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Full Length Research Paper

Application of Lentikats Biotechnology for removal of nitrates from ion-exchange brines: Implications for adaptation of encapsulated denitrifiers

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Denitrifying Bacterium *Paracoccus denitrificans* encapsulated in polyvinyl alcohol matrix (so called Lentikats Biocatalyst; LB) was applied for the removal of nitrates from simulated ion-exchange brines (12.14 g.L^{-1} Cl^{-}, 1.35 g.L^{-1} SO_4^{2-}, and 2.26 g.L^{-1} N-NO_3^{-}). The effect of brines dilution on the denitrification activity of fresh Lentikats Biocatalysts was tested simultaneously for four dilutions (100% = non-diluted, 80% = 4:1 brine:water, 60 and 40%) in four sequent repetitions per dilution without intermediate cultivation. In the first set, the denitrification of 100 and 80% brine was severely inhibited, while the denitrification activity in 60 and 40% brines was comparable to activity in non-inhibiting denitrification medium. The denitrification activity in 60 and 40% brines declined in later repetitions due to a lack of nutrients, while it increased in 80 and 100% brines before it declined as well. Such results are in contradiction to previous observations based on correlation analyses of data from longer experimental period. The detailed comparison implicates the adaptation of the encapsulated denitrification bacteria to higher salinity and emphasizes its importance for achievement of high denitrification activities of Lentikats Biocatalyst in industrial-scale applications even in non-diluted brines.

Key words: Denitrification, ion-exchange brines, Lentikats Biocatalyst, *Paracoccus denitrificans*, polyvinyl alcohol.

INTRODUCTION

Lentikats Biotechnology denotes a proprietary name for various applications of biological material encapsulated in polyvinyl alcohol (PVA) matrix (so called Lentikats Biocatalyst; LB) in accordance with the patented encapsulation method (Vorlop and Jekel, 1999; Jekel et al. 1998). Unlike other encapsulation techniques utilizing PVA matrix, this procedure is highly compatible and developed to industrial production scale, which enables biotechnological applications of encapsulated enzymes, living microbial cells or cellular extracts in numerous industrial fields such as food processing (Grosova et al., 2009; Rebros et al., 2007; Rosenberg et al., 2005), biotransformations (Bruss et al., 2002; Kubac et al., 2006; Vejvoda et al., 2006; Wilson et al., 2004), bioethanol production (Rebros et al., 2005a, b), or waste-water treatment (Bouskova et al., 2011, Sievers et al., 2002; Trogl et al., 2011a, b; Vackova et al., 2011). In the last field emphasis was given to nitrogen removal processes using encapsulated pure cultures of nitrification or denitrification bacteria. The latest research efforts are focused on the elimination of nitrogen from industrial waste-waters that are not easily addressed by existing technologies, for example toxic, nutrient-deprived, or of high-salinity (Bouskova et al., 2011; Trogl et al., 2011a,b).

Our previous studies (Bouskova et al., 2011; Trogl et al., 2011a) concerned the elimination of nitrates from brines (12.14 g.L^{-1} Cl^{-}, 1.35 g.L^{-1} SO_4^{2-}, up to 2.26 g.L^{-1} N-NO_3^{-}) originating from the regeneration of ion-exchange

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Abbreviations: LB, Lentikats Biocatalyst; **PVA,** polyvinyl alcohol; **MDM,** mineral denitrification medium.

columns. These are commonly used for the removal of nitrates from potable, basin, and similar types of waters, yet their regeneration, carried out using concentrated brines, results in the production of large quantities of high-salinity and high-nitrate effluents for disposal. The environmental impact of ion-exchange processes poses us with the challenge of developing a suitable regeneration technology. Biological denitrification of nitrates, a widely applied and efficient method in wastewater treatment, is very complicated in brines, due to synergistic negative effects of the high-salinity, high-nitrates, and the lack of nutrients important for the proliferation of denitrifying bacteria (Bae et al., 2004; McAdam and Judd, 2008; McAdam et al., 2010). However, encapsulation of denitrifiers into LB has previously consistently proven itself to be a beneficial step for the successful elimination of nitrates from such brines, both in batch (Trogl et al., 2011a) as well as in continuous operation (Bouskova et al., 2011). However, the denitrification activities of LB obtained in those studies varied in order of magnitude. Correlation analyses, based on the overall results of multiple batch experiments, revealed that the temperature and time elapsed from the last cultivation are the most significant factors affecting the denitrification activities of the LB. Substantial results variance however remained unexplained or hypothesized likely resulting from sideline of the initial and boundary problems. Among other factors, the adaptation of encapsulated denitrifying bacteria was hypothesized during long-term applications of LB on high-salinity and high-nitrates waters (Trogl et al., 2011a).

