ADDIS ABABA UNIVERSITY

SCHOOL OF GRADUATE STUDIES

ISOLATION AND CHARACTERIZATION OF ALKALOPHILIC CYANIDE- DEGRADING BACTERIA FROM ETHIOPIAN ALKALOPHILIC SODA LAKES (LAKE CHITU)

BY:

WAJANA LAKO LABISSO

A Thesis Submitted to the School of Graduate studies of the Addis Ababa University in partial fulfillment of the Degree of Master of Science in Biology

(Applied Microbiology)

July, 2007

Acknowledgments

I am very grateful to my adviser Dr. Amare Gessesse for introducing me to the fascinating fields of applied Microbiology. His motivation, inspiration, and optimistic-scientific approaches, without which I would not have come this far, were highly appreciable.

My sincere gratitude also goes to my co-advisor Dr. Seyoum Leta for his encouragement and peculiar sense of humor. He was always willing to help me whenever I was in need of his support and sharing his knowledge with me in very stimulating discussions.

My deepest gratitude is also extended to Fr. Tadewos Gizaw (Franciscan Capuchin Friary priest in Catholic Church) for his motivation and moral & financial support at very beginning of this program. He was really the pace- maker of my M. Sc. study. Fr. Tadewos, Whatever is said and whatever is done Never I forget you**!!**

In addition, special thanks to Frs. Fekadu Fako, Dejene Hidoto for taking care of me and being there for me at all times.

I owe special thanks to w/o Tigist Mengesha and Hirut ---- ,Lab. Assistants in Applied Microbiology lab for their invaluable consistent help throughout the research.

Many thanks to friends and colleagues in applied microbiology Lab., especially Ato Dereje Teshome & Tadesse Chane for their technical help and moral encouragements during lab times.

I want to thank all my families in wolayta soddo and my friend, Tsion Orlando (in Nazareth) for giving me all love, comforts, encouragements and for having the patience throughout the years.

Finally, Greatest thanks to my omnipotent & omniscient God for His boundless love and Care in my life!!

Declaration

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

Name: Wajana Lako Labisso

Place: Addis Ababa University

Signature…….

This thesis has been submitted for examination with our approval as advisors:

Amare Gessesse, Ph.D Signature…… Date……

Seyoum Leta, Ph.D Signature……. Date……

Acknowledgments

I am very grateful to my adviser Dr. Amare Gessesse for introducing me to the fascinating fields of applied Microbiology. His motivation, inspiration, commitment and optimistic- scientific approaches, without which I would not have come this far, were highly appreciable. My sincere and warmest gratitude also goes to my co-advisor Dr. Seyoum Leta for his encouragement and peculiar sense of humor throughout the study. He was always willing to help me whenever I was in need of his support and sharing his knowledge with me in very stimulating discussions.

I would like to greatly acknowledge Biology Department, Addis Ababa University, for letting me exploit all the available resources needed throughout my study.

My deepest gratitude is also extended to Fr. Tadewos Gizaw (Franciscan Capuchin Friary priest in Catholic Church) for his motivation and moral and financial support at very beginning of this study. He was really the pace- maker of my M. Sc. study. Fr. Tadewos, whatever is said and whatever is done Never I forget you**!!** In addition, special thanks to Frs. Fekadu Fako, Dejene Hidoto and Mengesha Toma for taking care of me and being there for me at all times.

I owe special thanks to w/o Tigist Mengesha and Hirut Teshome, lab assistants in Applied Microbiology lab for their invaluable technical help throughout the research. My deepest gratitude also goes to Ato Dawit W/Meskel for his unreserved technical assistance during sonication of bacterial cells. Many thanks to friends and colleagues in applied microbiology Lab., especially, Dereje Teshome, Tadesse Chane and others for their technical help and moral encouragements during lab times.

I want to thank all my families (in Wolayta soddo) and my friend, Tsion Orlando (in Nazareth) for giving me all love, comforts, encouragements and for having the patience throughout the years.

Finally, Greatest thanks to my omnipotent & omniscient God for His boundless Love and Care in my life!!

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Abstract

Currently, alkalophillic microbial cyanide degradation has gained greatest attention due to its vital advantages over the acidophilic and /or neutral and conventional cyanide removal methods from industrial wastes. The objectives of this study were to determine how readily alkalophillic cyanotrophic microorganisms could be isolated and screened from enrichment cultures supplied with cyanide as sole source of nitrogen. A total of 63 potential microorganisms were isolated from Lake Chitu mud sediments. Two relatively more active isolates (designated as AUCN-54 & AUCN-60) in resisting and utilizing cyanide were selected for morphological and physiological characterizations. Biomass (dry weight) and protein measurements were the bases for selection. The optimum cyanide concentrations (for maximum growth yield- dry weight and protein) were found to be 10.2mM and 17.34mM for AUCN-54 and AUCN-60, respectively. Thus, AUCN-60 was found to be better in utilizing and resisting cyanide. Both isolates were capable of managing to grow and utilize sodium cyanide a t a salt concentration of 0.25-10 % (w/v), which was consistent with the salinity of the sample site (microbial original habitat). Concerning the pH, temperature and antibiotics resistance study, clear variations were observed between the two isolates. Interestingly, both of the bacterial isolates were good in utilizing sugarcane molasses as carbon source during cyanide degradation, indicating the need for further study in large scale applications. In addition, both of the cyanotrophic isolates were capable of utilizing potassium thiocyanate as a sole source of nitrogen. On contrary, they were poor in utilizing $K_2CU(CN)_4$ as a sole source of nitrogen. This may be attributed to the stability of the cyanide complex due to strong attraction of CN^- group to the central atom in the ligand.The bacterial isolates screened and characterized in this particular study and in their original habitat (sample site) could be of great importance due to their good cyanide utilization capacity and high salt and pH tolerance, which are commonly encountered influential factors in cyanide contaminated waste treatments.

Key words: Alkalophiles, Cyanide, Cyanotrophic, Cyanide-degrading bacteria

Abbreviations and Acronyms

APHA	American Public Health Association
CA	carbonic anhydrase
CN	cyanide
CN^-	Cyanate
CNCl	Cyanogen chloride
CNN	cyanide hydratase
CNO	cyanide oxygenase
HCN	Hydrocyanic acid
LD50	Lethal Dose, 50%
mg	milli gram
ml	milli liter
NADH-	Nicotinamide adenine dinucleotide
OCN	cyanate
pH	potential hydrogenation
pKa	Acid dissociation constant
ppm	parts per million
WAD	weak acid dissociable
WHO	World Health Organization

1. Introduction

Environmental pollutions caused by industrial and domestic activities are the main threats to the surface and ground water qualities (Fernandez and Kunz, 2005). Majorities of industries in developing countries discharge their wastewater into nearby water bodies and open land without any treatment. Then, downstream inhabitants use such polluted streams and rivers for various purposes such as horticulture, drinking, washing and other domestic activities. Ground water from boreholes or spring is also the source of drinking water for the dwellers. These could lead to serious public health and ecological disasters (Nui and Volesky, 2000; Yamasaki *et al.*, 2002).

The hazardous materials released from industrial processes depend on the type of industries involved. Some of industries release recalcitrant, heavy metal-containing, toxic, and carcinogenic wastes. Particularly, mining operations and their waste disposal methods are considered as one of the main sources of environmental degradations (Logsdon *et al*., 1999). This is, because they use toxic chemicals for facilitating the processes of ore extraction. For example, the main important toxic extractant involved in gold and silver mining industries is cyanide. In addition to cyanide compounds, tailings from gold mining operations contain high levels of heavy metals such as arsenic, cadmium, mercury and lead (Avalos *et al.*, 1990). All of these become part of the discharge. Leaks or spills of this waste into the environment without treatment is extremely toxic to fish, plant life and human beings. A teaspoon full of 2% solution of cyanide is a lethal dose for a human. The most common environmental problems are likely to result from the chronic contamination of surface and ground waters by lower concentrations of cyanide and related breakdown compounds (Wong *et al.*, 2000)

In addition, introduction of mining activities affects a lot of people in the process areas. Waste disposals from process plants and sediment runoffs from open cut mines are dumped into rivers and oceans. Smothering of riverbeds and ocean floors, heavy metal contamination and acid mine drainages are consequences of mine waste disposal into the environment (Zlosnik and Williams, 2005).

1.1. Physical and Chemical properties of Cyanide

Cyanide refers to numerous compounds, both natural and man-made, having the chemical group CN. It is a colorless solid with a slight odor of bitter almonds. Cyanide combines with up to 97 percent of gold, including particles of gold that are too small to be seen with naked eyes. This makes it one of the most efficient process chemicals for the extraction of metals (May and Alexander, 2005; Mineral council of Australia, 2005). It also removes other precious metals other than gold and base metals from ore. Zinc and copper are relatively weakly complexed by cyanide while gold, silver, iron and cobalt are strongly complexed (Avalos *et al.* and 1990; Hubb *et al.,* 2000).

The cyanide used in processing the ore is sodium cyanide (NaCN). This is white solid that dissolves readily in water, yielding sodium ion (Na+) and cyanide ion (CN^-). Some of the later then converted into hydrogen cyanide or hydrocyanic acid (HCN). The cyanide ion and hydrogen cyanide are often collectively called *free cyanides.* The relative amount of free cyanide present in certain environment is largely controlled by the pH of the environment with the percent of HCN rising as the pH falls. At pH seven, about 99.5 percent of the cyanide exists as HCN. At lower pH levels than this, essentially all dissolved cyanide is present as HCN. In natural waters most free cyanide present exists as HCN since the natural environmental pH range is in between 6 and 8.5 (Rychte *et al.,* 1979; May and Alexander, 2005).

Aqueous solutions of cyanide are not thermodynamically stable. However, in the absence of catalysts and at high pH, oxidation to cyanate is very slow. Thus, the cyanide solution should be kept at a pH well above the pKa of HCN (about 9.2) to prevent out gassing of HCN (McKinnon, 2002). Otherwise, hydrocyanic acid (HCN) will evaporate and pollute the atmospheric air, which could be inhaled with its pollutant and result in serious problems in living systems. Therefore, lime or sodium hydroxide is used to control the pH limit in cyanide containing environments (Andrews, 1999; Sorokin *et al.,* 2001).

Cyanide cannot only be lost through evaporation as HCN gas, but also through hydrolysis (oxidative pathway that breaks the carbon –nitrogen bond), conversion into thiocyanate, precipitation of cyanometallic compounds (notably with sulfide ores) and adsorption (coprecipitation) with ferric oxides and others (Avalos *et al.,* 1990, and Oudjehani *et al.,* 2002).

1. 2. Classification of cyanide and its compounds

The chemistry of cyanide is very complex and there are usually many different forms of cyanide in mining solutions. Toxicologically, these forms can be subdivided into four different groups as indicated in Table 1 below.

Table 1. Toxicological classification of cyanide species (Modified from Chen and Kunz, 1997; Brehaut, 2000; Akcil, 2003).

Cyanide species	**Toxicological Features**
Free cyanides (CN^-, HCN)	Absorbed by ingestion or inhalation. Causes cytotoxic hypoxia by inhibition of cytochrome oxidase, which disables the cells' oxygen utilization in the electron transport chain. At sufficient concentration this causes a lethal suppression of the central nervous system
WAD (weak acid dissociable**)** cyanides $Zn(CN)_4^{2-}$, $Ni(CN)_4^{2-}$, $Cu(CN)_3^{2-}$	These are less toxic than the free cyanide**,** but they generally persist longer in the environment. Chronic exposure can lead to accumulation of metals in the body, causing decreased growth, reduced reproduction and other physiological abnormalities.
Iron cyanides $Fe(CN)_6^{3-}$,$Fe(CN)_6^{4-}$	These shows the same kind of toxicity as the WAD cyanides, but should be considered more hazardous from an ecotoxicological perspective because of their high stability
Cyanide related compounds SCN^-, CNO^-	Thiocyanate and cyanate toxicity tests on fish show an irregular pattern. In some cases all fish died almost instantaneously, whereas others survived until they returned to clean water, but died within a week. Cyanate was shown to be somewhat more toxic to fish than thiocyanate

1.3. Industrial uses of cyanide

Cyanide is a widely used and valuable industrial chemical. Cyanide and cyanide containing compounds are used in pesticides and fumigants, plastics, electroplating, photographic application and mining industries. According to World Health Organization (WHO), over one million tones of CN (about 80% of the annual product of CN compounds) is used in the chemical industry for production of organic chemicals such as nylon and acrylics, in electroplating, metal processing, and photographic applications. Similarly, mining industry in the USA alone utilized about 70 million kilo grams of cyanide in 1989 alone. The current amount is expected to be much higher than this value (American Public Health Association (APHA), 1998; Whitlock and Mudder, 1998; McKinnon, 2002). Generally, the industrial application of cyanide and its compounds are summarized in Table1.

