Free Cyanide Degradation Kinetics of Cyanide Degrading Bacteria

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***Abstract*—This study reports on the cyanide degrading bacteria (CDB) identified from artisanal mining sites and their free cyanide (FCN) degradation kinetics in aqueous medium. CDB were isolated from soil and water samples contaminated with FCN. The CDB were mainly dominated by *Pseudomonas aeruginosa*, *Citrabacter* sp., *Providencia* sp. (BAB-6345), *Providencia staintii* (Bp-40), *Citrobacter sedlakii* (D5) and *uncultured bacterium*. . FCN removal rate by these CDB varied between 95 – 99 % during a 24h incubation at an initial FCN concentration of 60 mg CN^- L^{-1}. The degradation kinetic studies have shown a half-saturation constant (K_M), of 14 mmol CN^-. L^{-1} for FCN and a specific activity of 28.4 mmol NH_4^+ formed. mol^{-1} CN^-. min^{-1}. It was conclude that various CDB are available in the artisanal mining sites and the substrate FCN is well affined with the CDB activities.**

***Keywords*—**Cyanide degrading bacteria, Free cyanide, Biodegradation kinetics

I. Introduction

Cyanide could be present naturally in the environment through cyano-compound producing organisms such as plants, fungi and bacteria [1]. Anthropogenic activities such as industries using metallurgical processes are still the primary source of cyanide disposal into the environment[2] [3][4]. The industrial effluents containing cyanide could be treated by physical, chemical and biological treatment methods. Among these treatments, biological methods are less expensive and do not create secondary hazards as it often happens during physical and chemical treatments[5] [6].

Many microorganisms were identified as able to degrade free cyanide (FCN). Some of them are pathogenic for animals and plants. Mekuto *et al.* [8] have reported that *Klebsiella oxytoca* was detected as cyanide degrading bacteria (CDB). *Klebsiella oxytoca* is often a cause of bronchopneumonia, urinary tract infection and septicaemia in humans [9]. Similarity, Akinpelu *et al.* [10] have identified the fungi *Fusarium oxysporum* as a microorganism that have a high potential on cyanide biodegradation. Nevertheless, many plant diseases are caused by *Fusarium oxysporum* such as cortical, root and fruit rots, head blights, leaf spots and vascular wilt diseases [11], [12]. These plants' diseases could affect food quality as the presence of mycotoxins in such food can be toxic to humans and animals [13] and subsequently lead to deleterious effects leading to economic losses [12].

Therefore, the identification of CDB is important prior to their application in the bioremediation of cyanide containing effluents to minimise effects on human and environmental health. CDB identification is required to control their growth during their field application [14]. Once more, CDB enzyme characteristic supports the decision on the suitability of the CDB and allows its performance assessment of these CDB in terms of cyanide degradation rates and the affinity between the enzyme they produced and the substrate to be degraded [15] in order to optimize the bioremediation performance.

This research reports, firstly on the identification of CDB isolated from water and soil in the artisanal small scale gold mining catchment of Burkina Faso's Zougnazagmiline and Galgouli sites and secondly, on the FCN removal kinetics by the CDB isolated.

II. Materials And Methods

A. CDB source

Soil and water samples from the artisanal small scale gold mining areas were collected during the dry and wet season of the years 2015 and 2016, at the catchment areas of Zougnazagmiline and Galgouli sites,. These sites were reported in Razanamahandry *et al.*[14]. Physico-chemical parameters such as pH, redox potential, electrical conductivity and temperature were directly measured in-situ to ensure the reliability of the data obtained. Samples were collected in black

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plastic bags and in amber borosilicate bottles to avoid FCN volatilization. Soil and water samples were respectively stored at 4 °C to preserve the microbial flora. FCN concentrations were measured at laboratory scale according to pyridine pyrrazalon method by using spectrophotometer (Hach DR 5 000, Germany) at 600 nm. CDB were isolated from the contaminated samples with a higher FCN concentration, in order to recover the most resistant bacteria.

