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Coupling dark fermentation and microbial electrolysis to enhance biohydrogen production from agro-industrial wastewaters and by-products in a bio-refinery framework

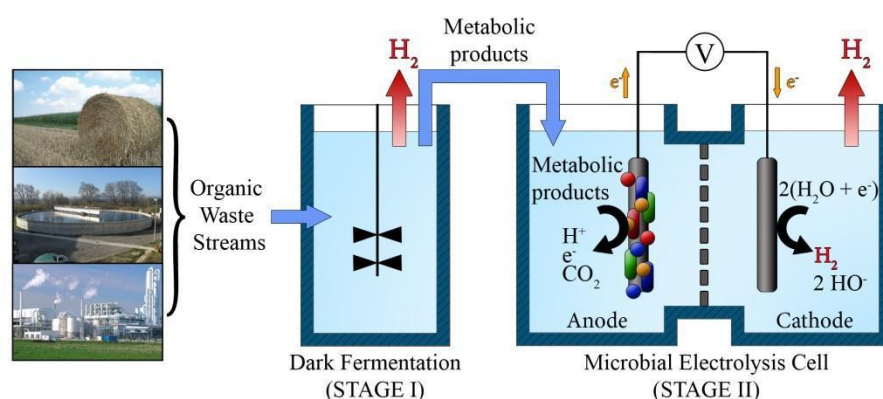
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ABSTRACT



The aim of this work is to evaluate biohydrogen production from agro-industrial wastewaters and by-products, by combining dark fermentation and microbial electrolysis in a two-step cascade process. Such coupling of both technologies constitutes a technological building block within a concept of environmental biorefinery where sustainable production of renewable energy is expected.

Six different wastewaters and industrial by-products coming from cheese, fruit juice, paper, sugar, fruit processing and spirits factories were evaluated for the feasibility of hydrogen production in a two-step process. The overall hydrogen production when coupling dark fermentation and microbial electrolysis was increased up to 13 times when compared to fermentation alone, achieving a maximum overall hydrogen yield of 1608.6 ± 266.2 ml H₂/ gCOD_{consumed} and a maximum of $78.5 \pm 5.7\%$ of COD removal.

These results show that dark fermentation coupled with microbial electrolysis is a highly promising option to maximize the conversion of agro-industrial wastewaters and by-products into bio-hydrogen.

Key words – Biohydrogen, Dark Fermentation (DF), Microbial Electrolysis Cell (MEC), agro-industrial wastewaters and by-products, two-stage process

1. INTRODUCTION

Nowadays alternative energy production chains are increasingly studied with the objective to reduce the dependence on oil, mitigate climate change originated by human activities and redirect the current production processes (open-cycles) toward natural closed ecological cycles. Such alternative production chains need a replacement of current raw materials, including energy, with renewable resources.

Currently, more than 60 Million Tons of hydrogen per year are produced in the World with about 10 MTH₂/y in the EU. Although H₂ is mostly used as chemical reactant in various industrial sectors, such oil refining (44%) or ammonia production (38%), hydrogen is considered as one of the most serious alternatives to fossil fuels for future transportation vehicles [1]. Indeed, the major advantage of energy from hydrogen is the absence of polluting emissions since the utilization of hydrogen, either via combustion or via fuel cells, results in pure water [2]. It has also the highest energy content per unit weight of any known fuel (142 kJg⁻¹) and can be transported for domestic/industrial consumption through conventional means [3]. However, hydrogen production at industrial scale is mainly based on natural gas reforming with high environmental impacts through the release of huge amounts of fossil carbon dioxide [4]. The two most important challenges are to generate sustainably enough H₂ from renewable sources and to reduce the production costs [5]. The development of green technologies is therefore crucial for the sustainability of a future H₂ transportation sector.

Currently, besides hydrogen production by water electrolysis based on renewable resources, the promising sectors of hydrogen production are those of biomass biorefinery.

Among diverse renewable biomass based H₂-producing technologies, Dark Fermentation (DF) using anaerobic microbial communities, has gained increased attention not only because it achieves high H₂ production rates, but also for its ability to produce H₂ at low costs when degrading complex and unsterilized substrates such as waste [6].

Among various organic-rich substrates, wastewaters and industrial by-products have gained a considerable attention due to their advantages such as high organic loads, low nutrient requirements and positive net energy gain [7]. Particularly, wastewaters represent abundant, cheap and widely available sources of biodegradable substrates not yet exploited to produce bio-hydrogen by dark fermentation (DF) [2]. The use of mixed consortia as biocatalyst for H₂ production, can be a practical and promising option for scaling up the DF technology, especially when wastewaters are used as substrates [8]. However, fermentative H₂ production provides only partial oxidation of the organic substrate. Therefore, it is likely to be industrially viable only if the fermentative bioprocesses are integrated within a stream that can utilize the fermentation by-products [9].

Bio-electrochemical systems have been proposed as a technology that can be coupled with fermentative hydrogen production [10]. More specifically, microbial electrolysis (ME) that is promising candidates for

the augmentation of classical, single-stage dark fermentation to generate hydrogen gas with better efficiency [11]. Indeed, microbial electrolysis is accomplished in an electrochemical reactor, with an anode at which organic substrates are microbially oxidized to provide an electric current that is then used at the cathode to electrochemically produce hydrogen from water. The anode and cathode may be or not separated by an ionic membrane. This process requires a small external power supply in order to make the hydrogen production thermodynamically favorable [12]. Combining DF with ME in a two-steps cascade process can result in a complete exploitation of biodegradable wastewaters, which maximizes at the same time both energy recovery and effluent treatment [10]. Combining necessities such as COD removal, material and energy recovery into a single cascade process increases the system overall efficiency and potential for deployment. This provides dual environmental benefits in the direction of wastewater treatment along with sustainable bioenergy generation [7]. In the field of ME cells for the production of hydrogen as a value-added product researches have explored all the parameters that might influence their performance, e.g.: electrode materials, pH, temperature, type of substrate, among many others [13–15].

To date, only a few studies have dealt with H₂ production in DF process coupled with ME. Mostly, single carbon sources were used as model substrates [8,16,17]. Nonetheless, more complex (fermentable) substrates, such as cellulose [18,19] have also been investigated.

Lalaurette et al. [20] were the first to propose a two-stage system (DF – ME) using organic waste (acid-pretreated corn stover) as substrate, but they observed methane generation in the ME.

Recently, different kinds of organic waste and by-products have been investigated for H₂ production by coupling DF and ME in a cascade process: acid-pretreated corn stalk [21]; crude glycerol [22] cheese whey [23] and waste activated sludge [24,25]. All these studies reported relatively high H₂ yields, with both overall H₂ yield and energy efficiency being much higher than the one obtained when only dark fermentation was used. Although all these works deal with bench-scale reactors, they clearly demonstrated the suitability of the use of dead-end fermentation products as substrates for microbial electrolysis, significantly increasing the overall production of hydrogen by coupling both technologies.

