KINETICS OF CALCITE PRECIPITATION BY UREOLYTIC BACTERIA UNDER AEROBIC AND ANAEROBIC CONDITIONS

by

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ABSTRACT

Carbonate precipitation is a natural phenomenon with a great importance in many chemical and engineering applications. Precipitation can be induced by bacteria as a by-product of common microbial processes, such as ureolysis. In this process, bacteria hydrolyze urea through a series of reactions which raise the pH of the system. In the presence of calcium ions, this rise in pH shifts the saturation state of the system, allowing for solid calcium carbonate ($CaCO_3$) to form. The use of these bacteria in biotechnical applications is appealing because urea is a fairly inexpensive substrate, and ureolytic bacteria are common in soil and aquatic environments. Bacteriogenic mineral plugging is an innovative use for this process. This technique controls subsurface fluid movement through the reduction of porosity and permeability of geologic formations, such as oil wells and aquifers. A potential use of this technology is in geologic carbon sequestration, which involves capturing CO_2 and storing it underground in deep saline aquifers.

The goal of this project is to determine the kinetics of urea hydrolysis and $CaCO_3$ precipitation for use in the deep subsurface to mitigate potential leakage pathways of sequestered CO_2. To achieve this goal, three species of ureolytic bacteria, *S. pasteurii*, *B. sphaericus* strain 21776, and *B. sphaericus* strain 21787, were grown in batch systems under static conditions. Kinetic analysis was performed on the data gathered in these experiments. Due to the potential lack of oxygen in the deep subsurface, experiments using *S. pasteurii* were also carried out under anaerobic conditions. Because of the potential need to manipulate the rate of $CaCO_3$ precipitation to allow maximum distribution in the deep saline aquifers, the rates of urea hydrolysis and $CaCO_3$ precipitation among species and between aerobic and anaerobic conditions were compared.

All three species studied were capable of inducing calcite precipitation. *B. sphaericus* strain 21776 exhibited the highest rate coefficient for both ureolysis and $CaCO_3$ precipitation, while *B. sphaericus* strain 21787 showed the lowest. *S. pasteurii* is capable of hydrolyzing urea and inducing calcite precipitation in anaerobic environments, although growth in these environments could not be shown conclusively.

INTRODUCTION

Carbonate Precipitation

Carbonate precipitation is a common natural phenomenon found in environments that are oversaturated in carbonate ions, such as marine water, fresh water, and soils. Precipitation can occur via abiotic and biotic pathways. Abiotic precipitation occurs in supersaturated solutions through evaporation, temperature increases, and pressure decreases (Castanier et al., 1999). Biotic precipitation can be either biotically controlled or biotically induced. When an organism exerts some sort of control over the location, size, and composition of the minerals formed, like skeletons and shells, the process is said to be biotically *controlled* (Frankel and Bazylinski, 2003). If the precipitation arises as a result of the metabolic activity of an organism, and the organism has little or no control over the mineralization, the process is biotically *induced* (Frankel and Bazylinski, 2003).

Carbonate precipitation has great importance in many environmental and chemical engineering applications. Abiotic precipitation has been used for purposes as wide ranging as permeability reduction in unconsolidated soils (Bird and Putman, 2008) to methods for carbon dioxide disposal (Lackner et al., 1995). Biologically induced carbonate precipitation by bacteria has been proposed for several biotechnological applications.

Microbial Carbonate Precipitation

Carbonate mineralization by bacteria can occur through active or passive pathways. Active precipitation occurs as a by-product of common microbial processes such as photosynthesis, urea hydrolysis, sulfate reduction, and iron reduction (Knorre and Krumbein, 2000). These processes can lead to an increase in pH in the environment surrounding the bacteria, which in turn alters the saturation state of carbonate and other ions, such as calcium and iron. This new saturation state allows for the carbonate to precipitate out of solution as calcium carbonate (calcite, aragonite, or vaterite), magnesite, siderite, dolomite, or any number of carbonate minerals. One engineering application for active carbonate precipitation is the use of iron (III) reducing bacteria to stabilize fly ash, a residue generated by the combustion of coal, into siderite ($FeCO_3$) and calcite ($CaCO_3$) (Roh et al., 2001). A proposed conservation treatment of carbonate stones in monuments and statues, which are deteriorating due to air pollution (Rodriguez-Navarro and Sebastian, 1996) uses *Myxococcus xanthus*, a bacterium capable of precipitating vaterite by active nucleation (Rodriguez-Navarro et al., 2003). The treatment is used to waterproof and/or strengthen stone surfaces to keep water and other weathering agents from entering the core of the stone. In passive carbonate precipitation, heterogeneous nucleation on negatively charged points of bacteria attracts positively charged ions, allowing for the precipitation of carbonate (Frankel and Bazylinski, 2003). Calcium carbonate is one of the most common products of carbonate precipitation, as both calcium and carbonate ions are abundant in natural environments.

Calcite Precipitation by Ureolysis

One of the most studied pathways of the microbial precipitation of calcite involves urea hydrolyzing bacteria. The ureolytic process is fairly straightforward. In the first step, urea ($CO(NH_2)_2$) is hydrolyzed to ammonia (NH_3) and carbonic acid (H_2CO_3) in the series of reactions outlined below (Equations 1-3) (Burne and Chen, 2000). The ammonia and carbonic acid equilibrate in water to form bicarbonate (HCO_3^-), ammonium (NH_4^+), and one hydroxide ion (OH^-) (Equations 4 and 5). It is at this step that the pH increase essential to calcite precipitation occurs. This rise in pH shifts the bicarbonate equilibrium to form carbonate ions (CO_3^{2-}) (Equation 6) which, in the presence of soluble calcium (Ca^{2+}), precipitates out of solution as calcium carbonate ($CaCO_3$) if saturation is exceeded (Burne and Chen, 2000; Castanier et al., 1999). The overall reaction from the hydrolysis of urea in the presence of calcium is described by Equation 8.

$$CO(NH_2)_2 + H_2O \rightarrow NH_2COOH + NH_3 \quad (1)$$

$$NH_2COOH + H_2O \rightarrow NH_3 + H_2CO_3 \quad (2)$$

$$CO(NH_2)_2 + 2H_2O \rightarrow 2NH_3 + H_2CO_3 \text{ (Overall, step 1)} \quad (3)$$

$$H_2CO_3 \leftrightarrow HCO_3^- + H^+ \quad (4)$$

$$2NH_3 + 2H_2O \leftrightarrow 2NH_4^+ + 2OH^- \quad (5)$$

$$HCO_3^- + H^+ + 2OH^- \leftrightarrow CO_3^{2-} + 2H_2O \quad (6)$$

$$CO_3^{2-} + Ca^{2+} \leftrightarrow CaCO_3 \quad (7)$$

$$CO(NH_2)_2 + 2H_2O + Ca^{2+} \leftrightarrow 2NH_4^+ + CaCO_3 \text{ (Overall process)} \quad (8)$$

Biotechnological Applications of Bacterial Ureolysis

The use of these bacteria in biotechnological applications is appealing for many reasons. One is that urease, the enzyme that catalyzes the hydrolysis of urea to ammonia and carbon dioxide, is common in a wide variety of soil and aquatic bacteria (Warren et al., 2001), and so the introduction or use of foreign bacteria may not be required. Another is that urea, an important nitrogen compound found in natural environments, is a fairly inexpensive substrate (Hammes et al., 2003a). Also, the use of bacteria to raise the pH in the environment is preferable to the direct injection of a base because the gradual hydrolysis of urea is likely to promote a wider spatial distribution of calcite, whereas the direct addition of base is likely to cause immediate precipitation at the injection site (Ferris et al., 2003).

Wastewater Treatment

One use of urea hydrolyzing bacteria is for the removal of soluble calcium from industrial wastewater associated with paper-recycling plants and landfills (van Langerak et al., 1997). The high calcium concentration in these waters can create problems associated with scaling, such as clogging of pipes and malfunctioning of reactors (Hammes et al., 2003b). One special consideration when treating these systems is that the wastewater needs to be low in nitrogen and kept at near neutral pH (Hammes et al., 2003a) to comply with the Clean Water Act (CWA) (U.S. Environmental Protection Agency, 1972). Calcium can be removed abiotically by chemical crystallization reactors, which involve the addition of base in the presence of nucleation sites, but these are

expensive and can promote high pH environments. Hammes et al. (2003a) investigated the use of bacteria as an alternative for chemical $CaCO_3$ precipitation in this type of environment. The amount of urea added to the system was adjusted to determine whether an adequate amount of calcium could be precipitated while at the same time keeping the pH and nitrogen concentrations at an optimal level. Their results were encouraging, but more studies need to be performed before this application can be put into use.

Soil Improvement

Improvement of soil strength is possible using ureolytic bacteria, but often the desired improvements have limited injection depth and result in a major reduction in permeability (Stocks-Fischer et al., 1999), an undesired characteristic that can cause the redirection of the natural groundwater, leading to soil failure. Reduced permeability has been shown to occur when a mixture of bacteria and reagents are either sprayed on the soil, or injected into the soil at high velocity and pressure (Whiffin et al., 2007). Whiffin et al. (2007) determined that soil strengthening can be achieved and permeability maintained when the bacteria and reagents are injected at low velocities, and the urea hydrolysis rate is balanced with the reactant flow rate to achieve soil strengthening in the desired locations.

Environmental Remediation of Radionuclides

Department of Energy (DOE) operations in the western U.S. have left groundwater contaminated with divalent metals (Pb, Zn, Cd), and radionuclides (^{90}Sr, UO_2^{2+}, and ^{60}Co) (Riley and Zachara, 1992). It is possible that some of these elements

can be incorporated into calcite crystals either by substituting for calcium or by occupying vacancies in the crystal lattice (Veizer, 1990). Strontium can readily substitute for calcium. There is evidence that the fine-grained carbonate minerals formed by microbial activity may incorporate more strontium than crystals formed abiotically (Ferris et al., 1995). Bacterial ureolysis is a particularly good application in this situation because the large volume of contaminated material deep in the subsurface calls for a cost effective, *in situ* method for containment and stabilization (Fujita et al., 2000, 2004; Mitchell and Ferris, 2005). Higher ureolysis and precipitation rates have been shown to allow for more Strontium to be incorporated into the carbonate minerals (Fujita et al., 2004; Mitchell and Ferris, 2005). Also, as opposed to abiotic injection of reagents, the bacterially induced precipitation can achieve a wider spatial distribution in the aquifers, allowing radionuclides to be precipitated over a larger area.

Bacteriogenic Mineral Plugging

An innovative use for calcite precipitation by ureolytic bacteria is the plugging of cracks and preferential pathways in porous media. This technique controls subsurface fluid movement through the reduction of porosity and permeability of both geologic formations (Ferris et al., 1996) and manmade structures like concrete and cement (Ramachandran et al., 2001).

Enhanced Oil Recovery: Heavy crude oil, found in Canada, Alaska, Venezuela and other areas, is highly viscous and does not flow well. Because water responds better to pumping than heavy oil, only a portion of the oil contained in some reservoirs can be

recovered. To increase the yield of oil, enhanced oil recovery techniques are used. The most common of these is the injection of gas (carbon dioxide, natural gas, and nitrogen are frequently used), which expands and thereby pushes oil into the well. The injected gas can also diffuse into the oil, thereby lowering its viscosity and making it easier to pump. Alternatively, selectively plugging high permeability areas in the reservoirs may be a way to control excess water production. Ferris et al. (1996) explored the possible use of ureolytic bacteria to precipitate calcium minerals in high permeability water channels. In their studies, indigenous bacteria were grown in sand cores, after which the permeability of the cores was tested. They found a significant reduction in permeability, suggesting that this is a very feasible method for enhanced oil recovery.

Carbon Sequestration: Geologic carbon sequestration involves capturing carbon dioxide (CO_2) from point sources like power plants, and storing it underground in deep saline aquifers instead of allowing its release into the atmosphere. Not much is known about how the CO_2, which will be in a supercritical phase ($scCO_2$) at the pressure and temperatures found in these aquifers, will behave. Zero Emissions Research and Technology (ZERT), a DOE funded project, is developing technologies to monitor and map the movement of CO_2 once it has been injected. There is concern that the CO_2 could migrate back to the Earth's surface via preferential pathways like old well bore holes and cracks in the cap rock. Bacteriogenic mineral plugging can potentially be used to seal these pathways, and keep the CO_2 underground. The $scCO_2$ may also be permanently sequestered if it can be converted into carbonate minerals such as $CaCO_3$.

Kinetics of Calcite Precipitation by Ureolysis

If microbial carbonate precipitation by ureolysis is to be used for biotechnological applications, the kinetics of bacterial growth, urea hydrolysis, and calcite precipitation must be determined. Knowledge of these rates will allow researchers to model the bacteria-mineral interactions for a wide variety of environments, as well as determine the feasibility of a certain application to solve a given problem. Some research in this area has been performed (Ferris et al., 2003; Fujita et al., 2000, 2008; Stocks-Fisher et al., 1999) and is summarized below.

Variation of Initial Calcium Concentrations

One of the earlier studies to look at the kinetics of calcite precipitation by ureolytic bacteria was performed by Stocks-Fischer et al. (1999). This group was interested in the potential use of bacteria for mineral plugging. The species they used was *Sporosarcina pasteurii* (ATCC 6453, formerly *Bacillus pasteurii*), a highly ureolytic, gram-positive, endospore-forming soil bacterium. Calcium precipitation experiments were carried out in a calcite mineralizing medium (CMM) containing 3 g/L Bacto nutrient broth, 333 mM urea, 187 mM ammonium chloride, 25 mM sodium bicarbonate, and different concentrations of calcium chloride (12.6, 25.2, and 50.4 mM). *S. pasteurii* was grown aerobically at 25°C for 54 hours with shaking. Changes in NH_4^+, Ca^{2+}, and cell concentrations were monitored over time, as well as pH, and are shown in Figure 1.

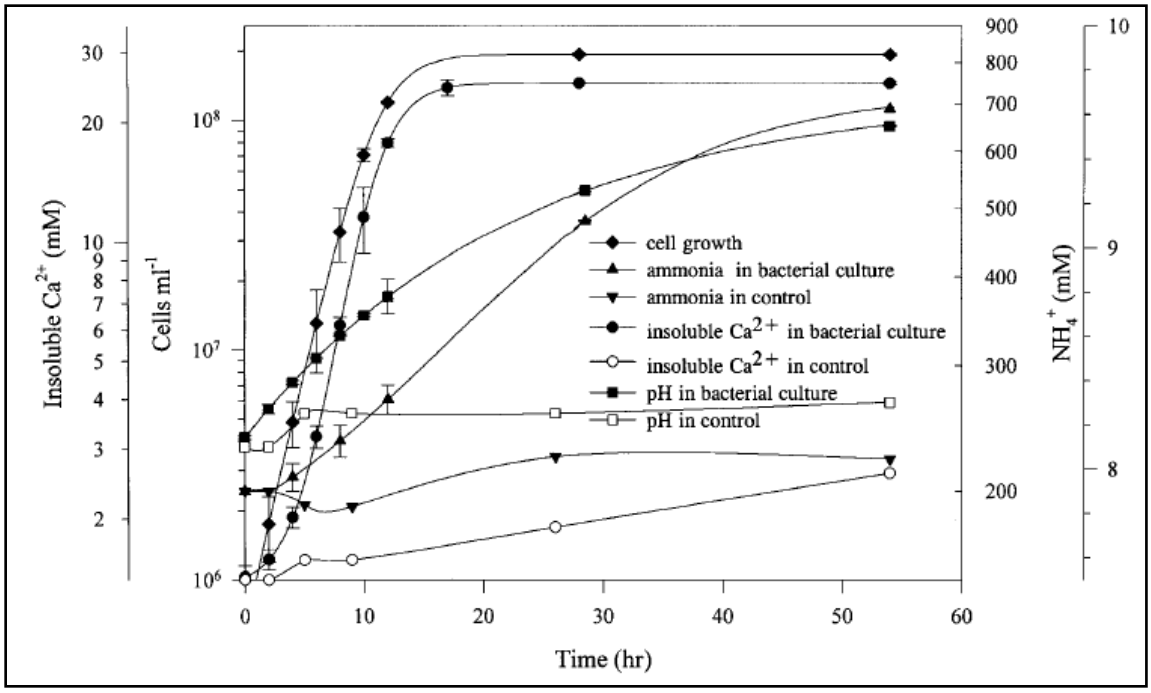


Figure 1: Microbially induced $CaCO_3$ precipitation in the presence of *S. pasteurii* (inoculum size $1x10^6$ cells/mL) at 25.2 mM $CaCl_2$ at 25°C. From Stocks-Fischer et al. (1999). Reprinted from Soil Biology and Biochemistry, vol. 31; S. Stocks-Fischer, J. Galinat, and S. Bang; *Microbiological Precipitation of $CaCO_3$;* pp. 1563-1571; 1999; with permission from Elsevier Science, Ltd.

This study found that the concentration of insoluble calcium was proportional to bacterial growth and inversely proportional to ammonia production. They proposed that the kinetics of calcite precipitation followed a modified logistic curve (Marquardt, 1963), and found their rate constants for $CaCO_3$ formation and ammonia production (Table 1) by regression analysis on the equation:

$$y = \left(\frac{a}{1 + e^{-b(x-c)}} \right) + d \quad (9)$$

where y is the concentration of Ca^{2+} or NH_4^+, a is the range of Ca^{2+} or NH_4^+ variation, b is the rate constant, x is time, c is the time at the maximum (dy/dt), and d is the initial

concentration of Ca^{2+} or NH_4^+. They determined that the rate constants at the different calcium concentrations were virtually the same, suggesting that the rate of urea hydrolysis and calcium precipitation were independent of the initial Ca^{2+} concentration.

Table 1: Rate coefficients for microbiologically-induced precipitation of $CaCO_3$ by *S. pasteurii* at different initial concentrations of $CaCl_2$[a]. From Stocks-Fischer et al. (1999).

	Initial Concentration of Ca^{2+} (mM)		
Rate Constant	12.6	25.2	50.4
$k_{ammonia}$ (hr^{-1})	0.76	0.77	0.72
k_{precip} (hr^{-1})	0.10	0.10	0.13

[a] Inoculum 1 x 10^6 cells/mL

In their experiments, Stocks-Fischer et al. also grew cells in columns filled with sand, which were fed media continuously from gravity. When the columns became plugged after 10 days, X-ray diffraction (XRD) and scanning electron microscopy (SEM) were performed to determine the morphology of the calcium carbonate as well as to get a sense of the interactions between $CaCO_3$ and cells. It was determined that the crystals formed were calcite. They also showed that the bacteria become embedded in the cemented material, a mixture of sand and calcite. The researchers hypothesized that at low cell concentrations, $CaCO_3$ mineral formation occurred due to passive precipitation, with cells being used as nucleation sites. After this initial precipitation, the crystals continued to grow due to the metabolic activities of the bacteria (active precipitation).

They proposed the following set of reactions to describe the precipitation of $CaCO_3$ at the surface of the cell:

$$Ca^{2+} + Cell \rightarrow Cell - Ca^{2+} \quad (10)$$

$$CO_3^{2-} + Cell - Ca^{2+} \rightarrow Cell - CaCO_3 \quad (11)$$

Variation in Initial Cell Concentrations

A similar study by Fujita et al. (2000) investigated calcite precipitation kinetics for the purpose of co-precipitation experiments of heavy metals and radionuclides. They determined the calcite precipitation and urea hydrolysis rates of *S. pasteurii* (ATCC 11859) and bacterial isolates from the Eastern Snake River Plain (ESRP) Aquifer. Instead of varying the calcium concentration in the medium, this study investigated the difference in rate coefficients for varying initial cell concentrations as measured by optical density at 600 nm (OD_{600}). Initial *S. pasteurii* OD_{600} values were 0.072 and 0.041, while that of the isolates (110AD, 110AF, and 9B) was 0.149. The media used was modified slightly from the media used by Stocks-Fischer et al. (1999). The calcium concentration was 25 mM and no ammonium chloride was added. Cultures were incubated at room temperature without shaking for 8 hours. NH_4^+, Ca^{2+}, and pH were measured over time (Figure 2).

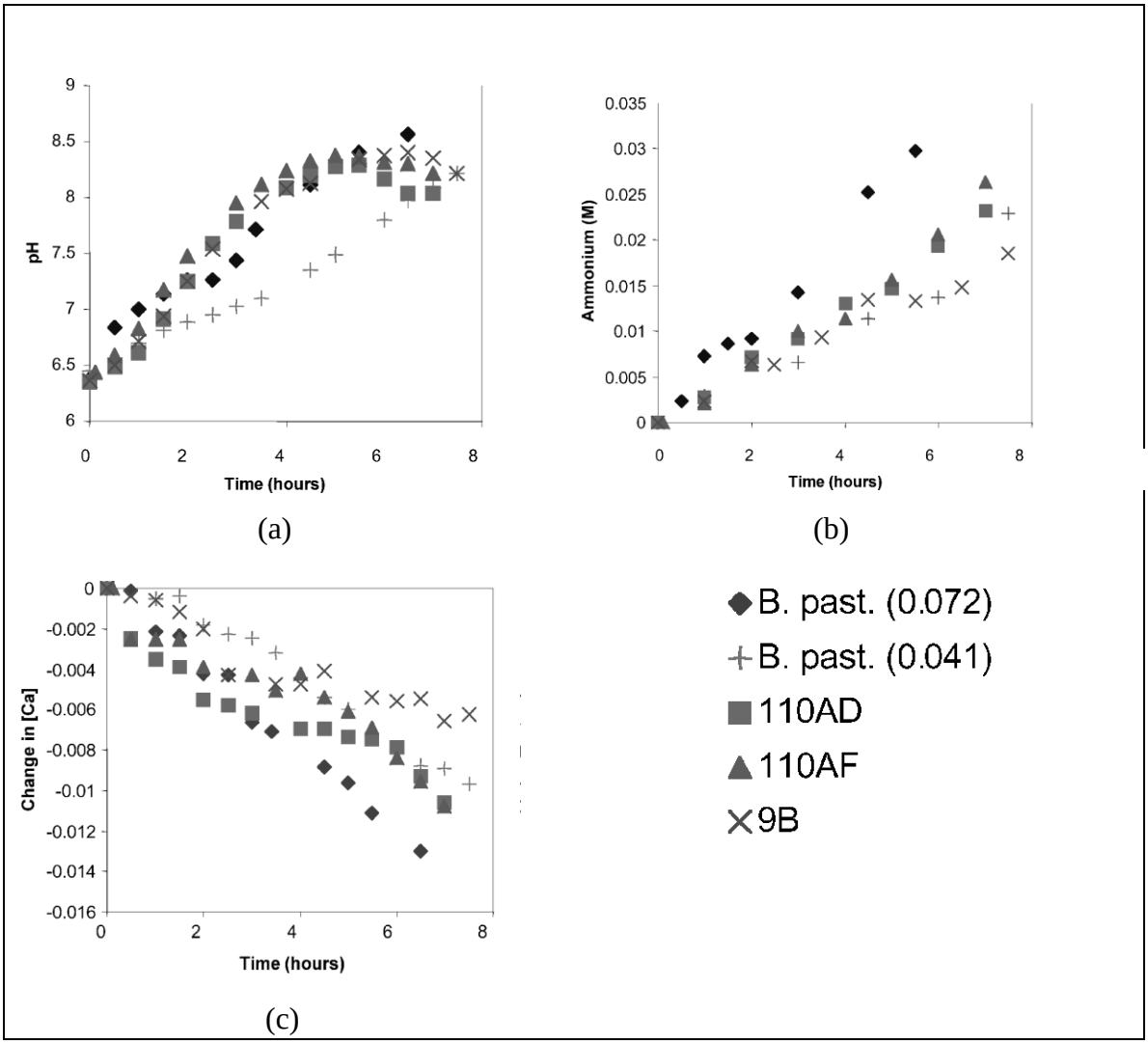


Figure 2: Change in pH (a), ammonium (b) and dissolved calcium (c) over time during calcite precipitation experiments with ESRP isolates and *S. pasteurii*. The units for the change in [Ca] are M. From Fujita et al. (2000). Reprinted from Geomicrobiology Journal, vol. 17; Y. Fujita, F.G. Ferris, R.D. Lawson, F. Colwell, and R. Smith; *Calcium Carbonate Precipitation by Ureolytic Subsurface Bacteria;* pp. 305-318; 2000; with permission from Taylor & Francis.

