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Kinetics of calcite precipitation by ureolytic bacteria under aerobic and anaerobic conditions

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S1. EXPERIMENTAL

S1.1 Preparation of calcite mineralizing medium

Kinetic experiments were carried out using the $CaCO_3$ Mineralizing Medium (CMM+) described by Ferris and Stehmeier (1996) (Table S1).

Table S1. Recipe for Calcite Mineralizing Medium (CMM+).

Ingredient	Manufacturer	Concentration	Na^+	Cl^-	NH_4^-	Ca_2^+	HCO_3^-
Nutrient broth	BD (Franklin Lakes, NJ)	3 g L^{-1}	--	--	--	--	--
Urea	Fisher Scientific (Fair Lawn, NJ)	330 mM	--	--	--	--	--
NH_4Cl	Fisher Scientific (Fair Lawn, NJ)	187 mM		187	187		
$NaHCO_3$	Fisher Scientific (Fair Lawn, NJ)	25.0 mM	25				25
$CaCl_2*2H_2O$	Acros Organics (Morris Plains, NJ)	25.2 mM		50.4		25.5	
Concentrated HCl	Mallinckrodt (Hazelwood, MO)	Adjusted to pH 6					
		Total	25	237.4	187	25.2	25

Calcium chloride dihydrate was omitted from the recipe to produce calcium free medium (CMM-).

S1.2 Bacterial growth

Plate Counts: Standard serial dilutions of 10^{-1} to 10^{-6} (10^{-1} to 10^{-8} for inoculum) were made in Phosphate Buffered Saline (PBS) solution, consisting of 8.5 g L^{-1} NaCl (Fisher, Fair Lawn, NJ), 0.61 g L^{-1} KH_2PO_4 (Fisher, Fair Lawn, NJ), and 0.96 g L^{-1} K_2HPO_4 (Fisher, Fair Lawn, NJ). Plates were made of a solution of 37 g L^{-1} BHI, 20 g L^{-1} urea, and 15 g L^{-1} granulated agar (Beckton, 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^{-1} (Herigstad et al., 2001):

$$\left[\frac{CFU}{mL}\right] = \left(\frac{CFU/drop}{dilution\ factor}\right) \quad \text{(S1)}$$

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 which relates absorbance to path length by the linear relationship:

$$A = \varepsilon l c \quad \text{(S2)}$$

where A is the absorbance (no units), ε is the molar absorptivity of the solution (L $mol^{-1}\cdot cm^{-1}$), l is the path length (cm), and c is the concentration (mol L^{-1}) (Ingle and Crouch, 1988). Thus, if the path length and molar absorptivity 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 absorptivity does not vary, and equation S2 becomes:

$$A = \alpha l \quad \text{(S3)}$$

where α is the absorption coefficient (cm^{-1}) of the solution. 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 later be tested for protein concentration. The protein content of the sample was determined using the Pierce Coomassie Protein Assay. Protein standards were made by diluting a 2.0 mg mL^{-1} 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 before 50 µL of each prepared sample was added to three separate wells of a 96 well plate. Coomassie Plus™ Protein Assay Reagent (150 µL, Pierce, Rockford, IL) was added to each well using a multichannel pippetter. The plate was incubated at room temperature for 15 minutes, and then read on the microplate reader at 595 nm. Protein concentrations were determined relative to the linear regression of standard samples.

S1.3 TEM Imaging

The samples extracted from the system by pipette were fixed by adding 100 µL of a 25% glutaraldehyde solution to 900 µL of the sample in a microcentrifuge tube to achieve a final concentration of 2.5% glutaraldehyde. After fixation, samples were centrifuged and re-suspended 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% glutaraldehyde and 0.05M phosphate buffered saline (PBS) solution. The cell pellets were then washed three times for ten minutes each with PBS 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. Samples were imaged on a Zeiss 912 Transmission Electron Microscope by Susan Brumfield in the Department of Plant Sciences & Plant Pathology, Montana State University.

S1.4 Geochemical modelling

PHREEQC is a speciation-solubility geochemical model that can predict the speciation and solubility of elements/compounds. PHREEQC was used (1) to determine the saturation index in the CMM+ medium in abiotic incubations, thus to estimate the potential precipitation of minerals due solely to the ingredients in the medium; and (2) to verify that precipitation of $CaCO_3$ in the medium was indeed induced by ureolysis.

