Bioresource Technology 147 (2013) 321–331

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Relative effect of different inorganic acids on selective enrichment of acidogenic biocatalyst for fermentative biohydrogen production from wastewater

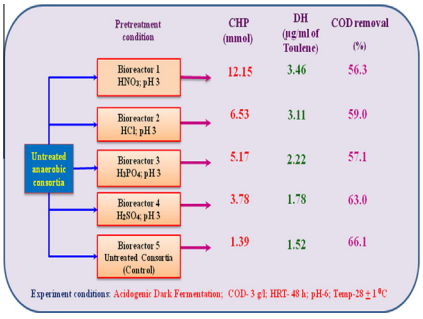
Omprakash Sarkar, R. Kannaiah Goud, G. Venkata Subhash, S. Venkata Mohan *

Bioengineering and Environmental Centre (BEEC), CSIR-Indian Institute of Chemical Technology (CSIR-IICT), Hyderabad 500 007, India

HIGHLIGHTS

- Selective enrichment of biocatalyst by different inorganic acids was studied.
- Acetate–butyrate type fermentation was observed with HNO_3 treated culture.
- HNO_3 pretreated consortia showed relatively more H_2 production.
- Dehydrogenase activity and bio-electro kinetics correlated well.
- Microbial diversity illustrated the enrichment of *Firmicutes*.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 15 July 2013
Received in revised form 1 August 2013
Accepted 4 August 2013
Available online 9 August 2013

Keywords:
Anaerobic consortia
Pretreatment
Dehydrogenase activity
FISH
Anaerobic sequencing reactors (AnSBR)

ABSTRACT

The effect of different inorganic acids viz., HNO_3, HCl, H_2SO_4 and H_3PO_4 on inoculum pretreatment to selectively enrich hydrogen (H_2) producing acidogenic bacteria was evaluated in anaerobic sequencing batch bioreactors. Relative positive efficiency of HNO_3 pretreated consortia in enhancing H_2 production (11.85 mol H_2/kg COD_R) was noticed compared to other acids (HCl, 5.64 mol H_2/kg COD_R; H_2SO_4, 7.65 mol H_2/kg COD_R; H_3PO_4, 6.90 mol H_2/kg COD_R) and untreated-parent consortia (control, 6.80 mol H_2/kg COD_R). On the contrary, substrate degradation (COD removal) was higher with the control operation (ξ_{COD}, 66.3%; substrate degradation rate (SDR), 1.42 kg COD_R/m^3-day) compared to pre-treated culture. HNO_3 pre-treatment resulted in a shift in the fermentation pathway towards more acetic acid production, while other acid pretreatment and untreated culture showed mixed type fermentation (acetic, butyric, propionic acids). The bio-electrochemical analysis and dehydrogenase activity supported the biocatalyst performance after HNO_3 pretreatment with specific enrichment of *Firmicutes* and *Bacillus*.

1. Introduction

Biological hydrogen (H_2) production via dark-fermentation using wastewater has generated significant attention in recent times (Ren et al., 2007; Venkata Mohan, 2009). Using anaerobic consortia as the biocatalyst for H_2 production from wastewater treatment facilitates operational flexibility, permits diverse biochemical functions, facilitates process stability and provides possibility of using a wider range of substrates/feed-stocks and restricts the need of sterile operation (Ren et al., 2007; Venkata Mohan, 2009; Kannaiah and Venkata Mohan, 2012a,b). Despite the advantages, the major limitation with mixed culture use is the consumption of H_2 produced in the fermentative microenvironment by hydrogenotrophic or H_2 consuming methane-producing bacteria (MB) including homoacetogens, and sulfate reducing bacteria present in mixed consortia (Kraemer and Bagley, 2007; Venkata Mohan, 2009; Kannaiah et al., 2012). Regulating the metabolic process towards acidogenesis by simultaneously inhibiting the H_2 consuming methanogenesis facilitates H_2 to become a metabolic end product (Wang et al., 2011; Kannaiah et al., 2012).

* Corresponding author. Tel.: +91 40 27191664.
E-mail address: vmohan_s@yahoo.com (S. Venkata Mohan).

0960-8524/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.biortech.2013.08.021

For the selective inhibition of H_2 consumers and enrichment of H_2-producing acidogenic bacteria (AB), pretreating the parent culture plays an important role (Kim et al., 2003; Venkata Mohan et al., 2008a,b; Kannaiah and Venkata Mohan, 2012a,b). Pretreatment facilitates a shift in metabolic functions of a mixed biocatalyst toward acidogeneic microenvironment from methanogenesis and simultaneously prevents the function of methanogenic bacteria (MB) without effecting the activity of the H_2-producing bacteria (Luo et al., 2010; Venkata Mohan and Kannaiah, 2012). Physiological differences existing between the AB and MB is the basis for pretreatment procedures (Zhu and Beland, 2006; Mohanakrishna and Venkata Mohan, 2013; Kannaiah and Venkata Mohan, 2012a,b). Among the various pretreatment methods available, acid-shock pretreatment method is widely used to control and regulate H_2 production both during inoculum preparation as well as during process operation (Venkata Mohan, 2009; Moreno-Davila et al., 2010; Venkata Mohan et al., 2008a; Kannaiah and Venkata Mohan, 2012b). Methanogenic activity is generally limited to a relatively narrow pH range, between 6.8 and 7.2, while most acidogenic bacteria producing H_2 can function over a broader pH range (Venkata Mohan et al., 2011a; Moreno-Davila et al., 2010; Venkata Mohan et al., 2008a,c). The acid-shock method suppresses methanogenic activity of the cultures by protecting the spore-forming bacteria and allowing H_2 to become a metabolic end product (Lee et al., 2009; Kannaiah and Venkata Mohan, 2012b). Long term studies (520 days) were reported with acid-shock pretreatment using orthophosphoric acid as redox controller showed enhanced and sustained H_2 production (Kannaiah and Venkata Mohan, 2012b). A shift in the fermentation process towards acetic acid production was observed after applying acid pretreatment, with the dominance of Clostridia and *Bacilli* classes (Wang and Wan, 2009; Liu et al., 2009; Kannaiah and Venkata Mohan, 2012b). The nature of the acid used for pretreatment will play an important role in selectively enriching the AB (Lee et al., 2009). Therefore, an attempt was made in this investigation to evaluate the relative influence of various acids reagents viz., HNO_3, HCl, H_2SO_4 and H_3PO_4 for acid-shock pretreatment of anaerobic consortia regarding both H_2 production and substrate degradation in compared to an untreated parent culture in order to identify the best inorganic acid reagent for acid-shock pretreatment. To comprehensive study the functional role of inorganic acid redox controller's on aciodgenic H_2 production, experiments were designed and performed in anaerobic suspended growth reactor. Relative changes in bioprocesses were evaluated based on H_2 production, substrate degradation and VFA composition along with dehydrogenase (DH) activity, bio-electro kinetics and quantification of the microbial community.

2. Methods

2.1. Acid-shock pretreatment

Anaerobic sludge was collected from an anaerobic bioreactor treating complex wastewater and was used as the parent inoculum. After sampling, the parent culture was sieved to separate the grit using a nylon filter. The resulting sludge was then used for the inoculum preparation as a biocatalyst. Four different inorganic acids viz., HNO_3, HCl, H_2SO_4 and H_3PO_4 with a concentration of 0.1 N were used for pretreating the parent inoculum. Parent inoculum (140 ml) was subjected to acid-shock treatment by adjusting the pH to 3 using each the selected inorganic acids (0.1 N) and for a period 24 h under anaerobic conditions. Subsequently, the pH of acid pretreated inoculum was re-adjusted to 6 using 0.1 N NaOH. Pretreatment operations were performed at room temperature (28 ± 1 °C) with provided continuous mixing by a magnetic stirrer (120 rpm). Untreated inoculum was evaluated as the control to assess the relative efficiency of inorganic acids as acid pretreating agents.

