

AN ABSTRACT OF THE THESIS OF

Christopher J. Neighbor for the degree of Honors Baccalaureate of Science in Biological

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Urea Hydrolysis by Bacteria for Use in Calcium Carbonate Precipitation in Aquifers.

Abstract Approved: _____

Frederick Colwell

The use of ureolytic bacteria for the remediation of contaminated groundwater aquifers by inducing calcium carbonate precipitation is being studied in order to establish a better understanding of the modeling and prediction of how the bacteria will act *in situ*. This research has pursued the use of various ureolytic bacteria in order to establish a comparison for selection of model bacteria. The naturally ureolytic bacterium *Sporosarcina pasteurii* performed at a very high rate of urea breakdown and this rate may be too fast for long term in vitro studies of calcium carbonate precipitation. The genetically modified organisms *Escherichia coli* DH5 α (pURE 14.8), *Pseudomonas aeruginosa* MJK1, and *E. coli* MJK2 were also measured in controlled batch experiments for urea breakdown. The rate of urea breakdown was modeled using a Michaelis-Menton equation; the observed V_{max} was determined using urea, ammonium, and cell concentrations. The relative level of activity in descending order was found to be *Sporosarcina pasteurii*, *E. coli* MJK2, *E. coli* DH5 α (pURE 14.8), and *Pseudomonas aeruginosa* MJK1. This ureolytic activity of bacteria may aid in a better understanding of how to produce an effective calcium carbonate precipitation result when introducing urea into a contaminated aquifer.

Keywords: Urea hydrolysis, bacterial urease, bioremediation, urease kinetics

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Analysis and Modeling of Kinetic Parameters for Urea Hydrolysis by Bacteria

for Use in Calcium Carbonate Precipitation in Aquifers

by

Christopher J. Neighbor

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Analysis and Modeling of Kinetic Parameters for Urea Hydrolysis by Bacteria for Use in Calcium Carbonate Precipitation in Aquifers

Introduction

Aquifers

An aquifer is an underground water-bearing permeable geologic formation which allows for groundwater extraction through the use of a well. Aquifers can occur in permeable rock or unconsolidated material (e.g., sand, gravel, silt). Aquifers are relied upon heavily for use by ecological systems, drinking water, irrigation, and industry. With the increase in human activity and population the potential for aquifer contamination has increased. Because usable water can be considered a limited resource, investment into the maintenance and restoration of aquifers is warranted (EPA "Groundwater").

There are several possible sources of human generated contamination including farming, mining operations or careless disposal of industrial or nuclear waste. The farming can cause nitrite contamination from the fertilizer. It can also cause accumulation of pesticides, herbicides and livestock waste. The mining and nuclear waste disposal may introduce heavy metals and/or harmful radionuclides to the environment (EPA "Groundwater").

These different kinds of aquifer contamination may need to be treated. Nitrite contamination of aquifers from fertilizers is typically untreatable making it so that the contaminated aquifer must be retired from use. Other contamination cases, such as with petroleum hydrocarbons, aromatic hydrocarbons, halogenated hydrocarbons contamination, can be treated and broken down by microbial enzymes (Wallrabenstein et al. 1995; Rahm et al. 2006; Roling et al. 2001; de Liphthay et al. 2002). Metal contaminants such as lead, zinc, and cadmium can be immobilized through

the co-precipitation of calcium carbonates. The divalent radionuclides of cobalt-60 and strontium-90 can also be immobilized by calcium carbonate co-precipitation (Fujita et al. 2000).

The radionuclide which contaminates groundwater, strontium-90, is directly below calcium on the periodic table indicating a similar number of unpaired electrons in the external orbit. This means that strontium-90 which is ingested in contaminated groundwater can replace the calcium in human bones (EPA "Radiation Protection: Strontium").

Current methods for treating these contaminated aquifers include the "pump and treat" method. This method entails pumping water out of the aquifer via a well, treating the water, and returning the water back to the aquifer (EPA "Basics of pump and treat groundwater remediation technology"). Such a method is often not financially feasible for radionuclide treatment as the amount of time and cost required to pump out the aquifer to a non-toxic level would be excessive.

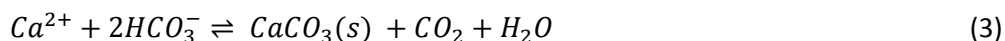
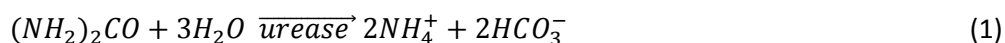
An alternative method of contamination treatment is through bioremediation. If the remediation of the aquifer is done utilizing the activity of microorganisms this is considered bioremediation (Griebler and Lueders 2009). There is also potential for use of microbes for induced calcium co-precipitation which can cause immobilization of heavy metals and/or radionuclides.

