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Evaluation of Pretreatment of Intermediate Solids from an *H-M-Z-S* Biorefinery on Biological Hydrogen Production and Saccharification

Leticia Romero-Cedillo (DNN, CINVESTAV, Mexico City, Mexico)
M. Teresa Ponce-Noyola, ***Héctor M. Poggi-Varaldo*** (r4cepe@yahoo.com), and Perla Sotelo-Navarro (CINVESTAV, Mexico City, Mexico)

ABSTRACT: The aim of this work was to evaluate the effect of pretreatment of intermediate solids (waste solids after the enzyme production stage *Z*, X_Z in a biorefinery processing the organic fraction of municipal solid wastes, *OFMSW)* on a second round of bio-H_2 production by dark fermentation and sugar production through enzymatic saccharification. The evaluated pretreatments were: dilute acid with H_2SO_4 1.5% (v/v), NaOH 1M, and Na_2CO_3 1M using a relation of 1:10 (w/v) and constant heating at 60 °C for periods of 1h. Solids treated with alkaline method NaOH 1M exhibited the highest cellulose availability (38.53%) compared to the unpretreated solids (30.51%) whereas lignin content decreased from 16.7% to 7.41%. Due to the above results, pretreatment with NaOH 1M was selected to conditioning the X_Z solids for further studies in biorefinery *H-M-Z-S.* In biological hydrogen production, the order of cumulative H_2 production was 100%*OFMSW* > 60% *OFMSW*/40% *Pr* X_z > 100% X_z > 60% *OFMSW*/40% *UPr* X_z > 100% *Pr* X_z. The highest cumulative yield was 2.2 mmolH_2/gTS using *OFMSW* as a substrate. In contrast, the lowest value 0.087 mmolH_2/gTS corresponded to *Pr* solids.

In the saccharification stage, the highest yield of reducing sugars (0.067 g sugar/g holocellulose) was observed in the tests that were loaded with 5 IU/mL of enzyme and 1% of *Pr* X_Z. The reducing sugars and bio-H_2 yields were generally very low indicating that the pretreated X_Z were not suited for further reuse in the biorefinery. It is likely that the presence of inhibitors released after the pretreatment, such as phenolic compounds from lignin degradation, could be linked to these results.

Keywords: Biohydrogen, biorefinery, intermediate solid stream, *OFMSW,* pretreatment, saccharification

INTRODUCTION

Recently, research of biofuels and renewable energy has gained more attention in the context of exploring a diversity of substrates and biomass composition, likely organic wastes. In this context, research on improving lignocellulosic residues accessibility through pretreatment and to increase their degradation has increased significantly in the last several years. It should be noted that the pretreatment of a great variety of substrates has been studied such as food wastes (Kim et al., 2009; Jang et al., 2015), vegetable wastes (Jia et al., 2014), sewage sludge (Jung et al., 2012), pulp and paper sludge (Yunqin et al., 2009), corn stover (Zhu et al., 2010), apple pomace (Wang et al., 2010), rice straw and spruce chips (Teghammar et al., 2012).

Bio-hydrogen is an attractive alternative to substitute fossil energies because of its high energy content. In addition, it does not generate any toxic byproducts in their combustion of fuel cell use. However, conventional technologies to generate H_2 are either costly and/or based on fossil

To increase the bio-hydrogen from biomass and wastes, efficiency of hydrolysis or degradation should increase. Lignocellulosic biomass must be conditioned so as to remove lignin (Lissens et al., 2004; Datar et al., 2007).

In former experiments reported in the open literature, carbohydrates such as glucose and sucrose were used as the main source of bio-H_2 and other bioenergy production. Yet, actual organic wastes such as *OFMSW,* are commonly used for economic reasons

(Robledo-Narváez et al., 2013, Escamilla-Alvarado et al., 2012). The use of these wastes is attractive for reducing pollution load and costs, through their integration into a biorefinery process option (Jang et al., 2015).

Dark fermentation still faces its own challenges, namely, the chemical complexity of feedstocks that could lead to poor biodegradability, among others. This drawback can be improved by the pretreatment of the organic wastes (Romero-Cedillo et al., 2016). For instance, Kang et al. (2012) reported a high solubilization of sewage sludge (85%) after the ammonia pretreatment, showing the best hydrogen yield at 175 mg/L of ammonia concentrations.

The pretreatment of lignocellulosic wastes alters the structure of cellulose and removes the lignin, allowing an increase of cellulose conversion into single sugars (Weimer et al., 1985). The removal of the lignin may be possible through physical, chemical or biological treatments or combination of methods. Acid or alkaline hydrolysis are the most typical. The chemical pretreatment used (Ogeda et al., 2010). Lignocellulosic is attacked by several enzymes. For instance, in filamentous fungi the cellulase complex consists of three major enzymes, an endo-1-4- β-glucanase (EC 3.2.1.4), a 1,4-β- D-cellobiohydrolase (EC 3.2.1.91), and a 1,4-β-glucosidase (EC 3.2.1.21), which act simultaneously in the various links of the polymer (Xu et al., 2010). In other research about saccharification of cornstalk, Zhao et al. (2013) found that 81.2% of substrate was converted by enzymatic saccharification process. The efficient conversion was obtained using 38.5 g/L of substrate and 35.7 IU/g for cellulases, at pH 5 and 49.7 °C; after saccharification, they used the liquor as substrate to dark fermentation and hydrogen production using *Thermoanabacterium thermosaccharolyticum* and resulted in 90.6 mLH_2/g cornstalk hydrolysate. (Zhao *et* al. 2013)

In previous works, Escamilla-Alvarado *et* al. (2013) evaluated the holocellulases production from fermented solids wastes derived from hydrogenogenic stage in the *H-M-Z-S* biorefinery using *Trichoderma reesei* MCG-80. They found higher cellulose activity when used fermented solids as substrate, compared to the pure cellulose as substrate (Solka floc) 2140 *FPU*/L and 2100 *FPU*/L, respectively. Their results were attributed because in dark fermentation a partial degradation of the substrate is caused due the microbial activity causing hydrolysis of the polymers and alter the crystallinity of cellulose (Escamilla-Alvarado et al. 2013). In order to increase the attention of bio-products, the pretreatment of solid wastes in biorrefinery could allow an increase in biohydrogen production trough dark fermentation and improve the saccharification process by using this pretreated waste as a substrate.

