FERMENTATIVE HYDROGEN PRODUCTION BY ANAEROBIC BIOFILMS FROM A PRETREATED MIXED MICROFLORA

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ABSTRACT

The present work was focused on fermentative production of hydrogen by anaerobic biofilms from a pretreated mixed microflora in a continuous fixed bed-reactor. Microbial mixed culture was thermally (100°C, by 30 min) and acidic (pH 3.0, 24 h) pretreated. Metagenomic analysis of the pretreated microbial cultured was conducted. Microbial cultured community was mainly formed by lactic acid bacillus from phylum *Firmicutes*. Dairy wastewater (COD 27.73 g L^{-1}, pH 11.3) at a temperature of 18±5°C was fed to a 1.5 L continuous fixed bed-reactor using *Opuntia imbricata* as substratum. Results obtained showed that at an HRT of 0.4 h the yield hydrogen production was 2.11 mmol H_2 g^{-1} $COD_{consumed}$, mean while an increase in HRT to 0.8 h caused a decrease in yield hydrogen production to 1.31 mmol H_2 g^{-1} $COD_{consumed}$.

Cartel presentation.

1.-Introduction

As a consequence of industrial development and population growth there has been an increase of 17-fold in the last century of energy consumption in the world. However, conventional energy resources, like fossil fuels, cannot meet the increasing energy demand [1].This excessive usage of fossil fuels has caused a considerable negative environment impact e.g. global warming and acid rain, resulting in serious effects on the earth's climate, weather conditions, vegetation, and aquatic ecosystems [2]. Therefore, the use of biofuels as alternative energy sources has many advantages, such as contribution to the reduction of CO_2 emission [3]. Hydrogen gas is a clean energy source with a high energy content of 122 kJ g^{-1}. Unlike fossil fuels hydrogen does not cause any CO_2, CO, SO_x and NO_x emissions, and producing water as its only by-product when it burns, reducing green house effects considerably [4].Hydrogen is considered to be a major energy carrier of the future and can directly be used in fuel cells for electricity generation [5]. However, currently 90% of H_2 is being produced from methane reformation or electrolysis of water and contributes to only 3% total energy consumption [6]. Biological hydrogen production from renewable resources using microorganisms appears to be the most attractive method compared to other hydrogen production processes because it has fewer environmental concerns [7].Fermentative hydrogen production with anaerobic bacteria is a promising way of economical and sustainable energy source generation [9]. Recently, experiments have been carried out to study the possibility of hydrogen production using organic wastes from various industries in combination with the wastewater treatment strategy [10]. Dairy wastewater contains complex organics, such as polysaccharides, proteins and lipids, which on hydrolysis form sugars, amino acids, and fatty acids. In subsequent acidogenic reaction, these intermediate products are converted to volatile fatty acids (VFA), which are further degraded by acetogens, forming acetate, CO_2, and H_2 [12].

The objective of this study was to investigate in a continuous flow fixed bed reactor the process of H_2 production from dairy wastewater with an anaerobic pretreated mixed microbial culture for biofilms formation on *Opuntia imbricata* to develop a stable anaerobic fermentation process for continuous H_2 production. *S*ubstratum used on this work has been tested to develop biofilm reactor systems useful for the treatment of different wastewaters. Contrary to some synthetic organic or inorganic polymer materials such as resins, gels and

fibers conventionally used as substratum, *Opuntia imbricata* is renewable with high grade of reusability and without disposal problems [13]. In addition 16S rDNA sequence analysis from anaerobic mixed cultures were carried out to establish predominant bacterial types that grew in the hydrogen process production in this particular work.

2.- Materials and methods

2.1.-Substratum pretreatment and preparation

Small pieces of dried stems of *Opuntia imbricata* were cut (previously wash and rinse) in segments (Fig. 1), and their characteristics are shown in Table 1.

