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ABSTRACT

In this work, different proportions of model fruit and vegetable wastes (MFVW) and acid hydrolyzed fish wastes (AHFW) were used for hydrogen production in a minimum culture medium based on seawater. Experiments were performed in pH-controlled Stirred Tank Reactor (STR) with or without the addition of nitrogen and sulfur sources. The total H_2 production and the maximum hydrogen productivity of *T. maritima* in the culture medium, containing MFVW and AHFW (45 mmol L^{-1} carbohydrates) at a C/N ratio of 12, were 132 mmol L^{-1} and 15 mmol h^{-1} L^{-1}, respectively. However, tripling the concentration of carbohydrates to reach a C/N ratio of 22, has increased two times the maximum H_2 productivity (28 mmol h^{-1} L^{-1}) due to the improvement in nutrient balance. The cumulative H_2 production was 285 mmol L^{-1}, yielding a potential energy generation of 0.1210^3 MJ ton^{-1} wastes, which could be an interesting alternative for energy recovery.

Introduction

Today, 80% of global energy demands depend on fossil fuels which have led to the greenhouse gas emissions and environmental pollution [1]. However, renewable energy sources have an increased interest in recent years because they have the aptitude to reduce the dependence on fossil fuels and their environmental impact [1]. Hydrogen seems to be the most attractive clean future energy vector for electricity generation in fuel cells, as well as an interesting gaseous biofuel for the transportation sector [2]. It is widely generated directly or indirectly from fossil fuel resources, but it can also be

Abbreviations: AHFW, acid hydrolyzed fish wastes; COD, chemical oxygen demand (g L^{-1}); FVW, fruit and vegetable wastes; FW, fish wastes; MFVW, model fruit and vegetable wastes; STR, stirred tank reactor; TS, total solids (%/wet basis); VS, volatile solids (%/TS).

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produced sustainably from renewable resources (sun, wind, hydropower, and biomass) [3].

H_2 production by dark fermentation is usually preferred due to its low energy requirements, a low operating cost, a higher rate of H_2 and the use of a wide range of organic wastes [4–6]. The highest fermentative H_2 yields were obtained with hyperthermophilic microorganisms to avoid the risk of contamination by other H_2-consuming microorganisms, to promote the reaction kinetics and to decrease the hydrogen solubility [7–10]. *Thermotoga maritima*, which belongs to the order *Thermotogales* has been considered an excellent candidate for H_2 production from various simple and complex carbohydrates [11–13]. It produces thermostable enzymes involved in the hydrolysis of complex polysaccharides into simple sugars [14]. Many studies showed a strong correlation between *T. maritima* cell growth and its ability to produce H_2. It showed the highest H_2 yield which is close to the Thauer limit (4 mol H_2 per mol glucose) with acetate as the main end product [15].

The fermentative H_2 production process needs a large amount of water. Therefore, using seawater instead of freshwater has many advantages. It is an alternative solution for scaling-up the fermentation process as reported by other studies [16–18]. It reduces the freshwater consumption and avoids the risk of contamination by non-halophilic microorganisms. In addition, seawater contains mineral compounds and salts that will be potentially used for bacterial growth. The average composition of seawater depends on the geographical areas. It contains (g L^{-1}) NaCl (27.133), $MgCl_2$ (2.504), $MgSO_4$ (3.382), $CaCl_2$ (1.167), KCl (0.742), $NaHCO_3$ (0.207), NaBr (0.085) and total salts (35.220) [18]. Therefore, Saidi et al. [19] reported that using a simplified culture medium composed of fruit and vegetable wastes (FVW) and natural seawater provided several nutrients which have replaced certain components necessary for *T. maritima* growth. The maximum hydrogen productivity and the hydrogen yield were 12.4 mmol h^{-1} L^{-1} and 3.89 mol H_2 mol^{-1} hexose, respectively [19].

Producing biohydrogen from biomass, rich in carbohydrates such as FVW and without chemical compounds addition, is an interesting cost-effective process. However, FVW are characterized by a low nitrogen content which limits the dark fermentation efficiency [20]. Bouallagui et al. [21] showed that the supply of co-substrates with high nitrogen content is a solution to adjust FVW nutrient balance for biogas production. Among these co-substrates, fish wastes (FW) were considered the highly biodegradable meat. In Tunisia, sardines are the most widely consumed blue fish species due to their availability and low cost [22]. However, they are susceptible to rapid deterioration [23]. Consequently, using the co-digestion process of FVW with FW is interesting technology for biohydrogen production.

Kim et al. [24] obtained H_2 yield of 2.11 mol H_2 mol^{-1} hexose by using food wastes to sewage sludge ratio equal to 10:1 (w/w on a COD basis). This production was 13% higher than that obtained by using food wastes alone [24]. The highest H_2 production rate (3.27 LH_2 L^{-1} d^{-1}) obtained by Tenca et al. [25] was achieved by using a mixture of FVW and swine manure with a ratio of 35/65. Also, Gomez-Romero et al. [26] were combined fruit and FVW with crude cheese whey at a C/N ratio of 21 to optimize H_2 production. The maximum volumetric H_2 production rate, and H_2 yield were 10.56 mmol H_2 h^{-1} L^{-1}, and 449.84 mL H_2 g COD^{-1}, respectively.

