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Dark fermentation effectiveness as a key step for waste biomass refineries: influence of organic matter macromolecular composition and bioavailability

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SUMMARY

In next generation bio-based refineries, hydrolysis and primary (or extractive) fermentations by undefined microbial cultures (UMC) are precursors of secondary bio-transformations, in which H_2, CO_2 and mixed carboxylates are used as substrate for achieving added-value target products (e.g. bio-based chemicals, bio-plastics and pigments). Dark fermentation (DF) is the most simple UMC-driven hydrolysis and primary fermentations to extract gaseous and soluble mixtures of compounds from raw biomass. Which solid fractions (types of macro-molecules) of mixed raw organic matter (OM) are efficiently hydrolyzed + fermented during DF is an aspect that was rarely considered in depth.

Here, a first attempt was made to propose a new approach for understanding the effects of DF on different fractions of biomass. A set of seven different biomasses underwent optimized DF tests and, for simplicity, only the gaseous main product, i.e. bio-hydrogen potential (BHP) production, was used as parameter to assess DF efficacy. BHP was studied in relation with OM characteristics: on one side, chemical composition (macro-molecular fractions) and, on the other side, bioavailability to UMC attack (using two different biological assays). BHP was found significantly correlated (Pearson's test for $p < 0.05$, $n = 7$) only to acid detergent lignin (negatively), soluble sugars and sugars + starch (positively). Bioavailability was negatively correlated with fibrous fractions and to fat-like fractions, but correlations with BHP were poorer ($p > 0.05$, $n = 7$). A statistical model (partial least square regression) was proposed for predicting BHP from OM characteristics, with interesting predictability. In the next future, the proposed approach should be widened to better understand the DF effectiveness not only referred to its gaseous products, but especially focusing on the wide range of soluble products (carboxylates), thought as substrates for secondary biorefinery. Copyright © 2015 John Wiley & Sons, Ltd.

KEY WORDS

dark fermentation; bio-hydrogen; carboxylates; biomass; waste; biorefinery

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1. INTRODUCTION

Fossil-based refineries and traditional chemical industry have been based on extracting energy and building blocks (process intermediates such as H_2, CO, CH_4, alkanes, fatty acids, alcohols and esters) essentially from oil, natural gas or other non-renewable carbon sources. Today, a new model of refinery is rising, based on renewable carbon and on the concept of circular, instead of linear materials/energy streams, as principle for the overall sustainability [1]. H_2 and other building blocks, intermediate-chain compounds derived from biomass transformations, can be produced and also used as a reactant (reducing agent) in renewable carbon refinery cycles based on biomass and renewable energy [2].

To close energy and carbon cycles, the reuse of impure organic substrates (e.g. organic waste, food-industry byproducts, agricultural residues and green waste), side streams of other bio-refinery chains and/or food industry, characterized by random and variable physical, chemical and biological properties, is of fundamental importance [3]. In this sector, the refinery approach must be deeply different from other bio-refineries based on dedicated homogeneous substrates (e.g. bioethanol/biodiesel from dedicated crops [4] or bio-hydrogen from enzymatic hydrolysis of purified carbohydrates [5]) and it was recently called 'carboxylate platform' [6]. After eventual chemical–physical pretreatments [7], undefined microbial cultures (UMC) are used for simultaneously hydrolyzing (through specific enzymes produced directly on-site by microbial activity) and transforming (through the so-called primary or extractive fermentations) the mixed organic matter (OM) into easily available substrates for downstream specific bioconversions [8].

Dark fermentation (DF) is the most common primary UMC-driven hydrolysis/fermentation and allows simultaneous production of H_2/CO_2 and a mixture of soluble carboxylates (short-chain carboxylic acids, aminoacids, amines, alcohols, etc.), available for further downstream bio-refineries, called secondary bioconversions [6,9]. A variety of secondary bioconversions are currently investigated to obtain target compounds of particular interest (e.g. carboxylic acids, biofuels, solvents, biopolymers, building blocks, etc.) [10] and/or further bio-hydrogen through either photo-fermentations (by cyanobacteria or green algae) or electrically-driven bioprocesses (i.e. microbial electrolysis cells) [11].

In this emerging sector, DF is the intermediate ring of the chain; in DF, the sequence hydrolysis + fermentation is simultaneously driven by UMC, able to produce specific enzymes directly on-site and to ferment the liberated monomers [6]. Hydrolysis is the *conditio sine qua non* to produce soluble monomers available to microbial conversions and often represents the rate-limiting step, because of recalcitrance of the OM structure and to limiting process bio-chemical conditions [11].

However, in literature the large majority of DF studies have focused on maximizing H_2 production, thereby dealing with (i) easily hydrolysable and (ii) sugar-rich substrates (i.e. milk/cheese whey, organic waste leachates, winery wastewaters and rice starch). The integrated chain hydrolysis–fermentation has been less (mostly recently) studied as-a-whole, regardless of type and quality of the organic substrate treated, especially for ligno-cellulosic or other hardly hydrolysable materials (e.g. complex proteins, fats, long-chain fatty acids, polyphenols and aromatics). Few contributions in recent literature have exhaustively examined the influence on DF of chemical composition, structural recalcitrance [12], inhibitory metabolites liberation and metabolic side paths [13] that may mislead the main 'road' of soluble-sugar glycolysis to pyruvate and NADH [14].

