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Alkaline cyanide degradation by *Pseudomonas pseudoalcaligenes* CECT5344 in a batch reactor. Influence of pH

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ABSTRACT

Water containing cyanide was biologically detoxified with the bacterial strain *Pseudomonas pseudoalcaligenes* CECT5344 in a batch reactor. Volatilization of toxic hydrogen cyanide (HCN) was avoided by using an alkaline medium for the treatment. The operational procedure was optimized to assess cyanide biodegradation at variable pH values and dissolved oxygen concentrations. Using an initial pH of 10 without subsequent adjustment allowed total cyanide to be consumed at a mean rate of approximately 2.81 mg CN^- L^{-1} O.D.$^{-1}$ h^{-1}; however, these conditions posed a high risk of HCN formation. Cyanide consumption was found to be pH-dependent. Thus, no bacterial growth was observed with a controlled pH of 10; on the other hand, pH 9.5 allowed up to 2.31 mg CN^- L^{-1} O.D.$^{-1}$ h^{-1} to be converted. The combination of a high pH and a low dissolved oxygen saturation (10%) minimized the release of HCN. This study contributes new basic knowledge about this biological treatment, which constitutes an effective alternative to available physico-chemical methods for the purification of wastewater containing cyanide or cyano–metal complexes.

1. Introduction

Cyanide is an important industrial chemical which is produced on a large scale for use by mining (gold extraction), electroplating and many other industries. Such industries discharge large amounts of cyanide-containing liquid waste which often contains significant amounts of heavy metals and is a highly toxic, hazardous effluent [1]. By way of example, the jewellery industry in Córdoba (Spain) produces about 10 tons per year of alkaline waste (pH >13) containing free cyanide at concentrations up to 20 g L^{-1} (*ca.* 0.77 M) in addition to large amounts of cyanide complexes of some metals (Fe, Co, Cu, Ni, Zn Ag, V and Au) [2].

Cyanide compounds are present in the environment in various forms including HCN, simple inorganic salts (NaCN, KCN), metal–cyanide complexes, thiocyanates and organic cyanides or nitriles [3]. The toxicity of cyanide depends on its particular form. Thus, whereas free cyanide is an extremely dangerous metabolic poison, metal–cyanide complexes vary in toxicity depending on the ease with which cyanide is released from them. The toxicity of free cyanide increases with decreasing pH; in fact, the acid form, HCN, is 2.3 times more toxic than the anionic form (CN^-) [4]. Cyanide exerts its toxic effects via at least three known mechanisms which involve the formation of (*a*) complexes with di- or tri-valent metals at the active site of metallo-enzymes such as cytochrome oxidase; (*b*) Schiff base intermediates leading to stable nitrile derivatives during enzyme catalysis; or (*c*) cyanohydrins with metabolic keto groups [5]. Thousands of sites worldwide are contaminated with cyanide as a result of past industrial activity and/or accidental spills [6]. Cyanide-containing effluents must not be discharged without previously reducing their cyanide content to very low levels (<1 mg L^{-1}).

Cyanide-rich waste is usually treated with physical and chemical methods that convert cyanide into less toxic products [7]. Most detoxification methods in wide use involved chemical oxidation. Such is the case with the alkaline chlorination–oxidation process, by which the cyanide-containing waste is initially treated with chlorine or hypochlorite to produce much less toxic cyanate that may be further oxidized if additional chlorine is added. Although this treatment may be efficient in detoxifying waste containing free cyanide, it has some disadvantages. Thus, chlorination is ineffective with metal–cyanide complexes owing to their low reactivity. Also, the process produces sludge, which requires licensed disposal; is relatively expensive owing to the large amount of chlorine it requires; and may cause the formation of hazardous products

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in the presence of some organic compounds [8]. Other methods for treating cyanide-containing waste involve the use of an SO_2/air mixture (INCO process), copper-catalysed hydrogen peroxide oxidation, ozonation, iron precipitation or electrolytic decomposition. These methods, however, are also expensive and require special equipment and maintenance [9]. Because none of the physical or chemical treatments currently in use appears to be fully acceptable for detoxifying cyanide-contaminated waste, a pressing need exists to develop an effective alternative providing a high degradation efficiency at a low cost.

