Green Chemistry

Accepted Manuscript

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/greenchem

Rice straw hydrolysate to fuel and volatile fatty acids conversion by *Clostridium sporogenes* BE01: Bio-electrochemical analysis of electron transport mediators involved

Lalitha Devi Gottumukkala*[a, c], Rajeev Kumar Sukumaran[a], Venkata Mohan S[b], Sajna Kuttuvan Valappil[a], Omprakash Sarkar[b], Ashok Pandey[a],

[a] Centre for Biofuels, Biotechnology Division, National Institute for Interdisciplinary Science and Technology –CSIR, Industrial Estate PO, Thiruvananthapuram 695019, India.

[b] Bioengineering and Environmental Centre (BEEC), Indian Institute of Chemical Technology -CSIR, Hyderabad 500 007, India.

[c] Dept. of Process Engineering, Stellenbosch University, Private Bag X1, Matieland, 7602, South Africa.

*Corresponding author

Lalitha Devi Gottumukkala

Dept. of Process Engineering

Stellenbosch University

Private Bag X1

Matieland, 7602

South Africa

Tel: +27 21 8089485

Fax: +27 808 2059

e-mail: lalithaniist@gmail.com, lalithadevi@sun.ac.za

Abstract

Clostridium sporogenes BE01, a non-acetone forming butanol producer could produce hydrogen and volatile fatty acids (VFAs) during butanol fermentation from rice straw hydrolysate. Bio-electrochemical analysis revealed the changes occurred in redox microenvironment and electron transport mediators during fermentation at different pH and $CaCO_3$ concentrations. $CaCO_3$ played a very important role in enhancing the production of hydrogen, volatile fatty acids and solvents by stimulating the changes in electron transport system. Electron transport system mediated by NAD/NADH, flavins, Fe-S clusters, protein bound FAD, cytochrome complex in *C. sporogenes* BE01 was analysed by cyclic voltammetry (CV). Electro kinetic analysis revealed that the favorability for redox reactions increased with increase in pH and polarization resistance reduced significantly with $CaCO_3$ supplementation.

1. Introduction

Biobutanol gained significant attention for its properties as liquid transportation fuel, but its production from lignocellulosic biomass is facing challenges both technically and economically.[1] VFAs and hydrogen produced during butanol fermentation can be considered as an added advantage, when an efficient process is in place. Formation of various industrial products from a single substrate in a single run could be considered beneficial for lignocellulosic biorefinery processes. Fluctuation in redox environment leads to change in bacterial growth pattern, glucose utilization, products formed etc[2] and hence it is essential to understand the redox microenvironment of the bioreactors operated with the desired biomass and microbes. Redox balance in a bioreactor is one among the many key components that controls carbon flux and change in metabolic activity of the organism.[3] In order to assess the bio-electric potential of a

bioreactor, it is essential to perform the analysis of bio-electro catalytic efficiency of the microbial catalyst. Electrochemical characterization of microbial bioreactors will help in understanding the redox active species participating in electron transfer reactions.[4]

The metabolic pathway of glucose to butanol conversion is complex and several enzymes are involved in diverting the pathway towards VFAs (acetic acid and butyric acid) and for assimilation of these VFAs to solvents. It is a highly interlinked chain of redox reactions with many electron transporters involved. Formation of hydrogen and volatile fatty acids is the intrinsic part of biochemical pathway for butanol fermentation by Clostridia.[5] Conversion of glucose to acetyl CoA through pyruvate route generates hydrogen and acetyl CoA is the precursor for VFAs and solvents production.[6,7] Hydrogen generated by Clostridial species is directly related to volatile fatty acids production. Conversion of acetyl CoA to acetate yields hydrogen twice the yield as the conversion to butyrate[8] and the ratio of acetic acid and butyric acid has tremendous effect on the ratio of solvents formed.[9] Hydrogen produced during the process is an energy rich gaseous fuel and VFAs can be used as precursors for Polyhydroxyalkanoates.[10] Solvents are the main metabolic products and can be used as biofuels.

This study presents the efficiency of *C. sporogenes* BE01, a novel non acetone producing bacteria to convert fermentable sugars generated from hydrolysis of lignocellulosic biomass to solvents, VFAs and hydrogen. It is also focused to understand the possible electron transport mediators and redox reactions involved during the process. This is the first report on hydrogen production from rice straw hydrolysate by using a pure strain of *C. sporogenes* and also it is the first report on demonstration of electron transporters mediated redox activity of *Clostridium species* during biobutanol fermentation from lignocellulosic biomass.

2. Results and discussion

2.1. Glucose utilization

Dilute acid pretreatment and enzymatic hydrolysis of rice straw generated 45 g/L of glucose. However, heat sterilizing the hydrolysate at 121 ºC for 10 min resulted in 15 g/L sugar loss and the final glucose obtained was 30 g/L. Pentoses were found in negligible concentrations in the hydrolysate, as hemicellulosic fraction was efficiently removed during dilute acid pretreatment. The redox microenvironment of *C. sporogenes* BE01 driven biobutanol fermentation in enzymatic rice straw hydrolysate was studied at various initial pH (5.8, 6.2, 6.4 and 6.8) and with different $CaCO_3$ concentrations (0 g/L, 5 g/L and 10 g/L). pH plays a very significant role in microbial catalysis and growth of the bacteria. Especially in butanol fermentation, pH has direct control over the acids formation and their assimilation.[11] *C. sporogenes* BE01 exhibited change in pattern of glucose utilization with change in microenvironment of the bioreactor. pH of the medium in the range of 5.8 to 6.4 had very less effect on sugar utilization, but the utilization rate enhanced with pH approaching near neutral. This signifies the increased metabolic activity and growth at near neutral pH (Fig. 1a).

Supplementation of $CaCO_3$ in the medium enhanced glucose utilization and it increased with increasing the concentration of $CaCO_3$ (0, 5 and 10 g/L) (Fig. 1b). Increased glucose consumption and ABE productivity in the presence of ≥ 4 g/L $CaCO_3$ was reported with *C. beijerinkii* grown in semi defined P2 medium.[12] Calcium ions has various effects at cellular level which could contribute to increased growth, though pH buffering effects might also contribute to stimulatory effects on butanol fermentation. It was reported by Richamond et al, that the presence of $CaCO_3$ increased protein synthesis in *Clostridium* species and this increase is proportional to the amount of $CaCO_3$ in the medium. It was also stated that these upregulated

proteins might be involved in glucose uptake and utilization.[13] Similar glucose utilization patterns were observed in our results with *C. sporogenes* BE01 (Fig. 1b).

2.2. Hydrogen and volatile fatty acids

During butanol fermentation, butyric acid production was relatively higher than the acetic acid production in all the conditions tested (Fig. 2). *C. sporogenes* is a known producer of butyric acid and was reported for cheese fermentation in combination with *C. butyricum* and *C. beijerinckii*.[14,15] In *C. tyrobutyricum* fermentation studies, butyrate at 15 g/L showed inhibitory effect on acetate formation.[16] In this study, when overall VFAs production was considered, near neutral pH 6.4 and 6.8 were found to be favourable with the production of 5.2 g/L and 5.5 g/L respectively. The reactor with $CaCO_3$ concentration of 5g/L performed considerably well for conversion of sugars to acids (Fig. 2b and 2d).

C. propionicum, an organic acid producer showed highest growth and organic acid productions at pH 7.[17] Acid forming enzymes are highly pH dependent. The activity of the enzymes would be inhibited or enhanced with increase or decrease in pH.[16] Acidogenic process was highly active in the first 24h and became stable throughout the fermentation time, except at pH 6.8, VFAs production was in increment till 48h and had a sharp decrease at 72h but raised at 96h (Fig. 2a and 2c). The same was observed with 5g/L and 10 g/L $CaCO_3$ supplementation, which could be attributed to efficient assimilation of acids to solvents at 72h. Hydrogen production was in accordance with acids production and the highest percentage of hydrogen (20%) of the total gas produced was at pH 6.4 and 6.8 (Fig. 3a). Though, there was no considerable difference in the percentage of hydrogen produced, there was a notable difference in the total gas produced at different pH and that showed marked variation when represented in

terms of cumulative hydrogen (Fig. 3c and 3d). The total hydrogen production increased with increase in pH till 6.4 but reduced at 6.8. Total hydrogen production at pH 6.4 continued till 96h and there was no much reduction in the gas production with respect to time (Fig. 3c).

