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**Tannery wastewater treatment using alkaliphilic Sediment
Inoculum in Anoxic-Oxic Bioprocess**

**A thesis submitted to the School of Graduate Studies in partial
fulfillment for the degree of Masters of Science in Biotechnology**

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Acronyms and symbols

AMO = Ammonia Monooxygenase

AOB = Ammonia Oxidizing Bacteria

COD = Chemical Oxygen Demand

DC = Denitrifying isolates obtained from Lake Chitu

DS = Denitrifying isolates obtained from the treatment system

DO = Dissolved Oxygen

F/M = Food to Microorganism ratio

HAO = Hydroxylamine Oxidoreductase

HRT = Hydraulic Retention Time

Lab = Laboratory

MCRT = Mean Cell Retention Time

meq = milliequivalent

MLSS = Mixed Liquor Suspended Solid

MLVSS = Mixed Liquor Volatile Suspended Solid

ms = millisiemens

NOB = Nitrite Oxidizing Bacteria

Q = Flow rate

SOB = Sulfur Oxidizing Bacteria

SRB = Sulfur Reducing Bacteria

SV₃₀ = Sludge Volume after 30 minutes of settling

TDS = Total Dissolved Solids

TWW = Tannery Wastewater

Abstract

Tanning is one of the oldest industries known by its voluminous wastewater and the use of huge type and amount of chemicals. In Ethiopia there are over twenty tanning industries. Most of which release their wastes without pretreatment. TWW is known particularly by its high organic load, nitrogenous, sulfide and chromium contents and by its salinity and alkalinity. In the presence of these toxic substances and saline and alkaline conditions, TWW treatment by biological methods using conventional neutrophilic organisms is hardly efficient. The objective of this study was to treat TWW by naturally alkaliphilic sludge obtained from Soda Lake Chitu water and sediment using lab-scale anoxic-oxic activated sludge treatment system and to evaluate the adaptability of the inoculated sludge to the toxic tannery wastes. The efficacy of the alkaline sludge and performance of the reactors was evaluated by analyzing COD, TN, NH_4^+ , S^{2-} , SO_4^{2-} , NO_3^- , Cr_{tot} , MLVSS, MLSS and pH of the raw and treated TWW. The system was operated on continuous basis, but physicochemical data was obtained at specific OLR of 1.92gCOD/L/d and 1.64gCOD/L/d at feed 1 and feed 2 respectively. The system was able to remove 99% S^{2-} , 93%TN and 89% NO_3^- at feed 1 and 96% NH_4^+ , 93% COD and 92% Cr_{tot} at feed 2 from raw TWW. The result showed that at relatively high OLR there was slightly better removal of sulfides, TN and nitrate but higher removal of COD, ammonia and chromium was observed at comparatively lower OLR. In addition, the treatment process was effective in removing the color and notorious odor of TWW. Among the key factors of the activated sludge in treating the nitrogenous TWW are denitrifying bacteria. Adaptability of inoculated microorganisms to the TWW was evaluated by isolating denitrifying bacteria from the inoculant lake sediment and from the steady state treatment system. Comparative characterizations of these isolates showed that there was little difference in their pH optima (9.5-10.5) and range, chromium tolerance (250-2500mg/l) and preferences to energy sources. Also the differences in their cell wall chemistry (<3%), colony morphology, starch hydrolyzing ability and catalase reaction were minimal. But remarkable difference was evident in nitrate reducing ability and salt tolerance. Denitrifying isolates obtained from the treatment system were found to be complete nitrate reducers, incomplete nitrate reducers, exclusively nitrite reducers and both nitrite and nitrate reducers, but isolates from the inoculum were only complete and incomplete nitrate reducers. Presence of exclusively nitrite reducers, both nitrite and nitrate reducers and tolerance to high salinity by the denitrifying isolates obtained from the treatment system (DS) showed a shift in denitrification function by the inoculated microbial community. The shift in denitrification function and tolerance to high salinity conditions by the DS isolates was supposed to be associated to the adverse nature of TWW and strict anoxic condition to which the bacteria adapt the new environment than resisting it.

Key words: *Tannery wastewater, Lake Chitu, alkaliphiles, denitrifying bacteria, lab scale activated sludge.*

1. Introduction

1.1. Status quo of tannery wastewater treatment in Ethiopia

Tanning generates voluminous wastewater containing different types of chemicals that have adverse ecological impacts. These chemicals came from the processes of converting raw hide in to leather. Tannery wastewater (TWW) is loaded with harmful organic and inorganic chemicals which made its rank among the adverse industrial pollutants (Javaid *et al.*, 2000). In Ethiopia there are over 20 tanneries and tanning is a relatively old industry. Most of these tanneries release their wastes in to the environment without any prior treatment (Seyoum Leta, 2004). Introduction of tannery wastes to the environment through raw wastewater has destructive effects to the natural ecosystem (Forney *et al.*, 2000; Mishra and Doble, 2007).

Ethiopia has comparative potential for leather production from the perspectives of its livestock wealth and growing future of industrialization. However, there is a tradition of disposing tannery wastes without pretreatment as can be observed in Modjo tannery, one of the oldest tanneries in Ethiopia, which discharges its untreated effluent to the nearby Modjo river. Therefore, it is an absolute requirement to promote sustainable wastewater management and ensure the protection of environmental quality by employing proper TWW treatment mechanisms (Altaf *et al.*, 2008). TWW can be treated by the cost effective biological treatment strategies such as activated sludge systems (Perxas, 2005).

1.2. Origin and impacts of tannery wastewater

Tanning generates solid, liquid and gaseous wastes that are of serious consequence in polluting fresh water and soil (Javaid *et al.*, 2000). There are two adopted methods of tanning hide/skin: vegetable tanning and chrome tanning. Chrome tanning accounts nearly 90% of leather production (Ram *et al.*, 1999). Tanning processes comprise the beamhouse, tanning and finishing stages which collectively result in wastewater containing chemically diverse components of organic matter, heavy metals, nitrogen and sulfur compounds (Naumezyk and Rusiniak, 2005).

Wastewater refers to water that has been used and disposed into the environment with altered physicochemical properties (Gallert and Winter, 2005). Chemicals utilized in tanning processes include lime, salts of sodium, chromium and ammonium, fat liquors, bacterial and fungicides, dyes, etc (Mohanta *et al.*, 2010). The presence of these chemicals in TWW can create adverse pollution to the environment (Lefebvre *et al.*, 2005).

Most of the pollutants of tannery wastewater are released from the beam house and tanning processes. TWW emanating from beamhouse is rich in sulfides and organic matter, whereas tanning stage is known by inorganic salts of chlorine, ammonia, chromium and sulfate (Goltara and Mendez, 2003). The discharge of TWW in to the surface water and soil let these chemicals get into the food chain and accumulate in the environment to disrupt the biological processes (Mohanta *et al.*, 2010). TWW is linked to high salinity, alkalinity and specific pollutants such as chromium, ammonia and sulfides (Reemtsma and Jekel, 1997; Cooman *et al.*, 2003). Lefebvre *et al.* (2005) depicted tanneries to generate saline effluents with TDS above 35 g/l.

1.3. Management of tannery wastewater

Wastewater treatment stands as a last line of defense against water pollution (Stenstrom and Song, 1991). Most of the pollutants in TWW are in solution and suspension forms. Therefore, physical, chemical and biological treatment methods can be adopted as a method to treat TWW either by their own or in combinations (Naumezyk and Rusiniak, 2005). The physical aspects of wastewater treatment such as sedimentation, filtration and coagulations are collectively called **unit operations**. Those treatment methods that involve chemical and biological processes are called **unit processes**. Biological unit processes involve microbial activity which is very much important in nutrient removal and organic matter biodegradation (Bitton, 2005).

1.3.1. Biological wastewater treatment

Biological treatment refers to methods of cleaning polluted wastewater using microorganisms so that it can be returned to the environment without safety concerns (Kabdasli *et al.*, 2003). Biological approaches of wastewater treatment are preferred over physical and chemical approaches in feasibility and affordability. As a result, biological methods of wastewater treatment are the commonest approaches for effective removal of organic matter and nutrients

(Mrozik and Piotrowska-Seget, 2009). Biological wastewater treatment takes advantage of the catabolic versatility of microorganisms to detoxify wastes into harmless end products (Kabdasli *et al.*, 2003). As the major fraction of organic matter in wastewater is in particulate form (Kong *et al.*, 2008), the strategy in biological wastewater treatment relied on the ability of microorganisms to breakdown compounds for their growth and energy demand (EFB, 1999). Biological wastewater treatment depends on operational factors such as sludge age (MCRT), pH, temperature, DO, electron donor type and concentration, OLR and presence of toxic substances. The impact of these factors to chemoautotrophs is more sensitive than heterotrophs and hence they require more conducive environmental conditions (Stenstrom and Song, 1991).

1.3.1.1. Activated sludge process

There is a general argument that all industrial wastewaters can be treated biologically provided that there is proper analysis and control of environment (Cheremisinoff, 1996). Biological nitrogen removal from wastewater by activated sludge is a large biotechnological approach worldwide (Kampfer *et al.*, 1996; Drysdale *et al.*, 1999; Ramdhani, 2005; Fernandez *et al.*, 2008; Kong *et al.*, 2008). The concept of biological wastewater treatment through activated sludge was introduced in 1914 by Locktt and Ardern at the city of Manchester. The name *activated sludge* was adapted from the capacity of the sludge to increase the removal rate of organic material by circulating sludge to either in batch or continuous feeding system (Bitton, 2005).

Activated sludge is secondary biological suspended growth process by which particulate and dissolved substances are changed into settleable mass called *sludge* under suitable conditions. It provides one of the highest degrees of treatment obtainable in the wastewater treatment technology by which organic matter converted into carbon dioxide, water, ammonia (NH₃) and new cell biomass that constitute the sludge with concomitant elimination of nutrients (Bitton, 2005; Ramdhani, 2005). Activated sludge process depends on groups of microorganisms, mainly bacteria along with protozoa and rotifers. Protozoa play crucial roles by grazing dispersed bacteria thereby reduce the turbidity of the effluent (Samara *et al.*, 2009). In activated sludge process the diverse bacteria organized in aggregates known as *flocs* (Bourrain, 1999).

Activated sludge is generally composed of 70 to 90% organic and 10 to 30% inorganic matter. Organic material in the wastewater serve as a food to microorganisms that collectively with the inert matter and non biological organic matter form the biological solids or mixed liquor suspended solids (MLSS) (Cheremisinoff, 1996). During treatment operation, microbial biomass needs to be maintained to suitable food to microorganism (F/M) ratio and sludge age by recycling settled sludge from the clarifier and excess sludge is regularly wasted (Cakci and Bayramoglu, 1995).

1.3.1.2. Modified Ludzack-Ettinger process

Modified Ludzack-Ettinger (MLE) is a basic activated sludge process developed mainly to comprise biological denitrification. The process was designed by Ludzack and Ettinger and modified by Bernard (Bitton, 2005; Padayachee, 2006). The MLE design utilizes readily biodegradable organic matter from the influent wastewater as energy source for denitrification. Its anoxic basin receives influent wastewater from feed tank and mixed liquor from the aerobic basins which are rich in organic matter and nitrate respectively. It also receives returned sludge from the clarifier. The denitrified effluent is further aerated in the oxic reactor where ammonia oxidized into nitrate. The most frequently adopted schemes of treatment for biological removal of nitrogen and organic matter by activated sludge via nitrification-denitrification processes are two-sludge and single-sludge processes (Szpyrkowicz and Kaul, 2004).

In the two-sludge activated sludge process, each nitrification step is preceded by denitrification and followed by sludge sedimentation step (Szpyrkowicz and Kaul, 2004). Two-sludge process was developed by Barnard in South Africa, known by Bardenpho process (Bitton, 2005). The two-sludge process is said to be efficient in nutrient removal, but its drawback is the need for an external carbon source to sustain denitrification process. Single-sludge process comprises a series of aerobic and anoxic systems with one settling tank. The nutrient removal efficiency of single sludge process is lower than the two-sludge process (Szpyrkowicz Kaul, 2004; Bitton, 2005).

1.4. Biological nitrogen removal from tannery wastewater

Nitrogen exist in different forms with its most oxidized state nitrate and reduced forms ammonia and organic nitrogen compounds. In untreated TWW excess nitrogen occur as ammonia and organic nitrogen in soluble and particulate forms (Sabalowsky, 1999; Kabdasli *et al.*, 2003). Almost all nitrogenous compounds, except magnesium ammonium phosphate, are easily soluble in water and thus cannot be removed by chemical precipitation. Some amino-nitrogen and heterocyclic nitrogen compounds must also convert into ammonia in aerobic and anaerobic processes to be eliminated in the form of gaseous nitrogen (Gallert and winter, 2005). The most commonly applied treatment scheme for nitrogenous wastewater is biological methods of nitrification and denitrification (Szpyrkowicz and Kaul, 2004). For instance, in Europe where annual leather production is about 190 million m², biological treatment methods are the most predominantly used methods of treatment (Zupancic and Jemec, 2009).

1.4.1. Environmental hazards of nitrogen compounds

The different forms of nitrogen in tannery wastewater have various environmental and health concerns. Some of the most important effects of nitrogen compounds are summarized as follows.

1. **Toxicity:** Among the nitrogen compounds released from the leather production processes is ammonia. Ammonia in its unionized form is toxic to aquatic life particularly to fish (Bitton, 2005, USEPA, 2009).
2. **Oxygen depletion:** Releasing high amount of ammonia nitrogen in to the environment imparts nitrogenous oxygen demand in aquatic environments (Campose *et al.*, 2002). Every mg of ammonia exerts 4.6 mg oxygen demand leading to oxygen depletion and hence affect aquatic ecosystem (Cheremisnif, 1996; Kabdash *et al.*, 2003; Bitton, 2005; USEPA, 2009).
3. **Eutrophication:** Nitrate is one of the important nutrients for algal growth. Its direct release or formation from ammonia oxidation in water bodies promotes eutrophication (Cheremisnif, 1996, USEPA, 2009).
4. **Reducing chlorination efficiency:** Chlorine can combine with ammonium to form chloramines (NH₂Cl) which has a lower germicidal effect than free chlorine (Bitton, 2005).

5. **Corrosion:** If present at a concentration of 1mg/l or more, nitrogen compounds especially ammonia can instigate corrosion of metals (Bitton, 2005).

6. **Health impacts:** The occurrence of nitrate and nitrite in streams of water beyond the permitted limit can cause methemoglobinemia (“blue babies” syndrome) in infants and even in certain susceptible segments of the adult population (USEPA, 1993; Kabdash *et al.*, 2003; Bitton, 2005).

1.4.2. Nitrogen sources of tannery effluents

Tannery wastewater consists of compounds extracted from skin, chemical reagents applied in tanning and products from their degradation. In processing raw hide/skin only the middle layer (corium) is the one to react with the tanning agents and constitute leather. The outer and inner layers are removed and account for the majority of organic content of the TWW. Nitrogen in TWW exists as ammonia and organic nitrogen. Proteins mainly collagen and their hydrolysis products are predominant components of organic nitrogen in TWW (Gallert and Winter, 2005; Naumezyk and Rusiniak, 2005). Generally nitrogen in TWW originated from soaking-liming-delimiting-bathing-pickling-tanning processes and their washings (Kabdasli *et al.*, 2003).

1.4.3. Mechanisms of biological nitrogen removal from wastewater

Nitrogen removal from wastewater has become an inevitable task due to the adverse impacts of excess nitrogen in receiving water bodies. In biological wastewater treatment nitrogenous compounds are eliminated by combined or sequential microbial actions of proteolysis, ammonification, nitrification, and denitrification (Zielinska and Wojnowska-Baryla, 2006). Depending on the kind of nitrogen compounds present in wastewater, nitrogen removal particularly requires the processes of ammonification, nitrification and denitrification in their respective sequences (Gallert and Winter, 2005). Biological wastewater treatment plants achieve nitrogen removal by population of bacteria that includes nitrifiers and denitrifiers exposing them alternatively to oxic and anoxic conditions (Lee *et al.*, 2010).

1.4.3.1. Ammonification

Among the processes supposed to happen in wastewater treatment is hydrolysis of protein to peptides and amino acids using extracellular proteolytic enzymes secreted by ammonifying

bacteria such as *Pseudomonas*. Peptides and amino acids further mineralized to ammonia by microbial actions called ammonification. Some of the ammonia-nitrogen is incorporated into cell mass, but the majority of ammonia nitrogen is oxidized by dissimilatory processes (USEPA, 1993; Bitton, 2005; Gallert and Winter, 2005). The rough estimate of protein component of bacteria is about 50% from which 16% is nitrogen and the synthesis of 1g of bacteria biomass require about 0.08g of ammonia-N. According to Gallert and Winter (2005), proteolysis of organic nitrogen results in the production of ammonia via *hydrolytic* (1), *oxidative* (2), *reductive* (3) and *desaturative deamination* (4).



