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A NOVEL BIOREFINERY APPROACH FOR BIOFUELS AND HOLOCELULLOLYTIC ENZYMES PRODUCTION FROM ORGANIC WASTES

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ABSTRACT

The organic fraction of municipal solid waste (OFMSW) was fed to a two-stage solid substrate anaerobic digestion (2S-SSAD) process evaluated at 54 and 35°C on bioreactors running at 21, 14 and 10 d mass retention time (MRT). The fermented solids from these operational units were fed to corresponding individual methanogenic bioreactors at 28 d MRT. The spent solids from the 2S-SSAD process were evaluated as substrates for cellulase and xylanase production using the mutant eubacterium strain *Cellulomonas flavigena* PR-22 in submerged cultures in flask experiments. Hydrogen productivity in mesophilic bioreactors almost doubled that in thermophilic ones ($p < 0.05$). On the other hand, the effect of MRT of the hydrogenogenic stage (levels 10, 14, 21 d) was not significant ($p = 0.103$). The best performance was at 35°C and 10 d MRT (hydrogen productivity = 13.2 $NmL_{H2}/kg_r/d$). In the second (methanogenic) stage, the methanogenic productivity was significantly superior in thermophilic regime. Maximum methane productivity was 341 $NmLCH_4/kg_r/d$ that corresponded to the thermophilic process operated at 42 d total MRT (total MRT defined as hydrogenogenic MRT plus methanogenic MRT). Xylanolytic and cellulolytic activities with spent solids as sole substrate reached up to 5000 and 980 IU/L, respectively. We conclude that integration of enzyme production as an additional stage using spent solids from bioenergy processes is possible, attractive and properly fits in the *biorefinery* concept, towards which environmental and process biotechnology is focusing. The scale-up from Erlenmeyer flasks to fermenters is strongly suggested to evaluate the possible increase and feasibility of enzyme production.

Key words: biorefinery, enzymes, hydrogen, methane, organic wastes

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Introduction

The generation of solid wastes amounts to thousands of tons per day in megalopolis of developed and underdeveloped countries. In Mexico, 102,000 ton/day are produced (INEGI, 2009) where the organic fraction (OFMSW, paper and organic wastes) represents up to 60% of the total. The OFMSW is a potential raw material for the production of biofuels, intermediary chemicals, proteins, enzymes, as well as other added-value products (Holtzapple *et al*., 1992; Silva *et al*., 1995, Lay *et al*., 1999, Poggi-Varaldo *et al*., 2005; Valdez-Vazquez *et al*., 2005). Bioproducts are actually obtained through biotechnology, but few works have dealt with the problem of highly organic-loaded effluents from these processes. On a biorefinery perspective such problems are to be solved by the maximization of end products (Zeikus, 1980, Kamm and Kamm, 2004).

Two-stage digestion is used in the production of hydrogen and methane as products that have widespread industrial application. Hydrogen is considered a highly energetic clean fuel. Besides, it is used as a reactant in petrochemical and chemical industries (Das and Veziroglu, 2001; Kapdan and Kargi, 2006). Methane, on the other hand, has direct application as natural gas and may be also used in chemical synthesis (Reith *et al*., 2003).

When using solid substrate digestion, digestates from this process still have an important residual organic content, thus they have been commonly used as soil amenders (Poggi-Varaldo *et al*., 1999). However, research on obtaining other added-value products from digestates is still scarce (Robledo-Narváez *et al*., 2008). In this regard, cultures of aerobic bacteria have been used for holocellulases production using agroindustrial and municipal wastes (Pérez-Avalos *et al.*, 2002; Xin and Geng, 2010). This may be an indication that organic digestates from bioenergy processes could be used for the same purpose.

Therefore, the objective of this work was to assess a novel process that consisted in two-stage solid substrate anaerobic digestion process for hydrogen and methane generation followed by the production of added-value xylanases and cellulases from the digestates of a two-stage bioenergy process.

Materials and methods

Two-Stage Solid Substrate Anaerobic Digestion. A two-stage solid substrate anaerobic digestion (2S-SSAD) was used for hydrogen and methane production (Figure 1). Inocula for both stages were obtained from solid substrate anaerobic digesters as reported elsewhere (Poggi-Varaldo *et al*. 1997, Valdez-Vazquez *et al*., 2005). Conditions for hydrogenogenic bacteria selection were fostered inducing an organic loading shock, which reduced pH in bulk to 5-6, thus inhibiting methanogenic archaea. In this way, the bioreactors became acidogenic-hydrogen producing units.