In this study, it is the first time where the hyperthermophilic bacterium *T. maritima* was used for biohydrogen production from FVW and FW co-fermentation. In fact, FW are characterized by a high content of nitrogen which is important for a better C/N ratio establishment. In addition, marine hyperthermophilic microorganisms are able to grow better on culture medium prepared with seawater rather than freshwater. Using hyperthermophilic co-digestion has several benefits. It increases the hydrolysis rate of the polymeric feedstock because hyperthermophilic hydrolytic enzymes have a highly stable activity with a better affinity to their substrates [27,28]. Therefore, the use of hyperthermophilic conditions has a positive effect on the solubilization of the co-substrate mixture which is better for higher and faster production of hydrogen. The potential of fish wastes (*Sardina pilchardus*) as an alternative source of nitrogen and sulfur was evaluated to avoid the addition of chemical products (NH_4Cl and cysteine HCl). Different C/N ratios, obtained by mixing different proportions of MFVW and AHFW, were used to evaluate the potential of hydrogen production and the metabolic pathway of *T. maritima*.

Material and methods

Substrates composition and preparation

In order to obtain reproducible conditions, model fruit and vegetable wastes (MFVW) were used with a specific composition. The main constituents of MFVW (g/kg) were: plums (207), peaches (207), apples (207), carrots (138), potatoes (130) and tomatoes (110). MFVW were crushed with an electric blender into small pieces measuring less than 2 mm in length and width. They were filtered, fully mixed and directly frozen at −20 °C until further use.

Sardines (*Sardina pilchardus*) were obtained from the Bir Kassa wholesale market located in Tunis (Tunisia). The whole fish (heads, bones, skin, and viscera) was grounded with an electric blender and homogenized. Viscera Fish (stomach, pancreas, and intestine) contain endogenous enzymes that can contribute to the hydrolysis of proteins. However, sardine muscles are composed of several groups of proteins (sarcoplasmic, myofibrillar, and stroma proteins) that are difficult to be used for bacteria growth [29]. Therefore, a pretreatment of sardines is required to obtain a suitable liquid easily used as a nitrogen source.

Acid-hydrolysis of sardines was combined with the water extraction as described by Gao et al. [30]. The minced sardines were mixed with water of equal weight. They were pretreated at 121 °C for 20 min and centrifuged at 3000 g for 20 min to separate the residue and the supernatant. The obtained residue was diluted with water to a ratio of 1/10 and the pH was set at around 2 by the addition of 6 M HCl. The acidified sample was pretreated again at 121 °C and centrifuged at 3000 g for 20 min. The first and the second supernatants were mixed and stored at −20 °C for later use. The degree of organic matter acidification, coming from pretreated sardines, was calculated:

Acidification yield (%) = $((TSS_i - TSS_f)/TSS_i)*100$

where, TSS_i is the initial suspended solids concentration ($g\,L^{-1}$) before the pretreatment and TSS_f is the final suspended solids concentration after the pretreatment.

Bacterial strains and culture conditions

T. maritima DSM 3109 was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ). The culture medium of *T. maritima* contained FVW, natural seawater, NH_4Cl (1 g L^{-1}), and cysteine HCl (0.3 g L^{-1}) as reported by Saidi et al. [19]. The pH was adjusted at 7.0 with 1M NaOH and distributed into 250 mL serum bottles with a working volume of 60 mL. The serum bottles were made in anaerobic conditions by flushing with sterile nitrogen gas and were reduced with Na_2S (0.4 g L^{-1}). After inoculation with *T. maritima* (10% v/v), the serum bottles were incubated at 80 °C without shaking. These precultures were used to inoculate the bioreactor.

Stirred tank reactor and operating conditions

Batch fermentations of *T. maritima* were performed in a STR of 2.5 L as described by Saidi et al. [19]. The STR with a working volume of 1.1 L was inoculated with a preculture of *T. maritima* (10% v/v). The mixture was kept anaerobic by sparging with continuous sterile nitrogen gas at a flow rate of 50 mL min^{-1}. The bioreactor was stirred at 150 rpm with an electric motor (IKA EUROSTAR 20 digital). It was heated (80 ± 0.5 °C) by thermal recirculation of water in the double envelope jacket of the bioreactor using a heat bath. The outlet gas was condensed in a water cooler condenser (7 °C) to avoid the liquid loss in the STR. A probe (Mettler Toledo InPro 3253, Switzerland) was placed in the bioreactor to monitor pH, temperature and redox potential. The adjustment of pH at 7.0 ± 0.1 was automatic by the addition of 1M NaOH. The measurements of carbon dioxide content in the STR outlet gas were done via a probe connected to a transmitter (Vaisala Series GMT221, Finland).

The culture medium of *T. maritima* contained natural seawater, MFVW, AHFW, NH_4Cl and cysteine HCl. The details of the experimental conditions of the experiments performed in triplicate are presented in Table 1.

