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Ureolytic bacteria isolated from Sarawak limestone caves show high urease enzyme activity comparable to that of *Sporosarcina pasteurii* (DSM 33)

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ABSTRACT

Aims: Microbial induced calcite precipitation (MICP) is a natural occurring biological process that employs the usage of ureolytic bacteria for a wide range of applications such as improving the mechanical properties of soils. The aim of this study was to isolate and identify local urease-producing bacteria from the limestone caves of Sarawak and characterise their specific urease activities.
Methodology and results: Enrichment culture technique was used to isolate urease-producing bacteria. These local isolates were identified using phenotypic and molecular characterisation. Conductivity method and biomass (OD600) measurements were conducted to analyze and determine the specific urease activities of the local isolates. 16S rRNA gene sequence analysis identified the bacterial isolates as *Sporosarcina pasteurii*, *Sporosarcina luteola* and *Bacillus lentus*.
Conclusion, significance and impact of study: This is the first study reporting the isolation and identification of urease-producing bacteria from Fairy and Wind Caves situated in Bau, Sarawak, Malaysia. The findings in this study suggest the bacterial isolates are capable of inducing calcite precipitation and serve as alternative microbial ureolytic agents.

Keywords: ureolytic bacteria, urease activity, *Sporosarcina pasteurii*, microbial induced calcite precipitation (MICP), biocementation

INTRODUCTION

Urease (urea amidohydrolase, EC 3.5.1.15) is a nickel-containing enzyme which catalyses urea substrate to form carbon dioxide and ammonia (Cheng and Cord-Ruwisch, 2013). This enzyme has been well studied from a clinical perspective as urease is related to the virulence of pathogenic microorganisms (Collins and D'Orazio, 1993; Mobley *et al.*, 1995; Lee and Calhoun, 1997). However, the study on the application of using the production of microbial ureases in civil and geotechnical engineering has increased because of the abilities of these microorganisms to induce calcite precipitation in the presence of urea and calcium ions (Cheng and Cord-Ruwisch, 2013). This process is termed microbial induced calcite precipitation (MICP), an innovative technique that harness the activity of bacteria to improve the physical properties of soils as a sustained and environmentally responsible method for soil strengthening and other engineering applications (DeJong *et al*., 2011; Gat *et al*., 2014).

Urea hydrolyzing microorganisms are abundant in natural soils, thus, making the urea hydrolysis process common to soils worldwide (Mobley and Hausinger, 1989). A study on *Sporosarcina pasteurii* showed that the hydrolysis of urea may be associated with the generation of adenosine triphosphate (ATP) and that other bacteria might utilize urea for the same process (Jahns, 1996). In as much as many soil bacteria are able to suppress urease in the presence of ammonia and other nitrogen compounds, a few bacteria are known to be able to produce urease in the presence of urea (Mobley *et al*., 1995).

A well-documented application of ureolytic microorganism has been the improvement on the mechanical properties of soil for construction and environmental purposes, known as biocementation. Biocementation enhances the strength and stiffness properties of soil through microbial activities and products (Ivanov and Chu, 2008). Various studies have reported the isolation of indigenous ureolytic bacteria through the use of enrichment culture from a variety of soil samples, ground water samples and cement samples (Rivadeneyra *et al*., 1993; Hammes *et al*., 2003; Achal and Pan, 2011; Burbank *et al*., 2012; Elmanama and Alhour, 2013). Most *Bacillus* species are able to hydrolyse urea using urease enzymes (Hammes *et al*., 2003). Previous studies have adopted and reported *B. sphaericus* (LMG22557) and *S.*

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pasteurii (DSM33) as the preferred ureolytic bacteria strain for biocementation applications (De Muynck *et al*., 2008; Chunxiang *et al*., 2009).

Studies on alternative non-pathogenic bacterial species used for urea hydrolysis are very limited. This research gap forms the basis for the initiation of this study. The aim of this study was to isolate, identify and characterize locally isolated bacteria as potential alternative microbial ureolytic agents capable of inducing calcite precipitation. This is the first study reporting the isolation and identification of ureolytic bacteria from limestone caves of Sarawak. These isolated bacteria serve as good alternative to *S*. *pasteurii* (DSM33), for biocementation applications.

MATERIALS AND METHODS

Sample collection

Samples used in this study were collected from Fairy and Wind Caves situated in Bau, Kuching division, Sarawak, East Malaysia, on the island of Borneo (N 01°22'53.39" E 110°07'02.70") and (N 01°24'54.20" E 110°08'06.94"). Soil samples obtained from Fairy cave were taken at a depth of 5-10 cm underground in regions surrounded by plants and limestone formation. Each sample were collected using sterile tools and placed inside sterile containers, properly sealed and stored in an ice box at 4 °C (at the sampling site) before being transported to the research laboratory.

Biological material

Sporosarcina pasteurii (DSM33) type strain was purchased from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). This bacterial strain was used as a positive control for subsequent experiments in this study. It was aseptically grown under aerobic batch conditions according to the DSMZ instruction and stored on Petri plates containing nutrient agar (HiMedia, Laboratories Pvt. Ltd) at 4 ˚C in the fridge until when usage was necessary.

