J. Viet. Env. **2014**, Vol. 6, No. 3, pp. 196-200
DOI: 10.13141/jve.vol6.no3.pp196-200

JOURNAL of VIETNAMESE ENVIRONMENT

Physiological properties of new species of *Acidithiobacillus* isolated from abandoned Tin mine in Ha Thuong, Thai Nguyen province

Một số đặc điểm sinh lý của vi khuẩn Acidithiobacillus spp. phân lập được từ mỏ thiếc bỏ hoang ở Hà Thượng, tỉnh Thái Nguyên

Research article

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Acidophilic bacteria are able to tolerate acidic environment and also contribute to the lowering of environmental pH value, implying potential applications in metal-leaching technology extracting metals from tailings and electronic wastes. In this study, we conducted a sampling campaign in abandoned Tin mine in Ha Thuong, Thai Nguyen province, to isolate acidophilic bacteria and to study physiological characteristics of the isolated bacteria. As a result, two acidophilic bacteria were successfully isolated and identification by 16S rDNA gene sequences showed that the two bacteria are similar to *Acidithiobacillus ferrooxidans* and *Acidithiobacillus thiooxidans* (98% and 94 % of similarity, respectively). Both strains are tolerant of pH in the range of 3 and have the ability to grow optimally at temperatures of 30°C.

Vi khuẩn ưa axit có ý nghĩa ứng dụng trong công nghệ tách rút kim loại từ quặng đuôi và ngay cả từ rác thải linh kiện điện tử. Trong nghiên cứu này, chúng tôi đã tiến hành thu mẫu ở mỏ thiếc bỏ hoang ở Hà Thượng, Thái Nguyên nhằm phân lập được nhóm vi khuẩn ưa axit và qua đó nghiên cứu một số đặc điểm sinh lý của vi khuẩn này. Kết quả chúng tôi đã phân lập được hai chủng vi khuẩn ưa axit. Định dạng bằng nhận diện trình tự gen 16S rADN cho thấy hai vi khuẩn này có độ tương đồng là 98% với vi khuẩn Acidithiobacillus ferrooxidans và 94% Acidithiobacillus thiooxidans. Cả hai chủng vi khuẩn đều có tính chịu pH trong khoảng 3 và có khả năng sinh trưởng tối ưu ở nhiệt độ 30°C.

Keywords: *Acidithiobacillus*, acid mining drainage, bioleaching, morderate temperature tolerance

1. Introduction

The disposal of mining waste (such as tailing, waste rocks, slugs, mill, etc.) is a major environmental problem nowadays. Mining waste consists of many heavy metals (Au, Zn, Cd, Hg, As, Co, etc.) what are most frequent grounds of hydrometallurgical waste changed into hazardous waste. In recent years, the bioleaching of heavy metals has been more and more interested in because of its application to mining waste and electronic waste (Willner *et al.*, 2013)(Wang *et al.*, 2009)(Sugio *et al.*, 2008)

In the bioleaching process, microorganisms reduce the pH value of the environment and dissolve solid compound containing metals. The process was applied to recover the metals from mining waste or electronic waste. Due to the microbial flexibility, microorganisms adapt easily to changing and extreme living conditions of the process (Willner *et al.*, 2013). It's environmental friendly, cheaper and easier to control than conventional techniques.

Acidophilic microorganisms are key players in the bioleaching techniques, because of its ability to utilize both Fe^{2+} and sulfur moieties in sulfide ores as energy source (Rzhepishevska *et al.*, 2007)(Sharma *et al.*, 2012). They include four groups of following bacteria: *Acidithiobacillus ferrooxidans*, *Acidithiobacillus thiooxidans*, *Acidithiobacillus albertensis*, *Acidithiobacillus caldus* (Willner,

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http://dx.doi.org/10.13141/JVE
ISSN: 2193-6471

2012). *Acidithiobacillus ferrooxidans* and *Acidithiobacillus thiooxidans* are the well-studied representatives of all. These bacteria were usually found in both natural and artificial environments such as the mining area of metal ores. The groups are a Gram-negative, γ-proteobacterium, rod-shape, motile by flagella. Relevant grow temperature for them is 30^{o}C and pH is less than four, however, it can't be lower than one (Sharma *et al.*, 2012). All groups are autotrophic species which utilize inorganic compounds as energy sources. For example, *Acidithiobacillus thiooxidans* and *Acidithiobacillus ferroxidans* can oxidize inorganic sulphur compounds as sole energy substrate to grow both aerobically and anaerobically (Rzhepishevska, 2008). And A*cidithiobacillus ferroxidans* is the only group that can also oxidize Fe^{2+}.

