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Urease activity in microbiologically-induced calcite precipitation

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Abstract

The role of microbial urease in calcite precipitation was studied utilizing a recombinant *Escherichia coli* HB101 containing a plasmid, pBU11, that encodes *Bacillus pasteurii* urease. The calcite precipitation by *E. coli* HB101 (pBU11) was significant although its precipitation level was not as high as that by *B. pasteurii*. Addition of low concentrations (5–100 μM) of nickel, the cofactor of urease, to the medium further enhanced calcite precipitation by *E. coli* (pBU11). Calcite precipitation induced by both *B. pasteurii* and *E. coli* (pBU11) was inhibited in the presence of a urease inhibitor, acetohydroxamic acid (AHA). These observations on the recombinant urease have confirmed that urease activity is essential for microbiologically-induced calcite precipitation. Partially purified *B. pasteurii* urease was immobilized in polyurethane (PU) foam to compare the efficacy of calcite precipitation between the free and immobilized enzymes. The immobilized urease showed higher K_m and lower V_{max} values, which were reflected by a slower overall calcite precipitation. However, scanning electron micrographs (SEM) identified that the calcite precipitation occurred throughout the matrices of polyurethane. Furthermore, PU-immobilized urease retained higher enzymatic activities at high temperatures and in the presence of a high concentration of pronase, indicating that immobilization protects the enzyme activity from environmental changes.

Keywords: Urease; Calcite precipitation; *Bacillus pasteurii*; Recombinant *Escherichia coli*; Polyurethane

1. Introduction

Urease (urea amidohydrolase; EC 3.5.1.5) catalyzes the hydrolysis of urea to ammonia and CO_2. The urease from jack bean is the first enzyme to be crystallized (Sumner, 1926). Urease activity is found in a wide range of microorganisms and plants, some of which produce the enzyme in large quantities (Mobley et al., 1995a,b; Ciurli et al., 1996). In particular, *Bacillus pasteurii*, a soil microorganism, produces intracellular urease constituting close to 1% of the cell dry weight. The urease from *B. pasteurii* consists of three different subunits with two nickel atoms in individual active sites (Benini et al., 1999). Several urease

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accessory gene products are required to incorporate the nickel ions into the apourease, which is essential for the expression of urease as a catalytically active holoenzyme (McGee et al., 1999). The urease genes from numerous microorganisms have been sequenced and expressed in the recombinant plasmids (Maeda et al., 1993; Morsdorf et al., 1994).

Recently, we introduced *B. pasteurii* to induce microbial $CaCO_3$ precipitation, which was applied as a microbial sealant to remediate cracks and fissures in structural formations of granite and concrete (Gollapudi et al., 1995; Ramachandran et al., 2001). It seems that the ammonia produced by the microbial enzymatic hydrolysis of urea increases the pH in surroundings, subsequently inducing $CaCO_3$ precipitation. There are several additional steps involved in completion of this microbiologically-induced precipitation of $CaCO_3$, mainly as a form of calcite (Stocks-Fischer et al., 1999). Our previous studies demonstrated the effective microbial plugging of cracks and fissures in granite, but not in concrete. It is mainly due to the fact that an extremely high pH of concrete (pH 12.5) inhibits the growth of *B. pasteurii* (optimum pH 9.0). Thus, we adopted a cell immobilization technique utilizing polyurethane (PU) in the remediation of concrete cracks to protect cells from the high pH of concrete (Bang et al., 2001). Porous matrices of PU not only increase the surface areas but also minimize the diffusion limitation for substrates and products (Klein and Kluge, 1981). However, the major drawback of whole cell immobilization in PU is that the viability of cells encapsulated in PU polymer is uncertain. It is understood that metabolic activities of cells remain high, but there is no sign of cell growth or reproduction detected (Sumino et al., 1992).

In this study, urease enzyme has been immobilized in PU foam as an alternative to the whole cell immobilization (Fukushima et al., 1978; Wang and Ruchenstein, 1993; Bang et al., 2001). We hypothesized that the immobilized enzyme could overcome the loss of viability of the whole cells in PU. In addition, the use of immobilized enzyme will be environmentally safer than that of immobilized microorganisms. Although *B. pasteurii* is a common soil microorganism, it is not desirable to apply a large quantity of microorganisms in the environment. Attempts have been made to identify the changes of kinetic properties of urease due to the variations in structural and functional integrity of the enzyme upon immobilization. We have utilized *Escherichia coli* HB101 that harbors a plasmid encoding *B. pasteurii* urease genes (You et al., 1995) to confirm our assumption that urease would be the primary factor to initiate calcite precipitation. *B. pasteurii* and the recombinant *E. coli* were tested for the effects of Ni^{2+}, the urease cofactor, and acetohydroxamic acid (AHA) that functions as a competitive inhibitor of urease by chelating nickel atoms at the active site. Further the efficacy of calcite precipitation by both organisms was compared. Abundance of pronase, a proteolytic enzyme, in soil and water from various sources draws additional concerns to the application of enzyme in the natural environment (Ciurli et al., 1996). To understand the fate of urease under different environmental conditions, the stability of the urease enzyme at different temperatures and in the presence of pronase has been also examined.

