Journal of Environmental Management 169 (2016) 34–45

Contents lists available at ScienceDirect

Journal of Environmental Management

journal homepage: www.elsevier.com/locate/jenvman

Research article

Physiological and functional diversity of phenol degraders isolated from phenol-grown aerobic granules: Phenol degradation kinetics and trichloroethylene co-metabolic activities

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ARTICLE INFO

Article history:
Received 2 July 2015
Received in revised form
11 December 2015
Accepted 14 December 2015
Available online 22 December 2015

Keywords:
Aerobic granules
Phenol
Trichloroethylene
Co-metabolism
Diversity

ABSTRACT

Aerobic granule is a novel form of microbial aggregate capable of degrading toxic and recalcitrant substances. Aerobic granules have been formed on phenol as the growth substrate, and used to co-metabolically degrade trichloroethylene (TCE), a synthetic solvent not supporting aerobic microbial growth. Granule formation process, rate limiting factors and the comprehensive toxic effects of phenol and TCE had been systematically studied. To further explore their potential at the level of microbial population and functions, phenol degraders were isolated and purified from mature granules in this study. Phenol and TCE degradation kinetics of 15 strains were determined, together with their TCE transformation capacities and other physiological characteristics. Isolation in the presence of phenol and TCE exerted stress on microbial populations, but the procedure was able to preserve their diversity. Wide variation was found with the isolates' kinetic behaviors, with the parameters often spanning 3 orders of magnitude. Haldane kinetics described phenol degradation well, and the isolates exhibited actual maximum phenol-dependent oxygen utilization rates of 9–449 mg DO g DW^{-1} h^{-1}, in phenol concentration range of 4.8–406 mg L^{-1}. Both Michaelis–Menten and Haldane types were observed for TCE transformation, with the actual maximum rate of 1.04–21.1 mg TCE g DW^{-1} h^{-1} occurring between TCE concentrations of 0.42–4.90 mg L^{-1}. The TCE transformation capacities and growth yields on phenol ranged from 20–115 mg TCE g DW^{-1} and 0.46–1.22 g DW g $phenol^{-1}$, respectively, resulting in TCE transformation yields of 10–70 mg TCE g $phenol^{-1}$. Contact angles of the isolates were between 34° and 82°, suggesting both hydrophobic and hydrophilic cell surface. The diversity in the isolates is a great advantage, as it enables granules to be versatile and adaptive under different operational conditions.

1. Introduction

Aerobic granules, a relatively novel form of microbial aggregate, have been drawing increasing research and application interests. Formed under aerobic conditions and without carriers, aerobic granules have round shape, compact structure, high settling speed, and ability to withstand toxic substances (Marques et al., 2013). Aerobic granular sludge (AGS) reactors have successfully treated various toxic, recalcitrant and inhibitory substrates, including phenol (Jiang et al., 2002), *p*-nitro phenol (Yi et al., 2006), halogenated phenols (Carruci et al., 2009; Duque et al., 2011; Wang et al., 2007), and various industrial wastewaters (Val del Río et al., 2012). Though toxic, these substances could all support microbial growth as either sole or partial carbon/energy sources. However, some synthetic compounds like di- and tri-chloroethylene (TCE) can seldom serve as growth substrates for indigenous microorganisms (Morono et al., 2004; Mukherjee and Roy, 2012), but can be efficiently removed via non-growth linked mechanisms such as co-metabolism (Nzila, 2013).

Co-metabolism is the biotransformation catalyzed by a non-specific enzyme or cofactor generated in the concurrent metabolism of a growth-supporting substrate (Dalton and Stirling, 1982). TCE has been widely used as the model compound to study aerobic co-metabolism, and phenol has been identified as a good growth substrate, due to the high growth and TCE degradation rates of phenol degraders (Wang et al., 2015). Phenol cultures

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http://dx.doi.org/10.1016/j.jenvman.2015.12.021
0301-4797/

also exhibited high tolerance to TCE transformation product toxicity (TPT), which is an effect caused by certain product(s) of TCE transformation that covalently bind cellular materials (Hopkins et al., 1993). TPT can cause cellular function loss and cell death, therefore certain amount of biomass could only transform a finite quantity of TCE before total inactivation occurs (Alvarez-Cohen and McCarty, 1991). The ratio of TCE transformed to biomass is defined as the transformation capacity (T_C).

As aerobic granules show remarkable potential in degrading toxic substances, their possible application in co-metabolism has been investigated. In previous studies, aerobic granulation was conducted with phenol as the sole growth substrate (Zhang and Tay, 2012). The rate limiting factors were identified, and the inhibitory and toxic effects of phenol/TCE were comprehensively studied (Zhang and Tay, 2014, 2015). In this study, it is intended to further explore the potential of phenol-grown aerobic granules in terms of the individual microbial populations therein. Phenol degraders previously isolated from aerobic granules had exhibited diverse phenol degrading activities (Jiang et al., 2004), indicating a large pool of microbial population therein that can be exploited for co-metabolic application. Therefore pure cultures were isolated, purified, and screened using stable phenol-grown aerobic granules as the seed. The objective was to obtain preliminary knowledge on the physiological and functional diversity of phenol-degrading bacteria in the aerobic granules, to identify their phenol and TCE degradation activities, and to select candidate(s) for possible TCE co-metabolism application.

