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Dispersion of Small Ceramic Particles (Al_2O_3) with *Azotobacter vinelandii*

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The high surface charge of small ceramic particles such as alumina particles prevents them from dispersing evenly in aqueous suspensions and forming high-density compacts. However, suspensions of 400-nm-diameter alumina particles treated with alginate from the bacterium *Azotobacter vinelandii* were well dispersed. The alginate bound firmly to the particle surface and could not be removed by repeated washing with distilled water (2.82 mg of the bacterial alginate adsorbed to 1 g of the alumina particles). Furthermore, *A. vinelandii* grew and produced alginate in the presence of up to 15% (vol/vol) alumina particles. These results suggest that an in situ process using this bacterium to coat ceramic particles with alginate might be developed. In in situ processing experiments, the particle-packing densities were significantly increased and the viscosities of 5 and 10% (vol/vol) suspensions were reduced 4- and 60-fold, respectively, over those of controls. The bacteria were readily removed from the alumina particles by washing.

Since prehistoric times, potters have prepared clay beds by amending them with organic materials such as urine, manure-water mixtures, and tannin. This practice of "aging" made the clay materials more plastic and therefore more readily workable for pottery production.

Early in the 20th century, several scientists performed experiments that implicated microorganisms in clay aging. In 1902, Stover noted that clays which were sterilized did not exhibit the plastic qualities of properly aged clays containing bacteria (23). Furthermore, when the sterilized clay was inoculated with previously aged clay, it acquired the workability properties of normally aged clays within 2 to 4 weeks. Spurrier demonstrated the growth of filamentous algae in aging clay and hypothesized that the products of their growth contributed to the increase in plasticity of the clay body (22).

Glick (7, 8) and Baker and Glick (3) noted that clays to be used in the manufacture of high-quality ceramics were aged in commercial cellars for several weeks to a year. New cellars were prepared by inoculating fresh clay material with small of amounts of well-aged clays. Several bacteria were isolated from the seasoned clays and identified as species of *Bacillus* and *Pseudomonas* as well as of other genera.

One is naturally led to inquire how microorganisms are able to increase the plasticity of clays. Since current ceramic processing employs synthetic polymers as dispersants, we hypothesized that microbial exopolymers, such as polysaccharides or polypeptides, are responsible.

Advanced ceramic techniques for the production of highly dense, fine-grained products employ a system in which particles of a very small size (<1 μm in diameter) or powders are evenly dispersed within some solvent system and then concentrated to a high density in the green body (i.e., the ceramic object before firing or sintering). Particles of such small size are subject to strong interparticle interactions, such as the van der Waals force. Alumina in particular has a mixture of positive and negative surface charges which at certain pHs (for instance, pH 8) causes agglomeration in rough aggregates and results in a porous product. To prevent this, a polyelectrolyte, which acts by providing a significant electrical repulsion between particles, may be used as a dispersant. Currently, synthetic polymers from petrochemicals, such as polymethacrylic acid (PMAA) and polyacrylic acid (PAA), are used as dispersants (5, 6). However, commercial PMAA may contain formaldehyde, and PAA may contain residual amounts of acrylic acid; both of these chemicals are toxic. Furthermore, they are produced from precursors that are toxic and/or carcinogenic. In contrast, naturally occurring polymers are nontoxic and do not pose problems of disposal, since they are readily degraded by natural processes.

Earlier studies from this laboratory showed that although the bacterial glycan dextran was ineffective as a dispersant, it became a satisfactory dispersant of alumina when it was modified chemically to produce dextran sulfate (9a). Like the acrylate polymers, dextran sulfate has an acidic side group which is thought to be the important feature for alumina dispersion. However, because of the sulfate groups, dextran sulfate is not completely removed by sintering.

Therefore, we set out to determine whether naturally occurring acidic polymers could be used as dispersants. The first naturally occurring acidic polymer that we studied was alginate, an α-1-4-linked linear copolymer of β-D-mannuronic (M) and β-D-guluronic (G) acids (10–12, 15) synthesized by several species of marine algae (4). Polymers with molecular weights of about 5,000 were found to be satisfactory dispersants for alumina processing (17a). The purpose of this study was to determine whether bacterial alginate from *Azotobacter vinelandii* (9, 18) would also serve as an effective dispersant of ceramic particles and, if so, whether an in situ process could be developed to produce bacterial alginate in the presence of ceramic particles.