Final composition of the calcite mineralizing medium and the ion concentrations used in the model are shown in Table S1. To calculate the species in the experimental solutions, salts were first equilibrated with the partial pressure of atmospheric $CO_{2(g)}$ at 0.00039 atm and 30°C. From the initial conditions in the CMM+ medium in abiotic incubations (without adding urea to the system, Table S1), the saturation indices obtained showed negative values, indicating undersaturated conditions in the medium, which suggests that no precipitation of minerals would occur under the initial conditions (Table S2).

Table S2. Saturation indices determined with PHREEQC in the calcite mineralizing medium in abiotic incubations

Phase in solution	Saturation index
Aragonite	-0.22
Calcite	-0.08
$CO_{2(g)}$	-0.29
$H_{2(g)}$	-14.06
$H_2O_{(g)}$	-1.38
Halite	-4.08
$NH_{3(g)}$	-5.71
$O_{2(g)}$	-53.53

For a gas, S=log10(fugacity)

Fugacity=pressure*phi/1 atm
For ideal gases, phi=1

The initial conditions in the calcite mineralizing medium in abiotic incubations (without adding urea to the system) were used in the model to verify that precipitation of calcite was indeed induced by ureolysis (Table S2). Urea hydrolysis proceeded in steps of 1mM (333 mM of initial urea concentration in 300 steps). The solution was allowed to equilibrate for each step and calcite was allowed to precipitate when supersaturated (Figure S1). From the results obtained, 25.45 mM of urea need to be added for calcite to precipitate (Figure S1).

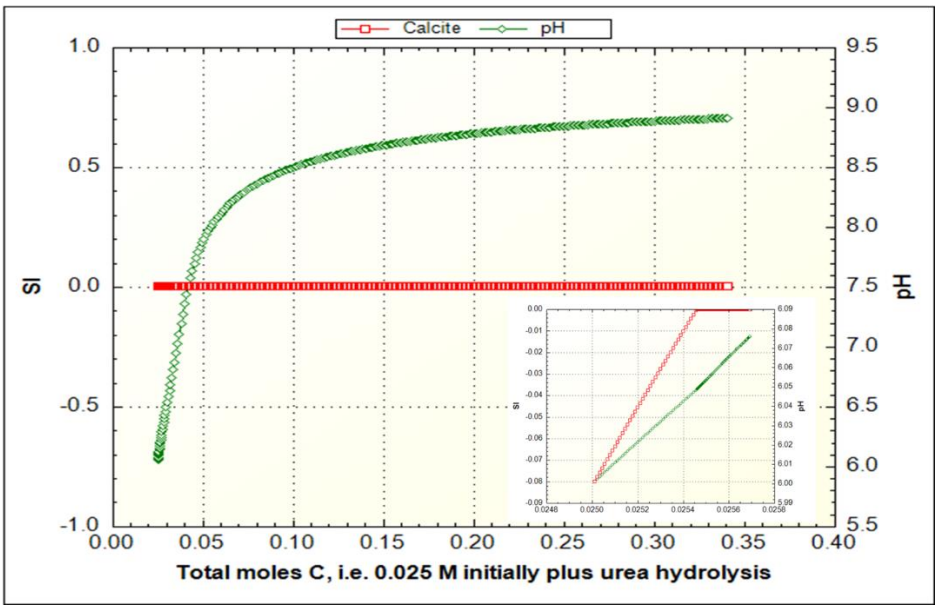

Figure S1. Simulation of calcite precipitation and pH change as ureolysis occurred in the calcite mineralizing medium. S= saturation index. The inset plot shows the small amounts of urea added to the system until precipitation was observed.

S2 DATA PROCESSNG

S2.1 Kinetic analysis

Figure S2 shows the fitting of the kinetic model for aerobic experiments (section 2.6, main paper) with *S. pasteurii* and the *B. sphaericus* strains in calcium inclusive, CMM+, (A) and calcium exclusive, CMM-. A summary of the estimated parameters for aerobic experiments are shown in Table S3. Figure S3 shows the fitting of the kinetic model for *S. pasteurii* under anaerobic conditions and different terminal electron acceptors. A summary of the estimated parameters for anaerobic experiments are shown in Table S4.

