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A feasible scale-up production of *Sporosarcina pasteurii* using custom-built stirred tank reactor for in-situ soil biocementation

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Highlights

- A custom-built reactor (3 m³) was used to cultivate *Sporosarcina pasteurii*.
- Urease activity was 11.1 ±0.48 mM urea hydrolysed.min⁻¹ at the end of cultivation.
- CaCO₃ content for treated mould samples ranged from 19.0 ±1.58% to 39.4 ±1.88%.
- SEM analysis showed irregular, elemental rhombohedral and polyhedral crystals.

Abstract

Production of urease enzyme from ureolytic bacteria (e.g. *Sporosarcina pasteurii*) is essential for precipitation of CaCO₃. This bioprocess is notably utilized for soil improvement, biocementation and bioremediation of heavy metals. Despite the viability for field-scale implementation as a suitable alternative to conventional treatment methods, most reports in the literature are constrained to laboratory-scale. Thus, this study demonstrates an economic strategy to scale-up the production and cultivation of ureolytic bacteria using a custom-built stainless steel stirred tank reactor (3 m³). Scalability of the bacterial cells was carried out from 214 L to 2400 L seed cultures for 90 h. Technical-grade ingredients (food-grade yeast extract media and chemicals) were used for scale-up production of the bacterial cells. The growth profile, pH and urease activity were subsequently monitored throughout the in-situ production period. Biocementation on sand mould specimens was also performed using bacterial cultures obtained from the reactor and low-grade cementation reagents. The sand columns were grouped into different treatment cycles to determine the effect of ureolysis. CaCO₃ content, effluent pH and microstructural analyses (scanning electron microscopy with energy dispersive X-ray spectroscopy) of the treated soil samples were also evaluated. The results show that scale-up production of ureolytic bacteria cells under non-sterile conditions using a custom-built reactor for industrial MICP application is feasible.

Keywords: non-sterile cultivation; soil biocalcification; crystal morphology; large-scale production; urease enzyme.

1. Introduction

Microbially induced carbonate precipitation (MICP) relies on microbial agents for nucleation sites provided by the bacterial cells for sufficient urease production and CaCO_3 precipitation. Several studies have shown that *Sporosarcina pasteurii* is a preferred ureolytic bacterium for various MICP studies because it can tolerate harsh environmental conditions and produce desirable urease (Gowthaman et al., 2019; Singh et al., 2017; Stocks-Fischer et al., 1999; Whiffin et al., 2007). Despite the successful potential of MICP technology, only few field-scale trials are available in the literature (Esnault-Filet et al., 2012; Gomez et al., 2019, 2015; Phillips et al., 2018; van Paassen, 2009). The cost of cultivation materials and sterility requirement for ureolytic bacterial production adversely affect the implementation of MICP. Previous studies have reported that the cost to scale-up bacterial cells can reach 60% of the total operating costs and commercial growth medium sterilization (i.e. \$0.46 to \$0.66 per litre) which can be twice the cost of growth medium ingredients (Cheng and Cord-Ruwisch, 2012; Whiffin, 2004).

Most laboratory-based experiments on scalability of ureolytic bacteria are performed in chemostats (1 L to 5 L) (Zhu and Dittrich, 2016). However, for field-scale trials, larger reactors will be required which can be costly. Some researchers have recommended the use of custom-built reactors and non-sterile bacterial cultivation methods which may help minimize the cost by more than 50% (Cheng and Cord-Ruwisch, 2013; Wang et al., 2017; Zhu and Dittrich, 2016). However, only few studies have demonstrated potential strategies which can be adopted on cost-effective scale-up process of ureolytic bacteria. Whiffin (2004) reported using a custom-built fibreglass airlift pilot-scale reactor (120 L capacity) to scale-up the cells of *S. pasteurii* for 50 h at 30°C under non-sterile conditions. Cheng and Cord-Ruwisch (2013) showed that batch-scale chemostats (1 L capacity) could stimulate bacterial growth from activated sludge samples under non-sterile conditions at 28°C for 140 h. Recently, Aoki et al. (2018) reported using a low-technology down-flow hanging sponge reactor (170 cm³ capacity) to enrich ureolytic microorganism from a reservoir-collected sample at 25°C for 130 days under non-sterile conditions. However, MICP researchers are yet to report on in-

situ cultivation of ureolytic bacterial using a custom-built stainless steel stirred tank reactor (3 m³).

The aim of the study herein was to investigate the feasibility of scaling up the production of an *S. pasteurii* strain under non-sterile conditions for in-situ soil biocementation. The bacterial cells were scaled up from 214 L to 720 L and finally 2400 L for 90 h. Next, in-situ biocementation on sand mould samples via a simple surface percolation method and different treatment cycles was performed to determine the performance of the bacterial cells after cultivation. CaCO₃ content, effluent pH and scanning electron microscopy (SEM) with energy dispersive X-ray spectroscopy (EDX) analyses of the treated soil specimens were evaluated after soil biocementation.

