ISOLATION AND CHARACTERIZATION OF NATURALLY OCCURRING CALCITE-FORMING BACTERIA IN MALAYSIA

By

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DEDICATION

Dedicated especially to my lovely husband, Mr. Kalidasan, for his unlimited patience and lovely inspiration, which has kept my spirit burning all the while.....and to my dearest daughter Loseny... My Princess...Greatest gift of our life...

Also to my ever supportive family father, Mr. Thirumalai, mother, Mrs. Purna Chandirka, father-in law, Mr. Gopal, mother-in law, Mrs. Janaki, for their unconditional support and care.

May God bless us.

ABSTRACT

ISOLATION AND CHARACTERIZATION OF NATURALLY OCCURRING CALCITE-FORMING BACTERIA IN MALAYSIA

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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 this study is to identify calcite-forming bacteria (CFB) from limestone and soil samples. 18 isolates were identified to capable of calcite forming out of 54 total isolates. The mechanisms of calcite forming were successfully identified for all 18 CFB isolates. 16S rDNA gene sequencing with polymerase chain reaction (PCR) identified *B. sphaericus*, *B. cereus* and *B. pumilus* (2 isolates); *B. sphaericus*, and *B. cereus* falls into urea hydrolysis and CA type, whereas *B. pumilus* falls into CA type of calcite forming. *B. pumilus* was successfully characterized using both conventional biochemical characterization and 16s rDNA sequencing. Culture conditions of *B. pumilus* were further successfully optimized for temperature, *pH* and glucose concentration. Three types of experimental systems with *B. pumilus*, without *B. pumilus* and without continuous supply of carbon dioxide (CO_2) with the presence of *B. pumilus* were studied to determine the effects of produced CA by *B. pumilus* in

removing CO_2 as calcite. Finally, through our current study CO_2 sequestration ability of *B. pumilus* was proven.

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APPROVAL SHEET

This dissertation entitled "**ISOLATION AND CHARACTERIZATION OF NATURALLY OCCURRING CALCITE-FORMING BACTERIA IN MALAYSIA"** was prepared by KOMALA A/P THIRUMALAI and submitted as partial fulfillment of the requirements for the degree of Master of Science at Universiti Tunku Abdul Rahman.

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DECLARATION

I KOMALA A/P THIRUMALAI hereby declare that the dissertation is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UTAR or other institutions.

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GLOSSARY

Agarose gel electrophoresis	Highly purified form of agar gelled into a matrix to separate large DNA and RNA molecules that range from over 20,000 nucleotides
Calcite	A common rock forming mineral of calcium carbonate ($CaCO_3$), found primarily in limestone
Calcite forming bacteria (CFB)	A bacterium that forms calcite in the presence of calcium ions
Deoxyribonucleic acid (DNA)	The nucleic acid molecule that contains all the genetic information regarding the development and functioning of all living organism. DNA is also known as the blueprint of life
Optical light	The light that is detected by the human eye having an wave length of 4000 and 7000 angstroms
Polymerase chain reaction (PCR)	A method of making multiple copies of a DNA sequence, involving repeated reactions with a polymerase
Qualitative	This is scientific observation that is not based measurements and numbers
Quantitative	The scientific observations that are based on measurements and numbers
Scanning electron microscope (SEM)	An electron microscope in which the surface of a specimen is scanned by a beam of electrons that are reflected to form an image
X-ray diffraction (XRD)	The diffraction pattern seen after passing X-rays through a pure crystals substances

1 CHAPTER

INTRODUCTION

1.1 Background of Study

Six greenhouse gases have been identified to contribute to the climate change *i.e* carbon dioxide (CO_2), methane (CH_4), nitrous oxide (N_2O), hydrofluorocarbons (HFC), perfluorocarbons (PFC), and sulfur hexafluoride (SF_6) (Raju R. Yadav et al., 2014). Out of these gases, CO_2 play a major role. Large stationary facilities such as power plants, cement production, oil and gas refineries contributes to CO_2 emissions while cement production contributes to major CO_2 emissions (Worell et al., 2001). In addition to this, calcite is the main material used in cement production, and the fact is calcite has been extracted from the limestone resources in the natural environment. Ultimately naturally occurring limestone resources need to sacrified in the cement production process. This implicates that the production of cement requires significant substitute, which will certainly help towards reducing CO_2 emissions to effectively manage the environmental issue of climate change.

Using the latest bacterial biotechnology, bacterially induced calcite formation can be developed as an alternative to cement. Apparently, calcite formation by bacteria is sustainable as the bacteria can be found abundant in nature and reproduced easily at the lowest cost. A bacterium that forms calcite in the presence of calcium ions is known as calcite forming bacteria (CFB). There exist in nature four different processes that can be harnessed for calcite

formation: carbonic anhydrase (CA), sulphate reduction, nitrate reduction, and urea hydrolysis (Knorre and Krumbein, 2000; Henriques, 2011). The bacteria will act as a nucleation sites and will accelerate the calcite formation (Hammes and Verstraete, 2002). Enzyme urease produced by bacteria catalyzes substrate urea into ammonium and precipitates carbonate ions. These carbonate ions in the presence of a calcium source readily form calcite (Stocks-Fisher et al., 1999; Chahal et al., 2011).

Bacterial denitrification of calcium nitrate uses calcium salts of fatty acids as electron donor and carbon source. This process leads to calcite formation, bacterial growth and production of nitrogen gas and some excess CO_2 (Van Passen et al., 2010). Sulphate reduction mechanisms are carried out by sulphate reducing bacteria (SRB). The reaction often starts with dissolution of gypsum, which is a pure physicochemical process. Under these circumstances, organic matter consumed by SRB with the release of sulphide and metabolic CO_2 (Hammes and Verstraete, 2002). Castanier et al. (1999) further showed that removal of the produced hydrogen sulphide, and resultant *pH* increase, is a prerequisite for calcite formation to occur. The activity of SRB has been shown to facilitate dolomite formation (Bansal, 2011).

CA is very well-known enzymes that are ubiquitous in nature (Bansal, 2011). CA is a zinc-containing enzyme which catalyzes the reversible conversion of CO_2 to bicarbonate, which would then be available for calcite formation (Rahman and Oomori, 2010). Slow rate of CO_2 hydration overcomes in the presence of CA enzyme. CA enzyme promotes CO_2 hydration and

subsequently forms calcite in the presence of calcium ions (Rahman and Oomori, 2010). Generally, these metabolic processes increase the alkalinity by increasing the *pH* of the environment and thereby favor calcite formation in the presence of calcium ions (Hammes et al., 2003).

In this connection, CA-type of CFB is used to fix CO_2 by converting the CO_2 into carbonate which is formed as calcite by addition of appropriate calcium sources (Prabhu et al., 2011). Interestingly, biological CO_2 sequestration is an environmental friendly method and economical way of reducing CO_2. This calls for research study into the bacterial processes and feasibility study into applications of CO_2 sequestration by CFB. The approach of the present study was to screen for naturally occurring CFB with the concept of calcite formation by bacteria which were already well established in the literature. In conclusion, initial part of this study focuses on isolation and characterization of CFB. Subsequently the latter part of the study focuses on bacterial CO_2 sequestration by calcite formation.

1.2 Problem Statements

The proposed research objectives are set the following problem statements identified as:

- Are there CFB naturally occurring in Malaysia?
- Are the local bacteria different (new species) from literature?
- What are the conditions for culturing and maintaining the bacteria?
- Can the locally isolated CFB sequester CO_2?

To the best knowledge of the author, there is no published material describing CFB isolated from limestone or soil samples in Malaysia. Recently, CFB has been anticipated as an environmental friendly method for CO_2 sequestration. Therefore, this study will become a starting stone as the preliminary screening of CFB in Malaysia. The results of this study are promising to provide insight for CO_2 sequestration as the solution for climate change issue. In conclusion by proving the CO_2 sequestration ability of CFB will create a new scientific transition by locally isolated and identified bacteria in Malaysia.

1.3 Research Objectives

The present proposed study is part of a research study whose overall goal is to find the suitable CFB for CO_2 sequestration as a solution for climate change issue. Thus, this will require an initial investigation for the presence of naturally occurring CFB in Malaysia, followed by feasibility study of developing the calcite forming process for CO_2 sequestration. As the initial part of the research, this present research will meet the following objectives:

1. To determine the presence of CFB from limestone and soil samples including identification of calcite-forming mechanisms.
2. To characterize by molecular and biochemical characterization of the selected CFB.
3. To investigate the carbon dioxide sequestration ability of the selected CFB.

1.4 Scope of Study

To achieve the objectives of the research study, the first part of this study concentrates on isolation and identification of CFB from limestone and soil samples in Malaysia. The latter part of this study focuses on characterization of CFB mechanisms followed by molecular and biochemical characterization of isolated CFB. This is followed by the optimization of selected CFB isolates culturing conditions. The series of lab work are based on the range of three parameters comprising temperature, *pH*, and initial glucose concentration. Finally, the CO_2 sequestration ability of the selected CFB will be proved.

2 CHAPTER

LITERATURE REVIEW

2.1 Calcite

Calcite is one of the most abundant and reactive mineral on the earth's surface, constituting about 4% by weight of the earth crust (Whiffin, 2004). There are six calcium carbonate ($CaCO_3$) or calcite mineral polymorphs including calcite, aragonite, vaterite, $CaCO_3$ monohydrate, $CaCO_3$ hexahydrate and amorphous $CaCO_3$, among of them; calcite, aragonite and vaterite are non-hydrated crystals (Li et al., 2010b). Calcite is the most stable phase with the typical habits of rhombohedron, scalenohedron, and prismatic habits (Cizer et al., 2008). It has been found that calcite and aragonite have stable thermodynamic structures, while vaterite is thermodynamically unstable so that it is easily transformable to calcite or aragonite in aqueous solution (Li et al., 2010b).

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2.2 CFB Mechanisms

Three main existing groups of microorganisms that can induce calcite formation via their metabolic processes are (Castanier et al., 1999; Hammes and Verstraete, 2002; Ariyanti et al., 2012; Seshagiri Rao et al., 2013);

(i) photosynthesis organisms such as *cyanobacteria* and *algae* that remove carbon dioxide (CO_2)

(ii) sulphate reducing bacteria (SRB) that are responsible for the dissimilatory reduction of sulphate

(iii) several organisms which are involved in the nitrogen cycle via any one of the methods such as oxidative deamination of amino acids, nitrate reduction or urea hydrolysis

Potential microbial source for calcite formation stated in literature are Arthrobacter genera (Ercole et al., 2001), Actinomycetes (Cacchio et al., 2003; Baskar et al., 2006), *Pseudomonas aeruginosa*, *Pseudomonas putida* (De Muynck et al., 2010), *Vibrio* sp. (Rivadeneyra et al., 1993) and *Myxococcus xanthus* (Rodriquez-Navarro et al., 2003). Fungi and algae also have been involved in the calcite formation (Northup and Lavoie, 2001). Fungal hyphae may act as nucleation sites for crystal formation (Northup and Lavoie, 2001). It will be a great start of this preliminary screening on calcite formation with calcite forming bacteria as the bacteria are presence in abundance in nature and reproduced easily.

Bacteria forms calcite through four main metabolic processes such as: urea hydrolysis, denitrification, sulphate reduction and CA type of calcite formation (Dhami et al., 2013). Generally, there are four most important factors affect the bacterial calcite formation, *i.e* (1) the calcium concentration; (2) the concentration of dissolved inorganic carbon (DIC); (3) the *pH*; and (4) the availability of nucleation sites (Hammes and Verstraete, 2002). The primary role of bacteria is to create an alkaline environment (high *pH* increased DIC) through various metabolic processes (Hammes and Verstraete, 2002).

Urea will be hydrolyzed into ammonia and carbonate ions in the presence of urease enzyme (Castanier et al., 1999). Ammonia will increase the *pH* which favors the calcite formation in the presence of calcium ions (Castanier et al., 1999). For denitrification, the initial steps of calcite formation is to produce alkalinity and CO_2, where nitrate serves as an electron acceptor and it ultimately reduced to nitrogen gas (Castanier et al., 1999). The produced CO_2 with the alkaline environment results in the formation of calcite in the presence of calcium ions (Van Passen et al., 2010).

Sulphate reduction mechanisms carried out by the SRB in sulphur cycle under anoxic conditions. SRB produce carbonate, bicarbonate ions and hydrogen sulphide (Castanier et al., 1999). Hydrogen sulphide (will induce pH increase) and CO_2 are produced, resulting in the formation of calcite in the presence of calcium ions (Castanier et al., 1999). CA is a zinc-containing enzyme which catalyzes the reversible conversion of CO_2 to bicarbonate, which would then be available for calcite formation (Rahman and Oomori,

2010).

Bacterial calcite formation has wide range of industrial applications such as restoration of calcarous stone materials (Tiano et al., 1999), bioremediation (Ferris, 2003), wastewater treatment (Hammes et al., 2003), strenghtening of concrete (Ramachandran et al., 2001) and selective plugging for enhanced oil recovery (Ferris and Stehmeir, 1992; Gollapudi et al., 1995; Nemati and Voordouw, 2003).

2.3 Previous Reported Work on CFB

Numerous researchers have demonstrated the capability of microorganisms from various environments to induce calcite formation in laboratory (Boquet et al., 1973; Ferris and Stehmeier, 1992; Rivadeneyra et al., 1993; Erlich 1998; Rivadeneyra et al., 1998; Castanier et al., 1999; Stocks-Fisher et al., 1999; Tiano et al., 1999; Knorre and Krumbein, 2000; Ercole et al., 2001; Cacchio et al., 2003; Lee, 2003; Nemati and Voordouw, 2003; Rodriguez-Navarro et al., 2003; Whiffin, 2004; Nemati et al., 2005; Baskar et al., 2006; Dejong et al., 2006; Lian et al., 2006; Baskar et al., 2007; Whiffin et al., 2007; De Muynck et al., 2008; Ivanov and Chu, 2008; Achal et al., 2009; De Belie and De Muynck, 2009; Sarda et al., 2009; Achal et al., 2010; Arunachalam et al., 2010; De Muynck et al., 2010; Jonkers et al., 2010; Park et al., 2010; Van Passen et al., 2010; Ariyanti et al., 2012; Elmanama and Alhour, 2013; Seshagiri Rao et al., 2013).

This shows that there is a wealth of literature about CFB, and different studies have demonstrated different usage of CFB. A brief summary of some of the earlier research, on this topic is presented below. Most of the available literatures on the topic were reported by researchers abroad. However to the authors' best knowledge; there is no published material on CFB isolated in Malaysia. Recently Ng et al. (2012) from Malaysia did research on soil improvement by calcite formation of urease type *Bacillus megaterium*. There are some evidences on research done on cave in Malaysia as Foo and Tan (2012) study the diversity of culturable actinomycetes from a Kandu Cave (Perak, Malaysia) for the production of metabolites in pharmacology.

Nor Bakhiah (2010) did master study on hydrogeochemical of caves drip water throughout Peninsular Malaysia which covers Batu Caves Hill (Selangor), Gunung Tempurung (Perak), Gunung Senyum (Pahang), Gunung Jebak Puyuh (Pahang), Kaki Bukit (Perlis), Bukit Baling (Kedah). Ros Fatihah (2010) studied geomorphology and origin of Gua Tempurung, and her studies shown that the cave has undergone a complex and long history of development. Besides, its formation can be related to the evolution of the Kinta Valley landscape with evidences preserved in the cave. In summary, this is the pioneer research on CFB isolated in Malaysia.

Ercole et al. (2001) from Italy has isolated and identified *Bacillus sphaericus* and *Bacillus firmus* from cave samples; their research is more into finding of bacterial action in cave speleothem formation. Cacchio et al. (2003) from Italy also performed a study on CFB isolated from cave samples. They stated that *Bacillus megaterium* is an abundant species in their study. The objective of their study is to investigate the role of CFB in monument protection, one of the possible industrial applications of CFB. In addition, Lee (2003) from Korea studies calcite formation by *Bacillus amyloliquefacience* CMB01 for the application into restoration of deteriorated calcareous monuments.

Whiffin (2004) worked with two different types of bacteria, *Proteus vulgaris* and *Sporosarcina pasteurii* (*Bacillus pasteurii*) to find out the highest urease producer among these two type of bacteria. The objectives of the study was to develop an industrially suitable cost-effective bacterial process for the

production of urease active cells, and investigate the potential of urease active cells to act as a catalyst for the production of bio-cement.

Baskar et al. (2006, 2007) from India reported calcite formation by bacteria isolated from cave samples. Baskar et al. (2006) identified *Bacillus thuringiensis* and *Bacillus pumilus* strains from their study. They did propose that the optimum temperature and bacterial activity appears to be the important factors in calcite formation, eventually the formation of stalactites. Up to date, no further research was published on those identified bacteria. Their investigation mostly concentrated on finding out the origin of caves formation. Interestingly, Barabesi et al. (2007) from Italy suggested that there are link between calcite formation and fatty acid metabolism, but till date no further research article was published on further experiments to prove the link.

Sarda et al. (2009) demonstrated the ability of *Bacillus pasteurii* in biocalcification to improve the durability of bricks by reducing water absorption. In their studies, they included the bacterial screening for the maximum urease production which includes *Bacillus pasteurii*, *Brevibacteriummammoniagenes*, and *Bacillus lentus*. *Bacillus pasteurii* was found to have the maximum urease production. This justify the fact that most of the preliminary researches on calcite formation focused specifically on *Bacillus pasteurii* and generally on urease type of bacteria (Stocks Fisher et al., 1999; Bang et al., 2001; Ramakrishnan et al., 2001; Ferris et al., 2003; Whiffin, 2004; Whiffin et al., 2007; Achal et al., 2009; Sarda et al., 2009; Arunachalam et al., 2010; Li et al., 2011a; Patel and Patel, 2011).

Arunachalam et al. (2010) from India studied the calcite forming ability of *Bacillus sphaericus*, and the application of *Bacillus pasteurii* in bio-cement. *Bacillus pasteurii* (now known as *Sporosarcina pasteurii*) seems to be the first bacteria to record the calcite forming ability into application in building industry (Boquet et al., 1973; Stocks-Fisher et al., 1999). Beside *Bacillus pasteurii*, *Bacillus sphaericus* was another species with similar entity, having the capability of calcite formation (Arunachalam et al., 2010). De Muynck et al. (2008; 2010) from Belgium studied about *Bacillus sphaericus* for its application in building industry.

Recently, Hammad et al. (2013) studied the urease activity and induction of calcite formation for the bacterium, *Sporosarcina pasteurii*. They have tested the induction of calcite formation in both solid and broth media. Kumar et al. (2013) studied on *Bacillus flexus*, *Bacillus pasteurii* and *Bacillus sphaericus* calcite formation on improving compressive strength of mortar cubes. The authors found that *Bacillus flexus* have better potential of calcite production than *Bacillus pasteurii* and *Bacillus sphaericus*. This shows that the previous studies contain much of the laboratory work that dealt with CFB. Some of those bacterial strains found to be involved in calcite formation are listed in Table 2.1.

Most of the studied bacteria on calcite formation are based on urea hydrolyzing mechanism simply because they are easy to control and comply with three prerequisites needed: (i) provide abundant nucleation sites, (ii) high urea hydrolysis rate, (iii) resistant to cementation solution and resistant to

varying *pH* (Henriques, 2011). 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 (Van Passen et al., 2010). The use of aerobic bacteria in urea hydrolysis unable to grow in situ due to lack of oxygen, which will results in decay of the calcite formation in time (Van Passen et al., 2010). Van Passen et al. (2010) described the feasibility of calcite formation by denitrification, another process of calcite formation to overcome the problem occurred in urea hydrolysis mechanism. *Castellanielladenitrificans,* facultatively anaerobic organism was found through their research.

Table 2.1: List of calcite forming bacteria found in literature.

Author	Species
Bacillus pasteurii	Boquet et al., 1973 Erlich, 1998 Bang et al., 2001 Ramakrishnan et al., 2001 Ferris et al., 2003 Whiffin, 2004 Whiffin et al., 2007 Achal et al., 2009 Sarda et al., 2009 Arunachalam et al., 2010 Li et al., 2011a Patel and Patel, 2011 Hammad et al. 2013 Kumar et al., 2013
Bacillus sphaericus	De Muynck et al., 2008 De Belie and De Muynck, 2009 Arunachalam et al., 2010 Kumar et al., 2013
Bacillus subtilis	Erlich, 1998 Stocks-Fisher et al., 1999 Ramakrishnan et al., 2001 Baskar et al., 2006 De Muynck et al. 2008 SeshagiriRao et al. 2013
Bacillus cereus	Erlich, 1998 Stocks-Fisher et al., 1999 De Muynck et al. 2008
Bacillus pumilus	Boquet et al., 1973 Baskar et al., 2006 Yadav et al. 2011 Silva-Castro et al., 2013
Bacillus megaterium	Lian et al., 2006 Ng et al., 2012
Bacillus thuringiensis	Baskar et al., 2006
Bacillus amyloliquefaciens	Lee, 2003
Bacillus lentus	Dick et al., 2006
Bacillus pseudofirmus	Jonkers et al., 2010
Bacillus cohnii	Jonkers et al., 2010
Bacillus mycoides	Elmanama and Alhour, 2013
Bacillus flexus	Kumar et al., 2013

2.4 Carbon Dioxide Sequestration by CFB

Conversion of CO_2 into carbonates in the process of calcite formation is a environmentally safe and stable product for long time CO_2 sequestration (Dhami et al., 2013). Naturally, CO_2 sequestration is based on CO_2 sequestration in the form of calcite over geological time scale (Dhami et al., 2013). CA is a key enzyme in the chemical reaction of living organisms which linked with calcite formation (Rahman et al., 2007). CA is a zinc containing enzyme which catalyzes the reversible hydration of CO_2 (Kanbar, 2008). Generally, in order to capture CO_2 for calcite formation, the CO_2 should undergo a number of transformations such as the dissolution in an aqueous phase, hydration by water, ionization, and carbonate formation. Among these, the hydration of the CO_2 is the slowest step. It has been shown that the hydration of CO_2 can be enhanced by CA (Bond et al., 2001; Kanbar, 2008).

Yadav et al. (2011) summarized that bacterially produced CA specifically by *Bacillus pumilus* are capable to promote calcite formation. Whereas Li et al. (2011b) also found that that CA protein produced by *Bacillus* sp. isolated from karst soil able to promote calcite formation. Recently, CA was widely used for CO_2 sequestration. Bond et al. (2001) established an integrated system to accelerate the CO_2 hydration into carbonate mineral formation by using CA. Liu et al. (2005) studied the precipitation of $CaCO_3$ from produced waters (from oil and gas production) in the presence of the CA enzyme. Mirjafari et al. (2007) studied the CA application to improve the hydration of CO_2 in the solution. Favre et al. (2009) reported the detailed study of CA for $CaCO_3$ formation. In summary, CA-type of calcite formation is shall

be used to fix CO_2 which has a capability to reduce the amount of CO_2 in the earth.

2.5 Industrial Applications of *Bacillus pumilus*

Bacillus pumilus is one of the identified bacteria via both molecular and biochemical characterization in our current study. This strain shows the maximum CA activity and proven for CO_2 sequestration ability in our current study, therefore possible industrial applications of *Bacillus pumilus* stated in the literature is presented here. *Bacillus pumilus* industrial capability was proved for xylanase production (Duarte et al., 2000; Marta et al., 2000; Battan et al., 2007; Bindu et al., 2007; Ayesegul et al., 2008; Kapoor et al., 2008; Liu and Liu, 2008; Monisha et al., 2009; Buthelezi et al., 2010), cellulase production (Kotchoni et al., 2006), lipase production - biocatalyzed (Ruiz et al., 2002), antibacterial compounds production (Hassan et al., 2009), D-Ribose production – flavor enhancer in food, health food, pharmaceuticals, and cosmetics (Miyagawa et al., 1992), endoglucanase production (Hidayah et al., 2008), and bacteriocins production (Aunpad and Na-Bangchang, 2007). *Bacillus pumilus* are also able to degradate keratinous waste (Kumar et al., 2008), and reduction of chromium (Shakoori et al., 2010) apart from the ability of various enzymes production. Chromium is the toxic metal which poses toxic effects to human and the environments (Shakoori et al., 2010), whereby the chromium reduction capability of *Bacillus pumilus* will brings enormous benefits.

Xynalase are mostly produced from bacterial fermentation processes, which has wide industrial and biotechnological applications (Buthelezi et al., 2010). As stated in previous paragraph, numerous researchers have done studied on *Bacillus pumilus* xynalase production by considering the wide

industrial applications. There are numerous applications of cellulose produced by *Bacillus pumilus* in various industries such as brewery and wine, textile, detergent, pupl and paper industries (Kotchoni et al., 2006), in agriculture and in animal feeds (Bhat and Bhat, 1997; Jang and Chen, 2003).

In addition, bacteriocin has a potential application as natural preservatives (Klaenhammer, 1988) and therapeutic application as an antibacterial agent (Gray et al., 2006). Besides, bacteriocin usage as a replacement for currently used antibiotics is promising (Papagianni, 2003). In conclusion, *Bacillus pumilus* has a lot of industrial significance, by considering the nature of bacteria which are able to reproduce easily at the lowest cost.

