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ORIGINAL ARTICLE

Alternative techniques to improve hydrogen production by dark fermentation

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Abstract
The production of biofuels as an alternative to the fossil fuels has been mandatory for a cleaner and sustainable process. Hydrogen is seen as the fuel of the future because it has a very high energy density and its use produces only water instead of greenhouse gases and other exhaust pollutants. The biological synthesis of hydrogen by dark fermentation complies with these criteria. In the current work, the use of cheese whey permeate was evaluated aiming hydrogen production by dark fermentation using a microbial consortium in the semi-continuous process, with a reaction volume of 700 mL. The volume of the medium renewal and the frequency of replacements of fresh medium were evaluated to extend the production of H_2. It is important to note decreases in the hydrogen production after 84 h. The target-product content became higher particularly when 466 mL of medium were withdrawn, in every 24 h in the first two replacements and, subsequently, in every 12 h. Besides, it was observed lower lactic acid concentration under this condition, suggesting that the shorter removal time of the medium could inhibit lactic acid bacteria, which may secrete bacteriocins that inhibit the hydrogen-producing microorganisms.

Keywords Hydrogen · Dark fermentation · Microbial consortium · Sequential batch process

Introduction

The energy crisis and the environmental degradation have aroused a strong concern worldwide. Energy from fossil fuels accounts 80% of energy consumption and contributes to the climate changes; besides the rapid depletion of natural energy sources (Guo et al. 2010). Moreover, the interest in the world in searching new energy sources, which must be clean and renewable, increases continually. Nowadays, hydrogen gas represents a potential source of energy attracting the global interest. This gas has high energy content (286 kJ/mol). Moreover, it is a clean fuel since only water is produced in its combustion; there is no release of carbon monoxide and carbon dioxide which are the main gases responsible for the greenhouse effect. Furthermore, this fuel can be obtained from renewable feedstock (Bao et al. 2012).

Hydrogen can be obtained by several ways, but currently it has been produced mainly by the natural gas via steam reforming and the coal gasification. However, these processes are highly expensive and use fossil fuels producing greenhouse gases. Faced with these prospects, the hydrogen production by biological routes has assumed an attractive alternative to obtain clean energy (Sinha and Pandey 2011).

Hydrogen production by fermentative route makes it an advantageous process concerning economic and environmental aspects (Guo et al. 2010). However, in these processes, it is important to note that hydrogen yield can be affected by several parameters, such as inoculum preparation, substrate concentration, temperature, pH, presence of metals, type of reactor, among others that have been widely investigated (Sinha and Pandey 2011; Avcioglu et al. 2011; Amorim et al. 2009; Romão et al. 2014). In addition, industrial effluents, such as cheese whey, became an attractive substrate to microbial consortium on the hydrogen production. Lactose, a fermentable sugar, can

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be metabolized resulting in higher volumetric hydrogen productivity (Silva et al. 2016).

It should be emphasized that hydrogen production presents a reduced extension time as one of the bottlenecks of batch process, and fermentation period from 24 to 100 h by mesophilic consortium being recorded (Kargi et al. 2012; Matsumoto and Nishimura 2007). Therefore, this work aimed to evaluate the strategy to increase the hydrogen production and extend the process time using the semi-continuous mode (Padovani et al. 2016). It is rarely reported in the literature for sustainable hydrogen evaluation.

Materials and methods

Preparation of the inoculum culture

Microbial consortium was used as inoculum and provided by the NUCBIO Laboratory (Biotechnological Process Center of the School of Chemical Engineering) of the Federal University of Uberlandia (Brazil). It was previously adapted in a synthetic medium with the following composition: KH_2PO_4 3 g/L, K_2HPO_4 7 g/L, $MgSO_4$ 1 g/L, yeast extract 3 g/L, meat extract 0.5 g/L, $(NH_4)_2SO_4$ 1 g/L, and lactose 20 g/L (Romão et al. 2014).

