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Bioremediation of cyanide contaminated water

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DEDICATIONS:

To My Parents

THANKS!

While I'm excited about my graduation, I'm sad about leaving the TUEERU research Unit of Tuskegee University. I have enjoyed working with professors and students, each of them have provided me very useful advices and tips for the accomplishment of my research work.

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Abstract:

Degradation of various cyanide complex (sodium and potassium ferrocyanide, zinc cyanide and potassium cyanide argentate) by free cells of *Pseudomonas fluorescens* Pf5 *and P. resinovorans* ATCC®14235 in the presence of glucose was investigated as a function of initial pH and initial glucose concentrations. Bacterial growth, ammonia generation and glucose utilization were parameters assessed during the tests. *P. fluorescens* Pf5 and *P. resinovorans* were able to degrade potassium and sodium ferro cyanide using them as nitrogen source in M9 medium. However, potassium cyano argenate ($KAg(CN)_2$) was an inhibiting compound on the growth of *P. fluorescens Pf5* and *P. resinovorans.* The MIC of *P. fluorescens* Pf5 was 50 mM (21.1g/l) for $K_4Fe(CN)_6$ and 75mM (36.3/l) for $Na_4Fe(CN)_6$. The MIC values were 25mM (10.55g/l) and 75mM (36.3/l) for $K_4Fe(CN)_6$ and $Na_4Fe(CN)_6$, respectively for *P. resinovorans.* The maximum ammonia concentration was detected in the medium at pH 7 when using *P. fluorescens Pf5* and at pH 5 when using *P. resinovorans*. For both bacteria strain, the growth was higher when glucose concentration was raised; however, ammonia concentration decreased when glucose concentration was increased

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Keys Words:

Ferro cyanide – zinc cyanide – potassium cyano argenate - *P. fluorescens* Pf5 - *P. resinovorans* - Bioremediation

La dégradation d'une variété de compose complexes de cyanure (sodium et potassium ferrocyanide, cyanure de zinc et l'argenate de cyanure de potassium) par de souches libres de *Pseudomonas fluorescens* Pf5 et *P. resinovorans* ATCC®14235 en présence de glucose, a été étudiée en fonction du pH initial et de la concentration initiale de glucose. La croissance bactérienne, la generation d'ammoniac, et l'utilisation du glucose ont été les paramètres suivis durant les tests. *Pseudomonas fluorescens* Pf5 et *P. resinovorans* étaient capables de dégrader le ferro cyanure de potassium et de sodium, utilisant ces derniers comme source d'azote. Cependant, l'argentate de cyanure de potassium a inhibé la croissance des deux souches bactériennes. La Concentration Minimum Inhibitrice étaient 50 mM (21.1g/l) pour $K_4Fe(CN)_6$ et 75mM (36.3/l) pour $Na_4Fe(CN)_6$ *P.* Le CMI étaient 25mM (10.55g/l) et 75mM (36.3/l) pour $K_4Fe(CN)_6$ et $Na_4Fe(CN)_6$, respectivement pour *P. resinovoran*s. La concentration maximum d'ammoniac a été détectée dans le milieu à pH 7 quand *P. fluorescens* Pf5 était utilisé, et à pH 5 quand *P. resinovorans* était utilisé. Pour les deux souches bactériennes, la croissance était plus importante quand la concentration initiale de glucose était augmentée ; cependant la concentration d'ammoniac a diminué quand la concentration initiale de glucose était augmentée.

Mots-clés :

Ferro cyanure – cyanure de zinc – argenate de cyanure de potassium - *P. fluorescens* Pf5 - *P. resinovorans* – Biorestauration.

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CHAPTER 1

INTRODUCTION

Cyanide compounds are widely distributed on Earth; indeed they have been postulated to have played a key role in the prebiotic chemistry that led to the evolution of biological macromolecules and primitive life (Commeyras et al. 2004). As a result, these compounds have had a significant presence in the environment throughout the evolution of life. However, concerns have been focused recently on the potential toxicity of such compounds.

Cyanide is a group of compounds which contains a C≡N group: one atom of carbon linked with one atom of nitrogen by three molecular bounds. Cyanide compounds are usually categorized into 3 groups: the first group called free cyanide is related to the cyanide ion CN^- (produced by dissolving sodium or potassium cyanide in water) and hydrogen cyanide gas; the second group is related to weak and moderately strong complexes formed between cyanide ion and some metals such as Zn, Ni, Ag, Cd, Hg; the third group is related to strong complex formed between cyanide ion and Fe ion. Other forms of cyanide include cyanates and nitriles. Due to its composition cyanide compounds are widely used in several industries including electroplating, synthetic fiber production, steel and coal processing, mining industries. In the mining industry, cyanide is primarily used for leaching gold and silver from ores, but it is also used in low concentrations as a flotation reagent for the recovery of base metals such as copper, lead and zinc. Cyanide is used at nearly 90% of the gold mines, including most of the gold mining plant in West Africa (Burkina Faso, Ghana, Mali, etc). Therefore cyanide is present in wastes and slurries produced by these industries; then potentially hazardous for human health and for the environment. At high concentration cyanide compounds are toxic; the most toxic in all these compounds is hydrogen cyanide (HCN). The cyanide ion is one of the potent inhibitor of growth and cellular metabolism, including respiratory, nitrogen phosphate metabolism (Dubey and Holmes, 1995). It can also cause rapid breathing, tremors under short terms exposure, thyroid effects, nerve damage and death for long-term exposure. When exposed in the environment cyanide compounds are toxic to fish, invertebrates, mammals, algae, macrophyts, etc. by causing delayed mortality, pathology, disrupted respiration, osmoregulatory disturbances and altered growth patterns (Ingles and Scott, 2002).

Regardless to his hazardous behavior in the environment and for human health, appropriate technics for cyanide removal must be applied in waste water treatment before release in the environment. Several technics have been used by industries to remove cyanide. The physical and chemical methods are the most applied by industries. Physical and chemical methods include the alkaline chlorination process, the sulfide dioxide air process which utilize SO_2 and air at alkaline pH to oxidize the cyanide, the copper-catalyzed hydrogen peroxide process using the hydrogen peroxide, the Caro's acid process, ion exchange, reverse osmosis, etc. Although it exists all this range of chemical methods, the biological methods are more indicated for cyanide removal because of their low operation cost, their ability to remove a wide range of cyanide compounds, and their ability to produce high quality effluent (Botz et al, 2005).

Despite cyanide's toxicity to living organisms, biological treatments are feasible alternatives to chemical methods without creating or adding new toxic and biologically persistent chemicals. The biological degradation of metal cyanide complex anions is based upon functions carried out by living cells and involves the metabolism and/or transformation of cyanide complex anions to products less toxic to the environment. Different mechanisms contribute to cyanide complex ions degradation in an aerobic biological treatment such as biological metabolism, adsorption onto the biomass, and chemical reaction with organic compounds. Some microorganisms can adapt to grow in the presence of metal cyanide complex ions by inducing the synthesis of enzymes for the degradation of cyanide complex ions or by synthesizing cyanide-resistant enzymes (Baxter and Cummings, 2006).

The biological degradation of cyanide compounds generally occurs in two steps: the first step called the oxidative breakdown carries out in presence of oxygen and water to produce carbonate ion and ammonia. The second step converts ammonia to nitrate through the conventional two step nitrification process, where nitrite is generated as an intermediate (Botz et al. 2005).

Cyanide is used by the bacteria for their growth as nutrient. It acts as nitrogen source for the bacteria. So, an external carbon source such as glucose is added to the medium to allow the bacteria to grow. Some bacteria are able to use the cyanide compounds as both carbon and nitrogen source. Therefore, supply of external carbon source is no longer needed for these bacteria (Dursun et al. 1999).

Many studies were carried out to identify the ability of microorganisms to degrade simple or complex cyanide compounds. It has been proved that, not only Fungi such as

Fusarium solani (Barclay et al., 1998), *Trichoderma harzianum* (Muffadal et al., 2005), but also bacteria such as *Klebsiella oxytoca* (Chen et al., 2008), *Pseudomonas fluorescens* P70 *(*Dursun et al. 1999*), Pseudomonas stutzeri* AK61 *(*Ivorgsen et al. 1991*),* etc. Research is going on to identify microorganisms that have the ability to detoxify a variety of cyanide compounds from waste water.

Several works have been done on bioremediation of simple cyanide compounds however there's a lack of knowledge on bioremediation of complex cyanide compounds. Since the most commonly isolated microorganisms, which can degrade complex cyanide compounds, are the *Pseudomonas* species, the general objective of this research is to assess the removal ability of complex cyanide compounds by *Pseudomonas fluorescens* Pf5 and *Pseudomonas resinovorans*. The specific objectives are as follows:

- Exploration of the growth kinetics of the bacteria
- Determination of the minimum inhibitory concentration (MIC) of 4 ($KAg(CN)_2$, $Zn(CN)_2$, $K_4Fe(CN)_6$, $Na_4Fe(CN)_6$cyanide compounds using the bacteria
- Evaluation of the effects of the pH, and initial glucose concentration on the degradation of different cyanide compounds.

CHAPTER 2
LITERATURE REVIEW

2.1. Generality on cyanide

Cyanide is a group of compounds that contains the C≡N group. It is widely distributed in the environment. It is naturally produce in the environment by a wide range of taxa and it has been proven that cyanide compounds have played a major role in the biological evolution on earth (Oro and Lazcano-Araujo, 1981); they have been postulated to have played a key role in the prebiotic chemistry that led to the evolution of biological macromolecules and primitive life (Commeyras et al. 2004). Although cyanide is produced naturally by a wide range of taxa (fungi, arthropods, bacteria, etc), anthropogenic activities are responsible for the major part of cyanide found on the earth. Cyanide is present in wastewater produced by mining, metal cleaning, manufacturing, electroplating, coal cooking, automobile and pharmaceuticals industries (Baxter and al., 2006).

2.2 Utilization of Cyanide

Industries:

Cyanide is one of the main building blocks for the chemical industry because of its composition of carbon and nitrogen—both common elements—and the ease with which it reacts with other substances.

Cyanide compounds find a wide range of uses. For example, metal cyanides, chiefly potassium and sodium salts are used in electroplating processes, where they are used in the basic degreasing and the electroplating baths to control the concentration of metal ions (Baxter and Cummings, 2006). Significant application of iron cyanide is as anticaking agents both in road salt and fire retardants.

Gold mining:

Cyanide is used in mining to extract gold (and silver) from ores, particularly low-grade ores and ores that cannot be readily treated through simple physical processes such as crushing and gravity separation (Logsdon, 1999).

The use of water-based solutions to extract and recover metals such as gold is called “hydrometallurgy.” Gold mining operations use very dilute solutions of sodium cyanide (NaCN), typically in the range of 0.01% and 0.05% cyanide. The sodium cyanide dissolves in

water where, it dissolves the gold contained in the ore under mildly oxidizing conditions. The resultant gold-bearing solution is called “pregnant solution”. Either zinc metal or activated carbon is then added to the pregnant solution to recover the gold by removing it from the solution. The residual or “barren” solution (i.e. barren of gold) may be re-circulated to extract more gold or routed to a waste treatment facility (Logsdon, 1999).

2.3 Different forms of cyanide

The term cyanide generally refers to one of three classifications of cyanide, and it is critical to define the class of cyanide that is to be treated. The three classes of cyanide are: (1) total cyanide; (2) weak acid dissociable (WAD) cyanide; and (3) free cyanide. Only hydrogen cyanide and the cyanide ion in solution can be classed as "free" cyanide. The proportions of HCN and CN^- in solution are according to their equilibrium equation. This is influenced by the solution pH. WAD cyanide includes those cyanide species liberated at moderate pH of 4.5 such as HCN (aq), CN^- and cyanide Cu, Cd, Ni, Zn, Ag complexes and others with similar low dissociation constants. Total cyanide includes all free cyanide, all dissociable cyanide complexes and all strong metal cyanide including ferro-cyanide $Fe(CN)_6^{-4}$, ferri-cyanide $Fe(CN)_6^{-3}$, and portions of hexacyano cobaltate $Co(CN)_6^{-3}$ and those of gold and platinum (Botz et al. 2005).

Free cyanides are the most toxic to microorganisms, followed by WAD which are moderately toxic. Strong metals cyanide are the less toxic to microorganisms and the less harmful to the environment, (Botz et al. 2005)

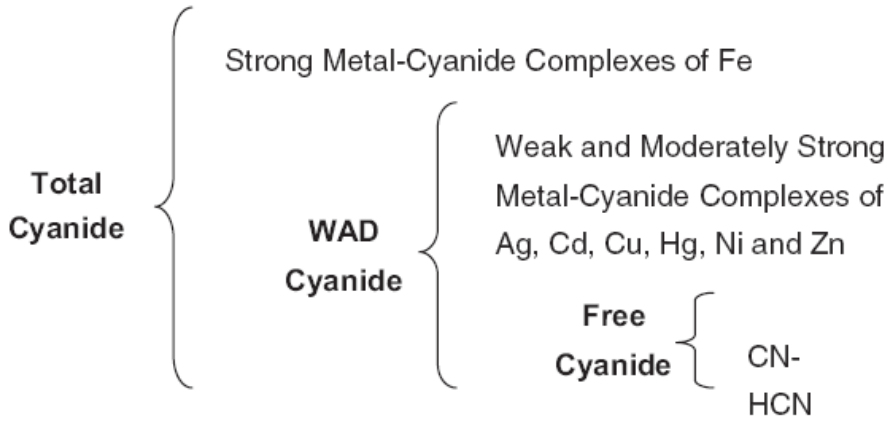


Fig1. General classification of cyanide compounds (Botz et al. 2005)

2.4. Effect of cyanide compounds on the human health and the environment

2.4.1 Effect on human health:

The cyanide ion is one of the potent inhibitor of growth and cellular metabolism, including respiratory, nitrogen phosphate metabolism. It can cause rapid breathing, tremors under short terms exposure, thyroid effects, nerve damage and death. (Dubey and Holmes, 1995). The most toxic form of cyanide is HCN gas. The American Conference of Governmental Industrial Hygienists (ACGIH) lists the ceiling threshold limit of HCN at 4.7 ppm. At concentrations of 20 to 40 ppm of HCN in air, some respiratory distress may be observed after several hours. Death occurs in minutes at HCN concentrations above approximately 250 ppm in air. (Logsdon et al. 1999)

2.4.2 Effect of cyanide compounds in the environment

Once released in the environment, the reactivity of cyanide provides numerous pathways for its degradation and attenuation: Complexation, adsorption, precipitation, volatilization, biodegradation. Although cyanide reacts readily in the environment and degrades or forms complexes and salts of varying stabilities, it is toxic to many living organisms at very low concentrations (Logsdon et al. 1999).

Fish and aquatic invertebrates are particularly sensitive to cyanide exposure. Concentrations of free cyanide in the aquatic environment ranging from 5.0 to 7.2 $\mu g.L^{-1}$ reduce swimming performance and inhibit reproduction in many species of fish. Other adverse effects include delayed mortality, pathology, disrupted respiration, osmoregulatory disturbances and altered growth patterns (Logsdon et al. 1999).

Algae and macrophyts can tolerate much higher environmental concentrations of free cyanide than fish and invertebrates. However, differing sensitivities to cyanide can result in changes to plant community structure, with cyanide exposures leaving a plant community dominated by less sensitive species (Logsdon et al. 1999).