The aim of this research was to evaluate and compare in the same conditions the potential of different agro-industrial effluents, wastewaters and by-products, for the application of a two-step biohydrogen production process, combining DF and ME in a biorefinery framework.

Six different substrates coming from cheese (CW), fruit juice (FJW), paper (PW), sugar (SW), fruit processing (FPW) and spirits (VB2) factories were selected to evaluate the suitability of two-step process at bench-scale. These substrates were representative of different agro-industrial sectors, with wastewater treatment systems (or by-products current use) that could be upgraded by producing biohydrogen. The effluents from the fermentation of these substrates were tested in two chambers ME cells to further recover H₂ and COD removal.

2. MATERIALS AND METHODS

2.1 Agro-industrial wastewaters and by-products

Samples of 20 different agro-industrial wastewaters and by-products were collected from a wide range of industrial sectors (i.e. agroindustry, food, manufacturing, biofuel, wastewater treatment). They were evaluated in terms of H₂ potential in DF and fully characterized before and after dark fermentation [26]. Six different wastewaters and by-products, representative of different agro-industrial sectors, with wastewater treatment systems (or by-products current use) that could be upgraded by producing biohydrogen, were selected on the basis of fermentative conversion efficiency (i.e., both H₂ and metabolic by-product accumulation) to be used as substrate for the present study. They were: deproteinized ricotta cheese whey (**CW**) namely “scotta”, mixed sheep and cattle, supplied from a dairy factory in central Italy; fruit processing wastewater (**FPW**) from a food manufacturing industry from south of France; sugar production wastewater (**SW**) provided by a French sugar factory; wastewater from an industrial fruit juice production plant (**FJW**) located in south France; concentrated vinasse residues from the distillation of sugar beets for spirits production, supplied by industrial plant (**VB2**) of a French cooperative and paper mill wastewater (**PW**) collected from a French paper factory.

It's worth to precise that, whilst FPW, SW, FJW and PW are typical industrial wastewaters which undergone to the classical (aerobic or anaerobic) wastewater treatment process, CW and VB2 should be considered as industrial by-products. **CW**, namely “scotta”, is the effluent from the Ricotta cheese production, unlike “classical” whey, which is rich in proteins and is currently used as a livestock feed, scotta is poor in proteins [27]. This makes *scotta* unattractive for further processing; therefore the liquid itself has been poorly studied so far and is actually considered as a waste [28]. **VB2**, instead, is the result of six fold concentration of the original distillation effluent and currently it is valued as fertilizer.

As shown in table 1, the substrates composition varied significantly both in terms of total organic matter content, and biochemical or chemical composition.

For CW, FPW and FJW most of the determined COD (Chemical Oxygen Demand) was explained by its sugars content (from 60 to 80%). Proteins were only present in a minor extent in CW where they represented max. 10% of the total COD. Surprisingly, in SW which came from a sugar factory, sugars represented only 5% of VSS (Volatile Suspended solids) while most of the organic matter content was related to organic acids and alcohol, mainly acetic acid, lactic acid and ethanol (data not shown). This suggests that this wastewater had already undergone a natural fermentation process. In VB2 the glycerol represented a significant component of COD, around 30%, the remaining COD was explained by approximately 30% of proteins and around 30% of organic acids (in part lactate) and alcohol, sugars represented only a minor content ($\leq 3\%$ of VSS). PW is a kind of wastewater which is difficult to treat by

conventional aerobic and anaerobic methods cause of its contents in complex molecules and the presence of toxic compounds. In PW the contents in reduced metabolites exceeded the 20% of total COD, including a little content of lactic acid. It is possible to assume that substantial amount of the organic matter is represented by complex molecules as cellulose.

Table 1. Chemical and biochemical characteristics of raw agro-industrial wastewaters and by-products: ricotta cheese whey (CW); fruit juice production wastewater (FJW); fruit processing wastewater (FPW); paper mill wastewater (PW); sugar production wastewater (SW) and vinasse residues from the distillation of sugar beets for spirits production (VB2).

Substrate	COD (g/L)	Sugars (g/L)	Proteins (g/L)	Glycerol (g/L)	Lactate (g/L)	Cl ⁻ (g/L)	PO ₄ ³⁻ (g/L)	SO ₄ ²⁻ (g/L)	Na ⁺ (g/L)	K ⁺ (g/L)	N Tot (g/L)	NO ₂ ⁻ (mg/L)	NO ₃ ²⁻ (mg/L)	NH ₄ ⁺ (mg/L)	Mg ²⁺ (mg/L)	Ca ²⁺ (mg/L)
CW	57.80	39.36	5.88	0.00	0.60	7.98	0.44	9.52	9.57	0.51	0.94	0.00	65.16	20.73	83.12	210.50
FJW	1.80	1.03	0.08	0.00	0.00	0.60	0.00	0.84	0.92	0.03	0.01	0.00	8.83	0.00	13.98	32.58
FPW	39.10	16.96	0.75	0.00	0.00	3.45	0.00	9.45	7.01	0.36	0.12	0.00	56.55	0.00	83.64	354.63
PW	4.24	0.07	0.15	0.00	0.25	0.64	0.00	1.02	0.82	0.03	0.02	13.03	5.76	0.00	11.79	203.49
SW	2.35	0.09	0.12	0.00	0.17	6.39	0.00	1.02	4.08	0.10	0.02	9.39	6.28	0.00	32.05	282.93
VB2	455.67	13.19	149.96	94.47	66.60	7.92	1.09	23.57	24.73	49.92	23.99	37.91	3243.77	270.07	217.58	1102.72

2.2 Dark fermentation experiments

Dark fermentation batch experiments were carried out in 500 mL serum flasks with a working volume of 200 mL. To uniform the experimental conditions, raw substrate samples were diluted to reach an organic content ≤ 3 gCOD L⁻¹. SW and FJW which were characterized by a lower organic matter content (2.35 and 1.80 gCOD L⁻¹ respectively) were used without dilution. MES buffer 50 mM and 1 mL of oligoelements solution [29] were added to each flask. Anaerobically digested sludge pretreated by heat/shock treatment (90°, 10 min) was used as inoculum at a substrate-to-biomass ratio (S/X) of 10. The initial pH was adjusted to 5.5 with NaOH 2N or 37% HCl. All batch tests were performed in triplicate and standard deviations are presented through all the manuscript. After inoculation, each bottle was flushed with nitrogen gas (>99.995%) for 10 min to ensure anaerobic conditions. The bottles were then capped with a rubber stopper and incubated at 37°C. Reactors headspace were sampled at 6h intervals and gas pressure and composition (O₂, H₂, N₂ and CH₄) were measured using an automated multiplexed micro-gas chromatograph (IGC R3000, SRA instrument) equipped of a thermal conductivity detector using 12 to 20 flasks in parallel, as previously described [30]. All volumes were expressed under temperature and pressure standard conditions. Cumulative hydrogen production was determined by mass balance as previously described in [31]. The experimental procedure ended when the pressure in the flask headspace started to drop, indicating hydrogen consumption. After this, liquid samples were taken at the end of batch operation for pH and metabolite analyses. The effluents of dark fermentation were biochemically characterized, and stored in sealed bottles at -20°C to preserve the final composition to be tested in the second stage of microbial electrolysis cells.

