Nitrate removal from water by immobilized bacteria

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ABSTRACT

Nitrate may reach surface and ground waters as a consequence of agricultural activity and discharge of domestic and industrial waste. Among the various methods used for nitrate removal, denitrification, a process in which nitrate is biologically reduced to elemental nitrogen, is relatively reliable and inexpensive as compared to other physical and chemical nitrate removal processes. Denitrification is generally conducted with biofilters in which bacteria are either immobilized to the surface of insoluble carriers or are entrapped within an immobilization matrix. We examined the use of non-toxic and biodegradable natural hydrocolloids for entrapment of denitrifying bacteria. Gel beads containing starch and alginate were used for this purpose. Three types of gel beads were examined: (1) wet gel beads; (2) porous wet gel beads; and (3) freeze-dried gel beads. With respect to nitrate removal, wet and dried gel beads showed similar removal capacities. Porous beads demonstrated an advantage over regular gel beads only during the first of the approximately 3-month incubation period. The viability of the immobilized bacteria was only slightly affected during prolonged refrigerated storage of the beads. Compared to freeze-dried beads, production costs of wet beads are significantly reduced, so it is anticipated that such carriers will eventually lead to a method that can be applied on an industrial scale.

Key words | aquariums, denitrification, hydrocolloids, immobilization

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INTRODUCTION

Nitrate is widespread in the aquatic environment. In recent years, elevated levels of nitrate in surface and ground water are increasingly experienced due to agricultural run-off and domestic waste discharge (WHO 2011; Wongsanit *et al.* 2015). High nitrate levels in drinking water have been associated with methemoglobinemia in infants (Greer & Shannon 2005; Richard *et al.* 2014) while long-term human exposure to relatively low levels of nitrate has been linked to alterations in thyroid gland function and an increased risk of gastric cancer (Kelley & Duggan 2003; Ward *et al.* 2010). In the aquatic environment, high nitrate levels may cause stress and mortality of fish and invertebrates as a result of methemoglobinemia (Lasier *et al.* 2016; Baker *et al.* 2017).

More stringent environmental restrictions with respect to nitrate discharge are driving the search for improved nitrate removal technologies. Today, most of these technologies comprise physical/chemical methods such as ion exchange, reverse osmosis, electrodialysis and biological methods. The latter methods are based on nitrate reduction by denitrifiers under anoxic conditions and generally employ immobilized biosystems, i.e. physical or physico-chemical bonding of denitrifiers to the surface of insoluble carriers such as sand, plastic or ceramic particles (van Rijn *et al.* 2006). Adsorbed microorganisms, immobilized by weak hydrogen bonds or by electrostatic interactions with the carrier (fixed-film processes), are easily washed from

the support carriers into the treated water, resulting in microbial pollution. A more effective immobilization of microorganisms can be accomplished by entrapping microorganisms in a matrix. Whereas in the bioindustry, entrapment of microorganisms has been practised since the early 1960s, in the waste water treatment industry this technique is relatively new. Here, mainly synthetic materials are used for entrapment. These recalcitrant agents can themselves be considered pollutants when released into the environment. Alternatively, natural hydrocolloids (water-soluble polymers) can be used to entrap the microorganisms; however, very little work has been conducted in this field.

In previous work, we examined the use of some non-toxic, FDA-approved, biodegradable natural hydrocolloids for entrapment of denitrifying bacteria (Tal *et al.* 1997, 1999, 2003). We found that the biological and mechanical properties of the entrapment complex (bead) were improved by including starch as an internal carbon source and matrix enhancer. In addition, we showed that freeze-drying ensures a long shelf life for the beads without mitigating their biological activity. The latter study provided a proof of concept for the technology but more practical issues, such as optimizing the bead performance and reducing their production cost, were not addressed. In the present study, three differently prepared bead types were examined for nitrate-removal capacity: (1) freeze-dried gel beads, (2) regular gel beads, and (3) porous gel beads with entrapped air pockets (aerated beads). The mechanical properties of all three bead types were compared and their nitrate-removal capacity was examined in an aquarium set-up over a period of 100 days. In addition, the effect of storing the un-dried beads prior to their use was studied.