High concentration of ammonia in wastewater is harder to treat than any other industrial wastes (Kabdasli *et al.*, 2003). Recently biological removal of ammonia from wastewater has been operated in traditional processes of nitrification and denitrification and by the newly developed anaerobic ammonium oxidation (anammox) methods (Morita *et al.*, 2008).

1.4.3.2. Nitrification

Nitrification refers to the dissimilatory processes of converting ammonia into nitrate by nitrifying bacteria (Sabalowsky, 1999; Takaya *et al.*, 2003). It was introduced in 1950's as a means of removing ammonia from wastewater by activated sludge process (Gallert and Winter, 2005). Nitrification could be autotrophic or heterotrophic and is an important prerequisite for total nitrogen removal in wastewater treatment via subsequent denitrification (Cheremisinoff, 1996; Juretschko *et al.*, 1998; Morita *et al.*, 2008). It is generally assumed that nitrification occur in conditions where dissolved oxygen exceeds 2mg/dm⁻³ (Szpyrkowicz and Kaul, 2004).

Nitrification occurs in two-steps. The first step is oxidation of ammonia to nitrite that in turn is divided in two stages. Initially ammonia is oxidized to hydroxylamine (NH₂OH) by *Ammonia Monooxygenase* (AMO). Then NH₂OH is oxidized to nitrite by *Hydroxylamine Oxidoreductase* (Limpiyakorn *et al.*, 2005; Whang *et al.*, 2008). The second step of nitrification is oxidation of nitrite to nitrate by nitrite oxidizing bacteria (NOB). The key

enzyme for oxidation of nitrite to nitrate is *nitrite oxidoreductase* (Gerardi, 2002; Bitton, 2005).

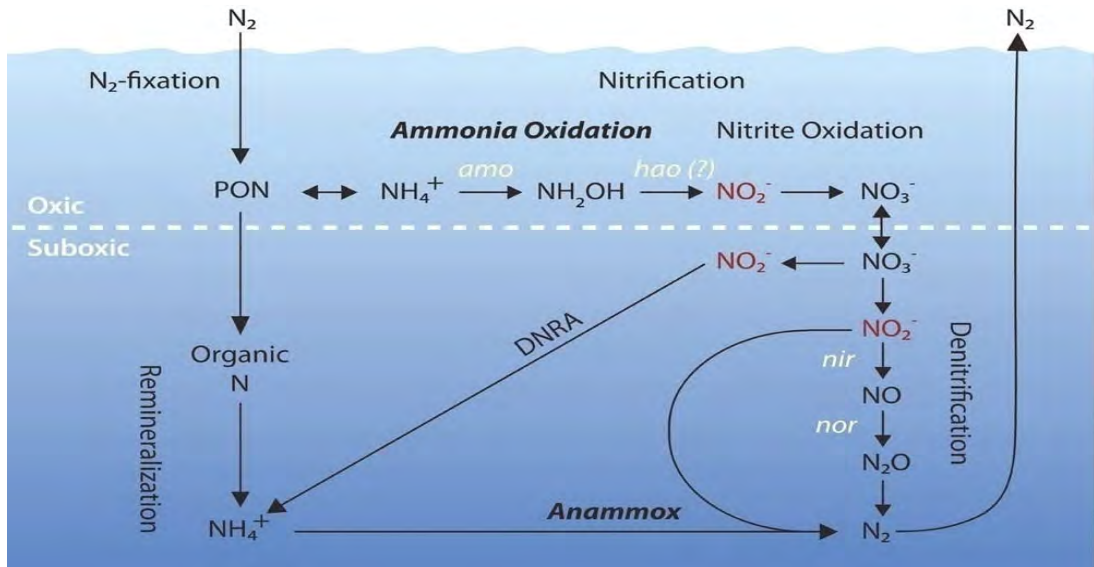
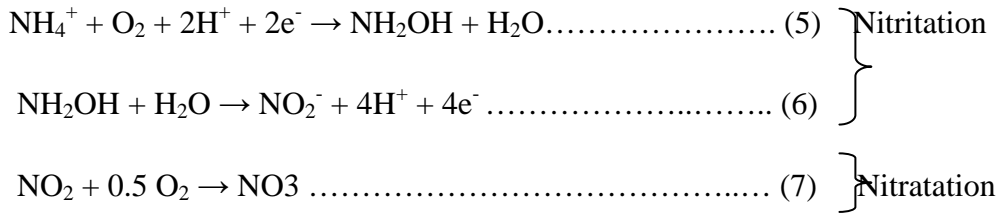
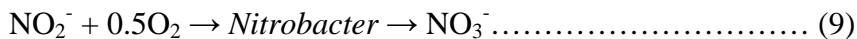
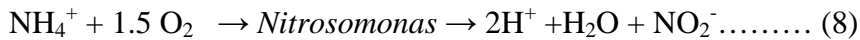


Figure 1. Microbial nitrogen transformations (adapted from Francis *et al.*, 2007)

The two principal bacterial genera of importance for nitrification are *Nitrosomonas* and *Nitrobacter* both classified as autotrophic organisms (USEPA, 1993). *Nitrosomonas* oxidize ammonium to nitrite and *Nitrobacter* carry out oxidation of nitrite to nitrate.



Though they are notoriously slow growing, ammonia oxidizing bacteria (AOB) are of universal importance to the removal of ammonia pollution from wastewater (Jonoud *et al.*, 2003; LaPara and Ghosh, 2006). Nitrifying bacteria are characterized by their oxygen requirement, production of small biomass and cause destruction of alkalinity through the consumption of CO₂ and production of H⁺. For each gram of ammonia-N converted to nitrate, 4.57g of oxygen

used, 7.14g of alkalinity removed and 0.08g of inorganic carbon utilized and formation of 0.16g of new cells (Bitton, 2005; Gallert and Winter, 2005; USEPA, 2009).

Nitrification is a sensitive process for several environmental and operational factors (Whang *et al.*, 2008). Some of the major limiting factors for nitrification are summarized as follows:

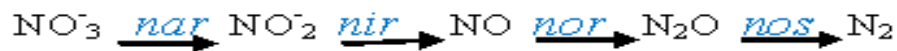
- 1. Alkalinity and pH:** The optimal pH for nitrification falls in narrow range beyond which it affects their growth and activities. Nitrification also causes substantial destruction of alkalinity (Wang *et al.*, 2009).
- 2. Temperature:** Nitrifying bacteria survive over a wide range of environmental temperature (4-45°C), but optimum temperature for their growth is in the range of 25-30°C. Temperature far from the range of the optimum is an important limiting factor for the growth and activity of nitrifiers. (Szyrkowicz and Kaul, 2004; Bitton, 2005; Wang *et al.*, 2009).
- 3. Dissolved oxygen:** Nitrification is an aerobic process such that molecular oxygen acts as a final electron acceptor. The critical dissolved oxygen (DO) concentration below which nitrification inhibited is around 2mg l⁻¹ (Bitton, 2005; Wang *et al.*, 2009).
- 4. BOD/TKN ratio:** The ratio of feed biodegradable organic carbon to nitrogen available for nitrification in the wastewater is one of the critical factors for nitrification (Zhou and Mancl, 2007; Wang *et al.*, 2009). As the *BOD/TKN* ratio increases, the fraction of nitrifiers decreases (Bitton, 2005).
- 5. Inhibitors:** In wastewater treatment process nitrifiers are subject to product and substrate inhibition. Particularly AOB are more susceptible than NOB to inhibitors (Bitton, 2005; Wang *et al.*, 2009).

1.4.3.3. Denitrification

Denitrification is a step wise dissimilatory reduction of nitrate to nitrogen gas with intermediate formation of nitrogen oxides each catalyzed by specific enzyme (Sameshima-Saito *et al.*, 2004; Ramdhani, 2005; Heylen, 2007). Efficient nitrogenous wastewater treatment relies on successive exposition of wastewater to nitrification and denitrification conditions (Gerardi, 2002; Lee *et al.*, 2002; Takaya *et al.*, 2003). Biological denitrification is achieved

under anoxic condition where certain heterotrophic bacteria are stimulated to use organic compounds from the wastewater using nitrate/nitrite as final electron acceptor (Bitton, 2005; Gallert and Winter, 2005; Ramdhani, 2005). Predenitrification or anoxic phase prior to aerobic one is a feasible process configuration for wastewaters with TN content in the range of 100-150mg/l to use the influent organic carbon (Artan *et al.*, 2004).

Denitrification involves a stepwise conversion of nitrate into molecular nitrogen in a series of respiratory steps coupled with biodegradation of organic pollutants (Drysdale *et al.*, 1999; Ramothokang *et al.*, 2006; Heylen, 2007). Denitrification start with the reduction of nitrate to nitrite catalyzed by a three-domain transmembrane enzyme *nitrate reductase (nar)* followed by reduction of nitrite to nitric oxide using *nitrite reductase (nir)*. Nitric oxide further converted into nitrous oxide by *nitric oxide reductase (nor)* and finally nitrous oxide is reduced to molecular nitrogen by *nitrous oxide reductase (nos)* (Schmidt *et al.*, 2003). Denitrifiers that did not possess the enzyme nitrous oxide reductase end denitrification by releasing nitrous oxide (Perxas, 2005). When summarized, denitrification involves the following four enzyme catalyzed reactions.



Denitrification is considered as a community activity as many denitrifiers do not produce the entire suite of enzymes to complete the reaction (Wallenstein *et al.*, 2006). Mostly denitrification is mediated by heterotrophic bacteria capable of utilizing nitrate/nitrite in the presence of organic matter (Park *et al.*, 2001). In other instance, some bacteria are also known with the ability of chemolithotrophic denitrification utilizing inorganic compounds such as ammonium, hydrogen sulfide and inorganic carbon as energy source in the presence of nitrate. Chemolithotrophic denitrification that reduces sulfur compounds offer great biotechnological potential since the process can attain simultaneous nitrogen and sulfur removal in anoxic conditions (Fernandez *et al.*, 2008)

1.4.3.3.1. Denitrification in alkaline conditions

Denitrifying bacteria defined by their ability to reduce nitrate or nitrite in to nitrogen consists of broad variety and ecological distributions in *Proteobacteria spp.* and archaea (Sameshima-

Saito *et al.*, 2004; Rich *et al.*, 2003). Denitrification can occur in alkaline and saline environments such as soda lakes which have valuable potential application for biological treatment of alkaline and saline industrial wastes (Sorokin *et al.*, 2006). One of the important attributes of denitrification for alkalinity is that it produces strong base by dissimilatory reduction of nitrate to gaseous nitrogen (Bitton, 2005; Mahmood *et al.*, 2007). Denitrification produces 3.57mg/l of alkalinity (as CaCO₃) for each mg/l of NO₃ consumed and 0.4g VSS for every g of COD (USEPA, 2009). Reduction of each mole of nitrate/nitrite to nitrogen gas consumes about 0.6 acid equivalents (H⁺) which brings alkalinity that partially restores the alkalinity lost in the aerobic reactor during nitrification (Mahmood *et al.*, 2007).

1.4.3.3.2. Fundamentals of denitrification

Total nitrogen removal from wastewater by the activated sludge treatment is only complete if it ends with denitrification. Since nitrates are eutrophic, health hazards and inhibitors for nutrient removal, denitrification is an obligatory phenomenon in wastewater treatment (Drysdale *et al.*, 1999; Kowalchuk and Stephen, 2000; Bitton, 2005; Gallert and Winter, 2005). Denitrification often associates with the concern of releasing nitrous oxide, a green house gas known to persist in atmosphere (Perxas, 2005). In wastewater treatment denitrification is often influenced by environmental factors. Among the major factors are nitrate concentration, diauxic lag, presence of anoxic condition and organic matter, pH, temperature and trace metals (Bitton, 2005). Diauxic lag is the time required for re-synthesis of *nitrate reductase* and other denitrifying enzymes when denitrifying bacteria are switched from oxic to anoxic conditions (Lee *et al.*, 2009).

1.5. Challenges in tannery wastewater treatment

Despite their wide spread, most microorganisms including bacteria grow best around neutral pH (Yumoto, 2002). Moreover, many of the wastewater treatment research reports still dealt with the ability of microorganisms to remove soluble and particulate wastes at neutral pH or conditions around there. Yet, tannery effluents have alkaline conditions to which neutrophilic organisms are hardly adaptive under ordinary circumstances (Ulukanil and Digrak, 2002). As a matter of fact tanneries generate alkaline and saline wastewater (>35 g/l TDS) containing different kinds of compounds (Lefebvre *et al.*, 2005). Thus, TWW is difficult to treat by

biological process using conventional neutrophilic microorganisms due to its alkalinity, salinity and toxic nature (Campose *et al.*, 2002; Lefebvre *et al.*, 2006).

Neutrophilic microorganisms are unable to operate efficiently at highly alkaline conditions and salinities above that of seawater (3.5%) and their capacity of adaptation to salinity is easily lost after exposition to low salinity conditions (Lefebvre *et al.*, 2006; LeBorgne *et al.*, 2008). Such environments have to be manipulated by groups of organisms that are specifically adapted to the alkaline conditions. Organisms that survive extreme environmental conditions are referred to as extremophiles (Ulukanil and Digrak, 2002). Extremophile microorganisms that are adapted to thrive in alkaline and saline environments are the best options for bioremediation of environments polluted by alkaline and saline wastes. Organisms that grow optimally at about pH 10 and at 2.0-5.2M salt (NaCl) concentration are known as alkaliphiles and halophiles respectively (LeBorgne *et al.*, 2008).

1.6. The use of alkaliphiles for tannery wastewater treatment

Tannery wastewater treatment using activated sludge can be enhanced by some groups of specialized bacteria obtained from natural ecosystems, genetically modified bacteria (GMB) or by using bacteria as plasmid donors for degradative pathways. However, selection of suitable specialized microorganisms from natural complex flora by adapting process parameters is thought to eliminate the public's concern of GMB (Gallert and Winter, 2005). The process of degrading toxic and recalcitrant compounds from polluted sites by microorganisms is known as bioremediation, a technique that uses living organisms in order to degrade or transform contaminants into harmless forms. One of the *in situ* bioremediation strategies is bioaugmentation by which the biodegradability of pollutants is improved by introduction of single strains or consortia of microorganisms with desired catabolic capabilities (Mrozik and Piotrowska-Seget, 2009). The use of naturally alkalophilic bacteria for biological treatment of TWW is a promising alternative for more stringent effluent quality (Campose *et al.*, 2002). Treating TWW by alkaliphilic and halophilic microorganisms obtained from natural soda lakes could be a noble approach due to the occurrence of stable microbial consortia in these environments (Lefebvre *et al.*, 2005; Sorokin and Kuenen, 2005; LeBorgne *et al.*, 2008).

Conventional microorganisms used for biological wastewater treatment showed poor degradative efficiency to tannery effluent due to high salinity and presence of inhibitory conditions (Ventosa, *et al.*, 1998; Lefebvre *et al.*, 2005). TWW treatment is said to be effective only if the bacteria are able to perform in the prevailing environmental conditions. However, studies on the impact of toxic substances to functional bacterial communities revealed that both the long-term and short-term exposures of bacteria to stress such as heavy metals (e.g. Cr and Zn) have an impact in microbial community stability (Turpeinen *et al.*, 2003).

Bacteria have the potential to adapt harsh environmental conditions. Adaptation by bacteria is such an important response to toxic and inhibitory conditions. The ability to adapt and response to toxicants may vary due to differences in their bioavailability and is an important attribute for effective and successful wastewater treatment (Turpeinen *et al.*, 2003). Alkaliphilic and halophilic bacteria have the capability to thrive in alkaline and saline conditions. Hence, they owe the ability to eliminate pollution under alkaline and saline environments (Yumoto, 2002; Sivaprakasam *et al.*, 2008; Zhuang *et al.*, 2010).

1.6.1. Soda lakes as sources for alkaliphiles

Soda lakes represent the most stable naturally occurring alkaline environments on earth having high concentration of sodium carbonates and pH values above 11.5 (Grant, 2006). In soda lakes Na^+ is the dominant cation and $\text{HCO}_3^-/\text{CO}_3^{2-}$, Cl^- , and SO_4^{2-} are the dominant anions. There is uniquely buffered habitat in soda lakes created by variable combinations of sodium carbonate with sodium chloride and sodium sulfate. This makes stable development of obligately alkaliphilic organisms growing optimally at pH around 10 (Sorokin and Kuenen, 2005). The alkalinity and salinity of the soda lakes made them an ideal source for alkaliphilic and halophilic bacteria that could have considerable economical, ecological and scientific values (Le Borgne *et al.*, 2008). One of the Soda Lakes in Ethiopia is Chitu located in Rift valley having pH, salinity, alkalinity and conductivity of 10.2, 45g/l, 581meq/l and 49mS/cm respectively (Elzabeth Kebede, 1997).

