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# Potential of biohydrogen production from effluents of citrus processing industry using anaerobic bacteria from sewage sludge

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ABSTRACT

Citrus crops are among the most abundant crops in the world, which processing is mainly based on juice extraction, generating large amounts of effluents with properties that turn them into potential pollution sources if they are improperly discarded. This study evaluated the potential for bioconversion of effluents from citrus-processing industry (wastewater and vinasse) into hydrogen through the dark fermentation process, by applying anaerobic sewage sludge as inoculum. The inoculum was previously heat treated to eliminate $H_2$-consumers microorganisms and improve its activity. Anaerobic batch reactors were operated in triplicate with increasing proportions (50, 80 and 100%) of each effluent as substrate at 37 °C, pH 5.5. Citrus effluents had different effects on inoculum growth and $H_2$ yields, demonstrated by profiles of acetic acid, butyric acid, propionic acid and ethanol, the main by-products generated. It was verified that there was an increase in the production of biogas with the additions of either wastewater (7.3, 33.4 and 85.3 mmol $L^{-1}$) or vinasse (8.8, 12.7 and 13.4 mmol $L^{-1}$) in substrate. These effluents demonstrated remarkable energetic reuse perspectives: 24.0 MJ $m^{-3}$ and 4.0 MJ $m^{-3}$, respectively. Besides promoting the integrated management and mitigation of anaerobic sludge and effluents from citrus industry, the biohydrogen production may be an alternative for the local energy supply, reducing the operational costs in their own facilities, while enabling a better utilization of the biological potential contained in sewage sludges.



## 1. Introduction

Energy plays a key role in the progress of human civilizations, and it has become increasingly vital to support the technological and globalized world nowadays. According to estimates from the International Energy Agency (EIA, 2011), between 2008 and 2035 there will be an increase of 53% on the energy consumption, with an average annual growth of 1.6%. However, this growth would not be followed by conventional energy sources such as oil, coal, and natural gas, as these reserves are estimated to deplete until the year of 2050 (Goyal et al., 2008). Recent projections (EIA, 2011) also reported that renewable energy is the fastest-growing energy source in the world, which may increase on an average rate of 3% per year from 2010 to 2035, reaching 14%.

About 200 billion tons of lignocellulosic biomass have been generated worldwide by the primary agricultural sector (Guo et al., 2010). Brazilian agroindustry occupies an area of 28,840,726 ha, producing about 597 million tons of residues from several crops per year (sugarcane, corn, rice, soybean, cassava, wheat, coconut, and citrus) (Ferreira-Leitão et al., 2010).

Regarding citrus crops (oranges, lemons, grapefruit, and mandarins), they are among the most abundant crops in the world, being orange the most typical one, accounting for about 82% of the global citrus crop production (Ferreira-Leitão et al., 2010). The processing of citrus is based mainly on juice extraction, but these fruits are also used to produce several derivatives, either in the chemical industry for the production of flavonoids, essential oils, biofuels, limonene, and pectin (Pourbafrani et al., 2010), or in food industry for canning, sweet and soluble dietary fiber production (Ferreira-Leitão et al., 2010 and Marín et al., 2007).

Brazil is the main citrus producer country in the world. The overall orange production reached 16.9 million tons in 2014, which

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represents 33% of the worldwide production. Within these statistics, São Paulo State is the most representative, with 12.3 million tons (73%). In the same period, the total amount of citrus waste generated from orange processing by Brazilian industries was about 8.4 million tons (USDA, 2015).

Citrus wastes consist of peels (60–75%), segment membranes (30–35%), and seeds (10%) (Crawshaw, 2001 and Wilkins et al., 2007), which are mainly composed by highly fermentable carbohydrates. Thus, the disposition of these wastes in landfills, besides being costly, can increase the production of leached and methane, causing severe environmental impacts (Negro et al., 2016).

Usually, after drying and pressing, this solid residue is used to produce the citrus pulp pellets, employed as a supplement for cattle feed, which is not a cost effective solution (Awan et al., 2013; Ferreira-Leitão et al., 2010 and Lohrasbi et al., 2010).

Second-generation ethanol (2G), through residues of pulp and citrus bagasse processing, may be a promising and profitable alternative (Awan et al., 2013; Lohrasbi et al., 2010; Pourbafrani et al., 2010 and Widmer et al., 2010) to the management and energy recovery from residues generated in agroindustry. Meanwhile, similar to sugarcane bioethanol production (Moraes et al., 2014), significant amounts of vinasse are produced in either first (1G) or 2G-ethanol producing processes.

This effluent needs further treatment due to its high content of organic matter and nutrients, besides heaving low pH and high corrosivity. Such properties might cause several environmental impacts if it is improperly disposed, including water and groundwater pollution, toxicity for aquatic organisms, proliferation of vectors for diseases, as well as greenhouse gases emissions during its degradation in soil (Christofoletti et al., 2013). Instead of harmful, the surplus organic load may turn the effluents of citrus industry into promising substrates for hydrogen generation through dark fermentation process.

Hydrogen is considered a promising energy source for the future due to its renewability, as well as for its clean end of usage. It has greater energy contents per unit of weight (142.35 kJ $g^{-1}$; 2.75 times) (Khamtib and Reungsang, 2014) in comparison to hydrocarbon fuels (Hu et al., 2013), and since water is the only by-product generated by its combustion, hydrogen is an alternative energy source more sustainable than fossil fuels (Guo et al., 2010). However, to make it competitive with conventional energy carriers, ensuring its sustainable benefits, further technological advancements (Kumar et al., 2016) as well as the improvement of practical and scientific knowledge are essential.

Dark fermentation is an environmental feasible process due to its simultaneous waste treatment and hydrogen production and is advantageous because of its high production rate with a low energy input (Liu et al., 2011), and because of the versatility in the use of carbohydrate-containing substrates as agricultural wastewater, food waste, domestic wastewater, industry wastewater, among others (Hu et al., 2013 and Khamtib and Reungsang, 2014). It may be a suitable alternative for energy production in small-scale from industrial plants with highly available and low-cost biomass (Das and Veziroglu, 2008), providing a low-cost local energy supply.

Tropical countries like Brazil, with average annual temperatures around 25 °C, favor the activity of hydrogen-producing communities during anaerobic fermentation and offer an opportunity to investigate the hydrogen productions potential (Maintinguer et al., 2015) through various substrates without requiring a significant energy input.

The clean energy production may represent an interesting alternative to the management of effluents from citrus industries that, up to now, it has not been performed. Therefore, the aim of the present study was to evaluate the potential reuse of different effluents (wastewater and citrus vinasse), generated in large amounts by the citrus processing industry in Brazil, as substrate for biological production of hydrogen, by employing the sewage sludge as inoculum source.

## 2. Material and methods

### 2.1. Inoculum source and adaptation conditions

The inoculum was obtained from full-scale UASB (Upflow Anaerobic Sludge Blanket) reactors used to treat the sanitary sewage of São José do Rio Preto (20°49′13″S 49°22′47″W, São Paulo State, Brazil), a city of 442,500 inhabitants.

The anaerobic granular sludge is a suspension with 2.5% of suspended solids and pH 6.8. After the collection, it was inoculated in natura (20% v/v) in anaerobic batch reactors (100 mL of total volume) containing 50 mL of culture medium, (PYG: glucose, 10 g $L^{-1}$; peptone, 5 g $L^{-1}$, yeast extract, 5 g $L^{-1}$, and meat extract, 5 g $L^{-1}$; pH 7.0) and 50 mL of the headspace filled with $N_2$ (100%). The reactors were maintained at 37 °C for 7 days, and the resulting biomasses were subjected to heat treatment (100 °C for 10 min) to inactivate the methanogenic archaea ($H_2$ consumers) and select endospore-forming anaerobic bacteria involved in $H_2$ production, such as *Clostridium* sp (Maintinguer et al., 2008).

### 2.2. Enrichment of hydrogen-producing bacteria

Before tests of hydrogen production, the cellular purification of the heat-treated inoculum was performed through serial dilutions (1/10) in anaerobic batch reactors containing a new sterile PYG media in pH 5.5, (at 37 °C). Even after the heat treatment, it is appropriate to maintain this pH value (Fang and Liu, 2002) to avoid methanogenesis.