METHODS

Culture medium and bacterial growth

For entrapment, *Pseudomonas* sp. strain JR12 (Tal *et al.* 2003) was used. The bacteria were grown in liquid Luria broth (LB) or on solid nutrient (1.5% w/v agar) agar (NA; Formedium Ltd, Hunstanton, UK). Cells were grown in LB for 24 h with aeration at 30 °C to a final concentration of 5×10^8 to 1×10^9 cell/mL.

Preparation of the immobilization complex

Alginate with a molecular mass of 60–70 kDa, containing 61% mannuronic acid and 39% guluronic acid (Sigma, LV, St Louis, MO), was dissolved in distilled water (2% w/w). A potato-starch suspension (Sigma) was added to the alginate solution at a final concentration of 20% (w/w). Strain JR12 ($\sim 10^{10}$ cell/mL) was then added at a 1:9 volumetric ratio to the alginate-starch solution. For aerated gel beads, a solution containing alginate, starch and bacteria was aerated for 1 h by means of compressed air from a membrane pump (model M2K3, Schego, Germany) at room temperature. Regular and aerated beads were obtained by dripping the non-aerated and aerated solutions into a stirred 1% (w/w) solution of calcium chloride (the volumetric ratio between the alginate mixture and the calcium chloride solution was 1:5). This procedure resulted in a spontaneous cross-linking reaction that produced spherical beads with an average diameter of about 4 mm. Each bead contained around 10^8 cells. Freeze-dried beads were prepared by freezing regular (non-aerated) gel beads for 1 h at −80 °C followed by freeze-drying at −50 °C at a pressure of 1.1 Pa (Martin Christ model ALFA I-5; Osterode am Harz, Germany).

Bacterial enumeration

Viable cells were counted by immersing 10 beads in 10 mL of 2% (w/w) sterile sodium citrate solution (three repetitions). The solution was vigorously shaken (400 rpm) for about 20 min until all beads were totally dissolved. The suspension was then immediately diluted, plated on NA and incubated at 30 °C. Bacterial colonies developing on the solid medium were counted after 24 h of incubation. Results are presented as colony-forming units per bead.

Experimental set-up

The denitrifying filter consisted of a plastic canister (height 33 cm, diameter 6 cm, working volume 950 mL) filled with ~2,000 beads and containing plastic spacers (K1, Kaldnes Ltd, Norway) to secure an even distribution of the beads.

Canisters, each containing a different bead type, were hooked up to 100-L freshwater aquariums equipped with compartments containing plastic substrates for enhancement of nitrifying activity (Figure 1). Canisters containing beads without immobilized bacteria or only plastic spacers were used in the control runs. Two aquariums were used for each treatment. The denitrifying filters, fed with effluent water from the nitrifying compartments, were operated at a flow rate of 1.5 L/h. Each freshwater aquarium was stocked with three ornamental koi carp (*Cyprinus carpio*) with a total biomass of 250 g. Fish in each aquarium were fed 5 g of 35% protein feed daily. Temperature and pH were periodically recorded and duplicate filtered water samples from the inlet and outlet of the denitrifying canisters were periodically analysed for nitrate according to Holm *et al.* (1997),

nitrite according to Strickland & Parsons (1968), and ammonia according to Scheiner (1976).