In this study, the course of initial batch denitrifications of simulated ion-exchange brine (12.14 $g.L^{-1}$ Cl^- + 1.35 $g.L^{-1}$ SO_4^{2-} + 2.26 $g.L^{-1}$ $N\text{-}NO_3^-$, denoted further as 100%-brines) and its dilutions (40, 60 and 80%-brines) was studied using fresh LB with encapsulated *Paracoccus denitrificans*. In order to minimize the influence of other factors (ambient temperature), denitrification tests of these four dilutions were carried out simultaneously.

MATERIALS AND METHODS

Encapsulation of *Paracoccus denitrificans* into LB was carried out by LentiKat's Inc using automatic industrial-scale manufacturing line according to the patented method of Vorlop and Jekel (1999). Prior to the experiments, the LB was cultivated under anoxic conditions (concentration of dissolved oxygen < 0.1 $mg.L^{-1}$) in mineral denitrification medium (MDM) (Trogl et al., 2011a) amended with KNO_3 (4.08 $g.L^{-1}$ ~ 569 $mg.L^{-1}$ $N\text{-}NO_3^-$) and with ethanol serving as a carbon source (2.9 $mL.L^{-1}$ ~ 8.4 g COD/g N) to achieve a final denitrification activity of ~1000 mg $N\text{-}NO_x^-.h^{-1}.kg^{-1}$ LB. The MDM was replaced with a fresh one after every three to four batches, when the turbidity (caused by free bacteria) increased to $OD_{600} > 1$.

All experiments were carried out at ambient (laboratory) temperature (~25°C), which was recorded at the time of sampling. Cultivations of LB (1 kg wet weight) were carried out in a 12-L cylindrical reactor (working volume 10 L) equipped with overhead stirring in order to maintain the LB suspended and evenly distributed throughout the whole reactor volume. Further denitrification experiments in brines were carried out in 1.2-L glass bottles (working volume of 1 L) with ~100 g of LB (wet weight; the exact weight was recorded for further calculations). The bottles were magnetically stirred.

Simulated brines were prepared from p.a. chemicals and distilled water. Further denoted 100%-matrix (that is, a solution of salts without nitrates) consisted of 20 $g.L^{-1}$ NaCl (~12.14 $g.L^{-1}$ Cl^-) and 2 $g.L^{-1}$ Na_2SO_4 (~1.35 $g.L^{-1}$ SO_4^{2-}). Further denoted 100%-brine (that is, matrix with nitrates) was prepared by amending the 100%-matrix with KNO_3 to reach the concentration of 10 $g.L^{-1}$ NO_3^- (~2.3 $g.L^{-1}$ $N\text{-}NO_3^-$). The 100%-brine was diluted with distilled water to 80, 60 and 40% brines (that is, 800, 600, and 400 ml of 100%-brine per liter, respectively).

Samples were withdrawn at appropriate intervals (1 to 15 h); 3 ml of each sample was used for OD_{600} determination, 1 ml was diluted in a 25-ml volumetric flask for determination of nitrates and nitrites. Analyses were carried out as described previously (Trogl et al., 2011a). Briefly, pH, and dissolved oxygen + temperature were recorded at the time of sampling using online probes PCL332 and KCL 153/t/SD, respectively (Gryf, Czech Republic). Nitrates were determined by ion-chromatography and nitrites were determined by means of spectrophotometry using a Merck kit for sea water (Merck GmbH).