2.3 Microbial electrolysis cell set up and operation

The electrochemical system used for hydrogen production from fermentation effluents was a cylindrical two-chamber reactor. Each chamber had a working volume of 400 mL. The anode was a 7.0 cm x 5.5 cm x 1.0 cm carbon felt (RVG4000, Mersen S.A., France), screwed onto a 2 mm diameter titanium rod (TI007910/13, Goodfellow SARL, France) as electron collector. The cathode was a 90% Platinum-10% Iridium mesh (Heraeus PSP S.A.S., France). The reactor was hermetically sealed with a silicone seal and crimping ring of stainless steel at each chamber. An anion exchange membrane (AEM, Fumasep FAA, FuMA-Tech BWT GmbH, Germany) separated both chambers. All ME tests were conducted at a set temperature of 37° C. A magnetic stirrer rotating at 250 rpm gently homogenized the mixture during all the experiments. ME tests were carried out in triplicate for FPW, PW and SW and in duplicate for FJW, CW and VB2; standard deviations are presented through all the manuscript.

All the bioelectrochemical experiments were conducted using a saturated calomel electrode (SCE, Hg / Hg₂Cl₂ +240 mV vs. SHE) (Orignalys Electrochem SAS, France). The anode potential was fixed at +200.0 mV vs. SCE (+444.0 mV vs. SHE) using a Potentiostat VMP3 (BioLogic Science Instruments, France) controlled by using the EC- Laboratory v.10.1 software. For all experiments, the current density was recorded every 60 s. The electroactive biofilm in the anode was previously enriched by using two chronoamperometric cycles with 2 g/L acetate as unique carbon and energy source. During these two chronoamperometric cycles a medium with the following composition was used: 2 g/L NH₄Cl, 0.5 g/L K₂HPO₄, 9.76 g/L MES (2-[N-morpholino] ethane sulfonic acid) buffer, trace vitamins/ minerals solutions [32] and 2-bromo-ethane sulfonate (BES) 10 mM to inhibit methanogens. At the beginning of each enrichment cycle, the ME reactors were inoculated with anaerobic sediments (10% v/v) as described in [33]. Duration of a chronoamperometric cycle during enrichment procedure varied from –6 to 7 days. Cyclic voltammetry (CV) was performed at the beginning of each ME enrichment batch cycle before reactor inoculation (control-CV) to assure that the electrochemical system was working adequately and at the maximum of current density production during chronoamperometry. An illustrative example of chronoamperometric cycles and relatives CVs performed during the enrichment procedure of electroactive biofilm is reported in Fig. S1 of Supplementary Information (SI).

After the enrichment cycles, the medium in the anode chamber was replaced with the effluents from the fermentation as carbon and energy source, neither inoculum nor additional nutrient components were added to the effluent during the test. Prior to use dark fermentation effluents, these were pretreated by removing microbial biomass through centrifugation (at 8000 rpm for 20 minutes) to avoid any possible influence. The pH was adjusted to 7.0 and effluents were flushed with nitrogen gas (purity ≥ 99.995%, Linde France S.A.) for 30 minutes and 10 minutes more within the sealed reactor to establish anaerobic conditions. The total duration of a chronoamperometric cycle during experiments varied from 7 - 20 days. The volume of biogas production in ME reactors was measured using water displacement method. The current, biogas composition, soluble organic molecules and volatile fatty acids (VFAs) concentration were monitored as described below.

2.4 Analytical measurements

The analysis of Total (TSS), Volatile Suspended Solids (VSS) and chemical oxygen demand (COD) were performed according to standard methods [34]. Total Nitrogen content (TKN) was analyzed using a Buchi digestion unit K438 and a Buchi 370-K distillator/titrator. Proteins were determined by multiplying Total Kjeldhal Nitrogen (TKN) by 6.25. The volatile fatty acids (VFA) composition was determined with a gas chromatograph (GC-580, Clarus) equipped with an automatic sampler, an Elite-FFAP column (PerkinElmer) and a flame ionization detector (FID) according to Quéméneur et al. [30]. The

concentrations of soluble sugars (glucose, fructose, sucrose, galactose, arabinose, lactose and xylose), glycerol and other soluble organic molecules such as ethanol, lactate, succinate and formate were measured by HPLC analysis and refractometric detection, as previously described in [35]. Ionic content was analyzed with ion chromatograph (ICS 3000, Dionex, USA) equipped with pre-columns NGI 2 mm and CG 11 2 mm followed by separation columns CS 16 3 mm and AG 15 2 mm for cations and anions, respectively. The eluents used for this analysis were HMSA (25– 40 mM) pumped at 0.3 mL/min for cations and KOH (10–74 mM) pumped at 0.35 mL/min for anions [36].

For ME experiments, the gas composition (CH₄, CO₂, H₂ and N₂) of the anodic and cathodic chambers was analyzed daily from a sample taken directly in the gaseous headspace of the reactor with a syringe which was analyzed by a gas chromatograph (Clarus 580 GC, Perkin Elmer, USA) coupled to thermal catharometer detector as described in [37]. All volumes were expressed under temperature and pressure standard conditions. Volatile fatty acids (VFA), residual sugars and non-VFA metabolic byproducts such as organic acids (lactate or formate) and ethanol in the liquid phase were analyzed every 2-3 days as described before.

2.5 Microbial community analysis of anodic biofilm

At the end of ME experiment, the anodic biofilm was sampled and genomic DNA was extracted and purified as previously described [32]. The V4 and V5 regions of the 16S rRNA genes were amplified and sequenced by Illumina MiSeq (get.genotoul.fr) and sequences were analysed as described elsewhere [38]. Sequences have been submitted to GenBank, with accession No. KU998264 - KU999067.