Electron micrographs and image analysis

Bead structure was examined by scanning electron microscopy (SEM). Gel beads were examined with a Quanta 200 Environmental Scanning Electron Microscope (FEI, USA). Freeze-dried beads were examined in a JEOL (model JSM-356, Japan) scanning electron microscope after sputtering with gold for 195 s at 5 °C in an argon atmosphere, using the SEM Coating System (Polaron 515, UK). SEM micrographs were obtained by cutting through the alginate beads with a double-edged razor blade to expose the internal surface features.
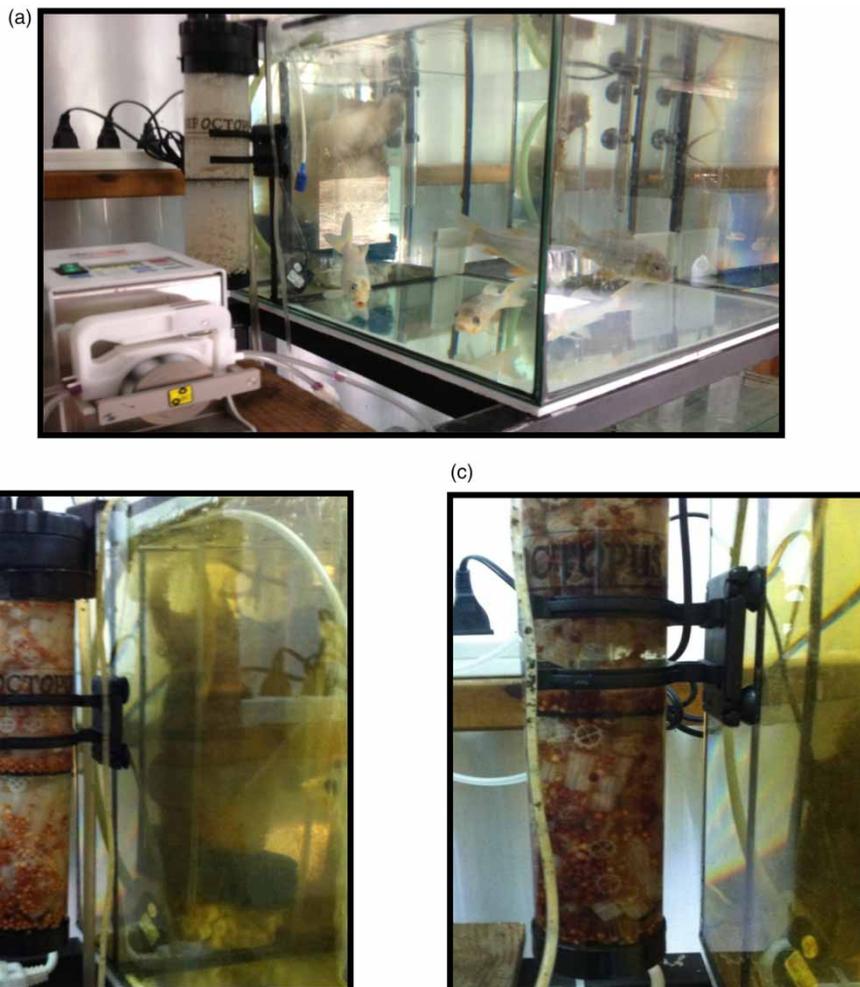

Figure 1 | Denitrification filters containing immobilized *Pseudomonas* sp. JR12. (a) First day of the experiment. (b) After 2 weeks. (c) After 8 weeks.

Physical properties of beads

Bead mechanical properties were determined by uniaxial compression between plates to ~80% deformation with an Instron universal testing machine (UTM, model 5544, Instron Co., Canton, MA) using an Instron card for the 5544 series. 'Merlin' software (Instron) was used for data acquisition and conversion of the UTM's continuous voltage versus time output into digitized force–deformation, force–time, stress–strain, or stress–time values with any desired definition of stress and strain. Six beads of each type and from at least two batches were compressed in every test.

For the different beads, the force versus time data were converted to a 'pseudo-stress' versus 'engineering strain' relationship using the following substitutions:

$$\sigma = \frac{F}{A_0} \tag{1}$$

$$\varepsilon_E = \frac{\Delta H}{H_0} \tag{2}$$

where σ is the 'pseudo-stress', ε_E, the dimensionless 'engineering strain', F, the force needed to compress the bead at a given time, H_0, the original diameter of the bead, ΔH, the total deformation and A_0, the cross-sectional area of the original bead. The porosity values of the freeze-dried beads was estimated using the relationship:

$$P = 1 - \left(\frac{\rho_\beta}{\rho_s}\right) \tag{3}$$

where ρ_β/ρ_s is the bulk density divided by the solid density. This ratio was calculated using the equation:

$$\varepsilon_D = 1 - 1.4\left(\frac{\rho_\beta}{\rho_s}\right) \tag{4}$$

where ε_D is the densification strain value derived from the stress–strain relationship of the compressed carriers (Ashby & Gibson 1988).