Optimal pH for hydrogen production varies with each species and strains. For *C. beijerinkii* DSM 1820, optimum pH reported was 6.7, for *C. Pasteurinum* it was 5.4 and for *C. butyricum* 5.1.[18] Hydrogen production varies with change in glucose concentration. In *C. acetobutylicum* ATCC824 glucose fermentation, hydrogen production rate ranged from 680-1270 ml/ g glucose per liter of reactor.[19] There was increase in total gas production and percentage hydrogen production when $CaCO_3$ concentration was reduced to 5 g/L (1260 ml/L), but in the absence of $CaCO_3$ and with increase in $CaCO_3$ to 10 g/L the total hydrogen produced was 591 ml and 698 ml respectively (Fig. 3b and 3d). Long acidogenic phase and higher acids production can be correlated with the increased hydrogen production with the supplementation of 5 g/L $CaCO_3$. The favorability of 10 g/L $CaCO_3$ was towards solvents assimilation rather than hydrogen production.

2.3. Solvents production

C. sporogenes BE01 was reported in our previous studies for its ability to produce solvents from rice straw hydrolysate.[20] Butanol and ethanol were the two solvents produced by *C. sporogenes* BE01, without forming acetone and their ratio varies with the change in the process parameters. Acetic acid and Butyric acid, produced in the acidogenic phase of the culture were assimilated at the later solventogenic phase of the culture.[21] *C. sporogenes* BE01 was able to form solvents at pH as low as 5.8, but the solvent production increased when pH approached near neutral (Fig. 4a and 4c). At 5.8 pH, though the rate of butanol and ethanol formation was high at first 48h and

24h respectively, solvent formation ceased later and total solvent produced was comparatively less (Fig. 4a and 4c). This could be due to lowered pH with VFAs production and inefficient assimilation of VFAs. At pH 6.2 and 6.4, relatively high solvent production was observed for the first 24h and 72h respectively. Further accumulation of solvents was not found from 72h to 96h. The efficient conversion of sugars and acids to solvents was achieved in a different mode by *C. sporogenes* BE01 at pH 6.8. The solvents formation in the first 24h was considerably low when compared to rest of the pH range, which signifies the acids accumulation did not lead to decrease in pH to the level where assimilation starts for solvents production, but the overall high solvent production 7.3 g/L was achieved with constant increase in solvent accumulation till 96h without attaining saturation (Fig. 4a and 4c).

$CaCO_3$ was used to maintain the pH in the range by neutralizing organic acids formed during fermentation. A preliminary study on the effect of $CaCO_3$ on butanol fermentation was mentioned in our previous report[20] and in the current study, we tried to correlate it with initial pH and solvent formation. Solvents production increased with increasing the concentration of $CaCO_3$ in the medium (Fig. 4b and 4d). This could be due to the effect of calcium ions and its effective buffering action resulted in efficient VFA assimilation. Without $CaCO_3$supplementation, the total yield of solvents was just 3.8 g/L which could be due to lower glucose utilization rate and less VFA formation. Supplementation of 10 g/L $CaCO_3$ increased the total solvent production to 7.4 g/L. Reduction in $CaCO_3$ concentration from 10 g/L to 5 g/L lead to 26% decrement in total solvents formed (Fig. 4d).

Final pH of all the experiments varied with change in $CaCO_3$ concentrations in the medium. In the absence of $CaCO_3$ and with the supplementation of 5 g/L $CaCO_3$, pH dropped from 6.8 (initial pH) to 5.9 (final pH) in 96 h, indicating the formation of acids and improper

assimilation to solvents. Whereas with 10 g/L $CaCO_3$, pH dropped to 6.3 in 48 h and then gradually increased to 6.5 in 96 h. $CaCO_3$ concentration was observed to effect the solvent production more than initial pH of the medium.

2.4. Electrochemical analysis

With the function of difference in initial pH and $CaCO_3$ concentrations in the rice straw hydrolysate medium, there was variation in voltammogram peaks obtained during cyclic voltammetry (CV) analysis and the change was with respect to time as well. Redox currents were found to be high with sterile rice straw hydrolysate alone, which signifies it as a potential substrate for bio-electro catalytic reactions. Redox currents were not differed predominantly and were mostly in the range of RC: -18 ± 3 μA to OC: 18 ± 4 μA. With the alterations in initial pH and with various $CaCO_3$ concentrations in the medium, the difference observed was marginal (Fig. 5). The observations indicate that the catalytic activity was more or less same in all the conditions provided, but the difference was with the shift in metabolic activity and the mediators/electron carriers involved.

2.4.1. Electron transport mediators

In all the different initial pH experiments except 5.8, Preliminary hours had shown redox potential for the biological reaction of NAD^+/ NADH (E^0 values -0.32 V) and cytochrome complex (0.1 to 0.25 V).[22] In the later hours peaks for flavins (-0.2 V to -0.29 V), protein bound FAD (0.00 V to 0.1) and Iron-sulphur clusters were observed.[23,24] For Iron-Sulphur clusters, with the E^0 values less than -0.05, they can be attributed to rubredoxin or any simplest type of Fe-S cluster and with the E^0 values close to -0.15 could be for iron-sulphur cluster of N2 [4Fe-4S] associated with complex 1.[25,26] Rubredoxin from *C.pasteurianum* was reported to have

reduction potential less than -0.05 V.[26] Association of Fe-S with Fe-Fe dehydrogenase enzyme for hydrogen production was also well reported.[27] Peaks for 4Fe-4S clusters were not prominent in pH 5.8, but were found at 24h in pH 6.2, extended till 72h in pH 6.4 and at near neutral pH the peaks were observed till 96h. There were reports on increased levels of Fe-S clusters during solvent formation and furfural challenged cultures.[28] Though, in bacteria their primary function is to mediate low potential electron transfer, their function is extended to several catalytic proteins.[29] Interference of protein bound FAD increased with increase in pH and that can be attributed to the reactions that occur in the presence of enzymes like ferredoxin reductases containing FAD as the prosthetic group.[30]

Distinct peaks were observed with change in $CaCO_3$ concentrations in the medium. Peaks for Cytochrome, quinone and simple Fe-S clusters were commonly observed throughout the fermentation. The major difference was with frequency of peaks for NADH, protein bound FAD, flavoproteins and 4Fe-4S clusters. Peaks were observed for all the mediators mentioned above with 10 g/L $CaCO_3$ containing medium. The presence of many electron transporters implies that the redox activity was occurring efficiently and this was also supported by high butanol production. While, in the absence of $CaCO_3$ peaks for the common mediators like Cytochcrome bc1 and 4Fe-4S clusters were observed. Rubredoxins like Fe-S clusters in bacteria are low potential electron transporters and this could be the reason for comparatively less acids and solvents production. With 5 g/L $CaCO_3$ peaks for Cytochcrome bc1, NAD/NADH, flavoproteins were found but protein bound FAD peaks were absent.

2.4.2. Electron transportation during butanol fermentation

Cyclic voltammetry analysis suggested that NADH, flavoproteins, protein bound FAD/FMN and 4Fe-4S cluster were involved in electron transport system that might be facilitating a key reaction that result in higher butanol yield. Peaks were prominent for 4Fe-4S and protein bound FAD with 10 g/L $CaCO_3$ supplementation, where high solvents production was observed. This indicates the stimulation of a membrane bound protein that has FAD and 4Fe-4S clusters. In the fermentation conditions, where high VFAs and hydrogen were produced, peaks for NADH, flavoproteins and Fe-S cluster were prominent and very interestingly peaks for protein bound FAD were very rare. Based on the redox potentials obtained for various electron transport mediators during tested fermentation conditions and the reported electron transport systems in *Clostridium* species, most probable electron transport system involved in hydrogen production, VFAs and butanol fermentation is postulated (Scheme 1).

In an electron transport system, electrons always flow from negative to positive redox potential. Based on the CV peaks, during solvents formation, electrons flow from NADH (-0.32 V) to an enzyme or protein that contains FAD (0.0 to 0.1 V) via flavins (FMN/FAD) (-0.2 to 0.29 V) and 4Fe-4S clusters (-0.15). Quinones and cytochromes (0.1 to 0.25 V) stabilizes the FAD bound enzyme in the reduced state. But, for fermentation conditions where the solvents formation was less and VFAs formation was more, peaks for FAD and 4Fe -4S clusters were less prominent, while peaks for simple Fe-S clusters were dominating. This indicates that there was shift or bifurcation in electron transportation during solventogenesis.

