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Performance evaluation and phylogenetic characterization of anaerobic fluidized bed reactors using ground tire and pet as support materials for biohydrogen production

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ABSTRACT

This study evaluated two different support materials (ground tire and polyethylene terephthalate [PET]) for biohydrogen production in an anaerobic fluidized bed reactor (AFBR) treating synthetic wastewater containing glucose (4000 mg L^{-1}). The AFBR, which contained either ground tire (R1) or PET (R2) as support materials, were inoculated with thermally pretreated anaerobic sludge and operated at a temperature of 30 °C. The AFBR were operated with a range of hydraulic retention times (HRT) between 1 and 8 h. The reactor R1 operating with a HRT of 2 h showed better performance than reactor R2, reaching a maximum hydrogen yield of 2.25 mol H_2 mol^{-1} glucose with 1.3 mg of biomass (as the total volatile solids) attached to each gram of ground tire. Subsequent 16S rRNA gene sequencing and phylogenetic analysis of particle samples revealed that reactor R1 favored the presence of hydrogen-producing bacteria such as *Clostridium*, *Bacillus*, and *Enterobacter*.

1. Introduction

Due to the indiscriminate use of fossil fuels and rising worldwide energy demand, CO_2 emission in the atmosphere has increased and generated serious environmental problems, such as the greenhouse effect and, consequently, global warming (Kapdan and Kargi, 2006). A promising alternative to fossil fuels is hydrogen, which is a source of clean, renewable energy that has been deemed "the fuel of the future", as it produces only water during its combustion, i.e., it produces no carbon when used as fuel. Hydrogen is very energy efficient (122 kJ g^{-1}), with 2.75 times more energy content than any hydrocarbon, and it can be converted into electrical and/or mechanical energy and heat (Kapdan and Kargi, 2006).

According to Das and Veziroglu (2001), hydrogen may be drawn from fossil fuels, water and biological matter. In the latter case, organic compounds are fermented by bacteria, which release H_2 by means of hydrogenases and eliminate electrons generated during the degradation of carbohydrates. These bacteria can produce H_2 at a high-rate and do so ad infinitum, day and night, without light. In addition, by growing and multiplying rapidly, they can provide the production system with additional microorganisms. In this process, a wide variety of carbon sources may be used, such as glucose, starch, sucrose, and xylose. Furthermore, dark H_2 fermentation is considered to be the most commercially viable process, because it yields high hydrogen production and may be coupled to a wastewater treatment plant. In short, it can treat wastewater while generating clean energy (Wu et al., 2007).

The use of mixed cultures is extremely important and requires the appropriate selection of cultures according to the requisite function that are well suited to the nonsterile, ever-changing, and complex nature of the substrate/wastewater. The main species associated with the biological production of hydrogen during acidogenesis of carbohydrates are *Enterobacter*, *Bacillus*, and *Clostridium* (Kapdan and Kargi, 2006; Mohan, 2009).

Several high-rate anaerobic reactors were successfully tested for the biological production of hydrogen. The anaerobic fluidized bed reactors (AFBR) are treatment systems that take advantage of the principle of fluidization to promote adequate mass transfer between the liquid to be treated and the microorganisms that act to degrade the organic matter. This type of reactor with adhered biofilm has been widely used as a biological treatment system for effluents with high efficiency and short hydraulic retention time (HRT) (Lin et al., 2009; Barros et al., 2010). In previous reports, various support materials were employed as carriers of microorganisms in AFBR, such as activated carbon (Zhang et al., 2007), Celite (Koskinen et al., 2007), expanded clay (Shida et al., 2009; Barros

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et al., 2010), ethylene–vinyl acetate copolymer (Lin et al., 2009) and polystyrene (Barros et al., 2010).

Reducing the cost of wastewater treatment and finding ways to produce useful products from wastewater are important concerns for environmental sustainability (Mohan, 2009). Therefore, there has been an increasing trend toward more efficient utilization of polymeric residues, including ground tires and polyethylene terephthalate (PET), as packing materials. In addition to the economic advantage of using these inexpensive raw materials, the use of these wastes as a packing material in an AFBR to produce hydrogen is particularly attractive from an environmental point of view.

In addition to the present overuse of fossil fuels, another serious environmental problem is the generation and inappropriate disposal of solid waste. The generation of huge quantities of used tires and scrap plastics, such as PET, which is a potential health hazard when stored, is a growing worldwide concern (Mondal and Warith, 2008). Under natural conditions, tires take considerable time to decompose; PET takes over 100 years. When accumulated in dumping grounds, this solid waste creates an ideal breeding place for mosquitoes, insects, and rodents; thus, it is becoming a health problem and an environmental issue. According to the National Tire Industry Association (http://www.anip.com.br), Brazil produced around 61.3 million tires in 2009, most of which were disposed of in landfills, riversides, roadsides, and even in backyards. However, according to the Brazilian PET Industry Association (http://www.abipet.com.br), Brazil was ranked second in the world, behind only Japan, with respect to PET recycling. In 2008, 253 kt of PET were recycled in Brazil, i.e., 54.8% of the original consumption.

