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Optimisation and Comparative Evaluation of Fermentative Hydrogen Production using Pure and Co-culture of *Clostridium beijerinckii* and *Enterobacter aerogenes*

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Abstract

Hydrogen, the only fuel to produce water as a by-product is seen as an ideal fuel for the future that can be produced from waste feedstock. The use of optimum bioreactor conditions and co-cultures with well-defined strains offer great potential to enhance hydrogen production due to diverse metabolic pathways. This study was aimed at determining the optimum pH and temperature on hydrogen production rate by *Clostridium beijerinckii* 6444 using glucose as substrate as well as investigating the influence of co-culture comprising of *C. beijerinckii* 6444 and *Enterobacter aerogenes* NCIMB 10102 on rate of hydrogen production and yield from glucose, molasses, crude glycerol and seed cake via dark fermentation. Batch fermentation in serum bottles was carried out in order to determine the total accumulative hydrogen gas, biomass concentration, and feedstock consumption in four days of fermentation. The co-culture was further scaled up to a 5 L fermenter. The optimum pH and temperature corresponding to 6.2 and 30 ^{o}C respectively were determined. The co-culture recorded the highest cumulative hydrogen volume (66.5 ± 4.2 ml) from crude glycerol though it did not

significantly increase the yields. The decrease in hydrogen production was observed as a result of formation of volatile fatty acids (acetic acid 1.5-3.1 g/l, butyric acid 3.2-5.6 g/l) among others which have inhibitory effects. The highest dead cell count of 3.4 x 10^8 cells/ml indicated that pH was inhibitory. The scale-up showed an increase in the hydrogen production rate from 13.38 ml/l/hr to 24.13 ml/l/hr and yield of 1.37 mol H_2/mol glycerol after 24 hrs. The study showed that the co-culture of *C. beijerinckii* and *E. aerogenes* can be used to enhance hydrogen production from crude glycerol as a cheap carbon source.

Keywords: Biohydrogen, Dark fermentation, Biofuel, Co-culture, Crude Glycerol

1. Introduction

Hydrogen produced from biological process (biohydrogen) is deemed to be the fuel for the future that will replace petrochemical fuels owing to less energy intensive technologies involved (Bader et al., 2010). Hydrogen has also been reported to have high energy yield of 122kJ/g which is three times more energy than same weight/weight ratio of petrol and diesel (Azbar & Levin, 2012). The energy can be converted to mechanical energy in combustion engines or used to generate electricity in proton exchange membrane fuel cells (Azbar & Levin, 2012). When undergoes combustion only water vapour and heat energy are liberated without carbon monoxide, carbon dioxide, hydrocarbons and fine particles which are a nuisance to the environment (Bader et al., 2010). The transition to hydrogen fuel consumption in the transportation sector accounts for 80% of the total projected increase over the next 30 years (EIA, 2013 and European Commissions, 2014; Masset et al., 2012). The increase in demand globally for hydrogen use has been bolstered considerably in the recent years mainly as a result of the threats posed by fossil fuels in contributing to environmental pollution such as high concentration of carbon dioxide in the atmosphere, global warming concerns as well as political and economic reasons associated with it (Ding et al., 2009). The Netherlands Environmental Assessment Agency, (2013) report shows that global carbon emissions have increased by 1.4% and had reached 34.5 billion tons in 2012. The report also indicates that carbon dioxide was a major constituent of the emissions contributing to the greenhouse effect, of which 57% was from combustion of fossil fuels.

Hydrogen is currently produced mainly from fossil fuels and energy-intensive processes such as gasification of coal, steam reformation-methane and electrolysis (Masset et al., 2012). The transition from non-renewable to renewable energy-based hydrogen has shown to provide a wide range of approaches such as light-dependent processes (e.g. photo-fermentation) or light-independent processes (i.e. dark fermentation) using microorganisms. However, dark fermentation is proving to be a more attractive process as it is capable of producing hydrogen without any constraints of light by utilising carbohydrates within a diverse range of organic waste feedstock and wastewater from sewage treatment, food, agriculture and municipal industries (Martínez-Pérez et al., 2007). Furthermore, dark fermentation has been reported to provide higher rates of production, more moderate reaction conditions and products that are easier to use in downstream processes compared to light dependant processes (Kothari et al., 2012; Levin et al., 2004).