Kinetic data for urea hydrolysis were determined by linear regression. These studies showed that the *S. pasteurii* culture with the higher OD_{600} value hydrolyzed urea more rapidly than the other two cultures, which exhibited similar hydrolysis rates (see Table 2). This indicates the urease activity in *S. pasteurii* is considerably higher than that in the isolates. With all strains, ammonium concentration increased linearly with time. When the ammonium production rate constants of the two *S. pasteurii* experiments are normalized to the starting OD_{600}, both cultures give similar values, signifying that the amount of biomass is critical in determining the rate of urea hydrolysis.

The trend seen in the calcium precipitation rates (Table 2) mimicked those of ammonium production, i.e., the *S. pasteurii* culture with the higher OD_{600} value exhibited a faster precipitation rate than the other two cultures, which showed similar precipitation rates. Kinetic data for calcium precipitation were determined by linear regression. Figure 3 shows that the rate of calcite precipitation was directly proportional to the rate of urea hydrolysis. This contrasts with the results of Stocks-Fischer et al. (1999), who stated that calcite precipitation was directly linked to cell growth and indirectly linked to ammonia production. One explanation for this is that the latter study started their experiments at a pH of 8, so the medium was already supersaturated with calcite; the former study initiated their experiments at a pH of 6.5.

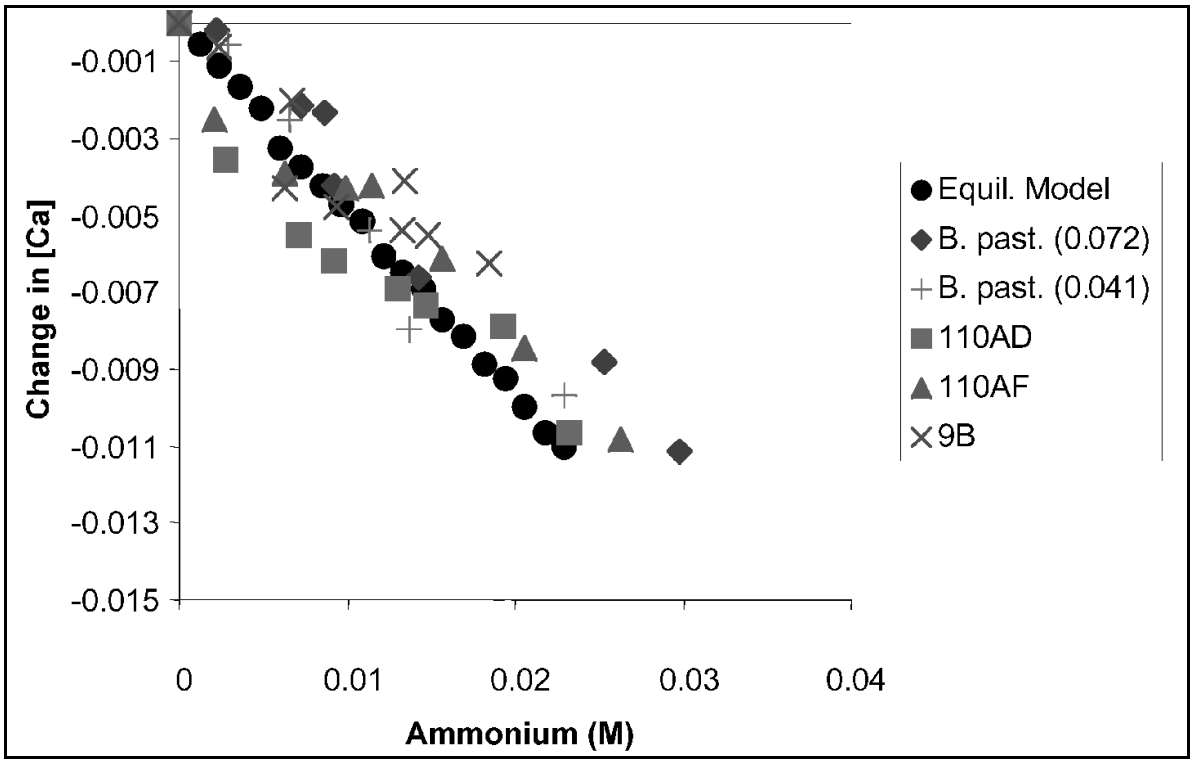


Figure 3: Change in dissolved calcium concentration as a function of ammonium concentration during calcite precipitation experiments with ESRP isolates and *S. pasteurii*. The units for the change in [Ca] are M. From Fujita et al. (2000). Reprinted from Geomicrobiology Journal, vol. 17; Y. Fujita, F.G. Ferris, R.D. Lawson, F. Colwell, and R. Smith; *Calcium Carbonate Precipitation by Ureolytic Subsurface Bacteria;* pp. 305-318; 2000; with permission from Taylor & Francis.

Table 2: Rate Coefficients for ammonium production and calcium precipitation. From Fujita et al. (2000).

Culture	$k_{ammonia}$ (mol/hr)	k_{Ca2+} (mol/hr)
S. pasteurii (0.072)[a]	0.0054	-0.0020
S. pasteurii (0.041)[a]	0.0027	-0.0012
110AD	0.0032	-0.0011
110AF	0.0034	-0.0014
9B	0.0025	-0.0010

[a] *S. pasteurii* (0.072) and *S. pasteurii* (0.041) had initial cell suspension OD_{600} of 0.072 and 0.041. OD_{600} of ESRP isolates was 0.149.

Urea Concentration and Temperature Variations

In a study expanding on the results found by Fujita et al. (2000), Ferris et al. (2003) investigated the kinetics of calcite precipitation by *S. pasteurii* (ATCC 11859) in artificial groundwater (AGW) based on the aqueous chemistry of the Snake River Plain Aquifer over varying temperatures (10, 15, and 20°C) and urea concentrations (6 mM for 20°C experiment, 25 mM for 10 and 15°C experiments). Initial pH for these experiments was 6.5, and they were run with static incubation for seven days. Changes in NH_4^+ and Ca^{2+} (Figures 4 and 5, respectively), as well as pH were determined over time.

A more comprehensive kinetic model was used on the data for this experiment than the linear regressions used in the previous two studies. Urea hydrolysis was assumed to be first order with respect to urea concentration:

$$\frac{d[urea]}{dt} = -k_{urea}[urea] \quad (12)$$

After integration and use of the stoichiometry of Equation 3, the concentration of urea at time *t* can be described in terms of measured and known variables:

$$[NH_4^+]_t = 2[urea]_o(1 - e^{-k_{ured}t}) \quad (13)$$

The change in ammonium ion concentration over time is shown in Figure 4.

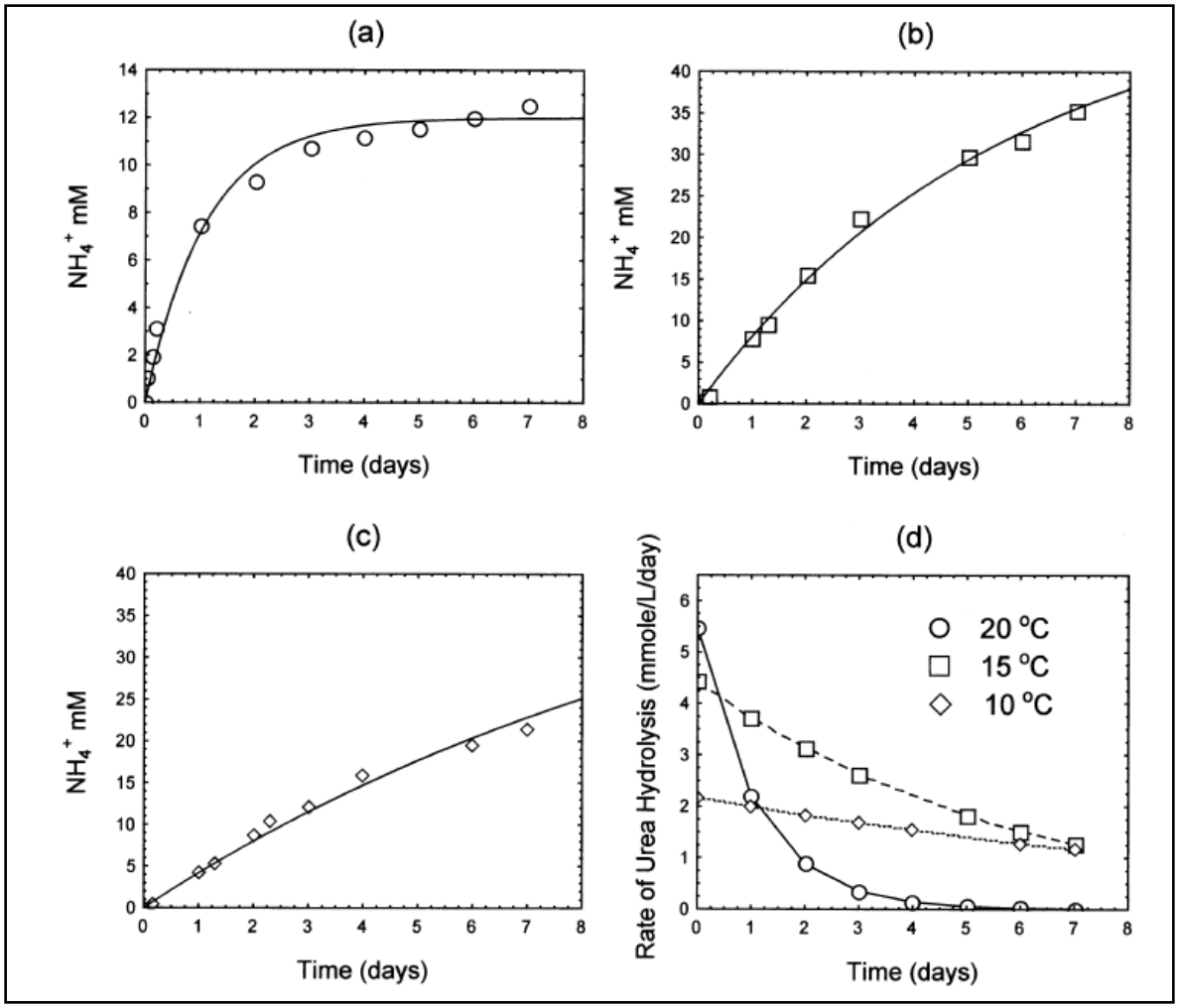


Figure 4: Dissolved ammonium concentrations over time from hydrolysis of urea by *S. pasteurii* at a) 20° C, b) 15°C, and c) 10°C. Data are the mean values of triplicate experiments. d) Calculated urea hydrolysis rates for each experimental temperature. From Ferris et al., 2003. Reprinted from Geochimica et Cosmochimica Acta, vol. 68(8); F.G. Ferris, V. Phoenix, Y. Fujita, and R. Smith; *Kinetics of Calcite Precipitation Induced by Ureolytic Bacteria;* pp. 305-318; 2000; with permission from Elsevier Science Ltd.

The precipitation of calcium was assumed to be a nonlinear function of the saturation state (S) of the solution with respect to calcite. S can be determined by the equation:

$$S = \frac{\{Ca^{2+}\}\{CO_3^{2-}\}}{K_{S0}} \quad (14)$$

where $\{Ca^{2+}\}$ and $\{CO_3^{2-}\}$ represent the activities of dissolved Ca^{2+} and CO_3^{2-} in the media, and K_{S0} is the solubility constant for calcite at each given temperature. The activity coefficients were found using the extended Debye-Hückel equation as given by Stumm and Morgan (1996). Experimental data were used to determine the concentration of the calcium ion in solution. The concentration of the carbonate ion was determined using the relationship:

$$[CO_3^{2-}] = \alpha_2 C_T \quad (15)$$

where

$$\alpha_2 = \left(\frac{[H^+]^2}{K_1 K_2} + \frac{[H^+]}{K_2} + 1 \right)^{-1} \quad (16)$$

K_1 and K_2 were calculated for each experimental temperature (Stumm and Morgan, 1996). H^+ concentrations were determined from pH measurements. C_T represents the amount of dissolved carbon in the system, which varied with time according to the relationship:

$$C_T = C_{Tinitial} + C_{Turea} - C_{Tcalcite} \quad (17)$$

where $C_{Tinitial}$ is the molar amount of dissolved inorganic carbon in the media, C_{Turea} is the amount of dissolved carbon produced by urea hydrolysis ($[urea]_o$-$[urea]_t$; $[urea]_t$ is

determined after finding k_{urea}), and $C_{Tcalcite}$ is the amount of dissolved inorganic carbon removed from solution by the precipitation of calcite. The decrease in the calcium ion concentration over time for the different experimental temperatures is shown in Figure 5.

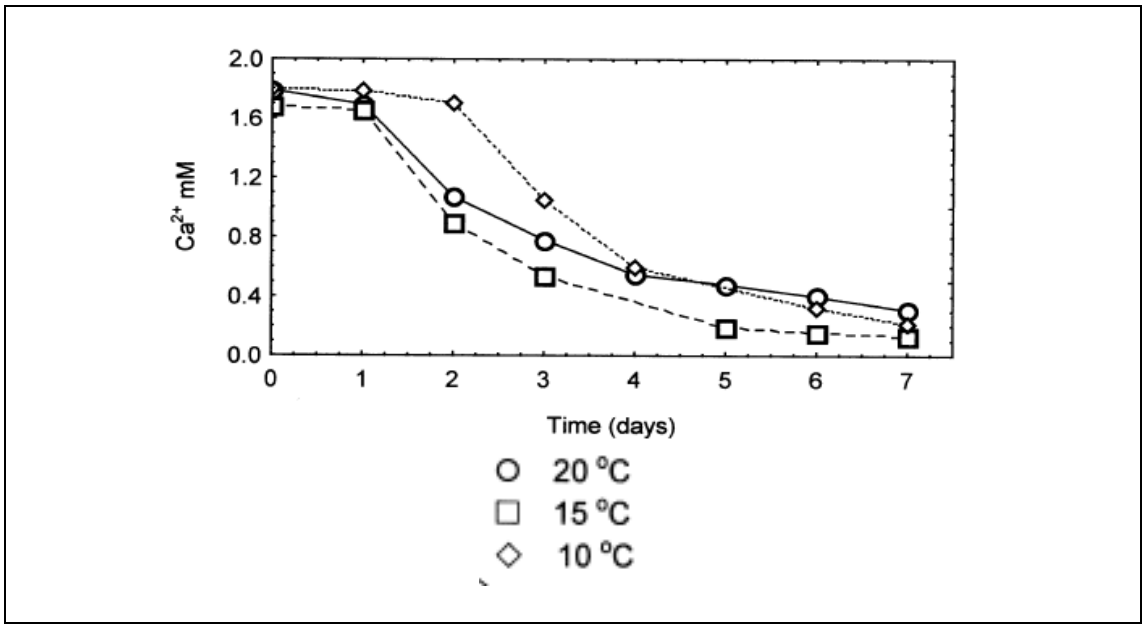


Figure 5: Dissolved calcium concentrations as a function of time. From Ferris et al., 2003. Reprinted from Geochimica et Cosmochimica Acta, vol. 68(8); F.G. Ferris, V. Phoenix, Y. Fujita, and R. Smith; *Kinetics of Calcite Precipitation Induced by Ureolytic Bacteria;* pp. 305-318; 2000; with permission from Elsevier Science Ltd.

The decrease in saturation index after the onset of calcite precipitation is assumed to be first order with respect to time:

$$\frac{dS}{dt} = -k_s S \quad (18)$$

Integration gives the expression:

$$S_t = S_o e^{-k_s t} \quad (19)$$

where S_o is the apparent saturation state if precipitation had occurred at time zero.

The rate of calcite precipitation is then determined by a nonlinear empirical relationship (Teng et al., 2000), where k_p is the rate coefficient of calcite precipitation:

$$\frac{d[Ca^{2+}]}{dt} = -k_p (S-1)^2 \quad (20)$$

The critical supersaturation, $S_{critical}$, is the saturation state where calcium precipitation actually begins. It is determined by integrating the expression:

$$\frac{d[Ca^{2+}]}{dS} = \frac{k_p (S-1)^2}{k_s S} \quad (21)$$

to get:

$$[Ca^{2+}]_s = \left(\frac{k_p}{k_s}\right)\left[S\left(\frac{S}{2}-2\right) - S_{critical}\left(\frac{S_{critical}}{2}-2\right) + \ln\left(\frac{S}{S_{critical}}\right)\right] + [Ca^{2+}]_{S_{critical}} \quad (22)$$

Ammonium production, calcite precipitation, and saturation rate coefficients found by Ferris et al. (2003), as well as the apparent saturation state and critical saturation state, are shown in Table 3. A strong dependence of temperature on urea hydrolysis was shown. It was also determined that calcite precipitation rates did not vary significantly at different temperatures, although there was a longer lag time before the onset of precipitation at the lower temperatures because urea hydrolysis rates at lower temperatures generated a longer lag time before critical supersaturation was attained. $S_{critical}$ was found to be independent of temperature.

Table 3: Summary of kinetic parameters for urea hydrolysis, change in saturation state, calcite precipitation, apparent saturation, and critical saturation for calcite precipitation at different temperatures. [a] From Ferris et al. (2003).

Temperature (°C)	k_{urea} (day^{-1})	k_s (day^{-1})	k_{precip} (μmol/L/day)	$S_{apparent}$	$S_{critical}$
10	0.09	0.27	0.17	121	72
15	0.18	0.25	0.15	96	76
20	0.91	0.26	0.16	90	70

[a] Inoculum cell suspension had an OD_{600} of 0.14

Research Objectives

This research was conducted as part of a Department of Energy (DOE) sponsored project (ZERT) at the Center for Biofilm Engineering to determine the kinetics of urea hydrolysis and calcite precipitation for use in the deep subsurface to mitigate potential leakage pathways of sequestered CO_2. Because of the potential need to manipulate the rate of calcite precipitation to allow maximum distribution in deep saline aquifers, three species of ureolytic bacteria were analyzed and compared, specifically *Sporosarcina pasteurii*, *Bacillus sphaericus* strain 21776 and *Bacillus sphaericus* strain 21787. Deep subsurface environments have the potential of being anaerobic in nature, and so comparing rates of anaerobic ureolysis and precipitation to aerobic rates is also beneficial. To study this, *S. pasteurii* was grown under both conditions, and rates were compared.

MATERIALS AND METHODS

Bacterial Growth Media and Inoculum Preparation

Bacterial Strains

S. pasteurii 11859 was purchased from the American Type Culture Collection (ATCC). *B. sphaericus* 21776 and 21787 were purchased from the Belgian Coordinated Collections of Microorganisms, Laboratory of Microbiology, Ghent University (BCCM/LMG). *Bacillus subtilis*, a non-ureolytic organism, was used as a control species. All strains were cultured by Adrienne Phillips, M.S. per instructions from their source. Stocks were prepared in solution of 37 g/L Brain Heart Infusion (BHI; Oxoid, Lenexa, KS), 20 g/L urea (Fisher Scientific, Fair Lawn, NJ) and deionized (DI) water, which was autoclaved for 25 minutes for sterility. Stocks were frozen at -70°C in 20% glycerol (Fisher Scientific, Fair Lawn, NJ).

Growth Media

Kinetic experiments were carried out in the Calcite Mineralizing Medium (CMM) described by Ferris and Stehmeier (1996) (Table 4). Both calcium inclusive (CMM+) and calcium exclusive (CMM-) versions of this media were used.

Table 4: Recipe for Calcite Mineralizing Media

Ingredient	**Manufacturer**	**Concentration**
Nutrient Broth	BD (Franklin Lakes, NJ)	3 g/L
Urea	Fisher Scientific (Fair Lawn, NJ)	333 mM
Ammonium Chloride	Fisher Scientific (Fair Lawn, NJ)	187 mM
Sodium Bicarbonate	Fisher Scientific (Fair Lawn, NJ)	25.0 mM
Calcium Chloride Dihydrate	Acros Organics (Morris Plains, NJ)	25.2 mM
Concentrated HCl	Mallinckrodt (Hazelwood, MO)	Adjusted to pH 6

Aerobic Medium: A double strength solution of nutrient broth was prepared and autoclaved. A separate solution of double strength urea, ammonium chloride, and sodium bicarbonate was prepared and stirred until completely dissolved. These two solutions were combined and adjusted to a pH of 6.0 using concentrated HCl. For CMM+, calcium chloride was added after the pH adjustment. The media was then filter sterilized into a sterile Pyrex media bottle using a 0.2 μm pore size bottle top filter (Nalgene, Rochester, NY).

Anaerobic Medium: Oxygen-free medium was made in a similar manner as the aerobic medium, but was made in an anaerobic chamber using anoxic water. The double strength solution of nutrient broth was adjusted to a pH of 6 before being autoclaved. The double strength solution of urea, ammonium chloride, and sodium bicarbonate was pH adjusted to 6, calcium chloride dihydrate was added for CMM+ media, and the solution was filter sterilized using a 0.2 μm pore size surfactant-free cellulose acetate (SFCA) syringe filter (Corning Inc., Corning, NY) into a serum bottle, capped and sealed. The solutions were then combined to reach the final concentrations listed in Table 4.

A growth survey of *S. pasteurii* was conducted using the media described above, with the addition of 10 mM nitrate, sulfate, and iron as terminal electron acceptors (TEAs). Concentrated stock solutions of each terminal electron acceptor were made in the following ways:

NO_3^-: A concentrated nitrate solution was made using 1M $NaNO_3$, which was mixed and filter sterilized in an anaerobic chamber.

SO_4^{2-}: A concentrated sulfate solution was made by combining 1M Na_2SO_4 and 1M Na_2S and filter sterilizing in the anaerobic chamber. Na_2S was added to quench any residual oxygen and make sulfate reduction possible.

Fe(III): A stock solution of iron (III) citrate was made by adding 1.225 g of ferric citrate to 70 mL of deionized (DI) water in a 100 mL volumetric flask. The flask was heated until the powder dissolved, and the solution was pH adjusted to a pH of 6.5 with $NaHCO_3$. The solution was sparged with N_2 gas for approximately 20 minutes and transferred to an anaerobic chamber. The flask was filled to 100 mL with DI water and placed on a stir plate for 2 hours. This solution was filter sterilized and sealed.

Appropriate amounts of each stock solution were added to separate solutions of CMM. The growth survey was also conducted in CMM without the addition of a TEA.

Anaerobic calcite precipitation experiments were performed with *S. pasteurii* and CMM as described above with nitrate as a terminal electron acceptor. A stock solution of 10M $NaNO_3$ was made by mixing and filter sterilizing in the anaerobic chamber, and an appropriate amount was added to the CMM to reach a final concentration of 1M NO_3^-. Calcite precipitation experiments were also conducted in CMM without the addition of a TEA.

