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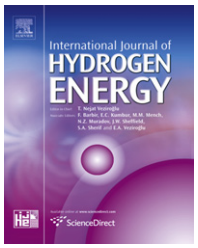

Bioconversion of crude glycerol from biodiesel production to hydrogen

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ABSTRACT

This study evaluates the potential of bioconversion of crude glycerol, discharged from biodiesel production plant, to hydrogen (H_2) by an enriched microbial community. Microbial community was enriched from activated sludge in a medium amended with 2.5 g/L of crude glycerol. Optimal cultivation parameters for H_2 production such as initial pH, cultivation temperature and substrate concentration were investigated. H_2 yields from raw glycerol at optimal conditions (pH 6.5; 40 °C and 1 g/L raw glycerol) were 1.1 ± 0.1 mol-H_2/mol-glycerol$_{consumed}$. H_2 production was associated with acetate-butyrate type fermentation, along with ethanol as one of the end products. Kinetic experiments on H_2 production from pure and crude glycerol indicated the absence of any inhibitory effects from the impurities present in crude glycerol. The community analysis revealed that the enriched microbial consortium was dominated mainly by *Clostridium* species.

1. Introduction

Trans-esterification of oils in biodiesel manufacturing process produces glycerol as a main by-product. Increasing amount of crude glycerol has led to waste disposable issues. Recently, researchers have been studying the possibilities to convert the waste glycerol to useful products, wherein, microbial conversion of residual glycerol has been considered as an economically viable process. Microbe-assisted conversion of waste glycerol to valuable compounds such as methane [1], 1, 3-propanediol [2,3], ethanol [4], succinic acid [5] and H_2 [6,7] have been reported. Ito et al. investigated the production of H_2 and ethanol from waste glycerol using *Enterobacter aerogenes* HU-101 strain [8]. Lately, Ngo et al. reported a yield 2.7 mol-H_2 mol−1 glycerol$_{consumed}$ from pretreated crude glycerol along with N_2 sparging and pH control using *Thermotoga neapolitana* DSM 4359 strain [6]. Saki and Yagishita demonstrated the bioconversion of discharged waste glycerol to H_2 and ethanol using bioelectrochemical cells [9]. Recently, bioconversion of crude glycerol has been studied using natural mixed inoculum. Selembo et al. reported H_2 and 1, 3-propanediol (1,3-PD) production from industrial glycerol using mixed microbial culture as inoculum [7]. Fernandes et al. investigated the effect of H_2 production from industrial glycerol waste using anaerobic sludge as inoculums and reported a yield of 200 ml-H_2 g^{-1} COD glycerin [10].

Physico-chemical parameters such as initial pH, cultivation temperature and substrate concentration greatly affect the biohydrogen production efficiency. It has been reported that medium pH and cultivation temperature are the most vital factors that affect microbial growth lag phase, enzyme activities, metabolite distribution and H_2 production [11].

In this study, optimal initial pH, cultivation temperature and substrate concentration were tested for maximal H_2 production by an enriched microbial community grown in minimal medium amended with industrial glycerol as the sole

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carbon source. Further, kinetic experiment was performed to study the effect of impurities in industrial glycerol on growth, H_2 production and substrate utilization. Finally, the enriched microbial community was analyzed.

2. Materials and methods

2.1. Glycerol source

Pure glycerol of molecular biology grade ($\geq$ 9) was obtained from Sigma. The crude glycerol, a by-product from biodiesel manufacturing process, was kindly provided by Savon Siemen Oy (Iisalmi, Finland), The crude glycerol had an alkaline pH (pH ~ 12) and contained 45% (v/v) glycerol, 30% (v/v) methanol.

2.2. Enrichment of microbial community

Activated sludge collected from waste water treatment plant (Viinikanlahti, Finland) was used as the seed inoculum. Modified HM100 medium (NH_4Cl 1.0 g/L, K_2HPO_4 0.3 g/L, KH_2PO_4 0.3 g/L, $MgCl_2.6H_2O$ 2.0 g/L, KCl 4.0 g/L, Na-acetate $3H_2O$ 1.0 g/L, tryptone 1.0 g/L, cystein-HCl 0.5 g/L and resazurin 0.002 g/L) was used as the enrichment and growth medium. The seed inoculum was enriched in 120 ml serum bottles with working volume of 50 ml sterile anoxic pure glycerol (100 g/L) amended enrichment medium at 37 °C. By the onset of H_2 production, 5 ml of culture was inoculated to similar media and the enrichment process was repeated twice. After two enrichment rounds, the culture was grown in enrichment media supplemented with anoxic crude glycerol (2.5 g/L). Enrichment was conducted twice with crude glycerol as the sole carbon source, resulting in H_2 producing enrichment inoculum that was used for subsequent experiments.

