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SHORT COMMUNICATION

Screening for Urease-Producing Bacteria from Limestone Caves of Sarawak

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ABSTRACT

Urease is a key enzyme in the chemical reaction of microorganism and has been found to be associated with calcification, which is essential in microbially induced calcite precipitation (MICP) process. Three bacterial isolates (designated as LPB19, TSB31 and TSB12) were among twenty-eight bacteria that were isolated from samples collected from Sarawak limestone caves using the enrichment culture technique. Isolates LPB19, TSB31 and TSB12 were selected based on their quick urease production when compared to other isolates. Phenotypic characteristics indicate all three bacterial strains are gram-positive, rod-shaped, motile, catalase and oxidase positive. Urease activity of the bacterial isolates were measured through changes in conductivity in the absence of calcium ions. The bacterial isolates (LPB19, TSB12 and TSB31) showed urease activity of 16.14, 12.45 and 11.41 mM urea hydrolysed/min respectively. The current work suggested that these isolates serves as constitutive producers of urease, potentially useful in inducing calcite precipitates.

Keywords: Bacterial isolation, conductivity, enrichment culture, microbial induced calcite precipitation (MICP), *Sporosarcina pasteurii*, urease activity

Microbial enzymes are more stable and have properties which are more diverse than other enzymes derived from plants and animals (Alves *et al.,* 2014). Reported studies have shown that there are increasing number of microorganisms being screened from extreme environments with capability to produce essential enzymes useful for various industrial applications (Elmanama & Alhour, 2013; Krishnapriya *et al.,* 2015; Omoregie *et al.,* 2015). This suggests the importance of natural and extreme environments investigation for microorganisms with potential industrial relevancy.

Caves are natural geological formations which are considered as extreme environments and forms ecological niches for specific microorganisms (Schabereiter-Gurtner *et al.,* 2004). The interaction between minerals and microorganisms play an important role in caves formations, specifically in structures such as stalactites and stalagmites (Tomcyzk-Żak & Zielenkiewicz, 2015). Some cave microorganisms are capable of inducing $CaCO_3$ precipitates on the surfaces of their cells, which conduce to the formation of limestones (Schabereiter-Gurtner *et al.,* 2004).

Sarawak is situated on the island of Borneo, known as the third largest island in the world and one of the twelve mega-biodiversity regions (Lateef *et al.,* 2014; Tan *et al.,* 2009). The rich mega-biodiversity in Sarawak has attracted the attention of researchers within and outside of Malaysia. The existing scientific studies have focused on peat soils, plants, corals, microbes in aquatic and forest environments (Cole *et al.,* 2015; Kuek *et al.,* 2015; Lateef *et al.,* 2014; Miyashita *et al.,* 2013; Sa'don *et al.,* 2015). Sarawak's limestone forest is one of the nine main types of forest documented in Sarawak,

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covering about 520 m^2 or 0.4% of the total area (Banda *et al.,* 2004; Julaihi, 2004). The caves or limestone areas in Sarawak have become the main focus for researchers to investigate the diversity of bats indigenous to the caves (Mohd *et al.,* 2011; Rahman *et al.,* 2010). Caves are usually limited in nutrient, secluded from the outside environment and may contain novel, diverse microbial populations (Sugita *et al.,* 2005). Hence, it is essential to carry out pioneering studies ventured at exploring the isolation of microbial species.

This paper reports the investigation of microorganisms isolated from Sarawak caves. These isolated microbes are known to survive in alkaline environments and some are able to produce urease, an enzyme which involve in microbial induced calcite precipitation (MICP). MICP is a natural phenomenon associated with a wide range of bacterial species in an alkaline environment rich in Ca^{2+} (Achal & Pan, 2011). MICP is resultant of complex biochemical reactions often governed by an enzyme urease (urea amidohydrolase; EC 3.5.1.5) produced by microorganisms (Achal, 2015; Zhang *et al.,* 2015). Urea hydrolysis is a reaction that can be controlled easily in the process of carbonate generation, with the potential to produce high concentrations of carbonate within a short time (Achal & Pan, 2011). The addition of urea to the microorganism system allows the conversion of urea to dissolved inorganic carbon and ammonium, subsequently releasing the ammonium to the environment (De Muynck *et al.,* 2010). The presence of calcium ion leads to a supersaturation condition and precipitation of calcite in the microorganism's cell wall, which later becomes encapsulated by calcite precipitate. The limitation of transferred nutrient to the microorganism results in cell death (Ariyanti *et al.,* 2011; Hammes & Verstraete, 2002).