Lactose as carbon source was obtained from cheese whey permeate purchased from *Sooro Concentrado Indústria de Produtos Lácteos Ltda* (Brazil). The cheese whey permeate was composed mainly of lactose (92.97%) and protein (1.42%). The inoculum vials were stored in the dark at room temperature. To keep anaerobiosis, the inoculum was flushed with nitrogen gas for 10 min.

Hydrogen production experiments

Fermentations were carried out in a Bio-Tec-Flex Tecnal™ batch reactor, with a 1.5 L with a reaction volume of 700 mL, equipped with mechanical stirrer and temperature control. The reaction volume was composed of inoculum (1.6% v/v) and synthetic medium (98.4% v/v). Fermentation medium, consisted of lactose (20 g/L) from cheese whey permeate, ferrous sulfate (0.6 g/L), ammonium sulfate (1.5 g/L), magnesium sulfate (1.28 g/L). The initial pH was adjusted to 7.0. After ensuring anaerobic conditions, fermentations assays were carried out at 30 °C. All these experimental conditions were defined previously by Romão et al. (2014). The fermentation process was controlled over time and hydrogen, non-converted lactose, extracellular metabolite concentrations and cell growth were measured. The biogas produced was measured by water displacement.

Semi-continuous mode assays

Semi-continuous system (SCS) was studied under different operating conditions as Fig. 1 illustrates: varying amounts of the medium culture that was partially replaced and the time used to withdraw the medium. It should be emphasized that the proportion of 1.6% (v/v) of inoculum was maintained in the fresh medium. Nitrogen was purged into the medium to keep it in anaerobic conditions every renewal of the medium. The pH was controlled at 5.5 and 30 °C, as it was proved that under this condition, higher productivity and yield were achieved (Romão et al. 2017). The renewal volume varied from 1/3 (233 mL) and 2/3 (466 mL) of the working volume (700 mL) and the renewal timing (intervals of 24 h or 12 h) was used when lactose was considerably consumed. The proposal of renewal medium was to eliminate the inhibition of the toxic metabolites and probable increase of microorganism population that are not hydrogen producing.

The first SCS fermentation was carried out as followed: the process started in batch using culture conditions described in "Hydrogen production experiments". After 24 h, the first renewal of the medium was performed. At this time, 233 mL of medium was withdrawn using a peristaltic pump with a flow rate at 0.2 mL/s. Afterward, a fresh synthetic medium was added to reset the initial substrate concentration as observed in the beginning of the bioprocess. Lactose concentration was adjusted aiming to keep its level around 20 g/L after the replacement of the medium. The following removals of the medium were performed after 48 h and after 60 h, subsequently.

In the second SCS fermentation, the medium was withdrawn every 28 h, approximately. In this assay, the amount of medium removed was 466 mL. Finally, in the third SCS fermentation, two cycles of medium removals were performed; being in the first 48 h with a step of 24 h for each removal. Thereon, after 48 h, the withdrawals were performed every 12 h (466 mL of removed medium).

Analysis

Biogas samples were collected and its composition was determined by gas chromatography using the chromatograph Shimadzu™ model GC 17A, equipped with a thermal conductivity detector (TCD) and a capillary column Carboxen™ 1010 (length 30 m, internal diameter 0.53 mm). The operating temperatures of the injection port, the oven and the detector were 230, 32 and 230 °C, respectively. Argon was used as a carrier gas (Romão et al. 2014).

Lactose, ethanol and organic acids were measured by high-performance liquid chromatography (HPLC). Samples were diluted with ultrapure water, filtered through a membrane (0.22 µm pore size, Millipore™) and injected into a chromatographic system (Shimadzu™ model LC-20A

