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2016 IOP Conf. Ser.: Mater. Sci. Eng. 136 012088

(http://iopscience.iop.org/1757-899X/136/1/012088)

Isolation and Identification of Concrete Environment Bacteria

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Abstract. This paper presents the isolation and molecular method for bacteria identification through PCR and DNA sequencing. Identification of the bacteria species is required in order to fully utilize the bacterium capability for precipitation of calcium carbonate in concrete. This process is to enable the addition of suitable catalyst according to the bacterium enzymatic pathway that is known through the bacteria species used. The objective of this study is to isolate, enriched and identify the bacteria species. The bacteria in this study was isolated from fresh urine and acid mine drainage water, Kota Tinggi, Johor. Enrichment of the isolated bacteria was conducted to ensure the bacteria survivability in concrete. The identification of bacteria species was done through polymerase chain reaction (PCR) and rRDNA sequencing. The isolation and enrichment of the bacteria was done successfully. Whereas, the results for bacteria identification showed that the isolated bacteria strains are *Bacillus sp* and *Enterococus faecalis*.

Keywords: Bioconcrete**,** effective microorganism, enrichment, 16S rDNA gene sequencing.

1. Introducion

Bacteria is a living micro-organism that are everwhere. From the smallest grain of soil to the small drop of water. Bacteria can also be found in organic matters such as in live bodies of plants and animals. These living micro-orginisms are known to live in harsh environemts and thrive in these living conditions [1]. The bacteria species isolated from different parts of the world and different environmet have unique characteristics. The uniqueness of these bacteria are slowly being discovered and used to improve problems of human kind. Some groups of bacteria have capabality in improving crops, health, degraded organic contaminants and others. An attempt of bacteria in concrete able to improve concrete properties and healing process. Concrete is an important building material. However, the drawback of this material is the deterioration in which intervention in terms of chemicals are normally used. The chemicals are used to repair cracks, reduce water permeability of concrete, early hardening and many more. [2] had stated in his study that bacteria are able to promote preciptation of calcium carbonate in the form of calcite. The

formation occurs as a by-product of a common microbial metabolic process which help in increasing alkalinity and produce microbial calcite precipitation. Many reseearchers added bacteria into concrete to allow precipitation of calcium carbonate within the concrete to improve its properties [3,4,5]. The bacteria used by previous researchers were isolated from local sources of temperate countries that different in environmental factors. Therefore, this study aims to isolate, enriched and identify a local source of bacteria which has potential to improve concrete properties.

Bacteria identification based on 16S ribosomal gene rDNA sequencing and polymerase chain reaction (PCR) is a popular technique of bacteria identification method [6,7,8,9,10]. This method for bacteria identification is the preferred method due to its speed in obtaining results and accuracy. The use of 16S rDNA gene sequencing are more commonly being employed in rountine laboratory diagnostics particularly after adopting PCR in biomedical science [10].

According to [9], PCR based method is a cheaper and more faster method to identify bacteria, the PCR typing assays is design from genome sequence data which uses the concept of observing variation in gene content of strains within a given species.

2. Experimental Procedure

2.1 Bacteria isolation and enrichment

2.1.1 Isolation

The bacteria used in this study are isolated from fresh urine and acid mine water, Kota Tinggi, Johor, Malaysia. Samples from acid mine water was taken over the dry season to avoid auto dilution on the sample via rainfall. Sample was collected from the discharge point of the mining retention pond. A PTFE bottle was used in order to keep the sample collected. To ensure that the sample collected is maintained at the right condition, the PTFE bottle containing the sample was kept in a box with ice cubes before being transported back to the lab. Upon arriving at the lab, the sample was kept at 4^0C until enrichment process. Bacteria isolated from fresh urine was temporarily name *Ureolytic bacteria* while bacteria isolated from acid mine water was named *Sulphate reduction bacteria*. The isolation process of both *ureolytic* and *sulphate reduction bacteria* (SRB) were conducted following a process of enrichment, serial dilution streaking plate, strain purification and gram staining. Isolation and purification method of bacteria was done according to [11]. All process and media used were autoclave at 121^0C for 15 minutes for sterilization.

