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Electro-extractive fermentation for efficient biohydrogen production

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Abstract

Electrodialysis, an electrochemical membrane technique, was found to prolong and enhance the production of biohydrogen and purified organic acids via the anaerobic fermentation of glucose by *Escherichia coli*. Through the design of a model electrodialysis medium using cationic buffer, pH was precisely controlled electrokinetically, i.e. by the regulated extraction of acidic products with coulombic efficiencies of organic acid recovery in the range 50-70% maintained over continuous 30-day experiments. Contrary to previous reports, *E. coli* produced H₂ after aerobic growth in minimal medium without inducers and with a mixture of organic acids dominated by butyrate. The selective separation of organic acids from fermentation provides a potential nitrogen-free carbon source for further biohydrogen production in a parallel photofermentation. A parallel study incorporated this fermentation system into an integrated biohydrogen refinery (IBR) for the conversion of organic waste to hydrogen and energy.

Keywords (5 keywords)

Fermentation; biohydrogen; electrodialysis; *Escherichia coli*; organic acid

Contents

1. Introduction	1
2. Materials and Methods	2
2.1 Extractive fermentation apparatus and operation	2
2.2 Bacterial strains and maintenance	3
2.3 Analysis	3
3. Results and Discussion	3
3.1 Preliminary extractive fermentation method development	3
3.2 Electrokinetic pH control in biohydrogen fermentations	4
3.3 Comparison with non-extractive fermentation	4
3.4 'Rescue' experiments: indication of ethanol limitation	5
3.5 Aerobic growth and H ₂ production in a single reactor	6
3.6 An indefinite extractive fermentation?	7
3.7 Extractive fermentation as a bioresource technology	8
4. Conclusions	9
Acknowledgements	9
References	9
Figure Captions	12
Tables and Figures	13

1. Introduction

Biohydrogen technology offers practical options for clean fuel gas production relying on only sustainable resources: organic material and sunlight (Redwood et al., 2009). Hydrogen gas can provide electricity with high efficiency in a fuel cell. BioH₂ from a fermentative culture has been shown to power proton exchange membrane fuel cells directly (Macaskie et al., 2005).

Dark hydrogen fermentations typically provide 2-3 mol H₂/mol hexose sugar, with maximal production rates in the range 10-50 mmol H₂/h/L culture (Hallenbeck and Ghosh, 2009; Redwood et al., 2009; and references therein). *E. coli* is a useful model organism for the study of bioH₂ fermentation because it is oxygen tolerant, mesophilic, non-sporulating, unaffected by the partial pressure of H₂ and highly amenable to metabolic engineering (Redwood et al., 2008; Sinha and Pandey, 2011 and references therein). Furthermore, as a facultative anaerobe, *E. coli* presents the opportunity for fast aerobic growth to high density followed by a longer period of anaerobic H₂ production with little additional growth, an approach which is reported here for the first time.

Sustained operation is a challenge for biohydrogen fermentations. In an excess of substrate, the first obstacle is acidification of the medium by newly formed organic acids (OA), which is normally overcome by adding caustic pH titrants. However, the fermentation is ultimately limited by the toxicity of accumulated end products (Warnecke and Gill, 2005). Chemostat operation (with suspended cells) is non-ideal for biohydrogen fermentation because of the continual discard of cells and unmetabolised nutrients in the outflow (e.g. Bisailon et al., 2006). Immobilisation of cells (particularly through granule formation) has been successful in retaining fermenting cells with improved substrate loading rates and H₂ production rates but the production rate is limited by diffusion into the immobilisation matrix or granule (Show et al., 2010).

Here, electrodialysis (ED) was identified as a possible means of sustaining indefinitely a free-cell biohydrogen fermentation with a high H₂ production rate, substrate loading rate and cell density, without the use of pH titrant or immobilisation (Figure 1). The key function of ED is the selective transport and removal of acidic fermentation products across a semi-permeable anion-selective membrane, thereby simultaneously controlling fermentation pH and forestalling the accumulation of OAs to limiting concentrations. Extractive fermentation (using ED) shares some common features with microbial fuel cells (MFC) in which the definitive feature is the exchange of current between living cells and a chemical electrode, either directly or via a mobile electrochemical mediator (Pant et al., 2010). Extractive fermentation (EF) differs in that the electrical terminals of the ED cell are shielded by flanking ion selective membranes and a large culture circulates through a small ED cell chamber, where cells experience the electric field transiently. Therefore, unlike in MFC, the bacteria 'store' electrochemical potential in charged organic molecules which are then removed in combination with pH regulation.