Table 2. Cyanide and its compounds with their industrial applications (Modified from Moran, 1998; May and Alexander, 2005)

Types of Cyanide compounds	Their corresponding industrial Applications
HCN	Primarily used in production of nylon, ryon, polyvinylchloride, modacrylic, polyester wadding, neoprene foam, rubber plastics, insulation and adhesive resins.
NaCN	The most prevalent use is in mineral processing industries. It is also employed in electroplating and case hardening of metals, extraction of gold and silver from ores, base metal floatation coal gasification, and the fumigation of ships.
KCN	Used for electrolytic refining of platinum, for metal coloring and as an electrolyte for the separation of gold, silver and copper from platinum.
Cyanide salts	Used as chelating agents, and the complex cyanides of copper, zinc and cadmium are used in electroplating processes, principally the plating of iron, steel, and zinc.

In mining industries in general and gold mining industries in particular, cyanide is used as a flotation reagent (Fg.1) (Avalos *et al.,* 1990). It is also used to separate gold –rich pyrite from arsenopyrite and to extract gold and silver from ores.

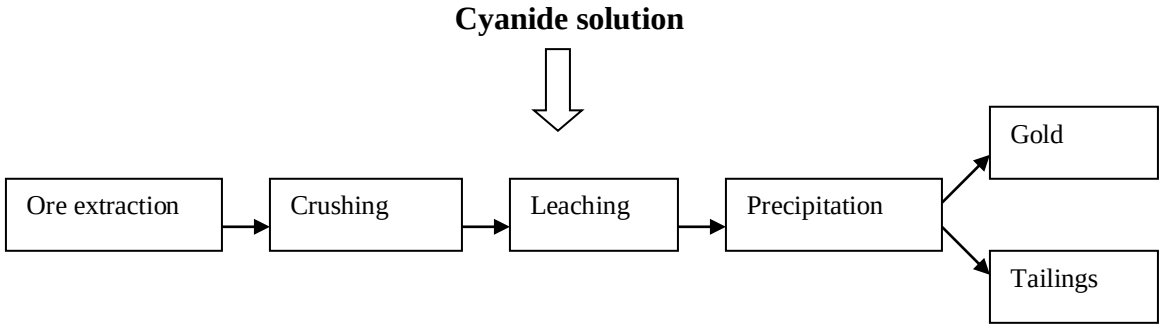


Figure1. The cyanide leaching method (adapted from Brinne, 2004)

The process in which gold and silver are extracted from their corresponding ores by the application of cyanide is called *cyanidation.* This is still the method of choice for most of the world's gold mines (Logsdon *et al.,* 1999; Moran, 1999; McKinnon, 2002).

1.4. Environmental impacts of cyanide

There have been several reports worldwide (Logsdon *et al.,* 1999; Brehaut, 2000; Almagro *et al.,* 2005) on environmental disasters associated with cyanide leakage from tailing dams. The most recent incident involves the leakage in February 2000 from Aurul mine in Baja Mare, Romania. The tailings flowed down the river killing fish along the way and forcing the communities nearby the river to find alternative sources of drinking water.

Cyanide is toxic to many living organisms at very low concentrations. Fish and aquatic invertebrates are particularly sensitive to cyanide exposure (U.S. Environmental Protection Agency, 1999). Concentrations of free cyanide in the aquatic environment ranging from 5.0 to 7.2 micro grams per liter reduce swimming performances and inhibit reproduction in many species of fish. Other adverse effects include delayed mortality, susceptibility to predation, disrupted respiration, osmoregulatory disturbances and altered growth patterns (McKinnon, 2002; May and Alexander, 2005).

A concentration of 20 to 75 micro grams per liter free cyanide causes the death of many species of fish. In addition, concentrations of in excess of 200 micro grams per liter are rapidly

toxic to most species of fish. Invertebrates experience no adverse lethal effects at 18 to 43 micro grams per liter of free cyanide but lethal effects at 43 to 100 micro grams per liter (Kenneth *et al.*, 1981; Brehaut, 2000)

Algae and macrophytes can tolerate much higher environmental concentrations of free cyanide than fish and invertebrates. Aquatic plants are unaffected by cyanide at concentrations that are lethal to most species of fresh water and marine fish and invertebrates (Burguillo and Nicolas, 1977; Kenneth *et al.*, 1981; Almond, 2000; Akcil, 2002b; Gurbuz *et al.*, 2004).

As different authors indicated (Moran, 1999; Akcil *et al.*, 2002; McKinnon, 2002) oral LD50 for birds is 0.8 milligrams per kilogram of body weight. Seriously poisoned birds may display symptoms, as panting, eye blinking, salivation and lethargy appear within one-half to five minutes after ingestion in more sensitive species. Exposure to higher doses resulted in deep, labored breathing followed by gasping and shallow intermittent breathing in all species. Birds may drink water containing WAD cyanide that is not immediately fatal. Gradually, this may be converted in to more toxic forms of cyanide (free cyanides) in acidic conditions of the stomach.

Mineral council of Australia (2005) indicated that the most common environmental problems are likely to result from the chronic contamination of surface and ground waters by lower concentration of cyanides and related breakdown compounds. Such releases are much more difficult to detect and evaluate than are acute. In the later cases, high concentration spills are often associated with rapid, observable deaths of aquatic organisms (Logsdon *et al.*, 1999 and Akcil,. 2001).

1.5. Public Health impacts of Cyanide

Cyanide is the most important genocidal and homicidal agent (Gurbuz *et al.*, 2004) that could be produced in human body and exhaled extremely in low concentrations with each breath. Relatively low concentrations of it could be highly toxic to people and wild life. Liquid or gaseous HCN and alkaline salts of cyanide can enter the body through inhalation, ingestion or adsorption through the eyes and skin. The rate of skin absorption could be enhanced when the skin is abraded or moist. Inhaled salts of cyanide are readily dissolved and absorbed upon contact with moist mucous membranes (Kitis *et al.*, 2005 and Lonesiy, 2006).

The toxicity of HCN to human is dependent on the nature of exposure. In addition, there is variability in dose – response effect between individuals. The LD50 for ingestion is 50 to 200 milligrams per kilogram of body weight. The LD50 for contact with abraded skin is 100 mili grams per kilogram of body weight (AKcil, 2006).

Although the time, dose and manner of exposure may differ, the biochemical action of cyanide is the same upon entering the body. Once in the bloodstream, cyanide forms stable complex with cytochromes c oxidase and other metal-enzymes that promote the transfer of electrons in the mitochondrion of cells during the synthesis of ATP. Without a properly functional cytochromes c oxidase, cells cannot utilize the oxygen present in the bloodstream. This results in cytotoxic hypoxia or cellular asphyxiation. The lack of available oxygen causes a shift from aerobic to anaerobic metabolism. This leads to the accumulation of lactic acid in the blood. The combined effects of hypoxia and lactic acidosis lead to depression of the central nervous system that can result in respiratory arrest death (McKinnon, 2002). At higher lethal concentrations, cyanide also affects other organs and systems in the body, including the heart. However there is no clear evidence that shows the presence of teratogenic, mutagenic or carcinogenic effects of chronic cyanide exposures (Kariman and Burkhart (1986).

According to the U.S.A. Environmental Protection Agency (1999) the primary source of cyanide in the air is from car exhaust. Other air borne sources include emissions from chemical processing industries and municipal waste incinerators. Similarly, Moran (1999) indicated that smoking cigarette could be another source of cyanide poisoning. Cyanide may be present in waters discharged from chemical industries, iron and steel works and wastewater treatment facilities. Moreover, exposure to cyanide may also occur in the work places, for example, the electroplating, metallurgical firefighting, steel manufacturing, and metal cleaning industries are a few of the work places where exposure may occur(Zlosnik and Williams, 2004).

Initial symptom of cyanide poisoning can occur from exposure to 20 to 40 parts per million (ppm) of gaseous hydrogen cyanide (HCN) (Mudder and Botz, 2004). It may include headache drowsiness, weak and rapid pulse, deep and rapid breathing, a bright-red color in faces, nausea and vomiting. Convulsions, dilated pupils, clammy skin, a weaker and more rapid pulse, and slower, shallower breathing can follow these symptoms. Finally, the heart beat becomes slow

and irregular. Body temperature falls. The lips face and extremities take on blue color. The person falls in a coma, and death occurs (Conley and Priest, 1980; Kariman and Burkhart, 1986).

The body has several mechanisms to effectively detoxify cyanide. The majority of cyanide reacts with thiosulfate to produce thiocyanate in reactions catalyzed by sulfur transferase enzymes such as rhodanase. Ingvorsen *et al.* (1991) indicated that sodium thiosulfate, administered intravenously, provides sulfur to enhance the sulfur transferase- mediated transformation of cyanide to thiocyanate. The thiocyanate is then excreted in the urine over a period of days. Although thiocyanate is approximately seven times less toxic than cyanide, increased thiocyanate concentrations in the body resulting from chronic cyanide exposure can adversely affect the thyroid. Cyanide has a great affinity for methemoglobin than for cytochrome oxidase. Thus, it will preferentially form cyanomethemglobin. According to Brehaut (2000) amyl nitrite, sodium nitrate and dimethyl amino phenol (DMAP) are used to increase the amount of methemoglobin in the blood, which then binds with cyanide to form nontoxic cyanomethemoglobin. Since cyanide can result in all these environmental and public health problems (Even though applied widely in several industries), process waters generated during the activities of such industries need to be treated before reuse or discharge into the environment (Andrews, 1999; McKinnon, 2002; Lonesiy, 2006).

1.6. Treatments of Cyanide containing wastes

A range of different techniques are available for the treatment of cyanides and its compounds in industrial waste-waters. Some of the most commonly employed methods are chemical procedures and Biological methods (Brinne, 2004; Mineral Council of Australia, 2005).

1.6.1. Chemical treatments of cyanide containing wastes

Most cyanide destruction processes operate on the principle of converting cyanide into one or less toxic compounds such as ammonia, cyanate (OCN^-) and nitrate through the oxidation reactions. There are several chemical destruction processes that are well proven to produce treated effluents with low levels of cyanide and metals (Augugliaro, 1997; Mudder *et al.*, 2001).

Among these, the commonly applied more important chemical treatment methods are:

- Oxidation by H_2O_2
- Oxidation by SO_2
- Alkaline Chlorination methods.

The choice of treatment options depend on the nature of cyanide compounds as only oxidizable cyanides can be treated by hydrogen peroxide or other oxidants. Hydrogen peroxide is the most common oxidant that requires high pH.The reaction between CN and H_2O_2 produce cyanate and water (Eqn.1)

$$CN^- + H_2O_2 \xrightarrow{Cu^{2+}\ \text{Catalyst}} OCN^- + H_2O \quad \text{.....Eqn. 1}$$

The hydrogen peroxide treatment process chemistry is similar to that described for the oxidation by SO_2 process, but hydrogen peroxide is used rather than sulfur dioxide and air. With this process, soluble copper is also required as a catalyst and the end product of the reaction is cyanate, which is regarded as being at least 1000 times less toxic than cyanide. The cyante will hydrolyze overtime depending on the pH to give CO_2 and ammonium salts or carbonate and ammonia. This reaction takes time but can be catalyzed by the presence of copper (Akcil, 2002b and Ackil *et al.*, 2003).

The principle behind oxidation by SO_2 process is the oxidation of cyanide and thiocyanate anions to cyanate. Metal cyanide complexes are also broken down resulting in precipitation of both the metal hydroxides (e.g., Those of Cu, Zn, Ni) and metal cyanide solid precipitates (e.g., $Cu_2Fe(CN)_6$). The reaction is catalyzed by the presence of copper, which may have to be added as copper sulphite. Air is also required as is lime to maintain the pH at optimum levels as the reaction proceeds (Whitlock and Mudder, 1998; Akcil, 2001).

$$CN^- + SO_2 + O_2 + H_2O \xrightarrow{Cu^{2+}\ \text{Catalyst}} OCN^- + SO2^{-4} + 2H^+ \quad \text{.....Eqn.2}$$

Alkaline chlorination at one time was the most widely applied of the cyanide treatment processes, but it has gradually been replaced by other chemical processes and is now used only occasionally. The cyanide destruction reaction in this process is a two-step process, the first step involving conversion to cyanogen chloride (CNCl) followed in the second step by hydrolysis of the cyanogen chloride into cyanate:

$Cl_2 + CN^- \longrightarrow CNCl + Cl^-$……Eqn.3

$CNCl + H_2O \longrightarrow OCN^- + Cl^- + 2H^+$……..Eqn. 4

If additional chlorine is added to attain breakpoint, this process can also oxidize thiocyanate and ammonia to nitrogen gas in the event these two cyanide related compounds must be removed as well in the treated effluent. Alkaline chlorination is effective for oxidation of all cyanides except ion complexes, noble metal, and cobalt complexes. Chlorine has the lowest chemical costs but requires high pH to avoid cyanogens chloride formation (Akcil, 2001; Akcil and Mudder, 2003).