2. Materials and methods

2.1. Ureolytic bacterial strain

S. pasteurii NB28(SUTS) (GenBank accession number **KX212192.1**), previously isolated by Omoregie et al. (2017) was used throughout this study. Freeze-dried culture stock was rehydrated in sterile nutrient broth (13 g L⁻¹, HiMedia, Laboratories Pvt. Ltd). The revived bacterial cells were then grown on Tryptone Soya Agar (TSA; 40 g L⁻¹, HiMedia, Laboratories Pvt. Ltd) and urea (20 g.L⁻¹, Merck, Shd. Bhd) overnight at 32°C.

2.2. Inoculum culture preparation

The production of microbial inoculum for bulk starter culture was adopted from Prabakaran and Balaraman (2006) with minor modifications. Initially, bacterial colonies from the TSA slant was inoculated into cultivation medium that contained technical-grade yeast extract (15 g.L⁻¹, Angel Yeast Co. Ltd.) ammonium sulphate (10 g.L⁻¹, Petronas Chemical Company Bhd), sodium acetate (4.1 g.L⁻¹, Petronas Chemical Company Bhd.), urea (40 g.L⁻¹, Petronas Chemical Company Bhd.) and pH 7.0 (Omoregie et al., 2019a). The cultures were then incubated for 48 h at 32 °C with shaking (150 rpm) on an orbital shaker (Smith, A3446). Next, freshly prepared sterile cultivation medium (1.5 L) were placed in 8 separate Erlenmeyer flasks (3 L capacity), inoculated with the previously grown bacterial cultures and incubated at 30°C for 24 h under shaking condition (110 rpm) (CERTOMAT[®] CT plus, Sartorius). Similar to

Gomez et al. (2016) and Phillips et al. (2018), the bacterial cultures (65 L) were cultivated daily and transferred for storage. Three separate vented anti-glug plastic water containers (30 L capacity) were selected and disinfected to temporarily house the accumulated bacterial cultures. After 120 h, 13 L were transferred into the plastic containers and stored in a cold room (4°C) before being used for in-situ scalability process. The bulk ureolytic bacterial cultures had optical densities (OD) ranging from 0.7 to 1.2 and pH ranging from 9.2 to 9.6.

2.3. Setup of the custom-built reactor

The strategy to scale-up the cells of *S. pasteurii* was conducted under non-sterile condition in a custom-built stirred tank reactor (3 m³) (**Fig. 1** and **Table 1**) and the vessel was fabricated by Creative Encounter Sdn Bhd, Malaysia. Total production cost for the main components used to fabricate the stirred tank reactor is USD 6574.97 (**Supplementary materials, Table AO1**). Prior to cultivation, the vessel interior section was chemically sterilized with liquid detergent and sodium hypochlorite solution (5%, v/v) (Whiffin 2004). The vessel was later rinsed with clean tap water to remove chloride residues (Seifan et al., 2017). From the total reactor volume, 80% was selected for bacterial cultivation to minimize possible build-up of gas bubbles which may occur during fermentation (Couper et al., 2009; Gossain and Mirro, 2010).

2.4. Large-scale ureolytic bacterial cultivation

For step-wise bacterial production from 214 L, 720 L and 2400 L seed cultures, approximately 30% (v/v) inoculate was used. The growth medium ingredients used in this study were of technical-grade and prepared in a disinfected plastic container (200 L capacity) with clean tap water. The physicochemical properties of the tap water (**Supplementary materials, Table AO2**) was analysed by EnviC Laboratory Sdn. Bhd. Malaysia. The initial seed culture (216 L) was prepared with yeast extract (30 g.L⁻¹), urea (40 g.L⁻¹), sodium acetate (4.1 g.L⁻¹), ammonium sulphate (10 g.L⁻¹) and transferred into the reactor via a liquid transfer pump (Pedrollo; PQ60, 40 L.min⁻¹). Bacteria were acclimatised for 30 h before subsequently increasing the volume to 720 L (20 g.L⁻¹ of yeast extract, 10 g.L⁻¹ of urea and 10 g.L⁻¹ of ammonium sulphate) and 2400 L (20 g.L⁻¹ of yeast extract and 10 g.L⁻¹ of ammonium sulphate). Both seed cultures were also incubated for 30 h with shaking condition (35 rpm). The cost analysis

for the ingredients used for the mass production of the ureolytic bacteria is shown in **Supplementary materials, Table AO3**.

2.5. Monitoring methods

Performance of the ureolytic bacterial cells was monitored throughout the fermentation period. Samples (40 mL) were taken every 6 h from the sampling collection outlet, placed in sterile 50 mL tubes and transported on ice to the laboratory. Analytical assay methods previously described by Omoregie et al, (2019a) were used to determine the growth profile (OD at 600 nm using a Thermo Scientific™ GENESYS™ 20 spectrophotometer), pH profile (SevenEasy™ (Mettler Toledo pH meter with precision of 0.01) and urease activity (electric conductivity meter (Milwaukee, MI806, with a precision of 1 $\mu\text{S}.\text{cm}^{-1}$) of the culture samples. Dissolved oxygen (DO), total dissolved solids (TDS) and salinity of bacterial cultures were also monitored during the cultivation period. The DO, TDS and salinity were measured with disinfected multipurpose meter (YSI Pro Plus multiparameter meter, Xylem) having a precisions of 0.05%, 0 $\text{mg}.\text{L}^{-1}$ and 0.1 ppt, respectively. In addition, the interior temperature (23-34°C) and relative humidity (60-100%,) of the custom-built reactor were observed throughout the cultivation period (**Supplementary materials, Table AO4**).