Alkaliphilic bacteria are classified into two categories: alkaline-tolerant and alkaliphilic bacteria. Alkaline-tolerant bacteria show optimum growth at pH below 9 and are able to grow around pH 9 or more, whereas alkaliphilic bacteria grow to their optimum at pH above 9 (Yumoto, 2002). The term alkaliphiles is generally restricted to microorganisms that actually require alkaline conditions for

growth (Ulukanli and Digrak, 2002). Alkaline lakes with high salt concentrations hold an additional factor to have halophilic properties. Alkaline bacteria that can adapt and live in saline environments are known as haloalkaliphiles requiring both an alkaline pH of 9 and above and high salinity (up to 33% (w/v)) (Sorokin and Kuenen, 2005). Treatment of TWW having high alkalinity and salinity is suggested to be effective by the concerted actions of alkaliphilic and halophilic microorganisms (Lefebvre *et al.*, 2006).

1.6.2. Biotechnological importance of alkaliphilic bacteria

Although most of the microorganisms occur predominantly in neutrophilic environments, there are also microorganisms that can survive and grow in extremely alkaline (>pH 9) and saline (>30%NaCl) environments (Yumoto, 2002). A diverse population of alkaliphiles and halophiles occur in stable natural soda lakes (Grant, 2006). Alkaliphilic microorganisms exhibit ability to maintain cytoplasmic pH much lower than external pH values of 10 to 11 by 3 to 4 units (Horikoshi, 1999). Wastewater treatment processes involving nitrification and denitrification are most sensitive to alkalinity and high salt concentrations. Research reports by Sivaprakasan *et al.* (2008) indicated that removal of COD by conventional biological wastewater treatment process reduced at salt concentrations above 8,000mg l⁻¹ and at high F/M. Therefore, for the degradation of organic materials from tannery effluents the biomass should be adaptive to its high pH and saline conditions (Campose *et al.*, 2002).

Tannery wastewater has an alkaline pH up to 11.5 and contains slowly biodegradable, refractory and toxic components of different concentrations (Ryu *et al.*, 2007). The unique survival strategies of alkalophiles and halophiles in adverse environments and the potential for robust biocatalysts and unique metabolic capabilities can be a great source of novel biochemical processes for bioremediation (Grant, 2006). Hence, it is possible to use naturally alkaline microorganisms from soda lakes in TWW treatment system for stringent removal of nitrogenous and organic wastes (Campose *et al.*, 2002).

1.7. Toxic impacts of tannery wastewater to activated sludge

Solid and liquid wastes emanating from the tanning industries are inevitable by-products of the leather manufacturing process (Naumezyk and Rusiniak, 2005). Although there is similarity in alkalinity and salinity, naturally alkaline environments are different from TWW environments for

some critical reasons. It is because TWW contain diverse types of chemical compounds in excess that can be serious ailments for the microorganisms meant to do the job of cleaning it (Javaid *et al.*, 2000). Major concerns of inorganic compounds in TWW for the biological treatment process are chromium, sulfides and ammonia (Reemtsma and Jekel, 1997). Another issue is the high organic load (7950-15240 mg/l COD) of TWW that could affect functionality of treatment system (Seyoum L., 2004).

1.7.1. Chromium

Chromium, the 17th most abundant element on earth (Sethunathan *et al.*, 2004), is able to exist in several forms. The insoluble trivalent (III) and soluble and mobile hexavalent (VI) forms of chromium are biggest ecological issues due to their persistence and stability in the environment (Cervantes *et al.*, 2001; Camargo *et al.*, 2004; Viti *et al.*, 2006; Benazir *et al.*, 2009). One of the major applications of chromium is in tanning industries from which it finds way to the environment through wastewater (Stassinakis *et al.*, 2002; Mishra and Doble, 2007). Of the amount (about 19,000mg/l) applied in tanning only 60-70% of chromate reacts to the skin. The remaining 30-40% is discharged to the environment via untreated tannery effluent (Viti *et al.*, 2006). Generally, chromium (VI) coming from tanning process amounts 40-25,000 mg/l (Goltara *et al.*, 2003; Benazir *et al.*, 2009).

Chromium (III) is among the essential metals in trace amount for life. It is metabolically important mainly for glucose and lipid metabolism (Sharma, 2002), yet at high level it can be toxic (Bruins, *et al.*, 2000). But chromium (VI) is considered as a priority pollutant in many countries including USA due to its carcinogenicity and mutagenicity (Stassinakis *et al.*, 2002; Viti *et al.*, 2006; Samaras *et al.*, 2009). The agency for toxic substances and disease registry accepts Chromium (VI) as one of the top 16th hazardous substance. The maximum concentration level permitted in wastewater is no more than 5mg/l for chromium (III) and 0.05mg/l for chromium (VI) (Chen and Gu, 2004; Kabir and Ogbeide, 2008).

The toxicity of chromium comes from the possibility of free diffusion of Chromium (VI) across the cell membrane and its strong oxidative potential (Srivastava and Thakur, 2005). Results of experiments on chromium toxicity showed that nitrifying microbial communities are more sensitive

to chromate than heterotrophic bacteria. In other instances, high concentration of chromium has effect on the structure of activated sludge floc in that it affect the abundance of filamentous microorganisms resulting in poor clarification (Stassinakis *et al.*, 2002). Short-term exposures of metal contaminants to microbial populations affect their activity, but contamination for an extended period may lead to population shift in favor of resistant microorganisms (Kamaludeen *et al.*, 2003).

For bacteria chromium (VI) has destructive effects in cell elongation, cell enlargement and inhibits cell division which eventually leads to prohibition of cell growth. The toxic impact of chromium is more pronounced in gram-negative bacteria (Sharma, 2002). Contamination by heavy metals is often irreversible and even may repress or kill parts of the microbial community. It is generally assumed that exposure to metals leads to the establishment of a stress tolerant/resistant microbial population (Viti and Giovannetti, 2000).

Chromium-resistant bacteria capable of reducing chromate to its harmless form have been reported from chromium contaminated environments (Pal and Paul, 2004). Microbial tolerance and ability to reduce chromium involve bioaccumulation, biosorption, biotransformation, entrapment in extracellular capsules, demethylations and methylation. Mechanisms for bacterial resistance to chromate may be conferred by genes located on chromosomes and plasmids (Rivera *et al.*, 2008). Microorganisms possessing chromate reductases can convert chromium (VI) in to less toxic trivalent form. The use of such microorganisms in chromium polluted environments is a good attribute for chromium bioremediation (Srivastava and Thakur, 2005). Study by Benazir *et al.* (2009) indicated that *Pseudomonas* species were more efficient (with 99.6% efficiency) than *Aeromonas*, *Bacillus*, *Micrococcus* and *Microbacterium* species in reducing chromium.

1.7.1.2. Sulfides and sulfates

Tannery wastewater contains high concentration of sulfate and sulfides that are potential inhibitors for biological wastewater treatment processes (Kabdasli *et al.*, 2003). The sulfide especially is suspected to be the most inhibiting substance in biological treatment of TWW (Genschow, *et al.*, 1997). In TWW, sulfides result from the use of sodium sulfide (Na_2S) and sodium hydrosulfide (NaHS). Of all the wastewater generated from tannery nearly 30% is due to the liming procedure with concentration of sulfide 630-880 mg/l (Mesdaghina and Yousefi, 1991). Under alkaline

conditions sulfides remain largely in solution. The release of sulfide to the environment is detected by its characteristic foul smell like that of rotten egg causing serious odor problems and is a public nuisance (Sayers and Langlais, 1977; Tudunwada *et al.*, 2007). Sulfides can be oxidized into sulfur and sulfate by sulfur oxidizing bacteria (Bosnic *et al.*, 2000). Besides its notorious smell, high sulfide content of tannery effluent can pose the following problems (Sayers and Langlais, 1977; Mesdaghinia and Yousefi, 1991).

1. Being a biological toxic substance, sulfides kill bacteria and upset the biological treatment process.
2. Hydrogen sulfide gas generated in concentrations above 1,500mg l⁻¹ is a deadly poison to human.
3. Hydrogen sulfide can cause corrosion of metal pipes.
4. Sulfides can cause elimination of DO in receiving water bodies.

1.8. Microbial community structure and dynamics in the activated sludge

Microbial communities are self-organizing and self-sustaining assemblages. Analysis of microbial structure in wastewater treatment plant since the last decade of the 20th century showed that *beta-*, *alpha-* and *gammaproteobacteria* as well as *bacterioides* and *actinobacteria* are the most frequently retrieved ones (Wagner and Loy, 2002). Studying population structure and function of activated sludge can yield information useful to assess the microbial dynamics associated to environmental factors. However, information concerning the function of each population cannot be obtained by elucidating changes in community structure. Hence, pure cultures are indispensable for detailed analysis of functionally dominant population. So, isolation of functionally dominant populations in activated sludge is quite important (Watanabe *et al.*, 2000)

Changes in microbial community in the activated sludge are supposed to be associated to the composition of influent wastewater and operational and environmental factors. TWW contains nitrogen species and toxic substances mainly chromium and sulfide that can affect population structure and function in the activated sludge (Gich *et al.*, 2000; Kabdasli *et al.*, 2003). Nonetheless, acclimatization has been claimed as a good strategy to adapt the impacts of

toxicity, but it may lead to the dominance of an environment by the species that have best adaptive resources (Mertoglu *et al.*, 2008).

In addition, any fluctuation in the concentration of the macro-and micronutrients, temperature, pH, DO can affect the population dynamics as microorganisms tend to adapt the change rather than tolerating it. Temperature and pH variation from the optimum to the extremes mostly influence metabolic pathways of bacteria, end products formed and hence population of activated sludge (Cheremisinoff, 1996; Burgess *et al.*, 1999). This usually results change in population functions towards the organisms' best suited to the existing conditions (Burgess *et al.*, 1999). The task of total nitrogen removal from TWW is mainly accomplished by nitrifiers and denitrifiers. There are wide varieties of denitrifying bacteria in wastewater treatment systems including the genera *Achromobacter*, *Aerobacter*, *Alkaligenes*, *Bacillus*, *Pseudomonas*, *Brevibacterium*, *Denitrobacillus*, *Flavobacterium*, *Lactobacillus*, *Micrococcus*, *Proteus*, *Spirillum*, and *Xanthomonas* all capable of dissimilatory nitrate reduction (Sabalowsky, 1999).

1.9. Mechanisms of studying bacterial community in an activated sludge

Bacteria exhibit an enormous level of morphological, physiological and metabolic diversities and are of great importance to global carbon, nitrogen and sulfur cycling (Spain *et al.*, 2009). One of the most pronounced differences among prokaryotes is their metabolic versatility. Diversity of microorganisms in activated sludge systems is vital for removing wastes from the wastewater. The description of bacterial species and their diversity in activated sludges is important for characterization of populations involved in the process performance of nitrogen and organic matter removal and floc structure for efficient wastewater treatment (Bourrain, 1999). The isolation and categorization approaches of bacteria are generally culture-based and culture-independent (molecular) methods (Juretschko *et al.*, 1998; Malik *et al.*, 2007).

1.9.1. Molecular approaches

Community structures of bacteria in wastewater treatment are difficult to describe by conventional cultivation approaches alone. The inadequacy of traditional methods to elucidate community structure can be compensated by molecular techniques emerged in 1990s (Juretschko *et al.*, 2002;

Sanz and Köchling, 2006; Malik *et al.*, 2007). Since then different molecular tools have been developed and applied to investigate the microbial community composition and dynamics in activated sludge systems. Molecular methods rely on the characterization of cellular constituents based on nucleic acids, proteins, fatty acids and other taxa-specific compounds. Identifying microorganisms at the molecular level provide an opportunity to understand microbial diversity and functionality (Malik *et al.*, 2007).

However, DNA-based techniques are still liable to biases. Among some of the pitfalls of molecular techniques are DNA extraction protocols may not be suitable for all bacteria, PCR primers can be biased towards specific bacterial groups, amplified genes might not be functional and extra-cellular DNA can persist in the environment. As a result there is a renewed interest in cultivation to isolate yet-uncultivated bacteria from different environments using modified approaches (Heylen, 2007).

1.9.2. Cultivation-based approaches

Isolation, characterization and classification of bacteria based on cultivation approaches are classical techniques carried out on the basis of microbial features. Some of the landmark tools for isolation are comparisons of cell morphology, Gram-staining, growth rate, tolerance to different conditions, physiological responses and biochemical reactions (Dale and Park, 2004). Most of these methods are still in use in combination with molecular methods or by their own. However, it is mandatory to be aware of the possible drawbacks of these methods to avoid some bias in elucidating the dynamics, diversity and abundance of bacterial communities (Malik *et al.*, 2007).

2. Objectives of the study

2.1. General objective

The major purpose of this study was to investigate the nitrogenous, sulfide and organic waste removal efficiencies of alkaliphilic bacteria by continuous feed lab-scale activated sludge treatment system and to evaluate the adaptability of bacterial community by isolating and characterizing denitrifying bacteria.

2.2. Specific objectives

1. To optimize nitrogen and sulfide removal efficiency of the inoculated bacterial consortia in alkaline condition using a lab scale activated sludge treatment system.
2. To evaluate the efficiency of treatment system and inoculated alkaline bacterial consortia in removing priority pollutants mainly TN, COD, S²⁻ and NH₄⁺.
3. To assess the adaptability of the inoculated alkaline denitrifying bacteria in the treatment process.
4. To elucidate the occurrence of any morphological and functional shift in the characteristic features of denitrifying alkaliphilic bacteria.

3. Materials and methods

3.1. Description of sampling sites

This study used water and sediment samples collected from Lake Chitu that was used as inoculum to prepare activated sludge for biological treatment of TWW. Lake Chitu is located in Ethiopian Rift valley at altitude of 1600m above sea level south of Lake Shalla. Chitu is a creator soda lake that host huge flocs of lesser flamingo (*Phoeniconaias minor*). The pH, salinity, conductivity and alkalinity of the lake are 10.53, 35g/l, 56 ms and 580 me/l respectively. The alkalinity of the lake is due to the occurrence of high sodium carbonate (Elizabeth Kebede, 1996). The rational to use Lake Chitu as source microbial seed to this lab-scale treatment plant is its alkaline and saline conditions which also prevail in TWW. The inoculum was taken at the surface and subsurface of the black sediment and was mixed with the lake water to encompass aerobic and anoxic microorganisms. The sample was brought to Addis Ababa university Biotechnology laboratory by sterile plastic containers and preparation of sludge follows before 48h of its sampling.

3.2. Influent tannery wastewater

Composite tannery wastewater was collected from the different outlets of Modjo tannery located about 80 kms south of the capital Addis Ababa. Modjo tannery is a medium sized factory situated near Modjo river with aggregate wastewater discharge of 3500 to 5500m³/d (Seyoum Leta, 2004). TWW was collected in a week time interval to be used as a continuous feed to the treatment system seeded with alkaliphilic biomass obtained from the soda lake. Composite TWW was characterized according to standard methods in terms of Chemical Oxygen Demand (COD), Ammonia (NH₃), nitrate (NO₃⁻), sulfate (SO₄²⁻), sulfide (S²⁻), total nitrogen (TN), total chromium (Cr_{tot}), and pH before and after biological treatment (APHA, 1998; USEPA, 1993; Seyoum Leta, 2004).

3.3. Start up and seed sample preparation

Since tannery wastewater is characteristically saline (Sivaprakasam *et al.*, 2008) and alkaline, biomass adapted to naturally saline and alkaline environment was used as a seed. The treatment operation began by preparing alkaliphilic seed sludge from the Chitu sediment and surface water sample. Both the anoxic and aerobic reactors were seeded with 10% (v/v) prepared sludge and set for acclimatization and optimization. The seed sample was evaluated for its capability to remove COD, TN, NH₄⁺, NO₃⁻, and S²⁻ during and after acclimatization (Tesfaye Minuta, 2006).

3.4. Experimental reactor set-up and operation

In this study Modified Ludzack Ettinger (MLE) predenitrification-nitrification continuous lab-scale activated sludge treatment process was used to remove nitrogen, sulfide and organic wastes from TWW. Activated sludge was used as a means of treatment as it is well suited to treat organically dominated wastewater (Cheremisinoff, 1996). The configuration of treatment system comprises four zones: four liter feeding tank, two liter anoxic reactor with one liter functional volume, four liter aerobic reactor with two liter functional volume and two liter clarifier (Fig 2). The aerobic reactor was provided with continuous aeration by using a Gallehamp modular fermenter aerator and was stirred to blend oxygen, microorganisms and organic matter forming mixed liquor. The anoxic reactor was also continuously stirred by

magnetic stirrer to keep its contents in suspension. The treatment system was installed at Addis Ababa University faculty of Science campus in the pilot treatment project room at room temperature.

