Impact of operating history on mixed culture fermentation microbial ecology and product mixture
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ABSTRACT

Mixed culture fermentation is an alternative to pure culture fermentation for production of biofuels and valuable products. A glucose-fed, continuous reactor was operated cyclically to a central pH of 5.5 from a number of precedent pHs, from 4.5 to 7.5. At each pH, stable chemical production was reached after 2 retention times and was held for least 2 further retention times prior to the next change. Bacterial groups were identified by phylogenetic analysis of 16S rRNA gene clones. Bacterial community dynamics were monitored by terminal-restriction fragment length polymorphism. More ethanol was produced at high pH, and more butyrate at lower pH. At pH 5.5, the product spectrum was not measurably influenced by precedent pH but showed seemingly random changes. The impact of precedent pH on community structure was more systematic, with clear indications that when the pH was returned to 5.5, the bacterial group that was dominant at the precedent pH remained at high abundance. This result is important, since it indicates a decoupling between microbial function (as indicated by product spectrum), and community structure. More work is needed to determine the longevity of this hysteresis effect. There was evidence that groups retained their ability to re-emerge even after times of low abundance.

Key words | ethanol, fermentation, glucose, hydrogen, hysteresis, mixed culture

INTRODUCTION

Fermentation is a widely applied biotechnology process worldwide because it can produce valuable chemicals and biofuels. Traditional pure culture fermentation requires sterile operating conditions as well as high quality raw materials. In contrast, Mixed Culture Fermentation (MCF), which does not rely on specific strains, can be operated continuously under non-sterile conditions without risk of contamination. It also has enhanced capacity to consume substrates of variable composition such as wastewater or agricultural wastes. MCF therefore has the potential to greatly reduce the cost of the fermentation process. Ethanol and hydrogen gained from mixed culture fermentation are important stock for renewable biofuels. Other products can include commodity chemicals such as lactic acid, acetic acid, and higher organic acids. The mechanism of product control for MCF however is completely different to that of pure culture fermentation. Whilst pure fermentation products are chosen by selecting microbes that only have limited metabolic pathways, mixed cultures have a wide variety of available pathways. Fermentation products therefore need to be regulated by control of environmental conditions. Understanding the link between environmental conditions and the resultant product mixture is a major goal of MCF research.

The major limitation in application of MCF is a lack of understanding of two key aspects. The first is the microbial community structure and the factors important in determining it. The limited studies available indicate that the dominant bacteria in MCF belong to the class Clostridia (Temudo et al. 2008), but there is an enormous range of organisms capable of fermenting glucose to organic acids, and presumably, if the feed stock used is more complex (e.g. wastewater), there will be a wider range of organisms carrying out other metabolic processes. The other key limitation is a lack of understanding of how operational factors affect the various pathways, and hence production of products. pH is known to have a strong impact on both the bacterial population and product mixture (Horiuchi et al. 1999; Batstone et al. 2002; Fang & Liu 2002). The bacterial
community generally demonstrates a shift towards *Clostridia* at high (7.5–8.0) and low pH (4.0–5.5) levels, with other populations at moderate pH (6.0–7.0) levels (Temudo et al. 2008). In terms of the production spectrum, high pH results in a decrease in butyric acid levels, and an increase in ethanol, with a shift from hydrogen to formic acid as external electron acceptor. However, whilst the product spectrum and bacterial community structure at different pHs has been investigated, there remain major questions about the impact of operating history. That is, how do long- or short-term changes influence the microbial community structure and capacity of microbes to produce specific products? In this study we address this issue by operating a continuous fermenter operated to a stable condition of pH 5.5 from a number of different intermediate pH levels, and analyse both the chemical product mix and bacterial community structure.

### METHODS

#### Fermenter inoculum

The inoculum was biomass from an anaerobic digester in Brisbane, Australia. It is a single low rate sludge digester, and is operated at 35°C with a retention time of 14–16 days. The feed to the digester is thermally hydrolysed waste activated sludge.

#### Fermenter operation

A continuously stirred bioreactor, with a working volume of 1.31 L, was operated anaerobically for a total of 14 days. The feed included autoclaved glucose (10 g L\(^{-1}\)) and Basal media solutions (in g L\(^{-1}\): NH\(_4\)Cl, 200; NaCl, 20; MgCl\(_2\) · 6H\(_2\)O, 20; CaCl\(_2\) · 3H\(_2\)O, 0.24; K\(_2\)HPO\(_4\) · 3H\(_2\)O, 400; trace metal and selenite solution and vitamins) held in different bottles, and fed separately through a peristaltic pump. The retention time was 6 h. pH was monitored by a pH probe and was automatically controlled by adding acid (2 mol/L HCl) or base (2 mol/L NaOH). Gas production was measured by a connected gas flow meter. The system was operated through a regime of pH points, from low pH (4.5) to high pH (7.5) with the central pH 5.5. pH 5.5 was selected as the central pH point since it was the native pH when the fermenter was not pH regulated during inoculum preparation. Each pH was maintained for around 4 retention times before changing, and the pH sequence is shown in Figure 1.

