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Biodegradation of nitroglycerin and ethylene glycol dinitrate by free and immobilized mixed cultures

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ARTICLE INFO

Article history:
Received 11 July 2013
Received in revised form
1 October 2013
Accepted 2 October 2013
Available online 12 October 2013

Keywords:
Nitroglycerin
Ethylene glycol dinitrate
Biodegradation
Mixed culture
Free and immobilized cells

ABSTRACT

Aerobic biodegradation of nitroglycerin (NG) and ethylene glycol dinitrate (EGDN), both as individual substrates and in their mixture, was tested using batch or fed-batch cultivation with free suspended cells enriched from a soil sample subjected to a long-term contamination with explosives. EGDN was degraded only in the presence of glycerol as a co-substrate whereas NG could serve as a sole carbon, energy and nitrogen source for growth, its degradation being only slightly boosted by either glycerol or pyruvate. NG was not sufficient as a co-substrate for microbial growth on EGDN; furthermore, the presence of EGDN inhibited the NG degradation. The growth inhibition by both NG and EGDN was alleviated by the addition of glycerol. At an optimum nitroglycerin concentration of 30 mg/L, a maximum specific degradation rate of 60.9 ± 1.8 mg/g_{dw}/h was observed. The biodegradation of both pollutants occurred with a release of nitrite. A method was developed for growing substantial amounts of NG-degrading biomass in the presence of glycerol for its immobilization on expanded slate in a pot-scale packed-bed reactor. Preliminary reactor tests were conducted in a continuous operation mode yielding a 70–90% NG biodegradation up to a load of 20 mg/L/h, with a removal rate up to 16 mg/L/h.

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1. Introduction

Nitric acid esters, e.g., nitroglycerin (NG) and ethylene glycol dinitrate (EGDN), are widely used in the pharmaceutical industry and as explosives; EGDN often replaces NG in dynamite formulations for the cold-climate use due to its lower freezing point. Combined with their well-known microbial toxicity and high persistence to biodegradation (White and Snape, 1993; Pesari and Grasso, 1993), this feature makes nitric acid esters dangerous environmental pollutants and necessitates their on-site degradation.

Several reports have been published on NG biodegradation in wastewater, either as a sole energy and carbon source (Accashian et al., 1998; Marshall and White, 2001) or with glycerol (White et al., 1996a, 1996b), ethyl acetate (Pesari and Grasso, 1993) and glucose (Zhang et al., 1997) as co-substrates. Marshall and White (2001) isolated several strains of four species from NG-contaminated soil but only

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0043-1354/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.watres.2013.10.004

one, *Rhodococcus* sp., was able to degrade NG completely, as a sole carbon and energy source. Several studies on efficient NG biodegradation in soil, either as a sole energy and carbon source (Clausen et al., 2011, 2010; Yost, 2004) or with glucose as a co-substrate (Yost, 2004), have been published.

Only Ramos et al. (1996) and Dario et al. (2010) studied EGDN biodegradation; both reported the necessity of an additional carbon source to achieve any EGDN removal. Ramos et al. (1996) reported an incomplete EGDN biodegradation by the isolates of *Arthrobacter ilicis* and *Agrobacterium radiobacter* using glucose as an additional source of carbon. The released EGMN was a dead-end metabolite. Then Dario et al. (2010) observed a complete EGDN mineralization while using ethylene glycol as a co-substrate, with a transient EGMN production, by several isolates identified as *Bacillus subtilis*, *Bacillus* sp., *Agrobacterium radiobacter* and *Serratia fonticola* (Dario et al., 2010).

