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Regulation of acidogenic metabolism towards enhanced short chain fatty acids biosynthesis from waste: Metagenomic Profiling

Omprakash Sarkar, A. Naresh Kumar, Shikha Dahiya, K.Vamshi Krishna, Dileep Kumar Yeruva, S.Venkata Mohan*

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

*E-mail: vmohan_s@yahoo.com; Tel: 0091-40-27161765

Abstract

Short chain carboxylic (volatile fatty) acids (VFA) production in mixed microbiomes is majorly limited by the prevalence of the methanogenic bacteria and availability of substrate from waste to the biocatalyst during the fermentation process. To enhance the VFA production from food waste, the present study evaluates a strategy for selective enrichment of biocatalyst by exposing to acid-shock followed by operation with alkaline conditions (pH 10). Comprehensive system based analysis was carried during bio-based platform chemicals synthesis from waste in conjugation with microbial profiling and bio-electrochemical analysis. After selective enrichment of biocatalyst enhanced VFA synthesis was recorded with pretreated biocatalyst (PT; 11.1 g/l) compared with untreated parent biocatalyst (UT) (6.1 g VFA/l). Additionally, both systems depicted biohydrogen production as a co-product. Variations in VFA profiles were documented with respect to biocatalyst used and thus influenced degree of acidification (DOA-PT: 37% and UT: 11%). Higher fraction of acetic acid (6.9 g/l) was observed followed by butyric acid (2.6 g/l) and propionic acid (1.3 g/l) in PT operation contrary to the control system (acetic acid, 3.9 g/l, butyric acid, 1.6 g/l; propionic acid, 0.9 g/l). Specifically in PT system showed biosynthesis of iso-valeric acid: 0.15 g/l (C5) and caproic acid: 1.9 g/l (C6) ascribes the possibility of chain-elongation through selective enriched microbial community. PT system specifically showed E_{peak} at -0.415 V on cyclic voltammogram which corresponds to

28 involvement of redox couple H^+/H_2 correlating with the enhanced acidogenic process, contrary
29 to UT. Tailoring of parent inoculum (pretreatment) resulted the enriching and enhancing the
30 capabilities of biocatalyst in secreting the redox mediators, which was not detected in the UT
31 system. Acidogenic firmicutes (spore formers) and fatty acid producing Bacteroides were
32 selectively enriched in PT system along with saccharolytic and proteolytic bacteria (*Bacillus*
33 *cellulosilyticuas* (alkalophile), *Soehngenia saccharolytica*, etc.). Presence of *Clostridium*
34 *autoethanogenum*, *Propionibacterium freudenreichii*, etc. in PT system supports effective
35 utilization of complex carbohydrates facilitating acidification.
36
37 **Keywords:** Chain elongation; Biohydrogen; Acetic Acid; Cytochrome; Platform chemicals
38

39 **1. Introduction**

40 Demand for renewable chemicals and fuels, are pushing the industry towards higher
41 sustainability to improve cost-effectiveness and meet customers demand with sustainability. [1, 2]
42 On the other hand waste is being considered as renewable and potential feedstock for production
43 of spectrum of bio-based production ranging from fuel to platform chemicals.[3] At present,
44 development of environmentally sound and innovative strategies to process such waste to useful
45 products is an area of increasing importance and relevance. Carboxylic acids or volatile fatty
46 acids (VFA) are important bulk chemicals that are used as building blocks for the production of
47 polymers, as acidulants, preservatives and flavouring agents or as precursors for the synthesis of
48 chemicals.[4,5,6,7] Carboxylic acids contain a functional group whose reactivity allows the
49 formation of e.g. esters and amides. Additional innovative applications for VFA include their
50 thermal conversion to ketones and subsequent hydrogenation to alcohol fuels, use as electron

donors in microbial fuel cells and use as feedstock for microalgae based biodiesel production. [8,9] As most other commodity chemicals, VFA are currently being produced from fossil fuels through chemical synthesis. US Department of Energy (DOE) identified the most interesting platform chemicals that can derived from lignocellulose are carboxylic acids with diverse green chemistry applications. [1, 2, 10]

Anaerobic process specifically acidogenic fermentation (acidogenesis) is one of the sustainable waste management route which is gaining significant importance due to its potential to generate spectrum of bio-based products. [2,4, 11, 12] Biochemical pathways involved in the acidogenesis results in formation of multi products including carboxylic acids (C2: Acetic acid, C3: Propionic acid, C4: Butyric acid, C5: Valeric acid, C6: Caproic acid), molecular di-hydrogen, lactic acids, ethanol, CO_2, etc. [1, 10, 13] Acidogenesis based VFA production and its composition gets influence by various physical, chemical and biological parameters viz., substrate nature and composition, organic load, operating pH and temperature, nature of biocatalyst, fermentation time, etc. Open microbiomes operation can establish a more robust and economical viable process than single strains.[4,14,15] However, mixed microbiome operation restrict the function of acidogenic fermentation towards VFA production due to synergetic interaction of methanogenic bacteria (MB) consuming them along with H_2 to methane (CH_4). Among the process parameters regulating VFA production, parent inoculum is one factor which play importance role on the final fermentation products specifically when mixed culture fermentation is adopted.[16,17] Mixed fermentation provides diversity of metabolic abilities that adapt to a wide variety of substrates and conditions and also offers the advantage of working with non-sterile conditions.[12,18,19]

Improve the production rate of carboxylic acids is important at present to make process viable economically. In the context of biocatalyst, this can be done by avoiding the loss of H_2 and VFA

to H_2-consuming anaerobes (methanogens). Pretreatment of mixed culture (inoculum) by selective strategies are often practiced to regulate specific requirement of fermentation end-products. [12, 16, 17] Regulating the metabolic pathway towards acidogenesis by simultaneously inhibiting MB to allow carboxylic acids to become a metabolic end product along with H_2 can facilitate good productivity. Pretreatment of the parent culture (biocatalyst) plays an important role in the selective enrichment of acidogenic bacteria (AB).[12, 17, 20, 21] Physiological differences between AB and H_2-consuming bacteria (MB) forms the main basis for the preparation of biocatalyst for acidogenic fermentation. And also, food waste valorisation has attracted a great deal of attention as a raw material for the production of fuels and chemicals. [10, 22] Harnessing valuable products, from food wastes in controlled acidogenic fermentation make this conversion process sustainable. In this context, the present communication reports comprehensive experimental results carried to decipher the dynamics of mixed culture acidogenic fermentation process by regulating metabolism for enhanced short chain mono carboxylic acids synthesis through food waste in conjugation with microbial community and bio-electrochemical analysis.

2. Materials and Methods

2.1 Selective enrichment of biocatalyst/ Acidogenic Bioreactors

Two identical borosilicate-glass bottles were used to fabricate bench scale anaerobic reactors with a total/working volume and gas holding capacity of 0.5/0.4 l and 0.1 l respectively. The reactors were operated in suspended growth configuration with fed batch flow mode at ambient temperature (28±2°C) with OL of 15 g COD/l d^{-1} for 45 cycles. All the 45 cycles were operated for 102 h. Proper anaerobic condition was maintained in the bioreactors by sparging nitrogen gas for 5 min after every feeding and sampling event. pH of feed was adjusted to pH 10 using 1 N

NaOH/1N HCl, prior to loading of waste. The reactors contents were kept in suspension by subjecting to mixing (100 rpm).

2.2 Food waste

Composite food waste collected from the institute campus canteen and the non-biodegradable fraction was separated used as renewable feedstock. The food waste was grinded using electrical blender and then the resulted slurry was filtered through a stainless steel sieve (approx 0.15mm pore size) to remove large particles. The filtered slurry was subjected to an oil-separating system (worked based on the gravity) to remove the oil. The resulting slurry was then diluted with domestic sewage water and brought down to the desired organic load (OL) before feeding to bioreactor.

2.3 Selective enrichment of biocatalyst

Parent inoculum (biocatalyst) for the experiment was collected from an anaerobic bioreactor treating complex wastewater. Nylon filter was used to sieve the collected biocatalyst to separate the grit from the biocatalyst and the resulting biocatalyst was used as the inoculum. For Acid shock pretreatment experiment, biocatalyst 10% v/v (0.05 L) was exposed to acid-shock treatment using 0.1 N HNO_3 by adjusting pH to 3 and the condition was maintained anaerobic by sparing with N_2 gas. The one time acid-shock exposed inoculum was subjected to this shock for 24 h. Pretreatment operations were performed at room temperature (28±1 $^{\circ}$C) by providing continuous mixing by a magnetic stirrer (120 rpm). Later, before starting the experiment, pH of acid pretreated inoculum was re-adjusted to 6 using 0.1 N NaOH/0.1 N HCl. For control, untreated biocatalyst (10% v/v (0.05 L)) was evaluated to assess the relative efficiency of selective enrichment procedure. Enrichment of the inoculum (for both pretreated and untreated)

120 was done with food waste (COD, 30 g/l; 48 h; pH 10) for four cycles prior to subjecting to the
121 bioreactors.

122 **2.4 Analysis**

123 Sampling of both liquid and gas was done at different time interval for analyses. Short chain
124 fatty acid (VFA) composition was analyzed using high performance liquid chromatography
125 (HPLC; Shimadzu LC10A) employing UV-Vis detector (210 nm) and C18 reverse phase column
126 (250 x 4.6 mm diameter; 5 µm particle size, flow rate: 0.5 ml/h; wave length: 210 nm). Mobile
127 phase of 40% acetonitrile (in 1 mN H_2SO_4; pH, 2.5–3.0) and 20 µl sample injection was used.
128 Biogas composition was monitored using gas chromatography (GC; NUCON 5765) using
129 thermal conductivity detector (TCD) with 1/8" x 2 m Heysep Q column employing Argon as
130 carrier gas. The injector and detector were maintained at 60°C each and the oven was operated at
131 40 °C isothermally. Different analyses were performed viz. chemical oxygen demand (COD-
132 closed refluxing titrimetric method), VFA and pH as per standard methods (APHA 1998).
133 Buffering capacity (β) was estimated based on the acid-base titrations employing auto-titrator
134 (Mettler Toledo DL50). The β can be calculated using the equation, where, C is the concentration
135 of acid or base (ml), V_s is the volume of sample (ml), m is the slope of tangent on curve (Eq-1).