Therefore, the present study focused on the performance evaluation of two AFBR using ground tire and PET as support materials, and it investigated the microorganisms involved in biohydrogen production using molecular biology techniques. The effect of HRT on the performance of AFBR treating synthetic wastewater containing glucose (4000 mg L^{-1}) was also investigated.

2. Methods

2.1. Anaerobic fluidized bed reactor

Fig. 1 shows a schematic representation of the two identical jacketed reactors used for H_2 production in this study. The reactors were constructed of transparent acrylic with the following dimensions: 190 cm tall, an internal diameter of 5.3 cm, and a total volume of 4192 cm^3. The temperature in the AFBR was maintained at 30 ± 1 °C by recirculating water from a heated bath through the column's water jacket.

2.2. Synthetic wastewater, support materials, and inoculum

The synthetic wastewater contained glucose as the main carbon source (4000 and 5000 mg L^{-1} DQO) and was supplemented with nutrients as described by Barros et al. (2010). The wastewater pH was approximately 7.0; accordingly, 1000 mg L^{-1} of sodium bicarbonate and 1 mL L^{-1} of hydrochloric acid (10 M) were added to maintain the reactor pH at approximately 5.5.

Particles of ground tire and PET were used in the AFBR as support materials for biomass immobilization in reactors R1 and R2, respectively. The particles were submitted to prior chemical treatment to effect their cleaning and enhance their surface roughness. For the ground tire particles, the treatment process consisted of soaking the particles in a sodium hydroxide solution (7.5 10^{-3} M) for 30 min, rinsing them in water, and oven drying them at 40 °C. For the PET particles, the treatment process consisted of soaking the particles in a hydrochloric acid solution (10 M) for 30 min, rinsing them in water, and oven drying them at 40 °C. The basic characteristics of the support materials are shown in Table 1.

The inoculum used in this study was obtained from the anaerobic sludge of upflow anaerobic sludge blanket (UASB) reactor treating effluent from swine wastewaters. The H_2 productivity of the sludge was enhanced by heat treatment according to the methodology of Kim et al. (2006). This treatment consisted of preheating the sludge for 10 min at 90 °C to inhibit the methanogenic activity.

2.3. AFBR startup and operational conditions for biohydrogen production

The two AFBR with ground tire and PET as support materials were fed with a medium containing glucose (4000 mg L^{-1}) and heat-treated sludge (10% v/v). Approximately 621 and 1375 g of particles of ground tire and PET were introduced into the reactors R1 and R2, thus creating an initial fixed bed of 50 and 80 cm in depth for the reactors, respectively. Nitrogen gas was used to

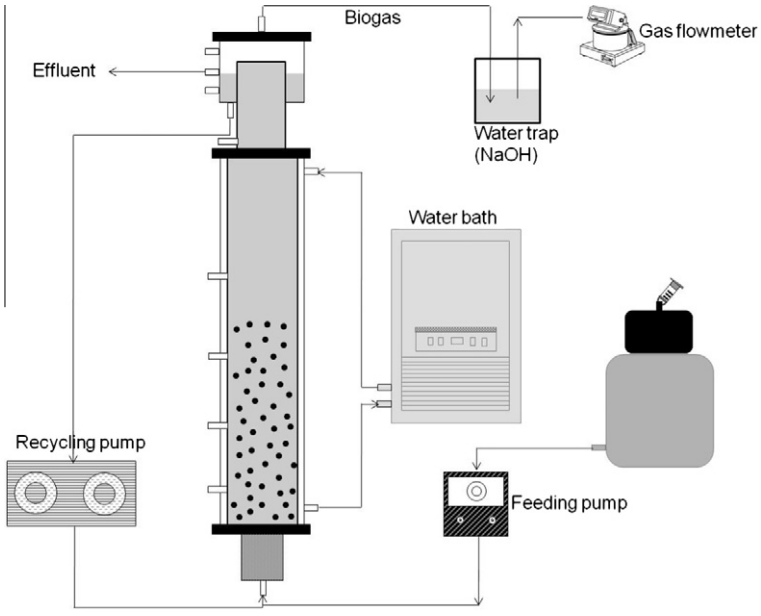

Fig. 1. Schematic representation of anaerobic fluidized bed reactor system.

Table 1
Support material characteristics.

Support material	Diameter (mm)	Density (g cm^{-3})	V_{mf} (cm s^{-1})	Roughness (%)
Ground tire	2.8–3.35	1.14	1.18	18.00
PET	2.2 × 2.2	1.25	1.35	10.23

V_{mf}: minimum fluidization velocity.

sparge the fermentation medium to create an anaerobic environment. For reactors R1 (ground tire) and R2 (PET), the total liquid flow (Q) was kept at 122 and 139 L h^{-1}, respectively. The bioreactors were initially operated on batch mode for 48 h to activate the H_2-producing sludge. Afterward, it was switched to a continuous mode at HRT of 8 h. The reactors R1 and R2 reached and average height of 120 and 92 cm, which corresponded to a working volume of 2646 and 2029 cm^3, respectively. When steady state was reached (based on a constant H_2 production rate with a variation of within 5–10% for 5–10 days), the HRT was decreased progressively from 8 to 1 h. The two reactors were operated for 191 days in five experimental phases, i.e., the five HRTs reported in Table 2. A gas–liquid separator was used at the effluent outlet to collect gaseous and soluble products separately. A gas meter (TG1; Ritter Inc., Germany) was used to quantify the amount of hydrogen generated.

