Isolation and Genetic Characterization of a Microbial Consortium Capable of Cyanide Degradation

by

Wilmarí Meyer

May 2010

University of the Free State
Universiteit van die Vrystaat

Isolation and Genetic Characterization of a Microbial Consortium Capable of Cyanide Degradation

by

Wilmarí Meyer

B.Sc. Hons. (UFS)

Submitted in fulfillment of the requirements for the degree

MAGISTER SCIENTIAE

In the Faculty of Natural and Agricultural Sciences

Department of Microbial, Biochemical and Food Biotechnology

University of the Free State

Bloemfontein

South Africa

May 2010

Supervisor: Prof. J. Albertyn

Co-Supervisors: Dr. L.A. Piater

Prof. E. van Heerden

This dissertation is dedicated to my husband (Bruce), my parents (Peet and Ria) and sister (Colette) for understanding and supporting my dreams.

"The great tragedy of science: The slaying of a beautiful hypothesis by an ugly fact."
Thomas Henry Huxley

ACKNOWLEDGEMENTS

I would like to express my appreciation to the subsequent people and institutions:

- Dr. Lizelle Piater for your guidance, assistance and encouragement but most of all, your dedication to your post-graduate students and always keeping an open door policy.

- Prof. Koos Albertyn for accepting me as a master's student in the second year of my project. Thank you for always making time to discuss a problem and to reach a solution that leads to positive results.

- Prof. Esta van Heerden for the advice and financial support for the duration of this study.

- Special thanks to Prof. Derek Litthauer for your patience and assistance with the editing and interpretation of the pyrosequencing results.

- Department of Biotechnology
    - Lecturers for all the advice and privilege to gain knowledge from people who already left footprints in the science community of South Africa.
    - Fellow students for the smiles and chats when passing each other in the hallway, specially the senior students of the Biochemistry group.
    - Friends for moral support and making my master's degree, in retrospect, an excellent experience.

- My husband, Bruce, for all your love and understanding. Thank you for accompanying me to the department in the early morning hours. I love you.

- My parents, Peet and Ria, for giving me the opportunity to pursue my dreams, all your love and understanding. My sister, Colette, for your support, encouragement and love. I love you all.

- The National Research Foundation for the financial support during my master's degree.

- Jesus Christ for giving me opportunities in life. For strength and endurance to reach my dreams and for all the people in my life who surrounds me with love and encouragement.

Table of contents

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

%	Percentage
℃	Degrees Celsius
APS	Ammonium persulphate
ATP	Adenosine 5-triphosphate
BLAST	Basic Local Alignment Search Tool
bp	Basepair
CDS	Coding sequence
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
DTPA	Diethylenetriaminepentaacetic acid
EDTA	Ethylene diaminetetraacetic acid
g	Gram
Gb	Giga basepair
$g.l^{-1}$	Gram per liter
gDNA	Genomic DNA
h	Hour
IPTG	Isopropyl β-D-1-thiogalactopyranoside
kb	kilobasepair
L	Litre
LB	Luria-Bertani broth
$\mu g.ml^{-1}$	Microgram per millilitre
µl	Microlitre
µM	Micromolar
m	Meter
M	Molar
Mb	Mega basepair
$mg.g^{-1}$	Milligram per gram
$mg.kg^{-1}$	Milligram per kilogram
$mg.l^{-1}$	Milligram per litre
$mg.\mu l^{-1}$	Milligram per microlitre
min	Minute
ml	Millilitre
mM	Millimolar

$mS.cm^{-1}$	Millisiemens per centimeter
NAD^+	Nicotinamide adenine dinucleotide
NADH	Reduced nicotinamide adenine dinucleotide
$NADP^+$	Nicotinamide adenine dinucleotide phosphate
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NCBI	National Center for Biotechnology Information
ng	Nanogram
$ng.\mu l^{-1}$	Nanogram per microliter
OD	Optical density
ORF	Open reading frame
PCR	Polymerase chain reaction
$pmol.\mu l^{-1}$	Picomol per microliter
PPi	Pyrophosphate
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revolutions per minute
RT	Room temperature
SDS	Sodium lauryl sulfate (Sodium dodecyl sulfate)
SNPs	Single-nucleotide polymorphisms
TAE	Tris (2-Amino-2-(hydroxymethyl)-1,3-propandiol)-HCl, Glacial acetic acid and EDTA
TE-buffer	Tris-EDTA buffer
TEMED	N,N,N',N'-tetramethylethylenediamine
Tris	2-Amino-2-(hydroxymethyl)-1,3-propandiol
$U.\mu l^{-1}$	Units per microliter
UV	Ultraviolet
V	Volt
v/v	Volume per volume
$V.cm^{-1}$	Volt per centimeter
w/v	Weight per volume
x *g*	Times gravity
x	Times
X-Gal	5-bromo-4-chloro-3-indolyl-beta-D-galactopyranosidehosphate

CHAPTER 1

1. LITERATURE REVIEW

1.1. Introduction

There are two viewpoints for the origin of life (Matthews, 2004). The first viewpoint suggests that ribonucleic acid (RNA) was present before deoxyribonucleic acid (DNA) based on some RNA molecules that can act both as a catalyst and a carrier of information. The second viewpoint has been proposed since the early 1800's. This statement suggests that cyanide based compounds did play an important role in the evolution of life on earth. It is believed that there is a connection between cyanide, proteins and life on earth through the polymerization of HCN. α-Amino acids are believed to be secondary products from the formation of HCN polymers that give rise simultaneously to polypeptides and polynucleotides which in turn are precursors for proteins (enzymes) and nucleic acids (DNA and RNA). This model is mainly based on the occurrence of abundant formation of HCN polymers in our Milky Way galaxy stating that the primitive earth was covered with these polymers. Life then emerged from these polymers due to the aqueous, reducing environment on earth (Matthews, 2004).

Cyanide can be defined as: *A triple-bonded molecule with a negative charge consisting of one atom of carbon in the 2^+ oxidation state and one atom of nitrogen in the 3^- oxidation state* (International Cyanide Management Code). In the world we are living in now cyanide is used in a variety of manufacturing protocols of various products as well as in the mining industries. Cyanide generally refers to one of three classifications which are: 1) Total cyanide (CN_T), 2) Weak acid dissociable cyanide CN_{WAD} and 3) Free cyanide (CN_F) as can be seen in Figure 1.1.1. These three forms of cyanide are measured differently in environmental samples and it is very important to understand the relationship before analysis is done. The different classes of cyanide can be measured either by primary or alternative analytical methods.

The ideal method for the analytical determination of CN_F is silver nitrate titration. The free cyanide ions and the silver ions will form a complex. When all the free cyanide is bound in the silver cyanide complex the endpoint of the titration will be indicated by the surplus silver ions. The preferred analytical method for determining the CN_{WAD} is by using manual

distillation at a pH of 4.5. The CN_{WAD} will be liberated as dissolved HCN (g). The HCN (g) is carried to a caustic soda absorption by an air stream and the CN_{WAD} will appear as CN_F. The CN_F can then be determined with the silver nitrate titration method as discussed previously. The preferred analytical method to determine the CN_T is similar to the method described for determining CN_{WAD}. Due to the presence of stable iron cyanide complexes for example, ferro- and ferricyanides, temperatures must be elevated and strong acidic conditions must be applied for the liberation of the cyanide ion from the stable complexes. Figure 1.1.1 indicates that the CN_F concentration is always smaller or equal to the CN_{WAD} concentration, and likewise, the CN_{WAD} concentration is always smaller or equal to the CN_T concentration.

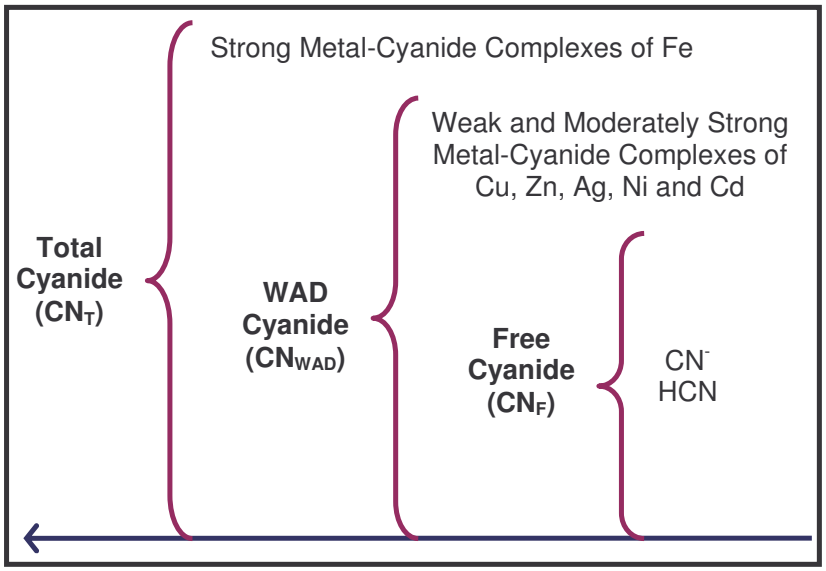

Figure 1.1.1: General classification of cyanide compounds (Taken from Global InfoMine).

Cyanide is highly reactive and will readily form complexes with 1) alkali earth cations forming simple salts or 2) numerous metal cations forming ionic complexes with varying strengths (International Cyanide Management Code). Sodium, calcium and potassium salts are toxic due to their high solubility in water and quick solubilization to form free cyanide. Metal-cyanide bond strength is classified by the pH which leads to dissociation (Sharma *et al.*, 2008). Cyanide complexed with metals such as copper, zinc and cadmium are classified as weak acid dissociable (WAD) complexes and dissociates at a ~ pH 4. These complexes are less toxic than the cyanide salts but can still dissociate into free cyanide and the metal cation which can also be toxic to the environment. Strong acid dissociable (SAD) complexes are with metals such as gold, mercury, cobalt and iron and dissociates at ~pH 0 (Sharma *et al.*, 2008).

Salt-type cyanide compounds defined as metal cyanide complexes combined with alkali cations, such as potassium ferrocyanide ($K_4Fe(CN)_6$), is more soluble in water as iron-cyanide complexes without the cation, and will form hydrogen cyanide gas when dissolved (Sharma *et al*., 2008). Thiocyanate (SCN^-) can also form when the cyanide ion combines with sulfur and this compound dissociates under weak acidic conditions. Thiocyanate is however not classified as a WAD complex due to the complexing properties that closely resemble that of cyanide. Cyanate (OCN^-) forms when cyanide is oxidized either by natural processes or treatment strategies of effluent waste water containing cyanide. Cyanate is less toxic than hydrogen cyanide (HCN) and is hydrolyzed readily to form ammonia (NH_3) and carbon dioxide (CO_2) in the environment (Sharma *et al*., 2008).

Cyanide is highly toxic to living organisms due to the potent inhibitory effect on the respiration system. The inactivation of the respiratory system is due to the tight binding of cyanide to cytochrome *c* oxidase (CcOX) which is the oxygen-reducing component of the mitochondrial electron transport (Leavesley *et al*., 2008). Anaerobic respiration will occur leading to lactic acidosis. The lethal dose of potassium or sodium cyanide is 200-300 mg and for hydrogen cyanide 50 mg. The toxicity of cyanide depends on the complex in which the cyanide molecule is bound (International Cyanide Management Code). The most toxic forms of cyanide are: 1) free cyanide (CN^-) or 2) hydrogen cyanide (HCN), which can be in a gaseous or aqueous state (referred to as hydrocyanic- or prussic acid). Usually, at a low alkaline pH (between 9.3 – 9.5), the amount of CN^- and HCN in the solution are in equilibrium. At a very high alkaline pH (>11), the equilibrium will shift and 99% of the cyanide in the solution will exist in the CN^- form. In contrast, at a neutral pH (~7) the solution will contain 99% HCN depending on the temperature and the pH. Both gaseous and aqueous HCN are colourless and have a bitter almond odour. It has been suggested in literature that the inability to smell cyanide is due to a sex-linked recessive gene (Allison, 1953). In general ~18% of males and ~5% of females are unable to smell cyanide solutions (Allison, 1953).

1.2. Natural occurring cyanides

Cyanide compounds can be synthesized (cyanogenesis) by various taxa including fungi, plants, bacteria and arthropods. Plants can synthesize cyanide compounds that are bitter tasting and called cyanogenic glucosides (Cummings and Baxter, 2006). The glucosides serve as a defense against pathogens and herbivores and in some plants, for example, cassava roots and potato-like tubers grown in tropical countries; cyanide occurs naturally (Dash *et al*., 2009). Some bacteria and fungi can utilize the toxicity of the cyanide

compounds to produce antibiotic cyanide compounds that also inhibit competing organisms (Cummings and Baxter, 2006). *Chromobacterium violaceum* ATCC 53434 and *Trichoderma harzianum* are two examples. *C. violaceum* produces an antibiotic, aerocyanidin (an isonitrile), that is active against Gram-positive organisms and *T. harzianum* produces homothallin II (a nitrile), that is active as an antibiotic against Gram-negative and –positive bacteria. These compounds can also be deployed in other roles, for example, as pheromones in insects to control mating behavior (Cummings and Baxter, 2006).

1.2.1. Cyanogenesis in plants

The first hydrogen cyanide (HCN) compound was isolated in 1802 from the leaves of a peach and bitter almond nuts. The process by which cyanide [CN^- or hydrogen cyanide (HCN)] is produced is described as cyanogenesis (Bais *et al*., 2008). In plants, cyanogenic glycosides (CG) can be chemically defined as glycosides of α-hydroxinitriles (cyanohydrins), amino acid derived plant constituents, belonging to the secondary metabolites (not essential metabolites for development and growth but important for the survival of the species based on the fact that they relate plants with the components of their environment for example, physical environment and the living community (Iriti and Faoro, 2009)), and are present in more than 2 500 plant species (Vetter, 2000).

The chemical nature of substituents, namely aromatic, aliphatic and glycosides with a free α-hydroxynitrile are the main characteristic of the groups into which CG's can be divided (Vetter, 2000). The control of cyanogenesis at genetic level has not yet been established. Variation is seen between different plant species in the amount of hydrogen cyanide (HCN) that is produced. This variation reflects the fact that differences exist in both the synthesizing of the CG's and the enzyme responsible for the degradation of these compounds. This can be caused by diverse ecological and physiological factors (Vetter, 2000).

The biosynthesis of cyanogenic glycosides is represented in Figure 1.2.1 (Vetter, 2000). n-hydroxylamino acid is formed after the hydroxylation of the α-amino acid. The n-hydroxylamino acid is converted to form an aldoxime and this is converted into a nitrile. An α-hydroxynitrile is formed after the hydroxylation of the nitrile. The final step is the glucosylation of the α-hydroxynitrile which yields the corresponding CG (Vetter, 2000).

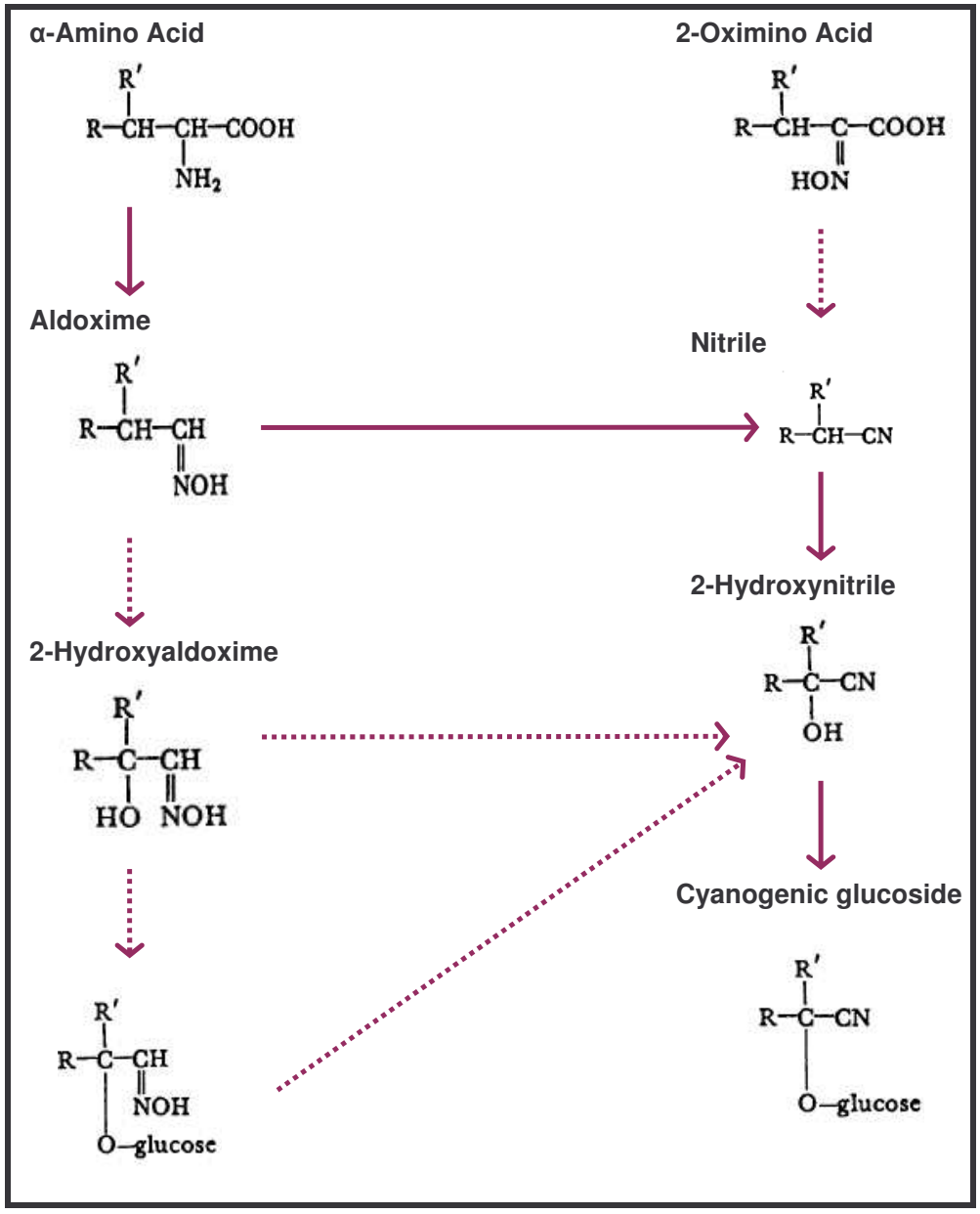

Figure 1.2.1: The pathways of cyanogenic glycoside biosynthesis. Alternative pathways are indicated with dotted arrows (Taken from Vetter, 2000).

1.2.2. Cyanogenesis in fungi

Hydrogen cyanide production by a microorganism was first demonstrated in 1871 by von Lösecke while observing the fungus *Marasmius oreades* (Knowles, 1976). Cyanide in fungi is generally produced during the late exponential to stationary growth phase in both the fruiting bodies and the mycelia. Glycine is directly used in cyanide production where the carbon of the methylene group of glycine produces the carbon of the cyanide molecule. Serine can also be used but less cyanide is formed. This is due to the conversion of serine

to glycine using the enzyme serine hydroxymethyl transferase. Cyanogens (compounds containing a CN^- group) are produced during active growth and the rate of free cyanide produced from the cyanogen is very slow. The most prominent cyanogen present is glyoxylic acid cyanohydrin.

1.2.3. Cyanogenesis in bacteria

Various bacteria have been identified to produce cyanogens for example, *Chromobacterium violaceum* and various *Pseudomonas* species including *P. auruginosa*, *P. fluorescens*, *P. putida* and *P. syringae* (Visca *et al*., 2007). However, glycine (as well as glutamate) must be added to the growth media as a nitrogen source for cyanogenesis to occur (Knowles, 1976). It is proposed that cyanogenesis of bacteria may occur to regulate the intracellular glycine levels as the growth rate slows down (Askeland and Morrison, 1983) and methionine regulates the glycine pool of the cell. Three other amino acids are involved in cyanogenesis namely serine, threonine and phenylalanine (Castric, 1977). Serine is a good precursor but does not stimulate cyanogenesis. Threonine can be converted to glycine and serves as a precursor. Phenylalanine is a poor precursor and does not stimulate cyanogenesis. It is proposed that the role of threonine is through an indirect route such as the regulating of the glycine pool or transport of glycine. Cyanogenesis is part of the secondary metabolism (Blumer and Haas, 2000). The characteristics of a secondary metabolite (cyanide) are (i) no function in primary metabolism, (ii) synthesis in limiting growth conditions (O_2 limitation), (iii) an ecological advantage to the producing organism as a result of synthesis and (iv) tolerance to cyanide of the synthesizing organism.

Hydrogen cyanide and carbon dioxide (CO_2) is formed by oxidative decarboxylation of glycine (Brandl and Faramarzi, 2006) through the three-subunit membrane-bound flavoenzyme encoded by *hcnABC* (cluster of three genes which presumably form a operon) (Blumer and Haas, 2000). In *C. violaceum* and *Pseudomonas* species the enzyme complex responsible for hydrogen cyanide formation is HCN synthase (Blumer and Haas, 2000). This enzyme is sensitive to oxygen and very labile. HCN synthase has only been partially purified from *Pseudomonas* species due to the instability of this enzyme. In Figure 1.2.2 a model, of glycine oxidation, is proposed.

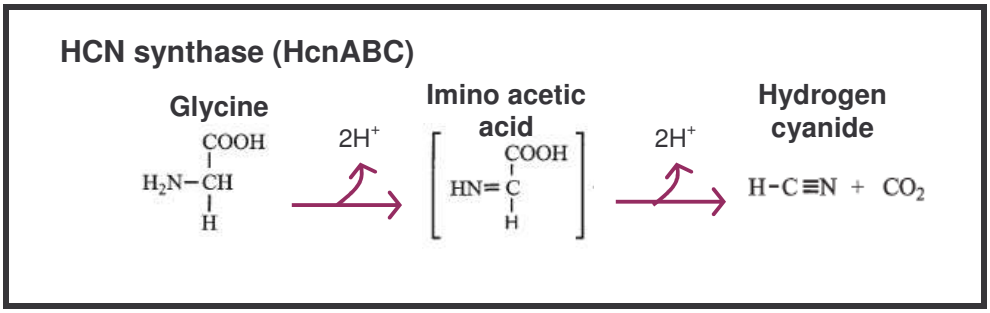

Figure 1.2.2: The oxidation of glycine occurs via two steps (Taken from Blumer and Haas, 2000).

The subunits of HCN synthase enzyme of *P. fluorescens* have similarities with known oxidases or dehydrogenases. For example, the glycine oxidase of *Bacillus subtilis* resembles the HcnC subunit. The reaction that is catalysed by glycine oxidase is given in Figure 1.2.3. This enzyme and related oxidases catalyse the oxidation of substrate amino acids to the corresponding imino acids. These imino acids are then hydrolysed to ammonia and the α-keto acids. These oxidases are monosubunit flavoproteins.

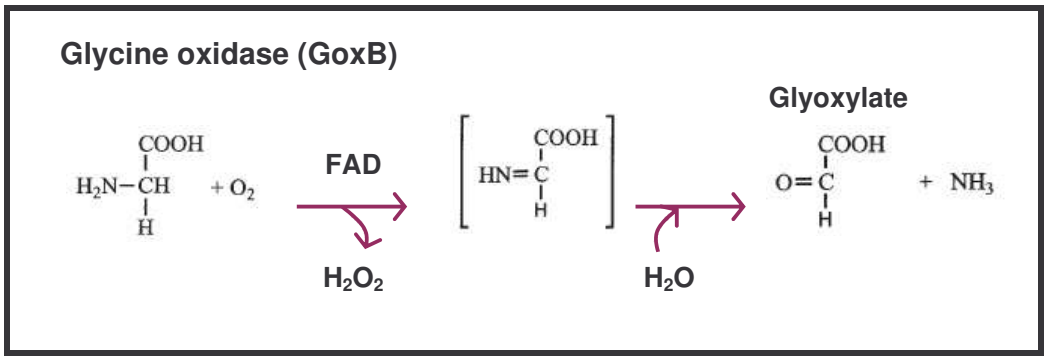

Figure 1.2.3: Glycine oxidase catalysed reaction (Taken from Blumer and Haas, 2000).

HcnA subunit is similar to a putitive octopine oxidase from *Rhizobium meliloti* and the putative nopaline oxidase from *Anabaena tumefaciens.* The reaction catalysed by the nopaline oxidase is given in Figure 1.2.4.

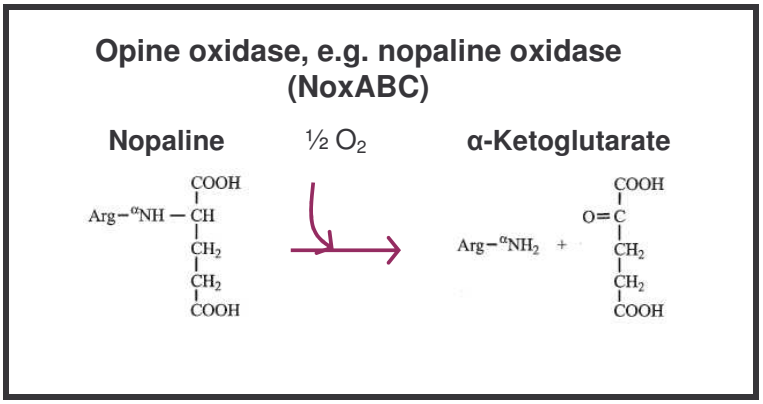

Figure 1.2.4: Reaction catalysed by an opine oxidase (Taken from Blumer and Haas, 2000).

Based on amino acid sequence comparisons it can be deduced that HCN synthase is basically an amino acid dehydrogenase/oxidase. Glycine oxidase and opine oxidase cleave C-N bonds but HCN synthase cleave the imino acetic acid at the C-C bond. The biological reason behind this cleavage is still unknown. Hydrogen cyanide can also be produced when peroxidase is coupled to imino acid oxidase. An example of this can be seen in *Anacystis nidulans*. l-Histidine is catalysed to form an imino derivative by an l-amino acid oxidase and hydrolysed to imidazole pyruvate. If coupled to peroxidase, various products form due to the limited hydrolysis of the derivative. The products include HCN, imidazole aldehyde and CO_2 (Figure 1.2.5). Peroxidase can be replaced by inorganic oxidants and various amino acids can also be used. Cyanide is a strong chelator of various metal ions such as (Co^{2+}, Cu^{2+}, Ni^{2+}, etc.) and a good nucleophile, thus, it is proposed that cyanide will be released into the periplasm rather than cytoplasm.

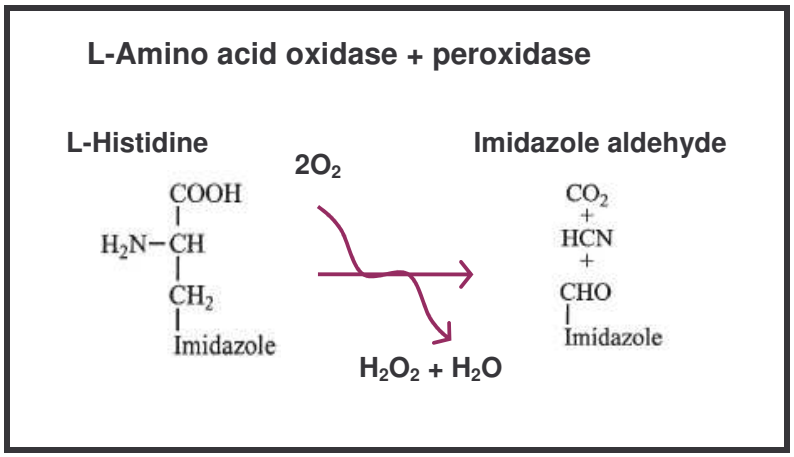

Figure 1.2.5: Coupled system for synthesizing HCN. (Taken from Blumer and Haas, 2000).

Cyanogenesis in bacteria is influenced by oxygen (O_2), phosphate (P) and iron (Fe) concentration (Brandl and Faramarzi, 2006) and occurs maximally in the late exsponential and early stationary phase under micro-aerophilic conditions (Gallagher and Manoil, 2001). The amount of hydrogen cyanide being produced by cyanogenesis can be as high as 300 µM (William *et al*., 2006). This process can assist the pathogenicity of the cyanogenic bacterium towards other bacteria which share their ecological niche (for example in the rhizosphere), and therefore serve as a biocontrol metabolite (Visca *et al*., 2007). Cyanide that is synthesized by cyanogenic organisms can also form complexes with transition metal ions (Blumer and Haas, 2000). These complexes can assist the bacteria in mobilizing the metal ions, e.g. from clay minerals in soil.

In *P. fluorescens* and *P. aeruginosa* the synthesis of hydrogen cyanide (HCN synthase) depends on the anaerobic regulator of arginine deiminase and nitrate reductase (ANR) protein that acts as a transcriptional regulator (Blumer and Haas, 2000). The ANR protein is part of the fumurate and nitrate reductase regulator (FNR) family and these proteins are oxygen-sensing and dimeric. These proteins are inactivated, in the presence of oxygen, when their two $[4Fe\text{-}4S]^{2+}$ clusters are converted to $[2Fe\text{-}2S]^{2+}$. The ANR proteins exhibit strong similarity to the structure and functionality of the FNR proteins. Oxygen tension on two levels plays an important role in the activity of HCN synthase. Control through oxygen tension is at the level of enzymatic activity and at the level of transcription *via* ANR. The global activator GacA is a secondary regulatory protein of cyanogenesis in *P. aeruginosa* and *P. fluorescens*. GacA is a response regulator of GacS. GacS plays an important role in a variety of extracellular product excretion. Through a cascade of reactions the end result of GacA is the activating or deactivating of the *hcnABC* genes (Blumer and Haas, 2000).

P. aeruginosa possesses an aerobic electron-transport chain that is branched and terminated by up to five terminal oxidases (Williams *et al*., 2006). Two of the five oxidases are like a cytochrome cbb_3 type, one is like a cytochrome aa_3 type, one is a quinol oxidase (related to cytochrome bo_3 in *Escherichia coli*) and the fifth oxidase is called the cyanide-insensitive oxidase (CIO). CIO is homologous to the cytochrome *bd* quinol oxidases, which are not members of the haem-copper oxidase family. However, the CIO of *P. aeruginosa* does not possess haem *d*, thus, the CIO belongs to a class of oxidases that are related to cytochrome *bd* oxidases but are grouped separately. CIO allows respiration to proceed in the presence of >1 mM potassium cyanide and are relatively insensitive to the oxygen concentration. The regulation of CIO is not yet fully understood, but cyanide is an inducer of the genes *cioAB* that codes for this oxidase. It is now proposed that the production of CIO

and cyanogenesis is coincidentally due to the role CIO plays in cyanide synthesis and growth of this organism under cyanogenic conditions (Williams *et al*., 2006).

1.3. Other sources of cyanide

1.3.1. Anthropogenic sources of cyanide

The cyanidation process is a major contributor to the anthropogenic contamination in our environment. This process is being used for the extraction of mainly gold (silver can also be extracted) from ore (Gönen *et al*., 2004). The basic principle of the process has been used for over a century but recently it has been refined for the recovery of low-grade gold ores down to the grain size of 10 µm. In Table 1.3.1 the cyanide species that can be present, in effluents from gold milling operations, are listed (White *et al*., 2005). The species are listed according to their stability (indicated by the blue arrow) with free cyanides the most unstable and ferricyanides the most stable. Thiocyanates (SCN^-) and cyanates (OCN^-) are also present due to the dissolution reaction of metal cyanides.

Table 1.3.1: Cyanide complexes (modified from White *et al*., 2005).

Category	Cyanide form
Free cyanide	CN^-, HCN
Simple cyanide salts	
• Readily soluble	NaCN, KCN, $Ca(CN)_2$, $Hg(CN)_2$
• Relatively insoluble	$Zn(CN)_2$, $Cd(CN)_2$, CuCN, $Ni(CN)_2$, AgCN
Weak complexes	$Zn(CN)_4^{-2}$, $Cd(CN)_3^-$, $Cd(CN)_4^{-2}$
Moderately strong complexes	$Cu(CN)_2^-$, $Cu(CN)_3^{2-}$, $Ni(CN)_4^{2-}$, $Ag(CN)_2^-$
Strong complexes	$Fe(CN)_6^{4-}$, $Co(CN)_6^{4-}$

During cyanidation gold is complexed to cyanide which is very stable (White *et al*., 2005). It has been demonstrated that microorganisms can degrade various metal-cyanide complexes and this led to the hypothesis that biological recovery of gold, from gold mill operation effluent waters, will be possible. Bacteria will degrade the aurocyanide complex (gold cyanide complex) and lead to the liberation of Au^+ (Equation 1.3.1), which in turn will

seek an electron from the nearby environment due to its galvanic properties. This will produce elemental gold (Equation 1.3.2). Iron and zinc will be good electron donors due to their low placement on the galvanic series. Metal gold will form a plate on the electron donor's surface due to the reduction that takes place on the metal's surface (White *et al.*, 2005).

Equation 1.3.1: $[Au(CN)_2]^- + aH_2O + bO_2 + \text{enzyme} \rightarrow Au^+ + cNH_4^+ + dCO_2 + \text{biomass}$

Equation 1.3.2: $Au^+ + e^- \rightarrow Au^0$

Other anthropogenic sources of cyanide can occur in the environment due to various types of industries.

- The food and feed industries also contributes to cyanide pollution (Siller and Winter, 1998). One example of the food industry is cassava starch production. The cassava plant is the staple food of 500 million people in the tropics and Africa. These plants produce cyanogenic glycosides, as mentioned earlier, and the hydrolysis of these products by plant-borne enzymes lead to concentrations of cyanide of up to 200 $mg.l^{-1}$ in the environment. The agriculture industry contributes significantly to cyanide pollution of the environment by the application of nitrile based pesticides such as chlorothalonil (a broad spectrum fungicide, and bromoxynil (Cummings and Baxter, 2006). Chlorothalonil, also used as an anti-fouling agent on boat hulls, leach into marinas and can lead to serious environmental damage to water species other than the target fouling organisms (Sakkas *et al.*, 2002). Bromoxynil is used as an herbicide to control diseases of broad leaf crops.

- Industrial activity in the past can also contribute to cyanide pollution. The highest percentage of contamination is due to former manufactured gas plant (MGP) facilities (Cumming and Baxter, 2006). Manufactured gas was synthesized from coal and the result of the manufacturing process was contaminants such as, tars, cyanides and hydrogen sulphide. The soil and ground water at old MGP's can have cyanide concentrations of up to 5000 $mg.kg^{-1}$ and 280$mg.l^{-1}$, respectively (Thomas *et al.*, 2003).

- Sewage treatment plants, especially those localized in industrial areas, discharge cyanide into the environment and the concentration of cyanide can be as high as 0.1 $mg.l^{-1}$.

- The process of aluminium metal smelting occurs in a reduction cell (a pot) which is composed of steel rods and lined with carbon cathodes called the pot lining (Pong *et al*., 2000). The reduction of aluminium occurs in a bath of cryolite (Na_3AlF_6) at 930ºC -1000ºC. Fluoride compounds and cyanide impregnate the carbon cathode lining. The cyanide production is caused by the reaction of carbon and nitrogen in the presence of sodium at very high temperatures. When iron occurs in the aluminium or the cell fails the potlining is replaced. The spent potlining (SPL) contains high levels of leachable cyanide and fluoride and is considered as an environmental hazardous material.

- Sodium and potassium salts are used in electroplating processes where they are used in the electroplating baths and in the basic decreasing to control the metal ions concentration (Smith, 2003).

- Iron cyanide is used as anticaking agents both in fire retardants and road salts (Cummings and Baxter, 2006). Each year 10 million tonnes of road salt are used in the United States of America (USA) and this account for an environmental input of 700 tonnes iron cyanide per year.

According to a recent study by the American Agency for Toxic Substances and Disease Registry (ATSDR), 834 000 tonnes hydrogen cyanide is required per year to satisfy the requirements of their chemical, mining, steel and electroplating industries (ATSDR, 2004).

Most of the above mentioned industries have established procedures for the handling, storage and distribution of cyanide compounds to eliminate the possible contamination of the soil and ground water (Cummings and Baxter, 2006). Unfortunately, accidental spillages do take place and lead to the contamination of the immediate environment as well as the ground water systems. The most significant example occurred in Romania at the Baia Mare mining operation (Soldán *et al*., 2001). Approximately 100 000 m^3 of cyanide was released into the Tizsa river system. This spillage led to the mortality of aquatic organisms, animals and plants living and growing close to the contaminated river.

1.4. Degradation of cyanide

1.4.1. Natural degradation

Attenuation or natural degradation of cyanide is all the processes that reduce cyanide concentrations without human involvement. Cyanide undergoes numerous reactions and transformations naturally (Figure 1.4.1) (Álvarez *et al*., 2004). These reactions include volatilization, bio-degradation, adsorption (onto surfaces of solids), oxidation and photodecomposition. Variables influence each mechanism differently and these variables include pH, water chemistry and temperature (Álvarez *et al*., 2004).

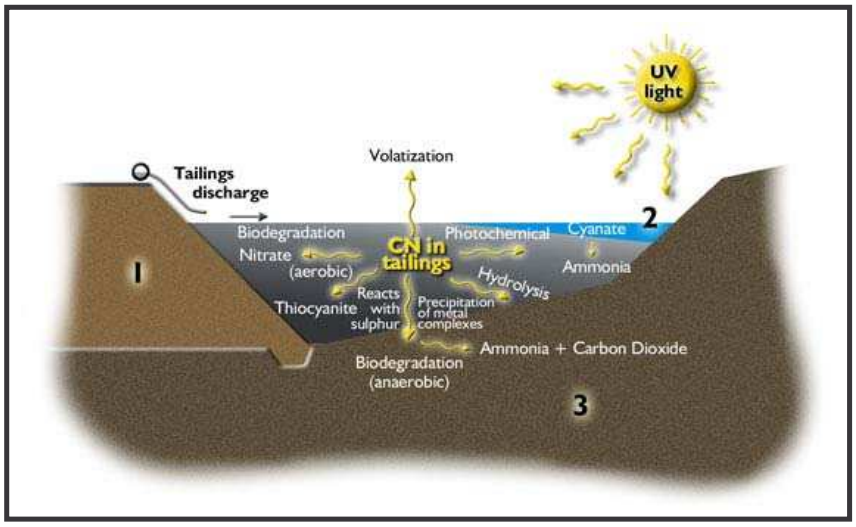

Figure 1.4.1: Natural cyanide degradation processes. 1) Waste Rock Embankment; 2) Decant pond and 3) Natural ground (Taken from Martha Mine, 2001).

The pH of the water, that is part of the tailings discharge, is initially high for the complexing of cyanide with gold. The pH will decrease naturally to a value of below 9. At this pH most of the free cyanide will occur as hydrogen cyanide (HCN). HCN evaporates (volatilizes) easily due to its high vapour pressure. If cyanide reacts with air to form bicarbonate and ammonia it is due to the process of oxidation. Naturally occurring microorganisms in the soil can degrade cyanide, and this is known as biodegradation. Sunlight (ultraviolet) can degrade cyanide complexes, and this process is called photodecomposition (photochemical reactions). Photodecomposition is effective against iron cyanide complexes. Factors contributing to the stability of iron cyanide complexes are pH, cyanide concentration in soil and ground water as well as the redox potential (Boopathy, 2000). In acidic soils the iron complexes are stable but in an alkaline pH, the various iron complexes such as Prussian blue, solubility is greatly increased and it allows the complexes

to become mobile. Under these conditions the iron complexes can also dissociate into free cyanide. Large surface areas and shallow ponds were used in the 1970's to increase the rate of natural degradation. This increased the contact between carbon dioxide (CO_2) of the atmosphere which will decrease the pH of the tailings water and in turn increase the hydrogen cyanide concentration which in turn will ensure a high volatilization rate. Natural degradation will destruct most of the free cyanide however; strongly complexed cyanides are highly unlikely to release their free cyanides at a tempo that is noteworthy. This realization led to the employment of chemical, and later on, biological degradation strategies (Álvarez *et al*., 2004).

1.4.2. Physical degradation

The Barren/Fresh Water Rinsate treatment is an accelerated version of natural degradation and this process is based on the washing of the heap from the barren pond by using fresh water (Mosher and Figueroa, 1996). The water is added to counter evaporation losses. During this method the cyanide concentrations decrease due to the native microbiota, volatilization and complexation and no reagents are used. This method will be best suited in climates that have access to an inexpensive source of fresh water and have a negative water balance (minimize volume of Rinsate generated). This method requires no additional engineering costs and no additional capital equipment. High cost for operation and maintenance can occur due to the closure standard for the height of the heap (Mosher and Figueroa, 1996).

1.4.3. Chemical degradation

Cyanide can either be treated or recovered by various processes. The process of cyanide treatment is classified as a destruction-based process whereas the process of recovery of cyanide is classified as a physical process (Akcil, 2003). By using either chemical or biological reactions, in a destruction process, the cyanide is converted to less toxic compounds.

To select the appropriate treatment process for the destruction of cyanide, various factors must be considered (Akcil, 2003). The factors include chemical characteristics of the solution or slurry that need to be treated, the volumes to be treated, the environmental setting and the applicable regulations. Mostly, the destruction processes of cyanide are based on the conversion of cyanide into less toxic compounds through an oxidation process.

One of the earliest methods developed for the destruction of cyanide were alkaline breakpoint chlorination (Cummings and Baxter, 2006). The destruction of cyanide is a two step process as can be seen in Equation 1.4.1 (Step 1) and Equation 1.4.2 (Step 2). The first step is the conversion of cyanide to cyanogen chloride and the second step is the hydrolysis of the cyanogen chloride into cyanate (Akcil, 2003).

Equation 1.4.1: $Cl_2 + CN^- \rightarrow CNCl + Cl^-$

Equation 1.4.2: $CNCl + H_2O \rightarrow OCN^- + Cl^- + 2H^+$

If thiocyanate and ammonia are also present in the treated effluent, additional chlorine can be added to attain the breakpoint which will lead to the oxidation of these two compounds to produce nitrogen gas [$N_2(g)$] (Akcil, 2003). This process has been replaced by other treatments due to the lack of effect on strong acid dissociable (SAD) complexes of cyanide, high utilization of chlorine as well as the high concentration in the discharge and the high reagent costs to control the pH (Cummings and Baxter, 2006).

The INCO (SO_2/air) process (patented) was developed in the 1980's by INCO (Akcil, 2003). This process combines sulfur dioxide (SO_2), oxygen (O_2) and cyanide compounds to oxidize the weak acid dissociable (WAD) cyanide complexes in the effluent water to the less toxic compound, cyanate (Cummings and Baxter, 2006). The iron cyanide complexes are reduced to the ferrous state and precipitates as insoluble iron-copper-cyanide complexes (Mudder *et al*., 2007). The residual metals (noted as M in the following equations) due to the liberation from the CN_{WAD} are precipitated as their hydroxides (Equation 1.4.5).

This process needs to occur in the presence of a copper catalyst and between a pH value of 8-9 (shown in Equation 1.4.3 and Equation 1.4.4).

Equation 1.4.3: $CN^- + SO_2 + O_2 + H_2O \xrightarrow{Cu^{2+}\ catalyst} OCN^- + H_2SO_4$

Equation 1.4.4: $M(CN)_4^{2-} + 4SO_2 + 4O_2 + 4H_2O \xrightarrow{Cu^{2+}\ catalyst} OCN^- + 8H^+ + 4SO_4^{2-} + M^{2+}$

Equation 1.4.5: $M^{2+} + 2OH^- \rightarrow M(OH)_2$

The main advantage of the process is the application in the treatment of tailings slurry as well as solution (Akcil, 2003) and the whole process can be done inside a simple stirred tank

reactor circuit (Mudder *et al.*, 2007). The disadvantage is the fact that the pH must be regulated constantly, due to the generation of acid, by the addition of lime (CaO) which leads to the formation of metal hydroxide sludge's and the process is ineffective against CN_{SAD} (Cummings and Baxter, 2006).

Hydrogen peroxide (H_2O_2) can also be used for the oxidation of cyanide compounds (Degussa process) as can be seen in Equation 1.4.6.

Equation 1.4.6: $CN^- + H_2O_2 \xrightarrow{\textbf{Cu}^{2+}\textbf{ catalyst}} OCN^- + H_2O$

When metal cyanide complexes must react with hydrogen peroxide, the rate of the reaction depends on the dissociation rate of the complex (Knorre and Griffiths, 1984). The metals (noted as M in the equation) are precipitated as hydroxides as can be seen in Equation 1.4.7.

Equation 1.4.7: $M(CN)_4^{2-} + 4H_2O_2 + 2OH^- \rightarrow M(OH)_2 + 4OCN^- + H_2O.$

Iron cyanide complexes can not be oxidized by hydrogen peroxide, but they can be precipitated by heavy metal ions, especially copper. The cyanate, formed by oxidation of cyanide complexes, is hydrolyzed to form ammonium carbonate (Equation 1.4.8).

Equation 1.4.8: $OCN^- + 2H_2O \rightarrow CO_3^{2-} + NH_4^+$

This process is used widely in North America in solutions rather than slurries (Akcil, 2003) and hydrogen peroxide is a more potent oxidant than oxygen and it is cheaper than sulfur dioxide (Cummings and Baxter, 2006). The process can be applied over a broad pH range. This process is also unsuccessful against CN_{SAD} and hydrogen peroxide will oxidize other substances such as, thiocyanates, sulphides and metal ions in low oxidation states, in the pulp (Knorre and Griffiths, 1984). This leads to the inaccurate calculation of hydrogen peroxide that is necessary for total detoxification of cyanide in solution.

Complexation, by acidification/volatilization, of cyanide compounds is another process that assists in the waste remediation of cyanide (Equation 1.4.9) where M indicates a metal ion (Cummings and Baxter, 2006).

Equation 1.4.9: $M(CN)_x^{y-x} + xH^+ \rightarrow xHCN(g) + M^{y+}$

Ozonation can also be used to treat cyanide effluents. Ozone produces non-toxic products and is a strong oxidant (Monteagudo *et al*., 2004). Oxidation, with ozone, of cyanide occurs rapidly and is limited by the transport of the gas to the aqueous phase. When ozone reacts with cyanide, the reaction produces cyanate and repeated reactions with ozone will transform cyanate (Equation 1.4.10) to nitrogen gas and bicarbonate ions (Equation 1.4.11).

Equation 1.4.10: $3CN^- + O_3(aq) \rightarrow 3OCN^-$

Equation 1.4.11: $2OCN^- + O_3(aq) + H_2O \rightarrow 2HCO_3^- + N_2$

Cyanate is not oxidized to nitrite or nitrate (no nitrification or denitrification are needed) due to the fact that continued ozonation prevents cyanate hydrolysis through the reaction given in Equation 1.4.12.

Equation 1.4.12: $OCN^- + 3H_2O \rightarrow NH_4^+ + HCO_3^- + OH^-$

This reaction is favourable at room temperature (RT) and pH 7 (Monteagudo *et al*., 2004).

1.4.4. Biological degradation

Destruction of cyanide by microorganisms was examined in the early twentieth century for the first time (Ackil and Mudder, 2003) and the first commercially implemented biological process in the gold mining industry was in 1980 at the Homestake Gold Mine in the United States of America (Cummings and Baxter, 2006). Industrial and hazardous liquid wastes can be treated with well established aerobic and anaerobic biological processes (Akcil *et al*., 2003). Biological processes have been used to treat liquid wastes containing a variety of metals, inorganic constituents and organic compounds. These processes provide an alternative to chemical processes and the processes provides simple implementation and a cost effective solution, while providing environmentally acceptable and high quality

effluents. Cyanide destruction by biological processes are overall less expensive than chemical processes, although the capital costs are initially higher the operation costs are considerably lower (Ackil, 2003). Biological processes also have the ability to couple both, denitrification of resulting ammonia and detoxification of cyanide, to ensure effluents that are less hazardous to the surroundings after it is discharged (Cummings and Baxter, 2006).

Factors that can lead to limiting the economic viability of biological processes are their vulnerability to environmental factors such as temperature and the occurrence of high organic carbon concentrations which can inhibit aerobic treatments. These factors can influence the quality of the effluents, thus, it is important to develop a biological process from bench scale in laboratories and later on full scale pilot studies. This adds cost to the biological process and can delay application of such system in the field (Mosher and Figueroa, 1996).

1.5. Background of the biological process

Microorganisms were shown to be a robust and viable in the biological process for the destruction of cyanide in mining waters (Akcil *et al*., 2003).The aerobic nitrification process involves two oxidation steps which in turn is followed by the denitrification step ensures the complete removal of ammonia from wastewaters (summary of process shown in Figure **1.5.1**).

