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Hydrogen and organic acids production from mesophilic and thermophilic dark fermentation of vinasse without buffers

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Abstract The main objective of this study was to evaluate the valorization of vinasse dark fermentation (DF) through bioH_2 and value-added metabolites production without buffers in two similar Upflow Anaerobic Sludge Blanket (UASB) reactors under mesophilic (U30) and thermophilic (U55) conditions. An average organic acids yield of (376 ± 52 and 248 ± 122) mg-COD_{OA}.$g^{-1}CODt_{in}$, and productivity of (17396 ± 5220 and 14024 ± 1642) mg-COD_{OA}.L^{-1}.d^{-1} was obtained for U30 and U55 reactors, respectively, with only vinasse as substrate. BioH_2 production was feasible with sucrose and pH below 3.0, but it ceased with substrate replacement by vinasse along with natural pH increase. A change in the structure of the microbial community was also observed: *Ethanoligenens*, *Clostridium* sensu stricto 12, and *Liquorilactobacillus* using sucrose, under pH <3.0 were replaced by *Prevotella*, *Megasphaera*, *Pectinatus*, *Clostridium* sensu stricto 11 and *Lactobacillus* using vinasse, under pH>4.0.

Keywords: dark fermentation, biohydrogen production, organic acids production, UASB reactor, vinasse.

1. Introduction

The sugar and ethanol industry is one of the most important in Brazilian agribusiness, with vinasse being the primary byproduct, with volume generated from 8 to 15 L of vinasse per liter of ethanol produced. Huge amounts of vinasse are produced every year in Brazil, and currently, fertigation is the main final destination because it is a simpler and cheaper solution besides being advantageous due to the high concentration of nutrients in vinasse. However, the continuous application of vinasse to the soil without caution may lead to its acidification and salinization, and also groundwater contamination (Fuess, Garcia, and Zaiat 2018)

Vinasse DF is a promising alternative to reduce its pollutants and can be advantageous for the recovery of different by-products. Several studies show the valorization of vinasse DF through: [i] biohydrogen production (Ferraz Júnior et al. (2015a; 2015b); Reis et al., 2015; Fuess et al. (2016; 2018a); Fuess, Zaiat, and Nascimento, 2019; Niz et al., 2019; Couto et al., 2020; Magrini et al., 2020); and [ii] value-added metabolites (such as ethanol, butanol and organic acids) (Couto et al., 2020; Eng Sánchez et al., 2021; Magrini et al., 2020). Nevertheless, vinasse has a low pH and several studies report that alkalinity supplementation is essential to its DF process stability, especially in the methanogenesis stage

(Ferraz Júnior et al. (2015a; 2015b; 2016); Fuess et al., 2016; Fuess, Zaiat, and Nascimento, 2019).

In the biorefinery concept, reduction or even elimination of alkalinity could be extremally advantageous, that said, the results obtained by Mota et al. (2018) proved to be quite promising, since the authors reported long-term bioH_2 production with satisfactory yields (3.4 mol-H_2.$mol_{sucrose}$), along with acetate and ethanol production, in a UASB reactor fed with sucrose-based wastewater, without medium buffering, under pH 2.7 environment. In addition to the advantages of not using buffers, these findings can dispense the need for pre-treatment of sludge to reduce competing microorganisms, decreasing treatment of vinasse costs on a real scale. Although these results seem promising, their applicability in real wastewaters needs to be evaluated. Since vinasse is an effluent that needs optimized strategies, mainly regarding alkalinity supplementation, the main objective of this study was to evaluate the valorization of vinasse DF through bioH_2 and value-added metabolites production without buffers.

2. Material and methods

Two similar continuous acidogenic Upflow Anaerobic Sludge Blanket (UASB) reactors (**Error! Reference source not found.**) of 1.4 L were operated in parallel and evaluated under two environmental conditions, mesophilic (30 °C) and thermophilic (55 °C) without any type of buffer. The reactors were identified respectively as U30

and U55. Initial operating conditions were 25 gCOD.L.d^{-1} for OLR and 4.6 h for HRT.

As an attempt to select a suitable microbiome to produce H_2 along with organic acids without the need of any buffering addition or sludge pre-treatment, the reactors were inoculated and initially fed with sucrose-based wastewater according to Mota et al. (2018).