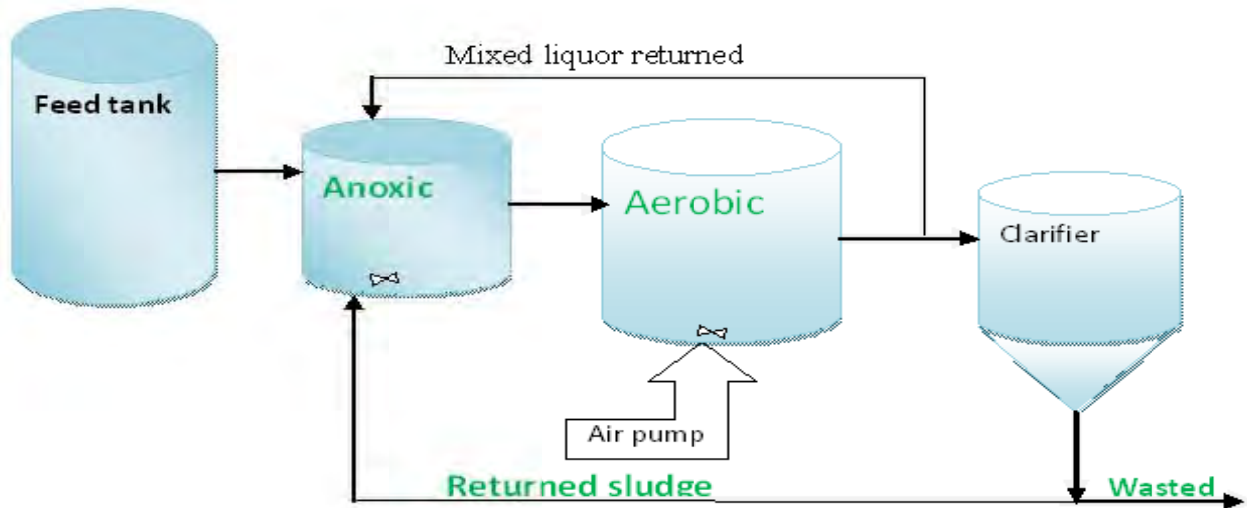


Figure.2 Schematic set up of the reactors

Table 1. Operational parameters of the treatment system

No	Operational parameters	Feed 1 (OLR = 1.92gCODL ⁻¹ d ⁻¹)		Feed 2 (OLR = 1.64gCODL ⁻¹ d ⁻¹)	
		ANOXIC	AEROBIC	ANOXIC	AEROBIC
1	MLSS	2901mg/l	3262mg/l	2666mg/l	3107mg/l
2	MLVSS	2284 mg/l	2510mg/l	1984mg/l	2418mg/l
3	Flow rate (Q)	1.2L/d	1.2L/d	1.4 L/d	1.4L/d
4	Sludge Return rate	0.6Q	-	0.6Q	-
5	HRT	20h	40h	16.67h	33.33h
6	pH(average)	10.04	9.98	10.04	9.98
7	SVI(maximum)	-	61ml/g	-	54ml/g
Operational mode		Influent tank → anoxic → oxic → clarifier → effluent tank			

All the values of operational parameters in table 1 were taken at steady state performance of the reactors. Steady state refers to the process performance at which there was stable removal of COD, NH₄⁺, NO₃⁻ and S²⁻ from the TWW.

3.5. Experimental procedures

This activated sludge treatment process was established after seeding alkaline sludge to the treatment system. The influent TWW was delivered to the anoxic reactor at a rate of 1.2L/d during feed 1 and 1.4L/d during feed 2 using a peristaltic pump and then to the aerobic reactor making the HRT 20h and 16.67h for anoxic and 40 and 33.33 hours to aerobic reactor for feed 1 and 2 respectively (Tesfaye Minuta, 2006). The concentration of suspended biomass was maintained by the returned sludge at a rate of 0.6Q (Q refers to flow rate) after a routine optimization processes. Returning sludge from the clarifier prevent wash out of active microbial consortia from the system. The treatment system had been acclimatized and optimized for 98 days before the actual data was generated in successive feeds and the total operation time was 128 days. Since the influent TWW was rich in organic matter and toxic elements, relatively longer period of acclimatization and optimization was done in order to allow the inoculated biomass adapt to the relatively inconvenient environment (Lefebvre *et al.*, 2005).

The experiment was carried out on a continuous basis, but the data that characterize performances of the treatment system in this study were obtained at two particular feeds of the steady state condition. Organic loading rate (OLR) was 1.92 gCOD/L/d for feed 1 and 1.64 gCOD/L/d feed 2 and performance of the system at the two feeds was evaluated by similar parameters (section 3.6.). Characterization of denitrifying bacterial community was done by using samples collected from the mixed liquor of the treatment system and the sediment inoculum. The pH of influent TWW was maintained in the range of 10.2 to 10.5 adjusting it by adding sodium carbonate according to Carrera *et al.* (2003). The reasonable maintenance of pH simulates bioreactors to the natural source of the inoculant and avoids unnecessary shocks to the sludge. Mixed liquor from the aerobic reactor returned to the anoxic reactor to replenish nitrate in the anoxic reactor (Bitton, 2005).

3.6. Physicochemical analysis and methods

Steady state behavior of the treatment system was monitored by determination and analysis of the pH, COD, TN, MLVSS, MLSS, NH_4^+ , NO_3^- , S^{2-} , Cr_{tot} and SO_4^{2-} according to Padayachee (2006). Samples taken from raw TWW, anoxic and aerobic reactors and final effluent were

taken during each feed test to evaluate the performances of the treatment system. Analysis of the physicochemical characteristics was carried out following standard HACH procedures using DR/2010 HACH Portable Datalogging spectrophotometer. The pH of the treatment system was regularly monitored by OAKTON[®] EUTECH instruments UK, digital calibrated pH meter. The MLSS and MLVSS were determined according to APHA's (1998) standard methods by collecting 100ml mixed liquor from aerobic reactor and centrifuged at 6000rpm. The pellet was desiccated at 105°C overnight on an evaporating dish to determine the MLSS. The weighed dry MLSS was incinerated at 600°C for 5 hours using Furnance-1300 to determine MLVSS.

Removal efficiency for each parameter was calculated by the following formulation.

$$\% \text{ removal efficiency} = \frac{C_{\text{inf}} - C_{\text{eff}}}{C_{\text{inf}}} \times 100$$

Where, C_{inf} = concentration in the influent and C_{eff} = concentration in the effluent

3.7. Determining Nitrification and Denitrification activities

The uptake rate for ammonia and nitrate by the ammonia oxidizing and denitrifying bacterial consortia in the aerobic and anoxic conditions was determined by batch system for 300 and 180 minutes, respectively. The mixed liquor adopted from the aerobic and anoxic reactors was complemented with external NH_4^+ and NO_3^- sources. The biomass (MLVSS) was quantified from each reactor to determine the NH_4^+ and NO_3^- uptake per VSS. The change in the concentration of NH_4^+ and NO_3^- over time was considered as ammonia and nitrate uptake rate and expressed as $\text{mg NH}_4^+ \text{ VSS}^{-1} \text{ h}^{-1}$ and $\text{mg NO}_3^- \text{ VSS}^{-1} \text{ h}^{-1}$, respectively.

3.7.1. Nitrification activity test

Nitrification activity test was conducted in batch reactor according to Chung and Liu, (2001) and Abdul-Talib *et al.* (2002). A 2L batch reactor was filled with 1.5L of mixed liquor which has been operated at stable conditions and ammonium sulfate was added in to the reactor to enrich the ammonium content to a final concentration of 70mg/l. The reactor was mixed with magnetic stirrer and continuously aerated. At every 30 minutes 20ml samples were taken and the sludge was sedimented at 8000rpm for 5 min (Schmidt *et al.*, 2004). The clear supernatant

was analyzed by HACH procedures (section 3.6.) for its ammonia content. MLVSS was also determined from the same sample to evaluate rate of nitrification or Ammonia Uptake Rate (AUR) of the nitrifying consortia.

$$\text{Rate of Nitrification} = \frac{(\text{NH}_4^+ - \text{N})_{\text{in}} - (\text{NH}_4^+ - \text{N})_{\text{out}}}{\text{HRT}(\text{VSS})}$$

3.7.2. Denitrification activity test

Denitrification activity of the biomass in the anoxic reactor was evaluated according to Chung and Liu (2001). The reactor was supplemented with sodium nitrate to give an initial nitrate concentration of 40.0mg/l. To create an oxygen-free condition, the 1.5L reactor was filled with the mixed liquor leaving no headspace. The reactor was sealed and completely mixed by magnetic stirrer. A mixed liquor sample of 20ml was taken at 30 minute interval for analysis of nitrate up take rate (NUR) and MLVSS was determined from 100ml of the same sample.

$$\text{Rate of Denitrification} = \frac{(\text{NO}_3^- - \text{N})_{\text{in}} - (\text{NO}_3^- - \text{N})_{\text{out}}}{\text{HRT}(\text{VSS})}$$

3.8. Characterization of sludge

Activated sludge collected from the aerobic reactor beginning from day of inoculation was observed for its flocculation and microorganism abundance. Similarly the sludge was also characterized for its settleability and Sludge Volume Index (SVI) according to Alleman et al. (1984). Flocculation and microbial abundance were followed by microscopic observation. SVI was extrapolated from SV_{30} and MLSS using the following formulation:

$$\text{SVI (ml/g)} = \frac{\text{settled sludge volume (ml)} \times \frac{1}{\text{sample volume (L)}} \times 1000 \text{ mg/g}}{\text{MLSS (mg/l)}}$$

3.9. Comparative characterization of denitrifying isolates

In this section microbiological examination of alkaliphilic denitrifying bacterial isolates obtained from lake sediment and activated sludge was done by the standard biological procedures of cultivation on denitrifying enrichment media (section 3.9.1). For each sample,

the total viable colony count was determined by 10fold serial dilution from which the cultivable microbial load was estimated using colony forming unit (CFU) count. Morphologically distinct bacterial colonies were picked from plates containing the countable dilutions. Colonies were particularly picked based on the differences in their color and morphology according to Christopher *et al.* (2010).

$$\text{CFU} = \frac{\text{Number of colonies} \times \text{Dilution of plate} \times 1\text{ml}}{\text{Volume of culture on the plate}}$$

3.9.1. Isolation, enumeration and characterization of denitrifying bacteria

Microbiological characteristics of sediment sample before and after running the treatment process was evaluated by cultivating isolates with due emphasis to denitrifying bacteria which play key role in completion of nitrogen removal from the wastewater. Isolation of denitrifying bacteria from the sediment sample and steady state activated sludge was done by using denitrifier enrichment media containing K_2HPO_4 (0.1g/l), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.1g/l), CaCl_2 (0.15g/l), $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (0.02g/l), $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ (0.076g/l), sodium acetate (2g/l), sodium nitrate (2g/l), nutrient broth (5g/l), trace elements (10ml) using acetate as electron donor (Heylen *et al.*, 2006; Shapovalova, *et al.*, 2008). The isolates were cultivated at pH 10.5 having the medium buffered with sodium carbonate according to Sorokin and Kuenen (2005).

The bacterial abundance of both the sediment and activated sludge was examined by standard pour plate count techniques capturing pure isolates based on Dunbar *et al.* (1999). Mixed liquor sample was taken from the treatment system and the sample was homogenized using vortex at high speed. One ml homogenized sample was added to sterile 9 ml 0.85% saline solution and serially diluted up to 10^{-9} . From each dilution 100 μl sample was spread onto the agar plate. After 6 days of incubation at 30°C , colonies were picked from countable dilutions and purified by repeated streaking until isolated pure colonies were obtained and purified cultures were stored at 4°C in agar slant (Yezza *et al.*, 2005).

3.9.2. Morphological characterization of denitrifying isolates

Microorganisms isolated from lake sediment and treatment system were continuously examined at 400x and 1000x magnification by wet mount microscope (GIMA Spa-Via Monza,

102-20060 Gessate (MI)-Italia). Indeed, colonies of morphologically distinct types were routinely characterized by their shape, margin, elevation, diameter, density and surfaces repeatedly (Christopher *et al.*, 2010). After a series of screening, selected denitrifying bacterial isolates from the lake sediment and activated sludge were subjected to further biochemical characterizations specifically using starch hydrolysis, motility, catalase and gram tests.

3.9.3. Comparative characteristics of selected denitrifying isolates

The adaptability of inoculant sample to the treatment process was assessed by comparative analysis of denitrifying isolates obtained from Chitu sediment (DC) and stable treatment system (DS) that were named as DC and DS isolates in this study. Five isolates from DC (inoculum) and five from DS (system) were characterized in terms of pH, salt and chromium tolerance, carbon source preference, motility, spore formation and cell shape. Gram test, catalase test, starch hydrolysis test and nitrate reduction test were done by taking all the isolated and purified DC and DS bacteria. In this study determination of biomass by turbidity measurement (optical density) was done using JENWAY 6300 spectrophotometer UK at 600nm, unless stated otherwise.

3.9.3.1. Determining pH optima and range

The optimum and range of pH for both DC and DS isolates was determined by the method described in Altaf *et al.* (2008) preparing culture using nutrient broth from each pure isolate. Different pH values from 7.0 to 11.5 were adjusted by using Na₂CO₃ (25% (w/v)) to determine the optimum and range of pH at which isolates showed growth. The broth culture was incubated at 37°C for 120hs. Growth of the isolates was observed by measuring turbidity (optical density). The variations in the growth rate of each isolate at different pH values was considered as a response to their pH optimum and ranges.

3.9.3.2. Determining salt tolerance

Salt tolerance of DC and DS isolates was compared by setting different molarities of NaCl concentrations in broth culture. The saline broth inoculated with 10% overnight grown log phase culture was incubated at 37°C for 120hs and growth in biomass was attended by measuring turbidity (optical density). The discrepancies in growth rate of each isolate to

different salt concentration were considered as their response to salt concentrations. Isolates from both the sediment and sludge were cultured using nutrient broth supplemented with sodium acetate (2g/l), sodium nitrate (2g/l) and different molar concentration of NaCl (Shapovalova *et al.*, 2008).

3.9.3.3. Determining Chromium tolerance

Chromium tolerance by DC and DS isolates was evaluated by supplementing the broth media with potassium dichromate ($K_2Cr_2O_4$) in a series of different concentrations according to the method described in Camargo *et al.* (2003). The minimum inhibitory concentration (MIC) and range of tolerance to chromium was determined by culturing the isolates at 37°C for 144hs. The ability of the isolates to grow in different concentration of chromium (0 to 3000mg/l) was tested at optimum pH. MIC was described by the absence of growth at elevated concentrations of chromate.

3.9.3.4. Nitrate reduction tests

The ability of denitrifying isolate to reduce nitrate/nitrite in to nitrogen gas through nitric oxide and nitrous oxide was considered as a means for differentiating isolates into functional groups. Based on this concept denitrifying isolates were characterized by their nitrate reducing ability using nitrate broths inoculated with pure isolates and incubated at 37°C for four days. Colorimetric biochemical nitrate reduction test was conducted using the reagents Sulphanilic acid (8g/L of 5N Acetic acid) (reagent A) and α -naphthylamine (5g/ L of 5N Acetic acid) (reagent B). The presence of nitrite was confirmed by the production of red precipitation up on addition of reagent A and reagent B drop by drop in their order. The nitrate and nitrite broth media was prepared using peptone (5g/L), meat extract (3g/L) $NaNO_3$ and $NaNO_2$ at pH 10.5 (Ramdhani, 2005).

3.9.3.5. Determining differences in carbon source utilization

Denitrifying isolates from each group were compared for their preferences to carbon sources. Their preference to utilize carbon source was evaluated using starch (2g/l), maltose (2g/l), acetate (2g/l), glucose (2g/l), glycerol (2ml/l) and ethanol (3ml/l). Each carbon source was added to the basal media after separately sterilized by autoclaving and dispensed in to sterilized

test tubes to which an overnight grown culture of the test isolates were aseptically inoculated and incubated at 37°C for 72h. Differences in the growth of each isolate on different carbon source were rated as differences to utilize the available carbon source.

In addition, characterization of denitrifying isolates by catalase test and starch hydrolysis test were done using standard procedures. Catalase test was conducted by using 3% hydrogen peroxide (H₂O₂). Isolates were labeled as catalase positive up on the formation of air bubbles when adding few drops of 3% (H₂O₂) on to young culture. Starch hydrolysis was confirmed by pouring potassium iodine solution on starch medium on which test isolates were streak grown for 48h at 30 °C. Formation of clear zone after addition of potassium iodine solution around the streak area showed starch hydrolyzing ability of the isolates by secreting enzyme maltase. Bacterial isolates from each sample were also assigned to cell wall groups on the basis of potassium hydroxide (KOH) string test (Duckworth *et al.*, 1996; Powers, 1995).

