Coproduction of hydrogen, ethanol and 2,3-butanediol from agro-industrial residues by the Antarctic psychrophilic GA0F bacterium

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Abstract

In this study, the simultaneous production of hydrogen, ethanol, and 2,3-butanediol was assessed using three agro-industrial residues: cheese whey powder (CWP), wheat straw hydrolysate (WSH) and sugarcane molasses (SCM), by the Antarctic psychrophilic GA0F bacterium [EU636050], which is closely related to *Pseudomonas antarctica* [KX186936.1]. The main soluble metabolites produced in all the fermentations were ethanol and 2,3-butanediol. CWP demonstrated to be the most effective carbon source, since fermentation of this substrate resulted in the highest yields of H_2 (73.5 ± 10 cm^3 g^{-1}), ethanol (0.24 ± 0.03 g g^{-1}) and 2,3-butanediol (0.42 ± 0.04 g g^{-1}), followed by the use of SCM, whereas WSH showed to have an inhibitory effect during the fermentation process, showing the lowest production values. Our results demonstrated the ability of the Antarctic psychrophilic GA0F bacterium to produce valuable products using low-cost substrates at room temperature conditions.

Keywords: Biofuels; Dark fermentation; Hydrogen; Ethanol; 2,3-butanediol.

1. Introduction

Biofuels have been considered as an option to replace fossil fuels. However, they must be derived from feed-stocks produced with much lower life-cycle and green-house emissions than traditional fossil fuels and with little or no competition with food production [1]. In this regard, renewable biomass is the most versatile non-petroleum-based resource that is generated from various industries as waste materials [2]. Lignocellulosic materials such as cereal straw, maize cob residues, food and starch-based materials, as well as organic industry wastewater, represent a vast source of raw materials that can be easily converted into sustainable energy carriers [3]. Among many alternatives, hydrogen and ethanol could emerge as important sustainable fuel sources in the foreseeable future. Biohydrogen can be used directly in combustion engines for transportation or in fuel cells for electricity generation, its high energy density (122 kJ/g), and the fact that water is the only by-product generated, makes hydrogen an ideal alternative to fossil fuels [4]. Furthermore, ethanol is the most employed liquid biofuel either as a fuel or as a gasoline enhancer; it has a high oxygen content that allows better oxidation of the gasoline hydrocarbons with the consequent reduction in the emission of CO_2 to the atmosphere [5]. 2,3-Butanediol is a high-value chemical with high heating value (27.20 kJ/g) which compares favorably with other liquid fuels (methanol 22.08 kJ/g, ethanol 29.06 kJ/g) [6]. Likewise, 2,3-butanediol is used as a precursor in the manufacture of a range of chemical products (*i.e*. perfumes, fumigants, moistening foods, antifreeze, and pharmaceuticals) [7, 8]. The production of hydrogen, ethanol, and 2,3-butanediol can be carried out throughout fermentative processes

such as dark fermentation. This method is environmentally friendly and more cost-effective compared to its chemical and thermochemical counterparts [9]. Different substrates such as corncob molasses, cheese whey and pre-treated lignocellulosic biomass have been used to produce H_2, ethanol and 2,3-butanediol [10-12]. Although the development of fermentation processes using economical carbon sources is an important issue for the production of these bio-commodities on a commercial scale, it is also desirable to find microorganisms with the ability to improve the production of these value-added compounds with the concomitant reduction in energy consumption. From this perspective, the study of Antarctic ecosystems and their microorganisms have received greater attention to produce hydrogen at temperatures close to room temperature [13, 14]. These microorganisms, which have the ability to grow at low temperatures (0-25°C) [15], are characterized by their high catalytic efficiencies, that make them attractive for different biotechnological areas [16]. These studies were carried out using pure simple carbon sources, while to our knowledge, there are no reports regarding biofuel production by Antarctic psychrophilic bacteria using complex substrates such as agro-industrial residues. Therefore, the aim of this study was to evaluate the dark fermentation of different complex substrates such as cheese whey (CW), wheat straw hydrolysate (WSH) and sugarcane molasses (SCM) by the Antarctic psychrophilic GA0F bacterium.

2. Materials and methods

2.1 Bacterium and substrates

Psychrophilic GA0F bacterium [EU636050] was used as fermentative organism. GA0F bacterium was previously isolated from glacier sediments from Antarctica [17] and it is closely related to *Pseudomonas antarctica* [KX186936.1] (according to NCBI). GA0F bacterium was routinely grown in solid YPG medium [13]. The agro-industrial residues CWP, SCM, and WSH were evaluated as potential carbon sources for GA0F bacterium for dark fermentation cultivations. CWP was purchased from Land O'Lakes Inc. (Arden Hills, Minnesota), and SCM was obtained from a local industry in San Luis Potosí, Mex, while WSH was obtained from CUCBA (University of Guadalajara, Jalisco, Mex). Fermentations using CWP 20 g dm^{-3} contained 13.5 g dm^{-3} of total sugars. SCM were diluted from a stock solution to a final total sugar concentration of 21 g dm^{-3}. For fermentations using WSH, the concentrated liquid fraction obtained from evaporation (at 70°C) of the slurred wheat straw that was pre-treated at 121°C for 1 h in a steam sterilizer in dilute H_2SO_4 (0.75% v/v) at 4% (w/v) was used. The WSH concentrated liquid fraction contained 20.4 g dm^{-3} of total sugars (composed of glucose 3.2 g dm^{-3}, xylose 14.2 g dm^{-3}, and arabinose 3.0 g dm^{-3}), organic acids such as formic acid 1.0 g dm^{-3}, and acetic acid 2.2 g dm^{-3}, and furfural 0.6 g dm^{-3}.