There are reports on a Clostridial electron transport bifurcation system, where cytoplasmic complex of butyryl coA dehydrogenase and electron transferring flavoprotein (BCD/ETF) that catalyses a key reaction(crotonyl coA to butyryl coA) in butanol pathway is coupled to membrane associated proton translocating NADH-ferridoxin reductase complex (Rnf

A-G or Rnf A-E).[30,31] Signals obtained for 4Fe-4S clusters, protein bound FAD/FMN, flavoproteins, NAD/NADH is the characteristic of electron bifurcation system involving Rnf complex, reported in few *Clostridium* species.[30] Quinones and cytochrome were reported for maintaining the enzymes in reduced active state.[32] NADH-ferredoxin reductase (RnfA-G) complex that couples ferredoxin oxidation by NAD^+ with proton/ Na^+ translocation is a membrane associated enzyme complex which contains 4Fe-4S clusters, covalently bound FMN, non-covalently bound FAD and 2Fe-2S cluster. The electrons are proposed to flow from NADH to Rnf complex resulting translocation of Na^+ or H^+ .[30,33] RnfA-G was reported in *C.tetanomorphum*, but whether Rnf is a real Na+ pump or H+ pump that translocates Na+ during proton gradient formation has to be still established. [30] In *C.ljungdahlii* and *C.kluvyeri*, proton translocating Rnf was reported. [30,33,34]

During butanol fermentation, glucose is converted to pyruvate, which is further oxidized to acetyl CoA and acetate. Hydrogenases linked to pyruvate:ferredoxin oxidoreductase produce hydrogen using proton as terminal acceptor. However, hydrogenases coupled to NADH: ferredoxin reductases produce hydrogen by using NADH as electron donor and proton as electron acceptor.[35] Rnf/Nqr (NADH:quinone oxidoreductase) was reported in the genome of all *C.botulinum* group 1, but absent from the group II genomes[36] and it is a known fact that *C.sporogenes* is considered as a non-toxigenic equivalent of *C.botulinum* group I.[37] This strengthens the idea of Rnf based electron transportation in *C.sporogenes* during glucose fermentation.

There are no detailed reports on *C.sporogenes* butanol fermentation and metabolic pathway involved. Correlation of CV analysis with the pattern of products formed during fermentation, broadly suggest the possible stimulation of electron transport chain associated with

Rnf and butyryl coA dehydrogenase in the presence of $CaCO_3$. Calcium ions were also reported to stabilize membrane bound proteins.[13] The increased production of butanol and butyric acid with the supplementation of $CaCO_3$ could be attributed to stimulation and stabilization of membrane proteins. However, a very detailed research is necessary to support this hypothesis.

2.5. Bio-electro kinetic analysis

The rate of electron transfer to the electrode by oxidized and reduced species can be interpreted by Tafel plot. According to Tafel equation, when the over potential is large the reverse reaction rate is negligible. The slope of Tafel plot reveals the value of electron transfer coefficient.[38] Low Tafel slope indicates the high current obtained at low over potential. So, higher the slope higher the activation energy required, that indicates the less favourability of the oxidation / reduction reaction.[39] The efficiency of the bioreactor towards reduction or oxidation reactions and the favourability for the membrane associated biochemical redox reactions can be analyzed by the redox slopes obtained under each condition with respect to time. Oxidative Tafel slope and reductive Tafel slope were derived from the Tafel plot obtained (Fig 6).

Though there was variation in Tafel slope obtained with respect to change in pH and $CaCO_3$ concentrations, in all the experimental conditions oxidative slope was higher than the reductive slope. This indicates that rice straw hydrolysate bioreactor with *C. sporogenes* BE01 was more favourable for the reduction metabolism that is solvents production. In relation to different initial pH experiments, oxidative slope increased with increase in pH and reductive slope decreased with increase in pH (Fig. 7a and 7b). This indicates that, reduction metabolism was favourable at pH 6.4 and 6.8 when compared to lower pH.

In the absence of $CaCO_3$ oxidation slope was low but increased with increase in $CaCO_3$ concentration in the medium (Fig. 7c). This suggests that addition of $CaCO_3$ was not favourable for oxidation reactions and in the case of reduction slopes, 5 g/L $CaCO_3$ showed comparatively less reduction potential followed by 10 g/L $CaCO_3$ and no $CaCO_3$ supplementation (Fig. 7d). Reduction in activation energy required for redox reactions in the presence of $CaCO_3$ could be responsible for increased production of hydrogen, acids and solvents.

Polarization resistance, Rp refers to the resistivity of the electrolytes around the electrode, this could be due to resistance of electron transfer by the microbe or the insulation effect of products formed on the electrode surface. For a reactor to be active in electron transfer and product formation, polarization resistance should be low.[39] Fermentation with initial pH 6.4 showed less Rp when compared with other pH range (Fig. 7e). Polarization resistance was high in the reactor without $CaCO_3$ supplementation and decreased with supplementation of 5 g/L $CaCO_3$, but the resistance increased slightly with increased $CaCO_3$ supplementation (Fig. 7f), as stated before it could be either due to the resistance or insulation effect.

3. Conclusion

Clostridium sporogenes BE01 is capable of producing 7 g/L of VFAs and 1.2 L/L of hydrogen during butanol fermentation in rice straw hydrolysate with 30 g/L of glucose. 7.3 g/L of total solvents were produced, of which 5 g/L was butanol. Butanol and ethanol production was in the ratio 7:3. Fe-S clusters, Cyt bc1 and quinones were the common electron transporters involved during butanol fermentation. $CaCO_3$ supplementation resulted in high solvent formation by stimulating the electron transport system mediated by protein bound FAD, 4Fe-4S, NADH and flavoproteins. Presence of peak for the protein bound FAD was found till 96h at 6.4 and 6.8 pH

with 10 g/L $CaCO_3$ supplemented medium. Involvement of 4Fe-4S cluster, NAD/NADH, protein bound FAD and flavoproteins during active butanol fermentation presents the possibility of electron bifurcation system mediated by a membrane bound complex, probably Rnf. Tafel plot revealed that rice straw hydrolysate supplemented with $CaCO_3$ had low polarisation resistance, Rp and required less activation energy for reduction metabolism, making it more favourable for solvents production during fermentation by *C. sporogenes* BE01

4. Experimental methods

4.1. Pretreatment and hydrolysis of rice straw

Dilute acid (0.4% w/w H_2SO_4) was used to pretreat the rice straw obtained from a local vendor. Rice straw was knife milled to reduce the size and the pretreatment was carried out for 1h at 120 ºC at the solid loading of 15% (w/w). The pentose fraction obtained (liquid stream) was separated from solids by filtration and centrifugation. Pretreated rice straw was dried at room temperature till excess moisture was removed. Commercial cellulase (Zytex India ltd, Mumbai) with the enzyme activity of 80 FPU/ml was used for enzymatic hydrolysis of pretreated rice straw at 50 °C. 30 FPU/gds was used for the hydrolysis and the run was continued for 48h. The suspended and unhydrolyzed solid mass was separated by centrifugation at 10,000 rpm for 20 min.

4.2. Fermentation

C. sporogenes BE01 was maintained in spore form at 4 ºC. The spores were heat shocked at 80 ºC for 2 min and the temperature was immediately brought down by placing in ice bath. The heat shocked spores were cultured in TGY medium to develop preinoculum. Actively growing cells were inoculated in to fresh TGY medium for inoculum generation. Highly motile, 12h old

culture was inoculated in to rice straw hydrolysate. Rice straw hydrolysate was made anaerobic by cooling under nitrogen atmosphere after heat sterilization at 120 ºC for 10 min. Bottles with loosened caps were placed inside the anaerobic chamber for 12 h before inoculation.