2.2. Experimental methodology

Five identical bench scale anaerobic sequencing reactors (AnSBR) were fabricated with borosilicate-glass to have a total/working volume of 1.2/0.84 L and gas holding capacity of 0.36 L. All the five reactors were operated in suspended growth configuration using sequencing/periodic discontinuous batch mode continuously for 10 cycles, accounting for a total operation period of 20 days with 48 h of retention time [20 min of FILL, 47 h of REACT (anaerobic), 20 min of SETTLE, 20 min of DECANT operations]. The reactors were kept in suspension mode during REACT phase (Sfig 1). At the beginning of each cycle a predefined volume (0.72 L) of wastewater was fed during the FILL phase and contents were subjected to continuous mixing (100 rpm). Each of the bioreactors was inoculated with culture pretested with different organic acids. During the start up phase, each reactor was fed 120 ml of designated inoculum along with 0.72 L of designed synthetic wastewater [DSW; glucose-3 g/L, NH_4Cl-0.5 g/L, KH_2PO_4-0.25 g/L, K_2HPO_4-0.25 g/L, $MgCl_2$-0.3 g/L, $CoCl_2$-25 mg/L, $ZnCl_2$-11.5 mg/L, $CuCl_2$-10.5 mg/L, $CaCl_2$-5 mg/L, $MnCl_2$-15 mg/L, $NiSO_4$-16 mg/L, $FeCl_3$-25 mg/L]. Prior to its operation, the pH of each reactor's contents was adjusted to 6 using 30% orthophosphoric acid. All the bioreactors were operated at ambient temperature (28 ± 2 °C) under similar conditions (OL, 2.14 kg COD/m^3-day; pH 6.0) to study the relative efficiency of H_2 production and substrate degradation in comparison with the control operation. Nitrogen gas was sparged to the bioreactor for 2 min after every feeding and sampling event to maintain anaerobic conditions. The reaction mixture during REACT phase was subjected to continuous mixing (100 rpm).

2.3. Analysis

Biogas volume and composition was monitored via water displacement and gas chromatography respectively. The fraction of H_2 in the biogas was determined by a gas chromatograph (NUCON 5765) using thermal conductivity detector (TCD) with 1/8″ × 2 m Heysep Q column employing nitrogen as carrier gas. The injector and detector were maintained at 60 °C each and the oven was operated at 40 °C isothermally. The pH of the solution was measured using a pH meter. Quantification of the H_2 gas was also carried out using a microprocessor based pre-calibrated H_2 sensor (ATMI GmbH Inc). The concentration and quantitative estimation of VFA was carried out by employing high performance liquid chromatography (HPLC; Shimadzu LC10A) with UV–Vis detector (210 nm) using C18 reverse phase column (250 × 4.6 mm diameter; 5 μ particle size) and 40% acetonitrile (in 1 mN H_2SO_4; pH, 2.5–3.0) as mobile phase with flow rate of 0.6 ml/min; sample injection 20 μl]. The performance of bioreactors was assessed by monitoring chemical oxygen demand (COD-closed refluxing titrimetric method), pH and VFA by the standard methods (APHA, 1998). Dehydrogenase (DH) enzyme activity was estimated by colorimetric procedure based on the reduction of 2,3,5-triphenyltetrazolium chloride (TTC) (Kannaiah and Venkata Mohan, 2012a,b). Cyclic voltammetry (CV) was recorded using potentiostat-galvanostat system (Autolab-PGSTAT12, Ecochemie) by transferring suspension (150 ml) from reactors to three electrode-electrochemical cell [working electrode, platinum wire; counter electrode, graphite rod; reference electrode, Ag/AgCl (S)] using a potential ramp between +0.5 and −0.5 V at a scan rate of 30 mV/s. Tafel analysis was carried out with CV profiles by processing through GPES-4.0 software supplied with CV.

2.4. Fluorescent in situ hybridization (FISH)

Dominance of microbial population was traced by a modified procedure of Fluorescent *in situ* hybridization (FISH) (Amann et al., 1995). The 5'-Cy3 modified oligonucleotide probes with the sequence of (16 S rRNA) and (23S rRNA) were used to the track the *Firmicutes* (pB-00195: 5′-TGG AAG ATT CCC TAC TGC-3′) (Meier et al., 1999), *Bacillus* (pB-01036: 5′- CTT CAG CAC TCA GGT TCG-3′) (Salzman et al., 2002) and *Pseudomonas* (pB-00375: 5′-GCT GGC CTA ACC TTC-3′) (Schleifer et al., 1992) respectively (Stable 1). The probes were purchased from MWG Biotech. Five ml of sample was subjected for three repeated washings (7500 rpm; 5 min; 28 °C) recovered with distilled water for removing the dead cells and contaminants, subsequently was suspended in phosphate-buffered saline (PBS; pH 7.4) for additional washing. The resulting cells were fixed with 4% para-formaldehyde and kept for incubation at 4 ^{0}C for 4 h. The fixed cells were washed twice with PBS and suspended in PBS:Ethanol (1:1) solution stored at −20 °C. Followed by smear preparation, 10 μl of the sample was applied in a well of gelatin-coated glass-side, dried for 1 h at 46 °C and subsequently dehydrated in solutions of 50%, 80% and 96% ethanol each for 3 min. To start hybridization, 9 μl of hybridization buffer and 1 μl of fluorescent labeled rRNA probe (50 ng/μl) were added to the well. Hybridization was carried out for 3 h at 46 °C in a humidified chamber. Following hybridization, washing was carried out using a washing buffer for 20 min at 48 °C. The hybridized bacterial cells were counterstained with 4',6-diamidino-2-phenylindole (DAPI) to observe total bacterial population binding with the A-T rich regions in DNA. The fluorescent stained smears were visualized with an epifluorescent microscope (Nikon Eclipse-80i) at 400X magnification. Photomicrographs were captured with an attached digital camera (YIM-smt, 5.5 mega pixels) and analyzed using NIS-elements software (D3.0). Counting of Cy3-stained bacterial cells was done in a single microscopic field using a Cy3 filter followed by DAPI-stain cell counting (DAPI-filter). The bacterial cell count was done randomly on the selected fields (more than 10) of the smears.

3. Results and discussion

3.1. Biohydrogen

Experimental results illustrated the specific function of acids used for pretreating biocatalyst for both H_2 production as well as substrate degradation. The H_2 production profiles derived from designed experimental operations in comparison with the control (untreated operation) is depicted in Fig. 1a. All the acid-shock pretreated experimental operations depicted higher H_2 production than the untreated anaerobic culture (inocolum/control). However, H_2 production rates varied based on the type of acid used for pretreating the biocatalyst. Among the experimental variations, HNO_3 pretreated culture documented higher H_2 production, 4.25 (5th cycle) times higher than that of the control operation. During the last cycle of operation, HCl pretreated biocatalyst also showed higher H_2 production efficiency (3.6 fold higher than the control's; Cycle 10). H_3PO_4 and H_2SO_4 pretreated culture showed 2.1 (Cycle 4) and 1.8 (Cycle 2) times higher H_2 production than the control operation's. Based on the cumulative H_2 production (CHP), the efficacy of the acid-shock pretreated culture, in descending order was HNO_3 > HCl > H_3PO_4 > H_2SO_4 > Control. When the CHP profiles were analyzed individually, the pattern showed a distinctive variation as a function of cycle operation. Operation with HNO_3 pretreated culture showed a rapid improvement and approached higher CHP during the 3rd cycle of operation, stabilizing thereafter. In the case of H_2SO_4 and H_3PO_4 pretreated-culture, the CHP showed rapid increment up to 2nd and 4th cycles of operation respectively with a rapid fall prior to stabilization from the 6th cycle onwards. On the contrary, HCl pretreated-consortia gradually approached towards higher H_2 production until 10th cycle of operation. Control operation showed initially higher H_2 production which dropped gradually and stabilized after 6th cycle at lower values.

During fermentation of organics, heterotrophic bacteria produce electrons which have to be disposed off, in order to sustain their electrical neutrality. In aerobic metabolism O_2 acts as the electron (e^-) acceptor. In anaerobic fermentation, other electron acceptors are necessary, where protons (H^+) act as an electron acceptors and get reduced to molecular H_2. Reduction equivalents, such as formate, reduced ferredoxin and NADH functions as electron donors. Dehydrogenase (DH) activity signifies the viability of bacteria with the function of metabolic activity. Redox reactions for the inter-conversion of metabolites and transferring protons (H^+) between metabolic intermediates are catalyzed by the (DH) enzyme using several mediators (NAD^+, FAD^+, etc.). These redox mediators are capable of carrying H^+ and electrons (e^-) and are known as energy carriers because they are involved in biological energy generation (ATP). DH activity of the biocatalyst showed a marked improvement after applying acid-shock pretreatment indicating the increased redox inter-conversion reactions leading to the higher proton gradient in the cell that may result in higher H_2 production (Fig. 1b). DH activity correlated well with the H_2 production profiles. It was found throughout the experiment that DH activity synchronized H_2 production. Similar to H_2 production, HNO_3 pretreated consortia showed high DH activity (3.56 μg/ml of Toulene; Cycle 10) followed by HCl (3.16 μg/ml of Toulene; Cycle 10), H_3PO_4 (2.89 μg/ml of Toulene; Cycle 10), control (1.66 μg/ml of Toulene; Cycle 10) and H_2SO_4 (1.5 μg/ml of Toulene; Cycle 10) operations correlating with H_2 production. HNO_3 treated culture showed higher DH activity throughout the operation. Dehydrogenase activity of the HNO_3 pretreated biocatalyst got stabilized towards increased proton shuttling between metabolic intermediates leading to higher H_2 production.

