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Use of DGGE for Identification and Descriptive Quantification of Xenobiotic Degrader in Activated Sludge

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Abstract **-**Activated sludge gains xenobiotic degradation capability through acclimation to the target. Acclimated activated sludge contains a two-part biomass of non-degrader and degrader, abundance of the latter being most important for activated sludge to successfully treat the inflow of xenobiotic pollutants. For the purposes of understanding and also possible improvement of xenobiotic treatment, xenobiotic degraders contained in the activated sludge biomass must be identified and quantified. This study employed the DGGE method which separates bacterial species into distinctive gel bands that allowed achievement of both purposes relatively efficiently.

Keywords**:** Xenobiotic degrader, activated sludge, degradative capacity, DGGE

1. Introduction

Activated sludge is a consortium of microorganisms that are generally cultured with biogenic organic substrates from the polluting streams. Under this natural situation, activated sludge microbes (indigenous microorganisms) are not able to metabolize a xenobiotic organic. Through the process of acclimation, in which indigenous sludge is put in reaction with the target xenobiotic until the sludge finally degraded the target, some originally indigenous activated sludge cells gain xenobiotic degradation capability and are converted degraders (Chong, 2005). In practical application of the activated sludge process in the treatment of a target xenobiotic pollutant(s), the kinetics of degradation is often a major concern. Since degradation rate is related to the amount of degrader present in the system, degrader quantity contained therein must be measured. In an activated sludge system where xenobiotic degraders have risen from acclimation, degraders and non-degraders co-exist (Chong, 2009). The dynamic changes between degraders and non-degraders, and the resultant degrader quantities in a xenobiotic degrading activated sludge reactor at some state of operation, must be fully monitored so that the correct description of the behaviour or performance of the bioreactor is possible. To fulfil this monitoring goal, identification and quantification of xenobiotic degraders become a mandatory task for scientists and engineers alike.

In the literature, xenobiotic degraders are frequently addressed with degrader identification conducted with modern molecular biology technologies such as Denaturing Gradient Gel Electrophoresis (DGGE), real-time quantitative PCR, or pyrosequencing. Examples of degrader identification works include those of Luo et al (2009) for benzo(a)pyrene (a PAH) degraders, Cho et al. (2013) for hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) degraders, and Chi et al. (2013) for para-, meta- and ortho-nitrophenol degraders. Chong and co-workers have based xenobiotic degradation on the quantification of degraders (Chong, 2009; Chong and Huang, 2012; Chong et al., 2008). Among the methods of identification of specific bacterial species (or genera) in mixed consortia, DGGE is often used; some example publications of DGGE application are Cea et al. (2013), Luo et al (2009), Tian et al. (2008), and Zhang et al. (2015).

The purpose of this study was to identify xenobiotic degraders contained in activated sludge that were cultivated in different types of acclimation reactors. The method of DGGE that separates bacteria species was employed and by comparisons of DGGE bands shown for the acclimated and indigenous sludge, degraders were identified. Band densities of the species identified to be degraders were analyzed

so that the proportions of degraders in the bulk sludge were qualitatively estimated. The applicability of the methods to practical treatment systems is commented.

2. Materials And Methods

2. 1. Sample Xenobiotic And Activated Sludge

The xenobiotic compound used as the activated sludge degradation/treatment target was the herbicide 2,4-dichlorphenoxyacetic acid (2,4-D).

Activated sludge seed that was originally indigenous to 2,4-D was obtained from a soil that did not have any record of 2,4-D nor metal (slag) contamination. The mixed culture from soil was first enlarged in Nutrient Broth and subsequently seeded to a long-term cultivation reactor that was operated in a fed-batch mode. The fed-batch reactor was re-fed once everyday with fresh biogenic substrates containing 100 mg/L sucrose and 25 mg/L peptone, and minerals listed below. One-tenth (1/10) volume of fed-batch reactor suspension was wasted everyday so that a mean cell resident time (θc) of 10 d was achieved. Sludge from the fed-batch reactor was used to seed all test reactors.

The types of test reactor used in this study to obtain activated sludge having different 2,4-D degradative characteristics were shown in Table 1. Activated sludge in RD was fully acclimated to 2,4-D by undergoing three (3) consecutive complete degradation of 2,4-D, after which activated sludge settled, supernatant discarded, and 2,4-D re-fed to the original reactor at the end of the first- and second-time degradation. For each of the continuous-flow reactor (CSTR) of RA and RC (reactor volume 5L, with cell-recycle from an external clarifier), an influent was introduced by peristaltic laboratory pump with flowrate adjusted to achieve the hydraulic residence time (θ). Mean-cell residence time (θc) was maintained by wasting sludge mass twice daily from the mixed liquor, each time one-twentieth of mixed liquor was wasted and refilled to the original level with the clear effluent. RA and RC were operated uninterrupted for more than four (4) θc (45 d) to reach a steady state. At steady state, 2,4-D from RC was undetectable and the RC activated sludge was considered fully acclimated to 2,4-D. Overall, RB contained indigenous microorganisms for comparison (control) to the acclimated microorganisms from RD; similarly, indigenous sludge of RA was to compare to that of RC.

