Bacillus sp. strains to produce bio-hydrogen from the organic fraction of municipal solid waste

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Abstract:

Bio-hydrogen, obtained by fermentation of organic residues, is considered a promising renewable energy. However, the industrial scale H_2 production from organic waste is far to be realized as technical and economical limitations have still to be solved. Low H_2 yields and lack of industrially robust microbes are the major limiting factors.

To look for bacteria with both interesting hydrogen fermentative traits and proper robustness, granular sludge from a brewery full scale Upflow Anaerobic Sludge Blanket (UASB) digester was selected as trove of bacteria processing complex substrates. One hundred and twenty bacterial strains, previously isolated from heat-treated granular sludge and genetically identified by 16S rDNA sequencing, were screened for extracellular hydrolytic profile on cellulose, hemicellulose, starch, pectin, lipids, protein. The most interesting hydrolytic strains were assessed for their H_2-production from glucose and soluble starch. Two *Bacillus* sp. strains, namely F2.5 and F2.8, exhibited high H_2 yields and were used as pure culture to convert Organic Fraction of Municipal Solid Waste (OFMSW) into hydrogen. The strains produced up to 61 mL of H_2 per grams of volatile solids and could be considered as good candidates towards the development of industrially relevant H_2-producing microbes.

Keywords: bio-hydrogen; dark fermentation; Organic Fraction of Municipal Solid Waste; *Bacillus* sp.; pure cultures; strain selection

1. INTRODUCTION

Biological hydrogen production from organic waste represents both an energy production process and a first stage of stabilization for organic biomass since it degrades complex substrates to readily biodegradable compounds or to metabolites of commercial interest (i.e. organics acids and solvents) [1-3].

Organic waste and low-cost organic by-products of food-processing industry have been already investigated as promising renewable materials to be converted into hydrogen and other fuels, polymers, enzymes and bulk chemicals [4-11]. However, to guarantee the economical sustainability of the organic waste-to-hydrogen route, one of the main requirements is linked to the availability of efficient H_2 producing microbes with proper robustness to be used at industrial scale [1]. To this purpose, several methods for pre-treatment of inoculums used in many biogas processes have been proposed, including heat-treatment, acidification, basification, aeration or freezing [12, 13], with the final aim of obtaining microbial consortia in which hydrogen

consuming and non-hydrogen producing bacteria are suppressed.

The Organic Fraction of Municipal Solid Waste (OFMSW), characterized by high moisture and high biodegradability due to a large content of food waste, kitchen waste and leftovers from residences, cafeterias and markets, has been previously evaluated for H_2 production through the addition of heat-treated inocula [5, 14-16]. Although this pre-treatment practice gave good H_2 performances in laboratory [1, 12], it could be not feasible at larger scale. Moreover, the use of exogenous inocula does not allow to guide properly the fermentation process [5, 12]. To address this issue, recent research advances have been reported indicating that OFMSW itself could produce, without any external inoculum supplementation, high H_2 yields [5]. Natural decomposition occurs to food waste when left for few days at room temperature due to the presence of indigenous microorganisms. In case of no or very low oxygen concentration, fermentation of organic matter takes place and methane production may also occur with time. Therefore, some species of indigenous microbial population of organic waste may have good characteristics for the hydrolysis of complex substrates into simple monomers and for an efficient conversion into H_2. As a result, food waste could serve both as substrate and source for H_2 production and H_2-producing bacteria, respectively [5, 17]. This novel approach paves the way for the development of inoculants to produce H_2 from OFMSW relying on the indigenous microbes.

Another recent research strategy is the use of selected microbe(s) for the conversion of organic waste into H_2 [16, 18]. The possibility to select strain(s) for their hydrolytic and fermenting abilities according to the main complex substrates available in the food waste makes this avenue very effective. However, it remains still unexplored as pure cultures have been so far mostly applied for H_2 production from simple sugars (i.e., glucose, sucrose and xylose) or laboratory-grade soluble starch [12, 19, 20].

In this paper, to search for microbes with both interesting hydrogen fermentative traits and proper robustness, granular sludge from a brewery full scale Upflow Anaerobic Sludge Blanket (UASB) digester was selected as promising environment because of being at industrial scale and processing complex substrates. One hundred and twenty bacterial strains, previously isolated from heat-treated granular sludge and selected for their high H_2 production [21], were screened for extracellular hydrolytic profile on cellulose, hemicellulose, starch, pectin, lipids, protein. The isolates exhibited a broad range of hydrolytic activities and the most interesting strains were assessed for their H_2-production from glucose. The top H_2-performing microbes were then evaluated in H_2-production trials using starch as main carbon source. Two *Bacillus* sp. strains showed high H_2 levels and were evaluated also on OFMSW, mainly composed by starch, lipids

and protein. The microbes gave promising H_2 yields and could be considered as good candidates towards the development of industrially relevant microbes for the processing of organic waste into H_2.

2. MATERIALS AND METHODS

2.1 Microbial strains

One hundred and twenty microbial strains were previously isolated from granular sludge samples heat-treated (100°C) with increasing residence times in order to inhibit indigenous methanogenic bacteria. All the strains were identified by 16S rDNA sequencing [21].

2.2 Screening for the production of extracellular hydrolytic enzymes

Calibrated suspensions (A_{600} =0.9, corresponding to an average concentration of 10^6 cells per mL) of bacterial cells, grown for 24 h at 37°C in NB broth at 100 rpm, were used to inoculate plates containing the appropriate media described below and purified agar (Sigma, Italy). Petri dishes were checked for the presence of enzymatic activity described below, after aerobic incubation at 37°C for 3 days. No discrepant results were recorded in repeated experiments.

