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Assessment of Biohydrogen Production Potential of Anaerobic Sludge Consortium and Clostridium acetobutilycum Strain 6441 via Dark Fermentation of Cheap Carbohydrate

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Abstract

The hydrogen production potential of *Clostridium acetobutylicum* strain 6441 and heat treated anaerobically digested sludge consortium (ADS) was studied. The inoculums' ability to metabolize some cheap substrates while producing hydrogen was used as assessment factors. Environmental conditions for the cultures were the same having a starting pH of 6.0 and a temperature of 35^{o}C. Growth kinetics, volumetric hydrogen production rate and the rate of catabolism of the sugars was studied. *C. acetobutyricum* showed greater production potential and metabolic plasticity; having a hydrogen production rate of 5.29, 1.337 and 1.058 mlh^{-1} and a substrate utilization rates of 149.7, 92.89 and 180.1 $mgl^{-1}h^{-1}$ from glycerol, xylose and lactose respectively. ADS on the other hand, showed hydrogen production rates of 1.476, 1.017 and 0.290 mlh^{-1} and substrate utilization rates of 56, 22.35 and 35.59 $mgl^{-1}h^{-1}$ from glycerol, xylose and lactose respectively. Upon scale up, a total of 19.8% and 11.297% hydrogen was produced via the fermentation of crude (untreated) glycerol and pure glucose using *C. acetobutylicum* and ADS respectively. The study revealed *C. acetobutylicum* and glycerol to have a greater hydrogen production potential than ADS and other substrates. More work was suggested to optimize the production potential of ADS since it is preferred to pure cultures for reasons of sterilization and energy cost.

Keywords: biohydrogen, Dark fermentation, Crude glycerol, Lignocellulosic biomass, UASB, Anaerobically digested sludge

1. Introduction

Energy security, environment-friendliness, renewability and sustainability, today, justify the need for an alternative source of energy to fossil-based fuels. This is owed to factors such as: the negative environmental impact of greenhouse gases (GHG) produced from their combustion (Lashof & Ahuja, 1990; Cox *et al*., 2000, Horowitz & Jacob, 1999), the decline in their production and forecast of their imminent exhaustion (Das & Veziroglu, 2001).

Although, some renewable alternatives (butanol, ethanol, nuclear power, wind power, etc.) have been suggested, hydrogen stands out due to its high energy content per unit weight (Chong *et al*., 2009), cost effectiveness and zero emission of pollutants. Hence, it has been identified as good alternative to fossil fuels and petroleum (Das & Veziroglu, 2001; Chong *et al*., 2009; Hung *et al*., 2011; Levin *et al*., 2004).

Hydrogen is at present mostly produced through unsustainable thermochemical processes such as hydrocarbon reformation, etc. (Sinha & Pandey, 2011). It is expected that for environment-friendliness and sustainability, biological processes must be resorted to. Biological production of hydrogen however is faced with challenges, most of which are detailed in the review of Hawkes *et al*., (2002) and Levin *et al.*, (2004).

Among these challenges are the production potential of hydrogen from suggested cheap carbon sources such as lignocellulosic materials as well as crude glycerol and the choice of inoculum. In this research, we aimed at finding out the most suitable inoculum and cheap substrate for use in the production of biohydrogen via dark fermentation.

Hence, our objective was to compare the yield of hydrogen from lignocellulosic biomass (cheese whey) and crude glycerol when fermented using hydrogenic consortium from anaerobically digested sludge and *Clostridium acetobutylicum* strain 6441. The substrates were mimicked using pure sugars. Lignocellulosic biomass was mimicked using xylose while cheese whey and crude glycerol were mimicked using lactose and pure glycerol respectively. The metabolic kinetics of the fermentations was also studied and the inoculum and substrate with the highest yield of hydrogen was scaled up.