For example, if mineral nitrogen (in form of ammonia) is commonly a fundamental nutrient for bacterial consortia [15], there is still poor description of complex proteins breakdown in DF that leads to ammonia liberation [16]. Again, it was reported that lipid hydrolysis leads to the reduction of glycerol, a substrate that bacteria are able to use for growing; in addition the formation of carbohydrates and volatile fatty acids (VFA) from lipids was reported to be involved in hydrogen production [17]; at the same time, long chain fatty acid formation from triglycerides hydrolysis was several times reported as a possible source of bacterial cell inhibition, because of negative interactions with cell membranes [18]. In general, hydrolytic/fermentative paths that involve proteins, lipids and other macromolecular classes are still an open field of study.

Finally, new tools should be developed to follow the path that in the past decades has been accompanying field applications of anaerobic digestion (the forefather of the upcoming 'carboxylate platform'), i.e. to allow full, efficient and versatile applications of DF to the widest variety of biomass types available, regardless of their chemical/physical/structural characteristics and variability [19].

In this work, a new approach is proposed to understand the effect/efficiency of DF, by given UMC at optimized process conditions, on waste/mixed OM depending on the combination of its chemical composition and recalcitrance. Here, we present only a first attempt in this path, with a limited number of samples and parameters analyzed: a statistical approach was proposed for a first set of differently chemically composed organic matrices, and ultimate H_2 generation yield alone was considered as parameter representing the efficiency of the overall chain hydrolysis + fermentation. In the future, also the wide spectrum of soluble organic compounds that are liberated from solid organic matter should be deeply studied as target.

2. MATERIALS AND METHODS

2.1. Set of biomass samples

A set of seven different organic substrates were considered to perform an optimized DF test: corn silage, malt powder, beet silage, giant cane (*Arundo donax* L.), olive pomace and rice bran. Pure glucose (100% soluble sugar C-6) was used as reference substrate with null recalcitrance to hydrolysis (100% soluble) and standard chemical characteristics for DF glycolysis. Samples were selected in order to obtain a variability in both chemical composition and recalcitrance to microbial degradation. All samples were dried at 40 °C until constant weight, then milled to pass through a 0.5-mm screen.

2.2. Biomass macromolecular characterization

Dry matter (DM) and volatile solids (VS) contents were evaluated according to standard procedures [20,21]. Total Kjeldhal Nitrogen (TKN) was detected on fresh material, while organic nitrogen content was used to evaluate the total protein content of samples [22]. Van Soest method [23] was used to evaluate fiber content on dry samples milled at 0.5 mm. NDF (Neutral Detergent Fiber), ADF (Acid Detergent Fiber) and ADL (Acid Detergent Lignine) data were used to calculate the content of lignin-like fraction, cellulose (ADF-ADL), hemicellulose (NDF-ADF) and soluble cell content (100-cellulose-emicellulose-lignine). Total fat content was determined by Soxhlet extraction with ether [24]. Hall method [25] was used for the determination of sugar content (TESC, Total Ethanol-Soluble Carbohydrate 80%) while the amyloglucosidase/α-amylase kit method was used for the determination of total starch [26]. The gross energy (GE) was measured using the adiabatic bomb calorimeter IKA 4000 (IKA®-Werke GmbH & Co. KG, Staufen, Germany).

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2.3. Evaluation of OM bioavailability to microbial hydrolysis

Two different tests were performed to obtain an estimation of the availability to microbial hydrolysis (in one word: bioavailability) of the given samples and to see how this is linked to the biomass macromolecular composition.

First, *in vitro* digestibility was measured, based incubation in rumen fluid inoculum obtained from cow digestive system, according to Robinson et al. [27]: dried samples were placed into individual *in vitro* incubation bags (multi-weave polyethylene polyester polymer cloth) and incubated in a DAISY® *in vitro* system (Ankom—Macedon, NY, USA).

The other approach was to measure short-term biodegradability according to Schievano et al. [28], by detecting the Specific Oxygen Uptake Rate (SOUR). Briefly, 0.2 g of dry matter was set in a flask to which the following were added: 500 ml of deionized water, 12 ml of phosphate buffer solution and 5 ml of nutritive solution [28]. To ensure optimum microbial activity and rates standard conditions were ensured and, to allow oxygen diffusion, the slurry was continuously stirred and intermittently aerated every 15 min. Potential oxygen uptake was reported as cumulative oxygen demand during the 20-h test (OD_{20}, g_{O2} $g^{-1}{}_{DM}$).

2.4. Optimized DF tests

UMC were used to incubate the selected organic matrices in lab-scale reactors set under optimal conditions for DF, as indicated by Tenca et al. [29]. Bio-hydrogen generation was measured and considered as parameter representing the effectiveness of DF. As soon as optimal conditions were ensured for DF, bio-hydrogen production was thought to be only dependent on substrate characteristics and thereby called bio-hydrogen potential (BHP). BHP was used as parameter to understand DF correlation to both macromolecular composition and OM bioavailability.