Medium enriched by agar noble, KCN and various minerals salts source as reported in Oudjehani *et al*.[15] were used to isolate the CDB subsequent to incubation at 28 °C for 7 days[3]. The isolated CDB colonies were streaked several times on the same agar plates. Different colony morphologies were hygienically pick and inoculated in nutrient broth subsequent to incubation at 37 °C for 24 h, followed by organism storage at 4 °C.

B. FCN removal by isolated bacterial

The isolated CDB were tested for their ability to biodegrade FCN. Biodegradation tests were conducted under the same conditions as that reported in Razanamahandry *et al*.[3], but the FCN concentration was set at 60 mg L^{-1} since the highest FCN removal rate was obtained at this FCN concentration.

C. Morphological and Biochemical Characterization

The CDB morphological characterization was studied by testing different cultural media which included Nutrient agar, the pigment on the nutrient agar, MacConkey's agar, Cetrimide agar, Blood agar and Deoxycholate agar. The Pseudomonas agar was also used to assess the presence of *Pseudomonas* species which have been detected in previous cyanide studies [16].

The biochemical characterization tests involved the use of different substrates (i.e. glucose, sucrose, lactose, xylose, maltose, mannitol) to assess the substrate utilization patterns of the isolated organisms.

The molecular characterization was realized by analyzing the genetic approach using PCR method.

D. Molecular biological techniques

Firstly, the DNA for each isolated bacteria was extracted by using the Promega Kit [19]. Cells were lysed by adding 600 µL of the "Nuclei Lysis" solution to the isolated bacteria followed by incubation at 80°C for 5 min and left at ambient temperature. A volume (3 µL) of the RNase solution was then added. After mixing for 5 min, the mixture was incubated at 37°C for 35 min and allowed to cool to ambient temperature. The DNA of precipitated by adding 200 µL of the protein precipitation solution to the cooled mixture. The mixture, containing the protein solution, was vortexed and incubated on ice for 5 min and then, centrifuged at 15,000 rpm for 4 min. Thereafter, the DNA was rehydrated and precipitated again, by transferring the supernatant from the centrifuged mixture to a tube containing 600 µL of isopropanol at ambient temperature and gently mixed by inversion. The mixture was centrifuged at 1500 rpm for 1 min. The supernatant was discarded, and 600 µL of 70% (v/v) ethanol was added to the remaining portion, mixed and centrifuged in the same manner as the preceding step. Ethanol was aspirated and the resulting DNA sample was dried in open air for 15 min. The dried DNA samples were rehydrated with 100 µL of rehydration DNA solution for 1 h at 65°C followed by overnight storage at 4 °C. The DNA was stored in an incubator at -80°C until their amplification by PCR.

A solution of the amplification reaction, prepared under the sterilized ventilated hood was composed of: 10 µL buffer (5 × phusion® HF Buffer # B0519S), 2 µL DNTPs, 2 µL of the primer "forward f515", 2 µL of the primer reverse r806 ", 0.65 µL of GoTaq and 32.35 µL of H_2O for each 1 µL of each DNA sample to have a final mixture volume of 50 µL. Two controls were used: the negative constituted by ultrapure water and the positive by *E.coli* DNA.

The mixture was introduced into the PCR instrument. The PCR was then configured according to the following reaction cycle: pre-warming at 98°C, initial denaturation for 5 min at 95°C followed by x30 cycles, of 1 min at 50°C, 2 min at 50°C and 30 min at 72°C with a final extension of 5 min at 72°C and a constant temperature of 12°C until the system shut down. This cycle was repeated for 2 h.

The DNA amplified by the PCR was then visualized using electrophoresis, at an intensity of 80 mA. A 1% (w/v) agarose gel which served as a support for the DNA to be visualized was prepared in 0.5 TAE. Negative and positive controls were also run on this agarose gel. The red gel was added prior to visualization producing the agarose gel image comprising the DNA bands.