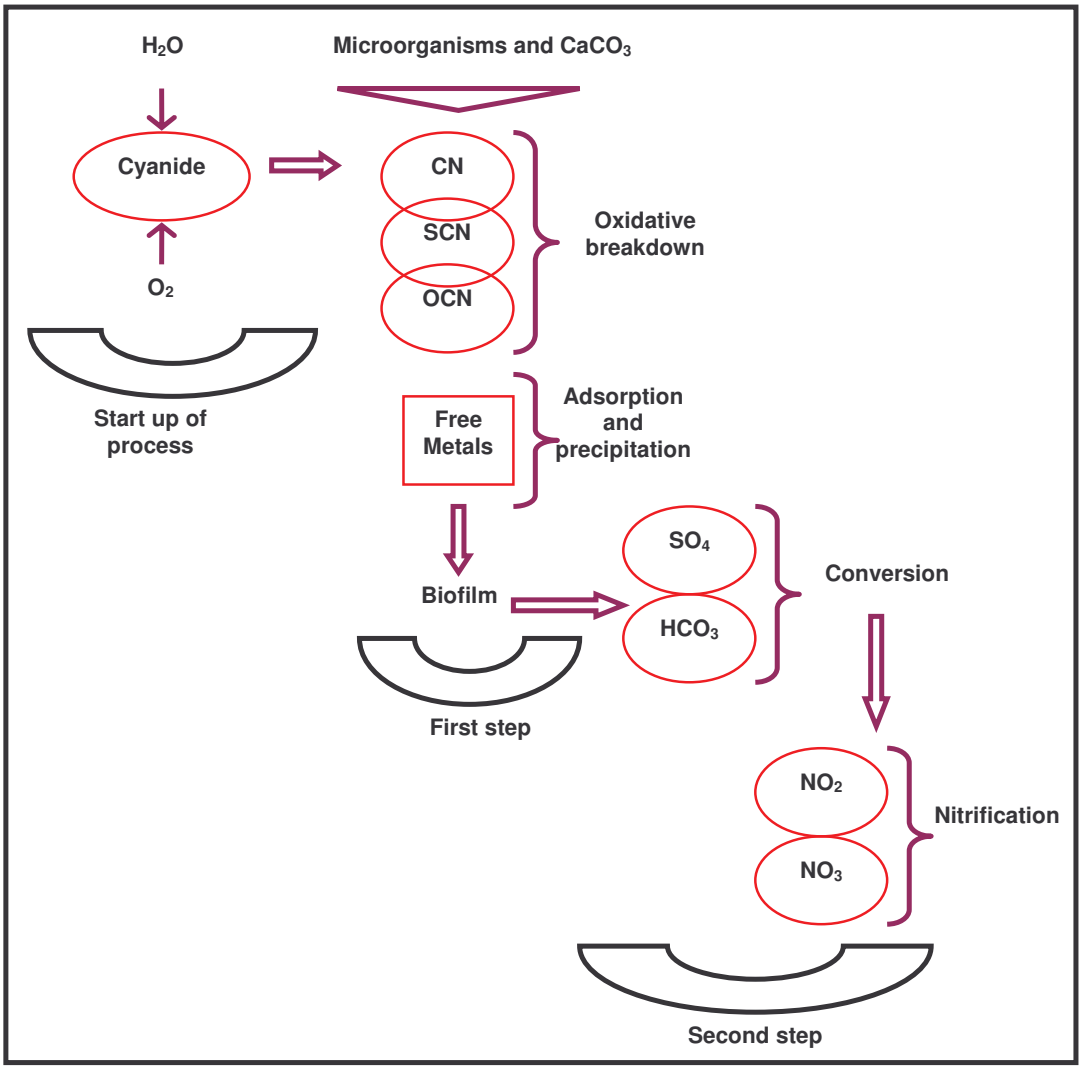

Figure 1.5.1: Aerobic biological treatment process (Taken from Akcil, 2003).

Based on this fact, it seems possible that the primitive microorganisms, which lived under anaerobic conditions of early earth, were capable of utilizing cyanide in conjunction with other nitrogen and carbon sources.

The general detoxification of complexed and free cyanides, by aerobic bacteria, lead to the formation of carbonate and ammonia and the biofilm will adsorb the free metals (indicated by the M) in Equation 1.5.1 (Ackil *et al.*, 2003).

Equation 1.5.1: $M_xCN_y + 4H_2O + O_2 \xrightarrow{\text{Bacteria}} M\text{-biofilm} + 2HCO_3^- + 2NH_3$

The degradation rates of metal complexed cyanides follow their respective chemical stability, thus zinc (Zn) was the most rapid followed by nickel (Ni), copper (Cu) and iron (Fe) (Akcil *et al*., 2003). Iron cyanides are degraded to a minor extent. Thiocyanide (SCN) degradation is rapid and the optimum pH is between 6.7- 7.2 (Equation 1.5.2).

Equation 1.5.2: $SCN^- + 5/2O_2 + 2H_2O \xrightarrow{\text{Bacteria}} SO_4^{2-} + HCO_3^- + NH_3$

A mixed population of bacteria is exposed to aerobic conditions for nitrification and anaerobic conditions for denitrification. After degradation of cyanide, thiocyanide and sorption of metals, the produced by-product, ammonia, must be degraded. Aerobic autotrophic bacteria for example, *Nitrosomas* and *Nitrobacter*, are used in the nitrification stage where the first oxidation (Equation 1.5.3) of ammonia occurs very slowly and leads to the formation of nitrite, an intermediate, followed by the rapid second oxidation (Equation 1.5.4) to nitrate (Akcil *et al*., 2003).

Equation 1.5.3: $NH_4^+ + 3/2O_2 \xrightarrow{\text{Bacteria}} NO_2^- + 2H^+ + H_2O$

Equation 1.5.4: $NO_2^{2-} + 1/2O_2 \xrightarrow{\text{Bacteria}} NO_3^{3-}$

The microbiota of the nitrification and destruction stages are usually non-competitive and limited by the change in concentrations of the cyanide, thiocyanide and ammonia (Akcil *et al*., 2003). The cyanide and the thiocyanide are used as nutrients in the destruction stage, but are toxic to the nitrifiers. The nitrifiers use ammonia as a nutrient and use either bicarbonate or other inorganic compounds as a carbon source. The recovery of the nitrifiers is much slower than the cyanide degraders if the effectiveness of cyanide degradation is disturbed. The nitrifiers are the rate-limiting factor in the plant design for the destruction of cyanides. The biological treatment processes can be applied in addition to aerobic and anaerobic growth, *in situ*, attached and suspended, and active and passive growth. The various bacterial species can utilize, take up and/or precipitate ammonia, nitrate, metals, sulphate, cyanide and thiocyanide (Akcil and Mudder, 2003). At the Nickel Plate Mine in British Columbia (Canada), a combined suspended growth biological process is employed. The nitrate, formed during nitrification, is removed via an included, extra denitrification step through the addition of methanol, serves as an organic substrate, which in turn will biologically convert the nitrate to nitrogen gas as shown in Equation 1.5.5 (Akcil *et al*., 2003).

Equation 1.5.5: $6NO_3^- + 5CH_3OH \xrightarrow{\text{Bacteria}} 5CO_2 + 3N_2 + 7H_2O + 6OH^-$

An example of a commercial operating biological process is at Homestake Mining Company's (HMC) Santa Fe mine (Akcil, 2003). The process is called the Biopass System. This system is a biological based process which is passive, *in situ* and contains an aerobic and anaerobic part (Figure 1.5.2). The components on the right hand side of the figure are added at different stages to the process to ensure that all the contaminants are removed from the effluent.

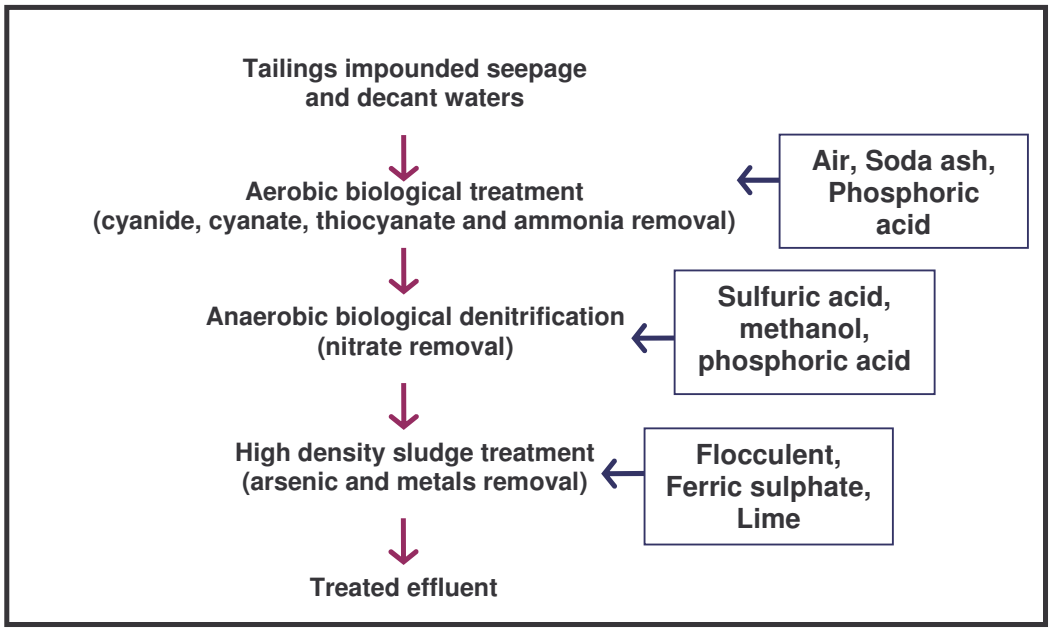

Figure 1.5.2: Flow sheet of a passive in situ biological treatment process (Adapted from Given *et al.*, 1998).

The microorganisms involved in the destruction of cyanide and thiocyanide, include indigenous soil bacteria and occur aerobically (Akcil, 2003). The anaerobic microorganisms are more susceptible to toxic upsets, therefore, this process requires either a suspended or an attached aerobic growth processes for the removal of free cyanide, especially thiocyanide. Different growth processes capable of removing cyanide and thiocyanide are available and include packed beds, sequencing batch reactors, activated sludge systems, biological filters and rotating biological contactors. The factors influencing the choice of the biological process is cyanide concentrations, temperature (minimum and maximum) and pH of the process environment as well as the environment (Akcil, 2003).

1.6. Degradation pathways: Cyanide and nitriles

There are four general pathways described in literature for the biodegradation of cyanide (Dash *et al*., 2009). Newly characterized cyanide biodegradation pathways are described regularly for additional organisms. Some organisms are capable of using more that one pathway (Ebbs, 2004). The pathway used by the organisms is dictated by external conditions like pH, cyanide concentration and oxygen. The solubility and bioavailability of cyanide in soil-water systems can also influence the selection of the desired pathway. The general pathways are: reductive, hydrolytic, substitution/transfer and oxidative (Dash *et al*., 2009).

1.6.1. Reductive pathway

This pathway is uncommon and only a few species contain the enzyme for this pathway (Dash *et al*., 2009). The two step enzymatic mechanism results in the formation of ammonia and methane (Figure 1.6.1). *Klebsiella oxytoca* is one of the few organisms that are able to degrade hydrogen cyanide to ammonia and methane (Kao *et al*., 2003).

1.6.2. Hydrolytic pathway

Cyanidase, cyanide hydratase, nitrilase and nitrile hydratase are enzymes capable of cyanide degradation through hydrolytic pathways (Dash *et al*., 2009). The substrates for the nitrilase and nitrile hydratase enzymes are nitriles and hydrogen cyanide is the substrate for the enzymes cyanidase and cyanide hydratase (Figure 1.6.1). Cyanide hydratase is responsible for cyanide destruction in fungal species and this enzyme is activated at low concentrations of cyanide in most fungi that are pathogens of cyanogenic plants such as *Gloeocercospora sorghi* and *Stemphylium loti* (Dash *et al*., 2009). Cyanide is degraded by *Fusarium* sp. and *Pseudomonas fluorescens* by the enzyme cyanidase (cyanide dihydratase).

Cyanide dihydratase leads to the irreversible formation of the intermediate product, formamide (R-$CONH_2$), due to cyanide degradation and finally formamide will be degraded to carbon dioxide and ammonia. Both cyanidase and cyanide hydratase are bacterial enzymes but share amino acid and structural similarities to nitrile hydratase and nitrilase, although nitrilase and nitrile hydratase are less substrate specific than cyanidase and cyanide hydratase. Organic cyanides (nitrites) are biodegraded *via* the two enzymes nitrilase and nitrile hydratase with amidase coupled to the reaction. These two enzymes convert aromatic as well as aliphatic nitriles to the corresponding amide or acid. *K.oxytoca* utilizes

nitrile hydratase for the degradation of assorted nitrile compounds. In *Brevibactenum* sp. R312, *Rhodococcus rhodochrous* J1, *Rhodococcus* sp. N-774 and *Pseudomonas chlororaphis* B23 and several other bacterial species, the presence of amidase and nitrile hydratase were confirmed (Dash *et al*., 2009).

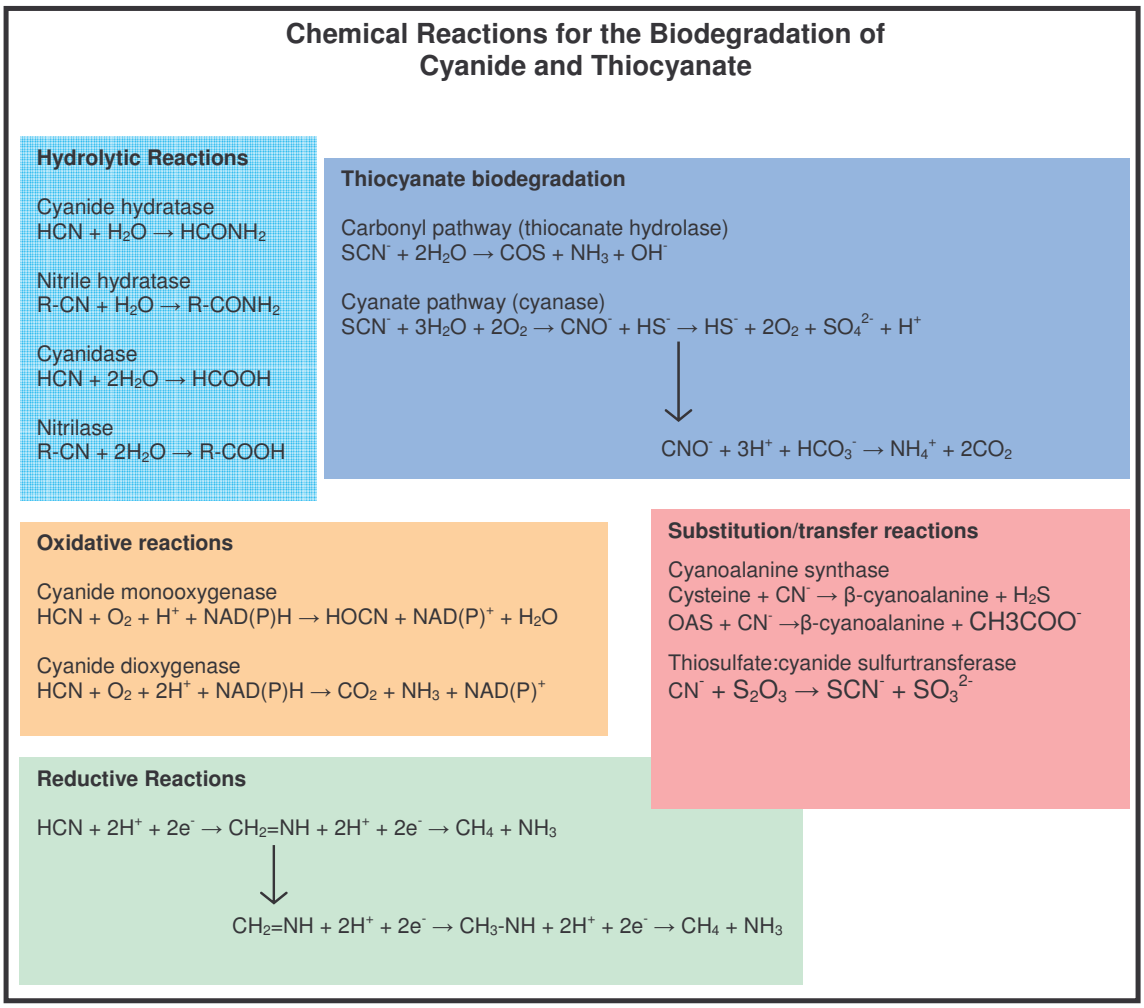

Figure 1.6.1: General pathways responsible for biodegradation of cyanide and thiocyanide. R represents either an aromatic or aliphatic group. Cyanoalanine synthase can use cystein as well as O-acetylserine (OAS) as a substrate (Taken from Ebbs, 2004).

1.6.3. Substitution/Transfer reactions

β-Cyanoalanine synthase (Figure 1.6.1) is able to convert cyanide to β-cyanoalanine or α-aminonitrile followed by the release of ammonia and an acid due to the hydrolysis of the intermediate products (Dash *et al*., 2009). Oxygen and NAD(P)H are not directly required and no carbon dioxide is released. Sulfurtransferase is another enzyme of this class and degrade cyanide to thiocyanate. The thiocyanate can be further degraded by either the

carbonyl or the cyanate pathway. The cyanate pathway leads to the formation of sulfate and carbon dioxide in the presence of cyanase, and the carbonyl pathway leads to the formation of carbonyl sulfide (COS) in the presence of thiocyanate hydrolase. The formation of ammonia occurs in both of the pathways. Cyanase has been detected in *Escherichia coli* and in a *Flavobacterium* sp. Thiosulfate:cyanide sulfurtransferase is also called rhodanese, which catalyse the transfer of a sulfane sulfur atom from a donor, thiosulfate, to cyanide yielding thiocyanate (Visca *et al*., 2007). Rhodaneses have been identified in *E. coli*, *P. aeruginosa* and *Azotobacter vinelandii*.

1.6.4. Oxidative reactions

The biodegradation of cyanide via oxidative reactions leads to the formation of ammonia and carbon dioxide [Figure 1.6.1] (Dash *et al*., 2009). There are two oxidative pathways. The first pathway converts cyanide to cyanate in the presence of cyanide monooxygenase. The cyanate is then converted to ammonia and carbon dioxide in the presence of cyanase (protects organism against cyanide poisoning). The second pathway converts cyanide to ammonia and carbon dioxide directly in the presence of cyanide dioxygenase. Recently, it has been proposed that this pathway is dependent on the co-factor pterin and the formation of cyanohydrins is important for cyanide degradation via the oxygenase-mediated pathways (Ebbs, 2004).

A summary of the diversity of the different cyanide degrading microorganisms with their respective enzymatic activities and specificities is given in Table 1.6.1. For example, microorganisms like *Pseudomonas fluorescens* can utilize the cyanide as a nitrogen source and convert hydrogen cyanide in the presence of cyanide oxygenase with the consumption of one mole oxygen per one mole cyanide with the use of NADH or NAD(P)H. Cell free extracts of *P. fluorescens* also contains the enzyme cyanide monooxygenase which can degrade cyanide to ammonia and carbon dioxide via the intermediate compound cyanate. Immobilized cells of *P. putida* can use the first pathway to produce ammonia and carbon dioxide (Dash *et al*., 2009).

Table 1.6.1: Summary of cyanide degrading microbial taxa. (Taken from Cummings and Baxter, 2006).

Microorganism	Enzymatic pathway (Associated metal ions)	Substrate specificity
Bacteria		
Rhodococcus rhodochrous J1	L-NHase[a] (Co)	Aromatic nitriles
	Nitrilase	Aliphatic and aromatic nitriles
Bacillus pallidus	NHase[a] (Co)	Aliphatic nitriles
	Nitrilase	Aromatic nitriles
Athrobacter sp. J1	NHase[a]	Aliphatic nitriles
	Nitrilase A	Aromatic nitriles
	Nitrilase B	Aromatic nitriles
Brevibacterium imperialis CBS489-74	NHase[a]	Acrylonitrile
Pseudomonas chlororaphis B23	NHase[a] (Fe)	Aliphatic nitriles
Pseudomonas putida	NHase[a] (Co)	Aliphatic nitriles
	Isonitrile hydratase	Isonitriles
Pseudomonas sp. S1	Nitrilase	Aliphatic, aromatic and arakyl nitriles
Pseudomonas fluorescens NCIMB 11764	Cyanide oxygenase	KCN
Pseudomonas stutzeri AK61	Cyanidase	KCN
Pseudomonas aeruginosa	Rhodanese	Cyanide
Escherichia coli	Cyanide dioxygenase	KCN
Klebsiella oxytoca	Nitrogenase	KCN
Klebsiella pneumoniae subsp. *ozaenae*	Nitrilase	Bromoxynil
Agrobacterium tumefaciens strain D3	NHase[a]	Aromatic nitriles
Fungi		
Myrothecium verrucaria	NHase (Zn)	Cyanamide
Fusarium solani	Nitrilase	Aromatic nitriles
	Cyanide hydratase	KCN, HCN, $K_2Ni(CN)_4$, $K_4Fe(CN)_6$
Fusarium laterium	Cyanide hydratase	Benzonitrile, HCN
Aspergillus niger	NHase	Aliphatic and aromatic nitriles
Gloeocercospora sorghi	Cyanide hydratase	HCN

[a]**Nitrile hydratase**

1.7. Biodegradation of metal-cyanide complexes

The greatest need in the degradation of cyanide is perhaps the degradation of metal-cyanide complexes due to their stability and resistance to cyanide remediation strategies (Ebbs, 2004). Cyanide can complex with copper, nickel, zinc and iron and these complexes restrain biodegradation strategies (Cummings and Baxter, 2006). In one strain of *Pseudomonas fluorescens* cyanide could no longer be utilized as a nitrogen source due to the complex formation of cyanide with copper (Silva-Avalos *et al*., 1990). In other *P. fluorescens* strains it was seen that the metal-cyanide complexes can be degraded to the equivalent levels than those reached by chemical methods (Akcil *et al*., 2003). At alkaline pH values metal-cyanide complexes can be degraded by organisms such as *Burkholderia cepacia* and when the free cyanide is liberated from the metal, enzymatic (as discussed in section 1.2.3.2.) or non-enzymatic mechanisms can be used by microorganisms to utilize the cyanide as a source of carbon and/or nitrogen. The free cyanide can react with pyruvate, α-keto acids and α-ketoglutamate to form the corresponding cyanohydrin. These cyanohydrins can be degraded by the catalyses of cyanide oxygenase to ammonia and carbon dioxide (Kunz *et al*., 2001). *E. coli* BCN6 can degrade iron, zinc and copper cyanide complexes and each metal ion has different effects on the growth due to the different toxicity of the metal ions. An *Acinetobacter* sp. can degrade silver, cadmium, zinc, gold, copper, gold, iron and cobalt cyanide complexes. This bacterium has a unique, single degrading complex that can degrade metal-cyanide complexes, simple cyanides and nitriles in contrast to other cyanide degrading enzymes that can only degrade a specific form of cyanide.

Several fungal species can degrade metal-cyanide complexes (Cummings and Baxter, 2006). These complexes include iron and nickel and the species include *Scytalidium thermophilum* and *Penicillium miczynski*. Yeast can grow and utilize potassium cyanide as sole nitrogen source and degrade up to 65 mM potassium tetracyanonickelate when supplied with an additional carbon source (Cummings and Baxter, 2006).

Biological processes for the treatment of metal-mining sites are used for the treatment of industrial effluents containing cyanide wastes. Various examples of these processes do exist. Homestake Mining Company (HMC) developed a process called Biopass. This process can remove ammonia, cyanate, thiocyanide, cyanide, nitrate and metals (Cummings and Baxter, 2006). At the USMX Green Springs gold mine in Nevada, USA an *in situ* biological process was used for the treatment of cyanide wastes. *P. pseudoalcaligenes* was isolated from the site and placed inside carbon adsorption tanks that acted as bioreactors. The tanks were used to recirculate the cyanide solution and this

process was able to decrease the cyanide concentration from 20 - 8.5 $mg.l^{-1}$ over 15 weeks. Pintail System Inc. developed a biological treatment process by isolating native bacteria, capable of cyanide degradation, from the soil of the Yellow Pine Mine in Idaho, USA. These bacteria were cultivated with the water from the tailings pond and at the end of the project the concentration of cyanide decreased from 46.6 - 0.2 $mg.l^{-1}$ (Cummings and Baxter, 2006).

The advances on biodegradation of cyanide are given in Table 1.7.1 (Dash *et al*., 2009). *Pseudomonas fluorescens* is capable of using ferrocyanide ions as the sole nitrogen source and was shown to remove 79% of the total cyanide at a pH of 5. *Pseudomonas* species are also capable of degrading up to 400 $mg.l^{-1}$ of cyanide. *Klebsiella oxytoca* can degrade cyanide in the presence of nitrogenase and this organism can also degrade nitriles. *P. putida* is able to utilize cyanates, cyanides and thiocyanates as sole nitrogen and carbon source (Wolfram *et al*., 1992). When the cells of *P. putida* were immobilized onto an adsorbent like zeolite, alginate beads and granular activated carbon (GAC), the rate of cyanide degradation was improved. When the cells were immobilized on GAC and used in the simultaneous adsorption and biodegradation (SAB) process, the efficiency of the rate of cyanide removal improves even further (Dash *et al*., 2008). Biosorption can also be used instead of biodegradation (Patil and Paknikar, 1999). This occurs when microorganisms' adsorp the toxic cyanide compounds and an example of this is the fungus *Rhizopus arrhizus*. This fungus can adsorp cyanide compounds at a pH of 13 with a high loading capacity of 612.2 $mg.g^{-1}$.

Cyanide can occur in the environment due to either natural or anthropogenic sources. The presence of cyanide in the environment is highly toxic and can affect the wildlife, aquatic environments and livestock. The degradation of cyanide can be achieved by natural degradation (very slow), physical and chemical degradation (introduce other hazardous chemicals into the environment) and biological degradation. Although cyanide degradation pathways have been established and proven in various microorganisms, there still exist organisms isolated from indigenous environments contaminated with cyanide compounds, which can contribute and broaden our understanding of cyanide removal strategies employed by these unique microorganisms. Thus, the aims of the current study were to i) isolate a microbial consortium or single microorganisms that are capable of utilizing cyanide as a sole carbon and nitrogen source, ii) identify the microorganisms involved in the degradation, iii) identify the gene(s)/protein(s) involved in cyanide degradation and iv) to employ pyrosequencing to identify the pathway(s) involved in cyanide degradation.

Table 1.7.1: Summary of microorganisms involved in bioremoval of various cyanide compounds (Taken from Dash *et al.*, 2009).

Compound to be removed	Microorganisms	Type of Reactor	Dependent parameters		
			Concentration ($mg.l^{-1}$)	pH	Temperature (ºC)
CN_{WAD}	*Pseudomonas* sp.	Batch	100 - 400	9.2 - 11.4	30
Ferrous (II) cyanide complex	*Pseudomonas fluorescens* immobilized on calcium algenate	Packed bed reactor	-	4-7	25-35
Ferrous (II) cyanide complex	*Pseudomonas fluorescens*	Batch	100	5	25
KCN	*Klebsiella oxytoca*	Batch	38	7	30
Nitriles	*Klebsiella oxytoca*	Batch	650 - 2 600	7	30
Cyanides	Consortium of bacteria	Continuous	20	7	22
NaCN	*Pseudomonas putida* immobilized on sodium alginate	Batch	100 - 400	6.7	25
NaCN, cyanates and thiocyanates	*Pseudomonas putida* immobilized on sodium alginate	Batch	196	7.5	25
Phenol and cyanides	*Pseudomonas putida* immobilized on ultrafiltration membranes	Batch	-	-	27
Copper- and zinc cyanides	*Citrobacter* sp., *Pseudomonas* sp.	Batch	52	7.5	35
Metallo-cyanide	Strains of *Trichoderma* spp.	Batch	2000	6.5	25
Tetra-cyano-nickelate (II)	*Pseudomonas fluorescens* immobilized on zeolite	Batch	26	-	30
KCN	*Bacillus pumilus*	Batch	162 790	8.5 - 9.0	40

1.8. References

- **Ackil, A.** (2003) Destruction of Cyanide in Gold Mill Effluents: Biological versus Chemical Treatments. *Biotechnology Advances* **21:** 501-511.

- **Ackil, A., Karahan, A.G., Cifci, H. and Sagdic, O.** (2003) Biological Treatment of Cyanide by Natural Isolated Bacteria (*Pseudomonas* sp.). *Minerals Engineering* **16:** 643-649.

- **Ackil, A. and Mudder, T** (2003) Microbial Destruction of Cyanide Wastes in Gold Mining: Process Review. *Biotechnology Letters* **25:** 445-450.

- **Allison, A.C.** (1953) Cyanide-Smelling Deficiency among Africans. *Man* **53:** 176-177.

- **Álvarez, R., Ordóñes, A., Martinéz, T., Loredo, J., Pendás, F. and Younger, P.** (2004) Passive Treatment for the Removal of Residual Cyanide in Drainage from Closed Gold Mine Tailing Ponds. *Mine Water* **1:** 35-42.

- **Askeland, R.A. and Morrison, S.M.** (1983) Cyanide Production by *Pseudomonas fluorescens* and *Pseudomonas aeruginosa*. *Applied and Environmental Microbiology* **45:** 1802-1807.

- **ATSDR** (Agency for Toxic Substances and Disease Registry (2004) http://www.atsdr.cdc.gov/toxpro2.html. Retrieved 10-01-2010.

- **Bais, H.P., Rudrappa, T., Splaine, R.E. and Biedrzycki, M.L.** (2008) Cyanogenic Pseudomonads Influence Multitrophic Interactions in the Rhizosphere. *PLoS ONE* **3:** 1-11.

- **Blumer, C. and Haas, D.** (2000) Mechanism, Regulation, and Ecological Role of Bacterial Cyanide Biosynthesis. *Archive of Microbiology* **173:** 170-177.

- **Bokanga, M.**, Du, L., Moller, B.L. and Halkier, B.A. (1995) The Biosynthesis of Cyanogenic Glucosides in Roots of Cassava. *Phytochemistry* **39:** 323-326.

- **Boopathy, R.** (2000) Factors Limiting Bioremediation Technologies. *Bioresource Technology* **74:** 63-67.

- **Brandl, H and Faramarzi, M.A.** (2006) Formation of Water-Soluble Metal Cyanide Complexes from Solid Minerals by *Pseudomonas plecoglossicida*. *Federation of European Microbiological Societies* **259:** 47-52.

- **Castric, P.A.** (1977) Glycine Metabolism by *Pseudomonas aeruginosa*: Hydrogen Cyanide Biosynthesis. *Journal of Bacteriology* **130:** 826-831.

- **Cummings, S.P. and Baxter J.** (2006) The Current and Future Applications of Microorganisms in the Bioremediation of Cyanide Contamination. *Antonie van Leeuwenhoek* **90:** 1-17.

- **Dash, R.R., Majumber, A. and Kumar, A.** (2008) Treatment of Metal Cyanide Bearing Wastewater by Simultaneous Adsorption Biodegradation (SAB). *Journal of Hazardous Materials* **152:** 387-396.

- **Dash, R.R., Gaur, A. and Balomajumder, C.** (2009) Cyanide in Industrial Wastewaters and it's Removal: A Review on Biotreatment. *Journal of Hazardous Materials* **163:** 1-11.

- **Ebbs, S** (2004) Biological Degradation of Cyanide Compounds. *Current Opinion in Biotechnology* **15:** 231-236.

- **Gallagher, L.A. and Manoil, C.** (2001) *Pseudomonas aeuruginosa* PAO1 Kills *Caenorhabditis elegans* by Cyanide Poisoning. *Journal of Bacteriology* **183:** 6207-6214.

- **Given, B., Dixon, B., Douglas, G., Mihoe, R. and Mudder, T.** (1998) Combined Aerobic and Anaerobic Biological Treatment of Tailings Solution at the Nickel Plate Mine. *In:* The Cyanide Monograph, Mudder, T.I. and Botz, M. (eds.) Mining Journal Books Limited, London, UK, 391-421.

- **Global InfoMine** (1990) http://technology.infomine.com/reviews/cyanide. Retrieved 06-07-2008.

- **Gönen, N., Kabasakal, O.S. and Özdil, G.** (2004) Recovery of Cyanide in Gold Leach Waste Solution by Volatilization and Absorption. *Journal of Hazardous Materials* **B113:** 231-236.

- **International Cyanide Management Code for the Gold Mining Industry** (2010) http://www.cyanidecode.org/cyanide_chemistry.php. Retrieved 12-02-2010.

- **Iriti, M. and Faoro, F.** (2009) Chemical Diversity and Defense Metabolism: How Plants Cope. *International Journal of Molecular Science* **10:** 3371-3399.

- **Kao, C.M., Liu, J.K, Lou, H.R., Lin, C.S. and Chen, S.C.** (2003) Biotransformation of Cyanide to Methane and Ammonia by *Klebsiella oxytoca*. *Chemosphere* **50:** 1055-1061.

- **Knorre, H. and Griffiths, A.** (1984) Cyanide Detoxification with Hydrogen Peroxide Using the Degussa Process, http://www.unr.edu/mines/mlc/conf_workshops/Cyanide/Chapter519.pdf. Retrieved 15-01-2010.

- **Knowles, C.J.** (1976) Microorganisms and Cyanide. *Bacteriological Reviews* **40:** 652-680.

- **Kunz, D.A., Fernandez, R.F. and Parab, P.** (2001) Evidence that Bacterial Cyanide Oxygenase is a Pterin-Dependent Hydroxylase. *Biochemical and Biophysical Research Communications* **287:** 514-518.

- **Leavesley, H.B., Li, L., Prabhakaran, K., Borowitz, J.L. and Isom, G.E.** (2008) Interaction of Cyanide and Nitric Oxide with Cytochrome *c* Oxidase: Implications for Acute Cyanide Toxicity. *Toxicological Sciences* **101:** 101-111.

- **Matthews, C.N.** (2004) The HCN World: Establishing Protein-Nucleic Augucid Life via Hydrogen Cyanide Polymers. *In*: Cellular Origin and Life in Extreme Habitats and Astrobiology 6 - Origins: Genesis, Evolution and Diversity of Life, Seckbach, J. (ed.) Kluwer Academic Publishers Netherlands, 121-135.

- **Martha Mine** (2001) http://www.marthamine.co.nz/cyanide.html. Retrieved 15-02-2010.

- **McMahon, J.M., White, W.L.B. and Sayre, R.T.** (1995) Cyanogenesis in Cassava. *Journal of Experimental Botany* **46:** 731-741.

- **MERG** (Mining Environment Research Group) (2001) http://www.geology.gov.yk.ca/pdf/MPERG_2001_2.pdf. Retrieved 21-02-2010.

- **Monteagudo, J.M., Rodríguez, L. and Villaseñor, J.** (2004) Advanced Oxidation Processes for Destruction of Cyanide form Thermoelectric Power Station Waste Waters. *Journal of Chemical Technology and Biotechnology* **79:** 117-125.

- **Mosher, J.B. and Figueroa, L.** (1996) Biological Oxidation of Cyanide: A Viable Treatment Option for the Minerals Processing Industry? *Minerals Engineering* **9:** 573-581.

- **Mudder. T.I., Botz, M.M. and Smith, A.** (2007) Treatment and Recovery of Cyanide. In: Mining Journal Books, Chapter **6:** 327-333.

- **Nicolet, Y., Piras, C., Legrand, P., Hatchikian, C.E. and Fontecille-Camps, J.C.** (1999) *Desulfovibrio desulfuricans* Iron Hydrogenase: The Structure Shows Unusual Coordination to an Active Site Fe Binuclear Center. *Structure* **7:** 13-23.

- **Patil, Y.K. and Paknikar, K.M.** (1999) Removal and Recovery of Metal Cyanides using a Combination of Biosorption and Biodegradation Process. *Biotechnology Letters* **21:** 913-919.

- **Pierik, A.J., Hulstein, M., Hagen, W.R. and Albracht, S.P.J.** (1998) A Low-Spin Iron with CN and CO as Intrinsic Ligands Forms the Core of the Active Site. *European Journal of Biochemistry* **258:** 572-578.

- **Pierik, A.J., Roseboom, W, Happe, R.P., Bagley, K.A. and Albracht, S.P.J.** (1999) Carbon Monoxide and Cyanide as Intrinsic Ligands to Iron in the Active Site of [NiFe]-Hydrogenases. *The Journal of Biological Chemistry* **274:** 3331-3337.

- **Pong, T.K., Adrien, R.J., Besida, J., O'Donnell, T.A. and Wood, D.G.** (2000) Spent Potling – A Hazardous Waste Made Safe. *Institution of Chemical Engineers* **78:** 204-208.

- **Sakkas, V.A., Lambropoulou, D.A. and Albanis T.A.** (2002) Study of Chlorothalonil Photodegradation in Natural Waters and in the Presence of Humic Substances. *Chemosphere* **48:** 939-945.

- **Sharma, V.K., Yngard, R.A., Filip, J. and Zboril, R.** (2008) Ferrate (VI) Oxidation of Weak-Acid Dissociable Cyanides. *Environmental Science and Technology* **42:** 3005-3010.

- **Siller, H. and Winter, J.** (1998) Degradation of Cyanide in Agroindustrial or Industrial Wastewater in an Acidification Reactor or in a Single-Step Methane Reactor by Bacteria Enriched from Soil and Peels of Cassava. *Applied Microbiology and Biotechnology* **50:** 384-389.

- **Silva-Avalos, J., Richmond, M.G., Nagappan, O. and Kunz, D.A.** (1990) Degradation of the Metal-Cyano Complex Tetracyanonickelate(II) by Cyanide-Utilizing Bacterial Isolates. *Applied and Environmental Microbiology* **56:** 3664-3670.

- **Smith, G.** (2003) Cyanides in Metal Finishing: Risks and Alternatives. *International Journal of Surface Engineering and Coatings* **81:** B33-B37.

- **Soldán, P., Pavonič, M., Bouček, J. and Kokeš, J.** (2001) Baia Mare Accident - Brief Ecotoxilogical Report of Czeck Experts. *Ecotoxicology and Environmental Safety* **49:** 255-261.

- **Thomas, K.V. McHugh, M., Hilton, M. and Waldock, M.** (2003) Increased Persistence of Antifouling Paint Biocides when Associated with Paint Particles. *Environmental Pollution* **123:** 153-161.

- **Vetter, J.** (2000) Plant Cyanogenic Glycosides. *Toxicon* **38:** 11-36.

- **Visca, P., Cipollone, R., Frangipani, E., Tiburzi, F., Imperi, F. and Ascenzi, P.** (2007) Involvement of *Pseudomonas auruginosa* Rhodanese in Protection from Cyanide Toxicity. *Applied and Environmental Microbiology* **73:** 390-398.

- **White, D.M., Aitimbetov, T. and Seth, I.** (2005) Biological Gold Recovery from Gold-Cyanide Solutions. *International Journal of Mineral Processing* **76:** 33-42.

- **Williams, H.D., Zlosnik, J.E., Tavankar, G.R., Bundy, J.G., Mossialos, D. and O'Toole, R.** (2006) Investigation of the Physiological Relationship Betwenn the Cyanide-Insensitive Oxidase and Cyanide Production in *Pseudomonas auruginosa*. *Microbiology* **152:** 1407-1415.

- **Wolfram, J.H., Babu, G.R.V. and Chapatwala, K.D.** (1992) Conversion of Sodium Cyanide to Carbon Dioxide and Ammonia by Immobilized Cells of *Pseudomonas putida*. *Journal of Industrial Microbiology* **9:** 235-238.

CHAPTER 2

2. ISOLATION AND CHARACTERIZATION OF CYANIDE DEGRADING MICROORGANISMS

2.1. Background

Cyanide is highly toxic to the environment and arises in the soil and water by natural and anthropogenic means (Fernandez *et al*., 2004). One of the main sources of anthropogenic contamination is the industrial recovery of gold and silver due to the use of sodium and potassium cyanide which can easily dissociate to free cyanide (Cipollone *et al*., 2006). This compound can inhibit the respiration of aerobic life forms by binding to cytochrome oxidase. Natural contamination, in turn, can be ascribed to the over 1000 plant species, fungi and bacteria that produce cyanide (cyanogenesis) as a product which aids in self defence mechanisms (Fernandez *et al*., 2004). Cyanogenesis in bacteria is strongly regulated and the cyanide produced can be tolerated by most living organisms but usually only at concentrations below 1 mM (Blumer and Haas, 2000). The formation of cyanide by cyanogenic organisms can be used for metal solubilization (Faramarzi *et al*., 2004). Here, cyanide forms water soluble metal complexes at pH 7 and this is a huge advantage in the mining industry where gold or silver surplus in the effluent water can be harvested due to the good water solubility of metal-cyanide complexes and high chemical stability (Faramarzi *et al*., 2004).

Usually, cyanide is used as the sole nitrogen source and organisms capable of utilizing cyanide can effortlessly extract it from the environment. Cyanide is mostly present as hydrogen cyanide under physiological conditions (pH 7) and, due to its volatile nature, can diffuse readily into the environment (Blumer and Haas, 2000). Bacteria can exhibit natural resistance to cyanide by synthesizing enzymes capable of cyanide degradation (Fernandez and Kunz, 2005). The two main pathways for the degradation of cyanide is either by the enzyme cyanide nitrilase (CNN) [also described as cyanidase or cyanide dihydratase] or cyanide oxygenase (CNO). CNN catalyzes the reaction responsible for the formation of ammonia and formic acid from cyanide through a single step hydrolysis reaction (Equation 2.1.1) while CNO catalyzes the reaction responsible for the formation of carbon dioxide and ammonia from cyanide through oxygenolytic conversion (Equation 2.1.2).

Equation 2.1.1: $HCN + 2H_2O \rightarrow HCO_2H + NH_3$

Equation 2.1.2: $HCN + O_2 + NADH + H^+ \rightarrow CO_2 + NH_3 + NAD^+$

Formamide is believed to be an intermediary product in the CNO reaction (Fernandez and Kunz, 2005) and the produced formate can be oxidized by formate dehydrogenase (FDH). The main role of CNN is to detoxify cyanide inside the microorganism and is found in numerous bacteria. CNO has also been found in a number of *Pseudomonas* spp. and the function is to support growth on cyanide (Fernandez *et al*., 2004).

In this chapter the sampling of a cyanide contaminated environment as well as isolation and characterization of microorganisms capable of cyanide degradation will be discussed. The aims included the identification of microorganisms capable of utilizing the cyanide as both a carbon and nitrogen source and to elucidate the mode of degradation. This was done by designing primers, specific for the known genes involved in cyanide utilization in control organisms, and screening isolates with these primers for the presence of the afore-mentioned genes. Hereto, we used three control organisms namely *Bacillus pumilus*, *Pseudomonas fluorescens* and *Pseudomonas stutzeri* known and described in literature to be capable of cyanide degradation.

2.2. Materials & Methods

2.2.1. Reagents and other consumables

Polymerase chain reaction (PCR) and deoxyribonucleic acid (DNA) isolation reagents were obtained from Fermentas Life Sciences. All chemicals used during this study were analytical or molecular grade and used without further purification. The chemicals were, unless otherwise stated, obtained from Merck or Sigma-Aldrich. Primers used during this study were obtained from either IDT (Integrated DNA Technologies, USA) or Inqaba Biotec™ (SA).

2.2.2. Site location and sampling

Klipspruit Calcium Cyanide Factory (Figure 2.2.1) is located in Pimville, Soweto, South Africa (S 26º 16.585'; E 027º 55.214'; Elevation 1471 m) and production of calcium cyanide was ended in 2002.

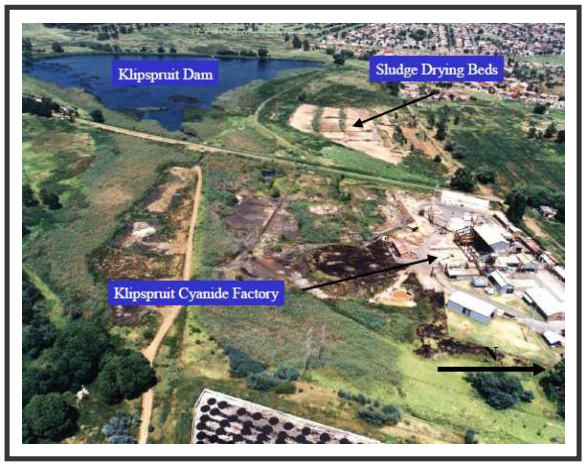

Figure 2.2.1: Photo of Klipspruit Calcium Cyanide site prior to remediation (Taken from Site Report JW132/05/A224, Jones & Wagener, Johannesburg, South-Africa).

In 2003 the remediation of the site was considered and Jones & Wagener (Consulting Civil Engineers) proposed the use of two possible remediation options, either the Conkey Filter Sludge or the addition of a small quantity of tar and the stored calcium cyanide was removed from the site. It was decided to implement the Conkey Filter Sludge approach in June, 2003. This site was used for the collection of soil and water samples in 2008 (Figure 2.2.2).

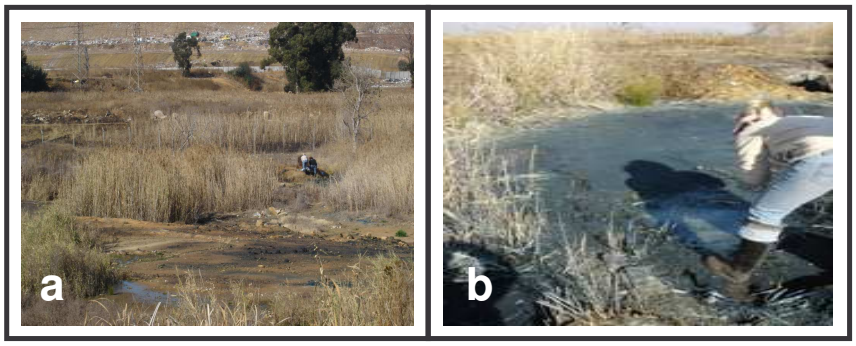

Figure 2.2.2: Factory site (2008). (a) One of the sludge drying beds and (b) South of the original Calcium Cyanide Factory buildings.

Seven soil samples (designated H1-H7) and three water samples (designated C1-C3) were obtained as illustrated in Figure 2.2.3. The seven soil samples were obtained in the vicinity of water sample, C1, based on the dark blue colour of the water [Figure 2.2.2 (b)]. The dark blue colour is due to the formation of Prussian blue with the chemical formula $Fe_4[Fe(CN_6)]_3$. C3 is a dam from which water was pumped to dilute the effluent of the old factory and C2 is water outside the perimeter of the old factory site. Field measurements were taken on the site.

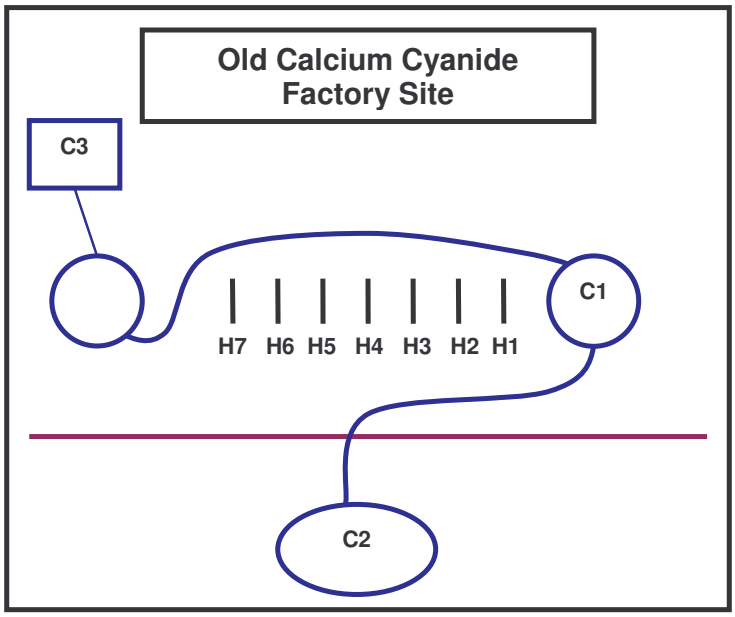

Figure 2.2.3: Schematic illustration of sample collection. C1 – C3, water samples and H1 – H7, soil samples. The purple line indicates the border fence between the old calcium cyanide factory site and government owned land. The blue line indicates the flow of the water from C3 to the perimeter of the Klipspruit Calcium Cyanide site.

2.2.3. Processing of samples

2.2.3.1. Analysis of water samples

The water samples were sent to the Institute for Groundwater Studies (IGS) situated on the campus of the University of the Free State (UFS) for analysis.

2.2.3.2. Inoculation and growth of isolates

The isolates used in this study were obtained from the Klipspruit Calcium Cyanide Factory site through the use of enrichment techniques. The three water and seven soil samples were stored at 4ºC and inoculated into Luria-Bertani (LB) media containing tryptone (10 $g.l^{-1}$), yeast extract (5 $g.l^{-1}$) and NaCl (10 $g.l^{-1}$). No NaCN was added and 1 g of the soil and 100 ml of the water samples was added to 500 ml of LB broth and incubated at 30ºC with aeration (180 rpm). The liquid samples were transferred consecutively (10% of the liquid cultures were transferred to fresh LB media in 500 ml Erlenmeyer flasks containing LB media) until the blue colour was no longer visible. The liquid cultures (10%) were transferred to Erlenmeyer flasks containing minimal media (pH 7.0) [Babu *et al*., 1996] containing K_2HPO_4 (4.3 $g.l^{-1}$), KH_2PO_4 (3.4 $g.l^{-1}$) and $MgCl_2$ (0.3 $g.l^{-1}$). The media was amended with 0.5 ml trace element solution containing $MnCl_2.4H_2O$ (1 $mg.l^{-1}$), $FeSO_4.7H_2O$ (0.6 $mg.l^{-1}$), $CaCl_2.H_2O$ (2.6 $mg.l^{-1}$) and $NaMoO_4.2H_2O$ (6 $mg.l^{-1}$). Where indicated, the media was supplemented with 4 mM or 10 mM NaCN as the sole carbon and nitrogen source. Phosphate buffer media plates were prepared by adding 15 g agar to 1 l of media supplemented with either 4 mM or 10 mM NaCN as the sole source of carbon and nitrogen.