Table 1. Characteristics of substratum

Parameter	Specification
Identification	*Opuntia imbricata*
Configuration	Fixed-bed
Origin	Natural
Height	2.5± 0.2 cm
Diameter	2.5±0.5 cm
Density	0.838 g cm^{-3}
Specific surface area	0.599 $m^2 g^{-1}$ (from BET)

2.2.-Dairy wastewater

Dairy wastewater was collected directly from the company's sewage located in Saltillo, Coahuila, Mexico. After recollection wastewater was stored at 4°C until characterization, and these parameters were determined according to standard methods [14] (Table 2).

Table 2. Dairy wastewater composition

Parameter	Dairy wastewater
pH	*11.32* ± 0.240
COD (g L^{-1})	27.73 ± 0.531
Conductivity (m S^{-1})	2640 ± 52.8
TSS (g L^{-1})	21.9 ± 0.557

2.3.-Pretreatment of anaerobic microbial mixed culture

Anaerobic microbial mixed culture (500 mL) was obtained from a UASB reactor that treated wastewater from brewery Modelo (Zacatecas, Mexico). Pretreatment was carried out as describe by Chen and Hu. In the heat pretreatment the sludge was heated in boiling

water bath for 30 min first, then cooled down and was followed by acidic pretreatment that involved decreasing the pH 3.0 using 0.1N HCl solution for 24 h and a readjustment of pH back to 7.0 by 0.1N NaOH solution.

2.4.-DNA extraction

Metagenomic DNA extraction from pretreated bacterial source sample was assayed according to the silica-based method proposed by Rojas *et al.*

2.5.-PCR amplification

Universal primers gc338 (forward) (5´-ACTCCTACGGGAGGCAGCAG- 3´) and gc518 (reverse) (5´- ATTACCGCGGCTGCTGG- 3´) for the domain Bacteria were used for amplification of the hypervariable V3 region on 16S rDNA. Both primers included a 40 base GC clamp and produced a 230 pb amplicon to be analyzed by DGGE [17]. The PCR reaction mixture (25 µL) consisted of 5 mM MgCl2, 200 µM each dNTP, 0.5 µM forward and reverse primers, 2.5 U *Taq* DNA polymerase (Bioline), 1-5 µL template (approximately 100–200 ng of metagenomic DNA), and 0.3% bovine serum albumin (BSA, Sigma-Aldrich) in a proprietary buffer (pH 8.5). All PCR amplifications were performed on a thermal cycler My Cycler (Bio-Rad Laboratories Inc., USA) using a touchdown program. An initial denaturation for 5 min at 94^{o}C was followed by a total of 30 cycles of amplification consisting of (1) denaturation at 94 ^{o}C for 60 s, (2) touchdown annealing (10 cycles from 65 ^{o}C to 55 ^{o}C, 1^{o}C decrement/cycle; 20 cycles at 55 ^{o}C) for 45 s, and extension at 72 ^{o}C for 60 s. The program ended with an extension step at 72 ^{o}C for 5 min. PCR products were examined and verified on 2% agarose gels and visualized by ethidium bromide staining (Sigma-Aldrich). The gels were photographed under UV light in a Bio-Rad GelDoc 2000 system (Bio- Rad Laboratories Inc., USA).

2.6.- DGGE analysis

Denaturing gradient gel electrophoresis was performed with the DCode^{TM} (Bio- Rad Laboratories Inc., USA) system and reagents from Sigma-Aldrich. Denaturation conditions were optimized based on preliminary results, but generally, gels had denaturation gradients of 40–60%, where 100% denaturant is defined as 7 M urea and 40% formamide. Gels were either 8% polyacrylamide or contained an 8-12% polyacrylamide gradient. Electrophoresis was at 60 V for 16 h. Gels were stained with Sybr\Gold (Invitrogen). Bands in DGGE images were identified visually on a presence–absence basis. PCR products were directly

sequenced by an external sequencing service (Macrogen, USA) with the same primers used for amplification. Contig assembling and sequence alignment was performed with the free software BioEdit [18], and taxonomic classification and nearest neighbors with the RDP database (release 9·1) [19, 20]. The RDP classifier uses a naïve Bayesian method [21]. Phylogenetic inference based on 16S rRNA gene sequences was carried out following the maximum parsimony method by using bootstrap values based on 100 replications with program Winclada [22].

