

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

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25 **Abstract:** Microbially induced carbonate precipitation (MICP) involves the hydrolysis
26 of urea by indigenous or introduced urease-producing bacteria, which induces carbonate
27 precipitation. By allowing this process to occur in the pores of unconsolidated sand,
28 sand particles bond together, creating a sandstone like material. Although MICP has
29 been explored recently for possible applications in civil and construction engineering,
30 this study examines its application to sand production control during hydrate gas
31 exploitation from subseafloor sediments. The major uncertainty is the ureolytic
32 activities of bacteria and associated enzyme under the subseafloor condition. The main
33 aim of this study was to quantify the ureolytic efficiency of a urease-producing
34 bacterium and purified urease enzyme in the oxic and anoxic conditions. The purified
35 urease enzyme and *B. megaterium* were subject to bench shaking ureolytic activity tests
36 in both conditions. Biochemical parameters including urea concentration, electric
37 conductivity (EC), pH, and optical density at 600 nm (OD₆₀₀) of the solution at different
38 time intervals were measured. As a quality control procedure, dissolved oxygen
39 concentration (DO) of the final solutions was also measured. Results show that the
40 effect of oxygen availability on ureolytic efficiency of purified urease enzyme is
41 marginal. However, anoxic ureolytic performance of *B. megaterium* is better than its
42 oxic counterpart. It is also found that higher concentration of urease and multi-
43 amendment of bacteria help raise ureolytic efficiency. In order to sustain ureolytic
44 efficiency and facilitate its up-scaled field application, several practice measures can be
45 implemented including growing bacteria aerobically to exponential stage before
46 implemented into the subseafloor sites, injecting larger bacteria cell number, and
47 repeatedly supplying fresh bacteria cells.

48

49 **Key words:** microbially induced carbonate precipitation; sand production; *B.*
50 *megaterium*; urease enzyme.
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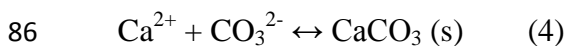
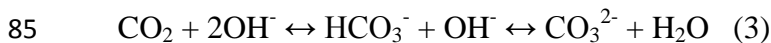
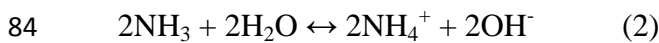
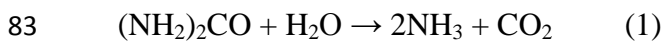
52 **1. Introduction**

53 Sand production has been a major obstacle for the successful exploitation of weakly
54 consolidated /unconsolidated oil and gas reservoirs worldwide. It is reported that 70%
55 of the global hydrocarbon reservoirs are susceptible to sand production (Fattahpour et al.
56 2012). Typically, sand production is defined as sand particles in weakly consolidated
57 subsea hydrocarbon-bearing sediments moving into the exploitation well along with the
58 hydrocarbon and water flows, due to drilling and completion activities. The detachment
59 of particles are usually induced by the combination of high pore fluid velocity and
60 material degradation behaviour (Rahmati et al. 2013). If it were to occur, sand
61 production could result in troubles such as plugging of the perforations or production
62 liner, wellbore instability, failure of sand control completions, and pipelines and surface
63 facilities erosion (Rahmati et al. 2013). Several sand production control approaches
64 have been developed by the petroleum industry and academia. These include the
65 construction of sand screen, injection of chemical inhibitors, and setting up solid-fluid
66 separation system. However, there is always a demand for more efficient, economic and
67 durable solution for sand production control.

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

75 2013; Soon et al. 2013). By allowing this process to occur in the pores of
76 unconsolidated sand, sand particles bond together, creating a sandstone like material.

