ISOLATION AND GENOTYPIC IDENTIFICATION OF*ACIDITHIOBACILLUS FERROOXIDANS*

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Abstract

In this study, bioleaching of mineral pyrite ore collected from Moehti Moemi mining area, Yamethin Township, Mandalay Region was conducted by using *Acidithiobacillus ferrooxidans*. Two Soil samples were collected from two sampling areas, (Sakhangyi, SKG and Shwesin, SHN mining sites), Moehti Moemi, Yamethin, Mandalay Region. *A. ferrooxidans* was isolated from these two soil samples. The principal objective of the present investigation was to isolate and identify the acidophilic sulphur oxidizing *A. ferrooxidans* and to grow the isolates on selected 9K medium. The most commonly studied pyrite oxidizing bacteria are thermophilic *A. ferrooxidans*, which oxidizes ferrous iron and sulphur. The isolated bacteria were identified by phenotypic and genotypic identifications. In phenotypic identification, twelve biochemical tests such as motility test, hydrogen sulphide test, catalase test, urease test, starch hydrolysis test, indole test, methyl red test, gelatin liquefaction test, Voges- Proskauer test, nitrate reduction test, citrate utilization test and triple sugar iron test were performed. In genotypic identification, *A. ferrooxidans* was detected and confirmed by using specific primers for 16S rDNA PCR.

Keywords: *Acidithiobacillus ferrooxidans*, 9K medium, phenotypic identification, genotypic identification

Introduction

Acidithiobacillus ferrooxidans oxidizes iron(II) and recovers metals from low-grade sulphide ores and catalyses the oxidation of sulphur compounds to sulphuric acid. The acidiphilic microorganisms that take part in dissolution of metals from the sulphide ores are autotrophic in nature and can grow in inorganic medium having low pH values and can tolerate high metal ion concentrations. *A. ferrooxidans* species are rod-shaped, gram-negative, non-spore forming and thermophilic (Rawlings, 2002). These microbes have a number of features in common including; (a) they grow autotrophically by fixing CO_2 from the atmosphere, (b) they obtain their energy by using either ferrous iron or reduced inorganic sulphur compounds (some use both) as an

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electron donor, and generally use oxygen as the electron acceptor, (c) they are acidophiles and grow in low pH environments, (d) they are remarkably tolerant to a wide range of metal ions (Tuovinen, 1971). *A. ferrooxidans* have been isolated from different sources, most of the strains showed the following optimum growth conditions, i.e. pH 1.5-6 and a temperature range of 28–37 ^{o}C. Cells of *A. ferrooxidans* is ranging in length from 0.9 to 2 μm (Rawlings, 2002).

Bioleaching, a general term refers to the conversion of an insoluble metal into a soluble form by biological oxidation and by applying microbes. Metals for which this technique is mainly employed for recovery includes, copper, cobalt, nickel, iron, sulphur, zinc and uranium. For recovery of gold and silver the activity of leaching bacteria is applied only to remove interfering metal sulphides from ores bearing the precious metals prior to cyanidation treatment. The application of bacterial leaching to metal recovery from mineral ores has progressed steadily in the last 20 years (Rohwerder *et al*., 2003).Bioleaching has improved the efficiency of the mineral procession industry by lowering of the overall capital and procession costs and by diminishing environmental concerns associated with the pollution derived from emission of smelting operations (Quatrini and Holmes, 2005).

In the last few years, several molecular techniques for typing of *A. ferrooxidans* have been developed using PCR methodology such as 16S rDNA analysis. Many techniques are used to characterize and monitor microbial population. Molecular techniques have been employed to interrogate natural environment with great success. Many of these techniques have relied on the use of DNA sequence information. The polymerase chain reaction (PCR) has shown promise in detecting the presence of bioleaching microbes. The use of PCR allows for the detection of a specific organisms or groups. The 16S rDNA PCR used in this study is a rapid and specific tool for identification of microorganisms (Ilieva*et al*., 2011). In the present work, *A. ferrooxidans* were isolated from two soil samples collected from Moehti Moemi, Yamethin Township, Mandalay Region. It was characterized by using morphology and biochemical tests according to Bergey's Manual of Systematic Bacteriology (Bergey and Gibbons, 1974) and identified by PCR method using 16S rDNA analysis.