The amplified DNA was inserted into a plasmid of a microorganism for purification and cloning. A ligation reaction formed by 5 µL 2X Rapid Ligation Buffer, T4 DNA ligase, 1 µL p GEM®-T or pGEM®-T Easy Vector (50 ng), from 3 µL PCR and T4 DNA Ligase (3 Weiss units/µL) were prepared. Then, the ligation reaction mixture was incubated at ambient temperature for 1 h. The microorganism called TOP10 was used as a transformation cell or as an amplified DNA support. A volume (2 µL) of the amplified DNA was inserted into this microorganism and gently mixed. The microorganism containing the DNA amplified was then incubated on ice for 30 min and heated at 42°C for 30 sec and placed again on ice for 2 min. The microorganism was cultured in a nutrient-rich medium called SOC Medium. A volume (250 µL) of this medium was added to the tube where the microorganism was present. This tube was incubated in a shaking incubator for 1 h at 37°C and at 225 rpm, with 100 µL of this culture being placed in petri dishes containing Luria-Bertani (LB) medium (medium composed of: Tryptone 10 g L^{-1}, Yeast extract 5 g L^{-1} and NaCl 10 g L^{-1}) with ampicillin/X-Gal (100 µg/mL) to grow overnight in an incubator at 37°C.

Microorganisms clone were cultured by adding 200 µL of 100 mg/mL of ampicillin in 200 mL of the LB liquid medium to obtain a mixture of a solution of LB and ampicillin. A volume (3 mL) of the LB and ampicillin solution was dispensed into 15 mL conical tubes to ensure that the microorganisms had sufficient dissolved oxygen. Two tubes were prepared for each sample. White colonies from two different parts of the petri dish were grown in each tube, conducted under sterile conditions.

Afterwards, the conical tubes were placed in a shaking incubator at 37°C at 160 rpm.

Plasmid DNA into the microorganism clone was extracted and purified. The Promega extraction kit was used for this part of the study. First, a lysate buffer was prepared by adding 2 mL of the bacterial culture to a 2 mL microcentrifuge tube and centrifuged at maximum speed for 2 min. To the supernatant 600 µL of H_2O was added to sedimented cells in the tube which were thoroughly mixed with H_2O subsequent to the addition of 100 µL of Lysis buffer (blue colour) followed by mixing by inverting the tube 6 times. A neutral solution (350 µL) between 4 to 8°C of neutralization was added and mixed by inverting the tube until a yellow solution was obtained. The tubes were then centrifuged at maximum speed using a microcentrifuge (Eppendorf, Germany) for 3 min. The supernatant was transferred to a pure tube with a mini-column, the "PureYieldTM Minicolumn (Promega)", without distributing cells to the bottom of the tube. The mini-column tube was centrifuged at a maximum speed for 30 sec. Thereafter, the liquid that passed through the mini-column was aliquoted out with the mini-column being put back into the same collection tube. Then, the mini-column was washed by adding 200 µL of "Endotoxin Removal Wash (ERB)" and centrifuged at maximum speed for 30 sec and the resultant liquid was decanted. The 400 µL of the "column wash solution (CWC)" was added to the mini-column and centrifuged for 30 sec. Finally, the plasmid DNA was eluted by transferring the mini-column into a sterile 1.5 mL tube, then 30 µL of the buffer elution solution was directly added to the mini-matrix column and allowed to rest for 1 min at ambient temperature. After 1 min, the mini-column with the 1.5 mL tube was centrifuged for 30 sec to elute the plasmid DNA.

E. Identification of the Isolates

Volumes (12 µL) of the plasmid DNA were transferred to a 1.5 mL tube which was sent to Microsynth, a Next Generation Sequencing company, for DNA sequencing. The capillary electrophoresis method was applied using "Sanger cycle sequencing" equipment. The remainder of the eluted DNA was stored at -20°C. The primer used for sequencing is the T7 provided by Microsynth.

The sequencing procedure was done according to Microsynth in-house procedures, which made use of the Sanger cycle sequencing method. The resultant sequences were compared using the Basic Local Alignment Search Tool (BLAST) site (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The program compares the sequence of nucleotides or proteins and gives the statistical significance of each comparison. Software MEGA version 6.0 was used to MEGA version 6.0 was used to construct the phylogenetic trees.