Enrichment culture of samples

To enrich the cave samples for urease producing bacteria, 1 g or 1 mL of each sample was inoculated into 50 mL of nutrient broth medium (HiMedia, Laboratories Pvt. Ltd) containing 6% urea (250 mL shaking flasks) and incubated under aerobic batch conditions at 30 °C for 120 h under shaking condition at 130 rpm. The initial pH of all media was adjusted to 8.0 using 0.1 M NaOH before sterilization. Sterile urea substrate (by 0.45 µm filter sterilization) was added post-autoclaving to prevent chemical decomposition under autoclave condition.

Isolation of urea hydrolyzing bacteria

For bacterial isolation, 1 mL of individual enriched culture samples were serially diluted (sixfold) and plated on nutrient agar (with 6% urea). 0.1 mL aliquot of serially diluted enrichment samples were inoculated onto Petri plates containing nutrient agar were then spread using a sterilized L-shaped spreader until the fluid was evenly distributed. The agar Petri plates were then incubated under aerobic conditions at 32 °C for 42 h. Upon the growth of isolates capable of hydrolysing 6% urea in Petri plates containing nutrient agar, subsequent sub-culturing was performed until single bacterial colonies were obtained.

Screening for ureolytic bacteria

Urea agar base (Oxoid, Thermo Scientific Microbiology Sdn Bhd) also known as Christensen's medium was used as a qualitative urease assay and to rapidly screen for urease producing bacteria. Preparation of the medium was performed following manufacturer's instructions. The media components contained the following; peptone (1.0 g/L), glucose (1.0 g/L), sodium chloride (5.0 g/L), disodium phosphate (1.2 g/L), potassium dihydrogen phosphate (0.8 g/L), phenol red (0.012 g/L) and agar (15.0 g/L). Urea solution (4%, w/v) was separately prepared by filtration with the use of 0.45 µm syringe and 10 mL of the urea solution was aseptically introduced to 990 mL of the urea agar base medium. To test for the production of urease, heavy inoculation was performed on the surface of the urea agar base medium in the test tubes and incubated under aerobic conditions at 35 °C for 48 h. The urease production test was studied through visual observation for color changes. The isolates that changed the medium from pale yellow to a pink-red color were selected for further studies based on their ability to rapidly produce urease qualitatively.

Phenotypic characterization

Morphological characterization such as colony and cell morphology, Gram stain reaction, endospore stain reaction, motility, catalase and oxidase tests were performed by standard methods (Holt *et al*., 1994; Bisen, 2004; Moyes *et al*., 2009; Akanbi *et al*., 2010; Shields and Cathcart, 2011; Olufunke *et al*., 2014).

Molecular characterization

Genomic DNA of isolated bacterial strains was extracted using the freeze and thaw method adapted from Muramatsu et al., (2003). Forward primer 8F: 5'-AGAGTTTGATCCTGGCTCAG-3' (Hughes et al., 2000) and reverse primer 1525R: 5'-AAGGAGGTGATCCAGCC-3' (Lane *et al*., 1985) were used to amplify the 16S rRNA gene from the genomic DNA by PCR. PCR amplification was performed using MyTaq Red Mix (Bioline) according to the manufacturer's instructions. The PCR master mix contained the following: template (200 ng, 2 µL), primers (1 µL, 20 µM), MyTaq Red Mix (25 µL) and sterile ddH2O (22 µL). Amplification was performed using a MasterCycler Gradient Thermal

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Cycler (Eppendorf 5331) with the following cycling conditions: 95 °C for 5 min for the initial denaturation of the template DNA followed by 95 °C denaturation for 60 sec, 55 °C annealing for 60 sec and 72 °C extension for 1 min 30 sec, followed by a final elongation at 72 °C for 7 min and a 4 °C hold and the process was repeated for 29 cycles. PCR products were sent to 1st Base Laboratory Malaysia for product purification and DNA Sequencing. Amplified DNA was purified using PCR Clean-up kit (SS1012/3) with procedure following manufacturer's instructions. The eluted solution (pure DNA) was then stored at −20 °C. Sequencing was performed on an Applied Biosystem 3130xl Genetic Analyser, using BigDye® Terminator v3.1 cycle sequencing kit according to the protocol in the user manual. Two PCR primers were used. 27F: 5'-AGAGTTTGATCMTGGCTCAG-3' (Heuer *et al*., 1997) was used as a forward primer and 1525R (5'-AAGGAGGTGATCCAGCC-3') as reverse primer. DNA purification and cycle sequencing of the PCR products were carried out by First BASE Laboratory Sdn. Bhd., Malaysia.