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



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

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

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

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

117

118 **2. Materials and Methods**

119 *2.1 Bacteria, Enzyme and Culture Media*

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

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

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

134 In this study, *B. megaterium* (ATCC 14581) is used as the urease-producing
135 microbe species. It is a Gram positive, rod-shaped soil bacterium with size ranging from
136 2 to 5 μm (Lian et al. 2006). Although past research has shown that *B. megaterium* has a
137 relatively lower ureolysis rate than *S. pasteurii* (Bachmeier et al. 2002; Whiffin 2004),
138 the selection of *B. megaterium* is more relevant to this study, as it is used under the
139 deep-sea conditions. This is due to the reason that *B. megaterium* can form endospores
140 that are highly resistant to extreme environmental conditions. More specifically: a) *B.*
141 *megaterium* can grow at temperatures from 3 °C to 45 °C (Vos et al. 2009). It means
142 that it can be potentially used at low temperature under deep sea condition while also
143 adaptive to the heating environment during hydrate dissociation; b) *B. megaterium* has
144 the ability to grow on many carbon sources even including some waste (Vary 1994); c)
145 *B. megaterium* is found to be able to survive toxic environments and may have potential

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

151 The culture media used in this study for the harvest of *B. megaterium* is ATCC-
152 Medium 3. In the initial stage, freeze-dried culture was rehydrated in the nutrient broth
153 solution, which consisted of 8.0 g nutrient broth in 1 L distilled water and had been
154 autoclaved at 121°C. Then, the rehydrated bacteria cells were grown on a plate which
155 also contained nutrient agar (23g in 1 L distilled water, sterilized at 121 °C) at 20 °C
156 overnight. Afterwards, a single colony was transferred to the liquid media solution,
157 which contained 8 g/L nutrient broth and 5 g/L NaCl. The bacteria solution was then
158 harvested in a constant-temperature incubator until a final OD₆₀₀ of 0.1 was achieved.

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160 2.2 Shaking Ureolytic Activity Test

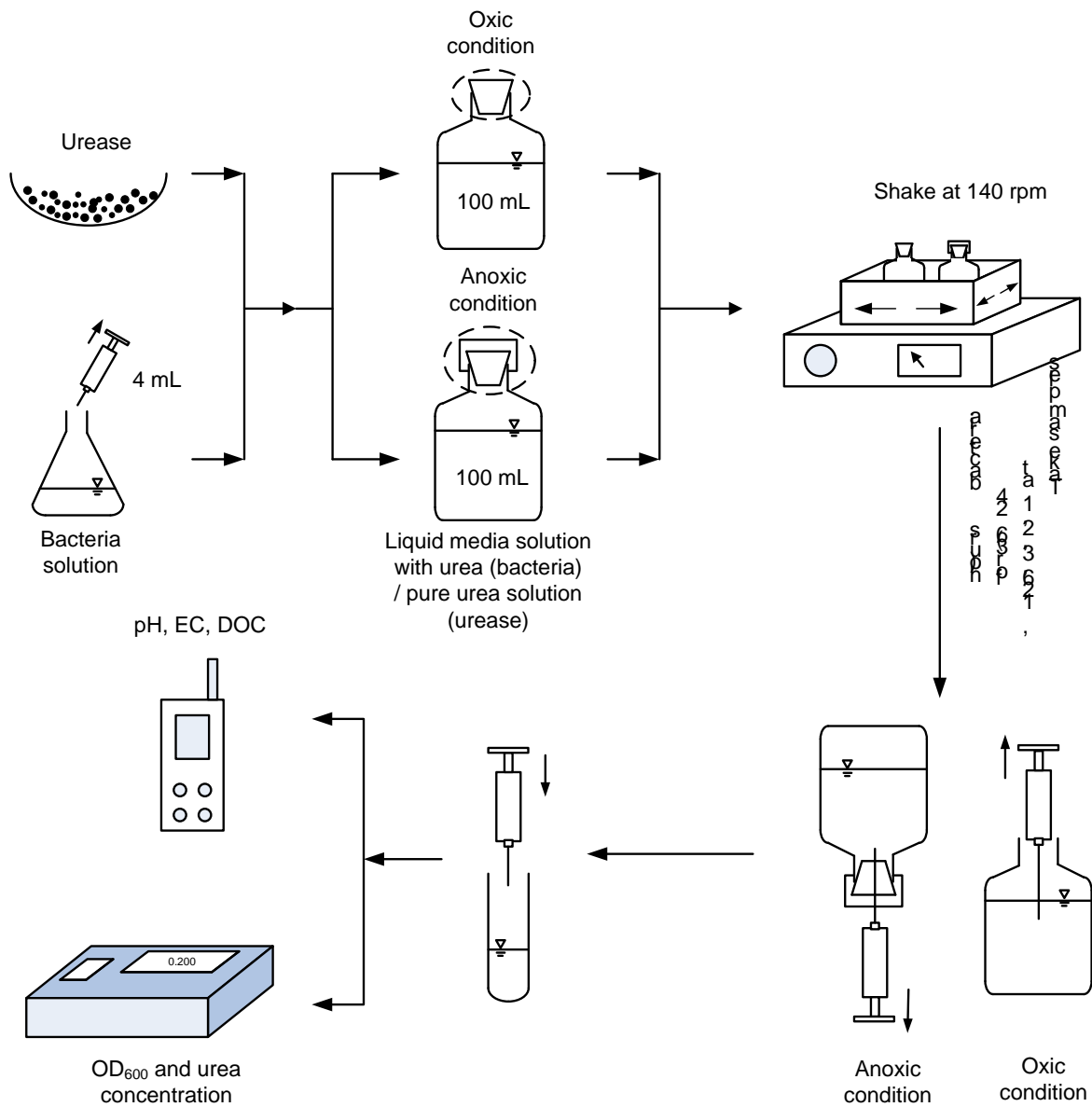
161 The ureolytic activities of both purified urease enzyme and *B. megaterium* were
162 investigated via the bench shaking test at constant ambient temperature of 20 °C. The
163 schematic of the test procedures are shown in **Fig. 1**. For the oxic case, either purified
164 urease enzyme in powder or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