Fermentation was carried out for 96 h in 250 ml bottles containing 200 ml of rice straw hydrolysate medium with 10% (v/v) inoculum. As described in the previous reports $CaCO_3$ and yeast extract (Himedia, India) were the only supplementation and cysteine HCl (Himedia, India) was added as reducing agent.[20] Initial pH of the hydrolysate was 4.8, but the addition of yeast extract and $CaCO_3$ increased the pH of the medium to 6, which was further adjusted to required pH by 1N NaOH and 1N HCl. Initial pH range of 5.8, 6.2, 6.4 and 6.8 with 10 g/L $CaCO_3$ was chosen to study the effect of initial pH on bioprocess. For analysing the effect of pH on glucose utilisation and fermentation, rice straw hydrolysate was supplemented with 10 g/L of $CaCO_3$ and pH was adjusted to 5.8, 5.2, 6.4 and 6.8 and three different concentrations of $CaCO_3$ (0 g/L, 5 g/L and 10 g/L) were tried to understand the role and effect of $CaCO_3$ on redox micro environment of rice straw hydrolysate inoculated with *C. sporogenes* BE01. pH change of the fermentation medium was monitored at 24 h interval and samples were collected for analysis.

4.3. Analytical methods

4.3.1. Total gas estimation and hydrogen analysis

Total gas produced by *C. sporogenes* BE01 was analysed by mounting 20 ml gradient syringe on every reactor by piercing it through rubber septum. The gas produced displaced the piston of the syringe and gradient helped to measure the gas produced. Head space volume of the reactor was also taken in to consideration.

Percentage hydrogen analysis in the total gas produced was done by gas chromatograph (Nucon) equipped with a thermal conductivity detector (TCD). 2.1/8" x 2 m SS column with molecular sieve stationary phase of size 60/80 mesh was used for gases separation at the temperature 60 °C. Carrier gas used was nitrogen at a flow rate 20 ml/min under 1kg/cm^2 pressure. The detector and injector were operated at 80 and 50 °C respectively.

4.3.2. Sugars and acids analysis

Glucose was quantified by using 100 x 7.8 mm Fast carbohydrate analysis column (Biorad) using HPLC (Shimadzu prominence UFLC) equipped with RI detector. Oven temperature was maintained at 85 °C and de-ionized water was used as mobile phase at the flow rate of 0.8 ml/min. Volatile fatty acids were analysed by using Rezex® ROA organic acid analysis column (Phenomenex) and PDA detector at oven temperature 50 °C. Mobile phase used for separation was 0.05M H_2SO_4 at the flow rate 0.6 ml/min.

4.3.3. Solvents analysis

Butanol and ethanol were analyzed by gas chromatograph (Chemito GC 8610). Poropak Q ® column was used for separation with the gradient oven temperature rise from 150 °C to 200 °C at the rate of 8 °C/min. Flame ionization detector (FID) was used for detection and the detector temperature was maintained at 250 °C. Sample was injected with injector temperature at 150 °C.

4.3.4. Electro chemical analysis

Cyclic Voltammetry (CV) was performed using potentiostat–glavanostat system (Autolab-PGSTAT12, Ecochemie) to understand the redox micro environment and electron transport mediators involved during the fermentation process. Analysis was performed during the

fermentation in real time conditions. Aliquots of 20 ml from the reactor were sampled and voltammograms were recorded under anaerobic fermentation conditions using platinum wire as the working electrode and a carbon rod as counter electrode against the reference electrode (Ag–AgCl(S)). Potential ramp was applied in the range of +0.5V to -0.5V at the scan rate of 30 mV/s. Redox currents and peaks were recorded for further analysis.

Electro kinetic analysis was performed by plotting Tafel slope using autolab software. Natural log of anodic current (lnI) was plotted against applied range (E/V) for presenting the Tafel plot. Oxidation slope and reduction slope for every voltamogram was recorded and plotted against time to understand the fluctuations in redox environment with respect to time.[39] Current versus voltage curve approximates a straight line and the slope obtained from this is polarisation resistance, Rp.[40]

Acknowledgements

LDG would like to acknowledge Council of Scientific and Industrial Research (CSIR) for providing senior research fellowship for her PhD work.

References

1. V. Garcia, J. Pakkila, H. Ojamo, E. Muurinen and R. L. Keiski, *Renew. Sust. Energ. Rev*., 2011, 15, 964-980.
2. E. M. Barnes and M. Ingram, *J. Appl. Bacteriol*., 1956, 19,117-128.
3. G. Rao and R. Mutharasan. *Appl. Environ. Microbiol*., 1987, 53, 1232-1235.
4. E. Marsili, J. B. Rollefson, D. B. Baron, R. M. Hozalski and D. R. Bond, *Appl. Environ. Microbiol*., 2008, 74, 7329-7337.
5. R. Geshlagi, J. M. Scharer, M. Moo-Young and C. P. Chao, *Biotech.Adv*., 2009, 764-781.

6. M. Juanita and W. Guangyi, *Int. J. Hydrogen. Energ*., 2009, 34, 7404–7416.
7. KEGG PATHWAY: Pyruvate metabolism - *Clostridium acetobutylicum*. Genome.jp. Retrieved 2010-07-01.
8. Y. Tao, Y. Chen, Y. Wu, Y. He and Z. Zhou, *Int. J. Hydrogen. Energ.*, 2007, 32, 200–206.
9. G. Matta-el-Ammouri, R. Janati-Idrissi, A. M. Junelles, H. Petitdemange and R. Gay, *Biochimie*., 1987, 69, 109-115.
10. W. S. Lee, A. S. May Chua, H. K. Yeoh and G. C. Ngoh, *J. Chem. Technol. Biotechnol*., 2103, DOI: 10.1002/jctb.4197.
11. X. Yang, M. Tu, R. Xie, S. Adhikari and Z. Tong, *AMB express*., 2013, 3:3, DOI:10.1186/2191-0855-3-3**.**
12. B. Han, V. Ujor, L. B. Lai, V. Gopalan and T. C. Ezeji, *Appl. Environ. Microbiol.,* 2013, 79, 282-293.
13. C. Richmond, B. Han and T. C. Ezeji, *Continental Journal of Microbiology*., 2011, 5, 18-28.
14. A. G Le Bourhis, J. Doré, J. P. Carlier, J. F. Chamba, M. R. Popoff and J. L. Tholozan, *Int. J. Food Microbiol*., 2007, 25, 113, 154-163.
15. T. J. Montville, N. Parris, and L. K. Conway, *Appl. Environ. Microbiol*., 1985, 49, 733–736.
16. Z. Ying and Y. Shang-Tian, *J. Biotechnol*., 2004, 110, 143–157.
17. E. E. Stinson and K. A. Naftulin, *J. Ind. Microbiol*., 1991, 8, 59-63.
18. J. Masset, M. Calusinska, C. Hamilton, S. Hiligsmann, B. Joris, A. Wilmotte and P. Thonart, *Biotechnol. Biofuels*., 2012, 5:35, DOI:10.1186/1754-6834-5-35.

19. H. Zhang, M. A. Bruns, B. E. Logan, *Water. Res.,* 2006, 40, 728-734.

20. L. D. Gottumukkala, B. Parameswaran, S. K. Valappil, K. Mathiyazhakan, A. Pandey, R. K. Sukumaran, *Bioresour. Technol.,* 2013, 145, 182-187.

21. S. Y. Lee, J. H. Park, S. H. Jang, L. K. Nielsen, J. Kim, K. S. Jung, *Biotechnol. Bioeng*., 2008, 101, 209-227.

22. Gerald Karp, in *Cell and Molecular Biology : Concepts and experiments*, John Wiley & Sons, 6th edn, 2009, Ch.4, pp. 173-205.

23. S. G Mayhew, in *Flavoprotein protocols,* ed. S. K. Chapman and G. A.Reid, Springer, 1999, Vol.131, Ch.4, pp. 49-60.

24. R. Klaus-Heinrich, in *eLS*. John Wiley & Sons Ltd, Chichester. 2001, http://www.els.net, doi: 10.1038/npg.els.0001373.

25. T. Ohnishi, *Biochim. Biophys. Acta*., 1998, 1364, 186-206.

26. I-J Lin, E. B. Gebel, T. E. Machonkin, W. M. Westler, J. L. Markley, *PNAS.*, 2005, 102, 14581-86.

27. P. Berto, M. Di Valentin, L. Cendron, F. Vallese, M. Albertini, E. Salvadori, G. M. Giacometti, D. Carbonera and P. Costantini, *Biochim. Biophys. Acta*., 2012, 1817, 2149-57.