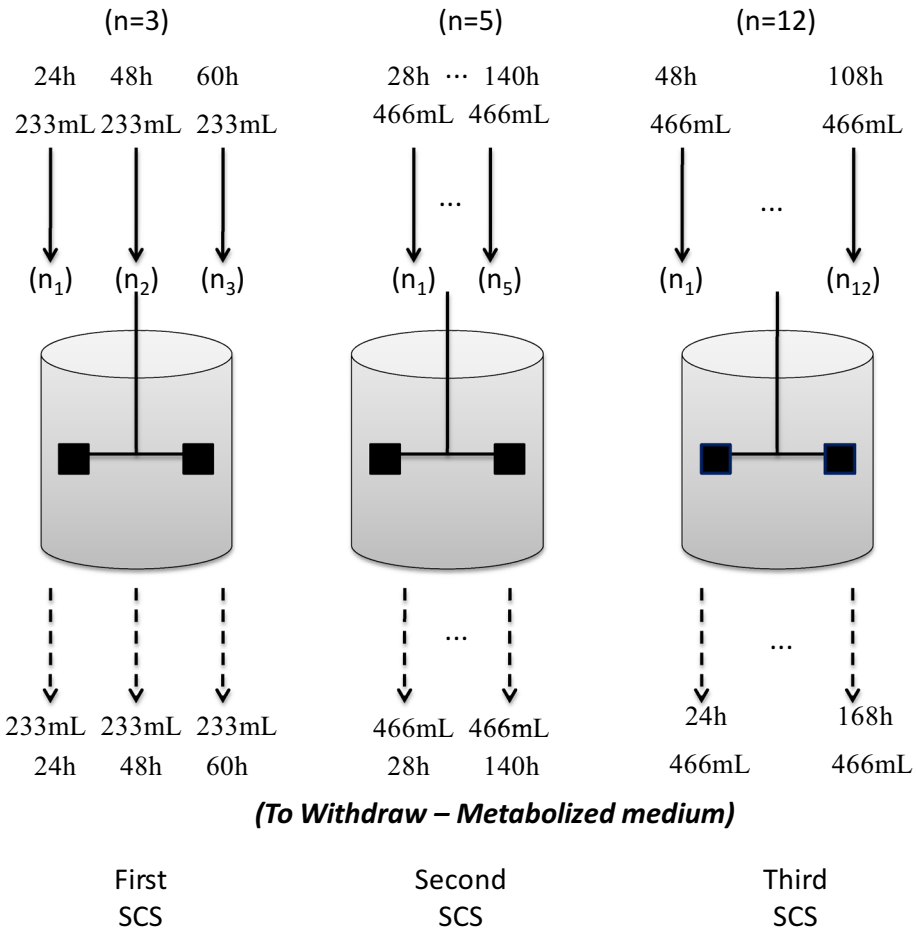

Fig. 1 Scheme of semi-continuous system (SCS) for hydrogen production by varying renewal volume and timing of fresh nutrient medium

Prominence, Supelcogel™ C-610H column), equipped with UV–Vis and Infrared detectors. Analyses were carried out using phosphoric acid 0.1% as a carrier solution, pump flow rate at 0.5 mL/min, temperature oven at 32 °C. The sample volume injected into the chromatograph was 20 µL (Romão et al. 2014). The sample standard deviation was ± 0.08 (g/L) for the concentration of organic acids, ethanol and sugars. The cell density (g_{vs}/L) was determined by spectrophotometry at an optical density of 660 nm and the sample standard deviation was ± 0.11 (g/L). on the hydrogen

Results and discussion

As observed in previous batch fermentations (Romão et al. 2014), volumetric hydrogen productivity and hydrogen yield from substrate consumption reached a peak and then decreased over time. To avoid that, a semi-continuous mode fermentation (part of the culture medium was removed including bacteria cells and replaced with fresh medium) was performed.

After 24 h, 233 mL of medium was withdrawn and then fresh nutrient medium (233 mL) was added. This procedure was repeated after 48 h and then after 60 h. It was not detected methane production into the biogas, but hydrogen and CO_2. Figure 2a introduces the findings to the variable responses (volumetric productivity and yield) and Fig. 2b illustrates the kinetic behavior of organic acids and ethanol production.

Figure 2a showed that productivity and conversion decreased to 50% from maximum values after 60 h. Regarding to the formed metabolites (Fig. 2b), ethanol concentration remained low and concentrations of acetic and butyric acids were constant from the first and second medium replacement and lactic acid concentration increased from 2.11 to 5.61 g/L in second medium replacement. It is important to note that these metabolites presented the highest concentration up to 60 h of fermentation. However, the propionic acid concentration increased after the first medium renewal and it reached 2.2 g/L.