2.5 Physical and chemical treatment of cyanide

The physical and chemical treatments of cyanide operate on the principle of converting cyanide into a less toxic compound through an oxidation reaction. Several destruction processes are well proven to produce treated solutions or slurries with low levels of cyanide as well as many metals: alkaline chlorination process, sulfur dioxide and air process, copper-catalyzed hydrogen peroxide process, Caro's acid process, the iron-cyanide

precipitation, activated carbon polishing, ion exchange, reverse osmosis, ozonation, etc. (Ackil, 2003).

2.5.1 Alkaline chlorination process:

The cyanide destruction reaction occurs in two-steps. The first step in which cyanide is converted to cyanogen chloride (CNCl) and the second step in which cyanogen chloride hydrolyses to yield cyanate according to the following reactions:

$Cl_2 + CN^- \rightarrow CNCl + Cl^-$

$CNCl + H_2O \rightarrow OCN^- + Cl^- + 2H^+$.

Cyanate is then hydrolized to ammonia in the presence of a slight excess of chlorine at alkaline pH.

$OCN^- + 3H_2O \rightarrow NH_4^+ + HCO_3 + OH^-$. (Botz et al. 2005)

In the presence of excess chlorine, ammonia is oxidized to nitrogen gas (N_2). The process is typically applied to treat low solutions flows initially containing low to high levels of cyanide. It is effective for the treatment of solutions for the oxidation of free and WAD cyanides, but a lesser amount of iron cyanides are removed. (Botz et al. 2005).

2.5.2 Sulfur dioxide and air process

The process developed by INCO Ltd in 1980, utilizes SO_2 and air at an alkaline pH in the presence of a soluble copper catalyst to oxidize cyanide to the less toxic compound cyanates (OCN) according to the following reaction:

$SO_2 + O_2 + H_2O + CN^- \rightarrow OCN^- + SO^{2-}_4 + 2H^+$ (Botz et al. 2005)

The process uses SO_2 (supplied either as compressed liquid sulfur dioxide or a reduced-sulfur salt such as sodium metabisulfite ($Na_2S_2O_5$), sodium sulfite (Na_2SO_3) or ammonium bisulfite (NH_4HSO_3). Also supply of oxygen, lime and copper (Cu^{2+}) are required for the reaction. It is able to treat a cyanide solution with an initial concentration of 450mg/l up to 0.1 – 2.0mg/l, (Ingles et Scott, 1987). The primary application of the sulfur dioxide and air process is with slurry tailings, but it is also effective for the treatment of solutions for the oxidation of free and WAD cyanides.

2.5.3 Copper-catalyzed hydrogen peroxide process

The treatment process is similar to that described in the sulfur dioxide and air process, but hydrogen peroxide is utilized rather than sulfur dioxide and air. Soluble copper is also required as a catalyst and the end product is cyanate according to the equation shown below:

$H_2O_2 + CN^- \rightarrow OCN^- + H_2O$

Although the reaction can be carried out over a wide pH range, it is usually conducted at a pH of about 9.0–9.5 for optimal removal of residual metals such as copper, nickel and zinc initially complexed to cyanide. (Botz et al. 2005)

The primary application of the hydrogen peroxide process is with solutions rather than slurries due to the high consumption of hydrogen peroxide that occurs in slurry applications. The process is typically applied to treat moderate to low levels of cyanide. The hydrogen peroxide process is effective for the treatment of solutions for the oxidation of free and WAD cyanides, and iron cyanides. As indicated, copper (Cu_2^+) is required as a soluble catalyst, which is usually added as a solution of copper sulfate ($CuSO_4.{}_5H_2O$) (Botz et al. 2005)

2.5.4 Caro's acid process:

Peroxymonosulfuric acid, also known as Caro's acid is produced by reacting concentrated hydrogen peroxide and sulfuric acid in a controlled temperature environment as follows:

$H_2O_2 + H_2SO_4 \rightarrow H_2SO_5 + H_2O.$

Caro's acid reacts with cyanide trough an oxidative reaction which is shown below:

$H_2SO_5 + CN \rightarrow OCN^- + SO_4^{2-} + 2H^{+}.$

In this process there is no need to supply copper catalyst to the reaction. Caro's acid is normally used in slurry treatment applications where the addition of a copper catalyst is not desirable (Botz et al. 2005).

2.5.5 Activated carbon polishing

Activated carbon has an affinity for many metal-cyanide compounds, including the soluble cyanide compounds of copper, iron, nickel and zinc. Activated carbon is suitable for use as a polishing process to remove cyanide to low levels when the initial cyanide concentration is already below about 1–5 mg/L. This is a simple and effective process,

convenient for installation at sites where activated carbon is used in metallurgical processes for precious metals recovery. (Botz et al. 2005).

It has been shown that this method was able to treat a solution with initial total cyanide solution concentration of 0.98mg/l to a concentration of 0.2mg/l (Botz and Mudder, 1997).

2.5.6 Other cyanide treatment processes:

There are several other techniques for cyanide removal, which have been applied at full scale but their implementation has been limited for several reasons: Ion exchange and reverse osmosis could be used for cyanide removal, but with both of these techniques waste brine is generated as by-product; it's disposal or treatment is difficult and expensive, and in some cases the brine is hazardous and require special handling. An advantage of these techniques is the simultaneous removal of cyanide, cyanates, thiocyanate, ammonia and nitrate. (Botz et al. 2005)

Ozone is a strong oxidant and capable of oxidizing free and WAD cyanides to cyanates, ammonia and nitrate (Carillo-Pedroza and Soria-Aguilar, 2001; Nava et al., 2003). Low-effluent cyanide concentrations can be achieved with ozone, with release of cyanate, ammonia and nitrate. Iron cyanides are also oxidized by ozone. Ozone is relatively expensive to produce and this has limited its use for cyanide destruction, particularly for large water flows, but may find application in small-volume polishing applications. (Botz et al. 2005)

2.6 Natural cyanide attenuation

It is well known that cyanide solutions placed in ponds or tailings impoundments undergo natural attenuation reactions, which result in the decrease of the cyanide concentration. These attenuation reactions are dominated by natural volatilization of hydrogen cyanide, but other reactions such as biological degradation, oxidation, hydrolysis, photolysis and precipitation also occur (Botz et al. 2005). At several sites, ponds or tailings impoundments are intentionally designed to maximize the rate of cyanide attenuation. Advantages of natural attenuation include lower capital and operating costs when compared to chemical-oxidation processes. (Ackil, 2003)

2.7 Bioremediation of cyanide:

An alternative to the treatment of cyanide contaminated wastewater is the biological treatment. The biological methods are more indicated for cyanide removal because of their

low operation cost, their ability to remove a wide range of cyanide compounds, and their ability to produce high quality effluent (Botz et al, 2005). Microbial destruction of cyanide from tailings and wastewaters is proven and viable alternative to physical and chemical treatment processes. (Ackil, 2003)

2.7.1 Bioremediation of cyanide contaminated wastewater

Several reviews have summarized the biological treatment of cyanide in the mining industry (Mudder et al., 2001). Biological treatment of cyanide has been shown a viable process for destroying cyanide in mining process waters; the process can be readily exploited and engineered to accommodate both large flows and the elevated cyanide containing solutions generated at commercial precious metals operations.

2.7.1.1 Background of the process:

Cyanide destruction by microorganisms was first commercially demonstrated in the gold mining industry at the Homestake Gold Mine, USA in the middle 1980s (Mudder & Whitlock 1984). The attached growth fix film biological facility consisted of five stages of forty eight rotating biological contactors (RBCs) for the removal of cyanide, ammonia, and metals (Mudder *et al.* 1998, Whitlock & Mudder 1998). The biodegradation occurred into two steps:

The first step is the oxidative breakdown of cyanides, and subsequent sorption and precipitation of free metals into the biofilm. Cyanide and thiocyanate are then converted to ammonia, carbonate and sulfate (Ackil, 2003)

$CN^- + ½\ O_2 + 2H_2O \rightarrow HCO_3^- + NH_3$

$SCN^- + 2O_2 + 3H_2O \rightarrow SO_4^{2-} + NH_4^+ + HCO_3- + H^+$

$M(CN)_x^{y-x} + 3xH_2O + x/2O_2 \rightarrow M^{y+} + xNH_4^+ + xHCO_3^- + H^+$

In the second step, ammonia is converted to nitrate through the conventional two step nitrification process shown below:

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

$$NO_2^{2-} + ½\ O_2 \rightarrow NO_3^{3-}$$

The ease of degradation of metal cyanides depends on their chemical stability: free cyanide are the most readily degradable, followed by metal cyanide complexes of Zn, Ni, and Cu; iron cyanide the least degradable (Mudder *et al.* 1998).

The various microbial flora (*Actinomyces, Alcaligenes, Arthrobacter, Bacillus, Micrococcus, Neisseria, Paracoccus, Pseudomonas*, and *Thiobacillus)* which are involved in the different degradation stages are normally non-competitive, the presence of which are limited by changes in various constituent concentrations (Given *et al*. 1998, Mudder *et al.* 1998). Cyanide act as a nutrient for the destruction stage of bacteria and are toxic to the nitrifying bacteria in elevated levels. Ammonia acts as a nutrient for the nitrifiers, which utilize inorganic compounds such as bicarbonate as their source of carbon (Ackil, 2003).

Biological treatment can be applied in many situations under many conditions. It has been employed in full-scale facilities worldwide both for conventional cyanidation and heap leach applications. The microbial species can develop in multiple environments allowing for uptake, treatment, sorption, and/or precipitation of cyanide, ammonia, nitrate, sulfate, and metals (Akcil and Mudder, 2003).

2.7.1.2 Review on previous research on cyanide bioremediation:

It has been shown that most of these microorganisms have the ability to degrade cyanide compounds, by converting the compounds to ammonia. The enzymatic pathways generated by these species include hydrolytic, oxidative, reductive, substitution and transfer reactions inside the bacteria (Ebbs 2004). Different enzymes, which are involved in theses pathways contribute to make the cyanide removal possible, are cyanide hydratase, nitriles hydratase, cyanidase, cyanide dioxygenase, nitrogenase, etc. (Gupta et al, 2010).

***Biodegradation of simple cyanide compounds*:**

The degradation of cyanide to ammonia and formate catalysed by a cyanide dihydratase has been observed in several bacteria including *Pseudomonas stutzeri* AK61, *Alcaligenes xylosoxidans* and *Bacillus pumilus* C1 (Ingvorsen et al. 1991; Meyers et al. 1991; Watanabe et al. 1998). Other pathways utilizing cyanide dioxygenase and nitrogenase have also been identified in *E. coli* and *Klebsiella oxytoca*, respectively (Figuera et al. 1996; Kao et al. 2003).

The degradation of simple cyanides has also been demonstrated in fungi, for example, a purified cyanide hydratase from *Fusarium lateritium* catalyses the hydration of cyanide to formamide (Cluness et al. 1993). Cyanide hydratase activity was also demonstrated by several *Trichoderma strains*, which metabolised cyanide by rhodanese activity (Ezzi and Lynch 2002). Immobilized cells of *Pseudomonas putida* also shown their ability to degrade

simple cyanide ($Na^{14}CN$) to NH3 and CO2. Results shown that 70% of the carbon was converted to CO2, and only 10% was associated to the cell biomass (Chapatawla et al, 1998.) Another study performed on *Trametes versicolor* (white rot fungus) showed that the cells were able to remove 130 mg/l of KCN in 42 hours ~~h~~ at 30°C and pH of 10.5 with a stirring rate at 150 rpm (Cabuk et al, 2006). In the application, microbial technologies have already been applied to the detoxification of simple cyanides in industrial wastewaters: Immobilized forms of *Alcaligenes denitrificans* (Basheer et al. 1992).

***Bioremediation of metal cyanide complex*:**

The increasingly stable complexation of cyanide with Zn, Cu, Ni and Fe can inhibit the application of biodegradation technologies to their remediation) Complexation of copper with cyanide can completely stop the use of the cyanide complex as a source of nitrogen in the biodegradation test of *P. fluorescens.* (Raybuck, 1992)

However, many strain of *P. fluorescens (*strain BKM B-5040, strain NCIMB 17764) have been shown to be able to degrade zinc, copper, silver and iron cyano complexes; degradation of nickel cyanide have been performed by *P. fluorescens* NCIMB 17764 with the mediation of cyanide oxygenase activity (Dorr and Knowles 1989).

In optimum conditions (pH 5, stirring rate: 150rpm, aeration rate : 0.15 vvm, glucose concentration 0.465g/l) *Pseudomonas fluorescens* P70 is able to remove 78.9% of ferrous (II) cyanide with an removal rate of 30 $mg.g^{-1}.h^{-1}$ (Dursun et al, 1999) .

Escherichia coli BCN6 was able to use iron, zinc and copper cyanide complexes, as nitrogen sources. Each metal-cyanide complex was observed to have a different effect on growth due to the varying toxicities of the metal ions on the biosorption mechanism taken up by the cells (Figuera et al. 1995).

Burkholderia cepacia C-3 was demonstrated to degrade a range of metal complexed cyanide compounds at alkaline pH, which is representative of those found in heap leach effluents. The highest removal rate observed was 1.85 mg CN. h^{-1} (Adjei and Ohta 2000).

An *Acinetobacter sp*. strain RBPI isolated from gold mine effluents was shown to be capable of degrading gold, silver, cadmium, zinc, copper, cobalt and iron cyanide complexes; by using a single purified cyanide degrading complex that was able to degrade either simple or metal cyanide complexes (Finnegan et al. 1991).

Pure cultures and mixed consortia of fungi isolated from a contaminated gasworks soil, including *F. solani, F. oxysporum, Trichoderma polysporum, Scytalidium thermophilum* and *Penicillin miczynski*, could degrade iron and nickel cyanides (Barclay et al. 1998)

A yeast, *Cryptococcus humicolus* MCN2, isolated from cokeplant wastewater and grown on KCN as a sole nitrogen source, degraded concentrations of potassium tetracyanonickelate up to 65 mM when supplied with sufficient carbon (Kwon et al. 2002) .

In the bioremediation tests, either free cyanide or metals cyanide compounds are used, but relatively little work appears to have been done on the ability of microbial strains to degrade metal cyanide complexes. The bacteria strain usually grows in M9 medium, or in a medium providing the essential nutrient for the microbial growth. Some microorganisms are able to grow in the medium using the cyanide compound as sole source of carbon and nitrogen. But in other cases the microbe uses cyanide compounds as source of nitrogen, and then there's a need to provide an external carbon source, usually glucose, dextrose, or fructose. The temperature, the aeration rate or the shaking effect are others parameters which are important for implementation of the test. Different parameters can be used to verify the removal of cyanide: microbial growth, generation of ammonia, cyanide or glucose utilization. (Dursun et al. 1999, Cabuk et al. 2006, Chapatawala et al. 1998, Adjei and Ohta, 1999).

2.7.1.3 Factors affecting the bioremediation of cyanide

Many factors involved in the biodegradation process will affect greater or lesser the rate of the cyanide removal. The pH of the bioremediation medium, the initial concentration of glucose, the initial concentration of cyanide compounds, the temperature and even the initial quantity of microorganisms presents in the medium, are the factors which affect significantly the biodegradation parameters. (Dursun et al. 1999, Cabuk et al. 2006, Chapatawala et al. 1998, Adjei and Ohta, 1999 ; Figueira et al. 1996)

Effect of pH:

At neutral acidity, most of the cyanide compounds release hydrogen cyanide gas (HCN) when they are put in solution. To stabilize it, the cyanide solutions must be prepared at basic pH (10-12) by adding sodium hydroxide before being added to the biodegradation .medium. Release of HCN is then avoided, and the solution can be used for several days (i.e. 10-14 days).