Considering that an acidogenic and hydrogenogenic microbioma was enriched, after 57 days of operation, sucrose-based wastewater was gradually replaced by vinasse collected from a full-scale sugar-, ethanol- and electricity-producing sugarcane biorefinery located in Pradópolis, São Paulo, Brazil. The vinasse was diluted to an initial substrate concentration (S_0) of approximately 5 g.L^{-1} and by the end of the operation, S_0 was increased to 10 g.L^{-1} to obtain an OLR of 50 gCOD.L.d^{-1}.

Microbial characterization was performed for U30 under three reactor's feeding regimes: 100% sucrose-based effluent, 50% sucrose-based and 50% sugarcane vinasse-based effluent, and 100% sugarcane vinasse-based effluent. For U55, microbial characterization was performed only when it was fed exclusively with sugarcane vinasse-based effluent. Sludge samples were taken from the middle of the reactors, where the bed was located and consequently also most of the biomass concentration. Samples were taken from U30 on days 37, 79, and 114 and from U55 on day 114. The higher number of samples from U30 is justified because the microbial characterization of this reactor was subjected to omics analysis in the scope of other studies.

Further details regarding materials and methods are described in Ribeiro et al. (2021).

3. Results and discussion

The strategy of initially feeding reactors with sucrose in order to promote an acidogenic and hydrogenogenic enrichment of the microbiome was effective mainly for U30, since it showed satisfactory performance in the production of bioH_2, with an average volumetric hydrogen production rate (VHPR) of 3174 ± 961 mLH_2.L^{-1}.d^{-1}, while U55 had an average VHPR of 1124 ± 634 mLH_2.L^{-1}.d^{-1} (Table 1). In addition, U30 reactor showed values comparable to the VHPR value presented by Mota et al. (2018) for sucrose-fed UASB reactor, 4200 ± 1056 mLH_2.L^{-1}.d^{-1}. Another indication that such strategy was effective was the high fraction of H_2 observed in biogas, respectively 71 ± 17% and 68 ± 12% for U30 and U55, when sucrose was the only substrate. However, respective values of 1.5 ± 0.7 and 1.5 ± 1.1 mol-H_2.$mol^{-1}CH_{consumed}$ (Table 1) of hydrogen yield (HY) for U30 and U55 reactors, indicate that the biomass adaptation may not have been sufficient to eliminate other microorganisms that may have been responsible for H_2 consumption or for inhibition of hydrogenotrophic microorganisms through competition, since these values were lower than 3.4 ± 0.7 mol-H^2.$mol^{-1}sucrose_{consumed}$ reported by Mota et al. (2018).

From the moment vinasse was introduced into the feed substrate of both reactors, there was a decrease in the production of bioH_2 directly proportional to the concentration of the vinasse until its cessation, when the substrate consisted only of vinasse. Such results were consistent with the spectrum of observed soluble metabolites. Hydrogen-producing associated metabolites: acetic, butyric acid, ethanol and lactic acid (only in U30), were predominant in the first phase of the study when feed substrate consisted only of sucrose, and as it was gradually replaced by vinasse, there was also a drop in concentrations and acidification levels corresponding to such metabolites, followed by a gradual increase in propionic acid in both reactors and valeric acid (only in U30).

An instability tendency was observed throughout the study in bioH_2 production. Production peaks were observed as soon as operating conditions were changed, followed by significant decrease in bioH_2 production. Ferraz Júnior et al. (2015; 2015b) also observed this tendency. Reis et al., (2015) operated two anaerobic fluidized bed reactors and the authors also started adapting the reactors to produce hydrogen with a simpler substrate, glucose, and vinasse was added to the feed gradually. R10 (10 gCOD.L^{-1}) also had a peak in hydrogen production at the beginning of the operation and a downward tendency at the end of the operation until cessation along with susbtrate replacment by vinasse.

A punctual HY of 4.07 mol-H_2.$mol^{-1}CH_{consumed}$ was observed in U55 in 100%-vinasse condition and although this value is higher than the yields described by other studies (Magrini et al., 2020; Fuess, Zaiat, and Nascimento, 2019; Niz et al., 2019; Ferraz Júnior et al., 2015b; Reis et al., 2015) it is believed that yield values could be lower if other relevant substrates were considered, since only conversion of carbohydrates was used, neglecting the role of lactate and glycerol, for instance (Fuess, Zaiat, and Nascimento, 2019). A change in the preferred substrate can be observed mainly in the U55 reactor from the 75%-vinasse condition, since there was a decrease in CH conversion from 60 ± 7 to 48 ± 10% and a significant increase in the glycerol conversion from 2 ± 4 to 75 ± 11%. It was also observed in U30 that showed glycerol conversion of at least 98%, from the 75%-vinasse condition, reaching 100% when only vinasse was being used (**Error! Reference source not found.**). The greater abundance of *Pectinatus* and *Prevotella* in U30 compared to U55 could explain the better glycerol removal from vinasse under mesophilic conditions (Figure 1).

