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

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Clostridium sporogenes BE01, a non-acetone forming butanol producer, can produce hydrogen and volatile fatty acids (VFAs) during butanol fermentation from rice straw hydrolysate. Bio-electrochemical analysis revealed the changes that occurred in the 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 the electron transport system. The electron transport system mediated by NAD/NADH, flavins, Fe–S clusters, protein bound FAD, and cytochrome complex in *C. sporogenes* BE01 was analysed by cyclic voltammetry (CV). Electrokinetic analysis revealed that the favorability for redox reactions increased with an increase in pH, and the polarization resistance reduced significantly with $CaCO_3$ supplementation.

1. Introduction

Biobutanol has gained significant attention for its properties as a 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. The formation of various industrial products from a single substrate in a single run can be considered beneficial for lignocellulosic biorefinery processes. The fluctuation in the redox environment leads to change in the bacterial growth pattern, glucose utilization, and products formed,[2] and hence it is essential to understand the redox microenvironment of the bioreactors operated with the desired biomass and microbes. The redox balance in a bioreactor is one among the many key components that controls carbon flux and changes in the metabolic activity of the organism.[3] In order to assess the bio-electric potential of a bioreactor, it is essential to perform an analysis of the bio-electro catalytic efficiency of the microbial catalyst. The electrochemical characterization of microbial bioreactors will help in understanding the redox active species participating in the 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 the assimilation of these VFAs to solvents. It is a highly interlinked chain of redox reactions with many electron transporters involved. The formation of hydrogen and volatile fatty acids is an intrinsic part of the 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 solvent production.[6,7] Hydrogen generated by Clostridial species is directly related to volatile fatty acid production. The conversion of acetyl CoA to acetate yields hydrogen in twice the yield than the conversion to butyrate[8] and the ratio of acetic acid and butyric acid has a tremendous effect on the ratio of the solvents formed.[9] Hydrogen produced during the process is an energy rich gaseous fuel and VFAs can be used as precursors for polyhydroxyalkanoates.[10] The 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 the hydrolysis of lignocellulosic biomass to solvents, VFAs and hydrogen. The study also focuses on understanding the possible electron transport

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mediators and redox reactions involved during the process. This is the first report on hydrogen production from rice straw hydrolysate using a pure strain of *C. sporogenes* and also the first report on demonstrating the electron transporter mediated redox activity of *Clostridium species* during biobutanol fermentation from lignocellulosic biomass.

2. Results and discussion

2.1. Glucose utilization

The dilute acid pretreatment and enzymatic hydrolysis of rice straw generated 45 g L^{-1} of glucose. However, heat sterilizing the hydrolysate at 121 °C for 10 min resulted in 15 g L^{-1} sugar loss and the final glucose obtained was 30 g L^{-1}. Pentoses were found in negligible concentrations in the hydrolysate, as the 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^{-1}, 5 g L^{-1} and 10 g L^{-1}). pH plays a very significant role in the microbial catalysis and growth of the bacteria. Especially in butanol fermentation, pH has direct control over the formation and assimilation of acids.[11] *C. sporogenes* BE01 exhibited a change in the pattern of glucose utilization with changes in the microenvironment of the bioreactor. pH of the medium in the range of 5.8 to 6.4 had a very low effect on sugar utilization, but the utilization rate enhanced with pH approaching near neutral. This signifies the increased metabolic activity and growth at a near neutral pH (Fig. 1a).

Supplementation with $CaCO_3$ in the medium enhanced glucose utilization and it increased with an increasing concentration of $CaCO_3$ (0, 5 and 10 g L^{-1}) (Fig. 1b). Increased glucose consumption and ABE productivity in the presence of ≥4 g L^{-1} $CaCO_3$ was reported with *C. beijerinkii* grown in semi defined P2 medium.[12] Calcium ions have various effects on a cellular level, which can contribute to increased growth though pH buffering effects might also contribute to stimulatory effects on butanol fermentation. It was reported by Richmond *et al.* that the presence of $CaCO_3$ increased protein synthesis in *Clostridium* species and this increase was 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 under all the conditions tested (Fig. 2). *C. sporogenes* is a known producer of butyric acid and has been reported for cheese fermentation in combination with *C. butyricum* and *C. beijerinckii*.[14,15] In *C. tyrobutyricum* fermentation studies, butyrate at 15 g L^{-1} showed an 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 a production of 5.2 g L^{-1} and 5.5 g L^{-1}, respectively. The reactor with a $CaCO_3$ concentration of 5 g L^{-1} performed considerably well for the conversion of sugars to acids (Fig. 2b and d).

C. propionicum, an organic acid producer, showed the highest growth and organic acid production at pH 7.[17] Acid forming enzymes are highly pH dependent. The activity of the enzymes can be inhibited or enhanced with an increase or decrease in pH.[16] The acidogenic process was highly active in the first 24 h and became stable throughout the fermentation period, except at pH 6.8, VFAs production increased until 48 h, sharply decreased at 72 h and increased again at 96 h (Fig. 2a and c). The same was observed with 5 g L^{-1} and 10 g L^{-1} $CaCO_3$ supplementation, which could be attributed to efficient assimilation of acids in the solvents at 72 h. Hydrogen production was in accordance with acid production and the highest percentage of hydrogen (20%) of the total gas produced was at pH 6.4 and 6.8 (Fig. 3a). Although there was no considerable difference in the percentage of hydrogen pro-