Cyanide bioremediation occurs either in acid or in basic conditions, but in most cases there is an optimum value. The optimum initial pH is specific to the type of microbe used and also to the cyanide compounds. Using *Pseudomonas putida*, Chapatawala et al 1998) observed an optimum initial pH of 7.5 for the removal of CN^-. The initial pH play a major role in the bioremediation of ferrous cyanide by *Pseudomonas fluorescens* P70; the maximum ferrocyanide removal ($30mg.g^{-1}.h^{-1}$) and specific growth rate were observed at pH 5. At pH 3 and 9, the microbial activity was almost inhibited. No significant cyanide removal was observed (Dursun et al, 1999). Strain C3 of *Bulkholderia cepacia* is able to remove cyanide in a pH range from 8 to 10, with a maximum cyanide removal (1.85mg CN.h-1) at pH 10 (Adjei and Ohta, 2000).

Studies of HCN degradation using the fungus *F. solani* under alkaline conditions (pH 9.2 –10.7) demonstrated that the cyanide was degraded via a cyanide hydratase and amidase pathway. Alkaline pH reduces the risk of cyanhydric acid volatilisation, making the use of alkaline tolerant organisms and enzymes more attractive for degradation of cyanide containing effluents (Dumestre et al. 1997).

The pH of the biodegradation medium can change during the test. Chapatawala et al, (1998) observed a slight increase of the initial pH up to the 120^{th} hour during the biodegradation of cyanides, cyanates and thiocyanate by immobilized cells of *Pseudomonas putida*; they attributed this increase, to the accumulation of NH_3 formed due to the cleavage of CN^- group. After the 120^{th}, the pH started increasing due to the neutralizing effect of carboxylic acid formed during the experience.

Initial cyanide concentration

The cyanide compounds concentration is one of the most important factors regulating its removal. Usually, cyanide compounds are used as nutrient source for the microbes. As seen previously, cyanide is a group of compounds containing the C≡N group. The presence of carbon and nitrogen in the formula suggested that compounds may provide these two elements for the growth of the bacteria. Some bacteria have demonstrated the ability to grow in M9 medium containing only cyanide compounds as carbon and nitrogen source.

As cyanide acts as a nutrient for bacteria, cyanide removal is usually favorable for the growth of bacteria and cyanide removal up to a certain concentration, at which it does not show an inhibiting effect on the growth of the bacteria. The lowest cyanide concentration at which it inhibits the growth of the bacteria is called Minimum Inhibitory Concentration (MIC).

Using *Pseudomonas fluorescens* P70 to degrade ferrocyanide compounds, Dursun et al, (1999) noticed that the removal rate increased with increasing initial ferrocyanide concentration up to 50 $mg.l^{-1}$ for microbial growth and 100 $mg.l^{-1}$ for ferrocyanide biodegradation at a glucose concentration of 0.465 $g.l^{-1}$. Maximum ferrocyanide removal and grow rates were determined as 30.7 $mg.g^{-1}.h^{-1}$ and 0.08 h^{-1}, respectively under these conditions. At higher ferrocyanide concentrations, microbial growth and substrate removal rates were decreased because ferrocyanide ions had a potentially inhibitory effect on cell growth. Also there was no growth of bacteria by using ferrocyanide as a sole source of carbon and nitrogen.

The increase of the concentration of KCN on cyanide removal by *Trametes versicolor,* showed a decrease of the cyanide removal rate. At initial KCN concentration of 25mg/l, the cyanide removal was about 84%, and for 400mg/l of initial KCN the removal rate was about 27%. (Cabuk et al. 2006).

Initial concentration of glucose*:*

Some microorganisms are able to grow in medium containing only cyanide compounds as nutrients (i.e. carbon and nitrogen). But other microorganism such as *Pseudomonas fluorescens* P70, *Bhurkholderia cepacia* strain C3, could not grow in medium containing only cyanide as nutrient. In these cases there is a need to supply an external carbon source generally provided as glucose (Dursun et al, 1999), arabinose, fructose, galactose, mannose and xylose , but it seems that hexoses (fructose and glucose) readily support the utilization of cyanide; with pentose (arabinose and xylose), poor cyanide utilization of *B. Cepacia* was observed (Adjei and Ohta, 2000).

Using *Pseudomonas fluorescens* P70 lower initial glucose concentrations favored higher removal yields for both the glucose and ferrous (II) cyanide complex ions. The optimum glucose concentration was 0.465 $g.L^{-1}$ at which the bacteria remove 98% of the glucose and 60% of the ferrocyanide. At lower glucose concentration the glucose was totally used (100%) but the cyanide removal rate was lowered. Over 0.465 $g.L^{-1}$ of glucose concentration, generally a decrease in removal yields of both glucose and cyanide was observed (Dursun et al. 1999).

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials:

3.1.1 Microorganisms:

The microorganisms used in the study were *Pseudomonas resinovorans* ATCC®14235 and *Pseudomonas fluorescens* Pf-5. Both bacteria were purchased at ATCC (USA).

Pseudomonas fluorescens is a common Gram-negative, rod-shaped bacterium. It is found in soil and especially in water and wastewater. Several strains of *Pseudomonas fluorescens*, have been used to remove cyanide from water.

Pseudomonas resinovorans is a Gram-negative, soil bacterium that is commonly found in the lubricating oils of wood mills. It has been shown to be able to degrade carbazole, which is produced by petrochemicals industries. The bacteria were isolated from activated sludge of a coking wastewater treatment and the removal efficiency of carbazole was up to 97% in 36 hours, using carbazole as sole source of carbon, energy and nitrogen (Zhao et al. 2011). The use of this species of bacteria to remove carbazole, a recalcitrant compound present in wastewater, with production of ammonia, suggested that we could use the same species for the bioremediation of cyanide containing in wastewater.

3.1.2 Cyanide compounds:

Four cyanide compounds have been used in this study. These are two strong iron complexes of cyanide (i.e. Potassium hexacyanoferrate(II) trihydrate, $K_4Fe(CN)_6.3H_2O$ and sodium hexacyanoferrate(II) decahydrate, $Na_4Fe(CN)_6.10H_2O$) and two moderately strong metal complexes of cyanide (i.e. Potassium dicyanoargentate $KAg(CN)_2$) and zinc cyanide $Zn(CN)_2$). All the cyanide compounds used in the study were purchased from SIGMA-ALDRICH (California, USA).

3.1.3 Growth Media:

The growth media were prepared by adding 30g of powdered tryptic soy broth to 1 liter of deionized (DI) water, and 25g of nutrient broth in powdered form to 1 liter of DI water; The solutions were then sterilized in the autoclave at 121°C for 15 min. As indicated by the bacteria manufacturer *P. fluorescens* Pf5 grown in tryptic soy broth and *P. resinovorans* in nutrient broth.

An aliquot of *Pseudomonas fluorescens* Pf-5 and *Pseudomonas resinovorans* was inoculated to the tryptic soy broth and nutrient broth, respectively. The inoculum was then placed in the shaker incubator (Max Mini 4450) at 30°C, 230 rpm and grown for 72 hours in order to get a complete growth curve for the bacteria. The bacterial growth was measured at every hour by using a spectrophotometer (NanoDrop ND 100) at an optical density of 600nm. To avoid any latency during the biodegradation test, the bacteria used were supposed to be harvested during the log phase of their growth.

3.2 Biodegradation assessments:

3.2.1 Preparation of resting cells

A preliminary growth curve obtained during 72 hours showed that, both of the bacteria started their log phase after 24 hours in the incubator. Therefore, the bacteria were harvested after 24 hours of growth in the incubator at 230 rpm and 30°C. The bacteria were harvested by centrifugation at 4000*g for 5 min using a Centrifuge (Eppendorf 5810 R). After harvesting, the bacteria were washed twice in a phosphate buffer solution (PBS). Finally, the cells were suspended into the PBS solution for further tests. The PBS solution was prepared by using 23.4 ml of 5M NaCl, 18 ml of 0.5M $NaPO_4$ buffer solution and 258.6 ml of DI water.

3.2.2 Biodegradation medium

The resting cells were grown in M9 medium supplemented with glucose and cyanide compounds. For the preparation of M9 medium, M9 Minimal salt solution (5×) was prepared first: 1 liter of deionized water, 33.9g of Disodium phosphate NA_2HPO_4, 15g of monopotassium phosphate KH_2PO_4, 2.5g of Sodium Chloride (NaCl) and eventually 2ml of

1M $MgSO_4$ (when there was a need to supply an external source of nitrogen). Then 2ml of 1M $MgSO_4$ and 0.1ml of 1M $CaCl_2$, were added to 200 ml of M9 minimal salts 5× prepared above. Finally, the solution was adjusted to 1l by adding DI water.

Preparation of 20% glucose:

Glucose solution was prepared by adding 20g of powdered glucose to 80g of DI water (20%). The glucose solution prepared was used as a stock solution. When needed, glucose stock solution was added to M9 medium to get the desired concentration: 20 ml of glucose solution was added to 1 liter of M9 medium to get 4g/l; 10g was added to get 2g/l; 5g was added to get 1g/l, etc.

Preparation of cyanide compounds:

Stock solutions of cyanide compounds were prepared at a concentration of 0.5M and pH 10 to avoid release of HCN gas during the experiments. The stock solutions were prepared every 14 days and stored at 4°C or colder. Then, 625µl of cyanide stock solution was added to 50ml of M9 medium to get a final cyanide concentration of 6.25mM.

pH adjustment:

The pH of the biodegradation medium is also adjusted during the test. The acidic conditions were adjusted by adding several drop of 0.25N hydrochloric acid (HCl) and the basic condition was adjusted by using 0.25 N sodium chloride (NaCl).

3.3 Preliminary tests:

3.3.1 MIC tests

MIC tests were performed to identify the concentration of cyanide compounds, which inhibits the growth of the bacteria. In these tests cyanide compounds were added to the M9 medium as a nitrogen source. M9 medium with different cyanide compounds concentrations from 100mM to 1.56mM were introduced into culture tubes with a volume of 2ml for each sample.

About 50µl of resting cells suspended in PBS solution (OD ≈1) were added to the 2 ml of M9 medium containing cyanide compounds. The solution was then placed into the shaker

incubator at 30°C and 230 rpm for 24 hours. The bacterial growth was assessed at the beginning and after 24 h of growth by measuring the absorbance at 600nm using a spectrophotometer.

For each bacterial strain a control was made by using M9 medium with ammonium chloride (5.0g/l) as a nitrogen source. Cyanide compound was not added to the M9 medium. The bacterial growth was also assessed and compared to growth in M9 medium containing cyanide compounds.

3.4 Growth in M9 medium using cyanide as sole source of carbon and nitrogen

To test the ability of the two bacterial strains to use cyanide compounds as carbon and nitrogen source, bacterial strains were added to the M9 medium which contains only cyanide compounds. Glucose and ammonium chloride were not added to the solution. At the end of the test, the bacterial growth was assessed by measuring the absorbance of the sample at 600 nm using the spectrophotometer.

3.4 Biodegradation tests using cyanide as nitrogen source and glucose as a carbon source

Since the bacterial strains didn't show any growth in the M9 medium without glucose and ammonium chloride, all the biodegradation tests were performed using cyanide compounds as nitrogen source and glucose as a carbon source.

Biodegradation tests were performed in a 250 ml glass flask. 50 ml of M9 medium was poured into the flask. Then 20% glucose and stock solution of cyanide compounds were added to the medium (as mentioned in II.2.b and II.2.c). Finally, 5ml of suspended resting cells at OD value of 0.5 were added to the solution and mixed properly. The fermenter was coated in black color to prevent the photodecomposition of ferrocyanide ($Fe(CN)_6^{-4}$) ions to ferrocyanide ($Fe(CN)_6^{-3}$) ions in the presence of light (Bodek et al, 1998). The fermenter was then put into the shaker incubator and the different parameters (bacterial growth, glucose concentration, ammonia concentration) were assessed for 72 hours.

3.5 Parameters assessed during the tests:

3.5.1 Bacterial growth:

The bacterial growth was assessed by measuring the absorbance at 600 nm using the Spectrophotometer. The blank used was prepared by taking a small amount of the solution (M9 + cyanide solution + glucose) before adding the resting cells. The absorbance was measured at every 4 hours until 20 hours and then every 12 hours up to the 72nd hour.

Specific growth rate:

The Specific growth rate μ is defined as the increase in cell population per unit time. It was determined from the growth curve of the bacteria. A linear trend line was generated using the optical density data collected during the exponential phase. The slope of this linear line represents the specific growth rate of the bacteria as shown in Figure 2.

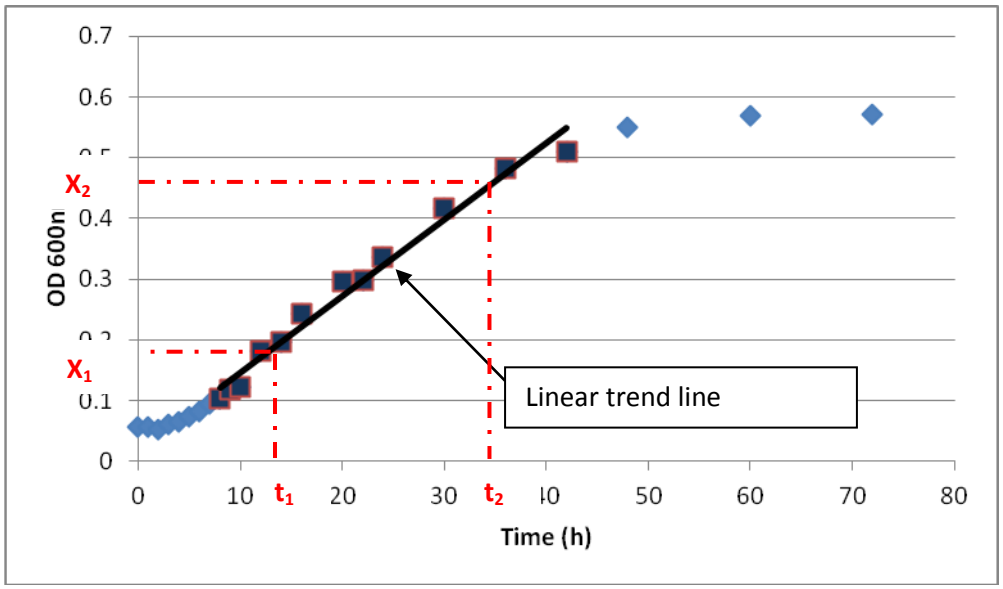


Figure2. Calculation of specific growth rate

$\mu = (LnX_2 - LnX_1) / (t_2-t_1)$

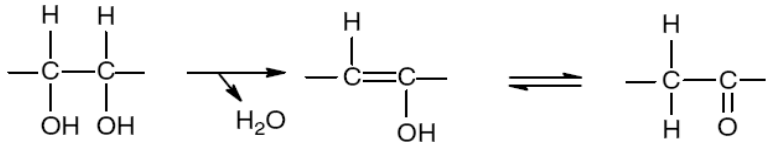
(Levasseur et al. 1993)

3.5.2 Glucose analysis.

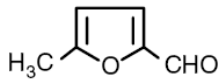
Glucose concentration was determined by using the "Phenol-sulfuric acid assay" method.