Chemical and analytical analysis

Total solids (TS), volatile solids (VS), total organic carbon (TOC), total kjeldahl nitrogen (TKN), chemical oxygen demand (COD) and pH of the substrates were determined according to standard methods [31]. Determination of carbohydrate concentrations was performed using the anthrone sulfuric acid method as described by Saidi et al. [19]. Cellulose and hemicelluloses concentrations were measured according to Sun et al. [32].

During fermentation, the residual sugars (glucose and fructose) and metabolic products (acetate and lactate) concentrations were analyzed by high performance liquid chromatography HPLC (Agilent 1200 series, USA) equipped with a quaternary pump coupled to a refractive index detector (RID) and an Aminex HPX-87H ion-exchange column (300 × 7.8 mm, Bio-Rad). Liquid samples resulting from batch fermentations were centrifuged at 14000×*g* for 5 min. The supernatants were filtered through a 0.45 μm cellulose acetate Minisart syringe filter (Sartorius Stedim). Sulfuric acid 5 mmol L^{-1} was used as a mobile phase with a flow rate of 0.5 mL min^{-1}. The HPLC was connected to a computer running ChemStation software.

Hydrogen measurements were performed at regular intervals of 30 min by a gas chromatograph (GC, Perichrom Company, France) which was connected to a computer running WINILAB III software. The GC was equipped with a thermal conductivity detector (TCD) and a concentric CTR1 column (Alltech, USA). The operating temperatures of the detector, the injector, and the oven were 100 °C, 100 °C, and 40 °C, respectively. Argon was used as the carrier gas at a flow rate of 20 mL min^{-1}.

Results and discussion

Physical and chemical characterization of substrates

The average characteristics of the MFVW and AHFW used in this study and their standard errors are listed in Table 2. The VS content in MFVW and AHFW were 94.6% and 91%,

Table 1 – Experimental conditions for the STR batch fermentations.

Experiments	MFVW (mL)	AHFW (mL)	Seawater (mL)	Cysteine HCl	NH_4Cl	C/N
E1	100	0	890	+	+	47
E2	100	50	840	+	+	32.4
E3	100	100	790	+	+	25.1
E4	100	250	640	+	+	15.7
E5	100	400	490	+	+	12
E6	100	50	840	–	–	32.4
E7	100	100	790	–	–	25.1
E8	100	250	640	–	–	15.7
E9	100	400	490	–	–	12
E10	200	400	390	–	–	17.8
E11	300	400	290	–	–	22

+: Present.
–: Absent.

Table 2 – Physical and chemical characterization of MFVW and AHFW.

Parameters	MFVW	AHFW
Total solids (TS) (% wb)	10.1 ± 0.1	4.4 ± 0.4
Volatile solids (%/TS)	94.6 ± 1.7	91 ± 1.2
Total COD (g L^{-1})	148 ± 2.5	50 ± 1.3
Particulate COD (g L^{-1})	52 ± 4.1	–
Soluble COD (g L^{-1})	96 ± 4	–
Total organic carbon (g L^{-1})	103.6 ± 4.5	39 ± 1.2
Carbohydrates (g L^{-1})	106 ± 1.9	13.5 ± 0.3
Total Kjeldahl Nitrogen (g.L^{-1})	2.2 ± 0.04	12 ± 2.8
Lipids (g/100g)	2.5 ± 1.5	1.8 ± 0.6
Reducing sugars (g L^{-1})	83.5 ± 5.6	0
pH	4.07 ± 0.09	2 ± 2.05
Cellulose (g L^{-1})	4.5 ± 0.7	0
Hemicellulose (g L^{-1})	1.9 ± 0.2	0
Reducing sugars (HPLC) (g.L^{-1})	84.1 ± 0.3	0
C/N	47	3.25

wb: wet basis.

respectively. The total organic carbon concentrations in MFVW and AHFW were 103.6 g L^{-1} and 15 g L^{-1}, respectively. The high protein content of AHFW (12 g L^{-1}) is the result of proteins solubilization during acid hydrolysis and the removal of insoluble solid matter by centrifugation [33]. Several studies reported that the protein content of fish protein hydrolysates is ranging between 60% and 90% of the total composition [33].

