Ureolytic activities of a urease-producing bacterium and purified urease enzyme in the anoxic condition: Implication for subseafloor sand production control by microbially induced carbonate precipitation (MICP)

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Abstract: Microbially induced carbonate precipitation (MICP) involves the hydrolysis of urea by indigenous or introduced urease-producing bacteria, which induces carbonate precipitation. By allowing this process to occur in the pores of unconsolidated sand, sand particles bond together, creating a sandstone like material. Although MICP has been explored recently for possible applications in civil and construction engineering, this study examines its application to sand production control during hydrate gas exploitation from subseafloor sediments. The major uncertainty is the ureolytic activities of bacteria and associated enzyme under the subseafloor condition. The main aim of this study was to quantify the ureolytic efficiency of a urease-producing bacterium and purified urease enzyme in the oxic and anoxic conditions. The purified urease enzyme and *B. megaterium* were subject to bench shaking ureolyic activity tests in both conditions. Biochemical parameters including urea concentration, electric conductivity (EC), pH, and optical density at 600 nm (OD_{600}) of the solution at different time intervals were measured. As a quality control procedure, dissolved oxygen concentration (DO) of the final solutions was also measured. Results show that the effect of oxygen availability on ureolytic efficiency of purified urease enzyme is marginal. However, anoxic ureolytic performance of *B. megaterium* is better than its oxic counterpart. It is also found that higher concentration of urease and multi-amendment of bacteria help raise ureolytic efficiency. In order to sustain ureolytic efficiency and facilitate its up-scaled field application, several practice measures can be implemented including growing bacteria aerobically to exponential stage before implemented into the subseafloor sites, injecting larger bacteria cell number, and repeatedly supplying fresh bacteria cells.

Key words: microbially induced carbonate precipitation; sand production; *B. megaterium*; urease enzyme.

1. Introduction

Sand production has been a major obstacle for the successful exploitation of weakly consolidated /unconsolidated oil and gas reservoirs worldwide. It is reported that 70% of the global hydrocarbon reservoirs are susceptible to sand production (Fattahpour et al. 2012). Typically, sand production is defined as sand particles in weakly consolidated subsea hydrocarbon-bearing sediments moving into the exploitation well along with the hydrocarbon and water flows, due to drilling and completion activities. The detachment of particles are usually induced by the combination of high pore fluid velocity and material degradation behaviour (Rahmati et al. 2013). If it were to occur, sand production could result in troubles such as plugging of the perforations or production liner, wellbore instability, failure of sand control completions, and pipelines and surface facilities erosion (Rahmati et al. 2013). Several sand production control approaches have been developed by the petroleum industry and academia. These include the construction of sand screen, injection of chemical inhibitors, and setting up solid-fluid separation system. However, there is always a demand for more efficient, economic and durable solution for sand production control.

Recently microbially induced carbonate precipitation (MICP), a bacteria-generated bio-mineralization process, has been investigated extensively in geotechnical and environmental applications (Cuthbert et al. 2013; Jiang et al. 2014; Montoya et al. 2013; Al Qabany and Soga 2013; Soon et al. 2014). The hydrolysis of urea by indigenous or introduced urease-producing bacteria (e.g., *Sporosarcina pasteurii* (*S. pasteurii*), *Sporosarcina aquimarina* (*S. aquimarina*) and *Bacillus megaterium* (*B. megaterium*)) is one of the most popular pathways used to induce carbonate precipitation (Hata et al.

2013; Soon et al. 2013). By allowing this process to occur in the pores of unconsolidated sand, sand particles bond together, creating a sandstone like material.

The carbonate precipitation via ureolysis involves several stages: synthesis of urease enzyme through bacteria metabolic activities (Krajewska 2009); formation of ammonia (NH_3) and dissolved inorganic carbon (DIC) after urea catalysed by urease enzyme (eq. 1); increase in alkalinity at the proximity of bacteria cells (eq. 2 and 3); formation of carbonate precipitation on bacteria cell surfaces in the presence of available calcium source (eq. 4) (Ferris et al. 2004).

$$(NH_2)_2CO + H_2O \rightarrow 2NH_3 + CO_2 \quad (1)$$

$$2NH_3 + 2H_2O \leftrightarrow 2NH_4^+ + 2OH^- \quad (2)$$

$$CO_2 + 2OH^- \leftrightarrow HCO_3^- + OH^- \leftrightarrow CO_3^{2-} + H_2O \quad (3)$$

$$Ca^{2+} + CO_3^{2-} \leftrightarrow CaCO_3\,(s) \quad (4)$$

The distribution of produced carbonate precipitation has a preference around particle-particle contacts, which is primarily attributed to the microbe's preference to remain away from exposed particle surfaces and stay near smaller surface features (DeJong et al. 2010). Therefore, the particle-particle contacts contribute to stronger cementation within soils. Past studies show that MICP technique has the following highlighted features: (1) Enhancing soil strength and stiffness (Montoya et al. 2013; Al Qabany and Soga 2013); (2) Retaining soil permeability (Martinez et al. 2013; Whiffin et al. 2007); (3) Creating expanded treatment zone (Martinez et al. 2013); (4) Fast bio-geochemical reaction rate (Martin et al. 2012).

The cementation tends to occur at particles and hence the pore spaces are kept open (DeJong et al. 2010). Therefore, MICP-treated sand provides resistance to erosion, but keeps the flow characteristics (i.e. permeability) similar to the original state for oil/gas

production. This unique characteristic of MICP technique can benefit for the subseafloor sand production control, provided necessary technical issues are addressed.

The main issue for the application of MICP in the deep sea conditions is the degree of ureolysis activity of bacteria and pure enzyme at low temperature, high pressure and limited oxygen supply conditions. Hence, the primary objective of this study was to investigate the ureolytic activities of urease-producing bacteria and urease enzyme in oxic and anoxic conditions. The commercially purified urease enzyme and *B. megaterium* were subject to bench shaking ureolytic activity tests in both oxic and anoxic conditions. Biochemical parameters including urea concentration, electric conductivity (EC), pH, and optical density at 600 nm (OD_{600}) of the solution at different time intervals were measured. As a quality control procedure, dissolved oxygen concentration (DO) of the final solutions was also measured. By employing these variables, the ureolysis capacities of both purified urease enzyme and *B. megaterium* in the anoxic condition were assessed against in the oxic condition. It should be noted that, in this study, no cementation reagents were amended into the bacteria solution afterwards. This is to eliminate interference from precipitating calcium, as only ureolysis efficiency was under investigation. The process of calcite precipitation in deep sea conditions will be presented in future publication.