Microbial treatments (biodegradation and bioremediation) may be a potentially inexpensive, environmentally friendly alternative to the previous conventional processes [8,10]. Biological detoxification of cyanide by microorganisms has several advantages over physical and chemical treatments that include the low cost of *in situ* treatments and the ability to thoroughly convert toxic products into natural, non-toxic derivatives [7,11]. However, much remains to be done as regards the development and transfer of effective biological treatment processes from the laboratory bench to full industrial scale via appropriate pilot studies. Although cyanide is toxic to living organisms, biological treatments are feasible alternatives to chemical methods for its removal since a wide range of microorganisms are known to metabolize it through different enzymatic reactions [2,12–22].

Microorganisms were first used for cyanide degradation in the gold mining industry at the Homestake Gold Mine (Lead, SD) [23]. Since then, microbial destruction of cyanide has been the subject of much research at the laboratory, pilot and full scales [7,24–28]. The greatest hindrance encountered so far is that the neutral pH required for optimum performance of most enzymes is unsuitable for cyanide-containing waste, which is normally alkaline. Some authors have succeeded in degrading cyanide with microorganisms under neutral or acid conditions [29,30]; also, some microbial strains can survive in the presence of alkaline cyanide, but are unable to degrade it under such conditions [31]. In previous work, we isolated and characterized the bacterial strain *Pseudomonas pseudoalcaligenes* CECT5344, which can grow on cyanide as its sole nitrogen source under alkaline conditions [2,22,32]. In this work, which is part of a more comprehensive optimization study, we examined the influence of various growth-related variables such as oxygen supply and pH on the efficiency of the biotreatment of cyanide-containing wastewater with *P. pseudoalcaligenes* strain CECT5344 under alkaline conditions in a batch bioreactor.

2. Material and methods

2.1. Chemicals

Sodium cyanide (NaCN) (purity ≥97%), ammonium chloride (≥99.5%) and sodium acetate (≥99%) were supplied by Sigma–Aldrich (USA). Nitrogen was purchased from Air Liquid (Paris, France). All other chemicals used in the study were analytical grade. Solutions were prepared in water that was previously passed through a Milli-Q system from Millipore (Bedford, MA).

2.2. Microorganisms and growth conditions

The alkaliphilic cyanotrophic bacterium *P. pseudoalcaligenes* CECT5344 [2] was grown in Luria-Bertani (LB) medium or a minimal medium [33] (which is going to be referred as M9) with 50 mM sodium acetate as carbon source and 2 mM ammonium chloride or 2 mM NaCN as the sole nitrogen source. The pH of the medium was adjusted to 9.5 or 10.0 with 0.1N sodium hydroxide (NaOH). Stock solutions containing 200 mM NaCN were prepared by dissolving NaCN with the aid of NaOH. Solid media were prepared with Difco Bacto-agar at concentrations up to 1.5%, w/v. The bacterium was routinely grown aerobically at 30 °C at 250 rpm in a New Brunswick Scientific C24 orbital incubator (Edison, NJ). Inocula were prepared in 100 mL Erlenmeyer flasks that were filled to 10% of their total volume and incubated at 30 °C at 250 rpm. Inoculum purity was checked in Petri dish cultures.

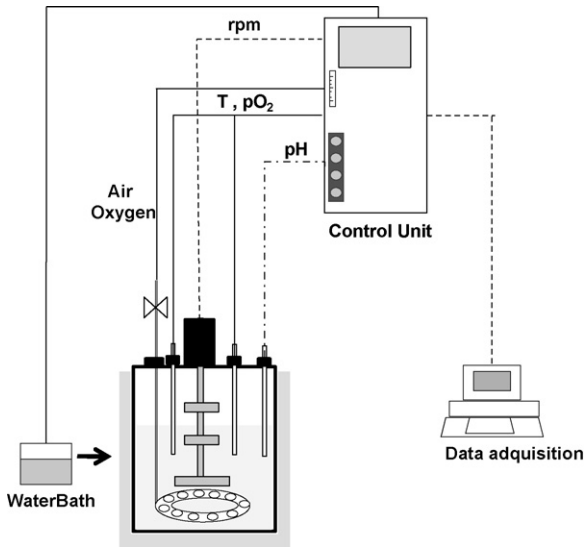

Fig. 1. Bioreactor schematic representation. Batch experiments were conducted in a Biostat® B plus (Sartorius BBI systems) bioreactor that is a cylindrical glass reactor with a maxim capacity of 5 L with a cap on the top that permits hermetic conditions to be obtained. Mixing is conducted by a stirrer. The gassing system consists in a rotameter and a solenoid valves. The system is thermostated at 30 °C with a circulation pump or a water bath. pH is controlled with a peristaltic pumps adding base solution. It is equipped with pH, pO_2 and temperature probes. A software MFCS/DA for visualization, data acquisition and trend display is used.

