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Abstract

Solid organic waste represents an abundant, cheap, and available source of biodegradable substrates not yet exploited to produce biohydrogen by dark fermentation. The impact of the composition of solid organic waste on microbial metabolic pathways and subsequently on biohydrogen production, has not been clearly elucidated. The aim of this study is to determine the compositional features of different substrates that influence bioH2 production. For this, we measured Biological hydrogen potentials (BHP) on 26 different substrates and performed a multivariate statistical analysis of the experimental data using a partial least square regression. The results showed that the BHP values correlated well with the initial carbohydrate content measured after mild hydrolysis. A predictive model explaining more than 89% of the experimental variability was then built to predict the maximal biohydrogen yield with a high accuracy and for a large spectrum of organic waste. An explicative model showed that only carbohydrates, butyrate and lactate concentrations were significant variables explaining more than 98% of biohydrogen yield variability. Interestingly, an interaction term between carbohydrates and lactate concentrations was required to explain microbial pathways producing hydrogen.

Keywords

Biohydrogen, Biological Hydrogen Potential (BHP), Correlation analysis, Dark fermentation, PLS regression.

1. Introduction

Anaerobic digestion is a microbial and ecological process widespread in nature that degrades complex organic matter to methane and carbon dioxide in strict anaerobic environments. The microbial anaerobic digestion process is composed of four steps: hydrolysis, acidogenesis, acetogenesis and methanogenesis. This natural process has been used for many years to treat liquid effluents as well as solid waste, and produce concomitantly methane used as renewable energy source in its biogas form. This latter can be further converted into electricity and heat by combustion [1-2]. Moreover, as an intermediate of the whole anaerobic process, biohydrogen can accumulate when methanogenesis is inhibited and thus can be produced from various sources of biomass [2]. Interestingly, adding 5 to 20% of biohydrogen to methane biogas generates a new biogas, so called biohythane, that, after combustion, could reduce nitrogen oxide emission and increase engine combustion performances as compared to CH_4 combustion alone [3]. Additionally, hydrogen is considered as one of the most interesting alternative energy carrier since it has the highest energy content (122 $kJ.g^{-1}$) and produces only water after combustion [4].

Besides its production under anaerobic conditions, biohydrogen is a key central metabolic intermediate and can therefore be consumed very efficiently by many microorganisms, such as methanogens, homoacetogens and sulfate-reducing bacteria [2]. It is thus necessary to limit the microbial anaerobic process to its first three steps, in a process called "dark fermentation". Besides biohydrogen, some metabolic end-products are produced concomitantly such as acetate, butyrate, propionate, ethanol and lactic acid. Similarly to biohydrogen, these end-products could be highly valuable for industrial purposes, e.g. lactate can be reused for PLA (PolyLactic Acid) bioplastic production, a renewable and biodegradable plastic [5]. Metabolic end-products, so-called metabolites, could also serve to improve further methane production in a second step [6]. Liu *et al*. (2006) showed that methane yield was 21 % higher in a two-step than in one step anaerobic biogas process, likely due to an advanced hydrolysis of the substrate during the hydrogen production step [7]. Better description of the distribution of these end-products according to the kind of organic matter is therefore essential to better evaluate the biomass conversion potentiality in a bio-refinery concept, where biohydrogen, methane as well as metabolic intermediates are recovered as valuable industrial products.

Among potential and easily available substrates, agricultural solid waste represents an abundant, cheap and highly biodegradable source of substrates that can produce hydrogen by dark fermentation. About 1.5 million tons of agricultural, forestry and fishing waste are annually produced in France [8]. Worldwide, lignocellulosic biomass residues are evaluated to exceed 220 billion of tons produced per year [9]. Over the past decades, the possibility to convert many different types of waste into biohydrogen has been investigated. In particular, food waste showed relative high performances of conversion to biohydrogen. So far, a range of 2.68 - 8.75 $mmol_{H2}\ g_{VS}^{-1}$ has been reported for food waste collected in food restaurants [10-13]. Nevertheless, the "food waste" category, when investigated as feedstock for hydrogen production, represents a wide variety of substrates including kitchen refuse, municipal waste, food industry co-products such as oil mill, cheese whey and starch-manufacturing waste [2]. Similarly, a wide group of agri-industrial waste has been investigated for hydrogen production and constitutes a very promising category of feedstock. Indeed, high biohydrogen production yields were observed from 3.77 $mmol_{H2}\ g_{VS}^{-1}$ to 11.67 $mmol_{H2}\ g_{hexose}^{-1}$ and 12.95 $mmol_{H2}\ g_{VS}^{-1}$ for palm oil mill effluent, molasses and cheese whey, respectively [14-17]. The use of a third type of waste, called agricultural residues that are generated from the primary agricultural sector, such as maize stalks or rice straws is usually reported in association with acidic, enzymatic or microwave pretreatments. This results in few available data dealing with hydrogen production from raw agricultural waste. Overall, reported hydrogen production yields vary a lot for a given category of feedstock, mainly because of the high variability in nature and composition of the substrates, as well as the differences in experimental procedures, *e.g.* batch or continuous reactors [10-12]. Moreover, structural features as well as chemical composition of organic substrates could have both an important role on hydrogen production in dark fermentation bioprocesses [18]. When considering the whole anaerobic digestion process of complex organic solid waste, only few studies dealt with the link existing between the complex chemical composition of such organic substrates and methanogenesis. Buffiere *et al.* (2006) reported a strong correlation between the composition in cellulose and lignin of seven solid organic substrates and their biological methane potential [19]. They showed that the sum of cellulose and lignin of organic substrates presented a linear and negative impact on methane production yields. The authors suggested that such linear correlation was mainly due to bio-accessibility constraints for hydrolysis of complex solid substrates.