MATERIALS AND METHODS

Bacterial cultivation. *A. vinelandii* NCIB 8789 (National Collection of Industrial Bacteria, Aberdeen, Scotland) was

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maintained on Larsen's broth medium (14) in cyst-stage cultures. Cyst-stage cultures (stock cultures) were subcultured every 2 months to fresh broth, grown at 30°C for 2 days, and stored at 4°C. Working cultures were subcultured from the stock culture when needed. The composition of normal Larsen's broth medium is as follows: sucrose (or mannitol), 20 g; K_2HPO_4, 1.0 g; $MgSO_4 \cdot 7H_2O$, 1.0 g; $FeSO_4 \cdot 7H_2O$, 50 mg; $Na_2MoO_4 \cdot 2H_2O$, 5 mg; $CaCl_2$, 50 mg; CH_3COONH_4, 2.3 g; distilled water, 1 liter; the pH is 6.5. For plate cultures, 1.8% agar was added to the broth.

Bacterial counts and cyst observations. Bacterial cells were counted by standard plate count techniques on solid Larsen's medium. Cyst formation of *A. vinelandii* was observed directly by using a phase-contrast microscope.

Determination of polysaccharide in culture suspensions. Bacteria were removed from the culture by centrifugation at 22,000 × *g* for 40 min. Polysaccharide in the supernatant was determined by the *meta*-hydroxydiphenyl-sulfuric acid assay (17). Mannitol (20 g/liter) was used in place of sucrose as a carbon source in the growth medium because of its minimal interference with the colorimetric analysis when this assay was used. Low-viscosity kelp alginate (Sigma) was used as the standard for this assay. Spectrophotometric work was conducted with a Gilford Response II spectrophotometer (Gilford Instrument Laboratories, Oberlin, Ohio) or a Spectronic 20 spectrophotometer (Bausch & Lomb, Rochester, N.Y.).

Polysaccharide harvest. *A. vinelandii* NCIB 8789 was shaken in the Larsen's broth at 30°C for 5 days. Bacterial cells were removed by centrifugation at 22,000 × *g* for 40 min. Three volumes of 2-isopropanol were then added to the supernatant for precipitation (13). The precipitate was washed two to four times with 2-isopropanol and then dissolved in distilled water before being dialyzed against distilled H_2O overnight. After dialysis, the polysaccharide was lyophilized.

Kelp alginate. The polymer used was a low-viscosity (approximately 250 cps for a 2% solution at 25°C) kelp alginate (Sigma Chemical Company, St. Louis, Mo.) produced from *Macrocystis pyrifera* (molecular weight, 75,000 to 100,000) M/G (mannuronic acid/guluronic acid) ratio of alginate from *M. pyrifera* has been reported independently as 1.56 (20).

Ceramic particles. The ceramic particles used in this study were high-purity (99.99%) Al_2O_3, with an average particle diameter of 400 nm and a density of 3.96 g/ml (AKP-30, Sumitomo Chemical America, Inc., New York, N.Y.).

pH adjustment of particle suspensions. Because the pH of the particle suspension is affected by the extent of mixing (2), suspensions for sedimentation tests, rheological measurements, and in situ cultures were mixed for more than 0.5 h (in some situations, overnight) with a magnetic stirrer. The pH was initially adjusted to the experimental value and then readjusted to the same value after mixing. For sedimentation tests, the pH was adjusted to between pH 8.0 and 8.5, a pH range which is the best for observing the dispersion effects of the bacterial alginate because there is no net charge on the surfaces of the alumina particles.