28. Y. Zhang and T. C. Ezeji, *Biotechnol. Biofuels*., 2013, 6:66. DOI:10.1186/1754-6834-6-66.

29. K. Brzoska, S. Meczynska, M. Kruszewski, *Acta Biochim. Pol*., 2006, 53, 685-91.

30. W. Buckel and R. K. Thauer, *Biochim. Biophys. Acta*., 2013, 1827, 94-113.

31. B. Ward *in Molecular medical microbiology: Bacterial energy metabolism*, ed. Y-W Tang, M. Sussman, D. Liu, I. Poxton, J. Schwatrzman, Academic press, 2nd edn, 2014, Vol.1, Ch.11, pp 201-234, 2002.
32. M. Calusinska, T. Happe, B. Joris, A. Wilmotte, *Microbiology.,* 156, 1575-88, 2010.
33. H. Seederf, W. F. Fricke, B. Veith, H. Bruggemann, H. Liesegang, A. Strittmatter, M. Miethke, W. Buckel, J. Hinderberger, F. Li, C. Hagemeier, R. K. Thauer, G. Gottschalk, *PNAS*., 2008, 105, 2128-33.
34. H. Nagarajan, M. Sahin, J. Nogales, H. Latif, D. R. Lovley, A. Ebrahim, K. zengler, *Microb. Cell. Fact*., 2013, 12, 118.
35. M. Calusinska, T. Happe, B. Joris, A. Wilmotte, *Microbiology*., 2010, 156, 1575-88.
36. H. Bruggemann, A. Wollherr, C. Mazuet, M. R. Popoff, in *Genomes of foodborne and waterborne pathogens: Clostridium botulinum*, ed. P. Fratamico, Y. Liu, S. Kathariou, ASM press, 2010, Ch.13.
37. A.T. Carter and M. W. Peck, *Res. Microbiol*., 2014, doi:10.1016/j.resmic.2014.10.010
38. A. J. Bard and L. R. Faulkner, in *Electrochemical Methods: Fundamentals and Applications*, Wiley, 2nd edn, 2000, Ch.3, pp. 105-134.
39. S.V. Mohan, C. N. Reddy, A.N. Kumar and J. A. Modestra, *Bioresour. Technol*., 2013, 147, 424-433.
40. Metrohm Autolab B.V, http://www.ecochemie.nl/download/Applicationnotes/Autolab_Application_Note_COR03.pdf (accessed February 2015).

Captions and legends

Figure 1. Glucose consumed by *C. sporogenes* BE01 from rice straw hydrolysate. (a) At different initial pH supplemented with 10 g/L $CaCO_3$. (b) With the supplementation of $CaCO_3$ at two different concentrations (5 g/L and 10 g/L) and without the supplementation of $CaCO_3$ (0 g/L).

Figure 2. Volatile Fatty acids (VFA) production by *C. sporogenes* BE01 in rice straw hydrolysate at different initial pH and $CaCO_3$ concentrations. (a) Butyric acid production at different initial pH. (b) Butyric acid production with the supplementation of $CaCO_3$. (c) Acetic acid production at different initial pH. (d) Acetic acid production with different $CaCO_3$ concentrations.

Figure 3. Hydrogen production by *C. sporogenes* BE01 in rice straw hydrolysate. (a) Percentage hydrogen production at different initial pH. (b) Percentage hydrogen production with different $CaCO_3$ concentrations. (c) Cumulative hydrogen production at different initial pH (d) Cumulative hydrogen production with different $CaCO_3$ concentrations.

Figure 4. Solvents produced by *C. sporogenes* BE01 in rice straw hydrolysate. (a) Butanol produced at different initial pH. (b) Butanol produced with different $CaCO_3$ concentrations. (c) Ethanol produced at different initial pH. (d) Ethanol produced with different $CaCO_3$ concentrations.

Figure 5. Cyclic voltammogram profiles recorded at various pH and $CaCO_3$ concentrations: 0 - No $CaCO_3$, 0.5% - 5 g/L $CaCO_3$, 1% – 10 g/L $CaCO_3$.

Figure 6. Electro kinetic analysis of butanol fermentation by *C.sporogenes* BE01 in rice straw hydrolysate medium at various pH and $CaCO_3$ concentrations based on Tafel plot.

Figure 7. Oxidative slope, reductive slope and polarization resistance during butanol fermentation. (a) Oxidative slopes at different initial pH. (b) Oxidative slopes with different $CaCO_3$ concentrations. (c) Reductive slope produced at different initial pH. (d) Reductive slope with different $CaCO_3$ concentrations. (e) Polarization resistance at different initial pH. (f) Polarization resistance with different $CaCO_3$ concentrations.

Scheme 1: Electron transporters involved and possible electron flow during butanol fermentation VFA and hydrogen production from rice straw hydrolysate by *C. sporogenes* BE01. Green: 5 g/L $CaCO_3$ supplementation; Yellow: 10 g/L $CaCO_3$ supplementation; Pink: common mediators in all the fermentation conditions tested; ↑ high concentration; ↓ Low concentration.