The common feed of minerals and their concentrations were: $FeCl_3$, 1.2 mg/L; $CaCl_2$, 12 mg/L; MgSO4.7H_2O, 65 mg/L; NH_4Cl, 125 mg/L; K_2HPO_4, 200.0 mg/L; and KH_2PO_4, 156.6 mg/L.

Activated sludge concentration was measured as suspended solids (SS), 2,4-D was measured with HPLC.

2. 2. PCR-DGGE Analysis

Denaturing gradient gel electrophoresis (DGGE) is a molecular fingerprinting method that separates multiple DNA products of polymerase chain reaction (PCR). Different DNA sequences from different microorganisms, when generated by PCR, are similar in size (base-pair). When all of those products were run on conventional agarose gel electrophoresis, only a single DNA band will result. During DGGE, PCR products encounter increasingly higher concentrations of chemical denaturant as they migrate through a polyacrylamide gel. Differing sequences of DNA denature at different denaturant concentrations thus resulting in a series of bands, with each band theoretically representing a different bacterial population present in the community.

Activated sludge biomasses from multiple reactors of the same type were mixed in equal proportions and equal masses were sampled from the suspension of each reactor. Bacterial DNA from each mixture suspension was extracted using the EZ-10 spin column genomic DNA miniprep kit (Bio Basic Inc., Canada), according to the manufacturer's instructions. PCR amplification of the V3 region of the bacterial 16S rRNA gene was performed using primers 338F-GC (5'-CGCCCGCGCGCGGCGGGCGGGGCGGGGGCACG GGGGGACTCCTACGGGAGGCAGCAG-3'; GC-clamp underlined)) and 518R (5'-TTAC CGCGGCTGCTGG -3'). PCR reaction was performed under the following conditions: 94°C for 5 min

(initial denaturation), 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 20 s. The PCR products were confirmed by running 5 μL aliquots of the reaction mixtures on 1~3% agarose gels. PCR amplicons were separated using DGGE, carried out using the Dcode Universal Mutation Detection System (Bio-Rad Laboratories, Inc., USA). The PCR products were loaded onto 8% (w/v) polyacrylamide gel with a linear gradient of denaturants, including urea and formamide. A 30%-60% denaturing gradient was used, in which the 100% denaturant contained 7M urea and 40% (v/v) formamide. Electrophoresis was initially run at 60°C for 30 min, then for 6 h at a constant voltage of 160 V. After electrophoresis, the gels were stained using silver stain (gel bond) for 30 min. Images were acquired by visualization and photographed on a UV transilluminator.

Relative band position and band density of the DGGE gel image were analyzed by the software GelAnalyzer 2010 (freeware, see Ref. Web-1).

Table. 1. Reactor types use in this study.

Designation	Type	Feed (mg/L) [a,b]	Operation	Resulting Sludge
RB	Batch	Sucrose: 100; peptone: 25	100 rpm, 25±2°C [c]	Batch grown indigenous
RA	CSTR	Sucrose: 100; peptone: 25	θ = 8 h; θc = 10 d [d] 25±2°C [c]; diffused air aeration	Continuous grown indigenous
RD	Batch	2,4-D: 100	100 rpm, 25±2°C [c]	Acclimation to 2,4-D
RC	CSTR	2,4-D: 100	θ = 8 h; θc = 10 d [d] 25±2°C [c]; diffused air aeration	Acclimation to 2,4-D

[a] Microbial seeded: indigenous sludge from fed-batch reactor, ≈ 100 mg-SS/L initially; [b] common minerals; [c] Room temperature; [d] θ: Hydraulic residence time; θc=Mean-cell residence time.

3. Results And Discussion

Fig. 1a shows four (4) lanes of DGGE images of activated sludge species, part of whose 16SrRNA were separated into bands. Lane X represents RA indigenous sludge grown in CSTRs, Lane A represents RA acclimated sludge from 2,4-D fed CSTRs, Lane Y represents RB indigenous sludge grown in shake-flasks, and Lane B represents RD acclimated sludge from 2,4-D fed flasks. Lane X and Y must show all non-degraders. Comparisons made between Lanes X and A, and Lanes Y and B facilitate identification of degrader evolved in sludge from batch and steady state CSTR acclimation, respectively. From Fig. 1b, those species (marked N) that existed in both indigenous and acclimated sludge were considered all or partially non-degrader containing, while degraders were identified from those bands that had density darkened (quantity enlarged) or newly appeared in the acclimated sludge.

Lanes A and B were also compared to show the different patterns of degrader evolution between batch and CSTR acclimations. Shown from the number of bands and/or band intensity (Fig. 1a), the batch acclimation mode indeed put a heavier acclimation force for selecting a few highly capable degraders and thus microbial diversity was distinctively less than that of the CSTR-acclimated biomass. Furthermore, 2,4-D acclimated activated sludge from CSTR shows the existence of non-degraders that have abundances quite similar to activated sludge of the biogenic CSTR (Lane X and A).

