BIOHYDROGEN PRODUCTION BY EXTRACTIVE FERMENTATION AND PHOTOFERMENTATION

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ABSTRACT

Electrodialysis (ED), an electrochemical membrane technique, prolonged and enhanced the production of biohydrogen and purified organic acids (OAs) via anaerobic fermentation of glucose and food wastes. In this extractive fermentation, pH was precisely controlled by the regulated extraction of acidic products. The solid particles and inorganic salts present in real wastes were not problematic when hydrothermal pre-treatment was incorporated. The selective separation of OA from waste-fed fermentations provides a nitrogen-free carbon source for further biohydrogen production in a parallel photofermentation. H_2 yield could be up to 10-fold enhanced by combining extractive fermentation and photofermentation. Therefore, ED provides the key link in an *integrated process* (IP) for efficient bioH_2 production from organic wastes.

INTRODUCTION

Biohydrogen provides a route to clean energy from bioregradable wastes in a sustainable and environmentally benign process.

Part of the value of bioH_2 is its intrinsic purity, making it ideal for fuel cells, which are seen as a key technology in a future H_2 economy, with much higher efficiencies of electricity generation than combustion generators. Whereas reformed H_2 requires extensive purification to remove CO (a potent catalyst poison for PEM fuel cels), bioH_2 is intrinsically free of CO and requires little or no purification for use in PEM fuel cells [1].

The UK is committed to providing 15% of its energy requirement from renewable sources by 2020. Meanwhile, we produce annually over 100 million tonnes of biodegradable wastes [2]. Most is landfilled, a route which is unsustainable, increasingly costly and fails to recover the whole value of this significant renewable resource. Plans are underway to divert much of this biowaste into biogas production (anaerobic digestion), whereby a maximum of about 5-10% of the UK's energy demand could be met with existing technology.

This value could be more than doubled using an '*integrated process*' (IP) incorporating extractive fermentation and solar photofermentation (Fig 1). In the IP organic acids generated by anaerobic fermentation are converted to H_2 in a photo-fermentation. Dual bioreactor systems of this type were recently the subject of a focused review [3], which highlighted the high sensitivity of the photofermentation to inhibitory nitrogenous substances and the challenge of producing a nitrogen-free OA stream from nitrogen-rich organic wastes. The proposed IP addresses this challenge using a fermentation system incorporating selective anion separation by electrodialysis (ED).

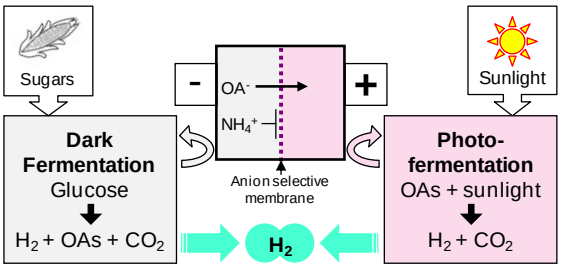

Fig 1. Concept diagram for integrated process (IP) for bioH_2 production using electrodialysis (ED).

LITERATURE REVIEW

E. coli is well-studied in bioH_2 production. It is a facultative anaerobe capable of rapid aerobic growth, non-sporulating, insensitive to H_2 partial pressure and highly amenable to metabolic engineering which has been applied to enhance H_2 production [4-7] and extend the substrate range [8,9]. H_2 production by anoxygenic photosynthetic bacteria such as *Rhodobacter sphaeroides* strain OU001 has been documented previously [10-13]

ED has been explored for the purification of many fermentation products [14-19] but this study describes the novel application of ED to *E. coli* bioH_2 fermentation, the impact on fermentation performance and the photobiological conversion of extracted OA to further H_2.

EXPERIMENT

Materials & Methods

Extractive fermentation methods

The H_2-overproducing strain *Escherichia coli* HD701 [6] was maintained, revived and routinely grown using nutrient broth with sodium formate (5% w/v) as decribed previously [4].