2.2 Batch dark fermentation experiments

For dark fermentation experiments, preinocula of GA0F bacterium were grown in liquid YPG medium and incubated at 25°C and 120 rpm. After overnight growth cells were harvested by centrifugation, washed and then inoculated into 120 cm^3 anaerobic serological bottles (Prisma, DF, Mex) containing 110 cm^3 of production

105 medium containing 0.25 g dm^{-3} yeast extract and 2.75 g dm^{-3} Bacto-tryptone
106 supplemented with each of the agro-industrial substrates (CW, WSH or SCM).
107 Serological bottles were rubber stopper capped with an aluminum crimp cap to
108 avoid gas leakage. The production medium was supplemented with 1 cm^3 dm^{-3}
109 trace elements solution [13]. The cultures were started at an optical density at 600
110 nm wavelength (OD_{600nm}) of 0.1. Initial pH was adjusted at 7, and incubated at
111 25°C and 180 rpm. All the experiments were carried out in triplicate.

112

113 **2.3 Analytical methods**

114 The volume of produced biogas was measured by the water displacement method
115 using an inverted burette with acidic water (pH <2). The percentage of hydrogen in
116 the biogas was determined by gas chromatography using a thermal conductivity
117 detector (Agilent Technologies Wilmington, DE, USA) as previously described [13].
118 1 cm^3 samples were taken at different times during fermentation, then were diluted
119 and filtered using a 0.22 µm syringe filter (Millipore, Bedford, Massachusetts,
120 USA). End-fermentation metabolites such as succinic acid, lactic acid, formic acid,
121 acetic acid, ethanol, and 2,3-butanediol were quantified by High-Performance
122 Liquid Chromatography (HPLC, Infinity LC 1220, Agilent Technologies, Santa
123 Clara CA, USA) using a Refractive Index Detector, with a column Phenomenex
124 Rezex ROA (Phenomenex Torrance, CA, USA) at 60°C, and 0.0025 M H_2SO_4 as
125 mobile phase at 0.5 cm^3 min^{-1}. The carbohydrates content in each agro-industrial
126 substrate (CWP, WSH, and SCM) was analyzed by the colorimetric method for
127 determination of sugars and related substances [18, 19]. Furfural present in WSH

was spectrophotometrically determined by the method established by Mexican standard regulation NMX-V-004-1972 [20].

2.4 Statistical analysis

The statistical analysis of the different experiments was determined by analysis of variance (ANOVA) and unpaired Student's *t*-test. Treatments with $p < 0.05$ were considered as statistically significant. The statistical analysis was performed using Excel v16 and GraphPad Prism v5.

3. Results and discussion

3.1 Cheese whey fermentation

Cheese whey is a cheap substrate and raw material nutritionally rich used for biofuel production [21]. This by-product is the liquid remaining from cheese production and represents about 85-95% of the milk volume. Typically, this residue contains lactose (4.5-5% w/v), soluble proteins (0.6-0.8% w/v) and lipids (0.4-0.5% w/v) [22]. Cheese whey powder (CWP) is a dried and concentrated form of cheese whey, it has some obvious advantages, such as reduced volume, concentrated source of lactose (75-80%), long term stability and ease of storage and transportation [23, 24]. In this work, 20 g dm^{-3} of CWP, which contained 13.5 g dm^{-3} of total sugars, were used as the substrate for batch fermentations. Fig. 1 shows the hydrogen production kinetics using CWP as substrate. As it is noted, most of the lactose present in CWP was rapidly consumed within the first 48 h of

fermentation. After lactose was depleted from the medium, approximately at 150 h, the maximum hydrogen production attained by GA0F bacterium was 923.2 ± 130 cm^3 dm^{-3}. The use of CWP as substrate turned out to be beneficial for the psychrophilic bacterium, which was probably due to the nutrients present in the solution, including nitrogen and minerals. The hydrogen production observed can be compared to those attained by mesophilic and thermophilic bacteria. For example, Kargi et al. [25] reported the hydrogen production by anaerobic sludge using CWP under mesophilic (35°C) and thermophilic (55°C) conditions showing that the highest hydrogen production of 1,144 cm^3 H_2 dm^{-3} was reached under thermophilic conditions with a maximum production rate of 3.46 cm^3 H_2 dm^{-3} h^{-1}. Instead, in this study psychrophilic GA0F bacterium reached 923.20 ± 130 cm^3 H_2 dm^{-3}, with a maximum production rate of 7.60 ± 0.4 cm^3 H_2 dm^{-3} h^{-1}, which represents two-fold the production rate reported for the thermophilic sludge. Furthermore, the process required 30°C less than the thermophilic fermentation, which denotes an economic advantage, since it is possible to carry out the process at room temperature. Several studies [25, 26] cheese whey has proved to be a suitable substrate for hydrogen production using mesophilic and thermophilic bacteria. Nevertheless, there are few reports regarding the use of cheese whey for hydrogen production by psychrophilic bacteria. Recently, Debowski et al. [27] reported the evaluation of hydrogen production by psychrophilic bacteria isolated from underground water and from demersal lake water using cheese whey as substrate. From 12 strains evaluated, *Rhanella aquatilis* (RA7) reached the highest hydrogen production of 134 cm^3 dm^{-3}, while the hydrogen production achieved by GA0F bacterium was almost 7 times higher than the production attained by RA7.