Figure 2a also indicated that the medium replacement provided the hydrogen production for a longer period of time than simple batch mode. Romão et al. (2014) obtained a maximum productivity around 15 h, also using microbial consortium, at 30 °C and milk whey permeate as substrate, and the hydrogen production conducted by Matsumoto and

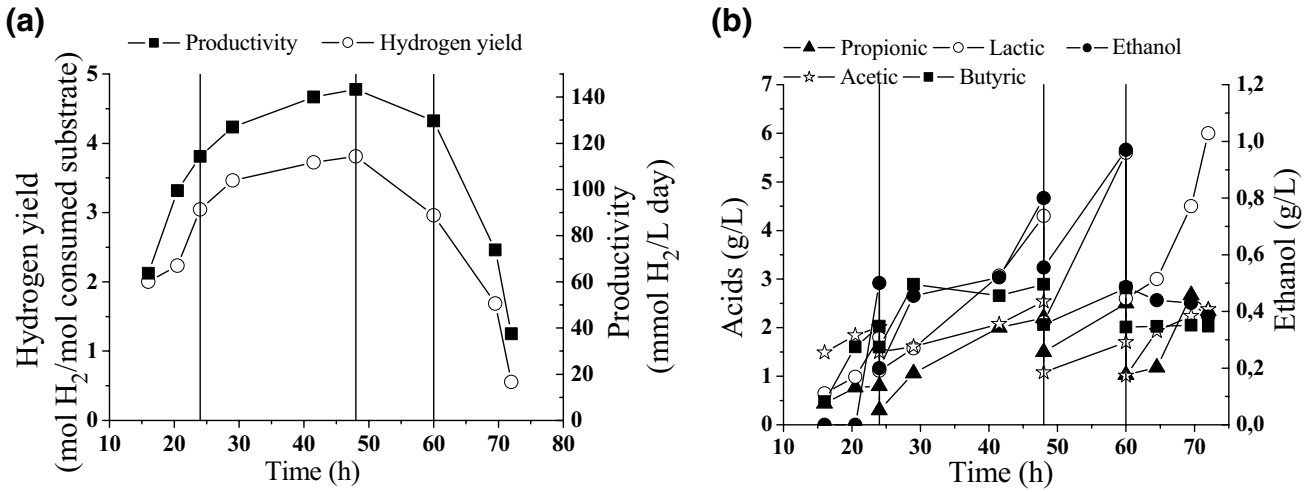

Fig. 2 Evaluation of the volumetric hydrogen productivity and the hydrogen yield (**a**) as well as metabolite concentration (**b**) obtained of the fermentation process in the optimized conditions with 233 mL of medium removed and pH of 5.5

Nishimura (2007) with pure culture (*Clostridium diolis* JPCC H-3) and medium composted of sho-chu post-distillation slurry solution reached its maximum at 24 h.

At 60 h, the drop in the hydrogen productivity was 9.5% and at 69.5 h it was observed almost 48.5%. The findings also suggested that increases in the hydrogen evolution may be related with the removal of toxic metabolites. After 60 h, lactic acid concentration increased, in contrast to the other acids. It indicated that the medium replacement stimulated the lactic acid production by bacteria and inhibited the target-product synthesis. Calli et al. (2008) discussed the influence of pH on the hydrogen production using lactose and xylose as substrates during dark fermentation by the same operation mode used in this work. These authors observed that metabolic route was modified depending on the pH used. Further investigation showed that the pH kept at 5 provided decreases in the hydrogen yield from 3.4 to 1.7 mol H_2/mol of lactose. It might be associated with the deviation of acetate and butyrate production to ethanol and lactate. To improve the system, anaerobic fermentation was performed and the amount of medium removed was changed from 233 to 466 mL. It was performed every 28 h. The data are presented in Fig. 3.