A sum of 16 batch denitrification tests was carried out. Four simultaneous tests for each brines dilution (100, 80, 60 and 40%) were repeated four times which included delays without intermediate cultivation. One portion of LB was used for a single dilution only. At the beginning of each test, LB was washed three times with ~200 ml of potable water, appropriate brine was then added and the pH was adjusted to initial ~7.0 with a few drops of hydrochloric acid (1:1). No further pH adjustments were performed during the course of the tests. Each test was started by adding a single dose of ethanol, serving as a substrate for denitrification and dosed at the ratio of 4.2 g COD/g $N\text{-}NO_3^-$.

The activity of LB, expressed as the amount of $N\text{-}NO_x^-$ (that is, sum of $N\text{-}NO_3^-$ and $N\text{-}NO_2^-$) removed per hour by one kilogram of wet LB, was calculated from the time needed to reduce the initial $N\text{-}NO_x^-$ concentration to a final $N\text{-}NO_x^-$ concentration, predominantly below the determination limit (5 $mg.L^{-1}$).

Statistical comparisons (t-tests) were calculated using Microsoft Excel.

RESULTS

Cultivation of Lentikats Biocatalyst

An increase in denitrification activity within the course of batch cultivation tests (using MDM) is depicted on Figure 1. The maximum activity achieved was ~1000 mg N. $h^{-1}.kg^{-1}$ LB similar to previously carried experiments (Trogl et al., 2011a). The denitrification activity did not increase during the last three batch tests, hence a maximum achievable activity of LB. A comparison of the achieved denitrification activities with values of OD_{600} (that is, the concentration of free bacteria) shows that the vast majority of the overall activity can be attributed to the encapsulated bacteria. The estimated contribution of free bacteria was less than ~25%.