Inocula Preparation

Pilot cultures were started by adding 100 μL of thawed stock to 100 mL of autoclaved BHI/2% urea. *S. pasteurii* and *B. sphaericus* were grown on an incubator shaker (New Brunswick Scientific, Edison, NJ) at 30°C, while *B. subtilis* was grown on an incubator shaker at 37°C. 100 μL of pilot cultures were transferred at 24 hours and 48 hours to new flasks containing 100 mL of BHI/2% urea. The last transfer occurred 13-15 hours before inoculation of the experimental systems.

Aerobic Inoculum: Once the pilot cultures were ready for inoculation, 40 mL (± 12%) of culture was added to a 50 mL microcentrifuge tube. This tube was centrifuged at a relative centrifugal force (RCF) of 4303 x g using a Sorvall Instruments (Asheville, NC) RC-5C centrifuge with a Sorvall PTI F15S 8x50c rotor for 10 minutes at 4-6°C. Once centrifuged, the supernatant was poured off the cell pellet, and about 40 mL (± 12%) of CMM- was added. The centrifuge tube was then gently shaken to resuspend the cells, and again centrifuged for 10 minutes. This process was repeated once more. After the third run in the centrifuge, the supernatant was poured off and enough CMM- was

added to achieve a final absolute OD_{600} reading of 0.4 (± 12%) on 100 μL of inoculum in a 96-well plate. Plate counts and OD_{600} measurements were performed on inocula to estimate the initial concentration of biomass.

Anaerobic Inoculum: Pilot cultures for anaerobic experiments were grown in the same manner as for those used in aerobic work. However, cells were resuspended in an anaerobic chamber with anaerobically prepared CMM-. The final suspension was transferred to a serum bottle, sealed and crimped. Optical density measurements were taken after the final suspension.

Experimental Procedure

System Setup and Design

Kinetic experiments were run using a batch system, consisting of 150 mL of media (either CMM+ or CMM-) for aerobic experiments and 100 mL for anaerobic experiments and inoculated with 1.5 mL of prepared culture (1.0 mL for aerobic experiments) in 250 mL Pyrex bottles for aerobic experiments or in 150 mL serum bottles for anaerobic experiments. After inoculation, the systems were statically incubated at 30°C in a Nuaire (Plymouth, MN) incubator. Samples were taken over the course of 35 hours.

At each sample time, 3 mL of sample (2 mL for aerobic experiments) was aseptically extracted from the system and measurements were made of pH, ammonia concentration, dissolved calcium concentration, and growth via plate counts, optical density, and protein assay.

Bacterial Growth

Growth of bacteria was determined using three methods: plate counts, optical density at 600 nm (OD_{600}), and protein assay. These methods are described below. Optical density was used to adjust the inoculum to the preferred absorbance, and as a growth indicator in experiments carried out in the absence of calcium. Calcite crystals interfered with absorbance readings, and were not an accurate gauge of growth in CMM+ experiments.

Plate Counts: Standard serial dilutions of 10^{-1} to 10^{-6} (10^{-1} to 10^{-8} for inoculum) were made in Phosphate Buffer Saline (PBS) solution, consisting of 8.5 g/L NaCl (Fisher, Fair Lawn, NJ), 0.61 g/L KH_2PO_4 (Fisher, Fair Lawn, NJ), and 0.96 g/L K_2HPO_4 (Fisher, Fair Lawn, NJ). Plates were made of a solution of 37 g/L BHI, 20 g/L urea, and 15 g/L granulated agar (Becton, Dickinson and Co., Franklin Lakes, NJ), which was autoclaved for 30 minutes and cooled to approximately 55°C before pouring. Five 10 μL drops of each dilution were plated in rows on the agar plate, allowed to dry, and the plates were placed in the 30°C incubator. Plates were counted after 48 hours of incubation, and the dilution with 3-30 colony forming units (CFU) per drop was counted. Cell numbers for each plate were determined as follows to obtain CFU/mL:

$$\left[\frac{CFU}{mL}\right] = \left(\frac{CFU/drop}{dilution\ factor}\right) \quad (23)$$

where the dilution factor is the final volume divided by the aliquot volume.

Optical Density: The optical density at 600 nm was used to quantify the turbidity of a solution. Three x 100 μL of sample were added to separate wells of a 96-well plate and read by BioTek Instruments (Winooski, VT) Synergy HT Microplate Reader using KC4 software. The absorbance of a media blank was also measured in this manner. The absorbance of the blank was subtracted from the average of the triplicate readings of the sample to give the relative absorbance at each time point.

The relative absorbance readings from the 96-well plates, where the path length is 0.26 cm, were converted to path lengths of 1 cm using the Beer-Lambert Equation (Appendix B.1). Initial biomass concentrations in the systems were calculated using the absorbance readings from the inoculum and multiplying by the dilution factor of (volume of inoculum)/(total volume).

Protein Concentration: At each sample point, 500 μL of culture was frozen to be tested at a later time for protein concentration. The protein content of the sample was determined using the Pierce Coomassie Protein Assay. Protein standards were made using a 2.0 mg/mL Albumin Standard (ThermoScientific, Waltham, MA). To prepare samples and standards, 200 μL of 1 N NaOH (Fisher Scientific, Fair Lawn, NJ) was added to 200 μL of sample in a microcentrifuge tube to achieve a final concentration of 0.5 N NaOH. Samples were then vortexed (Thermolyne MaxiMix II, Waltham, MA) and digested at 90°C in a water bath (Fisher Scientific, Fair Lawn, NJ) for 10 minutes. After another round of vortexing, the samples were allowed to cool down, whereupon 28 μL of a 6:10 v/v solution of concentrated HCl (Mallinckrodt, Hazelwood, MO) was added. The samples were vortexed again. 50 μL of each prepared sample was added to three separate

wells of a 96 well plate. 150 μL of Coomassie Plus™ Protein Assay Reagent (Pierce, Rockford, IL) was added to each well using a multichannel pipetter. The plate was incubated at room temperature for 15 minutes, and then read on the microplate reader at 595 nm. To quantify protein concentration, the average of the readings for each standard was plotted against concentration, and a linear regression was performed. Using the y = mx + b equation from this standard curve, where y is the absorbance and x is the concentration, the concentration for each sample could be found by solving for x.

pH

The pH of the media and samples was determined using a Fisher Scientific (Fair Lawn, NJ) pH/ion/conductivity meter. The meter was calibrated daily using pH 7 and pH 10 buffer solutions (Fisher Scientific).

Ammonia Concentration

Ammonia concentration was determined using the Nessler Assay. Standards were made using a stock solution of 1000 mg/L of N (3.82 g/L of NH_4Cl). Samples were diluted 1:1000 before running the assay. Three 250 μL samples were added to separate wells of a 96-well plate. Then 3 μL of Mineral Stabilizer (HACH, Loveland, CO), 3 μL of Polyvinyl Alcohol (HACH, Loveland, CO) and 10 μL of Nessler Reagent (HACH, Loveland, CO) were added to each well. The plate was allowed to incubate at room temperature for 13 minutes, and was then read on the microplate reader at 425 nm. Ammonium-N concentrations were determined using the y = mx + b equation from the linear regression of the standard curve, where y is the absorbance and x is the

concentration. The concentration for each sample could be found by solving for x. These concentrations were then converted into ammonium concentrations by multiplying by (18 g NH_4^+ /14 g N).

Dissolved Calcium Concentration

Dissolved calcium concentrations were determined using an Agilent 7500ce Inductively Coupled Plasma Mass Spectrometer (ICP-MS) in Montana State University's Environmental and Biofilm Mass Spectrometry Facility. An aliquot of 100 μL of sample was filtered through a 0.2 μm syringe tip filter (Corning, Inc., Corning, NY) and added to 4.9 mL of 10% HNO_3. The diluted acid was made from Trace Metal Grade Nitric Acid, 67-70% (Fisher Scientific, Pittsburgh, PA). Samples were again diluted 1:2 to reach a final dilution of 1:100 and 5% HNO_3 before being measured on the ICP-MS.

Imaging

Scanning Electron Microscope (SEM)

SEM images were taken of the calcite crystal structures formed in some of the experiments. In these experiments, fiberglass coupons approximately 3 mm x 3 mm were placed in the bottom of the batch cultures. At the end of the experiments, these coupons were extracted from the bottle, frozen in a cryogenic freezing chamber, and imaged on a JOEL Model 6100/NORAN/Röntec/Oxford SEM in the Image and Chemical Analysis Laboratory (ICAL) at Montana State University by Dr. Andrew Mitchell.

Transmission Electron Microscope (TEM)

Images were also taken using a LEO 912AB TEM and photographed with a Proscan 2048x2048 CCD camera in Montana State University's Plant Science and Plant Pathology Laboratory to determine the effects of calcium on bacterial growth. Samples were taken from a batch culture in CMM+ inoculated with *S. pasteurii*. At the point of crystal formation (after approximately 2.5 hours), a mixture of calcite crystals and cells were extracted from the system by pipette. Separate samples of *S. pasteurii* grown in the absence of calcium were also collected and imaged. The samples were fixed by adding 100 µL of a 25% gluteraldehyde solution to 900 µL of the sample in a microcentrifuge tube to achieve a final concentration of 2.5% gluteraldehyde. After fixation, samples were centrifuged and resuspended in a small amount of 2% noble agar. Once the agar had solidified, the cell pellet was removed from the microcentrifuge tube and cut into smaller pieces, which were fixed overnight in a 3% gluteraldehyde and 0.05M potassium sodium phosphate buffer (PSPB) solution. The cell pellets were then washed three times for ten minutes each with PSPB and stained with 2% osmium tetroxide at room temperature for 4 hours. The samples were dehydrated in a series of ethanol washes and propylene oxide, and cell pieces were set in Spurrs resin and baked overnight at 70°C. Thin sections (60-90 nm) were cut with a Diatome diamond knife on a Reichert OM-U2 ultramicrotome and stained with uranyl acetate and Reynolds lead citrate. The samples were prepared and imaged by Susan Brumfield, M.S.

Kinetics of Calcite Precipitation

The rate coefficient for urea hydrolysis was determined by integrating the following first order differential equation:

$$\frac{d[urea]}{dt} = -k_{urea}[urea] \cdot X \quad (24)$$

to get:

$$[Urea]_t = [Urea]_o e^{-k_{urea}Xt} \quad (25)$$

Similarly, a first order differential equation was assumed for calcite precipitation, assuming that for every mole of Ca^{2+} removed from solution one mole of $CaCO_3$ formed:

$$\frac{d[Ca^{2+}]}{dt} = -k_{precip}[Ca^{2+}] \quad (26)$$

Integration of the above equation yields:

$$[Ca^{2+}]_t = [Ca^{2+}]_o e^{-k_{precip}t} \quad (27)$$

where k_{urea} and k_{precip} are urea hydrolysis and dissolved calcium precipitation rate coefficients, respectively and X is the concentration of biomass. Urea hydrolysis rates were calculated in two ways:

1) Under the assumption that the reaction is zero order with respect to biomass (X = 1)

2) Rates were normalized to the initial biomass of the system ($X = X_0$).

Rate constants were found using the least square fit method on Microsoft Excel. The data used for analysis excluded lag phases of urea hydrolysis and calcite precipitation.

RESULTS AND DISCUSSION

Solution Chemistry

The experiments inoculated with ureolytic species demonstrated an increase in pH and NH_4^+ over time. Table 5 outlines the pH found at different times for each species during aerobic experiments. *S. pasteurii* and *B. sphaericus* 21776 show similar trends in pH over time. The higher initial pH in the *B. sphaericus* 21776 experiments could be due to the media being made well enough in advance of inoculation to allow the sodium bicarbonate in the media to act as a buffer. Evidence supporting this can be found in the change in pH for the sterile control, which rises to 7.5 after 24 hours. *B. sphaericus* 21787 rises to a lower pH after both 10 hours and 30 hours, indicating that it may not have as strong of an ureolytic capability as the other two species. *B. subtilis*, the control species, and the sterile controls showed only a small pH increase, indicating that *B. subtilis* is not capable of increasing the solution pH and that the increase in pH is caused by the activities of the ureolytic bacteria and not abiotic chemical reactions. Appendix A lists the results of these pH measurements in detail.

Table 5: Average pH from triplicate aerobic experiments. Data for hour 0 was taken immediately after inoculation

Species	0 Hours	10 (±1) Hours	24 (±3) Hours*
S. pasteurii	6.66 (0.06)	8.87 (0.08)	9.33 (0.02)
B. sphaericus 21776	7.24 (0.30)	8.80 (0.20)	9.23 (0.09)
B. sphaericus 21787	6.87 (0.15)	8.06 (0.12)	8.70 (0.26)
B. subtilis[§]	6.80	---	7.50
Sterile Control	7.08 (0.04)	---	7.31 (0.05)

*Data taken from only two experiments.
[§] Data taken from only one experiment.
() standard deviation.

The change in urea concentration in aerobic experiments over time caused by ureolysis is shown in Figure 6. These values are calculated from measured ammonium concentrations using the stoichiometry in Equation 3. One mole of urea is assumed to hydrolyze into two moles of ammonium, and therefore the amount of urea hydrolyzed at any given time is assumed to be equal to half the amount of ammonia produced. The initial value of urea at hour 0 is taken to be the amount added to the medium, 0.333 M. After 30 hours, *S. pasteurii* had hydrolyzed 58-82% of the available urea and *B. sphaericus* 21776 hydrolyzed 72-80% of available urea. *B. sphaericus* 21787, however, hydrolyzed only 12-15% of all urea. This supports the hypothesis formed by analysis of the pH data—*S. pasteurii* and *B. sphaericus* 21776 seem to have similarly high ureolytic capabilities compared to that of *B. sphaericus* 21787 under the conditions employed in this study. Data from *B. subtilis* and the sterile control show no marked decrease in urea concentrations over time.

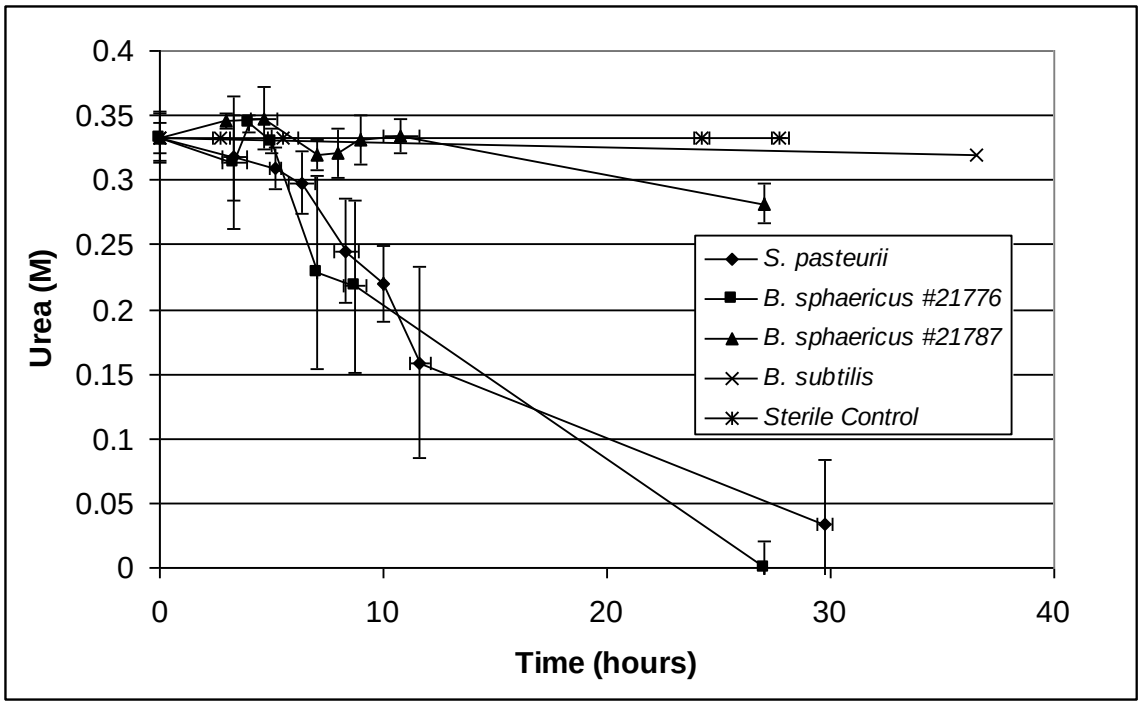


Figure 6: Urea hydrolysis over time in aerobic experiments. Data shown is the average of at least two experiments for *S. pasteurii* and the *B. sphaericus* species, the average of duplicate experiments for the sterile control, and the data from one experiment for *B. subtilis*. Error bars represent the standard deviation of averaged data.

The average dissolved calcium concentration over time for the three ureolytic species analyzed is shown in Figure 7. Again, a similar trend is seen for *S. pasteurii* and *B. sphaericus* 21776. After a lag time of 3-4 hours, dissolved calcium concentrations decrease to 10-15% of the initial concentrations by hour 12, and to <2% by 27 hours. *B. sphaericus* 21787, on the other hand, shows a decrease to only 81% of the initial concentration after 12 hours; after 27 hours it decreases to 5%. Data from *B. subtilis* and the sterile control show no obvious decrease in calcium concentration over the sampling period. Dissolved calcium concentrations in systems inoculated with *B. subtilis* are lower than those for the other systems. This could be due to passive precipitation of calcite, with *B. subtilis* cells acting as nucleation points, or due to sorption of calcium to the cells. No data was taken until nine hours after inoculation, so the initial Ca^{2+} concentration was not known. Dissolved calcium concentrations in the sterile control were higher than those of the other systems. This could be a result of an error when preparing the medium or performing dilutions.

SEM images were taken of calcite crystals formed in some of the experiments (Figure 8). These images show calcite crystals in close association with cells, indicating that the precipitation of calcium may interfere with the growth of the organisms.

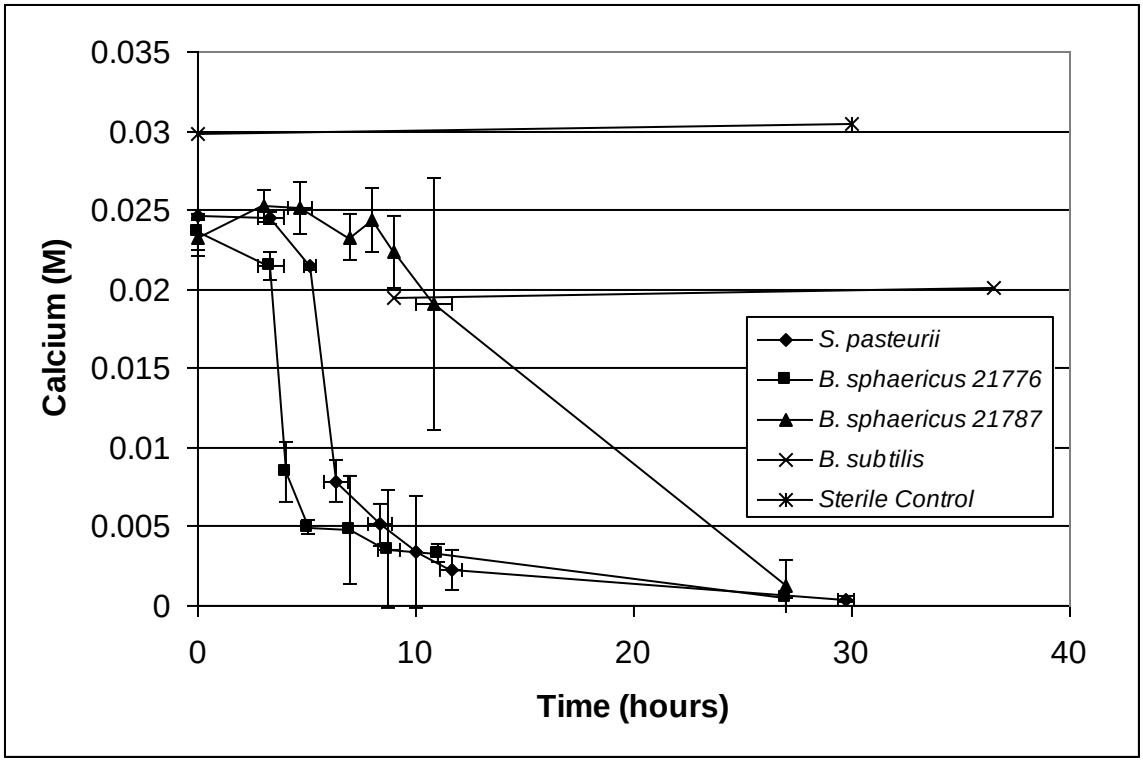


Figure 7: Dissolved calcium data over time in aerobic experiments. Data shown is the average of at least two experiments for *S. pasteurii* and the *B. sphaericus* species, and the data from one experiment for *B. subtilis* and the sterile control. Error bars represent the standard deviation of averaged data.

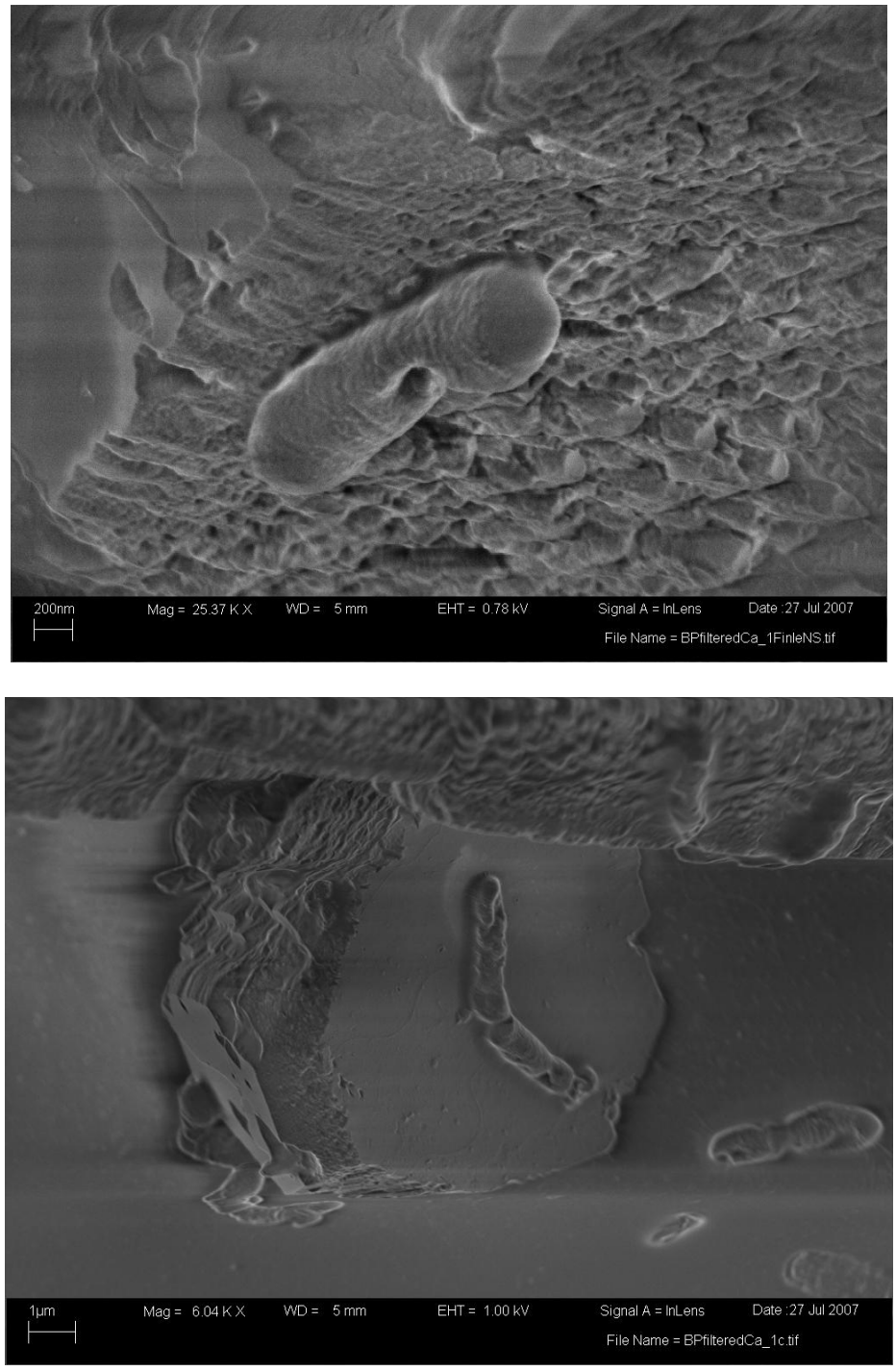


Figure 8: SEM images of *S. pasteurii* cells in close association with or embedded in $CaCO_3$ crystals.

