Biodegradation of cyanide-containing wastes by *Pseudomonas pseudoalcaligenes* CECT5344

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Cyanide is a toxic nitrogen compound for almost every living organism since it binds irreversibly to haem-proteins, (i.e. cytochromes involved in all known respiratory processes [1]). In the nature, there are bacteria, algae, fungi, and many plants, which are able to produce cyanide. However, the contamination of the environment with cyanide is mainly due to human activities. In this sense, mining, electroplating and jewellery industries generate effluents with a high concentration of cyanide. There are many possible methods for treating wastes containing cyanide. The latest cyanide disposal method is cyanide biodegradation, which is an environmentally friendly and quite inexpensive treatment method [2].

Some microorganisms have been described to be able to degrade cyanide at a neutral pH, but under this condition a high concentration of cyanide evaporates as hydrocyanic acid (HCN), a weak acid with a pK_a value of 9.2. Thus, it is very important to isolate cyanotrophic microorganisms that work at alkaline pH. In this sense, our research group has isolated a native bacterium, *Pseudomonas pseudoalcaligenes* CECT 5344 from the Guadalquivir River in Córdoba, which is able to degrade free cyanide and cyano-metalic complexes under alkaline conditions (up to an initial pH of 10) [3,4]. The aim of this work was to check if *P. pseudoalcaligenes* CECT 5344 is able to tolerate and degrade the cyanide present in the lixiviate from a gold mining industry located in Asturias, Spain.

Keywords: Cyanide; *Pseudomonas*; Biodegradation.

1. Introduction

Mining, metallurgic and jewellery industries produce residues containing high amounts of cyanide and its very stable metal complexes. Cyanides are highly toxic and their toxicity is related to their physicochemical speciation. The free cyanide form (HCN, CN^-) is classified as the most toxic because of its high metabolic inhibition potential whereas metal–cyanide complexes (e.g. $Fe(CN)_6^{3-}$, $Fe(CN)_6^{4-}$) are considered relatively less toxic [5]. The acute toxicity of complex cyanide species is related to the relative ease with which free cyanide can be dissociated from the complex, with comparatively weaker complexes being more toxic than stronger complexes [6].

In the mining of precious metals, cyanide is widely used to leach gold and silver from the ore. The cyanidation process converts gold and silver to water soluble cyanide complexes [7]. Each year over a billion tons of gold ore are treated using this process, which means a significant demand for cyanide by these industries. [8].

The bacterium *Pseudomonas pseudoalcaligenes* CECT 5344, which was isolated in Córdoba, Spain [4], tolerates and assimilates high cyanide concentrations under alkaline conditions (up to pH 10), thus minimizing its volatilization as hydrocyanic acid. This bacterium was isolated by enrichment cultivation in media containing the cyanide from the electroplating industry as the sole nitrogen source. In this work we have checked the capability of CECT5344 strain to use the cyanide-containing lixiviate from the Rio Narcea Gold mine as nitrogen source in the hope that the process could be used to detoxify the effluent

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as an alternative to the existing chemical treatment used by the Company in order to eliminate the cyanide.

2. Methods for detoxification of cyanide from wastes.

Cyanide-containing wastes are usually eliminated by chemical or physical treatments. In the following section we will introduce briefly the most popular methods ending with the biodegradation as an alternative methodology. In each case we will focus in the advantages and inconveniences of the corresponding treatment.

2.1 Alkaline chlorination

In alkaline chlorination hypochlorite ions oxidize cyanide to form CO_2 and N_2 gas. The treatment destroys free cyanide and cyanide complexes, except the more stable such as ferricyanide. This technology is well known and is widely used. One of the major disadvantages of this method is the potential formation of chlorinated organics, which are extremely toxic. In addition, some of these products (e.g. cyanogen $(CN)_2$) are even more harmful and toxic than cyanide itself [2]. Reagents used in the process are very toxic and therefore the conditions must be controlled very carefully [2].