2.3. Experimental procedure

Batch experiments were performed in 120 ml serum bottles with a working volume of 50 ml. Crude glycerol (2.5 g/L) was used as the carbon source unless otherwise stated. Batch experiments to investigate the effect of initial medium pH (5.0–8.0) on H_2 production were performed at 37 °C and 150 rpm. H_2 production efficiencies at different temperatures (25–46 °C) were studied temperature gradient incubator (Test Tube Oscillator, Terra-tec, Australia) at optimal pH and 150 oscillations/min. Twenty-five milliliter tubes with 10 ml culture medium, supplemented with crude glycerol (2.5 g/L) was inoculated with 100 ml pre-culture and incubated in temperature gradient at the optimal initial pH (observed from pH experiment). Optimal substrate concentration was tested with different crude glycerol concentrations (0.5, 1, 2.5, 3.5 and 5 g/L) at optimal pH and cultivation temperature. The cultivation time for all the experiments was 72 h. The experiment was conducted in triplicates and the data were averaged.

Carbon material balance and H_2 yield values was calculated as reported previously [12]. Carbon dioxide in the liquid phase and carbon associated with biomass was excluded from the carbon mass balance calculations. The crude glycerol used in this study contained 30% methanol. To confirm methanol utilization by the enriched microbial community, a growth-curve test was performed and analyzed the end metabolites produced. It was observed that the microbial consortium did not utilize methanol. Hence, methanol was excluded from the carbon mass balance calculations.

Kinetic experiments on pure and crude glycerol were performed at pH 6.5, 40 °C and 1 g/L crude glycerol. Cumulative H_2 production was calculated as previously described [13]. The fermentation time for kinetic study was 194 h and was conducted in triplicates.

2.4. Molecular characterization of microbial community

In order to analyze the presence of dominant microbes, 16s RNA gene from 10^1 to 10^6 dilutions of the enriched inoculum was amplified using polymerase chain reaction (PCR). The amplified products were separated by denaturing gradient gel electrophoresis (DGGE). Amplification of 16s RNA gene of the bacterial community and DGGE was performed as previously described [14]. Prominent bands from DGGE gel were cut and re-amplified using 5′-CCT ACG GGA GGC AGC AG-3′ and 5′-CCG TCA ATT CMT TTG AGT TT-3′ primers. The amplified products were sequenced (Macrogen, Korea) and compared with existing sequences available in GenBank using BLAST program.

2.5. Analytical techniques

Organic acids, alcohols and carbon substrate were analyzed using High-Performance Liquid Chromatography (HPLC) (LC-20AD, Shimadzu, Japan) and the gaseous content were analyzed using Gas chromatograph (GC-2014, Shimadzu GC) as described previously [12]. Measurements were repeated thrice and averaged. H_2 content mentioned in the study refers to the H_2 concentration (%) in the biogas present in the headspace of the serum bottle.

The modified Gompertz equation was used to fit the cumulative hydrogen production data obtained from batch cultivations to obtain the major parameters [15].

$$H = P\exp\left\{-\exp\left[\frac{R_m * e}{P}(\lambda - t) + 1\right]\right\} \quad (1)$$

Where H presents the cumulative hydrogen volume (ml), P the hydrogen production potential (ml), R_m responds to the maximum hydrogen production rate (ml/h), λ is the lag phase time (h) and e is 2.718 and t .the incubation time (h). The values for P, Rm λ were determined by fitting the hydrogen production data for Equation (1) using the Solver function in Microsoft Excel 2007 by congregating the residual sum of squares between the experiment and estimation data to a minimum value.