This paper reports the findings on the role of urease in calcite precipitation and the behavior of the immobilized urease by examining: (1) effects of AHA and Ni^{2+} on calcite precipitation by the recombinant *E. coli* and *B. pasteurii*; (2) kinetic properties of free and immobilized ureases; (3) calcite precipitation induced by the free and immobilized enzymes; and (4) effects of the temperature and the pronase on the free and immobilized enzymes.

2. Materials and methods

2.1. Microorganisms and growth conditions

This study utilized *B. pasteurii* ATCC 11859 and two recombinant *E. coli* HB101 (*supE44 recA ara14 proA2 lacY1 galK2*) containing plasmids pBU11 and pBR322, respectively (You et al., 1995). *B. pasteurii* ATCC 11859 was purchased from the American Type Culture Collection (Bethesda, MD) and maintained in ATCC 1832

medium (BPU). Details of the growth conditions are described elsewhere (Bang et al., 2001). Recombinant *E. coli* strains were provided by S.D. Kim (Yeungnam University, Korea). Plasmid pBR322 contains no urease gene, while plasmid pBU11 has been constructed with the entire sequence of the urease gene cluster from *B. pasteurii* ATCC 11859 including a segment of the plasmid pBR322 sequence (Lee and Kim, 1992). Both plasmids encode the ampicillin resistant gene. *E. coli* HB101 (pBR322) and (pBU11) strains were maintained in Luria–Bertani (LB) broth containing 50 μM $NiCl_2$ for urease activity and ampicillin (100 μg ml^{-1}) for maintenance of the plasmid. Broth cultures for $CaCO_3$ precipitation experiments were prepared in urea–$CaCl_2$ medium as previously reported (Stocks-Fischer et al., 1999). Throughout the study, *E. coli* strains were grown at 37 °C and *B. pasteurii* at 30 °C.

2.2. Enzymes and chemicals

Partially purified urease (type IV) from *B. pasteurii* ATCC 11859 and pronase E from *Streptomyces griseus* were purchased from the Sigma Co. (St. Louis, MO). Acetohydroxamic acid (AHA) and dimethylglyoxime ($dmgH_2$) were purchased from Aldrich Chemicals (St. Louis, MO). Water-based prepolymer of polyurethane (HYPOL® 3000) was provided by the Hampshire Chemical Corp. (Boston, MA). PU prepolymer consists of hydrophilic prepolymer, 91% (w/w), and toluene diisocyanate, 9% (w/w). All other chemicals used were reagent grade.

2.3. Experimental procedures

2.3.1. Effects of acetohydroxamic acid (AHA) on calcite precipitation and ammonia production

The culture of *B. pasteurii* was grown overnight in BPU medium. *E. coli* HB101 (pBU11) and (pBR322) strains were grown in LB broth supplemented with antibiotic and nickel as described above. Cells were inoculated into 20 ml of urea–$CaCl_2$ medium to a final concentration of 1×10^7 cells ml^{-1}, in which nickel (5 μM) and ampicillin (100 μg ml^{-1}) were supplemented for the recombinant *E. coli* strains. Inhibition of urease activity in the presence of 5 mM AHA was determined by measuring $CaCO_3$ precipitation induced by *B. pasteurii* and two strains of the recombinant *E. coli*. All samples were prepared in triplicate. The experiment was carried out in a water-bath shaker at 37 °C for *E. coli* strains and at 30°C for *B. pasteurii*. At each interval (0, 2, 4, 6, 8, 10, 12, 24, and 72 h), replicate flasks were removed to determine the insoluble Ca^{2+}, NH_4^+ production, and pH. The EDTA titration method was used to determine the concentration of calcium ions and a colorimetric method was used for NH_4^+ measurement (Stocks-Fischer et al., 1999).