The obtained microbiological information on NG and EGDN biodegradation justified the current proof-of-concept biotechnology study, in which two different mixed cultures were prepared and applied for NG and EGDN biodegradation as either sole energy and carbon sources or with co-substrates, documenting the resulting substrate interactions. Unlike the previous studies, the focus of the current study was on exploring the feasibility of NG removal as a sole carbon, energy and nitrogen source by immobilized cells, which is essential for practical applications. Using the results of microbiological experiments with co-substrates, we developed a way of producing larger amounts of efficiently NG-degrading biomass sufficient for immobilization and then applied the obtained immobilized culture in a packed-bed bioreactor to achieve, for the first time, a near-complete NG mineralization in a continuous cultivation mode, without any metabolite accumulation.

2. Material and methods

2.1. Microorganisms and medium

Soil and sediments collected near the Explosia ammunition plant, Pardubice, Czech Republic, which had been subject to a long-term contamination with explosives, including NG, were used as a microbial source. A soil sample suspended in a BSM medium was placed on a rotary shaker and cultivated for 7 days with the addition of NG and EGDN in concentrations of 30 mg/L and 10 mg/L, respectively. The cells were then detached from the soil using a laboratory vibrator followed by an ultrasound bath treatment. The cell suspension was decanted from the remaining soil particles and harvested by centrifugation. Using this procedure, we isolated two mixed cultures (labeled 1 and 2 henceforth) capable of degrading nitrated organics. Both cultures were adapted and enriched using five weeks multiple repeated cultivations with either NG (culture 1) or mononitrotoluenes (MNTs) and subsequently 2,4-dinitrotoluene (culture 2, used in our earlier study on biodegradation of these pollutants – Hudcova et al., 2011) as sole carbon, energy and nitrogen sources. Culture 1 was used in most of the experiments including those with immobilized cells. Culture 2 was used only in the experiments with co-substrates and on substrate interactions.

All experiments were conducted using a basal salt medium (BSM) containing (g/L) K_2HPO_4, 0.23; KH_2PO_4, 0.18; $MgSO_4$, 0.2 and trace elements $FeSO_4$, 2.7×10^{-3}; $ZnSO_4$, 2.8×10^{-3}, $MnSO_4$, 4.5×10^{-3}; $CuSO_4$, 3.2×10^{-3}; $CoCl_2$, 5.4×10^{-5}; $Na_2B_4O_7$, 5.3×10^{-5}; Na_2MoO_4, 8.5×10^{-5}. The experiments were conducted at pH = 6.0 and temperature of 21 °C unless otherwise stated. Both the NG and EGDN used were of a p.a. grade; they were obtained from Explosia as solutions in methanol.

2.2. Reactor configuration and operation

The Erlenmeyer flasks used had a total volume of 250 mL and a working (liquid) volume of 50 mL. Except for the initial growth parameterization studies documented in Table 1, the free suspended cell cultivations were conducted in specifically modified Erlenmeyer flasks on a rotary shaker at 120 rpm at a laboratory temperature of 21 °C and initial medium pH of 6. These modified Erlenmeyer flasks contained extra glass baffles blown out of the flask sides to allow for greater aeration and mixing. NG and/or EGDN were used as the sole carbon, nitrogen and energy sources except for the experiments with co-substrates and those in which large amounts of biomass were grown for immobilization, as specified. In long-term cultivations essential for microbial adaptation to toxic chemicals such as NG and EGDN, cells were centrifuged once a week to prevent a possible inhibition by by-products such as

Table 1 – Influence of experimental conditions on NG degradation by free cells of culture 1, with a starting NG concentration of 15 mg/L an agitation rate of 120 rpm for both the rotary shaker and magnetic stirrer.

Optimized parameters	Parameter values			q_s (mg/g_{dw}/h)
	T (°C)	pH	Configuration	
Configuration	21	7	Plain flask, rotary shaker	39.5 ± 0.6
			Plain flask, magnetic stirrer	46.9 ± 0.2
			Modified flask, rotary shaker	47.4 ± 0.5
pH	21	6	Modified flask, rotary shaker	51.9 ± 0.7
		7		40.4 ± 0.6
		8		27.9 ± 0.4
T	21	7	Modified flask, rotary shaker	39.4 ± 0.6
	27			48.5 ± 0.4

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