3. Results and discussion

3.1. Effect of initial medium pH on H_2 production

The optimal initial medium pH that yielded the maximum H_2 production on crude glycerol as the sole carbon source by the enriched inoculum was investigated. The enriched

community was observed to utilize crude glycerol and produce H_2 at wide pH ranges. Though H_2 was produced, a drastic decrease in H_2 yield was observed at pH 5.0 as well as minimal utilization of substrate. Substrate utilization and H_2 yield increased with an increase in pH up to pH 6.5, after which an opposite trend was observed. Substrate utilization also followed the similar trend as H_2 production. The substrate utilization increased from 4.6% ± 0.5 (pH 5.0) to 36.7% ± 3.5 (pH 6.5) and thereby remained stable at higher pH ranges (Table 1). The optimal initial medium pH was identified to be pH 6.5.

At optimal pH value, the substrate utilization and H_2 yield was observed to be 36.7% ± 3.5 and 0.7 ± 0.0 mol-H_2/mol-glycerol$_{consumed}$ respectively. Similarly, initial pH significantly affected the H_2 content. The H_2 content in the gas phase increased from 0% at pH 5.0 with an increase in initial pH and was 12.5% ± 0.3 at pH 6.5. Further increase in initial pH decreased the H_2 content. Increased H_2 yields from crude glycerol at slightly alkaline conditions have been previously reported [7,9,16].

VFA and ethanol was produced as a function of initial pH. The inoculum followed acetate-butyrate fermentation along with ethanol as one of the end metabolites. Onset of H_2 production co-occurred with acetate and butyrate while an increase in ethanol concentrations negatively affected the H_2 production. At pH 5.0, the inoculum showed low substrate utilization efficiency (4.6% ± 0.5) and produced very little end metabolites, negatively affecting the carbon and electron recovery. Growth at low pH values results in reduced formation of intracellular ATP, inhibiting the substrate utilization. Hydrogen production and substrate utilization was observed to increase with an increase in pH (5.0–6.5) in the presence of higher concentrations of acetate and butyrate. Further increase in pH resulted in a shift in the end-metabolite production, with an increase in ethanol production and decreased H_2 yield. 1, 3-PD was not observed in any pH ranges. At pH 6.5, acetate (1.2 ± 0.2 mM), butyrate (3.6 ± 0.8 mM) and ethanol (4.1 ± 0.6 mM) were produced. Acetate is the main by-product of cell growth and an increase in acetate concentration usually indicates the improvement in cell growth [23]. Acetate and butyrate production can be directly linked with efficient H_2 production, whereas ethanol formation utilizes the reducing equivalents, thus decreasing H_2 production efficiency [17].

3.2. Effect of cultivation temperature on H_2 production

Effect of cultivation temperature on H_2 production was investigated within the range of 25–46 °C with crude glycerol (2.5 g/L) as the sole carbon source. The initial pH was adjusted to be 6.5. The H_2 yield increased from 0.3 ± 0.0 to 1.0 ± 0.3 mol-H_2/mol-glycerol$_{consumed}$ with an increase in temperature from 25 to 40 °C. Any further increase in cultivation temperature decreased the H_2 yields (Fig. 1). In terms of H_2 yield, much difference was not observed within the temperature range 31 °C–43 °C. Utilization of crude glycerol followed the similar trend as of the H_2 yield. Substrate utilization improved from 2.9% ± 0.7–35.7% ± 1.4 when the temperature was raised from 25 °C to 40 °C. In terms of substrate utilization efficiency, 40 °C was observed to be optimal. At optimal conditions (40 °C), the H_2 content and H_2 yield was calculated to be 14.0% ± 0.2 and 1.0 ± 0.3 mol-H_2/mol-glycerol$_{consumed}$ respectively. Absence of growth and utilization of carbon source was observed at 46 °C.

As shown in Table 2, the H_2 evolution was accompanied by the production of acetate and butyrate along with ethanol. At optimal conditions for H_2 production (40 °C), acetate (3.8 ± 0.0 mM), butyrate (3.3 ± 0.1 mM) and ethanol (3.0 ± 0.3 mM) was produced. Acetate, butyrate and ethanol concentrations remained unchanged when the cultivation temperature was raised from 35 °C to 40 °C, indicating the temperature range for efficient substrate utilization and growth. An increase in temperature from 40 °C to 43 °C resulted in a sharp decrease in H_2 content and acetate concentration, signifying that acetate is accompanied with H_2 production. These results indicate that the enriched microbial community produced H_2 proficiently at mesophilic temperature and higher temperatures impaired growth and metabolite production.

