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DIVERSITY AND EFFICIENCY OF CYANIDE DEGRADING ALKALIPHILIC BACTERIA FROM ETHIOPIAN SODA LAKES

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To GOD be the glory!!!

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Abbreviations

16S rDNA ---------- 16S ribosomal Deoxyribonucleic Acid

16S rRNA ---------- 16S ribosomal Ribonucleic Acid

APHA ---------- American Public Health Association

ARDRA---------- Amplified Ribosomal DNA Restriction Analysis

ATSDR ---------- Agency for Toxic Substances and Disease Registry

BLAST---------- Basic Local Alignment Search Tool

BS---------- Basal Solution

CICAD---------- Concise International Chemical Assessment Document

CN^- ---------- Cyanide

CNO---------- Cyanate

CTAB ---------- Cetyltrimethylammonium Bromide

DNA---------- Deoxyribonucleic Acid

ECETOC---------- European Centre for Ecotoxicology and Toxicology of Chemicals

HCN---------- Hydrogen Cyanide or Hydrocyanic acid

IAEA ---------- International Atomic Energy Agency

MEGA ---------- Molecular Evolutionary Genetics Analysis

MERG---------- Mining Environment Research Group

NaCN---------- Sodium Cyanide

NCBI ---------- National Center for Biotechnology Information

NUFU ---------- Norwagian Universities Programme for Development, Research and Education

OD ---------- Optical Density

PCR---------- Polymerase Chain Reaction

pKa ---------- Acid Dissociation Constant

RDP ---------- Ribosomal Database Project

SAD ---------- Strong Acid Dissociable Cyanide

SCN ---------- Thiocyanates

WAD ---------- Weak Acid Dissociable Cyanide

Abstract

The aim of this study was to isolate and characterize cyanide degrading alkaliphilic bacteria from Ethiopian Soda Lakes. Seventy two isolates capable of growing on a medium provided with cyanide as a sole nitrogen source has been found. Based on their morphological features, thirty isolates were screened for subsequent identification. ARDRA of the 16S ribosomal genes amplified by PCR was used to screen these isolates. Restriction analysis was done using three endonucleases namely; *AluI, HaeIII* and *RsaI*. ARDRA revealed the presence of nine polymorphic groups among the thirty. 16S rDNA amplicons of representative strains were sequenced and compared with sequences from NCBI and RDP databases. This revealed the presence seven bacterial strains from which three belonged to genus *Bacillus,* three to genus *Halomonas,* and the other one to uncultured bacterium clone, FJ152630. Based on phylogenetic analysis, the unidentified strain was more related to and clustered with the *Halomonas* sub-lineages. Strains CNS_{10}, CNA_{12} and CNC_1 isolated from Lake Shala, Lake Abijata and Lake Chitu respectively were found to be better candidates in that they have utilized up to 99.33% of 200mg/l cyanide and tolerated up to cyanide concentration of 600mg/l in batch mode with pH of 10.22. In addition to cyanide, these strains used other organic and inorganic nitrogen sources, yeast extract being the most utilized. Acetate was being provided as a source of carbon and energy source but most interestingly all the strains have aggressively grown utilizing cyanide when provided with cheap molasses as carbon source. In general, this study indicated the presence of diverse cyanide utilizing bacteria with visible potential for practical application.

1. Introduction

Cyanide compounds, both organic and inorganic, are found widely distributed on our planet. Indeed they have been postulated to have played a key role in the prebiotic chemistry that led to the evolution of biological macromolecules and primitive life (Commeyras *et al.,* 2004). However, in recent years there is a growing concern on the potential toxicity due to huge amount of cyanide containing wastes generated through anthropogenic activity.

Cyanides are set of compounds that contain a carbon-nitrogen group (connected with three molecular bonds, $[:C\equiv N:]^-$) found in several forms, HCN being the most common and extremely deadliest metabolic poison. Despite cyanide's toxicity to living organisms, a greater number are capable of degrading cyanide and utilizing it primarily as a nitrogen source (Dash *et al.,* 2009).

The existence of such pathways has allowed the development of biotechnologies to degrade cyanide compounds in industrial waste streams. The latest cyanide removal method by this time is cyanide biodegradation, which is an environmentally friendly and quite inexpensive treatment method (Dubey and Holmes, 1995).

There are many organisms isolated for cyanide degradation most of which are active at neutral pH. From the practical point of view, however, free cyanide gets out from aqueous solutions as poisonous gas and due to this most of cyanide containing wastes have high pH. Therefore it is important to isolate and characterize alkaliphilic organisms that can breakdown cyanide compounds at highly alkaline conditions.

1.1. Sources of Cyanide wastes

Naturally, cyanide containing chemicals are produced by a wide range of life forms including plants, fungi, bacteria, algae and arthropods as part of their normal metabolism (MERG Report, 2001), as defensive metabolite (Møller and Seigler, 1999; Knowles, 1976) or with invasive purposes (Gallagher and Manoil, 2001). However, the large scale presence of cyanide compounds in the environment is attributed to anthropogenic activities: as they are extensively

used in many industries. Cyanide is commonly found in wastewaters from various applications and industries including metal cleaning, electroplating, metal processing, automobile parts manufacture, steel tempering, mining, nuclear processing, photography, pharmaceuticals, coal coking, ore leaching, plastics, pesticides, fumigants, fire retardants, etc (IAEA, 2006; Akcil, 2003; MERG Report, 2001).

Over one million tons of cyanide (about 80% of the annual product of cyanide compounds) is used in the chemical industries for the production of organic chemicals such as nylon and acrylics, in electroplating, metal processing and photographic applications (Akcil, 2003; Botz and Mudder, 2002; McKinnon, 2002).

Cyanide has been the preferred lixiviant worldwide since 1887 in extracting gold and other metals such as silver, copper and zinc from ores. Each year, over a billion tons of gold ore are treated using this process (Young and Jorda, 1995), which means a significant demand for cyanide by these industries. According to some estimates up to 90% of gold is produced using cyanide (Gurbuz *et al.,* 2009).

In the mining of precious metals, a process known as cyanidation process (I), converts gold and silver to water soluble cyanide complexes (Hagelstein, 1997).

$$4\ Au + 8\ NaCN + O_2 + 2\ H_2O \rightarrow 4\ Na[Au(CN)_2] + 4\ NaOH \quad \text{……..….. (I)}$$

Gold is recovered from the cyanidation solution by cementation with zinc (II) leaving a solution which bears cyanide, metallo-cyanide complexes, cyanates, and thiocyanates (SCN) complexes along with other chemical species dissolved from the ore.

$$2\ Na[Au(CN)_2] + Zn \rightarrow 2\ Au + Na_2[Zn(CN)_4] \quad \text{..…… (II)}$$

The amount of NaCN generated annually, the land area needed to dump the waste and the quantity of water used for processing 250,000 tons of ore per year are 125 tons, 1.56 ha and 365.000m^3 respectively (Korte, 1993: *cited in* Gurbuz *et al.,* 2009)

The industrial effluents generally contain between 0.01 and 10 mg/L of total cyanide. However, some cyanide wastes from individual operations at electroplating and metal finishing plants can be stored for periods of years, after which the effluent may contain from1% to 3% (10,000–30,000 mg/L) of cyanide. In fact some industrial effluents from electroplating plants have been found to contain even higher cyanide levels of 100,000 mg/L. Effluents from these industries have very high concentration of cyanide as compared to cyanide levels of about 0.001–0.05 mg/L found in unpolluted stream and lake water (Dash *et al.,* 2009).

1.2. Toxicological properties of cyanide and its compounds

Aqueous solutions of cyanide at neutral and acidic conditions are not thermodynamically stable as cyanide ion combines with hydrogen and form Hydrogen cyanide. HCN is a colorless or pale blue liquid or gas with a faint bitter almond like odor. It is a very weak acid, with a pK_a value of 9.22 at 25^{o}c and soluble in water and alcohol (ATSDR, 1997; CICAD, 2004). At pH 7.0, about 99.5 % of the cyanide exists as HCN and at lower pH than this; essentially all dissolved cyanide is present as HCN (May and Alexander, 2005; Rychte *et al.,* 1979).

Cyanide ion also combines with metals and other compounds in various degrees. The alkali and alkaline metals form readily soluble cyanide salts in water. The cyanide used in the processing of ore is sodium cyanide (NaCN) that dissolves readily in water, yielding sodium and cyanide ions. Transitional metals form various complexes with different stability. Zinc and copper form relatively weak complex while gold, silver, iron, and cobalt form strong complexes with cyanide. Although thiocyanate (SCN) and cyanate (CNO) are weak acid dissociable cyanide (WAD), they often considered in their own categories. Cyanides also combine with some substances to form organic cyanides like acrylonitrile, propinonitrile. Generally, the chemistry of cyanide is very complex and there are usually many different forms of cyanide in the environment.

Toxicologically, these different forms of cycanide can be subdivided into four groups listed in Table 1 below.

Table 1: Toxicological classification of cyanide species (Mekonnen Meaza, 2008; Environment Australia, 2003; MERG Report, 2001; Smith and Mudder, 1991).

Cyanide Compounds	Toxicological properties
Free cyanides: CN-, HCN	Absorbed by ingestion or inhalation and causes cytotoxic hypoxia by inhibition of cytochrome oxidase, which disables the cells' oxygen utilization in the electron transport chain. At sufficient concentrations this causes a lethal suppression of the central nervous system.
WAD cyanides: $Zn(CN)_4^{-2}$, $Cu(CN)_3^{-2}$, $Ni(CN)_4^{-2}$	They are less toxic than the free cyanides, 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.
$Fe(CN)_6^{-4}$ $Fe(CN)_6^{-4}$	Show the same kind of toxicity as the WAD cyanides, but should be considered more hazardous from an ecotoxicological perspective because of their high stability in the environment.
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 then died within a week. Cyanate was shown to be somewhat more toxic to fish than thiocyanate.

1.3. Impacts of Cyanide on environment and human health

There is a controversy and debate between environmentalists and mining companies about the toxicity of different cyanide compounds (Brinne, 2000) due to underestimation or exaggeration. However, various studies have evidently investigated the potential toxic effects of cyanide compounds to the whole environment and human health, emphasizing on their dangerous consequences. Accordingly, they stress on the need to give attention for the issue in two angles; one to find out some alternative means in their industrial applications and second to explore ways to break down them to less- or non-toxic forms.

1.3.1. Environmental Impacts of Cyanide

Several incidents have been reported worldwide on environmental disasters associated with cyanide leakage from processing industries and tailing dams (Gurbuz *et al.,* 2009; Bosecker and Blumenroth, 2001; Brehaut, 2000; Logsdon *et al.*, 1999; Dumestre *et. al.,* 1997).

As an example, in 1990, a leak in Colorado (USA) was reported to have destroyed aquatic life along a 17-mile stretch of one river, and in the same year, 10 million gallons of cyanide solution spilled into the South Carolina River (USA), killing thousands of fishes (Young, 1992). In some cases like leakage from Arnul mine in Baja Mare, Romania, down to a river has forced the communities nearby to find alternative sources of drinking water, apart from killing aquatic life along the way.

Aquatic organisms especially fishes being very sensitive even to small concentrations, cyanide is toxic to most species in varying amounts. Exposure to cyanide in solution through consumption of surface water is the main exposure route for most animals affected by cyanide poisoning, but concurrent exposure through inhalation and skin absorption may also occur. Cyanide contamination of rivers, lakes, and seas can permanently damage some species. Toxicological studies have indicated that short-term acute exposure to high levels of cyanide can harm the nervous, respiratory and cardiovascular system of animals and can finally cause death at higher concentrations. Migratory birds have suffered from cyanide poisoning associated with heap leaching facilities and tailings ponds. They may absorb cyanide through their skin when wading or swimming, or ingest it through drinking. Small mammals, such as rodents, foxes, rabbits, bat have been found dead near cyanide containing tailings ponds, which they may have used as a drinking water source (MERG Report, 2001).

However, the most common environmental problems are due to chronic contamination of surface and ground water by low concentration of cyanide and related water breakdown compounds like metal cyanide complexes, cyanate, thiocyanate, ammonia and nitrate. Such releases are much more difficult to detect and evaluate than the acute (Brehaut, 2000; Logsdon *et al*, 1999).

1.3.2. Public Health Impact

Cyanide is a toxic compound for almost all organisms since it binds irreversibly to many metalloproteins, such as the cytochromes involved in all known respiratory processes. Due to this, cell and tissue utilization of oxygen is impaired, and with time, a state of cytotoxic and histotoxic anoxia occurs (oxidative metabolism is brought to complete cessation). Because of these, cells shift from aerobic to anaerobic metabolisms and lead to accumulation of lactic acid. Due to its high dependency on oxidative metabolism and limited anaerobic capacity, the central nervous system is particularly vulnerable to cyanide intoxication (Way, 1984). Even the product of anaerobic respiration (the lactic acid) fastens the depression of the central nervous system and result in respiratory arrested death (McKinnon, 2002). It is a very fast acting poison capable of killing a person within minutes if exposed to a lethal dose without prompt first aid treatment. Cyanide can also affect other organs and systems in the body, including the heart and spleen when exposed to higher lethal concentrations. People exposed to non-fatal doses normally recover in a short time, and there has been no evidence to suggest that it causes mutation, cancer or affects the reproduction system (MERG Report, 2001).

The principal routes for cyanide absorption to human body are inhalation, ingestion, and eye and dermal exposure. The amount and speed of cyanide arrival to particular body parts differ based on routes of absorption as well as external and internal body conditions. Following cyanide exposure, regardless of its type, toxic amounts of hydrogen cyanide is absorbed with great rapidity through the bronchial mucosa and alveoli (ATSDR, 1997). In other words, essentially all cyanide ingested as cyanide salts will form hydrogen cyanide and quickly absorbed to the respiratory system. This is because of the physiological pH condition of human body that is much lower than the pk_a value of cyanide, 9.2. (ECETOC, 2004; CICAD, 2004).

Approximately 40 enzymes are found to be inhibited by cyanide. This includes a number of important metalloenzymes containing iron, copper, or molybdenum for their most part. Alkalinephosphatase, carbonicanhydrase, catalase, peroxidase, ascorbicacidoxidase, xanthine oxidase, and succinicdehydrogenase are some of the common examples (CICAD, 2004; ATSDR, 1997; US EPA, 1990; Ardelt et al., 1989).

