Saccharification of Fermented Solids for a Hydrogen Producing Biorefinery

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ABSTRACT

Biorefineries may be used to decrease dependence on fossil fuels by producing biofuels, or to produce new bioproducts and commodities. The production of hydrogen has been successfully coupled to a biorefinery model coined H-M-Z, providing advantages such as the generation the environmental friendly clean fuel hydrogen, and surprisingly an intermediate organic by-product coined as fermented solids (FS) that showed interesting properties for more biofuel and enzymes production. The objective in this work was to add a new stage for the saccharification of lignocellulosic substrates in a hydrogen producing biorefinery model.

Two saccharification experiments were performed. In the first one (SS-1) two factors were evaluated: type of substrate and type of holocellulolytic enzymes according to a 2x3 factorial design. The substrates were OFMSW, FS, and filter paper Whatman No. 1 (FP). The holocellulolytic enzymes used were the enzyme extract from Z-stage (*Trichoderma reesei* extract) of the biorefinery and the commercial enzyme Celluclast (Sigma-Aldrich, USA). In the second experiment (SS-2), the FS were subjected to evaluation at four different levels of enzyme:substrate ratio [40, 60 80, 100 and 120 FP units (g volatile solids)$^{-1}$].

In SS-1 all substrates were best saccharified with the *T. reesei* extract, whereas the most saccharified substrate, independently of the enzyme used, was the FP [up to 18 g of glucose and xylose (L)$^{-1}$]. The FS had up to 34 % higher saccharification than OFMSW, with up to 13.09 g of glucose and xylose (L)$^{-1}$. The highest saccharification efficiency for FS was 75 % on holocellulose basis. In the SS-2, we found that contrary to expected, the highest enzyme:substrate ratio had no significant positive effect on saccharification yields. Indeed, the ratio 40 FP units (g volatile solids)$^{-1}$ had the highest saccharification yield [11 mg sugars (filter paper unit)$^{-1}$].

In conclusion, the hydrogen production in a biorefinery is transcendental because on the one hand, it provides a renewable clean fuel, and on the other hand, it provides a pre-degraded substrate (saccharified liquors) easily convertible into valuable bioproducts, or even into more biofuels.

Keywords: biohydrogen; cellulases; municipal organic solid wastes; saccharification.

1. Introduction

Biorefineries may contribute to decrease dependence on fossil fuels and to produce new bioproducts. One of the most acknowledged advantages of biorefineries is its flexibility and wide spectrum of possible products. There is not only one possible configuration for biorefineries; this depends on feedstock, aim and bioproducts or biofuels orientation [1]. The most common biofuels produced in expected biorefineries are ethanol, methane and vegetable or microbial oils [2, 3]; whereas the bioproducts include enzymes, proteins, resins, biomolecules, carbohydrates, fertilizers, among others [1, 4, 5].

Due to the thermodynamic and ecological characteristics of hydrogen, it is considered to be a promising bioproduct in biorefineries [2, 6]. Hydrogen may be produced via biological processes such as dark fermentation or photo fermentation, with axenic cultures or consortia [7]. The consortia have metabolic interactions that can degrade more exhaustively a complex substrate and may use unsterilized substrate. In contrast, axenic cultures have a reduced metabolic capability and often need microbial-contamination free substrate. When biomass is hard to assimilate by microorganisms of interest, a common approach is to hydrolyze its polymeric chains using enzymes. For instance, the efficient hydrogen producing *Clostridium pasteurianum* is unable to assimilate starch directly [8]. However if starch is hydrolyzed either by physical-chemical or biological methods, *C. pasteurianum* effectively evolves hydrogen from this hydrolyzate.

Holocellulases are an important group of enzymes for hydrolyzing cellulose and hemicellulose [9]. Cellulose is the most abundant natural polymer on earth and it has been identified as a robust feedstock for biofuels and bioproducts [10]. However, the forms in which cellulose is found in nature are as diverse as the number of plant species. Residual biomass from agriculture and from the organic fraction of municipal solid wastes (OFMSW) is regarded as a renewable and non-food competing substrate.

The saccharification is the depolimerization of the carbohydrates that constitute the vegetable biomass cell walls into simple sugars [11]. One of the most common applications of the hydrolyzates from saccharification, is its fermentation into compounds such as ethanol, butanol, lactic acid, and even hydrogen [12]. When converting lignocellulosic biomass to hydrogen, glucose and xylose are the conducive reducing sugars [13].