After ten serial transfers, the samples which produced turbidity were streaked out on minimal agar plates supplemented with NaCN as the sole carbon and nitrogen source. Repetitive streaking of colonies obtained on cyanide-containing minimal media agar plates ensured that pure cultures capable of cyanide utilization were isolated. Isolates were stored as described in section 2.2.3.3.

2.2.3.3. Cryopreservation of bacteria

Cryopreservation was performed according to the method of Perry (1995). Sterile glycerol solution (40% v/v) was prepared. Single colonies were inoculated into nutrient agar (section 2.2.5.1) and cultivated overnight at 30ºC with aeration. The cells were diluted in a 1:1 (v/v) ratio with the sterile glycerol solution in cryo-tubes and stored at -80ºC.

2.2.3.4. Gram staining

The gram stain was performed to confirm the gram-staining ability of the bacteria and to ensure that the cultures were pure. A glycerol stock of each of the cultures was inoculated into LB broth and grown for 24 h at 30ºC. The sample cultures were streaked on LB agar plates, incubated overnight at 30ºC and a gram stain subsequently performed. This procedure consists out of four parts and the dyes used were freshly prepared. The sample was mounted and fixed on a microscope slide. The slide was flooded with crystal violet, allowed to stand for 1 min and rinsed with dH_2O. The sample was then flooded with an iodine solution, allowed to stand for 1 min and rinsed with dH_2O. Ethanol, a decolourizer, was added drop wise to the slide until the blue-violet colour was no longer emitted from the sample and the slide was rinsed with dH_2O. In the final step safranin, the counter stain was used to flood the slide, allowed to stand for 1 min and rinsed with dH_2O to remove the excess dye. The slide was blotted dry and viewed under a microscope.

2.2.4. General molecular techniques

2.2.4.1. Genomic deoxyribonucleic acid isolation

A glycerol stock of the control and isolated organisms was inoculated into 100 ml of nutrient agar (section 2.2.5.1) and cultivated at 30ºC while shaking (180 rpm) for 48 h. The genomic deoxyribonucleic acid (gDNA) was isolated using the method described in Labuschagne and Albertyn (2007). The culture was centrifuged (20 000 x *g*; 1 min; 4ºC) in 2 ml Eppendorf tubes and the supernatant removed by means of aspiration. The isolation of gDNA entailed the addition of 500 µl DNA isolation buffer [100 mM Tris-Cl [2-amino-2-(hydroxymethyl)-1,3-propandiol] (pH 8.0); 50 mM EDTA (ethylene diaminetetraacetic acid); 1% SDS (sodium dodecyl sulfate)] followed by the addition of 200 µl glass beads (425 – 600 µm diameter). The mixture was vortexed vigorously for 4 min and immediately cooled on ice. Ammonium acetate (275 µl; 7M; pH 7) was added to the tube, vortexed, incubated at 65ºC for 5 min and cooled on ice for 5 min. Chloroform (500 µl) was added, the tube vortexed and centrifuged (20 000 x *g*; 5 min; 4ºC). Isopropanol was added to the supernatant at a 1:1 (v/v) ratio for precipitation [5 min; RT (room temperature)]. The tube was centrifuged (20 000 x *g*; 5 min; 4ºC) and the supernatant discarded. The pellet was washed with 70% (v/v) ethanol and again centrifuged (20 000 x *g*; 5 min; 4ºC). The pellet was dried, redissolved in 150 µl TE [Tris-EDTA buffer (10 mM Tris-Cl; 1 mM EDTA; pH 8.0)] containing 50 $\mu g.ml^{-1}$ RNase (ribonuclease) and incubated at 37ºC for 30 min on a shaking incubator (180 rpm). The DNA concentration was determined using the NanoDrop Spectrophotometer ND-1000 (NanoDrop Technologies, Inc., USA) and verified by loading 5 µl DNA sample onto a 1% (w/v) agarose

gel supplied with a constant 7.5 $V.cm^{-1}$ for 1 h. The bands were visualized using a GelDoc XR (Bio-Rad) [high UV source].

2.2.4.2. 18S rDNA polymerase chain reaction

An eukaryal 18S rDNA PCR was performed using the eukaryotic-specific primers EukA and EukB (Table 2.2.1) to amplify the ~ 1 700 bp rDNA product.

Table 2.2.1: Eukaryotic universal primers.

Primer	Sequence	Reference
EukA	5'-AAC CTG GTT GAT CCT GCC AGT-3'	Behnke *et al*., 2006
EukB	5'-TGA TCC TTC TGC AGG TTC ACC TAC-3'	Behnke *et al*., 2006

The reaction mixture contained 1 µl gDNA template (50 $ng.\mu l^{-1}$), 1 µl EukA primer (10 µM), 1 µl EukB primer (10 µM), 0.5 µl dNTPs (10 mM), 0.4 µl (5 $U.\mu l^{-1}$) of Kapa *Taq* DNA polymerase (Kapa Biosystems, SA), 2 µl of 10x buffer and 14.1 µl sterile dH_2O. The thermal cycling was performed in the Thermo Electron Corporation PXE 0.2 Thermal Cycler as indicated in Table 2.2.2.

Table 2.2.2: Thermal cycling for the 18S rDNA PCR reaction.

Step	Temperature (ºC)	Time	Cycle number
Denaturation	**94**	**90 sec**	**1**
Denaturation	94	30 sec	
Annealing	65	60 sec	30
Elongation	72	45 sec	
Final elongation	72	5 min	1
Hold	4	∞	

PCR products were visualized on a 1% (w/v) agarose gel containing 2.5 $mg.\mu l^{-1}$ ethidium bromide, either using a GelDoc XR (Bio-Rad) [high UV source] or a Dark Reader® Transilluminator DR-45M (Clare Chemical Research, USA) [non-UV transillumination] for DNA to be excised from agarose for further downstream applications, after electrophoresis at 90 V for 1 h. Excised DNA products were purified using the BioSpin Gel Extraction Kit

(BioFlux) following the manufacturer's recommendations. The purified bands were used directly in sequencing reactions to determine the nucleotide composition of the various genes.

2.2.4.3. 16S rDNA polymerase chain reaction and Sanger sequencing

Polymerase chain reaction (PCR) amplification was carried out using SuperTherm *Taq* DNA polymerase (Southern Cross Biotechnology, SA) according to the manufacturer's recommendations. Bacterial specific universal primers 27F and 1492R (Table 2.2.3) were used to amplify the ~ 1500 bp product.

Table 2.2.3: Bacterial universal primers.

Primer	Sequence	Reference
27F	5'- GGT TAC CTT GTT ACG ACT T-3'	de Lillo *et al*., 2006
1492R	5'-AGA GTT TGA TCC TGG CTC AG-3'	de Lillo *et al*., 2006

The reaction mixture contained, unless otherwise stated, 2.5 µl of the 10x buffer, 2.5 µl 15 mM $MgCl_2$, 0.5 µl of each primer (10 µM), 0.5 µl of 10 mM dNTPs (deoxynucleotide triphosphate), 0.25 µl (5 $U.\mu l^{-1}$) of *Taq* DNA polymerase, 1 µl of template DNA (50 $ng.\mu l^{-1}$), filled to a final volume of 25 µl using sterile distilled water (dH_2O). Thermal cycling was performed using the Thermo Electron Corporation PXE 0.2 Thermal Cycler with the thermal cycle followed indicated in Table 2.2.4, unless otherwise stated.

Table 2.2.4: Thermal cycling for the 16S rDNA PCR reaction.

Step	Temperature (ºC)	Time	Cycle number
Denaturation	95	5 min	1
Denaturation	95	30 sec	
Annealing	49	45 sec	30
Elongation	72	60 sec	
Final elongation	72	10 min	1
Hold	4	∞	

PCR products were visualized on a 1% (w/v) agarose gel containing 2.5 mg.µl^{-1} ethidium bromide, either using a GelDoc XR (Bio-Rad) [high UV source] or a Dark Reader® Transilluminator DR-45M (Clare Chemical Research, USA) [non-UV transillumination] for DNA to be excised from agarose for further downstream applications, after electrophoresis at 90 V for 1 h. Excised DNA products were purified using the BioSpin Gel Extraction Kit (BioFlux) following the manufacturer's recommendations. The purified bands were used directly in sequencing reactions to determine the nucleotide composition of the various genes.

The products were sequenced (Table 2.2.5) using the ABI Prism® BigDye™ Terminator Cycle Sequncing Ready Reaction Kit v. 3.1 (Applied Biosystems, USA) following the instructions of the manufacturer. The final concentration of the DNA template in the reaction, for a fragment between 1000 – 2000 bp, was between 10 – 40 ng. A sixteenth of a reaction was done and the reaction mixture contained 0.5 µl premix, 1µl 27F primer (3.2 pmol.µl^{-1}) or 1 µl 1492R primer (3.2 pmol.µl^{-1}), 2µl dilution buffer, x µl template (depending on the concentration of DNA) and x µl dH_2O depending on the amount of template added. The total reaction volume was 10 µl. A control reaction was performed and the reaction mixture contained 0.5 µl premix, 4 µl control sequencing primer, 2 µl dilution buffer, 2 µl control plasmid [pGEM-3Zf(+)] and 1.5 µl sterile dH_2O for a total reaction volume of 10 µl.

Table 2.2.5: Thermal cycling for the sequencing PCR reaction.

Step	Temperature (ºC)	Time	Cycle number
Denaturation	96	1 min	1
Denaturation	96	10 sec	
Annealing	50	5 sec	25
Elongation	60	4 min	
Hold	4	∞	

Sequencing PCR products were purified using the EDTA/Ethanol precipitation protocol. The volume of the sequencing reaction was adjusted to 20 µl with dH_2O and transferred to a 1.5 ml Eppendorf tube that contained 60 µl absolute ethanol and 5 µl of a 125 mM EDTA solution. This mixture was vortexed for 5 sec and precipitated at room temperature for 15 min. The tube was centrifuged for 10 min at 20 000 x *g* at 4ºC. The

supernatant was aspirated without disturbing the pellet. Ethanol (70%, 60 µl) was added to the pellet and centrifuged for 5 min at 20 000 *x* g at 4ºC. The supernatant was completely aspirated without disturbing the pellet and dried in the Eppendorf Concentrator 5301 for 5 min. The samples were stored in the dark before analysis was performed on the samples. The samples were subjected to the sequencing reaction in a thermal cycler from Thermo Electron Corporation PXE 0.2. The sequence data was analysed with Vector NTI Suite 9 (ContigExpress) and used to obtain BLAST results (NCBI) either to ensure that the correct microorganism was used in further applications or to identify unknown isolates from environmental samples.

2.2.4.4. Denaturing Gradient Gel Electrophoresis

Genomic DNA (gDNA) was isolated using the FastDNA® Spin Kit For Soil (MP Biomedicals, LLC) and used in the Denaturing Gradient Gel Electrophoresis (DGGE). A 16S rDNA PCR (section 2.2.4.2) was performed on the isolated gDNA. The correct size (~1 500 bp) bands were excised from the agarose gel, purified using the BioSpin Gel Extraction Kit (BioFlux) and used as template for the follow up PCR. The appropriate percentage (8%) of acrylamide/bisacrylamide gel (16x16x1 mm) was selected based on the size of the partial fragment of a bacterial gene. The size bands to be separated by an 8% gel is 200 bp and 341F-GC and 517R primers were used in the thermal cycling reaction performed in the Thermo Electron Corporation PXE 0.2 Thermal Cycler with the thermal cycling indicated in Table 2.2.6. The PCR reaction mixture (total volume 20 µl) contained 2 µl 10x buffer, 0.4 µl dNTPs (10 mM), 0.4 µl 341F-GC (10 µM), 0.4 µl 517R (10 µM), 10 µl template, 0.25 µl SuperTherm *Taq* DNA polymerase (Southern Cross Biotechnology, SA), 2 µl 15 mM $MgCl_2$ and 4.55 µl dH_2O.

Table 2.2.6: Thermal cycling for the DGGE PCR reaction.

Step	Temperature (ºC)	Time	Cycle number
Denaturation	95	5 min	1
Denaturation	95	45 sec	
Annealing	55	45 sec	25
Elongation	72	60 sec	
Final elongation	72	10 min	1
Hold	4	∞	

The separation gel (gradient-portion) was composed of different gradients of urea-formamide (UF) solutions. The deionized formamide (100 ml formamide, ~5 g mixed red resin, shaken overnight protected from light and filter sterilized) and acrylamide/bisacrylamide (38.93 g acrylamide and 1.07 bisacrylamide was dissolved in 100 ml Milli-Q dH_2O, filer sterilized and stored in the dark) were prepared. Working solutions (0% and 80%) were prepared as indicated in Table 2.2.7.

Table 2.2.7: Working solutions of urea-formamide solutions.

0% UF (50 ml)	80% UF (50 ml)
10 ml 40% acrylamide/bisacrylamide	10 ml 40% acrylamide/bisacrylamide
1 ml 50x TAE	1 ml 50x TAE
1 ml 100% glycerol	16 ml formamide
38 ml Milli-Q H_2O	16.8 g urea
	1 ml 100% glycerol
	Fill up to 50 ml with Milli-Q dH_2O

APS (1 g APS dissolved in 10 ml Milli-Q dH_2O and filter serilized) [63 µl] and N,N,N',N'-tetramethylethylenediamine (TEMED) [7 µl] was added to the gradient solutions and the separation gel cast into a clean gradient delivery system. The top of the gel was covered with a thin layer of water-saturated tert-amyl alcohol. The TAE (242 g Tris-HCl, 57.1 ml glacial acetic acid, 18.6 g EDTA, fill to 1 L with dH_2O and filter sterilize) buffer in the tank was pre-heated to 60ºC. The tert-amyl alcohol was rinsed with Milli-Q dH_2O and removed with filter paper when the separation gel polymerized. The stacking gel was composed of 10 ml 0% UF solution containing APS (63 µl) and TEMED (7 µl) and cast on top of the separation gel. The comb was inserted without introducing air bubbles. Following polymerization the comb was removed, the casting stand attached to the core and inserted into the pre-treated tank containing TAE buffer. The samples were loaded and the DGGE performed at 200 V for 3 h. The gel was stained with SYBR Gold solution for 15 min, washed with dH_2O and visualized on a GelDoc XR (Bio-Rad). The bands were excised from the gel and incubated overnight at 50ºC in 50 µl dH_2O.

Re-amplification was performed on the overnight incubated excised bands. The reaction mixture contained 2 µl 10x buffer, 2 µl 15 mM $MgCl_2$, 0.4 µl 341F primer (10 µM), 0.4 µl 571R primer (10 µM), 0.4 µl dNTPs, 10 µl template, 0.25 µl SuperTherm *Taq* DNA

(Southern Cross Biotechnology, SA) and 4.55 µl dH_2O. The thermal cycling was performed as discussed in section 2.2.4.2. The products of the re-amplification were used for the reaction mixture for the thermal cycling for the sequencing PCR discussed in section 2.2.4.2.

2.2.4.5. Polymerase chain reactions and Sanger sequencing

A 16S (section 2.2.4.3) and 18S rDNA PCR (section 2.2.4.2) were performed on the pure cultures and the products were loaded onto a 1% agarose gel. The concentration of the 16S products were measured on the NanoDrop Spectrophotometer ND-1000. The 16S rDNA PCR products were used in the sequencing PCR reaction (section 2.2.4.3) following the thermal cycle. The sequencing PCR was cleaned using the EDTA/Ethanol precipitation protocol (discussed in section 2.2.4.3) for sequencing cleanup.

2.2.4.6. Minimal inhibition concentration determination

Two methods were used to determine the minimal inhibition concentration (MIC). The first method was performed by inoculating glycerol stocks into 50 ml of minimal media supplemented with 4 mM NaCN. The flasks were incubated at 30ºC for 24 h. The turbid cultures, measured at 600 nm, were transferred to 500 ml Erlenmeyer flasks containing fresh minimal media supplemented with final NaCN concentrations ranging from 10 mM to 100 mM. Growth was obtained in all the flasks and the MIC experiment was performed at higher concentrations of NaCN starting at 0.5 M to 4 M.

The second method was performed in the same manner as in method one. The turbid cultures were plated out on minimal media agar plates containing NaCN concentrations ranging from 1 M to 4 M.

2.2.5. Control organisms

2.2.5.1. Media and growth conditions

Bacillus pumilus (Skowronski and Strobel, 1969), *Pseudomonas fluorescens* (Palleroni, 1984) and *Pseudomonas stutzeri* (Rius *et al*., 2001) were inoculated into nutrient agar containing peptone (5 $g.l^{-1}$) and meat extract (3 $g.l^{-1}$) or supplemented with agar (15 $g.l^{-1}$) for solid media (DSMZ) and cultivated at 30ºC with aeration.

2.2.5.2. gDNA isolation

The gDNA was isolated from the three control organisms as discussed in section 2.2.4.1. The isolated gDNA was used in various downstream applications for example 16S rDNA PCR.

2.2.6. Growth studies

2.2.6.1. Five selected isolates

The growth studies were performed in minimal media supplemented with either 4 mM or 10 mM NaCN as the sole source of carbon and nitrogen. The growth studies were only performed for the five most promising microorganisms. A glycerol stock of each microorganism was streaked on an agar plate containing minimal media supplemented with NaCN. The agar plate was incubated for 24 h at 30ºC. After the incubation a single colony was inoculated into 50 ml of media (pre-inoculum 1). The pre-inoculum 1 was incubated at 30ºC for 24 h on an orbital shaker (180 rpm). After 24 h, 10 ml of the culture was inoculated into 90 ml (pre-inoculum 2) of fresh media and incubated at 30ºC for 48 h. After incubation, 5 ml of the culture was inoculated in 95 ml (inoculum) of fresh media and samples were taken every 12 h until stationary phase was reached. The maximum growth rate (μ_{max}) [Equation 2.2.1] and the doubling time (t_d) [Equation 2.2.2] were calculated for the growth curves performed on the five selected isolates as well as the three control organisms (Perni *et al*., 2005).

Equation 2.2.1: $\mu_{max} = (\ln(x_t/x_0))/t_t\text{-}t_0$

Equation 2.2.2: $t_d = \ln 2/\mu_{max}$

2.2.6.2. Control organisms

Growth studies were performed in Luria-Bertani (LB) medium as well as minimal media supplemented with either 4 mM or 10 mM NaCN as the sole source of carbon and nitrogen. The concentration NaCN was selected based on information obtained from literature (Fernandez *et al*., 2004). Also, 4 mM NaCN is the average concentration utilized by microorganisms employed in bioremediation of cyanide contaminated sites (Meyers *et al*., 1991). The higher concentration (10 mM) was used as a selective parameter for microorganisms capable of utilizing cyanide at higher concentrations. A glycerol stock of each control organism (*Bacillus pumilus*, *Pseudomonas fluorescens* and *Pseudomonas stutzeri*) was streaked on an agar plate either containing LB media or minimal media

supplemented with NaCN. The agar plate was incubated for 24 h at 30ºC after which a single colony was inoculated into 50 ml of media (pre-inoculum 1). The pre-inoculum 1 was incubated at 30ºC for 6 h of growth in LB-medium and 24 h in minimal medium on an orbital shaker (180 rpm). After 6 h (LB-medium) and 24 h (minimal medium), 10 ml of the culture was inoculated into 90 ml (pre-inoculum 2) of fresh media and incubated at 30ºC for 4 h (LB-medium) and 48 h (minimal medium). After incubation, 5 ml of the culture was inoculated in 95 ml (inoculum) of fresh media and samples were taken, every 2 h for LB media and every 4 h for the minimal media supplemented with NaCN, until stationary phase was reached.

2.2.7. Specific primers to identify genes involved in cyanide degrading

Primers, for the specific cyanide degrading gene present in each of the three control organisms, were designed by aligning multiple sequences of the same cyanide degrading gene present in the same genus represented by the control organisms (Table 2.2.8).

Table 2.2.8: Specific primer sequences designed for the cyanide degrading genes.

Control organisms	Gene	Forward primer	Reverse primer
Bacillus pumilus	Cyanide dihydratase	5'-TCATGTGAACTGATCGACGAGGCA-3'	5'-ACAACAGGAGTGGGCTGCTGATTA-3'
Pseudomonas fluorescens	Hydrogen cyanide synthase	5'-ATTTCGCCGACCACGAACAGATGA-3'	5'-TGAAGTTGTGTTCCAGGCCCATCT-3'
Pseudomonas stutzeri	Cyanide degrading enzyme	5'- CTGTACTGCCGCAGCCTTGAATTT-3'	5'-AGCGATGAAGAGCCAGTCGTTCGG-3'

The designed primers were used to identify the cyanide degrading genes in the three control organisms in thermal cycling using the Bio-Rad MJ-Mini Personal Thermal Cycler. The gradient PCR cycle (annealing temperature between 48 - 55ºC) was used to determine the optimum temperature for the primers to anneal to the gDNA. The designed primers were also used in the thermal cycling reaction with the gDNA isolated from the five selected isolates. The optimum temperature used was 48ºC for the annealing phase of the PCR reaction. The products were loaded onto a 1% agarose gel. The bands were excised from the gel and cleaned using the BioSpin Gel Extraction Kit (BioFlux).

2.2.8. Cyanide assay: Picric acid assay

2.2.8.1. Reagent preparation

This assay is a colometric procedure to determine the concentration of weak acid dissociable (WAD) cyanide present in samples (International Cyanide Management Code for the Gold Mining Industry, 2010). Picric acid is reduced in the presence of free cyanide to form a brownish coloured compound known as isopurpuric acid. The concentration of the free cyanide is directly proportional to the colour formation. The pH of the sample is closely controlled (checked periodically) due to the variance in colour formation outside of the pH range between 9.0 - 9.5. This assay is reliable for the determination of WAD cyanide concentrations above 0.5 $mg.l^{-1}$. Three reagents were prepared; (i) a nickel solution by dissolving 0.2 g of $NiCl_2.6H_2O$ and 1 g NaCl in 100 ml of dH_2O and dilutions to 500 ml with dH_2O, (ii) a picric acid solution by dissolving 40 g of DTPA (diethylenetriaminepentaacetic acid) in 800 ml of dH_2O with the addition of 20 ml NaOH (prepared by dissolving 16 g in 50 ml of dH_2O) and (iii) a NaCN solution by adding 0.189 g to 50 ml NaOH solution (prepared by dissolving 2 g NaOH in 50 ml dH_2O) with further dilution to 100 ml with dH_2O. Picric acid (6 g), $Na_2B_4O_7.10H_2O$ (26.54 g) and Na_2CO_3 anhydrous (8 g) was added to the DTPA solution. The pH of the DTPA solution was adjusted to 8.7 using the remaining NaOH solution. The DTPA solution was diluted to 1 L, in a volumetric flask, by adding dH_2O.

2.2.8.2. Standard curve

The standard curve was constructed in 1.5 ml Eppendorf tubes in triplicate. Different cyanide concentrations (samples) were prepared in the detection range of the assay. The tubes contained 100 µl of sample, 10 µl of the nickel solution, 300 µl of dH_2O and 250 µl of picric acid. These tubes were boiled for 20 min and left on a work bench to cool to RT afterwhich 340 µl of dH_2O was added and the mixture read on the Spectronic®GENESYS™ 5 spectrophotometer at 520 nm.

2.2.8.3. Assay

A glycerol stock of the five selected isolates as well as the three control organisms was inoculated into minimal media supplemented with 100 mM NaCN (higher concentrations are outside the detection limit). The cultures were grown for 24 h at 30ºC and 10 ml of the growth was transferred to 90 ml fresh minimal media supplemented with 100 mM NaCN. Samples were obtained and the growth monitored (600 nm) over six days. Samples were taken at 6 h intervals. The samples were centrifuged at 14 000 x *g* in an Eppendorf Centrifuge 5810 R and the supernatant (50x diluted) used in the assay.

2.3. Results & Discussion

2.3.1. Site location and sampling

2.3.1.1. Field measurements

The name, field measurements and GPS coordinates of the water samples (KSSO07042008C1-C3) are given in Table 2.3.1. The soil samples were designated KSSO07042008H1-H7. The soil samples were taken from the surrounding vicinity of KSSW07042008C2 with the coordinates of S 26˚16.604', E 027˚55.211' and an elevation of 1569 m.

Table 2.3.1: Physicochemical properties of the water samples collected from the Klipspruit Calcium Cyanide Factory site**.**

Sample name	GPS coordinates	Field measurements		
Water samples:		**pH**	**Temperature (ºC)**	**EC (mS.cm^{-1})**
KSSW07042008C1	S 26˚16.585'; E 027˚55.214'; Elevation – 1471 m	3.57	15.5	4.36
KSSW07042008C2	S 26˚16.604'; E 027˚55.211'; Elevation – 1569 m	6.37	18.8	1.97
KSSW07042008C3	S 26˚16.531'; E 027˚55.173'; Elevation – 1667 m	7.96	14.5	0.22

* EC (Electronic Conductivity)

2.3.2. Processing of samples

2.3.2.1. Analysis of water samples

Water analysis (section 2.2.3.1) was performed at the IGS (UFS) and the results from the analysis are summarized in Table 2.3.2 (a) and (b). The CN_T concentration is highlighted in yellow on Table 2.3.2 (b). The values of the concentrations are given in mg.l^{-1}.

Table 2.3.2 (a): Results obtained represent the mineral composition of the three water samples collected from Klipspruit Calcium Cyanide Factory site after a water analysis was performed.

Sample	pH	Ca	Mg	Na	K	F	Cl	Br	NO_3(N)	PO_4	SO_4
KSSW0704200C1	3.2	469	106	103	20.5	1.96	427	0.44	0.54	<1	3884
KSSW07042008C2	6.45	297	59	83	4.91	0.17	86	0.31	1	<1	1425
KSSW07042008C3	7.34	23	5.3	66	3.6	0.2	26	0.07	2.27	<0.1	30

Table 2.3.2 (b): Results obtained represent the mineral composition of the three water samples collected from Klipspruit Calcium Cyanide Factory site after a water analysis was performed (continued).

Sample	Al	Fe	Mn	As	Ba	TDS	U	Zn	CN_T	Ni	$NO_2(N)$
KSSW0704200C1	180	605	46	<0.006	0.007	5865	<0.050	1.74	0.027	0.312	<0.01
KSSW07042008C2	0.025	334	4.11	0.006	0.041	2477	<0.050	0.08	0.017	0.033	<0.01
KSSW07042008C3	0.029	0.111	0.004	<0.006	0.048	240	<0.050	<0.004	0.011	<0.010	<0.01

* TDS, Total Dissolved Solids

The results of the water analysis indicate that a low concentration of cyanide was present in the water samples obtained from the Klipspruit Calcium Cyanide Factory site. These concentrations are in excess of 3 500 fold lower than the 4 mM NaCN used in the enrichment media. This may be as a result of the specificity of the detection method for the concentration of the various classes of cyanide including CN_{WAD} and CN_F or that the amount of heavy metals inside the sample inhibits the detection method. The Klipspruit Calcium Cyanide Factory was remediated in 2003 which could explain the low concentration of cyanide in the water samples.

2.3.2.2. Inoculation and growth of isolates

The seven soil and three water samples were inoculated (section 2.2.3.2) into LB medium and consecutively transferred until the blue colour, due to the presence of Prussian blue, was no longer visible [Figure 2.3.1 (a)]. The turbid samples were transferred to minimal media supplemented with NaCN as sole carbon and nitrogen source [Figure 2.3.1 (b)].

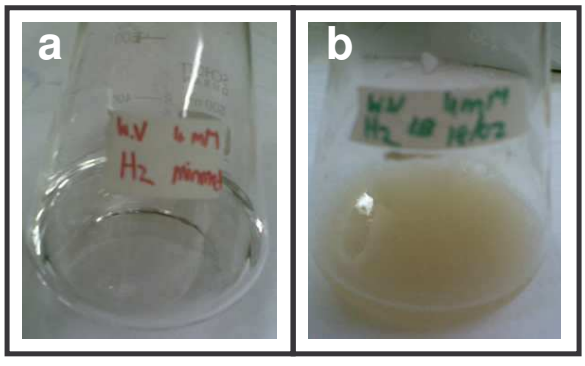

Figure 2.3.1: Turbidity in LB medium (a) and sample transferred into minimal medium (b).

Following growth in the minimal media supplemented with either 4 mM or 10 mM NaCN samples were streaked on solid minimal medium agar plates supplemented with the same NaCN concentration as the liquid sample to ensure that the turbidity observed was positive growth and to obtain single colonies (Figure 2.3.2).

Figure 2.3.2: Turbid samples streaked out on minimal agar plates containing NaCN.

Single colonies were picked and re-streaked on solid minimal medium agar plates supplemented with either 4 mM or 10 mM NaCN to obtain pure cultures. One isolate from each sample enriched with either 4 mM or 10 mM NaCN were selected. A total of twenty isolates were selected.

2.3.2.3. gDNA isolation

gDNA isolated (section 2.2.4.1) from the single colonies obtained on the minimal medium agar plates supplemented with either 4 mM (Figure 2.3.3) or 10 mM (Figure 2.3.4) [discussed in section 2.2.4.1] was loaded onto a 1% (w/v) agarose gel and visualized with a GelDoc XR (Bio-Rad). The figures depict the pure and intact gDNA isolated from the environmental samples.

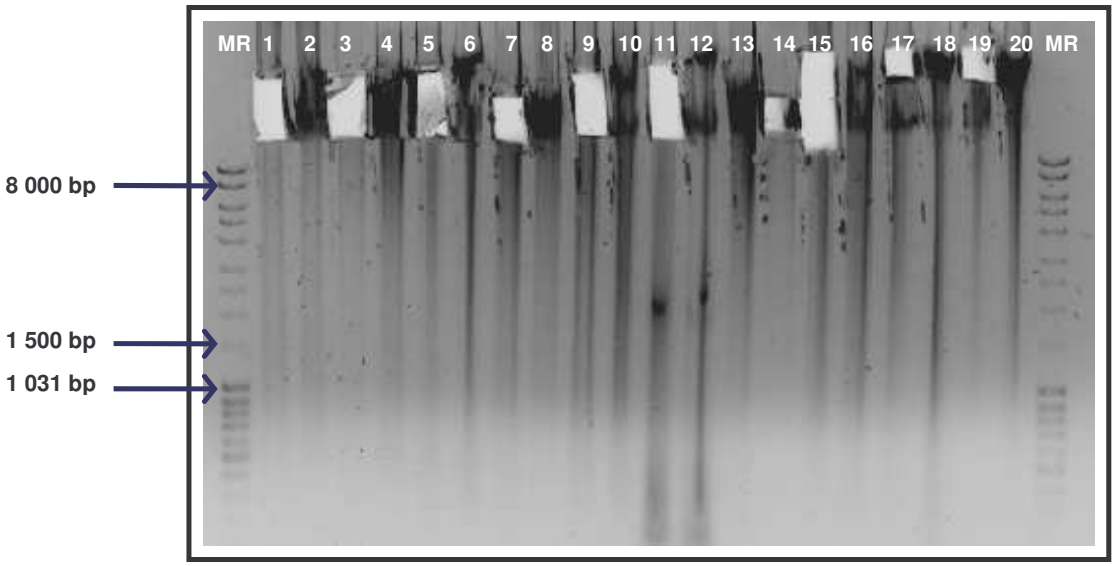

Figure 2.3.3: gDNA isolation from samples grown in minimal medium supplemented with 4 mM NaCN. H1, Lane 1 and 2; H2, Lane 3 and 4; H3, Lane 5 and 6; H4, Lane 7 and 8; H5, Lane 9 and 10; H6, Lane 11 and 12; H7, Lane 13 and 14; C1, Lane 15 and 16; C2, Lane 17 and 18; C3, Lane 19 and 20 and MR, MassRuler^{TM} DNA Ladder Mix (Fermentas).

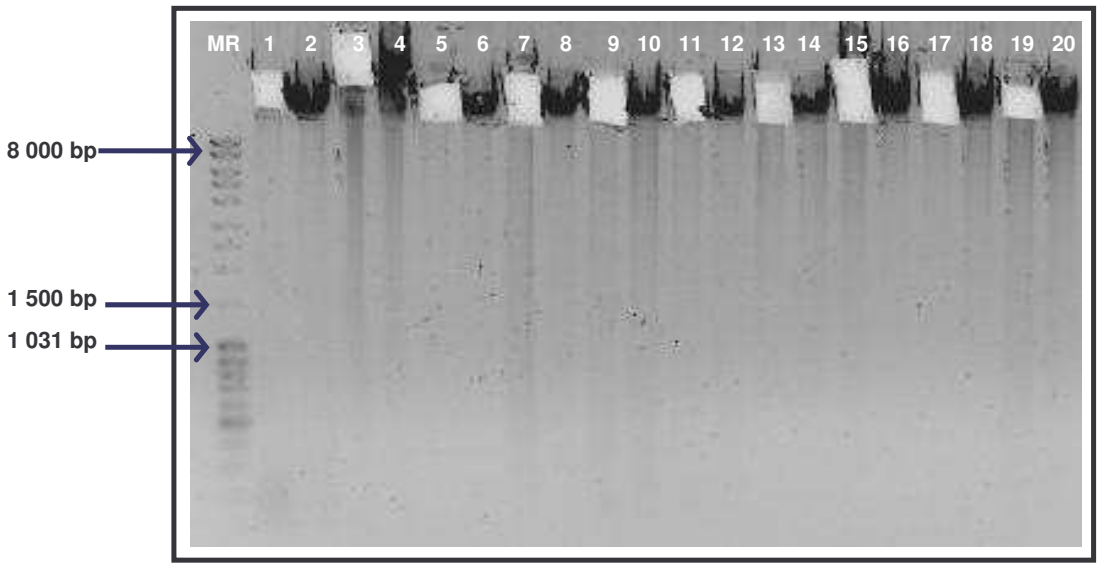

Figure 2.3.4: gDNA isolation from samples grown in minimal medium supplemented with 10 mM NaCN. The sampes were loaded in duplicate. H1, Lane 1 and 2; H2, Lane 3 and 4; H3, Lane 5 and 6; H4, Lane 7 and 8; H5, Lane 9 and 10; H6, Lane 11 and 12; H7, Lane 13 and 14; C1, Lane 15 and 16; C2, Lane 17 and 18; C3, Lane 19 and 20 and MR, MassRuler^{TM} DNA Ladder Mix (Fermentas).

The concentrations (in triplicate) of the isolated gDNA (ng.µl^{-1}) from the isolated organisms ranged from 300 - 2 700 ng.µl^{-1}.

2.3.2.4. DGGE analysis

Genomic DNA (Figure 2.3.5) was used in a 16S rDNA PCR thermal cycling reaction (section 2.2.4.3) with the universal bacterial primers (27F and 1492R). gDNA from six soil samples were isolated with the FastDNA® Spin Kit For Soil (MP Biomedicals, LLC). No gDNA was extracted from soil sample KSSO07042008H7 due to the small quantity left after the enrichment experiments.

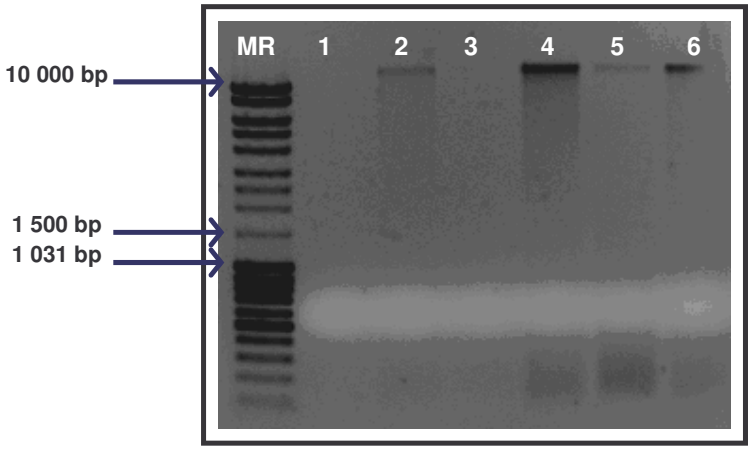

Figure 2.3.5: gDNA isolated from the soil samples. Lane 1, KSSO07042008H6; Lane2, KSSO07042008H3; Lane 3, KSSO07042008H5; Lane 4, KSSO07042008H2; Lane 5, KSSO07042008H1; Lane 6, KSSO07042008H4 and MR, MassRuler™ DNA Ladder Mix (Fermentas).

The products obtained from the 16S rDNA cycling were loaded onto a 1% (w/v) agarose gel (Figure 2.3.6).

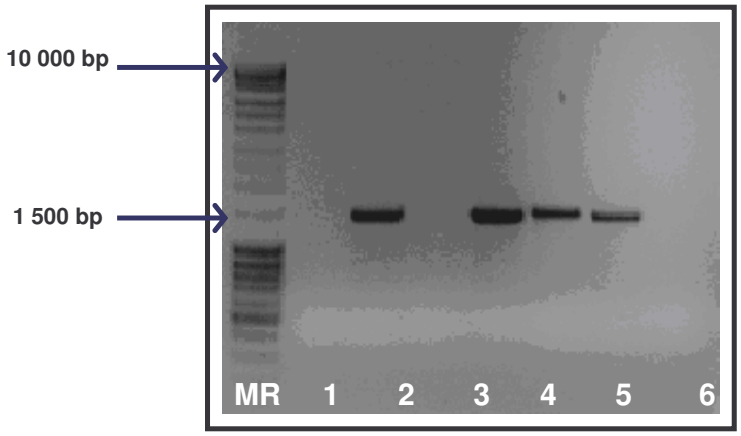

Figure 2.3.6: Amplification of bacterial 16S rDNA fragments. Lane 1, KSSO07042008H6; Lane 2, KSSO07042008H3; Lane 3, KSSO07042008H5; Lane 4, KSSO07042008H2; Lane 5, KSSO07042008H1; Lane 6, KSSO07042008H4; Lane 7, Negative control and MR, MassRuler^{TM} DNA Ladder Mix (Fermentas).

A PCR product of the correct product size (~1 500 bp) were obtained for four of the six soil samples (Figure 2.3.6). The positive products were used as templates for the PCR thermal cycling reaction (section 2.2.4.3) using the DGGE specific primers 341F-GC and 517R. These primers result in products that are ~200 bp in size (Figure 2.3.7).

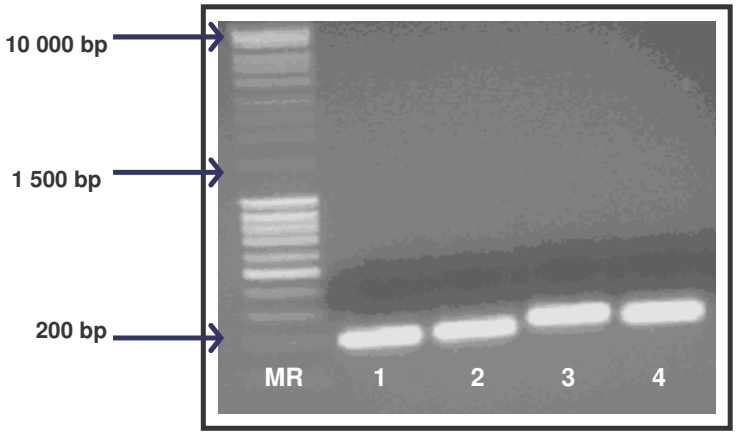

Figure 2.3.7: Amplification of bacterial 16S rDNA fragments. Lane 1, KSSO07042008H3; Lane 2, KSSO07042008H2; Lane 3, KSSO07042008H1; Lane 4, KSSO 07042008H4 and MR, MassRuler^{TM} DNA Ladder Mix (Fermentas).

The ~ 200 bp products were used in the DGGE analysis discussed in section 2.2.4.4 (Figure 2.3.8).

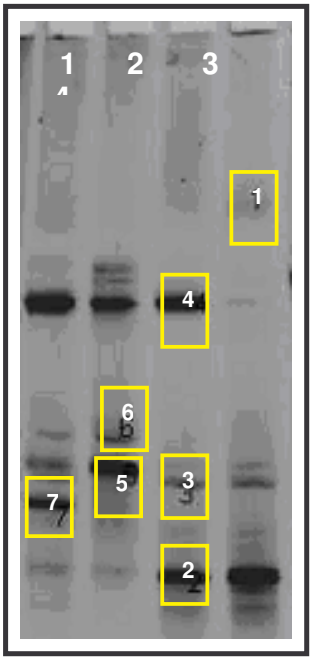

Figure 2.3.8: DGGE analysis of the soil samples. Lane 1, KSSO07042008H3; Lane 2, KSSO07042008H2; Lane 3, KSSO07042008H1 and Lane 4, KSSO07042008H4.

The numerical numbers on the gel are the bands that were excised from the gel and cleaned for the sequencing reaction as discussed in section 2.2.4.2. The sequencing results are given in Table 2.3.3. These initial results (highlighted in the blue block in Table 2.3.3) confirmed the distinctive biodiversity present in the soil obtained from the cyanide contaminated Klipspruit site.

Table 2.3.3: The BLAST results of the products obtained from the DGGE analysis.

Band	Accession number	Organism	Query Coverage (%)	E-value	Maximum Identity (%)
1	CP000697.1	*Acidiphilium cryptum* JF-5, complete genome	100	2e-16	80
2	AB552191.1	Uncultured bacterium gene for 16S rRNA, partial sequence, clone: OY04C1-058	100	1e-42	94
3	GU630034.1	Uncultured bacterium clone HF4525 16S ribosomal RNA gene, partial sequence	98	1e-08	74
4	AB552174.1	Uncultured bacterium gene for 16S rRNA, partial sequence, clone: OY04C1-038	100	2e-47	95
5	FJ669022.1	Uncultured bacterium clone 318-194-HRB1 16S ribosomal RNA gene, partial sequence	100	6e-52	95
6	GU568261.1	Uncultured bacterium clone N26b 16S ribosomal RNA gene, partial sequence	98	1e-48	95
7	FP475956.1	*Thiomonas* sp. str. 3As, chromosome, complete genome	100	5e-53	94

* Bands 1 and 2 produced no significant similarity and therefore a discontiguous megablast was performed on the two sequences.

No gDNA was isolated from the three water samples, thus the DGGE analysis was not performed on these samples.

2.3.2.5. Gram staining

The twenty isolates obtained from the minimal medium agar plates were subjected to gram staining (section 2.2.3.4). The gram staining protocol preliminarily confirmed that the single colonies were pure. Five isolates were selected for further experiments based on the MIC and the maximum identity (section 2.3.2.7). The five isolates included three gram-positive (4H3, *Bacillus* sp.; 4C1, *Paenibacillus* sp. and 10H4, *Leifsonia* sp.) [Figure 2.3.9] and two gram-negative organisms (4C2, *Achromobacter* sp. and 10C3, *Brevundimonas* sp.) [Figure 2.3.10]. The three gram positive organisms (4H3, 4C1 and 10H4) and the two gram negative organisms (4C2 and 10C3) were identified as rod shaped which corresponds with data available on these organisms in literature (Fan *et al*., 1972; Reddy *et al*., 2003; Saha *et al*., 2005; Chester and Cooper, 1979 and Han and Andrade, 2005).

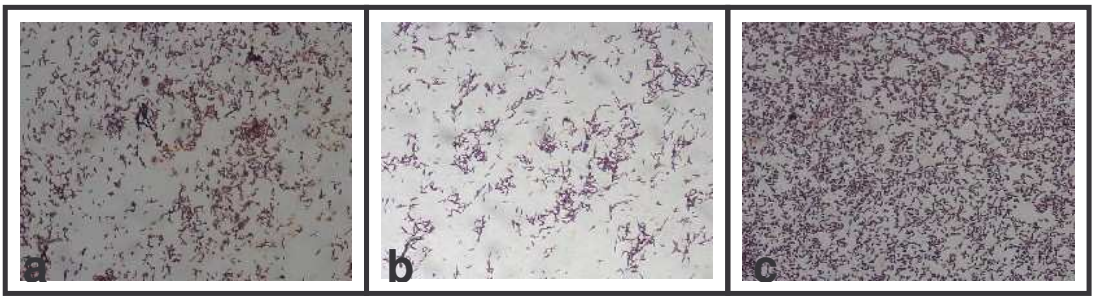

Figure 2.3.9: Gram staining photos obtained from the gram positive organisms a) 4H3, *Bacillus* sp.; b) 4C1, *Paenibacillus* sp. and c) 10H4, *Leifsonia* sp..

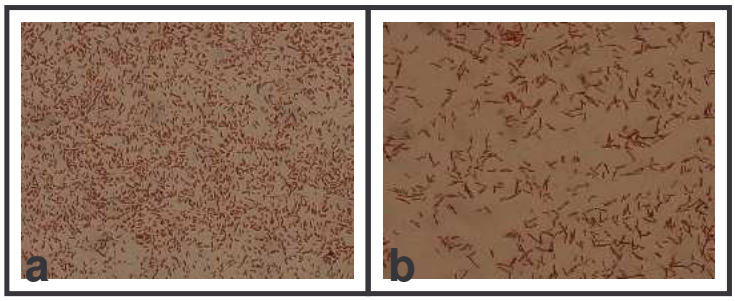

Figure 2.3.10: Gram staining photos obtained from the gram negative organisms a) 4C2, *Achromobacter* sp. and b) 10C3, *Brevundimonas* sp..

2.3.2.6. 16S and 18S rDNA PCR

PCR reactions to amplify the 16S (section 2.2.4.3) or 18S (section 2.2.4.2) rRNA gene were performed using purified gDNA as template. The twenty isolates obtained from the minimal medium supplemented with 4 mM NaCN (Figure 2.3.11) as well as the microorganisms isolated from the minimal medium supplemented with 10 mM NaCN (Figure 2.3.12) were used as the template in the PCR reactions. PCR products were loaded onto a 1% agarose gel and subjected to non-UV transillumination (DarkReader). No positive results were obtained with the 18S universal primers (EukA and EukB) thereby suggesting that none of the isolates are eukaryotic organisms. PCR products were gel purified using the BioSpin Gel Extraction Kit (BioFlux) and used in sequencing analysis.

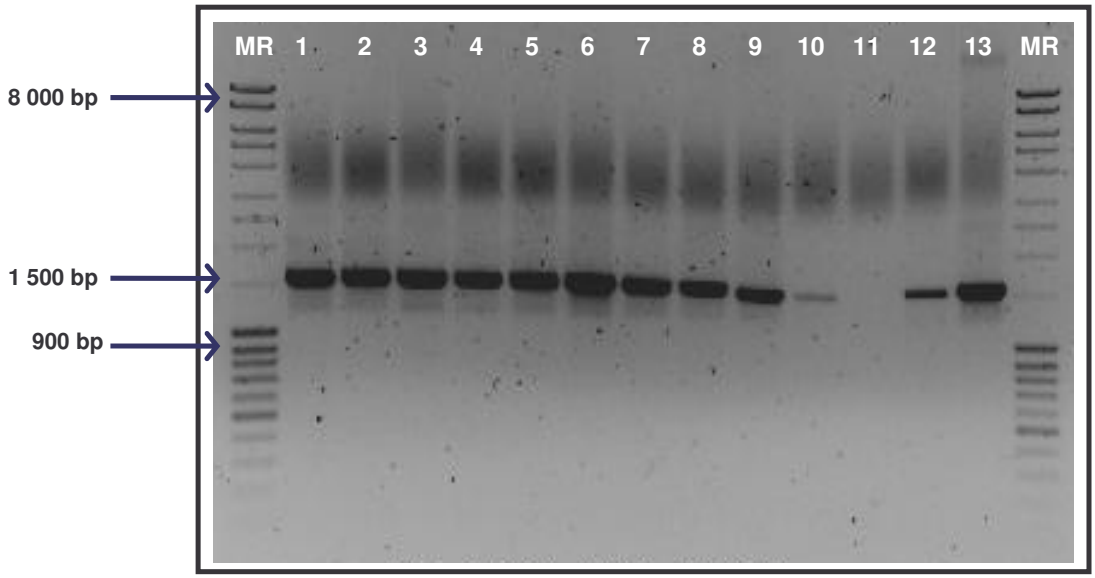

Figure 2.3.11: 16S rDNA PCR performed on the gDNA isolated from the minimal media supplemented with 4 mM NaCN. H1, Lane 1; H2, Lane 2; H3, Lane 3; H4, Lane 4; H5, Lane 5; H6, Lane 6; H7, Lane 7; C1, Lane 8; C2, Lane 9; C3, Lane 10; Negative control, Lane 11; *Serratia marcescens* (positive control 1), Lane 12; *Thermus scotoductus* (positive control 2), Lane 13 and MR, MassRuler^{TM} DNA Ladder Mix (Fermentas).

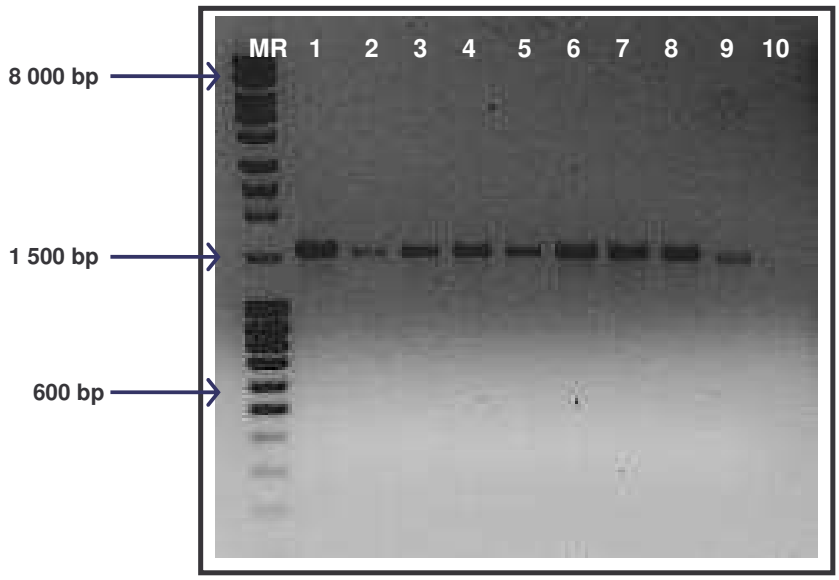

Figure 2.3.12: 16S rDNA PCR performed on the gDNA isolated from the minimal medium supplemented with 10 mM NaCN. H1, Lane 1; H3, Lane 2; H4, Lane 3; H5, Lane 4; H6, Lane 5; H7, Lane 6; C1, Lane 7; C3, Lane 8; *T. scotoductus* (positive control), Lane 9; Negative control, Lane 10 and MR, MassRuler^{TM} DNA Ladder Mix (Fermentas).