2.6 Characterization of Bacteria

2.6.1 Molecular Characterization

A molecular characterization method often depends on the analysis of chromosomal or extrachromosomal DNA (deoxyribonucleic acid) (Farber, 1996). These methods have many advantages over phenotypic methods in terms of better discriminatory power and reproducibility (Farber, 1996). The most common genotypic characterization methods used are; plasmid profiling, nucleotide sequencing, pulsed-field gel electrophoresis, PCR (polymerase chain reaction) based methods, REP (repetitive sequence-based) PCR, PCR ribotyping, RAPD (random amplified polymorphic DNA), 16S-ITS (internally transcribed spacer) rDNA region-RFLP (restriction fragment length polymorphism) (Farber, 1996).

PCR is based on the *in vitro* amplification of DNA by a thermostable DNA polymerase enzyme (usually *Taq* polymerase from *Thermusaquaticus*). Special primers are used in order to amplify the region of interest. PCR includes repeated cycles of high temperature for denaturing the DNA, primer annealing and an extension step in which the complementary DNA is synthesized by the action of a heat stable polymerase. At the end of each cycle the number of copies of the chosen sequence is doubled. Thus the amount of the target sequence is increased exponentially (Bush and Nitschko, 1999). The amplified target sequence of a given bacteria can be used for 16S rDNA sequencing.

16s rDNA is a powerful tool to trace phylogenetic relationship between bacteria and to identify bacteria from various sources (Mignard and Flandrois, 2006). Identification based on the 16S rDNA sequence is popular because 16S rDNA sequence exists universally among bacteria and includes regions with species-specific variability which makes it possible to identify bacteria to the genus or species level by comparing with databases in the public domain (Vandamme et al., 1996; Mignard and Flandrois, 2006).

2.6.2 Phenotypic Characterization

For determining new isolate belongs to which of the major groups, certain phenotypic characteristics have primary importance shown below (Smibert and Krieg, 1994);

- Morphology – rod, coccus, vibrioid, helical or other
- Gram status – positive or negative
- Motility
- Pigment formation
- Nutritional classification – phototrophic, chemoautotrophic, chemoheterotrophic
- Oxygen demand – aerobic, anaerobic, microaerophilic
- Presence of spores – endospores, exospores

- Antibiotic sensitivity
- Ability to use various carbon, nitrogen, and sulfur sources

An organism can be classified at genus and/or species level using various biochemical reagents. The use of phenotypic techniques is limited by the appearance of phenotypic differences of isolates of the same strain (Bush and Nitschko, 1999). Therefore, classical phenotypic methods are often not suitable or insufficient for differentiation of microorganisms (Farber, 1996). Therefore genotypic and phenotypic methods must be used together in order to classify a bacterium.

Conventional biochemical assay is used for the identification of wide variety of bacteria based on the biochemical properties of bacteria (Cacchio et al., 2003). It is a system that utilizes automated biochemical methodologies that tests the bacteria's ability to utilize a panel of 95 carbon sources in microplate (Bochner, 2009). This test includes positive and negative control (Morgan et al., 2009). Each well of the microplates contains a different carbon source along with tetrazolium violet (Miller and Rhoden, 1991). The chemistry is rehydrated by the inoculation of the well with a cell suspension of the test organism. Utilization of the carbon source in a well results in a redox reaction, which forms a purple colour, or a turbidity change (Morgan et al., 2009). The pattern of utilization of carbon sources plays an important role in differentiating species.

The system uses a computer and software to interpret the pattern of purple wells in the microplate (Miller and Rhoden, 1991; Bochner, 2009). Different strains of bacteria yield distinct patterns, and different species will give distinct families of patterns that are recognized by software (contains bacterial database). The conventional biochemical assay technique is simple and user friendly in operation with minimum sample preparation protocols and the technology exhibits high level of accuracy in identification of aerobic bacteria, anaerobic bacteria, yeast and filamentous fungi (Morgan et al., 2009).

2.7 Concluding Remarks

Considering the climate change issue which caused by excess amount of CO_2, CA type of CFB shall be utilized for CO_2 sequestration. Therefore, this study primarily deals with the CFB isolated from limestone and soil sample which includes CFB isolation, CFB mechanism identification, characterization of selected bacterial species by molecular and biochemical identification, and optimization of selected bacterial growth. Finally, CO_2 sequestration ability of selected bacteria will be studied.

3 CHAPTER

METHODOLOGY

3.1 Research Framework

Figure 3.1 shows the flow chart of the overall experimental works. This research was divided into six major stages: limestone and soil samples collection, isolation of CFB, distinguishing the CFB mechanisms, bacterial characterization by molecular and biochemical identification, determination of growth conditions of selected bacteria, and finally carbon dioxide (CO_2) sequestration by selected bacteria. Growth conditions were optimized for temperature; *pH* and glucose concentration as those parameters plays a vital role in calcite formation. Lastly, CO_2 sequestration ability was examined in three different treatments, with bacteria, without bacteria and without continuous supply of CO_2 to understand the effects of bacterially produced carbonic anhydrase (CA) in CO_2 sequestration.

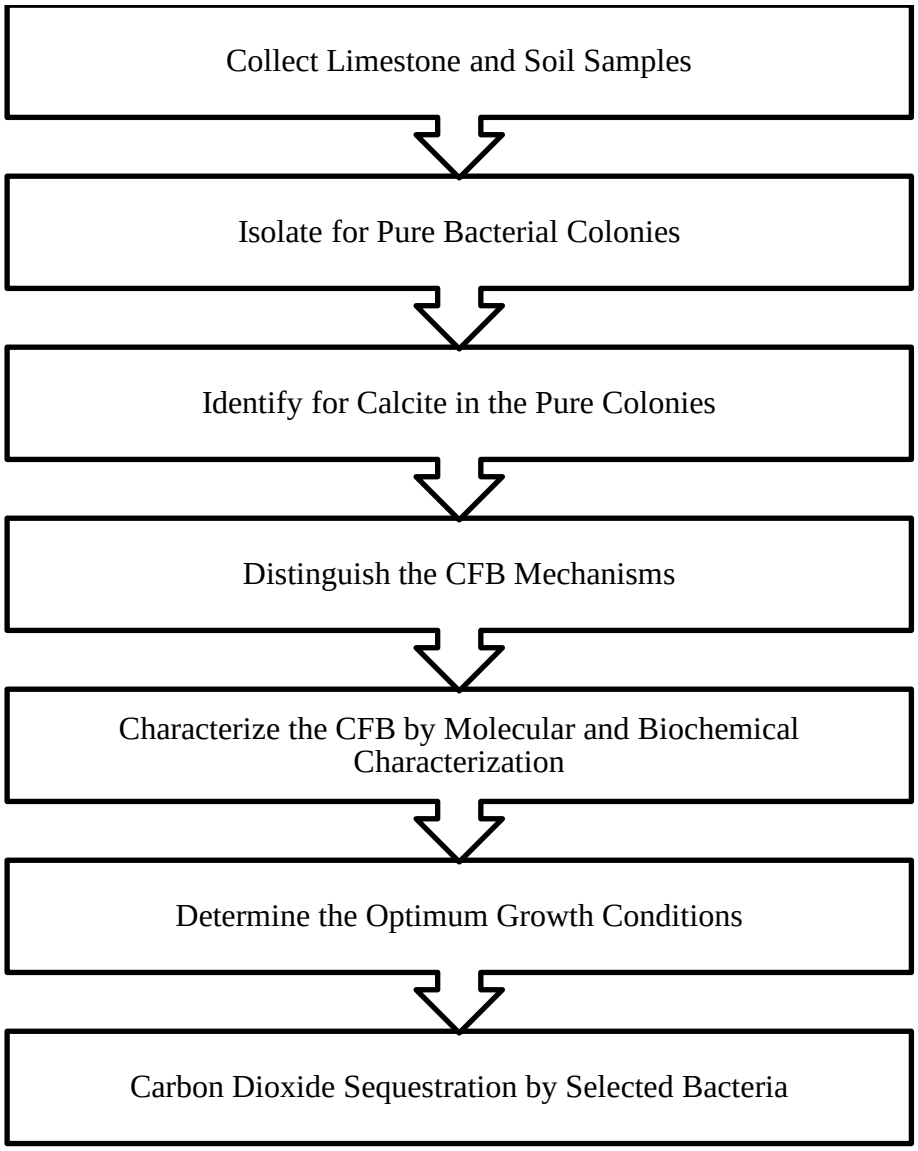

Figure 3.1: Flow chart of the overall experimental works in this study.

3.2 Materials

3.2.1 Chemicals

The media used for bacterial isolation were shown in table 3.1. Isolation media was used for primary bacterial isolation, broth culture media was used for the bacterial culture conditions optimization, and nutrient agar was used to maintain bacterial culture.

Table 3.1: Media used in isolation and maintaining of bacteria.

Media	Quantity
Isolation Media (g/L)	
Calcium acetate	2.5
Yeast Extract	4.0
Glucose	10.0
Agar	18.0
Broth Culture Media (g/L)	
Calcium acetate	2.5
Yeast Extract	4.0
Glucose	10.0
Media Used for Maintain Bacteria (g/L) – Nutrient Agar	
Peptone	5.0
Meat Extract	3.0
Agar-agar	15.0
pH 7.0 $\pm$ 0.2	

The stains and indicators used for gram staining are shown in table 3.2. Solutions A and B were mixed to obtain crystal violet staining reagent. For the iodine solution, iodine and potassium iodine were grinded. Water was added slowly and the solution was stirred until the iodine was dissolved. The solution was stored in an amber bottle. Table 3.3 shows the overall chemicals used for DNA extraction experiment for molecular identification by 16s rDNA sequencing method.

Table 3.2: Stains and indicators used in gram staining method.

Stains and Indicators	Quantity
Crystal Violet Staining Reagent (Solution A)	
Crystal violet	2 g
Ethanol (95%)	20 ml
Crystal Violet Staining Reagent (Solution B)	
Ammonium oxalate	0.8 g
Distilled water	80 ml
Iodine Solution	
Iodine	1 g
Potassium iodine	2 g
Distilled water	300 ml
Safranin Solution	
Safranin (2.5% in 95% alcohol)	10 ml
Distilled water	100 ml

Table 3.3: Chemicals used in DNA extraction method.

DNA Extraction	Quantity
Extraction Buffer	
TE Buffer	460 µl
10% SDS	30 µl
Proteinase K	10µl
Total	**500 µl**
Phenol: Chloroform: Isoamyl alcohol (25: 24: 1)	
Phenol	5 ml
Chloroform	4 ml
Isoamyl alcohol	1 ml
Total	**10 ml**
1M Tris-HCI	
Tris (hydroxymethyl) aminomethane	60.57 g
Distilled water	500 ml
Total	**500 ml**
0.5M EDTA	
EDTA	18.6 g
Distilled water	100 ml
Total	**100 ml**
TE Buffer	
1M Tris-HCI	10 ml
0.5M EDTA	2 ml
Total	**12 ml**

Table 3.4 shows the buffers used for electrophoresis gel to run the DNA extraction and PCR product. For the 10X TBE buffer, firstly, all the chemicals are dissolved in slightly less than 1 liter of water. The buffer was adjusted to 1 liter of volume by adding distilled water and there is no requirement of sterilization.

Table 3.4: Buffer used for agarose electrophoresis gel.

Buffer	Quantity
10X TBE Buffer	
Tris Base	108.0g
Boric Acid	55.0 g
0.5 M EDTA	20.0 ml
Distilled water	1.0 L
Total	**1.0 L**
1 X TBE Buffer	
10X TBE Buffer	100.0 ml
Distilled water	900.0 ml
Total	**1.0 L**

3.3 Isolation of CFB

3.3.1 Isolation of crystals forming colonies

Bacteria from the collected limestone and soil samples were isolated and screened for calcite crystals formation in B4 medium. B4 medium is made of 4 g yeast extract, 2.5 g calcium acetate, 10 g glucose and 18 g agar per liter of distilled water. The B4 medium contains calcium acetate as a calcium source to induce the calcite formation. Initially, the collected samples were powdered in a porcelain mortar and pestle, and 1 g of the powdered samples was suspended in 9 ml sterile saline solution and mix vigorously. Triplicate B4 medium spread plates on petri plates with sample dilutions ranging from 10^1 to 10^5 were inoculated. Inoculated plates were incubated at 37 $^{\circ}$C 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 incubated together with experimental samples. The individual colonies were selected and purification of the individual colonies carried out by repeated streaking on B4 agar plates. For ease of reading, the isolated strains were assigned specific names according to the sample ID, followed by SC stands for subculture, and the strains were numbered to differentiate between the different isolated colonies from the same sample.

3.3.2 Crystals Identification

To identify whether the crystals formed by bacteria were calcite, firstly a simple test with diluted hydrochloric acid (HCI) was carried out. If calcite was present, it will dissolve in the presence of HCI, with the release of tiny gas bubbles of carbonate. More detailed crystals characterization relied on X-ray Diffraction (XRD) analyses as it is a fast and reliable tool for routine mineral identification.

XRD was used to determine the mineralogy of crystals formed by bacteria by qualitative and quantitative manners (El-Hajji, 2006). The qualitative analysis revealed the mineralogy make up in sample, while the quantitative analysis was used to measure the abundance of those minerals in the sample. Following the qualitative analysis, the identification can be accomplished by comparing the diffraction with known standard mineral (El-Hajji, 2006). In the direct comparison method, the unknown mineral can be identified by comparing the resulting strongest diffraction peak (s) with those of known mineral standards (El-Hajji, 2006). Quantitative analysis requires deliberate and careful sample preparation, good quality data and a detailed understanding of the material being analyzed.

For this research study, the qualitative method was used for the mineral identification as the intent of the research is to identify the type of mineral present in the samples. For this, the diffraction was compared with standard mineral (Calcite) and commercial software was used to identify the minerals associated with the strongest peaks following the diffraction process. XRD

measurements were done on both collected limestone and soil samples and collected crystals formed by bacteria.

The limestone samples were powdered using porcelain mortar and pestle, before subjected to XRD analysis. Then, the crystals containing solid media was cut into flat square blocks and placed in clean microscopic glass slides (75 x 25 mm). The glass slides together with crystals were dried in dryer (70 °C) for 3 days to make sure that the agar was evaporated and left with crystals. In all experiment, the un-inoculated agar blocks were included as control. It was suspected that there were background signals due to the presence of agar. The supply voltage of the X-ray tube was set at 50 kV and 30 mA. The 2 θ scan range was between 22 ° and 50 °; each scan was done in steps of 0.05 °. The crystalline phases were identified using the International Center for Diffraction Data (ICDD) database (Joint Committee on Powder Diffraction Standards, JCPDS).

3.4 Characterization of CFB

3.4.1 Gram staining

Gram staining is the fundamental to the phenotypic characterization of bacteria which is a differential staining procedure, based on the bacterial ability to retain the colour of the stains used in the procedure. Gram positive cells have a thick peptidoglycan layer and stain blue to purple, whereas gram negative cells have a thin peptidoglycan later and stain red to pink.

A loopful of tap water was placed on a slide and a small sample of colony (grown on agar plates) was transferred to the drop and emulsified. The smear on the slide was fixed by passing the slide three times through the flame of a Bunsen burner. The smear was stained with crystal violet solution for up to one minute. The slide rinsed under running tap water for some seconds (note: after each staining solution the slide rinsed running tap water). Then, immersed with iodine solution, and allowed to act (as a mordant) for about one minute, rinsed with running tap water. Followed by 95 % alcohol for 10 seconds, and rinsed with running tap water. Then stained with safranin solution and allows counterstaining for 30 seconds and dried on paper towels. Finally, the cells morphology was then examined under oil immersion lens using binocular microscope. Gram positive cells will appears purple/blue while gram negative cells seemed pink/red.

3.4.2 Characterization of CFB mechanisms

As stated in chapter 2, section 2.2 CFB mechanisms, bacteria forms calcite through four main metabolic processes such as: urea hydrolysis, denitrification, sulphate reduction and CA type of calcite formation (Dhami et al., 2013). Therefore, urea hydrolysis, hydrogen sulphide (H_2S) production, denitrification, nitrate reduction, nitrite reduction, ammonification, and extracellular CA assay were done on all the isolates to determine the calcite forming mechanisms.

Firstly, urea hydrolysis test is performed by growing the test organisms on urea broth containing phenol red as *pH* indicator. During the incubation, the urease enzyme produced by bacteria will hydrolyze urea into ammonia that raises the *pH* of the medium which indicates by the change in colour to pink (Vashist hemraj et al., 2013). Secondly, H_2S production can be detected by incorporating a heavy metal salt containing ion (Fe^{++}) as a H_2S indicator. H_2S a colourless gas when produced reacts with metal salts ($FeSO_4$) forming visible insoluble black ferrous sulphide (FeS) precipitates.

Thirdly, the production of ammonia from organic compounds is called ammonification, for this the bacteria will be inoculated into peptone broth and the amount of ammonia produced interpreted by the change in colour. Fourthly, bacteria produce the enzyme nitrate reductase and nitrite reductase. These two enzymes catalyze two reactions involving the conversion of nitrate compound into end product of nitrogen gas. Principally, nitrate (NO_3^-) is reduced to nitrite (NO_2^-) by nitrate reductase and nitrite reduced to nitrogen gas

by nitrite reductase. Presence of nitrate and nitrite reductase indicates by the change of colour. Fifthly, the reduction of nitrate to nitrogen gas (N_2) or nitrous oxide (N_2O) is called denitrification (Elmer W. Koneman. 2005) and the presence of gas bubbles indicates positive reaction.

CA activity was determined by measuring the esterase activity, which is by measuring the amount of *p*-nitrophenol (*p*-NP) produced by spectrophotometrically. The concept of esterase activity refers to *p*-nitrophenyl acetate substrate hydrolyzed by the CA enzyme into *p*-NP, whereby the change can be observed spectrophotometrically at 348nm to estimate the amount of *p*-NP produced. In all experiments, media un-inoculated with bacterial cells were included as control. All the assays were done in triplicates.

3.4.2.1 Enzymatic Hydrolysis of Urea

The samples from bacterial broth culture were inoculated in urea broth and incubated in incubator shaker at 30 °C, 200 rpm. A positive test indicated a change in colour from red to hot pink or magenta.

3.4.2.2 Hydrogen Sulphide (H_2S) Production

The samples from bacterial broth culture were inoculated in Sulfide – Indole – Motility (SIM) media and incubated at 30 °C, 200 rpm. If sulphide is produced, it combines with iron compounds to produce FeS, a black precipitate.

3.4.2.3 Ammonification

The bacterial culture was added to a tube of peptone broth, and incubated at 30 °C for 12 – 48 hours. A drop of Nessler's reagent was deposited into a depression of spot plate, and a drop of inoculated peptone broth culture was added to the Nessler's reagent in the depression. The production of ammonia was interpreted by the changes in colour. Brown precipitate indicates the presence of ammonia by a large amount, while orange colour indicates a relatively lower concentration of ammonia.

3.4.2.4 Nitrate Reduction Test

The bacterial culture was added to a tube of ammonium sulfate broth, and incubated at 30 °C for 12 – 48 hours. Three drops of Trommsdorf's solution was placed on spot plate. By using separated pipet tips, one drop of diluted sulfuric acid was mixed together. Then, a drop of bacterial culture was added with a pipet. The amount of nitrate present was interpreted by colour of the bacterial culture. Blue/black colour indicates the presence of a large amount of nitrate.

3.4.2.5 Nitrite Reduction Test

The bacterial culture was added to a tube of nitrite broth, and incubated at 30 °C for 12 – 48 hours. 200 ml reagent N-(1-naphthyl) ethylene diamine was added followed by 200 ml of α-naphthol reagent. A red-violet colour indicates the presence of nitrite.

3.4.2.6 Denitrification

The bacterial culture was added to a tube of nitrate broth durham (containing an inverted vial), and incubated at 30 °C for 12 – 48 hours. After the incubation, the nitrate broth culture durham tube was observed for a gas bubble inside the inverted vial. The results were interpreted by observation of presence (positive) or absence (negative) of gas.

3.4.2.7 Extracellular Carbonic Anhydrase Assay

For the extracellular CA test by esterase activity, the extracellular bacterial cells were harvested at 48 hours by centrifugation at 8,000 rpm for 10 minutes at 25 °C. Esterase enzymatic activity was determined by using the spectrophotometric assay described by Prabhu et al. (2009). In brief, the assay consisted of 1.8 ml of 0.1 M phosphate buffer and 1.0 ml of 3 mM *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 348 nm. The change in absorbance at 348 nm was recorded over first 5 minutes to estimate the amount of the *p*-NP released. The non-enzymatic hydrolysis was subtracted by using a blank without enzyme. The absorbance values of *p*-NP were measured in UV/VIS spectrophotometer at 348 nm. One unit of activity represents the amount of enzyme catalyzing to produce 1 µM *p*-NP per minute under the assay conditions. To convert UV/VIS spectrophotometer data of bacteria into concentration values, a calibration curve of *p*-NP were measured. One of the products of *p*-nitrophenyl acetate degradation is the *p*-NP. A concentration of 0.01 to 0.10 µM of *p*-NP was

dissolved in 0.1 M phosphate buffer. Then absorbance values were measured in UV/VIS spectrophotometer at 348 nm. At the end, the calibration curve of concentration versus absorbance was established.

3.4.3 Molecular Characterization by Amplification of 16s rDNA Region

3.4.3.1 Extraction of Genomic DNA

24 hour cultures grown in nutrient broth were pelleted by centrifugation for 10 minutes at 10 000 rpm and supernatant was discarded. The pellet was re-suspended in extraction buffer (TE Buffer, SDS, and Proteinase K). The eppendorf tubes were mixed thoroughly and the samples were incubated for 1 hour at 37 $^{\circ}$C. An aliquots of 500 µl Phenol: Chloroform: Isoamyl alcohol (25:24:1) was added and the tube was inverted gently 5 to 6 times before centrifuged at 14 000 rpm for 10 minutes. By using wide bore pipette tips, 500 µl of the top aqueous layer was removed carefully to a new microcentrifuge tube without disturbing tissue debris at the interface. 250 µl phenol and 250 µl Chloroform: Isoamyl alcohol were added and inverted 5 to 6 times. 500 µl top aqueous layers were transferred to a new tube using wide bore tips, and 500 µl Chloroform: Isoamyl alcohol was added; centrifuged at 14 000 rpm for 10 minutes. This step was repeated once again to make sure the purified DNA was extracted. 500 µl of the top aqueous layer was transferred to a new microcentrifuge tube, and two volumes of cold absolute ethanol (1 ml) and 40 µl of NaCl were mixed with the top aqueous layers by several rapid abrupt inversions of the tubes for 5 to 6 times.

Then, the tubes were centrifuged at 14 000 rpm for 15 minutes. The precipitated genomic DNA was collected at the bottom of the tube after pouring out the ethanol carefully. The pellet was washed with 1 ml of 70 %

ethanol for 1 hour. The ethanol was decanted and the remaining ethanol was removed with a micropipette. The pellet was dried at room temperature for about 15 to 20 minutes. Finally, 50 µl TE buffer (1 M Tris-HCI, 0.5 M EDTA, and sterile distilled water) was added and kept at 4 $^{\circ}$C for overnight.

3.4.3.2 Agarose Gel Electrophoresis of Extracted Genomic DNA

Genomic DNA quality was checked in 0.8 % agarose gel. For this purpose 0.8 gram agarose were dissolved in 100 ml 0.5 X TBE buffer by boiling. Then it was poured into the gel casting stand and combs were placed. After the gel solidified the combs were removed. The casting tray carrying the gel was placed into the tank. TBE buffer was carefully added until the buffer was 2 – 3 mm above the surface of the gel. 2 µl of extracted genomic DNA were taken and mixed with 1 µl of loading buffer. The samples were loaded into the wells of agarose starting from the second well. Finally 3 µl Lamda DNA/ Hind III were loaded into the first well. Electrophoresis was performed for approximately 60 minutes at 90 mA. The extracted genomic DNA was visualized on an UV transilluminator (Electrophoresis Documentation and Analysis System 120) with Kodak Digital Science 1D – Imaging Software.

3.4.3.3 Optimization of 16s rDNA Region Amplification

Polymerase Chain Reaction (PCR) was performed for the amplification of 16s rDNA region. A series of optimization of conditions were conducted to ensure the best PCR conditions for amplification by determining two parameters namely $MgCl_2$ concentration and annealing temperature, T_a. The optimization

of $MgCl_2$ and T_a were performed using PCR Machine (Thermal Cycler PTC-200 DNA Engine). Amplification was performed using the following amplification conditions:

Step 1: 94 °C for 2 minutes (initial denaturation)

Step 2: 94 °C for 1 minute (denaturation)

Step 3: 50 °C – 60 °C for 1 minute (annealing) } 34 cycles

Step 4: 72 °C for 1 minute (elongation)

Step 5: 72 °C for 1 minute (final extension)

DNA oligoprimers used in this experiment were:

Forward Primer = BacF (5'-GGGAAACCGGGGCTAATACCGGAT-3')

Reverse Primer = R1378 (5'-CGGTGTGTACAAGGCCCGGGAACG-3')

The forward primers (BacF) are especially for *Bacillus* sp. and related taxa, whereas reverse primers (R1378) are universal bacterial 16s rDNA (Heuer et al., 1997; Garbeva et al., 2003). There were four different $MgCl_2$ concentrations used for optimization; 1.5 mM, 2.0 mM, 2.5 mM, and 3.0 mM. Concentrations of each reagent for master mix in each different concentrations of $MgCl_2$ were shown in table 3.5. The annealing temperature range was between 50 °C – 60 °C, including 50 °C, 50.4 °C, 51.2 °C, 52.5 °C, 54.2 °C, 56.4 °C, 58.9 °C, 61.0 °C, 62.7 °C, 63.9 °C, 64.7 °C, and 65 °C. Finally, the PCR product was left to cool to 10 °C for about 10 minutes before taking out from the PCR machine to avoid temperature shock for the products. The $MgCl_2$ and annealing temperature that provide very clear and consistent banding pattern

were selected as optimal reaction condition.