Although the conversion of glycerol was high and stable, it may not have been a determining factor for bioH_2 production from vinasse, most likely due to the pH conditions obtained in this study, which were always below 4.5 in both reactors. Mangayil et al. (2012) obtained an optimal production of bioH_2 from crude glycerol at pH 6.5 and the authors observed a drastic decrease in H_2 yield at pH 5.0, as well as minimal use of substrate. Fuess, Zaiat, and Nascimento, 2019 also report that glycerol fermentation might not be a determining aspect for direct bioH_2 production from vinasse since authors observed stable and high levels of glycerol conversion (usually>95%) accompanied by relatively low bioH_2 production (521 ± 132 mLH_2.$L^{-1}d^{-1}$) under pH <5.

Table 1. Average values of pH, carbohydrate conversion (EC_{CH}), glycerol conversion (EC_{Gly}), hydrogen yield (HY), volumetric hydrogen production rate (VHPR), acidification degree (AD), organic acids yield (Y_{OA}) and productivity (P_{OA}) of each condition in U30 and U55.

Period[a]	1-57	58-67	68-79	80-86	87-104	105-114
S_0[b]	5	5	5	5	5	10
S fraction[c]	100	75	50	25	0	0
V fraction[c]	0	25	50	75	100	100
pH U30	2.9 ± 0.1	3.2 ± 0.1	3.9 ± 0.2	4.2 ± 0.0	4.5 ± 0.1	4.5 ± 0.1
pH U55	3.2 ± 0.1	3.7 ± 0.0	3.9 ± 0.1	4.0 ± 0.2	4.5 ± 0.2	4.4 ± 0.3
EC_{CH} U30[c]	69 ± 19	88 ± 9	88 ± 4	90 ± 0	71 ± 11	73 ± 8
EC_{CH} U55[c]	27 ± 12	44 ± 19	60 ± 7	48 ± 10	47 ± 10	60 ± 11
EC_{Gly} U30[c]	0[f]	0 ± 0	64 ± 45	99 ± 1	99 ± 0	100 ± 0
EC_{Gly} U55[c]	0[f]	0 ± 0	2 ± 4	75 ± 11	80 ± 20	71 ± 13
HY U30[d]	1.5 ± 0.7	0.8 ± 0.7	0.8 ± 0.7	0.6 ± 0.4	0.0 ± 0.1	0.0 ± 0.0
HY U55[d]	1.5 ± 1.1	2.1 ± 0.5	1.6 ± 1.0	2.2 ± 1.0	1.0 ± 1.5	0.1 ± 0.1
VHPR U30[e]	3174 ± 961	1193 ± 836	886 ± 353	639 ± 367	17 ± 50	0.0 ± 0.0
VHPR U55[e]	1124 ± 634	1517 ± 315	782 ± 445	595 ± 230	82 ± 163	34 ± 41
AD U30[c]	47 ± 17	58 ± 15	54 ± 20	51 ± 24	40 ± 5	27 ± 9
AD U55[c]	17 ± 7	31 ± 7	45 ± 14	35 ± 7	28 ± 13	23 ± 1
Y_{OA} U30[g]	373 ± 135	443 ± 170	377 ± 181	456 ± 232	376 ± 52	259 ± 84
Y_{OA} U55[d]	98 ± 36	241 ± 69	291 ± 61	209 ± 24	248 ± 122	212 ± 12
P_{OA} U30[h]	12996 ± 5377	14492 ± 5686	10765 ± 3793	16814 ± 8000	13286 ± 1556	17396 ± 5220
P_{OA} U55[e]	3320 ± 1039	6469 ± 1798	8327 ± 3063	6327 ± 690	7703 ± 3453	14024 ± 1642

[Notes: [a]days, [b]$g.L^{-1}$, [c]percentage, [d]$mol\text{-}H_2.mol^{-1}CH_{consumed}$; [e]$NmL\text{-}H_2.L^{-1}.d^{-1}$; [f]virtually 0; [g]$mg\text{-}COD_{OA}.g^{-1}CODt_{in}$, [h]$mg\text{-}COD_{OA}.L^{-1}.d^{-1}$; S = sucrose; V = vinasse].