The focus of this study was the community structure and chemical products at each pH 5.5, with chemical and biomass samples taken when the pH had been maintained for 2 retention times, which is considered as approaching steady state, and acid/base dosing rate had been stable.

#### Chemical analysis

VFAs were measured in triplicate by High Performance Liquid Chromatography-UV (HPLC-UV) and Gas Chromatography with Flame Ionization Detector (GC-FID) by the AWMC analytical services laboratory. Gas samples were analyzed by Gas chromatography-thermal conductivity detector (GC-TCD).

#### DNA extraction

Biomass was pelleted by centrifugation at 15.366g for 5 min. Genomic DNA was extracted using the Fast DNA Spin for Soil kit (Q-biogene, Australia) according to the manufacturer’s protocol. Extracted DNA was kept at −20°C until further use.

#### Terminal-Restriction Fragment Length Polymorphism (T-RFLP)

T-RFLP was carried out on samples from each pH point. Near full-length 16S rRNA genes were amplified by polymerase chain reaction (PCR) with the bacteria-specific PCR primer pair 5’-CAG GCC TAA CAC ATG CAA GTC-3’ (Marchesi et al. 1998) with a fluorescent label on the 5’ end, and 1389R (5’-ACG GGC GGT GTG TAC
AAG-3′) (Marchesi et al. 1998). The amplification protocol was based on that of Osborn et al. (2000) with adaptations. Each 50 μL reaction consisted of 200 μmol/L of each dNTP, 1 × PCR buffer II (Applied Biosystems), 3 mmol/L MgCl₂, 400 nmol/L of each primer, 0.05 U μL⁻¹ of AmpliTaq Gold DNA polymerase (Applied Biosystems) and 15 ng of extracted DNA. The PCR program consisted of an initial denaturation step at 95 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 2 min, with a final extension step at 72 °C for 10 min. The PCR products were then purified using the QIAquick PCR purification kit (QIAGene, Australia). The restriction enzymes MspI (5′-C^C G G-3′) (Fermentas, Canada) and Sau3AI (5′^-G A T C-3′) (Fermentas, Canada) were used for digestion. Each restriction enzyme digestion reaction contained 10 μL purified PCR product diluted to 15 ng μL⁻¹ and 0.32U restriction enzyme. Each digestion was performed in duplicate. Ethanol precipitation of the digestion product was then performed. Samples were analysed using capillary electrophoresis in a DNA sequencer at the Australian Genomic Research Facility. The resulting electropherograms were analyzed by GeneMarker (SoftGenetics, USA).

**Clone library and T-RFLP identification**

Three 16S rRNA gene clone libraries were constructed from biomass samples collected at pH 4.5, 7.5 and pH 5.5 with precedent pH 7.0. PCR products were cloned into the pGEM®-T Easy Vector (Promega, USA) through the ligation procedure described in the manufacturer's manual. Vectors were then transformed into TOP10 competent *Escherichia coli* cells (Invitrogen, USA) to construct the clone libraries. T-RFLP analysis was performed on 40 clones which were randomly picked from every clone library to identify the major operational taxonomic unit groups. Duplicate representative clones from each group were sequenced with primers T7 (5′-TAA TAC GAC TCA CTA TAG GG-3′) (Pavco & Steege 1991) and SP6 (5′-TAT TTA GGT GAC ACT ATA G-3′) (Pavco & Steege 1991). A total of 20 clones were sequenced. Primer sequences were removed in MEGA4 (Kumar et al. 2008) and contigs were produced from the trimmed sequence reads using DNAbaser (Heracle Software, Germany). The sequences were aligned using the NAST aligner in Greengenes (DeSantis et al. 2006). Contigs were imported into ARB (version 2.5b, Ludwig et al. 2004). Identification of the major bacterial groups was performed using phylogenetic analysis within ARB.

**RESULTS**

**Chemical product spectrum**

The consumed glucose was converted into 2–11% hydrogen, 10–20% biomass and 62–75% soluble products at all pH values (Figure 2). The range of products is similar at all pHs but there are some differences in the proportions of each product.
product, particularly between the high pHs (7.0 and 7.5) and lower pHs (4.5, 6.0 and 6.5). At high pHs, ethanol is the major product (>30%) and butyric acid is a minor product (<3%). At other pHs, ethanol production is lower (5–10%) but butyric acid production is higher (10–40%). Likewise, at high pHs (7.0 and 7.5), formic acid production is >5% and lactic acid is <1%. At other pHs (4.5, 6.0 and 6.5), the reverse is true, with lactic acid >5% (and at pH 6.0 >10%) and formic acid <3%. Acetic acid is the major product at pH 6.0, making up almost 40% of the product spectrum, but is a minor product at lower and higher pHs (15–25%).