2.2 SO_2/AIR Oxidation (INCO)

This process oxidizes with O_2 free cyanide and weak acid dissociable cyanide complexes (e.g., Ag, Ni, Cu, and Zn complexes) as well as the added SO_2 to cyanate and sulfuric acid, respectively. The reaction is carried out under alkaline conditions by the addition of $Ca(OH)_2$, thus allowing the neutralization of sulfuric acid as well as the precipitation of metals as hydroxides. Iron cyanide complexes (hexacyanoferrates) cannot be oxidized, but they are removed by precipitation [2]. This method is quite effective but its inconvenience is that it is relatively expensive.

2.3 Electrolytic oxidation

Electrochemical oxidation is an alternative process for destroying cyanide ions at the anode and collecting heavy metals from the cathode. Free cyanide, cyanide complexes and concentrated cyanide solution can be handled with the electrochemical oxidation method [9]. In the anode, cyanide and its metal complexes become oxidized to cyanate, which is further oxidized to CO_2 and N_2. In the anode, the metallic cations are reduced and deposited as metals. This method is also considered environmentally friendly.

2.4 Biodegradation

Although chemical and physical processes can be employed to degrade cyanide and its related compounds, they are often expensive and complex to operate. Biotreatment can be less expensive than chemical and physical methods, but much faster than natural degradation. Since cyanide is a natural biodegradable compound, biological treatments are more indicated to eliminate it from industrial effluents [10]. The success of biodegradation depends upon the presence of microbes with the physiological and metabolic capabilities to degrade the pollutants in the contaminated environment [2]. The biological treatment relies upon on the acclimation and enhancement of indigenous microorganisms such as bacteria, but most of the time the environmental conditions, mainly the chemical composition, must be previously modified. The addition of nutrients to a contaminated site in order to enhance the activity of indigenous microorganisms is called biostimulation. By contrast, the inoculation with specialized microflora is called bioaugmentation. In any case, the process can be done *in situ* or *ex situ*,

depending on many factors such as the amount of contaminated area or the toxicity of the pollutant. The biological treatment of cyanide is a relatively new phenomenon in the gold mining industry and has been used only in a few instances [11].

3. Selection and characterization of cyanide degrading bacteria

In the cyanide molecule, the oxidation state of C (+2, like that in CO) and N (-3, like that in NH_4^+) make this compound a bad C source but a good N source for bacterial growth. Taking also into account the relatively high concentration of cyanide in the industrial effluents, this means from a practical point of view that the diluted residues can be used as N-sources by specialized microorganisms in media supplied with a suitable C-source and other mineral nutrients (N, P, K, fundamentally). Nevertheless, a microorganism can metabolize cyanide only when, in addition to a biodegradative pathway to convert cyanide into an assimilable product (NH_4^+), it also harbors a cyanide-resistance mechanism (generally a cyanide-insensitive oxidase) [12]. Finally, a microorganism thriving in cyanide containing media will need a system for taking up Fe from the medium (siderophores), since Fe forms very stable complexes with cyanide and it is not available for the organism. From a chemical point of view, the biological treatment of industrial effluents contaminated with cyanide requires an alkaline pH in order to avoid the volatile HCN (pK_a=9,2) formation [4]. Thus, the first step in the biological treatment process is the selection of bacteria able to tolerate and degrade cyanide in the millimolar range at alkaline pH.