Figure 3a showed that removing 466 mL, the hydrogen productivity and yield remained at values close to maximum during 71 h. In this case, the highest value was 148.2 mmol H_2/L day and 3.9 mol H_2/mol of consumed substrate, respectively. After 71 h, the volumetric hydrogen productivity and hydrogen yield were strongly decreased (around 50% of maximum). In relation to the formed metabolites (Fig. 3b), there is a low concentration of them up to 50 h of fermentation, except for the lactic acid. It was also noted that increases in the renewal volume of the medium replacement resulted in increases of the lactic acid concentration. In the first SCS the maximum lactic acid concentration was 6 g/L and, in the second SCS fermentation lactic acid content reached 17 g/L and, at the end, its concentration reached 13 g/L.

Although the higher content of lactic acid, it was also observed that replacing 466 mL of medium, the system kept higher productivity and yield for longer period of time than using 233 mL of removed medium (Fig. 3a). It confirmed the hypothesis that the removal of metabolites avoided decreases in the variable responses. Moreover, it was also noted biomass was reduced after each withdrawal of the medium (Fig. 3c), immediately after the renewal the cell concentration increased. It should contribute to hydrogen production for a longer period of time, since previous experiments showed there is a limited amount of biomass which favors the target-product synthesis (data not shown). Besides, after 150 h the cell growth was neglected. It could be indicate cell death by lysis caused by inhibition for some metabolite that accumulated despite the medium removal.

For instance, high lactic acid concentration indicates the presence of bacteria producing this metabolite in the microbial consortium. Several strains of lactic acid producing bacteria secrete bacteriocins and it can inhibit the hydrogen-producing bacteria. This fact was very clear in the work developed by Noike et al. (2002). These authors realized that substantial hydrogen production ceases after the concentration of lactic acid was high into the medium. Therefore, they evaluated the hydrogen production using polypeptides and proteases to verify if bacteriocins affected the hydrogen-producing bacteria. In their findings, the addition of nisin did not result in hydrogen production. But, if nisin and chymotrypsin were used simultaneously, the target-product was synthesized. Further, the investigation was conducted also using the culture supernatant of *Lactobacillus paracasei* and