2.6. Biocementation by surface percolation

The sandy soil (pH 7.16 ± 0.02) used herein was sourced from Phua Kheng Heng while polystyrene boxes served as moulds for the biocementation experiment. The treatment had different cycles for each mould group. Mould 1 (24.38 cm height, 34.04 cm length and 25.15 cm width) mould 2 (13.97 cm height, 22.10 cm length and 22.10 cm width) and mould 3 (21.59 cm height, 22.86 cm length and 16.51 cm width) were each filled with 15 kg, 5 kg and 5 kg of sand, respectively. According to *Unified Soil Classification System*, the sand used in this study was classified as poor-grade soil (**Supplementary materials, Table AO5**). Four silicone tube hoses (30 cm length) and scouring pads (porous plastic pads) were attached to all moulds before discharging the effluent. The cementation solution (CS, pH 5.01 ± 0.03) used for MICP treatment via surface percolation consisted of technical-grade urea ($66.06 \text{ g}.\text{L}^{-1}$), calcium chloride

(110 g.L⁻¹, Lianyungang Longyi Industry Co. Ltd) and yeast extract (5 g.L⁻¹). The moulds were first percolated with bacterial cultures and allowed to settle for 2 h before adding the technical-grade CS. The bacterial cultures were added into the mould sets twice only on the first day while the CS were added once per day. Repeated treatment cycles (8 days, 5 days and 2 days for mould 1, mould 2 and mould 3, respectively) were carried out with only CS until the percolation period was complete (**Table 2**). Effluent samples (50 mL) were periodically taken from the ejection points for pH analysis while the rest were properly discarded. Each mould was then flushed with clean tap water to remove any soluble ammonium salt formed after which they were allowed to cure for 8 weeks before further analyses.

2.7. Measurement of CaCO₃ content

Acid washing was used to estimate the amount of CaCO₃ deposited in the sand mould (Omorieg et al., 2019c). Specimens (100 g) were taken within 20-30 mm from the top, middle and bottom sections before dissolving in 200 mL of 1.5 M HCl with continuous stirring to completely dissolve the CaCO₃ (Mahawish et al., 2019). The specimens were then washed with sterile deionized water for 10 min through a Whatman® filter paper (11 µm pore size) and oven-dried at 90°C for 24 h (Osinubi et al., 2019). CaCO₃ content was determined by weight difference before and after being subjected to acid wash. For each test, nine treated soil specimens were measured to achieve average values.

2.8. Microstructure characterization

For investigation of the MICP performance in the sand moulds after biocementation treatment, samples were imaged using SEM-EDX (Hitachi S-3400N) analyses. Samples were sputter-coated with a thin layer of gold prior to analysis. The SEM analysis was performed at an accelerating voltage 15 kV, a spot size of 3.0, magnification 5x up to 300,000x and resolution of 3.0-4.0 nm, respectively. EDX spectroscopy was used to determine the elemental composition of samples.

2.9. Statistical analysis

The experimental results were analysed using the statistical analysis software GraphPad Prism® (version 8) to determine the significance of differences between the

means groups obtained in this present study. One-way analysis of variance (ANOVA) and Tukey-Kramer post hoc test were performed using Minitab® (version 19). The level of significance was set at 0.05.

3. Results

3.1. Scale-up cultivation of ureolytic bacteria

Prior to the in-situ bacterial cultivation, the performance of *S. pasteurii* NB28(SUTS) in sterile (OD of 1.1 ± 0.02 and urease activity of 19.2 ± 0.04 mM urea hydrolysed.min⁻¹) and non-sterile (OD of 0.9 ± 0.07 and urease activity of 14.5 ± 0.02 mM urea hydrolysed.min⁻¹) cultivation medium using technical-grade ingredients in tap water was investigated under laboratory-conditions (data not shown). Cultivation in non-sterile medium resulted in 18% and 25% reduction for OD and urease activity, respectively, when compared to sterile cultivation medium. Hence, the 65 L inoculum was prepared under sterile laboratory-condition to minimise contamination. **Fig. 2** shows the scale-up process of the ureolytic bacterial cultures.