Degrader contents (percentages) in Lane A and Lane B were estimated by dividing the units for the sums of degrader band density by that of the total band density; sums of degrader density were measured from all independent bands, and the extra darkness of bands common with the respective control. Degrader contents were estimated to be approximately 63 and 45% for the batch- and steady state CSTR-acclimated sludge, respectively.

4. Conclusions

Xenobiotic degradative capability acquired from acclimation of activated sludge was found to arise from evolution of degrader species. DGGE method is a feasible method by which the identification and descriptive quantification of degrader are achievable. Sufficient insights into the results of xenobiotic acclimation reactions were gained serving scientific and practical purposes. Refinement of the method for more accuracy can be useful for better understanding and thus improvement of the xenobiotic treatment bioprocess.

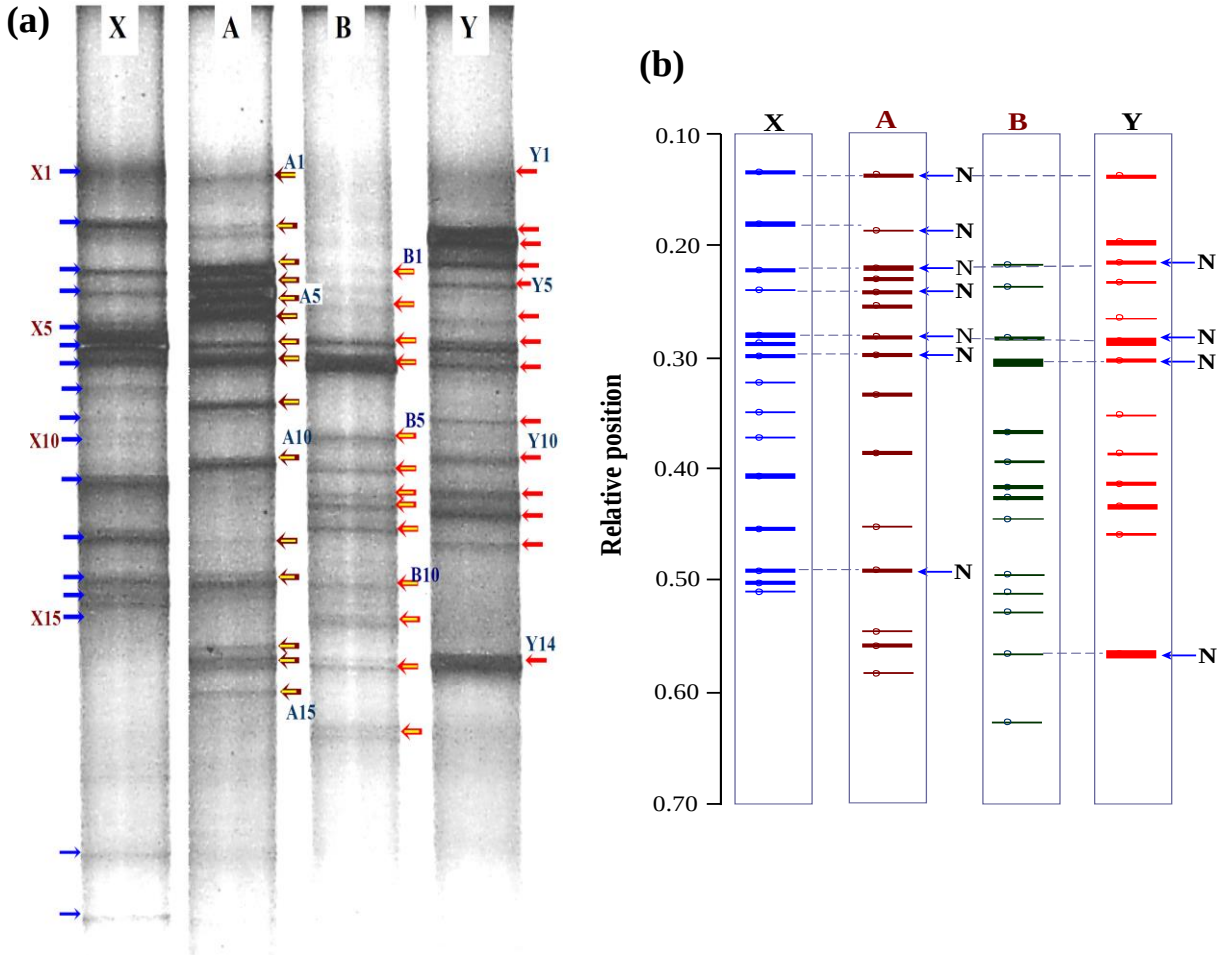

Fig. 1 (a) Gel images of DGGE analyses of activated sludge and (b) relative positions of bands to identify degrader bacterial genera. Activated sludge shown are: Lane X: RA (CSTR, indigenous) at steady state; Lane A: RC (CSTR, acclimated) at steady state; Lane B: RD (batch, three-time acclimated); Y: RB (batch, indigenous). Lanes X and Y contain all non-degraders.

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