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Abstract

The main objective of this work was to study the feasibility of biohydrogen production by heat-treated anaerobic sludge with glucose as carbon substrate and ammonium as nitrogen source, obtained from the electrochemical reduction of concentrated nitrate solutions (3 g L^{-1} NO_3^-), namely model solutions simulating those obtained after physicochemical denitrification of industrial wastewaters. Experiments were realized in bacth systems involving 125 mL (without pH control) and 3 L (with pH control) reactor, respectively.

In order to find the optimal conditions for H_2 production, the first set of experiments were conducted without pH control at various initial pH (pH 5.5, 6.5 and 7.5) and glucose concentrations (5-30 g L^{-1}); and the highest H_2 yield (1.1 mol H_2 $mol^{-1}_{glucose\ consumed}$) was reached for a glucose concentration of 25 g L^{-1} and at pH 6.5. These optimal conditions were then considered to examine the effect of pH control on biohydrogen production, in a 3 L bioreactor. The pH control allowed a considerable improvement of the hydrogen gas conversion efficiency, which reached 2.02 mol H_2 $mol^{-1}_{glucose\ consumed}$.

Introduction

Hydrogen is considered as an attractive future clean and renewable energy carrier due to its environmental-friendly conversion. Indeed, its combustion product is water and it has a high energy content [1], since the energy yield of hydrogen is 122 kJg^{-1}, namely 2.75 times that of fossil fuel [2]. During the last decades, several processes have been used for hydrogen production [3], including electrolysis of H_2O, thermocatalytic reformation of hydrogen rich substrates and various biological processes [4,5]. Among these methods, microbiological hydrogen production has attracted considerable attention because it has the potential to convert low cost residues or organic waste/wastewater to hydrogen [1−3]. According to the type of employed microorganisms, biological H_2 production under anaerobic conditions can be classified into two different categories: dark and photo fermentation processes [6]. The most promising and widely investigated process is dark fermentation. It has more advantages than photofermentative H_2 production, including lower costs since energy provided by sunlight is not required, high hydrogen production rate and simple process control with a wide range of temperature and pressure conditions which can be implemented [7,8]. Theoretically, a maximum of 4 moles of hydr*ogen per mole glucose converted via the acetate pathway can be generated via dark hydrogen fermentation; however, the reported yields are typically below this value, due to the generation of by-products [9]. In addition to acetate, small organic compounds such as butyrate and ethanol are also produced, and are termed ''dead-end'' or ''fermentation barrier,'' and limit the hydrogen yield to a maximum of 2−3 mol H_2 [7]. Usually, no more than one third of the total potential electrons in complex biomass can be transferred to hydrogen, while the remaining, namely two thirds, end up in the form of these fermentation by-products, enhancing therefore the total costs of biohydrogen production process. High hydrogen yields are however required to make the process economically feasible [10].

The most noteworthy study is that lately reported by Rivera *et al.* [11] in which they proved that the bio-electrochemical systems fed with dark fermentation effluents is a way forward to get higher H_2 production efficiency.

Many factors must be taken into consideration for enhancing hydrogen production in fermentation systems, such as pH, substrate concentration, inoculum, temperature and nitrogen sparging [12,13]. Abiotic factors such as the type of bioreactor is also of key interest, since it was demonstrated by Kumar *et al.* [14] that advanced fermenter configuration incorporating upstream, such as H_2 production and subsequent downstream including biohydrogen recovery and purification may lead to novel and promising biohydrogen systems.

A broad spectrum of carbohydrate rich substrates have been considered to producebiohydrogen, such as starch [15], xylose, agricultural wastes [16], lignocellulose [14] and glucose [17–22]. However, the use of glucose or the conversion of raw material to glucose is favored because it is easily biodegradable by most of bacteria [12]. Nevertheless, the chosen glucose concentrations must be low enough in order to minimize pH changes resulting from acids produced during fermentation [23]. Indeed, it was shown that pH is among the major environmental factors which affects biohydrogen production due to its effect on the hydrogenase activity, the enzyme involved in the metabolism of hydrogen in most bacteria [24]; optimal initial pH value for biological hydrogen production is generally found to be between 5.0 and 6.5 [25]. However, optimum pH value can vary according to the carbon substrate used, from pH 9 for sucrose [26] to pH 5.5 for glucose [27].

Consequently, lowering production cost is still a key issue to project this technology to an industrial-scale. Nitrogen and carbon feedstock represents 30–40% total costs of fermentative hydrogen production [28]. Nitrogen is one of the most essential nutrients needed for the growth of hydrogen-producing bacteria. It is a very important component for nucleic acids, proteins and enzymes that are of great significance to the growth of hydrogen-producing bacteria. Thus, an

appropriate level of nitrogen addition is beneficial to the growth of hydrogen-producing bacteria and to dark fermentative hydrogen production accordingly [29].

One way to make biological hydrogen production providing simultaneously economic and environmental benefits consist of the coupling the biohydrogen production to another physicochemical depollution process, such as that of nitrate ions (NO_3^-).

NO_3^- contamination of water sources has become a serious problem around the world [30]. Nitrate causes methemoglobinemia in infants and eutrophication of water bodies [31].

The transformation of NO_3^- to the non-toxic nitrogen gas (N_2) is the most widely used electrochemical method for nitrate removal from contaminated water [32–35], however the rate and the selectivity of the reaction are usually low, limiting therefore their utility for large scale application [36]. In this context the use of coupling process can allow to overcome these limitations.

Electrochemical and biological processes were coupled for the removal of heavy metals [37] and biorecalcitrants compounds [38,39]. However and to the authors' knowledge, their previous work was the sole study dealing with the feasibility of such coupled process to produce biohydrogen while treating nitrate ions from water [36]. On the one hand, according to the literature copper cathode (Cu) is known to be the most efficient electrocatalyst for the electro-reduction of nitrate by mainly producing ammonium as final product [30,40]. On the other hand, inorganic ammonium (NH_4^+) was the most widely investigated nitrogen source for fermentative hydrogen production [18,22,23,41,42], owing to its lower cost if compared to organic nitrogen sources, such as for instance yeast extract [43,44] and polypepton [45,46].

For these reasons, from an environmental and economical point of view, an electrochemical reduction of nitrate to NH_4^+ over Cu cathode can be considered as a promising and an attractive way, since it allows not only the treatment of nitrate, but also energetic valorization of the nitrate treated effluent.

