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Enrichment of anodophilic nitrogen fixing bacteria in a bioelectrochemical system

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ARTICLE INFO

Article history:
Received 5 March 2014
Received in revised form
15 June 2014
Accepted 30 June 2014
Available online 9 July 2014

Keywords:
Microbial fuel cell
Bioelectrochemical system
Nitrogen fixation
Nitrogen fixing bacteria
Diazotroph

ABSTRACT

We demonstrated the ability of a bio-anode to fix dinitrogen (N_2), and confirmed that diazotrophs can be used to treat N-deficient wastewater in a bioelectrochemical system (BES). A two-compartment BES was fed with an N-deficient medium containing glucose for >200 days. The average glucose and COD removal at an anodic potential of +200 mV vs. Ag/AgCl was 100% and 76%, respectively. Glucose removal occurred via fermentation under open circuit (OC), with acetate as the key byproduct. Closing circuit remarkably reduced acetate accumulation, suggesting the biofilm could oxidise acetate under N-deficient conditions. Nitrogen fixation required an anode and glucose; removing either reduced N_2 fixation significantly. This suggests that diazotroph utilised glucose directly at the anode or indirectly through syntrophic interaction of an N_2-fixing fermenter and an anodophile. The enriched biofilm was dominated (68%) by the genus *Clostridium*, members of which are known to be electrochemically active and capable of fixing N_2.

1. Introduction

Industrial wastewaters, including wastewater produced from pulp and paper industries, are carbon (C) rich but nitrogen (N) deficient (Pratt et al., 2007; Pokhrel and Viraraghavan, 2004). To enable efficient biological treatment, a C:N ratio of 100:5 in the raw influent is usually recommended (Peng et al., 2003; Slade et al., 2011). Hence, external supplementation of N (as ammonium or nitrate) is needed to treat N-deficient wastewater (Dennis et al., 2004). N supplementation incurs costs, and intense monitoring is required to prevent discharge of excess N to the environment (Gauthier et al., 2000).

As an alternative to supplementing N, the use of diazotrophic (N_2-fixing) bacteria has been proposed as a method for treating N-deficient wastewater in activated sludge systems (Pratt et al., 2007; Gauthier et al., 2000). N_2-fixing bacteria are capable of converting atmospheric nitrogen (N_2) to ammonia (NH_3) as a means of supplementing N requirements for growth (Nair, 2010). Biological N_2 fixation is catalysed by the nitrogenase enzyme complex, and the reduction of N_2 to NH_3 takes place according to reaction (1) (Nair, 2010):

$$N_2 + 8H^+ + 8e^- + 16ATP \rightarrow 2NH_3 + H_2 + 16ADP + 16Pi \quad (1)$$

Although N_2-fixing bacteria could be used in activated sludge processes to oxidise carbon in N-deficient wastewater, the widespread use of this approach has not been possible because nitrogenase is irreversibly inhibited by oxygen (O_2),

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http://dx.doi.org/10.1016/j.watres.2014.06.046
0043-1354/

and conventional activated sludge processes require aeration to facilitate oxidation of organic carbon (Nair, 2010). To prevent O_2 inhibition of nitrogenase, diazotrophs often secrete extracellular polymeric substances (EPS; also known as slime) to limit O_2 diffusion into cells (Nair, 2010). Excessive EPS production can cause sludge bulking, resulting in poor solid/liquid separation and reduced effluent quality (Peng et al., 2003). Hence, the use of N_2-fixing activated sludge processes to oxidise carbon in N-deficient wastewater is problematic.

One approach to eliminating the negative impact of O_2 on N_2 fixation is to combine N_2-fixing microorganisms with bioelectrochemical systems (BESs). A typical BES consists of an anode and a cathode chamber (Logan et al., 2008). The anodic chamber facilitates the growth of microorganisms (anode respiring bacteria; ARB) under anaerobic conditions, using the electrode (anode) as the sole electron acceptor. If the ARB are diazotrophs, oxidation of N-deficient wastewater in the absence of O_2 becomes feasible because a solid electrode (not O_2) is the final electron acceptor. The anode potential regulates the thermodynamics of bacterial metabolism. Therefore, N_2 fixation in diazotrophic ARB is likely to be regulated by the anodic potential. Consequently, inhibitory effects of O_2 on N_2 fixation may be eliminated because of the maintenance of anaerobic conditions in the anode chamber. The electrons donated by the ARB flow to the cathode via an external circuit, where they combine with protons and O_2 to form water (Logan et al., 2008).