3.3. Effect of substrate concentration on H_2 production

The initial substrate concentration is also a crucial parameter for optimizing H_2 production [18]. Effect of substrate concentration on substrate utilization, H_2 production and metabolite distribution was tested with different concentrations of crude glycerol (0.5, 1, 2.5, 3.5, 5.0 g/L) by the enriched inoculum (Table 3). The experiments were conducted in batch cultivations at pH 6.5 and 40 °C. It was observed that the H_2 yield increased positively with crude glycerol concentration up to

Table 1 – Glycerol utilization (%), H_2 content in the gas phase (%), H_2 yield (mol-H_2/mol-glycerol$_{consumed}$), carbon recovery (%), electron recovery (%) and end metabolite concentration (mM) as a function of initial medium pH (standard deviations in parenthesis).

pH	Crude glycerol utilization (%)	Metabolites (mM)[a]			H_2 content in the gas phase[a] (%)	H_2 yield (mol-H_2/mol-glycerol$_{consumed}$)	Carbon recovery[b] (%)	Electron recovery[b] (%)
		Acetate	Ethanol	Butyrate				
5.00	4.6 (0.5)	0.1 (0.0)	0.1 (0.0)	0.1 (0.0)	0			
6.00	10.7 (2.0)	0.5 (0.1)	0.4 (0.1)	1.7 (0.1)	0.8 (0.5)	0.1 (0.0)	91.3	86. 7
6.50	36.7 (3.5)	1.2(0.2)	4.1 (0.6)	3.6 (0.8)	12.5 (0.3)	0.7 (0.0)	85.8	80.9
7.50	30.8 (1.0)	1.0 (0.2)	3.7 (0.2)	3.1 (0.2)	9.9 (0.2)	0.5 (0.0)	90.8	69.4
8.00	30.5 (0.4)	1.1 (0.2)	4.1 (0.3)	3.0 (0.1)	9.3 (0.2)	0.6 (0.0)	92.0	78.0

a Calculated for the serum-bottle culture with the working volume of 50 ml, corresponding to 49 ml modified HM100 media amended with 2.5 g/L of crude glycerol and 1% inoculum. Each value was measured after 48 h cultivation and was an average of triplicate cultivations.
b CO_2 in the liquid phase and carbon content in biomass was ignored during carbon recovery and electron recovery calculations.

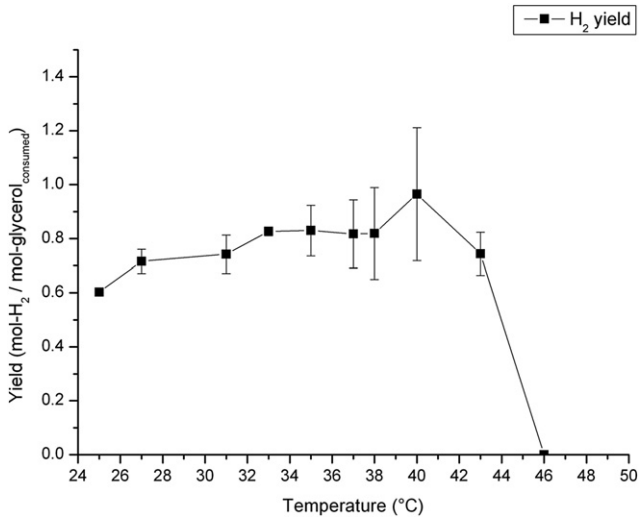

Fig. 1 – Effect of H_2 yield on cultivation temperature. Error bars indicate standard deviation from triplicate experiments. In some cases, the error bars are smaller than the symbol.

