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# Scaling Up Biocement Production in the Department of the Air Force

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## ABSTRACT

Bacterial-based biomineralization is currently actively under active investigation in academic, industrial, and government labs around the globe. Particularly, Microbially Induced Calcium Carbonate Precipitation (MICP) is a biomineralization strategy that relies on targeted metabolisms to produce $CO_3^{2-}$ that can complex with exogenously added $Ca^{2+}$ to produce $CaCO_3$. In commercial applications, the $CaCO_3$ can serve to cement aggregates together to form hardened surfaces and/or industrial building materials. This presentation will describe the investigations and the challenges associated with producing bacteria at commercial scale, including optimizing bacterial growth media within the limitations of commercial facilities while remaining cost effective. The work presented here highlights considerations and methods for identifying media and growth properties related to cost effective commercial scale growth of bacteria. Also presented is an example of the strategies and methods for isolating and characterizing environmental bacteria that are unencumbered by intellectual property rights. The results will enable biomineralized materials to be developed for large scale applications.

## INTRODUCTION

Civil infrastructure such as buildings and roadways are commonly constructed from concrete that is produced through mixing granular substrates with Portland cement. Upon addition of water, the Portland cement is chemically converted to minerals that cement the substrate grains together to form hardened materials. The process of producing Portland cement is energy intensive, and the processes for placing cementing mixtures in place for forming concrete is logistics intensive.
Biocement is a material that is generated by biological activity that produces minerals to cement substrate grains together. Biocement is commonly produced via Microbially Induced Calcium carbonate Precipitation (MICP) that converts chemical feedstocks into $CaCO_3$ minerals as cement.

Specifically, in ureolytic MICP the feedstocks are urea and a $Ca^{2+}$ salt. A urease source is provided to the system and produces $CO_2$ and $NH_3$ in a process commonly called ureolysis. In solution, the generated $NH_3$ drives up the local pH by collecting protons to form $NH_4^+$. The generated $CO_2$ is dissolved as $HCO_3^-$, which equilibrates to $CO_3^{2-}$ in the basic environment. The $CO_3^{2-}$ complexes with the provided $Ca^{2+}$ to generate $CaCO_3$, cementing the substrate grains together (Figure 1). In our studies, the urease source is a urease-producing bacterium that we mix into the substrate as a preserved powder.

Current commercial applications of MICP commonly rely on the bacterium *Sporosarcina pasteurii* for stabilizing soil to mitigate dust and erosion (Fattahi et al. 2020; Jiang et al. 2018; Li et al. 2019; Peplow 2020). Other applications and other bacteria are under active investigation but are not widely in use. After further optimization, MICP has the potential to contribute to production of industrial infrastructure as building and roadway materials. Current practices commonly rely on growing MICP-capable bacteria onsite in open top containers. These sites already require civil engineering and construction engineering expertise, and the current biocement production practice requires adding microbiology expertise to manage tasks such as aseptic technique, $O_2$ control, and temperature control.