Sequence analysis

The raw chromatogram sequences provided by First BASE Laboratory Sdn. Bhd., Malaysia were viewed using Chromas lite program, edited using BioEdit Programme (Hall, 1999) and stored in FASTA format. The forward and reverse primer sequences were removed before the sequence were blasted with existing sequences in NCBI GenBank database (Zhang *et al*., 2000) using BLAST nucleotide collection database program to search for closest best match sequence. The top similarity to the query sequence was used as an estimated reference species of the identity of the isolate.

Phylogenetic analysis

Phylogenetic analysis were performed using MEGA (Molecular Evolutionary Genetic Analysis) version 6 (Tamura *et al*., 2013). Prior to phylogenetic analysis, ambiguous sequences at the start and the end were deleted and gaps were manually adjusted to optimize alignment (Mahidi, 2015). The evolutionary distances were inferred by using the Neighboring Joining Method (Saitou and Nei, 1987). Bootstrap replicates (1000) were taken into account to infer the bootstrap consensus tree for the representation of evolutionary history. For investigation of the taxonomic composition of the microbial strains, ribosomal database project (RDP-II) using the SeqMatch tool to search the taxonomy database classification and nomenclature for all of the organisms in the public sequence databases.

Enzyme activity assay

Urease activity was measured immediately after incubating an overnight bacterial culture. In the absence of calcium ions, urease activity was determined by a conductivity method (Harkes *et al*., 2010). According to Whiffin *et al*. (2007), the urease reaction involves the hydrolysis of non-ionic substrate urea to ionic products thus generating a proportionate increase in conductivity under standard conditions. One millilitre (1 mL) of bacterial suspension of an overnight grown culture was added to 9 mL of 1.11 M of urea (reaction concentration 1 M urea) and conductivity (mS/cm) was measured for a duration of 5 min at 25 °C by immersing the probe of the conductivity meter (WalkLAB conductivity pro meter, Trans Instruments COMPRO) into the bacterial-urea solution. The conductivity variation rate (mS/cm/min) was obtained from the gradient of the graph. The conductivity variation rate was then multiplied by a dilution factor (df). The df was taken as the ratio of the stock bacteria culture to the sampling bacteria culture before inoculating into the urea solution (Zhao *et al*., 2014). These values were then used to calculate urease activity, by converting the conductivity variation rate (mS/cm/min) to urea hydrolysis rate (mM urea hydrolysed/min), based on the correlation that 1 mS/cm/min corresponds to a hydrolysis activity of 11 mM urea/min in the measured range of activities (van Paassen, 2009). Specific urease activity (mM urea hydrolysed/min/OD) which reflects the urease catalytic abilities of the urea hydrolysis (Zhao *et al*., 2014) was derived by dividing the urease activity (mM urea hydrolysed/min) by the bacterial biomass (OD600). Biomass concentration was determined by measuring the optical density of bacterial suspension with a spectrophotometer (GENESYSTM 20, Thermo Fisher Scientific) at a wavelength of 600 nm.

RESULTS AND DISCUSSION

Enrichment culturing of cave samples

A total of 12 samples were collected from Sarawak limestone caves region. The sample descriptions are shown in Table 1. To screen for highly active ureolytic bacteria, it was essential to select the conditions (urea as a substrate and alkaline pH) at which the desired types of microbes could grow and produce urease (Al-Thawadi and Cord-Ruwisch, 2012). Stabnikov *et al*. (2013) suggested that urease producing bacteria are common inhabitants of soils with regular supply of urea, which is the final product of nitrogen metabolism of mammals. Hence, enrichment culture designed to select urease producing bacteria suitable for MICP should be supplemented with sufficient amount of urea substrate (Hammes *et al*., 2003; Chu *et al*., 2011; Burbank *et al*., 2012). Although caves are considered as extreme environments, they are inhabited by microbial communities with unexpected diversity (Tomczyk-Żak and Zielenkiewicz, 2015). These makes Sarawak limestone caves suitable sampling locations as microbial communities in these environments are enriched and exposed to alkaline and limestone conditions.

To screen for highly active urease producing bacteria, enrichment culture technique was used as it brings about competition among the microbiota for available nutrients

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Table 1: Description of samples collected from Fairy and Wind caves, Bau Sarawak.

No	Location	Type of sample collected	Colour	Texture
1	Wind Cave	Sediment from drapery -1	Grey	Coarse
2	Wind Cave	Sediment from bat's urine	Brown	Silk
3	Wind Cave	Sediment from a bird's nest	Brown	Coarse
4	Wind Cave	Sediment from drapery-2	White	Coarse
5	Wind Cave	Sediment from stalactite	White	Fine
6	Wind Cave	Soil from mud-bank	Brown	Coarse
7	Wind Cave	Water dropping from stalactite	none	none
8	Fairy Cave	Soil around limestone area-1	Black	Silk
9	Fairy Cave	Soil around limestone region -2	Grey	Coarse
10	Fairy Cave	Soil around limestone region -3	Brown	Clay
11	Fairy Cave	Soil around plant region -1	Brown	Fine
12	Fairy Cave	Soil around plant region -2	Brown	Coarse

and against growth inhibitors (Gorski, 2012). The enrichment culture used in this study was designed to target bacteria which are able to degrade urea, as the main source of nitrogen and energy. Six percent (6%) urea was selected in order to target bacteria able to survive at high urea concentration and potentially able to produce urease. The enrichment samples were cultured for a duration of 120 h, it was observed that during the incubation period (24-48 h) there was a pungent smell, indicating the release of ammonia gas.