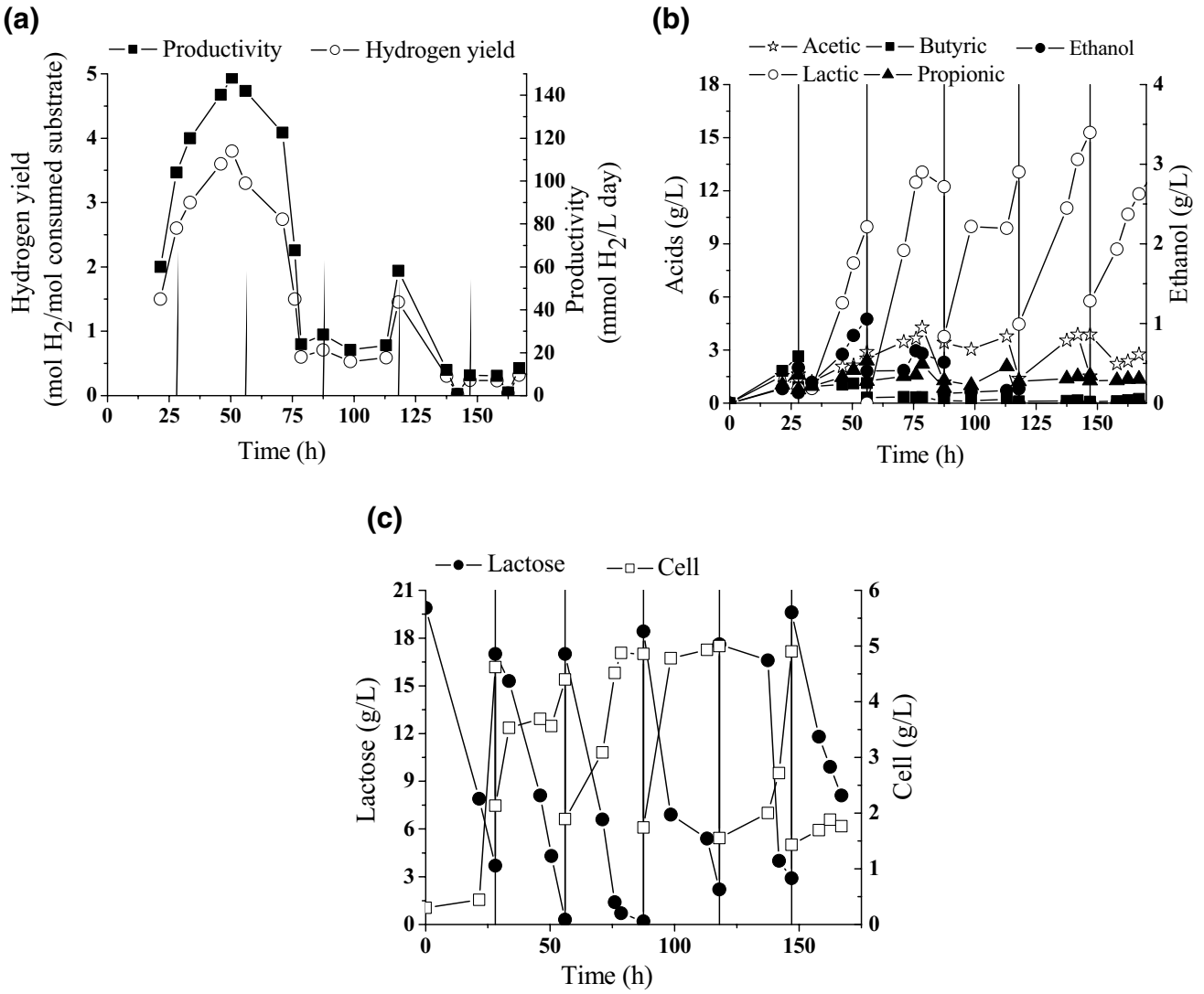

Fig. 3 Evaluation of the volumetric hydrogen productivity and the hydrogen yield (**a**), metabolites concentration (**b**) and cell growth (**c**) obtained of the fermentation process in the optimized conditions with 466 mL of medium removed and pH of 5.5

trypsin. The authors concluded that the formed polypeptides caused inhibition on the H_2 production. On the other hand, if a protease was added the inhibition was lessened.

Since the increase of renewal volume succeeded to extend the H_2 production, a different procedure was applied in the process modifying the time in which the medium replacement was effective in an attempt to reduced lactic acid concentration, mainly after 28 h. In the third assay, medium replacement was performed every 24 h in the first two 24 h. Afterwards, the medium replacement was performed every 12 h until 168 h of fermentation. This procedure was carried out to achieve low concentration of lactic acid in the medium. The amount of the medium replacement was 466 mL and the results are shown in Fig. 4.

Figure 4a pointed that by reducing the time in which the medium was replaced, the volumetric productivity and yield decreased strongly after 84 h (around 50%), later than in the previous assays. It is important to note that changes in the time in which the medium was replaced increased the time of fermentation, comparing to the assays in which the medium renewal occurred every 28 h.

In relation to the metabolites (Fig. 4b), it was observed that the concentration of lactic acid remained low up to 40 h, and around 4 g/L lower in comparison to the second SCS assay. After the second medium replacement, the lactate concentration reached values above 8.7 g/L. Finally, at the end of the fourth medium replacement, the lactic acid concentration reached high values, around 11 g/L. Despite the high acid concentration, the hydrogen production was sustained until the fifth medium replacement (100 h). After that it was observed decreases in the cell density, probably, due to cell death and lysis.