These data prove the feasibility of Antarctic psychrophilic microorganisms to convert complex substrates such as CWP into hydrogen. Besides hydrogen, fermentation of CWP resulted in the production of several soluble metabolites. As shown in Fig. 2, the main metabolite produced was 2,3-butanediol (5.3 ± 0.5 g dm^{-3}) followed by ethanol (3.0 ± 0.04 g dm^{-3}), succinic acid (0.5 ± 0.08 g dm^{-3}), and acetic acid (0.28 ± 0.06). This metabolite profile is typical of the mixed acid fermentation by sugar fermenting bacteria belonging to genus *Enterobacter, Klebsiella, Bacillus, Serratia*, and others. [28]. Guo et al. [23] reported the 2,3-butanediol production from CWP by *Klebsiella pneumoniae* CICC 10781 reaching a yield of 0.42 g g^{-1}, likewise, another study by Lee and Maddox [29] showed a high 2,3-butanediol yield of 0.46 g g^{-1} using rennet whey permeate as substrate. Meanwhile, in this study, the 2,3-butanediol yield of 0.42 g g^{-1} lactose, which represents 78% of the maximum theoretical 2,3-butanediol yield of 0.538 g g^{-1} carbohydrate.

3.2 Wheat straw hydrolysate fermentation

Wheat straw is an abundant agro-industrial residue with low commercial value. Like any other biomass of lignocellulosic composition, it is composed by a complex mixture of cellulose (40-50%), hemicellulose (25-35%) and lignin (15-20%), therefore, this biomass requires to be hydrolyzed to expose the carbohydrates and make them accessible for the microorganisms [30]. After pretreatment, a broth rich in glucose, xylose, and arabinose is produced; in addition, other compounds such as furfural, phenolic compounds, and acetic acid are formed [31]. In this work, the

composition of WSH was 20.4 g dm^{-3} total sugars (which included 3.2 g dm^{-3} glucose, 14.2 g dm^{-3} xylose, 3.0 g dm^{-3} arabinose), 1.0 g dm^{-3} formic acid, 2.2 g dm^{-3} acetic acid and 0.6 g dm^{-3} furfural. Fig. 3 depicts the hydrogen production kinetics by the GA0F bacterium using WSH as substrate. As it can be seen, hydrogen production started at 24 h, followed by a lag phase from 100 to 192 h. After that, hydrogen production restarted and continued until 336 h. When bacterial cells are exposed to multiple sugars, they do not metabolize all sugars simultaneously, but rather a sequential utilization of carbon sources is carried out. This phenomenon is characterized by two growth phases often separated with lag periods. Fig. 3 shows that total sugar concentration decreased by almost half of the initial concentration at the first 48 h of fermentation. Afterward, the total sugar concentration was maintained at the same concentration in agreement with the diauxic shift in hydrogen production, subsequently, another portion of the carbon source was consumed. The maximum hydrogen production and hydrogen production rate reached were 744.8 ± 36 cm^3 H_2 dm^{-3} and 5.4 ± 0.5 cm^3 H_2 dm^{-3} h^{-1}, respectively (Table 1). This hydrogen production was low compared to other studies reported in the literature (Table 2). For instance, Sagnak et al. [32] reported the production of 2,785 cm^{-3} H_2 dm^{-3} by mesophilic anaerobic sludge (37°C) using hydrolyzed waste ground wheat containing 27.5 g dm^{-3} total sugar . In the same way, Khamtib et al. [33] reported the production of 1,947 cm^{-3} H_2 dm^{-3} by hot spring enriched culture from oil palm trunk hydrolysate at 55°C using an initial substrate concentration of 22.07 g dm^{-3}, while Cakir et al. [34] at the same temperature using heat-treated anaerobic sludge produced 3,008 cm^{-3} H_2 dm^{-3} from acid-hydrolyzed ground wheat starch with an initial total sugar concentration of 18.5 g dm^{-3}. One of