2.1.2 Enrichment

The enichment of bacteria was conducted after the bacteria were isolated. The enrichment process is conducted to ensure the bacteria survivability in concrete. The process involves adding bacteria into media composition. A control flask without addition of urine and acid mine water were also prepared for ureolytic and sulphate reduction bacteria respectively. Composition of *sulphate reduction bacteria sample* (SRB) consists of 25 ml (nutrient broth) added with 10 ml (Mg $SO_4.7H_2O$) and 1 ml (water from Sg pelepah Kota Tinggi). U*reolytic bacteria* composition including 25 ml (nutrient broth), 10ml (urea 40%) and 1 ml (urine). Two environmental condition namely pH and oxygen concentration were controlled in the isolation process. Realising that concrete environment is a high alkaline environment, the pH value of the isolation flask is maintained in alkaline condition for ensuring self survival of the isolated strains. The high pH value contributes to high urease activity which leads to high carbonate precipitation rate [12]. The pH of each sample were adjusted to an alkaline condition by adding NaOH until the pH value reaches the range of 9-11.

Oxygen concentration in the isolation flask was monitored to be in anaerobic condition. This is to ensure only anaerobic or facultative bacteria will be isolated. Adjusment of the oxygen is needed due to lack of oxygen in concrete material. Nitrogen gas was purged in the enrichment

flask before enrichment started to remove the remaining oxygen in the flask. The concentration of oxygen was monitored with a dissolved oxygen meter to ensure very low oxgen or 0 ppm oxygen.The flask was shaked at room temperature. The adjustment of pH and anaerobic condition were done every day over the enrichment period of 20 days. The set-up of the enrichment flask as shown in Fig.1

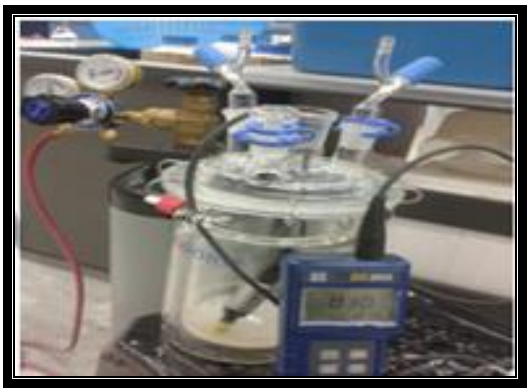

Figure 1. Enrichment flask.

2.1.3 Dilution and Pure Streaking Plate Method

Along 20 days of enrichment, the flask with sample will turn turbid. Every 10 days 0.1 ml of the culture was taken and added to 9.9 ml of sterile distilled water. The serial dilution process was done for both samples using yeast extract and sulphate API agar which were prepared for *Ureolytic* and *Sulphate reduction bacteria* respectively. Each of the dilution was aseptically plated on yeast extract and sulphate API agar which were appropriately labeled. The plates were incubated at 37^0C in the incubator until the desired strain growth. Colonies appeared were picked up and cultured. Fig. 2 illustrates agar with colonies of bacteria.

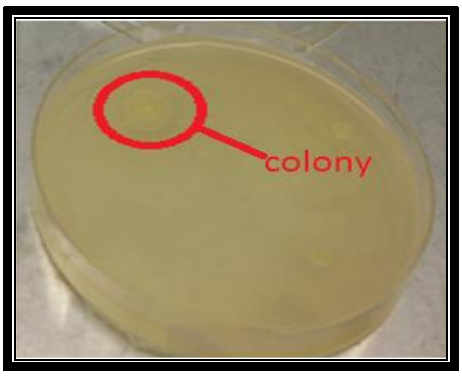

Figure 2. Plate with colony.

2.1.4 Strain Purification and gram staining

Purification step was performed until pure strain was obtained. Purification involves a streak plate method was done to ensure only single strain obtained in each plating. Gram staining is used to investigate the major categories of microorganism or examine the morphology. In this study simple stains including crystal violet, iodine and safranin were applied. Two groups of bacteria can be distinguished with gram staining. Using this method, gram positive bacteria cell wall will retain crystal violet while gram negative change.