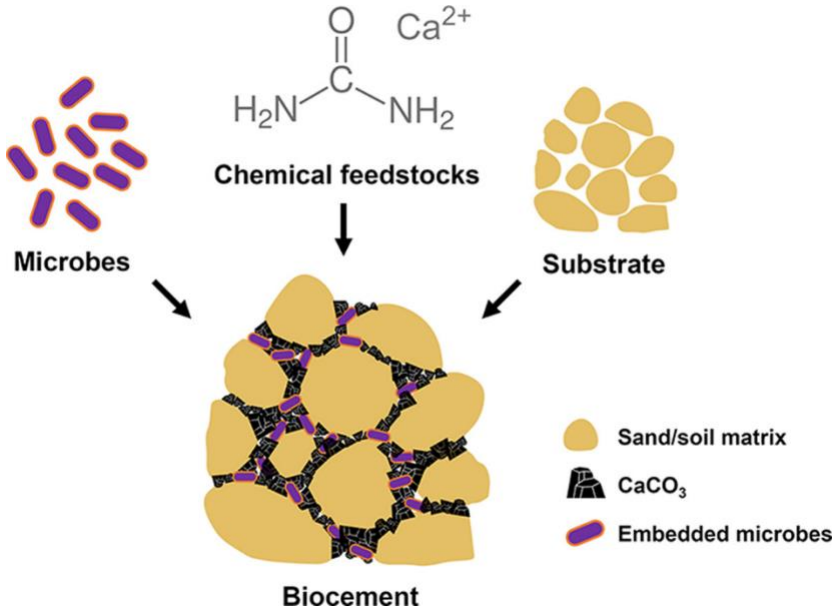


**Figure 1. Biocement production via ureolytic microbially induced calcium carbonate precipitation. Bacterial cells are embedded within a substrate or aggregate material. The bacteria use provided chemical feedstocks to generate $CaCO_3$ minerals that form bridges among the substrate grains, cementing the grains together. The product is a hardened material that can be used for infrastructure purposes. Reproduced with permission from (Carter et al. 2023).**

A commercial product that operates instead like a ready-mix concrete powder would allow operators to divorce production of the bacteria from the site of operation and the application of the bacteria. To develop a ready-mix, bacteria must be produced at the commercial scale and have methods for preserving the bacteria in a powder form. Preservation by Commercial Bio-Manufacturing Organizations (CMOs) is most commonly achieved via lyophilization (freeze drying) or spray drying. Lyophilization preserves more cell viability, but spray draying tends to be

less expensive. Here, we present some of the troubleshooting that we have done as we work to scale up production of MICP-capable bacteria for commercial ready-mix-like applications.

## METHODS

### *Bacterial strains and growth conditions*

*Sporosarcina pasteurii* DSM33 (ATCC 11859) was obtained from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures. Medium for plates was prepared with Brain Heart Infusion (BHI) medium and 330 mM urea. In 96-well plates, cultures were grown with continuous shaking at 30 °C in 300 µL and inoculated with 3 µL of rinsed cells from early stationary cells grown in BHI with 330 mM or 110 mM urea. Final OD values of cultures from 96-well plates were calculated from 20-fold dilutions that were measured in 1 cm pathlength cuvettes.

### *Urea concentration measurements*

Urea was detected by colorimetrically comparing sample dilutions to a standard curve of known concentrations. Standard samples ranged from 2 mM to 0.25 mM. For a single measurement, 10 µL of sample was mixed well with 200 µL of chromogenic reagent and incubated at 80 °C for 10 min before observing the absorbance of a 150 µL aliquot at 525 nm. Chromogenic reagent was 1 part Reagent UA (per 100 mL: 500 mg diacetylmonoxime, 10 mg thiosemicarbazide) and 2 parts Reagent UB (per 600 mL: 100 mL 85% phosphoric acid, 300 mL 95 – 98% sulfuric acid, 100 mg ferric chloride).

### *Urease activity measurements*

Urease activity was measured discontinuously as the time-dependent and enzyme-dependent production of ammonia upon addition of urea to a reaction. Master mixes of reactions were built on ice in a single tube containing a buffer solution (100 mM HEPES pH 7.5, 100 mM KCl, 1 µM $NiCl_2$) and cells or enzyme contained in the same buffer solution. Reactions were started with 10 µL of 1 M urea addition. Reactions were stopped via addition of 100 µL volume of 10% HCl. Concentrations of $NH_3$ were detected in each of the five timepoint tubes via the Berthelot method in which ammonia concentrations are measured colorimetrically and compared to a standard curve (Weatherburn 1967).

## RESULTS

### *Identification of appropriate urea concentrations for S. pasteurii growth*

*Sporosarcina pasteurii* DSM33 is a workhorse organism for MICP (Carter et al. 2023). In media, urea can primarily serve two roles. Urea can serve as a nitrogen source for some bacteria in some environmental conditions, and urea hydrolysis can acclimate a medium to an elevated pH for growth of organisms such as *S. pasteurii* that require a high pH for optimal growth.

Reports suggest that *S. pasteurii* growth requires urea concentrations up to 330 mM in nutrient rich media (Gibson 1934; Pei et al. 2021). However, the control and the role of urease in *S. pasteurii* is distinct compared to other bacteria. Independent of the presence of urea, urease

activity is similar in *S. pasteurii* cells grown in media rich with other nitrogen-containing molecules (Mörsdorf and Kaltwasser 1989). Also, in *S. pasteurii* the two enzymes that are responsible for collecting the urease-generated $NH_3$ as a nitrogen source are either absent (glutamine synthetase) or low in activity (glutamate dehydrogenase). Instead, the $NH_3$ accumulates in the growth medium (Mörsdorf and Kaltwasser 1989). Altogether, these facts suggest that the $NH_3$ is useful to *S. pasteurii* cells but not for the same roles as in other bacteria.