A wide range of charged products and inhibitors have been extracted from fermentations and other complex solutions using ED (KiBeom, 2005; Strathmann, 2010; Wong et al., 2010; Xu et al., 2007; and references therein) but to the authors' knowledge, this study represents the first investigation of sustained, pH-regulated electrodialysis fermentation applied to bioH₂ production. In this context ED is doubly effective as it also generates a concentrated stream of separated OAs suitable for additional bioH₂ production by purple non-sulphur (PNS) bacteria (to be reported subsequently).

ED techniques employ cation-selective (CSM), anion-selective (ASM) and bi-polar (BP) membranes, to achieve the charge-selective separation and the generation of acid and alkali, applicable in processes such as seawater desalination and OA production (see above).

This study describes the adaptation of fermentation techniques to incorporate electroseparation and the performance of long-term glucose-fed extractive fermentations for the production of OAs and H_2 by *E. coli*. Modes of operation were examined and the challenge of achieving high current efficiency was identified. The proposed solutions, electrokinetic pH control with a customised ED medium and start-up protocol, are described.

2. Materials and Methods

All media and solutions were prepared using deionised water and analytical grade reagents.

2.1 Extractive fermentation apparatus and operation

Thin-cell ED apparatus (C-Tech Innovation Ltd, Capenhurst, UK) was configured as shown in Figure 1, comprising stainless steel electrodes of which the anode was Pt-coated. The electrical terminals of the ED cell were connected to the output of a power supply (Thurlby Thandar Instruments, PL330 TP). The cell was divided into four chambers (named C, M, MA, and A from cathode to anode) separated by 3 membranes; bi-polar (BP: Fumasep FBM), anion-selective (ASM: Fumasep FAB) and cation-selective (CSM: Fumasep FKB), respectively (Figure 1). Fresh membranes were used in each experiment. Silicone rubber gaskets (1 mm thickness) were cut to expose membrane areas of 200 cm^2 (128x157 mm).

Compartment M represented the ‘main’ compartment (source of anions for recovery) and MA represented the space separating compartments M and A into which anions were recovered. The flanking membranes (CSM and BP) prevent direct contact between bacterial cells and the electrodes, as the extremes of pH at the electrode surfaces would result in unwanted reactions (Li et al., 2004; Mustacchi et al., 2005). The BP membrane also splits water, generating H^+ on the cathode side and OH^- on the anode side, a function exploited to provide pH control simultaneously during extractive fermentations (Wong et al., 2010). The CSM also functions to transport Na^+ from the C chamber, resulting in the formation of sodium salts in the MA chamber. A 4-channel peristaltic pump was used to circulate solutions and medium constantly at 300 mL/min through all four chambers. Chambers A and C (flanked by the anode and cathode, respectively) were in contact with a single solution of 0.5 M Na_2SO_4 (1 L), the M chamber was in contact with the *E. coli* culture (initially 3 L) and MA was in contact with the permeate vessel containing initially 2 L of stirred phosphate buffer (0.366 g K_2HPO_4 , 0.443 g KH_2PO_4 /L; pH 6.8).

Fermentations used an Electrolab, UK; 300-series system. In accordance with Table 1, macronutrients and buffers were sterilised by autoclaving in a volume of 2.7 L inside the fermentation vessel (‘M’) before sterile $MgSO_4$, thiamine, antifoam, microelements and water were added aseptically to make a final volume of 3 L at pH 6.5 ± 0.1 . The medium was heated to 30 °C and aerated (1 L/min) with turbine agitation (600 rpm) before adding 1% (v/v) inoculum from a pre-culture. Pre-cultures were incubated for 16-18 h (30 °C, 180 rpm) using 100 mL of nutrient broth (no. 8; Oxoid UK) with 5 g/L added sodium formate.

pH control was not required during aerobic growth for the initial 24 h, after which the transition to anaerobic fermentation was made by purging with oxygen-free N_2 (30 min, 1 L/min) and the culture was also connected to the M chamber of the ED cell (Figure 1), which had been cleaned by circulating 75% ethanol (15 min) and washing three times with sterilised water. The pH was allowed to fall to 6.0, where it was maintained automatically by the removal of acidic products across the ASM in a pH-sensing feedback loop (C-Tech

Innovation, Capenhurst, UK). As shown in Figure 2, the software increased the applied current in response to a declining trend in pH below the setpoint and decreased the current in response to an increasing pH trend. V and I were recorded via the power supply's PC interface using the same software. Glucose was fed starting from the end of the N₂ purge at a constant rate of 0.15 mol/day (1.5 M solution, 0.1 L/day, sterile and under air).