2. Materials and methods

2.1. Media Composition

The media used for H_2 fermentation in serum vials contained lactose, xylose and glycerol in their pure forms as sole carbon substrates (25 g/l) and of sufficient inorganic supplements made up according to the formulary from (Ginkel *et al*., 2001; Sharma *et al*., 2011). However, NH_4HCO_3 and $FeCl_2$ were replaced with NH_4Cl and

$FeSO_4.7H_2O$ respectively. The sugars and the salts were sterilized separately to avoid the reaction between ammonium salts and the sugars.
Except for LB used during the inoculum development, all media used were buffered to pH 6.0 (Tao *et al.,* 2007) using 0.1 M $C_6H_8O_7$ and 0.2 M Na_2HPO_4. Lignocellulosic biomass (wheat straw) used in another subset of serum vial fermentations was pretreated by grinding, treatment with dilute acid and steam explosion at a temperature of 121^{o}C for 15 minutes. During scale up, the medium composition was modified to the recipe used byKyazze *et al.* (2006).

2.2. Microbial Inoculum Development

2.2.1 Consortium From Anaerobically Digested Sludge (ADS)

The anaerobic treated sludge used as seed sludge was obtained from a municipal sewage treatment plant in London. The pH and total suspended solids (TSS) was found to be 7.74 and 116.4 g/l respectively.
The sludge was selectively treated in favour of hydrogenic bacteria by steaming at 100^{o}C for 15 minutes. This inhibited hydrogen-consuming methanogens and initiated sporulation of hydrogen-producing acidogens (Ginkel *et al*., 2001, Chang, 2004). The heat treated sludge was acclimated in lysogeny broth (LB) at 35^{o}C for 12 hours without sieving. Anaerobiosis was ensured by sparging the broth with nitrogen gas for 5 minutes. Prior to incubation in a shaker incubator, the culture vessel was slightly opened to eliminate the possible effect of shear pressure and/ or product inhibition due to gas production. Oxygen interference was avoided Oxoid Anaerogen® sachets.

2.2.2 Clostridium acetobutylicum Strain 6441

Inoculum of C. acetobutylicum was developed from the stock from University of Westminster culture collection using LB medium as explained above.

2.2.3 Acclimatization

This experiment was carried out in order to find out the effect of acclimatization on the lag phase of the inoculums in glycerol. During inoculums development, instead of using LB medium, 80 ml of the production medium (section 2.1) was spiked with 10 ml of glycerol (10 g/L) and 10ml of the heat treated ADS (*h*ADS) and was incubated for about 48 hours. This inoculum was used for glycerol fermentations only.

2.2.4 Viability of inoculums

Prior to use of the developed inoculums, viability test was carried out. From the microbial suspension, 100 µl was transferred into an Eppendorf tube. The suspension was diluted two folds using 100 µl of Trypan blue stain and mixed gently.
Aliquots of the mixture was transferred into a haemocytometer by capillary action. The cells were focused on using 10X magnification. Cells in 4 sets of 16 micro-squares in the haemocytometer was counted under 40X objective of the microscope. Non-viable cells were stained blue while viable cells were unstained.
The inoculums were used only when viability was up to 80% (usually between 18 – 24 hours incubation). Viability was calculated using the expression:

$$\frac{\text{Viable}}{\text{Total cell count}} \times 100 \quad \ldots\ldots (1)$$

2.

2.3.1 Batch Fermentation for H_2 Production in Serum Vials Using Pure Sugars

Aliquots (10 ml) of the sludge inoculum were placed in 250 ml serum vials; each containing 80ml of the fermentation medium and 10 ml of the test carbon source. Nitrogen was sparged into the vials to create anaerobiosis and also to pressurise the gas outlet tubing which was used to collect the off gas over water in a 250ml volumetric flask. The pH of the water over which the off gas was collected was acidified to pH 2.0 using dilute H_2SO_4 in order to reduce the loss of the produced gases in water due to solubility.
The fermentation was run at a temperature of 35^{o}C, pH 6.0 and horizontal rotation rate of 120 rpm in a shaker water bath. For improved accuracy and as proof of reproducibility, each fermentation condition (varying sugars) was set up in duplicates. During the course of the experiments, the volume of gas (CO_2 and H_2) produced from each vial was monitored using an inverted volumetric flask. This was measured by the volume of water displaced from the volumetric flask as a result of capillary pressure from the produced gas (Figure 1). Eachfermentation was run until gas production stopped. Nitrogen was sparged into each vial in-between samples to strip out trace oxygen which might have entered into the vials during sampling and to maintain pressure by compensating for the volume of samples drawn.
Experimental controls contained the medium (without the test carbon sources) and inoculum.