Figure 1 depicts a general biorefinery diagram which consists of four main stages: *H* hydrogen production from *OFMSW* (organic fraction of municipal solid wastes), *M* methane production from fermented organic wastes (X_h), *Z*, holocellulases production from X_h, and *S*, saccharification process using holocellulases and pretreated solids X_z (*Pr* X_z). In our work will be pay attention to the stream of solids that exit the *Z* stage, i.e., X_z.

The objectives of this work were to evaluate *(i)* the effect of chemical pretreatments (alkaline and dilute acid) on removal of lignin from the solid stream X_z, derived from the enzume production of an *OFMSW* biorefinery; *(ii)* the effect of using these pretreated solids in dark fermentation for further bio-H_2 generation; and *(iii)* to determine the effect of using the pretreated solids for obtaining saccharified liquors that could be fed to additional bioprocesses in the biorefinery.

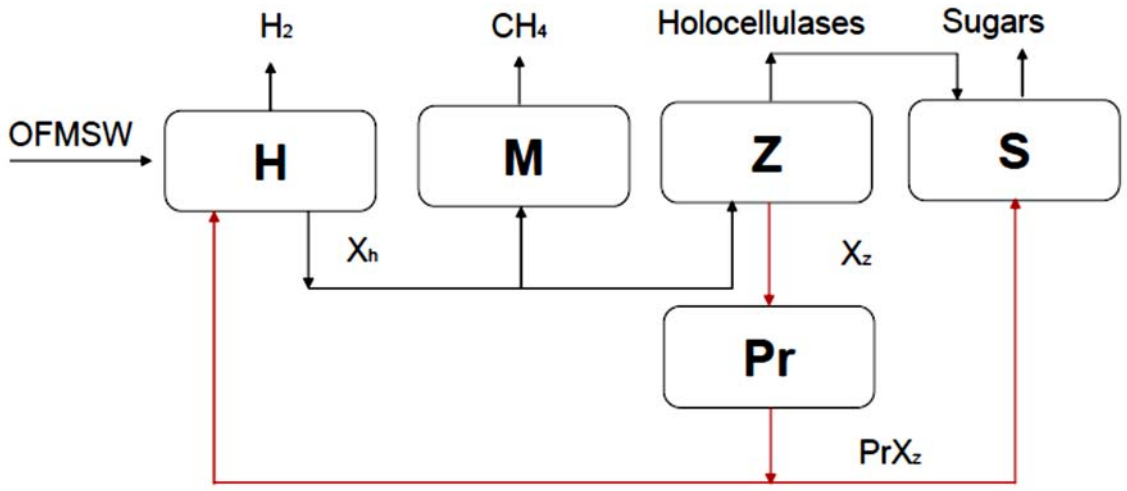

FIGURE 1. Diagram of the Biorefinery *HMZS, H*: Hydrogen production from the *OFMSW, M*: methane production, *Z*: enzymatic process using holocellulases and fermented organic wastes (X_h) derived from *H* stage; *Pr* pretreatment stage; and *S*, sacharification of *Pr* X_z (pretreated X_z solids).

MATERIALS AND METHODS

Holocellulases production and recovery of intermediate solids X_z. *Trichoderma reesei* MCG-80 was used for the production of holocellulases. The strain was spread from 200µL of samples with spore concentration $1*10^6$ spores/mL, this volume was poured on agar plates supplemented with *PDA* (Potato dextrose agar) and incubated at 30 °C for 5 days until a radial growth was observed.

For inoculation in flasks, 3 cm^2 of mycelial growth were sampled using a sterile Pasteur pipette and then added to 500 mL Erlenmeyer flasks containing 150 mL of Mandels mineral medium and fermented organic wastes (*Xh*) (3% w/v). The complete composition of the medium (g/L) was: Urea 0.3, $(NH_4)_2SO_4$ 1.4, KH_2PO_4 (2), $CaCl_2{\cdot}2H_2O$ (0.3), $MgSO_4{\cdot}7H_2O$ (0.3), $FeSO_4{\cdot}7H_2O$ (0.005), $MnSO_4{\cdot}4H_2O$ (0.001), $ZnSO_4{\cdot}7H_2O$ (0.001), $CoCl_2$ (0.002), Peptone (1) and Tween-80 (1).

At the end of this period, new mineral medium (1L) was inoculated using 100 mL from the previous growth culture. Once inoculated, the Erlenmeyer flasks were incubated at 30 °C during five days at 150 rpm. The cellulolytic activity was monitored by quantification of reducing sugars according to Miller method (1959) and then reported as paper filter units per unit volume (*FPU*/mL). After the holocellulases production, the remaining biomass was recovered by centrifugation at 7,000 rpm for 20 min; the enzymes present in the supernatant were concentrated by ultrafiltration using a 40 kDa membrane. The biomass was washed twice and then pretreated by further use as substrate in saccharification process.

Pretreatment of intermediate solids X_z. The target material were solids derived from Z stage of our biorefinery. They were recovered at the end of the kinetics with *Trichoderma reesei* MCG-80 (the operational conditions were according Escamilla *et* al. 2013). Recovered X_z were subjected to alkaline and dilute acid pretreatment. The pretreatments consisted in two separate alkali solutions NaOH 1M and Na_2CO_3 1M, for dilute acid we

used HCl 1.5% (%v/v). The solids were added to each of the three treatments at a one mass:volume ratio 1:10, heated for 1 h at 60 °C with constant stirring every 15 min.