2.2.1 Cellulase activity (CelA)

Cellulase production was detected on Hankin and Anagnostakis Medium containing 5 g/L carboxymethyl-cellulose (CMC). After cell growth, the presence of cellulolytic activity (CelA) was detected by Congo red method [22].

2.2.2 Lipolytic activity (LipA)

Strains were tested on tributyrin agar medium containing (g/L): peptone, 5; yeast extract, 3; tributyrin, 10; agar, 15; pH 6.0. Lipase activity (LipA) of the strains were indicated by a clear halo around the colony in an otherwise opaque medium as previously described [23].

2.2.3 Pectinolytic activity (PecA)

The secretion of extracellular pectic enzymes was tested on polygalacturonic acid medium (g/L): yeast nitrogen base, 6.7; glucose, 5; polygalacturonic acid (Fluka, Italy), 7.5; pH 7.0 [24]. The screening was performed using polygalacturonic acid medium with or without glucose (10 g/L). After cell growth, plates were flooded with a solution of 6N HCl. The appearance of a degradation halo around bacterial colony was considered an indication of the polygalacturonic acid hydrolysis [25].

137

138 *2.2.4 Proteolytic activity (PrA).*

139 Extracellular protease production was determined on protein medium with skim milk (Difco,
140 Italy), pH 6.5. A clear zone around the colony indicated protease activity (PrA) as described in
141 literature [24, 26].

142

143 *2.2.5 Starch-degrading activity (StA)*

144 Microbial strains were screened for the ability to hydrolyze soluble potato starch (Sigma,
145 Italy) on Wollum medium containing (g/L): Yeast Extract (Difco), 1; Na_2NO_3, 1; KCl, 0.5;
146 $MgSO_4$, 0.5; starch, 10; agar, 17 [25]. After incubation, Petri dishes were flooded with iodine
147 solution. A pale yellow zone around colonies in a blue medium indicated starch degrading
148 activity (StA) [27].

149

150 *2.2.6 Xylan-degrading activity (XylA)*

151 Cultures were screened for xylan degrading activity by growth on modified Hankin and
152 Anagnostakis Medium containing 0.5% xylan from oat-spelt (Fluka, Italy). Colonies showing
153 xylan-degrading activity (XylA) were identified by a clear hydrolysis zone around the colony
154 after treatment with Congo Red.

155

156 **2.3 Amylolytic enzymes characterization**

157 The starch degrading strains were tested for their amylolytic activity once cultivated in NB
158 with 20 g/L soluble starch or Starch Production Medium (SPM) supplemented with (g/L):
159 peptone, 5; soluble starch, 20; Na_2HPO_4, 2; KH_2PO_4, 1. The pH was set to 7.0 for both media.
160 The strains were aerobically grown at 37 °C for up to 168 h. Ten mL samples were withdrawn at
161 24 h intervals and, after centrifugation (10 min, 5,500 x g), the supernatant was used for
162 enzymatic assays.

163 Total amylase activity was determined in liquid assays using the reducing sugar method with
164 glucose as standard [28]. The optimal enzyme pH was assessed at 50°C with 50 µL of the
165 supernatant and 450 µL of the substrate (0.1% soluble potato starch) suspended in 0.05 M
166 citrate-phosphate or sodium-phosphate buffer at pH values ranging from 5.5 to 8.0. The optimal
167 assay temperature was determined at pH 6.0 and 7.0 using temperatures ranging from 30 to
168 60°C. The enzymatic reactions were conducted for 10 min and terminated by boiling in a
169 waterbath for 15 min.

Enzymatic activities were expressed as unit (U) per mL of supernatant, which is defined as the amount of enzyme which releases 1 μmol of reducing end groups per min. All experiments were carried out in triplicate.

2.4 Batch test for hydrogen production from glucose

To evaluate the H_2-potential from glucose of the twenty strains with the most promising hydrolytic phenotype, 100 mL Pyrex vessels, were filled with 50 mL of Nutrient Broth (NB, Oxoid, pH 6.0) with or without glucose (5 g/L) and sterilized by autoclave (121° C, 20 min). Each strain was pre-grown overnight in NB and inoculated into the batch reactors at an optical density (600nm) value of 0.2. After inoculation, the reactors were hermetically closed using a silicon plug. Once flushed with N_2 gas for 3 min, the vessels were incubated without stirring in a thermostatic chamber at 37° C.

The amount of biogas produced was recorded daily, using the water displacement method [21]: the biogas accumulated in reactors headspace is released in a second bottle filled with an acidified (pH<3) and saline (NaCl 25%) solution, which avoids the dissolution of gas into the liquid. The biogas moves an equivalent volume of liquid that was subsequently measured with a graduated cylinder. Biogas composition in terms of hydrogen, carbon dioxide and methane were measured by gas chromatography as indicated in the "Analytical methods and calculations" paragraph.

At the end of fermentation, liquid samples were kept at -20°C to analyse the volatile fatty acids (VFAs) concentration and the amount of residual glucose or starch as described below in the "Analytical methods and calculations" paragraph.

All experiments were carried out in triplicate and the results averaged.

2.5 Batch test for hydrogen production from soluble starch and OFMSW

The most promising starch-hydrolyzing strains were evaluated for their ability to convert soluble starch into H_2. The strains were grown in SPM for 72 h and then used to inoculate 50 mL fresh SPM into Pyrex bottles as described above. Sodium phosphate buffer (pH 6.0 and 7.0) was used.