2.6 Calculations

A modified Gompertz equation was used to model hydrogen production in dark fermentation experiments [39], Excel Add-in Solver was used to fit this model to the experimental data as previously described in [40]. The performance of the ME reactors were characterized and compared based on the following parameters: Coulombic efficiency (CE), cathodic hydrogen recovery (r_{cat}), hydrogen yield (Y_{H_2}), maximum current density (A/m²) and electrical energy recovery yield (YE). All calculations were based on COD removal because of the complexity of the substrate used. The coulombic efficiency (CE, %) was calculated as $CE = n_{ce} / n_{th}$, where n_{ce} is the number of hydrogen moles that could be recovered based on the measured current and n_{th} is the theoretical maximal production based on COD removal. The cathodic hydrogen recovery (r_{cat} , %) was calculated as $r_{cat} = n_{H_2} / n_{ce}$, where n_{H_2} is number of moles of hydrogen actually produced at the cathode, which were calculated from the volume of hydrogen measured during the experiment and the ideal gas law. Calculations for hydrogen yield (Y_{H_2} , mL H₂/ g COD) were based on the hydrogen volume produced for unit of consumed COD as $Y_{H_2} = V_{H_2} / \Delta COD$.

The performances in terms of energy recovery (YE, %) were based on the energy content of the hydrogen produced, compared to the electric energy input added into the system by the potentiostat (W_e , kJ), as $YE = -W_{H_2} / W_e$. The amount of energy recovered by the hydrogen produced over a batch cycle (W_{H_2} , kJ) was calculated as $W_{H_2} = n_{H_2} \times \Delta H_{H_2}$ on the basis of the hydrogen heat combustion energy, $\Delta H_{H_2} = -285.8$ kJ/mol. The net electric energy yield has been calculated as electric energy input (Wh) consumed per mole of H_2 produced as $W_e / n_{H_2} = \Delta H / YE$ where ΔH is the heat of combustion of hydrogen = 79.3972 Wh/mol. The overall hydrogen yield was calculated as the sum of the cumulated volume of hydrogen per unit of COD consumed in each stage (Dark Fermentation + Microbial Electrolysis).

Analysis of variance (ANOVA) was performed for comparison of mean with *R* software (version 3.3.0, available in CRAN website) by using *Rcmdr* library.

3. RESULTS AND DISCUSSION

3.1 Dark Fermentation

3.1.1. Hydrogen production from fermentation of different agro-industrial wastewaters and by-products

Table 2 summarizes the results of the fermentation of different agro-industrial wastewaters and by-products, including H₂ production performances parameters and metabolic end products accumulation.

Table 2. Fermentation parameters of raw agro-industrial wastewaters and by-products: ricotta cheese whey (CW); fruit juice production wastewater (FJW); fruit processing wastewater (FPW); paper mill wastewater (PW); sugar production wastewater (SW) and vinasse residues from the distillation of sugar beets for spirits production (VB2).

Substrate	Time (h)	pH _F	cond _F (mS.s ⁻¹)	Yield	Gompertz parameters				ASuc	EtOH	AAce	APro	ABut	13Prop
				pH ₂ (mL)	r (mLH ₂ h ⁻¹)	λ (h)	R ²							
CW	37	5.5	7.4	95.1	53.0	3.30	17	1.00	12	101	35	0	81	0
FJW	35	5.1	6.1	74.9	25.6	2.73	13	0.99	0	235	105	0	92	0
FPW	34	4.8	5.9	57.6	39.5	2.48	12	1.00	36	77	279	34	209	0
PW	37	5.5	7.4	19.3	14.2	0.82	24	1.00	0	0	147	0	261	0
SW	37	5.4	15.8	18.8	8.5	1.08	21	1.00	19	0	94	9	286	154
VB2	59	5.6	6.6	35.4	15.5	0.81	26	1.00	127	62	74	57	117	0

Notes: time required to reach maximum production of hydrogen (Time), final pH (pH_F), final conductivity (cond_F) and H_2 production yields ($\text{mLH}_2 \text{ gCOD}_{\text{initial}}^{-1}$); H_2 Gompertz equation parameters: production potential (PH_2), rate (r), lag phase (λ) and Pearson correlation coefficients (R^2) between observed and predicted data; and metabolic end-products accumulation in $\text{mgCOD gCOD}^{-1}\text{L}^{-1}$ (ASuc= succinic acid, EtOH= ethanol, AAce= acetic acid, AProp= propionic acid, ABut= butyric acid, 13Prop=1,3 propanediol) when the maximum hydrogen was reached. (nd = not determined).

Depending on the substrate, the maximum cumulative hydrogen production yield varied from 18.8 ± 4.8 to $95.1 \pm 26.4 \text{ mLH}_2 \text{ gCOD}_{\text{initial}}^{-1}$ (Fig. 1). The highest yield was obtained with CW, which was the richest substrate in soluble sugars. Interestingly, CW also showed the highest variability between replicates (Fig. 1 and Table 2). This result is consistent with the yield of $102 \pm 33 \text{ mL H}_2 \text{ gVS}^{-1}$ previously reported for the same kind of substrate [41]. The wastewater issued from a sugar factory (SW), showed the lowest yield at $18.8 \pm 4.8 \text{ mLH}_2 \text{ gCOD}_{\text{initial}}^{-1}$, which is not surprising when considering the low amount of sugars content present (i.e. 5% of organic content) in the raw wastewater. Indeed, carbohydrate-rich substrates are mostly referred in the literature to favor biohydrogen production through fermentative pathways [6,8,42]. Our results are consistent with these findings since a significant linear correlation ($R^2 = 0.89$) was found between the cumulated volume of H_2 and the initial soluble sugars content in substrates.

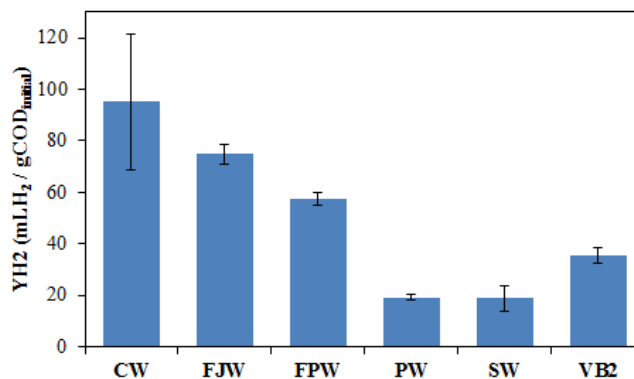


Figure 1. Maximum Bio-Hydrogen production yield ($\text{mLH}_2 / \text{gCOD}_{\text{initial}}$) obtained for various organic agro-industrial wastewaters and by-products: deproteinized ricotta cheese whey (**CW**); wastewater from an industrial fruit juice production plant (**FJW**); fruit processing wastewater (**FPW**); paper mill wastewater (**PW**); sugar production wastewater (**SW**) and vinasse residues from the distillation of sugar beets for spirits production (**VB2**). Error bars represent standard deviation of triplicates.

