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Denitrification of industrial wastewater: Influence of glycerol addition on metabolic activity and community shifts in a microbial consortium

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HIGHLIGHTS

- The wastewater are characterized by a high concentration of nitrates.
- The denitrification rate was at 44 mg N g^{-1} d m d^{-1}.
- The microbial consortium were characterized by a diverse metabolic activity.
- Shifts in the bacterial community dynamics of the consortium could be observed.

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ABSTRACT

The wastewater originating from explosives manufacturing plants are characterized by a high concentration of nitrates (3200 mg N L^{-1}), sulfates (1470 mg L^{-1}) and low pH (1.5) as well as the presence of organic compounds, such as nitroglycerin (1.9 mg L^{-1}) and nitroglycol (4.8 mg L^{-1}). The application of glycerol (C/N = 3) at such a high concentration enabled complete removal of nitrates and did not cause the anaerobic glycerol metabolic pathway of the DNC4 consortium to activate, as confirmed by the low concentrations of 1,3-propanediol (0.16 g L^{-1}) and acetic acid (0.11 g L^{-1}) in the wastewater. Increasing the glycerol content (C/N = 5) contributed to a notable increase in the concentration of both compounds: 1.12 g L^{-1} for acetic acid and 1.82 for 1,3-PD (1,3-propanediol). The nitrate reduction rate was at 44 mg N g^{-1} biomass d^{-1}. In order to assess the metabolic activity of the microorganisms, a method to determine the redox potential was employed. It was established, that the microorganisms can be divided into four groups, based on the determined denitrification efficiency and zero-order nitrate removal constants. The first group, involving *Pseudomonas putida* and *Pseudomonas stutzeri*, accounts for microorganisms capable of the most rapid denitrification, the second involves rapid denitrifying microbes (*Citrobacter freundi* and *Pseudomonas alcaligenes*), the third group are microorganisms exhibiting moderate denitrification ability: *Achrobactrum xylosoxidans*, *Ochrobactrum intermedium* and *Stenotrophomonas maltophila*, while the last group consists of slow denitrifying bacteria: *Rodococcus rubber* and *Sphignobacterium multivorum*.

1. Introduction

During biological denitrification processes nitrates are utilized by bacteria as ultimate electron acceptors in the electron transport chain. As a consequence the nitrates are reduced to gaseous nitrogen forms. Organic compounds are utilized by denitrifying bacteria as sources of carbon and energy. When considering the efficiency of denitrification processes, it is essential to select a carbon source which allows for an optimal reduction of nitrates. At the same time, the selected compound should be relatively cheap and commonly available. This issue is especially important during denitrification of industrial wastewater originating from plants producing explosive materials (Marecik et al., 2013). The wastewater from such plants contains nitrating mixtures during the production of explosives use of nitric acid and sulfuric acid (Hauptmanns, 2009). Due to this, the wastewater is typically characterized by a high concentration of nitrates and sulfates as well as a highly acidic pH value. Another problem corresponds to their specific and unbalanced composition is a high composition of inorganic compounds,

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lack of phosphates and microelements and the presence of poorly biodegradable organic compounds, which pose a significant threat to the environment and may potentially disrupt the functioning of conventional municipal wastewater treatment plants. Such wastewater often contains organic compounds with toxic, mutagenic or even cancerogenic properties (Podlipna et al., 2008, 2010). To date most of the scientific experiments focused on the biodegradation of nitroglycerin and nitroglycol were carried out either under aerobic conditions with the use of bacteria (Ye et al., 2004; Dario et al., 2010), fungi (Sundaram et al., 1997) and plants (Meagher, 2000; Eapen et al., 2007; Rylott and Bruce, 2008) or under anaerobic conditions with the use of anaerobic sludge (Christodoulatos et al., 1997).

However, the applicability of these methods may often be limited by the high concentration of nitrates (Andalib et al., 2011). Due to this reason it was necessary to employ microorganisms which displayed an ability to efficiently reduce nitrates as well as notable tolerance towards the toxic compounds present in the wastewater. In order to maintain optimal growth conditions for such microorganisms and achieve a high treatment efficiency, a constant supply of essential nutrients must be provided (either by direct addition or by mixing with different wastewater streams).

In the case of industrial wastewater with a considerably higher concentration of nitrates the above-mentioned carbon sources were no longer a feasible strategy, hence other viable carbon sources were sought. Numerous different organic compounds were tested – i.e. cellulose (Godini et al., 2011), alcohols, glucose (Aesoy et al., 1998; Elefsiniotis and Li, 2006), formic acid, as well as industrial waste products – i.e. molasses, whey, post-distillation waste (Bernet et al., 1996), fatty waste (Cyplik et al., 2012b), activated sludge hydrolysis products (Kim et al., 2009; Biradar et al., 2010) and many others. Glycerol, a by-product obtained during production of biofuels from plant oils (biodiesel), is considered as a particularly promising carbon source (Hájek and Skopal, 2010). The biotechnological processes which enable bioconversion of waste glycerol into valuable products are a perfect example (Da Silva et al., 2009), however it is also plausible to use this compound as a carbon source during denitrification of industrial wastewater. It should be pointed out that glycerol is a viable carbon source for the denitrification process, as confirmed by the reports of other authors. Hallin et al. (2006) did not observe any significant differences when comparing the denitrification rate with either glycerol or methanol as a carbon source. Bodík et al. (2009) used waste from biodiesel production processes containing 60% glycerol for denitrification of municipal wastewater. Application of this carbon source at a COD/N ratio equal to 1.7 allowed for a complete removal of nitrates from the wastewater.