Simultaneous batch denitrification of brines under various dilutions

Figure 2 shows the kinetics of four sequent sets of

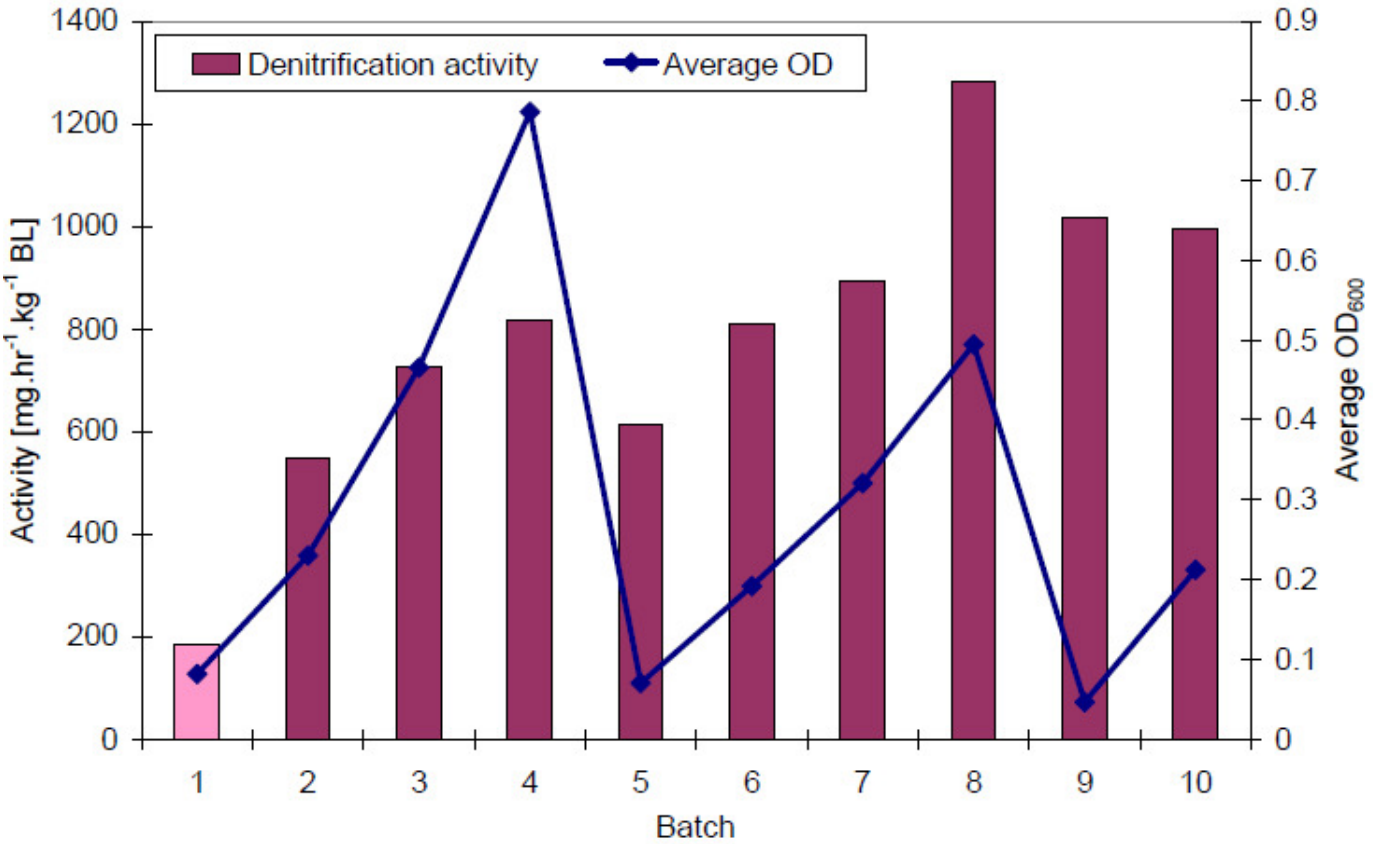

Figure 1. Increase of denitrification activities in the course of batch cultivations in mineral denitrification medium. Secondary axis shows the average OD_{600} (that is, concentrations of free bacteria) of each experiment. Note that the medium was not changed regularly between the each batch tests, therefore the OD_{600} increased due to free bacteria accumulation in the liquid medium. The average temperature of the batch tests varied between 24.4 and 26.8°C, without any significant effect on the activity.

simultaneous batch denitrification tests complemented by their pH profiles (Figure 3). Concentration of dissolved oxygen dropped to ~0 within the first hour of each tests and did not rise until nitrates and nitrites were completely eliminated. The temperature generally increased in the course of the batch tests from the initial 20 to 23°C to a final 25 to 28°C.

As observed previously (Glass and Silverstein, 1998, 1999; Trogl et al., 2011a; Vackova et al., 2011), the time course of the denitrification reaction followed approximately zero-order kinetics with nitrite peaks and a pH increase copying the elimination of nitrites. Two significant exceptions from this general pattern were obtained during the first tests in 100 and 80%-brines (Figure 2a). In both cases, the denitrification was severely inhibited and the denitrification kinetics did not follow the general pattern. Stagnating concentrations of nitrites and even a reverse increase of nitrate concentrations were recorded. This inhibition was related to a somewhat unusual pH profile (Figure 3a). As opposed to other denitrification experiments, the pH in these tests decreased to as low as 5.5 and later stabilized at pH ~6. However, in the second batch tests using 80 and 100%-brine, the denitrification profile stabilized again and also the denitrification activity increased significantly (Figures 2b and 3b).

A summary of obtained denitrification activities (Figure 4) reveals different time courses of denitrification activities in 40 and 60%-brines from those obtained in 80 and 100%-brines. The activity course in 40 and 60%-brines indicates no inhibition; the initial denitrification activities in brines were comparable to activities achievable in the non-inhibiting cultivation MDM (~1000 mg N. $hr^{-1}.kg^{-1}$ LB, Figure 1). The consequent decrease of denitrification activity in those brines was similar to previously described ones (Bouskova et al., 2011; Trogl et al., 2011a) ascribed partially to the absence of nutrients and a consequent decline of vivid bacterial population and partially to an escape of the encapsulated bacteria from the flexible PVA gel. On the other hand, in the cases of 80 and 100%-brine, the first batches resulted in almost zero denitrification activity. The inhibition was partially suppressed in the second set of batches, leading to an increase in the denitrification activity, yet the activity remained below the values obtained in 60 and 40%-brines in all further repetitions.