In the case of H_2 production from OFMSW, each vessel was supplemented with 10 g VS/L (which corresponds to 150 g/L of fresh weight), instead of soluble starch. The experiments was monitored until biogas production stopped. At the end of H_2 fermentation, liquid samples were withdrawn and kept at -20°C for further analysis. All the experiments were carried out in triplicate and the results averaged.

The sample of OFMSW used for batch tests was obtained in May 2015 from separate collection of MSW in Padova (Italy). Approximately 200 kg of organic waste was manually sieved, sorted and divided into the following fractions: fruits (F), vegetables (V), meat–fish–cheese (MFC), bread–pasta-rice (BPC), undersieve 20 mm (U) and rejected materials. Undersieve 20 mm was composed of materials smaller than 20 mm. The rejected materials were shoppers, plastics, metals, glass, bones, paper and cardboard, shells and fruit kernels.

Using the sorted fractions, a sample of organic waste was prepared maintaining the same proportion of the single fractions without the rejected materials. The prepared sample of OFMSW was ground in a kitchen mill prior to be used as substrate for H_2-production. The shredded OFMSW had total solid (TS) concentration of 146±11 gTS/L and volatile solid (VS) and total organic carbon (TOC) concentration of 93±1% and 45±1%, respectively, referred to dry weight. Total Kjeldahl nitrogen (TKN), ammonium and total phosphorus concentration was 2861±113 mg N/L, 408±35 mg N/L and 375±18 mg P/L, respectively. Concentrations (of dry weight)of lipids, proteins, cellulose, hemicellulose, lignin, starch and pectin in OFMSW sample were also detected as follow: 18±1, 17±1, 5.0±0.6, 6.0±0.5, 2.0±0.2, 19±1, 8.0±0.7, respectively.

2.6 Analytical methods and calculations

TS, VS, TKN, ammonium and total phosphorous concentrations were analysed according to standard methods [29]. TOC values were obtained by difference between Total carbon (TC) and inorganic carbon (IC). TC and IC were analysed by a TOC analyser (TOC-V CSN, Shimadzu). Concentration of lipids, proteins, pectin, lignin, cellulose, hemicellulose and starch were analysed according to official methods [30].

VFAs concentrations (acetic, propionic, butyric, isovaleric acids) were analysed by a gas chromatograph (Varian 3900) equipped with a CP-WAX 58 WCOT fused silica column (Varian) and a Flame Ionization Detector (FID). Nitrogen was used as carrier gas at a flow of 4 mL/min in column. The oven temperature programme was initially set at 80 °C for a min, then increased at a rate of 10 °C/min to 180 °C (finally maintained for 2 min). Injector and detector temperatures were both set to 250 °C.

Residual glucose and soluble starch in the NB or SPM broths were measured using the peroxidase-glucose oxidase method with the D-glucose and starch assay kit, respectively (Boehringer Mannheim).

Biogas composition in the headspace of reactors, in terms of hydrogen (H_2), carbon dioxide (CO_2) and methane (CH_4) concentrations, was analysed by gas chromatography using a micro-GC (Varian 490-GC) equipped with i) a 10-meter MS5A column (to analyse H_2 and CH_4) ii) a

10-meter PPU column (to analyse CO_2) and iii) two Thermal Conductivity Detectors (TCDs). Argon was used as carrier gas at a pressure of 60 kPa in columns. Injector and column temperatures were both set to 80 °C.

Data on biogas and hydrogen productions was expressed at a temperature of 0 °C and pressure of 1 atm. Hydrogen volumes produced in the time interval between each measurement [*t - (t-1)*] during dark fermentation batch tests, were calculated using a model considering i)the hydrogen gas concentration at times *t* and *t-1*, together with the total volume of biogas produced at time *t*, ii) the concentration of the specific gas at times *t* and *t-1*, and iii) the volume of the head space of reactors [13]. The following equation was applied:

$$V_{H2,t} = C_{H2,t} \cdot V_{BG,t} + V_{HS} \cdot \left(C_{H2,t} - C_{H2,t-1}\right)$$

Where:

$V_{H2,t}$: volume of hydrogen produced in the interval between *t* and *t-1*;

$C_{H2,t}$, $C_{H2,t-1}$: hydrogen concentrations measured at times *t* and *t-1*;

$V_{BG,t}$: volume of biogas produced between time *t* and *t-1*;

V_{HS} : volume of the headspace of reactors.

Cumulative hydrogen production (V_{H2cum}) was calculated as sum of hydrogen productions between each measurement ($V_{H2,\ t}$) during dark fermentation batch tests, according to the following equation:

$$V_{H2cum} = \sum_{t=1}^{n} V_{H2,t}$$

Where:

V_{H2cum}: cumulative hydrogen production at the end of the dark fermentation test;

$V_{H2,t}$: hydrogen production between times t and t-1.

Hydrogen yields, expressed as $NmLH_2/g$ VS and $molH_2/mol$ glucose, were calculated according to the following equations:

$$Hydrogen\ yield\ (NmlH2/g) = \frac{V_{H2cum}}{W_{sub}}$$

Where:

V_{H2cum}: cumulative hydrogen production at the end of the dark fermentation test;

W_{sub}: weight of added VS.

$$Hydrogen\ yield\ (molH2/molglucose) = \frac{\frac{V_{H2cum}}{22.414\ L/mol}}{\frac{W_{glucose}}{180\ g/mol}}$$

Where:

V_{H2cum}: cumulative hydrogen production at the end of the dark fermentation test;

22.414 L/mol: volume occupied by 1 mole of ideal gas at 1 atm pressure and 0° C;

$W_{glucose}$: weight of glucose equivalent added at the beginning of the batch test;

180 g/mol: weight of 1 mole of glucose equivalent.