Biogas composition analysis documented the dominance of H_2 over methane with the acid-shock pretreated operation due to the selective enrichment of specific H_2 producing bacteria (Fig. 1c). HNO_3 pretreated operation documented a higher fraction of H_2 (41–46%) in biogas followed by HCl (27–42%), H_3PO_4 (28–36%) and H_2SO_4 (18–26%). On the contrary, control operation showed higher fraction of methane (36–41%) than H_2 (13–21%) in the biogas indicating metabolism more inclined towards methanogenesis presumably due to the co-existence/domination of MB. Among all the pretreated operation, co-existence of methane was more with the H_2SO_4 operation (26–33%).

3.2. Acid metabolites Vs redox microenvironment

3.2.1. VFA concentration

Dark-fermentation process associates with metabolic conversion of organic substrate to H_2 along with acid metabolic intermediates i.e. volatile fatty acids (VFA). During the first six cycles of operation, all the experimental variations documented inconsistencies in VFA production pattern, varying between 950 and 1400 mg/l (Fig. 2a). A higher concentration of VFA production was observed during the initial cycle (2 and 3 rd cycles) operation irrespective of the experimental variations studied. The untreated culture showed rapid VFA synthesis and attained the highest concentration of 1380 mg/l (3rd cycle) prior to a rapid drop before stabilizing at 8th cycle (1195 mg/l). Similar to the control operation, H_3PO_4 pretreated culture followed the same trend but with lesser VFA concentration (1380 mg/l; cycle 3) prior its stabilization at the 8th cycle relatively higher VFA concentration (1210 mg/l).

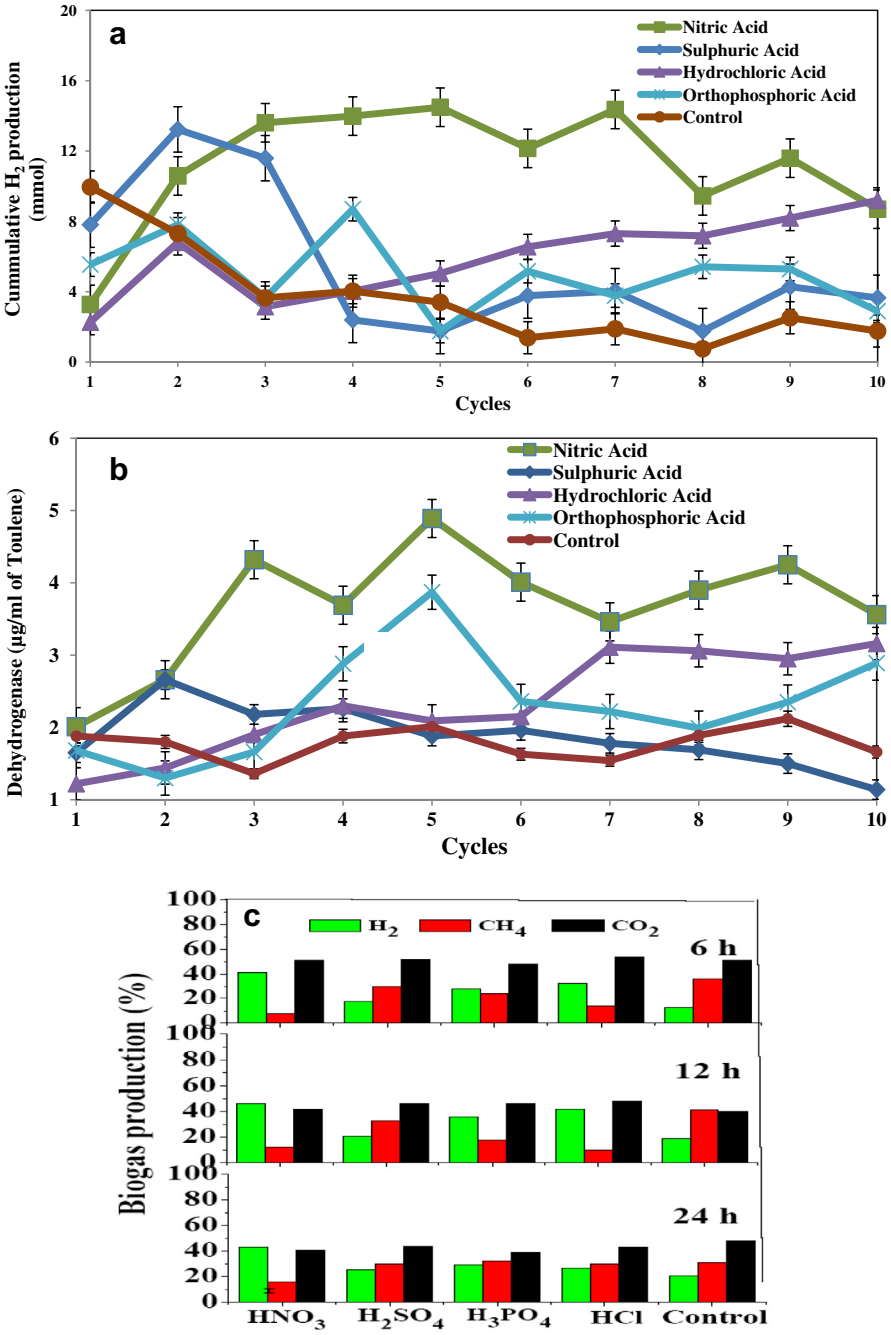


Fig. 1. (a) Cumulative H_2 production of different inorganic acid pretreated and untreated (control) anaerobic inoculum with the function of reactor operation time. (b) Dehydrogenase activity of different acid and untreated (control) anaerobic inoculum with the function of reactor operation time. (c) Percentage of H_2 production analysed through gas chromatography.

HNO_3 pretreated biocatalyst approached a peak during the 2nd cycle in VFA concentration (1260 mg/l) followed by a sharp drop (next two cycles) and approached stabilized higher concentration at 6th cycle (1280 mg/l). Next to HNO_3 culture, HCL pretreated biocatalyst showed higher VFA concentration during stabilized phase of operation (cycle 10, 1263 mg/l). H_2SO_4 pretreated culture showed low VFA production (1102 mg/l; 10th cycle) during stabilized phase of operation, even compared to the control operation (1198 mg/l; 10th cycle). During the stabilized phase of operation, the VFA concentration varied in a narrow range (1150–1300 mg/l). Higher VFA production in the case of HNO_3 and HCl demonstrates the effective functioning of the acidogenic metabolic process towards higher H_2 production.

3.2.2. VFA profile

The composition and concentration of VFA showed distinct variation based on the nature of the biocatalyst used (Fig. 2b). All the experimental variations showed the presence of acetic acid, butyric acid and propionic acid with variable concentrations. Acetic acid was found in major fraction compared to butyrate and propionate in acid pretreated cultures than untreated biocatalyst. Acetate composition showed an increase with time in all the experimental conditions except in the case of the HCl and control operations. HNO_3 pretreated operation showed higher acetate production (57.2%), followed by HNO_3 (38.2%), H_3PO_4 (43.8%) and H_2SO_4 (32.7%). The control operation documented lower acetic acid production (16.9%) followed by butric acid (10%) and propoinic acid (8.6%).