170 solution). The crimp vials were then quickly crimp-sealed with sterile butyl septa and
171 subject to a further deoxygenation using oxygen-free N₂ and CO₂. After the mixture of
172 purified urease enzyme or bacteria solution with urea, the Erlenmeyer flasks and sealed
173 crimp vials were subject to bi-directionally horizontal shaking at 140 rpm. In some
174 bacteria cases, bacteria solution was amended into solutions with additional
175 deoxygenation again at 1, 2, 3, 6, 12, and 24 hours after the start of shaking. This multi-
176 amendment mode is regarded as a possible enhancement method based on current
177 engineering practice. Actually, there were already a few field trials of MICP technique
178 (van Paassen et al. 2010; Gomez et al. 2015). In particular, Gomez et al. (2015) applied
179 the bacteria solution every four days for five identical cycles in the field implementation
180 of MICP for improving loose sand. This bacteria implementation method is very similar
181 to the “multi-amendment” mode adopted in this study.

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

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

194 Neupane et al. 2013), indicating that these concentrations are feasible for normal MICP
 195 implementations. To ensure the repeatability of the test results, all experimental cases
 196 were conducted in triplicate.
 197



198
 199 **Fig. 1** Schematic of bench shaking test

200
 201
 202

203 **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

204 ^a Single-amendment at the start of test

205 ^b Multi-amendment at the start and then 1, 2, 3, 6, 12, and 24 hours after the start of test

206 ^c Not applicable

207

208 *2.3 Urease enzyme degradation test*

209 Urease enzyme degradation test is conducted to investigate the effect of urease
 210 enzyme degradation on the EC and pH profiles of the aqueous solutions. The purified
 211 urease enzyme concentration used for this test is 8 g/L. Firstly, the purified urease
 212 enzyme was dissolved in 100 mL distilled water under both oxic and anoxic conditions
 213 at three different ambient temperatures (i.e., 4, 20, 35 °C). Triplicate samples were
 214 prepared to demonstrate the repeatability of the test results. Then, 1 mL of the solution
 215 was sampled at 1h, 3h (not for 4 °C), 6h (not for 4 °C), 1d, 2d, 3d (not for 4 °C), 4d, 5d
 216 (not for 4 °C), 6d, 7d (not for 4 and 35 °C), 8d (not for 35 °C), and 9 d (not for 4 and 35
 217 °C). The EC and pH values of the aqueous samples were then measured. The end of
 218 enzyme degradation is determined based on the termination of increase of EC or
 219 decrease of pH.

