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# Simultaneous species-specific PCR detection and viability testing of poly(vinyl alcohol) cryogel-entrapped *Rhodococcus* spp. after their exposure to petroleum hydrocarbons

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ABSTRACT

A method of simultaneous species-specific PCR detection and viability testing of poly(vinyl alcohol) cryogel-entrapped *Rhodococcus* spp. was developed that allowed the estimation of immobilized *Rhodococcus opacus* and *Rhodococcus ruber* survival after their exposure to petroleum hydrocarbon mixture. Spectrophotometric INT assay revealed high tolerance of gel-immobilized rhodococci to petroleum hydrocarbons, while among two *Rhodococcus* strains studied, *R. ruber* tolerated better to hydrocarbons compared to *R. opacus*. These findings were confirmed by respirometry results that showed increased respiratory activity of gel-immobilized *Rhodococcus* strains after 10-day incubation with 3% (v/v) petroleum hydrocarbon mixture. Moreover, jointly incubated rhodococcal strains demonstrated higher oxidative activities toward petroleum hydrocarbons than individual strains. Both *Rhodococcus* species were recovered successfully in cryogel granules using 16S rDNA-targeted PCR, even though the granules were previously stained with INT and extracted with ethanol. The method developed can be used for rapid detection and monitoring of gel-immobilized bacterial inocula in bioreactors or contaminated soil systems.



## 1. Introduction

The genus *Rhodococcus* exhibits a wide variety of catabolic pathways for the transformation and degradation of various organic substrates, such as aliphatic and aromatic hydrocarbons, chlorinated phenols, heterocyclic compounds, nitriles, sterols, pesticides, lignin, coal, and crude oil (Van der Geize and Dijkhuizen, 2004; Larkin et al., 2005). Furthermore, rhodococci are promising candidates for the bioremediation of contaminated environments because of their physiological plasticity and robustness, lack of pathogenicity and ecotoxicity (Warhurst and Fewson, 1994; Kuyukina and Ivshina, 2010).

Microorganisms immobilized in polymeric gels are being extensively used for biocatalysis, biosensor and, especially, bioremediation applications due to their increased metabolic activity and tolerance to environmental stresses compared to suspended microbial cultures (Cassidy et al., 1996; Rathore et al., 2013). Among natural and synthetic gels, poly(vinyl alcohol) (PVA) cryogels formed through the freeze–thaw technique, are promising matrices for microbial immobilization due to their high micro- and macro-porosities, water-retention capacity, thermal resistance, slow biodegradability and excellent rheological characteristics (Lozinsky, 2002). Previously, we have developed a method of *Rhodococcus* cell immobilization into PVA-cryogel granules, which resulted in improved bacterial survival under long-term storage and contaminated soil conditions (Kuyukina et al., 2006, 2012).

Many papers showed that microbial consortia consisting of two or more strains exhibit better biodegradation performance than single strains (reviewed by Mikesková et al., 2012). Various co-cultures of *Rhodococcus* species alone or in combinations with other bacterial/fungi strains used for bioremediation of contaminated environments are listed by Kuyukina and Ivshina, 2010. However, sometimes mixed bacterial cultures show less efficient biodegradation compared to single strains (Larcher and Yargeau, 2011) presumably due to strain competition or antagonistic effects. To avoid a competitive inhibition of co-immobilized microorganisms, bacterial strains with various biodegradation peculiarities are immobilized separately into different gel granules, which are then added at different proportions to the contaminated medium (Kuyukina et al., 2012). Such separate strain immobilization allows maintaining an optimal inoculant ratio in bioreactor or soil systems simply by varying the amounts of gel granules. While this method could be used for studying the comparative survival and biodegradation efficiencies of immobilized inoculants, it would require simultaneous identification and viability assessment of cultures entrapped in different gel granules.