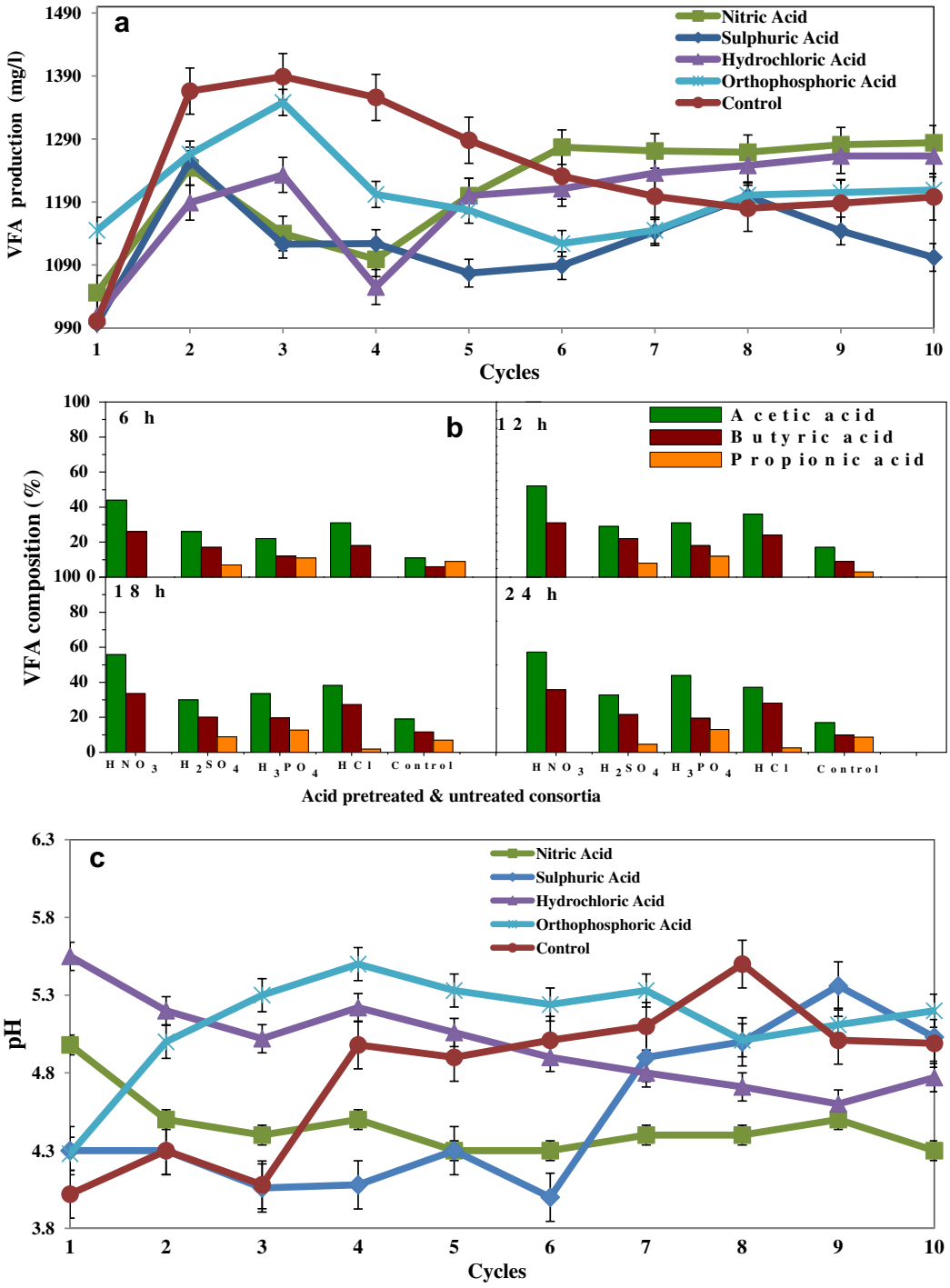


Fig. 2. (a) VFA production of different inorganic acid pretreated and untreated inoculum at the end of the cycle. (b) VFA composition analysis showing production in acetic acid, butyric acid, propionic acid in reaction from 6th h to 24th h. (c) pH profile of acid pretreated and untreated culture at the end of the cycle.

Based on the metabolic pathway used by the microorganism and the corresponding end-products, H_2 yields were variable. Highest theoretical yield of four moles of H_2 per mole of glucose can be obtained when acetate is the fermentation end-product (Venkata Mohan and Kannaiah, 2012). Higher acetic acid synthesis in acid treated cultures corobrated well with the H_2 production observed. Along with acetate, the butyrate fraction was also found more in acid pre-treated cultures than the control operation. Theoretically, along with butyrate, two moles of H_2 can be generated per mole of glucose. The co-existence of butyrate with acetate indicates suitable microenvironment for acidogenic fermentation. Higher butyric acid concentration was also observed with the HNO_3 operation. The absence of propionate in HNO_3 and presence of very less composition in HCl both also provide a positive microenvironment towards acidogenic H_2 generation. The propionic acid pathway demands additional two moles of H_2 (Mohanakrishna and Venkata Mohan, 2013). The presence of relatively higher concentration of propionate observed with H_3PO_4, H_2SO_4 and control operations might be probable reason for less observed H_2 production. Variation in the VFA production profile with different pretreated operations suggested changes in microbial composition and metabolic shift of the biocatalyst. The acidogenic effluents of H_2 production process

were used as the main substrate for producing of biodiesel by microalgae (Venkata Mohan and Prathima, 2012) and bioplastics through microaerophilic operation (Venkateswar Reddy et al., 2012; Venkateswar Reddy and Venkata Mohan, 2012).

3.2.3. System pH

System pH plays a crucial role in carrying out the metabolic reactions in all know living systems. The accumulation of VFA observed during acidogenic fermentation in all the experimental variations, studied effects the system redox microenvironment. More acidic microenvironment prevailed during HNO_3 (pH 4.3) operation followed by HCl (pH 4.7), H_2SO_4 (pH 5.03) and H_3PO_4 (pH 5.2) due to the production of metabolic acid intermediates (Fig. 2c). Prevailing acidic microenvironments corroborated well with the VFA composition profiles. The control operation also documented shift towards acidic redox microenvironment (pH, 5.0), but with a relatively less degree due to less acid metabolites generation. Highly acidic redox conditions (less than pH 4) are generally unfavorable for H_2 production as they inactivate H_2 producing bacteria. The rapid depletion of pH will cause a metabolic alteration of the microorganisms resulting in the shift of intermediates production pathway with consequent decrease in H_2 production (Lee et al., 2009). Depending on organism and growth conditions, changes in external pH can bring about subsequent alterations in several primary physiological parameters, including internal pH, concentration of other ions, membrane potential and proton-motive force.

3.3. Substrate degradation

Substrate degradation during H_2 production was evaluated by estimating substrate (as COD) removal efficiency (ξ_{COD}) and substrate degradation rate (SDR). These experiments documented a marked variation between the pretreated and untreated biocatalysts (Fig. 3a). Contrary to H_2 production, substrate degradation was effective with untreated consortia due to the presence of homoacetogens, sulfate reducing bacteria (SRB) and MB. Untreated inoculum showed relatively higher substrate degradation efficiency (ξ_{COD}, 66.30%; SDR, 1.42 kg COD_R/m^3-day) compared to acid-pretreated biocatalysts, which might be attributed due to the co-existence of methanogenic bacteria (MB). On the contrary, all the acid-pretreated operations registered low substrate degradation efficiency, wherein, H_2SO_4 operation depicted higher degradation (ξ_{COD}, 66.2%; 1.41 kg COD_R/m^3-day) followed by HCl (ξ_{COD}, 59.9%; 1.28 kg COD_R/m^3-day), H_3PO_4 (ξ_{COD}, 61.13%; 1.30 kg COD_R/m^3-day) and HNO_3(ξ_{COD}, 56.66%; 1.21 kg COD_R/m^3-day) (Fig. 3b). Lower substrate degradation observed with HNO_3 pre-treated biocatalyst reiterates the higher H_2 production due to the elimination of MB. Elimination of MB restricts the further substrate degradation towards methane and therefore lower SDR was noticed with higher H_2 yield (Srikanth et al., 2010; Luo et al., 2010). An untreated culture has higher bacterial population with a wide variety of biochemical functions facilitating diverse metabolic activities. On the contrary, pretreating the biocatalysts allows for the selective enrichment of bacterial population specific towards acidogenesis with relatively less diversity (Kannaiah and Venkata Mohan, 2012a,b). Higher specific H_2 yield (SHY) calculated during 10th cycle of operation showed higher yield with HNO_3 (6.64 moles/kg COD_R) followed by HCl (4.95 moles/kg COD_R), H_2SO_4 (2.42 moles/kg COD_R), H_3PO_4 (1.8 moles/kg COD_R) and control (1.03 moles/kg COD_R) operations (Fig. 3c).