1 g/L. Fermentation with substrate concentrations above 1 g/L resulted in decreased substrate to H_2 conversion. Decrease in substrate utilization was observed at higher pure glycerol concentrations (data not shown). Theoretical yield of H_2 from glycerol is 3.0 mol-H_2/mol-glycerol [7]. At optimal conditions (1 g/L), the hydrogen yield was 1.1 ± 0.1 mol-H_2/mol-glycerol$_{consumed}$, corresponding to 37% of the theoretical maximum. Previous studies on crude glycerol fermentations have also reported a decrease in hydrogen production with an increase in crude glycerol concentration [8,19]. These observations suggest that surplus of organic compounds in higher concentrations of crude glycerol has an inhibitory effect on hydrogen production.

At glycerol concentrations 0.5–1 g/L the substrate utilization efficiency of 54.8%–56.5% was observed. Optimization of substrate concentration improved the substrate utilization efficiency by 35% from the optimal temperature experiment. Carbon source utilization decreased with an increase in crude glycerol over 1 g/L. Similar effect was also observed for the H_2 content. This decrease in H_2 production can be co-related with the end metabolites produced. At crude glycerol concentration of 1 g/L, H_2 production was accompanied mainly acetate – butyrate type fermentation at the concentrations of 2.1 ± 0.1 mM and 2.6 ± 0.3 mM respectively. An increase in carbon concentration resulted in a stable acetate production and higher ethanol and butyrate concentrations. At crude glycerol concentration of 5 g/L, acetate (2.2 ± 0.3 mM), butyrate (4.5 ± 0.1 mM) and ethanol (4.7 ± 0.4 mM) were produced. Fermentation with 5 g/L of crude glycerol resulted in an increased ethanol production. Similar results were obtained at higher concentrations of pure glycerol (data not shown). Ethanol production consumes the reducing equivalents, in turn affecting the H_2 yield. This is in proportion with the low H_2 yield at 2.5, 3.5 and 5 g/L of crude glycerol.

3.4. *Comparison on H_2 production by crude and pure glycerol*

In order to determine the effect of impurities present in crude glycerol on H_2 production, the enriched microbial community was subjected to kinetic analysis of cumulative H_2 and end metabolite production with pure and crude glycerol as the sole carbon sources. The effect was investigated at 40 °C, pH 6.5 with a substrate concentration of 1 g/L. The H_2 production potential (P, ml), maximum H_2 production rate (R_m, ml/h) and the lag phase (λ) were estimated by fitting the cumulative H_2 production data with the modified Gompertz equation (Fig. 2) [15]. This equation was suitable in describing the progress of cumulative H_2 production during batch fermentation tests. The experiment was conducted in triplicate experiments for

Table 2 – Glycerol utilization (%), H_2 content in the gas phase (%), carbon recovery (%), electron recovery (%) and end metabolite concentration (mM) as a function of cultivation temperature (standard deviations in parenthesis).

Temperature (°C)	Glycerol utilization (%)	Metabolites (mM)[a]			H_2 content in the gas phase[a] (%)	Carbon recovery[b] (%)	Electron recovery[b] (%)
		Acetate	Ethanol	Butyrate			
25	2.9 (0.7)	0.3 (0.0)	0.1 (0.0)	0.3 (0.0)	0.5 (0.0)	58.7	39.8
27	5.7 (0.9)	0.4 (0.0)	0.2 (0.1)	0.8 (0.1)	0.5 (0.0)	85.8	75.4
28	10.2 (0.5)	0.8 (0.0)	0.2 (0.1)	1.7 (0.1)	4.9 (0.2)	82.1	88.7
31	15.3 (1.5)	2.2 (0.1)	0.6 (0.0)	1.9 (0.3)	6.2 (0.6)	79.3	91.8
33	15.1 (0.6)	2.0 (0.1)	1.3 (0.1)	1.8 (0.2)	6.8 (0.1)	83.6	89.9
35	22.6 (0.4)	2.3 (0.1)	2.7 (0.1)	2.7 (0.2)	9.2 (0.3)	86.1	98.3
37	26.2 (1.2)	2.5 (0.1)	2.7 (0.1)	3.1 (0.0)	10.3 (1.0)	81.4	93.4
38	28.9 (0.4)	2.8 (0.1)	3.2 (0.5)	3.2 (0.0)	11.5 (1.8)	79.4	91.8
40	35.7 (1.4)	3.8 (0.0)	3.0 (0.3)	3.3 (0.1)	14.0 (0.2)	66.0	78.5
43	19.7 (2.3)	1.9 (0.2)	2.4 (0.1)	2.8 (0.2)	8.1 (0.2)	86.8	98.0
46	ND[c]	ND	ND	ND	ND	ND	ND

a Calculated for the serum-bottle culture with the working volume of 10 ml, corresponding to 9 ml modified HM100 media amended with 2.5 g/L of crude glycerol and 1% inoculum. Each value was measured after 48 h cultivation and was an average of triplicate cultivations. CO_2 in the liquid phase was ignored.

b CO_2 in the liquid phase and carbon content in biomass was ignored during carbon recovery and electron recovery calculations.

c Not detected.