2.4. Chemical analyses

The biogas hydrogen content was determined by gas chromatography (GC-2010, Shimadzu, Japan) using TCD with argon as the carrier gas and a column packed with Supelco Carboxen 1010 Plot (30 m × 0.53 mm i.d.) (Maintinguer et al., 2008).

Concentrations of volatile fatty acids (VFA) and alcohols were also measured by gas chromatography (GC-2010, Shimadzu, Japan) equipped with FID and COMBI-PAL headspace injection (AOC 5000 model) as well as a HP-INNOWAX column (30 m × 0.25 mm i.d. × 0.25 μm film thickness) (Maintinguer et al., 2008).

The glucose concentration was measured with an enzymatic GOD-PAP (Shida et al., 2009). Chemical oxygen demand (COD), pH, and solids (total solids, TS; volatile suspended solids, VSS; and total volatile solids, TVS) were measured in accordance with Standard Methods (1998).

Biomass adhesion to the ground tire and PET particles was determined according to the methods of Chen and Chen (2000).

2.5. Molecular biology analysis

Total genomic DNA was obtained after cell lysis with glass beads and phenol–chloroform extraction as described by Griffiths et al. (2000).

The amplification of the polymerase chain reaction (PCR) was performed with a bacterial domain primer set for the 16S rRNA gene, 27 forward (5′-AGAGTT TGATCCTGGCTCAG-3′) and 1100 reverse (5′-AGGGTTGCGCTCGTTG-3′) (Lane, 1991). The PCR amplification was carried out with initial denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 45 s, annealing at 55 °C for 45 s, and extension at 72 °C for 1.45 min, with a final extension step at 72 °C for 7 min and cooling at 4 °C (Thermocycler Eppendorf AG – Hamburg 22,331).

The PCR products were purified with an Illustra GFX PCR DNA kit and Gel Band Purification (GE Healthcare). The clone library was generated with pGEM ®-T Easy Vector Systems (Promega) and transformed into *Escherichia coli* competent cells following the manufacturer's instructions. After extraction of plasmid DNA was carried out, amplification of PCR with primers M13F–M13R (Messing, 1983). The PCR amplification was carried out with initial denaturation at 94 °C for 2 min, followed by 25 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 1 min, with a final extension step at 72 °C for 7 min and cooling at 4 °C (Thermocycler Eppendorf AG – Hamburg 22,331).

The nucleotide sequencing was performed in an ABI Prism® 310 Genetic Analyzer (Applied Biosystems). The assembly sequences were determined using the SeqMan – DNA-STAR (Lasergene sequence analysis), and the phylogenetic affiliations of the obtained sequences were determined by using the BLAST search program at the NCBI web site for comparison with the 16S rRNA gene sequence in the database at Genbank (http://www.ncbi.nlm.nih.gov) and the Ribosomal Data Base Project (http://rdp.cme.smu.edu). The phylogenetic tree was constructed by the neighbor-joining method (Saitou and Nei, 1987) using MEGA version 4.1 software (Kumar et al., 2008). Bootstrap resampling analysis for 1000 replicates was performed to estimate the confidence of tree topologies. *Methanosarcina mazei* (NC 003901) was used as an outgroup.

3. Results and discussion

3.1. Glucose conversion and hydrogen production

Fig. 2 shows the comparison between average glucose conversion and H_2 content in AFBR containing ground tire (R1) and PET (R2) in different HRT. Influent glucose was the same in the two reactors, i.e., 4000 mg L^{-1} ± 300.

Glucose conversion in reactor R1 (ground tire) remained virtually constant, i.e., around 90% at HRT 2 h. It dropped to 64% at HRT 1 h. Moreover, glucose conversion in R1 was more efficient at HRT 1, 2, and 4 h when compared to reactor R2 (PET). At HRT 8 and 6 h, the average efficiency of R2 (90%) was almost equal to that of R1. Efficiency decreased to 85%, 71%, and 60% at HRT 4, 2, and 1 h, respectively. The reduced glucose removal efficiencies at HRT 1 h were probably due to glucose overload in the reactors.

Table 2
Production of soluble metabolites during H_2 production under different operating conditions in AFBR.

Support material	HRT (h)	EtOH/SMP (%)	Hac/SMP (%)	Hbu/SMP (%)	HPr/SMP (%)	HLa/SMP (%)	HAc/HBu
R1 (ground tire)	8	25.40	28.71	27.66	1.57	16.65	1.04
	6	29.82	30.36	28.35	2.32	9.15	1.07
	4	22.16	34.40	31.27	1.61	10.57	1.10
	2	7.43	42.02	36.47	1.70	12.38	1.15
	1	12.81	34.58	31.34	0.88	20.37	1.10
R2 (PET)	8	25.74	25.38	27.39	1.37	20.11	0.93
	6	28.43	25.54	25.54	0.88	19.61	1.00
	4	16.12	29.93	28.81	1.25	23.89	1.04
	2	7.93	35.96	29.97	0.52	25.61	1.20
	1	10.72	17.26	15.55	1.67	54.80	1.11

EtOH: ethanol; HAc: acetate; HBu: butyrate; HPr: propionate; HLa: lactate; SMP = HAc + HBu + HPr + HLa + EtOH; EtOH/SMP, molar ethanol to SMP ratio; HAc/SMP ratio, molar acetate to SMP ratio; HBu/SMP ratio, molar butyrate to SMP ratio; HLa/SMP ratio, molar lactate to SMP ratio; HAc/HBu ratio, molar acetate to butyrate ratio.

