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Native Cyanide Degrading Bacterial Consortium and its Potential for Gold Mine Tailings Tertiary Biotechnological Treatment

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Gold extraction by cyanidation generates toxic effluents stored in tailing dams which are an environmental hazard due to the presence of metal-cyanide complexes and sulfates. We aimed to isolate a native bacterial consortium from a gold mine tailing dam and study its cyanide biodegradation potential as part of tertiary biotechnological treatment. The cyanide-degrading consortium was streaked by serial dilutions on specific medium R2A. MALDI-TOF MS proteomic fingerprint of the isolates showed that bacterial strains belonged to *Microbacterium paraoxydans*, *Brevibacterium casei*, *Brevundimonas vesicularis*, *Bacillus cereus,* and *Cellulosimicrobium cellulans*. The first four genera were previously identified as cyanide degrading bacteria. *Microbacterium*, *Brevibacterium.* and *Bacillus cereus* were previously found in alkaline conditions. The five genera were also previously reported as resistant to several heavy metals. *Cellulosimicrobium* was previously described as cyanogenic, and, to our knowledge, this would be the first report to relate it also to cyanide biodegradation. These genera were identified as functional bacteria for cyanide degradation and might be suitable for mine tailing biotechnological tertiary treatment.

1. Introduction

Cyanides are a highly toxic group of compounds characterized by cyano ion (C≡N) (Jaszczak et al., 2017). They are used in gold leaching for cyanidation (Sharma et al., 2019). Cyanides are highly reactive with heavy metals forming metal complexes, useful for precious metals extraction from ores, besides toxicity due to inactivation of metalloenzymes. It is estimated that the cyanide toxicity threshold may be two ppm for some anaerobes and 200 ppm for most aerobic microorganisms (Kuyucak & Akcil, 2013). WHO (2003) indicates a maximum acceptable limit of 0.05 mg L^{-1} for total cyanides in wastewaters. The mining industry produces large amounts of wastewaters from cyanidation, containing heavy metals such as arsenic, lead, mercury, cadmium, chromium, copper, iron, zinc, and cyanides (Seyyed Alizadeh Ganji and Hayati, 2018). Thus, these wastewaters, stored at tailing ponds, must be treated to minimize toxicity. For instance, physicochemical methods often are efficient, use hazardous and expensive reagents, require costly equipment, maintenance, and high initial investment costs (Park et al., 2017; Sharma et al., 2019). On the other hand, microorganisms that can degrade cyanides could be a viable alternative for mine tailing treatment. Cyanotrophic bacteria are capable of using cyanides as C and N sources for metabolism. Native bacteria of polluted environments, such as mine tailing ponds, have metabolic abilities to deal with contaminants (Moradkhani et al., 2018). Therefore, the hypothesis is that the best bacteria for gold mine tailings biotreatment are native, due to their adaptation; consequently, the aim is to isolate a native bacterial consortium from a gold mine tailing dam and study its cyanide biodegradation potential.

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2. Material and methods

Native cyanide degrading bacterial consortium was obtained from a gold mine tailing pond, cultured, and microbiological characterized as described below.

2.1 Sampling

The native cyanotrophic bacterial consortium was obtained by aerobic collection from mine tailing pond "Mastrantos II" in Guanajuato, Mexico with a total cyanide concentration of 40 mg L^{-1}. Five sediment samples were diluted 1:10 with sterile peptone isotonic solution and vortexed. After that, solutions were sedimented in Imhoff cone for 1 h. Dilution, vortex, and sedimentation were repeated two more times. Then, supernatants were stored at 4 °C overnight. After that, supernatants were centrifuged at 10 000 rpm, 5 min until pellets were formed. Five pellets were recovered and stored in glycerol (30%, Karal, Mexico) at -20 °C until further use.

2.2 Bacterial consortium isolation

Pellets were activated in peptonized broth (BD Bioxon) with orbital agitation (150 rpm), 30 °C for 2 days. After that, the synthetic cyanided solution was inoculated (10 % V/V), with sodium cyanide (Sigma), 10 mg L^{-1} and incubated at same conditions as the activation step for 3 days. The synthetic cyanided solution composition is presented on Table 1. Preliminary biodegradability tests (data not shown) were carried out to select proper consortium from pre-screening among 5 native consortia allowed to select the consortium which degraded the highest cyanide initial concentration tested in other experiments (120 mg L^{-1} unpublished results).