Therefore, the interest of this work does not reside only in the biological hydrogen production, but also in the finding of an alternative nitrogen source, ammonium solution resulting from nitrate electrochemical treatment, as substitute for expensive and commercial nitrogen products usually investigated in fermentative hydrogen production studies [41,44,45,47]. This should also allow to simultaneously decrease the global cost of the denitrification and the hydrogen production cost, making the hydrogen gas more available and cheaper.

In a previous study [48] the feasibility of coupling electrochemical and biological processes to destroy nitrate ions (NO_3^-) from water while producing biohydrogen were proved. However, the comparison of the H_2 yield obtained in this study using aerobic activated sludge and ammonium solution generated electrochemically to those of the literature involving anaerobic activated sludge and performed under similar experimental conditions (batch study, glucose as a carbon source and similar optimal pH) shows lower hydrogen yields. Indeed, 0.35 mol H_2 mol^{-1}glucose consumed was obtained by the authors [36] versus for example 1.8 mol H_2 mol^{-1} mol^{-1}glucose and 2.2 mol H_2 mol^{-1}glucose obtained by Wang and Wan [49] and Lee et al. [50], respectively. The optimization of hydrogen production efficiency using more adequate inoculum, anaerobic activated sludge, and by controlling the pH medium was therefore the purpose of the present work.

2. Materials and Methods

2.1. Electrochemical nitrates pretreatment

All the chemicals used in this study were purchased as reagent grade from Alfa-Aesar (Schiltigheim, France).

The electrochemical pre-treatment of nitrate solutions (3 g L^{-1} NO_3^-) prepared in phosphoric acid (H_3PO_4 10^{-2} mol.L^{-1}) and adjusted to a pH of 1.1 using sulfuric acid 96% has been carried out as described in a previous study [48]. These high nitrate concentrations were chosen to be similar to real effluents obtained after the treatment of drinking water contaminated by nitrate using ion

exchange treatment methods [51,52]. This electrochemical step was based on a home-made flow cell using a copper porous material (5.6 cm diameter and 0.3 cm thickness) as a working electrode prepared as previously described [53]. The electrolyte solution (3 g L^{-1} NO_3^- in 10^{-2} M $H_3PO_{4)}$ percolated the porous copper electrode at a constant flow rate of 2 mL min^{-1} monitored by a Gilson minipuls 2 peristaltic pumps (Middleton, WI, USA). The current intensity was generated by a home-made power supply (30 V/3 A).

To calculate the applied current intensity (A) the following relation was used:

$$i \text{ (A)} = [NO_3^-]_{t=0} \times d \times (10^{-3}/60) \times n \times F \tag{1}$$

where $[NO_3^-]_{t=0}$ is the initial nitrate concentration (mol L^{-1}), d is the flow rate (mL min^{-1}), n is the number of exchanged electrons, $8e^-$, for the conversion of nitrate to ammonium and F is the Faraday's constant (96500 C).

After only one pass through the electrochemical flow cell, the performance of the electrochemical conversion of nitrate into ammonium was evaluated using the following parameters: the chemical yield of ammonium formation (% X_{NH4+}) (Eq. 2) and the selectivity based on the ammonium yield (% S_{NH4+}) (Eq. 3).

$$\% \ X_{NH4+} = ([NH_4^+]_t \times 100) \ / \ [NO_3^-]_{t=0} \tag{2}$$

$$\% \ S_{NH4+} = ([NH_4^+]_t \times 100 \ ) \ / \ ([NO_3^-]_{t=0} - [NO_3^-]_t) \tag{3}$$

where $[X]_{t=0}$ and $[X]_t$ are the concentrations of NH_4^+ or NO_3^- in solution at initial time t_0 and a given time t (s). All the concentrations are in mol L^{-1}.

The effluents collected after nitrate electrochemical reductions were used for subsequent biohydrogen production tests.

2.2. Seed sludge and medium composition

The anaerobic microflora used in this study was collected from an anaerobic digester for methane production located in a local municipal wastewater treatment plant (Beaurade, Rennes, France). Prior to use, the seed sludge was first washed four times with tap water, and was then

sieved to remove sand, stone and other coarse matters. Thereafter a heat treatment of sludge at 102°C for 90 min was performed in order to enrich H_2-producing bacteria and to inhibit the hydrogenotrophic methanogens activity.

One liter of the hydrogen production medium for this inoculum contained:

360 mg $MgSO_4.7H_2O$, 48 mg $NiCl_2.6H_2O$, 66 mg $CaCl_2.2H_2O$, 50 mg $FeCl_3$, 30 mg $MnCl_2.4H_2O$, 21 mg $CoCl.6H_2O$, 23 mg $ZnCl_2$, 10 mg $CuCl_2.2H_2O$, 175 mg K_2HPO4 and 175 mg K_2HPO4 [27].

In all experiments the glucose and the electrolyzed solution collected after electrochemical treatment of the nitrate solution (3 g L^{-1} NO_3^-, namely 484 mmol L^{-1}) were used as a carbon and nitrogen sources, respectively.

2.3. Batch tests in 125 mL glass bottles

The batch tests were carried out to investigate the effects of initial pH and glucose concentration on biohydrogen production experiments. 125 mL glass bottles with a working volume of 100 mL were used for the batch tests. All experiments were carried out in duplicate; the mean values with the corresponding error were reported in the figures.

(1) The pH of the medium culture was buffered using 0.07 mol L^{-1} of 2-(N-morpholino) ethanesulfonic acid monohydrate (MES), an inexpensive and nontoxic buffer [10,23].

(2) The reactors were inoculated with the heat-treated sludge at 4 g L^{-1}.

(3) To provide anaerobic conditions, the headspace was replaced with nitrogen gas (purged for 5 min).

(4) Finally, bottles were incubated in a shaker (New Brunswick, Innova 40) at 180 rpm and at a constant temperature (37 °C± 1). This temperature was adopted because the majority of biohydrogen production studies was conducted under mesophilic conditions, typically in the range of 35-37 °C [18,21,27,41,54]. This pH range is favorable for the cultivation and the

hydrogen-producing activity of microorganisms. Consequently, it increases the production yield of hydrogen from residual organic substrates [55].

Two series of experiments were conducted to study H_2 production.