***Reaction theory**:*

In the presence of strong acids and heat, carbohydrates undergo a series of reactions that leads to the formation of furan derivatives such as furanaldehyde and hydroxymethyl furaldehyde.



The initial reaction, a dehydration reaction is followed by the formation of furan derivatives. For the hexoses, the Furan derivate has this formula:



The Furan then condenses with themselves or phenolic compounds to produce dark colored complexes. The developed complex absorbs UV-VI light, and the absorbance is proportional to the sugar concentration in a linear fashion. An absorbance maximum is observed at 490 nm for hexoses as measured by a UV-VI spectrophotometer. (Brumer and Cui, 2005)

***Operating procedure**:*

About 0.5 ml of the sample is taken in a microtube, and centrifuged at 7000*g for 1 min using the micro centrifuge (Eppendorf 5415R); 10 µl of the supernatant is then taken and put into a 10 ml glass test tube.

About 0.5ml of phenol in a 4% solution was added to the test tube containing the clear sample solution of 10µl. About 2.5 ml of 96% concentrated sulfuric acid was then added in a rapid stream directly to the surface of the liquid in the test tube (Fournier, 2001). The mixture was shaken vigorously and waits until 30 min for color development. The solution absorbance was measured at 490 nm using a spectrophotometer (HACH DR 2800). Mixing and standing time are kept the same for all samples to assure reproducible results.

***Quantification**:*

A calibration curve was prepared using the sugar being assayed. Glucose stock solution of 1 mg/ml was used to prepare 10 standards such as 5, 10, 15, 20, 25, 30, 35, 40, 45, 50µl of glucose solution added in each glass of 10 ml. A graph of absorbance vs. concentration was constructed and the concentration was derived from the calibration curve.

The residual glucose concentration of the samples was measured at every 4 hours until 20 hours and then every 12 hours up to the end of the test.

3.5.3 Analysis of Ammonia:

The background of biological removal of cyanide compounds showed a production of ammonia during the degradation of cyanide by the microorganisms. Thus, the assessment of ammonia concentration in the sample could be a strong parameters to prove the biodegradation of cyanide compounds. Ammonia was measured according to the Method 10023 (Salicylate method for Nitrogen, Ammonia Low Range (0.02 to 2.50mg/l NH_3-N)) provided by the HACH company.

3.6 Assessment of the effects of different parameters

3.6.1 Effect of initial pH:

The tests were conducted to assess the effect of the initial pH of the biodegradation medium on bio-removal of cyanide. The biodegradation medium was prepared at constant cyanide concentration of 6.25mM and at constant glucose concentration of 4g/l. The initial pH was maintained at 5, 7 and 9. Resting cells were then added to the medium and placed in the shaker incubator at 30°C and 230 rpm for 72hours. For each initial pH, bacterial growth, glucose concentration, and ammonia generation were measured during the test, and the results were then compared.

3.6.2 Effect of initial glucose concentration:

The effects of the initial glucose concentration of the biodegradation medium were investigated. The biodegradation medium was prepared in flasks with different glucose concentrations of 2, 4, 6, 8 g/l. The cyanide concentration of the medium was 6.25mM. The

initial pH was the optimum pH value obtained from the previous tests. Resting cells were then added to the medium and placed in the shaker incubator at 30°C and 230 rpm for 72 hours. For each initial glucose concentration, bacterial growth, glucose concentration, and ammonia generation were determined during the test and the results were then compared.

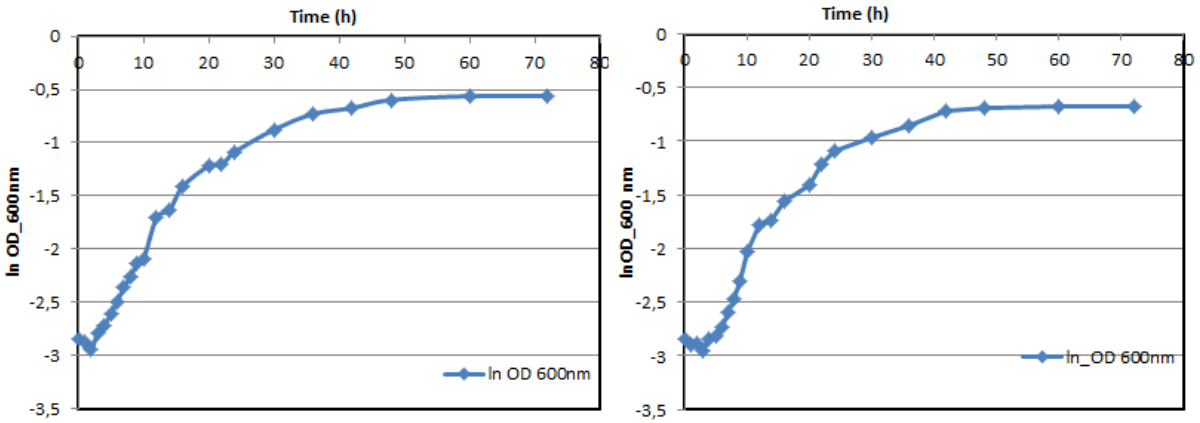
CHAPTER 4

RESULTS AND DISCUSSIONS

4.1 Growth curves:

The growth curve of the bacteria shows three main stages (Fig 3 a and b): The first stage was a **lag phase** which last about 4 to 6 hours after the beginning of the experiment, for both bacteria. In this stage, there was no growth of bacteria. It is the period where the individual bacteria are maturing by synthetizing RNA, enzymes and other molecules. The second stage is the logarithmic phase which last from the 6^{th} hour to the 42^{nd} hour. It is characterized by cell doubling due to the presence of sufficient nutrient in the medium. The third **stage** is called the stationary phase, which start from the 42^{nd} hour; it was due to depletion of the nutrient, and/or the formation of inhibitory products such as organic acids which do not allow the bacteria to grow.

In the biodegradations test of cyanide, in order to get useful results it's important to avoid the lag phase of the bacteria growth. Therefore, bacteria were harvested during the logarithmic phase (i.e. after 24h) for using in the biodegradation test of cyanide.



a- P. fluorescens* Pf5** ***b- P. resinovorans

Fig3: Growth curves

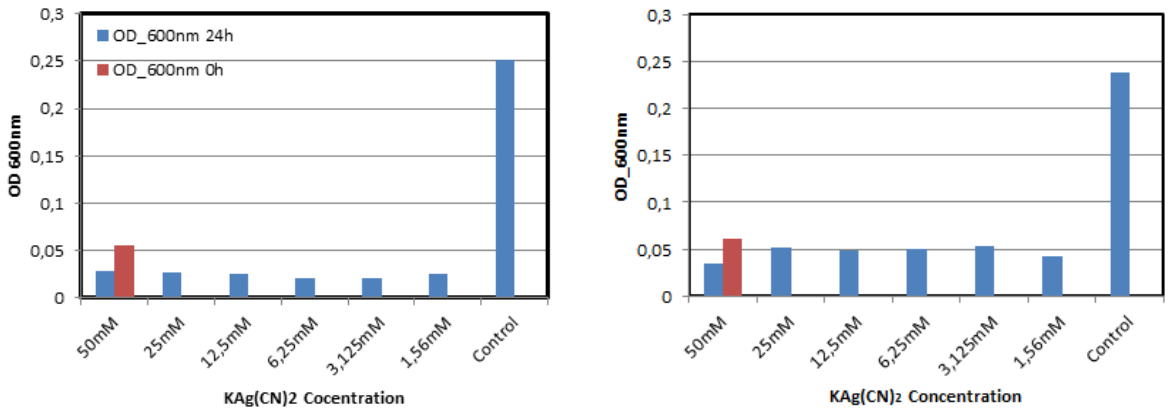
Specific growth rate for *P. fluorescens* Pf5: μ= 0.0825

Specific growth rate for *P. resinovorans*: μ= 0.0595

4.2 MIC Tests:

4.2.1 *Potassium dicyanoargentate*

Figure 4 shows that there was no growth of the bacteria for different concentrations (1.56mM, 3.125mM, 6.25mM, 12.5mM, 25mM) of potassium dicyanoargentate ($KAg(CN)_{2)}$. For each concentration there was a decrease of both bacterial population after the 24 hours of the test compared to its initial concentration which suggests an inhibitory effect of $KAg(CN)_2$. This is probably due to the presence of silver (Ag) in this compound. Silver is generally known as a bactericide, and it is used for disinfecting drinking water (Buktus et al. 2004). Therefore, these results indicate that, bioremediation process using *Pseudomonas fluorescens* Pf5 and *P. resinovorans* is not effective for the removal of the silver cyanide complexes.



a- P. fluorescens* Pf5** ***b- P. resinovorans

Fig4: MIC tests for $KAg(CN)_2$

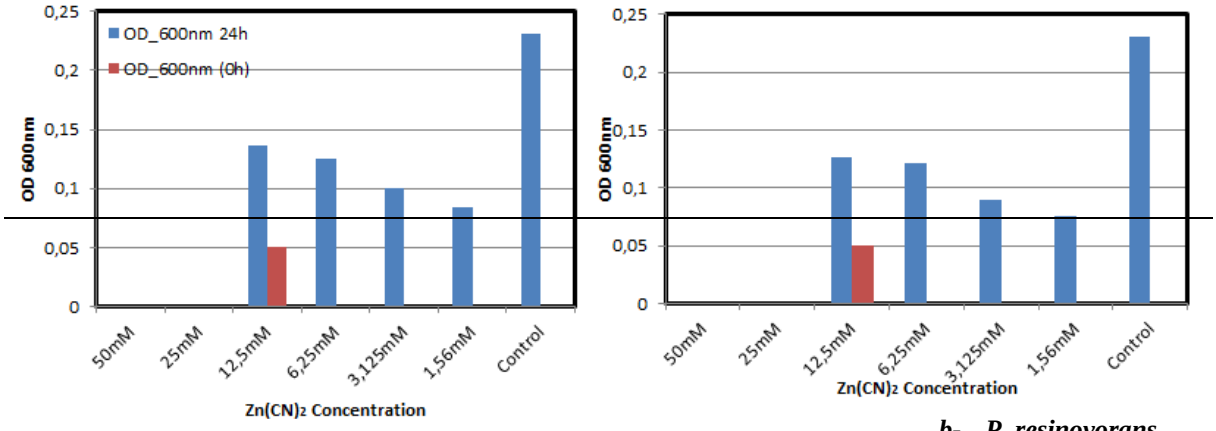
Silver is not the sole element able to inhibit bacterial growth when complexed with cyanide ion; copper also showed an inhibiting effect on the growth of one strain of *Pseudomonas fluorescens* when copper makes complex-compound with cyanide. The ability of microorganisms to grow and utilize cyanide in the presence of these metals is important, and may allow its use in the biological remediation of waste streams from industries such as ore refining and electroplating (Silvos-Avilos et al. 1990).

4.2.2 Zinc cyanide

Results in Figure 5 show an increase of optical density in the M9 medium supplemented with resting cells, glucose, and zinc cyanide after 24 hours in the incubator

compared to its initial value. The initial optical density was 0.05. After 24 h, the optical densities increased to 0.136, 0.125, 0.1, and 0.84 for 12.5, 6.25, 3.125, 1.56mM of zinc cyanide, respectively. For *P. resinovorans* the initial OD_600nm increased to 0.127, 0.12, 0.089, and 0.075.

In the MIC test, zinc cyanide did not dissolve completely even at the lowest concentration of 1.56 mM because the solubility of zinc cyanide in water is 0.5 $mg.L^{-1}$ at 25°C. It is thought that the non-dissolved particles of zinc cyanide might interfere during the measurement of the optical density of the solution. Therefore, it was difficult to determine the MIC value of Zinc cyanide using *P. fluorescens* Pf-5 *or P. resinovorans*, and zinc cyanide was not used for the biodegradation test.



c- P. fluorescens **Pf5** *b- P. resinovorans*

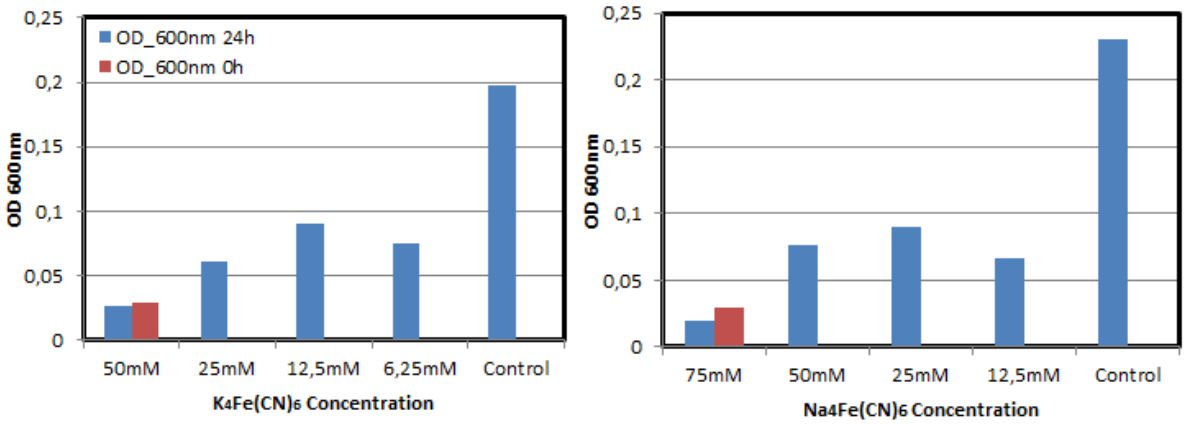
Fig5: MIC tests for $Zn(CN)_2$

4.2.3 Potassium and sodium ferrocyanide

Figure 6 shows the bacterial growth in M9 medium supplemented with glucose and potassium ferro cyanide at different concentration with an initial optical density of 0.029 in the medium *(P. fluorescens* Pf5). Results show an increase of optical density in the medium after 24 hours compared to its initial value. The optical densities were 0.075, 0.09 and 0.06 for potassium ferrocyanide of 6.25mM, 12.5mM, and 25mM, respectively. For the sample containing 50 mM of potassium ferrocyanide a decrease in the optical density of 0.027 was observed after 24 h. The optical densities of the medium was 0.067, 0.09, 0.076 and 0.02 for 12.5mM, 25mM, 50mM and 75 mM of sodium ferrocyanide, respectively after 24 hours

An increase of the optical density of solution containing ferrocyanide after 24 hours indicates the growth of bacteria in the medium. The decrease of the optical density after 24

