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Mixed Continuous Suspended and Immobilized Culture of Diazotrophic Isolates from Root-free Soil and the Endorhizosphere of *Leptochloa fusca* L. Kunth

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Abstract

Two diazotrophic isolates from Kallar grass were grown in binary mixed continuous culture. Under otherwise identical conditions, the dominance of one particular strain depended on the suspended or immobilized cultivation type, and probably on the ability to release exopolysaccharides. Ammonium accumulation in the supernatant was at least twice as high in immobilized culture, where the growth rate was lower.

Keywords: Alginate beads, associative bacteria, immobilization, Kallar grass, mixed continuous culture, nitrogen fixation, rhizosphere

1. Introduction

Microbial physiology in the rhizosphere is characterized particularly by slow growth rates, limiting carbon and nitrogen availabilities, low O_2 – partial pressure, cell to cell contact and microbial competition or antagonism. In the laboratory, these conditions can hardly be met by batch cultures, but approximately by continuous fermentor systems. Mixed culture experiments have to be performed under well-defined conditions known to be significant in the habitat of interest.

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The purpose of this work is the comparison of mixed suspended and immobilized cultures. Immobilization is introduced as a further step towards natural conditions since rhizosphere bacteria adhere to roots and soil particles or are entrapped in the small closed habitat of the endorhizosphere.

Azospirillum brasilense BS52, a reisolation of strain Sp7, is capable of exopolysaccharide formation (Del Gallo and Haegi, 1990), which can be stimulated by immobilization in gel. This strain was chosen to investigate whether exopolysaccharide formation has an effect on the population dynamics in binary mixed cultures with the endorhizosphere isolates H6a2 and *Azoarcus* BH72.

2. Materials and Methods

The diazotrophic rods H6a2 and *Azoarcus* BH72 were isolated from the endorhizosphere of Kallar grass (Reinhold et al., 1987), *Azospirillum brasilense* BS52 from adjacent non-rhizosphere soil (Hurek et al., 1987). The bacterial strains were precultured 24 hr at 35°C in a modified Nfb-medium at pH 6.8 (Barak, 1982).

All experiments were carried out in a nitrogen-free medium containing ($g \cdot l^{-1}$): L-malate, 1.0 (neutralized by NaOH); MOPS-buffer, 0.5; $CaCl_2 \cdot 2H_2O$, 0.3; $MgSO_4 \cdot 7HH_2O$, 0.2; $Na_5O_{10}P_3$, 0.05; $MnSO_4 \cdot H_2O$, 0.01; $FeSO_4 \cdot 7H_2O$, 0.01; $Na_2MoO_4 \cdot 2H_2O$, 0.002; pH 6.8. Gel beads for embedded cultivation were prepared as follows: The cells were mixed with a sterile 3% (w/v) sodium alginate solution (Protan GmbH, Norderstedt, FRG) and dropwise added to a stirred 2% (w/v) $CaCl_2$-solution. Bacterial bead inoculation was 0.5% (wet weight/vol.). Ionotrophic gelation took place immediately. The gel has a pore network allowing gases, nutrients and metabolic products to diffuse in and out without limitations, when the beads are smaller than 1 mm in diameter (Tanaka, 1983). For continuous suspended culture of strains BH72 and H6a2 a 1.0 l Biostat-V fermentor (Braun Melsungen, FRG) was used. For continuous immobilized culture of strains BH72 and BS52 a 3.0 l SG 2000 fermentor (Chemap, Switzerland) was applied, which was especially re-designed to provide minimal shear forces and an oxygen transfer rate comparable to that of the Biostat. In both setups the temperature was 35°C, the pH 6.8. The fermentor was flushed with nitrogen gas and artificial air to obtain a constant dissolved oxygen concentration of 6 μM.

In order to determine the number of cells in the immobilized culture, the bacteria were released by dissolving the beads in 5% (w/v) tripolyphosphate and counted visually by light microscopy. Viable counts were employed for the suspended culture. Bacterial protein in the suspended culture was determined by the micro Goa method (Bergersen, 1980), immobilized protein by the method

of Freeman et al. (1982). NH_3 formed was detected by the enzymatic UV-test no. 542946, L-malate by test no. 139068 (both Boehringer, Mannheim). Kjeldahl digestion revealed the nitrogen content (Bergersen, 1980). To prove carbon-limitation in suspended culture, additional malate was injected, which led to an increase in optical density and protein content. Dilution rates were adjusted to 0.08 h^{-1} in suspended and 0.22 h^{-1} in immobilized culture in order to prevent bacterial growth in the medium surrounding the beads. Data of μmax- and k_s-values refer to the carbon source L-malate.