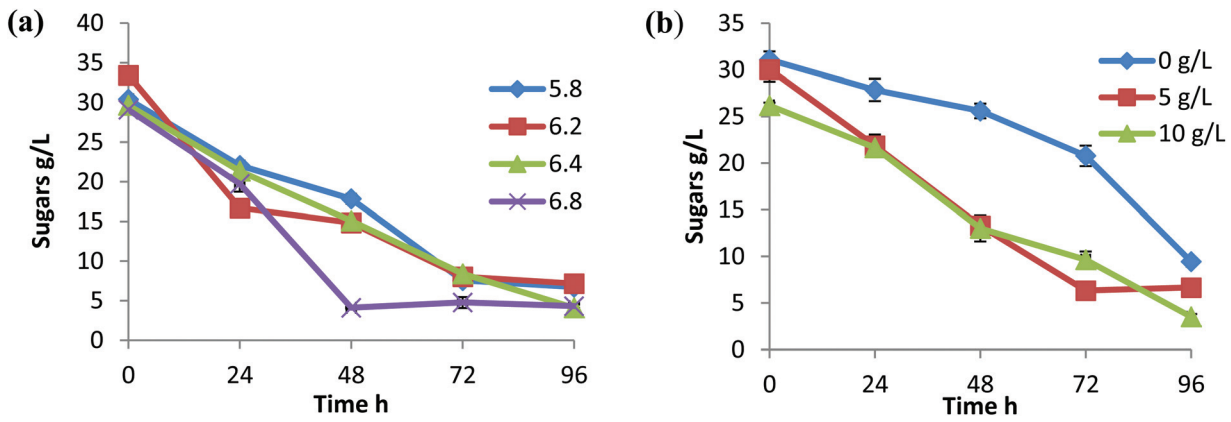


Fig. 1 Glucose consumed by *C. sporogenes* BE01 from rice straw hydrolysate. (a) At different initial pH supplemented with 10 g L^{-1} $CaCO_3$. (b) With the supplementation of $CaCO_3$ at two different concentrations (5 g L^{-1} and 10 g L^{-1}) and without the supplementation of $CaCO_3$ (0 g L^{-1}).

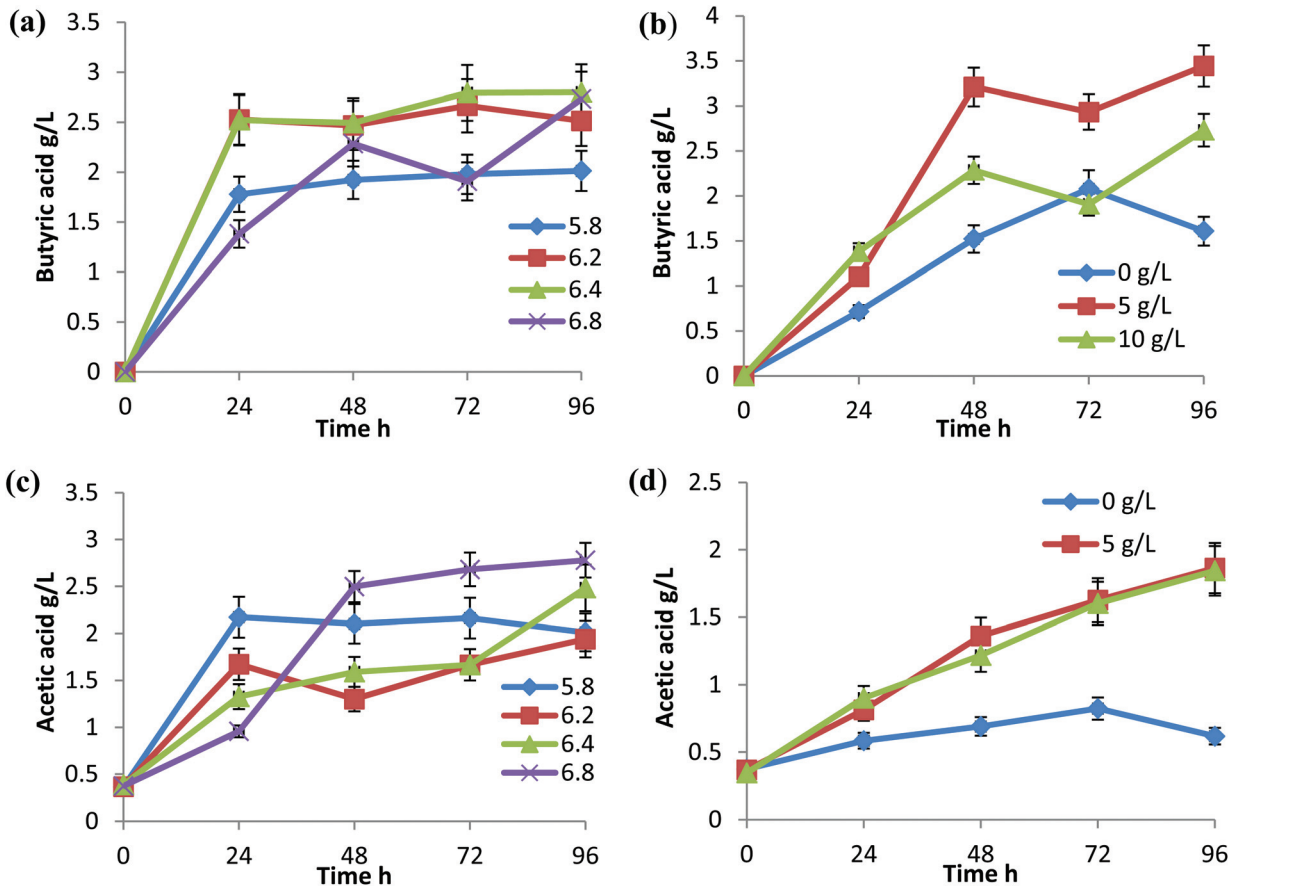


Fig. 2 Volatile fatty acid (VFA) production by *C. sporogenes* BE01 in rice straw hydrolysate at different initial pH and $CaCO_3$ concentrations. (a) Butyric acid production at different initial pH. (b) Butyric acid production with the supplementation of $CaCO_3$. (c) Acetic acid production at different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

duced, there was a notable difference in the total gas produced at different pH and showed marked variation when represented in terms of cumulative hydrogen (Fig. 3c and d). The total hydrogen production increased with an increase in pH to 6.4 but reduced at pH 6.8. The total hydrogen production at pH 6.4 continued for 96 h and there was little reduction in the gas production with respect to time (Fig. 3c).

