



**HAL**  
open science

## Isolation and characterisation of an environmental *Clostridium beijerinckii* strain for biohydrogen production from dairy wastes

M. Mete, P. Pattyn, A. Robidart, G. Beringuier, H. Thomas, Cyrille  
Grandjean, R. Irague, Yves Andres

► **To cite this version:**

M. Mete, P. Pattyn, A. Robidart, G. Beringuier, H. Thomas, et al.. Isolation and characterisation of an environmental *Clostridium beijerinckii* strain for biohydrogen production from dairy wastes. *International Journal of Hydrogen Energy*, 2024, 49, pp.371-383. 10.1016/j.ijhydene.2023.08.274 . hal-04762612

**HAL Id: hal-04762612**

**<https://hal.science/hal-04762612v1>**

Submitted on 31 Oct 2024

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Distributed under a Creative Commons Attribution 4.0 International License

# Isolation and characterization of a novel environmental *Clostridium beijerinckii* strain for dihydrogen production from dairy wastes

METE M.<sup>1,2,3</sup>, PATTYN P.<sup>2</sup>, ROBIDART A.<sup>2</sup>, BERINGUIER G.<sup>2</sup>, THOMAS H.<sup>2</sup>, GRANDJEAN C.<sup>3</sup>, IRAGUE R.<sup>2</sup>, ANDRES Y.<sup>1</sup>

<sup>1</sup> IMT Atlantique, CNRS, Génie des procédés environnement agroalimentaire (GEPEA), UMR 6144, 2 rue Alfred Kastler 44300 Nantes, France

<sup>2</sup> Athena Recherche et Innovation, 49170 Saint-Georges-sur-Loire, France

<sup>3</sup> Nantes Université, CNRS, Unité des Sciences Biologiques et des Biotechnologies (US2B), UMR 6286, 2 chemin de la Houssinière, BP92208, 44000 Nantes, (France)

## Abstract

Biological dihydrogen (H<sub>2</sub>) production is a promising alternative to the conventional non-renewable energies. H<sub>2</sub> production by dark fermentation is particularly studied regarding its independence from fossil fuel and electricity, its cultivation into bioreactors and the broad range of substrate that can be used. A major goal application of dark fermentation is the valuation of wastes. In this context, cheese whey wastes are particularly adapted, since rich in organic matter, abundant and low cost.

A novel H<sub>2</sub>-producing strain was isolated and identify as a *Clostridium beijerinckii* strain based on morphological and physiological characteristics, and 16S rDNA sequencing. The optimum temperature and pH for H<sub>2</sub> production was 40°C and pH 7, respectively. Substrate and stress tolerance tests showed that *C. beijerinckii* C.sp.1.3 could produce H<sub>2</sub> from glucose, lactose, maltose, cellobiose, galacturonic acid, xylose, and sucrose, and to a lesser extent from starch, cellulose and glycerol. The H<sub>2</sub> production potential from a mix of dairy industrial wastes of this strain has been evaluated. The highest productivity in batch for 5 g.L<sup>-1</sup> of Total Organic Carbon (TOC) was 833.1 mL H<sub>2</sub>.L<sup>-1</sup>.d<sup>-1</sup>, with a yield of 2.03 mM<sub>H<sub>2</sub></sub>.mM<sub>lactose</sub><sup>-1</sup>, demonstrating the potential of this strain for H<sub>2</sub> production from industrial wastes.

## Key words

*Clostridium beijerinckii*, biohydrogen, dark fermentation, dairy wastes

## Introduction

The world energetic dependence on fossil fuels, and their impacts on environment drastically increased the development of new alternatives and renewable energy sources. Among the most promising sources, biohydrogen is standing out.

Hydrogen (H<sub>2</sub>), is a powerful energy vector with a calorific capacity of 122 kJ.g<sup>-1</sup> which is 2.75 times higher than oil. Its combustion is clean, emitting water steam. Last but not least, H<sub>2</sub> has a wide range of potential use from electricity to transportation fuel. However, according to the International Energy Agency Global Hydrogen Review 2022, 99.96% of H<sub>2</sub> produced in 2021 derived from side reactions of non-renewable energy industry (coal, fossil fuels, gas, oil) . This H<sub>2</sub> is consequently associated with high carbon emissions. However, low carbon H<sub>2</sub> production processes exist, such as water electrolysis (0.04% of H<sub>2</sub> produced in 2021), biomass gasification and biological H<sub>2</sub> production.