The volumetric productivity (Q) was based on as $NmLH_2$/g VS per litre of culture medium per day ($NmLH_2$/L/d) and the maximum volumetric productivity (Q_{max}) was compared as the highest volumetric productivity displayed by the strains.

3. RESULTS AND DISCUSSION

3.1 Screening for extracellular enzymatic activities

One hundred and twenty microbial strains were previously isolated and identified from samples of heat-treated granular sludge used to perform hydrogen production batch tests [21]. The heat-treatment (100°C for increasing residence times of 0.5, 1, 2 and 4 hours) strongly affected the microbial viability in the sludge and the heat-treated sludges produced high and variable hydrogen yields from glucose, with the microbial consortia surviving after 2 and 4 hour boiling times having the most promise [21]. All isolates were screened for the production of industrially relevant extracellular enzymes and exhibited a broad range of hydrolytic activities (Table 1).

Fifty-seven strains were found proteolytic with a great majority of positive isolates belonging to *Bacillus* genus. A high number of pectinolytic strains has been also detected: the fact that only four out of 34 strains confirmed their potential once grown in the presence of both glucose and polygalacturonic acid (PecA+glucose) clearly indicates that, in the screened microbial collection, the production of pectinolytic enzymes is mainly not constitutive. This finding is in accordance with the related literature on microbial pectinases [31]. Twenty-seven microbes gave positive results for starch-degrading activities. As reported in Table 1, three strains produced active xylanases meanwhile only a *B. licheniformis* isolate was found to be cellulolytic. No lipolytic microbes were recovered.

Table 1. Extracellular enzymatic activity of 120 microbial strains isolated from samples of heat-treated granular sludge (CelA: cellulolytic activity; LipA: lipolytic activity; PecA: pectinolytic activity; PecA + glucose: pectinolytic activity screened in the medium supplemented also with glucose; PrA: proteolytic activity; StA: starch-degrading activity; XylA: xylan-degrading activity).

		number of positive strains						
Strains	n. of strains	CelA	LipA	PecA	PecA + glucose	PrA	StA	XylA
Bacillus sp.	31	-	-	8	-	16	6	1
Bacillus badius	20	-	-	7	-	11	5	-
Bacillus berjingensis	6	-	-	3	-	2	-	-
Bacillus farraginis	8	-	-	-	-	-	-	-
Bacillus flexus	1	-	-	-	-	-	1	-
Bacillus licheniformis	3	1	-	2	1	1	3	1
Bacillus megaterium	3	-	-	3	-	3	3	-
Bacillus subtilis	3	-	-	3	-	1	3	-
Bacillus tequilensis	4	-	-	2	3	1	4	1
Brevibacillus sp.	3	-	-	-	-	-	-	-
Brevibacillus agri	3	-	-	-	-	1	-	-
Brevibacillus brevis	2	-	-	-	-	1	-	-
Brevibacillus parabrevis	1	-	-	-	-	-	-	-
Enterobacter sp.	2	-	-	-	-	-	-	-
Enterobacter cloacae	1	-	-	-	-	-	-	-
Lysinibacillus sp.	16	-	-	5	-	5	3	-
Paenibacillus sp.	6	-	-	2	-	-	-	-
Paenibacillus cookii	3	-	-	-	-	-	1	-
Sporosarcina sp.	4	-	-	-	-	-	-	1
Total n. of strains	120							
Total n. of positive strains		1	-	34	4	57	27	3

The majority of the catalytic activities were found to be protease, amylase and pectinase. This outcome could be explained considering that the strains have been isolated from an anaerobic digester of a brewery whose fed by-products are usually rich in starch, pectin and protein [32]. Overall, the isolates belonging to *Bacillus* sp. genus displayed the highest number of hydrolytic activities. They are attractive species for the industry as they are rarely pathogenic, grow fast and secrete high amounts of proteins. These properties make bacilli very useful in industrial

315 applications where they contribute up to 50% of the enzyme market [33].
316
317 **3.2 Hydrogen potential from glucose by selected microbial strains**
318 The presence of different extracellular enzymatic activities in many screened isolates was
319 considered promising towards the definition of a proper inoculum for the conversion of complex
320 organic waste into hydrogen. In literature, indeed, *Bacillus* species are known as strong
321 candidates for biological H_2-production because (i) they can survive under harsh conditions,
322 hence could compete with other microbes (ii) they have large and versatile enzymatic activities,
323 therefore a diverse range of bio-waste could be used as substrate for bio-hydrogen production,
324 (iii) they do not require light for H_2 production, (iv) *Bacillus* sp. spores are being used as
325 probiotics in humans and animals; thus, they may not pose environmental health concerns [33,
326 34].
327 Twenty strains belonging to *Bacillus* sp. and *Brevibacillus* sp. were selected for their
328 hydrolytic activities and evaluated for H_2 potential. Firstly, the microbes were screened in NB
329 supplemented with 5 g/L glucose and compared in terms of hydrogen yield and glucose
330 consumption after 48 hours of incubation. The microbes produced H_2 with variable yields (0.16-
331 1.53 mol of H_2 per mol of consumed glucose) which were in agreement with the yield range so
332 far reported in literature by *Bacillus* sp. under dark fermentative conditions (0.20-2.04 mol/mol
333 glucose used) [34]. The most proficient microbes are reported in Table 2 together with other H_2-
334 performances recently described for *Bacillus* sp. grown on the same amount of glucose.
335 **Table 2.** Comparison of hydrogen production potential of *Bacillus* sp. and *Brevibacillus* sp.
336 strains from glucose (5 g/L) as carbon source.
337