2. Materials and Methods

2.1 Bacteria, Enzyme and Culture Media

The investigation involves two series of tests. The first test series involve examination of activities of purified urease enzyme in oxic and anoxic conditions,

whereas the second test results examination of ureolytic activities of urease-producing bacteria in oxic and anoxic conditions.

Urease enzyme is often found naturally in algae, fungi, bacteria, plants, and invertebrates (Krajewska 2009). Commercially, urease has been commonly manufactured through beans purified from the jack bean meal. In this study, purified urease enzyme was supplied by Kishida Chemical, Osaka, Japan, which has an enzyme activity of 2950 U/g (Neupane et al. 2013). The investigation of purified urease enzyme in this work is based on the following two considerations: (1) the fundamental mechanism of MICP is ureolysis by urease enzyme regardless of originating from bacteria or industrial production; (2) the use of purified urease enzyme (instead of urease-producing bacteria) could be an alternative and straightway pathway to trigger carbonate precipitation.

In this study, *B. megaterium* (ATCC 14581) is used as the urease-producing microbe species. It is a Gram positive, rod-shaped soil bacterium with size ranging from 2 to 5 μm (Lian et al. 2006). Although past research has shown that *B. megaterium* has a relatively lower ureolysis rate than *S. pasteurii* (Bachmeier et al. 2002; Whiffin 2004), the selection of *B. megaterium* is more relevant to this study, as it is used under the deep-sea conditions. This is due to the reason that *B. megaterium* can form endospores that are highly resistant to extreme environmental conditions. More specifically: a) *B. megaterium* can grow at temperatures from 3 °C to 45 °C (Vos et al. 2009). It means that it can be potentially used at low temperature under deep sea condition while also adaptive to the heating environment during hydrate dissociation; b) *B. megaterium* has the ability to grow on many carbon sources even including some waste (Vary 1994); c) *B. megaterium* is found to be able to survive toxic environments and may have potential

as a detoxifying agent (Vary 1994). d) The large and elongated rod-shaped *B. megaterium* cell may provide the advantage of avoiding being flushed out during depressurization process during hydrate dissociation. Considering the adaptability of *B. megaterium* in the severe environment, it is a more reliable decision to use *B. megaterium* as a potential candidate for MICP application under deep sea conditions.

The culture media used in this study for the harvest of *B. megaterium* is ATCC-Medium 3. In the initial stage, freeze-dried culture was rehydrated in the nutrient broth solution, which consisted of 8.0 g nutrient broth in 1 L distilled water and had been autoclaved at 121ºC. Then, the rehydrated bacteria cells were grown on a plate which also contained nutrient agar (23g in 1 L distilled water, sterilized at 121 ºC) at 20 ºC overnight. Afterwards, a single colony was transferred to the liquid media solution, which contained 8 g/L nutrient broth and 5 g/L NaCl. The bacteria solution was then harvested in a constant-temperature incubator until a final OD_{600} of 0.1 was achieved.

2.2 Shaking Ureolytic Activity Test

The ureolytic activities of both purified urease enzyme and *B. megaterium* were investigated via the bench shaking test at constant ambient temperature of 20 ºC. The schematic of the test procedures are shown in **Fig. 1**. For the oxic case, either purified urease enzyme in powder or bacteria solution were added into sterile Erlenmeyer flasks, which had been filled with 100 mL urea solution (for urease) or liquid media solution with urea (for bacteria solution). The flasks were then stoppered with foam plugs. For the anoxic case, either purified urease enzyme in powder or bacteria solution were added into sterile crimp vials, which had been filled with 100 mL oxygen-free urea solution (for urease) or oxygen-free liquid media solution with urea (for bacteria

solution). The crimp vials were then quickly crimp-sealed with sterile butyl septa and subject to a further deoxygenation using oxygen-free N_2 and CO_2. After the mixture of purified urease enzyme or bacteria solution with urea, the Erlenmeyer flasks and sealed crimp vials were subject to bi-directionally horizontal shaking at 140 rpm. In some bacteria cases, bacteria solution was amended into solutions with additional deoxygenation again at 1, 2, 3, 6, 12, and 24 hours after the start of shaking. This multi-amendment mode is regarded as a possible enhancement method based on current engineering practice. Actually, there were already a few field trials of MICP technique (van Paassen et al. 2010; Gomez et al. 2015). In particular, Gomez et al. (2015) applied the bacteria solution every four days for five identical cycles in the field implementation of MICP for improving loose sand. This bacteria implementation method is very similar to the "multi-amendment" mode adopted in this study.

It should be noted that 0.1 mL of resazurin solution (0.1 g/L) was also added into test vessels as an oxidation-reduction indicator. When oxygen content is poor or expelled, the colour of solution changes from the blue to the pink.

During the shaking, solution samples were taken for various biochemical measurements at 1, 2, 3, 6, 12, and 24 hours (36 hours for bacteria case) after the start of shaking. The entire test terminated after 24 hours of shaking for the purified urease enzyme case and 36 hours for the bacteria case. As a quality control procedure, DO of the final solutions was also measured. The detailed experimental conditions are summarized in **Table 1**, showing the various amounts of urease, bacteria and urea in the solutions, oxygen availability, and amendment mode used in this study. In particular, the purified urease concentrations reported in this paper has also been used by other research in MICP applications (Fidaleo and Lavecchia 2003; Yasuhara et al. 2012;

Neupane et al. 2013), indicating that these concentrations are feasible for normal MICP implementations. To ensure the repeatability of the test results, all experimental cases were conducted in triplicate.