The rising of environmental questions and the critical decrease of fossil fuel stocks led to the development of biological H<sub>2</sub> production processes: bio-photolysis, photo-fermentation and dark fermentation. These bioprocesses gained a lot of interest these past years for their ability to use organic waste, vegetal biomass and/or sunlight to generate H<sub>2</sub> (Hallenbeck and Benemann, 2002; Dincer, 2012; Kumar *et al.*, 2018). If H<sub>2</sub> production from biomass is still at a research scale, this resource aims at being strongly developed through the environmental objectives of world instances.

Indeed, light-dependent processes rely on the operation of photosynthesis, a fundamental and critical process with great potential for mitigating carbon emissions. These bioprocesses are carried out by microalgae, cyanobacteria and anoxygenic photosynthetic bacteria. During bio-photolysis, photosynthetic organisms such as cyanobacteria (*Anabaena sp.*, *Nostoc sp.*) and microalgae (*Chlorella sp.*, *Chlamydomonas sp.*, *Nannochloropsis sp.*, *Scenedesmus sp.*) convert light energy and water into H<sub>2</sub> using hydrogenases and nitrogenases (Suresh, Kumari and Venkata Mohan, 2023). Photo-fermentation, on the other hand, involves anoxic photosynthetic bacteria such as *Rhodobacter spp.*, *Rhodovulum spp.*, *Rhodopseudomonas spp.*, and *Rhodospirillum spp.* These organisms convert organic molecules into H<sub>2</sub> and CO<sub>2</sub> by fixing nitrogen with nitrogenases (Suresh, Kumari and Venkata Mohan, 2023).

Dark fermentation corresponds to the conversion of metabolizable organic matter into H<sub>2</sub>, CO<sub>2</sub> and volatile fatty acids (VFAs) using hydrogenases, under anaerobic conditions. It is carried out by strict aerobic organisms (*Clostridium spp.*, and *Rumminococcus spp.*), facultative aero-anaerobes (*Enterobacter sp.*, *Escherichia coli* and *Citrobacter sp*) and anecdotally by strict aerobes (*Azotobacter vinelandii*, *Alcaligenes eutrophus* and *Termococcus sp.*)(Cao *et al.*, 2022). Dark fermentation offers several advantages over other bioprocesses. It does not require light energy (although it does require thermal energy to maintain the reactors at the right temperature), production yields can reach high values and cultivation in a conventional bioreactor is possible. Dark fermentation has been studied using various approaches. To date, the impact of culture parameters or bioreactors optimisation on pure culture or complex communities has been reported abundantly (Chin, Chen and Chou, 2003; Jung *et al.*, 2011; Kim *et al.*, 2012; Xiao *et al.*, 2013). It also has the advantage of being able to exploit abundant and inexpensive resources as substrate sources, such as food wastes (De Gioannis *et al.*, 2013; Das and Basak, 2021; François *et al.*, 2021), agricultural crop residues (Rabelo *et al.*, 2018), chemical wastes (Cripa *et al.*, 2020), human wastewaters(Wang *et al.*, 2018), and agrifood wastewaters (Davila-Vazquez *et al.*, 2009; Rosa *et al.*, 2014; Silva *et al.*, 2018; Dinesh *et al.*, 2020), which represent a non-exhaustive list of wastes studied for H<sub>2</sub> production.

Dairy wastes, due to its physicochemical characteristics, is a well-suited source for H<sub>2</sub> production at an industrial scale (H. J. Kim *et al.*, 2008; Karadag *et al.*, 2014; Patel *et al.*, 2016). However, the exploitation of dark fermentation on a large scale has not been fully developed. Different cultivation strategies are feasible to achieve dark fermentation, the choice of inoculum being one of

them. Dark fermentation on dairy wastes has been mainly studied with the exploitation of complex microbial population through the use of sludge or indigenous microorganisms (Davila-Vazquez *et al.*, 2009; Abreu *et al.*, 2010; Karadag *et al.*, 2014). Even though few microorganisms of the community are known as H<sub>2</sub> producers, the microbial structure and how it impacts H<sub>2</sub> production are still to be elucidated. These questions have gained more interests these past years (Francois-Lopez, 2016; Kumar *et al.*, 2018; Wang *et al.*, 2018; Yang and Wang, 2019). Pure cultures are not predominant for dark fermentation on dairy wastes, even though they remain a subject of interest (Elsharnouby *et al.*, 2013; Patel *et al.*, 2016; Dinesh *et al.*, 2020).

The aim of this study was to isolate, characterize and identify a strain capable of producing H<sub>2</sub> in pure culture, from a mix of dairy wastes, without supplements. Sludge from a dairy farm have been harvested, and enriched on dairy wastes for H<sub>2</sub> production. After isolation of an H<sub>2</sub> producer bacteria, the characterization of the strain and its H<sub>2</sub> potential on dairy wastewaters has been established.