Cyanide toxicity to human depends on the nature of exposure and variability's and dose-response effect among individuals (Wajana Lako, 2007). A person exposed to higher dose of cyanide shows symptoms which may include; headache, drowsiness, weak and rapid pulse, deep and rapid breathing, nausea and vomiting. Convulsions, dilated pupils, clammy skin, a weaker and more rapid pulse, and slower, shallower breathing can follow these symptoms. Finally, the heartbeat becomes slow and irregular, body temperature falls, the lips face and extremities take on blue color. The person falls in coma and death may occur (McKinnon, 2002).

2. Theory and Principles

2.1. Approaches to treat Cyanide-containing wastes

To protect the environment and the public from adverse impact of cyanide compounds, effluents containing cyanide from various industries must be treated before discharging into the environment (Dash *et al*., 2009; Raybuck, 1992). Hence, many countries and environmental protection agencies have put regulations that impose limiting standards for discharge of cyanide bearing wastewater. To mention few, the US Environmental Protection Agency has proposed a limit for drinking and aquatic biota waters regarding total cyanide (free and metal-complexed cyanides) to be 0.2 and 0.05 mg/l respectively (US EPA, 1985). The German and Swiss regulations have also set limit of 0.01 mg/l cyanide for surface water and 0.5 mg/l for sewers (Parga *et al*., 2003). Therefore, developing and applying cyanide removal technologies is necessary for lowering the concentration of cyanide compounds to a level that can have least possible effect to the environment and human health.

There are a range of techniques available for the treatment of cyanide and its compounds in industrial waste waters (Dash *et al.*, 2009; Akcil, 2003, 2002a,b and 2001; Botz, 2001). These treatment processes are classified as physical processes (usually have less preference) versus destruction-based processes of cyanide recovery and activated carbon sorption. In a destruction process, either chemical or biological reactions are utilized to degrade cyanide compounds. And, most of these destruction processes operate on the principle of converting cyanide into one or more less toxic compounds through an oxidation reaction.

Determining the most appropriate method of cyanide removal should be based on consideration of many factors such as the nature and volume of the waste, environmental setting, and review of regulatory framework that governs its discharge into the environment.

There are several chemical destruction processes that are well proven to produce effluents with low levels of cyanide and metals. However, the current available chemical methods are not well adapted and efficient with regard to economical, technological, and environmental costs (Akcil and mudder, 2003; Akcil *et al.,* 2003; Akcil, 2001). These methods are often expensive and sophisticated to operate. They are also designed to target free cyanide and are generally less efficient against other cyanide forms, such as thiocyanate and metal cyanide complexes (Rorke and Mühlbauer, 1999). Some of them create additional toxic and biological persistent chemicals (Akcil *et al*., 2003).

Based on these economical and environmental evaluation, biological treatment of cyanide from wastewater has been shown a viable, efficient and cost-effective alternative to treatment processes available for cyanide (Akcil and Mudder, 2003; Akcil *et al*., 2003; Botz, 2001).

2.2. Biodegradation of Cyanide compounds

Despite the well-known toxicity of cyanide to living organisms (Akcil and Mudder, 2003; Dumestre *et. al.,* 1997), biological treatments are feasible alternatives to physical and chemical methods (Akcil, 2003), because a wide range of organisms are known to utilize such toxic chemicals as sources of energy or nutrition (Brinne, 2000; Castric, 1981: *cited in* Dumestre *et. al.,* 1997; Harris *et al,* 1987). Some of these organisms use cyanide as a nitrogen source and some others as both nitrogen and carbon source (Zlosnik and Williams, 2006; Mekonnen Meaza, 2008).

In biological treatment of cyanide, the microorganisms convert free and metal-complexed cyanides to bicarbonate and ammonia, while the free metals (if found complexed) are either adsorbed within the biofilm or precipitated from solution. The ease with which the metal cyanide complexes are degraded generally follows their order of chemical stability with free cyanide being the most readily degradable and iron cyanide the least.

These biological processes have been proven at large scale in well-understood and engineered systems in several countries. There are examples of biological processes finding applications in the treatment of industrial effluents containing cyanide (Patil and Paknikar 2000; Dictor *et al.,* 1997). One aerobic biological treatment process that has been successfully applied in the detoxification of industrial effluents at a Homestake Mining Co. operation in South Dakota, USA utilizes a strain of *Pseudomonas paucimobilis* specifically acclimatized to the waste (Baxter and Cummings, 2006).

As indicated in Dubey and Holmes (1995), an outline for a framework for the development of processes for biological cyanide degradation includes:

- Identification of microorganisms able to degrade cyanide completely or partially, and having cyanide resistance.
- Determination of the criteria for optimal growth of these organisms and for their efficient degradation of cyanide.
- Establishment of the metabolic pathways involved in cyanide degradation for the organisms, in order to determine the rate-limiting steps in the degradation process.
- Identification and localization of genes for cyanide degradation, to see if they are chromosomal or located on plasmids, organized as an operon etc.
- Identification of microbial strains suitable for practical applications.
- Development of practical engineering processes that is suited to the chemical and physiological characteristics of the microorganisms and to the properties of the materials to be processed.

Biological treatment of cyanide contaminated wastewater is an option with many advantages. Unlike chemical treatment methods the end products are non-toxic, the costs are relatively small and all forms of cyanide can be treated. They have a higher capital cost, but a significantly lower operating cost, so that the present-worth cost is significantly lower for the biological methods (Akcil 2003; Desai and Ramakrishna, 1998). Biological treatment of cyanide polluted wastes has its own advantages and disadvantages compared to physical and chemical methods (Table 2).

Table 2. Advantages and disadvantages of biological treatment method (Dash *et al*., 2009; Mekonnen Meaza, 2008; Ebbs, 2004; Smith and Mudder, 1991).

Advantages:
• The process is simple in design and process control is minimal. • Generally inexpensive, reagent costs for nutrients are comparatively very low. • All forms of cyanide are treatable. • Superior resistance to shock and upsets. • No chemical handling equipment or expensive control needed. • Heavy metals are removed through a combination of adsorption and precipitation. • Concomitant biogas generation in anaerobic conditions • Removes thiocyanate and ammonia • environmentally acceptable treatment effluent
Disadvantages:
• It tends to be very site specific and specific study require for each type. • Capital costs are slightly higher than those of other treatment processes. • Cannot treat high concentrations. • The performance of the process is affected more adversely by cold temperature than other processes. • Suitable microbial populations may not be found. • Additional treatment may be required if the residual effluent metal concentrations exceed acceptable levels. • The cyanide is not recovered. • The process will only operate under a fairly continuous flow of tailings water and is less suitable for batch treatment. • The technology is not well established. There are too few operational full-scale reactors.

Biological treatment can be applied in many situations, under many conditions, and in various configurations including in situ, aerobic and anaerobic, active and passive, and suspended and attached growth (Gurbuz *et al.,* 2009; Mekonnen Meaza, 2009).

However, it has to be kept in mind that none of the chemical, physical and biological treatment methods are suitable under all conditions, and some treatments may require a combination of processes in order to attain the intended environmental goals.

2.3. Mechanism and pathway of Cyanide biodegradation

Organisms growing in the presence of cyanide must have a cyanide-insensitive metabolism, such as the cyanide-insensitive oxidase (or the cytochrome *bd*) in bacteria (Richardson, 2000; Matsushita *et al.,* 1983; both cited in Almagro *et al*., 2005b). Another problem due to the presence of cyanide in the environment is the formation of extremely stable metal-cyanide complexes that make essential metals unavailable to the organisms. Therefore, bacterial proliferation in the presence of cyanide requires specific metal uptake systems such as the strategy for iron assimilation which consists of the production of organic compounds, and generically called siderophores (Huertas *et al.,* 2006). Finally, a cyanotrophic microorganism requires an assimilatory pathway able to convert cyanide into ammonium and other less toxic compounds (Almagro *et al*., 2005b; Dubey and Holmes, 1995; Raybuck, 1992).

Common biodegradation pathways of cyanide are described in several reports. However, additional organisms with the capacity for cyanide biodegradation with new pathways are still being reported. More than one pathway can also be utilized for cyanide biodegradation in some organisms (Ebbs, 2004). Generally, there are four general pathways for the biodegradation of cyanide: hydrolytic, oxidative, reductive, and substitution/transfer (Dash *et al.,* 2009; Ebbs, 2004; Kunz *et al.,* 2001).

***Hydrolytic reactions*:** are catalyzed by cyanide hydratase (primarily a fungal enzyme), forming formamide, or cyanidase, which produces formate and ammonia. Cyanide hydratase and cyanidase are bacterial enzymes and have recently been shown to have similarity at both the amino acid and structural levels to nitrilase and nitrile hydratase enzymes (O'Reilly and Turner,

2003), which are found in a wide variety of bacterial, fungal and plant species. Nitrilases and nitrile hydratases convert both aliphatic and aromatic nitriles to the corresponding acid or amide, respectively, but show less substrate specificity than cyanide hydratase and cyanidase.

Cyanidase: $HCN + 2H_2O \rightarrow HCOOH + NH_3$

Cyanide hydratase: $HCN + H_2O \rightarrow HCONH_2$

Nitrile hydratase: $R–CN + H_2O \rightarrow R–CONH2$

Nitrilase: $R–CN + 2H_2O \rightarrow R–COOH$

R represents either an aliphatic or aromatic group

***Oxidative reactions*:** the oxidative reactions for the biodegradation of cyanide produce either CO_2 and NH_4^+ in a single step (in the presence of cyanide dioxygenase) or cyanate in the presence of cyanide monoxygenase. Then Cyanase further catalyzes the bicarbonate-dependent conversion of cyanate to ammonia and carbon dioxide (Ebbs, 2004; Harris and Knowles, 1983a,b). Cyanases have been identified in numerous bacteria, fungi, plants and animals.

Cyanide dioxygenase: $HCN + O_2 + 2H^+ + NAD(P)H \rightarrow CO_2 + NH_3 + NAD(P)^+$

Cyanide monoxygenase: $HCN + O_2 + H^+ + NAD(P)H \rightarrow HOCN + NAD(P)^+ + H_2O$

***Reductive reactions*:** this is not much common and enzyme required for such pathway is found in rare species. These reactions follow a two step mechanism (Raybuck, 1992) resulting in the formation of methane and ammonia.

$$HCN + 2H^+ + 2^{e-} \rightarrow CH_2{=}NH + H_2O \rightarrow CH_2{=}O$$
$$\downarrow$$
$$CH_2{=}NH + 2H^+ + 2e^- \rightarrow CH_3–NH + 2H^+ + 2e^- \rightarrow CH_4 + NH_3$$

***Substitution/transfer reactions*:** in this reaction pathway cyanide, in the presence of serine or cysteine residues, may be converted into β-cyanoalanine or α-aminonitrile, catalyzed by β-cyanoalanine synthase, followed by hydrolysis of the products to release NH_3 and an acid (Castric and Strobel, 1969; cited in Almagro *et al.,* 2005b; Blumenthal *et al.,* 1968; Dunnill and

Fowden, 1965). During this process there is no direct requirement for O_2 or NAD(P)H, nor is CO_2 released.

Another pathway catalyzed by sulfurtransferase enzymes results in the conversion into thiocyanate which is less toxic than cyanide compounds. The thiocyanate could then be biodegradaded either by carbonyl pathway or cyanate pathway. The biodegradation of thiocyanide by carbonyl pathway (in the presence of thiocyanate hydrolase) produces carbonyl sulfide (COS) and by cyanate pathway (in the presence of cyanase) results in the formation of sulfate and CO_2 (Ebbs, 2004).

Cyanoalanine synthase:

$$\text{Cysteine} + CN^- \rightarrow \beta\text{-cyanoalanine} + H_2S$$

Thiosulfate: cyanide sulfurtransferase

$$CN^- + S_2O_3 \rightarrow SCN^- + SO_3^{2-}$$

2.4. Factors affecting cyanide biodegradation

The process of microbial cyanide degradation is known to be influenced by a number of factors (Gurbuz *et al.,* 2009). In one or another way, since the process of cyanide biodegradation is catalyzed by enzymes and many conditions affect their activities, the efficiency of microbial biodegradation is influenced by:

- pH of the solution
- Temperature of the environment
- Oxygen level
- Nutrient availability
- Concentrations of cyanides
- Oxidation-reduction potential
- The presence of additional pollutants at contaminated sites
- Microbial population

From the cyanide chemical properties point of view, the biological treatment of industrial effluents contaminated with cyanide requires an alkaline pH above its pK_a value, 9.2, in order to avoid the formation of volatile HCN. In addition, since cyanide is known to react chemically with some keto groups (the Kiliani reaction), the use of glucose or similar carbon sources should be avoided and mostly (including in this work) sodium acetate is used as a carbon source for isolation of cyanide degrading microorganisms.

As indicted by Patil and Paknikar (2000), temperature variation is also another factor that highly influence microbial cyanide degradation. According to their report, the activity of cyanide degrading enzymes, microbial growth, and the stability of cyanide and its compounds are all influenced by temperature differences.

Most organisms capable of biodegrading cyanide are sensitive to cyanide concentration, with biodegradation and/or growth rate decreasing above specific thresholds for each organism (Raybuck, 1992). As effluents from some industries can have concentrations above the maximum range for most organisms, biological treatment may not be a viable option. The formation of degradation products, such as ammonia, or the presence of other nitrogen sources (e.g. nitrate and nitrite) or contaminants (e.g. phenol) may also limit the efficacy with which some waste streams can be treated (Ebbs, 2004).

Addition of nutrients to the contaminated sites and supply of sufficient oxygen to the microbes (since aerobic cyanide degradation is much more efficient) greatly enhance the rate of cyanide biodegradation. The presence of metals found complexed with cyanide have also effects on the biodegradation process. According to Moran (1998), some metal ions like the trivalent metal cations (Sc^{3+}, Fe^{3+}, Cr^{3+}, and Tb^{3+}) enhanced the enzymatic activity of cyanide dehydratase approximately two fold, where as Hb^{2+} and to a less extent Pb^{2+} and Ag^{2+} were inhibitory.

