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NITRIFICATION AND AUTOTROPHIC DENITRIFICATION IN CALCIUM ALGINATE BEADS

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ABSTRACT

Immobilization of nitrifiers and autotrophic denitrifiers (*Thiobacillus denitrificans*) within calcium alginate gel was demonstrated. Calcium carbonate reagent was immobilized along with bacteria as the stabilizing agent. Protons released as a result of microbial respiration reacted with calcium carbonate producing calcium ions which internally stabilized the calcium alginate gel. The microbially active gel beads were mechanically stable and active for three months in a continuous flow system without addition of calcium.

KEYWORDS

Immobilization, calcium alginate gels, nitrification, denitrification, gel stabilization.

INTRODUCTION

Microorganisms immobilized on substrata have significant advantages over traditional planktonic forms in wastewater treatment reactors. Immobilized cell reactors permit greater liquid throughputs without the potential for cell washout. In addition, immobilized cell reactors sometimes present less cell separation problems as compared to slurry reactors.

Biofilms

Biofilm or fixed film reactors are the most common form of immobilized cell reactors in wastewater treatment. Trickling filters, rotating biological contactors, packed towers, and fluidized beds are receiving increasing attention for carbonaceous removal as well as nitrification and denitrification. These fixed film reactors are characterized by high specific surface areas, permit high liquid throughputs, and are relatively easy to operate. The biofilm is generally adsorbed, rather than entrapped, to the substratum.

Entrapped Cells

Cells entrapped in porous substrata or in polymer gels are another example of immobilized cells. This paper focuses on microbial cells entrapped in calcium alginate beads. The beads can be used in a packed bed reactor, a fluidized bed reactor, or in a continuous stirred tank reactor. The gel bead reactors have the same advantages as the biofilm reactors plus some additional benefits. For example, nutrients and buffering agents can be immobilized with cells. Microbial succession, which causes problems in nitrification biofilm reactors does not occur in alginate bead reactors. In biofilm reactors, erosion or sloughing maintains a significant cell concentration in the liquid phase. Since cell detachment from gel beads is insignificant, even potable water treatment applications are more plausible. The stated advantages of entrapped cells provided the impetus for this study.

Immobilization in Polymer Gels

Immobilization of living microorganisms like bacteria, fungi, yeast, and algae within natural gels has received considerable attention in the literature recently. Two of the natural polymers, calcium alginate and κ-carrageenan, offer promising advantages in application to biological waste water treatment. Calcium alginate gels are formed by crosslinking alginate monomer ions with calcium ions while carrageenan is crosslinked with potassium ions to form κ-carrageenan gels. Immobilization procedures are similar in both gels. Monomers are dissolved in water free of calcium or potassium, respectively. The monomer solution is mixed with the suspension of microorganisms and the mixture is exposed to the crosslinking agent (calcium or potassium) to form the gel with entrapped living cells.

The calcium alginate and the κ-carrageenan gels both have a major problem; after a few days in a continuous flow reactor, the beads lose stability due to loss of calcium or potassium from the gel to the liquid phase. To prevent instability, the calcium or potassium ions concentration in the feed solution is artificially increased to decrease or invert the ion concentration gradient between beads and bulk liquid medium. Chotani and Constantinides (1983) used a feed concentration of 1.5 g l^{-1} $CaCl_2 \cdot 2H_2O$, when they immobilized yeast in calcium alginate gel to produce ethanol. Karkare *et al.* (1985) used 10 g l^{-1} KCl in the feed solution for yeasts immobilized in κ-carrageenan gel.

To utilize microorganisms immobilized in natural polymers for biological water and waste water treatment, a method for polymer stabilization, other than artificially increasing the concentration of selected ions in the feed solution, must be developed. The goal of the presented work was to develop such a method for bacteria immobilized within calcium alginate gel. The idea for this method originated from the work with nitrifiers immobilized on marble surfaces (Kowalski and Lewandowski, 1982; Lewandowski, 1985). In this work, nitrification in Packed Bed Reactors and Activated Sludge Reactors using microorganisms immobilized on marble surfaces was possible because of the neutralization reaction between protons released during nitrification and calcium released from the marble. Immobilization of calcium carbonate along with the nitrifiers within calcium alginate gels should similarly serve as an internal source of calcium ions to stabilize calcium alginate gels. Generally, any kind of microorganisms producing protons in their respiratory processes, immobilized along with calcium carbonate in calcium alginate gel, should stabilize the gel by releasing calcium ions.