The optimal pH for hydrogen production varies with each species and strains. For *C. beijerinkii* DSM 1820, the optimum pH reported was 6.7, for *C. pasteurianum* it was 5.4 and for *C. butyricum* it was 5.1.[18] Hydrogen production varies with a change in glucose concentration. In *C. acetobutylicum* ATCC824 glucose fermentation, the hydrogen production rate ranged from 680 to 1270 ml g^{-1} glucose per liter of reactor.[19] There was an increase in total gas production and percentage hydrogen production when the $CaCO_3$ concentration was reduced to 5 g L^{-1} (1260 ml L^{-1}), but in the absence of $CaCO_3$ and with an increase in $CaCO_3$ to 10 g L^{-1} the total hydrogen produced was 591 ml and 698 ml, respectively (Fig. 3b and d). The long acidogenic phase and higher acid production can be correlated with the increased hydrogen production found with the supplementation of 5 g L^{-1} $CaCO_3$. The favorability of 10 g L^{-1} $CaCO_3$ 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 solvent production increased when pH approached near neutral (Fig. 4a and c). At 5.8 pH, though the rate of butanol and ethanol formation was high for the first 48 h and 24 h, respectively, solvent formation ceased later and the total solvent produced was comparatively less (Fig. 4a and c). This could be due to the lowered pH with VFAs production and inefficient assimilation of the VFAs. At pH 6.2 and 6.4, a relatively high solvent production was observed for the first 24 h and 72 h, respectively. Further accumulation of solvents was not found from 72 h to 96 h. The efficient conversion of

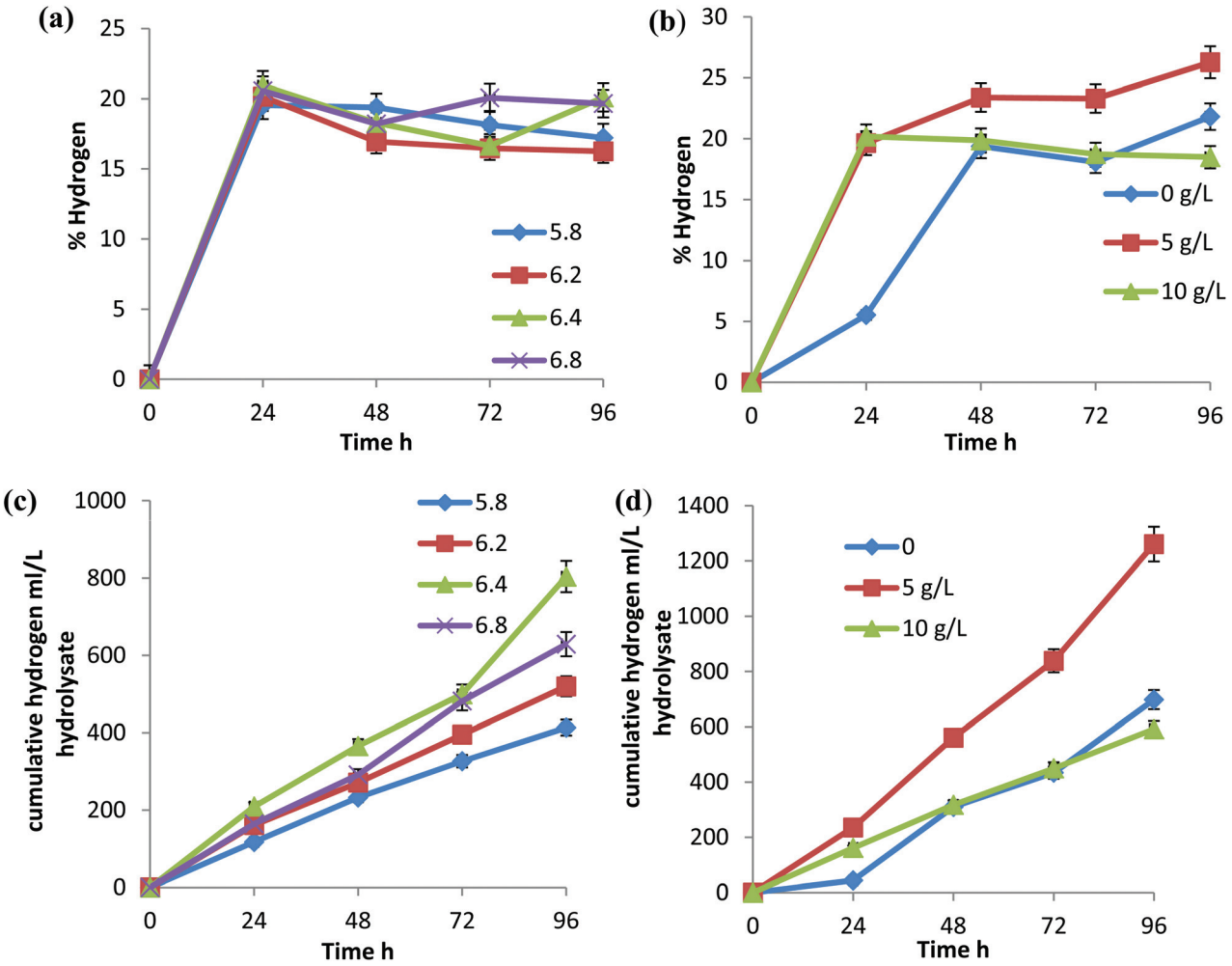


Fig. 3 Hydrogen production by *C. sporogenes* BE01 in rice straw hydrolysate. (a) Percentage hydrogen production at different initial pH. (b) Percentage hydrogen production with different $CaCO_3$ concentrations. (c) Cumulative hydrogen production at different initial pH (d) Cumulative hydrogen production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

sugars and acids to solvents was achieved in a different mode by *C. sporogenes* BE01 at pH 6.8. Solvent formation in the first 24 h was considerably low compared to rest of the pH ranges, which signifies that the acid accumulation did not lead to a decrease in the pH to a level wherein assimilation starts for solvent production, but the overall high solvent production 7.3 g L^{-1} was achieved with a constant increase in solvent accumulation until 96 h without attaining saturation (Fig. 4a and c).

$CaCO_3$ was used to maintain the pH in the range by neutralizing the 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 the initial pH and solvent formation. Solvent production increased with an increase in the concentration of $CaCO_3$ in the medium (Fig. 4b and d). This could be due to the effect of calcium ions and its effective buffering action, which resulted in efficient VFA assimilation. Without $CaCO_3$ supplementation, the total yield of solvents was just 3.8 g L^{-1}, which could be due to the low glucose utilization rate and low VFA formation. The supplementation of 10 g L^{-1} $CaCO_3$ increased the total solvent production to 7.4 g L^{-1}. A reduction in $CaCO_3$ concentration from 10 g L^{-1} to 5 g L^{-1} lead to a 26% decrease in the total solvents formed (Fig. 4d).