Strain	Enzymatic profile	H_2 yield (mol/mol glucose)	Residual glucose (%)	Reference
Bacillus sp. F2.5	StA	1.53	nd	This study
Bacillus sp. F2.7	PrA, StA	0.88	2.9	This study
Bacillus sp. F2.8	PrA, StA	1.47	nd	This study
B. farraginis F4.10	PrA, StA	0.31	nd	This study
B. megaterium F1.22	PectA, PrA, StA	0.57	nd	This study
B. tequilensis F2.16	PectA, StA, XylA	0.36	2.5	This study
Brevibacillus sp. F4.12	PectA, PrA	0.75	nd	This study
Brevibacillus sp. F4.16	PrA	0.69	nd	This study
Bacillus sp. EGU444	PrA	0.35	na	[35]
B. thuringiensis EGU378	LipA, StA	0.26	na	[35]
B. megaterium ATCC15374	StA	0.60	1.0	[36]
B. thuringiensis EGU45	nd	1.67	24.0	[37]
B. cereus EGU44	nd	1.92	23.2	[37]
B. cereus EGU43	PrA	1.12	21.6	[37]
B. cereus EGU3	nd	0.96	22.4	[37]
Bacillus sp. FS2011	nd	2.04	0.5	[38]

338 na: not available; nd: not detectable

Interestingly, the glucose-to-H_2 conversion efficiencies of the newly isolated bacteria were comparable to those of the literature and the highest yields were exhibited by two *Bacillus* sp. strains (namely F2.5 and F2.8) with 1.53 and 1.47 mol of H_2 per mol of used glucose, respectively. The majority of the microbes investigated in this study completely utilize the glucose available in the system meanwhile other *Bacillus* sp. strains, although exhibiting high H_2 yields, did not convert all the substrate [37]. This finding is of great interest since a microbial strain should have both high substrate utilization and H_2 yield for being implemented in the industrial bio-hydrogen technology.

As reported in Table 2, the strains selected in this study showed one to three hydrolytic capabilities whereas only few *Bacillus* sp. microbes with high H_2 potential were described in literature also for enzymatic activities. The most efficient strains, *Bacillus* sp. F2.5 and F2.8, were selected for further studies. Their amylolytic enzymes could be very useful for the H_2-conversion of food waste, where starch can account up to 30% of the TS [15, 39, 40].

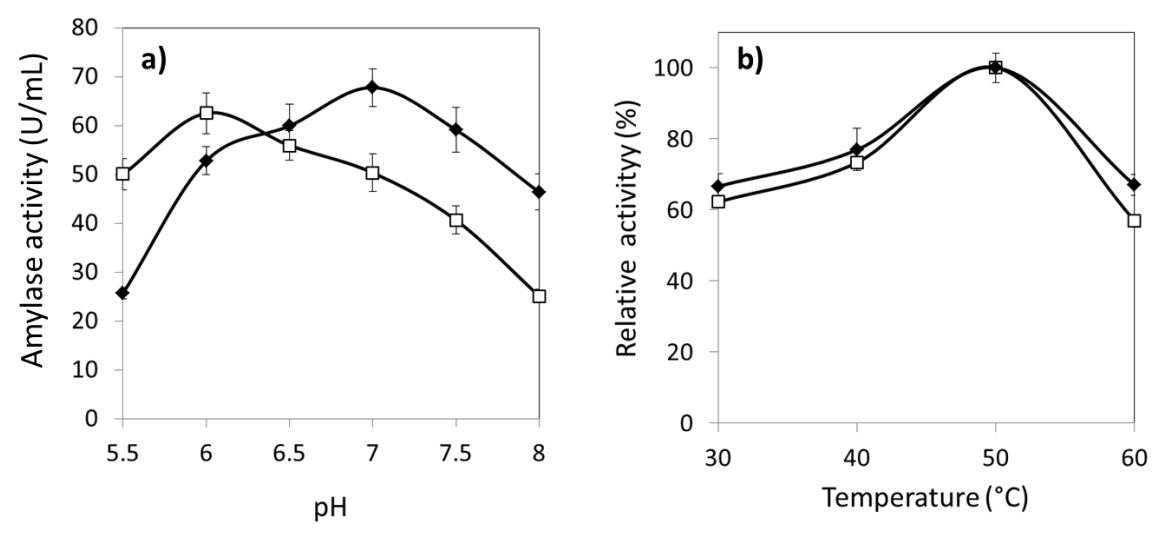
3.3 Characterization of amylolytic enzymes secreted by *Bacillus* sp. F2.5 and F2.8

To study the degrading activity of *Bacillus* sp. F2.5 and F2.8, the strains were grown both in NB and SPM supplemented with 20 g/L soluble starch. The highest enzymatic activities were detected in SPM broth after 72 h of incubation at 37°C (data not shown), thus this medium was selected to deeply investigate their amylolytic abilities. The activity of both microbes after 72 h incubation in SPM was firstly assessed at 50°C using different pH values (Fig. 1a). The two strains displayed comparable amylase activities: *Bacillus* sp. F2.8 showed the most promise with the highest enzymatic activities (67.8 U/mL) detected at pH 7.0 meanwhile the uppermost catalytic ability of *Bacillus* sp. F2.5 was found at pH 6.0 (62.5 U/mL). pH greatly influenced the enzymes of both strains: the total amylase activity of *Bacillus* sp. F2.5 at higher pH progressively dropped to 25.1 U/mL at pH 8.0, which stand for almost 40% of the highest value. The amylase activity of *Bacillus* sp. F2.8 was found high in the pH range of 6.0-8.0.