Colour determination

Layers of beads before and after application were analysed for colour with a Minolta Chroma Meter CR-100 (Minolta Camera Co. Ltd, Osaka, Japan). Readings are reported using the L^*, a^*, b^* system, where L^* corresponds to lightness, a^* to the red/green scale and b^* to the yellow/blue scale.

RESULTS AND DISCUSSION

Bead strength and colour

The experimental set-up of aquariums and the canister with denitrifying beads is presented in Figure 1. The porosity of the freeze-dried beads was determined from the densification strain value (ε_D), which was derived from the stress–strain relationship of the compressed carriers (~0.88, see Figure 2) and the ratio between the bulk and solid density (Equation (4)). Inserting the calculated ρ_β/ρ_s (0.086) into Equation (3) resulted in a porosity value for the freeze-dried carriers of around 91%, similar to values reported for other freeze-dried gel products (Ashby & Gibson 1988; Nussinovitch 1997, 2003; Zohar-Perez *et al.* 2005).

Regular gel beads were stronger than the modified aerated beads, probably due to the latter's entrapment of air and consequent disruption of the gel matrix. For example, at 45% strain, the pseudo stress values were 0.11 and 0.07 MPa and at 55% strain, they were 0.19 and 0.15 MPa for regular and modified beads, respectively (Figure 3). Note that during incubation of the aerated beads, we observed coalescence of some air bubbles within the gel bead while a portion of those bubbles left the gel and induced mini-cracks within its matrix. A similar observation was reported for agar, alginate and carrageenan gels by
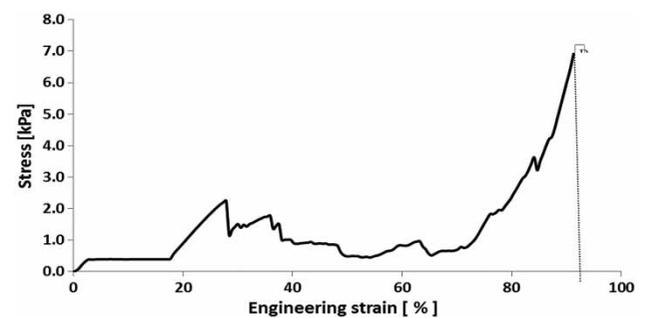

Figure 2 | Stress–strain relationship for compressed freeze-dried alginate beads entrapping *Pseudomonas* sp. JR12.
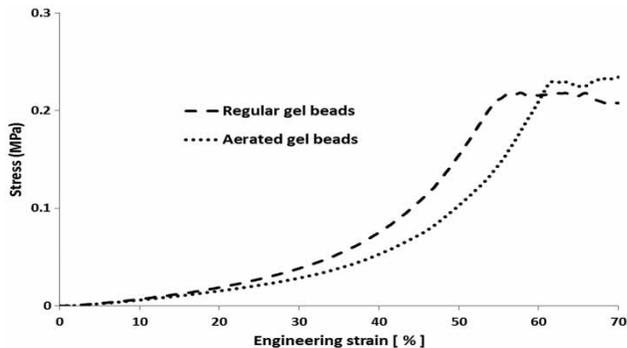
Figure 3 | Stress–strain relationship for compressed regular and aerated alginate gel beads entrapping *Pseudomonas* sp. JR12.

Figure 4 | Survival of *Pseudomonas* sp. JR12 immobilized in regular alginate gel beads during storage at 4 and −18 °C.

Nussinovitch *et al.* (1992). Regular and modified (aerated) gel beads reached identical stress values (∼0.2 MPa) at 55 and 60% strain values, respectively. In other words, to reach similar stress values, more strain had to be applied to the aerated beads. Consequently, aerated beads were less resistant to compression due to their higher porosity. Stiffer carriers are expected to be subject to slower disintegration. However, when applied in the aquarium set-up, the aerated beads were weaker than the regular beads, but were in fact less influenced by the formation and escape of nitrogen gas flow from the carriers. Probably this was due to gas paths created within the beads upon aeration as a result of escaping gas bubbles.