Recent studies usually use a thiosulfate and a polysulfide as electron donors to increase the bioleaching of metal sulfides. The ore of metal sulfides is first degraded by chemical attack of Fe^{3+} and/or protons. Thiosulfate is the main intermediate degraded the disulfides pyrite (FeS_2), molybdenite (MoS_2), and tungstenite (WS_2). The result of thiosulfide oxidation generated H^+ (or sulfuric acid) that dissolute metal in mining waste. So, the bacteria involving in this process are important members of microbial consortia used to recover copper, zinc, lead, niken, gold, etc. via bioleaching (Valdés *et al.*, 2008)(Olson *et al.*, 1990).

In our study, we have conducted the sampling campaign to isolate the key players in bioleaching process in abandoned Tin mining area, at Ha Thuong commune, Thai Nguyen province. Our aims of this study were to isolate acidophilic bacteria and characterize their growth properties, which will be the underlining knowledge for further applications in mining or electronic wastes treatment.

2. Materials and method

2.1 Sampling sites

The total of 5 samples was collected from Ha Thuong tin mine (Latitude 105◦41'427'' E, Longitude 21◦38'015'' N). Among five samples, there are two soil samples from fern-farming, tailing and mining soils, one sludge sample from the wastewater pond, and two water samples from the wastewater pond and canal (table 1).

2.2 Isolation and identification of acidophilic bacteria

2.2.1 Isolation of acidophilic bacteria

The collected samples were enriched in mineral medium. The component of the mineral medium included KH_2PO_4: 3 g/L, $MgSO_4.7H_2O$: 0.5 g/L, $(NH_4)_2SO_4$: 3 g/L, $CaCl_2.2H_2O$: 0.25 g/L, $Na_2S_2O_3.5H_2O$: 5 g/L with the pH value adjusted to pH 4.4 ~ 4.7 by H_2SO_4 10N. After seven days of enrichment at 30^{o}C, the samples were transferred into new flasks of the mineral medium and incubated at 30^{O}C for 7 days without shaking. The enrichment protocol was repeated three times, and the presence of acidophilic bacteria in liquid medium was detected via the lowering of pH value, approximately pH of 2 or 3. The acidophilic bacteria were isolated by spreading enriched liquid medium to petri disk of the mineral medium agar. The colonies of bacteria on each petri disk were picked up and isolated.

2.2.2 Identification of acidophilic bacteria

Morphological identification: The isolated bacteria culture was collected by centrifugation at 6000 x g and washed four times by deionized water. Cell culture was stained by Gram's stain for identification (Manual of Clinical Microbiology 3ed) and observed at optical microscopy at 1000 x magnification (with oil).

Extraction and purification of bacterial genomic DNA: The bacterial cells harvested by centrifugation at 9000 x g were washed with deionized water. The total genomic DNA was purified by using Biobasic Canada kit. The total DNA was dissolved in 100µl 10 mM Tris HCl pH 8.0 and stored at 4^{o}C.

PCR amplification and sequencing of 16S rDNA gene: PCR amplification of the 16S rDNA gene was carried out to generate approximately a 1.5 Kb band on the electrophoresis using the forward primer FD1 (5' – AGAGTTTGACCTGGCTCAC – 3') and the reverse primer RP2 (5' – CGGCTACCTTGTTACGACTT – 3'). The PCR amplification was carried out with 25 µl of 2X Taq PCR Mastermix, 2 µl of 5 nM each of primers, purified DNA genome 20 ng/µl, and the reaction mixture made to a final volume 50 µl with deionised water in Mastercycler personal (Eppendorf, Germany). The conditions used for PCR were as following: predenaturation at 95^{o}C for 2 min, followed by denaturation at 95^{o}C for 25 seconds, annealing at 54^{o}C for 1 min, and extension at 72^{o}C for 70 seconds, in which thermal cycling was 35 cycles. Gel electrophoresis was used to test the PCR products by 1.2% dyed with Ethidium bromine agarose gel, the band was observed at λ 320nm. The product band was cut and purified by Quiaquick gel axtraction kit (Quiagen, Germany). The purified 16S rDNA was sequenced by Macrogen Inc (Korea) with the same forward and reverse primers in PCR. The obtained sequences were analyzed and aligned with other published sequences from Genbank by software Mega 6.06.