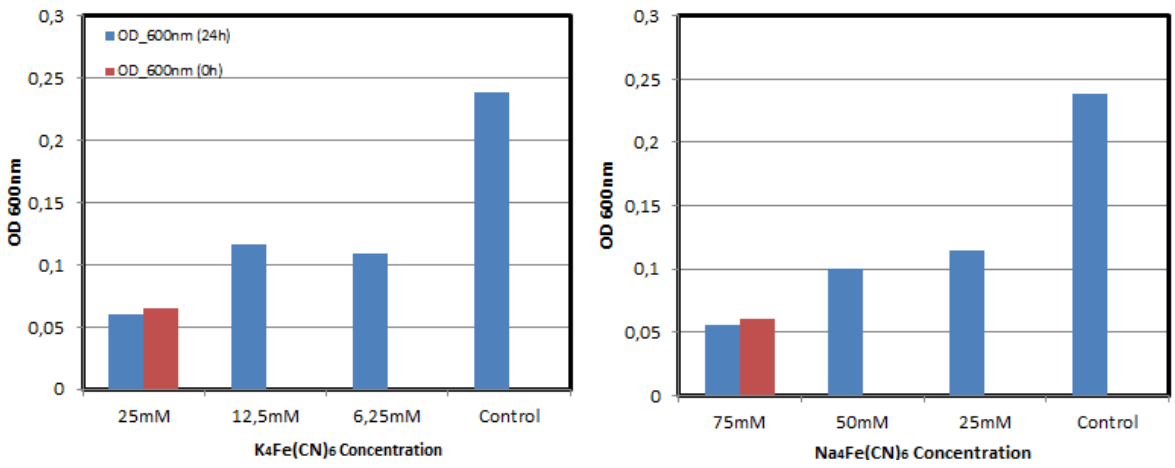
hours suggests an inhibiting effect of the cyanide compound to the growth of the bacteria. Therefore, 50 mM (i.e. 21.1 $g.L^{-1}$) could be considered as the MIC value of $K_4Fe(CN)_6$ and 75 mM (i.e. 36.3 $g.L^{-1}$), the MIC values of $Na_4Fe(CN)_6$, for the growth of *P. fluorescens Pf5*.



a- $K_4Fe(CN)_6$ **b- $Na_4Fe(CN)_6$**

Fig6: MIC tests for *P. Fluorescens* Pf5 using ferrocyanide

For *P. resinovorans*, from an optical density of 0.065, the optical densities were 0.125, 0.118, 0.109 and 0.116 for ferrocyanide of 1.56, 3.25, 6.25, and 12.5mM. For the sample containing 25mM of potassium ferro cyanide a decrease in the optical density of 0.06 was observed after 24 hours. For sodium ferrocyanide, the optical density was 0.115 and 0.1 for 25 and 50mM of, respectively after 24 hours, and 0.006 for 75 mM. Therefore, 25 mM (i.e.10.55 $g.L^{-1}$) could be considered as the MIC value of $K_4Fe(CN)_6$, and 75mM (i.e. 36.3 $g.L^{-1}$) the MIC value of $K_4Fe(CN)_6$, for the growth of *P. resinovorans.* (Fig. 7)



a- $K_4Fe(CN)_6$ **b- $Na_4Fe(CN)_6$**

Fig7. MIC tests for *P. resinovorans* using ferrocyanide

4.3 Biodegradation of cyanide compounds without external carbon and nitrogen sources:

Results of bacterial growth in the M9 medium supplemented with cyanide compounds as carbon and nitrogen source are presented in Figure 8. The initial optical density of the medium was 0.061 using $K_4Fe(CN)_6$, $Na_4Fe(CN)_6$, $KAg(CN)_2$ and $Zn(CN)_2$. After 24 hours, the optical densities were 0.059 for $K_4Fe(CN)_6$, 0.062 for $Na_4Fe(CN)_6$ and 0.057 for $Zn(CN)_2$. In M9 medium containing $KAg(CN)_2$, the optical density decreased to 0.038 after 24 hours. Therefore, *Pseudomonas fluorescens* Pf5 was not able to grow in the M9 medium supplemented with $K_4Fe(CN)_6$, $Na_4Fe(CN)_6$, $KAg(CN)_2$ or $Zn(CN)_2$, because the bacteria was not able to use these cyanide compounds as both carbon and nitrogen sources. For *P. resinovorans* also, there was no bacterial growth in different cyanide compounds after 24 hours.

Other bacterial strain such as *Pseudomonas fluorescens* P70 was also not able to use cyanide as a sole carbon and nitrogen source for their growth (Dursun el al. 1999). These results suggest that an external carbon source is needed to allow the growth of bacteria.

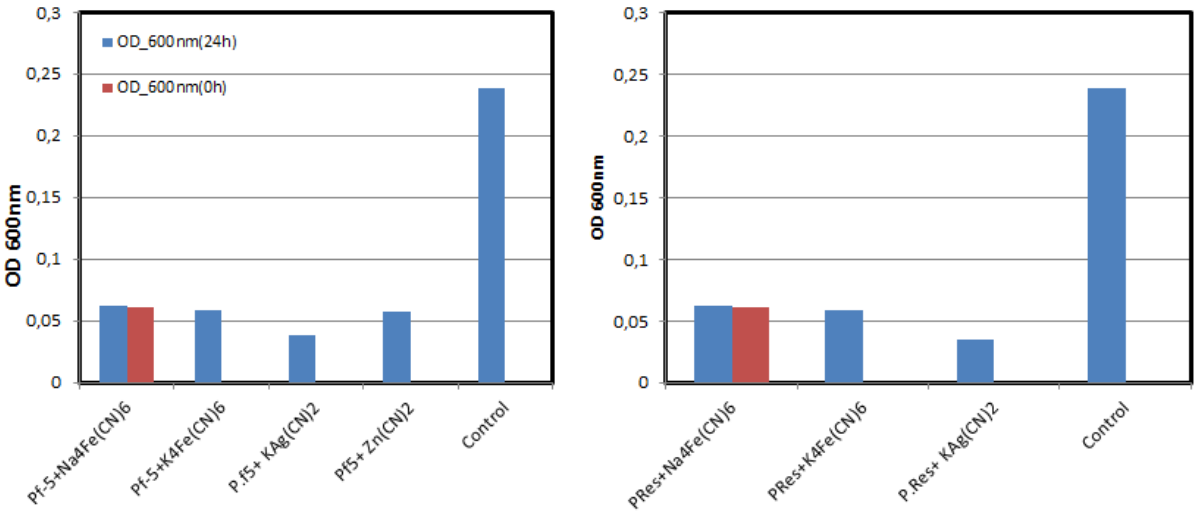


Fig8. Growth of bacteria in different cyanide compounds

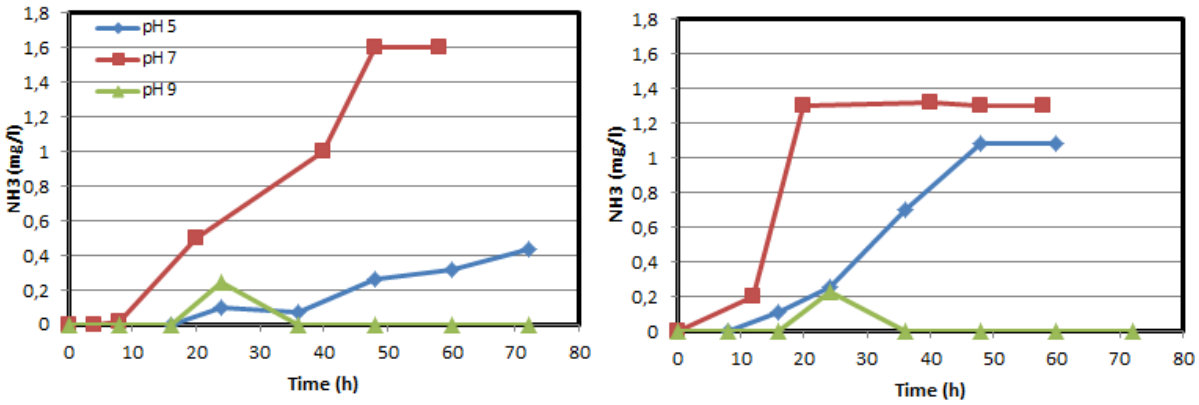
4.4 Biodegradation of cyanide compounds with glucose as external carbon source

Since $Zn(CN)_2$ didn't dissolve totally in the medium and then influenced the bacterial growth assessment, and since $KAg(CN)_2$ inhibited the bacterial growth, further biodegradation tests were done with only $K_4Fe(CN)_6$ and $Na_4Fe(CN)_6$.

4.4.1 Effect of pH:

Effect of pH on ammonia generation

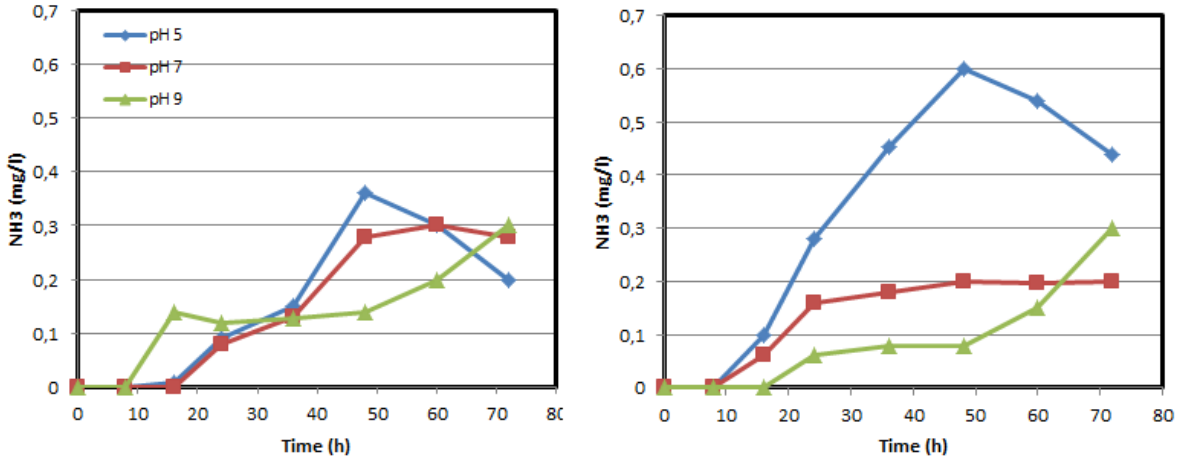
Using *P. fluorescens* Pf5 initial pH played a major role in the biodegradation of the cyanide compounds. At pH 7, ammonia was first detected at the 8th hour at a concentration of 0.02 mg/l using $K_4Fe(CN)_6$. Then the ammonia concentration increased to 1.6 mg/l at the 48th hour. After 48th hour, the ammonia concentration was stable at 1.6 mg/l in the sample. At pH 5, ammonia was detected after 24 hours and then increased slowly during the test. At the end of the test the ammonia concentration was 0.44 mg/l. For $Na_4Fe(CN)_6$, the ammonia concentration was 0.2 mg/l at the 12th hour and increased to 1.3g/l after 20 hours, at pH 7. The ammonia concentration was relatively constant during the rest of the test. At pH 5, ammonia was first detected at the 16th hour at 0.11 mg/l. Then, the ammonia concentration increased up to 1.08 mg/l at the 48th hour and finally showed a relatively constant value. For both ferro cyanide ammonia concentration at pH 9 was 0 mg/l during the test except at the 24th hour where ammonia concentration was 0.22 mg.l^{-1} and 0.24 (Fig. 9).



a) **$K_4Fe(CN)_6$** **b) $Na_4Fe(CN)_6$**

Fig 9: Effect of pH on ammonia generation by *P. fluorescens* Pf5

Using *P. resinovorans* , at pH 7, ammonia was first detected at the 24th hour at a concentration of 0.08 mg/l using $K_4Fe(CN)_6$. Then the ammonia concentration increased to 0.28mg/l at the 48th hour. After 48 hours the ammonia concentration was stable in the sample at 0.28mg/l. At pH 5, ammonia was detected after 16 hours (0.008 mg/l) and then increased slowly to 0.36 mg/l at the 48th hour. After the 48th hour ammonia concentration decreased progressively to 0.2 mg/l at the 72nd hour. Ammonia concentration at pH 9 was first detected at 0.14mg/l at the 16th hour, and then stabilized up to the 48th hour. Then the concentration increased up to 0.3 mg/l at the 72nd hour (Fig 10a) . At pH 7, ammonia was first detected at the 16th hour at a concentration of 0.06mg/l using $Na_4Fe(CN)_6$. Then the ammonia concentration increased to 0.16mg/l at the 24th hour. After 24th hour the ammonia concentration was stable in the sample at 0.3 mg/l. At pH 5, about 0.1 mg/l of ammonia was detected after 16 hours and increased to 0.6 mg/l at the 48th hour. After the 48th hour ammonia concentration decreased slowly to 0.44mg/l at the 72nd hour. Ammonia concentration at pH 9 was first detected at 0.06 mg/l at the 24th hour, and then stabilized up to the 48th hour. Finally, the concentration increased up to 0.3 mg/l at the 72nd hour (Fig 10b).



a) **$K_4Fe(CN)_6$** **b) $Na_4Fe(CN)_6$**

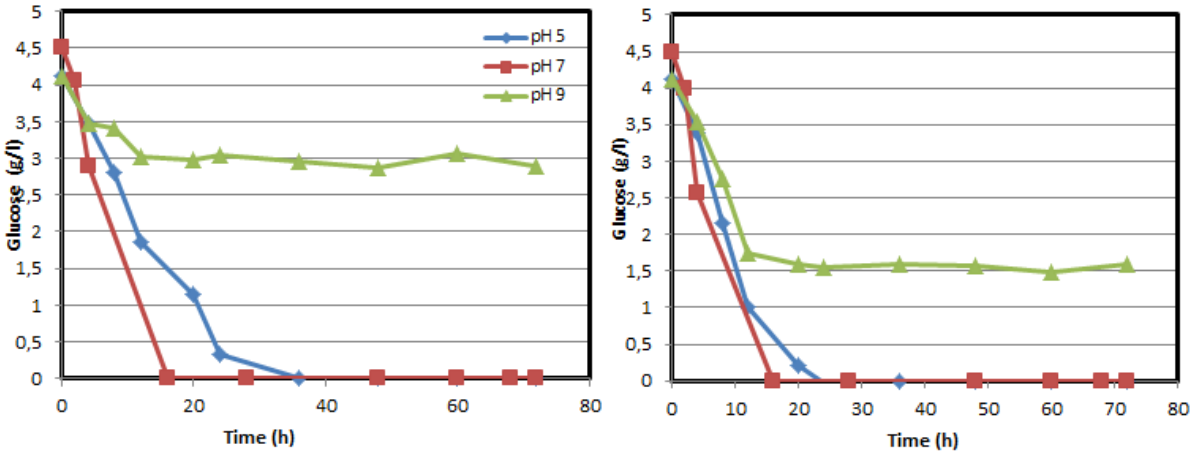
Fig 10: Effect of pH on ammonia generation by *P. resinovorans*

For *P. fluorescens* Pf5, maximum ammonia generated (1.6 mg/l for $K_4Fe(CN)_6$, and 1.32mg/l for Na4Fe(CN)6) at pH 7 suggesting a maximum bacterial activity and cyanide degradation at this pH. For *P. resinovorans*, maximum ammonia generated (0.36 mg/l for $K_4Fe(CN)_6$, and 0.6mg/l for Na4Fe(CN)6) at pH 5.

Dursun et al (1999) reported a maximum ferro cyanide degradation rate at pH 5 using *Pseudomonas fluorescens* P70; the bacterial activity was significantly reduced at pH 7 and was almost inhibited at pH 9.

Effect of pH on glucose utilization.

