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Biohydrogen production from chemical wastewater as substrate by selectively enriched anaerobic mixed consortia: Influence of fermentation pH and substrate composition

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Abstract

Influence of fermentation pH and substrate composition (composite chemical wastewater as primary carbon source) on molecular H_2 production was studied in batch experiments using sequentially pretreated [heat-shock (100 °C; 2 h) and acid (pH 3; 24 h)] anaerobic mixed consortia as inoculum. Sequentially coupled repeated pretreatments showed positive influence on the overall H_2 generation. Effective H_2 production was evidenced at fermentation pH 6 (1.25 mmol H_2/g COD) compared to 5 (0.71 mmol H_2/g COD) and 7 (0.27 mmol H_2/g COD). Fermentation pH of 6.0 was found to be optimum for effective H_2 generation with the pretreated inoculum. The feed consisting of only glucose as primary substrate showed low H_2 yield, while feed with chemical wastewater admixed either with glucose or sewage wastewater as co-substrates demonstrated high H_2 yield. Addition of co-substrate (glucose or sewage wastewater) along with chemical wastewater showed enhanced H_2 yield. Glucose concentration exceeding 2 g/l resulted in reduced H_2 yield. Higher VFA concentrations were recorded in experiments carried out at fermentation pH 5 than 7. At fermentation pH 6 VFA composition showed the presence of acetate, butyrate, and propionate with relatively lower concentration of ethanol. Acid-forming pathway with acetic acid as a major metabolite dominated the metabolic flow during the H_2 production.

Keywords: Hydrogen; Anaerobic fermentation; Composite chemical wastewater; Sewage; Co-substrate; Mixed microflora; Heat-shock treatment; Acid treatment

1. Introduction

Global energy requirements at present are mostly dependent on the fossil fuels, which eventually lead to foreseeable depletion of limited fossil energy resources [1,2]. Current utilization of hydrogen (H_2) is equivalent to 3% of the energy consumption and is expected to grow significantly in the near future [2]. Recently, a great deal of attention is being paid to the usage of H_2 as an alternative and eco-friendly fuel throughout the world. The advantages of this sustainable energy source are numerous: it is clean, efficient, renewable, and does not generate any toxic byproduct, as it can be produced by water decomposition [3]. In spite of its green nature, today most of the H_2 is produced from nonrenewable sources, such as natural gas, oil, and coal [4]. These methods mainly use fossil fuels as energy sources, which are considered to be energy intensive and not always environment friendly.

Biological production of H_2 is one of the alternative methods, which is being focused extensively by the research fraternity more recently. Broadly, biological H_2 production processes were classified as biophotolysis of water using algae and cyanobacteria, photodecomposition of organic compounds by photosynthetic bacteria and fermentative H_2 production from organic compounds [2,5]. These processes were mostly operated at ambient temperatures and pressures, which were less energy intensive and environmental friendly. The fermentative evolution of H_2 is more advantageous than the photochemical evolution especially for mass production of H_2 by microorganisms. However, comparatively little attention is given to fermentative H_2 production so far [2,6]. In addition,

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they can also use various waste materials and wastewater from industrial processes as substrates and the process can be operated at ambient temperature (30–40 °C) and normal pressure. Use of industrial wastewater as substrate facilitates both treatment and renewable extraction of clean gas at the same time. Very recently, H_2 production through anaerobic fermentation using wastewater has attracted attention [2,7–14] and this leads to open avenues for the utilization of renewable energy sources, which are inexhaustible.

Fermentative H_2 production is considered to be a complex process and needs optimization with respect to the type of inoculum and pretreatment, substrate nature and composition, co-substrate addition, fermentation pH, fermentation period, etc. prior to upscaling. Inoculum selection and its pretreatment is one of the important aspects which have a vital role in selecting the requisite microflora for efficient H_2 production. Several types of pretreatment procedures (heat treatment, chemical treatment, pH treatment, etc.) were reported in literature for a variety of inocula [11–14]. Additionally, system operating conditions (operating pH, short fermentation period and sludge retention time) will also have significant effect on H_2 evolution [4]. In this paper, we report experimental data pertaining to the batch studies performed on selectively enriched mixed consortia to study the effect of fermentation pH and substrate composition on H_2 production.

2. Experimental

2.1. Selective enrichment of H_2 producing mixed microflora

Anaerobic mixed microflora acquired from an operating laboratory scale upflow anaerobic sludge blanket (UASB) reactor treating composite chemical wastewater for the past 3 years were used as the inoculum. Dewatered sludge from UASB reactor was subjected to repetitive pretreatment sequences (four times) with shock treatment (100 °C; 2 h) followed by acid treatment (pH 3 adjusted with orthophosphoric acid; 24 h) to selectively enrich the H_2 producing mixed microflora by inhibiting the growth of methanogenic bacteria (MB) and facilitating the growth of spore-forming bacteria. The characteristics of enriched H_2 producing mixed consortia after pretreatment prior to use in the experiments is depicted in Table 1.