Table 3.5: Concentration of each reagent at each different $MgCl_2$ concentration.

Reagent	$MgCl_2$ concentration (mM)			
	1.5	**2.0**	**2.5**	**3.0**
$MgCl_2$	0.75	1.00	1.25	1.50
5 x PCR Buffer	2.50	2.50	2.50	2.50
2mM dNTP	1.25	1.25	1.25	1.25
10µM Forward Primer	0.50	0.50	0.50	0.50
10µM Reverse Primer	0.50	0.50	0.50	0.50
Taq polymerase	0.05	0.05	0.05	0.05
DNA template	0.75	0.75	0.75	0.75
Total volume	12.50	12.50	12.50	12.50

3.4.3.4 Amplification of 16s rDNA Region

The PCR was conducted to amplify 16s rDNA region for identifying the isolated bacteria. Sequence of 16s rDNA of isolated bacteria was amplified using a pair of primers, forward primer BacF and reverse primer R1378. The reaction was carried out in a master mix 12.5 µl reaction containing dNTPs buffer, $MgCl_2$, primers and *Taq* DNA polymerase. The DNA template was added after the master mix was distributed into individual tubes. The PCR tubes were placed in the PCR machine using the programmed thermal cycling profile as follows: initial denaturation at 94 $^{\circ}$C for 2 minutes, followed by 35 cycles of denaturation at 94 $^{\circ}$C for 1 minute, annealing at 50 $^{\circ}$C for 1 minute and DNA extension at 72 $^{\circ}$C for 1 minute, and final extension at 72 $^{\circ}$C for 1 minute.

3.4.3.5 Agarose Gel Electrophoresis of Amplified 16s rDNA Region

2 µl of optimized and amplified 16s rDNA region product was run on a 2 % agarose gel electrophoresis with 1 kb ladder marker was used as the marker. Electrophoresis was conducted at 120 V for about 30 – 45 minutes and it was completed when the bromophenol blue dye approached the end of the gel or three quarter of the gel. The current flow can be confirmed by the observation of bubbles formed in the electrode. Then, the gel was stained using ethidium bromide for 10 – 15 minutes. Finally, the gel was visualized under UV light and the image was captured using the digital camera which was connected to a UV transilluminator (Electrophoresis Documentation and Analysis System 120) with Kodak Digital Science 1D – Imaging Software.

3.4.3.6 Purification of Amplified 16s rDNA Region

The PCR product was purified using Wizard^{R} SV Gel and Binding PCR Clean-Up System by Promega. The purification step serves to separate the PCR product from salt, nucleotides, and PCR primers (Graham and Hill, 2001). Equal volume of membrane binding solution was added to the amplified 16s rDNA region. Then, minicolumn was inserted into the collection tube, and the prepared amplified 16s rDNA region was transferred to the minicolumn assembly and incubated at room temperature for 1 minute. After 1 minute, the mixture centrifuged at 12 000 rpm for 1.5 minutes. Flowthrough was discarded and reinserted the minicolumn into the collection tube. 700 µl membrane wash solution (ethanol added) was added and centrifuged at 12 000 rpm for 1.5 minute. Flowthrough was discarded and reinserted the minicolumn into

collection tube. The previous step was repeated with 500 µl membrane wash solution C and centrifuged at 12 000 rpm for 1.5 minutes. The collection tube was emptied and re-centrifuges the column assembly for 1 minute with the microcentrifuge lid open (or off) to allow evaporation of any residual ethanol. The minicolumn was carefully transferred to a clean 1.5 ml microcentrifuge tube. 50 µl of nuclease-free water was added to the minicolumn and incubated at room temperature for 1 minute. Centrifuged at 12 000 rpm for 1.5 minutes. The minicolumn was discarded and purified 10 µl of amplified 16s rDNA region with specific primers was sent to First Base Laboratories Sdn. Bhd for sequencing.

3.4.3.7 16s rDNA Region Sequence Analysis

All the forward and reverse 16S rDNA sequences from each sample obtained were compared and aligned using MEGA 4.0 where the sequences were edited to the correct nucleotide according to the highest peak on the chromatogram. Alignments were made for all samples using DNA weight matrix ClustalW (1.6) and saved in MEGA format. Some of the noisy data were checked once again by referring to the sequence chromatogram.

Phylogenetic trees were generated using Neighbouring-Joining (NJ) analysis (Saitau and Nei, 1987). NJ analysis was done based on Kimura 2-parameter (Kimura, 1980). The support for the clades within a tree was determined using bootstrap analysis (Felsenstein, 1985). 1000 bootstrap replicates were used in NJ analysis.

3.4.4 Biochemical Characterization by Biolog Microplate

For taxonomic identification, the isolates were subjected to a series (94 tests) of biochemical tests provided in 96-well plates by Biolog (Harward, USA), which includes 71 carbon source and 23 chemical sensitivity assays. The Biolog plates are based on colorimetric method whereby the reduction of used tetrazolium redox dye indicate the utilization of carbon source and resistance to inhibitory chemicals which provide the identification of bacterial strain compared with those of known bacteria whose profiles are entered into a database of Biolog. The Biolog system produces printout of identification of the species followed by the next 10 closest species. In building a database, correct identification within the top five choices of closest species is considered reasonable. Method wise, inoculation and reading of the Biolog plate carried out based on instruction of the manufacturer. Initially, inoculation was prepared at specified cell density (28 % for gram positive bacteria) from the bacterial culture. Then, inoculated into Biolog plate, and incubated. Lastly, the plate was observed for the metabolic patterns to obtain the bacterial identification.

3.5 Culture Conditions Optimization of S3 SC_1

Growth conditions of S3 SC_1 were optimized for 3 parameters; temperature, *pH* and glucose concentration individually. The biomass was measured using spectrophotometer at 600 nm absorbance (OD_{600}). All experiments were performed in triplicates. In all experiments, broth cultures un-inoculated with bacterial cells were included; and measured as controls. The range and the levels of culturing conditions variables under study are given in table 3.6. The range of temperature is chosen based on available literature, such minimum optimum temperature for calcite formation was found by Baskar et al. (2006) was 25 $^{\circ}$C; besides the practical availability of the incubator also affect the choice of chosen temperature. *pH* plays an vital role in calcite formation, therefore acidic, neutral and alkalic condition was chosen. Whereas, Boquet et al. (1973) stated that the medium without glucose gave good growth and crystals formation, but added glucose speeded the processes. Therefore, we choose glucose concentration as one of the parameters for optimization. Initially, the glucose concentration in B4 medium was 10 g/L, therefore, minimum 1 g/L to maximum 30 g/L was chosen ranges of glucose concentration.

Table 3.6: Experimental range and levels of the test variables.

Variable	Range and Level			
Temperature ($^{\circ}$C)	25	30	37	40
pH ($\pm$ 0.2)	5	7	9	-
Glucose concentration (g/L)	1	10	20	30

3.5.1 Temperature Optimization

An aliquots of the pre-cultured (1 ml) was taken and inoculated in 10 ml of the sterilized B4 broth medium followed by incubation at different temperatures as shown in table 3.6. Subsequently, the OD_{600} was read for each temperature at every time interval to determine the biomass concentration formed.

3.5.2 *pH* Optimization

Aliquots of the pre-cultured of 1 ml was inoculated in 10 ml of the sterilized B4 medium having different *pH* as shown in table 3.6 followed by incubation at 30 $^{\circ}$C. The OD_{600} of each medium with different *pH*'s was read and the bacterial growth was compared in each case.

3.5.3 Carbon Source Optimization

Aliquots of the pre-cultured of 1 ml was used to inoculate 10 ml of the sterilized B4 medium. Each medium (*pH* 7 $\pm$ 0.2) was supplemented with different amount of glucose as shown in table 3.6 and incubated at 30 $^{\circ}$C, and the OD_{600} of each medium was read accordingly.

3.6 Carbon Dioxide Sequestration Experiment of S3 SC_1

Prior to experiment, CO_2 gas was bubbled through 500 ml of sterile water for 3 hours at room temperature, and the gas was supplied throughout the experiment. A volume of culture containing adequate number of cells grown in CA producing medium was centrifuged at 8000 x g and the pellet was suspended with 1 M Tris-HCl buffer (*pH* 8.3) for the extracellular CA production. CO_2 sequestration by S3 SC_1 was examined in 3 different treatments; treatment A (with bacteria), treatment B (without bacteria) and treatment C (without continuous supply of CO_2).

40 ml of CO_2 saturated water was transferred into a 250 ml conical flask. Aliquots of 10 ml of Tris-HCl (1 M, *pH* 8.3) were added into the conical flask. For treatment B, the S3 SC_1 extracellular CA extract was replaced with 10 ml of distilled water. Aliquots of 40 ml of calcium acetate were added into the solution. CO_2 was slowly let diffused into the flask throughout the experiment for an hour. For treatment C, CO_2 was not continuously supplied as in treatments A and B. At the end of the experiments, the mixture was centrifuged and dried overnight at 70 $^{\circ}$C. Finally, dried crystals weight was measured by using weighing machines, and crystals morphology and presence of bacterial cells and zinc element was determined by Scanning electron microscope (SEM) /Energy dispersive X-ray spectroscopy (EDS).

3.6.1 SEM Sample Preparation

SEM is a powerful technique applied in micro-imaging of a variety of samples surface. This technique is very helpful to explore the texture of the surface of samples. Besides that, SEM incorporated with EDS help to determine the elemental composition of surfaces as well (Zunbul, 2005). In this work, SEM was used to visually examine images of calcite formed by bacteria, while EDS was used to perform spot analysis of the elemental composition.

The SEM/EDS characterization was carried out using a Field Electron Scanning Electron Microscope (JSM-6701F FESEM). Prior to analysis, the dried calcite crystals were sprinkled onto adhesive carbon taps supported on aluminium stubs. The aluminium stubs were coated with platinum to prevent accumulation of static electric charge on the specimen during electron irradiation. Images of the samples surfaces were then recorded at different magnifications. Elemental analysis was performed at different points randomly selected on the solid surface and EDS mapping was conducted.

4 CHAPTER

RESULTS AND DISCUSSION

4.1 Sampling Area

Samples were collected on 18th of March 2010 from 4 sites: limestone cave (Gua Tempurung, Kampar), from offside area of Rock Chemical Industries (RCI) (M) Berhad, Gopeng, river (near Gua Tempurung), hill slope (near Tesco Distribution center). For the ease of reading, the samples were given sample ID following the sequence of sample collected. S1 stands for sample collected at first and followed. For the isolated bacteria, begins with the sampling ID followed by SC stands for sub culture and numbered to differentiate the isolates from the same sub culture.

4.1.1 Site 1

Gua Tempurung (4° 25’ 04.9” N - 101° 11’ 14.3” E) is the largest limestone cave in the peninsular Malaysia (figure 4.1), located in Lembah Tempurung (Tempurung Valley) near Gopeng, Perak, Malaysia. It is 25 km to the south of

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T. Komala, Tan C. Khun (2013). Calcite-Forming Bacteria Located in Limestone Area of Malaysia. *Journal of Asian Scientific Research*, 3(5), pp. 471-484 [**Indexed by SCOPUS**]

T. Komala, Tan C. Khun (2014). Biological Carbon Dioxide Sequestration Potential of *Bacillus pumilus*. *Sains Malaysiana*, 43(8), pp. 1149–1156 (**Indexed by ISI Thomson Reuters**)

Ipoh, the capital of the state of Perak. It is a massive cavern inside Gunung Tempurung (Tempurung Mountain) standing at 497 metres high. Gua Tempurung is visible from the North-South Expressway near Gopeng. It is a cave of more than 400 million years old marble limestone (calcite calcium) of the Kinta limestone type measuring 1.9 km length and 120 meters height. There are four limestone samples collected from site 1 (figure 4.2).

4.1.2 Site 2

The sampling river is located near the Gua Tempurung (4° 25’ 04.9” N - 101° 11’ 14.3” E), Kampar, Perak (figure 4.1). It is a slow moving river with a shallow water level. The soil sample from river was taken from river bed and river side. River bed is the ground the river flows over whereas river side is the area of land by the side of the river (figure 4.3).

4.1.3 Site 3

Stalactites sample collected from offsite area of Rock Chemical Industries (RCI) Berhad (4° 25’ 49.4” N - 101° 09’ 46.6” E), primarily involved in the extraction, processing and distribution of limestone based products which is located in Gopeng (figure 4.1).

4.1.4 Site 4

The laterite slope sample was taken from hill slope near the Tesco Distribution Centre, Gopeng, Perak (4° 29’ 27.7” N - 101° 09’ 30.4” E) (figure 4.3).

Figure 4.1: Locations of all four sampling sites.

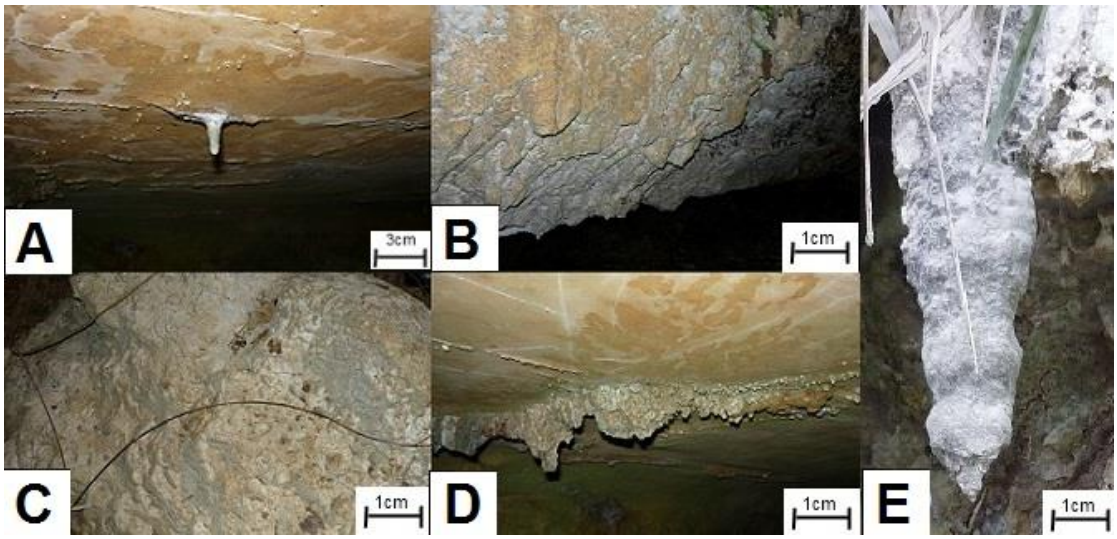


Figure 4.2: Captured images of limestone samples (a) S1 (b) S2 (c) S3 (d) S4 (e) S5.

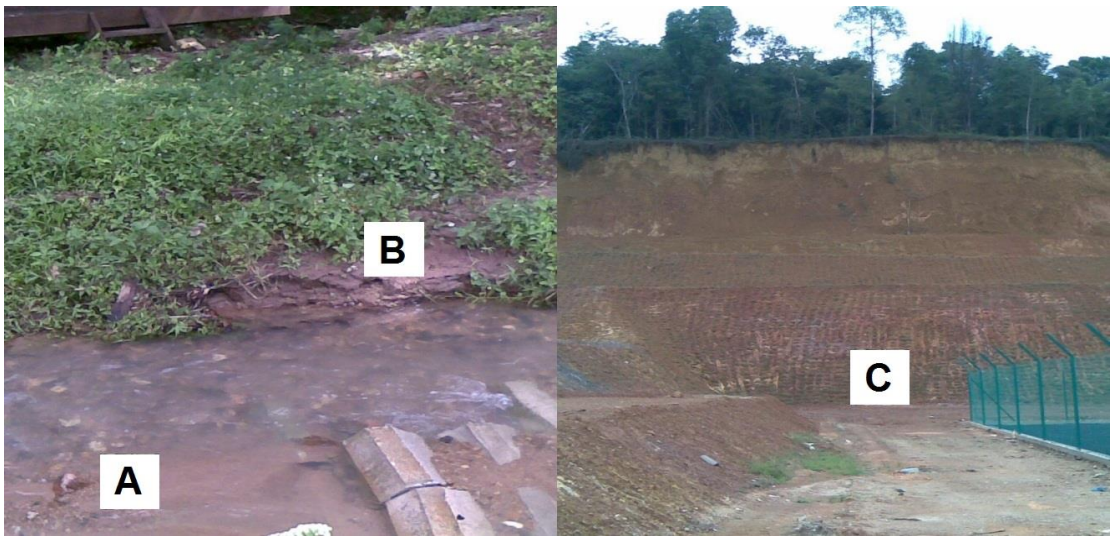


Figure 4.3: Captured images of soil sample. (A - S6) Soil sample from river bed, (B - S7) from river side, and (C – S8) Laterite soil from hill slope.

4.2 Mineral Identification of Limestone and Soil Samples

Table 4.1 and 4.2 shows the simplified result of mineral identification from limestone and soil samples respectively. The positions of the diffraction lines of limestone samples were close to those of pure calcium carbonate powder (Merck) as shown in figure 4.4. In this connection, quantitative estimates based on relative peak intensities also confirm that calcite present in abundance as shown in table 4.1. Although calcite was the most common mineral observed, the presences of other minerals were also detected such as sodium nitrate, silicon sulfide, cadmium neodymium thorium arsenate, and cadmium bismuth thorium arsenate.

In general, mineral identification of soil samples was difficult due to the presence of many mineral phases in the sample. Secondly, due to the small percentage of each of the phases (in principle, diffractometry identifies mineral phases greater than 5 % of the sample). This makes the interpretation of each of them in the diffractometer difficult; therefore the values were presented in table 4.2.

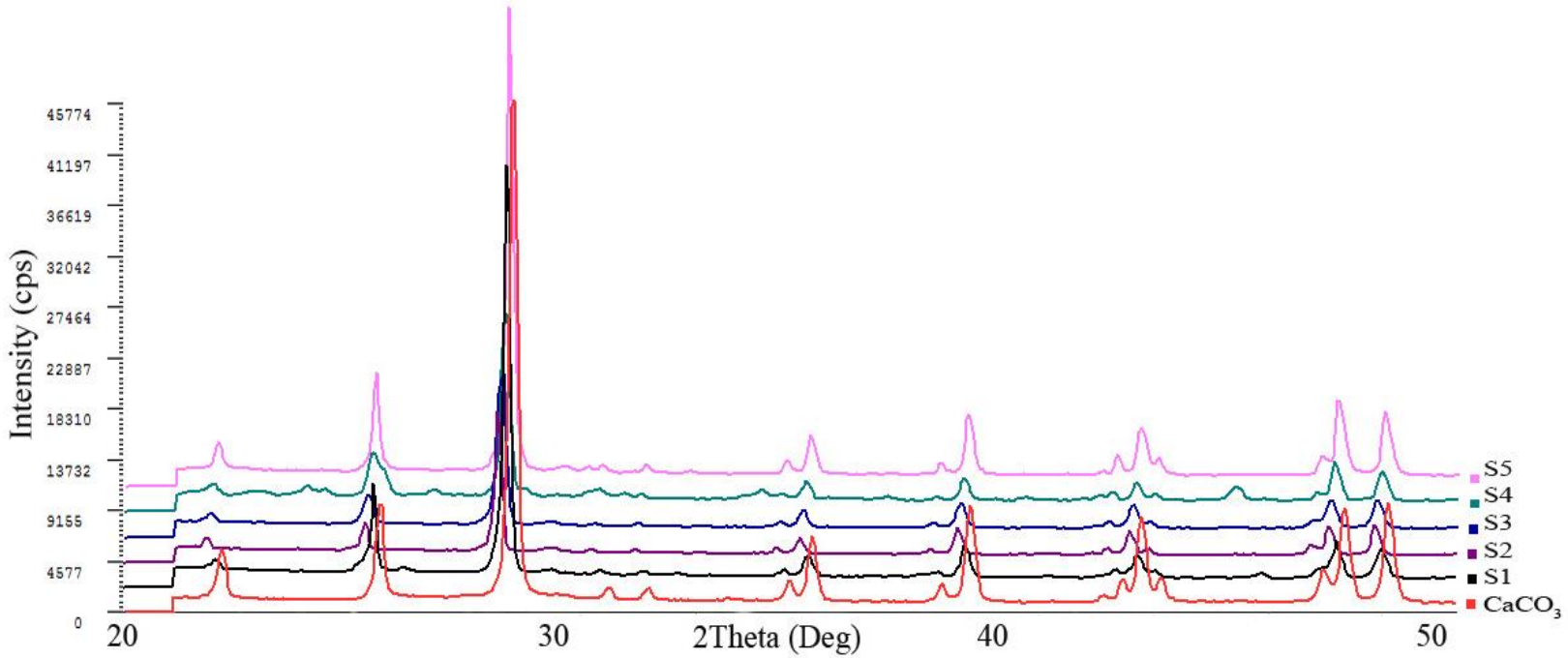


Figure 4.4: X-ray diffraction spectra of all the limestone samples compares with pure $CaCO_3$ spectra sample.

Table 4.1: Quantitative analysis of the final weight fractions[a] of limestone samples.

Sample ID	**Calcium carbonate (Calcite, syn) ($CaCO_3$)**	**Calcium carbonate (Calcite) ($CaCO_3$)**	**Sodium nitrate (Nitratine) ($NaNO_3$)**	**Silicon sulfide (SiS_2)**	**Cadmium neodymium thorium arsenate (CdNdTh(AsO4)3)**	**Cadmium bismuth thorium arsenate ($CsGdMO_2O_8.H_2O$)**
S1	0.761	0.714	0.866	0.815	0.826	0.822
S2	0.902	0.926	0.930	0.904	0.959	0.954
S3	0.870	0.874	0.892	0.862	0.945	ND
S4	0.925	ND	0.843	0.856	ND	ND
S5	0.905	0.873	0.804	0.850	0.904	0.877

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

Table 4.2: Quantitative analysis of the final weight fractions[a] of soil samples.

Sample ID	**Boron nitride (BN)**	**Potassium borate (K_2B8O_{13})**	**Rubidium yttrium telluride ($RbYTe_2$)**	**Potassium gadolinium oxide fluoride (K0.31Gd0, 69F1, 8400. 27)**	**Sulfur bromide nitride (SNBr0.33)x**	**Titanium oxide (Anatase, syn)**	**Rubidium nitrate ($RbN0_3$)**
S6	0.925	0.865	0.744	ND	ND	ND	ND
S7	0.975	ND	0.756	0.824	0.819	ND	ND
S8	ND	ND	ND	ND	ND	0.880	0.765

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

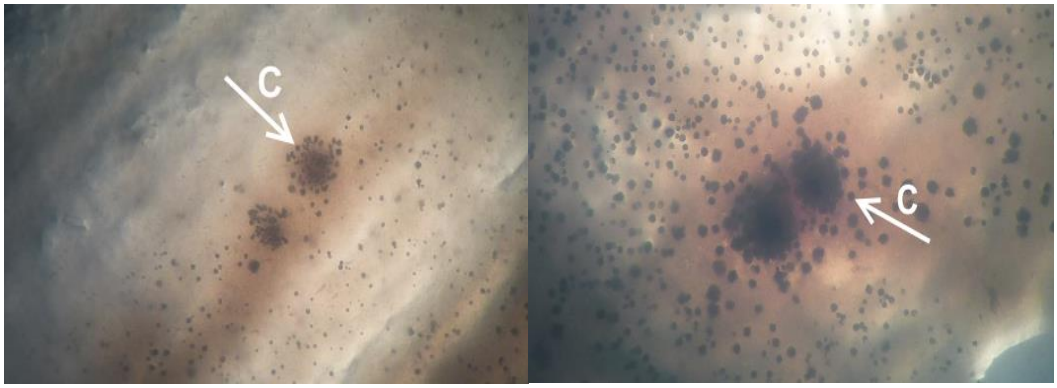
4.3 Isolation of CFB

4.3.1 Isolation of crystals forming colonies

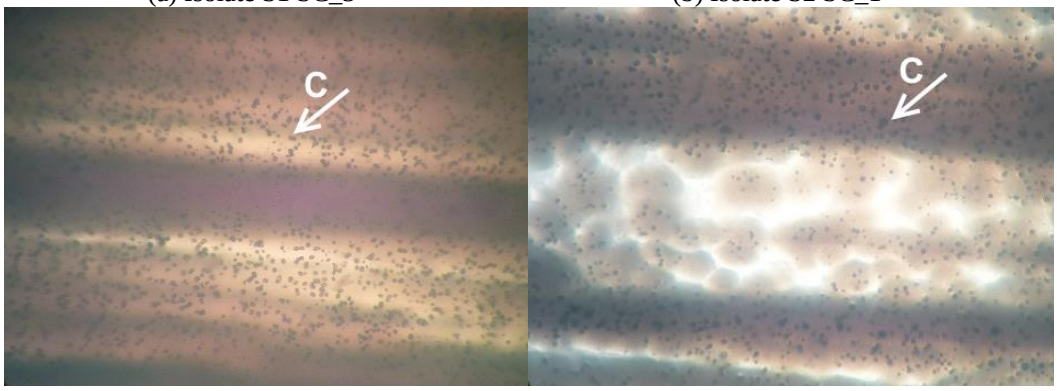
Out of 54 total bacterial isolates, 18 isolates were detected for the presence of crystals by observation using the light microscope. Collected samples, number of colonies isolated and crystals forming colonies were shown in table 4.3. It was found that the number of bacterial colonies varied with the sampling location. Light microscope images of colonies showing the crystals formation were shown in figure 4.5. It was observed that the calcite formation always formed within the presence of bacterial colonies on the agar surface.