The hydrogen production and the absence of methane in headspace of reactors were confirmed by chromatographic analysis, after 72 h of incubation.

The hydrogen-producing bacteria (10 mL of the cellular suspensions) were inoculated in triplicate of batch reactors (2 L of total volume), containing 1 L of a culture medium (Del Nery, 1987) with the following composition (expressed in mg $L^{-1}$): fructose (5000), peptone (1000), urea (40.0), and 2.5 mL $L^{-1}$ of solutions A, B, C, and D, which are: A – $NiSO_4{\cdot}6H_2O$ (0.50); $FeSO_4{\cdot}7H_2O$ (2.5); $FeCl_3{\cdot}6H_2O$ (0.25); $CoCl_2{\cdot}2H_2O$ (0.04); B – $CaCl_2{\cdot}6H_2O$ (2.06); C – $SeO_2$ (0.14); D – $KH_2PO_4$ (5.36); $K_2HPO_4$ (1.3); $Na_2HPO_4H_2O$ (2.76).

In addition, 2.5 mL of solutions of $B_{12}$ vitamin (0.04 g $L^{-1}$), p-amino benzoic acid (0.04 g $L^{-1}$), and biotin (0.01 g $L^{-1}$) were added to supplement the synthetic medium (Maintinguer et al., 2008), and the initial pH was adjusted for 5.5. The synthetic medium and the vitamin solutions were previously sterilized through filtration in a 0.22 μm membrane. The headspace of the reactors were filled with $N_2$ (100%) and they were maintained at 37 °C for 72 h. After this period, the cellular suspension of enriched consortia was separated by centrifugation (9000 rpm at 4 °C, for 10 min) and the resulting biomass was used as inoculum in batch tests with citrus effluents.

### 2.3. Substrates for hydrogen bioproduction

Two effluents from citrus processing industry were employed as a substrate for $H_2$ production: the raw wastewater and citrus vinasse. They were provided by one of the leading companies of citrus juice production, located in the city of Matão (21°36′12″S 48°21′57″W), São Paulo State, Brazil.

The wastewater represents the liquid residue of the entire production process, including the steps of juice extraction, concentration, as well as the production of derivatives from citrus bagasse.

The wastes' recycling in the aforementioned industry is carried out through the ethanol production from the liquor obtained with the pressing of citrus bagasse. The final effluent of this process is designated as citrus vinasse (herein referred only as vinasse). The characteristics of the raw effluents are presented in Table 1.

Previously to tests, both residues were maintained at −20 °C. Then, they were filtered to remove insoluble suspended solids and sedimented inorganic compounds.

Vinasse and wastewater were also subjected to dilutions to supply a concentration of carbohydrates similar to that used for cell enrichment (5.0 g $L^{-1}$ of fructose). Potable water was utilized for the dilution procedure, since it could contribute with additional essential nutrients, such as calcium, magnesium, and sulfate.

### 2.4. Operation of the anaerobic batch reactors

Three different tests were performed in triplicate with wastewater and vinasse applied separately in anaerobic batch reactors (2 L of total volume), at 37 °C, using 1 L of substrate (pH 5.5) with the following composition [effluent + synthetic medium], in respective proportions: (1) [50% + 50%]; (2) [80% + 20%]; (3) [100% + 0%]. Tests using wastewater were defined as W1, W2, and W3, while tests using vinasse were defined as V1, V2, and V3, respectively.

It is worth noting that in tests with 100% of effluent neither additions of nutrients nor adjustments in carbon and nitrogen content (C/N ratio) were made to ensure the proper evaluation of their potential in raw form as well as their effects on the activity of hydrogen-producing bacteria. The headspace (1 L) of the reactors was filled with $N_2$ (100%). The reactors were sealed with sterilized screw caps and then inoculated with the previously reactivated cellular suspension of inoculum, described in Section 2.2.

From the start of tests up to the end of operation, individual samples (6 mL) of substrate were taken from the reactors, by inserting a syringe through the seal rubber to determine carbohydrate consumption, cellular growth, and the fermentation byproducts.

### 2.5. Chemical and chromatographic analysis

#### 2.5.1. Cellular growth and carbohydrate consumption

The cellular growth was monitored through optical density at 600 nm ($OD_{600}$) and expressed in the form of volatile suspended solids (VSS, g $L^{-1}$). Both cellular growth and COD (chemical oxygen demand) were measured in accordance to APHA (2005).

The consumption of carbohydrates (TSC) of wastewater was monitored through a phenol-sulfuric acid colorimetric method (Herbert et al., 1971). In the case of vinasse, the consumption of TSC was determined through high-performance liquid chromatography (HPLC) because it presented color, which is a result of the suspended solids (Table 1) and the insoluble by-products of ethanol processing (naturally present in this residue) that remained after filtration, thus preventing the measure by colorimetric method.

**Table 1**
Characteristics of raw citrus effluents.

| Composition | Wastewater | Vinasse |
|---|---|---|
| Glucose (g $L^{-1}$) | 12.454 | 41.016 |
| Fructose (g $L^{-1}$) | 3.862 | 62.213 |
| Total soluble solids, TSS (° Brix)[a] | 1.00 | 8.94 |
| Suspended solids, SS (%) | 0.60 | 6.00 |
| COD (g $L^{-1}$) | 19.47 | 77.70 |
| pH | 11.92 | 4.07 |

[a] % by weight; include carbohydrates, protein, acids.

The measurement by HPLC was carried out in the LC-20AT equipment (Shimadzu, Kyoto, Japan), according to the following methodology: isocratic method in oven at 40 °C, with 100% of acetic acid (10 mmol $L^{-1}$) as the mobile phase, at a flow rate of 0.8 mL $min^{-1}$, by adopting a Shim-pack SCR-102H column (7.9 mm × 30 cm), and a refractive index detector (Model RID-10A) both from Shimadzu (Kyoto, Japan). Before the measurements, the samples were filtered in a Chromafil PVDF 0.45 μm-pore size filter (Macherey-Nagel, Düren, Germany).

#### 2.5.2. pH

The adjustments of pH were made with the additions of hydrochloric acid (1.0 mol $L^{-1}$) or sodium hydroxide (1.0 mol $L^{-1}$), and the measurements were made at the beginning and at the end of the tests, according to APHA (2005).

#### 2.5.3. Gaseous components in biogas

The gaseous components present in headspace of anaerobic reactors were simultaneously evaluated using a TOGA system (Transformer Oil Gas Analyzer) coupled with TRACE™ GC Ultra, Ultra Gas Chromatograph (Thermo Scientific, Rodano, Italy), equipped with split/splitless injectors and two detectors; thermal conductivity detector (TCD) and flame ionization detector (FID), with methanizer. Argon was used as the carrier gas (1.5 mL $min^{-1}$, in splitless mode). The presence of methane was investigated during the stages of inoculum preparation. Carbon dioxide and hydrogen (biogas) generated in fermentative process were evaluated during the anaerobic batch tests.

The fraction of headspace collected (0.1 mL) were analyzed in an Rt-MSieve 5A column (30 m × 0.53 mm i.d.; Restek, PA, USA). Hydrogen and nitrogen were detected by TCD, and methane was detected by FID, after passing through the methanizer. The temperatures of the TCD and the injector were adjusted to 150 °C and for FID, 250 °C. After the sample had passed through the methanizer and had, subsequently, been eluted from a porous polymer Carboxen 1006 PLOT column (30 m × 0,53 mm i.d.; Supelco, PA, USA), carbon dioxide was detected by FID.

The oven complete programming was performed as it follows: 50 °C for 4.5 min, heating at 40 °C $min^{-1}$ up to 180 °C, for 1.5 min, and then cooling at 50 °C $min^{-1}$ up to 50 °C (3.15 min).

#### 2.5.4. Short-chain volatile organic by-products

The by-products of hydrogen generation, such as volatile fatty acids (VFA) and alcohols, were determined by gas chromatography, using the GC 2010 (Shimadzu, Kyoto, Japan) equipped with split/splitless CombiPAL AOC-5000 autosampler (CTC Analytics, Zwingen, Switzerland) and a high-frequency FID detector, adjusted to 250 °C. The oven programming was conducted at 45 °C for 1.0 min, followed by heating at 50 °C $min^{-1}$ up to 250 °C, for 3.0 min.