2.3 Growth conditions of isolated bacteria

2.3.1 pH condition

The isolated bacteria were cultured in 250ml flaks containing 200 ml mineral medium at pH: 2, 4 and 6 at room temperature (27^{o}C). The bacterial growth was monitored by sampling 5 ml of cultures everyday and measuring the optical density at the wavelength of 600 nm.

Table 1. Metal concentrations and pH values in the sampling sites

No	Label	Type of samples	pH	Zn (ppm)	Pb (ppm)	Cd (ppm)	Cu (ppm)
1	DBDX-HT	Soil	3.64	120.7	213.5	0.16	364
2	BCHC-HT	Sludge	3.15	99.9	384.5	0.25	1172.9
3	DHP-HT	Soil	2.61	107	135.5	0.2	357.8
4	NHC-HT	Water	2.8	2.796	0.134	0.0019	14.88
5	NKT-HT	Water	2.91	7.217	0.626	0.0062	13.771

2.3.2 Temperature condition

The isolated bacteria were cultured in 250 ml flaks containing 200 ml mineral medium at temperatures: 25^{O}C, 30^{O}C, 35^{O}C, 40^{O}C. The bacterial growth was monitored by sampling 5 ml of cultures everyday and measuring the optical density at the wavelength of 600 nm.

3. Results and discussion

2.1 Isolation and identification of acidophilic bacteria

After enrichment cultures, two strains of acidophilic bacteria was isolated from DBDX and NKT samples. The Gram's stain showed that two isolates (DBDX and NKT) belong to two different groups, the DBDX isolate was Gram-positive, other of NKT sample was Gram-negative (Figure 1). Although the image obtained at 1000 magnification in figure 1 are not clear enough to clarify whether the isolate from DBDX sample is spherical or rod shape, the isolate from NKT sample is rod shape.

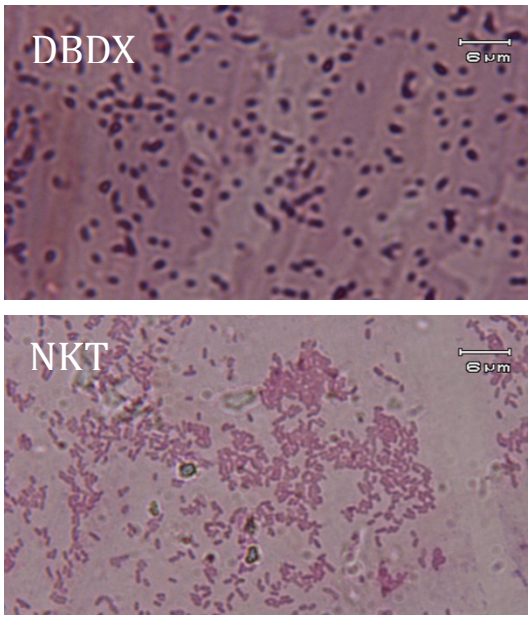

Figure 1. Images of Gram's stain isolates at optical microscopy

PCR products of NKT and DBDX isolates were checked by electrophoresis (Figure 2). The bands of 16S rDNA appeared at approximately 1.5 Kb as expected. The sequences of two isolates were used to construct the dendrogram with other similar sequences retrieved from GenBank (Figure 3). Based on the phylogenetic tree, two isolates are distinguished from each other. The strain DBDX is identical to that of the *Acidithiobacillus ferrooxidans* (98%) and the nucleotide sequences of 16S rDNA from NKT isolate was identical to that of the *Acidithiobacillus thiooxidans* (94%) from Genbank. The sequencing results confirmed the Gram's staining of the cells because *A. ferrooxidans* and *A. thiooxidans* are Gram-positive and negative bacteria, respectively. The two bacteria are phylogenetically different.

