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CALCITE-FORMING BACTERIA LOCATED IN LIMESTONE AREA OF MALAYSIA

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ABSTRACT

Calcite-forming by bacteria has been reported in various geological environments including limestone caves, and soil. There are four natural processes by which calcite is formed: carbonic anhydrase (CA), sulphate reduction, nitrate reduction, and urea hydrolysis. The aim of study is to identify calcite-forming bacteria occurring in limestone areas of Malaysia. Ten bacilli were identified to capable of calcite forming out of 23 total isolates. The processes of calcite forming were found to be either CA or urea hydrolysis, as none of the isolates falls into nitrate or sulphate reducing type of calcite-forming bacteria. Identification using 16S ribosomal DNA gene sequencing with polymerase chain reaction (PCR) identified B.sphaericus, B.cereus and B. pumilus; B.sphaericus, and B.cereus falls into urea hydrolysis and CA type, whereas B.pumilus falls into CA type of calcite forming.

Keywords: Calcite-forming bacteria, Limestone areas, Carbonic anhydrase, Urea hydrolysis

INTRODUCTION

Calcite-formation by bacteria has been reported in various environments that include limestone caves (Baskar et al., 2006; Cacchio et al., 2003; Chalmin et al., 2008) and soils (Baskar et al., 2006; Cacchio et al., 2003; Chalmin et al., 2008; Boquet et al., 1973). Calcite-formation by bacteria is governed by four main factors; (1) the calcium concentration, (2) the carbonate concentration, (3) the pH of the environment (which affects carbonate speciation and calcium carbonate solubility) and (4) the presence of nucleation sites (Hammes and Verstraete, 2002). The bacterial cells themselves have shown to be act as nucleation sites (Whiffin, 2004). Boquet et al. (1973) has concluded that calcite crystals formation is a common phenomenon and that its occurrence is simple a function of the composition of the medium used. Indeed, four different processes whereby calcite is formed by bacteria have been found. They include carbonic anhydrase (CA) (Achal and Pan, 2011), urea hydrolysis (Achal and Pan, 2011; Whiffin, 2004), sulphate reduction (Castanier et al., 1999; Hammes and Verstraete, 2002), and nitrate reduction (Castanier et al., 1999; Hammes and Verstraete, 2002). Most of the studied bacteria on calcite formation are based on urea hydrolysis process, as it is controlled by urease enzyme (Whiffin, 2004). Urea will be hydrolysed into ammonia and carbonate ions in the presence of urease enzyme (Whiffin, 2004). Ammonia will

increase the pH which favours the calcite formation in the presence of calcium ions (Whiffin, 2004). But still there are some limitation on enzymatic hydrolysis of urea, whereby the side product produced are detrimental and it is required removal which is again an application of another process (Passen et al., 2009).

Recently, CA type of calcite formation by bacteria has been used extensively for sequestration of carbon dioxide (CO_2) by converting it into mineral calcite (Prabhu et al., 2011). CA is a zinc containing enzyme, has a capability to sequester CO_2 by converting it into carbonate which is further precipitated as calcite by addition of appropriate calcium sources. CO_2 as well as other greenhouse gases, has been emitted by anthropogenic (human induce) activities into the atmosphere since the industrial revolution (Mirjafari et al., 2007). The fact is CO_2 is the major greenhouse gases emitted from the industries that utilize fossil fuels, such as power plants that burn coal (Mirjafari et al., 2007). The rising of CO_2 emission leading to global climate change is one of the greatest environmental challenges that the world faces today (Mirjafari et al., 2007). Therefore, sequestration of CO_2 into calcite mineral using CA as a bio-catalyst appears to be a promising option as calcite mineral are abundant in nature and environmentally benign and stable (Prabhu et al. 2011).

The present study is part of a research study whose overall goal is to develop a green technology for global climate change using bacterially formed calcite. Therefore, the development of green technology will require an initial investigation for the presence of naturally occurring calcite-forming bacteria (CFB) in Malaysia. Besides, Malaysia has abundant of limestone areas especially peninsular Malaysia, where the major limestone areas include Langkawi island, Kedah-Perlis, Kuala Lumpur, Kinta Valley, Perak, Selangor, Gua Musang, and Kelantan (Bakhshipouri et al., 2009). However to the authors' best knowledge; there has been no published material on CFB isolated from limestone cave or soil samples in Malaysia. Nevertheless, recently Ng et al. (2012) from Malaysia did a research on soil improvement by calcite formation of urease type *Bacillus megaterium*. The purpose of the present study is to screen for naturally occurring CFB which included determination of geo-sample areas, isolation of CFB, X-ray diffractometer (XRD) analysis to determine the mineral composition, identification of CFB mechanisms, and lastly on CFB identification through 16S rDNA sequencing.