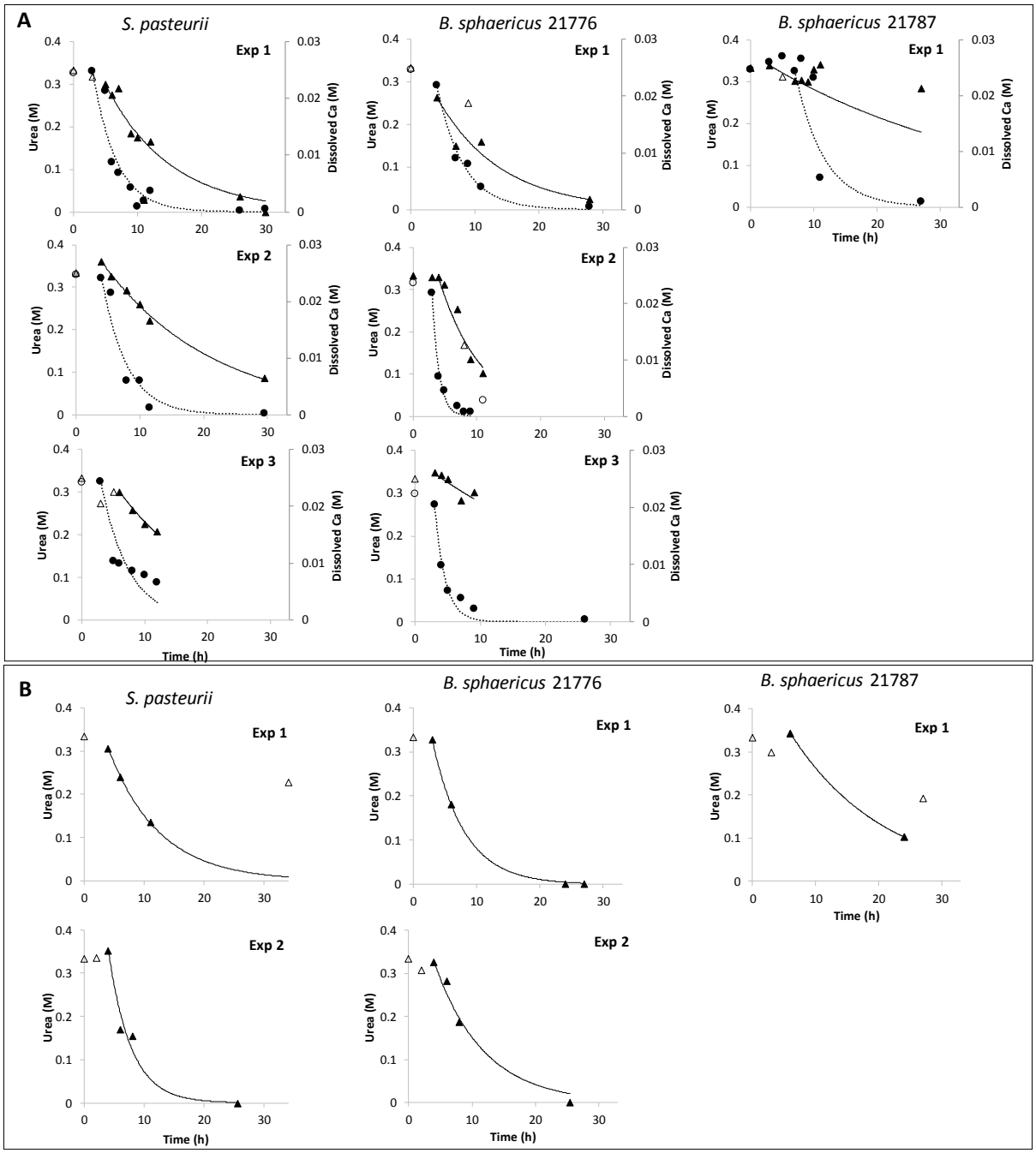

Figure S2. Changes in urea (▲) and dissolved calcium (●) concentrations over time from individual aerobic experiments with *S. pasteurii* and the *B. sphaericus* strains in (A) CMM+ and (B) CMM- medium. Curves are the lines of best fit kinetic model through minimizing the sum of the squared error. Solid data points were used to determine best fit.