The headspace samples were analyzed under these conditions in an RTX-1 column (30 m × 0,32 mm × 3,0 μm; Restek, PA, USA), using helium as carrier gas (1.0 mL $min^{-1}$).

### 2.6. Experimental data fitting

The experimental data of hydrogen production obtained during the anaerobic batch tests (average values of triplicates) were fitted to obtain the parameters $P$, $Rm$, and $\lambda$ through a non-linear sigmoidal adjustment of the modified Gompertz function (Lay et al., 1998) (Eq. (1)), using the software STATISTICA® 8.0 (Statsoft, Inc., Tulsa, OK, USA).

$$H(t) = P \cdot \exp\left\{-\exp\left[\frac{Rm \cdot e}{P}(\lambda - t) + 1\right]\right\} \quad (1)$$

where $H$ represents the cumulative hydrogen production (mmol $H_2$ $L^{-1}$ of substrate), $P$ is the hydrogen production potential (mmol $H_2$ $L^{-1}$), $Rm$ is the maximum rate of hydrogen production (mmol $H_2$ $L^{-1}$ h), $t$ is the period of incubation (h), $\lambda$ is the period of lag phase (h), and $e$ is Euler's number (2.71828).

### 2.7. Characterization of hydrogen-producing bacteria

The morphological characteristics of anaerobic consortia were performed by Gram staining test (Maintinguer et al., 2008), using a Motic AE31 optical microscope, coupled with Moticam 2000 camera (Ted Pella, Sweden). The steps comprising the experimental procedure described are illustrated in Fig. 1.

### 2.8. Energetic reuse perspectives

The energetic reuse perspectives (ERP) of each effluent were evaluated from different points of view, using the data obtained in the tests conducted with 100% of effluent in substrate (W3 and V3). These approaches were performed taking into account the hydrogen yields (HY) in mols of $H_2$ generated per liter of substrate ($ERP_1$, Eq. (2)), per mol of TSC consumed ($ERP_2$, Eq. (3)), as well as per gram of COD influent ($ERP_3$, Eq. (4)), considering the energy content of $H_2$ as 284 kJ $mol^{-1}$ (or 142 kJ $g^{-1}$) (Khamtib and Reungsang, 2014 and Ren et al., 2014).

$$ERP_1 = \left(\frac{\text{mol } H_2 \text{ produced}}{\text{L of substrate}}\right) \times \left(\frac{\text{Energy content}}{\text{mol of } H_2}\right) \quad (2)$$

$$ERP_2 = \left(\frac{\text{mol } H_2 \text{ produced}}{\text{mol of TSC consumed}}\right) \times \left(\frac{\text{Energy content}}{\text{mol of } H_2}\right) \quad (3)$$

$$ERP_3 = \left(\frac{\text{mol } H_2 \text{ produced}}{\text{g of COD influent}}\right) \times \left(\frac{\text{Energy content}}{\text{mol of } H_2}\right) \quad (4)$$

## 3. Results and discussion

The reactors operated with additions of wastewater and vinasse had different effects on cellular growth (Table 2).

It was observed an increase in the period of inoculum adaptation, as increased from 50% (test W1) to 80% (test W2) the proportion of wastewater added to the substrate. Nevertheless, after overcoming the relatively longer period of adaptation (lag phase), the inoculum achieved a cellular growth about two times higher from W1 to W2 and W3 in less than 40 h of operation (Table 2).

Regarding to vinasse, the inoculum showed similar performance in cell growth for all the tests, despite the long period of stagnation when the proportion of this effluent in substrate increased from 50% to 80%.

Tests with vinasse presented relatively lower cellular growth when compared to the ones conducted with wastewater. This fact, as well as the stagnation from test V1 to test V2, are probably due to the presence of higher amounts of solids (TSS and SS, Table 1), inorganic substances, and a variety of recalcitrant chemical compounds such as phenols, furfural, and melanoidin, which are inherently present in vinasse.

These compounds are generated from the acid-catalyzed hydrolysis pretreatment of lignocellulosic biomass for 2G-ethanol processes from both sugarcane (Lazaro et al., 2014; Moraes et al., 2015 and Siles et al., 2011) and citrus waste (Grohmann et al., 1995 and Widmer et al., 2010), and might be toxic for the anaerobic bacteria, hindering its growth.

Nevertheless, the absence of a lag phase, especially in tests V1 and V3, suggests that pretreatment procedures, as well as the environmental conditions applied to anaerobic tests (initial pH, temperature), were suitable to promote the inoculum adaptation with both low concentration and high concentration of vinasse in substrate.

The heterogeneity of the mixed cultures from sewage sludge is also a factor to be taken into account when evaluating the cellular growth performances. Although the reactors have been inoculated with the same amount of biomass, it is not possible to ensure that the enriched anaerobic consortia will present the same adaptation ability, neither the same $H_2$ production performance in all tests.

### 3.1. Effect of wastewater and vinasse concentration on hydrogen production

The potential of hydrogen production was observed for both residues in all conditions tested. However, in each case, the increase in residue concentration had different effects on the hydrogen production (Fig. 2).

The anaerobic reactors that were operated with additions of wastewater showed an increase in the hydrogen production with the increase in wastewater concentration (Fig. 2a, Table 2), ranging from 7.3 to 33.4, and 85.3 mmol $H_2$ $L^{-1}$ for tests W1, W2, and W3, respectively, for a period of 48 h, 42 h, and 61 h. It represented a raise of 11.7 times on hydrogen production when the concentration of wastewater on the substrate increased from 50 to 100%. These results prove that the wastewater from citrus industry was not inhibitory to hydrogen-producing bacteria. Instead of inhibitory, the wastewater composition was highly favorable to the biogas fermentative production.

Considering the reactors operated with vinasse, the production of hydrogen also followed the increase of vinasse concentration in the substrate, but in a lower proportion in relation to the tests performed with wastewater. In this case, the rise in hydrogen production was only of 1.5 times from V1 to V3 (Table 2). Tests V2 and V3 showed very similar performances, including even the period of production (96 h) (Fig. 2b).

Regarding the HY, the values obtained are very significant when compared to other studies (Table 3) developed for a wide variety of wastewaters, under similar operational conditions. Lazaro et al. (2014) obtained a maximum HY of 2.23 mmol $H_2$ $g^{-1}$ COD, using a mesophilic consortium from anaerobic sludge (UASB) as inoculum, and sugarcane vinasse as substrate. These results are close to the ones obtained, in the present study, for citrus vinasse. However, citrus wastewater showed a hydrogen yield 6.4 times higher than the one reported for sugarcane vinasse.

It is interesting to note that the production of hydrogen by citrus vinasse presented a higher yield when compared to other wastewater substrates as well as synthetic (Mohan et al., 2007), domestic, confectionary (Van Ginkel et al., 2005), and even other kinds of sugarcane vinasse, as reported by Peixoto et al. (2012).

Ren et al. (2014) evaluated the potential of hydrogen generation by a pure culture (B49) in a high-strength synthetic wastewater, and obtained a higher yield when compared to the one from vinasse in the present study. However, in the case of wastewater (W3), the reported values are 2.0 times lower. The same relation is valid for the yield obtained by other researchers with potato, candy processing (Van Ginkel et al., 2005), brewery (Shi et al., 2010), and synthetic wastewaters Ren et al. (2014), as well as domestic sewage and glycerin wastewaters (Fernandes et al., 2010).

The relatively high performance of citrus wastewater in such comparisons could be attributed not only to the inoculum enrichment conditions but also to the inherent presence of fruit nutrients in this effluent, taking into account that it is remaining from the whole process of juice extraction.