2.5. Cyanide degrading microbes

A number of microorganisms that are able to degrade cyanide and its metal complexes have been identified to date (Dash *et al.,* 2009; Baxter and Cummings, 2006; Gurbuz *et al*., 2004; Akcil *et al.,* 2003; Adjei and Ohta, 1999; Barclay *et al.,* 1998; Goncalves *et al.,* 1998; Pereira, *et al.,*

1996; Dubey and Holmes, 1995; Raybuck, 1992; Harris and Knowles, 1983a) and majority of these are bacteria and fungi. They show a wide range of metabolic functions and can thrive in multiple environments allowing for uptake, treatment, sorption and precipitation of cyanide, its related compounds and metals (Akcil *et al*., 2003; Akcil, 2002a,b; Nelson *et al*., 1998; Oudjehani *et al*., 2002).

Previous works have reported microbial degradation of cyanide at neutral or acidic conditions (Suh *et al.,* 1994; Raybuck, 1992; Barclay *et al.,* 1998). But the major challenge for biodegradation of cyanide compounds is the alkalinity of cyanide containing wastewaters and the volatilization of cyanide under neutral or acidic conditions to hydrocyanic acid (HCN), a weak acid with a pK_a value of 9.2. Also, strains adapted to survive in the presence of cyanide at alkaline pH but unable to degrade it have also been described (Dumestre *et. al.,* 1997).

By contrast, references describing cyanide biodegradation at alkaline pHs are scarce. A fungus *F. solani* (Dumestre, *et. al.,* 1997) and bacteria *P. pseudoalkaligenes* (Igeño *et al.*, 2007; Almagro *et al.,* 2005a,b), *Marinobacter* species (Brinne, 2000), and Burkholderia *cepacia* (Adjei and Ohta, 1999) are examples among those few which can produce novel enzymes capable of degrading cyanide at highly alkaline pH. Such microbes are the ones suitable for practical applications of cyanide biodegradation. Therefore, continuous search for cyanotrophic microorganisms capable of degrading cyanide at alkaline conditions is the principal element for the effort being made to develop efficient biotechnological methods of cyanide removal.

As part of such research works, it is relevant to focus on alkaline soda lakes as sample sources to isolate alkaliphilic bacteria which can thrive in high alkalinity conditions of cyanide waste waters and simultaneously can utilize it as sole nitrogen source. The rationale behind using this sample for treatment of cyanide containing synthetic waste was the pH similarity between alkaline soda lake and cyanide containing industrial effluents. To avoid volatilization of hydrogen cyanide, processing industries involving cyanide use at alkaline condition and generate alkaline effluents. Therefore, cyanide biotreatment of these alkaline effluents require alkaline condition (greater than 9.2) and the alkaline environment adapted sample is assumed to be fittest for this purpose.

Mekonnen Meaza (2008) and Wajana Lako (2007) have isolated some alkaliphilic bacteria capable of degrading cyanide; from one of the Ethiopian Soda Lakes, Lake Chitu. The former has reported two alkaliphilic cyanotrophic isolates and the later applied mixed culture from the lake to a synthetic waste water containing cyanide and maintained for long time in anaerobic-aerobic continuous reactor. From this, some cyanotrophic microbes have been isolated. These studies have shown us the potential of these soda lakes as sources of cyanide degrading microorganisms. Based on the information, three Ethiopian Soda Lakes located in the Great Rift Valley namely: Lake Abijata, Lake Chitu and Lake Shala have been selected to search more bacteria capable of cyanide degradation and characterize them using molecular techniques so that is possible to assess their diversity and to infer their exact taxonomic positions.

2.6. Molecular Identification of microorganisms

Traditionally, bacterial identification has been conducted using a variety of morphological and biochemical tests that allow the grouping of microbial isolates into genera and species. However, phenotypic identification is over dependent on a small number of subjectively chosen properties, which might lead to placing the bacteria in the wrong genus and the properties looked for are themselves inadequate for identification. Moreover phenotypic identification is time consuming, laborious and also affected by culture conditions. Now a days, molecular methods of microbial identification are often used in addition to or instead of morphological and biochemical techniques. Molecular methods involve examining the DNA of the bacterium in question, either by using a technique to map certain important characteristics of a bacterial genome, or by sequencing a portion of the bacterial DNA. Results are then compared to a database of known bacteria; hopefully resulting in a "match" that allows identification. DNA sequencing has become so standard and straightforward that it is now often easier and quicker than traditional biochemical methods. Particularly, molecular identification by 16S rDNA sequence is a valuable tool for identifying and characterizing bacterial diversity and it overcomes many of the shortcomings mentioned above (Babu *et al.*, 2004).

2.6.1. The suitability of rRNA gene for identification and phylogenetic studies

16S rRNA gene is the excellent and extensively used choice to identify any kind of an unknown organism specially, on the basis of no priori knowledge. For a number of reasons, no gene has shown as broad applicability over all the taxonomic groups as the 16S rRNA gene (Clarridge, 2004), The new edition of *Bergey's Manual of Systematic Bacteriology*, the most widely used and authoritative reference on bacterial taxonomy, is organized using 16S rRNA gene sequence analysis as the backbone (Garrity and Holt, 2001; Krieg and Garrity, 2001 both cited in Clarridge, 2004).

The ribosomal RNA molecules are responsible for protein synthesis which is one of the most basic biological processes in the cell and therefore these molecules are highly conserved (Woese, 1987). There are lots of investigations made about the 16S rRNA gene being conserved in wide range of organisms and vary in an orderly fashion throughout the phylogenetic tree. The degree of conservation is assumed to result from the importance of the 16S rRNA as a critical component of cell function. This is in contrast to the genes needed to make enzymes where mutations in these genes can usually be tolerated more frequently since they may affect structures not as unique and essential as rRNA. In other words if a bacterium does not have the gene to make the enzymes needed to utilize lactose, it can use an alternative sugar or protein as an energy source.

The 16S rRNA gene sequence is about 1,550 bp long and is composed of both variable and conserved regions. The gene is large enough, with sufficient interspecific polymorphisms of 16S rRNA gene, to provide distinguishing and statistically valid measurements. When no specific primers are designed, the 16S rDNA genes could be amplified using universal primers and digested with selected restriction enzymes (Espinoza *et al.*, 2008).

As indicated in Clarridge (2004) and references therein, although the absolute rate of change in the 16S rRNA gene sequence is not known, it does mark evolutionary distance and relatedness of organisms. Problems in assigning a numerical value to this rate of change include the possibility that this rate of change of 16S rRNA gene may not be identical for all organisms (different taxonomic groups could have different rates of change), the rates could vary at times during

evolution, and the rates could be different at different sites throughout the 16S rRNA gene. There are so-called “hot spots” which show larger numbers of mutations; these areas are not the same for all species. Generally, as indicated by Woese (1987) who defined important properties of 16S rRNA gene, it seems to behave as a molecular chronometer.

16S rRNA gene is universal in bacteria therefore relationships can be measured among all bacteria. In general, the comparison of the 16S rRNA gene sequences allows differentiation between organisms at the genus level across all major phyla of bacteria, in addition to classifying strains at multiple levels, including what we now call the species and subspecies level. The occasional exceptions to the usefulness of 16S rRNA gene sequencing usually relate to more than one well-known species having the same or very similar sequences (Clarridge, 2004).

2.6.2. Amplified ribosomal DNA restriction analysis (ARDRA)

It is a molecular technique based on the restriction fragment length polymorphism of the 16S ribosomal RNA gene amplified by a polymerase chain reaction (PCR) and usually referred as amplified 16S ribosomal DNA restriction analysis (ARDRA). Since sequence analysis of 16S rRNA is relatively expensive and time-consuming, ARDRA technique is used for routine application before sequencing when rapid identification of the diversity of large numbers of strains is required. Based on this technique, species could be differentiated in to different ARDRA groups on the basis of their characteristic 16S rRNA gene restriction patterns (Yoon *et al.*, 1997). As explained in many reports (Cruz *et al,* 2001; Jawad *et al.*, 1998), ARDRA gives a rapid, economical definitive result when simply attempting to determine diversity and genospecies to which isolates of certain bacteria belong.

2.6.3. DNA sequence databases and sequence search

The 16S rRNA gene sequence has been determined for a large number of strains. GenBank (http://www.ncbi.nlm.nih.gov/), the largest databank of nucleotide sequences, has over 20 million deposited sequences, of which over 90,000 are of 16S rRNA gene (Clarridge, 2004). This means that there are many previously deposited sequences against which to compare the sequence of an unknown strain.

The generated DNA sequences are usually assembled by aligning the forward and reverse sequences. This consensus sequence is then compared with a database library by using analysis software. Some systems allow comparisons of the single forward or reverse sequences. Other well-known databases of 16S rRNA gene sequences that can be consulted via the World Wide Web are the Ribosomal Database Project (RDP) (http://rdp.cme.msu.edu/), European Molecular Biology Laboratory (http://www.ebi.ac.uk/embl/), Smart Gene IDNS (http://www.smartgene.ch), and Ribosomal Differentiation of Medical Microorganisms (RIDOM) (http://www.ridom.com/).

2.6.4. Phylogenetic tree for inferring evolutionary relatedness

The purpose of phylogenetic analysis is to infer evolutionary relationships among organisms. It can be used to establish evolutionary trees showing common ancestry between different known organisms and is also a powerful tool to position uncharacterized organisms in relation to previously characterized ones.

There are many different methods for reconstructing phylogenetic trees. These include the Unweighted Pair-Group Method with Arithmetic Mean (UPGMA) Method (Sokal and Michener, 1958), the Transformed Distance Method (Klotz *et al*., 1979; Farris, 1977), the Neighbor-Joining Method (Saitou and Nei, 1987), Maximum Parsimony Methods (Fitch, 1977) and the Maximum Likelihood Methods (Felsenstein, 1981).

3. Objective of the study

The overall objective of the study was to assess diversity and efficiency of cyanide degrading alkaliphilic bacteria from Ethiopian Soda Lakes and contribute to the effort being made towards alleviating problems of environmental cyanide waste contamination.

Specifically, this work was intended to:

- Isolate cyanide degrading alkaliphilic bacteria using enrichment culture supplied with cyanide as a sole nitrogen source.
- Characterize these isolates using molecular techniques; ARDRA and 16S rDNA sequencing.
- Identify the phylogenetic relationship of these bacteria among themselves and with other related bacterial strains.
- Examine the level of tolerance and degradation efficiency of these bacteria against different cyanide concentrations.
- Evaluate their preference to use different carbon sources for better cyanide degradation and utilization of different nitrogen sources in comparison with cyanide.

4. Materials and Methods

4.1. Description of sample site and sample collection

Samples were collected from three Great Rift Valley Soda Lakes of Ethiopia for isolation of cyanide degrading alkaliphilic bacteria. These are Lake Abijata, Lake Chitu and Lake Shala located in southern part of the country and known for their high alkalinity and salinity.

Mud and water samples of the three soda lakes were collected with sterile plastic bottles and stored at 4^{o}c until used.

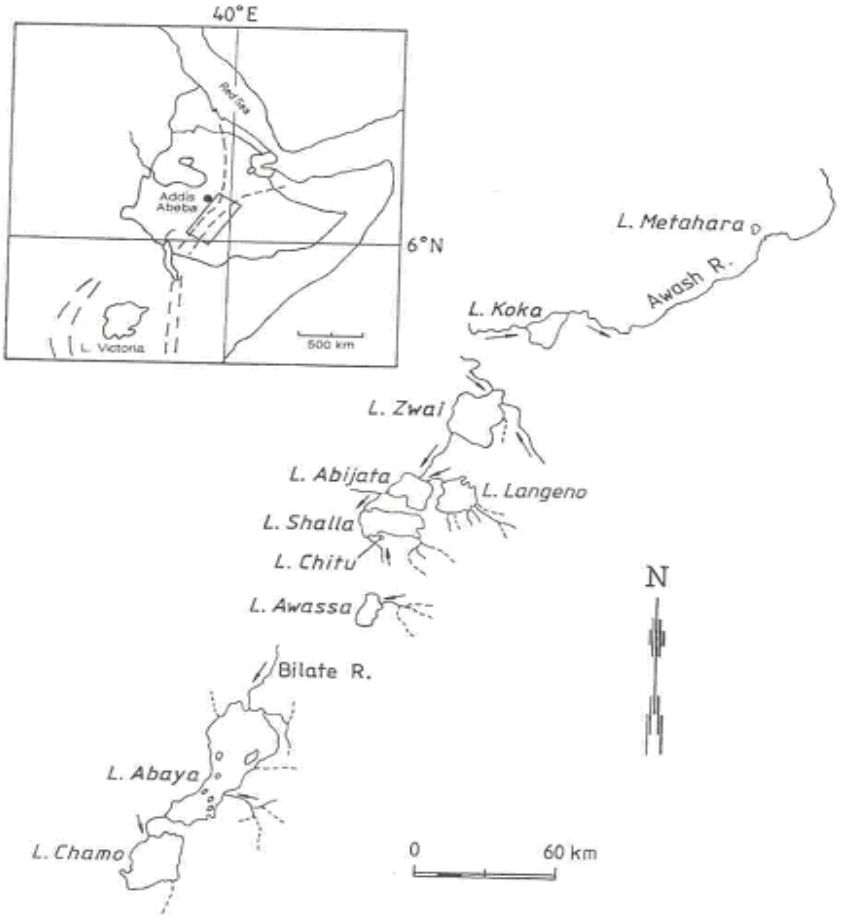

Figure 1: Location of sample sites (Elizabeth Kebede, 1996)

4.2. Enrichment, culture conditions, isolation and purification of isolates

Enrichment culturing procedure was done according to Ingvorsen *et al*., (1991) by inoculating serially diluted samples from the three lakes in to autoclave-sterilized basal solution (BS) containing NaCN as a sole nitrogen source. It had the following ingredients (amount given per liter): KH_2PO_4. $2H_2O$, 3 gm; $MgSO_4.7H_2O$, 0.2 gm; NaCl, 0.25 gm; $CaCl_2.2H_2O$, 0.02 gm; $MnSO_4.4H_2O$, 0.01 gm; $FeCl_3.6H_2O$, 0.045 gm; $ZnSO_4.7H_2O$, 0.01 gm; $CuSO_4.H_2O$, 0.002 gm; $CoCI_2.6H_2O$, 0.003 gm; $NiCI_2.6H_2O$, 0.003 gm; $NaMoO_4.2H_2O$, 0.002 gm; NaCN, 0.2 gm; and Na_2CO_3, 10 gm.