2.2 Bacteria identification process

2.2.1 Preparation of Lysogeny Broth

The bacteria sample is first inoculated into Lysogeny Broth (LB) and incubated for 18 hours with temperature of 37º C and 400 RPM. The samples were then centrifuged to collect pellet for gDNA extraction.

2.2.2 Genomic DNA isolation of bacteria

The DNA extraction of the bacteria used in this study was done based on modified phenol-chloroform protocol. Based on this method, 1.5ml of bacterial cell suspensions was centrifuged at 13,000 RPM, 4ºC for 2 min.The bacterial pellets were resuspended with TEN extraction buffer (0.1M NaCl, 10mM Tris-Cl; pH 8.0, 1mM EDTA; pH 8.0). They were then mixed by gently pipetting up and down till homogenized. RNase (10mg/ml) was added to the tube followed by the addition of lysozyme (50µg/ul). The tubes were gently inverted and incubated at 37°C for 30 min. After that, TENST buffer (0.1M NaCl, 10M Tris-Cl; pH 8.0, 1mM EDTA; pH8.0, 0.12% TritonX-100, 1.6% N-lauryl sarcosine) was added to the tubes followed by further 1 hour incubation at 37°C. Proteinase K (10mg/ml) was later added, followed by incubation at 55°C for 1 hour. The almost clear suspensions were treated with 15% SDS prior to further 30 min incubation at 55°C. An equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) was added and mixed gently followed by centrifugation at 13,000 RPM, 4°C; for 5 min. The supernatants basically, the clear aqueous layer was transferred to sterile 1.5ml tubes. This processes were repeated until the supernatants were clear without any white protein precipitate. The recovered supernatants were added to an equal volume of chloroform/isoamyl alcohol (24:1) with careful mixing prior to centrifugation at 13,000 RPM, 4°C; for 5 min. The clear aqueous layers were transferred to new tubes. 0.1 volumes of sodium acetate were added to the supernatants followed by the addition of absolute ethanol and DNA precipitation is allowed to take place overnight at -20°C. DNA was pelleted by centrifugation at 13,000 RPM, 4°C; for 10 min and the supernatants were discarded. This is then followed by 70% ethanol wash, recentrifuged at 13,000 RPM, 4°C; for 5 min, discarded the supernatants and air dried. The dry DNA pellet was resuspended with Tris-HCl, pH 8.0 buffer and kept overnight at -20°C.

3. Results and Discussion

3.1 Bacteria

The bacteria strains were successfully isolated and enrichment process was conducted. The bacteria was purified by conducting serial dilution streaking plate. The pure strain of bacteria obtained was analyze by gram staining. The Gram staining results obtained showed that both bacteria was gram positive. Gram positive bacteria denoted that bacteria remain coloured with gram staining even after washing with alcohol or acetone. The characteristic for Gram positive bacteria is absent of outer membrane, 20-30mm thick cell wall, the wall contains 70-80 % murein and teichoic acids. The Gram-positive lineage of bacteria are species which have a common phylogeny and cell wall structure, among the many Gram positive bacteria are *Bacillus, Clostridium, Lactobacillus, Mycoplasma* species.

3.2 DNA Quantification of bacteria

DNA quantification was quantified spectrophotometrically using a Nanodrop 2000 spectrophotometer (Fisher Scientific). Absorbance measurements were taken at 260nm and 280nm and used to calculate the DNA purity and concentration. DNA samples were considered free from protein contamination if the A_{260} to A_{280} ratio was between 1.8 and 2.0. The result of the quantification is in Table 1.

Table 1. The concentration and the quality of DNA using Nanodrop.