Table 3 – Glycerol utilization (%), H_2 content in the gas phase (%), H_2 yield (mol-H_2/mol-glycerol$_{consumed}$), carbon recovery (%), electron recovery (%) and end metabolite concentration (mM) as a function of substrate concentration (standard deviations in parenthesis).

Glycerol concentration (g/L)	Glycerol utilization (%)	Metabolites (mM)[a]			Cumulative H_2 volume (ml)	H_2 content in the gas phase[a] (%)	H_2 yield (mol-H_2/mol-glycerol$_{consumed}$)	Carbon mass balance[b] (%)	Electron balance (%)[b]
		Acetate	Ethanol	Butyrate					
0	ND[c]	0.6 (0.1)	2.8 (0.2)	2.3 (0.3)	2.7 (0.2)	3.7 (0.3)	ND	ND	ND
0.5	54.8 (3.9)	1.8 (0.2)	1.8 (0.4)	2.2 (0.0)	10.3 (1.6)	12.1 (1.9)	0.8 (0.1)	68.6	69.2
1	56. 5 (3.9)	2.1 (0.1)	2.4 (0.2)	2.6 (0.3)	16.1 (0.5)	15.2 (0.5)	1.1 (0.1)	71.5	82.9
2.5	36.1 (1.0)	1.8 (0.2)	3.8 (0.2)	3.1 (0.3)	11.9 (1.8)	12.9 (0.8)	0.8 (0.1)	85.6	93.0
3.5	31.7 (0.6)	2.2 (0.1)	4.2 (0.0)	4.5 (0.0)	10.6 (1.2)	13.1 (1.4)	0.6 (0.1)	84.6	86.2
5.0	27.2 (1.3)	2.2 (0.3)	4.7 (0.4)	4.5 (0.1)	5.5 (0.2)	7.4 (0.3)	0.3 (0.0)	82.6	76.1

a Calculated for the serum-bottle culture with the working volume of 50 ml, corresponding to 49 ml modified HM100 media amended with corresponding concentrations of crude glycerol and 1% inoculum. Each value was measured after 48 h cultivation and was an average of triplicate cultivations.
b CO_2 in the liquid phase and carbon content in biomass was ignored during carbon recovery and electron recovery calculations.
c Not detected.

194 h. The lag parameter (λ) was 6 h and 0.7 h for crude and pure glycerol, respectively. The H_2 production potential parameter (P) for crude glycerol (14.9 ml) was more than triple than that for pure glycerol fermentation (4.9 ml). The maximum H_2 production rate parameter was 20.5 ml/h and 14.2 ml/h for crude and pure glycerol fermentation, respectively.

A lag phase of 6 h for H_2 production was observed when crude glycerol was used as the sole carbon source. The enriched community produced H_2 immediately when pure glycerol was used as the sole carbon source. The increased lag phase by the inoculum can be due to two reasons. Firstly, the pre-culture used for the experiment was grown in pure glycerol indicating the adaptation time for the inoculum when grown in crude glycerol. The second reason can be due to the presence of impurities in the crude glycerol. After the lag phase, the culture produced H_2 in similar fashion as observed for pure glycerol fermentation. The H_2 volume increased from 4.1 ± 0.0 ml to 20.4 ± 0.9 ml from 7th to 121st hour. Further fermentation did not result in a marked increase in H_2 volume, producing 20.9 ± 1.5 ml at the end of fermentation. After 194 h, fermentation with pure glycerol resulted in a H_2 volume of 14.8 ± 0.9 ml. The substrate utilization efficiency was similar for the microbial consortium under crude glycerol and pure glycerol mediated fermentations. Increased cumulative H_2 volume in crude glycerol fermentation might be explained by the presence of free fatty acids and non-glycerol organic matter in the raw glycerol. The pH adjustment of crude glycerol converts the soaps to free fatty acids. Seifert et al. obtained higher hydrogen yield from crude glycerol fermentation [16]. Similar observation was reported by Marques et al. on hydrogen production from crude glycerol by *E. aerogenes* [20].