Fermentative hydrogen producing microbes are capable of producing hydrogen in the exponential growth phase by oxidising glucose (Ding et al., 2009). Acetate and butyrate are

associated to hydrogen production with theoretical maximum yields of 4 moles H_2/mol glucose and 2 moles H_2/mol glucose, respectively (Hawkes et al., 2002). Biohydrogen has reportedly been synthesised by several species of fermentative bacteria: *Clostridium*, *Bacillus*, *Klebsiella*, and *Citrobacter* (Lee et al., 2011). Pure cultures, in particular *Clostridium* species, are found to be promising fermentative hydrogen producers among other genera (e.g. *Enterobacter*, *Bacillus)* with the highest hydrogen yields of 1.61-2.36 mol H_2/mol hexose (Hawkes et al., 2002). In order to eliminate the use of reducing agents for oxygen removal to maintain anaerobic condition in the fermenter, a co-culture with well-defined hydrogen producers and a consortium of micro-organisms can be employed. Co-culture is reported to poses metabolic diversity and enzymatic reactions which allow co-cultures to utilise a variety of complex sugars (polysaccharides, disaccharides etc.) compared to pure cultures. Bader et al. (2010) reported that co-cultures are more robust to alterations in environmental conditions and are capable of metabolising pentose and hexose simultaneously. However, the 'creation' of stable artificial co-cultures has been reported to be problematic due to differences in growth rates, pH requirements, consumption and excessive production of metabolites between micro-organisms (Weibel, 2008). In other studies, syntrophic associations between clostridia species and facultative aerobes *Enterobacter* and *Bacillus* have significantly enhanced hydrogen production by consuming oxygen and preventing inhibition of H_2 producing enzymes of the clostridia strain (Chou et al., 2011). The broad spectrum of applicable substrates in fermentative hydrogen production facilitates the possibility of combing the energetic utilization of biomass to hydrogen with the simultaneous treatment of waste materials (Quéméneur et al., 2011).

The study conducted by Hamilton et al. (2010) shows that hydrogen producing microbes are capable of regulating their metabolic pathway based on the concentration of liquid metabolites (volatile fatty acid and solvent) which is greatly influenced by environmental factors such as initial pH, temperature and type of substrate. In an attempt to improve hydrogen production via dark fermentation, optimisation of these factors and careful selection of microbial consortia play a critical role in developing bioprocesses. In this study, the strategy to investigate the influence of co-culture comprising of *C. beijerinckii* and *E aerogenes* from glucose, molasses, crude glycerol and seed cake was developed which included; 1) Optimisation of pH and temperature using a pure strain of *C. beijerinckii* from glucose. 2) Comparing the effect of the co-culture and their pure strains on hydrogen productivity and yield from different substrates using the optimum pH and temperature obtained in the previous set up. 3) Scaling up of the batch fermentation using the culture and the feedstock that gave the highest hydrogen yield so as to achieve better control of pH and agitation.

2. Materials and Methods

2.1 Strains and Growth Media

Pure strains of *Clostrium beijerinckii* 6444 and *Enterobacter aerogenes* NCIMB 10102 used for this study were purchased in powdered form from National Collection of Industrial Marine Bacteria (NCIMB, UK). *C. beijerinckii* was grown in a 125 ml serum bottle containing 90 ml tryptone yeast extract medium (TYG). The medium consisted of 3g/l

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tryptone, 5g/l yeast extract and 5g/l of glucose. Prior to inoculation, the serum vials were sealed with butyl rubber stoppers and flashed with nitrogen. The culture was then incubated at 37 ^{o}C for 48 hrs. *E. aerogenes* cells were revived using nutrient broth medium containing 15 g/l peptone, 3 g/l yeast extract, 6 g/l sodium chloride and 1 g/l D(+)-glucose. The cells were then aerobically incubated at 30°C in a shaken flask on a rotary shaker at 150 rpm for 48 hours.

2.2 Resources

All the materials and regents were purchased from Sigma Aldrich.

2.3 Substrates

In this study glucose, molasses, crude glycerol and seed cake were used as the carbon sources. The media for all the batch experiments were prepared by diluting glucose, molasses, crude glycerol and seed cake with distilled water to a concentration of 10 g/l and autoclaved separately from MDT medium. The MDT medium used comprised of 5 g/l casein peptone, 0.5 g/l yeast extract, 1.2 g/l KH_2PO_4, 5.1 g/l Na_2HPO_4, 0.5 g/l L-cysteine.