Sedimentation tests. Sedimentation columns were prepared by adding 2% (vol/vol) AKP-30 powder (for the in situ tests, this was 5 to 10% [vol/vol]) to aqueous solutions with various concentrations of the polymer (5, 6). The suspensions were sonicated for 5 min and mixed with a magnetic stirrer for 0.5 h. The pH of the suspension was adjusted to the experimental value (8.0 to 8.5) before the final volume was brought to 10 ml. The suspension was decanted into a conical-bottom, graduated polystyrene tube (Falcon 2095; Becton Dickinson) and left undisturbed at room temperature for 3 to 4 weeks. Final sedimentation heights (cake height in milliliters) were measured to +/−0.1 ml and converted into wet densities in the following way. The theoretical cake height of a 2% (vol/vol) AKP-30 particle suspension in 10 ml of water is 0.2 ml. One milliliter of AKP-30 particles weighs 3.96 g; therefore, the theoretical packing volume of 0.792 g of AKP-30 (2% [vol/vol]) is 0.2 ml, assuming the particles are completely packed. This value was divided by the measured cake height (in milliliters) of the samples, and the percentage was then calculated. For example, if the measured cake height of one sample is 0.5 ml, its wet density is 40% of the theoretical value ([0.2 ml/0.5 ml] × 100%). In highly packed ceramic suspensions, the packing density approaches 100% (theoretical) after sintering.

Viscosity measurements. Viscosity measurements were obtained by using a Rheometrics Fluid Spectrometer (model 8400; Rheometrics Inc., Piscataway, N.J.). Suspensions (about 15 ml) for viscosity measurements were prepared as described for the sedimentation tests.

Washing of the bacterial alginate from the AKP-30. A 200-ml volume of Larsen's broth with mannitol as a carbon source was inoculated with a 1-ml suspension of *A. vinelandii* cells and shaken at 30°C for 5 days. The culture was centrifuged at 22,000 × *g* for 40 min to remove the bacterial cells. The concentration of the polymer in the suspension was determined by the *meta*-hydroxydiphenyl-sulfuric acid assay. Alumina (5% [vol/vol]) (5.94 g of AKP-30) was added to 28.5 ml of the supernatant. The mixture was then ultrasonicated (with a probe) for 2 min for complete mixing. The suspension was adjusted to pH 8, and after centrifugation the concentration of the alginate remaining in the supernatant was determined by the *meta*-hydroxydiphenyl-sulfuric acid assay. The pellet was then washed three times. After each washing, the polymer concentration of the supernatant was assayed. The amount of the polymer bound to the particles was calculated by determining the difference between the total amount of polymer and that in the supernatant.

Removal of bacterial cells from the in situ system. The bacterium was cultured with a 5% (vol/vol) ceramic particle suspension at 30°C for 5 days on a shaker and then centrifuged for 40 min at 22,000 × *g*. This resulted in three layers: the ceramic particle cake at the bottom, a very thin layer of bacterial cells in the middle, and the spent broth (supernatant) at the top. The supernatant was decanted, and the cell layer at the top part of the particle pellet was removed by a sterile pipette. The newly exposed surface was then washed 8 to 10 times with sterile saline solution (0.85% NaCl) to remove loosely associated bacteria before the pellet was resuspended in sterile water to the original volume. Bacterial numbers were determined in this suspension by plate counting.

Nutrient limitation tests. *A. vinelandii* was cultivated in normal Larsen's broth for about 24 h, and the cells were harvested by centrifugation. The cell pellet was washed twice and then suspended in sterile saline. A sample was removed for bacterial enumeration, and 1 ml of this suspension was inoculated into 99 ml of modified nutrient-deficient Larsen's broth. Three modified Larsen's media were used. Modified medium 1 (mannitol only) contained only mannitol (20 g/liter). All other components were deleted. Modified medium 2 (mannitol plus buffer) contained mannitol (20 g/liter), but the K_2HPO_4 was reduced to 65 mg and 32 mg of KH_2PO_4 was added; $MgSO_4 \cdot 7H_2O$ was reduced to 40 mg, and 50 mg of $FeSO_4$ was replaced by 1.7 mg of