Growth

The kinetic models used to interpret the data assume constant cell density. Careful analysis of the data was necessary to validate this assumption. Experiments indicate that the presence of calcium had some effect on the measured growth rate of the bacteria. Figure 9 shows the averaged protein and CFU data from *S. pasteurii* and *B. sphaericus* aerobic experiments. There is a clear difference in the rate of growth in experiments carried out in the presence of calcium as opposed to those performed in the absence of calcium. This trend is most obvious in the *S. pasteurii* experiments, and much muted in the *B. sphaericus* 21787 experiments. Both *B. sphaericus* strains exhibited less growth than *S. pasteurii*, growing to a maximum of around 90 mg/L protein for strain 21776 and 50 mg/L for strain 21787, as opposed to near 140 mg/L of protein for *S. pasteurii*. Culturable cell counts show similar trends. *S. pasteurii* grown in the absence of calcium reached a maximum of near 1.3×10^8 CFU/mL during the first 8 hours after inoculation, while cultures grown in the presence of calcium show a decrease in CFU/mL for the first 8 hours, after which they start to increase.

The protein and CFU measurements along with the SEM imaging led to the hypothesis that bacterial cells can act as nucleation sites for calcium crystals to form and at least a fraction of the encased cells precipitates out of solution with the calcite. Figure 10 shows averaged protein and calcium concentrations from all experiments. In *S. pasteurii* experiments, protein remained constant until most of the dissolved calcium precipitated, and then started to increase. A similar trend was seen in *B. sphaericus*

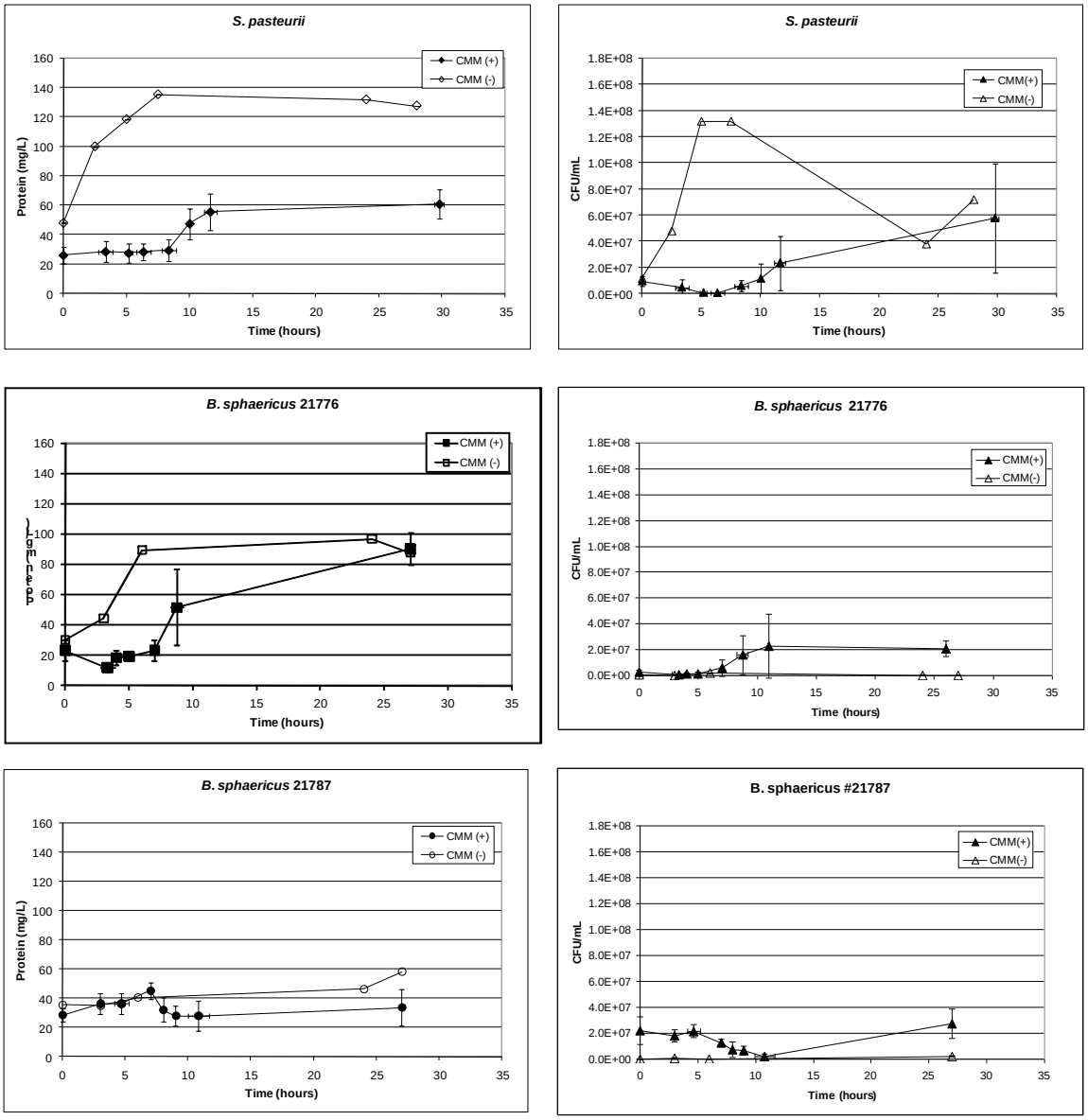


Figure 9: Change in protein concentration and CFU/mL over time for CMM+ and CMM- aerobic medium. Data shown is the average of triplicate experiments for calcium inclusive experiments, while the data for calcium exclusive experiments was from one experiment per species. Error bars represent the standard deviation of triplicate experiments for CMM+.

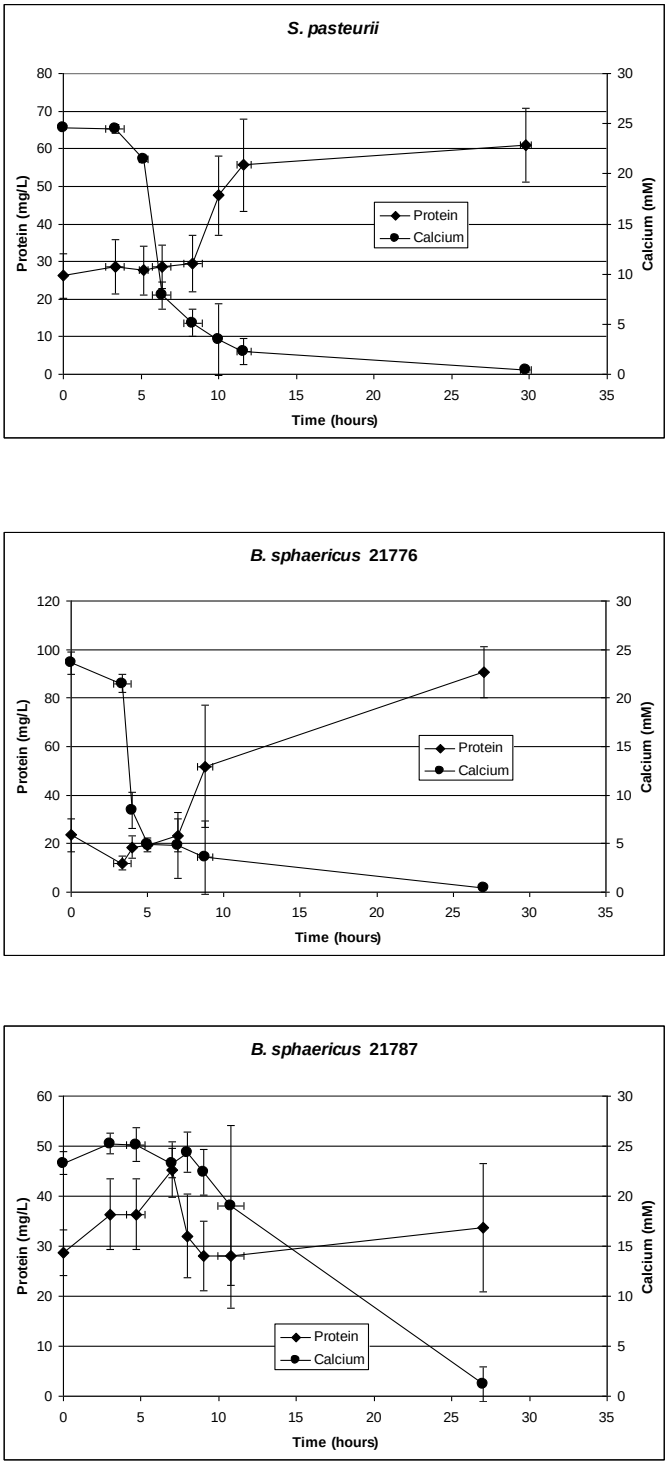


Figure 10: Protein and dissolved calcium concentration as a function of time. Data shown is the average of triplicate experiments. Error bars represent the standard deviation of triplicate experiments.

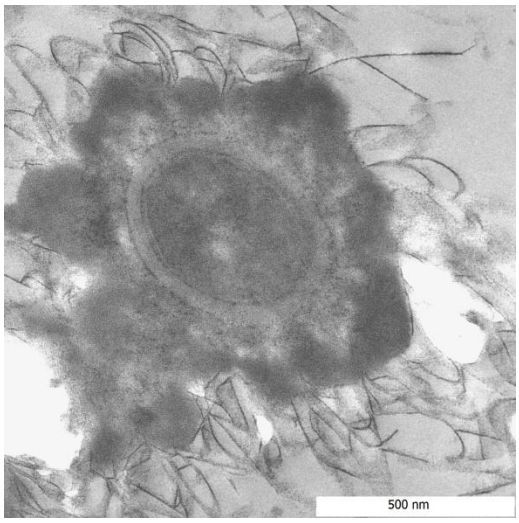
21776. *B. sphaericus* 21787 did not grow as quickly as the other strains tested, and calcium did not precipitate to any appreciable extent over the sampling period, most likely because the cells are not as ureolytically active as the other two strains. Calcium precipitated out of solution after 30 hours, but this occurred after sampling had stopped.

Activity of Encased Cells

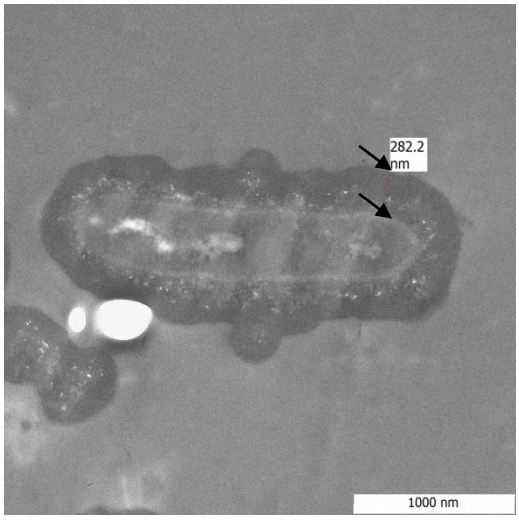
To further validate the assumption that cell growth can be neglected in kinetic analysis, it must also be shown that encased cells are either not metabolically active, or that the calcite surrounding the cells effectively acts as a barrier to solutes such as urea getting to the cell or to hydroxide ions, formed by the hydrolysis of urea, from getting through the calcite to the bulk solution and effecting a change on the solution pH.

TEM Imaging

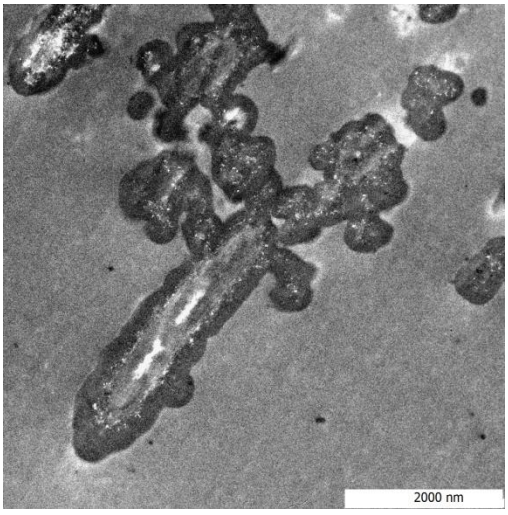
To further explore the possibility of cells getting encased in calcite crystals, and determine whether these cells can still be metabolically active, images were taken using transmission electron microscopy (TEM), which allows imaging of thin slices of samples. Figure 11 a-c shows images of samples taken from experiments run with *S. pasteurii* in CMM+. The cells are surrounded by a layer of calcium-containing precipitates, as determined using parallel electron energy loss spectroscopy (EELS). Figure 11d shows *S. pasteurii* grown in CMM- for a comparison. By measuring the thickness of calcite, as in Figure 11b, the rate of urea diffusion through the calcite can be calculated.



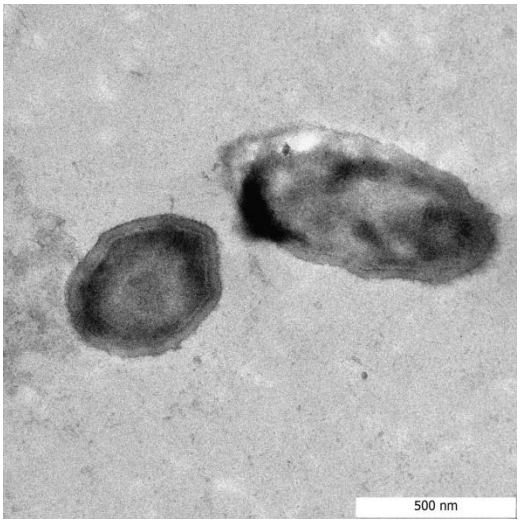
(a)



(b)



(c)



(d)

Figure 11: TEM images of *S. pasteurii* in CMM+ (a-c) and CMM- (d).

Diffusion of Urea Through Calcite

To determine whether the encased cells were still capable of ureolysis, a detailed analysis of urea diffusion in calcite was performed. The literature supplies information regarding the diffusion of oxygen in calcite at high temperatures (Farver 1994), but does not have information of urea diffusion in calcite at 30°C. Therefore a number of assumptions were made. First, since urea has a lower diffusivity coefficient than oxygen in aqueous solutions at 25°C (Stewart, 2003), it was assumed that this will hold true at other temperatures and through other substances, like calcite. Second, since the diffusion coefficient of oxygen through calcite at 400°C and 100 MPa is 2.66×10^{-22} m^2/s (Farver 1994), and diffusion coefficients generally increase with increasing temperature and pressure, it can be assumed that the diffusion coefficient of urea in calcite at atmospheric pressure and 30°C is smaller than 2.66×10^{-22} m^2/s.

Assuming that the geometry of the calcite is a uniformly thick slab with a thickness of 200 nm, as determined from the TEM images (Figure 11b), the time it will take to reach 5% of the bulk urea concentration can be calculated using the relation (Carslaw and Jaeger 1959):

$$t_5 = 0.1\frac{L^2}{D_e} \quad (28)$$

where L is the slab thickness, D_e is the effective diffusion coefficient in calcite, and t_5 is the amount of time it will take to reach 5% of the bulk concentration. Using the above assumptions, it would take at least 175 days for urea to diffuse through the calcite surrounding the cells. Because calcite precipitation takes place over the course of

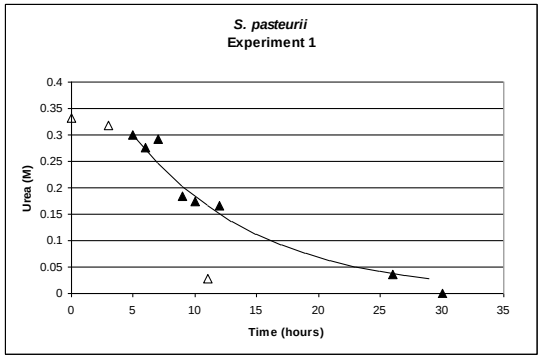
approximately one day, it can safely be assumed that if the encased cells are still alive, urea is not able to diffuse through the calcite fast enough for the cells to hydrolyze it and contribute to the rise in solution pH.

Kinetics

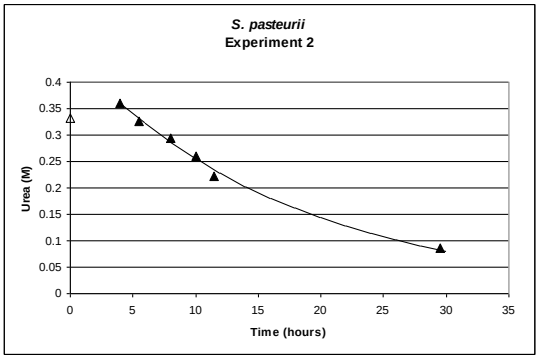
A first order rate law with respect to urea concentration was used to determine the rate of urea hydrolysis in the systems. This is an appropriate choice of model, since the biomass analysis indicated that the cell density was constant for the duration of calcite precipitation. The precipitation of calcite from the system is dependent on the saturation state of the system, as well as the growth mechanism of the calcite (Teng et al., 2000). The literature is ambiguous on defining a set rate expression for the precipitation, so a first order rate law was applied to these studies for both its simplicity and the fact that it seems to describe the systems well.

Sporosarcina pasteurii

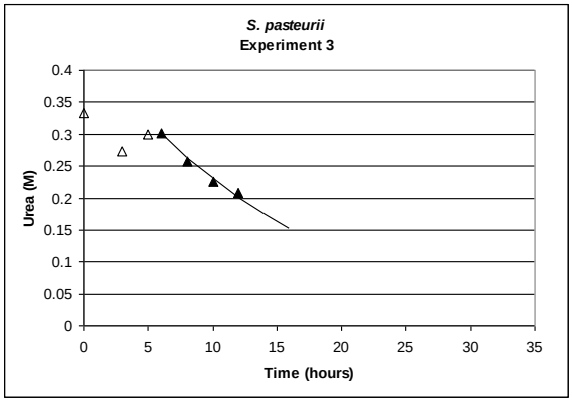
The first order kinetic expression for urea hydrolysis (Equation 24) in systems inoculated with *S. pasteurii* yielded the individual curves shown in Figure 12. Each individual experiment is shown with its correlating best fit line. Table 6 lists the corresponding k_{urea} values, the number of data points used to determine rate coefficients, the R^2 value between the model and the points used, and the lag time before hydrolysis started. The value of k_{urea} has also been normalized to the absorbance reading of initial biomass and CFU/mL, as extrapolated using the technique outlined in Appendix B.2.



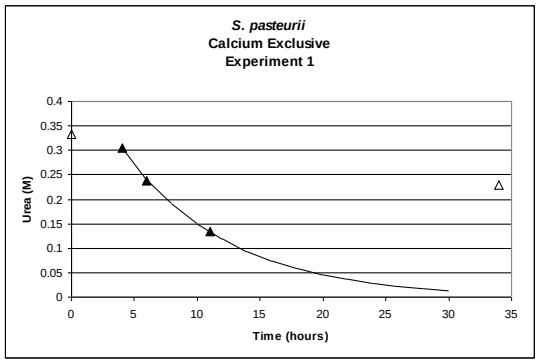
(a)



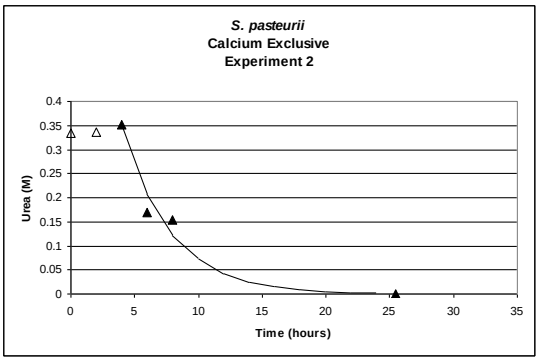
(b)



(c)



(d)



(e)

Figure 12: Urea concentrations over time from experimental data for *S. pasteurii* in calcium inclusive (a-c) and calcium exclusive experiments (d-e). Individual data points are experimental data and the curves are the lines of best fit. Filled in data points were used to determine best fit.

Table 6: Summary of kinetic parameters for urea hydrolysis (k_{urea}) in experiments inoculated with *S. pasteurii*, including R^2 values, lag time, and number of data points used in evaluation.

CMM+	Initial Biomass OD_{600}	k_{urea} (hr^{-1})	R^2	Lag Time (hours)	# of Data Points (Total Points)	k_{urea} normalized to: OD_{600} (OD_{600}^{-1} hr^{-1})	k_{urea} normalized to: CFU (mL CFU^{-1} hr^{-1})
Exp 1	0.015	0.0986	0.9698	5.0	7(9)	6.72	3.96E-08
Exp 2	0.013	0.0575	0.9948	4.0	6(7)	4.44	3.00E-08
Exp 3	0.015	0.0672	0.9858	6.0	4(7)	4.59	2.71E-08
Average:	**0.014**	**0.0744**		**5.0**		**5.25**	**3.22E-08**
Std Dev:	0.001	0.0215		1.0		1.27	6.54E-09
CMM-							
Exp 1	0.017	0.118	0.9996	4.0	3(5)	6.99	3.65E-08
Exp 2	0.017	0.266	0.9626	4.0	4(6)	15.5	7.99E-08
Average:	**0.017**	**0.192**		**4.0**		**11.2**	**5.88E-08**
Std Dev:	0.000	0.104		0.0		5.99	2.02E-08

The average rate coefficient for urea hydrolysis in calcium inclusive media was 0.0744/hr with an average lag time of five hours. The relative standard deviation for the three replicate experiments was 29%. The variations among experiments could be due to many factors. No two experiments were run at the same time, so minor differences in pilot cultures, laboratory conditions, and inoculum preparation could contribute to the high standard deviation. Sampling times also varied slightly from culture to culture, which could have affected the way the data was analyzed. The initial biomass in all three systems was nearly the same, so the rate coefficients normalized to cell numbers have approximately the same percentage of standard deviation. The average rate coefficient for the calcium exclusive media was 0.192/hr, with a lag time of four hours. This is more than two times higher than the rate in calcium inclusive media, which indicates that the presence of calcium influences the rate of urea hydrolysis, most likely through the encasement of cells in calcite. The concentration of urea 34 hours after inoculation in Experiment 1 in CMM- appeared to increase from previous point, and was not used in the

kinetic analysis in this experiment. Urea concentrations were calculated by converting measured ammonium ion concentrations using the stoichiometry of Equation 3, where for every 1 mole of urea hydrolyzed, 2 moles of ammonium ions are formed. Therefore, the increase in urea concentration at this sample point corresponds to a decrease in ammonium ion concentration. The pH at this sample point is 9.28, but the pKa of ammonia/ammonium ion is 9.25. This indicates that the system is becoming oversaturated in ammonia, resulting in the off-gassing of ammonia and the apparent decrease in ammonium ion concentration.