Although changes in the numbers of immobilized bacteria during the incubation period were not recorded, changes in bead colour during this period provided a strong indication of bacterial growth. Bead colour changed during the experimental period from a white to brown hue. Minolta chromo readings indicated a loss of bead brightness from initial L^* values of 59 ± 3 to 44 ± 3 after 2 weeks and 32 ± 2 after 4 weeks. Furthermore, changes in the red/green (a^*) and yellow/blue (b^*) spectra of the beads were observed: from initial a^* values of -5 ± 2 to 14 ± 1 after 4 weeks of incubation and initial b^* values of 6 ± 4 to 17 ± 3 after 4 weeks of incubation.

Storage of beads

Storage of the regular beads for up to 100 days at 4 °C did not decrease the viability of the entrapped cells, whereas storage at −18 °C did result in loss of viability (Figure 4).
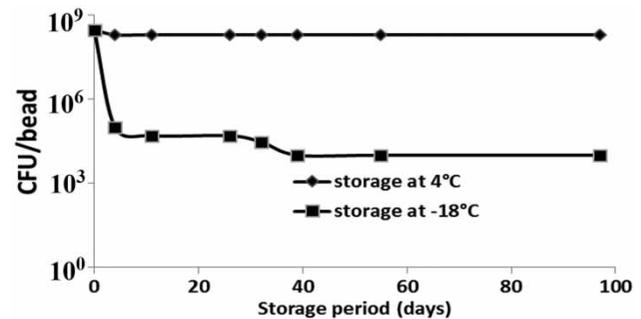
Regular and aerated beads were found to sustain viable cells for at least 200 days of storage at 4 °C (Figure 5). These results confirmed earlier studies (Trivedi & Pandey 2008; Schoebitz *et al.* 2013) in which it was found that storage of alginate beads at 4 °C preserved the viability of entrapped cells. The practical implication of this latter finding is that the more expensive freeze-drying process previously used for bead preservation (Tal *et al.* 1997) is not strictly necessary for the production of beads with a long shelf life.

Nitrate removal by beads

Nitrate-removal capacity of regular, aerated and freeze-dried gel beads was compared in two different runs. Canisters containing beads without immobilized bacteria or only plastic spacers were used in the control runs. During the experimental period, temperature in the various aquariums fluctuated between 28 and 30 °C and the pH values between
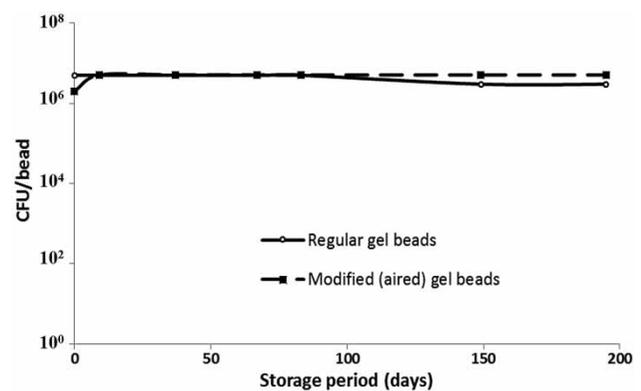

Figure 5 | Survival of *Pseudomonas* sp. JR12 immobilized in regular and aerated alginate gel beads during storage at 4 °C.

7.4 and 8.5. Nitrite and ammonia concentrations in both runs were below 0.1 and 0.5 mg/L, respectively, in all treatments (not shown).

In the first run, lasting 100 days, the effects of regular and freeze-dried beads on ambient nitrate concentrations in the aquariums were examined. Nitrate removal, as inferred from ambient nitrate concentrations in the aquarium water, was similar in both treatments (Figure 6). Therefore, under these specific conditions, there was no advantage in using freeze-dried over regular wet beads for nitrate removal. Knowledge of the specific nitrate removal of the beads at different incubation times was required to more accurately discern possible differences in nitrate-removal capacity between the two bead types.

