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Biological Carbon Dioxide Sequestration Potential of *Bacillus pumilus*

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

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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*

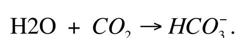
ABSTRAK

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*

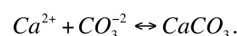
INTRODUCTION

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.

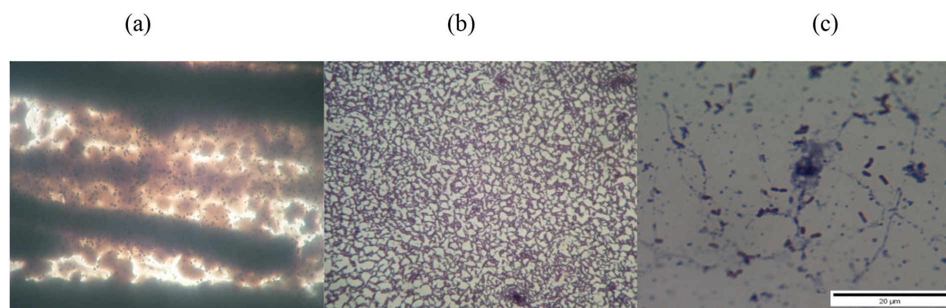


FIGURE 1. (a) Optical microscopic images of crystals formed by S3 SC_1, (b) appearance of Gram (+) cell under binocular microscope, S3 SC_1 and (c) appearance of Gram (+) and rod shaped cells under light microscope using oil immersion (100X magnification)

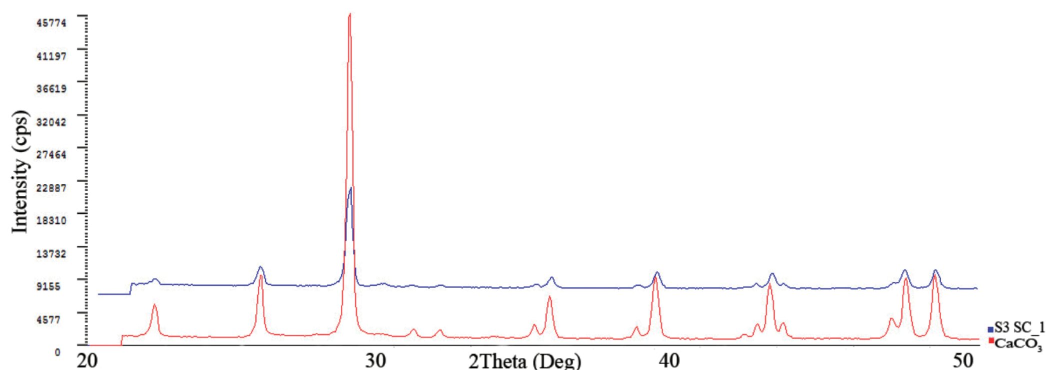


FIGURE 2. X-ray Diffraction spectra from S3 SC_1 (blue line) sample (counting time = 1 s/step) showing a close similarity with pure $CaCO_3$ (red line) spectra

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

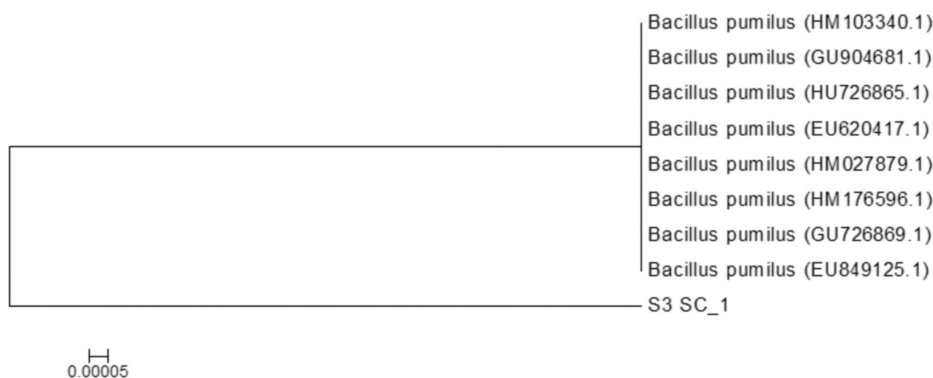


FIGURE 3. Dendogram estimated phylogenetic relationship on the basis of 16s rDNA gene sequence data of the S3 SC_1 with eight reference isolates (98% similarity). The scale bar represents 0.005% divergence