221 the factors that could have affected hydrogen production is the adverse effect of
222 inhibitory compounds present in WSH. Van Ginkel and Logan [35] reported the
223 addition of 25 mM of acetic acid to the fermentation resulted in a decrease in
224 hydrogen yield by 13%. During acid hydrolysis, acetic acid is released from
225 acetylxylan from hemicellulose [36]. The unfavorable effect of acetic acid is
226 attributed to its diffusion into the cytosol where the dissociation of the acid occurs,
227 decreasing the cytosolic pH [37]. Likewise, furfural produced from pentoses inhibits
228 dark fermentation by decreasing the enzyme activities, inhibiting protein and RNA
229 synthesis and also breaking down DNA [38]. An initial concentration of 2.2 g dm^{-3}
230 (36.6 mM) acetic acid and 0.6 g dm^{-3} furfural could have had a negative effect on
231 dark fermentation by psychrophilic GA0F bacterium. Cao et al. [39], demonstrated
232 that a concentration of 1 g dm^{-3} furfural and hydroxymethylfurfural (HMF) exerted a
233 large negative influence on growth and hydrogen production. While
234 Panagiotopoulos et al. [40] observed inhibition of the fermentation of mild-acid
235 pretreated corn stalks by furfural concentrations in a range of 0.08-0.17 g dm^{-3}.
236 Likewise, Bellido et al. [41] described a complete inhibition of ethanol fermentation
237 by using WSH due to the presence of 1.5 g dm^{-3} acetic acid, 0.15 g dm^{-3} furfural
238 and 0.05 g dm^{-3} HMF. As stated by Sivagurunathan et al. [42] the threshold
239 inhibition concentration of the by-products released during the pretreatment of
240 lignocellulosic biomass is specific to the type of microorganism applied as
241 inoculum. To our knowledge, there are no previous reports regarding the use of
242 psychrophilic bacteria using lignocellulosic hydrolysates for biofuel production,
243 therefore more research is needed to characterize the psychrophilic bacteria
244 tolerance to this kind of fermentation inhibitors. The application of several

mesophilic and thermophilic microorganisms using different hydrolysates of lignocellulosic materials such as wood [43], oil palm frond [44], wheat straw [11], corn stover [45], sugarcane bagasse [46], has been widely studied for 2,3-butanediol or ethanol production. Perego et al. [47] reported a 2,3-butanediol production of 8.8 g dm^{-3} using starch hydrolysate, likewise, Hazeena et al. [48] reached 7.2 g dm^{-3} using oil palm frond hydrolysate. Another study by Yu et al. [43] shows the production of 1.12 g dm^{-3} ethanol and 3.37 g dm^{-3} 2,3-butanediol at 30°C by *Klebsiella pneumoniae* from steam-exploded aspen presoaked in acid. While in this study, GA0F bacterium attained a 2,3-butanediol and ethanol production of 3.7 ± 0.3 g dm^{-3} and 3.1 ± 0.07 g dm^{-3}, respectively (Fig. 2). The yields of 2,3-butanediol and ethanol reported in the literature are in a range of 0.2 to 0.5 g g^{-1} carbohydrate consumed. In this study, ethanol (0.19 ± 0.01 g g^{-1}) and 2,3-butanediol (0.23 ± 0.05 g g^{-1}) yields using WSH were within the range mentioned above, although low with respect to the theoretical yield of 0.5 g g^{-1}. This issue could be further improved as suggested by Palmqvist and Hahn-Hagerdal [37] through an optimization of the pretreatment and hydrolysis conditions of wheat straw and by detoxification methods for the removal of inhibitors prior to fermentation, as well as by acclimatization of the strains to hydrolysates through serial sub-culturing [43].

3.3 Sugarcane molasses

Sugarcane molasses are an agro-industrial by-product of the sugar manufacturing process, which contain sucrose as the most abundant sugar and small quantities of

glucose, fructose and raffinose [49]. SCM are also rich in nutrients required by most microorganisms (metals, vitamins and nitrogen compounds) [50]. This by-product represents a cheap raw material, readily available, and accessible for conversion with limited pretreatments as compared to starchy or lignocellulosic materials, since all sugars are present in an easily fermentable form [51]. In this work, the use of diluted SCM (21 g dm^{-3} total sugars) led to a hydrogen production of 979.3 ± 74 cm^3 dm^{-3} and a production rate of 8.5 ± 0.8 cm^3 dm^{-3} h^{-1} (Table 1). Similar hydrogen production parameters are found in the literature. For instance, Kumar et al. [52] reported 1,800 cm^3 H_2 dm^{-3} by *Enterobacter aerogenes* at 30°C using 40 g dm^{-3} cane molasses. da Silva et al. [53] evaluated the use SCM combined with leachate, which originates from the disposal of plastics, batteries and mercury lamps, for hydrogen production under mesophilic conditions (35°C). Their results showed that hydrogen production was improved from 663 cm^3 H_2 dm^{-3} using SCM plus a nutrient solution, to 1,770 cm^3 H_2 dm^{-3} upon addition of the leachate to SCM. In our study, psychrophilic GA0F bacterium reached 979.3 ± 74 cm^3 H_2 dm^{-3} using SCM only with the addition of a nutrient solution (see section 2.2), similar to the one used in the aforementioned study, this represents an advantage since GA0F bacterium required 10°C less to carry out the fermentation. Fig. 4 illustrates hydrogen production kinetics using SCM as substrate. Similarly, as observed in fermentations using WSH, a diauxic behavior was present on hydrogen evolution from soluble sugars in SCM. Hydrogen production started at 24 h and continued until 96 h, after that a lag phase of 100 h was observed. Subsequently, the hydrogen production restarted until 408 h. The analysis of soluble metabolites showed that hydrogen production was accompanied principally