Figure 1

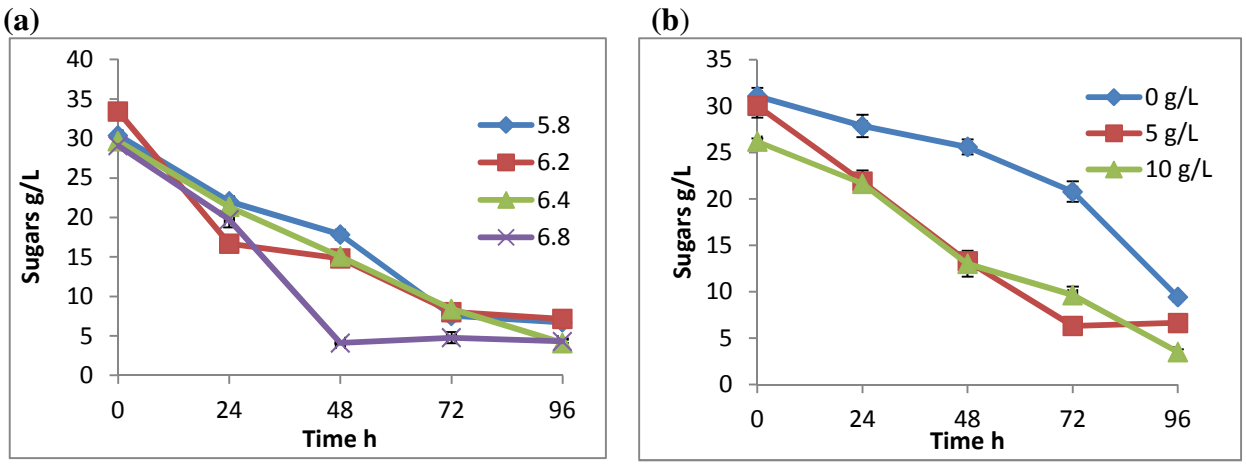


Figure 2

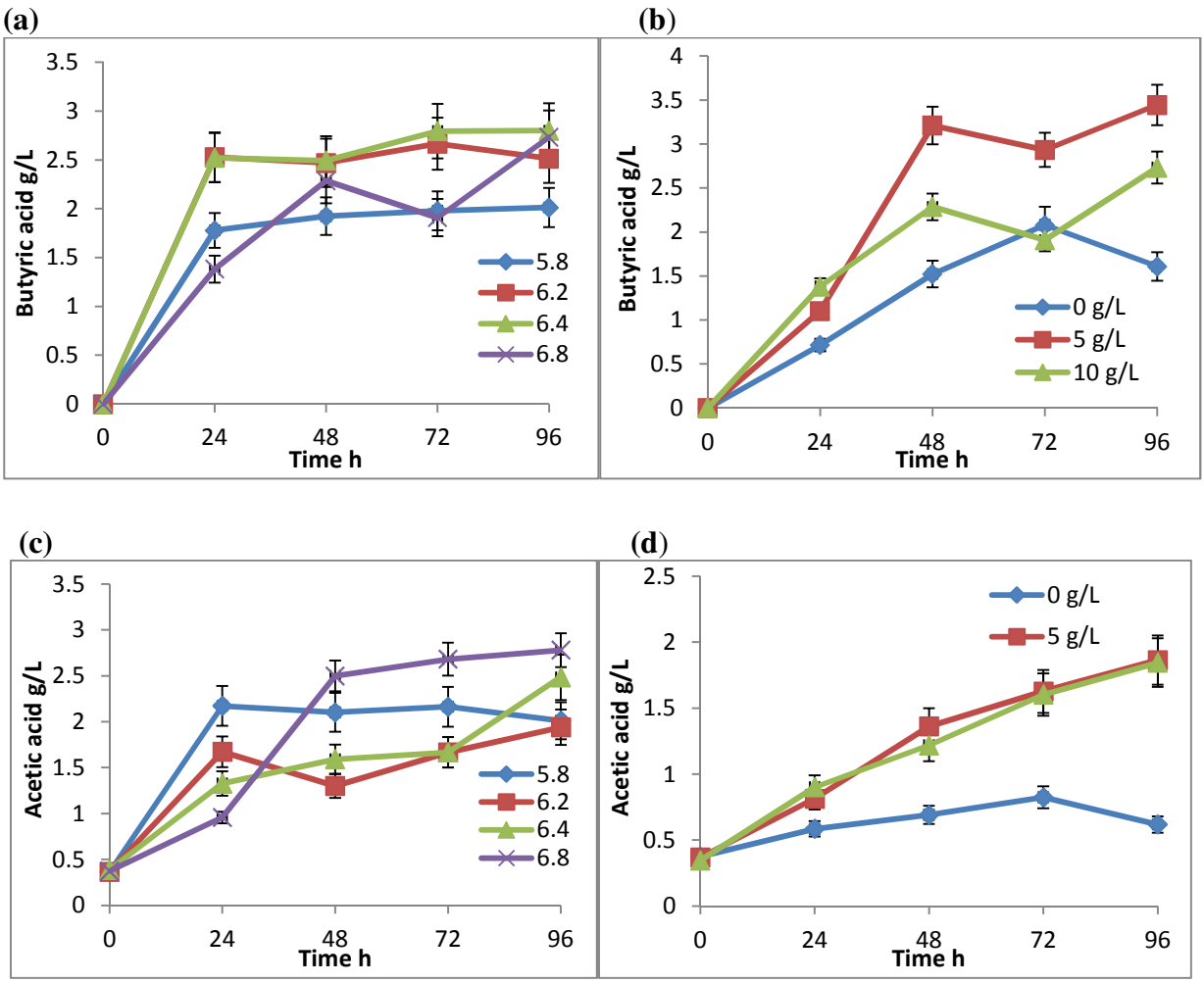


Figure 3

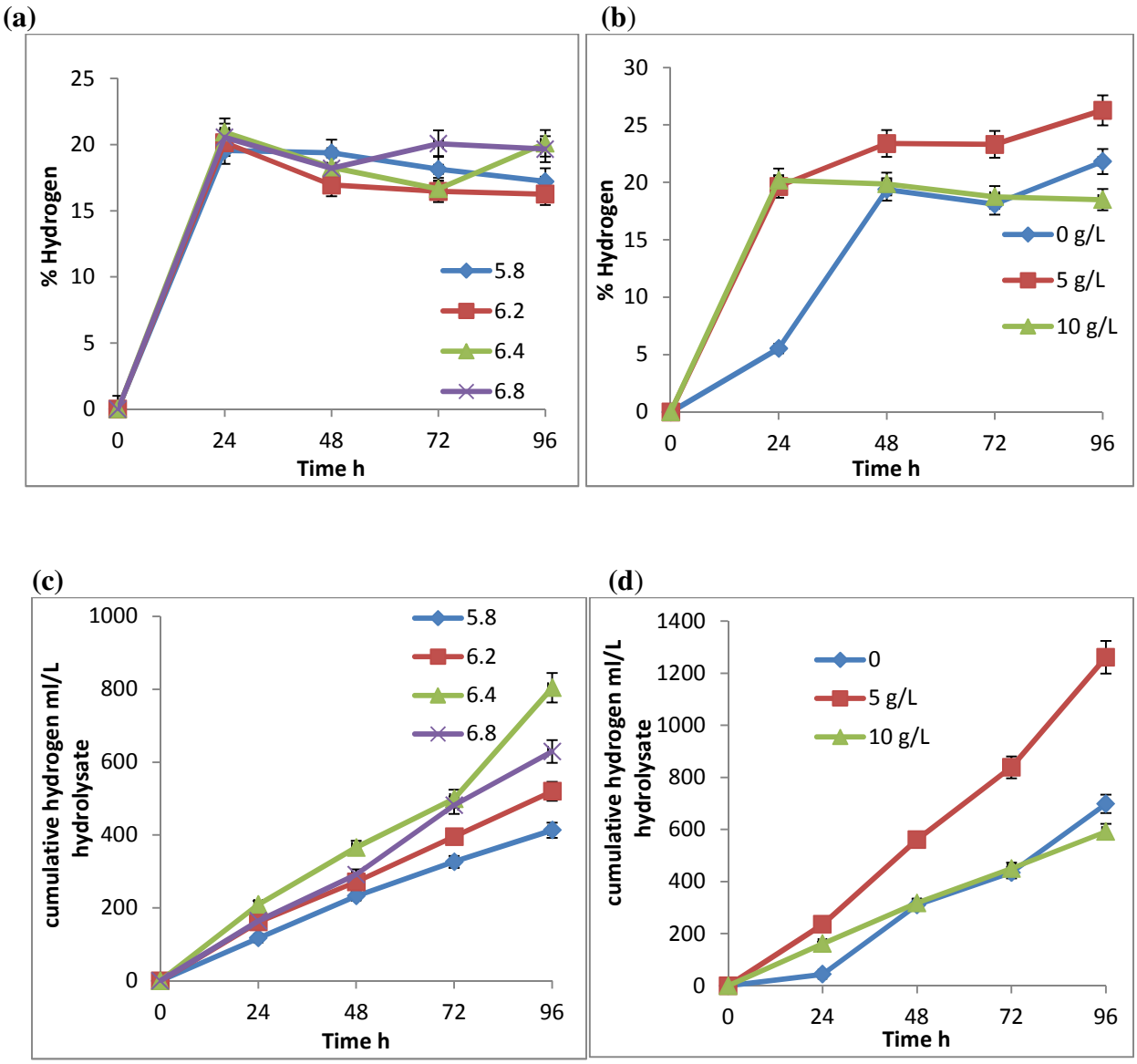


Figure 4

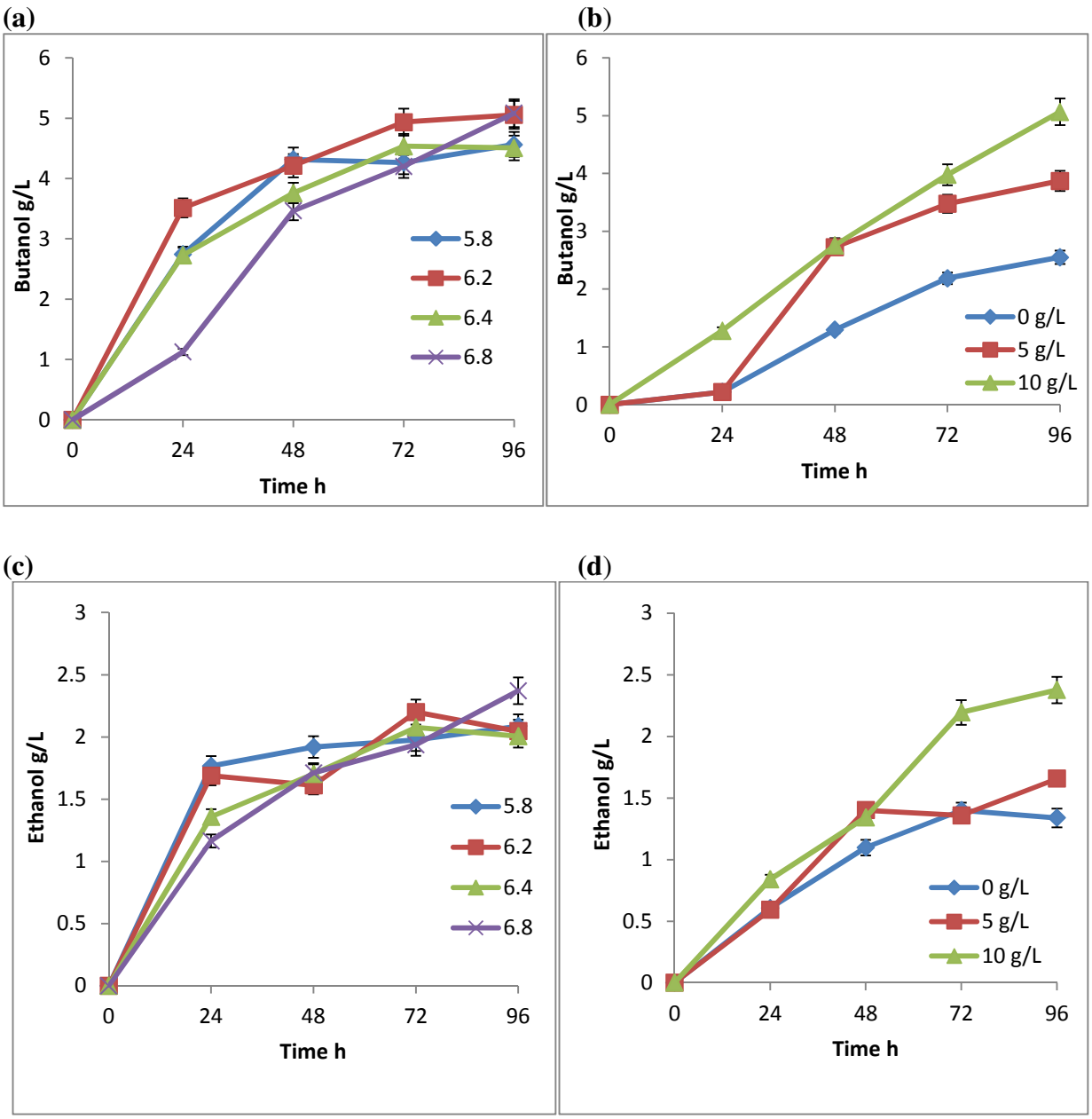


Figure 5