2.4 Experimental Procedure

2.4.1 Optimization of pH and Temperature

In order to determine the optimum pH and temperature for hydrogen production rate, a set of batch experiment were carried out in the 125 ml serum vials using pure culture of *C. beijerinckii* 6444 and glucose. A working volume of 80 ml was used consisting of 8 ml of culture placed in nine serum bottles each containing 8 ml glucose with a concentration of 10 g/l in 64 ml of fermentation medium (MDT).

Nitrogen was flushed into the vials to create anaerobic environment for culture. The experiments were in triplets with each set incubated at 20, 30 and 40 ^{o}C on a rotary shaker at 150 rpm with initial pH adjusted to 5.5, 6.2 and 7.0 for each set.

2.4.2 Investigating the influence of Co-culture on hydrogen

To study the influence of co-culture on hydrogen yield, four sets of experiment were used each comprising of a co-culture of *C. beijerinckii* and *E. aerogene*, a pure culture of *C. beijerinckii* and *E. aerogenes* and abiotic set up as controls. A working volume of 80 ml was used with each set of experiment employing a different type of substrate as carbon source. The substrates used consisted of 8 ml of 10 g/l of glucose, molasses, crude glycerol and seed cake in 64 ml MDT medium. The experiments were conducted aseptically under anaerobic conditions as described above at pH of 6.2 and temperature of 30 ^{o}C on a rotary shaker at 150 rpm.

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2.4.3 Scale-up of Hydrogen Production Using Co-culture

The co-culture was scaled up to a 5 L fermenter operated at 150 rpm. The working volume was 4 L with 10 g/L of glycerol concentration + MDT which was inoculated with 10% v/v of *C. beijerinckii* and *E. aerogenes* inoculum. All the parameters were measured as before: cell concentration, metabolites produced, pH, and viability. The pH and percentage of hydrogen was measured using data logger National Instruments Lab VIEW programme.

2.5 Sampling Analysis

Biogas samples from the head-space of the serum vials were collected using a 1 ml syringe fitted with a needle at pre-determined time intervals (every 24 hours) for four days. The optical density of the cultures was measured at 600 nm using spectrophotometer JENWAY model 6305. The pH changes over the experimental period were determined using a calibrated pH meter (Oakton benchtop series 700).

The phenol sulphuric acid method, as described by Dubois et al (1956), was used to determine the glucose concentration and sugars remaining in the culture on a daily basis. The concentration of glucose in molasses, crude glycerol and seed cake were measured in relation to sugar consumption. A standard curve with known concentrations of glucose (0-100 mg/L) was used to determine the amount of glucose remaining in the pure and co-culture medium. Residual liquid in samples from centrifugation at 13,000x g for 5 minutes was used in this assay.

The viability of cells in pure and co-cultures was determined from final samples using the LONZA trypan blue viability test. Samples were diluted tenfold and stained using 0.4% trypan blue (Sigma Aldrich). Dead cells were counted using haemocytometer at x 40 magnification under light microscope (Zeiss primo star).

The hydrogen composition in 1 ml of biogas injected from the head space was analysed and measured by means of gas chromatograph (SRI, model 310C) equipped with a thermal conductivity detector (GC-TCD) with the operational temperatures of injection port, oven, and the detector all set at 150°C. The GC-TCD employs nitrogen as carrier gas at flow rate of 3.8 ml/min. A one point calibration was performed using 50% hydrogen /50% nitrogen to determine the peak area which corresponded to 50% hydrogen within 1 ml of injected calibration gas. Accordingly, the peak area represented the percentage composition of hydrogen gas within gaseous samples analysed

The liquid metabolites (fatty acids and solvents) concentration were analysed using the gas chromatography (Varian CP-3800) fitted with flame ionisation detector (GC-FID). The liquid samples were centrifuged at 13,000g (Thermo Scientific-Heraeus Pico) and filtered using a 0.2 µm filter to remove collides and cells. Supernatant was stored in 1 ml GC vials. The GC contained TCD with a column size of 30 mm length x 0.320 mm diameter x 0.50 µm film - HP INNOWax (Agilent J & W GC columns).The mobile phase was hydrogen and helium gas with a flow rate of 2 ml/min. The programme innowax was used, with the injector temperature of 220 ºC and detector temperature of 250 ºC. The temperature of the chamber was maintained at 35 ºC from 0 - 5 min after which it increased constantly to 170 ºC for 15 min. Unknown concentrations of metabolites were determined using analysed standard solutions of acetic acid, butyric acid and ethanol.