In the initial step of the biorefinery model coined as H-M-Z [14, 15], the dark fermentation of OFMSW produced hydrogen and an organic by-product termed as fermented solids (FS) that showed good biodegradability properties in contrast to the OFMSW. Consequently, methane and holocellulases were produced in parallel from the FS. The objective in this work was to add a new stage for the saccharification of lignocellulosic substrates in a hydrogen producing biorefinery model.

2. Materials and methods

2.1. Biorefinery H-M-Z set up

This biorefinery H-M-Z was comprised by an in-series process for hydrogen and methane production and by the parallel process for holocellulases production (Fig. 1).

The hydrogen-producing process (H-stage) was fed with the organic fraction of municipal solid wastes (OFMSW). The hydrogenogenesis was performed at 55 °C and semi-continuous feeding at 21 d MRT, with a maximum productivity of 202 NmL $H_2/kg_r/d$ [15]. The fermented solids (FS) were the purges from

the H-stage, which were fed as they were to the methanogenic stage, and washed and then fed to the enzyme producing stage.

The methanogenic stage (M-stage) was operated at 55 °C and semi-continuous operation at 28 d MRT. The productivity was 2 023 NmL $CH_4/kg_r/d$ [15].

The enzymes production (Z-stage) was performed using *Trichoderma reesei* MCG 80 in 450 mL working volume fed with FS at 1.5 %VS. The operation was fed batch at 30 °C, 450 rpm, 9 d. The enzyme titers were 2.06 FPU/mL and 1.23 IU_{CMC}/mL [14].

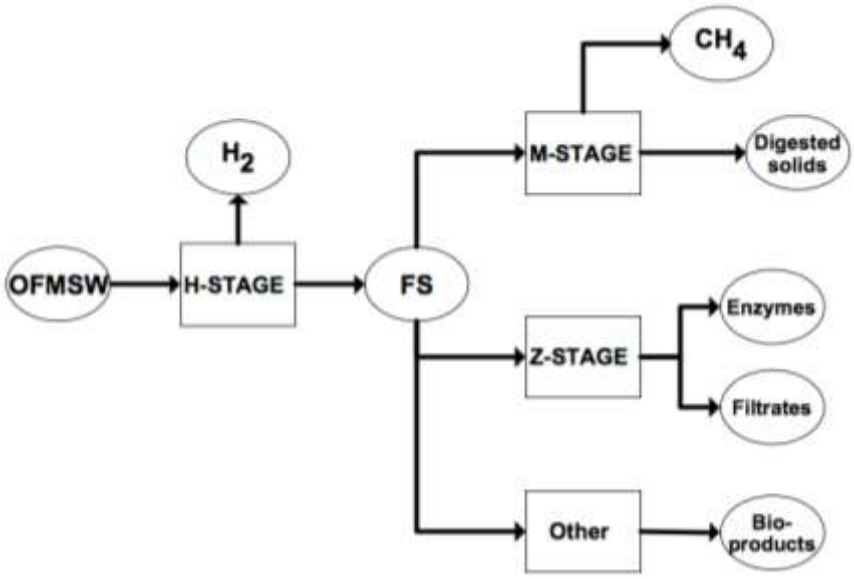

Fig. 1. Biorefinery model H-M-Z

2.2. Saccharification experiments evaluating types of enzyme and substrate

Two factors were evaluated: type of substrate and type of holocellulolytic enzymes according to a 3x2 factorial design. The substrates were OFMSW, FS, and filter paper Whatman No. 1. As holocellulolytic enzymes, we used the enzyme extract from Z-stage from biorefinery model (*Trichoderma reesei* extract) and the commercial enzyme Celluclast (Sigma-Aldrich, USA).

Saccharification was carried out in 125 mL Erlenmeyer flasks with 25 mL working volume at 50 °C, 150 rpm. The substrates concentration was 2 %VS in 0.05 M citrate buffer (pH 5.0). The enzyme loading was 60 FPU/g VS. The enzymes had the composition described in Table 1.

Table 1. Enzyme activities of Celluclast and *Trichoderma reesei* enzyme extract.