Table 1: Synthetic cyanided solution composition

Salt solution (89 % v/v)	Trace element Solution (1 % v/v)	BBC Buffer (10 % v/v) pH=9.6	Carbon and Nitrogen Source
KH_2PO_4, 1.7 mg L^{-1} NaCl, 0.5 g L^{-1} $MgSO_4.7H_2O$, 0.5 g L^{-1} $CaCl_2$, 0.1 g L^{-1}	$ZnSO_4{\cdot}7H_2O$, 0.05 g L^{-1} $MnCl_2{\cdot}4H_2O$, 0.05 g L^{-1} $CuCl_2{\cdot}2H_2O$, 0.005 g L^{-1} $Na_2MoO_4{\cdot}2H_2O$, 0.005 g L^{-1} $Na_2B_4O_7{\cdot}10H_2O$, 0.002 g L^{-1} $CoCl_2{\cdot}6H_2O$, 0.0003 g L^{-1}	Na_2CO_3, 1.59 g L^{-1} $NaHCO_3$, 2.93 g L^{-1}	NaCN, 10 mg L^{-1}

A synthetic cyanided solution sample from the previous culture of the selected consortium was serially diluted (10^{-5}, 10^{-6}, 10^{-7}), and spread over sterile R2A (Merck, Germany) plates incubated at 30 °C for 7 days. Colonies were re-streaked. Pure colonies were microbiological characterized (Prescott and Harley 2002), Gram and spore stained (Hycell, Mexico) with a standard procedure. Slides were observed under a light microscope to determine shape and cells arrangement. Pure colonies were stored in Glycerol (Karal, Mexico) at -20 °C until further utilization.

2.3 Bacterial identification

For 16S rRNA analyses, after 3 days of each pure colony from the consortium was cultured in cyanide synthetic medium (NaCN = 10 mg L^{-1}), a sample was taken, and genomic DNA was extracted using DNeasy Ultraclean (Qiagen) extraction kit. After DNA extractions samples were stored at -20 °C until used. Genomic DNA was used as a template for polymerase chain reaction (PCR) amplification of 16S rRNA with universal primers 27F-YM and 1492R (Frank et al., 2008), Thermal cycling (Bio-Rad, C-1000 Touch Thermal Cycler) conditions are shown in table 2. All reactions were set in triplicate. Amplification products were purified with Gel/PCR DNA Fragments extraction kit (IBI Scientific). DNA was stored at -20 °C for subsequent analysis.

Table 2: 16S rRNA PCR conditions

Step	Cycles	Temperature (°C)	Duration (s)
Initial denaturalization	1	98	120
Denaturalization		98	10
Annealing	28	48	15
Extension		72	12
Final Extension	1	72	300

PCR products purified were used for an amplified ribosomal DNA restriction analysis (ARDRA). ARDRA was performed using AluI and HaeIII (NEB) restriction enzymes. Digestion was carried out at 37 °C for 2 h with CutSmart buffer (NEB) and 30 ng from 16S rRNA PCR products. Same 16S rRNA restriction patterns were identified as the same bacterial strain and grouped for sequencing. At least three strains with the same restriction pattern were selected for sequencing (future experiments). For mass spectrometry proteomic fingerprint,

colonies were streaked in BAS for 3 days at 30 °C, and samples preparation for matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) was carried out according to direct transfer protocol recommended by manufacturer. If the score did not match a probable identification, MALDI-TOF MS was carried out in compliance with ethanol/formic acid extraction protocol recommended by manofacturer. MALDI-TOF MS was conducted using an IVD MALDI Biotyper mass spectrometer (Bruker Daltonics) equipped with an N_2 laser. All spectra were recorded in linear, positive ion mode. Spectra were collected as a sum of 500 shots across a spot. A mass range of 2000-20,000 m/z was used for analysis. Main spectra were calculated from 3 spectra per strain and used for a score with BioTyper software (2.2).

3. Results and discussion

After serial dilution from native consortium isolated, colonies were re-streaked, and 23 different pure colonies were obtained. Figure 1 shows characteristics observed of the isolated microorganisms. Pure colonies were classified into 5 groups. Group 1 was formed with strains C1, C2, C3, C4, C5, C9, C11, C13, C14, C15; group 2 with strains C6, C7, C8, C18, C20, C21, C23; group 3 C10, C12, C17, C19 strains. Strains C16 and C22 had unique microbiological characteristics thus they were considered as a single group each.