## Material and methods

### Origin of biological samples

Bacterial strains have been isolated from a sample of a stagnant liquid mixture of bovine manure and cheese whey, collected in the vicinity of a cheese-producing dairy farm of La Chapelle sur Erdre, Pays de La Loire, France. The environmental sample was heat treated at 105°C during 30 min for H<sub>2</sub> producers' pre-enrichment and then, used to inoculate a sterile dairy wastewater F1. The dairy wastewater was chosen in order to favour the emergence of strains able to grow in a rigorous environment. Culture was conducted until H<sub>2</sub> was detected in the produced gas. Then, the H<sub>2</sub>-producing strains were isolated by plating an appropriate dilution of the culture broth on CNM agar.

The strains *Clostridium acetobutylicum* DSM 792 and *Clostridium beijerinckii* DSM 971 were provided from DSMZ, (German collection of microorganisms and cell cultures).

Two types of industrial wastes were used for this study, a cheese whey (CW) and a wastewater (F1). They were collected from an industrial dairy located at Ancenis, Pays de la Loire, France. Fresh samples were conserved at -20°C after sampling until utilisation.

### Strain identification

**DNA extraction.** Cells were grown on 10 mL of anoxic Clostridial Nutrient Medium (CNM) during 24 h at 37°C, without agitation, in a culture tube sealed with rubber cap and aluminium ring. Pellets were resuspended in sterile distilled water and disrupted by heat treatment, 95°C during 5 min. The heat treatment was repeated one time after a cooling period of 5 min. The mixture was then centrifuged (10 000 rpm, 5 min) and the supernatant was used for PCR amplification.

**PCR amplification and 16s rDNA sequencing.** Supernatant of extracted DNA was used as PCR template for 16s rDNA gene amplification with the primers 27f (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') and a high-fidelity polymerase. The PCR was performed as follow: denaturation step at 98°C during 30 s, 30 cycles of amplification step at 98°C for 30 s, 55°C for 30 s and 72°C for 30 s, a final elongation step at 72°C for 1 min. The PCR amplification product was purified and sequenced (Microsynth, Switzerland). The sequence was compared with the sequences available in the NCBI and EZbiocloud databases. Mega 11 was used for phylogenetic tree construction.

**Genome sequencing.** Cells pellets were prepared as mentioned above and used for sequencing. The genome of the *Clostridium beijerinckii* C.sp.1.3 strain was sequenced and assembled using the Illumina HiSeq technology and *de novo* assembly (Eurofins, France). Annotation and genome analysis were performed using the RAST server (<https://rast.nmpdr.org/>) and the MicroScope platform (<https://mage.genoscope.cns.fr>), respectively (Aziz *et al.*, 2008; Vallenet *et al.*, 2019).

## Strain characterization

Optimal temperature, optimal pH, carbon phenotype and organic acids toxicity were evaluated on a basal media according to Mechichi et al., (1997) and composed of  $\text{NH}_4\text{Cl}$  1 g.L<sup>-1</sup>,  $\text{KH}_2\text{PO}_4$  0.3 g.L<sup>-1</sup>,  $\text{K}_2\text{HPO}_4$  0.3 g.L<sup>-1</sup>,  $\text{NaCl}$  0.6 g.L<sup>-1</sup>, ( $\text{CaCl}_2$ ,  $\text{H}_2\text{O}$ ) 0.1 g.L<sup>-1</sup>, ( $\text{MgCl}_2$ ,  $6\text{H}_2\text{O}$ ) 0.2 g.L<sup>-1</sup>, cysteine HCl 0.5 g.L<sup>-1</sup>, yeast extract 1 g.L<sup>-1</sup>, trace element solution 1.5 mL.L<sup>-1</sup>. The trace element solution is HCl 25 % 10 mL.L<sup>-1</sup>, ( $\text{FeCl}_2$ ,  $4\text{H}_2\text{O}$ ) 1.5 g.L<sup>-1</sup>, ( $\text{CoCl}_2$ ,  $6\text{H}_2\text{O}$ ) 190 mg.L<sup>-1</sup>, ( $\text{MnCl}_2$ ,  $4\text{H}_2\text{O}$ ) 100 mg.L<sup>-1</sup>,  $\text{ZnCl}_2$  70 mg.L<sup>-1</sup>,  $\text{H}_3\text{BO}_3$  62 mg.L<sup>-1</sup>, ( $\text{NaMoO}_4$ ,  $2\text{H}_2\text{O}$ ) 36 mg.L<sup>-1</sup>, ( $\text{NiCl}_2$ ,  $6\text{H}_2\text{O}$ ) 24 mg.L<sup>-1</sup>, ( $\text{CuCl}_2$ ,  $2\text{H}_2\text{O}$ ) 17 mg.L<sup>-1</sup>. The media was supplemented with 5 g.L<sup>-1</sup> of carbon source according to the experiment. The experiments were performed into serum bottles with 50 mL of working volume, sealed with rubber caps and flushed with filtered nitrogen to create anaerobic conditions. For each condition, duplicates were made.