No.	Sample ID	Nucleic Acid Conc.	Unit	260/280	260/230
1	S	165.1	ng/µl	1.85	2.15
2	U	159.7	ng/µl	1.84	2.12

3.3 Polymerase Chain Reaction (PCR)

PCR primers have been employed to confirm the presence or absence of microorgisms.Primers which are used in both directions;forward and reverse such as in this study are longer primers. This forward and reverse primers sequence are designed based on the short general primers. The PCR reaction condition was a 10X PCR Buffer, 25 mm $MgCl_2$, 2.5 mm dNTPs, 10 µmol each primer (27F; 1492R), 5U Taq DNA polymerase. Universal 16SrDNA PCR was performed with forward and reverse primers, which are 27 (forward primer)5'-AGAGTTTGATCMTGGCTCAG-3' and 1492 (reverse primer) 5'-GGGTTACCTTGTTACGACTT-3'. After 35 cycles consisting of denaturation at 95^{o} C (30 Seconds), Annealing at 50^{o} C (30 Seconds) and extension at 72^{o} C (1 min and 30 seconds), the PCR product is analyzed on an agarose gel. A band indicating a fragment was shown by agarose gel electrophoresis. A PCR product of about 600 kb in size was successfully amplified from the two samples. The agarose gel product is as shown in Figure 3.

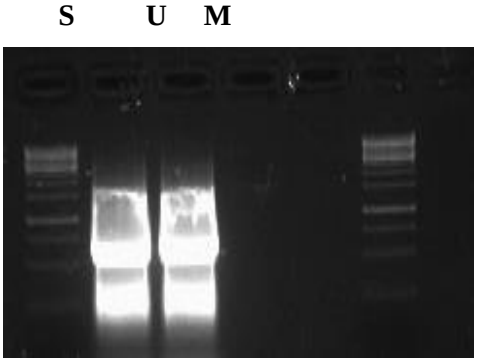

Figure 3. Agarose gel of PCR products.

3.4 Molecular Identification

After purification of the PCR product using the Qiagen PCR Purification Kit.Identification. The DNA isolated. Analysis of DNA sequencing was performed using the BLASTX software (BLAST), National center for biotechnology information. Both DNA sequences for sulphate reduction and ureolytic bacteria resulted to tree of phylogenetic as presented in Figure 7 and 8. The Figures will result to the most promising strains.

3.5 DNA sequences for bacteria

The DNA sequences for both *sulphate reduction bacteria* and *ureolytic bacteria* are provided in 3.5.1 and 3.5.2. The basic unit of DNA structure is nucleotide, composed of phosphate, deoxyribose sugar and a nitrogen base. Each deoxyribose sugar bonds convalently in a repeating pattern with two phosphates, One of the bond is to number 5' (five primer) another bond is to 3' carbon on deoxyribose. This specifies the order and direction of each strand. The nitrogen bases, purines and pyrimidines attach by covalent bonds at the 1' position of the sugar. Pairing of purines and pyrimidines is dictated by the formation of hydrogen bonds.Thus DNA sequence, the purine Adenine (A) pairs with pyrimidine Thymine (T) and the purine Guanine (G) pairs with pyrimidine Cytosine (C) [13]. These the bases that made up a strand of DNA. The DNA sequence is then compared to similar or any other DNA sequences by uploading the sequence to BLAST

programs, which is a program that is supported by the National Center for Biotechnology Information (NCBI).