4. Result

4.1. Process performance of the treatment system

The data presented and analyzed in this study were obtained from continuous lab-scale tannery wastewater treatment reactors. Samples collected from the four sites (feed, anoxic, aerobic and sedimentation tanks) of the treatment system were analyzed for physicochemical and microbiological characteristics. Composite TWW collected from Modjo tannery was found to be alkaline having pH in the range of 7.8 to 11.5. Consecutive sampling of influent TWW to determine COD/TN ratio showed that it was in the range of 5.2 to 11.86. Maximum removal of COD, TN, NH₄⁺ and S²⁻ was obtained in the COD/TN ratio range of 10.78-11.86 (Table 2).

The influent TWW was found to contain very high amount of COD (1780-9200 mg/l), TN (90-432 mg/l), NH₃ (143-222 mg/l), S²⁻ (103-561 mg/l), SO₄²⁻ (392.3-858 mg/l) and Cr_{tot} (20.03-39.2 mg/l) (see appendix F). This indicates that there were higher COD, TN, NH₃, S²⁻, SO₄²⁻ and Cr_{tot} contents than the environmental discharge limit set for TWW. Amount of similar parameters measured from effluent of the treatment system showed minimum of 234 mg/l COD, 28.9 mg/l TN, 9 mg/l NH₃, 5 mg/l S²⁻, 14.65 mg/l NO₃⁻, 1.25 mg/l Cr_{tot}, and 1243 mg/l

SO₄²⁻. In the final effluent pH of the treated wastewater was also found to be between 9.71 and 9.75 (Table 2).

Despite the variability in the COD, TN, NH₃, SO₄²⁻ and S²⁻ contents of the influent TWW, the concentration of similar environmental parameters have been reduced in the treated effluent except SO₄²⁻ which rises up to its 2.5 fold (Table 2). Total alkalinity of the anoxic and aerobic conditions remained in the range of 250-280 meq/l and 160-190 meq/l respectively. Dissolved oxygen amounts 0ppm in anoxic reactor where as it was up to 3.94ppm in aerobic reactor.

Table 2. Summary of the characteristics of raw (influent) and treated (effluent) TWW

Parameters	Feed 1(OLR=1.92gCODL ⁻¹ d ⁻¹)			Feed 2 (OLR=1.64gCODL ⁻¹ d ⁻¹)		
	Influent	Effluent	% removal	Influent	Effluent	% removal
Color	Dark blue	No color		Dark blue	No color	
Odor	Foul smell	No odor		Foul smell	No odor	
pH	10.45	9.71		10.50	9.75	
COD	4659mg/l	544.42 mg/l	88.3	3416 mg/l	234 mg/l	93.15
NH ₃	143 mg/l	15.5 mg/l	89.2	222 mg/l	9 mg/l	95.9
TN	432 mg/l	28.9 mg/l	93.3	288mg/l	48.2 mg/l	83.3
NO ₃ ⁻	-	14.65 mg/l		-	21 mg/l	
SO ₄ ²⁻	392.3 mg/l	1243 mg/l		858 mg/l	1712 mg/l	
S ²⁻	561 mg/l	5 mg/l	99.1	192 mg/l	6 mg/l	96.9
Cr _{tot}	39.2mg/l	8mg/l	74	20.03mg/l	1.485mg/l	92.6
COD/TN ratio	10.78	-		11.86	-	

Note: pH was reported on average

4.1.1. Color and turbidity contrasts of raw and treated TWW

The color and turbidity of the influent TWW changed during the treatment process as it was demonstrated by the difference in the appearance of influent and effluent (Appendix D). Treated TWW became almost color less and clear in the effluent. Similarly, the notorious smell of the influent TWW that was caused by hydrogen sulfides faded in the final effluent. The change in the color of TWW after anoxic and aerobic stages of treatment indicate the color provided due to the presence of chromium and other dye stuffs was removed.

4.1.2. Nitrogen dynamics in the treatment processes

During this study the high ammonia and TN content of the influent TWW decrease to acceptable ranges in the effluent after biological treatment processes (Table 2). The difference in ammonia and TN content of influent TWW and treated effluent demonstrated for the occurrence of oxidation of NH_3 in to NO_3^- (Fig 3). This was exemplified in the reduction of ammonia from its highest state in the anoxic reactor (216 and 268mg/l) to its lowest in the effluent (15.5 and 9mg/l). Nitrate and TN also showed similar pattern of decrement in the effluent after the raw TWW was treated by the system.

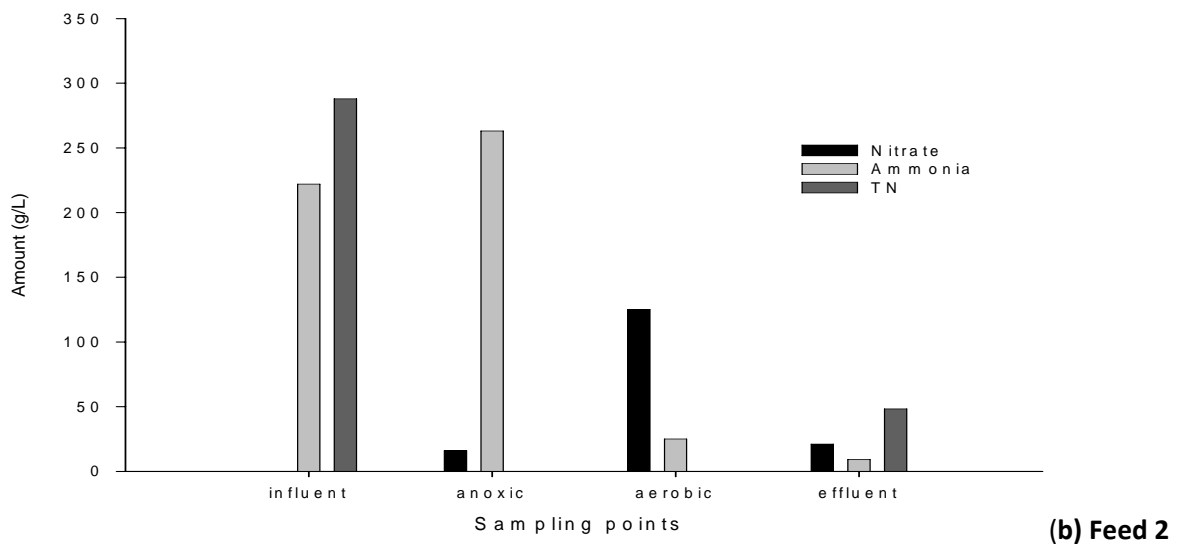
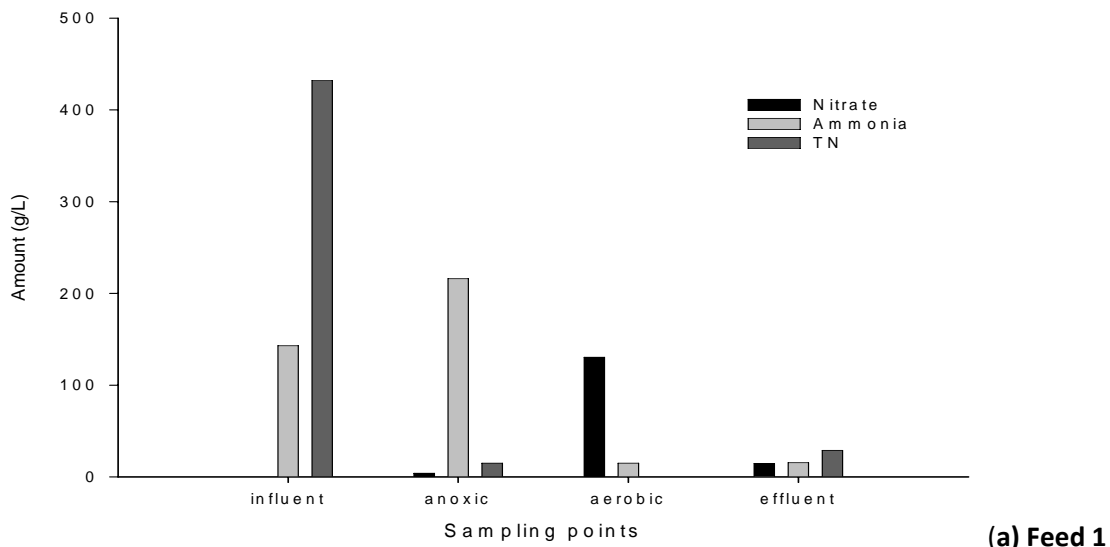


Figure 3. Nitrogen profile of the treatment system at two different feeds

4.1.3. Sulfide-sulfate dynamics of the treatment process

The results in sulfide and sulfate content of the treatment system showed that amount of sulfide under anoxic condition was greater than that of the influent by 4.6% during feed 1, but it was reduced by 15% at feed 2. The decrease in sulfide during feed 2 means there was sulfide oxidation in anoxic condition. On the other hand sulfate tremendously increased by 50 to 68% extraordinarily in the aerobic condition (data extrapolated from Table 2) than it was in the influent and anoxic conditions and was also high in the final effluent (Fig 4).

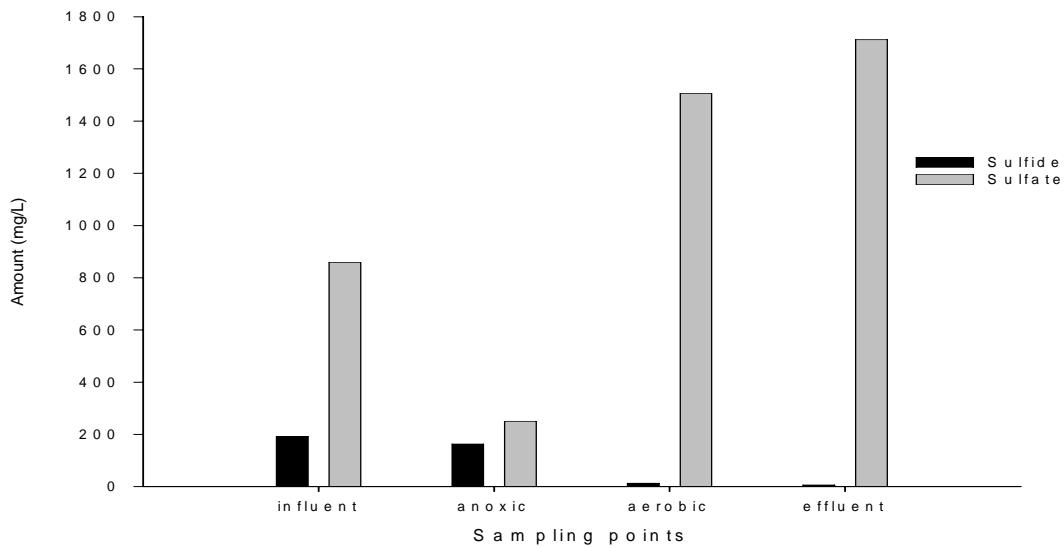


Figure 4. Sulfide and Sulfate dynamics at the last feed (feed 2)

4.1.4. Overall removal efficiency of the treatment system

The removal efficiency of the alkaliphilic activated sludge was relatively higher for S^{2-} , NO_3^- and TN during feed 1, where as NH_3 , COD and Cr_{tot} reduced by relatively higher percentages at feed 2 (Fig 5). The highest COD/TN ratio of the influent TWW at which maximum removal of COD achieved was 11.86 (Table 2). Assuming that the difference in the COD of influent TWW and treated effluent is the biodegradable fraction, a maximum of 93.15% of the COD was removed leaving only 6.85% of the initial.

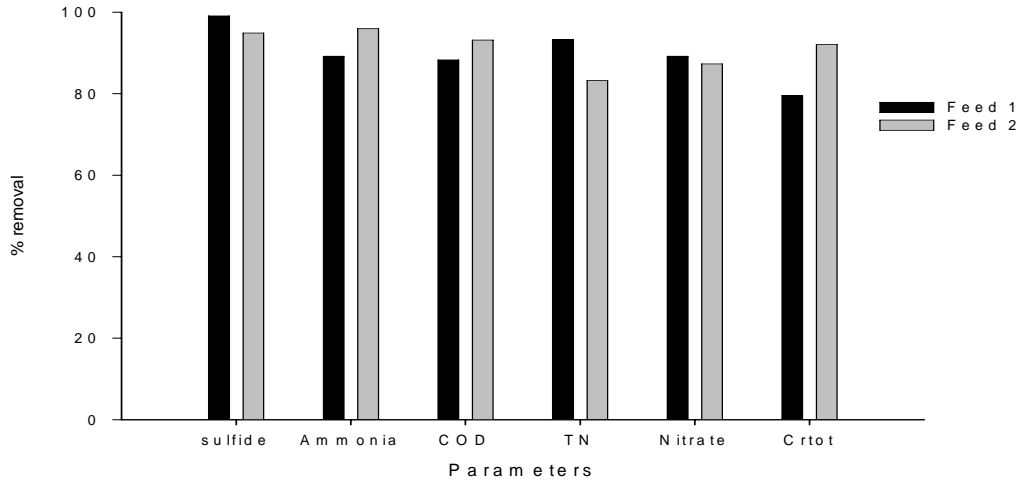


Figure 5. Removal efficiency of the treatment system for selected parameters

4.1.5. Nitrification and denitrification activities of the activated sludge

The rate of denitrification and nitrification carried out by the denitrifying and nitrifying alkaliphilic bacterial consortia was illustrated in batch reactors. There was complete uptake of nitrate in the batch reactor in less than 3h at a rate of $5.21 \text{ mgNO}_3 \text{ gVSS}^{-1}\text{h}^{-1}$ as shown in Fig 6. This showed that nitrate up take rate (NUR) was not limited by the important conditions for denitrification particularly by oxidizable organic matter of the TWW provided that other conditions (pH, agitation, and anoxic condition) were monitored throughout the batch test.

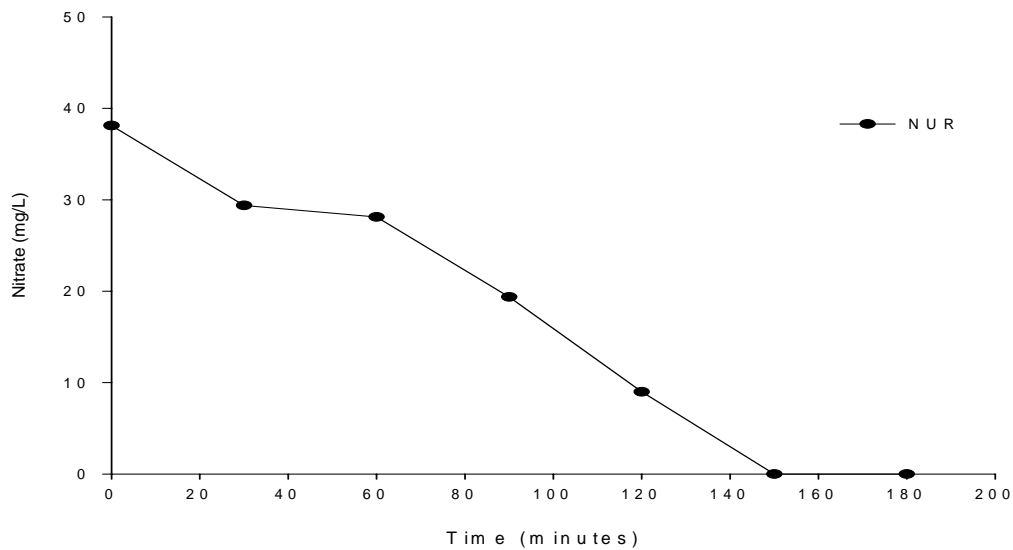


Figure 6. Nitrate uptake rate (NUR) by activated sludge

On the other hand, ammonium uptake rate (demonstrated in Fig 7) was $3.58 \text{ NH}_3 \text{ gVSS}^{-1}\text{h}^{-1}$ provided that pH and aeration conditions were regulated at operational values meant for the continuous feed treatment system.