The major nutrients present in orange fruit are K, Ca, Mg, B, Fe and Zn, whose concentrations vary during fruit development and depend on both seasonal and genetic factors (Storey and Treeby,

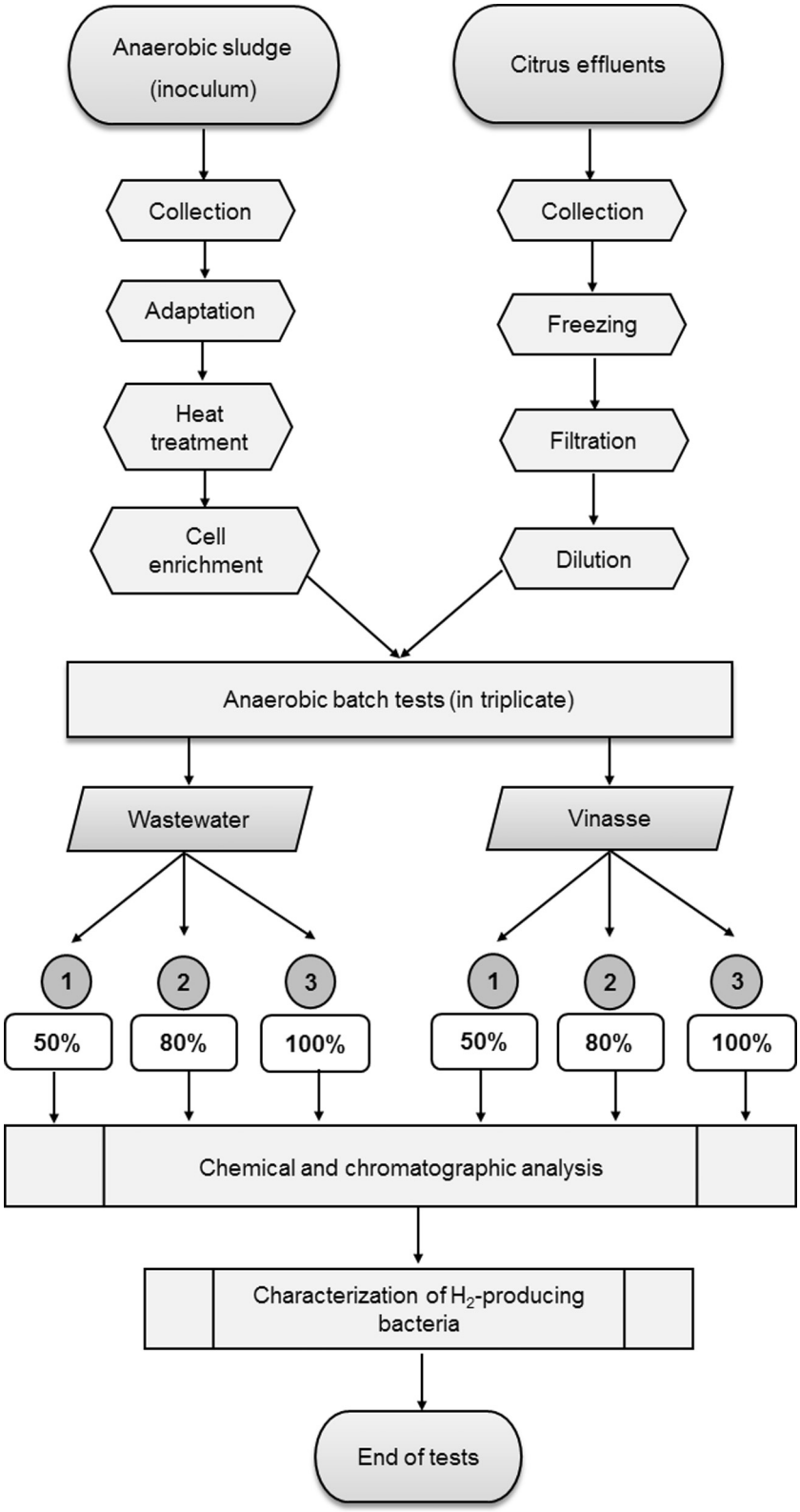


**Fig. 1.** Flowchart of the experimental steps conducted for biohydrogen production from anaerobic sewage sludge using the citrus effluents in substrate.

2000). These nutrients significantly affect the $H_2$ production through anaerobic sewage sludge, being Mg, Zn and Fe the most important (Lin and Lay, 2005).

Furthermore, as previously mentioned, vinasse is composed by a wide variety of chemical compounds that can cause toxic effects on cell growth and metabolic activity of inoculum and, therefore, can severely depress the performance of $H_2$ production. The magnitude of these impacts depends on both the concentration of inhibitory substances and the specific tolerance of microorganisms towards them (Kumar et al., 2015).

**Table 2**
Results obtained in batch tests with effluents from citrus processing industry.

| Parameters | Wastewater | | | Vinasse | | |
|---|---|---|---|---|---|---|
| | W1 | W2 | W3 | V1 | V2 | V3 |
| Influent COD (g $L^{-1}$) | 3.00 ± 0.07 | 5.40 ± 0.10 | 6.00 ± 0.30 | 5.30 ± 0.13 | 7.10 ± 0.21 | 6.70 ± 0.35 |
| Operation time (h) | 90.0 ± 0.5 | 90.0 ± 0.5 | 90.0 ± 0.5 | 186.0 ± 0.5 | 186.0 ± 0.5 | 186.0 ± 0.5 |
| TSC consumption (%) | 86.5 ± 2.2 | 88.0 ± 1.5 | 86.0 ± 3.5 | 100.0 ± 5.3 | 88.0 ± 4.2 | 68.0 ± 3.0 |
| VSS (g $L^{-1}$)[a] | 0.70 ± 0.08 | 1.30 ± 0.04 | 1.40 ± 0.04 | 0.8 ± 0.04 | 0.9 ± 0.08 | 0.8 ± 0.07 |
| Period (h)[a] | 48.0 | 35.0 | 37.0 | 41.5 | 24.0 | 18.5 |
| HY (mmol $H_2$ $g^{-1}$ COD)[b] | 2.4 | 6.2 | 14.2 | 1.7 | 1.8 | 2.0 |
| HY (mol $H_2$/mol TSC)[c] | 0.6 (14.0%)[d] | 1.3 (31.6%) | 3.0 (73.2%) | 0.3 (8.0%) | 0.5 (13.0%) | 0.7 (17.7%) |
| P (mmol $H_2$ $L^{-1}$)[e] | 7.3 ± 0.1 | 33.4 ± 0.4 | 85.3 ± 4.0 | 8.8 ± 0.2 | 12.7 ± 0.3 | 13.4 ± 0.5 |
| Period (h)[e] | 48.0 | 42.0 | 61.0 | 96.0 | 96.0 | 84.5 |
| Rm (mmol $L^{-1}$ h) | 0.20 ± 0.1 | 3.7 ± 0.2 | 2.1 ± 0.1 | 0.22 ± 0.04 | 1.3 ± 0.2 | 0.32 ± 0.06 |
| Lag phase (h) | 0.0 ± 1.4 | 9.0 ± 0.3 | 9.7 ± 1.3 | 0.0 ± 1.2 | 15.5 ± 0.89 | 0.0 ± 0.6 |
| $R^2$ | 0.99 | 0.99 | 0.99 | 0.97 | 0.98 | 0.97 |
| Final pH[f] | 4.65 ± 0.08 | 4.64 ± 0.05 | 4.32 ± 0.03 | 5.49 ± 0.01 | 5.10 ± 0.10 | 5.19 ± 0.04 |

[a] Higher cellular growth.
[b] HY = Hydrogen Yield, considering the mean influent COD in each case; used to calculate $ERP_3$.
[c] HY = Hydrogen Yield based on the mean consumption of TSC; used to calculate $ERP_2$.
[d] HY (%) = Relation between the experimental and the theoretical maximum $H_2$ production, based on the total TSC consumption verified by HPLC.
[e] Maximum $H_2$ production, calculated by the modified Gompertz function; used to calculate $ERP_1$.
[f] pH measured at the end of tests.