To evaluate the effect of temperature on  $\text{H}_2$  production by the isolated strain, the serum bottles have been incubated at 20, 30, 37, 40, 45, 50, 55°C, for 48 h with an initial pH 7.

To evaluate the effect of pH on  $\text{H}_2$  production by the strain, the serum bottles have been incubated at 37°C, with initial pH set at 5, 6, 7, 8, 9 and 10 for 48 h. For pH 5 to 7, the media was supplemented with 50 mM of 2-(N-morpholino)ethane sulfonic acid (MES). For pH 8 to 10, the media was supplemented with 50 mM 3-(cyclohexyl amino)-2-hydroxy-1-propanesulfonic acid (CAPSO). The pH was adjusted with a pure HCl solution and a 10 M NaOH solution.

The level of toxicity of acetic and butyric acid on *C. beijerinckii* C.sp.1.3 were tested with different concentrations of acetic and butyric acid from 0 to 100 mM.

The carbon phenotype of the strain was established. The fermentation was performed at 37°C, initial pH 7, during 48 h. The different carbon sources tested were: xylose, glucose, lactose, maltose, saccharose, cellobiose, cellulose, starch, galacturonic acid, glycerol and inositol. For each carbon source, duplicates were made. A negative control without any additional carbon source was also performed. The same experiment was performed with *Clostridium acetobutylicum* DSM 792 (DSMZ) *Clostridium beijerinckii* DSM 971 (DSMZ) as references.

## Industrial wastes fermentation for $\text{H}_2$ production

$\text{H}_2$  production ability of *C. beijerinckii* C.sp.1.3 has been tested on a mix of CW and F1, with a concentration of 5 g.L<sup>-1</sup> of Total Organic Carbon (TOC). Kaldnes K1 were used as culture media. The experiment was assessed in a bioreactor with a working volume of 2 L, and a porosity of 93 %. The bioreactor and the media were heated at 110°C for 5 min, and kept for 30 min in the autoclave. Then the media was flushed with nitrogen. Bioreactors are inoculated with a 24 h inoculum, with a final concentration at 7 % v.v<sup>-1</sup>. The fermentation was performed at 40°C, with a pH 7 regulation, under agitation 100 rpm. Sample of the fermentation process were collected to analyse the biogas composition, the sugar and organic acids.

## Analytical methods

Chemical Oxygen Demand (COD) was determined using a commercial kit (CHEMetrics LLC). Sugars and organic acids were analysed by HPLC (Nexera LC40 Shimadzu ©) equipped with a RID detector, and a RESEX ROA column (Phenomenex ®). TOC was determined by TOC-meter analysis Analytik Jena multi-N/C® 2100S, controlled by multiWin. C,H,N,S elementary analysis were performed with a Thermo Finnigan FLASH EA 1112 Series. Micronutrients were measured by ionic chromatography with a 940 Professional IC Vario (Metrohm), paired with 919 IC Autosampler plus.

Cell density was evaluated at 600nm using UV-vis spectrophotometer (Thermo Scientific™ Orion™ AquaMate 7000 Vis). pH measure was established by pH meter (Bioblock scientific™). The flow rate of the biogas produced was continuously measured with milliGascounter® MGC-1 V3.4 PMMA (Ritter©) connected to a computer running Rigamo data acquisition soft-ware (V3.1). The proportion of  $\text{H}_2$  gas was determined using a gas analyser (micro-GC) INFICON micro-GC FUSION®, equipped an inlet heater at 70°C, and two modules. Module A is equipped with an injector at 70°C, in series with Rt-Molsieve 5A 0,25 mm at 50°C, 30 psi and a thermal conductivity detector (TCD) at 70°C.

The carrier gas in the column is argon, the back flush time is 10 s, and the ramp temperature in the column during analysis goes from 50°C (hold 10 s) to 110°C (hold 90 s). Module B is equipped with an injector at 70°C, in series with Rt-U-Bond 12 m at 70°C, 20 psi and a thermal conductivity detector (TCD) at 70°C. The carrier gas in the column is helium, the inject time is 50 ms, and the ramp temperature in the column during analysis goes from 70°C (hold 30 s) to 120°C (hold 80 s).