The final pH of all the experiments varied with the changes in the $CaCO_3$ concentration in the medium. In the absence of $CaCO_3$ and with the supplementation of 5 g L^{-1} $CaCO_3$, the pH dropped from 6.8 (initial pH) to 5.9 (final pH) in 96 h, indicating the formation of acids and their improper assimilation into the solvents; whereas, with 10 g L^{-1} $CaCO_3$, the pH dropped to 6.3 in 48 h and then gradually increased to 6.5 in 96 h. The $CaCO_3$ concentration was observed to affect the solvent production more than the initial pH of the medium.

2.4. Electrochemical analysis

The variations in the voltammogram peaks obtained during cyclic voltammetry (CV) analysis and the change with respect

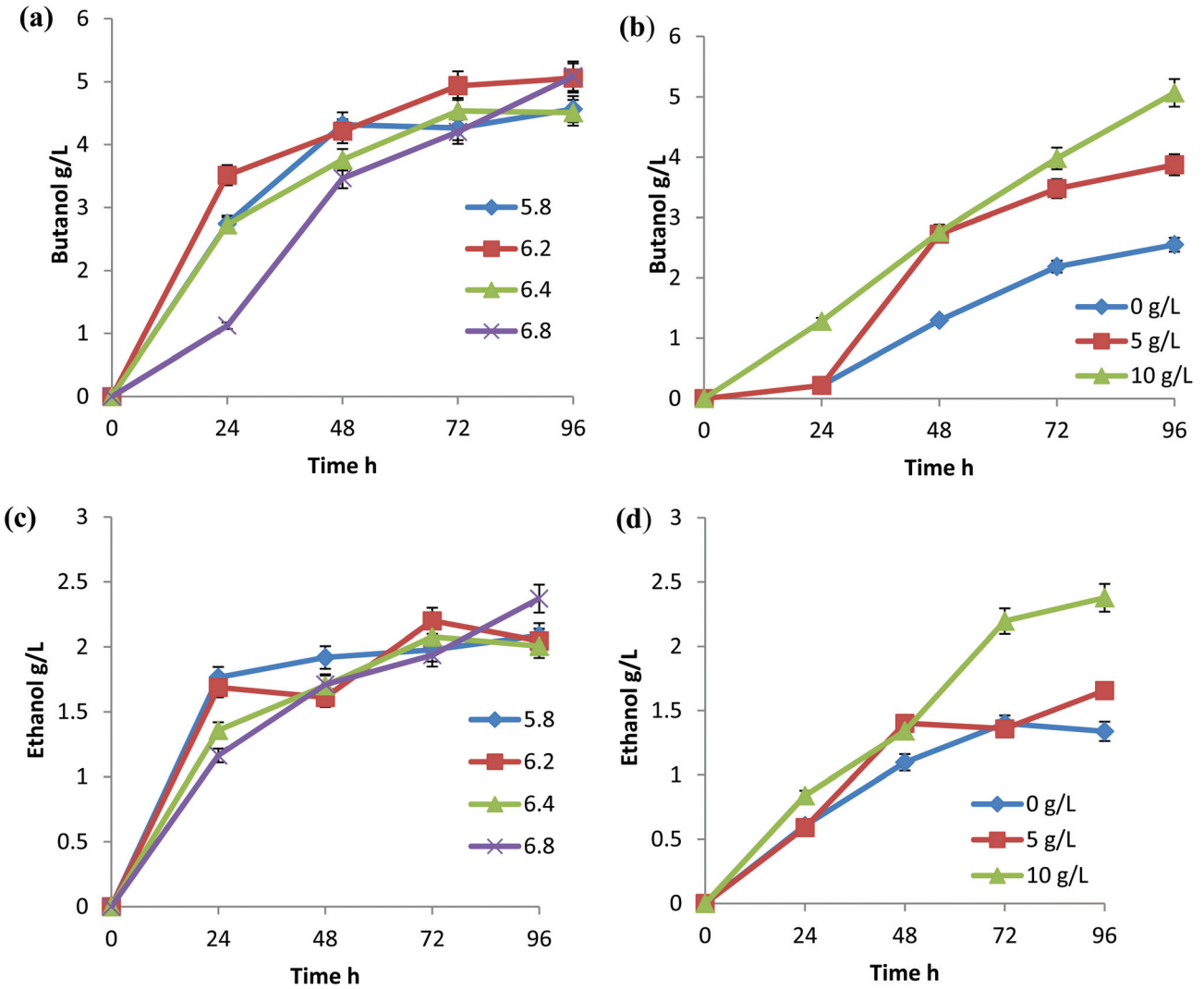


Fig. 4 Solvents produced by *C. sporogenes* BE01 in rice straw hydrolysate. (a) Butanol produced at different initial pH. (b) Butanol produced with different $CaCO_3$ concentrations. (c) Ethanol produced at different initial pH. (d) Ethanol produced with different $CaCO_3$ concentrations.

to time were due to the differences in the initial pH and $CaCO_3$ concentrations in the rice straw hydrolysate medium. The redox currents were found to be high with sterile rice straw hydrolysate alone, which signifies it is potential substrate for bio-electro catalytic reactions. The redox currents did not differ predominantly and were mostly in the range from RC: −18 ± 3 μA to OC: 18 ± 4 μA. With the alterations in the initial pH and 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 the 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 at pH 5.8, the preliminary hours had shown redox potential for the biological reaction of NAD^+/NADH (E^0 values −0.32 V) and the 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 V) and iron–sulphur clusters were observed.[23,24] Iron–sulphur clusters with E^0 values less than −0.05 can be attributed to rubredoxin or any of the simplest type of Fe–S clusters and those with E^0 values close to −0.15 could be attributed to the iron–sulphur cluster of N2 [4Fe–4S] associated with complex 1.[25,26] Rubredoxin from *C. pasteurianum* was reported to have a reduction potential of less than −0.05 V.[26] The association of Fe–S with Fe–Fe dehydrogenase enzyme for hydrogen production is also well reported.[27] The peaks for the 4Fe–4S clusters were not prominent at pH 5.8, but were found after 24 h at pH 6.2, which extended up to 72 h at pH 6.4 and at near neutral pH the peaks were observed after 96 h. There have been reports on increased levels of Fe–S clusters during solvent formation and furfural challenged cultures.[28] Although in bacteria their primary function is to mediate low potential electron transfer,