Cultures (1.5 L) were harvested (4435 × g, 20 ºC, 10 min), washed twice in phosphate buffered saline (PBS: 1.43 g Na_2HPO_4, 0.2 g KH_2PO_4, 0.8 g NaCl, 0.2g KCl per L, pH 7.0) and pellets were resuspended to 0.2 L in sterile PBS. For prolonged operation, the fermentation vessel (5 L, Electrolab 300 series) was sterilised by autoclaving containing

2.8 L aqueous fermentation medium (42.6 g Na_2SO_4, 10.456 g K_2HPO_4, 0.204 g KH_2PO_4, 0.297 g $(NH_4)_2SO_4$, and 0.5 mL polyethylene glycol pH 5.5). Subsequently, the following were added aseptically: 6 mL 1 M $MgSO_4$, 30 mL 2 M glucose and 9 mL trace elements solution [20]. The solution was warmed to 30 °C before 0.2 L cell suspension was added to give a final volume of 3 L, and a final concentration of 20 mM glucose. Adjustment to pH 5.5 was made immediately and maintained automatically.

Feeding began after 24 h operation. The feed (0.6 M glucose, 0.15 M $(NH_4)_2SO_4$, pH 5.5) was sterilised by autoclaving and rendered anaerobic by purging with oxygen-free nitrogen before being pumped into the reactor at a constant rate of 100 mL/d to provide 60 mmol glucose and 30 mmol NH_4 per day. The feed-bottle and vessel headspaces were connected so that the feed input would not affect the measurement of H_2 production.

At intervals, the culture was checked for contamination by dilution plating. As a derivative of strain MC4100, *E. coli* HD701 cannot utilise lactose [21] and produced white colonies on MacConkey agar (Sigma).

H_2 was measured by the displacement of 1 M NaOH (which trapped CO_2) from a graduated cylinder. Other than CO_2, H_2 was the only gas found the in the off-gas from the culture (Gas chromatographic analysis).

Electrodialysis methods

An thin-cell elecrodialysis system was purchased from C-Tech Innovation, Capenhurst (UK) and configured as shown in Fig. 2. The four chambers of the ED cell (C, M, MA, and A) were separated by 3 membranes; bi-polar (BPM: Neosepta BP-1E), anion (ASM: Neosepta AHA), and cation (CSM: Nafion 324), respectively, purchased from Eurodia, France. The fermentation culture was circulated continuously through the 'M' chamber (0.45 L/min). A constant 400 mA was applied over a membrane area of 200 cm^2. The stack resistance was typically 10 Ω. Current efficiency (CE) for OA separation was calculated as [14].

Photofermentation methods

Rhodobacter sphaeroides strain O.U. 001 (DSM 5864) was grown in SyA medium [22], which included 1 g/L yeast extract (Merck) and Na-succinate (30 mM). *R. sphaeroides* was pre-cultured in full bottles (30 ºC, 40 µE/m^2/s tungsten-halogen lamp). Cells were harvested from late exponential phase (4435×g; 10 min) and resuspended in AA-b medium [22] omitting sources of NH_4^+ and using 50 mg/L $MgSO_4$•$7H_20$ and 25 mg/L $CaCl_2$•$2H_20$.

OAs from the MA chamber of the ED cell were transferred into a 3 L photobioreactor (PBR; Fig. 3) by continuous dilution with basal medium (0.366 g K_2HPO_4, 0.433 g KH_2PO_4, 0.05 g $MgSO_4$•$7H_20$, 0.025 g $CaCl_2$•$2H_2O$, 1 g yeast extract/L) at 1 L/day.

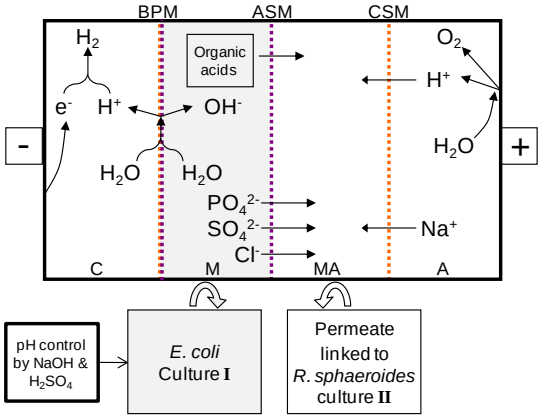

Fig. 2. Extractive fermentation using constant BPAC electrodialysis (this study). BPM: bipolar membrane, ASM: Anion selective membrane, CSM: cation-selective membrane; C, cathode chamber; M, main chamber; MA, permeate chamber; A, anode chamber; -, cathode; +, anode. A and C chambers were connected to a shared reservoir.

