Hydrogen Production from Organic Wastes and Re-Use of Fermented Solids to Produce Holocellulases: A Crucial Combination for Biorefinery of Organic Wastes

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ABSTRACT

This work reports results from semi-continuous hydrogen production (H-stage) followed by a methanogenic stage (M-stage), and the re-use if its fermented and digested solids for the production of holocellulolytic enzymes (Z-stage) with the purpose of performing a holistic evaluation of a biorefinery model. The H-stage, fed with organic fraction of municipal solid wastes (OFMSW) at 20.9 % total solids, operated at 21 d mass retention time (MRT) and 55 ºC. It exhibited a hydrogen productivity (I_{H2}) of 202 NmL H_2/kg_{wmr}/d. The fermented solids (FS) from this stage were either washed and conditioned to serve as substrate to *Trichoderma reesei* MCG 80 (Z-stage), or were used directly to feed methanogenic digesters (M-stage). The M-stage was operated at 28 d MRT and 55 ºC, yielding 2 023 $NmLCH_4/kg_{wmr}$/d. Its digestates (DS) were also washed and conditioned to serve as substrate in Z-stage. The Z-stage also was fed with OFMSW as a positive control. Regarding the Z-stage, OFMSW was compared against OFMSW supplemented with lactose, FS, and DS. No positive effect on enzyme production from ODMSW due to lactose was found. The highest enzymatic titres were in the order FS > OFMSW ≈ OFMSW + lactose > DS. The use of FS from hydrogenogenic fermentation for enzyme production holds promise for waste management. It promotes energy and added-value bioproducts generation, a green alternative to common practice of management and disposal of organic wastes. Our results also demonstrate the key role of hydrogen production from solid wastes in biorefinery approaches.

1. Introduction

Biorefineries based on the cascade principle aim at obtaining as many bioproducts as possible (biofuels and added-value products) from biomass through series and/or parallel bioprocesses [1, 2]. In direct cascading, the biorefinery is focused on obtaining firstly bioproducts and lastly biofuels. For instance, industrial and agricultural wastewater conversion to bioenergy (hydrogen, methane, microbial fuel cells) is deferred to favor biochemicals production (enzymes, biomass protein, organic acids and antibiotics) which have a higher added value compared to biofuels [3].

Yet as a consequence of energetic crisis and unfavorable fossil fuel forecasts [4], biorefinery may also be regarded on a different perspective that we have coined as inverse cascading. In this concept the main products are first biofuels, followed by reclaiming the processed biomass waste for generating added-value products (for instance, enzymes, pharmaceuticals). Lastly, the waste streams discharged from the latter, can be used for more biofuel production [5]. In this way the biorefinery increases the energy yields and use part of this energy for self-sustainable operation.

Some biorefinery prospects appeal to prioritize trendy bioethanol production and to use residual biomass and wastes either to obtain methane or to burn it directly for heat production [6, 7]. Others have proposed coupled hydrogen and methane generation from biomass, which is based on previous acidogenic-methanogenic process [8] and indeed feasible since both gases are produced from the anaerobic putrefaction of the biomass ([9]. In such processes, hydrolysis of the organic matter and its conversion to short-chain organic acids and hydrogen occurs in the hydrogen producing bioreactor, and so, microorganisms in the coupled methanogenic reactor profit from organic acids and metabolites already produced so methane generation is hastened and further biomass degradation occurs at the same time. This, has proved to enhance methane yields when compared to one-phase methanogenic process [10, 11].

However hydrogen and methane producing processes also produce effluents containing considerable amounts of organic matter, which consequently must be managed and disposed off [12]. Recently, digestates proved to be feasible substrates for xylanases and cellulases induction [13]. Indeed, other wastes or refuse materials such as animal manure [14] or Kraft paper mill sludge [15] have also proven to be adequate substrates for cellulases and xylanases induction.

The cellulases and xylanases are enzymes with recognized industrial applications to the conditioning of fabrics in textile industry, biobleaching of Kraft pulp in the pulp and paper industry, animal feed digestibility and more recently to the saccharification of lignocellulosic substrates for biofuel production [16-18]. The cellulolytic complex constituted by β-1,4 endoglucanases, β-1,4 exoglucanases (or cellobiohydrolases) and β-glucosidases perform the synergic function of breaking through β-1,4 glucosidic linkages in the polymeric structure of amorphous cellulose [19-21]. On their behalf, β-1,4-endoxylanases that cleave the β-(1-4)-D-xylopyranosyl linkages of xylans in hemicellulose, ß-xylosidases that hydrolyse short xylooligosaccharides to yield xylose, and other enzymes such as α-L-arabinofuranosidases, α-glucoronidases and acetyl xylan esterases, are engaged in breaking down hemicellulose polymers to simple sugars [22-24].