The breakdown of urea by the urease enzyme allows the release of ammonium gas to the environment. It is highly recommended to work using a facial mask when handling urease producing bacteria inside the incubation room. Another precaution which should be taken is to incubate these bacteria in a small incubator (MMM Incucell 55, MMM Medcenter Einrichtungen GmbH) and place the incubator inside a fume hood (BASIX 52, LABCRAFT) to prevent the discharge and spread of ammonia gas in the laboratory.

Isolation and selection of ureolytic bacteria

After incubation, the enriched samples were aseptically diluted six-folds and spread onto nutrient agar containing urea substrate. These agar plates were incubated at 32 °C for 42 h to allow the growth of urea degrading bacteria. Various potential ureolytic bacteria isolates were seen in the nutrient agar plates. Each bacterial isolate was subcultured on nutrient agar to obtain pure colonies. These isolates were then tested for their abilities to survive on growth media containing high urea concentration. Bacteria that could not grow on nutrient agar supplemented with 6% urea were discarded. Among all the isolated bacteria, seven bacterial isolates designated as LPB4, TSB31, TSB12, NB40, TSB55, TSB29 and LB31 were selected based on their quick urease production when tested on urea agar base medium. Urea agar base media is used for the differentiation of a variety of microorganisms, especially on the basis of urease production (Atlas, 2010).

Bacterial isolates which were able to produce urease turned the urea agar base medium in the test tube from pale orange to pink, while the isolates which were unable to produce urease turned the media yellow. This medium contains urea and a pH indicator, phenol red. When urea is hydrolyzed, ammonia accumulates in the media which results in an increase in the pH of the environment (Hammad *et al*., 2013). This increase in pH causes the pH indicator to change from pale orange to pink, confirming a positive result for urea hydrolysis. Several studies have reported using urea agar base media as a preferred qualitative urease assay for isolation and differentiation of ureolytic microorganisms (Hammes *et al*., 2003; Achal *et al*., 2010; Achal and Pan, 2011; Burbank *et al*., 2012; Dhami *et al*., 2013; Elmanama and Alhour, 2013; Hammad *et al*., 2013).

Table 2: Physiological characteristics of urease producing bacterial isolates.

Characteristic	LPB4	TSB31	TSB12	NB40	TSB55	TSB29	LB31
Form	Filamentous	Circular	Circular	Circular	Circular	Irregular	Circular
Size (mm)	12	8	6	6	1	4	4
Surface	Rough	Rough	Smooth	Smooth	Smooth	Smooth	Smooth
Chromogenesis	Whitish-yellow	Brownish-white	Brownish-white	Brown	White	Brownish-white	Brown
Gram stain	+; rod	+; rod	+; rod	+; rod	-; rod	+; rod	-; rod
Endospore staining	Spore forming	Spore forming	Spore forming	Spore forming	Spore forming	Spore forming	Spore forming
Catalase	+	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+	+
Motility	+	+	+	+	+	+	+

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Preliminary bacterial identification

LPB4, TSB31, TSB12, NB40, TSB55, TSB29 and LB31 were characterized using standard methods. All seven isolates were Gram-positive rod-shaped and smooth surfaces (Table 2). There were noticeable morphological differences among the bacterial isolates. The limited diversity of the bacterial community in limestone environment is not surprising because of its alkaline condition, only organisms capable of growing in these conditions can survive in such an environment (Achal *et al*., 2010). The close relationship of bacterial isolates observed among the isolated strains might be as a result of the dominant species which might occur during enrichment culturing period since *Bacillus* species are usually selected by isolation and cultivation methods (Stocks-Fischer *et al*., 1999). The phenotypic and biochemical properties of the bacteria isolates have a similar resemblance of those *Bacillus* species reported previously by (Achal *et al*., 2010).

Achal and Pan (2011) reported in their study that one of the reasons ureolytic bacteria are able to survive at high pH might be due to their alkaline habitat of which the aforementioned bacterial strains were also isolated from. Study from Aono *et al*., (1999) on contribution of the cell wall component teichuronopeptide to pH homeostasis and alkaliphily in the alkaliphilic *B*. *lentus* C-125 reported that the teichurono-peptide may contribute to a pH homeostasis at alkaline pH and support the bacteria strains to survive in an alkaline environment.

Bacterial identification using 16S rRNA sequence analysis

The seven selected urease producing bacteria isolated from Sarawak limestone region were identified and characterized by the sequencing of 16S rRNA. These sequences were BLAST searched against the GenBank database using the BLASTN program. The nucleotide BLAST analysis of the 16S rRNA region showed a reasonable degree of correlation with the physiological characterization especially the morphological classification schemes of species within the genus.