MICP is used in civil and geotechnical engineering to improve the mechanical properties of soil for construction and environmental purposes by enhancing the strength and stiffness properties of soil through microbial activity and products (Ivanov & Chu, 2008). Numerous reported studies have mainly adopted the use of *Sporosarcina pasteurii* as their preferred ureolytic bacteria for MICP process because it is non-pathogenic and has quick capability to produce urease (Achal *et al.,* 2009; Al-Thawadi, 2008; Cheng & Cord-Ruwisch, 2013; Cuzman *et al.,* 2015b; Kang *et al.,* 2014; Wei *et al.,* 2015; Whiffin, 2004). However, studies on alternative species used for urea hydrolysis are very limited. This current study is aimed at using enrichment culture technique to isolate urease-producing bacteria from samples collected from limestone caves with the potential of inducing calcite precipitates.

Samples used in this study were collected from Fairy and Wind Caves situated in Bau, 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"). Cave samples were collected using a sterile spatula and were aseptically transferred into sterile polyethene bags before transferring the samples to the laboratory in an ice box which was maintained at 4°C for microbiological study. *Sporosarcina pasteurii* type strain DSM33 was purchased from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (Braunschweig, GERMANY). The bacterial strain was used as a positive control for subsequent experiments in this study.

To enrich the cave samples, 1 g or 1 ml of each sample was inoculated into 50 ml growth medium (250 ml shaking flasks, at 30°C for 120 hrs at 130 rpm). Urea substrate (6%) was supplemented into the growth media using a sterile filtered 0.45 µm syringe. The pH of the broth solution was adjusted to 8.0 using 1 N HCL and 1 N NaOH. For bacterial isolation, the enriched culture samples were diluted (sixfold) and plated on nutrient agar media (with 2% urea). The agar petri plates were then incubated at 32°C for 42 hrs. Upon the growth of isolates in the petri plates, subsequent sub-culturing was performed until single bacterial colonies were obtained.

Christensen's medium also called urea agar base (Oxoid) was used to screen for urease producing bacteria. The media components contained the following; 1.0 g/l of Peptone, 1.0 g/l of Glucose, 5.0 g/l of Sodium chloride, 1.2 g/l of Disodium phosphate, 0.8 g/l of Potassium dihydrogen phosphate, 0.012 g/l of Phenol red and 15.0 g/l of Agar. Urea solution (40%) 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. The medium was carefully mixed by gently swirling the Schott bottle containing the urea agar base so as to avoid bubble formation and 10 ml was then distributed into sterile test tubes. The bacterial isolates were heavily inoculated on the surface of the medium and then incubated at 37ºC for 72 hrs. The urease production test was studied through visual observation for colour changes. The isolates that changed the medium from pale yellow to a pink-red colour were selected for further studies based on their ability to quickly produce urease qualitatively. Morphological characterization such as colony and cell morphology, gram stain reaction, motility, catalase ad oxidase tests were performed by standard methods.

Conductivity (mS/cm) method was used to determine urease activity (mM urea hydrolysed/min) in this study. For enzyme assay, 1.0 ml of overnight cultured bacterial culture was added to 9.0 ml of 1.11 M urea solution (Harkes *et al.,* 2010) and the conductivity in was recorded. The assay was measured by using an electric conductivity meter (WalkLAB conductivity pro meter) for a duration of 5 min at 25ºC. The urease activity was then calculated by taking the dilution factor into account. The relative conductivity (mS/cm/min) was then converted to urea hydrolysis rate, urease activity from a standard curve generated by determining the conductivity change resulted from complete hydrolysis of several concentration of urea by purified urease (Sigma Cat. No. U-7127) according Whiffin (2004). Urease activity was determined from the following equation;

