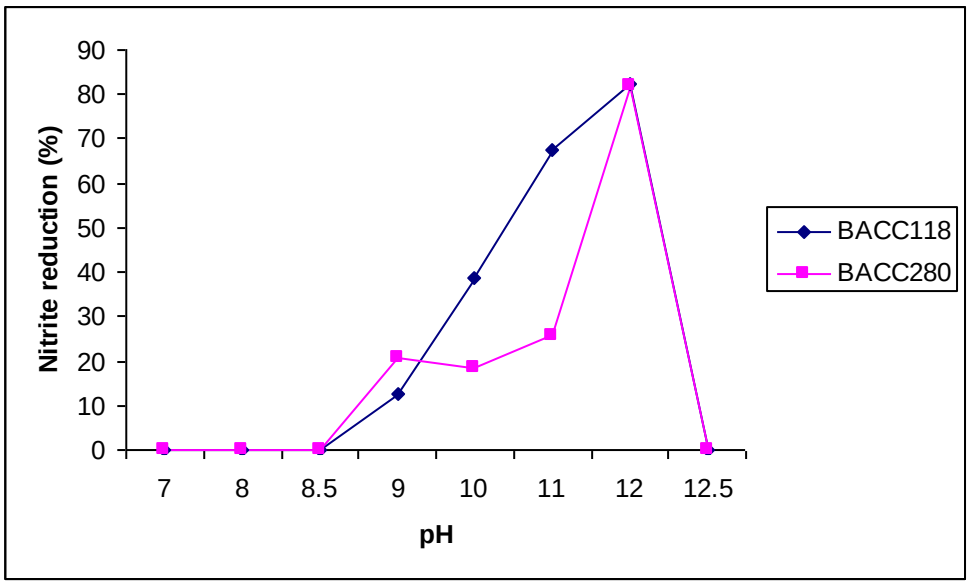


Figure 10. Denitrification activity at different pH (buffered)

3.3.2.7 Denitrifying activity at different pH (not buffered)

The two selected bacterial isolates display different behavior against different pH (un-buffered) values. Isolate BACC280 starts activity beginning from pH 9 and the maximum denitrification is noticed at pH 12. However, isolate BACC118's activity is gradual starting from pH 7 and the maximum activity is at around pH 12.

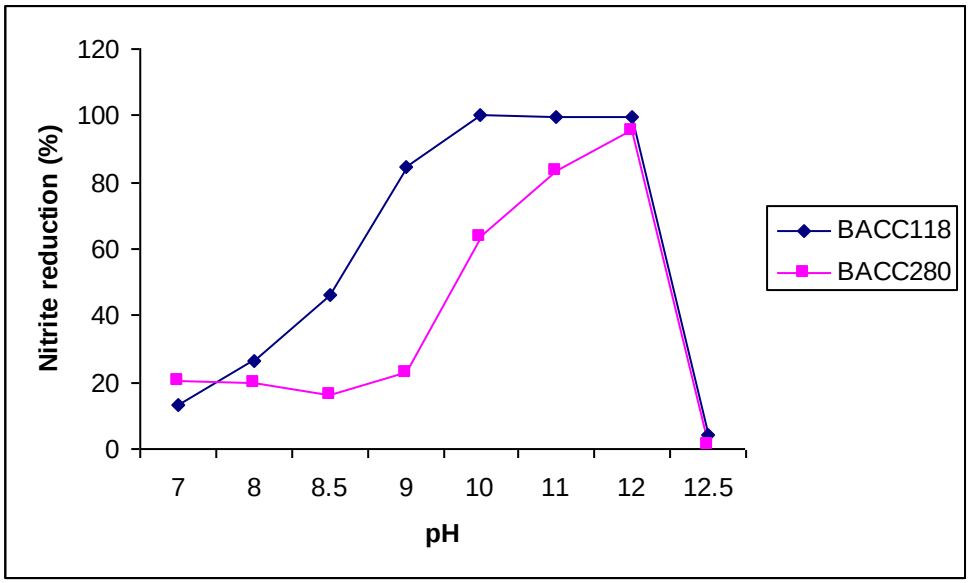
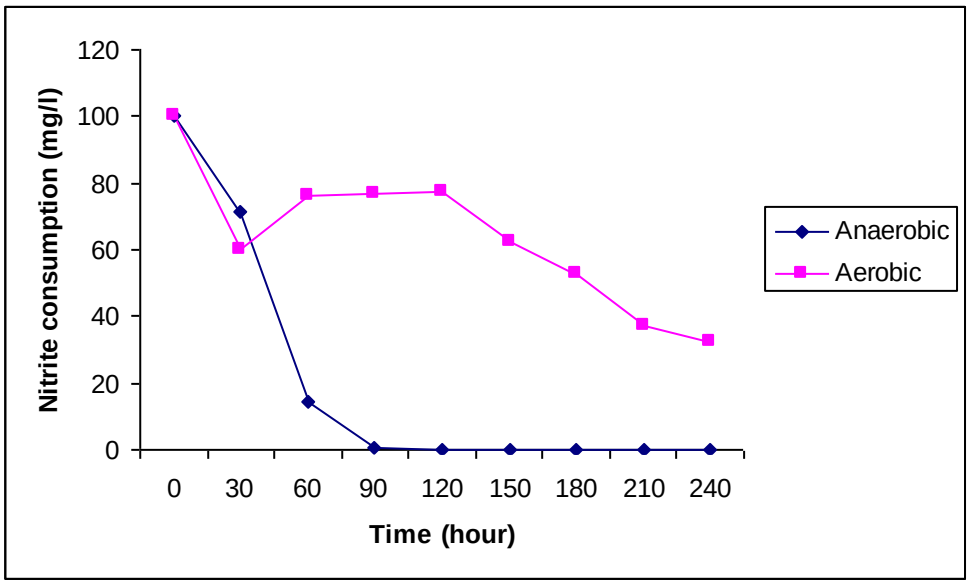