Eighteen of the isolates yielded the expected ~1 500 bp band which corresponds to the size of the 16S rRNA gene of bacteria. No 16S rRNA products were obtained for samples H2 and C2 from minimal medium supplemented with 10 mM NaCN.

2.3.2.7. Sanger sequencing

The eighteen purified 16S rDNA products were subjected to the thermal cycling depicted in the sequencing PCR reaction (section 2.2.4.2). The sequencing results were edited and subjected to a BLAST analysis on the National Center for Biotechnology Information (NCBI) database. The highest BLAST result of each of the eighteen isolates is summarized in Table 2.3.4. The number in front of each sample name indicates the concentration of NaCN added to minimal media when the microorganisms were isolated.

Table 2.3.4: The BLAST analysis results obtained from the16S rDNA sequences of the eighteen bacterial isolates obtained from Klipspruit Calcium Cyanide Factory site.

Sample	Accession number	Organism	Contig size (bp)	Query coverage (%)	E-value	Maximum Identity (%)
4H1	GQ246654.1	*Lysinibacillus* sp. M1T1B8 16S ribosomal RNA gene, partial sequence	1 332	100	0.0	100
4H2	HM068891.1	*Lysinibacillus fusiformis* strain CBII-2 16S ribosomal RNA gene, partial sequence	1 323	100	0.0	99
4H3	FJ554665.1	*Bacillus marisflavi* strain SU1 16S ribosomal RNA gene, partial sequence	1 372	100	0.0	99
4H4	FJ554665.1	*Bacillus marisflavi* strain SU1 16S ribosomal RNA gene, partial sequence	1 383	100	0.0	99
4H5	FJ554665.1	*Bacillus marisflavi* strain SU1 16S ribosomal RNA gene, partial sequence	1 337	100	0.0	99
4H6	HM224386.1	*Bacillus pumilus* strain B1 16S ribosomal RNA gene, partial sequence	1 377	100	0.0	100
4H7	FJ554665.1	*Bacillus marisflavi* strain SU1 16S ribosomal RNA gene, partial sequence	1 334	100	0.0	100
4C1	AB363733.1	*Paenibacillus lautus* gene for 16S rRNA, partial sequence, strain: NBRC 15380	1 257	100	0.0	99
4C2	FJ169468.1	*Achromobacter xylosoxidans* 16S ribosomal RNA gene, partial sequence	1 359	100	0.0	99
4C3	GU198196.1	*Microbacterium schleiferi* strain HBSD-C 16S ribosomal RNA gene, partial sequence	542	100	0.0	96
10H1	AM162341.1	*Paenibacillus* sp. GR13-04 partial 16S rRNA gene	699	99	0.0	96
10H3	FJ872401.2	*Leifsonia* sp. MMA-AI-3 16S ribosomal RNA gene, partial sequence	1 270	100	0.0	98
10H4	FJ872401.2	*Leifsonia* sp. MMA-AI-3 16S ribosomal RNA gene, partial sequence	1 360	100	0.0	99
10H5	FJ959372.1	*Microbacterium* sp. 0-20 16S ribosomal RNA gene, partial sequence	1 285	99	0.0	99
10H6	FJ784125.1	*Leifsonia* sp. L89 16S ribosomal RNA gene, partial sequence	1 369	100	0.0	98
10H7	AJ717356.1	*Microbacterium oxydans* 16S rRNA gene, isolate AC94	1 377	99	0.0	99
10C1	FJ009389.1	*Microbacterium oxydans* strain D6 16S ribosomal RNA gene, partial sequence	1 371	100	0.0	99
10C3	DQ177489.1	*Brevundimonas* sp. Tibet-IX23 16S ribosomal RNA gene, partial sequence	1 337	99	0.0	95

After the sequences were analyzed (section 2.2.4.3) using Vector NTI Suite 9 (ContigExpress) contigs were obtained as indicated in Table 2.3.4. No contigs were obtained for the two isolates highlighted in yellow (Table 2.3.4). The results obtained were different from the expected results after the DGGE analysis (section 2.3.2.4) were performed.

2.3.2.8. MIC determination

Of the two methods used to determine the MIC (section 2.2.4.6) the best results were obtained using the first method. In the second method, at cyanide concentrations above 2.5 M, the agar plates remained semi-solid and the cultures could not be plated out on the soft agar. The results obtained from the MIC determination for the organisms isolated from minimal media supplemented with 4 mM and 10 mM NaCN are summarized in Table 2.3.5 (a) and Table 2.3.5 (b). The three gram-positive organisms (highlighted in yellow) and the two gram-negative organisms (highlighted in green) were selected for further studies based on the MIC calculated, maximum identity obtained from Sanger sequencing (section 2.3.2.7) and gram-staining results discussed in section 2.3.2.5.

Table 2.3.5 (a): Summary of the minimal inhibition concentration for the isolated microorganisms from minimal media supplemented with 4 mM NaCN.

Isolate	0.5 M	1 M	1.5 M	2 M	2.5 M	3 M	3.5 M	4 M	4.5 M	Highest concentration* (M)
4H1	+	+	+	-						1.8
4H2	+	+	+	-						1.7
4H3	+	+	+	+						2.1
4H4	+	+	+	+	+	+	+	+	-	4.2
4H5	+	+	+	-						1.8
4H6	+	+	+	-						1.7
4H7	+	+	+	-						1.6
4C1	+	+	+	-						1.8
4C2	+	+	-	-						1.3
4C3	+	-	-	-						0.8

* The highest concentration is the last concentration where growth was observed

Table 2.3.5 (b): Summary of the minimal inhibition concentration for the isolated microorganisms from minimal media supplemented with 10 mM NaCN.

Isolate	0.5 M	1 M	1.5 M	2 M	2.5 M	3 M	3.5 M	4 M	4.5 M	Highest concentration* (M)
10H1	+	+	+	+	+	+	+	-		3.7
10H3	+	+	+	+	+	+	+	-		3.8
10H4	+	+	+	+	+	+	+	-		3.1
10H5	+	+	+	+	+	+	+	-		3.1
10H6	+	+	+	+	+	+	+	-		3.1
10H7	+	+	+	+	+	-	-	-		2.6
10C1	+	+	+	+	+	+	+	-		3.4
10C3	+	+	+	-						1.8

* The highest concentration is the last concentration where growth was observed

2.3.3. Control organisms

2.3.3.1. gDNA isolation

gDNA was isolated (section 2.2.4.1) from *B. pumilus*, *P. fluorescens* and *P. stutzeri*. The isolated gDNA was evaluated on a 1% (w/v) agarose gel (Figure 2.3.13). The figure depicts the pure and intact gDNA isolated from the control organisms.

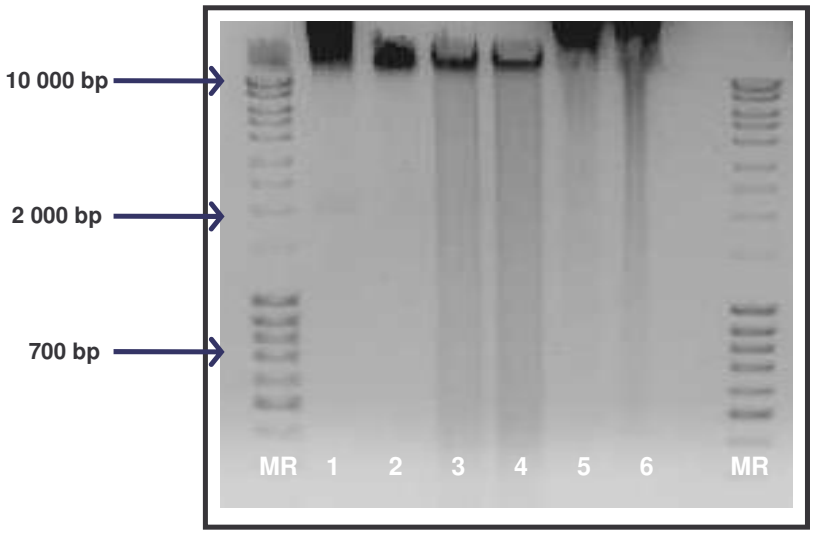

Figure 2.3.13: Isolated gDNA from *B. pumilus* (Lane 1 and Lane 2); *P. fluorescens* (Lane 3 and Lane 4); *P. stutzeri* (Lane 5 and Lane 6) and MR, MassRuler™ DNA Ladder Mix (Fermentas).

The concentrations (in triplicate) of the isolated gDNA (ng.µl^{-1}) from the three control organisms ranged from 100 - 970 ng.µl^{-1}.

2.3.3.2. 16S rDNA PCR

To confirm the identity of the control organisms the isolated gDNA was used in the 16S rDNA thermal cycling reaction (section 2.2.4.3) with the universal bacterial primers (27F and 1492R) [Figure 2.3.14].

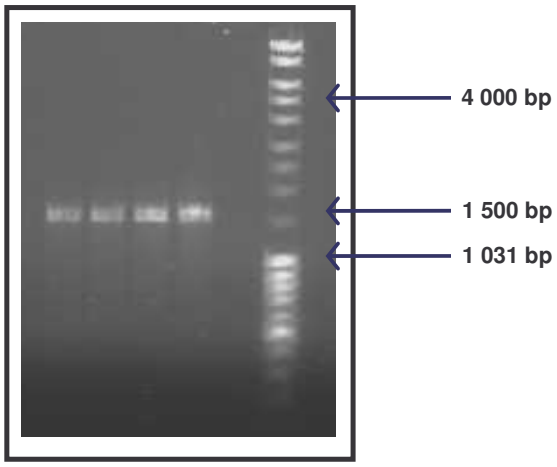

Figure 2.3.14: The 16S rDNA PCR products (~1 500 bp). Lane 1, *B. pumilus*; Lane 2, *P. fluorescens*; Lane 3, *P. stutzeri*; Lane 4, *S. marcescens* (positive control); Lane 5, negative control and Lane 6, MassRuler^{TM} DNA Ladder Mix (Fermentas).

The ~1 500 bp PCR products were excised from the gel, purified and used in the Sanger sequencing reaction.

2.3.3.3. Sanger sequencing

The purified products obtained from the 16S rDNA PCR reaction were used in the thermal cycling sequencing reaction (Table 2.2.5). The sequencing reaction was cleaned with the EDTA/ethanol protocol as discussed in section 2.2.4.2. Sequencing results confirmed the identity of the three control organisms (Table 2.3.6).

Table 2.3.6: The sequencing results obtained from the three control organisms.

Accession number	Organism	Query Coverage	E-value	Maximum Identity
EU333893.1	*Bacillus pumilus* strain K22-10 16S ribosomal RNA gene, complete sequence	100%	0.0	100%
U26417.1	*Pseudomonas stutzeri* strain LS401 16S rRNA gene	100%	0.0	99%
GU475123.1	*Pseudomonas fluorescens* strain R31 16S ribosomal RNA gene, partial sequence	100%	0.0	99%

2.3.4. Growth profiles

2.3.4.1. Five selected isolates

Growth studies were performed on the five selected isolates (section 2.2.3.2) in minimal medium supplemented with either 4 mM (Figure 2.3.15) or 10 mM (Figure 2.3.16) NaCN as the sole carbon and nitrogen source. The maximum growth rate (μ_{max}) and doubling time (t_d) are summarized in section 2.3.4.3.

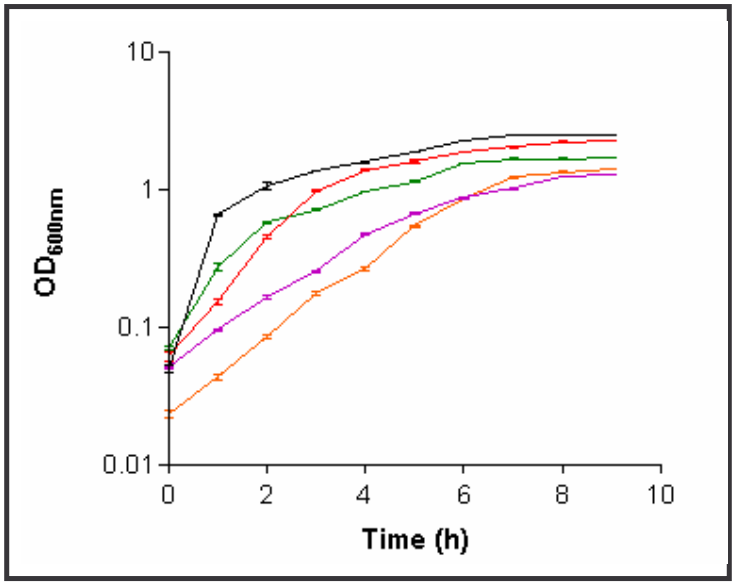

Figure 2.3.15: Growth curves of the five selected isolates (4H3, 4C1. 4C2, 10H4 and 10C3) in minimal medium with 4 mM NaCN added at 30ºC. Error bars indicate standard deviations. Black, 4H3; Red, 4C1; Green, 4C2; Purple, 10H4 and Orange, 10C3.

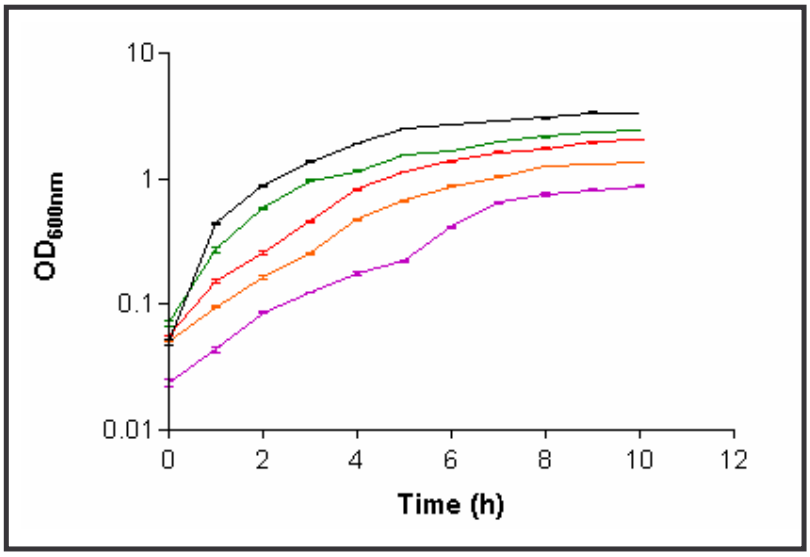

Figure 2.3.16: Growth curves of the five selected isolates (4H3, 4C1, 4C2, 10H4 and 10C3) in minimal medium with 10 mM NaCN added at 30ºC. Error bars indicate standard deviations. Black, 4H3; Red, 4C1; Green, 4C2; Purple, 10H4 and Orange, 10C3.

2.3.4.2. Control organisms

Growth studies (section 2.2.6.2) were performed in LB medium without NaCN (Figure 2.3.17). The three control organisms reached stationary phase after 8 h of growth at 30ºC in a shaking incubator.

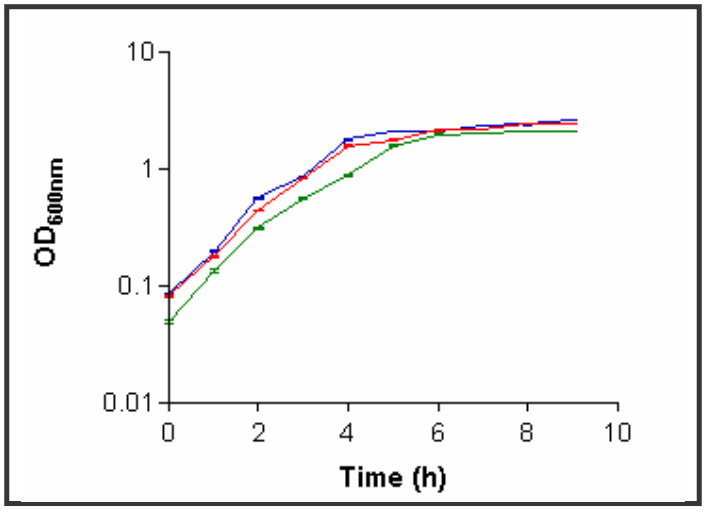

Figure 2.3.17: Growth study without the addition of NaCN in LB medium. Error bars indicate standard deviations. Green, *B. pumilus*; Red, *P. fluorescens* and Blue, *P. stutzeri*.

The growth studies (section 2.2.6.2) were also performed in minimal media supplemented with 4 mM NaCN (Figure 2.3.18) or 10 mM NaCN (Figure 2.3.19). The NaCN was added as the sole carbon and nitrogen source.

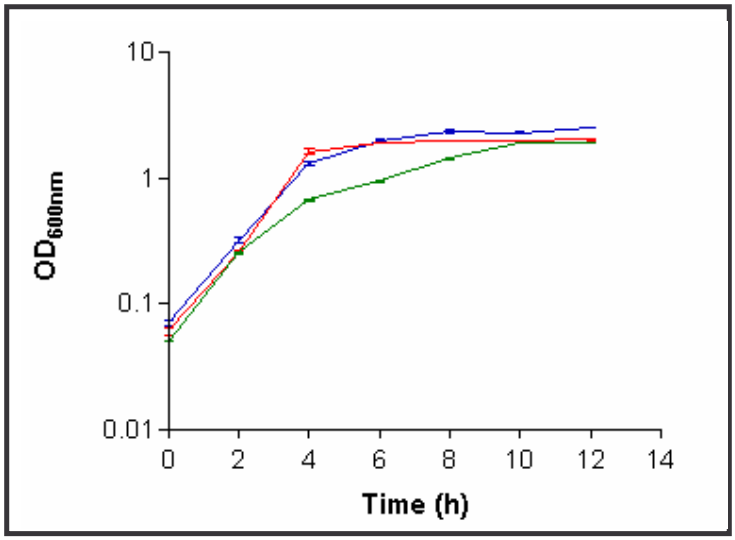

Figure 2.3.18: Growth study with the addition of 4 mM NaCN to minimal medium. Error bars indicate standard deviations. Green, *B. pumilus*; Red, *P. fluorescens* and Blue, *P. stutzeri*.

The maximum growth rates (μ_{max}) and doubling times (t_d) of the growth curves represented in Figure 2.3.18 are given in Table 2.3.8.

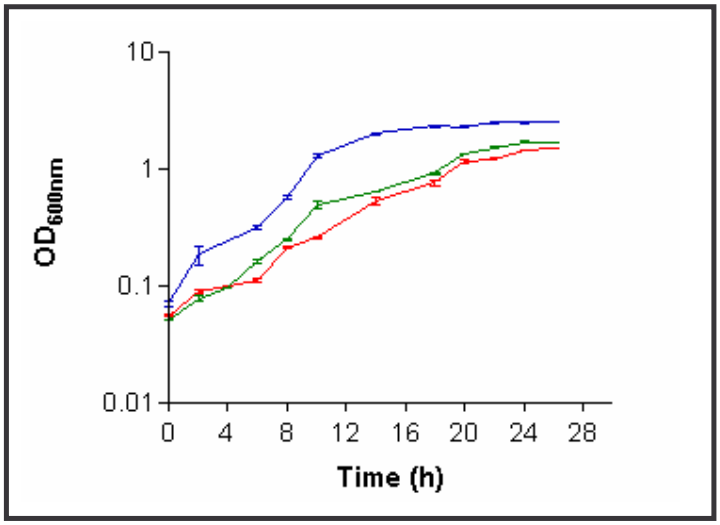

Figure 2.3.19: Growth study with the supplementation of minimal media with 10 mM NaCN. Error bars indicate standard deviations. Green, *B. pumilus*; Red, *P. fluorescens* and Blue, *P. stutzeri*.

2.3.4.3. Comparison of maximum growth rates and doubling time

The five selected isolates grown in minimal medium supplemented with 4 mM NaCN reached stationary phase in 9 hrs. The maximum growth rate of the control organisms (Table 2.3.9) was, on average, higher than the five selected isolates (Table 2.3.7) and the doubling time was, on average, lower in the control organisms than in the selected isolates. Thus, the control and isolated organisms are capable of utilizing the 4 mM NaCN as sole carbon and nitrogen source without the inhibition of growth due to the toxicity of cyanide to the bacterial cell. Isolates 4H3 and 4C2 (Table 2.3.7) utilized the NaCN slower that the control organisms (Table 2.3.8). This can either be due to the existence of different cyanide degradation pathways present in the different organisms or that the activation of these pathways in the isolates are slower than the pathways present in the control organisms.

Table 2.3.7: The maximum growth rate and doubling time of the selected isolates.

4 mM			10 mM		
Selected Isolates	**μ_{max} (h^{-1})**	**t_d (h)**	**Selected Isolates**	**μ_{max} (h^{-1})**	**t_d (h)**
4H3	0.224	3.090	4H3	0.380	1.823
4C1	0.538	1.289	4C1	0.603	1.149
4C2	0.260	2.668	4C2	0.328	2.111
10H4	0.543	1.277	10H4	0.360	1.925
10C3	0.569	1.218	10C3	0.543	1.277

Table 2.3.8: Growth parameters calculated from the growth curves of the three control organisms in mimimal medium supplemented with either 4 or 10 mM NaCN.

4 mM			10 mM		
Control organism	**μ_{max} (h^{-1})**	**t_d (h)**	**Control organism**	**μ_{max} (h^{-1})**	**t_d (h)**
Bacillus pumilus	0.488	1.420	*Bacillus pumilus*	0.275	2.524
Pseudomonas fluorescens	0.879	0.789	*Pseudomonas fluorescens*	0.202	3.431
Pseudomonas stutzeri	0.708	0.979	*Pseudomonas stutzeri*	0.354	1.959

The five selected organisms reached stationary phase after 10 hrs in minimal medium supplemented with 10 mM NaCN as the sole carbon and nitrogen source. The growth rates, as well as the doubling time, of the isolates (Table 2.3.7) are faster in comparison with the growth rate and doubling time of the control organisms (Table 2.3.8).

From Figure 2.3.15 and Figure 2.3.16 it can be concluded that the selected isolates can comfortably utilize 10 mM NaCN as the sole source of carbon and nitrogen and that the concentrations are not toxic for the organisms. The selected isolates presumably showed better adaptation skills than the control organisms due the faster maximum growth rates presented when the concentration of the NaCN was increased. The maximum growth rates (μ_{max}) and doubling times (t_d) of the growth curves represented in both Figure 2.3.18 and Figure 2.3.19 are given in Table 2.3.8.

The maximum growth rates (μ_{max}) and doubling times (t_d) of the growth curves represented in Figure 2.3.17 are given in Table 2.3.9.

Table 2.3.9: Growth parameters calculated from the growth curves of the three control organisms in LB medium.

Control organism	μ_{max} (h^{-1})	t_d (h)
Bacillus pumilus	0.520	1.330
Pseudomonas fluorescens	0.647	1.071
Pseudomonas stutzeri	0.594	1.167

From Table 2.3.8 and Table 2.3.9 it can be seen that the μ_{max} and t_d of *B. pumilus* stayed the same in LB medium without the addition of NaCN and minimal medium supplemented with 4 mM NaCN. When the minimal medium was supplemented with 10 mM NaCN the μ_{max} decreased and the t_d increased. The same tendency was observed in *P. fluorescens* and *P. stutzeri*. It can be concluded that the additional stress factor introduced by the presence of 10 mM NaCN in the medium inhibits the growth of the three control organisms.

2.3.5. Screening of isolates for genes involved in cyanide degradation

Primers were designed (section 2.2.7) for the specific cyanide degradation genes namely cyanide dihydratase, hydrogen cyanide synthase and a cyanide degrading enzyme present in the three control organisms. These were tested on the gDNA isolated from the control organisms (Figure 2.3.20).

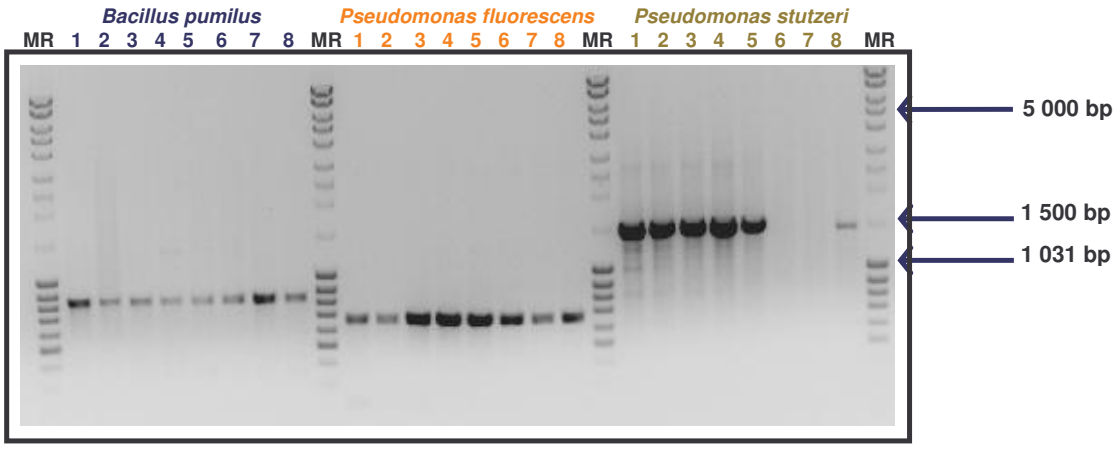

Figure 2.3.20: Gradient thermal cycling with specifically designed primers tested on gDNA isolated from control organisms. The temperatures used in the thermal cycling were ranged between 48-55°C.

The size of the gene products are 800 bp for the cyanide dihydratase of *B. pumilus*, 600 bp for the hydrogen cyanide synthase of *P. fluorescens* and 1 200 bp for the cyanide degrading enzyme of *P. stutzeri* (gene sizes obtained from NCBI).

The designed primers were used in the PCR reaction on the gDNA isolated from the eighteen isolated organisms with the selected annealing temperature of 48ºC (Figure 2.3.21, Figure 2.3.22 and Figure 2.3.23).

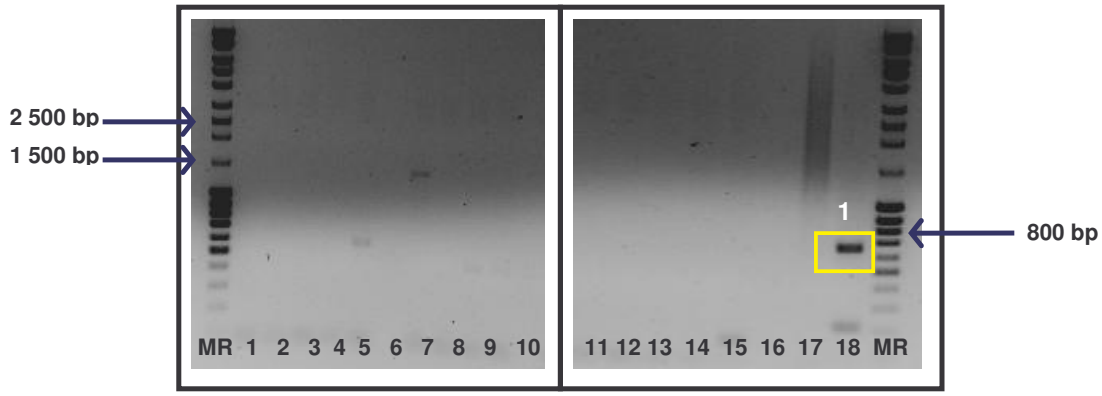

Figure 2.3.21: In the figure the gDNA extracted from the eighteen organisms isolated from minimal medium supplemented with either 4 mM or 10 mM NaCN was used as template for the primer designed for the gene present in *B. pumilus* (800 bp). Lane 1, 4H1; Lane 2, 4H2; Lane 3, 4H3; Lane 4, 4H4; Lane 5, 4H5; Lane 6, 4H6; Lane 7, 4H7; Lane 8, 4C1; Lane 9, 4C2; Lane 10, 4C3; Lane 11, 10H1; Lane 12, 10H3; Lane 13, 10H4; Lane 14, 10H5; Lane 15, 10H6; Lane 16, 10H7; Lane 17, 10C1 and Lane 18, 10C3. The yellow square indicates the band excised from the gel for Sanger sequencing analysis.

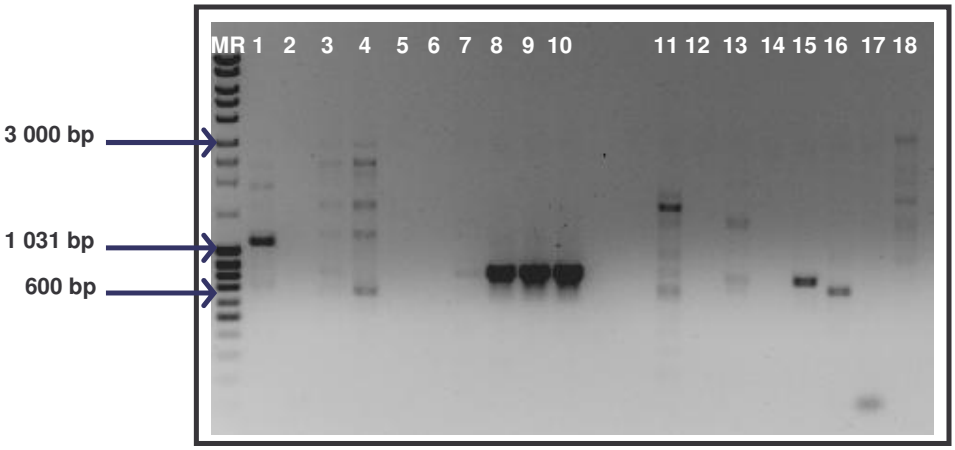

Figure 2.3.22: In the figure the gDNA extracted from the eighteen organisms isolated from minimal medium supplemented with either 4 mM or 10 mM NaCN was used as template for the primer designed for the gene present in *P. fluorescens* (600 bp). Lane 1, 4H1; Lane 2, 4H2; Lane 3, 4H3; Lane 4, 4H4; Lane 5, 4H5; Lane 6, 4H6; Lane 7, 4H7; Lane 8, 4C1; Lane 9, 4C2; Lane 10, 4C3; Lane 11, 10H1; Lane 12, 10H3; Lane 13, 10H4; Lane 14, 10H5; Lane 15, 10H6; Lane 16, 10H7; Lane 17, 10C1 and Lane 18, 10C3.

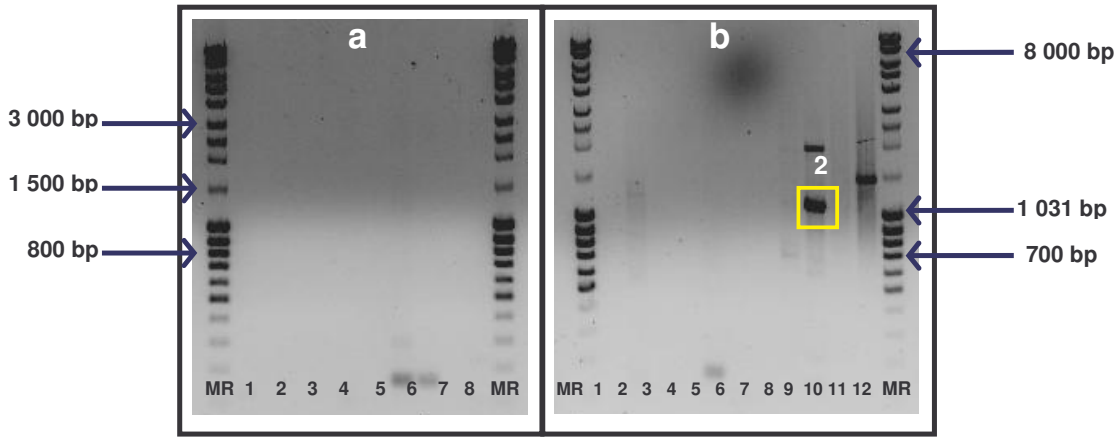

Figure 2.3.23: In figure (a) the gDNA extracted from the eight organisms isolated from minimal supplemented with 10 mM NaCN and in figure (b) the gDNA extracted from the ten organisms isolated from minimal medium supplemented with 4 mM NaCN were used as the template for the primer designed for the gene present in *P. stutzeri* (1 200 bp). Lane 1, 10H1; Lane 2, 10H3; Lane 3, 10H4; Lane 4, 10H5; Lane 5, 10H6; Lane 6, 10H7; Lane 7, 10C1 and Lane 8, 10C3. In figure (b) Lane 1, 4H1; Lane 2, 4H2; Lane 3, 4H3; Lane 4, 4H4; Lane 5, 4H5; Lane 6, 4H6; Lane 7, 4H7; Lane 8, 4C1; Lane 9, 4C2 and Lane 10, 4C3. The yellow block indicates the band excised from the gel for Sanger sequence analysis.

Some of the organisms did contain the correct size band but various non-specific product formations did occur and in many of the samples no product was amplified. The correct size bands were excised from the gel and purified. The purified bands were used in the thermal cycling as discussed in section 2.2.4.5 and summarized in Table 2.3.10.

Table 2.3.10: Sanger sequencing results summarizing the positive results obtained from the designed primers on the isolated gDNA from the eighteen isolates.

Band	Accession Number	Organism	Query Coverage	E-value	Maximum Identity
1	FJ204434.1	Groundwater biofilm bacterium U3b 16S ribosomal RNA gene, partial sequence	62%	6e-67	72%
2	EU884439.1	*Enterobacter* sp. 12 16S ribosomal RNA gene, partial sequence	26%	2e-28	71%

* Band 1, obtained from the primers designed for the cyanide dihydratase gene present in *B. pumilus* and Band 2, obtained from the primers designed for the cyanide degrading gene present in *P. stutzeri*.

Although the two bands corresponded to the correct size product, the results obtained from the BLAST search were inconclusive and can thus not be said that the genes responsible for cyanide degradation are present in the isolates tested with the specific designed primer pairs. This results can possibly indicate that the predicted cyanide degradation pathways present in the three control organisms are either not present in the isolates or that the degradation of cyanide in the isolates occur through unidentified pathways.

2.3.6. Cyanide assay

The cyanide assay (section 2.2.8) is referred to as the picric assay due to the formation of a red-brown colour when free cyanide is bound to picric acid. This colour complex absorbs maximally at 520 nm.

2.3.6.1. Standard curve

For the picric acid assay, a standard curve (Figure 2.3.24) was constructed to indicate where the absorbance (520 nm) would deviate from linear kinetics. The picric acid assay was performed as discussed in section 2.2.8.2.

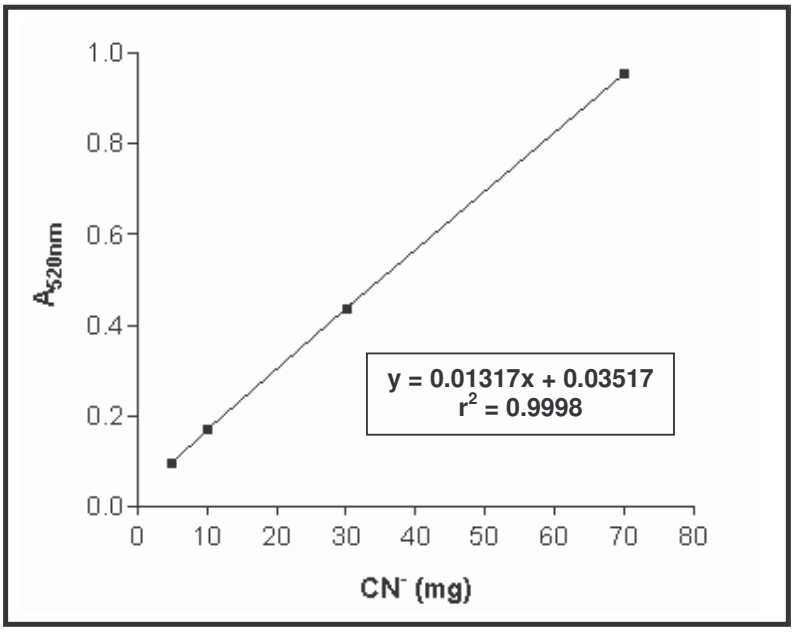

Figure 2.3.24: The standard curve for the picric acid assay. The data is representative of standards prepared in triplicate and then averaged. Standard deviations (in triplicate) are indicated as bars on the graph but were smaller than the symbols used.

2.3.6.2. Assay

The picric assay (section 2.2.8.3) was performed following the growth of the five selected organisms. The supernatants of the samples used in the assay were collected at various time intervals while the organisms were grown at 30ºC. In (Figure 2.3.25) the results indicate a decrease in NaCN concentration over time (h). The substrate concentration decreased from ~ 950 - ~ 10 mg.l^{-1} over ~ 145 h.

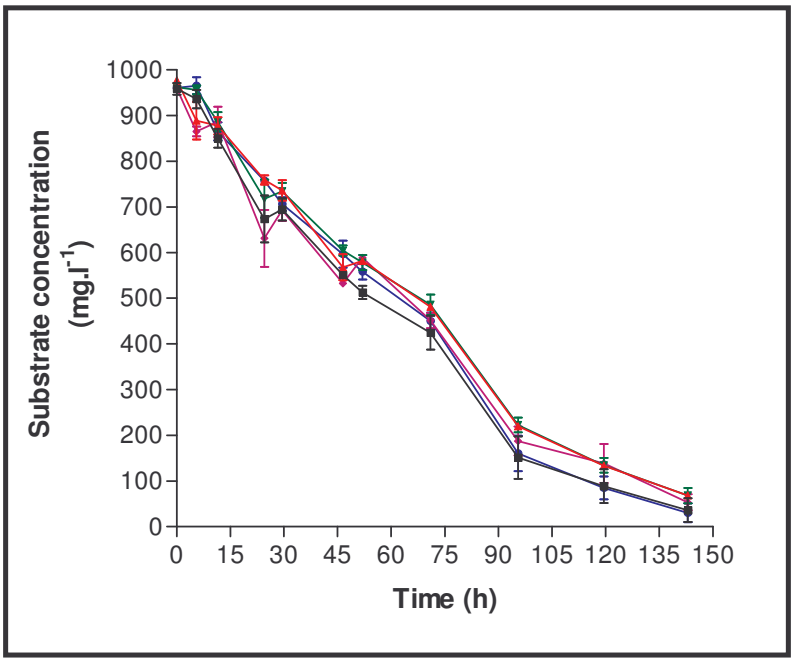

Figure 2.3.25: The substrate concentration (mg.l^{-1}) was calculated over time. The graph represents substrate utilization over time in the five selected organisms. Standard deviations (in triplicate) are indicated as bars on the graph. Black, 4H3; Red, 4C1; Green, 4C2; Purple, 10H4 and Blue, 10C3.

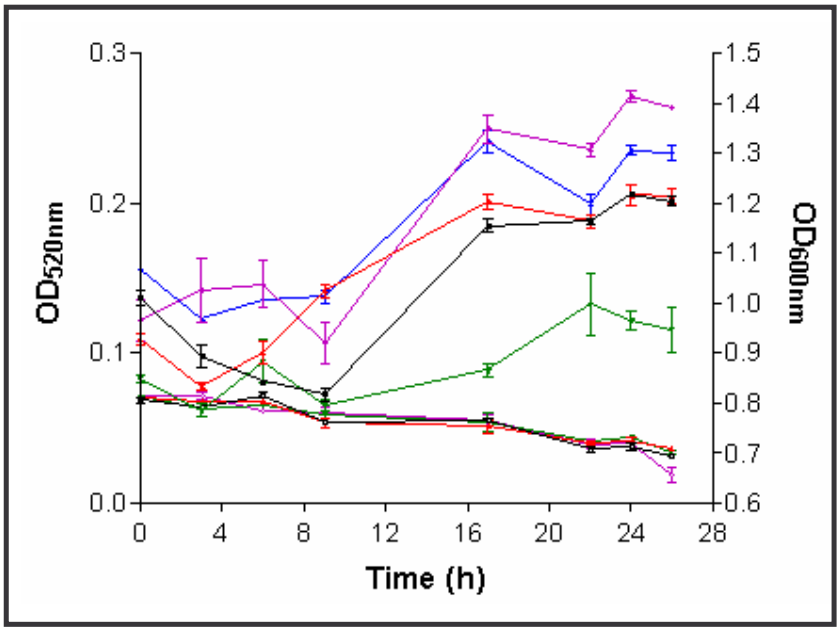

Figure 2.3.26: The growth (OD_{600nm}) increased over time (h) as the absorbance (520 nm) decreased. Standard deviations (in triplicate) are indicated as bars on the graph. Black, 4H3; Red, 4C1; Green, 4C2; Purple, 10H4 and Blue, 10C3.

In Figure 2.3.26 it can be seen that the absorbance decreases over time and simultaneously the optical density (600 nm) of the five organisms increased. This illustrates that the amount of cyanide, added as sole carbon and nitrogen source, decreased from ~ 55 - ~ 5 mg CN^- with a concomitant increase in growth over 28 h. This indicates that the five selected microorganisms are capable of utilizing the NaCN as the sole carbon and nitrogen source.

The three control organisms and five selected isolates were subjected to the same conditions during the cyanide assay. The control organisms were unable to utilize the NaCN as the sole carbon and nitrogen source.

2.4. Conclusions

Samples for this study were obtained from the Klipspruit Calcium Cyanide Factory site in Soweto, near Johannesburg in South Africa (section 2.2.2). This site was used to produce calcium cyanide for the use in extracting gold from ore in the cyanidation process. Enrichment techniques were employed to isolate microorganisms capable of utilizing the NaCN added to the minimal medium as the sole carbon and nitrogen source. The eighteen isolates were sequenced (Table 2.3.4) and the results obtained indicated that most of the

organisms have maximum identities of 97% or lower to a described specie. This can indicate that the species identified are unique. Gram staining and MIC determinations were performed to complement the novel results obtained in the sequencing results. The concentrations achieved in the MIC experiments indicate that these organisms possess the necessary adapting mechanisms to utilize the NaCN at high concentrations and as the sole carbon and nitrogen source. The isolated organisms were able to survive at concentrations of NaCN up to 3.5 M.

The maximum growth rate achieved by the five selected microorganisms (Table 2.3.7) in minimal media supplemented with 10 mM NaCN was higher than the control organisms at the same concentration (Table 2.3.8). When the minimal medium was supplemented with 4 mM of NaCN the maximum growth rate of the control organisms (Table 2.3.8) was faster than the five selected isolates (Table 2.3.7). The difference in the capability of utilizing the NaCN can indicate that the isolates are adapted to survive and even thrive in a harsh environment.

The picric acid assay indicated that the five selected microorganisms can utilize the supplemented NaCN as the sole carbon and nitrogen source (Figure 2.3.25). In Figure 2.3.26 it can be seen that the optical density (600 nm) increased as the NaCN decreased. It can be concluded that the bacteria are capable of detoxifying the cyanide in their immediate environment and degrading the cyanide to survive on a single carbon and nitrogen source.

The primers specifically designed (section 2.2.7) for the cyanide degrading genes, present in the three control organisms, were tested on the gDNA obtained from the isolates. Most of the isolates produced multiple bands but the products obtained were the incorrect size. The few correct size bands were sequenced but the BLAST results did not indicate that the gene was present in the specific isolate. It was then decided to select one organism (4H3, *Bacillus* sp.) of the selected five isolates to undergo pyrosequencing (Chapter 3). This organism was selected based on the MIC and Sanger sequencing results as well as the versatility of *Bacillus* spp. currently used in bioremediation plants. The various enzymes present in the metabolic pathway involved in cyanide degradation and cyanogenesis could be identified utilizing the sequence data obtained from pyrosequencing.

2.5. References

- **Babu, G.R.V., Vijaya, O.K., Ross, V.L., Wolfram, J.H. and Chapatwala, K.D.** (1996) Cell-free Extract(s) of *Pseudomonas putida* Catalyzes the Conversion of Cyanides, Cyanates, Thiocyanates, Formamide, and Cyanide-containing Mine Waters into Ammonia. *Applied Microbiology and Biotechnology* **45:** 273-277.

- **Behnke, A., Bunge, J., Barger, K., Breiner, H., Alla, V. and Stoeck, T.** (2006) Microeukaryote Community Patterns Along an O_2/H_2S Gradient in a Supersulfidic Anoxic Fjord (Framvaren, Norway). *Applied and Environmental Microbiology* **72:** 3626-3636.

- **Blumer, C. and Haas, D.** (2000) Mechanism, Regulation, and Ecological Role of Bacterial Cyanide Biosynthesis. *Archive of Microbiology* **173:** 170-177.

- **Burri, R. and Stutzer, A.** (1895) Über Nitrat Zerstörende Bakterien und den durch dieselben bedingten Stickstoffverlust. Zentrbl Bakteriol Parasitenkd II Abt. **1:** 350-367.

- **Chester, B. and Cooper, L.H.** (1979) *Achromobacter* Species (CDC Group Vd): Morphological and Biochemical Characterization. *Journal of Clinical Microbiology* **9:** 425-436.

- **Cipollone, R., Ascenzi, P., Frangipani, E. And Visca, P.** (2006) Cyanide Detoxification by Recombinant Bacterial Rhodanese. *Chemosphere* **63:** 942-949.

- **de Lillo, A., Ashley, F.P., Palmer, R.M., Munson, M.A., Kyriacou, L., Weightman, A.J. and Wade, W.G.** (2006) Novel Subgingival Bacterial Phylotypes Detected Using Multiple Universal Polymerase Chain Reaction Primer Sets. *Oral Microbiology Immunology* **21:** 61-68.

- **DSMZ** (2007) German Collection of Microorganisms and Cell Cultures.

- **Fan, D.P., Pelvit, M.C. and Cunningham, W.P.** (1972) Structural Difference Between Walls from Ends and Sides of the Rod-Shapes Bacterium *Bacillus subtilis*. *Journal of Bacteriology* **109:** 1266-1272.

- **Faramarzi, M.A., Stagers, M., Pensini, E., Krebs, W. and Brandl, H.** (2004) Metal Solubilization From Metal-Containing Solid Materials by Cyanogenic *Chromobacterium violaceum*. *Journal of Biotechnology* **113:** 321-326.

- **Fernandez, R.F., Dolghih, E. And Kunz, D.A.** (2004) Enzymatic Assimilation of Cyanide *via* Pterin-Dependent Oxygenolytic Cleavage to Ammonia and Formate in *Pseudomonas fluorescens* NCIMB 11764. *Applied and Environmental Microbiology* **70:**121-128.

- **Fernandez, R.F. and Kunz, D.A.** (2005) Bacterial Cyanide Oxygenase is a Suite of Enzymes Catalyzing the Scavenging and Adventitious Utilization of Cyanide as a Nitrogenous Growth Substrate. *Journal of Bacteriology* **187:** 6396-6402.

- **Han, X.Y. and Andrade, R.A.** (2005) *Brevundimonas diminuta* Infections and Its Resistance to Fluoroquinolones. *Journal of Antimicrobial Chemotherapy* **55:** 853-859.

- **International Cyanide Management Code for the Gold Mining Industry** (2010) http://www.cyanidecode.org/cyanide_chemistry.php. Retrieved 12-02-2010.

- **Labuschagne, M. and Albertyn, J.** (2007) Cloning of an Epoxide Hydrolase-Encoding Gene from *Rhodotorula mucilaginosa* and Functional Expression in *Yarrowia lipolytica*. *Yeast* **24:** 69-78.

- **Meyers, P.R., Gokool, P., Rawlings, D.E. and Woods, D.R.** (1991) An Efficient Cyanide-Degrading *Bacillus pumilus* Strain. *Journal of General Microbiology* **137:** 1397-1400.

- **Palleroni, N.J.** (1984) *Pseudomonadaceae*. *In:* Bergey's Manual of Systematic Biology, Kreig, N.R. and Holt, J.G. (eds.) The Williams and Wilkens Co., Baltimore, 141-199.

- **Perni, S., Andrew, P.W. and Shama, G.** (2005) Estimating the Maximum Growth Rate from Microbial Growth Curves: Definition is Everything. *Food Microbiology* **22:** 491-495.

- **Perry, S.F.** (1995) Freeze-Drying and Cryopreservation of Bacteria. *Methods in Molecular Biology* **38:** 21-30.

- **Reddy, G.S.N., Prakash, J.S.S., Srinivas, R., Matsumoto, G.I. and Shivaji, S.** (2003) *Leifsonia rubra* sp. nov. and *Leifsonia aurea* sp. nov., Psychrophiles from a Pond in Antarctica. *International Journal of Systematic and Evolutionary Microbiology* **53:** 977-984.

- **Rius, N., Fusté, M.C., Guasp, C., Lalucat, J. And Lorén, J.G.** (2001) Clonal Population Structure of *Pseudomonas stutzeri*, a Species with Exceptional Genetic Diversity. *Journal of Bacteriology* **183:** 736-744.

- **Saha, P., Mondal, A.K., Mayilraj, S., Krishnamurthi, S., Bhattacharya, A. and Chakrabarti, T.** (2005) *Paenibacillus assamensis* sp. nov., a Novel Bacterium Isolated From a Warm Spring in Assam, India. *International Journal of Systematic and Evolutionary Microbiology* **55:** 2577-2581.