The primary objective of this study was to evaluate the influence of glycerol addition on the denitrification efficiency of industrial wastewater originating from an explosives production plant carried out by a novel bacterial consortium. The secondary objective was focused on assessing the metabolic activity and bacterial community dynamics during the denitrification process. Additionally, the formation of specific glycerol metabolites (i.e. 1,3-propanediol, 1,3-PD) was also analyzed in the framework of this study.

2. Materials and methods

2.1. Characterization of wastewater

The nitrate processing wastewater was collected from Polish explosives production plant. The determined composition of treated wastes was: 3200 mg N L^{-1}, 48 mg L^{-1} chlorides, 1470 mg L^{-1} sulfates. Moreover, the wastes contained organic compounds: 4.8 mg L^{-1} nitroglycol, 1.9 mg L^{-1} nitroglycerin, and a number of other unknown organic compounds (COD ranged from 1100 to 3700 mg L^{-1}). An addition of microelements was necessary in order to ensure that the denitrification process proceeded properly (g L^{-1}): KH_2PO_4 2.8, NaCl 0.5, NH_4Cl 1.0, $MgSO_4{\cdot}7H_2O$ 0.01, $FeSO_4{\cdot}7H_2O$ 0.001, $MnSO_4{\cdot}4H_2O$ 0.0005, $ZnCl_2$ 0.00064, $CaCl_2{\cdot}6H_2O$ 0.0001, $BaCl_2$ 0.00006, $CoSO_4{\cdot}7H_2O$ 0.000036, $CuSO_4{\cdot}5H_2O$ 0.000036, H_3BO_3 0.00065, H_2MoO_4 0.005, EDTA 0.001, 0.0146 ml L^{-1} HCl (37%).

Due to a low pH value, the wastewater has been alkalized to a value of 9.0 by the addition of NaOH, according to the procedure described in Cyplik et al. (2012a).

2.2. Isolation of microorganisms

The denitrification of industrial wastewater originating from explosives production plant was carried out with the use of the DNC4 microbial consortium isolated from the area of Polish Carpathian Mountains. The isolation was carried out according to the procedure described in Owsianiak et al. (2009).

2.3. Denitrification of wastewater in the bioreactor

The denitrification process was conducted in a BioFlo III type bioreactor (New Brunswick Scientific, USA) with a working volume of 5 L, equipped with an electrode to measure the pH value (Ingold). Stirring was obtained by employing a Rushton turbine. The bioreactor was filled with 5 L of wastewater and afterwards inoculated with the DNC4 consortium (initial concentration of biomass was approx. at 10 ± 0.5 g L^{-1}). The denitrification process was carried out at a temperature of 25 ± 0.5 °C, a pH value of 9.0 and a stirring rate of 100 rpm. Samples were taken once every 24 h.

2.4. Analytical methods

2.4.1. Determination of metabolic activity and separation of microorganisms

Flow cytometric analyses were performed using BD FACS Aria III (Becton Dickinson) flow cytometer (cell sorter), equipped with 4 lasers (375, 405, 488 and 633 nm), 11 fluorescence detectors, forward scatter (FSC) and side scatter (SSC) detectors. Cytometric analysis of metabolic activity among microbial cells, expressed as the redox potential, was conducted with the use of the BacLight Redox Sensor Green Vitality Kit (Invitrogen). The determination of cellular redox potential of analyzed microorganisms was based on comparison of green fluorescence intensity from redox potential-sensitive reagent (RedoxSensor Green) between negative and positive samples. Negative samples represented the reference samples for the normalization of fluorescence signals due to addition of the electron transport chain uncoupler which reduces the redox potential of analyzed cells. The difference in medians of green fluorescence signals expressed as relative fluorescence units (RFU) between positive and negative samples corresponds to the redox potential of analyzed cells.

The optical alignment and the functional stability tests were carried out with the use of the Cytometer Setup and Tracking system (Becton Dickinson). FACS Flow solution (Becton Dickinson) was used as the sheath fluid. The cytometer operated under the following conditions: 70 μm sorting nozzle and sheath fluid pressure at 483 kPa. The characterization of cells was conducted based on two non-fluorescent parameters – FSC and SSC and two fluorescent parameters – green fluorescence (FITC detector) from the RedoxSensor Green reagent (detection with the use of a 530/30 optical filter) and red fluorescence (PE-Texas Red detector) from propidium iodide (detection with the use of a 616/23 optical filter). The excitation of both fluorochromes employed in the analysis was obtained by using a 288 nm blue laser. The cytometric analysis was