Materials and Methods

Collection of Soil Samples

Two soil samples from Sakhangyi(SKG) and Shwesin (SHN) were collected from Moehti Moemi, Yamethin Township, Mandalay Region. The collected soil samples were dried in air, ground and sieved through 200 mesh size and pH of the soil samples were determined.

Isolation of *A. ferrooxidans*

A. ferrooxidans was cultured on selected 9K medium, which mixed with solution A and solution B (Silverman and Ludgren, 1959). Solution A was prepared by adding 0.3 g of $(NH_4)_2SO_4$, 0.01 g of KCl, 0.05 g of K_2HPO_4, 0.05 g of ($MgSO_4$) $7H_2O$ and 0.001 g of $Ca(NO_3)_2$to 100 mL of distilled water. Solution B was prepared by 2.2 g of $FeSO_4$ in 50 mL of distilled water. In order to cultivate *A. ferrooxidans*, 50 mL of solution B was added to the prepared media. Two soil samples containing 9K medium were placed on a rotary shaker with 200 rpm for 8 days. After 8 days, bacteria were taken with a sterilized inoculation loop from the two conical flasks (9K medium) and streaked in Glucose Yeast Beef (GYB) medium plate by streak plate method. Then, it was incubated in an incubator at 37^{o}C for 7 days. After 7 days, single colonies of *A. Ferrooxidans* species were grown on GYB plate. Next, single colonies of *A. ferrooxidans* species were transferred to nutrient agar medium for culturing the pure colonies (Silverman and Ludgren, 1959). Five strains (two strains from SKG soil sample and three strains from SHN soil sample) of *A. ferrooxidans* subcultured on nutrient agar medium were stored in an incubator and transferred weekly for further studies.

The present research work was conducted at Myanmar Pharmaceutical Research Department and Microbiology Laboratory Department of Zoology, West Yangon University.

Phenotypic Identification of the Isolated *A. ferrooxidans*

The bacteria were identified by morphological examinations, gram staining and biochemical tests (Cruickshank, 1968).After confirming by microscopic examination, the isolated bacteria were identified by phenotypic method. Twelve biochemical tests such as motility test, hydrogen sulphide test, catalase test, urease test, starch hydrolysis test, indole test, methyl red

test, gelatin liquefaction test, Voges-Proskauer test, nitrate reduction test, citrate utilization test and triple sugar iron (TSI) test were conducted in accordance with Bergey's Manual of Determinative Bacteria. From phenotypic identification, among five isolated bacteria, one from SKG and one from SHN were identified as *A. ferrooxidans* (Shahroz *et al*., 2012).

Genotypic Identification of the Isolated *A. ferrooxidans*

The two selected bacteria were also characterized by genotypic identification. These bacteria were cultured in nutrient broth (NB) and laurain broth (LB) and allowed to stand for overnight. DNA was extracted from two A. *ferrooxidans*. Genomic DNA extraction was done by purelink genomic extraction kit according to protocol.DNA yield was checked by electrophoresis in 1 % agarose gel (35 min at 135 V and 60 mA) stained with 5μL dye (bromophenol blue and xylene cyanol). *A. ferrooxidans* was detected by using specific primers for 16S rDNA PCR. The nucleotide sequences of the primers used in PCR reactions for *A. ferroxidans* were F 5′ -AGA-GTT-TGA-TCM-TGG-CTC-AG-3′ and R 5′ -CGG-TTA-CCT-TGT-TAC-GAC-TT-3′ . Amplification conditions were carried out using Dream Taq PCR master mix, according to protocol. The volume of the reaction mixture was 25μL. The PCR program consisted of one cycle of DNA denaturation at 94 ^{o}C for 3 min, primer annealing at 50 ^{o}C for 3 min and a final extension at 72 ^{o}C for 8 min for 35 cycles gave a large number of products. The reaction were carried out in an Eppendorf Thermocycler. PCR products were separated by 1 % agarose gel and electrophoresis stained with 5 μL gel stain. Bands were visualized by illuminating ethidium bromide (Grigorii *et al*., 2003).

Results and Discussion

Sampling Sites

In this study**,** two soil samples: Sakhangyi (SKG) and Shwesin (SHN) collected from Moehti Moemi, Yamethin Township, Mandalay Region (Figure 1) were used to isolate *A. ferrooxidans*. The SKG soil was found to be moderately acidic (pH 5.7) and SHN soil was slightly acidic (pH 6.4).

