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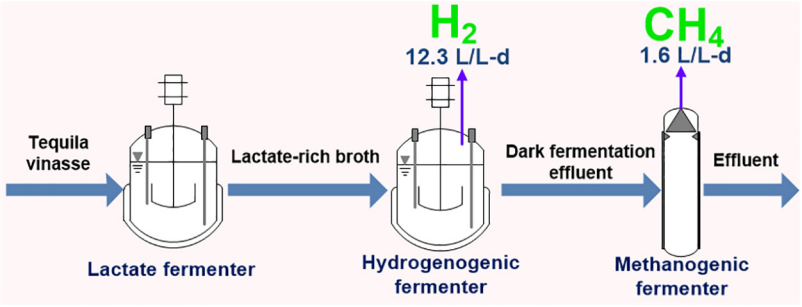
GRAPHICAL ABSTRACT



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ABSTRACT

This study evaluated a novel three-stage process devoted to the cascade production of lactate, biohydrogen and methane from tequila vinasse (TV), with emphasis on attaining a high and stable biohydrogen production rate (HPR) by utilizing lactate as biohydrogen precursor. In the first stage, tailored operating conditions applied to a sequencing batch reactor were effective in sustaining a lactate concentration of 12.4 g/L, corresponding to 89% of the total organic acids produced. In the second stage, the stimulation of lactate-centered dark fermentation which entails the decoupling of biohydrogen production from carbohydrates utilization was an effective approach enabling stable biohydrogen production, having HPR fluctuations less than 10% with a maximum HPR of 12.3 L/L-d and a biohydrogen yield of 3.1 L/L_{TV}. Finally, 1.6 L CH_4/L-d and 6.5 L CH_4/L_{TV} were obtained when feeding the biohydrogen fermentation effluent to a third methanogenic stage, yielding a global energy recovery of 267.5 kJ/L_{TV}.

1. Introduction

Tequila vinasse (TV) is generated in large amounts during the elaboration of tequila. It is estimated that the total volume of TV generated by Mexican tequila factories in 2019 was around 3630 million liters, which agrees with 10–12 L of TV generated per each liter of

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tequila produced (López-López et al., 2010). This distillery effluent is characterized by a low pH (3.4–4.5), high concentrations of chemical oxygen demand (COD; 25–100 g/L) and total solids (TS; 20–50 g/L), as well as by the presence of salts, metal ions, phenolic compounds and melanoidins in lower concentrations (López-López et al., 2010). TV is commonly treated by anaerobic digestion (AD); however, the AD of TV may result in acidification of the cultivation broth due to its high content of easily degradable compounds along with its lack of alkalinity (López-López et al., 2015). In this context, a two-stage AD process with separate acidogenesis and methanogenesis might enhance process stability, energy recovery and digestate quality provided proper reactor control of the separated stages is at place, particularly for feedstocks that undergo rapid acidification such as vinasses (Fuess et al., 2018; Lindner et al., 2016; Schievano et al., 2014). Furthermore, the hydrolytic/acidogenic stage separated from the methanogenic stage may foster waste biorefinery for producing high value-added by-products such as biohydrogen ($bioH_2$), a clean energy carrier derived from renewable sources, which is foreseen to play a major role in a biorefinery framework (Venkata Mohan et al., 2016).

Dark fermentation (DF) processes have long been proven to be a very promising alternative to produce $bioH_2$ (Ghimire et al., 2015). The DF process also induces a rise in the alkalinity of TV, provided that regulated pH conditions exist, which would be beneficial for the development of integrated DF-AD schemes. However, the bioconversion of organic wastes, including TV, into $bioH_2$ is often limited by the poor stability of the hydrogenogenic stage, which typically results in shortfalls in $bioH_2$ production rates (HPR) and yields (YH_2), and ultimately in process failure (Bakonyi et al., 2014). Indeed, most of the challenges involved in the implementation of two-stage $bioH_2$ and CH_4 schemes are concerned with the $bioH_2$-producing reactor (Guwy et al., 2011). In addition, instabilities in the hydrogenogenic stage can potentially upset the methanogenic reactor by disrupting the availability of CH_4 precursors. Therefore, the development of innovative operational strategies during DF that enable an effective and stable $bioH_2$ conversion is crucial to guarantee the implementation of integrated DF-AD schemes.

Besides the conventional acetate and butyrate H_2-producing pathways, $bioH_2$ production from lactate has been attracted more interest than at first expected. At this point, it should be stressed that there is strong evidence of the beneficial impact of producing $bioH_2$ from lactate mainly in terms of ensuring process stability, likely due to the availability of lactate as a simpler $bioH_2$ precursor derived from the fermentation of more complex compounds (Asunis et al., 2019; Blanco et al., 2019; Fuess et al., 2019; Juang et al., 2011), a phenomenon in which the lactate produced by lactate producers, e.g. lactic acid bacteria (LAB), is apparently cross-fed to some specialized H_2-producing bacteria (HPB) (Schwalm et al., 2019). In batch processes, a dual-phase lactate-based fermentation has been consistently imposed as the dominant DF metabolic pattern using TV as the feedstock, wherein complex carbohydrates are transformed mainly into lactate and acetate in a first step, and then both intermediates are subsequently converted mainly to butyrate and $bioH_2$ in a second step (hydrogenogenic stage) (Diaz-Cruces et al., 2020). However, the number of studies devoted to exploring the potential of lactate type fermentation for $bioH_2$ production is still scarce, especially more research using continuous $bioH_2$-producing systems is needed to engineer new enhanced DF process configurations.

The key objective of this study was to enhance the continuous $bioH_2$ production from TV by exploiting the lactate-driven DF. For this purpose, a two-stage lactate-centered process was developed and examined, focusing on improving $bioH_2$ productivity and stability. The acidogenic effluent was further evaluated in a third fermentation stage for CH_4 production, and the total energy recovery derived from the co-production of $bioH_2$ and CH_4 was estimated. It is expected that the lactate-driven DF approach, which entails the production of $bioH_2$ in carbohydrate-shortage conditions, can help to open up new perspectives for engineering more efficient and robust $bioH_2$-producing processes. The results herein discussed shed new light on the mechanisms underlying the lactate-type fermentation from TV, which in turn can be expanded to other feedstocks, such as food waste, distillery wastewater, molasses, cheese whey, among others.