Due to the fact that an initial glucose concentration of 15 g L^{-1} was previously found as the optimal carbon concentration for biological hydrogen production using aerobic activated sludge [48], this glucose concentration was considered for the first series of experiments, which was dedicated to examine the effect of initial pH values on H_2 production efficiency. Three different pH values (5.5, 6.5 and 7.5) were chosen, which were adjusted using 0.07 mol L^{-1} MES buffer.

In the second experimental series, the effect of the initial glucose concentration was studied using different amounts of glucose (5, 10, 15, 20, 25 and 30 g L^{-1}).

The pH measurements were performed using a 9165BN pH electrode connected to an Orion-828 pH Analyzer (Orion Research Inc., Beverly, MA, USA).

Hydrogen yields were calculated based on the following equation:

H_2 yields (mol H_2 mol^{-1} $_{glucose\ consumed}$) = H_2 produced (mol) / Glucose consumed (mol)   (4)

2.4. Batch reactor experiments in 3L bioreactor

The optimal conditions (glucose concentration and pH) determined above in the section 2.3, were considered thereafter to produce biohydrogen in a 3L fermentor (New Brunwick Scientific BioFlo 110) operated in batch mode and an inlet substrate concentration of 25 g glucose L^{-1}. The bioreactor was filled with 1.5. L (working volume) with culture medium beforehand sparged with nitrogen gas to achieve anaerobic conditions and inoculated with heat-treated anaerobic sludge (4 g L^{-1}). The pH of the mixed liquor was kept constant at 6.5 by feeding 4 mol L^{-1} NaOH or 2 mol L^{-1} HCl solutions via respective peristaltic pumps. The temperature was kept at 37°C with a temperature controller, and the agitation rate was 180 rpm. A biogas sampling port was installed in the reactor to allow direct biogas sampling with a syringe. For kinetic studies of batch anaerobic

hydrogen production, samples (2 ml) were withdrawn periodically from the reactor with a needle inserted through a port on the top surface).

2.5. Analytical methods

The liquid samples were analyzed for biomass concentration, residual glucose concentration, volatile fatty acids and solvents. Biogas percentages in the reactor headspace were also measured.

2.5.1. Biomass concentration (Microbial growth analysis)

Biomass growth was measured in terms of Suspended Solids (SS, g L^{-1}). Where 5 mL of sludge was disposed in a previously weighed porcelain cup (P1) and placed in an oven at 105 ° C until having a constant weight (P2). The Suspended solids were calculated according to Eq.5:

$$SS \ (g \ L^{-1}) = (P2-P1) \ / \ 0.005 \qquad (5)$$

2

2.5.2. Liquid samples

NH_4^+ concentrations in solution resulting from nitrate electroreduction and that remained after biological hydrogen production were measured by UV−vis spectroscopy as reported elsewhere [56–58]. NO_3^- and NO_2^- ions were determined by ion chromatography (Dionex 120) equipped with a chemical suppressor (ASR 300 -ultra 4 mm) and a Dionex on Pac AS19 Anion Exchange column (4x250mm). 2 mol L^{-1} KOH was used as eluent at a flow rate of 1.04 mL min^{-1}.

Gas chromatography−mass spectrometry (GC/MS) measurements of the soluble microbial products (SMPs) in the fermentation solution were performed with a Perkin Elmer Clarus 500 apparatus (California, United States) coupled with a Variance CB-FFAP 0.25 μm column (25 m × 0.15 mm). Compounds identification was conducted with the help of a list database (Nist). GC/MS analysis showed that acetic, butyric, isobutyric acids and ethanol were the main liquid metabolites (data not shown). Other compounds were detected at trace levels, such as acetaldehyde.

Analysis of fermentation by-products (volatile fatty acids (VFAs), lactic acid and ethanol) contained in the aqueous phase was carried out by gas chromatography (GC Thermo Focus) equipped with a flame ionization detector (FID) and a FFAP-CB column (15 m $\times$ 0.1 mm, 0.1 µm) specially adapted to the separation of volatile fatty acids. In order to reduce the presence of suspended solids, liquid samples were centrifuged at 8000 rpm for 10 min at 4ºC then acidified by 6 mol L^{-1} HCl and filtered through 0.25 µm membrane before analysis. The addition of HCl solution to the sample allows the protonation of the volatile fatty acids to be analyzed. The injector and detector were operated at 200 and 250 °C, respectively. The oven was held at 90°C for 0.5 min; then heated to 200 °C with a ramp of 40 °C min^{-1} and maintained at 200°C. Nitrogen was used as carrier gas with a flow rate of .2 mL min^{-1}. The column head pressure was controlled at 35 Pa.

Glucose consumption was quantified using a high performance liquid chromatography (HPLC) system (Shimadzu LC-20AD) which was equipped with an ions exclusion column HPX-87H (300 x7.8 mm; Bio-Rad, Hercules, CA, USA), a Shimadzu RIO-6A refractive index (RI) detector and a photodiode array (PDA) analyzer. 0.5 mol L^{-1} H_2SO_4 was used as mobile phase at a flow rate of 0.7 cm^3 min^{-1} (waters pump, Milford, MA, USA).

CO_2 and N_2O were measured as specified in the following section (2.5.3).

2.5.3. Biogas content

The proportion of hydrogen and methane in the produced gas was determined by a µGC (Agilent Micro GC3000, SRA Instruments) equipped with a thermal conductivity detector (TCD) and a stainless steel column (30m) packed with molecular sieve 5Å. The temperatures of the column and detector were maintained at 60°C and 100°C, respectively. In order to determine the proportion of carbon dioxide and nitrous oxide, the same gas chromatograph (Agilent Micro GC3000, SRA Instruments) was used, equipped with a TCD and a stainless steel column (10 m) packed with Porapak Q. The temperatures of the column and the detector were maintained at 70°C and 100°C,

respectively. Pure helium (6.0 quality) was used as carrier gas and the pressure through the columns was fixed at 40 psi.

3. Results and Discussion

3.1. Batch study in 125 mL glass bottles without pH control

3.1.1. Effect of the initial pH

Nitrate electrolysis was performed with NO_3^- solutions of 3 g L^{-1}, phosphoric acid (10^{-2} M H_3PO_4) was used as supporting electrolytes. It is noteworthy that such electrolytes were chosen to prevent the addition of a phosphorus source, needed for the subsequent bacterial culture. Using the optimal conditions determined in a previous study [53] namely a current intensity of (1.24 A) applied between the copper porous electrode and two counter electrodes made of fine platinum grids and a flow rate of 2 mL min^{-1}. After only one pass of concentrated nitrate solutions through the flow cell, the NO_3^- electroreduction to ammonium was quasi quantitative in the acidic solution with a % X_{NH4+} of 92.5%, namely a very high NH_4^+ ions selectivity (%S_{NH4+} = 98.7%) corresponding to 0.86 g L^{-1} NH_4^+. Ammonium solutions were then tested as a nitrogen source to produce H_2 in a batch system.