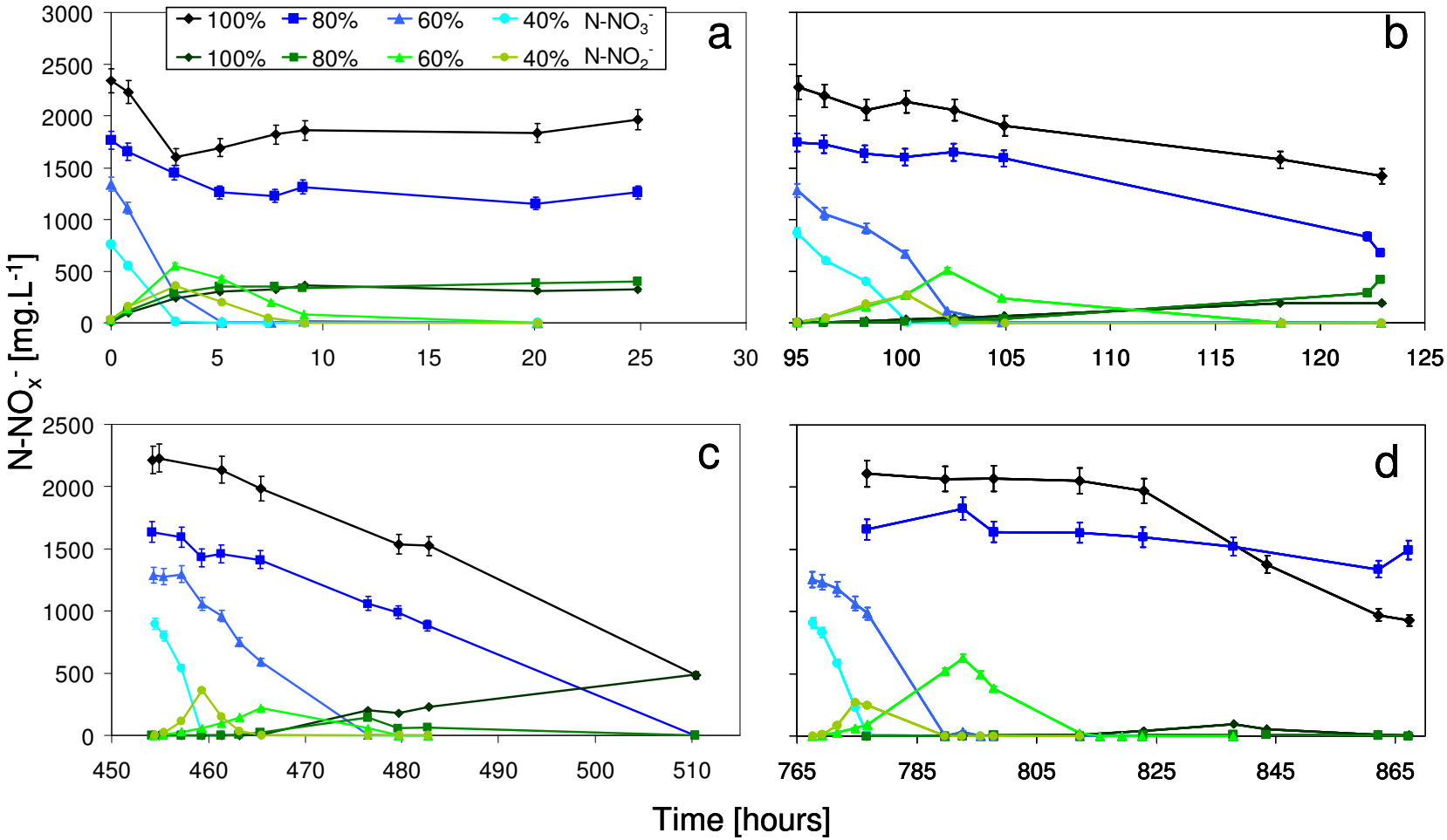

Figure 2. Kinetics of batch denitrification tests with diluted brines. Four simultaneous tests with different brines dilution (indicated by the intensity of blue and green colour) were repeated four times with included delays (a to d = 1st to 4th test).