Parameter	Celluclast [a]		
	Volumetric	Specific	Specific normalized[b]
Filter paper activity	98.5 ± 2.7 FPU/mL	0.80 ± FPU/mg	1 FPU/FPU
Carboximethylcellulase activity	65.1 ± IU/mL	0.53 ± IU/mg	0.66 IU/FPU
Xylanase activity	156.3 ± U/mL	1.26± U/mg	1.58 U/FPU
β-glucosidase activity	14.0 ± µmol/min/mL	0.11 ± µmol/min/mg	0.14 µmol/min/FPU
Protein	123.6 ± mg/mL	-	-
Parameter	*Trichoderma reesei* enzyme extract [c]		
	Volumetric	Specific	Specific normalized[b]
Filter paper activity	37.01 ± 3.0 FPU/mL	0.87 ± 0.07 FPU/mg	1 FPU/FPU
Carboximethylcellulase activity	35.25 ± 0.01 IU/mL	0.83 ± 2.3E-4 IU/mg	0.95 IU/FPU
Xylanase activity	87.6 ± 0.42 U/mg	2.07 ± 0.01 U/mg	2.4 U/FPU
β-glucosidase activity	1.63 ± µmol/min/mL	0.04 ± µmol/min/mg	0.04 µmol/min/FPU
Protein	42.3 ± 1.2 mg/mL	-	-

Notes: [a] commercial enzyme Celluclast (Sigma-Aldrich); [b] specific enzyme normalized at 1 FPU/mL; [c] enzyme extract from *T. reesei* MCG 80 in Z-stage.

2.3. Saccharification experiments evaluating enzyme:substrate ratio

The FS were subjected to evaluation at four different levels of enzyme:substrate ratio: 40, 60 80, 100 and 120 FPU/g VS. The experiments were performed in 10 mL working volume assay tubes at 50 °C and 150 rpm, with enzyme extract from *T. reesei* at corresponding loadings. The substrate were the FS at 2 %VS.

2.4. Residual enzyme analysis

Supernatant from the saccharification experiments of the enzyme:substrate ratio evaluation was recovered, centrifuged at 5 000 g and assayed for filter paper activity, in order to determine the potential of reusing the enzymes after 72 h saccharification.

2.5. Analysis

Samples were first centrifuged at 5 000 g, 10 min and 4 ºC. Glucose and xylose analysis were performed by triplicate using a biochemical analyzer YSI 2700 Select (YSI Inc, OH, EUA). The response variables used are listed in Table 2.

Determination of enzyme titers as paper filter activity was performed as previously described [14] by the dinitrosalicylic acid method [16] according to Mandels et al. [17]. Filter paper activity units (FPU) expressed the amount of glucose released per min under proper assay conditions. All determinations were made by triplicate along with respective enzyme and substrate blanks.

Table 2. Response variables

Response variables	Units	Equation	
Sugar concentration	g/L	$[GX] = [glucose] + [xylose]$	(1)
Xylose/glucose ratio	-	$X/G = \frac{[xylose]}{[glucose]}$	(2)
Saccharification efficiency in volatile solids basis	$\%_{VSb}$	$h_{s,VS} = \frac{[GX]}{[VS]_{substrate}[substrate]} * 100$	(3)
Saccharification efficiency in holocellulose basis	$\%_{holocel}$	$h_{s,h} = \frac{[GX]}{[Holocellulose]_{substrate}[substrate]} * 100$	(4)
Saccharification yield	g sugars/FPU	$Y_s = \frac{[GX] * Vr}{EL * m_{VS}} * 100$	(5)

Notes: [glucose], glucose concentration (g/L); [xylose], xylose concentration (g/L); $[VS]_{substrate}$, concentration of volatile solids in substrate (%); [substrate], substrate concentration (g/L); $[Holocellulose]_{substrate}$, concentration of holocellulose in substrate (%); Vr, reaction volume (L); EL, enzyme loading (FPU/g VS); m_{VS}, amount of VS (g VS).

3. Results and discussion

3.1 Saccharification experiments evaluating types of enzyme and substrate

The OFMSW, paper filter and FS were used as substrates for saccharification using enzyme extract and the commercial enzyme Celluclast on a 3x2 experimental design.

Both enzyme preparations were load in the same proportion: 1.2 FPU/mL or 60 FPU/g VS. The profile of all the saccharification curves was asymptotic (Fig. 2). When using the enzyme extract, all the saccharifications were higher than those obtained with Celluclast in all the substrates, whereas the best saccharifications were obtained with filter paper, independently of the enzyme used (Table 3).