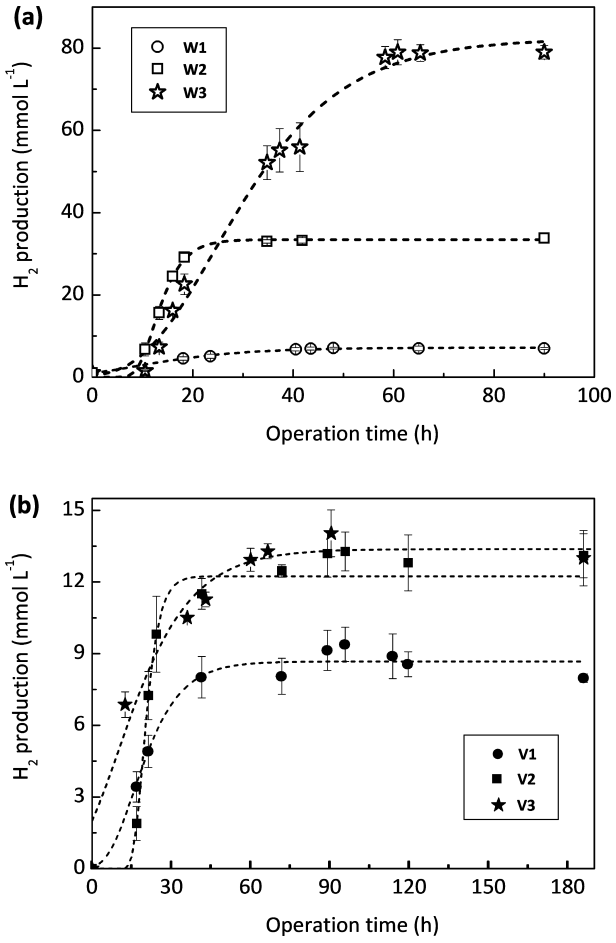


**Fig. 2.** Production of hydrogen during the operation of batch reactors filled with different proportions of (a) wastewater (W1: 50%; W2: 80%; W3: 100%) and (b) vinasse (V1: 50%; V2: 80%; V3: 100%). The bars represents the standard deviation of mean values.

Similar to presented in this study, Moreno-Andrade et al. (2015) demonstrated the feasibility of the hydrogen production from various industrial wastewaters containing inhibitory compounds such as vinasses from sugar and tequila industries, raw and physicochemical-treated wastewater from plastic industry and toilet aircraft wastewater.

In general, the data presented in Table 3 show the lack of foreseeability in hydrogen yield, achieved by employing the same kind of wastewater as substrate in fermentation processes. As it can be seen in Table 3, when sugar cane vinasse is employed as substrate, the yield in hydrogen production varies from 0.7 to 25.0 mmol $H_2$ $g^{-1}$ COD influent, which represents a rise of 36 times.

During the production of hydrogen through a soil heat-treated inoculum in a glucose-based medium, Van Ginkel and Logan (2005a) observed that the hydrogen yield is a function of the substrate organic load (initial sugar concentration), since their highest yields (2.8 mol $H_2$ $mol^{-1}$ glucose) were obtained by decreasing the influent glucose concentration from 10 to 2.5 g COD $L^{-1}$. They also stated that glucose concentration has a greater effect on $H_2$ yield than the hydraulic retention time (HRT), and it is most noticeable for values lower than 10 g $L^{-1}$. According to the authors, high sugar concentrations can lead to the inhibition of hydrogen production, causing a decrease in carbon loading rate, statement supported by Mohan et al. (2007).

Maintinguer et al. (2008) reported a similar effect on hydrogen production from the synthetic sucrose-based medium by anaerobic seed sludge. They attributed the lower $H_2$ yield to the inhibition of the inoculum through an increase from 1.8 g $L^{-1}$ to 4.1 g $L^{-1}$ in substrate sugar concentration.

In fact, Fernandes et al. (2010) obtained a strong performance in hydrogen production for all wastewaters evaluated, working with a low organic load (expressed as influent COD). However, with an overview of Table 3, it becomes clear that it is not a rule. Either low $H_2$ yields from substrates containing low organic load (Peixoto et al., 2012) or high $H_2$ yields achieved from substrates with high organic content (Ren et al., 2014; Shi et al., 2010 and Van Ginkel et al., 2005) have been reported in the related literature.

Also, by testing different industrial effluents and domestic wastewater, Van Ginkel et al. (2005) concluded that the amount of $H_2$ produced per liter of food processing wastewater varied widely under the test conditions, and it was not a function of the initial COD applied. On the other hand, Chuang et al. (2012) observed that high substrate concentration could support the growth of anaerobic consortia from sewage sludges as well as their fermentative production of $H_2$ through the hydrolysate from cellulosic waste.

**Table 3**
Comparative study on hydrogen production in anaerobic batch reactors under mesophilic conditions.

| Inoculum | | Reactor | Substrate | Temperature (°C) | Organic load (COD influent/ g $L^{-1}$) | Hydrogen yield (mmol $H_2$ $g^{-1}$ COD influent) | Reference |
|---|---|---|---|---|---|---|---|
| Type | Source | | | | | | |
| Activated sludge | Domestic Wastewater | Batch[a] | Olive mill wastewater | 35 | 68.1 | 0.54 | Lin et al. (2012) |
| Anaerobic (fixed-bed) | Synthetic wastewater | Batch | Sugarcane vinasse | 25 | 0.37 | 0.7[b] | Peixoto et al. (2012) |
| Anaerobic mixed microflora | Chemical wastewater | Batch[c] | Synthetic wastewater + domestic sewage wastewater | 29 | 4.5[d] | 0.71 | Mohan et al. (2007) |
| Anaerobic consortium | Soil | Batch[e] | Confectionary B<br>Domestic wastewater<br>Apple processing<br>Potato processing<br>Confectionary A | 37 | 10.0<br>6.2<br>9.0<br>10.5<br>0.6 | 0.8[f]<br>1.57[f]<br>3.14[f]<br>5.5[f]<br>6.7[f] | Van Ginkel et al. (2005) |
| Anaerobic sludge (UASB) | Poultry slaughterhouse | Batch | Sugarcane vinasse | 37 | 7.1 | 2.23 | Lazaro et al. (2014) |
| Anaerobic sludge (UASB) | Citrate-producing wastewater | Batch[g] | Brewery wastewater | 36 | 6.05 | 6.04 | Shi et al. (2010) |
| Pure strain (B49) | Anaerobic activated sludge | Batch | Synthetic wastewater (glucose-based) | 25 | 15.4 | 6.52[b] | Ren et al. (2014) |
| Anaerobic (fixed-bed) | Synthetic wastewater | Batch | Domestic sewage<br>Glycerin wastewater<br>Sugarcane vinasse | 25 | 0.25[h]<br>0.25[h]<br>0.25[h] | 6.01<br>6.03<br>25.0 | Fernandes et al. (2010) |
| Anaerobic granular sludge | Municipal sewage | Batch | Citrus vinasse | 37 | 6.7 | 2.0 | This study |
| Anaerobic granular sludge | Municipal sewage | Batch | Wastewater | 37 | 6.0 | 14.2 | |

[a, c, e, g] Initial pH adjusted to 6.8, 5.0, 6.1 and 5.95 respectively.
[b] The calculation of data was made considering the reported COD influent and the conversion from mL to mmol of $H_2$ was performed by the equation: pV = nRT; at 25 °C and 1 atm.
[d, h] Assuming this value as COD influent of all tests, as the author did not specify.
[f] The conversion from mL to mmol of $H_2$ was performed by the equation: pV = nRT (at 37 °C and 1 atm), considering the values of HY informed by the authors.

Therefore, the related data elucidate that there is no consensus on the optimum experimental conditions to achieve the highest hydrogen production yields through mixed cultures, especially from complex substrates.

Thereby, in order to propose the application of a waste material for energy recovery it is essential to conduct a previous assessment of the most suitable operational conditions for the desired conversion process. In case of dark fermentation process, besides the concentration of carbohydrates, factors such as reactor setup, temperature, pH, period of fermentation, source of inoculum, as well as its hydrogen-producing bacteria (Hu et al., 2013) play key roles in an efficient $H_2$ production, with emphasis on the type of inoculum and its pretreatment procedure (Mohan et al., 2007).

### 3.2. Main byproducts and metabolic pathways of $H_2$ production

The main byproducts generated during the operation of batch reactors were acetic acid, butyric acid, propionic acid and ethanol. It is interesting to note that the tests with wastewater (Fig. 3a) did not present similar profiles about the metabolites generated. In other words, the increase in the concentration of wastewater leads to different pathways of hydrogen production, but all of them presented high hydrogen yields. This behavior was different from the observed for vinasse, in which the increase in the concentration seems not to affect the pathways of hydrogen production (Fig. 3b).

