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Title: Assessing ureolytic bacteria with calcifying abilities isolated from limestone caves for biocalcification

Running title: Ureolytic bacteria obtained from limestone caves

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SIGNIFICANCE AND IMPACT OF THE STUDY

Ureolytic bacteria continues to play an important role as microbial tools used in geotechnical engineering for soil biocalcification. Microbial strains with the ability to produce urease enzyme and induce calcium carbonate mineral are often isolated from soil, water and sludge samples. However, screening of these essential microbes from extreme regions such as caves are rarely investigated. In this current study, native bacteria which were isolated from limestone cave samples were identified and characterized. The findings suggested that these ureolytic bacterial isolates have the potential to serve as suitable alternative microbial agents for soil strengthening and stabilization.

ABSTRACT

Biocalcification through the use of ureolytic bacteria and biochemical activities has evolved in recent decades into a fervent resourceful effective technology suitable for soil stabilization, crack repair and bioremediation. Extensive studies have been carried out numerous ureolytic bacterial species isolated from soils and sewage samples. However, very limited attention has been given to limestone caves with natural calcite formations, as a possible source for isolation of ureolytic bacteria. In this present study, bacterial isolates were recovered from limestone cave samples to determine their suitability for biocalcification. Twenty-seven morphologically distinct bacterial isolates were identified by partial 16S rRNA gene sequencing and their various genetic diversity was characterized according to their phylogenetic affiliations. Based on the molecular identification, *Sporosarcina* was the most abundant genus among all the ureolytic isolates, while the rest belonged to *Pseudogracilibacillus* and *Bacillus* genera. Analytical analysis on urease measurement showed that urease activities for the isolates ranged from 1.130 to 21.513 mol urea hydrolysed min^{-1}, with isolate NB33 achieving the highest value and TSB4 achieving the lowest value. The estimated $CaCO_3$ precipitates for the isolates ranged from 4.04 to 17.26 mg ml^{-1}, with isolate NB30 achieving the highest value and TSB20 achieving the lowest value. The growth and pH performances of four

selected isolates from *Sporosarcina Pseudogracilibacillus* and *Bacillus* genera were also studied up to 24 h alongside the control strain. The findings in this study demonstrated that the ureolytic bacteria from limestone caves are promising bio-calcifying agents.

Keywords: ureolytic bacteria, urease, 16S rRNA sequencing, caves, biocalcification

INTRODUCTION

Biocalcification which is also known as microbial-induced carbonate precipitation (MICP) has become a relevant field in recent decades due to its versatile commercial applications in various science and engineering practices (Li *et al.*, 2013). To date, ureolysis is the most widely reported MICP process for geotechnical engineering applications because it is easily understandable, controllable and aids in inducing high contents of calcium carbonate ($CaCO_3$) precipitates in the desired period (Dhami, Reddy & Mukherjee, 2013). Ureolysis-driven MICP process relies on the use of living microorganisms which has the abilities to hydrolyse urea through urease enzyme to ammonia and carbonic acid, hence increasing the pH surrounding of the microorganism due to the production of hydroxide ions (Kantzas *et al.*, 1992). The released carbonic acid is then converted into bicarbonate ions to form carbonate ions and in the presence of calcium ions such as calcium chloride, an interaction occurs on the surface of the microbial's cell which promotes calcification to occur as pH subsequently increases hence resulting in $CaCO_3$ precipitates (Kantzas *et al.*, 1992; Ferris *et al.*, 1996).

Microbial activities were previously regarded by engineers as a causal factor of clogging in groundwater wells (Mitchell & Soga, 2005). However, in the last two decades, the use of microbial urease has switched from clinical relevance to geotechnical engineering and applied biotechnology. Although, *Sporosarcina pasteurii* (*S. pasteurii*), a non-pathogenic bacterium preferably used as a

microbial cementing agent to precipitates $CaCO_3$ to consolidate loose soil particles, there are other known ureolytic microorganisms with calcifying abilities (Anbu *et al.*, 2016). However, most of these bacterial species are not suitable for engineering purposes because they are disease causative agents.

Cave region serve as a suitable alternative region for the screening of desired ureolytic strains. Generally, caves are natural geological formations which are known to be an extreme environment unfavourable for microbial development due to severe abiotic conditions present, thus allowing the survival of microbial species that are adapted to the availability of low-level nutrients (Wu *et al.*, 2015). Some of these cave microbes are capable of precipitating $CaCO_3$ on the surfaces of their cells which have helped in shaping cave structures such as stalactites, stalagmites and bristles (Tomczyk-Żak & Zielenkiewicz, 2016). There are very limited studies on the exploitation of ureolytic microorganisms from cave regions (Omoregie *et al.*, 2016). Numerous bacterial species capable of producing urease were previously isolated from soils, water and sludge samples (Hammes *et al.*, 2003; Al-Thawadi & Cord-Ruwisch, 2012). Hence, it is noteworthy for more scientific studies to be performed on the diversity of essential microbes from extreme regions such as caves for numerous biotechnological and engineering applications.