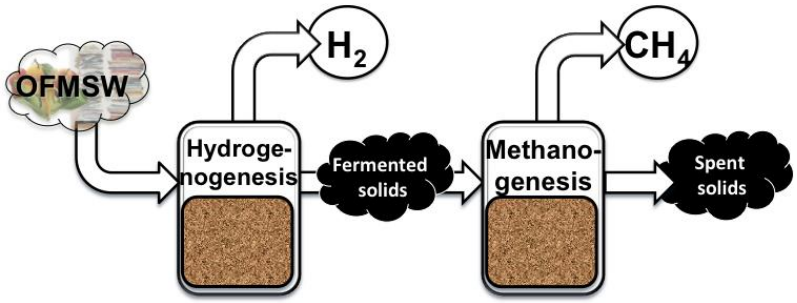

FIGURE 1. **Process schematic of the 2S-SSAD process.**

The hydrogenogenic stage was fed with the organic fraction of municipal solid waste (OFMSW). For reproducibility sake, a model OFMSW was prepared by mixing dried food wastes from the cafeteria of the CINVESTAV (60% w/w) with used office paper (40% w/w). Mixture was stored at 4ºC for later use (Valdez-Vazquez *et al*., 2005). Previous to feeding, OFMSW was conditioned with a $NaHCO_3$-K_2HPO_4 buffer (55.7 $gCaCO_3/L$) to a total solids concentration of 35% w/w. Hydrogen productivity was evaluated at three mass retention times (MRT): 21, 14 and 10 d; at mesophilic (35ºC) and thermophilic (55ºC) regimes. The second stage consisted of the methanogenic process, fed with the fermented solids from the purges of the first stage. Each stream of fermented solids was fed as it was to a methanogenic bioreactor working at the same temperature at which the solids were obtained. Methanogenesis at mesophilic and thermophilic regimes were evaluated at 28 d MRT. Solid digestates from methanogenic stage were coined as spent solids.

The two-stage process was compared to MRT equivalent methanogenic controls working at the same temperatures and equivalent MRT: 21+28, 14+28 and 10+28 d. These controls were fed with OFMSW at 35% total solids.

One-liter glass jars were used as bioreactors, and working mass inside bioreactors was 500 g. Bioreactors were drawed-and-filled twice a week at corresponding MRT. Treatments and controls were run by duplicate. The main response variables used for evaluating the process performance were H_2 and CH_4 productivity ($NmL/kg_{wmr}/d$), yield ($NmL/g\ VS_{rem}$), and organic acids and solvent accumulation ($mgCOD/kg_{wmr}$). Due to the extensive of experimentation, selected results are shown here; yet, they all are available upon request.

Analyses were performed to solid samples taken from OFMSW and bioreactors. Volatiles solids (VS) and total solids (TS), pH, volatile organic acids (VOA), lactic acid and solvents were analysed as reported elsewhere (Poggi-Varaldo *et al*., 1999; Valdez-Vazquez *et al*., 2005; Baghcherahsaraee *et al*., 2009). Biogas production was measured by acid brine displacement (Valdez-Vazquez *et al.,* 2005); gas volumes were normalized to 273 K and 1 ata (NmL or NL). H_2 and CH_4 contents were determined by gas chromatography in a GOW-MAC chromatograph model 350 fitted with TCD and a Molecular Sieve 5A packed column (injector, detector and column temperatures were 37, 100 and 70ºC, respectively). Argon was the carrier gas.

Holocellulases Production. The mutant eubacterium strain *Cellulomonas flavigena* PR-22 was used for the production of cellulases and xylanases from the spent solids of the 2S-SSAD. OFMSW and a 1:1 mixture spent solids- OFMSW were also assessed as substrates. Substrates were tyndalized in mineral medium prior to inoculation with *C. flavigena* PR-22. Inoculation ratio was 10% (v/v) to 250 ml Erlenmeyer flasks containing 50 ml of mineral medium and 1.0% (w/v) of either the substrates. Composition of the mineral medium was the following (in g/L; Ponce-Noyola and de la Torre, 1993): NaCl (5.5); $(NH_4)_2SO_4$ (2.5); K_3PO_4 (3.5); $CaCl_2$ (0.1); $MgSO_4$ (0.1); yeast extract (0.2).

Experiments lasted for 65 h at 37 ºC in an orbital shaking at 150 rpm. Experiments were made by triplicate. Samples from the medium for xylanase and cellulase analyses were taken at 0, 24, 48 and 65 h. Enzymatic activities were assayed by measuring reducing sugars by the dinitrosalicylic acid method (Miller, 1959) according to Mandels *et al*. (1974) and as reported by (Ponce-Noyola and de la Torre, 1995). Activities were expressed in International Units (IU), which were defined as the amount (µmol) of xylose or cellobiose (determined as reducing sugars) released per minute under assay standard conditions. Cellulose, lignin and hemicellulose contents in substrates were determined as reported elsewhere (Poggi-Varaldo *et al*., 1999). All determinations were made by triplicate.