2.7.- Biofilm formation on *Opuntia imbricata*

Biofilm formation was carried out in a 1.5 L continuous fixed bed reactor (Fig.1), 520 g (dried weight) of *Opuntia imbricata* (substratum preparation describe previously) was added and 500 mL of pretreated anaerobic microbial mixed culture. Reactor was fed by a peristaltic pump (Manostat - division of Barnant Co.) for a period of 30 days with following conditions; initial pH 7.0, temperature 20 ± 3 °C, HRT=2 h and initial wastewater concentration was 27.73 g COD L^{-1}, maintaining an initial concentration of substrate up to 12 g/l thus to control a rank of pH of 4-5.5 by means of the effect of the substrate concentration; during this period all parameters mentioned above were monitored on daily basis. Subsequently HRT was decreased to 1 hr for a period of 30 days, maintaining rest of conditions.

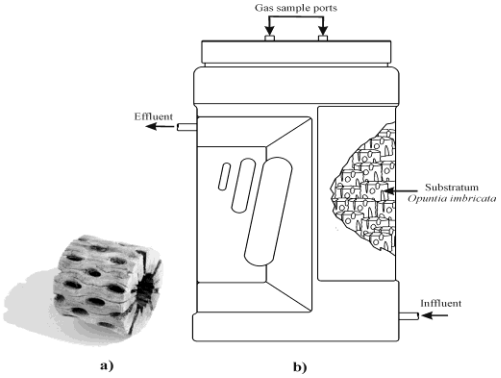

Figure 1. Continuous fixed bed up-flow reactor packed with Opuntia imbricata used for hydrogen producing biofilm formation.

2.8.- HRT influence on hydrogen production

Two HRT's (0.4 h and 0.8 h) were assessed for this stage of the work. Dairy wastewater concentration was 27.73 gL^{-1}, pH 11.32 and temperature (ambient) 18 ± 5°C.

2.9.- Analysis.

The analysis of hydrogen and methane were determined by gas chromatography (GC TCD) Varian 3400, equipped with a Molecular Sieve 5ª packed column injecting 25 µl using a 1 ml syringe. GC conditions were as followed: injector and detector temperature 200 °C, column temperature 50°C, using helium as carrier gas with flow rate 6 ml/min. Removal of COD was determined according to analytical methods described in standard methods [14]. The pH was determined from effluent and measured by potentiometer (WTW, Inolab-pH/ION Level 2). All data collected represents the means of three replications.

3.0- Results and discussions

3.1.-Metagenomic analysis of pretreated mixed microbial consortium pretreated

The microbial community of the biofilm used for biohydrogen production was studied by PCR and DGGE analysis of the pretreated mixed microbial consortium. The metagenomic DNA extracted from the consortium had a high molecular weight, with a size near to 20 kb and show scarce evidence of degradation. Molecular analysis using PCR-DGGE followed by sequencing enabled characterization of the bacterial population in the biofilm reactor (Figure 2a).This approach allows detection of the dominant bacteria present in the samples by sequencing the interesting gel bands (Figure 2b). Excised DGGE bands were compared with BLAST references based on the phylogenetic relationship of the ~230 bp partial 16S rDNA sequence of the hypervariable V3 region. The V3 region is regarded as a good choice when it comes to length and species–species heterogeneity [23,24], the region is also considered to be highly variable and have a high grade of resolution. Comparison of the 16S rDNA sequences to databases showed most belonged to the *Firmicutes phylum*, being lactic acid bacteria the predominant population in the mixed microbial consortium. These sequences share a high identity to different taxonomic ranks such as Bacillales and Lactobacillales. Recent reports of hydrogen production by microbial mixed culture made same approach by metagenomic analysis, [25]. carried out the 16S rDNA analysis of DNA extracted from bioreactors during periods of high H_2 production from cheese processing wastewater observing that more than 50% of the bacteria present were members of the genus *Lactobacillus* and about 5% were *Clostridia*; Angelidaki *et al.* carried out same analysis for identification of hydrogen producing bacteria from the granules obtained of thermophilic anaerobic reactors (60 and 70 °C) with different carbon source, they found

that *Firmicutes phylum* had a population density of 15-27% at 60 °C and 17-20% at 70 °C, confirming early reports by Cheong *et al.* whom mentioned that some species of *Bacillus* and *Clostridium***,** are hydrogen producing bacteria, and resistant to high temperatures and desiccation due to formation of spores, remaining only microorganisms capable of forming spores and eliminating methanogens that do not posses this capability.