At pH 7, initial glucose concentration in the medium was 4.5 g/l when $K_4Fe(CN)_6$ was used as a nitrogen source (Fig 11a). The *P. fluorescens* Pf5 used glucose completely (100%) within 16 hours. At pH 5, the initial glucose concentration was 4.12 g/l and showed a decrease up to 0 g/l at the 36th hour. At pH 9, the glucose was reduced from an initial value of 4.12 to 3.03 g/l within 12 hours, and then, it was relatively constant up to the end of the test (i.e. 2.89 g/l) . Glucose utilization by *P. fluorescens* Pf-5 using $Na_4Fe(CN)_6$ is shown in Figure 12. At pH 7, initial glucose concentration in the medium was 4.5 g/l. Glucose utilization was completed by the bacteria within 16 hours. At pH 5, the glucose concentration was 4.12g/l and showed a decreasing trend up to 0 g/l at the 24th hour. At pH 9, the initial glucose concentration was 4.12 g/l and then, reduced to 1.59 g/l at the 20th hour. After that, the glucose concentration was relatively constant up to the end of the test (Fig 11b).



a) $K_4Fe(CN)_6$ **b) $Na_4Fe(CN)_6$**

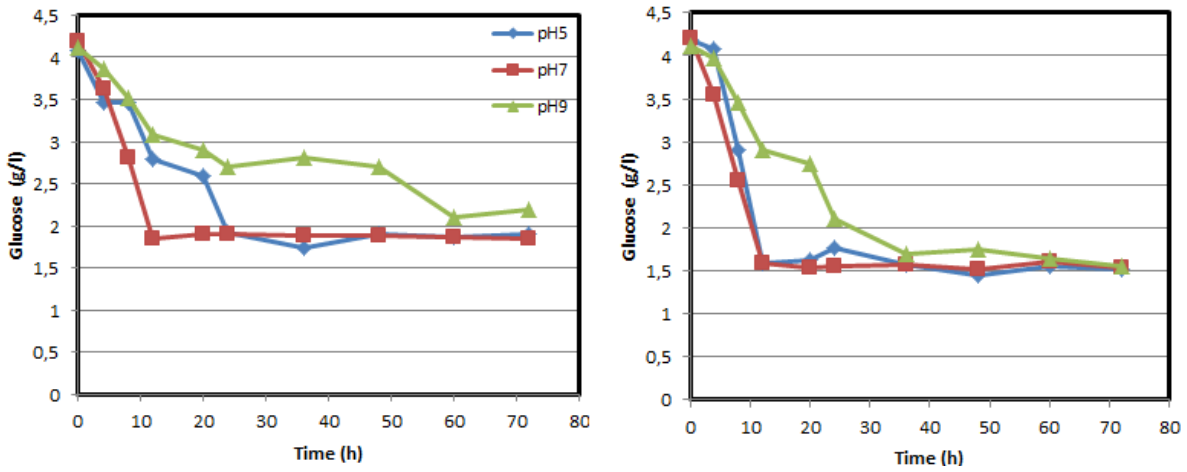
Fig 11: Effect of pH on glucose utilization by *P. fluorescens* Pf5

Higher bacterial activity at pH 7 induced higher glucose utilization. Therefore, the glucose concentration rapidly dropped to 0 g/l for both ferro cyanides. At pH 5, all the glucose was used even though it took more time. This may be due to the reduction of bacterial

activity mentioned above. At pH 9, the activity of bacteria was significantly reduced which explains the non-generation of ammonia during the test. However, a few amount of glucose have been used suggesting that the bacterial activity was not completely inhibited. pH 9 may have a complete inhibition effect on ammonia generation but would moderately inhibit the use of glucose by the bacteria.

At pH 7, initial glucose concentration in the medium was 4.5g/l when $K_4Fe(CN)_6$ was used as a nitrogen source. *Pseudomonas resinovorans* used glucose up to 1.85g/l within 16 hours. At pH 5, the bacteria used glucose up to 1.89 mg/l within 36 hours. At pH 9, the glucose utilization was subsequently reduced ; from an initial value of 4.12 to 3.03g/l within 12 hours, and then it was relatively constant up to the end of the test (i.e. 2.89g/l) (Fig 12a).

At pH 7 and 5, initial glucose concentration in the medium was 4.5g/l when $Na_4Fe(CN)_6$ was used as a nitrogen source. *Pseudomonas resinovorans* used glucose up to 1.59 g/l within 16 hours. At pH 9, the glucose utilization was subsequently reduced from an initial value of 4.12 to 1.69 g/l within 36 hours, and then it was relatively constant up to the end of the test (i.e. 1.856mg/l) (Fig 12b)



a- $K_4Fe(CN)_6$ b- $Na_4Fe(CN)_6$

Fig 12: Effect of pH on glucose utilization by *P. resinovorans*

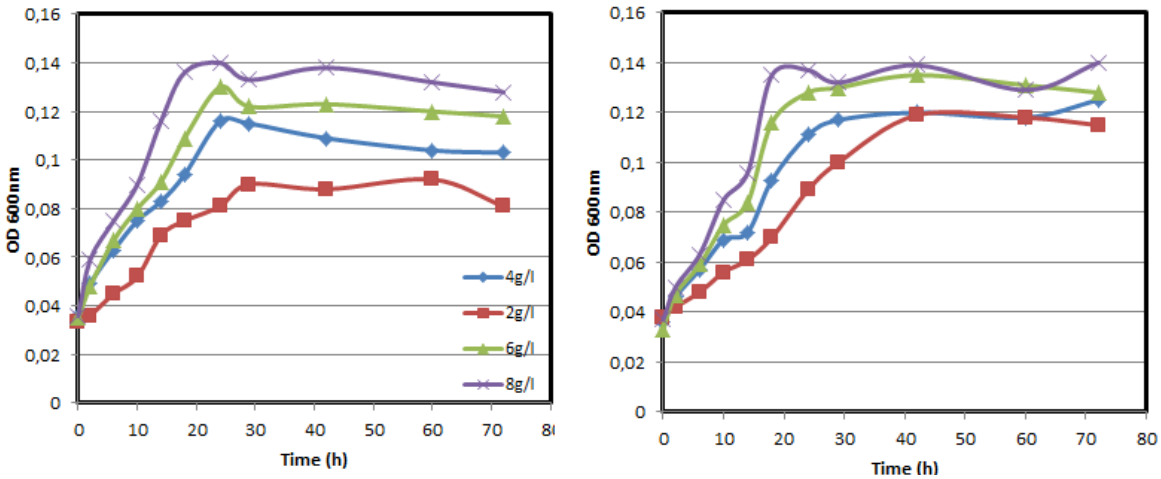
For both ferro cyanide compounds, glucose concentration at the end of the test was about the same. Although maximum ammonia generation occurs at pH 5, glucose utilization at pH 5 and 7 was similar. At pH 9, the same amount of glucose was used even though it took more time.

4.4.2 Effect of initial glucose concentration

Effect of initial glucose concentration on bacterial growth

Bacterial growth showed an increase as the initial glucose concentration was raised when $K_4Fe(CN)_6$ was used as a nitrogen source (Fig 13a). At 2g/l of glucose concentration, the OD value increased from 0.033 to 0.09 within 29 hours, and then relatively stable at 0.081 at the end of the test. The growth of bacteria showed a similar trend for 4 g/l, 6g/l and 8 g/l of glucose concentrations. In ease cases, OD values increased from the beginning to 24th hour, and then stabilized until the end. However, the optical density generally increased when the initial glucose concentration was increased for all the cases. The maximum OD value was 0.128 at 8 g/l of glucose concentration.

Figure 13b shows the bacterial growth using $Na_4Fe(CN)_6$ as a nitrogen source. At 2g/l of glucose concentration, the initial OD value was 0.038 and then increased to 0.119 at the 42th hour. After that, it was relatively stable at 0.117 up to the end of the test. For other concentrations (i.e. 4, 6, 8g/l), the bacterial growth showed the same trend with a sharp increased from the beginning up to the 24th hour, and then stabilized until the end of the test. However, the optical density generally increased when the initial glucose concentration was increased. The maximum OD value was 0.14, which was detected at 8g/l of glucose concentration.

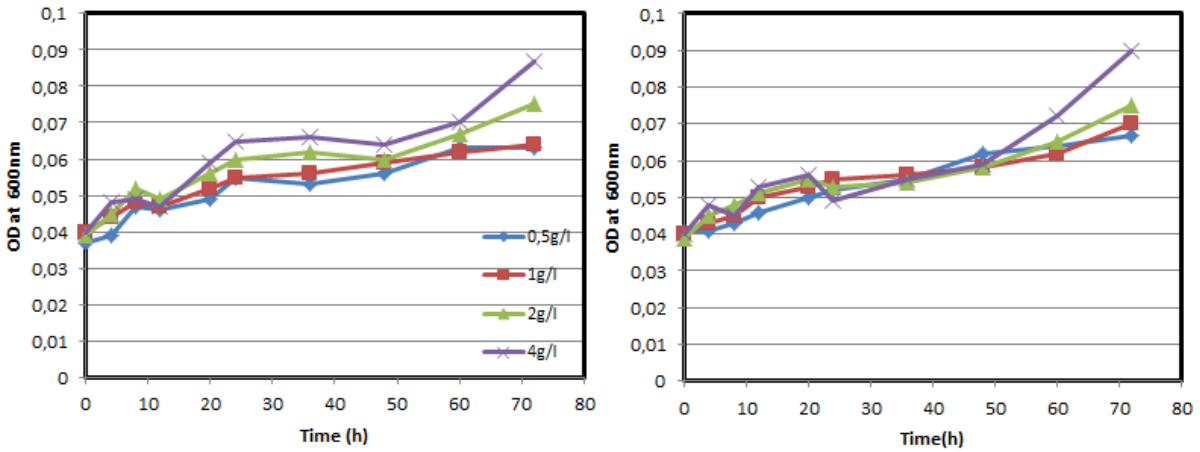


a) $K_4Fe(CN)_6$ b) $Na_4Fe(CN)_6$

Fig 13: Effect of initial glucose concentration on *P. fluorescens* Pf5 growth

Bacterial growth showed an increase as the initial glucose concentration was raised when $K_4Fe(CN)_6$ was used as a nitrogen source (Fig 14a). At 0.5 g/l of initial glucose concentration, the OD increase slowly during the test from 0.041 to 0.063. The growth of bacteria showed a similar trend for 1g/l, 2g/l and 4g/l of glucose concentrations. In each case, OD values increased from the beginning to 24th hour, and then stabilized until the end. However, the optical density generally increased when the initial glucose concentration was increased for all the cases. The maximum OD value was 0.87 for 4g/l of glucose concentration.

The growth of bacteria also increased with the increase of initial glucose concentration when $Na_4Fe(CN)_6$ was used as a nitrogen source (Fig 14b). At 0.5g/l of initial glucose concentration, the OD increase slowly during the test from 0.041 to 0.067. The growth of bacteria showed a similar trend for 1g/l, 2g/l and 4g/l of glucose concentrations. In each case, OD values increased from the beginning to 24th hour, and then stabilized until the end. However, the optical density generally increased when the initial glucose concentration was increased for all the cases. The maximum OD value was 0.9 for 4g/l of glucose concentration.



a- $K_4Fe(CN)_6$ **b- $Na_4Fe(CN)_6$**

Fig14. Effect of initial glucose concentration on P. resinovorans growth

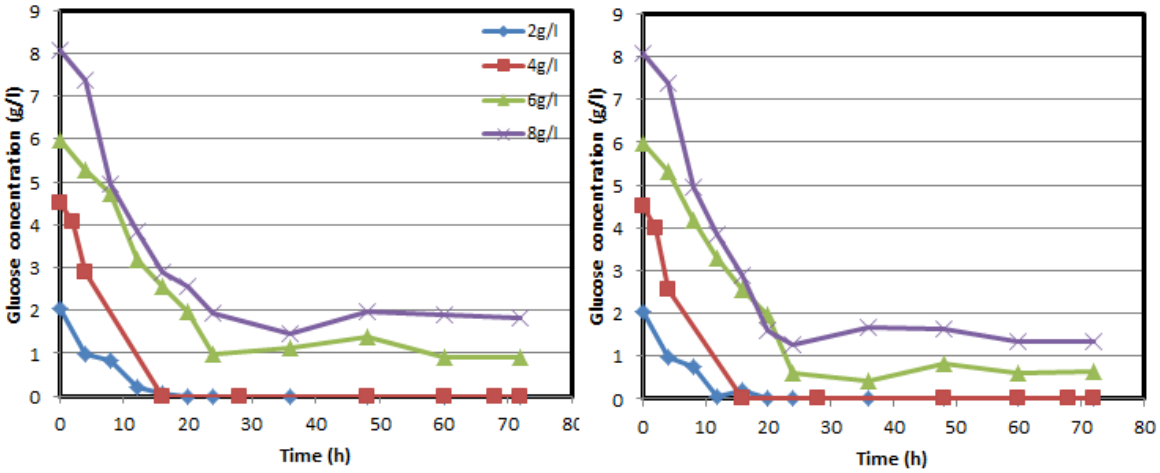
Glucose is used as nutrient (carbon source) for the growth of the bacteria. For both bacteria, an increase of the initial glucose concentration favored an increase of bacterial growth. Dursun et al. (1999) obtained a similar result when glucose concentration was raised: the

bacterial growth rate was increased when glucose concentration was raised up to 0.465 $g.L^{-1}$; but above this concentration the bacterial growth didn't change significantly.

Effect of initial glucose concentration on glucose utilization

Initial glucose concentration had a significant effect on glucose utilization by *P. fluorescens* Pf5 using K4Fe(CN)6 as a nitrogen source (Fig 15a). Using potassium ferrocyanide, the bacteria used 100% of the glucose from the medium containing 2g/l and 4g/l after 16 hours. In medium containing 6g/l of initial glucose, the glucose concentration rapidly decreased to 0.94g/l after 24 hour, and then stable until the end of the test. In the medium containg 8g/l of initial glucose, glucose concentration rapidly reduced to 1.44g/l after 36 hours, and then stable at 1.84 g/l up to the end of the test.