2. Materials and methods

2.1. Feedstock

TV was kindly provided by a tequila factory located in Tequila, Jalisco, Mexico, which produces tequila “100% agave” through the autoclave cooking method. A 300-L sample of fresh TV was collected in plastic containers. TV was cooled to ambient temperature (ca. 30 °C) and centrifuged using a continuous centrifuge (Gea Westfalia, model asD2-06–107, Germany) operated at 10000 rpm with a feed flow rate of 15 mL/s. The liquid fraction was stored at 4 °C until use. The centrifuged TV was characterized as follows: pH 3.9 ± 0.2, COD 42.2 ± 0.6 g/L, TS 29.4 ± 0.51 g/L, volatile solids (VS) 26.7 ± 0.7 g/L, total nitrogen 69.3 ± 1.2 mg/L, total phosphorous 1298.3 ± 98.3 mg/L, and iron 22.7 mg/L. More details concerning the physicochemical composition of the TV used is available as Supplementary material.

2.2. Inocula

The inoculum coded as PTA-124566 by the American Type Culture Collection was used as the biocatalyst to perform both the primary lactate fermentation and hydrogenogenesis of TV. This biocatalyst, mainly encompassing HPB, LAB and acetic acid bacteria, was re-activated according to the procedure used by García-Depraect and León-Becerril (2018). In brief, 50 mL of the PTA-124566 inoculum was cultivated for 12 h, at 35 °C, pH 5.5–6.5 and 100 rpm, in a mechanically stirred reactor holding 450 mL of a growth medium containing (in g/L) lactose 10, NH_4Cl 2.4; K_2HPO_4 2.4; $MgSO_4{\cdot}7H_2O$ 1.5; KH_2PO_4 0.6; $CaCl_2{\cdot}2H_2O$ 0.15 and $FeSO_4{\cdot}7H_2O$ 0.05. On the other hand, active anaerobic granular sludge harvested from a properly operating full-scale up-flow anaerobic sludge blanket (UASB) reactor treating TV under mesophilic conditions was used as the methanogenic inoculum. The digestate of the large-scale biogas plant had a pH of 7.2 ± 0.2, an organic acids content of 0.12 ± 0.01 g acetate equivalent/L, an ammonium concentration of 26.5 ± 0.7 mg/L, a total alkalinity of 3032.4 ± 166.2 mg $CaCO_3$/L, and a VS/TS ratio of 0.8 ± 0.06, which implies the use of sludge with a suitable metabolic activity.

2.3. Experimental set-up and process operation

The experimental set-up consisted of three sequential stages, namely, primary lactate fermentation, hydrogenogenesis, and methanogenesis, as shown in the Supplementary material. A 20-L Bioclave bioreactor (Applikon Biotechnology, The Netherlands) with a working volume of 8 L was used to perform the primary lactate fermentation from TV. The lactate producing reactor was operated for 41 cycles in sequencing batch mode with a total cycle operation time of 12 h: 5 min filling, 11.5 h reaction, 20 min settling and 5 min discharging. The volume exchanged corresponded to 90% of the working volume, which resulted in a hydraulic retention time (HRT) of 13.3 h. Initially, the fermenter was filled with 10% of re-activated PTA-124566 inoculum and 90% (*v/v*) of TV. With the aim of favoring lactate formation in the primary lactate fermentation stage, the centrifuged TV was supplemented only with 2.4 g/L NH_4Cl to avoid nitrogen limitation. Temperature, pH and agitation rate were automatically maintained constant at 35 ± 0.5 °C, 5.5 ± 0.1 (using 10 N NaOH) and 100 rpm, respectively. The fermented TV was collected into a storage tank located into an ice-bath and employed to produce $bioH_2$.

A continuously stirred tank reactor (CSTR) with a total volume of 3 L (2 L working volume; Applikon Biotechnology, The Netherlands) was

Table 1
Operational parameters of the hydrogenogenic reactor.

	Period						
Parameter	I	II	III	IV	V	VI	VII
Days	[a]1.7–12	12–18.5	18.5–43.6	43.6–54.6	54.6–58.1	58.1–60.5	60.5–65.75
OLR (g COD/L-d)	42	56	85	113	169	253	169
OLR (g VS/L-d)	27	35	53	71	107	160	107
HRT (h)	24	18	12	9	6	4	6
Cycles	10.2	8.6	50.2	29.3	14.0	14.4	21.0

Notes: [a] Start-up batch phase: 0–1.7 days. Nomenclature: OLR: organic loading rate; HRT: hydraulic retention time.

used to carry out bioH_2 production via DF using the effluent derived from the anaerobic sequencing batch reactor (AnSBR). This effluent rich in lactate was supplemented only with 0.05 g/L $FeSO_4{\cdot}7H_2O$ prior to feeding it to the bioH_2 producing reactor due to the low iron content of the TV used in the present study, which is a key nutrient required for bioH_2 production (Lee et al., 2001). The hydrogenogenic fermenter was continuously operated for 65 d at different HRTs in seven periods (I–VII). During periods I–VI, the HRT was decreased stepwise from 24 to 18, 12, 9, 6 and 4 h by increasing the feed flow rate while maintaining the substrate concentration constant at 42 g COD/L, which entailed organic loading rates (OLRs) in the range of 42–253 g COD/L-d. In period VII, the CSTR was operated at an HRT of 6 h (169 COD/L-d) to evaluate the resilience of the process. Temperature, pH and agitation rate were automatically maintained at 35 ± 1 °C, 5.8 ± 0.1 (using 10 N NaOH or 3.5 N H_2SO_4) and 500 rpm, respectively, by using the ez-Control system (Applikon Biotechnology, The Netherlands). The operational conditions of the hydrogenogenic fermenter are summarized in Table 1. The hydrogenogenic fermenter was inoculated with 10% *v/v* of re-activated PTA-124566 inoculum and operated in batch mode for 1.7 d prior continuous operation (exponential bioH_2 production was taken as a switch-over criterion which was assumed to take place once the lag phase ended and the bioH_2 flow rate increased). The DF effluent obtained during period VII was collected into a storage tank located into an ice-bath. This effluent was diluted twice with distilled water and the pH-value was adjusted to 6.7 ± 0.16 using $NaHCO_3$ before feeding into the UASB reactor.