### Data analysis

Cumulated H<sub>2</sub> production was analysed with modified Gompertz equation (Equation 1) (Van Ginkel, Sung and Lay, 2001). With nls package of Rstudio, model factors were determined: latency period of biogas production of the strain (L), maximum production of H<sub>2</sub> (R<sub>max</sub>), and maximum productivity (H<sub>0</sub>). Then, the duration to produced 95 % of the H<sub>2</sub> was calculated (t<sub>95</sub>) according to Equation 2.

$$H = H_0 * \exp \left[ - \exp \left( \left( \frac{R_{max} * e}{H_0} \right) * (L - t) + 1 \right) \right] \quad (1)$$

$$t_{95} = \left[ \frac{H_0}{R_{max} * e} * (1 - \ln(-\ln(0.95))) \right] + L \quad (2)$$

## Results and discussion

### Physicochemical characteristics of the dairy wastes

The main physicochemical characteristics of the two dairy wastes are summarized in **Table 1**. Overall, CW appears to be rich in organic matter close to 57 mg<sub>COD</sub>.L<sup>-1</sup> and a high sugar concentration (43.40 g.L<sup>-1</sup>). On the contrary, F1 is quite poor in organic matter, with 4.8 mg<sub>COD</sub>.L<sup>-1</sup> and negligible sugar concentration (0.89 g.L<sup>-1</sup>). In addition, CW has an acid pH (5.70), a low acidity (451 mg<sub>CaCO3</sub>.L<sup>-1</sup>) and negligible alkalinity; while F1 has a basic pH (11.88), a negligible acidity, and a moderate alkalinity (1260 mg<sub>CaCO3</sub>.L<sup>-1</sup>). The ratio C/soluble TOC show that CW is more recalcitrant (2.96) than F1 (0.99), which means that it is more difficult to degrade CW than F1.

According to Meena et al. (2020), if high organic content (COD), and acidic pH may enhance any bacterial growth turning the waste into a potential contaminated source, the high lactose content turns them into a suitable media for dark fermentation. Besides, a low alkalinity implies a rapid acidification, which affects the process. Dark fermentation is associated with mainly acetic and butyric acid production. This causes a strong pH decrease if the media is not buffered, which can lead to a premature interruption of the fermentation.

As macro and microelements are essential for microorganisms and dark fermentation, ratios C/N, and iron concentration were also analysed for both wastes. F1 and CW showed a TOC/N of 10.6 and 82.2 respectively, which illustrates a deficiency in nitrogen, mainly for CW. This deficiency is consistent with data of the literature (Farizoglu *et al.*, 2004). Both of the substrates showed limitation of iron, which is a key element for the hydrogenase activity. The only organic acid in presence for both wastes is the lactic acid, with respectively 0.46 g.L<sup>-1</sup> for F1 and 2.71 g.L<sup>-1</sup> for CW.

CW is representative of other cheese whey wastes found in the literature (Farizoglu *et al.*, 2004; Patel *et al.*, 2016), while dairy wastes like F1 has not been reported for H<sub>2</sub> production.

Even though few macro and microelements are negligible, these wastes, and particularly CW, are rich and can be exploited for H<sub>2</sub> production from dark fermentation. Consequently, the mix CW and F1 (CW+F1) with a TOC of 5 g.L<sup>-1</sup> COT is selected as the model of wastewaters for the validation of the isolated strain.

**Table 1: Characteristics of the dairy wastewaters CW and F1. “bdt” is used for “below detection threshold”.**

		<b>F1</b>	<b>CW</b>
<b>pH</b>		11.88	5.70
<b>Alkalinity (mg<sub>CaCO3</sub>.L<sup>-1</sup>)</b>		1,260	bdt
<b>Acidity (mg<sub>CaCO3</sub>.L<sup>-1</sup>)</b>		bdt	451
<b>Density (g.mL<sup>-1</sup>)</b>		1.0004	1.041
<b>Dry mass (g.L<sup>-1</sup>)</b>		4.65	56.37
<b>Volatile mass (g.L<sup>-1</sup>)</b>		3.26	50.96
<b>Chemical Oxygen Demand (COD) (g.L<sup>-1</sup>)</b>		4.81	56.99
<b>Soluble Total Organic Carbon (TOC) (g.L<sup>-1</sup>)</b>		1.49	7.53
<b>Elementary analysis (g.L<sup>-1</sup>)</b>	N	0.139	0.271
	C	1.474	22.266
	H	0.242	3.338
	S	bdt	bdt
<b>Soluble sugar (g.L<sup>-1</sup>)</b>		0.89	43.40
<b>Soluble protein (g.L<sup>-1</sup>)</b>		0.87	1.53
<b>Lactic acid (g.L<sup>-1</sup>)</b>		0.46	2.71
<b>Micronutrient (g.L<sup>-1</sup>)</b>	PO <sub>4</sub> <sup>3-</sup>	0.030	1.05
	Ca <sup>2+</sup>	0.075	0.650
	Mg <sup>2+</sup>	0.0071	0.083
	K <sup>+</sup>	0.05	1.6
	Na <sup>+</sup>	0.380	0.320
	Fe <sup>2+</sup>	bdt	0.00005
	Mn <sup>2+</sup>	bdt	0.000002