The same experimental set-up was used in a second run (80 days), this time including aerated wet beads in addition to the regular wet beads and freeze-dried beads. It was found (Figure 7) that nitrate concentrations were considerably
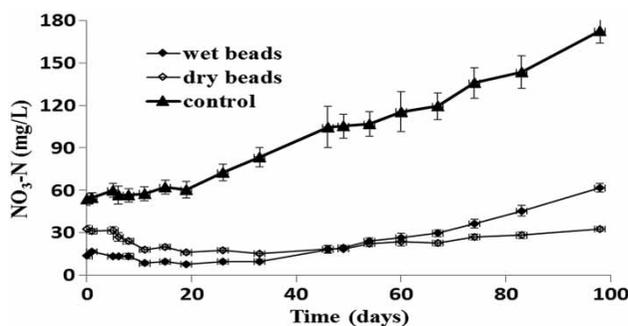

Figure 6 | First run: Nitrate removal from aquariums by immobilized *Pseudomonas* sp. JR12.
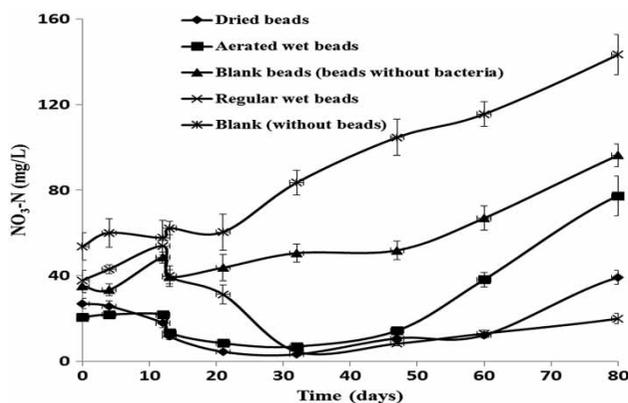

Figure 7 | Second run: Nitrate removal from aquariums by immobilized *Pseudomonas* sp. JR12.

lower in aquariums hooked up to canisters containing any of the bead types than those in the control aquariums. On day 80, aquariums equipped with regular beads and freeze-dried beads revealed the lowest ambient nitrate concentrations. The aquarium hooked up to a canister with control beads (i.e. without bacteria) exhibited lower ambient nitrate concentrations than the control aquarium (without beads), probably due to the development of a native denitrifying consortium on and within the control beads.

Ambient nitrate concentrations in aquariums equipped with aerated beads were relatively low up to day 50 of incubation. Thereafter, nitrate concentrations increased as compared to aquariums equipped with regular and freeze-dried beads. The higher porosity of aerated beads might explain their relatively efficient initial nitrate-removal capacity. Subsequent rupture of the gel matrix as a result of expanding air pockets, leading to starch leakage from the immobilization complex, might have caused their inferior nitrate-removal capacity as the experiment proceeded. A similar weakening of aerated beads was observed by Nussinovitch et al. (1992).

The inclusion of starch granules in the immobilized complex is clearly seen in the SEM micrographs (Figures 8–10), as are the entrapped bacteria attached to those starch granules (Figure 10). The inclusion of starch granules in the immobilized complex supported the entrapped bacteria with a carbon source and increased the solidity and stability of the carrier (as also reported by Tal et al. 1999). The bright-appearing areas in the SEM micrographs (Figures 8 and 9) are those where the alginate matrices entrap the starch granules and immobilized bacteria.