F. FCN Removal Kinetics Studies

Eight (n = 8) liquid media formed by at varying FCN concentrations of 10, 20, 30, 40, 50, 60, 70, and 80 mg/L using the media described in Razanamahandry et al [3]. A volume (1 mL) of the CDB colonies being added to each medium and stirred thereafter. A volume (35 mL) of the BDC mixture and cyanide solution were analysed for the cyanide concentration, while the density of the living BDC and NH_4^+ production at the initial phase were tested (at t = 0 min) and the final phase (at t = 10 min) being determined. A kinetic model was then established from the measured parameters. The kinetic model described by Michaelis-Menten was used according to Eq. 1:

$$v = -\frac{dS}{dt} = \frac{dP}{dt} = \frac{v_{max}\,S}{K_M + S} \quad (1)$$

Where:

v : initial rate in the absence of product of the enzymatic reaction for a concentration of susbtrate (S) which is the concentration of free cyanide (mol min^{-1}),

v_{max} : maximum initial velocity in (mol min^{-1}),

K_M : Michaelis constant is the concentration in (S) for which $v = \frac{v_{max}}{2}$ (mol L^{-1}),

P: concentration of the product which is NH_4^+ and

v_{max} and K_M express the characteristics of the enzyme and were determined using the Lineweaver-Burk curve with y = $1/v$ and $x = 1/S$ whereby $v_{max} = 1/b$ and $K_M = a/b$.

with a and b being coefficients of the linear trend curve of the Lineweaver-Burk curve $y = ax + b$.

III. Results And Discussion

A. CDB Isolated and FCN Removal Rate

Thirty-five (n = 35) isolates were obtained and coded according to: (i) the source (Zougnazagmiline or Galgouli site), (ii) the sample type (water or soil), (iii) the season and the year of sample collection (dry season or wet season, year 2015 or 2016), and (iv) forms of colonies of individual species in petri dishes.

The isolated CDB demonstrated FCN biodegradation capabilities with a removal rate of 99% after 24 h and a biodegradation rate ranging from 0.15 to 39.42 mol CN^-min^{-1} Bacterium $^{-1}$ where determined to be suitable for this study.

As results of morphological and biochemical studies, Table 1 shows the CDB species isolated, that were detected, which include: *Pseudomonas* sp., *Klebsiella* sp., *Staphylococcus* sp., *Providencia* sp., *Serratia* sp. and *Citrobacter* sp.

The bacteria in the water at both sites were identified: *Pseudomonas* sp., *Klebsiella pneumoniae, Providencia* sp. and *Providencia thailandensis*. These species were also identified in the soil at both sites with the dominance of the *Pseudomonas aeruginosa, Citrobacter sedlakii, Serratia* sp., *Klebsiella oxytoca, Providencia stuartii* and *Staphylococcus* sp. The same species appeared regardless of the different season and year of the collected sample.

The species *Klebsiella pneumoniae, Pseudomonas aeruginosa, Serratia* sp., *Klebsiella oxytoca* and *Staphylococcus* sp. have been proven in the literature to be cyanide degraders [20]–[22].

TABLE I: CDB SPECIES ISOLATED

Sites	Sample type	Species
Zougnazagmiline	Water	*Klebsiella pneumoniae, Pseudomonas* sp.
		Providencia sp.
	Soil	*Klebsiella oxytoca,*
		Providencia stuartii
		Pseudomonas sp.
		Staphylococcus sp.
Galgouli	Water	*Providencia* sp.
		Klebsiella pneumoniae Providencia thailandensis
	Soil	*Pseudomonas aeruginosa*
		Citrobacter sedlakii
		Klebsiella oxytoca
		Serratia sp.