There do seem to be minor impacts of precedent pH on the proportion of products at central pH 5.5 but these are not systematic. For example, acetic acid is the major product at precedent pH 6.0 and also at the following central pH 5.5. Likewise, butyric acid is the major product at precedent pH 6.5 and also at the following central pH 5.5. However, ethanol is the major product at precedent pHs 7.0 and 7.5 but is only a minor product at both central pH 5.5s following these pHs. Likewise the formic acid/hydrogen balance, which indicates whether hydrogen ions or carbon dioxide are principal electron acceptor (Batstone et al. 2006), is completely dominated by the central pH, with no impact from the precedent pH. Specifically, formic acid is always the dominant electron acceptor at high pH (7.0 and 7.5), while hydrogen is always the dominant electron acceptor at pH 5.5.

Identification of major bacterial groups

Figure 3 summarises the major operational taxonomic unit groups identified in this experiment. Only a limited number of unique groups were identified, implying relatively low evenness. The majority of clones analysed (16 of 29) were related to the genus Clostridium within the phylum Firmicutes, with three different groups (A, B & C; 5, 1 & 10 clones respectively) identified. Three other groups were identified; one related to the genus Klebsiella within the phylum Proteobacteria (10 clones), one related to genus Bifidobacterium within phylum Actinobacteria (2 clones), and the third related to the genus Enterococcus within phylum Firmicutes (1 clone).

T-RFLP RESULTS

Bacterial community dynamics, as determined by T-RFLP analysis of peaks that corresponded with operational taxonomic unit groups identified from phylogenetic analysis, are shown in Figure 4. Clostridium A was the dominant organism at pH 4.5 and 6.0, Clostridium B was dominant at pH 6.5 and Klebsiella-related organism was dominant at pH 7.0 and 7.5 (Figure 4(a)). Therefore in terms of abundance, Clostridium B was associated with production of butyric acid and the Klebsiella-related organism was associated with production of ethanol, formic acid and succinic acid (Figure 2). The relative abundance of different products is quite different at pH 4.5 and 6.0 and so it is difficult to associate Clostridium A with particular chemicals. For each of these major groups, a hysteresis effect was observed at the following central pH 5.5, i.e. when an organism was dominant at a particular precedent pH, it also had a higher relative abundance at the following central pH 5.5 than at other pH 5.5s (Figure 4(a)). Other, minor peaks include two...
unknown groups and Clostridium C (Figure 4(b)). These all show highest abundance at one or more of the pH 5.5s, suggesting that at pH 5.5 a greater diversity of organisms was able to co-exist than at higher or lower pHs. However, there is considerable variation in each of their abundances at the different central pH 5.5s suggesting that, whilst the shift to a more diverse community structure is systematic, there is not a shift towards particular community members.

**DISCUSSION**

While the fermenter had a community with low evenness, meaning that it was dominated by often only 1 or 2 groups, it is evident that sub-dominant members remained in the system, as they repeatedly re-emerged at their pH optimum. For example, Clostridium C had low abundance (<10%) at each pH point except at the pH 5.5 following the precedent pH 6.5, where it was in high abundance (>40%) (Figure 4(b)) Therefore, there is obviously some capacity to retain subdominant members within the community despite the relatively low retention time (6 h).

T-RFLP is suitable for routine analysis of large sample numbers due to its reproducibility and potential for automation (Smalla et al. 2007). Whilst it is not a truly quantitative technique, relative changes in structure and diversity between T-RFLP profiles can be detected reliably (Hartmann & Widmer 2008) and it is therefore useful for monitoring trends in community structure over space, time and, in this instance, pH. T-RFLP is a PCR-based method and is therefore prone to all of the biases inherent to PCR (amplification bias, lack of primer specificity, etc.). It is also possible that pseudo-peaks are created from incomplete restriction enzyme digestion, possibly due to shielding of the terminal restriction site as a result of the formation of secondary structure in the DNA molecule (Meyerhans et al. 1990; Pallansch et al. 1990). Therefore, future work will involve verification of community structure with another quantitative method, such as fluorescence in situ hybridization (FISH) or real-time PCR.

As shown in Figure 2, there is a small and varying proportion of total products missing. This could be due to basic errors in the COD balance versus amount fed (though consistently negative in this case), or could be due to undetected compounds in the liquid (e.g. unmeasured organic acids and alcohols), or in the gas (e.g. CO).

Based on our results, it appears that the precedent pH has little influence on the bacterial community function (as measured by chemical production) at central pH 5.5 but that it does have a measurable influence on the bacterial community structure. This is important, since it indicates that to a certain extent, function can be decoupled from diversity. However, there also appear to be elements of the community structure at central pH 5.5 that are not influenced by precedent pH. Further work is needed to assess whether the influence of precedent pH on community structure remains after a greater number of retention times.

**CONCLUSIONS**

Precedent pH appears to have no measurable impact on chemical products, with apparently random variations at central pH 5.5 after a precedent pH point. However,
precedent pH does appear to have an impact on the microbial community structure, with characteristics of the precedent pH community identifiable in the subsequent central pH 5.5 community.

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REFERENCES


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