- **Skowronski, B. and Strobel, G.A.** (1969) Cyanide Resistance and Cyanide Utilization by a Strain of *Bacillus pumilus*. *Canadian Journal of Microbiology* **15:** 93-98.

CHAPTER 3

3. PYROSEQUENCING: ELUCIDATION OF THE CYANIDE METABOLISM IN A *BACILLUS* SP.

3.1. Background

The removal of chemicals, released in the environment in large quantities, can be achieved by biodegradation (Babu *et al*., 1996). Microorganisms play an important role in the removal of anthropogenic waste. Cyanide is toxic to the environment but it can also be utilized as a sole carbon and/or nitrogen source by various microorganisms (Baxter and Cummings, 2006; Dash *et al*., 2009). Enzymes involved in cyanide metabolism are cyanide hydratase, rhodanese and oxygenase (Babu *et al*., 1996). Three control organisms were used to monitor and compare cyanide degradation as well as the genes involved in cyanide degradation of a new *Bacillus* isolate. The known enzymes characterized and involved in cyanide degradation were discussed in the literature overview (Chapter 1, section 1.6).

3.2. Cyanide degradation pathways

3.2.1. *Bacillus pumilus*

In 1969 a microorganism was isolated and identified as a *Bacillus pumilus* strain that could survive in concentrations of 2.5 M cyanide (Skowronski and Strobel, 1969). It was stated that the cyanide was degraded to ammonia and carbon dioxide and that the organism could grow comfortably in 1 M cyanide-containing media. *Bacillus pumilus* C1 was described as an aerobic, gram-positive and endospore-forming microorganism isolated for its ability to degrade cyanide in the presence of free cyanide (CN^-/HCN) concentrations of up to 3.8 mM (Meyers *et al*., 1991). Here, Meyers and co-workers (1991) purified and characterized an enzyme of *Bacillus pumilus* C1 and identified it as a **cyanide dihydratase** (EC 4.2.1.66) [Meyers *et al*., 1991]. This enzyme catalyzes the formation of ammonia and formate from cyanide (Equation 3.2.1) and no cofactor is required (Gupta *et al*., 2010).

Equation 3.2.1: $HCN + H_2O \leftrightarrow HCOOH + NH_3$

Cyanide dihydratase (cyanidase) is highly stable, specific in activity towards cyanide and easy to purify. These characteristics make this enzyme a very good candidate for use in industrial processes to detoxify cyanide biologically. Jandhyala *et al*. (2003) determined the first quaternary structure of cyanide dihydratase in *Bacillus pumilus* C1, and the gene (*cyn*D) is only the second cyanide dihydratase that was cloned and sequenced. This enzyme present in *Pseudomonas stutzeri* and *Bacillus pumilus* share a high level of sequence identity (80%), the protein can be expressed in *Escherichia coli* and gene disruptions lead to the loss of activity. All of these factors suggest that *cyn*D is the sole cyanide dihydratase gene in *Bacillus pumilus* C1 (Jandhyala *et al*., 2003).

3.2.2. *Pseudomonas fluorescens*

Pseudomonas fluorescens is a non-pathogenic saprophytic, gram-negative, obligate aerobe (Palleroni, 1984). This microbe is commonly found in soil, plant and water environments and is motile by means of multiple polar flagella. This microbe is well-known for the diversity in its metabolic system and is often used in bioremediation applications due to its capacity to degrade various pollutants such as polycyclic aromatic hydrocarbons, cyanide and styrene (Palleroni, 1984).

P. fluorescens was isolated in 1983 by Harris and Knowles, due to the fact that this strain could convert cyanide into ammonia (NH_3) and carbon dioxide (CO_2). White and co-workers (1988) isolated a pseudomonad, described as a methylotroph, capable of degrading cyanide to ammonia and formate instead of carbon dioxide. In 1992 Kunz and co-workers proposed that *P. fluorescens* may have more than one pathway that leads to the degradation of cyanide (Figure 3.2.1). The production of formate (HCO_2H) and formamide ($HCONH_2$) were detected both aerobically and anaerobically, and both products were constantly detected simultaneously.

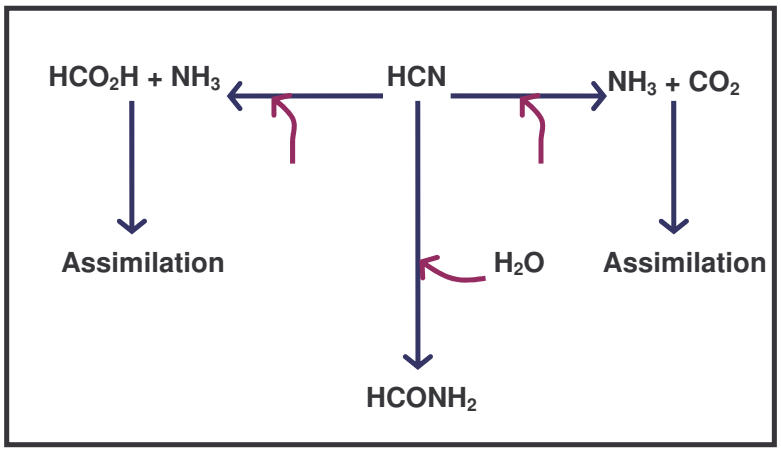

Figure 3.2.1: Cyanide conversion metabolic pathways by *Pseudomonas fluorescens* NCIMB 11764 (Taken from Kunz *et al*., 1992).

This illustrates that there are two separate pathways for cyanide degradation, both including the chemical conversion to ammonia which can be degraded further and utilized as a nitrogen source. An oxygenase-type enzyme could be responsible for the conversion of cyanide to carbon dioxide and ammonia. Direct cyanide hydrolysis leads to the formation of formate and it is proposed that cyanidase is responsible for the reaction. **Cyanide hydratase** (described for cyanide degradation in phytopathogenic fungi) could be responsible for the conversion to formamide.

In 1994 Kunz *et al*. proposed a hypothesis that cyanide degradation in *P. fluorescens* occurs *via* multiple pathways and that a third enzyme, **formate dehydrogenase**, could also be involved in cyanide metabolism (Figure 3.2.2).

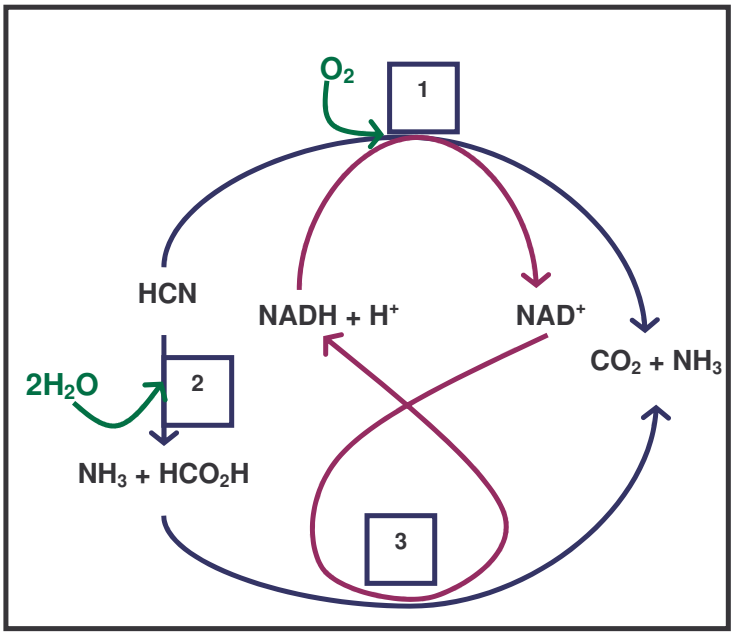

Figure 3.2.2: Cyanide metabolism in *P. fluorescens* indicating the role of cyanide oxygenase (1), cyanide nitrilase [cyanidase, cyanide dihydratase] (2) and formate dehydrogenase (3) *via* the regeneration of the pyridine cofactors (Taken from Kunz *et al*., 1994).

The recycling of NADH implies the involvement of this oxygenase-type mechanism due to the increased putative cyanide oxygenase activity during formate oxidation. Thus, formamide and formate is produced simultaneously under anaerobic conditions *via* **cyanide hydratase** and **cyanide nitrilase** (dihydratase). It is further hypothesized that **formate dehydrogenase** is induced by cyanide and may function under cyanide poisoning conditions by preserving NADH. Respiratory chain enzymes will be inhibited by cyanide and the NADH generated from glucose (energy and carbon source) will be utilized by the cyanide oxygenase reaction (non-energy yielding). This will lead to growth and energy limitation which can be overcome if the NADH molecules are cycled *via* formate dehydrogenase (Kunz *et al*., 1994).

Dash and co-workers (2009) stated that most *Pseudomonas* species are capable of the complete oxidation of thiocyanate and cyanide. These species can utilize the cyanide as a sole nitrogen source and **cyanide monooxygenase** catalyzes the conversion of hydrogen cyanide to ammonia and carbon dioxide with the formation of a metabolic intermediate product, cyanate. This conversion includes the consumption of one mole oxygen for every mole cyanide along with either NAD(P)H or NADH.

Cyanide in *Pseudomonas fluorescens* can also be consumed by non-enzymatic reactions with a reaction between a metabolite, excreted into the media, and cyanide (Kunz *et al*., 1998). The responsible metabolite can be a siderophore due to co-purification with iron chelating activity. The siderophores were identified as pyruvate (Pyr) and α-ketoglutarate (αKg) due to their ability to act as iron chelators.

When *P. fluorescens* is grown in cyanide containing media it leads to a growth rate that always lags behind the rate of cyanide utilization. This can be explained by the chemical reaction that occurs between cyanide and excreted α-keto acids. The chemical reaction leads to the formation of the corresponding cyanohydrin, in the extracellular space, which can further be degraded by cyanide degrading enzymes, in the intracellular space, to ammonia and carbon dioxide (Figure 3.2.3). The ammonia is then used as cellular nitrogen from cyanide as growth substrate. It is now suggested that the preferred substrates for cyanide oxygenase are cyanohydrins and not free cyanide due to the lower toxicity of the cyanohydrins compared to free cyanide. Keto acids are excreted under nitrogen limiting factors and are a natural occurrence that happens irrespective of the ability to degrade cyanide. The occurrence of keto acids on the out side of cells in nature can explain how microorganisms can acquire tolerance towards cyanide but if the microorganisms can utilize the cyanide as a nitrogen source physiological pathways are used and not the cyanide tolerance pathways. The utilization of cyanide still requires the ability to elaborate enzymes like cyanide oxygenase but the presence of keto acids will provide a selective advantage in the biosphere where cyanide is usually the only source of nitrogen (Kunz *et al*., 1998).

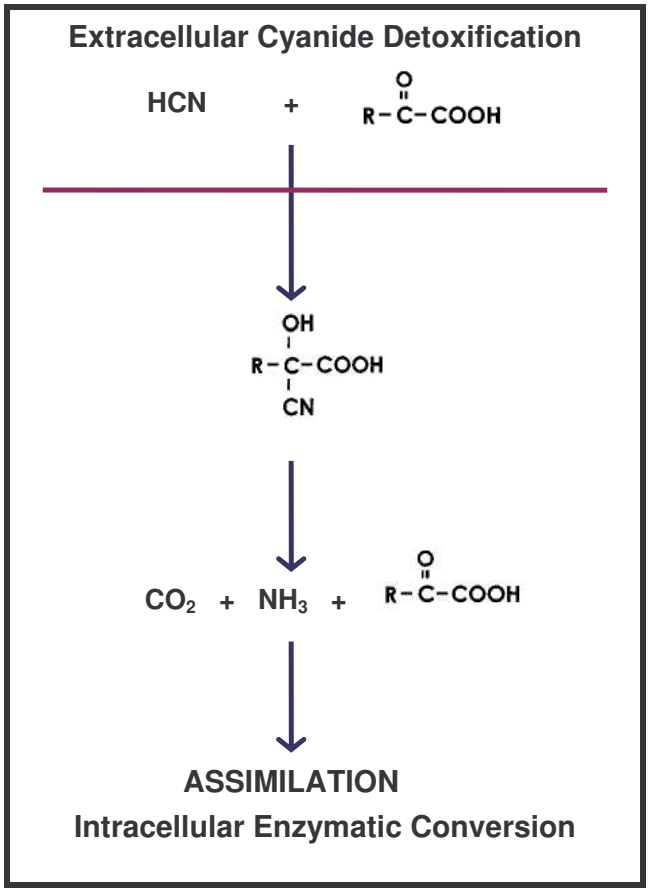

Figure 3.2.3: The assimilation pathway of cyanide in *Pseudomonas fluorescens* NCIMB 11764. R is CH_2CH_2COOH for Kg-CN and CH_3 for Pyr-CN (Taken from Kunz *et al*., 1998).

Fernandez and Kunz (2005) described that the **cyanide oxygenase** (CNO) of *Pseudomonas fluorescens* consists of four protein components and may exist as a multi-enzyme complex inside the cell. The enzyme's activity is dependent on the presence of oxygen, the reduced pterin molecule (a co-factor) and NADH. The four protein complexes are P1, an NADH oxidase (oxidoreductase) [Nox]; P2, an NADH peroxidase (Npx); P3, a cyanide dihydratase (CynD) [similar to the cynD enzymes found in *Bacillus pumilus* and *Pseudomonas fluorescens*] and P4, a carbonic anhydrase (CA). Nox, Npx and CA contain oxidative stress functions and CA protects against oxyradicals and this maybe the key to explain why *P. fluorescens* can utilize cyanide even when cyanide is known as an oxidative stress inducer (Fernandez and Kunz, 2005).

3.2.3. *Pseudomonas stutzeri*

Pseudomonas stutzeri was first isolated in 1895 as *Bacillus denitrificans* II but renamed as *P. stutzeri* in 1952 (Burri and Stutzer, 1895; Van Niel and Allen, 1952). When the organism is isolated it has unusual shape that can be described as dry and wrinkly (Rius *et al*., 2001). It is widely distributed in the environment but mainly found in water and soil. It is a gram-negative, mobile, rod-shape and non-fluorescent bacterium. It is metabolically versatile and this versatility leads to the degradation of pollutants in the environments due to mining and natural pollution (Rius *et al*., 2001).

The cyanide degrading ability of *P. stutzeri* is independent of the presence or absence of cyanide-containing media during cultivation (Watanabe *et al*., 1998). Thus, the **cyanide degrading enzyme** is present whether cyanide is present or absent and the enzyme will not be inhibited by the addition of cyanide. This enzyme can degrade cyanide without the addition of a co-enzyme and is homologous to the N-terminus of **cyanidase** (cyanide dihydratase) from *Bacillus pumilus*. The cyanide degrading enzyme catalyzes the conversion of cyanide to formate and ammonia (Watanabe *et al*., 1998). The cyanide dihydratase behaves as a true nitrilase due to the formation of ammonia and formate directly from cyanide (Sewell *et al*., 2003).

Certain *Pseudomonas* species contain **rhodaneses** (thiosulfate:cyanide sulfurtransferases) that can possibly aid in the detoxification of cyanide (Cipollone *et al*., 2007). These enzymes catalyze the conversion of cyanide to thiocyanate by transferring a sulfane sulfur atom from a suitable donor (thiosulfate) to cyanide. *Pseudomonas* species are capable of the degradation of metal-cyanide complexes (Baxter and Cummings, 2006). These metal-cyanide complexes include metals like copper, iron, zinc and silver. The enzyme involved in the degradation of these metal-cyanide complexes are **cyanide oxygenase** (Baxter and Cummings, 2006).

3.3. DNA pyrosequencing

3.3.1. Background

The biological sciences have been transformed due to the use of genome sequencing (Kartalov and Quake, 2004). Sanger sequencing was developed 25 years ago and has undergone relatively few changes since then, the most important being automation and an increased throughput. In recent years, Sanger sequencing has been replaced by new generation technologies for high-throughput genome sequencing. One of the most widely accepted next generation sequencing technologies is the pyrosequencing platform as developed by 454 and marketed by Roche Diagnostics. Reads of up to 1000 bases of sequence with a total of 1 Gb per run will be routine by the end of 2010.

This DNA sequencing technique (Figure 3.3.1) is based on detection of the released pyrophosphate (PPi) when a correct nucleotide is incorporated into DNA by the enzyme DNA polymerase. Due to an enzymatic cascade visible light is generated and the light produced is proportional to the number of nucleotides that are incorporated (Ronaghi, 2001). The enzymatic cascade starts with the incorporation of a nucleotide by DNA polymerase. The incorporation results in the release of inorganic PPi which is used to convert APS to ATP, catalyzed by the ATP-sulfurylase enzyme. The released ATP serves as energy source for luciferase which oxidizes the luciferin and light is generated. The nucleotide added is known, thus the sequence of the template can be determined. The light transmitted from the pyrosequencing reaction can be detected by a charge-coupled device camera.

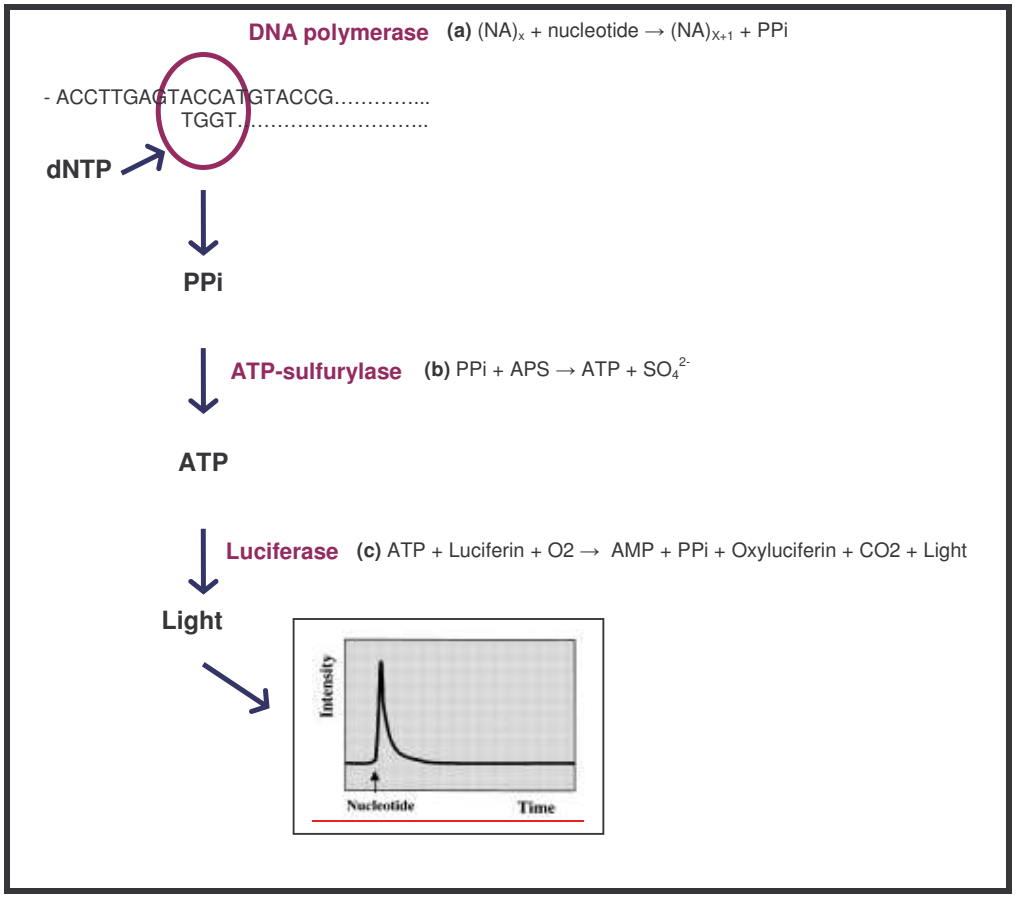

Figure 3.3.1: The enzymatic cascade involved in pyrosequencing. The enzymes involved and the corresponding catalyzed reactions are (a) DNA polymerase, (b) ATP-sulfurylase and (c) Luciferase. $(NA)_x$, nucleic acid chain and $(NA)_{x+1}$, one nucleotide added to the chain.

In this chapter a *Bacillus* sp. was subjected to high-throughput 454-pyrosequencing (GS FLX Titanium Series) at Inqaba Biotec™, Pretoria (SA). The DNA library was constructed and sequenced at Inqaba Biotec™. The assembled contigs were used for the construction of an internal BLAST database. The KEGG PATHWAY Database was employed for the identification of all enzymes involved in cyanide degradation as well as cyanogenesis. These enzymes were used in a BLAST analysis against the constructed internal database for the identification of enzymes involved in cyanide metabolism in the *Bacillus* sp. The complete coding sequences (CDS) of some of the identified enzymes were identified in the genome of the selected isolate and these enzymes were subjected to a BLAST analysis against the NCBI database. BLAST analyses were performed to confirm that the complete CDSs of all the identified enzymes were obtained and to verify the function of these enzymes.

3.4. Materials & Methods

3.4.1. Strain verification

3.4.1.1. Genomic deoxyribonucleic acid isolation

A glycerol stock of the *Bacillus* sp. B4H3 was inoculated into 100 ml of nutrient broth (Chapter 2, section 2.2.4.2) and cultivated at 30ºC while shaking (180 rpm) for 48 h. The genomic deoxyribonucleic acid (gDNA) was isolated using the method described by Labuschagne and Albertyn (2007) as discussed in Chapter 2, section 2.2.4.1. The concentration of the gDNA was determined using the NanoDrop Spectrophotometer ND-1000 (NanoDrop Technologies, Inc., USA).

3.4.2. High-throughput 454-pyrosequencing (GS FLX Titanium Series)

3.4.2.1. Library construction and pyrosequencing

The GS FLX Titanium Series produces 400-500 bp reads. The DNA library of the *Bacillus* sp. was constructed and sequenced at Inqaba Biotec, Pretoria, South Africa. The gDNA was fractionated into small fragments (300-800 bp) using standard protocols developed specifically for Roche 454 GS-FLX sequencer. Short adaptors were added to each fragment by using standard molecular biology techniques. The single-stranded fragments in combination with the added adaptors made up the library that was used in subsequent steps. The DNA library containing the single-stranded fragments was immobilized onto specifically designed DNA Capture Beads. The beads and the amplification reagents were emulsified in a water-in-oil mixture (emulsion PCR amplification). This resulted in one bead with one clonally amplified fragment attached to it. The emulsion PCR was broken while the amplified fragments remained bound to the specific bead. The fragments were loaded onto a Pico Titer Plate device where the diameter of the wells of the plate only allow one bead per well. Individual nucleotides flowed over the whole plate in a fixed order. The addition of a nucleotide, complementary to the fragment sequence, resulted in a chemiluminescent signal that was recorded by the CCD camera of the instrument. The *de novo* assembly was performed at Inqaba Biotec using the Newbler software (Roche Applied Science).

3.4.2.2. 16S rRNA gene sequence

The nucleotide sequence of the 16S rRNA gene (1 661 bp) present in the genome of the *Bacillus* sp. was used in an alignment against the 16S rRNA gene sequence obtained in Chapter 2, section 2.2.4.3 to verify that the same organism was used in the pyrosequencing analysis.

3.4.2.3. Specific primers to identify genes involved in cyanide degradation

The primers, for the specific cyanide degrading gene present in each of the three control organisms, were designed by aligning multiple sequences of the same cyanide degrading gene present in the same genus represented by the control organisms (Chapter 2, section 2.2.7). These primers (section 2.2.4.2) were used in a BLAST analysis against the constructed internal database (section 3.4.2.4).

3.4.2.4. Data analysis

A BLAST database of the nucleotide sequences of the assembled contigs was constructed using the formatdb command as described in the BLAST manual. The KEGG PATHWAY Database (Kyoto Encyclopedia of Genes and Genomes) was employed to identify enzymes involved in cyanide metabolism in a wide variety of microorganisms. The enzymes identified in the KEGG PATHWAY Database were used in a BLAST search (tblastn) against the constructed nucleotide database. The assembled sequence was loaded into Artemis (Wellcome Trust Sanger Institute). Artemis, a free genome viewer, was used to view the genome data, and to identify putative coding sequences. The amino acid sequences of the enzymes that were identified in the constructed database were used in Artemis to obtain the coding sequences (CDSs) of the specific enzymes. The amino acid sequences of the CDSs were used in a BLAST search (blastp) on the NCBI server to confirm the function of the identified enzymes and to ensure that the complete coding sequence was obtained.

3.5. Results & Discussion

3.5.1. Strain verification

3.5.1.1. Genomic deoxyribonucleic acid isolation

The gDNA isolated (section 3.4.1.1) from the *Bacillus* sp. was loaded onto a 1% agarose gel and visualized under the GelDoc XR (Bio-Rad). Figure 3.5.1 depicts the pure and intact gDNA isolated from this microorganism.

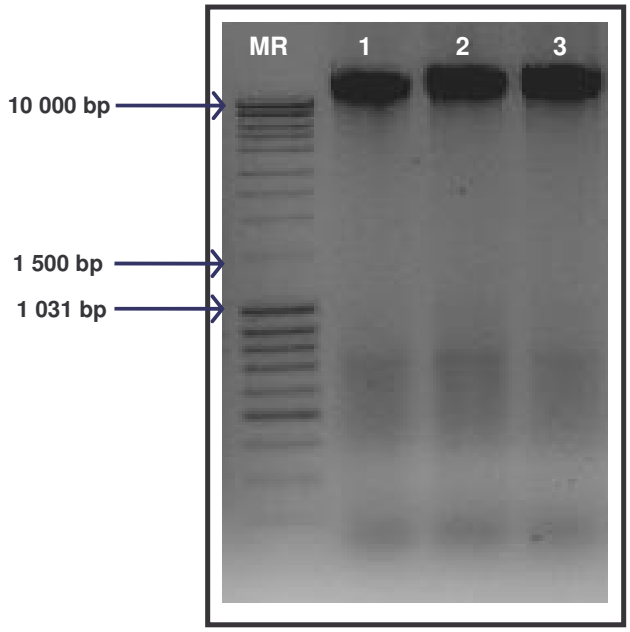

Figure 3.5.1: gDNA isolated from the *Bacillus* sp. Lane 1, 2 and 3, *Bacillus* sp. and MR, MassRuler^{TM} DNA Ladder (Fermentas).

The concentrations (in triplicate) of the isolated gDNA (ng.µl^{-1}) from the *Bacillus* sp. ranged from 1 000 – 2 200 ng.µl^{-1}.

3.5.2. High-throughput 454-pyrosequencing (GS FLX Titanium Series)

3.5.2.1. Newbler Metrics Results

The gDNA (section 3.4.1.1) of the *Bacillus* sp. was sequenced at Inqaba Biotec™ (section 3.4.2) and the specifications for the *de novo* assembled data are presented in Table 3.5.1.

Table 3.5.1: Specifications of the d*e novo* assembled data obtained from Inqaba Biotec™.

Read status	
Number of reads	197 511
Number of bases	60 155 359
Number of aligned reads	191 998
Number of aligned bases	57 197 128
Inferred read error	1.06%
Large contig metrics	
Number of contigs	1 262
Number of bases	6 152 220
Average contig size	4 874
N50 contig size	8 382
Largest contig size	56 062
All contig metrics	
Number of contigs	1 450
Number of bases	6 203 737

The total number of bases sequenced was ~60 Mb and the total contigs obtained were ~1 300. The average contig size was ~4 900 bp and the largest contig comprised ~56 000 bp. The genome is approximately 6.15 Mb large. Genome sizes of other *Bacillus* species are listed in Table 3.5.2. The Newbler assembled data was used to construct an internal BLAST database.

Table 3.5.2: Genome size of various *Bacillus* species.

Species	Genome size
Bacillus coahuilensis (Alcaraz *et al*., 2008)	3.35 Mb (smallest *Bacillus* sp. genome)
Bacillus subtilis (Trevors, 1996)	4.1 Mb
Bacillus halodurans (Takami *et al*., 2000)	4.2 Mb
Bacillus licheniformis (Rey *et al*., 2004)	4.2 Mb
Bacillus megaterium (Trevors, 1996)	4.6 Mb
*Bacillus thuringiensis (*Alcaraz *et al*., 2008)	5.2 Mb
Bacillus anthracis (Radnedge *et al*., 2003)	5.23 Mb
Bacillus sp. B4H3	6.15 Mb

The genome size of the *Bacillus* sp. used in this study is slightly larger in comparison with the genome sizes of other *Bacillus* species. This can be due to either gaps or repeat sequences in the sequencing data. The pyrosequencing data needs to be analyzed completely to ensure that the whole genome was indeed sequenced.

3.5.2.2. 16S rRNA gene sequence

The nucleotide sequence of the 16S rRNA gene obtained in Chapter 2, section 2.3.2.7 was used in a BLAST analysis against the constructed internal database to ensure that the correct organism was used in the pyrosequencing experiment (Table 3.5.4). The 16S rRNA gene sequence from Chapter 2, section 2.3.2.7 was not present in the genome sequence data obtained from the pyrosequencing analysis. The 16S rRNA gene present in the genome of the *Bacillus* sp. was subjected to a BLAST analysis (Table 3.5.3) against the NCBI database to determine the identity of the organism used in the pyrosequencing analysis.

Table 3.5.3: The BLAST analysis results obtained from the 16S rRNA gene sequences of the *Bacillus* sp. selected for pyrosequencing.

Number	Accession number	Organism	Query coverage (%)	E-value	Maximum identity (%)
1	FJ009378.1	*Bacillus cereus* strain G10 16S ribosomal RNA gene, partial sequence	100	0.0	99
2	AB363736.1	*Bacillus nealsonii* gene for 16S rRNA, partial sequence, strain: NBRC 100169	100	0.0	99
3	EF488087.1	*Bacillus anthracis* strain QD232 16S ribosomal RNA gene, partial sequence	99	0.0	99

The BLAST results indicated that the organism that was subjected to pyrosequencing was likely a *Bacillus cereus*. Due to the close relationship of various *Bacillus* species towards each other, species of this genus can not be distinguished with the use of universal bacterial primers (Chapter 2, section 2.2.4.3). Although the organism that was subjected to the pyrosequencing analysis was not the *Bacillus marisflavi* identified in Chapter 2, section Table 2.3.4, the results obtained from the genome sequence data revealed interesting enzymes involved in the cyanide metabolism of the *Bacillus* sp.

The 16S rRNA gene present in the genome of the *Bacillus* sp. was situated on contig 01513 (Table 3.5.4) and produced a maximum identity of 99%.

Table 3.5.4: BLAST results obtained for the 16S rRNA gene of the *Bacillus* sp. against the constructed internal database.

Contig	E-value	Maximum identity (%)
01513	0.0	99
01169	1.7	2
01045	1.7	2
01179	6.7	1
00567	6.7	1
00216	6.7	1

3.5.2.3. Specific primers to identify genes involved in cyanide degradation

The primers (Chapter 2, section 2.2.7) used for screening in Chapter 2, section 2.3.5 were subjected to a tblastn analysis against the constructed internal database. The results obtained from the BLAST analysis indicated that the primers were not specific (E-value = 0.0) towards the amino acid sequences present in the genome of the *Bacillus* sp. that was used in the pyrosequencing analysis. The primers annealed non-specifically to various contigs indicating that non-specific product formation will occur. It can be hypothesized that the multiple bands observed on the 1% agarose gels in Chapter 2, section 2.3.5 are due to non-specific product formation.

3.5.2.4. The KEGG PATHWAY Database

The KEGG PATHWAY database (section 3.4.2.4) was used to obtain all enzymes involved in cyanogenesis as well as cyanide degradation reactions. The enzymes were identified from six established metabolic pathways present in microorganisms as well as plant species and are represented by their Enzyme Commission numbers (EC numbers). The six metabolic pathways used as reference were i) Cyanoamino acid metabolism (Entry: ko00460), ii) Nitrogen metabolism: reduction and fixation (Entry: ko00910), iii) Glycine, serine and threonine metabolism (Entry: ko00260), iv) Glyoxylate and dicarboxylate metabolism (Entry: ko00630), v) Carbon fixation metabolism (Entry: ko00710) and vi) Methane metabolism (Entry: ko00680). The metabolic pathways are shown in Appendix A.

3.5.2.5. BLAST search: Enzymes identified in KEGG PATHWAY database

The amino acid sequences of enzymes involved in cyanogenesis and cyanide degradation identified by employing the KEGG PATHWAY database were downloaded from the Universal Protein Resource (UniProt) database and were used in a BLAST search against the constructed internal database to identify the enzymes present in the *Bacillus* sp. involved in the cyanide metabolism. The enzymes that produced significant hits with the assembled contigs were used in a NCBI BLAST search and are summarized in Table 3.5.5 – Table 3.5.10.

Table 3.5.5 (a): Enzymes possibly involved in cyanoamino acid metabolism of the *Bacillus* sp. as identified using the KEGG database.

EC number	Name and Reaction catalysed	BLAST result (Internal database)	E-value	Accession number (NCBI)	Highest BLAST result (NCBI)	E-value
1.4.99.5	**hydrogen cyanide synthase (HcnC)** glycine + 2 acceptor ↔ hydrogen cyanide + CO_2 + 2 reduced acceptor	contig 00553 contig01199	4e-37 6e-26	YP_259686.1	hydrogen cyanide synthase HcnC [*Pseudomonas fluorescens* Pf-5]	0.0
2.3.2.2	**γ-glutamyl transferase/peptidase** 3-cyano-L-alanine + L-glutamate ↔ γ-glutamyl-β-cyano- alanine + H_2O	contig00258 contig00409	2.1 3.7	-	-	-
2.5.1.47	**cysteine synthase** O_3-acetyl-L-serine + hydrogen sulfide ↔ L-cysteine + acetate	contig00507 contig01117	e-141 3e-99	ZP_04298522.1	Cysteine synthase [*Bacillus cereus* MM3]	2e-172
3.5.5.1	**nitrilase** nitrile + $2H_2O$ ↔ carboxylate + NH_3	contig01211 contig01059	3.2 3.2	-	-	-
3.5.5.4	**cyano-alanine nitrilase** 3-cyano-L-alanine + $2H_2O$ ↔ L-aspartate + NH_3	contig00039	6.2	-	-	-
4.1.2.10	**mandelonitrile lyase** mandelonitrile ↔ cyanide + benzaldehyde	- -	- -	-	-	-
4.1.2.11	**hydroxymandelonitrile lyase** (S)-4-hydroxymandelonitrile ↔ cyanide + 4-hydroxybenzaldehyde	- -	- -	-	-	-
4.1.2.37	**hydroxynitrilase** acetone cyanohydrin ↔ cyanide + acetone	- -	- -	-	-	-
4.2.1.65	**3-cyano-alanine hydratase** L-asparagine ↔ 3-cyano-L-alanine + H_2O	- -	- -	-	-	-

Table 3.5.5 (b): Enzymes possibly involved in cyanoamino acid metabolism of the *Bacillus* sp. as identified using the KEGG database (continue).

EC number	Name and Reaction catalysed	BLAST result (Internal database)	E-value	Accession number (NCBI)	Highest BLAST result (NCBI)	E-value
4.2.1.66	**cyanide hydratase/formamide dehydratase** formamide ↔ hydrogen cyanide + H_2O	contig00039	2e-66	ZP_04235304.1	Carbon-nitrogen hydrolase [*Bacillus cereus* Rock3-28]	7e-145
		contig01105	0.34			
4.4.1.9	**L-3-cyanoalanine synthase/cysteine synthase** L-cysteine + hydrogen cyanide ↔ hydrogen sulfide + 3-cyano-L-alanine	contig00507	e-139	ZP_04298522.1	Cysteine synthase [*Bacillus cereus* MM3]	2e-172
		contig01117	e-100			

Table 3.5.6: Enzymes possibly involved in the nitrogen metabolism of the *Bacillus* sp. as identified using the KEGG database.

EC number	Name and Reaction catalysed	BLAST result (Internal database)	E-value	Accession number (NCBI)	Highest BLAST result	E-value
2.7.2.2	**carbamate kinase** $ATP + NH_3 + CO_2 \leftrightarrow ADP + carbamoyl\ phosphate$	contig00200	2e-85	ZP_04229542.1	Acetylglutamate kinase [*Bacillus cereus* Rock3-29]	9e-76
		contig00056	3e-04			
3.5.1.49	**formamidase** $formamide + H_2O \leftrightarrow formate + NH_3$	contig00039	3e-08	ZP_04235304.1	Carbon-nitrogen hydrolase [*Bacillus cereus* Rock3-28]	7e-145
		contig00658	5.3			
4.2.1.1	**carbonate dihydratase/carbonic anhydrase** $H_2CO_3 \leftrightarrow CO_2 + H_2O$	contig00414	5e-17	YP_003654533.1	carbonate dehydratase [*Arcobacter nitrofigilis* DSM 7299]	2e-36
		contig00476	4.5			
4.2.1.104	**cyanase/cyanate hydratase** $cyanate + HCO_3^- + 2H^+ \leftrightarrow NH_3 + 2CO_2$	contig00854	2e-32	YP_002448650.1	cyanate hydratase [*Bacillus cereus* G9842]	9e-81
		contig01251	5.6			
6.3.4.16	**carbomoyl-phosphate synthase** $2ATP + NH_3 + CO_2 + H_2O \leftrightarrow 2ADP + phosphate + carbamoyl\ phosphate$	contig00866	e-126	ZP_04187821.1	Biotin carboxylase [*Bacillus cereus* AH1271]	0.0
		contig00340	e-113			

Table 3.5.7: Enzymes possibly involved in the glycine, serine and threonine metabolism of the *Bacillus* sp. as identified using the KEGG database.

EC number	Name and Reaction catalysed	BLAST result (Internal database)	E-value	Accession number (NCBI)	Highest BLAST result	E-value
1.4.2.1	**glycine dehydrogenase** glycine + H_2O + 2ferricytochrome c ↔ glyoxylate + NH_3 + 2ferrocytochrome c + $2H^+$	- -	- -	-	-	-
1.4.3.3	**D-amino-acid oxidase** D-amino acid + H_2O + O_2 = 2-oxo acid + NH_3 + H_2O_2	contig00553 contig00541	0.0 2e-17	ZP_04226369.1	Glycine oxidase ThiO [*Bacillus cereus* Rock3-29]	0.0
2.1.2.1	**glycine hydroxymethyl transferase** 5,10-methylenetetrahydrofolate + glycine + H_2O = tetrahydrofolate +L-serine	contig00365 contig01477	0.0 e-149	ZP_04303486.1	Serine hydroxymethyltransferase [*Bacillus cereus* MM3]	0.0
2.6.1.4	**glycine transaminase** glycine + 2-oxoglutarate = glyoxylate + L-glutamate	contig00596 contig00091	6e-17 9e-10	-	-	-
2.6.1.44	**alanine glyoxylate transaminase** L-alanine + glyoxylate = pyruvate + glycine	contig00999 contig00325	1e-53 1e-52	ZP_03107439.1	4-aminobutyrate transaminase [*Bacillus cereus* NVH0597-99]	0.0

Table 3.5.8 (a): Enzymes possibly involved in the glyoxylate and dicarboxylate metabolism of the *Bacillus* sp. as identified using the KEGG database.

EC number	Name and Reaction catalysed	BLAST result (Internal database)	E-value	Accession number (NCBI)	Highest BLAST result	E-value
1.1.1.60	**2-hydroxy-3-oxopropionate reductase** (R)-glycerate + $NAD(P)^+$ = 2-hydroxy-3-oxopropanoate + NAD(P)H + H^+	contig01091 contig00963	7e-49 5e-46	ZP_00236963.1	2-hydroxy-3-oxopropionate reductase [*Bacillus cereus* G9241]	7e-166
1.2.1.2	**formate dehydrogenase** formate + NAD^+ = CO_2 + NADH	contig00442 contig00320	0.0 0.0	ZP_04184506.1	formate dehydrogenase [*Bacillus cereus* AH1271]	0.0
1.2.2.1	**formate dehydrogenase** formate + 2ferricytochrome b1 = CO_2 + 2ferrocytochrome b1 + $2H^+$	contig00075 contig00381	3e-27 4e-23	ZP_04299718.1	2-hydroxyacid dehydrogenase [*Bacillus cereus* MM3]	1e-171
1.2.3.4	**oxalate oxidase** oxalate + O_2 + $2H^+$ = $2CO_2$ + H_2O_2	- -	- -	-	-	-
1.2.3.5	**glyoxylate oxidase** glyoxylate + H_2O + O_2 = oxalate + H_2O_2	contig00459 contig00815	e-132 1e-37	ZP_04174566.1	2,3-dihydroxybenzoate-AMP ligase [*Bacillus cereus* AH1273]	0.0
2.7.1.31	**glycerate kinase** ATP + (R)-glycerate = ADP + 3-phospho-(R)-glycerate	contig00366 contig00044	e-119 3e-83	ZP_04298604.1	Glycerate kinase [*Bacillus cereus* MM3]	0.0
2.8.3.2	**oxalate-CoA transferase** succinyl-CoA + oxalate = succinate + oxalyl-CoA	- -	- -	-	-	-
3.1.2.10	**formyl-CoA hydrolase** formyl-CoA + H_2O = CoA + formate	- -	- -	-	-	-
4.1.1.2	**oxalate decarboxylase** oxalate + H^+ = formate + CO_2	- -	- -	-	-	-

Table 3.5.8 (b): Enzymes possibly involved in the glyoxylate and dicarboxylate metabolism of the *Bacillus* sp. as identified using the KEGG database (continue).

EC number	Name and Reaction catalysed	BLAST result (Internal database)	E-value	Accession number (NCBI)	Highest BLAST result	E-value
4.1.1.8	**oxylyl-CoA decarboxylase** oxalyl-CoA = formyl-CoA + CO_2	contig00470	1e-43	ZP_04299217.1	Acetolactate synthase [*Bacillus cereus* MM3]	0.0
		contig00305	1e-37			
4.1.1.47	**tartranate-semialdehyde synthase** 2 glyoxylate = tartronate semialdehyde + CO_2	contig00305	7e-84	ZP_04299700.1	hypothetical protein bcere0006_12490 [*Bacillus cereus* MM3]	0.0
		contig00089	1e-78			
6.2.1.8	**oxalyl-CoA synthatase/oxalate-CoA ligase** ATP + oxalate + CoA = AMP + diphosphate + oxalyl-CoA	-	-	-	-	-

Table 3.5.9: Enzyme possibly involved in the carbon fixation metabolism of the *Bacillus* sp. as identified using the KEGG database.

EC number	Name and Reaction catalysed	BLAST result (Internal database)	E-value	Accession number (NCBI)	Highest BLAST result	E-value
2.7.2.3	**phosphoglycerate kinase** ATP + 3-phospho-D-glycerate = ADP + 3-phospho-D-glyceroyl phosphate	contig00491	0.0	YP_001647706.1	phosphoglycerate kinase [*Bacillus weihenstephanensis* KBAB4]	0.0
		contig01494	0.78			

Table 3.5.10: Enzymes possibly involved in the methane metabolism of the *Bacillus* sp. as identified using the KEGG database.

EC number	Name and Reaction catalysed	BLAST result (Internal database)	E-value	Accession number (NCBI)	Highest BLAST result	E-value
1.2.1.2	**formate dehydrogenase (NADH dependant)** formate + 2ferricytochrome b1 = CO_2 + 2ferrocytochrome b1 + $2H^+$	contig00442 contig00320	0.0 0.0	ZP_04184506.1	formate dehydrogenase [*Bacillus cereus* AH1271]	0.0
1.2.1.43	**formate dehydrogenase (NADPH dependant)** formate + $NADP^+$ = CO_2 + NADPH	contig00442 contig00320	e-143 1e-83	ZP_04184506.1	formate dehydrogenase [*Bacillus cereus* AH1271]	0.0
1.2.7.4	**carbon monoxide dehydrogenase** CO + H_2O + 2 oxidized ferredoxin = CO_2 + 2 reduced ferredoxin + $2H^+$	contig01088	4.3	-	-	-
1.2.99.2	**carbon monoxide dehydrogenase/acetyl-CoA synthase** CO + H_2O + Acceptor ↔ CO_2 + Reduced acceptor	contig00548 contig00524	0.87 1.1	-	-	-
1.12.1.2	**hydrogen dehydrogenase** H_2 + NAD^+ = H^+ + NADH	contig00378 contig00401	5.1 6.7	-	-	-
1.12.7.2	**ferrodoxin hydrogenase** H_2 + 2 oxidized ferredoxin = 2 reduced ferredoxin + $2H^+$	contig00442 contig0020	1e-20 1e-20	ZP_04146788.1	formate dehydrogenase [*Bacillus thuringiensis*]	0.0

The BLAST results confirmed the existence of a complete cyanide degradation pathway in the selected *Bacillus* sp. The complete amino acid sequences of the enzymes are given in appendix B.

At least five enzymes had multiple forms of the gene present (Table 3.5.11).

Table 3.5.11: Multiple forms of the five identified genes.

EC number	Enzyme	Number	Different forms	Contig	Coding sequence
1.2.1.2	formate dehydrogenase	1	1.2.1.2_2_aa	00320	1 497 217
		2	1.2.1.2_4_aa	00442	2 122 022
2.1.2.1	glycine hydroxymethyl transferase	3	2.1.2.1_aa	00365	1 728 904
		4	2.1.2.1_2_aa	01477	6 071 450
2.5.1.47	cysteine synthase	5	2.5.1.47_aa	00507	2 446 602
		6	2.5.1.47_3_aa	00780	3 688 226
		7	2.5.1.47_4_aa	00089	485 145
4.2.1.66	cyanide hydratase/formamide dehydratase	8	4.2.1.66_aa	00160	837 920
		9	4.2.1.66_2_aa	00039	226 339
4.4.1.9	L-3-cyano-alanine synthase	5	4.4.1.9_aa	00507	2 446 602
		6	4.4.1.9_3_aa	00780	3 688 226
		10	4.4.1.9_4_aa	00091	501 082
		11	4.4.1.9_5_aa	00322	1 526 615

* EC 2.5.1.47_aa and EC 4.4.1.9_aa (highlighted in pink) are the same enzyme as well as EC 2.5.1.47_3_aa and EC 4.4.1.9_3_aa (highlighted in grey). The numerical numbers after the underscore indicate a different CDS in a different contig if the amino acid sequences were used in a BLAST search against the internal database.

In order to verify that these were not artifacts brought about by the assembly of the sequence data, the context in which the genes were found was checked in Artemis.

Figure 3.5.2 illustrates the positions and different flanking coding sequences of the eleven genes on the different contigs ruling out the possibility of assembly artifacts. The presence of different forms of some enzymes in different contexts in the genome is an indication that these genes may be crucial in the cyanide resistance and metabolism of the organism.

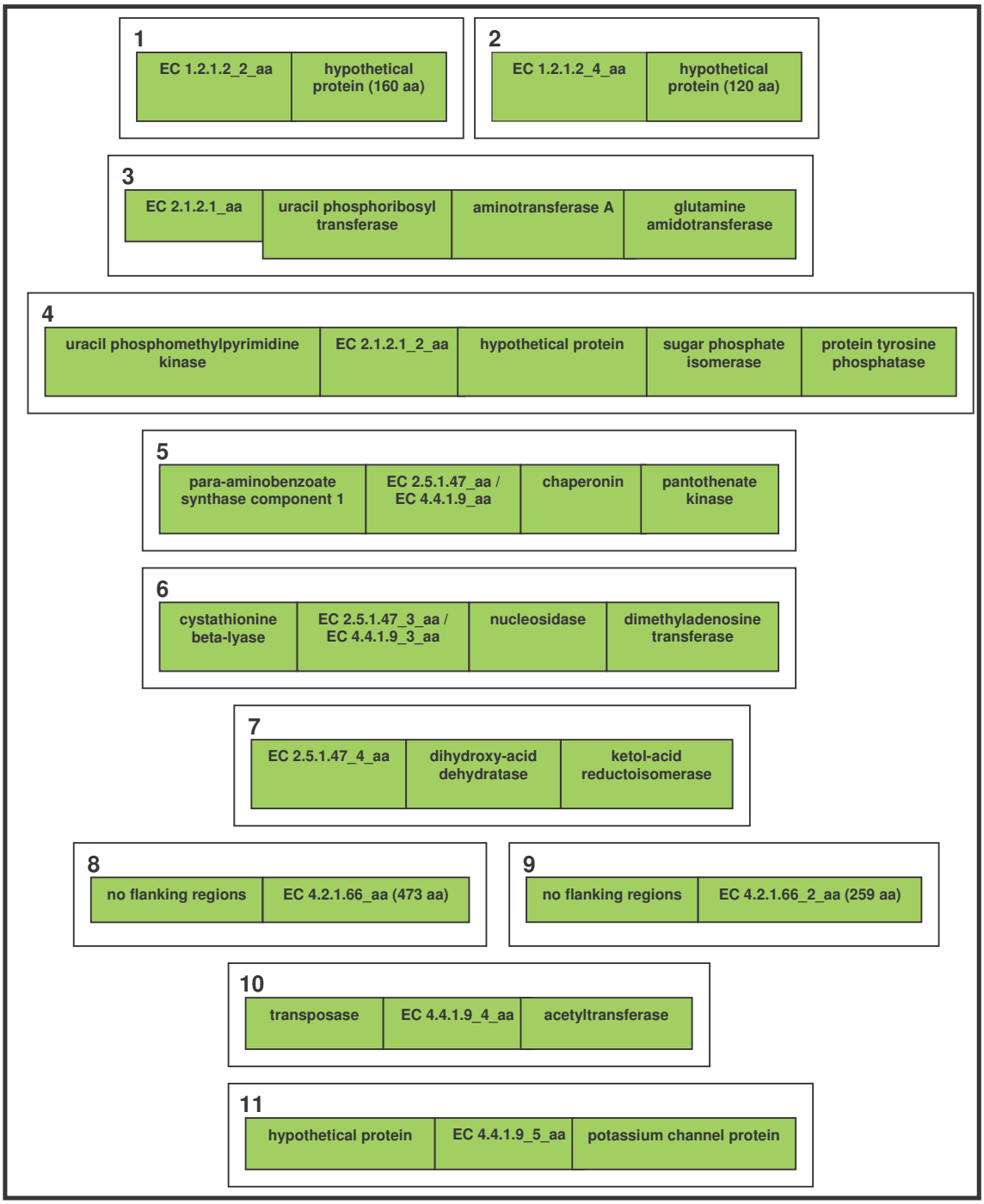

Figure 3.5.2: Different flanking regions of the genes which are represented by more than one coding sequence.