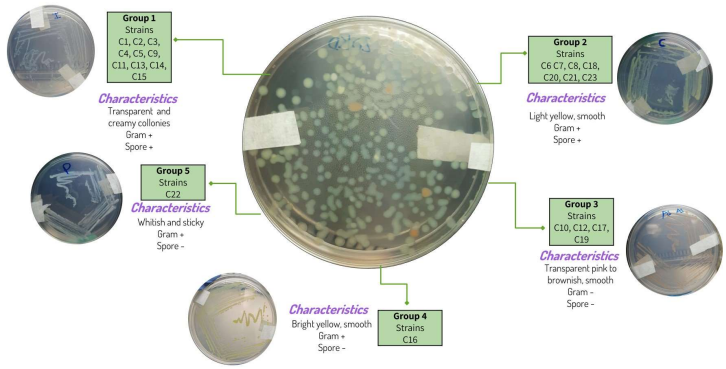

Figure 1: Microbiological characteristics from the native cyanotrophic consortium.

Literature reports regarding the isolation of native cyanotrophic consortia from gold mine tailings (Khamar et al., 2015; Mekuto et al., 2018; Moradkhani et al., 2018; Tiong et al., 2015) described the identification, on average, of 10.33 ±11 species using 16S rRNA sequencing. The number of potential cyanotrophic species identified in this study (23) agrees with previous reports. After 16S rRNA PCR amplification and purification from 23 isolated strains, electrophoresis showed that the approximate size of DNA fragments was about 1.2 kb. ARDRA analysis of 16S rRNA confirmed previous microbiological clustering, as shown in figure 2.

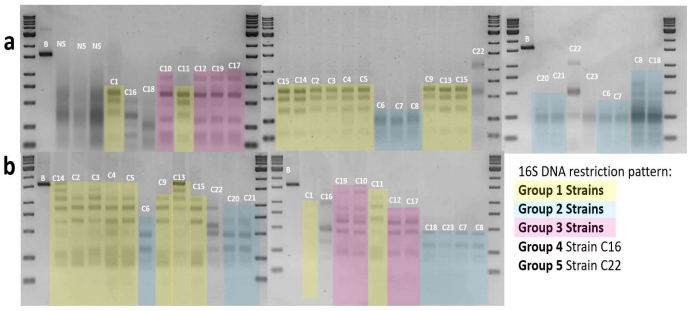

Figure 2: 16S rRNA restriction pattern. a) HaeIII b) AluI. B: 16S rRNA from C14 strain without digestion enzymes. NS: Not successful digestion. C1, C2, C3-C23 are the 16S rRNA restriction patterns with each restriction enzyme tested for every strain isolated. Colors represent different groups of bacterial strains depending on the microbiological characterization.

Figure 2a demonstrates that strains from group 1 (shadowed in yellow) presented the same digestion patterns for each one. Strains from the second group (blue shadow) had similar patterns except for strains C18 and C8. The third group of strains had also the same digestion pattern (pink shadow) for every strain of the group. C16 and C22 had unique restriction patterns each. Restriction analysis with AluI (Figure 2b) shows similar results: strains from the first group (yellow shadow) showed similar restriction patterns except for incomplete digestions

for strains C3, C13, and C14; those restriction profiles had a band of approximately 1.2 kb at the top of the gel. Strains for group 2 (blue shadow) presented the same digestion pattern confirming previous microbiological classification for this group. The third group had also the same restriction pattern for each strain of the group. C16 and C22 had distinctive profiles for each one. Thus, there were selected three strains from each group, C16 and C22 for 16S rRNA PCR products sequencing by triplicate in both senses and confirm identification and axenic cultures from future experiments. MALDI-TOF MS fingerprint of each microorganism is presented in table 3. Strains C2, C3, C4, C4, C9, C13, C14, C15, and C6 presented scores higher than 2.3 which are considered as highly probable species identification. Strains C7, C18, C20, C21, and C10, with a score from 2 to 2.299 are appraised as a secure genus and probable species identification. Strains C23, C22, and C16 with scores from 1.7 to 1.999 represent a probable genus identification. Strains C2 and C19 are not reliable identifications and for strains C11, C8 and C17 there were no peaks found on mass spectra. MALDI- TOF MS-based accurate identification depends on an adequate spectrum quality and close database reference matches. Like ribosomal RNA, ribosomal proteins are universally conserved; the correct identification ppercentages varies from 93 to 100% compared to 16S rRNA identification (Strejcek et al., 2018) This preliminary identification will be confirmed with 16S rRNA PCR products sequencing.