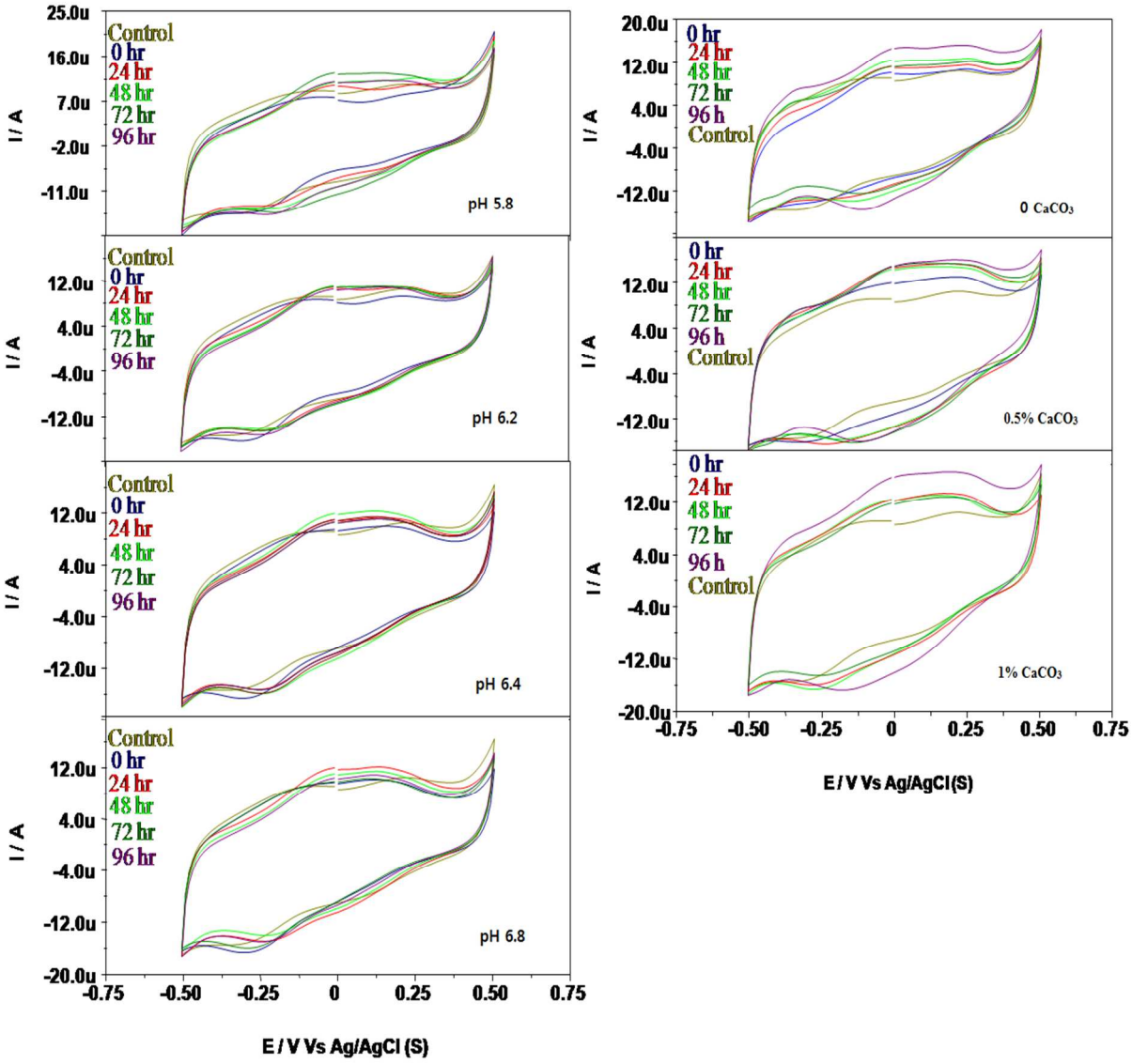


Figure 6

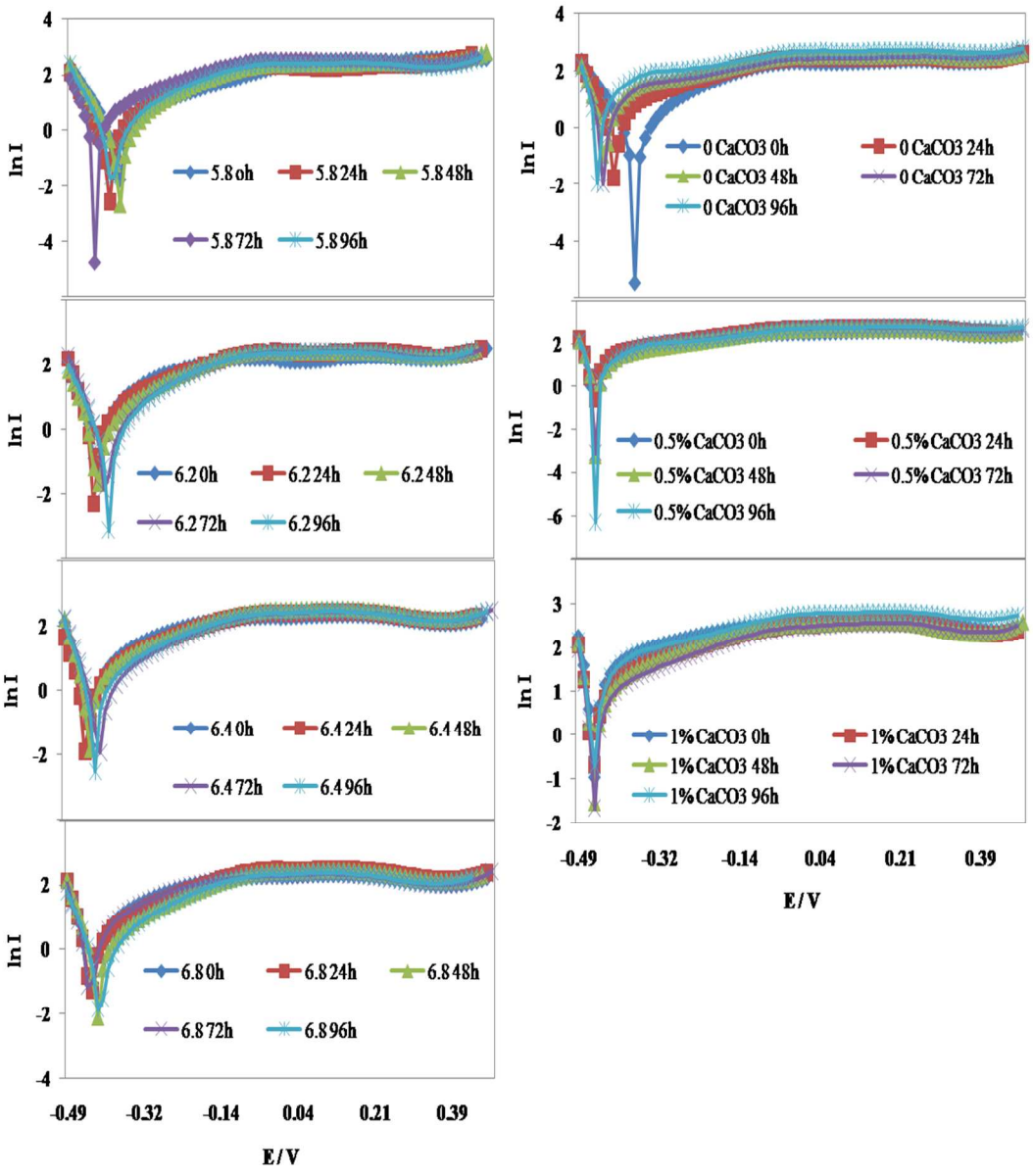
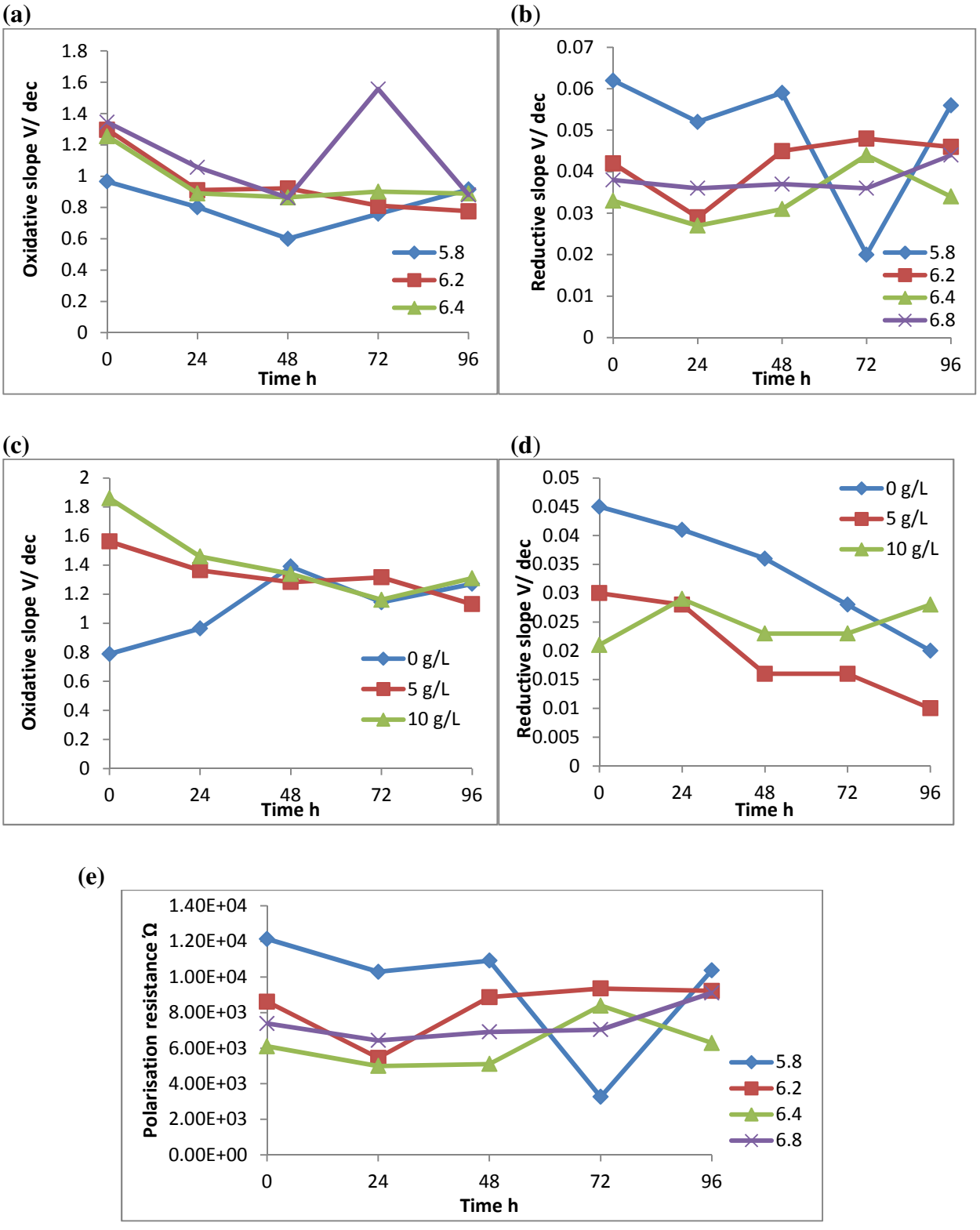
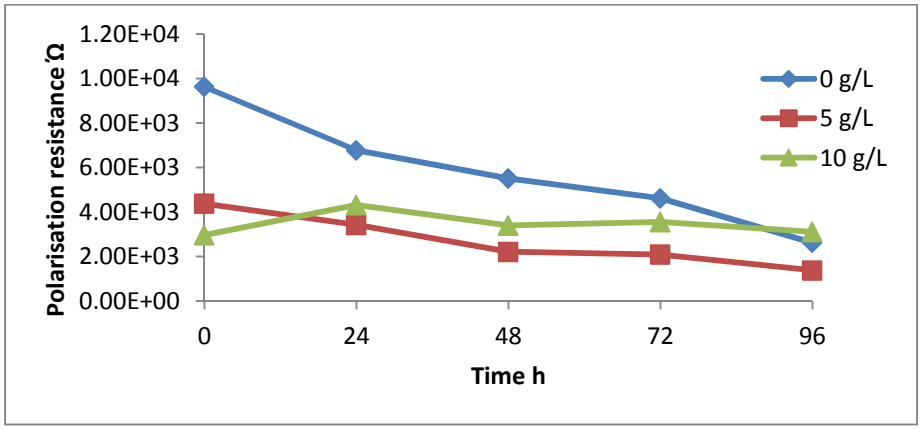


Figure 7



(f)



Scheme 1