2.6 Statistical Analysis

Statistics is used in analysing the results and average figures are expressed as mean values±SD.

3. Results and Discussion

3.1 Optimisation of pH and Temperature for Hydrogen Production

Table 1 shows the cumulative volume of hydrogen produced in four (4) days of fermentation using pure strain of *C. beijerinckii* from glucose in MDT fermentation medium. The buffering action of the MDT medium prevented any significant change in pH, hence a constant pH range was observed in the four days of fermentation in all the cultures. The results indicate that the highest volume of hydrogen of 58.9±1.8 ml was recorded at the temperature of 30 °C and pH of 6.2 compared to second highest performance of 52.4±2.0 ml which was obtained at temperature of 40 °C and pH of 6.2. The lowest cumulative hydrogen yield (7.8±8.5 ml) was measured at the temperature of 20 °C and pH of 5.5 for the same period of time. Butyric and acetic acid were the major metabolites produced in the cultures. The VFA showed an inhibitory effect on the cultures and hydrogen gas production, which was assessed by measuring dead cell counts.

Table 1. Cumulative hydrogen production at various temperature and pH

Temperature (°C)	pH	Cumulative H_2 Production (ml)
20	5.5	7.8±8.5
	6.2	2.02±3.0
	7.0	8.8±5.2
30	5.5	40.5±2.2
	6.2	58.9±1.8
	7.0	51.8±5.0
40	5.5	29.3±4.2
	6.2	52.4±2.0
	7.0	38.9±1.9

The results obtained compare well with the work of Tanisho et al. (1987) who reported hydrogen producing ability of *C. beijerinckii* from glucose to yield above 50% at optimum pH of 6.0-6.5 and temperature 30-37 °C compared to pH and temperature outside the respective specifications. Temperature and pH have been extensively studied and reported to be critical parameters which have an effect on hydrogen production by altering metabolic pathways through inhibiting the enzymatic activity of microbes (Khanal et al., 2004; Masset et al., 2010). The activities of the essential enzyme hydrogenase and ionization state of the amino acids functional groups, capable of taking part in catalysis, are mainly dependant on changes in temperature and pH respectively. It has been reported that optimal pH and

temperature promotes the production of acetic acid and butyric acid, both of which are associated with increased hydrogen yields (Khanal et al., 2004). However, the study conducted by Temudo et al. (2007) revealed that under unoptimal pH, the hydrogen fermentation process shifted to solvent production or prolonged the lag phase. Furthermore, the lactate production was always observed together with sudden change of environment parameters, such as pH, and temperature, which indicated the culture was not adapted to the new environment conditions.

3.2 Comparative Hydrogen Production by Pure and Co-culture

3.2.1 Cumulative Hydrogen Production

As represented in figure 1, there is significant variation in the hydrogen volume produced in four days of fermentation by co-culture of *C. beijerinckii* and *E. aerogenes* and pure culture of the same strains from glucose, molasses, glycerol and seed cake. The graphs generally indicate a pattern of lag phase, followed by a rapid hydrogen production phase and minimal production towards the end. The highest cumulative volumes of hydrogen gas production of 66.5±4.2 ml and 61.2±3.2 ml after 72 hrs were recorded by co-culture from glycerol and molasses as shown in fig. 1C and 1B respectively. Figure 1A and 1D show that there is no significant difference in total cumulative H_2 gas obtained between co-culture and pure culture of *C. Beijerinckii.* The lowest cumulative H_2 gas production of 36.5±5.3 ml was recorded using *E. aerogenes* from glucose as shown in Figure 1A.