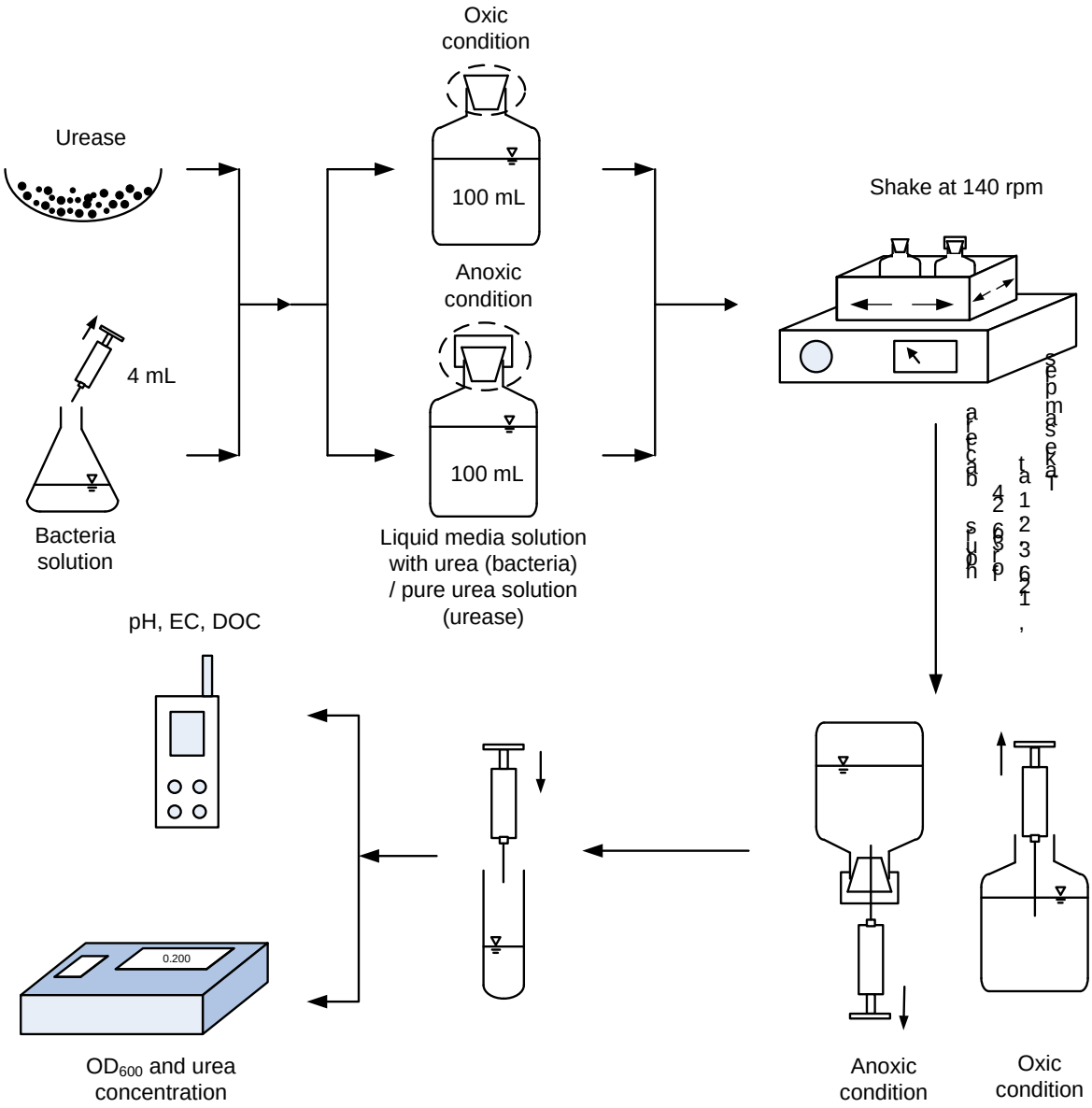

Fig. 1 Schematic of bench shaking test

Table 1 Experimental conditions of bench shaking ureolytic activity tests

Sample	Oxygen availability	Amount of urease (g/L)	Amount of bacteria solution (mL)	Initial urea concentration (mM)	Amendment mode
E-AT-2	Oxic	2	N.A. [c]	500	S [a]
E-AT-4	Oxic	4	N.A.	500	S
E-AT-8	Oxic	8	N.A.	500	S
E-AN-2	Anoxic	2	N.A.	500	S
E-AN-4	Anoxic	4	N.A.	500	S
E-AN-8	Anoxic	8	N.A.	500	S
B-AT-S	Oxic	N.A.	4	500	S
B-AT-M	Oxic	N.A.	4	500	M [b]
B-AN-S	Anoxic	N.A.	4	500	S
B-AN-M	Anoxic	N.A.	4	500	M

[a] Single-amendment at the start of test
[b] Multi-amendment at the start and then 1, 2, 3, 6, 12, and 24 hours after the start of test
[c] Not applicable

2.3 Urease enzyme degradation test

Urease enzyme degradation test is conducted to investigate the effect of urease enzyme degradation on the EC and pH profiles of the aqueous solutions. The purified urease enzyme concentration used for this test is 8 g/L. Firstly, the purified urease enzyme was dissolved in 100 mL distilled water under both oxic and anoxic conditions at three different ambient temperatures (i.e., 4, 20, 35 ºC). Triplicate samples were prepared to demonstrate the repeatability of the test results. Then, 1 mL of the solution was sampled at 1h, 3h (not for 4 ºC), 6h (not for 4 ºC), 1d, 2d, 3d (not for 4 ºC), 4d, 5d (not for 4 ºC), 6d, 7d (not for 4 and 35 ºC), 8d (not for 35 ºC), and 9 d (not for 4 and 35 ºC). The EC and pH values of the aqueous samples were then measured. The end of enzyme degradation is determined based on the termination of increase of EC or decrease of pH.

2.4 Monitoring methods

The biochemical variables measured in this study include pH, EC, DO, OD_{600}, and urea concentration. pH of solution was measured using a HORIBA LAQUAtwin Compact pH Meter, which has a precision of 0.1. EC is a good indicator of the ionic content in solution. It was measured using a HORIBA Compact Conductivity Meter, which has a minimum range of 1 μS/cm. DO is a sensitive variable indicating oxygen availability within the final solution. It was measured via HACH HQ40d portable Optical Dissolved Oxygen meter (LD0101 probe). OD_{600} is an indicator of bacteria concentration in solution and was measured at the wavelength of 600nm using a SmartSpec™ Plus Spectrophotometer. Urea concentration was measured using the colorimetric urea analysis method (Knorst et al. 1997). A solution (0.5 mL) containing 4% (w/v) of p-dimethylaminobenzaldehyde and 4% (v/v) sulphuric acid in absolute ethanol was added to 2mL of sample solutions in a cuvette. After 10 min, the absorbance of the solution was measured at 422nm against a reagent blank using a SmartSpec™ Plus Spectrophotometer. A calibration had been made between absorbance and standard urea solution before the test.