In case of sodium ferrocyanide, the *P. fluorescens* Pf5 used 100% of the glucose after 16 hours and 12 hours for 2g/l and 4g/l of initial glucose concentrations, respectively (figure 17). In the medium containing 6g/l of initial glucose, the glucose concentration decreased to 0.6 g/l after 24 hours, and then stable until the end of the test. In the medium containg 8 g/l of initial glucose, glucose concentration rapidly decreased to 1.28 g/l after 24 hours and then stable at 1.34 g/l up to the end of the test (Fig 15b)

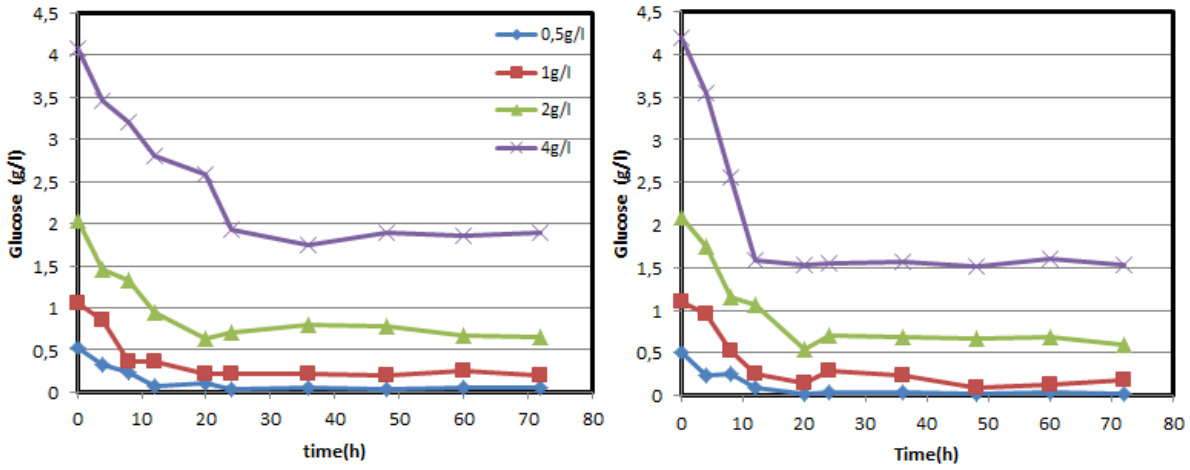


a- **$K_4Fe(CN)_6$** b- **$Na_4Fe(CN)_6$**

Fig 15. Effect of initial glucose concentration on glucose utilization by *P. fluorescens* Pf5

Initial glucose concentration had a significant effect on glucose utilization by *P. resinovorans* using potassium ferro cyanide (Fig 14a) and sodium ferro cyanide (Fig 14b) . At 0.5 g/l of initial glucose in the medium bacteria used the glucose up to 0.043 g/l after 24hours; then the glucose concentration was relatively stable during the rest of the test: at the end 0.045g/l of glucose was present in the medium. For other initial glucose concentration 1g/l, 2g/l and 4g/l utilization of glucose was similar. At 4g/l of initial glucose, 1.9g/l of glucose was remaining in the medium.

When sodium ferro cyanide was used, the bacteria used the glucose up to 0.022 g/l within 24 hours at 0.5mg/l of initial glucose concentration in the medium. Then the glucose concentration was relatively stable during the rest of the test with the end glucose concentration of 0.026g/l in the medium. For other initial glucose concentrations (i.e. 1g/l, 2g/l and 4g/l) utilization of glucose was similar. At 4g/l of initial glucose, 1.53g/l of glucose was remaining in the medium (Fig 16).



a- $K_4Fe(CN)_6$ **b- $Na_4Fe(CN)_6$**

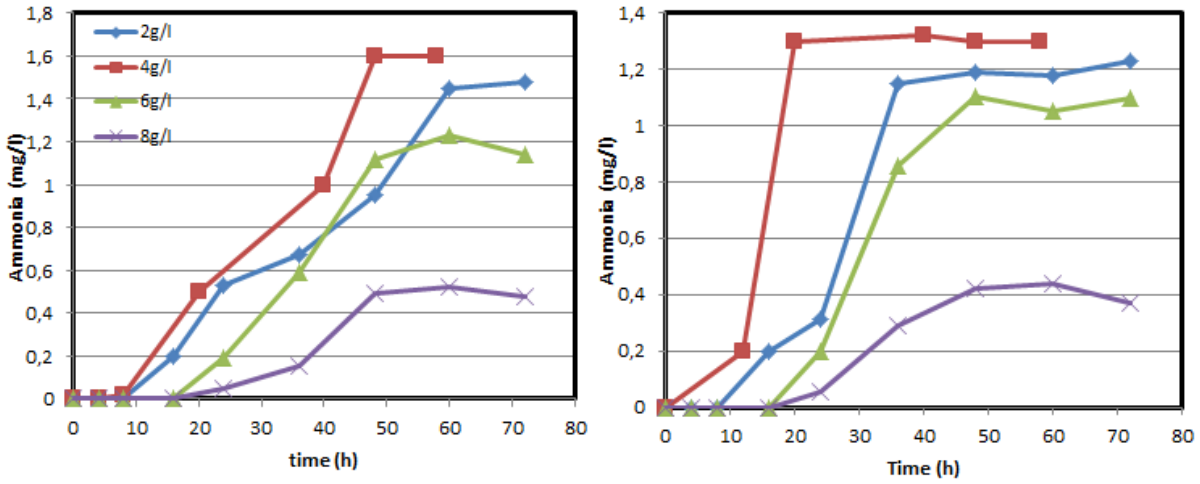
Fig 16. Effect of initial glucose concentration on glucose utilization by *P. resinovorans*

Effect of initial glucose concentration on ammonia generation

The ammonia generation was maximum at 4 g/l of initial glucose concentration using $K_4Fe(CN)_6$. Ammonia concentration was 0.02mg/l at the 8th hour. The ammonia concentration increased to 1.6 mg/l at the 48th hour, then stable until the end of the experiment . For initial glucose concetrations of 2g/l and 6g/l, ammonia generation followed a similar trend with the first ammonia detection at the 8th hour. After that, it reached a stable value after 60 and 48 hours. At 8g/l of glucose concentration, ammonia generation reduced significantly . It was

detected after 24 hours. The maximum ammonia generation was 0.49 mg/L after 48 hours (Fig 17a.)

Maximum ammonia was generated at 4g/l of initial glucose concentration using $Na_4Fe(CN)_6$ (Fig 17b). Ammonia was first detected at 12th hour and the measured value was 0.2 g/l. The ammonia concentration rapidly increased to 1.3 mg/l at the 16th hour, and then stable until the end of the experiment . For initial glucose concetrations of 2g/l and 6g/l, ammonia generation followed a similar trend with a first ammonia detection at the 8th and the 16th hours respectively , and a stabalization of the ammonia generation was observed after 36 and 48 hours. At 8g/l of initial glucose concentration, ammonia generation was significantly low. The ammonia generation was first detected at the 24th hour. Then, it increased slowly to 0.42 mg/l at 48 hours and became stable for the rest of the experimental period.



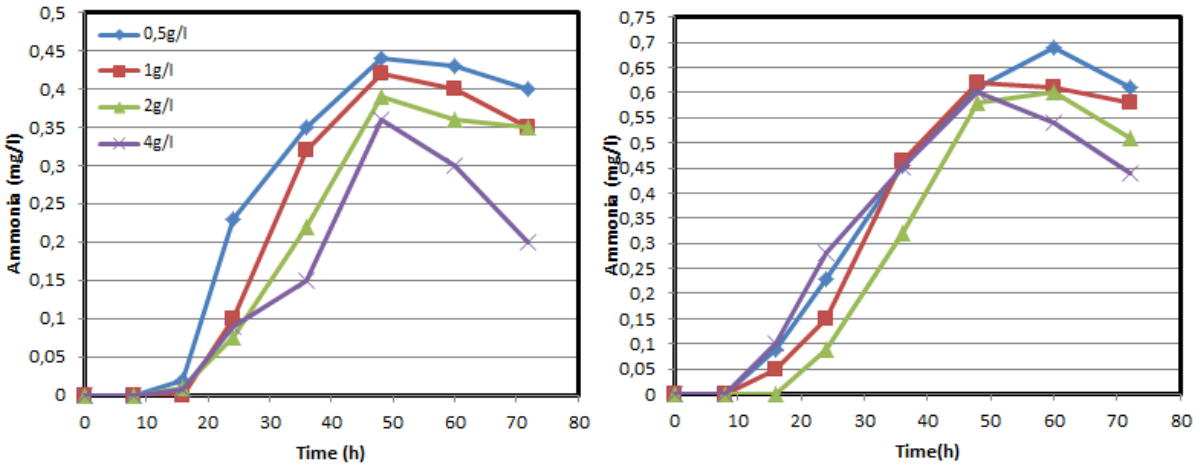
a- $K_4Fe(CN)_6$ **b- $Na_4Fe(CN)_6$**

Fig 17. Effect on initial glucose concentration on ammonia generation by *P. fluorescens* Pf5

For *P. resinovorans* Maximum ammonia in the medium was detetcted at 0.5g/l of initial glucose concentration using $K_4Fe(CN)_6$ (Fig 18a) and $Na_4Fe(CN)_6$ (Fig 18b). Ammonia was first detected at 16th hour and the measured value was 0.019 mg/l. The ammonia concentration rapidly increased to 0.44 mg/l at the 48th hour, and then started deacreasing up to 0.4mg/l at the end of the test. For initial glucose concetrations of 1, 2 and 4g/l, ammonia generation followed a similar trend with a first ammonia detection at the 24th hour for 1g/l and the 16th hour for 2g/l and 4g/l. Then an increase of ammonia concentration occured up to 48th hour and the and finally a decrease of this concentration.until the end of the

test. At 4g/l of glucose, ammonia concentration was the lowest. The maximum concentration (i.e. 0.36 mg/l) was detected after 48 hours and then decrease to 0.2mg/l after 72 hours.

About 0.09 mg/L of ammonia was detected at 16th hour using $Na_4Fe(CN)_6$ as a nitrogen source. The ammonia concentration rapidly increased to 0.69 mg/l at the 60th hour, and then started deacreasing up to 0.61mg/l at the end of the test. For initial glucose concetrations of 1, 2 and 4g/l, ammonia generation followed a similar trend with a first ammonia detection at the 24th and the 16th hours respectively , then an increase of ammonia concentration and finally a decrease of this concentration. At 4g/l of glucose, ammonia concentration reached a lowest value. The maximum ammonia concentration (i.e. 0.6 mg/l) was detected after 48 hours and then decrease to 0.44 mg/l after 72 hours.



a-$K_4Fe(CN)_6$ **b- $Na_4Fe(CN)_6$**

Fig18. Effect of initial glucose concentration on ammonia generation by *P. resinovorans*

Table 1 presents the ammonia generation and glucose utilization during biodegradations tests when using *P. fluorescens* Pf5. For both ferro cyanides, maximum ammonia concentration was detected at 4g/l of glucose (i.e. 1.3 mg/l for $Na_4Fe(CN)_6$ and 1.6mg/l for $K_4Fe(CN)_6$). At 2g/l of glucose, the ammonia concentrations were 1.23 mg/l and 1.48 mg/l for sodium and potassium ferro cyanide, respectively. Above 4g/l of glucose, ammonia generation decreased as the glucose concentration was raised up to 8g/l. The ammonia concentrations were 0.44 mg/l for sodium ferro cyanide and 0.52mg/l for potassium ferro cyanide. All the glucose in the medium was used when the initial glucose concentration was under 4g/l. Above 4g/l of glucose, the glucose utilization decreased. At 6g/l of initial

glucose, 90% of the glucose was used for $Na_4Fe(CN)_6$ and 85% for $K_4Fe(CN)_6$. At 8g/l of glucose, 83.42% and 77% of glucose utilization were observed for sodium and potassium ferro cyanide, respectively.

Table 2 presents the effect of initial glucose concentration on glucose utilization and ammonia generation, when using *P. resinovorans*. For both compounds, maximum ammonia detected (i.e. 0.69 and 0.44 $mg.L^{-1}$) and maximum glucose utilization occurred at 0.5g/l of initial glucose concentration. When the initial glucose concentration was raised, the ammonia concentration decreased, so did the glucose utilization. Minimum glucose utilization (i.e 64% for $Na_4Fe(CN)_6$ and 56% for $K_4Fe(CN)_6$) and ammonia concentration (i.e. 0.6 $mg.L^{-1}$for $Na_4Fe(CN)_6$ and 0.36mg/l for $K_4Fe(CN)_6$) was detected at 4g/l of initial glucose.

The decrease of ammonia generation with the increase of glucose concentration might be due to the increase of bacteria population in the medium when the glucose concentration is raised. The ammonia produced during the oxidative breakdown of cyanide compounds, acts as a nutrient (nitrogen source) for the bacteria (Ackil, 2003). The higher bacteria population present when glucose concentration was raised up, would have incorporated more ammonia as nutrient.

Dursun et al, (1999) also noticed a similar result according to glucose utilization: they found that 98% of glucose was used at the optimum glucose concentration of 0.456 $g.L^{-1}$. About 100% of the glucose was used when the glucose concentration was below the optimum value and the glucose utilization decreased above 0.456 $g.L^{-1}$.

Using *Pseudomonas fluorescens P70* to remove ferrocyanide, the maximum cyanide removal (i.e. 60%) occurred at 0.465 $g.L^{-1}$ of glucose (Dursun et al. 1999). The cyanide removal generally decreased when the glucose concentration was either decreased or increased from 4 $mg.L^{-1}$**.** Adjei and Ohta (1999), also noticed an increase in KCN removal up to 1.85mg $CN^- h^{-1}$ when the initial fructose was increased up to 0.25% w/v (2.5 $g.L^{-1}$). Above 0.25% of fructose, the degradation rate of KCN decreased as the initial fructose concentration was raised.

Table 1: Effect of Initial glucose concentration on ammonia generation and glucose utilization by *P. fluorescens* Pf5

	$Na_4Fe(CN)_6$		$K_4Fe(CN)_6$	
Glucose concentration (g/l)	Glucose utilization	Maximum ammonia produced	Glucose utilization	Maximum ammonia produced
2	100%	1.23	100%	1.48
4	**100%**	**1.3**	**100%**	**1.6**
6	90%	1,1	85%	1,23
8	83,42%	0,44	77%	0,52

Table2. Effect of initial glucose concentration on glucose utilization and ammonia generation by *P. resinovorans*

	$Na_4Fe(CN)_6$		$K_4Fe(CN)_6$	
Glucose concentration (g/l)	Glucose removal	Maximum ammonia produced	Glucose removal	Maximum ammonia produced
0.5	95%	0.69	91	0.44
1	90%	0.62	80%	0.42
2	70%	0.6	67%	0,39
4	**64%**	**0.6**	**56%**	**0,36**

CONCLUSIONS AND RECOMMENDATIONS

This research work has been focused in different aspects of bioremediation of cyanide in water. The first aspect was to identify the MIC values of *P. fluorescens Pf5* and *P. resinovorans* using potassium ferrocyanide and sodium ferrocyanide and the second aspect was the assessment of different factors (i.e. initial pH and initial glucose concentration) on biodegradation of cyanide.

P. fluorescens Pf5 and *P. resinovorans* were able to degrade potassium and sodium ferro cyanide as nitrogen source in M9 medium when glucose was added as a carbon source.. However, potassium cyano argenate ($KAg(CN)_2$) was an inhibiting compound on the growth of *P. fluorescens Pf5* and *P. resinovorans.*

The MIC of *P. fluorescens Pf5* was 50 mM (21.1g/l) for $K_4Fe(CN)_6$ and 75mM (36.3/l) for $Na_4Fe(CN)_6$ *P.* In the biodegradation of potassium and sodium ferrocyanide, maximum ammonia concentration was detected at initial pH 7. Initial glucose concentration had a positive effect on the bacterial growth. Bacterial growth was higher when glucose concentration was raised. However, ammonia concentration decreased when glucose concentration was increased.

The MIC values were 25mM (10.55g/l) and 75mM (36.3/l) for $K_4Fe(CN)_6$ and $Na_4Fe(CN)_6$, respectively for *P. resinovorans.* In the biodegradation of potassium and sodium ferrocyanide, maximum ammonia concentration was detected at initial pH 5 for *P. resinovorans.* The growth of bacteria was higher when glucose concentration was raised. However, ammonia concentration decreased when glucose concentration was increased.