To demonstrate this principle two experiments were conducted: (1) immobilization of nitrifiers, and (2) immobilization of autotrophic denitrifiers, *Thiobacillus denitrificans*, within calcium alginate along with calcium carbonate reagent. The effectiveness of the proposed method was evaluated based on the stability of calcium alginate beads containing immobilized microorganisms in continuous flow and batch reactors.

MATERIALS AND METHODS

Cultivation of Microorganisms for Immobilization

Nitrifiers. Nitrifiers were cultivated in a continuous flow reactor, 3 ℓ volume, containing suspended calcium carbonate (30 g l^{-1} $CaCO_3$). The continuous flow reactor contained an internal settling tank to avoid washout of suspension. The nitrifier inoculum was activated sludge from a municipal waste water treatment plant. Aeration was provided by compressed air. Suspended calcium carbonate served as the solid support for the nitrifiers and neutralized protons released during the nitrification process to buffer the system. Feed solution contained 50 mg l^{-1} $NH_4 \cdot N$ (as NH_4Cl) in tap water. Liquid detention time was 1 day. Nitrification in the reactor proceeded according to the following stoichiometric equations:

$$NH_4^+ + 1.5O_2 \xrightarrow{\text{Nitrosomonas}} NO_2^- + 2H^+ + H_2O \quad (1)$$

$$NO_2^- + 0.5O_2 \xrightarrow{\text{Nitrobacter}} NO_3^- \quad (2)$$

Protons released during the first stage of the nitrification process react with calcium carbonate:

$$CaCO_3 + 2H^+ \longrightarrow H_2CO_3 + Ca^{2+} \quad (3)$$

The nitrification process was monitored by analyzing $NH_4 \cdot N$ and calcium concentrations in the influent and ammonia, nitrite, nitrate and calcium concentrations in the effluent. According to the stoichiometry, every mg $N\text{-}NH_4$ oxidized results in the release of 7.14 mg calcium carbonate.

Denitrifiers

Autotrophic denitrifiers (*Thiobacillus denitrificans*) were cultivated in a continuous flow reactor (5 ℓ volume) containing a suspension of sulphur particles and calcium carbonate. The continuous flow reactor was supplied with an internal settling tank to avoid washout of the slurry. The reactor and the feed solution were continuously purged with nitrogen (N_2) to maintain anoxic conditions. Mixing was supplied with a magnetic stirrer and a mechanical stirrer. Sulphur served

as solid support and as the electron donor for the microorganisms. Calcium carbonate served as a neutralizing agent for protons released in microbial respiration. Sulfur concentration was maintained at 30 g l^{-1} and calcium carbonate at 30 g l^{-1}. The denitrifier inoculum was activated sludge from a municipal wastewater plant. The feed solution contained 50 mg l^{-1} $N\text{-}NO_3$ (as KNO_3) and 5 mg l^{-1} $NH_4\text{-}N$ (as NH_4Cl) in tap water. Liquid detention time was 1 day. The denitrification process proceeded as follows (Batchelor and Lawrence, 1978):

$$0.2NO_3^- + 0.167S + 0.0667H_2O \xrightarrow[\textit{denitrificans}]{\textit{Thiobacillus}} 0.1N_2 + 0.167SO_4^{2-} + 0.133H^+ \qquad (4)$$

Protons released in the process reacted with suspended calcium carbonate according to Eq. 3. The denitrification process was monitored by analysis of nitrate nitrogen, calcium and sulphate concentrations in the influent and nitrate, nitrite, calcium and sulphate in the effluent. According to stoichiometric calculations, denitrification of 1 mg $NO_3\text{-}N$ results in release of 5.73 mg SO_4^{2-} and 1.38 mg $CaCO_3$.