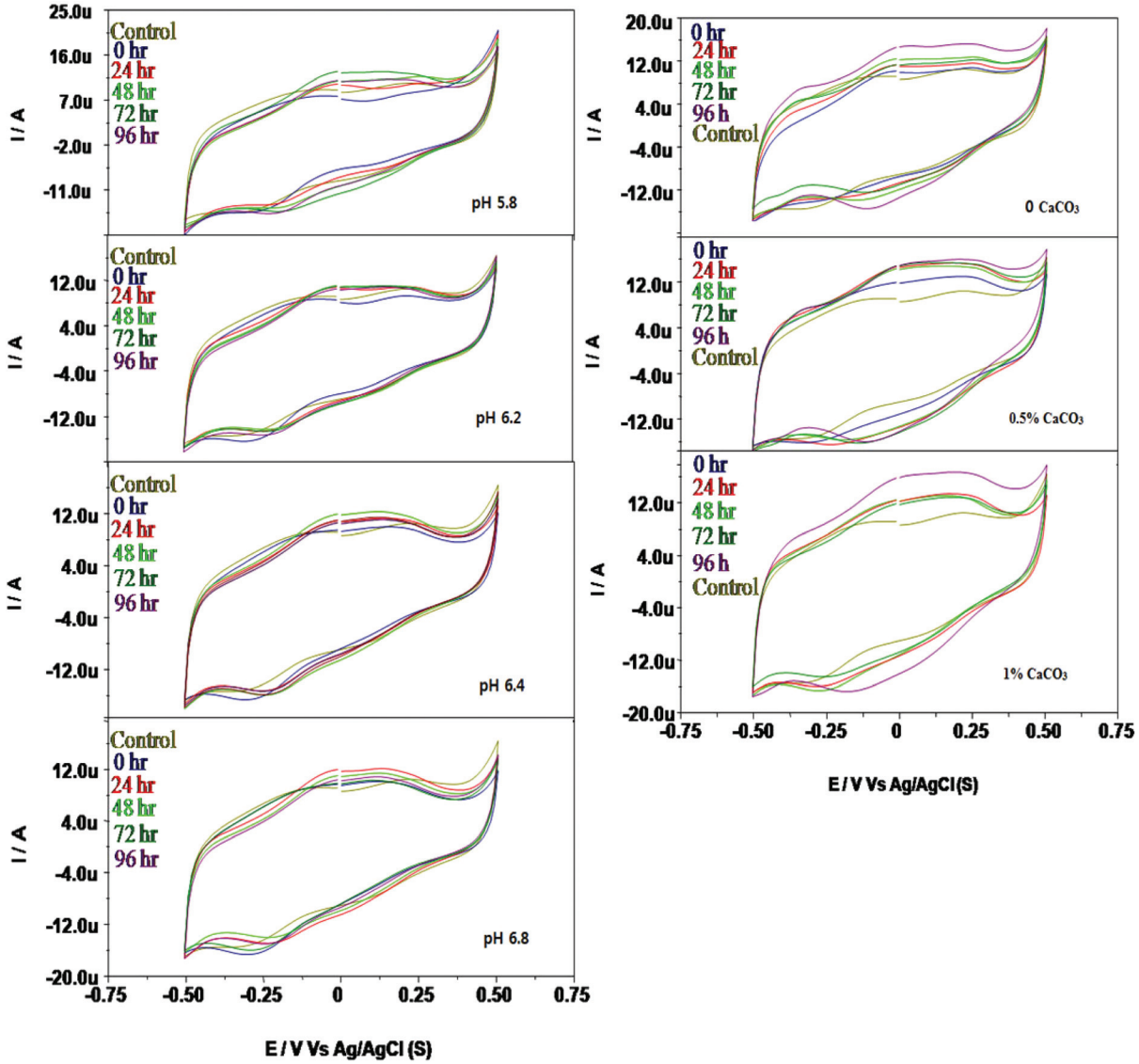
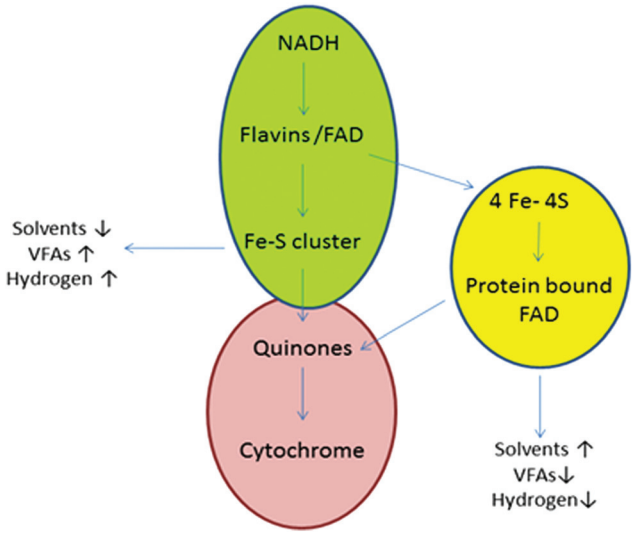


Fig. 5 Cyclic voltammogram profiles recorded at various pH and $CaCO_3$ concentrations: 0% – No $CaCO_3$, 0.5% – 5 g L^{-1} $CaCO_3$, 1% – 10 g L^{-1} $CaCO_3$.