Better saccharification results were obtained with FS than with the OFMSW. Similarly, the FS had given better results than OFMSW in the methane and enzyme production stages. Several explanations may be formulated such as an effective pre-hydrolysis of cellulose resulting in more accessible and readily digestible polysaccharide chains.

Saccharification efficiencies (η_s) were near 50 % for OFMSW, and up to 65 % for FS. The higher η_s was obtained with filter paper, a result almost expectable being this refined cellulose with no presence of inhibitors [18, 19].

It was found that both the substrates and the enzymes had significant effect on saccharification, yet no interaction was found amidst the factors (Table 4). Moreover, Fig. 4 shows that the substrates where best saccharified in the order filter paper > FS > OFMSW, whereas the enzyme extract was statistically superior to Celluclast.

Compared to literature our results fell in the superior range (Table 5). Most of the saccharification studies used chemical, thermal or mechanical pretreatments, being the most common the diluted acid hydrolysis, steam pressure explosion, ball milling and ozonation. For instance, Ballesteros et al. [20] applied thermal pretreatment (160 ºC, 30 min) to municipal solid waste (MSW) 20 %TS, obtaining 35 g glucose/L after saccharification with a cellulose mix of Celluclast 1.5L and ß-glucosidase Novozyme 188. Clanet et al. [21] used enzyme extract from *T. reesei* CL847 achieving 48 $\%_{VSb}$ of saccharification from MSW at 6 %TS. After applying sequential diluted acid (1% v/v H_2SO_4, 3 h, 60 ºC) and thermal (120 ºC, 15 min) pretreatments to MSW, Li et al. [22] obtained a higher saccharification of 72.8 % with an enzymatic load of 60 FPU/ g from *T. viride* applied to 1 %TS MSW.

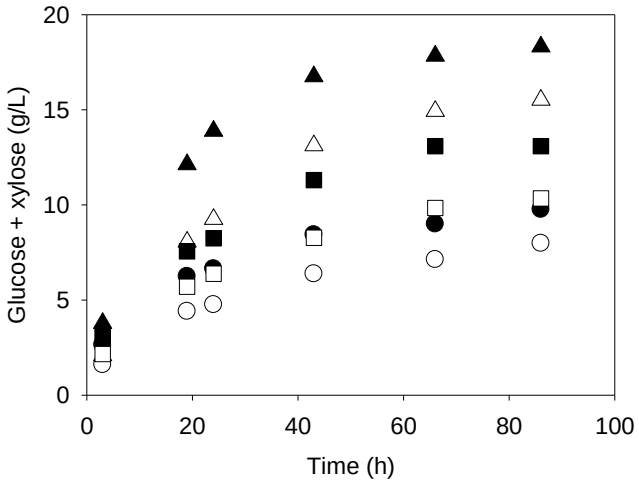

Fig. 2. Saccharification profiles. Solid symbols, *T. reesei* enzyme extracts; open symbols, Celluclast; Triangles, filter paper; Squares, FS; Circles, OFMSW.

Table 3. Maximum saccharifications at 60 FPU/g VS

Substrate	[GX] (g/L)	X/G (-)	$\eta_{s,VS}$ ($\%_{VSb}$)	$\eta_{s,h}$ ($\%_{holocel}$)
OFMSW + *T. reesei* enzyme extract	9.77 ± 0.63	0.30 ± 0.011	48.9 ± 3.2	54.3 ± 3.5
OFMSW + Celluclast	7.99 ± 0.52	0.28 ± 0.010	39.9 ± 2.6	44.4 ± 2.9
Filter paper + *T. reesei* enzyme extract	18.32 ± 0.56	0.13 ± 0.002	91.6 ± 2.1	96.4 ± 2.4
Filter paper + Celluclast	15.51 ± 0.22	0.09 ± 0.025	77.6 ± 2.6	83.3 ± 2.9
FS + *T. reesei* enzyme extract	13.09 ± 0.79	0.18 ± 0.009	65.4 ± 3.8	72.7 ± 4.2
FS + Celluclast	10.34 ± 2.61	0.24 ± 0.029	51.7 ± 12.6	57.4 ± 14.1