136 $$\beta = \frac{C}{V_s \times m} \text{ ---------- } [1]$$

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2.4 Bio electrochemical Analysis

Electrochemical behaviour of the biocatalyst before and after pretreatment was evaluated by employing potentiostat–galvanostat system (Biologic, EC Lab). Cyclic voltammetry was performed at a potential ramp using a scan rate of 30 mV/s over an applied voltage range of - 0.5 to 0.5 V. All the electrochemical assays were performed using platinum wire as working electrode and graphite rod as counter electrode against reference electrode (SHE) in wastewater (as electrolyte).[16]

2.5 Metagenomic analysis

Both treated and untreated biocatalyst samples were collected at the end of the operation of the experiment and the genomic DNA (gDNA) was extracted using the NucleoSpin® Soil kit (Macherey-Nagel GmbH & Co, Germany). Primers [Forward primer 341F, 5I -AGG CCT AAC ACA TGC AAG TC- 3I; Reverse primer 517R, 5I - ATT ACC GCG GCT GCT GG- 3I; GC clamp is added to forward primer] were used to highly conserved region (V3) of 16 rRNA gene was amplified using 16S rRNA universal by using thermal cycler (Eppendorf, Germany) (Table 1).[12] Amplified PCR product was purified by using QIAquick PCR purification kit (Qiagen, USA). With amplified PCR product, denaturing gradient gel electrophoresis (DGGE) was performed on Bio-Rad DCode system. Purified PCR product was separated on 8% (w/v) polyacrylamide gel with 30-80% denaturant gradient and the electrophoresis of the gel was performed at a constant voltage of 80 V at 60°C for 14 h. Ethidium bromide was used to stain polyacrylamide gel and the image was captured by a molecular imager (Syngene G-Box, UK). Selected bands were eluted in ultra pure water overnight and eluted DNA bands were re-amplified, with the same primers (341F, 517R) but without GC clamp in forward primer.

Amplified bands were sequenced. Amplified bands were sequenced at Bioserve seq, India. All the 16S rDNA partial sequences obtained were aligned with NCBI GenBank database using the BLAST server (NCBI BLAST) to obtain closest relatives. MEGA 6.0 package was used to align obtained sequences (ClustalW) and evolutionary relation between relatives was studied by constructing phylogenetic trees by the using neighbour-joining statistical method. Bootstrap analysis based on 500 iterations was used as a test for constructed phylogenetic trees.

3. Results and discussion

3.1 Short chain fatty acids production

Operation data documented distinct variations in VFA production signifying the apparent change in the metabolic behaviour of selectively enriched biocatalyst (PT) compared to the control inoculums (UT) (Fig1). More than 50% increment in VFA productivity was evidenced after applying acid-shock pretreatment to the anaerobic inoculum. Up to initial six cycles of operation, both the systems showed marginal variation in VFA synthesis (PT, 6 g/l; UT, 4.1 g/l). A steep raise in VFA production was recorded after seventh cycle onwards till 15^{th} cycle of operation (PT, 6.6 to 10.6 g/l; UT, 4.1 to 5.1 g/l). VFA production remained more or less the same up to 25^{th} cycle (10.6 g/l) documenting stabilized performance up to 45^{th} cycle (11 ± 0.9 g/l). Maximum VFA productivity (total) of 11.1 g/l was observed with PT compared to 6.1 g/l for UT operation. Alkaline pH initiates the hydrolysis of the substrate in both PT and UT. However, selective enrichment of microbial consortia documented significant regulatory influence on short chain mono carboxylic acids synthesis from waste. Initially systems were operated for 48^{th} h of cycle period, followed by 72^{nd} h, and 102^{nd} h. With increase in cycle period (24^{th} h), total VFA production of 3 g/l and 2.7 g/l was noticed with PT and UT respectively. In the 2^{nd} phase

operation (25^{th}-48^{th} h), rapid increase in VFA production was recorded particularly with pretreated operation [PT/UT; 24^{th} h (3/2.7 g/l) to 48^{th} h (7.9/3.8 g/l). In the case of 3^{rd} phase (49^{th} h -84^{th} h) a gradual increment in VFA production was observed (PT, 10.6 g/l; UT, 5.6 g/l). Extending the cycle period for another 12 h, PT operation resulted in highest carboxylic acid productivity (11.1 g/l; 102^{nd} h), whereas reduction in fatty acid was noticed with UT operation (from 5.7 to 5.3 g/l) due to its consumption by methanogenic bacteria (MB) to produce CH_4 as evidenced with biogas profile. Increased fermentation time enhanced accumulation of fatty acid to certain extent with the function of biocatalyst nature.

Fig 1

3.1.1 Finger printing of carboxylic acids

Carboxylic acid profile showed the presence of acetic acid (H_{Ac}, C2) in major fraction followed by propionic acid (H_{Pr}, C3), butyric acid (H_{Bu}, C4) and traces of iso-valeric acid (H_{Va}, C5) and caproic acid (H_{Ca}, C6) in PT system (Fig 2). PT operation documented relatively higher concentrations of H_{AC} [PT, 7.9 g/l (102 h) ; UT=3.9 g/l (96 h)] (Eq 2), followed by H_{Bu} [PT, 3.9 g/l (102 h); UT, 1.29 g/l (96 h)] (Eq 3), H_{Pr} [PT, 1.3 g/l (102 h); UT =0.9 g/l (102 h)] and H_{Va} [PT, 0.15 g/l (12 h); UT =0.1 g/l (48 h)]. H_{AC} accumulated in UT system consumed due to the dominance of MB in UT system. H_{Bu} production showed gradual increment up to 102^{nd} h (4.0 g/l). In the case of UT operation also H_{Bu} was more or less same (3.9 g/l). Higher Relatively higher synthesis of H_{Pr} was observed during 102h PT operation (1.3 g/l) compared to UT (0.9 g/l). H_{Pr} concentration showed marked improvement with increase in the fermentation time from

12 to 72 h (PT, 0.2- 1.3 g/l; UT, 0.4-0.9 g/l). Increment in propionic acid concentration at the end of fermentation might be attributed to the utilization of H_2 (Eq 4).

$$C_6H_{12}O_6+2H_2O \longrightarrow 2\ CH_3COOH+4\ H_2+2\ CO_2 \text{ ---------- (2)}$$

$$C_6H_{12}O_6 \longrightarrow CH_3CH2CH_2COOH+2\ H_2+2\ CO_2\text{---------- (3)}$$

$$C_6H_{12}O_6+2H_2 \longrightarrow 2\ CH_3CH_2COOH+2\ H_2O \text{ ---------- (4)}$$

Interestingly, PT system showed the presence of medium chain carboxylic acids from 36 h onwards (MCFA) viz., caproic acid (C6: 36 mg/l), contrary to the UT system. MCFAs are straight carboxylic acids which contains chain length of six to eight carbon atoms (C6-C8). The production of caproic acid continued till the end of cycle operation (102 h: 48 mg/l). The reductions of short carboxylic acids are tailored by H_2 or ethanol as single electron donor through reverse β-oxidation process. The reverse β oxidation process is a recurring process and adds an acetyl-CoA molecule thus derived from the ethanol oxidation to a carboxylate (Eq-3), elongating its carbon chain length.[6, 14] Suppression of MB in PT system, *in situ* H_2 pressure and accumulated ethanol (1.1 mg/l) might have induced the production of MCFA (caproic acid). In contrast, inoculum of UT was not subjected to pretreatment (for methanogens inhibition), hence it might have consumed the accumulated H_2 and acetate for the production of CH_4.

Fig 2

3.1.2 Carboxylic acids distribution with fermentation time

Acidogenic process is dynamic and gets influenced by quantity and composition of carboxylic acid present at that time and their consumption pattern. In order to understand the dynamics of process, the production/consumption rates of carboxylic acids are calculated based on the following equations.[10] (Eq 5 and 6).

Production rate of carboxylic acids (PR_{Ca}) = (VFA_{max} - VFA_{int})/T_{Prod} [5]

Consumption rate of carboxylic acids (CR_{Ca}) = (VFA_{Drop} - VFA_{max})/T_{Drop} [6]

Where, VFA_{max} represents maximum VFA concentration (g/l), VFA_{int} is initial VFA concentration (g/l), VFA_{Drop} denotes drop/consumption in VFA concentration due to its consumption (g/l), T_{Prod} represents fermentation time in hours and T_{Drop} represents VFA consumption time (hours). The positive and negative values explain the rate of production and consumption of carboxylic acids respectively. Relatively higher PR_{Ca} was observed with PT operation. Based on $H_{Ac,}$ PR_{Ca} showed higher values with PT (+0.085 g/h) than UT (+0.03 g/h) operation. H_{Ac} concentration was 0.7 g/l at 12^{th} h of cycle period gradually increased till the end of the cycle operation [2.0 g/l (24 h) to 7.9 g/l (102 h)]. This accumulation of H_{AC} with time was due to the absence of MB and presence of other acid oxidizing bacteria like *Acetobacter pasteurianus.* These classes of bacteria have the capability of producing VFA in high acids enriched media. Similarly, based on H_{Bu}/H_{Pr} also PT showed higher PR_{Ca} (+0.038 g/h, +0.0126 g/h) compared to UT operation (+0.0117/+0.0043 g/h) respectively. H_{Va} had relatively lower PR_{Ca} with UT. CR_{Ca} with PT operation was observed to be very low compared to its production (H_{Ac}, -0.007 g/h) which facilitated fatty acids accumulation. CR_{Ca} with controlled system was relatively higher (H_{Ac}, 0.02 g/h) indicating relatively higher consumption. In UT system, VFA synthesis was found till 96^{th} h (3.9 g/l) followed by consumption from 102^{nd} h (3.6 g/l). Consumption of H_{AC} after 96^{th} h can be attributed to the dominance of methanogenic activity in the later stage of operation.