2.3.2. Effects of Ni^{2+} on urease expressed by recombinant E. coli

The effect of nickel on urease was determined by measuring the amount of $CaCO_3$ precipitation induced by the recombinant *E. coli* encoding urease genes in the presence of $NiCl_2$. The culture of *E. coli* (pBU11) grown in LB was prepared as described above. Samples for $CaCO_3$ precipitation were prepared in triplicate with urea–$CaCl_2$ medium containing ampicillin (100 μg ml^{-1}), to which $NiCl_2$ was added to final concentrations of 0, 5, 100, 500, and 1000 μM, respectively. The experiment with the recombinant *E. coli* (1×10^7 cells ml^{-1}) was carried out at 37 °C in a water-bath shaker. At intervals (0, 2, 4, 6, 8, 10, 12, 24 and 72 h), the amounts of insoluble Ca^{2+} and NH_4^+ produced were determined. A nickel chelator, $dmgH_2$, was added at a ratio of 10:1 ($dmgH_2$:Ni^{2+}) to the medium containing higher concentrations of nickel (500 and 1000 μM) prior to EDTA titration.

2.3.3. Immobilization of urease in PU

For immobilization of the partially purified *B. pasteurii* urease enzyme, 0.5 ml of urease in a buffer solution (25 U ml^{-1}) containing 100 mM phosphate (Na_2H/NaH_2PO_4) and 1 mM EDTA, pH 7.7, was added to 0.5 g polyurethane prepolymer (HYPOL® 3000) in a 5 ml test tube to produce a cylindrical-shaped sponge with an average dimension of 10 mm (d) × 50 mm (l). The blank was prepared in the same manner, using phosphate buffer without enzyme. The polymerized PU foam was cured for 2 h in ice before use.

Details of polymerization has been described elsewhere (Bang et al., 2001). Physical properties of the polymerized PU such as tensile strength, elastic modulus, and elongation were determined in our laboratory using Minimat 2000 (Rheometric Scientific, Piscatway, NJ), while bulk density and percent porosity were determined by the Micrometrics Instrument Corp. (Norcross, GA). For further experiments described below, the PU foam was aseptically cut into eight equal-sized cylindrical foam pieces with a semi-circular cross section ($d = 10$ mm, $l = 12.5$ mm) before use.

2.3.4. Urease assay and protein determination

Both the free and immobilized enzymes were assayed in buffer containing 100 mM phosphate and 1 mM EDTA, pH 7.7, and using 66 mM urea as the substrate. The volume of the reaction mixture was 1 ml for the free enzyme and 5 ml for the immobilized enzyme assay. PU-immobilized enzyme was incubated for 5 min at 30 °C prior to urease assay. The reaction was initiated upon addition of the free or immobilized enzyme and urease activity was determined by measuring the amount of ammonia released by urea according to the phenol-hypochlorite assay method (Natarajan, 1995). One unit of urease is defined as the amount of enzyme hydrolyzing one μmol urea per min. Michaelis–Menten kinetic constants, K_m and V_{max}, were estimated by graphing the data in the Lineweaver–Burk plot. Protein concentration was determined by following the procedure of Bradford (1976), using a Bio-Rad protein assay solution. Bovine serum albumin was used as the standard.

2.3.5. Calcite precipitation induced by free and immobilized urease

Urease-induced calcite precipitation experiments were carried out in a 20 ml reaction mixture containing 25.2 mM $NaHCO_3$, 25.2 mM $CaCl_2$, and 66 mM urea, at 30 °C with shaking (130 rpm). The precipitation was initiated by adding the free and immobilized enzymes (0.5 and 5.0 U ml^{-1}) separately into the sample mixture prepared in triplicate. At every 2 h for 12 and 24 h, the replicates were terminated to determine the amount of insoluble Ca^{2+} according to the method described previously (Stocks-Fischer et al., 1999).

2.3.6. Effects of temperature on free and immobilized ureases

Free and PU-immobilized enzymes were prepared in phosphate buffer to final concentrations of 0.05 and 5.0 units ml^{-1}, respectively, and maintained at three different temperatures of 4, 30 and 60 °C for 7 days. Both free and immobilized urease samples were prepared in triplicate. Each day, the replicates from the individual temperatures were assayed for the remaining urease activity by adding 66 mM of urea. The enzyme assay procedures for the both free and immobilized samples were the same as described above.

2.3.7. Effects of pronase on free and immobilized urease

S. griseus pronase (EC 3.4.24.4) was reconstituted in the same phosphate buffer as for *B. pasteurii* urease. One unit of pronase activity is defined as the amount of enzyme used for hydrolyzing casein to produce Folin-based color equivalent to one μmol tyrosine per min. Effects of pronase on the free and immobilized urease were examined by incubating one unit of pronase per 2500 U of urease at 30 °C for 7 days. All samples were prepared in triplicate. Each day, the remaining urease activity from the replicates was determined as described above.