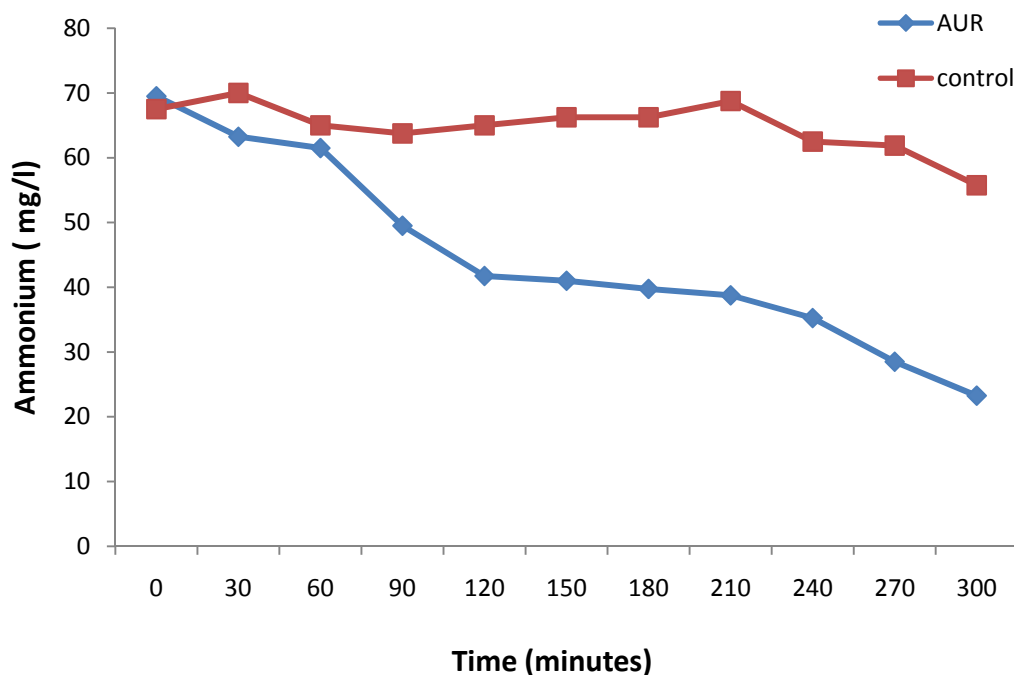


Figure 7. Ammonia uptake rate (AUR) by the activated sludge

4.1.7. Sludge characteristics

4.1.7.1. Flocculation and Bacterial abundance

Activated sludge sample collected from the treatment system at regular intervals since the beginning of the experiment was continuously observed by light microscope with magnifications of 400x and 1000x and the result showed that rod shaped bacteria were abundant throughout the treatment process. Diatoms, axe-shaped and linear protozoa-like organisms were also abundantly observed (Fig 8).

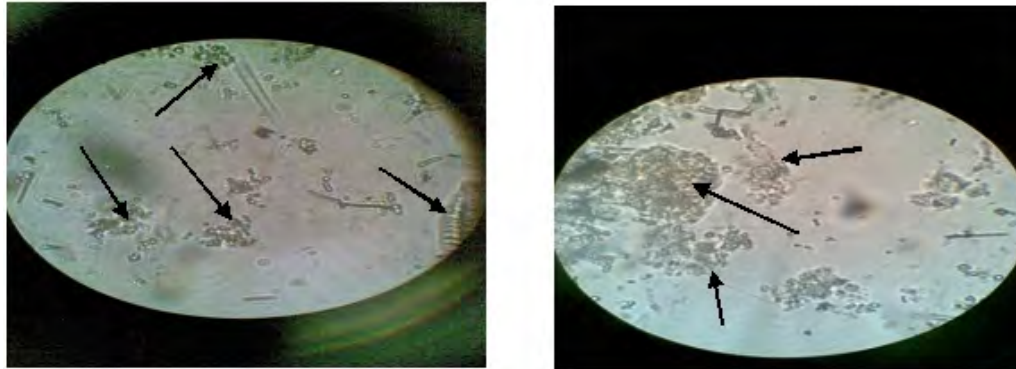


Figure 8. Flocculation at the early stage (left) and end of acclimatization (right)

Flocculation of the activated sludge was also followed starting from the date of actual operation. It has been noted that small aggregates began to appear in just 7 days of the treatment performance that gradually grown into relatively larger mass of flocs as depicted in Fig 8.

4.1.7.2. Sludge Settleability

The tendency of flocs in the mixed liquor to settle and concentrate in the clarifier was evaluated by its settleability. There was very high settling tendency by the concentrated flocs (Fig9). Activated sludge was also characterized by MLSS, MLVSS and SVI to accommodate the settling behavior of the flocs. Concentration of suspended solids of the aerobic reactor measured as MLSS and MLVSS for five samples on average was 2897.2mg/l and 2195.5mg/l. The SVI of mixed liquor was in the range of 32 to 61ml/g.

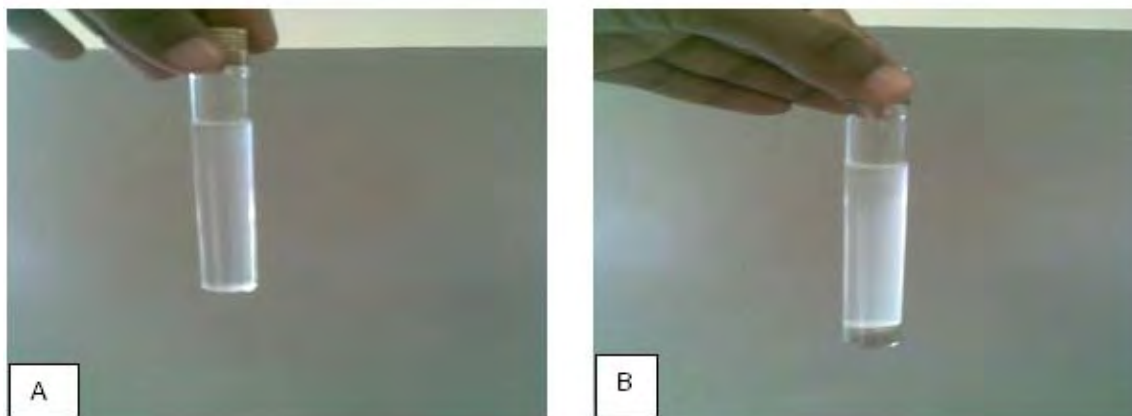


Figure 9. Settleability of the sludge: Treated effluent (A) and settled mixed liquor (B)

4.2. Microbiological characteristics of denitrifying bacterial isolates

In this study the anoxic-oxic treatment process was supposed to comprise different categories of bacteria mainly nitrifiers, sulfate reducers, sulfide oxidizers and denitrifiers. Nevertheless, this study paid special attention to denitrifying bacteria isolates named as denitrifying isolates cultivated from Chitu (DC) and denitrifying isolates cultivated from the treatment system (DS) to characterize them comparatively.

4.2.1. Isolation and verification of denitrifying bacterial isolates

A total of 290 DC and 420 DS colonies were picked under similar isolation conditions. After repeated subculturing and screening 42 isolates from the sediment sample and 58 isolates from the treatment system were taken for further characterizations. In this study the maximum cultivable bacterial isolates of lake sediment and activated sludge were 6.5×10^8 and 9.4×10^9 CFU/ml respectively showing that bacterial abundance obtained in the treatment system was higher in an order of 10 times magnitude than the lake sediment.

4.2.2. Morphological properties of isolated bacteria cells and colonies

Denitrifying bacterial isolates obtained from lake sediment and treatment system were compared by their colony size, shape, density, surface, elevation and margin from cultures grown on denitrifiers' enrichment media after 6 days of incubation. According to the colony phenotypes, DC and DS isolates showed similarities (Table 3). The colony morphology showed that most of the DC and DS colonies were 3-4mm in colony diameter, concentric shape, entire margin, convex in elevation and have smooth surface.

Table 3. Colony morphologies of DC and DS isolates according to Christopher *et al.* (2010)

Colony diameter	≤1mm	2mm	3mm	4mm	≥5mm
DC	10	5	32.5	30	22.5
DS	9	22	28	24	17
Colony shape	Spreading	Concentric	Irregular		
DC	7.5	75	12.5		
DS	3	83	11		
Margin	Entire	Undulate	Lobate	Filamentous	
DC	70	10	10	2.5	
DS	79	5	9	2	
Elevation	Effuse	Flat	Convex	Umbonate	
DC	5	10	82.5	2.5	
DS	2	21	72	3	
Density	Opaque	Translucent	Uncertain		
DC	45	47.5	7.5		
DS	79	21	0		
Surface	Smooth	Filamentous	Wrinkled	powdery	
DC	87.5	2.5	5	5	
DS	90	2	2	3	

Note: (1) All the numerical values are given in percentages (%)

4.2.3. The pH tolerance

The pH of composite tannery wastewater was found to vary between 7.8 and 11.5. However, an 80% of the tested denitrifying bacteria isolated from the treatment system showed maximum growth at pH 10.0 and 10.5, whereas 60% of DC isolates grow best at pH 10.0 (% data extrapolated from Table 4). The rest of tested DS and DC isolates showed maximum growth at pH 9.5 (Table 4). The range of pH at which all the isolates exhibit growth was from 7.0 to 11.5 for both DC and DS isolates.

Table 4. The pH optimum and range of DC and DS isolates

Isolates	Optimum	Range	Isolates	Optimum	Range
DS2-7	9.5	7.0 -11.5	DC17	10.0	7.0 – 11.0
DS2-9	10.0	7.0-10.5	DC18	10.0	7.0 – 11.5
DS2-24	10.5	7.0 -11.5	DC21	10.0	7.5 – 11.5
DS13	10.0	7.0 -10.5	DC22	9.5	7.5 – 11.0
DS16	10.5	7.0 - 11.0	DC24	9.5	8.0 – 11.5

4.2.4. Salt tolerance

With regard to their salt tolerance, DC and DS isolates showed different response in their optimum and range of growth with respect to different salt concentrations. Most of the DC isolates (60%) grew best at 0.5M NaCl and the rest (40%) of them were comfortable to 1.0M salt (% extrapolated from Table 5). However, 40% of DS isolates respond well to relatively high salt concentration (1.5M), but only 20% of DS were comfortable to 0.5M salt (Table 5). Average optimum salt concentration in which the isolates grow best was 1.1M for DS and 0.7M for DC isolates showing that DS isolates have superior salt tolerating ability than DC isolates.

Table 5. Salt optimum and range of some DC and DS isolates

DS-Isolates	Optimum	Range	DC-Isolates	Optimum	Range
DS2-7	1.0M	0 – 3.0M	DC17	1.0	0 – 2.0M
DS2-9	1.5M	0 – 3.5M	DC18	0.5M	0 – 2.0M
DS2-24	1.5M	0 – 3.0M	DC21	0.5M	0 – 1.5M
DS13	0.5M	0 – 2.5M	DC22	1.0	0–1.5M
DS16	1.0M	0 – 1.5M	DC24	0.5M	0 – 2.0M
Average	1.1M		Average	0.7M	

4.2.5. Chromium tolerance

On average the Minimum Inhibitory Concentration (MIC) of DC isolates for chromium was higher than those of DS isolates. Similarly, there was maximum growth by most tested DC and DS isolates at relatively higher concentration of chromate under the same growth condition (Fig.10). Most of the tested DC isolates grown well at the relatively high concentration of chromate (1500-2750mg/l) and the total chromium detected in the TWW (20-40mg/l) was far lower than the MIC. Tolerances to high concentration of chromate by DC isolate signify that the chromium found in the raw tannery wastewater was tolerable by the inoculated bacteria.

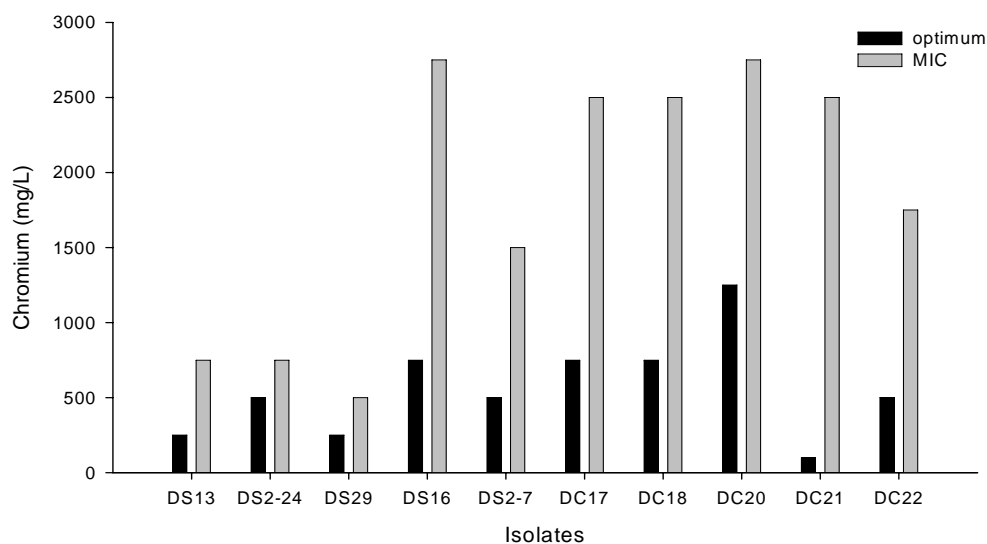


Figure 10. MIC of Chromium (VI) to DS and DC isolates in 72h cultures.

4.2.6. Nitrate reducing abilities of DC and DS isolates

Based on their ability of reducing nitrate, denitrifying isolates were found to be complete nitrate reducers, incomplete nitrate reducers, exclusively nitrite reducers and inconclusives (Table 6). Inconclusive refers to those isolates that showed ambiguous nitrate reducing characteristics. Incomplete denitrifiers were found to be predominant in both sediment (47.5%) and activated sludge (50%) isolates (Table 6). Most of the isolates were able to reduce nitrate to nitrite and it was confirmed by forming red precipitate (prontosil) in the colorimetric nitrate reduction test (Fig 12). There were no exclusively nitrite reducers and both nitrate and nitrite reducers among DC isolates.

Table 6. Nitrate/nitrite reduction by DC and DS isolates

Characteristic features	DC isolates	DS isolates
Complete (true) nitrate reduction	12.5%	15.5%
Incomplete nitrate reduction	47.5%	50%
Exclusively nitrite reduction	0	1.7%
Both nitrate and nitrite reduction	0	8.6%
Inconclusives	40%	24.2%

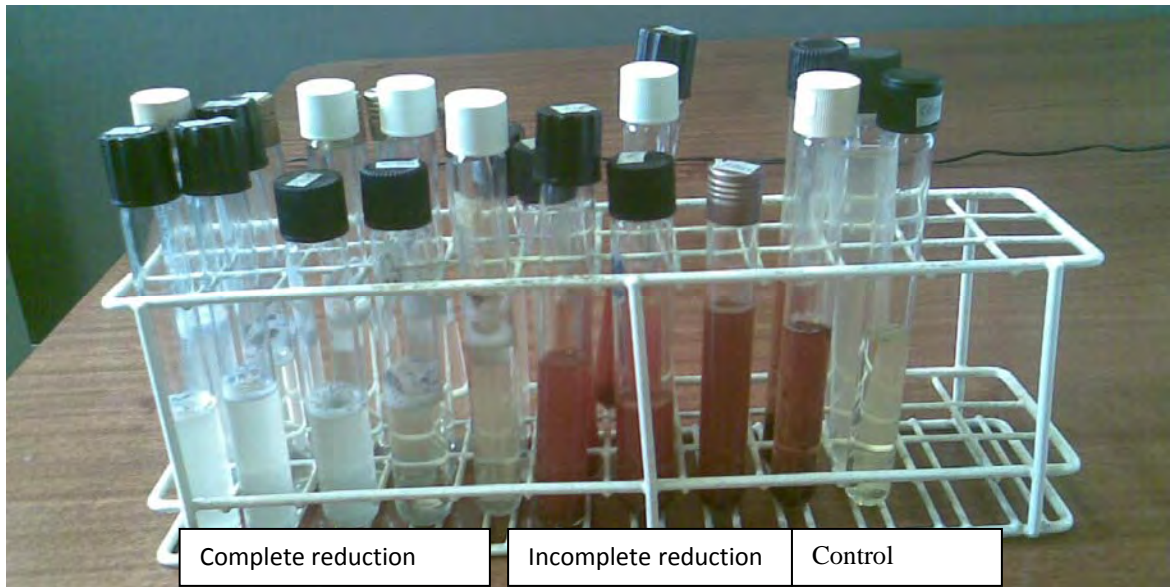


Figure 12. Differences in nitrate reduction capacity of denitrifying isolates.

On the other hand, result of biochemical tests showed that compared by their ability to hydrolyze starch, DC isolates show only 7% superiority to DS isolates. In terms of gram reaction, there was only 3% difference between DC and DS isolates. However, there was 14-17% difference in catalase reaction where most of the DC and DS isolates were catalase positive (Table 7).

Table 7. Some biochemical tests for DC and DS isolates

Isolates	Starch hydrolysis (amylase)		Gram test		Catalase test	
	+ve	-ve	+ve	-ve	+ve	-ve
DC	67.5%	32.5%	47.5%	52.5%	90%	10%
DS	60.34%	39.66%	44.8%	55.2%	72.4%	24.14%

With regard to cell shape and spore formation, all the tested isolates were rod-shaped and spore formers, but in their motility both DC and DS test isolates constitute fewer non-motile strains (Table 8). Most of the isolates were motile and there was no difference in their spore formation among the DC and DS isolates.