These findings are in accordance with those described in literature regarding *Bacillus* sp. amylases, where the optimal pH values were reported to be within the broad range of 3.5-12 and the pH was found to deeply affect their catalytic activity on starch [41-43].

The amylolytic enzymes were assayed at temperatures from 30 to 60°C at the optimal pH for each strain, namely pH 6.0 and 7.0 for *Bacillus* sp. F2.5 and F2.8, respectively. Enzyme activity increased with temperature up to 50°C, which was found to be the optimum for the two microbes (Fig. 1b).

Figure 1. The effect of pH (a) and incubation temperature (b) on the amylase activity of *Bacillus* sp. F2.5 (□) and *Bacillus* sp. F2.8 (◆) grown for 72 h in SPM containing 20 g/L soluble starch.



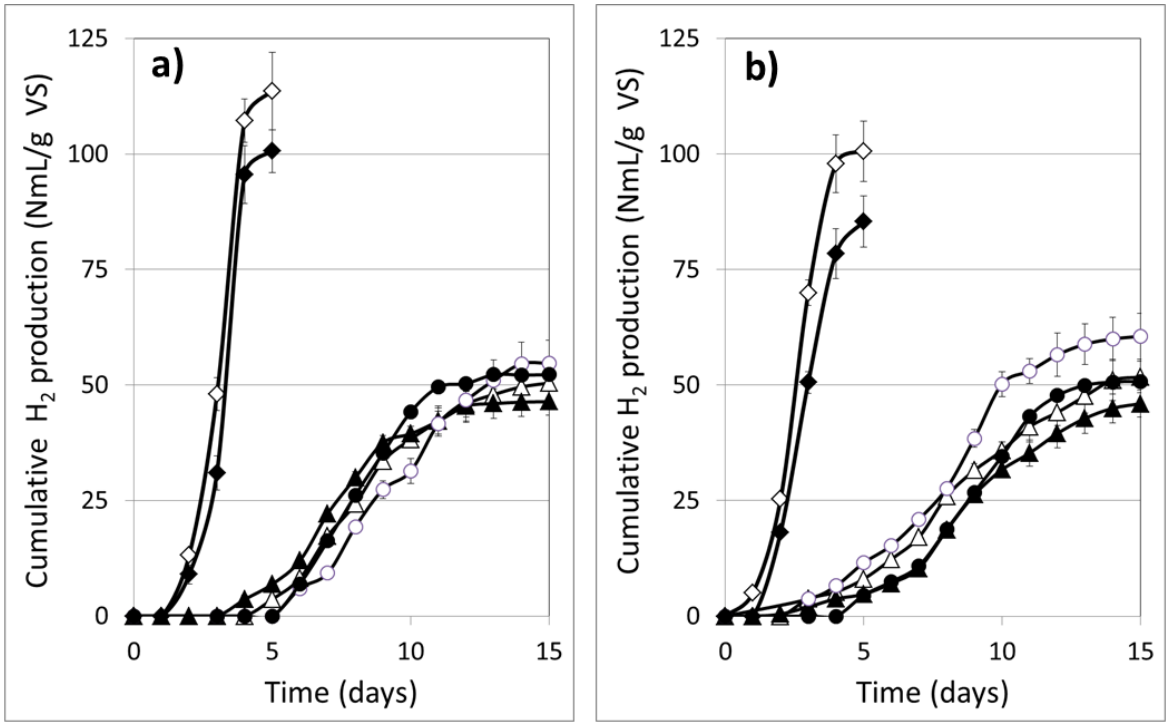
At 60°C, the enzymatic values were lower, 57 and 67% of the highest activity detected at 50°C for *Bacillus* sp. F2.5 and F2.8, respectively. Both microbes had high relative activity at 30 and 40°C (on average 64 and 74%, respectively) and their optimal temperature values were inferior than those usually reported for other *Bacillus* sp. amylases (60-70°C) [42, 44-46]. Overall, *Bacillus* sp. F2.5 and F2.8 produced amylase with high potential with enzymatic activities comparable to those recently reported by efficient amylolytic *Bacillus* sp. strains [34, 42]. Moreover, the high enzymatic activities registered at thermal levels near to those optimal for growth (37°C) could be beneficial for the saccharification of starchy substrates into glucose during the starch-to-hydrogen fermentation.

3.4 Hydrogen production from glucose and soluble starch by *Bacillus* sp. F2.5 and F2.8

Considering that OFMSW is usually quite rich in starch [15, 39], with the final aim of assessing their ability to convert OFMSW into H_2, *Bacillus* sp. F2.5 and F2.8 were firstly evaluated for their H_2 potential from soluble starch (20 g/L) at pH 6.0 and 7.0, selected as the optimal values for the amylase secreted by each strain (Fig. 1a). The microbes were also cultivated in the presence of the equivalent amount of glucose (22 g/L), as reference medium.

No methane was detected throughout the experiments whereas the strains were able to produce H_2 from glucose and soluble starch (Fig. 2a,b).