1.6.2. Biological treatments of Cyanide containing wastes

Although several chemical processes can be employed to degrade cyanide and its related compounds, they are often expensive and complex to operate. A better alternative to these processes is biological treatment, which typically relies upon microorganisms such as bacteria, fungi and some algae. Biological treatment of cyanide has been shown a viable and robust process for removing cyanide in the mine and other industrial process waters. Cyanide could be degraded by aerobic and anaerobic microorganisms, the later being much more slow and inefficient because of the following points:

- Undesirable compounds like ammonia are the major form end products
- Strong cyanide complexes are poorly removed
- Failure to remove cyanide to very low levels

Thus, the most promising application for an anaerobic system may be as a pretreatment step for an aerobic system. Such pretreatment is likely to improve aerobic system efficiency by substantially lowering inflow cyanide concentrations (Fallon *et al.*, 1991; Ackil *et al.*, 2001; Ackil, 2003). The classic aerobic biological process involves two separate bacterial oxidation steps to facilitate complete assimilation of the wastewater (Fig.2).

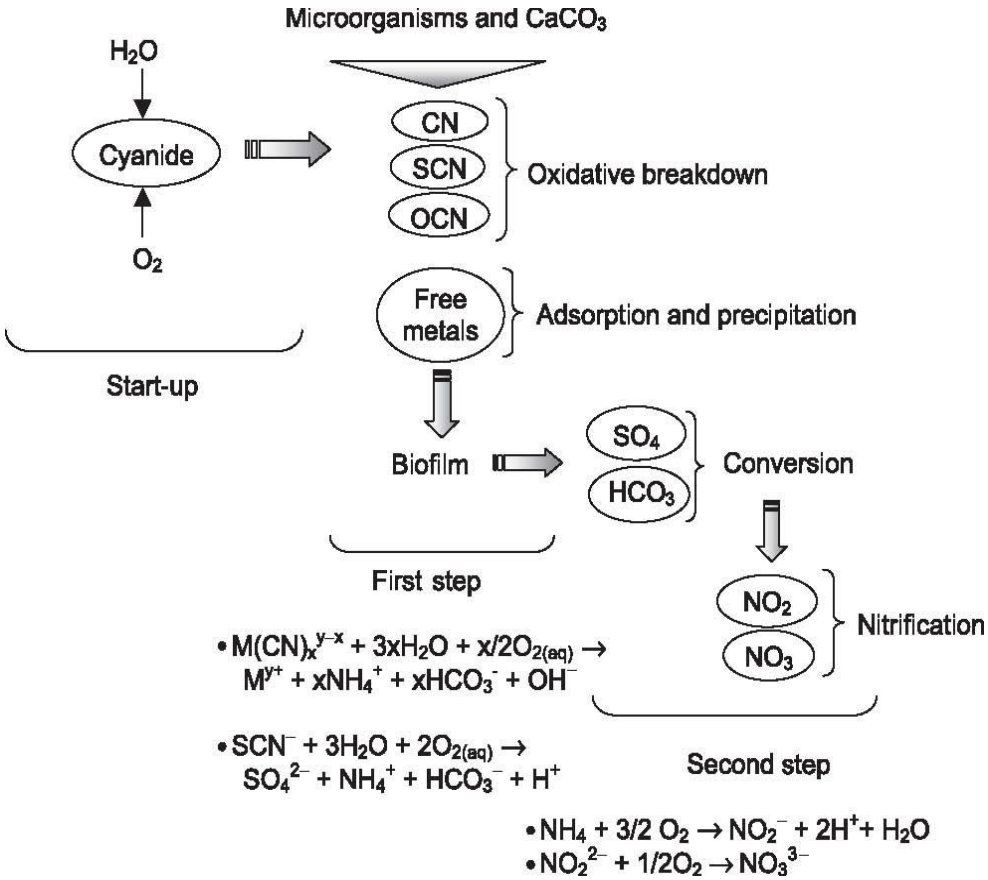


Fig.2. Mechanism of the aerobic biological cyanide treatment process (adapted from Akcil, 2003).

According to Akcil (**2003),** aerobic biological cyanide treatment process is considered as the basis of the first full-scale application of biological treatment in the mining industry and other industries that release cyanide wastes into the environment. The first step in the biological treatment process is the oxidative breakdown of cyanides and thiocyanate, and subsequent sorption and precipitation of free metals into the biofilm. Cyanide and thiocyanate are degraded to a combination of ammonia, carbonate, and sulfate. The second step converts ammonia to nitrate through the conventional two-step nitrification process with nitrite as the intermediate (Figs. 2 and 3).

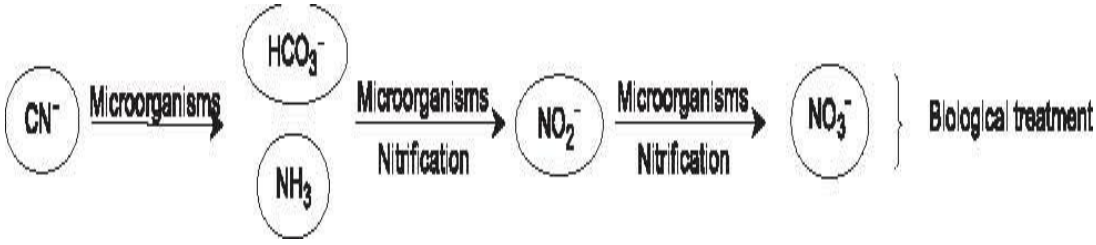


Fig. 3. A schematic view of the biological treatment process Adapted from Akcil (2003)

Various *Pseudomonas* species (Andrews, 1999; Almagro *et al.,* 2005) and other bacterial strains (Brinne, 2004) and several fungal populations (Dumestre *et al*, 1997) were involved in biological cyanide conversion processes. Degradation of cyanide complexes like tetracyanonickelate by *Cryptococcus* (Hyouk *et al.,* 2002; Kao *et al.,* 2005) was also reported for complete detoxifications of the cyanide waste-water. Efficient removal of cyanide from industrial wastes is possible in presence of oxygen molecule (Eqns.5, 6, 7, 8, 9).

Biological treatment provides the most efficient and economical means of removing nitrogen from waste water in the form of ammonia or nitrate. Typically, a mixed population of bacteria are alternatively exposed to aerobic conditions for nitrification and then anoxic conditions for denitrification (Eqn. 8 and 9) (Gallert and Winter, 2005; Dereje Teshome, 2006).

$$SCN^- + 2H_2O + 5/2O_2 \longrightarrow SO_4^{2-} + HCO_3^- + NH_3 \text{.....Eqn.5 (thiocyanate degraders)}$$

$$CN^- + 1/2O_2 + 2H_2O \longrightarrow HCO_3^- + NH_3 \text{.....Eqn. 6 (cyanide degraders)}$$

$$NH_3 + 3/2O_2 \longrightarrow NO_2^- + H^+ + H_2O \text{ .....Eqn. 7 (Ammonia Oxiders\}}$$

$$NO_2^- + 1/2O_2 \longrightarrow NO_3^- \text{.....Eqn. 8 (Nitrite Oxidizers\}}$$

$$3NO_3^- + 2.5CH_3OH \longrightarrow 1.5N_2 + 2.5CO_2 + 3.5H_2O + 3OH^- \text{.....Eqn. 9 (Denitrifiers)}$$

During nitrification, ammonia is first oxidized to the intermediate nitrites slowly and rapidly to nitrate. In denitrification, the nitrate is then reduced to nitrogen gas resulting in complete

removal of nitrogen from the solution being treated (MERG, 2000 and Akcil, 2003).
The high cost of chemical detoxification of cyanide –containing industrial wastes has generated interests in the biological detoxification of these wastes. Despite the well known toxicity of cyanide to a number of cellular processes (Andrews, 1995;Akcil and Mudder, 2003), several plants, bacteria and fungi produce cyanide or cyanide- containing compounds, for instances as a defense mechanisms. Partly as a response to the former, other organisms exist either by neutralizing the toxicity or utilizing cyanide compounds as a substrate. The later can be the base for biological cyanide treatment on –site (Balaguer *et al.,* 1997.

According to Brinne (2004) many organisms possess metabolic pathways by which they can utilize cyanide-contaminated wastes as a source of energy or nutrition. At any sites polluted with organic wastes there will be a resident and natural microbiota specialized in breaking down the pollutants, depending up on the amount and characteristics of the toxic wastes, as well as the age of the site. The most powerful microorganisms discovered yet to detoxify cyanide and its compounds are bacteria, fungi and algae (Knowles, 1976; Adjei and Ohta, 1999; Ackil *et al.,* 2003).

1.6.2.1.1. Bacterial cyanide degradation

Despite the occurrence of cyanide in the biosphere, our understanding of the role that microbes play in cyanide recycling is incomplete. This is somewhat surprising since bacteria able to use cyanide as a natural sole nitrogen source were readily isolated from nature (Fernandez *et al.,* 2004). Growth on cyanide requires enzymatic conversion to ammonia that is then assimilated by well-established biochemical pathway. Bacterial isolates like *Pseudomonas psedoalkaligens* strains were able to grow by using cyanide as a sole nitrogen source under alkaline conditions and acetate as a carbon source. This is more preferable as far as the chemistry of cyanide is concerned. They were able to tolerate up to 30 mM (Almagro *et al.,* 2005) of free cyanide and may use several cyanometal complexes.

Another *Pseudomonas* strains involved in cyanide and cyano-metal complex degradation were *P. fluorescens*. These were powerful to degrade $Ni(CN)_4^{-2}$ and use the product as a sole

nitrogen source. Some authors (Fernandez *et al*., 2004; Almagro *et al*., 2005) also showed that Cu $(CN)_4^{2-}$ is also a suitable nitrogen source for these bacteria, but to a lesser extent than the nickel complexes. In addition, a number of other *Pseudomonas species* were considered as principal agents in a biotreatment processes for cyanide destructions in mining wastes. Among these, commonly reported ones were *P. Putida, P.pickettii,* and *P. paucimobilis (*Ingvorsen *et al.,* 1991).

According to Avalos (1990) the bacterium, *Burkholderia cepacia* is able to utilize cyanide optimally at pH 10, but it needs glucose as a carbon source. In addition, it is sensitive to metal ions, such as iron and copper. Some of the *Klebsiella species* were surprisingly evolved to resist cyanide. *Micrococcus, Achromobacter and Bacillus species* were also cyanotropic in nature (Kao *et al.,* 2003). According to Meyers *et al.* (1993) several bacteria were capable to utilize thiocyanate, particularly, *Thiobacillus thiocyanooxidans, T. thioparus, and T. denitrificans* were the most important bacteria to degrade thiocyanates as illustrated in reactions(Eqn. 8 ,9 and 10) (Katayama *et al.,*1998).

$$SCN^- + 2H_2O + 2O_2 \longrightarrow SO_4^{2-} + NH^{4-} + CO_2 \ldots\ldots \text{Eqn . 10.}$$

1.6.2.1.2. Fungal cyanide degradation

Many workers have reported (Fallon *et al.,* 1991; Gurbuz *et al.,* 2003) microbial cyanide degradation at neutral or acidic conditions. Strains adapted to survive the toxicity of cyanide at alkaline pH but unable to degrade it has also been described. By contrast, literatures describing cyanide biodegradation at alkaline pH are scarce (Sorokin *et al.,* 2001).

One of the most powerful fungal strain to breakdown cyanide and its related compounds at extremely alkaline conditions is *Fusarium solani* (Dumestr *et al.,* 1997). It utilizes cyanide as a sole nitrogen source. Other authors (Almagro *et al.,* (2005) showed that a yeast strain of *Cryptococcus hurmcolus* was able to metabolize cyanide. He also isolated a powerful mold strain of *Acremonium strictum* that was capable of degrading thiocyante.

Kao *et al.,* (2003) identified an efficient strain of *F. solani* and *Trichoderma polysporum* that were endowed with ability to degrade even a cyano-metal complex known as

tetracyanonickelate ($Ni(CN)_4^{2-}$). They also cultured mixtures of *F. oxisporum*, *Scytalidium thermophilum* and *Penicillium miczynski* on hexacyanoferrate, and all cultures were able to utilize $K_4Fe(CN)_6$ as sole nitrogen source. The cultures also rapidly degraded $K_2Ni(CN)_4$ at neutral pH. This was the weakness of the strains because of the volatility of cyanide.