Regardless of the vinasse fraction present in the substrate, U30 reactor showed a better organic acids production (P_{OA}) performance than U55 reactor, it also can be seen through acidification degree (AD) results in all stages of operation (**Error! Reference source not found.**1). These findings are in agreement with the results described by Eng Sánchez et al. (2021) in their study with DF of sugarcane vinasse to recover soluble metabolites, the authors also obtained maximized yield (401 $mg\text{-}COD_{OA}.g^{-1}CODt^{initial}$) and productivity (653 $mg\text{-}COD_{OA}.L^{-1}.d^{-1}$) of organic acids in a mesophilic/alkaline (40 °C; pH = 8.8–10.0) condition. Considering the phase in this study, where only vinasse was being used as substrate, it was possible to obtain an organic acid yield (Y_{OA}) of (376 ± 52 and 248 ± 122) $mg\text{-}COD_{OA}.g^{-1}CODt_{in}$ for reactors U30 and U55 respectively, and it is important to highlight that these results were observed without the addition of any buffering agent and in a pH range between 4.1 and 4.8, therefore the monetary savings that the non-need of buffer agents can generate in a real scale process may compensate for the slightly lower yield.

Characterization of the microbial community from the reactors at the genus level is depicted in Figure 1. A great reduction in diversity was observed. Shannon-index values of the inoculum used in U30 and U55 were 6.54 and 6.25, respectively. However, when U30 was fed with 100% sucrose-based effluent, the Shannon-index value of its sludge sample was 2.61. This sharp reduction of microbial diversity was accompanied by extremely low pH values (<3.0). As vinasse was added to the substrate, microbial diversity increased; Shannon-index values were 3.87, 3.59, and 4.33 for U30 fed with sucrose and vinasse, with vinasse only and U55 fed with vinasse, respectively.

Successful $bioH_2$ production obtained at pH below 3.0 using sucrose-based effluent did not sustain when a more complex substrate was used as feeding and pH naturally increased to values above 4.0, as reported in our previous study on cheese whey DF without adding buffers (Ribeiro et al. 2021). This can be attributed to the change in microbial community from non-homoacetogenic and hydrogen-producing bacteria (related to *Ethanoligenens* and *C. acidisoli*) to propionate-producers (*Pectinatus*), lactate-producers (*Lactobacillus*, *Bifidobacterium*), and other non-hydrogenogenic bacteria (*Megasphaera*, *Prevotella*) as well as potential homoacetogenic bacteria (within *Clostridium* genus).

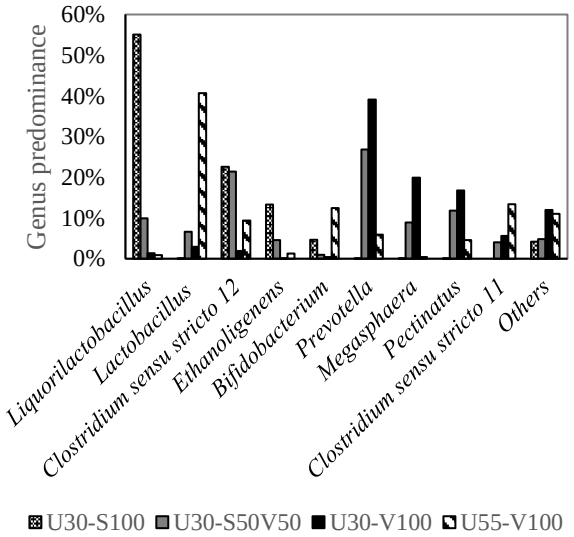

Figure 1. Relative abundance of prevalent genera in U30 fed with effluent based on 100% sucrose (U30-S100), 50% sucrose and 50% vinasse (U30-S50V50) and 100%

vinasse (U30-V100), and in the U55 fed with effluent based on 100% vinasse (U55-V100).

4. Conclusions

Results obtained in this study showed that DF of vinasse without any buffering addition might be a promising approach for the valorization of real wastewaters through the production of organic acids, however, regarding bioH_2 production, further strategies would be needed to maintain hydrogen-producing bacteria when sucrose was replaced by real effluent along with a natural pH increase, since a pH below 3.0 environment may be a key factor for bioH_2 production without buffers.

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