As shown in Table 2, the overall fermentation behaviors, including the time required to reach maximum production of hydrogen (Time), the final pH (pH_F), the final conductivity (cond_F) and the metabolic end-

products patterns (succinic acid, ethanol, propionic acid, butyric acid and 1,3 propanediol), varied in a large extent according to the origin of the wastewater. Interestingly, the Gompertz model was highly suitable to describe the kinetics of the BHP tests for all wastewaters (Fig. 2), as shown by the R^2 coefficients (>0.99) generated after fitting the Gompertz model to experimental data (Table 2).

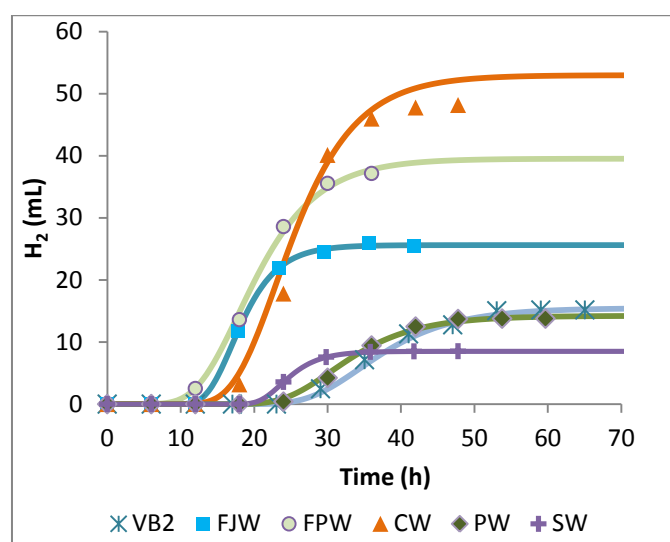


Figure 2. Cumulative hydrogen production (mL) from dark fermentation of different agro-industrial wastewaters and by-products: deproteinized ricotta cheese whey (CW); wastewater from an industrial fruit juice production plant (FJW); fruit processing wastewater (FPW); paper mill wastewater (PW); sugar production wastewater (SW) and vinasse residues from the distillation of sugar beets for spirits production (VB2). Points represent experimental data, while the curves are calculated by Gompertz equation.

3.1.2. Metabolic end products from fermentation of different agro-industrial wastewaters and by-products

At the end of fermentation experiments, no sugars were detected, nor lactate, initially contained in VB2 and CW. This shows that sugars and lactate were effectively consumed as substrates in the fermentative metabolism. Meanwhile, other reduced organic compounds, such as succinic acid, acetic acid and ethanol, which were detected in some of the raw wastewaters (FPW, SW and PW), were not degraded and their concentrations remained unchanged at the end of fermentation or were slightly accumulated during the process. Values reported in Table 2 are the ones observed in the samples at the end of the batch fermentation. Butyrate was the main end-product, followed by acetate and/or ethanol depending on the origin of the substrate. This main metabolic pathway was likely a result of initial heat-shock treatment of

the microbial inoculum which favorably selected spore-forming anaerobic bacteria, belonging mainly to the *Clostridium* genus. Indeed, fermentative metabolism of *Clostridium* species produces mainly butyrate and acetate as primary soluble metabolites [9]. Interestingly, ethanol was the main metabolite produced during CW fermentation, which was the substrate which yielded the max H₂. Such difference in metabolic pathways could be explained from the different initial composition of substrates. For example, CW was the sole substrate containing lactose. As reported elsewhere, the fermentation of lactose by a *Clostridium* strain (*Clostridium Thermolacticum*) produces mainly ethanol and acetate as end-products [43].

The amounts and the relative abundances of acetate, ethanol or butyrate varied depending on the type of substrate; propionate was produced in some cases but only in a poor quantity. Although lactate was not detected as end-product, it was initially detected in some substrates (Table 1). Probably, lactate was converted into butyrate with concomitant H₂ production. This former metabolic pathway has been widely observed in mixed culture hydrogen fermentative systems [44–46]. Guo et al. [47] reported that lactate had even a positive effect on bioH₂ production for carbohydrate-poor substrates, while it showed a strong negative effect for carbohydrate-rich substrates. The authors proposed an interaction term between carbohydrates and lactate concentrations in a model explaining the microbial pathways for hydrogen production from a large spectrum of organic waste [47]. In addition, 1,3 propanediol was also detected as end-product during the fermentation of VB2, which is not surprising since this substrate initially contained glycerol. Such metabolite, 1,3 propanediol, could be produced as by-product of glycerol fermentation, but this metabolic pathway is not linked to the production of hydrogen [48].

3.2 Further H₂ production from fermented agro-industrial wastewaters and by-products in microbial electrolysis cells

3.2.1 Coulombic efficiency and current density generation

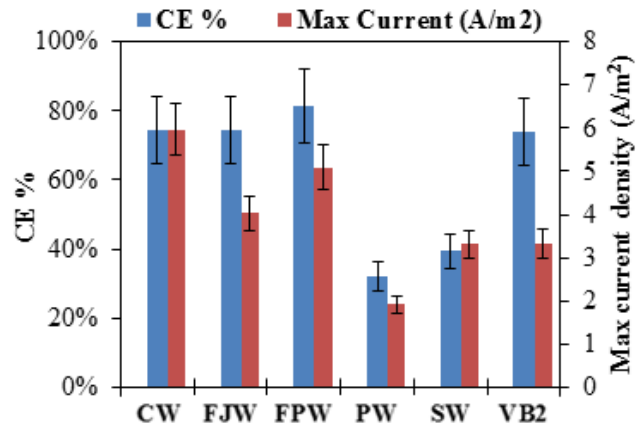


Figure 3. ME stage performance in terms of coulombic efficiency (CE, %) and maximum current density (A/m^2) from diverse fermented agro-industrial wastewaters and by-products: ricotta cheese whey (**CW**); fruit juice production wastewater (**FJW**); fruit processing wastewater (**FPW**); paper mill wastewater (**PW**); sugar production wastewater (**SW**) and vinasse residues from the distillation of sugar beets for spirits production (**VB2**).

Overall, ME results showed satisfactory performances in terms of electrical current production for almost all of the effluents when comparing to other works using a similar experimental system, ie. a carbon felt electrode colonized by an electroactive biofilm fed with an easy degradable substrate such as acetate [49]. The highest value of $7.46 \pm 1.76 A m^{-2}$ was obtained with deproteinized ricotta cheese whey (CW) effluent. Interestingly, a complete conversion of metabolic by-products to acetate was first observed before significant acetate consumption was observed and current started to increase.