The PBR was cylindrical (105 mm ID x 346 mm wetted height, 0.107 m^2 lit area). The average light intensity (400-950 nm) at the culture surface was 334.3 µE/m^2/s, provided by 3 equipositioned tungsten lamps (40 W). The culture was mixed at 1200 rpm using a magnetic stirrer and follower and kept at 30.0±0.2 ºC using a submerged cooling coil.

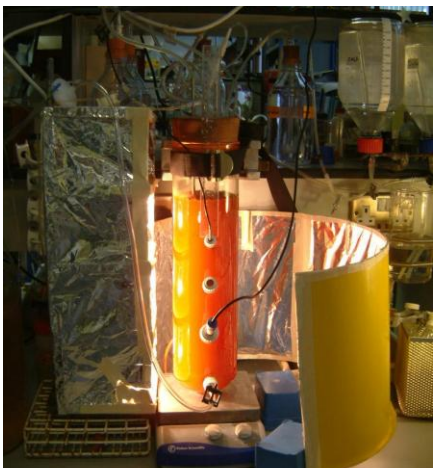

Fig. 3. Photobioreactor (PBR) for Rhodobacter sphaeroides (shown with the reflector unfurled)

The vessel was sterilised by autoclave and filled with 3 L of a mixed OA medium based on SyA containing 16 mM acetate, 14 mM succinate, 8 mM lactate, 5 mM butyrate and 1 g/L yeast extract, to match the typical OA profile of short *E. coli* fermentations (data not shown). The medium was inoculated with 30 mL late-exponential phase pre-culture and purged with argon (30 min) before H_2 production was measured as with *E. coli*. After growing for 72 h, the culture was diluted constantly

(1 L/day) with permeate from the MA chamber of the ED cell.

Analysis methods

Samples were filtered (0.2 μm) and stored at –20 °C before analysis. Anion HPLC and glucose analysis were as described previously [23]. OA identification was validated using a Waters ZMD mass spectrometer using electrospray ionisation.

DISCUSSION AND RESULT ANALYSIS

Extractive fermentation

In fermentations without ED (Fig. 4), *E. coli* HD701produced H_2 at maximally ~300 mL/h.

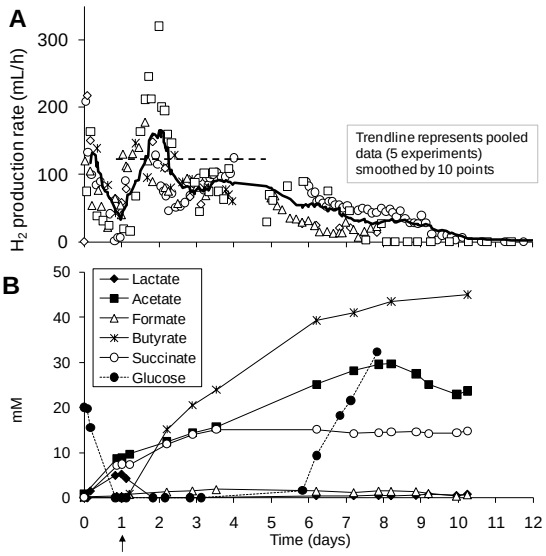

Fig. 4. E. coli HD701 fermentations without ED. A shows 5 replicates; B shows representative data. Arrows indicate the start of glucose feeding at 24 h. The dotted line (A) indicates 120.3 mL H_2/h equivalent to2 mol H_2/mol hexose; 100% yield; [4]. *Controls without glucose feeding ceased H_2 production after 24 h. Note that at 6 d glucose consumption ceased and H_2 production fell.*

During the initial 24 h lactate, acetate, formate and succinate were produced as in mixed acid fermentation (MAF) [24] but after 24 h lactate disappeared, the rates of acetate and succinate formation declined and butyrate became the main fermentation product. This apparent switch from MAF to a butyrate fermentation [25] was reproducible in timing and common to several MC4100-derived *E. coli* strains (data not shown). The fermentations continued to consume all fed glucose, producing H_2 and butyrate until ~6 days.