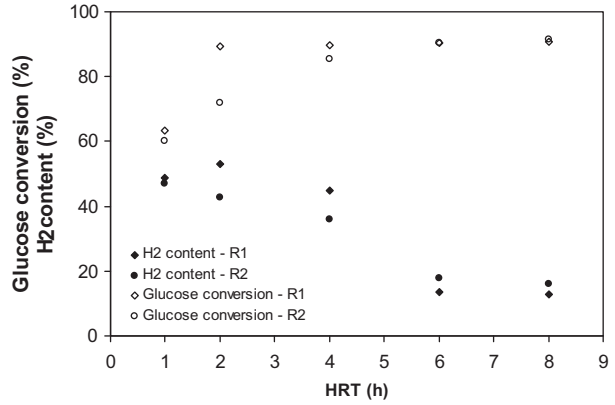

Fig. 2. Effect of HRT on the glucose conversion and H_2 content in AFBR containing ground tire (R1) and PET (R2).

Values of pH were stable and fell within the operating range of an anaerobic acidogenic system, i.e., between 4.47 and 5.85 in reactor R1 (ground tire) and between 4.44 and 5.67 in reactor R2 (PET). Influent pH was between 6.59 and 7.07 in both reactors.

Hydrogen and carbon dioxide were present in the biogas of both reactors, while methane was not detected during any phases of the experiment. The absence of methane in the biogas may be attributed to the heat treatment of the inoculum and the maintenance of the pH at around 5.5, which inhibits the methanogenic activity that is responsible for the consumption of hydrogen in the system. Hydrogen content in the biogas ranged from 12.7% to 53.0% in reactor R1 (ground tire) and between 12.3% and 47.1% in reactor R2 (PET) (Fig. 2). H_2 content in the biogas rose with HRT reduction to 2 h in R1 and R2 and increased further with HRT reduction to 1 h. Even though the H_2 content in R2 rose with an HRT reduction to 1 h (47.1%), R1 produced more H_2 in the biogas at the same HRT (48.7%). These results are consistent with the literature on AFBR with glucose (Zhang et al., 2007; Shida et al., 2009) and sucrose (Lin et al., 2006).

Fig. 3 shows the effect of HRT and organic loading rate (OLR) on the hydrogen yield (HY) and hydrogen production rate (HPR) of the reactors containing ground tires (R1) and PET (R2).

HY values ranged from 1.20 to 2.15 mol H_2 mol^{-1} glucose in reactor R1 (ground tire) and 1.14 to 1.87 mol H_2 mol^{-1} glucose in reactor R2 (PET), increasing with HRT reduction in both reactors up to HRT 2 h and decreasing at HRT 1 h. R1 and R2 showed optimum HYs of 2.15 mol H_2 mol^{-1} glucose and 1.87 mol H_2 mol^{-1} glucose, respectively, at HRT 2 h (Fig. 3). This may be an indication that metabolic changes occurred when the HRT was reduced from 2 to 1 h and that more substrate was used for the growth and maintenance of biomass than for the formation of end products (Zhang et al., 2007).

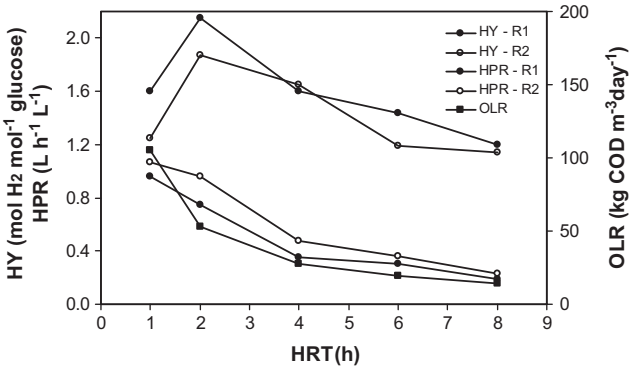

Fig. 3. Effect of HRT and ORL on the HY and HPR in AFBR containing ground tire (R1) and PET (R2).

Moreover, it should be noted that HY values rose as OLR increased when HRT was reduced from 8 to 2 h in both reactors. OLR and HY correlated linearly in reactors R1 (ground tire) and in R2 (PET) up to an OLR of 52.70 kg COD m^{-3} day^{-1} at HRT 2 h. However, at HRT 1 h in both reactors, HY dropped with the increase of OLR, which suggests that there was a reactor overload at this OLR and, as a result, a diversion of the metabolic pathway and/or a kinetic limitation (Fig. 3). This behavior was also observed by Lin et al. (2006), Zhang et al. (2007), and Shida et al. (2009).

Considering that the maximum theoretical HY for glucose is and 4 mol H_2 mol^{-1} glucose, the results obtained in the present study correspond to 53.8% and 46.8% yields, respectively.