3. Results

Fig. 2 shows that magnitude of remaining urea concentration within the solution, which is the most straightforward indicator for the urea hydrolysis rate. In general, it can be observed that the pure enzyme (**Fig. 2(a)**) could trigger a much higher urea hydrolysis rate than the bacteria solution (**Fig. 2(b)**) regardless of oxygen availability, enzyme concentration and bacteria amendment modes.

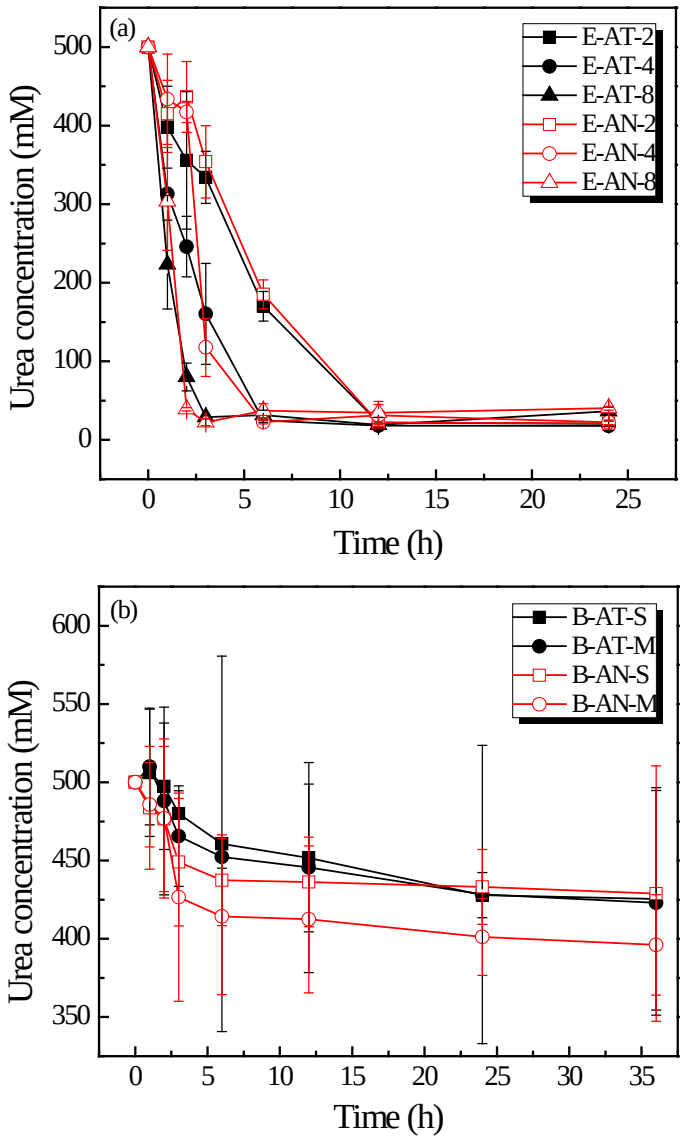

Fig. 2 Variations of the concentration of remaining urea in solution with time ((a). urease enzyme; (b). bacteria solution)

For the purified enzyme case (**Fig. 2(a)**), it is found that higher concentration of enzyme yielded faster urea degradation rate, (i.e., a higher ureolytic rate) (Yasuhara et al. 2012). This positive correlation between ureolytic rate and urease enzyme concentration is also confirmed by Fidaleo and Lavecchia (2003). On the other hand, the oxygen availability only had a marginal effect on the urea degradation rate, if the error bar is considered.

For the bacteria solution case (**Fig. 2(b)**), it is apparent that anoxic condition contributed to a lower remaining urea concentration in the solution, especially at the initial 12 hours. This indicates that ureolytic rate by bacteria solution in anoxic condition is faster than that in oxic one, regardless of amendment modes. Furthermore, in both oxic and anoxic conditions, urea degraded faster when multi-amendment of bacteria had been applied.

The time-dependent behaviours of solution EC for the urease enzyme case and the bacteria solution case are shown in **Fig. 3(a)** and **3(b)**, respectively. As the magnitude of EC reflects the amount of electrolytic ions in the solution, its evolution could demonstrate the magnitude and rate of ureolytic reactions as can be derived from **Equations (1), (2) and (3)**.

In both purified urease enzyme and bacteria cases, the values of EC in anoxic conditions were higher than those in oxic conditions at any time during the shaking tests. This phenomenon is possibly attributed to the following three factors: (a) the ureolytic rate by the purified urease enzyme was faster in anoxic conditions than in oxic conditions; (b) extra electrolytic substances other than ureolytic products were produced during the bacteria cell growth in the anoxic conditions; and (c) the carbonate (CO_3^{2-}) and bicarbonate (HCO_3^-) ions were made in the oxygen-free medium through bubbling with N_2 and CO_2.

Considering the results of the remaining urea concentration within the solution, it can be at least confirmed that, in the bacteria solution case, the higher ureolytic reaction rate in anoxic conditions contributes to the high EC magnitude. However, it is difficult to define whether the last two factors also contribute. Further confirmation can only be made when the pH results are also considered.