The change in dissolved calcium concentration over time for each individual experiment is shown in Figure 13 along with its correlating best fit line. Table 7 lists the corresponding k_{precip} values along with the R^2 value, as well as the number of data points used to determine rate coefficients and the lag time before precipitation started. The average rate coefficient for calcium precipitation was 0.253/hr, with a fairly low standard deviation (8.5%), and a lag time of 3.3 hours. Experiment 3 had a low R^2 value, and the fit in Figure 13c does not seem to fit the data well. This experiment was the only experiment where dissolved calcium concentrations did not decrease to <0.0004M. The concentration decreased sharply at the onset of calcite precipitation in a manner similar to the previous two experiments, but then showed only a gradual decrease to 0.006M. These samples were analyzed two times by the ICP-MS, both tests resulting in similar dissolved calcium concentrations. Possible explanations for the difference in Experiment 3 are dilution errors or differences in pilot cultures. Calcite precipitation and urea hydrolysis started at approximately the same time.

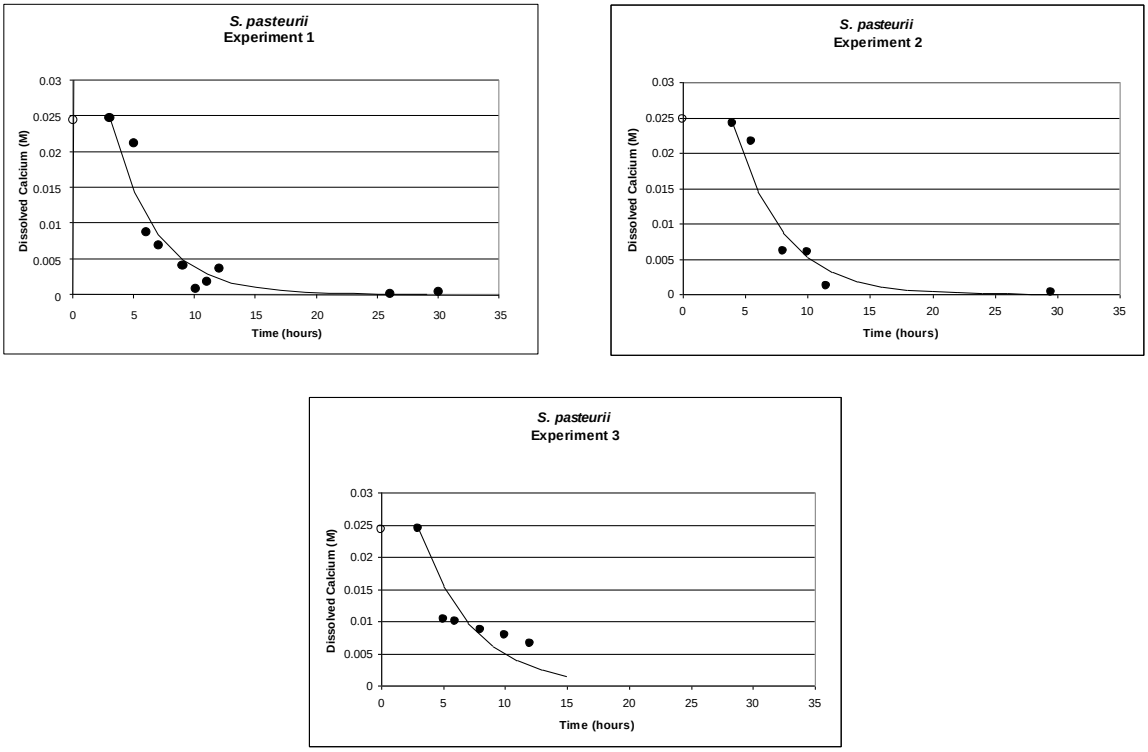


Figure 13: Dissolved calcium concentrations over time from experimental data for *S. pasteurii* in calcium inclusive media. Individual data points are experimental data and curves are the lines of best fit. Filled in data points were used to determine best fit.

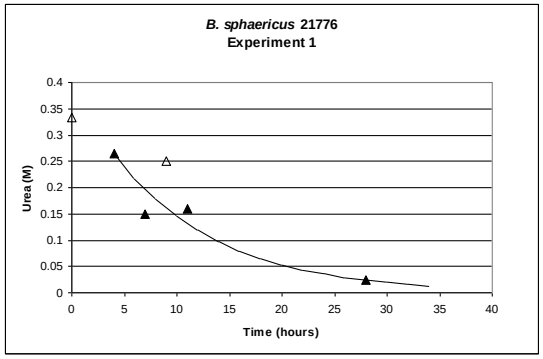
Table 7: Summary of kinetic parameters for calcite precipitation (k_{precip}) in CMM+ experiments inoculated with *S. pasteurii*, including R^2 values, lag time, and number of data points used in evaluation.

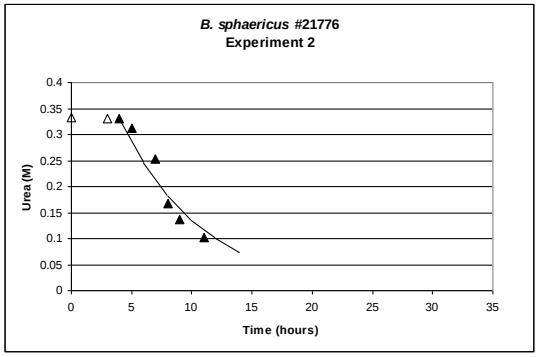
	k_{precip} (hr^{-1})	**R^2**	**#Data Points (Total Points)**	**Lag time (hours)**
Experiment 1	0.272	0.9103	10(11)	3
Experiment 2	0.257	0.9378	6(7)	4
Experiment 3	0.230	0.8403	5(7)	3
Average	**0.253**			**3.3**
Std Deviation	0.022			**0.6**

Bacillus sphaericus 21776

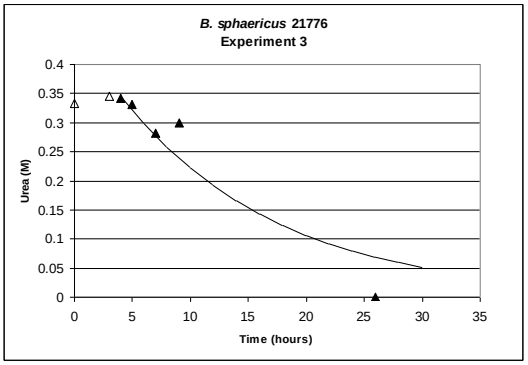
Analysis of kinetics of urea hydrolysis for *B. sphaericus* 21776 yielded the individual curves shown in Figure 14. Each individual experiment is shown with its correlating best fit line. Table 8 lists the corresponding k_{urea} values along with the R^2 value, as well as the number of data points used to determine rate coefficients and the lag time before hydrolysis started.

The average rate coefficient for urea hydrolysis was 0.108/hr, with a standard deviation of 35.8%. In Experiment 1, the 9 hour data from was not used in kinetic analysis because of a large standard deviation among triplicate absorbance readings on the spectrophotometer. Experiment 2 had the lowest concentration of initial biomass, and the largest rate coefficient for urea hydrolysis, resulting in a comparatively large rate coefficient when normalized to cell numbers. The rate coefficient found in Experiment three was much lower than those found in the previous two experiments. There was not a significant difference in the initial conditions of these experiments, such as pH or medium preparation, but dilution errors or differences in pilot cultures could explain the variations among experiments. The average lag time before urea hydrolysis begins is 4.0 hours. The rate coefficient in calcium exclusive media was 0.168/hour, with a lag time of 3.5 hours.





(a) (b)



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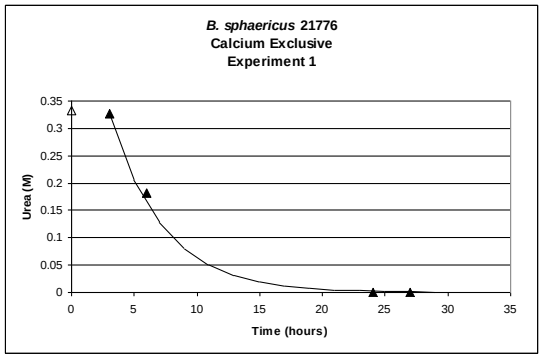
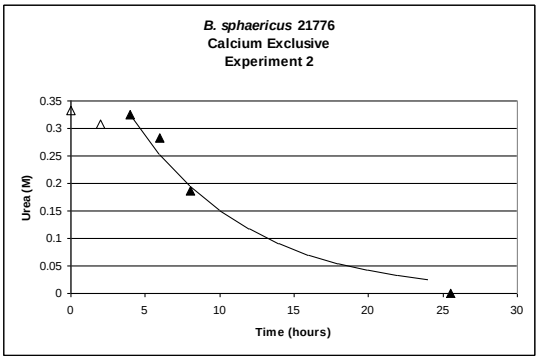




(d) (e)

Figure 14: Urea concentrations over time from experimental data for *B. sphaericus* 21776 in calcium inclusive (a-c) and calcium exclusive experiments (d-e). Individual data points are experimental data and the curves are the lines of best fit. Filled in data points were used to determine best fit.

Table 8: Summary of kinetic parameters for urea hydrolysis (k_{urea}) in experiments inoculated with *B. sphaericus* 21776, including R^2 values, lag time, and number of data points used in evaluation.

CMM+	Initial Biomass OD_{600}	k_{urea} (hr^{-1})	R^2	# of Data Points (Total Points)	Lag Time (hours)	k_{urea} normalized to: OD_{600} (OD_{600}^{-1} hr^{-1})	k_{urea} normalized to: CFU (mL CFU^{-1} hr^{-1})
Exp 1	0.015	0.100	0.9063	4(6)	4.0	6.73	3.91E-08
Exp 2	0.012	0.150	0.9420	6(8)	4.0	12.3	8.99E-08
Exp 3	0.015	0.074	0.9446	5(7)	4.0	5.05	2.99E-08
Average:	**0.014**	**0.108**			**4.0**	**8.02**	**5.30E-08**
Std Dev:	0.001	0.039			0.0	3.79	3.23E-08
CMM-							
Exp 1	0.016	0.199	0.9999	4(5)	3.0	12.8	7.14E-08
Exp 2	0.015	0.129	0.9870	4(6)	4.0	8.84	5.26E-08
Average:	**0.015**	**0.168**			**3.5**	**10.8**	**6.20E-08**
Std Dev:	0.001	0.050			0.71	2.80	1.33E-08

The change in dissolved calcium concentration over time for each individual experiment is shown in Figure 15 along with its correlating best fit line. Table 9 lists the corresponding k_{precip} values along with the R^2 value, as well as the number of data points used to determine rate coefficients and the lag time before precipitation started.

The average rate coefficient for calcium precipitation in experiments inoculated with B. sphaericus 21776 was 0.604/hr. The standard deviation among the three experiments was 57%, which is quite high. The first experiment had a very low rate coefficient compared to the last two experiments. Samples were not taken on as fine a time scale in this experiment as with the others, which could account for a lower calculated rate coefficient. The average lag time was 3.3 hours. The onset of calcite precipitation occurred at approximately the same time as urea hydrolysis.

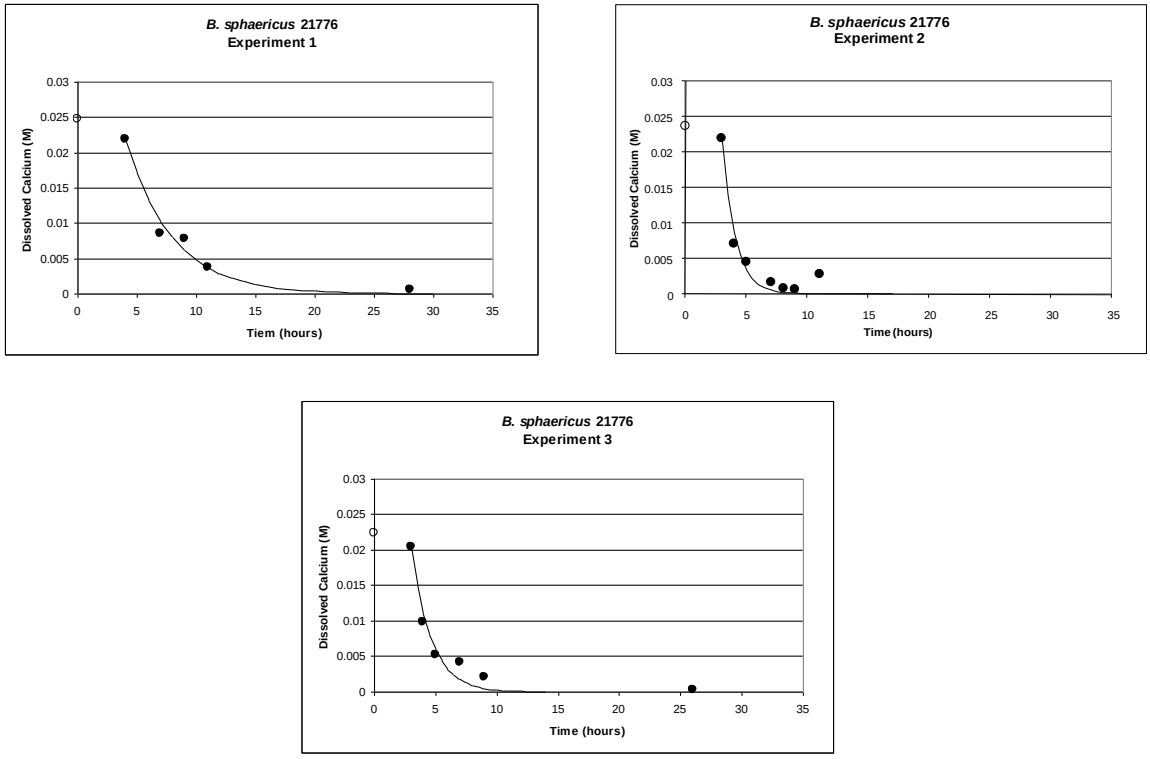


Figure 15: Dissolved calcium concentrations over time from experimental data for *B. sphaericus* 21776 in calcium inclusive media. Individual data points are experimental data and curves are the lines of best fit. Filled in data points were used to determine best fit.

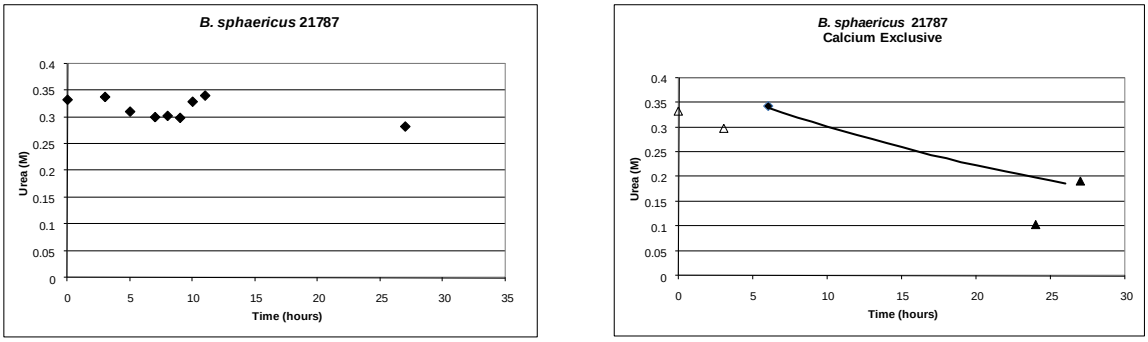
Table 9: Summary of kinetic parameters for calcite precipitation (k_{precip}) in CMM+ experiments inoculated with *B. sphaericus* 21776, including R^2 values, lag time, and number of data points used in evaluation.

	k_{precip} (hr^{-1})	R^2	#Data Points (Total Points)	Lag time (hours)
Experiment 1	0.254	0.9791	5(6)	4
Experiment 2	0.942	0.9772	7(8)	3
Experiment 3	0.617	0.9741	6(7)	3
Average	**0.604**			**3.3**
Std Deviation	0.344			**0.6**

Bacillus sphaericus 21787

Of the three experiments run with B. sphaericus 21787, only one showed a decrease dissolved calcium concentrations within the twelve hours after inoculation. No noticeable decrease in urea concentration was seen in this experiment. The other two experiments did show a decrease in calcium concentration after twelve hours, but the systems were not adequately sampled during this period, and kinetic analysis could not be performed for these systems. Figures 16 and 17 show the changes in urea and dissolved calcium over time, as well as the best fit line for calcite precipitation. Table 10 shows the kinetic parameters for this experiment.

The rate coefficient for urea hydrolysis in CMM- was 0.030/hr. The urea concentration in the CMM+ does not decrease below 0.3M until 27 hours after inoculation, when they drop to 0.28M. This does not conclusively show that urea hydrolysis is occurring, and no rate coefficient was determined for this system. Calcite precipitation did not start until about 8 hours. In the other two experiments run with this species, calcite precipitation did not start until after 12 hours, indicating that while *B. sphaericus* 21787 is capable of precipitation, it does not occur for quite awhile after inoculation compared to *S. pasteurii* and *B. sphaericus* 21776.



(a) (b)

Figure 16: Urea concentrations over time from experimental data for *B. sphaericus* 21787 in calcium inclusive (a) and calcium exclusive experiments (b). Individual data points are experimental data and the curves are the lines of best fit.

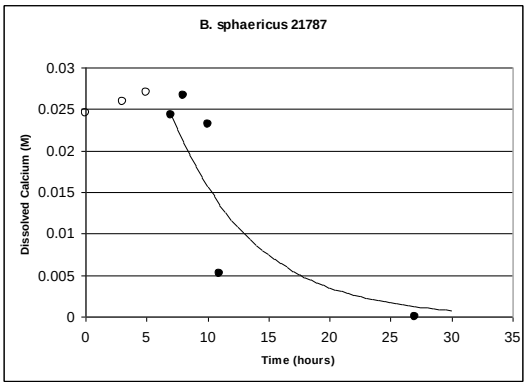


Figure 17: Dissolved calcium concentrations over time from experimental data for *B. sphaericus* 21787 in calcium inclusive media. Individual data points are experimental data and curve is the line of best fit.

Table 10: Summary of kinetic parameters for urea hydrolysis (k_{urea}) and calcite precipitation (k_{precip}) in experiments inoculated with *B. sphaericus* 21787, including R^2 values, lag time, and number of data points used in evaluation.

Urea Hydrolysis	Initial Biomass OD_{600}	k_{urea} (hr^{-1})	R^2	# of Data Points (Total Points)	Lag Time (hours)	k_{urea} normalized to:	
						OD_{600} (OD_{600}^{-1} hr^{-1})	CFU (mL CFU^{-1} hr^{-1})
CMM+	0.0156	n/a	n/a	n/a	n/a	n/a	n/a
CMM-	0.0159	0.030	0.7914	3(5)	6	1.89	1.03E-08
Calcite Precipitation		k_{precip} (hr^{-1})					
CMM+		0.219	0.6426	3(8)	8		

Comparison of Kinetics

A summary of the kinetic coefficients for each species is outlined in Table 11. *B. sphaericus* 21776 shows the highest rates and shorter lag times for both urea hydrolysis and calcite precipitation. *B. sphaericus* 21776 also has the highest rate of urea hydrolysis per unit biomass. If the amount of urease present in a species determines its ureolytic capabilities, this indicates that *B. sphaericus* 21776 has a higher concentration of urease than *S. pasteurii*.

Table 11: Summary of kinetic parameters of three ureolytic species. Standard deviations for triplicate experiments of *S. pasteurii* and *B. sphaericus* 21776 are shown in parenthesis.

Species	k_{urea} (hr^{-1})	k_{urea} (OD_{600}^{-1} hr^{-1})	k_{urea} (mL CFU^{-1} hr^{-1})	Lag time (hrs)	k_{precip} (hr^{-1})	Lag time (hrs)
S. pasteurii	0.0744 (0.0215)	5.25 (1.27)	3.22E-08 (6.54E-09)	5.0 (1.0)	0.2530 (0.022)	3.3 (0.6)
B. sphaericus 21776	0.108 (0.039)	8.02 (3.79)	5.30E-08 (3.23E-08)	4.0 (0.0)	0.604 (0.344)	3.3 (0.6)
B. sphaericus 21787	n/a	n/a	n/a	n/a	0.219	8

It is also desirable to compare the results of these experiments to those conducted in the literature described in the introduction of this paper. In order to accurately compare the kinetic parameters of the experiments, the kinetic coefficients were re-evaluated according to Equations 24 and 26. The initial cell concentration for each experiment also had to be standardized, as some were reported as optical densities (Fujita et al., 2000 and Ferris et al., 2003) and some were reported as CFU/mL (Stocks-Fischer et al., 1999). Data from calcium exclusive experiments were used to correlate CFU/mL to OD_{600} (Appendix B.2). Table 12 shows the re-evaluated data for the experiments, as well as the averaged data from aerobic *S. pasteurii* experiments performed for this project.

Table 12: Summary of kinetic coefficients and initial growth conditions for experiments performed for this thesis and those described in the literature review.

	S. Parks (2009)	Stocks-Fischer et al. (1999)	Fujita et al. (2000)	Ferris et al. (2003)
S. pasteurii strain	ATCC 11859	ATCC 6453	ATCC 11859	ATCC 11859
Temperature (°C)	30	25	20	20
Initial pH	6.7	8.0	6.5	6.5
$[Ca^{2+}]_o$ (mM)	25.2	25.2	25	1.75
$[Urea]_o$ (mM)	333	333	333	6
$[cells]_o$ (OD_{600})	0.0141	0.0102	0.072	0.070
k_{urea} (hr^{-1})	0.0744	0.0282	0.0081	0.0381
k_{urea} (OD_{600}^{-1} hr^{-1})	5.251	2.76	0.112	0.540
k_{urea} (mL CFU^{-1} hr^{-1})	3.22E-08	2.82E-8	3.75E-10	1.81E-09
k_{precip} (hr^{-1})	0.253	0.116	0.113	0.014

The highest rate coefficients were found in the experiments performed for this thesis. The media used in these experiments was the same as that used by Stocks-Fischer et al. (1999), and very similar to that used by Fujita et al. (2000). The differences in rates between our data and those of Stocks-Fischer et al. (1999) can be attributed to temperature and pH differences, and well as a difference in bacterial strains used for the experiments. The experiments performed for this thesis were conducted at a higher temperature, 30°C, which is the optimum temperature for growing *S. pasteurii* (Gibson, 1934).

Fujita et al. (2000) ran experiments for 8 hours, with the last published ammonium ion data taken 5.5 hours after inoculum. The urea concentration never dropped below 0.3M. The pH at 5.5 hours was 8.2, but calcite precipitation in the experiments run for this thesis occurred at an approximate pH of 8.5. In our experiments, calcite precipitation and urea hydrolysis appear to occur at approximately the same time, so urea hydrolysis may not have started in the Fujita et al. (2000) systems, which could explain why their k_{urea} was so low. The medium used in both experiments were similar, with the exception that Fujita et al. (2000) did not add NH_4Cl.

The medium used for the experiments performed in this thesis is quite different than that used by Ferris et al. (2003), which could account for differences in these rate coefficients. Ferris et al. (2003) used artificial ground water, which is a non-growth medium. The addition of nutrient broth to the CMM makes it a rich media that provides a lot of nutrients for the bacteria to grow on. Ferris et al. (2003) also ran their

experiments at a lower temperature and added a smaller initial concentration of urea, which could account for the lower rate coefficients.