Results and discussion

Hydrogenogenic Stage of the 2S-SSAD. A typical dynamic performance of hydrogenogenic bioreactors is shown in Figure 2. Table 1 shows that mesophilic bioreactors doubled the hydrogen productivity (I_{H2}) in thermophilic ones. Temperature also had a considerable effect on solvent accumulation ($p< 0.0001$). In contrast, other works have shown that thermophilic hydrogenogenesis had better performance than that of mesophilic (Shin *et al*., 2004; Valdez-Vazquez *et al*., 2005; Liu *et al*., 2006).

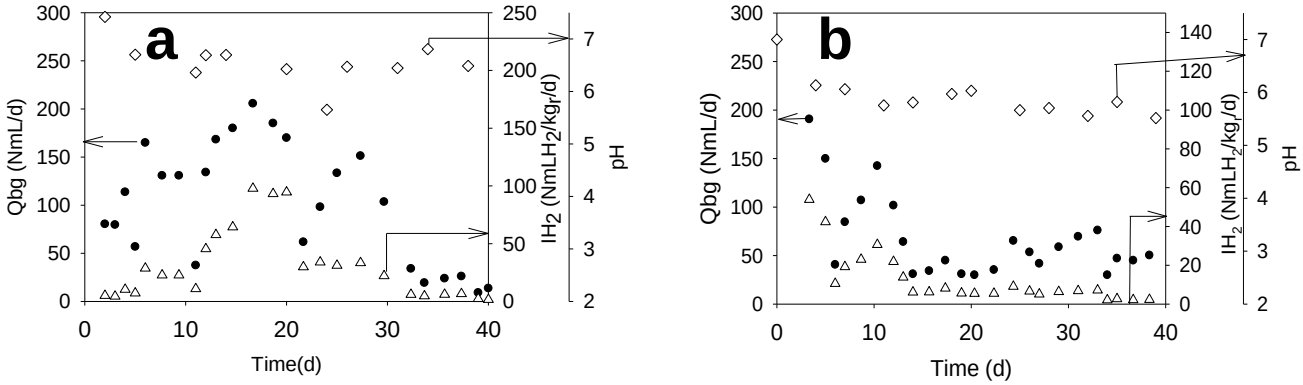

FIGURE 2. Typical time courses in hydrogenogenic stage at 21 d. (a) mesophilic, (b) thermophilic. (●) Q_{bg}: Biogas production (△), I_{H2}: hydrogen productivity (◊) pH.

TABLE 1. Performance of hydrogenogenic stage by its effects.

		Hydrogenogenic stage					
		I_{H2}[a] (3.98)[b]	VOA[c] (1166)[b]	Lactic[d] (3413)[b]	Solvent[e] (253)[b]	VOA/Solvents[f] (1.1)[b]	A/B[g]
MRT[h]	10 d	8.14	8089	32117	3610	2.37	0.58
	14 d	6.55	7944	22024	3025	4.45	0.62
	21 d	9.58	6392	19734	3046	2.37	0.68
Thermal regime	Mesophilic	11.22	6908	21364	1866	4.38	0.61
	Thermophilic	4.97	8042	27887<	4568	1.76	0.65

Notes: [a]Hydrogen productivity (NmL_{H2}/kg_r d); [b] standard error of the experimental design (EED = $(MSS_{error}/r)^{0.5}$) Montgomery (1991); [c]Volatile organic acids accumulation ($mgCOD_{VOA}/kg_r$); [d]Lactic acid accumulation ($mgCOD_{lactic\ acid}/kg_r$); [e]Solvent accumulation (mgCODsolvent/kg_r); [f]Volatile organic acids to solvents accumulation relation; [g]Acetic acid to butyric acid relation ($mgCOD_{acetic\ acid}$/$mgCOD_{butyric\ acid}$); [h]mass retention time (d).

This could be related to lactic and solvent fermentation deviations in our thermophilic units, whereas hydrogenogenesis of the above references was associated with no or low content of lactic acid. It is known that lactic acid and solventogenic shifts are hydrogen sinks. Moreover, no significant effect of MRT on process performance was found (p = 0.103).