MATERIALS AND METHODS

Sampling Sites

Calcite formation by bacteria from limestone areas are the main interest of this study, where focused on deposits on the limestone cave surfaces. The samples were taken from two sites, a cave at location 4°25'50.0" N, 101°10'51.9" E (**Fig. 1**) and limestone quarry at location 4°25'48.9" N, 101°9'55.5" E. The cave is limestone, and is located in Tempurung Valley, Perak. Malaysia. More than 400 million years old with 1.9 km length and 120 m height. The limestone quarry is located at Gopeng, is the limestone supply for Rock Chemical Industry (M) Berhad, which is primarily involved in the extraction, processing, and distribution of limestone based products. In total, there are five limestone samples were collected as shown in **Fig. 2**, for ease of reading, the samples were given sample ID as S1 until S5. S1 stands for sample collected at first and followed. The collected samples were brought to the laboratory and preserved at 4°C before subjected into further analysis. Mineralogy was determined by x-ray diffractometer (XRD) analysis on all the five powdered limestone cave samples collected.

Isolation of Calcite-Forming Bacteria (CFB)

Bacteria from the five collected limestone samples were isolated and screened for calcite crystals formation in B4 medium (2.5g calcium acetate, 4g yeast extract, 10g glucose and 18g agar per litre of distilled water); agar plates containing calcium acetate as a calcium source to induce the calcite

formation by the bacteria. Initially, the collected limestone samples were powdered in a porcelain mortar and pestle, and 1g of powdered sample suspended in 9 ml sterile saline solution and mix vigorously. Triplicate B4 medium spread plates on petri plates were inoculated with sample dilutions ranging from 10^1 to 10^5 and incubated at 37^0C for 2 weeks. B4 agar plates were daily examined for the presence of crystals by binocular microscope up to 2 weeks. Controls consisted of un-inoculated culture medium along with experimental samples. Individual colonies were selected and purified by repeated streaking on B4 agar plates. For ease of reading, the isolated strains were designed specific names according to the sample ID, followed by SC stands for subculture, and the strains were numbered to differentiate between the other isolated colonies from the same sample.

X-ray diffractometer (XRD) Analysis

X-ray diffraction measurements were done on both collected limestone samples and collected crystals formed by bacteria in petri plates. The limestone samples were powdered using porcelain mortar and pestle, before subjected into XRD analysis. Then, the crystals containing solid media was cut into flat square blocks and placed in clean microscopic glass slides (75 x 25mm). The glass slides together with crystals were dried in dryer (70^oC) for 3 days to make sure the agar dried and left with crystals. The supply voltage of the X-ray tube was set at 50kV, 30mA. The 2θ scan range was between 22^o and 50^o; each scan was done in steps of 0.05^o. The crystalline phases were identified using the International Centre for Diffraction Data (ICDD) database (Joint Committee on Powder Diffraction Standards, JCPDS).

Figure 1. Location of sampling sites in the limestone caves of Gua Tempurung, Gopeng, Perak

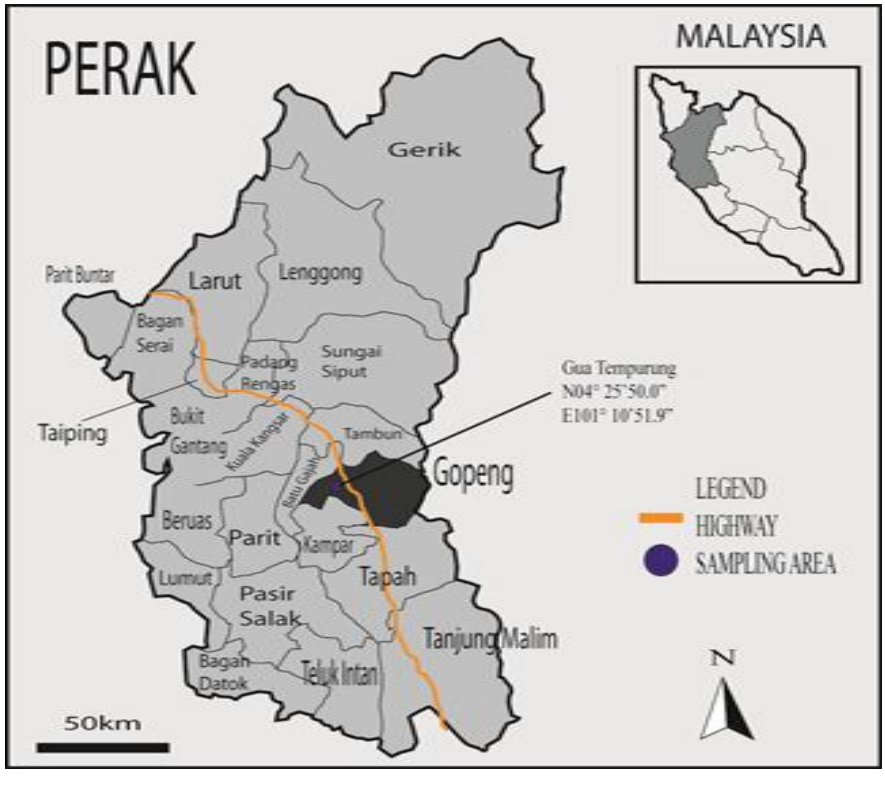
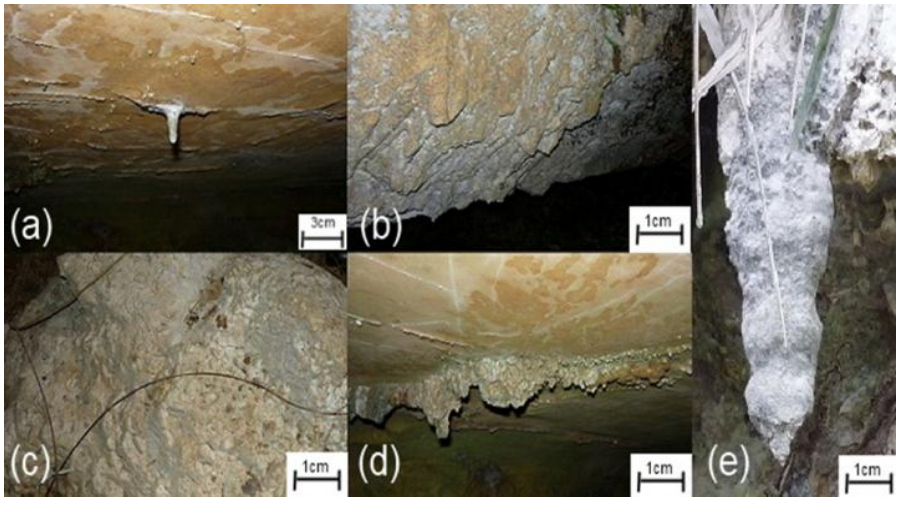