Although many different types of substrates have already been assessed for their potential of hydrogen production, the impact of experimental set up, type of substrate as well as microbial ecosystem variability make difficult to predict accurately biohydrogen yields when using different categories of waste. Furthermore, indicators of well-established microbial metabolic routes are still missing. Therefore, the purpose of the present study was to investigate biological hydrogen yields of a large range of solid organic waste to further evaluate the impact of the biochemical composition of these substrates on fermentative microbial processes. For this, we performed a multivariate analysis by partial least square regression method to build a predictive model of biohydrogen production yield according to the biochemical composition of the solid waste. Concomitantly, we built an explicative model emphasizing the role of the main microbial metabolic pathways occurring in dark fermentation.

2. Material and methods

2.1. Experimental substrates and chemical composition analysis

Samples of 26 different solid organic substrates were used in this study. We distinguished four groups : (i) the first group corresponded to the substrates rich in carbohydrates, including apples (*royal gala*), carrots, Jerusalem artichoke roots, maize flour, oats, potatoes, and wheat flour; (ii) the second group corresponded to the substrates rich in proteins, i.e. soybean milk cake, chicken meat, cow manure with straw, fish residues, and meat waste from restaurants, (iii) the third group corresponded to agri-industrial waste, including food waste from restaurants, rapeseed oil cakes, sunflower oil cakes, grape marc, vegetable waste from restaurants, fruit peels (orange peels and banana peels) and maize cob, (iv) and the last group corresponded to agricultural end-products, such as Jerusalem artichoke leaves and stalks, giant reed stalks and leaves, maize stalks, rice straw and sorghum stalk.

To homogenize the samples, all substrates were freeze-dried for 48 hours and milled (particule size < 3 mm) in a blender. Total solids (TS) were quantified according to standard methods [20]. Carbohydrates were extracted using a gentle acid-extraction method with starch as reference substrate. For this, around 500 mg of samples were added to 40 mL of 2 N HCl in a sealed glass vial and put in an ultrasonic bath for one hour at room temperature. The liquid fraction was then centrifuged and carbohydrate concentration was measured using the

anthrone method [21]. The carbohydrate concentration was expressed in glucose(Glc)-equivalent. Protein composition was assessed by extraction in sodium hydroxide solution. For this, around 500 mg of substrate were treated with 40 mL of 0.5M NaOH in an ultrasonic bath for one hour. The supernatants were then centrifuged and protein concentrations were measured by the Lowry method and expressed in bovine serum albumin (BSA) equivalent [22].

2.2. Biological hydrogen potential (BHP) test

Batch experiments, so called biological hydrogen potential (BHP) tests, were carried out at 37°C in sealed 600 mL serum flasks in duplicates. Each flask contained from 1 to 9 gTS of substrate, in order to observe a minimal volume of 10 mL of hydrogen production for subsequent analysis. At start of the experiment, 200 mL of MES (2-[N-morpholino]ethane sulfonic acid, 40 mmol.L^{-1}) buffer and 3 mL of seed sludge (final concentration of 225 mg-$_{COD}$.L^{-1}) were added to the flask. The seed inoculum, corresponding to an outlet sludge sample from a semi-industrial anaerobic digester treating vinasses, was heat treated at 90°C for 10 minutes to deactivate methanogens prior to inoculation. After heat-shock treatment, the inoculum was stored at -20°C to provide the same inoculum to all BHP experiments. No additional nutrient medium solution was added. The initial pH value was adjusted to 5.5 with NaOH 2N or 37% HCl. The headspace of the flasks was flushed with nitrogen gas for 5 minutes to provide anaerobic conditions. The experimental procedure ended when the pressure in the flask headspace started to drop off indicating hydrogen consumption. Each experiment was performed in duplicates.

Total biogas production was daily measured with a water displacement method, and biogas volume corresponded to the volume of acidified water displaced (pH=2). Biogas composition was determined using a gas chromatograph coupled to catharometric detection (Shimadzu GC-8A), as described elsewhere [23].

To evaluate the biohydrogen yield, cumulative hydrogen production was determined for all substrates. A modified Gompertz equation model (Equation 1) was fitted to experimental data of cumulative hydrogen production as shown in Figure 1.

$$H(t) = H_{max} \cdot \exp\{-\exp[R \cdot e/ H_{max} (T_{lat} - t) + 1]\} \qquad \text{(Equation 1)}$$

where H_{max} is the maximum cumulated hydrogen amount (hydrogen potential) expressed in mL of hydrogen per gram of total solids (mL g_{TS}^{-1}), R is the maximum hydrogen productivity (in mL $L^{-1} d^{-1}$), T_{lat} is the lag-phase (in days) and t is the incubation time (in days). This model was fitted to the experimental data in using a non-linear regression algorithm (Matlab V6.5, Mathworks).