Calcium carbonate precipitation

This precipitation of calcium carbonate can be initiated within aquifers by increasing the pH. The concept of using co-precipitation of calcium carbonate has been explored for bioremediation of aquifers previously (Colwell et al. 2005). It can be used to treat lead, zinc, and cadmium contamination in aquifers.

Bacterial breakdown of urea, increases the pH of the aquifer causing calcium carbonate to precipitate, trapping the strontium in mineral form and removing it from the aqueous phase. In theory, by using bacteria already in place in the aquifer to increase the pH, rather than injecting a base, this type of remediation can allow for a slower, more evenly distributed precipitation to occur in the aquifer instead of a localized reaction at the injection site.

The ureolytic organism induces precipitation through urea breakdown which increases the pH and creates reactive ions. The stoichiometric reactions involved in the process are represented below.



The $CaCO_3$ solubility in the medium increases with decreasing temperature. The solubility of the CO_2 increases with lower temperatures as well. The proportion of carbonates present in the medium is based on the pH of the solution (Figure 1).

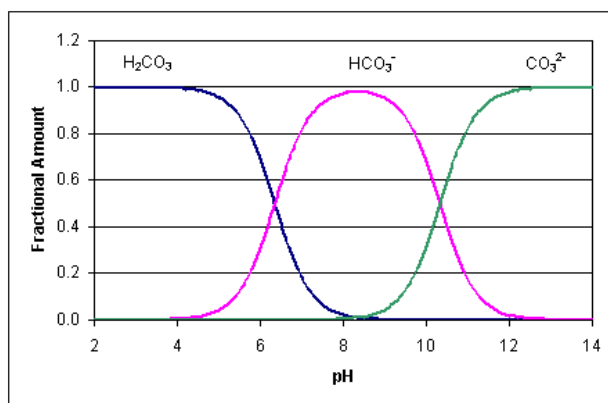


Figure 1. The relative fractional state of carbonates in aqueous solution with relation to pH (USU "Dependent Carbonate Equilibrium")

The precipitation of calcium carbonate will occur more vigorously at a basic pH value. The pKa value of CaCO_3 is 9.0. The ammonia and ammonium are also present in proportion to the pH value of the medium. The pKa value of NH_4^+ is 9.24 (Ferris et al. 2003). The Henderson-Hasselbalch equation allows for the calculation of monoprotic acid ratios at various pH values:

$$pH = pKa + \log \frac{A^-}{HA} \quad (4)$$

Recently, an *in situ* experiment was performed in the Snake River Plain aquifer in which the native ureolytic bacteria population was stimulated using molasses (Fujita et al. 2008). Urea was then injected into the aquifer, however the ureolysis which took place was too aggressive resulting in increased calcium carbonate precipitation which plugged and reduced the porosity of the aquifer (Fujita et al. 2008). Further understanding of the ureolytic capacity of bacteria within aquifers would increase the feasibility of using this form of bioremediation in other aquifers.

In the laboratory, the use of model bacteria which can approximate similar ureolytic rates as that of the local naturally ureolytic bacteria would be beneficial in establishing a better understanding of how to model calcium carbonate precipitation from ureolysis *in situ*.

Ureolytic bacteria

Urease is an enzyme which can be produced by certain naturally occurring bacteria in order to survive in a nitrogen poor environment. Urease can also be found in various plants such as jack bean (Mobley et al. 1995), and is used to break down urea into ammonia and carbonate as described in *Equation 1*.

The subsurface bacterial ecology may contain several varieties of naturally occurring ureolytic bacteria (Fujita et al. 2000, Mobley et al. 1995). Some bacteria with constitutive urease

production exhibit constant production of urease, while others can be controlled with a promoter. Some bacterial species only produce urease under nitrogen-starved conditions. Nitrogen regulation of gene expression occurs in a number of ureolytic organisms including *Pseudomonas aeruginosa*. Exogenous nickel may be needed for full expression of recombinant urease. Two nickel ions are required per active site on the urease (Moblely et al. 1995).

In order to perform lab studies under controlled conditions to provide scalable rates of urea hydrolysis a model microbe capable of CaCO_3 precipitation is desirable. Several model bacteria have been genetically modified to contain a plasmid with the urease producing gene (Kaufman 2011). The plasmid of the *P. aeruginosa* uses arabinose as a promoter. Resistance to ampicillin or gentamicin was also included on the plasmid to allow for selective pressure to be placed on the bacteria in culture. The model bacteria used in these experiments all have an aerobic metabolism.