their function is extended to several catalytic proteins.[29] The interference of protein bound FAD increased with an increase in pH and 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 the change in $CaCO_3$ concentration in the medium. The peaks for cytochrome, quinone and simple Fe–S clusters were commonly observed throughout the fermentation. The major difference was with the 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^{-1} $CaCO_3$ containing medium. The presence of many electron transporters implies that the redox activity occurred efficiently and this was also supported by high butanol production. While in the absence of $CaCO_3$ peaks for the common mediators, such as cytochrome 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 the comparatively lower acid and solvent production. With 5 g L^{-1} $CaCO_3$, the peaks for cytochrome bc1, NAD/NADH, and 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 clusters were involved in the electron transport system, which might be facilitating a key reaction that results in higher butanol yields. Peaks were prominent for 4Fe–4S and protein bound FAD with 10 g L^{-1} $CaCO_3$ supplementation, wherein high solvent production was observed. This indicates the stimulation of a



Scheme 1 Electron transporters involved and the possible electron flow during butanol fermentation, VFA and hydrogen production from rice straw hydrolysate by *C. sporogenes* BE01. Green: 5 g L^{-1} $CaCO_3$ supplementation; yellow: 10 g L^{-1} $CaCO_3$ supplementation; pink: common mediators in all the fermentation conditions tested; ↑ high concentration; ↓ low concentration.

membrane bound protein that has FAD and 4Fe–4S clusters. In the fermentation conditions, wherein high VFAs and hydrogen were produced, the peaks for NADH, flavoproteins and Fe–S cluster were prominent and very interestingly, whereas the peaks for protein bound FAD were very rare. Based on the redox potentials obtained for various electron transport mediators during the tested fermentation conditions and the reported electron transport systems in *Clostridium* species, the most probable electron transport system involved in hydrogen production, VFAs and butanol fermentation was postulated (Scheme 1).

In an electron transport system, electrons always flow from a negative to positive redox potential. Based on the CV peaks, during solvent formation, the 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 the 4Fe–4S clusters (−0.15). Quinones and cytochromes (0.1 to 0.25 V) stabilize the FAD bound enzyme in the reduced state. However, for fermentation conditions wherein the solvent formation was low and VFAs formation was high, the peaks for FAD and 4Fe–4S clusters were less prominent, while peaks for simple Fe–S clusters were dominant. This indicates that there was a shift or bifurcation in electron transportation during solventogenesis.

There are reports on a Clostridial electron transport bifurcation system, wherein the cytoplasmic complex of butyryl CoA dehydrogenase and electron transferring flavoprotein (BCD/ETF) catalyses a key reaction (crotonyl CoA to butyryl CoA) in the butanol pathway and is coupled to a membrane associated proton translocating NADH-ferredoxin reductase complex (Rnf A-G or Rnf A-E).[30,31] Signals obtained for the 4Fe–4S clusters, protein bound FAD/FMN, flavoproteins and NAD/NADH are characteristic of the electron bifurcation system, involving the Rnf complex, and have been reported in few *Clostridium* species.[30] Quinones and cytochrome were reported for maintaining the enzymes in their reduced active state.[32] The 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 clusters. The electrons are proposed to flow from NADH to the Rnf complex, resulting in the 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. kluyveri*, proton translocating Rnf has been 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 a terminal acceptor. However, hydrogenases coupled to NADH: ferredoxin reductases produce hydrogen using NADH as an electron donor and proton as an 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 the *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 the metabolic pathway involved. A correlation of the CV analyses with the pattern of products formed during fermentation broadly suggest the possible stimulation of the 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 $CaCO_3$ can be attributed to stimulation and stabilization of membrane proteins. However, detailed research is necessary to support this hypothesis.

2.5. Bio-electro kinetic analysis

The rate of electron transfer to the electrode by the oxidized and reduced species can be interpreted by a Tafel plot. According to the Tafel equation, when the over potential is large the reverse reaction rate is negligible. The slope of the Tafel plot reveals the value of the electron transfer coefficient.[38] A low Tafel slope indicates the high current obtained at low over potential. Therefore, the higher the slope, the higher the activation energy required, which 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 slopes and

reductive Tafel slopes were derived from the Tafel plot obtained (Fig. 6).

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

In the absence of $CaCO_3$, the oxidation slope was low but increased with an increase in $CaCO_3$ concentration in the medium (Fig. 7c). This suggests that the addition of $CaCO_3$ was not favourable for the oxidation reactions and in the case of the reduction slopes, 5 g L^{-1} $CaCO_3$ showed a comparatively lower reduction potential followed by 10 g L^{-1} $CaCO_3$ and no $CaCO_3$ supplementation (Fig. 7d). Reduction in the activation energy required for the redox reactions in the presence of $CaCO_3$ could be responsible for the increased production of hydrogen, acids and solvents.

Polarization resistance, R_p, refers to the resistivity of the electrolytes around the electrode. This could be due to resistance of the electron transfer by the microbe or the insulation