Effect of increasing the amount of AHFW on H_2 production

The hydrogen production by *T. maritima* from MFVW with the supply of a gradually increased amount of AHFW in the minimum medium was evaluated either with (E_2 to E_5) or without (E6 to E9) the addition of NH_4Cl and cysteine HCl (Table 1). Results are presented in Fig. 1 and Table 3. Experiment E_1 was performed using MFVW as a substrate with the addition of NH_4Cl and cysteine HCl. After the addition of AHFW in the presence of NH_4Cl and cysteine HCl, the hydrogen production was improved. The cumulative H_2 production increased from 109 mmol L^{-1} (E_1) to 176 mmol L^{-1} reaching H_2 yield of 3.87 mol H_2 mol^{-1} hexose in experiment E_5 where the C/N ratio was 12 (Fig. 1, Table 3). However, the maximum H_2 productivity increased approximately threefold times than that in E_1. These results confirm the positive effect of using the AHFW as co-substrate on H_2 production by *T.maritima*, despite the appearance of a lag phase of approximately 2–3 hours (Fig. 2). In fact, *T. maritima* takes some time to adapt to its new substrate in the culture medium. In previous studies, yeast extract was considered the best organic nitrogen source for hydrogen production. It provides amino acids, peptides, nitrogen, carbohydrates, vitamins, mineral elements and other growth compounds necessary for the growth of microorganisms [34]. In this study, despite the absence of yeast extract in co-digestion assays, the maximum H_2 productivity (24.6 mmol h^{-1} L^{-1}) was higher than that (7.3 mmol h^{-1} L^{-1}) achieved by Saidi et al. [19] in the presence of yeast extract (1 g L^{-1}). In fact, fish protein hydrolysates were considered an important source of nitrogen (enzymes, proteins, and nucleic acids) for maintaining the growth of different microorganisms [35]. Moreover, AHFW are rich in peptides and free amino acids which are taken by the cell and directly used for protein synthesis or for their transformation into other nitrogenous cellular constituents [36].

The effect of using different C/N ratios on co-digestion assays in the absence of NH_4Cl and cysteine HCl was investigated (E_6, E_7, E_8, and E_9) (Table 1). Increasing the amount of

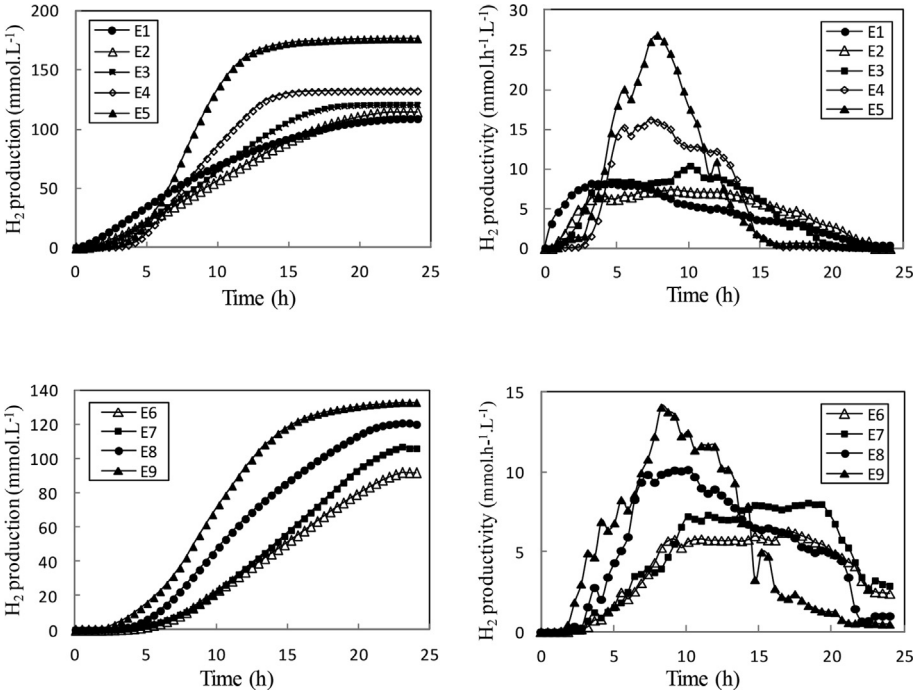

Fig. 1 – Cumulative H_2 production and H_2 productivity versus time in batch experiments with increasing amounts of AHFW with (E_1, E_2, E_3, E_4 and E_5) or without (E_6, E_7, E_8 and E_9) Cysteine HCl and NH_4Cl addition.

Table 3 – Main results obtained for experiments E1 to E11.