Figure 2. Limestone cave samples (a) S1 (b) S2 (c) S3 (d) S4 (e) S5.



Calcite Forming Mechanisms Determination

Urea hydrolysis, H_2S production, denitrification, nitrate reduction, nitrite reduction, ammonification, and extracellular carbonic anhydrase (CA) assay were done on all the isolates to determine the calcite forming mechanisms. For the extracellular CA test by esterase activity, the extracellular bacterial cells were harvested by centrifugation at 8,000 rpm for 10 minutes at 25^{o}C.

CA Analysis by Esterase Activity

Esterase enzymatic activity was determined by using the spectrophotometric assay described by Prabhu et al. (2009) with slight modification. In brief, the assay consisted of 1.8 ml of 0.1M phosphate buffer and 1.0 ml of 3mM p-nitrophenyl acetate with 0.2 ml of bacterial extracellular CA extract. 1 ml of sample from this solution was taken and measured its absorbance in UV/VIS spectrophotometer at 348nm in triplicates. The change in wavelength absorbance at 348 nm was recorded over first 8 min to estimate the amount of the p-nitrophenol released. The non-enzymatic hydrolysis was subtracted by using a blank without enzyme. One unit of enzymatic activity represents the amount of enzyme catalysing to produce 1µmol p-nitrophenol per min under the assay conditions.

Calibration Curve of p-nitrophenol

To convert UV/VIS spectrophotometer data of bacteria into concentration values, a calibration curve of p-nitrophenol were measured. One of the products of p-nitrophenyl acetate degradation is the p-nitrophenol. A concentration of 0.01 to 0.10 µmol of p-nitrophenol was dissolved in 0.1M phosphate buffer. Then absorbance values were measured in UV/VIS spectrophotometer at 348nm. At the end, the calibration curve was established as concentration versus absorbance.

Identification of Bacterial Strains by 16S rDNA Sequencing

The bacteria genomic DNA was extracted by using the phenol-chloroform method by Taggart et al. (1992) with some modification. The polymerase chain reaction (PCR) was conducted to amplify 16s rDNA to identify the isolated bacterial strains. The 16S rDNA was amplified by polymerase chain reaction (PCR) using the primer BacF (5'-GGGAAACCGGGGCTAATACCGGAT-3'; which is specific for *Bacilli* and related taxa) and R1378 (5'-CGGTGTGTACAAGGCCCGGGAACG-3'; which is the universal reverser primer for bacterial 16S rDNA) (Garbeva et al., 2003). Sequence analysis was done by sending the samples to First Base Laboratories Sdn. Bhd for sequencing. The 16S rDNA gene sequences of the most closely related to our isolated bacterial strains were retrieved from the database and aligned by using the

Clustal X program; and the phylogenetic tree was constructed by the neighbor-joining method using software package MEGA 4.0 (Adiguzel et al., 2009; Tamura et al., 2007; Thompson et al., 1997). Sequences with a percentage identify of 96% or higher were considered to represent the same species (Ana & Baltasar, 2006).