2. Materials and methods

2.1. Source of microbial seeds and pure cultures

Two rounds of granulations had been conducted, by Jiang et al. (2002) and Zhang and Tay (2012). For the first batch, isolation of pure cultures had been done, and 10 phenol-degrading isolates were obtained (Jiang et al., 2004). They were designated PG01 to PG10, and their phenol degradation kinetics were studied, but their TCE transformation ability was unknown. Isolation of phenol-degraders was performed again in this study, using the 2nd batch of phenol-grown aerobic granules as the seed. Mature granules were obtained from a stably maintained AGS reactor fed with phenol for several months. The details of the reactor configuration, operation, granulation process and the changes in the biomass' phenol and TCE degradation activities have been described in detail in the previous study (Zhang and Tay, 2012).

2.2. Chemicals and media

Phenol and TCE stock solutions were prepared as previously described (Zhang and Tay, 2012). Bacterial counting and isolation were performed on four sets of agar plates, the first of which was total plate counting (TPC, 2.35% plate count agar, Difco 247940). For the other 3 sets, 1.2% American bacteriological agar (Pronadisa) was used, supplemented with minerals, vitamins and micronutrients according to Jiang et al. (2004). Phenol was supplied to the 2nd and 3rd sets (phenol and phenol-TCE, respectively) at 500 mg L^{-1}. The TPC and phenol sets were incubated in normal air, and the phenol-TCE set in an 8 L desiccator together with a mixture of 30 mL TCE-saturated water and 20 mL plain water. Being volatile, TCE evaporates and distributes between liquid and gas, the equilibrium of which is described by its Henry's law constant (Gossett, 1987). The equilibrium TCE concentration in the desiccator' air was calculated as approx. 5 mg L^{-1}, and the TCE solution was replaced every 2–3 days. The 4th set, mineral phosphate buffer (MP)-TCE plates was also incubated under TCE atmosphere, but without phenol in the agar. After isolation, the cultures were grown and maintained in liquid MP medium supplied with 250 mg L^{-1} phenol for further investigation. All chemicals used were of analytical or molecular biology grade.

2.3. Preparation of inoculums

Aerobic granules were harvested from the stably operated reactor, mixed and washed twice with sterile MP medium, then grinded to small particles with a sterile mortar and pestle for at least 20 min. The grinded sludge was checked with microscope to ensure that the cells were sufficiently separated, then collected and resuspended in fresh MP medium, and the biomass concentration was measured. 100 μL of the same sample was serial diluted and spread on agar plates. The colonies that appeared on triplicate plates were periodically counted according to Barnett (1990).

2.4. Purification and isolation

After the colonies appeared on phenol and phenol-TCE plates, some were picked for purification (TPC plates were not used), and the principles for selection were:

- Fast growth, big colony, early appearance
- Representative morphology
- Growth on the highest dilution plates

Accordingly, colonies were picked and streaked on phenol plates in duplicates. The plates sourced from phenol-TCE plates were incubated in normal and TCE atmosphere each. Colonies were then examined by eyes and light microscopy after gram staining. When uniform morphology was obtained on one plate, and the colony on it was uniform under microscopy, the culture was considered pure. The differentiation and description of colony morphologies were done according to Benson (2002).

2.5. Differentiation between isolates of similar morphology

Repetitive extragenic palindromic sequence-polymerase chain reaction (REP-PCR) was adopted as a culture-independent method to confirm the results of visual inspection (Jiang et al., 2004). DNA was extracted from colonies and amplified with BOX-air primers, and the products visualized on agarose gel. When all the bands aligned, two cultures were considered the same bacterium. The detailed procedure is supplied in the supplementary materials.

2.6. Growth and preparation of pure culture for kinetic studies

MP medium with 250 mg L^{-1} phenol was inoculated with the isolates, then shaken at 150 rpm. A dilution rate of 0.33–0.67 d^{-1} was adopted to ensure the cultures were in healthy growth. Phenol concentrations were measured before the dilution to ascertain its depletion. Cells were collected by centrifugation (3300 rpm, 15 min), washed with MP medium, and resuspended in the same medium at various concentrations for kinetic studies. Cell purity was checked at the beginning and end of incubation by microscopy after gram staining, and no significant contamination was observed.

2.7. Phenol and TCE kinetic studies and TCE transformation capacities

Phenol kinetics was expressed as specific oxygen utilization rates (SOUR) versus phenol concentrations, determined according to standard method (APHA, 2005). TCE removal was indicated by its