2.3.3 Batch fermentation for H_2 production in serum vials using pre-treated wheat straw and crude glycerol

After the fermentation ran with pure sugars, crude glycerol and pre-treated wheat straw were fermented with both *h*ADS and *C. acetobutylicum* Strain 6441. All the conditions adopted during the fermentation of the pure sugars were maintained. However, 20 g of both the wheat straw and crude glycerol were used. This set up was observed only for gas production. No samples were drawn due to the absence of nitrogen gas.

2.3.4 Scale-up

At the end of the batch operations in serum vials, scale up of the fermentation was initially carried out in a 4 L up-flow anaerobic sludge blanket (UASB) reactor using 20 g/l glucose and 10% v/v *h*ADS inoculum. The pH was monitored and maintained between 5.3 and 6.0 using a Mettler Toledo PID controller, 1 M NaOH and 0.5 M HCl. The temperature was maintained at 35^{o}C by circulating warm water through the thermal insulating layer of the UASB. The broth was circulated using a peristaltic pump to achieve effective mixing as well as agitation.

The contents of the UASB were later transferred to a continuous stirred tank reactor (CSTR) for effective agitation to enhance release of the produced gas. Agitation was set at 100 RPM to avoid vortex. To avoid corrosion of the online analytical sensors, the off gas was bubbled through dreschel bottles containing concentrated copper II sulphate and silica gel to strip out hydrogen sulphide and dry the gases respectively (Kyazze *et al*., 2006).

After this, fermentation involving 20 g/l crude glycerol and *C. acetobutylicum* in CSTR was started. At the stop of gas production, another 20 g/l was fed into the system for the process to continue.

2.4 Offline Analyses

Samples from serum vials, UASB and CSTR were analysed offline between 24 and 48 hours for biomass concentration (spectrophometrically at 600 nm), volatile fatty acids (VFA) and sugar metabolism.

2.4.1 Determination of Sugar Metabolism

Metabolism of the sugars during the fermentations was monitored by determining the change in chemical oxygen demand (COD) using standard closed reflux titrimetric methods described in Environment Agency (UK) Standard method 5220D as referenced by (Fernando *et al*., 2012). A total of 2 ml of diluted samples were used and the COD was calculated using the mathematical expression:

$$COD\ (mg/L) = (V_b - V_s) * DF * M * 4000 \text{ ---------- } (2)$$

where V_s and V_b are ferrous ammonium sulphate (FAS) titrant volumes for the samples and blank respectively while M is the molarity of ferrous ammonium sulphate (0.025M) and DF, the dilution factor of the samples

2.4.2 Determination of VFA Production

Samples for GC analysis were spun in a centrifuge at 13.2 RPM for 10 minutes and filtered using MIlipore® filter of pore size, 0.22 µm mesh. Analyses for volatile fatty acids (Butyric , acetic and propionic acids) and other solvents (ethanol, butanol and acetone) was carried out using Varian™ CP-3800 gas chromatograph with flame ionization detector and HP-Innowax column (40^{o}C - 260^{o}C; Column length, diameter & film – 30 m, 0.320mm & 0.50µm respectively), Carrier gas – Helium with a flow rate of 1 ml/ min.

2.5 Online Analyses

The fermentation temperature was maintained at 35^{o}C by circulating warm water through silicone tubing wound around the reactor. Heating of the water was achieved using Grant water bath. Temperature and pH were simultaneously monitored using a Mettle Toledo pH probe and monitor.

The off gas from the fermenter (4 L) was analysed using Phoenix Contact gas flow meter and Hy-Optima 700 H_2 sensor for gas flow rate and % hydrogen respectively. An average of 60 measurements/ minute was logged using a National Instruments datacard to LabView® software (student Edition).

2.6 Statistical Analysis

All experimental data were analysed using Microsoft Excel and were shown as mean ± SD (standard deviation).