To better understand the effects of urea in *S. pasteurii* cultures, we monitored the growth of *S. pasteurii* in multiple common lab-scale media with various urea concentrations and various initial pH values (Figure 2).

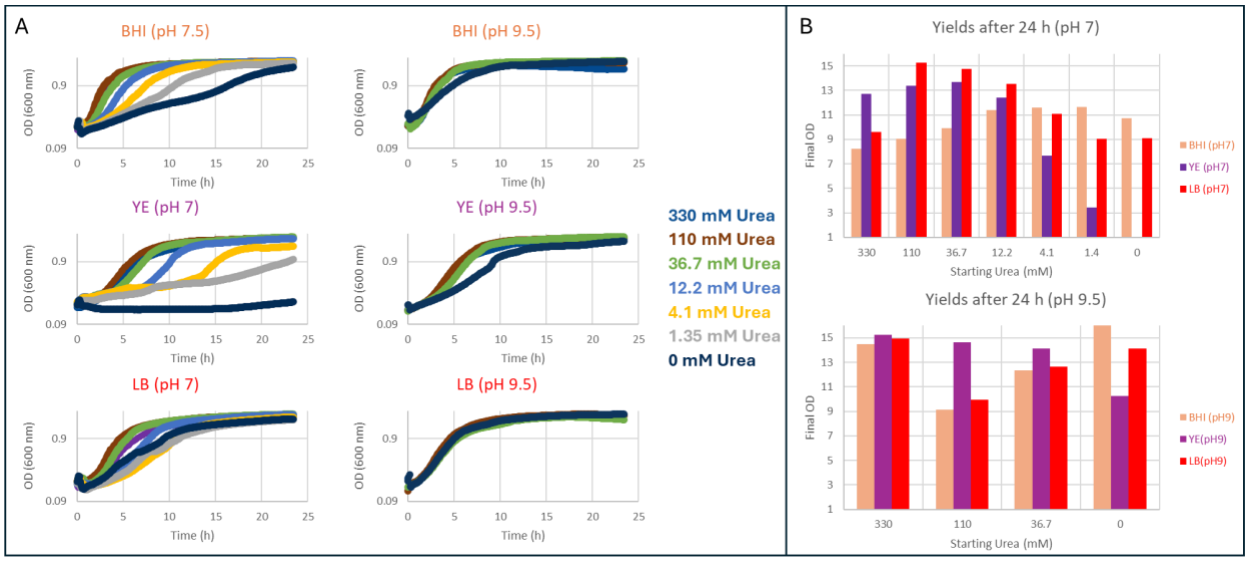


**Figure 2. Growth rates and growth yields were observed for *S. pasteurii* in various lab-scale growth conditions. A) Growth rates were measured in lab scale media with various urea concentrations and at pH 7.0/pH 7.5 or pH 9.5. Only 330, 110, 36.7, and 0 mM urea concentrations were investigated in pH 9.5 media. B) Because the upper limit of the linear range of the plate reader was OD600 ~1, final OD600 values (growth yields) were calculated by measuring OD600 of dilutions of the cultures represented in A. Abbreviations: BHI, Brain Heart Infusion; YE, Yeast Extract; LB, Lysogeny Broth.**

In all media investigated at pH 7 or 7.5, we observed higher growth rates in cultures with urea concentrations between 36.7 mM and 110 mM urea than in the more commonly used 330 mM urea media. In LB, growth rates were least dependent on urea concentrations.

In media initiated at pH 9.5, all growth rates were similar for a given medium, except during growth with 0 mM urea in BHI or YE. Again, growth rates with LB were least dependent on urea concentration.

Growth yields in pH 7.0 or 7.5 media were higher at urea concentrations lower than the commonly used 330 mM. In LB, the growth yield was nearly 50% greater with 110 mM urea than in media with 330 mM. Growth yields in pH 9.5 media however were reduced in cultures with urea concentrations lower than 330 mM.