DISCUSSION

The data obtained reveals several obvious trends, which are to some extent contradictory to previously reported results from similar tests (Trogl et al., 2011a). However, previous conclusions have been based predominantly on the correlation analyses of the overall results of two sets of batch experiments from a period of approximately

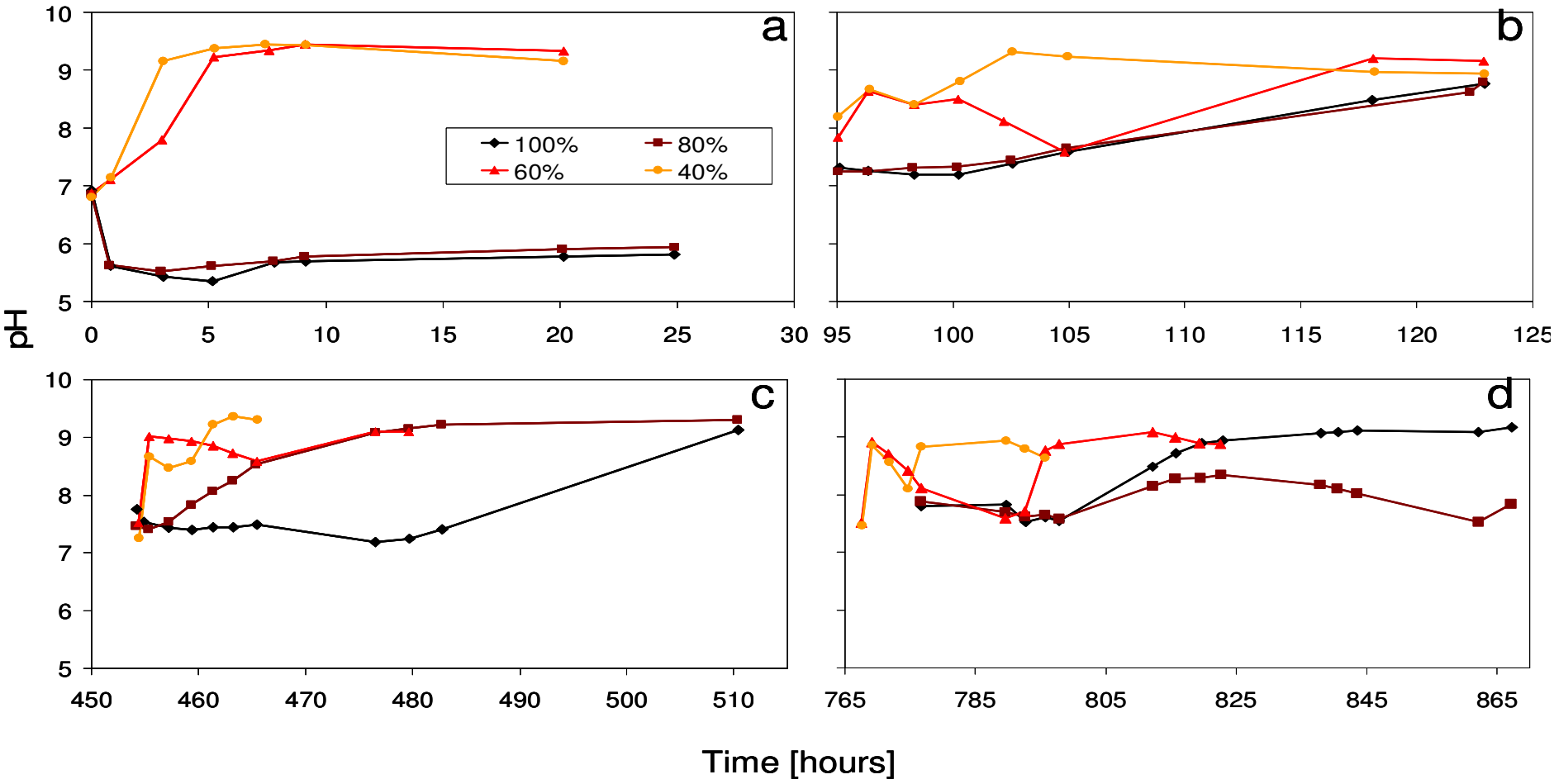

Figure 3. pH profiles during the course of repetitive batch denitrification tests with simulated ion-exchange brines (simultaneous tests with different brines dilution are indicated by the colour intensity; a to d denotes 1st to 4th test.