3.1.3 Degree of Acidification

Degree of acidification (DOA) represents the extent of acidification achieved due to the production of carboxylic acids in relation to substrate (as COD) degradation (Eq 7).[5, 23]

$$\text{Degree of acidification (DOA, \%)} = \frac{S_f}{S_i} \times 100 \quad ...(7)$$

Where, S_i represent initial substrate concentration measured in COD as mg/l and S_f is net VFA concentration (final-initial) expressed as theoretical equivalents of COD (in mg/l, H_{Ac}, 1.066; $H_{Pr,}$, 1.512; $H_{Bu,}$1.816). DOA was calculated in terms of individual carboxylic acid concentrations and also with the mixture of three fatty acids. As valeric and caproic acid concentration was low, it was not included in the DOA calculations. VFA composition and fermentation time showed marked influence on the acidification (Fig 3). Higher DOA of 37% was observed with PT operation compared to UT (10%). It is interesting to notice that higher H_{Ac} concentration contributed for higher acidification in PT system (22.47%) compared to UT (4.95%). It is obvious that higher VFA production and substrate degradation influence the DOA. DOA is mainly influenced by the composition of the VFA produced in the system and enumerates the capacity of the given system to produce the carboxylic acids. Lower H_{Bu} (10.3%) and H_{Pr} acid (5.04%) also contributed to increase in DOA in pretreated operation. Comparatively lower acetate (4.9%), butyrate (3.9%) and propionate (2.02%) were seen in control which contributed to total DOA of 10.89%.

Fig 3

3.2 Biohydrogen (H_2)

Relatively three folds higher H_2 production was observed with PT than untreated biocatalyst (Fig 4). With the course of time cumulative hydrogen production (CHP) gradually increased in both the reactors, but total volume of H_2 varied. PT system showed threefold higher production efficiency (CHP, 0.73 l; HCE, 20.3%) than corresponding UT operation (0.29 l; 6.6%) at operating pH 10. Alkaline conditions facilitate dominance of phosphoroclastic pathway resulting in increased acetate as well as H_2 production. Mixed culture acidogenic fermentation involves the synergistic and competitive interactions of a wide diversity of microbial groups. Pretreatment of the parent inoculum (biocatalyst) plays a vital role in shifting the metabolic function of a biocatalyst towards acidogenesis with simultaneous inhibition of MB without affecting the activity of the H_2-producing acidogenic bacteria (AB).[12, 20] Physiological differences between AB and H_2-consuming bacteria (MB) form the main basis for the preparation of biocatalyst.[21] Acidogenic H_2-producing bacteria can form spores in adverse environmental conditions viz., high temperature, extreme redox conditions, etc., which is specifically absent in the MB. [12, 24] Propionic acid and lactic acid pathways demand additional H_2 of two and one moles, respectively. Biogas composition analysis showed higher fraction of H_2 (45%) from PT than UT (14%) system at 38th h of cycle operation. H_2 production correlated well with the formation of acetate and butyrate in PT system.[16,17] Apart from the H_2 production, PT system resulted relatively lower methane production (14% of total biogas) than corresponding UT system (36% of total biogas). This signifies the tailoring of parent inoculum with acid shock results the enrichment of specific acidogenic bacteria contrary to methanogenic bacteria (consume VFA/H_2) to produce methane. Enriched microbial community in PT also correlated well with observed acidogenic microbial community profiling.

Fig 4

3.3 Bio-electrochemical Analysis

Cyclic voltammogram of pretreated (PT) and untreated (UT) systems were recorded during the course of operation to enumerate the bioprocess based redox couples involved in the electron transfer process. Marked variations in the voltammogram profiles were observed with the function of PT and UT inoculum (Fig 5). Voltammogram profiles also showed specific peaks attributed to the involvement of redox mediators. The corresponding peak potentials (E_{peak}, vs SHE) were compared with the standard redox potentials (E_o, Redox).[25, 26]

In the case of PT system, E_{peak} was identified during 12 h at -0.035 V, corresponding to the involvement of quinine as redox mediator. This peak was observed to be consistent during the entire cycle of operation (till 80 h) depicting its role in the electron transfer during the bioprocess. Besides, an additional peak was detected at 0.205 V, corresponding to the involvement of cytochromes-bc1 complex. The electron transfer in cytochrome-b complex occurs from a low-potential quinone to a higher-potential C-type cytochrome and links this electron transfer to proton translocation.[27] This can be ascribed to the involvement of protons either towards H_2 production or facilitating the carboxylic acids synthesis. Interestingly, PT system resulted E_{peak} at -0.415 V, which depicts H_2 formation during the bioprocess, confirmed by the presence of redox couple H^+/H_2. During the course of fermentation time (30 h), E_{peak} at -0.415 V was significantly decreased (peak current) and was not observed at the end of operation (80 h). Pretreatment of biocatalyst showed specific influence on process efficiency in terms of carboxylic acids and H_2 production. Significant and specific peaks depicted during the operation were found to have a specific role on the bioprocess. Secreted redox couples viz., cytochrome b-complex, quinone, H^+/H_2 by the biocatalyst during the operation aided in the electron transfer efficiency towards the reduction of protons either towards H_2/volatile fatty acids. In contrast to

PT, UT biocatalyst showed a single E_{peak} at 0.205V during 30h, which corresponds to the involvement of cytochrome-bc_1 complex which can be attributed to the lower H^+ shuttling due to their reduction to CH_4 (due to methanogenesis).[16] These results show the specific influence of pretreatment in enriching and enhancing the capabilities of biocatalyst in secreting the redox mediators, which was not detected in the UT system, corroborating with the microbial community fingerprinting as well.

Fig 5

3.4 Metagenomic profile

Structure and function of microbial communities greatly influenced by its biodiversity and can differ in composition both qualitatively and quantitatively. Studying metagenomic profile can give information on various aspects of diversity, taxonomy and resources provided by microbial populations.[28] Thus for the present study where there is possibility to control and direct the fermentation towards specific metabolites requires knowledge on the microbial composition and metabolism. For microbial diversity identification performed PCR DGGE demonstrated and confirmed the presence of volatile fatty acid and H_2 producers like members of *Bacillaceae* and *Clostridiaceae* in the acid treated reactors. Members of these families are known to be key players in production various volatile fatty acids and H_2 in anaerobic conditions. Thus the increased production of H_2 was most probably occurred due to acid shock, whereas subsequent reduction of hydrogen to methane was mostly decreased. DGGE patterns also suggested the presence of new bands in the acid-shock biocatalyst indicative of either new species emerging or domination of species which are less abundant in the parent inoculums (UT) (Fig 6). However, certain bands retained both in UT and PT suggesting that certain species have adapted to live in the low acidic pH of inoculum.

Major phylums related to the DGGE bands of PT reactor can be divided into four groups viz., firmicutes, bacteroides, actinobacteria and proteobacteria while the UT was also dominated by four major groups viz., bacteroides, firmicutes, actinobacteria and other unculturables (Table 2). Firmicutes have dominated the acid-shock reactor which might be due to their ability to resist stress by various pathways like endosporulation.[17] Similar results were obtained in the previous experiments where the inoculum was pre-treated with acid for H_2 production. [29] Most of these bacteria are acidogenic in nature and versatile H_2-producing bacteria that can form spores in adverse environmental conditions viz., high temperature, extreme redox conditions, etc., which is specifically absent in methanogens.[30] Moreover, acidogenic pH inhibits the growth of hydrogentrophic methanogens and thereby reducing the CH_4 production from H_2.[29] Enriched firmicutes were mostly acidogenic like *Lactobacillus delbrueckii (NC_008054.1), Lactobacillus harbinensis (NR_041263.1), Clostridium acidurici (CP003326.1)* and *Clostridium autoethanogenum (NC_022592.1)*, which are well known for their ability to produce various fatty acids (Fig7). Clostridia were previously reported to be major part of the consortium producing H_2 under anaerobic conditions supporting their enrichment. [24] Along with these bacteria, relatives of saccharolytic bacteria like *Bacillus cellulosilyticus (NR_074904.1)* and *Soehngenia saccharolytica (NR_117382.1)* were also found. *Clostridium autoethanogenum* is a facultative chemolithoautotrophic, anaerobic, endospore forming gram positive bacteria.[31] It is one of the few bacteria which can ferment carbon monoxide (CO) and can also utilize few other carbon sources to form ethanol and acetate. *Clostridium acidurici* which is homoacetogen can form acetic acid from purines. *Soehngenia saccharolytica* is a gram positive spore forming clostridia which forms acetate, H_2 and CO_2 as key products of metabolism.[32] Other bacteria relating to lactobacillus group of the acidophilus complex were also observed. *Bacillus*

cellulosilyticus produces range of carbohydrate degrading enzymes and can produce VFA from range of substrates including fructose, glucose, mannose, sucrose, lactose, etc. which is hydrolytic products of complex compounds present in food waste.