Altogether, these results suggest that the classical growth of *S. pasteurii* in pH 7.0 or pH 7.5 media with 330 mM urea media is mildly detrimental to growth rate and more detrimental to

growth yield. Instead, growth in LB with a concentration between 36.7 and 110 mM urea is preferable for rapidly obtaining the greatest number of cells.

### *Optimizing for commercial-scale growth*

Common bench-scale media are too expensive to use at the commercial scale. Common commercial scale media are yeast peptones or byproducts of agricultural or food production streams (Babakhani et al. 2021; Fang et al. 2019; Omoregie et al. 2018). CMOs tend to charge customers either per liter of production or per production time. In both cases, there is commonly no overnight crew for harvesting cells. Therefore, growth must only be fast enough to reach final yields during windows of time within a work-day or during subsequent work-days. Therefore, if cells grow fast enough, producing cells faster is not necessarily advantageous.

Also, at the commercial scale, CMOs tend to seek strategies to avoid excessive production of toxic gases such as $NH_3$. Based on our work in lab-scale media, we were confident that we could identify a growth medium that would mitigate $NH_3$ without sacrificing growth rate or growth yield. Similar to our investigations with lab-scale media, we observed 24 h growth yields in media with nutrient components that were up to 100X less expensive than the common lab-scale media and with 12 mM urea (Figure 3), the lowest urea concentration that was effective in each tested lab-scale medium.

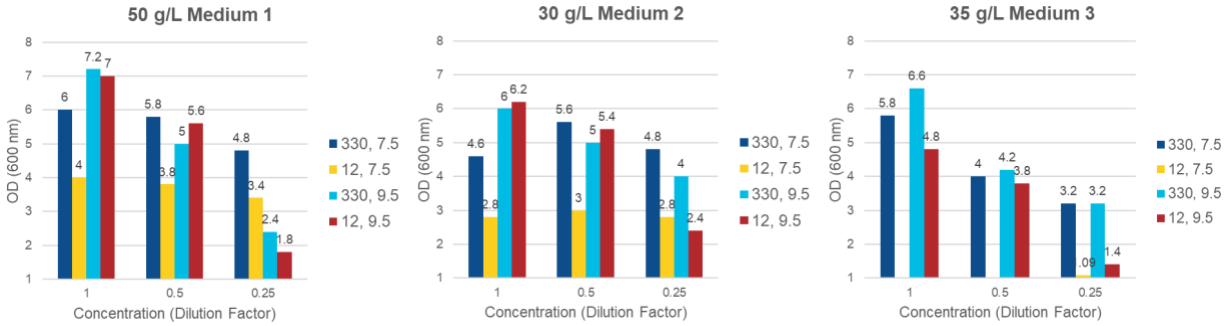


**Figure 3. Growth yields after 24 h of growth in media containing various nutrient sources that are cost effective for commercial-scale bacterial growth. Each nutrient source (distinct from media in Figure 2) was provided at dilutions indicated below each plot relative to the concentration listed above each plot. Media were prepared at pH 7.5 or pH 9.5 with 330 mM or 12 mM urea as indicated in the key for each plot.**

In all cases, except Medium 1 at the 0.25X concentration, cultures in pH 9.5 media performed roughly as well or better than equivalent pH 7.5 cultures. This allowed us to develop culturing conditions that will potentially produce >25-fold less $NH_3$ than more traditional conditions used at the lab-scale.

### *Isolation of other MICP-capable bacteria*

Beyond the cost of production, reducing commercialization costs can include reducing costs associated with intellectual property rights. All of the aforementioned work was performed with *S. pasteurii* DSM33, a strain whose intellectual property rights belong to the American Type Culture Collection (ATCC). Producing commercial products using bacteria from national strain collections

requires negotiating royalty rates that are not required for bacterial strains that are isolated from the environment. To expand our commercialization options to include a strain unencumbered by intellectual property rights, we isolated soil bacteria from rural areas in Ohio and Indiana. Our isolation scheme is described in Figure 4.