3.1 Bacterial isolation

When natural environments are contaminated with pollutants, the indigenous microbial communities are likely to contain microbial populations of different taxonomic characteristics, which are capable of degrading the contaminating chemicals [13]. For this reason, a way to isolate cyanide degrading bacteria is to use samples of areas which might have been in contact with polluted waste, as a source of microorganisms for enrichment cultivation. *Pseudomonas pseudoalcaligenes* CECT5344 was isolated by this procedure. Basically, the medium used was the M9 minimal medium [14], at pH 9.5 with 2 mM of free cyanide (using the diluted residue from the electroplating industry) and 50 mM acetate as the sole added nitrogen and carbon sources, respectively. The medium was inoculated with sludge from the left margin of the Guadalquivir River (Córdoba, Spain) and incubated in an Erlenmeyer at 30 ^oC in a rotatory shaker. Cyanide was completely depleted after two weeks and the process was repeated four times by reinoculating in fresh medium with 1% (vol/vol) of the previously grown culture. Samples of the enriched culture were plated on LB medium solidified with 1.8 % bacto agar and individual colonies were purified and tested for axenic growth in liquid cultures with cyanide as the sole nitrogen source. Only one type of colonies was able to assimilate cyanide. Therefore, a pure culture was grew form a single colony and kept for further analysis [3-4]. This bacterium is an excellent tool for degrading the high amounts of cyanide present in the residue of the jewellery industry. The bacterium tolerates cyanide up to a concentration of 30 mM at alkaline pH (up to an initial pH = 10), thus minimizing cyanide volatilization as HCN, and also produces siderophores that allow the bacterium to thrive in media containing stable metal-cyanide complexes [4]. In addition, this alkalophilic strain uses alternative nitrogen sources such as nitrate, nitrite, cyanate, cyanoacetamide, nitroferricyanide (nitroprusside) and several cyano-metal complexes. All these characteristics make this strain a model organism to be used in bioremediation processes and biotreatment of industrial residues with cyanide and its derivatives [3-4].

4. *Pseudomonas pseudoalcaligenes* CECT5344 as a possible tool for degrading the cyanide present in the gold mining lixiviate.

P. pseudoalcaligenes CECT534 was able to grow and tolerate the cyanide present in the diluted residue form the jewellery industry (0.76 M free cyanide in the original residue) [3, 4] which makes it a good candidate for the biological treatment of the gold mining lixiviate. The mining industry of Asturias (Rio

Narcea Gold Mines, RNGM, Spain) generates a cyanurated lixiviate containing up to 20 mM (520 mg/l) of free cyanide. In addition to free cyanide, this lixiviate contains cyano-metallic complexes that are also a suitable N-source for the bacterium. Nowadays RNGM uses a chemical method (INCO) for the treatment of the lixiviate generating an effluent containing less than 5 ppm cyanide that is further recycled in the extraction of the ore. In the present communication we report the laboratory-scale experiments checking the possibility of a biological treatment as an alternative to the INCO process.

As shown in Figure 1, *P. pseudoalcaligenes* CECT5344 was able to grow at the expenses of the diluted lixiviate as the sole nitrogen source. The maximum growth reached was inversely proportional to the dilution, being the maximal cell yield obtained with the lixiviate diluted 10 times (Figure 1). Lower dilutions (less than 10 times) did not result in higher cell-yields (not shown). This is probably due to the limitation of the carbon source present in the M9 medium (50 mM acetate). In agreement with this hypothesis is the fact that ammonium was detected in the supernatant of media after the biological treatment, when the lixiviate was used as N-source at a dilution lower than 10 times. Consequently, a dilution of 10-times was fixed as the minimal possible (2 mM cyanide in the culture medium) in order to avoid the presence of untreated N-sources, mainly cyanide, at the end of the bio-treatment. *P. pseudoalcaligenes* used both the ammonium and some of the cyanide when the lixiviate (10 times diluted) was used as the sole N-source (Figure 1). Under these conditions the cyanide concentration decreases until it reaches a constant level that was almost identical to that in uninoculated media, thus indicating that some cyanhydric acid evaporates at this pH. The cyanide present in the lixiviate was not completely degraded, even when a 20 or 30-times diluted lixiviate was used as the N-source (Figure 1B and 1C, respectively).