2.3.8. Scanning electron microscopy (SEM)

Urease-laden PU foam samples were prepared for SEM at 0 and 24 h after incubation in urea–$CaCl_2$ medium. All samples were completely dried at room temperature and cut into approximately 0.5 cm cube pieces prior to fixing for SEM analysis. Procedures for SEM sample preparation were reported previously (Bang et al., 2001). The samples on the stub were sputter-coated with gold and examined by scanning electron microscopy (JEOL JSM-840A) at accelerating voltages ranging from 15 to 25 K_v.

3. Results

3.1. Effects of AHA on calcite precipitation and ammonia production

Fig. 1 shows the effects of a urease inhibitor, AHA, on the calcite precipitation induced by *B. pasteurii* and *E. coli* (pBU11) with the urease gene. *E. coli* (pBR322) encoding no urease genes served as the negative control. In the absence of AHA, the recombinant *E. coli* induced the calcite precipitation even though its precipitation level was not as high as that by *B. pasteurii*, while there was no detectable calcite precipitation by *E. coli* (pBR322). The same trends were also noted for the ammonia production by the same cells (data not included), corresponding to the patterns observed in the calcite precipitation. Calcite precipitation was completed in medium at pH close to 9.4. In the presence of AHA, *B. pasteurii* or *E. coli* (pBU11) induced little calcite precipitation. Subsequently, there was neither pH increase nor ammonia production under the condition where AHA inhibited the urease activity in the cell. However, the turbidity of both cultures containing AHA still increased indicating that the cell growth was not affected.

3.2. Effects of nickel on calcite precipitation

The presence of nickel ions in the active site of urease is essential for the functional activity as well as the structural integrity of the enzyme. Effects of nickel on the calcite precipitation patterns of *E. coli* (pBR322) are depicted in Fig. 2. The calcite precipitation rate is increased dramatically by the addition of nickel (5–100 μM), showing the highest rate in the presence of 5 μM Ni^{2+} ion. However, there was no increase in the calcite precipitation by *B. pasteurii* in the presence of additional Ni^{2+} (data not shown). At the concentrations above 500 μM Ni^{2+}, calcite precipitation by the urease-producing cells drastically decreased, resulting in no apparent precipitation at 1000 μM Ni^{2+}. Higher concentrations of Ni^{2+} seemed to inhibit not only the urease activity but also the cell growth, as there was no increase in the turbidity of the growth medium. During the assays for calcite precipitation, the chelating agent removed the excess nickel ions that interfered with EDTA titration.

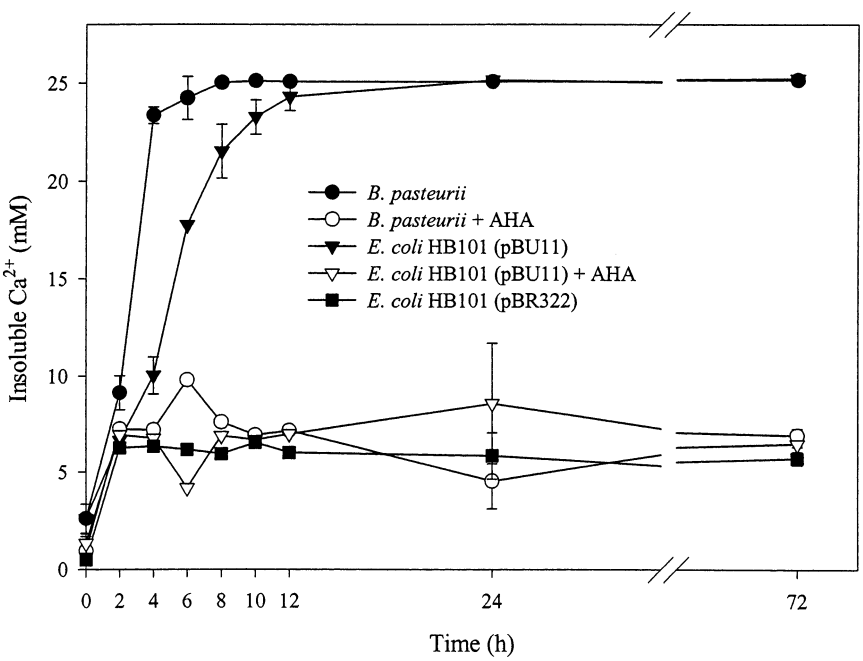

Fig. 1. Effects of AHA on urease-induced calcite precipitation by *B. pasteurii* and the recombinant *E. coli* HB101 strains.