2.2. Substrate composition

Designed synthetic wastewater (SW) [(g/l) of glucose: 2.5, NH_4Cl: 0.5; KH_2PO_4: 0.25; K_2HPO_4: 0.25; $MgCl_2 \cdot 6H_2O$: 0.3; $FeCl_3$: 0.025; $NiSO_4$: 0.016; $CoCl_2$: 0.025; $ZnCl_2$: 0.0115; $CuCl_2$: 0.0105; $CaCl_2$: 0.005 and $MnCl_2$: 0.015; Chemical Oxygen Demand (COD) of 4.50 g/l] was used. Composite chemical wastewater (CW) having pH of 7.6, total alkalinity of 1.20 g/l, total dissolved inorganic solids (TDIS) of 25.45 g/l, COD of 6.24 g/l, biochemical oxygen demand (BOD_5) of 1.14 g/l, total nitrogen of 0.129 g/l and total phosphorus of 0.361 g/l was used. The selected wastewater was a combined mixture of different types of chemical wastewaters collected from a common effluent treatment plant (CETP) in Hyderabad, India. The wastewater was a composite one aggregated from chemicals, drugs, pharmaceuticals, pesticides and various chemical processing units. Characteristically, the wastewater is low biodegradable (BOD/COD $\sim$ 0.30) in nature. Domestic sewage wastewater (DSW) which had a pH of 7.2, COD of 0.43 g/l and BOD_5 of 0.28 g/l was also used as co-substrate.

Table 1
Characteristics of selectively enriched H_2 producing mixed consortia

S. No.	Parameter	Value
1	pH (1:10)	7.7 ± 0.2
2	ORP (1:10)	-62.18 ± 11 mV
3	Total solids	18.53 ± 0.44 g/l
4	Suspended solids (SS)	13.54 ± 0.32 g/l
5	Volatile suspended solids (VSS)	7.62 ± 0.16 g/l

2.3. Batch fermentation experiments

Experiments were designed to evaluate the influence of substrate composition and fermentation pH on the H_2 evolution. In total, 16 experimental sets were designed and performed with variable substrate composition and fermentation pH values (Table 2). Except experiment CE40S, all other experiments were studied separately at three fermentation pH values. All the experiments were performed in batch mode using a series of 250 ml conical flasks (working volume of 200 ml). Each flask prior to experimentation was inoculated with 20 ml of pretreated anaerobic H_2 producing mixed consortia (VSS of 7.6 g/l) under aseptic anaerobic conditions. pH optimization experiments were performed at various fermentation pH values [5–7] taking 180 ml of feed (Table 2). Aqueous phase pH before feeding was adjusted employing concentrated orthophosphoric acid or 3 N NaOH solution to the desired initial levels of 5.0, 6.0 and 7.0. After pH adjustment the flasks were flushed with oxygen-free nitrogen gas for 30 s and capped tightly with a rubber septum (butyl rubber) and placed in an incubator with orbital shakers (110 rpm). All the experiments were performed at a constant mesophilic temperature (29 ± 2 °C). Glucose and DSW and in some cases SW was used as co-substrate along with

Table 2
Experimental variations studied

Experimental variation	Feed composition				Fermentation pH studied
	CW (%)	SW (%)	DSW (%)	Glucose (g)	
CE01	–	100	–	2.5	5, 6, 7
CE401	40	60	–	1	5, 6, 7
CE402	40	60	–	2	5, 6, 7
CE403	40	60	–	3	5, 6, 7
CE402S	40	30	30	2	5, 6, 7
CE40S	40	–	60	–	6

CW: chemical wastewater; SW: synthetic wastewater; DSW: domestic wastewater (sewage).