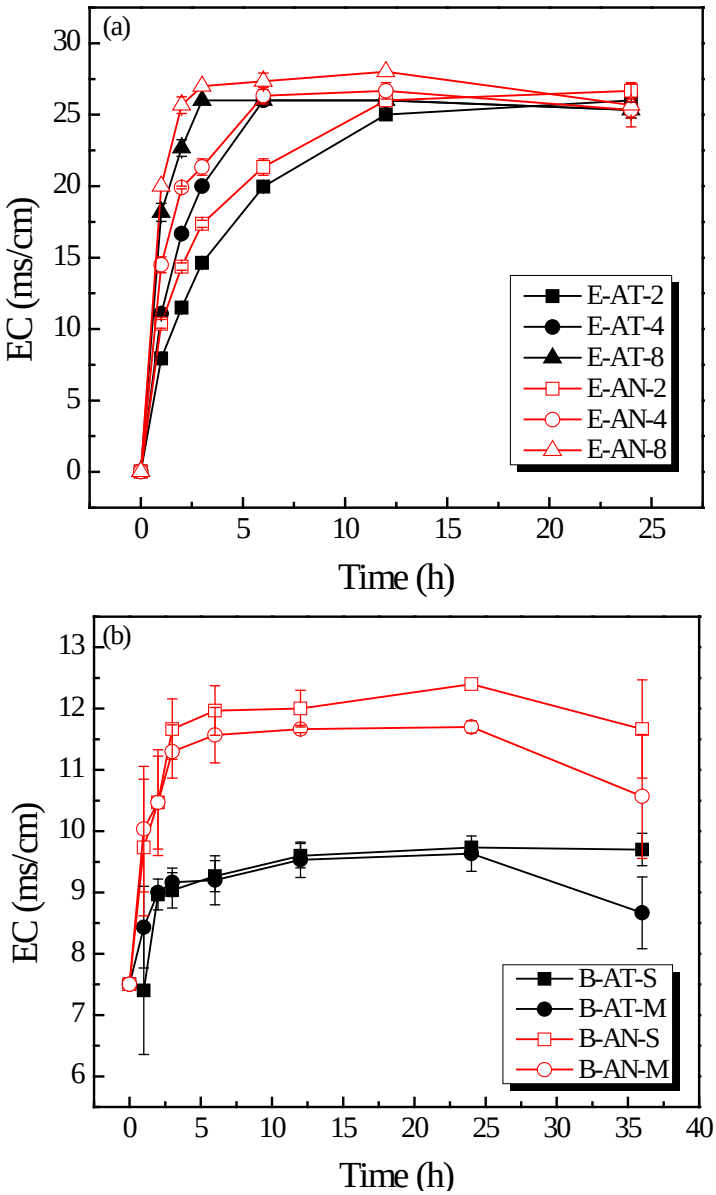

Fig. 3 Variations of solution EC with time ((a). urease enzyme; (b). bacteria solution)

For the purified urease enzyme case, since the ureolytic rate was found to be almost equivalent in both conditions and there cannot be extra cell-related substance synthesized by the purified enzyme, the high EC values in anoxic conditions could only be attributed to the CO_3^{2-} and HCO_3^- ions. As shown in **Fig. 3**, compared with the cases of low enzyme concentration and single-amendment of bacteria solutions, respectively,

the high enzyme concentration case and multi-amendment of bacteria solution case resulted in high values of EC at the same shaking time before the final equilibrium was reached. This is consistent with the results of remaining urea concentration with time, which is triggered by the high ureolytic rate as well.

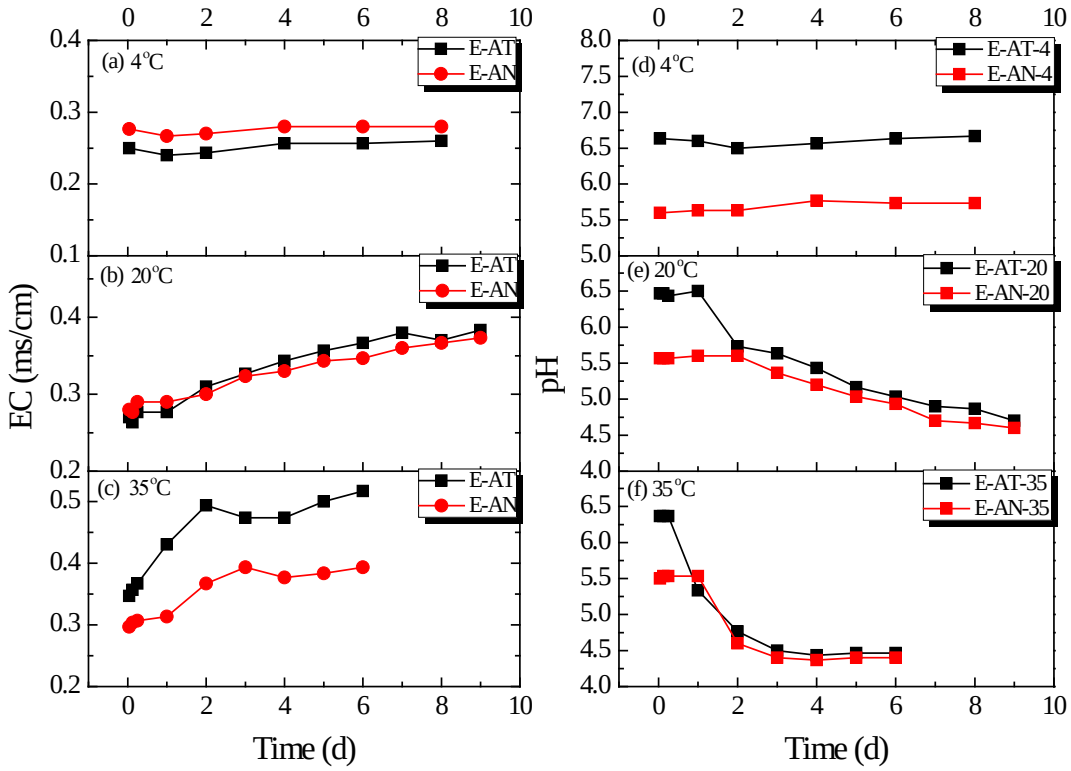

Fig. 4 Degradation behavior of purified urease enzyme (8 g/L) in distilled water at 4 °C, 20 °C and 35 °C

It should be noted that the urease enzyme itself may degrade with time, which could potentially contribute to the increase in EC values. Therefore, the degradation behavior (life-time) of the purified urease enzyme (8 g/L) in distilled water at 4 °C, 20 °C and 35 °C were characterized, as shown in **Fig. 4**. The end of enzyme degradation is determined based on the termination of increase of EC (**Fig. 4 (a), (b), (c)**) or decrease of pH (**Fig. 4 (d), (e), (f)**). It is found that at 4 °C, no distinguishable enzyme degradation occurs at least during the entire experimental period (8 days). At 20 °C and

35 °C, the life-time of the urease enzyme is found to be 7 and 3 days, respectively. Therefore, within the testing time (1d) in this study at 20 °C, the enzyme degradation should be insignificant. Moreover, it is found that the oxygen availability has no distinguishable influence on the degradation behavior of urease enzyme in distilled water. The measured life-time of urease enzyme in this study is consistent with those reported in the previous studies. For example at 25 °C, the reported life-time of urease can be 5 to 8 days (Krajewska et al. 1990; Petti et al. 1976). At 4 °C, its half-time can be 18 to 24 days (Krajewska et al. 1990; Petti et al. 1976).