Table S3. Summary of kinetic parameters for aerobic urea hydrolysis (k_{urea}) in calcium inclusive (CMM+) and calcium exclusive (CMM-) experiments, and calcite precipitation (k_{precip}) in CMM+ experiments inoculated with *S. pasteurii*, *B. sphaericus* 21776 and *B. sphaericus* 21787.

Aerobic	Initial Biomass OD_{600}	k_{urea} (h^{-1})	R^2	Lag time (h)	# Data points (total)	k_{urea} normalized to: OD_{600} (OD_{600}^{-1} h^{-1})	k_{urea} normalized to: CFU (mL CFU^{-1} h^{-1})	k_{precip} (h^{-1})	R^2	Lag time (h)	#Data points (total)
***S. pasteurii* CMM+**											
Exp 1	0.015	0.098	0.969	5.0	8(10)	6.718	3.96E-08	0.272	0.910	3	10(11)
Exp 2	0.013	0.057	0.994	4.0	6(7)	4.441	3.00E-08	0.256	0.937	4	6(7)
Exp 3	0.015	0.067	0.985	6.0	4(7)	4.594	2.71E-08	0.229	0.840	3	6(7)
Average:	**0.014**	**0.074**		**5.0**		**5.251**	**3.22E-08**	**0.253**		**3.3**	
Std Dev:	0.001	0.021		1.0	-	1.273	6.54E-09	0.021		**0.6**	
***S. pasteurii* CMM-**											
Exp 1	0.017	0.118	0.999	4.0	3(5)	6.993	3.65E-08	-	-	-	-
Exp 2	0.017	0.265	0.962	4.0	4(6)	15.462	7.99E-08				
Average:	**0.017**	**0.192**		**4.0**		**11.227**	**5.88E-08**	-	-	-	-
Std Dev:	0.000	0.104		0.0	-	5.988	2.02E-08				
***B. sphaericus* 21776 CMM+**											
Exp 1	0.015	0.100	0.906	4.0	4(6)	6.734	3.91E-08	0.253	0.979	4	5(6)
Exp 2	0.012	0.149	0.942	4.0	6(8)	12.282	8.99E-08	0.941	0.977	3	7(8)
Exp 3	0.015	0.073	0.944	4.0	5(6)	5.045	2.99E-08	0.616	0.974	3	6(7)
Average:	**0.014**	**0.107**		**4.0**		**8.020**	**5.30E-08**	**0.604**		**3.3**	
Std Dev:	0.001	0.038		0.0	-	3.786	3.23E-08	0.344		**0.6**	
***B. sphaericus* 21776 CMM-**											
Exp 1	0.016	0.199	0.999	3.0	4(5)	12.796	7.14E-08	-	-	-	-
Exp 2	0.015	0.128	0.987	4.0	4(6)	8.840	5.26E-08				
Average:	**0.015**	**0.168**		**3.5**		**10.818**	**6.20E-08**	-	-	-	-
Std Dev:	0.001	0.050		0.71	-	2.797	1.33E-08				
***B. sphaericus* 21787**											
CMM+	0.015	0.023	0.842	3	5(9)	1.526	8.52E-09	0.219	0.642	8	5(8)
CMM-	0.015	0.050	1	3	2(5)	3.1961	1.75E-08	-	-	-	-