Table 4.3: Samples, number of isolates, crystal forming colonies.

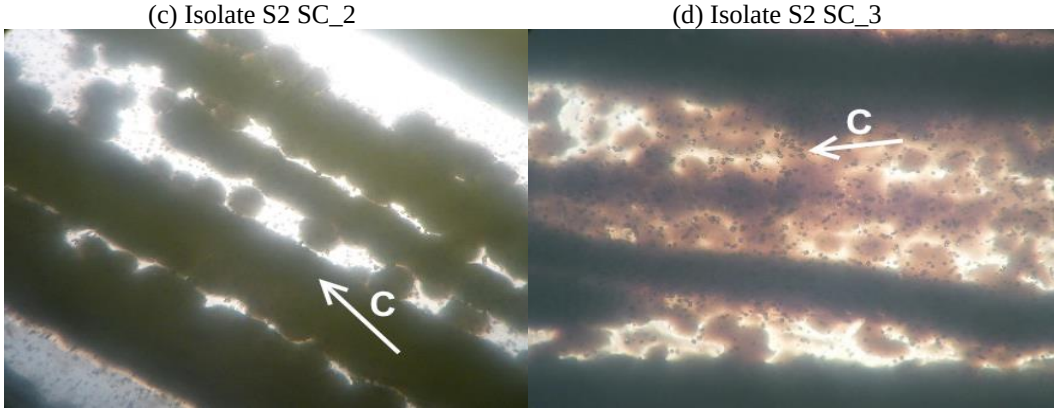
Sample ID	**Number of colonies**	**Crystals Forming Colonies**
S1	5	1 (S1 SC_3)
S2	7	4 (S2 SC_1, S2 SC_2, S2 SC_3, S2 SC_4)
S3	5	3 (S3 SC_1, S3 SC_2, S3 SC_3)
S4	2	-
S5	4	2 (S5 SC_1, S5 SC_2)
S6	11	4 (S6 SC_1, S6 SC_2, S6 SC_3, S6 SC_4)
S7	9	1 (S7 SC_1)
S8	11	3 (S8 SC_1, S8 SC_2, S8 SC_3)
Total	**54**	**18**



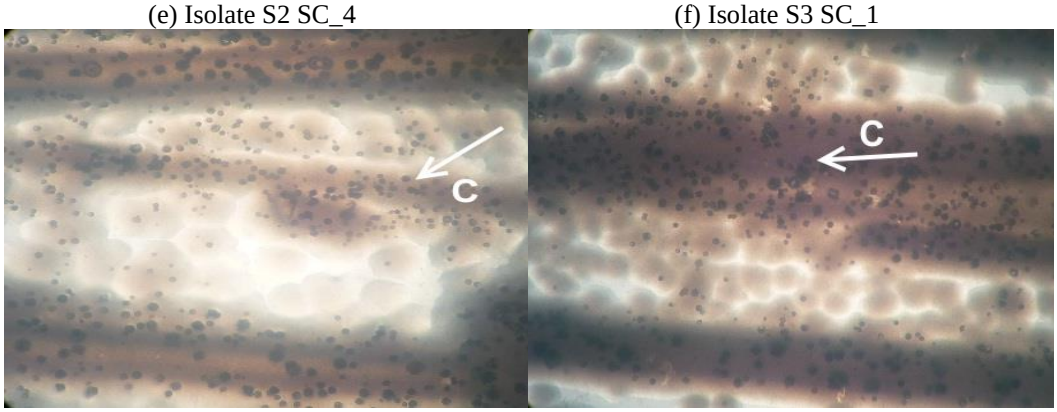
(a) Isolate S1 SC_3 (b) Isolate S2 SC_1



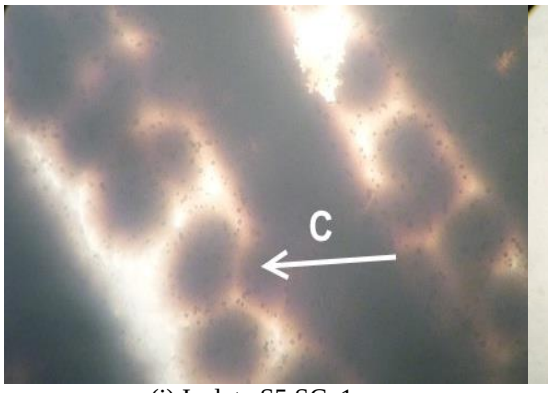
(c) Isolate S2 SC_2 (d) Isolate S2 SC_3



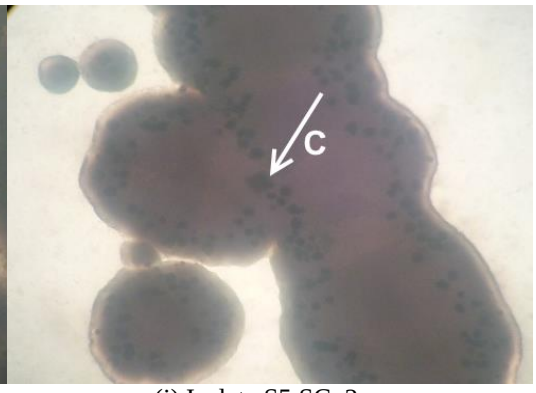
(e) Isolate S2 SC_4 (f) Isolate S3 SC_1



(g) Isolate S3 SC_2 (h) Isolate S3 SC_3

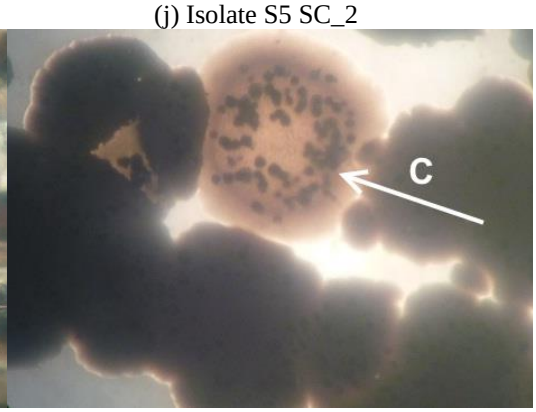


(i) Isolate S5 SC_1

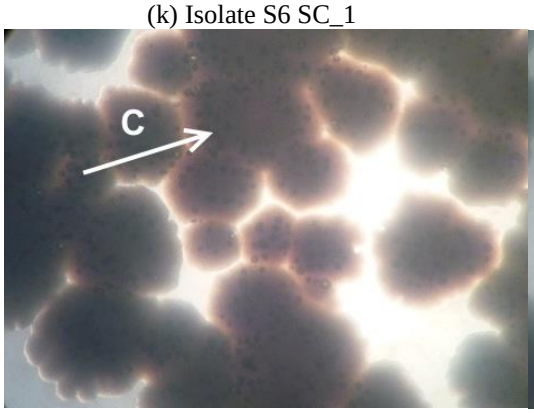


(j) Isolate S5 SC_2

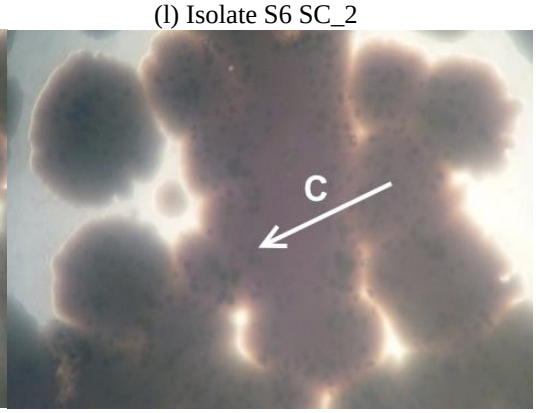
(k) Isolate S6 SC_1



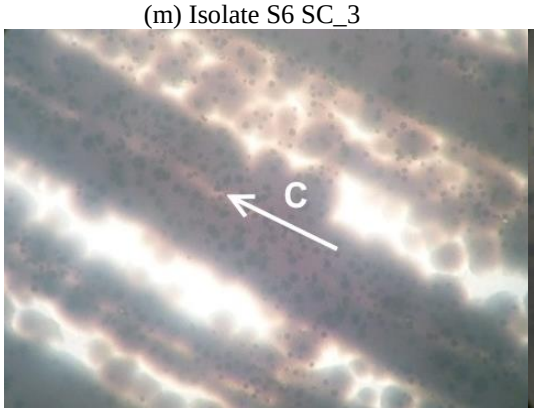
(l) Isolate S6 SC_2



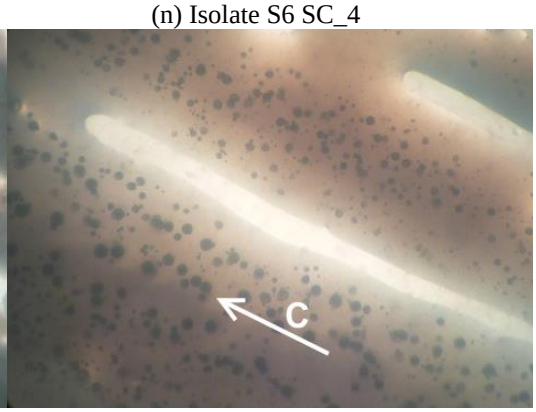
(m) Isolate S6 SC_3



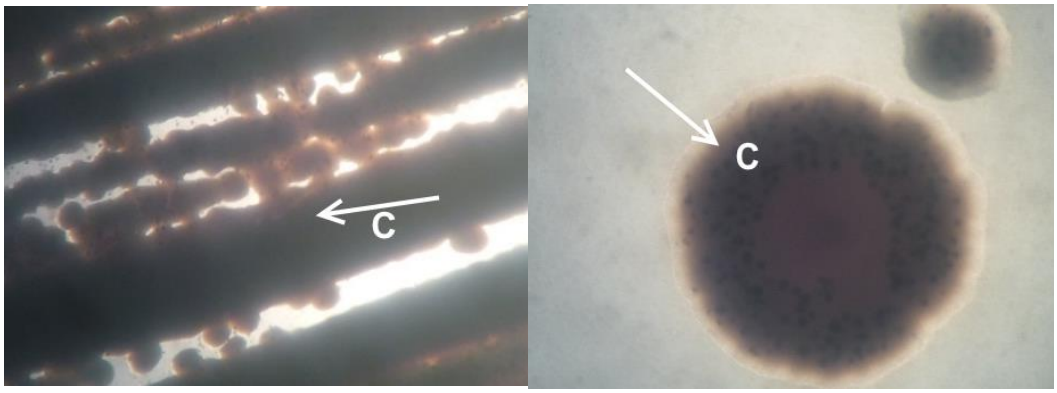
(n) Isolate S6 SC_4



(o) Isolate S7 SC_1



(p) Isolate S8 SC_1



(q) Isolate S8 SC_2 (r) Isolate S8 SC_3

Figure 4.5: Light microscopic images of crystals forming colonies from different isolate which is indicated by C. Generally, the mineralization areas within the colonies appear dark in colour. C = Crystals. Magnification 100 x.

4.3.2 Crystals Identification

The crystals formed by bacterial isolates were characterized by XRD; referring to calcium carbonate or calcite powder (Merck) as standard. The positions of the diffraction lines were close to those of pure calcite, which indicates the presence of calcite. Figure 4.6 shows one of the samples x-ray diffraction spectra. Nevertheless, quantitative estimates based on relative peak intensities also confirmed that calcite is abundant as shown in table 4.4. Mainly, there are three different types of calcite were found, namely calcite synrhombohedral 05-0586, calcite rhombohedral 24-0027 or Calcium carbonate 47-1743.

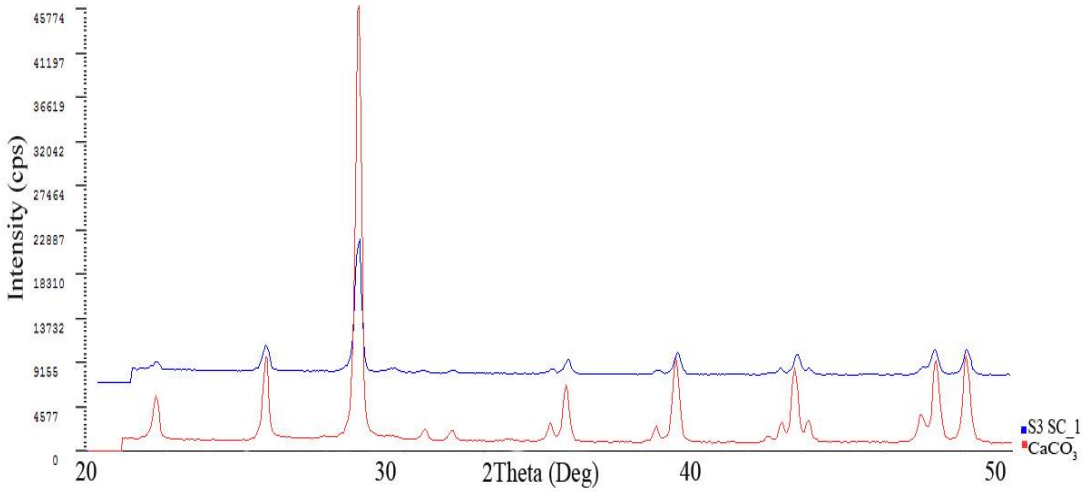


Figure 4.6: X-ray diffraction spectra of S3 SC_1 sample (counting time = 1 sec/step) compares with pure $CaCO_3$ spectra.

Table 4.4: Quantitative analysis of the final weight fractions[a] of crystals.

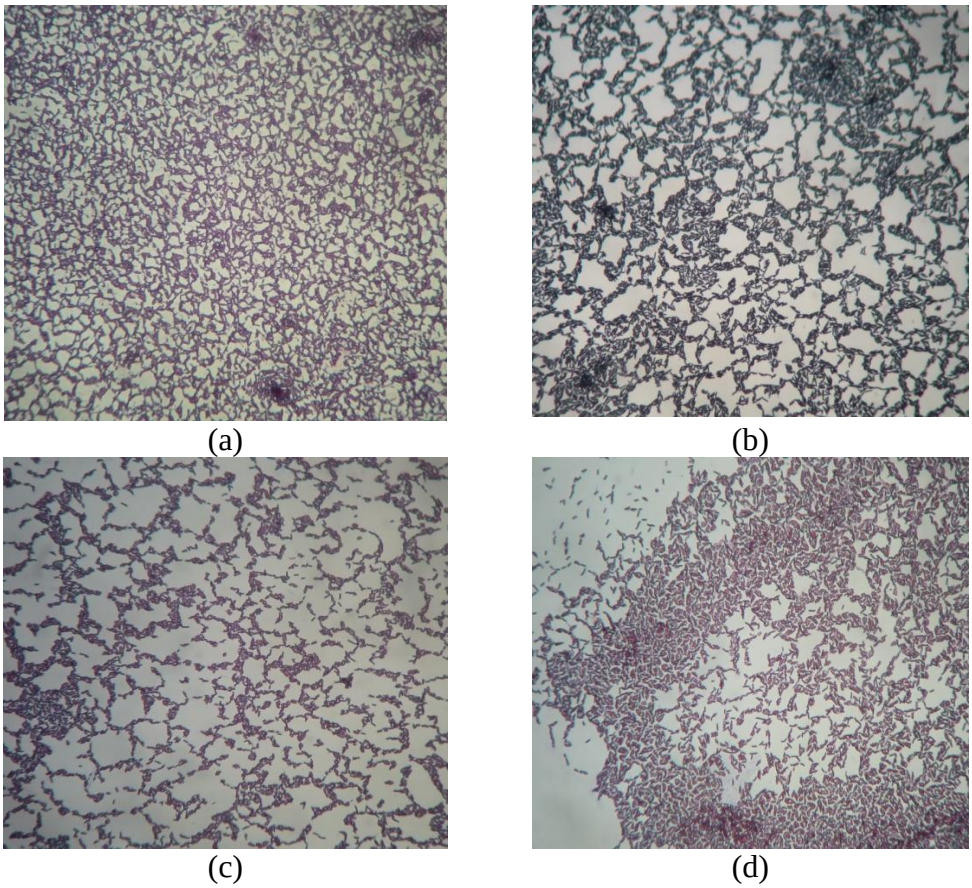
Isolates ID	**Calcium carbonate (Calcite, syn) ($CaCO_3$)** (ICDD 05-0586)	**Calcium carbonate (Calcite) ($CaCO_3$)** (ICDD 24-0027)	**Calcium carbonate ($CaCO_3$)** (ICDD 47-1743)
S1 SC_1	0.744	0.725	0.871
S2 SC_1	0.214	0.205	0.825
S2 SC_2	0.934	0.883	0.866
S2 SC_3	0.781	0.737	0.877
S2 SC_4	0.311	0.298	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
S6 SC_1	0.984	ND	ND
S6 SC_2	0.779	0.739	0.908
S6 SC_3	0.310	0.288	0.903
S6 SC_4	0.755	0.707	0.853
S7 SC_1	0.774	0.744	0.972
S8 SC_1	0.989	0.958	0.602
S8 SC_2	0.231	0.217	0.797
S8 SC_3	0.814	0.768	0.847

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

4.4 Characterization of CFB

4.4.1 Gram Staining

Calcite forming bacterial colonies was gram stained. All observed as purple/blue cells under light microscope and thus accepted to be Gram positive. Some of the Gram (+) cells are shown in figure 4.7.



(a) (b) (c) (d)

Figure 4.7: Appearance of Gram (+) cells under light microscope (a) S3 SC_1, (b) S3 SC_2, (c) S3 SC_3; (d) S5 SC_2. Magnification 100 x.

4.4.2 Characterization of CFB Mechanisms

There were a total of seven biochemical tests were performed to identify the CFB mechanisms including urea hydrolysis, hydrogen sulphide production, nitrate reduction, nitrite reduction, denitrification, ammonification, and CA analysis by esterase activity. Out of 18 CFB isolates, 12 isolates were found to be urease positive, one isolates (S8 SC_2) positive to denitrification (Table 4.6).

4.4.2.1 CA Analysis by Enzymatic Esterase Activity

The powder *p*-NP was dissolved in phosphate buffer, and the blank solution was also chosen as the same phosphate buffer. There was a control for every sample, and the value was after deduction of those controls value. R2 value of the line 0.998 and slope of the calibration curve was calculated as 0.02 as shown in figure 4.5.

Table 4.5 shows the amount of *p*-NP produced at 48 hours of bacterial growth which shows the presence of significant variations in the amount of *p*-NP produced. One unit of esterase activity represents the amount of enzyme catalysing to produce 1 μM *p*-NP per min under the assay conditions. The highest *p*-NP/min was shown as 5.750, 5.658, 5.874, and 6.017 mM by S3 SC_1, S3 SC_3, S5 SC_1 and S8 SC_2 respectively.

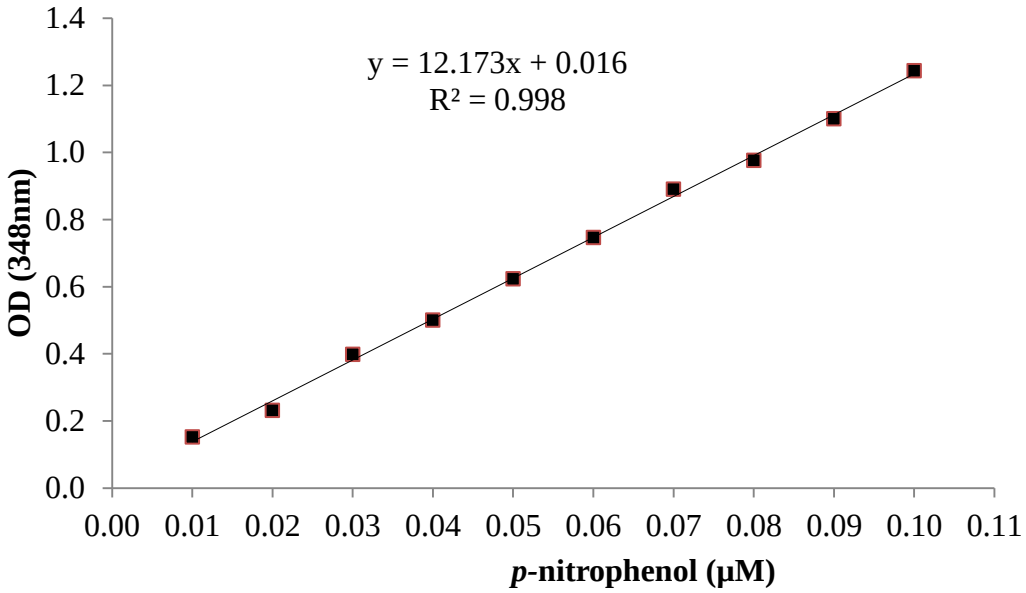


Figure 4.8: Calibration curve of *p*-nitrophenol in 0.1 M phosphate buffer.

Table 4.5: *p*-nitrophenol/min (mM) of 18 bacterial isolates at 48 hours of bacterial growth.

Isolates	mM p-nitrophenol/min
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
S6 SC_1	3.543
S6 SC_2	1.140
S6 SC_3	0.698
S6 SC_4	0.370
S7 SC_1	2.105
S8 SC_1	6.017
S8 SC_2	1.150
S8 SC_3	0.267

Table 4.6: Results of six biochemical test done on all the bacterial isolates. The entire tests were done in triplicates and one the results shown in table.

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	+	-	-	-	-	-
S6 SC_1	+	-	-	-	-	-
S6 SC_2	-	-	-	-	-	-
S6 SC_3	-	-	-	-	-	-
S6 SC_4	+	-	-	-	-	-
S7 SC_1	+	-	-	-	-	-
S8 SC_1	-	-	-	-	-	-
S8 SC_2	-	-	+	+	-	+
S8 SC_3	+	-	-	-	-	-

1: Urea hydrolysis; 2: Hydrogen sulphide production; 3: Nitrate reduction; 4:Nitrite reduction; 5: Ammonification; 6: Denitrification.

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

4.4.3 Molecular Characterization by 16s rDNA Region Amplification

*** All the images were taken using camera-phone, as the malfunction of the build-in camera which used to capture the gel images ***

4.4.3.1 Extraction of Genomic DNA

There are only four isolates were selected for molecular identification, 2 isolates (S2 SC_2 and S3 SC_3) shows positive to urea hydrolysis test and 2 isolates (S3 SC_1and S5 SC_1) shows the presence of carbonic anhydrase (CA). This is due to insufficient grant to carry out molecular characterization on all the isolates. Genomic DNA of four isolates (S3 SC_1, S5 SC_1, S2 SC_2, and S3 SC_3) were successfully extracted using phenol-chloroform technique. The extracted DNA was transferred into another small tube to prevent contamination; and the stock DNA was preserved at - 4 $^{\circ}$C. Figure 4.9 shows the results of samples of total genomic DNA extract. The presence of total genomic DNA was confirmed by satisfactory bands on 0.8 % agarose gel.

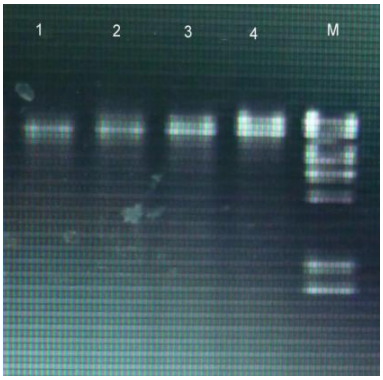


Figure 4.9: Genomic DNA extraction. Lanes 1 represent S3 SC_1; Lane 2 represents S5 SC_1; Lane 3 represents S2 SC_2; Lane 4 represents S3 SC_3. Lane M represents Lamda Hind III Marker.

4.4.3.2 Optimization of 16s rDNA Region Amplification

The BacF forward primer and 1378R reverse primers were optimized for $MgCl_2$ concentration and annealing temperature, Ta. The optimization of $MgCl_2$ concentration for PCR was carried out within the range of 1.5 mM to 3.0 mM. The annealing temperature was optimized using the gradient option of the thermal cycler. 2.0 mM $MgCl_2$ and 50 $^{\circ}$C was chosen as the optimal condition for amplification since it gives a nice single band at approximately 1300 bp without a lot of smearing compare to the rest of the wells as shown in figure 4.10.