The bio-safety level of the model organisms is also an important consideration. The bio-safety level of the *Pseudomonas aeruginosa* is level 2 indicating that certain additional safety precautions are required in order to work with this bacterium. *Pseudomonas aeruginosa* is also an opportunistic pathogen. It was recommended during the operations within the lab that one should avoid working with the bacterium if a primary infection has weakened the worker's immune system.

Enzymes and enzyme kinetics

Enzymes are a form of protein which is produced by the cells. They provide an active reaction site for a specific substrate. The quantity and activity of an enzyme is unique to the organism producing it (Moblely et al. 1995).

A standard model for enzyme kinetics is the Michaelis-Menton equation. A general description of the equation is as follows.

$$V = \frac{V_{max}*[S]}{K_m + [S]} \quad (5)$$

Where V is the rate of substrate breakdown, S is the concentration of substrate, and K_m is a constant concentration of the substrate at which the rate of substrate breakdown is equal to half of the V_{max} value. The Michaelis-Menton equation parameter of V_{max} is based on the ϵ_0 and k_s , where ϵ_0 is the concentration of the enzyme and k_s is the specific activity of the enzyme. If the concentration of enzyme is increased, then the rate of ureolysis should increase by the same magnitude (i.e. if the concentration of urease is doubled, the observed V_{max} is expected to also double)(Fidaleo et al. 2003). The specific activity is unique to the specific variety and origin of the urease.

The kinetics of urea hydrolysis have been described by means of a modified Michaelis-Menton reaction rate expression, which incorporates pH-dependent kinetics, substrate inhibition, and noncompetitive product inhibition by NH_4^+ (Schultz 2010, Fidaleo et al. 2003).

$$\frac{d[S]}{dt} = \frac{V_{max}[S]}{[K_m + [S] * (1 + \frac{[S]}{K_s})][1 + \frac{[P]}{K_p}]} \quad (6)$$

K_m = concentration of substrate at which rate is $1/2 V_{max}$

K_s = substrate inhibition constant

K_p = noncompetitive product inhibition constant

The substrate (S) being used is urea and the product (P) that causes the inhibition is ammonium.

The urea can act as a protein denaturant in high enough concentrations hence the potential for substrate inhibition. The inhibition effect is negligible for concentrations of urea less than 100mM. (Mobley et al. 1995) The K_m value is only significant when operating at low substrate

concentrations. The literature K_m value for urease is 3mM (Fidaleo et al. 2003). The ammonium is produced as a product according to the reaction in *equation 1*. Two ammonium molecules are produced per each urea broken down by the urease.

In the laboratory, the use of a homogeneous model bacteria which can approximate similar ureolytic rates as that of the local naturally ureolytic bacteria would be beneficial in establishing a better understanding of how to model calcium carbonate precipitation from ureolysis *in situ*. The objective of this research was to provide both a relative and a quantitative comparison of the ureolytic rates of bacteria to be used for *in vitro* modeling purposes. Both naturally occurring and genetically modified bacteria were modeled for urea breakdown in order to provide a general prediction for the possible activity of the bacteria in a 2-D flowcell experiment and *in situ* (Kaufman 2011). The parameters determined in this research would provide a reference point when selecting a bacteria with the desired level of activity for the experiments or modeling.

Materials and Methods

Microbes used

The bacteria used in this experiment were *Sporosarcina pasteurii*, *Escherichia coli* DH5 α , *Escherichia coli* DH5 α (pURE14.8), *Pseudomonas aeruginosa* AH298, *Pseudomonas aeruginosa* MJK1 and *Escherichia coli* MJK2. The cell lines *E. coli* DH5 α and *Pseudomonas aeruginosa* AH298 were used as negative controls for urea hydrolysis activity. The construction of the urease modified bacteria was performed by various sources (see Table 1). All of the bacteria used are aerobic. The *Pseudomonas aeruginosa* MJK1 uses L-arabinose as a promoter.

Media

The CMM- medium is the modified calcite mineralizing formula (Ferris et al. 1996) without the added calcium. Modifications to the media are noted in Table 1.

Preparation of inoculums

A series of batch cultures were initiated in order to prepare inoculums for the flask experiments. The inoculums for the flasks were prepared as overnight duplicate cultures. The overnight broths of the organisms were grown in 4ml of growth media as indicated in Table 1. The strains were grown with shaking at 200 rpm at 37 °C except for the *S. pasteurii* which was grown at 30 °C. Successful growth was determined based on observed turbidity of the media. Cells were removed from the broths by centrifugation at 2500 rcf for 5 min and then washed with 1ml CMM- base to remove spent media. After being centrifuged and washed twice, the cells were resuspended in 500 μ l of a CMM- media. These washed cultures were then transferred into 30 ml CMM- base for flask studies. The appropriate antibiotic was added to the various cultures, and 50 mM L-arabinose (Sigma) was added to the *Pseudomonas aeruginosa* MJK1.