In addition, a remarkable number of algal strains were also found to be detoxifying cyanide. Mostly encountered speceies of algae utilizing cyanide are *Chlorella sp*., *Arthrospira maxima*, and *Scenedesmus obliquus* (Gurbuz *et al.,* 2003).

1.6.3. Mechanisms of microbial cyanide degradation

Several biochemical pathways have been suggested (Meyers *et al.,* 1993; Zlosnil and Williams, 2004) for the degradation of cyanide in bacteria and other microbes. *Bacillus pumilus Pseudomonas fluorescens* and *P. paucimobils* have all been shown to convert cyanide to ammonia and carbon dioxide. In the case of *P. fluorrescens* strain, the reaction may proceed by means of an NAD(P)H-dependent deoxygenase. Similarly, cyanide dehidratase (formamide hydrolyase) catalyzed hydration of cyanide to formamide, which is further decomposed into formic acid and ammonia, has been reported to occur in fungi as well as in *P. fluorescensstrains* (Mihaylov and Hendrix, 1994; Fernandez *et al.,* 2004; Fernandez and Kunuz, 2005)

Varieties of enzymatic pathways for cyanide degradation have also been described from aerobic and anaerobic microorganisms (Ingvorsen *et al.,* 1991). The most common microbial enzymes important in converting cyanide into compounds, which may serve as carbon and nitrogen substrates, are formamide dihydrolyase, L-3-cyanoalanine synthase, thiosulfate sulfur transferase, cyanide oxygenase (CNO), NADH oxidase (NOX), NADH peroxidase, cyanide hydratase (CNN) and carbonic anhydrase (CA)(Ingvorsen *et al*.,1991).

P. fluorescens cleaves cyanide oxygenatively to formate and ammonia via reactions involving formamide as an intermediate. The enzyme responsible for cleavage is cyanide oxygenase (CNO), which is located in the cytosolic fractions of cells induced with cyanide. It requires both reduced pyridine nucleotide (NADH) and a source of reduced pterin as a cofactor (Fernandez and Kunz, 2005).

$HCN + O_2 + 2NADH + 2H^+ \longrightarrow [HCONH_2] + H_2O + 2NAD^+$…….Eqn.11.

$[HCONH_2] + H_2O \longrightarrow HCO_2H + NH_3$……..Eqn. 12

$HCO_2H + NAD^+ \longrightarrow CO_2 + NADH + H^+$……Eqn. 13

Sum: $HCN + O_2 + NADH + H^+ \longrightarrow CO_2 + NH_3 + NAD$……...Eqn. 14

Formate is further oxidized by a soluble formate dehydrogenase (FDH) simultaneously elevated in cyanide –induced cells (eqn.12.) These two enzymes accomplish the complete oxidation of cyanide to carbon dioxide and ammonia. In this process each molecule of NADH and oxygen are consumed (Ingvorsen *et al.,* 1991).

1.6.4. Factors affecting microbial cyanide degradation

A number of factors influence the pace of cyanide degradation by microbes. Because enzymes catalyze cyanide degradation and many conditions affect the activities of enzymes, the efficiencies of microbes are influenced by the following circumstances:

- pH
- Complexation of cyanide compounds
- Temperature
- Oxidation- reduction potential

Patil and Paknikar (2000) indicated that microbial cyanide degradation is highly influenced by variation in temperature. According to the authors report the activities of cyanide degrading enzymes, microbial growth and the stability of cyanide and its compounds are influenced by temperature differences. For example, they were able to isolate bacterial strains capable of degradation of silver cyanide efficiently at 35^{o}C, although the degradation efficiency was considerable at 20^{o}Cand 30^{o}C.

The optimum pH for microbial activities depends on the type of microbes involved. Some microbes are active at acidic or neutral pH (Graham *et al.,* 1994; Fernande *et al.,* 2004) while others are able to grow and produce functional enzymes at alkaline pH. For example, the *F. solani* and *P. pseudoalkaligenes* produce novel enzymes that degrade cyanide at pH 10.1. In general, from the cyanide chemical and physical properties point of view, the biological treatment of individual effluents contaminated with cyanide requires an alkaline pH in order to

avoid the formation of volatile HCN. Therefore, the main factor accounting for cyanide elimination in biodegradation process is the evaporation of HCN due to the neutral or acidic conditions of the medium, its toxicity as well (Fernande and Kunz, 2005).

A wide variety of metal ions are known (McKinnon, 2002) to interact with cyanide, forming both simple binary salts and multiligand complexes. Some metal ions have positive effect on the rate of cyanide degradation by *cyanide dehidratase*. Moran (1999) indicated that the trivalent metal cations as Sc^{3+}, Fe^{3+}, Cr^{3+}, and Tb^{3+}all enhanced the enzymatic activity of *cyanide dehydratase* approximately two fold, where as $Hg^{2+}$and, to a less extent $Pb^{2+}$and Ag^{2+}were inhibitory. Based on Brinne' s (2004) report, complexed cyanide often remains mobile and dose not adsorb, partly because it is negatively charged. Iron complexes tend to be relatively harmless as long as they do slightly breakdown.

The strongly complexed cyanide is perhaps best kept complexed; the weaker complexes should be allowed to break down and converted to other forms as rapidly as possible. However, the former are not available for microbial activities. While growth on free cyanide (KCN, NaCN) by bacteria has been demonstrated in several cases, the utilization of cyanide complexes is rare. This indicated that strong complexes like – iron cyanide and cobalt cyanide complexes are very stable (Avalos *et al.,* 1990; Fallon *et al.,* 1991; Andrews, 1999; Souza *et al.,* 2002; Zlosnik *et al.,* 2004; Fernandez and Kunz 2005).

The most commonly proposed mechanisms of cyanide resistance and detoxifications by living organisms are inductions of enzymes for degradation and detoxifications of cyanide or form cyanide-resistant enzymes (Moreau and Romani, 1982). Because cytochrome oxidase is sensitive to cyanide poisoning and respiration is central to functioning of cells, the evolution and development of cyanide-resistant respiratory system is of particular interest. Various eukaryotic microorganisms were also found to evolve cyanide-resistant "alternative oxidases", which are not cytochromes. In addition, branched respiratory systems also developed in bacteria, with one branch less cyanide-sensitive than the other. According to Knowles (1976) a shift from aerobic growth in the presence of cyanide to aerobic growth in its absence caused cytochrome a_1 and d to disappear and the ctochrome *b* content to halve but had little effect on the cytochrome *o* content. Conversely, addition of very small amount of cyanide to cells

resulted in a massive induction of cytochrome a_1 and d and small decrease in the cocentraios of cytochhrome *b* and *o* (Barber, 1978; Kariman and Burkhart, 1986; John *et al.,* 1987; Lien and Astringe, 1997; Jandhyala *et al.,* 2003).

Generally, the biological assimilation of cyanide needs, at minimum, the occurrence of three separate processes, i.e., a cyanide assimilation pathway, a cyanide resistance mechanism and a system for metal acquisition (Rissler *et al.,* 1977; Rychte *et al.,* 1979; Rustin *et al.,* 1980; Zlosnik and Williams, 2004).

1.6.5. Advantages of microbial cyanide degradation

Although a range of different techniques is suggested for the treatment of cyanide-contaminated wastes, microbial removal is preferred to others (Akcil, 2005) in a number of ways. One reason is that it is less expensive and environmentally friendly. In general, the advantageous aspects of biological cyanide removal methods over chemical removal methods are described in Table 3.

Table 3. Comparisons between Biological and chemical cyanide waste treatments (Modified from Akcil, 2003; Akkcil and Mudder, 2003)

Properties	**Biological degradation**	**Chemical detoxification**
Rate of removal	Relatively faster	Slower than its counterparts
Effluent water reuse	Effluent water reuse is potentially possible.	Since the end products themselves are hazardous, waste-water reuse is not recommended
Environmental aspects	Environmentally friendly because the end products and intermediate forms are utilized by the involved organisms	The input chemicals andend products have their own impact on the environment
Cost wise	More economical	Less economical

According to MERG (2000) biological method was more successful at removing cyanide than peroxide treatment method. Ammonia concentrations were more observed in the chemical treatment systems than the biological counterparts, indicating the need for further removal method of ammonia from the process. The biological treatment also had high levels of nitrate, indicating that microbes in the column were oxidizing ammonia as well as the cyanide. This confirmed that biological treatment can attain cyanide removal rates superior to a chemical treatment. Thus, biological breakdown of ammonia is one of several benefits of a biological treatment method. Similarly, Brinne (2004) indicated that cyanide treatments by microorganisms and associated species in gold mill effluents is a natural process and can be easily exploited to handle large flows and cyanide concentrations found in commercial gold operations.

In general, cyanide containing wastes are released from a number of mining and related industrial processes and discharged in to the environment. The free cyanide (CN^- and HCN) with its related compounds may result in strong ecological disasters. Among the several detoxification methods involved, biological cyanide removal method is suggested as more preferable to chemical methods and it is environmentally friendly ways of cyanide waste treatment method. However, it is affected by several factors that influence the activities of microorganisms and the stability of cyanide compound itself. The most important of all is the effect of pH on the physical and chemical properties of cyanide. That is, free cyanides readily escape (outgas) from media at neutral and acidic pH, enhancing environmental pollution and being not available for microbial degradation. Thus, cyanide solutions must always be kept at alkaline conditions. Even though microbes that degrade cyanide at acidic and neutral pH are reported, due to the above reasons, alkaliphillic cyanide- degrading microbes are being considered as feasible options for biological cyanide-degradations. This study, therefore, was carried out with the following objectives:

1.7. The objectives of the study

The general objective of this study was to determine how easily alkaliphillic cyanide-degrading microorganisms could be isolated, screened and characterized from enrichment cultures supplied with cyanide as sole source of nitrogen

Specifically, the study was carried out:

- To isolate alkaliphilic cyanide-degrading bacteria from Ethiopian rift valley alkaline soda lake (Lake Chitu).

- To characterize the growth of cyanotrophic alkalophilic strains by using cyanide as sole nitrogen source

2. Materials and Methods

2.1. Samples and sampling sites

Soil, water and mud sediments were collected from Lake Chitu, which is located in Southern part of Ethiopia, at an altitude of 1600 m with maximum depth of 21 m. Lake Chitu is one of the most saline and alkaline lakes among Ethiopian Rift valley lakes with average pH of 10.2 and salinity of 44.9 g/l (Elizabeth Kebede, 1996).

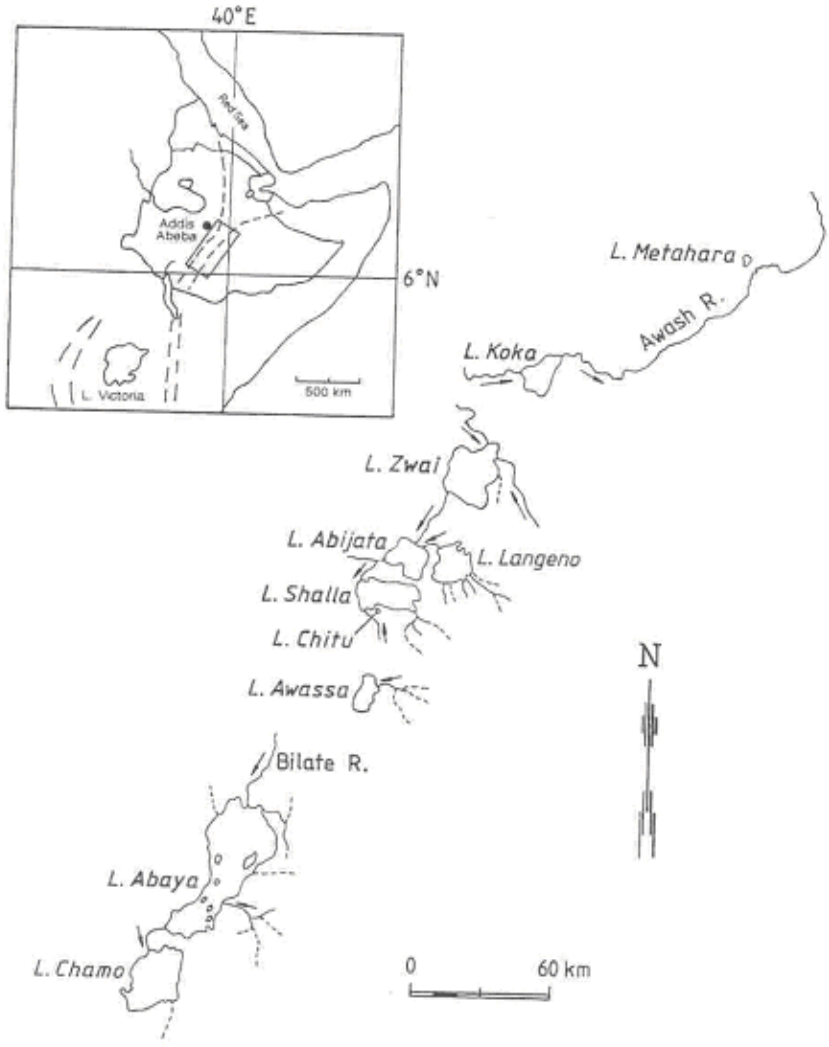


Fig.4.Location of the study area (Adapted from Elizabeth, 1996)

2.2. Enrichment, culture conditions, isolation and purification

A 0.25 g of soil samples, mud sediments and 0.25 ml lake water samples were serially diluted (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and $10^{-5)}$ in test tubes. One ml of the corresponding diluted samples were inoculated into 100 ml sterilized enrichment liquid medium (composition shown in Table 4) in 500 ml Erlenmeyer flask and placed on orbital shaker at 120 revs/min at ambient temperature. At 48 h interval, one ml of the culture was transferred into 100 ml sterilized enrichment liquid medium (identical to the previous media) in 500 ml Erlenmeyer flask and placed on orbital shaker at 120 revs/min at ambient temperature. The growth and activity of bacteria was observed microscopically.