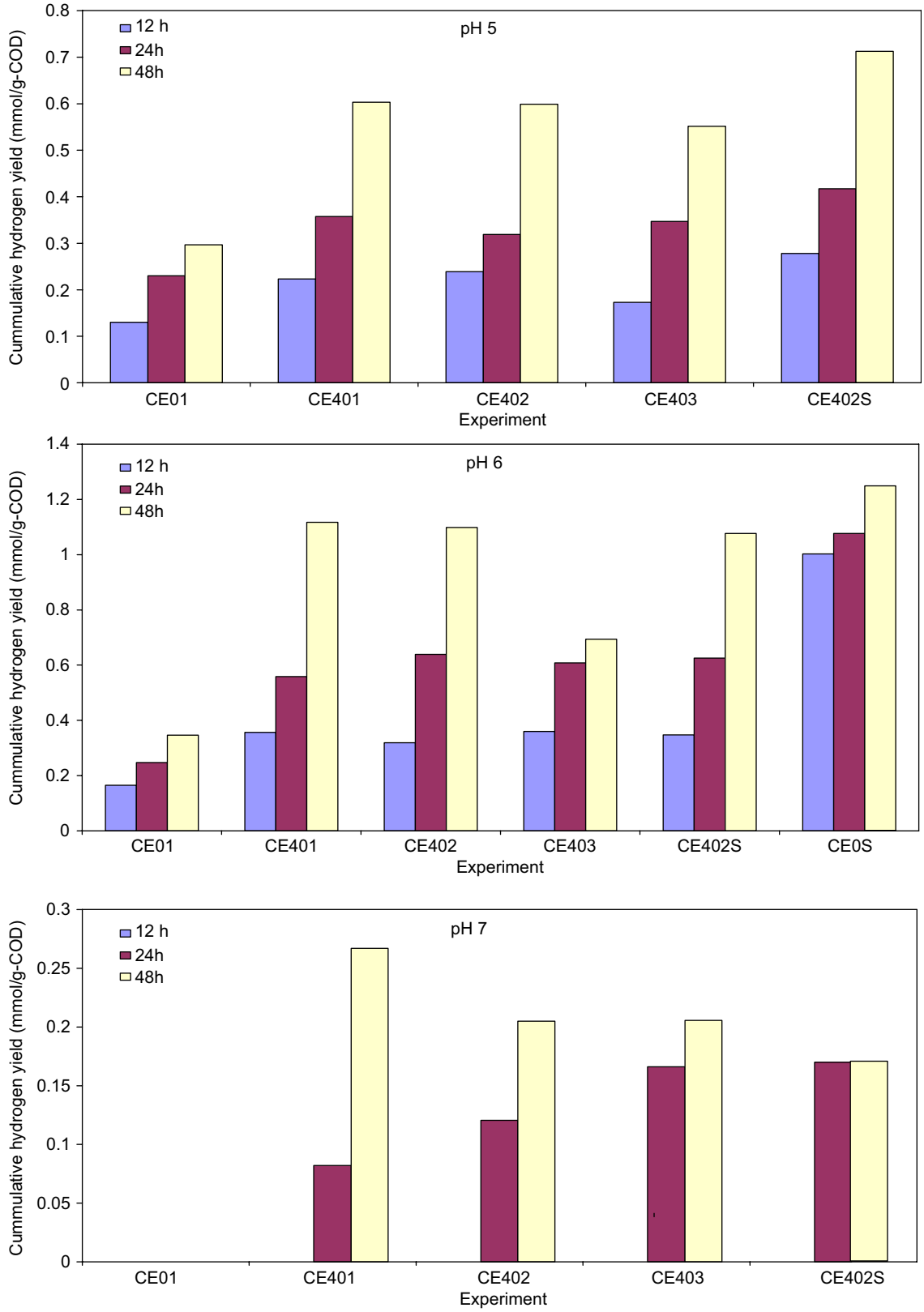

Fig. 1. H_2 production pattern with the function of fermentation pH during experimental variations studied.

chemical wastewater to study the influence of co-substrate on H_2 evolution. For each set of experimental variation three separate flasks were operated. After monitoring H_2 and collecting samples, the flasks were discarded. Control flask without the addition of the feed was used for all experimental sets.

2.4. Analysis

H_2 generated during batch experimental studies was estimated using a microprocessor-based pre-calibrated H_2 sensor (electrochemical 3 electrode H_2 sensor, FMK satellite 4–20 mA version, ATMI GmBH Inc., Germany). The output signal displayed the % volume of H_2 in the headspace, which was further converted to mmol. The system was calibrated once in two days using calibration cap provided with the instrument, and sensor had a measuring range of 0.01–10% H_2 with 5 s response time in a temperature range of 20–80° C. Oxidation–reduction potential (ORP) and pH values were determined by a pH meter (Model 20, Denver instruments Ltd.). Total alkalinity, TSS, VSS, volatile fatty acids (VFA) and BOD_5 were determined according to Standard Methods [15]. Soluble COD was analyzed employing dichromate closed refluxing method performed according to the Standard Methods [15]. The separation and quantitative determination of VFA composition were carried out by high performance liquid chromatography (HPLC) (UV–VIS detector; C18 column: reverse phase column, 250 × 4.6 mm and 5 μm particle size; flow rate: 0.5 ml/h; wave length: 210 nm; mobile phase: 40% of acetonitrile in 1 mN H_2SO_4 (pH 2.5–3.0); sample injection: 20 μl). The anaerobic mixed consortium was subjected to scanning electron microscopy (SEM). Prior to SEM imaging samples were fixed in glutaraldehyde (2.5%) in 0.05 M phosphate buffer (pH 7.2) for 24 h at 4 °C and post-fixed in aqueous osmium tetroxide (2%) in the same buffer for 2 h. After post-fixation samples were dehydrated in a series of graded alcohol and dried. Dried samples were mounted over the stubs with double-sided conductivity tape, and a thin layer of platinum metal was applied over the sample using an automated sputter coater for about 2 min and scanned in SEM (JOEL-JSM 5600).

3. Results and discussion

Batch experiments performed with the pretreated and selectively enriched anaerobic mixed microflora revealed the influence of fermentation pH, and feed composition on H_2 generation (Fig. 1). It is apparent from the experimental data that the nature and composition of the substrate had significant influence on the overall H_2 production. Among the studied substrate compositions, the feed consisting of glucose as only primary carbon source (CE01) resulted in low H_2 yield. On the contrary, feed with chemical wastewater admixture either with glucose or sewage wastewater as co-substrates (CE401, CE402, CE403, CE402S) evidenced comparatively higher H_2 yield (Table 3). Presence of co-substrate (simple molecule) might have assisted in activating the initial anaerobic metabolism, which resulted in enhanced performance. However, when concentration of glucose exceeded 2 g/l (optimum co-substrate concentration in this study) it resulted in reduced performance of the reactor in some cases. This might be attributed to the phenomena of carbon repression encountered during the complex anaerobic metabolism. Ginkel and Logan [16] reported that high sugar concentrations were susceptible to product inhibition and in such cases a decrease in the carbon loading rate showed