A UASB reactor with a working volume of 2 L was devoted to CH_4 production from the effluent of the DF-CSTR. A detailed description of the UASB reactor can be found elsewhere (López-López et al., 2015). Inoculation was performed with 20% (*v/v*) of the anaerobic sludge (López-López et al., 2015). The UASB was continuously operated for 30 d at 48 h of HRT (OLR = 8.7 g COD/L-d) and 35 ± 2 °C. The pH in the UASB remained in the range of 7.1–7.5. Recirculation of the treated effluent was maintained at a recycling flowrate to influent flowrate ratio of 1.0. Anaerobic conditions were assured naturally by facultative microorganisms, disregarding gas flushing and the addition of reducing agents during the entire three-stage operation. The biogas flow rate and composition (bioH_2 and CH_4), COD removal, carbohydrate conversion, and distribution of metabolic intermediates were monitored in the three units. Stabilized operational conditions were assumed to occur when the variation in HPR (or CH_4 production rate) was less than 10% (Kumar et al., 2016a). All chemicals used were of ACS reagent grade.

2.4. Analytical methods

The physicochemical composition of TV was characterized using standard methods (APHA, 2005). Total carbohydrates, total reducing sugars, protein, total phenolic content, and biogas composition (H_2, CH_4 and CO_2) were measured as previously reported by García-Depraect et al. (2017). Soluble metabolic products, including lactate, acetate, butyrate, and propionate, were measured by high-performance liquid chromatography (Varian ProStar, model 230, USA) according to the procedure used by García-Depraect and León-Becerril (2018). Biomass concentration in the AnSBR and CSTR was estimated from the intracellular protein content as previously outlined by García-Depraect and León-Becerril (2018). Gas production was measured using a μFlow® gas flow meter (Bioprocess control, Sweden). Gas volume was corrected to standard temperature (0 °C) and pressure (1 atm) conditions.

2.5. Data analysis

Energy recovery (ER, kJ/L_{TV} or $kJ/g\ VS_{added}$) was calculated for bioH_2 and CH_4 considering H_2 (12.74 kJ/L) and CH_4 (35.16 kJ/L) superior heat of combustion according to Schievano et al. (2014). The total ER was estimated as the sum of the ER from bioH_2 and CH_4. BioH_2 production stability index (HPSI) was calculated using Eq. (1), which considers variations in HPR during all operation time of a given period (excluding results from the first 3 HRTs of operation), as previously reported by Tenca et al. (2011). The non-parametric Mann-Whitney test, with a significance level of 5%, was used to compare the bioH_2 production performance attained in periods I–VII in terms of HPR and YH_2.

$$HPSI = 1 - \frac{standard\ deviation\ HPR}{average\ HPR} \quad (1)$$

3. Results and discussion

3.1. Primary lactate fermenter performance

The primary lactate fermentation constituted the first stage of the proposed three-stage fermentation system. Interestingly, there was no removal of COD during the primary lactate fermentation, whereas the consumption of reducing sugars and total carbohydrates in this stage was 55.2 ± 4.5% and 64 ± 10.8%, respectively. The need for a continuous supply of an alkaline agent to maintain the desired pH was 3.2 ± 0.6 mL/L of culture, which is indicative of the accumulation of soluble metabolites due to bacterial activity. Indeed, biomass grew from 0.2 to 0.6 g/L of cell dry weight, shaping a metabolic profile where lactate was the dominant organic acid (89.4 ± 6.2% of the total organic acids) with a concentration of 12.4 ± 2.9 g/L (Table 2).

It has been shown, in DF batch cultures using TV as the substrate, that the hydrogen production performance is strongly impacted by operational pH (García-Depraect et al., 2019). In this study, besides the inoculum used, the low pH of 5.5 and reaction times below the critical retention time for lactate production were found to be of utmost importance to steer the metabolic pathway toward lactate production. Thus, no loss of reducing equivalents and carbon in the form of bioH_2 and CO_2, respectively, occurred as indicated by the null production of biogas recorded. Little to no biogas production during the first days of hydrolysis/acidogenesis has also been observed in previous studies using mixed consortia as biocatalyst (Asunis et al., 2019; Sträuber et al., 2012).

In this context, control of fermentation time and pH has been used as a strategy to steer the process to a targeted metabolic pathway (Asunis et al., 2019; Fuess et al., 2019). The selective production of

Table 2
Operation performance of the lactate producing reactor under semi-continuous mode.

Parameter	Feed	Effluent
COD (g/L)	42.2 ± 0.6 (3)	44.0 ± 1.5 (25)
10 N NaOH consumption (mL/L)	5.8 ± 0.5 (41)	3.2 ± 0.6 (41)
Reducing sugars (g/L)	10.0 ± 0.1 (3)	4.4 ± 0.4 (65)
Total carbohydrates (g/L)	14.4 ± 0.2 (3)	5.1 ± 1.5 (18)
Biomass (g CDW/L)	0.23 ± 0.05 (3)	0.6 ± 0.2 (67)
Lactate (g/L)	2.5	12.4 ± 2.9 (41)
Acetate (g/L)	2.2	1.4 ± 0.8 (35)
Butyrate (g/L)	BDL	BDL (41)
Propionate (g/L)	0.3	BDL (41)

Notes: Mean values ± standard deviation. The number of samples is indicated in parenthesis. Organic acids of the feed were assessed from only one measure. Nomenclature: CDW: cell dry weight; BDL: Values were below the analytical detection limit in the assay (10 mg/L).

lactate by pH control has been reported in the pH range of 3.5–4.0, whereas higher pH values in the range of 5.0 to 7.0 could trigger mixed-acid fermentation (Gu et al., 2018; Itoh et al., 2012; Wu et al., 2016). The differences in lactate selectivity between this work and the others are attributed to several factors. The key operating parameters that govern lactate formation efficiency are still not fully understood, but the outcomes of this study indicate that a selective production of lactate could be achievable by using tailored environmental and operational conditions, i.e. pH, nutrients supplementation, reaction time, and inoculum, the latter being the most determining factor.