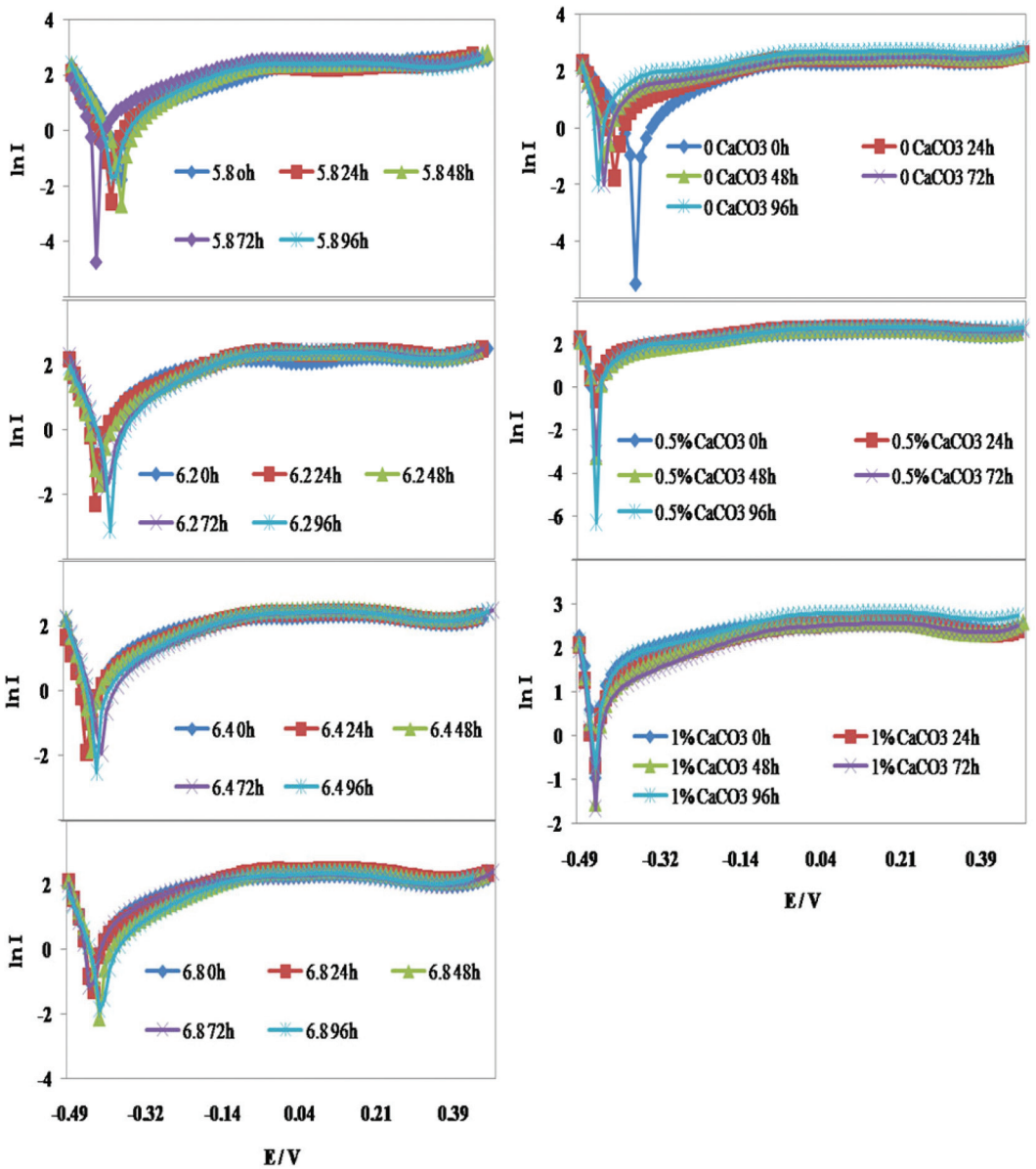


Fig. 6 Electrokinetic analysis of butanol fermentation by *C. sporogenes* BE01 in rice straw hydrolysate medium at various pH and $CaCO_3$ concentrations based on the Tafel plot.

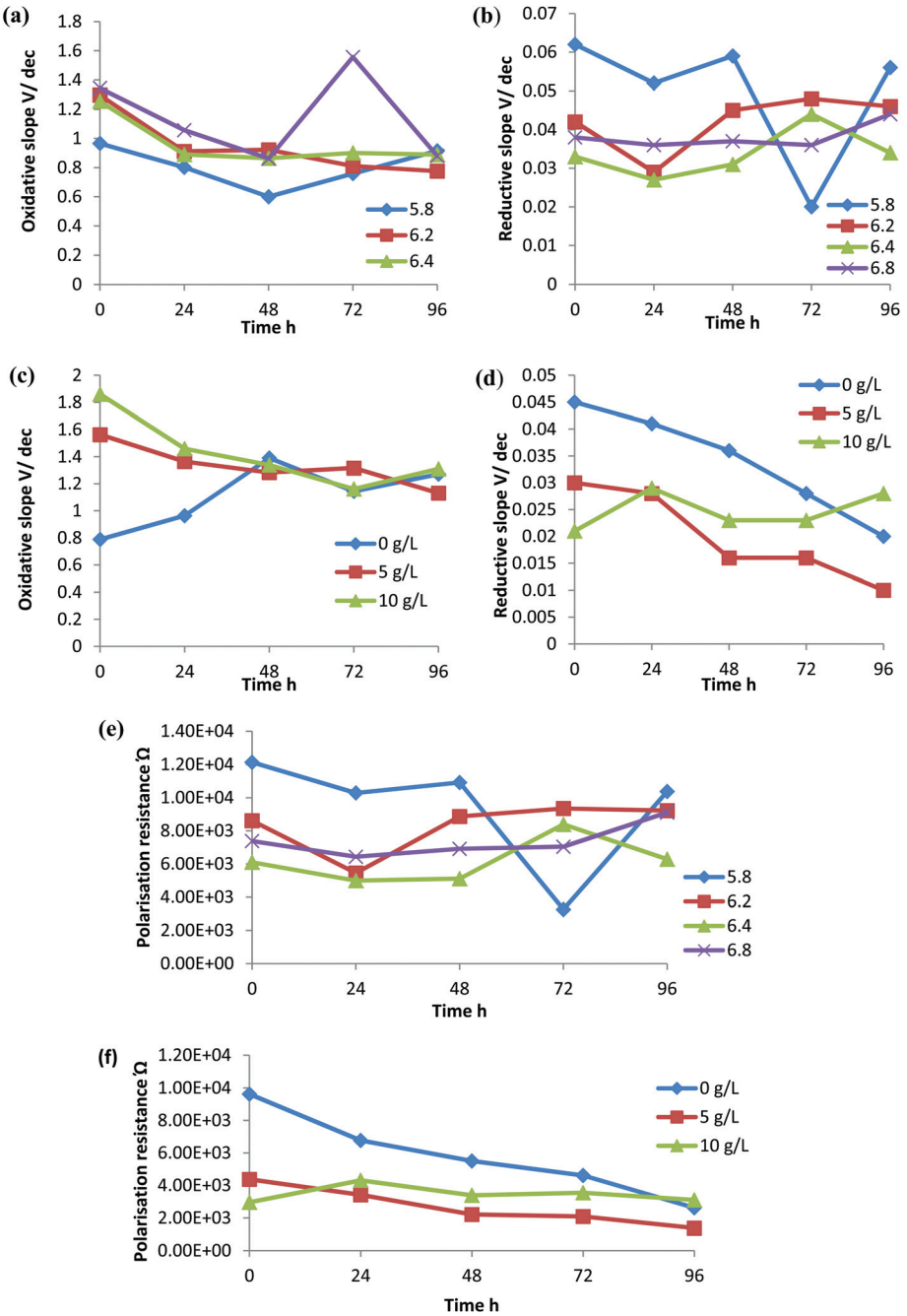


Fig. 7 Oxidative slope, reductive slope and polarization resistance during butanol fermentation. (a) Oxidative slopes at different initial pH. (b) Oxidative slopes with different $CaCO_3$ concentrations. (c) Reductive slopes produced at different initial pH. (d) Reductive slopes with different $CaCO_3$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