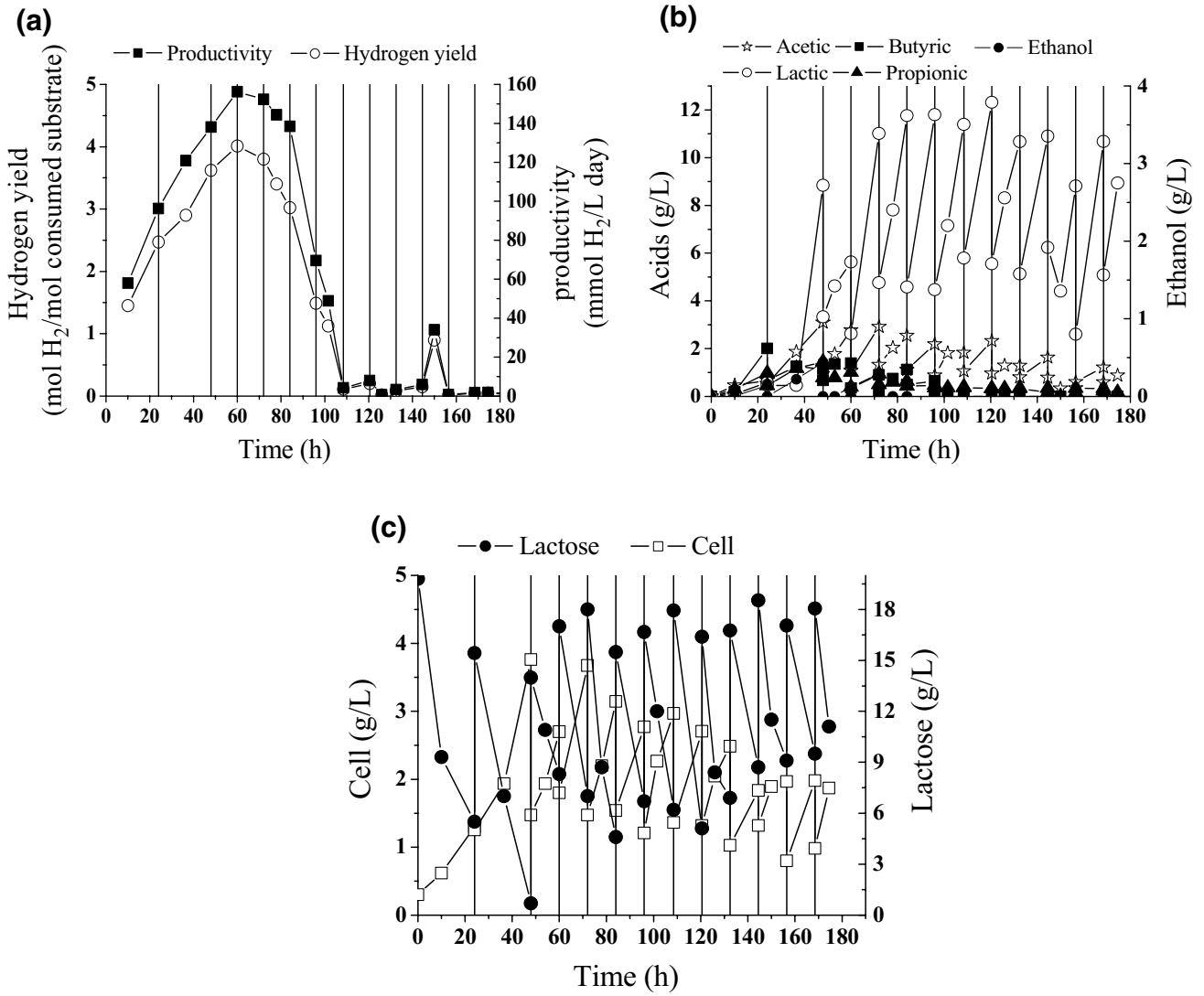

Fig. 4 Evaluation of the volumetric hydrogen productivity and the hydrogen yield (**a**), metabolites (**b**) and cell growth (**c**) obtained of the fermentation process in the optimized conditions with 466 mL of medium removed every 24 h and, then, every 12 h. The pH was kept in 5.5

Therefore, the results obtained in the current work showed higher hydrogen production by semi-continuous mode fermentation, mainly when the time used to replace the medium was shorter. By replacing 466 mL of the medium twice every 24 h and then every 12 h, a significant decrease was observed only after 84 h of fermentation.