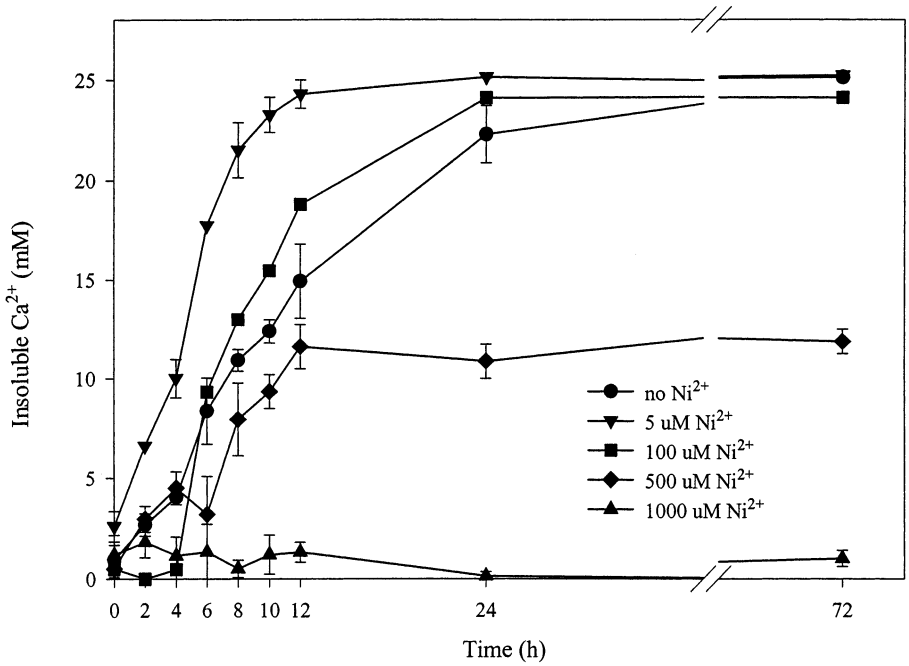

Fig. 2. Effects of Ni^{2+} on urease-induced calcite precipitation by *E. coli* HB101 (pBU11).

Table 1
Physical properties of polyurethane used for immobilization

Density (g ml^{-1})[a]	Tensile strength (kPa)	Elastic modulus (kPa)	Porosity (%)[a]	Elongation (%)
0.12 ($\pm$0.001)	103.00 ($\pm$7.07)	1.05 ($\pm$0.04)	89.78	62.56

All data were obtained from the average of triplicate samples. Values in parentheses are standard deviations.
[a] Tests were done by the Micrometrics Inc., Norcross, GA.

3.3. Immobilization of urease in PU

Upon polymerization, PU foam with the open cell structure becomes pliable and elastic. Physical properties of PU polymer are summarized in Table 1. PU foam that was prepared with or without urease showed no difference in its physical properties. Fig. 3 includes scanning electron micrographs showing the cross-sections of porous PU foam that forms the open-cell matrix. The PU polymer is smooth and less porous close to the surface (Fig. 3A), while the polymer matrix has a higher porosity toward the inside (Fig. 3B).

3.4. Kinetics of immobilized urease

The apparent K_m and V_{max} were determined using a Lineweaver–Burk plot for urea of the free and immobilized ureases. The K_m value of the immobilized urease (22.99 mM) somewhat increased when compared to that of the free one (17.30 mM), indicating a slight decrease in enzyme affinity to the substrate urea upon immobilization. That is, the immobilized urease requires more urea to achieve its maximum velocity. The V_{max} of the immobilized urease also showed a lower value, 0.73 mM min^{-1} mg^{-1} protein,

which is approximately 50% of that of the free enzyme (1.57 mM min^{-1} mg^{-1} protein).