Figure 2. Cumulative hydrogen productions of *Bacillus* sp. F2.5 (a) and *Bacillus* sp. F2.8 (b) grown in SPM supplemented with 22 g/L of glucose (◆), 20 g/L soluble starch (▲) or 10 gVS/L of OFMSW (●). Filled and empty symbols report values obtained at pH 6.0 and 7.0, respectively. VFAs profiles (mg/L and % TVFA, Total Volatile Fatty Acid), maximum volumetric H_2 productivity (Q_{max}), (NmL/L/d), and relative H_2 concentration (%) of the biogas produced on different substrates are also reported (c). Data shown are the mean values of three replicates and standard deviations are included.



c)

Substrate	Strains	pH	H_2 %	Q_{max} mL/L/d	TVFA mg/L	Acetate mg/L	%	Propionate mg/L	%	Butyrate mg/L	%
Glucose	F2.5	6	45	23.9	1774	973±85	55	247±48	14	554 ± 60	31
		7	45	26.8	2115	1134±99	54	367±26	17	613 ± 62	29
	F2.8	6	45	19.6	1548	873±68	56	182±18	12	493± 48	32
		7	45	24.0	1861	1087±99	58	205±16	11	569 ± 55	31
Starch	F2.5	6	45	3.7	896	490±69	55	123±25	14	282 ± 30	31
		7	45	3.9	1058	568±99	54	183±40	17	307 ± 51	29
	F2.8	6	45	3.8	774	437±45	57	91±19	12	247 ± 45	31
		7	44	4.1	1106	637±29	58	123±27	11	345 ± 43	31
OFMSW	F2.5	6	38	4.2	1117	625±71	56	158±20	14	334 ± 39	30
		7	38	3.9	1131	601±55	53	199±17	18	331 ± 28	29
	F2.8	6	39	4.0	945	527±49	56	122±15	13	296 ± 30	31
		7	39	5.0	1277	737±58	58	144±13	11	396 ± 35	31

The two microbes completely utilized glucose within five days yielding high levels of hydrogen. *Bacillus* sp. F2.5 obtained the uppermost H_2 concentrations both at pH 7.0 and 6.0, with 114 and 101 mL of H_2, respectively whereas *Bacillus* sp. F2.8 produced lower volumes:

101 and 85 mL at pH 7.0 and 6.0, respectively. As a result, the top fermenting abilities were achieved at pH 7.0, with the H_2 yield of 0.91 and 0.81 mol per mol of consumed sugar for *Bacillus* sp. F2.5 and F2.8, respectively. Lowering the pH resulted in a reduced efficiency, mostly for *Bacillus* sp. F2.8 whose yield was 0.69 mol per mol of consumed sugar meanwhile the other strain produced 0.82 mol of H_2 per mol of used glucose. *Bacillus* sp. F2.8 displayed the most efficient fermenting profile with the highest H_2 productivity attained at pH 7.0 (26.8 mL of H_2 per day), which was 1.12-fold that of *Bacillus* sp. F2.5 (24.0 mL of H_2 per day). Relative H_2 concentration was found to be similar (about 45%) for the two strains (Fig. 2c).

In the presence of soluble starch, *Bacillus* sp. F2.5 and F2.8 produced high H_2 levels, too (Fig. 2), consuming all the available polysaccharide. At pH 7.0, *Bacillus* sp. F2.8 confirmed the most efficient hydrolyzing ability, obtaining the highest amount of H_2 (51.8 per gram of consumed starch) in a shorter timeframe (Fig. 2b). Similar performances but with lower productivity were detected for *Bacillus* sp. F2.5 (Fig. 2a): in the first days of fermentation, higher amounts of hydrogen were produced at pH 6.0 while, at the end of incubation, pH 7.0 supported slightly better the H_2 potential of *Bacillus* sp. F2.5.

The relative concentration of H_2 was similar for the two microbes (Fig. 2c): 44 and 45 % for *Bacillus* sp. F2.8 and F2.5, respectively and the highest H_2 efficiencies were found at pH 7.0: 0.42 and 0.41 mol of H_2 per mol of consumed starch for *Bacillus* sp. F2.8 and F2.5, respectively. Their yields from soluble starch were 51 (0.81/0.42) and 44% (0.91/0.41), respectively, of those above presented in the same broth from glucose. Interestingly, although the two strains had similar starch-to-H_2 efficiency, *Bacillus* sp. F2.8 showed H_2 potential from glucose lower than *Bacillus* sp. F2.5 (Table 2, Fig. 2). This could be associated with the most efficient starch-degrading activity described for *Bacillus* sp. F2.8 at pH 7.0 (Fig. 1a). Nevertheless, both strains exhibited promising H_2 yields which were found to be comparable with those described in literature mainly by mixed consortia [47, 48]. The highest H_2 yield from starch reported so far by a strain belonging to the *Bacillus* genus was recently disclosed as 0.70 mol H_2 per mol of reducing sugar [49]. On the other hand, as reported in Fig. 2c, both *Bacillus* sp. strains described in the present work showed productivity (about 4 mL of H_2 per day) lower than those found in other studies on H_2 production from starch. However, their limited H_2-production rate, which could be mainly influenced by their low inoculum size and static incubation, are likely to be improved by optimizing the growth conditions and other environmental factors such as micronutrients availability, buffers and temperature which were reported as key parameters to boost H_2 productivity [47, 48, 50].

3.5 Hydrogen potential from OFMSW

The fractions analysis of the OFMSW obtained from manual sorting procedure (Table 3) revealed a composition similar to those of other OFMSW recently described in literature [5, 15].