2.3. Measurement of bacterial growth

Bacterial growth was measured in three ways, namely: (*a*) via the optical density at 600 nm [O.D. (600 nm)]; (*b*) by estimating the number of colony forming units (CFU) from plate counts of tenfold diluted samples in LB-rich solid medium (plates were incubated at 30 °C for 24 h and colonies of viable bacteria counted); data are given as CFU mL^{-1} and (*c*) by determining dry weight (DW) in duplicate 5 mL culture samples following passage through Whatman™ No. 1 filter papers and drying to a constant weight at 100 °C; data are given as g cell L^{-1}.

2.4. Batch culture conditions

Experiments were conducted in a Biostat® B plus (Sartorius BBI systems) 5 L bioreactor (Fig. 1), using the following operational procedure: the reactor was loaded with media containing 50 mM sodium acetate and 2 mM ammonium chloride as carbon and nitrogen source, respectively. Bioreactor and media were autoclaved, with exclusion of the $MgSO_4$ and $FeSO_4$ solutions, which were sterilized by filtration and added to the M9 trace solution after autoclaving. A volume of 5 mL of inoculum from a *P. pseudoalcaligenes* overnight started culture in M9 medium with 2 mM ammonium chloride as nitrogen source was used; the inoculum contained approximately 3×10^5 CFU mL^{-1} and had a dry weight of 0.010 g cell L^{-1} at the start of incubation. Once ammonium ion was fully depleted after 24 h, a NaCN stock solution was added in the volume required to obtain a 45 mg L^{-1} concentration of cyanide. The temperature was kept at 30 °C and the pH initially adjusted to 10.0 or 9.5 with NaOH. Where stated, the pH was controlled and