RESULTS AND DISCUSSION

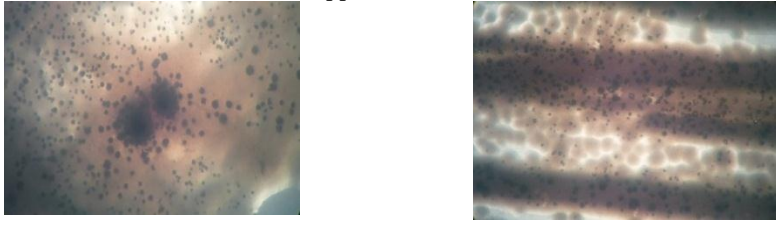
CFB Isolation

Out of 23 total bacterial isolates from all the isolated samples, 10 isolates were detected for the presence of crystals by observation using the binocular microscope (**Table 1**). The images taken from binocular microscope of isolates S2 SC_1 and S3 SC_3 were shown in **Figure 3**.

Table 1. Samples, number of bacterial colonies, isolate names

Sample ID	Number of Bacterial Colonies	CFB Colonies
S1	5	1 (S1 SC_3)
S2	7	4 (S2 SC_1, S2 SC_2, S2 SC_3, S2 SC_4)
S3	5	3 (S3 SC_1, S3 SC_2, S3 SC_3)
S4	2	-
S5	4	2 (S5 SC_1, S5 SC_2)
Total	23	10

Figure 3. Optical microscopic images of colonies from different isolate showing crystals formation. (a) Isolate S2 SC_1: intense cystals formation within the colony, and also in the surrounding culture medium (b) S3 SC_3: colonies showing a remarkable production of crystals, the mineralization areas within the colonies appear dark



This preliminary study involved the isolation of CFB in limestone area of Malaysia. Our findings confirmed that there is CFB found in limestone cave samples in Malaysia. As such the original hypothesis of presence of calcite formation by bacteria in limestone caves were supported by this research. Similar research on bacterial calcite formation from limestone caves were conducted by Baskar et al. (2006), Cacchio et al. (2003), and Ercole et al. (2001). It is found that number of bacterial colonies found varied between the different samples been collected (**Table 1**). Ercole et al. (2001) stated that the bacteria might have been scarce in some area due to their presence having a trivial origin, percolation, contamination due to the presence of cave-dwelling animals and visitors.

X-ray diffractometer (XRD) Analysis

The crystals formed were characterized by XRD by using calcium carbonate powder (Merck) as standard. It can be clearly seen from the XRD graphs that all the major peaks of cave samples were at the same 2Θ with calcium carbonate powder as reference (**Fig. 4**). Quantitative estimates based on relative peak intensities also confirmed that calcite is more abundant (**Table 2**). X-ray diffraction analysis showed that the caves are composed of calcium carbonate as it is the dominant

mineral. For the crystals formed by the bacteria, all of the samples the X-ray diffraction patterns indicated the presence of calcite (**Table 3** and **Fig. 5**; shows one of the samples x-ray diffraction spectra). There are three different types of calcite was found by XRD analysis are calcite rhombohedral (calcite, card number 05-0586), calcite rhombohedral (calcite, card number 24-0027) and calcium carbonate (calcite, card number 47-1743) (**Table 2, 3**). Overall, the mineral identification of cave samples shows the calcite mineral was dominant (**Table 2**). Therefore, this is an evidence that the Gua Tempurung was formed by mineral action which takes millions years to form. Besides, microscopic observations on bacterial calcite formation showed that the calcite formation is takes place in the presence of bacterial colonies (**Fig. 3**). The XRD analysis shows that only the calcite element was found in the crystals collected formed by bacterial isolates. According to Boquet et al. (1973) bacterial calcite formation is a general phenomenon in the bacterial world, and under appropriate conditions many bacteria are capable to form calcite crystals.

Table 2. XRD quantitative analysis of the final weight fractions [a] of powdered limestone cave samples

Sample ID	Calcium Carbonate (Calcite, syn) ($CaCO_3$) (ICDD 05-0586)	Calcium Carbonate (Calcite) ($CaCO_3$) (ICDD 24-0027)
S1	0.761	0.714
S2	0.902	0.926
S3	0.870	0.874
S4	0.925	ND
S5	0.905	0.873

[a] Numbers represent an average of weight fraction values obtained from energy-dispersive XRD quantitative analysis. (ND: not detected).

Figure 4. Shows the X-ray Diffraction spectra which compares all the samples with standard $CaCO_3$ (counting time = 1 sec/step) showing a close similarity: S1 (black line), S2 (purple line), S3 (blue line), S4 (green line), and S5 (pink line)