In this context, three ammonium solutions at different initial pH values (5.5, 6.5 and 7.5) were inoculated with anaerobic sludge pretreated thermally. For each one, the initial glucose concentration was set at 15 g L^{-1}, optimal glucose concentration for hydrogen production previously recorded in the case of aerobic sludge [48]. Analysis of the residual glucose concentration after 48 hours of dark fermentation shows similar trend as the yields of H_2 related to the initial pH, namely a peak for 6.5 initial pH (Figure 1).
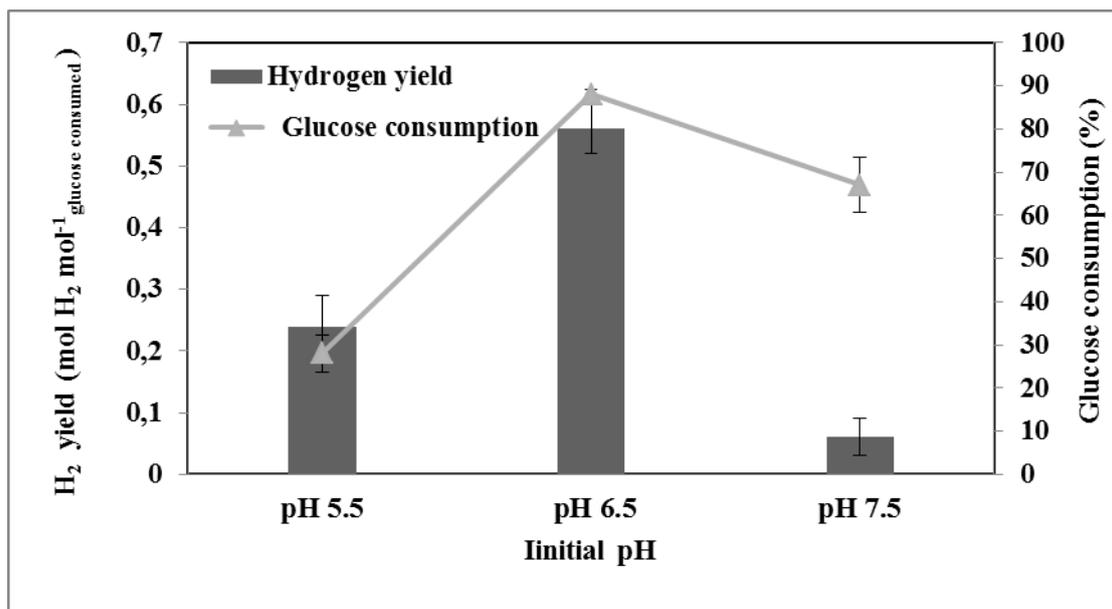
Figure 1 : H_2 yields and glucose consumption versus the initial pH (Error bars are based on duplicate experiments. Initial glucose concentration: 15g L^{-1}).

Indeed, at pH = 6.5, 88% of the initially added glucose (15 g L^{-1}) was consumed and hydrogen yield reached 0.56 mol H_2 $mol^{-1}_{glucose\ consumed}$ (Figure 1).

In parallel with hydrogen production, high ammonium assimilation yields were also observed, they varied between 75% and 85% after 48 h culture (Table 1), with a maximum of 85% obtained at pH 6.5.

Another important parameter to be considered is the quality of the produced biogas, that should mainly contain H_2; it is therefore important to minimize as much as possible other gases. Analyses of biogas by microGC showed the absence of H_2S (hydrogen competitor), CH_4 and N_2O formation throughout experiments, including the biotic controls. But in contrast to the control realized without addition of glucose and nitrogen sources wich was free of H_2 and CO_2, at the three pH values the produced gas contained only H_2 and CO_2. The molar ratio (CO_2/H_2) is a good indicator for the purity of biogas; according to table 1, a sharp minimum (0.1) was obtained for an initial pH of 6.5.

Table 1 : Summary of the results obtained after 48 h culture in 125 mL batch reactors at different initial pH values. Error bars are based on duplicate experiments; Initial glucose concentration: 15 g L^{-1}.

Initial pH	NH_4^+ assimilation (%)	Final pH	[a] R (CO_2/H_2)	[CH_4] (mol.L^{-1})	SS (g L^{-1})
5.5	75 ± 2.6	5.1 ± 0.1	1.1 ± 0.1	0.0	15.0 ± 1.5
6.5	85 ± 3.2	4.98 ± 0.2	0.1 ± 0.01	0.0	14.0 ± 4.5
7.5	80 ± 2.4	5.53 ± 0.3	1.5 ± 0.2	0.0	15.5 ± 2.8

[a]R (CO_2/H_2)) is the ratio between the molar concentrationsof produced CO_2 to H_2 at different pH.

In the studied pH range (Table 1), whatever the initial pH value, the final biomass concentration remained roughly similar, namely close to 14-15 g L^{-1}. However, the differences between the final pH, even if limited, can lead to significant changes in the metabolic activity of hydrogen producing bacteria, characterized by very different amounts of produced hydrogen and carbon dioxide. This behavior is probably due to a change in metabolic pathways of the bacterial consortium; pathway that promotes or not hydrogen production. To confirm this hypothesis the results of an analytical monitoring of soluble microbial products (SMPs) resulting from glucose fermentation under different initial pH were shown in table 2. As shown in table 2, at pH 5.5 the main soluble products were acetic acid followed by butyric and isobutyic acids; while low amounts of acetaldehyde were detected in the three media. At pH 5.5, hydrogen production was mainly accompanied by acetic acid and ethanol formation and then, with the increase of the medium pH, the metabolism was gradually shifted to the acetic acid / butyric acid pathway with a decrease of ethanol formation. In summary and as illustrated by table 2, with the increase of the initial pH, the

concentrations of acetic and butyric acids progressively increased, while that of ethanol decreased rapidly.

Table 2: Residual acids, solvents and alcohols in solution produced after 48 h culture under different initial pH values. Uncertainties are based on duplicate experiments. Initial glucose concentration: 15 g L^{-1}.