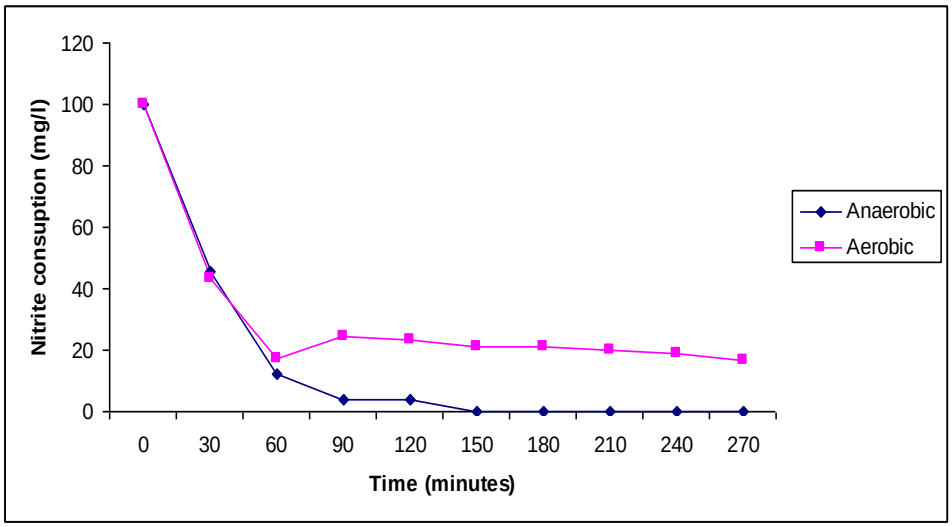
Figure 11. Denitrification activity of isolates at different pH (un buffered)

3.3.2.8 Measurement of denitrification Aerobically and Anoxically

Isolates BACC280 and BACC118 showed similar characteristics when exposed to anaerobic and anoxic environment. Both Isolates utilized and completed the nitrite fast in anoxic environment but nitrite consumption was gradual in aerobic condition of BACC118 (Fig. 12).



a) BACC118



b) BACC280

Figure 12 Aerobic and anaerobic nitrite up take rate for a) BACC118 and b) BACC280

4. Discussion

Soda lakes are essentially closed systems which have different microorganisms that circulate the nutrient in that environment (Baumagrite, 2003; Ulukanli, 2002). Lake Chitu is one of the productive alkaline soda lakes in the Ethiopian Rift Valley. It consists of high algal bloom and feathers of the lesser flamingo with protein and other nitrogenous compounds. As a result high amount of partially degraded organic nitrogenous and carbon compounds are accumulated in the lake.

However, the organic nitrogen compounds are hydrolyzed and converted to NH_4^+- N aerobically. The presence of large concentration of nitrate in the lake also indicates the presence of active and very fast ammonia oxidizing bacteria (AOB) or nitrifires. Finally, denitrifying bacteria reduce nitrite and nitrate to nitrogen gas. These can indicate that there is active nitrification and denitrification that occurs in the lake.

In this study, 78 alkaliphilic organisms were isolated from Lake Chitu samples indicating that denitrification is a common phenomenon in the lake. Several studies indicated that the optimum pH for denitrification to be in the range of 7 - 8.5 and almost all the organisms known are neutralophilic (USEPA, 1993; Gerardi, 2002). To date there are only few reports on alkaliphilic denitrifying bacteria (Sorokin *et al*. 2003a & b). The presence of denitrifying bacteria in alkaline lakes may indicate the presence of several new denitrifying bacteria hitherto unknown.