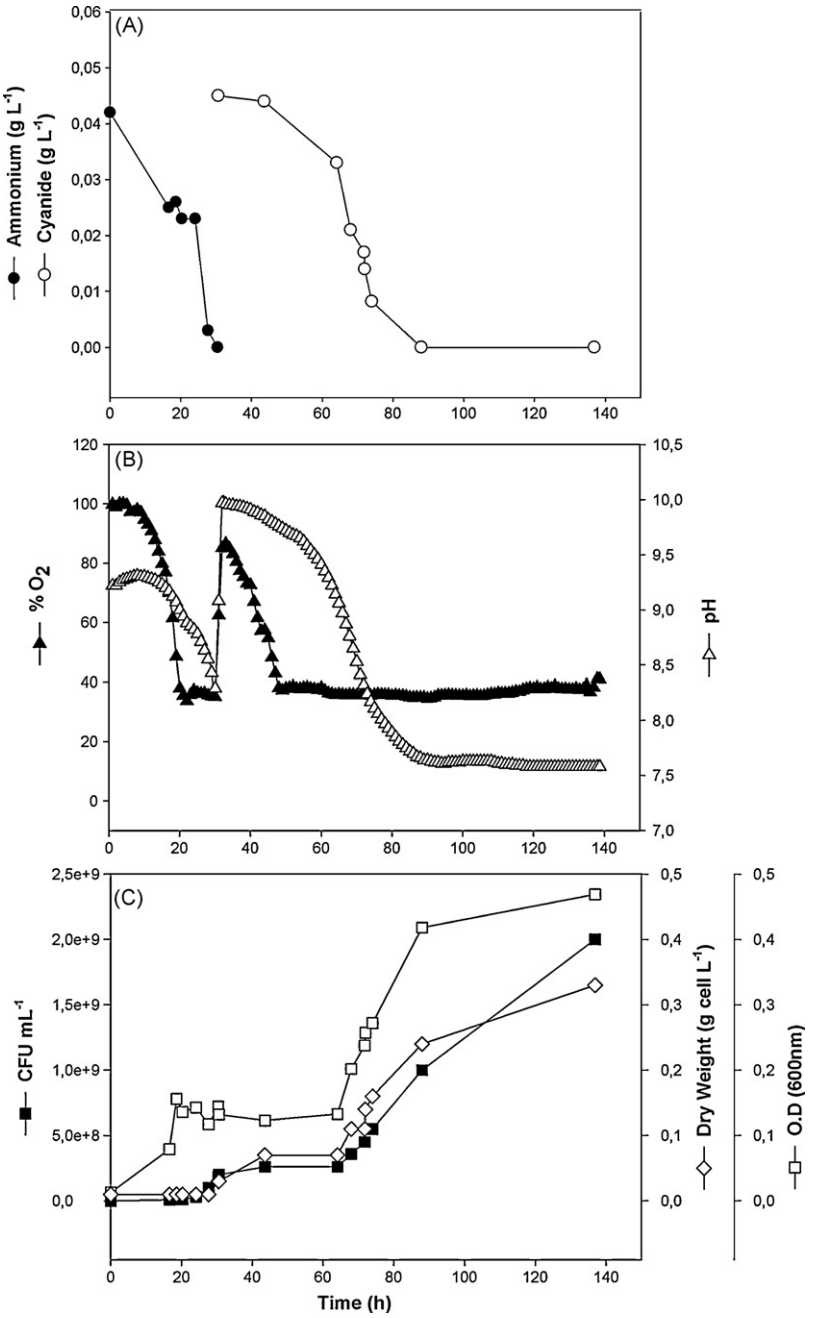

Fig. 2. Degradation of cyanide by *Pseudomonas pseudoalcaligenes* CECT5344 at pH 10.0 without further pH control. (A) Ammonium and cyanide consumption. (B) Variation of pH and O_2. (C) Bacterial growth as monitored by measuring optical density at 600 nm [O.D. (600 nm)], CFU mL^{-1} and DW g cell L^{-1}. Cyanide (45 mg L^{-1}) was added after 24 h, once the nitrogen source (ammonium chloride) was depleted. pH was initially adjusted to 10.0 but subject to no further control.

kept at 10.0 or 9.5 by automatic addition of 0.1N NaOH. Continuous agitation at 450 rpm was provided. Using a sterilizable pO_2 probe and the bioreactor main control unit facilitated aeration by means of pulse air control valves in order to keep the dissolved oxygen saturation at either 35% or 10%. In order to prevent HCN losses, the bioreactor exhaust cooler was connected to a washing flask containing a concentrated NaOH solution. The absence of cyanide in samples from this flask confirmed the effectiveness of this aeration procedure.

2.5. Analytical methods

Ammonium ion was quantified with Nessler's reagent as described elsewhere [34] and cyanide colorimetrically [35].

2.6. Statistics

All plotted data were the averages of three independent experiments each. The mean standard deviations never exceeded 3%.

3. Results and discussion

In this work, we examined the influence of pH and dissolved oxygen on the viability of the alkaliphilic strain *P. pseudoalcaligenes* CECT5344 in a batch reactor (Fig. 1) containing cyanide. This strain can survive up to pH 11.5 and tolerates up to 780 mg CN^- L^{-1} [22].

The effect of pH was examined in a series of tests (see Section 2) conducted at an initial pH of 10 which was controlled at no time