Photofermentation

Photofermentations gave 0.85 (±0.1) g dry weight (DW)/L on the initial substrate for 3 days after which dilution began. Controls diluted with 'basal medium' (lacking OAs) ceased H_2 production after 5-6 days as the initial substrate was exhausted (Fig. 5). Conversely, when diluted with mixed OA medium (see above) H_2 production settled to ~65 mL/h, and was monitored for 12 days. Substrate was in excess and substrate conversion efficiency was ~15% whereas light conversion efficiency was ~5%.

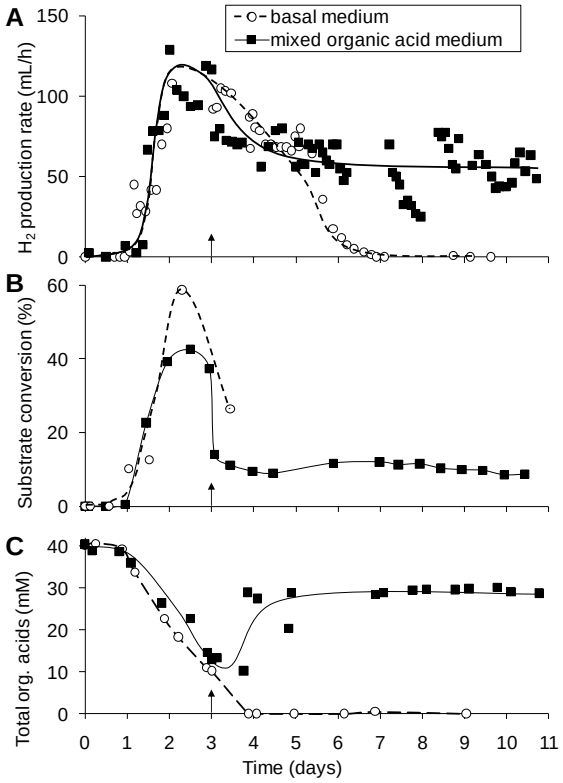

Fig. 5. Continuous cultures of R. sphaeroides, diluted with synthetic OA medium. H_2 production at 150 mL/h (A) corresponded to light conversion at 7.84%. Substrate conversion efficiency (B) was calculated according to [26] based on consumed substrate. Arrows indicate the onset of dilution.

Integrated process

As shown in Table 1, the integrated process was compared to two different controls. Ec1/Rs1 were without electrodialysis (ED). Ec2/Rs2 used ED fitted with an inactive ASM (inactivated at 15 V, 24 h), to check for any influence of the ED system other than via OA extraction. Ec3/Rs3 represent the integrated process with an active ASM in the ED cell. Fig. 6 shows, firstly, that stable and continuous H_2 production occurred only with ED (Ec3) attributed to OA removal, forestalling OA toxicity. Secondly, Applying DC to *E. coli* without product removal (Ec2) shortened the period of H_2 production, but also provided short-term stimulation. Fig. 6A shows a reproducible spike in the H_2 production rate at days 2-3. The production of H_2 at >100% apparent yield is due to the uptake of lactate (see Fig. 4B). In Ec1 experiments the peak H_2 production rate was 150 mL/h, whereas 320 mL/h was recorded for Ec2. A further stimulatory effect was the prolonged period of com-

Table 1: Integrated process experiments

CONDITION & DESCRIPTION	DARK FERMENTATION	PHOTOFERMENTATION
1. Control – no ED	Ec1 (n=3)	Rs1 (n=1)
2. Control – DC only - ED with inactive ASM	Ec2 (n=4)	Rs2 (n=2)
3. Integrated process - ED with active ASM	Ec3 (n=2)	Ec3 (n=2)