Acetate, with the exception of carbon-source utilization experiments where different carbon sources, was used as carbon source at final concentrations of 0.25% (w/v). pH of the medium was adjusted at about 10.22 using 26% (w/v) Na_2CO_3 sterilized alone. Sodium cyanide was added from a filter-sterilized (0.2 µm-pore-size filter) solution to the cooled medium immediately before inoculation. Cotton plugged shake flasks with BS medium were inoculated with environmental samples and placed on orbital shaker.

After a series of sub-culturing in liquid medium, one ml of the culture from each dilution were dispensed on to sterile enrichment agar medium (1.5%) with the same composition and spread thoroughly. The plates were incubated at 30ºC to examine formation of colonies.

All the plates were observed carefully and single colony of each isolate, based on cell and colony morphology, was picked using alcohol flamed sterile inoculating loop and streaked on to enrichment agar medium. A series of re-streaking was performed to ensure the purity of each isolate. Pure cultures were labeled and transferred to slant culture tubes and stored at 4^oC until used for other experiments.

4.3. Preliminary selection of effective cyanide degrading isolates

The isolated bacteria were analyzed for their effectiveness based on the use of cyanide as the sole nitrogen source. In all the cases, except for bacterial growth response to different cyanide concentrations experiment, 0.2 gm/l of cyanide was used. Bacterial isolates that grow faster and

have reasonable biomass after repeated re-culturing on cyanide containing medium were selected and used for further experiments.

4.4. Molecular identification of cyanide degrading bacteria

4.4.1. Genomic DNA extraction

Genomic DNA extraction from the cyanide utilizing alkaliphilic bacterial isolates was done using freeze-and-thaw (Barberio *et al.,* 2001) and CTAB (Ausubel *et al.,* 2003; Nishiguchi *et al.,* 2002) methods. Briefly, 1 to 5 colonies were added in to 50µl of sterile water and mixed well. The cell suspension was heated at 98°C for 10 min and shifted at -20°C for 15 min. this was done for two cycles. Lysates were maintained at -20°C until used.

For the CTAB method, 1 to 5 colonies were suspended in TE buffer. Sodium dodecyl sulfate (10% (w/v)), and proteinase K (20 mg/ml) were added and incubated at 37°C for 1 hr. After incubation, 5 M NaC1 and CTAB/NaC1 solution (0.7 M NaC1, 10% CTAB) were added and incubated again at 65°c for 10 min to remove polysaccharides and other contaminant macromolecules. Equal volumes of chloroform: isoamyl alcohol (24:1) and Phenol: Chloroform: Isoamyl were used for further purification. Finally, isopropanol was used to precipitate DNA and then resuspended in Tris-HCl buffer after washing the pellets using 70% ethanol. DNA was checked by electrophoresis using 1% agarose stained with ethidium bromide and stored until used for PCR amplification experiment.

4.4.2. PCR amplification of 16S rRNA gene

The genomic DNA from each isolate was used to amplify a bacterial specific ca.1,500 bp 16S rRNA gene with the bacteria universal primers E9F (5' – GAGTTTGATCCTGGCTCAG – 3') (Farely *et al*., 1995) and U1510R (5' – GGTTACCTTGTTACGACTT –3') (Reysenbach and Pace, 1995) using a ThermoHybaid PCR Express Thermal Cycler (Applied Biosystems).

The PCR reaction mixture contained 19µL of ultrapure sterile H_2O, 25µL of HotStar Taq Master Mix (Qiagen, Germany), 2µL of each primer (5uM), and 2µL of template DNA(~150ng) to make up a final reaction volume of 50µL.

The thermal profile used was a pre-heat step at 95°c for 15 min, 30 cycles each consisting of 30 sec denaturation at 95°C, 30 sec of primer annealing at 52°C, and 1 min of chain extension at 72°C. Final extension was for 6 min at 72°C. Products of amplification were analysed by electrophoresis through 1.2 % agarose gel (w/v) stained with ethidium bromide and visualized under UV transilluminator. Gel picture was captured using Molecular Imager Gel Doc XR+ system (BIO-RAD) based on high resolution CCD technology. 1kb plus molecular weight standard (Invitrogen, Germany) was used to estimate the approximate size of the amplified fragment.

4.4.3. Restriction digest of PCR amplified product

Based on the number of restriction site identified using the Web cutter program (http://Tools.NEB.com// NEBcutter2/index.PHP; New England Biolabs, Ipswich, MA) inside the 16S rRNA bacterial gene and availability in our laboratory *AluI, HaeIII* and *RsaI* (Fermentas) were selected for separate endonuclease digestion. These restriction enzymes are good choices for studying 16S rRNA based bacterial identification because they have several cutting points in the sequence, give unique restriction patterns for each species, and demonstrate DNA fragments greater than 100bp which allows for easier visualization and interpretation.

8 µl (~ 5ug) of each amplified PCR product was added in to a reaction mixture containing 0.4 µl enzyme (10U/µl), 1.5µl 10X buffer (either Tango yellow or Tango red buffer depending on the type of the enzyme), and 6.2µl of ultrapure sterilized H_2O and was incubated overnight at 37°C. Digestion fragments were electrophoresed on 3% agarose gel (w/v) stained with ethidium bromide, creating a 'unique' banding pattern. The molecular size of the DNA fragments was estimated using 1kb-plus DNA molecular weight marker. Similar to the PCR products, pictures of restriction patterns were captured using Molecular Imager Gel Doc XR+ system (BIO-RAD) and used for ARDRA analysis.

4.4.4. 16S rRNA gene sequencing and database search

One representative bacterial isolate from each of the ARDRA group was selected for 16S rRNA gene sequencing. In addition, isolates with ambiguous restriction band pattern as well as isolates within the same ARDRA group with some how different colony morphology from the rest of the group members were also taken for sequencing. A total of 14 bacterial isolates were selected for sequencing. 16S rRNA gene sequencing was done using the sequencing facility at University of Bergen, Norway.

Sequences were fed to DNADOT Restriction map Toolkit (http://www.vivo.colostate.edu/molkit/mapper/index.html) and theoretical restriction sites for the selected endonucleases were analyzed to validate the correct match of ARDRA and sequence results.

Classifier of RDP version 2.2 (http://rdp.cme.msu.edu/) of Michigan State University was then used to roughly assess how diverse the sequences are at the genus level.

Following, Sequence identification was estimated using BLAST of the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/BLAST/) as well as Ribosomal Database Project (RDP) (http://rdp.cme.msu.edu/).

4.4.5. Multiple sequence alignment and phylogenetic tree construction

Multiple sequence alignment and phylogenetic tree construction were performed using MEGA4 software package (Tamura *et al.,* 2007). Evolutionary history was inferred using Neighbor-Joining method (Saitou and Nei, 1987) and evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.,* 2004). The bootstrap consensus tree inferred from 1000 replicates was taken to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985).

4.5. Screening for efficient cyanotrophic strains in batch culture

4.5.1. Cell growth determination

Apart from using microscopic observation and colony count on plates, bacterial growth determination in suspension culture was carried out in flask culture based on the optical density at 600nm (Almagro *et al*., 2005a & b; Akcil, 2003). One ml of starter culture of test bacteria grown in 10ml enrichment liquid medium in a test tube containing NaCN as sole nitrogen source were transferred in to duplicates of 30ml sterilized media (containing 200mg/l cyanide) in cotton plugged 250ml Erlenmeyer flasks. They were all placed on incubator adjusted at 30^{o}C with periodic shaking for two weeks. The same enrichment media, but without inocula, were used as control for measuring absorbance.

4.5.2. Determination of residual cyanide

Residual cyanide was measured spectrophotometrically using the picric acid method according to Bilger and Wolf (1987). Free cyanide and weak acid cyanide reacts with the picric acid reagent (in alkaline solution) to produce a salt of *iso*-purpuric acid having an orange color that can be measured colorimetrically at a wavelength of 520nm. Alkaline picrate reagent was prepared by dissolving 3gm picric acid and 8gm NaOH in 400ml of distilled water. In to this, 7g of sodium carbonate and 7gm borax were added and filled to a final volume of 500ml, then stirred well to make sure that everything is dissolved.

Samples taken from the cyanide containing growth media inoculated with bacteria were centrifuged with 6,000 rpm for 10 minutes to precipitate biomass. Supernatants were then diluted using alkaline water (pH of 10.8) to make the cyanide concentration on the range 1-5mg/l. Accordingly, cell-free supernatants from 0hr and 48 hrs were diluted 30X, 96 hrs and 144 hrs were diluted 15X, and the last three from 192 hrs, 240 hrs and 288 hrs were diluted 10X. Then 7ml of each diluted supernatant, after vortexing, was transferred to another test tube. 2.5ml alkaline picrate solution was added to it, mixed well and incubated at 90°C for15 min. After cooling down, absorbance of the cyanide-picric acid reaction product was measured at a wavelength of 520nm. The same procedure was followed for the control (growth media without inocula) and for the blank (without cyanide) that remained yellow under this condition.

4.5.3. The effect of different cyanide concentration on bacterial growth

The tolerance and performance of the bacterial isolates were tested at various concentrations of cyanide in batch mode. The concentrations were 100, 200, 400, 600, and 800mg/l cyanide. One ml of starter cultures were transferred in to duplicates of 30 ml sterilized media (containing either of the cyanide concentrations) in cotton plugged 250ml Erlenmeyer flasks. And, the same media with no inocula were used as control. All were placed for two weeks on incubator adjusted at 30ºC with periodic shaking and then growth was measured.

4.5.4. Comparison of bacterial growth on different carbon-sources

The preference of the strains to utilize different nitrogenous compounds other than cyanide was examined, while acetate was provided as carbon source. Different nitrogenous compounds, both organic (peptone, urea and yeast extract) and inorganic (NH_4Cl and $NaNO_3$), and NaCN were used for the experiment. All were added in to the rest of the media by filter sterilizing. Therefore, sterilized enrichment liquid medium containing the nitrogenous compounds for each strain is prepared. The experiment was carried out in similar way as in the cases of bacterial growth determination on cyanide except the use of different nitrogenous compounds here. Their preference to use these nitrogen sources was evaluated by measuring bacterial growth using absorbance at 600nm. Liquid media containing corresponding nitrogenous compounds were prepared without inocula for use as a control.

4.5.5. Bacterial preference to use nitrogen-sources other than Cyanide

Besides sodium acetate, alternatives carbon sources such as glucose, xylose, sucrose, cheap molasses, and maltose were examined for their suitability. 0.2 gm/l of NaCN was used as nitrogen source and each of the above carbon sources were evaluated based on their importance for better utilization and degradation of cyanide. All the experimental set up was carried out as indicated in Wajana Lako (2007). Because cyanide is known to react with compounds like sugars with their keto groups at alkaline condition, measuring bacterial growth rather than residual cyanide was preferred.

5. Results

5.1. Selected microbial isolates of Cyanide degraders

A total of 72 cyanide degrading alkaliphilic microorganisms were isolated from Lakes Abijata, Chitu, and Shala samples using medium containing cyanide as a sole nitrogen source. From these, 30 isolates were selected based on morphological features (shape, color, size, margin, elevation and texture of the colonies) as shown in the appendix, rate of growth and amount of biomass which can also be explained by the ability to utilize cyanide. Seventeen of these were from Lake Abijata, six from Lake Chitu, and the remaining seven were isolated from samples of Lake Shalla.

5.2. PCR amplification products of 16SrRNA gene

The 16S rRNA genes were successfully amplified via PCR from genomes of the selected bacterial isolates. PCR amplifications with the same oligonucleotide primers were performed with *E. coli* as a positive control and a single band of similar size was obtained. Fig. 2 below shows sample image for PCR amplification.

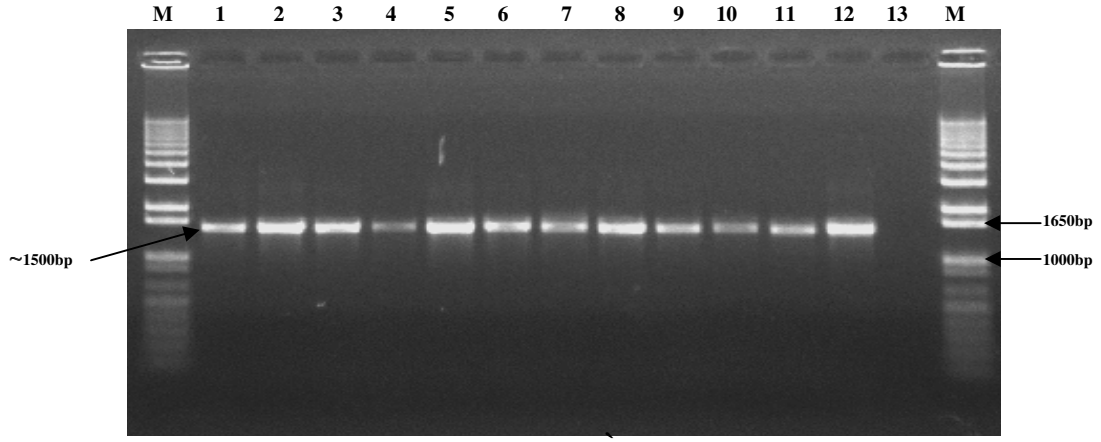

Figure 2: PCR amplification products of 16S rDNA gene. Lanes M 1kb plus DNA ladder; lane 1, CNA_1; lane 2, CNA_2; lane 3, CNA_4; lane 4, CNA_5; lane 5, CNA_6; lane 6, CNC_1; lane 7, CNC_2; lane 8, CNC_3; lane 9, CNS_4; lane 10, CNS_3; lane 11, CNS_{10}; Lane 12, positive control (*E. coli* 16S rDNA) and Lane 13, negative control (without template DNA).

5.3. Amplified rDNA restriction pattern analysis

In order to differentiate similar isolates, all the 30 isolates were examined by Amplified Ribosomal DNA Restriction Analysis (ARDRA). The 16S rDNAs PCR amplification products were cleaved with restriction enzymes *Alu I, Hae III* and *Rsa I* displaying their own restriction patterns, as shown in Fig. 3 (I, II and III). ARDRA profile obtained with these restriction enzymes revealed the presence of nine different polymorphic groups among the thirty isolates.