HPR values ranged from 0.19 to 0.96 L h^{-1} L^{-1} in R1 and from 0.23 to 1.07 L h^{-1} L^{-1} in R2. In both reactors, HPR increased with HRT reduction from 8 to 1 h. R2 displayed better performance in terms of HPR. In both reactors, HPR was higher at HRT 1 h. When HRT was reduced from 8 to 1 h in both reactors, there was a correlation between OLR and HPR (Fig. 3). OLR and HPR correlated linearly in R1 (ground tire) and in R2 (PET) up to an OLR of 104.96 kg COD m^{-3} day^{-1} at HRT 1 h. These results suggest that changes in the metabolism of microorganisms occur when HRT is reduced. The linear relationship between HPR and OLR was also observed in other studies that used AFBRs (Lin et al., 2006; Zhang et al., 2007; Shida et al., 2009; Barros et al., 2010).

Hydrogen production in this study (Fig. 3) was similar to that obtained by Zhang et al. (2007) and Shida et al. (2009) at pH values below 4.0. However, the results obtained by this research were also similar to those of Barros et al. (2010), who kept pH values around 5.5. Yet, in comparison to the results obtained by Lin et al. (2006) under pH conditions between 6.0 and 7.0, which are deemed as favorable for hydrogen production (Fang and Liu, 2002), the hydrogen production rate was lower in this study. Mohan et al. (2007) observed that reduction of the pH from 6.0 to 4.5 favored the emergence of acidogenic bacteria, which inhibit the activity of methanogenic archaea.

The difference between this study and other studies demonstrates the need for proper maintenance of acidogenic populations and prevention of competition for substrate in the system by other microorganisms that do not produce hydrogen. Besides the thermal treatment of the inoculum according to methodology adapted from Maintinguer et al. (2008), it appears that maintaining a closed circuit system for 48 h at a glucose concentration of 4000 mg L^{-1} favored the performance of the reactors because of the stage of biomass adaptation to glucose during the cell immobilization phase.

3.2. Soluble microbial products

During the operation of both reactors, a predominance of acetic acid (HAc), butyric acid (HBu), and lactic acid (HLa) as well as a low production of propionic acid (HPr) and ethanol (EtOH) was observed in all experimental phases (Table 2).

It may be noticed that reduction of HRT from 8 to 2 h increased the HAc concentration from 28.71% to 42.04% and from 25.38% to 35.96% in reactors R1 and R2, respectively. However, when HRT was reduced to 1 h, this concentration decreased to 34.58% and 17.26% in R1 and R2, respectively. Similarly, the HBu concentration increased in reactor R1 (ground tire) and R2 (PET) with HRT reduction from 8 to 2 h, but the concentration decreased to 31.44% and 15.55% in R1 and R2, respectively, when HRT was reduced to 1 h. However, while HLa production was unrelated to HRT in R1, there was increased production of this acid in R2 when HRT was reduced from 8 to 1 h. EtOH production in both reactors rose with HRT reduction from 8 to 6 h but

dropped when HRT was reduced from 6 to 1 h. Generally, EtOH concentrations dropped with HRT reduction. Lin et al. (2006) did not find a correlation between the production of organic acids and HRT reduction in AFBR. However, Zhang et al. (2007) reported a decrease in the production of acids and alcohols with HRT reduction for the same type of reactor.

The HPr concentration in the system was lower than 2.32% in both reactors (0.52% to 2.32%) at HRT from 8 to 1 h. This finding may promote hydrogen yield, given that whenever the pathway of HPr production is favored, 2 mol of H_2 are consumed for every 2 mol of HPr produced. This phenomenon may also be associated with inhibition caused by low pH and sensitivity at short HRT, which has been reported by other researchers (Zhang et al., 2007).

When comparing this study to others, it may be noted that Zhang et al. (2007), Koskinen et al. (2007), and Barros et al. (2010) found that the presence of HPr was insignificant, which may be attributed to the use of low pHs. These authors suggested that the activity of HPr-forming microorganisms is inhibited under conditions of low pH.

Another important issue worthy of examination is the HAc/HBu ratio presented in Table 2. Several authors claim that this ratio is indicative of hydrogen production in acidogenic systems (Lin et al., 2006; Koskinen et al., 2007; Barros et al., 2010). In general, a higher HAc/HBu ratio gives a higher theoretical H_2 yield, according to reaction stoichiometry, bioconversion of 1 mol of glucose into HAc yields 4 mol H_2 mol^{-1} glucose, but only 2 4 mol H_2 mol^{-1} glucose is formed when HBu is the end product.

In this study, the HAc/HBu ratio in reactor R1 (ground tire) rose from 1.04 to 1.15 when HRT was reduced from 8 to 2 h. However, it dropped to 1.10 at HRT 1 h. Reactor R2 (PET) performed similarly; its HAc/HBu ratio increased from 0.93 to 1.20 with HRT reduction from 8 to 2 h and dropped to 1.11 with HRT reduction to 1 h (Table 2).

The HAc and HBu production is indicative of good H_2 production, in contrast to the HPr production, which consumes hydrogen. Other studies also indicated the production of HAc and HBu as the main soluble metabolites (Lin et al., 2006; Zhang et al., 2007; Koskinen et al., 2007; Shida et al., 2009; Barros et al., 2010). EtOH and HLa are considered to be unfavorable metabolites in hydrogen production, as no hydrogen is consumed or produced in their production.