Fig 6

Majority of the bands next to the firmicutes were bacteroidetes viz., *Bacteroides graminisolvens (NR_113069.1), Proteiniphilum acetatigenes (NR_043154.1), Paludibacter propionicigenes (NC_014734.1), Macellibacteroides fermentans (NR_117913.1)* and *Bacteroides helcogenes (NC_014933.1).* Bacteroidetes make the bulk of microbial consortium along with firmicutes which can treat wastewater/waste. They are acidogens and can produce fatty acids depending on the substrate available [(propionate/acetate; *Proteiniphilum acetatigenes*, *Bacteroides graminisolvens* and *Paludibacter propionicigenes*) (lacatate/ acetate/butyrate/isobutyrate; *Macellibacteroides fermentans*)].[33] Other groups of bacteria which showed relatedness to the acid-shock sequences were *Propionibacterium freudenreichii (NC_014215.1)* and *Acetobacter pasteurianus (NC_013209.1).* Major product of *P. freudenreichii* metabolism is propionic acid. [28,34] Major product of *A. pasteurianus* is acetic acid. This bacterium is known to survive high acidity stress conditions which explain its survival in acid shock reactor.[35] Few of these reported bacteria are known to produce soluble external mediators which were evident as peaks in cyclic voltammograms during various hours of operation. High propionic acid production observed in PT might be attributed due to the presence of *Propionibacterium freudenreichii* and *Paludibacter propionicigenes* bacteria. Presence of *Macellibacteroides fermentans in* PT leads to higher production of butyrate and iso-butyrate as the main fermentation products of this bacteria are lactate, acetate, butyrate and iso-butyrate from glucose metabolism.

Fig 7

Microbial diversity of parent inoculum or UT was mostly dominated by the uncultured bacteroides and firmicutes along with actinobacteria and several unidentified bacteria. Bacteria relating to class Bacillaceae and Clostridia were dominant among firmicutes viz., *Enterococcus saccharolyticus LMG 11427 (NR_114786.2), Bacillus toyonensis BCT-7112 (CP006863.1), Bacillus coagulans DSM 1 ATCC 7050 (CP009709.1), Brassicibacter mesophilus BM (NR_108841.1)* and *Clostridium ultunense DSM 10521 (NR_117379). Bacillus coagulans* is a spore forming lactic acid producing facultative anaerobic bacteria while *Bacillus toyonensis* and *Brassicibacter mesophilus* are a strict anaerobe found in food industry wastewater and are known to produce acetic acid and propionic acid.[16] Among phylum Bacteroidetes, relatives of parabacteriodes (NR_109439.1) and uncultured bacteroides were found to be dominant. Other relatives observed are *Amycolicicoccus subflavus (NC_015564.1)* along with several unidentified and uncultured bacteria. *Amycolicicoccus subflavus* is an actinobacteria which is known for growing in harsh environments.[36] In UT most of the bacteroides identified are unculturables whose functional role in the fatty acid production is unknown. It has also been observed that many species which grow on various types of food wastes are identified in UT owing to the substrate give.

On the basis of these results, it is can be proposed that the acid pretreatment of inoculum has enriched the bacteria related to the families *Clostridiacea*e, *Bacteriodes and Bacillaceae* which are in turn involved in production of volatile fatty acids and H_2 from wide range of carbon sources while surviving the acid stress. While in the case of UT, along with acidogens certain non-specific and VFA utilizing bacteria also survived which might have reduced the yield of VFA and H_2. Lower production of VFA in the UT might be attributed to the prevailing of methanogenic activity, which utilizes VFA along with H_2 towards CH_4 conversion which is

evident from the presence of *Clostridium ultunense*. *Clostridium ultunense* can oxidize acetic acid to produce CH_4 in syntrophic association with H_2 utilizing MB at low partial pressure of H_2,[37] which is particularly true in case of UT where the H_2 production rates are low making this reaction possible. Elevated levels of CH_4 indicate the syntrophic association between this clostridium species and methanogens. PT suppresses the growth of MB resulting in regulating the acidogenic activity facilitating higher VFA production.

3.5 System Redox microenvironment

Prior to start-up, aqueous phase pH of both biosystems were adjusted to alkaline condition (pH 10), based on previous study,[10] which aided higher solubilisation of food waste. Alkaline conditions prevent the specific activity of methanogenic archaea that prefer a neutral pH and eventually consume VFA. With the course of time both the bioreactors showed decrement in pH due to accumulation of VFA in system (Fig 8). Initially marginal drop in pH was observed up to 6 h followed by a marked drop (PT/UT: 8.76/8.91) which continued till the end of the cycle operation (102 h: 5.5/6.9). PT system showed higher pH drop (5.5) than corresponding UT system (6.9). Drop in system pH indicated fatty acid accumulation, and their effect are more evident when the pH is below 7.0. UT system, showed increment in pH at end of cycle period due to degradation of the fatty acids by obligate proton reducing bacteria (methanogens/heterotrophic acetogens). The pH imposes altered dynamics between different groups of microorganisms, but also facilitates a metabolic shift within the same group. Change in pH may also influence the enzyme activities and in turn metabolic activities of the biocatalyst. Neutral pH assists methanogens to grow over acidogens. In the case of UT operation more or less neutral redox microenvironment was observed from 78 to 102 h, which invariably favours

the methanogenic activity and lead to CH_4 evolution. Sudden increase in pH to 7.3 at 90th h might be due to consumption of accumulated VFA as a primary substrate by the homoacetogens. Alkaline pH improves the acetic acid accumulation/production and its fraction in the total VFA.[12] Production and/or consumption of butyric acid was inconsistent with pH and time.

Fatty acids production influence on the system's buffering capacity (BC) as well. System's BC correlated well with the VFA production and system pH. The PT system showed improved buffering until 90th h of cycle operation (12 h (0.021 β mol); 102 h (0.04 β mol; 102 h)). On the contrary, UT system showed increment in BC till 72nd h and then decreased thereafter till the end of cycle. UT system has higher BC at 72 h (0.028 β mol) which reduced to 0.022 β mol at 102 h. The pH is kept stable by the buffer effect of the protein residues and other macromolecules present.[38] In situ buffering systems like ammonium bicarbonate (pKa 6.35/ 9.35), free ammonia present in the system, which in association with fatty acids like viz., formic acid (pKa 9.25) and acetic acid (pKa 4.75/9.25) and bicarbonate (pKa 6.35/9.35), carbonate (pKa 6.35/9.25) play a great role in buffering biological systems.

Fig 8

3.6 Waste Remediation

COD removal helps in elucidating the substrate consumption by the system microbiomes (Fig 9). Comparatively acid-shock pretreated system depicted less substrate (COD) removal (45-54%) than the UT system (58-65%), which can directly attributed to the specific enrichment of aciodgenic bacteria to terminate metabolic reaction until fatty acid production. Prevalence of

462 *Proteiniphilum acetatigenes, Bacteroides graminisolvens, Paludibacter propionicigenes* and
463 *Macellibacteroides* make the system more effective in utilizing carbohydrate sources commonly
464 found in food based wastewater. Interestingly, in PT system *Proteiniphilum acetatigenes a*
465 proteolytic bacterium was also found. It helps to degrade proteins and grows on very limited
466 substrates to produce acetate and propionate. Most of the bacteria belonging to firmicutes have
467 wide nutritional choice to act upon enabling them to live on wide range of substrates.
468
469 **Fig 9**
470
471 **4. Conclusion**

472 The selectively enriched acidogenic bacteria enhanced the short chain carboxylic acids
473 biosynthesis in pre-treated system contrary to untreated biocatalyst. Additionally, alkaline
474 operation conditions (pH 10) induced the hydrolysis of food based wastewater and resulted in
475 enhanced short chain carboxylic acids along with biohydrogen (H_2) production as co-product in
476 pretreated system. Additionally, high concentration of acetic acid with simultaneous production
477 of medium chain caproic acid in pretreated system allocates the efficiency to produce high rate
478 carboxylic acids. This also induced the high rate degree of acidification (DOA) in pretreated
479 system contrary to the untreated system. Microbial community profiling showed dominance of
480 spore forming acidogenic firmicutes and short chain fatty acid producing bacteroides in
481 pretreated system. The present study highlights the application of pretreatment to parent
482 biocatalyst which holds promise as a viable option for enhancing production of valuable platform
483 chemicals through valorisation of food based wastewater in sustainable way.
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Acknowledgments

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Abbreviations

VFA-	Volatile Fatty acid
UT-	Untreated Biocatalyst
PT-	Pretreated Biocatalyst
DOE-	Department of Energy
AB-	Acidogenic Bacteria
MB-	Methanogenic Bacteria
OL-	Organic Load
SHE-	Standard Hydrogen Electrode
MCFA-	Medium Chain Fatty Acid
DOA-	Degree of Acidification
BC-	Buffering Capacity

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584 **Table: 1Primes used for the PCR amplification of V3 region of 16s rRNA gene for DGGE.**

585

Name	Target group	Function	Sequence
341FGC	Eubacteria	Forward	CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCCTACGGGAGGCAGCAG
341F	Eubacteria	Forward	CTACGGGAGGCAGCAG
517R	Eubacteria	Reverse	ATTACCGCGGCTGCTGG

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597 **Table 2: Sequence based identification, classification and taxonomic affiliation of various bands excised**
598 **from DGGE gels derived from bacterial 16S rRNA gene from PT and UT.**