3.4. Bacterial community structure

3.4.1. Gram Staining

In this study, the gram staining technique was used as a tool or a marker to enumerate the shift/distribution in the bacterial community during H_2 production. Interestingly, Gram staining illustrated distinct changes in the community in correlation to experimental variation studied. The dominance of gram positive bacteria was evidenced in the HNO_3 treated operation (2.1×10^5 cells/ml), which yielded higher H_2 production (Tables 1 and 2). The total number of gram positive bacteria (2.1×10^5 cells) was two folds higher than the gram negative bacteria (0.12×10^5 cells/ml). Gram positive bacteria is a bacteria that includes *Firmicutes* generally associated with H_2 producing *Bacillus* and *Clostridia* classes. Many of these species also have the ability to form endospores which are able to tolerate various stress conditions viz., high temperature, extreme pH, nutrient stress, etc. and revert when normal conditions regained back. In the case of HCl pre-treated culture (1.06×10^5 cells/ml; 1.1×10^5 cells/ml) and control (1.01×10^5 cells/ml; 1.16×10^5 cells/ml) both gram positive and gram negative bacteria are more or less the same. On the contrary, H_2SO_4 operation showed lower gram positive bacterial cells (0.89×10^5 cells/ml) than the gram negative bacteria (1.24×10^5 cells/ml) which also yielded less H_2 production compared to other pre-treatment strategies. This trend was noticed with control operation in the distribution between gram positive (1.16×10^5 cells/ml) and gram negative bacterial cells (1.16×10^5 cells/ml).

3.4.2. Diversity quantification using FISH

The phylogenetic compositions of bacterial communities in different acids pretreatment were analyzed by fluorescent *in situ* hybridization (FISH) (Amann et al., 1995). FISH facilitates phylogenetic analysis as well as provides the quantitative abundance of the bacteria. Phylum and genus specific Cy3 labeled probes targeted to 16S rRNA sequences of *Firmicutes* (phyla), *Pseudomonas* (genus) and 23S rRNA sequences of *Bacillus* (genus) were used in this study. Simultaneously total bacterial population count was done by counterstaining the smear with a DAPI stain which served to differentiate the specific bacteria among the total bacterial population. The distribution of the bacterial community after acid-shock pretreatment induced significant changes (Sfig 2). Direct cell counting of microscopic images revealed that Gram-positive bacteria (*Firmicutes*) were frequently detected in all the acid-shock pretreated cultures associated with *Bacillus* as prevailing genus. However, based on FISH counting, the abundance of the probe pB-00195-hybridized *Firmicutes* group accounted for 81.1% of the total cells, which was higher than that of probe pB-01036-hybridized *Bacillus* and probe pB-00375-hybridized *Pseudomonas* genus. HNO_3 acid pre-treated application affected the species composition of the microbial communities significantly by enriching potentially diverse microflora compared to other acids and control, especially pertaining to H_2 production. HNO_3 pretreated inoculum showed highest population of bacteria belonging to phylum *Firmicutes* (81.1%) along with *Bacillus* (61.9%) with relatively less fraction of *Pseudomonas* (14.7%). More or less a similar trend was observed with HCl where *Pseudomonas* showed higher number of bacterial population compared to HNO_3 pretreated inoculum (*Firmicutes*, 50.7%; *Bacillus*, 64.3%; *Pseudomonas*, 58.8%). H_3PO_4 (*Firmicutes*, 51.7%; *Bacillus*, 30.8%; *Pseudomonas*, 84.7%) and H_2SO_4 (*Firmicutes*, 60.7%; *Bacillus*, 30.0%; *Pseudomonas*, 79.2%) pretreatment, the florescence intensity was very low when no significant bio-H_2 production was observed, presumably because the cells in the inoculum were damaged by the strong acid pretreatment. on the contrary, untreated culture (control) showed relatively less bacterial population (*Firmicutes*, 18.8%; *Bacillus*, 20.3%; *Pseudomonas*, 30%) which is correlating well with the substrate degradation (COD removal) rates of the control.

Type of acid used for pre-treating parent inoculum showed marked influence on the community structure and collaborates well with the H_2 production data as well. Higher fraction of gram positive bacteria also correlates well with selective enrichment

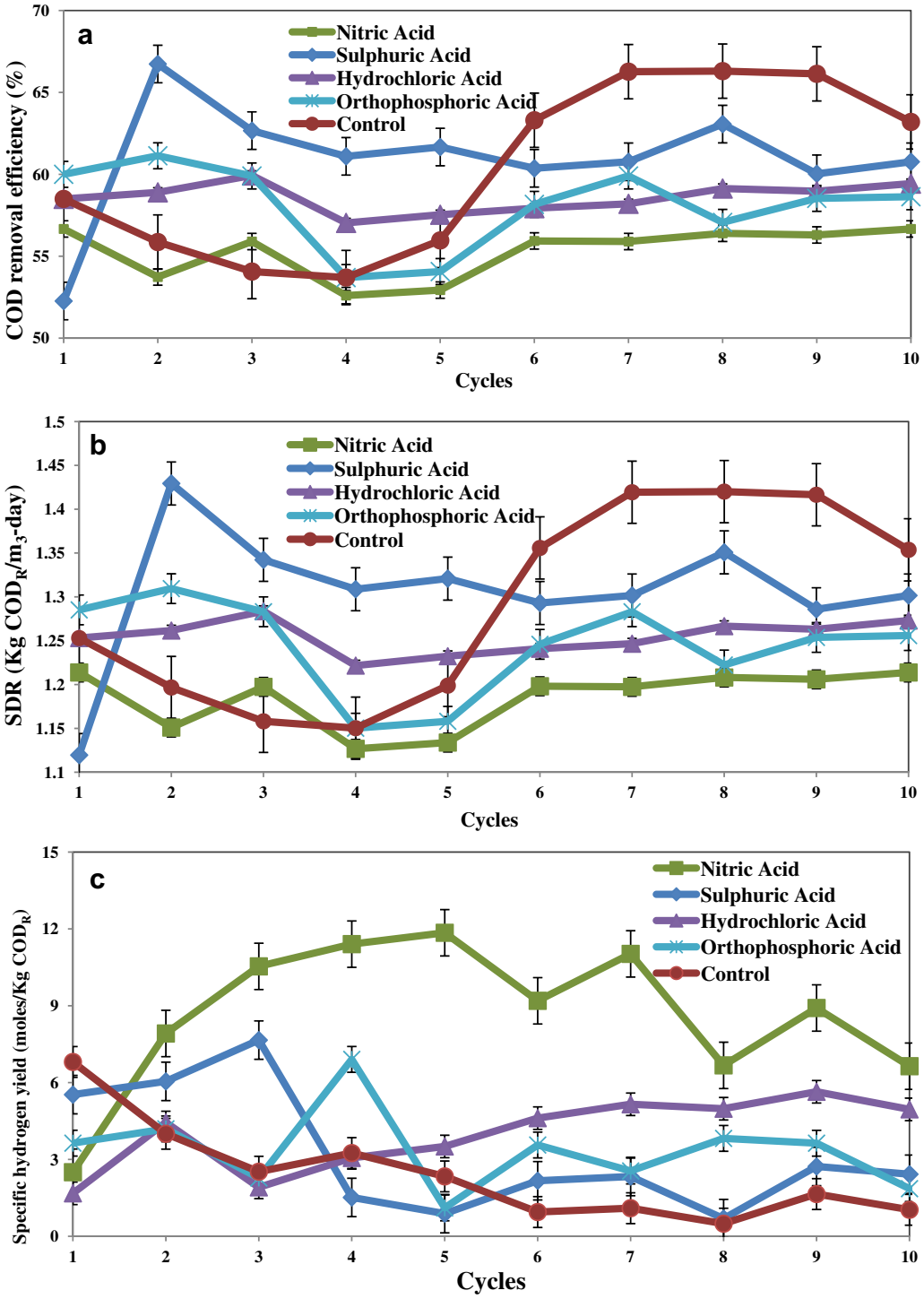


Fig. 3. (a) Substrate degradation pattern as COD removal efficiency (b) substrate degradation rate of different inorganic acids pretreated and untreated (control) anaerobic inoculum with the function of reactor operation time. (c) Specific hydrogen yield (SHY) of different inorganic acids pretreated and untreated (control) inoculums with the function of reactor operation time.

Table 1
Gram staining of the pretreated consortia showing the gram positive and gram negative bacterial population count.

Total population count	HNO_3	HCl	H_3PO_4	H_2SO_4	Control
Gram +ve bacteria (ml^{-1})	2.1×10^5	1.06×10^5	1.22×10^5	0.89×10^5	1.01×10^5
Gram −ve bacteria (ml^{-1})	0.12×10^5	1.1×10^5	1.08×10^5	1.24×10^5	1.16×10^5
Ratio of Gram −ve/Gram +ve	0.05	1.04	0.88	1.39	1.15
Ratio of Gram +ve/Gram −ve	17.5	0.96	1.13	0.72	0.87

Table 2
Total count of the bacterial population with DAPI and Cy3.