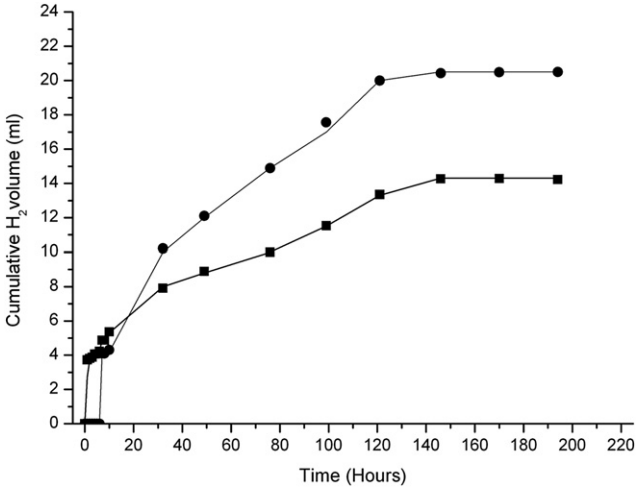

Fig. 2 – Curve fitting of cumulative H_2 volume experimental data from pure and crude glycerol fermentations with modified Gompertz equation. Symbols (●) plots the experimental data obtained from crude glycerol fermentation, (■) implies the data obtained from pure glycerol fermentation and (–) modified Gompertz fit.

As observed in previous experiments, acetate, butyrate and ethanol were the fermentation metabolites observed. Hydrogen production commenced with the acetate production. It can be observed from Fig. 3 that acetate concentration decreased gradually with an increase in time for

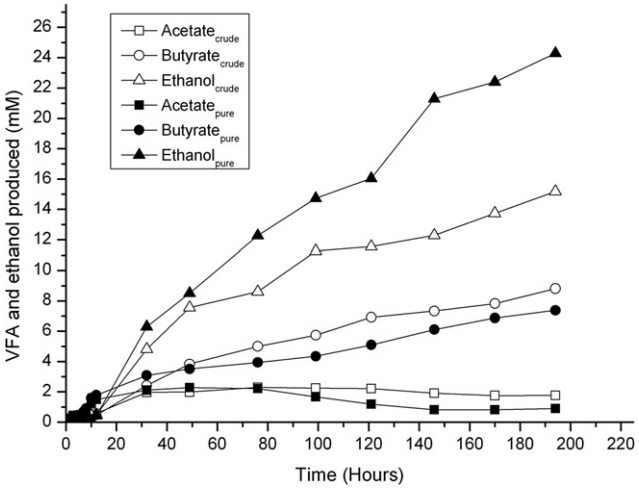

Fig. 3 – Kinetic profiles of metabolic products from pure and crude glycerol fermentation.

fermentations with pure and crude glycerol. During fermentation with crude glycerol, the acetate concentration increased from 0 mM to 2.3 ± 0.2 mM when the cultivation time reached 121 h. Further incubation resulted in a reduction in acetate concentration. Decrease in acetate production can be directly linked with stable cumulative H_2 volume. Similar decrease in acetate production was observed with pure glycerol mediated fermentation. The acetate concentration improved from 0 mM to 2.1 ± 0.2 mM when the cultivation time reached 76 h. Acetate concentration reduced to 0.8 ± 0.1 mM at the end of fermentation.

Butyrate production can be the reason for the negative effect on acetate production and cumulative H_2 volume with prolonged fermentation time. The butyrate concentrations were observed to increase for both pure and crude glycerol fermentations. During the cell growth phase, in order to meet the high energy requirement, cells produce acetate as the main metabolite [21,22]. At late exponential stage, the cells produce less acetate and re-utilizes and converts the external acetate to butyrate, resulting in medium detoxification and reduction in hydrogen ion concentration [21,23]. Thus the cells shift from more ATP producing acetate production to butyrate formation.