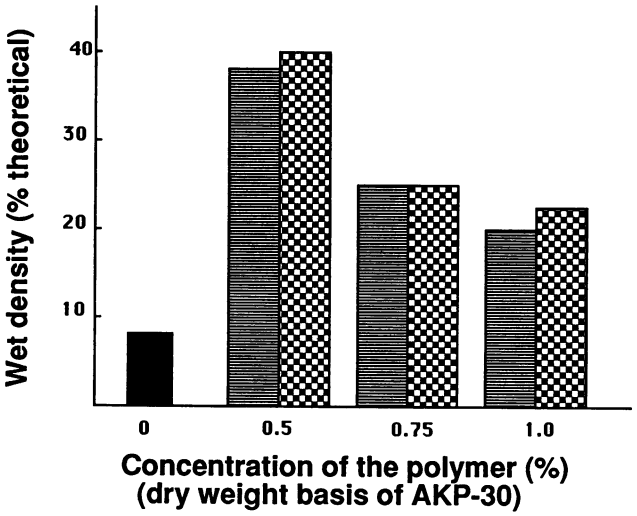

FIG. 1. Sedimentation (wet packing density) of 2% (vol/vol) suspensions of AKP-30 alumina (theoretical wet packing density, 100%) with kelp ([17a]) and bacterial ( ) alginate. All samples were adjusted to pH 8.0 to 8.5.

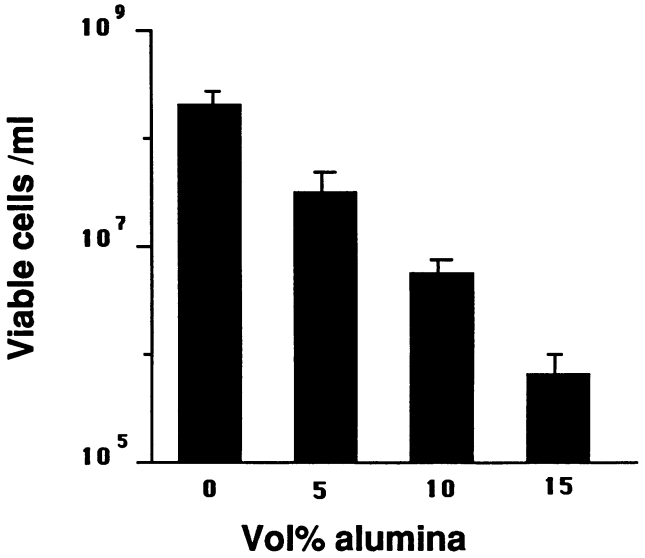

FIG. 2. Growth of *A. vinelandii* at various solids loadings of alumina inoculated with 3.4×10^3 cells per ml.

$FeCl_2 \cdot 4H_2O$. Medium 3 (mannitol plus buffer and acetate) contained the same ingredients as medium 2 but was also supplemented with 1.5 g of sodium acetate. After incubation for 96 h, the number of bacteria and the concentration of bacterial alginate were determined.

RESULTS

Production of alginate by *A. vinelandii*. The production of alginate during growth was determined by sampling batch cultures periodically and analyzing them for viable cell counts and alginate production. Bacterial cell numbers peaked after 20 to 40 h, and cyst production (19) began toward the end of logarithmic growth (between 40 to 60 h). Bacterial alginate secretion corresponded closely with the onset of cyst formation, i.e., in the late logarithmic to early stationary phase of growth. The yield of the alginate, which was harvested by precipitation from culture supernatants with 2-isopropanol, ranged from 0.8 to 1.2 g (dry weight) per liter of broth at the end of 4 days of culture.

The range of the M/G ratio of alginate from *A. vinelandii* was reported to be 0.53 to 0.58 (14), which is lower than that of the kelp alginate from *Macrocystis pyrifera*, reported to have an M/G ratio of 1.56 (20). The viscosity of the bacterial alginate was reported to be influenced by the addition of salts, the M/G ratio, and the molecular weight (16).

Bacterial alginate as a dispersant. The alginate obtained from *A. vinelandii* cultures was tested for its ability to act as a dispersant by preparation of 2% (vol/vol) suspensions of alumina particles. The resulting wet particle-packing densities showed clearly that bacterial alginate was an effective dispersant (allowing for increased wet packing density relative to the control) and acted in a manner similar to that of kelp alginate (Fig. 1). The maximum wet packing density occurred with a 0.5% concentration of the alginate (dry weight basis of polymer to particles) for both alginates.