CONCLUSIONS

In the present study, nitrate removal from aquariums by means of gel beads was shown to be similar to that with freeze-dried beads. It was further demonstrated that the beads' activity, i.e. the viability of the entrapped bacteria, could be effectively preserved by placing the beads in refrigerated storage for an extended period. Based on these results, we conclude that gel beads containing immobilized
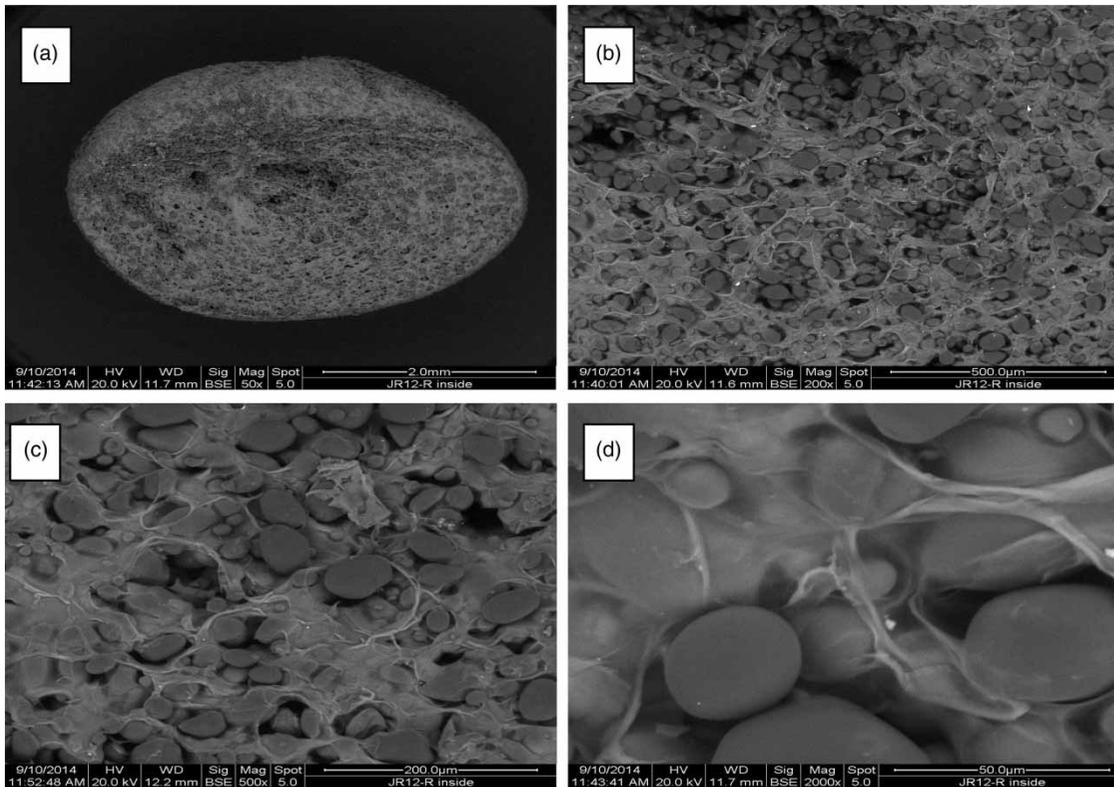
Figure 8 | Electron micrographs of halved regular gel beads entrapping *Pseudomonas* sp. JR12. Magnification: (a) ×50, (b) ×200, (c) ×500, (d) ×2,000.
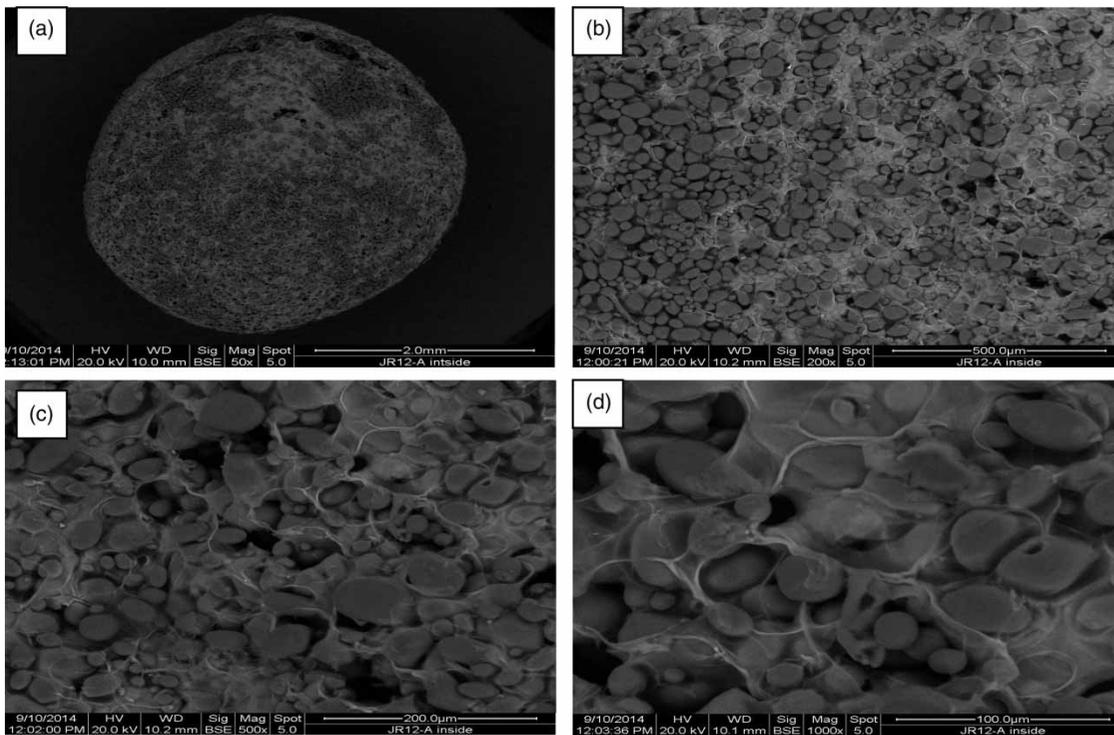