The best performance in individual bioreactors was attained at 35°C and 10 d MRT (I_{H2} = 13.2 NmL_{H2}/kg_r/d, data not shown). Even though the pH in all bioreactors fell in the range 5.5 - 6.5, *i.e.* the range favorable for hydrogenogenesis (Valdez-Vazquez and Poggi-Varaldo, 2009), the lowest H_2 yields were associated to the lowest pH in thermophilic regime (data not shown). Hydrogen concentration in biogas of our bioreactors was poor to moderate; hydrogen content was up to 22% (corresponding to 21 d mesophilic bioreactor). Submerged cultures reported biohydrogen contents ranging from 10% (Han and Shin, 2004) to 69% (Shin *et al*., 2003). However it is not clear whether this could be due to CO_2 solubilization or to a higher biological activity. As for solid substrate fermentation, Valdez-Vazquez *et al*. (2005) achieved 58% and 42% hydrogen in biogas in thermophilic and mesophilic regimes, respectively.

In general, hydrogenogenic process performance in this research was poor compared to other works (Valdez-Vazquez *et al*., 2005; Liu *et al*., 2006). This could be attributed to high lactic acid and solvents accumulation as mentioned previously. VOA accumulation in thermophilic regime was slightly higher than in mesophilic one. Acetic-butyric acid relation (A/B) on COD basis, was in a proportion 0.61 and 0.65 for mesophilic and thermophilic respectively, meaning that butyric acid production was favored over acetic acid, thus yielding lower hydrogen results. If we assume that 50% of glucose is fermented to H_2 plus acetic acid whereas the other 50% is fermented to H_2 plus butyric acid, then A/B relation would be 0.802. Consequently a value greater than 0.802 would mean that acetic acid fermentation was favored, whereas a lower value would mean that butyric acid fermentation was the prevailing one (Liu *et al*., 2006), as in our case. This partially

justifies low hydrogen production. It is well known that, ideally, from each molecule of hexose 4 mol of hydrogen are produced in acetic fermentation whereas in butyric fermentation only 2 mol of hydrogen may be obtained. So far, there is not a known method for favouring acetic fermentative microorganisms over butyric fermentative ones when using consortia, due to inherent microbial interactions and also because some microorganisms may present both pathways, such as *Clostridium acetobutylicum* (Madigan *et al*., 1997). Other metabolites were present in different proportions. A significant production of propionic acid was also present in all bioreactors. Acetone was present only in mesophilic regime, whereas only thermophilic regime showed butanol in a small amount. Solventogenic deviation was more evident in thermophilic regime since solvent concentration was two-fold higher compared to mesophilic (Table 1).

As stated previously, lactic acid production was also related to low hydrogen productivities. Thermophilic regime exhibited the highest effect on lactic acid accumulation. Overall, concentrations of lactic acid in all bioreactors were superior to those commonly reported in hydrogenogenic processes (Noike *et al*., 2002; Wang and Zhao, 2009). Their values were also higher than those of the solvents and VOA, and therefore could be the main cause of such unexpected low hydrogen results. Lactic acid presence is mainly due to lactic acid bacteria (LAB). *Lactobacillus* sp*.*, *Enterococcus* sp*.* and *Bifidobacterium* spp*.* have been reported to be present in fermentative processes. LAB are known to produce bacteriocins which affect other microorganisms. Bacteriocins are proteins with bactericidal activity directed against many Gram-positive bacteria, including *Clostridium*. These compounds are frequently found as secondary metabolites produced by LAB and genus *Bacillus*, among others (Noike *et al*., 2002; Godic and Bogovic, 2003; Noike *et al*., 2005). Furthermore, lactic acid pathway is by itself an electron sink that avoids hydrogen production (Madigan *et al*., 1997). In order to eliminate the development of hydrogen consuming microorganisms, such as methanogenic archaea and LAB, thermal shock pre-treatment can be used (Noike *et al*., 2002; Valdez-Vazquez and Poggi-Varaldo, 2009). In continuous processes, by using low retention times organic overloading is performed and inhibition of hydrogen-consuming methanogenic archaea by low pH occurs. However this procedure did not seem to affect lactic generating bacteria. Other researches have also employed organic overloading but no lactic acid was reported (Valdez-Vazquez *et al*., 2005). Overall, our results clearly suggest that fermentation underwent a lactic acid deviation, favored by a low pH and the inexistence of a specific inhibition pre-treatment for LAB.