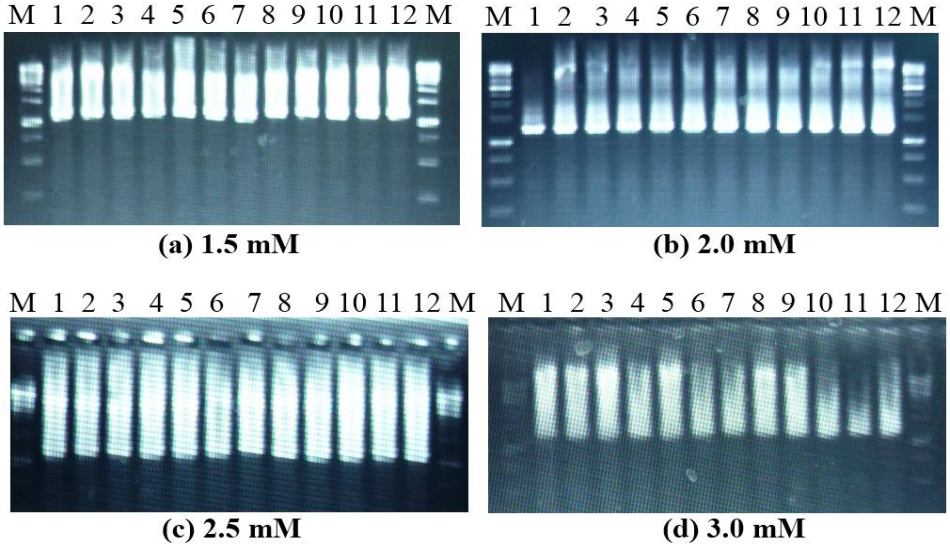


Figure 4.10: Optimization of 16s rDNA Region Amplification at different $MgCl_2$ concentration and 12 different annealing temperatures (a) 1.5 mM; (b) 2.0 mM; (c) 2.5 mM; and (d) 3.0 mM. Lanes 1-12 represents different temperature. Lane M is a 1 kb DNA ladder.

4.4.3.3 Amplification of 16s rDNA Region

Figure 4.11 shows the amplification of 16s rDNA region of 4 isolates. Lane 1 to 12 represents the results of replicates for each particular sample. The replicates were done to make sure enough samples for purification process since it was advisable to have at least 100 µl of amplification products. All the isolates give a clear band at approximately 1300 bp.

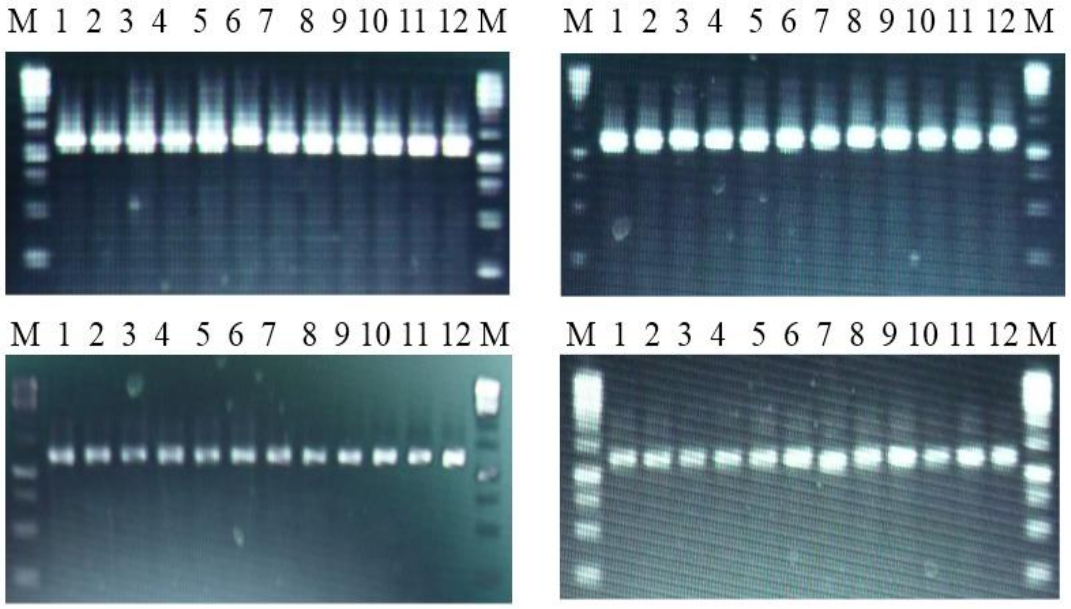


Figure 4.11: Amplification of 16s rDNA Region. (a) Isolate S3 SC_1; b) Isolate S5 SC_1 (c) Isolate S2 SC_2 (d) Isolate S3 SC_3. Lanes 1-12 represents the replicates of amplification. Lane M represents 1 kb ladder.

4.4.3.4 Purification of Amplified 16s rDNA Region

The amplification product was purified using MEGA quick-spin PCR and Agarose Gel DNA Extraction System to remove excess nucleotides and primers. This spin column-based allows the recovery of PCR products within as little as 20 minutes. Figure 4.12 shows the PCR product purification electrophoresis gel.

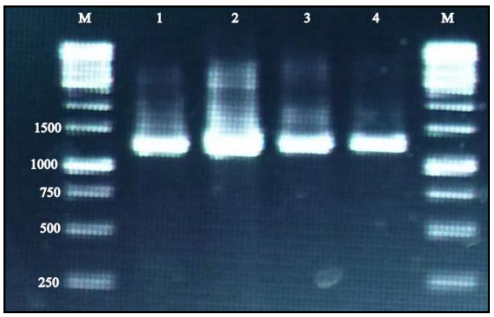


Figure 4.12: Purification of Amplified 16s rDNA Region. Lane 1 represents S3 SC_1; Lane 2 represents S5 SC_1; Lane 3 represents S2 SC_2; Lane 4 represents S3 SC_3. M represents 1 kb ladder.

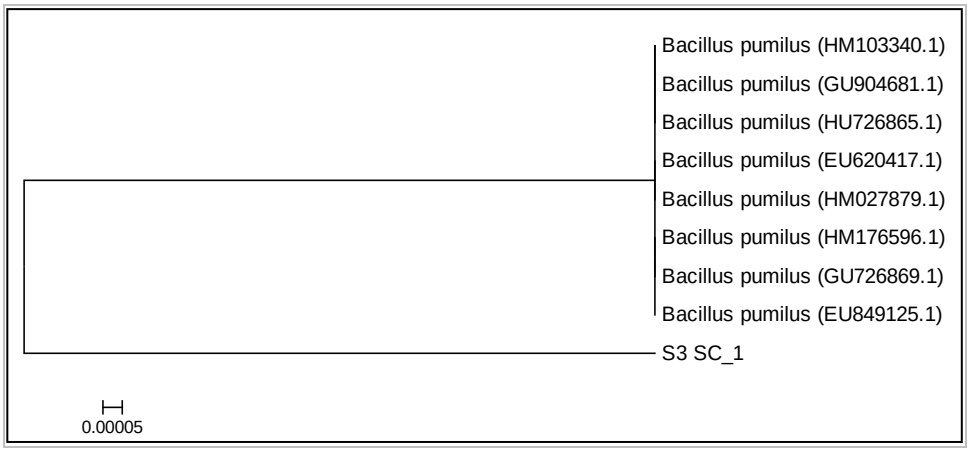
4.4.3.5 16s rDNA Region Sequence Analysis

16s rDNA sequence analysis showed that there was a strong similarity (≥95 % - ≥98 %) between the bacterial isolates and representative isolates in gene bank of *Bacillus* spp. The results indicate that 16s rDNA gene sequence data is helpful for identification of CFB at species level. The software package MEGA 4 was used for the analysis. Overall the identification results of four isolates are shown in Table 4.7 followed by the dendogram estimated phylogenetic relationships with eight reference strains, using neighbor-joining method in figure 4.13.

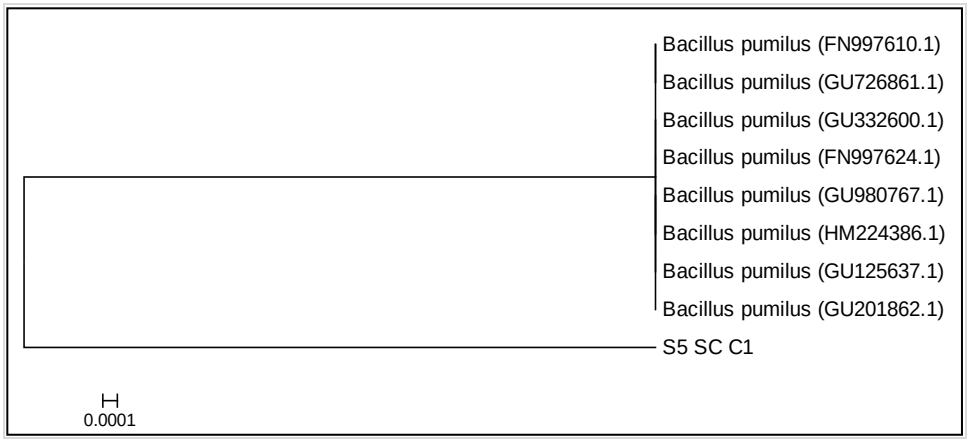
Table 4.7: Overall identification results of four isolates.

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

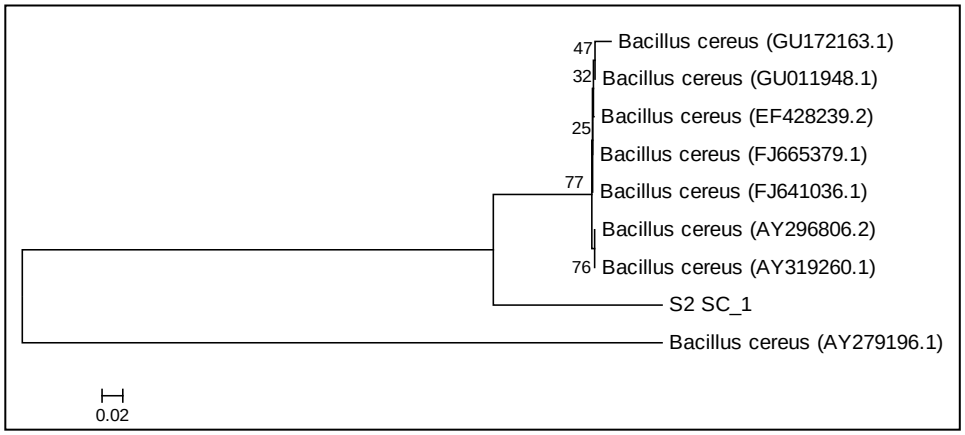
(a)


Bacillus pumilus (HM103340.1)
Bacillus pumilus (GU904681.1)
Bacillus pumilus (HU726865.1)
Bacillus pumilus (EU620417.1)
Bacillus pumilus (HM027879.1)
Bacillus pumilus (HM176596.1)
Bacillus pumilus (GU726869.1)
Bacillus pumilus (EU849125.1)
S3 SC_1
0.00005

(b)


Bacillus pumilus (FN997610.1)
Bacillus pumilus (GU726861.1)
Bacillus pumilus (GU332600.1)
Bacillus pumilus (FN997624.1)
Bacillus pumilus (GU980767.1)
Bacillus pumilus (HM224386.1)
Bacillus pumilus (GU125637.1)
Bacillus pumilus (GU201862.1)
S5 SC C1
0.0001

(c)


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

(d)

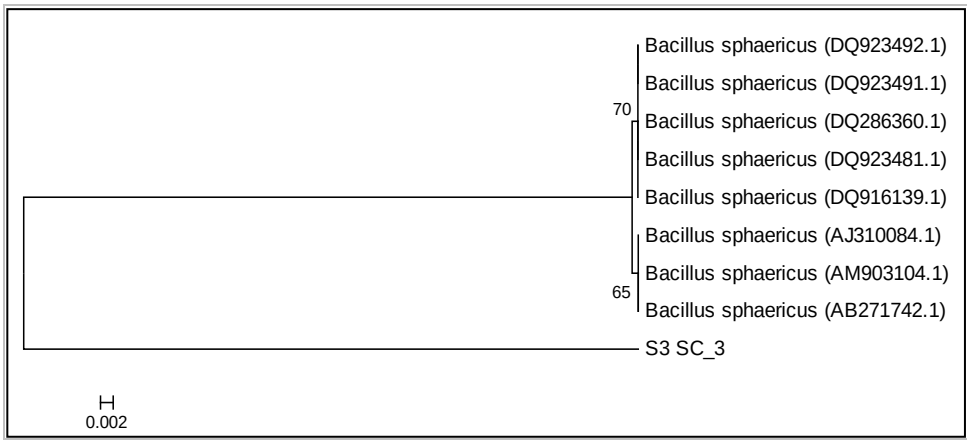


Figure 4.13: Phylogenetic position of the bacterial strain with eight references isolates. Phylogenetic tree was inferred by using the neighbour-joining methods and MEGA 4 software package was used for the analysis (a) S3 SC_1 showing 98% similarity with *Bacillus pumilus*, 0.005% divergence (b) S5 SC_1 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.

4.4.4 Biochemical Characterization by Biolog Microplate

Biochemical characterization tests were carried out using Biolog microplate. Out of three samples tested, S3 SC_1 was successfully identified as *Bacillus pumilus*. Biolog strip of *Bacillus pumilus* identification shown in figure 4.14. Identification details of all the tested isolates were shown in table 4.8. Biochemical characterizations of identified *Bacillus pumilus* results were presented in table 4.10.

Table 4.8: Identification details of the isolates by GEN III (Biolog System).

Isolates	Level of ID	Identified species	Probability (%)	Similarity	Distance
S3 SC_1	Species	*Bacillus pumilus*	0.997	0.581	7.417
S1 SC_3	-	NI	-	-	-
S2 SC_2	-	NI	-	-	-

*NI – Not Identified.

Table 4.9: *Bacillus pumilus* S3 SC_1 growth at different NaCl concentration.

Salt Concentration (%)	4	8	10	12	20	30
Bacillus pumilus growth	++	++	++	++	-	-

++: Positive growth; -: No growth.

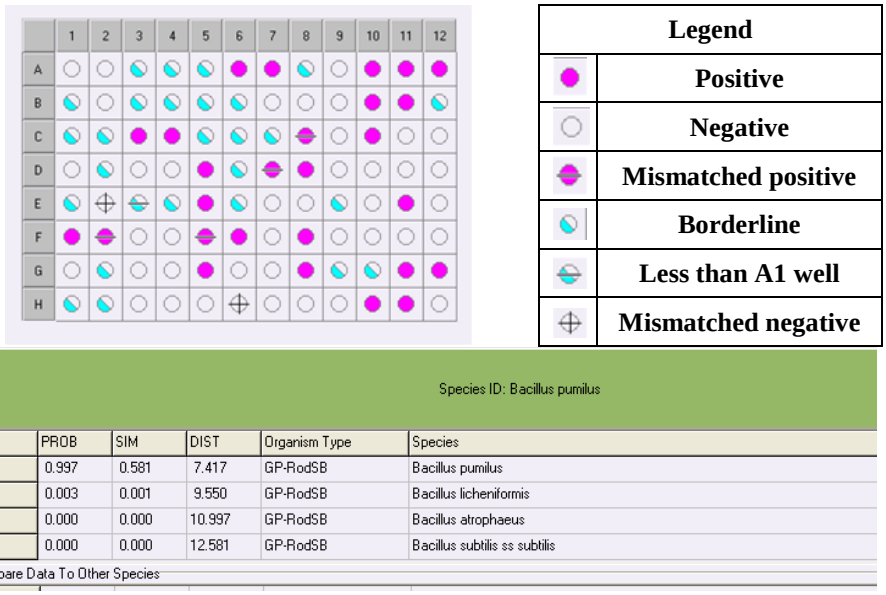


Figure 4.14: Biolog strip showing isolate S3 SC_1 identifies as *Bacillus pumilus*.

Table 4.10**:** Biochemical Characterization of *Bacillus pumilus* S3 SC_1.

Characteristics	*Bacillus pumilus*	Characteristics	*Bacillus pumilus*
pH 6	++	L-Galactonic Acid Lactone	-
pH 5	++	D-Gluconic Acid	-
1% NaCI	++	D-Glucuronic Acid	-
4% NaCI	++	Glucoronamide	++
8% NaCI	+	Mucid Acid	-
1% Sodium Lactate	++	Quinic Acid	++
Fusidic Acid	-	D-Saccharic Acid	-
D-Serine	-	Vancomycin	-
D-Sorbitol	-	Tetrazolium Violet	-
D-Mannitol	+	Tetrazolium Blue	-
D-Arabitol	-	p-Hydroxy-Phenylacetic Acid	-
myo-Inositol	-	Methyl Pyruvate	+
Glycerol	++	D-Lactic Acid Metyl Ester	-
D-Glucose-6PO_4	+	L-Lactic Acid	-
D-Fructose-6PO_4	-	Citric Acid	++
D-Aspartic Acid	++	α-Keto-Glutanic Acid	-
D-Serine	-	D-Malic Acid	-
Troleandomycin	-	L-Malic Acid	++
Rifamycin SV	-	Bromo-Succinic Acid	+
Minocycline	-	Nalidixic Acid	+
Gelatin	+	Lithium Chloride	++
Glycyl-L-Proline	-	Potassium Tellurite	++
L-Alanine	-	Tween 40	+
L-Arginine	+	γ-Amino-Butryric Acid	+
L-Aspartic Acid	++	α-Hydroxyl Butryric Acid	-

Characteristics	*Bacillus pumilus*	Characteristics	*Bacillus pumilus*
L-Glutamic Acid	+	β-Hydroxy-D,L-Butyric Acid	-
L-Histidine	-	α-Keto-Butyric Acid	-
L-Pyroglutamic Acid	-	Acetoacetic Acid	-
L-Serine	+	Propionic Acid	-
Lincomycin	-	Acetic Acid	-
Guanidine HCl	++	Formic Acid	-
Biaproof 4	-	Aztreonam	++
Pectin	++	Sodium Butyrate	++
D-Galacturonic Acid	-	Sodium Bromate	-
Dextrin	-	N-Acetyl-D-Glycosamine	+
D-Maltose	+	N-Acetyl-β-D-Monnosamine	-
D-Trehalose	+	N-Acetyl-D-Galactosamine	-
D-Cellobiose	+	N-Acetyl Neuraminic Acid	-
Gentiobiose	++	α-D-Glucose	+
Sucrose	++	D-Mannose	+
D-Turanose	+	D-Fructose	++
Stachyose	-	D-Galactose	++
D-Raffinose	+	3-Methyl Glucose	+
α-D-Lactose	-	D-Fucose	+
D-Melibiose	+	L-Fucose	+
β-Methyl-D-Glucoside	+	L-Phamnose	-
D-Salicin	+	Inosine	-

++: Positive result for the test, +: Partially positive for the test, -: Negative result for the test.

Overall biochemical identifications were accepted as correct if the assigned identity met or exceeded 92% probability. Therefore, the identification of S3 SC_1 as *Bacillus pumilus* was accepted with 99.7 % probability (table 4.8). *Bacillus pumilus* grows at both *pH* 5 and 6. *Bacillus pumilus* strain is able to ferment Gentiobiose, Sucrose, D-Fructose, and D-Galactose sugar components and partial metabolism of these sugar substrates: D-Maltose, D-Trehalose, D-Cellobiose, D-Turanose, D-Raffinose, D-Melibiose, β-Methyl-D-Glucoside, D-Salicin, N-Acetyl-D-Glycosamine, α-D-Glucose, D-Mannose, 3-Methyl Glucose, D-Fucose and L-Fucose. Negative values for α-D-Lactose indicated that the *Bacillus pumilus* strain does not

ferment lactose. When tested with 3 different concentrations of NaCl, the growth of the *Bacillus pumilus* S3 SC_1 strain was found to be positive at 1 % and 4 %, but decreased at 8 % (table 4.10). The *Bacillus pumilus* S3 SC_1 tested in laboratory at different salt concentration (from 4 % to 30 %) indicated positive growth until 12 % salt concentration and, the growth was inhibited at more than 20 % salt concentration during the test period (table 4.9).

4.5 Culture Conditions Optimization

Bacillus pumilus S3 SC_1 were chosen for both culturing conditions optimization and CO_2 sequestration studies, cause this isolates was successully identified by both molecular and biochemical characterization. Besides, *Bacillus pumilus* shows the presense of CA enzyme which is the enzyme used to enhance the calcite formation process by utilizing the CO_2.

The culture conditions of *Bacillus pumilus* S3 SC_1 were optimized for temperature, *pH* and glucose as these conditions play an important role in enhancing calcite forming ability (Boquet et al., 1973; Hammes and Verstraete, 2002). The entire tests were done in triplicates and the value in all the graph represents average of absorbance measured.

4.5.1 Temperature Optimization

Figure 4.15 shows the effects of different temperature on *Bacillus pumilus* S3 SC_1 bacterial mass production. Optimum temperature for growth of *Bacillus pumilus* S3 SC_1 was 30 ˚C.

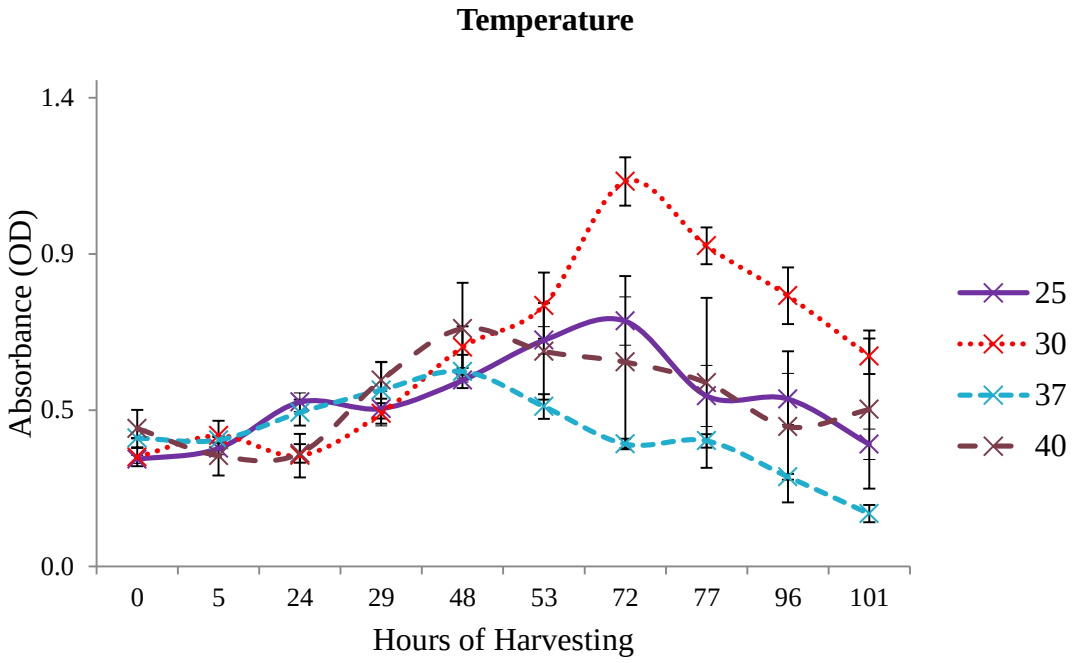


Figure 4.15: Growth profile of *Bacillus pumilus* S3 SC_1 at different temperature.

4.5.2 *pH* Optimization

Figure 4.16 shows the effects of different *pH* on *Bacillus pumilus* S3 SC_1 bacterial mass production. Optimum *pH* for growth of *Bacillus pumilus* S3 SC_1 is *pH* 7.

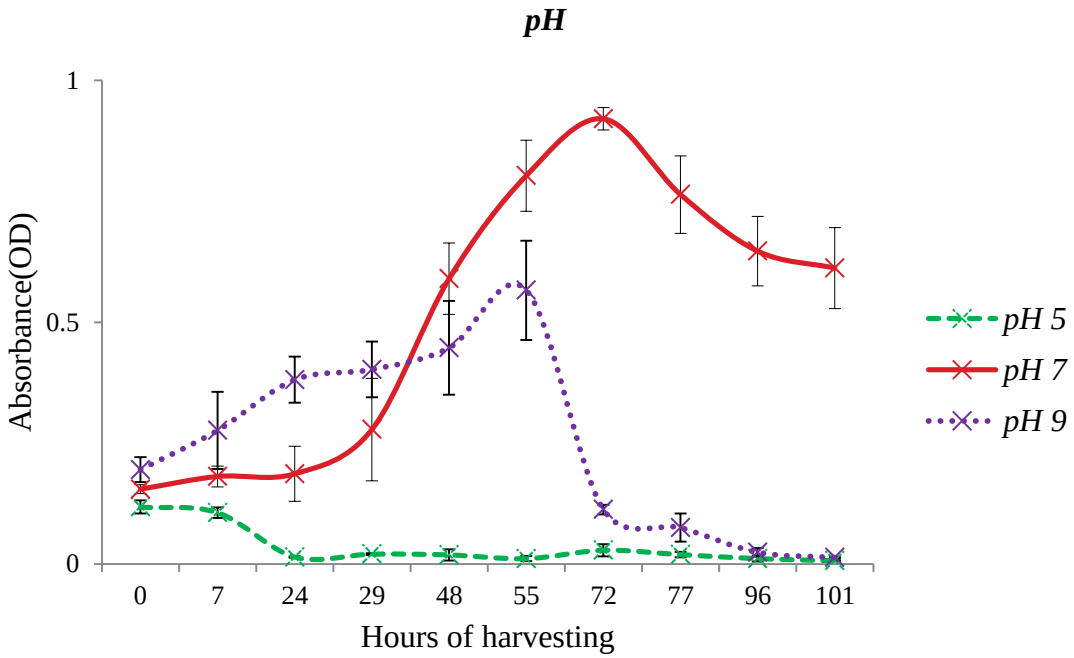


Figure 4.16: Growth profile of *Bacillus pumilus* S3 SC_1 at different *pH*.