Table 4. Components of the enrichment media for cyanotrophic bacterial isolation (Ingvorsen *et al*., 1991).

Component	Amount(g/l)
$Na_2HPO_4.2H_2O$	7
KH_2PO_4	3
$MgSO_4.7H_2O$	0.3
NaCl	0.25
$CaCl_2.\ 2H_2O$	0.02
$MnSO_4.4H_2O$	0.01
$FeCl_3.6H_2O$	0.045
$ZnSO_4.7H_2O$	0.01
$CuSO_4.H_2O$	0.002
$CoCl_2.6H_2O$	0.003
$NiCl_2.6CH_2O$	0.003
$NaMoO_4.2H_2O$	0.002

After series of subculturing at every 48 h, one ml of the culture from each dilution were inoculated into 9ml sterilized enrichment liquid medium containing test tubes and kept on orbital shaker at 120 rev/min. One percent (v/v) of 48hrs old culture was dispensed onto sterile agar (1.5%, Difco laboratory) medium and spread by using alcohol flamed glass rod spreader

bent into a hockey stick to examine the formation of colonies. Then, the plates were incubated at 30^0C for about 3 to 5 days.

All the plates were observed carefully and single colony of each isolate was picked with alcohol flamed sterile inoculating loop and transferred into 10 ml sterile enrichment liquid medium in a test tube and then placed on rotary shaker at ambient temperature at 120 rev./ min. After observation of cloudy bacterial suspensions (after 48 h), streaking was performed on sterile plates of enrichment agar (1.5%, Difco laboratory) media and then the plates were incubated at 30^0C. Four times of re-streaking were carried out to observe purity and uniformity of colony types. In all cases in this study, pH of the bacterial growth medium was adjusted by Na_2CO_3 and 0.25 % (w/v) of sodium acetate was used as a carbon source, except in carbon sources utilization experiments, where different carbon sources were supplied. Similarly, sodium cyanide was supplied as a sole nitrogen source, except in nitrogen sources utilization experiment, in which case different nitrogen sources were supplied.

2.4. Bacterial designation preservation

A single well isolated colony was picked to enrichment agar slant that contains sodium cyanide as only source of nitrogen and incubated at 30^0C. After adequate growth was taken place, the culture slant was stored at 4^0C for further study. The isolates were designated as AUCN followed by their corresponding numbers, indicating that they are cyanide utilizing bacterial strains isolated in Addis Ababa University.

2.5. Selection of potential cyanide degrading isolates

All of the 63 isolates were subjected to the same amount of cyanide concentration and incubated in similar laboratory conditions to screen potentially more powerful isolates in utilizing cyanide. As suggested by Almagro *et al.* (2005), the selection was based on the physical (morphological) observation, microscopical observation, growth pattern of the isolates and comparison of biomass (dry weight) and protein content of cells.

2.6. Morphological and physiological characterization

2.6.1. Morphological characterization: - The selected bacterial isolates were gram stained and examined for the gram reaction and shape. The shape of colony of both strains were determined by streaking a loopful of 48 h old cultures on to sterile agar plates and incubating at 30^0C for 4-6 days. In each case, colonies were touched with a wire loop and lifted to examine the texture. In addition, the appearance of the colonies of both strain was examined and recorded as shiny (translucent) or opaque. Similarly, the presence and pattern of spores in the isolates were also tested. In addition, the selected bacterial isolates were examined under an epfluorescence microscope (Olympus, Japan) attached to a CCD digital camera. Analysis®DOCU software (CC12DOCU, Germany) was used for image acquisition of the isolates.

2.6.2. Physiological characterization

2.6.2.1. Effects of different cyanide concentrations on bacterial growth

Both of the isolates were subjected to different concentration of NaCN (20% - w/v dissolved in 0.1 M NaOH) solution to avoid the risk of HCN formation and volatility. Various concentration of NaCN starting from 1.02 mM to 19.38 mM was tested in both isolates. Starter culture of the selected test strains (AUCN-54, and AUCN-60) were grown in test tubes containing 10 ml enrichment sterilized liquid medium on orbital shaker at 120 rev/min at ambient temperature for 48 hours. One percent (v/v) of each bacterial culture was transferred into 100 ml sterilized enrichment liquid medium in 500 ml Erlenmeyer flask and placed on orbital shaker at 120 revs/min at ambient temperature for 6 days. Duplicates of experiments were carried out for each isolates and the mean dry weight and mean protein content of cells (mg/ml) were obtained with a filter paper (Whatman international, Made in England- pore size of 0.45 µm) and modified Lowery method in combination with NaOH methods (after sonication for 15 minutes), respectively (Lowry, 1951; Hasegawa *et al.,* 2003). The later was to increase the efficiency of protein extraction from cells. A 100 ml of enrichment liquid medium (without inocula) in 500 ml Erlenmeyer flask was kept as control for each isolates in dry weight and protein measurements.

2.6.2.2. Time-course for bacterial growth

One ml of a 48 hours old starter culture of test isolates grown in 10 ml enrichment liquid medium in a test tube containing NaCN as sole nitrogen source were transferred into duplicates of 100 ml sterilized media in 500 ml Erlenmeyer flasks. Cells were filtered (According to Almagro *et at.,* 2005) with Whatman filter paper (0.45 μm pore size) and the biomass retained was heated at 100^0c till constant weight was obtained. Cells were harvested from equal volumes of cultures of dry weight by centrifugation and cells in the pellets were lysed by NaOH in combination with sonication for 15 minutes. Homogenized cell suspensions were centrifuged (for 30 min) and supernatants (after Lowery procedure) were taken for OD reading at 750 nm (Lowery, 1951). Dry weight and protein measurement of each isolates were carried out at the interval of 12 hours starting from 0hr to 168hrs. A 100 ml of enrichment liquid medium (without inocula) in 500 ml Erlenmeyer flask was used as control for each isolates in dry weight and protein measurements.

2.6.2.3. Salt tolerance.

The ability of the bacterial isolates to tolerate salt (NaCl) was tested by inoculating the organisms with 100 ml enrichment liquid medium containing (0, 0.25, 1, 5, 10, 15, and 20% (w/v)) in 500 ml Erlenmeyer flasks, in duplicates. The experiment was carried out in similar culture media as in above cases except for the salt concentration differences. Finally, growth was measured by determining the dry weight of the organisms as described under growth rate determination case.

2.6.2.4. Growth at different concentrations of Na_2CO_3

The effect of increasing concentrations of Na_2CO_3 on growth of the two isolates was determined by inoculating the isolates in duplicates of 100 ml media containing 0.25, 0.50, 0.75, 1, 1.5, 2, 2.5, and 3 % (w/v) Na_2CO_3. The pH values of each liquid culture in a flask were measured just after inoculation and right before harvesting cells. Finally, the culture was harvested and growth was evaluated quantitatively by measuring the biomass of the culture.

2.6.2.5. Growth at different Temperature

A loopful of 48 hrs liquid culture of cyanide-degrading bacterial isolates was streaked onto sterile enrichment agar media in plates in duplicates. The plates were then incubated at various temperature (5, 10, 15, 20,30,35,40 and 45^0_C) and growth of isolates was evaluated qualitatively as (-) for no growth and (+) for positive growth (Tiwari *et al.,* 2004). Non-streaked plates of each isolates were also incubated at 30^0c as control.

2.6.2.6. Comparison of bacterial growth on different carbon-source

Eight different carbon sources were taken to examine the utilization potential of the isolates (while sodium cyanide serves as nitrogen-source). These are cyanide (as sole carbon and nitrogen source), molasses, sodium-acetate, D-xylose, D-fructose, sucrose, D-glucose and maltose. Ten percent (w/v) distilled water solution of each carbon -source except molasses and sodium acetate (that were directly autoclaved with other medium) was prepared and temporarily stored in a refrigerator after filter sterilization using a Millipore filter (0.22 µm). Then, 90 ml of carbon-source- free sterilized liquid medium was mixed with each of its corresponding carbon-source in 500 ml Erlenmeyer flasks. Finally, 1% (v/v) of 48 h starter culture in a test tube was inoculated into the already prepared media and incubated at ambient temperature for 5 days. Growth of the isolates was quantitatively evaluated by measuring the dry weights of the culture. Duplicates of culture flasks were kept for each isolates and control flask containing sodium-acetate as carbon source but without any inocula was kept in the orbital shaker at 120 rev/min. for the same period of time as inocula containing media.

2.6.2.7. Intrinsic Antibiotic Resistance (IAR)

Five different antibiotics (chloroamphenicol, streptomycin, Neomycin, kanamycin and ampicillin were selected to test the natural resistance of the strains to the antibiotics. The stock solution of each antibiotic was prepared according to Almagro et al. (2005) by dissolving 2.0 g of chloramphenicol in 100ml absolute ethanol while distilled water was used as a solvent for each of 2.0 g remaining four antibiotics. The stock solution of each antibiotics was filter sterilized using a Millipore filter (0.22µm) and aseptically added to autoclaved enrichment agar media kept at 50^0c in water bath at the final concentration of 10 (for chloroamphenicol), 25 (for

kanamycin monosulphat), 30 (for each of streptomycin-sulfate and Neomycin) and 100 (for ampicillin) in μg/ml and finally poured separately into agar plates. Duplicate plates were used for each isolates with the mentioned antibiotic concentrations and antibiotics- free agar medium plates were used as control. All the plates were kept at room temperature for 24 hrs before inoculation. Eventually, loopful of both cyanide- utilizing test isolates were streaked on enrichment medium on agar plates supplemented with corresponding antibiotics of test and incubated at 30^0c for 5 days and bacterial growth was evaluated visually as (+) for positive growth and (-) for absence of growth.

2.6.2.8. Comparisons of bacterial growth using cyanide, organic nitrogen and inorganic nitrogen sources

Growth of cyanide utilizing bacterial isolates was tested in eight different nitrogenous compounds (while acetate was provided as carbon source). These are organic nitrogen sources (urea, peptone and yeast extract), cyanide containing inorganic nitrogenous compounds (NaCN, $K_2Cu(CN)_4$ and KCNS) and other inorganic nitrogenous compounds (NH_4Cl and $NaNO_3$). Urea, NaCN and KCNS were filter sterilized by using Millipore filter (0.22 μm). All others were sterilized by autoclaving before use and 12.5 mM stock solution of $K_2Cu(CN)_4$ was prepared by mixing equal volumes of 100 mM KCN and 25 mM sterilized $CuSO_4$ as suggested by Almagro et al. (2005).

Duplicates of 100 ml enrichment sterilized liquid medium containing each test nitrogenous compounds were prepared for each isolates. The experiment was carried out in similar way as to the growth rate determination cases except for nitrogen sources. Growth of test isolates was evaluated quantitatively by determining the biomass (mean dry weight) and soluble protein in (mg/ml) from equal volumes of culture media in the same procedures as described in other cases.

3. Results

3.1. Selection of potential cyanide Degrading isolates

A total of 63 alkaliphilic cyanide-degrading microorganisms were isolated from Lake Chitu mud sediment samples in a medium supplemented with sodium cyanide as a sole nitrogen source. Based on the rate of growth, among 63 cyanotrophic bacterial strains isolated from the provided alkaline soda lake samples, two strains (designated as AUCN-54 and AUCN-60) were selected for further study.