The startup UMC was collected from a 10-l lab-scale reactor producing hydrogen under anaerobic thermophilic conditions (55 °C) and maintained in the pH range 5 – 6 [30]. Before the test, the inoculum was heat-shocked in oven at 100 °C for 1 h to inhibit non-spore forming bacteria and methanogens and diluted with sterile water to obtain optimal residual volatile fatty acid (VFA) concentration ($VFA < 800\ mg\ l^{-1}$). Batch reactors of 500 ml were inoculated with 300 ml, fed with 0.3 g of dried biomass; substrate/inoculum ratio and overall dilution were chosen to avoid metabolite-driven inhibition [29]. All reactors headspaces (200 ml) were flushed with N_2 and incubated at 55 °C till no further biogas was produced. All tests were performed in triplicate.

Volumetric production of biogas was daily monitored through graduated syringes. H_2, CH_4 and CO_2 concentrations were daily measured through gas chromatography (Micro GC 3000, Agilent Technology, Santa Clara, CA, USA).

2.5. Statistical approach

The data set employed to perform statistical study was composed by the chemical and physical parameters characterizing the considered biomass samples. A Pearson correlation matrix was performed by using the SPSS statistical software (version 17; SPSS, Chicago, IL). Data were transformed to normality according to the literature [31].

Multiple linear regressions to predict bio-hydrogen potential production (BHP) *versus* chemical and physical variables were detected using the Partial Least Square method (PLS) by applying NIPALS algorithm [32]. The cross-validation 'leave-one-out' approach of scaled variables was applied to calculate the goodness of regressions (goodness of fit coefficient—R^2 and goodness of prediction coefficient—R^2cv, respectively). Taking into consideration all variable values the best PLS regression was calculated and the importance of each independent variable (importance coefficient) defined. Then PLS analysis was repeated excluding the variables characterized by the smallest importance coefficient [32]. This procedure was repeated until a final regression model with high regressions coefficients (R^2 and R^2cv) and the smallest number of variables was achieved. PLS was performed using SCAN software (Minitab Inc., State College, PA).

3. RESULTS AND DISCUSSION

3.1. Chemical properties

A wide diversity in chemical–physical composition in the organic matrices studied was evidenced (Table I). Pure glucose (100% OM and soluble sugar content), as expected, undergoing SOUR test, gave the highest biodegradability ($OD_{20} = 250 \pm 8\ g_{O2}\ kg^{-1}_{DM}$, Table I), and the *in vitro* digestibility resulted of $1000\ g\ kg^{-1}$ (i.e. 100% digestible).

The other substrates showed relatively high OM content ($834–950\ g\ kg^{-1}_{DM}$), the rest of DM being ash. Rice bran, corn silage and olive pomace showed higher raw protein contents ($131–159\ g\ kg^{-1}_{DM}$, Table I) as compared to the other samples (all below $62\ g\ kg^{-1}_{DM}$, Table I). Olive pomace showed the highest content of total lipids, with an ethereal extract of $127\ g\ kg^{-1}_{DM}$, while the rest of the organic matrices were below $40\ g\ kg^{-1}_{DM}$ (Table I). Olive pomace also showed the highest ADL content ($383 \pm 1\ g\ kg^{-1}_{DM}$), probably because of the presence of the olive kernel, and in general high fiber contents (669 ± 2 and $584 \pm 2\ g\ kg^{-1}_{DM}$ of NDF and ADF, respectively). Giant cane had comparatively less ADL, similar ADF, while sensibly higher NDF contents (Table I). On the other side, malt powder showed the lowest fiber contents, even if NDF still represented over 30% of the DM (Table I). The rest of malt powder was almost starch (over $600\ g\ kg^{-1}_{DM}$), with low protein and fats contents (Table I). Starch as also found in considerable amount in corn silage ($151 \pm 1\ g\ kg^{-1}_{DM}$), while in concentrations below $30\ g\ kg^{-1}_{DM}$ in the other

Table I. Organic matter content, chemical composition and bioavailability (represented by *in vitro* digestibility and OD_{20}) of the considered set of organic matrices. Results of optimized DF batch tests reported as ultimate bio-hydrogen potential (BHP).

			Corn silage	Malt powder	Beet silage	Olive pomace	Rice bran	Giant cane	Pure glucose
Total organic matter		$g_{VS}\ kg^{-1}{}_{DM}$	914 ± 0	852 ± 0	898 ± 0	940 ± 0	834 ± 0	950 ± 0	1000
Organic matter chemical composition	Raw proteins	$g\ kg^{-1}{}_{DM}$	150 ± 1	61.7 ± 0.1	43.2 ± 0.1	131 ± 0	159 ± 0	59.8 ± 3.2	udl*
	Ethereal extract (EE)	$g\ kg^{-1}{}_{DM}$	18.2 ± 0.1	79.8 ± 0.1	4.40 ± 0.02	127 ± 0	32.5 ± 0.2	38 ± 0	udl
	NDF	$g\ kg^{-1}{}_{DM}$	522 ± 1	320 ± 2	446 ± 1	669 ± 2	534 ± 2	826 ± 3	udl
	ADF	$g\ kg^{-1}{}_{DM}$	301 ± 1	173 ± 1	254 ± 1	584 ± 2	375 ± 2	558 ± 3	udl
	ADL	$g\ kg^{-1}{}_{DM}$	57.6 ± 0.3	35 ± 0	120 ± 1	383 ± 1	131 ± 1	128 ± 1	udl
	Cell soluble (CS)	$g\ kg^{-1}{}_{DM}$	478 ± 2	680 ± 3	554 ± 2	331 ± 2	466 ± 2	174 ± 2	1000
	Starch	$g\ kg^{-1}{}_{DM}$	151 ± 1	637 ± 3	30.3 ± 1	8 ± 0.01	4.6 ± 0	28.5 ± 0.1	udl
	Soluble sugars	$g\ kg^{-1}{}_{DM}$	140 ± 1	1.5 ± 0	1.7 ± 0.03	udl	udl	100 ± 0	1000
	Gross energy	$MJ\ kg^{-1}{}_{DM}$	19.3 ± 0.2	17.2 ± 0.2	17.1 ± 0.2	23.9 ± 0.3	17.9 ± 0.2	18.7 ± 0.2	14.2 ± 0.1
Organic matter bioavailability	*In vitro* digestibility	$g\ kg^{-1}{}_{DM}$	771 ± 2	393 ± 1	942 ± 4	362 ± 2	524 ± 2	347 ± 1	1000
	OD_{20}	$g_{O2}\ kg^{-1}_{DM}$	102 ± 5	128 ± 5	94 ± 4	72 ± 2	95.4 ± 2.2	53.4 ± 1.7	250 ± 8
Dark fermentation test	BHP	$Ndm^3_{H2}\ kg^{-1}_{DM}$	106 ± 23	107 ± 13	69.9 ± 3.1	48.7 ± 8.9	96.6 ± 5.9	102 ± 10	186 ± 10