	E1	E2	E3	E4	E5	E6	E7	E8	E9	E10	E11
Initial total carbohydrates (mmol L^{-1})	48.1 ± 1.9	44 ± 1.6	43 ± 2.2	43.3 ± 2.3	50 ± 0.9	41 ± 0.8	42.02 ± 1.2	43.05 ± 2.7	43 ± 1.9	77.11 ± 2.9	113 ± 3
Carbohydrates Consumption (mmol L^{-1})	33.6 ± 0.5	35.6 ± 0.8	35.5 ± 2.4	36.1 ± 2.6	48 ± 1.7	31.02 ± 0.9	32.8 ± 2.2	35.75 ± 0.5	36.5 ± 1.2	53.4 ± 1.7	78 ± 2.5
Carbohydrates Consumption (%)	69.85	80.9	82.5	83.5	96	75.65	78	83	84.8	69.2	69
Total H_2 production (mmol L^{-1})	109 ± 2.9	115 ± 2.4	120 ± 1.9	132 ± 3.1	176 ± 3.6	89 ± 1.7	107 ± 2.1	121 ± 2.6	132 ± 2.3	187 ± 3.1	285 ± 2.9
Total H_2 production (mL H_2 g^{-1} (COD)	27.5 ± 3.2	28.5 ± 1.5	29 ± 3.5	31 ± 2.9	39 ± 1.6	24 ± 2.4	27 ± 3.4	29.5 ± 2.6	31 ± 1.4	40.5 ± 2.5	57 ± 2.7
H_2 Yield (mol H_2 mol^{-1}hexose)	3.24 ± 0.1	3.39 ± 0.04	3.58 ± 0.07	3.85 ± 0.3	3.87 ± 0.1	3.01 ± 0.9	3.26 ± 0.5	3.57 ± 0.1	3.8 ± 0.05	3.84 ± 0.1	3.86 ± 0.5
Maximum H_2 productivity (mmol h^{-1} L^{-1})	7.3 ± 1.3	7.3 ± 0.5	10.4 ± 1.2	16.4 ± 0.7	24.6 ± 1.3	6.3 ± 1.2	8 ± 0.9	10.2 ± 0.5	15 ± 1.2	18.9 ± 1.3	28.2 ± 1.5
Acetate production (mmol.L^{-1})	56 ± 1.5	42.9 ± 1.5	63.9 ± 0.9	51.75 ± 1.3	99.5 ± 2.6	42.44 ± 2.2	61.7 ± 0.9	62.2 ± 1.7	74 ± 1.9	103.9 ± 2.2	148 ± 3.5
Lactate production (mmol L^{-1})	10.1 ± 1.1	24 ± 0.5	25 ± 0.9	30.4 ± 1.1	33.4 ± 2.9	26.7 ± 0.8	27.3 ± 0.7	34.7 ± 0.5	36.4 ± 2.2	41.1 ± 1.4	49 ± 1.3
Acetate/hexose (mol mol^{-1})	1.67	1.51	1.84	1.33	1.96	1.13	1.12	1.82	1.93	1.93	1.94
H_2/acetate (mol mol^{-1})	1.95	1.92	1.95	1.95	1.98	1.95	1.96	1.96	1.97	1.99	1.99
CO_2 production (mmol L^{-1})	49.3 ± 1.8	49 ± 1.5	49.9 ± 2.5	60.4 ± 2.4	75.9 ± 1.1	46.11 ± 0.9	49.7 ± 2.3	56.3 ± 1.6	58.8 ± 1.5	83.7 ± 2.4	125 ± 3.1
H_2/CO_2 (mol mol^{-1})	2.21	2.34	2.42	2.19	2.33	2.02	2.15	2.14	2.24	2.23	2.29

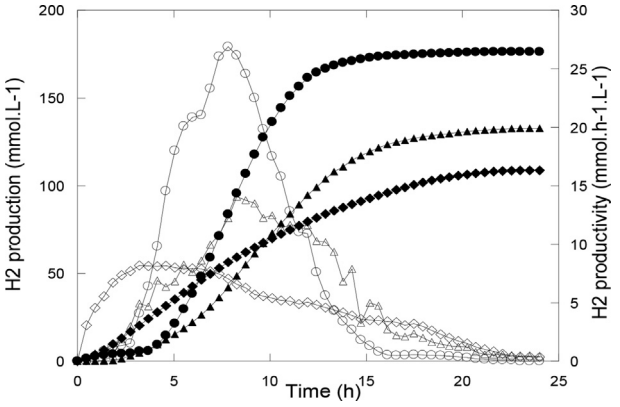

Fig. 2 – Cumulative H_2 production (E_1: ◆, E_5: ●, E_9: ▼) and H_2 productivity (E_1: ◇, E_5:○, E_9: ▽) versus time.

AHFW to obtain a C/N ratio of 12 (E_9), has improved the H_2 production from 109 mmol L^{-1} (E_1) to 132 mmol L^{-1}, despite the absence of NH_4Cl and cysteine HCl (Fig. 1, Table 3). Therefore, this enhancement could be attributed to the presence of AHFW that contain compounds able to provide nitrogen and sulfur indispensable for *T. maritima* growth as reported by some studies [19,37]. Indeed, the acid hydrolysis of fish wastes has broken complex proteins into small peptides and single amino acids which could be consumed by this halophilic bacterium. Moreover, many studies showed that aquatic organisms can use easily the low molecular weight peptides which can be absorbed rapidly than single amino acids and whole proteins [38,39]. In addition, Rinker and Kelly [37] reported that *T. maritima* did not significantly consume individual amino acids added to culture media. *Sardina pilchardu* is also characterized by the presence of sulfur amino acids (methionine, cysteine) and sulfur compound indispensable for *T. maritima* growth. These compounds are essential for protein, Fe-S clusters, and ferredoxin synthesis. Also, Prost et al. [40] showed that dimethyl sulfide which was considered one of the most important sulfur compounds is present in *Sardina pilchardu* (18.0%). This component was obtained from amino acid degradation [40].

The comparison between experiments showed a similarity between E_7 and E_1 with H_2 yield of 3.25 mol H_2 mol^{-1} hexose. This result showed that using 100 mL of AHFW could substitute compounds coming from NH_4Cl (g L^{-1}) and cysteine HCl (0.3 g L^{-1}) with a preference to use organic compounds. In addition, many studies showed that the synthesis of amino acids for protein synthesis from organic nitrogen is rapid and required few energy compared to that performed from inorganic sources [34,36].