one year. Such a rough method leaves space for boundary problems, exceptions, and unresolved hypothesis such as those studied here. A discussion and reciprocal comparison of these results is therefore beneficial.

Physiological adaptation of encapsulated bacteria

The results noted in this paper emphasize the importance of initial adaptation of the encapsulated denitrifiers to the extreme salinity in order to achieve the high (full) denitrification activity of LB. Firstly, this conclusion is supported by observed denitrification activities of LB in 100 and 80%-brines. The atypical course of denitrification

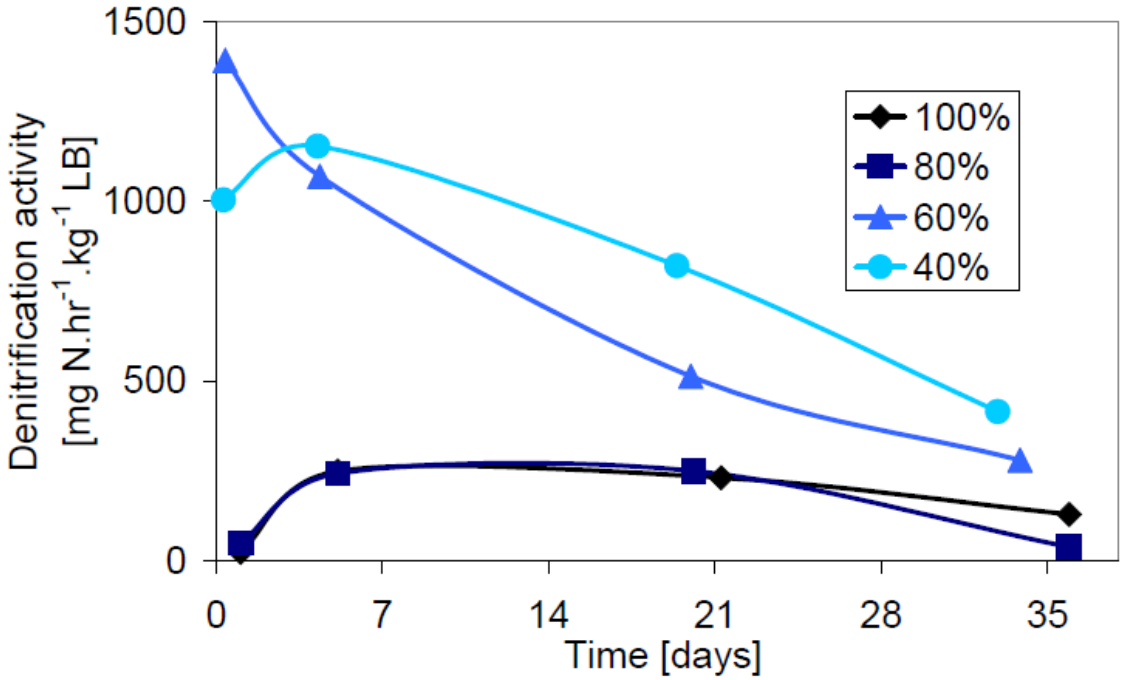

Figure 4. Time course of achieved denitrification activities of Lentikats biocatalyst applied to diluted ion-exchange brines.