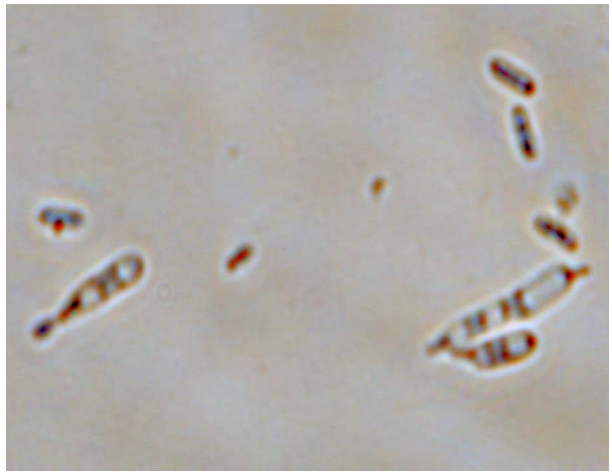
3.2. Morphological and physiological characterizations

Based on the different cultural, morphological and physiological features of the isolates (Table 5) and identification keys set for comparison in the Bergey's manual of systematic bacteriology (9^{th} ed.), isolate AUCN-60 was grouped under *Bacillus sp.* The remaining isolate was not possible to identify to species level based on this biochemical test; however, depending on the morphological and other general physiological characters, it was classified as *Sporosarcina sp*. The isolates were gram positive and oxidase and catalase positive, indicating that they are aerobic.

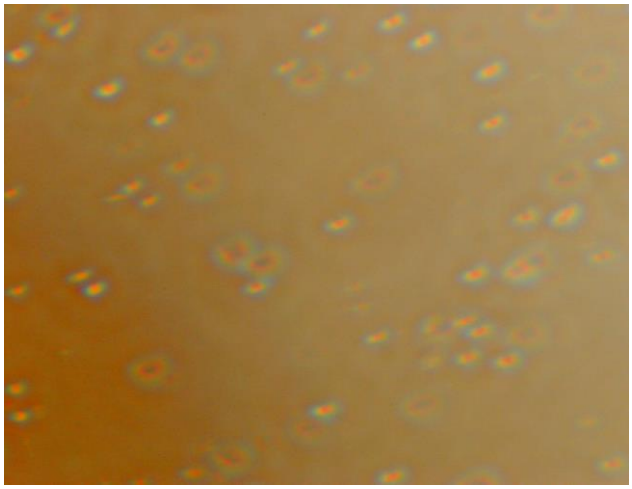
3.2.1. Morphological appearances of isolates

AUCN-54 was found to be coccoid shaped while AUCN-60 was rode shaped. AUCN-60 was white chalky filamentous bacteria (Fig. 5 and Table 5) with sticky nature on the surface of agar media. Both of the strains are motile, each possessing one ellipsoidal terminal spores.

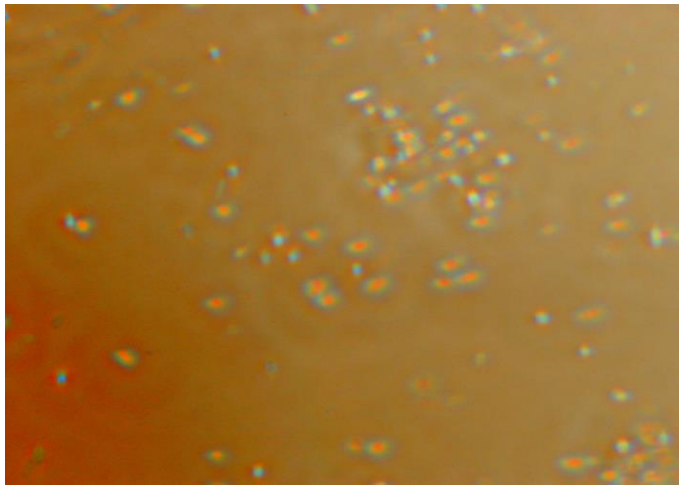
a) AUCN-60 Grown on NaCN and



b) AUCN-60 Grown on Peptone



c) AUCN-54 Grown on NaCN



d) AUCN-54 Grown on Peptone

Fig 5. Cyanide-degrading Bacterial isolates viewed under epfluorescence Microscope (a-d).

Table 5. Biochemical test results for the selected Cyanide degrading Bacterial isolates

Tests		Bacterial Isolates	
		AUCN-54	AUCN-60
Gram reaction		+	+
Oxidase		+	+
Catalase		+	+
Sucrose		+	+
Mannitol		+	+
Lactose		+	+
Gas production		+	+
H_2S oxidation		-	-
Urea		-	-
Colony colour		Light yellow	White
Colony appearance		Translucent	opaque
Colony shape		Circular	Circular
Colony texture		Buttery	sticky
Cell shape		Cocci	Rod
Cell arrangement		Diplococcic	single
Spore staining	Shape	Ellipsoidal	Ellipsoidal
	Location	Terminal	Terminal
	Number	1	1
Motility		Motile	Motile
Identification		*Sporosarcina sp.*	*Bacillus sp.*

3.2.2. Physiological characterizations of the isolates

3.2.2.1. Effects of different cyanide concentrations on bacterial growth

For all cyanide degrading bacteria, there is always a maximum concentration of cyanide above which no growth is observed (Avalos *et al.*, 1990). This varies from one microorganism to the other. The bacterial isolates under study responded differently to increasing concentration of cyanide in the growth media. AUCN-54 was capable to withstand cyanide concentration up to 13.26 mM while AUCN-60 was found to tolerate cyanide concentration up to 19.38 mM. Optimum growth for AUCN-54 and AUCN – 60 was observed at 10.2 mM and 17.34 mM for, respectively (Fig.6). But when grown on agar plates both isolates formed viable colonies up to cyanide concentration of 61.23 mM (data not shown).

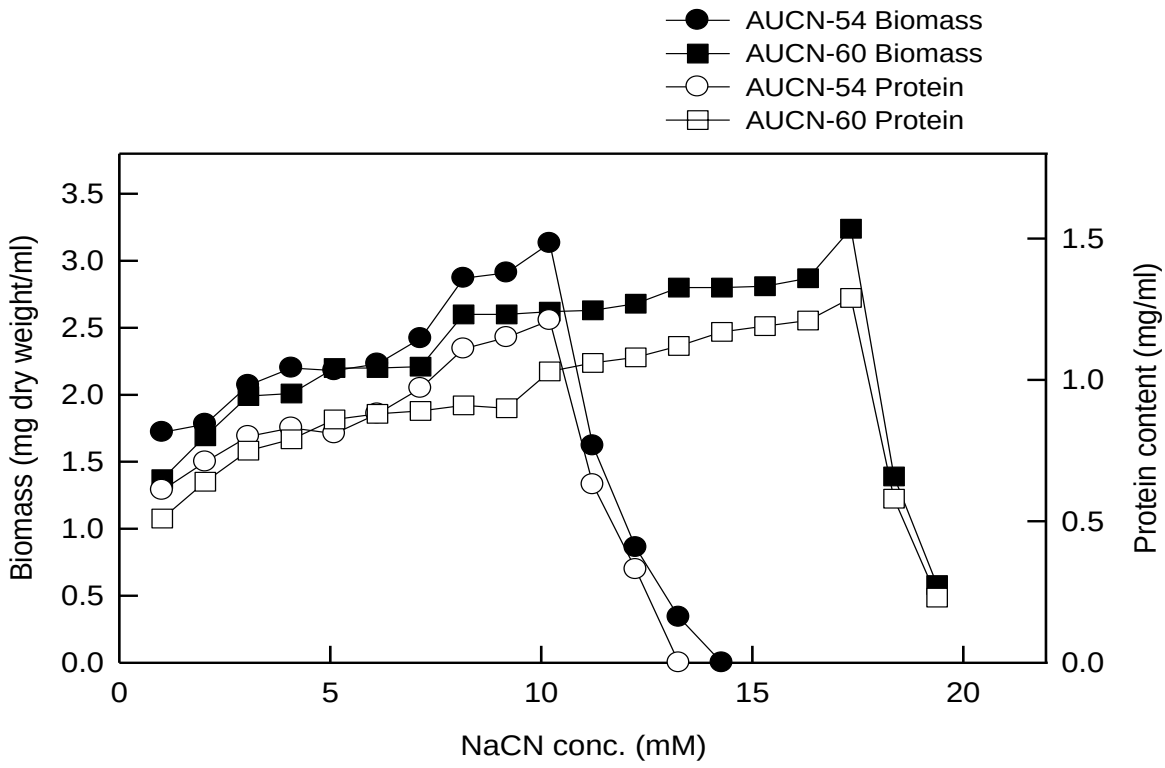


Fig.6. Biomass and protein measurement of isolates as function of NaCN concentrations

3.2.2.2. Time-course for bacterial growth

Quantitative study was carried out on the bases of biomass and protein quantification to evaluate the growth rate of the two isolates under study. Both isolates showed characteristic exponential growth starting from the second day of inoculation day to the end of fifth day. The growth pattern of the two isolates clearly differed from each other **(Fig.7)**. That is, AUCN-54 grew fast but immediately inhibited by increased cyanide concentrations. On the contrary, AUCN-60 grew slowly and adapted higher concentrations of cyanide.

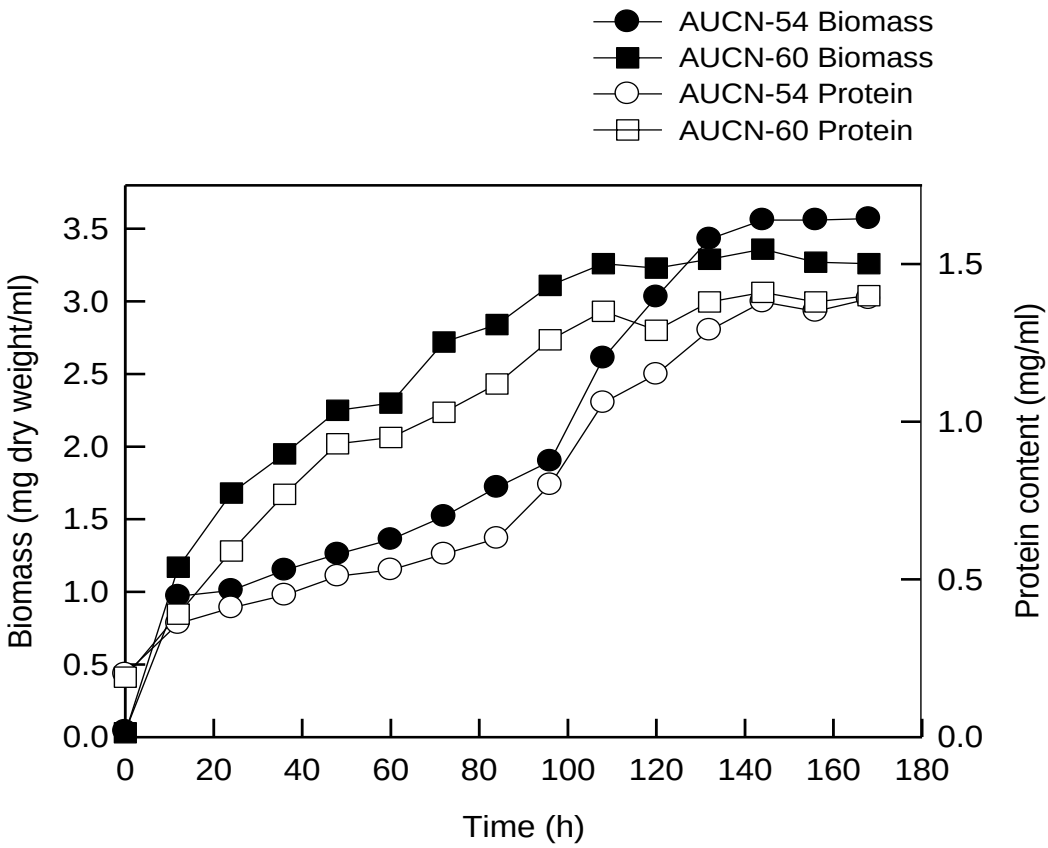


Fig. 7 Time-course for bacterial growth

3.2.2.3. Salt (NaCl) tolerance

Both of the bacterial isolates displayed similar properties towards NaCl tolerance test. They were capable of producing optimal dry weight at a salt concentration ranging between 0.25-10 percent (w/v). Above 10 %, negligible biomass was recorded for both isolates.