Current knowledge of diazotrophic ARB (DARB) is limited. Although potential diazotrophs including *Azoarcus*, *Clostridium* and *Geobacter* have been reported in association with anodes of microbial fuel cells (MFCs) under N supplemented conditions (Phung et al., 2004; Kim et al., 2004), it is unclear whether these bacteria met their N requirements via fixation of atmospheric N_2. Belleville et al. (2011) and Clauwaert et al. (2007) operated BESs to treat N-deficient wastewater, but did not provide direct experimental evidence of N_2 fixation, although they assumed that this was how the N requirements of the ARB were met, and did not investigate the bacterial diversity in their systems.

The objectives of this study were to: (1) investigate the efficiency of anodic oxidation of an N-deficient wastewater by an enriched microbial biofilm community; (2) elucidate the possible routes of glucose metabolism by the anodic biofilm; (3) assess the influence of anodic current production on N_2 fixation; and (4) characterise the enriched anodic bacterial community using 454 sequencing.

2. Materials and methods

2.1. Composition of the N-deficient medium

The synthetic N-deficient medium used in this study contained glucose as the sole source of carbon and energy, and represents wastewater characteristic of pulp and paper, and sugar refining industries. The medium contained (per litre of DI water): $MgSO_4 \cdot 7H_2O$ (25 mg), $CaCl_2 \cdot 2H_2O$ (25 mg), glucose monohydrate (374–1684 mg), KH_2PO_4 (2300 mg), K_2HPO_4 (5750 mg) and 0.40 mL of trace element solution. The trace element solution contained (per litre): nitrilotriacetic acid (5000 mg), H_3BO_3 (310 mg), $FeSO_4 \cdot 7H_2O$ (267 mg), $CoSO_4 \cdot 7H_2O$ (128 mg), $CuSO_4 \cdot 5H_2O$ (11 mg), $MnCl_2 \cdot 4H_2O$ (9.6 mg), $Na_2MoO_4 \cdot 2H_2O$ (267 mg) and $ZnSO_4 \cdot 7H_2O$ (128 mg). The addition of nitrilotriacetic acid (0.15 mg-N L^{-1}) resulted in a measurable dissolved organic N level of approximately 0.30 ± 0.14 mg L^{-1} in the anodic feed. The medium was continuously supplied to the anodic chamber of the BES at a flow rate of 0.30–1.20 mL min^{-1}. The cathodic medium was identical to the anolyte, with the exception of glucose. The catholyte was replaced biweekly to avoid accumulation of ionic species.

2.2. Construction and operation of the bioelectrochemical system

A two-chamber BES described by Cheng et al. (2012) was used to enrich an electrochemically active biofilm in the anodic chamber exposed to N-deficient conditions. Fig. 1 provides a schematic of the BES reactor setup. The BES was continuously operated for more than 200 days. The anodic and cathodic chambers were filled with granular graphite as the electrode material. The electrode covered with the anodophilic biofilm is referred to as the working electrode, and the cathode is referred to as the counter electrode. An Ag/AgCl reference electrode (MF-2079 Bioanalytical Systems) was embedded among the graphite granules of the working electrode. The reference electrode was intermittently checked against a new reference electrode, and was found to remain functional throughout the experimental period. Anolyte (400 mL) and catholyte (2000 mL) were continuously recirculated through the anodic and cathodic chambers, respectively, at a flow rate of 160 mL min^{-1} using a peristaltic pump (Console drive, Cole–Parmer). The anodic chamber of the BES was inoculated with soil microorganisms. The inoculum was prepared by incubating approximately 200 g of soil (obtained from a local garden in Perth, Australia) in 800 mL of anolyte medium at 35 °C overnight.

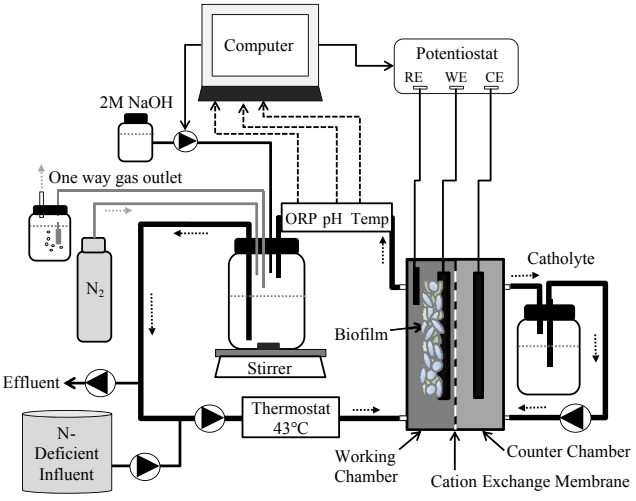

Fig. 1 – Schematic diagram of the two-chamber BES operated in continuous mode. RE = reference electrode; WE = working electrode; CE = counter electrode.