From a microbiological point of view, the selective production of lactate has been strongly linked to the presence of LAB, mainly to the genera *Lactobacillus* and *Streptococcus* (Gu et al., 2018; Sträuber et al., 2012; Wu et al., 2016). In our particular study, *Lactobacillus* and *Streptococcus* were dominant in the microbial consortium seeded to the lactate producing reactor (García-Depraect and León-Becerril, 2018), thus it is possible that they were the main responsible for lactate production in the first stage that supported the activity of lactate-consuming HPB in the following hydrogenogenic fermenter. In this context, the null biogas production and the accumulation of lactate strongly suggest that the low pH and short reaction time used in this study led to the decoupling of the primary lactate fermentation dominated presumably by LAB from the second lactate fermentation, in which higher proliferation of HPB is expected to occur.

3.2. Hydrogenogenic fermenter performance

The lactate-rich TV produced in the AnSBR was fed into the hydrogenogenic reactor, which was continuously operated for 65 d at decreasing HRTs while keeping the COD concentration constant. The results showed that the HRT/OLR exhibited a strong effect on HPR and YH_2 (Fig. 1, Table 3). The gradual decrease in the HRT from 24 h to 6 h (corresponding to OLRs from 42 to 169 g COD-L/d) resulted in increasing HPR (0.12–11.7 L H_2/L-d) and YH_2 (4.7–128 mL H_2/g VS_{added}), which indicated that the lactate-consuming HPB enriched in the hydrogenogenic fermenter were able to metabolize lactate, likely along with acetate, mainly into $bioH_2$ and butyrate. Lactate removal efficiencies ranged from 38.6% to 99.6% depending on the HRT applied (Fig. 2). However, the decrease in HRT to 4 h during period VI (at an OLR of 253 g COD/L-d) mediated a decrease of 71.7% and 81.1% in the HPR and YH_2 (on VS_{added} basis), respectively, compared to those attained at an HRT of 6 h. This deterioration in process performance was consistent with a decrease in biomass concentration from 1.65 to 1.2 g cell dry weight/L and an increase in lactate concentration. This process collapse was attributed to cell washout. This adverse effect on process performance at low HRT was also observed in previous works (Kumar et al., 2016b; Roy et al., 2014; Santos et al., 2014).

Overall, the HPR of 11.7 L H_2/L-d attained during period V (6 h-HRT, 169 g COD/L-d) was found to be statistically higher than those achieved in periods I–IV, as indicated by the nonparametric Mann-Whitney test with a significant level of 5%. As shown in Fig. 1E, the $bioH_2$ content in the biogas generated in the DF-CSTR varied along the entire process from 15.6% to 90.7% *v/v*. On the other hand, there was no clear trend in COD removal efficiency, which averaged 13.1%. The COD-based mass balance considering the soluble effluent COD, biomass growth and $bioH_2$ formation ranged from 91.9% to 99.3%. The maximum $bioH_2$ content here recorded was higher than the majority of values reported in the literature, and similar to that (92%) reported by Kumar and Das (2000) using *Enterobacter cloacae* IIT-BT 08 and sucrose in batch cultures.

Interestingly, based on the initial amount of carbohydrates in TV, an incomplete carbohydrate conversion with an average value of 75.5 ± 6.5% was found throughout the whole three-stage system, which agrees with the conversion efficiencies reported by other studies fermenting vinasse (Ferraz Júnior et al., 2014; Santos et al., 2014). However, the carbohydrates conversion in DF-CSTR barely reached 8.5% of the total carbohydrates conversion. In this regard, the role of lactate as the direct $bioH_2$ precursor explains the mismatch between $bioH_2$ production and carbohydrates consumption, since in this particular case $bioH_2$ is produced from the consumption of lactate rather than from carbohydrates, a metabolic pattern also observed in other studies (Asunis et al., 2019; Blanco et al., 2019; Detman et al., 2019, Fuess et al., 2018, 2019; García-Depraect et al., 2017, 2019; Juang et al., 2011; Kim et al., 2012). In this context, it has been shown that in this metabolic pathway, lactate and acetate serve as the electron donor and acceptor, respectively (Tao et al., 2016).

The HPR recorded in the present study at an HRT of 6 h was very similar to the 12.4 L H_2/L-d obtained by García-Depraect et al. (2020) using a TV-fed CSTR operated at an HRT of 4 h and an OLR of 309.0 g COD/L-d, which together are rank at the top of $bioH_2$ productivities so far reported in the literature using TV as the feedstock. When compared to HPRs obtained from sugarcane vinasse, the maximum HPR observed in this study was approximately 11.5 times higher than that obtained by Ferraz Júnior et al. (2014) under 55 °C and an HRT of 12 h (72.4 g COD/L-d), similar to that of dos Reis et al. (2015) under 22 °C and a HRT of 1 h (5 g COD/L), and 0.4 times lower than the one reported by Santos et al. (2014) under 55 °C and a HRT of 1 h (720 g COD/L-d). Such differences could be attributed to variations in the type of microbial populations, vinasse characteristics, reactor configuration and environmental and operating conditions.

3.2.1. $BioH_2$ production stability

The stability of the hydrogenogenic reactor was assessed by monitoring biogas composition, HPR, YH_2, HPSI and the distribution of soluble metabolites. Period VII served to evaluate the capacity of the DF-CSTR to return to the previous pseudo-steady state following the process deterioration induced by the decrease in the HRT from 6 to 4 h. The DF-CSTR showed a remarkable instability and low process performance during period I (HPSI = 0.35; Table 3), which experienced a sharp drop in $bioH_2$ content, YH_2 and HPR from 61 to 15%, 34.7 to 4.4 mL H_2/g VS_{added} and 0.9 to 0.1 L H_2/L-d, respectively (Fig. 1C and D). The decrease in HRT during period II which took place in order to avoid operational failure resulted in an initial increase in the $bioH_2$ content, YH_2 and HPR up to 34%, 16.6 mL H_2/g VS_{added} and 0.6 L H_2/L-d, respectively, which gradually declined to 21%, 4.1 mL H_2/g VS_{added} and 0.1 L H_2/L-d, respectively, by the end of this period. In this context, HPSI slightly increased up to 0.42. Interestingly, the $bioH_2$ content, YH_2 and HPR rapidly increased up to steady-state values of 70%, 64 mL H_2/g VS_{added} and 3.4 L H_2/L-d, respectively, during period III, along with an increase in the HPSI up to 0.7. The further decrease in HRT to 9 and 6 h mediated HPSI indices of 0.9–0.94 along with a significant increase in the $bioH_2$ content, YH_2 and HPR up to 90.1%, 109.8 mL H_2/g VS_{added} and 11.7 L H_2/L-d, respectively.

