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Research paper

Development of a bacterial propionate-biosensor for anaerobic digestion monitoring

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ABSTRACT

Monitoring anaerobic digestion (AD) leachate for changes in acetate and propionate concentrations is essential for effective AD operation. In this paper the development of a novel propionate cell-based biosensor is described. A previously designed *E. coli* mutant (IMD Wldgy) that could selectively determine acetate concentrations in synthetic leachates, based on oxygen uptake measurements, was used as a starting point in the development of a propionate biosensor. However, the propionate-grown IMD Wldgy cells exhibited extremely low propionate:acetate O_2 consumption ratios (1:2.4). Screening for alternative propionate-grown *E. coli* strains naturally possessing a more favourable propionate:acetate O_2 consumption ratio identified strain IMD 1, which exhibited a positive ratio (1.6:1). To improve the selectivity of the strain, successive gene knockouts were performed generating the IMD 1 hldgyep mutant. However, propionate-grown IMD 1hdlgyep's O_2 consumption ratio was deemed too low to be considered as a propionate detecting bio-element. It was reasoned that the mechanisms by which *E. coli* activates acetate had to be removed. Deleting *acs* (acetyl-CoA synthesase) and *ackA* (acetate kinase) from IMD Wldgyep, resulted in an *E. coli* IMD Wldgyepak knockout mutant that, when grown on propionate, produced a mean propionate:acetate O_2 consumption ratio of approx. 13:1. The resulting IMD Wldgyep and IMD Wldgyepak strains, which formed the acetate- and propionate-biosensor, respectively, were capable of detecting acetate and propionate concentrations ranging from 0.05 mM to 4.5 mM within two-phase AD synthetic leachates.

1. Introduction

The primary objective of any Anaerobic Digestion (AD) plant is to maximise methane yields, organic loading rates (OLR) and the destruction of volatile solids (VS), while minimising reactor volume sizes and hydraulic retention times [1–3]. High throughput two-phase AD systems adhere most closely to this primary objective but require extensive monitoring and control (MC), which currently requires specialised instrumentation and its assessments are generally subsequent to the activities in the reactor, so are not done in most instances. Therefore, many commercial AD systems function sub-optimally [3]. It has been demonstrated that the deviation of the propionate degradation rate in relation to that of acetate in a two-phase AD reactor occurs before a change in chemical oxygen demand (COD) reduction efficiency or drop in pH is observed [4], which are the parameters current MC technologies monitor to determine the efficient functioning of an AD system. Thus the rapid, accurate measurement of the utilisation of these two compounds could be used to indicate reactor performance. The use of acetate and propionate concentrations as indicators of performance was further supported since AD batch reactors that were supplied with acetate and propionate concentration ratios below 2:1 required extended HRTs to produce comparable VS destruction and methane yields, when compared to ratios above this value [5]. There have been repeated calls for a means to accurately measure an AD system's individual volatile fatty acid (VFA) concentrations as a means to identifying near real time AD system health [4,6]; however, current monitoring techniques are not suitable for in-situ deployment. A sensor capable of detecting an AD system's acetate and propionate concentrations is required to be accurate, possess a suitably long operational lifespan (stability) and not require extensive sample pre-treatment.

An *E. coli* acetate-biosensor based on whole cell biological oxygen demand sensors [7,8], capable of catabolising acetate with the exclusion of all other organic acids in synthetic leachate (SL), has been developed [9]. It was shown to be accurate, remained stable for three to four days and did not require sample filtration. However, the *E. coli* strain, although capable of differentiating between propionate concentrations in propionate-only samples, was incapable of selectively

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catabolising propionate when acetate was present in solution. Thus, further development of a selective propionate biosensor is required. In this paper two approaches to this were explored: firstly, the identification of an *E. coli* strain that has a naturally low acetate utilisation when grown on propionate and secondly, the deletion of key genes associated with acetate activation in the cell. Acetate- and propionate-selective *E. coli* strains were further optimised by immobilisation and the removal of metabolic pathways that are necessary for the catabolism of organic acids present in two-phase AD system-biological leachates (BL). Immobilisation significantly extended the biosensors stability periods, while the removal of organic acid-specific metabolic pathways allowed for changes in the ratio of acetate and propionate to be accurately measured by oxygen electrode in the presence of additional organic acids. It is proposed that the ability to identify a two-phase AD's individual acetate and propionate concentrations will allow for timely adjustments to the operational parameters to be made, thus ensuring AD system health is maintained.