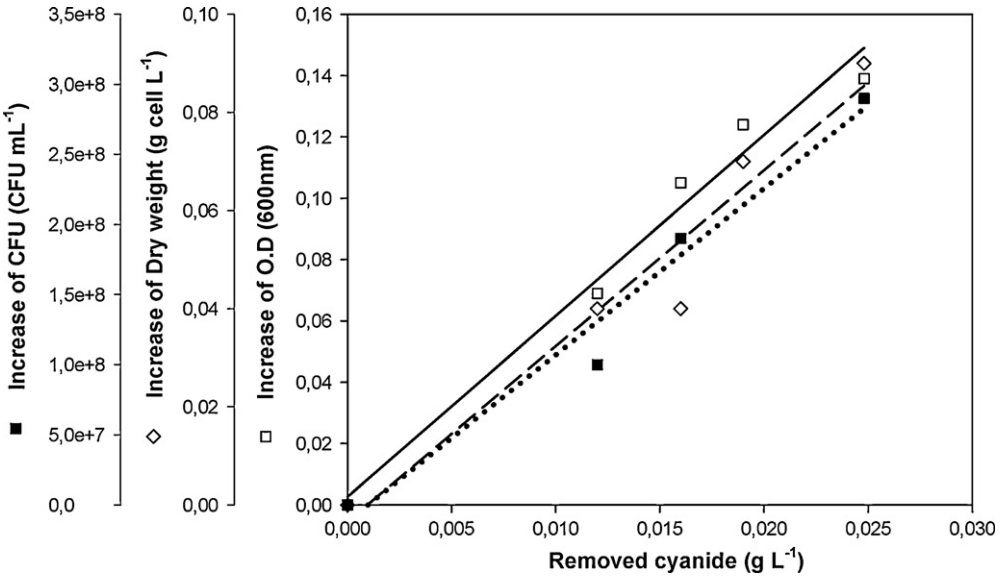

Fig. 3. Bacterial growth as a function of cyanide removal. Bacterial growth as a optical density 600 nm [O.D. (600 nm)] (—), DW (g cell L^{-1}) (- - -), and CFU mL^{-1} (···) was obtained from cyanide batch bioreactors at pH 10.0 without further pH control and is represented as a function of cyanide removal (g L^{-1}). A Linear regression was obtained.

afterwards. Cyanide degradation and cell growth (O.D. at 600 nm, CFU mL^{-1} and DW as g cell L^{-1}) were measured over a period of 140 h. Fig. 2A–C shows the variation of cyanide, ammonium ion, oxygen, pH and bacterial growth. In order to avoid oxygen limitation and minimize HCN losses by air stripping, oxygen saturation was set at 35% (Fig. 2B). Under these conditions, an amount of cyanide of 45 mg CN^- L^{-1} was removed within 57.6 h (mean rate *ca.* 0.78 mg CN^- L^{-1} h^{-1}), which is equivalent to a mean cyanide consumption of 2.81 mg CN^- L^{-1} O.D.$^{-1}$ h^{-1} (Fig. 2A). As can be seen from Fig. 2B, the oxygen concentration and pH decreased by effect of cyanide being consumed by *P. pseudoalcaligenes* cells. Thus, pH decreased from 10.0 to a final value of around 7.5 (Fig. 2B); however, roughly 60% of cyanide was degraded by the bacteria before the pH fell below 9.0 (Fig. 2A and B). Therefore, cyanide degradation occurs between pH 9.0 and pH 10.0.

Some microorganisms are known to degrade cyanide under alkaline conditions. Thus, the fungal strain (IHEM 8026) of *Fusarium solani* was found to degrade 50 mg CN^- L^{-1} in 96 h (mean rate *ca.* 0.52 mg CN^- L^{-1} h^{-1}) under alkaline conditions, but the medium contained a 125 mg L^{-1} concentration of yeast extract [31]. This cyanide consumption rate is similar to that obtained with strain CECT5344 here, but it required the presence of yeast extract. Also, the bacterium *Burkholderia cepacia* is seemingly capable of degrading cyanide at an initial pH of 10, but requires glucose as carbon source. However, glucose reacts with cyanide, so it acts as a chemical reagent [18,36]. The alga *Scenedesmus obliquus* was found to efficiently degrade cyanide, about 0.10 mg CN^- L^{-1} h^{-1}, at an initial pH 10.3 that was subject to no subsequent control; this could be resulted in the release of HCN [36].

Other authors succeeded in bioeliminating cyanide at neutral pH by using a strain of *Pseudomonas* sp. in a medium consisting of yeast extract (Kin B™) and glycerol as carbon source; under these conditions, however, the biological degradation of cyanide may in fact have been rested on a chemical transformation [28].

Bacterial growth, as explained, was measured in terms of CFU, O.D. (600 nm) and DW; all three exhibited a similar variation pattern (Fig. 2C). A substantial lag phase occurred after cyanide was added to the ammonium-growing cells. In fact, no growth was observed, but some cyanide removal was detected – the cyanide concentration was reduced to approximately 30 mg CN^- L^{-1} – during such a phase. This preliminary phase may be required to induce various phenomena leading to cyanide degradation such as cyanide-insensitive respiration [37], a siderophore-based mechanism for iron acquisition in the presence of cyanide [32] and specific enzymes or metabolic intermediates involved in the biodegradation pathway [22]. As shown later on, the lag phase was pH-dependent and followed by exponential growth and maximal cyanide degradation 60–80 h after cyanide was added. Cyanide removal paralleled the exponential growth phase and the greatest rise in oxygen consumption (Fig. 2B).