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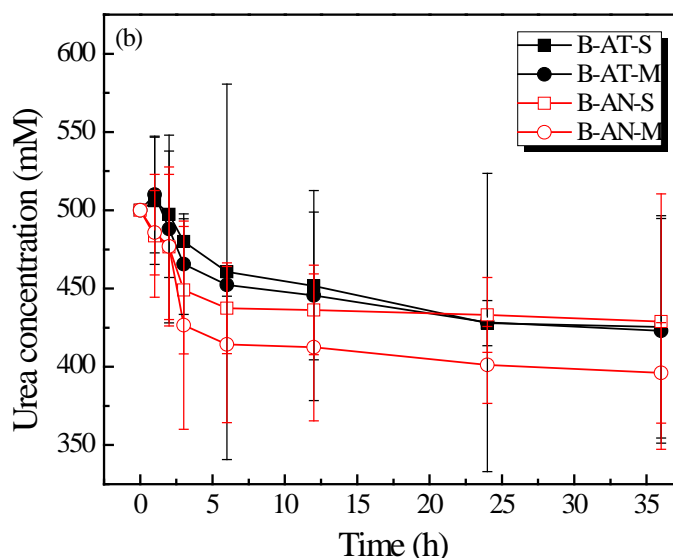
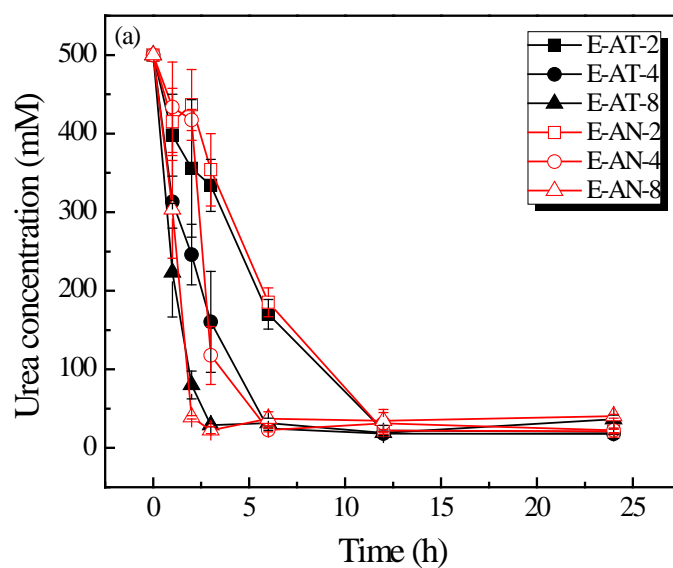
221 *2.4 Monitoring methods*

222 The biochemical variables measured in this study include pH, EC, DO, OD₆₀₀, and
223 urea concentration. pH of solution was measured using a HORIBA LAQUAtwin
224 Compact pH Meter, which has a precision of 0.1. EC is a good indicator of the ionic
225 content in solution. It was measured using a HORIBA Compact Conductivity Meter,
226 which has a minimum range of 1 μ S/cm. DO is a sensitive variable indicating oxygen
227 availability within the final solution. It was measured via HACH HQ40d portable
228 Optical Dissolved Oxygen meter (LD0101 probe). OD₆₀₀ is an indicator of bacteria
229 concentration in solution and was measured at the wavelength of 600nm using a
230 SmartSpec™ Plus Spectrophotometer. Urea concentration was measured using the
231 colorimetric urea analysis method (Knorst et al. 1997). A solution (0.5 mL) containing
232 4% (w/v) of p-dimethylaminobenzaldehyde and 4% (v/v) sulphuric acid in absolute
233 ethanol was added to 2mL of sample solutions in a cuvette. After 10 min, the
234 absorbance of the solution was measured at 422nm against a reagent blank using a
235 SmartSpec™ Plus Spectrophotometer. A calibration had been made between
236 absorbance and standard urea solution before the test.