4.5.3 Carbon Source (Glucose) Concentration Optimization

Figure 4.17 shows the effects of different glucose concentration on *Bacillus pumilus* S3 SC_1 bacterial mass production. Optimum glucose concentration for growth of *Bacillus pumilus* S3 SC_1 was 20 g/L.

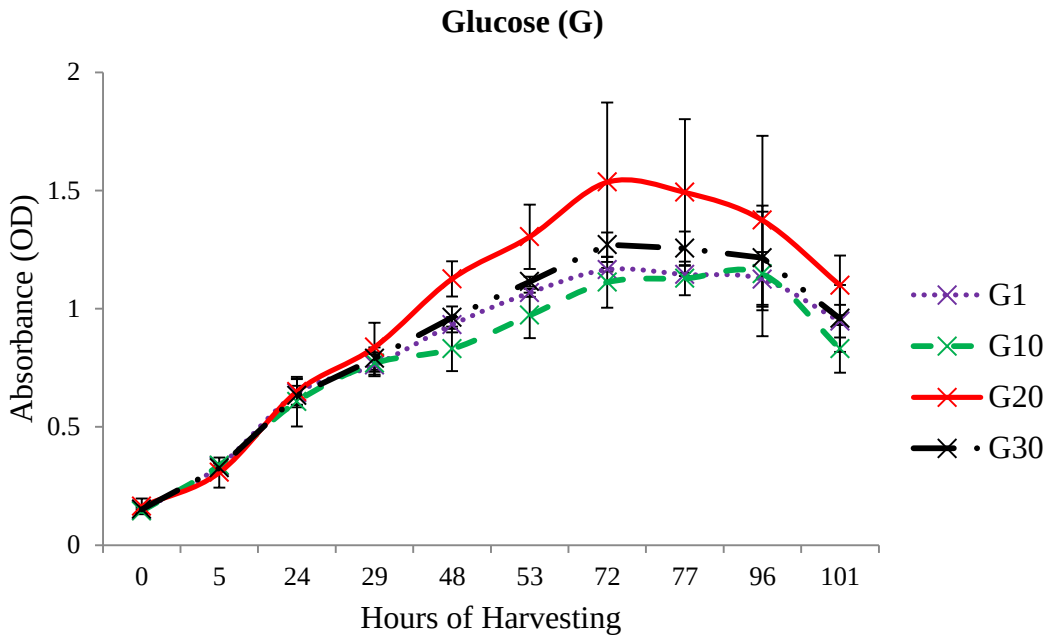


Figure 4.17: Growth profile of *Bacillus pumilus* S3 SC_1 at different glucose concentration.

4.6 Carbon Dioxide Sequestration Experiment of *Bacillus pumilus* S3 SC_1

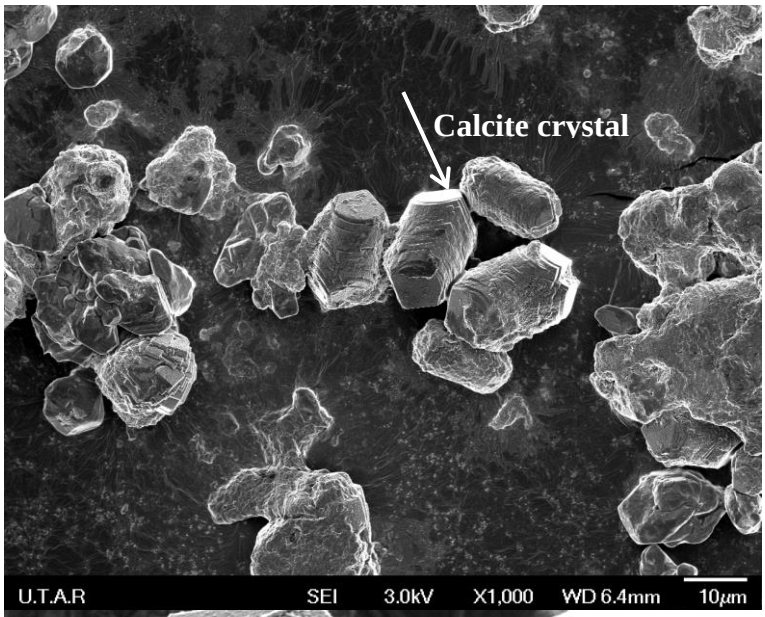
CO_2 sequestration by *Bacillus pumilus* S3 SC_1 was examined in 3 different treatments; treatment A (with bacteria), treatment B (without bacteria) and treatment C (without continuous supply of CO_2). The reasons of selecting these treatment regimes are explained as follows:

- Treatment A is to understand the effects of bacteria in CO_2 sequestration
- Treatment B acts as a control test
- Treatment C is to understand the effects of amount of CO_2 supplied

Table 4.11 shows the calcite crystals weight (mg) and EDS element (weight %). It was observed that the calcite crystals weight was higher in the treatment A compared to treatment B, and C. Besides, the amount of calcite crystals weight of treatment B was lesser than treatment C. According to SEM analysis in treatment A, prismatic layer of calcite was dominant in three replicates (figure 4.18). Treatment C shows amorphous shape of crystals (figure 4.19), while rhombohedral calcite crystals were found in treatment B (figure 4.20). As the prismatic layer of calcite crystal was dominant in treatment A, close view images were taken and shown in figure 4.21; from top (a) and from side view (b). There were obvious differences in the size and morphology of calcite crystals in all the treatments based on the photos taken by SEM.

Besides, the bacterial imprints on the surface of the calcite crystals in treatment A were observed (figure 4.23). These results suggested that CA produced by bacteria is fastening the CO_2 sequestration process. The elemental mapping on the prismatic crystals formed in treatment A shown in figure 4.22 shows calcium was dominant mineral which inferred to be as calcite.

(a)



(b)

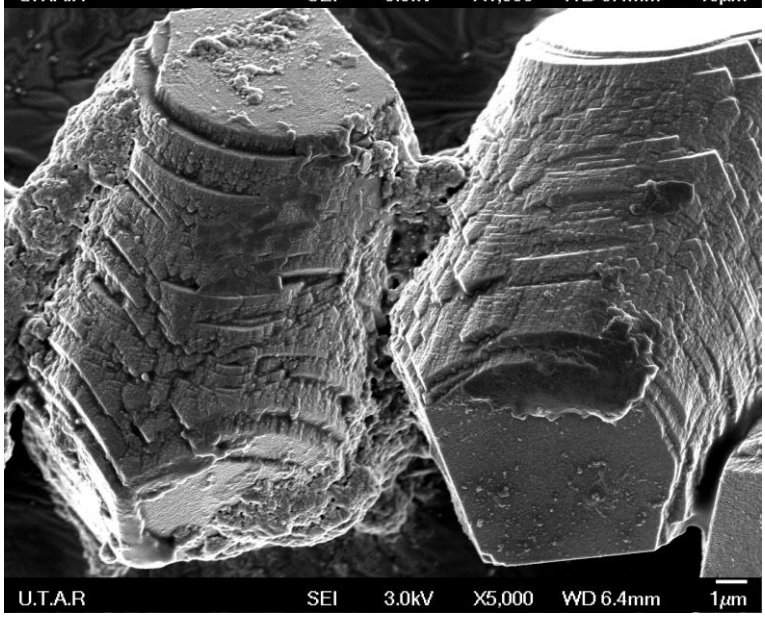
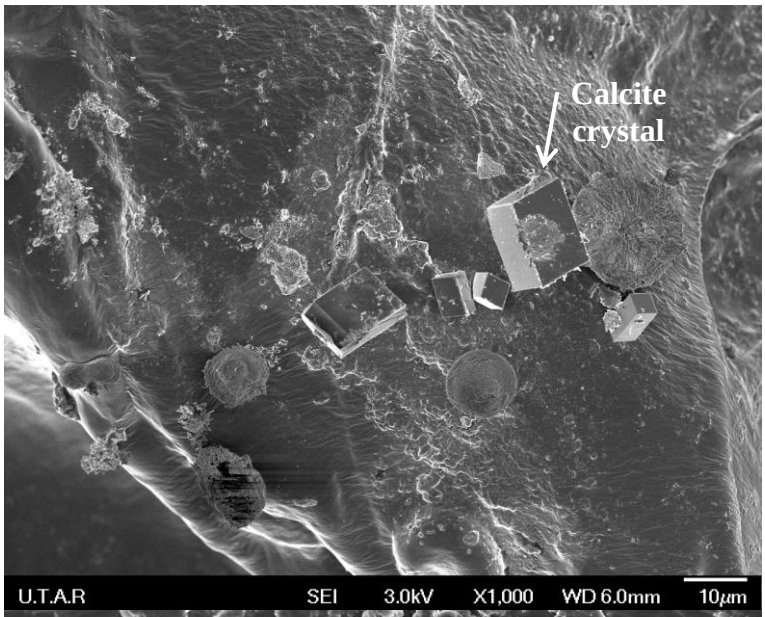


Figure 4.18: SEM images of treatment with bacteria and a close view of crystals. Magnification (a) 1000 x (b) 5000 x.

(a)



(b)

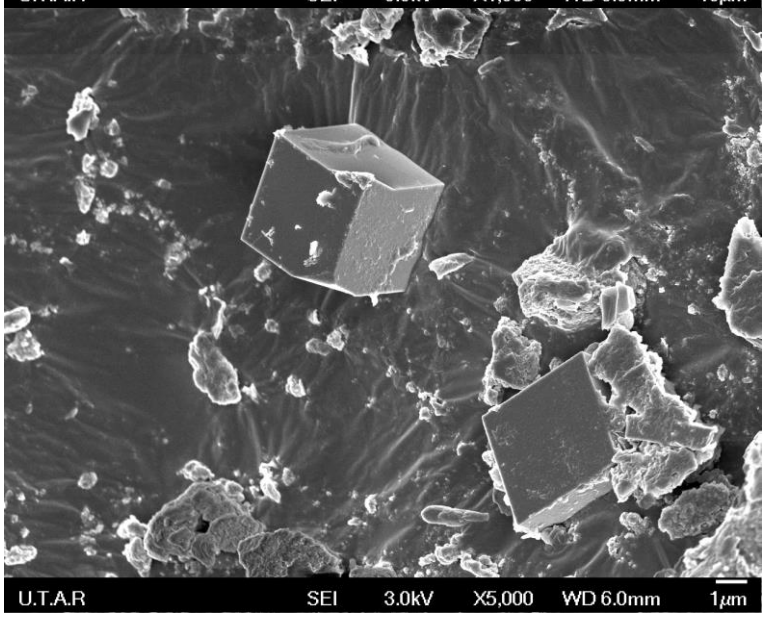
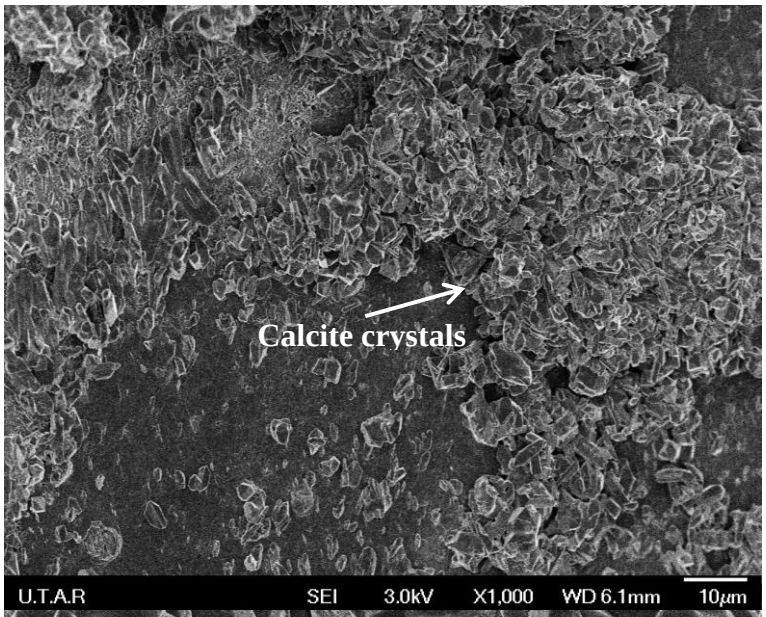


Figure 4.19: SEM images of treatment without bacteria and a close view of crystals. Magnification (a) 1000 x (b) 5000 x.

(a)



(b)

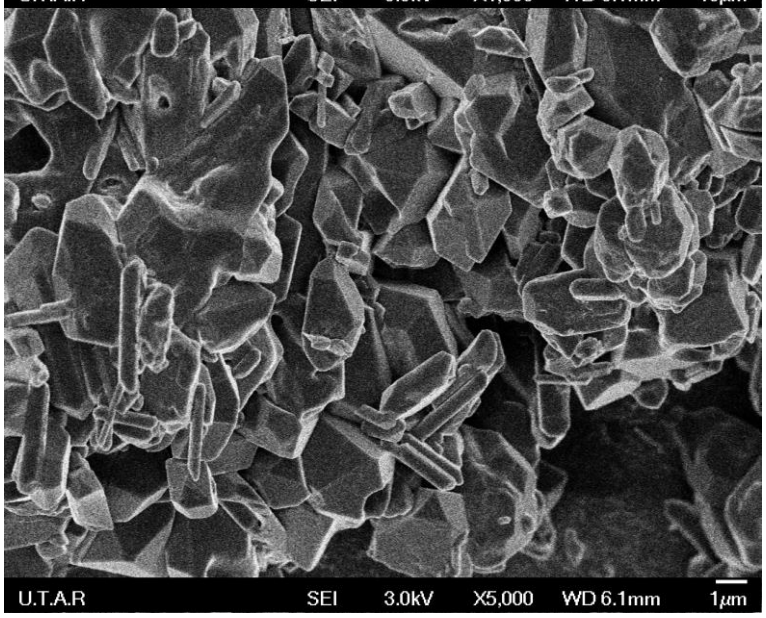
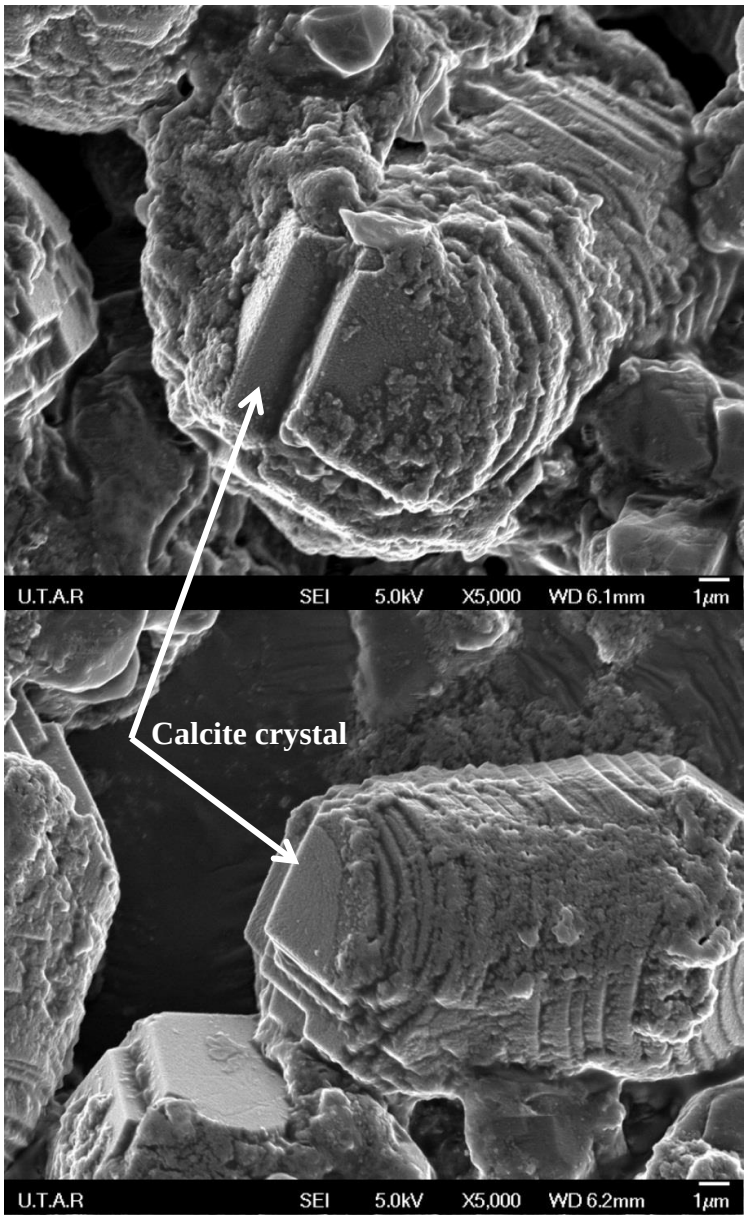


Figure 4.20: SEM images of treatment without continuous supply of carbon dioxide and a close view of crystals. Magnification (a) 1000 x (b) 5000 x.

(a)



(b)

Figure 4.21: Top (a) and side (b) view of dominant prismatic calcite crystal (5000 x magnification).

Table 4.11: Calcite crystals weight (g) and EDS element (weight %).

Condition		Treatment A	Treatment B	Treatment C
Calcite crystals weight (mg)		80	10	30
EDS Element (Weight %)	Calcium	15.62	2.12	2.76
	Oxygen	47.67	38.26	33.74
	Carbon	34.48	54.70	59.83

* Treatment A with bacteria, treatment B without bacteria and treatment C without continuous supply of carbon dioxide.

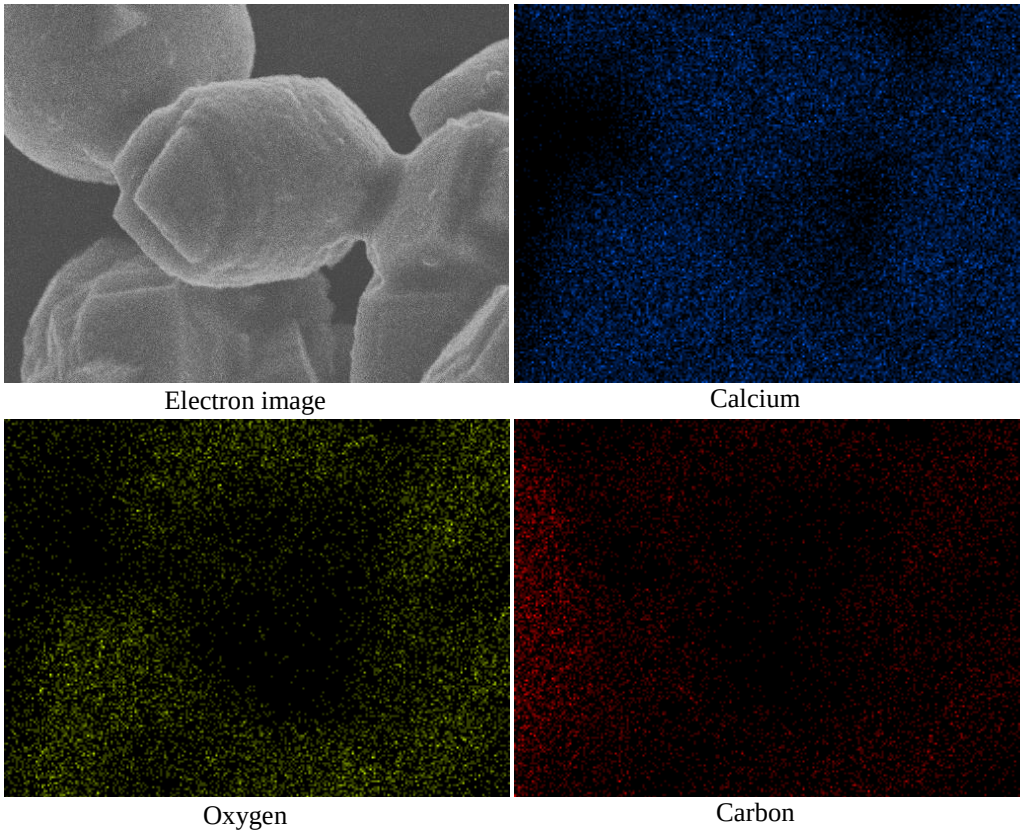


Figure 4.22: Elemental mapping of calcite crystals in treatment A showing calcium, oxygen and carbon elements.

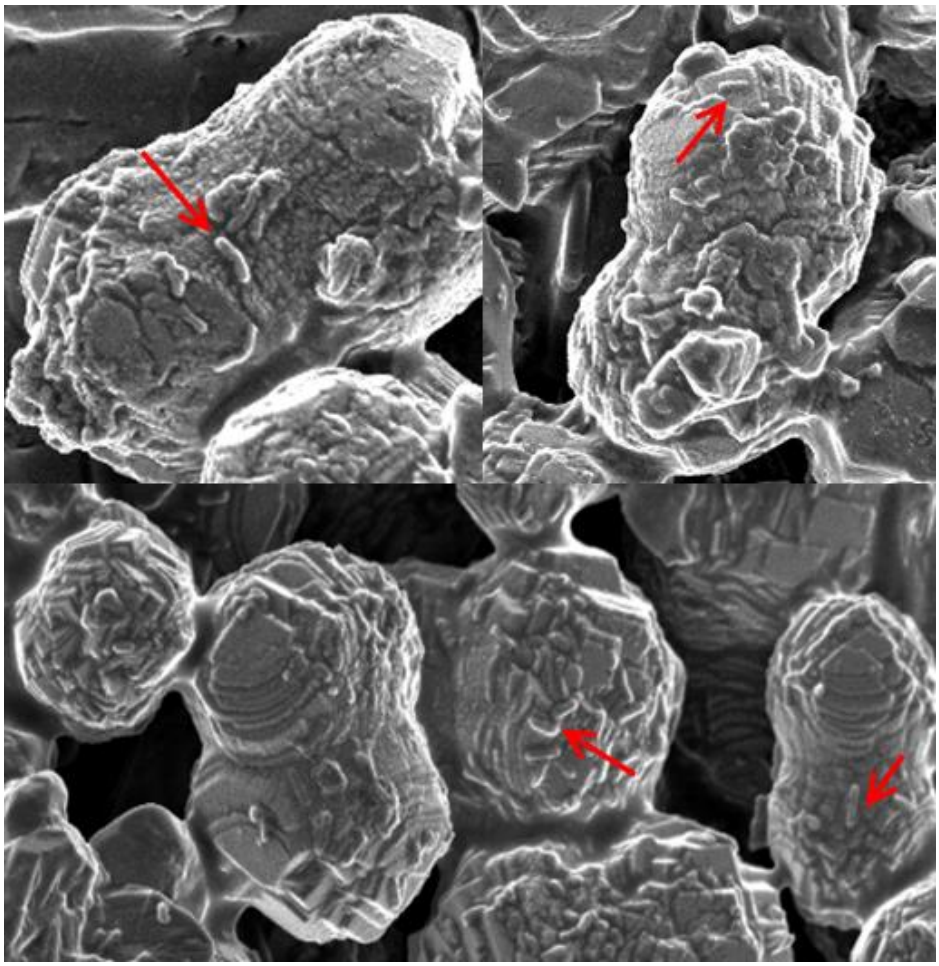


Figure 4.23: Bacterial imprints on the surface of calcite crystals in treatment A as indicated by red arrows.

4.7 Discussion

Our findings confirmed that there are CFB found in limestone and soil samples in Malaysia. As such the original hypothesis of presence of calcite formation by bacteria isolated from limestone and soil samples were supported by the results of preliminary screening in this study. Bacteria from various natural habitats have frequently been reported to form calcite both in natural and laboratory conditions (Alhour, 2013). Similar research on bacterial calcite formation from limestone samples were conducted by Ercole et al. (2001), Cacchio et al. (2003), and Baskar et al. (2006).

In this present study it was found that the samples containing the most bacteria isolated tended to contain a higher proportion of CFB, and number of bacterial colonies found varied with the sampling location (Table 4.3). 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. Boquet et al. (1973) has isolated 210 bacterial from soils, and they stated that all the bacteria are able to form calcite. Shinano (1972) isolated 13% CFB isolates out of 5000 isolates examined. Our present study shows similar results as Shinano (1972) since out of 54 isolates, only 33% CFB isolates were present.

Mineral identification of soil samples was difficult due to presents of many mineral phases in the samples. Mineral identification using XRD was accomplished via qualitative and quantitative manners, and this was solely dependent on the intent of the research. Intent of this study is more on isolation

and characterization of CFB from natural samples. Therefore, the mineral identification of soil samples was not a vital criterion for in-depth discussion.

Overall, the mineral identification of limestone samples shows the calcite mineral was dominant (table 4.1). Besides, microscopic observations on bacterial calcite formation showed that the calcite formation takes place in the presence of bacterial colonies (figure 4.2). According to Boquet et al. (1973), in right situation most of the bacteria are capable to form calcite. The XRD analysis shows that only the calcite element was found in the crystals collected formed by bacterial isolates. This again proves that the bacterial cells act as nucleation sites for the calcite formation (Stocks-Fisher et al., 1999; Hammes and Verstraete, 2002; Li et al., 2011b).

Caves are normally in an environment of nutrient-limited, dark due to the absence of solar energy, geologically isolated and contain low levels of available organic carbon to support diverse bacterial communities (Barton and Northup, 2004). Malaysia has some of the biggest and longest limestone cave, especially in Peninsular Malaysia comprising Langkawi, Kedah-Perlis, Kuala Lumpur and Kinta Valley. The practical applications of cave microbiology are wide and varied; application of calcite forming bacterial isolated from limestone and soil samples has been investigated in our current project as one of the solution for climate change issue.