Total microbial count	*Firmicutes* (per ml)		*Bacillus* (per ml)		*Pseudomonas* (per ml)	
	DAPI	Cy3	DAPI	Cy3	DAPI	Cy3
HNO_3	0.21×10^5	0.13×10^5	0.37×10^5	0.30×10^5	0.34×10^5	0.05×10^5
HCl	0.75×10^5	0.38×10^5	0.56×10^5	0.36×10^5	0.17×10^5	0.10×10^5
H_3PO_4	0.42×10^5	0.24×10^5	0.26×10^5	0.08×10^5	0.19×10^5	0.16×10^5
H_2SO_4	0.28×10^5	0.17×10^5	0.20×10^5	0.06×10^5	0.24×10^5	0.19×10^5
Control	0.84×10^5	0.09×10^5	0.75×10^5	0.38×10^5	0.20×10^5	0.06×10^5

observed in this study. Change in the diversity of microorganisms also infers change in the size and composition of the population. In the case of the control operation, none of the studied bacterial groups were dominant. This might be due to the greater diversity of the population containing both the producers and utilizers of H_2. Bacteria belonging to *Firmicutes*, especially *Bacillus* and *Clostridia* are spore formers and well known potential H_2 producers (Esteso et al., 1996; Robert et al., 2007; Karakashev et al., 2009), which might have survived the harsh acidic conditions by forming endospores. *Firmicutes* grow in acidogenic microenvironment (between pH 4.5 and 6.0) and form endospores in adverse conditions (Myoung et al., 2009; Venkata Mohan et al., 2011b). Under acidic and high temperature conditions, the cells of *Firmicutes* undergo desiccation and forms spores (Robert et al., 2007; Thong et al., 2008; Kannaiah et al., 2012). Microorganisms which are not capable of spore forming (mostly probably methanogens) get eliminated from the parent culture after the application acid-shock pretreatment. *Bacilli* species viz., *B. Subtilis*, *B. licheniformis* and *B. Coagulans* were reported to be capable of producing H_2. Clostridia species viz., *C. butyricum, C. Kluyveri* and *C. pasteurianum* (Esteso et al., 1996), *C. acetobutylicum* (Esteso et al., 1996; Karakashev et al., 2009) were also reported to be capable of producing H_2 effectively.

3.5. Bio-electrochemical activity

Bio-electrochemical behavior of the biocatalyst in terms of oxidation and reduction catalytic currents was studied by employing cyclic voltammetry (CV) (Rabaey et al., 2004; Raghavulu et al., 2012). Voltammograms (Vs Ag/AgCl (S) visualized distinct variations in biocatalyst behavior between acid-shock pretreated and control operation (Fig. 4). Acid-shock pretreated biocatalysts recorded higher redox catalytic currents than the control operation. Voltammograms recorded with untreated biocatalyst visualized simultaneous redox catalytic currents with a marginal inclination towards reduction behavior (oxidative catalytic current (OC): 0.02 ± 0.08 μA; reductive catalytic current (RC): -0.05 ± 0.10μA). Redox currents remained more or less similar during the operation. Though the reduction currents were marginally higher, lesser H_2 production was observed as the electron and proton losses will be higher due to the multiple metabolic interactions within the diverse microflora that would have drifted the liberated redox equivalents to get scavenged by MB as an electron sink or the shift in pathway towards methane. Reversible peaks were visualized on the voltamogram at 6 h and 12 h with peak potentials of 0.282 mV and 0.403 mV corresponding to cytochrome-c and nitrate reductase respectively. Cytochrome-c is a mobile carrier

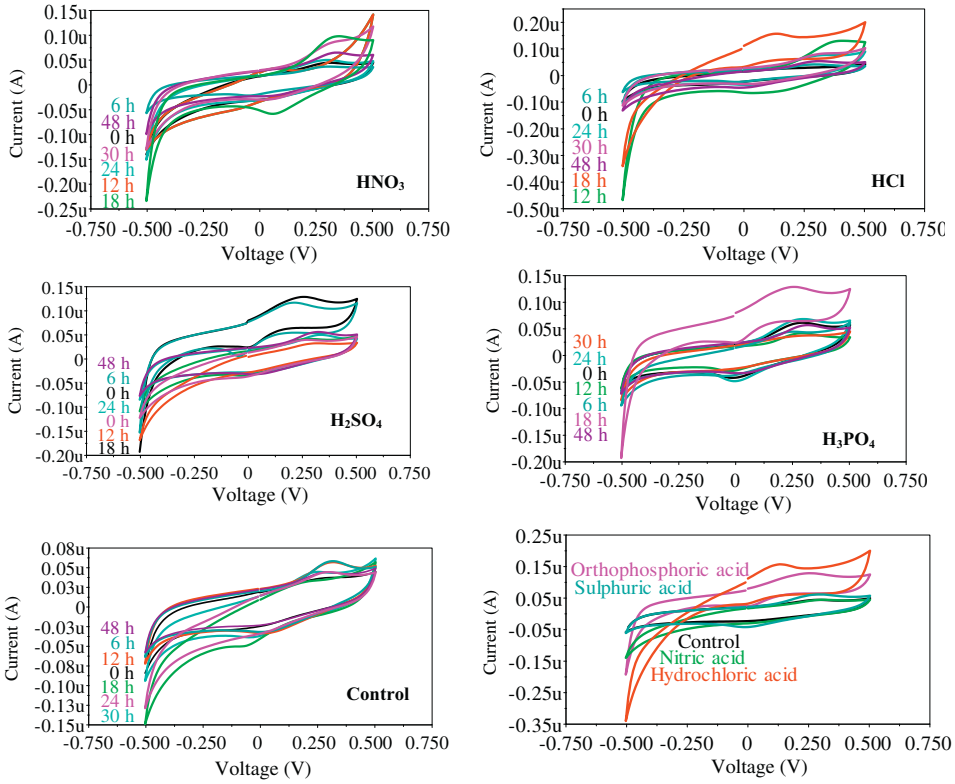


Fig. 4. Cyclic voltammograms profile of different inorganic acids pretreated and untreated inoculums with the function of operation time, voltammograms of HNO_3, HCl, H_2SO_4, H_3PO_4 and control.

protein aiding in the electron transfer which is found to be associated with almost all kinds of bacterial species. As the control operation includes diverse microflora, nitrate reductase detected during the 12 h would have been involved in the metabolism of nitrifying bacteria. Nitrate reductase (assimilatory nitrogen reductase) is a mononuclear Mo-containing enzyme, which reduces nitrate (NO_3^-) to nitrite (NO_2^-) using NADH + H^+ as source of reducing equivalents (Gonzalez et al., 2006).

Among the pre-treated operations, the HNO_3 biocatalyst showed higher redox currents followed by HCl, H_3PO_4, H_2SO_4 and control operations, which was in line with the H_2 production data and DH enzyme activity. A significant change in biocatalyst behavior towards reduction was observed after applying of acid-shock pretreatment. All the acid pre-treated biocatalysts showed the typical tendency towards reduction contributing for the reduction of available free protons [H^+] which can be correlated with the observed higher H_2 production. Relatively, higher redox catalytic currents were observed with HNO_3 operation during 6 h (OC: 0.08 μA; RC: −0.24 μA) with a reversible peak (0.292 mV) attributing to the participation of cytochrome-c during both oxidation and reduction. RC (−0.10 ± 0.15 μA) was relatively higher than OC (0.05 ± 0.10 μA) depicting the influence of the pretreatment method in enriching the acidogens towards the reduction of available free protons which resulting in higher H_2 production. Distinct reversible peaks were recorded on the voltammograms during 8 h and 48 h with peak potentials of 0.322 mV and 0.262 mV corresponding to the participation of ferricyanide and cytochrome-c respectively. The involvement of ferricyanide as the mediator would have favored more H_2 production because of its capability of neutralizing the liberated electrons with the shuttling free protons as it is a strong oxidizing agent (reduction participant).