### Isolation and identification of *Clostridium beijerinckii* C.sp.1.3

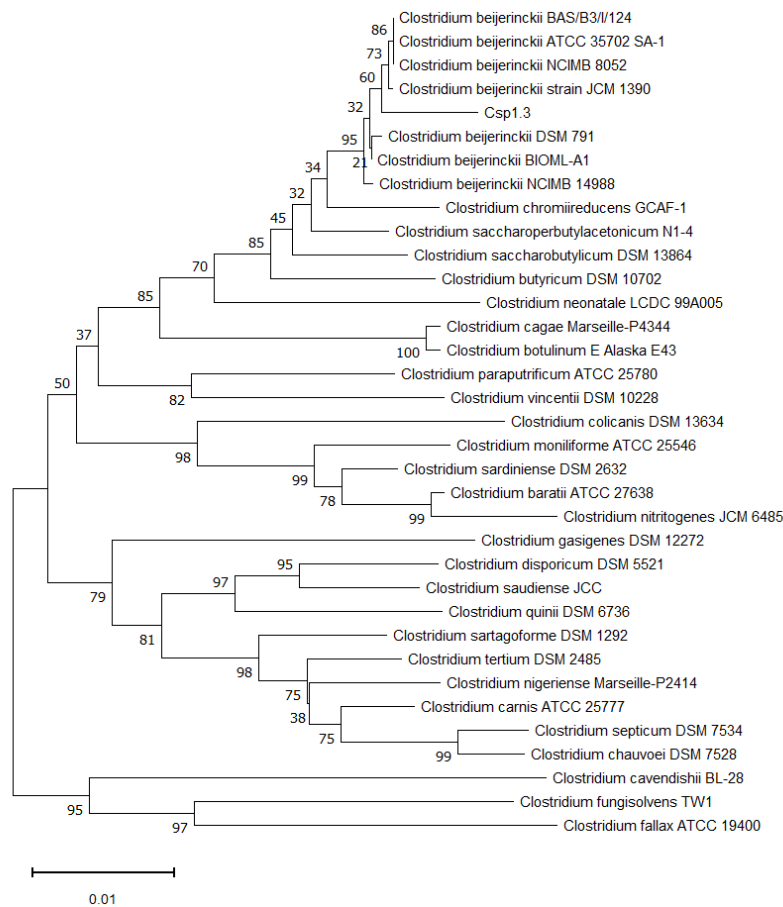
Ten isolated colonies, with morphological differences, were purified by successive plating on the same media. Finally, the ten purified strains were cultivated on various liquid rich medium to evaluate their gas production potentials (**Table 2**). From the ten isolated colonies, only one strain, named C.sp.1.3, shows a high H<sub>2</sub> production without the production of unwanted gas such as H<sub>2</sub>S in all tested conditions. For these reasons, the strain C.sp.1.3 was selected as the best candidate for H<sub>2</sub> production.

The 16S rRNA gene of the strain C.sp.1.3 was sequenced and blasted in the NCBI and EzBioCloud databases for sequence comparison. Both databases showed that C.sp.1.3 share 99.69 % identity with *Clostridium beijerinckii* DSM 791. The neighbour-joining phylogenetic tree (**Figure 1**) was constructed using 16S rRNA sequences of the closest *Clostridium* species as revealed by NCBI and EzBioCloud databases, supplemented with 16S rRNA sequences of sequenced *Clostridium beijerinckii* strains. It clearly shows that C.sp.1.3 fell in the cluster of *Clostridium beijerinckii* strains, suggesting that the isolate should belong to *Clostridium beijerinckii*.