Initial pH	Acetaldehyde (mg L^{-1})	Ethanol (mg L^{-1})	Acetic acid (mg L^{-1})	Butyric acid (mg L^{-1})	Isobutyric acid (mg L^{-1})
5.5	26.6 ±1.5	693.0±32.0	574.5 ±4.3	141.6±12.0	45.6±12.0
6.5	7.1±0.5	153.9±11.0	1520.7 ±24.0	520.1±16.0	299.9±8.0
7.5	1.4±0.1	40.0±4.0	3200.0±18.0	802.5±5.4	416.0±5.4

It is noteworthy that the final pH values were very close (Table 1), despite different initial pH. The pH change increased from 7.3% to 26.0% for pH increasing from 5.5 to 7.5. These pH changes corresponded to the production of high concentrations of organic acids (Table 2) decreasing therefore the pH of the buffered (0.05 mol L^{-1} MES) solutions. From the comparison of the soluble fermentation products (Table 2) and hydrogen yield profiles (Figure 1) under different initial pH, it is clear that the low hydrogen production recorded at pH = 5.5 can be related to the high production of ethanol (639 mg L^{-1}). Indeed, this ethanol production in the reactor indicates that at this initial pH value, either spore-forming hydrogen producers were not the dominant community, or that the bacteria began utilizing an ethanol producing metabolic pathway. This pathway, via acetaldehyde as an intermediate, does not produce H_2 (Eq.6) like the acetate and butyrate pathways do as shown in Eq.7 and Eq.8

$$C_6H_{12}O_6 \longrightarrow 2CH_3CH_2OH + 2CO_2 \tag{6}$$

$$C_6H_{12}O_6 + 4H_2O \longrightarrow 2CH_3COO^- + 2HCO_3^- + 4H_2 + 4H^+ \tag{7}$$

$$C_6H_{12}O_6 + 2H_2O \longrightarrow CH_3CH_2CH_2COO^- + 2HCO_3^- + 2H_2 + 3H^+ \tag{8}$$

The latter supposition was clearly confirmed by table 2, which showed that the highest amount of acetaldehyde (26.6 mg L^{-1}) was recorded at pH 5.5. The low levels of acetic and butyric acids detected at pH 5.5 proved that these hydrogen-producing pathways were not being utilized. In contrast, the high levels of acetic acid in the reactor running at pH 6.5 were indicative of the highest hydrogen yield (0.56 mol H_2 mol^{-1} $_{glucose\ consumed}$). These results are in agreement with those obtained by Fang and Liu [17], which proved that acetic acid production was favored at pH 6.5, while a pH decrease in the biohydrogen productivity accompanied by a significant increase in the level of volatile fatty acids (VFA) (Table 2) and a pH decrease of two units from the initial pH were observed (Table 1). These results can be interpreted by the fact that the excessive self-production of volatile fatty acids, especially acetic acid at pH 7.5, inhibited the growth of hydrogen-producing bacteria reducing therefore the H_2 production efficiency [59].

Accordingly, studying the pH effect on biological hydrogen production and hydrogenase activity in the range of pH values varying from 5 to 8 and in similar conditions than those considered in the present study (glucose as carbon source, 37ºC and in batch mode). Xiao et al. [22] found that the activity of hydrogenase decreased significantly with increasing culture pH and that weak acidic conditions favored hydrogen production.

All of these results proved that the initial pH have a considerable effect on the hydrogen production metabolism of heat-treated anaerobic sludge.

H_2 could be directly utilized during the formation of propionic acid according to the following equation (Eq.9) [60] :

$$C_6H_{12}O_6 + 2H_2 \longrightarrow 2CH_3CH_2COOH + 2H_2O \tag{9}$$

In this study, propionic acid was absent, which suggests that the thermal pre-treatment was successful and that the conditions used were not favorable for the growth of bacteria producing this acid. Similarly lactic acid, associated with zero-H_2 pathways [61] was also not detected.

Concerning the variation of the ratios R (CO_2/H_2) obtained at various pH, table 1 showed that the lowest ratio (0.1) was recorded at pH 6.5, which can be linked to the formation of a significant amount of organic acids, particularly in the form of acetic acid (Table 2). However, the increase of this ratio for an initial pH higher than 6.5, reflected an increase in the amount of CO_2 produced and hence a decline of the organic carbon, also mostly found in the form of acetic acid (Table 1). The increase of this ratio R (CO_2 / H_2) for pH below 6.5 indicated a CO_2 increase to the detriment of organic carbon which was predominantly found in the form of ethanol. Therefore at an initial pH equal to 6.5 the lowest amount of CO_2 and the highest biohydrogen production yields were recorded. Moreover, the absence of hydrogen production in the biotic control illustrated the absence of nutrient solubilization by thermal pretreatment of sewage.

In conclusion, an initial pH of 6.5 seems favorable to the production of hydrogen by heat treated anaerobic sludge. This result is in agreement with those of Karadag *et al*. [18] and Oh et *al.* [23] ; in similar conditions to those considered in this work they obtained 6.2 and 6.5 as optimal initial pH values, respectively. Therefore a pH of 6.5 was considered afterward to study the effect of the initial glucose concentration on hydrogen production.