In situ production of alginate. The foregoing results indicate that bacterial alginate can be used as a dispersant for ceramic particles. The question arises as to the most expedient method that could be used to deliver the alginate to the particles. The most direct means of doing this would be an in situ process in which the bacterium is grown with the particles while it produces the alginate. Therefore, we initiated work to determine whether the bacterium could be grown in the presence of the particles, while producing the alginate at the same time. This concept was tested using 5, 10, and 15% (vol/vol) alumina suspensions inoculated with 3.0×10^3 bacterial cells per ml of Larsen's medium and incubated at 30°C for 4 days. Before inoculation, the pHs of all samples were adjusted to 7.4 (for bacterial growth) and were readjusted to 8.0 to 8.5 (for sedimentation and viscosity measurements) after culturing.

Organisms grew at all concentrations of alumina, although bacterial yields were progressively reduced at higher alumina concentrations (Fig. 2). The bacterial numbers in the cultures increased to 5.1×10^7, 7.5×10^6, and 8.2×10^5 per ml of suspension from the inoculum concentration of 3.0×10^3/ml for the 5, 10, and 15% (vol/vol) suspensions, respectively. A control culture without alumina increased to 3.1×10^8/ml during the 4-day incubation period. As the concentration of the particles increased incrementally by 5% (vol/vol), the bacterial cell yield was reduced about 10-fold. This reduced yield may be attributable to reduced oxygen concentrations, to pH effects of the alumina, or to some other unknown factor. The polymer was detected in the supernatant of each culture after incubation.

We next evaluated whether the particles became coated with the polymer during growth of the bacterium. The 5 and 10% (vol/vol) suspensions which had been incubated with *A. vinelandii* and their sterile controls were adjusted to the same pH before being tested. Sedimentation test results showed that the particle packing was twice as dense as that in the untreated sterile control for the 5% (vol/vol) suspension and two and one-half times as dense than that in the untreated sterile control for the 10% (vol/vol) suspensions (Fig. 3). These results indicated that the polymer was being produced, coating the particles, and facilitating more dense packing of the particles in the sedimented cake.

Suspension viscosity is another method of assessing the effectiveness of a dispersant. Well-dispersed systems are

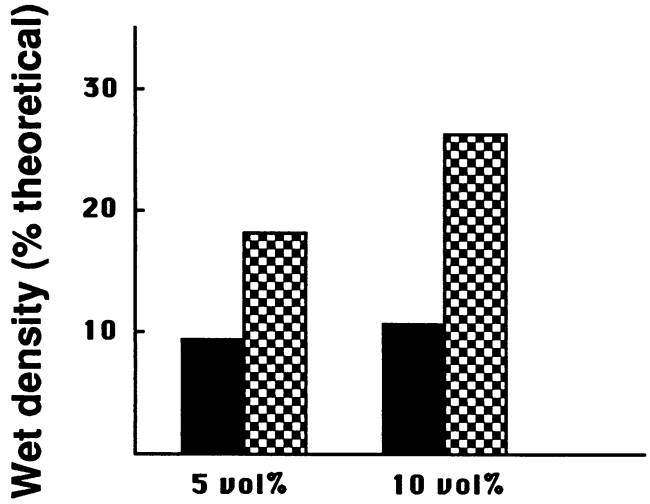

FIG. 3. Particle-packing density resulting from in situ processing of alumina particles by growing cultures of *A. vinelandii* in the presence of 5% (vol/vol) alumina (▩) versus that in the sterile control (■) and in the presence of 10% (vol/vol) alumina (▩) versus that in the sterile control (■). Before sedimentation columns were prepared, the pH was adjusted to the same value as that of the sterile control.

characterized by low viscosities due to repulsive particle-particle interactions in suspension (1, 6). The viscosities of the inoculated 5 and 10% (vol/vol) suspensions were reduced fourfold (from 0.48 to 0.12 P 10 sec^{-1}) and 60-fold (from 6.0 to 0.1 P 10 sec^{-1}) compared with those of the controls.