DGGE Band	Closest relative	Accession number	Similarity	Phylum	Metabolic function
VFA SVM 1	Bacillus cereus	NC_004722.1	83	Firmicutes	Soil dwelling bacteria
	Lactobacillus delbrueckii subsp. bulgaricus	NC_008054.1	82	Firmicutes	Lactic acid production
VFA SVM 2	Lactobacillus harbinensis	NR_041263.1	96	Firmicutes	Lactic acid production
	Uncultured Lactobacillus sp. clone	JX099832.1	96	Firmicutes	Lactic acid production
VFA SVM 3	Propionibacterium freudenreichii subsp. shermanii	NC_014215.1	88	Actinobacteria	Propionic acid
VFA SVM 4	Clostridium autoethanogenum	NC_022592.1	81	Firmicutes	Ethanol and Acetate from acetic acid
	Acetobacter pasteurianus	NC_013209.1	93	Proteobacteria	Acetic acid
VFA SVM 5	Paludibacter propionicigenes	NC_014734.1	85	Bacteroidetes	Propionic and Acetic acid
	Bacteroides helcogenes	NC_014933.1	85	Bacteroidetes	Acetic acid, succinic acid, propionic acid and isobutyric acid
VFA SVM 6	Bacteroides graminisolvens	NR_113069.1	93	Bacteroidetes	Propionic and Acetic acid
	Bacteroides graminisolvens	NR_041642.1	93	Bacteroidetes	Propionic and Acetic acid
VFA SVM 7	Proteiniphilum acetatigenes	NR_043154.1	80	Bacteroidetes	Propionic and Acetic acid
	Bacteroides graminisolvens	NR_041642.1	81	Bacteroidetes	Propionic and Acetic acid
VFA SVM 8	Bacillus cellulosilyticus	NR_074904.1	84	Firmicutes	Can degrade various celluloses and produce acetic acid
	Uncultured Brevibacillus sp.	AM263548.1	82	Firmicutes	-
VFA SVM 9	Soehngenia saccharolytica	NR_117382.1	94	Firmicutes	Acetate, H_2 and CO_2
	Clostridium acidurici	CP003326.1	95	Firmicutes	Acetate from purines
VFA SVM 10	Macellibacteroides fermentans	NR_117913.1	86	Bacteroidetes	lacatate, acetate, butyrate and isobutyrate
VFA SVM 11	Uncultured low G+C Gram-	DQ252446.1	84	-	-

	positive bacterium				
VFA SVM 12	Bacterium enrichment culture	KJ629451.1	90	-	-
	Uncultured Bacteroides	EU882476.1	90	Bacteroidetes	-
VFA SVM 13	Uncultured bacterium	KM105837.1	87	-	-
	Uncultured bacterium	KM042872.1	87	-	-
VFA SVM 14	Enterococcus saccharolyticus	NR_114786.2	95	Firmicutes	Grows on various carbon sources to produce acetic acid
	Uncultured bacterium clone	KM105837.1	95	-	-
VFA SVM 15	Brassicibacter mesophilus	NR_108841.1	95	Firmicutes	Acetic acid and propionic acid from food waste
	Clostridium ultunense	NR_117379	95	Firmicutes	Utilizes acetate and hydrogen in association with methanogen to produce methane
VFA SVM 16	Uncultured Bacteroides	EU882476.1	87	Bacteroidetes	-
	Uncultured Bacteroidetes	CU918067.1	87	Bacteroidetes	-
VFA SVM 17	Bacillus toyonensis	CP006863.1	92	Firmicutes	Used for synthesising toyonesin
	Bacillus coagulans	CP009709.1	86	Firmicutes	Lactic acid
VFA SVM 18	Uncultured Bacteroidales	KM059722.1	82	Bacteroidetes	-
	Uncultured Bacteroidetes	KM058988.1	82	Bacteroidetes	-
VFA SVM 19	Amycolicicoccus subflavus	NC_015564.1	81	Actinobacteria	Grows in harsh conditions like saline and oil contaminated soils
VFA SVM 20	Parabacteroides chartae	NR_109439.1	86	Bacteroidetes	Grows on various carbon sources to produce acetic acid
VFA SVM 21	Uncultured Bacteroidales	FJ219718.1	93	Bacteroidetes	-
	Uncultured Bacteroidetes	AB742116.1	91	Bacteroidetes	-

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Figures

Fig 1: Variations in total VFA production in acid-shock pretreated (PT) and untreated (UT) operations at initial pH of 10 with respect to operation time (45^{th} cycles).

Fig 2: (a) Pattern of in VFA biosynthesis in pretreated (PT) and untreated system (UT) with respect to time (0^{th} h to 102^{nd} h). (b) Positive and negative profiles showing the production and consumption rate of individual fatty acids with the function of initial pH and fermentation time.

Fig 3: Acidification achieved due to the production of VFAs in relation to substrate degradation, Composition of individual VFA's produced in the reactor and degree of acidification (DOA).

Fig 4: (a) Enhanced H_2 evolution and suppressed CH_4 production in acid-shock pretreatment, higher CH_4 content in UT; (b) Higher cumulative biohydrogen production (CHP) from acid-shock pretreatment (PT) than untreated culture (UT) (c) Biohydrogen conversion efficiency (HCE) of pretreated and untreated culture at initial pH 10.

Fig 5: Variations in cyclic Voltammogram profiles with respect to course of time and pretreatment strategy.

Fig 6: PCR DGGE profiles of bacterial communities associated with A) PT and B) UT. Bands were excised and sequenced for further evolutionary analysis.

Fig 7: The evolutionary profiles of enriched microbial community inferred using the Neighbour-Joining method (a) Acid pretreated culture (PT), (b) Untreated control (UT) system.

Fig 8: Change in pH from 0^{th} h to 102^{nd} h of operation. Influence of acid-shock pretreatment on buffering capacity (shown as insert).

Fig 9: Substrate degradation in terms of COD removal (15 kg COD/m^3/day) at pH 10 for pretreated and untreated operations; Change in substrate removal pattern in the reactor with respect to time (shown as insert).

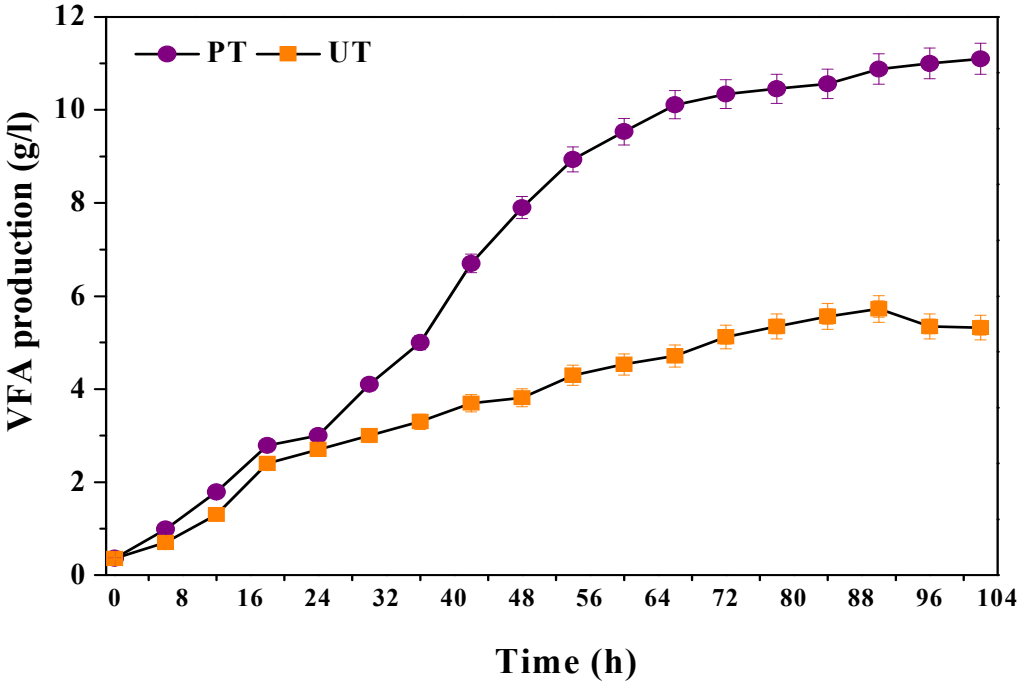
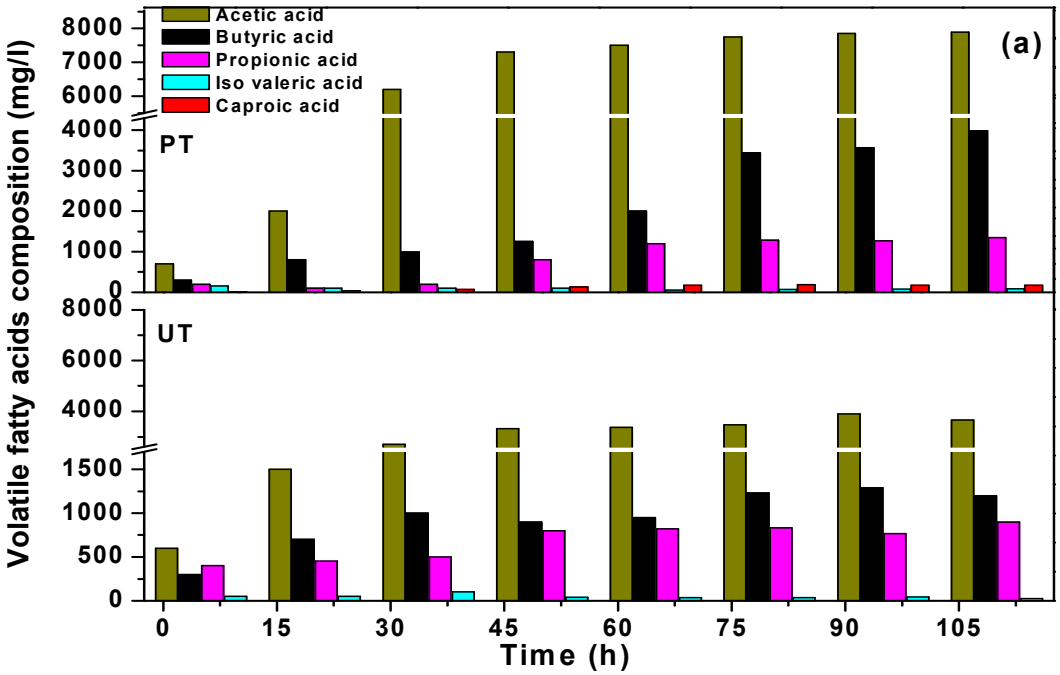