Sarawak has many unexplored regions rich in microbial diversity potentially useful for various biotechnological applications. This provided the opportunity for us to screen for highly active ureolytic bacteria with calcifying abilities suitable for biocalcification. Hence, the objectives of the present study were to a) isolate ureolytic bacteria from two caves situated in Sarawak, b) identify these isolates using phenotypic and molecular characterizations, c) investigate the ureolytic activities and calcifying abilities of these isolates.

RESULTS AND DISCUSSION

Isolation and identification

A total of 201 bacterial isolates were obtained from Petri plates containing nutrient agar and 2% urea (w/v). Out of these 201 bacterial isolates, 90 were able to grow on the Petri dishes containing 6% urea (w/v) which were then screened for urease production. However, only 27 isolates able to produce urease enzyme having different morphological characteristics. The microscopic analysis showed that all the ureolytic bacterial isolates were Gram-positive except for three of the isolates (A63, B53, and A62) which were Gram-negative bacteria. The endospore-staining, catalase, oxidase and motility results showed that all these isolates tested positive except for isolate LPB41 and TSB21 which tested negative for catalase, oxidase and motility.

As per BLAST analysis, most of the ureolytic bacteria showed high degrees of similarity (97-99%) with bacterial sequences in the database (Table 1). The obtained 16S rRNA gene sequences were deposited in GenBank (accession numbers KX212190.1 to KX212216.1) and were identified based on the partial 16S rRNA gene sequencing. The isolates had good-quality sequences with mean length ranging from 920-1385 base pairs. In accordance with the investigative result from phylogenetic analysis of the ureolytic bacteria, we classified all the strains into two phyla: *Firmicutes* and *Bacilli*. Bacteria from these phyla are known to be urease positive (Burbank *et al.*, 2012; Stabnikov *et al.*, 2013). The phylogenetic tree (Fig. 1) showed that the ureolytic bacteria were identified as *Sporosarcina pasteurii*, *Pseudogracilibacillus auburnensis*, *Bacillus lentus* and *Bacillus fortis*. It is noteworthy some of the 16S rRNAs for the cave bacteria reported in our showed less than 97% similarity with the existing sequences in the GenBank database. Since similarity values of 97-100% between sequences are considered for species assignment (Stackebrandt & Goebel, 1994; Rosselló-Mora & Amann, 2001), it is, therefore, possible that these cave ureolytic bacteria with similarity value below 97% might represent new type strain of the bacterial species.

Urease activity

The ureolytic bacteria having the highest urease activity as shown in Fig. 2 was bacterial isolate NB33 (21.513 mol urea hydrolysed min^{-1}) while TSB4 (1.130 mol urea hydrolysed min^{-1}) had the lowest urease activity value when compared to all the isolates and control strain (14.087 mol urea hydrolysed min^{-1}). The urease activities of the isolated bacteria in this study were similar to existing reported results for other ureolytic bacterial strains used for MICP application ranging from 5 to 20 mol urea hydrolysed min^{-1} for *S. pasteurii* (DSM33) (Harkes *et al.*, 2010); 2.2 to 13.3 mol urea hydrolysed min^{-1} for *S. pasteurii* (ATCC11859) (Whiffin, 2004); 3.3 to 8.8 mol urea hydrolysed min^{-1} for *Bacillus* strains (Al-Thawadi & Cord-Ruwisch, 2012; Stabnikov *et al.*, 2013). Urease from bacteria in growth medium is extracellularly produced, hence it is rather a preferred choice than requiring the whole cell to consolidate $CaCO_3$ precipitation or other forms of urease which are rather expensive (Kantzas *et al.*, 1992). Typically, ureolysis results in accumulation of both bicarbonate and ammonia in the bacterial cells favouring the physiological regulatory links between urea a bicarbonate metabolism which used their respective pH environment or utilizes urea for nitrogen source or energy (Hammes *et al.*, 2003; Dhami *et al.*, 2013).