Immobilization Procedure

Suspensions of cells and particles from the reactors were settled for 2 h. The suspension volumes used were determined to provide 50 g l^{-1} as suspended matter in the beads. The settled sulphur and/or calcium carbonate particles with the active biomass were mixed with 2% sodium alginate (Protanal LF 20/60 14303181—product of Protan A/S, 3000 Drammen, Norway) dissolved in double distilled water. Then, 5 mg l^{-1} $NH_4\text{-}N$ (as NH_4Cl) and 5 mg l^{-1} $NO_3\text{-}N$ (as KNO_3) were added to the nitrifying and the denitrifying solutions, respectively, for the microorganisms during the immobilization procedure. The mixtures were continuously mixed by magnetic stirrers. The mixtures were pumped through pipets to two separate, continuously mixed, tanks containing 2% solutions of $CaCl_2 \cdot 2H_2O$. Discrete droplets, formed at the pipet tips, dripped into the $CaCl_2$ $2H_2O$ solution, and formed 4 mm diameter beads upon contact. The beads remained in the $CaCl_2$ solutions for 24 hours to allow for complete crosslinking.

Reactor Operation

The beads were transferred to 2 ℓ batch reactors containing solutions of 50 mg l^{-1} $NH_4\text{-}N$ for nitrifiers or 50 mg l^{-1} $NO_3\text{-}N$ and 5 mg l^{-1} $NH_4\text{-}N$ for denitrifiers. The solutions were prepared in tap water. The immobilized nitrifiers were aerated with compressed air. The immobilized denitrifiers were purged with nitrogen to maintain anoxic conditions.

After 24 hours batch operation, continuous flow at a detention time of 24 h was begun. The feed solution compositions supplied to the reactors were 50 mg l^{-1} $NH_4\text{-}N$ for nitrifiers and 50 mg^{-1} $NO_3\text{-}N$ along with 5 mg l^{-1} $NH_4\text{-}N$ for denitrifiers. The solutions were prepared using tap water. Reactor mixing was supplied with compressed air for nitrification or nitrogen in the case of denitrification. The processes were monitored by analyzing the same components as in the case of the respective slurry reactors. After two weeks of continuous flow operation, the kinetic parameters were determined.

Kinetic parameters for the processes were determined in *batch mode* at 22°C. The flow to the reactors was stopped, the liquid was decanted, and the reactors were filled with new solutions containing the desired concentrations of substrate (electron acceptor and electron donor). Samples from the reactors liquid were taken at equal time intervals and analyzed for substrate, intermediates, and products concentrations. The data were graphically analyzed to determine the appropriate reaction coefficients.

The reactors containing nitrifying and denitrifying beads were operated with continuous flow (liquid detention time 24 h) for three months to determine long term bead stability and activity.

Analytical Methods

Concentrations of all components were determined according to Standard Methods (1985).

RESULTS AND DISCUSSION

The bead stabilization principle is presented in Figure 1. According to the model, the reaction between the hydrogen ions released as a metabolic product and the calcium carbonate should produce calcium ions to stabilize the calcium alginate gel. The results confirmed the expectations. The beads containing calcium carbonate were stable throughout the testing period (3 months). Calcium alginate beads without calcium carbonate, operated under the same operating conditions, lost stability within 2-4 days.

The reactions occurring within the nitrifying and denitrifying beads are presented in Figures 2 and 3, respectively. The interactions between the substrates (electron acceptor and electron donor), products and bead components during the investigated processes are described in these figures. The analysis of the reactor operation supports the model proposed

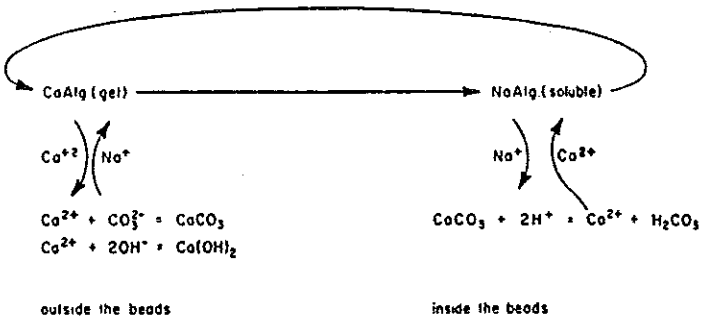

Fig. 1. The relationship of external and internal Ca^{++} to alginate bead stability.

in these figures. For example, in the reactor effluent, the products of both microbial reactions and neutralization reactions were found in concentrations close to the stoichiometric predictions. In both reactors, calcium ion concentration in the effluent increased, demonstrating that $CaCO_3$ was dissolved within the beads. In the denitrification reactor effluent, sulfate concentration increased simultaneously with calcium ion concentration. Bead stability, along with chemical analysis, confirms the usefulness of the method for bead stabilization.