*udl = under detection limit.

samples (Table I). Soluble sugars were found in relatively high concentrations (10% of DM) only in corn silage, as well as in giant cane (Table I), while undetectable in rice bran and olive pomace and negligible in beet silage (Table I).

The gross energy content varied from 17.1 MJ kg^{-1}_{DM} (beet silage) to 23.9 MJ kg^{-1}_{DM} (olive pomace).

3.2. Bioavailability *versus* chemical properties

The bioavailability to microbial hydrolysis sensibly varied for the considered set of biomass samples. *In vitro* digestibility ranged from a minimum of 347 g kg^{-1}_{DM} (giant cane) to a maximum of 942 g kg^{-1}_{DM} (beet silage). Malt powder showed a surprisingly low digestibility compared to what expected for a starch-rich substrate (Table I). This may be caused by a low amylase activity that may occur in rumen and/or, more probably, by partial inhibition of the ruminal activity because of acidosis, as often reported for starch-rich diets in bovines [33]. On the other hand, the SOUR test applied on malt powder resulted in relatively high OD_{20} (128 $g_{O2}\ kg^{-1}_{DM}$), as compared to the other organic matrices (Table I), that were in the range 50 – 100 $g_{O2}\ kg^{-1}_{DM}$ (Table I). Pure glucose gave 250 $g_{O2}\ kg^{-1}_{DM}$, i.e. double OD_{20} than malt powder. Being malt mostly composed of starch (Table I), i.e. of carbohydrate polymers, these results highlight the difference in bioavailability between carbohydrate polymers and the corresponding readily soluble monomers (glucose).

Interestingly, when Pearson's correlation matrix was performed, significant ($p < 0.05$, $n = 7$) negative correlations were found for digestibility *versus* ADF ($r = -0.750$), NDF ($r = -0.786$) and *versus* EE ($r = 0.893$) (Figure 1). This confirms that, among chemical components, fibrous and fat fractions (complex and insoluble polymers) negatively influence hydrolysis and reduce carbon bioavailability. Similarly, OD_{20} gave significant negative correlations especially with fibrous fractions, i.e. with ADL ($r = -0.821$), ADF ($r = -0.857$) and NDF ($r = -0.893$) (Figure 1).

Even if this highlights relatively obvious links between the most recalcitrant fractions of OM and its bioavailability, it is important to confirm that OD_{20}, in particular, can be considered as a representative variable for describing bioavailability of OM to microbial hydrolysis, as already reported in previous literature contributions [34]. Interesting is also the significant negative correlation found between digestibility and EE. This highlights, in particular, the difficulty of lipid-like fractions to be readily bioavailable in aqueous media, as described by other authors [18,32].

3.2.1. Dark fermentation tests

All incubated bioreactors gave consistent biogas productions, with relatively high H_2 contents (35–50% *v/v*). No detectable methane was found in the biogas produced by all bioreactors, as expected for optimal

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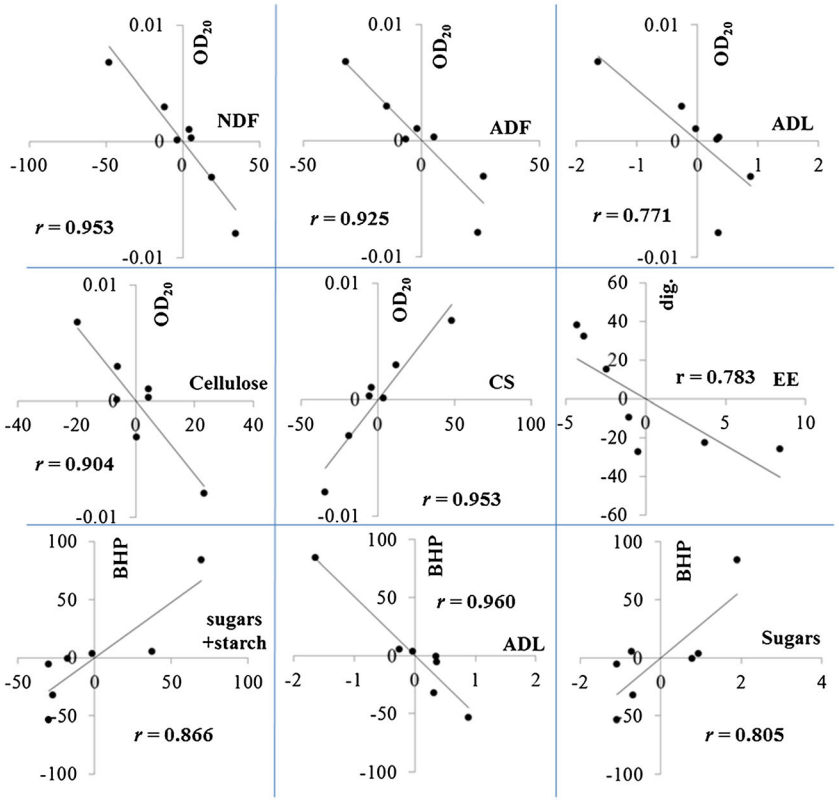