Urea hydrolyzed (mM) = Conductivity (mS/cm/min) x 11.11 (Whiffin, 2004)

A total of twenty-eight bacteria were isolated from selective enrichment culture medium. For the purpose of screening for high urease producing bacteria, it was necessary to select appropriate conditions at which these desired microbes would survive. Enrichment culture technique is widely used to isolate bacteria in clinical, biotechnological and environmental studies because it brings about competition among microbiota for available nutrients and against growth inhibitors by favouring specific bacterial type strains or subgroups (Gorski, 2012; Muniesa *et al.,* 2005). It was observed that during the incubation period (24 hrs) there was a unique pungent smell, indicating the release of ammonia gas. The breakdown of urea by the urease enzyme allows the release of ammonium gas to the bacteria's environment. This gas can be poisonous to humans if inhaled and can cause serious respiratory (Gueye *et al.,* 2001; Woto-Gaye *et al.,*1999). Hence, it is 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 inside an incubator kept in a fume hood so as to minimize the emancipation of ammonia gas to the environment.

The screening for urease producing bacteria was conducted using urea agar base medium in test tubes. The colour changes from pale yellow to pink-red indicated positive urease activity. Out of the twenty-eight bacteria isolated from the cave samples, three bacterial isolates (LPB19, TSB31 and TSB12) were selected based on the ability of the isolates to completely turn the urea agar base medium pink in comparison with other isolated urease producing bacteria and the control strain used in this study. In Table 1, the control strain was able to completely turn the urea agar base medium from yellow to pink within 24 hrs of incubation period while the selected isolates (LPB19, TSB31 and TSB12) changed their respective urea agar base medium to pink between 30-36 hrs of incubation. However, the rest of the locally isolated urease-producing bacteria turned their medium to pink within an incubation period of 48-72 hrs. Several studies have reported that urea agar base can be used as a quick method to primarily screen for urease-producing bacteria which can be suitable for biocementation purposes (Burbank *et al.,* 2012; Chahal *et al.,* 2011; Hammes *et al.,* 2003). Urea agar base contains urea and phenol red which acts as a pH indicator. When urea is hydrolysed by the bacteria, ammonia is released and becomes accumulated in the medium which increases the pH of the environment making it alkaline (Hammad *et al.,* 2013).

Table 1. Urea hydrolysis observed in urea agar base medium.

Bacteria	Time (hrs)
Control	24
LBP19	30
TSB12	32
TSB31	36

Bacterial isolate LPB19, TSB31 and TSB12 were characterized under standard methods. All three isolates were gram-positive, rod-shaped, motile, catalase and oxidase positive as shown in Table 2. There were noticeable morphological differences among the isolated urease-producing bacteria. The limited diversity of the bacterial community in limestone environment is not surprising because of its extreme alkaline condition, only organisms capable of growing in these conditions can survive in such an environment (Achal *et al.,* 2010). The close morphology of bacterial isolates were observed among the isolates and it might be as a result of the dominance species which might occur during enrichment culturing period since *Bacillus* species are usually selected by the isolation and cultivation methods (Stocks-Fischer *et al.,* 1999).

Urease activity was measured through changes in conductivity (mS/cm) in the absence of calcium ions (Whiffin, 2004). Conductivity measurement is a suitable method to measure urease activity, because urease turns the urea molecule (non- conductive) into two charged ions: ammonium (NH_4^+, positively charged) and carbonate (CO_3^{2-}, negatively charged) (Cuzman *et al.,* 2015a). The relative average in conductivity (mS/cm/min) for isolated strains LPB19, TSB12 and TSB31 were 0.15, 0.11 and 0.10 mS/cm/min respectively as shown in Figure 1. In comparison to isolate TSB12, TSB31 and control (0.13 mS/cm/min), isolate LPB19 showed the highest average change in conductivity which is 0.15 mS/cm/min.

The average change in conductivity for *Sporosarcina pasteurii* (DSM 33) reported in other studies were in a range of 0.083-0.23 mS/cm/min (Cuzman *et al.,* 2015b; Whiffin *et al.,* 2007). A study by Hammad *et al.* (2013) reported using *Sporosarcina pasteurii* (NCIMB 8841) which had an average change in conductivity of 0.05 mS/cm/min. However, another study conducted by Chu *et al.* (2012) reported having an average change in conductivity of 0.06 mS/min/cm for halotolerant and alkaliphilic urease-producing strains which were isolated from tropical beach sand and later identified as *Bacillus* sp.