Table 8. Summary of few characteristic features of tested isolates

Isolates	Cell shape	Motility	Spore Forming	Catalase	Salt Optimum	Optimum growth in Cr medium	pH Optimum	Starch hydrolysis
DC17	Rod	Non-motile	+	+	1.0M	2.5mM	10.0	+
DC18	Rod	Motile	+	+	0.5M	3.4mM	10.0	+
DC21	Rod	Motile	+	+	0.5M	4.2mM	10.0	+
DC22	Rod	Motile	+	+	1.0M	3.4mM	9.5	+
DC24	Rod	Non-motile	+	+	0.5M	1.7mM	9.5	+
DS2-7	Rod	Non-motile	+	+	1.0M	1.7mM	9.5	-
DS2-9	Rod	Non-motile	+	+	1.5M	0.85mM	10.0	-
DS2-24	Rod	Motile	+	+	1.5M	0.85mM	10.5	+
DS13	Rod	Motile	+	+	0.5M	0.85mM	10.0	+
DS16	Rod	Motile	+	+	0.5M	2.5mM	10.5	+

4.2.7. Contrasts in carbon source utilization by denitrifying isolates

In carbon and energy source utilization, almost all DC and DS isolates were able to grow comfortably in maltose, acetate and glucose supplemented media, except isolate DC22 which showed little growth response to the presence of these three carbon and energy sources. Comparatively there was lower growth response by the isolates to ethanol, starch and glycerol than the above three, except isolate DC21 that showed good growth response to glycerol (Fig12).

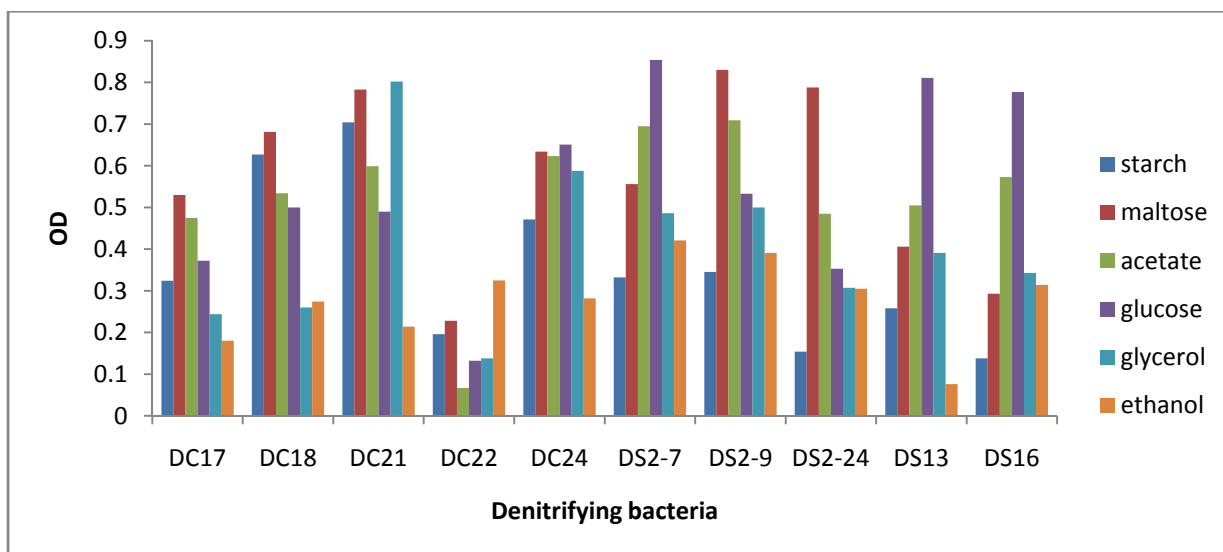


Figure 12. Carbon source preferences of DC and DS isolates reflected by maximum growth (48 hrs)

4. Discussion

This study was conducted to evaluate the treatability of TWW by alkaliphilic microbial consortia and to analyze the performances of the continuous feed treatment system. Biological treatment process in this study was targeted to remove organic matter and nutrients most notably nitrogen (TN and NH_3) and sulfide from TWW and to assess the impact of the feed wastewater to the inoculated bacterial community. The impact of TWW on the alkaliphilic denitrifying consortia was evaluated by comparative microbiological and biochemical analysis of denitrifying isolates cultivated from the inoculum and treatment system.

Wastewater collected from Modjo tannery was found to be variable in the concentrations of key parameters throughout the study. There was also dissimilarity in the composition range of pollutants given by different authors (Naumezyk and Rusiniak, 2005). Variability in the composition of influent wastewater might be associated to the chemical intensive temporal activities and by the differences in the amount of organic matter removed from hide and skin in each batch of tanning processes. Results of physicochemical analysis showed that influent TWW was up to 8 fold COD, 6 fold TN, 6 fold NH_4^+ , 376.5 fold S^{2-} , and 28.5 fold Cr_{tot} more than the provisional discharge limit set by EEPA (2003) (Table 2). However, the concentration of similar parameters in the final effluent reduced by 93.15% in COD, 93.3% in TN, 95.95% in NH_3 , 99.11 % in S^{2-} and 92.04% in Cr_{tot} after treatment in the last two feeds (Table 2).

In the literature, pH of tannery wastewater was reported to vary from 3.0 to 11.5 (Naumezyk and Rusiniak, 2005; Ryu *et al.*, 2007). However, in this study the pH of composite TWW was between 7.8 and 11.5 showing that its total alkalinity is greater than the total acidity. Alkaline condition of the tannery wastewater was supposed to be favored by the seed alkaline biomass. Since the pH of influent TWW was often lower than the natural environment for the inoculum, it was regularly monitored and adjusted between 10.2 and 10.5 by adding sodium carbonate (Na_2CO_3). Adjustments of the influent TWW by Na_2CO_3 have additional advantage for nitrifiers as it adds the merit of providing carbon dioxide (Carrera *et al.*, 2003), and used as a source of Na^+ that is usually required by alkaliphilic bacteria (Horikoshi, 1999). After treatment, pH of the TWW was reduced to narrow range (9.71- 9.76) in the final effluent. Being able to maintain the pH to narrow range could avoid detrimental effects caused by its fluctuation in receiving water bodies.

Organic content of the raw TWW are suggested to be mainly proteins resulted from the processed hide/skin and its hydrolysis. In this study maximum COD and NH_3 removal efficiency was 93.15% and 95.95% at feed 2 where as S^{2-} and TN were removed by 99.11% and 93.3% at feed 1 (Fig 5). This illustrated the elimination of TN and S^{2-} at relatively higher OLR and that of NH_4^+ and COD at lower OLR by the activated sludge. Removal of 93.15% means that the organic matter is used up by heterotrophic activated biomass using bound oxygen from nitrate/nitrite and the free oxygen in the anoxic and aerobic reactors in their order. By degrading organic matter heterotrophic bacteria obtain their energy demand in the anoxic and aerobic reactors. The treatment process was also able to remove total chromium by 92.59% in the effluent.

In a batch test done in this study, average nitrate uptake rate by the activated sludge was 5.21 $\text{mg NO}_3\text{gVSS}^{-1}\text{h}^{-1}$ using TWW as a sole energy source (Fig 6). This recapitulates the situation happened in the anoxic reactor where nitrate was exhaustively eliminated using organic matter from the mixed liquor as carbon and energy source. The high nitrate concentration recirculated from the aerobic reactor was decreased to 4.02mg/l (a removal by 97%) in the anoxic condition at feed 1(Fig 3). Reduction in the amount of COD and NO_3^- together demonstrated that dissimilatory nitrate reduction has been carried out by heterotrophic denitrifying bacteria. According to Ramdhani (2005), this dissimilatory nitrate reduction process was most likely to be denitrification. Nevertheless, NO_3^- in the final effluent was slightly higher (14.65mg/l) making the overall nitrate removal efficiency 89.29% (Fig 6).

On the other hand there was a rise in the concentration of nitrate in aerobic reactor after nitrate in the returned mixed liquor has been depleted in the preceding anoxic reactor. There was also significant decrease in the concentration of ammonia in the aerobic reactor than it was in the anoxic condition (Fig 3). This shows the occurrence of oxidative activities by nitrifying bacteria in the aerobic reactor. The difference in ammonia contents of influent and effluent TWW suggested that the alkaliphilic nitrifying bacterial consortia carried out ammonia oxidation under the operational conditions (Table 1). As to Carrera *et al.* (2003), it is achievable to decontaminate ammonium from TWW even at elevated amount (200-500mg/l) by biological nitrogen removal (BNR) process. Other reports on high nitrogen contents of industrial wastewater also showed that it is possible to remove as high as 5000mg/l NH_4^+ at

high rate by two sludge nitrification and denitrification (Szpyrkowicz and Kaul, 2004). However, the single sludge process used in this study presents fascinating alternative for ammonia elimination from TWW. Determination of ammonia uptake rate by batch test showed an uptake of ammonia by the nitrifying consortia at a rate of $3.58 \text{ mg NH}_4^+\text{-N gVSS}^{-1}\text{h}^{-1}$. Within 5h HRT nitrifying microbial community was able to take up 66.5% of the available ammonia (Fig 7).

The concentration of ammonia in the anoxic reactor was higher by 33.8% than it was in the influent. This increment in ammonia concentration is believed to result from respiratory ammonification by which some nitrite converted to ammonia or due to hydrolysis of organic nitrogenous compounds into amino acids and subsequent ammonification. According to Chen *et al.* (2007) and Gallert and Winter (2005), biological degradation of organic nitrogenous compounds produce ammonia under suitable conditions.

The nitrogen and COD removal efficiencies obtained in this study were comparable to different previous works (Table 9). A study by Goltara *et al.* (2003) to remove nitrogen and carbon from TWW by using membrane bioreactor showed 60-90% TN, 90% COD and 100% NH_4^+ removal efficiency. Cheng and Liu, (2001) achieved TN and NH_4^+ removal of 91% and 92% respectively by continuous-flow intermittent aeration process of nitrification-denitrification with 8.0 mg/l nitrate content of the final effluent. Carrera *et al.* (2003) also achieved 90-100% ammonia removal efficiency by two sludge suspension growth that is comparable to the efficiency of the treatment system in this study.

Study on saline tannery wastewater by Sivaprakasam *et al.* (2008) also reported 83% COD removal efficiency by using mixed salt tolerant bacterial consortia. Lefebvre *et al.* (2005) as well showed 95% COD removal efficiency from tannery liquor using SBR which is very close to the result of this study. The removal efficiency of the treatment plant and microbial biomass of this study also agreed to the work of Seyoum Leta (2004) whose report showed removal efficiency of 95-98% COD, 82-98% TN, 46-95% NH_4^+ and 95-99% S^{2-} . The biological nitrogen and COD removal obtained in this study was even superior to the chemical coagulation and ozone oxidation processes by which Naumezyk and Rusiniak (2005) achieved 62-64% removal efficiency for NH_4^+ and 85-88% COD reduction at alkaline condition.

Table 9. Removal efficiency of the treatment system compared to different previous works

Methods of treatment	Parameters	Maximum removal (%)	References
Continuous activated sludge	COD, TN, NH ₄ ⁺ , S ²⁻	93, 93, 96, 99	This study
Membrane bioreactor	COD, TN, NH ₄ ⁺	90, 60-90, 100	Goltara <i>et al.</i> , 2003
Intermittent continuous flow	TN, NH ₄ ⁺	90, 92	Chung and Liu, 2001
Two sludge suspension growth	NH ₄ ⁺	90-100	Carrera <i>et al.</i> , 2003
SBR	COD	95	Lefebvre <i>et al.</i> , 2005
Chemical coagulation	COD, NH ₄ ⁺	62-64, 85-88	Naumezyk, 2005
Continuous activated sludge	COD, TN, NH ₄ ⁺ , S ²⁻	98, 98, 95, 99	Seyoum Leta, 2004
Continuous activated sludge	COD, TN, NH ₄ ⁺ , S ²⁻	97, 95, 97, 92	Tesfaye Minota, 2006

The standard provisional discharge limit for COD, TN and NO₃⁻ for tannery wastewater in Ethiopia is 500mg/l, 60mg/l (80% removal) and 30 mg/l respectively (EEPA, 2003). From the perspectives of stringent effluent discharge limits, treatment of TWW by alkaliphilic consortia was found to be compliant and effective in reducing the concentration of important pollutants to the range of permitted discharge limits. It also indicates the adaptability of the inoculated sludge to the relatively harsh chemistry of TWW. This put forth the notion that inoculated alkaliphilic microbial consortia in the treatment system had successfully carried out its biological role of removing the target pollutants TN, ammonia and COD.

Another interesting aspect of the treatment was removal of color and odor from the influent TWW. There was significant difference in the color and turbidity of the raw and treated TWW. The raw TWW that was light to deep blue color became nearly colorless after it has been treated. Likewise, the notorious odor of the wastewater was almost eliminated in the final effluent. Removal of bluish color of the wastewater implied the elimination of chromate from the treated effluent as the blue color is thought to be attributed by chromium where as the absence of odor has to be a result of oxidation of sulfide in to sulfur or sulfate.

There are claims that nitrogen removal by biological process has problems due to self inhibition, sensitivity to pH and temperature for industrial application (Kabdasli *et al.*, 2003). However, in this study the possibility of treating TWW by alkaliphilic activated sludge using

anoxic-oxic process configurations works effectively under saline and alkaline conditions. In the presence of high concentration and variability of COD, TN, NH_4^+ and S^{2-} in the influent TWW, the treatment system performed at a steady state condition after its acclimatization. The ability of the system to remove high concentration of pollutants from TWW indicated the adaptability of the inoculated alkaline bacterial consortia to the treatment conditions.

As to the report by Szpyrkowicz and Kaul (2004), COD/TN mass ratio of 12.5 is sufficient to ensure function of the treatment process without limitation by low content of organic matter for nitrogen removal in the anoxic-oxic system. But in this study COD/TN ratio of 10.89 g CODgN^{-1} during feed 1 and 11.9g CODgN^{-1} in feed 2 was sufficient to attain appreciable nitrogen removal without limitation of organic matter. The MLSS and MLVSS determined from the mixed liquor also showed presence of high organic matter.

Sulfide and sulfate content of the influent TWW was found considerably high (Table 2). Amount of sulfide in the anoxic reactor decreased during the last feed (Feed 2) than in the influent TWW (Fig 4). Reduction in the amount of sulfide is thought to happen due to the presence of sulfide oxidizing nitrate reducing bacterial consortia. Sulfate also decrease in the same reactor. According to Genschow *et al.* (1997) and Gallert and winter (2005), the decrease in the amount of sulfate was suggested to be due to its reduction to sulfur by Sulfur Reducing Bacteria (SRB). The sulfate content of aerobic reactor increased by twofold more than the influent TWW with aggregate sulfide removal efficiency of 99.11% (Fig 5). The work of Mahmood *et al.* (2008) on the removal of sulfide in anoxic condition agreed to this suggestion. Accordingly, there was a steady state of 99% sulfide oxidation at pH 11.0 by sulfide oxidizing nitrate reducing consortia in anoxic conditions.

Analysis of tannery wastewater for Cr_{tot} content showed that it is in tenfold more than the toxicological discharge limit. But the Cr_{tot} detected in the raw TWW has been reduced by 92% in the final effluent after a series of anoxic and oxic conditions. The reduction in the amount of total chromium was speculated to be due to some physical and biological mechanisms such as precipitation to the compact sludge, bioreduction, biosorption and bioaccumulation. In the chromate tolerance test, denitrifying isolates respond their resistance to chromium by growing up to 2500mg/l. The minimum inhibitory concentration (MIC) of chromate for 10 selected denitrifying isolates was more than 63 times higher than it is in the raw TWW (Fig 10). Based

on the results of chromium tolerance of DC and DS isolates, it can be logically argued that amount of Cr_{tot} detected in the raw TWW was far less than the MIC of the test isolates. The ability of the isolates to grow at relatively high concentration of chromate indicates the capability of inoculum alkaliphilic microorganisms to operate chromium removal activities in chromium contaminated conditions.

Denitrifying capability of the sludge determined by batch test under anoxic condition showed that nitrate was exhaustively eliminated in just less than 3h HRT (Fig 6). Compared to the batch test, the continuously feed denitrification process required longer duration for removal of nitrate. This demonstrates the possible effects of diauxic lag during oxic-anoxic cycles which is the time required for re-synthesis of enzymes needed for nitrate reduction after certain period of exposure to oxic condition. Lee *et al.* (2009) depicted that in alternative aerobic-anoxic continuous treatment, bacteria required relatively longer period of anoxic condition and they retained the ability to re-synthesize denitrifying enzymes after an anoxic-oxic cycle.