Table 4. Analysis of variance of the effects on saccharification

Source	Sum of squares	Degrees of freedom	Mean square	F value	p-value (prob > F)	Effect
Model	151.7	5	30.34	11.42	0.005	Significant
Substrate	132.95	2	66.47	25.03	0.0012	Significant
Enzyme	18.08	1	18.08	6.81	0.0402	Significant
Substrate x Enzyme	0.67	2	0.34	0.13	0.8832	Not significant
Pure error	15.94	6	2.66			

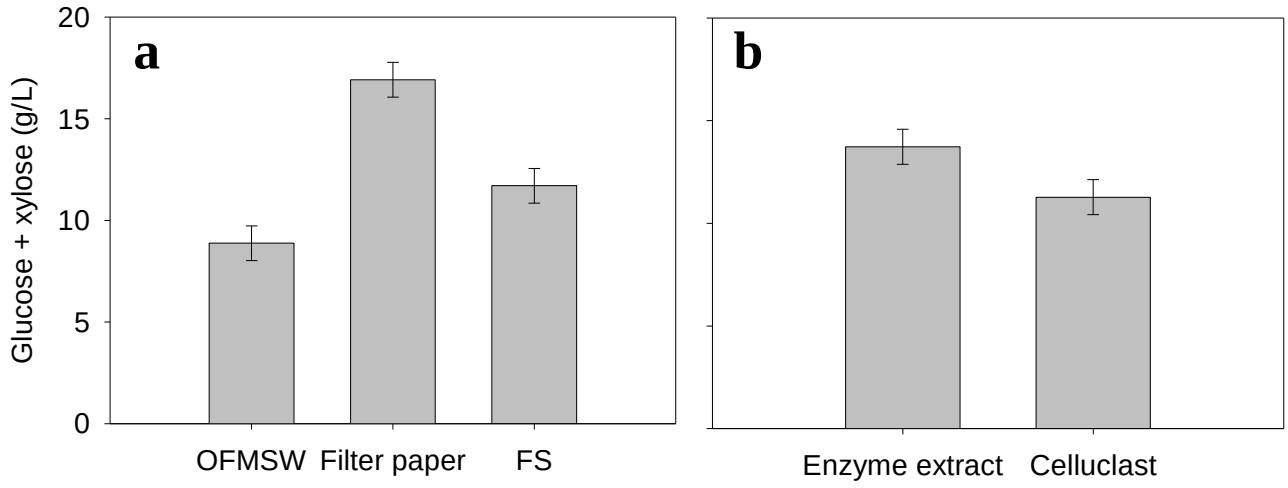

Fig. 3. Effects on saccharification. a) substrates, b) enzymes

Most pretreatments increase the saccharification of lignocellulosic substrates, however it must be considered their impact on environment and their costs. Diluted acid is regarded to have lower costs than short duration thermal pretreatments and a higher effect on saccharification [22]. Some pretreatments produce inhibitors to enzymes, mainly furaldehydes, organic acids, and phenolic compounds derived from the hydrolysis of lignin [19].

Recalling Table 1 (Materials and methods), it is observed that probably the reason for the better results with the enzyme extracts was due to its higher content of hemicellulases and endocellulases, despite of the lower content of glucosidases compared to the Celluclast.

Table 5. Saccharification of organic residuals

Substrate	Pretreatment	Enzymes	Results	Ref
OFMSW 20 %TS	Thermal (160 ºC, 30 min)	Cellulase (NS50013) and ß-glucosidase (NS50010)	$\eta_{s,VS}$ [b] = 41.6 % 35 g glucose/L	[20]
Kitchen waste 1 %TS	Thermochemical (1.5 % HCl, 120 ºC, 120 min)	NU	$\eta_{s,VS}$ = 57.3 %	[23]
OFMSW 6 %TS	NU [a]	Extract from *Trichoderma reesei* CL847 at 9 FPU/g substrate	$\eta_{s,VS}$ = 48 %	[21]
Sugarcane bagasse 5 %TS	Ball milling	*Trichoderma reesei* QM9414	$\eta_{s,VS}$ = 50.4 %	[17]
MSW 1 %TS	Acid (H_2SO_4 1 %v/v, 3 h, 60 ºC)+ thermal (120 ºC, 15 min)	*Trichoderma viride* at 60 FPU/g	$\eta_{s,cel}$ [c] = 72.8 %	[22]
Mixed solid waste 2.7 %TS	Acid (H_2SO_4 0.4 %v/v, 3 h, 60 ºC)+ steam pressure explosion (210 ºC, 3 min)	Cellulase at 66 FPU/g cellulose	$\eta_{s,cel}$ = 80 %	[24]
Milled cotton stalks 10 %TS	Ozone (45 mg O_3/L at 0.37 L/min, 150 min)	Cellulase at 20 FPU/g, ß-glucosidase at 45 IU/g and pectinase at 15 IU/g	$\eta_{s,VS}$ = 43.1 %	[25]
Fermented organic waste (FS) 2 %VS	Dark fermentation (55 ºC, 21 d mass retention time)	*Trichoderma reesei* MCG 80 60 FPU/g	$\eta_{s,VS}$ = 65.4 % $\eta_{s,h}$ = 72.7 %	This work