Table 2. Physiological characteristics of urease-producing bacteria strains.

Characteristics	Isolate LPB19	Isolate TSB12	Isolate TSB31
Form	Circular	Circular	Circular
Size (mm)	4	6	8
Surface	Smooth	Smooth	Smooth
Chromogenesis	Brown	Brownish-white	Brownish-white
Gram stain	positive; rod	positive; rod	positive; rod
Endospore stain	Spore forming	Spore forming	Spore forming
Catalase	positive	positive	positive
Oxidase	positive	positive	positive
Motility	positive	positive	positive

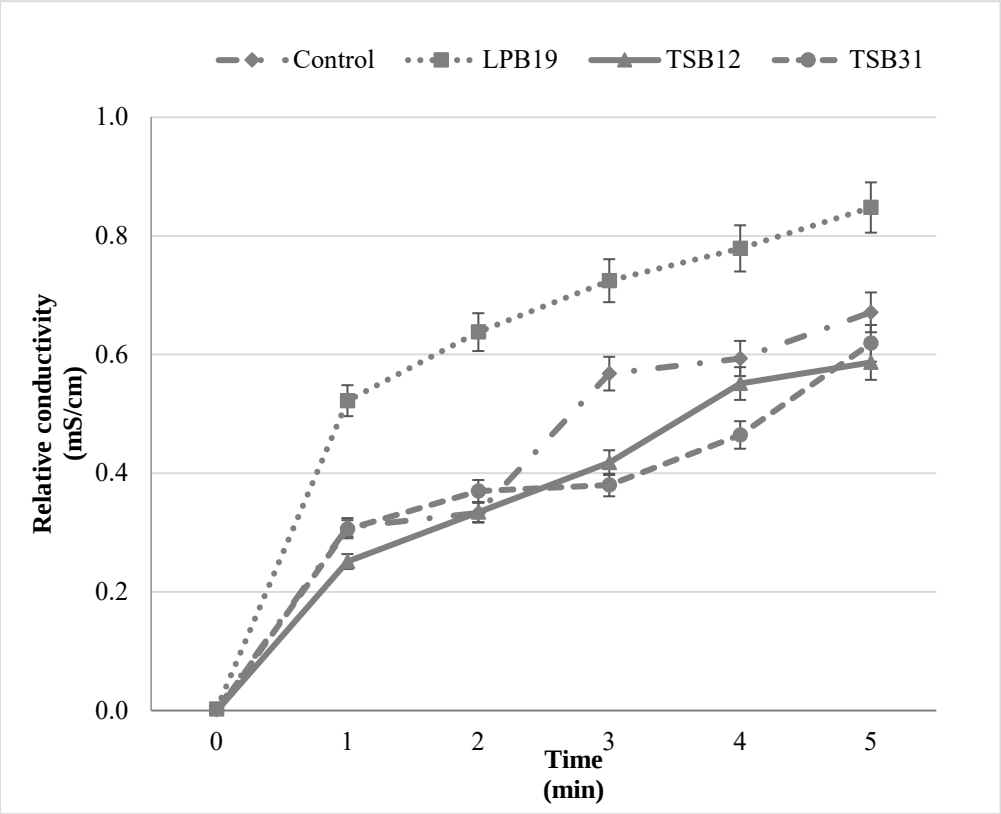

Figure 1. A plot of change in conductivity for a period of 5 minutes. Error bars represent standard error of the mean.