effect of the products formed on the electrodes surface. For a reactor to be active in an electron transfer and product formation, the polarization resistance should be low.[39] Fermentation with an initial pH of 6.4 showed less R_p compared with the other pH ranges (Fig. 7e). Polarization resistance was high in the reactor without $CaCO_3$ supplementation and decreased with a supplementation of 5 g L^{-1} $CaCO_3$, but the resistance increased slightly with increased $CaCO_3$ supplementation (Fig. 7f) and as stated before it could be either due to the resistance or insulation effect.

3. Conclusions

Clostridium sporogenes BE01 is capable of producing 7 g L^{-1} of VFAs and 1.2 L L^{-1} of hydrogen during butanol fermentation

in rice straw hydrolysate with 30 g L^{-1} of glucose. 7.3 g L^{-1} of total solvents were produced, of which 5 g L^{-1} was butanol. Butanol and ethanol production was in a ratio of 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. The presence of peak for protein bound FAD was found until 96 h at pH 6.4 and pH 6.8 with 10 g L^{-1} $CaCO_3$ supplemented medium. The involvement of 4Fe–4S clusters, NAD/NADH, protein bound FAD and flavoproteins during active butanol fermentation presents the possibility of an electron bifurcation system mediated by a membrane bound complex, probably Rnf. The Tafel plot revealed that rice straw hydrolysate supplemented with $CaCO_3$ had a low polarisation resistance, R_p, and required less activation energy for the reduction metabolism making it more favourable for solvent 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 1 h at 120 °C with a solid loading of 15% (w/w). The pentose fraction obtained (liquid stream) was separated from the solids by filtration and centrifugation. Pretreated rice straw was dried at room temperature till the excess moisture was removed. Commercial cellulase (Zytex India ltd, Mumbai) with an enzyme activity of 80 FPU mL^{-1} was used for the enzymatic hydrolysis of the pretreated rice straw at 50 °C. 30 FPU per gds was used for the hydrolysis and the run was continued for 48 h. 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 its spore form at 4 °C. The spores were heat shocked at 80 °C for 2 min and the temperature was immediately brought down by placing in an ice-water bath. The heat shocked spores were cultured in a TGY medium to develop the preinoculum. Actively growing cells were inoculated into a fresh TGY medium for inoculum generation. The highly motile 12 h old culture was inoculated into rice straw hydrolysate. Rice straw hydrolysate was made anaerobic by cooling under an nitrogen atmosphere after heat sterilization at 120 °C 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 a reducing agent.[20] The 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 the required pH using 1 N NaOH and 1 N HCl. The initial pH of 5.8, 6.2, 6.4 and 6.8 with 10 g L^{-1} $CaCO_3$ was chosen to study the effect of the initial pH on the bioprocess. For analysing the effect of pH on glucose utilisation and fermentation, rice straw hydrolysate was supplemented with 10 g L^{-1} of $CaCO_3$ and the pH was adjusted to 5.8, 5.2, 6.4 and 6.8 and three different concentrations of $CaCO_3$ (0 g L^{-1}, 5 g L^{-1} and 10 g L^{-1}) were investigated to understand the role and effect of $CaCO_3$ on the redox microenvironment of rice straw hydrolysate inoculated with *C. sporogenes* BE01. The pH change of the fermentation medium was monitored at 24 h intervals 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 a 20 mL gradient syringe on every reactor by piercing it through a rubber septum. The gas produced displaced the piston of the syringe and the gradient helped to measure the gas produced. The head space volume of the reactor was also taken in to consideration.

Percentage hydrogen analysis of the total gas produced was carried out by gas chromatography (Nucon) equipped with a thermal conductivity detector (TCD). A 2.1/8″ × 2 m SS column with a molecular sieve stationary phase of size 60/80 mesh was used for gas separation at a temperature of 60 °C. The carrier gas used was nitrogen at a flow rate of 20 mL min^{-1} under 1 kg 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 using a 100 × 7.8 mm fast carbohydrate analysis column (Biorad) by HPLC (Shimadzu prominence UFLC) equipped with a RI detector. The oven temperature was maintained at 85 °C and de-ionized water used as the mobile phase at a flow rate of 0.8 mL min^{-1}. Volatile fatty acids were analysed using a Rezex® ROA organic acid analysis column (Phenomenex) and PDA detector at an oven temperature of 50 °C. The mobile phase used for the separation was 0.05 M H_2SO_4 at a flow rate of 0.6 mL min^{-1}.

4.3.3. Solvents analysis. Butanol and ethanol were analyzed by gas chromatography (Chemito GC 8610). A Poropak Q® column was used for the separation with a gradient oven temperature rise from 150 °C to 200 °C at a heating rate of 8 °C min^{-1}. A flame ionization detector (FID) was used and the detector temperature was maintained at 250 °C. The sample was injected with an injector temperature of 150 °C.

4.3.4. Electrochemical analysis. Cyclic Voltammetry (CV) was performed using a potentiostat–glavanostat system (Autolab-PGSTAT12, Ecochemie) to understand the redox microenvironment 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 the counter electrode against the reference electrode (Ag–AgCl(S)). A potential ramp was applied in the range of +0.5 V to −0.5 V at a scan rate of 30 mV s^{-1}. The redox currents and peaks were recorded for further analysis.

Electrokinetic analysis was performed by plotting the Tafel slope using autolab software. The natural log of the anodic current ($\ln I$) was plotted against the applied range (E/V) for presenting the Tafel plot. The oxidation slope and reduction slope for every voltammogram was recorded and plotted against time to understand the fluctuations in the redox environment with respect to time.[39] The current *versus* voltage curve approximates a straight line and the slope obtained from this is the polarisation resistance, R_p.[40]

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