3. Results

3.1 Experiment to monitor growth rates, substrate utilization rates and hydrogen yield from test substrates using ADS and C. acetobutylicum 6441

3.1.1 Growth rates

Figures 1a and 1b show the microbial growth dynamics in relation to their utilization of the test sugars. In figure 1a, it could be noticed that there was a 24 hour lag phase in the growth of ADS consortium on glycerol; however, this lag phase was avoided in the experiment involving *C. acetobutylicum* (fig. 1b) by using inoculums

developed by acclimatizing the test organism in glycerol. This shows that acclimatization can be used as a tool in the elimination of unwanted lag phases and to hasten the start-up of the process which according to Kyazze *et al.*, (2006) should not be time consuming.

In figures 1a and 1b, it could also be noticed that the growth pattern looked similar with points I and II showing a lag. This possibly could be due to the effect of metabolites production evident from GC analyses.

Also worthy of note is the reduction in the COD of the sugars present in the fermentation medium. A model of the growth rates and metabolic rates of the test inoculums against specific sugars is given below to reveal the most promising inoculums for hydrogen production.

Table 1. Growth and metabolic rates modelled using monod's equation and 1st order kinetics respectively

Organism	**Substrate**	**Spec. growth rate, μ (hr^{-1})**	**Metab. Rate $mgl^{-1}h^{-1}$**
C. acetobutylicum	Xylose	0.074	92.89
	Lactose	0.072	180.1
	Glycerol	0.087	149.7
ADS consortium			
	Xylose	0.059	22.35
	Lactose	0.052	35.59
	Glycerol	0.054	56.00

The table above shows that between both inoculums, C. acetobutylicum has the highest ability to metabolize the sugars. It however does not show any relationship between metabolic rate and growth rate as the specific growth rate, μ, of *C. acetobutylicum* on glycerol is 0.087 (highest) but its metabolic rate is lower than that of lactose. The same can be deduced from the rest of the variables in the table.

The table below shows the concentrations (mg/l) ±SD, n=2 of some identified metabolites produced as a reslt of the anaerobic fermentation

Table 2. Metabolites produced as a result of fermentation of various sugars by *C.acetobutylicum*

Organism	**Substrate**	**Acetic acid (mg/l)**	**Ethanol (mg/l)**	**Propionic acid (mg/l)**	**Butyric acid (mg/l)**
C. acetobutylicum	Xylose	223.397 ± 10.62	-	1.989 ± 0.014	1.89 ± 0.06
	Lactose	121.78 ± 8.3	2.431 ± 0.001		1.974 ± 0.02
	Glycerol	38.152 ± 0.67	-	17.794 ± 0.36	1.936 ± 0.1

Note: the unavailability of ADS metabolites GC results is as a result of time constraints, erratic power supply and set-backs due to long queue of the GC users.