Effect of using AHFW on metabolic pathway of T.maritima

T. maritima is known to metabolize a broad range of sugars. It uses for glycolysis the Embden-Meyerhof (85%) and the Entner-Doudouroff (15%) pathway to produce H_2, acetate, and CO_2 as major by-products [15]. Frock et al. [14] reported that H_2 production is associated with the conversion of all hexoses to acetate because there is no fleeing of electrons in other reactions.

The evolution of cumulative H_2 production and H_2 productivity in experiments E_1, E_5 and E_9 versus time is presented in Fig. 2. Results showed a significant improvement in the production of H_2 using co-digestion of MFVW and AHFW, suggesting a complementary effect between these two substrates. However, co-digestion assays in the absence of NH_4Cl and cysteine HCl showed a lag phase of 2–3 h. In fact, the delay of H_2 production was a result of these nutrients absence. The higher H_2 productivity (24.6 mmol h^{-1} L^{-1}) was obtained in E5 containing NH_4Cl and cysteine HCl which indicate that despite the importance of using AHFW as a co-substrate, the supply of NH_4Cl and cysteine HCl would further improve *T. maritima* growth and H_2 production kinetics. In addition, results indicate that cysteine HCl was used as reducing compound [41]. It reduces rapidly the redox potential (Eh) in the culture medium down to −350 mV due to the establishment of anaerobic conditions (data not shown). This condition could induce earlier the metabolic activity initiation of *T. maritima*. Ravot et al. [42] reported that the addition of sulfured compounds, such as dimethyl sulfide, methionine, cysteine, and thiosulfate, relieve the inhibition of H_2 and enhance the cellular growth. Consequently, the small amount of sulfur will be given as a priority to protein synthesis rather than the Fe-S clusters formation of hydrogenases involved in the production of hydrogen [43].

The fermentative end products (acetate and lactate) and the cumulative H_2 production in experiments E_1, E_5 and E_9 are presented in Table 3 and Fig. 3. The production of lactate was higher in the co-digestion experiments (E_5 and E_9) than that achieved in E_1. The concentration of lactate in E_9 was 36.4 mmol L^{-1} which is approximately four-fold compared to E_1. The production of hydrogen in E_9 remained high (3.8 mol H_2 mol^{-1} hexose) (Table 3) despite high lactate production. Therefore, the presence of AHFW as co-substrate without NH_4Cl and cysteine HCl addition induced *T. maritima* to shift its metabolism from hydrogen and acetate to lactate production via lactate dehydrogenase as described by Willquist and van Niel [44]. Moreover, many studies indicated that during fermentative H_2 production by *Thermotoga* species, lactate production can vary from trace amounts to levels resembling that of acetate [14,15]. Schröder et al. [15] showed that lactate formation was detected in particular by *T. maritima*. Also, Verhaart et al. [45] confirmed that some hyperthermophiles were able to allow H_2 yields close the thauer limit, suggesting that, despite the more production of lactate and ethanol, nicotinamide adenine dinucleotide dehydrogenase (NADH) is used for proton reduction to form hydrogen.

The evolution of lactate production versus time is illustrated in Fig. 4. During the growth phase, the production of lactate started slowly and accelerated after the transition from the exponential growth phase to the stationary phase (Fig. 4). This production is correlated with the total consumption of carbohydrates by *T. maritima* and the reduced production of H_2 and acetate.

In general, hydrogenases which constitute a family of metalloenzymes, reduce protons to hydrogen by low-potential electrons in order to dispose of excess reductants that are generated during fermentation [46]. Consequently, hydrogen is a waste product that its formation serves as a physiological mechanism to protect the bacterial cells [46]. Dipasquale et al. [47] explained the formation of lactate as a manner to improve the disposal of the surplus by reducing power. However, the production of hydrogen and lactate compete for the utilization of reducing power. They reported that the conversion of 1 mol of pyruvate to lactate by lactate dehydrogenase requires oxidation of 1 mol of nicotinamide adenine dinucleotide dehydrogenase (NADH), as the production of 2 mol of hydrogen. Lakhal et al. [41] observed that the metabolism of *T. maritima* shifts towards lactate production under oxidative conditions. Furthermore, the presence of substrates difficult to hydrolyze could be responsible for the distribution of catabolic and anabolic fluxes leading to different metabolite levels that modulate lactate dehydrogenase activity [44]. In addition, the production of lactate depends on culture conditions such as high H_2 partial pressure, the transition to stationary phase and the shift of metabolic of pyruvate towards lactate production via lactate dehydrogenase [44].