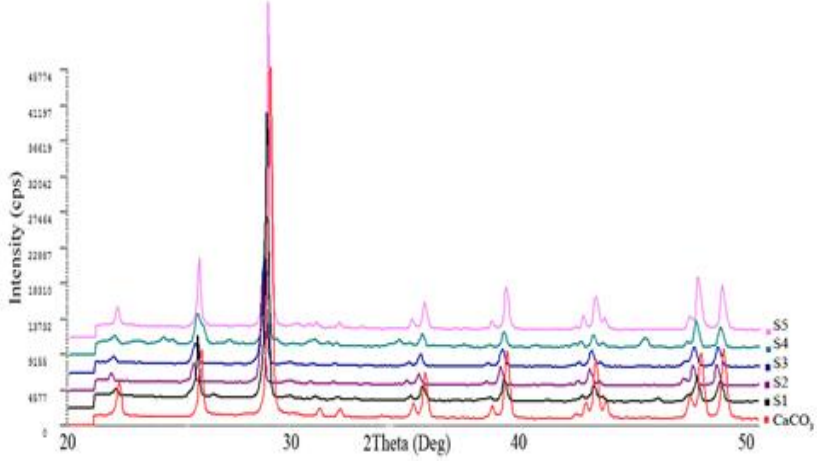


Figure 5. Shows the X-ray Diffraction spectra from one of the isolate, S1 SC_1 (blue line) (counting time = 1 sec/step) showing a close similarity with standard $CaCO_3$ (red line) spectra

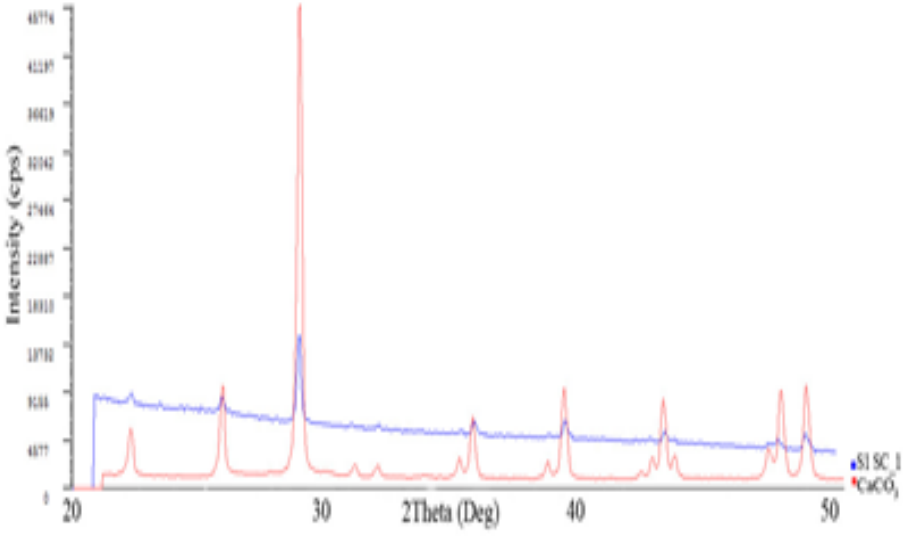


Table 3. XRD quantitative analysis of the final weight fractions [a] of crystals collected from all of the CFB bacterial isolates

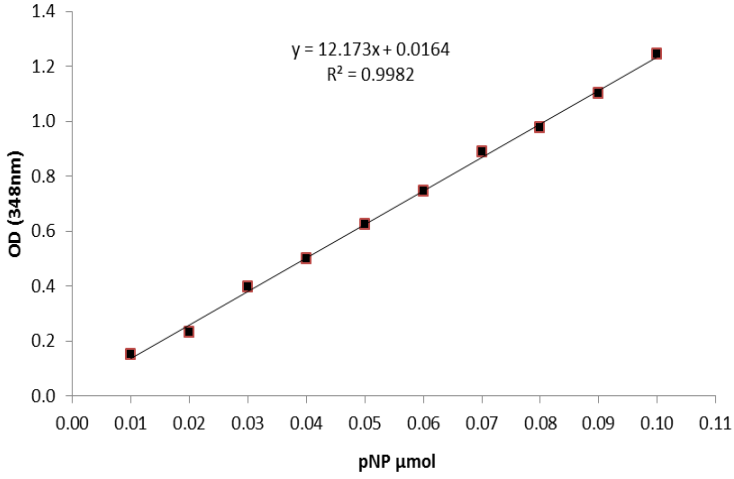
Bacterial Strain ID	Calcium carbonate (Calcite, syn) ($CaCO_3$) (ICDD 05-0586)	Calcium carbonate (Calcite) ($CaCO_3$) (ICDD 24-0027)	Calcium carbonate ($CaCO_3$) (ICDD 47-1743)
S1 SC_1	0.744	0.725	0.871
S2 SC_1	0.214	0.205	0.825
S2 SC_2	0.934	0.883	0.866
S2 SC_3	0.781	0.737	0.877
S2 SC_4	0.311	0.298	0.876
S3 SC_1	0.372	0.338	0.901
S3 SC_2	0.740	0.707	0.835
S3 SC_3	0.789	0.763	0.903
S5 SC_1	0.765	0.717	0.901
S5 SC_2	0.849	0.807	0.912

[a] Numbers represent an average of weight fraction values obtained from energy-dispersive XRD quantitative analysis.