Trichoderma reesei (*T. reesei*) is a brown-rot filamentous fungus capable of growing on both cellulose and hemicellulose [25] through the production of a cellulolytic complex conformed of two exoglucanases, five endoglucanases and two β-glucosidases [20, 26]. *T. reesei* MCG 80 is a 4th generation mutant variety from the parent strain *T. reesei* QM 6a [27], isolated from military facilities in Natick, U.S.A. Although widely used on residual biomass such as wheat straw, rice hulk and others, information on enzyme production using OFMSW and digestates from anaerobic fermentation of OFMSW is still limited [13, 28-31].

Considering this, we evaluated the production hydrogen, methane and holocellulases from the exhaustion of OFMSW on a biorefinery conception.

2. Materials and methods

2.1. Substrate Formulation

The OFMSW was the substrate for the H-stage. It was prepared of dried food wastes from local dinning hall and waste office paper, in a mass concentration of 60 and 40 % w/w, respectively [11, 32]. Table 1 shows the characteristics and composition of OFMSW as well as those of the feremented and digested solids.
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 20.9 %w/w.

Table 1. Substrates characterization and composition.

Parameter	OFMSW [a]	FS [b]	DS [c]
pH	6.41 ± 0.05	7.43 ± 0.04	10.48 ± 0.05
VS [d] (%db)	85.8 ± 0.24	81.6 ± 0.33	30.3 ± 0.20
Alkalinity [e] ($mgCaCO_3/kg_{wb}$)	2956 ± 750	2309 ± 45	3595 ± 323
TKN [f] (%db)	0.69 ± 0.10	0.84 ± 0.11	1.1 ± 0.09
Cellulose (%db)	46.6 ± 0.96	66.2 ± 0.24	14.8 ± 0.81
Holocellulose (%db)	77.7 ± 0.24	71.6 ± 0.24	16.9 ± 0.24
Lignin (%db)	8.1 ± 0.98	9.9 ± 0.24	13.5 ± 0.51
Ashes (%db)	14.2 ± 0.24	18.4 ± 0.24	69.7 ± 0.24

Notes: [a] organic fraction of municipal solid waste; [b] fermented solids from the hydrogen production stage; [c] digestates from the methanogenic stage; [d] volatile solids; [e] alkalinity is expressed as mg of $CaCO_3$ equivalents per wet sample; [f] total Kjeldahl nitrogen.

The fermented solids (FS) and the digestates (DS) coming out of the H and M stages were first washed with distilled boiling water, filtered, washed with ethanol 50 % v v^{-1}, and then again washed with boiling water, similarly to the

procedure for determination of extractives in biomass [33]. Washed digestates were then dried in an oven at 60 °C, 24 h, and milled (mesh No. 35). FS presented similar characteristics to OFMSW, whereas DS was markedly different in most characteristics (Table 1). Indeed, contents of biodegradable organic matter such as cellulose and hemicellulose were lower in DS than those of FS and OFMSW. Also, after washing, the pH of DS was in the alkaline side, in contrast to the neutral and slightly acidic pH of FS and OFMSW. In anyway, the initial pH of all the substrates plus media was set at 4.8 in the fermentation runs, so the pH of the substrate would not be a noisy factor. Characterization and composition of FS and DS is shown also in Table 1. All substrates for Z-stage were tyndalised in mineral medium prior to inoculation.

2.2. Hydrogen and Methane Production (H and M stages)

Hydrogen and methane production was performed on one-liter glass jars used as bioreactors, containing 500 g working mass volume (Fig. 1). The H-stage, was fed with 20.9% TS OFMSW at 55 ºC and 21 d mass retention time (MRT). For the M-stage, the fermented solids from the first stage were fed to the methanogenic bioreactor working at 55 ºC and 28 d MRT. Bioreactors were placed in an insulated wood-incubator with temperature control set at 55 ± 1 ºC.