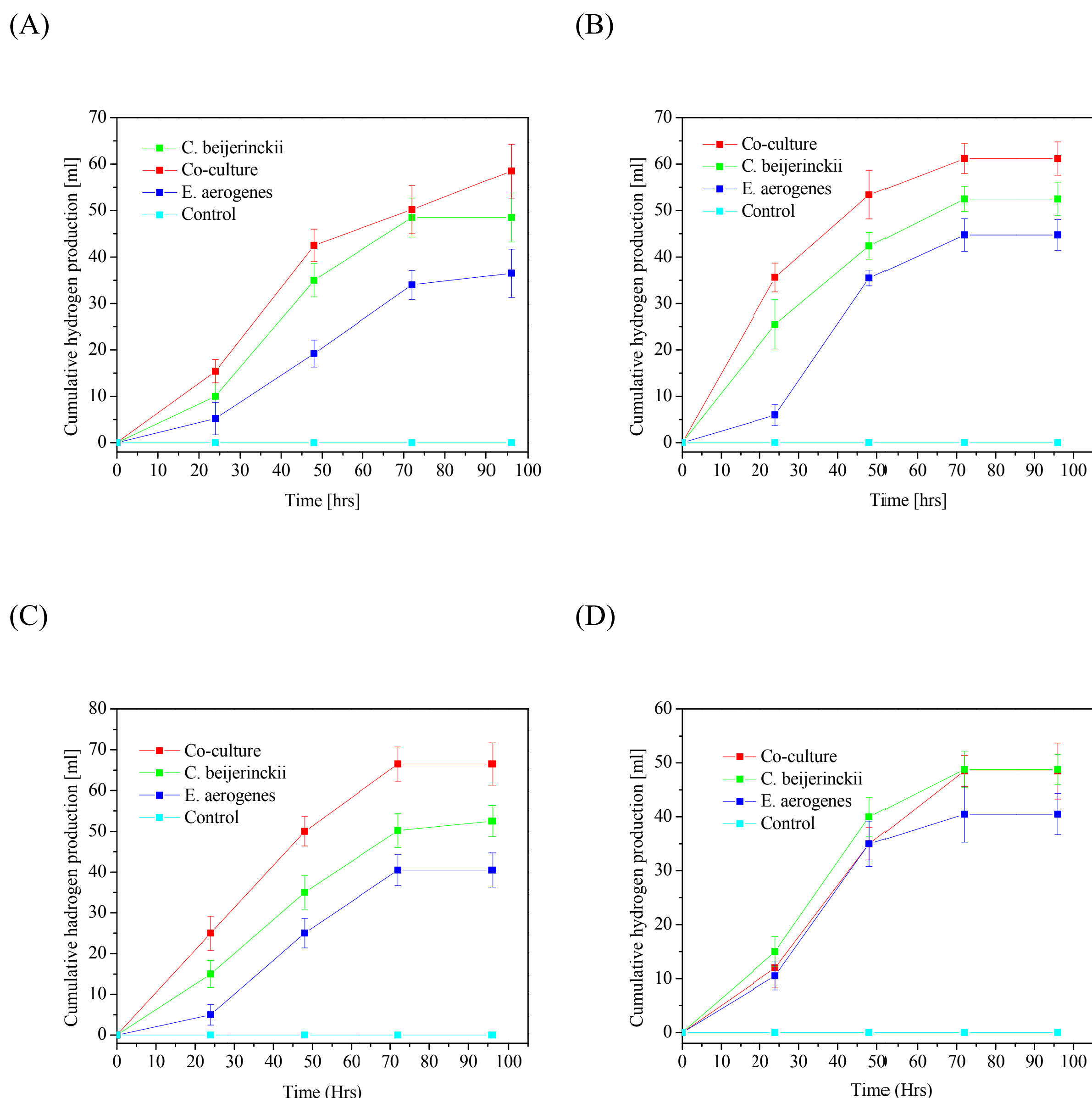

Figure 1. Investigation of cumulative hydrogen yield using co-culture of *C. beijerinckii* and *E. aerogenes* and their pure strains from different substrates (A) glucose (B) molasses (C) crude glycerol and (D) seed cake

It can be observed from the study that co-culture is capable of enhancing hydrogen productivity from glycerol. This could be attributed to the stability of the two strains in the co-culture as co-culture recorded the higher cell density compared to pure strains. Co-culture has been shown to have the ability to metabolise diverse range of sugars such as monosaccharides, disaccharides (fructose maltose, xylose etc.) from crude glycerol and molasses compared to pure strains (Chang et al., 2008). This is supported by the work conducted by Wang et al. (2008) which showed higher H_2 production rates and yields from

crude glycerol and molasses than glucose. In addition, Wang and Wan, (2009) observed that hydrogen yield could be improved by co-culturing a strict anaerobe *Clostridium* with a facultative *Enterobactor* that acts as scavengers of oxygen in the fermentation medium as oxygen sensitivity is a major limiting factor of most anaerobes. On the other hand, hydrogen production by pure cultures from waste feedstock has been a challenge as a result of the necessity for sterile conditions and in most cases incomplete utilisation of substrate (Temudo et al., 2007). However, the findings by Masset et al. (2012) showed a higher productivity of hydrogen of 1.5 L biogas/h using artificial co-cultures of known *Clostridium* species compared to pure cultures without the need for pre-treatments to hydrolyse complex compounds. This study demonstrates that all the cultures were capable of producing H_2 gas from the substrates used. The decline in H_2 production after day three could be as a result of nutrient and carbon source limiting environment along with inhibitory effects of low pH and VFA (acetic and butyric acids (Weibel, 2008).