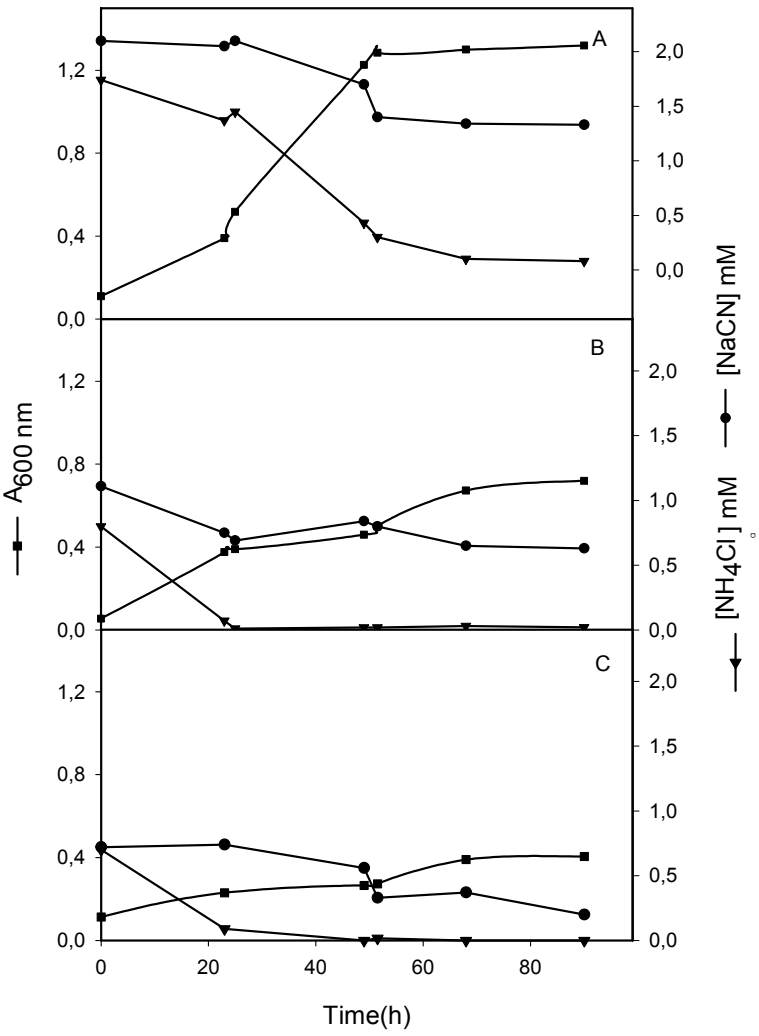

Figure 1. Utilization of the lixiviate from gold mining industry as N-source, by *Pseudomonas pseudoalcaligenes* CECT5344. The bacterial strain was cultured as indicated in Materials and Methods with the lixiviate (containing 20 mM free cyanide and 16 mM ammonium) as a nitrogen source. The cell growth was measured at the indicated times. Lixiviate was diluted: (A) 10 times, (B) 20 times and (C) 30 times.

Similar data were obtained when the lixiviate was diluted 40, 50 and 60 times (results not shown). These results clearly indicate that cyanide was not assimilated in a media that contained enough carbon and nutrients for growing. Moreover, and since the strain CECT5344 is able to grow with the residue generated by the jewellery industry up to 30 mM free cyanide concentration [4], the poisoning with cyanide (2 mM maximum) does not seems to be the problem of the unassimilated cyanide at the end of the growth curve (Figure 1). On the other hand, the presence of contaminating elements in the lixiviate could inhibit cyanide degradation. To avoid this problem and to optimize the cyanide biodegradation, the lixiviate was filtrated (0.22 μm-pore filter) in order to eliminate any possible impurities present in the medium. By using the filtrated and diluted lixiviate as N-source, the strain CECT5344 again assimilated efficiently the ammonium, but not the cyanide present in the media (Figure 2).