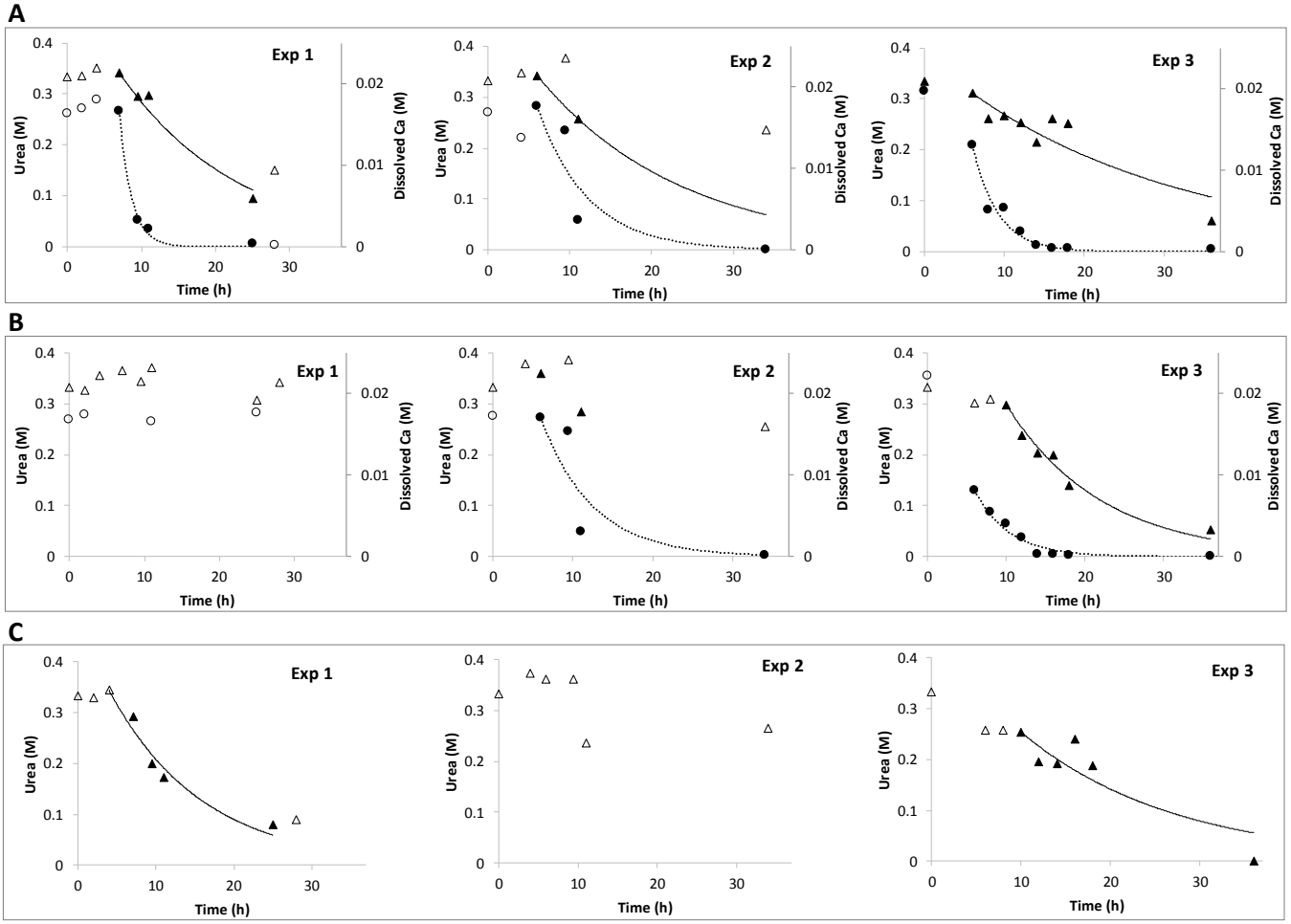

Figure S3. Changes in urea (▲) and dissolved calcium (●) concentrations over time from experimental data for *S. pasteurii* under different anaerobic conditions: (A) calcium inclusive medium (CMM+) with NO_3^-, (B) CMM+ without terminal electron acceptors, and (C) calcium exclusive media (CMM-) with NO_3^-. Individual data points are experimental data and curves are the lines of best fit for the minimum residual for the sum of squares. Solid data points were used to determine best fit.

Table S4. Summary of kinetic parameters for urea hydrolysis (k_{urea}) and calcite precipitation (k_{precip}) in anaerobic experiments inoculated with *S. pasteurii* in calcium inclusive (CMM+) and calcium exclusive (CMM-) media, with and without nitrate as the terminal electron acceptor (TEA).

