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SCIENCE DIRECT
Journal of Microbiological Methods 65 (2006) 551–556
Journal of Microbiological Methods
www.elsevier.com/locate/jmicmeth

Development of a microtiter plate-based method for determination of degradation profile of nitrophenolic compounds

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Received 15 September 2005; received in revised form 30 September 2005; accepted 30 September 2005
Available online 8 November 2005

Abstract

A microtiter plate-based assay was developed for the automatic monitoring of degradation profile of the yellow-coloured nitrophenolic compounds. The method enables to reduce the intervals between measurements of substrate concentration to minutes and to overcome the problem of discontinuity of sampling typical for conventional methods. The concentrations of nitrophenolic compounds were calculated from the absorbance values determined automatically by BIOSCREEN C. Verification of the method was based on the comparison of results with the conventional HPLC method results. The values of the rate and saturation constants were comparable for both the microtiter plate-based assay and the conventional HPLC method. The automatic method described here seems to be efficient for the screening degradation studies, which requires the treatment of quantity of samples.

Keywords: BIOSCREEN C; Degradation; Microtiter plate-based assay; Nitroaromatic; Microculture

1. Introduction

Nitroaromatic compounds are known as widely distributed environmental pollutants. Their occurrence in the environment is associated with the chemical and pharmaceutical industry and agriculture. An important group of the nitroaromatics are nitrophenolic compounds, regulated priority pollutants dispersed as agriculture pesticides, above all (Nishino et al., 2000). Moreover to the industrial production, nitrophenols are produced by combustion processes of motor vehicles (Lüttke et al., 1997) and enter the aquatic and terrestrial environment through rainwater (Tremp et al., 1993). Some of them are applied as chromogenic substrates due to their yellow colour. Nitrocatechols and their derivatives are important in the pharmaceutical industry—as selective inhibitors of catechol-*o*-methyltransferase useful for treatment of Parkinson disease (Learmonth and Freitas, 2002) or building blocks for the production of antihypertensive pharmaceuticals (Ennis and Ghazal, 1992).

The yellow or orange-coloured nitrophenolic compounds are easy detectable by spectrophotometric method (Leung et al., 2005). It is very advantageous for the application of a microtiter plate-based assay, although the spectrophotometric method is often replaced by more accurate chromatographic techniques

Abbreviations: K_s, saturation constant; k_{deg}, degradation/growth rate constant; MM, mineral medium; 4-NP, 4-nitrophenol; 4-NC, 4-nitrocatechol; 4-NG, 4-nitroguaiacol; CCM, Czech Collection of Microorganisms.

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doi:10.1016/j.mimet.2005.09.019

used in the course of "conventional" monitoring of degradation of nitrophenols. Microtiter plate-based assays using automated microculture systems with optical density measurement have been developed for a wide range of applications aimed on the quantitative growth curve analysis (Weiss et al., 2004; McKellar et al., 2002). Nevertheless, the use of a microculture method for quantitative determination of degradation profile of environmental pollutants has not been reported to the present.

In this work we describe the use of the microtiter plate-based assay for determination of degradation of nitroaromatics. Three nitroaromatic compounds from the group of nitrophenolics were chosen for the assessment of the microtiter plate-based method: 4-nitrophenol (4-NP), an environmental pollutant contaminating soil and wastewater and accumulating in food chain (Gemini et al., 2005); 4-nitrocatechol (4-NC), the intermediate of 4-NP metabolism (Raymond and Alexander, 1971; Hanne et al., 1993; Jain et al., 1994); and 4-nitroguaiacol (4-NG). The degradation activity of soil bacteria of the genera *Arthrobacter* and *Rhodococcus* was utilized for degradation. Bacteria of these genera have been commonly described as degraders of environmental pollutants (Westerberg et al., 2000; Pandey et al., 2003; Backman and Jansson, 2004) including nitroaromatics (Kotoučková et al., 2004; Navrátilová et al., 2005). The reliability of the automatic spectrophotometric microplate method for determination of degradation profiles of nitroaromatics was assessed by the simultaneously used conventional HPLC method.

2. Materials and methods

2.1. Test organisms and culture conditions

For the degradation experiments, there were chosen three bacterial strains isolated from soil by selective enrichment on nitrophenolic compounds. The method of the strains isolation was described previously, so as the classification of the strain P1P as a representative of the species newly described for the degradation of nitrophenolics, *Arthrobacter nitroguajacolicus* (Kotoučková et al., 2004). 16S rDNA sequence analysis (performed as described by Tvrzová et al., 2005) was used as the basic method for taxonomic classification of the strains C6-1 (CCM 7084) and J7 (CCM 7242).

The bacteria were cultivated in a sterile mineral medium (MM, Kotoučková et al., 2004) supplemented with a nitroaromatic substrate as a source of carbon and energy. 4-Nitrocatechol (Fluka) was the substrate for C6-1; 4-nitrophenol (Fluka) for *A. nitroguajacolicus* P1P; and 4-nitroguaiacol (Aldrich) for strain J7 (Table 1). To prepare starting cultures for degradation experiments, the cells of individual strains were cultivated in MM supplemented with a selected substrate in the concentration presented in Table 1. After the substrate degradation (medium decolourization), 5 ml aliquots were transferred to the 50 ml of the fresh medium and cultivated to the medium decolourization. Considering the fact that growth curve of the cells of three chosen degrading strains differs, it was essential to find the optimal length of starting flask culture preparation and to adapt the beginning of the cell cultivation to synchronize inoculation of the BIOSCREEN experiments. By this way, the cells of all three chosen strains were ready for inoculation in the same time.

2.2. Determination of degradation profile by microtiter plate-based assay

BIOSCREEN C (Labsystems) automatic cultivator/reader was used for the determination of degradation profile by the use of the microtiter plate-based assay. MM medium supplemented with nitroaromatic substrates (for concentrations see Table 1) was dispensed to the honeycomb microtiter plates (Labsystems). 300 μl of the medium and 30 μl of a starting flask culture were used for separated wells, 5 wells were used for every testing concentration. The microtiter plates were cultivated in aerobic conditions, at 28 °C, and aerated with shaking (duration 30 s, before and after measurement). Absorbance values were read at 420 nm every 10 min. The final data are average of 5 measurements. The concentration of a substrate was calculated from

Table 1
Bacterial strains and nitroaromatic substrates used for determination of degradation profile

Strain		Substrate		
Number	Classification	Designation	Concentration used for starting culture preparation (mM)	Experimental concentrations (mM)
P1P	*Arthrobacter nitroguajacolicus*	4-NP	0.5	0.05; 0.10; 0.15
C6-1	*Rhodococcus wratislaviensis*	4-NC	0.05	0.016; 0.04; 0.08
J7	*Rhodococcus wratislaviensis*	4-NG	0.05	0.0125; 0.025