Regarding the coulombic efficiency (CE) obtained from the diverse effluents tested (Fig. 3), it is important to point out the high range observed between the substrates. Such variability might be due to the diverse metabolic pathways that bacteria used to degrade the organic matter. Lower values of CE% mean an important loss of electrons from the substrate. A possible explanation of such loss is that the fed substrate, as in the case of PW, might have been partly used for biosynthesis of cellular components, e.g. biofilm formation, instead of direct substrate oxidation providing electrons to the anode. The best result in terms of coulombic efficiency was reached when the ME reactor was fed with fruit processing wastewater (FPW) with a value of $81.4 \pm 4.7\%$. Moreover, the average CE from the diverse effluents was $62.7 \pm 10.4\%$, which is a high value compared with CEs reported in previous studies dealing with pre-fermented effluents [20,22,23].

Chronoamperometric batch cycle performed with diverse fermented effluents are showed in Figure S2 of Supplementary Information.

3.2.2 Hydrogen generation and yield in the MEC

The results of the hydrogen production in MEC are shown in Figure 4 and Table 3. The highest hydrogen yield (Y_{H_2}) was observed with fruit juice wastewater (FJW), *i.e.*, 1478.2 ± 267.6 mL H_2 $gCOD_{consumed}^{-1}$. The best hydrogen cathodic recovery (r_{cat}) at $101.4 \pm 0.9\%$ was also found with fruit juice wastewater (FJW).

As reported in Table 3, a high variability of the performances was observed between substrates. These results show that carbon degradability and substrate conversion mainly depended on the different metabolites fractions fed to the ME reactors. Interestingly, the FJW, which showed the best performances in MEC, had the simplest initial composition of all the effluents, *ie.* only acetate and butyrate. Meanwhile, the PW, showing the worst result, had the most complex initial composition. Finally, a successful substrate to hydrogen conversion was obtained when the initial composition of the effluent had a significant quantity of ethanol (see Table 2).

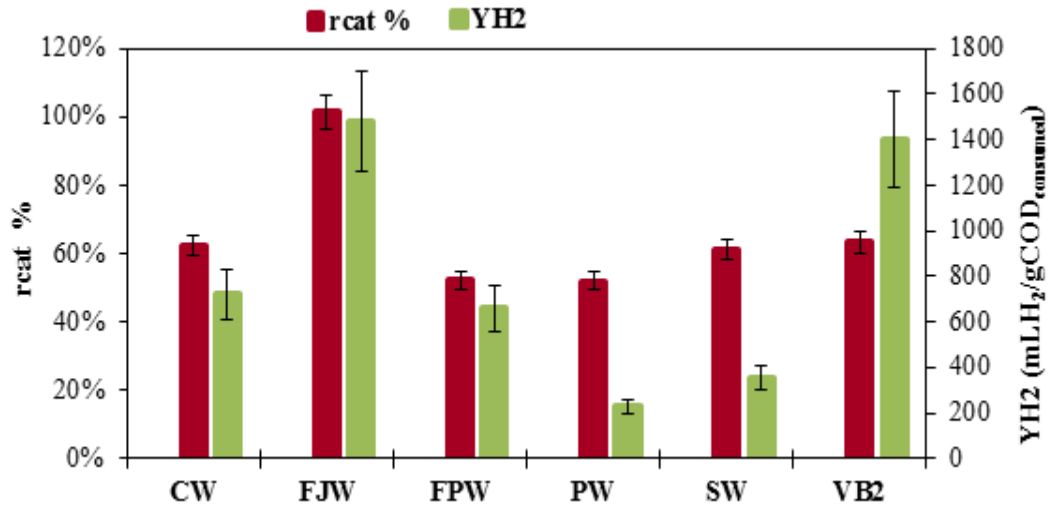


Figure 4. Hydrogen yield (Y_{H_2} , mL H₂/gCOD_{consumed}) and cathodic hydrogen recovery (r_{cat} , %) obtained in ME cells from fermentation effluents of diverse agro-industrial wastewaters and by-products: ricotta cheese whey (CW); fruit juice production wastewater (FJW); fruit processing wastewater (FPW); paper mill wastewater (PW); sugar production wastewater (SW) and vinasse residues from the distillation of sugar beets for spirits production (VB2).

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Values of cathodic hydrogen recovery ranged between $52.1 \pm 0.1\%$ and $101.4 \pm 0.9\%$. In all tested effluents, at least half of the current generated by the anode was used to produce hydrogen at the cathode. The lowest values of r_{cat} (Fig. 4) implied an important loss of electrons. As the cathodic chamber was abiotic, a low r_{cat} means that electrochemical side-reactions, such as reduction of metal ions, occurred in the cathodic chamber. It is likely that some ions of the substrates present in the anodic chamber diffused to

the cathode chamber through the membrane. It is worth mentioning that no methane was detected in the anode or in the cathode chambers and therefore could not explain electrons loss.

Table 3. MEC hydrogen production parameters of raw agro-industrial wastewaters and by-products:

Substrate	time (days)	H₂ (mL)	H₂ Yield (mLH₂ gCODconsumel⁻¹)	Electric Energy Yield (Wh/H₂ mol)
CW	14 ± 0	128.9 ± 4.8	714.7 ± 58.0	18.1 ± 0.8
FJW	16 ± 6	158.1 ± 14.4	1478.2 ± 267.6	11.0 ± 0.2
FPW	13 ± 7	56.9 ± 22.3	652.4 ± 38.5	21.5 ± 0.5
PW	9 ± 3	20.1 ± 20.3	219.4 ± 139.9	23.3 ± 3.0
SW	12 ± 5	51.3 ± 53.4	344.3 ± 175.7	25.2 ± 8.2
VB2	16 ± 0	176.7 ± 73.0	1399.57 ± 388.4	18.4 ± 1.8

CW = ricotta cheese whey; FJW = fruit juice production wastewater; FPW = fruit processing wastewater; PW = paper mill wastewater; SW = sugar production wastewater and VB2 = vinasse residues from the distillation of sugar beets for spirits production.