Anaerobic	Initial Biomass OD_{600}	k_{urea} (h^{-1})	R^2	Lag time (h)	#Data points (total)	k_{urea} normalized to:		k_{precip} (h^{-1})	R^2	Lag time (h)	#Data points (total)
						OD_{600} (OD_{600}^{-1} h^{-1})	CFU (mL CFU^{-1} h^{-1})				
***S. pasteurii* CMM+ NO_3^-**											
Exp 1	0.014	0.062	0.977	7.0	4(7)	4.389	2.67E-08	0.603	0.995	7.0	4(7)
Exp 2	0.016	n.d.	n/a	n/a	n/a	n/a	n/a	0.163	0.820	6.0	4(6)
Exp 3	0.012	0.035	0.813	6.0	8(9)	2.844	2.01E-08	0.314	0.955	6.0	6(9)
Average	**0.014**	**0.048**		**6.5**		**3.617**	**2.34E-08**	**0.360**		**6.5**	
Std. Dev	0.002	0.018	-	0.7	-	1.092	4.67E-09	0.223	-	0.6	-
***S. pasteurii* CMM- NO_3^-**											
Exp 1	0.014	0.083	0.92	7.0	5(7)	5.897	3.59E-08				
Exp 2	0.016	n.d.	n/a	n/a	n/a	n/a	n/a				
Exp 3	0.012	0.058	0.84	10.0	6(9)	4.659	3.30E-08	-	-	-	-
Average	**0.014**	**0.071**		**8.5**		**5.278**	**3.45E-08**				
Std. Dev	0.002	0.017	-	2.1	-	0.875	2.05E-09	-	-	-	-
***S. pasteurii* CMM+ no TEA**											
Exp 1	0.014	n.d.	n/a	n/a	n/a	n/a	n/a	n.d.	n/a	n/a	n/a
Exp 2	0.016	n.d	n/a	n/a	n/a	n/a	n/a	0.155	0.760	6.0	4(5)
Exp 3	0.012	0.082	0.96	10.0	6(9)	6.616	4.68E-08	0.227	0.970	6.0	8(9)
Average	**0.014**	**0.082**		**10.0**		**6.616**	4.68E-08	**0.191**		**6.0**	
Std. Dev	0.002	n/a	-	n/d	-	n/d	n/d	0.050	-	0.0	-

*Converted to 1 cm path length from 96 well plate measurements

n.d. = not determined

n/a = not available

S2.2 Technique for calculating the value of k_{urea} normalized to the absorbance reading of initial biomass and CFU mL^{-1}

Stocks-Fischer et al. (1999) reported initial biomass concentrations in terms of CFU mL^{-1}. 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 was determined between CFU mL^{-1} and OD_{600}. Data from calcium exclusive experiments inoculated with *S. pasteurii* performed for this paper were used to find that correlation. Figure S4 shows the plot of absorbance readings versus CFU mL^{-1}. The relationship found by linear regression analysis of this data was:

$$y = \left(3 \times 10^{-9}\right)x + 0.0072 \qquad \text{(S4)}$$

where y is the absorbance at 600 nm for a 1 cm path length and x is CFU mL^{-1}. This equation was used to convert the CFU mL^{-1} values given by Stocks-Fischer et al. (1999) to OD_{600} values and vice versa for the Fujita et al. (2000) and Ferris et al. (2003) papers.

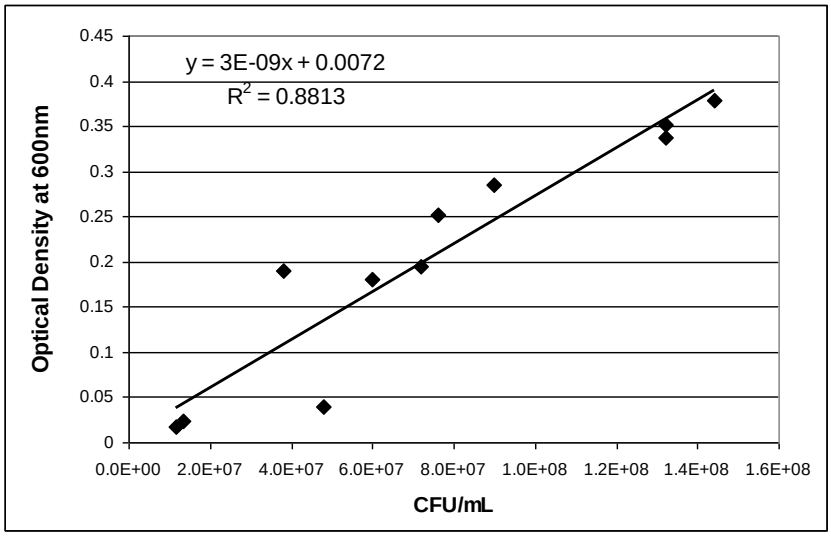

Figure S4. Optical density (600 nm) versus CFU mL^{-1} for *S. pasteurii* in CMM-.

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