At the end of pretreatment the samples were centrifuged at 7,000 rpm for 20 min, washed with distilled water and neutralized at pH of 7.0, then dried at 60°C for 24h until dehydration and subsequently sieved with a mesh No. 40. The solids were stored at room temperature until further use.

For cellulose determination, 1g of pretreated sample (previously dried) was used. It was placed in a flat-bottomed flask and then 15 mL of acetic acid (80%) and 1.5 mL of nitric acid were added. The samples were refluxed for 20 min and then the mixture was filtered (Gooch filters) and washed with concentrated ethanol. The samples were dried for 2 h at 105 °C until constant weight was obtained. Dry samples were incinerated at 550 °C.

Biohydrogen production from pretreated intermediate solids X_z. The biohydrogen production was conducted in serum bottles containing either different concentrations of *OFMSW* (organic fraction of municipal solid wastes), ´or pretreated residual solids from enzyme production X_z pretreated previously by alkaline method using NaOH 1M and unpretreated X_z solids. The total operational mass consisted of 40 g (wet weight) for each treatment and their respective controls. The relation of substrate and inoculum was 80% and 20% respectively; the inoculum was previously inhibited by heat shocked pretreatment at 95 °C for 1h to avoid methanogenic activity. Once inoculated, the head spaced in reactors were gassed using nitrogen to maintain anoxic environment. The reactors were incubated at 35 °C for a period of 38 days.

In Table 1, shows the composition of experimental units for each treatment used in the biological hydrogen production.

TABLE 1. Feeds to dark fermentation bioreactors using different substrates and proportions. Pretreatment of X_z was NaOH 1 M.

Bioreactor	Composition
T1	100% *OFMSW*
T2	100% *Pr* X_z
T3	100% *UPr* X_z
T4	40%*Pr* X_z + 60% *OFMSW*
T5	*40% UPr* X_z *+ 60% OFMSW*

Notes: X_z, Residual solids from cellulase production; *Pr* X_z: pretreated intermediate solids; *UPr* X_z: unpretreated intermediate solids.

The hydrogen production was measured daily until the stationary phase was presented, H_2 concentration in biogas was determined by gas chromatography using a GOW-MAC 350 chromatograph equipped with a thermal conductivity detector. The temperature of injector, column and detector were 25, 25 and 100 °C respectively and argon was the carrier gas. A packed silica gel column of diameter 60/80 was used. Once the stationary phase was observed and there was not a substantial increase in hydrogen production, the reactors were subjected to headspace flushing with inert N_2 and reincubated for a new cycle of bio-H_2 production (no substrate and no inoculum were added). The intermittent venting was performed for 2 minutes and a water seal was used to avoid the release of gas in reactors and maintain the system closed.

Saccharification process using pretreated intermediate solids X_z as substrate. The reaction was carried out in 120 mL serum bottles with a working volume of 20 mL

consisted in a phosphate buffer citrates 0.05M at pH 4.8. The bottles were maintained at 50 °C, 150 rpm for 48 h. Every 24 h, 1mL of sample was taken and quantification of reducing sugars were analyzed according Miller method (1959). The experimental design consisted in a factorial design 3^2, where the factors were enzyme and substrate concentrations. The levels for enzyme concentration were: 3 *FPU,* 5 *FPU* and 7 *FPU* (IU/mL), whereas for substrate concentration the levels were: 1%, 3% and 5% (%w/v).

RESULTS AND DISCUSSION

Holocellulases production and recovery of intermediate solids X_z. The results obtained from holocellulases production showed the highest specific activity of cellulases at 72 h (0.56 IU/mg protein or 0.52 IU/mL) (Figure 2). Our results were lower than those reported in previous research using *T. reesei* MCG-80 and *FOW* (fermented organic wastes) as substrate (2.14 IU/mL cellulose activity, Escamilla-Alvarado *et* al. 2013). This could be due to differences in substrates (our X_z is more degraded than X_h), higher agitation rate (600 rpm) and process carried out in controlled fermenters in the experiments of Escamilla-Alvarado et al. (2013). Regarding the xylanolytic activity, the values obtained in the present work using *T. reesei* MCG-80 were very close to results previously reported for *Cellulomonas flavigena* PR-22, (5.3 IU/mL and 8 IU/mL, respectively) (Escamilla-Alvarado *et* al. 2013).

It has also been observed that xylo-oligomers derived from xylan degradation played a crucial role in cellulase inhibition, and xylanases could reduce this inhibition by increasing the hydrolysis of xylo-oligomers to xylose (Kumar *et* al., 2009). The hydrolysis of lignocellulosic biomass can be significantly enhanced by addition of cellulase enzyme complexes with xylanases (Berlin et al., 2005). This could be ascribed to the partial removal of the hemicellulose in the cell wall and further increased access to the enzymes, resulting in greater glucose yields.