Table 3: Native cyanotrophic consortium identification by MALDI-TOF MS

Group	Best Match	Strains and Score
1	*Bacillus cereus*	C1: 1.656 C2: 2.482 C3: 2.426 C4: 2.408 C5: 2.351 C9: 2.444 C11: NPF C13: 2.394 C14: 2.404 C15: 2.367
2	*Microbacterium paraoxydans*	C6: 2.322 C7:2.29 C8: NPF C18: 2.079 C20: 2.157 C21: 2.099 C23: 1.974
3	*Brevundimonas vesicularis*	C10: 2.063 C12: 1.136 C17: NPF C19: 1.639
4	*Cellulosimicrobium cellulans*	C16: 1.793
5	*Brevibacterium casei*	C22: 1.714

NPF: No peaks found on the mass spectrum.

Preliminary identification showed that the cyanotrophic consortium is dominated by bacteria belonging to *Bacillus cereus* (group 1), *Microbacterium paraoxydans* (group 2), *Brevundimonas vesicularis* (group 3), *Brevibacterium casei*, and *Cellulosimicrobium cellulans*. Microbiological characteristics and MALDI-TOF-MS identification were consistent. Other native cyanotrphic consortia from gold mine tailings were identified as *Halomonas* (Khamar et al., 2015), *Pseudomonas* (Moradkhani et al., 2018), and *Bacillus* (Mekuto et al., 2018) which in this study were not found. This observation is coherent with previously published literature that suggests that the microbial composition of native cyanotrophic consortia depends on the isolation source.

3.1 Potential for gold mine tailings biotreatment

In other experiments (data not showed) it was demonstrated its capability to degrade up to 120 mg L^{-1} of free cyanide in synthetic water. Four of the five genera preliminary identified were previously known as cyanotrophic: *Microbacterium* (Han et al., 2014; Sankaranarayanan and Gowthami, 2015), *Bacillus cereus* (Dwivedi et al., 2016), *Brevibacterium* (He et al., 2014), and *Brevundimonas* (Han et al., 2014; Zhao et al., 2011). However, to the best of our knowledge, this study is the first to report bacteria closely related to *Cellulosimicrobium* as cyanotrophic, besides the description of this bacteria as cyanogenic (Yadav et al., 2015). Four genera from the consortium were previously related to mining environments, as shown in table 4.

Table 4: Potential for gold mine tailings biotreatment of the native cyanotrophic consortium

Bacteria	Mining related environments	Heavy metal resistance	Alkaline resistance
Bacillus cereus	Nearby mining soil, lead-zinc factory, nickel mine (Dwivedi et al., 2016)	Pb, Cd, Ni, Cd, Cr (Venugopal et al., 2019)	4.5-9.5 (Duport et al., 2016)
Microbacterium	Coal mine (Steinbock et al., 2019)	Zn, Ni, Fe, Cr, Co, Se, Sn, As (Sathyavathi et al., 2014; Zhou et al., 2015)	8-10 (Buczolits et al., 2008)
Brevundimonas	Soil of Pongkor Village gold mine, Bogor (Iriwati et al., 2012)	Cu, Hg, Pb (Rathi and K N, 2021)	Not reported
Cellulosimicrobium	Not reported	$ZnSO_4$, $NiSO_4$, $CuSO_4$, $Fe_2(SO_4)_3$, $Pb(C_2H_3O_2)$, $CdCl_2$ (Bhati et al., 2019)	6-9 (Karthik et al., 2017)
Brevibacterium	The root of *Prosopis laegivata* growing at the edge of a gold-mine tailing in Mexico(Román-Ponce et al., 2015)	Zn, Ni^{2+} Cu^{2+} As^{5+}, As^{3+}, Cr (Román-Ponce et al., 2015)	10-11 (Román-Ponce et al., 2015)

In addition to the ability of the consortium to resist and degrade cyanide, two more conditions are needed: the capability to grow in highly alkaline conditions (pH>9.2) to avoid volatilization of HCN and resistance to heavy metals. Four of the species identified were previously reported to resist pH higher than 9, and the five genera are known to resist heavy metals as presented in table 4. Nevertheless, additional information is required to evaluate their tolerance to both conditions combined.

4. Conclusions

Cyanide degrading consortium isolated from a gold mine tailing was preliminarily identified as *Bacillus cereus*, *Microbacterium paraoxydans, Brevundimonas vesicularis*, *Cellulosimicrobium cellulans,* and *Brevibacterium casei*. *Cellulosimicrobium* is described for the first time as cyanotrophic. Bacterial species of the identified consortium presented resistance to cyanide, heavy metals, and alkaline conditions. These characteristics are needed for any viable gold mine tailing biotreatment. Additionally, it showed potential to produce valuable by-products, which could improve the biotreatment's sustainability and economic viability.

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