Figure 1. Significant Pearson's correlations ($p < 0.05$, $n = 7$) found for chemical composition *versus* bioavailability (represented by *in vitro* digestibility and OD_{20}) and BHP (r = correlation coefficient).

DF process conditions. Ultimate bio-hydrogen productions were reported in Table I, as average of the triplicate tests.

Pure glucose produced 186 ± 10 Nl_{H2} kg_{DM}^{-1}, which corresponds to 1.49 mol_{H2} $mol_{glucose}^{-1}$; this result is in line with the results obtained by various authors in similar conditions, reported for comparison by Tenca et al. [29]. All other matrices resulted in lower H_2 production, with similar results (102–107 Nl_{H2} kg_{DM}^{-1}) for malt powder, corn silage and giant cane. Slightly lower BHP resulted from rice bran and the lowest was found for olive pomace (Table I).

3.2.2. BHP *versus* physical–chemical properties

A series of Pearson's correlations between BHP and different chemical components was performed on normalized data (Table II). Pearson's analysis showed strong negative correlations of BHP *versus* recalcitrant compounds, especially with the less digestible portions of fibers, i.e. ADL ($r = -0.893$, $p < 0.01$). Significant positive correlations for BHP were found also with soluble sugars + starch ($r = 0.893$, $p < 0.05$) and with OD_{20} ($r = 0.786$, $p < 0.05$).

On the other hand, no significant correlations of BHP were found with neither starch nor soluble sugars, when considered as single variables. Furthermore, no significant correlations were found for other fractions of the OM, such as proteins and lipids, NDF, ADF, cellulose and hemicellulose.

This means that while OM bioavailability (represented here as *in vitro* digestibility and OD_{20}) is strongly linked to the presence of the most recalcitrant fractions such as fibrous fractions and the most insoluble hydrophobic molecules (EE) (Figure 1), the fate of the same OM in an optimized DF environment follows slightly different paths. Bioavailability is still an important factor, as confirmed by the positive correlation found for BHP and OD_{20} (Figure 1) and the negative correlation for BHP and ADL (Figure 1), but it is not the only factor. Sugars and starch (caught together as bioavailable carbohydrates) were the only specific chemical fractions that significantly correlated to BHP (Figure 1). On the other hand, sugars and starch were not significantly correlated to bioavailability, because they are not the only bioavailable fractions of the OM; it is well known that part of proteins, hemicellulose, part of cellulose, part of EE, are also readily available to microbial hydrolysis [35]. This means that a specific dependency of bio-hydrogen generation during DF is to be ascribed to the initial presence of sugars + starch. Furthermore, this dependency is not particularly linked to their bioavailability (no significant correlation found), i.e. to their behavior during microbial hydrolytic mechanism, but it must regard to the other mechanism driven by DF, i.e. fermentative metabolism itself. In other words, not all the easily hydrolysable

Table II. PLS cycles for prediction of BHP *versus* chemical composition and bioavailability of the organic matter.

PLS cycle	Predictor variables	Importance of the predictor	Optimal number of component	R^2	R^2_{cv}
	Organic matter	0.05	1	0.77	0.08
1	Raw proteins	−0.06			
	Ethereal extract	−.06			
	NDF	−0.08			
	ADF	−0.08			
	ADL	−0.11			
	Hemicellulose	−0.05			
	Cellulose	−0.05			
	Cell Soluble	0.08			
	Starch	−0.03			
	Soluble sugars	0.09			
	Energy content	−0.06			
	Digestibility	0.05			
	OD_{20}	0.07			
	Starch + soluble sugars	0.1			
2	Raw proteins	−0.07	1	0.78	0.46
	Ethereal extract	−0.07			
	NDF	−0.09			
	ADF	−0.09			
	ADL	−0.12			
	Cell soluble	0.09			
	Soluble sugars	0.1			
	Energy content	−0.07			
	Digestibility	0.06			
	OD_{20}	0.08			
	Starch + soluble sugars	0.11			
3	NDF	−0.04	2	0.91	0.61
	ADF	−0.04			
	ADL	−0.27			
	Cell soluble	0.04			
	Soluble sugars	0.39			
	OD_{20}	−0.03			
	Starch + soluble sugars	0.21			
4	ADL	−0.29	1	0.91	0.81
	Soluble sugars	0.25			
	Starch + soluble sugars	0.26			

[a]PLS cycles (from 1 to 4) performed step by step, excluding variables characterized by the smallest *importance coefficient*.

fractions of OM (e.g. proteins, hemicellulose, etc.), that are fermented during acidification process, can be related to metabolic pathways driving to H_2 release, as previous literature often highlighted [17,36–38].