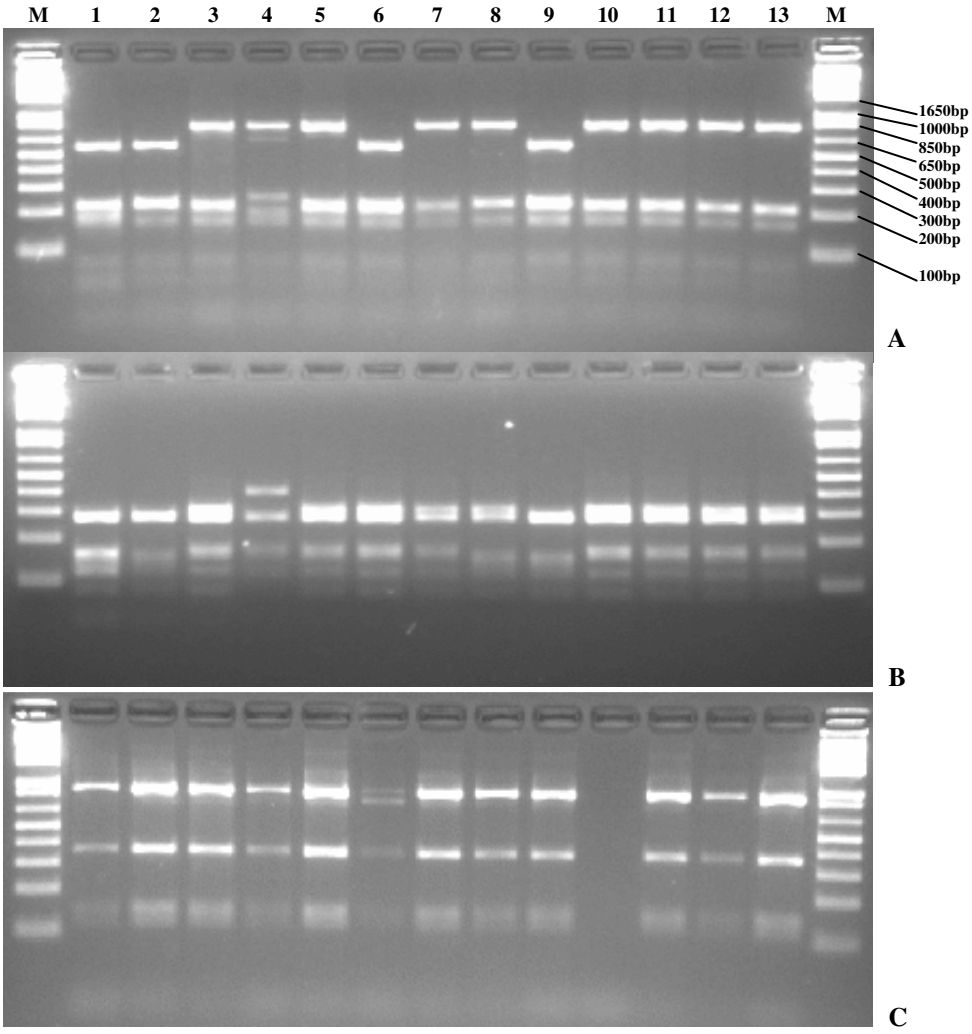

Figure 3(I): Restriction patterns of 16S rDNAs from cyanide degrading bacterial isolates in 3% agarose gel (A) 16S rDNAs digested with *AluI*. (B) 16S rDNAs digested with *HaeIII*. (C) 16S rDNAs digested with *RsaI*. Lanes M 1kb plus DNA ladder; lane 1, CNA_1; lane *2,* CNA_2; lane 3, CNA_4; lane 4, CNA_5; lane 5, CNA_6; lane 6, CNA_7; lane 7, CNA_8; lane 8, CNA_9; lane 9, CNA_{10}; lane 10, CNA_{11}; lane 11, CNA_{12}; lane 12, CNA_{13}; lane 13, CNA_{14}.

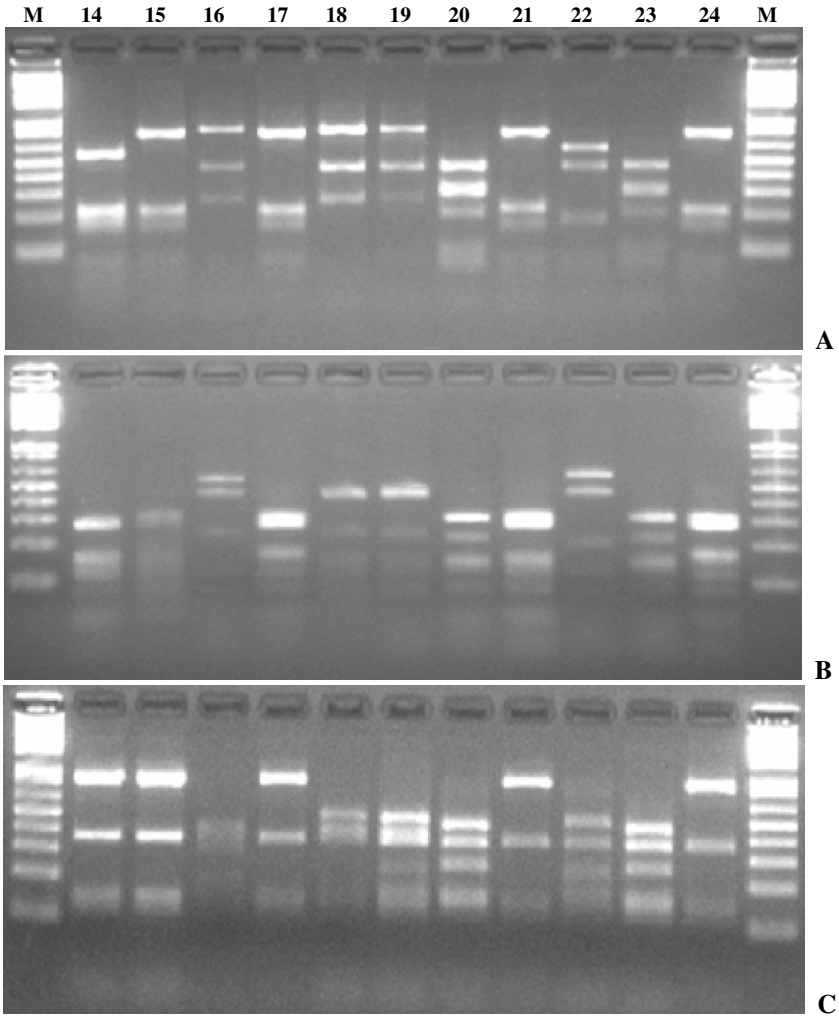

Figure 3(II): Restriction patterns of 16S rDNAs from cyanide degrading bacterial isolates in 3% agarose gel (A) 16S rDNAs digested with *AluI*. (B) 16S rDNAs digested with *HaeIII*. (C) 16s rDNAs digested with *RsaI*. Lanes M 1kb plus DNA ladder; lane 14, CNC_1; lane 15, CNC_2; lane 16, CNC_3; lane 17, CNC_4;lane 18, CNC_5; lane 19, CNC_6; lane 20, CNS_1; lane 21, CNS_{10}; lane 22, CNS_3; lane 23, CNS_4; lane 24, CNS_5.

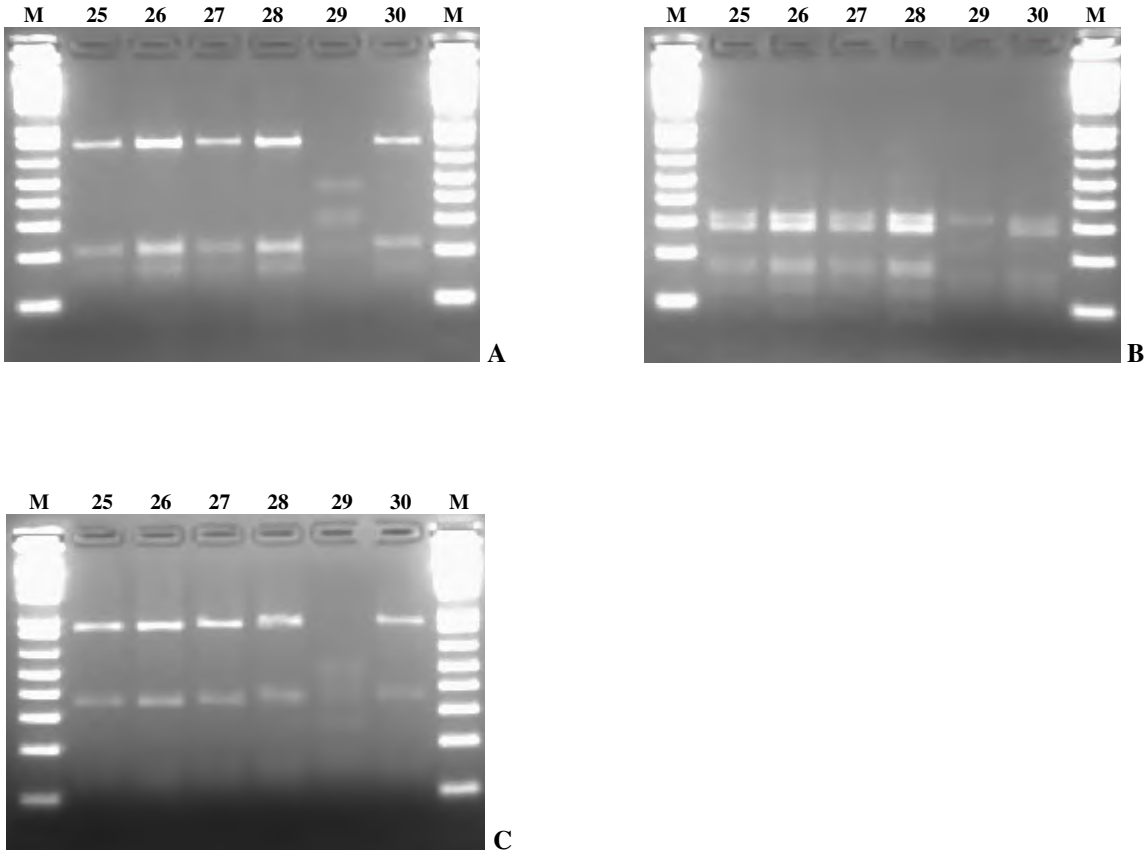

Figure 3(III): Restriction patterns of 16S rDNAs from cyanide degrading bacterial isolates in 3% agarose gel (A) 16S rDNAs digested with *AluI*. (B) 16S rDNAs digested with *HaeIII*. (C) 16s rDNAs digested with *RsaI*. Lanes M 1kb plus DNA ladder; lane 25, CNA_{15}; lane 26, CNA_{16}; lane 27, CNA_{17}; lane 28, CNA_{19}; lane 29, CNS_{7}; lane 30, CNS_{8}.

Of all the 30 isolates, 56.7% were from Abijata, 23.4% and 20% from Shala and Chitu respectively. The biggest ARDRA group (B) common in all the three lakes comprised 56.7% of the isolates, majority being from Lake Abijata. As shown in Fig.3 above, there are also some isolates having unique restriction pattern found confined in one of the three lakes. Table 3 below shows all the ARDRA groups based on restriction band pattern after endonuclease digestion.

Table 3: Distribution of cyanide degrading microbes based on their ARDRA profile

ARDRA Group	Isolates from Lake Abijata	Isolates from lake Chitu	Isolates from lake Shala	Remark
A	**CNA_1, CNA_2, CNA_{10},**	**CNC_1**		A group common to Lake Abijata and Chitu
B	**CNA_4, CNA_6, CNA_8,CNA_9, CNA_{11}, CNA_{12}, CNA_{13}, CNA_{14}, CNA_{15}, CNA_{16}, CNA_{17}, CNA_{19}**	**CNC2, CNC_4**	**CNS_5, CNS_8, CNS_{10}**	Common in all the three lakes
C		**CNC_5, CNC_6**		Both found in Lake Abijata
D			**CNS_1, CNS_4**	Both from Lake Shala
E		**CNC_3**		Unique to Lake Chitu
F			**CNS_3**	Only found in Lake Shala
G			**CNS_7**	Unique to Lake Shala
H	**CNA_5**			Unique to Lake Abijata
I	**CNA_7**			Only found in Lake Abijata

5.4. GenBank search and sequence submissions

16S rRNA gene sequences were obtained from total of 14 cyanide utilizing isolates. To get an idea of their diversity, we have used classifier of RDP version 2.2 of Michigan State University for our sequences and all of them came out being 100% bacteria (Fig. 4). Of these, 4 were under the genus *Bacillus* while the rest 10 were grouped under the genus *Halomonas*.