B. CDB Identification by DNA Sequencing

The concentration of the extracted DNA ranged from 29.5 to 792.5 µg mL^{-1}. The purity of the DNA was also verified from the ratio DNA/RNA and DNA/Proteins which were close to 2. Fig. 1 shows the result of the amplification of some DNA samples (MP, EI, NF, FI and EC) by PCR with both negative and positive controls. All DNA bands had a base pair of 291 which proved the conformity between the primers (R806 - F515 = 291) used and the DNA sample studied. The negative control also demonstrated that the sample had not been contaminated during preparation. These were the desired DNA bands required and were confirmed by the positive control. The isolates (n = 35) were formed by 10 species of bacteria from the sample collection sites. Table 2 summarizes the link between these isolates and the identified species. All identified CDB species were directly related (99%) similar identity and 100% recovery rate to the isolates in the repository. The species obtained were also marked by a *Providencia* sp. type dominance.

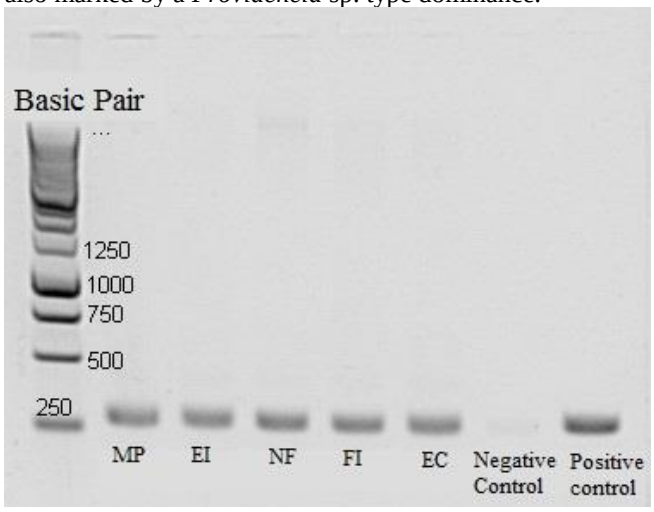


Fig. 1 DNA amplified visualized through the agarose gel

Fig. 2 and 3 show the phylogenetic tree of species of bacteria present in water and soil samples from each study site. Bacteria (n = 10) were detected from the two sites (Zougnazagmiline and Galgouli). The samples were composed of: *Uncultured bacterium clone* (OTU7),*Uncultured bacterium*, *Providencia* sp. (BAB-6345), *Providencia stuartii* (MRB-44), *Providencia stuartii* (Bp-40), *Uncultured bacterium clone* (LIB049 C04 1646), *Providencia* sp. (BAB-6345), *Pseudomonas aeruginosa*, *Citrobacter sedlaki* (D5) and *Uncultured bacterium clone* (Otu01369).

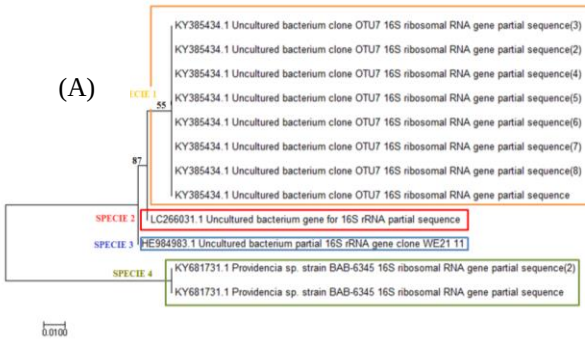
TABLE II: CDB SPECIES IDENTIFIED BY DNA SEQUENCING

Isolate Codes	Identity (%)	Recovery (%)	Species
MP, EI	99	100	*Uncultured bacterium clone* (OTU7)
NF, FI, EC	99	100	*Uncultured bacterium*
BP,HF, FF,KC,CF	99	100	*Providencia* sp. (BAB-6345)
GR, NC, IC, CI, FC, LC, DI, BC	99	100	*Providencia stuartii* (MRB-44)
CC, EF, JE, JI, GC	99	100	*Providencia stuartii* (Bp-40)
DR, IN, DC	99	100	*Uncultured bacterium clone* (LIB049 C04 1646)
IP,OF, HI	99	100	*Providencia* sp.(BAB-6345)
MC, LP, BI	99	100	*Pseudomonas aeruginosa*
OP	99	100	*Citrobacter sedlakii* (D5)
AC, AP	99	100	*Uncultured bacterium clone* (Otu01369)