In the urease enzyme degradation test, the EC is found to increase with enzyme degradation. However, the maximum observed EC increase is only around 0.2 ms/cm, which is significantly smaller than the variations of solution EC during the ureolytic activity test (**Fig. 3**). Therefore, it can be confirmed that the contribution of enzyme degradation on EC increase can be negligible.

The variations of solution pH are shown for the purified enzyme case and the bacteria case in **Figs. 5 (a)** and **5 (b)**, respectively. Regardless of oxic or anoxic conditions, solution pH increases sharply within the initial 1 hour. This is attributed to the immediate hydrolysis of urea (see **Equation (1), (2) and (3)**). Solution pH then increases only marginally or just keep constant, indicating that the aqueous system has reached equilibrium. The only exception is the B-AN case (Bacteria solution, anoxic), which experiences slightly reduction. This is attributed to the continuous production of acidic substances in the solution.

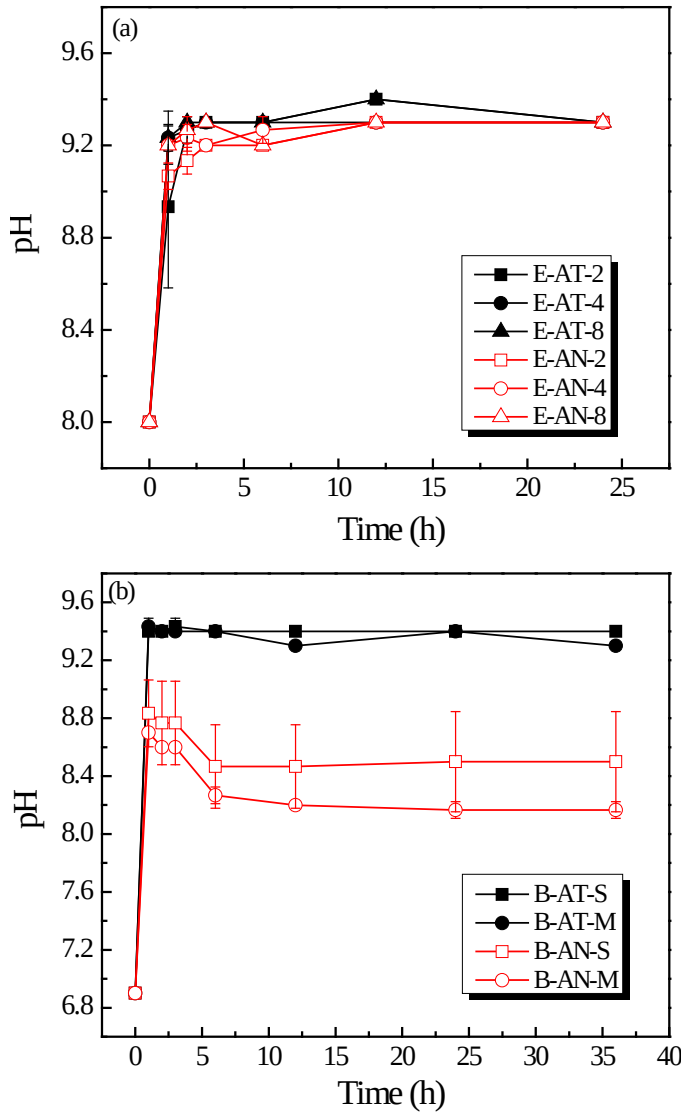

Fig. 5 Variations of solution pH with time ((a). urease enzyme; (b). bacteria solution)

In the purified urease enzyme case, the solution pH in anoxic conditions is only slightly lower than that in oxic ones. This is possibly caused by the CO_2 gas used for deoxygenation and is consistent with the EC results. In the bacteria case, however, the solution pH in anoxic conditions stabilizes around 8.2-8.5, which is significantly lower than that of 9.4 in oxic conditions. This suggests that not only the CO_3^{2-} and HCO_3^- ions but also extra acidic electrolytic substances synthesized by the bacteria (other than ureolysis-related ones) in anoxic conditions contribute to the low pH and high EC

values. Both Ferris et al. (2004) and Clancy and Burne (1997) found that bacteria cell growth during ureolysis process produced extra acidic substances. Results also show that a higher initial urease enzyme concentration yields faster increase in solution pH in the initial 2 hours, indicating a high ureolytic rate. This is consistent with the results of remaining urea concentration and EC values.

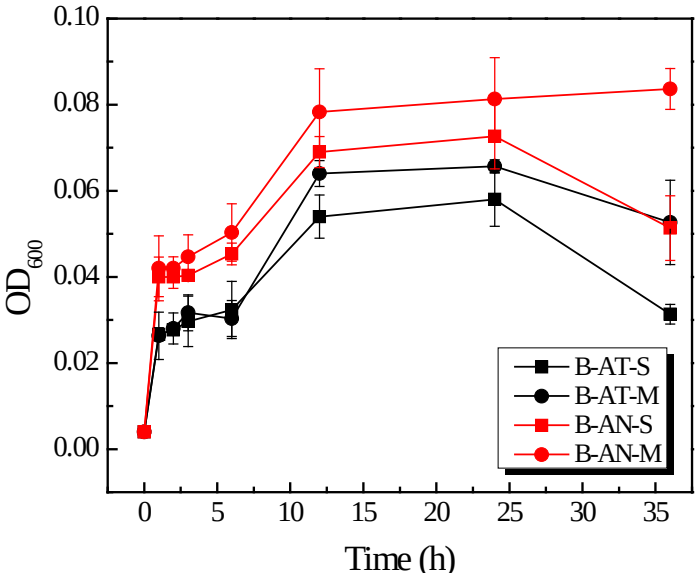

Fig. 6 Variations of the optical density at 600 nm (OD_{600}) in bacteria solution with time

In the bacteria case, the OD_{600}, an indicator of the bacteria cell number in the solution, was also monitored as shown in **Fig. 6**. In both oxic and anoxic conditions, the bacteria cell number increases steadily in the initial 24 hours, indicating that the growth of bacteria is active throughout this period. In the next 12 hours, however, the OD_{600} begins to decline, suggesting that the bacteria are in the death phase. In addition, anoxic conditions can stimulate a better growth of bacteria than oxic conditions. Moreover, although it is not so significant at the initial a few hours, multi-amendment is found to contribute to a higher bacteria cell number than single-amendment. The above two

findings are consistent with the observations of remaining urea concentration with time under different oxygen availability conditions. Therefore, it can be inferred that higher bacteria cell number in the aqueous system could help maintain a higher ureolysis rate.