[FIGURE 1]

2.3. Analytical methods for determination of metabolic products

Volatile fatty acids (VFAs), i.e. acetic(C_2), propionic (C_3), butyric and iso-butyric (C_4 and iC_4), valeric and iso-valeric (C_5 and iC_5) and caproic (C_6) acids were determined with a gas chromatograph coupled to flame ionization detection (FFAP column, VARIAN 3900 chromatography systems, Middelburg, The Netherlands) [24]. Organic acids (lactate), ethanol were quantified by high-pressure liquid chromatography (HPLC) coupled to a refractometer (Waters R410). The analysis was performed with an Aminex column (HPX-87H, 300x7.8mm) at 35°C (Bio-rad) under an isochratic elution of 0.005M H_2SO_4 with a flow rate at 0.4 mL/min [24].

2.4. Multivariate analysis of experimental data by Partial Least Square regression method

The partial least square (PLS) regression method corresponds to a multivariate analysis of experimental data based on the construction of explicative PLS factors, also called principal components, by minimizing the covariance between dependent variables (Y block: BHP values) and the explicative variables (X block: substrate and product concentrations). The algorithm constructs orthogonal PLS factors of each block by minimizing the covariance between X and Y blocks. The first PLS factor contains the highest percentage of variance, and the following factors account for decreasing amounts of variance. We developed a predictive model of hydrogen yield by considering only the initial biochemical composition of the substrates, while metabolic end-products were additionally considered in an explicative model. Prediction of the Y block was assessed with a multivariable linear regression on X block through PLS1 models using the software R version R 1.2.2 for Windows and using PLS functions developed by Durand *et al.* (1998) [25]. We used a PRESS criterion (predicted residual error sum of square) in a leave-two-out cross-validation procedure to choose the final dimension of the predictive model. The p-values of the regression coefficients were calculated by using the PLS algorithm with the Unscrambler software (version 10.2).

3. RESULTS AND DISCUSSION

3.1. Biochemical characteristics of the 26 solid organic substrates

The distribution in biochemical components of the 26 organic substrates is given in Table 1. In the group of substrates rich in carbohydrates, i.e. apples, carrots, wheat flour, Jerusalem artichoke roots, maize flour, and oats, carbohydrates represented from 51.3 % to 95.2 % of total solids and from 70 % to 100 % of volatile solids. In the "protein-rich" group, *i.e.* chicken meat, cow manure, fish residues and meat residues from restaurant, total proteins represented from 14.3% to 19.2% of total solids and 69% to 100% of volatile solids. As the highest protein-rich substrate, grape marc was composed of more than 72% of proteins in terms of total solids. The other types of substrates corresponded to complex mixtures of agri-industry organic waste and agricultural end-products with a balanced proportion of carbohydrates and proteins contents. In this study, lipids and lignin contents were not characterized since these two compounds do not represent a potential source of fermentative biohydrogen [18]. Moreover, monomeric constituents of cellulosic polymers are hard to solubilize when embedded within the lignocellulosic matrix and were likely not totally extracted by the method used in this study. That might explain the very low carbohydrate contents found in several lignocellulosic substrates, such as giant reed stalks, maize stalks and rice straws.

[TABLE 1]

3.2 Substrate mapping based on hydrogen yields

The biogas produced in all batch cultures was only composed of H_2 and CO_2, with no detectable CH_4, confirming that the heat-shock pretreatment applied on the inoculum was efficient to suppress all methanogenic activity. The R^2 coefficients generated by fitting the Gompertz model to experimental data of hydrogen accumulation were all over 0.98, indicating that this model was highly suitable to describe the kinetics of the BHP tests. Maximum cumulated hydrogen production yields (H_{max}) are presented in Figure 2. An analysis of variance (one-way ANOVA) of the data clearly shows that the category of substrate had a highly significant effect on hydrogen yield (p-value<0.001). The average of H_{max} for carbohydrate-rich-content substrates, protein-rich-content substrates, agri-industrial waste and agricultural end-products were 155 ± 38 mL$_{H2}$ g$_{TS}^{-1}$, 6 ± 5 mL$_{H2}$ g$_{TS}^{-1}$, 42 ± 24 mL$_{H2}$ g$_{TS}^{-1}$, 29 ± 20 mL$_{H2}$ g$_{TS}^{-1}$, respectively. Carbohydrate-rich substrates are mostly referred in the

literature to favour biohydrogen production through fermentative pathways, and our results are consistent with these findings [2; 26]. In our study, the carbohydrates content corresponded more precisely to the fraction of sugars measured after mild hydrolysis, indicating that not only soluble sugars are susceptible to be degraded but also carbohydrates readily accessible for microorganisms. This suggests that pretreatment method deteriorating the inner structure of lignocellulosic materials should be preferentially used to make more accessible the carbohydrates, although this could be counterbalanced by the release of inhibitory compounds [23]. Maize flour which is only composed of soluble sugars showed the highest carbohydrate content (952 mg_{glc} g_{TS}^{-1}) and consequently the highest BHP value (224 mL_{H2} g_{TS}^{-1}). Interestingly, this value is close to the theoretical hydrogen yield in mixed culture proposed by Hawkes *et al.* (2007) , *i.e.* 2.5 mol $_{H2}$ g $_{hexose}^{-1}$ equivalent to 311 ml_{H2} g $_{hexose}^{-1}$ [27].