3.5. Calcite precipitation by free and immobilized ureases

Fig. 4 compares patterns of the calcite precipitation induced by the free and immobilized enzymes. At two different concentrations (0.5 and 5.0 U ml^{-1}) of urease, the free enzyme induced calcite precipitation at a higher rate. The rate of the calcite precipitation by the immobilized enzyme was slower, but reached the maximum within 24 h even at a lower concentration of the enzyme (0.5 U ml^{-1}). After 24 h of incubation with the immobilized enzyme at which the calcite precipitation completed, calcite crystals were observed throughout the matrices of the urease-laden PU (Fig. 5). Calcite crystals precipitated on the surface of the PU are less organized in shape (Fig. 5A), while calcite precipitation that occurred within the matrices shows a typical pattern of calcite crystals with

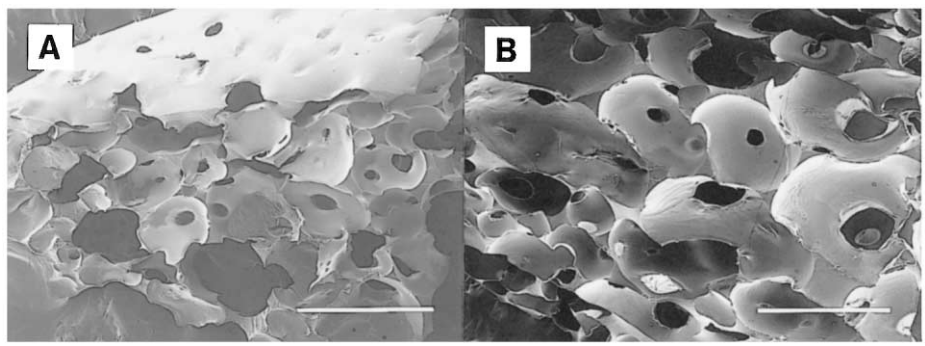

Fig. 3. Scanning electron micrographs of polyurethane foam showing porous matrices. (A) PU matrices close to the surface area (bar, 1 mm). (B) PU matrices toward the inside (bar, 1 mm).

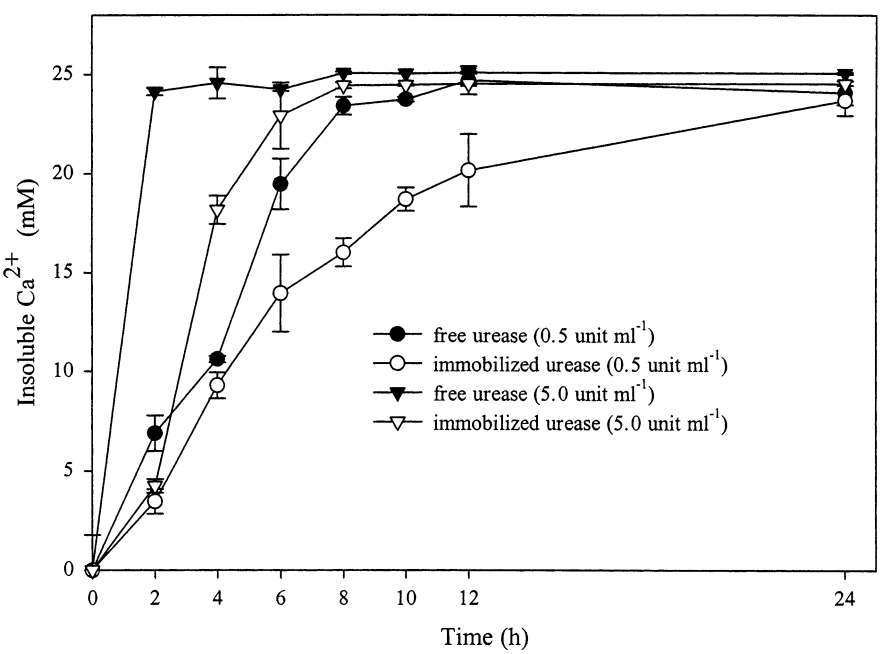

Fig. 4. Calcite precipitation induced by the free and immobilized urease.

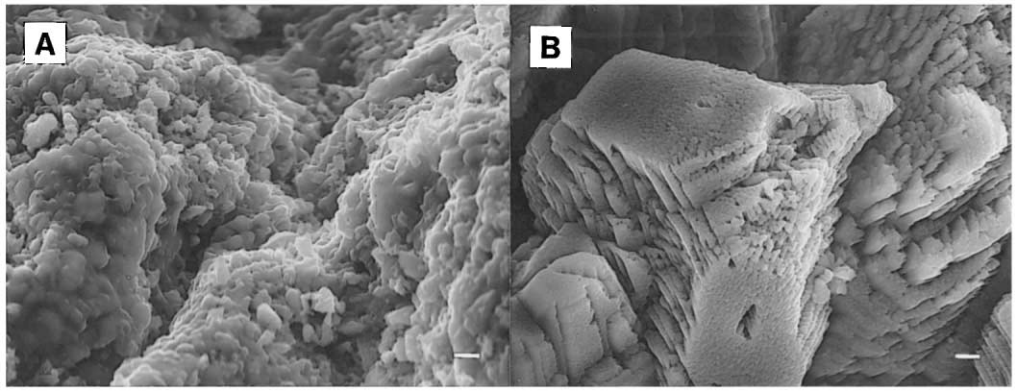

Fig. 5. Scanning electron micrographs of calcite precipitation induced by the PU-immobilized urease. (A) Calcite precipitation on the surface area of PU foam showing small and less organized crystals (bar, 1 μm). (B) Calcite precipitation within the matrices of PU foam showing typical and well organized crystals (bar, 1 μm).