Table 3
Variation of H_2 yield during studied experimental variations

Experiment	Fermentation pH	Relative H_2 production rate (mmol H_2/g COD-h)		
		0–12 h	12–24 h	24–48 h
CE01	5	0.0109	0.0085	0.0026
	6	0.0137	0.0068	0.0041
	7	0	0	0
CE401	5	0.0185	0.0112	0.0103
	6	0.0298	0.0168	0.0234
	7	0	0.0068	0.0077
CE402	5	0.0199	0.0067	0.0117
	6	0.0265	0.0267	0.0192
	7	0	0.0101	0.0035
CE403	5	0.0144	0.0145	0.0086
	6	0.0300	0.0507	0.0036
	7	0	0.0138	0.0017
CE402S	5	0.0232	0.0116	0.0123
	6	0.0289	0.0232	0.0188
	7	0	0.0142	0
CE40S	6	0.0835	0.0062	0.0071

enhancement in H_2 yield. The addition of sewage as co-substrate facilitated effective H_2 yield due to the presence of readily usable carbon source. Presence of chemical wastewater as primary carbon source has positive effect on the H_2 production. Its participation in the metabolic reactions involving molecular H_2 generation was evident from reduction in substrate concentration (as COD) in all the experimental variations studied (Fig. 2). Decrease in the COD concentration was observed irrespective of the experimental variations studied with a degree of variance.

Over the three fermentation pH values studied, relatively high H_2 yield was evidenced at fermentation pH 6 (1.25 mmol H_2/g COD) (Fig. 1). Next higher values of H_2 yield were documented at pH 5.0 (0.71 mmol H_2/g COD), while pH 7 showed relatively low H_2 production (0.27 mmol H_2/g COD). One of the possible reasons for the observed lower H_2 yield at fermentation pH 5 might be attributed to the decline in the system pH values below 5.0 due to acid production. This might lead to inhibition of the acidogenic metabolism thereby shifting the metabolic path to solventogenesis which might result in suppression of the H_2 production. Generally, acid accumulation in the system causes a sharp drop in the system pH inhibiting the H_2 production [4,17,18], and bacteria cannot sustain its metabolic activity at pH values less than 5.0 [19]. It was also reported that the optimum pH for H_2 production was 5.5, while the optimum pH for solvent production was in and around 4.5 [11,20,21]. Production of solvents was often considered to cause negative effects on H_2 production, whereas acid formation was favorable especially for H_2 production [22]. The concentrations of the undissociated forms of acetic or butyric acid were greater at pH 4.5 which caused inhibition [16]. Complete inhibition in H_2 production was reported in the pH range of 4–5 [23,24]. Maximum H_2 yield was documented