There were common species at these 2 sites; these were formed by: *Uncultured bacterium clone* (OTU7), *Uncultured bacterium* and *Providencia* sp. (BAB-6345). The composition of the culture medium of two first species is still unknown. They are likely to be new species. These three species were all present in the water of the two sites, but the species *Uncultured bacterium clone* (LIB049 C04 1646) was added for the case of Galgouli.

The species of bacteria present in the soil were much diversified for the Galgouli site compared to those of Zougnazagmiline. In Galgouli, the bacteria identified were: *Uncultured bacterium clone* (OTU7), *Uncultured bacterium,* and *Pseudomonas aeruginosa, Citrobacter sedlakii* (D5) and *Uncultured bacterium clone* (Otu01369), while at Zougnazagmiline, they were: *Uncultured bacterium clone OTU7, Uncultured bacterium, Providencia stuartii* (MRB-44) and *Providencia stuartii* (Bp-40).

Based on the ability of these 10 identified species to degrade cyanide, 4 groups of bacteria can be obtained according to Fig. 4.



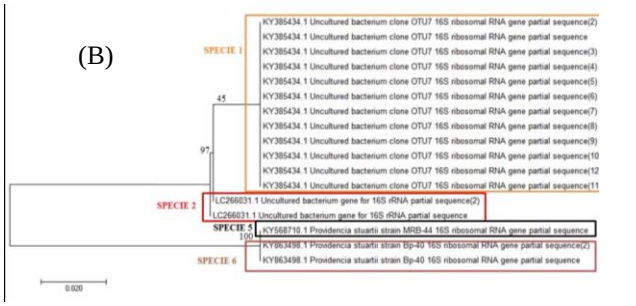


Fig. 2 Phylogenetic Trees of CDB species in Zougnazagmiline sites (A) from water samples (B) from soil samples

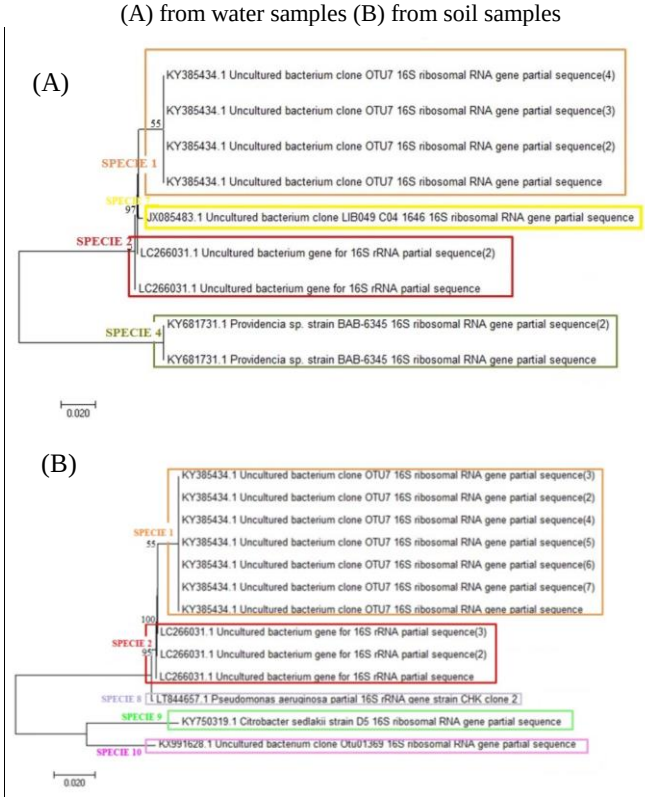


Fig. 3 Phylogenetic Trees of CDB species in Galgouli sites (A) from water samples (B) from soil samples