3.2.3. Statistical model to predict BHP with physical–chemical properties

Multiple partial least square (PLS) analysis performed on normalized data gave a selection, among chemical components, of those with higher influence on H_2 production and their relevance. The cycles performed for statistical selection were 4, and they are reported in Table II, together with the importance of each predictor selected, the optimal number of component, R^2 and R^2_{cv}. Multiple PLS resulted in a linear regression model able to predict BHP according to the following equation, based on three important variables:

$$\begin{aligned}\text{BHP} = {} & 110.24\text{–}20.04\ \arcsin\sqrt{\text{ADL}} \\ & +11.4\ \arcsin\sqrt{\text{soluble sugars}} \\ & +0.3749*(\text{starch} + \text{soluble sugars});\end{aligned}$$

in which BHP is expressed as Nl kg^{-1}_{DM} and ADL, soluble sugar and starch as % (*w/w*) of DM.

The equation showed good regression coefficient ($R^2 = 0.91$, $p < 0.05$) and high predictability ($R^2cv = 0.84$, $p < 0.05$). This was confirmed by the comparison between experimental and modeled data, considering a dataset of literature data and two new samples (Figure 2). A statistical validation was performed by applying the method used by Schievano et al. [19]. A double validation was done, first considering both the original dataset alone ($n = 7$) and then adding the new dataset ($n = 14$). The calculated validation indexes (Figure 2) were acceptable, when compared to typical ranges found in literature [19].

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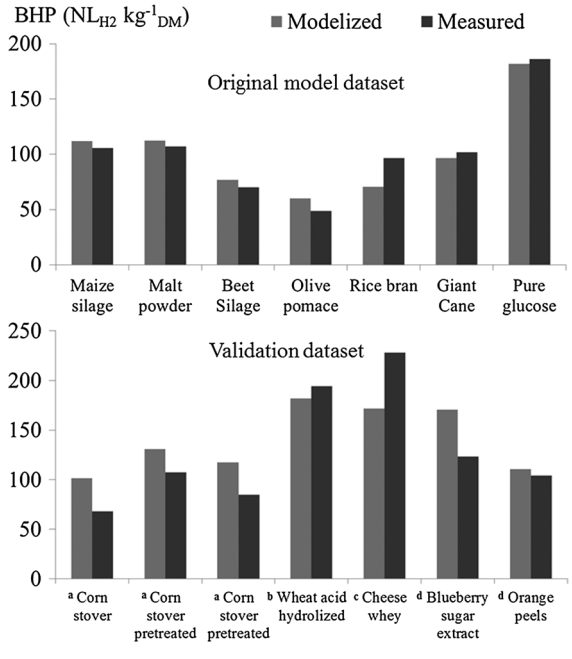

Validation Parameter:	[e] RRMSE	[f] EF	[g] R^2	Signif.
Best	0.00	1.00	1.00	< 0.05
Model (initial dataset)	22.40	0.66	0.67	0.02
Validation (improved dataset)	26.49	0.60	0.72	0.02

Figure 2. Comparison between modeled and measured bio-hydrogen potential (BHP); validation of the proposed model using the original and a new dataset. [a] Li et al. [39]; [b] Ozmihci et al. [40]; [c] Davila-Vazquez et al. [41]; [d] New samples; [e] Root mean squared error [19]; [f] Modeling efficiency [19]; [g] Regression coefficient [19].

This model allows obtaining a quick prediction of the efficiency (in terms of BHP) of DF process, when applied to heterogeneously composed biomass, by measuring few characteristics of its OM quality. This idea has been proposed in the past decades for full-scale applications of anaerobic digestion, to deal with the large variety of waste types available on market [19,28,42]. The same approach is here proposed as path to widen DF applicability to mixed waste materials, which composition normally vary consistently depending on seasonal factors, origin (industrial/agricultural process), pretreatment efficiency, etc.

This equation is a first example of a new way of understanding/modeling the complex pool of physical, chemical, biochemical and biological mechanisms involved in DF. However, it can only be considered as an initial attempt in developing the proposed new approach, for at least three reasons:

a. the set of samples here used was relatively limited (seven samples); the proposed statistical approach should in the next future be developed with larger numbers of samples, to ensure more generalized results;
b. the characterization of the OM should be refined by introducing more peculiar analytical variables, such as specific enzymatic attack assays and/or more detailed chemical characterization;
c. here, BHP was used, for simplicity reasons, as sole parameter to discriminate the effect of DF on a given OM. However, bio-hydrogen generation should not be the sole focus of DF, in future research. Instead, DF should mainly be considered as a pre-step to transform waste/mixed solid biomass into soluble carboxylates for secondary bio-based and waste-based refinery processes, such as microalgal mixotrophic cultivations, anodic current generations for MECs, microbial electrosynthesis, intracellular and extracellular polymeric compound accumulation and secondary fermentations towards target building blocks, as recently indicated by Rabaey and Ragauskas [43]. This, even if little bio-hydrogen is evolved and other fermentation pathways are naturally followed by UMC. For these reasons, future efforts in describing the relationship between the composition of a mixed waste-based OM and its fate in DF should also be focused on the production of target spectra of soluble carboxylates, in accordance to recent advances in this field [1,3,6,8,43].