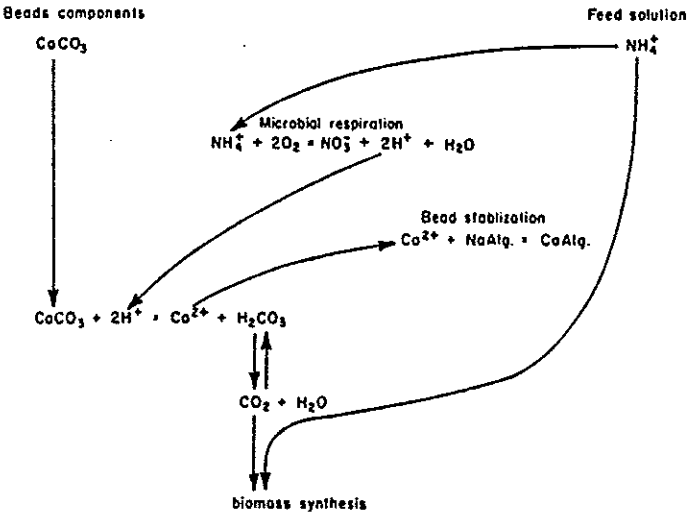

Fig. 2. Processes occurring in the nitrifying Ca-alginate bead reactor.

Figure 4 presents the results of the ammonia nitrogen oxidation rate by immobilized nitrifiers. The measurements were done in the batch reactor containing 0.5 l of calcium alginate beads and 1.5 l of solution of NH_4-N in tap water. The results reflect the activity of Nitrosomonas immobilized within the calcium alginate beads. The calculated reaction rate was 3.1 mg NH_4-N l^{-1} h^{-1}.

Figure 5 presents the results of the nitrite nitrogen, NO_2-N, oxidation rate by immobilized nitrifiers. The conditions were as described above except nitrite nitrogen solution replaced nitrate. Nitrite-nitrogen oxidation reflects Nitrobacter activity within the calcium alginate beads. The calculated reaction rate was 2.9 mg NO_2-Nl^{-1} h^{-1}.

Calculations of denitrification reaction rates for immobilized denitrifiers revealed some specific problems. Denitrification is a sequence of reactions. Nitrate is reduced to nitrite and subsequently nitrite is reduced to nitrogen gas. Using autotrophic denitrification with elemental sulfur as an electron donor within the beads, only *Thiobacillus denitrificans* is able to denitrify. When nitrate is the electron acceptor at the beginning of the experiment, all of the biomass is involved in nitrate reduction. As a result, nitrite concentration increases and part of the biomass switches to nitrite as the electron acceptor. This results in decreased nitrate conversion rate. After a certain time during which nitrite concentration increases, the biomass involved in nitrite reduction increases as well and a balance between the biomass involved

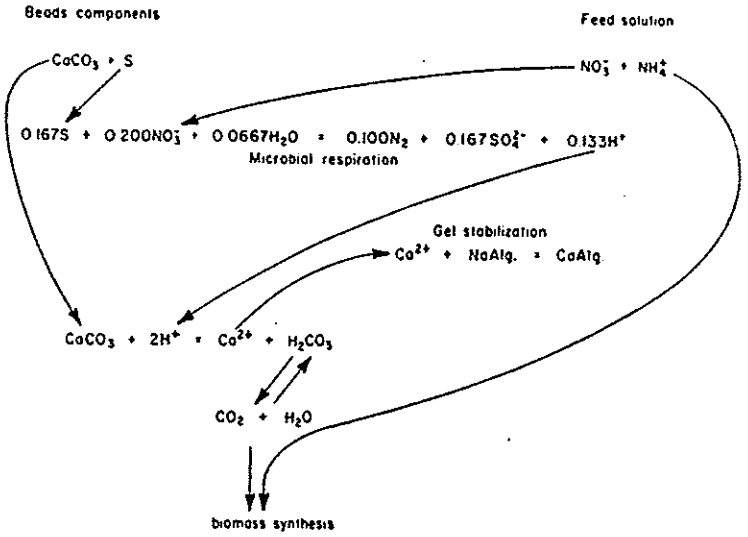

Fig. 3. Processes occurring in the denitrifying Ca-alginate bead reactor.