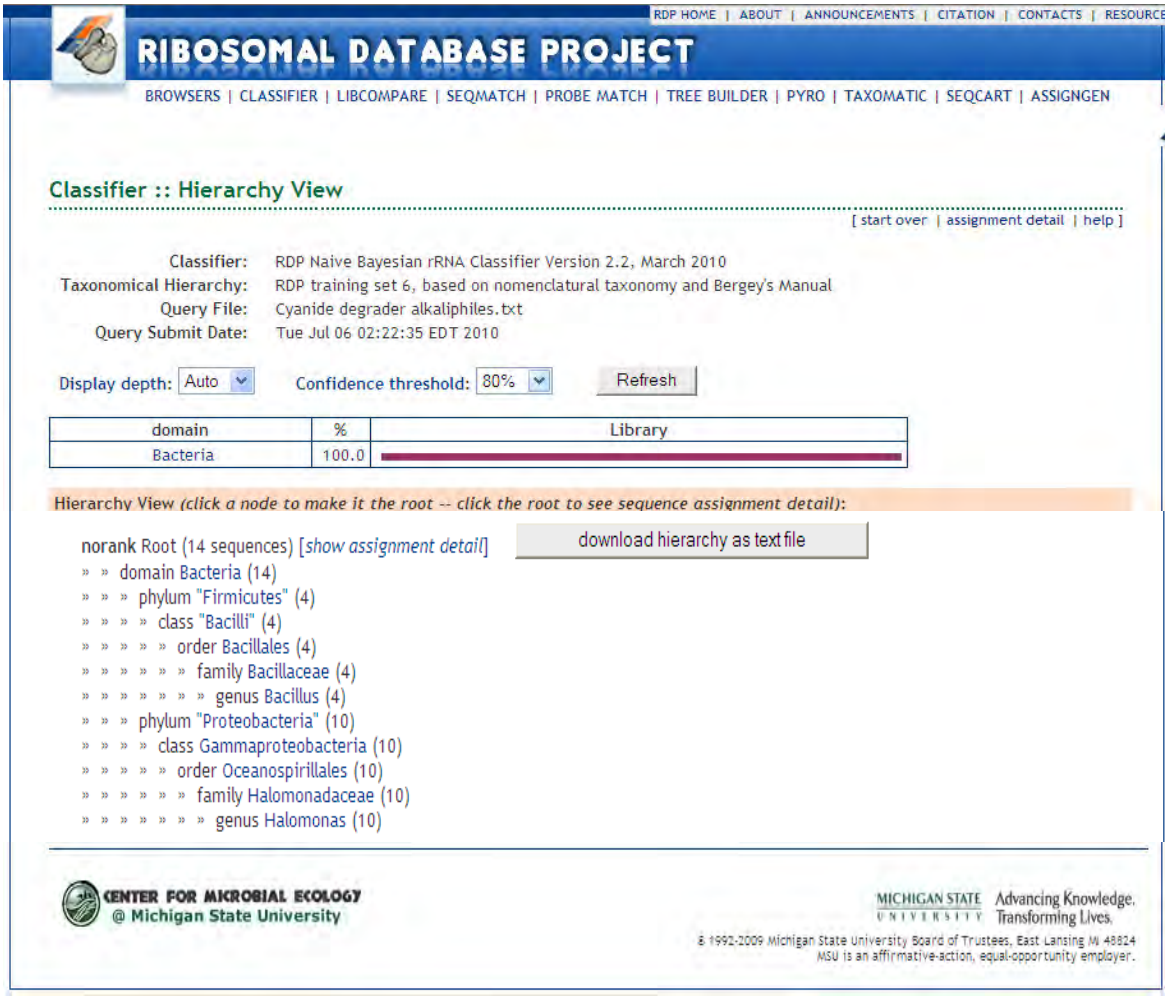

Figure 4: RDP page giving an idea of sequence diversity

These sequences were matched with previously published 16S rDNA sequences available in NCBI and RDP databases. As indicated in Table 4, two matches with the highest similarity were selected for each of the sequences. Accordingly, sequence analysis results revealed that from the total 14 sequences, seven different polymorphic groups were found (Table 5). Among which, six sequences (CNA_{12}, CNA_{15}, CNA_{19}, A_{21}, CNA_{17} and S_8) matched with *Halomonas campisalis*, two sequences (S_1 and S_{10}) matched with *Halomonas sp.*IB-O18, two other sequences (CNC_5 and CNC_6) matched with *Bacillus agaradhaerens*, and each of the remaining CNC_3, CNS_3, CNA_{16} and CNC_1 matched with *Bacillus beveridgei*, *Bacillius pseudofirmus*, uncultured *Halomonas sp.*clone and uncultured bacterium clone respectively (Table 4). All matches were with 99% similarity except one having 98% sequence match.

Table 4: GenBank sequence homology search results

Cyanide degraders	Closest match and its accession number	Sequence similarity (%) *	Taxon	Habitat of the match
CNA_{17}	*Halomonas campisalis* LL6 (DQ077911)	99 (1042/1051)	Gammaprot	Soda Lake, Lonar lake, India
	Lake Bogoria isolate 8B1 (X92138)	98 (1027/1052)	Gammaprot	Soda Lake
CNS_{10}	*Halomonas sp.*IB-O18. (AM490136)	99 (1003/1012)	Gammaprot	Soda Lake China
	Halomonias campisalis LL6 (DQ077911)	97 (989/1016)	Gammaprot	Soda Lake, Lonar lake, India
CNC_6	*Bacillus agaradhaerens* (FN432808)	99 (960/967)	Firmicutes	Sediment, salt lake
	Bacllus clarkia DSM 8720 (NRO36141)	97 (946/968)	Firmicutes	Not known
CNA_{16}	Uncult. *Halomonas sp.*clone (EU440679)	99 (992/998)	Gammaprot	Agricultural soil
	Halomonas nitritophilus (AJ309564)	98 (989/999)	Gammaprot	Not known
CNC_3	*Bacillus beveridgei* MLTeJB (FJ825145)	98 (1017/1030)	Firmicutes	Mono Lake
	Bacillus agaradhaerens (FN432808)	97 (1004/1029)	Firmicutes	Sediments, Salt Lake
CNA_{21}	*Halomonas campisalis* LL6 (DQ077911)	99 (960/961)	Gammaprot	Soda lake, Lonar lake, India
	Lake Bogoria isolate 8B1 (X92138)	99 (957/9620	Gammaprot	Soda Lake
CNA_{19}	*Halomonas campisalis* LL6 (DQ077911)	99 (939/940)	Gammaprot	Soda Lake, Lonar lake, India
	Lake Bogoria isolate 8B1 (X923138)	99 (934/943)	Gammaprot	Soda Lake
CNS_8	*Halomonas campisalis* LL6 (DQO77911)	99 (1028/1032)	Gammaprot	Soda lake, Lonar Lake, India
	Lake Bogoria isolate 8B1 (X923138)	99 (1023/1033)	Gammaprot	Soda Lake
CNS_1	*Halomonas sp.* IB-O18 (AM490136)	99 (988/995)	Gammaprot	Soda Lake China
	Halomonas campisalis LL6 (DQ077911)	97 (981/996)	Gammaprot	Soda Lake, Lonar lake, India
CNC_1	Uncultured bacterium clone (FJ152630)	99 (865/868)	Gammaprot	Alkaline Lake Teacoco
	Halomonas shengliensis SL014B-85 (EF121853)	98 (852/868)	Gammaprot	Crude oil
CNC_5	*Bacillus agaradhaerens* (FN432808)	99 (1014/1020)	Firmicutes	Sodiment, salt lake
	Bacillus clarkii DSM 8720 (NR026141)	97 (1000/1021)	Firmicutes	Not known
CNA_{12}	*Halomonas campisalis* LL6 (DQ077911)	99 (1009/1014)	Gammaprot	Soda Lake, Lonar
	Lake Bogoria isolate 8B1 (X92138)	99 (1004/1015)	Gammaprot	lake, India Soda Lake
CNS_3	*Bacillius pseudofirmus* (AB201799)	99 (1013/1014)	Firmicutes	Not known
	Bacillius hemicellulosilyticus (AB043846)	98 (1003/1015)	Firmicutes	Not known
CNA_{15}	*Halomonas campisalis* LL6 (DQ077911)	99 (962/964)	Gammaprot	Soda Lake, Lonar
	Lake Bogoria isolate 8B1 (X92138)	99 (957/964)	Gammaprot	lake, India Soda Lake

* Numbers in brackets under this column show sequence coverage for the blast results

Restriction pattern analysis showed CNS_1 and CNS_{10} being very different hence laid in different ARDRA groups. However 16S rRNA gene sequences of these isolates were found to be similar. DNADOT results for verifying size of restriction fragments (data not shown) revealed that CNS_{10} has almost similar band pattern with the rest of the group (B) member with only slight difference. This created confusion on how CNS_1 sequence could have similar sequence while having different restriction pattern. Therefore, it is decided in the future to sequence CNS_1again together with other isolates which are not sequenced.

Table 5: Strain identification based on closest database sequence matches

Sequenced isolates	ARDRA group	Best sequence matches from database
CNC_1	A	Uncultured bacterium clone, FJ152630 (99% similarity)
CNA_{12}, CNA_{15}, CNA_{19}, CNA_{21}, CNA_{17}, CNS_8	B	*Halomonas campisalis* LL6, DQ077911 (99% similarity)
CNA_{16}	B	Uncultured *Halomonas sp.*clone, EU440679 (99% similarity)
CNS_{10}, CNS_1	B and D respectively	*Halomonas sp.*IB-O18., AM490136 (99% similarity)
CNC_5, CNC_6	C	*Bacillus agaradhaerens,* FN432808 (99% similar)
CNC_3	E	*Bacillus beveridgei* MLTeJB, FJ825145 (98% similarity)
CNS_3	F	*Bacillius pseudofirmus*, AB201799 (99% similarity)

Based on this result CNC_1, CNA_{12}, CNA_{16}, CNS_{10}, CNC_5, CNC_3 and CNS_3 were selected as different cyanide degrading strains. The 16S rDNA nucleotide sequences obtained from these strains were submitted to the GenBank database and assigned the following accession numbers.

5.5. Phylogenetic analysis

As mentioned in the materials and methods, phylogenetic analysis was conducted in MEGA4 software package using study sequences and their closest matches. Bootstraps analyses were performed from 1,000 random resamplings. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) were shown next to the branches. The tree constructed placed the sequences under *Halomonas* and *Bacillus* major clusters (Fig. 5).

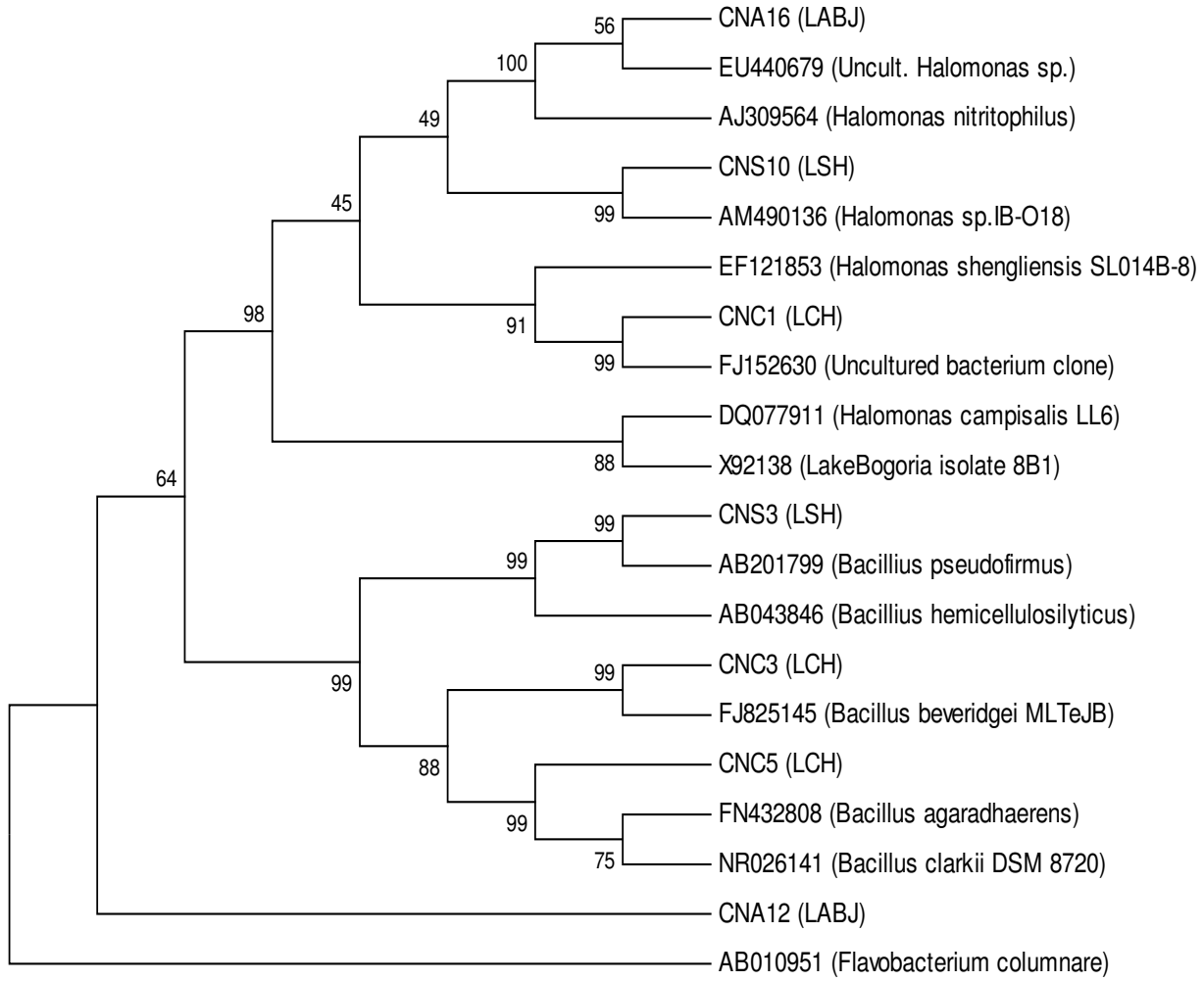

Figure 5. Phylogenetic tree based on a comparative analysis of the 16S rDNA sequences with reference sequences from BLAST analysis. LABJ, LCH and LSH are sequences obtained from Lake ABijata, Lake CHitu, and Lake SHala. Bootstrap values (1000 replicates) are shown at the nodes. *Flavobacterium columnare* was used as an outgroup taxon.

5.6. Screening for efficient cyanide utilizing bacterial strains

5.6.1. Determination of growth curve on cyanide

The seven strains identified above were found to grow reasonably on cyanide containing medium (Fig. 6). Particularly, CNA_{12} and CNS_{10} were found to have earlier lag phase and grew more abundantly than the others. While, CNS_3 is slow growing strain which only grew about half the biomass of the top growing strains. Other than CNA_{12} and CNS_{10}, strains start to have significant biomass increase after 96 hrs of incubation. Strains CNA_{12} and CNS_{10} grew exponentially up to 8^{th} and 10^{th} days of incubation respectively and start to decline after then. All the other five strains showed relaxed growth after day 8 and started to have more stabilized biomass increase.