In addition, we observed the lowest range of hydrogen yields from only 2 to 7 mL_{H2} g_{TS}^{-1} with protein-rich substrates, suggesting that the protein composition was unfavourable to biohydrogen production. Our results are consistent with the low hydrogen yields found in the literature from similar protein-rich substrates, *e.g.* 7 mL_{H2} g_{TS}^{-1}, 8 mL_{H2} g_{TS}^{-1} and 21 mL_{H2} g_{TS}^{-1} from eggs, lean meat and bean curd manufacturing waste, respectively [28-29]. Xiao et al. (2010) reported previously that the optimal pH for biohydrogen production from proteins is alkaline (>8.5) which is also consistent with our experiments [30]. In the groups of agri-industry waste and agricultural end-products, the selected substrates showed a wide range of hydrogen yields with several interesting hydrogen production potentials, reaching 71 mL_{H2} g_{TS}^{-1} and 68.7 mL_{H2} g_{TS}^{-1} for foodwaste and Jerusalem artichoke stalks, respectively (Table 1). Interestingly, sunflower and rapeseed oil cakes that are the main waste generated from the oil industry had significant hydrogen yields of respectively 15.8 mL_{H2} g_{TS}^{-1} and 43.3 mL_{H2} g_{TS}^{-1}, offering a new way of utilization of these two agri-industrial by-products.

[FIGURE 2]

3.3 Substrate mapping considering biochemical composition, metabolic end-products and hydrogen yields.

A PLS (Partial Least Square) regression was carried out to map the substrates according to their biochemical composition, the metabolic end-products generated as well as the hydrogen production yields. The biohydrogen yields (H_{max}) of 21 substrates, also called

biological hydrogen potential (BHP) values, were used as Y-block variables. Six explicative variables constituted the X-block such as the biochemical fractions defined by initial carbohydrate and protein compositions of the substrates, and the metabolic end-product concentrations such as acetate, butyrate, propionate and lactate determined at the end of fermentation. The visual representation of the 21 substrates is presented in Figure 3A and 3B after projection in the plans formed by the three first principal components or latent variables. The three first latent variables t1, t2 and t3 represent respectively 41 %, 21 % and 19% of the variance of X-block variables among a total variance of 81%. All the carbohydrate-rich substrates are located on the right side of the figures and the other substrates on the left corroborating the link between high hydrogen production yields and carbohydrate-rich substrates. The t2 latent variable discriminates the protein-rich substrates having low t2 values from agricultural substrates with high values on t2 (Figure 3A). Interestingly, agricultural substrates have low values on t3 (Figure 3B), whereas agri-industry waste is the most spread group with high and low values on t3 (Figure 3B).

[FIGURE 3]

Figures 4A and 4B plot the correlation circles of the original variables (X-block and Y-block variables) with the three first latent variables t1, t2 and t3. By convention, the Y variable (BHP) is located on the right side of the circle following the first latent variable indicating that the variability of the BHP value can be well evaluated by the PLS model. The correlation circle shows that the carbohydrate content as well as the butyrate concentration correlate well with the BHP values. Surprisingly, the acetate concentration is close to the t2 latent variable and is not correlated with hydrogen production (Figure 4A). In contrast, and as expected, propionate concentration presents the same pattern, with no correlation to hydrogen production. The proximity of acetate and propionate productions might be explained by the propionate metabolism related to acetate pathway in *Propionibacterium* sp. The t3 latent variable is mostly explained by lactate and protein concentrations (Figure 4B), and is oppositely correlated to the BHP values. Because all the latent variables are independent and orthogonal between each other, the lactate production could be considered as an independent variable with no correlation with butyrate production.

[FIGURE 4]