by the production of solvents, and to a low extent by volatile fatty acids such as acetic acid, succinic acid and lactic acid (Fig. 2). 2,3-butanediol production attained was 4.4 ± 0.4 g dm^{-3}, whereas the ethanol production was 3.7 ± 0.4 g dm^{-3}. Considering the substrate consumption, the yield achieved for both alcohols was 0.24 ± 0.02 g g^{-1} and 0.20 ± 0.02 g g^{-1}, respectively (Table 1). Perego et al. [47] reached a similar 2,3-butanediol yield from raw molasses (0.20 g g^{-1}) and decolored molasses (0.26 g g^{-1}) using *Enterobacter aerogenes* at 39°C. Dai et al. [54] reported 0.39 g g^{-1} by *Enterobacter cloacae* (GMCC 6053) at 37°C. Likewise, Afschar et al. [55], achieved 0.42 g g^{-1} using *Klebsiella oxytoca*. In addition, Cazetta et al. [56] reported an ethanol yield of 0.33 g g^{-1}, using *Zymomonas mobilis*, whereas Razmovski et al. [57] attained 0.49 g g^{-1} using *Saccharomyces cerevisiae*. These studies successfully achieved a high 2,3-butanediol or ethanol yield using SCM. In our study, the low ethanol and 2,3-butanediol production are compensated by the fact that hydrogen, ethanol, and 2,3-butanediol are produced simultaneously under room temperature conditions.

3.4 Comparison of hydrogen, ethanol and 2,3-butanediol production from CWP, HWS, and CSM by the GA0F bacterium

In this study, three different substrates CWP, WSH, and SCM were compared to determine the most suitable carbon source for the production of biofuels by GA0F bacterium. Hydrogen, ethanol, and 2,3-butanediol were produced in all cases; nevertheless, hydrogen yield (73.5 ± 10 cm^3 g^{-1}) from CWP was significantly ($p < 0.05$) higher compared to the yield achieved using the other two substrates (Table

315 1). This could be attributed to the fact that CWP is composed of a single carbon
316 source plus nutrients like vitamins and proteins, which makes it easily and rapidly
317 metabolized; also, CWP solution was probably nutritionally richer than the other
318 substrates resulting in higher hydrogen yields. Moreover, this substrate is free from
319 inhibitory compounds unlike WSH, which clearly affected the fermentation of
320 hexoses and pentoses available in the medium. In the same way, a significantly (p
321 < 0.05) higher 2,3-butanediol yield was obtained by the use of CWP, where the
322 GA0F bacterium reached 0.42 ± 0.04 g g^{-1}, which corresponds to 78% of the
323 theoretical yield. 2,3-butanediol is an important intermediate in diverse industrial
324 areas such as printing, cosmetics, food processing, fumigants, antifreeze, etc. [58],
325 also, 2,3-butanediol is a potentially valuable fuel additive with a heating value of
326 27.20 kJ g^{-1} which compares favorably with other liquid fuels (methanol 22.08 kJ g^{-1}
327 and ethanol 29.06 kJ g^{-1}) [6]. Bacteria belonging to *Enterobacter, Klebsiella,*
328 *Bacillus* and *Serratia* genus can produce this solvent through fermentation.
329 Through the synthesis of this diol, bacterial cells regulate intracellular NADH/NAD+
330 and also prevent the medium acidification by changing the metabolism from acid
331 production to the formation of neutral compounds [28]. The production of 2,3-
332 butanediol by mesophilic and thermophilic bacteria is well documented; on the
333 contrary, except by an earlier study of our group [13], no previous studies
334 regarding to the production of 2,3-butanediol by cold-loving bacteria have been
335 published so far, therefore, more studies are needed to understand the
336 fermentative aspects of psychrophilic bacteria. As mentioned above, 2,3-butanediol
337 is used as an anti-freeze in the industry due to its chemical properties; this fact
338 may provide clues as to why psychrophilic bacteria synthesize 2,3-butanediol apart

339 from the redox potential regulation. As described by Hubálek [59], 2,3-butanediol
340 can act as a cryoprotectant in harsh environments, preventing the formation of
341 large ice crystals within the cell and also reducing salt toxicity and excessive
342 dehydration. On the other hand, ethanol yields achieved by GA0F bacterium
343 ranged from 0.19-0.24 g g^{-1} where the highest value corresponds to CWP
344 fermentation and the lowest to the WSH fermentation (Table 1). However,
345 statistical analysis showed that there are not significant differences between the
346 ethanol yields achieved. The fact that the psychrophilic GA0F bacterium used in
347 this study preferentially produced solvents and hydrogen instead of acids
348 represents a competitive advantage over other processes since it could be
349 possible to establish an alcohol-rich fermentation in which the end products are not
350 toxic, as happens in ethanol or acetone-butanol fermentations.