In the case of the HCl pre-treated biocatalyst, simultaneous redox currents were observed all through the operation (OC: 0.08 ± 0.12; RC: 0.10 ± 0.35 μA) except for a marked increment during 12 h (0.34 μA) and 18 h (0.46 μA) in reduction followed by oxidation (12 h: 0.20 μA; 18 h: 0.09 μA) respectively. The increment in redox currents recorded corroborated well with the H_2 production profiles where the DH activity for the proton shuttling as well as their reduction towards H_2 was also higher. Reversible peaks were detected during 12 h and 30 h of operation with peak potentials of 0.262 mV and 0.322 mV analogous to the involvement of cytochrome-c and ferricyanide. The redox catalytic currents of H_3PO_4 and H_2SO_4 biocatalysts were lower compared to the other acid-shock treatments. H_3PO_4 operation documented almost similar catalytic currents throughout the operation with a minimum variation. A marked increment in both reduction as well as oxidation currents was recorded during 18 h (OC: 0.13 μA; RC: 0.16 μA) which correlated well with the DH activity. Quasi reversible peaks which can participate either in oxidation or reduction were detected during 12 h and 30 h during oxidation with a potential of 0.191 mV and 0.292 mV attributing to the involvement of cytochrome-c. In the case of H_2SO_4, the redox catalytic currents (OC: 0.04 ± 0.03 μA; RC: 0.08 ± 0.07 μA) remained with a minimal variation throughout the operation. Quasi reversible peaks were detected during reduction (18 h) and oxidation during (24 h and 30 h) with peak potentials of −0.04 mV, 0.04 mV and 0.031 mV contributing to the involvement of fumerate respectively. Fumerate acts as final electron acceptor in few anaerobic bacteria where it gets reduced to succinate by fumerate reductase (Iverson et al., 1999). Reduced menaquinone acts as donor of reducing equivalents.

3.5.1. Bio-electro kinetics

Bio-electrokinetic behavior of the biocatalyst can be studied using kinetic parameters in the form of Tafel slopes and polarization resistance (R_p) derived from semi-empirical Tafel Equations (Raghavulu et al., 2012; Kannaiah and Venkata Mohan, 2012b) where, i represents current (A), E is applied voltage (V), β_a is the oxidative Tafel slope ($\alpha_a nF/RT$), and β_c is the reductive Tafel slope ($\alpha_c nF/RT$). This equation simplifies the kinetics of electron transfer process controlled by two parameters viz., exchange current density (i_0) and redox Tafel slopes (β_a, β_c). Tafel plots also provide a visual understanding of the losses present in the system, which helps to interpret the biocatalytic activity based on the derived kinetic parameters, oxidative Tafel slope (β_a), reductive Tafel slope (β_c) and polarization resistance (R_p in Ω). Tafel slopes were generated using the voltammograms recorded with all experimental variations during different time intervals (Sfig 3). Lower redox Tafel slopes along with lower R_p were observed with pretreated biocatalyst compared to control operation.

Oxidation slopes were comparatively lower than the reduction slopes depicting a dominance of oxidation reactions over reduction reactions. The oxidation slope (β_a) varied in a similar pattern with all the pretreatment methods studied. Initially, the slopes were 0.4 ± 0.2 V/dec which showed increase followed by decrease untill the end of operation with all the experimental conditions studied. A sudden increment in slope was noticed in the control operation after 24 h (1.39 V/dec) and can be attributed to the shift in the pathway towards methanogenesis. Comparatively, lower and consistent oxidation slopes were recorded with HNO_3 operation (0.018 V/dec) that was in correlation to the observed higher H_2 profiles. The specifically enriched acidogens might minimally contribute to the losses in the system. The oxidation slopes in all the acid-shock pre-treated operations including control showed an increment by the end of operation indicating the occurrence of less oxidation reactions possibly due to exhaustion of substrate which subsequently requires higher activation energy for the electron transfer from the biocatalyst towards the working electrode.

Lower reduction slope (βc) was observed with HNO_3 (0.223 V/dec) pre-treatment biocatalyst followed by the control operation (0.226 V/dec), H_3PO_4 (0.314 V/dec) and HCl (0.335 V/dec), H_2SO_4 (0.245 V/dec). This pattern might be attributed to the requirement of less activation energy by the HNO_3 treated biocatalyst followed by the respective acid treated operations and control indicating reduction of the protons towards effective H_2 production. An higher reduction slope was observed during 12 h in the case of all the pretreatment methods (H_3PO_4: 0.768 V/dec; HCl: 0.732 V/dec; H_2SO_4: 0.711 V/dec; HNO_3: 0.676 V/dec; Control: 0.555 V/dec) while the increment was significant with H_3PO_4 and HCl indicating less number of reduction reactions, possibly due to relatively less availability of the free protons visualized also with the lower DH activity. Though higher reduction (lower βc) was observed with control operation, less H_2 production was achieved which correlates well with the electron loss or the pathway towards methanogenesis.

The resistance for the electron transfer from the biocatalyst at the solution electrode interface can be understood through polarization resistance (R_p) derived from Tafel analysis (Wang et al., 2011). At lower resistance electrons mobility will be higher to get reduced with a proton facilitating H_2 formation. Lower R_P was recorded with HNO_3 (1.87 KΩ) pre-treated biocatalyst followed by HCl (6.7 KΩ), H_2SO_4 (33.2 KΩ), H_3PO_4 (37.0 KΩ) and control (37.0 KΩ). This indicates that the resistance offered by the enriched acidogenic bacteria to transfer electrons by HNO_3 operation was comparatively less than the other acid-shock pretreated operations and control towards effective H_2 production by controlling the electron transfer kinetics. The control operation contains diverse species; the probability to undergo many possible metabolic interactions has higher electron losses subsequently resulting in the less transfer of redox equivalents. Electro kinetic evaluation enumerated that the acid-shock pretreated biocatalyst has specific functions over the control operation by controlling the kinetics of

electron transfer as well as the redox reactions by the minimization of activation losses to succeed over the thermodynamic barrier towards effective H_2 production. The viability of acid-shock pretreated biocatalyst towards H_2 production was also visible by the selective enrichment of H_2 producers viz., *Firmicutes* and *Bacillus*.

4. Conclusions

Performance of selectively enriched anaerobic consortia by different inorganic acids in comparison with an untreated parent culture showed marked variation in H_2 production and wastewater treatment efficiency. The study documented the positive influence of HNO_3 pretreated biocatalyst on H_2 production. Dehydrogenase activity improved the after application of acid pretreatment, indicating increased redox inter-conversion reactions leading to a higher proton gradient in the cell. Higher acetate production observed in acid pretreated cultures coraborated well with the H_2 production. VFA production suggested shifts in the metabolic activity of the biocatalyst, microbial community indicated selective enrichment of *Firmicutes* and *Bacillus* sp. after pre-treatment application.

Acknowledgements

The authors wish to thank the Director, CSIR-IICT for the kind support and encouragement received in carrying out this work. Funding from Ministry of New and Renewable Energy (MNRE, Project No. 103/131/2008-NT) and CSIR in the form of XII five year plan project SETCA (CSC-0113) are gratefully acknowledged.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2013.08.021.

References

Amann, R.I., Ludwig, W., Schleifer, K.H., 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol. Rev. 59 (1), 143–169.

APHA, 1998. Standard Methods for the Examination of Water and Wastewater, 20th ed. Washington D.C.

Esteso, M.A., Estrella, C.N., Podesta, J.J., 1996. Evaluation of the absorption on mild steel of hydrogen evolved in glucose fermentation by pure cultures of *Clostridium acetobutylicum* and *Enterobacter*. Sens. Act. B Chem. 32 (1), 27–31.

Gonzalez, P.J., Correia, C., Moura, I., Brondino, C.D., Moura, J.J.G., 2006. Bacterial nitrate reductases: molecular and biological aspects of nitrate reduction. J. Inorg. Biochem. 100 (5–6), 1015–1023.

Iverson, T.M., Chavez, C.L., Cecchini, G., Rees, D.C., 1999. Structure of the *Escherichia coli* fumarate reductase respiratory complex. Science 284, 1961–1966.

Kannaiah, Goud, Venkata Mohan, S., 2012a. Regulating biohydrogen production from wastewater by applying organic load-shock: Change in the microbial community structure and bio-electrochemical behavior over long-term operation. Int. J. Hydrogen Energy 37 (23), 17763–17777.

Kannaiah, Goud, Venkata Mohan, S., 2012b. Acidic and alkaline shock pretreatment to enrich acidogenic biohydrogen producing mixed culture: long term synergetic evaluation of microbial inventory, dehydrogenase activity and bio-electro kinetics. RSC Adv. 15, 6336–6353.