Biomineralization test

The result in Fig. 3 indicates the estimated insoluble $CaCO_3$ precipitated due to ureolysis-process after being incubated at 30°C for the duration of 7 days. The moment the bacterial cultures were inoculated into the broth media, white precipitates appeared instantly at the bottom of the conical flasks and its density increased with incubation. However, the samples were allowed to incubate in the aqueous medium but at the end of the incubation period, the precipitates were collected and weighed. All the bacterial isolates were able to induce $CaCO_3$ precipitates irrespective if they were high or low urease producers. The bacteria which induced the highest estimated $CaCO_3$ precipitate was isolated NB30 (17.26 mg ml^{-1}), while TSB20 (4.04 mg ml^{-1}) had the lowest $CaCO_3$ precipitate

result when compared all the isolates and control strain (11.02 mg ml^{-1}). The lack of correlation between the urease activity in Fig. 2 and biocalcification result in Fig. 3 showed that bacteria with the highest urease production does not necessarily induce the highest amount of $CaCO_3$. Hence, this $CaCO_3$ depleted over time did not appear to have a correlation with the rates of urea hydrolysis. Hence, it is suggested urea hydrolysis may also be used for other metabolic activities, thus confirming the complexity of MICP process (Raut, Sarode & Lele, 2014). Bacterial species are able to use protons which constitute an essential role required in the metabolic assimilation of bicarbonate and nutrients. A calcium ATPase-based trans mechanisms allow rapid supersaturation of high amount of supersaturations and carbonate precipitation (McConnaughey & Whelan, 1997). Hence, it is suggested urea hydrolysis may also be used for other metabolic activities, thus confirming the complexity of MICP process (Raut *et al.*, 2014).

MATERIALS AND METHODS

Sampling site and sample collection

The samples to elucidate ureolytic bacterial diversity were obtained from Fairy Cave Nature Reserve (N 01°22’53.39” E 110°07’02.70”) and Wind Cave Nature Reserve (N 01°24’54.20” E 110°08’06.94”) located in Bau, Kuching Division, Sarawak, Malaysia. Samples were collected at depth of 5-10 cm from these caves using sterile spatulas, chisels and forceps. The collected cave samples were then kept in sterile 50 ml Falcon™ centrifuge tubes (Fisher Scientific) and temporarily stored in an ice box (at the sampling site) before being transported to the Research laboratory for further microbiological analysis. The samples were later preserved in the fridge (4°C) before performing the isolation of desired bacteria until further analysis was performed.

Isolation of ureolytic bacteria

Rich media supplemented with high urea concentration was used as a selective medium for enrichment culture and isolation of ureolytic bacteria. The following growth medium were selected: nutrient broth (13 g l^{-1}, HiMedia Laboratories Pvt. Ltd); tryptic soy broth (30 g l^{-1}, Merck Millipore); lactose peptone broth (35 g l^{-1}, Becton, Dickinson and Company); luria broth (20 g l^{-1}, HiMedia Laboratories Pvt. Ltd) and brain heart infusion broth (37 g l^{-1}, Oxoid Thermo Scientific Microbiology) were each supplemented with sodium acetate (8.2 g l^{-1}, HiMedia Laboratories Pvt. Ltd), ammonium sulphate (10 g l^{-1}, HiMedia Laboratories Pvt. Ltd) and urea (20 g l^{-1}, Bendosen Laboratory Chemicals). The initial pH of all broth medium was adjusted to 8.0. Sterilized urea (by 0.45 µm filter sterilisation) was added post-autoclaving to prevent chemical decomposition under autoclave condition. The culture medium was then incubated aerobically incubated (CERTOMAT® CT plus – Sartorius) under 30°C for 120 h at 130 rpm. Post incubation, aliquots from ten-fold dilution (10^{-1} to 10^{-7}) were plated in non-selective nutrient agar (28 g l^{-1}, HiMedia Laboratories Pvt. Ltd) containing 2% urea (w/v) and incubated (MMM Incucell) under aerobic conditions at 32°C for 42 h. Pure bacterial colonies from each morphotype grown on the agar plates were picked and subsequently sub-cultured for further colony purification.

Selection of ureolytic bacteria

Christensen's medium (25 g l^{-1}, Oxoid Thermo Scientific Microbiology Sdn Bhd) was used as a differential media to screen for positive ureolytic bacteria (Omoregie *et al.*, 2016). The medium was aseptically introduced 5 ml of sterile 40% (v/v) and carefully mixed before gently transferred into separate sterile test tubes. The bacterial isolates were heavily inoculated on the surface of the medium and then incubated at 37°C for 72-120 h. The bacterial isolates which were able to turn the medium from pale yellow to pink during the incubation period were selected while others were discarded. *S. pasteurii* (DSM33) was used as a positive control for urease production test and as well as subsequent parts of this study.