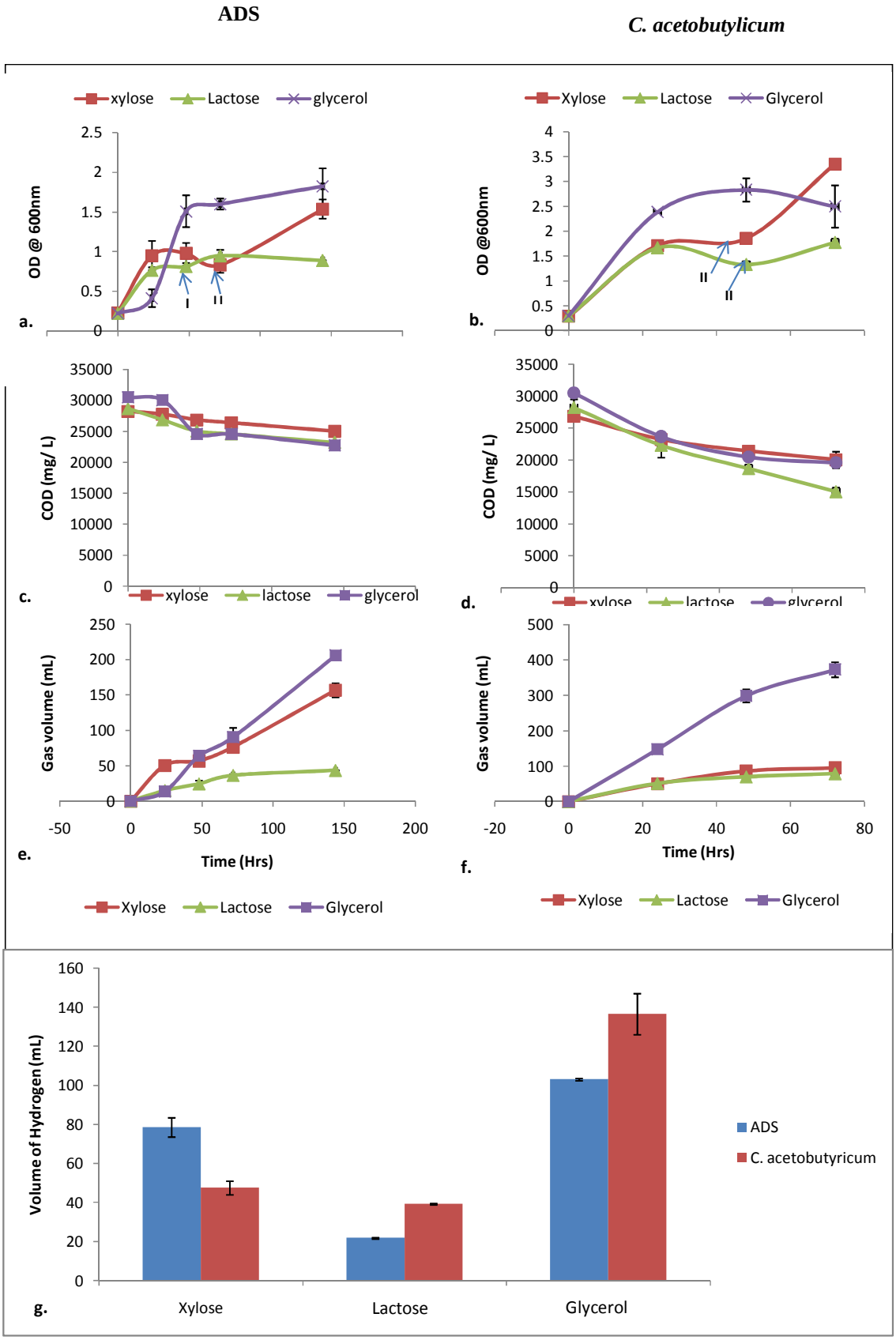


Figure 2. **a,b:** Growth kinetics of the fermentation using *C. acetobutylicum* and ADS; **c,d:** catabolic rate of various sugars by the test inoculums**; e,f:** time variation of volumetric gas production from various sugars using the test inoculums; g:comparative yield of hydrogen from from the test substrates and organisms. **g.** Comparison