237

238 3. Results

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



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

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

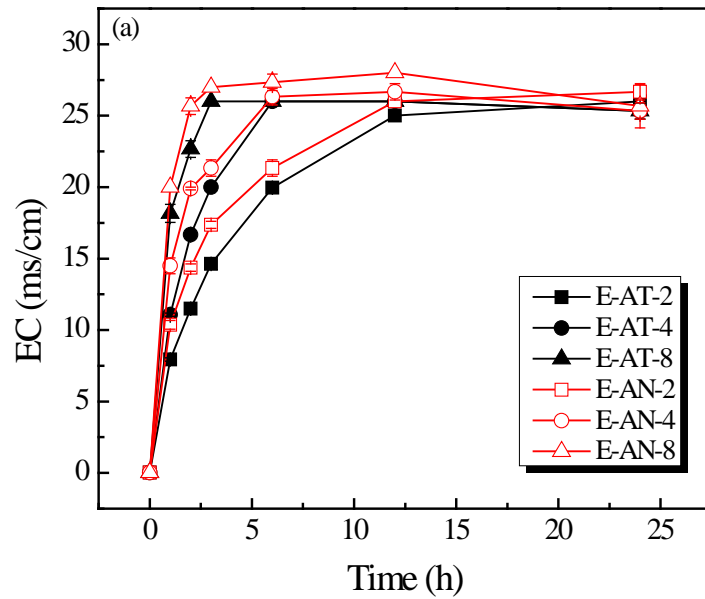
255 For the bacteria solution case (**Fig. 2(b)**), it is apparent that anoxic condition
256 contributed to a lower remaining urea concentration in the solution, especially at the
257 initial 12 hours. This indicates that ureolytic rate by bacteria solution in anoxic
258 condition is faster than that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

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

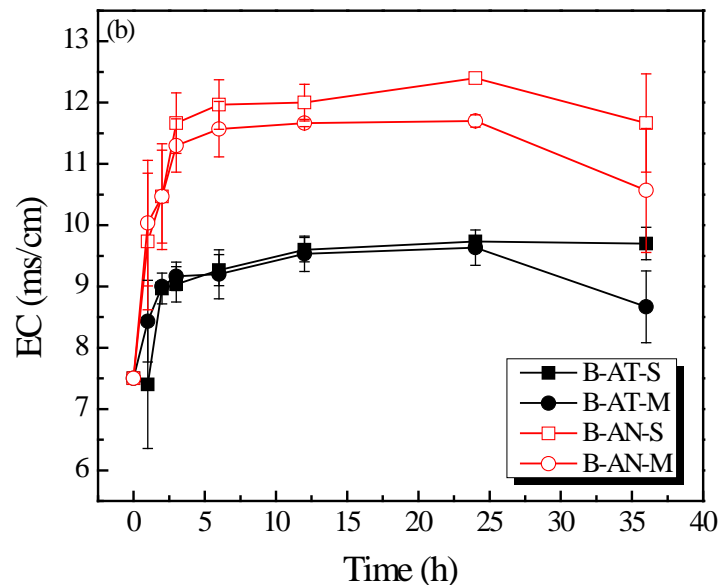
266 In both purified urease enzyme and bacteria cases, the values of EC in anoxic
267 conditions were higher than those in oxic conditions at any time during the shaking tests.
268 This phenomenon is possibly attributed to the following three factors: (a) the ureolytic
269 rate by the purified urease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