3. Results

To obtain a competitive isolate from the endorhizosphere, strain H6a2 and *Azoarcus* BH72 were cultivated in suspended continuous culture up to 5 culture vessel volume changes (Figs. 1 and 2). Strain BH72 was dominant over H6a2. The overall efficiency in nitrogen fixation was 10–15 mg N/g malate (Fig. 2).

In order to determine the impact of entrapment (in comparison to suspension) on the population dynamics within the mixed culture, the prevailing endorhizosphere-isolate BH72 and strain BS52 from root-free soil, most likely a reisolation of *Azospirillum brasilense* sp7 (Reinhold et al., 1986), were first cultivated in suspension together, with again BH72 being dominant, this time over BS52 (Figs. 3 and 4). The overall efficiency was lower, between 5 and 10 mg N/g malate.

In the second step the same combination (BS52/BH72) was cultivated embedded in alginate beads. This type of culture is not true continuous, since it is not carbon limited at high dilution rates, because of restricted bacterial growth in alginate beads and washout of free suspended cells (see malate concentration in Fig. 5). The dilution rate was adjusted to 0.22 h^{-1}, consequently there were no free cells in the medium surrounding the beads after 5 volume changes (protein in suspension, Fig. 5).

In contrast to the suspended culture, immobilized mixed cultivation of the same strains caused reversed results. This time strain BS52, the ability of which to form expolysaccharides is triggered by immobilization, dominated over strain BH72. With increasing volume changes (Fig. 5), protein in suspension decreased due to washout-conditions for the suspended part of the culture (D=0.22 h^{-1}), correspondingly malate in suspension increased, also due to the low growth rate of the immobilized bacteria. The increase in biomass (Fig. 6) was higher for strain BS52 than for BH72. The rise in ammonia-concentration in the medium (Figs. 6 and 7) with time (or volume changes) seems to be due in the major part to the decrease of bacteria in suspension, which would metabolize it and the increase of immobilized BS52 cells, which release it. Considering