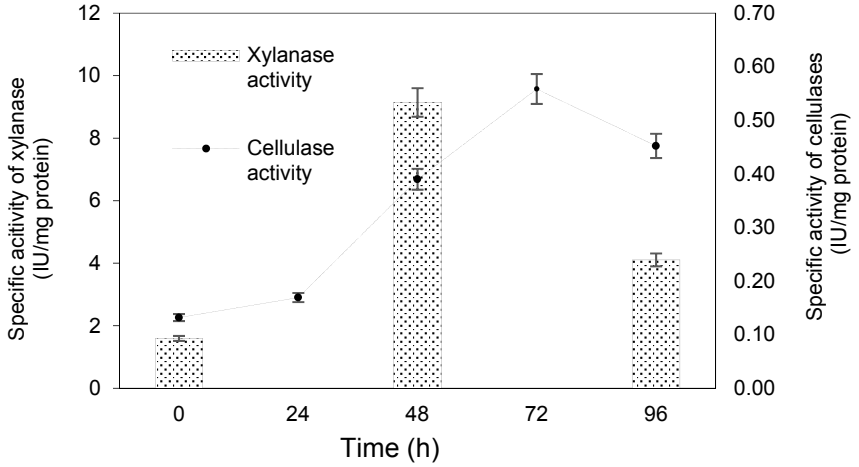

FIGURE 2. Specific activity of cellulases and xylanases from *T. reesei* MCG-80 grown in X_h solids (fermented organic wastes).

Piovesan *et* al. (2013) found a significant xylanolytic activity (60 IU/mL) in experiments using delignified steam-explosion pretreated sugar cane bagasse and the strain *Penicillium echinulatum*. Regarding this, within the context of the *H-M-Z-S* biorefinery, the

pretreatment of several solid streams could improve the holocellulases production as long as there is still a significant cellulose content in the substrate, greater than 80%. After the holocellulases production, the enzymatic complex was concentrated by ultrafiltration for further use.

Pretreatment of intermediate solids X_z. Favorable results were observed in samples pretreated by alkaline solutions; increases in the cellulose availability respect to the control (*UPr* X_z) were obtained. In figure 3, it can be appreciated a remarkable increase in cellulose were samples pretreated by NaOH 1M (from 29.75 up to 39.7%, Figure 3), represented almost a 30% improvement. In this case. Second place was for the Na_2CO_3 1M pretreatment, and last the dilute acid treatment (actually, there was a loss of cellulose in the latter, Figure 3).

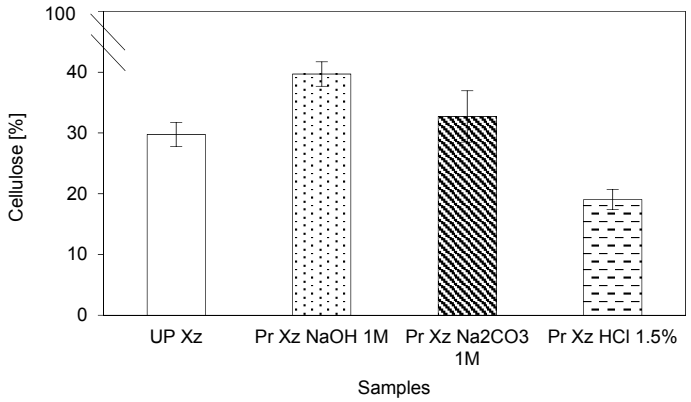

FIGURE 3. Cellulose content in X_z solids subjected to alkali or dilute acid pretreatment.

Keys: *UP* X_z, Unpretreated X_z, *Pr* X_z NaOH 1M, *Pr* X_z Na_2CO_3 1M and *Pr* X_z 1.5% HCl.

Most alkaline pretreatments are effective in removing lignin, unlike acid treatments in which hemicellulose is removed and thus lignin remains intact and apparently concentrated. Figure 4 shows the effect of pretreatments on lignin content. The reduction of lignin is associated to improvement of cellulose degradation in fermentation processes (Tarkow and Feist, 1969; Gaspar et al., 2007; Karp et al., 2015; Xiao et al., 2015) likely caused by the increase of cellulose availability for cellulases attack and further degradation. The alkyl aryl bonds are broken under alkaline conditions at pH greater than 10 (Park et al., 2012); this, in turn, promotes the increase in surface area and the reduction of polymer stability. Crystalline structure deteriorates and breakdown of linkages between lignin and carbohydrates occur, leading to a severe disruption of the lignin structure (Mosier et al., 2005).

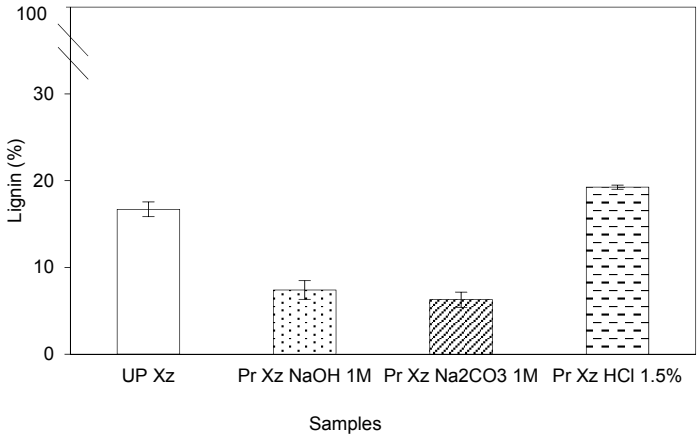

FIGURE 4. Lignin content in X_z samples subjected to alkali or dilute acid pretreatment.

Keys: *UPr* X_z, Unpretreated X_z, *Pr* X_z NaOH 1M, *Pr* X_z Na_2CO_3 1M and *Pr* X_z 1.5% HCl.

It can be seen that samples pretreated by dilute acid method with HCl 1.5%, did not show any improvement of cellulose content (Table 2). On the contrary, when used alkaline treatment NaOH 1M the results shown an increase in cellulose (up to 38.53% from 30.51% in the *UP* X_z), and lmost the cellulose content in samples of *OFMSW* (39.63%) that were not subjected to any previous degradation process (cellulose-consuming).

TABLE 2. Characterization of different substrates derived from biorefinery stages.