One of the problems encountered in the in situ processing of ceramic materials is the possible deleterious effect of the bacterial cells on the quality of the final ceramic product. Ideally, cells should be either grown separately from the particles by using a filter system or removed after they have been grown in the presence of the particles. Since we grew cells in direct contact with the particles, we were interested in determining whether cells could be readily removed without removing the polymer. To study this, successive washings were undertaken to determine whether bacterial cells could be removed from the particles (Table 1). After cultivation for 5 days at 30°C, bacterial cell numbers in the in situ system were 5.6×10^7 per ml of particle suspension (a mixture of bacterial cells, particles, and nutrient solution) before washing. Cell counts in the pellet gradually decreased after each successive washing with sterile saline solution. After the fifth washing, there were only 85 cells per ml of the pellet suspension. Therefore, the bacterial cells do not bind strongly to the particles and are readily removed by successive washings.

TABLE 1. Removal of *A. vinelandii* cells from alumina particles by washing

Treatment stage	Viable cells per ml of suspended pellet[a]
Before washing	5.6×10^7
Wash no.:	
1	3.2×10^5
2	1.3×10^3
3	8.2×10^2
4	4.8×10^2
5	8.5×10^1

[a] Viable counts are averages of three replicate plates and three repeated experiments.

TABLE 2. Adsorption of alginate to alumina particles[a]

Treatment stage	Alginate (mg) in 28.5 ml of supernatant	Alginate adsorbed (%)
Before addition of alumina	26.42	
After addition of alumina[b]	9.66	
Wash no.:		
1	0.143	99.1
2	0	99.1
3	0	99.1

[a] Data are averages of three tests.

[b] The alumina was removed from suspension by centrifugation. The amount of alginate adsorbed by the particles was determined by subtracting the amount remaining after the alumina was removed from the amount present initially, i.e., 16.76 mg of bacterial alginate was adsorbed by 5.94 g of AKP-30 particles.

Separate experiments using particles and cell-free culture supernatant containing bacterial alginate were conducted to assay the amount of polymer adsorbed to the particles and the strength of that adsorption (Table 2). In contrast to the results obtained for the bacterial cells, washing had little effect on the removal of polymer bound to the alumina surface. Thus, when the alumina (5.94 g) was added to the supernatant containing the polymer (26.42 mg in 28.5 ml of supernatant) and removed subsequently by centrifugation, it had adsorbed 16.76 mg of the alginate. By calculation, it was determined that a total of 2.82 mg of bacterial alginate was adsorbed per g of AKP-30 particles. After the first washing of the adsorbed particles, only 0.143 mg was detected in 28.5 ml of supernatant, and none could be detected after further washing. Therefore, more than 99% of the polymer that was initially bound remained adsorbed to the particles even after three successive washes.

Use of nongrowing cells for in situ processing. One logical route to consider for in situ ceramic processing is the use of pregrown bacterial suspensions that are capable of alginate production under nongrowing conditions. This would allow polymer production under conditions of greater control over pH, carbon source concentration, mineral base medium composition, and cell concentrations, all of which would be expected to have some influence on an in situ process. Furthermore, a condition might be found in which polymer production could be accomplished by a minimum bacterial biomass, since bacterial cells would be undesirable in the final product.

For this purpose, washed suspensions of *A. vinelandii* cells were inoculated into the three different nutrient-deficient media (Table 3). Growth did not occur in either modified medium 1 (mannitol only) or modified medium 2

TABLE 3. Production of alginate in nutrient limitation tests[a]

Medium	Final cell yield (cells/ml)	Amt of polyuronic acid present [μg/ml (μg/1,000 cells)]
Larsen's	3×10^8	810 (2.7)
Modified medium:		
1	3.0×10^5	5.8 (20)
2	3.2×10^5	30.5 (100)
3	5.0×10^6	495.4 (100)

[a] Experimental cultures were inoculated with 2.8×10^5 cells per ml and incubated for 96 h; data are averages of two experiments.