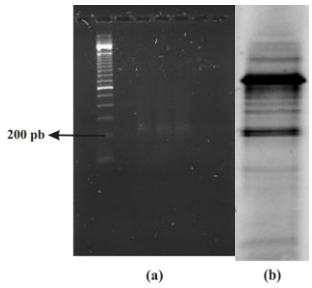

Figure 2. a) PCR product amplified with universal primers gc318 and gc518. ; b) DGGE analysis of a pretreated mixed microbial consortium for the fermentative hydrogen production from dairy wastewater. The $_{mg}$ADN extracted was PCR amplified using primers gc318/gc518.

Some other works have mentioned the effect of acidic pretreatment on microbial community, causing important change on community microbial distribution, as mentioned by Hwang *et al***,** that used FISH method and the LIVE/DEAD cell viability test and observed that after the acid pre-treatment (HCl, HNO_3, and H_2SO_4), the rate of bio-H_2 production increased rapidly, and the number of viable cells belonging to *Clostridium sp.* also increased.

3.2.- Influence of HRT on hydrogen production in fixed bed up-flow reactor

Hydrogen production on a continuous fixed bed up-flow reactor at different HRT‘s (0.8 and 0.4 hr), with initial COD concentration 27.33 gL^{-1}, pH 11.32 at ambient temperature (18 ± 5°C) is shown on figure 3a. Hydrogen production was higher at HRT of 0.4 h, with maximum of 283 mL of hydrogen after 12 hours (1.2 fold higher than HRT 0.8 h) and only 128 mL of hydrogen after 20 hours for the case of HRT of 0.8 hours. Figure 3b shows same behavior of cumulative hydrogen production at different HRT´s, where at HRT of 0.4 hours there was an accumulated production of 880 mL of hydrogen after 60 hours, whereas at HRT of 0.8 hours there was only 353 mL of hydrogen after 48 hours. These differences were reflected directly on hydrogen yield production, showing that at an HRT of 0.4 h,

hydrogen yield production was 2.11 mmol H_2 g^{-1} $COD_{consumed}$, whereas an increase on HRT caused a drop on hydrogen yield production to 1.31 mmol H_2 g^{-1} $COD_{consumed}$ (Table 3). Yang *et al.*, obtained a hydrogen yield production of 1.8-2.3 mmol H_2 g^{-1} $COD_{consumed}$ at an HRT of 24 hours at a pH range of 4.0-5.0 using synthetic dairy wastewater under mesophilic conditions.

Table 3. COD removal (%) and H_2 yield production by a continuous fixed-bed up-flow reactor with Opuntia imbricata as substratum at different HRT's

HRT (h)	**H_2 yield production (mmol H_2 g^{-1} COD_c)**
0.4	2.11
0.8	1.31

COD_c – Chemical Oxygen Demand consumed

Figure 3a shows a lag phase of 4 hours when an HRT's of 0.8 hours was used, this behavior is due to a lower decrease rate of pH level in the medium (see figure 4). Behavior of pH can be observed in figure 4. For HRT of 0.4 hours the decrease rate pH level was faster, reaching a pH of 5.4 after 12 hours, whereas with a HRT of 0.8 hours, pH only decreases to 7.01 during the same period of time