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Since conventional cultural methods of viability testing are not suitable for bacteria entrapped into water insoluble rigid gels, development of rapid and non-destructive methods for the determination of viability of immobilized bacterial cells is therefore necessary. Various biochemical methods allowing detection of bacterial metabolic activity are used for gel-entrapped cells, e.g. luminometric determination of intracellular ATP produced by living bacteria (Rathore et al., 2013). While this method showed good results with microencapsulated microorganisms (de Vos et al., 2009), it could not provide reproducible data with bacteria entrapped into macro-size granules due to ATP extraction limitations.

Viability of gel-encapsulated cells has been assessed using different fluorescent dyes, such as SYTO-green, ethidium bromide and 6-carboxyfluorescein (de Vos et al., 2009). It was concluded that microgranules with higher retention of the dye indicated better retention of water-soluble substrates, and therefore, higher cell viability. Also the applicability of relatively simple colorimetric 3-(4,5-dimethylthiozol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay to measure the viability of HepG2 liver cells encapsulated in alginate granules was investigated (Khattak et al., 2006). The MTT assay was effective in measuring alginate-encapsulated cell growth and viability, demonstrating less variance and higher throughput capability than cell cytometry. In the present study, we used 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT), widely applied for bacterial dehydrogenase activity tests (Kim et al., 1994; Wrenn and Venosa, 1996; Caravelli et al., 2004), which was previously explored for the viability assessment of cryogel-entrapped *Rhodococcus* cells (Kuyukina et al., 2006).

16S rDNA-targeted PCR has been increasingly used for rapid, sensitive and accurate detection of bacterial species and individual strains in clinical and environmental samples (Rodrigues et al., 2002; Matsuki et al., 2004; Clifford et al., 2012). We previously developed species-specific PCR primers for ecologically important *Rhodococcus* (*Rhodococcus globerulus*, *Rhodococcus erythropolis*, *Rhodococcus opacus* and *Rhodococcus ruber*) which enabled their direct detection in mixed cultures and environmental samples (Bell et al., 1999). The present paper is aimed to develop a rapid, relatively simple method of simultaneous 16S rDNA-targeted PCR detection and viability testing of *Rhodococcus* strains entrapped in PVA cryogel granules. A combination of INT staining and tetrazolium extraction followed by DNA extraction and species-specific detection of the microorganisms in one cryogel granule was applied to compare the survival of immobilized *R. opacus* and *R. ruber* after their exposure to model petroleum mixture. Additionally, respirometry analysis was performed to evaluate catalytic activities of separately and jointly incubated rhodococcal strains toward petroleum hydrocarbons.

## 2. Materials and methods

### 2.1. Microorganisms and culture conditions

Two strains *R. opacus* IEGM 249 and *R. ruber* IEGM 615 were from the Regional Specialized Collection of Alkanotrophic Microorganisms of the Institute of Ecology and Genetics of Microorganisms, Perm, Russia (IEGM; www.iegm.ru/iegmcol/index.html). The strains were grown in the following hydrocarbon-containing medium. A mineral salts medium contained (per liter of distilled water): $KH_2PO_4$, 2.0 g; $K_2HPO_4$, 2.0 g; $KNO_3$, 1.0 g; $(NH_4)_2SO_4$, 2.0 g; NaCl; 1.0 g; $MgSO_4 \times 7H_2O$, 0.2 g; $CaCl_2 \times 2H_2O$, 0.02 g; and $FeCl_3 \times 7H_2O$, 0.01 g; trace elements solution. *n*-Hexadecane (99% pure from Sigma-Aldrich) was used as carbon source and added to the mineral medium at a concentration of 3% (v/v). Cultivation was carried out at 28 °C on a rotary shaker at 160 rpm for 3 days. Broth culture of each strain was settled for 15 min, and the top hydrophobic layer containing bacterial cells and residual hydrocarbon was ultrasonically treated (0.1 A, 23 kHz) for 2 min and used for immobilization. Nutrient agar plate counts of the top hydrophobic layers revealed $8.2 \times 10^6$ CFU $ml^{-1}$ for *R. opacus* IEGM 249 and $7.3 \times 10^6$ CFU $ml^{-1}$ for *R. ruber* IEGM 615 (data not shown).