Recently, some studies reported that the use of an atmosphere enriched in carbon dioxide stimulated the synthesis of lactate by *T. neapolitana*, which has similar characteristics to *T. maritima*. This process was named capnophilic lactic fermentation (fermentative CO_2-dependent) where lactate formation was the result of the association of exogenous CO_2 with acetyl-CoA [9,47,48]. Dipasquale et al. [47] showed that the high production of lactate (up to 0.6 mol mol^{-1} glucose)

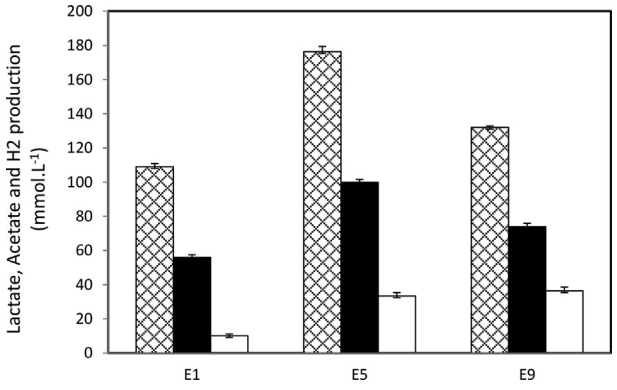

Fig. 3 – Comparative production of H_2 (⊠), Acetate (■) and Lactate (□) at 24 h between experiments E_1, E_5 and E_9.

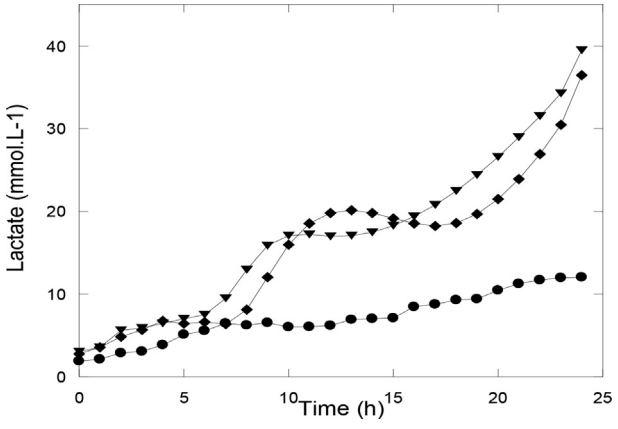

Fig. 4 – Lactate production versus time for experiment E_1: ●, E_5: ◆ and E_9: ▼.

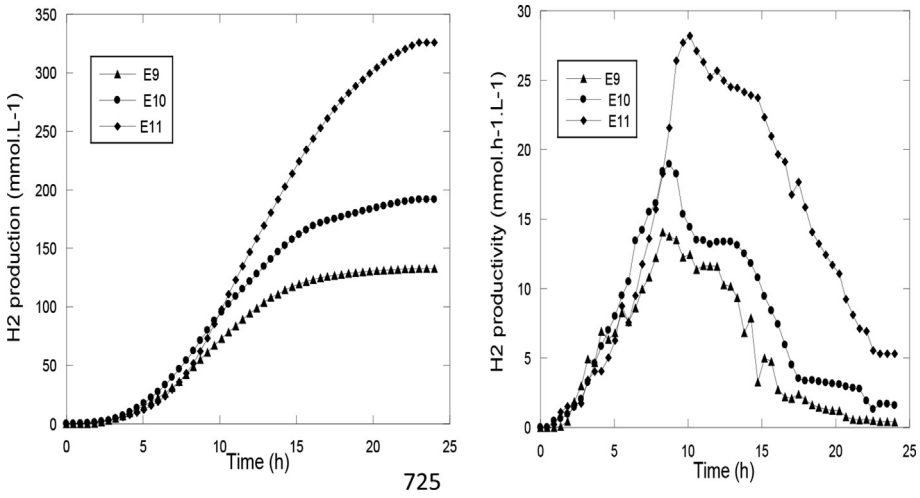

Fig. 5 – Cumulative H_2 production and H_2 productivity in batch experiments at different proportions of MFVW in the absence of Cysteine HCl and NH_4Cl.

does not affect the production of H_2 which was around 3.6 mol mol^{-1} glucose. These results were confirmed by d'Ippolito et al. [48] those observed the shift of the metabolism of *T. neapolitana* from acetate to lactate formation during the capnophilic lactic fermentation. The production of H_2 remains high through an additional consumption of reducing equivalents deriving from other cellular processes [48].