Table 1. Bacterial strains and media used in this work.

Strain	Relevant properties	Growth media
<i>Pseudomonas aeruginosa</i> AH298	GFP on chromosome	Luria-Bertani (LB) (MoBio Laboratories)
<i>Pseudomonas aeruginosa</i> MJK1	AH298 with urease added on pJN105 plasmid	LB plus 100 µg/ml gentamicin (Sigma)
<i>Escherichia coli</i> DH5α(pURE14.8)	Urease operon on pUC19 plasmid	LB plus 50 µg/ml ampicillin (Sigma)
<i>Escherichia coli</i> MJK2	GFP on chromosome	LB plus 100 µg/ml ampicillin
<i>Sporosarcina pasteurii</i>	Urease constitutive organism	Brain Heart Infusion (BHI) (Fluka) plus 2% urea (Mallinckrodt Chemicals)

Batch studies of urease kinetics

To determine urease kinetics the respective bacterial cultures were grown in a series of 150ml Erlenmeyer flasks. The CMM- medium was prepared according to Table 1 and was filter sterilized using filters with pore size 0.2µm (filter type, Company, city). The flasks were inoculated with 1 ml of inoculums which had been prepared the day before as described above. The optical density of the inoculating cells was measured at 600nm absorbance using the Picodrop photometer (NanoDrop ND-1000).

The flasks were prepared with 30 ml of CMM- media. For *P. aeruginosa* MJK1, 50 mM L-arabinose was added to the medium as a promoter. Additionally, antibiotics, as described in Table 1, were used to pressure the cells into retaining their urease plasmids. The Petri dish drop plates were prepared with LB auger and the same levels of antibiotics as mentioned in Table 1.

Since the bacteria used are aerobic, ensuring adequate oxygen concentration within the media was required to prevent unexpected cell die off. If media is autoclaved, it was then allowed to equilibrate with the atmosphere. The flasks were loosely topped with foil, which should have allowed the media to reach atmospheric equilibrium in terms of oxygen.

Sampling during the urease kinetics experiments

To measure pH, a 1 mL sample was aseptically removed from the flasks and then measured using a pH meter (SympHony). These pH measurements were made at the 0,3, and 6 hr time points and once every 24 h thereafter..

The cell density in the beakers was determined using a drop plate method (Herigstad et al. 2001). This was performed by first resuspending the cells by forceful swirling of the flask for several seconds. A 100- μ l sample was taken and placed in a microfuge tube. A series of serial dilutions were then performed on the sample using PBS buffer as the diluting solution. A dilution factor of either 10 or 100 was used. The dilutions used for the platings were the 10^3 and 10^5 - 10^8 . The cellular density was determined by placing a 10 μ l sample of the dilutions onto a prepared Petri dish (see Table 1). The cells were then grown overnight in a 37°C incubator. The dilution with distinguishable colonies was counted and multiplied by 100x the dilution factor to determine the original cell density in CFU/ml.

Ammonia was measured using the Nessler assay (Whiffin, van Paassen, and Harkes 2007) and was also compared to the expected concentration based on the breakdown of the urea and the stoichiometric ratio of 2:1.

The urea was measured by removing 1mL of sample from the flask with a syringe. Samples were taken at the 0, 6, and 12 hr time points and once every 24 h thereafter. The sample was then

filtered using a 0.2 µm syringe filter to remove cells, enzyme and other unwanted components.

The samples were then stored in the refrigerator until they were shipped on ice overnight to Montana State University for high-performance liquid chromatography (HPLC) evaluation.

Results

During the runs the cell, substrate, and product concentrations were measured as well as the pH value. The medium used was CMM- without ammonium and modified according to Table 1. The legend names of the organisms are shortened. The positive control organism *Sporosarcina pasteurii* is *S.past*. The tested ureolytic organisms *Escherichia coli* DH5 α (pURE 14.8) is pURE and *Pseudomonas aeruginosa* MJK1 is MJK1. The negative control organisms *E. coli* DH5 α is DH5 α and *Pseudomonas aeruginosa* AH298 is AH298.

Cell density

In order to examine the growth of the cells within the medium a drop plate analysis was performed from each of the flasks to determine the active cell density. The cell measurements are represented in figure 2. An upward trend in growth can be seen for the control organisms of the DH5 α and the AH298, however, for the ureolytic organisms, the cell density remained relatively constant or showed little sustained growth.