Parameter (% db)	*OFMSW* [a]	X_h [b]	*UPr* X_z [c]	*Pr* X_z NaOH [d]	*Pr* X_z Na_2CO_3 [e]	*Pr* X_z HCl [f]
Holocellulose	74.52±0.78	69.84±0.66	60.19±0.89	60.47±0.41	48.86±0.98	62.94±0.78
Cellulose	39.69±0.84	46.13±0.54	30.51±1.12	38.53±0.89	35.01±0.46	19.75±0.51
Hemicellulose	19.21±0.37	6.41±0.35	6.57±0.43	ND	ND	25.39±0.35
Lignin	9.85±0.25	12.86±0.67	16.70±0.84	7.41±1.06	6.29±0.87	19.25±0.24
Ashes	15.63±0.35	17.3±0.48	23.11±0.7	32.13±0.43	44.85±0.6	17.81±0.54
Volatile solids	71.36±0.56	72.55±0.34	61.76±0.25	55.00±0.36	51.22±0.43	94.34±0.57

Notes: [a] Organic fraction of municipal solid wastes; [b] Fermented organic wastes from H stage; [c] Unpretreated X_z; [d] Pretreated X_z by NaOH 1M; [e] Pretreated X_z by Na_2CO_3 1M; [f] Pretreated X_z by HCl 1.5%.

In Figure 5, *a* and *d images* show no change on surface texture of samples (*UPr* X_z versus dilute acid treated X_z). On the other hand, alkaline pretreated samples using NaOH 1M (image *b*) and sodium carbonate 1M (image *c*) showed regions of fiber rupture and amorphous areas. These amorphous regions are the result of the breakage of the links C-C and C-O that are present in the lignin (Grabber *et* al. 2004). This, in turn, releases of smaller organic molecules and greater access to the cellulose that now has an irregular structure (Zeng et al., 2014).

After the dilute acid pretreatment using HCl 1.5%, no breaking occurs in lignin polymer because there was only degradation of hemicellulose. The comparison between pretreatments by analyzing lignin removal, cellulose quantification and electronic microscopy, lead to the selection of 1 M NaOH pretreatment as the most appropriate in our work.

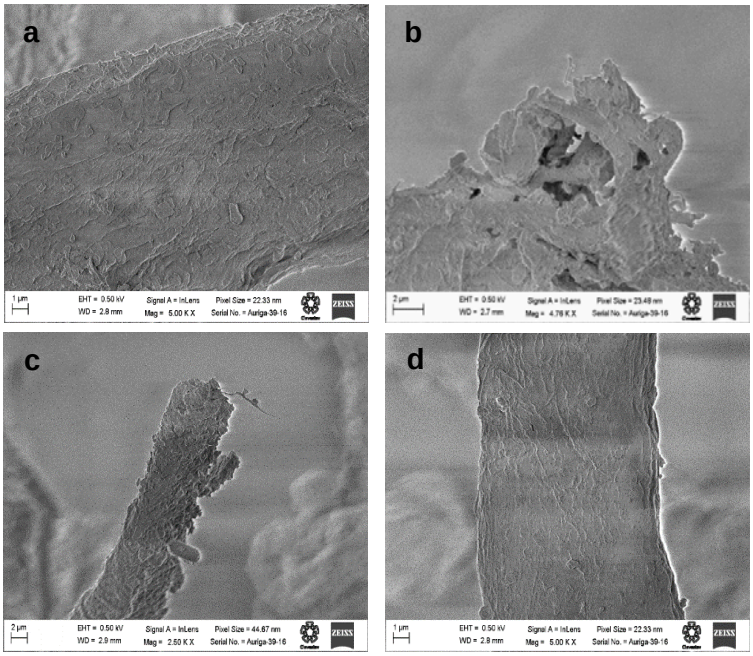

FIGURE 5. Scanning electron microcopy (*SEM)* before and after different pretreatments (a) *UPr* X_z; (b) *Pr* X_z by alkaline NaOH 1M; (c) *Pr* X_z by Na_2CO_3 1M; (d) *Pr* X_z by HCl 1.5%.

Biohydrogen production from pretreated intermediate solids X_z. Table 1 depicts the feed composition of each treatment in this experiment. When pretreatment was used, it corresponded to 1M NaOH. Table 3 shows the contents of solids of substrates for each treatment or experimental condition. *T1* (composed of 100% *OFMSW*) and *T4* (substrate containing 40% *Pr* X_z and 60% *OFMSW*) presented the highest content of volatile solids (61% and 60.2%, respectively) and a content of ashes of 38.9% and 39.8%, respectively.

The treatment *T2* that consisted of 100% of pretreated solids X_z, presented the lowest values regarding volatile solids (42.5%) and a large amount of ashes (57.48%). The above could be due to degradation/removal of some carbohydrates such starch and other organic molecules during the pretreatment. In our work, the composition and nature in the substrates seemed to be determinant factors that affected the performance of reactors.

T1 and *T4* exhibited the highest H_2 yields (2.19 and 1.11 mmoLH_2/g*TS,* respectively, Figure 6 and Table 4) and highest H_2 productions. The lowest production of hydrogen was observed in *T2* whose feed consisted of 100% *Pr* X_z. This could be due to the presence of inhibitors generated after the pretreatment. For instance, Reginatto et al. (2015) obtained yields of 2.94 mmoL/H_2 g*TS* when used agroindustrial lignocellulosic wastes previously pretreated with alkali; their results were lower than those obtained by the same group when fermenting no pretreated feed (4.54 mmoL/H_2 g*TS*).

TABLE 3. Proximal analysis of feedstocks loaded to batch bioreactors (*T1*-*T5* in mesophilic conditions) for biological hydrogen production.