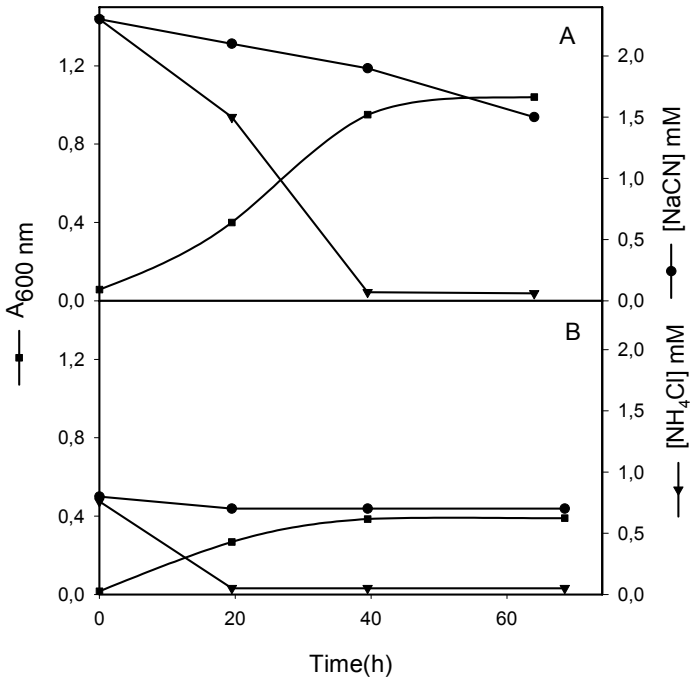

Figure 2. Growth of *Pseudomonas pseudoalcaligenes* CECT5344 in the filtrated lixiviate as N-source. The bacterial strain was cultured as indicated in Materials and Methods with the filtrated lixiviate as N-source. The cell growth was measured at the indicated times. Lixiviate was diluted: (A) 10 times, (B) 20 times.

Independently of the lixiviate filtration, the growth curves were very similar (Figures 1 and 2). These results suggest that some not-filterable compound(s) present in the lixiviate are responsible for the inhibition of cyanide assimilation in *Pseudomonas pseudoalcaligenes* CECT534.

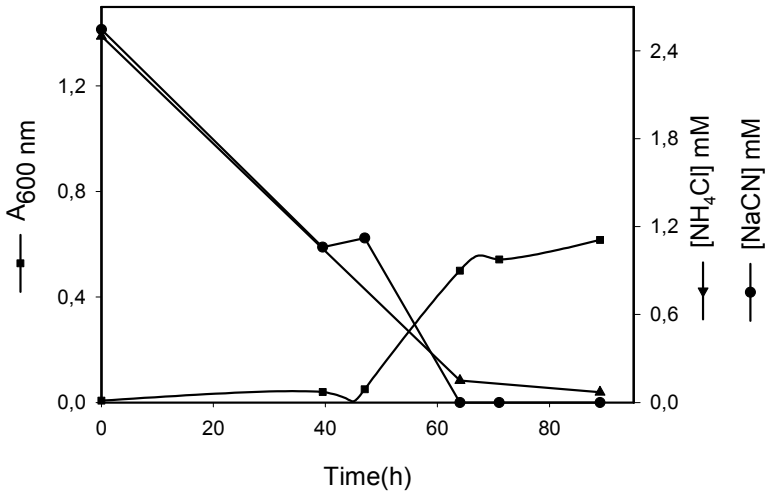

Figure 3. Growth of *P. pseudoalcaligenes* CECT5344 in a synthetic medium with cyanide and ammonium. Cells were cultured as indicated in Materials and Methods with 50 mM sodium acetate as carbon source, and 2.5 mM cyanide plus 2.5 mM ammonium chloride as N-sources (both added from sterilized stocks at the indicated concentrations). In non-inoculated samples (controls) cyanide and ammonium evaporation were less than 20%.

The next sets of experiments were designed to check this possibility. First, the bacterium was cultured in a synthetic medium containing similar concentration of ammonium and cyanide than those found in the diluted lixiviate. The results obtained unequivocally demonstrated that the bacterium is able to grow in this media and simultaneously assimilate both ammonium and cyanide (Figure 3).
By contrast, when the synthetic media was composed by a mixture of the diluted lixiviate plus added ammonium chloride and potassium cyanide, the bacterium was unable to assimilate all the cyanide present in the media (Figure 4). These results strongly suggest that the lixiviate from the mine contains

some non-filterable compound(s) that inhibit cyanide assimilation by *Pseudomonas pseudoalcaligenes* CECT5344.