Some interesting results can be seen when looking at rate coefficients that have been normalized to absorbance readings. Fujita et al. (2000) starts with the highest initial biomass concentration, but exhibits the lowest rate of urea hydrolysis per absorbance unit. The opposite trend is seen in the results from this experiment, which had the lowest initial biomass concentration but the highest rate of urea hydrolysis per absorbance unit. This suggests that at some point, the systems become oversaturated with organisms, and ureolysis becomes zero order with respect to biomass.

In summary, the experiment with the highest rate for urea hydrolysis and calcite precipitation was performed at the highest temperature and urea concentration and the lowest biomass concentration. The experiment with the lowest rate coefficient for urea hydrolysis was performed at a lower temperature and over a shorter duration of time, while the experiment with the lowest rate coefficient for calcite precipitation had the lowest initial concentration of urea and Ca^{2+}.

Anaerobic Experiments

Not much information can be found in the literature regarding the function of *S. pasteurii* in the absence of oxygen. Reboli and Farrar (1988) showed that *S. pasteurii* was capable of growing on anaerobic agar and reducing nitrate to nitrite, but did not elaborate on growth rates or conditions. Because of the lack of concrete scientific data on whether *S. pasteurii* could be a facultative aerobe, an experimental survey was performed to determine whether it was capable of growth and/or pH increase in CMM- with the addition of nitrate, sulfate, and iron as terminal electron acceptors. *S. pasteurii* was also grown in CMM- without an added electron acceptor. In this survey, pH and OD_{600} measurements were taken. After approximately 120 hours, the anaerobic systems were opened to the atmosphere and placed on a shaker to allow for oxygen diffusion. Figure 18 shows the change in pH over time for each media type. A pH increase to > 9 was seen with all types of TEA, as well as in the medium that did not have an external TEA added. The pH of the sterile controls did not exceed a pH of 7, with the exception of the media with sulfate as the added TEA, which had an initial pH of 7.7. There is not a significant difference in the change in pH between the inoculated aerobic and anaerobic media, suggesting that the rate of urea hydrolysis is not appreciably altered by the change in growth conditions.

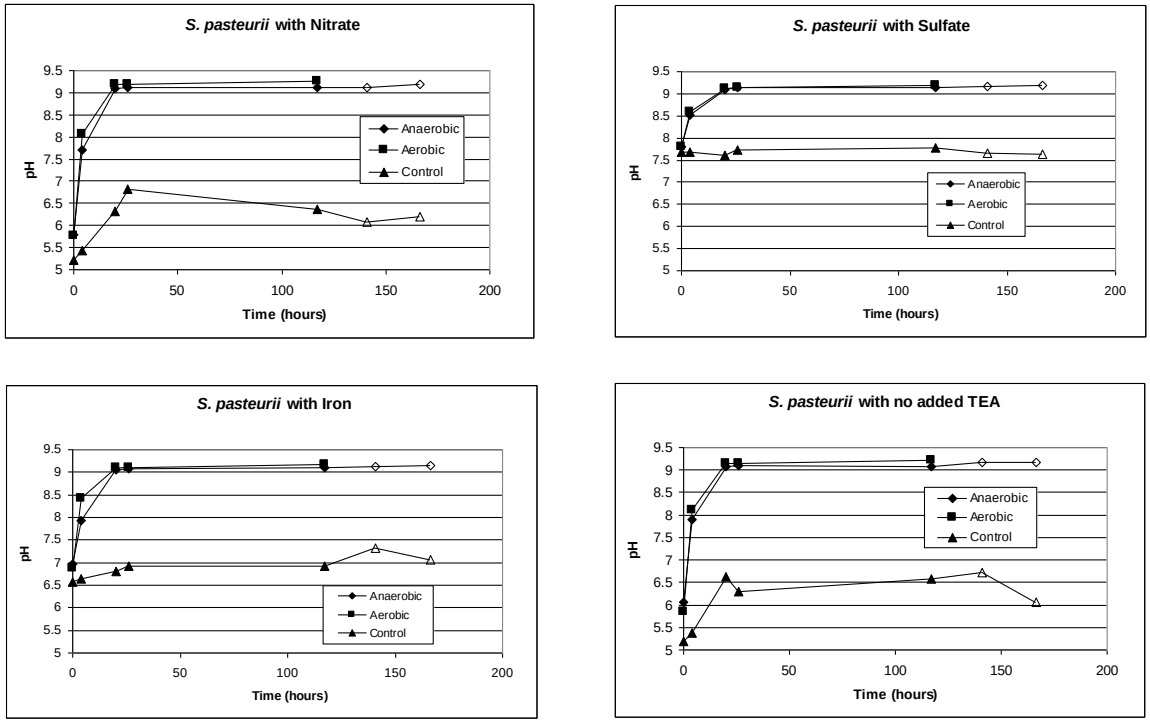


Figure 18: Change in pH over time in anaerobic and aerobic calcium exclusive media with various added terminal electron acceptors. Hollow points indicate where previously anaerobic media was opened to the environment.

Growth in the anaerobic experiments was quantified by OD_{600} measurements. Figure 19 shows the change in absorbance over time in each type of media. Absorbance readings in aerobic media increase much faster than those in anaerobic media, indicating that growth is slower under anaerobic conditions. The absorbance of inoculated anaerobic media with nitrate and without the addition of a TEA increased 20 hours after inoculation, and then appeared to level off and even decrease. The absorbance of inoculated anaerobic media with iron increases until the systems are opened to the atmosphere, but not at the same rate as the aerobic systems. No change is seen in systems where sulfate was added as an electron acceptor. Once oxygen is allowed to

diffuse into the systems, absorbance in inoculated systems increases, indicating that the bacteria are still viable after 120 hours of oxygen depletion. Coupled with the increase in pH, it can be concluded that *S. pasteurii* is capable of hydrolyzing urea under anaerobic conditions.

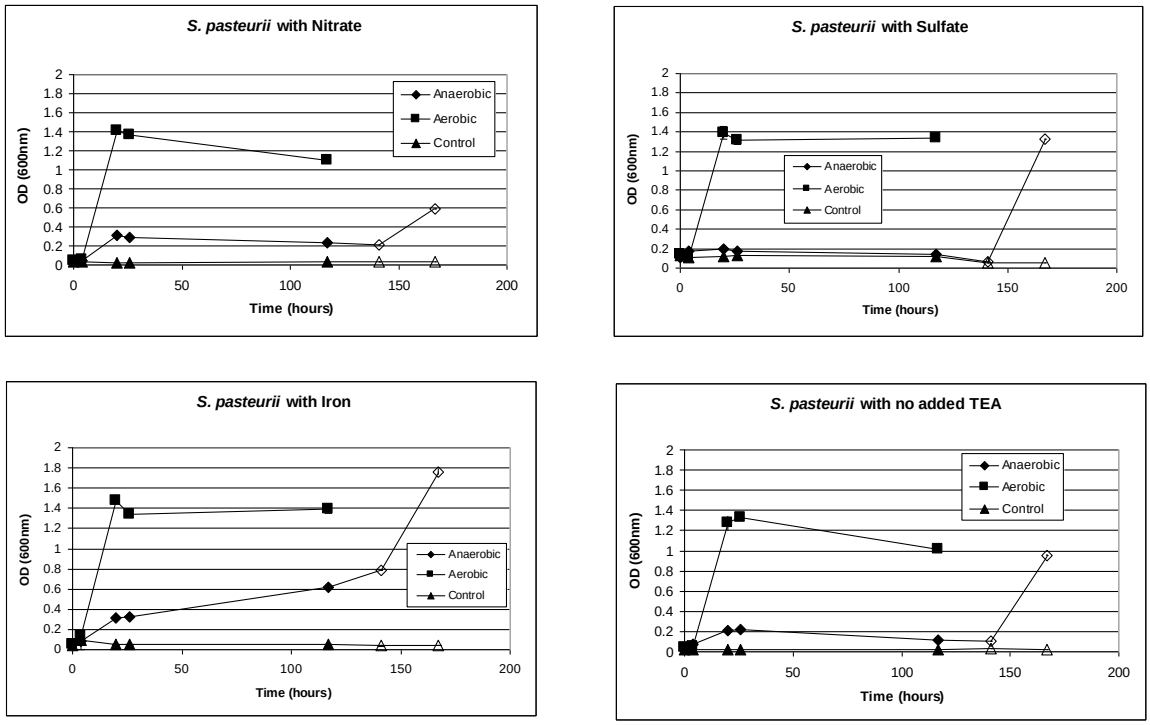


Figure 19: Change in OD_{600} over time in anaerobic and aerobic calcium exclusive media with various added terminal electron acceptors. Hollow points indicate where previously anaerobic media was opened to the environment.

Calcite Precipitation Experiments

Calcite precipitation experiments were carried out with nitrate added as a TEA, as well as without an external electron acceptor. Experiments were also run in CMM- with no added electron acceptor. Figure 20 shows the changes in urea and dissolved calcium concentrations over time for each experiment in CMM+ and NO_3^-, as well as the corresponding best fit line. Table 13 summarizes the kinetic coefficients extracted from this data.

The average rate coefficient for urea hydrolysis was 0.0489/hr, with a standard deviation between experiments of 38.6%. In Experiment 2, the urea concentration did not conclusively decrease, and so a urea hydrolysis kinetic coefficient was not determined for this experiment. The average lag time before the onset of hydrolysis was 6.5 hours. The average rate coefficient for calcite precipitation was 0.361/hr. The lag time before calcite precipitation began was 6.3 hours, approximately the same as the apparent onset of urea hydrolysis.

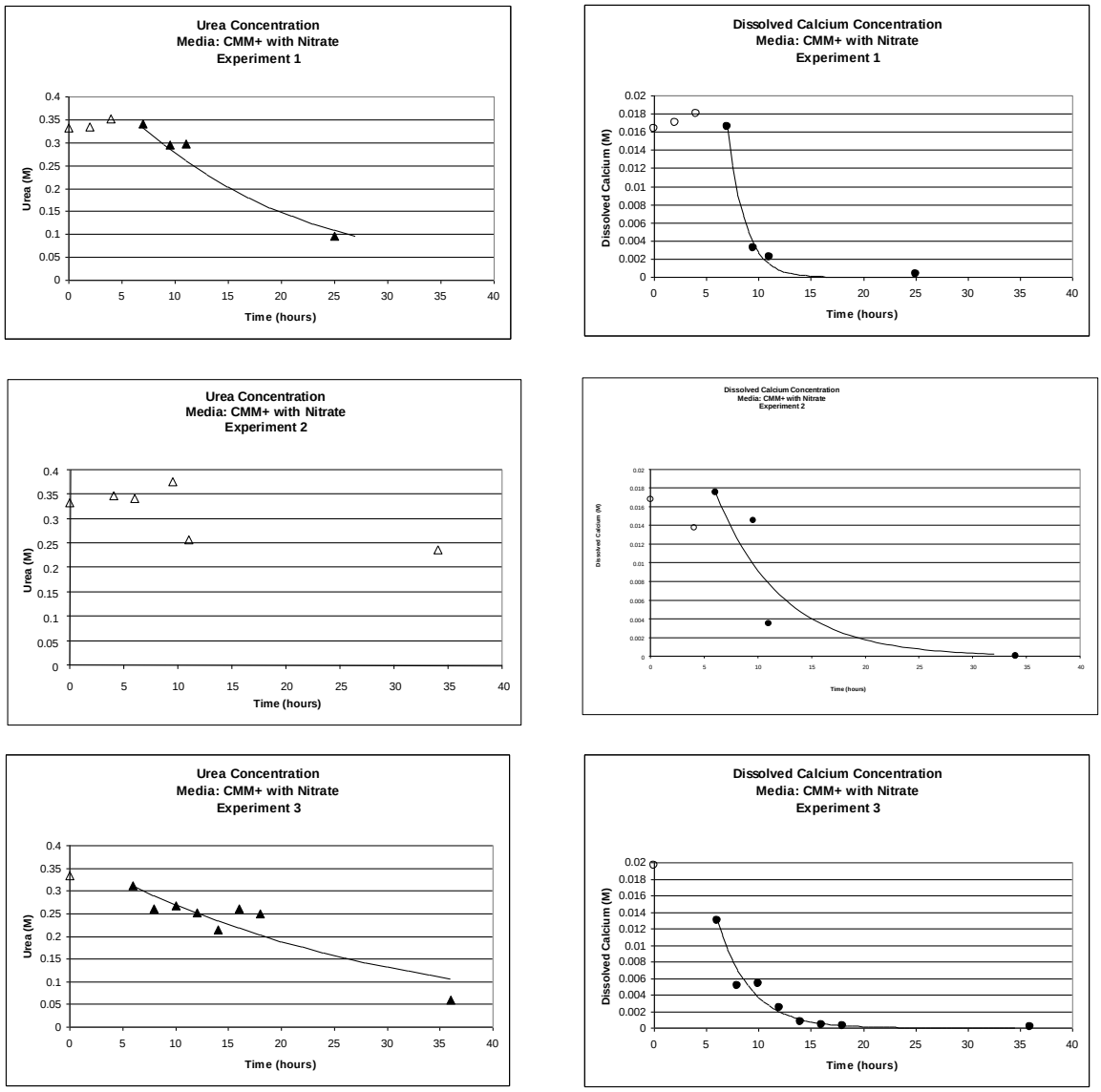


Figure 20: Urea and dissolved calcium concentrations over time from experimental data for *S. pasteurii* in anaerobic CMM+ with NO_3^-. Individual data points are experimental data and curves are the lines of best fit. Filled in data points were used to determine best fit.

Table 13: Summary of kinetic parameters for urea hydrolysis (k_{urea}) and calcite precipitation (k_{precip}) in anaerobic experiments inoculated with *S. pasteurii* in CMM+ and nitrate, including R^2 values, lag time, and number of data points used in evaluation.

Urea Hydrolysis	Initial Biomass OD_{600}	k_{urea} (hr^{-1})	R^2	# of Data Points (Total Points)	Lag Time (hours)	k_{urea} normalized to: OD_{600} (OD_{600}^{-1} hr^{-1})	k_{urea} normalized to: CFU (mL CFU^{-1} hr^{-1})
Exp 1	0.0142	0.0623	0.9771	4(7)	7.0	4.39	2.67E-08
Exp 2	0.0169	n.d.	n/a	n/a	n/a	n/a	n/a
Exp 3	0.0125	0.0356	0.8130	8(9)	6.0	2.84	2.01E-08
Average	**0.0145**	**0.0489**			**6.5**	**3.62**	**2.34E-08**
Std. Dev	0.0022	0.0189			0.7	1.09	4.67E-09
Calcite Precipitation		k_{precip} (hr^{-1})	R^2	# of Data Points (Total Points)	Lag Time (hours)		
Exp 1		0.604	0.9958	4(7)	7.0		
Exp 2		0.164	0.8202	4(6)	6.0		
Exp 3		0.314	0.9552	6(9)	6.0		
Average		**0.361**			**6.5**		
Std. Dev		0.224			0.6		

The changes in urea and dissolved calcium over time for each experiment in CMM+ without an additional TEA are shown in Figure 21. Table 14 summarizes the kinetic coefficients found from this data. Experiment 1 demonstrated no decrease in urea or dissolved calcium concentrations, and so no kinetic analysis was performed on the data. CFU analysis showed that there were cells in this culture. One possible explanation for lack of urea hydrolysis in this experiment is the possibility that urea was accidentally omitted from the system. Experiment 2 also showed no marked decrease in urea concentration, so a kinetic coefficient for urea hydrolysis was not determined. However, calcium concentrations decreased in this experiment. The average rate coefficient for urea hydrolysis in the remaining experiment was 0.0827/hr, with lag time of 10 hours.

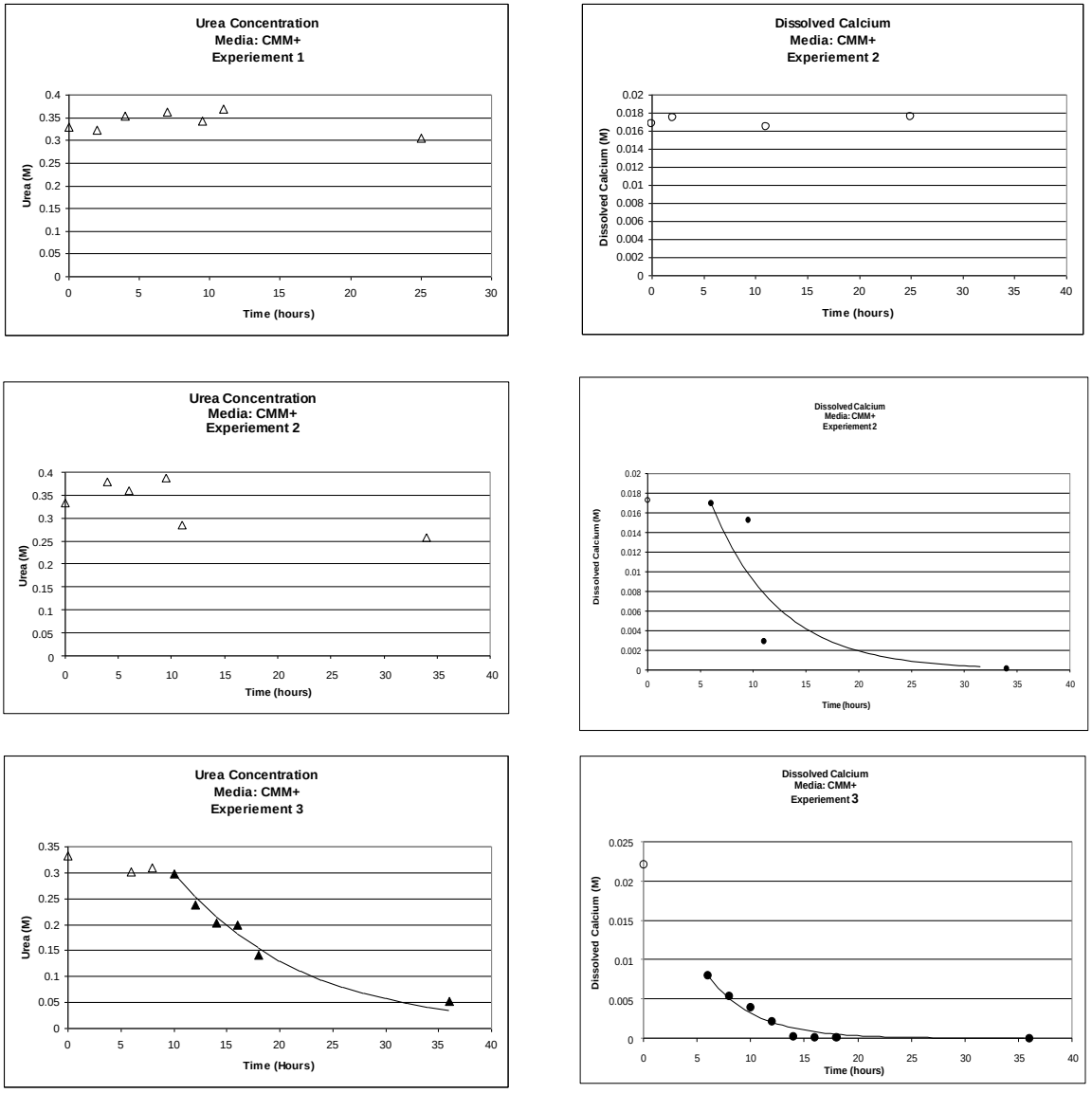


Figure 21: Urea and dissolved calcium concentrations over time from experimental data for *S. pasteurii* in anaerobic CMM+ no added TEA. Individual data points are experimental data and curves are the lines of best fit. Filled in data points were used to determine best fit.

The average rate coefficient for calcite precipitation was 0.192/hr, with a lag time of 6 hours. No data points were taken between 0 and 6 hours in Experiment 3, and the actual start of precipitation is hard to determine. This could be a source of error on the kinetic analysis of calcite precipitation and urea hydrolysis. If the onset of precipitation actually occurred at five hours, the value for k_{urea} would be higher.

Table 14: Summary of kinetic parameters for urea hydrolysis (k_{urea}) and calcite precipitation (k_{precip}) in anaerobic experiments inoculated with *S. pasteurii* in CMM+ with no additional TEA, including R^2 values, lag time, and number of data points used in evaluation.

Urea Hydrolysis	Initial Biomass OD_{600}	k_{urea} (hr^{-1})	R^2	# of Data Points (Total Points)	Lag Time (hours)	k_{urea} normalized to: OD_{600} (OD_{600}^{-1} hr^{-1})	k_{urea} normalized to: CFU (mL CFU^{-1} hr^{-1})
Exp 1	0.0142	n.d.	n/a	n/a	n/a	n/a	n/a
Exp 2	0.0169	n.d	n/a	n/a	n/a	n/a	n/a
Exp 3	0.0125	0.0827	0.9765	6(9)	10.0	6.62	4.68E-08
Calcite Precipitation		**k_{precip} (hr^{-1})**	**R^2**	**# of Data Points (Total Points)**	**Lag Time (hours)**		
Exp 1		n.d.	n/a	n/a	n/a		
Exp 2		0.156	0.7604	4(5)	6.0		
Exp 3		0.227	0.9704	8(9)	6.0		
Average		**0.192**			**6.0**		
Std. Dev		0.051			0.0		

Figure 22 shows the change in urea concentration over time for experiments performed in CMM- with nitrate added as a TEA, and Table 15 lists the corresponding kinetic parameters. Experiment 2 did not conclusively show a decrease in urea concentration, so kinetic analysis was not performed on this data. The average rate coefficient for urea hydrolysis was 0.0710/hour, with a standard deviation of 25.3%. The average lag time before onset of hydrolysis was 8.5 hours.

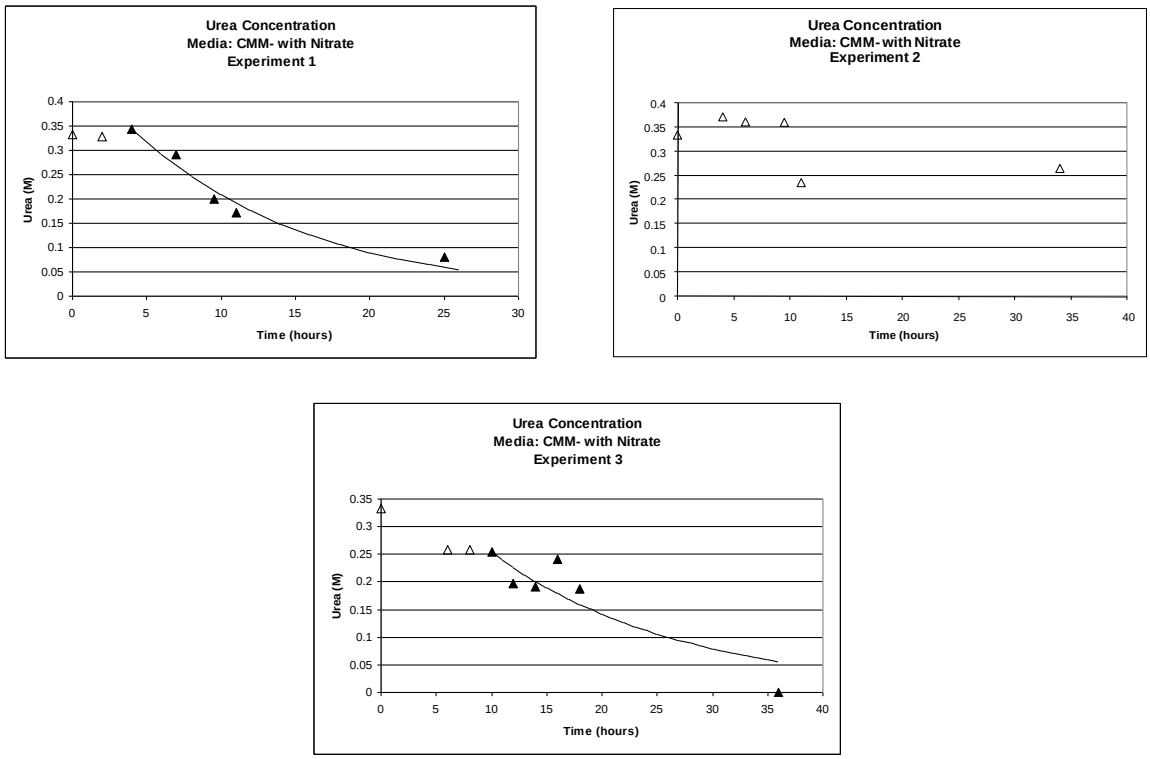


Figure 22: Urea concentration over time from experimental data for *S. pasteurii* in anaerobic CMM- with nitrate. Individual data points are experimental data and curves are the lines of best fit. Filled in data points were used to determine best fit.