Parameter (%db [a])	*T1* [b]	*T2* [c]	*T3* [d]	*T4* [e]	*T5* [f]
Holocellulose	74.52±0.78	60.47±0.41	60.19±0.89	68.89±0.59	68.78±0.83
Cellulose	39.69±0.84	38.53±0.89	30.51±1.12	39.22±0.98	36.02±0.98
Hemicellulose	19.21±0.37	-	6.57±0.43	-	14.14±0.4
Lignin	9.85±0.25	7.41±1.06	16.70±0.84	8.874±0.54	12.59±0.54
VS	84.37± 1.36	67.87± 1.25	76.89± 1.27	77.77±1.26	81.37±1.2
TS	23.59±0.52	23.11± 0.23	23.35± 0.62	23.39±0.57	23.49±0.57
Ashes	15.63±0.35	32.13±0.43	23.11±0.7	22.23±0.52	18.62±0.52
pH	7.31±0.15	7.54±0.67	7.38±0.44	7.40±0.21	7.22±0.60

Notes: [a] on dry basis; [b]100% *OFMSW*; [c]100% *Pr* X_z by NaOH 1M; [d]100% *UPr* X_z; [e]40% *Pr* X_z + 60% *OFMSW* ; and [f]40% *UPr* X_z+ 60% *OFMSW*.

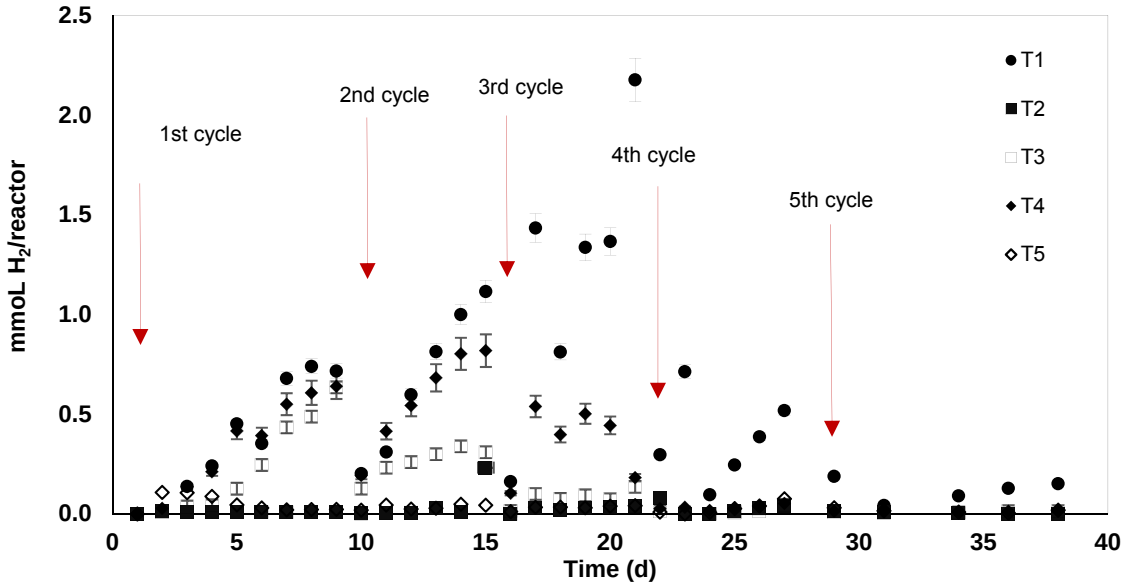

FIGURE 6. Time course of hydrogen production in batch reactors with intermittent venting and headspace flushing at each cycle.
Keys: (*T1*) 100% *OFMSW,* (*T2*) 100% *Pr* X_z by NaOH 1M, (*T3*) 100% *UPr* X_z, (*T4*) 40% *Pr* X_z by NaOH 1M + 60% *OFMSW* and (*T5*) 40% *UPr* X_z+ 60% *OFMSW*. The red vertical lines indicate flushing the bioreactor headspaces with gas N_2.

TABLE 4. Cumulative hydrogen production and yields obtained after 38 days of operation, corresponding to five treatments using different composition of substrate.

Treatment	Composition	ΣP_{H_2} (mmol H_2/reactor)	Y'_{H_2} (mmol H_2/g*TS*)
T1	*OFMSW* 100%	17.532	2.192
T2	*Pr* X_z 100%	0.699	0.087
T3	*UPr* X_z [c] 100%	4.209	0.526
T4	40% *Pr* X_z plus 60% *OFMSW*	8.882	1.110
T5	40% *UPr* X_z plus 60% *OFMSW*	1.093	0.137

Notes: (*T1*) 100% *OFMSW*; (*T2*) 100% *Pr* X_z by NaOH 1M; (*T3*) 100% *UPr* X_z; (*T4*) 40% *Pr* X_z by NaOH 1M + 60% *OFMSW*; (*T5*) 40% *UPr* X_z+ 60% *OFMSW*; (ΣP_{H2}) Bio-H_2 production in mmol/reactor; Y'_{H2} Bio-H_2 yield in mmol/g*TS*.

Saccharification process using *Pr* X_z as substrate. In the saccharification tests of *Pr* X_z the dosage of 5 IU/mL of enzyme effected the highest production of reducing sugars, even at lower concentrations of substrate (Table 4, Figure 6). For instance, the obtained concentrations of reducing sugars were 0.4, 0.9, and 1.31 g/L in experiments with initial concentrations of 1, 3, and 5% of pretreated X_z. These results suggest that there is a linear relationship between yield and initial substrate concentration. Yet, the influence of enzyme concentration is clearly non-linear; when the enzyme concentration was 7 IU/mL, there was a significant decrease of reducing sugars obtaining almost 50% less (0.81 g/L) (Figure 6).

Unexpected results at high enzyme dosages were also obtained by Escamilla-Alvarado *et al.* (2015). They reported 10 g/L of reducing sugars by the addition of 40 *FPU*/gVS, and when increased the amount of enzyme until 120 *FPU*/g*VS* the production of sugars had no significant increase (11.6 g/L), that is, a plateau or saturation level of the products.