Phenotypic characterization of bacteria

Phenotypic characterization was used to initially identify the bacteria through morphological and microscopic analysis. Colony appearance of the overnight isolates was studied through visual observation and the results were recorded. The cell morphology of the isolates was observed via a microscope (Nikon, Eclipse E200) after Gram-staining and endospore-staining were performed using standard staining protocols (Moyes, Reynolds & Breakwell, 2009).

DNA extraction and amplification and purification

The bacterial isolates were identified based on their respective 16S rDNA gene sequencing using universal primer set (8F: 5'-AGAGTTTGATCCTGGCTCAG-3' and 1525R: 5'-AAGGAGGTGATCCAGCC-3' primers) using standard procedures (Muramatsu *et al.*, 2003). Briefly, overnight grown bacterial colonies were suspended in sterile 96 wells plate containing 100 µl Tris-EDTA (TE) buffer solution and then kept in a freezer at -80°C for 24 h and thawed by immersing it into a 60°C a circulating water bath (Thermo Scientific™ Precision™) for 5 min to release DNA from the microbial cells. A 1 µl of the aliquot of the obtained lysate served as templates for PCR amplification of 16S rDNA gene (El-Sayed *et al.*, 2018). Each PCR reaction (50 µl) contained template (200 ng, 2 µl); primers (1 µl, 20 µm); MyTaq Red Mix (25 µl,) (Biolin, Cat#BIO-25043) and sterile ddH_2O (22 µl). PCR was performed in a MasterCycler Gradient Thermal Cycler (Eppendorf 5331) using a PCR program consisting of an initial denaturation at 95°C for 5 min, annealing at 55°C for 60 sec, extension at 72°C for 1 min 30 sec and a final elongation at 72°C for 7 min. The process was set to 30 cycles and the system was held at 4°C. The PCR amplification products were later verified by electrophoresis on 1.5% agarose gels in 1X TAE buffer. The PCR products were purified and sequenced using the DNA sequencing service of Apical scientific Sdn. Bhd., Malaysia (previously First BASE Laboratory).

Sequence and phylogenetic analyses

The raw DNA chromatogram sequences were viewed using Chromas lite programme edited with BioEdit Programme (Hall, 1999) and stored in FASTA format. The bacterial 16S rDNA nucleotide sequences were aligned with existing sequences currently available in the databases of National Centre for Biotechnological Information using BLASTn database (Zhang *et al.*, 2000). Phylogenetic analysis of the ureolytic bacteria isolates was conducted using the Molecular evolutionary genetic analysis (Mega) version 6 software (Tamura, Nei & Kumar, 2004). The phylogenetic tree was constructed using *a Neighbour-Joining* method based on the Tamura 3-parameter model with 1000 bootstrap replications (Tamura *et al.*, 2013). The Biodiversity of the bacteria originating from the limestone caves belonging to different genera were then determined.

Urease measurement

Urease activity was determined by using conductivity analytical method (Whiffin, 2004). The hydrolysis of urea can be measured by quantifying the relative changes in conductivity over time when urea is exposed to purified urease enzyme (Duraisamy, 2016). The ion concentration of ammonia during the process of urea hydrolysis increases due to the urease, hence the electrical conductivity of urea-bacterial solution increases proportionally to the amount of urease present (Zhao *et al.*, 2014). Each bacterial isolate was grown overnight in nutrient broth medium at 32 °C with shaking (130 rpm). 1.0 ml of the bacterial culture was inoculated into sterile universal bottles containing 9.0 ml of urea solution (1500 mmol l^{-1}) at 25^{o}C (Whiffin, 2004). The changes in electric conductivity (mS cm^{-1}) were monitored with a conductivity meter (Walk LAB conductivity pro meter, Trans Instruments COMPRO) for a duration of 5 min. The conductivity values were recorded and used to plot the gradient (mS cm^{-1} min^{-1}) before being converted to urease activity (mol urea hydrolysed min^{-1}) with regard to the dilution factor (Omoregie *et al.*, 2017). A calibration curve was previously performed by Whiffin (2004) under standard condition at 25^{o}C with purified urease

(Sigma Cat. No. U-7127) and different urea concentrations (0.025-0.25 mol). The calibration was performed to determine ammonium concentrations produced due to urea hydrolysis (Duraisamy, 2016). The gradient value (11.11) with linear correlation (R^2 = 0.9988) was used to multiply the rate of conductivity for urease activity. In the measured range of activities, 1 mS min^{-1} corresponds with a hydrolysis activity of 11 mol urea min^{-1} (Harkes *et al.*, 2010).