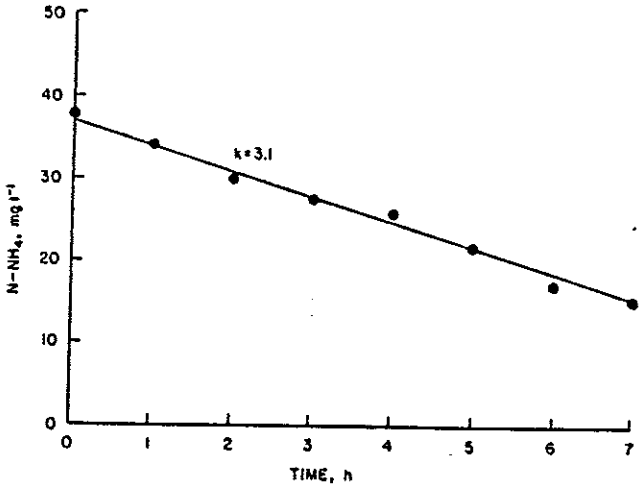

Fig. 4. Removal of NH_4-N in a batch nitrifying Ca-alginate bead reactor.

in nitrite and nitrate reduction was observed. Equation 4 is insufficient to describe the sequential reaction phenomena, but it can be described by the following stoichiometric model:

$$3NO_3^- + S + H_2O \xrightarrow{x_1, k_1} 2NO_2^- + SO_4^{2-} + 2H^+ \quad (5)$$

and

$$2NO_2^- + S \xrightarrow{x_2, k_2} N_2 + SO_4^{2-} \quad (6)$$

where: x_1 - biomass concentration involved in reduction of nitrate to nitrite
x_2 - biomass concentration involved in reduction of nitrite to nitrogen gas
k_1 and k_2 - reactions rates for reduction of nitrate to nitrite and nitrite to nitrogen gas, respectively.

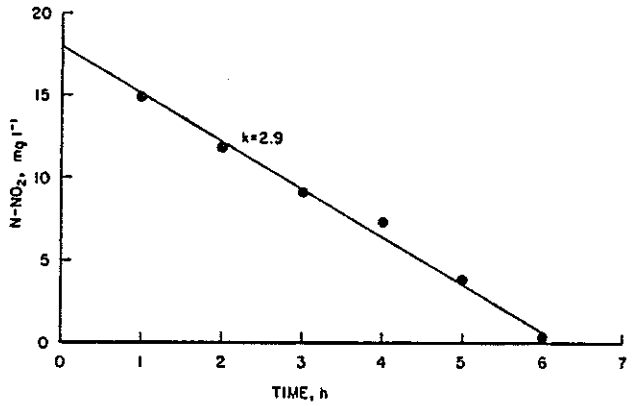

Fig. 5. Removal of NO_2-N in a batch nitrifying Ca-alginate bead reactor.

Results obtained during measurements of denitrification kinetics are presented in Figure 6. The rate of nitrate removal at the very beginning of the process is 4.6 mg $NO_3 \cdot N \cdot l^{-1} \cdot h^{-1}$ and decreases to the 2.4 mg $NO_3 \cdot N \cdot l^{-1} \cdot h^{-1}$ by the end of the experiment. Denitrification consists of two sequential reactions, conversion of nitrate to nitrite and conversion of nitrite to nitrogen gas. The first reaction does not cause changes in total nitrogen concentration in the system. The only reaction which reflects nitrogen removal from the system is conversion of nitrite to nitrogen gas. Thus, changes in total nitrogen concentration in Figure 6 reflect the reaction rate of nitrite nitrogen to nitrogen gas. According to the data, the reaction rate was 1.6 mg $NO_2 \cdot N\ l^{-1}\ h^{-1}$ initially and later increased to 4.8 mg $NO_2 \cdot N \cdot l^{-1} \cdot h^{-1}$.