A general comparison of our results with those from the open literature further confirm that our saccarification yields were very poor (Table 5). The highest amount of reducing sugars from *Pr*X_z was only 1.4 g/L using 3 IU/mL (*FPU*) for a yield of 4.52%. The highest yield was 6.72% with 5 IU/mL of enzyme and 1% *VS* substrate. In previous work, Escamilla-Alvarado *et* al. (2013) used holocellulases from *Trichoderma reesei* MCG 80 at concentration of 60 *FPU*/g and observed 13 g/L of reducing sugars (glucose and xylose) and a reducing sugar yield of 72.7% (on holocellulose basis), when using fermented solids (X_h, Figure 1). Regarding the conversion efficiency, in our study the most efficient conversion of the pretreated substrate was 6.72% (g sugar/g holocellulose $_{db}$) (Table 5). It should be noted that yields reported by Escamilla-Alvarado et al. (2013) were obtained using higher enzyme concentrations. Yet, this approach of increasing enzyme concentration in our work is not feasible since the reducing sugar yields plummeted for enzyme dosages of 7 IU/mL (Figure 6).

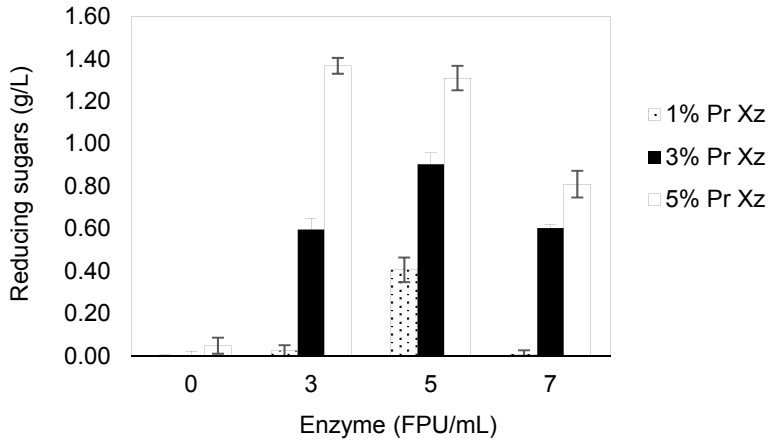

FIGURE 6. Evaluation of reducing sugars after saccharification using holocellulaes from *T. reesei* MCG-80 and pretreated solids X_z.

TABLE 5. Conversion efficiency of saccharification and chemical hydrolysis from different pretreatments and organic wastes.

Waste	Pretreatment	Enzyme	Substrate	Product	Y_{RS} [a] (%)	Reference
Cornstalk	None	35.7(IU/g)	40.1 (g/L)	36 g/L (glucose)	81.2	Zhao et al. 2013
Wheat straw	H_2SO_4 0.75% (v/v), 1h, 121ºC	Celluclast and Novozyme	7.83% (w/v),	565 mg sugar/g substrate	56.5%	Saha *et* al, 2005
Cassava bregs	Water/(Mmm) *DMP*/HCl, wt% 20/78.5/1.5; 130 ºC, 30 min	*CMCase* 52.8 U/mL *FPase* 26.2 U/mL	20 (g/L)	10.48 g/L (glucose)	52.4%	He *et* al. 2014
Rice straw	Microwave (360 W, 6 min) and alkali NaOH1% (w/v)	Cellulase from *Trichoderma viride* 5 U/g	12.5% (w/v) 61.3% Cellulose 9.7% Hemicellulose	56 g/L (reducing sugars)	NA	Badawi et al., 2012
OFMSW[c]	Steam, 160 ºC , 30 min (thermal active hygienization)	40-60 FPU[d]/g cellulose 20 FPU/g celllulose	20% (w/v) 10% (w/v)	36 g/L (reducing sugars) 30 g/L (reducing sugars)	NA	Ballesteros et al., 2010
Pinneaple solid waste	Microwave 8.5G/W, 4 min	Cellulase 1.13 U/mg Hemicellulase	NA[c]	47.67 g sugars/kg substrate	4.76%	Conesa *et* al., 2016
Orange peels	0.2 M acetic acid, pH=2.61, soaking in oven at 100 ºC for 1h	NApplic[b]	779 mg total carbohydrates/g substrate	694 mg total carbohydrates/g substrate	89.08%	Saha *et* al. 2016
Pinneaple waste	0.2 M acetic acid, pH=2.61, soaking in oven at 100 ºC for 1h	NApplic	752 mg total carbohydrates/g substrate	681 mg/ g substrate	90.55%	Saha *et* al. 2016
Grape	0.2 M acetic acid,	NApplic	674 mg total	622 mg/g	92.28%	Saha *et* al.