4. CONCLUSIONS

This work was aimed at proposing and gives only a first hint of a new approach, which can be explored by more comprehensive studies, for better understanding the behavior of UMC during DF, as obligate step towards the use of waste OM as substrate for bio-refineries. The fate of all different fractions of the OM in DF (i.e. proteins, hemicellulose, cellulose, aromatics, complex alcohols, triglycerides and waxes) should be better investigated, especially emphasizing the influence of their bioavailability and focusing mainly on soluble products obtainable from hydrolysis–fermentation of solid organic matter, because in the near future, this process is expected to be no longer utilized with the sole focus of bio-H_2 generation. In the next step of this approach, H_2 should be considered only one among a large variety of substances that can be separated by DF from waste OM. DF, itself, should be considered as a versatile UMC-based intermediate ring of new bio-refinery chains, aimed at optimizing transformation of complex OM into soluble or gaseous cocktails of compounds that can be utilized as substrate in the upcoming 'carboxylate platform'.

REFERENCES

1. Rabaey K, Rozendal RA. Microbial electrosynthesis—revisiting the electrical route for microbial production. *Nature Reviews Microbiology* 2010; **8**:706–716.

2. Mohan SV, Pandey A. Biohydrogen production: an introduction. In *Biohydrogen*. Elsevier: Burlington, 2013; 1–24.
3. Redwood MD, Orozco RL, Majewski AJ, Macaskie LE. Electro-extractive fermentation for efficient biohydrogen production. *Bioresource Technology* 2012; **107**:166–174.
4. Dahlqvist E (Ed.). *Biomass as Energy Source: Resources, Systems and Applications*. CRC Press, Taylor & Francis: London, UK, 2013.
5. Percival Zhang YH, Xu JH, Zhong JJ. A new high-energy density hydrogen carrier-carbohydrate-might be better than methanol. *International Journal of Energy Research* 2013; **37**(7):769–779.
6. Agler MT, Wrenn BA, Zinder SH, Angenent LT. Waste to bioproduct conversion with undefined mixed cultures: the carboxylate platform. *Trends in Biotechnology* 2011; **29**(2):70–78. doi:10.1016/j.tibtech.2010.11.006.
7. Adani F, Papa G, Schievano A, Cardinale G, D'Imporzano G, Tambone F. Nanoscale structure of the cell wall protecting cellulose from enzyme attack. *Environmental Science & Technology* 2011; **45**:1107–1113.
8. Redwood MD, Orozco RL, Majewski AJ, Macaskie LE. An integrated biohydrogen refinery: synergy of photofermentation, extractive fermentation and hydrothermal hydrolysis of food wastes. *Bioresource Technology* 2012; **119**:384–392.
9. Lee WE, May Chua AS, Yeoh HK, Ngoh GC. A review of the production and applications of waste-derived volatile fatty acids. *Chemical Engineering Journal* 2014; **235**:83–99.
10. Singhania RR, Patel AK, Christophe G, Fontanille P, Larroche C. Biological upgrading of volatile fatty acids, key intermediates for the valorization of biowaste through dark anaerobic fermentation. *Bioresource Technology* 2013; **145**:166–174.
11. Hallenbeck PC. Fundamentals of biohydrogen. In *Biohydrogen*. Elsevier: Burlington, 2013; 25–43.
12. Liu K, Atiyeh HK, Stevenson BS, Tanner RS, Wilkins MR, Huhnke RL. Mixed culture syngas fermentation and conversion of carboxylic acids into alcohols. *Bioresource Technology* 2014; **152**:337–346.
13. Schievano A, Tenca A, Scaglia B, Merlino G, Rizzi A, Daffonchio D, Oberti R, Adani F. Two-stage vs single-stage thermophilic anaerobic digestion: comparison of energy production and biodegradation efficiencies. *Environmental Science & Technology* 2012; **46**:8502–8510. doi: 10.1021/es301376n
14. Monlau F, Sambusiti C, Barakat A, Quéméneur M, Trably E, Steyer JP, Carrère H. Do furanic and phenolic compounds of lignocellulosic and algae biomass hydrolyzate inhibit anaerobic mixed cultures? A comprehensive review. *Biotechnology Advances* 2014; **32**:934–951.
15. Yokoi H, Saitsu A, Uchida H, Hirose J, Hayashi S, Takasaky Y. Microbial hydrogen production from sweet potato starch residue. *Journal of Bioscience and Bioengineering* 2001; **91**:58–63.
16. Kapdan IK, Kargi F. Bio-hydrogen production from waste materials. *Enzyme and Microbial Technology* 2006; **38**:569–582.
17. Lay J, Fan K, Chang J, Ku K. Influence of chemical nature of organic wastes on their conversion to hydrogen by heat-shock digested sludge. *International Journal of Hydrogen Energy* 2003; **28**:1361–1367.
18. Silvestre G, Illa J, Fernández B, Bonmatí A. Thermophilic anaerobic co-digestion of sewage sludge with grease waste: effect of long chain fatty acids in the methane yield and its dewatering properties. *Applied Energy* 2014; **117**:87–94.
19. Schievano A, Scaglia B, D'Imporzano G, Malagutti L, Gozzi A, Adani F. Prediction of biogas potentials using quick laboratory analyses: upgrading previous models for application to heterogeneous organic matrices. *Bioresource Technology* 2009; **100**:5777–82.
20. Sluiter A. *Determination of Total Solids in Biomass Natl Renew Energy Lab*. : Golden, CO, USA, 2005.
21. Lahav O, Morgan B, Loewenthal RE. Rapid, simple and accurate method for measurement of VFA and carbonate alkalinity in anaerobic reactors. *Environmental Science & Technology* 2002; **36**:2736–3274.
22. Bremner JM. Nitrogen—total. Methods of soil analysis, part 3—chemical methods. 1996; 1085–1121.
23. Van Soest PJ, Robertson JB, Lewis BA. *Methods for Dietary Fiber, Neutral Detergent Fiber, and Nonstarch Polysaccharides in Relation to Animal Nutrition*. Cornell University, Department of Animal Science and Division of Nutritional Science: Ithaca, NY, 1991.
24. AOAC. *Official Methods of Analysis* (13th). Association of Analytical Chemist: Washington, 1980.
25. Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F. Colorimetric method for determination of sugar and related substances. *Analytical Chemistry* 1956; **28**:350–356.
26. MEGAZYME Assay Procedure (Amyloglucosidase/α-Amylase method), K-TSTA 04/2009.
27. Robinson PH, Campbell Mathews M, Fadel JG. Influence of storage time and temperature on in vitro digestion of neutral detergent fibre at 48 h, and comparison to 48 h in sacco neutral detergent fibre digestion. *Animal Feed Science and Technology* 1999; **80**:257–266.
28. Schievano A, Pognani M, D'Imporzano G, Adani F. Predicting anaerobic biogasification potential of ingestates and digestates of a full-scale biogas plant using chemical and biological parameters. *Bioresource Technology* 2008; **99**:8112–8117.