The sequence analysis for the 16S rDNA gene of the selected four isolates were determined and compared with those of reference *Bacillus* spp

strains from 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 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.

Boquet et al. (1973) and Baskar et al. (2006) identified *Bacillus pumilus* strains from their study. Baskar et al. (2006) suggested that the optimum temperature and bacterial activity appears to be the main factors in the formation of calcite, 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.

Arunachalam et al. (2010) studied about the calcite forming ability of *Bacillus sphaericus*. *Bacillus sphaericus* was another species with similar entity of *Bacillus pasteurii* in calcite forming capability. Apparently, *Bacillus 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; 2010) from Belgium studied application of *Bacillus sphaericus* in building industry. Ercole et al. (2001) isolated and identified *Bacillus sphaericus* from cave samples; their research is more in finding of bacterial action into cave speleothem formation.

We found that the four identified bacterial strains as CA type of CFB to be *Bacillus pumilus* (2 isolates), *Bacillus sphaericus* and *Bacillus cereus*. 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 CO_2 to bicarbonate, which would then be available for calcite formation (Rahman et al., 2007). Therefore, 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 CA from the atmosphere.

Yadav et al. (2011) summarized that CA production specifically by *Bacillus pumilus* can promote calcite formation. Li et al. (2011b) also found that that the CA produced by *Bacillus* spp. isolated from karst soil are able to promote calcite formation. It was found that the isolated and identified *Bacillus pumilus* and *Bacillus sphaericus* were the best candidate of CA type of CFB. It is interesting to note, identified *Bacillus sphaericus* S3 SC_ 3 and *Bacillus cereus* S2 SC_1 fells into both urease and CA type of CFB. *Bacillus sphaericus* shows the highest esterase activity and so long was studied for urease type of CFB applications only.

Besides, *Bacillus pumilus* industrial capability was proved for xylanase production (Duarte et al., 2000; Marta et al., 2000; Battan et al., 2007; Bindu et al., 2007; Ayesegul et al., 2008; Kapoor et al., 2008; Liu and Liu, 2008; Monisha et al., 2009; Buthelezi et al., 2010), cellulase production (Kotchoni et

al., 2006), lipase production - biocatalyzed (Ruiz et al., 2002), antibacterial compounds production (Hassan et al., 2009), D-Ribose production – flavor enhancer in food, health food, pharmaceuticals, and cosmetics (Miyagawa et al., 1992), endoglucanase production (Hidayah et al., 2008), and bacteriocins production (Aunpad and Na-Bangchang, 2007).

Besides the various enzymes production ability, *Bacillus pumilus* are able to degradate keratinous waste (Kumar et al., 2008), and reduce chromium (Shakoori et al., 2010). Chromium is a metal which poses toxic effects to human and the environments (Shakoori et al., 2010), whereby the chromium reduction capability of *Bacillus pumilus* will brings enormous benefits.

The activity of CA can be estimated by following procedures; manometric, colorimetric, electrometric, titrimetric and spectrometric (Wilbur and Anderson, 1948; Sierra, 1957). CA activity in this study was determined by spectrophotometric method described by Prabhu et al. (2009). This method firstly described by Armstrong et al. (1996), which further modified by Polat and Natbantoglu (2002) and recently by Prabhu et al. (2009) where the procedure has been simplified.

Matthews et al. (2007) studied the biochemical characterization of the esterase activities of wine lactic acid bacteria by spectrophotometrically using *p*-nitrophenyl substrate. Esterase activity was examined through *pH* range of 3.0 to 8.0. Study findings shows that most of the lactic acid bacteria found to have the highest esterase activity at *pH* close to 6.0. Recently, Kanth et al.

(2014) studied the CA from *Persephonella marina* for CO_2 sequestration applications. CA isolated from *Serratia* sp. from marble rock for CO_2 sequestration by calcite formation has been studied by Bharti et al. (2014). Luca et al. (2013) reported the cloning and characterization of an α-CA from thermophilic bacterium *Sulfurihydrogenibium azorense*, concluded that it is the most catalytic effective CA ever investigated after human superoxide dismutase which makes this enzyme an interesting candidate for application in CO_2 sequestration and biofuel production. Sharma et al. (2009) studied the extracellular CA from *Pseudomonas fragi* isolated from calcite enriched soil, and emphasized the significant role CO_2 sequestration.

Temperature, *pH* and glucose play an important role in enhancing calcite forming ability (Boquet et al., 1973; Hammes and Verstraete, 2002). Bacterial growth at optimum conditions tends to have a higher number of bacteria, and subsequently high nucleation site for the calcite formation (Hammes and Verstraete, 2002). The rate of bacterial calcite formation is correlated with cell growth (Alhour, 2013). The present study only measures the bacterial absorbance, whereby future research could focus on quantification of calcite formation affected by those parameters.

Baskar et al. (2006) who isolated and identified *Bacillus pumilus* suggested that the bacterial activity and optimum temperature appear to be the key factors in calcite formation. From our result, the optimum growth was achieved at 30 $^{\circ}$C. Baskar et al. (2006) found that the optimum temperature for calcite formation was 25 $^{\circ}$C. Parvathi et al. (2009) stated that the growth

temperature of *Bacillus pumilus* ranged from 5 – 50 °C. It is apparent that the optimum temperature as obtained from the present study still shows reasonably good comparisons with previous reported values.

Kumar et al. (2008) tested *Bacillus pumilus* within the temperature range of 25 – 45 °C and *pH* range of 7.5 – 10. The optimum conditions were *pH* 8 and temperature 35 °C of keratinase production. Ariffin et al. (2006) found that cellulose production by *Bacillus pumilus* was optimally active at *pH* 6.0 and temperature 60 °C. Boquet et al. (1973) stated that the medium without glucose gave good growth and crystals formation, but added glucose speeded the processes. Therefore, we choose glucose concentration as one of the parameters for optimization. From our result, it shows that 20 g/L of glucose contributes to the optimum growth. Therefore, future study on calcite formation by *Bacillus pumilus* should take this parameter into consideration.

There were obvious differences in the size and morphology of calcite crystals in all the treatments based on the photos taken by SEM. Calcite crystals principally found as three polymorphs: calcite, needle-like aragonite and spherical vaterite (Cizer et al., 2008). Calcite is the most stable phase with the typical habits of rhombohedron, scalenohedron, and prismatic habits. Aragonite, vaterite and amorphous calcite are the metastable phases which may transform into calcite as the carbonation proceeds (Cizer et al., 2008). Treatments A and B show a stable form of calcite as prismatic and rhombohedral crystals, respectively. Treatment C shows amorphous shape crystals which are metastable phase of calcite. This may due to insufficient

amount of CO_2 supplied throughout the experiment. Therefore, thorough experimental design needs to be planned in future to confirm this. Overall from this study, it's concluded that the presence of bacteria influences the morphology of the calcite crystals.

These results suggested that bacteria serve as nucleation sites for calcite formation, which is in agreement with the earlier research results of Hammes and Verstraete (2002) and Henriques (2011). Through our current study, *Bacillus pumilus* S3 SC_1 mediated the sequestration of CO_2 via formation of calcite. Yadav et al. (2011) summarized that CA produced by *Bacillus pumilus* has ability towards CO_2 sequestration by calcite formation. Mirjafari et al. (2007) stated that the CA enzyme is used to enhance the hydration and subsequent formation of calcite. These previous findings showed good comparison with the present results in which the amount of calcite crystals formed in absence of bacteria are lesser compared to that with the presence of bacteria. A bacterium is the source of CA enzyme, and the formation of calcite was promoted in the presence of the enzyme. CA is well-known enzyme that is ubiquitous in nature and found in animals and plants and even in the human erythrocyte.

Apparently, CA produced by bacteria is beneficial as the bacteria are abundant in nature and can be reproduced easily at the lowest cost. CFB is highly desirable because it is natural and pollutants free (Alhour, 2013). Mirjafari et al. (2007) also found that in the absence of enzyme the calcite formation still takes place, but at a significantly slower rate compared to the

condition with the presence of enzyme. The reaction takes place in 1 hour, but the amount of calcite crystals formed is 80 mg in the presence of bacteria (bacteria is the source of CA enzyme) and 30 mg without the presence of bacteria. Therefore it was proven that CA produced by bacteria enhances the calcite formation. Moreover, SEM analysis shows the presence of bacterial imprints on the surface of calcite crystals in treatment A (with the presence of bacteria).

4.8 Concluding Remarks

This chapter presents the results of isolation and characterization of naturally occurring CFB isolated from limestone and soil samples in Malaysia. Following are the summary of the key findings from this chapter;

a) A total of 18 CFB were isolated from limestone and soil samples.

b) CFB mechanisms of all the 18 CFB isolates were successfully identified. 12 falls into urease type, one into denitrifying type and 4 (S3 SC_1, S3 SC_3, S5 SC_1, and S8 SC_1) isolates the maximum level of esterase activity of CA analysis.

c) *Bacillus pumilus* (2 isolates), *Bacillus cereus,* and *Bacillus sphaericus* were identified using 16s rDNA sequencing.

d) Culturing condition of *Bacillus pumilus* is optimum at;

   1) Temperature : 30 $^{\circ}$C

   2) *pH* : 7

   3) Glucose : 20 g/L

e) CO_2 sequestration ability of *Bacillus pumilus* was proven.

5 CHAPTER

CONCLUSIONS

The study site in a limestone area of Malaysia was found to have a considerably high population of calcite forming bacteria (CFB), 43.5% out of 23 isolates whereas soil samples shows low population of CFB, 25.8% out of 31 isolates. Through this preliminary screening, it can be concluded that by providing sufficient nutrient and calcium ions some of 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 18 CFB isolates, four isolates was identified of carbonic anhydrase (CA) type (shows highest amount of esterase activity), 12 isolates were identified to be urease type of CFB, and one isolates shows positive to denitrification. *Bacillus sphaericus*, *Bacillus pumilus* (2 isolates), and *Bacillus cereus* were identified through 16s rDNA sequencing. *Bacillus sphaericus* and *Bacillus cereus* falls into both categories of CFB; urease and CA type. As to the utilization of bacterial calcite formation of CA type for carbon dioxide (CO_2) sequestration to overcome climate change, this preliminary study reported permits a consideration of possible bacterial species. Further research was conducted in order to prove the CO_2 sequestration ability of *Bacillus pumilus*. The results showed that in the presence of bacteria the calcite crystals formed are higher with the fixed crystals morphology.

Overall, the summary of the findings listed below;

a. 18 CFB isolates were isolated from limestone and soil samples.

b. All the CFB mechanisms were characterized successfully.

c. Four selected isolates were identified via molecular characterization as *Bacillus pumilus* (2 isolates), *Bacillus sphaericus*, and *Bacillus cereus*.

d. *Bacillus pumilus* were further characterized using biochemical assay.

e. Growth condition of *Bacillus pumilus* were optimized for temperature, *pH* and glucose concentration.

f. CO_2 sequestration ability of *Bacillus pumilus* was proven.

In conclusion, climate change is the problem addresses at the beginning of this study which caused by excess amount of CO_2. At the end of this study, locally isolated and identified *Bacillus pumilus* ability towards CO_2 sequestration was proven. Therefore bacterial calcite formation of CA type can be utilized for CO_2 sequestration to overcome the issue of climate change. Finally it is concluded that sequestration of anthropogenic CO_2 into calcite mineral using CA gives the promising option of CO_2 sequestration.

Besides, the study presented here is only the beginning of the efforts of using bacterially produced CA for CO_2 sequestration. In future, additional

studies on addressing various issues should be taken for the industrial application to reduce the issue of climate change.

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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

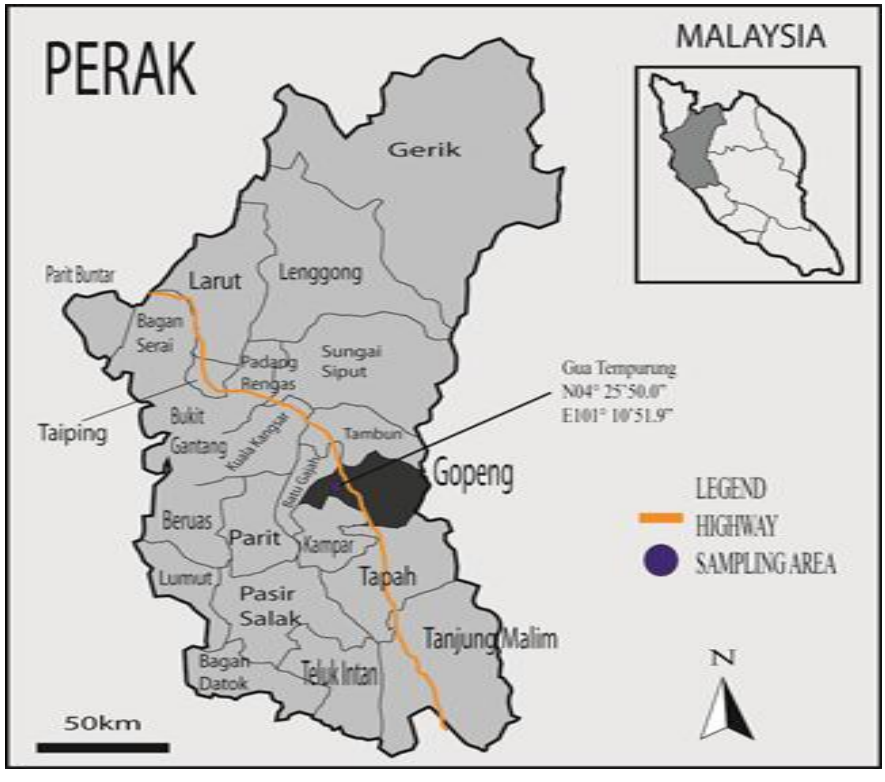
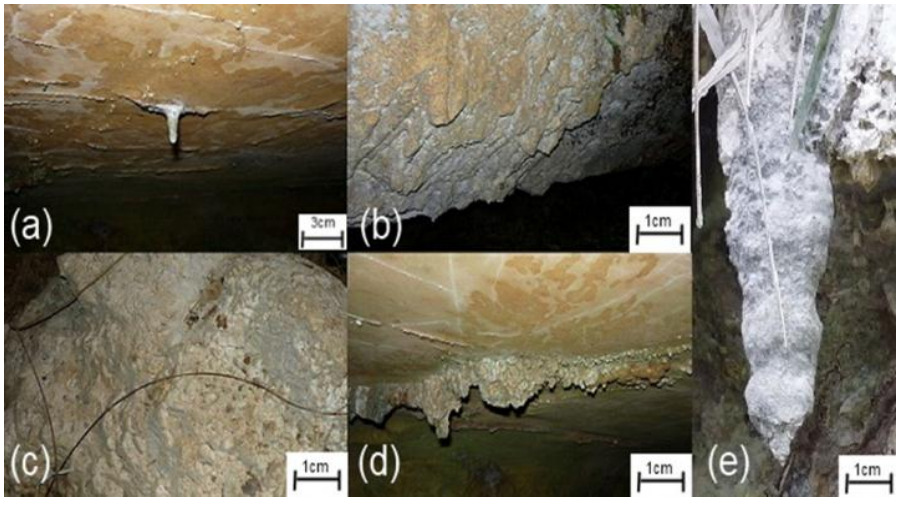


Figure 2. Limestone cave samples (a) S1 (b) S2 (c) S3 (d) S4 (e) S5.



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

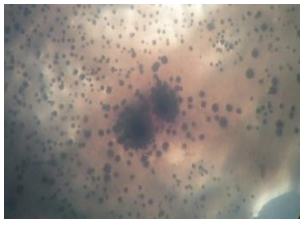
CFB Isolation

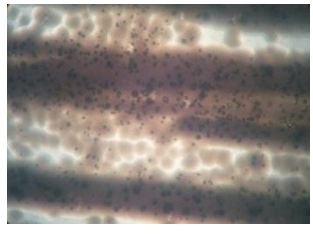
Out of 23 total bacterial isolates from all the isolated samples, 10 isolates were detected for the presence of crystals by observation using the binocular microscope (**Table 1**). The images taken from binocular microscope of isolates S2 SC_1 and S3 SC_3 were shown in **Figure 3**.

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)

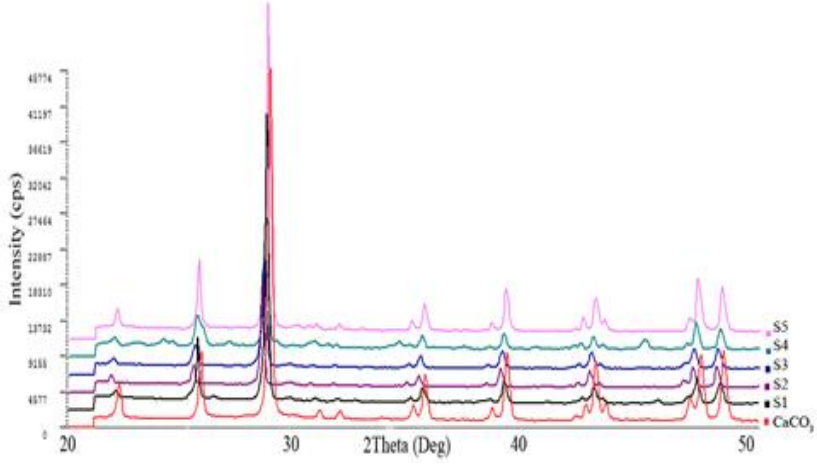


Figure 5. Shows the X-ray Diffraction spectra from one of the isolate, S1 SC_1 (blue line) (counting time = 1 sec/step) showing a close similarity with standard $CaCO_3$ (red line) spectra

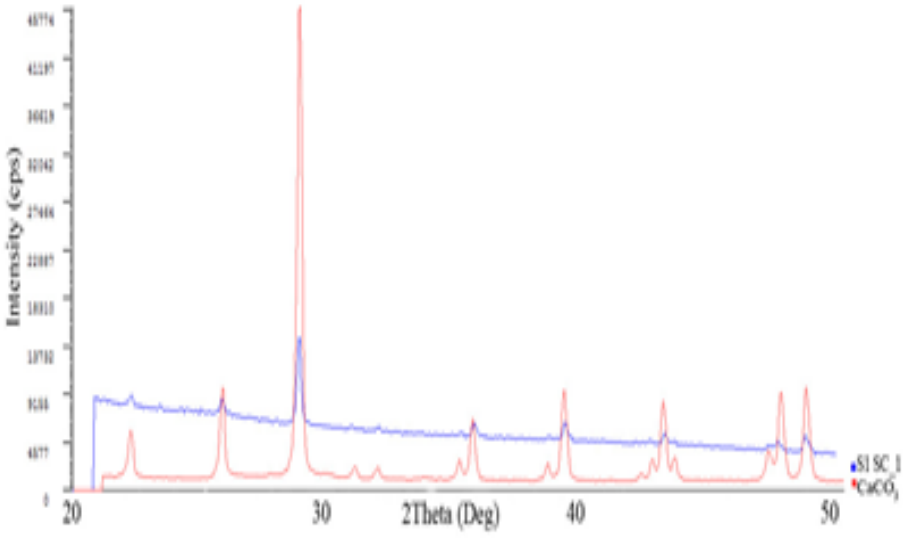


Table 3. XRD quantitative analysis of the final weight fractions [a] of crystals collected from all of the CFB bacterial isolates

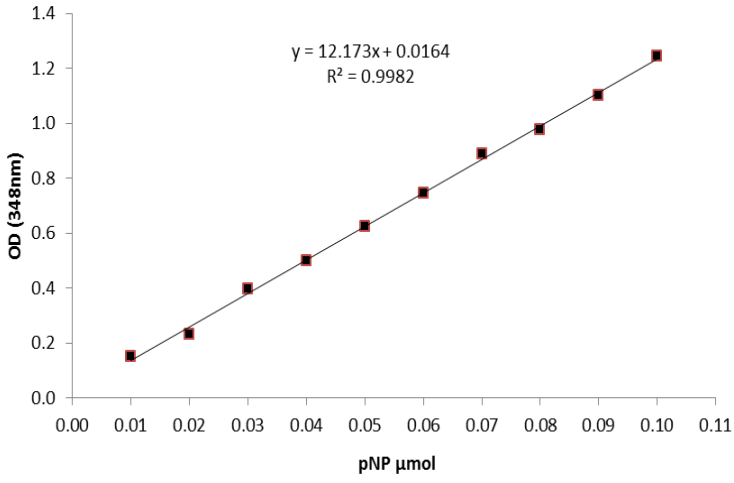
Bacterial Strain ID	Calcium carbonate (Calcite, syn) ($CaCO_3$) (ICDD 05-0586)	Calcium carbonate (Calcite) ($CaCO_3$) (ICDD 24-0027)	Calcium carbonate ($CaCO_3$) (ICDD 47-1743)
S1 SC_1	0.744	0.725	0.871
S2 SC_1	0.214	0.205	0.825
S2 SC_2	0.934	0.883	0.866
S2 SC_3	0.781	0.737	0.877
S2 SC_4	0.311	0.298	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)**.

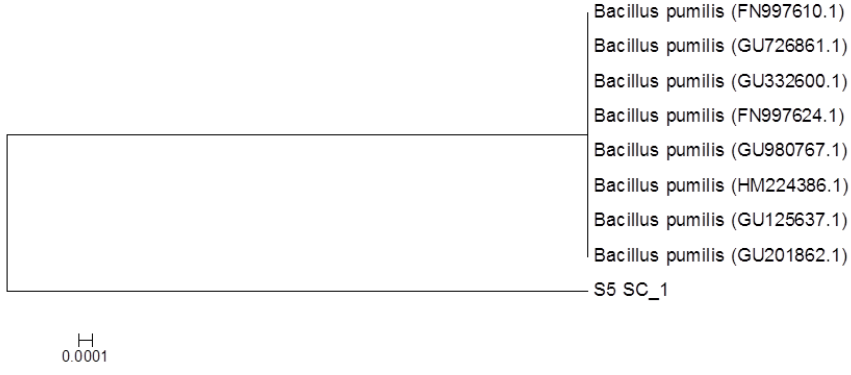
Table 6. shows the overall identification results of four isolates

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 (GU904881.1)
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)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Bacillus pumilis (GU980767.1)
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Bacillus pumilis (GU125637.1)
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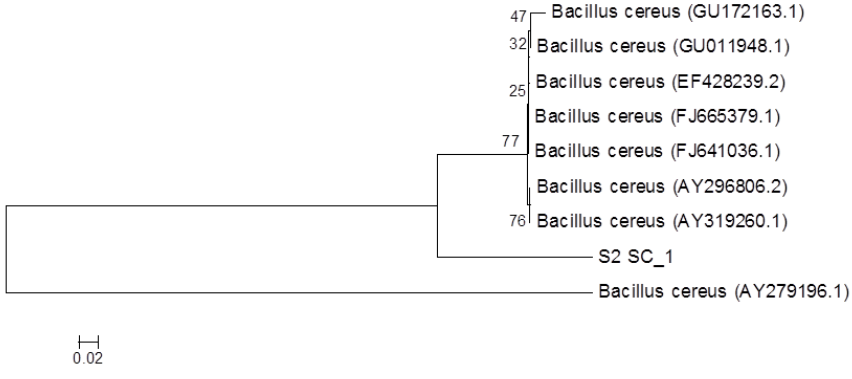
S5 SC_1
0.0001

(b)


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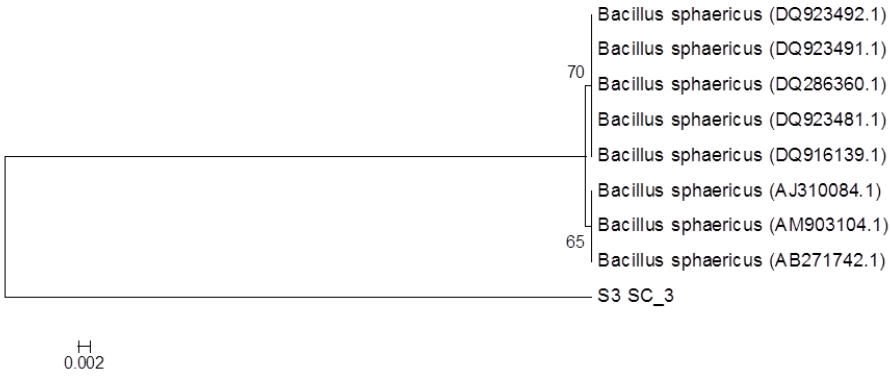
76
Bacillus cereus (GU172163.1)
Bacillus cereus (GU011948.1)
Bacillus cereus (EF428239.2)
Bacillus cereus (FJ665379.1)
Bacillus cereus (FJ641036.1)
Bacillus cereus (AY296806.2)
Bacillus cereus (AY319260.1)
S2 SC_1
Bacillus cereus (AY279196.1)
0.02

(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.