Table 4 summarizes the main metabolic pathways involved in hydrogen production during tests with wastewater and vinasse substrates. They were proposed taking into account: the relation between the byproducts generated (Fig. 3a and b); the hydrogen evolution, including production as well as consumption (Fig. 2a and b); the TSC consumption (Table 2) in each period of batch tests: from the beginning up to the maximum $H_2$ production, and from this point up to the end of operation.

Considering the tests with lower concentration of wastewater (W1), HBt was the main by-product (47.0 mg $L^{-1}$) generated, which corresponds to 61% of the total amount (76.3 mg $L^{-1}$) of soluble products generated. These results were consistent with the lower yield of hydrogen obtained for this residue. The metabolic pathway of hydrogen production through HBt generation may be expressed by reaction (1).

$$C_6H_{12}O_6 \rightarrow CH_3(CH_2)_2COOH + 2CO_2 + 2H_2 \quad (1)$$

EtOH was generated either in test W2 or test W3 (reaction (2)), until the maximum production of hydrogen. However, for test W2, the production of this alcohol was remarkable (318.0 mg $L^{-1}$; 55%) during the same period, as well as butyric acid generation (217.0 mg $L^{-1}$). Additionally, in both tests, there has been consumption of the propionic acid originally present in the effluent.

EtOH production, in this case, probably occurred from the consumption of carbohydrates present in wastewater, according to reaction (2), and not by $H_2$ consumption, as demonstrated in Table 4. It is because the $H_2$ production only increased in the same period, reaching 33.4 mmol $H_2$ $L^{-1}$, which is an expressive value taking into account the type of substrate employed (Table 3).

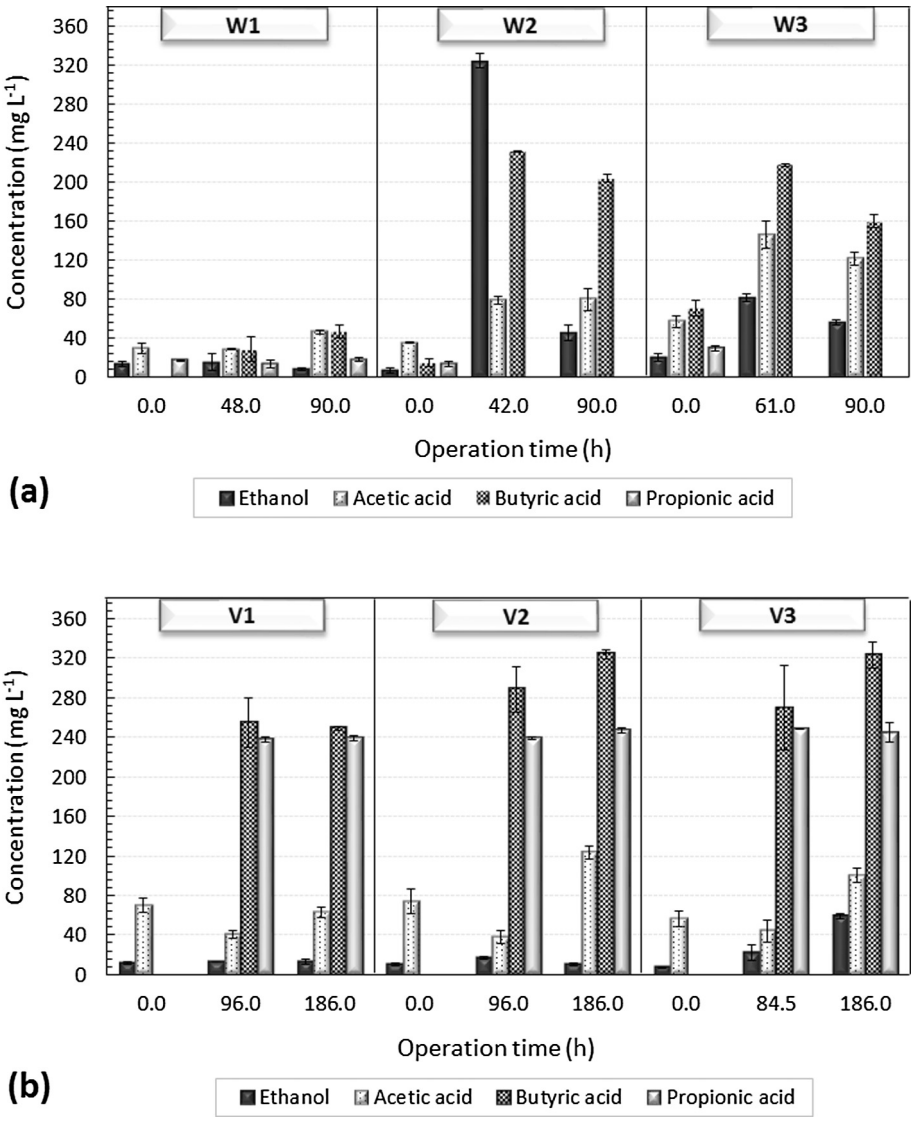


**Fig. 3.** Main by-products obtained during the biohydrogen production from (a) wastewater (W1: 50%; W2: 80%; W3: 100%) and (b) vinasse (V1: 50%; V2: 80%; V3: 100%) measured at the beginning, at the point of maximum $H_2$ and at the end of the tests.

$$C_6H_{12}O_6 \rightarrow 2CH_3CH_2OH + 2CO_2 \quad (2)$$

HAc production (reaction (3)) increased from 17.0 (W1) to 89.0 mg $L^{-1}$ (W3), when 100% of wastewater was tested, which might have contributed to the increase observed in hydrogen production (Fig. 2a), as its reaction provides the maximum yield of $H_2$ per mol of glucose (Angenent et al., 2004; Das and Veziroglu, 2008; Hawkes et al., 2007 and Saady, 2013). The total amount of byproducts generated (HBt + HAc) by employing wastewater was 76.3, 287.0 and 261.0 mg $L^{-1}$ in tests W1, W2, and W3, respectively.

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2 \quad (3)$$

Regarding to W3, the additional $H_2$ production may be due to the acetogenic reaction (Saady, 2013) accounted for the consumption of 30.0 mg $L^{-1}$ of propionic acid (HPr) (reaction (4)) as well as from syntrophic HAc degradation (24.4 mg $L^{-1}$), according to reaction (5) (Angenent et al., 2004 and Guo et al., 2010). The consumption of 1.0 mol of HPr produces 3.0 mols of $H_2$, while HAc oxidation yields 4.0 mols of $H_2$. In mixed anaerobic cultures, syntrophic association follows many metabolic pathways, since they are thermodynamically favorable (Saady, 2013), as acetogenic bacteria that can convert VFA and alcohols into HAc during its heterotrophic growth on different types of substrates.

$$CH_3CH_2COOH + 2H_2O \rightarrow CH_3COOH + 3H_2 + CO_2 \quad (4)$$

$$CH_3COOH + 2H_2O \rightarrow 4H_2 + 2CO_2 \quad (5)$$

The hydrogen production also occurred mainly through HBt pathway, (reaction (1)) in the anaerobic reactors fed with vinasse (Fig. 3b). However, the joint production of HPr (reaction (6)) could explain the lower hydrogen yield obtained from vinasse in relation to wastewater, since its production occurs through hydrogen consumption.

$$C_6H_{12}O_6 + 2H_2 \rightarrow 2CH_3CH_2COOH + 2H_2O \quad (6)$$

The mechanism of HPr accumulation is not clearly understood, but it could be attributed to many reasons, including the overloading in the start-up phase, the high hydrogen partial pressure in biogas, as well as the shift in the dominant species of acidogenic populations due to a stress condition because of the medium instability (Saady, 2013 and Sivagurunathan et al., 2014).