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

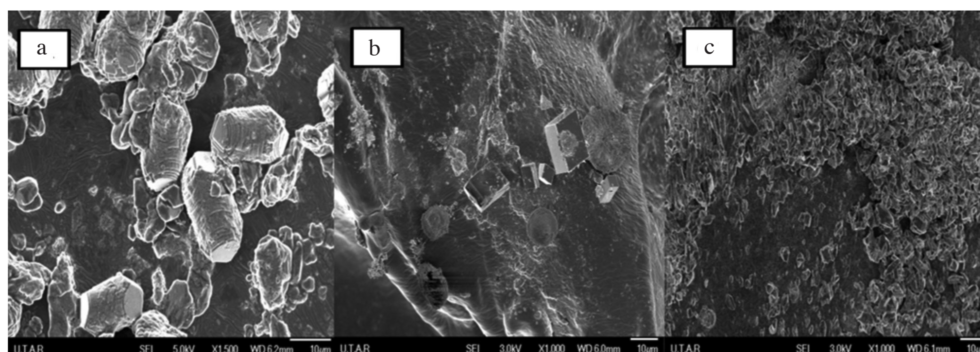


FIGURE 4. FESEM images of calcite crystal; (a) Prismatic layer, (b) rhombohedral and (c) amorphous

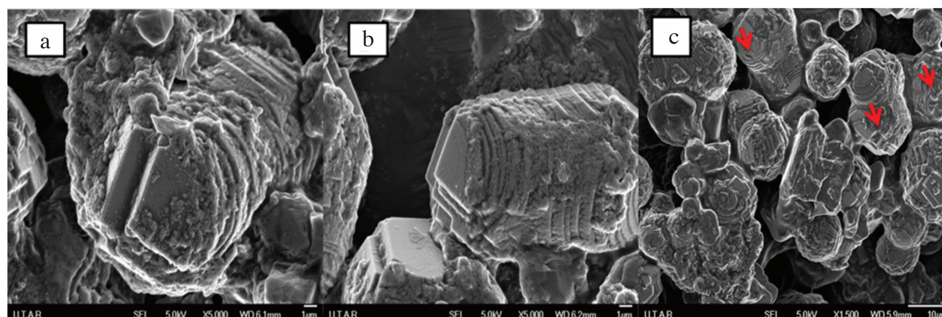


FIGURE 5. Dominant prismatic calcite crystal formed by *B. pumilus* S3 SC_1 (a) Top view, (b) side view and (c) bacterial imprints in calcite crystals surface indicated by arrows

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.

Acknowledgements

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References

Achal, V. & Pan, X. 2011. Characterization of urease and carbonic anhydrase producing bacteria and their role in calcite precipitation. *Current Microbiology* 62: 894-902.

Adiguzel, A., Ozkan, H., Baris, O., Inan, K., Gulluce, M. & Sahin, F. 2009. Identification and characterization of thermophilic bacteria isolated from hot springs in Turkey. *Journal of Microbiological Methods* 79: 321-328.

Ana, B. & Baltasar, M. 2006. PCR DGGE as a tool for characterizing dominant microbial populations in the Spanish blue-veined Cabrals cheese. *International Dairy Journal* 16: 1205-1210.

Aunpad, R. & Na-Bangchang, K. 2007. Pumilicin 4, a novel bacteriocin with anti-MRSA and anti-VRE activity produced by newly isolated bacteria *Bacillus pumilus* strain WAPB4. *Current Microbiology* 55(4): 308-313.

Ayesegul, E.Y., Feride, I.S. & Mehmet, H. 2008. Isolation of endophytic and xylanolytic *Bacillus pumilus* strains from zea mays. *Brazilian Archieves of Biology and Technology* 14: 374-380.