Estimated $CaCO_3$ precipitates

A method by Wei *et al.*, (2015) was used to test the ability of ureolytic bacteria to precipitate $CaCO_3$ in broth medium. In brief, nutrient broth (13. g l^{-1}, HiMedia Laboratories Pvt. Ltd) was supplemented with urea 2% (w/v), calcium chloride 2% (w/v) solution and inoculated the medium was inoculated with 2% (v/v) overnight grown bacterial cultures. The culture flasks were incubated under shaking condition (130 rpm) at 30°C for the duration of 168 h. The bacterial cultures were then suspended by centrifugation (10,000 g for 60 sec). The supernatants were then discarded and dH_2O (pH 8.5) was added to the centrifuge tubes to wash the pellets and later air-dried at 37°C for 24 h. The pellets obtained were then weighted to estimate the amount of calcium carbonate mineral precipitated (Walter *et al.*, 2000).

Statistical analysis

The data obtained in this study are shown as the mean and standard deviation for three replicates. Statistical analyses were performed using Excel spreadsheets available in the Microsoft Excel (version 2016).

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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Figure legends

Fig. 1: Phylogenetic tree based on the nucleotides of partial 16S rRNA sequences. The isolated ureolytic bacterial strains and their position based on the partial 16S rRNA sequence comparison were obtained using a Neighbour-Joining method within MEGA 6. *Escherichia coli* NBRC 102203 was used as an outgroup.

Fig. 2: Urease measurement by the ureolytic bacteria. The vertical error bars represent standard error of the mean.

Fig. 3: Precipitation of $CaCO_3$ by ureolytic bacteria in a medium containing nutrient broth (13. g l^{-1}), urea 2% (w/v) and calcium chloride 2% (w/v) after incubation for 168 h at 30°C. The vertical error bars represent standard error of the mean.

Table 1: Molecular identification of ureolytic bacteria based on 16S rRNA sequencing

Isolate ID	**GenBank Accession number**	**Bacterial specie**	**Base pair (bp)**	**Query cover (%)**	**Similarity (%)**
NB33	KX212190.1	*Sporosarcina pasteurii* strain WJ-4	1198	100	97
LPB21	KX212191.1	*Sporosarcina pasteurii* strain fwzy14	1385	95	97
NB28	KX212192.1	*Sporosarcina pasteurii* strain WJ-5	1280	90	96
NB40	KX212193.1	*Sporosarcina pasteurii* strain WJ-5	1200	99	97
LPB4	KX212194.1	*Sporosarcina pasteurii* strain WJ-3	1298	98	97
TSB21	KX212195.1	*Pseudogracilibacillus auburnensis*	1050	99	93
NB30	KX212196.1	*Sporosarcina pasteurii* strain fwzy14	1279	99	98
TSB4	KX212198.1	*Sporosarcina pasteurii* strain WJ-4	1219	98	96
TSB46	KX212199.1	*Sporosarcina pasteurii* strain WJ-4	1200	93	97
BHIB17	KX212200.1	*Sporosarcina pasteurii* strain WJ-4	920	99	91
TSB55	KX212201.1	*Bacillus lentus strain* NBRC 16444	1219	99	97
TSB31	KX212202.1	*Sporosarcina pasteurii* strain WJ-5	1159	100	98
TSB40	KX212203.1	*Sporosarcina pasteurii* strain WJ-5	1250	99	98
TSB29	KX212204.1	*Sporosarcina pasteurii* strain WJ-4	1200	100	99
TSB12	KX212200.1	*Sporosarcina pasteurii* strain	920	99	91

		fwzy14			
LPB22	KX212205.1	*Sporosarcina pasteurii* strain WJ-3	1151	99	96
BHIB15	KX212206.1	*Sporosarcina pasteurii* strain fwzy14	1198	100	99
TSB20	KX212207.1	*Sporosarcina pasteurii* strain WJ-4	1250	99	95
LB6	KX212208.1	*Sporosarcina pasteurii* strain WJ-4	1110	100	99
LB48	KX212209.1	*Sporosarcina pasteurii* strain fwzy14	1269	92	98
LB1	KX212210.1	*Sporosarcina pasteurii* strain WJ-5	1374	93	97
LPB41	KX212211.1	*Pseudogracilibacillus auburnensis*	1298	99	95
LB31	KX212212.1	*Sporosarcina pasteurii* strain fwzy14	1149	99	99
TSB2	KX212213.1	*Sporosarcina pasteurii* strain WJ-3	1267	98	97
A63	KX212214.1	*Bacillus fortis* strain R-6514	1250	98	96
B53	KX212215.1	*Bacillus fortis* strain R-6514	1325	99	97
A62	KX212216.1	*Bacillus fortis* strain R-6514	1248	100	97