The results obtained for biomass, pH and urease activity for the ureolytic bacteria cells are presented in **Fig. 3** and **Table 3**. The maximum OD for 216 L, 720 and 2400 L seed cultures were 0.5 ± 0.00 , 1.6 ± 0.02 and 1.8 ± 0.09 , respectively. The maximum pH and urease activity values for 216 L seed culture were $.4 \pm 0.05$ and 9.3 ± 0.14 mM urea hydrolysed.min⁻¹, while those of 720 L seed culture were 9.1 ± 0.02 and 1.1 ± 0.13 mM urea hydrolysed.min⁻¹, then finally those of 2400 L seed culture were 9.2 ± 0.05 and 11.1 ± 0.48 mM urea hydrolysed.min⁻¹, respectively. Measurements of DO, TDS and salinity for samples collected from the reactor during incubation are shown in **Table 4**. Also, culture samples collected from the reactor were screened for the presence of bacterial contaminants. Phenotypic and molecular characterizations were carried out following methods described by Omoregie et al., (2016) and Omoregie et al. (2019b). Four ureolytic bacterial isolates with accession numbers **MK595743**, **MK595744**, **MK595745** and **MK595747** were identified as *Pseudogracilibacillus marinus*.

3.2. Soil biocementation and CaCO₃ content

After MICP treatment, white precipitates of crystals manifested on the surfaces of some sand mould specimens as a result of ureolysis (**Supplementary materials, Fig. AO1**). This outcome meant that the bacterial cells grown under non-sterile conditions could produce sufficient urease to induce cementitious material for future large-scale MICP trials (**Supplementary materials, Fig. AO2**).

Table 5 shows pH analysis of effluent samples that were spatially collected during soil biocementation to monitor ureolysis activity as the treatment period progressed. In summary, the pH from mould 1 samples decreased from 8.7 ± 0.09 to 8.0 ± 0.05 after 3 days, while pH from mould 2 samples decreased from 8.7 ± 0.18 to 7.4 ± 0.10 after 6 days. The result also showed that pH from mould 3 samples decreased from 8.7 ± 0.11 to 7.2 ± 0.06 .

The distribution of CaCO_3 at top, middle and bottom layers of the treated sand specimens are shown in **Fig. 4**. Total CaCO_3 contents increased progressively with treatment cycles which was applied on different mould sets. The difference of CaCO_3 content between the top and bottom layers of mould 1 (3.8%) was lower than those of mould 2 (7.2%) and mould 3 (7.0%). The distribution of CaCO_3 in mould 1 was $9.2 \pm 0.54\%$ (top-layer), $4.5 \pm 0.44\%$ (middle-layer) and $5.4 \pm 0.60\%$ (bottom-layer). For mould 2, the CaCO_3 distribution was $14.4 \pm 0.51\%$ (top-layer), $7.2 \pm 0.64\%$ (middle-layer) and $7.2 \pm 0.44\%$ (bottom-layer). Finally, that of mould 3 was $18.2 \pm 0.70\%$ (top-layer), $10.0 \pm 0.50\%$ (middle-layer) and $11.2 \pm 0.68\%$ (bottom-layer). In addition, **Fig. 4** shows that moulds 1, 2 and 3 had CaCO_3 content of $19.0 \pm 1.58\%$, $28.8 \pm 1.59\%$ and $39.4 \pm 1.88\%$, respectively. Statistical analysis using one-way ANOVA showed that there were significant differences (P-value is 0.00) among the group means of the results.

3.3. Microstructure investigation

SEM with EDX analyses were performed on the treated samples collected from the top layer of the crushed biocemented sand specimens. The SEM micrographs showed that the treated specimens were coated with CaCO_3 mesocrystals which, in some cases, bridged the sand particles together (**supplementary materials, Fig. AO3** and **Fig. AO4**). The SEM micrographs (**Fig. 6A-C**) also showed that different treatment periods influenced the crystals morphological formations. In **Fig. 6A**, crystals ranging from 15

275 μm to 25 μm with topography showing an elongated flower-like crystals having smooth
 276 appearances clustered together were seen on mould 1 specimen. In **Fig. 6B**, several
 277 regular crystals ranging from 15 μm to 35 μm with topography showing a cubic-like
 278 crystals having smooth appearances which are clustered together were seen on mould 2
 279 specimen. However, **Fig. 6C** shows several irregular crystals structures ranging from
 280 2.5 μm to 30 μm with topography showing small cubic-like crystals scattered on the
 281 surfaces of specimen 3. Additionally, a few larger projecting cubic-like crystals
 282 clustered together with rough appearance were also seen. More so, higher SEM
 283 magnification (**supplementary materials, Fig. AO5**) showed morphological shape
 284 with elongated rhombohedral, elemental rhombohedral and polyhedral CaCO_3 crystal
 285 structures for moulds 1, 2 and 3, respectively.

286 4. Discussion

287 4.1. *Scale-up of ureolytic bacteria in the custom-built reactor*

288 Non-sterile growth of microorganisms has been widely used for various bioprocess
 289 such as biogas production and wastewater treatment but the use of non-sterile
 290 conditions to cultivate bacteria for enzyme production (i.e. urease) has not been widely
 291 explored (Gorski, 2012). It is worth noting that this is the first study reporting the use of
 292 a custom-built reactor (3 m^3) for scalability of ureolytic bacteria cells. The reactor was
 293 specifically built for simple, economic aerobic microbial scale-up cultivation and was
 294 situated outdoor to mimic field-scale conditions. It was equipped with a top-driven
 295 agitator system (stirrer motor) supported with a stand, stirrer shaft, impellers, sampling
 296 port, air delivery system and dump port for the discharge of culture.