3.2.2 Metabolites, Culture Growth, Substrate Consumption and Cell Viability during Fermentation

Figure 2, shows the graph of the main identified aqueous metabolites produced using co-culture of *C. beijerinckii* and *E. aerogenes* and their pure strains from glucose, crude glycerol, molasses and seed cake. The metabolites measured include acetic acid, butyric acid and ethanol. The graph shows similarities in the pattern of the aqueous metabolites produced with the highest concentration being that of butyric acid compared to that of acetic acid and ethanol. The co-culture from glycerol and molasses produced a greater quantity of butyric acid (5.6 and 5.4 g/l respectively) while *E. aerogenes* from seed cake recorded the lowest (3.2 g/l).

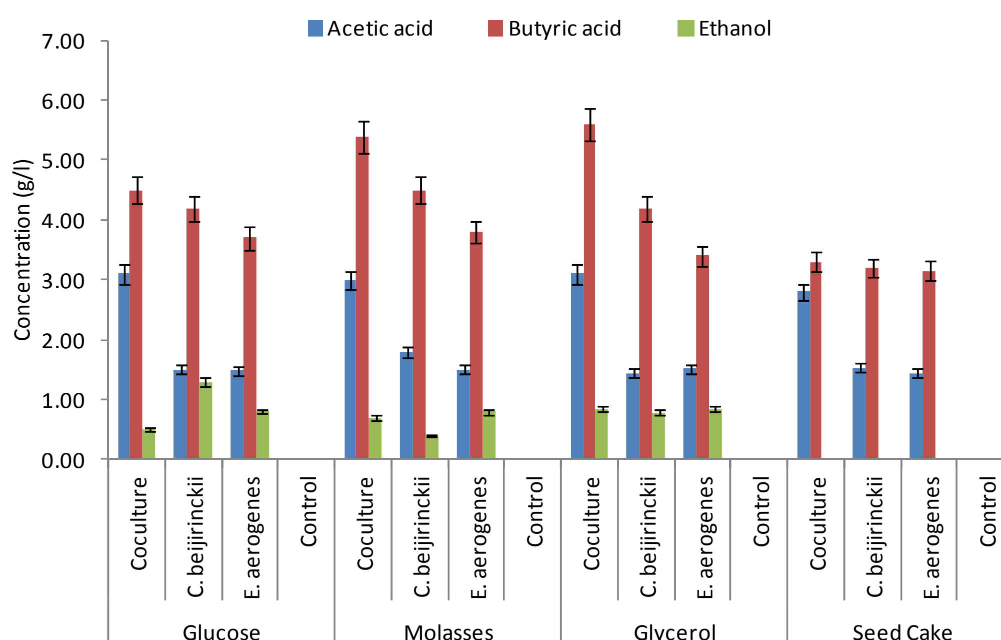

Figure 2. Aqueous metabolites produced using co-culture of *C. beijerinckii* and *E. earogenes* and their pure strains from glucose, molasses, crude glycerol and seed cake

The figure suggests that hydrogen was directly produced through the butyric acid metabolic pathway and explains why higher total cumulative gas was measured by co-culture from glycerol and molasses and lowest by *E. aerogenes* from seed cake. The results indicate that the concentration of butyric acid produced was about twice higher than that of acetic acid, hence only 2 moles of H_2 per hexose was produced. The high concentration of butyric acid obtained in the co-cultuture from crude glycerol and molasses correspond to high hydrogen yields. Masset et al. (2012) reported that C. *beijerinckii* strains have the ability to re-consume formate and lactate, producing additional hydrogen with carbon dioxide and butyrate as major by-products. The study carried out by Hu et al. (2013) also revealed that higher ratio of butyrate and acetate are associated with higher hydrogen yield while solvent reduced compounds such as ethanol, lactate and acetone are linked with lower hydrogen yield.