Feeding strategy for both H and M stages was performed by a draw-&-fill mode, which was performed twice a week at corresponding MRT. Treatments and controls were run by duplicate. The main response variables used for evaluating the process performance was H_2 and CH_4 productivity (NmL/(kg_{wmr} d)). Operation of bioreactors was over 50 d.

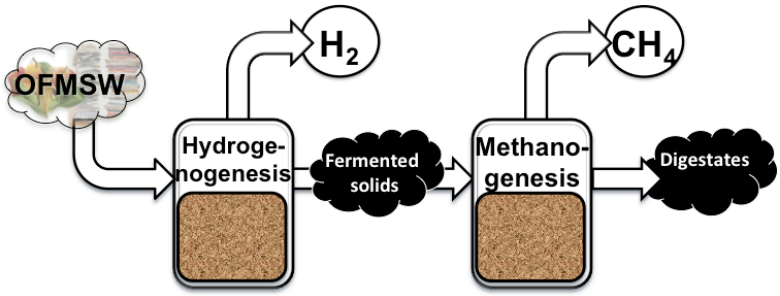

Figure 1. Flow diagram of the H and M stages

2.3. Holocellulases production (Z-stage)

Trichoderma reesei MCG 80 propagation was carried out on Petri plates containing potato-dextrose agar (PDA) prepared according to manufacturer (Bioxon, Becton Dickinson, Mexico), and incubated at 30 ºC. Plates completely sporulated with greenish conidia were harvested or maintained at 4 ºC. Spores were harvested by washing the spores

from PDA plates in four milliliters of distilled water containing Tween 80 at 0.1 %v v^{-1}. Then, 1 x 10^6 - 10^7 spores were then germinated in 250 mL Erlenmeyer flasks containing 50 mL of Mandels medium at 1 %w/v Solka floc BW 100 as substrate, 30 ºC and 300 rpm. Posterior seed cultures were inoculated at 10 % v/v with suspended mycelium from previous Solka floc cultures.

The studies on enzymatic production using the substrates FS, DS and OFMSW at 1 %VS were carried out in Erlenmeyer flasks at similar cultivation conditions than those used for seed cultures. Also, experiments on fermenters were run using 1.5 % VS FS as substrate, in 450 mL working volume Sixfors fermenters (Infors HT, Switzerland) at 30 ºC, 350 – 450 rpm, pH 4.8. Inoculation was performed at 10 % v/v from suspended mycelium seed cultures. Agitation was effected through two propellers Rushton, each with 6 blades. The pH was measured with an electrode Fermprobe (Broadley-James Corp., USA). Duration of fermentation was over 5 d.

Composition of mineral medium (pH 4.8) was (in g/L; [34]): 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); soy peptone (1); Tween-80 (1).

The enzyme extracts were recovered by centrifuging the samples at 6 000 *g*, 10 min and taking the supernatant. Fermenters conditions were run by duplicate.

2.4. Analyses

Volatiles solids (VS) and total solids (TS), pH, volatile organic acids (VOA), lactic acid and solvents were analysed as reported elsewhere [11, 35-37]. Biogas production in H and M stages was measured by acid brine displacement [36]; gas volumes were normalized (NmL or NL) to 273 K and 101.325 kPa. H_2 and CH_4 contents were determined by gas chromatography using a Molecular Sieve 5A packed column in a GOW-MAC chromatograph model 350 fitted with TCD (injector, detector and column temperatures were 37, 100 and 70ºC, respectively), and argon as carrier gas.

Samples from the Z-stage for xylanases and cellulases analyses were taken during fermentation. Enzymatic activities were assayed by measuring reducing sugars by the dinitrosalicylic acid method [38] according to Mandels et al. [34] and as reported by Ponce-Noyola and de la Torre [39]. Absorbance was measured in a spectrophotometer Jenway 6320D (Barloworld Scientific LTD, U.K.) and compared to standard curves using xylan, cellobiose or glucose as reducing sugar. Enzymatic activities in samples were expressed in either international units (IU) for xylanase activity, enzymatic units (U) for endoglucanase activity, and filter paper units (FPU) for filter paper activity. These enzymatic units were defined as the amount (µmol) of xylose, cellobiose or glucose released per minute under assay

standard conditions. Cellulose, lignin and hemicellulose contents in substrates were determined as reported elsewhere [35, 40] following the methods of AOAC [41]. All determinations were made by triplicate.