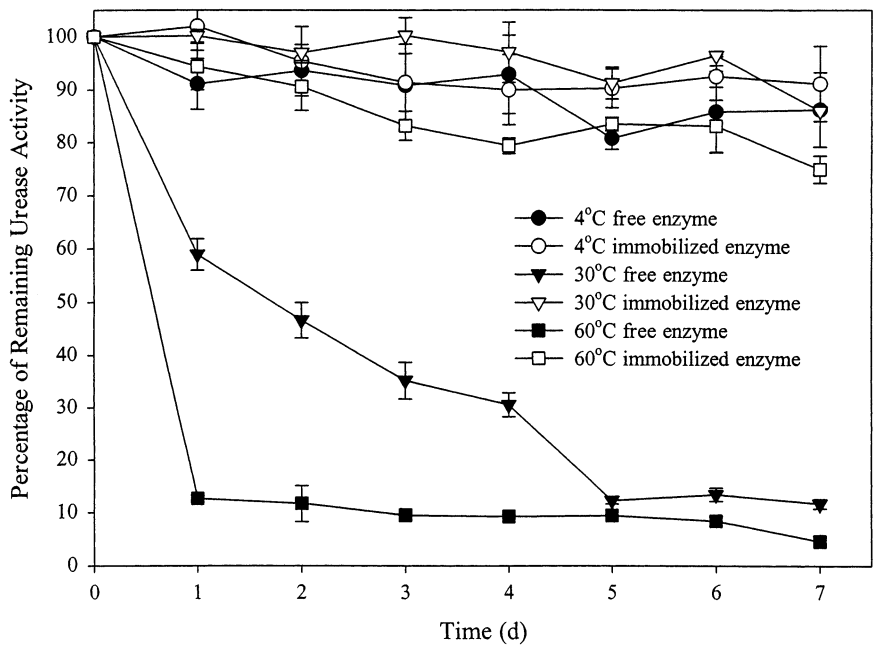

Fig. 6. Effects of temperature on the free and immobilized urease.

well formed faces and edges, which is a common shape of calcite growth induced by microorganisms (Fig. 5B) (Bang et al., 2001).

3.6. Effects of temperature and pronase on free and immobilized urease activities

Fig. 6 presents the effects of the temperature, 4, 30 and 60 °C, on the free and immobilized urease activities in final concentrations of 0.5 and 5.0 U ml^{-1}, respectively, showing the percentage of the remaining enzyme activities. At a lower temperature, 4 °C, there was no significant difference between the free and immobilized enzymes, both of which retained a high urease activity throughout the test period. However, after 7-day incubation at 30 and 60 °C, the immobilized enzyme activities remained high, showing approximately

86 and 75% of the enzyme activity remaining, respectively, while the free urease lost its enzymatic activity significantly, remaining at approximately 12 and 5%, respectively. In particular, the polyurethane matrix appeared to protect the enzyme activity more effectively at higher temperatures. Fig. 7 illustrates the stability of the free and PU-immobilized ureases with time in the absence and presence of pronase at 30 °C. Pronase effectively degraded the free urease, but not the immobilized urease. During the first 2 days, the degree of degradation by pronase was insignificant in the immobilized enzyme ($\sim$ 10%), while the effect was significant in the free enzyme ($\sim$ 50%).

4. Discussions

The data presented in this paper demonstrates that the urease enzyme plays a key role in microbiologically-induced calcite precipitation: (1) the calcite precipitation by *B. pasteurii* and *E. coli* (pBU11) expressing *B. pasteurii* urease was inhibited in the presence of a urease inhibitor; (2) a significant amount of calcite precipitation was induced by *E. coli* (pBU11), while little by *E. coli* (pBR322) that lacks urease genes; and (3) the calcite precipitation by the recombinant urease was significantly increased in the presence of lower concentrations of Ni^{2+} ion (5–100 μm). However, the requirement of additional nickel ions by the recombinant urease is mainly due to the fact that *E. coli* has a low-affinity nickel transporter system in the cytoplasmic membrane compared to *B. pasteurii* (Mobley et al., 1995a,b). The recombinant *E. coli*, therefore, requires nickel supplement in medium for the expression of catalytically active urease, while *B. pasteurii* does not.