Fig 1



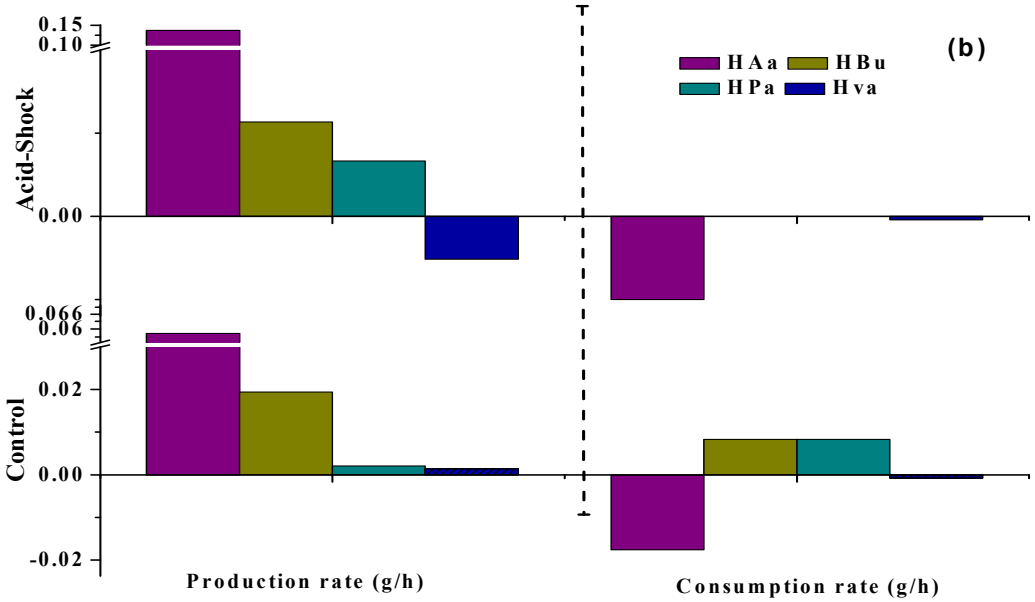


Fig 2

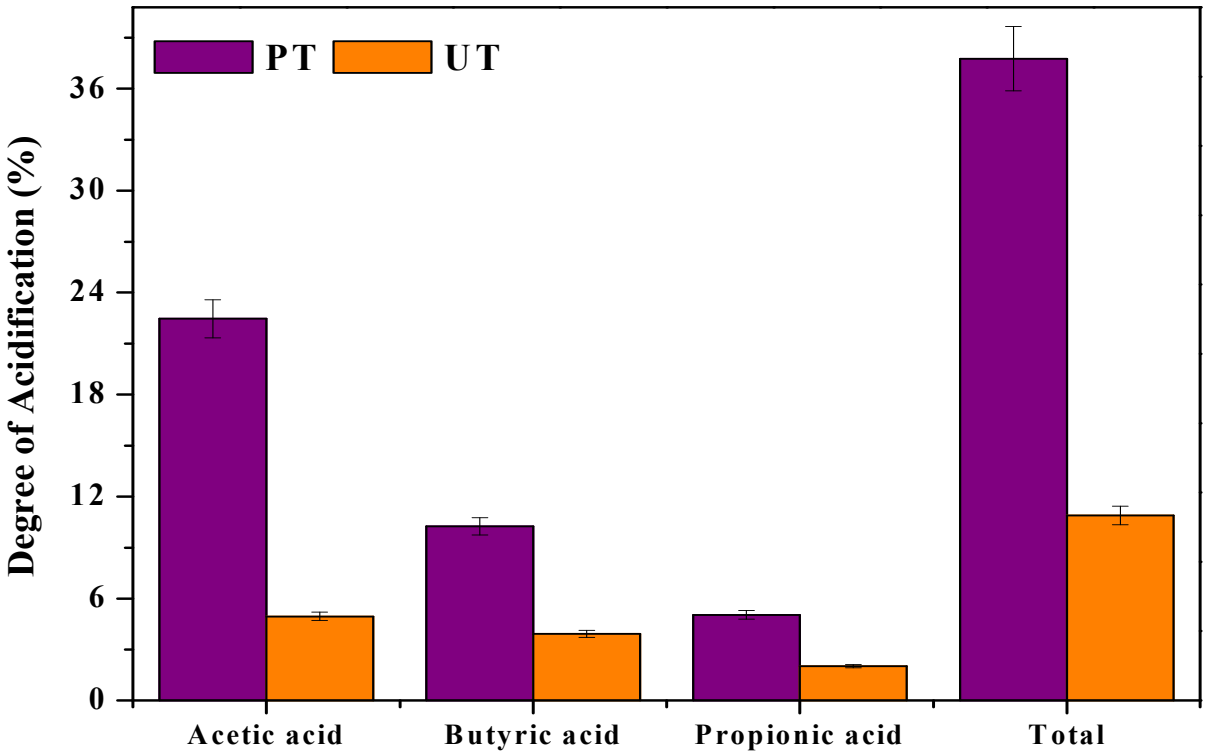
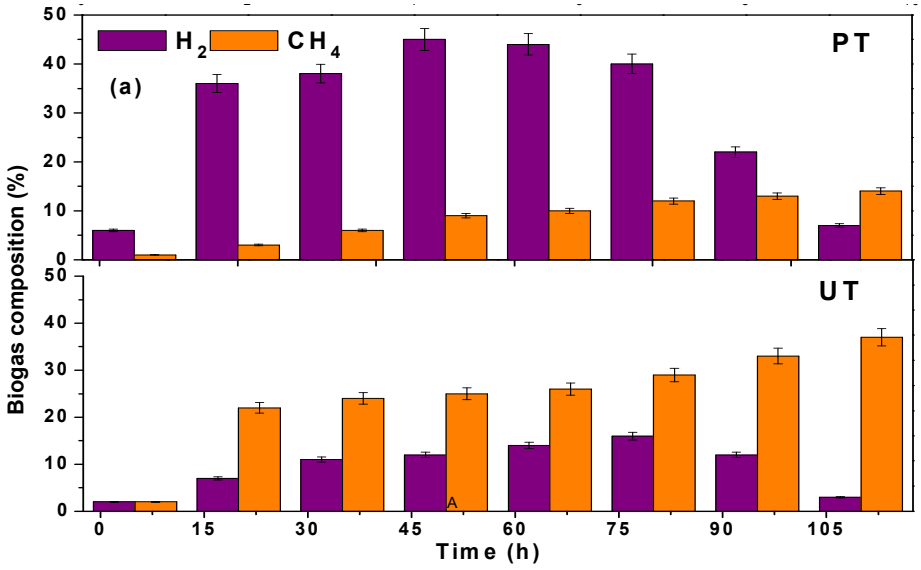
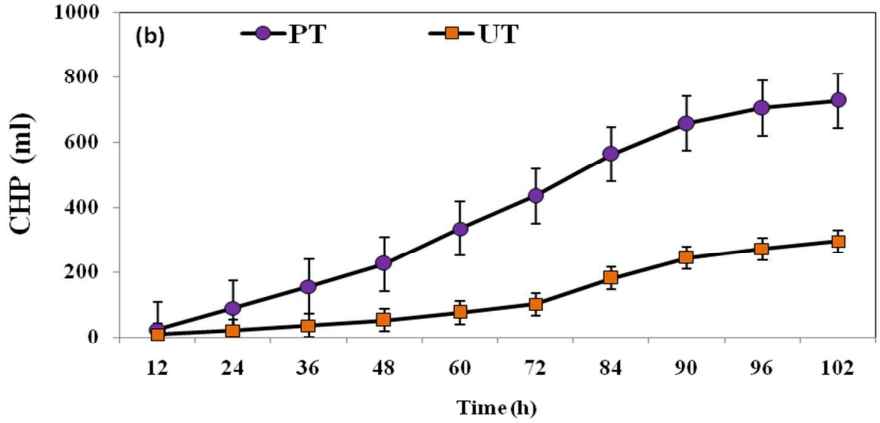