In electroseparation of any type, current efficiency (or Faraday efficiency) represents the fraction of charge passed in a time period, which can be attributed to the detected transfer of target anion (Madzingaidzo et al., 2002).

$$\text{Current Efficiency (\%)} = \frac{100NF}{i} \quad (\text{equation 1})$$

where N is the charge flux as target anion (here, organic acid) in mol/s/m², F is the Faraday constant (96485.38 sA/mol) and *i* is the current density in A/m².

2.2 Bacterial strains and maintenance

Escherichia coli strain FTD67 (provided by Dr F. Sargent, University of Dundee) was selected as previous studies showed its lack of uptake hydrogenase to be conducive to H₂ production (Redwood et al., 2008).

2.3 Analysis

For H₂ and CO₂ measurement, gas outflow from a port in the top plate of the fermentation vessel was connected, via Tygon hose, to continuous gas flow meters (Walker et al., 2009). As shown in Figure 1B, meters were placed upstream and downstream of a 'scrubber' solution containing 2 M NaOH so that the CO₂ fraction could be calculated by subtraction. The scrubber solution also contained universal indicator so that its depletion would be apparent by colour change. This method was shown by GC to remove CO₂ to below 0.05% (v/v). Anions were analysed by HPLC and glucose was measured colorimetrically as described previously (Redwood and Macaskie, 2006).

3. Results and Discussion

3.1 Preliminary extractive fermentation method development

Preliminary work showed that electrodialysis (ED) can recover organic acids (OA) from *E. coli* fermentations without causing significant inhibition but that automatic, demand-based regulation is necessary. The preliminary tests showed that intermittent high intensity separation (9.6 A, 1 h per day) caused inhibition of H₂ production which lasted for 16-20 h, perhaps due to cell membrane disruption, thermal shock (temperature reached 50 °C) or pH shock (maintained using H₂SO₄) or the generation of toxic chemicals. Conversely, a slow constant-current (400 mA) was not inhibitory. Glucose was fed continuously from 24 h onwards and pH was controlled by the automatic addition small amounts of titrants. Cells were grown aerobically then resuspended in a fermentation medium (containing Na₂SO₄ and (NH₄)₂SO₄ as the main salts) and made anaerobic by N₂ purging. H₂ production (0.7 L/L culture/day) and glucose utilisation (20 mmol/L/day) continued for ~10-15 days, as compared to 7 days in controls without ED but the coulombic efficiency of OA separation was poor; initially 5%, rising steadily to 12% over 14 days. This poor current efficiency was attributed to competitive ion transfer, i.e. the movement of inorganic anions (particularly SO₄²⁻) and the increasing trend in current efficiency with respect to the separation of OAs was attributed to their increasing concentration in the fermentation medium, which reached 90 mM and ultimately limited fermentation despite an excess of glucose and favourable pH (pH 5.5).

Therefore, to improve the current efficiency and maintain non-limiting OA concentrations the fermentation medium was redesigned (Table 1) and the ED was put under pH-linked automatic feedback control. This provided electrokinetic pH control in place of pH titrant additions (Figure 2).

An 'ED' medium was designed (Table 1) and tested in a new protocol for aerobic growth and subsequent H₂ production in a single vessel. Growth took place over 23 h in 3 L reactors (aerated at 1 L/min; pH 7.0). Using ED media with either formic acid or lactic acid (23 mM) the cultures reached the same density of 4.15 g DW/L in 23 h aerobic cultivation, followed by H₂ production after transition to the anaerobic fermentation mode (see further discussion below).

3.2 Electrokinetic pH control in biohydrogen fermentations

As shown in Figure 2, pH was controlled within 0.01 pH units using custom software provided by C-Tech Innovation (Capenhurst, UK). A baseline current of 10 mA was maintained in order to prevent back-diffusion of separated anion but when the fermentation pH decreased as a result of acid production, the applied current was increased until the pH increased (due to the removal of OA across the ED membrane with simultaneous OH⁻ generation by the BP membrane; Figure 1) prompting a return to the baseline current. At steady state the pH typically oscillated by ~0.0005 units with a period of ~5 min, while the current remained predominantly at the baseline (10 mA) rising briefly up to 20-fold several times per min (Figure 2). No attempt was made to optimise the frequency or responsiveness of current variation.