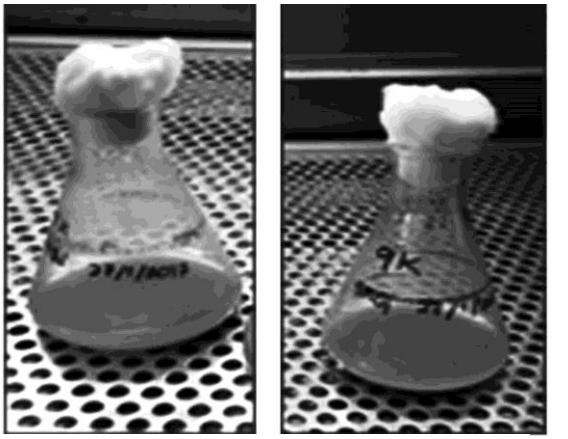
Figure 1: Location of Moehti Moemi Gold Mine in Yamethin Township, Mandalay Region

Isolation of *A. ferrooxidans*

For isolation of acidophilic iron and sulphur-oxidizing bacteria(*A. ferrooxidans*), the collected two soil samples: SHN and SKG were activated in 9K acid medium. After shaking for 8 days, the cultured medium was observed by a change in colour to turbid brown (SHN soil sample) and reddish brown (SKG soil sample) due to the oxidation of Fe^{2+} to Fe^{3+} (Figures 2 and 3).

(a) SHN Soil (b) SKG Soil

Figure 2: Activation of soil samples in 9K acid medium (initial state)



(a) SHN Soil (b) SKG Soil

Figure 3: Activation of soil samples in 9K acid medium (after 8 days)

It was streaked onto Glucose Yeast Beef medium (GYB). After 7 days of incubation at 37 °C, pale-yellow colonies of iron-oxidizing bacteria were developed on the GYB plate.

These pale-yellow colonies growing on GYB plates were picked, examined with microscope and cultivated separately on nutrient agar medium. Such ordinary procedures were repeated several times, finally pure cultured were obtained (Shahroz *et al*., 2012). From SKG soil samples, two strains of bacteria (SKG 1, SKG 3) and three strains of bacteria (SHN 2, SHN 4, SHN 5) from SHN soil sample were isolated.

Cell Morphology and Characterization of the Isolated Strains

According to microscopic observation, the five isolated strains (SKG 1, SHN 2, SKG 3, SHN 4 and SHN 5)were found to be motile and single rod-shape bacteria in pale yellow colour (Figure 4).

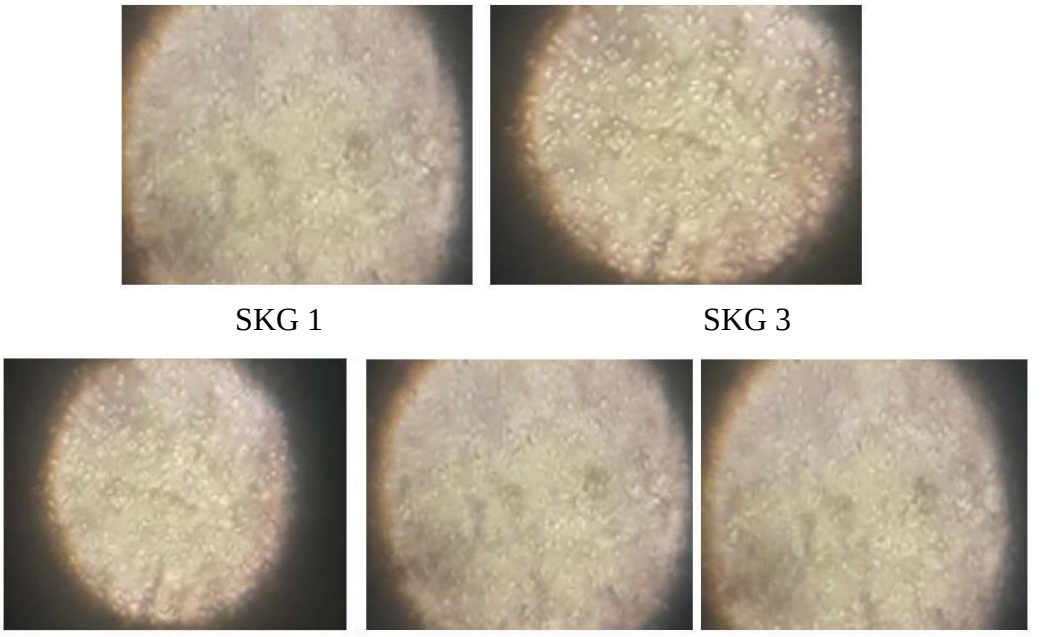


Figure 4: Morphology of five isolated bacterial strains

After the isolation of pure culture from nutrient agar medium, the bacteria were individually stained with 1 % crystal violet solution, gram's iodine solution, neutral red solution and then examined microscopically. It is the preliminary identification ofthe isolated strains. The strains gave red colour indicating to be Gram-negative.