According to Koskinen et al. (2008), H_2 production from carbohydrates occurs when acetate or butyrate is produced, while the production of ethanol does not result in H_2 production. This implies that ethanol production decreases when H_2 production is optimized (production of acetate), and vice versa. Depending on the organism, the ethanol (and hydrogen) yields vary substantially, from traces to nearly quantitative amounts.

The high production of HAc and HBu in the reactor containing ground tire as the support medium (R1) can explain why this reactor showed higher HY and greater hydrogen content in its biogas than the reactor that employed PET (R2) as the support material.

Table 3 shows a comparison of the present study and previous research regarding the production of hydrogen and soluble metabolites as well as the substrate and the support medium employed in the studies. These results indicate that ground tire (R1) and PET (R2) seem to be successful and feasible for continuous fermentative H_2 production in AFBR.

3.3. Biomass

The concentration of biomass adhering to the support medium at different HRTs in R1 (ground tire) and R2 (PET) was measured. As HRT was reduced from 8 to 2 h, the amount of biomass adhering to the support medium rose from 0.9 to 1.3 mg TVS g^{-1} ground tire and from 0.5 to 0.8 mg TVS g^{-1} PET.

According to Barros et al. (2010), biofilm accumulation on a support is a dynamic process that is the net result of growth and detachment. Biofilm formation is affected by several external factors, including the composition and the concentration of the feed, the velocity of the liquid phase (shear stress), the concentration of particles, particle–particle collisions, and particle–wall collisions. In addition, the nature and the concentrations of the substrates may affect biofilm growth and composition.

Zhang et al. (2008) concluded that biofilm thickness decreases with increasing granular biomass in the biofilm due to high activity of hydrogen-producing bacteria. When biofilm thickness increases, microorganism adherence to the medium becomes weaker. As a consequence of the particles colliding, the biofilm detaches from the support medium but leaves biofilm fragments on the medium.

For this reason, it may be concluded that the greater amount of biomass adhered to the medium probably caused the biofilm to thin out due to high activity of hydrogen-producing microorganisms, thus leading to the high HY at HRT 2 h, which displayed greater attached biomass. Biomass reduction at HRT 1 h in both reactors may have contributed to HY reduction in the reactors at HRT 1 h. Furthermore, the decreasing HRT (with increasing OLR) may have increased the thickness of the biofilm, and therefore attachment to the support material might have become weaker. As a result, some biofilm may have separated from support materials due to particle–particle collisions, causing a decrease in the observed values of TVS/support, when the lowest HRT value was reached. These effects would subsequently result in reduced HY. Another hypothesis is that once the AFBRs became overloaded, the systems were limited with respect to glucose conversion, while

Table 3
Comparative study on the efficiency of hydrogen fermentative production in AFBR.

Support material	Substrate	Maximum HPR, optimal HRT	Maximum HY, optimal HRT	HAc/Hbu (maximum HY)	Reference
Alginate gel	Sucrose	0.93 L h^{-1} L^{-1}, 2 h	2.67 mol H_2 mol^{-1} sucrose, 2 h	–	Wu et al. (2007)
Silicon gel	Sucrose	2.27 L h^{-1} L^{-1}, 2.2 h	4.98 mol H_2 mol^{-1} sucrose, 8.9 h	0.65	Lin et al. (2006)
Polyethylene–octane elastomer	Sucrose	1.49 L h^{-1} L^{-1}, 4 h	0.64 mol H_2 mol^{-1} sucrose, 4 h	1.88	Wu et al. (2007)
Polyethylene–octane elastomer	Glucose	1.34 L h^{-1} L^{-1}, 4 h	1.04 mol H_2 mol^{-1} glucose, 4 h	2.10	Wu et al. (2007)
Polyethylene–octane elastomer	Fructose	0.83 L h^{-1} L^{-1}, 4 h	0.56 mol H_2 mol^{-1} fructose, 4 h	2.14	Wu et al. (2007)
Activated carbon	Glucose	2.36 L h^{-1} L^{-1}, 0.5 h	1.19 mol H_2 mol^{-1} glucose, 0.5 h	1.48	Zhang et al. (2007)
Celite R-633	Glucose	0.46 L h^{-1} L^{-1}, 1.8 h	1.35 mol H_2 mol^{-1} glucose,[a] 1.8 h	1.67	Koskinen et al. (2007)
Expanded clay	Glucose	1.28 L h^{-1} L^{-1}, 1 h	2.29 mol H_2 mol^{-1} glucose, 2 h	1.34	Shida et al. (2009)
Expanded clay	Glucose	1.21 L h^{-1} L^{-1}, 1 h	2.59 mol H_2 mol^{-1} glucose, 2 h	1.21	Barros et al. (2010)
Polystyrene	Glucose	0.95 L h^{-1} L^{-1}, 1 h	1.90 mol H_2 mol^{-1} glucose, 2 h	1.28	Barros et al. (2010)
Ground tire	Glucose	0.96 L h^{-1} L^{-1}, 1 h	2.15 mol H_2 mol^{-1} glucose, 2 h	1.15	This study
PET	Glucose	1.07 L h^{-1} L^{-1}, 1 h	1.87 mol H_2 mol^{-1} glucose, 2 h	1.20	This study

[a] Based on the article data.

the HPR continued to increase as the HRT decreased (OLR increased).