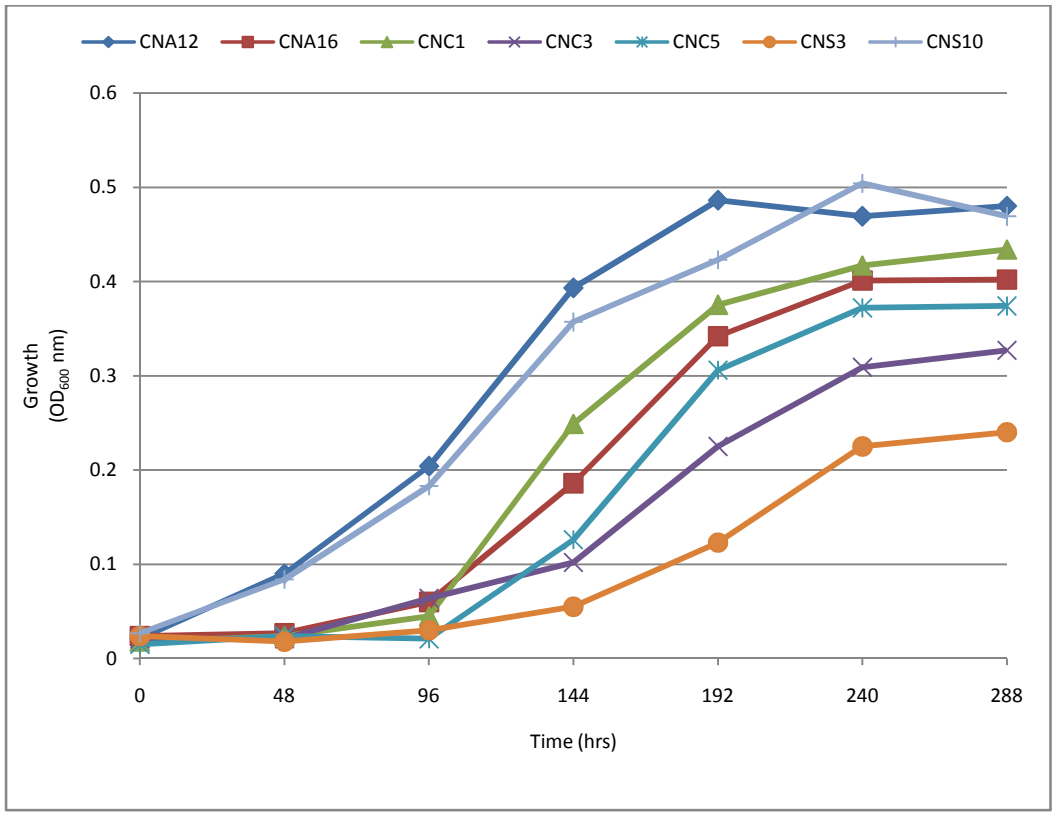

Figure 6: Growth of cyanide degrading strains in a synthetic medium containing sodium cyanide as a sole N-source and sodium acetate as C-source. OD_{600} for cell growth was measured at the indicated times.

5.6.2. Cyanide removal efficiency of the strains

The media used for determination of cell growth was also evaluated for rate of cyanide detoxification simultaneously by directly detecting residual cyanide left in the medium after bacterial cultivation. Table 6 shows the pattern and rate of cyanide removal for all the strains and the control which was without inoculum along the course of time. In all the media except the control, residual cyanide decreased significantly to lower levels during the incubation period. Accordingly, strains CNA_{12} and CNS_{10} were found to deplete up to 99.33% of cyanide. This result coincides with their growth rate which indicates the increase in biomass of bacterial strains is the result of utilizing cyanide as a nitrogen source for their metabolism. CNA_{16}, CNC_1 and CNC_5 were also good candidates that depleted more than 90% of the total cyanide found in the medium. The control which was kept without inoculum was found to lose some amount of cyanide but it was relatively not significant.

Table 6: Results for depletion rate of residual cyanide. * Co is for control.

Cyanide degrader strains	Percentage depletion of cyanide (200 mg/l of cyanide was provided for all)						
	0 hr	48 hrs	96 hrs	144 hrs	192 hrs	240 hrs	288 hrs
Co*	0	0	5.82	7.96	9.95	25.93	28.94
CNA_{12}	0	13.27	40.94	75.89	94.44	97.53	99.33
CNA_{16}	0	2.1	13.37	49.76	80.68	89.75	95.17
CNS_{10}	0	2.46	31.75	67.54	94.21	98.36	98.89
CNC_3	0	7.24	7.99	43.29	64.56	83.66	85.37
CNC_5	0	9.84	19.08	60.8	80.33	86.84	92.7
CNC_1	0	18.22	17.17	48.04	88.7	90.06	95.13
CNS_3	0	1.4	6.81	16.4	31.1	68.76	78.53

Based on determination of growth rate on cyanide and their removal efficiency, three strains, namely; CNA_{12}, CNC_1 and CNS_{10} were selected for further experiments.

5.6.3. Bacterial growth response to different cyanide concentrations

The bacterial strains under this study responded differently to increasing concentration of cyanide in growth media. As shown below in Fig. 8, CNS_{10} was able to utilize cyanide concentration of 400mg/l and tolerate up to 600mg/l cyanide. CNA_{12} was also the next best strain which has tolerated cyanide concentrations up to 600 mg/l and grew well at 200mg/l with slight decrease at 400mg/l of cyanide. Strain CNC_1 showed a kind of irregular growth response to concentration difference, however, had good tolerance against 600mg/l cyanide concentration. All the three strains had lowest activity at 800 mg/l followed by 100 mg/l of cyanide concentration.

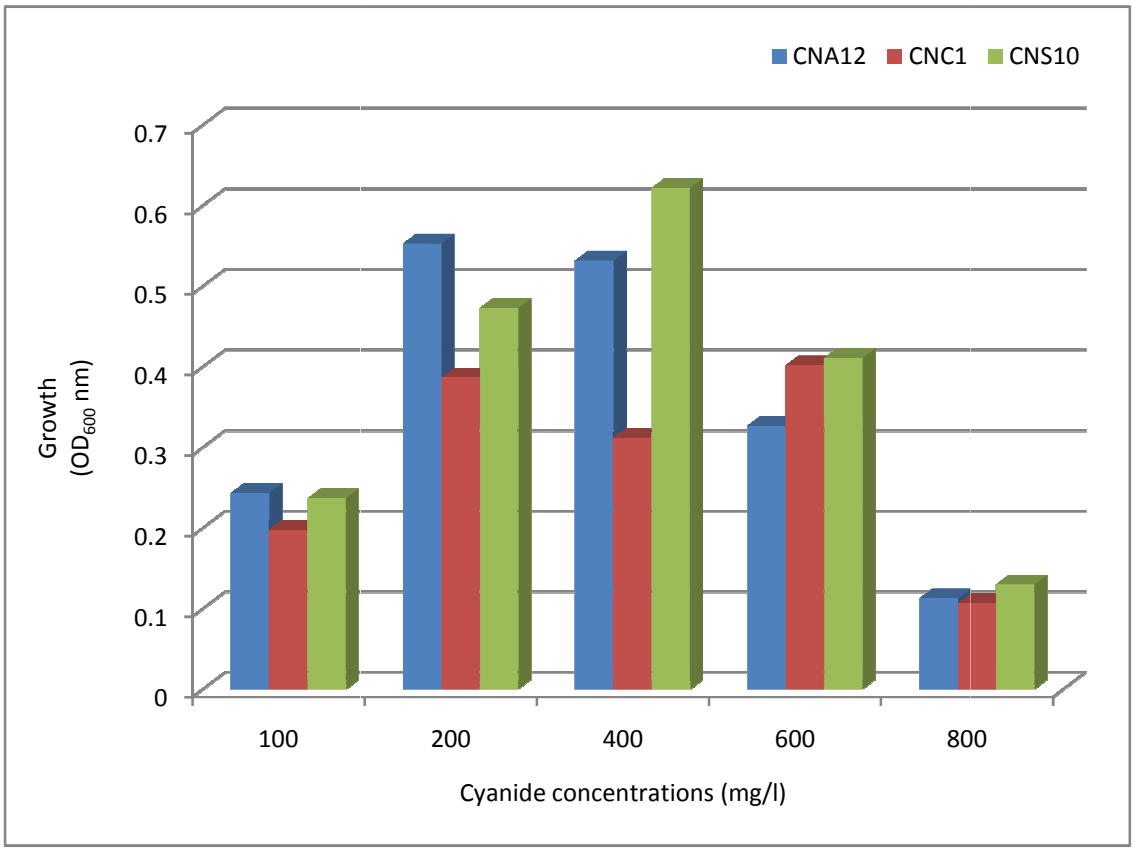

Figure 7: Biomass measurement as function of NaCN concentrations at the end of 240hrs incubation period.

5.6.4. Comparison of different carbon sources utilization

All of the three strains under study were capable of utilizing most carbon sources provided when NaCN was used as the only source of nitrogen (Fig. 9). Interestingly, better growth was observed when the isolates were provided with molasses as a carbon source. Sodium acetate is the second most preferred carbon source for all the three strains. They grew moderately when sucrose (except CNA_{12} with poor growth) and xylose were provided as carbon sources. On the other hand, growth yield of all strains was hardly recorded when maltose (except for CNC_1 which grew moderately) and glucose were used as sources of carbon.

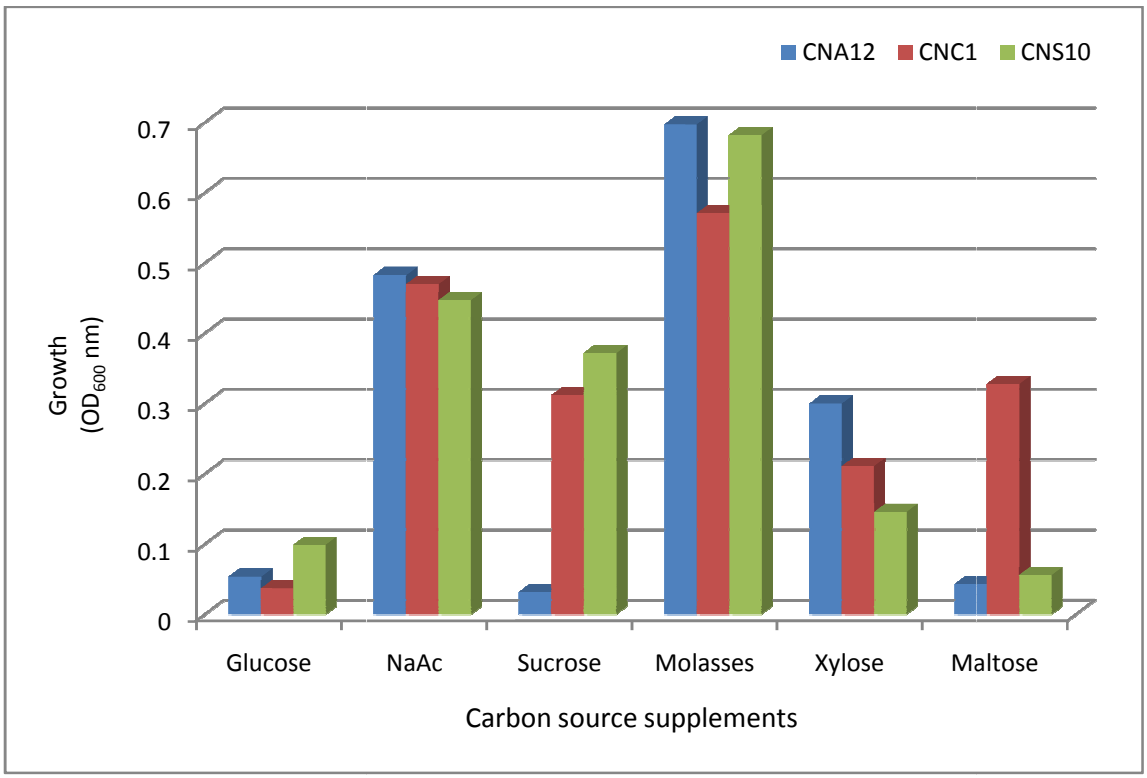

Figure 8: Growth on different carbon sources shown at the end of 240hrs incubation period.

Molasses is known to have traces of nitrogen in it requiring to make sure that cyanide is being depleted while cell biomass increases. The supernatant after centrifuging the growth medium (two weeks old) supplemented with molasses and cyanide as carbon and nitrogen sources respectively was taken and measured for the residual cyanide. Interestingly, there was a slight improvement in the depletion of cyanide for CNC_1 (97.5%) and CNS_{10} (98.92%), but a slight decrease for CNA_{12} (99.01).

5.6.5. Bacterial preference to use different nitrogen-sources

Nitrogen sources, both organic and inorganic, other than NaCN were used to learn how their preference is to utilize these different nitrogen sources. Accordingly, the largest growth yield was obtained when peptone and yeast extract were used as sources of nitrogen (Fig. 10). NaCN was the next best utilized nitrogen source for all the three strains. Except with moderate growth of CNS_{10}, $NaNO_3$ was poorly utilized by both CNA_{12} and CNC_1. Similarly, all of the three test strains were failed to utilize urea and NH_4Cl as a sole source of nitrogen.

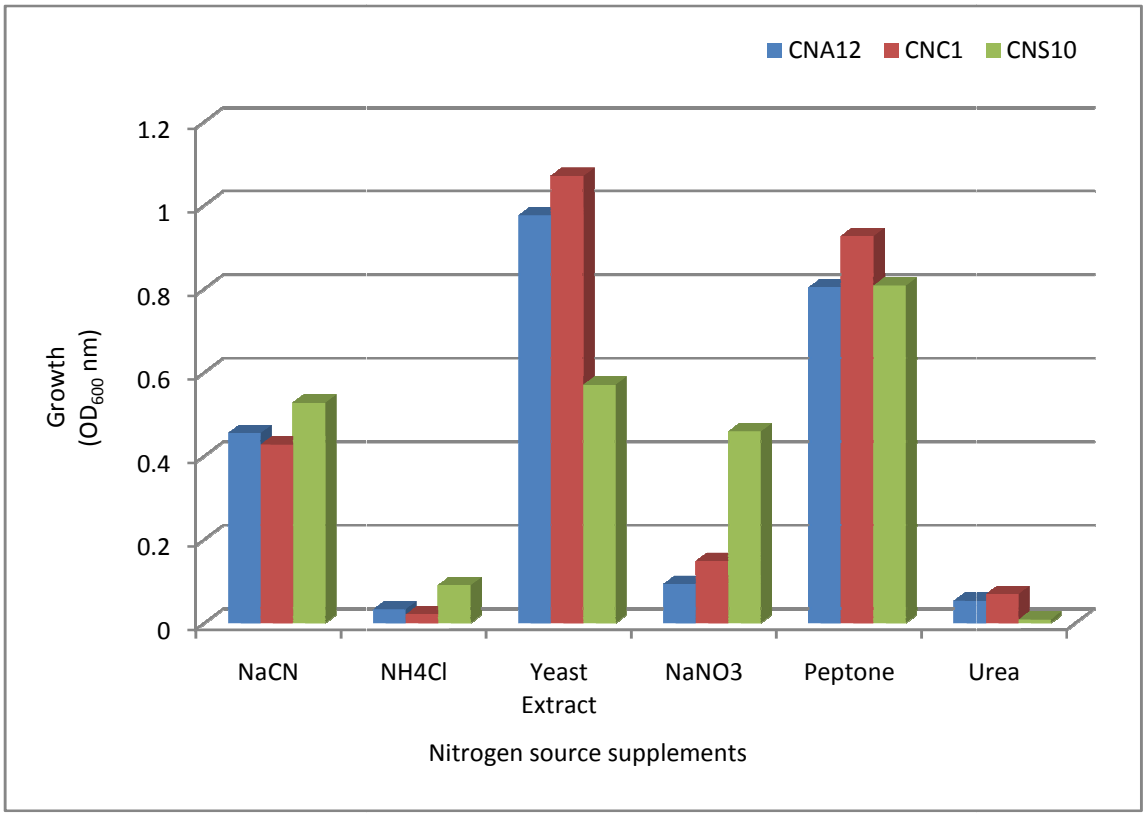

Figure 9: Bacterial biomass as function of different nitrogen sources at the end of 240hrs incubation period.