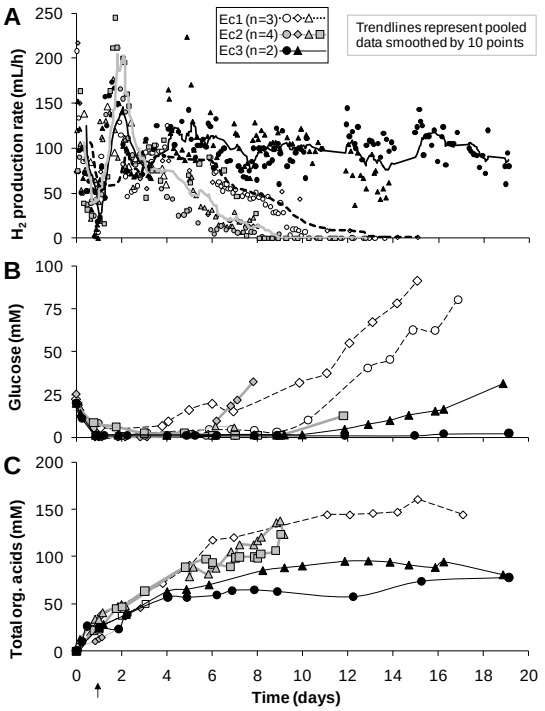

Fig. 6. Integrated process part I: Extractive E. coli fermentation (I; Fig. 2). Details in Fig. 4 legend. Ec3 and Rs3 (Fig. 7) were synchronised.

plete utilisation of continuously fed glucose (Fig. 6B). A similar phenomenon was described previously [27], but remains unexplained. Direct electron transfer between cells and the ED cell's electrodes is excluded as cells were separated from the electrodes by membranes.

Ec3/Rs3 experiments were synchronised. A single diluent ran through the MA compartment of the ED cell and through the PBR. Fig. 7 shows photofermentative H_2 production sustained for 16 days by OAs from extractive *E. coli* fermentations. Conversely, control experiments (Rs1/Rs2), diluted with media lacking OAs, ceased H_2 production within 3 days of dilution.

Photofermentative H_2 production was sustained despite the presence of 15 mM NH_4^+ in the *E. coli* feed. NH_4^+ was undetectable in permeates entering the PBR. Therefore ED enabled process integration by retaining NH_4^+ in the dark fermentation while transferring fermentation products to the PBR.

Substrate conversion efficiency (Fig. 7B) in the integrated system (Rs3) was higher than in Fig. 5B, attributed to the lower substrate loading associated with OA separation by ED.

In these experiments, current efficiency (CE) gradually increased from 5% to 28% (Fig 8), losses

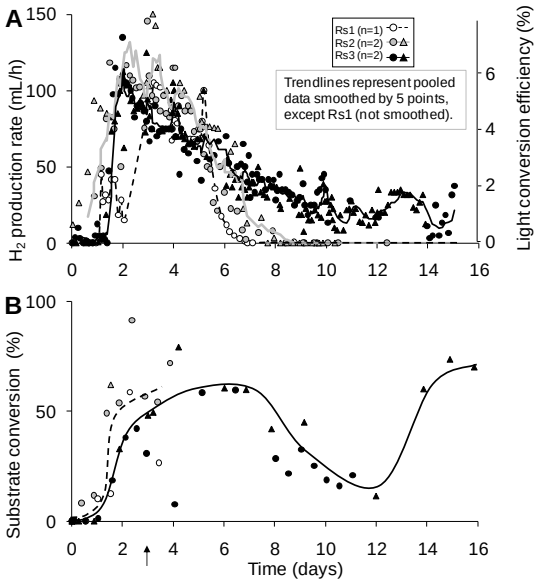

Fig. 7. Integrated process part II: R. sphaeroides photofermentation of organic acids from extractive fermentation (II; Fig. 2). Details in Fig. 5 legend.

being attributed to competitive ion transfer, i.e. the movement of inorganic anions such as SO_4^{2-}, which do not contribute to H_2 production. The increase is attributed to the increasing concentration of OAs in the fermentation medium (Fig. 6**).**

CE values of 78-99% were reported previously [14,15,18]. However, the low CE values observed here (5-28%) are sufficient for an effective integrated process. It was calculated that, to offset the electrical energy input for OA separation, the minimum required CE would be 13% where butyrate is the main OA (Fig. 4B).

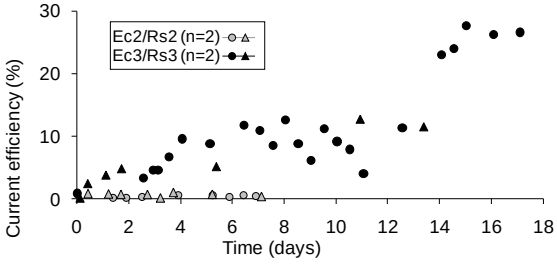

Fig. 8. OA transfer efficiencies in integrated process experiments (Ec3/Rs3) and controls (see Table 1). Current efficiency (CE) indicates the efficiency of OA transfer; charge of OAs transported over electrical charge passed.