Notes: [a] not used, [b] saccharification efficiency VS basis, [c] saccharification efficiency cellulose basis

3.2. Saccharification experiments evaluating enzyme:substrate ratio

Increasing the enzyme:substrate ratio gave higher saccharifications (Table 6). The maximum saccharification was 11.59 g/L at 120 FPU/g VS. However the highest yield 11.06 mg sugar/FPU was observed at 40 FPU/g VS. This means that the saccharification did not increase proportionally to the enzyme:substrate ratio. Ballesteros *et al*. [20] also assayed the effect of different enzyme loads on 20 %TS MSW, observing a higher but no proportional saccharification when increasing enzyme loads from 20 to 60 FPU/g cellulose. In fact, the 3-fold higher enzyme load of 60 FPU/g cellulose only increased 16 % the saccharification of the 20 FPU/g cellulose enzyme load. In our case the doubling of the enzyme load increased 20 % the saccharification. Considering the high cost of enzymes, it would be better to find a enzyme:substrate ratio that sets a compromise solution between efficiency, yield and costs of the enzyme and substrate.

The unsaccharified organic matter may be used as substrate for other processes. For instance, Singh et al. [18] used the saccharified potato pulp to induce enzyme production from *T. reesei* Rut C30, with slightly better results than the enzyme induction from fresh potato pulp.

Table 6. Enzyme:substrate ratio saccharification results

Enzyme:substrate (FPU/g SV)	[GX] (g/L)	$\eta_{s,VS}$ ($\%_{VSb}$)	$\eta_{s,h}$ ($\%_{holocel}$)	Y_s (mg sugars/FPU)
40	10.02 ± 0.26	50.1 ± 1.0	55.7 ± 1.33	11.06
60	9.60 ± 0.29	48.0 ± 1.9	53.3 ± 2.2	6.80
80	9.65 ± 0.40	48.2 ± 3.6	53.6 ± 3.9	5.43
100	10.49 ± 0.29	52.5 ± 3.4	58.3 ± 3.7	4.59
120	11.59 ± 0.13	58.0 ± 1.5	64.4 ± 1.9	4.31
Control Celluclast 60 FPU/g VS	7.13 ± 0.23	35.6 ± 1.4	39.6 ± 1.8	5.34

Confirmatory assays showed that enzyme loads of 40 and 60 FPU/g VS did not show significant differences in saccharification results (data not shown), leading to infer that 40 FPU/g VS is an adequate load for saccharifying FS with satisfactory results.

Saccharification time could be reduced to less than 72 h in order to decrease energy expenses, just down to the necessary time to reach an acceptable result, which could be 80 % of the maximum experimental saccharification at 72 h. For instance, when using OFMSW after 29 h of reaction the saccharification reached 80 % of the maximum experimental saccharification, whereas for filter paper the same percentage was achieved at 40 h. Ballesteros *et al*. [20] using enzyme loads of 40 and 60 FPU/g VS, reported that maximum saccharification was reached at 48 h. Kaur et al. [25] also reported that for alkali or ozone pretreated cotton stalks, their respective maximum saccharification was reached at 48 h.