Moreover, the better performance of the reactor containing tire particles (R1) may be attributed to the characteristics of this support medium, including higher roughness (18.0%) than PET (10.2%). The greater amount of biomass attached to the ground tires may also explain the better hydrogen production performance of the reactor containing this material as a support medium, as more acidogenic hydrogen-producing bacteria can adhere to this medium. Moreover, ground tire particles have more creviced surfaces than PET particles, and these crevices protect developing biofilms from shear forces, allowing more uniform biomass colonization (Barros et al., 2010).

Thus, as presented in Table 2, R1 (ground tires) showed a better HY (2.15 mol H_2 mol^{-1} glucose) than those reported by Zhang et al. (2007), Koskinen et al. (2007), and Barros et al. (2010) using polystyrene particles. In addition, R2 with PET particles showed a better yield (1.87 mol H_2 mol^{-1} glucose) than that presented by the reactors of Zhang et al. (2007), and Koskinen et al. (2007) and was virtually equal to that of Barros et al. (2010), who used polystyrene particles. Thus, it may be claimed that tire particles constitute a better support medium in AFBRs than activated carbon, Celite R-633, polystyrene, polyethylene-octane elastomer, and expanded clay. Similarly, PET was shown to be better than activated carbon, Celite R-633, polyethylene-octane elastomer, and expanded clay in AFBR behaved similarly to polystyrene with respect to HY.

3.4. Bacterial community composition

Analyses of bacterial community composition were conducted only for the ground tire biofilm, as this support medium was more suitable for hydrogen production. A hundred clones were obtained from R1 through cloning analyses and sequencing of 16S rRNA gene fragments of the microbial consortium. Clones with sequences smaller than or equal to 200 base pairs were not used in phylogenetic analyses. The clones obtained are shown in Fig. 4.

The similarity coefficient values found between clones and the NCBI database ranged from 96% to 100% and indicated the presence of phylogenetically related bacteria based on partial evaluation of 16S rRNA gene sequences.

Most of the clones, i.e., 61%, were related to *Clostridium*, whereas 32% were related to *Bacillus*, 5% to *Enterobacter*, and 3% to *Sporolactobacillus*. *Enterobacter*, *Bacillus*, and *Clostridium* were the main fermentative hydrogen-producing bacteria in a batch reactor fed with glucose as carbon source (Kawagoshi et al., 2005).

Fig. 4 shows the consensual phylogenetic tree obtained with primers for the bacteria domain from the sequences derived from cloning and sequencing of the microbial consortium in the AFBR containing ground tire as a support material (R1). Most clones (95%) belonged to the *Firmicutes phylum* (Clostridia and Bacilli classes) and only 5% to the *Proteobacteria phylum* (Gammaproteobacteria). *Clostridium* belongs to the Clostridia class, whereas *Bacillus* and *Sporolactobacillus* belong to the Bacilli class. *Enterobacter* belongs to the Gammaproteobacteria class (Fig. 4).

Clostridia are straight, Gram-positive, endospore-forming bacilli that thrive at pH values around 4.0. For most species, growth is most rapid at pH 6.5–7 and at temperatures between 30 and 37 °C. They are usually chemoorganotrophic; some species are chemoautotrophic or chemolithotrophic as well. They also usually produce mixtures of organic acids and alcohols from carbohydrates or peptones. The Clostridia may metabolize carbohydrates, alcohols, amino acids, purines, steroids, or other organic compounds. Most species are obligately anaerobic, although tolerance to oxygen varies widely; some species will grow but not sporulate in the presence of air at atmospheric pressure (Rainey et al., 2009). These bacteria produce hydrogen and organic acids through fermentation, and butyric acid and alcohol are the main compounds (Lin et al., 2008) formed from carbohydrates (Ueno et al., 2001).

Large amounts of butyric acid and acetic acid as well as H_2 and CO_2 are some of the products of the fermentation of carbohydrates by *Clostridium* species (Cohen et al., 1979).

Iyer et al. (2004) claim that when anaerobic sludge is subjected to thermal treatment, *Clostridium acetobutyricum* prevails and is responsible for the formation of butyric acid from glucose.