Methanogenic Stage of the 2S-SSAD. Results in this stage were comparable to other works on solid substrate and submerged digestion (Poggi-Varaldo *et al.,* 2005; Gomez *et al.,* 2006; Mohan *et al.,* 2008). As inocula came from mesophilic habitats it was speculated that mesophilic regime was to be superior (Poggi-Varaldo *et al*., 1997). However, thermophilic methanogenic productivity was significantly superior to mesophilic as shown in Table 2. ANOVA of methane productivity showed that the effects of thermal regime and fed origin (according to its MRT in first stage) were significant ($p < 0.05$). The highest methane productivity

in mesophilic regime was observed in bioreactors fed with fermented solids from H stage at 10 d MRT (Table 2).

TABLE 2. Performance of methanogenic stage by its effects.

		Methanogenic stage				
		I_{CH4} [a] (50)[b]	Y_{CH4} [c] (27)[b]	VOA [d] (453)[b]	η_{SV} [e] (12.5)	Ē[f]
MRT [g]	10H+28M [h]	267	153	2479	67.0	1239
	14H+28M [i]	248	180	3055	65.6	1413
	21H+28 M[j]	129	93	3556	65.4	789
Thermal regime	Mesophilic	157	111	4282	66.1	613
	Thermophilic	272	173	1778	65.8	1680

Notes: [a]Methanogenic productivity (NmL_{CH4}/kg_r d); [b]standard error of the experimental design (EED = $(MSS_{error}/r)^{0.5}$) Montgomery (1991); [c]Methanogenic yield (NmL_{CH4}/g VSrem); [d]volatile organic acids accumulation ($mgCOD_{VOA}/kg_r$); [e]volatile solids removal efficiency(%); [f]specific energetic potential (J/g_{db}); [g]mass retention time (d); [h]for H stage working at 10d MRT, for M stage working at 28d MRT fed with fermented solids from H stage at 10d MRT; [i]for H stage working at 14d MRT, for M stage working at 28d MRT fed with fermented solids from H stage at 14d MRT; [j]for H stage working at 21d MRT, for M stage working at 28d MRT fed with fermented solids from H stage at 21d MRT.

As solids from higher MRT were fed into the methanogenic digesters, methane productivity diminished. Specifically in thermophilic regime there was a maximum productivity with solids from the intermediate 14 d MRT. In fact, the maximum methane productivity overall digesters was 341 $NmLCH_4/kg_r/d$ and belonged to this thermophilic bioreactor (data not shown).

In both thermal regimes it was observed an apparent solvent removal (concentrations were below the limit of detection) and total consumption of lactic acid. In mesophilic regime acetic acid was kept in the same concentration as in hydrogenogenesis stage, whereas in thermophilic methanogenesis its concentration diminished considerably. An apparent consumption of propionic and butyric acid was also noted. Overall, apparent acids consumption was around 80% for thermophilic regime, whereas mesophilic regime just had 4-50%, indicating a higher metabolic activity correlated very to better results. Mesophilic metabolism appeared to address solvent consumption and organic acids production, whereas thermophilic consumed both organic acids and solvents.

Equivalent methanogenic controls were run. Comparing the 2S-SSAD to the controls we found out that methanogenesis was improved in 2S-SSAD. Controls in thermophilic regime had a better performance than those in mesophilic, a similar behavior of the methanogenesis in 2S-SSAD. Methanogenic productivity, methanogenic yield, and VOA apparent production in methanogenic controls diminished as MRT increased for both thermal regimes. This indicated that biological activity was slowed down due to a minor organic load in higher MRT units.

We performed an energetic potential analysis according to Escamilla-Alvarado (2009) for a deeper insight (Figure 3). Total hydrogen and methane produced during operation were converted into energy values (according to its enthalpy of combustion) and then they were summed to obtain the total energetic potential.

This model assumes that the mass flow of organic loading is constant through the first stage and second stage, meaning that all the matter coming out from the first stage goes completely and unchanged into the second stage. Results show that the energetic potential from H-M grouped in thermophilic and mesophilic processes were 61 and 21% higher, respectively, than the corresponding to equivalent methanogenic controls. The 2S-SSAD thermophilic series had higher energetic potential than mesophilic ones (averaging 1680 and 613 $kJ/kg_{dry\ substrate}$ respectively, Figure 3). The highest contribution to the energetic potentials corresponded to the methanogenic stage both in mesophilic and thermophilic operation. On average, methanogenesis accounted for *ca.* 95% of the overall energetic potential. Because the 2S-SSAD yielded higher energetic potentials when compared to a sole methanogenic process operated at the same equivalent retention time, we believe that separation of acidogenic stage (represented here by the hydrogenogenic stage) in the anaerobic digestion process could have had a positive effect over the total energy harvested from substrate. Although in this work hydrogenesis contributed the least to the total energetic potential, it has been demonstrated its positive input (Ueno *et al*., 2007, Xie *et al*., 2008). Surely, an improvement in the hydrogen production of this process (as previously shown in other works, Valdez-Vazquez and Poggi-Varaldo, 2009) will translate into a more attractive and effective 2S-SSAD process.