In this study, acetate and butyrate were the two mainly end-products besides hydrogen and carbon dioxide biogas. This main metabolic route was likely resulting from the initial thermal treatment of the inoculum which leads to a strong selection of spore-forming bacteria, belonging mainly to the *Clostridium* genus [2, 31]. Indeed, fermentative metabolism of *Clostridium* species produces mainly butyrate and acetate as primary soluble metabolites [32]. From a biochemical point of view, the metabolic route from glucose to acetate gives the highest yield of hydrogen with 4 molH2.molglucose^{-1} and the pathway from glucose to butyrate leads to a yield of only 2 molH2.molglucose^{-1}. These theoretical values give the illusion that higher accumulation of acetate would lead to higher hydrogen production. However, our results showed that acetate production was not a good indicator of the biohydrogen yields. In fact, acetate is also a product of hydrogen consumers, such as homoacetogens belonging to the same *Clostridium* genus, such as *Clostridium aceticum*. Since homoacetogenesis could occur concomitantly with biohydrogen production, this could have resulted in an immediate hydrogen and carbon dioxide consumption to produce acetate, as previously shown by Siriwongrungson et al. (2007) [33]. In addition, several spore-forming bacteria acetogens originated from the same *Clostridium* genus, such as *Clostridium scatologens*, *Clostridium magnum* or *Clostridium coccoides* could use the carbohydrates to produce acetate with no release of hydrogen gas, which outcompete directly hydrogen producers when growing on the same substrate [34]. In contrast, butyrate pathway is inevitably linked to hydrogen-producing fermentation in mixed culture, and no direct hydrogen consumption pathway related to butyrate production has been yet reported. Therefore, and in accordance with our results, butyrate accumulation can be considered as a better indicator of fermentative biohydrogen production than acetate production. Hawkes et al. (2007) previously suggested that Butyrate/Acetate (B/A) ratio might be used as a quantitative indicator between microbial metabolisms associated to hydrogen production, especially in continuous bioprocesses [27]. Nevertheless, these authors proposed to clarify the usefulness and the nature of the relationship between B/A ratio and hydrogen yields. By using statistical tools, we showed that this ratio cannot be applied in the case of lignocellulosic materials and more particularly in batch tests likely because of the activity of homoacetogenic bacteria.

Only low lactate and propionate concentrations were observed with regards to the sum of the other volatile fatty acids (acetate, propionate and butyrate). Lactate production could result from either a metabolic shift of hydrogen-producing bacteria or a population change to

lactic acid bacteria. Indeed, Lin and Wang (2007) showed that when glucose was the limiting source of carbon, *Clostridium butyricum* shifted from acetate/butyrate pathway to lactate/ethanol, and even when glucose was further supplied, the metabolic routes did not return to initial hydrogen production pathways [32]. Moreover, some thermal resistant lactic acid bacteria such as *Bacillus racemilacticus* could outcompete with hydrogen producers to use carbohydrates [35]. Such metabolic shift or microbial population change might explain why lactate concentration was independent to butyrate concentration.

In addition, propionate was the main fermentation end-product in relation with acetate accumulation suggesting the occurrence of microbial metabolisms as found in *Propionibacterium* species which are able to use carbohydrate-based feedstocks and glycerol as sole carbon sources. The ratio propionate to acetate is twice higher when using glycerol rather than glucose as substrate under fermentative conditions. This might explain why propionate concentration was in such high amounts in flasks fed with sunflower oil cakes, rapeseed oil cakes, meat residue from restaurant, chicken meat and fish residues [36,37].

3.4. Building of a predictive model of biological hydrogen potential (BHP)

Considering that the highest influential variable was the carbohydrate content, a simple linear regression model was established and led to Equation 2 where the BHP value was predicted from the carbohydrate content (Carb) expressed in expressed in $g.g_{TS}^{-1}$.

BHP= 1.3052 + 199.46*Carb   (Equation 2)

The R^2 determination coefficient of this simple model was equal to 0.89 confirming that more than 89% of the experimental variability could be explained by the model that predicts biohydrogen yields with a high accuracy. Based on the total carbohydrate content, which can be determined by a gentle acid extraction method, this model could be used as a rapid tool of substrate evaluation. This model is in good accordance with Monlau et al. (2012) who reported a similar predictive model based on carbohydrate contents of other types of substrates [18]. Nevertheless, the RMSEC/MAX value was equal to 21.2/224=0.09 indicating a low accuracy of 9% of the simple predictive model compared to the accuracy of 6% determined for the PLS model with all variables. This comparison suggested that carbohydrate content, after mild hydrolysis, was the main influential variable.

3.5 Building of an explicative model of biohydrogen production by dark fermentation

PLS analysis is not only able to supply a mapping of the substrates and variables but can also provide an explicative model of the BHP value from experimental variables corresponding to substrate characterization and microbial metabolic products. In order to achieve good robustness, a two-leave-out cross-validation technique was applied to determine the best number of latent factors which was found equal to 3. This model did include the experimental values of the fruit peels experiment because of its very high value in lactate concentration. The estimated regression coefficients led to a multi-linear equation including interaction terms given the calculated BHP value as follows (Equation 3):

BHP= 2.885 + 38.3557*Carb − 25.3034*Prot + 104.332*Acet +123.1281*But − 370.5045*Prop +302.39*Lact - 2.91584*But*Lact   (Equation 3)

where Carb, Prot, Acet, But, Prop and Lact variables correspond respectively to carbohydrate content and protein composition of the initial substrate, acetate, butyrate, propionate and lactate concentrations determined at the time of maximum cumulated hydrogen was achieved. These variables are expressed in $g.g_{TS}^{-1}$ and BHP values in $mL_{H2}.g_{TS}^{-1}$. The p-values of the regression coefficients were respectively equal to $3.4.10^{-6}$, 0.66, 0.15, $7.24.10^{-7}$, 0.65, 0.96, 0.01 indicating than only the 3 terms Carb, But and But*Lact were highly significant.