**Table 4**
Main pathways of anaerobic digestion involved in $H_2$ production during the tests with wastewater and vinasse.

| Test | Parameters evaluated | Period of test | | | Reaction[b] |
|---|---|---|---|---|---|
| | | Beginning | Maximum $H_2$ production | End | |
| *Wastewater* | | | | | |
| W1 | Operation time (h) | 0 | 48 | 90 | |
| | Pathways | HBt production (28.5 mg $L^{-1}$) | | HBt production (18.2 mg $L^{-1}$) | (1) [c] |
| | | | | HAc production (17.0 mg $L^{-1}$) | (3) |
| | $H_2$ evolution | Production (7.3 mmol $L^{-1}$) | | Stabilization | |
| | TSC (%)[a] | 86.5 | | – | |
| W2 | Operation time (h) | 0 | 42 | 90 | |
| | Pathways | HBt production (217.0 mg $L^{-1}$) | | | (1) [c] |
| | | HAc production (43.5 mg $L^{-1}$) | | | (3) |
| | $H_2$ evolution | Production (33.4 mmol $L^{-1}$) | | Stabilization | |
| | TSC (%)[a] | 88.0 | | – | |
| W3 | Operation time (h) | 0 | 61 | 90 | |
| | Pathways | HBt production (147.3 mg $L^{-1}$) | | | (1) [c] |
| | | HAc production (89.0 mg $L^{-1}$) | | | (3) |
| | | HPr consumption (30.0 mg $L^{-1}$) | | | (4) |
| | | | | HAc degradation (24.4 mg $L^{-1}$) | (5) |
| | $H_2$ evolution | Production (85.3 mmol $L^{-1}$) | | Stabilization | |
| | TSC (%)[a] | 86.5 | | – | |
| *Vinasse* | | | | | |
| V1 | Operation time (h) | 0 | 96 | 186 | |
| | Pathways | HBt production (255.0 mg $L^{-1}$) | | | (1) [c] |
| | | HPr production (238.0 mg $L^{-1}$) | | | (6) |
| | | HAc degradation (30.0 mg $L^{-1}$) | | | (5) |
| | $H_2$ evolution | Production (8.8 mmol $L^{-1}$) | | Stabilization | |
| | TSC (%)[a] | 100.0 | | – | |
| V2 | Operation time (h) | 0 | 96 | 186 | |
| | Pathways | HBt production (289.0 mg $L^{-1}$) | | | (1) [c] |
| | | HPr production (239.0 mg $L^{-1}$) | | | (6) |
| | | HAc degradation (36.0 mg $L^{-1}$) | | | (5) |
| | | | | HBt production (35.0 mg $L^{-1}$) | (1) |
| | | | | HAc production (85.0 mg $L^{-1}$) – Homoacetogenesis | (7) |
| | $H_2$ evolution | Production (12.7 mmol $L^{-1}$) | | Consumption (3.5 mmol $L^{-1}$) | |
| | TSC (%) | 88.0 | | 12.0 | |
| V3 | Operation time (h) | 0 | 85.4 | 186 | |
| | Pathways | HBt production (270.0 mg $L^{-1}$) | | | (1) [c] |
| | | HPr production (36.0 mg $L^{-1}$) | | | (6) |
| | | | | HBt production (53.0 mg $L^{-1}$) | (1) |
| | | | | HAc production (56.0 mg $L^{-1}$) - Homoacetogenesis | (7) |
| | $H_2$ evolution | Production (13.4 mmol $L^{-1}$) | | Consumption (3.5 mmol $L^{-1}$) | |
| | TSC (%)[a] | 68.0 | | 32.0 | |

[a] TSC consumption.
[b] Presented in descending order of production, according to Fig. 3.
[c] Main pathway for $H_2$ production in considered test.

The accumulation of the undissociated parts of soluble metabolites in the substrate may disrupt the physiological balance in cells of hydrogen-producing bacteria and then inhibit the fermentative hydrogen production (Wang et al., 2008). This inhibition causes a shift in acidogens' metabolism from acetate towards HPr, HBt, lactate, and alcohols generation, called solventogenesis (De Gioannis et al., 2013; Guo et al., 2010; Hawkes et al., 2007 and Saady, 2013), lowering the $H_2$ production yield. Solvent production usually occurs in the stationary growth phase, after the main $H_2$ production event, during the exponential growth phase of *Clostridia* (De Gioannis et al., 2013).

Wang et al. (2008) reported that the inhibitory effect on glucose fermentation by mixed cultures is stronger in the presence of added HAc, HPr, and HBt than in the presence of EtOH, and according to Guo et al. (2010), the accumulation of VFA is the main factor for the solventogenesis process, instead of hydrogen partial pressure. If the self-production of HBt becomes excessive, its inhibitory effect is greater than HAc's, in a hydrogen saturated medium (Van Ginkel and Logan, 2005b).

These compounds were originally present in the effluents evaluated in this study (Fig. 3a and b), but the overproduction of EtOH (W2) in wastewater and HPr in vinasse seems to be a result of HBt accumulation.

After the point of maximum $H_2$ production, HBt had an increase of 35.0 mg $L^{-1}$ and 53.0 mg $L^{-1}$ (Fig. 3b), coupled with the consumption of remaining TSC (Table 4), for V2 and V3, respectively. In contrast, the production of hydrogen dropped around 3.5 mmol until the end of the tests. This disagreement might be related to homoacetogenesis process, in which autotrophic acetogenic microorganisms (homoacetogens) consume $CO_2$ and $H_2$ to form HAc (reaction (7)) (Saady, 2013).

$$4H_2 + 2CO_2 \rightarrow CH_3COOH + 2H_2O \quad (7)$$

The occurrence of homoacetogenesis may be evidenced by the further production of 85.0 and 56.0 mg $L^{-1}$ of HAc in tests V2 and V3, respectively, coupled with the $H_2$ consumption observed. In fact, the similar behavior of mixed cultures' metabolic pathways in V2 and V3 reflects on the quite similar hydrogen yields obtained (Table 2).

Homoacetogenesis is a very common process during dark fermentation, consuming 11% of the $H_2$ yield in a batch operation (Saady, 2013). Under stress conditions, as HPr accumulation,

acetogenic bacteria shift their metabolism to autotrophic growth to relieve the inhibition effect, becoming homoacetogens what probably occurred in the tests.

This process has also been reported during $H_2$ production from sugarcane vinasse in either mesophilic (Lazaro et al., 2014) or thermophilic dark fermentation (Luo et al., 2010 and Santos et al., 2014). By employing an isolated strain, Lazaro et al. (2014) observed higher acetic acid generation associated to the lower $H_2$ yield, which was closely related to *Clostridium carboxidivorans*, a known homoacetogen.

Chuang et al. (2012) also reported the consumption of hydrogen with the subsequent production of acetate as the cause of the low $H_2$ production obtained through mixed cultures in a hydrolysate from the distillers grains.

It is plausible to argue that the production of $H_2$ from both effluents here evaluated took place through an HBt pathway, with the maximum theoretical yield of 2.0 mol $H_2$ $mol^{-1}$ glucose. However, the $H_2$ consuming reactions determined the lower yield of $H_2$ in the final balance of vinasse fermentation process.

In this case, the pretreatment conditions were not able to avoid the presence of homoacetogenic bacteria, whose activity was favored by the accumulation of acid metabolites during $H_2$ production processes, lowering the yield of vinasse in relation to wastewater.

The total amount of by-products generated in tests with vinasse (in mg $L^{-1}$) was in the order of 6.3, 2.2, and 2.5 times higher than wastewater. As previously discussed, the accumulation of such compounds could also be responsible for the inhibition of $H_2$-producers and the start-up in the activity of homoacetogens, affecting both the cellular growth and the hydrogen production.

Kawagoshi et al. (2005) stated that pH considerably affected hydrogen production and may cause a transition in the bacterial community. However, it became clear that the differences in hydrogen yields were not related to pH media, since the biggest change in pH was verified for W3 (Table 2), in which the highest hydrogen yield was achieved. On the other hand, despite the lower $H_2$ yields in tests with vinasse, the pH media remained virtually unchanged.

According to Khanal et al. (2004), with the adaptation of hydrogen producing consortia to the environmental conditions, such as pH, they started producing hydrogen at a moderate rate, in which the acid by-products are insufficient to cause a depletion of the buffering capacity and, hence, inhibitory effects. Therefore, the activity of hydrogen producers could be longer in a relatively consistent environment.

Given the above consideration, it is clear that the $H_2$ production from a mixed culture depends not only on the hydrogen producers, but also on the co-metabolism of the entirely anaerobic community (Hawkes et al., 2007). Thus, the comprehension of such metabolic relations might contribute to increase the biogas yield and, therefore, to improve the performance of energy recovery systems.