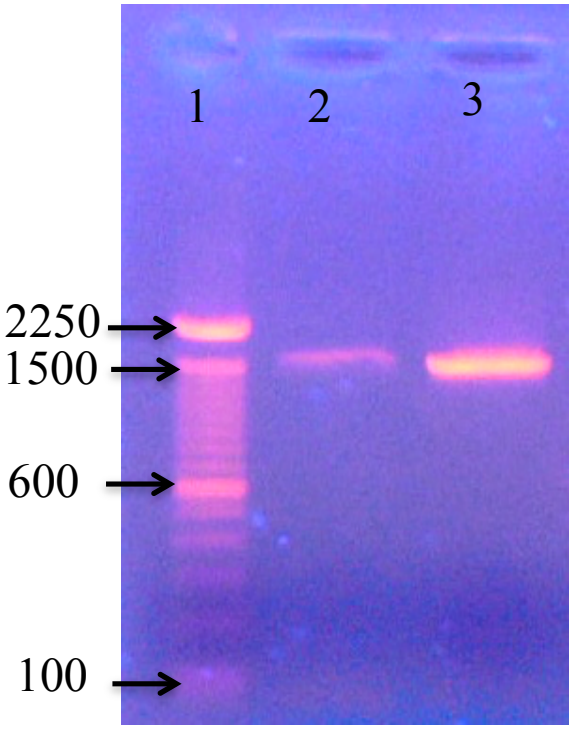

Figure 2. The image from the electrophoresis of the 16S rDNA PCR amplification, 1: NKT and 2: DBDX

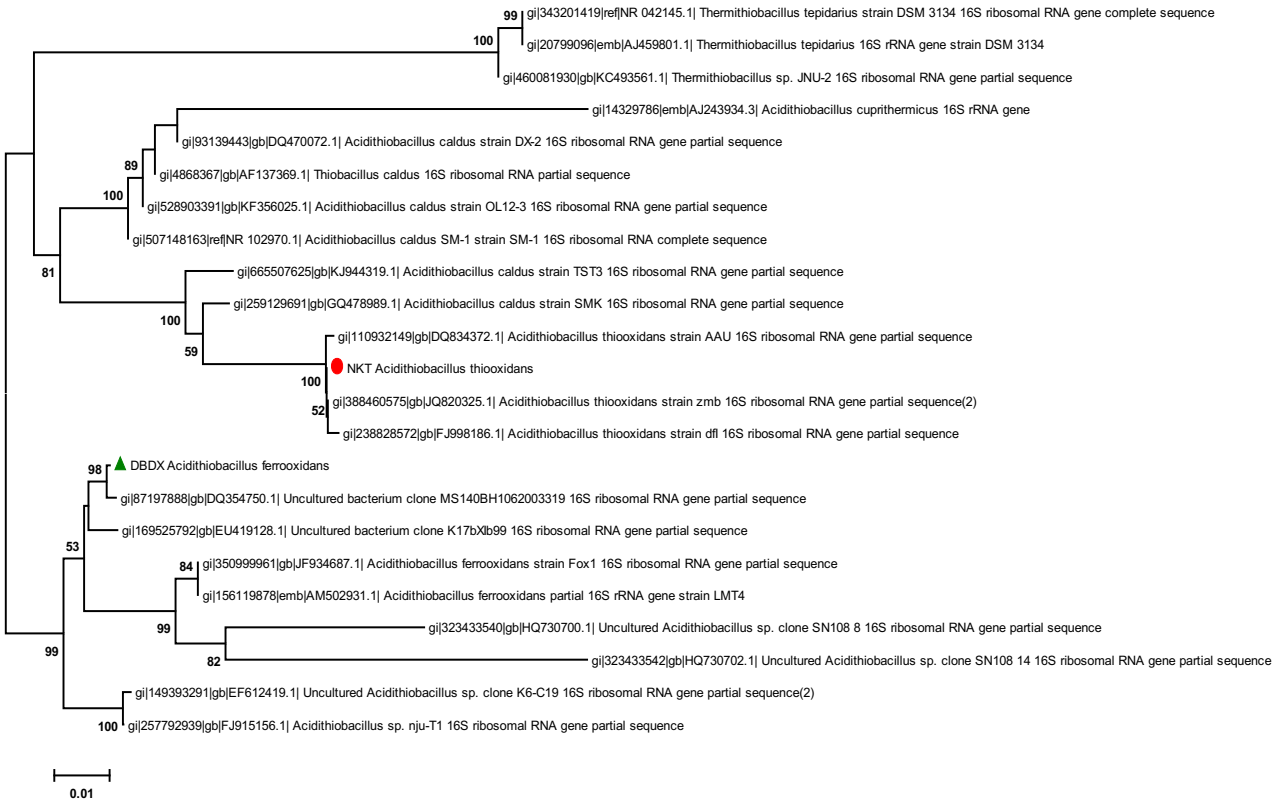

Figure 3. Phylogenetic tree of two strains (NKT and DBDX) based on the sequences of 16S rDNA

2.2 Effect of pH values on the growth of two strains

Figure 4 showed that the optimal pH value for the growth of two isolated strains was at pH 4 after five day incubation at room temperature. The growth of both strains was low at pH 2 and 6. The data on the figure 4 also demonstrated that the NKT strain was more sensitive to high pH value than the DBDX strain. The growth curves of two isolates also supported that the DBDX strain biomass (optical density (OD)) still increased at day 6 whereas the NKT strain had a biomass decrease (data not showed).