3.2.3 COD Removal and Energy yield in MEC

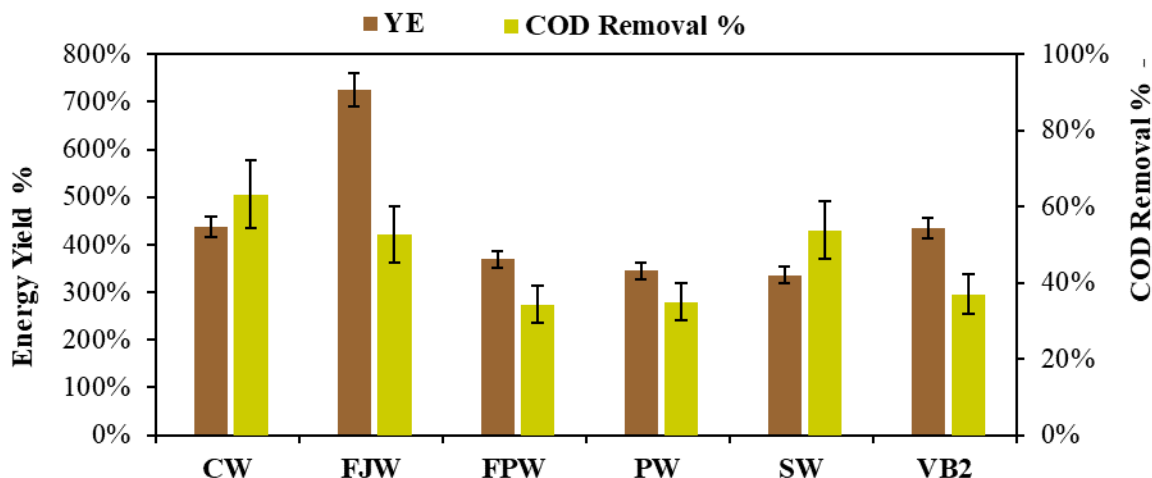


Figure 5. Energy yield (YE, %) and COD removal (%) in MEC stage from diverse fermented effluents: ricotta cheese whey (CW); fruit juice production wastewater (FJW); fruit processing wastewater (FPW); paper mill wastewater (PW); sugar production wastewater (SW) and vinasse residues from the distillation of sugar beets for spirits production (VB2).

Regarding the energy yield (Fig. 5), the total electrical input energy that was recovered from all effluents was always higher than 100%. In other words, the energy that could be recovered by combustion of H₂ was always higher than the supplied electrical needs. The minimum value was 336.3 ± 16.0 % meaning that the energy produced was at least three times higher than the supplied energy. The maximum energy recovery was 725.0 ± 7.8% from the fruit juice wastewater (FJW). Both high hydrogen yield and low electricity supply contributed to an increase of the overall energy yield.

Although the results presented in this study are obtained at bench scale, therefore they are not representative of an industrial process, it is interesting to compare them to the consumption of abiotic electrochemical electrolysis, as actual industrial alternative to the proposed bio-based H₂ production process. Commercial low temperature electrolyzers have system efficiencies of 56–73% (70.1–53.4 kWh/kg H₂ at 1 atm and 25 °C) [1] it means that the net electric energy (Wh) consumed per mole of H₂ produced ranged from 107 to 147. As reported in Table 3, the net energy yields of this study ranged from 11.0 ± 0.2 to 25.2 ± 8.2 Wh per mole of produced H₂, which is very lower. Thus, although these results

are not representative of an industrial process, they support the development of ME technologies as alternative for H₂ production, especially when combined with organic waste valorization.

Anyway an effective comparison should be done only with system in comparable conditions. Battle-Vilanova et al. [50], compared biotic and an abiotic (pure electrochemical reactions) MEC for hydrogen production in the identical operative conditions [50]. In that study, hydrogen was produced in a more efficiently way in the biotic ME and the energy demand of the abiotic MEC (170.1 - 995.5 kWh kg⁻¹ H₂) was higher than the biotic MEC (33.2 - 117.0 kWh kg⁻¹ H₂).

COD removal efficiency observed in MEC stage ranged from 35 to 63% in average and showed a great variability between replicates (Fig. 4). High variability of the energy yields and COD removals in MEC were likely resulting from the initial effluent COD concentration and composition. As shown in Fig. 4, the best results were obtained for CW, FJW and VB2. When comparing the data in Table 2 and Fig. 4, a relationship was found between high energy recovery and simple initial composition or a high initial amount of butyrate. The removal sequence of by-metabolites was as follows: ethanol > butyrate > acetate, which supports the previous conclusion.

3.2.4 Microbial community analysis of the anodic biofilms

MiSeq sequencing was performed on anodic biofilm samples after the end of the ME experiments. All the communities found on the anodes were highly diverse, with no clear dominance of any species (Simpson diversity indexes ranging from 0.91 to 0.93) except for the community found on the anode fed with FJW wastewater (Simpson diversity index of 0.73). This anode was the one exhibiting the best performances (see Table 3). ME reactor anodic biofilm fed with FJW was dominated by an OTU related to *Geovibrio ferrireducens* (99% sequence similarity) representing 50.3% of the total abundance. This OTU is found in most of the anodic biofilm microbial communities but at a lower abundance. Similarly to other electroactive bacteria belonging to the *Geobacteracea* family, *G.ferrireducens* is known to be a Fe(III)-reducing bacterium able to consume acetate and propionate, but unable to degrade ethanol and butyrate [51] which is consistent with the fact that the anodes were initially fed with acetate only. However, at this stage, the *G.ferrireducens* electrochemical activity cannot be definitively confirmed [52,53]. A detailed investigation of the electrochemical activity of *G.ferrireducens* should be specifically carried out as usually performed for other electrochemically active microorganisms [54]. Statistical analysis revealed that *G.ferrireducens* OTU abundance was correlated with a good overall H₂ production yield ($r = 0.76$, $p \leq 0.05$). Therefore this suggests that the bacteria corresponding to this OTU was able to perform an efficient conversion of the organic substrates into electricity. Butyrate and ethanol degradation and conversion into electricity was probably carried out by bacteria able to convert these substrates into acetate. Such conversion, as observed during the experiments, could have been supported by species related to the

Clostridiales order, since their total abundance was positively correlated to a good overall hydrogen yield ($r = 0.75$, $p \leq 0.05$). Another OTU of interest was revealed by statistical analysis and was related to an uncultured bacterium issued from the *Coriobacteriaceae* family. This OTU was present on all the anodes (1 to 10% of the total abundance) and its abundance positively correlated to a good CE ($r = 0.92$, $p \leq 0.01$) and a high current density ($r = 0.81$, $p \leq 0.05$). However, this OTU functions are still unknown.

3.3 Energy recovery from tested agro-industrial wastewaters and by-products by coupling dark fermentation and microbial electrolysis

Overall, combining dark fermentation and microbial electrolysis into a two-stage process resulted in a good overall hydrogen yield for the six tested effluents (Table 4). There was a significant effect of the wastewater type on the overall hydrogen yield at the $p < 0.05$ level for the six tested effluents [$F(5,9) = 4.959$, $p = 0.0186$]. A maximum value of $1608.6 \pm 266.2 \text{ mLH}_2 \text{ gCOD}_{\text{consumed}}^{-1}$, around $9.82 \text{ mol H}_2 \text{ mol glucose}^{-1}$ was obtained for fruit juice wastewater, which is one of the best results when compared with literature values (See Table 4). Such hydrogen yield is very close to the theoretically maximum value of $12 \text{ mol H}_2 \text{ mol glucose}^{-1}$. Additionally, a satisfactory COD removal (47.3% to 78.5%) was obtained when coupling both processes for all tested substrates.