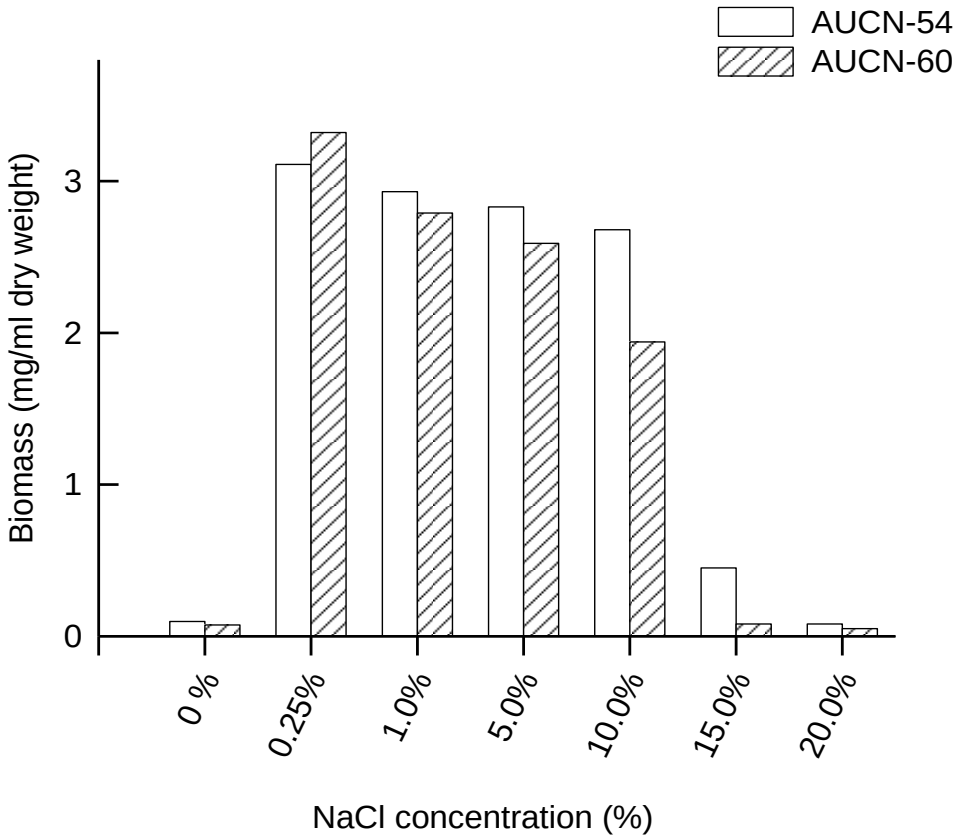


Fig. 8 Growth of bacterial isolates at various salt (NaCl) concentrations

3.2.2.4. Growth at different Na_2CO_3 Concentrations

Both of the bacterial isolates failed to grow at Na_2CO_3 concentration of 3% (W/V) and above. Growth below 0.25% Na_2CO_3 (W/V) was not tested. This is to avoid the risk of cyanide leaking in the form of HCN (Almagro *et al.*, 2005). Maximum biomass measurement for AUCN-54 was recorded at 1% Na_2CO_3 (w/v) and AUCN-60 produced the largest biomass at Na_2CO_3 concentration of 2 % (Fig.9).

Table 6. Variation in pH values of cultures just before incubation and right before harvesting

Na_2CO_3 (%)	AUCN-54			AUCN-60		
	pH values just after inoculation	pH value right before harvesting	Dry weight (mg/ml)	pH values just after inoculation	pH value right before harvesting	Dry weight(mg/ml)
0.25	7.34	9.11	2.7	7.37	9.29	2.73
0.50	8.96	8.61	2.7	8.71	9.71	2.79
0.75	10.10	9.92	2.94	9.86	9.83	2.82
1.00	10.05	9.96	3.22	10.05	10.02	2.94
1.50	10.31	10.15	2.54	10.26	10.16	3.23
2.00	10.36	10.21	2.78	10.39	10.22	3.6
2.50	10.47	10.25	1.67	10.45	10.35	2.04
3.0	10.51	10.45	1.26	10.54	10.50	1.69

Control pH changed from 10.05 $\Rightarrow$ 10.00

3.2.2.5. Growth at various temperatures

Bacterial isolates under study were found to display different properties towards temperature tests. Both of the bacterial isolates failed to show growth at 5^{o}C and 45^{o}C. In addition, isolate AUCN-60 was not able to resist and grow at 35 ^{o}C and above, whereas isolate AUCN-54 adapted and managed to grow at all the tested levels of temperatures except at 5^{o}C and 45^{o}C.

Table 7. Growth of bacterial isolates at various temperatures

Bacterial Isolates	Temperature(^{o}C)							
	5	10	15	20	30	35	40	45
AUCN-54	-	+	+	+	+	+	+	-
AUCN-60	-	+	+	+	+	-	-	-

Keys: (-) No growth, (+) Growth

3.2.2.6. Comparison of bacterial growth on different carbon-sources

Both isolates under study were capable of utilizing most carbon- sources provided when NaCN was used as the only sources of nitrogen. Comparatively, better growth was observed when the isolates were provided with sodium acetate as carbon source. On the other hand, growth yield of both isolates hardly recorded when cyanide was used as sole source of carbon and nitrogen. Moderate growth of both bacterial isolates was evident in a media containing molasses, fructose, glucose and maltose as a source of carbon whereas the isolates were poor in utilizing sucrose and xylose as source of carbon (Fig. 10).

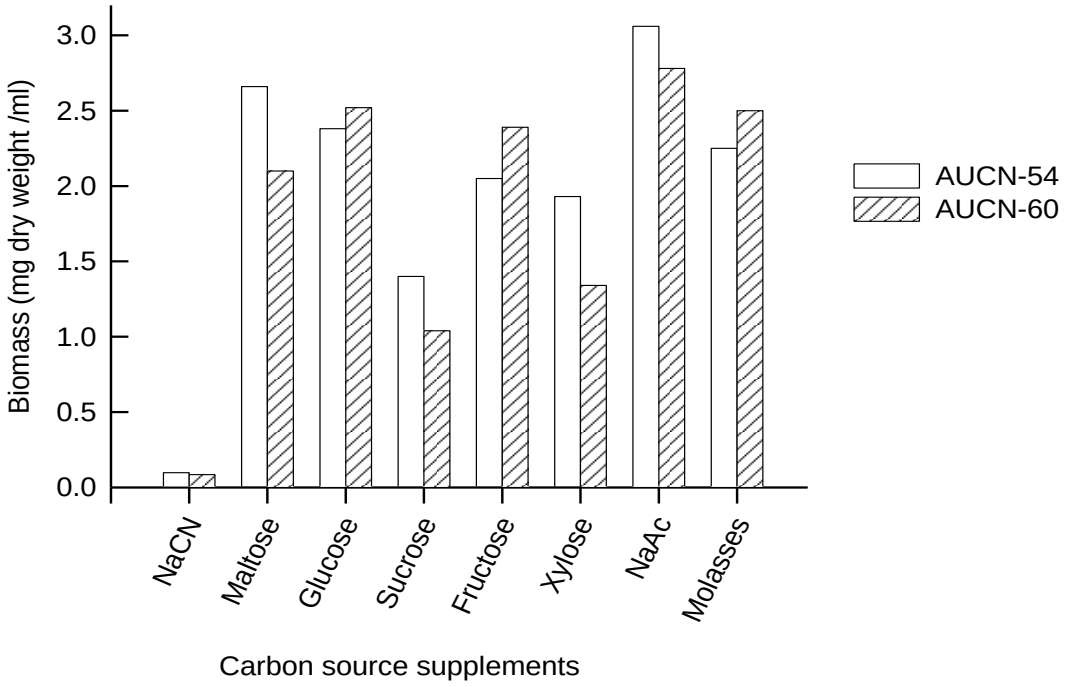


Fig.9 Growth on different sources of carbon

3.2.2.7. Intrinsic antibiotic resistance (IAR)

The evaluation of intrinsic antibiotic resistance of the bacterial isolates revealed that AUCN-60 was resistant to none of the test antibiotics whereas AUCN-54 was able to cope up with Kanamycin, ampicillin and neomycin but inhibited by chloramphenicol and streptomycin sulphate antibiotics at the indicated concentration (Table 8).

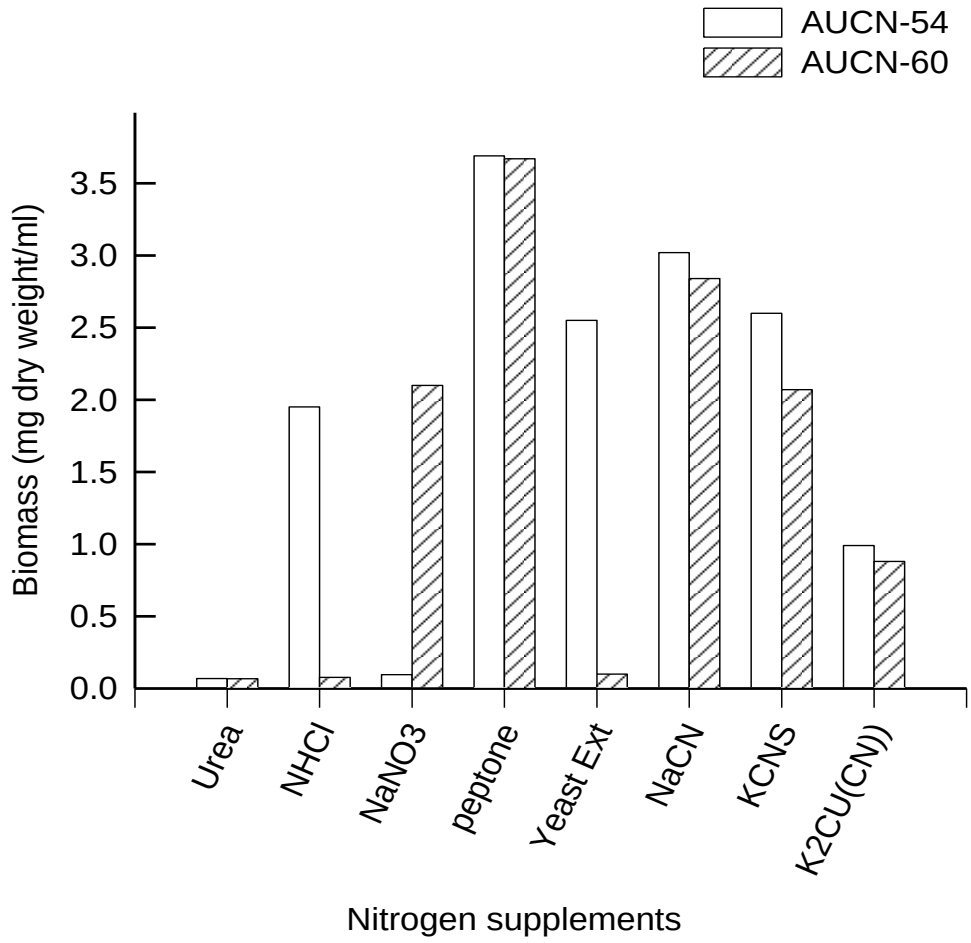
Table 8. Antibiotic resistance of bacterial isolates

Bacterial Isolates	Antibiotics and Their Concentrations(µg/ml)				
	Kanamycin (25)	Streptomycin (30)	Ampicill-in (100)	Neomycin (30)	Chlor-amphenicol (10)
AUCN-54	+	_	+	+	-
AUCN-60	-	-	-	-	-

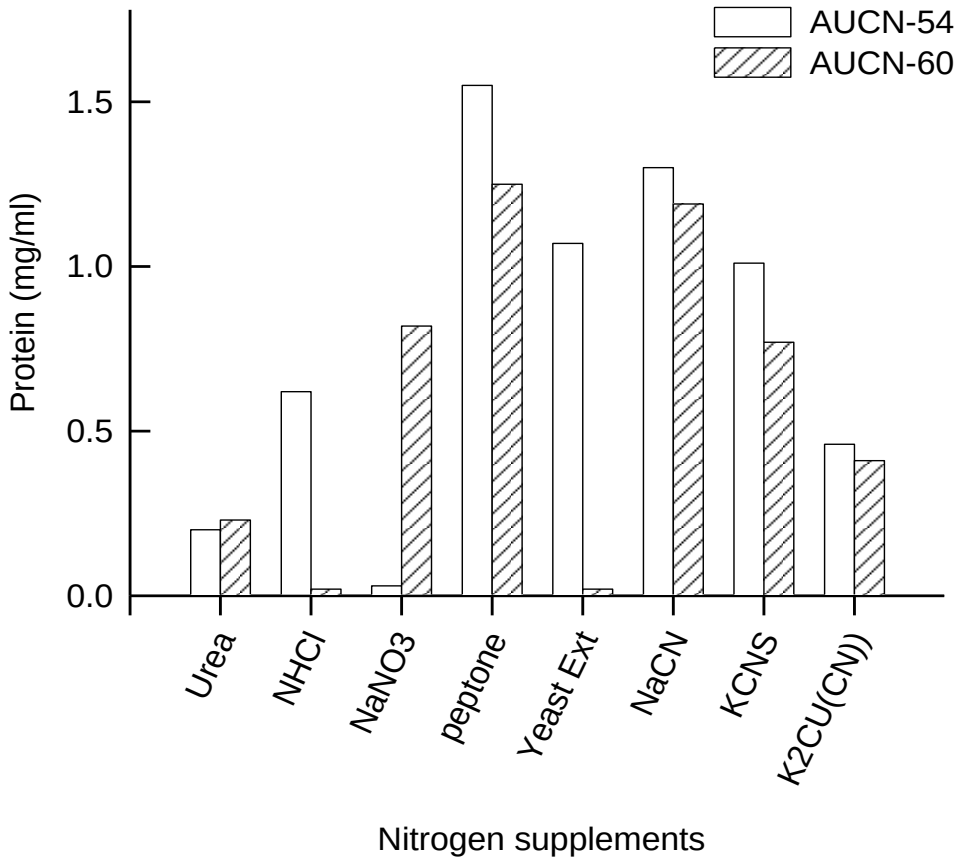
Keys: (-) No growth, (+) Growth

3.2.2.8. Comparisons of bacterial growth using cyanide, organic nitrogen and in organic nitrogen sources

Interestingly, the second largest growth yield, next to peptone, was obtained when sodium cyanide was used as a sole nitrogen source. Both of the isolates were capable of utilizing thiocyanate and cyanide-copper complex as a sole nitrogen sources. However, the growth yield obtained in all cases was not identical (Fig.11). Comparing the different cyanide compounds as nitrogen-sources, sodium cyanide was preferable nitrogen source for both isolates than others, while cyanide-copper complex was poorly utilized. In addition, both isolates were failed to utilize urea as a sole source of nitrogen.