pomace	pH=2.61, soaking in oven at 100 ºC for 1h		carbohydrates/ g substrate	substrate		2016
Banana peels	0.2 M acetic acid, pH=2.61, soaking in oven at 100 ºC for 1h	NApplic	310 mg/ g substrate	216 mg/g substrate	69.67%	Saha *et* al. 2016
Kitchen wastes	1.5% HCl (v/v), 100 ºC, 30 min	NApplic	514 mg total sugar/g	489 mg/g substrate	95%	Vavouraki *et* al. 2013
Kitchen wastes	1.5% H_2SO_4 (v/v), 100 ºC, 30 min	NApplic	580.26 mg total sugar/g	441 mg/g substrate	76%	Vavouraki *et* al. 2013
Sugarcane trash	Crude glycerol 6%(w/w), ferric chloride 1%(w/w), NaOH 5% (w/w), biomass loading 10% (w/w), 45 min	80 *FPU*, 48 h, 0.25% Tween 80 (w/w), 5% Biomass loading (w/w)	NA	0.796 g reducing sugars/g dry biomass	79.6%	Raghavi *et* al. 2016
OFMSW	Heat pretreatment, 85 ºC an 1h	NA	NA	57 g sugars/g TS	57	Nwobi et al. 2015
Kitchen wastes	1.5% HCl (v/v), H_2SO_4 1% (v/v), 3 h at 90 ºC	85 U/mL Glucoamylase from *Aspergillus niger*	NA	93.15 g/L (glucose)	79	Hafid et al. 2015
Fermented organic wastes (*FOW*) [d]	Dark fermentation (55 ºC, 21 d mass retention time)	60 *FPU*/g *Trichoderma reesei* MCG-80	2% *VS*	13 g/L (glucose and xylose)	73.7	Escamilla-Alvarado et al. 2015
Residual solids (*Pr* X_z) [e] from OFMSW biorefinery	NaOH 1M, 60 ºC, 1h solid/alkaline solution ratio(1:10)	5 IU/mL *FPU* from *Trichoderma reesei* MCG-80	1% (w/v) $_{db\ holocellulose}$	0.41 g/L (reducing sugars)	6.72 (g *RS*/g holocell)	This work

Notes: [a] yield of reducing sugars per unit of substrate, in (g/g)*100; [b] not applicable; [c] not available; [d] Fermented organic wastes; [e] Residual pretreated solids from *OFMSW* biorefinery

Thus, it is likely that other factors could be influencing our low reducing sugars yields. Degradation or denaturing of sugars, inhibitory co-products and remaining salts are factors that likely related to the yield pattern shown in Figure 6 and deserve further research (Cardoso-Coimbra *et* al. 2016; Duque *et* al. 2013; McIntosh *et* al. 2011).

The above results also suggest that the enzyme-substrate ratio should be explored in smaller intervals in the range 3 to 7 IU/mL. Also, presence of inhibitors (and eventually their removal) and other factors related to the efficient conversion holocellulose to reducing sugars should be further searched in order to improve the performance of the saccharification process of *Pr* X_z.

CONCLUSION

An apparent "increase" of cellulose was achieved after alkaline pretreatment of X_z, using NaOH 1M, due to lignin removal. This result occurred in spite that X_z had been subjected to previous degradation in other stages of the biorefinery (such as dark fermentation and cellulase production). Regarding the reduction of lignin, this was an indirect measure of crystallinity degree. It can be inferred that the X_z pretreated by alkaline method would be an easier substrate to degrade in further processes in biorefinery. Thus, the pretreatment of intermediate solids streams in *H-M-Z-S* biorefinery apparently could lead to an improvement of products yields, either H_2, CH_4, or sugars.

Yet, this potential did not occur as expected for additional bio-H_2 and reducing sugars production. Despite a favorable performance occurs in one of the treatments containing a fraction of pretreated X_z (*T4* treatment), the positive result on bio-H_2 generation was due the presence of *OFMSW* in the mixture. This was observed in treatment *T2*, in which only

pretreated solids were the substrate, where bio-H_2 production was relatively low. Likely, recalcitrance of this *Pr* X_z (low content of volatile solids and large amount of ashes) would have offset the potential benefits of the pretreatment. Results from saccharification of pretreated X_z, were lower compared to the saccharification of X_h (the fermented solids stream exhiting the hydrogenogenic bioreactor). Possibly, this could be due to X_z before pretretament was an intermediate solids stream that was subjected to two previous degradation processes (dark fermentation and holocellulases production) within the biorefinery *H-M-Z-S,* decreasing the levels of organic degradable matter to a such extent that even the alkaline pretreatment could not surmount. So, the saccarification potential of *Pr* X_z was lower compared to *FOW*, for example. Finally, it is recommended to further work on the effect of enzyme concentration on reducing sugars yields, either to confirm or overcome the sudden fall of sugars production at the 7 IU/mL level of enzymes, as well as other factors that could have negatively affected the conversion *Pr* X_z to reducing sugars in our work.

So far, it seems that in spite of pretreating the solid stream X_z, reuse of these solids in further yield improvements of bio-H_2 and sugars is not attractive. That is, likely a limit to the Principle of Cascading in the *H-M-Z-S* biorefinery has been met. Yet, the *Xz* still can be used as fertilizer/soil amender in agriculture.

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NOTATION

CMCase	*C*arboxymethylcellulases
FOW	Fermented organic wastes
FPase	Filter paper cellulases
FPU	Filter paper enzyme units
H	Bio-hydrogen production stage

H-M-Z-S	Stages in biorefinery for hydrogen, methane, enzymes and sugar production
M	Methanogenic stage
OFMSW	Organic fraction of municipal solid wastes
PDA	Potatoe dextrose agar
Pr	Pretreated
Pr X_z	Pretreated residual solids derived from holocellulases production
S	Sugars production stage
SEM	Scanning electron microscopy
T1	Substrate composition in fermentative batch hydrogen production using only *OFMSW*
T2	Substrate composition in fermentative batch hydrogen production using Pretreated solids X_z
T3	Substrate composition in fermentative batch hydrogen production using Unpretreated solids X_z
T4	Substrate relation in fermentative batch hydrogen production using 40% of Pretreated X_z solids and 60% *OFMSW*
T5	Substrate relation in fermentative batch hydrogen production using 40% of Unpretreated solids X_z
UPr X_z	Unpretreated residual solids from *Z* stage
X_h	Fermented residual solids derived from *H* stage
X_z	Residual solids from holocellulases production
Y_{RS}	Yield of reducing sugars (g sugar/g holocellulose $_{db}$)
Y'_{H2}	Bio-hydrogen yield expressed in mmol H_2/g TS)
Z	Holocellulases production stage using *Trichoderma reesei* MCG-80

Greek characters

ΣP_{H2}	Cumulative bio-hydrogen production *(*mmol H_2/reactor)