Phenotypic and Genotypic Identification of *A. ferrooxidans*

Afterthegram's staining, different biochemical properties of the isolated bacteria were studied by motility test, hydrogen sulphide test, catalase test, urease test, starch hydrolysis test, indole test, methyl red test, gelatin liquefaction test, Voges-Proskauer test, nitrate reduction test, citrate utilization test and triple sugar iron test (TSI test) (Shahroz *et al*., 2012). In motility test, the growth of all organisms going out away from the stab line with surrounding medium was clearly transparent. So, all of the organisms gave positive results. In hydrogen sulphide test, black ferrous sulphide was not found so the bacteria can not produce hydrogen sulphide gas. The absence of

black precipitate was the sign of negative result. In the case of catalase test, formation of oxygen gas bubble were observed. This was positive in catalase test while the absence of bubble formation was a negative catalase test (Macfaddin, 2002).

In urease test, the organisms were grown in the urea broth medium containing the pH indicator phenol red (Jang *et al*., 1980). The colour did not turn into deep pink showing negative result. If the colour turned into deep pink it showed positive result (Cruickshank, 1968). In starch hydrolysis test, the medium colour was blue-black indicated the absence of starch-splitting enzymes and represented a negative result and a clear zone of hydrolysis around the growth of organisms, showed a positive result. In indole test, the absence of red colouration demonstrated the substrate tryptophan was not hydrolysed, giving negative result (Jang *et al*., 1980).

In methyl red test, the addition of methyl red caused the medium remained red indicating positive result. If the medium turns yellow it shows negative because pH of the medium increases. In gelatin liquefication test, the medium remained solid on refrigeration at 4 ^{o}C indicating the lack of gelatinase. In this test, the bacteria was not able to hydrolyse gelatin because the medium remained solid so gelatin liquefication test gave negative result. In the case of Voges-Proskauer test, it gave cherry red colour indicating positive result. In the study of nitrate reduction test, the colour did not turn into red indicating negative result. In citrate utilization test, the colour did not change which indicated that the citrate was not used as a carbon source and the result was negative. In triple sugar iron test (TSI test), the colour change showed the carbohydrate fermentation had not taken place and the result was positive (Shahroz *et al*., 2012).

According to the result from physiological, morphology and microscopic examinations, five isolated bacteria (SHN 2, SHN 4, SHN 5, SKG 1 and SKG 3) were selected. In biochemical test results, it was observed that bacterial strains SHN 2 and SKG 3 were generally in agreement with theoretical characters of *A. ferrooxidans*. The cell lengths of these bacteria were respectively found to be 1.2 µm and 2 µm. The results of biochemical tests for five isolated bacteria strains are described in Table 1.

Table 1:Results of Five Isolated Bacterial Strains by Biochemical Tests

Biochemical Tests	Observation	Results of observation of five isolated bacteria					*A. ferrooxidans*
		SKG 1	SHN 2	SKG 3	SHN 4	SHN 5	
Motility test	Growth of organisms	-	+	+	+	-	+
Hydrogen sulphide test	No precipitate	-	-	-	-	-	-
Catalase test	Bubble of O_2 gas	+	+	+	+	-	+
Urease test	No deep pink colour	+	-	-	+	-	-
Starch hydrolysis test	Blue to black	-	-	-	+	+	-
Indole test	No red colouration	-	-	-	-	-	-
Methyl red test	Yellow colour	+	-	-	+	+	-
Gelatin lique-Faction test	Remain solid	+	-	-	+	+	-
Voges-proskauer test	Red layer	+	+	+	+	+	+
Nitrate reduction test	No colour change	+	-	-	-	+	-
Citrate utilization test	No colour change	+	-	-	-	-	-
Triple sugar iron test	Colour change	+	+	+	+	+	+

(+) = positive, (-) = negative

* Shahroz*et al.,* 2012

The two isolated bacteria SHN 2 and SKG 3 were so further identified by partial nucleotide sequence of 16S rDNA. Amplification conditions were optimized using genomic DNA from pure cultures of two isolated strains. Total DNA fragments amplified from the genomic DNA of base pairs were revealed with the previous study (Ilieva *et al*., 2011).All of the base pairs were observed to be above the 950 level (Figure 5).