Nitrate reducing ability of isolates obtained from the sediment and treatment plant indicated that 15% DC and 19% DS isolates were complete nitrate reducers, whereas 45 % DC and 50% DS isolates were able to reduce nitrate to nitrite with no further activities (Fig 12). This shows that majority of the isolates lack key enzymes to complete reduction of nitrate and incomplete nitrate reducers were most predominant in both the treatment system and natural sediment (Table 6). According to Drysdale *et al.* (1999), this reflects the fact that the treatment system has represented the natural ecosystem where most of the denitrifying organisms are capable of only incomplete nitrate reduction. Predominance of incomplete nitrate reducers suggested either these bacteria naturally lack enzymes for complete nitrate reduction or nitrite reductases of these isolates were affected by substrates or may the enzymes be alkaline pH sensitive. However, DS isolates were represented by isolates that can reduce nitrite and both nitrate and nitrite, a case not found in DC isolates. This indicates that the inoculated consortia undergo adaptation as a response to the treatment condition and cause a shift in denitrification function. In addition, although influent tannery wastewater is saline, it was converted into environmentally less harmful effluent by alkaliphilic biomass obtained from the soda lake. This showed that the inoculum was naturally able to tolerate high salinity or it has the capability of adapting saline conditions. Sivaprakasam *et al.*, (2008) asserted that sludge biomass adapted to

saline condition is required to degrade the organics present in saline and often toxic tannery effluent.

In literature, it has been reported that an elevated salt concentration could affect the COD removal and flocculation efficiency of the activated sludge (Kargi and Dincer, 1996). However, the performances of the treatment system in this study showed that COD removal is as high as 93.15% and the settleability of the sludge was excellent (Fig 9). Indeed, aggregate results on salt tolerance test of DC and DS isolates indicated that isolates were able to grow from 3% to 20% salt concentrations. This reinforced the reality that inoculated alkaline microorganisms have additional halophilic nature to survive TWW treatment conditions. It also gives practical response to the claims made to use alternative processes other than the biological methods to treat TWW with the main contention of its saline and toxic nature that would not be survived by the bacteria (Şengil *et al.*, 2009).

Growth properties of denitrifying isolates in different gradients of salt (NaCl) showed that they can survive at high salt concentrations. Maximum growth of the isolates was found in the range of 0.5M to 1.5M NaCl for DS isolate and 0.5M to 1.0M for DC isolates at pH 10.5. Range of growth for the DC and DS isolates was up to 2.0M and 3.5M respectively (Table 5). When aggregated, DS isolates show relatively wider range and maximum growth at relatively high concentration of salt than DC isolates. For isolates that grow best at elevated salt concentration, their growth was correlated to high rate of denitrification and COD elimination. The fact that denitrifying isolates tolerate salt concentrations up to 3.5M means it is six times more than the salinity of the inoculum source Lake where salinity was 35g/l or about 0.6M. Test on the influence of increasing salt concentration in nitrate reducing ability of isolates showed that at relatively high salt concentrations (>2M) there is a marked decline in nitrate reducing capability except few isolates (DS2-7, DS2-9 and DS13) which were able to reduce nitrate at 2.5M salt concentration. Indeed, batch tests for chromium tolerance by the selected isolates also showed that biological nitrate reduction can function in the presence of 500-2500mg/l chromate.

Although there is a considerable difference in the lower limit for bacteria to be called halophiles, according to Oren (2002), Le Borgne *et al.* (2008) and Zhuang *et al.* (2010) bacteria are to be called moderate and extreme halophiles if they are able to grow optimally at 0.5-2.5M and 2.5-5.5M salt respectively. Accordingly, both DC and DS isolates of this study are

moderately halophiles since they grow optimally in the range of 0.5M-2.5M salt concentration. However, as to the definition given by Bowers *et al.* (2009), some of the DS bacterial isolates may be labeled extreme halophiles since they grow optimally above 1.7M NaCl concentration.

The pH range and optimum for the growth of DC and DS isolates showed similar trend. All the experimental isolates exhibit maximum growth and complete reduction of nitrate at pH 9.5 to 10.5. At pH below 9.5 and above 10.5, there was limited growth by the isolates and nitrate was not exclusively utilized. This indicated that nitrate reducing activity by these isolates is strictly pH dependant and according to Le Borgne *et al.* (2008), both DC and DS isolates are to be called alkalophiles.

When compared, DC and DS isolates showed little difference in their optimum and range of tolerance to alkaline pH (Table 4). But DS isolates were better than DC isolates with regard to salt tolerance because DS isolates were able to reduce nitrate at salinity levels above 2.5M beyond which no single DC isolate was able to do so. The fact that DS isolates were able to survive high salinity condition than DC isolates could reflect the more saline characteristics of TWW than salinity of soda lakes that instigate the bacteria to develop adaptive mechanisms to saline conditions. Optimum growth of the isolates at salt concentration of 0.5 to 1.5M and pH of 9.5 to 10.5 suggested that the isolates have halophilic and alkalophilic nature according to the definition given by Ventosa, (1998) and Bowers *et al.* (2009). Theoretically, if the adaptive resources of organisms are utilized to deal with one type of environmental stress there will be less available resources to deal with other environmental stresses. However, the pH (up to 11.0) and salt tolerances (>2M NaCl) of the isolates in this study verified extraordinarily higher showing that the isolates have both alkaline and halophilic nature.

The maximum number of morphologically distinct hetrotrophic isolates obtained from lake sediment and treatment system was in the range of 10^8 and 10^9 CFU/ml respectively. This showed that compared to lake sediment, the mixed liquor contained more viable denitrifying bacteria in ten times order of magnitude. This may be due to the maintenance of suitable condition for the bacteria once acclimatized to the treatment condition.

Analysis of colony morphology showed that that denitrifying isolates obtained from lake sediment and mixed liquor showed most characteristics in common, except differences by few

orders of percentages in opacity, colony size, shape, margin and surface (Table 3). Isolates obtained from lake sediment and treatment plant also exhibit remarkable similarity in Gram reaction, enzyme catalase, starch hydrolysis and carbon source preferences. When compared, 47.7% DC and 44.8% DS isolates were gram positive and the respective 52.5% and 55.2% were gram negative (Table 7). Similar comparison in starch hydrolyzing ability showed that DC and DS isolates differ only in 7.16%. Thus the similarities in starch hydrolyzing ability, enzyme catalase and gram test by 92.84%, 70% and 97.3% respectively suggest that most of (>70%) the microorganisms in the inoculum have survived in the treatment condition and retained these features. Compared by their preference to different carbon sources, both DC and DS isolates showed relatively lower growth in starch, glycerol and ethanol supplemented media, but they were more favorite to acetate, glucose and maltose with no pronounced difference (Fig 12).

On the other hand, comparison on the basis of nitrate reducing ability showed difference between DC and DS isolates (Table 6). This was demonstrated by the absence of exclusively nitrite reducing and both nitrate and nitrite reducing DC isolates. In addition, DS isolates were able to grow at relatively high salt concentration than DC isolates. These dissimilarities in DC and DS isolates advocate for the occurrence of adaptation by the denitrifying bacteria that result shift in denitrification function and salt tolerance. The shift in nitrate reduction function and salt tolerance by the DC isolates is suggested to be associated with the saline nature of influent TWW and strict anoxic condition present in the treatment system.

6. Conclusion and recommendation

6.1. Conclusion

In this study biological removal of nitrogen, sulfide and organic matter from TWW by alkaliphilic bacterial consortia and microbiological study on denitrifying bacterial isolates was investigated. In the light of obtained results and related previous works, the following are major conclusions.

The biological treatment processes and performance of alkalophile biomass has resulted effluent properties that complied with the country's discharge limit for TWW. Reduction of the pollutants in to the range of acceptable discharge limits was claimed to be a result of concerted actions of inoculated alkaliphilic bacterial consortia under the harsh TWW environment. A removal of COD, TN, ammonia and sulfide from the feed wastewater connotes the establishment of stable populations of nitrifying, denitrifying, and other microorganisms in the reactors.

Microbiological analysis of denitrifying isolates obtained from the inoculum and treatment plant showed:

- High salt, pH, and chromium tolerance that reinforced the adaptive attributes of inoculated alkaliphilic consortia to TWW treatment conditions.
- Those isolates with the ability to tolerate high concentration of the toxic chromium could be important for detoxification of environments contaminated with chromium.
- There were differences in nitrate reducing function and in salt tolerance among DC and DS isolates.
- There was high similarity in gram reaction, enzyme catalase, starch hydrolysis, pH optima and preference to energy and carbon sources.

The exhibition of much of the characteristics in common by isolates obtained from inoculum and reactors illustrate that the inoculant alkaliphilic consortia have survived the treatment conditions. But, there was a difference in nitrate reduction function and salt tolerance by the DC and DS alkaliphilic denitrifying bacteria. The difference in denitrification function is thought to happen due to adaptation to environment in the reactors.

6.2. Recommendations

In addition to the current poor waste management capacity, industrialization could be an upcoming must to Ethiopia with the expansion of hide and skin processing and leather finishing factories. Tanning ultimately produces environmentally harmful wastes. However, this study showed that TWW is treatable by alkaline biomass obtained from naturally alkaline environments. Accordingly the following recommendations are forwarded.

- There is a possibility of successful application of alkaliphilic microbial consortia obtained from natural environment for efficient removal of pollutants from TWW. It is advisable to intensify similar researches having controls in parallel to the experimental one in order to come up with more specific conclusions about the potential of naturally alkaline organisms and with respect to the native consortia to treat TWW.
- The occurrence of viable abundant denitrifying isolates from alkaline environmental condition has to be further studied in search of more effective microbes to exploit their adaptive mechanisms which give insight to bioremediate environments polluted by industrial and domestic wastes.
- Microbial analysis shall be further supported by molecular elaborations for comprehensive description of microbial community dynamics.
- The treatment process of this study come up with significant reduction of NO_3 in anoxic condition. However, there is a limitation for further description of the fates of consecutive stages of denitrification with specific concern of releasing the green house gas N_2O . Further studies to describe the state of denitrification under anoxic conditions have to be very much encouraged.

Observations

- Installing and operating TWWT reactors by metallic equipments is a challenge due to the corrosive nature of the wastes.
- The temporal and seasonal variability in its organic load, saline, alkaline and toxic nature of tannery wastewater offers tough tasks of regular monitoring of the feed wastewater and extended optimization and acclimatization. Otherwise, there is a possibility to make shock load to the treatment system.
- The notorious smell and carcinogenic nature of the components of TWW made it terrible to carry on close manual operational monitoring and follow up.
- On a continuous basis of treatment there is a change in the pH and alkalinity across the reactors. Thus it was an absolute requirement to regulate the pH of the treatment operation on a daily basis.

7. References

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8. Appendices

A. Medium formulated for the cultivation of denitrifying bacterial isolates

Component compounds	Amount (g/l)	Trace elements	Amount (g/l)
K ₂ HPO ₄	0.1	EDTA	0.05
MgSO ₄ .7H ₂ O	0.1	ZnSO ₄ .7H ₂ O	0.02
CaCl ₂	0.15	MnCl ₂ .2H ₂ O	0.05
FeCl ₃ .6H ₂ O	0.02	MnCl ₂ .4H ₂ O	0.055
MnSO ₄ .H ₂ O	0.076	CaSO ₄ .5H ₂ O	0.015
Sodium acetate	2	CaSO ₄ .2H ₂ O	0.01142
Sodium nitrate	2	CoCl ₂ .6H ₂ O	0.02
Nutrient broth	5		
Nutrient agar	20		
Trace element	10ml		
pH	10.5		
Total salinity	13.5g/l		

B. Medium formulation for nitrate/ nitrite broth

Nitrate broth		Nitrite broth	
Component	Amount (g/l)	Component	Amount (g/l)
Peptone	5	Peptone	5
Meat extract	3	Meat extract	3
Sodium nitrate	1	Sodium nitrite	1
pH	10.5	pH	10.5

C. Colorimetric biochemical nitrate reduction test reagents

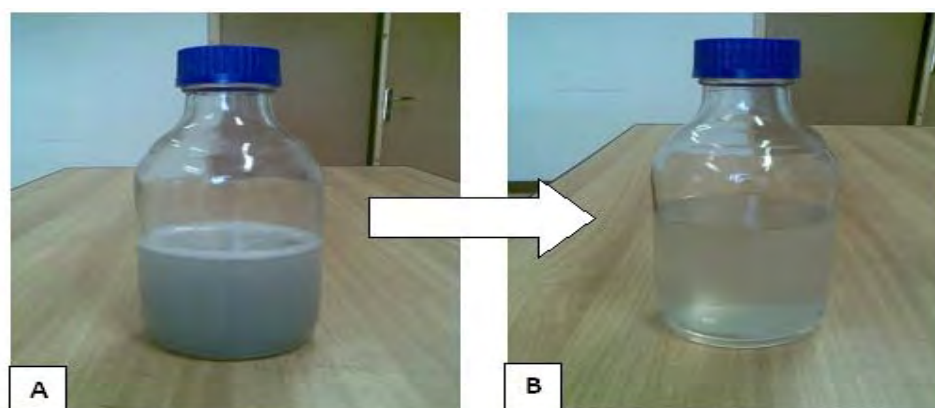
Reagent A		Reagent B	
Chemical	Amount	Chemical	Amount
Sulphanilic acid	8g	Alpha-naphthylamine	5g
Acetic acid (5N)	1L	Acetic acid (5N)	1L

Procedures for preparation of reagent A and reagent B

- ❖ The test organisms inoculated into nitrate/nitrite broth and incubated at 37°C for 3 days.
- ❖ After incubation, 5 drops of solution A was added followed by 5 drops of solution B to the cultures.

- ❖ Development of a red color indicates that the nitrate is only reduced to nitrite.
- ❖ If no red color develops then a small quantity of zinc powder should be added.
- ❖ Development of a red color after the addition of zinc indicates that no nitrate has been reduced at all. However, if there is no development of a red color, then this indicates that nitrate is reduced to nitrite, which is in turn reduced to either ammonia, nitrogen gas or a less oxidized form of nitrogen.

D. Color and turbidity contrast of tannery wastewater before (A) and after treatment (B)



E. MIC of Chromium for DC and DS isolates

Isolates	Maximum growth	Range	MIC
DS13	250mg/l	0- 500 mg/l	750mg/l
DS2-24	500mg/l	0-500 mg/l	750mg/l
DS2-9	250mg/l	0-750mg/l	500mg/l
DS16	750mg/l	0-2500mg/l	2750mg/l
DS2-7	500mg/l	0-1250mg/l	1500mg/l
DC17	750mg/l	0-2250mg/l	2500mg/l
DC18	750mg/l	0-2250 mg/l	2500mg/l
DC20	1250mg/l	0-2500mg/l	2750mg/l
DC21	1000mg/l	0-2250mg/l	2500mg/l
DC22	500mg/l	0-1500mg/l	1750mg/l

F. Physicochemical determination of the five samples of different times

Influent	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Average
COD	9200	1780	4917	4659	3416	
TN	144	198.6	90	432	288	
NH ₄	146.4	169	174.2	143	222	
S ²⁻	119	103	254	561	192	
SO ₄ ²⁻	399	416	408	392.3	858	
pH	8.91	8.44	10.30	9.46	8.71	
Cr _{tot}	27.45	29.2	31	39.2	20.03	
Anoxic	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Average
NH ₄	139	172.2	173.8	216.12	263	
S ²⁻	168	112.3	262	588.32	163	
NO ₃	88.4	41	38.9	4.02	16	
SO ₄ ²⁻	387.7	464	379.9	360	250	
MLVSS(mg/l)	1906	2002	2110	2284	1984	2057.2
MLSS(mg/l)	2611	2814	2828	2901	2666	2764
pH	10.24	10.30	10.21	10.36	10.41	
Aerobic	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Average
NH ₄	76.8	54.7	37.2	15.10	25	
S ²⁻	77.24	46	30.98	69.74	13	
NO ₃	90.47	105.6	113.2	130.4	125	
SO ₄ ²⁻	389.91	524	890.86	1205	1506	
MLVSS(mg/l)	1892	2022	2134	2510	2418	2195.5
MLSS(mg/l)	2552	2776	2789	3262	3107	2897.2
pH	9.98	9.99	9.97	9.98	10.02	
Effluent	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Average
COD	4736	566	712.89	544.42	234	
TN	89.9	65.45	43.83	28.9	48.2	
NH ₄	77.23	49.9	22.9	15.5	9	
S ²⁻	72.76	36.6	8	5	6	
NO ₃	61.65	47.4	17	14.65	21	
SO ₄ ²⁻	378.6	517	883.8	1243	1712	
pH	9.75	9.73	9.75	9.72	9.71	
Cr _{tot}	12.21	14.33	14.99	8	1.485	

Declaration

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

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