3.1.2. Effect of the initial glucose concentration

In order to find the optimal glucose concentration for biohydrogen production, five different glucose concentrations (5, 10, 15, 20, 30 g L^{-1}) were evaluated. The initial pH was set at 6.5. According to figure 2, it is clear that the biohydrogen productivity increased with glucose concentration to reach a maximum value of 1.1 mol H_2 $mol^{-1}_{glucose\ consumed}$ equivalent to 0.128 mol L^{-1} of hydrogen at an initial glucose concentration of 25 g L^{-1}.
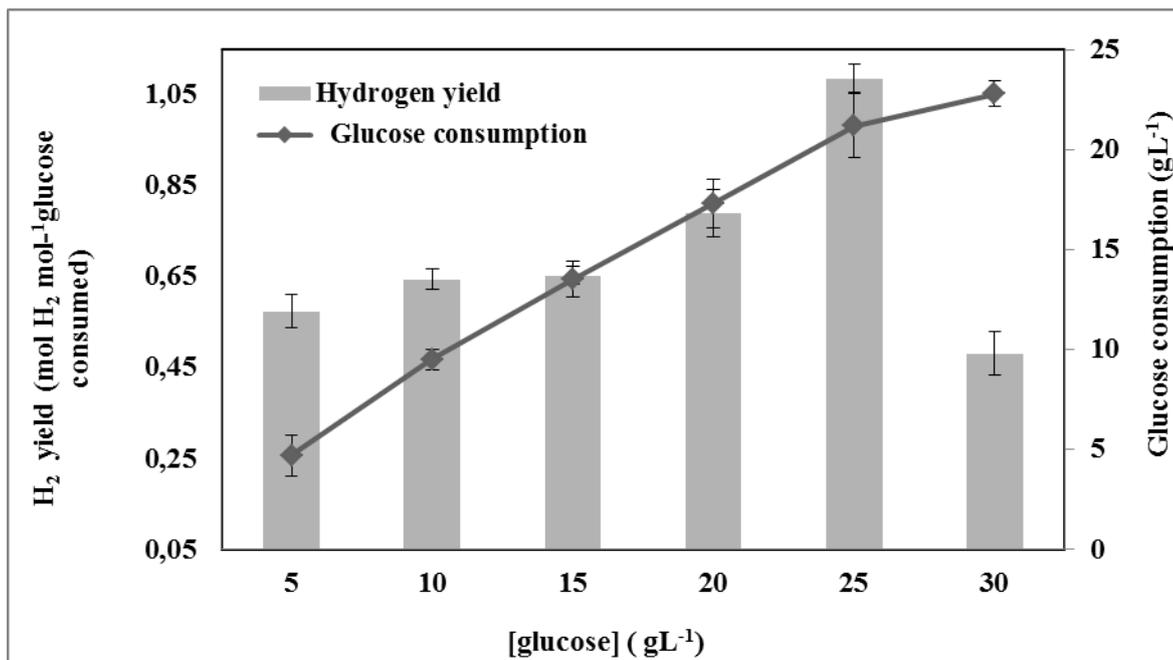
Figure 2: Effect of initial glucose concentration on H_2 production by heat treated anaerobic sludge after 48 h culture. Errors are based on duplicate experiments (Initial pH = 6.5).

Beyond 25 g L^{-1} glucose, biohydrogen production dropped very strongly and was quickly halved, at 30 g L^{-1} of glucose (this decrease will be discussed below). From this, the increase of hydrogen production can be related to the amount of carbon substrate. Table 3 showed that up to 10 g L^{-1}, glucose is the limiting factor. Ammonium assimilation profiles (Table 3) were in accordance with hydrogen production (Figure 2). Ammonium became the limiting factor from 25 g L^{-1} of glucose (98%). Bacterial growth was also favored; since it increased from 10.5 g L^{-1} to 24 g L^{-1} with the increase of the initial glucose content from 5 to 25 g L^{-1}.

Table 3: Biohydrogen production by heat treated anaerobic sludge after 48 h culture as function of the initial concentration of glucose. Uncertainties are based on duplicate experiments. (Initial pH = 6.5).

Glucose (g L^{-1})	NH$_4^+$ assimilation (%)	Final pH	^{a} R$_{CO2/H2}$	[CH$_4$] (mol L^{-1})	SS (g L^{-1})
5	41.1 ± 2.1	5.8 ± 0.1	0.4 ± 0.002	0.0	10.5 ± 3.9
10	59.7 ± 3.5	4.7 ± 0.2	0.2 ± 0.005	0.0	16.0 ± 0.8
15	74.4 ± 0.8	4.8 ± 0.3	0.2± 0.002	0.0	18.0 ± 0.5
20	78.2 ± 4.6	4.9 ± 0.2	0.1 ± 0.003	0.0	20.0 ± 3.3
25	98.8 ± 3.1	5.0 ± 0.4	0.025 ± 0.0006	0.0	21.0 ± 1.9
30	98 ± 0.3	4.8 ± 0.1	0.13 ± 0.007	0.0	21.5 ± 0.5

^{a}R (CO$_2$/H$_2$)) is the ratio between the concentration in mole L^{-1} of produced CO$_2$ and H$_2$ at different pH.

In conclusion for an initial glucose concentration lower than 15 g L^{-1}, glucose was the growth-limiting factor; while ammonium was not limiting until 25 g L^{-1} glucose.

Bacterial growth increased with the increase in substrate concentration up to 25 g L^{-1} glucose. A maximum of 21 g L^{-1} SS was attained at 25 g L^{-1} glucose. It is noteworthy that whatever the initial glucose concentration, biogas consisted mainly of hydrogen and carbon dioxide (Table 3); nitrogen gas was probably also formed but it could not be quantified. In fact each culture medium was previously deaerated with nitrogen gas, making therefore the analysis of nitrogen gas impossible because this latter was formed in very small amount compared to that used for deaeration. Finally and as above (first series of experiments), since no trace of methane gas or N$_2$O was detected during all the experiments, the parameter R (CO$_2$ / H$_2$) can characterize the purity of biogas. At pH = 6.5 and independently of the initial glucose concentration, the amount of the produced carbon dioxide

remained very low compared to the quantity of biohydrogen. Ratio values must match glucose concentration which gives: R (CO_2/H_2) varied from 0.025 to 0.4 for initial values of glucose ranging from 5 to 25 g L^{-1} (Table 3). Beyond 25 g L^{-1} glucose, the ratio R (CO_2/H_2) decreased again until 0.13. These ratios were much lower than those registered at pH 5.5 and 7.5 (Section 3.1.1), which justifies once more the choice of the pH (6.5). The concentrations of the various metabolites measured in the liquid phase are shown in Table 4.

Table 4: Residual acids, solvents and alcohols in solution produced after 48 h at different initial glucose concentrations. Uncertainties are based on duplicate experiments (initial pH 6.5).