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

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

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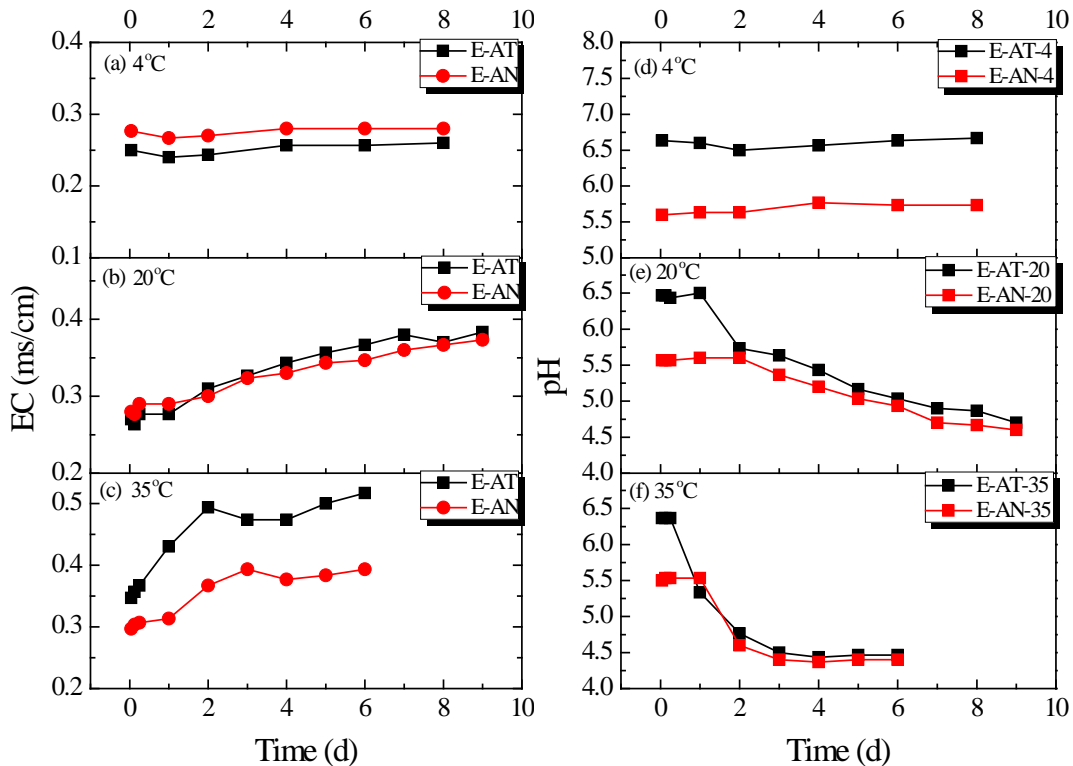
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For the purified urease enzyme case, since the ureolytic rate was found to be almost equivalent in both conditions and there cannot be extra cell-related substance synthesized by the purified enzyme, the high EC values in anoxic conditions could only 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,

289 the high enzyme concentration case and multi-amendment of bacteria solution case
 290 resulted in high values of EC at the same shaking time before the final equilibrium was
 291 reached. This is consistent with the results of remaining urea concentration with time,
 292 which is triggered by the high ureolytic rate as well.
 293



294
 295 **Fig. 4** Degradation behavior of purified urease enzyme (8 g/L) in distilled water at 4 °C,
 296 20 °C and 35 °C
 297

298 It should be noted that the urease enzyme itself may degrade with time, which could
 299 potentially contribute to the increase in EC values. Therefore, the degradation behavior
 300 (life-time) of the purified urease enzyme (8 g/L) in distilled water at 4 °C, 20 °C and
 301 35 °C were characterized, as shown in **Fig. 4**. The end of enzyme degradation is
 302 determined based on the termination of increase of EC (**Fig. 4 (a), (b), (c)**) or decrease
 303 of pH (**Fig. 4 (d), (e), (f)**). It is found that at 4 °C, no distinguishable enzyme
 304 degradation occurs at least during the entire experimental period (8 days). At 20 °C and

305 35 °C, the life-time of the urease enzyme is found to be 7 and 3 days, respectively.
306 Therefore, within the testing time (1d) in this study at 20 °C, the enzyme degradation
307 should be insignificant. Moreover, it is found that the oxygen availability has no
308 distinguishable influence on the degradation behavior of urease enzyme in distilled
309 water. The measured life-time of urease enzyme in this study is consistent with those
310 reported in the previous studies. For example at 25 °C, the reported life-time of urease
311 can be 5 to 8 days (Krajewska et al. 1990; Petti et al. 1976). At 4 °C, its half-time can be
312 18 to 24 days (Krajewska et al. 1990; Petti et al. 1976).