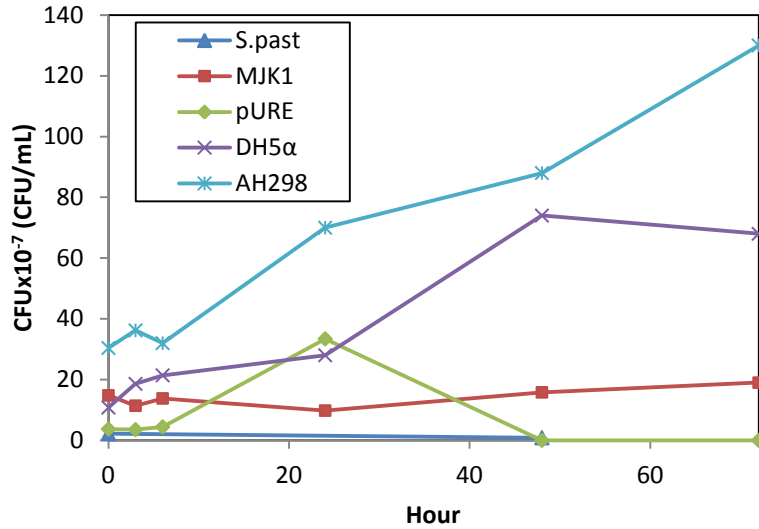


Figure 2. Numbers of active cells per ml plotted vs. time for five cultures of bacteria used in this study. The cells were cultivated in CMM- medium and cell numbers were determined by the drop plate method. For the legend the positive control organism *Sporosarcina pasteurii* is *S.past*. The tested ureolytic organisms *Escherichia coli* DH5α(pURE 14.8) is pURE and *Pseudomonas aeruginosa* MJK1 is MJK1. The negative control organisms *E. coli* DH5α is DH5α and *Pseudomonas aeruginosa* AH298 is AH298.

pH

The pH for the flasks was measured for the duration of the experiment. Equation 7 was used to model the pH within the flasks. The constants were found using the following equation and least squares fitting.

$$pH = -C_1 e^{-kt} + C_2 \quad (7)$$

The values of C_1 , k , and C_2 are calculated pH model constants. This model was chosen based on observed fit. The organisms which are most active at breaking down urea are predicted to have the quickest rate of pH increase.

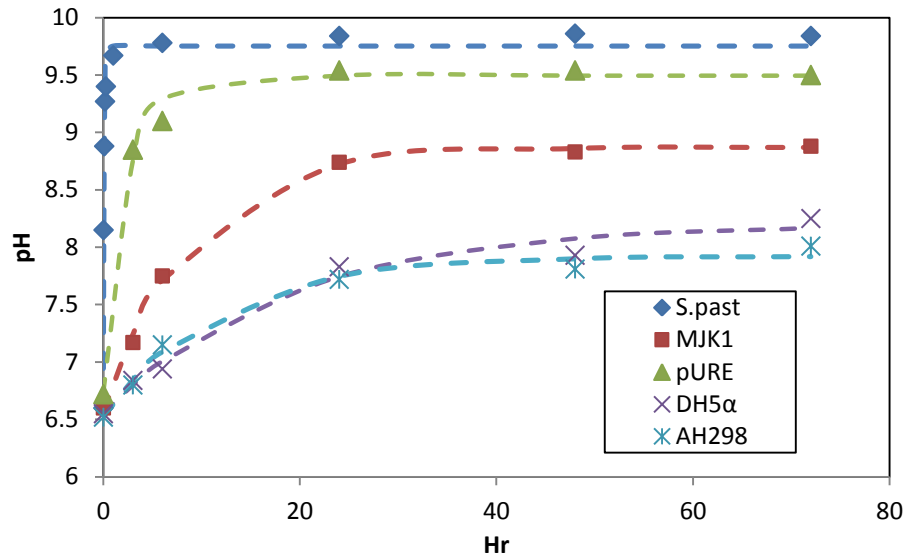


Figure 3. The measured pH values within the media vs. time. Values were measure using a pH meter. The dashed lines represent the fit model.

From the data in figure 3, the constants were determined for equation 7 and are represented in the Table 2 below.

Table 2. The constants values of the pH model in equation 7.

	S.past	pURE	MJK1	DH5α	AH298
C_1	9.75	9.49	8.87	8.20	7.92
k (1/hr)	13.185	0.436	0.115	0.053	0.087
C_2	3.12	2.76	2.31	1.65	1.40

The higher k values indicate a more rapid rate of rise in pH.