ACKNOWLEDGEMENTS

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Biological Carbon Dioxide Sequestration Potential of *Bacillus pumilus*

(Potensi Pemencilan Biologi Karbon Dioksida oleh *Bacillus pumilus)*

T. KOMALA* & TAN. C. KHUN

ABSTRACT

Bacillus pumilis *was isolated and identified from limestone and the ability towards carbon dioxide (CO_2) sequestration was demonstrated*. B. pumilus *(S3 SC_1), isolated from Gua Tempurung, Gopeng, Perak was able to form calcite in the presence of calcium ions*. B. pumilus *was successfully characterized by using conventional biochemical characterization and 16s rDNA sequencing. Three types of experimental systems with* B. pumilus, *without* B. pumilus *and without continuous supply of CO_2 with the presence of* B. pumilus *which could produce extracellular carbonic were studied to determine the effects of bacterially produced carbonic anhydrase (CA) by* B. pumilus *in removing CO_2 as calcite. Through our current study, CO_2 sequestration ability of* B. pumilus *was proven.*

Keywords: B. pumilus*; carbon dioxide sequestration*; *carbonic anhydrase; characterization*

ABSTRAK

Bacillus pumilis *telah diasingkan dan dikenal pasti daripada batu kapur dan keupayaan ke arah pemencilan karbon dioksida (CO_2) telah dijalankan*. B. pumilus *(S3 SC_1) diasingkan dari Gua Tempurong, Gopeng, Perak mampu membentuk kalsit dengan kehadiran ion kalsium*. B.pumilus *berjaya dicirikan dengan menggunakan pencirian biokimia konvensional dan 16s rDNA. Tiga jenis sistem percubaan dengan* B. pumilus, *tanpa* B. pumilus *dan tanpa bekalan berterusan CO_2 dengan kehadiran* B. pumilus *yang boleh menghasilkan ekstrasel carbonik telah dikaji untuk menentukan kesan bakteria hasilan karbonik anhidrase (CA) oleh* B. pumilus *dalam menghapuskan CO_2 sebagai kalsit. Melalui kajian ini, CO_2 keupayaan pemencilan oleh* B. pumilus *telah dibuktikan.*

Kata kunci: B. pumilus*; karbonik anhidrase; pemencilan karbon dioksida; pencirian*

INTRODUCTION

Carbon dioxide (CO_2) plays a major role as a greenhouse gas emitter. The world is getting hotter, owing to the greenhouse gas emissions. Greenhouse gases absorb and emit thermal radiation that brings about climate change. The climate change issue has attracted the attention from all the countries due to tremendous climate change. Biological carbon sequestration is environmental friendly and cost-effective means of reducing carbon dioxide from the atmosphere (Prabhu et al. 2011). Carbonic anhydrase (CA; Carbonate hydrolyase, EC 4.2.1.1) is produced by bacteria that catalyzes the reversible hydration/dehydration reactions of carbon dioxide and is involved in the calcite forming process (Siktar 2009). CA catalysts the inter-conversion between carbon dioxide and bicarbonate, with H^+ ions being transferred between the active site for the enzyme and the surrounding buffer (Achal & Pan 2011). This result in a change of pH from 8.2 to 8.4 as the reaction proceeds towards equilibrium which also contributes to calcite formation in the presence of calcium ions. CA provides a viable means to accelerate CO_2 into calcite in presence of suitable cation at moderate pH values (Sharma et al. 2008).

$$H2O + CO_2 \rightarrow HCO_3^-.$$

Calcite formation in solution occurs through overall equilibrium reaction of:

$$Ca^{2+} + CO_3^{-2} \leftrightarrow CaCO_3.$$

The production of CO_3^{-2} from bicarbonate (carbon dioxide conversion into bicarbonate via carbonic anhydrase activity) in water is strongly pH dependent; an increase in CO_3^{-2} concentration occurs under alkaline (pH8.2 to 8.4) conditions (Lee 2003). *B. pumilus* has been detected for CA production (Prabhu et al. 2009; Yadav et al. 2011). Yadav et al. (2011) has developed a single enzyme nanoparticles of carbonic anhydrase (SEN_CA) formed by modifying the surface of CA isolated from *B. pumilus* with a thin layer of organic/inorganic hybrid biopolymer such as chitosan. This is due to the instability of the enzyme. They have proven in their research that SEN_CA enhances the rate of carbon dioxide hydration further compared with free CA. Prabhu et al. (2009) on the other hand, study on the different chitosan based materials to immobilize CA enzyme extracted from *B. pumilus* for carbon sequestration.

Besides that, *B. pumilus* industrial capability was proven in xylanase production (Aysegul et al. 2008; Battan et al. 2007; Buthelezi et al. 2010; Duarte et al. 2000; Kapoor

et al. 2008; Liu & Liu 2008; Monisha et al. 2009), cellulose production (Kotchoni et al. 2006), lipase production - biocatalyzed (Ruiz et al. 2002), antibacterial compounds production (Hasan et al. 2009), D-Ribose production – flavor enhancer in food, health food, pharmaceuticals and cosmetics (De Wulf & Vandamme 1997; Miyagawa et al. 1992), endoglucanase production (Hidayah et al. 2008) and bacteriocins production (Aunpad & Na-Banhchang 2007). Besides the various enzymes production ability, *B. pumilus* are able to degrade keratinous waste (Kumar et al. 2008) and reduce chromium capability (Shakoori et al. 2010). Chromium is the toxic metal which poses toxic effects to human and the environments (Shakoori et al. 2010), whereby the chromium reduction capability of *B. pumilus* will brings enormous benefits.

Xynalase are mostly produced from bacterial fermentation processes, which has wide industrial and biotechnological applications (Buthelezi et al. 2010). As stated in previous paragraph, numerous researchers have been done on *B. pumilus* xynalase production by considering the wide industrial applications. There are numerous applications of cellulose production by *B. pumilus* in various industries such as brewery and wine, textile, detergent, pupl and paper industries (Kotchoni et al. 2006) and in agriculture and animal feeds (Bhat & Bhat 1997; Jang & Chen 2003). In addition, bacteriocin has a potential application as natural preservatives (Klaenhammer 1988) and therapeutic application as an antibacterial agent (Gray et al. 2006). Besides that, bacteriocin usage as a replacement for currently used antibiotics is promising (Papagianni 2003). In summary, *B. pumilus* has a lot of industrial significance, by considering the nature of bacteria which are able to reproduce easily at the lowest cost.

The purpose of this study was to determine the ability of *B. pumilus* (S3 SC_1) isolated from Gua Tempurung, Gopeng Perak to remove carbon dioxide as calcite which includes the identification by using biochemical and 16s rDNA sequencing methods. The effects of bacterially produced CA enzyme in calcite formation through two types of experimental system with and without the bacteria are also studied. In the end, the mass of calcite formed was determined together with morphology and mineralogical composition.

Materials and Methods

Bacterial strain

The S3 SC_1 isolates, which initially isolated from cave samples, was maintained in a slants of B-4 medium (2.5 g/L calcium acetate, 4.0 g/L yeast extract, 10 g/L glucose) at 4°C for further studies. The crystals formed by S3 SC_1 were observed using binocular microscope and gram stained cells were observed under binocular and light microscope, respectively.

X-ray diffractometer (XRD) analysis

X-ray diffraction measurements were done on collected crystals formed by bacteria in petri plates. The crystals formed by bacteria in agar plates were observed under binocular microscope, and the spot rich in crystals was cut into flat square blocks and placed in clean microscopic glass slides (75×25 mm). The glass slides together with crystals were dried in dryer (70°C) for 3 days to make sure the agar dried and left with crystals. The supply voltage of the X-ray tube was set at 50 kV and 30 mA. The 2θ scan range was between 22° and 50°; each scan was done in steps of 0.05°. The crystalline phases were identified using the International Centre for Diffraction Data (ICDD) database (Joint Committee on Powder Diffraction Standards, JCPDS) where the identification accomplished by comparing the diffraction spectra with known standard mineral, in this case calcium carbonate (Mercks) powder.

Biochemical characterization

Conventional biochemical characterization using Biolog GP plates (Biolog, Hayward, CA, USA) were carried out for S3 SC_1 isolates. Inoculation and reading of the microplates were carried out according to the instructions of manufacturer using a Biolog microplate reader, with Biolog MicroLog 3 and Release 3.50 computer Software. Comparison was made against a database containing identification patterns of bacteria species. A standard inoculum was determined with a turbimeter. A suspension was prepared by removing cells from a plate with a sterile swap into inoculating fluid (GP-IF for Gram positive and GN-IF for Gram negative). Cell density was adjusted to 28, 52 and 61% transmittance, respectively, for Gram positive rod-shaped spore-forming bacillus, Gram negative non-enteric and Gram negative enteric bacteria, respectively. Aliquots of 150 µL suspension was inoculated into the microplate with an 8-channel repeating pipette and incubated for 16 to 24 h at 30°C. The biochemical fingerprint was automatically read with the MicroStation Reader using MicroLOg 3 software.

16S rDNA sequencing

The bacteria genomic DNA was extracted by using the traditional phenol-chloroform method described by Taggart et al. (1992) with some modification. The amplification of 16S rDNA was as described by Garbeva et al. (2003). The 16S rDNA was amplified by using BacF (5'-GGGAAACCGGGGCTAATACCGGAT-3'; specific for Bacillus and related taxa) and R1378 (5'-CGGTGTGTACAAGGCCCGGGAACG-3'; universal bacterial 16S rDNA reverse primer). 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 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).

CA Analysis by Esterase Activity

Extracellular CA Enzyme Preparation

The bacterial was inoculated in 250 mL flask contained 100 mL CA producing medium (basic liquid beef-proteose-NaCl medium with 10 µM zinc sulfate, pH7.2), then these flask were incubated in a rotary shaker at 180 rpm, 30°C. A volume of culture containing adequate number of cells was centrifuged a 2000× g for 10 min and washed twice with 0.1 M phosphate buffer. The pellet was re-suspended with the same buffer, in which the measurements of enzymatic activity were made immediately after the re-suspension of the cells. For experimental system of carbon dioxide sequestration, a volume of culture containing adequate number of cells was centrifuged at 2000× g and the pellet suspended with 1M Tris-HCl buffer (pH8.3).

Esterase Activity

Esterase enzymatic activity was determined by using the spectrophotometric assay described by Polat and Nalbantoglu (2002) with slight modification. In brief, the assay consisted of 1.8 mL of 0.1 M phosphate buffer and 1.0 mL of 3 mM p-nitrophenyl acetate with 0.2 mL of bacterial extracellular CA extract. One mL of sample from this solution was taken and measured its absorbance in UV/VIS spectrophotometer at 348 nm. All the assays were done in triplicates. The change in absorbance at 348 nm was recorded over the first 5 min to estimate the amount of the p-nitrophenol (*p*-NP) released. The non-enzymatic hydrolysis was subtracted by using a blank without enzyme. One unit of activity represents the amount of enzyme catalyzing to produce 1 µmol p-nitrophenol per min under the assay conditions.

Experimental System for Carbon Dioxide Sequestration

Three types of experimental systems were designed; with *B. pumilus* (S3 SC_1) (Treatment A), without *B. pumilus* (Treatment B) and without continuous supply of CO_2 with the presence of *B. pumilus* (Treatment C). All the experiments were done in triplicates. First, 40 mL of CO_2 saturated water were transferred into 250 mL conical flask. Next, aliquots of 10 mL bacterial suspension were introduced into the mixture for treatment A, 10 mL of distilled water was replaced for treatment B. After that, aliquots of 10 mL of Tris-HCl (1 M, pH8.3) were added into the test tubes followed by aliquots of 40 mL 200 mM calcium acetate. CO_2-saturated water was supplied throughout the experiment for 1 h for treatment A and B. At the end of the experiment, the mixture was centrifuged and dried overnight at 70°C.

Analytical Methods

Dried crystals weight was measured by using the weighing machine. The precipitated crystals were analyzed using field emission scanning electron microscopy (FESEM/EDX) to determine the morphology and mineralogical composition.

Results

Bacterial Strain

The calcite crystals formation was observed using binocular microscope which shows the intense calcite formation within the colony (Figure 1(a)). This bacterial strain observed as purple cells under light microscope, thus accepted to be Gram positive (Figure 1(b), 1(c)).

X-ray Diffractometer (XRD) Analysis

The calcite formed by S3 SC_1 was characterized by XRD by referring to calcium carbonate powder (Merck) as standard. It can be clearly seen from the XRD graph in Figure 2 that all the major peaks were at the same position (2θ).

Biochemical Characterization

Overall biochemical identifications were accepted as correct if the assigned identity met or exceeded 92% probability. Therefore, the identification of S3 SC_1 as *B. pumilus* (S3 SC_1) was accepted with 99.7% probability.

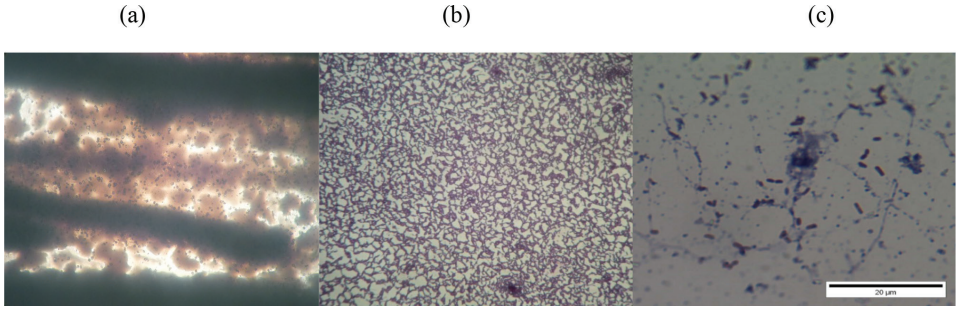

FIGURE 1. (a) Optical microscopic images of crystals formed by S3 SC_1, (b) appearance of Gram (+) cell under binocular microscope, S3 SC_1 and (c) appearance of Gram (+) and rod shaped cells under light microscope using oil immersion (100X magnification)

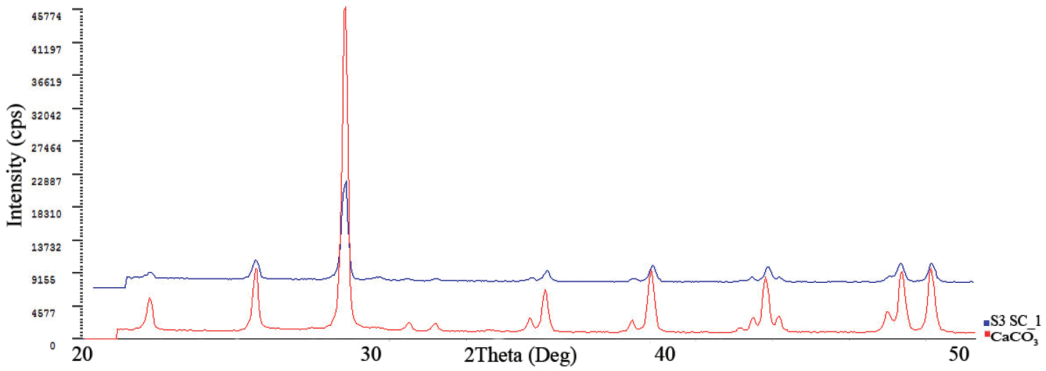

FIGURE 2. X-ray Diffraction spectra from S3 SC_1 (blue line) sample (counting time = 1 s/step) showing a close similarity with pure $CaCO_3$ (red line) spectra

B. pumilus strain is able to ferment Gentiobiose, Sucrose, D-Fructose and D-Galactose sugar components and partial metabolism of these sugar substrates: D-Maltose, D-Trehalose, D-Cellobiose, D-Turanose, D-Raffinose, D-Melibiose, β-Methyl-D-Glucoside, D-Salicin, N-Acetyl-D-Glycosamine, α-D-Glucose, D-Mannose, 3-Methyl Glucose, D-Fucose and L-Fucose. Negative values for α-D-Lactose indicated that the *B. pumilus* strain does not ferment lactose.

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. The sequence analysis for the 16S rDNA gene of the isolate S3 SC_1 was determined and compared with those of reference *Bacillus* spp strains. 16s rDNA sequence analysis showed that there was a strong similarity (≥95% - ≥98%) between the test strains and representative strains in the gene bank of *Bacillus* spp strains, which may indicate that 16s rDNA gene sequence data is helpful for identification of bacteria at species level. The sequence of isolate S3 SC_1 showed 98% homology with *B. pumilus* strain. Figure 3 shows the dendogram estimated phylogenetic relationship on the basis of 16s rDNA gene sequence data of the S3 SC_1 with eight references strain (98% similarity).

CA ANALYSIS BY ESTERASE ACTIVITY

p-nitrophenylacetate substrate is hydrolysed by the carbonic anhydrase into p-nitrophenol (A_{348}), whereby the change can be observed spectrophotometrically. To convert UV/VIS spectrophotometer data into concentration values, a calibration curve of p-nitrophenol was prepared (not shown). The blank solution was also chosen as the same buffer. R^2 value of the line 0.9982 and slope of the calibration curve was calculated as 0.02. The highest p-nitrophenol/min was shown as 5.8 mM by S3 SC_1 and esterase activity of 0.006 U/min was shown compared to the rest of the bacterial strain tested where the results are

TABLE 1. Sugar substrates utilization of *B. pumilus* S3 SC_1 observed in this study

Sugar substrates utilization			
Characteristics	*B. pumilus* S3 SC_1 phenotype	Characteristics	*B. pumilus* S3 SC_1 phenotype
Dextrin	-	N-Acetyl-D-Glycosamine	+
D-Maltose	+	N-Acetyl-β-D-Monnosamine	-
D-Trehalose	+	N-Acetyl-D-Galactosamine	-
D-Cellobiose	+	N-Acetyl Neuraminic Acid	-
Gentiobiose	++	α-D-Glucose	+
Sucrose	++	D-Mannose	+
D-Turanose	+	D-Fructose	++
Stachyose	-	D-Galactose	++
D-Raffinose	+	3-Methyl Glucose	+
α-D-Lactose	-	D-Fucose	+
D-Melibiose	+	L-Fucose	+
β-Methyl-D-Glucoside	+	L-Phamnose	-
D-Salicin	+	Inosine	-

++: Positive result for the test, +: Partially positive for the test, -: Negative result for the test

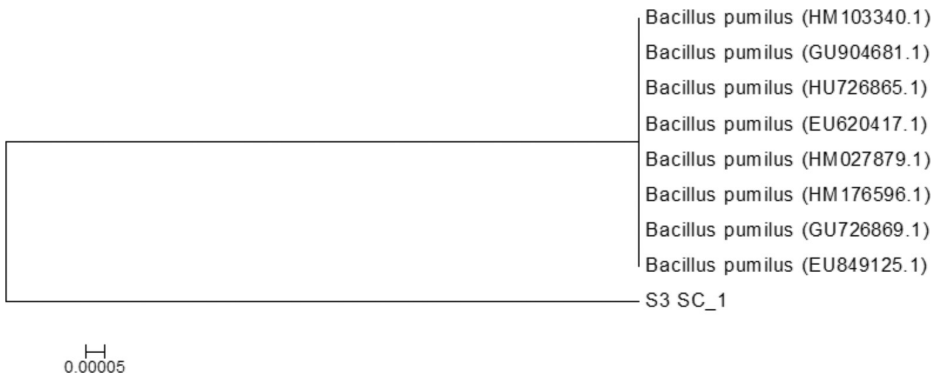

FIGURE 3. Dendogram estimated phylogenetic relationship on the basis of 16s rDNA gene sequence data of the S3 SC_1 with eight reference isolates (98% similarity). The scale bar represents 0.005% divergence

not shown here (for esterase activity of bacterial strain, refer to Komala and Khun (2013)).

EXPERIMENTAL SYSTEM FOR CARBON DIOXIDE SEQUESTRATION

Table 2 shows the calcite crystals weight (g) and EDX element (wt. %). It was observed that the calcite crystals weight in g was higher in Treatment A compared with Treatment B and C. Besides, there were obvious differences in the size and morphology of calcite crystals formed in three different treatments based on the photos taken by FESEM. According to FESEM analysis in Treatment A, the prismatic layer of calcite was dominant in all three replicates (Figure 4(a)). Treatment B shows rhombohedral calcite crystals, whereas Treatment C shows the amorphous shape of crystals observed in FESEM. As the prismatic layer of calcite crystal was dominant, close view images was taken and shown in Figure 5(a) and 5(b). Besides, FESEM analysis also indicates some bacterial imprints on the surface of calcite crystals in the experimental system with the bacteria (Figure 5(c)). These results suggested that CA produced by *B. pumilus* sequester supplied CO_2 into calcite minerals. In summary the objectives of biological CO_2 sequestration potential of locally isolated and identified *B. pumilus* was proven.

DISCUSSION

B. pumilus (S3 SC_1) isolated from cave samples was further confirmed by biochemical characterization analysis with a similarity index of 99.7%. The 16S rDNA sequences (~1300bp) of these bacteria showed 98% similarity with *B. pumilus* 16S rDNA sequences in the GenBank. Thus, a combination of conventional biochemical tests and genetic analysis enabled unambiguous identification of *B. pumilus* form Gua Tempurung, Gopeng Perak. Boquet et al. (1973)

TABLE 2. Calcite crystals weight (g) and EDX element (wt. %)

Condition		Treatment A	Treatment B	Treatment C
Calcite crystals weight (g)		0.08	0.01	0.03
EDX Element (wt. %)	C	34.48	54.7	59.83
	O	47.67	38.26	33.74
	Ca	15.62	2.12	2.76

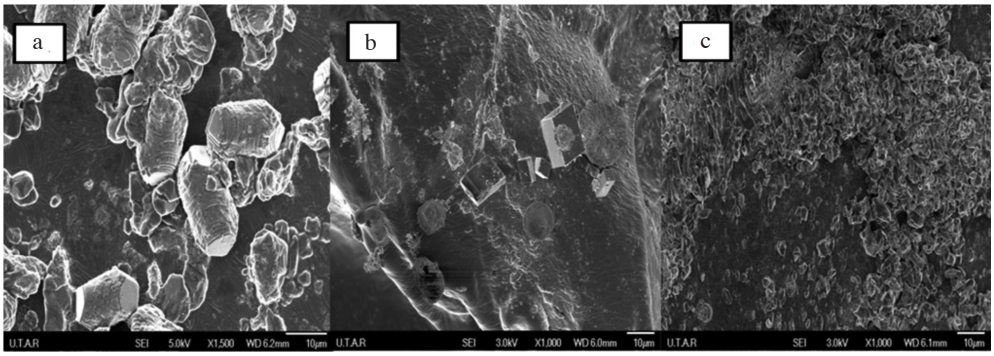

FIGURE 4. FESEM images of calcite crystal; (a) Prismatic layer, (b) rhombohedral and (c) amorphous

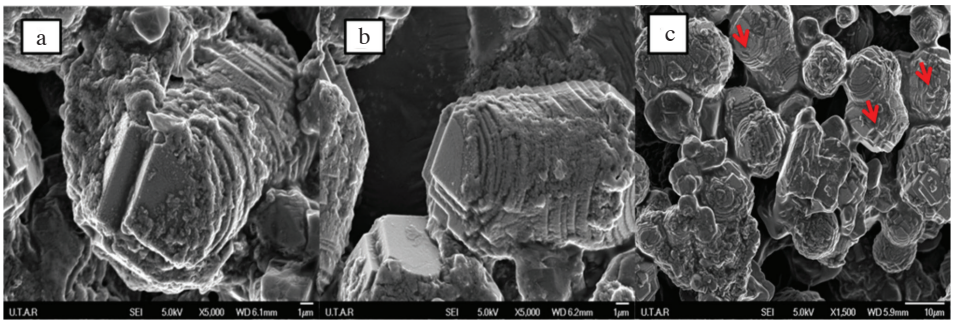

FIGURE 5. Dominant prismatic calcite crystal formed by *B. pumilus* S3 SC_1 (a) Top view, (b) side view and (c) bacterial imprints in calcite crystals surface indicated by arrows

has isolated calcite forming *B. pumilus* from soil and stated that this strain shows extensive crystal formation. Whereas, Baskar et al. (2006) has demonstrated that *B. pumilus* isolates from cave samples mediate the calcite formation. 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.

Through our current study, *B. pumilus* mediated the sequestration of CO_2 via the formation of calcite. These results suggested that bacteria serve as nucleation sites for calcite formation, which is in agreement with the earlier research results of other researchers. Yadav et al. (2011) summarized that bacterially produced CA specifically by *B. pumilus* ability in CO_2 sequestration by calcite formation. Whereas Li et al. (2011) also found that CA protein produced by *Bacillus* sp. isolated from karst soil able to promote calcite formation. CA is a key enzyme in the chemical reaction of living organisms which linked with calcite formation (Rahman et al. 2007). CA is a zinc-containing enzyme that catalyzes the reversible conversion of CO_2 to bicarbonate (HCO_3), which would then be available for calcite ($CaCO_3$) formation (Rahman et al. 2007). Importantly, our locally isolated *B. pumilus* proved the CO_2 sequestration ability; therefore it will bring a significant amount of economic and environmental benefit in future by reducing anthropogenic CO_2 from the atmosphere.

CONCLUSION

In this study, CFB *B. pumilus* (S3 SC_1) were successfully isolated and identified by using both molecular and biochemical identification. The primary objective of this research was to study the calcite formation in the presence of bacterially produced CA. The effects of bacterially produced CA on calcite formation were studied. The results showed that in the presence of bacteria the calcite crystals formed are higher with the fixed crystals morphology. In conclusion, climate change is the problem addresses at the beginning of this study which caused by excess amount of CO_2. At the end of this study, locally isolated and identified *B. pumilus* ability towards CO_2 sequestration was proven. Therefore bacterial calcite formation of CA type can be utilized for carbon sequestration to overcome the issue of climate change. Finally it was concluded that sequestration of anthropogenic CO_2 into calcite mineral using CA appears to be a promising option of CO_2 sequestration.

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