Figure 9 | Electron micrographs of halved aerated gel beads entrapping *Pseudomonas* sp. JR12. Magnification: (a) ×50, (b) ×200, (c) ×500, (d) ×1,000.

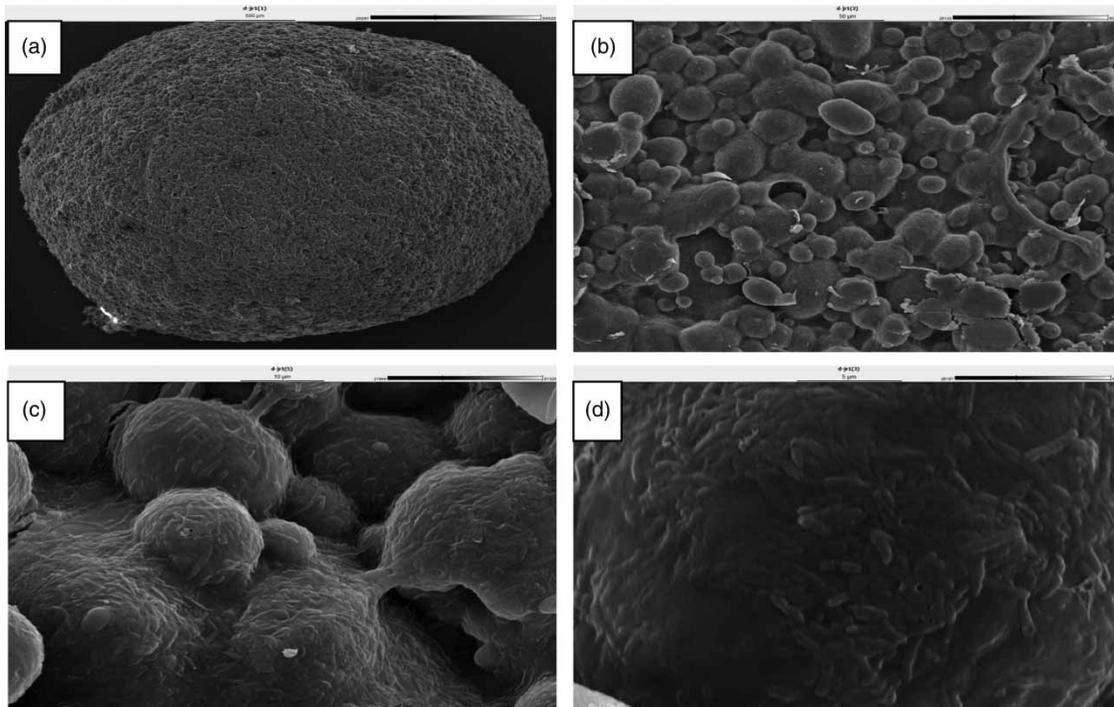**Figure 10** | Electron micrographs of outer surface of freeze-dried beads entrapping *Pseudomonas* sp. JR12. Magnification: (a) ×35, (b) ×350, (c) ×2,000, (d) ×5,000.

denitrifying bacteria and incorporating starch as a carbon source for these bacteria can be successfully applied for nitrate removal in nitrate-contaminated water such as aquariums. The efficient nitrate removal exhibited by wet gel beads, as well their relatively low production costs, warrant their further testing on a commercial/industrial scale.

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