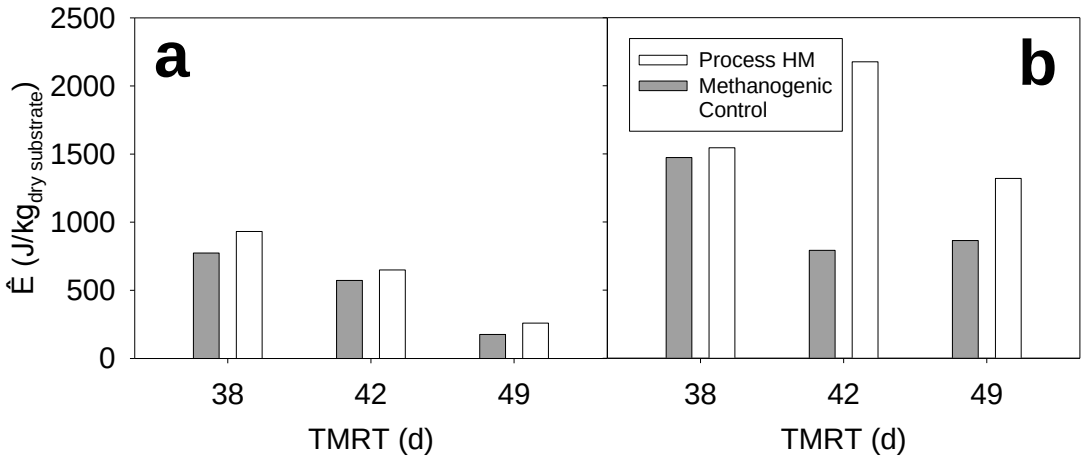

FIGURE 3. Specific energetic potential of hydrogenogenic-methanogenic process (H-M) and control processes. (a) mesophilic regime, (b) thermophilic regime.

Holocellulases Production. The spent solids proved to be a good cellulases and xylanases inducer. According to Figure 4 (a, c), maximum enzymatic activities in all substrates were reached at 48 h; at 65 h enzymatic activities decreased in all cases.

When *C. flavigena* was grown on spent solids the best xylanolytic and cellulolytic activities achieved were 5.00 (± 0.16) and 0.98 (± 0.04) IU/ml respectively. With OFMSW as control, activities of 6.69 (± 0.40) xylanases and

1.19 (± 0.04) IU/ml cellulases were obtained, which represented increments of 34 and 22%, respectively, over the values of those obtained for spent solids. No significant difference was found between enzyme activities from spent solids and the mixture spent solids-OFMSW. Due to the content of OFMSW (as a non-degraded material) in this mixture, higher activities were expected than just using only spent solids; instead, activities remained of the same order.

It may be speculated that although spent solids had 40% less cellulose than OFMSW, enzyme activity was not affected proportionally probably because the 2S-SSAD acted as a pretreatment, thus enhancing the accessibility to cellulose and hemicellulose bonds. Furthermore, it is also suggested that spent solids still contained a substantial amount of degradable cellulose and hemicellulose, in spite of their consumption during the 2S-SSAD. This is more evident in Figure 4 (b, d), where values of enzyme activities at 48 h incubation expressed on the basis of grams of initial dry holocellulose are showed, since it is also common to report activity results on initial mass of dry substrate basis (Senthilkumar *et al*., 2005).

Interestingly, results of enzyme production per initial holocellulose content using spent solids displayed the highest activities for both xylanolytic and cellulolytic, nearly 40% higher than the activities obtained with 100% OFMSW. Moreover, activity values of xylanase obtained in this work (*ca.* 2 562 IU/g initial holocellulose) were superior to those reported in an optimization experiment of xylanase production by solid substrate fermentation of wheat bran using the fungus *Aspergillus fischeri* Fxn1 (Senthilkumar *et al*., 2005).

Indeed, they found an optimized (maximum) activity of 1 024 IU/g substrate, whereas their background values were as low as 392 IU/g substrate.