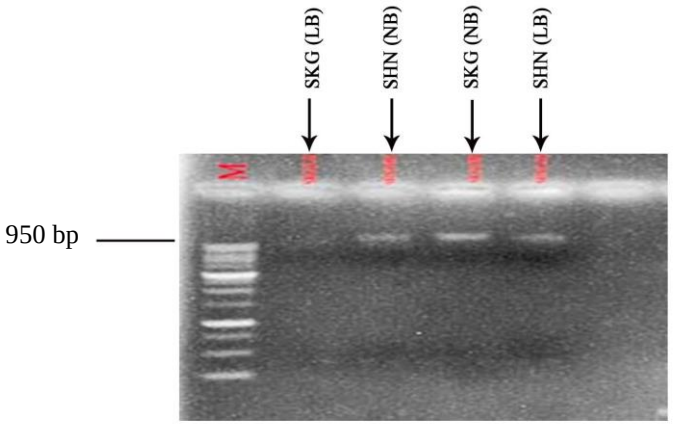


Figure 5:DNA fragments amplified from the genomic DNA of two isolated bacteria(SHN 2 and SKG 3)

Thus, the preliminary study of genomic DNA confirmed the isolated bacteria to be *A. ferrooxidans*. PCR was performed on the thermocycler. PCR under low stringency conditions gave a large number of products and several of which were of the anticipated size approx: 100 bp. This produced a few bands after gel electrophoresis. Each band were correspondedin size to the expected product of 118 bp which contained the desired sequences (Figure 6). PCR with 5′ -AGA-GTT-TGA-TCM-TGG-CTC-AG-3′ and R 5′ -CGG-TTA-CCT-TGT-TAC-GAC-TT-3′ primers gave single product of sizes upper the 100 bp (John *et al*., 1996). This is the first step in the molecular sequencing for the species identification. And also, this provide further impetus to study the commerical application of *A. ferrooxidans* in bioleaching process.

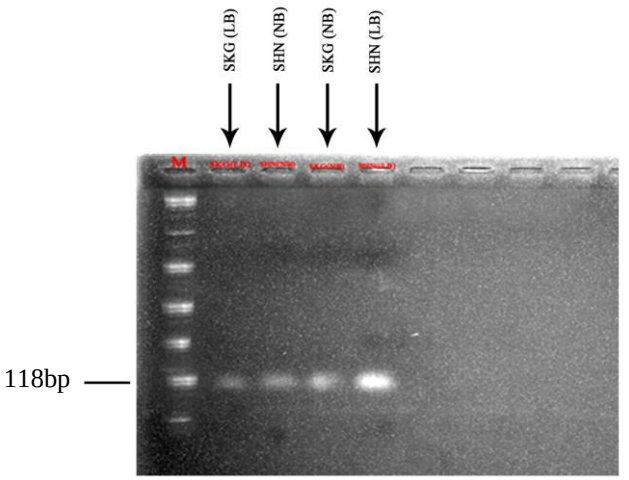


Figure 6: 16S rDNA amplification by PCR of two isolated bacteria (SHN 2 and SKG 3)

Conclusion

The results indicated that *A. ferrooxidans* was isolated from two soil samples collected from Sakhangyi and Shwesin mining sites, Moehti Moemi, Yamethin Township, Mandalay Region by 9K basal salt medium. The isolated *A. ferrooxidans* was identified phenotypically and genotypically. A. *ferrooxidans* is the main microbial species associated with the bioleaching process. Bioleaching is based on the ability of microorganisms to extract metal. The advantages of this technique are low cost, high efficiency and environmental friendliness.

According to molecular characterization, amplification conditions were optimized using genomic DNA from pure culture of the isolated bacteria (SHN 2 and SKG 3). The electrophoretic analysis of the PCR products showed that the size of the fragments amplified from the isolated bacteria matched with 118 bp. Also, no other amplification band was observed, which demonstrated the specificity of the chosen primers pairs. According to the result of this analysis, this extraction of genomic DNA could be done for molecular characterization and detection of sequencing for identification of *A. ferrooxidans*.

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