The stable $bioH_2$-producing periods were mostly characterized by

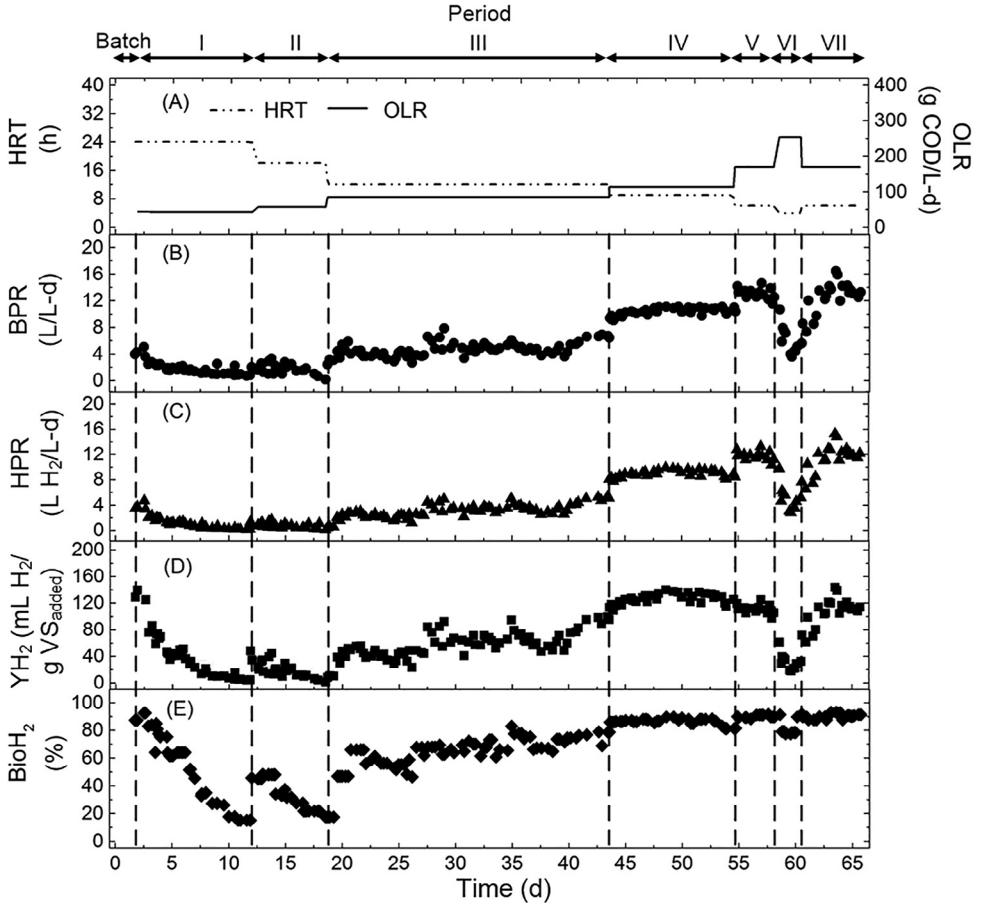

Fig. 1. Time course of biogas production rate (BPR), biohydrogen ($bioH_2$) production rate (HPR) and yield (YH_2) and $bioH_2$ content in the hydrogenogenic fermenter. YH_2 was calculated based on the initial volatile solid content of TV. Nomenclature: HRT: hydraulic retention time; OLR: organic loading rate.

lower lactate and propionate concentrations than unstable periods. A rapid decrease in process performance was recorded at an HRT of 4 h; however, the increase in HRT from 4 to 6 h during period VII mediated HPR and YH_2 statistically similar to those obtained in period V after 3 HRTs, revealing that the fermenter rapidly recovered the previous pseudo-steady state (Table 3). In this context, the HPSI value attained in period VII was 0.90 (Table 3), which demonstrated that the DF-CSTR performed under highly stable conditions and supported the fact that high and stable $bioH_2$ production can be derived from the utilization of lactate rather than from carbohydrates. Finally, there is a possibility that the recorded different fermentative $bioH_2$ production performances were caused by a gradual shift in the microbial community structure. However, further investigations will be necessary to elucidate microbial ecology and for a better understanding of the relationships between microbial community dynamics and process performance.

Table 3
Steady-state performance of the hydrogenogenic reactor under different operational conditions using lactate-rich tequila vinasse (TV) as feedstock.