Figure 3 shows the effectiveness of electrokinetic pH control in two independent experiments ('EF1, EF2'). From a neutral starting point, the pH fell towards the end of the aerobic growth phase (<1 days) and was subsequently maintained at pH 6.0 by the action of ED. H₂ production started on the third day and 40 L H₂ was produced over 20 days at maximally ~4.7 L/day/L culture. Initially a set maximum of 4 V was sufficient to achieve pH control but as growth continued into the anaerobic phase the rate of acid formation increased and the limit was increased to 10 V after 6 days.

The permeate solutions (MA chamber) remained clear (as shown in Figure 1B) during 30 days of uninterrupted activity in which the ASM was the only barrier separating permeates from dense (~5 g DW/L) *E. coli* cultures. The current efficiencies representing the main part of the fermentations (days 2-20) were 57% and 55% for EF1 and EF2, respectively. It is noteworthy that the current efficiency increased after 22 days in EF1, which was 'rescued' from ethanol toxicity (see below) but declined slightly in EF2, in which ethanol was not removed (see below).

Therefore, ASM are capable of simultaneous cell retention and charge-selective ion separation in glucose-fed *E. coli* fermentations and, furthermore, this was confirmed in extractive fermentations producing H₂ from food wastes (M. D. Redwood et al.; unpublished; Environ. Sci. Technol. in submission).

3.3 Comparison with non-extractive fermentation

Figure 4 shows that standard fermentations could consume up to 1 mol glucose, whereas extractive fermentations consumed 2.5 mol. Furthermore, standard (non-extractive) fermentations tolerated ~1 mol of accumulated OA (190 mM in 5.5 L final volume) within the culture, whereas 2.5-5 mol was extracted from fermentations with ED (Figure 4A). The extracted liquors contained 39.5% butyrate, 26.3% acetate, 23.8% formate, 7.9% lactate, 2.2% succinate and 0.4% propionate (averaged molar fractions; 70 analyses). The dominance of butyrate, rather than acetate and lactate, is a further unusual feature (Lugg et al., 2008)

apparently enhanced by this culture technique. Short (24-48 h) *E. coli* fermentations in batch mode followed the well-described fermentation balance of *E. coli* (e.g. Redwood et al., 2008), whereas when fermentation was prolonged by traditional pH control a switch to butyrate production (with lactate uptake) occurred 1-2 days into the anaerobic stage. The identity of butyrate was cross-validated by HPLC and mass spectrometry. Lugg et al. (2008) discussed the sparse previous reports of butyrate production and molecular support for a putative metabolic pathway in *E. coli*.

While the non-extractive fermentations (fed-batch) gained fluid volume as additional substrate was added, the volume of extractive fermentations varied very little due to the movement of water into MA across the anion-selective membrane. Show et al. (2010) stated, "*The key to successful application of anaerobic fermentation is to uncouple the liquid retention time and the biomass retention time in the reactor system*" and they discussed granules, biofilms and flocs as potential solutions. Using extractive fermentation the two retention times were uncoupled but with suspended cells, thereby avoiding the diffusion limitations of immobilised or granular fermentations. Due to the absence of outflow, feed rates need not be carefully controlled to minimise outflow of excess substrate and the longer adaptation times (typically 48h) required for adaptation to new substrates would not cause washout, as is problematic in chemostat cultures (Kyazze et al., 2006).

As shown in Figure 4B experiments EF1 and EF2 produced 1.6 and 1.9 mol H₂ from 2.3 and 2.8 mol glucose, respectively. Therefore, fermentative H₂ yield was low at 0.7 mol H₂/mol glucose in both cases, as yields close to the theoretical maximum (2 mol/mol) have been reported previously (Bisaillon et al., 2006). The low yield is attributed in part to the extraction of formate, the sole precursor of H₂ in *E. coli* (Redwood et al., 2008). The extracted formate amounted to 1.1 mol in EF1 and 0.9 mol in EF2 (as measured in MA solutions). Adding this potential H₂ to the actual H₂ (i.e. if extracted formate had been converted to H₂) would increase the yield to 1.0-1.2 mol/mol. Therefore, the extraction of formate cannot account for the whole yield deficit. Although similar in reactor volume, the earlier experiments (Bisaillon et al., 2006) operated as chemostats from which ethanol would be diluted continuously. The accumulation of ethanol (not actively removed by ED) is the most likely cause of the lower yield in the current work.