3. Results and discussion

3.1. Hydrogen and methane production in series

Hydrogen and methane productions were according to expected ones for solid substrate fermentation. Fig. 2 shows for both H and M stages, a first profile of probable acclimatization to operation condition, with a posterior tendency to stabilization of their productivities. Average productivities were I_{H2} = 202 NmL $H_2/kg_{wmr}/d$ for H-stage (Table 2), and I_{CH4} = 2 023 NmL$CH_4/kg_{wmr}/d$ for M-stage (Table 3). This was a result of applying the operation conditions found to be the best from previous reports [11, 36, 40]. Several authors coincide with thermophilic bioreactors to give better results than mesophilic ones [36, 42, 43]. Youn and Shin [44] reported that thermophilic operation had higher hydrogen production, ascribing this to a reduction of the dissolved hydrogen and to the consequent release of higher amounts of hydrogen due to the higher temperature in thermophilic reactors. However, other authors consider that mesophilic regime leads to a more economically feasible process [42, 45, 46]. It is noteworthy that mesophilic bioreactors are predominant in the context of wastewater treatment [47, 48].

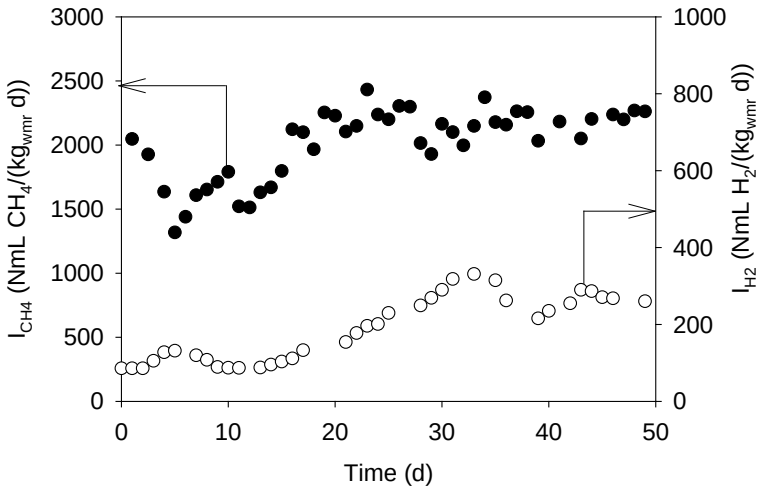

Figure 2. Time courses of H and M stages. (●) methane productivity, (○) hydrogen productivity.

Table 2. Average results from hydrogenogenic stage

Parameter	Units	H-stage (std dev)[a]
Hydrogen content	%	42.2 (5.4) [a]
Biogas production	NmL/d	212 (69)
I_{H2} [b]	NmL H_2/(kg_{wmr} d)	202 (69)
pH	-	6.29 (0.12)
COD_{VOA} [c]	mgCOD/ Kg_{wb}	7647 (515)
$COD_{solvents}$ [d]	mgCOD/ Kg_{wb}	371 (18)
Alkalinity [e]	mg$CaCO_3$/ Kg_{wb}	1070 (14.1)
A/B ratio [f]	-	0.89 (0.06)
Lactic acid concentration	mgCOD/Kg_{wb}	2924 (987)

Notes: [a] standard deviation, [b] hydrogen productivity, [c] chemical oxygen demand due to volatile organic acids, [d] COD due to solvents, [e] total alkalinity added for intervention of pH, [f] acetate to butyrate ratio.

Table 3. Average results from methanogenic stage

Parameter	Units	M-stage (std dev)[a]
Methane content	%	69.2 (9.4) [a]
Biogas production	NmL/d	1 190 (52)
I_{CH4} [b]	NmL CH_4/(kg_{wmr} d)	1 688 (30)
Y'_{CH4} [c]	NmL CH_4/ (g VS_{rem})	755 (32)
pH	-	8.16 (0.21)
Alkalinity	mg $CaCO_3$/ kg_{wmr}	11 396 (265)
Alpha[d]	-	0.40 (0.18)
η_{VS} [e]	%VS_{rem}	58.6 (1.0)

Notes: [a] standard deviation, [b] methane productivity, [c] methane yield, [d] alpha ratio (intermediate alkalinity/partial alkalinity), [e]volatile solids removal efficiency.