Ammonium concentration

In order to calculate the inhibitory effects due to ammonium, the ammonium concentration was measured during the course of the incubations. The ammonium was modeled to have an inhibitory effect on the urease activity based on the Michaelis-Menton equation (Equation 6). Direct measurement of ammonium by the Nessler assay provided inconsistent results. Accordingly, ammonium values were derived by measuring urea using HPLC and then stoichiometrically determining that two moles ammonium were produced per mole of urea broken down. This concentration for ammonium was then used to explore the potential for non-competitive product inhibition to occur with the reaction.

The lack of calcium precipitation during the experiment was designed to observe the potential change to the pH associated with the activity of the cells. The measured ammonia concentration using the Nessler assay is represented in figure 4.

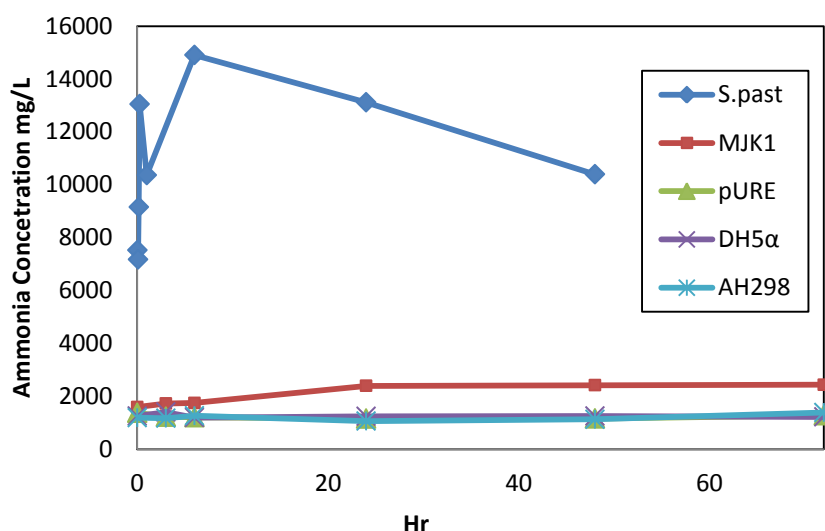


Figure 4. Ammonia concentration in mg/L plotted vs. time for the bacteria species used in this study. The ammonia was measured using the Nessler assay. The increased ammonia concentration results from the urea breakdown.

The ureolytic organisms *S. past* and MJK1 showed increased ammonia concentrations. No significant increase was observed for the control organisms and the pURE.

Urea concentration

For the final batch run the kinetics model was applied to the values for urea concentration which were collected in order to obtain an effective rate of activity.