297 The growth profile showed that there was a long lag phase which occurred only in
 298 the 216 L seed culture. The lag phase is the delay before the start of exponential growth
 299 and represents the earliest and most poorly understood stage of microbial growth cycle
 300 due to limited studies which describe the fundamental physiological and molecular
 301 processes (Rolfe et al., 2012). When cells in stationary phases are introduced into a new
 302 enriched-nutrient environment, it is desirable for the cells to rapidly obtain biomass to
 303 secure resources before their competitors (Schultz and Kishony, 2013). It is not known
 304 why the 216 L seed culture displayed an extended lag phase and took a considerably

longer period to reach exponential phase when compared to 720 L and 2400 L seed cultures. It is likely that *S. pasteurii* required a longer period to acclimatize to its new environment and compete for nutrients with other microbial cells.

During the scale-up process, urea and ammonium sulphate were omitted in the final stage of the microbial seed cultivation to reduce build-up of ammonia (due to ureolysis) and decreased alkalinity during the fermentation period. Unfortunately, one of the major problems affecting MICP is the production ammonia during treatment of bacterial cultivation which can have adverse impacts on human health and the environment (Ivanov et al., 2019). It was previously determined that 62 kg of calcium carbonate per 1 m³ from soil biocementation can result in the production of 10.5 kg and 11.2 kg of ammonia into the air, groundwater or surface water (Ivanov and Stabnikov, 2017). If a significant amount of urea was used during the large-scale bacterial production (0.27 m³) in the reactor, it would release up to 0.3 km³ of ammonia gas into the atmosphere. Besides, the custom-built reactor had no integrated systems for absorbing or treating the released gas, hence low urea concentration was used for 217 L and 720 L seed cultivation to minimize ammonia production. Interestingly, Aoki et al. (2018) recently showed the ammonia gas could be treated during cultivation of ureolytic bacteria in a non-sterile bioreactor system by transferring the ammonia gas from an effluent reservoir tank (after collection from the reactor) and then into a sulphuric acid solution tank.

After final cultivation (2400 L), the seed culture had an OD of 1.8 ± 0.09 which is within the recommended cell density (OD 0.8 to 2.0.) for MICP experiments (Al Qabany et al., 2012; Gat et al., 2014; Kashizadeh et al., 2020). The urease activity (11.1 ± 0.48 mM urea hydrolysed.min⁻¹) and pH (9.2 ± 0.05) measurements showed that bacterial cells were still able to produce urease needed for CaCO₃ precipitation. The results also indicated that under non-sterile cultivation conditions and without increasing the pH of the medium or using high a urea concentration, ureolytic bacterial cells can be produced in large volume. The use of this custom-built reactor and technical-grade reagents for large-scale bacterial production under non-sterile operation can serve as a suitable concept for future practical experiments that have financial constraints and are not concerned with the possibility of contaminating species.

4.2. MICP treatment

The entire soil mould specimens achieved cementation and were subjected to further analyses after curing (8 weeks). For successful biocementation, it is important to introduce bacterial cells into the soil for proper immobilization before applying the cementation solution (Cheng and Cord-Ruwisch, 2012). The bacterial cells were able to hydrolyse urea which later allows precipitation of CaCO_3 upon the presence of a calcium source. The soil biocementation experiment undertaken in this study was performed outdoors to mimic field-scale conditions akin to field-scale MICP trials which were carried out by other researchers (Esnault-Filet et al., 2012; Gomez et al., 2016, 2015; Phillips et al., 2018; van Paassen et al., 2010; Van Paassen et al., 2009). Small-scale moulds were used in the present study which is contrary to the large-scale moulds (i.e. 1.7 m^3 diameter tanks and 100 m^3 sandbox) reported in the literature. The small-scale soil biocement test was done to determine the performance of the bacterial cultures after cultivation in the custom-built reactor. Also, the use of surface percolation method for MICP treatment is widely known and has been reported suitable for columns below 2 m height and one-dimensional vertical cementation treatment because they are often confined by the column walls (Cheng and Cord-Ruwisch, 2014, 2012). Therefore, in-situ surface percolation was utilized since the heights of the moulds in this present study ranged from 0.14 m to 0.24 m.

The effluent pH was monitored during treatment to monitor ureolysis MICP treatment which is an effective indicator for urease activity changes (Gomez et al., 2016; Porter et al., 2017; Stocks-Fischer et al., 1999). In our study, the effluent pH ranged from 7.2 ± 0.06 to 8.7 ± 0.11 which is similar to the results reported by Porter et al. (2017) and Gomez et al. (2016). It was suggested that the loss of urease activity may be attributed to biological and chemical reactions (van Paassen, 2009). Therefore in situations where urease activities are low, more urease activity can be supplied to the soil to improve biocementation by percolating more bacterial cells (Cheng et al., 2017). However, the disadvantage of such an approach is formation of unwanted clogging at the surfaces of the soil mould that can inhibit further penetration of CaCO_3 precipitates during treatment.