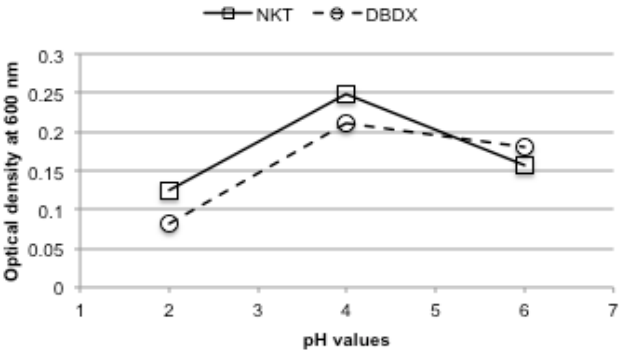

Figure 4. The effect of pH on the OD of two strains at different pH values at after five day incubation

It is interesting that the two strains grew slowly at low pH (pH 2) while the acidophilic bacteria can perform bioleaching of metals best at pH lower than 2 (Zhou *et al.*, 2007)(Bayat *et al.*, 2009)(Gao *et al.*, 2007). Zhou *et al.* (2007) reported the isolate from coal heap drainage grew optimally at initial pH 2 and its 16S rDNA gene sequence was more than 99% similar to *Acidithiobacillus caldus*. When isolating acidophiclic bacteria from an extreme acid drainage mine site, Gao *et al*., (2007) found and identified a strain of *Leptospirillum ferriphilum* that has an optimal growth at the pH of 1.6. In addition, the bacteria *Acidithiobacillus ferrooxidans* can leach Zn at maximum rate at the pH of 1.3. Our two strains were isolated from the samples with the pH around 3 (table 1), thereby adapting to the pH of 3, however, the pH of medium can reach at the pH of 2 after seven days of incubation.

2.3 Effect of temperature on the growth of two strains

Two strains of isolated acidophilic bacteria were inoculated in the mineral medium and incubated at different temperatures, ranging from 25 to 40^{o}C. Figure 5 demonstrated the result of the growth of two strains after five day incubation. The result clearly pointed out that two strains are not tolerant to high temperature, the optimum temperature is around 30^{o}C.

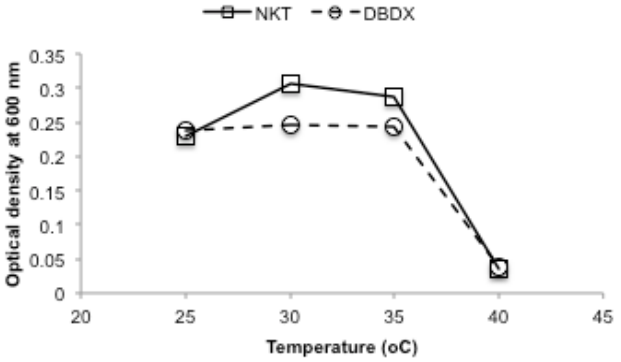

Figure 5. The effect of temperature on the OD of two isolated strains growing at different temperatures after five days of incubation

Hallberg *et al*. (2011) investigated in a comprehensive physiological and phylogenetic characterization of *Acidiferrobacter thiooxydans*, an acidophilic, thermo-tolerant, facultatively anaerobic iron- and sulfur-oxidizer of the family Ectothiorhodospiraceae and reported the optimal temperature for this bacteria was 38^{o}C and the maximal tolerance temperature was 47^{o}C. However, the bioleaching of Zn by *Acidithiobacillus ferrooxidans* was normally carried out at the temperature of 30^{o}C (Bayat *et al.*, 2009).

4. Conclusions

The isolation of acidophilic bacteria was successful with two new strains NKT and DBDX, the NKT strain can be identified as *Acidithiobacillus sp.*and the DBDX strain as *Acidithiobacillus ferrooxidans*. Both strains possess the different physiological properties in comparison with other *Acidithiobacillus* bacteria, low tolerance to temperature and pH values, implying the new species.

5. Acknowledgement

This research is funded by the Vietnam National Foundation for Science and Technology Development (NAFOSTED) under grant number 106.16-2011.65 and the program "Young scientist's fund" from Vietnam Academy of Science and Technology.

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