3.4 'Rescue' experiments: indication of ethanol limitation

After 22 days EF1 and EF2 had ceased activity in terms of both H₂ production and OA production (Figures 3 and 4A) despite continuing favourable conditions (excess glucose, optimal pH and very little OA accumulation). Hence the cessation may be attributed either to the depletion of another nutrient (e.g. N or P) or to the accumulation of an inhibitory end-product, not removed by ED, such as ethanol. To preclude the former, EF2 was re-dosed with the initial provisions of vitamins, trace elements and ammonium citrate (Table 1). This had no effect on the production of H₂ or OA (Figures 3D and 4), indicating that nutrient limitation was not responsible.

Zaldivar et al. (1999) reported growth inhibition by ethanol at 20 g/L for the *E. coli* strain LY01 having enhanced ethanol tolerance. Therefore, it is reasonable to suggest that *E. coli* FTD67 (derived from MC4100), could be inhibited in H₂ production by ethanol at 14-17 g/L (peak values, Figure 3) and ethanol toxicity may also inhibit growth and promote cell lysis causing the observed decline in biomass. In further support of this hypothesis EF1 was 'rescued' by harvesting cells from 50% of the culture, and resuspending in an equal volume of a solution containing concentrations of OAs matched precisely to those measured in the culture at 20 days. The solution contained (mM) lactic acid, 0.85; acetic acid, 8.37; formic acid, 4.57; butyric acid, 29.23; succinic acid, 0.23 and bis-tris base to pH 6.0

(~50 mM). Upon returning the resuspended cells into EF1, the result would be a ~50% dilution of all other soluble products. The ethanol concentration fell from 12.3 g/L (267 mM) to 5.2 g/L (Figure 3B) and this was followed by resurgence in H₂ production (Figures 3B and 4B) and current efficiency (Figure 3B) and OA production (Figure 4A). Unknown end-products cannot be excluded but ethanol is the only known major uncharged product (Clark, 1989) and was detected in potentially inhibitory concentrations (see above). Therefore it is most likely that ethanol was the cause of limitation. In this work EF prolonged fermentation time from ~3 days to 3 weeks and increased H₂ production per culture by 3-fold.

Losses in H₂ yield due to formate extraction would not be significant in a larger integrated system because the extractive fermentation would function primarily as an OA generator, rather than as the primary H₂ producing reactor (see later).

3.5 Aerobic growth and H₂ production in a single reactor

It has been accepted that “[*E. coli*] cells cultivated aerobically ... lack the ability to produce hydrogen” (Yoshida et al., 2007). Furthermore, it was reported that amino acids are necessary as anaerobic growth on defined media, with NH₄⁺ as the sole nitrogen source resulted in the absence of H₂ production activity unless amino acids (particularly glutamate) were added (Gest, 1954). This was confirmed more recently as *E. coli* cultivated aerobically on nutrient broth (growing up to ~0.8 g DW/L), would produce H₂ when mixed directly with phosphate buffer but not when harvested, washed and resuspended in phosphate buffer (Penfold et al., 2006). Later, this was overcome by adding formate (100 mM) to the aerobic pre-growth medium, which enabled H₂ production by washed cells in a range of nutrient-poor buffers (Orozco et al., 2011; Redwood et al., 2008). Similarly, Yoshida et al. (2007) designed a 3-step process in which cells were grown aerobically, then activated with formate before entering anaerobic H₂ production. Therefore, it would appear that either amino acids or formate are necessary as inducers of H₂ production for aerobically grown *E. coli*. Hence, the results of the present study are surprising as high H₂ production rates (maximally 8 mmol/h/L culture) were observed after aerobic growth in minimal media. The phenomenon was not strain specific as the parent strain MC4100 and its derivatives HD701 and FTD67 (Redwood et al., 2008), were all capable of H₂ production after aerobic growth in a single vessel using ED medium.

Furthermore, when formic acid in the ED medium (Table 1) was replaced with lactic acid H₂ production took place normally in the anaerobic phase, whereas formate was previously found to be a necessary inducer of H₂ production after aerobic growth.

The capability for H₂ production without induction by formate or amino acids may result from the properties of the single-reactor technique, in which there is a gradual progression from oxygen saturation at pH 7, into oxygen limitation with growth and concomitant OA formation and resultant fall to pH 6 (before a 30 min purge with N₂ to ensure anaerobiosis). Hewitt et al. (2000) reported progressive changes in cell physiology throughout aerated *E. coli* fermentations. Hence, this transition is a natural property of the culture and appears to promote metabolic reconfiguration (for H₂ production) whereas sudden artificial transitions were unsuccessful.