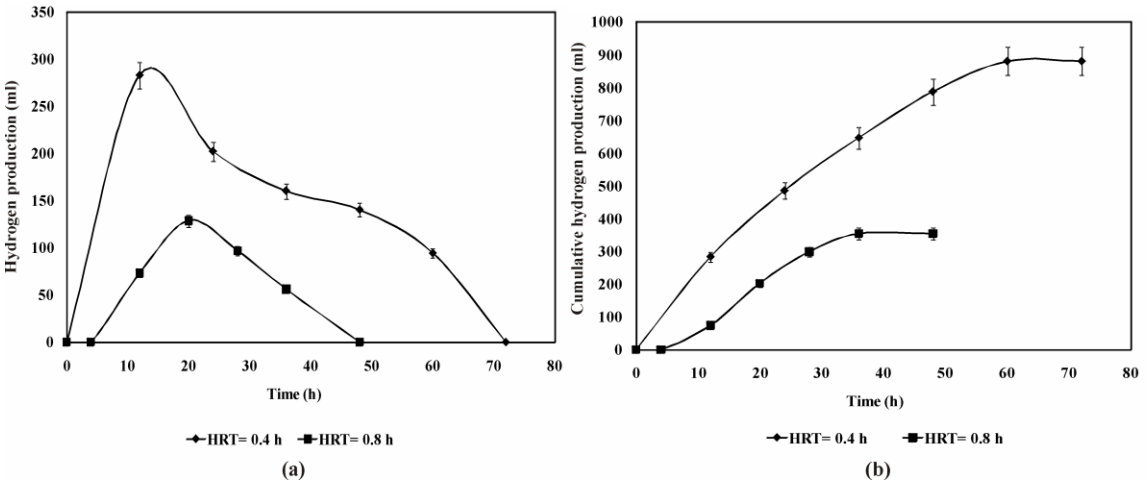

Figure 3. a) Hydrogen production (mL) and b) cumulative hydrogen production (mL) at different HRT's (0.4 y 0.8 h), by a continuous fixed-bed up-flow reactor with Opuntia imbricata using dairy wastewater (COD 27.73 g L^{-1}; initial pH 11.32; temperature 18 ± 5°C).

It is mentioned when using a microbial mixed culture and drastic changes on pH, HRT, temperature and substrate concentration occurs, this can affect the metabolism of hydrogen

producing bacteria or a shift on the microbial community can take place, leading to a change in fermentation sub-products and decreasing the hydrogen yield production [29].

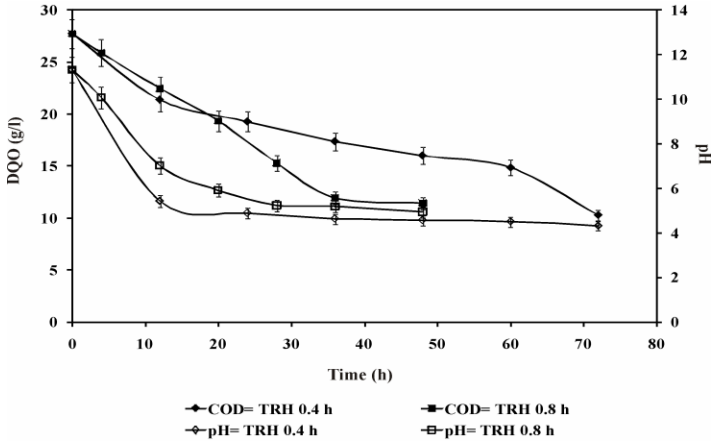

Figure 4. COD removal and behavior of pH of the effluent of a continuous fixed-bed up-flow reactor with Opuntia imbricata as substratum at different HRT's (0.4 and 0.8 h) using dairy wastewater (COD 27.73 g L^{-1}; initial pH 11.32; temperature 18 ± 5°C).

Fermentative hydrogen production with microbial mixed culture can take place in a wide range of pH, from 3.3 to 5.0 [30,31] contributing to inhibition of methanogenic bacteria that cannot tolerate pH below 5 according to different reports [32]. However, Kim *et al.*, 2003, reported that *hydrogenotrophic* methanogens can tolerate pH below 5 but was not the case for acetoclastic methanogens that were completely inhibit. It also mentioned by Kim *et al.*, 2003, that at high HRT´s *hydrogenotrophic* methanogens can tolerate acidic conditions more than other methanogens groups. Chen *et al.*, 2001, reported a slight methanogenic activity under high dilution rates conditions (HRT of 1 hr), however they mentioned if the characteristics of wastewater required a large time of hydrolysis, methanogenic activity may not be inhibit if HRT is not short enough. *Chen et al., 2001, also mentioned* that a shift in pH from 4.3 to 6 caused a cease in hydrogen production and only carbon dioxide was produced. These results indicate that a reduction in microbial activity occurs or a shift on the microbial community took place.