Period							
Parameter	I	II	III	IV	V	VI	VII
BPR (L/L-d)	0.8 ± 0.07 (5)	1.4 ± 0.2 (6)	4.8 ± 0.3 (26)	10.5 ± 0.4 (28)	12.9 ± 0.8 (11)	4.3 ± 0.2 (3)	13.6 ± 1.2 (16)
HPR (L H_2/L-d)	0.12 ± 0.01 (5)	0.4 ± 0.04 (6)	3.4 ± 0.3 (26)	9.1 ± 0.4 (28)	11.7 ± 0.7 (11)	3.3 ± 0.2 (3)	12.3 ± 1.2 (16)
[a]YH_2 (mL H_2/g VS_{added})	4.7 ± 0.5 (5)	10.8 ± 1.2 (6)	64.2 ± 6.0 (26)	128.1 ± 6.5 (28)	109.8 ± 7.2 (11)	20.7 ± 1.5 (3)	115.9 ± 11.2 (16)
[a]YH_2 (mmol H_2/g COD_{added})	0.13 ± 0.01 (5)	0.3 ± 0.03 (6)	1.8 ± 0.1 (26)	3.5 ± 0.3 (28)	3.1 ± 0.2 (11)	0.5 ± 0.04 (3)	3.2 ± 0.3 (16)
[a]YH_2 (mmol H_2/g $COD_{removed}$)	1.1 ± 0.1 (5)	3.6 ± 0.4 (6)	18.6 ± 1.7 (26)	21.3 ± 1.1 (28)	16.1 ± 1.1 (11)	5.1 ± 0.3 (3)	19.2 ± 1.8 (16)
YH_2 (L H_2/L_{TV})	0.1 ± 0.01 (5)	0.3 ± 0.3 (6)	1.7 ± 0.1 (26)	3.4 ± 0.1 (28)	2.9 ± 0.2 (11)	0.5 ± 0.04	3.1 ± 0.3 (16)
*[a]YH_2 (mol H_2/mol carbohydrate)	0.13 ± 0.01 (5)	0.3 ± 0.03 (6)	1.8 ± 0.16 (26)	3.6 ± 0.2 (28)	3.1 ± 0.2 (11)	0.5 ± 0.04 (3)	3.3 ± 0.3 (16)
$BioH_2$ content (% *v*/*v*)	15.6 ± 1.1 (5)	27.5 ± 5.1 (6)	70.5 ± 6.3 (26)	86.4 ± 2.3 (28)	90.1 ± 1.1 (11)	78.1 ± 0.5 (3)	90.7 ± 1.7 (16)
HPSI	0.35	0.42	0.70	0.94	0.93	0.75	0.93
Total COD removal (%)	11.7 ± 5.7 (5)	8.5 ± 2.4 (4)	9.7 ± 3.7 (5)	16.9 ± 4.3 (4)	18.7 ± 1.1 (3)	11.4 ± 5.5 (2)	16.9 ± 2.0 (4)
[a]Carbohydrate conversion (%)	74.3 ± 8.6 (3)	79.1 ± 0.5 (3)	80.2 ± 5.0 (3)	80.9 ± 0.6 (3)	74.1 ± 7.4 (3)	72.1 ± 6.8 (2)	67.1 ± 3.4 (3)

Notes: Average values ± standard deviation. The number of samples is indicated in parenthesis. *Values calculated using the molar mass of sucrose (342.29 g/mol) as a reference, according to Ferraz Júnior et al. (2014) and Fuess et al. (2017). [a]Parameters calculated based on the initial composition of TV and not from the composition of TV coming from the lactate producing reactor. Nomenclature: HRT: hydraulic retention time; OLR: organic loading rate; BPR: biogas production rate; HPR: $bioH_2$ production rate; YH_2: $bioH_2$ yield; HPSI: $bioH_2$ production stability index; COD: chemical oxygen demand.

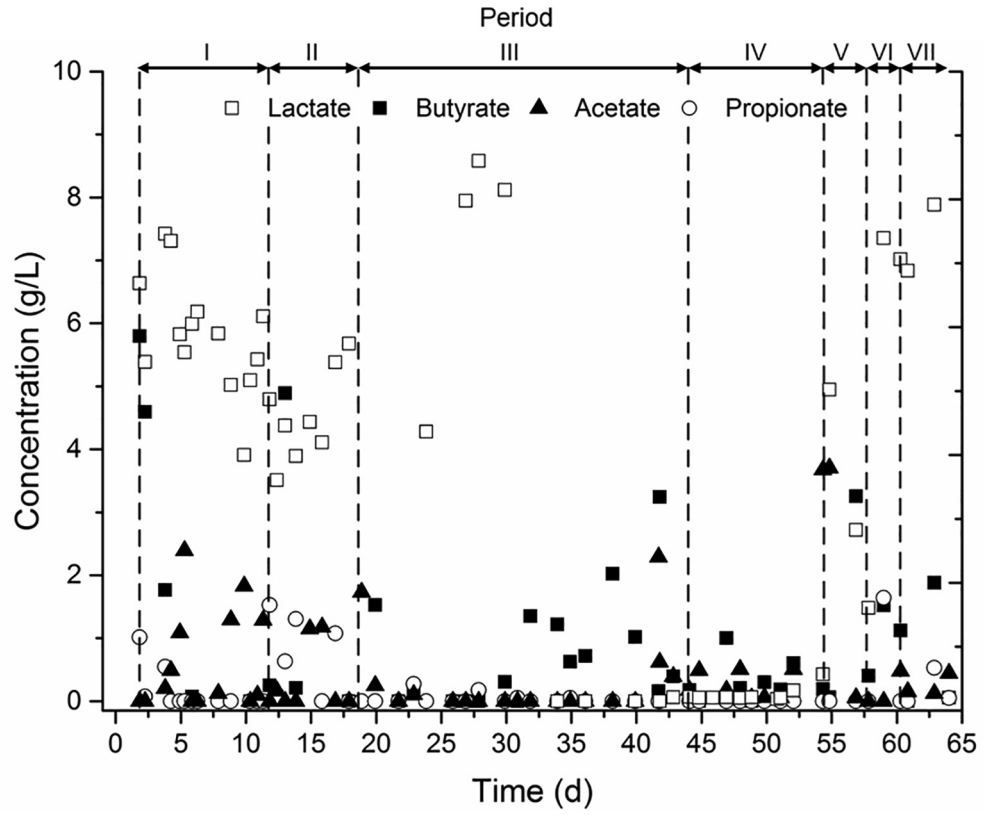

Fig. 2. Time course of lactate, butyrate, acetate and propionate concentrations in the hydrogenogenic reactor.

3.3. Methanogenic reactor performance

The hydrogenogenic reactor effluent obtained during period VII was treated using AD in order to boost the amount of energy recovered and COD removal efficiency. Under stabilized operational conditions, CH_4 production rate was 1.6 L CH_4/L-d, corresponding to a CH_4 yield of 6.5 L CH_4/L_{TV} (or 0.3 L CH_4/g $COD_{removed}$). The CH_4 content of the biogas averaged 68.5 ± 6.5% (*v/v*). It is worth noting that neutral pH in the cultivation broth of the UASB was maintained throughout the entire operation, with average total alkalinity of 5.1 ± 0.8 g $CaCO_3$/L. COD removal in the UASB averaged 61.3%, corresponding to an elimination capacity of 5.4 g COD/L-d and a COD effluent concentration of 6.7 ± 0.9 g/L. The COD recovery considering the soluble COD of the effluent, biomass growth, and CH_4 formation was of 96.4% assuming a COD loss due to biomass growth of 5% (van Lier et al., 2008). López-López et al. (2015) reported COD removal efficiencies of 61.4%–75.6% in a mesophilic UASB digester treating TV at 48 h of HRT under different OLRs (7.5–20 g COD/L-d) and recirculation ratios (1–10). In another study, Buitrón et al. (2014) reported removal efficiencies of 56%, 65% and 67% when TV at initial concentrations of 400, 1085 and 1636 mg COD/L, respectively, was continuously fed to a mesophilic UASB digester, operating at an HRT of 24 h. The authors found that no organic acids or very low concentrations were present in the digestate, which suggested that the treated TV contained recalcitrant compounds. Likewise, the organic acids in the effluent of the UASB here operated were also barely detectable at stabilized operational conditions (Table 4), which confirmed that lactate was efficiently degraded (Wu et al., 2016). Overall, the total COD removal in the integrated cascade process averaged 67.9%, implying the need for further methanogenic optimization.