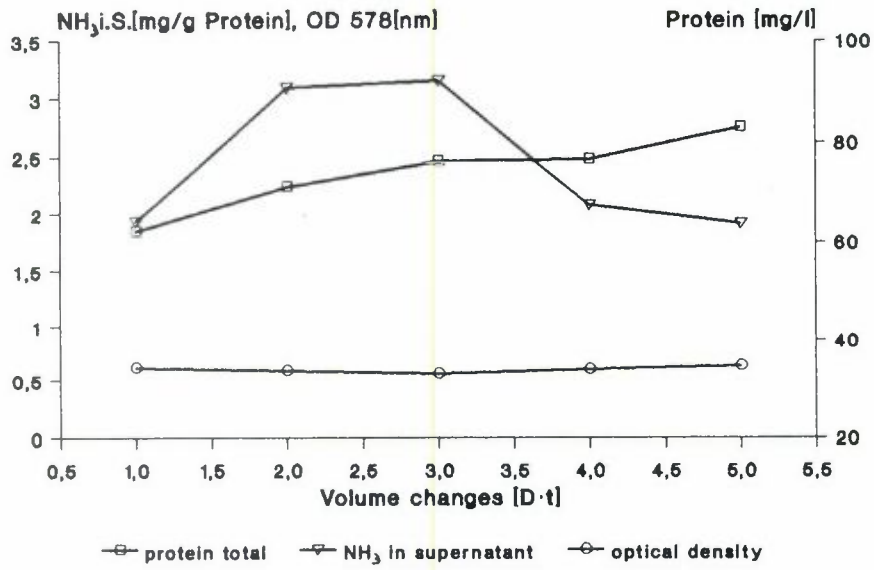


Figure 1. Mixed culture H6a2 + BH72 — Isolates from the endorhizosphere

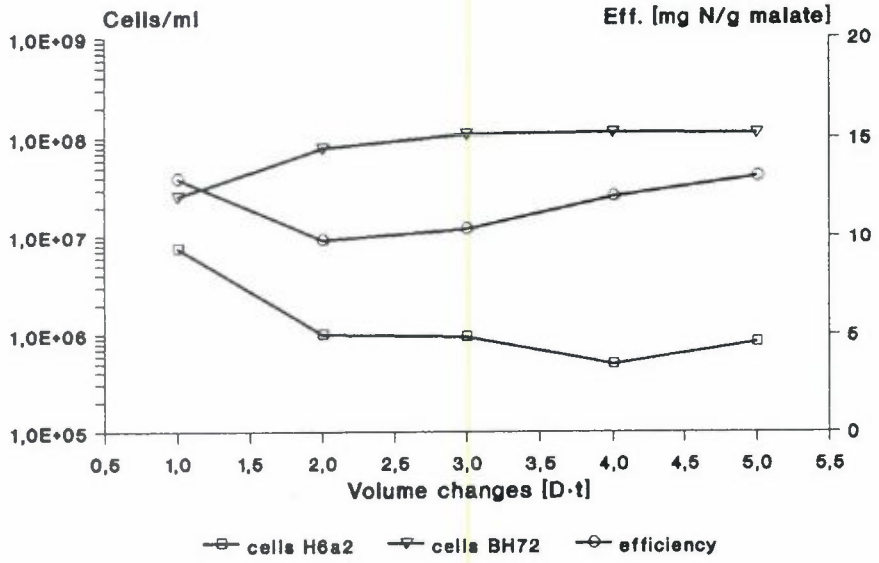


Figure 2. Mixed culture H6a2 + BH72 — Isolates from the endorhizosphere

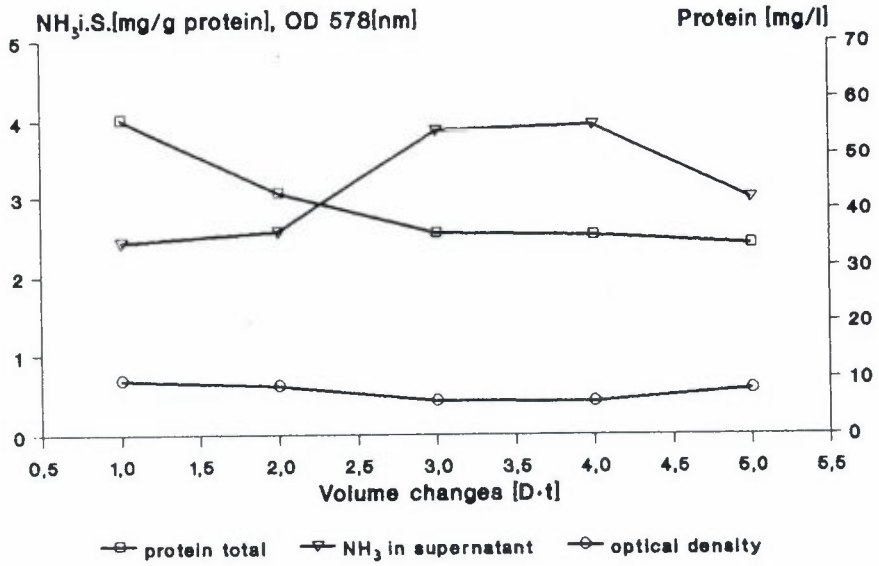


Figure 3. Mixed culture BS52 + BH72 — Isolates from ecto- and endorhizosphere