(Figure 2a) together with the unexpected pH decrease (Figure 3a), the reverse of nitrate reduction and the almost zero denitrification activity indicate a possible shock for the encapsulated bacteria upon the first contact with the concentrated brines. A normalization of the denitrification kinetics and an increase of the denitrification activity in the following tests (although not to the levels obtained in the non-inhibiting 40 and 60%-brines) suggest that the encapsulated bacteria were able to perform a form of physiological adaptation to the imposed stresses.

A more detailed insight into results of the very first batch tests in brines obtained in the previous study (Trogl et al., 2011a) revealed a similar behavior. In the previous study, one portion of LB was introduced into the same 100%-matrix with 114 $mg.L^{-1}$ $N\text{-}NO_3^-$ and showed inhibited kinetics and a pH decrease, very similar to the one observed here. Identically, another LB portion introduced into 40%-brine (that is, 909 $mg.L^{-1}$ $N\text{-}NO_3^-$) exhibited standard denitrification kinetics in its very first test.

The fact that no atypical denitrification kinetics was observed in 40 and 60%-brines disregards the absence of nutrients as a possible sole cause for the unusual behavior in the more concentrated brines. High concentrations of nitrates are also an unlikely cause as the phenomenon was observed at only 114 $mg.L^{-1}$ $N\text{-}NO_3^-$ (in 100%-matrix) and as the LB was capable of performing a standard high-rate denitrification at concentrations as high as 4500 $mg.L^{-1}$ $N\text{-}NO_3^-$ during its very first cultivation after encapsulation. Therefore, the high salinity (osmotic shock) or possibly the synergic effect of high-salinity and high-nitrates remain the most likely cause of the observed inhibition.

Microevolutionary adaptation

Despite the physiological adaptation, the denitrification remained substantially lower in 80 and 100%- brines in comparison to 60 and 40%-brines in the whole sequence of the four batch repetitions. Similarly, significant (t-test, $\alpha = 0.05$) differences between activities in 40 and 60%-matrices (294±92 mg N. $h^{-1}.kg^{-1}$ LB) and in 80 and 100%-brines (164±37 mg N. $h^{-1}.kg^{-1}$ LB) were also observed in previous study (Trogl et al., 2011a) during the first application of LB in brines. After re-cultivation of LB, these differences became insignificant (t-test, $\alpha = 0.05$, activities 599±59 mg N. $h^{-1}.kg^{-1}$ in 40 and 60%-brines versus 636±97 mg N. $h^{-1}.kg^{-1}$ in 80 and 100%-brines). Also, overall correlation between salinity and activity became insignificant. While the general increase of denitrification activities after cultivation can be explained simply by an increase of the encapsulated microbial population in LB, elimination of differences between higher and lower brines dilutions can not be. This comparison supports the hypothesis that the process of alternating periods of brines denitrification (no nutrients, decrease of encapsulated population and denitrification activity) with periods of cultivation (proliferation of

surviving bacteria) drives the microevolution of *P. denitrificans* towards a better adaptation to a higher salinity. This possible "halotolerant" strain of *P. denitrificans* was isolated from the adapted LB and its difference from the original strain is currently being studied.

Application consequences

It has been proven that *P. denitrificans* encapsulated in LB can be successfully applied towards the elimination of high concentrations of nitrates from high-salinity ion-exchange brines both in batch (Trogl et al., 2011a) as well as in continuous arrangement (Bouskova et al., 2011). The results obtained in this study support these findings and implicate the importance of adaptation of encapsulated denitrifiers to fit extreme conditions. This adaptation is to be carried out over at least one period of denitrification in brines followed by a re-cultivation step. Previous results indicate that a single brine-cultivation period might be sufficient for a significant improvement of denitrification activities of LB in brines. Optimization of the adaptation process is currently under investigation.

Conclusions

This study demonstrates the initial behavior of denitrifyng bacterium *P. denitrificans* encapsulated in PVA gel (Lentikats Biocatalyst) during the batch elimination of nitrates from high-salinity nutrient-free ion-exchange brines. The importance of adaptation of encapsulated denitrifiers in order to achieve high denitrification activities of LB was discussed. Results indicate that both physiological as well as microevolutionary mechanisms might be involved in these adaptations but further research is needed to understand them properly.

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