For future works it would be interesting to assess the cyanide bioremediation by measuring directly the cyanide concentration; for complex cyanide compounds, distillation will be necessary to liberate free cyanide ion before cyanide concentration assessment. Effect of initial cyanide concentration, initial glucose concentration, initial pH or even initial bacterial population on cyanide removal could be assessed. In order to conduct research work close to the fields conditions, a preliminary identification of microorganisms growing in cyanide contaminated waste water produced by industries is necessary. The identification of these microorganisms could contribute to improve the knowledge on bacteria able to degrade cyanide compounds. Biodegradation assessment will also need to be done directly on

industries waste water to figure out how bacteria are able to degrade a blend of various cyanide compounds, or associated with several other contaminants in the industries waste water. Finally an implementation of the technics at a pilot scale would be important to figure out additional parameters which could influence the bioremediation of the cyanide compounds.

References:

1. Akcil , A.G. Karahan, H. Ciftci, O. Sagdic, « Biological treatment of cyanide by natural isolated bacteria (Pseudomonas sp.) » Minerals Engineering 16 (2003) 643–649
2. A.Y. Dursun, A. C¸ alık, Z. Aksu, « Degradation of ferrous(II) cyanide complex ions by *Pseudomonas fluorescens* » Process Biochemistry 34 (1999) 901–908
3. Ahmet Cabuk *Æ* Arzu Taspinar Unal Nazif Kolankaya, « Biodegradation of cyanide by a white rot fungus, *Trametes versicolor,* » Biotechnology Letter 28 (2006)1313–1317
4. Akcil, A., Mudder, T. « Microbial destruction of cyanide wastesin gold mining: Process review » Biotechnology Letters 25 (2003) 445– 450.
5. Ata Akcil, « Destruction of cyanide in gold mill effluents: biological versus chemical treatments» Biotechnology Advances 21 (2003): 501– 511
6. Basheer S., Kut O.M., Prenosil J.E. and Bourne J.R.. « Kinetics of enzymatic degradation of cyanide » . Biotechnol. Bioeng. 39(1992) 629 –634
7. Baxter, Joanne, et Stephen P. Cummings. « The current and future applications of microorganism in the bioremediation of cyanide contamination ». Antonie van Leeuwenhoek 90, (mai 9, 2006): 1-17.
8. Bodek H, Ehnfholt DJ, Glazer AE, Loretti P, Lyman: « Properties, Processes and Estimation Methods ».W. Inorganic Chemistry USA, (1998) Chap. 10.13
9. Butkus M. A., Labare M. P., Starke J. A., Moon K., Talbot M. « Use of aqueous silver to enhance inactivation of coliphage MS-2 by UV disinfection ». Appl Environ Microbiol. 70 (2004) 2848-53.
10. C.Y. Chen a, C.M. Kao a, S.C. Chen, «Application of Klebsiella oxytoca immobilized cells on the treatment of cyanide wastewater » Chemosphere 71 (2008) 133–139
11. Carillo-Pedroza, F.R., Soria-Aguilar, « Destruction of cyanide by ozone in two gasliquid contacting systems » Eur. J. Miner. Proc. Environ. Prot. 1 (2001) 55–63.
12. Cluness M.J., Turner P.D., Clements E., Brown D.T. and O Reilly C. « Purification and properties of cyanide hydratase from Fusarium lateritium and analysis of the corresponding Chy1 gene » J. Gen. Microbiol. 139 (1993): 1807 –1815
13. Commeyras A., Taillades J., Collet H., Bioteau L., Vandenabeele- Trambouze O., Pascal R., Rousset A., Garrel L., Rossi J.-C., Biron J.-P., Lagrille O., Plasson R., Souaid E., Danger G., Selsis F., Dobrijevic M. and Martin H. « Dynamic co-evolution of peptides and chemical energetics, a gateway to the emergence of homochirality and the catalytic activity of peptides » Origin Life Evolution Biosphere 34 (2004) 35 –55.
14. Cui Zhaoa, Yin Zhangb, Xiaobao Li a, Donghui Wena,∗, Xiaoyan Tanga , « Biodegradation of carbazole by the seven Pseudomonas sp. strains and their denitrification potential », Journal of Hazardous Materials 190 (2011) 253–259
15. Dorr P.K. and Knowles C.J. « Cyanide oxygenase and cyanase activities of Pseudomonas fluorescens NCIMB 11764 » FEMS Microbiol. Lett. 60 (1989) 289 –294.

16. Dumestre A., Chone T., Portal J.M., Gerard M. and Berthelin J. « Cyanide degradation under alkaline conditions by a strain of Fusarium solani isolated from contaminated soils ». Appl. Environ. Microbiol. 63 (1997) 2729 –2734.
17. Eric Fournier, « Colorimetric Quantification of Carbohydrates » *Current Protocols in Food Analytical Chemistry* (2001) E1.1.1 - E1.1.8
18. Ezzi M. and Lynch J.M. « Cyanide catabolizing enzymes in Trichoderma spp.» Enz. Microb. Technol. 31 (2002): 1042 –1047.
19. Figueira M.M., Ciminelli V.S.T. and Linardi V.R. 1995. « Bacterial degradation of metal cyanide complexes » In: Jerez C.A., Vargas T., Toledo H. and Wiertz J.V. (eds), « Biohydrometallurgical Processing » University of Chile, Chile, 333 –339.
20. Figueira M.M., Ciminelli V.S.T., de Andrade M.C. and LinardiV.R. « Cyanide degradation by an Eschericia coli strain » Canadian Journal of Microbiology 42 (1996) 519 –523.
21. Finnegan I., Toerien S., Abbot L., Smit F. and RaubenheimerH.G. « Identification and characterisation of an *Acinetobacter sp.* capable of assimilation of a range of cyano-metal complexes, free cyanide ions and simple organic nitriles ». Appl. Microbiol. Biotechnol. 36 (1991) 142 –144.
22. Given B, Dixon B, Douglas G, Mihoc R, Mudder T. « Combined aerobic and anaerobic biological treatment of tailings solution at the Nickel Plate Mine. The cyanide monograph » London: Mining Journal Books; 1998.
23. Ingles, J., Scott, J.S., 1987. State-of-the-Art of Processes for the Treatment of Gold Mill Effluents. Environment. International Cyanide Management Institute (ICMI), 2002. Draft International Cyanide, Management Code for the Manufacture, Transport and Use of Cyanide in the Production of Gold. Available from www.cyanidecode.org.
24. Ingvorsen K., Hojerpedersen B. and Godtfredsen S.E.. « Novel cyanide-hydrolysing enzyme from Alcaligenes xylosoxidanssubsp. denitrificans» Applied Environmental Microbiology. 57 (1991): 1783 –1789
25. Kao C.M., Liu J.K., Lou H.R., Lin C.S. and Chen S.C. « Biotransformation of cyanide to methane and ammonia by Klebsiella oxytoca » Chemosphere 50 (2003): 1055 –1061.
26. KD Chapatwala, GRV Babu, OK Vijaya, KP Kumar and JH Wolfram « Biodegradation of cyanides, cyanates and thiocyanates to ammonia and carbon dioxide by immobilized cells of Pseudomonas putida » Journal of Industrial Microbiology & Biotechnology 20 (1998), 28–33
27. Kwon H.K., Woo S.H. and Park J.M. « Degradation of tetracyanonickelate(II) by Cryptococcus humicolus MCN2 ». FEMS Microbiology Letter 214 (2002) 211 –216.
28. Mark J. Logsdon, MSc Karen Hagelstein, PhD, CIH Terry I. Mudder, PhD, « The management of cyanide in the gold extraction » International Council On Metals And The Environment (April 1999)

29. Maurice Levasseur; Peter A. Thompson ; Paul J. Harrison «Physiological acclimation of marine phytoplankton to different nitrogen sources» Journal of Phycology 29 (1993) 587–595
30. Meyers P.R., Gokool P., Rawlings D.E. and Woods D.R.. « An efficient cyanide-degrading Bacillus pumilus strain » J. Gen. Microbiol. 137 (1991) 1397 –1400.
31. Michael D. Adjei and Yoshiyuki Ohta « Factors Affecting the Biodegradation of Cyanide by Burkholderia cepacia Strain C-3 » Journal Of Bioscience and Bioengineering 89 (2000) 274-277.
32. Michelle Barclay, Alwyn Hart, Christopher J. Knowles, Johannes C. L. Meeussen: and Vanessa A. Tetdl, « Biodegradation of metal cyanides by mixed and pure cultures of fungi » Enzyme and Microbial Technology 22 (1998) 223-231
33. M.M. Botz, T.I. Mudder, and A.U. Akcil, « Cyanide treatment: Physical, chemical and biological processes ». Developments in Minéral Processing, 15 (2005) 672 – 701.
34. M.M.Botz, Mudder, T.I., « Mine water treatment with activated carbon » Proceedings Randol Gold Forum, (1997). Randol, Golden, Colorado, 207–210.
35. Mudder T, Botz M, Smith A. The cyanide compendium. London: Mining Journal Books; 2001a. On CD-ROM.
36. Mudder T, Fox F, Whitlock J, Fero T, Smith G, Waterland R, et al. « The Homestake wastewater treatment process » : Part 2. Operation and performance. The cyanide monograph London: Mining Journal Books; (1998) 365-88
37. Mufaddal I. Ezzi, James M. Lynch « Biodegradation of cyanide by *Trichoderma* spp. and *Fusarium* spp» Enzyme and Microbial Technology 36 (2005) 849–854
38. Nava, F., Uribe, A., Perez, R. « Use of ozone in the treatment of cyanide containing effluents » Eur. J. Miner. Proc. Environ. Prot. 3 (2003) 316–323.
39. Neha Gupta, Chandrajit Balomajumder, V.K. Agarwal, « Enzymatic mechanism and biochemistry for cyanide degradation: A review » Journal of Hazardous Materials 176 (2010) 1–13
40. Oro´ J, Lazcano-Araujo A: « The role of HCN and its derivatives in prebiotic evolution ». In B, Conn EE, Knowles CJ, Westley J, Wissing F: London: Academic Press; (1981) 517-541.
41. Neha Gupta, Chandrajit Balomajumder, V.K. Agarwal « Enzymatic mechanism and biochemistry for cyanide degradation: A review » Journal of Hazardous Materials 176 (2010) 1–13
42. Raybuck S.A. « Microbes and microbial enzymes for cyanide degradation » Biodegradation 3 (1992) 3 –18.
43. S.K. « Dubey and D.S. Holmes, Biological cyanide destruction mediated by micororganisms» World Journal of Microbiology and Biotechnology 11 (1995), 257-265
44. Silvos-Avilos J., Richmond M.G., Nagappan O. and Kuntz D.A. « Degradation of metal-cyanide complex tetracyanonickelate(II) by cyanide utilising bacterial isolates ». Appl. Environ. Microbiol. 56 (1990) 3664 –3670.
45. Stephen Ebbs « Biological degradation of cyanide compound » Current Opinion in Biotechnology 15 (2004) 231–236

46. U.S. Department Of Health And Human Services, Public Health Service, Agency for Toxic Substances and Disease Registry, « Toxicological Profile for Cyanide » (July 2006).
47. Watanabe A., Yano K., Ikebukuro K. and Karube I. « Cyanide hydrolysis in a cyanide-degrading bacterium, Pseudomonas stutzeri AK61, by cyanidase ». Microbiology 144 (1998) 1677 –1682.
48. Yolanda Brummer and Steve W. Cui, « Understanding Carbohydrate Analysis » Taylor & Francis Group, LLC, 2 (2005)

APPENDICES:

EXPERIMENTAL PROTOCOLS:

I. **Bacterial growth**:

- **Material:**
  - ✓ Eppendorf culture tube (or glass flask of 250ml)
  - ✓ Micropipette Eppendorf 2 – 20 µl
- **Equipment:**
  - ✓ NanoDrop Spectrophotometer ND 1000
  - ✓ Computer
- **Operative**:
  - ✓ Using a micropipette, take 5 – 10µl of the distilled water, put it on the platform of the spectrophotometer and make the calibration.
  - ✓ Using a micropipette, take 5 – 10µl of the medium without the bacteria, and make the blank at 600nm.
  - ✓ Using a micropipette, take 5 – 10µl of the medium containing the bacteria, the absorbance at 600nm is directly showed on the computer

II. Glucose assessment:

- **Materials:**
  - ✓ Eppendorf Micropipette 5-50µl
  - ✓ Eppendorf Micropipette 1ml
  - ✓ 10ml test tubes

- ✓ 500ml volumetric flask
- ✓ Eppendorf Microcentrifuge tubes

- **Equipment**:
  - ✓ Spectrophotometr HACH 2800
  - ✓ Eppendorf centrifuge 5415R

- **Reagents**
  - ✓ Phenol 4% (4g of phenol dissolved in 96g of deionized water)
  - ✓ Glucose 1g/l (500mg of glucose dissolved in 500ml of DI water)
  - ✓ Sulphuric acid 96%

- **Operative:**

❖ Standard curve:

- ✓ Prepare ten standards by adding 5, 10, 15, …50µl of glucose (1g/l) in the tests tube.
- ✓ Add 0.5 ml of phenol 4% to each tube
- ✓ Add 2.5ml of sulfuric acid 96%
- ✓ Make a blank by adding only phenol and sulfuric acid to one test tube
- ✓ Shake vigorously and let the tubes undisturbed for 30min
- ✓ Take the absorbance at 590nm with the spectrophotometer
- ✓ Make a graph representing the absorbance as a function of the glucose concentration
- ✓ Generate the linear trend of this graph

❖ Sample measurement:

- ✓ Take 0.5ml of the sample in a Eppendorf micro centrifuge tube
- ✓ Centrifuge at 7000 g for 1min
- ✓ Take 10µl of the supernatant and put it in a 10ml test tube
- ✓ Add 0.5ml of phenol 4% to the test tube

- ✓ Add 2.5ml of sulfuric acid 96%
- ✓ Take the absorbance at 490nm with the spectrophotometer
- ✓ The glucose concentration is given by plotting the absorbance on the linear trend got in the previous test.

III. Ammonia assessment:

- **Materials**:
  - ✓ Low Range Test 'N Tube AmVer ™ Nitrogen Ammonia Reagent
  - ✓ Funnel, micro (for adding reagent)
  - ✓ Eppendorf micropipette 1ml
  - ✓ Pipette Tips
  - ✓ Eppendorf 4µl filter
- **Equipment:**
  - ✓ Spectrophotometer HACH 2800
  - ✓ Reagents:
  - ✓ Ammonia Salicylate Reagent Powder Pillow
  - ✓ Ammonia Cyanurate Reagent Powder Pillow
- **Operative**:
  - ✓ Select the stored program 342, Ammonia LR TNT on the spectrophotometr
  - ✓ Take 3ml of the sample and filter using a Eppendorf 4µl pore sized filter.
  - ✓ Add 2.0 ml of filtered sample to one AmVer ™ Diluent Reagent Test 'N Tube
  - ✓ Blank preparation: Add 2.0ml of DI water to one AmVer ™ Diluent Reagent Test 'N Tube

- ✓ Add the content of one Ammonia Salicylate Reagent Powder Pillow to each vial
- ✓ Add the contents of one Ammonia Cyanurate Reagent Powder Pillow to each vial
- ✓ Cap the vials tightly and shake thoroughly to dissolve the powder
- ✓ Start the spectrophotometer timer; a 20 minutes reaction time will begin
- ✓ After 20 minutes wipe the tubes, and zero the instrument: the displays will show 0.00 mg/l NH3-N.
- ✓ Read the results for the other(s) tube(s); the results are given in mg/l NH3-N