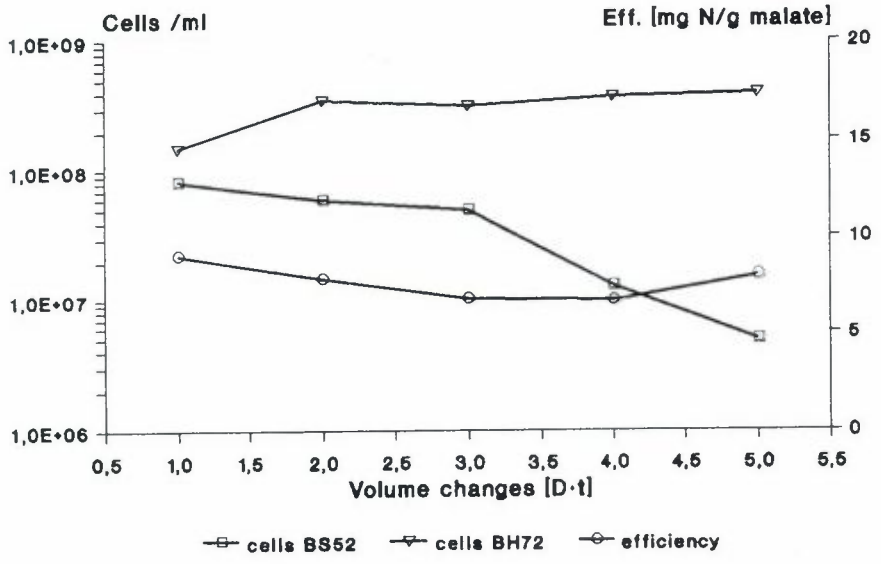


Figure 4. Mixed culture BS52 + BH72 — Isolates from ecto- und endorhizosphere

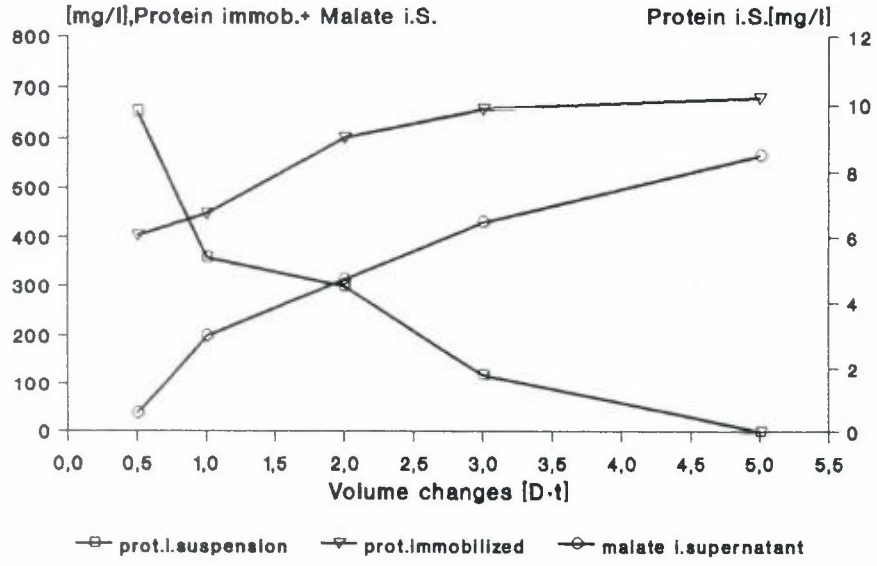


Figure 5. Immobilized mixed culture BS52 + BH72 — Isolates from ecto- and endorhizosphere

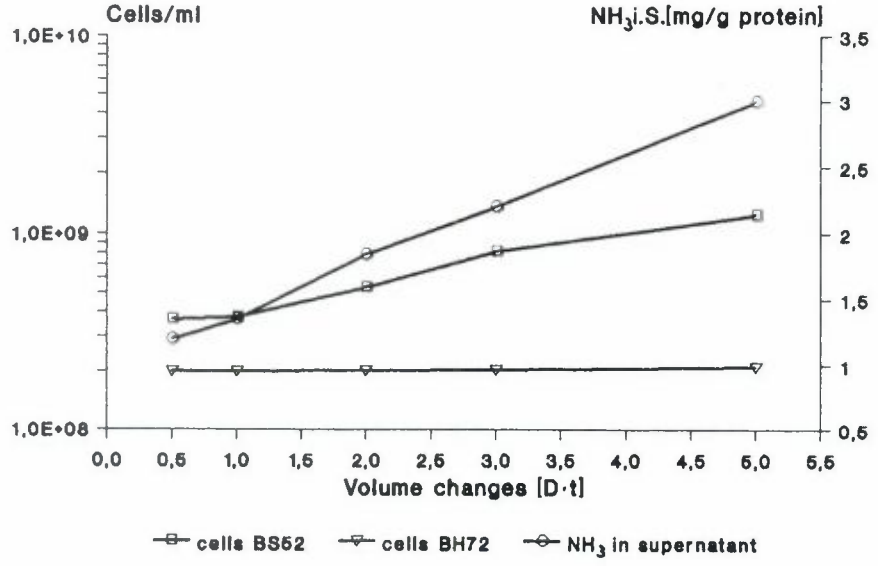


Figure 6. Immobilized mixed culture BS52 + BH72 — Isolates from ecto- and endorhizosphere