The BLAST analysis revealed that LPB4 had 97% similarity (99% coverage) with *S*. *pasteurii*, TSB31 had 97% similarity (99% coverage) with *S*. *pasteurii*, TSB12 had 99% similarity (100% coverage) with *S*. *pasteurii*, NB40 had 97% similarity (99% coverage) with *S*. *pasteurii*, TSB55 had 91% (99% coverage) with *B*. *lentus*, TSB29 had 98% similarity (99% coverage) with *S*. *pasteurii*, and LB31 had had 99% similarity (99% coverage) with *S*. *luteola*. Based on sequence data of the 16S of the rRNA region, all bacterial isolates from Sarawak limestone caves showed a high degree of similarity (91-99%) to their closest species. A recent study by Wei *et al*. (2015) reported isolating ureolytic bacteria from marine sediments of which majority of the identified bacteria were *Sporosarcina* sp. The nucleotide sequences which were obtained in the present study have been deposited in NCBI GenBank database. The provided GenBank accession numbers for the submitted nucleotide sequences for bacterial isolates LPB4, TSB31, TSB12, NB40, TSB55, TSB29 and LB31 are KX212194, KX212201, KX212204, KX212193, KX212200, KX212203 and KX212212.

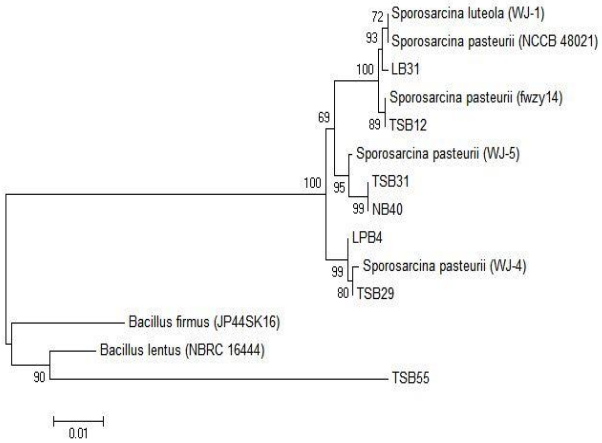

Figure 1: Phylogenetic tree based on the bacterial 16S rRNA gene sequence data sequence from different isolates of the current study along with sequences available in the GenBank database. The results show that the highly active isolates were identified as *S*. *pasteurii*, with *B*. *lentus* and *S*. *luteola.* The tree was constructed using Molecular Evolutionary Genetic Analysis (MEGA) version 6 (Tamura *et al.*, 2013). Numerical values indicates bootstrap percentile from 1,000 replicates. Bar, 0.005 substitution per nucleotides.

The relationship between the locally isolated ureolytic bacteria and their closest species are shown in the phylogenetic tree (Figure 1). Phenotypic and physiological properties of these isolate resemble those of Bacillus and Sporosarcina species previously reported (Gordon *et al*., 1973; Gordon and Hyde, 1982; Stocks-Fischer *et al*., 1999; Tominaga *et al*., 2009) which were further confirmed by 16S rRNA gene analysis. The genus *Bacillus* and *Sporosarcina* seem to be well suited for long-term survival in extreme environments (Achal and Pan, 2011). This may explain these isolates from Sarawak limestone cave samples. It is noteworthy that the phylogenetic analysis revealed LPB4, TSB31, NB40 and TSB55 showed less than 98% similarity with different species of *Bacillus* and *Sporosarcina*.

This is the first study to show the presence of at least one cultivable bacteria from Sarawak region (Fairy and Wind caves) capable of producing urease in the presence of high concentration of urea. The isolation of a few urease producing bacteria from the collected samples suggests that a small percentage of environmental bacteria are capable of participating in the precipitation of calcite through urea hydrolysis (Burbank *et al*., 2012).

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Measurement of specific urease activity

Conductivity and biomass of all bacterial isolates were measured at the end of incubation period (24 h). The conductivity variation rate (mS/cm/min) of each bacterial culture was measured and converted to specific urease activity. Among all the bacterial culture, TSB12 had the highest specific urease activity (17.400 mM urea hydrolyzed/min/OD) when compared to other local cultures, while TSB55 showed the lowest specific urease activity (12.715 mM urea hydrolyzed/min/OD) as shown in Figure 2. Several studies have reported the usage of *S. pasteurii* as a preferred urease producing bacteria, especially *S. pasteurii* ATCC 11859 which has been used in numerous studies of MICP (Bang *et al*., 2001; Bachmeier *et al*., 2002; Whiffin, 2004; Whiffin *et al*., 2007). Specific urease activity for *S. pasteurii* (DSM33) reported by Harkes *et al.* (2010) was between the range of 5 to 20 mM urea hydrolysed/min/OD. Another report from Whiffin (2004) on the specific urease activity of *S. pasteurii* (ATCC11859) was between 2.2 to 13.3 mM urea hydrolysed/min/OD. However, other studies reported locally isolated *Bacillus* strains had urease activity between 3.3 to 8.8 mM urea hydrolysed/min/OD (Al-Thawadi and Cord-Ruwisch, 2012; Stabnikov *et al*., 2013).