Glucose (g L^{-1})	Acetaldehyde (mg L^{-1})	Ethanol (mg L^{-1})	acetic acid (mg L^{-1})	Butyric acid (mg L^{-1})	Isobutyric acid (mg L^{-1})
5	4.2 ± 1.6	825 ±101.5	437 ± 29.6	60 ± 13.0	78 ± 23.9
10	2.1 ± 0.6	515 ±16.6	874 ± 84.4	30 ± 4.2	156 ± 18.2
15	7.1 ± 3.8	154 ± 50.3	1521 ± 203.4	300 ± 36.4	520 ± 59.7
20	4.3 ± 0.3	126 ± 25.1	1755 ± 219.1	554 ± 56.0	1945 ± 191.6
25	10.7 ± 3.0	111 ± 17.2	2101 ± 419.5	419 ± 85.0	1158 ± 265.8
30	3.5 ± 1.1	25 ± 2.3	3157 ± 321.0	1320 ± 199.4	1543 ± 79.4

At an initial glucose concentration of 5 g L^{-1} bioydrogen production was accompanied mainly by the formation of ethanol (825 mg L^{-1}); with the increase of the glucose concentration the metabolism gradually moved towards acetic and butyric acids pathway. As shown in table 4, the concentrations of acetic, butyric and isobutyric acids increased with the initial content of glucose, while that of ethanol decreased rapidly beyond 5 gL^{-1} glucose. This behavior is in agreement with the increase of the hydrogen production yield with a maximum (1.1 mol H_2 mol^{-1}_{glucose consumed}) obtained for 25 g L^{-1} of glucose (Figure 2). However, for 30 g L^{-1} glucose the excessive production

of acetic acid (3157 mg L^{-1}) inhibited the hydrogen production metabolism, which resulted in a drop of hydrogen production efficiency.

Finally, a pH of 6.5 and an initial glucose concentration of 25 g L^{-1} appeared to be the optimal conditions for the production of hydrogen. These conditions oriented the glucose metabolism toward the production of acetic acid, which corresponds to the biohydrogen production pathway. To allow pH control (at pH 6.5), to maintain pH at its optimal value, a larger bioreactor (3 L working volume) was used and experiments were performed in the optimal conditions determined in batch mode using 125 mL glass bottles.

3.2. Batch study in 3 L bioreactor with pH control

The degradation profiles of glucose and ammonium with heat treated anaerobic sludge and that of the biomass concentration expressed by the Suspended solids (SS) are illustrated in Figure 3. Glucose was completely (98.5 %) consumed after 48 h of fermentation, with a maximum conversion efficiency of glucose to hydrogen (based on a theoretical yield of 4 mol H_2 $mol^{-1}_{glucose\ consumed}$) of 50.7% for heat-treated samples at a pH of 6.5 (Figure 3).

Concerning the ammonium curve, Figure 3 shows that ammonium assimilation yields increased with time to reach an optimal of 86.4% at 48 h which remained then approximately constant until the end of culture. Suspended solids showed a peak (22.5 g L^{-1}) at 48 h. Inhibition of growth and cessation of NH_4^+ consumption after 48 h of fermentation s can be attributed to a carbon limitation, since glucose depletion (98.8 %) was observed after 48 h of culture.
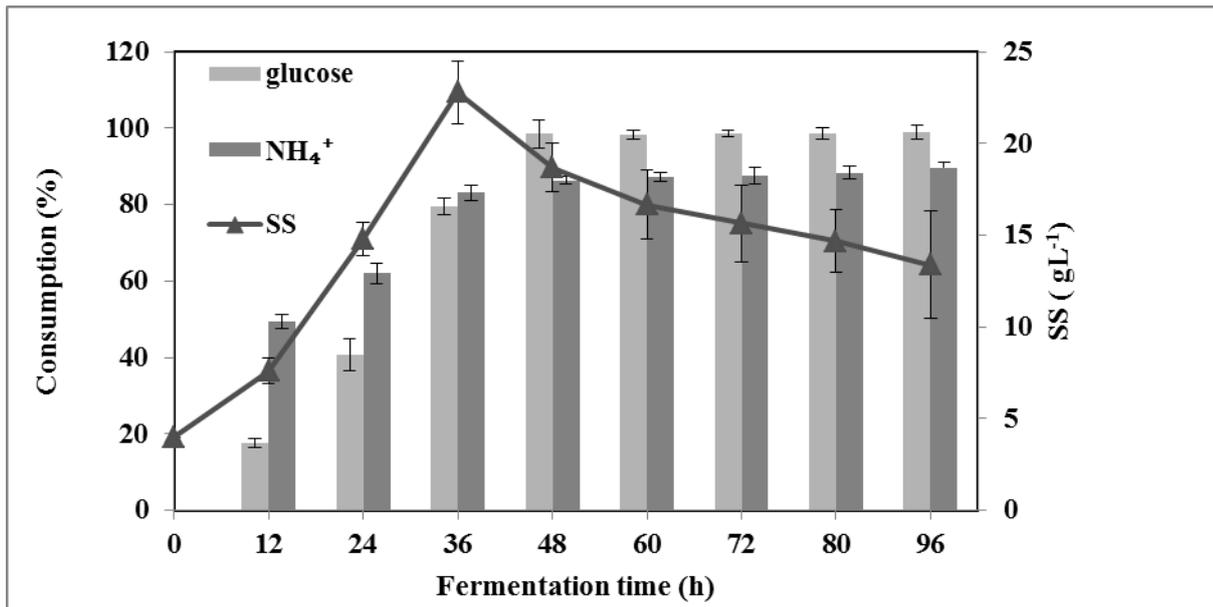
Figure 3: Time-courses of biomass growth, ammonium and glucose consumption during dark fermentation by heat treated sludge cultivated in a 3 L bioreactor with pH controlled at 6.5 and containing 25 g L^{-1} glucose. Errors are based on duplicate experiments.