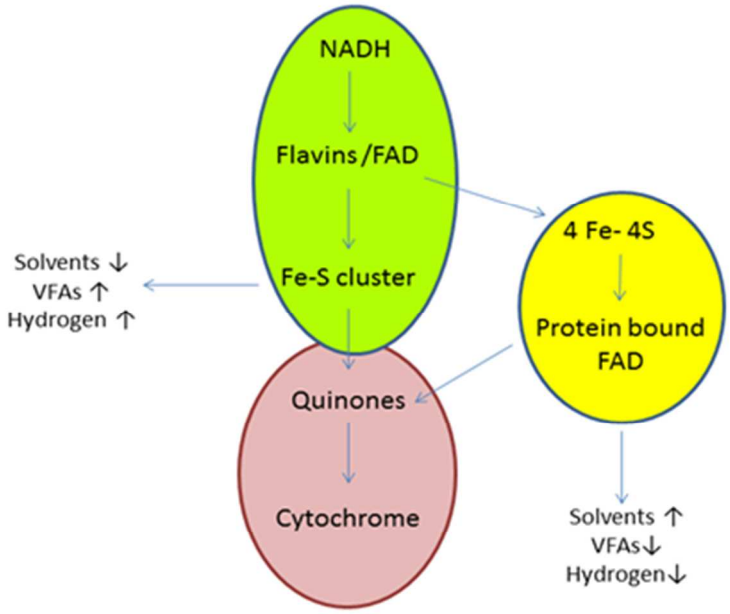


Table of contents entry

Hydrogen and VFAs production during butanol fermentation in rice straw hydrolysate by *Clostridium sporogenes* BE01 and electron transport mediators involved. ‘