(mannitol plus buffer). In modified medium 1, the yield of the alginate was very low (only 5.8 μg/ml), but in modified medium 2 alginate production was 30.5 μg/ml, or 100 ng per 1,000 cells. This yield compared favorably with the yield per cell in modified medium 3 (mannitol plus buffer plus acetate); however, greater growth occurred in medium 3 because of the added acetate. In contrast, under normal growth conditions in Larsen's medium the bacterium produced only 2.7 ng of alginate per 1,000 cells. In all media tested, the bacteria lost motility and developed to the cyst stage.

DISCUSSION

Clay aging resembles other empirical processes developed by humans, such as alcoholic and lactic fermentations and bread leavening, that have unwittingly involved microorganisms. However, the actual mechanism that underlies this microbial aging process is still only poorly understood. The results of this study suggest that the production of natural acidic bacterial exopolymers is the underlying explanation for this phenomenon.

The kelp and the bacterial alginates had comparable suspension behaviors, suggesting that they have similar physiocochemical properties. The alginate from *A. vinelandii* is not only structurally similar to the alginate from marine algae (both alginates contain blocks of M, blocks of G, and blocks of MG), but it also contains the same ratio of poly(G) to poly(M) that the alginate from marine algae does (21). The primary difference between the alginate from *A. vinelandii* and algae is that *O*-acetyl groups are associated with some of the M residues of the former. However, the ester-linked acetate groups do not contribute to the charge on the polymer (24, 25).

It is of interest that the polysaccharide concentration for best sedimentation is about 0.5% (Fig. 1), which is close to the concentration at which the polymer was fully adsorbed to the particles (0.3%) (Table 2). Too little or too much polysaccharide resulted in poorer sedimentation. This result also agrees with the results obtained with other polymers (17a). If insufficient polymer is present, dense packing cannot occur. If excess polymer is present, the polymer interacts with itself to cause gelling, leaving void spaces in the cake. Thus, the poorer packing density found at the lower concentration of alumina, i.e., 5 versus 10% (vol/vol) in the in situ growth experiment, is likely due to excess alginate production. It was also observed that the adsorption of polymer can be influenced somewhat by other experimental conditions, including ultrasonication time and the pH of the sample suspension (data not shown).

The high affinity of the polymer for the particles was demonstrated by the experiments in which the coated particles were subjected to several successive washings in distilled water (Table 2). Over 99% of the polysaccharide remained adsorbed to the particles even after three washings.

From the results of these experiments, it is possible to calculate the number of ceramic particles that can be coated with the alginate produced by a single *A. vinelandii* cell (assuming growth on modified medium 2 and an alumina particle radius [*r*] of 200 nm). From the polysaccharide adsorption data (Table 2), we know that about 2.82 mg of alginate are required to coat 1 g of particles and that this amount of polymer is produced by about 2.82×10^7 cells of *A. vinelandii* (Table 3). Thus, in theory, the alginate produced by a single bacterial cell from medium 2 can coat about 2.8×10^5 AKP-30 particles.

The results of this study indicate that *A. vinelandii* can be used in an in situ process. We have demonstrated that *A. vinelandii* can grow and produce the alginate in 15% (vol/vol) aqueous suspensions of alumina (60 g of particles per 100 ml of suspension). Furthermore, whereas bacterial cells do not adhere strongly to the particles and can be easily removed by washing, the alginate they produce is strongly bound to the particles and cannot be removed by repeated washings. Moreover, only a small number of nongrowing cells is required to coat a large number of particles. While it was not the intent of this study to design a specific procedure for a commercial in situ process for coating advanced ceramic materials, the results of this study should be useful for anyone who wishes to develop such a process using the alginate-producing bacterium *A. vinelandii*.

In addition, the results of this study suggest that commercial pottery manufacture with natural clays could benefit from a fuller understanding of the microbiology of the aging process. Perhaps the use of appropriate bacterial inocula, carbon sources, and other nutrients could improve polymer production, dramatically hasten the seasoning process, and make it more reproducible and efficient, like commercially controlled alcoholic and lactic fermentations.

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