3.2. Holocellulases production

The highest enzymatic titres were in the order FS > OFMSW ≈ OFMSW + lactose > DS (Table 4). The FS was the best substrate, as its filter paper activity (FPx) was superior to that of DS and OFMSW in about 180 and 23 %, respectively. The highest enzyme activities obtained from FS suggest that the microbial hydrolysis that occurred during its hydrogenogenic fermentation might have contributed to expose cellulose and/or to favored amorphous regions, which made FS a more degradable/inducer substrate. This effect may be compared to that observed in H-M processes, where methane generation has been improved as a result of being fed with partially degraded substrate from hydrogenogenic stage [11].

Regarding the low results from DS, explanations might be the inhibitory effect from compounds that were not correctly removed during washes, low availability of holocellulose (16.9 %db, Table 1) or even high contents of ashes (69.7 %db). Wang *et al*. [15] reported that high ash content (36 %db) in Kraft paper mill sludge fed for enzyme production with *T. reesei* RUT C30 was deterrent to the growth of this microorganism and a strong inhibitor to cellulases enzyme production. They improved their cellulases production by removing the ash content of the sludge to less than 4% by means of a de-ashing treatment with H_2SO_4 (concentration not specified). Moreover, partial improvement of their > 2 fold FPx results with de-ashed sludge could have been positively influenced by the acid hydrolysis from the use of H_2SO_4.

Unexpectedly, addition of lactose did not improve results for OFMSW. Although it is a weak inducer for *T. reesei* [49]), lactose is commonly preferred over other inducers because of its low cost [27].

Table 4. Results from holocellulases production evaluating different substrates in Erlenmeyer flasks

Substrate	Pe[a] (g/L)	CMCx[b] (IU/L)	FPx[c] (FPU/L)	Xylx[d] (IU/L)
FS[e]	1 737	1 130	1 319	7 555
DS[f]	338	720	466	5 760
OFMSW[g]	812	1 010	1 068	6 700
OFMSW + L[h]	826	1 010	1000	6 870

Notes: [a] extracellular protein, [b] carboxymethyl cellulase activity, [c] filter paper activity, [d] xylanase activity, [e] fermented solids, [f] digested solids, g organic fraction of municipal solid waste, h OFMSW supplemented with lactose as inducer.

Next, FS were tested as substrate in 450 mL fermenters. Fig. 3 displays the profile of the enzyme activities measured during the fermentation. At 132 the activities were 1 611 FPU/L, 1 067 IU/L for endocellulases (CMCx) and 3 131

IU/L for xylanases (Xylx). Productivity was 11.3 FPU/L/h. When comparing fermenter results to those obtained in Erlenmeyer flasks we observe that FPx was improved over 22 %, CMCx was similar, and Xylx was impaired in about 40 %. Table 5 shows different results from literature. We observe that FPx from fermenters fall in the upper side of similar non-optimization works. Indeed some other works show higher results than ours. For instance Rojas-Rejón et al. [50] reported higher CMCx and Xylx when working with *Cellulomonas flavigena* PR22, a mutated and improved actinobacteria. However comparison between enzyme activities from fungi and bacteria is not always feasible because cellulolytic systems differ and also because activity is also dependent on the substrate [51]. For correctly assessing enzyme extracts, saccharification studies should be needed. Other works in Table 5 deal with complex substrates, as manure, partially saccharified newspaper and de-ashed Kraft paper mill sludge. To work with pure substrates such as Solka floc and Avicel may give better results than using refuse or waste substrates, as in the case of Domingues et al. [52]. This may be partially due to the inexistence or a relatively low presence of inhibitor compounds, as already discussed above.

Further exploration of parameters in fermenter is needed in order to correctly assess its performance, potential and to improve results.