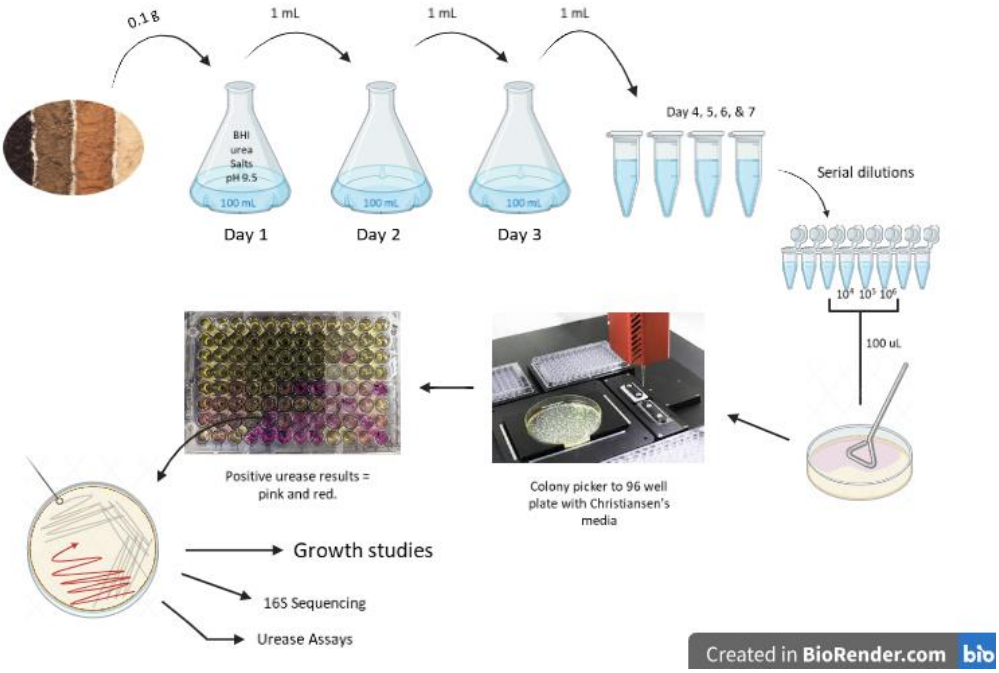


**Figure 4. MICP-capable bacterial strains were isolated and characterized from various soils. Soil was used to inoculate pH 9.5 media that contained complex nutrients, salts, and urea. In succession, a 7-day series of cultures (abbreviated to 3 days here for brevity) were incubated and were used to inoculate the next culture in the series. A sample of each culture was serially diluted and spread plated. Colonies from plates with isolated colonies were used as to inoculate wells of a 96-well plate that contained a medium that becomes pink in the presence of active urease enzyme. Cells from pink wells were streak plated, and isolated colonies were phenotypically and genetically characterized.**

We isolated >200 strains with 16S rRNA gene sequences that align with species in genera such as *Klebsiella*, *Bacillus*, and *Sporosarcina*. Of the strains that we isolated, strain AFRL 25 was a top candidate because it grew rapidly to high yields independent of urea concentrations (Figure 5). Without a urea dependence for growth, our CMO partners would not need to manage $NH_3$ production while growing AFRL 25. Urease activity, however, was variable for AFRL 25 cells depending on growth phase. Cells were grown in BHI with 110 mM urea and collected in mid-exponential phase (OD ~0.6) and early stationary phase. No urease activity was detectable from lyophilized cells or in cell extracts from cells harvested in mid-exponential phase, but urease activity was detectable in lyophilized cells and cell extracts from cells harvested in early stationary phase (Table 1).