of hydrogen yield

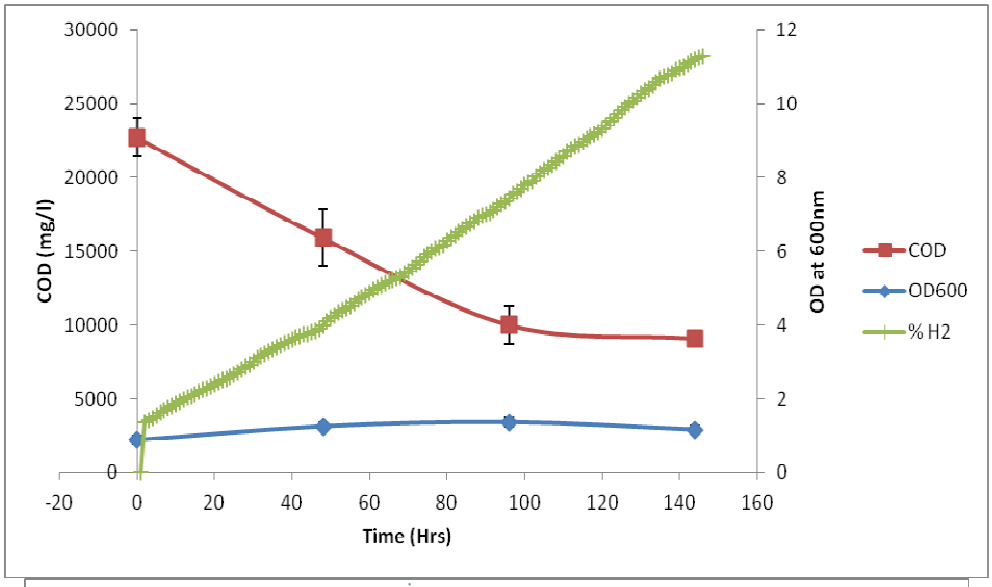
3.1.2 Comparative hydrogen Yield

A comparison of the yield of hydrogen is shown on Figure 1g. *C. acetobutylicum* as shown in the figure gave the highest yield of H_2 while metabolizing glycerol. From the histogram, it could be deduced that both inoculum readily produced hydrogen upon fermenting glycerol without the need for a co-substrate or bioaugmentation.

3.2 Production of Biohydrogen From Pretreated Wheat Straw and Crude Glycerol

In this experiment, the ability of both inoculums to breakdown pretreated lignocellulosic biomass (wheat straw) and crude (untreated) glycerol was assessed. Although samples were not drawn to estimate the amount of crude glycerol and wheat straw catabolised, about 49 ml and 68 ml of gas was produced by *C. acetobutylicum* and the ADS consortium respectively from wheat straw over a period of 144 hours. No gas was produced in the fermentation of crude glycerol with *C. acetobutylicum* until 120 hours of incubation. A total of 88 ml of gas was produced in the end of the fermentation (168 hours). Gas production was not observed in the fermentation of crude glycerol using the ADS sludge.

a



b

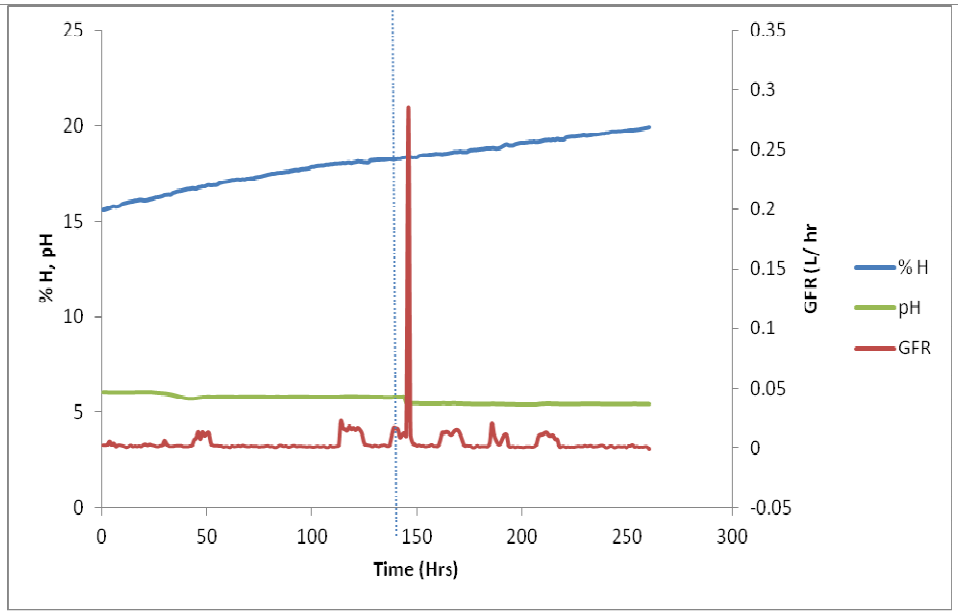


Figure 3. Graphical representation of the results from scale up experiments. **a:** growth, metabolic and hydrogen production kinetics using ADS on glucose. **b.** Hydrogen production kinetics with respect to the fermentation of untreated crude glycerol with *C. acetobutylicum* strain 6441

3.3 Experiment to Determine % Hydrogen Yield in Scaled-up Experiments

Over a fermentation period of 144 hours (Fig. 3a), on the average, 11.297 % of 21.73 L of the off gas was hydrogen. pH was perfectly buffered between 5.5 and 6. About 1.98 mg/ l butyric acid and 13.27 mg/l acetic acid was produced alongside a few other unidentified metabolites.