2. Materials and methods

2.1. Bacterial growth and immobilisation

The strains and plasmids used in this study are listed in Table 1; all new strains were deposited in the Industrial Microbiology Dublin (IMD) culture collection, School of Biomolecular and Biomedical Science, University College Dublin, Ireland. Acetate- and propionate-grown cell suspensions (20 mg wet cell mL^{-1}) were grown and extracted as described in a previous study [9]. Cell immobilisations were performed by adsorbing 1 mL of a cell suspension under a moderate vacuum (3.2×10^{-2} Torr) onto a 16 mm diameter cellulose acetate membrane (0.8 µm, GE Healthcare; Fig. 1) which had been cut to fit a 20 mL Coors™ Hirsch funnel (Fig. 1c). The cellulose acetate membrane which possessed a visible cell surface layer, was carefully removed and cut to fit the tip (Fig. 1b) of an Orion 5-Star Plus Dissolved Oxygen (DO) probe (Fig. 1a). The cell surface layer facilitated the adhesion of the cellulose acetate membrane onto the DO probe's tip (Fig. 1d), after which the joined edges were fixed into position with a tight Parafilm M seal (Fig. 1e). The immobilised-DO probe was submerged and clamped into position in Tris-buffer pH 7 solution (20 mL), and stirred at room temperature (Fig. 1g). To ensure measurement repeatability and differentiability, organic acids were added when a steady DO of at least 1.5 mg L^{-1} was observed. After each measurement the cellulose acetate membrane was carefully washed with deionised water and dried with blotting paper (Fig. 1f). Immobilised cells were stored overnight in Tris-buffer at 4 °C in between sampling and remained stable for six days.

2.2. Gene knockouts

E. coli IMD R and W3110 gene knockouts and all $P1_{vir}$ phage lysate generations were performed and confirmed using techniques described in a previous study [9]. The strains, plasmids and $P1_{vir}$ phage lysates created and used in the current study are listed in Table 1.

2.3. E. coli IMD1 $P1_{vir}$ phage transduction and $P1_{vir}$ phage generation

Successive IMD 1 transductions required *E. coli* B $P1_{vir}$ phage lysates, which had to be generated from Rel606 knockout mutants that had been transduced with a respective K-12 $P1_{vir}$ phage lysate. All transduction and $P1_{vir}$ phage generation techniques used were those reported previously [9]. However, the TSA-kanamycin concentration was increased to 100 µg mL^{-1} for IMD 1 transductions as the large number of satellite colonies made the identification of successful transductants difficult.

2.4. E. coli IMD 1 electro-transformation with the pcp20 plasmid

E. coli IMD 1 h (Δ*hsdR*) cells were made electro-competent [10] and electro-transformed using a modified electroporation protocol [11], whereby the electroporator was set to 1.8 mv (1 mm Molecular Bio-Products cuvette), 200 Ω and 25 µF. However, no successful transformations were observed until; (i) a high titre pcp20 (75 µg mL^{-1}) plasmid prep was prepared (QIAGEN plasmid midi-kit); (ii) the recovery period was extended from one to four hours; (iii) electroporated cells were centrifuged (11,000 rpm for 5 min), re-suspended in 150 µL TSB and plated on TSA plates with ampicillin concentrations that had been reduced from 100 to 50 µg mL^{-1}. Successive gene knockouts using the modified transduction and electro-transformation techniques outlined above were verified using the PCR protocol described in a previous study [9], with the exception, that Phusion® High-Fidelity DNA Polymerase (New England Biolabs) was used.

2.5. Synthetic leachate composition

The synthetic leachate (SL) used in a previous study [9] was modified and comprised of D-lactate, L-lactate, ethanol, butyrate, valerate and hexanoate in a 15:15:15:1:1:1 ratio, plus acetate and/or propionate as required.

2.6. Calculating initial O_2 consumption rates

The initial O_2 consumption rate was calculated from the time required to achieve two-fifths of the difference between the starting and minimum DO mg L^{-1} value, which corresponded to the linear portion of the O_2 response curve, after an initial DO response was observed. The time required to observe an initial response was experiment specific, cell suspensions required 15–20 s, whereas immobilised cells, due to reduced diffusion rates, required 45–65 s. Mean (µ) and standard deviation (SD) values were calculated from a minimum of four initial O_2 consumption rates unless otherwise specified. Statistical significance was determined with a two-tailed unequal variance *t*-test (Microsoft Excel).

3. Results and discussion

3.1. Propionate-grown E. coli K-12 strains as the propionate-biosensor

Acetate grown IMD W cell suspension experiments (acetate-biosensor), produced propionate:aceate O_2 consumption ratios that ranged from 1:8–1:39; however, to be able to estimate propionate concentration with the exclusion of all other organic acids, a propionate-specific propionate-biosensor is required. Previous observations [12–14] indicated that propionate-grown *E. coli* could preferentially catabolise propionate over acetate owing to expression of 2-methylcitrate synthase. However, the O_2 consumption of propionate-grown *E. coli* W3110 was greater with acetate than propionate (Table 2). This high O_2 consumption with acetate was thought to be as a consequence of an active glyoxylate shunt or citrate synthase. Therefore, two knockout strains, IMD Wi and IMD Wc, in which the genes for isocitrate lyase (*aceA*) and citrate synthase (*gltA)* were, respectively, deleted, were assessed for their ability to catabolise acetate and propionate. However, only a minor improvement in the ratio of propionate:acetate catabolism was observed in IMD Wi (Table 2), therefore attention then focussed on selecting another *E. coli* strain that might differentiate between the two acids.

3.2. Propionate grown E. coli B strains as the propionate detecting bio-element

E. coli B strain Rel606 was screened for its ability to catabolise propionate as it was hoped that the variances regarding the expression