Fig 3





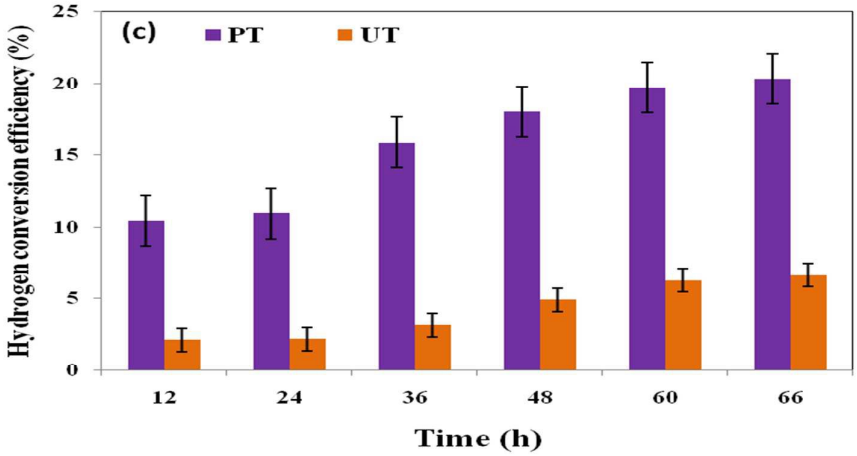


Fig 4

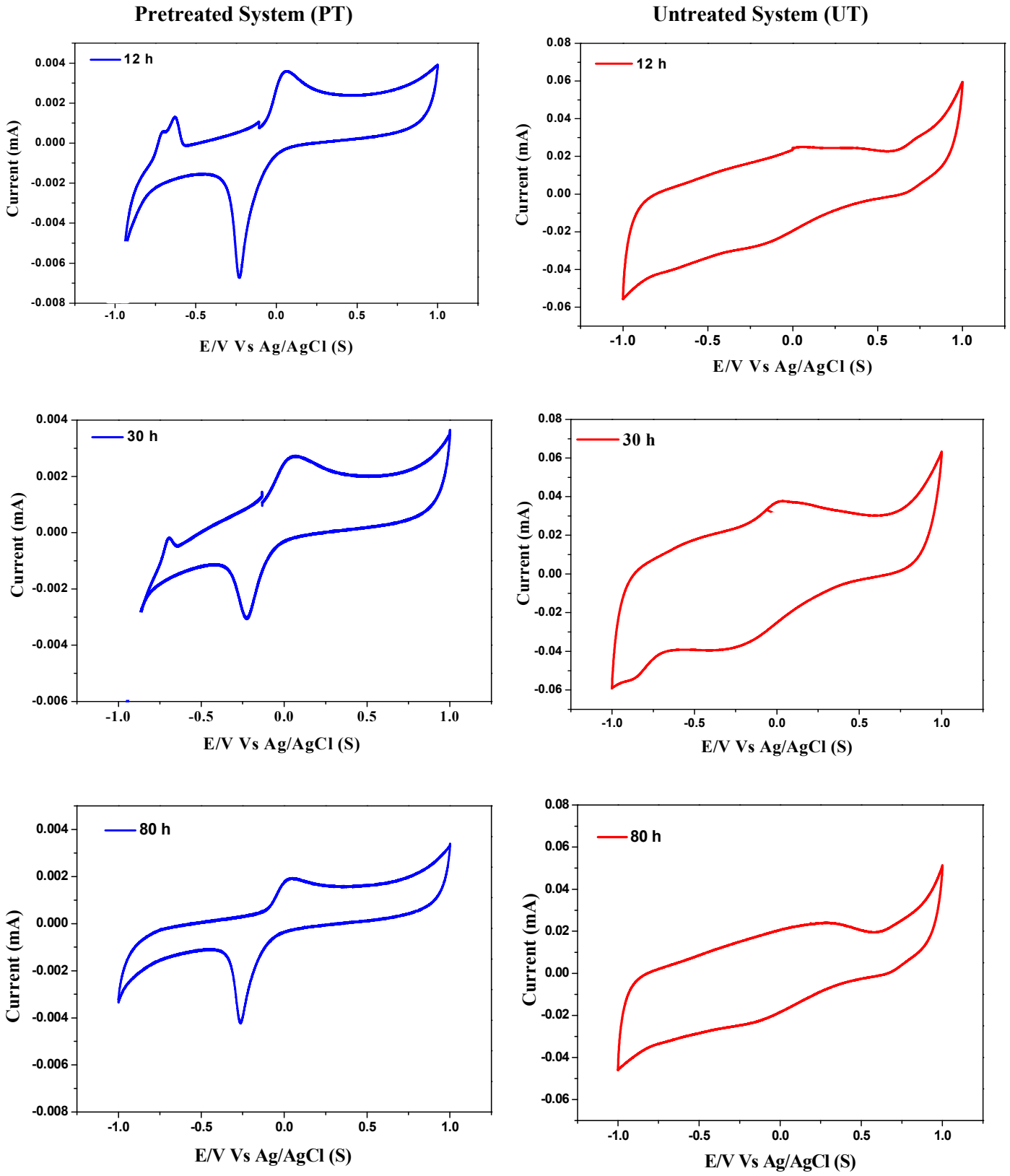


Fig 5

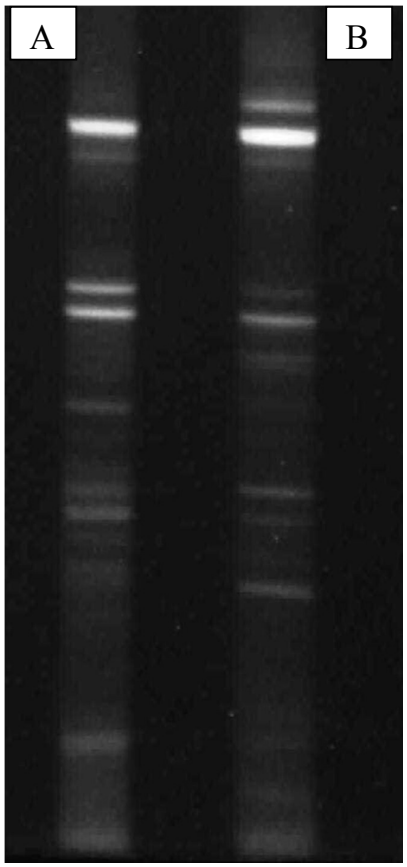
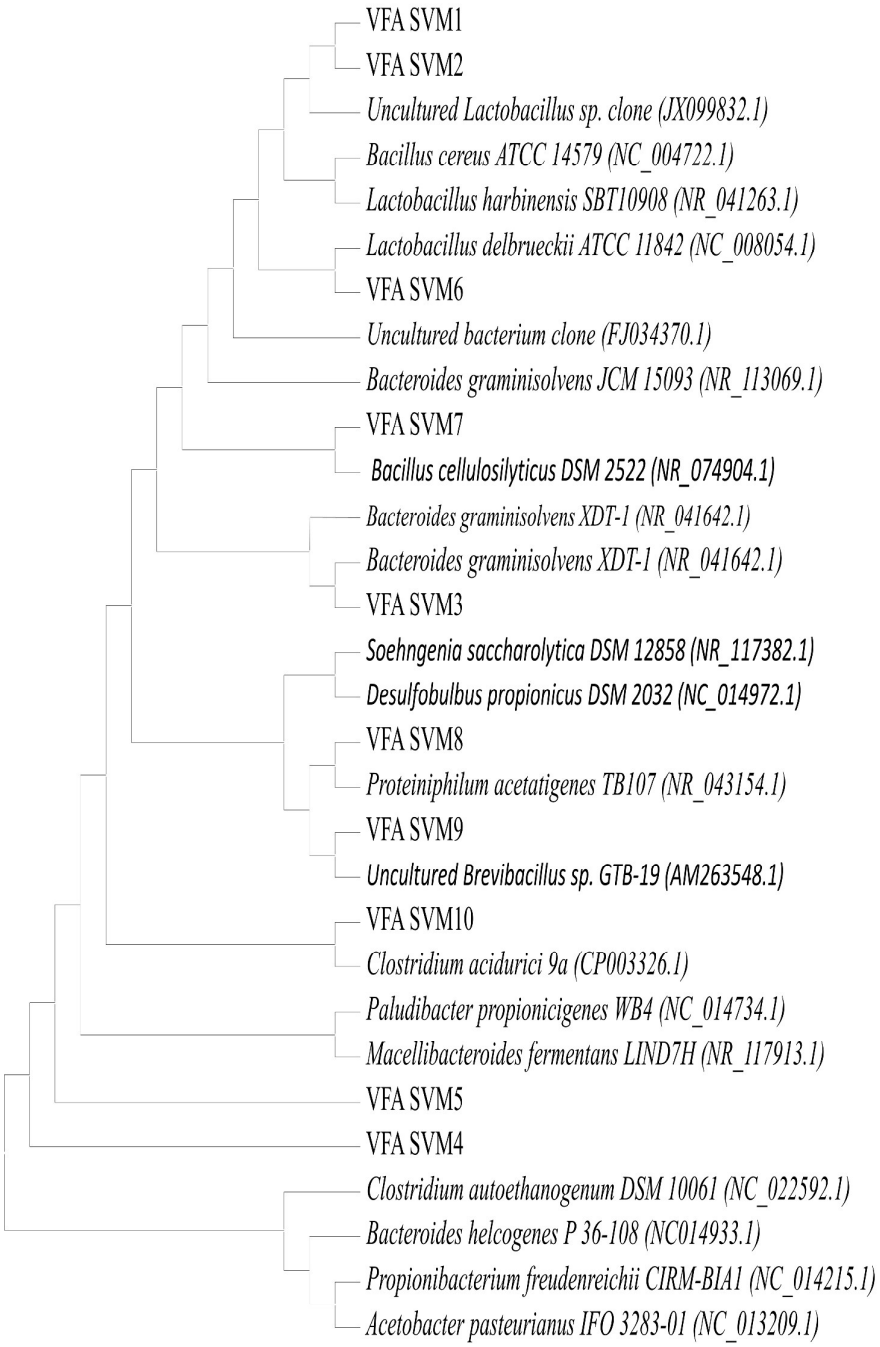