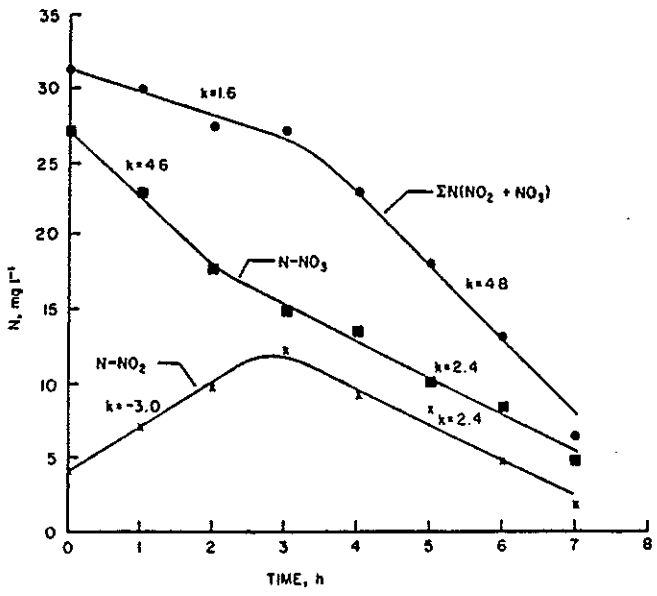

Fig. 6. Nitrogen dynamics in a batch, denitrifying Ca-alginate bead reactor.

A photo of two nitrifying beads are presented in Figure 7. The uniform distribution of calcium carbonate in these beads is illustrated in Figure 8. This is a photo micrograph of a 5 μm thick bead cross-section, using polarized light to enhance crystals in the sample.

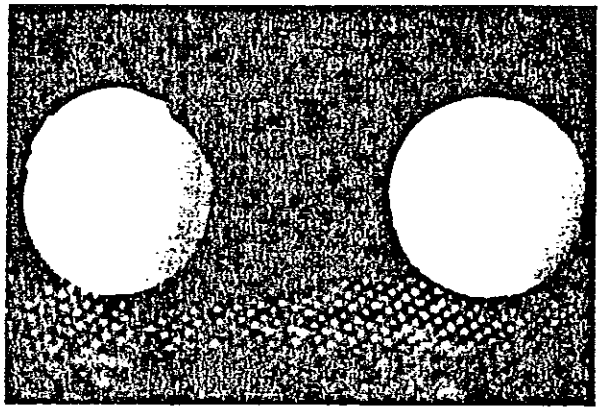

Fig. 7. Photo of two nitrifying beads. Bead diameter is 4 mm.

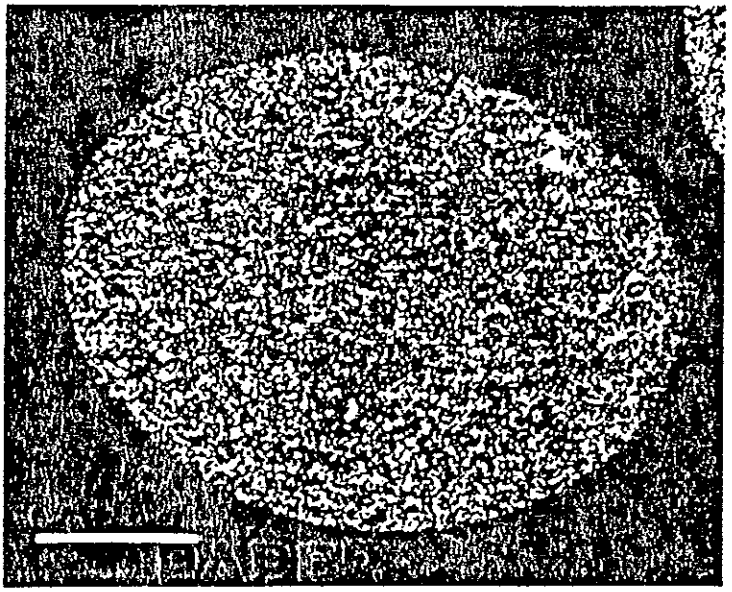

Fig. 8. Polarized light photo micrograph of a 5 μm thick nitrifying bead slice. Bar represents 1 mm.

The nitrifying and denitrifying processes were carried out by cells entrapped within the beads, rather than by cells growing on the bead surfaces. A scanning electron micrograph of a denitrifying bead, residing in the continuous flow reactor for three months, has been included (Figure 9) to illustrate the lack of significant biomass on the bead surface.

Fig. 9. Scanning electron micrograph of a denitrifying bead surface. Bar represents 2 μm.

CONCLUSION

Biomass, sulfur and/or calcium carbonate content in calcium alginate beads may be regulated to optimize nitrification and denitrification by the immobilized cells. The optimum composition depends on the specific application. Kinetic data for nitrification and autotrophic denitrification by microorganisms immobilized in calcium alginate cells were obtained.

A method was developed to improve the stability of calcium alginate gels. Calcium alginate can be stabilized by immobilization of calcium carbonate along with the microorganisms when protons are released as a metabolic product.

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