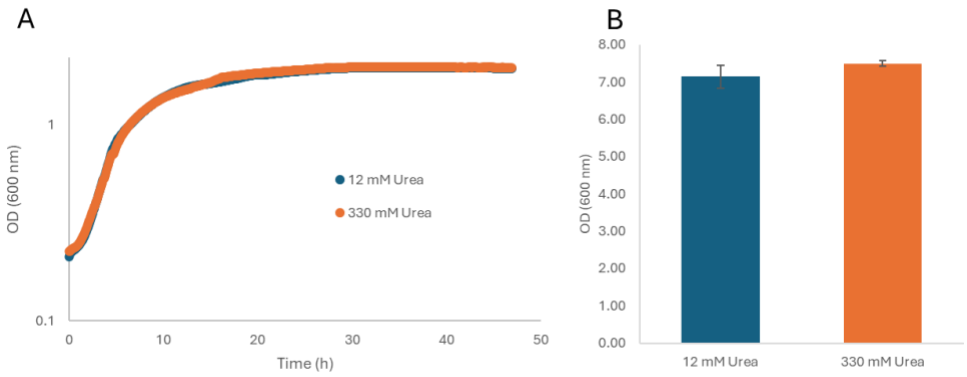


**Figure 5. Growth rates and growth yields of strain AFRL 25 in BHI with variable urea concentrations. A) Growth rates were measured in BHI with 12 or 330 mM urea. All displayed time points represent averages of values from three independent cultures. B) Because the upper limit of the linear range of the plate reader was OD600 ~1, final OD600 values (growth yields) were calculated by measuring OD600 of dilutions of each culture. The displayed values represent averages of three independent cultures. Error bars represent standard deviation.**

The results in Table 1 are best explained by the potential biological control of urease in AFRL 25. Urease in bacteria most commonly serves to scavenge nitrogen from the environment. Bacteria that produce urease commonly control urease production in response to the presence of other nitrogen sources (Liu and Bender 2007; Mobley and Hausinger 1989; Sachs et al. 2001). Although *S. pasteurii* is presumed to produce urease in all growth phases, most urease-producing bacteria only generate urease when the environment is depleted of molecules that can serve as better nitrogen sources and when urea is present.

**Table 1. Urease activity measurements of sources from AFRL 25**

| Urease Source | Growth Phase | Measured Activity* (nmol/min/mg†) |
|---|---|---|
| Cell Extract | Exponential | Undetectable |
| Cell Extract | Stationary | 120 ± 40 |
| Lyophilized Cells | Exponential | Undetectable |
| Lyophilized Cells | Stationary | 150 ± 40 |

* Activity values are reported as the average of two or three individual measurements ± the range of the measurements.
†For cell extract, mg = mg of protein; for lyophilized cells, mg = mg of lyophilized powder

To further investigate whether AFRL 25 controls urease production in response to environmental nitrogen availability, we grew AFRL 25 in BHI with 120 mM urea and monitored urea concentrations throughout growth (Figure 6). We observed that unlike *S. pasteurii* DSM33 (Figure 6B), strain AFRL 25 did not begin consuming urea until late into exponential growth (Figure 6A), suggesting that urease is only produced later in growth as other nitrogen sources are depleted from the medium.

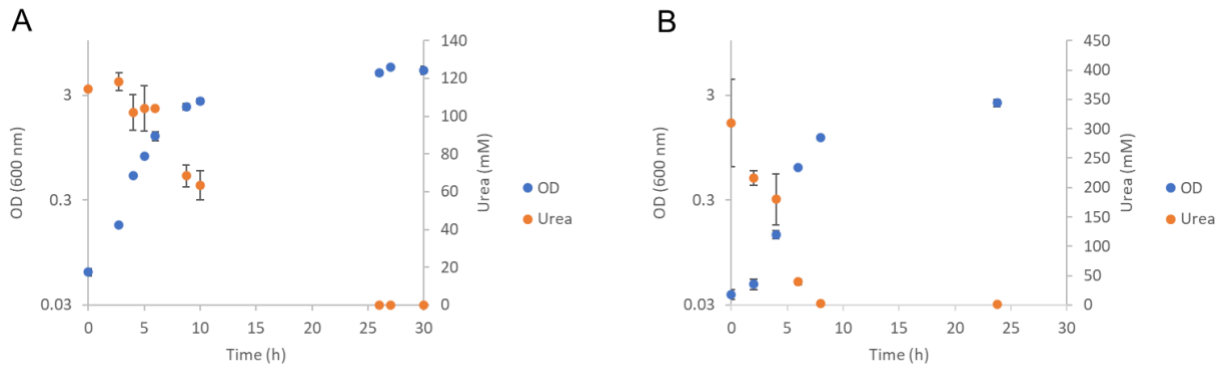


**Figure 6. Urea consumption of AFRL 25 and *S. pasteurii* DSM33 in different growth phases. AFRL 25 was grown in BHI with 120 mM urea (A), and *S. pasteurii* DSM33 was grown in BHI with 330 mM urea (B). All data were collected in triplicate cultures. Data points represent the average values of the triplicate measurements. Error bars represent the standard deviation for each average data point.**

We next wanted to ensure that strain AFRL 25 was capable of performing MICP. To investigate cementation, a suspension of bacterial cells was added to round cylindrical columns (2 in. x 0.75 in.) that were packed with hardware store play sand. After 4 h, 8 mL of cementation solution was added to the top of each column. See Table 2 for a list of cementation solution components. Seven additional (eight total) applications of cementation solution were added in 4 h intervals. The bacterial suspensions were either freshly grown cultures or suspensions of cells that had been preserved as a lyophilized powder. Our first attempt at performing cementation with AFRL 25 used Cementation Solution 1 and ended with no cemented material.