29. Tenca A, Schievano A, Lonati S, Malagutti L, Oberti R, Adani F. Looking for practical tools to achieve next-future applicability of dark fermentation to produce bio-hydrogen from organic materials in Continuously Stirred Tank Reactors. *Bioresource Technology* 2011; **102**:7910–7916.
30. Tenca A, Schievano A, Perazzolo F, Adani F, Oberti R. Biohydrogen from thermophilic co-fermentation of swine manure with fruit and vegetable waste: maximizing stable production without pH control. *Bioresource Technology* 2011; **102**:8582–8588.
31. Sheskin DJ. *Handbook of Parametric and Nonparametric Statistical Procedures* (3rd). CRC press: US, 2003.
32. Andries PMJ, Heyden YV, Buydens LMC. Improved variable reduction in partial least squares modelling based on Predictive-Property-Ranked Variables and adaptation of partial least squares complexity. *Analytica Chimica Acta* 2011; **705**:292–305.
33. Nagaraja TG, Titgemeyer EC. Ruminal acidosis in beef cattle: the current microbiological and nutritional outlook. *Journal of Dairy Science* 2007; **90**(1):17–38.
34. Schievano A, D'Imporzano G, Malagutti L, Fragali E, Ruboni G, Adani F. Evaluating inhibition conditions in high-solids anaerobic digestion of organic fraction of municipal solid waste. *Bioresource Technology* 2010; **101**(14):5728–5732.
35. Papa G, Scaglia B, Schievano A, Adani A. Nanoscale structure of organic matter could explain litter decomposition. *Biogeochemistry* 2013. doi:10.1007/s10533-013-9863-z.
36. Akutsu Y, Li Y, Harada H, Yu H. Effect of temperature and substrate concentration on biological hydrogen production from starch. *International Journal of Hydrogen Energy* 2009; **34**:2558–2566.
37. Noike T, Mizuno O. Hydrogen fermentation from municipal wastes. *Water Science and Technology* 2000; **42**:155–162.
38. Yu H, Zhu Z, Hu W, Zhang H. Hydrogen production from rice winery wastewater in an upflow anaerobic reactor by using mixed anaerobic cultures. *International Journal of Hydrogen Energy* 2002; **27**:1359–1365.
39. Li Q, Guo C, Liu CZ. Dynamic microwave-assisted alkali pretreatment of cornstalk to enhance hydrogen production via co-culture fermentation of *Clostridium thermocellum* and *Clostridium thermosaccharolyticum*. *Biomass and Bioenergy* 2014; **64**:220–229.
40. Ozmihci S, Kargi F, Cakir A. Thermophilic dark fermentation of acid hydrolyzed waste ground wheat for hydrogen gas production. *International Journal of Hydrogen Energy* 2011; **36**:2111–2117.
41. Davila-Vazquez G, Alatriste-Mondragón F, de León-Rodríguez A, Razo-Flores E. Fermentative hydrogen production in batch experiments using lactose, cheese whey and glucose: influence of initial substrate concentration and pH. *International Journal of Hydrogen Energy* 2008; **33**:4989–4997.
42. Gunaseelan VN. Regression models of ultimate methane yields of fruits and vegetable solid wastes, sorghum and napiergrass on chemical composition. *Bioresource Technology* 2007; **98**:1270–1277.
43. Rabaey K, Ragauskas AJ. Editorial overview: energy biotechnology. *Current Opinion in Biotechnology* 2014; **27**:5–6.