3.5.1 DNA Sequences for Sulphate Reduction bacteria

GNAGTTGCGGGCAGCTATACATGCAGTCGAGCGAATGGATTAAGAGCTTGCTCTTAT
GAAGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCCATAAGACTGGGA
TAACTCCGGGAAACCGGGGCTAATACCGGATAACATTTTGAACCGCATGGTTCGAAA
TTGAAAGGCGGCTTCGGCTGTCACTTATGGATGGACCCGCGTCGCATTAGCTAGTTG
GTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGG
CCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATC
TTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGCTTTCG
GGTCGTAAAACTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCACCT
TGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATAC
GTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGCAGGTGGTTTCT
TAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGAG
ACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGAG
ATATGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCTGTAACTGACACTGAGGC
GCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACG
ATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGAAGTTAACGCATTAAG
CACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAATTGACGGGGGC
CCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAG
GTCTTGACATCCTCTGAAAACCCTAGAGATAGGGCTTCTCCTTCGGGAGCAGAGTGA
CAGGTGGTGCATGGTTGTCGTCAACTCGTGCCNTGAAATGTTGGGTTAAGTCCCGCA
ACCGAGCGCAACCCTTTGACCTTAGTTGCCATCATTAAGTTTGGGCACTCTAAAGGTG
ACTGCCGGTTGACAAACCGGAAGGAAGGTGGGGGATGACGTCAAATCATCCTTGCCC
CTTATGACCTGGGGCTACCAACGTGGCTACAATGGGACGGGTACAAAGAGCTTCCAA
GACCCGCGAGGTGGAAGCTTAATTCCTTAAAAACCGTTTTCCANTTCCGGAATGGAA
GGCCGGCAACCCGCCCACATNGAAACCTGGGAATCCCTTAGNAACNNNTTCTGTTCC
CCTTATGCGGCTGGCTCCAAAAGGTTACCCCACCGACTTCGGGTGTTACAAACTCTCG
TGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGCGGCATGCTGAT
CCGCGATTACTAGCGATTCCAGCTTCATGTAGGCAGTTGCAGCCTACAATCCGAACT
GAGAACGGTTTTATGAGATTAGCTCCACCTCGCGGTCTTGCAGCTCTTTGTACCGTCC
ATTGTAGCACGTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTGACGTCATCCCC
ACCTTCCTCCGGTTTGTCACCGGCAGTCACCTTAGAGTGCCCAACTTAATGATGGCAA
CTAAGATCAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGC
TGACGACAACCATGCACCACCTGTCACTCTGCTCCCGAAGGAGAAGCCCTATCTCTA
GGGTTTTCAGAGGATGTCAAGACCTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACC
ACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTCAGCCTTGCGGC
CGTACTCCCCAGGCGGAGTGCTTAATGCGTTAACTTCAGCACTAAAGGGCGGAAACC
CTCTAACACTTAGCACTCATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTT
TGCTCCCCACGCTTTCGCGCCTCAGTGTCAGTTACAGACCAGAAAGTCGCCTTCGCCA
CTGGTGTTCCTCCATATCTCTACGCATTTCACCGCTACACATGGAATTCCACTTTCCTC
TTCTGCACTCAAGTCTCCCAGTTTCCAATGACCCTCCACGGTTGAGCCGTGGGCTTTC
ACATCAGACTTAAGAAACCACCTGCGCGCGCTTTACGCCCAATAATTCCGGATAACG
CTTGCCACCTACGTATTACCGCGGCTGCTGGCACGTAGTTAGCCGTGGCTTTCTGGTT
AGGTACCGTCAAGGTGCCAGCTTATTCAACTAGCACTTGTTCTTCCCTAACAACAGA
ATTTTACGACCCGAAAGCCTTCATCACTCAAGCGGCGTTGCTCCGTCAGACTTTCGTC
CATTGCGGAAGATTCCCTACTGCTGCCTCCCGTAAGAATCTGGGCCGTGTCCCAGTCC
CAGTGGGGCCGATCCCCTTCCCAGGCCGGCTACCCATCTTTGCCTTGGTGAGCCGTTA
CCTCACCAACTAGTTAAGGGGAAGCGGGGCCATCCTAAATGGAAACCCAAACCCGC
CTTTCAATTTCGAACCATGGGGTTCAAAAAGTTTCCGGGGTNTATCCCC