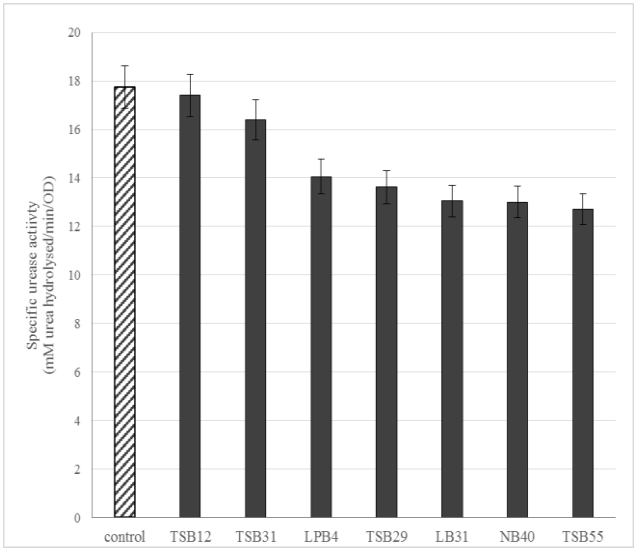

Figure 2: Comparison of specific urease activity among the isolated urease producing strains and the control strain. Two bacterial isolates, TSB2 and TSB31 show high urease activity, almost comparable to control strain.

The release of ammonia as a result of urea hydrolysis can be toxic and detrimental to most bacterial cells especially when the concentration is high (Cheng and Cord-Ruwisch, 2013). This production of ammonia is advantageous to certain bacteria specifically ureolytic bacteria such as *S. pasteurii*, which uses the ammonia production for the generation of adenosine triphosphate (ATP) (Cheng and Cord-Ruwisch, 2013). According to Stabnikov *et al*. (2013), some ureolytic bacteria can be pathogenic, especially strains such as *Helicobacter pylori*, *Proteus vulgaris*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*. Due to their level of their pathogenicity, they are not suitable for biocementation applications. Therefore, it is important to use non-pathogenic producers such as *S. pasteurii* and some species of *Bacillus*.

CONCLUSION

The BLAST analysis of the 16S rRNA gene sequence from the locally isolated ureolytic bacteria identified them as *Sporosarcina pasteurii*, *Bacillus lentus* and *Sporosarcina luteola*. The specific urease activity of the bacterial strains measured in comparison to the purchased control strain suggests that these ureolytic strains serve as a good urea hydrolytic agent, potentially useful for biocementation applications.

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REFERENCES

Achal, V. (2010). Characterization of two urease-producing and calcifying bacillus spp. Isolated from cement. *Journal of Microbiology and Biotechnology* **20, 1571-1576.**

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

Akanbi, T. O., Kamaruzaman, A. L., Abu Bakar, F., Hamid, S. A. N., Radu, S., Manap, A. M. Y. and Saari, N. (2010). Highly thermostable extracellular lipase-producing *Bacillus* strain isolated from a Malaysian hotspring and identified using 16S rRNA gene sequencing *International Food Research Journal* **17, 45-53.**

Al-Thawadi, S. and Cord-Ruwisch, R. (2012). Calcium carbonate crystals formation by ureolytic bacteria isolated from Australian soil and sludge. *Journal of Advanced Science and Engineering Research* **2, 12-16.**

Aono, R., Ito, M. and Machida, T. (1999). Contribution of the cell wall component teichuronopeptide to pH homeostasis and alkaliphily in the alkaliphile *bacillus lentus* c-125. *Journal of Bacteriology* **181, 6600-6606.**

Atlas, R. M. (2010). Handbook of microbiological media. 4th Edn. ASM press. Taylor and Francis group, LLC. London, England. **pp. 1868-1871.**

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Bachmeier, K. L., Williams, A. E., Warmington, J. R. and Bang, S. S. (2002). Urease activity in microbiologically-induced calcite precipitation. *Journal of Biotechnology* **93, 171-181.**

Bang, S. S., Galinat, J. K. and Ramakrishnan, V. (2001). Calcite precipitation induced by polyurethane-immobilized *Bacillus pasteurii*. *Enzyme and Microbial Technology* **28, 404-409.**

Bisen, P. S. (2004). Microbial Staining *In*: Microbes in Practice. IK International, New Delhi, India. **pp. 139-155.**

Burbank, M. B., Weaver, T. J., Williams, B. C. and Crawford, R. L. (2012). Urease activity of ureolytic bacteria isolated from six soils in which calcite was precipitated by indigenous bacteria. *Geomicrobiology Journal* **29, 389-395.**

Cheng, L. and Cord-Ruwisch, R. (2013). Selective enrichment and production of highly urease active bacteria by non-sterile (open) chemostat culture. *Journal of Industrial Microbiology and Biotechnology* **40, 1095-1104.**

