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Rapid fluorescence-based measurement of toxicity in anaerobic digestion

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ABSTRACT

A rapid fluorescence measurement based on resazurin reduction was developed and applied for the detection of toxicants/inhibitors to anaerobic digestion metabolism. By initially using a pure facultative anaerobic strain, *Enterococcus faecalis* as a model organism, this technique proved to be fast and sensitive when detecting the model toxicant, pentachlorophenol (PCP). The technique revealed significant metabolic changes in *Enterococcus faecalis* with a PCP spike ranging from 0.05 to 100 mg/L, and could detect PCP's toxicity to *E. faecalis* at a concentration of only 0.05 mg/L in 8 min. Furthermore, by extending this technique to a mixed anaerobic sludge, not only could the effect of 0.05–100 mg/L PCP be determined on anaerobic digestion metabolism within 10 min, but also its rate of biogas production. These results suggest that a resazurin-based fluorescence measurement can potentially be incorporated into a microfluidic system to develop a biosensor for the real-time monitoring, control and early warning of toxicant/inhibitor loads in the influent to an anaerobic digestion system.

1. Introduction

In recent years increasing attention is being focused on anaerobic wastewater treatment due to concerns about energy use, solids production and disposal, carbon footprint, and resource recovery. Anaerobic digestion utilizes a microbial consortium which breaks down biodegradable organic materials in the absence of oxygen, and can treat sewage as well as hospital and industrial effluent to recycle water and produce renewable energy (Rezaee et al., 2005; Show et al., 2010; Torres et al., 2013; Dereli et al., 2014). However, anaerobic digestion is sensitive to a wide range of toxicants, and when they are present in inhibitory concentrations in wastewater, digester upset or failure can occur (Chen et al., 2008). Off-line monitoring of toxicants using GC and HPLC has been widely used, however, measurement delays result in slow responses to these shocks. Such delayed feedback responses can lead to

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difficulties in preventing anaerobic digestion system failure. Real-time monitoring using RANTOX (rapid anaerobic load and toxicity tester) (Rozzi et al., 1999) or MFCs (microbial fuel cells) (Liu et al., 2011; Kaur et al., 2013) show some potential in detecting toxic loads, or reflecting real time microbial activity in anaerobic digestion. However, long response times, or their focus on measuring specific compounds, make these methods limited in scope.

Sensors which give advanced warning when shock/toxic loads are present in the feed are urgently needed in order to treat industrial wastewaters and prevent irreversible damage to the bioprocess. Optical methods are promising as they allow direct and rapid metabolic measurements of the culture activity. The resazurin reduction (RR) assay, previously commercialized under the trade names of Alamar Blue or CellTiter-Blue, utilize resazurin and resorufin as oxidation-reduction indicators that yield colorimetric changes and fluorescent signals in response to intercellular metabolic activity (Fig. 1) (Pratten et al., 2012). The RR assay has been shown to be a simple, fast and cheap way to continuously monitor living cell proliferation, and the viability of bacterial and eukaryotic cells due to its high water solubility, stability in culture medium, non-toxicity and permeability through cell membranes (O'Brien et al., 2000). As an indicator with fluorescent end-points, Alamar Blue was used to develop a rapid method to examine the antimicrobial efficacy of oral care formulations (Sreenivasan et al., 2003), and a rapid resazurin bioassay also proved valuable for screening chemical libraries for fungicides and as a biomarker for detecting the effects of fungicides on non-target fungi (Fai and Grant, 2009). In addition, many tetrazolium salts have become some of the most widely used tools in cell biology for measuring the metabolic activity of cells such as in the MTT assay (Berridge et al., 2005). However, despite yielding sensitive chromophores or fluorophores in response to intercellular metabolic activity, most of these assays with tetrazolium-based dyes were carried out under aerobic conditions.