The facility to produce a dense culture and then produce H₂ in a single reactor represents a significant advance with several advantages over methods reported to date. Firstly, it does not require cell harvest so it is more suitable for large scale implementation. Secondly, it achieves a higher culture density. Cultures grew to maximum densities of 2-4 g DW/L (as compared to 0.8 g/L using nutrient broth in shake flasks). Much higher densities could be reached by established techniques, i.e. monitoring the respiratory quotient

of the culture (RQ) while limiting the glucose supply to restrict ‘overflow metabolism’ (the aerobic formation of inhibitory OAs) (Hewitt et al., 2000). The single-reactor method described here uses the onset of overflow metabolism (and resultant fall in pH to 6.0) as a trigger for the switch to anaerobic fermentation. Therefore overflow metabolism is inherently limited, without the need for careful RQ and feed-rate monitoring.

3.6 An indefinite extractive fermentation?

According to an *ideal model*, an extractive fermentation (EF) could be sustained indefinitely. This would require the removal of charged products in balance with pH, while maintaining a constant ionic composition in the culture medium. In the ideal model the concentration of OA in the fermentation medium remains constant because each unit of organic acid generated by fermentation results in a detectable fall in pH, which triggers the exchange of 1 unit of anion for 1 unit of OH⁻ (with the corresponding rise in pH). This electrochemical balance is illustrated in Figure 1.

This situation was created in the glucose-fed extractive fermentations (EF1, EF2), where the duration of H₂ production was significantly extended from ~3 days to 3 weeks but was not indefinite. EF1 and EF2 were limited by the formation of non-ionic end products, which cannot be actively separated by the same method as charged products. ‘Rescue’ experiments (see above) confirmed that OA concentrations remained non-limiting, indicating that an unidentified fermentation product, and not the depletion of any key nutrient, was the cause. The most likely non-ionic end product is ethanol, given the well-described mixed acid fermentation in *E. coli* (Clark, 1989). Ethanol formation plays an important redox balancing role in *E. coli* and mutants defective in alcohol dehydrogenase cannot ferment glucose anaerobically (Clark, 1989). For lactic acid production, homofermentative lactic acid bacteria have been applied successfully in long-term EF (Pal et al., 2009), whereas for bioH₂ production non-solventogenic alternative species are elusive. *Enterobacter* spp. function similarly to *E. coli* (producing ethanol) while clostridia can produce mainly acetic and butyric acids but are prone to switching to acetone-butanol metabolism (and sporulation) in response to a variety of stresses, including OA toxicity (Logan, 2004). The threshold for OA-induced solventogenesis in clostridial-type fermentation is reportedly 19 mM free acid (at pH 5.5, glucose substrate) (Logan, 2004) which could be achieved by precisely controlled electroseparation, whereas *E. coli* tolerated up to 90 mM OAs in this work (while continuing to produce H₂). Certain thermophilic and hyperthermophilic bacteria (including some *Clostridium* spp.) and archaea are promising candidates for biohydrogen production (see Redwood et al., 2009) but the upper temperature limit for bipolar membranes is currently ~60°C, which precludes their use.

An indefinite extractive fermentation would also be affected by membrane fouling, which results from the adherence of particles (e.g. proteins or cell debris) or by scaling (Mondor et al., 2009; Strathmann, 2010). Therefore, fouling would be anticipated when treating fermentation broths and, indeed, a soft white layer was found on the M-side of the ASM after use. Membrane fouling limits ion transfer causing a reduction in the point of limiting current density (LCD) and an associated increase in stack resistance. In 30 days there was no sign of declining separation efficiency or capacity (Figure 3) but membrane fouling was indicated by stack resistance (not shown) which rose steadily from 10 Ω to 17 Ω over the initial 14 days and then remained at 17 Ω. This suggests that fouling reached a maximum and did not inhibit electrokinetic pH control. Therefore, membrane functionality out-last-ed biological activity. If ethanol accumulation can be overcome, fouling may be prevented by ultrafiltration (Zelic et al., 2004) reversed by defouling techniques such as hydraulic cleaning,

acid, base, ultrasound or electrodialysis reversal (Mondor et al., 2009; Strathmann, 2010; Wang et al., 2011).