**Table 2: Profile of the gas produced by the isolates when growth on various medium. Nd: not detected.**

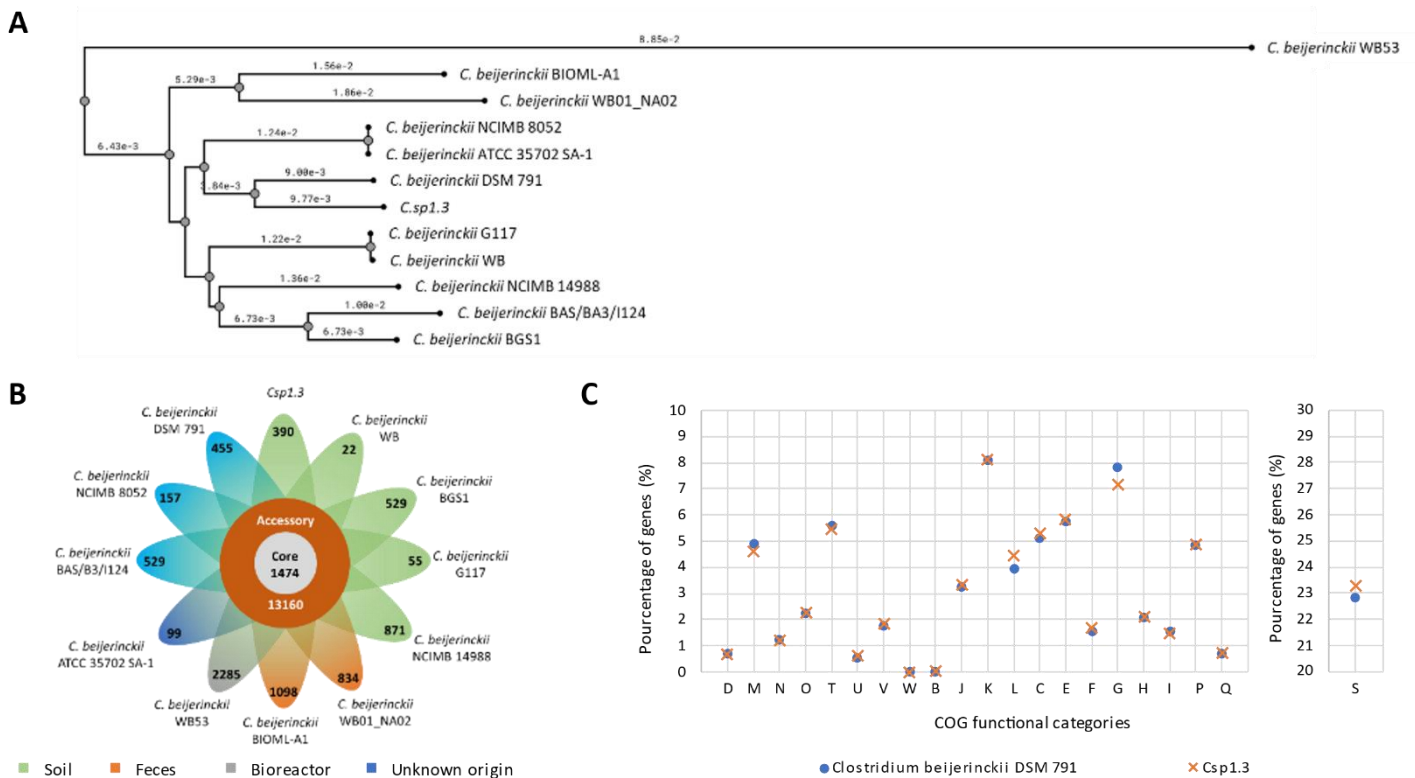
Strain	Gas production potential on various rich media		
	H <sub>2</sub>	CO <sub>2</sub>	H <sub>2</sub> S
C.sp.1.1	-	+	-
C.sp.1.2	++	+	+
C.sp.1.3	++	+	-
C.sp.1.4	nd	nd	nd
C.sp.1.5	++	+	+
C.sp.1.6	+	+	+
C.sp.1.7	+	+	+
C.sp.1.8	+	+	-
C.sp.1.9	-	-	-
C.sp.1.10	+	+	-



**Figure 1: Phylogenetic relationship of the selected H<sub>2</sub> production strain based on 16s RNA sequences. The tree was constructed using a neighbour-joining method with 1000 bootstraps. Bootstrap value (%) are indicated at the nodes.**



The genome of C.sp.1.3 was sequenced and deposited in the GenBank database with the accession number GCA\_029715615.1. The draft genome was 5,652,128 bp, consisting of 233 contigs. The longest scaffolds were 284,810 bp and the N50 length was 71,898 bp. Annotation predict a total of 5,022 CDS, 76 tRNA and 3 rRNA. The genomic distance of the C.sp.1.3 isolate with 11 other sequenced *C. beijerinckii* species (supplementary Table 1) was evaluated using the clustering algorithm available on the MicroScope platform. This enables to generate the phylogenomic tree depicted in **Figure 2A**. The genomic affiliation of C.sp.1.3 to the *C. beijerinckii* species was confirmed, as the ANI values were above the 94 % cut-off. This genomic investigation was completed by a pan-genomic analysis of the *C. beijerinckii* species (**Figure 2B**). A total of 18,482 genes distributed in 1,474 core genes groups were shared by all the 12 strains. With 47,274 genes for 13,160 genes groups, the variable genome represents 60% of the total genomes. Specific genes number varied from 22 to 2,285 for a total of 7,324 genes, i.e., 10 % of the genomes. These data demonstrate the great plasticity and adaptability of *C. beijerinckii* strains, allowing them to thrive in diverse environments. Overall, the C.sp.1.3 strain had a relatively average genomic structure among the *C. beijerinckii* species with 1,547, 3,774 and 390 genes belonging to the core, variable and specific genes, respectively. These analysis highlights the very close distance and structure between *C. beijerinckii* C.sp.1.3 and *C. beijerinckii* DSM 791. However, discrepancies appear between these two strains, in particular for the genes belonging to the COG functional categories of replication, recombination and repair, carbohydrate transport and metabolism, and of unknown functions (**Figure 2C**). Finally, all these results allow us to designate the isolate C.sp.1.3 as *Clostridium beijerinckii* C.sp.1.3.