### 3.3. Morphology of hydrogen-producing consortia

The characterization analysis of anaerobic consortia revealed the predominance of Gram-positive rods and rods with endospore (Fig. 4) in the anaerobic consortia employed for hydrogen production from both residues. These morphologies are characteristic of hydrogen-producing bacteria, mainly in case of *Clostridium* species, which are highly efficient for this purpose (Lazaro et al., 2014; Lin et al., 2012 and Maintinguer et al., 2015).

The pretreatment conditions applied proved to be suitable to inactivate methanogenic archaea and to select the hydrogen-producing consortia, once they provide fast growing behavior, high $H_2$ production rates, and high yields, especially for wastewater.

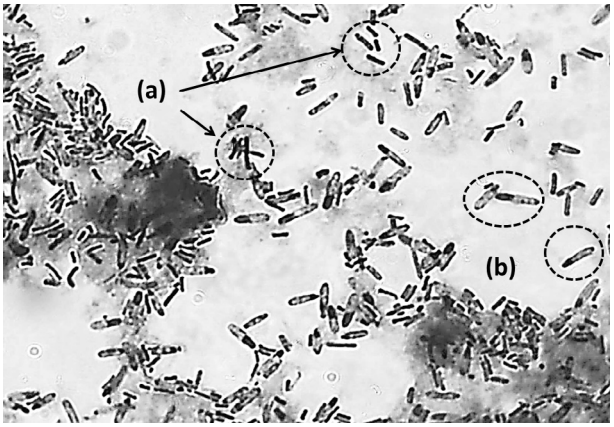


**Fig. 4.** Microscopic analysis in Gram staining of anaerobic bacteria, during the batch tests of hydrogen production, with emphasis to the presence of: (a) bacilli and (b) bacilli with endospores, at the magnification of 1000×.

However, complementary isolation and identification tests may be performed to elucidate the diversity of organisms involved in $H_2$ production processes, as suggested by the metabolic pathways obtained.

### 3.4. COD removal in effluents

The influent COD did not follow the increase in the concentration of both residues (Table 2). It can be due to the contribution of all compounds present in synthetic medium, which were added to complement substrate in the proper ratio.

The overall organic matter concentration (expressed as COD) remained almost constant after the tests with wastewater and achieved the maximum removal of 41% (V1) with vinasse. This fact was expected since the influent TSC was converted into VFA and EtOH during the fermentative process of hydrogen production, contributing to the final balance of COD.

This low COD removal efficiency, rather than being a problem, might represent a strategy to improve the treatment of industry wastewater through a two-stage process of fermentative hydrogen production. In this case, the effluent of acidogenic stage composed mainly be VFA and alcohols could be further employed as substrate for methane generation (Peixoto et al., 2012), photofermentation (Eroglu et al., 2006 and Hu et al., 2013) or even for biotechnological applications such as microalgal cultivation (Ren et al., 2014) and biodiesel production by oleaginous yeast (Singhania et al., 2013).

Besides the improvement of COD removal by a two-stage fermentation process, the VFA from $H_2$ production could be further recovered, since they are chemical compounds with high-added value and diverse applications in industry, like the synthesis of aldehyde, ketones, esters, and olefins (Singhania et al., 2013).

As previously reported, high amounts of HBt and HPr were obtained (respectively, 324 and 247 mg $L^{-1}$) in reactors operated with vinasse. The major use of HBt in the chemical industry is for the production of thermoplastics as polyhydroxybutyrate (PHB) (Singhania et al., 2013). However, HPr is applied as an intermediate in the synthesis of several types of compounds, such as cellulose fibers, herbicides, perfumes, pharmaceuticals and preservatives in animal feed and human foods (Liu et al., 2015).

This integrated two-stage bioprocess meets the concept of biorefinery, which contemplates the achievement of many environmental benefits through the complete transformation and valorization of waste input (Kumar et al., 2015). In this sense, engineering strategies as bioaugmentation (specific culture addition) can contribute to enhance the overall feasibility of the integrated bioprocess (Kumar et al., 2016).

**Table 5**
Potential for energy recovery through effluents from citrus processing industry.

| Estimates | Overall hydrogen production potential | |
|---|---|---|
| | Wastewater[a] | Vinasse[b] |
| $ERP_1$ (kJ $L^{-1}$ or MJ $m^{-3}$/kW h $m^{-3}$) | 24.0/6.7 | 4.0/1.0 |
| $ERP_2$ (kJ $mol^{-1}$ TSC) | 852.0 | 199.0 |
| $ERP_3$ (kJ $g^{-1}$ COD) | 4.0 | 0.6 |

[a] Test W3, with 100% of wastewater.
[b] Test V3, with 100% of vinasse.

### 3.5. Potential for energy recovery

As elucidated in Table 3, the effluents of citrus processing industry are promising substrates for fermentative hydrogen production, since they provide high yields in comparison to those obtained with different kinds of industrial wastewaters.

Considering the reuse of both effluents here evaluated (W3 and V3), an amount of 85.3 and 13.4 mmol $H_2$ $L^{-1}$ of effluent was obtained with maximum conversion efficiency of the TSC to hydrogen of 73.2% and 17.7% respectively (Table 2), based on the theoretical yield of 4.0 mol $H_2$ $mol^{-1}$ of glucose.

The energetic reuse perspectives (ERP) of citrus effluents for biohydrogen production through anaerobic digestion were calculated by Eqs. (2)–(4), and the results are presented in Table 5.

Supposing a high-scale citrus-processing plant projected for an input of 14,000 ton/day, and considering that after the orange juice extraction about 50% of the fruit is left as bagasse (Awan et al., 2013 and Ferreira-Leitão et al., 2010), the total amount of waste generated would be 7000 ton/day. If this residue were applied for 2G-ethanol production through enzymatic hydrolysis, using commercial yeast strains (*S. cerevisiae*) (Awan et al., 2013), around 60 L of ethanol (4.7 wt%) and 1217 L of citrus vinasse would be generated per ton of orange waste in natura. In other words, about 8519 $m^{-3}$ of vinasse would be produced in a single day.

Within this scenario, if this total amount of citrus vinasse was applied for fermentative production of hydrogen, the biogas produced could be used for power generation in a cogeneration self-production plant, for instance. Considering the hydrogen yield achieved in this study, it would be possible to recover up to 20.4 GJ or 5.7 MW $h^{-1}$ (Table 5) through combustion in high-efficiency gas turbines (close to 60%) (Chiesa et al., 2005). In the case of wastewater, the energetic reuse perspective would be even better: 24.0 MJ $m^{-3}$ (or 6.7 kW $h^{-1}$ $m^{-3}$ of effluent generated).

Therefore, this integrated process would be able to provide a local energy supply, reducing external energy demand for steps of hydrolysis and distillation, for example, which are the two major energy-consuming steps in 2G-ethanol producing processes (Widmer et al., 2010).

Han et al. (2015) demonstrated the feasibility of a combined bioprocess of solid-state fermentation (SSF) and fermentative hydrogen production from enzymatic hydrolysis of food waste, another plentiful waste. The techno-economic evaluation designed by the authors (Han et al., 2016) proved the economic feasibility of this novel integrated technology that, similar to discussed for citrus waste recycling, may contribute not only to mitigate the problem of food and agroindustrial wastes disposal, but also to produce an alternative and sustainable energy source.

## 4. Conclusions

This study demonstrates the potential for biohydrogen production through the effluents generated in citrus processing industry as well as the biological potential of anaerobic sewage sludge as inoculum for the dark fermentation process, which may represent an interesting reuse perspective for both kinds of residues.

Citrus vinasse showed higher potential for $H_2$ production when compared to synthetic and domestic wastewaters. Through citrus wastewater, the $H_2$ bioconversion efficiency achieved 73% that is two times higher than many industrial wastewaters.

The generation of high-added value chemicals, such as butyric acid and propionic acid from industrial wastewaters, deserves attention, since it may be an alternative option as interesting as the own bioenergy production.

The improvement of waste management through the biohydrogen production can make the citrus processing industry more sustainable and cost-effective, while allowing the simultaneous effluent treatment and energy recovery. These findings may contribute to a future application of these matrices as feedstock for local power generation, which could sustain the activities in the own generating facility.

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