Each identified protein was used to search the non redundant (nr) database using BLAST. The highest scoring hit for each query protein was compared to its homologue in the genome of the *Bacillus* sp. (Appendix B).

The five enzymes (highlighted in yellow in Figure 3.5.3) with multiple forms (EC 1.2.1.2; EC 2.1.2.1; EC 2.5.1.47; EC 4.2.1.66 and EC 4.4.1.9) were aligned (Appendix B) using the European Bioinformatics Institute (EMI) tool namely ClustalW2. From the aligned data it can be concluded that the multiple forms of a specific gene are not duplications of that gene. The multiple forms are also not shorter versions of the same enzymes.

A detailed metabolic pathway was derived for *Bacillus* sp. using the identified enzymes (Figure 3.5.3). Interestingly, this isolate possesses the classical *Bacillus* as well as the *Pseudomonas* reactions for cyanide degradation. The nitrogen pathway proceeds through the degradation of cyanide to yield formamide. Formamide will in return be degraded to formate and ammonia which lead to the utilization of cyanide as the sole nitrogen source. The single carbon utilized as the sole carbon source by this organism is based on the hypothesis that the reaction of cyanide to glycine (red block in Figure 3.5.3) is reversible. Glycine will then be converted to glyoxylate which will enter the glyoxylate and dicarboxylate metabolism. After the conversion of 3-phospho-d-glycerate to 1,3-bisphosphoglycerate the organism will continue in the carbon fixation metabolism.

The five enzymes described in Figure 3.5.2 are highlighted in yellow in Figure 3.5.3. These enzymes can be involved in cyanide degradation but can exhibit additional functions which can possibly aid in the detoxification of cyanide at high concentrations (>2.5 M).

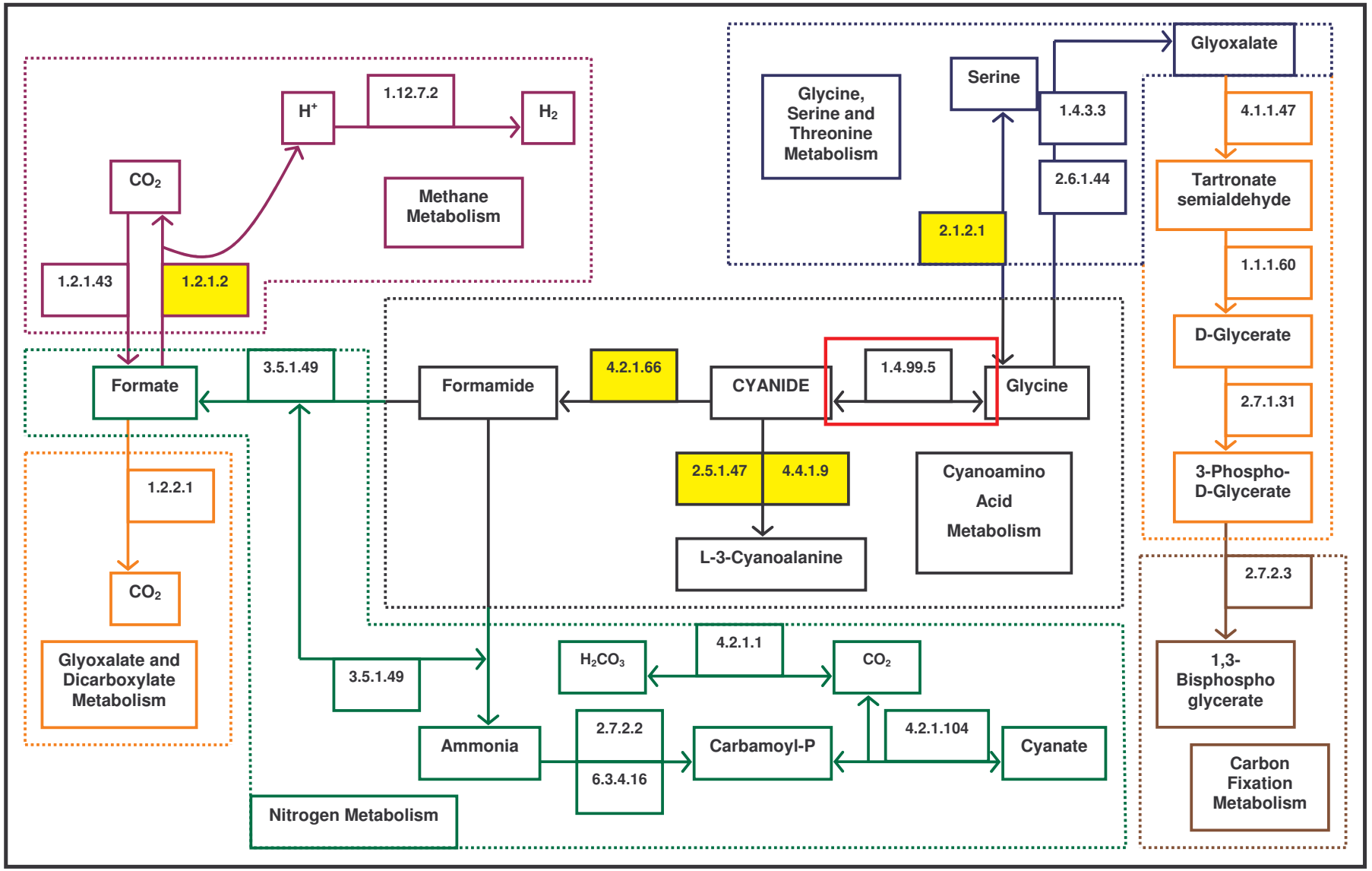

Figure 3.5.3: The detailed cyanide metabolic pathway derived from pyrosequencing results for the *Bacillus* sp. B4H3.

The acquisition of the *Pseudomonas* type of cyanide metabolism could have occurred by horizontal gene transfer (Alcaraz *et al*., 2008; Kidane *et al*., 2009). This should however be confirmed using appropriate bio-informatics analysis.

3.6. Conclusions

Initially the *Bacillus* sp. B4H3 was selected based on the results obtained in Chapter 2. These results included the MIC determination (Chapter 2, section 2.3.2.8), Sanger sequencing results (Chapter 2, section 2.3.2.7), tempo of cyanide degradation (Chapter 2, section 2.3.6.2) as well as the current knowledge obtained from literature stating that *Bacillus* and *Pseudomonas* species are commonly and successfully employed in bioremediation strategies at cyanide contaminated sites.

Genomic DNA was isolated from the *Bacillus* sp. and sent to InqabaBiotec™ (SA) for pyrosequencing analysis. The *de novo* assembly (section 3.4.2.1) was performed at InqabaBiotec™ using the Newbler software (Roche Applied Science). An internal BLAST database was constructed (section 3.4.2.4). The 16S rRNA gene present in the genome was different than the 16S rRNA gene sequence obtained in Chapet 2, section 2.3.2.7. It was decided to continue with the analysis of the genome. All the enzymes involved in either cyanide degradation or cyanogenesis were identified using the KEGG PATHWAY database. The identified enzymes were employed in a tblastn search against the constructed internal database. This BLAST search identified putative enzymes present in the *Bacillus* sp. genome. Artemis was employed to obtain the complete CDS of the enzymes present in the *Bacillus* sp. involved in the cyanide metabolism. The CDS was used in a blastp search on the NCBI server and the highest hit of each identified enzyme was aligned against the enzyme to ensure that the correct enzymes were identified. The identified enzymes were used to construct a possible cyanide degradation pathway metabolism that is present in this isolate (section Figure 3.5.3).

The specifically designed primers (Chapter 2, section 2.2.7) were subjected to a BLAST search against the internal constructed database (section 3.4.2.4). The results obtained can possibly explain the multiple bands observed on the 1% agarose gels in Chapter 2, section 2.3.5. This can indicate that the primers are not specific enough or that the selected isolate does not contain one of the three genes that were screened for.

A total of twenty one enzymes (complete CDS obtained) were identified in the *Bacillus* sp. (summarized in Figure 3.5.3). Five (EC 1.2.1.2; EC 2.1.2.1; EC 2.5.1.47; EC 4.2.1.6 and EC 4.4.1.9) of the total number of enzymes displayed more than one complete CDS in the genome of the isolate. To ensure that these genes were not duplications present in the genome or that the occurrence of the multiple forms is not an effect of sequencing errors, the multiple forms were aligned (Appendix B) against the highest hit obtained, for each enzyme, from the NCBI database. The alignments between each enzyme and the highest hit indicated that each enzyme is indeed a different form and can indicate that these enzymes have additional functions in the metabolism of cyanide. It is known that formate dehydrogenase (EC 1.2.1.2) can aid in the degradation of cyanide and possibly assist in detoxification of harmful compounds.

The pyrosequencing results indicated that the *Bacillus* sp. could utilize the single nitrogen through the conversion of cyanide to formamide which in turn can be converted to ammonia. The possible utilization of the single carbon is based upon a hypothesis that the reaction between cyanide and glycine is reversible (red block in Figure 3.5.3). This will lead to the carbon fixation metabolism which will prove that the isolate is capable of utilizing the single carbon as the sole carbon source.

Future research will include growth studies with the supplementation of NaCN as the sole carbon and nitrogen source as well as the supplementation of glycine as the sole carbon source to prove that the conversion of cyanide to glycine is reversible. The metabolism of cyanide can be annotated and the pyrosequencing results can be further analyzed to ensure that the whole genome of this *Bacillia* sp. is known. The *Bacillus* sp. can also be sent for species identification to DSMZ in Germany.

3.7. References

- **Ahmadian, A., Lundeberg, J., Nyren, P., Uhlen, M and Ronaghi, M.** (2000b) Analysis of the p53 Tumor Suppressor Gene by Pyrosequencing. *BioTechniques* **28:** 140-144.

- **Alcaraz, L.D., Olmedo, G., Bonilla, G., Cerritos, R., Hernández, G., Cruz, A., Ramírez, E., Putonti, C., Jiménez, B., Martínez, E., López, V., Arvizu, J.L., Ayala, F., Razo, F., Caballero, J., Siefert, J., Eguiarte, L., Vielle, J.P., Martínez, O., Souza, V., Herrera-Estrella, A. and Herrera-Estrella, L.** (2008) The Genome of *Bacillus coahuilensis* Reveals Adaptations Essential for Survival in the Relic of an Ancient Marine Environment. *Proceedings of the National Academy of Sciences* **105:** 5803-5808.

- **Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D.J.** (1997) Gapped BLAST and PSI_BLAST: A New Generation of Protein Database Search Programs. *Nucleic Acids Research* **25:** 3389-3402.

- **Babu, G.R.V., Vijaya, O.K., Ross, V.L., Wolffram, J.H. and Chapatwala, K.D.** (1996) Cell-free Extract(s) of *Pseudomonas putida* Catalyze the Conversion of Cyanides, Cyanates, Thiocyanates, Formamide, and Cyanide-containing Mine Waters into Ammonia. *Applied Microbiology and Biotechnology* **45:** 273-277.

- **Baxter, J. and Cummings, S.P.** (2006) The Current and Future Applications of Microorganisms in the Bioremediation of Cyanide Contamination. *Antonie van Leeuwenhoek* **90:** 1-17.

- **Burri, R. and Stutzer, A.** (1895) Über Nitrat Zerstörende Bakterien und den durch dieselben bedingten Stickstoffverlust. Zentrbl Bakteriol Parasitenkd II Abt. **1:** 350-367.

- **Cipollone, R., Frangipani, E., Tiburzi, F., Imperi, F., Ascenzi, P. and Visca, P.** (2007) Involvement of *Pseudomonas aeruginosa* Rhodanese in Protection from Cyanide Toxicity. *Applied and Environmental Microbiology* **73:** 390-398.

- **Dash, R.R., Gaur, A. and Balomajumder, C.** (2009) Cyanide in Industrial Wastewaters and Its Removal: A Review on Biotreatment. *Journal of Hazardous Materials* **163:** 1-11.

- **Fernandez, R.F. and Kunz, D.A.** (2005) Bacterial Cyanide Oxygenase is a Suite of Enzymes Catalyzing the Scavenging and Adventitious Utilization of Cyanide as a Nitrogenous Growth Substrate. *Journal of Bacteriology* **187:** 6396-6402.

- **Garcia, A.C., Ahamdian, A., Gharizadeh, B., Lundeberg, J., Ronaghi, M. and Nyren, P.** (2000) Mutation Detection by Pyrosequencing: Sequencing of Exons 5 to 8 of the p53 Tumour Suppressor Gene. *Gene* **253:** 249-257.

- **Gupta, N., Balomajumder, C. and Agarwal, V.K.** (2010) Enzymatic Mechanism and Biochemistry for Cyanide Degradation: A Review. *Journal of Hazardous Materials* **176:** 1-13.

- **Hanahan, D.** (1983) Studies on Transformation of *Esherichia coli* with Plasmids. *Journal of Molecular Biology* **166:** 557-580.

- **Harris, R. and Knowles, C.J.** (1983) Isolation and Growth of a *Pseudomonas* species That Utilizes Cyanide as a Source of Nitrogen. *Journal of General Microbiology* **20:** 337-341.

- **Jandhyala, D., Berman, M., Meyers, P.R., Sewell, B.T., Willson, R.C.and Benedik, M.J.** (2003) *Cyn*D, the Cyanide Dihydratase from *Bacillus pumilus*: Gene Cloning and Structural Studies. *Applied and Environmental Microbiology* **69:** 4794-4805.

- **Kartalov, E.P. and Quake, S.R.** (2004) Microfluidic Device Reads Up to Four Consecutive Base Pairs in DNA Sequencing-by-Synthesis. *Nucleic Acids Research* **32:** 2873-2879.

- **Kidane, D., Carrasco, B., Manfredi, C., Rothmaier, K., Ayora, S., Tadesse, S., Alonso, J.C. and Graumann, P.L.** (2009) Evidence for Different Pathways during Horizontal Gene Transfer in Competent *Bacillus subtilis* Cells. *PLoS Genetics* **5:** 1-13.

- **Kunz, D.A., Nagappan, O., Silva-Avalos, J. And Delong, G.T.** (1992) Utilization of Cyanide as a Nitrogenous Substrate by *Pseudomonas fluorescens* NCIMB 11764: Evidence for Multiple Pathways of Metabolic Conversion. *Applied and Environmental Microbiology* **58:** 2022-2029.

- **Kunz, D.A., Wang, C.S. and Chen, J.L.** (1994) Alternative Routes of Enzymatic Cyanide Metabolism in *Pseudomonas fluorescens* NCIMB 11764. *Microbiology* **140:** 1705-1712.

- **Kunz, D.A., Chen, J.L. and Pan, G.** (1998) Accumulation of α-Keto Acids as Essential Components in Cyanide Assimilation by *Pseudomonas fluorescens* NCIMB 11764. *Applied and Environmental Microbiology* **64:** 4452-4459.

- **Labuschagne, M. and Albertyn, J.** (2007) Cloning of an Epoxide Hydrolase-Encoding Gene from *Rhodotorula mucilaginosa* and Functional Expression in *Yarrowia lipolytica*. *Yeast* **24:** 69-78.

- **Lane, D. J.** (1991) 16S / 23S Sequencing. In E. Stackebrandt & M. Goodfellow (ed.), Nucleic Acid Techniques in Bacterial Systematics. p. 115-175. John Wiley and Sons, New York.

- **Meyers, P.R., Gokool, P., Rawlings, D.E. and Woods, D.R.** (1991) An Efficient Cyanide-Degrading *Bacillus pumilus* strain. *Journal of General Microbiology* **137:** 1397-1400.

- **Palleroni, N.J.** (1984) *Pseudomonadaceae*. *In:* Bergey's Manual of Systematic Biology, Kreig, N.R. and Holt, J.G. (eds.) The Williams and Wilkens Co., Baltimore, 141-199.

- **Radnedge, L., Agron, P.G., Hill, K.K., Jackson, P.J., Ticknor, L.O., Keim, P. and Andersen, G.L.** (2003) Genome Differences that Distinguish *Bacillus anthracis* from *Bacillus cereus* and *Bacillus thurengiensis*. *Applied and Environmental Microbiology* **69:** 2755-2764.

- **Rey, M.W., Ramaiya, P., Nelson, B.A., Brody-Karpin, S.D., Zaretsky, E.J., Tang, M., Lopez de Leon, A., Xiang, H., Gusti, V., Clausen, I.G., Olsen, P.B., Rasmussen, M.D., Andersen, J.T., Jørgensen, P.L., Larsen, T.S., Sorokin, A., Bolotin, A., Lapidus, A., Galleron, N., Ehrlich, S.D. and Berka, R.M.** (2004) Complete Genome Sequence of the Industrial Bacterium *Bacillus licheniformis* and Comparisons with Closely Related *Bacillus* species. *Genome Biology* **5:** R77.1-R77.12.

- **Rius, N., Fusté, M.C., Guasp, C., Lalucat, J. And Lorén, J.G.** (2001) Clonal Population Structure of *Pseudomonas stutzeri*, a Species with Exceptional Genetic Diversity. *Journal of Bacteriology* **183:** 736-744.

- **Ronaghi, M., Nygren, M., Lundeberg, J. and Nyren, P.** (1999) Analysis of Secondary Structures in DNA by Pyrosequencing. *Analytical Biochemistry* **267:** 65-71.

- **Ronaghi, M.** (2001) Pyrosequencing Sheds Light on DNA Sequencing. *Genome Research* **11:** 3-11.

- **Sambrook, J., Fritsh, E.F. and Maniatis, T.** (1989) Molecular Cloning. A Laboratory Manual Volume 1 (2nd ed.). Cold Spring Harbor Laboratory Press. Cold Spring Harbor, USA, New York.

- **Sewell, B.T., Berman, M.N., Meyers, P.R., Jandhyala, D. and Benedik, M.J.** (2003) The Cyanide Degrading Nitrilase from *Pseudomonas stutzeri* AK61 is a Two-Fold Symmetric, 14-Subunit Spiral. *Structure* **11:** 1413-1422.

- **Skowronski, B. and Strobel, G.A.** (1969) Cyanide Resistance and Cyanide Utilization by a Strain of *Bacillus pumilus*. *Canadian Journal of Microbiology* **15:** 93-98.

- **Takami, H., Nakasone, K., Takaki, Y., Maeno, G., Sasaki, R., Masui, N., Fuji, F., Hirama, C., Nakamura, Y., Ogasawara, N., Kuhara, S. and Horikoshi, K.** (2000) Complete Genome Sequence of the Alkaliphilic Bacterium *Bacillus halodurans* and Genomic Sequence Comparison with *Bacillus subtilis*. *Nucleic Acids Research* **28:** 4317-4331.

- **Trevors, J.T.** (1996) Genome Size in Bacteria. *Antonie van Leeuwenhoek* **69:** 293-303.

- **Van Niel, C.B. and Allen, M.B.** (1952) A Note on *Pseudomonas stutzeri*. *Journal of Bacteriology* **64:** 413-422.

- **Watanabe, A., Yano, K., Ikebukuro, K. and Karube, I.** (1998) Cyanide Hydrolysis in a Cyanide-Degrading Bacterium, *Pseudomonas stutzeri* AK61, by Cyanidase. *Microbiology* **144:** 1677-1682.

- **White, J.M., Jones, D.D., Huang, D. and Gauthier, J.J.** (1988) Conversion of Cyanide to Formate and Ammonia by a Pseudomonad Obtained From Industrial Wastewater. *Journal of Indian Microbiology* **3:** 263-272.

- **Zhang, Z., Schwartz, S., Wagner, L. and Miller, W.** (2000) A Greedy Algorithm for Aligning DNA Sequences. *Journal of Computational Biology* **7:** 203-214.

CHAPTER 4

4. SUMMARY

Cyanide is highly toxic to living organisms due to the potent inhibitory effect on the respiration system. This toxic compound can be deposited in the environment through various sources. Naturally occurring cyanide compounds can be synthesized (cyanogenesis) by various taxa including fungi, plants and bacteria. Cyanogenesis in bacteria is mostly linked to antagonistic activity against various microorganisms competing for the same nutrients in the same environment. Anthropogenic sources of cyanide include a wide variety of industries but the major contributor is the cyanidation process. This process extracts gold (silver can also be extracted) from ore and is responsible for the formation of metal-cyanide complexes in soil which can dissociate to form free cyanide under the correct conditions. Various microorganisms are capable to degrade free cyanide.

The aims of this study were to identify microorganisms capable of utilizing cyanide as both a carbon and nitrogen source and to elucidate the mode of degradation. Samples were obtained from the Klipspruit Calcium Cyanide Factory site and were inoculated into minimal medium supplemented with NaCN. Eighteen isolates were identified from the samples and included organisms that could possibly be novel isolates based on the maximum identity percentage obtained when the 16S rRNA gene sequences (~1 500 bp) were used in a BLAST analysis against the NCBI database. The MIC was calculated for each of the 18 isolates and indicated that most of the organisms were capable of degrading cyanide at concentrations of above 2 M. This, in correlation with literature, is far above average. Gram stains were performed on the eighteen isolates. Five isolates were chosen for further studies based on 16S rRNA sequencing results, MIC determinations as well as information from literature that states that *Bacillus* and *Pseudomonas* species are often employed in bioremediation strategies. The five selected organisms included three gram positive (*Bacillus* sp.; *Paenibacillus* sp. and *Leifsonia* sp.) and two gram negative (*Achromobacter* sp. and *Brevundimonas* sp.) isolates. For comparative studies three control organisms (*Bacillus pumilus, Pseudomonas fluorescens* and *Pseudomonas stutzeri*) that are known and described in literature to be capable of cyanide degradation, were included in this study.

The cyanide assay (100 mM NaCN) was performed on the five selected and three control organisms. The control organisms were unable to utilize the cyanide as the sole carbon and nitrogen source at this high concentration. In contrast, the selected organisms

were capable of increasing their biomass over time indicating that these organisms can utilize the NaCN as the sole carbon and nitrogen source.

To elucidate the mode of cyanide degradation primers were designed specific for the known genes involved in cyanide utilization in the three control organisms, and screening the five isolates with these primers for the presence of the these genes. The genes targeted were cyanide dihydratase (*Bacillus pumilus*), hydrogen cyanide synthase (*Pseudomonas fluorescens*) and cyanide degrading enzyme (*Pseudomonas stutzeri*). The specifically designed primers were used on the gDNA from the selected organisms and this led to various non-specific product formations and in many of the samples no product was obtained.

With the failure to identify the presence of known cyanide degrading genes in the five selected organisms, one of these organisms, *Bacillus* sp. B4H3, was selected for pyrosequencing to elucidate the complete cyanide metabolism in this microorganism. The sequencing data was analyzed and it was observed that the 16S rRNA gene sequence obtained in Chapter 2, section 2.3.2.7 was not present in the genome of the isolate after pyrosequencing. The pyrosequencing data was analyzed and a total of twenty one enzymes involved in the cyanide metabolism of this isolate were identified. From the complete metabolic pathway it can be concluded that the single nitrogen can be utilized through the conversion of cyanide to formamide which in turn can be converted to ammonia. The utilization of the single carbon is based upon the hypothesis that the reaction between cyanide and glycine is reversible. This will lead to the carbon fixation metabolism which will prove that the isolate is capable of utilizing the single carbon as the sole carbon source.

Key terms: cyanidation; cyanide degradation; cyanide metabolism; cyanogenesis; DGGE; MIC; pyrosequencing

CHAPTER 5

5. OPSOMMING

Sianied is toksies en inhibeer die respirasie sisteem van alle lewende organismes. Hierdie toksiese verbinding kan gedeponeer word in die omgewing deur middel van verskeie bronne. Sianied kom natuurlik in die omgewing voor deur die sintese van sianied deur verskillende organismes insluitende fungi, plante en bakterieë. Die sintese van sianied deur bakterieë help in die oorlewing van die organisme deur kompeterende organismes te inhibeer. Sianied kan ook deur verskeie industrieë in die omgewing opeindig en die hoof oorsaak van besoedeling is die gebruik van sianied in die sianidasie proses. Die proses word gebruik vir die ekstraksie van goud uit myn afval. Die oorblywende sianied vorm verbindings met verskeie metale en die verbindings kan onder die korrekte omstandighede dissosieer en tot die vorming van vrye sianied lei. Verskeie mikroörganismes beskik oor die vermoë om sianied te degradeer na minder skadelike komponente.

Die doelpunte van die verhandeling was om mikroörganismes te isoleer en te karakteriseer wat oor die vermoë besit om sianied as die enigste bron van beide koolstof en stikstof te gebruik en om die wyse van degradasie te beskryf. Monsters was geneem by die Klipspruit Kalsium Sianied Fabriek en is geïnokuleer in minimale medium wat NaCN bevat het as die enigste bron van koolstof en stikstof. Die 18 geïdentifiseerde isolate sluit moontlike unieke organismes in. Die stelling is gebaseer op die maksimum identiteit persentasie nadat die 16S rRNS geen (~ 1 500 bp) vergelyk is deur gebruik te maak van ‘n BLAST analise teen die NCBI databasis. Die minimale inhiberende konsentrasie was bereken vir elk van die 18 isolate en die meeste van die organismes het gegroei by konsentrasies hoër as 2 M sianied. In vergelyking met die literatuur is die konsentrasie ver bo die gemiddeld. Gram kleurings is gedoen op die 18 isolate. Vyf isolate was geselekteer vir verdere eksperimente gebaseer op die resultate verkry vanaf die Sanger basispaaropeenvolgingsbepaling, die minimale inhiberende konsentrasie bepalings asook bestaande kennis in literatuur waar dit bekend is dat *Bacillus* en *Pseudomonas* spesies huidiglik suksesvol geïmplementeer word in bioremediërings strategieë. Die geselekteerde vyf organismes het drie gram positiewe (*Bacillus* sp.; *Paenibacillus* sp. en *Leifsonia* sp.) en twee gram negatiewe (*Achromobacter* sp. en *Brevundimonas* sp.) organismes ingesluit. Drie kontrole organismes (*Bacillus pumilus*; *Pseudomonas fluorescens* en *Pseudomonas stutzeri*) wat beskryf word in die literatuur as sianied degraderende bakterieë, was gebruik in die studie vir vergelykende doeleindes.

Die drie kontrole en vyf geselekteerde organismes was gebruik in groei studies waar 4 mM of 10 mM NaCN bygevoeg is by die minimale medium. Die kontrole organismes het ‘n hoër maksimum groei tempo getoon by die laer sianied konsentrasie (4 mM) as die geselekteerde organismes. In die teenwoordigheid van 10 mM NaCN was die maksimum groei tempo’s van die geselekteerde organismes aansienlik hoër as dié van die kontrole organismes. Die sianied toets (100 mM NaCN) was uitgevoer op die kontrole en geselekteerde organismes. Die kontrole organismes het nie ‘n toename in sel konsentrasie getoon wat aandui dat dié nie die NaCN by die hoë konsentrasie kan gebruik as die enigste bron van koolstof en stikstof nie. Die geselekteerde organismes was wel in staat om die NaCN te gebruik as die enigste bron van koolstof en stikstof gebaseer op die toename in die sel konsentrasie.

Priemstukke spesifiek vir die gene teenwoordig in die drie kontrole organismes betrokke by sianied afbraak was gesintetiseerd om die manier van sianied degradering te ondersoek in die geselekteerde organismes. Die teiken sianied degraderende gene was sianied dehidratase (*Bacillus pumilus*), waterstof sianied sintase (*Pseudomonas fluorescens*) en die sianied degraderende ensiem (*Pseudomonas stutzeri*). Die spesifiek ontwerpte priemstukke was gebruik op die genomiese DNS van die geselekteerde organismes en dit het gelei tot verskeie nie-spesifieke of geen produk vorming.

Die onsuksesvolle identifisering van die bekende sianied degraderende gene het gelei tot die seleksie van die *Bacillus* sp. isolaat vir piro basispaaropeenvolgingsbepaling om die sianied metabolisme van hierdie isolaat uit te klaar. Gevolglik is een-en-twintig ensieme geïdentifiseer wat betrokke is by sianied metabolisme. Deur die metaboliese baan te analiseer kan die gebruik van die enkel stikstof bron as enigste bron van stikstof verduidelik word deur die omskakeling van sianied na formamied waarna dié omgeskakel word na ammoniak. Die gebruik van die enkel koolstof as enigste bron van koolstof is gebaseer op die hipotese dat die reaksie tussen sianied en glisien omkeerbaar is. Die omskakeling sal verseker dat die enkel koolstof die koolstof fikserende metabolisme sal binnegaan om sodoende energie te verskaf aan die isolaat.

Sleutel terme: DGGE; sianidasie; sianied degradering; sianied metabolisme; sianogenese; minimale inhiberende konsentrasie; piro basispaaropeenvolgingsbepaling

APPENDIX A

A. KEGG PATHWAY DATABASE: ELUCIDATION OF THE CYANIDE METABOLIC PATHWAY IN *BACILLUS* SP.

The six metabolic pathways used as reference were i) Cyanoamino acid metabolism (Figure A.1; Table A.1), ii) Nitrogen metabolism: reduction and fixation (Figure A.2; Table A.2), iii) Glycine, serine and threonine metabolism (Figure A.3; Table A.3), iv) Glyoxylate and dicarboxylate metabolism (Figure A.4; Table A.4), v) Carbon fixation metabolism (Figure A.5; Table A.5) and vi) Methane metabolism (Figure A.6; Table A.6).

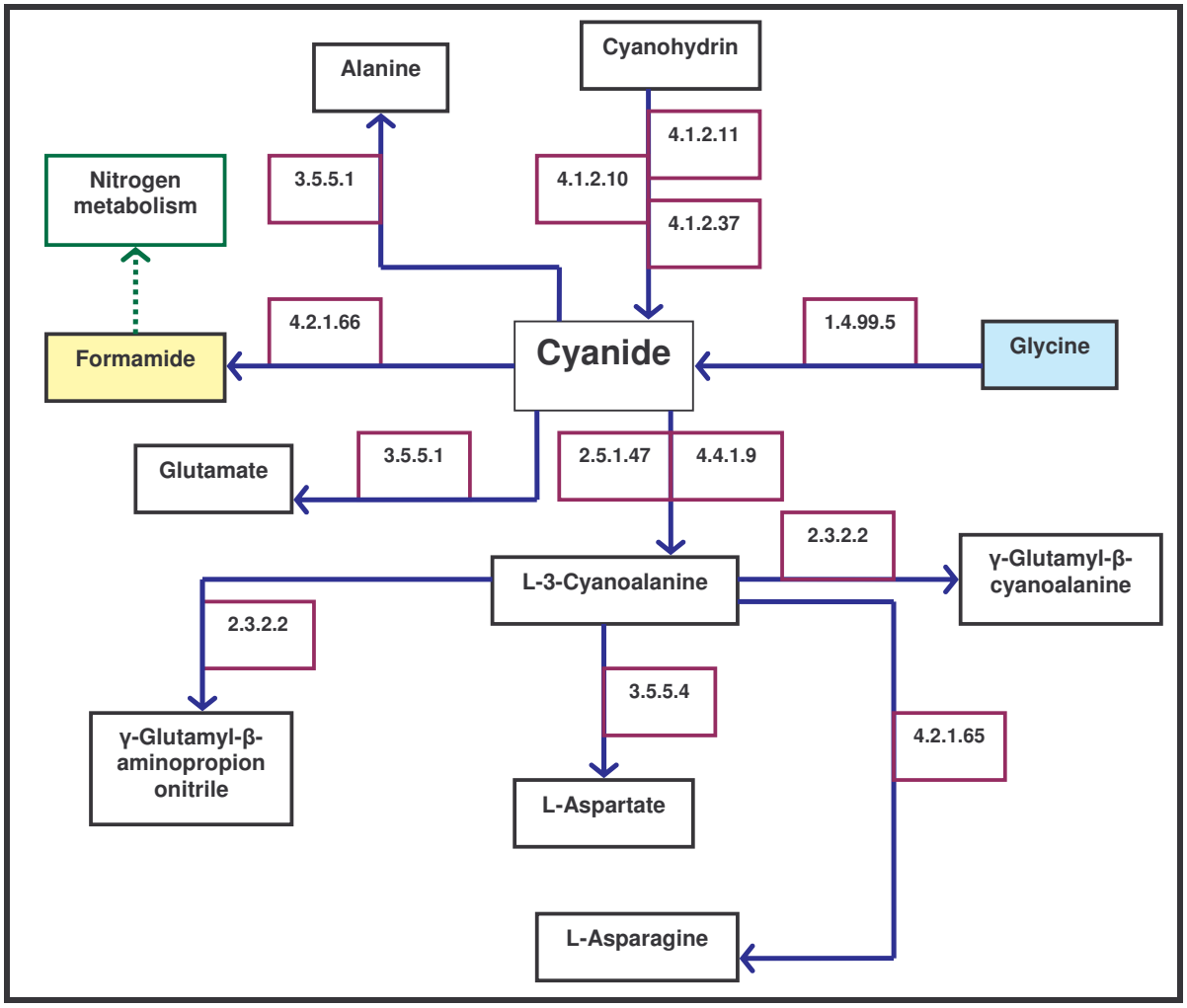

Figure A.1: The cyanoamino acid metabolism. The purple blocks indicate the enzymes selected for BLAST analysis against internal database.

Table A.1: Summary of enzymes involved in the cyanoamino acid metabolism with corresponding EC numbers.

EC number	Enzyme
1.4.99.5	Glycine dehydrogenase/Hydrogen cyanide synthase
2.3.2.2	Gamma-glutamyltransferase/peptidase
2.5.1.47	Cysteine synthase
3.5.5.1	Nitrilase (act on C-N other than peptide bonds)
3.5.5.4	Cyanoalanine nitrilase
4.1.2.10	Mandelonitrile lyase (plant enzyme)
4.1.2.11	Hydroxymandelonitrile lyase (plant enzyme)
4.1.2.37	Hydroxynitrilase (plant enzyme)
4.2.1.65	3-Cyanoalanine hydratase (plant enzyme)
4.2.1.66	Cyanide hydratase/Formamide dihydratase (fungal enzyme)
4.4.1.9	L-3-cyanoalanine synthase

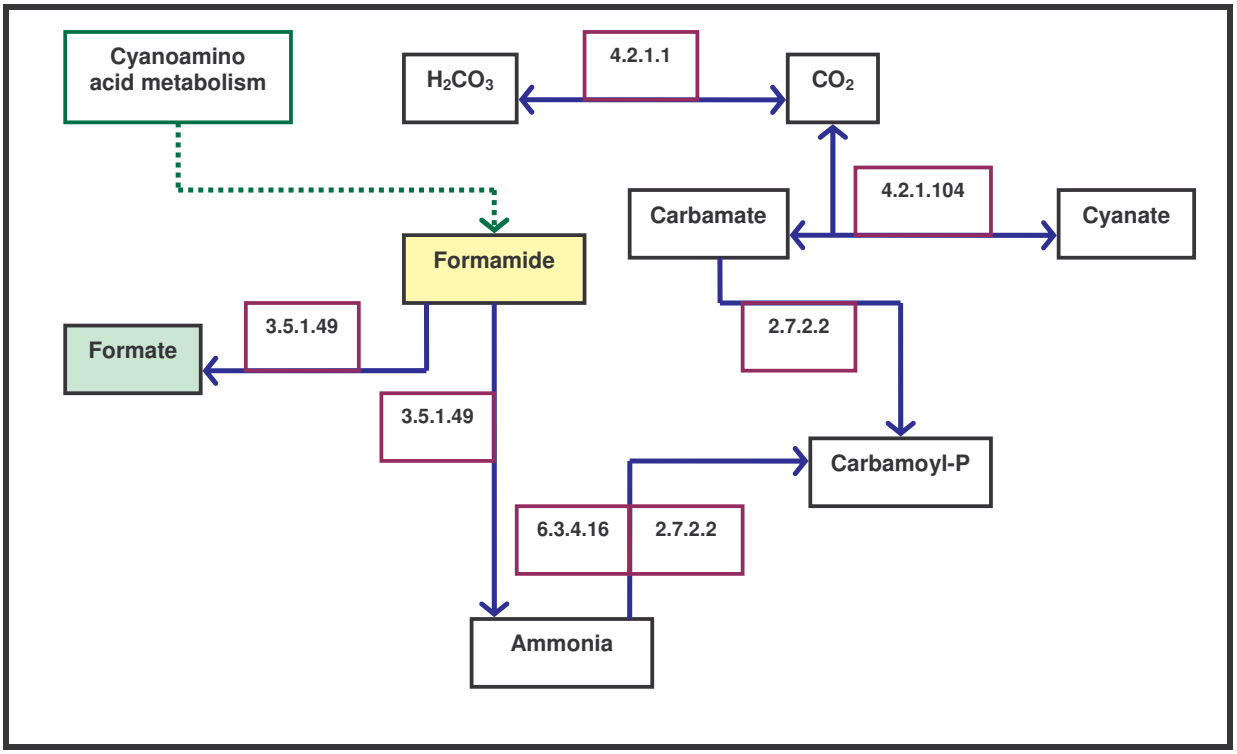

Figure A.2: The nitrogen metabolism: reduction and fixation. The purple blocks indicate the enzymes selected for BLAST analysis against internal database.

Table A.2: Summary of enzymes involved in the nitrogen metabolism: reduction and fixation with corresponding EC numbers.

EC number	Enzyme
2.7.2.2	Carbamate kinase
3.5.1.49	Formamidase (C-N bonds other than peptide bonds)
4.2.1.1	Carbamate dihydratase/Carbonic anhydrase
4.2.1.104	Cyanase/Cyanate hydratase
6.3.4.16	Carbamoyl-phosphate synthase

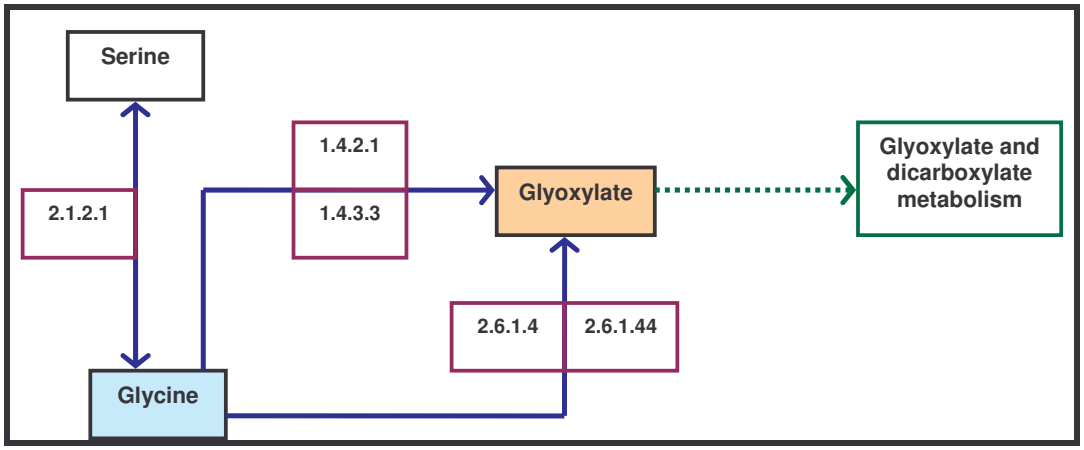

Figure A.3: The glycine, serine and threonine metabolism. The purple blocks indicate the enzymes selected for BLAST analysis against internal database.

Table A.3: Summary of enzymes involved in the glycine, serine and threonine metabolism with corresponding EC numbers.

EC number	Enzyme
1.4.2.1	Glycine dehydrogenase
1.4.3.3	D-amino-acid oxidase
2.1.2.1	Glycine hydroxymethyltransferase
2.6.1.4	Glycine transaminase (plant enzyme)
2.6.1.44	Alanine glyoxylate transaminase

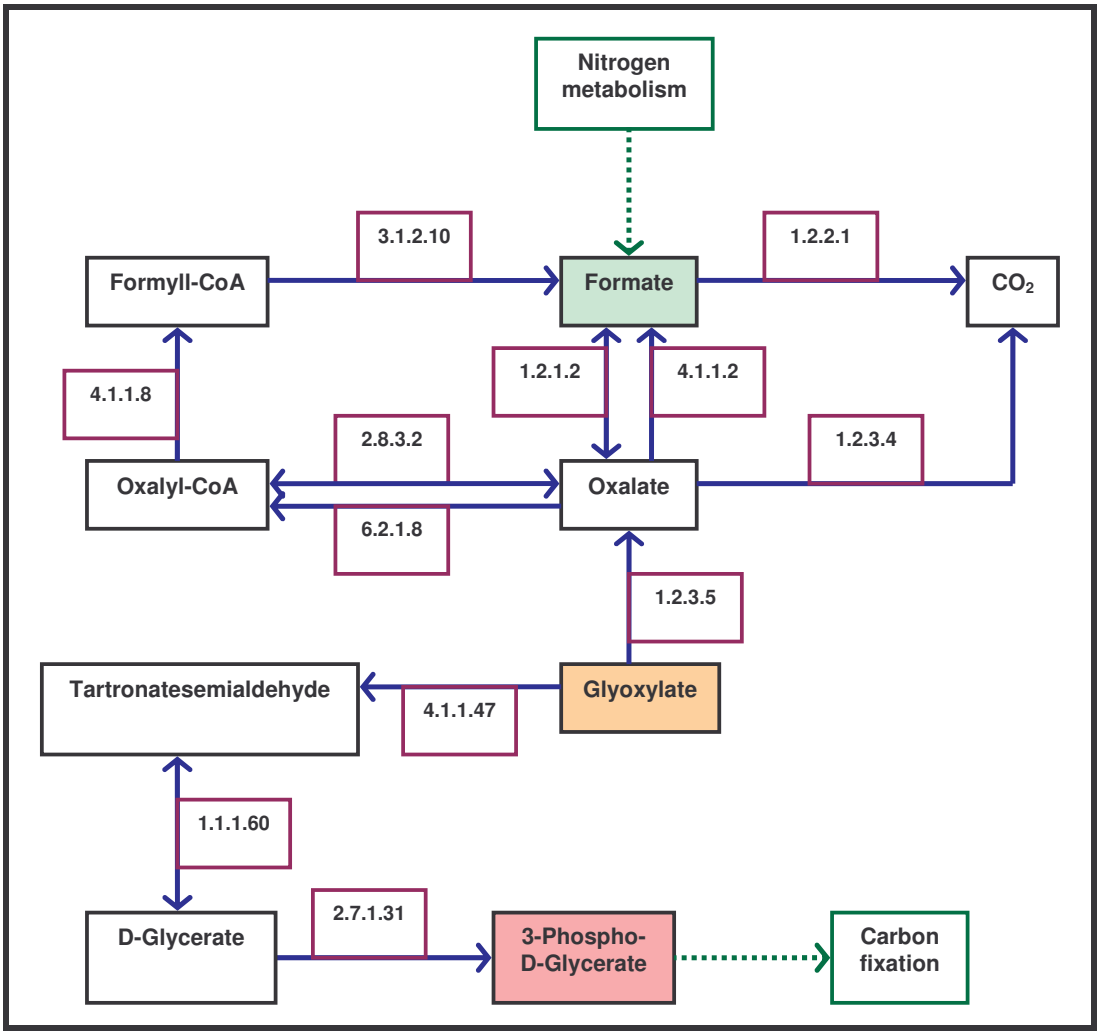

Figure A.4: The glyoxylate and dicarboxylate metabolism. The purple blocks indicate the enzymes selected for BLAST analysis against internal database.

Table A.4: Summary of enzymes involved in the glyoxylate and dicarboxylate metabolism with corresponding EC numbers.

EC number	Enzyme
1.1.1.60	2-Hydroxy-3-Oxopropionate reductase
1.2.1.2	Formate dehydrogenase
1.2.2.1	Formate dehydrogenase
1.2.3.4	Oxalate oxidase
1.2.3.5	Glyoxylate oxidase
2.7.1.31	Glycerate kinase
2.8.3.2	Oxylate CoA transferase
3.1.2.10	Formyl CoA hydrolase
4.1.1.2	Oxalate decarboxylase
4.1.1.8	Oxylyl CoA decarboxylase
4.1.1.47	Tartranate semialdehyde synthase
6.2.1.8	Oxalyl CoA synthetase/Oxalate CoA ligase

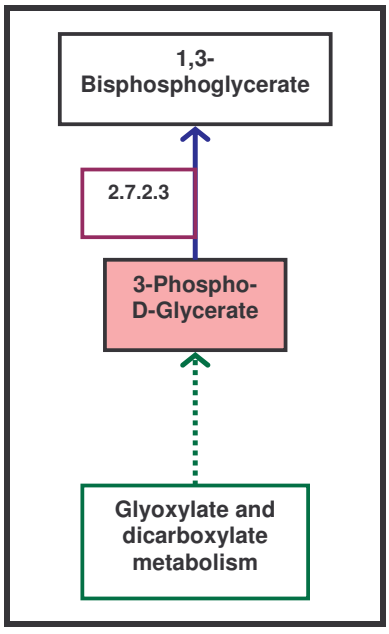

Figure A.5: The carbon fixation metabolism. The purple block indicates the enzymes selected for BLAST analysis against internal database.

Table A.5: Enzyme involved in the carbon fixation metabolism with corresponding EC numbers.

EC number	Enzyme
2.7.2.3	Phosphoglycerate kinase

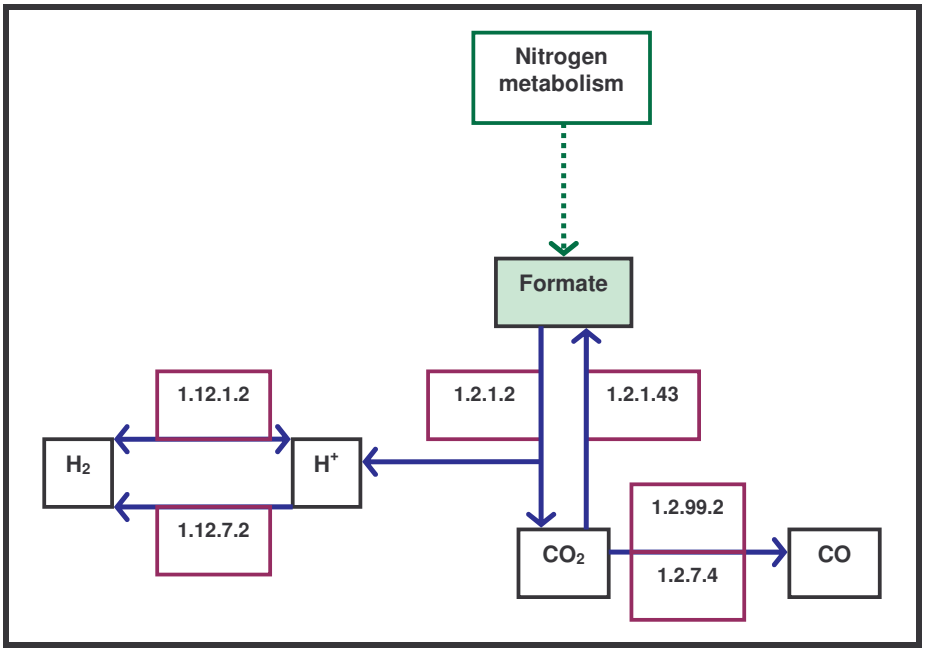

Figure A.6: The methane metabolism. The purple blocks indicate the enzymes selected for BLAST analysis against internal database.

Table A.6: Summary of enzymes involved in the methane metabolism with corresponding EC numbers.

EC number	Enzyme
1.2.1.2	Formate dehydrogenase
1.2.1.43	Formate dehydrogenase
1.2.7.4	Carbon monoxide dehydrogenase
1.2.99.2	Carbon monoxide dehydrogenase
1.12.1.2	Hydrogen dehydrogenase
1.12.7.2	Ferrodoxin hydrogenase

APPENDIX B

B. ELUCIDATION OF ENZYMES PRESENT IN *BACILLUS* SP. INVOLVED IN THE CYANIDE METABOLISM

B.1 Identifying complete coding sequences using Artemis

The enzymes obtained from the KEGG PATHWAY database was used in a BLAST search (tblastn) to identify the presence or absence of these enzymes in the selected organism. The enzymes that provided a significant E-value were subjected into Artemis to locate the complete CDSs present in the genome of the organism. Multiple alignments were performed using the enzymes identified in the *Bacillus* sp. and the highest hit obtained from NCBI (indicated in the tables).