The Figure 5 shows the effect of the interaction term between butyrate and lactate concentrations on hydrogen production. Surprisingly, lactate had a positive effect on bioH2 production for carbohydrate-poor substrates, and a strong negative effect for carbohydrate-rich substrates, which is in accordance with literature data. An interaction term between carbohydrates and lactate concentrations was even required to explain the microbial pathways producing hydrogen for a large spectrum of organic waste, giving new insight on biohydrogen-producing microbial pathways occurring in mixed cultures.

[FIGURE 5]

To evaluate the explanation capability of the model, the comparison between the calculated hydrogen potentials from substrate composition and metabolite production according to Equation 3 are presented in Figure 6. The bisector line was plotted in this graph showing a good repartition of the points around this line. The determination coefficient R^2 is equal to 0.97 confirming the good explanation capability of this model. The RMSEC/MAX

value (the root mean square error of calibration divided by the maximal value) is equal to 14.5/224=0.06 indicating a good accuracy at 6 % of the explicative model.

[FIGURE 6]

4. Conclusions

In this study, biological hydrogen potential (BHP) tests were performed for modeling the use of organic solids as substrates for producing hydrogen, for further prediction and better understanding of the associated fermentative pathways. We found that substrate composition and more particularly carbohydrate content had the most significant influence on hydrogen yields. By extension, a statistical PLS analysis showed that biohydrogen yields can be simply, rapidly and accurately assessed by simple measurement of the carbohydrate content after mild hydrolysis, suggesting that soluble and readily accessible sugars correspond to the sole fraction of the biomass that can be converted into biohydrogen. This suggests that pretreatment methods are required for making more accessible complex carbohydrates for further biohydrogen production. Furthermore, an explicative model was built and showed that, surprisingly, acetate did not correlate with hydrogen production suggesting that homoacetogenesis play a key role in H_2 production by dark fermentation. Only butyrate was associated to H_2-producing pathways and a small interaction with lactate at low concentration was observed implying that these two metabolites play a key role in mixed cultures producing H_2 by dark fermentation of organic matter. Further investigations are required for better understanding of this metabolic interaction between butyrate and lactate pathways.

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Legends

Table 1. Maximum cumulated hydrogen yield (Hmax), biochemical composition of solid organic wastes (carbohydrates and proteins) and metabolic end-product accumulation (acetate, butyrate, propionate and lactate) when the maximum hydrogen production was reached. (nd: not determined). Letter in appendix correspond to one of the four categories of substrates.

Fig. 1. Modified Gompertz model (solid line) fitting to experimental data (solid points). The parameters provided by the model - H_{max}, R and t_{lat} - correspond to the maximum cumulated hydrogen production (in $mL_{H2} \, g_{TS}^{-1}$), the maximum hydrogen production rate (in $mL \, L^{-1} d^{-1}$) and the lag-phase (in days), respectively.

Fig. 2. Maximum biohydrogen yields (in $mL_{H2} \, g_{TS}^{-1}$) of various organic solid waste. Considering their overall biochemical characteristics as well as their origins, these substrates were classified in four groups, ie. carbohydrate-rich substrates (Group1), protein-rich substrates, agri-industrial waste and agricultural end-products.

Fig. 3. Visual representation of 21 substrates by projection in plans formed by the three first principal components t1, t2 and t3 representing 41 %, 21 % and 19% of the total variance, respectively. (A) Projection according to the two first latent factors (t1, t2). (B) Projection according to the first and the third latent factors (t1, t3). Substrates from group 1 (carbohydrate-rich) are presented in black, Group 2 (protein-rich) in black and underlined,

Group 3 (agri-industrial waste) in grey and Group 4 (agricultural end-products) in grey and underlined.

Fig. 4. Correlation circles of measured variables and BHP predicted variable by projection in plans formed by the three first principal components t1, t2 and t3 representing 41 %, 21 % and 19% of the total variance, respectively. (A) Projection according to the two first latent factors (t1, t2). (B) Projection according to the first and the third latent factors (t1, t3).

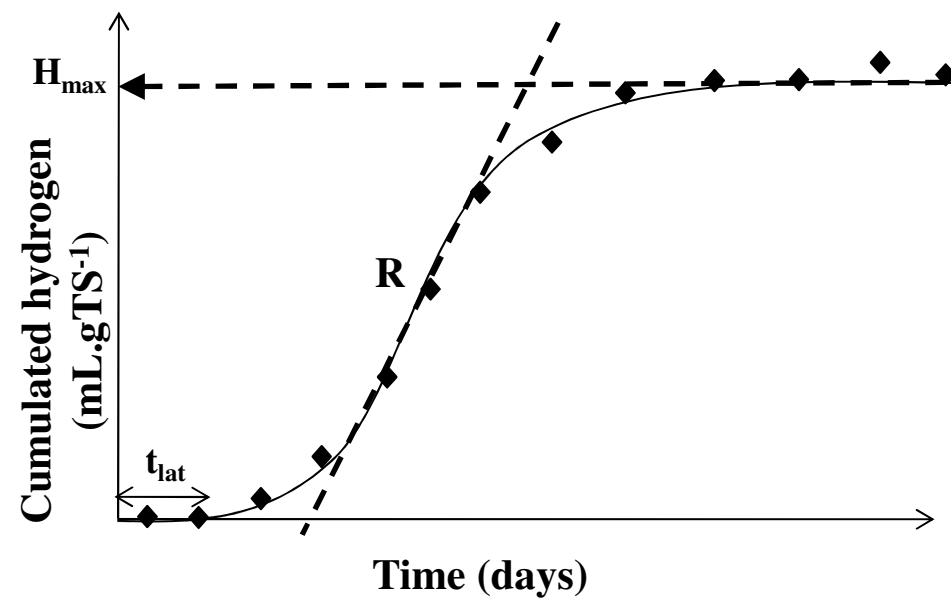**Fig.5.** Three dimensional representation of the Lactate and Butyrate variables on BHP prediction (Hmax)