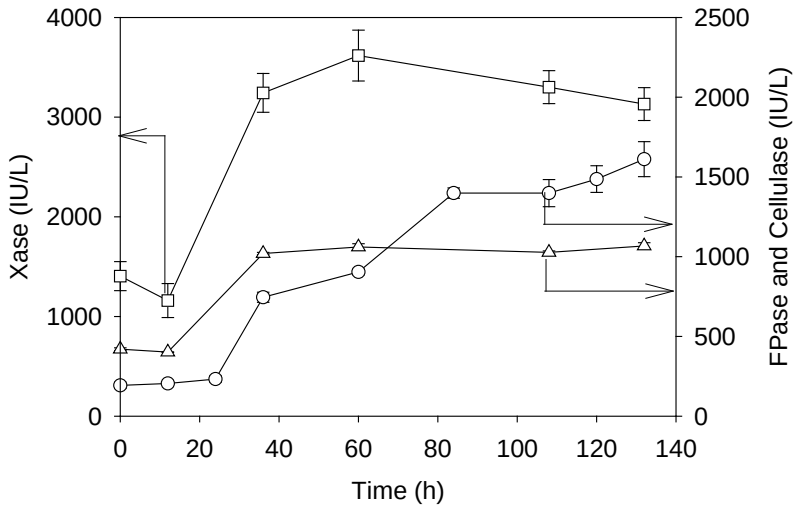

Figure 3. Enzyme activities using FS as substrate. (□) xylanase activity, (○) filter paper activity, (△) carboxymethyl cellulase activity.

Table 5. Summary of holocellulase production in open literature

Microorganism	Substrate	Process information	Results	Ref
Cellulomonas flavigena PR22	1 % w/v sugarcane bagasse	37 ºC, t_{inc} = 2 d, 1 vvm, 400 rpm, pH = 7	C[a]:1 630 U/L X[b]:21 700 IU/L	[50]
Trichoderma reesei Rut C-30	Partially saccharified newspaper 10 – 15 g/L	34 ºC, t_{inc} = 9 d, 200 rpm, pH = 5	F[c]: 2 000 FPU/L	[53]
Trichoderma reesei Rut C-30	Solka Floc 10g/L, glucose 10 g/L	28 ºC, t_{inc} = 5 d, 150 rpm, pH = 4.8	F: 2 200 FPU/L	[52]
Trichoderma reesei Rut C-30	Manure 6.7 g/L	27 ºC, t_{inc} = 6 d, 175 rpm, pH = 5.5	F: 800 FPU/L	[54]
Trichoderma reesei Rut C-30	Kraft paper mil sludge (de-ashed) 2.5 g glucan/L	28 ºC, t_{inc} = 3 d, 180 rpm, pH = 3.5	F: 1 600 FPU/L	[15]
Trichoderma reesei MCG 80	1.5 % VS FS	30 ºC, t_{inc} = 6 d, pH=4.8	F: 1 611 FPU/L C: 1 067 IU/L X: 3 113 IU/L	This work

Notes: [a] carboxymethylcellulase activity, [b] xylanase activity, [c] filter paper activity; t_{inc}, incubation time; VS, volatile solids; FS, fermented solids

4. Conclusion

In a biorefinery approach of three stages represented as H-M-Z for reclaiming OFMSW we have found the following:

- The H-stage was operated at 20.9 % total solids, 21 d mass retention time (MRT) and 55 ºC, thus yielding a hydrogen productivity (I_{H2}) of 202 NmL $H_2/kg_{wmr}/d$.
- The M-stage operated at 28 d MRT and 55 ºC yielded 2 023 $NmLCH_4/kg_{wmr}/d$.
- In the Z-stage, FS were compared to DS, OFMSW and OFMSW supplemented with lactose. No positive effect on enzyme production from OFMSW due to lactose was found. The highest enzymatic titres were in the order FS > OFMSW ≈ OFMSW + lactose > DS.

The production of added-value bioproducts along with bioenergy opens up a variety of possibilities for biorefinery configurations and for increasing the economic potential of OFMSW. Indeed favorable results from FS holds promise as a different approach for waste management and disposal of organic wastes, which would be a green alternative to the common practice of waste management.

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Notation

A/B	acetate to butyrate ratio
CMCx	carboxymethyl cellulase activity
COD	chemical oxygen demand
db	dry basis
DS	digestates or digested solids
FPx	filter paper activity
FS	fermented solids
H-stage	hydrogen producing stage
I_{CH4}	methane productivity
I_{H2}	hydrogen productivity
M-stage	methanogenic stage
MRT	mass retention time
OFMSW	organic fraction of the municipal solid waste
PDA	potato dextrose agar
t_{inc}	incubation time
TKN	total Kjeldahl nitrogen
TS	total solids
VS	volatile solids
wb	wet basis
Xylx	xylanase activity
Z-stage	holocellulases production stage