Chemically, calcification in aqueous medium produces two types of product: calcium carbonate and protons as shown below.

$$Ca^{2+} + HCO_3^- \rightarrow CaCO_3 + H^+ \quad (1)$$

In biological systems, many calcareous organisms couple calcification to their metabolic assimilation processes to scavenge protons (McConnaughey and Whelan, 1997). In urease-based reactions, NH_3 released by the enzymatic hydrolysis of urea uses the protons generated from calcite precipitation (reaction 1) to produce NH_4^+.

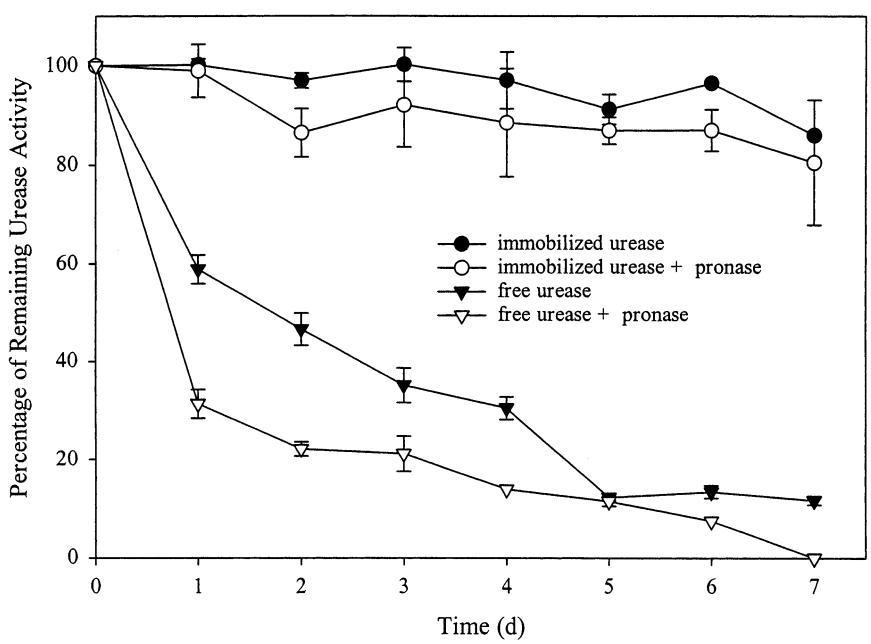

Fig. 7. Effects of pronase on the free and immobilized urease at 30 °C.

$$NH_3 + H^+ \rightarrow NH_4^+ \quad (2)$$

The subsequent increase of pH due to the presence of ammonia ions and the additional release of CO_2 from the enzymatic urea hydrolysis accelerate the rate of the urease-induced calcite precipitation. Thus, the biochemical calcite precipitation necessitates an active participation of urease.

The increased thermal stability of urease mediated by immobilization appears greater at higher temperatures (Fig. 6). It is also evident that immobilization protects the urease activity from pronase as shown in Fig. 7. Urease immobilization undoubtedly protects the structural and functional integrity of urease from the changes of environmental conditions. Nevertheless, the kinetic studies on microbial calcite precipitation by free and immobilized enzymes indicate that immobilization may affect the overall enzymatic activity, resulting in a reduction of the rate of urea utilization in PU polymer matrices. An increase in *Km* of the immobilized enzyme seems unavoidable mainly due to the partitioning effect of the polymer matrices on the substrate flow. In fact, the immobilized enzyme induced calcite precipitation at a slower rate than the free enzyme, which possibly results from diffusion limitation in PU matrices. However, the porous PU polymer served as the additional nucleation site for the calcite precipitation and enables the calcite crystals to grow throughout the matrices (Bang et al., 2001). It is also expected that mechanical supports from the calcite-laden PU matrix would enhance the strength of the crack-remediated structures.

In summary, our observation has detailed the urease-induced calcite precipitation and the stability of the immobilized urease in the environment. Data obtained from this study suggests the positive potential of using the immobilized enzyme instead of the immobilized whole cell in remediation. Currently, research is in progress to use the immobilized urease in remediation of surface cracks of concrete. Details of the experimental parameters involved in crack remediation with the urease-induced calcite are yet to be investigated.

Acknowledgements

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