Finally, water balance also presents a practical challenge for the indefinite fermentation. Ion separation is accompanied by a degree of water transport from the fermentation chamber so that the permeate chamber slowly gains volume via transport from the fermentation while water is constantly lost from the electrode wash solution by electrolysis. In this study, non-extractive fermentations gained 100 mL/day from glucose additions and typically ~110 mL/day from titrant additions, reaching the capacity of the fermentation vessel in 12 days. Conversely in EFs the rates of feed addition and water transport from the fermentation chamber ('M') were relatively in balance as the fermentation volumes (M chamber) changed by only ± 30 mL/day while over 100 mL was fed. Therefore, the retention times of fluid and biomass were uncoupled using suspended cells, aiding mixing and mass transfer. EF can be described neither as 'fed-batch' nor 'continuous' culture. Unlike 'fed-batch' there was a (reasonably) constant culture volume despite feeding and unlike 'continuous' culture there was very little dilution of biomass and no outflow of unused substrate or soluble products.

A precise water balance may be addressed by controlling the water-separation capacity of the ED cell using a moderate pressure gradient, preferably a small negative pressure to either A/C or MA, although this would dilute the contents of these vessels. This hypothesis could not be tested using the current apparatus due to the use of peristaltic pumps, which are sensitive to fluid pressures. However, water movement into MA was observed consistently. Alternative pump mechanisms (e.g. positive displacement or centrifugal) may enable such control but these are usually too large for bench-scale experiments. Since, in this study, ~30% of fed water was retained in the fermentation vessel, in practice an outflow would be required and the resultant dilution may be sufficient to control ethanol accumulation and achieve a continuous fermentation. Since ethanol accumulated to much higher concentrations than OAs only a small fraction of OAs would be lost in the outflow.

Hence, the technique described in this study offers practical advantages over traditional approaches (i.e. fed-batch, chemostat or cell immobilisation) but an indefinite extractive biohydrogen fermentation remains elusive mainly due to the solventogenic properties of suitable organisms.

3.7 Extractive fermentation as a bioresource technology

This study demonstrated the effectiveness of extractive bioH₂ fermentation using glucose substrate. However, the capacity to utilise bioresources is important for the technique to be useful in sustainable fuel production. Complex substrates would be more challenging for EF due to the presence of insoluble components and inorganic salts. The presence of inorganic salts could reduce the OA separation efficiency and also potentially distort the balance of pH regulation and OA removal, while the sugars of key bioresources exist primarily as polysaccharides (e.g. starch, cellulose and lignocellulose) requiring hydrolysis to enable rapid fermentation and also to prevent fouling of the narrow channels of the ED cell with solid particles. Such upstream hydrolysis of starch was achieved (Orozco et al., 2011) and EF took place with high separation efficiency with H₂ production from a range of biowastes. This work will be reported in subsequent publications.

Finally, and moving towards a zero waste high output process, the extracted OAs were coupled to a second phase of photofermentative bioH₂ production (M. D. Redwood et al., unpublished; Environ. Sci. Technol., in submission), while growth of *Spirulina*, a high

value foodstuff, on fermentation waste CO₂ was achieved in a parallel study (X. Zhang & L. E. Macaskie, unpublished).

4. Conclusions

- Aerobic cultivation and subsequent fermentation by *E. coli* to produce H₂ can occur in one vessel using a minimal medium without added inducers (formate or amino acids).
- Electrodialysis provides simultaneously the electrokinetic control fermentation pH while preventing the accumulation of organic acids (OA), thereby enhancing and prolonging H₂ fermentation.
- Electroseparation of OA from an active fermentation can be maintained over long periods (30 days) with cell retention while maintaining high OA separation efficiency.
- Extractive fermentation with suspended cells uncouples the retention times of fluid and biomass.
- The main limitation for extractive biohydrogen fermentation is solventogenesis.

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Figure Captions

Figure 1. Application of ‘BAC’ electrodialysis to fermentative and photofermentative biohydrogen production.

BP, bi-polar membrane; ASM, anion-selective membrane; CSM, cation-selective membrane; C, cathode chamber; M, main chamber; MA, permeate chamber; A, anode chamber; -, cathode; +, anode.

Figure 2. Automatic control of fermentation pH by extractive fermentation.

pH was regulated automatically using a program provided by C-Tech Innovation (Capenhurst, UK) which varied the applied current within set limits (10-3000 mA). pH and I were recorded at 1 s intervals and was not smoothed.

Figure 3. Electrokinetic pH control in extractive fermentation (EF1 and EF2).