The results with spent solids on volumetric basis were comparable to those reported using the parent wild strain *C. flavigena* CDBB-531 (Microbial Collection of the Biotechnology and Bioengineering Department, CINVESTAV Mexico) when grown on sugar cane bagasse (Ponce-Noyola and de la Torre, 1995): our xylanolytic activities were 2 fold higher while cellulolytic activity was similar. This strain was further mutated using N-methyl-N-nitro-N-nitrosoguanidine with the objective of enhancing its enzyme production capacity. Thus, *C. flavigena* PN-120 was obtained (Ponce-Noyola and de la Torre, 1995); later it was mutated into *C. flavigena* PR-22, a hyperproductive and substrate tolerant strain (Rojas-Rejón *et al*., 2007). Using sugar cane bagasse as substrate for *C. flavigena* PR-22 had spent solids in disadvantage. Sugar cane bagasse is a material with high cellulosic content (> 55% w/w cellulose) and it has been reported to be a very good xylanase and cellulase inducer Pérez-Avalos *et al*., 1996). Rojas-Rejón *et al.* (2007) grew *C. flavigena* PR-22 on sugar cane bagasse and reported maximum xylanolytic and cellulolytic activities of 21.7 and 1.63 IU/ml respectively. However we must consider that spent solids and OFMSW used in our work were not the best model substrates. For instance, the cellulose content of OFMSW was inferior to that of bagasse. Spent solids came from a previous biodegradative process (methanogenesis) and are in fact the digestates obtained from anaerobic digestion of OFMSW; thus the contents of cellulose and other degradable organic substances are lower in spent solids than in both OFMSW and bagasse. Therefore a lower enzymatic activity in both substrates compared to sugar cane bagasse could be expected. As for possible inhibitors present in spent solids, this possibility

could be discarded due to the fact that enzymes were obtained. If not used for enzyme production, the most common fate of spent solids would be as soil amenders (Poggi-Varaldo *et al.,* 1999).

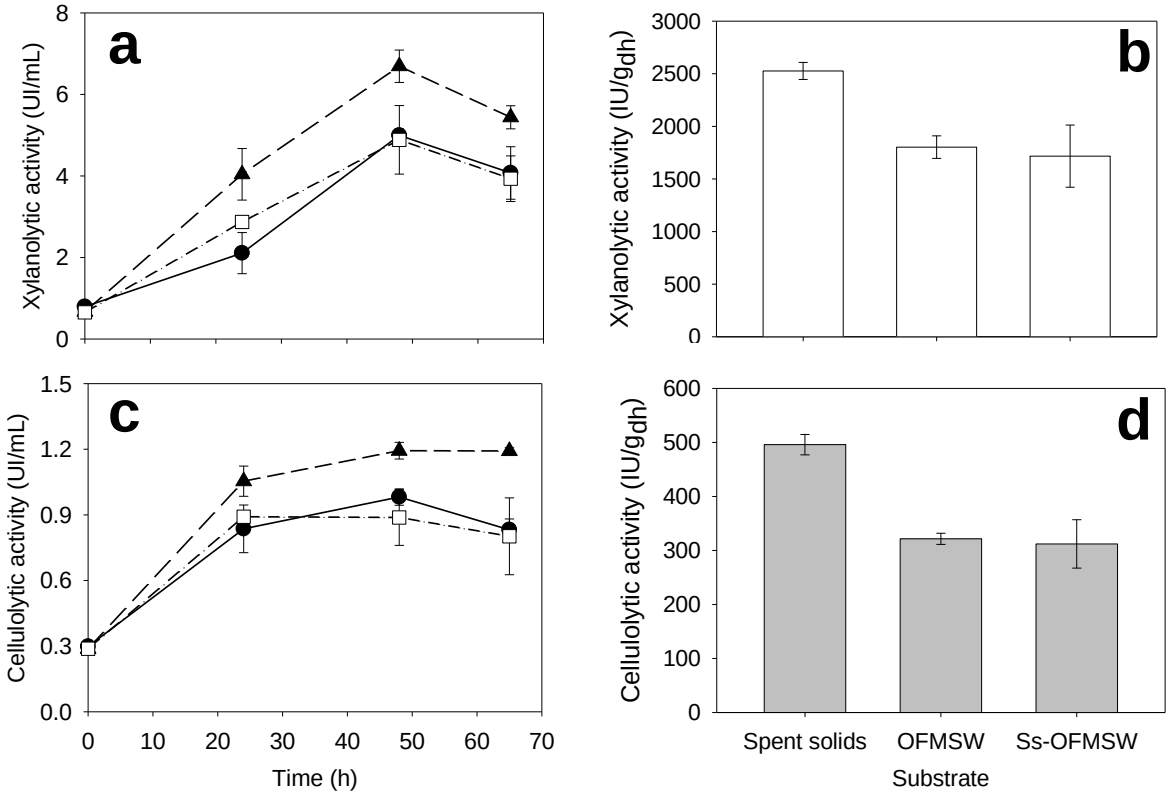

FIGURE 4. **Time courses of volumetric enzyme activities (a,c) and enzymatic activities at 48 h incubation based on initial holocellulose mass (b,d).** Symbols: (•) 100% spent solids, (□) 50-50% w/w organic fraction of municipal solid waste-spent solids, (▲) 100% organic fraction of municipal solid waste. Substrates (1) 100% spent solids; (2) 50% spent solids plus 50% w/w organic fraction of municipal solid waste; (3) 100% organic fraction of municipal solid waste.