The urease activity of the three bacterial isolates were calculated and compared to that of the control strain. The urease activity of Isolates LPB19, TSB 12 were 16.14, 12.45 and 11.41 mM urea hydrolysed/min respectively as shown in Figure 2, while the control strain had a urease activity of 14.09 mM urea hydrolysed/min. In comparison to isolate TSB12, TSB31 and control, isolate LPB19 showed the highest urease activity which is 16.14 mM urea hydrolysed/min. Urease activity for *Sporosarcina pasteurii* (DSM33) reported by Harkes *et al.* (2010) was between the range of 5 to 20 mM urea hydrolysed/min. Another report from Whiffin (2004) on the urease activity of *Sporosarcina pasteurii* (ATCC11859) was between 2.2 to 13.3 mM urea hydrolysed/min. However, another studies reported locally isolated *Bacillus* strains has urease activity between 3.3 and 8.8 mM urea hydrolysed/min. (Al-Thawadi & Cord-Ruwisch, 2012; Stabnikov *et al.,* 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 *Sporosarcina pasteurii* and some species of *Bacillus.* In addition, several studies have reported the usage of *Sporosarcina pasteurii* as a preferred urease producing bacteria, especially *Sporosarcina pasteurii* (ATCC 11859) which have been used in numerous studies of MICP (Bachmeier *et al.,* 2002; Bang *et al.,* 2001; Whiffin *et al.,* 2007; Whiffin, 2004).

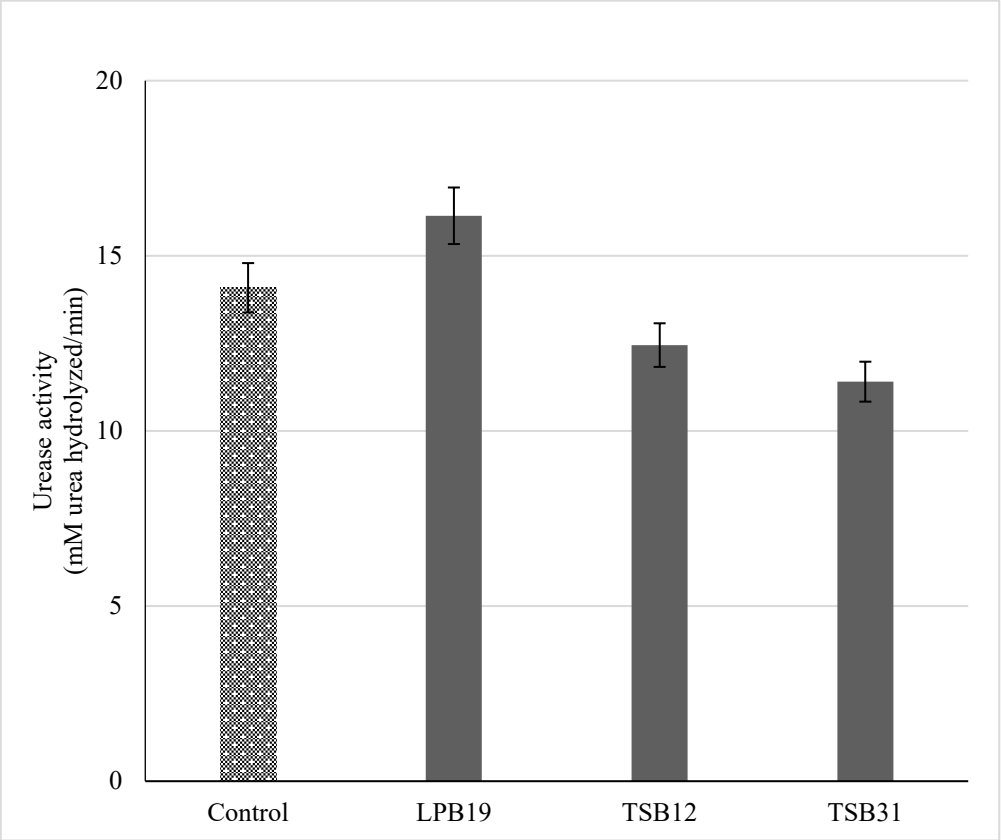

Figure 2. Measurement of urease activity. Error bars represent standard error of the mean.

The present study reports the isolation of urease-producing bacteria from samples collected from Sarawak limestone caves. The experiment indicates the presence of urease-producing bacteria in the cave sample. The urease activity detected suggests the potential use of the isolates in biocementation. Further works such as molecular identification and characterization of the enzyme need to be done to further understand the role of urease producing bacteria in the cave environment.

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