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Miniaturized kinetic growth inhibition assay with denitrifying bacteria *Paracoccus denitrificans*

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Abstract

A method to cultivate anaerobic bacteria on standard 96-well microplates with automatic recording of growth curves is presented. The method was used as a kinetic growth inhibition assay with denitrifying bacteria *Paracoccus denitrificans*, and applied to various heavy metal ions and selected agrochemicals. Incorporated in a battery of other biotest the assay could take into account effects of toxicants on denitrifying organisms. The results (EC_{50}) revealed that the assay was relatively sensitive. Performed in vials, the assay was also applied to toxicity testing of volatile compounds and represented a convenient method for assessing samples containing volatile constituents.

Keywords: Denitrification; Growth inhibition assay; *Paracoccus denitrificans*; Heavy metals; Pesticides; Volatile compounds

1. Introduction

Notwithstanding that laboratory one-organism ecotoxicological biotests are often criticized and stress is laid on the fact that they cannot cover the complexity of real ecosystems, due to the method's simplicity they are frequently used in ecotoxicity assessment. Some of those assays are well known, broadly applied and are even standardized as international or national standards (ISO 8692, 1989; EN ISO 10712, 1995). Biotests on bacteria are, without doubt, one of the most convenient because there is no need to keep the breed of testing organisms. In such tests growth of testing organisms is frequently followed by measuring an optical property, most often an optical density of the test culture.

To improve the significance of a sample assessment and to predict potential environment risk more reliably, a battery of several biotests should be used, preferably comprising organisms from various trophic levels. Because we aim to predict the impact of the sample on the complex ecosystem, the battery should also comprise organisms that perform major natural biochemical processes. Denitrification, a substantial part of the global nitrogen cycle, has a significant influence on all water and terrestrial ecosystems in a manner and scale not yet fully understood (Zumft, 1997). That is why it is advisable to incorporate a biotest with a denitrifying organism into the battery of biotests in use.

This work provides a method for anaerobic cultivation of bacteria on standard 96-well microplates, which is applicable as a growth inhibition test for water-soluble substances, liquid environmental samples or leachates of solid samples. It is also possible to perform the method in sealed vials. In this manner it is possible to test

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samples containing volatile constituents, which is problematic or impossible in biotests with aerobic organisms.

2. Materials and methods

2.1. Bacterial strains

Pseudomonas stutzery and *Paracoccus denitrificans* were obtained from The Czech Collection of Microorganisms, where they are stored as strains CCM 982 and CCM 2660 respectively. Both strains represent authentic natural facultative anaerobic bacteria.

2.2. Preparation of inoculum

Frozen bacterial suspension was used for the inoculation of test cultures. The bacteria were grown in a medium identical to the one described for the biotest. After 24 h of static cultivation optical density was measured at 550 nm, and 0.9 ml portions of the suspension were pipetted into 1.5 ml plastic tubes with 100 μl of glycerol as a cryo-preservative, thoroughly mixed, frozen and stored at −18 °C. For inoculation the frozen cryo-culture was thawed at room temperature and appropriately diluted, so that the given volume of inoculum brought the initial optical density (550 nm) of the test culture to 0.02. In our experience the frozen inoculum remained viable at least three months.

2.3. Composition of the test medium

The medium contained 7.5 mmol Na_2HPO_4, 15 mmol KH_2PO_4, 50 mmol KNO_3, 2 mmol $MgSO_4$, 15 mmol urea, 0.03 mmol Fe^{3+} citrate, 50 mmol glucose and 100 μl of trace element solution per litre. The trace element solution contained 0.20 g $MnSO_4$, 0.029 g H_3BO_3, 0.022 g $ZnSO_4 \cdot 7H_2O$, 1.0 g Na_2MoO_4, traces of $Co(NO_3)_2$, and traces of $CuSO_4$ in 500 ml of water.

2.4. Kinetic growth inhibition test on 96-well microplate

The concentrated medium solution was prepared from stock solutions of individual components, so that concentrations of all components were five-times higher than their, above cited, concentration for the final medium. 56 μl of the concentrated medium solution was pipetted into each well followed by a sample, dilution water and 15 μl of appropriately diluted inoculum. From four to six parallel wells were prepared for each sample concentration or growth control. The total volume in a well was 280 μl.

If the medium was amended with NH_4^+ ions, NH_4Cl was added in a separate solution into each well as the last one, to prevent oversaturation and precipitation, bringing the final NH_4^+ concentration to 30 mmol per litre.

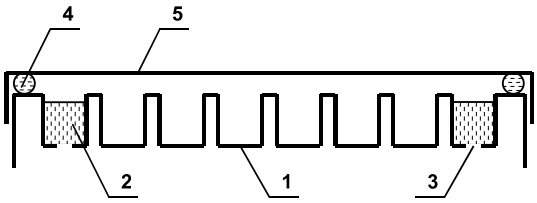

Fig. 1. Adaptation of a standard lid and 96-well microplate to anaerobic cultivation: 1. microplate; 2. septum; 3. hole; 4. silicon sealing; 5. transparent lid.

To close the microplate and create anaerobic conditions a standard transparent lid was used. Silicone paste (Lukopren T, VIA-REK, CZ) was applied between the lid and the microplate, around its rim, to ensure gas-tightness (Fig. 1). To prevent water condensation the inner surface of the lid was modified with an anti-fog solution for optics. After sealing it, the inner space of the microplate was flushed with nitrogen gas through septa, which had been prepared in two wells in opposite corners of the microplate by drilling through the bottom of the wells and filling them with a setting silicon cement (Lukopren S6410, LZK, CZ). Then the microplate was inserted into the microplate reader (Sunrise, TECAN), where it was cultivated without shaking for 24 h at 30 °C. Every 30 min optical densities at 550 nm were recorded in all wells.

For the EC_{50} evaluation the integral approach was used (Schmitz et al., 1998). From the data obtained, areas under the growth curves were calculated for each concentration of a sample and comparing the values to that of the growth control, the inhibition for each concentration was derived. The inhibition was subsequently transformed into probit values to linearize its dependence on concentration, and linear regression was used to deduce EC_{50} value.

2.5. Growth inhibition test for volatile compounds

In this embodiment the test was performed in 4.5 ml gas-tight vials and in the same total medium volume with virtually no headspace. 900 μl of the concentrated medium solution was pipetted into each vial followed by water and inoculum, and finally the sample solution was added using a gas-tight syringe and the vial immediately closed. This procedure is more useful than adding samples with a needle through septum, because volatiles can escape across penetrated septa during the cultivation period, especially when the septa are repeatedly used. Volatile compounds were added as DMSO solutions and an equal amount of DMSO was also present in the growth controls. The vials for each toxicant concen-