Crude glycerol fermentation using *C. acetobutylicum* was scaled up as well, however due to time limitations, complete analyses (GC, OD and COD) could not be carried out. However, average hydrogen yield was 19.425 % of 28.7 L off gas produced during the first run. An additional 20.055 % H_2 of 41 L off gas was also produced from the second 20 g/l crude glycerol fed into the fermentation system (fig 3b – from the dotted line). Thus giving a total hydrogen yield of 19.8% v/v of off gas produced throughout the fermentation run.

4. Discussion

From the results above, it can be established that both sets of inoculums (ADS and *C. acetobutylicum*) have the ability to produce hydrogen by fermenting cheap carbohydrate sources.This is evident from their ability to produce indicator-metabolites (VFAs) – predominantly acetic and butyric acids while reoxidizing reduced ferredoxin to gain energy.

The comparative hydrogen production potential between both inoculums is in favour of a pure bacterial strain thus supporting the citations by Wang *et al.*,(2003). The use of a pure culture in large scale or commercial production of biohydrogen is however undesirable due to the possibility of contamination. Its use will also lead to an increased process cost due to sterilization (Hung *et al.*, 2011; Kyazze *et al.*, 2006). However, since they are not fastidious, fermentation with them offers a clearer understanding of optimization measures and steps that must be followed to achieve higher yields of hydrogen. More so, since the sustainable production of biohydrogen depends on the fermentation of a large variety of complex cheap substrates (wastes), metabolic plasticity is very important. This is a very important characteristic of C. acetobutylicum evident from its ability to ferment both pure and complex substrates (crude glycerol and wheat straw) to produce hydrogen.

Although, naturally sourced hydrogenic consortium (ADS) is preferred to pure cultures,their inability to syntrophically produce more hydrogen or metabolize the substrates more than *C. acetobutylicum* is rather interesting. This justifies the suggestions of Hung *et al.*, (2011), that the first step to take in choosing a consortia for hydrogen production would be to understand the relationships between the microbial composition and their hydrogen production efficiency.

Fernando, E., a Ph.D student at University of Westminster, in his unpublished work identified the microbial strains present in the untreated sludge (the same as the one used in this research) using PCR-DGGE (polymerase chain reaction – denaturing gradient gel electrophoresis) as the following: *Clostridium sardiniensis* strain DSM 2632, *C. baratii* strain IP 2227, *C. uliginosum* strain CK55, *C. butyricum* strain VP13266, *C. colicanis* strain 3WC2, *C. saccharobutylicum* strain P262, *Eubacterium nitritogenes* strain JCM 6485 and *Pseudomonas delhiensis* strain RLD-1. Of these, only the *Clostridium* species are most likely to survive the selective pre-treatment of the sludge because of their ability to sporulate.

Although, strict anaerobes, the hydrogenic properties of all the afore mentioned *Clostridium* strains, especially as a consortium is unknown. Also unknown is their relationship; is there competition among the strains? Is any of them a non-hydrogenic carbohydrate consumer? This, according to Hung *et al.*, (2011) and Jo *et al.*, (2007), only render the process unfeasible and less productive. Low productivity of anaerobic consortia have also been blamed on the effect of overall population interaction which if altered as a result of fermentative metabolic changes, disturbs the microbial community function and perhaps their hydrogenic synthrophy. Thus, a more detailed study of the interaction and hydrogen production potential of each of the constituent strains in the ADS is needed.

The ability of both inoculums to produce hydrogen upon fermenting pretreated (partially) wheat straw as well as its mimicking substrate, xylose, is an indication that upon complete mineralization of lignocellulosic biomass, any of both inoculums could be used especially *C. acetobutylicum*.

5 Conclusion

Although the yield of hydrogen from the fermentation of the test substrates is small, this research has shown that hydrogen can be produced from the dark fermentation of cheap carbohydrate sources . Depending on cost considerations and the demand for metabolic plasticity, the choice of inoculums could vary between pure culture (*C. acetobutylicum* strain 6441) and ADS consortium. However, based on this research, *C. acetobutylicum* strain 6441 has a greater metabolic plasticity as well as hydrogen production potential than the consortium in the anerobically digested sludge

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