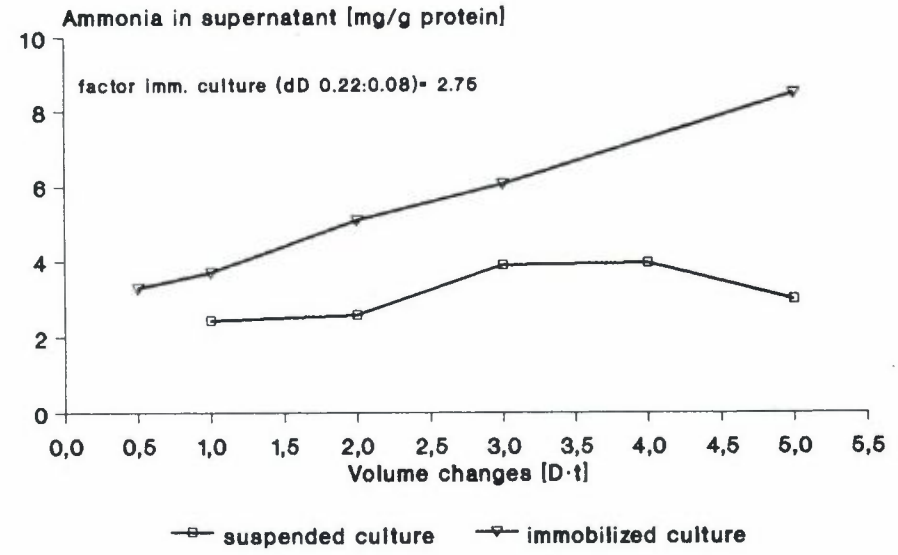
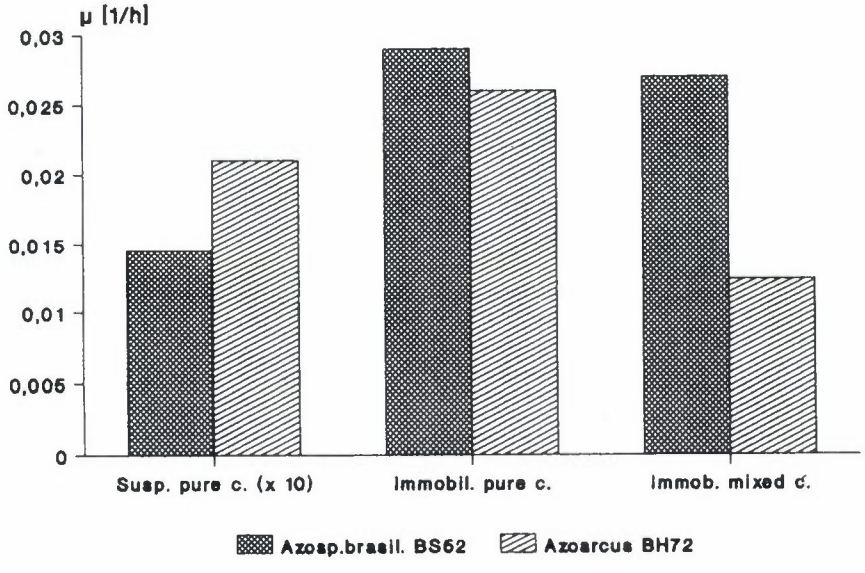


Figure 7. Nh in the supernatant of mixed cultures — Susp. and immob. cultures of BS52 + BH72



Suspended culture: μ = μmax

Figure 8. Azosp. brasil. BS52 and Azoarcus BH72 — Growth rates in continuous cultures

the different dilution rates, ammonia in the medium was approximately twice as high for the immobilized culture. Actually the difference is bigger, since the data of the suspended culture include ammonia from lysed cells as well as released ammonia. For the immobilized culture, the recorded values originate from exudation, at least at 5 volume changes (Fig. 7) since the protein content in the suspended part of the culture approached zero. The adjusted conditions in suspended and immobilized culture were identical with regard to pH, pO_2, temperature, stirring rate, even to the oxygen transfer rate with a K_La-value of 10 h^{-1}. The only difference was the culture volume (1 and 3 liters), which was not likely to affect the results.

4. Discussion

Competition is defined as the growth of two or more species limited by a common factor, in chemostat experiments, usually the carbon-source. When the dilution-rate applied is high enough, the prevailing organism in substrate-limited continuous mixed culture is always the one with the higher maximal growth rate (μ_{max}), regardless of the k_s-value (Veldkamp, 1972).

In suspended culture with *Azoarcus* BH72, the endorhizosphere isolate H6a2 had a lower μ_{max} and higher k_s than strain BH72 with a μ_{max} of 0.21 h^{-1} (unpublished data), and was therefore inferior in mixed culture.