Types of Calcite Forming Bacilli Determined

Firstly, urea hydrolysis test were done on 10 calcite forming colonies, whereby out of 10 isolates two isolates were identified as non-urease type bacterial isolates. All the isolates were further subjected to H_2S production, nitrate reduction, nitrite reduction, denitrification, and ammonification (**Table 4)**. Extracellular carbonic anhydrase (CA) assay by esterase activity was also done on all the isolates to detect the CA activity of the bacterial isolates.

Figure 6. Calibration Curve for p-nitrophenol in 0.1M phosphate buffer



CA Analysis by Esterase Activity

The concept of esterase activity is p-nitrophenyl acetate substrate will be hydrolysed by the carbonic anhydrase into p-nitrophenol (A_{348}), whereby the change can be observed spectrophotometrically. There was a control for every sample, and the value was after deduction of those controls value. To convert UV/VIS spectrophotometer data into concentration values, a calibration curve of p-nitrophenol was prepared (**Fig. 6**). The powder p-nitrophenol was dissolved in phosphate buffer, and the blank solution was also chosen as the same phosphate buffer. R2 value of the line 0.9982 and slope of the calibration curve was calculated as 0.02 (**Fig. 6**). Analysis of the amount of p-nitrophenol data showed the presence of significant variations in the different bacterial isolates (**Table 5**). One unit of esterase activity represents the amount of enzyme catalysing to produce 1µmol p-nitrophenol per min under the assay conditions. The highest p-nitrophenol/min was shown as 5.750, 5.658, and 5.874 mM by S3 SC_1, S3 SC_3, and S5 SC_1 respectively and an esterase activity was shown as 0.006 U/min. In our present study, isolate S3 SC_3 fell into two categories, urease and CA type of CFB.

Table 4. Shows the results of biochemical test done on all the CFB bacterial isolates. The entire tests were done in triplicates and the table below shows one of the results

Isolates	1	2	3	4	5	6
S1 SC_3	+	-	+	-	-	-
S2 SC_1	+	-	+	-	-	-
S2 SC_2	+	-	-	-	-	-
S2 SC_3	+	-	-	-	-	-
S2 SC_4	+	-	-	-	-	-
S3 SC_1	-	-	-	-	-	-
S3 SC_2	+	-	-	-	-	-
S3 SC_3	+	-	-	-	-	-
S5 SC_1	-	-	-	-	-	-
S5 SC_2	+	-	-	-	-	-

+: positive to the test; -: negative to the test.

1: Urea hydrolysis; 2: H_2S Production Test; 3: Nitrate Reduction Test; 4: Nitrite Reduction Test; 5: Ammonification; 6: Denitrification

Table 5. Shows the amount of p-nitrophenol/min (mM) hydrolyzed from p-nitrophenyl acetate from all of the CFB bacterial isolates

Isolates	p-nitrophenol/min (mM)
S1 SC_3	0.339
S2 SC_1	2.495
S2 SC_2	3.471
S2 SC_3	0.452
S2 SC_4	1.817
S3 SC_1	5.750
S3 SC_2	2.937
S3 SC_3	5.658
S5 SC_1	5.874
S5 SC_2	0.667

Urease type of CFB was found to be dominant in this present study. It was found that out of 10 isolates, eight isolates were urease type (**Table 4**). This is totally different compared to research done by Cacchio et al. (2003) where their results show that out of 22 isolates from limestone caves, only three isolates were urease type. Urea hydrolysis is the simplest mechanism in bacterial calcite formation, which results in the production of carbonate ions in the presence of ammonium. Calcite is readily formed under these conditions, in the presence of calcium ions (Whiffin 2004). Since our research found a number of urease type of CFB, it will be useful in future for further research on urease type of bacteria which is only controlled by only one enzyme. Applications of urease type of CFB are such as restoration of calcareous stone materials (Castanier et al., 1999; Stocks-Fisher et al., 1999), strengthening of concrete (Ramachandran et al., 2001), plugging of sand (Stocks-Fisherr et al., 1999), remediation of cracks in granite (Gollapudi et al., 1995), and ornamental stone (Dick et al., 2006).

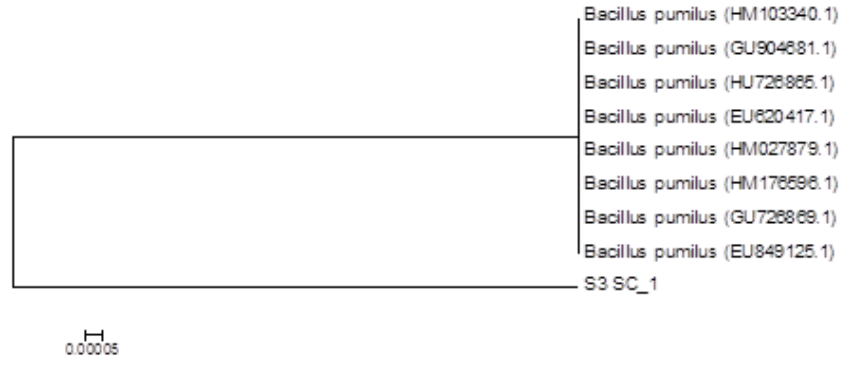
Identification of bacterial strains by 16S rDNA Sequencing

Phylogenetic tree was constructed based on comparison of 16S rDNA sequences of reference *Bacillus* spp strains in order to understand the phylogenetic position of our strain. For each sequences obtained from sequencing analysis were compared with eight reference strains, were further aligned by clustal W, and a dendogram was constructed from these aligned sequences by neighbor-joining method, using MEGA 4 software package. Overall the identification results of four isolates were shown in **Table 6** followed by the dendogram of samples in **Figure 7 (a, b, c, and d)**.