Instantaneously enhancing nutrient concentrations and diluting cell concentration in the semi-continuous mode can provide cell grow optimally, but the biosynthesis of the metabolites can be modified, such as organic acids or gas emission. The findings indicated that the time in which the medium is replaced has direct influence in the hydrogen evolution, since the management of biochemical reactions is essential for the cellular maintenance. Besides, it can highlight the enzymatic activity allowing a cell response to the environmental change. The operational conditions may be driven by medium replacement for a detoxification and sometimes bioactivation. What happens depends on the chemical structure of the intoxicant and its complementariness to the catalytic centre of the metabolizing enzyme.

Previous investigation (Romão et al. 2017) evaluated the effect of pH in the hydrogen production by dark fermentation in batch process using whey permeate as a substrate. It was reported a productivity of 129.33 mmol H_2/L day. However, after 40 h of fermentation, the productivity was reduced and reaches half of this value at the end of the process. It is important to note, maximum productivity (156.21 mmol H_2/L day) achieved in the current work was higher than reported before (Romão et al. 2017). Replacing part of the fermented medium contributed in keeping the culture activated for longer periods. Only after 80 h of fermentation, the hydrogen productivity

decreases, reaching 55% of the maximum value after 96 h. In a general, semi-continuous processes are able to keep higher levels of biomass when compared to continuous processes. This leads the former to have a greater potential in keeping high-yield H_2 production for longer times (Cheong et al. 2007).

As discussed in the literature, Saraphirom and Reungsang (2011) also evaluated the hydrogen production by replacing the medium in a defined interval of time, but using sweet sorghum syrup as substrate. The fermentation conditions were sugar (25 g/L), temperature at 30 °C, pH at 5.5 and ferrous sulfate concentration (1.45 g/L). The authors evaluated several hydraulic retention times (HRT) 96, 48, 24 and 12 h. The results showed that increasing the HRT from 12 h to 24 h resulted in yield increments and it was possible to reach a maximum of 0.68 mol H_2/mol hexose. However, after 24 h, increases in HRT caused decreases in the yield. Moreover, the highest volumetric hydrogen productivity (365.67 mmol H_2/L day) was obtained for the retention time of 12 h. For longer times, the variable response decreased.

Another alternative to extend hydrogen production by dark fermentation was reported by Moreira et al. (2017). The authors used milk whey permeate (initial lactose concentration of 20 g/L) and a mixed culture. Substrate was replaced (10 g/L of lactose) when carbon source was totally consumed. As the same volume used to withdraw the sample was used to add milk whey permeate, the volume was constant as in a batch bioreactor. The repeated batch fermentation allowed the process occurs for 449 h and produce 11.44 L of biogas.

In addition, Kim et al. (1987) cultivated *R. sphaeroides* with a dilution rate of 900 mL every 4 days. It was found to be very promising with respect to hydrogen production, as compared to the batch mode. These authors observed up to 650 mL/L day of the target-product. On the other hand, Fuess et al. (2016) verified that the packed-bed acidogenic reactor operated under thermophilic conditions is fully capable to maintain continuous hydrogen production rates under long-term operation (240 days). However, several studies have indicated unstable and decreasing in hydrogen production in acidogenic packed-bed systems after 60 days, regardless of the wastewater type and temperature conditions.

Similarly to this current work, these cited studies succeeded to improve hydrogen production by increasing the productivity and promoted longer time of fermentation. Nevertheless, none of them found out a certain cause for the bioreactor ceases the operation. Therefore, to definitely obtain a stable system operation to produce hydrogen continuously, for instance, by integrating it with a wastewater treatment, comprehension of the inhibitory effect on the culture must be evaluated.

Conclusion

The current work suggested that semi-continuous system is an attractive fermentation mode for hydrogen production. It occurs once this operation mode resulting low toxic product concentrations; besides refreshing conditioned medium in terms of substrate. H_2 productivity reached 148 mmol H_2/L days, although a strong reduction was systematically verified. It indicated the conduction of the fermentative process has a significant influence on the H_2 production. On the other hand, abrupt changes in the feeding were not able to sustain high productivity for a long time.

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Compliance with ethical standards

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

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