6. Discussion

In this study, a total of 72 isolates were isolated and purified on a cyanide-enrichment media. Out of which, 30 were selected based on colony characters, their rate of growth and biomass production. The majority of these (17) were isolated from Lake Abijata with high frequency which might indicate its higher potential with regard to cyanide degrading microbes. Earlier, Mekonnen Meaza (2009) and Wajana Lako (2007) isolated cyanide utilizing bacteria from one of the study sites (Lake Chitu).

The selected isolates were subjected to molecular identification process taking 16S rDNA as target gene. Different DNA profiles were obtained for the tested isolates using ARDRA technique. Among the thirty grown on cyanide containing medium, nine different restriction fragment length polymorphism groups were clearly identified. Some of the ARDRA groups were common to some or all of the lakes while the others being unique/ restricted to only one of the lakes (Table 3). Classifier of ribosomal database project (RDP) software showed sequence results from selected isolates came out all (100%) being bacteria (Fig. 4), specifically under genus *Bacillus* and *Halomonas*.

Sequence homology search using NCBI and RDP databases showed the presence of many bacterial strains that were closely related to the study organisms (Table 4). Majority of the matches were *Halomonas* and *Bacillus* sequences. With the exception of few, closest matches based on the search were isolated from habitats having basic similarity with sample sites of this study (Table 4). However, no cyanide utilizing bacterial species has came out as close matches to the study sequences.

Sequence result was concurrent with ARDRA grouping in showing sequence polymorphism. There were, however, cases that isolates showing similar restriction band pattern but having different sequence data. This, as confirmed using DNADOT restriction mapping (http://www.vivo.colostate.edu/molkit/dnadot/), was due to close similarity in their restriction sites for the selected endonucleases. The use of manual method for approximate estimation of fragment sizes might have also contributed for this perplexity.

The phylogenetic tree constructed using selected sequences and their closest matches from the databases, showed their phylogenetic position based on evolutionary relatedness. On the basis of this tree (Fig. 5), major clusters namely Gammaproteobacteria and Firmicutes have been found which comprised *Halomonas* and *Bacillus* groups respectively, as did the BLAST search.

The interesting thing here is that strain CNC_1 which have matched 99% with uncultured bacterium clone was clustered under the *Halomonas* group. This can be compared with the BLAST search result, shown in Table 4, where CNC_1 had 98% similarity with *Halominas shengeliensis*. The phylogenetic tree was biologically sounding in that three of the strains which had best sequence matches with *Bacillus* groups are clustered together and the others with the exception of CNA_{12} laid in a relatively different cluster. CNA_{12} sequence has exceptionally protruded from the rest of the group which could show, purely on the basis of this tree, the sequence being conserved for some time while the rest undergone many changes in the course of evolution.

Only very few species of genus *Bacillus* and *Halomonas* having cyanide utilization properties are reported in the literature (Castric and Strobel, 1969; Skowronski and Strobel, 1969; Sorokin *et al.,* 2000). This result can be a very good source for further study how degradation process is taking place in bacteria other than the well characterized ones such as the *Pseudomonas* group.

Further screening of strains for their cyanide degrading efficiency based on their growth pattern, biomass amount and depletion of cyanide in the medium was done. Accordingly, CNA_{12} and CNS_{10} were found to grow well earlier and have larger amount of biomass reading which might make them better candidates for practical treatment applications. Even there is difference among them in that CNA_{12} grew faster but immediately inhibited by increased cyanide concentrations. On the contrary, CNS_{10} grew slowly and adapted higher concentrations of cyanide (Fig. 6). Strains CNC_1 and CNA_{16} were also the next best growers on cyanide. CNS_3 was a slow grower strain with only less than half biomass readings of CNA_{12} and CNS_{10}.

Along with detection of cell survival and biomass increase, there was also up to 99.33% depletion of cyanide level in the medium by strain CNA_{12} (Table 6). CNS_{10} had also comparable cyanide removal efficiency with CNA_{12} about 98.89%. Three other strains namely CNC_3, CNA_{16} and CNS_5 were with next best efficiency removing more than 92% of the cyanide provided in the medium. Strain CNS_3 was the least efficient among them but still could degrade about 78.5% of the cyanide.

In other studies, there were cases where comparable percentage of cyanide removal attained using mixed cultures. The reason behind this is the presences of various groups of microorganisms which can utilize and reduce accumulation of inhibitory byproducts of cyanide breakdown (Mekonnen Meaza, 2009).

So cyanide reduction was accompanied with increase in bacterial population. Therefore, the possible mechanism responsible for reduction of cyanide concentration was biodegradation. Almagro *et al*., (2005a) also reported that cyanide bacterial degradation under alkaline condition is an assimilatory process.

Tolerance test against different cyanide concentrations was done using selected strains CNA12, CNS10 and CNC1 based on their cyanide removal performance. They are found to be capable of growing well up to cyanide concentration of 400 mg/l and tolerate up to 600 mg/l. Strain CNS_{10} had better growth response to 400 mg/l of cyanide as compared with CNA_{12} which was best at a cyanide concentration of 200 mg/l. There was a decreased response of strain CNC_1 to concentration of 400mg/l cyanide however was good at tolerating 600mg/l, even better than CNA_{12} (Fig.7). Only moderate growth was recorded for all the strains at the concentration of 100mg/l. This might suggest cyanide being the rate limiting component in the metabolic process. All of these strains were incapable of tolerating 800mg/l cyanide concentration which can be suggested with the record of low cell biomass. It is true that for all cyanide degrading bacteria, there is always a maximum concentration of cyanide above which no growth is observed (Avalos *et al.,* 1990).

There are studies reporting comparable or higher biodegradable cyanide concentrations (Almagro *et al.*, 2005a; Akcil *et al.,* 2003; Kao *et al*, 2003; Bosecker and Blumenroth, 2001; Watanabe *et al,* 1998; Dumestre *et al.,* 1997; Figueira *et al.,* 1996; Babu *et al.*, 1992; and Skowronski and Strobel, 1969). However majority of these are taking place in acidic, neutral or slightly alkaline conditions: below dissociation constant. Favorably, bacterial strains in this study are capable of degrading and tolerating higher concentrations at alkaline condition well above pKa value of cyanide.

In addition, isolates were further characterized on the basis of their efficient utilization of different carbon sources and their preference to use various nitrogen sources. Growth of some selected strains has been evaluated on cyanide as the only source of both carbon and nitrogen and there was no significant cell count observed (data not shown).

Among the others, cheap molasses was found to be the best carbon sources while cyanide still being nitrogen source (Fig. 8). According to Johnson *et, al.,* (1995), molasses has traces of nitrogen (0.5-1% (w/w)). This might have boosted initial cell biomass level and consequently improved cyanide removal efficiency of the strains.

It has been shown that growth of the strains was hardly recorded in maltose and glucose supplied media. One explanation for this could be a reaction between reducing sugars and cyanide, commonly taking place at highly alkaline pH and well documented as the Kiliani reaction (Hope and Knowles, 1991; Serianni *et al.,* 1980; Militzer, 1949). This reaction might have made nutrients unavailable and strains fail to utilize them. Dumestre *et al.* (1997) also reported rapid loss of cyanide observed in glucose, galactose, lactose, and maltose minimal media even in uninnoculated ones but not in saccharose minimal medium, yeast extract basal medium, or nutrient broth. According to Wajana Lako (2007), poor expression of invertase or low efficiency in breaking down of sucrose might also be the reason for the failure of utilizing this carbon source particularly for CNA_{12}.

In the nitrogen sources preference experiment, all of the three strains grew very abundantly when peptone and yeast extract were supplied as nitrogen sources instead of cyanide (Fig. 9). Interestingly, NaCN was the next nitrogen source utilized highest by the strains, even comparable in CNS_{10}. From these, it can be concluded that cyanide is still a very good source of nitrogen and can be utilized efficiently. This fact makes them very good candidates with high potential for the biotreatment of cyanurated residues.

Although ammonium is usually the preferred nitrogen source for bacteria, it is the least used nitrogen source in this study. At highly alkaline pH, ammonium has been described as unavailable due to evaporation or toxic to organisms (Almagro *et al*., 2005a; Harris and Knowles, 1983b). The absence of nitrate reductase could have also contributed for the least utilizability of sodium nitrate (Wajana Lako, 2007).

In another preliminary experiment, the strains (particularly CNA_{12}) were found to have the capability to utilize metal-cyanide complexes (data not shown). They were grown on agar medium with same composition used for enrichment except $Fe_2(CN)_3$ being used instead of NaCN as the only nitrogen source. The strains were capable of growing on this medium and there was halo formation around colonies which might suggest production of siderophores for adsorption of metals (Huertas *et al.,* 2006).

7. Conclusion and suggested future works

Alkaliphilic bacterial strains screened in this study evidentially showed the potential to possess degradative activities that can be harnessed to remediate cyanide wastes.

A total of about 72 cyanide degrading bacterial isolates were found from samples of three Ethiopian Soda Lakes. Phylogenetic analysis inferred from comparative 16S rRNA gene sequences indicated that the isolates fall in to seven sublineages which indicate the presence of diverse cyanide utilizing microbial lineages. They were categorized the genera *Bacillus* and *Halomonas*.

Strain CNA_{12} was able to deplete 99.33% followed by CNS_{10} and CNC_1 which removed more than 92% of cyanide provided in the medium. They were also capable of tolerating up to 600mg/l cyanide concentration. Another interesting merit of these strains was their preference to use cheap molasses as carbon and energy source for better utilization of cyanide. Cyanide was found to be preferable nitrogen source next to yeast extract and peptone.

Based on this study, the following points can be recommended.

1. Evaluation of the strains for their ability to utilize thiocyanate and metal-cyanide complexes.
2. Isolation and characterization of further strains from the lakes using varying combinations of culture compositions and conditions.
3. Immobilization of cells for improving their activity towards practical applicability.
4. Establishment of mechanism and biochemical pathways in degradation of both free and metal-cyanides.
5. Construction of laboratory-scale bioreactor.

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Appendix

Colony features of cyanide degrading isolates

Isolate	Source Lake	Colony size	Colony shape	Colony color	Margin	Elevation	Texture	Colony appearance
CNA1	Abijata	Medium	Circular	Light yellow/Cream	Entire	Raised	Moist	Opaque
CNA2	Abijata	Medium	Circular	Light yellow/Cream	Entire	Raised	Moist	Opaque
CNA4	Abijata	Medium	Circular	Brown creamy	Entire	Raised	Mucoid/ Moist	Opaque
CNA5	Abijata	Small	Circular	Brown creamy	Entire	Raised	Mucoid	Opaque
CNA7	Abijata	Medium	Circular	Brown creamy	Entire	Raised	Mucoid	Opaque
CNA8	Abijata	Large	Circular	Brown creamy	Entire	Raised	Mucoid	Opaque
CNA9	Abijata	Large	Circular	creamy	Entire	Raised	Mucoid	Opaque
CNA10	Abijata	Medium	Circular	Light yellow/Cream	Entire	Raised	Moist	Opaque
CNA11	Abijata	Large	Circular	Brown creamy	Entire	Raised	Mucoid	Opaque
CNA12	Abijata	Medium	Circular	Creamy	Undulate	Umbonate	Dry	Opaque
CNA13	Abijata	Small	Circular	Creamy	Entire	Convex ?	Moist	Opaque
CNA14	Abijata	Medium	Circular	Creamy	Undulate	Umbonate	Dry?	Opaque
CNA15	Abijata	Large	Circular	Creamy	Undulate	Umbonate	Dry	Opaque
CNA16	Abijata	Medium	Circular	Light brown	Undulate	Umbonate	Dry	Opaque
CNA17	Abijata	Medium	Circular	Creamy/White	Undulate	Umbonate	Dry	Opaque
CNA19	Abijata	Medium	Circular	Light yellow/Creamy	Entire	Raised	Moist	Opaque
CNC1	Chitu	Small	Circular	Light yellow/creamy	Entire	Raised	Moist	Translucent
CNC2	Chitu	Small	Circular	Light brown	Entire	Raised	Moist	Opaque
CNC3	Chitu	Small	Circular	Pink	Entire	Flat	Moist	Opaque
CNC4	Chitu	Medium	Circular	Creamy?	Undulate	Umbonate	Dry	Opaque
CNC5	Chitu	Medium	Circular	White	Filamentous	Flat (deep)	Dry	Translucent
CNC6	Chitu	Small	Circular	White	Filamentous	Flat (deep)	Dry	Translucent
CNS1	Shala	Punctiforms	Circular	Light yellow	Entire	Raised	Moist	Translucent
CNS3	Shala	Small	Circular	White	Entire	Raised	Moist	Translucent
CNS4	Shala	Punctiforms	Circular	Light yellow	Entire	Raised	Moist	Translucent
CNS5	Shala	Medium	Circular	White	Undulate	Umbonate	Dry	Opaque
CNS7	Shala	Large	Entire?	Creamy	Entire	Raised	Mucoid	Opaque
CNS8	Shala	Large	Circular	Dark brown	Entire	Umbonate	Mucoid	Opaque
CNS10	Shala	Large	Circular	Creamy	Entire	Raised	Mucoid	Translucent

? Confusing characters

Isolates which had excellent growth were selected regardless of their colony character.

Declaration

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

Birhanu Mekuaninte

Signature…… Date…….