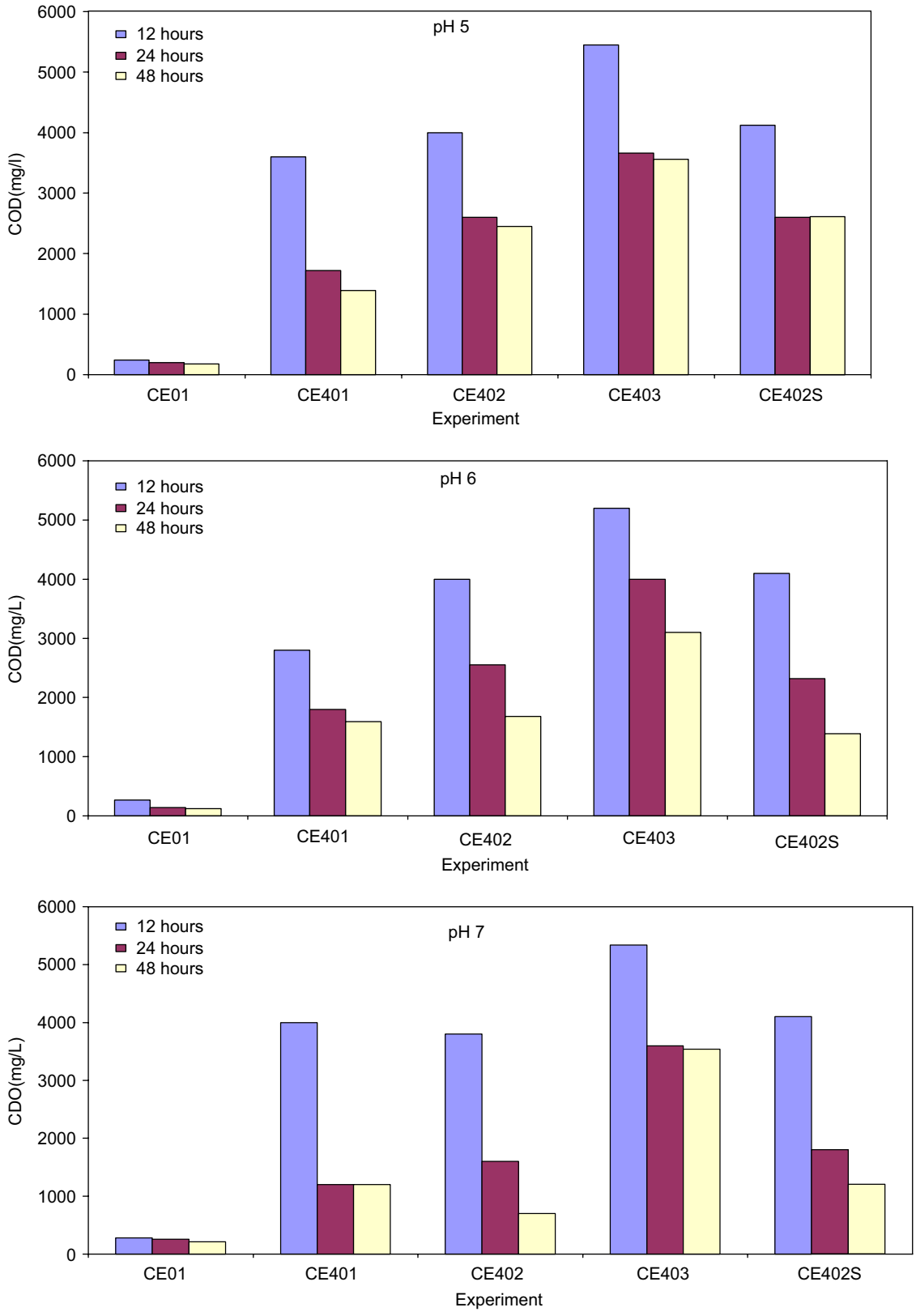

Fig. 2. Substrate concentration (COD) variation pattern with the function of fermentation pH during experimental variations studied.

between 0 and 12 h of fermentation period in all the experimental variations studied expect in CE403 at fermentation pH 6.0 (Table 3). This observation indicated that higher generation rates of H_2 were possible during the early stages of fermentation which further correlated with the data obtained on the metabolites of VFA as discussed below (Fig. 3).

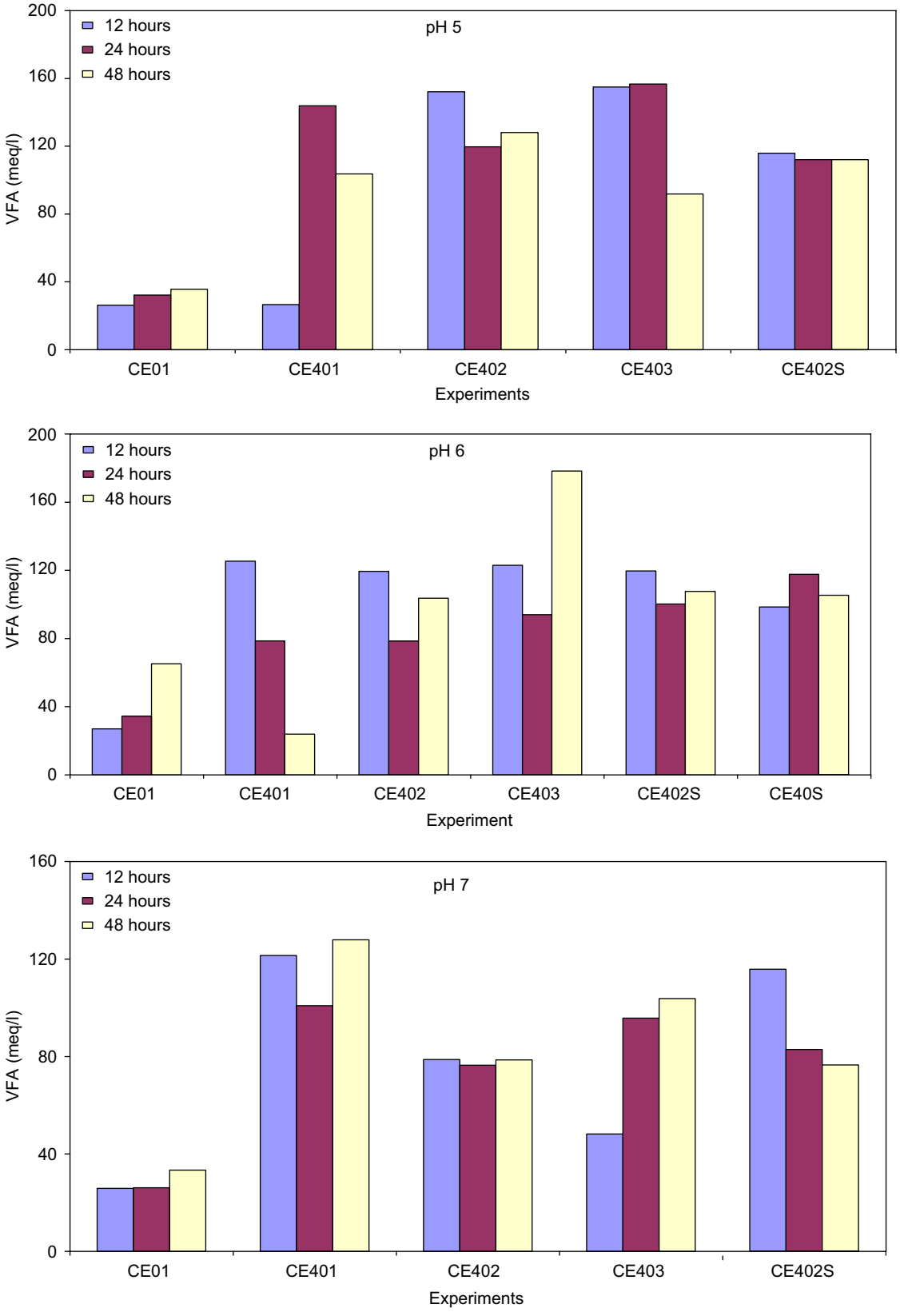

Fig. 3. VFA production pattern with the function of fermentation pH during experimental variations studied.