E. coli fermentations were sustained for 1 month by the removal of organic acid products by an electrodialysis (ED) cell controlled by pH. The voltage applied over the ED cell (thin solid lines in A and C) varied up to the set limit (thin broken lines in A and C). Arrows indicate the time of ‘rescue’ experiments (see text). The bracket in C indicates a temporary pump malfunction. Glucose was in excess throughout. Despite continuous substrate addition, culture volume remained relatively constant due to water loss via ED (see text). pH and V were recorded at 2 min intervals and data smoothing was applied (100 points; 200 min) because V fluctuated rapidly with I (see Figure 2). Current efficiency (equation 1) was derived from 2-3 daily recordings of the fluid volume in MA, the accumulated charge passed over the ED cell and analyses of the OA concentration in MA. Current efficiencies shown in B and D represent time-weighted averages smoothed by 5 points (i.e. ± 1 day) as individual outputs varied widely attributed to the combined error on several measured inputs.

Figure 4. Comparison of standard and extractive fermentations: Maintenance of low organic acid concentrations in extractive fermentations (A) and extension of fermentation (B).

EF1 and EF2: extractive fermentations using electrodialysis for simultaneous pH control and organic acid separation. SF1 and SF2: Standard fermentations took place under the same conditions as extractive fermentations except for the presence of the electrodialysis system. SF1 and SF2 were stopped at 14 days as H₂ production ceased at 7 days and organic acid formation ceased at 12 days. A shows the organic acids present in and extracted from fermentations. Standard fermentations could accumulate ~1 mol organic acids whereas 2.5-5 mol was extracted from fermentations with electrodialysis. Arrows indicate ‘rescue’ experiments showing that the cessation in H₂ production can be attributed to the accumulation of a secondary metabolite such as ethanol and not to cell age, nutrient depletion or organic acid toxicity. B shows the yield of H₂ from glucose in each experiment. All fermentations were fed glucose constantly (150 mmol/day) but standard fermentations could not consume >1 mol glucose, whereas extractive fermentations consumed 2.5 mol before requiring a ‘media refresh’ (see text).

Tables and Figures

Table 1. Development of ‘ED’ medium for extractive fermentation of *E. coli*.

Component class	Chemical	Final concentration (mM)	
		SM ^a	ED (this work)
Macronutrients and buffers	Glucose	27.75	as SM
	(NH ₄) ₂ SO ₄	20.43	3.5
	Na ₂ SO ₄	14.08	-
	NH ₄ Cl	9.348	-
	K ₂ HPO ₄	83.82	-
	NaH ₂ PO ₄	28.99	-
	(NH ₄) ₂ -H-citrate	4.782	-
	(NH ₄) ₂ HPO ₄	-	2
	Citric acid	-	5
	BIS-TRIS base	-	50
	Formic acid	-	23
	NH ₄ OH	-	44
Additions	MgSO ₄	2	as SM
	Thiamine (vitamin B1)	0.030	as SM
Micronutrients (Used as 3 mL/L)	Na ₂ -EDTA	0.1702	as SM
	ZnSO ₄	0.001878	as SM
	MnSO ₄	0.001775	as SM
	CuSO ₄	0.001201	as SM
	CoSO ₄	0.002241	as SM
	FeCl ₃	0.1743	-
	CaCl ₂	0.01721	-
	FeSO ₄	-	0.1743
	CaSO ₄	-	0.01721
Final inorganic anion charge (equivalents)		323.2	15.7

‘ED (electrodialysis)’ medium was formulated to minimise the influence of competing inorganic anion, while mimicking the nutrient content of SM^a medium (Hewitt et al., 2000) which was optimised for aerobic growth to high density. Phosphate buffer was replaced with the cationic buffer, bis-tris base (pK_a 6.15). 50 mM was found to be a suitable bis-tris concentration in short aerobic growth tests. Inorganic salts were replaced with formic acid, as an ‘inducer’ of H₂-producing metabolism (Redwood et al., 2008). The level of inorganic anion equivalent was reduced from 323 mM to 16 mM but could not be reduced to zero because of the minimum requirements for S and P, which are preferably supplied as phosphate and sulphate ion. *E. coli* K12 strains required only 0.1 mol P and 0.04 mol S per mol N (Bisaillon et al., 2006), which were matched. All media also contained 0.5 mL/L polyethylene glycol (antifoam). Citrate is an organic acid which does not serve as a carbon source for *E. coli*. It functions (along with EDTA) as a chelating agent and was monitored among organic acids for its contribution to current efficiency.

Figure 2

A: EF1 **B: EF1** **C: EF2** **D: EF2** **Figure 3**

Figure 4