Optimization studies on xylanase production working in submerged fermentation can yield better results. *Bacillus circulans* (Bocchini *et al*., 2002) and *Aspergillus fischeri* (Raj and Chandra, 1995) reported values of 22.45 IU/ml and 40.0 IU/ml, respectively. Overall, xylanase activity achieved with *C. flavigena* PR-22 and spent solids in our work fell in the middle of the range for xylanase values reported for a variety of bacteria and fungi in submerged fermentation mode (Raj and Chandra, 1995; Gessesse and Mamo, 1999; Bocchini *et al*., 2002).

As stated previously, comparisons of our results with those of literature should be made with caution. Several results from literature come from optimization studies whereas our experiments did not attempt any optimization but to prove the feasibility of using the spent solids from the 2S-SSAD to obtain an added-value product.

Conclusion

Hydrogen productivity in mesophilic bioreactors almost doubled that in thermophilic ones. Moreover, the effect of MRT of the hydrogenogenic stage was not significant. However, unexpectedly the hydrogen yields in all bioreactors were generally low.

As for the second stage, the methanogenic productivity was significantly superior in thermophilic regime. Integration of enzyme production as an additional stage to the 2S-SSAD is possible, attractive and properly fits in the *biorefinery* concept, towards which environmental and process biotechnology is focusing. Therefore this work presents a promising and original alternative in the biorefinery research. Thus, we believe that scale-up and enhancing the operating conditions should be further researched in order to evaluate more properly the full potential of this technology. Analysis tools such as life cycle assessment are strongly suggested to evaluate its real feasibility and for comparison reasons.

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References (incomplete, only a few examples of different type are shown)

Baghchehsaraee, B.; Nakhla, G.; Karamanev, D.; Margaritisk, A. (2009). Effect of extrinsic lactic acid on fermentative hydrogen production. *International Journal of Hydrogen Energy.* 34: 2473-2579.

Bocchini, D.A.; Alves-Prado, H.F.; Baida, L.C.; Roberto, I.C.; Gomes, E.; Silva, R.D. (2002). Optimization of xylanase production by *Bacillus circulans* D1 in submerged fermentation using response surface methodology. *Process Biochemistry.* 38: 727-731.

Das, D.; Veziroglu, T.N. (2001). Hydrogen production by biological processes: a survey of literature. *International Journal of Hydrogen Energy.* 26: 13-28.

Escamilla-Alvarado, C.; Ponce-Noyola, M.T.; Poggi-Varaldo, H.M. (2010). Energetic potential of two-stage hydrogenesis-methanogenesis fermentation of the organic fraction of municipal solid waste. *Proceedings of the 14th International Biotechnology Symposium*, Rimini, Italy, 2010.

INEGI (2009). Statistical yearbook of the United States of Mexico (in Spanish). National Geography Institute, Mexico.

Montgomery, D.C. (1991). *Design and analysis of experiments*. 3rd ed., John Wiley and Sons, NY.

Reith, J.H.; Wijffels, R.H.; Barten, H. (2003). *Bio-methane and bio-hydrogen. Status and perspectives of biological methane and hydrogen production*. The Hague, Holland.

Robledo-Narváez, P.; Escamilla-Alvarado, C.; Ponce-Noyola, M.T.; Poggi-Varaldo, H.M. (2008). Biohydrogen from wastes and combination of biological processes for energy yield improvement: a review. *In:* Sastre-Conde, I.; Macarie, H. (Eds.). *Book of Abstracts of the the Third International Meeting in Environmental Biotechnology Engineering*. pp. 130, Palma de Mallorca, Spain.

SEMARNAT (2011). www.semarnat.gob.mx/residuos_solidos/1424.html; accessed 22 January 2011.

Notation

ASSAD	acidogenic solid substrate anaerobic digestion
H-M	series process, hydrogen fermentation followed by a methanogenic stage
MRT	mass retention time
OFMSW	organic fraction of the municipal solid waste

Greek characters

α	parameter alpha, i.e., the ratio between intermediate alkalinity to partial alkalinity
η	removal efficiency of volatile solids

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