Fig 6

(a) PT



(b) UT

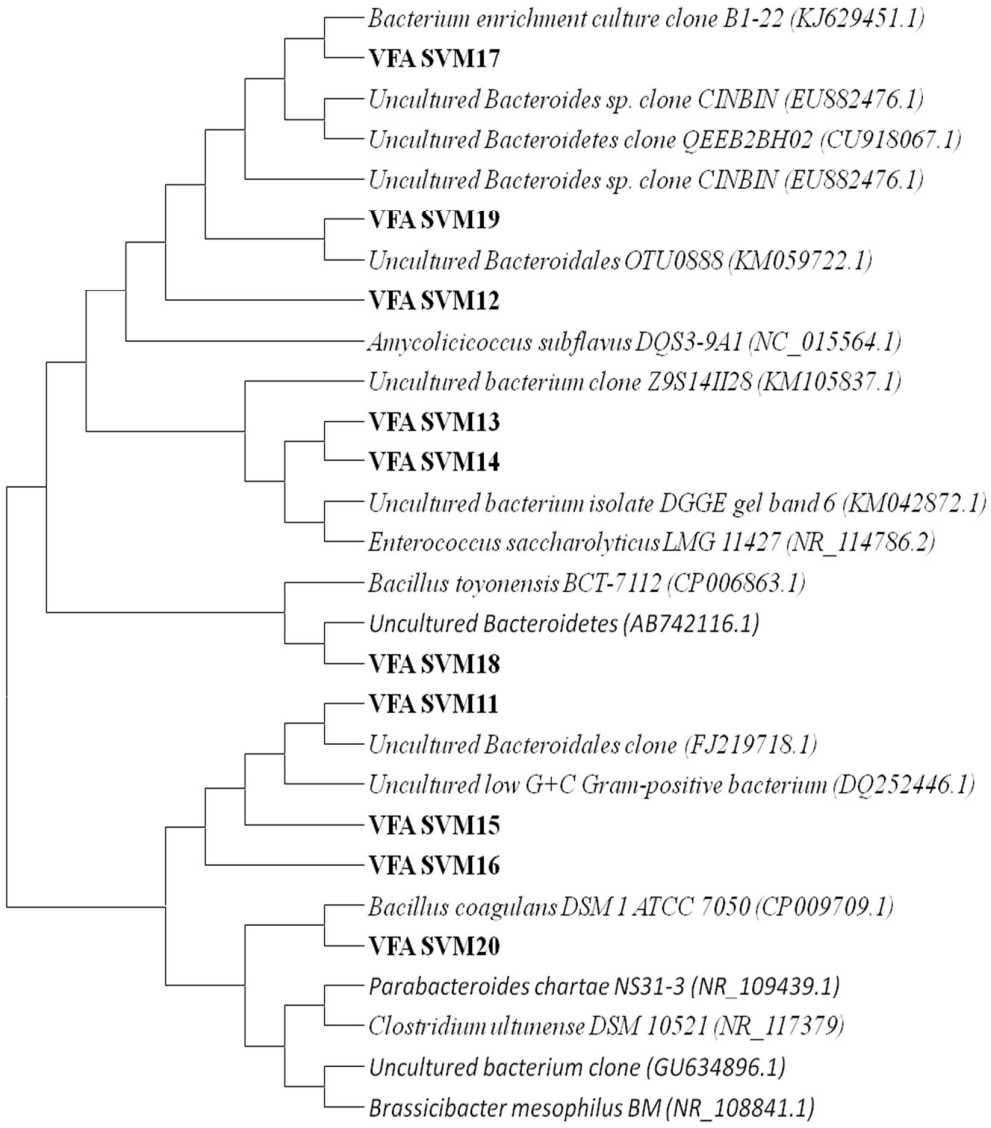


Fig 7

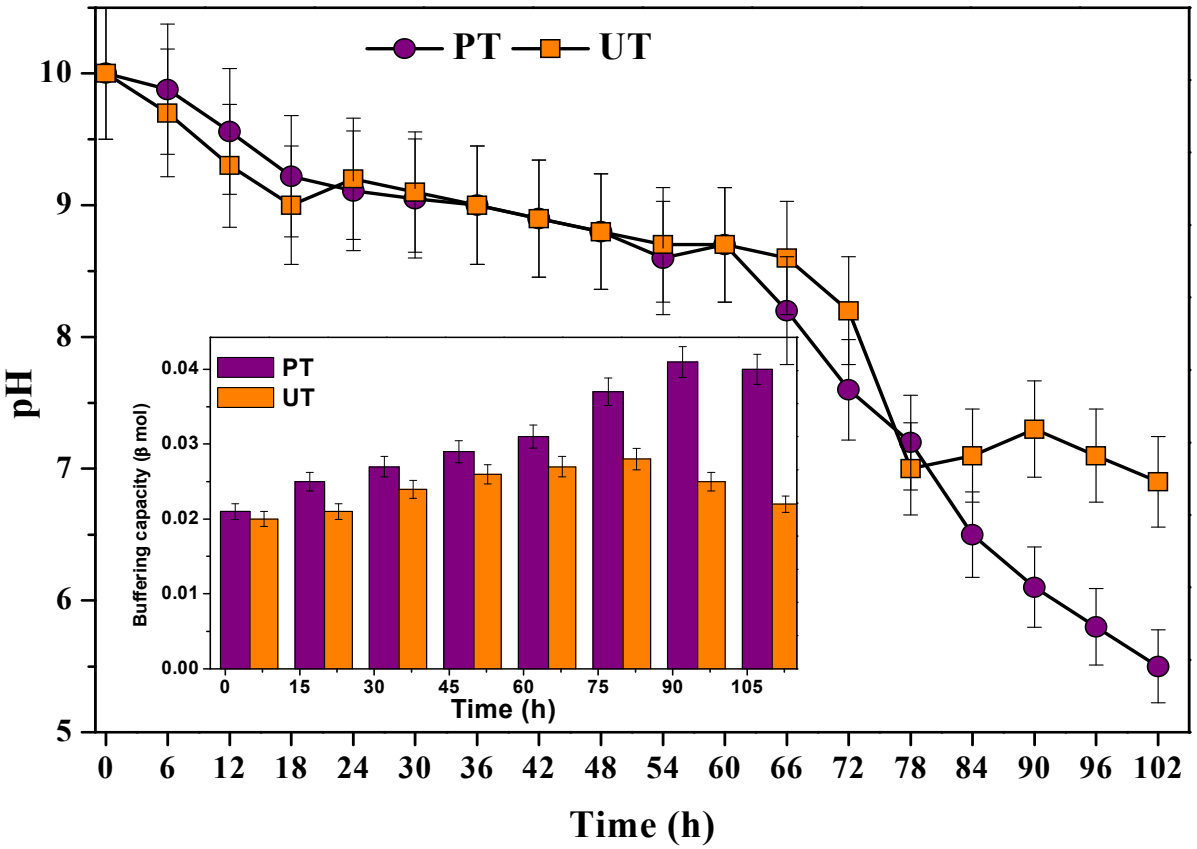


Fig 8

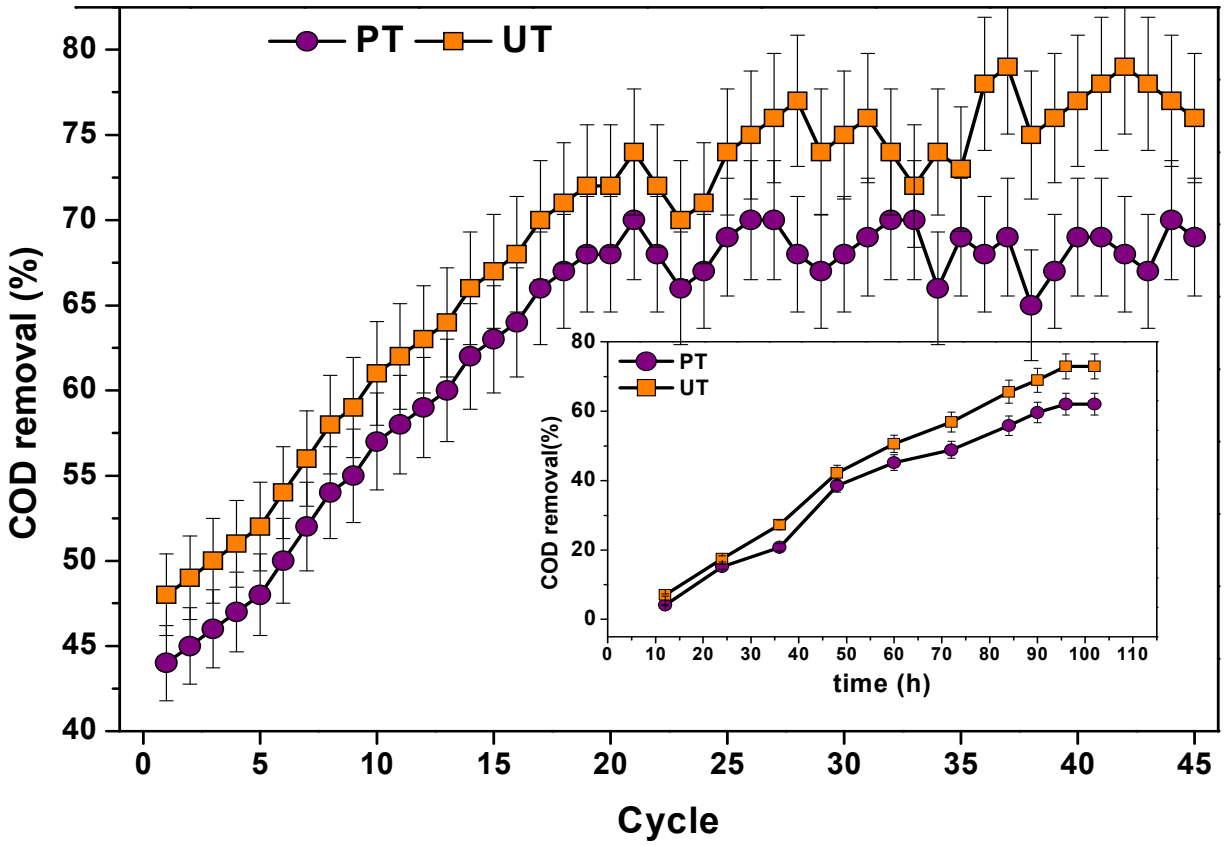


Fig 9