351

352 **4. Conclusions**

353 In this work, the simultaneous production of hydrogen, ethanol, and 2,3-butanediol
354 from different cheap substrates such as cheese whey powder, wheat straw
355 hydrolysate and sugar cane molasses by the psychrophilic GA0F bacterium is
356 demonstrated. The highest yields of hydrogen (73.5 ± 10 cm^3 H_2 g^{-1}), ethanol (0.24
357 ± 0.03 g g^{-1}) and 2,3-butanediol (0.42 ± 0.04 g g^{-1}) are obtained using cheese whey
358 powder, which is an economical, concentrated source of lactose. This study also
359 reveals the susceptibility of the GA0F bacterium to the inhibitory compounds
360 present in wheat straw hydrolysate, which result in the lowest production of the
361 three biofuels evaluated. Since fermentations carried out in this study resulted in a

rich solvent production with concomitant hydrogen production, the use of the GA0F bacterium could be considered for a further application at industrial scale under conditions of room temperature.

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Table 1. Hydrogen, ethanol and 2,3-butanediol production parameters obtained by the psychrophilic GA0F bacterium using CWP, WSH and SCM.

Substrate	H_2 (cm^3 dm^{-3})	Y_{H2} (cm^3 g^{-1})	EtOH (g dm^{-3})	Y_{EtOH} (g g^{-1})	BDO (g dm^{-3})	Y_{BDO} (g g^{-1})
CWP	923.2 ± 130	73.5 ±10	3.0 ± 0.04	0.24 ± 0.03	5.3 ± 0.5	0.42 ± 0.04
HWS	744.8 ± 36	43.6 ± 2	3.1 ± 0.1	0.19 ± 0.01	3.7 ± 0.3	0.23 ± 0.05
SCM	979.3 ± 74	52.4 ± 4	3.7 ± 0.4	0.20 ± 0.02	4.4 ± 0.4	0.24 ± 0.02

CWP: Cheese whey powder, WSH: Wheat straw hydrolysate, SCM: Sugarcane molasses, Y_{H2}: Hydrogen yield, EtOH: Ethanol, Y_{EtOH}: Ethanol yield, BDO: 2,3-butanediol, Y_{BDO}: 2,3-butanediol yield.

Table 2. Comparison of production and yield of hydrogen, ethanol, and 2,3-butanediol reported by different microorganisms using different substrates.

Substrate	Microorganism	T (°C)	H_2 ($cm^3\ dm^{-3}$)	Y_{H2} ($cm^3\ g^{-1}$)	EtOH ($g\ dm^{-3}$)	Y_{EtOH} ($g\ g^{-1}$)	BDO ($g\ dm^{-3}$)	Y_{BDO} ($g\ g^{-1}$)	Reference
CWP	GA0F	25	923.2	73.5	3.0	0.24	5.3	0.42	This study
	Rhanella aquatilis (RA7)	20	134*	NR	NR	NR	NR	NR	[27]
	Anaerobic sludge	55	1144	1.03[a]	NR	NR	NR	NR	[25]
	Klebsiella pneumoniae NCIB 8017	30	NR	NR	NR	NR	7.5	0.46	[29]
WSH	GA0F	25	744.8	43.6	3.1	0.19	3.7	0.23	This study
	Hot spring enriched cultured	55	1947	0.71	0.24	0.01	NR	NR	[33]
	Klebsiella pneumoniae	30	NR	NR	1.12	0.09	3.37	0.4	[43]
	Enterobacter aerogenes	39	NR	NR	NR	NR	8.8	0.88[a]	[47]
SCM	GA0F	25	979.3	52.4	3.7	0.20	4.4	0.24	This study
	Anaerobic sludge	35	1770	1.32[b]	NR	NR	NR	NR	[53]
	Enterobacter aerogenes	39	NR	NR	NR	NR	5.3	0.86[a]	[47]
	Klebsiella sp.	37	NR	0.67[a]	NR	0.59[a]	NR	0.59[a]	[12]

CWP: Cheese whey powder, WSH: Wheat straw hydrolysate, SCM: Sugarcane molasses, NR: Not reported, Y_{H2}: Hydrogen yield, EtOH: Ethanol, Y_{EtOH}: Ethanol yield, BDO: 2,3-butanediol, Y_{BDO}: 2,3-butanediol yield, [a]mol mol substrate^{-1}, [b]H_2_glu eq^{-1} (Glucose_equivalent: mmol of sugar as glucose).