Fig.6. Calculated versus experimental Hmax values (maximum cumulated hydrogen production in mLH2.gTS-1) of the PLS model based on biochemical characterization and metabolite production. Samples with missing biochemical analysis were not considered. The line represents the bisector.
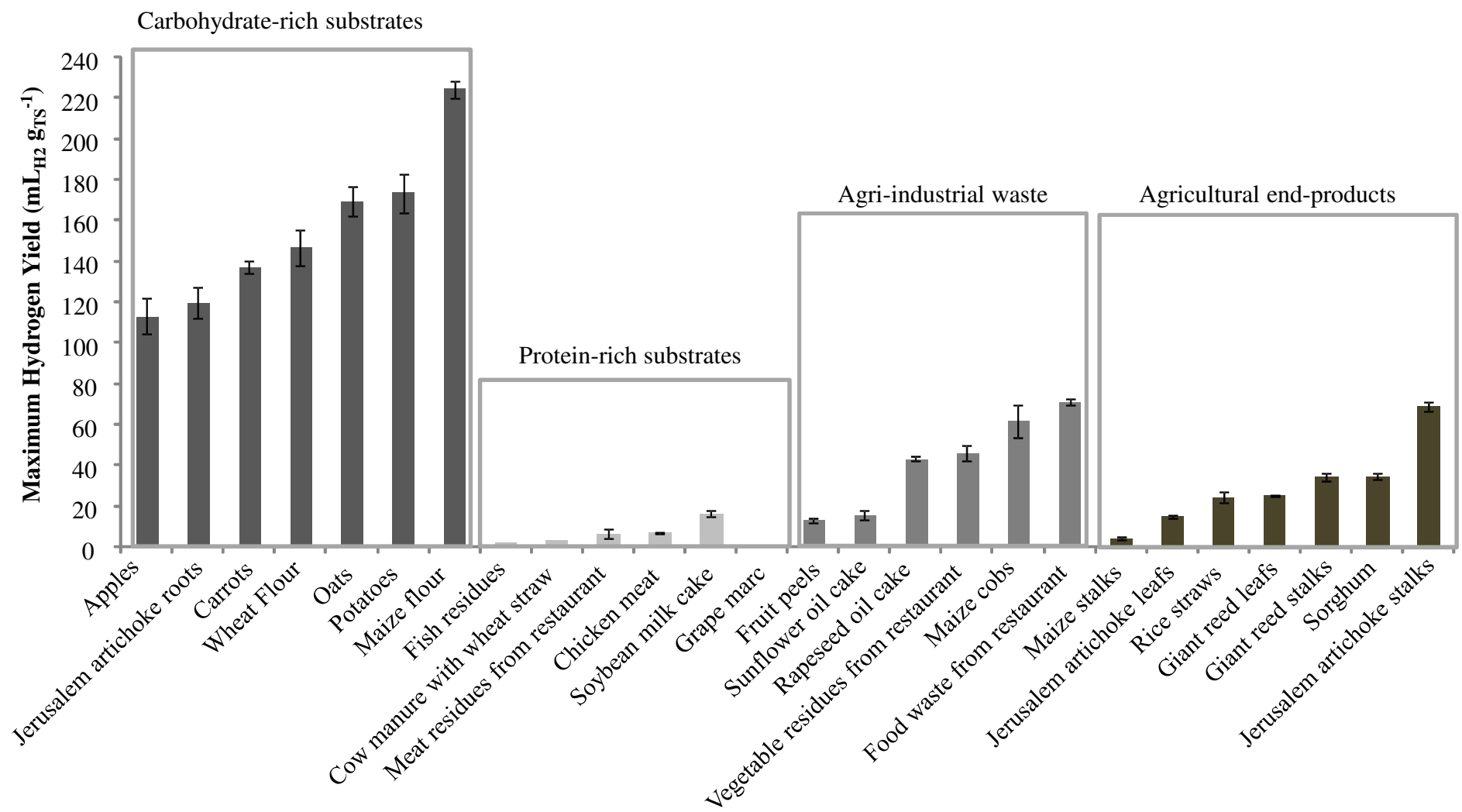
Table