The endorhizosphere-isolate *Azoarcus* BH72 was subsequently cultured in suspension with the root-free soil isolate *Azospirillum brasilense* BS52 (Figs. 3 and 4). Strain BH72 dominated again over BS52, which has a μ_{max} of 0.146 h^{-1}. The same strains were then tested in immobilized continuous culture, where a slow growth rate and cell to cell contact like in the rhizosphere is given. The slow growth rate in alginate beads is thought to be due to physical stress as a result of limited space. Immobilization caused reversed results in comparison to suspended culture: *Azospirillum brasilense* BS52 with a growth rate of 0.027 h^{-1} dominated over *Azoarcus* BH72 with 0.012 h^{-1} (Fig. 8). The prevailing strain in immobilized cultivation was not the endorhizosphere isolate BH72, as could be assumed, since the experimental setup is thought to resemble a microhabitat, but the root-free soil isolate BS52 (= *Azosp. brasil.* Sp7), which has the ability for cyst formation (Sadasivan and Neyra, 1987). Cyst formation is basically characterized by addition of layers of polysaccharides on the outer membrane of the cell. In immobilized pure culture both strains had each almost identical growth rates (Fig. 8), so this could not be the reason for the clear dominance in immobilized mixed culture alone. It is suggested that the ability of strain BS52 to form exopolysaccharides was responsible for its competitiveness, since it was shown that entrapment in alginate stimulated

cyst formation (Ueckert et al., 1990). It is suggested that exopolysaccharides may not only be involved in recognition and interaction with the host plant, but also in competition or antagonism with other habitat-specific bacteria, as was already assumed for other bacteria (Ingraham et al., 1983).

The release of nitrogenous compounds into the medium was roughly twice as high in immobilized culture. The increase in nitrogenase activity in immobilized culture may be correlated with the observed smaller growth rate (Fig. 7).

Obviously the outcome of mixed continuous culture trials strongly depends on the type of experimental setup. Therefore it is proposed to employ fermentor techniques like immobilization of bacteria, which may simulate more closely the natural environment in the rhizosphere.

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REFERENCES

Barak, R.I. 1982. Aerotactic response of *Azospirillum brasilense*. *J. Bacteriol.* **152:** 643–649.

Bergersen, F.J. 1980. Measurement of nitrogen fixation by direct means. In: *Methods for Evaluating Biological Nitrogen Fixation.* F.J. Bergersen, ed. Wiley, New York, pp. 65–110.

Del Gallo, M. and Haegi, A. 1990. Characterization and quantification of exocellular polysaccharides in *Azospirillum brasilense* and *Azospirillum lipoferum*. *Symbiosis* **9:** 155–161.

Freeman, A. et al. 1982. Protein determination of cells immobilized in cross-linked synthetic gels. *Europ. J. Appl. Microbiol. Biotechnol.* **14:** 13–15.

Hurek, T., Reinhold, B., and Niemann, E.-G. 1987. Effect of oxygen on NH_4^+ growth continuous cultures of *Azospirillum* spp. and diazotrophic rods closely associated with Kallar grass. *Can. J. Microbiol.* **33:** 919–922.

Ingraham, J.L., Maaloe, O., and Neidhardt, F.C. 1983. In: *Growth of the Bacterial Cell.* Sinaver Assoc., Sunderland, MA.

Reinhold, B., Hurek, T., Niemann, E.-G., and Fendrik, I. 1986. Close association of *Azospirillum* and diazotrophic rods with different root zones of Kallar grass. *Appl. Env. Microbiol.* **52:** 520–526.

Reinhold, B., Hurek, T., and Fendrik, I. 1987. Cross-reaction of predominant nitrogen-fixing bacteria with enveloped, round bodies in the root interior of Kallar grass. *Appl. Env. Microbiol.* **53:** 889–891.

Sadasivan, L. and Neyra, C.A. 1987. Cyst production and brown pigment formation in aging cultures of *Azospirillum brasilense* ATCC 29145. *J. Bacteriol.* **169:** 1670–1677.

Tanaka, H. 1984. Diffusion characteristics of substrates in Ca-alginate gel beads. *Biotechnol. Bioeng.* **26:** 53–58.

Ueckert, J., Stephan, M., Döbereiner, J., Niemann, E.-G., and Fendrik, I. 1990. Induction of cyst formation by entrapment of *Azospirillum* spp. in Ca^{2+}-alginate beads. In: *Nitrogen Fixation: Achievements and Objectives.* Chapman and Hall, London, p. 595.

Veldkamp, H. and Jannosch, H.W. 1972. Mixed culture studies with the chemostat. *J. Appl. Chem. Biotechnol.* **22:** 105–123.