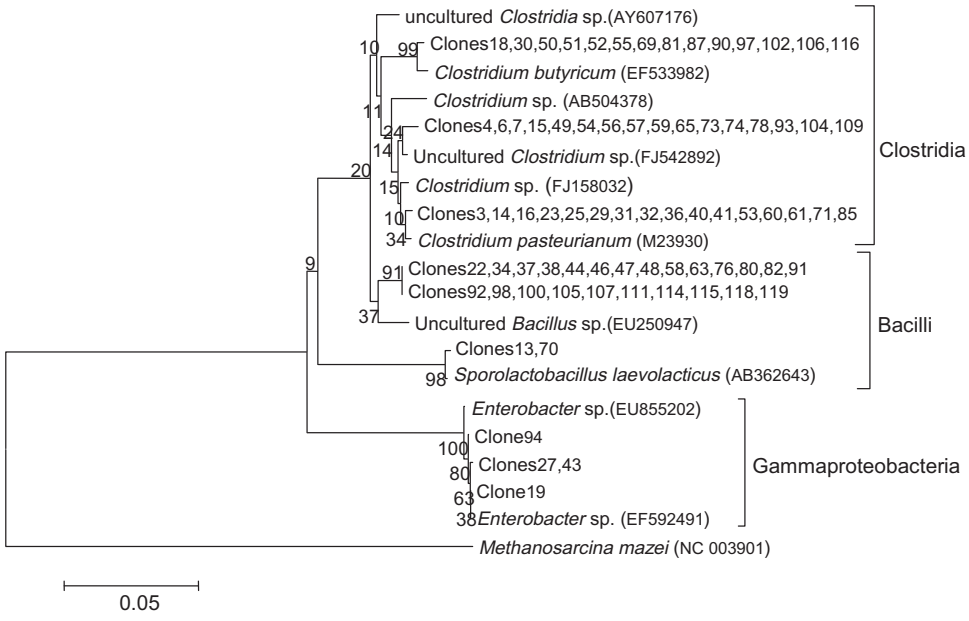

Fig. 4. Consensual phylogenetic tree based on sequences obtained from a sample of biomass adhering to support material (ground tire) in the AFBR (R1) fed with glucose to produce hydrogen. The values at the tree nodes indicate percentages of recurring branches (1000 bootstraps for resampling). *Methanosarcina mazei* was employed as an outgroup.

According to Cohen et al. (1979), the fermentation of butyric acid may be accomplished by *Clostridium butyricum*, *Clostridium tyrobutyricum*, and *Clostridium lacto-acetophilum*. Lin et al. (2008) mention that the fermentation of glucose by different *Clostridium* species predominantly yields acetic and butyric acid, carbon dioxide, hydrogen, and biomass.

Most species of *Bacillus* will use glucose and/or other fermentable carbohydrates as their sole sources of carbon and energy. Patterns of acid production from carbon substrates and patterns of assimilation of these substrates are of great value in the characterization and identification of *Bacillus* species. Diverse physiological abilities are exhibited, ranging from acidophilic to alkaliphilic. Endospores are formed, no more than one to a cell; these spores are resistant to many adverse conditions. They can be Gram-positive, Gram-positive only in the early stages of growth, or Gram-negative. The spherical-spored *Bacillus* species do not produce acid or gas from D-glucose or other carbohydrates. They are generally aerobes or facultative anaerobes, but a few species are described as strictly anaerobic (Schileifer, 2009).

A small percentage of clones (5%) were similar to Gram–Stain-negative *Enterobacter* sp. cells belonging to the Enterobacteriaceae family. These cells were facultatively anaerobic and chemoorganotrophic, having both a respiratory and a fermentative type of metabolism. D-glucose and other carbohydrates are catabolized with the production of acid and, in many species, gas (Holt et al., 1994). Kumar and Das (2000) inoculated an anaerobic batch reactor with *Enterobacter cloacae* (gram-negative, facultative anaerobic bacteria) and obtained an HY of 6 mol H_2 mol^{-1} sucrose at pH 6.0 and 36 °C. These bacteria also produced H_2 with glucose (2.2 mol H_2 mol^{-1} glucose) and cellobiose (5.4 mol H_2 mol^{-1} cellobiose). It is possible that the gram-negative bacilli identified in this study, favored under the operating conditions, consumed glucose and generated acid and hydrogen.

According to Shin et al. (2007), *Enterobacter* sp. uses the NADH pathway to generate hydrogen via the enzyme hydrogenase by reoxidizing glycolysis-produced NADH.

Some clones were related to Gram–Stain-positive *Sporolactobacillus*, with endospores resistant to heating for 10 min at 80 °C. Facultatively anaerobic or microaerophilic growth is observed under anaerobic cultivation, good growth occurs on media containing glucose, and D- or DL-lactic acid is produced homofermentatively. Acid is produced from glucose, fructose, galactose, mannose, maltose, sucrose, and trehalose. Carbohydrates are essential substrates for growth. However, acids are produced from a limited number of carbohydrates. For instance, *Sporolactobacillus laevolacticus* are responsible for lactic acid production and employed to ferment fructose and glucose at pH values below 4.0 (Yanagida and Suzuki, 2009).

Therefore, hydrogen production was related to the presence of *Clostridium*, *Bacillus*, and *Enterobacter*, which was favored by environmental conditions imposed on the reactor in question as well as its support medium (ground tire). Other authors (Koskinen et al., 2007; Maintinguer et al., 2008) have also observed these bacteria in anaerobic reactors employed to produce hydrogen.

4. Conclusion

The glucose fermentation in the AFBR containing ground tires as a support material was more suitable for hydrogen production, because besides presenting a higher hydrogen yield (2.15 mol H_2 mol^{-1} glucose), it showed higher hydrogen content in the biogas (52.97%), and improved production of acetic and butyric acids (39.3% and 34.1%, respectively). Based on the experimental results, the higher performance of this reactor may be attributed to the higher roughness of the ground tire particles when compared to PET particles. Therefore, these particles accumulate a greater amount of adhered biomass and, they favored the presence of hydrogen-producing bacteria such as *Clostridium*, Bacillus, and *Enterobacter*.

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