Table 15: Summary of kinetic parameters for urea hydrolysis (k_{urea}) in anaerobic experiments inoculated with *S. pasteurii* in CMM- with nitrate, including R^2 values, lag time, and number of data points used in evaluation.

Urea Hydrolysis	Initial Biomass OD_{600}	k_{urea} (hr^{-1})	R^2	# of Data Points (Total Points)	Lag Time (hours)	k_{urea} normalized to: OD_{600} (OD_{600}^{-1} hr^{-1})	k_{urea} normalized to: CFU (mL CFU^{-1} hr^{-1})
Exp 1	0.0142	0.0837	0.9627	5(7)	7.0	5.90	3.59E-08
Exp 2	0.0169	n.d.	n/a	n/a	n/a	n/a	n/a
Exp 3	0.0125	0.0583	0.8142	6(9)	10.0	4.66	3.30E-08
Average	**0.0145**	**0.0710**			**8.5**	**5.28**	**3.45E-08**
Std. Dev	0.0022	0.0179			2.1	0.88	2.05E-09

Comparison of Aerobic and Anaerobic Kinetics

Because of the possible use of ureolytic bacteria in anaerobic environments, it is desirable to know the difference in rates between conditions. Table 16 summarizes the average kinetic coefficients found in the above anaerobic experiments, as well as those found in the aerobic experiments using *S. pasteurii*. In calcium inclusive media, the rate of urea hydrolysis in the aerobic experiment is similar to that found in the anaerobic experiment without an external electron acceptor. These rates are close to two times higher that the calcium inclusive anaerobic experiment with nitrate as a TEA. One explanation for this is that nitrate could in some way hinder urea hydrolysis. Another explanation is that the nitrate interferes with the absorbance readings used to gather the data used in the analysis. In calcium exclusive media, the rate coefficient for urea hydrolysis in aerobic media is much greater than its calcium inclusive counterparts. The largest rate coefficient for calcite precipitation is found in the anaerobic media with nitrate added, although there is not a large difference among the three media. One explanation for the higher rate could be that the aerobic systems are open to the atmosphere, allowing for the release of ammonia and carbon dioxide, which could slow down the rate of increase in saturation state of the system. The anaerobic systems are crimped and sealed, and can be considered closed. Therefore, there is no release of molecules into the environment, potentially causing a faster increase in saturation state in the closed systems. The lag time the onset of urea hydrolysis and calcite precipitation in anaerobic media, however, is longer.

Table 16: Summary of kinetic coefficients found in aerobic and anaerobic experiments inoculated with *S. pasteurii*. Standard deviations for triplicate experiments, unless otherwise noted, are shown in parenthesis.

	Calcium Inclusive			**Calcium Exclusive**	
	Anaerobic CMM+/NO_3^-	Anaerobic CMM+	Aerobic CMM+	Anaerobic CMM-/NO_3^-	Aerobic CMM-
k_{urea} (hr^{-1})	0.0489 (0.0189) *	0.0827 (n/a) §	0.0744 (0.0215)	0.071 (0.0179) *	0.192 (0.1040) *
Lag Time (hrs)	6.5 (0.7) *	10.0 (n/a) §	5.0 (1.0)	8.5 (2.1) *	4.0 (0.0) *
k_{urea} (OD_{600}^{-1} hr^{-1})	3.62 (1.09) *	6.62 (n/a) §	5.25 (1.27)	5.28 (0.88) *	11.3 (5.99)
k_{urea} (mL CFU^{-1} hr^{-1})	2.34E-08 (4.67E-09) *	4.68E-08 (n/a) §	3.22E-08 (6.54E-09)	3.45E-08 (2.05E-09) *	5.88E-08 (2.02E-08) *
k_{precip} (hr^{-1})	0.361 (0.224)	0.192 (0.051)	0.253 (0.022)		
Lag Time (hrs)	6.5 (0.6)	6.0 (0.0)	3.3 (0.6)		

* Two experiments used for kinetic analysis

§ One experiment used in analysis

CONCLUSIONS

While all three ureolytic species studied, *S. pasteurii*, *B. sphaericus* 21776 and *B. sphaericus* 21787, were capable of inducing calcite precipitation, each did so at different rates. *B. sphaericus* 21776 had the highest rate coefficient for both urea hydrolysis and calcite precipitation, while *B. sphaericus* 21787 had the lowest rate coefficients. When rate coefficients are normalized to cell numbers, *B. sphaericus* 21776 demonstrated the highest rate of urea hydrolysis per cell, indicating that it may have a higher concentration of urease than *S. pasteurii* and *B. sphaericus* 21787. This information is valuable for applying this technology in the deep subsurface. In bacteriogenic mineral plugging, slow hydrolysis like that seen by *B. sphaericus* 21787 may allow for a larger spatial distribution of the bacteria before the onset of precipitation, and potentially less urea will be needed to induce precipitation. In the coprecipitation of radionuclides such as strontium, the high ureolysis and precipitation rates seen in systems inoculated with *B. sphaericus* 21776 could allow for more strontium to be incorporated into the calcite structure. As was shown by SEM imaging, bacterial cells become encased in calcite as precipitation occurs. Further analysis of TEM images and diffusion scales shows that these encased cells are most likely not ureolytically active, due to a relatively large time frame for urea diffusion through calcite to the cell.

S. pasteurii is capable of hydrolyzing urea in anaerobic environments in CMM with and without the addition of electron acceptors. However, it cannot be conclusively shown that *S. pasteurii* grows without oxygen. The onset of both urea hydrolysis and calcite precipitation occurs later under anaerobic conditions than under aerobic conditions.

Suggestions for Further Studies

The research performed for this thesis focused on the kinetics of calcite precipitation using known ureolytic bacterial species in a rich, undefined media. The findings from these experiments are to be used toward the goal of biologically plugging preferential CO_2 pathways in deep saline aquifers. Further studies must be performed to achieve this goal. One such study would be to isolate ureolytic bacteria from these aquifers to avoid the need to introduce foreign bacteria. It would also be beneficial to manipulate the growth and onset of ureolysis of the bacteria *in situ*. As the research presented in this paper has shown, different growth conditions can lead to different rates of urea hydrolysis and calcite precipitation. Using this knowledge, a study should be carried out to determine the best way to stimulate urea hydrolysis after an active bacterial community has been formed to ensure a good spatial distribution of precipitated calcite.

APPENDICES

APPENDIX A

Raw Data Tables

Table 17: Compiled data from *S. pasteurii* aerobic experiments in calcium inclusive media.

Aerobic Experiment 1, CMM+, Ran 1/6/2008-1/7/2008										
Hours	pH	Protein (mg/L)	Std Dev	Log CFU/mL	Std Dev	OD_{600}	Std Dev	NH4+ (M)	Std Dev	Dissolved Calcium (M)
0.0	6.7	30.872	0.588	6.748	6.290	0.043	0.001	0.1165	0.0075	0.0245
3.0	7.5	34.077	2.522	7.100	6.585	0.045	0.000	0.1463	0.0194	0.0248
5.0	8.4	35.744	1.175	4.778	4.739	0.218	0.071	0.1838	0.0133	0.0213
6.0	8.4	31.256	1.351	4.602	4.739	0.207	0.040	0.2312	0.0563	0.0088
7.0	8.6	32.667	2.473	5.255	5.216	0.122	0.031	0.2001	0.0738	0.0069
9.0	8.8	37.282	1.601	5.623	5.115	0.163	0.045	0.4138	0.0354	0.0042
10.0	8.9	55.872	8.740	6.009	5.705	0.176	0.060	0.4336	0.0272	0.0010
11.0	8.9	46.641	4.589	6.792	6.378	0.228	0.006	0.7250	0.0482	0.0019
12.0	9.1	47.410	4.219	6.991	6.663	0.251	0.059	0.4508	0.0347	0.0037
26.0	9.3	65.744	4.323	7.732	7.554	0.269	0.015	0.7119	0.1782	0.0002
30.0	9.3	69.205	2.118	7.330	6.224	0.190	0.011	0.8177	0.0361	0.0005
Aerobic Experiment 2, CMM+, Ran 2/22/2008-2/23/2008										
Hours	pH	Protein (mg/L)	Std Dev	Log CFU/mL	Std Dev	OD_{600}	Std Dev	NH4+ (M)	Std Dev	Dissolved Calcium (M)
Inoculum	n/a	n/a	n/a	9.556	9.259	0.377	0.072	n/a	n/a	n/a
0.0	6.6	19.194	3.664	7.064	6.545	0.061	0.002	0.1681	0.0592	0.0247
4.0	8.1	23.065	2.560	5.991	5.678	0.121	0.004	0.1138	0.0310	0.0242
5.5	8.2	24.032	3.414	6.000	5.530	0.168	0.005	0.1810	0.0514	0.0216
8.0	8.5	27.151	6.340	6.964	6.216	0.128	0.022	0.2467	0.0309	0.0061
10.0	8.8	35.108	1.304	7.338	6.636	0.104	0.002	0.3154	0.0407	0.0060
11.5	8.9	53.817	3.449	7.681	7.115	0.120	0.027	0.3901	0.0426	0.0012
29.5	9.3	52.527	5.215	7.973	7.416	0.096	0.001	0.6605	0.0714	0.0003
Aerobic Experiment 3, CMM+, Ran 5/26/2008										
Hours	pH	Protein (mg/L)	Std Dev	Log CFU/mL	Std Dev	OD_{600}	Std Dev	NH4+ (M)	Std Dev	Dissolved Calcium (M)
Inoculum	n/a	n/a	n/a	8.477	8.000	0.421	0.022	n/a	n/a	n/a
0.0	6.7	28.378	3.151	7.049	6.358	0.044	0.001	0.1364	0.0294	0.0242
3.0	8.2	28.600	9.955	5.188	4.517	0.146	0.014	0.2547	0.0051	0.0244
5.0	8.4	23.044	0.385	5.982	5.224	0.333	0.036	0.2019	0.0321	0.0104
6.0	8.5	21.489	3.672	6.033	5.378	0.107	0.004	0.2003	0.0062	0.0100
8.0	8.7	23.933	5.457	6.903	6.272	0.103	0.017	0.2866	0.0253	0.0087
10.0	8.9	51.711	0.770	n/a	n/a	0.202	0.048	0.3522	0.0135	0.0079
12.0	9.1	74.600	4.667	7.477	7.327	0.179	0.005	0.3879	0.0249	0.0066

Table 18: Compiled data from *S. pasteurii* aerobic experiments in calcium exclusive media.

Experiment 1, Ran from 9/30/2008-10/1/2008										
Hours	pH	Protein (mg/L)	Std Dev	Log CFU/mL	Std Dev	OD_{600}	Std Dev	NH4+ (M)	Std Dev	Dissolved Calcium (M)
0	7.630	n/a	n/a	7.127	6.382	0.043	0.001	0.1926	0.0069	n/a
4	8.910	n/a	n/a	8.505	8.378	0.101	0.004	0.2481	0.0318	n/a
6	9.170	n/a	n/a	n/a	n/a	0.121	0.004	0.3816	0.0169	n/a
11	9.250	n/a	n/a	7.954	7.327	0.111	0.003	0.5896	0.0431	n/a
34	9.280	n/a	n/a	7.881	7.545	0.102	0.003	0.4021	0.0394	n/a
Experiment 2, Ran from 3/4/2009-3/5/2009										
Hours	pH	Protein (mg/L)	Std Dev	Log CFU/mL	Std Dev	OD_{600}	Std Dev	NH4+ (M)	Std Dev	Dissolved Calcium (M)
Inoculum	n/a	n/a	n/a	n/a	n/a	0.488	0.010	n/a	n/a	n/a
0	6.19	n/a	n/a	n/a	n/a	0.043	0.001	0.2313	0.0098	n/a
2	7.32	n/a	n/a	n/a	n/a	0.044	0.002	0.2256	0.0632	n/a
4	8.47	n/a	n/a	n/a	n/a	0.064	0.001	0.1922	0.0581	n/a
6	8.95	n/a	n/a	n/a	n/a	0.128	0.007	0.5583	0.0958	n/a
8	9.07	n/a	n/a	n/a	n/a	0.149	0.004	0.5892	0.0469	n/a
25.5	9.16	n/a	n/a	n/a	n/a	0.119	0.007	1.0059	0.0816	n/a

Table 19: Compiled data from *S. pasteurii* anaerobic survey experiment in calcium exclusive media with various TEAs.

TEA: Nitrate, Ran 6/11/2008-6/17/2008									
	pH			OD_{600}					
Hours	Anaerobic	Aerobic	Control	Anaerobic	Std Dev	Aerobic	Std Dev	Control	Std Dev
0	5.79	5.770	5.220	0.048	0.001	0.048	0.001	0.045	0.004
4	7.7	8.060	5.440	0.049	0.001	0.050	0.002	0.045	0.001
20	9.09	9.180	6.310	0.118	0.003	0.405	0.006	0.044	0.001
26	9.12	9.180	6.810	0.112	0.003	0.394	0.003	0.044	0.000
117	9.12	9.250	6.370	0.097	0.001	0.323	0.006	0.045	0.001
141*	9.11	n/a	6.070	0.092	0.003	n/a	n/a	0.045	0.001
167*	9.19	n/a	6.200	0.190	0.002	n/a	n/a	0.044	0.001
TEA: Sulfate, Ran 6/11/2008-6/17/2008									
	pH			OD_{600}					
Hours	Anaerobic	Aerobic	Control	Anaerobic	Std Dev	Aerobic	Std Dev	Control	Std Dev
0	7.80	7.790	7.680	0.027	0.002	0.038	0.002	0.033	0.003
4	8.53	8.590	7.670	0.046	0.004	0.028	0.002	0.029	0.001
20	9.1	9.110	7.620	0.050	0.004	0.362	0.016	0.031	0.000
26	9.13	9.130	7.730	0.046	0.002	0.343	0.011	0.033	0.004
117	9.14	9.200	7.780	0.036	0.001	0.347	0.002	0.032	0.002
141*	9.16	n/a	7.660	0.018	0.001	n/a	n/a	0.015	0.001
167*	9.19	n/a	7.630	0.345	0.002	n/a	n/a	0.013	0.001
TEA: Iron, Ran 6/11/2008-6/17/2008									
	pH			OD_{600}					
Hours	Anaerobic	Aerobic	Control	Anaerobic	Std Dev	Aerobic	Std Dev	Control	Std Dev
0	6.97	6.880	6.570	0.014	0.002	0.015	0.002	0.012	0.001
4	7.94	8.420	6.650	0.021	0.002	0.034	0.001	0.025	0.025
20	9.05	9.090	6.800	0.083	0.003	0.385	0.008	0.012	0.002
26	9.07	9.100	6.920	0.083	0.004	0.349	0.010	0.013	0.002
117	9.09	9.180	6.930	0.160	0.003	0.363	0.013	0.014	0.000
141*	9.13	n/a	7.330	0.204	0.003	n/a	n/a	0.010	0.001
167*	9.14	n/a	7.070	0.458	0.010	n/a	n/a	0.010	0.000
TEA: none, Ran 6/11/2008-6/17/2008									
	pH			OD_{600}					
Hours	Anaerobic	Aerobic	Control	Anaerobic	Std Dev	Aerobic	Std Dev	Control	Std Dev
0	6.06	5.850	5.200	0.012	0.001	0.010	0.001	0.005	0.001
4	7.89	8.120	5.380	0.019	0.009	0.014	0.001	0.005	0.001
20	9.08	9.140	6.630	0.055	0.002	0.331	0.020	0.005	0.001
26	9.09	9.150	6.290	0.057	0.002	0.345	0.009	0.006	0.000
117	9.08	9.220	6.580	0.030	0.002	0.263	0.005	0.005	0.001
141*	9.17	n/a	6.710	0.027	0.002	n/a	n/a	0.008	0.003
167*	9.16	n/a	6.050	0.249	0.004	n/a	n/a	0.006	0.002

*Sample times where oxygen has been allowed to diffuse into solution.

Table 20: Compiled data from *S. pasteurii* anaerobic experiments in calcium inclusive media with NO_3^- as a terminal electron acceptor.

Hours	pH	Protein (mg/L)	Std Dev	Log CFU/mL	Std Dev	OD_{600}	Std Dev	NH4+ (M)	Std Dev	Dissolved Calcium (M)
Experiment 1, Ran from 9/18/2008-9/19/2008										
Inoculum	n/a	n/a	n/a	9.009	8.171	0.410	0.010	n/a	n/a	n/a
0	6.5	17.067	3.208	6.914	6.413	0.045	0.002	0.2251	0.0067	0.0164
2	6.8	18.000	3.940	6.914	5.651	0.042	0.002	0.2220	0.0067	0.0170
4	7.2	9.200	1.744	6.732	6.290	0.044	0.001	0.1891	0.0119	0.0181
7	7.9	18.800	4.214	6.792	6.470	0.104	0.001	0.2070	0.0120	0.0166
9.5	8.4	32.000	3.124	6.342	5.763	0.067	0.004	0.3026	0.0132	0.0032
11	8.5	34.267	3.717	6.384	5.701	0.077	0.002	0.2986	0.0377	0.0022
25	9.1	6.667	4.046	n.d.	n/a	0.170	0.150	0.7008	0.0216	0.0003
28*	9.2	0.000	1.386	5.279	4.477	0.072	0.002	0.5910	0.0097	0.0002
53*	9.1	0.000	2.540	n.d.	n/a	0.060	0.000	0.6963	0.1152	0.0001
Experiment 2, Ran from 9/30/2008-10/1/2008										
Inoculum	n/a	n/a	n/a	9.017	8.517	0.482	0.019	n/a	n/a	n/a
0	6.3	16.933	2.894	6.973	6.606	0.047	0.001	0.1917	0.0081	0.0169
4	7.0	15.467	2.663	6.428	5.877	0.046	0.001	0.1614	0.0228	0.0138
6	7.5	n.d.	n/a	6.602	6.389	0.047	0.001	0.1723	0.0220	0.0176
9.5	8.3	n.d.	n/a	6.477	6.088	0.124	0.003	0.1029	0.0268	0.0146
11	8.4	n.d.	n/a	5.914	5.396	0.091	0.001	0.3426	0.0397	0.0036
34	9.2	n.d.	n/a	n.d.	n/a	0.058	0.005	0.3838	0.0142	0.0001
Experiment 3, Ran from 1/30/2009-2/2/2009										
Inoculum	n/a	n/a	n/a	7.892	7.624	0.365	0.010	n/a	n/a	n/a
0	6.8	8.347	3.607	6.991	6.729	0.045	0.000	0.1818	0.0113	0.0197
6	8.0	4.480	2.800	4.000	4.000	0.197	0.008	0.2264	0.0258	0.0130
8	8.1	n.d.	n/a	4.301	4.088	0.193	0.013	0.3273	0.0190	0.0051
10	8.2	1.547	2.444	4.857	4.443	0.150	0.009	0.3147	0.0439	0.0053
12	8.3	2.480	2.227	5.140	4.515	0.043	0.001	0.3441	0.0166	0.0024
14	8.5	1.813	2.894	5.716	5.336	0.052	0.001	0.4209	0.0315	0.0007
16	8.6	6.747	2.723	6.204	5.841	0.058	0.003	0.3270	0.0255	0.0004
18	8.7	10.480	2.227	6.505	5.923	0.061	0.004	0.3469	0.0707	0.0003
36	8.9	38.347	1.890	7.107	6.744	0.071	0.003	0.7289	0.0443	0.0002
38*	8.9	13.547	1.973	6.892	6.253	0.064	0.001	1.1580	0.0653	0.0002
42*	9.0	10.347	0.924	7.049	6.171	0.064	0.002	0.8775	0.0631	0.0001
62*	9.0	n.d.	n/a	6.833	6.470	0.064	0.001	0.5871	0.0780	0.0001

*Sample times where oxygen has been allowed to diffuse into solution.

Table 21: Compiled data from *S. pasteurii* anaerobic experiments in calcium inclusive media without a terminal electron acceptor.

Hours	pH	Protein (mg/L)	Std Dev	Log CFU/mL	Std Dev	OD_{600}	Std Dev	NH4+ (M)	Std Dev	Dissolved Calcium (M)
Experiment 1, Ran from 9/18/2008-9/19/2008										
Inoculum	n/a	n/a	n/a	9.009	8.171	0.410	0.010	n/a	n/a	n/a
0	6.4	n.d.	n/a	6.914	6.413	0.045	0.001	0.2338	0.0017	0.0168
2	6.5	n.d.	n/a	6.914	5.651	0.043	0.001	0.2465	0.0166	0.0174
4	6.5	14.667	1.804	6.732	6.290	0.044	0.001	0.1886	0.0048	n/a
7	6.6	11.600	2.000	6.792	6.470	0.043	0.000	0.1683	0.0117	n/a
9.5	6.5	17.467	3.717	6.342	5.763	0.044	0.001	0.2107	0.0066	n/a
11	6.5	16.133	3.630	6.384	5.701	0.043	0.002	0.1571	0.0154	0.0165
25	6.6	n.d.	n/a	n.d.	n/a	0.044	0.001	0.2860	0.0045	0.0176
28*	6.7	25.600	3.487	5.279	4.477	0.044	0.003	0.2156	0.0104	n/a
53*	7.0	21.600	5.810	n.d.	n/a	0.051	0.014	0.2408	0.0186	n/a
Experiment 2, Ran from 9/30/2008-10/1/2008										
Inoculum	n/a	n/a	n/a	9.017	8.517	0.482	0.019	n/a	n/a	n/a
0	6.3	21.600	5.810	6.973	6.606	0.047	0.002	0.2164	0.0084	0.0173
4	6.9	20.000	4.915	6.428	5.877	0.046	0.001	0.1240	0.0215	n/a
6	7.3	20.267	7.204	6.602	6.389	0.047	0.001	0.1635	0.0057	0.0170
9.5	8.1	31.600	5.600	6.477	6.088	0.120	0.003	0.1077	0.0080	0.0153
11	8.4	30.533	6.516	5.914	5.396	0.145	0.039	0.3147	0.0348	0.0030
34	9.2	52.000	2.884	n/a	n/a	0.142	0.141	0.3700	0.0487	0.0002
Experiment 3, Ran from 1/30/2009-2/2/2009										
Inoculum	n/a	n/a	n/a	7.892	7.624	0.365	0.010	n/a	n/a	n/a
0	6.9	33.813	6.201	6.763	6.115	0.045	0.001	0.2402	0.0113	0.0222
6	7.9	16.480	5.810	3.602	3.739	0.324	0.091	0.3038	0.0051	0.0081
8	8.0	16.080	2.117	4.820	4.606	0.127	0.002	0.2881	0.0200	0.0055
10	8.2	15.013	2.013	5.763	5.115	0.089	0.004	0.3092	0.0624	0.0040
12	8.4	22.080	2.800	6.763	6.525	0.046	0.001	0.4301	0.0041	0.0023
14	8.7	33.280	3.940	7.057	6.768	0.110	0.008	0.5011	0.0219	0.0003
16	8.8	41.680	5.107	6.833	6.336	0.106	0.005	0.5080	0.0592	0.0003
18	8.9	52.613	2.948	6.914	6.493	0.090	0.003	0.6255	0.0714	0.0002
36	9.0	57.947	6.466	6.973	6.585	0.060	0.000	0.8022	0.1258	0.0001
38*	9.0	57.680	5.381	6.127	5.128	0.057	0.001	1.2089	0.0686	0.0001
42*	9.0	51.813	5.372	6.924	6.606	0.057	0.001	0.9486	0.0506	0.0001
62*	9.1	57.413	5.401	6.199	5.744	0.062	0.000	0.3487	0.1236	0.0001

*Sample times where oxygen has been allowed to diffuse into solution.