Table 4
Cumulative H_2 yield, system pH drop and VFA production rate with the function of fermentation pH and substrate composition

Experiment	Fermentation pH	System pH drop[a]	Total VFA (meq/l)	VFA production rate (meq VFA/h)	Time of VFA_{Max} (h)	Cumulative H_2 yield (mmol H_2/g COD)
CE01	5	−0.34	10.2 ± 0.8	0.21 ± 0.04	48	0.297 ± 0.05
	6	−0.76	39.8 ± 0.6	0.83 ± 0.02	48	0.346 ± 0.12
	7	−0.78	8.0 ± 1.1	0.17 ± 0.06	48	0.0
CE401	5	−0.66	118.4 ± 0.9	2.46 ± 0.02	24	0.603 ± 0.06
	6	−1.00	100.0 ± 1.0	2.08 ± 0.01	12	1.200 ± 0.06
	7	−0.69	102.5 ± 0.8	2.13 ± 0.04	48	0.267 ± 0.10
CE402	5	−0.77	118.4 ± 0.9	2.47 ± 0.04	12	0.599 ± 0.11
	6	−1.21	94.0 ± 0.9	1.96 ± 0.05	12	1.099 ± 0.08
	7	−0.70	53.4 ± 2.1	1.11 ± 0.06	12	0.205 ± 0.09
CE403	5	−0.55	131.3 ± 2.4	2.73 ± 0.07	24	0.553 ± 0.09
	6	−0.96	152.6 ± 1.2	3.18 ± 0.08	48	0.694 ± 0.10
	7	−0.80	77.9 ± 0.9	1.62 ± 0.04	48	0.206 ± 0.12
CE402S	5	−0.74	90.2 ± 1.4	1.88 ± 0.02	12	0.712 ± 0.15
	6	−1.19	94.3 ± 3.2	1.96 ± 0.01	12	1.077 ± 0.14
	7	−1.34	90.3 ± 2.8	3.55 ± 0.04	12	0.170 ± 0.12
CE40S	6	−1.02	128.3 ± 3.9	2.67 ± 0.03	12	1.248 ± 0.10

VFA_{Max}: Maximum VFA concentration.
[a]Indication of the reduction in pH value.

H_2 production is normally accompanied with the acid production coupled with solvent production due to the acidogenic metabolism where generation of these acidic intermediates reflects changes in the metabolic pathway of the microorganisms [25,26]. This also provides a better knowledge of such changes and conditions favorable for H_2 production. Here, VFA was represented as the total of all acids. VFA generation showed distinct variation with the function of substrate composition and fermentation pH studied (Fig. 3 and Table 4). Higher concentrations of VFA were observed in the experiments carried out at fermentation pH 5, while fermentation pH 7 resulted in comparatively lower VFA concentration. A steady decrease in the VFA concentration was observed with increase in the retention time, particularly at fermentation pH of 5 (except CE01 and CE40S) and 6 (except CE01 and CE40S). It was evident from the experimental data that the fermentation pH of 5 and 6 were found to be optimum for the effective functioning of enriched mixed microflora. Rapid conversion of organic substrate to fatty acids (12 h) was documented mainly with fermentation experiments CE401, CE402, CE402S and CE40S conducted at pH 6 (Fig. 3 and Table 4). Comparatively low VFA yield was observed in the experiments with glucose as only substrate (CE01) might be attributed to the substrate-limiting conditions. Low concentration and inconsistent pattern of VFA generation was observed at fermentation pH 7, which might be attributed to the non-supportive microenvironment for the proliferation of acidogenic bacteria (AB) to promote rapid acid production (Figs. 3 and 4). Higher production of VFA was documented in the studies using 3 g/l of glucose as co-substrate (CE 403) at all the pH variations studied.

The pH drop is generally considered as the index of VFA generation and the existing buffering capacity in the system. Production of acid intermediates (VFA) gradually reduces the system buffering capacity which further resulted in a concomitant decline in the system pH at all the experimental variations studied (Fig. 3 and Table 4). However, the pH drop showed a distinct trend in each of the experimental variations studied and was not closely coinciding with the VFA concentration. Maximum pH surge was documented in the case of experiment CE402S followed by CE40S and CE403. The lowest drop in the pH was observed in CE01. High production of VFA was visible with experiment CE403 followed by CE401, CE402 and CE40S, while a low amount of VFA production was evidenced with experiment CE01. Low pH drop and high VFA generation were documented at fermentation pH 5. However, maximum H_2 yield was observed at pH 6 in association with high pH drop and VFA generation. A higher pH drop represents rapid production of volatile acids. pH drop of 0.34–0.77, 0.76–1.21 and 0.69–1.34 were observed in the case of experiments with fermentation pH 5, 6 and 7, respectively. In spite of higher VFA production rate and pH drop, the H_2 production observed in the case of fermentation pH 5 was comparatively on the lower side to pH 6 (Table 4). The reason for this typical behavior might be attributed to the switch over of the reaction to solventogenesis from acidogenesis (H_2 consuming pathway) manifested due to the pH drop below 5.8 [27]. This phenomenon was considered as a negative metabolic shift as far as H_2 production was concerned. Drop in the pH could be because of accumulation of organic acids leading to process inhibition [28]. Accumulation of VFA was reported at pH 5 compared to pH 6 and pH range of 5.5–6 was considered to be ideal to avoid both methanogenesis and solventogenesis [21,27,31]. The optimum pH for MB was between 6.0 and 7.5 [29,30], while AB functioned well below pH 6. A drop in pH from 6 to 5 observed in the case of the experiment at pH 6 is considered to be the effective range for the functioning of AB and inhibition of MB. It can be