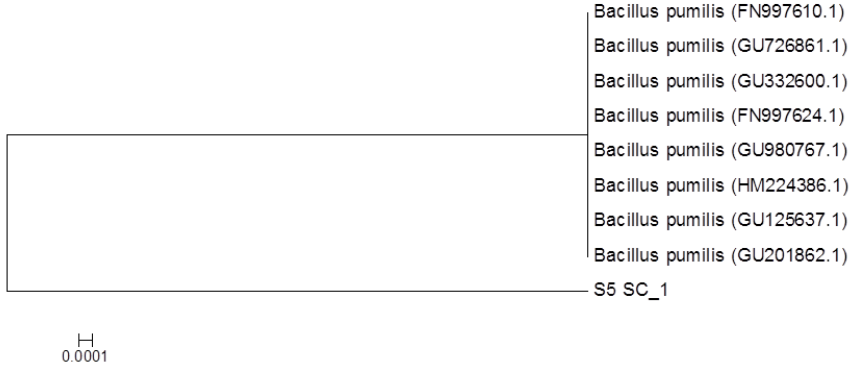
Table 6. shows the overall identification results of four isolates

Isolates	**Identified Species**
S3 SC_1	*B.pumilus* (98%)
S5 SC_1	*B.pumilus* (98%)
S2 SC_1	*B.cereus* (96%)
S3 SC_3	*B.sphaericus* (95%)

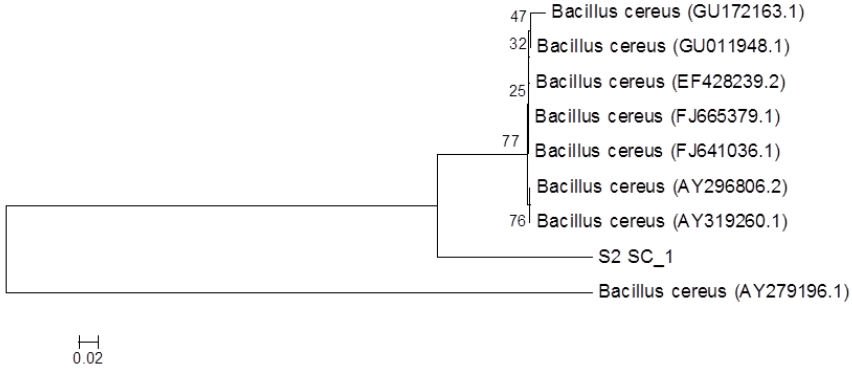
Figure 7. Phylogenetic position of the bacterial strain with eight references isolates. Phylogenetic tree was inferred by using the neighbour-joining methods and MEGA 4 software package was used for the analysis (a) S3 SC_1 showing 98% similarity with *Bacillus pumilus*, 0.005% divergence (b) S5 SC_1 showing 98% similarity with *Bacillus pumilus*, 0.01% divergence (c) S2 SC_1 showing 96% similarity with *Bacillus cereus*, 2% divergence (d) S3 SC_3 showing 95% similarity with *Bacillus sphaericus*, 0.2% divergence.


Bacillus pumilus (HM103340.1)
Bacillus pumilus (GU904681.1)
Bacillus pumilus (HU726865.1)
Bacillus pumilus (EU620417.1)
Bacillus pumilus (HM027879.1)
Bacillus pumilus (HM176696.1)
Bacillus pumilus (GU726869.1)
Bacillus pumilus (EU849125.1)
S3 SC_1
0.00005

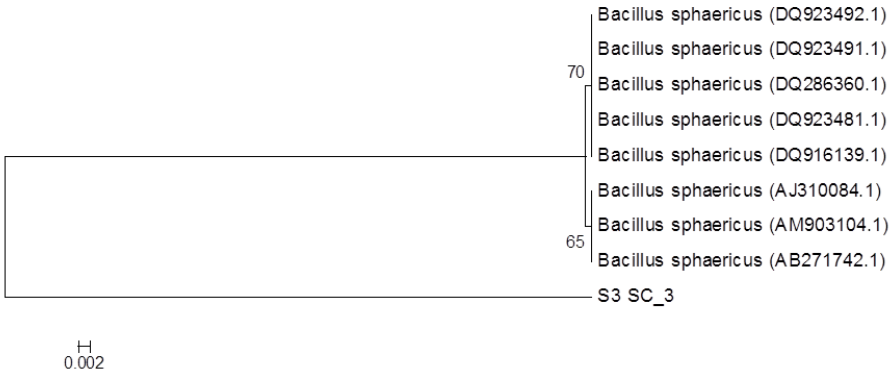
(a)