Baskar, S., Baskar, R., Mauclaire, L. & McKenzie, J.A. 2006. Microbially induced calcite precipitation in culture experiments: Possible origin for stalactites in Sahastradhara caves, Dehradun, India. *Current Science* 90: 58-64.

Battan, B., Sharma, J., Dhiman, S.S. & Kuhad, R.C. 2007. Enhanced production of cellulase-free thermostable xylanase by *Bacillus pumilus* ASH and its potential application in paper industry. *Enzyme Microbial Technology* 41: 733-739.

Bhat, M.K. & Bhat, S. 1997. Cellulose degrading enzymes and their potential industrual applications. *Biotechnological Advances* 15: 583-620.

Boquet, E., Boronat, A. & Ramos-Cormenzana, A. 1973. Production of calcite (calcium carbonate) crystals by soil bacteria is a general phenomenon. *Nature* 246: 527-529.

Buthelezi, S.P., Olaniran, A.O. & Pillay, B. 2010. Sawdust and digestive bran as cheap alternate substrates for xynalase production. *Journal of Microbiology Research* 5: 742-752.

De Wulf, P. & Vandamme, E.J. 1997. Production of D-ribose by fermentation. *Applied Microbial Biotechnology* 48: 141-148.

Duarte, M.C.T., Pellegrino, A.N.A., Portugal, E.P., Ponezi, A.N. & Franco, T.T. 2000. Characterization of alkaline xynalase from *Bacillus pumilus*. *Brazilian Journal of Microbiology* 31: 90-94.

Garbeva, P., Van Veen, J.A. & Van Elsas, J.D. 2003. Predominant *Bacillus* spp. in agricultural soil under different management regimes detected via PCR-DGGE. *Microbiology Ecology* 45: 302-316.

Gray, E.J., Lee, K.D., Souleimanov, A.M., Di Falco, M.R., Zhou, X., Ly, A., Charles, T.C., Driscoll, B.T. & Smith, D.L. 2006. A novel bacteriocin, thuricin 17, produced by plant growth promoting rhizobacteria strain *Bacillus thuringiensis* NEB17: Isolation and classification. *Journal of Applied Microbiology* 100: 545-554.

Hassan, F., Khan, S., Shah, A.A. & Hameed, A. 2009. Production of antibacterial compounds by free and immobilized *Bacillus pumilus* SAF1. *Pakistan Journal of Botany* 41: 1499-1510.

Hidayah, A., Mohd, A.H., Umi Kalson, M.S., Norhafizah, A., Farinazleen, M.G. & Yoshihito, S. 2008. Production of bacterial endoglucanase from pretreated oil palm empty fruit bunch by *Bacillus pumilus* EB3. *Journal of Bioscience and Bioengineering* 6: 231-236.

Jang, H.D. & Chen, K.S. 2003. Production and characterization of thermostable cellulases from Streptomyces transformant T 3-1. *World Journal of Microbiology and Biotechnology* 19: 263-268.

Kapoor, M., Nair, L.M. & Kuhad, R.C. 2008. Cost-effective xynalase production from free and immobilized *Bacillus pumilus* strain MK001 and its application in saccharification of *Proscopis juliflora*. *Biochemistry Engineering Journal* 38: 88-97.

Klaenhammer, T.R. 1988. Bacteriocins of lactic acid bacteria. *Biochemie* 70: 337-349.

Komala, T. & Khun, T.C. 2013. Calcite-forming bacteria located in limestone area of Malaysia. *Journal of Asian Scientifc Research* 3(5): 471-484.

Kotchoni, S.O., Gachomo, E.W., Omafuvbe, B.O. & Shonukan, O.O. 2006. Purification and biochemical characterization of Carboxymethyl cellulase (CMCase) from a catabolite repression insensitive mutant of *Bacillus pumilus*. *International Journal of Agriculture & Biology* 8: 286-292.

Kumar, G.A., Swarnaltha, S., Gayathri, S., Nagesh, N. & Sekaran, G. 2008. Characterization of an alkaline active - thio forming extracellular serine keratinase by the newly isolated *Bacillus pumilus*. *Journal of Applied Microbiology* 104(2): 411-419.