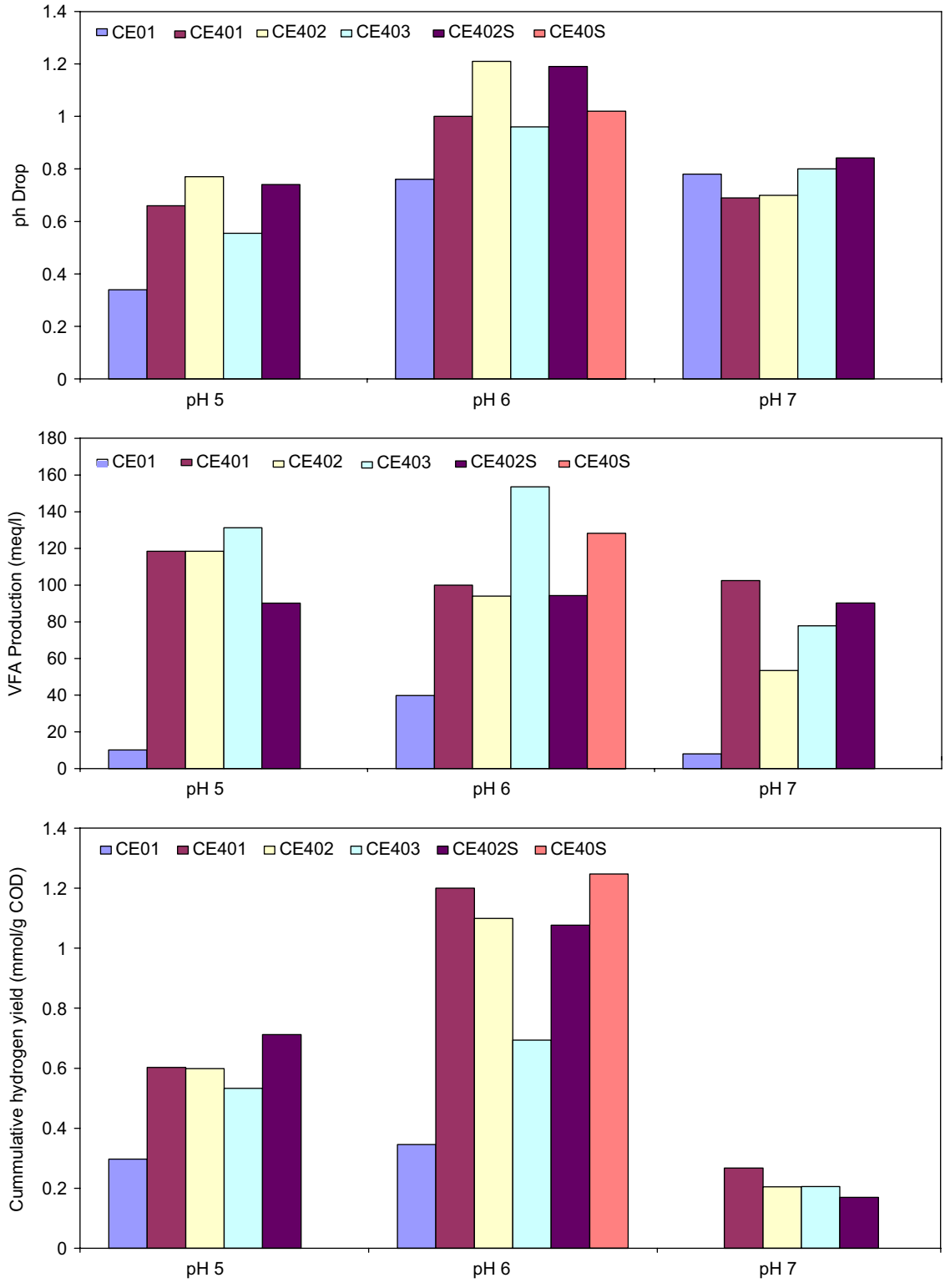

Fig. 4. System pH drop, VFA production and cumulative H_2 yield with the function of fermentation pH and substrate composition.

concluded from the observation that optimum fermentation pH for effective H_2 generation was 6.