B.1.1 Cyanoamino acid metabolism

EC 1.4.99.5: hydrogen cyanide synthase HcnC

Contig	Accession	Description	Query coverage	E value
00553	YP_259686.1	hydrogen cyanide synthase HcnC [*Pseudomonas fluorescens* Pf-5]	100%	0.0

Alignment

```
Bacillus_contig00553          MCKKYDVAIIGGGVIGSSVAHFLAERGHKVAIVEKQRIASEASKAAAG--
Pseudomonas_fluorescens       MIKHYDVVIAGGGVIGASCAYQLSKRKDLKVALIDAKRPGNASRASAGGL
                              * *:***.* ******:* *: *::* .  . : . : ..:**:*:**

Bacillus_contig00553          -----------------LLGVQAEWDAY---------------DPLFELA
Pseudomonas_fluorescens       WAIGESVGLGCGVIFFRMMSANRKREAQGSAVVVDSSTPHILPQSFFDFA
                                            ::..: : :*                :.:*::*

Bacillus_contig00553          RESRAIFPQLAAVLREKTGIDIGYEEKGIYRIAQNEDEKERILHIMEWQQ
Pseudomonas_fluorescens       LQSNELYPRLHRELMGLHNMDFKFEQTGLKFVIYDEEDRLYAEHIVGCIP
                               :*. ::*:*   *    .:*: :*:.*:  :  :*:::    **:

Bacillus_contig00553          KTGEDSYFLMGDRLREKEPCLSESIIGAVYYPKDGHVIAPELTKAFAHSA
Pseudomonas_fluorescens       HLSDQVRWLDQAALRASEPNVSHEAQGALEFLCDHQVNPFRLTDAYTEGA
                              : .::  :*    ** .** :*..  **: :  * :* . .**.*::..*

Bacillus_contig00553          SISGADIYEQTEVFDIRIENNKVTGIVTSEG-IMACEKVVIAGGSWSTKL
Pseudomonas_fluorescens       RQNGVDVYFNTNVTGVLHQGNRVSGVKTDVAGLFRCTTLINAAGAWAAEL
                                .*.*:* :*:* .:  :.*:*:*: *. . :: * .:: *.*:*:::*

Bacillus_contig00553          -LGYFHREWGTYPVKGEVVAVRSRKPLLKAPIFQERFYIAPKRGGRYVIG
Pseudomonas_fluorescens       SLQATGIEIPVKPVKGQILLTERMPKLLNGCLTTSDCYMAQKDNGEILIG
                               *     *  . ****::: ..    **:. :  .  *:* * .*. :**

Bacillus_contig00553          ATMKPHTFNKTVQPESITSILERAYTILPALKEAEWESAWAGLRPQSNHE
Pseudomonas_fluorescens       STTEDKGFDVTTTYPEINGLVQGAVRCVPELAHVNLKRCWAGLRPGSPDE
                              :* : : *: *.   .*..::: *   :* * ..: : .****** * .*
```

```
Bacillus_contig00553          APYMGEHEEIKGLYACTGHYRNGILLSPVSGQYMADLIEGKQENHLLDSL
Pseudomonas_fluorescens       LPILGPMDGVEGYLNACGHFRTGILTSAITGVLLDKLVNDEALPLDITPF
                               * :*  : ::*   . **:*.*** *.::*  : .*::.:     : .:

Bacillus_contig00553          LSKTV-----------
Pseudomonas_fluorescens       LARRFATTPVKKQPEPA
                              *:: .
```

EC 2.5.1.47: cysteine synthase

Contig	Accession	Description	Query coverage	E value
00507	ZP_04298522.1	cysteine synthase [*Bacillus cereus* MM3]	100%	2e-172
01117	ZP_04071420.1	cysteine synthase [*Bacillus thuringiensis* IBL 200]	100%	2e-174
00780	ZP_00237382.1	cysteine synthase/cystathionine beta-synthase family protein [*Bacillus cereus* G9241]	100%	2e-175

Multiple alignments

```
Bacillus_contig00570          -MRVAQSVSELIGKTPIVKLNRIVESNSADIYLKLEFMNPGSSVKDRIAL 49
Bacillus_cereus               MVRVANSITELIGNTPIVKLNRLADENSADVYLKLEYMNPGSSVKDRIGL 50
Bacillus_contig01117          -MKLCENVTELIGDTPVVRLSKFIPEDAADVYVKLEMFNPSRSVKDRAAY 49
Bacillus_thuringiensis        -MKLCENVTELIGDTPVVRLSKFIPEDAADVYVKLEMFNPSRSVKDRAAY 49
Bacillus_contig00780          -MNVYRGVHELIGHTPIVEITRFSLPEGVRLFAKLEFYNPGGSVKDRLGR 49
Bacillus_cereus1              -MNVYRGVHELIGHTPIVEITRFSLPEGVRLFAKLEFYNPGGSVKDRLGR 49
                               :.: ..: ****.**:*.:.::   :.. :: ***  **. ***** .

Bacillus_contig00570          AMIEDAEKKGLLKEGDTIIEPTSGNTGIGLAMVAAAKGYKAILVMPETMS 99
Bacillus_cereus               AMIEAAEKEGKLKAGNTIIEPTSGNTGIGLAMVAAAKGLKAILVMPDTMS 100
Bacillus_contig01117          NLLHVAEEHGLIKPGDTIIEPTSGNTGIGLAMNAAAKGYKAILIMPDNMS 99
Bacillus_thuringiensis        NLLHVAEENGLIKPGDTIIEPTSGNTGIGLAMNAAAKGYKAILIMPDNMS 99
Bacillus_contig00780          ELIEDALEKGLVTQGGTIIEPTAGNTGIGLALAALQHDLRVIVCVPEKFS 99
Bacillus_cereus1              ELIEDALEKGLVTQGGTIIEPTAGNTGIGLALAALQHDLRVIVCVPEKFS 99
                               ::. * :.* :. *.******:********: *  :. :.*: :*:.:*

Bacillus_contig00570          IERRNLLRAYGAELVLTPGPEGMGGAIRQATELAKEHG-YFIPQQFKNQS 148
Bacillus_cereus               MERRNLLRAYGAELVLTPGAEGMKGAIKKAEELAEKHG-YFVPQQFNNPS 149
Bacillus_contig01117          KERINLLKAYGAEVVLTPAEQRMPGAIAKALELQKQIPNSFIPQQFENPA 149
Bacillus_thuringiensis        KERINLLKAYGAEVVLTPAEQRMPGAIAKALELQKQIPNSFIPQQFENPA 149
Bacillus_contig00780          IEKQELMKALGATVVHTPTEQGMTGAIAKAKELVNEIPNSYSPSQFANEA 149
Bacillus_cereus1              IEKQELMKALGATVVHTPTEQGMTGAIAKAKELVNEIPNSYSPSQFANEA 149
                               *: :*::* ** :* **  : * *** :* ** ::    : *.** * :

Bacillus_contig00570          NPEIHRLTTGPEIVEQMG-EQLDAFIAGIGTGGTITGAGEVLKEAYKDIK 197
Bacillus_cereus               NPEIHRQTTGKEIVEQFGDDQLDAFVAGIGTGGTITGAGEVLKEAYPSIK 199
Bacillus_contig01117          NPNIHRYTTALEIYEQMD-GELDAFVATAGTGGTITGTGETLKEKLPNLY 198
Bacillus_thuringiensis        NPNIHRYTTALEIYEQMD-GELDAFVATAGTGGTITGTGETLKEKLPNLY 198
Bacillus_contig00780          NPRAYFKTLGPELWDALN-GEINIFVAGAGTGGTFMGTASYLKEKNVDIK 198
Bacillus_cereus1              NPRAYFKTLGPELWDALN-GEIDIFVAGAGTGGTFMGTASYLKEKNIDIK 198
                              **. :  * . *: : :.  ::: *:*  *****: *:.. ***   .:

Bacillus_contig00570          IYAVEPADSPVLSGGKPGPHKIQGIGAGFIPETLDVEVYDEIVQVKAEQA 247
Bacillus_cereus               IYAVEPSDSPVLSGGKPGPHKIQGIGAGFVPDILNTEVYDEIFPVKNEEA 249
Bacillus_contig01117          IAVVEPKGSPVLSGGVPGPHKLVGTSPGFIPKNLNTEVYNEIIQIADEEA 248
Bacillus_thuringiensis        IAVVEPKGSPVLSGGVPGPHKLVGTSPGFIPKNLNTEVYNEIIQIADEEA 248
Bacillus_contig00780          TVIVEPEGS-ILNGGKAGSHETEGIGLEFIPPFLKTSYFDEIHTISDRNA 247
Bacillus_cereus1              TVIVEPEGS-ILNGGKAGSHETEGIGLEFIPPFLKTSYFDEIHTISDRNA 247
                                 *** .* :*.** .*.*:  * .  *:*  *... ::**  :  .:*
```

```
Bacillus_contig00570          FEYARRVAKEEGILVGISSGAVIYAATEIAKKLGKGKKVLVIIPSNGERY 297
Bacillus_cereus               FEYARRAAREEGILGGISSGAAIYAALQVAKKLGKGKKVLAIIPSNGERY 299
Bacillus_contig01117          LTTMRNLARQEGLLVGPSSGASVYAAIMIAKRLGAGKKVLCIAPDTGERY 298
Bacillus_thuringiensis        LTTMRNLARQEGLLVGPSSGASVYAAIMIAKRLGAGKKVLCIAPDTGERY 298
Bacillus_contig00780          FLRVKELAQKEGLLVGSSSGAAFHASLLEAEQAAPGTNIVTIFPDSSERY 297
Bacillus_cereus1              FLRVKELAQKEGLLVGSSSGAAFHASLLEAEKAAPGTNIVTIFPDSSERY 297
                              :   :. *::**:* * **** .:*:   *:: . *.::: * *...***

Bacillus_contig00570          LSTPLYQFES 307
Bacillus_cereus               LSTPLYQFD- 308
Bacillus_contig01117          LSMGLFE--- 305
Bacillus_thuringiensis        LSMGLFE--- 305
Bacillus_contig00780          LSKDIYKGWE 307
Bacillus_cereus1              LSKDIYKGWE 307
                              **  :::
```

EC 4.2.1.66: cyanide hydratase/Formamide dihydratase

Contig	Accession	Description	Query coverage	E value
00039	YP_001646659.1	Nitrilase/cyanide hydratase and apolipoprotein N-acyltransferase [*Bacillus weihenstephanensis* KBAB4]	100%	6e-138
00160	ZP_04237723.1	Amino-acid permease rocC [*Bacillus cereus* Rock1-15]	100%	0.0

Multiple alignments

```
Bacillus_contig00160          -MQQPTNEKLHRTMKSRHLFMIALGGVIGTGFFLGSGYTINQAGPGGAIL 49
Bacillus_cereus               MMNQ--NQGLKRELKSRHIFMIALGGVIGTGLFLGSGYTIHEAGPGGAIV 48
Bacillus_contig00039          --------------------------------------------------
Bacillus_weihenstephanensis   --------------------------------------------------

Bacillus_contig00160          SYLVGGFIMYLTMLCLGELTVAMPVSGSFQKYATKFIGPGTGFMIGWLYW 99
Bacillus_cereus               AYLVGGFVMYLTMLCLGELAVAMPDAGSYQTYATKHISPAAGYVVGWMSW 98
Bacillus_contig00039          --------------------------------------------------
Bacillus_weihenstephanensis   --------------------------------------------------

Bacillus_contig00160          LGWAVTVGLELTSIGLMMKRWFPNVDVWVWCLVFGVILYASNAISAKSYA 149
Bacillus_cereus               LNWSATIGIELIAVSILMKRWFPDVPSWIWCVVFAVLLFAINALSSRSFA 148
Bacillus_contig00039          ---------------------------MKVACIQMDIVFGDVEKNIENAKN 24
Bacillus_weihenstephanensis   ---------------------------MKIALVQMDVQIGEPDVNFQKAEA 24
                                                          :  : : : :   :    .:

Bacillus_contig00160          ELEFWFSSIKVVTILAFVVLGGSALLGFISYDGKEAAPLFSNFVSDGGLF 199
Bacillus_cereus               EVEFWFASIKVITIIAFIILGGAAMFGFLDMKGNEPAPMFSSFTDYGGLF 198
Bacillus_contig00039          KISEAMKERPDVIVLPELWTTGYDLTRLTEIADRDGLQTKEKLKEWSKQY 74
Bacillus_weihenstephanensis   FLEEAIRQQPDLIILPEMWNTGYALEQADQLADVNGERTKQLFSSFAHKH 74
                               :.  : .   : ::. :   *  :    .  . :     . : . .  .

Bacillus_contig00160          PNGLAAVLLTMITVNFSFQGTELIGIAAGESENPEKTIPRAIRNTVWRIM 249
Bacillus_cereus               PNGLSAILITMIAVNFSFQGTELVGIAAGESENPEKTIPKAINNTVWRIL 248
Bacillus_contig00039          G----------------------VHIVGG-SIA-KQTEQG-VTNTMYVVN 99
Bacillus_weihenstephanensis   Q----------------------VILIAG-SVLNKRTEDEKITNTMYIFN 101
                                                     : : .* *   ::*    : **:: .
```

```
Bacillus_contig00160            LFFILTMTILVGLISWKEAGVIESPFVVVFDKIGIPYAADIMNFVIITAL 299
Bacillus_cereus                 VFFVLSIFILAGLFPWQQAGVIESPFVVVFDKIGIPYAADIINFVIITAV 298
Bacillus_contig00039            K--------------------EGELVNEYSKVHLFQLMDEHKYLIAGNS 128
Bacillus_weihenstephanensis     R--------------------QGELLVDYDKIHLFRLMDEHNYLTAGDQ 130
                                                     :. ::  :.*: :    *  :::

Bacillus_contig00160            LSVANSG--LYAATRILWSLANEGMAPTSFKKVNKRGIPITALVVTIAVA 347
Bacillus_cereus                 LSVANSG--LYATSRMLWSMSNQGMISPIFGKLSKNGVPIYALIVSTIVG 346
Bacillus_contig00039            TGEFKLD-DVECAGTICYDIRFPEWMRVHTAKGAKVLFVVAEWPLVRLAH 177
Bacillus_weihenstephanensis     LCLFDYDEDVKIGAMICYDLRFPQLSRTLVNKGAKVLINTAQWPSARVDH 180
                                    . .  :     : :.:           *  *  .

Bacillus_contig00160            GLSLFTSFLAEDTVYMYLLSIAGLSAVSSWIIIALSQLRFRSQYIKGGGK 397
Bacillus_cereus                 CLSLLSGIYAEDTVYLWLLSIAGFGAILVWASIALSNLLARRSYIKQGGD 396
Bacillus_contig00039            WRLLLQARAVENQCYVVACNRAGKDPSNEFAGHSLIVDPWGEVVVEANEE 227
Bacillus_weihenstephanensis     WRSLLIARAIENQSFMIAVNRTGTSRDTEFPGHSMVIDPLGRILLETNHE 230
                                   *: .   *:  ::   . :* .    :   ::         :: . .

Bacillus_contig00160            LEDLKYRTPLYPIVPILALITNSIVVISLAFIPEQRMALYCGIPFIIFCY 447
Bacillus_cereus                 VKDLKFKTPLYPFVPLLALVLNLTVIVGMAFIPEQRMALYCGIPFMIVCL 446
Bacillus_contig00039            ES---------NLFGELHFEKIKEVRKGIPVFADRRPELYK--------- 259
Bacillus_weihenstephanensis     ED---------IYYGDIDLQLVDEVRQQIPVMTDQRLDIY---------- 261
                                 .              : :     *   :..:.::*  :*

Bacillus_contig00160            IYYYMSK-KRKKPMKVEMEAKYENKN- 472
Bacillus_cereus                 LFYRATRNKARKVEHIEKTNTTEVESL 473
Bacillus_contig00039            --------------------------
Bacillus_weihenstephanensis     --------------------------
```

EC 4.4.1.9: L-3-cyano-alanine synthase

Contig	Accession	Description	Query coverage	E value
00507	ZP_04298522.1	Cysteine synthase [*Bacillus cereus* MM3]	100%	2e-172
01117	ZP_04071420.1	cysteine synthase [*Bacillus thuringiensis* IBL 200]	100%	2e-174
00780	ZP_00237382.1	cysteine synthase/cystathionine beta-synthase family protein [*Bacillus cereus* G9241]	100%	2e-175
01190	ZP_02467528.1	cysteine synthase A [*Burkholderia thailandensis* MSMB43]	98%	3e-44

Multiple alignments

```
Bacillus_contig00507          ------MRVAQSVSELIGKTPIVKLNRIVESNSADIYLKLEFMNPGSSVK 44
Bacillus_cereus               MLGVTGMRVAQSVSELIGKTPIVKLNRIVESNSADIYLKLEFMNPGSSVK 50
Bacillus_contig01117          ------MKLCENVTELIGDTPVVRLSKFIPEDAADVYVKLEMFNPSRSVK 44
Bacillus_thurengiensis        ------MKLCENVTELIGDTPVVRLSKFIPEDAADVYVKLEMFNPSRSVK 44
Bacillus_contig00780          ------MNVYRGVHELIGHTPIVEITRFSLPEGVRLFAKLEFYNPGGSVK 44
Bacillus_cereus1              ------MNVYRGVHELIGHTPIVEITRFSLPEGVRLFAKLEFYNPGGSVK 44
Bacillus_contig01190          ----MTLKHFNEFAKNVGNTPLIKLECEETKD-LSVYAKCEWHNPTGSIK 45
Burkholderia_thailandensis    ----------------------------------MYAKLESKNPSGSIK 15
                                                               :: * *  **  *:*

Bacillus_contig00507          DRIALAMIEDAEKKGLLKEGD-TIIEPTSGNTGIGLAMVAAAKGYKAILV 93
Bacillus_cereus               DRIALAMIEDAEKKGLLKEGD-TIIEPTSGNTGIGLAMVAAAKGYKAILV 99
Bacillus_contig01117          DRAAYNLLHVAEEHGLIKPGD-TIIEPTSGNTGIGLAMNAAAKGYKAILI 93
Bacillus_thurengiensis        DRAAYNLLHVAEENGLIKPGD-TIIEPTSGNTGIGLAMNAAAKGYKAILI 93
Bacillus_contig00780          DRLGRELIEDALEKGLVTQGG-TIIEPTAGNTGIGLALAALQHDLRVIVC 93
Bacillus_cereus1              DRLGRELIEDALEKGLVTQGG-TIIEPTAGNTGIGLALAALQHDLRVIVC 93
Bacillus_contig01190          DRAALGMINKYLQEADKKQ---PILEYSGGSLARSIGYICYCLGVQCTLV 92
Burkholderia_thailandensis    DRVAFGLFCDAINAHDFSSGPLKLLDSSGGNMAKALAHLGNLCGLPVHVV 65
                              ** .  ::    :    .     ::: :.*. . .:.      .    :

Bacillus_contig00507          MPETMSIERRNLLRAYGAELVLTPGPEGMGGAIRQATELAKEHG-YFIPQ 142
Bacillus_cereus               MPETMSIERRNLLRAYGAELVLTPGPEGMGGAIRQATELAKEHG-YFIPQ 148
Bacillus_contig01117          MPDNMSKERINLLKAYGAEVVLTPAEQRMPGAIAKALELQKQIPNSFIPQ 143
Bacillus_thurengiensis        MPDNMSKERINLLKAYGAEVVLTPAEQRMPGAIAKALELQKQIPNSFIPQ 143
Bacillus_contig00780          VPEKFSIEKQELMKALGATVVHTPTEQGMTGAIAKAKELVNEIPNSYSPS 143
Bacillus_cereus1              VPEKFSIEKQELMKALGATVVHTPTEQGMTGAIAKAKELVNEIPNSYSPS 143
Bacillus_contig01190          LSSGTGKSLLNELKHYGANVILVDKCMGFYAVIEKTIEISKQKPELYFLF 142
Burkholderia_thailandensis    IPDSASDELLQSLNDNKAIVTKVDRSHFLLGIIARSQQIAREEPGWTLLS 115
                              :..  . .  : :.   * :  .     : . * :: :: .:

Bacillus_contig00507          QFKNQSNPEIHRLTTGPEIVEQMGE-QLDAFIAGIGTGGTITGAGEVLKE 191
Bacillus_cereus               QFKNQSNPEIHRLTTGPEIVEQMGD-QLDAFIAGIGTGGTITGAGEVLKE 197
Bacillus_contig01117          QFENPANPNIHRYTTALEIYEQMDG-ELDAFVATAGTGGTITGTGETLKE 192
Bacillus_thurengiensis        QFENPANPNIHRYTTALEIYEQMDG-ELDAFVATAGTGGTITGTGETLKE 192
Bacillus_contig00780          QFANEANPRAYFKTLGPELWDALNG-EINIFVAGAGTGGTFMGTASYLKE 192
Bacillus_cereus1              QFANEANPRAYFKTLGPELWDALNG-EIDIFVAGAGTGGTFMGTASYLKE 192
Bacillus_contig01190          QHYNNANLLSHIEGTGREIITQMNGKPIHAWVASAGTGGTLMGVYKVVKN 192
Burkholderia_thailandensis    QHLNLVNTAVHQHHTGTEICRQLDGRRADAWVAAVGTGGTLAGVYAALAQ 165
                              *. *  *   :    . *:   :.    . ::*  *****: *.   : :

Bacillus_contig00507          AYKDIKIYAVEPADSPVLSGGKPGP----HKIQGIGAGFIPETLDVEV-- 235
Bacillus_cereus               AYKDIKIYAVEPADSPVLSGGKPGP----HKIQGIGAGFIPETLDVEV-- 241
Bacillus_contig01117          KLPNLYIAVVEPKGSPVLSGGVPGP----HKLVGTSPGFIPKNLNTEV-- 236
Bacillus_thurengiensis        KLPNLYIAVVEPKGSPVLSGGVPGP----HKLVGTSPGFIPKNLNTEV-- 236
Bacillus_contig00780          KNVDIKTVIVEPEGS-ILNGGKAGS----HETEGIGLEFIPPFLKTSY-- 235
Bacillus_cereus1              KNIDIKTVIVEPEGS-ILNGGKAGS----HETEGIGLEFIPPFLKTSY-- 235
Bacillus_contig01190          YTKKAELHLVMPAELPYGSEQPPNDLKKYAGSGGFGLGRKQHFIEQEDNL 242
Burkholderia_thailandensis    QNPDVRVVGCTPDEMPFGTMAPPNGSARFAGAGGLGYGFRQPFVSLIP-F 214
                                 .       *      .   ..         * .       :.

Bacillus_contig00507          YDEIVQVKAEQAFEYARRVAKEEGILVGISSGAVIYAATEIAKKLGKGKK 285
Bacillus_cereus               YDEIVQVKAEQAFEYARRVAKEEGILVGISSGAVIYAATEIAKKLGKGKK 291
Bacillus_contig01117          YNEIIQIADEEALTTMRNLARQEGLLVGPSSGASVYAAIMIAKRLGAGKK 286
Bacillus_thurengiensis        YNEIIQIADEEALTTMRNLARQEGLLVGPSSGASVYAAIMIAKRLGAGKK 286
Bacillus_contig00780          FDEIHTISDRNAFLRVKELAQKEGLLVGSSSGAAFHASLLEAEQAAPGTN 285
Bacillus_cereus1              FDEIHTISDRNAFLRVKELAQKEGLLVGSSSGAAFHASLLEAEKAAPGTN 285
Bacillus_contig01190          IEKQWTYSYEETLYEMARFYNETGMKIGTSAAANLLAAKQIGKEKGANFN 292
Burkholderia_thailandensis    AVPFQTVTHAESLKAMYRFLEETGIPIGGSAAANWIVACNVAAELGRNAT 264
                                        :::    .. .: *: :* *:.*   .:   . . . . .
```

```
Bacillus_contig00507            VLVIIPSNG---ERYLSTPLYQFES----------- 307
Bacillus_cereus                 ILVIIPSNG---ERYLSTPLYQFES----------- 313
Bacillus_contig01117            VLCIAPDTG---ERYLSMGLFE-------------- 305
Bacillus_thurengiensis          VLCIAPDTG---ERYLSMGLFE-------------- 305
Bacillus_contig00780            IVTIFPDSS---ERYLSKDIYKGWE----------- 307
Bacillus_cereus1                IVTIFPDSS---ERYLSKDIYKGWE----------- 307
Bacillus_contig01190            VVTVFPDAGSIEEWSDVKSLQQI------------- 315
Burkholderia_thailandensis      VVTVFADAGSDADRERGRQLMESDKNPIGNPDYANA 300
                                :: : .. .   :      : :
```

B.1.2 Nitrogen metabolism

EC 2.7.2.2: carbamate kinase

Contig	Accession	Description	Query coverage	E value
00200	ZP_04229542.1	Acetylglutamate kinase [*Bacillus cereus* Rock3-29]	100%	2e-63

Alignment

```
Bacillus_contig00200            --------------------------------------------------
Bacillus_cereus                 MSDYIVIKCGGSMLDQLNDVFFDCIKKLQQKYKVVIVHGGGPEIDAKLKE 50


Bacillus_contig00200            --------------------------------------------------
Bacillus_cereus                 CHIKVEKRDGLRVTPKEVMDVVQMVLCGSTNKKFVMNLQKHNLLAVGLSG 100


Bacillus_contig00200            -----------------VGEVSYVETALLKGLITLNYIPVIAPIGINDNE 33
Bacillus_cereus                 CDGNLLQVQPVSEEIGYVGEVSYVETTLLKKLIDMNYIPVIAPIGINGNE 150
                                         *********:*** ** :************.**


Bacillus_contig00200            IYNINADTAAAGIAAALSAKELIFITDVDGILHEGKLVKKTDEIEIVTLI 83
Bacillus_cereus                 VYNINADTAAAGIADALSAKELIFITDVDGILHEGKLVKKTDESEIATFI 200
                                :************* ***************************** **.*:*


Bacillus_contig00200            EKGVITGGMIPKVQAALASLKMGVQKISIVNGTKDFTEVTGECIGTTVTK 133
Bacillus_cereus                 EKGVITGGMIPKVQAALTSLKMGVQKVSIVNGTKDFTEVTGECIGTTVTK 250
                                *****************:********:************************


Bacillus_contig00200            GVSIV 138
Bacillus_cereus                 GVSIV 255
                                *****
```

EC 3.5.1.49: formamidase

Contig	Accession	Description	Query coverage	E value
00039	ZP_04235304.1	Carbon-nitrogen hydrolase [*Bacillus cereus* Rock3-28]	100%	7e-145

Alignment

```
Bacillus_contig00039          ----------------------MKVACIQMDIVFGDVEKNIENAKNKISEA 29
Bacillus_cereus               MTNDIIFPVFCKEKIKNGAEKMKVACIQMDIVFGDVEKNMENAKNKISEA 50
                                                   ******************:**********

Bacillus_contig00039          MKERPDVIVLPELWTTGYDLTRLTEIADRDGLQTKEKLKEWSKQYGVHIV 79
Bacillus_cereus               MKERPDVIVLPELWTTGYDLTRLSEIADRDGLETKEKLKEWSKQYGVHIV 100
                              ***********************:********:*****************

Bacillus_contig00039          GGSIAKQTEQGVTNTMYVVNKEGELVNEYSKVHLFQLMDEHKYLIAGNST 129
Bacillus_cereus               GGSIAKQTEQGVTNTMYVVNNEGDLVNEYSKVHLFQLMDEHKYLIAGNET 150
                              ********************:**:************************.*

Bacillus_contig00039          GEFKLDDVECAGTICYDIRFPEWMRVHTAKGAKVLFVVAEWPLVRLAHWR 179
Bacillus_cereus               GEFKLDDIECAGTICYDIRFPEWMRVHTAKGAKVLFVVAEWPLVRLAHWR 200
                              *******:****************************************

Bacillus_contig00039          LLLQARAVENQCYVVACNRAGKDPSNEFAGHSLIVDPWGEVVVEANEEES 229
Bacillus_cereus               LLLQARAVENQCYVVACNRSGKDPNNEFAGHSLIVDPWGEVVVEANKEES 250
                              ******************:****.**********************:***

Bacillus_contig00039          NLFGELHFEKIKEVRKGIPVFADRRPELYK 259
Bacillus_cereus               ILFGELTFEKIKEVRKGIPVFADRRPELYK 280
                               ***** ************************
```

EC 4.2.1.1: carbonic anhydrase/carbonate dihydratase

Contig	Accession	Description	Query coverage	E value
00414	YP_003654533.1	carbonate dehydratase [*Arcobacter nitrofigilis* DSM 7299]	93%	2e-36

Alignment

```
Bacillus_contig00414          MKGKVLVMKFK-PLKFDNCS------------------------------ 19
Arcobacter_nitrofigilis       MKKYVISLLVSGILLAANASENGEAKTSNETHKKHDSHWSYEGNNGPKFW 50
                              **  *: : ..  *   *.*

Bacillus_contig00414          --------TIDCGDFQSPIEIQPNCAIEMQDQGEILLNYDRTFISIDDKE 61
Arcobacter_nitrofigilis       GTINPSFQTCENGERQSPINITKEATVATNSLGNLFFDYKDTSISIINNG 100
                                      * : *: ****:*  :.::  :. *:::::*. * *** ::

Bacillus_contig00414          DTIKVHNDESGFAIINGRKFQLSEFHFHVGDKDKEQNESEHVLVGHCYKM 111
Arcobacter_nitrofigilis       HTIQVNSDNQSSALFQGKKFKLLQFHFHS--------KSEHTVNGEYYPL 142
                              .**:*:.*:.. *:::*:**:* :****         :***.: *. * :

Bacillus_contig00414          ELHLVHKSQVGRIAVLSVFFKIGCANSAIQTILDNINKKRGVTNTID--- 158
Arcobacter_nitrofigilis       ELHLVHQAEDGELGVVGVFFKLGDYNSSLQKVLKFMPKDAGGKNEIDKFS 192
                              ******::: *.:.*:.****:*  **::*.:*. : *. * .* **

Bacillus_contig00414          IDIKQLLPHETIYYHYLGSLTTNGFIENVEWYVFEEPIEMSQEQYNIFKT 208
Arcobacter_nitrofigilis       INPNDFLPKDRGYYHYLGSLTTPPCTQIVEWYVMKNPITLSQKQLEQFQN 242
                              *: :::**::  **********    : *****:::** :**:* : *:.

Bacillus_contig00414          RYSGNVRCIQNLNGRKILKKKIKLFTK 235
Arcobacter_nitrofigilis       LYNGNFRPTYPLNKRIVLEK------- 262
                               *.**.*    ** * :*:*
```

EC 4.2.1.104: cyanase/cyanate hydratase

Contig	Accession	Description	Query coverage	E value
00854	ZP_04303287.1	Cyanate lyase [*Bacillus cereus* MM3]	100%	7e-82

Alignment

```
Bacillus_contig00854          MKRQVLDMNRPEATKKILEAKITKGLTWEEIAKVSENSETWVVTALLGQA 50
Bacillus_cereus               MKKQVLDMNRQEATQKILEAKIKKGLTWEEIAKVSEHSETWVVTALLGQA 50
                              **:******* ***:*******.**************:**************

Bacillus_contig00854          TMTRPEAEKIGKLLELDEEVVQALTVVPLRGQVMEMPPTDPIAYRLYEMM 100
Bacillus_cereus               TMTRPEAEKIGKLLELDEEVVQALTVVPLRGQVMEMPPTDPIAYRLYEMM 100
                              **************************************************

Bacillus_contig00854          LQYAPTIRELILEKAGEGVMSAINFNLGVDTQEDPKGGDPRIVITLNGKF 150
Bacillus_cereus               LQYAPTIRELILEKAGEGVMSAINFNLGVDTQEDPKGGDPRIVITLNGKF 150
                              **************************************************

Bacillus_contig00854          LPFRTWRTW 159
Bacillus_cereus               LPF------ 153
                              ***
```

EC 6.3.4.16: carbomoyl-phosphate synthase

Contig	Accession	Description	Query coverage	E value
00866	ZP_04187821.1	Biotin carboxylase [*Bacillus cereus* AH1271]	100%	0.0

Alignment

```
Bacillus_contig00866          MIKKVLIANRGEIAVRIIRACKEMDIETVAIYSEADKESLHVQIADEAYC 50
Bacillus_cereus               MIKKVLIANRGEIAVRIIRACKEMDIETVAIYSEADKESLHVQIADEAYC 50
                              **************************************************

Bacillus_contig00866          VGPTISKESYLNLTNIISVAKLTGCDAIHPGYGFLAENADFAELCRECNL 100
Bacillus_cereus               VGPTISKESYLNLTNIISVAKLTGCDAIHPGYGFLAENADFAELCRECNL 100
                              **************************************************

Bacillus_contig00866          IFIGPSPEAISKMGTKDVARDTMKEAGVPIVPGSQGIIKNTEEAIELANQ 150
Bacillus_cereus               IFIGPSPEAISKMGTKDVARDTMKEAGVPIVPGSQGIIKNTEEAIELANQ 150
                              **************************************************

Bacillus_contig00866          IGYPVIIKATAGGGGKGIRVARHEEELVKGIQITQQEASTAFGNPGVYLE 200
Bacillus_cereus               IGYPVIIKATAGGGGKGIRVARHEEELVKGIQITQQEASTAFGNPGVYLE 200
                              **************************************************

Bacillus_contig00866          KYVEDFRHVEIQIMADTHGNAIHLGERDCTIQRRLQKLLEESPSPALDEN 250
Bacillus_cereus               KYVEDFRHVEIQIMADTHGNAIHLGERDCTIQRRLQKLLEESPSPALDEN 250
                              **************************************************

Bacillus_contig00866          VRKQMGDAAVKAAVAVDYTGAGTVEFIYEYRTKSFYFMEMNTRIQVEHPV 300
Bacillus_cereus               VRKQMGDAAVKAAVAVDYTGAGTVEFIYEYRTKSFYFMEMNTRIQVEHPV 300
                              **************************************************
```

```
Bacillus_contig00866          TEMVTGMDLIKEQIRVASGEKLSLQQEEVQFNGWAIECRINAENPAKKFM 350
Bacillus_cereus               TEMVTGMDLIKEQIRVASGEKLSLQQEEVQFNGWAIECRINAENPAKKFM 350
                              **************************************************

Bacillus_contig00866          PSPGKVEMYLPPGGFGIRVDSAVYPGYSIPPFYDSMVAKLIVHGKTREEA 400
Bacillus_cereus               PSPGKVEMYLPPGGFGIRVDSAVYPGYSIPPFYDSMVAKLIVHGKTREEA 400
                              **************************************************

Bacillus_contig00866          IAKMKRALSEFVIEGVHTTIPFHLQLLDHPDFVKGEFNTKFLEEHELVTQ 450
Bacillus_cereus               IAKMKRALSEFVIEGVHTTIPFHLQLLDHPDFVKGEFNTKFLEEHELVTQ 450
                              **************************************************
```

B.1.3 Glycine, serine and threonine metabolism

EC 1.4.3.3: D-amino acid oxidase

Contig	Accession	Description	Query coverage	E value
00553	ZP_04226369.1	Glycine oxidase ThiO [*Bacillus cereus* Rock3-29]	100%	0.0

Alignment

```
Bacillus_contig00553          MCKKYDVAIIGGGVIGSSVAHFLAERGHKVAIVEKQRIASEASKAAAGLL 50
Bacillus_cereus               MCEKYDVAIIGGGVIGSSVAHFLAERGHKVAIIEKQKIASEASKAAAGLL 50
                              **:******************************:***:**************

Bacillus_contig00553          GVQAEWDAYDPLFELARESRAIFPQLAAVLREKTGIDIGYEEKGIYRIAQ 100
Bacillus_cereus               GVQAEWDAYDPLFELARESRAIFPQLATVLREKTGIDIGYEEKGIYRIAQ 100
                              **************************:*********************

Bacillus_contig00553          NEDEKERILHIMEWQQKTGEDSYFLMGDRLREKEPCLSESIIGAVYYPKD 150
Bacillus_cereus               NEDEKERILHIMDWQQKTGEDSYFLTGDHLREKEPFLSESIIGAVYYPKD 150
                              ************:************ **:****** **************

Bacillus_contig00553          GHVIAPELTKAFAHSASISGADIYEQTEVFDIRIENNKVTGIVTSEGIMA 200
Bacillus_cereus               GHVIAPELTKAFAHSASISGADIYEQTEVFDIRMENNKVTGIVTSEGIIT 200
                              **********************************:***************::

Bacillus_contig00553          CEKVVIAGGSWSTKLLGYFHREWGTYPVKGEVVAVRSRKPLLKAPIFQER 250
Bacillus_cereus               CEKVVIAGGSWSTKLLGHFHREWGTYPVKGEVVAVRSRKPLLKAPIFQER 250
                              *****************:**********************************

Bacillus_contig00553          FYIAPKRGGRYVIGATMKPHTFNKTVQPESITSILERAYTILPALKEAEW 300
Bacillus_cereus               FYIAPKRGGRYVIGATMKPHTFNKTVQPESITSILERAYTILPALKEAEW 300
                              **************************************************

Bacillus_contig00553          ESAWAGLRPQSNHEAPYMGEHEEIKGLYACTGHYRNGILLSPVSGQYMAD 350
Bacillus_cereus               ESAWAGLRPQSNHEAPYMGEHEEIKGLYACTGHYRNGILLSPVSGQYMAD 350
                              **************************************************

Bacillus_contig00553          LIEGKQENHLLDSLLSKTV 369
Bacillus_cereus               LIEGKQENHLLDSLLSKTV 369
                              ********************
```

EC 2.1.2.1: glycine hydroxymethyltransferase

Contig	Accession	Description	Query coverage	E value
00365	ZP_04303486.1	Serine hydroxymethyltransferase [*Bacillus cereus* MM3]	100%	0.0
01477	ZP_02330312.1	Glycine hydroxymethyltransferase [*Paenibacillus larvae* subsp. larvae BRL-230010]	98%	5e-172

Alignment

```
Bacillus_contig00365        ----------------------------------------MEAQGSVLTNK 11
Bacillus_cereus             MDHLKRQDEKVFAAIEAELGRQRSKIELIASENFVSEAVMEAQGSVLTNK 50
Paenibacillus_larvae        MSKLAKQDPKILEAMNLELRRQRDKIELIASENFVSEAVMEAMGTVLTNK 50
Bacillus_contig01477        MKKLREKDYQVAEAIDYELQRQQKHIELIASENFVSLAVLEAMGTVLTNK 50
                                                             :** *:***** 

Bacillus_contig00365        YAEGYPGKRYYGGCEHVDVVEDIARDRAKEIFGAEHVNVQPHSGAQANMA 61
Bacillus_cereus             YAEGYPGKRYYGGCEHVDVVEDIARDRAKEIFGAEHVNVQPHSGAQANMA 100
Paenibacillus_larvae        YAEGYPGKRYYGGCECVDIVEGIARDRAKELFGAEHANVQPHSGAQANMA 100
Bacillus_contig01477        YAEGYPGKRYYGGCEYVDIVENLAIERAKQLFGADHANVQPHSGAQANMA 100
                            *************** **:**.:* :***::***:*.************* 

Bacillus_contig00365        VYFTILEQGDTVLGMNLSHGGHLTHGSPVNFSGVQYNFVEYGVDAESHRI 111
Bacillus_cereus             VYFTILEQGDTVLGMNLSHGGHLTHGSPVNFSGVQYNFVEYGVDAESHRI 150
Paenibacillus_larvae        VYLAALKPGDTVLGMNLSHGGHLTHGSPVNASGILYNFVEYGVSEEDFRI 150
Bacillus_contig01477        VYSACLKPGDTVLGMDLSHGGHLTHGSPVNSSGVLYNFISYGVNKRHFNI 150
                            ** : *: ******:************** **: ***:.***. . ..* 

Bacillus_contig00365        NYDDVLAKAKEHKPKLIVAGASAYPRVIDFKRFREIADEVGAYFMVDMAH 161
Bacillus_cereus             NYDDVLAKAKEHKPKLIVAGASAYPRVIDFKRFREIADEVGAYFMVDMAH 200
Paenibacillus_larvae        DYDKVRKLAFKHRPRLIVAGASAYPRTIDFEALGRIAQDVGALFMVDMAH 200
Bacillus_contig01477        DYDEVRTLALKHQPKMMVIGASAYSRIIDFERLSSIAKEVNAFFMADIAH 200
                            :**.*   * :*:*:::* *****.* ***: :  **.:*.* **.*:** 

Bacillus_contig00365        IAGLVAAGLHPNPVPHAHFVTTTTHKTLRGPRGGMILCEEQFAKQIDKSI 211
Bacillus_cereus             IAGLVAAGLHPNPVPHAHFVTTTTHKTLRGPRGGMILCEEQFAKQIDKSI 250
Paenibacillus_larvae        IAGLVAVGLHPSPVPHAHFVTTTTHKTLRGPRGGLILCKKPWAAAIDKAV 250
Bacillus_contig01477        IAGLVATGMHPSPVPYADFVTTTTHKTLRGPRGGIILCRRDWAATIDKSV 250
                            ******.*:**.***:*.****************:***.. :*  ***:: 

Bacillus_contig00365        FPGIQGGPLMHVIAAKAVAFGETLQDEFKTYAQNIINNANRLAEGLQKEG 261
Bacillus_cereus             FPGIQGGPLMHVIAAKAVAFGETLQDDFKTYAQNIINNANRLAEGLQKEG 300
Paenibacillus_larvae        FPGTQGGPLMHIIAAKAVALGEALQPEFKTYARNVIDNAAVLSQSLQAEG 300
Bacillus_contig01477        FPGIQGGPAMHMIAAKAVAFNEALQPEFKKYVSNTIKNAKVLAKELRELG 300
                            *** **** **:*******:.*:** :**.*. * *.**  *:: *:  * 

Bacillus_contig00365        LTLVSGGTDNHLILIDVRNLEITGKVAEHVLDEVGITVNKNTIPFETASP 311
Bacillus_cereus             LTLVSGGTDNHLILIDVRNLEITGKVAEHVLDEVGITVNKNTIPFETASP 350
Paenibacillus_larvae        LHVVSGGTDNHLILIDLRNLNITGKEAEHILDEVGITVNKNAIPFDPTSP 350
Bacillus_contig01477        FNVLTGGTDNHLLLIDLCNMNLTGKEAQILLEEVGITVNKNSIPFDKKGP 350
                            : :::*******:***: *:::*** *: :*:********:***:  .* 

Bacillus_contig00365        FVTSGVRIGTAAVTSRGFGLEDMDEIASLIAYTLKNHENEAALEEASKRV 361
Bacillus_cereus             FVTSGVRIGTAAVTSRGFGLEEMDEIASLIAYTLKNHENEAALEEASKRV 400
Paenibacillus_larvae        FITSGVRIGTPAATSRGMGREAMKDIARIISLTLKNPSDETALEKARAMV 400
Bacillus_contig01477        FITSGIRLGTPAVTTQGMGELEMIKIAEIICLVLKNPQNVRAKEKACSIV 400
                            *:***:*:**.*.*::*:*   * .** :*. .*** .:  * *:*   * 
```

```
Bacillus_contig00365          EALTSKFPMYTDL- 374
Bacillus_cereus               EALTSKFPMYTDL- 413
Paenibacillus_larvae          NELTSQYPL----- 409
Bacillus_contig01477          KELTKEFPIYKDIT 414
                              : **.::*:
```

EC 2.6.1.44: alanine glyoxylate transaminase

Contig	Accession	Description	Query coverage	E value
00999	ZP_03112552.1	4-aminobutyrate aminotransferase [*Bacillus cereus* 03BB108]	99%	0.0

Alignment

```
Bacillus_contig00999           MLEKNVGIHFATKRENNVKKRMGYVMNTKKFAKVNEQIPGSKAASLLERR 50
Bacillus_cereus               -MEKNVGIHFAIKRENNVKKRMGYVMNTKKFAKVNEQIPGPKAASLLERR 49
                               :********* ****************************.*********

Bacillus_contig00999          QNIVPKGVSNGIPTFVQSANGALLTDVDGNQYIDFAGAIGTINVGHCHPA 100
Bacillus_cereus               QNIVPKGVSNGIPTFVQSANGALVTDVDGNQYIDFAGAIGTINVGHCHPA 99
                              ************************:**************************

Bacillus_contig00999          VKEALHKQVDQYIHTGFNVMMYEPYIELAEKLAALAPGSFDKQVLFLNSG 150
Bacillus_cereus               VKEALHKQVDQYIHTGFNVMMYEPYIELAEKLAALAPGSFDKQVLFLNSG 149
                              **************************************************

Bacillus_contig00999          AEAVENAVKIARKYTKRPGIIAFSKGFHGRTLMTMTMTSKVKPYKFGFGP 200
Bacillus_cereus               AEAVENAVKIARKYTKRPGIIAFSKGFHGRTLMTMTMTSKVKPYKFGFGP 199
                              **************************************************

Bacillus_contig00999          FAPEVYKAPFPYEYRRPEGLTEEQYDDFMIEEFKNFFISEVAPETIAAVV 250
Bacillus_cereus               FAPEVYKAPFPYEYRRPEGLTEEQYDDFMIEEFKNFFISEVAPETIAAVV 249
                              **************************************************

Bacillus_contig00999          MEPVQGEGGFIVPSKKFVQEVRNICSEHGILFVADEIQTGFSRTGKYFAI 300
Bacillus_cereus               MEPVQGEGGFIVPSKKFVQEVRNICSEHGILFVADEIQTGFSRTGKYFAI 299
                              **************************************************

Bacillus_contig00999          DHYDVVPDLITVSKSLGAGVPISGVIGRKEIMNESAPGELGGTYAGSPLG 350
Bacillus_cereus               DHYDVVPDLITVSKSLGAGVPISGVIGRKEIMNESAPGELGGTYAGSPLG 349
                              **************************************************

Bacillus_contig00999          CAAALAVLDVIEKENLNDRAIELGKVVMNRFEEMKNKYNCIGDVRGLGAM 400
Bacillus_cereus               CAAALAVLDVIEKENLNDRAIELGKVVMNRFEEMKNKYNCIGDVRGLGAM 399
                              **************************************************

Bacillus_contig00999          CAFELVQDRKTKAPDKMLTANLCAEANKRGLLLLSAGTYGNVIRVLMPLV 450
Bacillus_cereus               CAFELVQDRKTKAPDKTLTANLCAEANKRGLLLLSAGTYGNVIRVLMPLV 449
                              *************** **********************************

Bacillus_contig00999          ITDEQLEEGLTIIEESLQVCYEKANTARV 479
Bacillus_cereus               ITDEQLEEGLTIIEESLQVCYEKANIARV 478
                              ************************* ***
```

B.1.4 Glyoxylate and dicarboxylate metabolism

EC 1.1.1.60: 2-hydroxy-3-oxo propionate reductase

Contig	Accession	Description	Query coverage	E value
01091	ZP_04302241.1	3-hydroxyisobutyrate dehydrogenase [*Bacillus cereus* MM3]	100%	2e-167

Alignment

```
Bacillus_contig01091          MERKNVSIGFIGIGVMGKSMVRHLMQDGYQVYVYNRTKMKTDSLVQDGAN 50
Bacillus_cereus               MERKNVSIGFIGIGVMGKSMVRHLMQDGYKVYVYNRTKTKTDSLVQDGAD 50
                              ****************************:******** **********:

Bacillus_contig01091          WCDTPKELVKQVDVVMTMVGYPHDVEEVYFGIDGILENANEGTIAIDFTT 100
Bacillus_cereus               WCDTPKELVKQVDVVMTMVGYPHDVEEVYFGIDGILENANEGTIAIDFTT 100
                              **************************************************

Bacillus_contig01091          STPTLAKRINETGKSKNVYTLDAPVSGGDVGAKEARLAIMVGGEKEIYEK 150
Bacillus_cereus               STPTLAKRINETGKSKNVYTLDAPVSGGDVGAKEARLAIMVGGEKEIYEK 150
                              **************************************************

Bacillus_contig01091          CLPLFEKLGTNIQLQGPAGSGQHTKMCNQIAIASNMIGVCEAVAYAKKAG 200
Bacillus_cereus               CLPLFEKLGTNIQLQGPAGSGQHTKMCNQIAIASNMIGVCEAVAYAKKAG 200
                              **************************************************

Bacillus_contig01091          LDPDKVLESISTGAAGSWSLSNLAPRMLKGDFEPGFYVKHFMKDMKIALD 250
Bacillus_cereus               LDPDKVLESISTGAAGSWSLSNLAPRMLKGDFEPGFYVKHFMKDMKIALD 250
                              **************************************************

Bacillus_contig01091          EAEKLELPVPGLSLAKELYEELIKDGEENSGTQVLYKKYIKG 292
Bacillus_cereus               EAEKLQLPVPGLSLAKELYEELMKDGEENSGTQVLYKKYIRG 292
                              *****:****************:******************:*
```

EC 1.2.2.1: formate dehydrogenase

Contig	Accession	Description	Query coverage	E value
00075	ZP_04299718.1	2-hydroxyacid dehydrogenase [*Bacillus cereus* MM3]	100%	1e-171