It should be noted that although the OD_{600} values measured in this study is low compared to some previous studies, the low magnitudes of OD_{600} with accompanying significant ureolytic activity for ureolytic bacteria were also reported by Martin et al. (2012) for the other ureolytic bacterium (*S. pasteurii*), proving that the bacteria still undergo growth at the low OD_{600}. Actually, Lauchnor et al. (2015) reported a linear relationship between OD_{600} and ureolytic rate for *S. pasteurii*. A significant ureolytic activity can be still observed under low OD_{600} (the specific activity is around 30 mM OD^{-1} min^{-1}). In this study, the specific ureolytic activity for the *B. megaterium* was around 3.3 mM OD^{-1} min^{-1} in the initial 5 hours, which is similar to that reported by Jiang and Soga (2014) and is deemed as sufficient to induce calcite precipitation (Whiffin et al. 2004).

After the termination of all tests, the DO of the final solutions was measured as a quality control procedure to examine whether the relevant oxygen availability conditions were well maintained. The corresponding results are shown in **Table 2**. The magnitudes of DO range from 7.8 and 8.0 mg/L in oxic conditions while they were less than 1.0 mg/L in anoxic conditions. Typically, the DO values in open-air shallow water range between 9.07-7.54 mg/L at the temperature between 20 and 30 ºC. On the other hand, the DO values in the seawater in the ocean around the world can be even lower than 1.6 mg/L in the Oxygen Minimum Zone (OMZ) (Garcia et al. 2010). Also the DO values in marine sediments have a similar magnitude with those in the seawater (Fischer et al. 2009). Therefore, this confirms that the DO values measured in this study match

well to those reported elsewhere and the relevant oxygen availability conditions are well preserved. Thus, all analysis based on different oxygen availability conditions are considered to be valid in this study.

Table 2 Dissolved oxygen content (DO) in final solution

Sample	DO (mg/L)	
	Mean	SD [a]
E-AT-2	8.05	0.03
E-AT-4	7.99	0.023
E-AT-8	7.84	0.05
E-AN-2	0.93	0.09
E-AN-4	0.85	0.07
E-AN-8	0.73	0.01
B-AT-S	7.81	0.28
B-AT-M	7.86	0.04
B-AN-S	0.69	0.08
B-AN-M	0.49	0.02

[a] Standard deviation

4. Discussion

4.1 Effect of oxygen availability on ureolytic efficiency of purified urease enzyme

Results show that the ureolytic efficiency of purified urease enzyme is marginally influenced by the oxygen availability within sealed crimp vials. This confirms that the purified urease enzyme can work in anoxic conditions as efficient as in oxic conditions. Although the EC values are greater in anoxic conditions than in oxic conditions, it is believed that this is attributed to CO_3^{2-} and HCO_3^- ions, which introduce additional electrolytic ions into the solution.

The satisfactory performance of purified urease enzyme in anoxic conditions is fundamentally determined by its catalyzing reaction mechanism as well as its adaptative nature for various oxidation-reduction environments. A widely-accepted catalytic mechanism of urease enzyme was proposed in the 1980's, and this involves the binding and activation by two nickels within the urease structure (Benini et al. 1999; Karplus et

al. 1997). As summarised by Karplus et al. (1997), one nickel ion is used to bind and activate urea while the second one is used to activate water molecule and bind hydroxide. The protein itself provides a nearby carboxylate to stabilize a urea resonance form. Then, the second nickel with binded hydroxide is activated for attack on the urea carbon by a protein residue. This results in the collapse of the tetrahedral intermediate, thus eliminating ammonia with the help of an active site thiol. Through the whole ureolytic reaction process, oxygen does not play a role on the catalytic function of urease enzyme. Furthermore, several studies in the first half of the 20th century investigated the effects of various oxidization-reduction potentials on the ureolytic activity of urease enzyme. Fischgold (1934) found that the activity of purified urease enzyme was independent of several reversible oxidation-reduction systems in different potential scales. Sizer and Tytell (1941) further confirmed that the activity of crude urease from jack bean was unaffected by the oxidizing and reducing regents. This is likely to be attributed to the impurities present in the crude enzyme, which protects urease from reacting with oxidizing-reducing agents.

In addition, urease enzyme is one type of hydrolases. It has been well reported that oxidase is strongly influenced by oxygen availability but hydrolases are not affected by it (Freeman et al. 2001). This can also explain why urease lacks the response to the oxic and anoxic conditions.

Based on the experimental data as well as the previous studies, it is concluded that oxygen availability has no significant effect on the ureolytic efficiency of purified urease enzyme. That is, urease activity is independent of oxidization-reduction environment. The efficiency is determined by the fundamental molecule functions of urease enzyme in ureolysis process.

4.2 Effect of oxygen availability on ureolytic efficiency of B. megaterium

Based on the results shown in **Figs. 2 and 6**, *B. megaterium* displays a better ureolytic performance in anoxic conditions than in oxic conditions. In fact, the bacterial ureases are homologous to plant ureases (e.g., from jack beans) in terms of amino acid sequence, which results in similar protein structures and enzymatic catalytic mechanisms (Balasubramanian and Ponnuraj 2010). It is also found that the urease enzyme degradation is not affected by the oxygen availability at least at the temperature range between 4 °C and 35 °C (Fig. 4). Therefore, the effect of oxygen availability on ureolytic activity of bacterial urease enzyme should be marginal. If this is the case, the high ureolytic activity of *B. megaterium* in anoxic conditions is possibly attributed to: (1) high urease synthesis capacity and (2) the effect of aqueous solution pH on ureolytic activity.