Kannaiah, Goud, Raghavulu, S.V., Mohanakrishna, G., Naresh, K., Venkata Mohan, S., 2012. Predominance of Bacilli and Clostridia in microbial community of biohydrogen producing biofilm sustained under diverse acidogenic operating conditions. Int. J. Hydrogen Energy 37 (5), 4068–4076.

Karakashev, D., Kotay, S.M., Trably, E., Angelidaki, I., 2009. A strict anaerobic extreme thermophilic hydrogen-producing culture enriched from digested household waste. J. Appl. Microbiol. 106 (3), 1041–1049.

Kim, J., Park, C., Kim, T.H., Lee, M., Kim, S., Kim, S., Wook, L.J., 2003. Effects of various pretreatmentsfor enhanced anaerobic digestion with waste activated sludge. J. Biosci. Bioeng. 95 (3), 271–275.

Kraemer, J.T., Bagley, D.M., 2007. Improving the yield from fermentative hydrogen production. Biotechnol. Lett. 29 (5), 685–695.

Lee, M.J., Song, J.H., Hwang, S.J., 2009. Effects of acid pre-treatment on bio-hydrogen production and microbial communities during dark fermentation. Bioresour. Technol. 100 (3), 1491–1493.

Liu, H., Wanga, G., Zhu, D., Pan, V., 2009. Enrichment of the hydrogen producing microbial community from marine intertidal sludge by different pretreatment methods. Int. J. Hydrogen Energy 34 (24), 9696–9701.

Luo, G., Xie, L., Zou, Z., Zhou, Q., Wang, J.Y., 2010. Fermentative hydrogen production from cassava stillage by mixed anaerobic microflora, effects of temperature and pH. Appl. Energy 87 (12), 3710–3717.

Meier, H., Amann, R., Ludwig, W., Schleifer, K.H., 1999. Specific oligonucleotide probes for in situ detection of a major group of gram-positive bacteria with low DNA G+C content. Syst. Appl. Microbiol. 22 (2), 186–196.

Mohanakrishna, G., Venkata Mohan, S., 2013. Multiple process integrations for broad perspective analysis of fermentative H_2 production from wastewater treatment. Appl. Energy 107, 244–254.

Moreno-Davila, I.M.M., Rios-GonZalez, L.J., Gaona-Llozano, J.G., Garza-Garcia, Y., Rodriguez-Martinez, J.G., 2010. Biohydrogen production by anaerobic biofilms from a pretreated mixed microflora. Appl. Sci. 5 (6), 376–382.

Myoung, J.L., Hyeon Song, J., Sun-Jin, H., 2009. Effects of acid pre-treatment on bio-hydrogen production and microbial communities during dark fermentation. Bioresour. Technol. 100, 1491–1493.

Rabaey, K., Boon, N., Siciliano, S.D., Verhaege, M., Verstraete, W., 2004. Biofuel cells select for microbial consortia that self-mediate electron transfer. Appl. Environ. Microbiol. 70 (9), 5373–5382.

Raghavulu, S.V., Babu, P.S., Kannaiah, R.K., Subhash, G.V., Srikanth, S., Venkata Mohan, S., 2012. Bioaugmentation of an electrochemically active strain to enhance the electron discharge of mixed culture: process evaluation through electro-kinetic analysis. RSC Adv. 2, 677–688.

Ren, N.Q., Chua, H., Chan, S.Y., Tsang, Y.F., Wang, Y.J., Sin, N., 2007. Assessing optimal fermentation type for bio-hydrogen production in continuous acidogenic reactors. Bioresour. Technol. 98 (9), 1774–1780.

Robert, C., Chassard, C., Lawson, P.A., Donadille, B., 2007. Bacteroides cellulosilyticus sp. a cellulolytic bacterium from the human gut microbial community. Int. J. Syst. Evol. Microbiol. 57 (7), 1516–1520.

Srikanth, S., Venkata Mohan, S., Lalit Babu, V., Sarma, P.N., 2010. Metabolic shift and electron discharge pattern of anaerobic consortia as a function of pretreatment method applied during fermentative hydrogen production. Int. J. Hydrogen Energy 35 (19), 10693–10700.

Salzman, N.H., De Jong, H., Paterson, Y., Harmsen, H.J.M., Welling, G.W., Bos, N.A., 2002. Analysis of 16S libraries of mouse gastrointestinal microflora reveals a large new group of mouse intestinal bacteria. Microbiol. 148, 3651–3660.

Schleifer, K.H., Amann, R., Ludwig, W., Rothemund, C., Springer, N., Dorn, S., 1992. Nucleic acid probe for the identification and insitu detection of pseudomonas. Am. soc. microbiol. 127–134.

Thong, S.O., Prasertsan, P., Karakashev, D., Angelidaki, I., 2008. 16S rRNA-targeted probes for specific detection of *Thermoanaerobacterium* spp., *Thermoanaerobacterium thermosaccharolyticum*, and *Caldicellulosiruptor* spp. by fluorescent in situ hybridization in biohydrogen producing systems. Int. J. Hydrogen Energy 32 (21), 6082–6091.

Venkata Mohan, S., 2009. Harnessing of biohydrogen from wastewater treatment using mixed fermentative consortia: process evaluation towards optimization. Int. J. Hydrogen Energy 34, 7460–7474.

Venkata Mohan, S., Kannaiah Kannaiah, R., 2012. Pretreatment of biocatalyst as viable option for sustained production of biohydrogen from wastewater treatment. In: Ackmez, Mudhoo (Ed.), Biogas production: pretreatment methods in anaerobic digestion. Wiley and Scrivener Publishing LLC, pp. 291–311.

Venkata Mohan, S., Prathima, M., 2012. Fatty acid rich effluent from acidogenic biohydrogen reactor as substrate for lipid accumulation in heterotrophic microalgae with simultaneous treatment. Bioresour. Technol. 123, 627–635.

Venkata Mohan, S., Babu, V.L., Sarma, P.N., 2008a. Effect of various pretreatment methods on anaerobic mixed microflora to enhance biohydrogen production utilizing dairy wastewater as substrate. Bioresour. Technol. 99 (1), 59–67.

Venkata Mohan, S., Lalit Babu, V., Srikanth, S., Sarma, P.N., 2008b. Bio-electrochemical evaluation of fermentative hydrogen production process with the function of feeding pH. Int. J. Hydrogen Energy 33 (17), 4533–4546.

Venkata Mohan, S., Mohanakrishna, G., Ramanaiah, S.V., Sarma, P.N., 2008c. Simultaneous biohydrogen production and wastewater treatment in biofilm configured anaerobic periodic discontinuous batch reactor using distillery wastewater. Int. J. Hydrogen Energy 33 (2), 550–558.

Venkata Mohan, S., Chiranjeevi, P., Mohanakrishna, G., 2011a. A rapid and simple protocol for evaluating biohydrogen production potential (BHP) of wastewater with simultaneous process optimization. Int. J. Hydrogen Energy 37 (4), 3130–3141.

Venkata Mohan, S., Agarwal, L., Mohanakrishna, G., Srikanth, S., Kapley, A., Purohit, H.J., Sharma, P.N., 2011b. Firmicutes with iron dependent hydrogenase drive hydrogen production in anaerobic bioreactor using distillery wastewater. Int. J. Hydrogen Energy 36, 8234–8242.

Venkateswar Reddy, M., Venkata Mohan, S., 2012. Influence of aerobic and anoxic microenvironments on polyhydroxyalkanoates (PHA) production from food waste and acidogenic effluents using aerobic consortia. Bioresour. Technol. 103 (1), 313–321.

Venkateswar Reddy, M., Nikhil, G.N., Venkata Mohan, S., Swamy, Y.V., Sarma, P.N., 2012. *Pseudomonas otitidis* as a potential biocatalyst for polyhydroxyalkanoates (PHA) synthesis using synthetic wastewater and acidogenic effluents. Bioresour. Technol. 123, 471–479.

Wang, J., Wan, W., 2009. Factors influencing fermentative hydrogen production: a review. Int. J. Hydrogen Energy 34 (2), 799–811.

Wang, Y.Y., Ai, P., Hu, C.X., Zhang, Y.L., 2011. Effects of various pretreatment methods of anaerobic mixed microflora on biohydrogen production and the fermentation pathway of glucose. Int. J. Hydrogen Energy 36 (1), 390–396.

Zhu, H., Beland, M., 2006. Evaluation of alternative methods of preparing hydrogen producing seeds from digested wastewater sludge. Int. J. Hydrogen Energy 31 (14), 1980–1988.