Table 4 reports a compilation of published works that report the production of hydrogen from complex substrates by coupling dark fermentation and microbial electrolysis. Although it is a difficult task to compare among groups due to the lack of standardization regarding the way of performing experiments and reporting data extracted from them [13], from Table 4 we can observe that there is a clear tendency to use raw sources of substrates such as real wastewaters, by-products and lingo-cellulose materials coming from different industrial processes. In spite of the fact that all these works deal with bench-scale reactors, they clearly demonstrated the suitability of the use of dead-end fermentation products as substrates for microbial electrolysis, significantly increasing the overall production of hydrogen by coupling both technologies. Another pending task to tackle in the near future is the scaling-up of both coupled technologies. So far, the works compiled in Table 4 deal with bench-scale reactors that although provide useful information about the functioning of the coupled technologies, it does not produce information about their success in the industrial scale.

However, there is an increase in interest in developing these bio-based technologies and when considered as single process there currently are tests carried out at least at pilot scale. Tapia et al. [55], reviewed the existing pilot scale hydrogen production dark fermentation experiences and concluded that improvement of energetic yields of dark fermentation is required to reduce system costs as this technology is competitive in the market. Extensive research has been conducted to overcome these limits and several strategies have been proposed, such as pretreatment of the seed sludge, design/retrofitting of bioreactors,

optimization of bioprocess parameters (e.g. pH, temperature, organic loading rate, hydraulic retention time, effluent recycling ratio, etc.), reduction of H₂ partial pressure, bioaugmentation [56]. Regarding ME technology, there are already some large- scale experiments under way but MECs are far from being a mature technology; important challenges, mostly techno-economic in nature (cost of materials, reactors design, hydrogen management, etc.) remain [13]. Therefore, although the results reported so far are very promising, a considerable research effort is still necessary to make these technologies feasible at industrial scale.

Table 4. Compilation of published works that report the production of hydrogen from complex substrates by coupling dark fermentation and microbial electrolysis.

Substrate Source	Type of DF reactor	Electrodes materials used in ME stage		Applied volt. (V)	YH ₂ DF I Stage (ml H ₂ / g COD)	YH ₂ ME II Stage (ml H ₂ / g COD)	YH ₂ Coupling (ml H ₂ / g COD)	Electric Efficiency (Wh / mol H ₂)	COD removal (%)	Ref.
		Anode	Cathode							
Domestic wastewater	Batch 35°C	Graphite brush	Carbon cloth with a Pt catalyst layer.	0.8	135.15*	1200.00	1335.15*	51.56*	50	[24]
Cellulose	CSTR 60°C	Brush carbon fibers	Carbon cloth with a Pt catalyst layer	0.44	57.58*	812.72*	870.30*	78.91*	15	[18]
Corn stalk	Batch 36°C	Two pieces of square graphite felts	Carbon cloth with a Pt catalyst layer	0.8	168.16*	1000.00	1168.16*	47.83*	44 ± 2	[21]
Crude glycerol	Batch 35°C	Heat-treated graphite brush	Carbon cloth with stainless steel mesh	1.0	124.10*	106.15	230.25*	NA	10.4 ± 1.4 / 40.6 ± 4.9%	[22]
Lignocellulose and cellobiose	Batch 50°C	Graphite fiber brush pretreated using an NH ₃	Flat carbon cloth containing a Pt catalyst	0.5	225.92*	900.00	1125.92*	36.09*	50	[20]
Ricotta cheese whey (CW)	Batch 37°C	Carbon felt	90% Platinum - 10% Iridium mesh	0.2	122.27	714.69	836.96	18.12	78.5 ± 5.7	This work
Fruit juice production wastewater (FJW)	Batch 37°C	Carbon felt	90% Platinum - 10% Iridium mesh	0.2	130.41	1478.22	1608.63	10.95	71.8 ± 1.6	This work
Fruit processing wastewater (FPW)	Batch 37°C	Carbon felt	90% Platinum - 10% Iridium mesh	0.2	101.85	652.42	754.27	21.50	73.8 ± 3.6	This work

Paper mill wastewater (PW)	Batch 37°C	Carbon felt	90% Platinum - 10% Iridium mesh	0.2	37.37	219.39	256.76	23.26	57.1 ± 6.1	This work
Sugar production wastewater (SW)	Batch 37°C	Carbon felt	90% Platinum - 10% Iridium mesh	0.2	51.91	344.33	396.24	25.16	67.0 ± 13.1	This work
Vinasse from spirits production (VB2)	Batch 37°C	Carbon felt	90% Platinum - 10% Iridium mesh	0.2	87.70	1399.57	1487.27	18.37	47.3 ± 6.4	This work

*= Calculated; NA= not available

CONCLUSIONS

Six different industrial wastewaters and by-products coming from cheese, fruit juice, paper, sugar, fruit processing and spirits factories were evaluated for their potential to generate hydrogen by coupling dark fermentation and microbial electrolysis in a two-step process. By considering both processes concomitantly (and not separately), a maximum total Hydrogen yield of 1608.6 ± 266.2 ml H₂/gCOD_{consumed} and a maximum of $78.5 \pm 5.7\%$ of COD removal were achieved. An increase up to 13 times of the total H₂ recovered from real wastewater was reached when considering both processes in comparison to the DF step alone. Fruit Juice Wastewater (FPW) provided the best results which could be due to the composition of the effluent in terms of easy degradable metabolites. Interestingly, this raw wastewater could be treated without the need of any dilution, in contrast with other wastewaters and by-products, and high H₂ yield could be recovered. Therefore the proposed biorefinery scheme could offer a captivating alternative to the current expensive wastewaters treatment technologies, by transform what is currently considered as a waste in a value added product.

Meanwhile, if considering the initial COD content of raw substrates, the most interesting substrates to be exploited for H₂ production in such two-stage system could be vinasse residue from spirits production (VB2) followed by deproteinized ricotta cheese whey (CW). Indeed, up to 28 ± 5 liters of H₂ could be produced per liter of raw VB2 and 8.1 ± 1.4 liters of H₂ could be produced per liter of raw CW. However, in the case of VB2 it is already available a valuing chain (i.e. it is used as fertilizer), it should be considered whether it is convenient to replace it with the proposed one. In this case, H₂ production potential of VB2 should be evaluated before this spirits production effluent undergoes concentration procedure. On the other hand, there are currently no exploitation systems for deproteinized ricotta cheese whey (CW), namely “scotta”, therefore the results presented here could open an interesting field of investigation for the valorization of this dairy by-product.

Overall, these results showed that coupling DF and MEC for organic wastewater treatment and/or by-products transformation constitutes not only a suitable but also a highly promising route for producing bio-H₂ within a scheme of environmental biorefinery.

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