As can be seen from Fig. 3, bacterial growth and cyanide removal were correlated throughout. This strain is therefore seemingly effective for the biological detoxification of cyanide-containing wastewater in various modes including continuous operation. The yields of cultures grown on 45 mg CN^- L^{-1} as their sole nitrogen source were determined and found to be $Y_{X/CN}$ = 5.9 O.D. (600 nm) g^{-1} CN^-, 3.58 g cell L^{-1} g^{-1} CN^- and 1.19×10^{10} CFU mL^{-1} g^{-1} CN^-. These values correspond to growth yields ($1/Y_{X/CN}$) of 0.17 g CN^- O.D. (600 nm)$^{-1}$, 0.28 g CN^- g^{-1} cell L and 8.4×10^{11} g CN^- CFU^{-1} mL, respectively.

Kinetically, the specific growth rate can be calculated from a mass balance for the cells under the assumption of a first-order

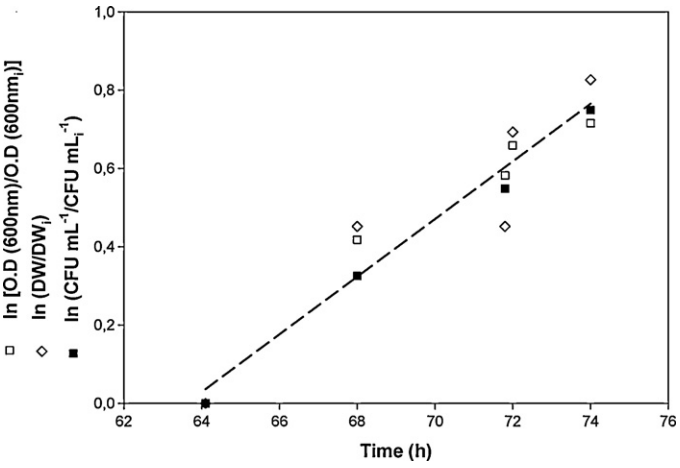

Fig. 4. Fitting of cell growth to a first-order kinetic law. A plot of $\ln(X/X_i)$ as a function of time is represented. Where X is cell concentration and X_i the initial cell concentration: ln[O.D. 600 nm/O.D. 600 nm_i] (□); ln[DW/DW_i] (◊); ln[CFU/CFU_i] (■).