4.3. CaCO_3 content

After soil biocementation, it is common to investigate the distribution of CaCO_3 content present within the treated sand body. The top, middle and bottom layers of the mould specimens did not show homogeneous distribution of CaCO_3 precipitates. This can often be linked to clogging during MICP treatments especially at the point of injection. The available urease from the bacterial cells situated at the injection points hydrolyses urea to form CaCO_3 precipitates which occurs continuously and results in a biofilm layer, thus preventing further cementation solutions from penetrating the soil particles. Such clogging can prevent sufficient cementation from reaching the depth of the mould specimens (van Paassen, 2009; Whiffin, 2004). Cheng and Cord-Ruwisch (2014) suggested clogging formation during surface percolation treatment is often due to immobilization of high concentrations of bacterial cells at the injection end. Several factors such as environmental temperature, relative humidity and reactions with native microorganisms during treatments can also influence the rate of CaCO_3 precipitation. The CaCO_3 content from the sample groups showed that significantly more crystal formation occurred in the soil moulds treated for 9 days than those treated for 3 and 6 days. It is important to give cementation cycle sufficient treatment interval to allow nucleation site and crystal formation essential for soil particle bridging to occur. Percolated soil columns effectively lead to more CaCO_3 or compressive strength when a lower degree of cementation treatment cycles and saturation zones are performed (Cheng et al., 2017; Cheng and Cord-Ruwisch, 2013).

4.4. Microstructural analysis

Microstructural analysis was undertaken to show the influence of different MICP treatment cycles on the sand mould specimens. SEM images revealed that the crystal deposits occurred on sand grains and at regions which cemented sand grains together. This happens because bacterial cells can readily attach themselves to the corners of sandy soil and not be easily disintegrated (Porter et al., 2018). The observed SEM images which displayed crystal formation on the soil particles are similar to other MICP studies (Cheng et al., 2017; Porter et al., 2018, 2017; Seifan et al., 2017). EDX results revealed peaks of calcium, oxygen and carbon elements which are indicative of CaCO_3 precipitation (Porter et al., 2018). The microstructural analysis of the treated biocement sand specimens suggested that MICP treatment performed under non-sterile conditions

could still precipitate a sufficient amount of CaCO_3 crystals. However, it is noteworthy that the CaCO_3 polymorphism and morphologies can be greatly influenced by bacterial metabolic activities, cementation reagents, extracellular polymeric substance and abiotic factors (i.e. pH, water, sunlight, oxygen, soil, humidity and temperature) (Seifan et al., 2017).

4.5. Cost implications

Using a custom-built reactor in this study can offer enormous economic advantage for in-situ MICP applications. Typically, large-scale production of ureolytic bacteria is essential for augmentation or injection of bacterial culture. The typical developmental phases associated with scale-up microbial cultivation (Petri dish, shake flask, bench-scale, pilot-scale and large-scale) are costly (Li et al., 2015). For example, in Malaysia, the fermentation platforms for large-scale production of bacterial cells (i.e. 3 L, 30 L, 300 L and 3000 L total capacities) cost USD 550,000 to 1.3 million (communication with Nursyahira Binti Mohd Ghazali from Fermetec Resources Sdn. Bhd. and Julian Lim from Medigene Sdn. Bhd.). Hence, a simple low-technology reactor (USD 6574.97) which could feasibly scale-up bacterial cells with minimal contamination was utilized in this study. Future improvement to reduce microbial contamination and increase biomass yield may include having boilers attached to interior part of the medium tank for sterilization. In addition, low-cost commercially procurable thermocirculator with heating coil could be incorporated into the custom-built reactor to aid temperature control.

5. Conclusion

This study investigated the feasibility of using a custom-built stirred tank reactor (3 m^3) to scale-up the production of *S. pasteurii* under non-sterile operation for various MICP applications. Using a sequential scale-up strategy, the bacterial cells were successfully grown from 216 L to 2400 L with suitable cell mass ($\text{OD} = 1.8 \pm 0.09$), pH (9.2 ± 0.05) and urease activity ($11.1 \pm 0.48 \text{ mM urea hydrolysed.min}^{-1}$). Furthermore, in-situ soil biocementation showed that bacterial cells grown in the custom-built reactor could induce CaCO_3 precipitation after being immobilized in sand specimens. CaCO_3 content and microstructural analyses were able to confirm the presence of CaCO_3 crystal formation within the treated sand particles.

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Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Fig. 1: Digital image of the custom-built stirred tank reactor (3 m³) used for large-scale production of ureolytic bacteria.

Fig. 2: An inner view of the custom-built reactor during scale-up process of the bacterial cells. Sequentially, volume of cultures was increased from 216 L (A) to 2400 L (B).

Fig. 3: Growth profile (A); pH profile (B) and urease activity (C) analyses for ureolytic bacterial cultivation. The vertical error bars represent mean $\pm$ SD (n = 9).