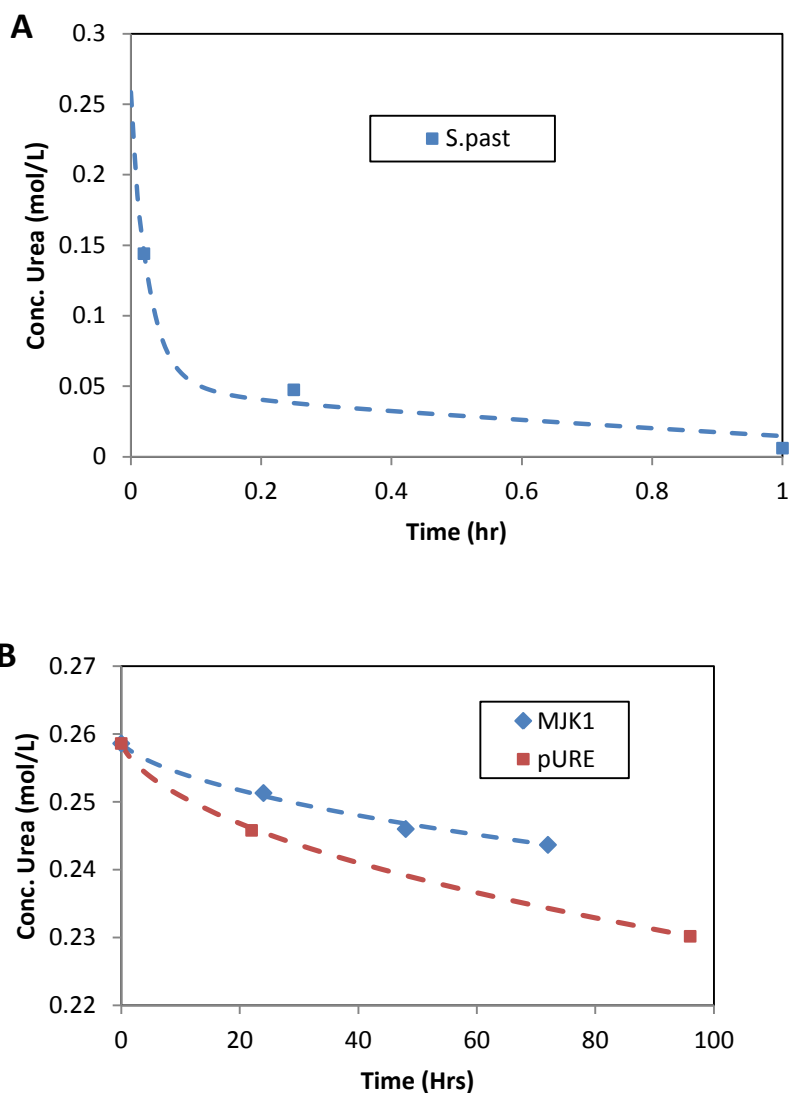


Figure 5. Urea concentration in mol/L plotted vs. time for the ureolytic bacteria species. The urea was measured using high-precision liquid chromatography (HPLC). The fit kinetics model is represented by the dashed lines. Note the differing time and concentration scales between A and B.

The least squares fitting to the data points allows for the determination of the apparent kinetic parameters from equation 6 which are shown in Table 3. The more bacteria with the highest rate of urea breakdown should have the highest V_{max} value.

Table 3. Enzyme kinetics model parameters for the ureolytic species

	S. past	pURE	MJK1
V_{max} (mol/L*hr)	8.08	0.00195	0.00082

K_m (mmol/L)	3.0
K_p (mmol/L)	5.0

The K_m and K_p values are assumed to be constant amongst the organisms. The K_m value was taken from literature (Fidaleo et al. 2003) and is found to not have a significant effect at these operating substrate concentrations. The K_p value was determined using the *S. pasteurii* and was assumed to be the same for the other organisms. While these constants can vary based on source, this was assumed because they share the similarity of being bacterial urease (Mobley et al. 1995).

Discussion

The performance of these bacteria in urea hydrolysis experiments provides insight into how these and other bacteria may perform in other experiments and for *in situ* studies.

Previous studies have determined the urease kinetics for isolated urease (Fidaleo et al. 2003). However, for *in vivo* bacterial urease, other considerations need also be considered such as cell membrane transport and environmental selective pressures. Cell growth can also cause pH change which effects the urease kinetics (Fidaleo et al. 2003, Moynihan et al. 1989).

Cell growth effects

The amount of cells within the flasks was monitored using the drop plate analysis. In order to reduce the complexity of the model the urease concentration of the batch was assumed to be constant. The concentration of the urease was assumed to be correlated to the concentration of cells present within the flask. The rate of ureolysis was divided by the initial cell concentration of cells within the flask. This allows the V_{max} rate to be given with the units of [mmol/(hr*cells)]. These units allow for the model to be much more easily scaled to fit other situations. For this flask study, the lack of cell growth for the ureolytic organisms in Figure 2 supports this assumption for the model.

pH effects

The breakdown of urea produces ammonia and carbonates. This has the effect of increasing the medium's pH. The relative rate of increase in pH was used as an initial indicator of potential ureolytic activity of the bacteria.

The pH effect is dependent on whether the urease is functioning in intracellular or extracellular locations. The effect of pH on the urease activity was at first pursued in this study because the urease was assumed to be extracellular. The enzyme activity of urease has been found to be strongly related to the pH of the solution in which it is acting (Fidaleo et al. 2003). In order to determine the V_{max} of the cell's urease, the pH of the solution in the flasks for the different organisms was modeled. The results presented below were for jack bean urease and were performed on the isolated enzyme in a buffered pH solution.

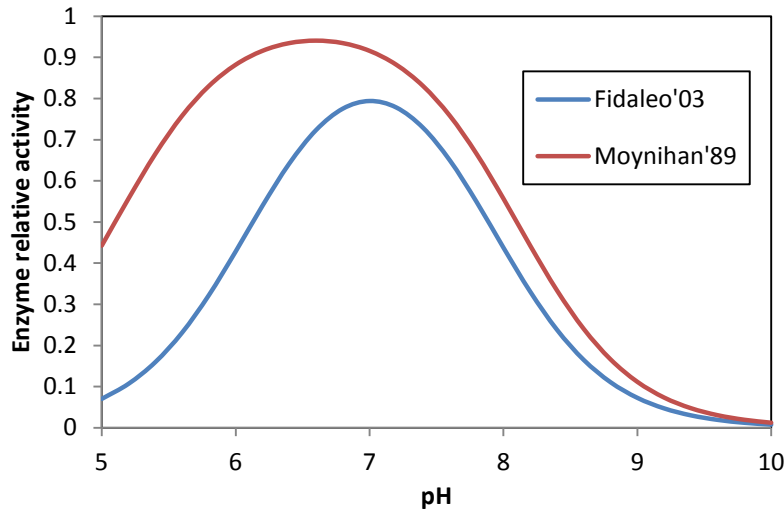


Figure 6. The effects of pH on the relative activity of the urease enzyme

Fidaleo et al. (2003) and Moynihan et al. (1989) also found that the K_m value was not significantly affected by the pH. The equation below was initially added into the kinetics modeling to account for the pH shift caused by the cells and the urea breakdown.