Alignment

```
Bacillus_contig00075          ---------------------------MYDKDALISLDELTDRVKDKDAL 23
Bacillus_cereus               MLVVAKILVAGKIPAIGLELLKDHDVEMYDKDALISLDELTDRVKDKDAL 50
                                                         ************************

Bacillus_contig00075          LSLLSTKVTKEVIDAAPNLKIVANYGAGYDNIDYSYAGEKGIAVTNTPKV 73
Bacillus_cereus               LSLLSTKVTKEVIDAAPNLKIVANYGAGYDNIDYSYAGEKGIAVTNTPKV 100
                              **************************************************

Bacillus_contig00075          STEATAELTFALLLAAARRIPEGDTLCRTTGFNGWAPLFFLGREVHGKTI 123
Bacillus_cereus               STEATAELTFALLLAAARRIPEGDTLCRTTGFNGWAPLFFLGREVHGKTI 150
                              **************************************************

Bacillus_contig00075          GIIGLGEIGKAVAKRAKAFGMNVLYTGPNRKPEAESELEATYVTLEELVQ 173
Bacillus_cereus               GIIGLGEIGKAVAKRAKAFGMNVLYTGPNRKPEAESELEATYVTLEELLQ 200
                              **************************************************:*

Bacillus_contig00075          TADFITINCAYNPKLHHMIDDEQFKMMKKTAYIVNASRGPIMNEAALAHA 223
Bacillus_cereus               TADFITINCAYNPKLHHMIDDEQFKMMKKTAYIVNASRGPIMNEAALAHA 250
                              **************************************************

Bacillus_contig00075          LKTNEIEGAALDVFEFEPKITEELKGLKNVVLAPHVGNATFETRDAMAEM 273
Bacillus_cereus               LKTNEIEGAALDVFEFEPKITEELKGLKNVVLAPHVGNATFETRDAMAEM 300
                              **************************************************

Bacillus_contig00075          AVRNILAVLKGEEPVTPVNQKVVVTK 299
Bacillus_cereus               AVRNILAVLNGEEPVTPVNQKVVVTK 326
                              *********:******************
```

EC 1.2.3.5: glyoxylate oxidase

Contig	Accession	Description	Query coverage	E value
00459	ZP_04174566.1	2,3-dihydroxybenzoate-AMP ligase [*Bacillus cereus* AH1273]	100%	0.0

Alignment

```
Bacillus_contig00459          MLTGYTEWPEEFANRYREVGCWPGETFGSLLRERAEKHGDEIAVVSGDTQ 50
Bacillus_cereus               MLTGYTEWPEEFANRYREEGCWLGETFGSLLRERAEKYGDQIAVVSGDTH 50
                              ****************** *** ***************:**:********:

Bacillus_contig00459          ITYSELDKKVDRLAAGLLDLGIKQEDRVVIQLPNIIEFFEICFALFRIGA 100
Bacillus_cereus               ITYSELDKKVDRLAAGLLNLGVKQEDRVVIQLPNIIEFFEICFALFRIGA 100
                              ******************:**:****************************

Bacillus_contig00459          LPVFALPSHRSSEISYFCEFGEASAYVISDKALGFDYRKLAREVKEKVPT 150
Bacillus_cereus               LPVFALPSHRSSEISYFCEFGEASAYVISDKALGFDYRKLAREVKEKVPT 150
                              **************************************************

Bacillus_contig00459          LQHVIVVGEEEEFVSINELYMDPVPLPEVQPSDVAFLQLSGGTTGLSKLI 200
Bacillus_cereus               LQHVIVAGEEEEFVNISDLYIDSVPLPEVKPSDVAFLQLSGGTTGLSKLI 200
                              ******.*******.*.:**:*.******:********************

Bacillus_contig00459          PRTHDDYIYSLRVSAEICNLNADSVYMAVLPVAHNYPMSSPGTFGTFYAG 250
Bacillus_cereus               PRTHDDYIYSLRVSAEICNLNAESVYMAILPVAHNYPMSSPGTFGTFYAG 250
                              **********************:*****:********************
```

```
Bacillus_contig00459          GKVVLATGGSPDEAFALIEKEKVTITALVPPLAMIWLDAASSRNNDLSSL 300
Bacillus_cereus               GKVVLATGGSPDEAFALIEKEKVTITALVPPLAMIWLDAASSRNNDLSSL 300
                              **************************************************

Bacillus_contig00459          EVIQVGGAKFSAEVAKRIHPTFGCTLQQVFGMAEGLVNYTRLNDPEEIII 350
Bacillus_cereus               QVIQVGGAKFSAEVAKRIRPTFGCTLQQVFGMAEGLVNYTRLNDPEEIII 350
                              :*****************:****************************

Bacillus_contig00459          HTQGRPMSTFDEVRVVDENDNDVKPGEVGSLLTRGPYTIRGYYKAEEHNA 400
Bacillus_cereus               HTQGRPMSTFDEVRVVDENDNDVKPGEVGSLLTRGPYTIRGYYKAEEHNV 400
                              **************************************************.

Bacillus_contig00459          RSFTEDGFYRTGDLVKVNEQGYIIVEGRDKDQINRGGEKVAAEEVENHLL 450
Bacillus_cereus               RSFTKDGFYRTGDLVKVNEQGYIIVEGRDKDQINRGGEKVAAEEVENHLL 450
                              ****:*********************************************

Bacillus_contig00459          AHDAVHDVAIVSMPDDYLGERTCAFVIARGQAPTVSELKMFLRERGIAAY 500
Bacillus_cereus               AHDAVHDVAIVSMPDDYLGERTCAFVIARGQAPTVSELKMFLRERGIAAY 500
                              **************************************************

Bacillus_contig00459          KIPDRIEFIDSFPQTGVGKVSKKELRKVITEKLITVKQ 538
Bacillus_cereus               KIPDRIEFIESFPQTGVGKVSKKELRKVITEKLITAKR 538
                              *********:**************************.*:
```

EC 2.7.1.31: glycerate kinase

Contig	Accession	Description	Query coverage	E value
00366	ZP_04298604.1	Glycerate kinase [*Bacillus cereus* MM3]	98%	0.0

Alignment

```
Bacillus_contig00366          MGIIVKVVIASDSYKESLKAIKVCEAIERGFEAIFPKAEYVKIPIGDGGE 50
Bacillus_cereus               ----MKVVIASDSYKESLKAIKVCEAIERGFGAIFPKAEYVKIPIGDGGE 46
                                  :************************ ******************

Bacillus_contig00366          GTVDSLVDATDGRMIPLHVTGPLRERIQSFYGMSKDKKTAFIEMAAASGL 100
Bacillus_cereus               GTVDSLVDATDGRMIPFHVTGPLRERIQAFYGMSKDKKTAFIEMAAASGL 96
                              ****************:************:**********************

Bacillus_contig00366          QHVPVKKRNPLITTTKGTGELILHALDEGAEHIILGLGGSATNDGGAGML 150
Bacillus_cereus               QHVPVKKRNPLITTTKGTGELILHALDEGAEHIILGLGGSATNDGGAGML 146
                              **************************************************

Bacillus_contig00366          SALGVRFINGKGEVIEPSGGTLHSIVAIDFSCMDSRLLHIKIEAACDVDN 200
Bacillus_cereus               SALGVRFINGKGEVIEPSGGTLHSIVAIDFSCMDARLMHIKIEAACDVDN 196
                              ***********************************:**:************

Bacillus_contig00366          PLVGIRGASFVFGRQKGANAEMMKELDENLKHYANILKQYLSCDVSKIPG 250
Bacillus_cereus               PLVGIRGASFVFGRQKGANAEMMKELDENLKHYANILKQYLSCDVAEIPG 246
                              **********************************************::***

Bacillus_contig00366          AGAAGGMGAAVIAVLKGNLRRGIEIVLDYTNFDKHIEGADLIITGEGRID 300
Bacillus_cereus               AGAAGGMGAAVIAVLKGSLRRGIEIVLDYTNFNKHIEGADLIITGEGRID 296
                              *****************.**************:*****************

Bacillus_contig00366          EQTAYGKAPVGVAERAKHFRIPVIAIGGSVSPNYPAVHEKGIDAVFSITA 350
Bacillus_cereus               EQTAYGKAPVGVAECAKHFRIPVIAIGGSVSPNYSAVHEKGIDAVFSITA 346
                              ************** ******************.***************
```

```
Bacillus_contig00366          SPMTLEEAYKVAEENIEMTAKNIAAVWKIASEKHF 385
Bacillus_cereus               SPMTLEEAYKVAEENIEMTAKNIAAVWKIASEKHF 381
                              ************************************
```

EC 4.1.1.8: oxylyl-CoA decarboxylase

Contig	Accession	Description	Query coverage	E value
00470	ZP_04299217.1	Acetolactate synthase [*Bacillus cereus* MM3]	100%	0.0

Alignment

```
Bacillus_contig00470          MISLSTGVKANDVKTKTKGADLVVDCLIKQGVTHVFGIPGAKIDSVFDVL 50
Bacillus_cereus               MISLSTGVKANDVKTKTKGADLVVDCLIKQGVTHVFGIPGAKIDSVFDVL 50
                              **************************************************

Bacillus_contig00470          QERGPELIVCRHEQNAAFMAAAIGRLTGKPGVCLVTSGPGTSNLATGLVT 100
Bacillus_cereus               QERGPELIVCRHEQNAAFMAAAIGRLTGKPGVCLVTSGPGTSNLATGLVT 100
                              **************************************************

Bacillus_contig00470          ANAESDPVVALAGAVPRTDRLKRTHQSMDNAALFEPITKYSVEVEHPDNV 150
Bacillus_cereus               ANAESDPVVALAGAVPRTDRLKRTHQSMDNAALFEPITKYSVEVEHPDNV 150
                              **************************************************

Bacillus_contig00470          PEALSNAFRSATSTNPGATLVSLPQDVMTAETTVESIGALSKPQLGIAPT 200
Bacillus_cereus               PEALSNAFRSATSTNPGATLVSLPQDVMTAETTVESIGALSKPQLGIAPT 200
                              **************************************************

Bacillus_contig00470          HEITYVVEKIKSAKLPVILLGMRASTNAVTKAVRELIADTELPVVETYQA 250
Bacillus_cereus               HEITYVVEKIKSAKLPVILLGMRASTNEVTRAVRELIADTELPVVETYQA 250
                              *************************** **:******************

Bacillus_contig00470          AGAISRELEDHFFGRVGLFRNQPGDILLEEADLVISIGYDPIEYDPKFWN 300
Bacillus_cereus               AGAISRELEDHFFGRVGLFRNQPGDILLEEADLVISIGYDPIEYDPKFWN 300
                              **************************************************

Bacillus_contig00470          KLGDRTIIHLDDHQADIDHDYQPERELIGDIALTVNSIAEKLPKLVLSSK 350
Bacillus_cereus               KLGDRTIIHLDDHQADIDHDYQPERELIGDIALTVNSIAEKLPKLVLSTK 350
                              **************************************************:*

Bacillus_contig00470          SEAVLERLRAKLSEQAEVPDRASEGVTHPLQVIRTLRSLISDDTTVTCDI 400
Bacillus_cereus               SEAVLERLRAKLSEQAEVPNRASEGVTHPLQVIRTLRSLISDDTTVTCDI 400
                              ********************:*******************************

Bacillus_contig00470          GSHSIWMARCFRSYEPRRLLFSNGMQTLGVALPWAIAATLVEPGKKVVSV 450
Bacillus_cereus               GSHSIWMARCFRSYEPRRLLFSNGMQTLGVALPWAIAATLVEPGKKVVSV 450
                              **************************************************

Bacillus_contig00470          SGDGGFLFSSMELETAVRLNAPIVHLVWRDGTYDMVAFQQMMKYGRTSAT 500
Bacillus_cereus               SGDGGFLFSSMELETAVRLNAPIVHLVWRDGTYDMVAFQQMMKYGRTSAT 500
                              **************************************************


Bacillus_contig00470          EFGDVDLVKYAESFGAKGLRVNTPDELEGVLKEALAADGPVIIDVPIDYR 550
Bacillus_cereus               EFGDVDLVKYAESFGAKGLRVNTPDELEGVLKEALAADGPVIIDVPIDYR 550
                              **************************************************

Bacillus_contig00470          DNIKLSEKLLPNQLN 565
Bacillus_cereus               DNIKLSEKLLPNQLN 565
                              ***************
```

EC 4.1.1.47: tartranate semialdehyde synthase

Contig	Accession	Description	Query coverage	E value
00305	ZP_04299700.1	hypothetical protein bcere0006_12490 [*Bacillus cereus* MM3]	100%	0.0

Alignment

```
Bacillus_contig00305          MSSKTEEKLATGAQLLLEALEKEGVEVIFGYPGGAVLPLYDALYDCEIPH 50
Bacillus_cereus               MSSKTEEKLATGAQLLLEALEKEGVEVIFGYPGGAVLPLYDALYDCEIPH 50
                              **************************************************

Bacillus_contig00305          ILTRHEQGAIHAAEGYARITGNPGVVIATSGPGATNVITGLADAMIDSLP 100
Bacillus_cereus               ILTRHEQGAIHAAEGYARITGNPGVVIATSGPGATNVITGLADAMIDSLP 100
                              **************************************************

Bacillus_contig00305          LVVFTGQVATTLIGSDAFQEADIMGLTMPVTKHNYQVRKASDLPRIIKEA 150
Bacillus_cereus               LVVFTGQVATTLIGSDAFQEADIMGLTMPVTKHNYQVRKASDLPRIIKEA 150
                              **************************************************

Bacillus_contig00305          FHIARTGRPGPVVIDLPKDMVVEQGERCNDVQMDLPGYNPNYEPNLLQIN 200
Bacillus_cereus               FHIARTGRPGPVVIDLPKDMVVEQGERCNDVQMDLPGYNPNYEPNLLQIN 200
                              **************************************************

Bacillus_contig00305          KLLQAIRAANKPLILAGAGVLHAKASKELTSFARKYEIPVVHTLLGLGGF 250
Bacillus_cereus               KLLQAIKAANKPLILAGAGVLHAKASKELTSFARKYEIPVVHTLLGLGGF 250
                              ******:*******************************************

Bacillus_contig00305          PPDDELFLGMGGMHGSYTANMALYECDLLINIGARFDDRLTGNLAYFAKK 300
Bacillus_cereus               PPDDELFLGMGGMHGSYTANMALYECDLLINIGARFDDRLTGNLAYFAKK 300
                              **************************************************

Bacillus_contig00305          ATVAHIDIDPAEIGKNVPTEIPIVASAKRALEVLLQPEGRKENHHKWLSL 350
Bacillus_cereus               ATVAHIDIDPAEIGKNVPTEIPIVASAKRALEVLLQPEGKKENHHEWISL 350
                              ***************************************:*****:*:**

Bacillus_contig00305          LKSRKEKYPLSYKRNSESIKPQYAIDMLYEITKGEAIVTTDVGQHQMWAA 400
Bacillus_cereus               LKSRKEKYPFSYKRNSESIKPQYAIDMLYEITKGEAIVTTDVGQHQMWAA 400
                              *********:******************************************

Bacillus_contig00305          QYYPLKTPDKWVTSGGLGTMGFGFPAAIGAQIAKREELVIAIVGDAGFQM 450
Bacillus_cereus               QYYLLKTPDKWVTSGGLGTMGFGFPAAIGAQIAKPEELVIAIVGDAGFQM 450
                              *** ***************************** ***************

Bacillus_contig00305          TLQELSVLKEHSLPVKVFILNNEALGMVRQWQDEFYNKRYSHSLLSCQPD 500
Bacillus_cereus               TLQELSVLKEHSLPVKVFILNNEALGMVRQWQDEFYNQRYSHSLLSCQPD 500
                              ************************************:*************

Bacillus_contig00305          FVALANAYGIKGVRIDDPLLAKKQIQQAIKLQEPVVIDCRVLQSEKVMPM 550
Bacillus_cereus               FVALANAYGIKGVRIDDPLLAKKQIQQAIELQEPVVIDCRVLQSEKVMPM 550
                              ****************************:*********************

Bacillus_contig00305          VAPGKGVHQMEGVEKR 566
Bacillus_cereus               VAPGKGVHQMEGVEKR 566
                              ****************
```

B.1.5 Carbon fixation metabolism

EC 2.7.2.3: phosphoglycerate kinase

Contig	Accession	Description	Query coverage	E value
00491	YP_001647706.1	phosphoglycerate kinase [*Bacillus weihenstephanensis* KBAB4]	100%	0.0

Alignment

```
Bacillus_contig00491            MNKKSIRDVDLKGKRVFCRVDFNVPMKEGKITDETRIRAALPTIQYLVEQ 50
Bacillus_weihenstephanensis     MNKKSIRDVDLKGKRVFCRVDFNVPMKEGKITDETRIRAALPTIQYLIEQ 50
                                **************************************************:**

Bacillus_contig00491            GAKVILASHLGRPKGQAVEELRLTPVAARLGELLGKDVKKADEAFGPVAQ 100
Bacillus_weihenstephanensis     GAKVILASHLGRPKGQAVEELRLTPVAARLGELLGKDVKKADEAFGPVAQ 100
                                **************************************************

Bacillus_contig00491            EMVAAMNEGDVLVLENVRFYAGEEKNDAELAKEFAALADIFVNDAFGAAH 150
Bacillus_weihenstephanensis     EMVAAMNEGDVLVLENVRFYAGEEKNDAELAKEFAALADIFVNDAFGAAH 150
                                **************************************************

Bacillus_contig00491            RAHASTAGIADYLPAVSGLLMEKELDVLGKALSNPERPFTAIIGGAKVKD 200
Bacillus_weihenstephanensis     RAHASTAGIADYLPAVSGLLMEKELDVLGKALSNPERPFTAIIGGAKVKD 200
                                **************************************************

Bacillus_contig00491            KIGVIRHLLDKVDNLIIGGGLAYTFVKALGHEIGLSLCENDKIELAKEFM 250
Bacillus_weihenstephanensis     KIGVIRHLLDKVDNLIIGGGLAYTFVKALGHEIGLSLCENDKIELAKEFM 250
                                **************************************************

Bacillus_contig00491            QLAKEKGVNFYMPVDVVITEEFSETATTQIVGIDSIPSTWEGVDIGPKTR 300
Bacillus_weihenstephanensis     QLAKEKGVNFYMPVDVVITEEFSETATTQIVGIDSIPSTWEGVDIGPKTR 300
                                **************************************************

Bacillus_contig00491            EIYADVIKNSKLVVWNGPMGVFEMTPFAEGTKAVGQALADAEDTYSVIGG 350
Bacillus_weihenstephanensis     EIYADVIKNSKLVVWNGPMGVFEMTPFAEGTKAVGQALADAEDTYSVIGG 350
                                **************************************************

Bacillus_contig00491            GDSAAAVEKFGMADKMSHISTGGGASLEFMEGKELPGVVCLNDK 394
Bacillus_weihenstephanensis     GDSAAAVEKFGMADKMSHISTGGGASLEFMEGKELPGVVCLNDK 394
                                *********************************************
```

B.1.6 Methane metabolism

EC 1.2.1.2: formate dehydrogenase

Contig	Accession	Description	Query coverage	E value
00442	ZP_04184506.1	formate dehydrogenase [*Bacillus cereus* AH1271]	100%	0.0
00320	ZP_04301767.1	formate dehydrogenase [*Bacillus cereus* MM3]	100%	0.0
00202	ZP_04146788.1	formate dehydrogenase [*Bacillus thuringiensis* BGSC 4Y1]	97%	0.0

Multiple alignments

```
Bacillus_contig00442          --------------------------------------------------
Bacillus_cereus               --------------------------------------------------
Bacillus_contig00202          --------------------------------------------------
Bacillus_thuringiensis        --------------------------------------------------
Bacillus_contig00320          --------------------------------------------------
Bacillus_cereus1              MVHITIDGKKYTAEPGSTVLGVINENGIEHPQICYVPEVDPIQTCDTCIV 50


Bacillus_contig00442          --------------------------------------------------
Bacillus_cereus               --------------------------------------------------
Bacillus_contig00202          --------------------------------------------------
Bacillus_thuringiensis        --------------------------------------------------
Bacillus_contig00320          --------------------------------------------------
Bacillus_cereus1              EVDGKLMRSCSTVTTEGMNIERNSARAKEAQTEAMDRLLENHLLYCTVCD 100


Bacillus_contig00442          --------------------------------------------------
Bacillus_cereus               --------------------------------------------------
Bacillus_contig00202          --------------------------------------------------
Bacillus_thuringiensis        --------------------------------------------------
Bacillus_contig00320          --------------------------------------------------
Bacillus_cereus1              NNNGNCKLHNTAELMEIEHQKYPYEPKVDVSEVDMTHPFYRYDPNQCIAC 150


Bacillus_contig00442          --------------------------------------------------
Bacillus_cereus               --------------------------------------------------
Bacillus_contig00202          --------------------------------------------------
Bacillus_thuringiensis        --------------------------------------------------
Bacillus_contig00320          --------------------------------------------------
Bacillus_cereus1              GQCVEVCQNLQVNETLSIDWEAERPRVIWDEGVNINDSSCVSCGQCVTIC 200


Bacillus_contig00442          --------------------------------------------------
Bacillus_cereus               --------------------------------------------------
Bacillus_contig00202          --------------------------------------------------
Bacillus_thuringiensis        --------------------------------------------------
Bacillus_contig00320          --------------------------------------------------
Bacillus_cereus1              PCNALMEKTMLGEAGFMTGLKPDILEPMVDLIKEVEPGYSGIFAVSEVEA 250


Bacillus_contig00442          --------------------------------------------------
Bacillus_cereus               --------------------------------------------------
Bacillus_contig00202          --------------------------------------------------
Bacillus_thuringiensis        --------------------------------------------------
Bacillus_contig00320          --------------------------------------------------
Bacillus_cereus1              AMRDTRTKKTKTVCTFCGVGCSFEVWTKGRKILKVQPSSNAPVNAISTCV 300


Bacillus_contig00442          --------------------------------------------------
Bacillus_cereus               --------------------------------------------------
Bacillus_contig00202          --------------------------------------------------
Bacillus_thuringiensis        --------------------------------------------------
Bacillus_contig00320          --------------------------------------------------
Bacillus_cereus1              KGKFGWDFVNSKERITKPLIRKNGTFVESTWEEALNVVASKLGSIKEEYG 350


Bacillus_contig00442          --------------------------------------------------
Bacillus_cereus               --------------------------------------------------
Bacillus_contig00202          --------------------------------------------------
Bacillus_thuringiensis        --------------------------------------------------
Bacillus_contig00320          --------------------------------------------------
Bacillus_cereus1              KDAVGFISSSKITNEDNYVIQKLARQVFETNNIDNCSRYCQSPATDGLFR 400
```


Bacillus_contig00442
Bacillus_cereus
Bacillus_contig00202
Bacillus_thuringiensis
Bacillus_contig00320
Bacillus_cereus1

```
Bacillus_contig00442          KFGWDFVNSEDRITKPLIRQGDMFVEASWEEALEVVASNMQHIKSEYGSD 356
Bacillus_cereus               KFGWDFVNSEDRITKPLIRQGDMFVEASWEEALEVVASNMQHIKSEYGSD 356
Bacillus_contig00202          KFGWDFVNSKERITKPLIRKNGAFVESTWEEALNVVASKLGSIKEEYGKD 409
Bacillus_thuringiensis        KFGWDFVNSKERITKPLIRKNGTFVESTWEEALNVVASKLGSIKEEYGKD 399
Bacillus_contig00320          WNSFHWG-SFDGTNTPLLFQDGFNFPD--KKARFAIADWVR--PAEFPAE 359
Bacillus_cereus1              WNSFHWG-SFDGTNTPLLFQDGFNFPD--KRARFAIADWVR--PAEFPAE 817
                                .:.:  * :  ..**: :..  .    :.*  .:*. :     *:  :

Bacillus_contig00442          AFGFISSSKVTNEENYLMQKLARQIYGTNNVDNCSRYCQSPATDGLFKTV 406
Bacillus_cereus               AFGFISSSKVTNEENYLMQKLARQIYGTNNVDNCSRYCQSPATDGLFKTV 406
Bacillus_contig00202          AIGFISSSKITNEDNYVIQKLARQVFETNNIDNCSRYCQSPATDGLFRTI 459
Bacillus_thuringiensis        AIGFISSSKITNEDNYVIQKLARQVFETNNIDNCSRYCQSPATDGLFRTI 449
Bacillus_contig00320          FDLHINNGRMLEH-----------FHEGNMTNKSTGIQTKVP-GVFVEV 396
Bacillus_cereus1              FDLHINNGRMLEH-----------FHEGNMTNKSTGIQTKVP-GVFVEV 854
                                 .*...:: :.            :  .*: * *   *: .. *:*  :

Bacillus_contig00442          GMGGDAGTVKDIAEAGLVIIVGANPTEGHPVLATRVKRAHKLHEQKLIVA 456
Bacillus_cereus               GMGGDAGTVKDIAEAGLVIIVGANPTEGHPVLATRVKRAHKLHEQKLIVA 456
Bacillus_contig00202          GMGGDAGTIKDIAKSGLVIIVG--------------------------- 481
Bacillus_thuringiensis        GMGGDAGTIKDIAQSGLVIIVGCNPTEGHPVLATRIKRAHKLHGQKLIVA 499
Bacillus_contig00320          SS--ALAKERGVKTGSLVRLVSP--------------------------- 417
Bacillus_cereus1              SP--ALAKERGVKTGSLVRLVSP--------------------------- 875
                              .     .. :.:  ..** :*.

Bacillus_contig00442          DLRKHEMAERADLFIHPRQGTDYVWLAGITKYIIDQDWHDKKFLAENVKN 506
Bacillus_cereus               DLRKHEMAERADLFIHPRQGTDYVWLAGITKYIIDQDWHDKKFLAENVKN 506
Bacillus_contig00202          ---------------------------------------------------
Bacillus_thuringiensis        DLRKTEMAERSDVFISPKQGTDQVWLMAVTKYMIDQGWHDQRFIDENVNF 549
Bacillus_contig00320          ---------------------------------------------------
Bacillus_cereus1              ---------------------------------------------------

Bacillus_contig00442          FDEYSKMLEKYTLDYTEEITGISKTQLKEMARMVYEADGTCILWGMGVTQ 556
Bacillus_cereus               FDEYSKMLEKYTLDYTEKITGISKENLKEMARMVYEADGTCILWGMGVTQ 556
Bacillus_contig00202          ---------------------------------------------------
Bacillus_thuringiensis        FEDFKESLAEYTLEYAEEVTGISQETLIQMAEMIRDADGTCILWGMGVTQ 599
Bacillus_contig00320          ---------------------------------------------------
Bacillus_cereus1              ---------------------------------------------------

Bacillus_contig00442          NTGGSTTSAAISNLLLVTGNYRRPGAGAYPLRGHNNVQGACDMATLPNWL 606
Bacillus_cereus               NTGGSTTSAAISNLLLVTGNYRRPGAGAYPLRGHNNVQGACDMATLPNWL 606
Bacillus_contig00202          ---------------------------------------------------
Bacillus_thuringiensis        NTGGSDTSAAISNLLLATGNYRRPGAGAYPLRGHNNVQGACDMGTLPGWL 649
Bacillus_contig00320          ---------------------------------------------------
Bacillus_cereus1              ---------------------------------------------------

Bacillus_contig00442          PGYQAVSDDTLRAKFEKAYGTTIPKAPGLNNIAMLLAAEEGKLRGMYVMG 656
Bacillus_cereus               PGYQAVSDDTLRAKFEKAYGTTIPKAPGLNNIAMLLAAEEGKLRGMYVMG 656
Bacillus_contig00202          ---------------------------------------------------
Bacillus_thuringiensis        PGYQHVTNDMERAKFEMAYGVKINSEPGLNNIEMLHAIDKGKMKAMYLVG 699
BacillusB_contig00320          -------------------------------------------------
Bacillus_cereus1              ---------------------------------------------------

Bacillus_contig00442          EEMALVDSNANHVQHILSNLDFLVVQDMFLSKTARFADVILPAAPSLEKE 706
Bacillus_cereus               EEMALVDSNANHVQHILSNLDFLVVQDMFLSKTARFADVILPAAPSLEKE 706
Bacillus_contig00202          ---------------------------------------------------
Bacillus_thuringiensis        EDMALVDSNANHVHEVLSSLDFFVVQDVFLSKTAQYADVILPAAPSLEKE 749
Bacillus_contig00320          ---------------------------------------------------
Bacillus_cereus1              ---------------------------------------------------
```

```
Bacillus_contig00442          GTFTNTERRIQRLYEVLKPLGDSKPDWWILQKVARALGGDWNYESPSEIM 756
Bacillus_cereus               GTFTNTERRIQRLYEVLKPLGDSKPDWWILQKVARALGGDWNYESPSEIM 756
Bacillus_contig00202          --------------------------------------------------
Bacillus_thuringiensis        GTFTNTERRVQRLYQVLPTLGDAKPDWWIVQEVANKLGANWSYSHPSEIF 799
Bacillus_contig00320          --------------------------------------------------
Bacillus_cereus1              --------------------------------------------------

Bacillus_contig00442          DEIASLAPLYSQATYDRLEGWNSLCWGSHDGSDTPLLYVDGFNFPDKLAR 806
Bacillus_cereus               DEIASLAPLYSQATYDRLEGWNSLCWGSHDGSDTPLLYVDGFNFPDKLAR 806
Bacillus_contig00202          --------------------------------------------------
Bacillus_thuringiensis        AEMASLSPLFSQANYEVLEGWNSFHWGSFDGTNTPLLFQDGFNFPDKKAR 849
Bacillus_contig00320          --------------------------------------------------
Bacillus_cereus1              --------------------------------------------------

Bacillus_contig00442          LSLDEWVPPVVAPDEYDLLLNNGRMLEHFHEGNMTNKSAGILSKVSEVFV 856
Bacillus_cereus               LSLDEWVPPVVAPDEYDLLLNNGRMLEHFHEGNMTNKSAGILSKVSEVFV 856
Bacillus_contig00202          --------------------------------------------------
Bacillus_thuringiensis        FAIADWVRPAEFPAEFDLHINNGRMLEHFHEGNMTNKSTGIQTKVPGVFV 899
Bacillus_contig00320          --------------------------------------------------
Bacillus_cereus1              --------------------------------------------------

Bacillus_contig00442          EISPELAIERNVKDGGLVELASPFGKIKVQALITDRVTGKELYLPMHATI 906
Bacillus_cereus               EISPELAIERNVKDGGLVELASPFGKIKVQALITDRVTGKELYLPMHATI 906
Bacillus_contig00202          --------------------------------------------------
Bacillus_thuringiensis        EVSPALAKERGVKTGSLVRLVSPFGALKLRALVTNRVKANELYLPMNSTD 949
Bacillus_contig00320          ----------------------FGALKLRALVTDRVKANELYLPMNSTD 444
Bacillus_cereus1              ----------------------FGALKLRALVTGRVKANELYLPMNSTD 902

Bacillus_contig00442          NEEAINILTGTATDLYTCTPAYKQTMVKMRVLREKGNRPLPSSNPRDKKR 956
Bacillus_cereus               NEEAINILTGTATDLYTCTPAYKQTMVKMRVLREKGNRPLPSSNPRDKKR 956
Bacillus_contig00202          --------------------------------------------------
Bacillus_thuringiensis        NETAINFLTGPAVDTRTNTPAYKQTKVRMEVLEVEGENPMPKVNPRNKKR 999
Bacillus_contig00320          NETAINFLTGPAVDVRTNTPAYKQTKVRMEVLEVDGANPMPKANPRNKKR 494
Bacillus_cereus1              NETAINFLTGPAVDVRTNTPAYKQTKVRMEVLEVDGANPMPKANPRNKKR 952

Bacillus_contig00442          NPQNGVEIEQKWQRKQYVSLVD- 978
Bacillus_cereus               NPQNGVEIEQKWKRKQYVSLVD- 978
Bacillus_contig00202          ----------------------
Bacillus_thuringiensis        HPQSGIEVHRKWARPGYVHLTDK 1022
Bacillus_contig00320          HPQAGIEINRKWARPGYVHLTDK 517
Bacillus_cereus1              HPQAGIEVNRKWARPGYVHLTDK 975
```

EC 1.2.1.43: formate dehydrogenase

Contig	Accession	Description	Query coverage	E value
00442	ZP_04184506.1	formate dehydrogenase [*Bacillus cereus* AH1271]	100%	0.0

Alignment

```
Bacillus_contig00442          MAEQTVRVTVDGKEFSTSGEKTILQLFNESNLEHPQICHVPEVDPIQTCD 50
Bacillus_cereus               MAEQTVRVTVDGKEFSTSGEKTILQLFNESNLEHPQICHVPEVDPIQTCD 50
                              **************************************************

Bacillus_contig00442          TCIVEVNGKLLRACSTKLESGMHIERQSQRAKEAQTEAMDRILENHLLYC 100
Bacillus_cereus               TCIVEVNGKLLRACSTKIENGMHIERQSQRAKEAQTEAMDRILENHLLYC 100
                              ******************:*.****************************

Bacillus_contig00442          TVCDNNNGNCKVHNTVHMMEIEEQKYPYEPKVSACEVDMSHPFYRYDPNQ 150
Bacillus_cereus               TVCDNNNGNCKVHNTVHMMGVEEQKYPYEPKVSACEVDMSHPFYRYDPNQ 150
                              ******************* :****************************

Bacillus_contig00442          CIACGQCVEVCQNLQVNETISIDWNLDRPRVIWDHGVSINDSSCVSCGQC 200
Bacillus_cereus               CIACGQCVEVCQNLQVNETISIDWNLDRPRVIWDHGVSINDSSCVSCGQC 200
                              **************************************************

Bacillus_contig00442          VTVCPCNALMEKTMLGEAGFMTGLKSDVLDPMIDFVKDVEPGYSSILAVS 250
Bacillus_cereus               VTVCPCNALMEKSMLGEAGFMTGLKPDVLDPMIDFVKDVEPGYSSILAVS 250
                              ************:************.************************

Bacillus_contig00442          EVEAAMRKTKVNKTKTVCTFCGVGCSFEVWTKDRHILKVQPVSDAPVNGI 300
Bacillus_cereus               EVEAAMRKTKVNKTKTVCTFCGVGCSFEVWTKDRHILKVQPVSDAPVNGI 300
                              **************************************************

Bacillus_contig00442          STCVKGKFGWDFVNSEDRITKPLIRQGDMFVEASWEEALEVVASNMQHIK 350
Bacillus_cereus               STCVKGKFGWDFVNSEDRITKPLIRQGDMFVEASWEEALEVVASNMQHIK 350
                              **************************************************

Bacillus_contig00442          SEYGSDAFGFISSSKVTNEENYLMQKLARQIYGTNNVDNCSRYCQSPATD 400
Bacillus_cereus               SEYGSDAFGFISSSKVTNEENYLMQKLARQIYGTNNVDNCSRYCQSPATD 400
                              **************************************************

Bacillus_contig00442          GLFKTVGMGGDAGTVKDIAEAGLVIIVGANPTEGHPVLATRVKRAHKLHE 450
Bacillus_cereus               GLFKTVGMGGDAGTVKDIAEAGLVIIVGANPTEGHPVLATRVKRAHKLHE 450
                              **************************************************

Bacillus_contig00442          QKLIVADLRKHEMAERADLFIHPRQGTDYVWLAGITKYIIDQDWHDKKFL 500
Bacillus_cereus               QKLIVADLRKHEMAERADLFIHPRQGTDYVWLAGITKYIIDQDWHDKKFL 500
                              **************************************************

Bacillus_contig00442          AENVKNFDEYSKMLEKYTLDYTEEITGISKTQLKEMARMVYEADGTCILW 550
Bacillus_cereus               AENVKNFDEYSKMLEKYTLDYTEKITGISKENLKEMARMVYEADGTCILW 550
                              ***********************:****** :******************

Bacillus_contig00442          GMGVTQNTGGSTTSAAISNLLLVTGNYRRPGAGAYPLRGHNNVQGACDMA 600
Bacillus_cereus               GMGVTQNTGGSTTSAAISNLLLVTGNYRRPGAGAYPLRGHNNVQGACDMA 600
                              **************************************************

Bacillus_contig00442          TLPNWLPGYQAVSDDTLRAKFEKAYGTTIPKAPGLNNIAMLLAAEEGKLR 650
Bacillus_cereus               TLPNWLPGYQAVSDDTLRAKFEKAYGTTIPKAPGLNNIAMLLAAEEGKLR 650
                              **************************************************

Bacillus_contig00442          GMYVMGEEMALVDSNANHVQHILSNLDFLVVQDMFLSKTARFADVILPAA 700
Bacillus_cereus               GMYVMGEEMALVDSNANHVQHILSNLDFLVVQDMFLSKTARFADVILPAA 700
                              **************************************************

Bacillus_contig00442          PSLEKEGTFTNTERRIQRLYEVLKPLGDSKPDWWILQKVARALGGDWNYE 750
Bacillus_cereus               PSLEKEGTFTNTERRIQRLYEVLKPLGDSKPDWWILQKVARALGGDWNYE 750
                              **************************************************

Bacillus_contig00442          SPSEIMDEIASLAPLYSQATYDRLEGWNSLCWGSHDGSDTPLLYVDGFNF 800
Bacillus_cereus               SPSEIMDEIASLAPLYSQATYDRLEGWNSLCWGSHDGSDTPLLYVDGFNF 800
                              **************************************************

Bacillus_contig00442          PDKLARLSLDEWVPPVVAPDEYDLLLNNGRMLEHFHEGNMTNKSAGILSK 850
Bacillus_cereus               PDKLARLSLDEWVPPVVAPDEYDLLLNNGRMLEHFHEGNMTNKSAGILSK 850
                              **************************************************
```

```
Bacillus_contig00442          VSEVFVEISPELAIERNVKDGGLVELASPFGKIKVQALITDRVTGKELYL 900
Bacillus_cereus               VSEVFVEISPELAIERNVKDGGLVELASPFGKIKVQALITDRVTGKELYL 900
                              **************************************************

Bacillus_contig00442          PMHATINEEAINILTGTATDLYTCTPAYKQTMVKMRVLREKGNRPLPSSN 950
Bacillus_cereus               PMHATINEEAINILTGTATDLYTCTPAYKQTMVKMRVLREKGNRPLPSSN 950
                              **************************************************

Bacillus_contig00442          PRDKKRNPQNGVEIEQKWQRKQYVSLVD 978
Bacillus_cereus               PRDKKRNPQNGVEIEQKWKRKQYVSLVD 978
                              ******************:*********
```

EC 1.12.7.2: ferrodoxin hydrogenase

Contig	Accession	Description	Query coverage	E value
00202	ZP_04146788.1	formate dehydrogenase [*Bacillus thuringiensis* BGSC 4Y1]	97%	0.0
00442	ZP_04184506.1	formate dehydrogenase [*Bacillus cereus* AH1271]	100%	0.0

Multiple alignments

```
Bacillus_contig00442          --------------------------------------------------
Bacillus_cereus               --------------------------------------------------
Bacillus_contig00202          MTCYESIVLFMNKWIGILARCANKLLHHDDNVPKCGAYIFVVEFFYSQKG 50
Bacillus_thuringiensis        ----------MNKWIGILARCANKLLHHDNNVPKCGAYIFVVEFFYSQKG 40


Bacillus_contig00442          ---MAEQTVRVTVDGKEFSTSGEKTILQLFNESNLEHPQICHVPEVDPIQ 47
Bacillus_cereus               ---MAEQTVRVTVDGKEFSTSGEKTILQLFNESNLEHPQICHVPEVDPIQ 47
Bacillus_contig00202          GKTMNNNMVHITIDGKKYTAEPGSTILGVINENGIEHPQICYVPEVDPIQ 100
Bacillus_thuringiensis        GKTMNNNMVHITIDGKKYTAEPGSTILGVINENGIEHPQICYVPEVDPIQ 90
                                 * :: *::*:***::::.  .*** ::**..:******:********

Bacillus_contig00442          TCDTCIVEVNGKLLRACSTKLESGMHIERQSQRAKEAQTEAMDRILENHL 97
Bacillus_cereus               TCDTCIVEVNGKLLRACSTKIENGMHIERQSQRAKEAQTEAMDRILENHL 97
Bacillus_contig00202          TCDTCIVEVDGKLVRSCSAVVTEGMNIERNSAHAKEAQTEAMDRLLENHL 150
Bacillus_thuringiensis        TCDTCIVEVDGKLTRSCSTVVTEGMNIERNSTRAKEAQTEAMDRLLENHL 140
                              *********:*** *:**: : .**:***:* :***********:*****

Bacillus_contig00442          LYCTVCDNNNGNCKVHNTVHMMEIEEQKYPYEPKVSACEVDMSHPFYRYD 147
Bacillus_cereus               LYCTVCDNNNGNCKVHNTVHMMGVEEQKYPYEPKVSACEVDMSHPFYRYD 147
Bacillus_contig00202          LYCTVCDNNNGNCKLHNTAELMEIEHQKYPYEPKVDASEVDMTHPFYRYD 200
Bacillus_thuringiensis        LYCTVCDNNNGNCKLHNTAELMEIEHQKYPYEPKVDVSEVDMTHPFYRYD 190
                              **************:***..:* :*.*********...****:*******

Bacillus_contig00442          PNQCIACGQCVEVCQNLQVNETISIDWNLDRPRVIWDHGVSINDSSCVSC 197
Bacillus_cereus               PNQCIACGQCVEVCQNLQVNETISIDWNLDRPRVIWDHGVSINDSSCVSC 197
Bacillus_contig00202          PNQCIACGQCVEVCQNLQVNETLSIDWEAERPRVIWDEGVNINDSSCVSC 250
Bacillus_thuringiensis        PNQCIACGQCVEVCQNLQVNETLSIDWEAERPRVIWDEGVNINDSSCVSC 240
                              **********************:****: :*******.**.*********

Bacillus_contig00442          GQCVTVCPCNALMEKTMLGEAGFMTGLKSDVLDPMIDFVKDVEPGYSSIL 247
Bacillus_cereus               GQCVTVCPCNALMEKSMLGEAGFMTGLKPDVLDPMIDFVKDVEPGYSSIL 247
Bacillus_contig00202          GQCVTICPCNALMEKTMLGEAGFMTGLKPDILEPMVDLIKEVEPGYSGIF 300
Bacillus_thuringiensis        GQCVTICPCNALMEKTMLGEAGFMTGLKPDILEPMVDLIKEVEPGYSGIF 290
                              *****:*********:************.*:*:**:*::*:*******.*:
```

```
Bacillus_contig00442          AVSEVEAAMRKTKVNKTKTVCTFCGVGCSFEVWTKDRHILKVQPVSDAPV 297
Bacillus_cereus               AVSEVEAAMRKTKVNKTKTVCTFCGVGCSFEVWTKDRHILKVQPVSDAPV 297
Bacillus_contig00202          AVSEVEAAMRDTRTKKTKTVCTFCGVGCSFEVWTKGRKILKVQPSSDAPV 350
Bacillus_thuringiensis        AVSEVEAAMRDTRTKKTKTVCTFCGVGCSFEVWTKGRKILKVQPSSDAPV 340
                              **********.*:.:*********************.*:****** *****

Bacillus_contig00442          NGISTCVKGKFGWDFVNSEDRITKPLIRQGDMFVEASWEEALEVVASNMQ 347
Bacillus_cereus               NGISTCVKGKFGWDFVNSEDRITKPLIRQGDMFVEASWEEALEVVASNMQ 347
Bacillus_contig00202          NAISTCVKGKFGWDFVNSKERITKPLIRKNGAFVESTWEEALNVVASKLG 400
Bacillus_thuringiensis        NAISTCVKGKFGWDFVNSKERITKPLIRKNGTFVESTWEEALNVVASKLG 390
                              *.****************::*********:.. ***::*****:****::

Bacillus_contig00442          HIKSEYGSDAFGFISSSKVTNEENYLMQKLARQIYGTNNVDNCSRYCQSP 397
Bacillus_cereus               HIKSEYGSDAFGFISSSKVTNEENYLMQKLARQIYGTNNVDNCSRYCQSP 397
Bacillus_contig00202          SIKEEYGKDAIGFISSSKITNEDNYVIQKLARQVFETNNIDNCSRYCQSP 450
Bacillus_thuringiensis        SIKEEYGKDAIGFISSSKITNEDNYVIQKLARQVFETNNIDNCSRYCQSP 440
                               **.***.**:*******:***:**::******:: ***:**********

Bacillus_contig00442          ATDGLFKTVGMGGDAGTVKDIAEAGLVIIVGANPTEGHPVLATRVKRAHK 447
Bacillus_cereus               ATDGLFKTVGMGGDAGTVKDIAEAGLVIIVGANPTEGHPVLATRVKRAHK 447
Bacillus_contig00202          ATDGLFRTIGMGGDAGTIKDIAKSGLVIIVG------------------- 481
Bacillus_thuringiensis        ATDGLFRTIGMGGDAGTIKDIAQSGLVIIVGCNPTEGHPVLATRIKRAHK 490
                              ******:*:********:****::*******

Bacillus_contig00442          LHEQKLIVADLRKHEMAERADLFIHPRQGTDYVWLAGITKYIIDQDWHDK 497
Bacillus_cereus               LHEQKLIVADLRKHEMAERADLFIHPRQGTDYVWLAGITKYIIDQDWHDK 497
Bacillus_contig00202          --------------------------------------------------
Bacillus_thuringiensis        LHGQKLIVADLRKTEMAERSDVFISPKQGTDQVWLMAVTKYMIDQGWHDQ 540


Bacillus_contig00442          KFLAENVKNFDEYSKMLEKYTLDYTEEITGISKTQLKEMARMVYEADGTC 547
Bacillus_cereus               KFLAENVKNFDEYSKMLEKYTLDYTEKITGISKENLKEMARMVYEADGTC 547
Bacillus_contig00202          --------------------------------------------------
Bacillus_thuringiensis        RFIDENVNFFEDFKESLAEYTLEYAEEVTGISQETLIQMAEMIRDADGTC 590


Bacillus_contig00442          ILWGMGVTQNTGGSTTSAAISNLLLVTGNYRRPGAGAYPLRGHNNVQGAC 597
Bacillus_cereus               ILWGMGVTQNTGGSTTSAAISNLLLVTGNYRRPGAGAYPLRGHNNVQGAC 597
Bacillus_contig00202          --------------------------------------------------
Bacillus_thuringiensis        ILWGMGVTQNTGGSDTSAAISNLLLATGNYRRPGAGAYPLRGHNNVQGAC 640


Bacillus_contig00442          DMATLPNWLPGYQAVSDDTLRAKFEKAYGTTIPKAPGLNNIAMLLAAEEG 647
Bacillus_cereus               DMATLPNWLPGYQAVSDDTLRAKFEKAYGTTIPKAPGLNNIAMLLAAEEG 647
Bacillus_contig00202          --------------------------------------------------
Bacillus_thuringiensis        DMGTLPGWLPGYQHVTNDMERAKFEMAYGVKINSEPGLNNIEMLHAIDKG 690


Bacillus_contig00442          KLRGMYVMGEEMALVDSNANHVQHILSNLDFLVVQDMFLSKTARFADVIL 697
Bacillus_cereus               KLRGMYVMGEEMALVDSNANHVQHILSNLDFLVVQDMFLSKTARFADVIL 697
Bacillus_contig00202          --------------------------------------------------
Bacillus_thuringiensis        KMKAMYLVGEDMALVDSNANHVHEVLSSLDFFVVQDVFLSKTAQYADVIL 740


Bacillus_contig00442          PAAPSLEKEGTFTNTERRIQRLYEVLKPLGDSKPDWWILQKVARALGGDW 747
Bacillus_cereus               PAAPSLEKEGTFTNTERRIQRLYEVLKPLGDSKPDWWILQKVARALGGDW 747
Bacillus_contig00202          --------------------------------------------------
Bacillus_thuringiensis        PAAPSLEKEGTFTNTERRVQRLYQVLPTLGDAKPDWWIVQEVANKLGANW 790


Bacillus_contig00442          NYESPSEIMDEIASLAPLYSQATYDRLEGWNSLCWGSHDGSDTPLLYVDG 797
Bacillus_cereus               NYESPSEIMDEIASLAPLYSQATYDRLEGWNSLCWGSHDGSDTPLLYVDG 797
Bacillus_contig00202          --------------------------------------------------
Bacillus_thuringiensis        SYSHPSEIFAEMASLSPLFSQANYEVLEGWNSFHWGSFDGTNTPLLFQDG 840


Bacillus_contig00442          FNFPDKLARLSLDEWVPPVVAPDEYDLLLNNGRMLEHFHEGNMTNKSAGI 847
Bacillus_cereus               FNFPDKLARLSLDEWVPPVVAPDEYDLLLNNGRMLEHFHEGNMTNKSAGI 847
Bacillus_contig00202          --------------------------------------------------
Bacillus_thuringiensis        FNFPDKKARFAIADWVRPAEFPAEFDLHINNGRMLEHFHEGNMTNKSTGI 890
```

```
Bacillus_contig00442          LSKVSEVFVEISPELAIERNVKDGGLVELASPFGKIKVQALITDRVTGKE 897
Bacillus_cereus               LSKVSEVFVEISPELAIERNVKDGGLVELASPFGKIKVQALITDRVTGKE 897
Bacillus_contig00202          --------------------------------------------------
Bacillus_thuringiensis        QTKVPGVFVEVSPALAKERGVKTGSLVRLVSPFGALKLRALVTNRVKANE 940


Bacillus_contig00442          LYLPMHATINEEAINILTGTATDLYTCTPAYKQTMVKMRVLREKGNRPLP 947
Bacillus_cereus               LYLPMHATINEEAINILTGTATDLYTCTPAYKQTMVKMRVLREKGNRPLP 947
Bacillus_contig00202          --------------------------------------------------
Bacillus_thuringiensis        LYLPMNSTDNETAINFLTGPAVDTRTNTPAYKQTKVRMEVLEVEGENPMP 990


Bacillus_contig00442          SSNPRDKKRNPQNGVEIEQKWQRKQYVSLVD- 978
Bacillus_cereus               SSNPRDKKRNPQNGVEIEQKWKRKQYVSLVD- 978
Bacillus_contig00202          --------------------------------
Bacillus_thuringiensis        KVNPRNKKRHPQSGIEVHRKWARPGYVHLTDK 1022
```