Chu, J., Ivanov, V., He, J., Naeimi, M., Li, B. and Stabnikov, V. (2011). Development of microbial geotechnology in Singapore. *In*: Proceedings of Geofrontiers 2011: Advances in Geotechnical Engineering. American Soceity of Civil Engineers, Geotechnical Special Publication 211, Dallas, Texas, U.S.A. **pp. 4070-4078.**

Chunxiang, Q., Jianyun, W., Ruixing, W. and Liang, C. (2009). Corrosion protection of cement-based building materials by surface deposition of $CaCO_3$ by *Bacillus pasteurii*. *Materials Science and Engineering* **29, 1273-1280.**

Collins, C. M. and D'Orazio, S. E. (1993). Bacterial ureases: Structure, regulation of expression and role in pathogenesis. *Molecular Microbiology* **9, 907-913.**

De Muynck, W., Cox, K., Belie, N. D. and Verstraete, W. (2008). Bacterial carbonate precipitation as an alternative surface treatment for concrete. *Construction and Building Materials* **22, 875-885.**

DeJong, J. T., Soga, K., Banwart, S. A., Whalley, W. R., Ginn, T. R., Nelson, D. C., Mortensen, B. M., Martinez, B. C. and Barkouki, T. (2011). Soil engineering *in vivo*: Harnessing natural biogeochemical systems for sustainable, multi-functional engineering solutions. *Journal of The Royal Society Interface* **8, 1-15.**

Dhami, N. K. (2013). Biomineralization of calcium carbonate polymorphs by the bacterial strains isolated from calcareous sites. *Journal of Microbiology and Biotechnology* **23, 707-714.**

Elmanama, A. A. and Alhour, M. T. (2013). Isolation, characterization and application of calcite producing bacteria from urea rich soils. *Journal of Advanced Science and Engineering Research* **3, 377-399.**

Gat, D., Tsesarsky, M., Shamir, D. and Ronen, Z. (2014). Accelerated microbial-induced $CaCO_3$ precipitation in a defined coculture of ureolytic and non-ureolytic bacteria. *Biogeosciences* **11, 2561-2569.**

Gordon, E. R. and Hyde, L. J. (1982). The bacillusfirmus-bacillus lentus complex and ph 7.0 variants of some alkalophilic strains. *Journal of General Microbiology* **128, 1109-1116.**

Gordon, R. E., Harynes, W. C. and Pang, C. H. (1973). The genus *Bacillus* (Agriculture Handbook no. 427). Agricultural Research Service, Department of Agriculture. **pp. 16-18.**

Gorski, L. (2012). Selective enrichment media bias the types of *Salmonella enterica* strains isolated from mixed strain cultures and complex enrichment broths. *PLoS One* **7(4), e34722.**

Hall, T. A. (1999). Bioedit: A user-friendly biological sequence alignment editor and analysis program for windows 95/98/nt. *Nucleic Acids Symposium Series* **41, 95-98.**

Hammad, I. A., Talkhan, F. N. and Zoheir, A. E. (2013). Urease activity and induction of calcium carbonate precipitation by *Sporosarcina pasteurii* NCIMB 8841. *Journal of Applied Sciences Research* **9, 1525-1533.**

Hammes, F., Boon, N., de Villiers, J., Verstraete, W. and Siciliano, S. D. (2003). Strain-specific ureolytic microbial calcium carbonate precipitation. *Applied and Environmental Microbiology* **69, 4901-4909**.

Harkes, M. P., van Paassen, L. A., Booster, J. L., Whiffin, V. S. and van Loosdrecht, M. C. M. (2010). Fixation and distribution of bacterial activity in sand to induce carbonate precipitation for ground reinforcement. *Ecological Engineering* **36, 112-117.**

Heuer, H., Krsek, M., Baker, P. and Wellington, E. M. H. (1997). Analysis of actinomycete communities by specific amplification of genes encoding 16S rRNA and gel-electrophoretic separation in denaturing gradients. *Applied and Environmental Microbiology* **63, 3233-3241.**

Holt, J. G., Noel, R. K., Peter, H. A. S., James, T. S. and Stanley, T. W. (1994). Determinative Bacteriology, 9 edn. Baltimore, USA. **pp. 284-288.**

Hughes, M. S., James, G., Ball, N., Scally, M., Malik, R., Wigney, D. I., Martin, P., Chen, S. and Love, D. N. (2000). Identification by 16s rrnagene analyses of a potential novel mycobacterial species as an etiological agent of canine leoproid granuloma syndrome. *Journal of Clinical Microbiology* **38, 953-959.**

Ivanov, V. and Chu, J. (2008). Applications of microorganisms to geotechnical engineering for bioclogging and biocementation of soil in situ. *Reviews in Environmental Science and Biotechnology* **7, 139-153.**

Jahns, T. (1996). Ammonium/urea-dependent generation of a proton electrochemical potential and synthesis of atp in *Bacillus pasteurii*. *Journal of Bacteriology* **178, 403-409.**