The optimum pH of the two isolates for denitrification was around pH 12. However, the environment in which they were isolated has pH around 10.4. In another study hydrogenase enzyme of haloalkaliphilic organism showed maximum activity at pH 10, a value that is higher than the optimal for the growth of bacterium (Detkova and Bolltanskaya, 2006). The optimum pH for

growth and for activity may be different. In the case of denitrification the reason is may be because of the process, which produces alkalinity by the reduction of nitrate to nitrogen gas (Sabalowsky, 1999) and the organisms are adapted to this extreme pH.

The concentration of sulfide is found to be very low despite the fact that there is large accumulation of sulfur compounds. This result suggested that, there are sulfur oxidizing bacteria that reduce sulfur compounds such as elemental sulfur, hydrogen sulfide and thiosulfate to sulfate while reducing nitrate and/ or nitrite to elemental nitrogen gas. Sulfur oxidation is also reported by Sorokin *et al.*, (2003a, 2003b). These indicate the presence of active nitrogen and sulfur cycle in soda lakes. In addition other authors also indicated the presence of the nutrient cycling in the lakes (Boltyanskaya, 2007; Sorokin, and Kuenen, 2005a).

The growth of isolate BACC280 with out salt was with long adaptation period. For this reason this organism can be taken as haloalkaliphilic organism. However, isolate BACC118 can grow starting from zero to 5% sodium chloride optimally, therefore, the organism can be considered as halo tolerant one (DasSarma and Arora, 2001).Generally, these two isolate can be considered as organisms that follow compatible solute strategy to avoid water loss to the environment. Because the salt in strategy is primarily employed by aerobic extremely halophilic archae and most other halophilic and halotolerant microorganisms employ the compatible solutes strategy (Detkova and Bolltanskaya, 2006). In addition, the result of conductivity measurement for Lake Chitu was found to be very high. And the growth of these isolates in a wide range of NaCl and Na_2CO_3 concentration might suggest the organisms' adaptation to climatic and seasonal changes in this extreme environment. Frequent climatic fluctuations results in changes in water availability and cause osmotic stress to the organisms and the organisms might develop

physiological adaptation to their original environment (Detkova and Bolltanskaya, 2007).

Denitrification activity of isolate BACC118 was most active at 0 – 10 % NaCl concentration and isolate BACC280 was active between 0 – 5% NaCl concentration. Similarly, the nitrate reductase of *H. campisalis and Halomonas* sp. AIR-2 was most active at 0.3-2.0 M NaCl concentration (Detkova and Bolltanskaya, 2006).

The denitrification activity of BACC118 requires similar NaCl concentration with that of its growth concentration. But Isolate BACC280's growth ability at different NaCl is not the same with denitrification activity of the organism. Growth optimum and activity optimum of Na_2CO_3 concentrations for the two isolates were also different. This is may be because the enzyme nitrite reductase has different or similar adaptation to the organisms' growth adaptation.

In general the presence of Na^+ ion is essential in the surrounding environment to survive in alkaline habitat. It helps for effective solute transport through the membrane. In Na^+ dependant transport system, the H^+ is exchanged with Na^+ ion by H^+/Na^+ antiporter system, thus generating sodium motive force, which drives substrate accompanied by Na^+ in to the cells. More over, H^+ and Na^+ will be adsorbed to the negative charge on the acidic nonpeptidoglycan component of the cell surface and repulse OH^- ion and as a consequence assist cells to grow in alkaline environment (Horkoshi, 1999).

The two isolates show active denitrifcation in an aerobic and anoxic condition. BACC118 has more affinity to O_2 than NO_2^- because in aerobic condition NO_2^- utilization was not fast. This means the organism uses more oxygen as a final electron accepter than nitrite. But BACC280 has more affinity for NO_2^- than O_2 because in the presence of oxygen the organism

prefer to use NO_2^- as a final electron accepter. This is may be because BACC118 is common inhabitant on the surface of the lake and it has adapted to utilize NO_2^- while going down due to mixing. However, isolate BACC280 may be bottom dweller organism (where there is no or little oxygen) for this reason it is affiliated to NO_2^- than O_2 and adapted to utilize O_2 while mixing. There are some reports showing aerobic denitrification that function as a mechanism of electron acceptance that is supplementary to or competitive with aerobic respiration (Chen, *et al.*, 2003).

5. Conclusion

☛ Lake Chitu is highly saline and alkaline lake with great nutrient recycling.

☛ The denitrifiying isolates reduce nitrogen compounds in to gaseous nitrogen both aerobically and anaerobically.

☛ The denitrifying isolates that are isolated from the lake have tolerance to very high sodium chloride and sodium carbonate concentrations.

☛ The two isolates may have the potential to treat wastes that contain high amount of nitrogen compounds, high salt concentration and high pH values.

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Declaration

I, the under signed, declare that this thesis is my original work. It has never been submitted in any institution and that all sources of materials used for thesis have been dully acknowledged.

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