Lee, Y.N. 2003. Calcite production by *Bacillus amyloliquefaciens* CMB01. *Journal of Microbiology* 41: 345-348.

Li, W., Liu, L.P., Zhou, P.P., Cao, L., Yu, L.J. & Jiang, S.Y. 2011. Calcite precipitation induced by bacteria and bacterially produced carbonic anhydrase. *Current Science* 100: 502-508.

Liu, M. & Liu, G. 2008. Expression of recombinant *Bacillus licheniformis* xynalase A in Pichia pastoris and xylooligosaccharides released from xylans by it. *Protein Expression Purification* 57: 101-107.

Miyagawa, K., Miyazaki, J. & Kanazaki, N. 1992. *Method of producing D-ribose*. Patent European patent 0501765A1.

Monisha, R., Uma, M.V. & Murthy, V.K. 2009. Partial purification and characterization of *Bacillus pumilus* xynalase from soil source. *Kathmandu University Journal of Science, Engineering and Technology* 5: 137-148.

Papagianni, M. 2003. Ribosomally synthesized peptide with antimicrobial properties: Biosynthesis, structure, function, and applications. *Biotechnology Advances* 21: 465-499.

Polat, M.F. & Nalbantoglu, B. 2002. *In vitro* esterase activity fo carbonic anhyrase on total esterase activity level in serum. *Turkish Journal of Medical Sciences* 32: 299-302.

Prabhu, C., Wanjari, S., Gawande, S., Das, S., Labhsetwar, N., Kotwal, S., Puri, A.K., Satyanarayana, T. & Rayalu, S. 2009. Immobilization of carbonic anhydrase enriched microorganism on biopolymer based materials. *Journal of Molecular Catalysis B: Enzymatic* 60: 13-21.

Prabhu, C., Valechha, A., Wanjari, S., Labhsetwar, N., Kotwal, S., Satyanarayanan, T. & Rayalu, S. 2011. Carbon composite beads for immobilization of carbonic anhydrase. *Journal of Molecular Catalysis B: Enzymatic* 71: 71-78.

Rahman, M.A., Oomori, T. & Uehara, T. 2007. Carbonic anhydrase in calcified endoskeleton: Novel activity in biocalcification in alcynonarian. *Marine Biotechnology* 10: 31-38.

Ruiz, C., Blanco, A., Pastor, F.I.J. & Diaz, P. 2002. Analysis of *Bacillus megaterium* lipolytic system and cloning of Lip A, a novel subfamily I.4 bacteril lipase. *Federation of European Material Societies Microbiology Letters* 217: a263-a267.

Shakoori, F.R., Tabassum, S., Rehman, A. & Shakoori, A.R. 2010. Isolation and characterization of Cr6+ reducing bacteria and their potential use in bioremediation of chromium containing wastewater. *Pakistan Journal of Zoology* 42: 651-658.

Sharma, A., Bhattacharya, A., Pujari, R. & Shrivastava, A. 2008. Characterization of carbonic anhydrase from diversified genus for biomimetic carbon-dioxide sequestration. *Indian Journal of Microbiology* 48: 365-371.

Siktar, E. 2009. The effect of L-cartinie on carbonic anhydrase level in rats exposed to exhaustive exercise and hypothermic stress. *African Journal of Biotechnology* 8(13): 3060-3065.

Taggart, J.B., Hynes, R.A., Prodohl, P.A. & Ferguson, A. 1992. A simplied protocol for routine total DNA isolation from salmonid fishes. *Journal of Fish Biology* 40: 963-965.

Tamura, K., Dudley, J., Nei, M. & Kumar, S. 2007. MEGA 4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. *Molecular Biology and Evolution* 24: 1596-1599.

Thompson, J., Gibson, T., Plewniak, F., Jeanmougin, F. & Higgins, D. 1997. The ClustalX Windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* 24: 4876-4886.

Yadav, R., Satyaranayanan, T., Kotwal, S. & Rayalu. S. 2011. Enhanced carbonation reaction using chitosan-based carbonic anhyrase nanoparticles. *Current Science* 100: 520-524.

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