The saccharification results from the assays of the FS and the enzyme extract from *T. reesei*, show that it is technically feasible to include this process in the biorefinery H-M-Z, thus becoming the biorefinery H-M-Z-S. Table 7 shows the compositional characteristics of the lignocellulosic materials produced/used in this proposed biorefinery H-M-Z-S. The main consumed component of the holocellulose was the hemicellulose, which constituted 40 % of the holocellulose in the OFMSW, and ended up constituting 7 and 12 % of the FS and of the digested solids, respectively. Moreover, in the re-fermented solids of the Z-stage and in the hydrolyzed solids from the saccharification process, the hemicellulose content was as low as 1 % of the total holocellulose. Due to the recalcitrance of the lignin, its concentration in the TS augmented as the holocellulose was degraded. The re-fermented solids had a cellulose concentration as low as that of the digested solids from the methane producing stage. In the case of the hydrolyzed solids, the cellulose concentration was found in a higher proportion, presumably in the form of crystalline cellulose [26].

To achieve an integral use of the hydrolyzates from a saccharification process, it is important that these are not inhibitory to other microbiological processes. The hydrolyzates obtained by Clanet et al. [21] were used successfully as substrate for bacteria, yeast and fungus, with growth comparable to those obtained from glucose as carbon source. On their behalf, Ballesteros et al. [20] also used hydrolyzates from MSW for the ethanol production (30 g/L) by simultaneous saccharification-fermentation in fed-batch. In the case reported by Nguyen et al. [24], who obtained hydrolyzates from a mixture of different solid residues, it was necessary an acclimatization of their *Saccharomyces cerevisiae* strain to this substrate. This was mainly because of a slight generation of toxic compounds during the pretreatment of acid hydrolysis and steam pressure explosion (0.4 % H_2SO_4, 210 ºC, 3 min). When the concentration of toxic compounds (e.g.

furaldehydes, phenolic compounds) is high, one of the strategies for their removal is by biological methods, as the addition of laccases or the use of microorganisms with affinity to those toxics [19].

Table 7. Compositional characteristics of lignocellulosic materials of H-M-Z-S biorefinery

Parameter	OFMSW	Fermented solids (FS) from H-stage	Digested solids from M-stage	Re-fermented solids from Z-stage	Hydrolyzed solids from S-stage
Volatile solids ($\%_{db}$)	85.8 ± 0.24	81.6 ± 0.33	30.3 ± 0.20	67.5 ± 0.06	80.7 ± 1.78
Ashes ($\%_{db}$)	14.2 ± 0.35	18.4 ± 0.24	69.7 ± 0.54	32.2 ± 0.50	19.3 ± 0.95
Cellulose ($\%_{db}$)	46.6 ± 1.40	66.2 ± 0.75	14.8 ± 0.35	19.7 ± 0.81	52.8 ± 0.44
Cellulose ($\%_{VSb}$)	54.3 ± 1.54	81.1 ± 0.88	48.8 ± 1.31	29.2 ± 1.20	65.4 ± 0.74
Holocellulose ($\%_{db}$)	77.7 ± 1.80	71.6 ± 0.83	16.9 ± 0.21	20.3 ± 0.45	53.0 ± 1.01
Lignin ($\%_{db}$)	8.1 ± 0.98	9.9 ± 0.54	13.5 ± 0.68	21.0 ± 0.02	28.7 ± 0.40

Regarding hydrogen production from hydrolyzates, Lo et al. [12] hydrolyzed starch with *Caldimonas taiwanensis* On1 to feed *Clostridium butyricum* CGS2 thus producing 0.22 L H_2/h/L. Moreover, they fed the metabolites from dark fermentation to a photo fermentation process using *Rhodopseudomonas palustris* WP3-5 for more hydrogen production. Ren et al. [13] assayed different mixtures of glucose and xylose with *Thermoanaerobacterium thermosaccharolyticum* W16. They found that both pure glucose and xylose, and their mixtures were effectively used to produce hydrogen, yet the preferred substrate was glucose (2.42 mol H_2/mol glucose). Consequently, they used corn stover hydrolyzates containing 5.8 g glucose/L, 4.2 g xylose/L and 0.6 g/L arabinose, which was successfully converted into hydrogen (120 mmol H_2/L, 2.3 mmol H_2/mol substrate) at similar yields than a synthetic medium containing glucose and xylose at the same proportion than the hydrolyzates. They also pointed out that apparently, the components in hydrolyzate of corn stover did not inhibit the growth of the microorganism used.