T   The H$_2$ yield profiles (Figure 4) in the reactor headspace were in agreement with the microbial growth (Figure 3), since it increased until reaching a maximum value of 2.03 mol H$_2$ mol^{-1}$_{glucose}$ $_{consumed}$ after 48 h culture, followed by a decrease until 1.13 mol H$_2$ mol^{-1}$_{glucose\ consumed}$ after 60 h and remained then nearly constant until end of culture. Such a decrease was likely to be associated with the consumption of H$_2$ from the headspace by homoacetogens (*Clostridium aceticum*) [18] and by hydrogenotrophic methanogens (archaebacteria) [23].
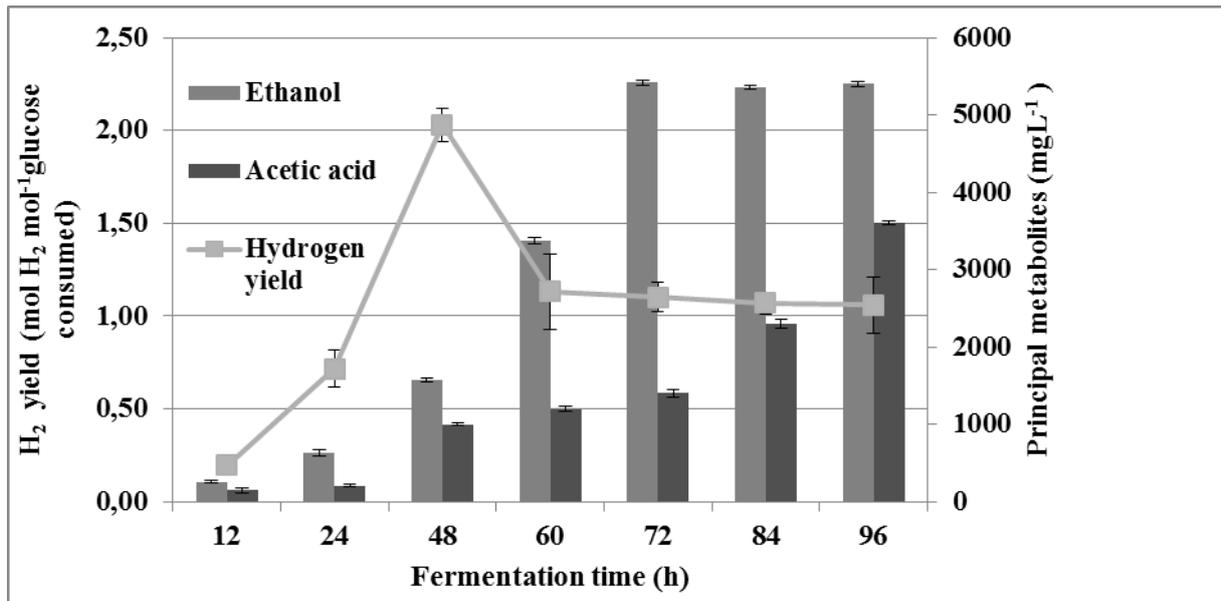
Figure 4: Time courses of the main metabolites and hydrogen production during dark fermentation by heat treated sludge cultivated in a 3 L bioreactor with pH controlled at 6.5 and containing 25 g L^{-1} glucose. Errors are based on duplicate experiments.

However, no methanogenesis was observed throughout the experiment as no CH_4 was detected in the samples. This Implies that H_2 might be utilized by some other microorganisms, such as homoacetogens. Spore-forming clostridia, e.g, *C. aceticum* and *C. thermoautrophicum*, are known to produce acetate from H_2 and CO_2 via the following reaction [62].

$$2HCO_3^- + 4H_2 + H^+ \longrightarrow CH_3COO^- + 4H_2O$$

This fact was confirmed from analysis of the biogas and the culture medium (Figure 4).The produced gas contained only hydrogen and carbon dioxide, and both compounds increased with the progress of the fermentation until reaching a maximum of 0.115 and 0.009 mol L^{-1} after 48 h respectively (data no shown). The biogas was free of methane; showing that the heat treated method was effective to remove methanogenesis and to enrich H_2-producing inoculum from mixed anaerobic sludge. Concerning, the culture medium, the main metabolites were acetic acid and ethanol, which increased with the progress of fermentation; after when glucose depletion, beyond

72 h, the amount of ethanol remained nearly unchanged, while that of acetic acid continued to increased. This production of acetic acid and the loss of hydrogen from the headspace despite the absence of organic carbon source, glucose, was most likely due to homoacetogenesis.

Finaly, the pH control of the culture medium led to a significant increase in the hydrogen gas conversion efficiency via heat-treated anaerobic activated sludge, 2.02 mol H_2 mol^{-1} glucose consumed, versus 1.1 mol H_2 mol^{-1} glucose consumed obtained without pH control (Figure 1) and 0.35 mol H_2 mol^{-1} glucose consumed (Table 5) previously obtained using aerobic activated sludge [48] .

Table 5: Summary of the H_2 production results obtained in 125 mL (without pH control) and 3 L batch reactors (with pH control). Error bars are based on duplicate experiments.

(Incubation time = 48 h, Ammonium solution generated electrochemistry = 8.6 g NH_4^+ .L^{-1})

	Inoculum	Bioreactor volume	H_2 yield (mol H_2 mol^{-1} glucose consumed)	Glucose consumption (%)	NH_4^+ assimilation (%)	Initial pH	Final pH	Reference
without pH control	heat-treated aerobic activated sludge	125 mL	0.35 ±0.01	82 ± 2.0	97.0 ± 3.5	5.5±0.2	3.5 ±0.1	previous study [48]
without pH control	heat-treated anaerobic sludge	125 mL	1.1 ± 0,01	85.1±1.5	98.8 ± 3.1	6.5±0.1	5.0 ± 0.4	This study
With pH control	heat-treated anaerobic sludge	3L	2.02 ±0.03	98.5± 2.2	86.4± 1.4	6.5±0.15	6.5 ±0.15	This study

The comparison of the H_2 yield obtained in this study using anaerobic activated sludge to those of the literature performed in similar conditions shows higher yields, namely 2.02 mol H_2 mol^{-1} glucose consumed in this work versus 1.17 mol H_2 mol^{-1} glucose consumed [27] and 0,96 mol H_2 mol^{-1} glucose consumed [23] or quite similar 2.00 mol H_2 mol^{-1} glucose consumed [63].

4. Conclusions

The results and findings of this study can be summarized as follows. Ammonium solutions resulting from nitrate electrochemical treatment can be substituted to the commercial products used as a nitrogen source to convert anaerobically glucose to H_2 in dark fermentation process.

The control of the culture medium pH had a strong influence on dark fermentative hydrogen production efficiency. In the optimal conditions corresponding to pH 6.5 and 25 g L^{-1} of initial glucose concentration and using heat treated anaerobic sludge, a hydrogen yield of 2.02 mol H_2 mol^{-1} glucose consumed was obtained by controlling the pH, versus 1.1 mol H_2 mol^{-1} glucose consumed recorded without pH control; it was also higher than the yield obtained in our previous study [48] with aerobic activated sludge (0.35 mol H_2 mol^{-1}glucose consumed).

A complete assimilation of the obtained NH_4^+ (0.86 g L^{-1} NH_4^+) and the added glucose (25 g L^{-1}) was achieved in both cases (with and without pH control). It is indeed a challenge to implement the process on an industrial scale but investigations at a pilot scale seems to be necessary prior to such implementation, especially considering that researches dealing with **dark fermentation** on a pilot scale are scarce, as well as the lack of studies on an industrial scale.

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