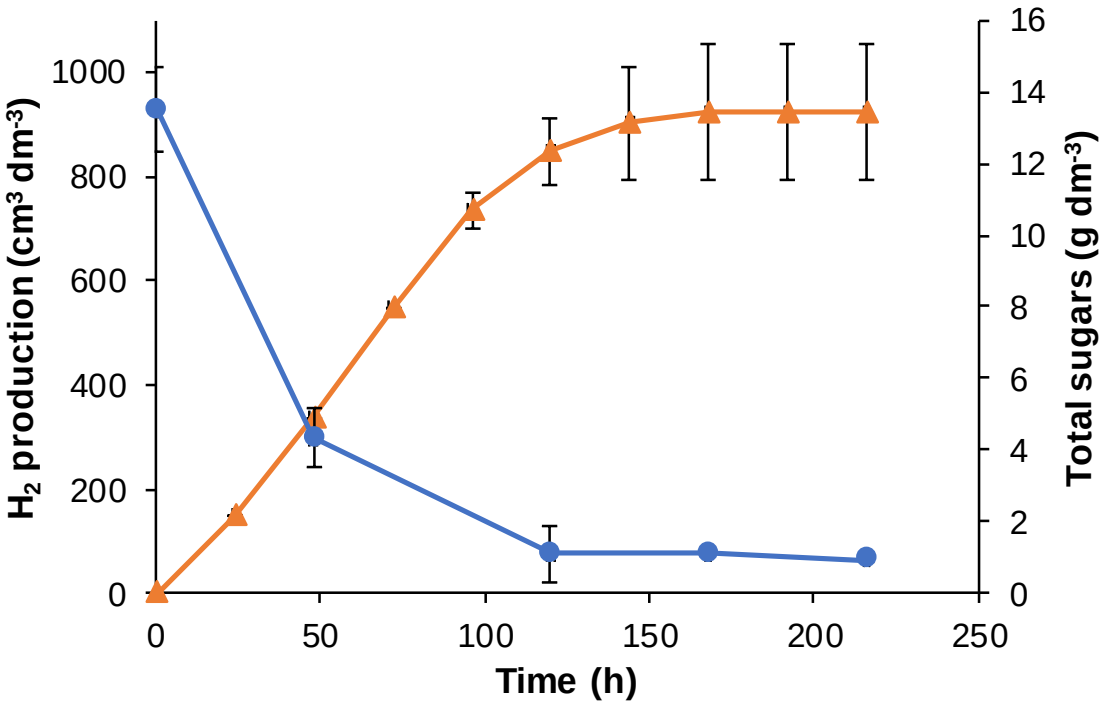
Figure captions

Fig. 1 Hydrogen production profiles of batch fermentations by the GA0F bacterium using CWP as substrate.

Fig. 2 Production of soluble metabolites at the end of the fermentation of CWP, WSH and SCM by the psychrophilic GA0F bacterium.

Fig. 3 Hydrogen production profiles of batch fermentations by the GA0F bacterium using WSH as substrate.

Fig. 4 Hydrogen production profiles of batch fermentations by the GA0F bacterium using SCM.



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586 **Fig. 1**

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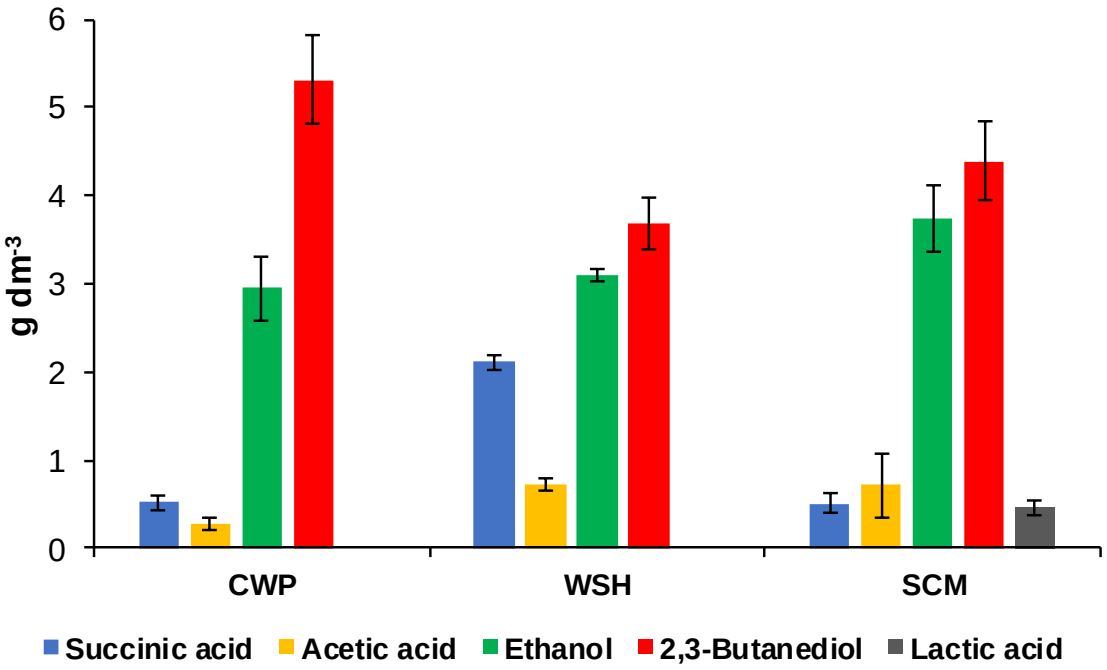
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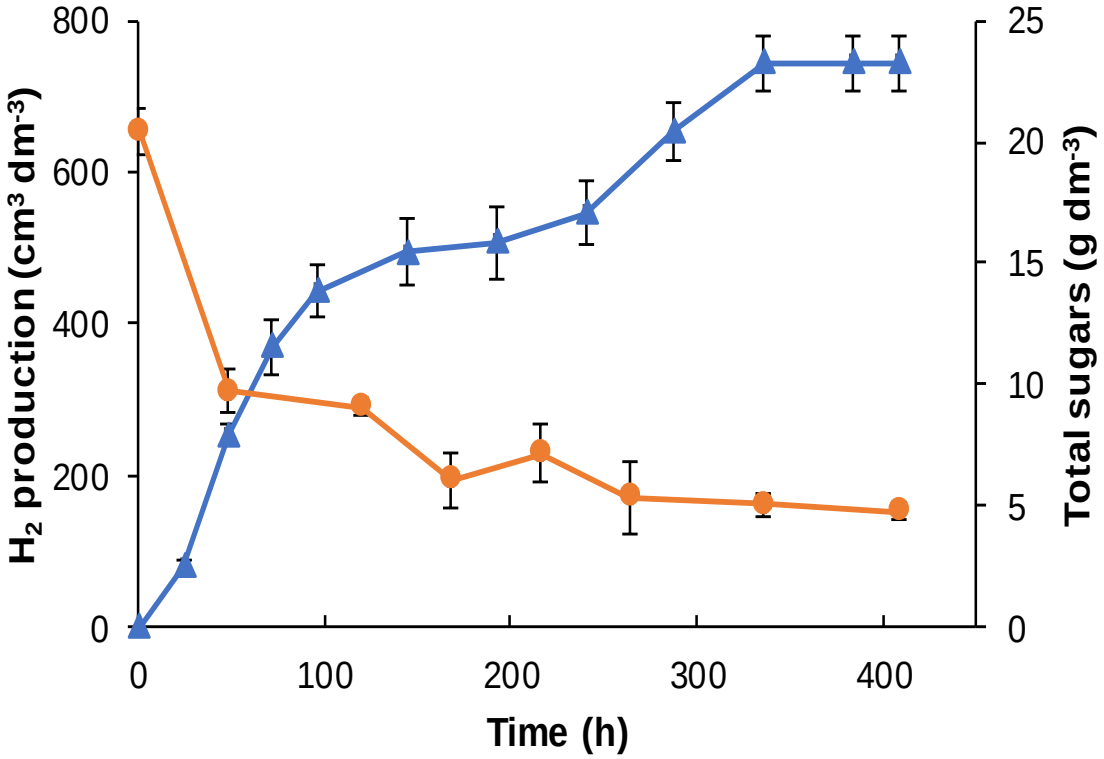
5
4
3
2
1
0
g dm-3
CWP
WSH
SCM
Succinic acid
Acetic acid
Ethanol
2,3-Butanediol
Lactic acid