Substrate	H_{max} $(mL_{H2}\ g_{TS}^{-1})$	Carbohydrates $(mg_{Glc}\ g_{TS}^{-1})$	Proteins $(mg_{BSA}\ g_{TS}^{-1})$	Acetate at H_{max} $(mg\ g_{TS}^{-1})$	Butyrate at H_{max} time $(mg.g_{TS}^{-1})$	Propionate at H_{max} time $(mg.g_{TS}^{-1})$	Lactate at H_{max} time $(mg.g_{TS}^{-1})$
Apples [a]	112.9	683	290	96	88	11	0
Carrots [a]	137.2	513	0	189	289	9	24
Wheat flour [a]	146.7	834	0	148	149	0	16
Jerusalem artichoke roots [a]	119.5	772	13	18	176	0	32
Maize flour [a]	224.3	952	3	158	336	6	0
Oats [a]	169.3	727	3	85	223	2	12
Potatoes [a]	173.3	nd	nd	126	151	2	189
Soybean milk cake [b]	16.1	7	765	111	69	3	0
Chicken meat [b]	6.9	0	190	89	38	6	0
Cow manure with wheat straw [b]	3.1	88	192	9	5	0	0
Fish residues [b]	2.0	1	143	177	20	14	0
Meat residues from restaurant [b]	6.4	40	151	75	44	3	0
Grape marc [b]	1.0	61	720	9	0	0	0
Foodwaste from restaurant [c]	71.0	230	100	55	71	1	0
Fruit peels [c]	13.1	289	11	28	2	0	286
Maize cobs [c]	61.7	243	76	55	106	0	48
Rapeseed oil cake [c]	43.3	103	97	15	67	4	0
Sunflower oil cake [c]	15.8	69	68	36	41	1	0
Vegetable residues from restaurant [c]	45.9	nd	nd	60	77	2	0
Giant reed leafs [d]	25.1	138	51	11	13	0	6
Giant reed stalks [d]	34.2	69	34	24	19	0	14
Jerusalem artichoke leafs [d]	14.9	nd	nd	207	141	35	0
Jerusalem artichoke stalks [d]	68.7	293	0	55	109	0	20
Maize stalks [d]	4.1	nd	nd	nd	nd	nd	nd
Rice straws [d]	24.3	152	33	8	5	0	4
Sorghum [d]	34.8	nd	nd	nd	nd	nd	nd

[a] carbohydrate rich group; [b] protein rich group; [c] agri-industrial waste; [d] agricultural residues
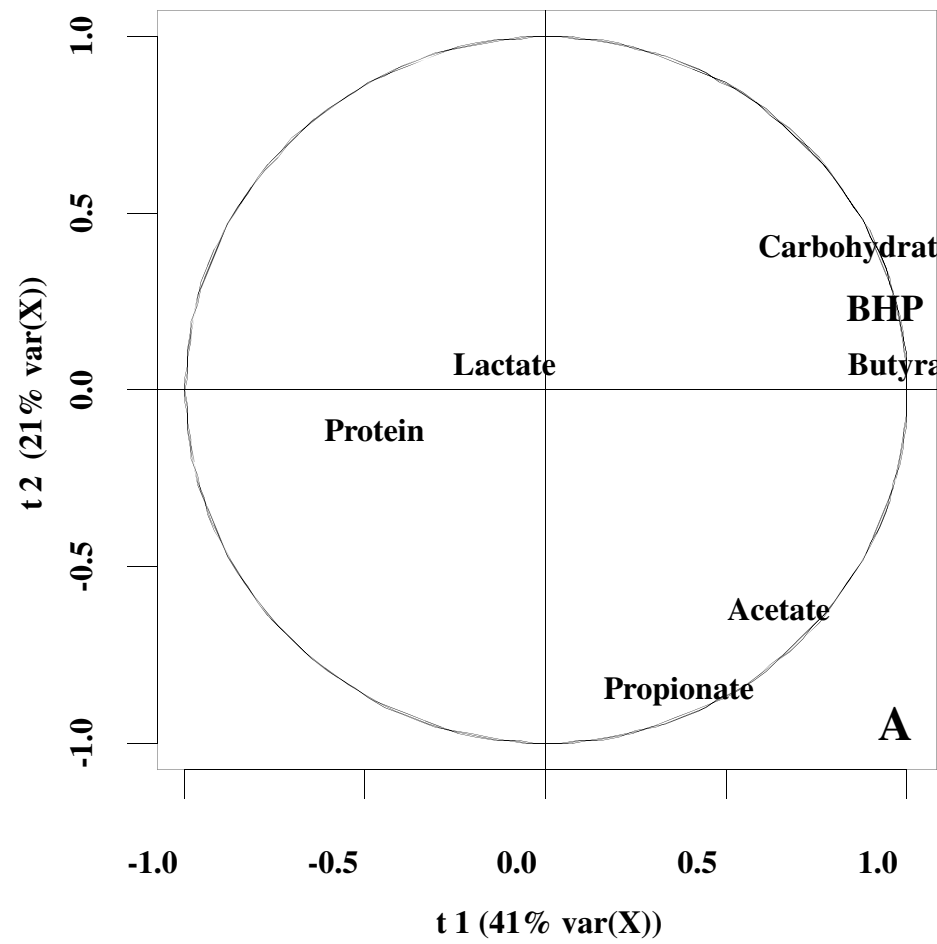
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