Effect of increasing the amount of MFVW on H_2 production

Experiments E_9, E_{10} and E_{11} were performed with the same volume of AHFW (400 mL) and different proportions of MFVW in the absence of cysteine HCl and NH_4Cl (Table 1). The corresponding cumulative H_2 production and H_2 productivity are presented in Fig. 5 and Table 3. The increase of the carbohydrates concentration in E_{11} (123 mmol L^{-1}) to reach a C/N ratio of 22, allowed a cumulative H_2 production 2.5 times higher than that obtained in E_9 (C/N ratio = 12) (Table 3). Consequently, the highest H_2 productivity (28 mmol h^{-1} L^{-1}) and H_2 yield (3.86 mol H_2 mol^{-1}hexose) achieved at C/N ratio of 22 were associated with a significant consumption of carbohydrates (69%) within 24 h of fermentation (Table 3). These results suggest that the improvement of hydrogen production by *T. maritima* could be a function of the C/N ratio and the proportions of MFVW and AHFW in the mixture. In addition, the high production of hydrogen is due to the high activity of *T. maritima* considered one of the most important H_2-producing bacterium. In fact, this bacterium produces a wide range of thermostable hydrolytic enzymes (cellulases, invertase, and xylanases) necessary for hydrolyzing a wide variety of simple and complex carbohydrates [28]. However, Nguyen et al. [13] observed that the concentration of glucose over 15 g L^{-1} has a negative effect on H_2 production and cell growth of *T. maritima* and *T. neapolitana* in batch cultures. Therefore, the use of fruit and vegetable wastes is better than glucose because they play an important role in the fermentation process. They provide mineral elements, amino acids and vitamins essential for the metabolic activity of *T. maritima* [19]. Moreover, this bacterium is able to use the glucose-based complex carbohydrates due to its genome which code for a large number of glycoside hydroxylases. These enzymes hydrolyze the glycosidic bond between carbohydrates or between a carbohydrate and a non-carbohydrate fraction [12].

H_2 potential of T. maritima and energy recovery efficiency

Table 4 summarizes the performance of H_2 production by some *Thermotoga* strains from various substrates reported in

Table 4 – The comparison of batch fermentative hydrogen production performances by some *Thermotoga*. sp from various substrates reported in the literature.

Strain	Carbon source	Substrate (g L^{-1})	H_2 yields (mol H_2 mol^{-1} substrate)	Maximum H_2 productivity (mmol h^{-1} L^{-1})	By-products	References
T.maritima	MFVW-AHFW	20	3.86	28.2	Acetate, lactate, CO_2	This study
T.maritima	Glucose	7.5	1.7	8.2	-	[13]
T.maritima	Arabinose	5	3.2	0.6	Acetate, lactate, CO_2	[49]
T.maritima	Xylose	5	2.7	0.4	Acetate, lactate, CO_2	[49]
T.maritima	MFVW	8.1	3.89	12.4	Acetate, lactate, CO_2	[19]
T.neapolitana	Potato steam peels	10	3.8	12.5	Acetate, lactate, CO_2	[50]
T.neapolitana	Miscanthus hydrolysate	10	3.2	13.1	Acetate, lactate, CO_2	[51]
T.neapolitana	Carrot pulp	10	2.8	12.5	Acetate, lactate, CO_2	[52]
T.neapolitana	Molasses	20	2.64	1.7	Acetate, lactate, CO_2	[28]
T.neapolitana	Cheese whey	12.5	2.4	0.94	Acetate, lactate, CO_2	[28]

the literature. In this study, the maximum H_2 productivity (28.2 mmol h^{-1} L^{-1}), obtained at C/N ratio of 22, is high when compared to the results of other studies. deVrije et al. [51] and Saidi et al. [19] used miscanthus hydrolysate and MFVW as substrates to obtain a maximum H_2 productivity of 13.1 mmol h^{-1} L^{-1} and 12.4 mmol h^{-1} L^{-1}, respectively. The maximum H_2 productivity of *T. Neapolitana* reached by Cappelletti et al. [28] on molasses and cheese whey were 1.7 and 0.94 mmol h^{-1} L^{-1}, respectively.

The energy recovery was calculated based on the hydrogen yield and the energy value of hydrogen which was reported to be 12.71 kJ L^{-1} [53]. In the optimal condition (E_{11}), the cumulative hydrogen yield was calculated as 89 L H_2 kg^{-1} VS. This yield compares well with the energy content of other lignocellulosic feedstocks as reported by Abreu et al. [54]. The use of lignocellulosic garden wastes by individual *T. maritima* and the co-culture of *T. maritima* and *C. saccharolyticus*, allowed a yield of 45.1 and 75.1 LH_2 kg^{-1}VS, respectively [54]. Moreover, the H_2 yield obtained from food wastes mixtures varied between 25 and 85 LH_2 kg^{-1} VS as shown by Alibardi and Cossu [55].

Consequently, the achieved energy recovery was 12.0310^3 MJ ton^{-1} wastes. Further studies on hydrogen production from organic wastes (Fish, fruit and vegetable wastes) should be considered at a pilot scale to include cost assessment investigation.

Conclusion

In this study, the feasibility of biohydrogen production by *T. maritima* from hyperthermophilic anaerobic co-digestion of MFVW and AHFW in seawater was investigated. The highest cumulative H_2 production of 285 mmol L^{-1}, leading to H_2 yield of 3.86 mol H_2 mol^{-1} hexose, was obtained in the experiment that was performed at C/N ratio of 22. The maximum H_2 productivity was 28 mmol h^{-1} L^{-1}. Therefore, the rich composition of MFVW and AHFW associated with the elements provided by seawater, allow an appropriate carbon to nitrogen ratio which enhanced the production of H_2. In fact, AHFW are an important source of nitrogen and sulfur. Consequently, using marine co-digestion process could bean interesting cost-effective alternative to produce biohydrogen without chemical compounds addition. Future development studies would be occurring at a pilot scale to obtain further data on the economic, technical and environmental implications of this promising process for biotechnological applications.

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