Lane, D. J., Pace, B., Olsen, G. J., Stahl, D. A., Sogin, M. L. and Pace, N. R. (1985). Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyse. *Proceedings of the National Academy of Sciences* **82, 6955-6959.**

Lee, S. G. and Calhoun, D. H. (1997). Urease from a potentially pathogenic coccoid isolate: Purification,

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

characterization, and comparison to other microbial ureases. *Infection and Immunity* **65, 3991-3996.**

Mahidi, N. (2015). Characterization of new *Muscodor padawan* and *Muscodor sarawak*, isolated from Sarawak, Malaysia: Evaluation of their potential as a biological control agent for *Ganoderma boninense,* a pathogenic fungus of *Elaeis guineensis*. PhD. Thesis. Swinburne University of Technology, Sarawak Campus, Malaysia.

Mobley, H. L. and Hausinger, R. P. (1989). Microbial ureases: Significance, regulation, and molecular characterization. *Microbiological Reviews* **53, 85-108.**

Mobley, H. L., Island, M. D. and Hausinger, R. P. (1995). Molecular biology of microbial ureases. *Microbiological Reviews* **59, 451-480.**

Moyes, R. B., Reynolds, J. and Breakwell, D. P. (2009). Differential Staining of Bacteria: Gram Stain. *Current Protocols in Microbiology* **15(3C), A.3C.1-A.3C.8.**

Muramatsu, H., Shahab, N., Tsurumi, Y. and Hino, M. (2003). A comparative study of Malaysian and Japanese actinomycetes using simple identification method based on partial 16S rRNA sequence. *Actinomycetol* **17, 33-43.**

Olufunke, O. A., Abiodun, A. O. and Dunah, F. C. (2014). Extended spectrum beta-lactamase-producing uropathogenic *Escherichia coli* in pregnant women diagnosed with urinary tract infections in South Western Nigeria. *Journal of Molecular Biology Research* **4, 34-41.**

Rivadeneyra, M. A., Delgado, R., Delgado, G., Moral, A. D., Ferrer, M. R. and Ramos-Cormenzana, A. (1993). Precipitation of carbonates by *Bacillus* sp. isolated from saline soils. *Geomicrobiology Journal* **11, 175-184.**

Saitou, N. and Nei, M. (1987). The neighbor-joining method: A new method for reconstructing phylogenetic trees. *FEMS Microbiology Reviews* **4, 406-425.**

Shields, P. and Cathcart, L. (2011). Motility test medium protocol. Available from : http://www.asmscience.org/content/education/protocol/protocol.3658 [Retrieved on: 16 October 2014].

Stabnikov, V., Jian, C., Ivanov, V. and Li, Y. (2013). Halotolerant, alkaliphilic urease-producing bacteria from different climate zones and their application for biocementation of sand. *World Journal of Microbiology and Biotechnology* **29, 1453-1460.**

Stocks-Fischer, S., Galinat, J. K. and Bang, S. S. (1999). Microbiological precipitation of $CaCO_3$. *Soil Biology and Biochemistry* **31, 1563-1571.**

Tamura, K., Stecher, G., Peterson, D., Filipski, A. and Kumar, S. (2013). Mega 6: Molecular evolutionary genetics analysis version 6.0. *Molecular Biology and Evolution* **30, 2725-2729.**

Tomczyk-Żak, K. and Zielenkiewicz, U. (2015). Microbial diversity in caves. *Geomicrobiology Journal* **1-19.**

Tominaga, T., An, S. Y., Oyaizu, H. and Yokota, A. (2009). *Sporosarcina luteola* sp. nov. isolated from soy sauce production equipment in Japan. *The Journal of General and Applied Microbiology* **55, 217-223.**

van Paassen, L. A. (2009). Biogrout Ground Improvement by Microbially Induced Carbonate Precipitation. PhD.Thesis. Delft University of Technology, Netherlands.

Wei, S., Cui, H., Jiang, Z., Liu, H., He, H. and Fang, N. (2015). Biomineralization processes of calcite induced by bacteria isolated from marine sediments. *Brazilian Journal of Microbiology* **46, 455-464.**

Whiffin, V. S. (2004). Microbial $CaCO_3$ precipitation for the production of biocement. PhD. Thesis. Murdoch University, Australia.

Whiffin, V. S., van Paassen, L. A. and Harkes, M. P. (2007). Microbial carbonate precipitation as a soil improvement technique. *Geomicrobiology Journal* **24, 417-423.**

Zhang, Z., Schwartz, S., Wagner, L. and Miller, W. (2000). A greedy algorithm for aligning DNA sequences. *Journal of Computational Biology* **7, 203-214.**

Zhao, Q., Li, L., Li, C., Li, M., Amini, F. and Zhang, H. (2014). Factors affecting improvement of engineering properties of MICP-treated soil catalyzed by bacteria and urease. *Journal of Materials in Civil Engineering* **26, 04014094-04014104.**

 ISSN (print): 1823-8262, ISSN (online): 2231-7538