Fig. 2



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601 **Fig. 3**

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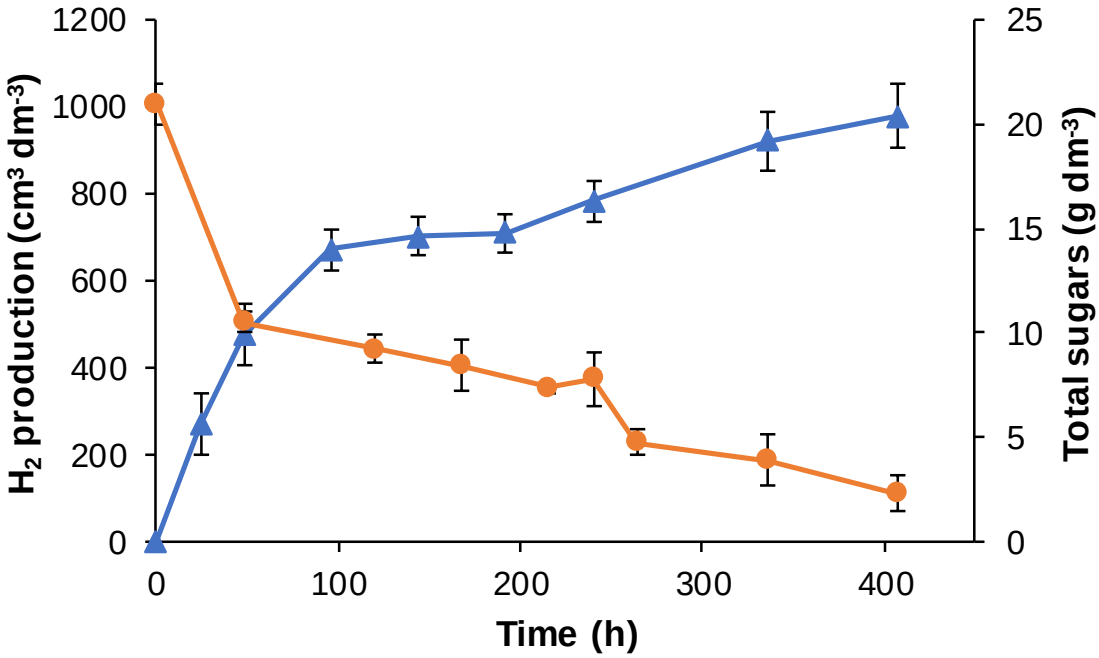

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607 **Fig. 4**

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