a) Bacterial biomass (dry weight) as function of different nitrogen sources



b) Bacterial protein as function of different nitrogen sources

Fig .10. **C**omparisons of bacterial growth using cyanide, organic nitrogen and inorganic nitrogen sources (a and b)

4. Discussion

Due to the drawbacks of chemical cyanide detoxification methods, the need for biological methods is highly increasing. Many microorganisms (bacteria, fungi and algae) using cyanide as sole nitrogen sources have been isolated from different environments (Akcil, 2001 and Ackil *et al.,* 2003). Such organisms offer an interesting potential for the development of an environmentally friendly method of cyanide removal from mining and related industrial wastes. The majority of cyanide-degrading microbes investigated so far grew in the neutral or acidic pH ranges (Souza *et al.,* 2002). However, at neutral pH and below, cyanide exists as HCN, which is highly volatile and pollutes the atmospheric air.

Thus, cyanide-removal processes based on cyanide-degrading microbes at a neutral pH or below could pose serious environmental problems for people and animals around the treatment sites and beyond. In addition, part of the cyanide molecules will not be available for microbial utilization. Therefore, to avoid the formation of volatile cyanide and its effects, cyanide solutions must be maintained above pKa value of 9.2. This leads to the need for application of alkaliphilic microbes for cyanide removal.

Since most alkaliphilic microbes optimally grow around pH 10, which is above the pKa of cyanide, application of such microbes could be environmentally safe in cyanide detoxification process. To date, there are few reports of alkaliphilic cyanide utilizing microbes (Sorokin *et al.*, 2001; Sorokin and Kuenen, 2005). Hence, the importance of their application in cyanide removal is rarely known. Even though alkalophilic microbes can be isolated from any environments, the diversity of such organisms is expected to be high in naturally occurring alkaline habitats.

In this study, 63 alkaliphilic cyanide-degrading bacterial strains were isolated from Lake Chitu mud sediment samples enriched with sodium cyanide as sole nitrogen source. This might show the importance of the lake as potential source of cyanide removing microbes, which are useful in mining and related industrial waste treatments. From the total of 63 isolates, only two were selected for further study. The biochemical characterization of selected isolates showed that both are aerobic cyanotrophic bacteria. This has significant implication from the application

points of view as aerobically growing microbes are more powerful in removing cyanide than their anaerobic parts (Fallon *et al*., 1991).

Exposure of cyanide degrading microorganisms to higher concentrations of cyanide is known to impair their growth. However, different organisms differ in their maximum cyanide concentration requirement that bring about growth without inhibition (Almagro *et al*., 2005). The two alkaliphilic isolates investigated in this study differed in their tolerance to the increasing concentrations of cyanide (Fig.6). The maximum concentrations of cyanide that brought about optimum growth for AUCN-54 and AUCN-60 were 10.2 & 17.34 mM, respectively. This is far higher than the optimal cyanide concentrations reported recently (Akcil, 2006). The ability of AUCN-54 and AUCN-60 to grow at higher cyanide concentration indicates their potential for cyanide detoxification from cyanide rich wastes. In addition, increasing cyanide concentration was directly related with increasing growth yield of the organisms until inhibitory concentration is reached. This pattern of growth relationship with increasing cyanide concentrations was reported in assimilatory caynotrophic microbes (Souza *et al*., 2002).

Both isolate showed optimum growth at high Na_2CO_3 concentrations, which was used to maintain the pH of the media alkaline. At lower Na_2CO_3 concentration, it is likely that the pH of the media changes (lowers) resulting in the formation of more HCN. But at high Na_2CO_3 concentration, the buffering capacity is high and there is no danger of pH shift to the neutral range (Avalos *et al.*, 1990). The two bacterial strains were isolated from Lake Chitu, an alkaline soda lake with high carbonate/bicarbonate concentrations (Elizabeth Kebede, 1996). This might indicate the potential of alkaline habitat for the isolation of alkaliphiles potentially useful for cyanide detoxification. The growth of both isolates beyond their optimum Na_2CO_3 concentration (1 % for AUCN-54 and 2 % for AUCN-60) was lowered.

Isolate AUCN-60 was found to be more sensitive to all the tested antibiotics than AUCN-54 (Table 8). The latter was tolerant to kanamycin, ampicillin and neomycin but failed to grow in the presence of chloramphenicol and streptomycin. Similarly, Almagro *et al*. (2005) isolated cyanide degrading *Pseudomonas* bacterial strains which were resistant to ampicillin and chloramphenicol but sensitive to kanamycin, streptomycin and gentamycin. Because of the absence of history that shows the exposure of the bacterial original habitat (Lake Chitu) to any antibiotics, resistance of isolate AUCN-54 to antibiotics might be innate /natural.

When cyanide was used as sole nitrogen and carbon source, very poor growth was observed for both organisms. This might indicate that the amount of formic acid released from cyanide was not sufficient as carbon and energy sources. Similarly, Almagro *et al*. (2005) reported that cyanide was not sufficient to serve both as a carbon and nitrogen sources. However, when additional carbon sources were included, better growth was observed, indicating that the organisms need additional carbon and energy sources for growth in cyanide wastes. This has significant implication from the application point of view, because in mining industry, cyanide is used alone and thus the waste does not contain enough carbon and energy sources. Thus, biological detoxification of cyanide from such wastes requires supplementation of carbon and energy sources.

The organisms were grown in the presence of different carbon sources to observe their preference to a particular carbon source. In all carbon sources tested, better growth was observed in sodium acetate, maltose, glucose, fructose, molasses and sucrose than when cyanide was used as sole nitrogen and carbon source. Moreover, the least growth was observed in sucrose. This may be due to poor expression of invertase or low efficiency in breaking sucrose or both.

The growth of the isolates on sugarcane molasses was interesting. Since cyanide alone is poor source of carbon and energy, the molasses utilization potential of the two isolates is important in large scale applications in further studies. Similarly, Patil and Paknikar (2000) isolated bacterial strains capable of utilizing cyanide as sole nitrogen sources when sugarcane molasses was used as carbon and energy sources.

When growth on different nitrogen sources was compared, next to peptone, highest growth was observed in the presence of sodium cyanide. It was very interesting to note that growth on cyanide is comparable to growth on organic nitrogen sources. Obviously, in their natural habitat both isolates are expected not to be exposed to cyanide. In addition, the metabolic machinery for peptone utilization and cyanide utilization are not the same. Thus, this may need further investigation to understand the biochemistry of cyanotrophic microbes.

In addition to growth on sodium cyanide, both isolates were found to utilize copper-cyanide complex as a sole nitrogen source, poorly. The difference in the assimilation of the two forms of cyanide is expressed to be related to their dissociation in water. That is, NaCN readily dissociates in water, yielding CN^- ions, which is easily available to microbial actions (Csonka and Hanson, 1991). Thus, microbes might utilize NaCN in better way to incorporate the nitrogen into their biomass. In contrast, the cyanide ligand in the copper-cyanide complex are tightly bound to the central copper atom and are thus unavailable for bacterial utilization. Therefore, the difference in the growth yield of bacteria when cultivated in NaCN and copper-cyanide complex may be attributed to the degree of substrate stability, which can be explained by the respective physical and chemical properties of the two compounds. In addition to NaCN, both of isolates were able to grow using thiocyanate as sole nitrogen sources. This might indicate that the organisms are capable of detoxifying the main toxic forms of cyanide released in mining and related industrial wastes. Earlier, Sorokin *et al*., (2001) isolated bacterial strains capable of utilizing thiocyanate as sole nitrogen source from the Kenyan and Siberian soda soils. This further shows the potential of the East Africa Soda lakes for isolation of cyanide degrading microorganisms that are powerful in detoxifying cyanide wastes.

Both bacterial isolates under study have managed to utilize cyanide at temperature range of 10 and 30^{o}C (Table 7). This could be very important from the application point of view because in most tropical countries the ambient temperature often ranges in between 25 and 40^{o}C. At pH values below 9.2 (pKa of NaCN), HCN is evolved from NaCN. HCN has a boiling point of 26^{o}C. Thus at high temperature and neutral pH, cyanide is not available for bacterial assimilation (Csonka and Hanson, 1991; Patil and Paknikar 2000). AUCN-60 and AUCN-54 grew at alkaline pH above the pKa of cyanide thus avoiding formation of the volatile HCN.

Thus the ability of the two organisms to grow at high temperature and high pH indicated their potential for biological cyanide detoxification in tropical regions.

The salt tolerance test (Fig.6) indicated that both of the bacterial isolates were able to utilize cyanide at NaCl concentration of 0.25 - 10 %. The isolation of these salt-tolerating cyanide-degrading bacteria may be related with their physiological adaptation to natural saline-alkaline habitat (Elizabeth, 1996; Venton *et al.*, 1998). This might be very interesting as far as the saline conditions of the wastes of mining and other cyanide related industries are concerned.

5. Conclusions and Recommendations

Cyanide is the most potent- poisonous chemical used in various industries. Acute or chronic exposure to it could perhaps be life –costing for workers in these industries and inhabitants nearby the industries. The importance of cyanide waste treatment, especially, by using microbial technology is inevitable. The alkaliphillic cyanide-degrading microbes screened in this study showed a clear indication for the potential for bioremediation of cyanide wastes.

One of the main objectives of this study was to determine the availability of alkalophillic microorganisms that utilize cyanide and its related compounds as a sole source of nitrogen. Consequently, 63 potential alkalophillic cyanotrophic bacterial strains were screened and characterized from one of Ethiopian alkaline soda lake (Lake Chitu). This could indicate the availability of other more powerful and diversified alkaline cyanotropic bacterial groups in these alkaline environments.

Despite incapability of bacterial efficiency measurement in this study, both of the bacterial isolates studied were competitive for resisting and utilizing higher concentration of cyanide (optimum growth yield for AUCN-54 is 10.20mM and AUCN-60 is 17.34mM) than any other microbes reported in the globe. One of the interesting aspects of these isolates is that they were managed to use sugarcane molasses as source of carbon while utilizing cyanide as a sole source of nitrogen. Even though it was not included in this study, this is bright hope in further applications of the isolates in laboratory and large scale applications.

In addition, both of the bacterial isolates were found to be tolerant to salt concentration of 0.25 - 10 %. This might be developed from the environment where they used to manage the highest saline habitat. This has great positive value (since mining wastes contain huge amount of salt and metals) in the ease with which microbes clear cyanide contaminated wastes.

Generally, it was possible to conclude this study with the following points:

- Cyanotrophic microbes are available in Ethiopian alkaline soda lakes
- They could develop the mechanism to resist and utilize cyanide.
- Cyanotrophic microbes were easily screened from their natural habitat and subjected to characterizations.
- The selected isolates were able to degrade all forms of cyanide provided
- Cumulatively, resisting and utilizing cyanide at higher pH and elevated salt concentrations with their ability to use molasses as sole source of carbon indicate that these microbes were potentially powerful candidates in degrading cyanide from industrial wastes.

Based on this study the following points are recommended on:

- determining the rate-limiting steps in the degradation process and supplying an amount of -limiting materials to enhance the growth rate of an organism, hence also the rate of degradations
- Further identification of alkalophilic microbial strains potentially powerful to degrade cyanide and its compounds at pH greater or equal to nine.
- Further isolation and characterization of cyanide – degrading microbial enzymes and their mechanisms of action
- Studying cyanide degrading microbial ecology and genetics

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