Ethanol production was observed for both pure and crude glycerol fermentation. Reduced H_2 production from pure glycerol can be explained by the ethanol concentration. For fermentation with pure glycerol, the ethanol concentration increased from 0 mM to 0.4 ± 0.0 mM as the incubation time reached 12 h. A drastic increase in ethanol concentration was observed in later time periods, producing 6.3 ± 0.1 mM to 24.3 ± 0.9 mM as the incubation time was increased from 32 to 194 h. Lower ethanol concentrations were observed when crude glycerol was used as the carbon source. This can be due to the metabolic shift inferred by the impurities present in the raw glycerol. The ethanol concentration increased from 0 mM to 0.5 ± 0.3 mM by 12 h of incubation. Though the microbial community produced 4.8 ± 0.1 mM of ethanol at 32nd hour, the ethanol production was slow when compared to pure glycerol mediated fermentation, yielding an ethanol concentration of 14.5 ± 0.6 mM in 194 h. The increased ethanol concentrations can be responsible for lower cumulative H_2 volume in fermentation with pure glycerol.

3.5. *Microbial community analysis*

Dominant bacteria in the enriched microbial community were analyzed using PCR-DGGE technique. The DGGE profiles were as illustrated in Fig. 4. In the bacterial profile of enriched culture, 4 prominent bands were detected. Following serial dilution, microbial diversity reduced, and bands 3, 4 and 5 were prominent. The microbial consortium was dominated by *Clostridium* sp. Band 3 and 4 were associated with uncultured bacterium clone (Accession no: FJ512181.1; Query coverage, 99%; Identity, 98%) and *Clostridium sporogenes* strain CL3 (Accession no: JF836014.1; Query coverage, 97%; Identity, 98%) and *Clostridium subterminale* isolate DSM 758 (Accession no: EU857637; Query coverage, 94%; Identity, 99%) respectively. Blast search showed identity of bands 1 and 2 with uncultured rumen bacterium clone GRC39 (97% identity) and uncultured firmicutes bacterium clone M0042_014 (93% identity) respectively. Further work should be conducted in order to understand the role of individual species in the microbial community on crude glycerol utilization.

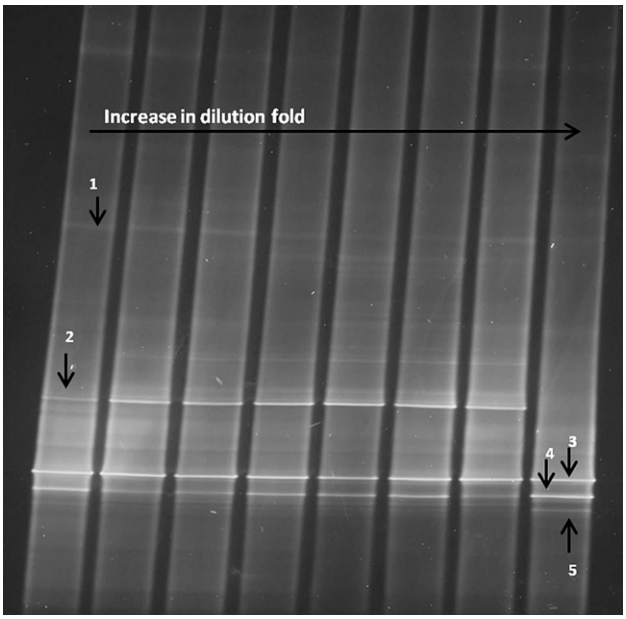

Fig. 4 – DGGE profiles of dominant bacteria in the enriched microbial community.

4. Conclusion

In this study, bioconversion of residual glycerol from biodiesel production process to H_2 by an enriched microbial consortium is described. The enriched bacterial community comprised mainly of *Clostridium* species. The inoculum produced H_2 at wide pH and temperature ranges. Optimization of physico–chemical parameters improved the substrate utilization efficiency by 35%. The maximal H_2 yield from crude glycerol at pH 6.5, 40 °C and 1 g/L of substrate was 1.1 ± 0.1 mol-H_2/mol-$glycerol_{consumed}$. Comparison of H_2 production with pure glycerol indicated that the inoculum growth and H_2 production were not affected by the impurities.

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