Table 22: Compiled data from *S. pasteurii* anaerobic experiments in calcium exclusive media with NO_3^- as a terminal electron acceptor.

Hours	pH	Protein (mg/L)	Std Dev	Log CFU/mL	Std Dev	OD_{600}	Std Dev	NH4+ (M)	Std Dev	Dissolved Calcium (M)
Experiment 1, Ran from 9/18/2008-9/19/2008										
Inoculum	n/a	n/a	n/a	9.009	8.171	0.410	0.010	n/a	n/a	n/a
0	6.6	23.200	2.263	6.973	6.416	0.044	0.001	0.2322	0.0045	n/a
2	7.1	25.000	3.111	7.009	6.115	0.043	0.001	0.2419	0.0038	n/a
4	8.0	31.400	0.849	5.845	5.389	0.044	0.002	0.2117	0.0081	n/a
7	8.9	29.000	5.940	6.176	5.686	0.042	0.001	0.3158	0.0070	n/a
9.5	9.0	39.000	3.111	5.982	5.496	0.046	0.001	0.4993	0.0510	n/a
11	9.1	43.000	3.677	5.447	5.284	0.044	0.002	0.5532	0.0654	n/a
25	9.2	n/a	n/a	5.283	4.413	0.043	0.001	0.7380	0.0434	n/a
28*	9.3	46.200	4.243	5.338	4.846	0.044	0.000	0.7176	0.0172	n/a
53*	9.2	n.d.	n/a	n/a	n/a	0.072	0.003	0.7392	0.0339	n/a
Experiment 2, Ran from 9/30/2008-10/1/2008										
Inoculum	n/a	n/a	n/a	9.017	8.517	0.482	0.019	n/a	n/a	n/a
0	6.5	24.800	3.857	6.914	5.923	0.047	0.002	0.2143	0.0214	n/a
4	7.2	26.267	2.663	6.924	6.416	0.047	0.000	0.1355	0.0096	n/a
6	7.8	33.200	2.227	6.699	6.548	0.048	0.003	0.1583	0.0176	n/a
9.5	8.8	35.067	2.203	7.107	6.312	0.053	0.003	0.1581	0.0602	n/a
11	9.0	45.200	2.433	7.041	6.477	0.059	0.001	0.4079	0.0275	n/a
34	9.2	47.600	1.386	n/a	n/a	0.054	0.001	0.3521	0.0424	n/a
Experiment 3, Ran from 1/30/2009-2/2/2009										
Inoculum	n/a	n/a	n/a	7.892	7.624	0.365	0.010	n/a	n/a	n/a
0	6.8	5.013	0.924	6.748	6.290	0.045	0.000	0.1942	0.0089	n/a
6	8.3	8.347	0.611	4.623	4.115	0.043	0.000	0.3445	0.0144	n/a
8	8.4	10.747	1.665	4.643	4.259	0.044	0.000	0.3443	0.0210	n/a
10	8.5	9.413	1.286	4.820	4.460	0.043	0.001	0.3526	0.0768	n/a
12	8.6	15.413	2.411	5.531	5.317	0.044	0.001	0.4676	0.0741	n/a
14	8.7	20.613	1.222	6.086	5.736	0.045	0.001	0.4785	0.0678	n/a
16	8.8	24.213	4.388	6.170	5.678	0.047	0.001	0.3785	0.0092	n/a
18	8.8	23.547	1.286	6.428	5.576	n/a	n/a	0.4851	0.1452	n/a
36	9.0	18.480	2.117	7.079	6.651	0.066	0.001	0.8723	0.3695	n/a
38*	9.0	15.547	1.286	7.033	6.378	0.064	0.002	1.3675	0.2733	n/a
42*	9.0	10.347	1.665	6.964	6.611	0.066	0.001	0.9663	0.1853	n/a
62*	9.0	5.013	3.946	7.204	6.406	0.078	0.001	0.4394	0.1520	n/a

*Sample times where oxygen has been allowed to diffuse into solution.

Table 23: Compiled data from *B. sphaericus* 21776 aerobic experiments in calcium inclusive media.

Hours	pH	Protein (mg/L)	Std Dev	Log CFU/mL	Std Dev	OD_{600}	Std Dev	NH4+ (M)	Std Dev	Dissolved Calcium (M)
Experiment 1, Ran from 2/2/2008-2/3/2008										
Inoculum	n/a	n/a	n/a	7.869	7.472	0.428	0.002	n/a	n/a	n/a
0	7.5	26.617	8.578	6.486	5.416	0.042	0.001	0.2313	0.0402	0.0247
4	8.3	14.117	3.753	4.833	4.647	0.156	0.004	0.3687	0.0500	0.0220
7	8.5	17.283	6.506	5.270	4.496	0.197	0.015	0.5989	0.0540	0.009
9	8.7	17.783	4.311	5.699	5.327	0.095	0.003	0.3966	0.1283	0.008
11	8.8	18.117	3.819	6.365	5.482	0.107	0.006	0.5769	0.1321	0.004
28	9.3	98.617	6.331	n/a	n/a	0.182	0.023	0.8484	0.0351	0.0006
Experiment 2, Ran on 7/8/2008										
Inoculum	n/a	n/a	n/a	8.350	7.710	0.357	0.024	n/a	n/a	n/a
0	6.9	24.378	3.355	6.556	6.057	0.043	0.002	0.1654	0.0212	0.0237
3	8.2	12.600	1.333	6.210	5.552	0.147	0.006	0.1701	0.0101	0.0220
4	8.3	n.d.	n/a	6.279	5.695	0.337	0.035	0.1720	0.0108	0.0071
5	8.5	21.711	2.037	6.369	5.400	0.174	0.021	0.2085	0.0256	0.0046
7	8.8	30.378	1.925	7.057	6.625	0.069	0.005	0.3233	0.0257	0.0018
8	8.9	45.489	2.143	7.435	6.683	0.082	0.004	0.4951	0.0194	0.0009
9	9.0	60.156	3.672	7.521	6.668	0.093	0.001	0.5586	0.0652	0.0008
11	9.1	84.156	0.770	7.683	6.839	0.120	0.003	0.6272	0.1012	0.0029
Experiment 3, Ran from 7/12/2008-7/13/2008										
Inoculum	n/a	n/a	n/a	8.250	7.336	0.420	0.013	n/a	n/a	n/a
0	7.3	19.711	7.813	6.369	5.181	0.045	0.001	0.1945	0.0161	0.0224
3	8.2	9.267	1.155	3.775	2.894	0.138	0.001	0.1689	0.0137	0.0204
4	8.4	18.600	4.667	5.079	4.452	0.181	0.019	0.1776	0.0178	0.0098
5	8.4	17.267	1.155	5.204	4.727	0.224	0.017	0.1969	0.0126	0.0053
7	8.6	22.600	3.528	5.716	5.515	0.070	0.005	0.2958	0.0123	0.0041
9	8.7	24.822	5.389	6.431	5.661	0.049	0.001	0.2608	0.0088	0.0021
26	9.2	82.378	6.049	7.318	6.761	0.081	0.000	0.8819	0.0465	0.0003

Table 24: Compiled data from *B. sphaericus* 21776 aerobic experiments in calcium exclusive media.

Experiment 1, Ran from 2/2/2008-2/3/2008										
Hours	pH	Protein (mg/L)	Std Dev	Log CFU/mL	Std Dev	OD_{600}	Std Dev	NH4+ (M)	Std Dev	Dissolved Calcium (M)
0.000	7.5	30.283	14.145	5.531	5.506	0.043	0.002	0.251	0.027	n/a
3	8.1	44.450	21.254	4.778	5.128	0.052	0.002	0.2630	0.0453	n/a
6	8.8	89.617	0.764	6.297	5.534	0.121	0.008	0.5544	0.1145	n/a
24	9.4	97.117	2.566	5.380	5.317	0.098	0.004	1.1688	0.2013	n/a
27	9.4	88.117	3.617	4.301	4.651	0.094	0.002	1.1591	0.3733	n/a
Experiment 2, Ran from 3/4/2009-3/5/2009										
Hours	pH	Protein (mg/L)	Std Dev	Log CFU/mL	Std Dev	OD_{600}	Std Dev	NH4+ (M)	Std Dev	Dissolved Calcium (M)
Inoculum	n/a	n/a	n/a	n/a	n/a	0.419	0.012	n/a	n/a	n/a
0	6.2	n/a	n/a	n/a	n/a	0.041	0.000	0.2176	0.0361	n/a
2	7.0	n/a	n/a	n/a	n/a	0.043	0.001	0.2685	0.0119	n/a
4	8.0	n/a	n/a	n/a	n/a	0.048	0.001	0.2317	0.0109	n/a
6	8.8	n/a	n/a	n/a	n/a	0.085	0.003	0.3199	0.0073	n/a
8	9.1	n/a	n/a	n/a	n/a	0.106	0.003	0.5088	0.1346	n/a
25.5	9.2	n/a	n/a	n/a	n/a	0.100	0.010	1.2493	0.2033	n/a

Table 25: Compiled data from *B. sphaericus* 21787 aerobic experiments in calcium inclusive media.

Experiment 1, Ran from 1/10/2008-1/11/2008										
Hours	pH	Protein (mg/L)	Std Dev	Log CFU/mL	Std Dev	OD_{600}	Std Dev	NH4+ (M)	Std Dev	Dissolved Calcium (M)
Inoculum	n/a	n/a	n/a	9.563	8.761	0.446	0.011	n/a	n/a	n/a
0	6.8	30.872	0.801	7.505	7.115	0.044	0.001	0.1458	0.0131	0.0246
3	7.4	41.385	6.706	7.326	6.358	0.045	0.001	0.1354	0.0149	0.0260
5	7.9	40.359	4.809	7.365	6.504	0.048	0.001	0.1897	0.0233	0.0270
7	8.0	42.923	1.387	7.079	6.349	0.089	0.001	0.2100	0.0149	0.0243
8	8.1	42.026	3.316	7.041	6.666	0.095	0.003	0.2055	0.0694	0.0266
9	8.2	36.385	3.669	6.944	6.413	0.109	0.002	0.2127	0.0121	0.0487
10	8.2	43.949	2.618	6.681	6.115	0.116	0.002	0.1531	0.0117	0.0232
11	8.3	42.667	0.222	6.505	6.253	0.126	0.006	0.1305	0.0185	0.0052
27	8.9	45.359	0.968	7.505	7.171	0.073	0.017	0.2448	0.0342	n.d.
Experiment 2, Ran on 7/8/2008										
Hours	pH	Protein (mg/L)	Std Dev	Log CFU/mL	Std Dev	OD_{600}	Std Dev	NH4+ (M)	Std Dev	Dissolved Calcium (M)
Inoculum	n/a	n/a	n/a	9.260	8.457	0.321	0.018	n/a	n/a	n/a
0	6.8	28.600	4.619	7.220	6.659	0.042	0.000	0.1884	0.0226	0.0226
3	7.2	31.267	1.764	7.170	6.605	0.046	0.003	0.1384	0.0120	0.0245
4	7.5	33.933	10.414	7.246	6.803	0.047	0.001	0.0971	0.0061	0.0238
5	7.6	34.822	5.389	7.384	6.504	0.053	0.001	0.1056	0.0192	0.0247
7	7.8	47.711	7.727	7.107	6.611	0.078	0.000	0.1709	0.0109	0.0223
8	7.8	29.489	2.143	7.033	6.618	0.084	0.002	0.1681	0.0127	0.0228
9	7.9	24.822	1.018	6.643	6.472	0.093	0.004	0.1549	0.0115	0.0240
11	8.0	23.044	1.388	6.270	5.664	0.099	0.000	0.1690	0.0107	0.0249
Experiment 3, Ran from 7/24/2008-7/25/2008										
Hours	pH	Protein (mg/L)	Std Dev	Log CFU/mL	Std Dev	OD_{600}	Std Dev	NH4+ (M)	Std Dev	Dissolved Calcium (M)
Inoculum	n/a	n/a	n/a	n/a	n/a	0.431	0.009	n/a	n/a	n/a
0	7.1	26.600	6.667	6.288	5.669	0.043	0.002	0.1549	0.0091	0.0227
8	8.0	24.600	4.372	7.225	6.504	0.105	0.004	0.1895	0.0054	0.0238
9	8.1	23.044	4.914	5.944	5.591	0.110	0.004	0.1313	0.0192	0.0208
10	8.1	16.600	4.372	5.602	5.151	0.119	0.012	0.1536	0.0197	0.0216
12	8.2	13.489	0.385	5.193	4.472	0.115	0.002	0.1951	0.0233	0.0205
27	8.5	22.156	2.037	7.354	6.578	0.046	0.001	0.2851	0.0136	0.0024

Table 26: Compiled data from *B. sphaericus* 21787 aerobic experiment in calcium exclusive media.

Experiment 1, Ran from 1/10/2008-1/11/2008										
Hours	pH	Protein (mg/L)	Std Dev	Log CFU/mL	Std Dev	OD_{600}	Std Dev	NH4+ (M)	Std Dev	Dissolved Calcium (M)
0	7.5	35.283	2.887	4.903	5.115	0.044	0.002	0.233	0.039	n/a
3	7.8	34.950	3.000	5.889	5.925	0.048	0.001	0.3000	0.1162	n/a
6	8.0	40.450	1.732	4.903	4.923	0.058	0.007	0.2120	0.0395	n/a
24	9.0	46.283	1.756	n/a	n/a	0.064	0.004	0.6928	0.0584	n/a
27	9.1	58.117	1.258	6.365	5.653	0.061	0.002	0.5141	0.0272	n/a

Table 27: Compiled data from *B. subtilis* aerobic experiment in calcium inclusive media.

Experiment 1, Ran on 5/26/2008										
Hours	pH	Protein (mg/L)	Std Dev	Log CFU/mL	Std Dev	OD_{600}	Std Dev	NH4+ (M)	Std Dev	Dissolved Calcium (M)
0	6.8	n/a	n/a	6.447	3.923	n/a	n/a	0.087	0.030	n/a
9	7.4	n/a	n/a	6.806	10.259	0.049	0.001	0.2281	0.0107	n/a
36.5	7.6	n/a	n/a	6.869	9.181	0.053	0.002	0.2833	0.0002	0.020
58	7.4	n/a	n/a	7.057	8.648	0.052	0.001	0.1032	0.0634	0.020
85	7.2	n/a	n/a	6.158	34.144	0.052	0.000	0.1619	0.0472	0.020
101	7.2	n/a	n/a	7.456	7.309	0.050	0.002	0.1224	0.0680	0.019
121	6.8	n/a	n/a	n/a	n/a	0.047	0.003	n/a	n/a	n/a
168	6.9	n/a	n/a	n/a	n/a	0.046	0.002	n/a	n/a	n/a
194	7.5	n/a	n/a	n/a	n/a	0.067	0.017	0.1215	0.0636	n/a
239	7.4	n/a	n/a	7.456	7.309	0.089	0.012	0.1127	0.0650	0.019

Table 28: Compiled data from sterile control aerobic experiment in calcium inclusive media.

Experiment 1, Ran from 10/17/2007-10/26/2007										
Hours	pH	Protein (mg/L)	Std Dev	Log CFU/mL	Std Dev	OD_{600}	Std Dev	NH4+ (M)	Std Dev	Dissolved Calcium (M)
0	5.9	n/a	n/a	n/a	n/a	0.038	0.001	0.106	0.032	0.030
3	6.1	n/a	n/a	n/a	n/a	0.040	0.012	0.1043	0.0363	n/a
7	6.2	n/a	n/a	n/a	n/a	0.038	0.000	0.1061	0.0348	n/a
10	6.2	n/a	n/a	n/a	n/a	0.039	0.000	0.1387	0.0200	n/a
24	6.4	n/a	n/a	n/a	n/a	0.038	0.001	0.1129	0.0382	n/a
30	6.4	n/a	n/a	n/a	n/a	0.039	0.001	0.1224	0.0418	0.031
150	6.6	n/a	n/a	n/a	n/a	0.039	0.001	0.1717	0.0166	n/a
222	6.5	17.489	4.914	n/a	n/a	n/a	n/a	n/a	n/a	n/a
Experiment 2, Ran on 5/26/2008										
Hours	pH	Protein (mg/L)	Std Dev	Log CFU/mL	Std Dev	OD_{600}	Std Dev	NH4+ (M)	Std Dev	Dissolved Calcium (M)
0	6.6	18.600	1.764	n/a	n/a	0.038	0.002	0.087	0.030	n/a
3	6.9	n/a	n/a	n/a	n/a	0.040	0.002	0.2281	0.0107	n/a
5	7.0	n/a	n/a	n/a	n/a	0.048	0.006	0.1398	0.0321	n/a
6	6.9	n/a	n/a	n/a	n/a	0.040	0.002	0.1529	0.0198	n/a
8	6.9	n/a	n/a	n/a	n/a	0.045	0.008	0.1864	0.0057	n/a
10	6.9	n/a	n/a	n/a	n/a	0.043	0.006	0.1476	0.0067	n/a
12	7.0	17.489	4.914	n/a	n/a	0.042	0.003	0.0699	0.0125	n/a
Experiment 3, Ran from 3/4/2009-3/5/2009										
Hours	pH	Protein (mg/L)	Std Dev	Log CFU/mL	Std Dev	OD_{600}	Std Dev	NH4+ (M)	Std Dev	Dissolved Calcium (M)
0	6.4	n/a	n/a	n/a	n/a	0.038	0.001	0.2156	0.0050	n/a
2	6.4	n/a	n/a	n/a	n/a	0.037	0.000	0.2138	0.0175	n/a
4	6.5	n/a	n/a	n/a	n/a	0.039	0.001	0.1814	0.0161	n/a
6	6.6	n/a	n/a	n/a	n/a	0.039	0.002	0.1000	0.0156	n/a
8	6.6	n/a	n/a	n/a	n/a	0.040	0.003	0.0802	0.0259	n/a
25.5	6.8	n/a	n/a	n/a	n/a	0.040	0.002	0.3938	0.0481	n/a

Table 29: Compiled data from sterile control aerobic experiment in calcium exclusive media.

Experiment 1, Ran from 7/31/2007-8/1/2007										
Hours	pH	Protein (mg/L)	Std Dev	Log CFU/mL	Std Dev	OD_{600}	Std Dev	NH4+ (M)	Std Dev	Dissolved Calcium (M)
0	7.2	n/a	n/a	n/a	n/a	0.428	0.002	0.224	0.068	n/a
3	7.2	n/a	n/a	n/a	n/a	0.042	0.001	0.4079	0.0262	n/a
6	7.3	n/a	n/a	n/a	n/a	0.156	0.004	0.3054	0.0956	n/a
24.5	7.6	n/a	n/a	n/a	n/a	0.197	0.015	0.3086	0.0452	n/a
27.5	7.5	n/a	n/a	n/a	n/a	0.095	0.003	0.3698	0.1210	n/a

APPENDIX B

Growth Measurement Comparisons

Beer-Lambert Law

The Beer-Lambert equation relates absorbance to path length by the linear relationship:

$$A = \varepsilon l c \quad (29)$$

where A is the absorbance (no units), ε is the molar absorbtivity of the solution (L/mol·cm), l is the path length (cm), and c is the concentration (mol/L) (Ingle and Crouch, 1988). Thus, if the path length and molar absorbtivity are known, and absorbance can be measured, it is possible to determine concentration. When comparing different path lengths in media that is similar in composition inoculated with the same type of media, it can be assumed that the molar absorbtivity does not vary, and equation 28 becomes:

$$A = \alpha l \quad (30)$$

where α is the absorption coefficient (cm^{-1}) of the solution.

Absorbance vs. CFU/mL

Stocks-Fischer et al. (1999) reported initial biomass concentrations in terms of CFU/mL. To be able to compare the kinetic coefficients reported in Stocks-Fischer et al. (1999) to those found in this paper, Fujita et al. (2000), and Ferris et al. (2003), a relationship needs to be found between CFU/mL and OD_{600}. Data from calcium exclusive experiments inoculated with *S. pasteurii* performed for this paper were used to find that correlation. Figure 23 shows the plot of absorbance readings versus CFU/mL. The relationship found by linear regression analysis of this data was:

$$y = (3 \times 10^{-9}) + 0.0072 \quad (31)$$

where y is the absorbance at 600 nm for a 1 cm path length and x is CFU/mL. This equation was used to convert the CFU/mL values given by Stocks-Fischer et al. (1999) to OD_{600} values.

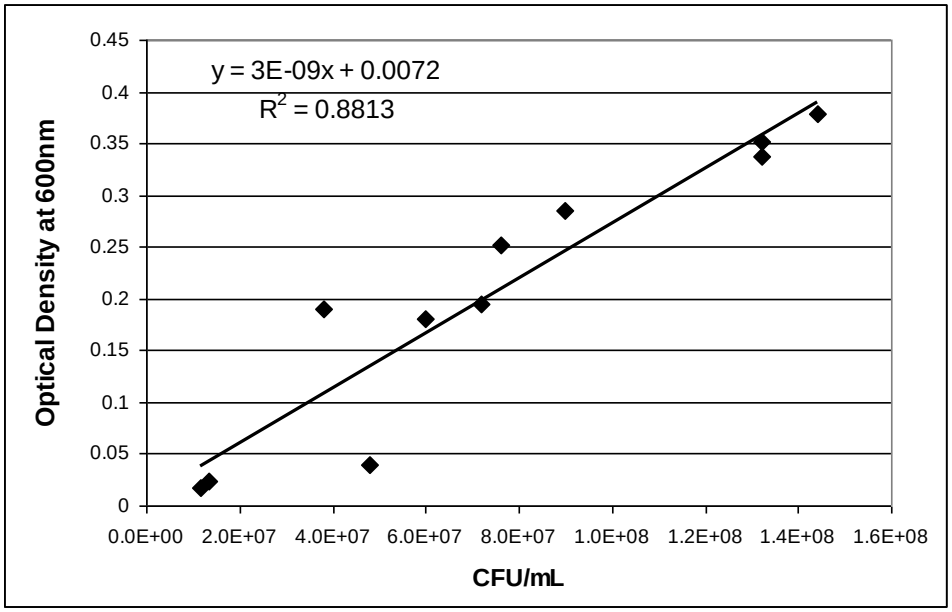


Figure 23: Optical density versus CFU/mL for *S. pasteurii* in CMM-.

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