To the best of our knowledge, the use of resazurin as a biotoxicity indicator for anaerobic digestion has not been rigorously investigated. The properties of resazurin may allow for the development of a rapid assessment sensor which measures the real time metabolic activity change in anaerobic cultures exposed to toxicants. In this work we present a rapid and flexible assay based on optical measurements of resazurin reduction by a pure strain of facultative anaerobes (*Enterococcus faecalis*) and mixed anaerobic sludge with and without a spiked toxicant, pentachlorophenol (PCP). We suggest that the resazurin reduction assay has considerable potential to be developed into a biotoxicity sensor to rapidly assess the inhibition of anaerobic digestion sludge for biogas production.

2. Material and methods

2.1. Chemicals

Resazurin (7-hydroxy-3H-phenoxazin-3-one 10-oxide), brain heart infusion (BHI) broth and pentachlorophenol (PCP) were of high purity and were from Sigma–Aldrich, Singapore. Other reagents and chemicals used were the highest available purity, and were obtained from Sigma–Aldrich, Singapore.

2.2. Bacterial strain and anaerobic sludge

Enterococcus faecalis (E. faecalis) OG1RF (ATCC® 47077™) stored at −80 °C in BHI broth/25% glycerol was gently thawed and 100 μL injected anaerobically into 10 mL of N_2 bubbled BHI sterile medium. The culture was incubated overnight and 100 μL was inoculated into another 10 mL of BHI medium and subcultured until the middle log phase (~3 h). A 2 L anaerobic reactor was operated at ~30 °C as the seed for the toxicity and activity test. The reactor was fed weekly for 4 circles with a synthetic medium (Table 1) and acetic acid to give a final COD (chemical oxygen demand) around 2 g/L. The methane production was monitored daily by the displacement of a water column after trapping CO_2 with 0.5 M NaOH.

2.3. E. faecalis spiked with PCP: fluorescence and absorbance measurements

Subcultured *Enterococcus faecalis* in their middle log growth phase described above were mixed with autoclaved BHI broth in a 96-well microplate to get a final OD_{600nm} of 0.1 in a 300 μL/well. Resazurin was added to each cell to a final concentration of 25 mg/L, and PCP was spiked to yield final concentrations of 0 (control), 0.05, 0.5, 5, 10 and 100 mg/L. The 96-well microplate was sealed immediately under anaerobic conditions by a sterile film. Triplicate experiments for each test were carried out at 37 °C, and fluorescence was read once every 2 min over 30 min by an Infinite M200 Pro microplate reader (TECAN, Singapore) with TECAN i-control software at $\lambda_{ex} = 530$ nm and $\lambda_{em} = 590$ nm with a gain of 30. For the reaction described in Fig. 1, many researchers, e.g. Zhou et al. (1997) and Perrot et al. (2003), found a maximum emission at 590 nm for resorufin but a negligible fluorescence for resazurin. Although 570 nm was once proposed as the optimal excitation wavelength for resorufin (Perrot et al., 2003), 530 nm is more commonly used (Nakayama et al., 1997; Springer et al., 1998; Evans et al., 2001), while resorufin can be metabolized to dihydroresorufin, a colorless and non-fluorescent metabolite (Natto et al., 2012).

Another aliquot of subcultured bacteria was mixed with autoclaved BHI broth in a 96-well microplate to a final volume of 300 μL, and PCP was spiked into the wells to result in concentrations of: 0 (control), 0.05, 0.5, 2.5, 5, 10, 20, 50 and 100 mg/L. The 96-well microplate was sealed and cultured in a 37 °C incubator, and triplicate experiments were carried out at each PCP concentration. OD_{600nm} was measured at intervals of 6 h by an Infinite M200 Pro microplate reader (TECAN, Singapore) with TECAN i-control software.

2.4. Anaerobic sludge spiked with PCP: fluorescence measurements and methane production

Five mL of fresh anaerobic sludge was collected from the anaerobic reactor one hour after feeding and transferred into a 15 mL test tube in triplicate. PCP was spiked into the tubes, yielding final concentrations of 0 (control), 0.05, 0.5, 5, 10 and 100 mg/L 150 μL PCP spiked anaerobic sludge was collected immediately from these tubes, transferred into a 96-well