3.4. Bioenergy recovery and mass balance

At optimum conditions for $bioH_2$ production (HRT of 6 h and OLR of 169 g COD/L-d), the HPR and YH_2 of 12.3 ± 1.2 L H_2/L-d and 115.9 ± 11.2 mL H_2/g VS_{added} were obtained, respectively. Taking into consideration the superior heat of combustion of H_2 (12.74 kJ/L), the hydrogenogenic reactor yielded 39.28 kJ/L_{TV} (1.47 kJ/g VS_{added}), corresponding to 14.6% of the total ER. This ER from $bioH_2$ was quite similar to the 1.5 kJ/g VS_{added} obtained in our previous batch study on $bioH_2$ production from TV under the same temperature and pH conditions (García-Depraect and León-Becerril, 2018). Meanwhile, the EPR from the hydrogenogenic reactor varied with respect to the operational condition applied with a maximum of 150.3 kJ/L-d achieved in periods V and VII. On the other hand, the methanogenic reactor under the conditions evaluated (HRT of 48 h and OLR of 8.7 g COD/L-d) showed a

Table 4
Performance obtained from the methanogenic reactor under stabilized operational conditions.

Parameter	Value
Daily biogas production (L/d)	4.7 ± 0.4 (22)
CH_4 content (% *v/v*)	68.5 ± 6.5 (19)
Biogas production rate (L/L-d)	2.3 ± 0.19 (22)
CH_4 production rate (L CH_4/L-d)	1.6 ± 0.13 (22)
pH influent	6.7 ± 0.16(19)
pH digester	7.3 ± 0.2 (19)
pH effluent	7.47 ± 0.15 (18)
Total alkalinity (TAlk; mg $CaCO_3$/L)	5161.1 ± 837.5 (6)
Total organic acid concentration (TA; mg/L)	504.8 (16)
TA/TAlk	0.1
Ammonia nitrogen in influent (mg/L)	95.5 ± 14.5 (16)
Ammonia nitrogen in effluent (mg/L)	102.3 ± 16.1 (16)
Lactate (mg/L)	313.3; Max:1380; Min: 0 (16)
Acetate (mg/L)	13.1; Max:90; Min: 0 (16)
Butyrate (mg/L)	104.2; Max:333; Min: 0 (14)
Propionate (mg/L)	73.8; Max:670; Min: 0 (16)

Notes: Mean values ± standard deviation. The number of samples is indicated in parenthesis.

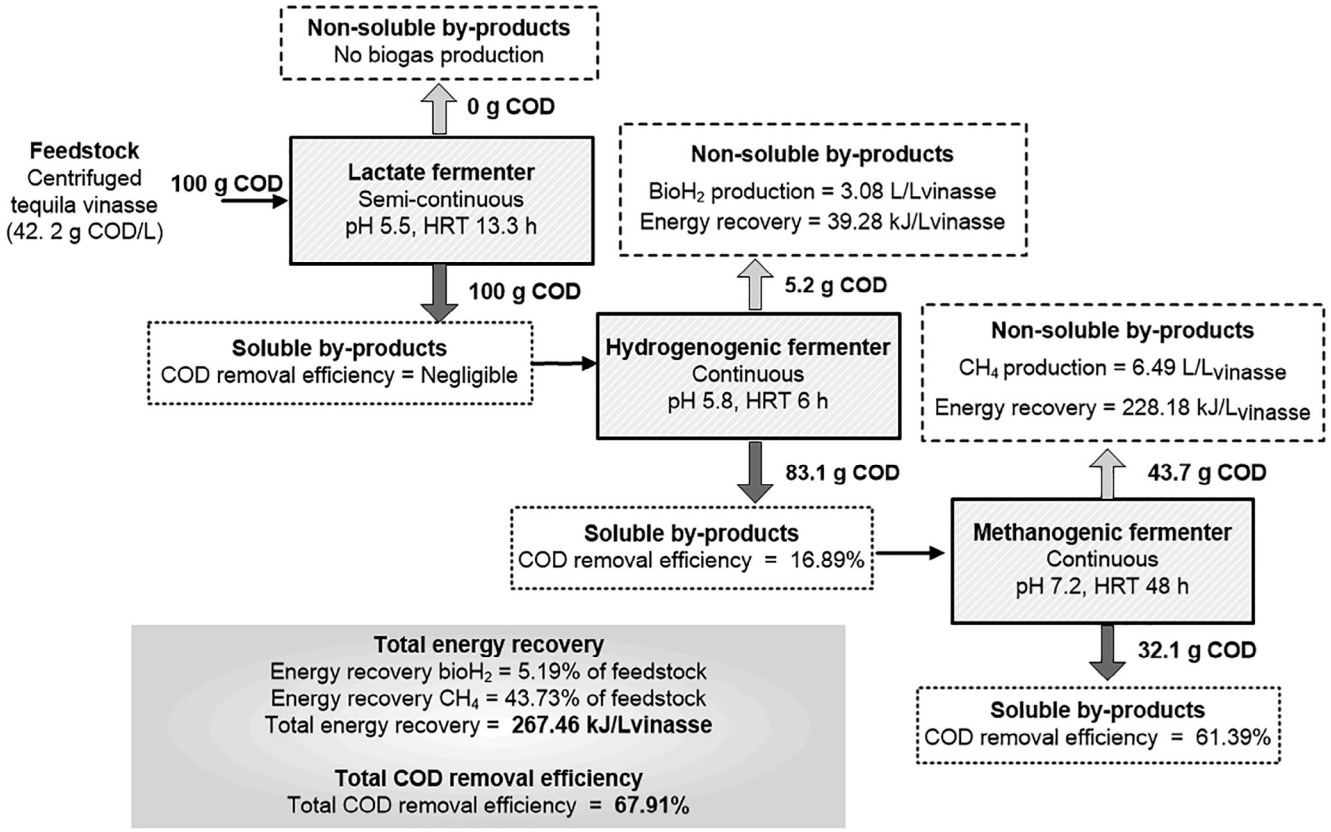

Fig. 3. Energy recovery and mass balance of the three-stage fermentation system.