3.3. Residual enzyme analysis

The potential reuse of the enzymes after 72 h saccharification of the FS was evaluated (Table 8). At the end of saccharification, the residual enzyme activity in the hydrolyzates was assayed. When dividing this residual activity by the initial enzyme activity, the enzyme recovery was obtained. In general good enzyme recoveries were attained (> 70 %) It could be appreciated that the enzyme recovery was higher at lower enzyme loads (Table 8). In the saccharification with 40 FPU/g VS the highest enzyme recovery of 86 % was obtained. The enzyme adsorption may have been the cause of this phenomenon. In deed, it has been reported that the proportion of the enzyme adsorbed to the residual substrate is depending of the enzyme:substrate ratio, of the hydrolysis efficiency and of the lignin content in the substrate [26]. The adsorption and desorption phenomena are very important for the hydrolysis and enzyme recovery, as there is a strong relation amidst enzyme adsorption and substrate hydrolysis. It is considered that a stronger adsorption leads to a higher hydrolysis [26]. Consequently when there is a strong adsorption in the endo and exoglucanases to the cellulosic substrate, the desorption is not realized unless the substrate has been degraded [27]. The exoglucanases may be bound to the insoluble substrate by means of two elements, the catalytic dominion and the cellulose-binding domain. The adsorption only by the cellulose binding domain

does not allows the hydrolysis of the substrate; to achieve this it is necessary the binding of the catalytic domain.

The observed enzyme recoveries of 70 – 86 % open up the possibility to recover and recycle the enzyme extract for new saccharification. The enzyme recycle is observed as an alternative to reduce the costs associated to the enzyme hydrolysis, as it is considered that the cost of the enzymes is nearly 60 % of the total cost of the process [18, 24]. Some enzyme recycling or reuse techniques are its immobilization, recovery by ultrafiltration of the hydrolyzate or by desorption from the residual substrate using fresh phosphate buffer, or reuse by contact of the adsorbed enzymes in the hydrolyzed substrate with fresh substrate [18, 24].

Table 8. Residual enzyme activity analysis

Enzyme load (FPU/g SV)	FPx,i [a] t = 0 h	FPx,f [b] t = 72 h	FPx,r [c] (%)
40	1.00	0.86	86
60	1.31	1.08	82
80	1.68	1.28	76
100	2.03	1.57	77
120	2.45	1.77	72
Control Celluclast 60 FPU/g SV	1.34	1.00	75

Notes: [a] FPx initial at 0 h, [b] FPx final at 72 h, [c] Amount of enzyme activity recovery (FPx,r = FPx,f/FPx,i *100). The filter paper activity of the *T. reesei* extract and of Celluclast were 17.55 and 111.2 FPU/mL respectively.

The hydrolyzed solids from FS may even have further application in other biotechnological processes. One of such could be its reuse in combination with fresh substrate to increase the enzyme production as proposed by Hogan and Mes-Hartree [26], who combined hydrolyzed residual poplar with steam pressure explosion pretreated fresh poplar. They reported higher enzyme activities (0.72 – 0.85 FPU/mL) when compared to fresh substrate (0.55 0.67 FPU/mL).

4. Conclusions

- The substrates were best saccharified in the order filter paper > FS > OFMSW.
- The enzyme extract from *Trichoderma reesei* MCG 80 was statistically superior to Celluclast.
- Saccharification efficiencies were near 50 % for OFMSW, and up to 65 % for FS.
- Increasing the enzyme:substrate ratio gave higher saccharification.
- The highest saccharification yield was obtained with enzyme load at 40 FPU/g VS.
- The enzyme loads of 40 and 60 FPU/g VS did not show significant differences in saccharification results (data not shown), leading to infer that 40 FPU/g VS could be an adequate load for saccharifying FS with satisfactory results.

- Results show that it is technically feasible to add a saccharification stage to the H-M-Z biorefinery model, thus achieving an integral use of the enzyme produced in Z-stage and the FS from H-stage.
- Hydrolyzates from FS need to be assayed to confirm its potential use to hydrogen production.

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Notation

GX	glucose plus xylose concentration
H-M-Z	three-stages biorefinery model for the production of hydrogen, methane and holocellulases.
FPU	filter paper unit
FS	fermented solids
IU	international enzyme activity unit
MSW	municipal solid waste
NmL	volume in millilitres, normalised at 273 K and 101.325 kPa

OFMSW	organic fraction of municipal solid waste
TS	total solids
VS	volatile solids
X/G	xylose to glucose ratio
Ys	saccharification yield

Greek characters

η_s	saccharification efficiency

Subindices

db	dry basis
h	holocellulose basis