The distribution of metabolites formed during H_2 generation was often considered as a crucial signal in assessing the efficiency of H_2 producing cultures [32,33]. Samples during the course of experiments (CE40S) were collected and analyzed for VFA composition (Table 5). To have a better understanding of the change in the metabolic pathway, the ratio of acetate (HAc) to butyrate (HBu), propionic acid (HPa) and ethanol (HEt) were calculated. Determination of the composition

Table 5
VFA composition with the function of fermentation time in experiment CE40S

Fermentation time (h)	HAc/HBu	HAc/HPa	HAc/HEt
0	0	0	0
2	0	0	0
4	4.2	0	0
6	4.9	0	0
8	5.3	6.1	0
10	6.6	5.4	0
12	7.6	4.2	0.08
18	8.1	3.1	0.12
24	8.9	3.8	0.19
48	9.2	2.7	0.24

HAc: acetic acid; HPa: propionic acid, HBu: butyric acid, HEt: ethanol.

of VFA by chromatography revealed the presence of acetate, butyrate, and propionate with relatively lower concentration of ethanol suggesting that, HAc was the major metabolite in the H_2 producing bacterial population. The butyrate concentration was observed after 4 h of fermentation period and the HAc/HBu ratio showed a gradual decline signifying an increasing concentration of butyrate. The generation of HPa was found after 8 h. Relatively lower concentration of ethanol was observed compared to acetate concentration. It is evident from the above discussion that the metabolic phenomena observed at fermentation pH 6 in the present study might be associated with the acidogenesis in spite of solventogenesis which was considered as optimum environment for H_2 generation. This observation was in agreement with the earlier reports [11,36]. Lay [12] suggested that a pH of 5.6 was optimum because a lower pH produced transition from acid to alcohol for mixed cultures. After 10 h of fermentation time a slight switch over to solvent production was observed by a decrease in acetic acid concentration and an increase in ethanol concentration (Table 5). However, this phenomenon would not affect the H_2 production when fermentation period was kept under control. Ginkel and Logan [34] observed a switch over to solventogenesis after 10 h of fermentation. Butyric acid might be more inhibitory than acetic acid when the liquid was saturated with H_2 since availability of electron sinks was reduced [34,35]. The distribution of metabolites suggested that the acid forming pathway dominated the metabolic flow during H_2 production at fermentation pH 6 with the selectively enriched culture.

Experimental data showed that applied sequentially coupled heat-shock and acid pretreatment procedure performed on the anaerobic mixed inoculum showed positive influence on the H_2 generation by selectively enriching the required H_2 producing mixed microflora. Typical anaerobic cultures could not produce H_2 as it acted as an intermediate in the methane formation and was rapidly consumed by MB in the population [37–39]. One of the effective ways to enhance H_2 yield from the anaerobic culture is to restrict or terminate the methanogenesis process to allow H_2 to become an end-product in the metabolic flow [39]. The heat-shock treatment facilitated elimination of non-spore forming methanogens from inoculum and acid treatment permitted elimination of MB group. This process facilitated

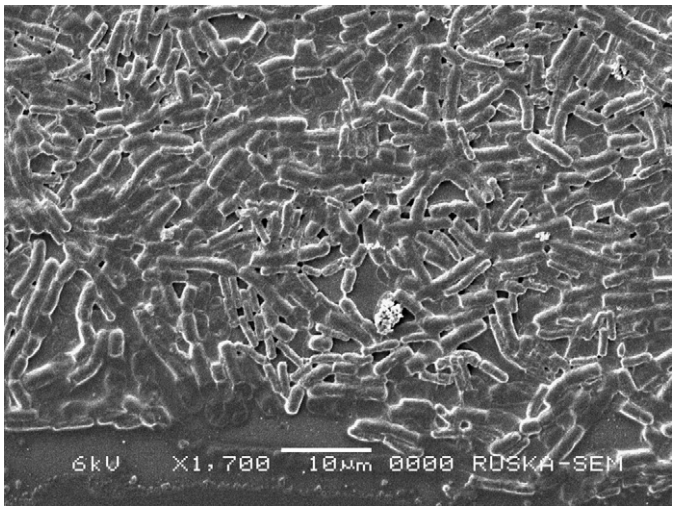

Fig. 5. SEM image (X 1.7 K) of selectively enriched anaerobic mixed consortia.

selective enrichment of spore forming AB group inhibiting the methanogenic activity, which led to the production of H_2 associated with acid generation. The biogas composition showed methane well below the detectable limit at all the fermentation pHs. Hence it could be confirmed that methanogenic population was inhibited and/or killed due to the adopted pretreatment of the inoculum. Moreover, operation of system at acid fermentation pH (acidogenesis) helped to limit the methanogenic activity and maximize biological H_2 production in batch tests. SEM images ($\times$1.7 K; Fig. 5) of the anaerobic mixed culture acquired from experiments (pH 6) visualized slightly bent, scattered and short chain rods along with relatively low frequency of cocci shaped bacteria of 10 µm (approximate length). Images of mixed consortia showed proliferation of morphologically similar group of bacteria. The selective enrichment procedure adopted in this study might have resulted in enrichment of specific group of bacteria capable of producing H_2.

4. Conclusions

The batch studies demonstrated the feasibility of H_2 generation from chemical wastewater as primary substrate using selectively enriched mixed consortia. The adopted pretreatment procedure on anaerobic mixed inoculum [heat-shock treatment and acid treatment] showed positive influence on the overall H_2 production. Chemical wastewater used as a primary carbon source documented its metabolic participation in H_2 evolution. Adding glucose and sewage wastewater as co-substrates along with chemical wastewater showed positive influence on the H_2 generation rate. Fermentation pH 6 was found to be optimum for the overall process efficiency of H_2 generation.

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