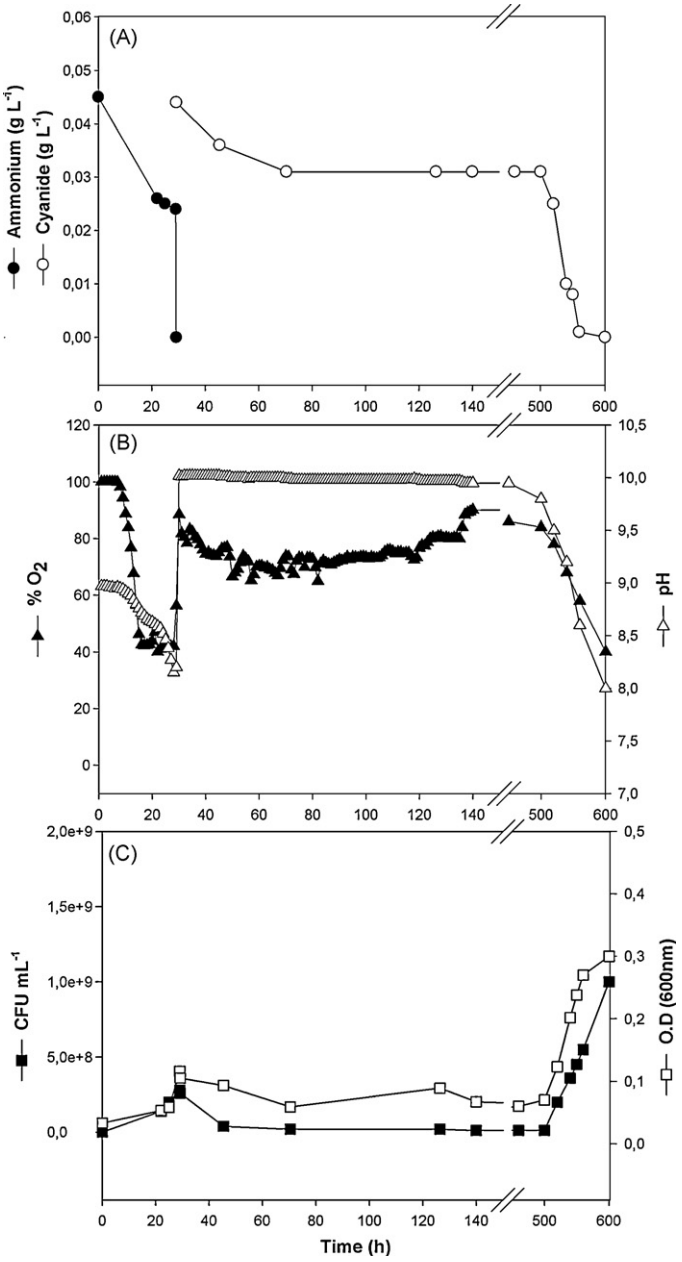

Fig. 5. Degradation of cyanide by *Pseudomonas pseudoalcaligenes* CECT5344 at a controlled pH of 10.0. (A) Ammonium and cyanide consumption. (B) Variation of pH and O_2. (C) Bacterial growth as monitored by measuring optical density at 600 nm [O.D. (600 nm)] and CFU mL^{-1}. Cyanide (45 mg L^{-1}) was added after 24 h, once the nitrogen source (ammonium chloride) was depleted. pH was adjusted and kept constant at 10.0 until 500 h that pH control was removed.

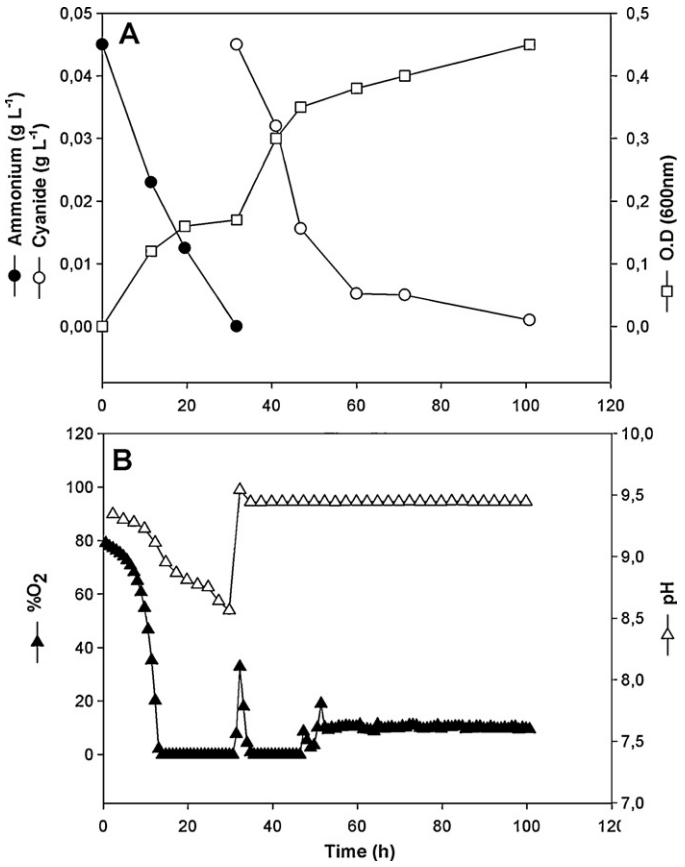

Fig. 6. Degradation of cyanide by *Pseudomonas pseudoalcaligenes* CECT5344 at a controlled of pH 9.5. (A) Ammonium and cyanide consumption, and bacterial growth as monitored by measuring optical density at 600 nm [O.D. (600 nm)]. (B) Variation of pH and O_2. Cyanide (45 mg L^{-1}) was added after 24 h, once ammonium chloride was depleted. pH was adjusted and kept constant at 9.5 throughout.