The overall H_2 yield of the integrated process was 5.7 mol H_2/mol glucose, derived as follows. Per mol glucose, *E. coli* produced 1.6 mol H_2, 0.6 mol ethanol (not used) and OAs equivalent to 6.8 mol

potential H_2. Substrate converson efficiency in the PBR was ~60% hence photofermentation of the fully extracted OAs would produce a further 4.1 mol H_2, enhancing the total yield by ~4-fold in comparison to dark fermentation.

Further development

ED shows promise to support a continuous bioH_2 fermentation by removing OAs in balance with production while simultaneously producing a photofermenation substrate rich in OAs and low in NH_4^+. Combined with the use of organic waste as feedstocks this could potentially offer an efficient route to sustainable energy from waste. To achieve this, several challenges remain:-

Firstly, previous attempts have failed to switch *E. coli* to anaerobic H_2 production after growth in an aerated reactor. It was shown [4,28] that formate can be added as an inducer of anaerobic H_2 production in aerobically grown *E. coli* and this principle can be applied in a single vessel method, obviating cell harvest [29].

Secondly, CE, initially poor (Fig. 8), increased during extractive fermentations, as the accumulation of OAs reduced competitive ion transfer. Future work would use high biomass *E. coli* will optimise an 'ED medium' for extractive fermentation, using (counterintuitively) OAs, to provide the required osmotic strength replacing most of the inorganic anion (PO_4^{2-}. SO_4^{2-}, Cl^-).

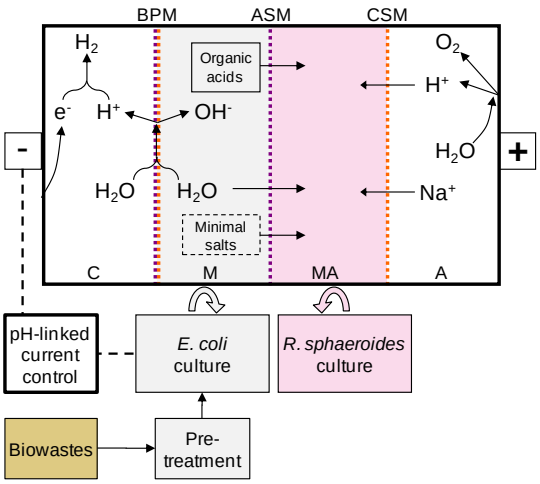

Fig. 9. Proposed extractive fermentation using pH-responsive electrodialysis and ED medium to maintain optimal fermentation pH by efficient OA separation. For labels see Fig. 2.

Thirdly, pH control was achieved, here, mainly through the automatic addition of NaOH but with a contribution from the ED system as the BPM generated OH^-. pH-responsive OA separation potentially replaces pH titrants (Fig. 9).

Finally, this study used glucose as a model substrate but renewable organic resources would be targeted commercally. *E. coli* (used as a convenient model) produced H_2 using various sugary wastes [1] but pre-hydrolysis will be required for optimal use of most resources. Potential methods include enzymatic, chemical and hydrothermal [30]. However, the application of ED to waste-fed fermentations would require the removal of solid particles, which may block the narrow channels of the cell and the presence of inorganic anion in wastes may prevent the establishment of the chemical balance between pH and OA concentrations as illustrated in Fig. 9. However in practice hydrothermal pre-hydrolysis of starchy and cellulosic wastes has been shown to produce hydrolysates suitable for *E. coli* fermentations (Redwood, Orozco & Macaskie, unpublished).

CONCLUSION

Electrodialysis can prolong dark fermentation and produce suitable feeds for photofermentation which could increase H_2 yields up to 10-fold. This could potentially underpin a new route to hydrogen energy from renewable resources. The *integrated process* (dark fermentation and photofermentation integrated by electrodialysis) holds promise for future biohydrogen production from renewable organic resources and wastes but several challenges must be overcome, including the proper regulation of OA extraction from fermentations in balance with formation and the use of biomass resources.

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