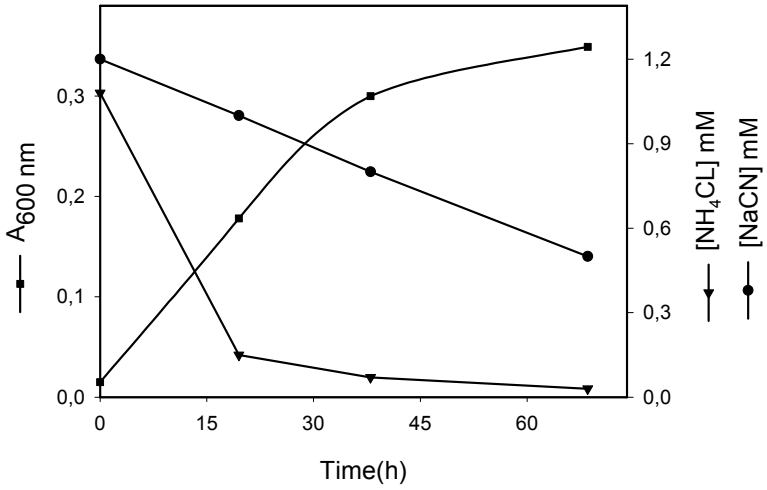

Figure 4. Simultaneous utilization of the cyanide present lixiviate from the gold mine by *P. pseudoalcaligenes* CECT534, in the presence of commercial cyanide and ammonium. Cells were grown in a medium containing 50% of a synthetic medium (containing the same cyanide and ammonium concentration that the 20-times diluted lixiviate) and 50% of lixiviate (20 times diluted).

Perhaps the learning of this work is that when looking for microorganism able to thrive and biodegrade a complex mixture is preferable to use the target mixture in the enrichment cultivation instead of using similar compounds. The same conclusion can be extracted from many publications concerning biodegradation of PCBs, where the bacteria were isolated with the natural analog biphenyl. As a result, most of these strains are able to metabolize biphenyl but only able to transform some PCB congeners.
Experiments are in progress looking for mutants of the parent strain able to use the cyanide present in the lixiviate from the gold mine industry both for their direct use in bioremediation experiments as well as for the elucidation of the molecular mechanism involved in the process. At the same time, we are looking for new organisms able to grow at the expenses of the lixiviate in the surrounding of the mine.

5. Materials and Methods.

5.1 Culture media.

The bacterium was grown either in M9 minimal medium adjusted to pH 9.5 or in LB medium on a rotatory shaker at 230 rpm and 30 ^{o}C. 50 mM acetate was used as the carbon source. When the residue generated in the jewelry electroplating baths or the mining lixiviate were used as the nitrogen sources, only the free cyanide was taken into account since the method used routinely for cyanide determination only detect free cyanide [16]. When the synthetic medium was used, the appropriate nitrogen sources were added from sterilized stocks at the indicated concentrations.

5.2 Analytical determinations

Bacterial growth was monitored by following the absorbance at 600 nm. Ammonium concentrations were determined as previously described [15]. Free cyanide oncentration was determined colorimetrically [4].

5.3 Chemicals

The lixiviate from the mining industry was kindly supplied by Rio Narcea Gold Mines Ltd. (Oviedo, Spain). The analysis of the sample used in this study revealed the presence of up to 20 mM free cyanide

(520 ppm), and ammonium 16 mM (272 ppm). The lixiviate also contained sulphur, 1170 ppm; copper 23 ppm, and molybdenum 2,5 ppm, as main components. When used as the nitrogen source, the lixiviate was diluted in the culture medium to give the desired concentration of free cyanide. All the rest of reagents were of the maximal purity commercially available.
The residue from the electroplating industry was kindly supplied by GEMASUR (Córdoba, Spain). The total cyanide concentration varies from 15 M to 37 M, depending on the sample whereas the free cyanide varies from 25 mM to 1 M. The difference between free and total cyanide is due to the presence of metals that complex most of the cyanide [4].

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