The trends from the OD indicated that the cell density was highest for co-cultures and remained stable during the last two days of fermentation compared to pure cultures. This suggests that the co-culture had reached the stationary phase within two days of fermentation. To the contrary, the growth of *C. Beijerinckii* and *E. aerogenes* showed a steady growth during the four days of fermentation implying that there was a slow growth. The trends of substrate consumption also revealed that the highest utilisation of glucose from glycerol was recorded after two days in all the cultures resulting in poor cell growth. In order to assess the viability of the cultures, dead cell count was used. Table 2 below shows the number of dead cells obtained at the end of fermentation. It can be seen that all the cultures showed high number of dead cells as a result of inhibitory effect toxic metabolites which leads to cell death.

Table 2. Dead cells at the end of fermentation

Culture	**Dead Cell count x 10^8 (cells/ml)**
Co-culture	3.4 ± 2.32
C. beijerinckii	3.1 ± 1.94
E. earogenes	2.3 ± 0.96
Control	0 ± 0

3.2.3 Scale up: Hydrogen Production Rate, Yield and Metabolites

The hydrogen production by co-culture from glycerol was scaled up to 5 L batch fermenter so as to increase hydrogen production rate per litre. The profile on figure 3 indicates a lag phase which was followed by rapid evolution of hydrogen from 18% to 97% during exponential phase between 20-27 hrs. This was accompanied by decrease in pH from 7.2 to around 6.1-6.3 optimal range. The highest gas composition of 99% hydrogen was recorded after 28 hrs at approximately maximum biomass level of 7900 mg/l as shown in figure 4. This shows that as the biomass concentration increases, the hydrogen production increases correspondingly. After 48 hrs, a decrease in % hydrogen production was measured as a result of exhaustion of carbohydrate coupled with toxic effects from acidic metabolites. Liquid samples taken after 16, 32 and 48 hrs gave 524.5, 164.6 and 40.2 mg/l carbohydrate content

and 0.412, 0.289 and 0.025 absorbance readings for biomass analysis (x 50 dilution factor) respectively.