Figure 3a also shows that in both HRT cases there was a decrease in hydrogen production as a result of substantial COD consumption (see Figure 4), where at an HRT of 0.8 hours COD after 36 hours diminish to 11.9 g L^{-1} and for the case of HRT of 0.4 hours it was 17.3 g L^{-1}. Early studies on batch showed that hydrogen production was detected at COD concentration over 12 g L^{-1}.

Previous studies on batch proved that hydrogen production took place on concentrations of COD higher than 12 g L^{-1}, however on concentrations below 12.0 g L^{-1} of COD, methane production is enable leading to a decrease on hydrogen production [34]. This can be observed on figure 6 where removal of COD from 14.8 g L^{-1} to 10.3 g L^{-1} after 60 to 70 hours at an HRT of 0.4 hours, influence the hydrogen production process causing a decrease in hydrogen as mentioned before (figure 3a). COD removal efficiency for both cases was 62.34 % after 72 hours and 58.78 % after 48 hours for HRT of 0.4 and 0.8 hours respectably. This concurs with early reports by [35,36] in which using high concentration of substrate (glucose 15-25 g L^{-1}) hydrogen production was detected, and Kyazze *et al.*, 2006, reported hydrogen production even at higher concentrations of substrate using sacarose up to 40 g L^{-1}.

Figure 5 shows that an HRT of 0.4 hours, methanogenic activity was completely inhibit due to fast drop on pH (4.5- 5.0) favoring hydrogen producing bacteria. However, in spite of being a short HRT (0.8 h) and taking in account early reports in which HRT's similar to the those use on this study (0.5 h to 24 hours), in where it was reported a high yield hydrogen production [37, 38], for our case (0.8 h) it was observed that methanogenic activity was not completely inhibit. This behavior can be explained due the use of biofilms system in which regularly the specific activity is increased and also this type of systems offer a higher operational stability [39].Also this is mentioned by Wu and Chang, in which the microbial community is protected by the polymeric matrix and allows tolerating extreme environmental conditions.

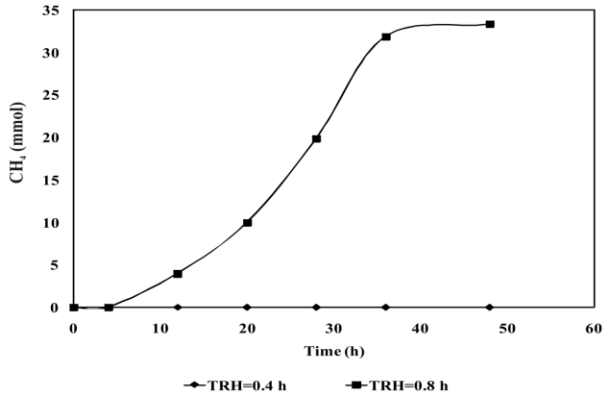

Figure 5. Methane production (mmol) on a continuous fixed-bed up-flow reactor with *Opuntia imbricata* as substratum at different HRT's (0.4 and 0.8 h) using dairy wastewater (COD 27.73 g L^{-1}; initial pH 11.32; temperature 18 ± 5°C).

4.- Conclusions

Results obtained by metagenomic analysis of the microbial mixed culture concluded that the main bacterial microbiota was found to be lactic acid bacteria of *Firmicutes phylum*.

HRT had a strong influence on yield hydrogen production, showing that the change of HRT from 0.8 to 0.4 hours enhanced the yield hydrogen production form 1.31 mmol H_2 g^{-1} CODconsumed to 2.11 mmol H_2 g^{-1} CODconsumed.

The use of *Opuntia imbricata* as substratum for biofilm formation enhanced the hydrogen production, offering economical and technical advantages over the use of pure culture, avoiding expensive sterilization requirements, extra use of energy for temperature and pH control.

5.- Acknowledgements

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