CH_4 yield of 6.5 L CH_4/L_{TV}. Taking into consideration the superior heat of combustion of CH_4 (35.16 kJ/L), the methanogenic reactor yielded 228.18 kJ/L_{TV} (8.53 kJ/g VS_{added}), corresponding to 85.4% of the total ER (Fig. 3). The EPR from the methanogenic reactor was estimated as 57.1 kJ/L-d. At this point, it should be noted that the overall ER is expected to increase when applied optimum operational conditions for the methanogenic stage. Based on mass balance calculation, 1 L of TV (42.2 g COD/L) could produce 3.4 L of H_2-rich biogas with a $bioH_2$ content of 90.7% and 9.48 L of CH_4-rich biogas with a CH_4 content of 68.5% (Fig. 3). Considering the coproduction of $bioH_2$ and CH_4, the maximum total ER was estimated as 267.46 kJ/L_{TV} or 10.02 kJ/g VS_{added}. Thus, about 29.7 kWh of electricity may be obtained from a ton of TV, assuming a 40% electricity conversion efficiency. The results obtained in this work were in good agreement with other studies that showed that methanogenesis coupled with hydrogenogenesis enables further recovery of energy from the DF effluent (Buitrón et al., 2014; Juang et al., 2011; Schievano et al., 2014). For instance, Fuess et al. (2017) reported a total ER of 181.5–187.2 kJ/$L_{vinasse}$ (2.1–2.5% derived from $bioH_2$) in a thermophilic two-stage AD process treating sugarcane vinasse. Similarly, Schievano et al. (2014) achieved total ERs of 9.7–19.0 kJ/g VS_{added} from four different organic wastes, the ER obtained by the hydrogenogenic stage (4–16% of the total ER) is also comparable to that obtained in the present study.

3.5. Implications of this work and future perspectives

The integration of fermentative $bioH_2$ production with methanogenesis has received increasing attention to overcome the limitations typically encountered in single-stage AD. In this context, the three-stage fermentation system involving lactate fermentation + DF + AD here evaluated can enhance the robustness of the hydrogenogenic stage, which in turn would positively impact on the performance of the methanogenic stage. At this point, it is worth mentioning that AD is only an option for the valorization of DF by-products since there are other alternative routes, e.g. microalgae systems, photofermentation, microbial fuel cells, microbial electrolysis cells, that can be used for such a purpose (Bakonyi et al., 2018; Ghimire et al., 2015). A cost-benefit assessment should be performed to determine the cost-competitiveness of the different integrative schemes (Venkata Mohan et al., 2016).

Process instability/$bioH_2$ inhibition is a common operational problem encountered in DF reactors devoted to $bioH_2$ production, which has been related in several cases to the over-proliferation of LAB. Lactate producers thrive in DF processes due to their ubiquitous nature and flexible and diverse metabolic machinery conferring them growth advantage. In this regard, this study aimed at exploiting the "unwanted" lactate type fermentation to efficiently produce $bioH_2$, while bringing forth practical and economical operational advantages. Hence, this study contributes to the scarce information regarding the continuous lactate-derived $bioH_2$ production which can be useful in other DF systems treating distillery wastewater (Couto et al., 2020; Fuess et al., 2018), molasses (Freitas et al., 2020; Oliveira et al., 2020), food waste (Noblecourt et al., 2018; Santiago et al., 2019), cheese whey (Asunis et al., 2019), winery effluents (Buitrón et al., 2020), and hydrolysates of lignocellulosic biomass (Muñoz-Páez et al., 2020), whose composition is suitable to undergo the lactate type fermentation.

The supply of additional nutrients and alkalinity in the whole three-stage process, as well as substrate dilution in the methanogenic stage, were identified as the main challenges of this innovative process configuration. Besides, the maintenance and well-functioning of the entire three-stage process should be assessed as an integrated configuration since, in cases where one reactor fails due to (un)foreseen operating difficulties, the other reactors may be impaired at least temporary. In our experience, proper acidogenic inoculum selection and tailored environmental (e.g. pH, nutrients) and operating conditions (e.g. HRT, OLR) are of high concern to ensure not only selective lactate production but also high $bioH_2$ production performance. Similarly, high microbial

diversity degrading DF by-products to CH_4 precursors but with a proper balance between acidogenic and methanogenic microorganisms should be pursued to assure proper methanogenic activity. Finally, an optimization of the methanogenic stage (e.g. using different HRT and OLR), and a more in-depth investigation of the microbial ecology of the whole fermentation units are needed to fully exploit the untapped energy potential of TV.

4. Conclusions

This study represents the first attempt to develop a novel three-stage fermentation system for the valorization of TV through sequential lactate, bioH_2 and CH_4 production. It was confirmed that tailored environmental and operational conditions were effective to sustain selective lactate production while preventing loss of reducing equivalents. A high and stable HPR was achieved using lactate as the main bioH_2 precursor at short HRTs and under carbohydrate-shortage conditions. Finally, the integration of hydrogenogenesis and methanogenesis enabled further recovery of energy from the DF effluent, the latter stage requiring further optimization to increase organic matter removal and ER efficiency.

CRediT authorship contribution statement

Octavio García-Depraect: Conceptualization, Methodology, Investigation, Formal analysis, Writing - original draft. **Raúl Muñoz:** Visualization, Writing - review & editing. **Jules B. van Lier:** Supervision, Writing - review & editing. **Eldon R. Rene:** Supervision, Writing - review & editing. **Víctor F. Diaz-Cruces:** Investigation, Formal analysis. **Elizabeth León-Becerril:** Conceptualization, Supervision, Writing - review & editing, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2020.123160.

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