3.5.2 DNA Suquences for Ureolytic bacteria

NNNGANNTNGGGCTGCTATAATGCAGTCGACGCTTCTTTCCTCCCGAGTGCTTGCACTCA
ATTGGAAAGAGGAGTGGCGGACGGGTGAGTAACACGTGGGTAACCTACCCATCAGAGGG
GGATAACACTTGGAAACAGGTGCTAATACCGCATAACAGTTTATGCCGCATGGCATAAGA
GTGAAAGGCGCTTTCGGGTGTCGCTGATGGATGGACCCGCGGTGCATTAGCTAGTTGGTG
AGGTAACGGCTCACCAAGGCCACGATGCATAGCCGACCTGAGAGGGTGATCGGCCACAC
TGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAAT
GGACGAAAGTCTGACCGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAAC
TCTGTTGTTAGAGAAGAACAAGGACGTTAGTAACTGAACGTCCCCTGACGGTATCTAACC
AGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTG
TCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCC
CCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGAGAGT
GGAATTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAGGAACACCAGTGGCGAAGG
CGGCTCTCTGGTCTGTAACTGACGCTGAGGCTCGAAAGCGTGGGGAGCAAACAGGATTA
GATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTGGAGGGTTTCCGCCCT
TCAGTGCTGCAGCAAACGCATTAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAA
ACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGC
AACGCGAAGAACCTTACCAGGTCTTGACATCCTTTGACCACTCTAGAGAATAGAGCTTTC
CCTTCGGGGACAAAGTGACAGGTGGTGCATGTTTGTCGTCAGCTCCGGGCCGTGAGATGT
TGGGTTAAGTCCCGCAACGAGCGCAACCCTTATTGGTAGTTGCCATCATTTAGTTGGGGC
ACTCTAACCGAGACTGCCCGGTGACAAACCCGAAGAGANNNNATCTATCCACCTTAGGC
GGCTGGCTCCAAAAGGTTACCTCACCGACTTCGGGTGTTACAAACTCTCGTGGTGTGACG
GGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGCGGCGTGCTGATCCGCGATTACTAG
CGATTCCGGCTTCATGCAGGCGAGTTGCAGCCTGCAATCCGAACTGAGAGAAGCTTTAAG
AGATTTGCATGACCTCGCGGTCTAGCGACTCGTTGTACTTCCCATTGTAGCACGTGTGTAG
CCCAGGTCATAAGGGGCATGATGATTTGACGTCATCCCCACCTTCCTCCGGTTTGTCACCG
GCAGTCTCGCTAGAGTGCCCAACTAAATGATGGCAACTAACAATAAGGGTTGCGCTCGTT
GCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAACCATGCACCACCTGTCAC
TTTGTCCCCGAAGGGAAAGCTCTATCTCTAGAGTGGTCAAAGGATGTCAAGACCTGGTAA
GGTTCTTCGCGTTGCTTCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAA
TTCCTTTGAGTTTCAACCTTGCGGTCGTACTCCCCAGGCGGAGTGCTTAATGCGTTTGCTG
CAGCACTGAAGGGCGGAAACCCTCCAACACTTAGCACTCATCGTTTACGGCGTGGACTAC
CAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGAGCCTCAGCGTCAGTTACAGACCAG
AGAGCCGCCTTCGCCACTGGTGTTCCTCCATATATCTACGCATTTCACCGCTACACATGGA
ATTCCACTCTCCTCTTCTGCACTCAAGTCTCCCAGTTTCCAATGACCCTCCCCGGTTGAGC
CGGGGGCTTTCACATCAGACTTAAGAAACCGCCTGCGCTCGCTTTACGCCCAATAAATCC
GGACAACGCTTGCCACCTACGTATTACCGCGGCTGCTGGCACGTAGTTAGCCGTGGCTTT
CTGGTTAGATACCGTCAGGGGACGTTCAGTTACTAACGTCCTTGTTCTTCTCTAACAACAA
AGTTTTACGATCCGAAAAACTTTCTTCACTCCAGCGGGGGTTGCTCGGGCAAAATTTTCGT
CCATTGGCGAAAAATTCCCTACTGGCTGCCTCCCCGTAAGGAATCTGGGGCCGGGGCTCC
AATCCCAAGTGTGGGCCGAATCACCCCTTCCAAGTGGGGNNTAGGCATCGGGGGCCTTGG
GGAAGCCCGTTACCTCCACCAACTTAGNTTAATGGACNCCGGGGGGTCCTCCATTTANGG
GGACCCCCAGA

Figure 4. Phylogenetic position of *Bacillus SP* and closely related taxa (*Sulphate reduction bacteria*).

4. Conclusion

The bacteria in this study was isolated from fresh urine and acid mine drainage water.The isolated bacteria was enriched to suit concrete environement. After enrichment, the bacteria was purify by serial dilution and gram staining was conducted. The Gram staining results revealed that both bacteria was gram positive. The bacteria was identified through PCR and 16S rDNA sequencing as *Bacillus sp* and *Enterococcus faecalis*.

Acknowledgement

This research was supported by Universiti Tun Hussein Onn Malaysia (UTHM) and The Ministry of Higher Education Malaysia through Fundamental Research Grant Scheme (FGRS), Vot 1211.

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