Fig. 4: CaCO_3 content of different layers (top, middle and bottom) for the biocemented sand samples. One-way ANOVA with Tukey-Kramer post hoc analyses were used to determine statistical significant ($P < 0.05$). The rectangular bars that share same letter indicates there are no statistical significant differences between their means. The vertical error bars represent mean $\pm$ SD ($n=9$).

Fig. 5: SEM micrographs showing different morphological crystal formations for Mould 1 (A); Mould 2 (B) and Mould (C) after successful MICP treatment (3, 6 and 9 days respectively).

635 **Table 1:** Geometric parameters of the custom-built stirred tank reactor

Parameter	Result
Brand/Catalog of tank	King Kong/HR300
Shape of tank	Vertical round bottom
Typical usage of tank	Water storage
Total capacity	3000 L
Working capacity	2400 L
Diameter of tank	1470 mm
Height of tank	3230 mm
Ratio height of liquid to height of reactor (H_L/H_t)	0.8
Ratio height of reactor to diameter of tank (H_L/D_t)	2.2
Ratio diameter of impeller to diameter of tank (D_a/D_t)	0.5
Thickness of tank	2 mm
Diameter of tank cover	40 mm
Diameter of outlet	25 mm
Agitation speed	35 rpm
Agitator power	0.75 kW
Quantity of aeration pods	4
Quantity of impeller	2
Material of impeller	Stainless steel
Diameter of impeller	800 mm
Height of impeller shaft	2286 mm
Thickness of elastomeric nitrile rubber insulation	6 mm

645 **Table 2:** Experimental conditions for the sand mould sets

Mould sets	Sand	Cementation solution	Bacterial culture	Treatment duration
1	5 kg	3 L (once per day)	2.5 L (twice only on first day)	3 days
2	5 kg	3 L (once per day)	2.5 L (twice only on first day)	6 days
3	15 kg	9 L (once per day)	7.5 L (twice only on first day)	9 days

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650 **Table 3:** Growth kinetics of ureolytic bacteria after cultivation

Kinetics parameters	Microbial seed cultures		
	216 L	720 L	2400 L
Specific growth rate (h^{-1})	0.5±0.00	0.0±0.00	0.0±0.00
Doubling time (h)	14.4±0.7	55.1±2.4	23.6.1±2.5
Maximum OD ₆₀₀	0.5 ±0.00	1.6 ±0.02	1.8 ±0.09
Maximum pH	9.4 ±0.05	9.1 ±0.02	9.2 ±0.05
Urease activity (mM urea hydrolysed.min ⁻¹)	9.3 ±0.14	11.1 ±0.13 ¹	11.1 ±0.48

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661 **Table 4:** DO, TDS and salinity of the bacterial seed culture samples

Time (h)	216 L seed culture			720 L seed culture			2400 L seed culture		
	DO (%)	TDS (mg.L ⁻¹)	Salinity (ppt)	DO (%)	TDS (mg.L ⁻¹)	Salinity (ppt)	DO (%)	TDS (mg.L ⁻¹)	Salinity (ppt)
0	11.2 ±1.13	4830.0 ±7.91	8.5 ±0.07	7.5 ±0.07	5639.9 ±6.15	11.7 ±0.13	6.4 ±0.05	9813.4 ±4.76	12.5 ±0.05
6	7.7 ±0.08	5092.6 ±7.67	11.9 ±0.04	5.5 ±0.07	7840.2 ±5.45	14.5 ±0.12	4.3 ±0.11	11425.0 ±7.21	17.4 ±2.12
12	6.3 ±0.03	9535.0 ±6.45	15.3 ±0.09	5.1 ±0.05	9457.4 ±4.51	16.8 ±0.14	3.3 ±0.04	17114.3 ±2.52	22.8 ±0.35
18	6.1 ±0.02	9869.3 ±14.36	18.3 ±0.10	5.4 ±0.05	11387.0 ±18.95	22.9 ±0.47	3.0 ±0.12	21005.0 ±3.46	35.2 ±0.19
24	5.9 ±0.15	11019.8 ±14.59	20.6 ±0.09	4.9 ±0.11	15683.4 ±8.78	23.8 ±0.42	3.1 ±0.09	23112.7 ±3.06	44.3 ±1.08
30	5.4 ±0.05	13937.7 ±10.25	27.4 ±0.14	4.6 ±0.10	18686.8 ±25.65	24.6 ±0.14	3.1 ±0.04	23955.0 ±3.46	51.2 ±0.86

Table 5: pH analysis of effluents samples collected from the mould sets

Treatment day	Mould 1	Mould 2	Mould 3
1	pH 8.7 $\pm$ 0.09	pH 8.7 $\pm$ 0.18	pH 8.7 $\pm$ 0.11
2	pH 8.5 $\pm$ 0.07	pH 8.5 $\pm$ 0.19	pH 8.5 $\pm$ 0.10
3	pH 8.0 $\pm$ 0.05	pH 8.0 $\pm$ 0.13	pH 8.1 $\pm$ 0.09
4	ND	pH 7.8 $\pm$ 0.10	pH 7.7 $\pm$ 0.10
5	ND	pH 7.5 $\pm$ 0.10	pH 7.5 $\pm$ 0.06
6	ND	pH 7.4 $\pm$ 0.10	pH 7.5 $\pm$ 0.07
7	ND	ND	pH 7.5 $\pm$ 0.12
8	ND	ND	pH 7.4 $\pm$ 0.06
9	ND	ND	pH 7.2 $\pm$ 0.06

ND = not determined

688 **Supplementary Materials**

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690 **Fig. AO1:** Biocemented samples after cured for 8 weeks.