### 2.2. Immobilization procedure

Poly(vinyl alcohol) (PVA) (99% hydrolyzed, with an average molecular weight of 89,000–98,000) was obtained from Sigma-Aldrich. The suspension of PVA (12%, w/v) in distilled water was made and sterilized by autoclaving (112 °C, 15 min). Bacterial cell immobilization into PVA cryogel was performed as described previously (Kuyukina et al., 2006). The sample of the top hydrophobic layer obtained from either *R. opacus* or *R. ruber* culture was added at the ratio 1:2 (v/v) to the PVA solution cooled down to 40 °C. The resulting mixture was vortexed for 2 min, and 100-μl aliquots were filled into the wells of 96-well round-bottom microplates (well working volume — 200 μl). Negative control cryogels that contained no bacterial cells were produced with appropriate amount of distilled water added to the PVA solution. The microplates were frozen at −18 °C for 12 h and subsequently thawed at 4 °C for 5 h. After the gel formation, the PVA-cryogel granules with immobilized *R. opacus* or *R. ruber* cells were stored at −20 °C, and were rehydrated overnight in 0.5% NaCl before use.

### 2.3. Incubation of immobilized bacteria with petroleum hydrocarbons

Model petroleum hydrocarbon mixture (modified from Walker and Colwell, 1974) consisted of (%): *n*-decane (12), *n*-undecane (12), *n*-dodecane (12), *n*-tetradecane (12), *n*-hexadecane (12), *n*-heptadecane (12), *n*-nonadecane (12), pristane (10), naphthalene (2), phenanthrene (2), and anthracene (2). All hydrocarbons were of HPLC grade (Sigma-Aldrich). To increase water solubility of polyaromatic hydrocarbons, they were initially dissolved in small amount of acetone and then added to alkane mixture. Hydrocarbon emulsion in mineral medium (3%, v/v) was prepared with 0.125% (v/v) of Tween 60 by 2-min sonication.

PVA cryogel-entrapped *Rhodococcus* cells (16 granules with either *R. opacus* or *R. ruber* solely, or *R. opacus* and *R. ruber* in equal proportion) were placed in 250-ml Erlenmeyer flasks containing 50 ml of mineral medium supplemented with model petroleum (3%, v/v). Additionally, similar flasks with cryogel-immobilized rhodococci but containing no petroleum were set up to evaluate bacterial survival in the absence of hydrocarbon substrate. Each experimental variant was performed in triplicates. Incubation was carried out on a rotary shaker at 160 rpm and 28 °C for 10 days.

### 2.4. Viability analysis of immobilized Rhodococcus cells

After the exposure to model petroleum, the PVA-cryogel granules with entrapped bacteria were collected from the flasks. Granules were washed from petroleum hydrocarbons with sterile sodium phosphate buffer (pH 7.4) for 30 min using ultrasonic bath, and examined for bacterial viability. Reduction of iodonitrotetrazolium violet, INT (Sigma-Aldrich), into insoluble formazan, visible as a dark-red precipitate, was used as an indicator of bacterial viability (Wrenn and Venosa, 1996). Freshly prepared PVA-cryogel granules with entrapped *Rhodococcus* cells or similar granules after 10-day incubation with/without model petroleum were placed into 96-well microplates, stained with INT solution ($2\ g\ l^{-1}$) and incubated overnight in darkness at room temperature. After incubation, the microplates with stained cryogel granules were examined using a microplate reader (Multiskan Ascent, Thermo, Finland) at 630 nm. Empty PVA cryogel granules containing no bacterial cells were used as optical density controls. A minimum of ten stained cryogel granules with entrapped bacteria were used in parallel for the INT assay. Alternatively, formazan reduced from INT was extracted from the granules with 96% ethanol for 4 h, and the optical density (OD) of extracts was determined at 492 nm. The viability of immobilized