$$V_{max}f(pH) = \frac{k_s [E_0]}{1 + \frac{10^{-pH}}{K_{E,1}} + \frac{K_{E,2}}{10^{-pH}}} \quad (7)$$

The values of $K_{E,1}$ and $K_{E,2}$ are 0.757 $\mu\text{mol/L}$ and 0.0127 $\mu\text{mol/L}$ respectively. (Fidaleo et al. 2003).

The impact the pH had on the V_{max} kinetics of the *S. pasteurii* was removed, this resulted in a much more reasonable product inhibition constant than originally calculated, indicating that the assumption of an intracellular urease was valid. A literature value of K_p for jack bean urease was found to be 12.2 mmol/L (Fidaleo et al. 2003) which is very similar to the calculated value. Additionally many other bacteria have been found to have intracellular urease (Mobley et al. 1995)

Kinetic parameters

The kinetic parameters determined in this study were compared to the literature and expected values to examine the effectiveness of the modeling. The kinetics parameter of K_m , with a value of 3 mM, is negligible compared to the concentration at which these experiments are operating. The flasks are prepared with 333 mM which is significantly more than the K_m value. This value is approximately six times larger than what was used for an injectate in a field application (Fujita et al. 2008). The larger value was used in the laboratory to ensure substrate concentration would not be a limiting factor in the reaction rate.

In some cases when there was no determinable product inhibition the kinetics model would be simplified to $V = V_{max}(S)/(K_m + (S))$. In this case the $V = V_{max}$ because the substrate concentration (S) is much larger relative to K_m . This results in a 0th order formula for the rate of urea hydrolysis, $dS/dt = V$. For some of the runs there did not seem to be detectable product inhibition (i.e. the rate of urea hydrolysis is either steady or increasing at the end). This could have been because of cell growth or increased urease concentration. It could also be that the HPLC was unable to measure at these concentrations. The calculations for kinetic activity in this experiment assume constant urease concentration.

Relative rates

The activity levels of urease can be compared between organisms using kinetic model approximations and are represented below in Table 4.

Table 4. Ureolytic rates of organisms reported for various studies in literature.

Organism	Rates of Ureolysis
	fmol urea hydrolyzed/cell/hr
<i>Sporosarcina pasteurii</i>	1.2×10^{-4} (Colwell et al. 2005) 4,000
<i>Escherichia coli</i> DH5 α (pURE14.8) (Collins and Falkow 1990)	9.89
<i>Pseudomonas aeruginosa</i> MJK1 (Werner et al. 2004 and Kaufman 2011)	3.19
<i>Escherichia coli</i> MJK2 (Folkesson et al. 2008 and Kaufman 2011)	26.9
Groundwater samples from Idaho well	8×10^{-5} (Colwell et al. 2005)

The experiments performed in this study yielded much higher rates than those compared in the literature. This is possibly due to the differing growth and substrate concentrations.

Conclusion

This study examined the rates of urea hydrolysis by the modified bacteria cells. Possible next steps for the study could be to extend the length of time over which the urea hydrolysis is measured. The inoculating of the flasks at a higher concentration of cells could also increase the amount of urea which was hydrolyzed making the drop in urea concentration more significant for the modified bacteria. Observing the effect of varying the concentration of the substrate urea within the flask is also a further potential step for this study.

In this study, the production of the urease by the bacteria was induced with a promoter. The urease concentration was assumed to be correlated to the cell concentration. A process to either measure the enzyme concentration present within the medium or a method to separate the bacterial urease from the cell could aid in more effective assumptions for modeling of the organisms.

There is potential use of these bacteria for work in *in vitro* flow cell experiments. The model bacteria which more closely represents the *in situ* bacteria ureolysis rates may be used to provide a more controlled experiment system. The lower ureolytic rates of the genetically modified organisms may be preferred for longer term experiments where the clogging of systems by *S. pasteurii* may be an issue.

There are many potential benefits for using bioremediation to treat contaminated aquifers. Microbial enabled co-precipitation of calcium carbonate can be used for certain cases of remediation. The optimal way in which to apply this method is still not well understood. This research provides a measure of the ureolytic activity of bacteria which may aid in a better understanding of how to produce an effective calcium carbonate precipitation result when introducing urea into a contaminated aquifer.

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