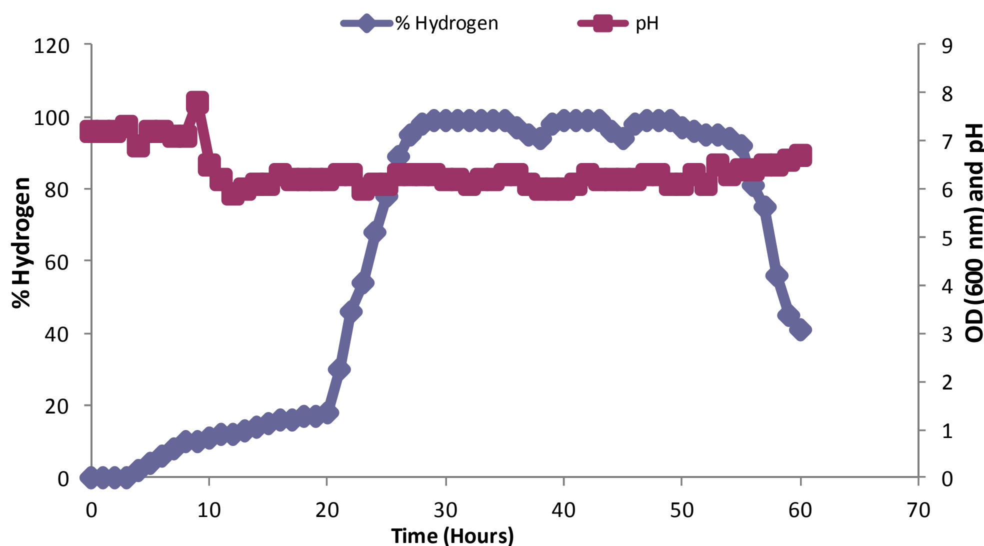

Figure 3. Cumulative hydrogen production using co-culture from crude glycerol

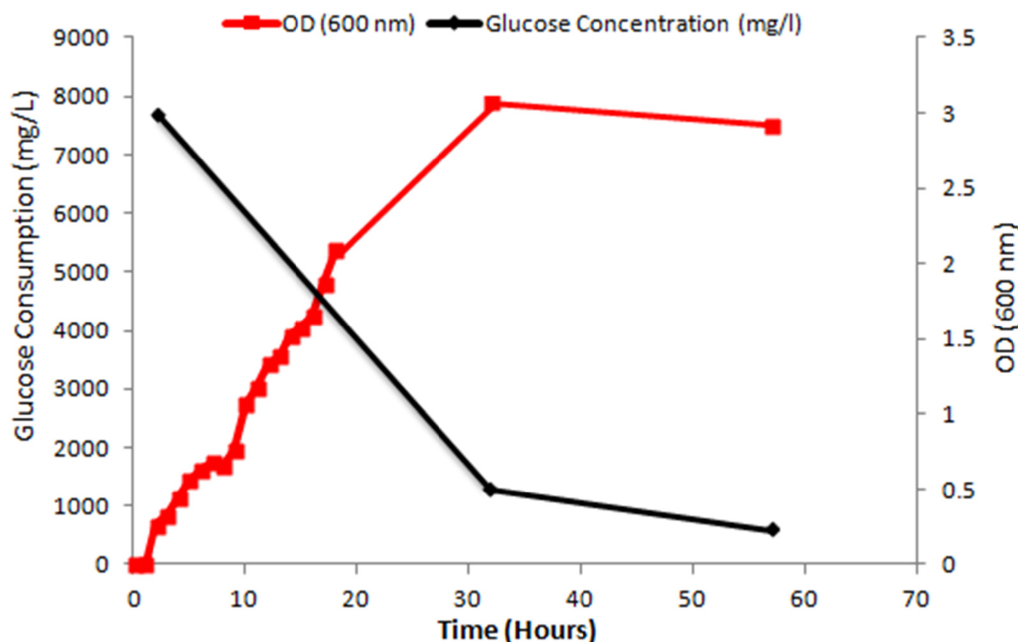

Figure 4. OD and glucose consumption of co-culture 5-litre batch fermentation

The study recorded an increase in hydrogen productivity by 10.75 ml/l/hr from 13.38 ml/l/hr and hydrogen yield (1.37 molH_2/mol glycerol) after 24 hrs. This could be attributed to slow release of glucose from the breakdown of crude glycerol. This was followed by an increase in

hydrogen generation and consequently a formation of VFA which led to decline in hydrogen production. The build up in partial pressure of hydrogen could have caused the alteration in metabolic pathway from acid production to ethanol production as evident from the rise of ethanol concentration. A report carried out by Argun and Kargi, (2011) showed that large-scale batch fermentation reduce the hydrogen yields due to inhibition of H_2 formation by excessive feedstock and metabolites produced. Table 3 below shows hydrogen production rate and yield obtained during batch fermentation at time 24 and 48 hrs. The final cell dead count is also indicated.

Table 3. Concentration of metabolites, hydrogen production rate, yield and final dead cell count in a 5-litre batch fermentation

Time (Hours)	Acetic acid (g/l)	Butyric acid (g/l)	Ethanol (g/l)	H_2 Production rate ml/hr)	H_2 yield (mol H_2/mol glycerol)	Final dead cell count (cells/ml)
0 – 24	3.65	7.26	0.41	13.38		
24 - 48	2.14	8.91	3.07	24.13	1.37	2.12×10^8

The GC analysis for the scale-up study showed variations higher concentration of acetic acid compared to butyric acid and this corresponds to higher H_2 yield. 65% of metabolite was acetic acid, thus hydrogen was produced with theoretical maximum value of 4 mol H_2/mol glucose (Reith et al., 2003). A variation in metabolites was observed as crude glycerol comprises complex mixtures of different sugars. Quéméneur et al. (2011) reported various metabolite concentrations i.e. acetic acid, butyric acid and ethanol from different carbohydrates and improved hydrogen production from long chain-length sugars. The amount of acetic acid produced between 24-48 hrs is directly proportional to the rate of production of hydrogen (24.13 ml/hr) and yield (1.37 mol H_2/mol glycerol). The dead cell count indicates the inhibition of cell culture due to toxic metabolites.

4. Conclusion

The investigation shows that co-culture of *C. beijerinckii* and *E. aerogenes* together with its pure strains are capable of producing hydrogen from glucose, molasses, crude glycerol and seed cake. The results also indicate that co-culture can be used to enhance hydrogen productivity and yield from crude glycerol at optimum pH and temperature of 6.2 and 30 °C respectively. Hydrogen production from all the feedstock used follows a similar pattern of lag phase, followed by a rapid hydrogen production phase and minimal production towards the end due to exhaustion of glucose and toxic effects acidic metabolites. The highest cumulative volume of hydrogen gas production of 66.5 ml and 61.2 ml after 72 hrs was recorded by co-culture from glycerol and molasses respectively. The scale-up showed an increase in the hydrogen production rate from 13.38 ml/l/h to 24.13 ml/l/h in 24 hrs. The yield was determined to be 1.37 mol H_2/mol glycerol.

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