Among these species, *Pseudomonas aeruginosa* has been reported by [18] as a soil-inhabiting species capable of degrading cyanide. Ibrahim *et al.* [1] also shown the faculty of the genus *Citrabacter* sp. to degrade total cyanide, especially metal cyanide. Nevertheless, minimal studies have shown the ability of the following species to degrade cyanide: *Providencia* sp. (BAB-6345), *Providencia staintii* (Bp-40) and *Citrobacter sedlakii* (D5). However, Mekuto *et al.* [8] have recently reported the ability of *Providencia* sp. to degrade FCN.

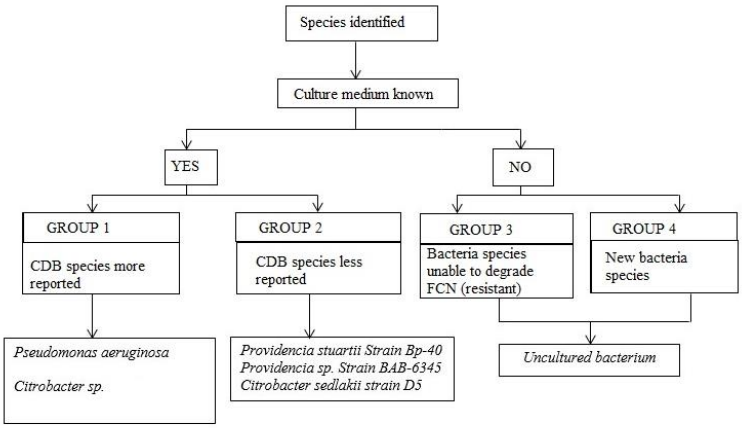
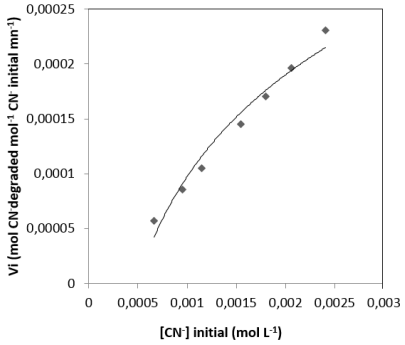
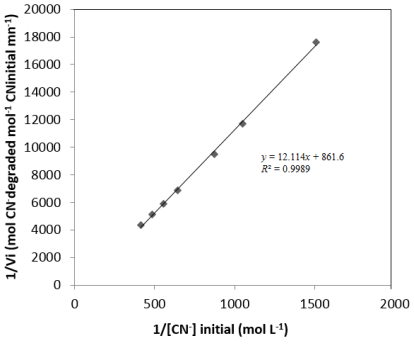


Fig. 4 Classification of the ten CDB species identified based on the CDB already reported

C. FCN Removal Kinetics

Fig. 5 presents the Michaelis– Menten kinetic parameters' values, which describes enzymatic activity kinetics when the tested FCN concentrations have been increased. A linear regression analysis, using Lineweaver–Burk type linearization (Fig. 6b), showed a FCN half saturation constant (K_M) of 14 mM. In addition, CDB tested have revealed 28.4 mmol NH_4^+ formed mol^{-1} CN^- initial min^{-1} as a value of the specific activity of enzymes produced by these species.





V_{max} = 1.16 mmol CN^- degraded mol^{-1} CN^- initial min^{-1}

K_M = 14 mM CN^- degraded

Fig. 5 Michaelis–Menten parameters for FCN degraded (A) Non-linear regression, (B) Lineweaver– Burk

IV. Conclusion

Various CDB were identified from water and soil samples contaminated by FCN from mining areas in Burkina Faso's

Zougnazagmiline and Galgouli sites. Ten (n = 10) bacteria species were obtained in which new CDB were detected and new microorganisms species were suspected. The affinity between the FCN substrate and the CDB enzyme was revealed by the FCN removal kinetics. Future works should be focused on the field application of the CDB identified.

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