Bacillus pumilis (FN997610.1)
Bacillus pumilis (GU726861.1)
Bacillus pumilis (GU332600.1)
Bacillus pumilis (FN997624.1)
Bacillus pumilis (GU980767.1)
Bacillus pumilis (HM224386.1)
Bacillus pumilis (GU125637.1)
Bacillus pumilis (GU201862.1)
S5 SC_1
0.0001

(b)


47
32
25
77
76
Bacillus cereus (GU172163.1)
Bacillus cereus (GU011948.1)
Bacillus cereus (EF428239.2)
Bacillus cereus (FJ665379.1)
Bacillus cereus (FJ641036.1)
Bacillus cereus (AY296806.2)
Bacillus cereus (AY319260.1)
S2 SC_1
Bacillus cereus (AY279196.1)
0.02

(c)



(d)

The sequence analysis for the 16S rDNA gene of the isolates were determined and compared with those of reference *Bacillus* spp strains in the database using NCBI blast (http://www.ncbi.nlm.nih.gov). 16s rDNA sequence analysis showed that there was a strong similarity (≥95% - ≥98%) between our test strains and representative strains in database of *Bacillus* spp strains, which may indicate that 16s rDNA gene sequence data is helpful for identification of bacteria at species level. *Bacillus pumilus* S3 SC_1; S5 SC_1, *Bacillus cereus* S2 SC_1, and *Bacillus sphaericus* S3 SC_3 were identified via 16S rDNA sequencing in our present research. Recently, Arunachalam et al. (2010) studied about the calcite forming ability of *B.sphaericus*. *B.sphaericus* was another species with similar entity of *Bacillus pasteurii* in calcite forming capability. Apparently, *B.pasteurii* was the first bacteria to record the calcite forming capability into application in building industry (Boquet et al., 1973; Stock-Fisher et al., 1999). De Muynck et al. (2008; 2009; 2010) from Belgium studied about *B. sphaericus* for its application in building industry as well. Baskar et al. (2006) identified *B. pumilus* strains from their study. They did propose that the bacterial activity and optimum temperature appears to be the key factors in calcite formation, ultimately the stalactites formation. Up to date, no further research was published on further application of those identified bacteria. Their investigation mostly concentrated on finding out the origin of caves formation. Ercole et al. (2001) isolated and identified *B. sphaericus* from cave samples; their research is more in finding of bacterial action into cave speleothem formation.

We found that isolated and identified two bacterial strains as CA type of CFB to be *B.pumilus* S3 SC_1 and *B.sphaericus* S3 SC_3. CA is a key enzyme in the chemical reaction of living organisms which linked with calcite formation (Rahman et al. 2007). It is a zinc-containing enzyme produced by bacteria that catalyses the reversible conversion of carbon dioxide (CO_2) to bicarbonate, which would then be available for calcite formation (Rahman et al. 2007). Therefore, carbonic anhydrase (CA) type of calcite formation acts as biological CO_2 sequestration which will bring significant amount of economical and environmental benefit in future by reducing anthropogenic CO_2 from the atmosphere. Yadav et al. (2011) summarized that CA production specifically by *B.pumilus* can promote calcite formation. Whereas Li et al. (2011) also found that that the CA produced by *Bacillus* spp. isolated from karst soil are able to promote calcite formation. It is interesting to note that from our research, identified *B.sphaericus* S3 SC_ 3 was fells into both urease and CA type of CFB. It shows the highest esterase activity and so long *B.sphaericus* was studied for urease type of CFB applications only. Therefore from this point, research can be conducted on *B.sphaericus* in future for application into CO_2 sequestration.

CONCLUSION

The study site in a limestone area of Malaysia was found to have a considerably high population of calcite forming bacilli, 43.5% out of 23 isolated. Through this preliminary screening for CFB, can be concluded that by providing the nutrient and calcium ions in appropriate amount, the bacteria are able to form calcite. The mineralogy identification of crystals formed by bacteria provide a concrete result that only the calcite mineral was formed by the isolated bacteria. Out of 10 CFB isolates, three isolates was identified of CA type (shows highest amount of esterase activity), and eight isolates were identified to be urease type of CFB. *B.sphaericus*, *B. pumilus*, and *B. cereus* were identified through 16s rDNA sequencing. *B.sphaericus* falls into both categories of CFB; urease and CA type. As to the utilization of bacterial calcite formation of CA type for carbon fixation to overcome global warming issues, the screening reported in the present paper allows a consideration of possible candidates. Since, identified *B.pumilus* shows highest amount of esterase activity, therefore further research are in the process of proving carbon sequestration ability.

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