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

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

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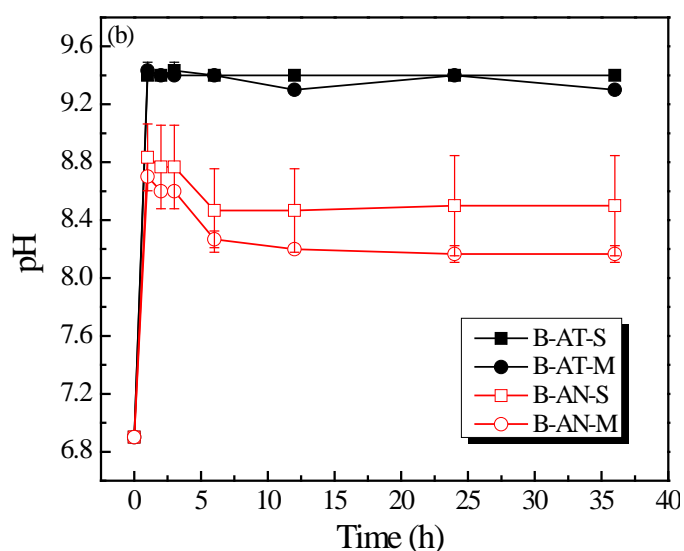
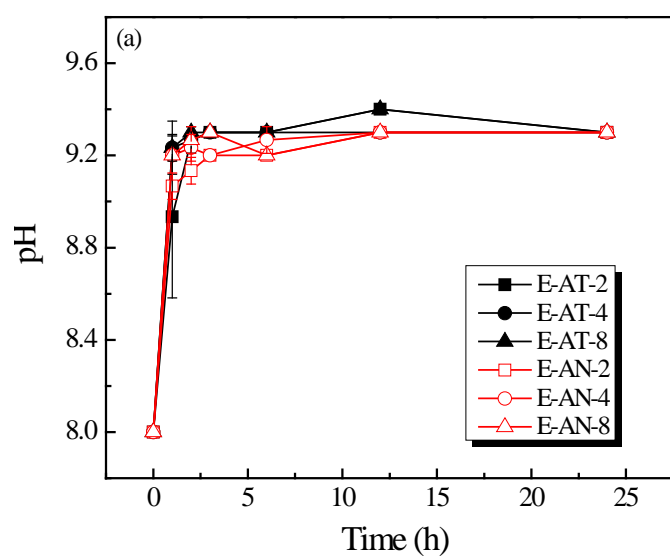


Fig. 5 Variations of solution pH with time ((a). urease enzyme; (b). bacteria solution)