There is no report with regards to the urease synthesis capacity of *B. megaterium*. However, there are some published data on the urease synthesis capacity of *S. pasteurii*, which is a commonly used urease-producing bacterium. Tobler et al. (2011) show that, if *S. pasteurii* is grown in large numbers aerobically in nutrient-rich conditions and then injected into anoxic oligotrophic groundwater, its ureolytic efficiency has no difference compared with that in oxic conditions at least for the initial 1.2 days. Mortensen et al. (2011) report that the ureolytic rate of *S. pasteurii* pellet incubated anaerobically had a roughly 1.5-fold of that in aerobic conditions. This is possibly due to the lysis of bacteria cells in anoxic conditions and direct release of enzyme from inside cytoplasm. On the other hand, Martin et al. (2012) state that the de novo synthesis of urease enzyme is impossible for *S. pasteurii* under anoxic conditions. The ureolytic activity in

448 the oxic condition is only due to the urease enzyme which has already been produced in
449 the aerobically grown inoculum. Soon, metabolism inhibition, enzyme degradation and
450 cell death could lead to the irreversible decline of urease enzyme activity in the oxic
451 condition.

452 Both *B. megaterium* and *S. pasteurii* are urease-producing aerobes and hence their
453 responses to anoxic conditions are considered to be similar. In this study, *B. megaterium*
454 was cultivated aerobically to the exponential stage before being transferred to anoxic
455 environment. Thus, a substantial amount of active urease enzyme was already within the
456 bacteria solution. If other possibilities (e.g., lysis of cells in anoxic conditions) are
457 accounted for, it is possible that more active urease enzyme are available in the solution,
458 contributing to greater ureolytic activity in anoxic conditions than in oxic conditions. A
459 study that provides a direct phenomenological evidence (such as microscopic
460 observations) is needed to confirm the aforementioned mechanism.

461 Aqueous solution pH can influence ureolytic activity. As shown in **Fig. 5(b),** the pH
462 of the bacteria solution in anoxic conditions is less than 8.8, whereas that in oxic
463 conditions is higher than 9.3. Hence, the effect of lower alkalinity in anoxic conditions
464 on a higher ureolytic activity needs to be examined. Howell and Sumner (1934) verified
465 that the ureolytic activity of plant urease peaked around pH of 6.5~7.5 and dropped fast
466 with further alkalinity. Achal (2010) reports that the bacterial urease enzyme produced
467 by *B megaterium* has a similar urease activity between pH = 8 and 9. Paladino (2009)
468 suggests that the optimal growth pH for growth of *B megaterium* is around 6.0.
469 Krulwich et al. (2011) state that *B megaterium* is not alkaliphilic bacteria, which
470 suggests that its optimal growth pH is between 5 and 9. In this study, bacteria cell
471 number was greater under lower pH condition (i.e. anoxic case), as shown in **Fig. 6**. The

lower pH in anoxic conditions could promote more synthesis of urease enzyme by *B megaterium*, resulting in more active urease enzyme.

In summary, the ureolytic efficiency of *B. megaterium* in anoxic conditions is greater than that in oxic conditions. This is attributed to the presence of more available active urease enzyme and higher ureolytic activity. The fundamental mechanism of this could be large amount of existing urease from initial aerobic growth, lysis of cells to release enzyme direct from inside cytoplasm, and the effect of aqueous solution pH.

4.3 Ureolytic efficiency of purified urease enzyme and B. megaterium

Results in **Figs. 2, 3, and 5** indicate that the purified urease enzyme can trigger higher ureolytic efficiency than *B. megaterium*. However, it is difficult to conclude that this difference is attributed to the different types of urease involved. Bacterial and plant ureases have high amino acid sequence similarity and hence similar catalytic mechanisms. If the reaction environment and the urease concentration are the same, similar ureolytic rates are expected. It is also difficult to conclude that the difference results from the discrepancy between viable cells and free enzyme outside cells. Mortensen et al. (2011) show that the urease activity of free enzymes and the viable cells are equivalent. In this study, it is hypothesized that this difference between the purified urease enzyme and *B. megaterium* is attributed to the difference in the concentration of available active urease enzyme in the aqueous system. Actually, if the concentration of purified urease enzyme is reduced, it is possible that the bacteria solution could be superior to the purified urease enzyme in terms of ureolytic activity. Further study is needed to quantify the time-dependent synthesized enzyme concentration in *B. megaterium* during testing.

5. Conclusions

In this study, the ureolytic activities of purified urease enzyme and *B. megaterium* were investigated in both anoxic and oxic conditions for their potential application in subseafloor sand production control. The following conclusions are drawn:

(1) The test results confirm that the effect of oxygen availability on ureolytic efficiency of purified urease enzyme is marginal. This is attributed to the fundamental molecule functions of urease enzyme in ureolysis process and the independence of urease activity on diverse oxidization-reduction environment.

(2) Based on the data of remaining urea concentration, EC, pH, and OD_{600}, the ureolytic activity of *B. megaterium* in anoxic conditions is greater than that in oxic conditions. This is attributed to the presence of more available active urease enzyme and higher ureolytic activity of urease enzyme. The fundamental mechanisms could be large amount of existing urease from initial aerobic growth, lysis of cells, and the effect of aqueous solution pH.

(3) The ureolytic efficiency of purified urease enzyme was greater than that of *B. megaterium.* This is likely due to difference in the concentration of available reactive urease enzyme in the aqueous system.

The test results obtained from this study indicate that it is feasible to use *B. megaterium* in anoxic conditions to induce controlled carbonate precipitation for subseafloor sand production control. In order to sustain ureolytic efficiency and facilitate its up-scaled field application, potential practice measures can be implemented including growing bacteria aerobically to exponential stage before implemented into the

519 subseafloor sites, injecting larger bacteria cell number, and repeatedly supplying fresh
520 bacteria cells.

521 Further work is currently conducted to facilitate this engineering application as
522 separate study. This includes: (i) the effect of anoxic environment on the precipitation
523 efficiency, (ii) the performance of the bacteria under low-temperature and high-pressure
524 conditions, and (iii) the interaction between bacteria and carbohydrate fuel.

525

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535

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