**Table 2. Cementation solution components for various experiments**

| Cementation Solution | Urea (mM) | $CaCl_2$ (mM) | Nutrient Broth (g/L) | Yeast Extract (g/L) |
|---|---|---|---|---|
| 1 | 330 | 330 | 3 | 0 |
| 2 | 330 | 330 | 0 | 1 |
| 3 | 330 | 330 | 0 | 0 |

After considering that urease is unlikely to be produced in AFRL 25 while other better nitrogen sources are available, we performed a second attempt at cementation, adjusting the composition of the cementation solution to remove nutrient broth, which contains animal peptones rich in nitrogen-containing amino acids. Columns that were cemented in the absence of nutrient broth were more solid than columns cemented in the presence of nutrient broth (Figure 7). Unfortunately, we have not yet been able to make quantitative measurements of the unconfined compressive strength of the columns to compare the strength to columns cemented with *S. pasteurii*.

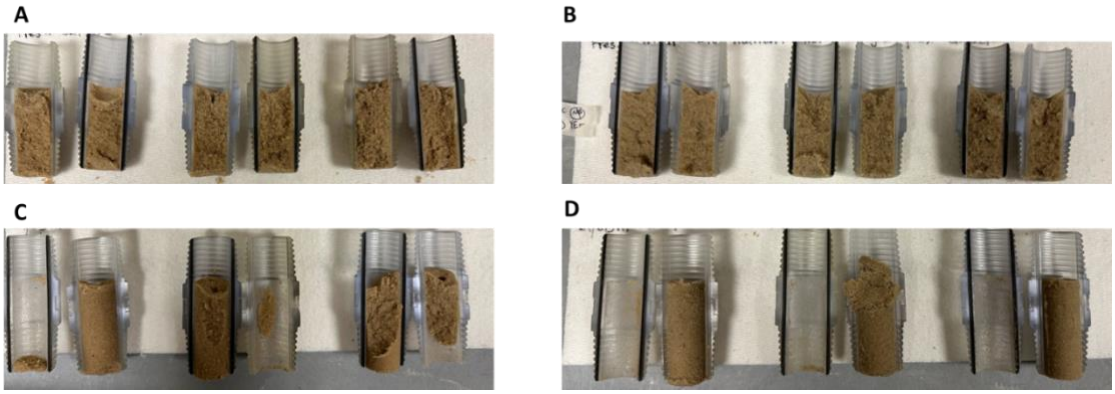


**Figure 7. Sand columns following cementation attempts with AFRL 25. Columns were treated with cells harvested directly from stationary cultures (A and B) or a suspension of lyophilized cells from the same culture volume (C and D). Columns were then treated eight times with Cementation Solution 3 (A and C) or Cementation Solution 2 (B and D) in 4 h intervals. The columns were incubated at room temperature for 24 h, disassembled, and photographed.**

## CONCLUSION

While there are a nearly overwhelming number of publications describing ways in which *S. pasteurii* can be used for commercial infrastructure, many of the publications employ conditions that are not financially scalable. We have worked to identify media and growth parameters that are more financially scalable and more practical at scale.

We observed that while most work in the field uses media with 330 mM urea, concentrations as low as 12 mM urea can support growth that is sufficiently rapid and produces equivalent growth yields. Lower urea concentrations enable successful commercial growth by mitigating $NH_3$ production that is otherwise challenging for CMOs to manage. We further identified nutrient sources for *S. pasteurii* growth that are up to 100-fold more cost effective than traditional lab scale media.

We have also begun to isolate strains such as AFRL 25 that are unencumbered by royalty rights and that do not require urea during growth. Without a urea requirement, AFRL 25 and similar strains can minimize the toxic $NH_3$ off gassing that challenges the scaled production of *S. pasteurii* by many CMOs.

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