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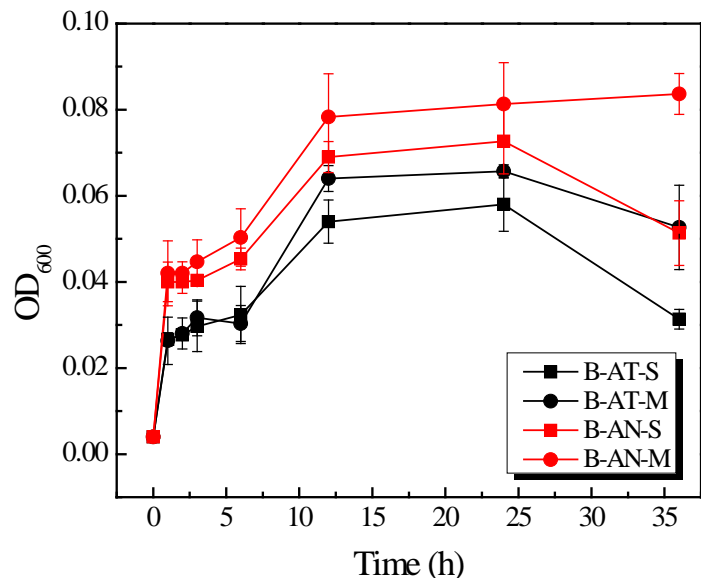
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332 In the purified urease enzyme case, the solution pH in anoxic conditions is only
 333 slightly lower than that in oxic ones. This is possibly caused by the CO₂ gas used for
 334 deoxygenation and is consistent with the EC results. In the bacteria case, however, the
 335 solution pH in anoxic conditions stabilizes around 8.2-8.5, which is significantly lower
 336 than that of 9.4 in oxic conditions. This suggests that not only the CO₃²⁻ and HCO₃⁻ ions
 337 but also extra acidic electrolytic substances synthesized by the bacteria (other than
 338 ureolysis-related ones) in anoxic conditions contribute to the low pH and high EC

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

344



345 **Fig. 6** Variations of the optical density at 600 nm (OD_{600}) in bacteria solution with time
 346

347

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

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

359 It should be noted that although the OD₆₀₀ values measured in this study is low
360 compared to some previous studies, the low magnitudes of OD₆₀₀ with accompanying
361 significant ureolytic activity for ureolytic bacteria were also reported by Martin et al.
362 (2012) for the other ureolytic bacterium (*S. pasteurii*), proving that the bacteria still
363 undergo growth at the low OD₆₀₀. Actually, Lauchnor et al. (2015) reported a linear
364 relationship between OD₆₀₀ and ureolytic rate for *S. pasteurii*. A significant ureolytic
365 activity can be still observed under low OD₆₀₀ (the specific activity is around 30 mM
366 OD⁻¹ min⁻¹). In this study, the specific ureolytic activity for the *B. megaterium* was
367 around 3.3 mM OD⁻¹ min⁻¹ in the initial 5 hours, which is similar to that reported by
368 Jiang and Soga (2014) and is deemed as sufficient to induce calcite precipitation
369 (Whiffin et al. 2004).

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

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

383

384 **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

385 ^a Standard deviation

386

387 **4. Discussion**

388 *4.1 Effect of oxygen availability on ureolytic efficiency of purified urease enzyme*

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

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

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

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

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

424

425 4.2 Effect of oxygen availability on ureolytic efficiency of *B. megaterium*

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

437 There is no report with regards to the urease synthesis capacity of *B. megaterium*.
438 However, there are some published data on the urease synthesis capacity of *S. pasteurii*,
439 which is a commonly used urease-producing bacterium. Tobler et al. (2011) show that,
440 if *S. pasteurii* is grown in large numbers aerobically in nutrient-rich conditions and then
441 injected into anoxic oligotrophic groundwater, its ureolytic efficiency has no difference
442 compared with that in oxic conditions at least for the initial 1.2 days. Mortensen et al.
443 (2011) report that the ureolytic rate of *S. pasteurii* pellet incubated anaerobically had a
444 roughly 1.5-fold of that in aerobic conditions. This is possibly due to the lysis of
445 bacteria cells in anoxic conditions and direct release of enzyme from inside cytoplasm.
446 On the other hand, Martin et al. (2012) state that the de novo synthesis of urease
447 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

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

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

479

480 4.3 Ureolytic efficiency of purified urease enzyme and *B. megaterium*

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

496

497 **5. Conclusions**

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

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

505 (2) Based on the data of remaining urea concentration, EC, pH, and OD₆₀₀, the
506 ureolytic activity of *B. megaterium* in anoxic conditions is greater than that in
507 oxic conditions. This is attributed to the presence of more available active urease
508 enzyme and higher ureolytic activity of urease enzyme. The fundamental
509 mechanisms could be large amount of existing urease from initial aerobic
510 growth, lysis of cells, and the effect of aqueous solution pH.

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

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

519 seafloor 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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