kinetics for the exponential growth phase:

$$\ln\left(\frac{X}{X_i}\right) = \mu t$$

where X is the cell concentration, X_i the initial cell concentration in the exponential phase, μ the specific growth rate and t time. Based on this equation, a plot of $\ln(X/X_i)$ as a function of t should be a straight line of slope coinciding with the specific growth rate. In fact, as can be seen from Fig. 4, a straight line was obtained irrespective of the way the cell concentration was expressed which allowed a specific growth rate of 0.074 h^{-1} to be calculated. From this rate, a doubling time of 9.4 h can be calculated for the strain CECT5344, as it could be expected, higher than doubling times obtained when a cyanate (5 h), ammonium (2.5 h) and nitrate (2.6 h) as used as nitrogen source [38]. A specific growth rate of 0.013 h^{-1} for a strain (CP1) of *Pseudomonas putida* in the presence of the pollutant 4-chlorophenol [39], and one of 0.2 d^{-1} for a strain (KPN) of *P. pseudoalcaligenes* under optimum conditions and the presence of pyridine as carbon and nitrogen source [40], were obtained in previous work.

pH is a key factor in cyanide biodegradation. In fact, a high pH is required to minimize HCN formation ($pK_a = 9.2$) [31], in order to increase the efficiency and safety of the biological process. This led us to assess bacterial growth and cyanide assimilation at a high, constant pH. To this end, cells were pre-grown as described under Section 2 and, after cyanide was added, the pH was adjusted and maintained at a constant value of 10.0 (Fig. 5 up to 500 h). As can be seen from Fig. 5C, though these conditions prevented growth, cells remained viable at a constant concentration of 5×10^8 CFU mL^{-1}. Similarly to Fig. 2, some cyanide consumption was observed in the previous phase – the cyanide concentration decreased from 45 to 30 mg L^{-1} – , but controlling pH did not suffice to further degrade cyanide (Fig. 5A). Minor changes in oxygen concentration were also observed (Fig. 5B). On the other hand, not controlling the pH (Fig. 5 from 500 h to end) allowed the bacteria to grow and cyanide to be removed. Although a high constant pH ($\geq$10) makes cyanide biodegradation less efficient or even impossible, the microorganisms can still survive, at a constant CFU mL^{-1} level, under these conditions (Fig. 5C).

These results suggest that a different, controlled pH value might be needed to ensure thorough biodegradation of cyanide while

avoiding the release of HCN. This led us to examine cyanide degradation at a controlled pH of 9.5. Also, 10% oxygen saturation (lower than before) was maintained throughout in order to avoid potential HCN stripping problems. As before, cells were pre-grown under ammonium-limiting conditions. With this procedure, an amount of cyanide of 44.9 mg L^{-1} was removed in 69.1 h (Fig. 6A), so the mean consumption rate was *ca*. 0.64 mg CN^- L^{-1} h^{-1}, equivalent to 2.31 mg CN^- L^{-1} $O.D.^{-1}$ h^{-1} (Fig. 6A). The last value is only slightly lower than that obtained by using an initial pH 10.0, with no further adjustment and 35% oxygen saturation in the first experiment; however, statistical differences between the two experiments were virtually zero. A comparison of Figs. 2 and 6 reveals some differences in the profiles for cyanide consumption and cell growth; also, the pH seemingly had a marked effect on efficiency. As can be seen from Fig. 2A and C, a lag phase arose when an initial pH of 10 was used that lasted until a pH around 9.5 was reached. On the other hand, no such lag phase occurred when the initial pH was set at 9.5 (see Fig. 6A and C). In any case, the results show that using a constant pH of 9.5 and 10% oxygen saturation affords complete biodegradation of cyanide at virtually the same rate as with no pH control and 35% oxygen saturation. In addition, the risk of stripping cyanide as HCN is reduced by effect of the high pH and lower dissolved oxygen concentration used.

4. Conclusions

The bacterium *P. pseudoalcaligenes* CECT5344 was found to degrade cyanide at a rate of about 0.64 mg CN^- L^{-1} h^{-1} (equivalent to a consumption of 2.31 mg CN^- L^{-1} $O.D.^{-1}$ h^{-1}) under controlled alkaline conditions in a batch reactor loaded with a minimal medium containing acetate as carbon source. Cyanide degradation was critically affected by pH, which must therefore be strictly controlled. Using a constant pH of 10 is ineffective towards cyanide degradation, which requires employing an initial pH of 10.0 without further control or a constant pH of 9.5 throughout instead. The risk of HCN volatilization can be minimized by using a high pH and a low dissolved oxygen concentration. Cyanide degradation by the strain CECT5344 in reactors operating at a constant pH of 9.5 may thus provide an effective alternative to existing physico-chemical treatments for the detoxification of wastewater containing cyanide or cyano-metal complexes with the need for no chemical pretreatment.

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