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695 **Fig. AO2:** Overall set up of the *in situ* biocement treatment trial to be performed in a sand
696 box (10 m³) using ureolytic bacterial cultured in the custom-built reactor.

Fig. AO3: SEM micrograph showing biocement sand grains coated with CaCO_3 crystals.

Fig. AO4: SEM micrograph showing the bridging of biocemented sand particles by CaCO_3 precipitation.

Fig. AO5: SEM micrographs showing topographical crystal structures of the biocemented sand specimens obtained from Mould 1 (A), Mould 2 (B) and Mould (C).

732 **Table AO1:** Cost analysis of the reactor

Features	Cost (USD)
Custom-built reactor (Stainless steel water tank, support stand, painting, PVC level gauge, elastomeric nitrile rubber insulation, air pump with pods, outlet values, tubing)	3377.97
Stirrer system (Stirrer motor, impeller shaft, paddle impeller)	1471.83
Control panel set-up (metal control panel, electronic inverter, magnetic contactor and 3 phase induction motor)	1049.58
Transfer pump and pipping system	675.59
Total	6574.97

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736 **Table AO2:** Physiochemical properties of tap water used in this study

Parameter	Result
Dissolved oxygen	6.47 mg.L ⁻¹
Biochemical oxygen demand	1.94 mg.L ⁻¹
Chemical oxygen demand	39.5 mg O ₂ .L ⁻¹
Total suspended solids	1.00 mg.L ⁻¹
Total dissolved solids	51.28 mg.L ⁻¹
Salinity	0.25 %
pH	7.46
Chloride	5.14 mg.L ⁻¹
Fluoride	0.02 mg.L ⁻¹
Sodium	3.48 mg.L ⁻¹
Magnesium	1.11 mg.L ⁻¹
Iron	0.02 mg.L ⁻¹
Aluminium	3.89 mg.L ⁻¹
Nitrate	1.34 mg.L ⁻¹
Ammonium nitrogen	0.02 mg.L ⁻¹

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738 **Table AO3:** Cost analysis of ingredients used for cultivation of *S. pasteurii*

Ingredients	216 L seed culture		720 L seed culture		2400 L seed culture	
	Concentration (kg)	*Cost (USD)	Concentration (kg)	*Cost (USD)	Concentration (kg)	*Cost (USD)
Yeast extract	4.47	84.26	20.16	380.02	67.20	1266.72
Urea	1.49	1.95	5.04	6.60	-	-
Ammonium sulphate	1.49	5.72	5.04	19.35	16.08	61.74
Sodium acetate	0.61	2.56	-	-	-	-
Total cost	94.49		405.97		1328.46	

755 **Table AO4:** Temperature and humidity measurements during *S. pasteurii* cultivation

Time (h)	Temperature (°C)			Relative humidity (%)		
	216 L	720 L	2400 L	216 L	720 L	2400 L
0	25	26	31	100	92	83
1	26	28	32	100	87	79
2	27	29	30	93	75	79
3	27	32	30	94	75	85
4	27	33	30	94	65	87
5	26	34	28	95	65	87
6	26	34	28	94	63	87
7	27	34	28	93	63	89
8	28	33	28	94	63	89
9	28	32	27	96	76	97
10	29	32	28	94	76	99
11	29	32	27	83	79	99
12	30	33	27	83	83	99
13	30	33	27	60	86	99
14	31	32	26	60	87	97
15	31	32	26	60	94	97
16	33	31	25	60	98	98
17	33	28	24	60	98	98
18	34	28	28	62	99	97
19	32	29	28	75	99	96
20	30	29	28	80	97	96
21	29	26	32	83	96	87
22	29	25	33	99	96	67
23	29	25	33	99	99	67
24	26	25	33	95	97	65
25	25	26	32	95	96	66
26	25	26	30	95	89	68
27	25	25	33	93	87	79
28	24	24	31	95	76	85

29	24	26	28	95	77	88	756
30	24	26	29	96	76	86	757

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Table AO5: Physical properties of the sand used in this study

D₁₀	D₃₀	D₆₀	C_u	C_c	pH	Specific gravity (G_s)	Maximum dry density (ρ_d max)	Soil classification USCS
0.22 mm	0.25 mm	0.35 mm	1.60	0.95	7.16	2.67	1.78 g/cm ³	Poorly graded sand

Fig. 5: SEM micrographs showing different morphological crystal formations for Mould 1 (A); Mould 2 (B) and Mould (C) after successful MICP treatment (3, 6 and 9 days respectively).

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