Table 3. Results from manual sorting procedure of the OFMSW used in this study

Fraction	Weight (Kg)	Percentage (%)
Fruit	52.01	25.9
Vegetable	42.21	21.0
Meat-Fish-Cheese	8.95	4.5
Bread-Pasta-Rice	44.44	22.1
Rejected materials	33.52	16.7
Undersieve 20 mm	19.67	9.8
Total	200.80	100

Fruit, vegetable and bread-pasta-rice were the most abundant shares on wet weight basis, meanwhile, as reported in Materials and Methods (section 2.1), starch, protein and lipids were found to be the main components of TS, with 19, 18 and 17% of TS, respectively. Fermentative H_2 production of OFMSW was found to be feasible with both strains: H_2 concentrations were slightly higher for *Bacillus* sp. F2.8 (Fig. 2b), which produced almost 61 mL of H_2 per g VS, at pH 7.0. At pH 6.0, the strain achieved slightly lower H_2 levels and productivity. On the other hand, *Bacillus* sp. F2.5 exhibited fermenting abilities comparable for both tested pH values and H_2-production was found 55 and 53 mL per g VS for pH 6.0 and 7.0, respectively (Fig. 2a).

Bacillus sp. F2.8 confirmed the most efficient productivity already described from soluble starch. At pH 7.0, the strain produced 5.0 mL of H_2 per day whereas 4.0 mL of H_2 were daily produced at lower pH (Fig. 2c). *Bacillus* sp. F2.5 had similar H_2 productivity at pH 6.0 (4.3 mL of biogas and H_2) while, at pH 7.0, its productivity was lower resulting in 3.9 mL of H_2 (Fig. 2c). Both strains produced comparable relative H_2 concentrations (nearly 38%) which were inferior than those above reported from soluble starch and glucose (Fig. 2c).

As shown in Table 4, the hydrogen levels produced in this study were consistent with those previously described for batch H_2 fermentation of OFMSW or food waste. Further, in the present study, the pre-treatment of inoculum was not required. Moreover, this is one of the earliest accounts on a single microbe capable of converting organic waste into H_2 with a high rate and yield. Only recently, Marone and colleagues described few strains, isolated by the bioaugmentation of vegetable waste (*Rahnella* sp. 10, *Buttiauxella* sp. 4 and *Raoultella* sp. 47),

for their promise in producing H_2 from vegetable kitchen waste collected from a cafeteria [17]. However, this is the first successful application of pure microbial cultures in bio-hydrogen production from OFMSW.

3.6 VFAs profiles from glucose, soluble starch and OFMSW fermentations

H_2 production is coupled with production of VFAs and/or solvents. The composition of VFAs generated is a useful indicator for monitoring the H_2 production pathways. The high VFAs concentrations achieved in this study indicate that favourable conditions for the growth and the activity of both strains were established during the course of the experiments (Fig. 2c). The detected soluble metabolites were acetate, butyrate and propionate. In all batch experiments the acetate was the major component (53-58%) with butyrate as the second most abundant acid (29-32%). This finding proved that similar metabolic pathways were involved and the acetate-butyrate was the predominant fermentation mode, which was reviewed as favouring H_2 production [1, 12]. As a result, supplementing different substrates significantly changed only the VFAs quantity rather than their shares: the highest amount of Total VFA (TVFA) was obtained from glucose meanwhile starch and OFMSW supported similar TFVA values. The higher the level of VFA accumulation (Fig. 2c), the higher H_2 production was achieved (Fig. 2a,b).

Table 4. Comparison of hydrogen production from OFMSW achieved in this study and other performances previously reported from OFMSW and food waste

Feedstock	Inoculum	Pre-treatment inoculum	Pre-treatment feedstock	Temperature (°C)	Yield (mL H_2/g VS)	Reference
OFMSW	*Bacillus* sp. F2.5	NO	Sterilized	35	61	This study
OFMSW	*Bacillus* sp. F2.8	NO	Sterilized	35	55	This study
OFMSW	pre-adapted H_2-producing bacteria	NO	NO	37	180	[16]
OFMSW	pre-treated digested sludge	100 °C 15 min	NO	37	140	[16]
OFMSW	NO	NO	NO	35	42	[5]
OFMSW	granular sludge	100°C 4 h	NO	35	70	[5]
OFMSW	granular sludge	100°C 4 h	Sterilized	35	57	[5]
OFMSW	granular sludge	100°C 4 h	NO	35	25-85	[15]
Food waste	anaerobic sludge	na	NO	35	39	[51]
Food waste	anaerobic sludge	na	NO	50	57	[51]
Food waste	grass compost	180°C 3 h	NO	35	77	[52]
Food waste	NO	NO	NO	35	4	[53]
Food waste	Food waste	90 °C 20 min	60-90 °C 20 min	35	26-149	[53]
Vegetal waste	Vegetal waste	NO	NO	28	22	[18]
Vegetal waste and potato peels	Vegetal waste and potato peels	NO	NO	28	18	[18]
Vegetal waste	*Rahnella* sp. 10	NO	NO	28	47	[18]
Vegetal waste	*Buttiauxella* sp. 4	NO	NO	28	71	[18]
Vegetal waste	*Raoultella* sp. 47	NO	NO	28	70	[18]

na: not available

4. CONCLUSIONS

This study demonstrated for the first time the effective conversion of OFMSW into H_2 by using pure cultures of *Bacillus* sp. strains properly selected for both their proficient enzymatic activities and their high fermenting abilities from glucose and starch. Future studies will further increase their H_2 performances and techno-economical evaluations will determine the actual feasibility of the whole process. Taken together, the results of this work gave advances in knowledge towards the development of microbial inoculants for the industrial processing of organic waste in H_2.

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