**Figure 2: Genomic comparison of various *Clostridium beijerinckii* strains.** A) Phylogenomic relationship between the selected species. B) Pangenome of *Clostridium beijerinckii* species with the number of cores, dispensable and specific CDS of each strain. C) Comparison of genes repartition in each COG functional categories between *C. beijerinckii* DSM791 and the isolate Csp1.3 genomes.

## Characterization of *C. beijerinckii* C.sp.1.3.

### Phenotypic characterization

The strain *C. beijerinckii* C.sp.1.3. is a gram positive, spore forming rod-shape, motile, anaerobic fermentative bacillus.

### Effect of temperature

The effect of varied temperatures on cell growth and metabolism was tested in batch culture on liquid minimal media using glucose as sole carbon source and an initial pH set at 7 (**Figure 3**). Elevation of temperature from 20 to 40°C showed no drastic impact on cell growth, as the OD 600nm fluctuated weakly with temperature variation, after 48 h. However, the highest optical density was significantly reached at 37°C. The impact of temperature was much more significant on the metabolism. Gas and secondary metabolite production increased gradually with increasing temperature until a maximum was reached at 40°C. Thus, the change from 20 to 40°C resulted in a 6.62 and 5.35-fold increase in H<sub>2</sub> and butyric acid production, respectively. An optimum H<sub>2</sub> production at 40°C is consistent with the results previously observed for the strain *C. beijerinckii* KCTC 1785 (J. K. Kim *et al.*, 2008). Beyond the temperature threshold of 40°C, cell growth was strongly affected, resulting in an almost fully inhibited metabolic activities.

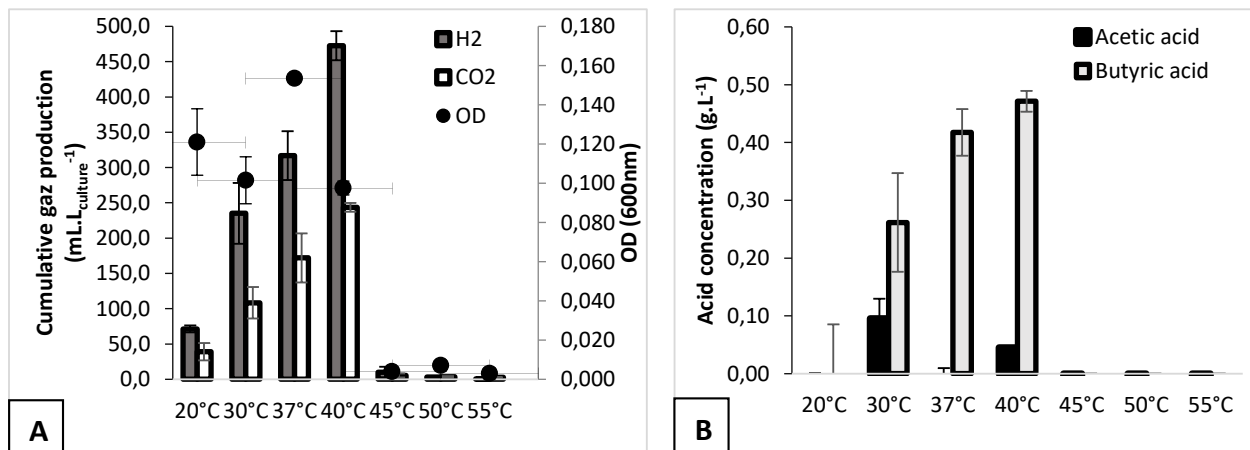


Figure 3: Effect of temperature on *C. beijerinckii* C.sp.1.3. growth and on H<sub>2</sub> and CO<sub>2</sub> production (A), and on acetic and butyric production (B), after 48h culture.

### Effect of pH

The pH effect on dark fermentation was carried out in batch mode during 48h, in minimal medium supplemented with glucose as sole carbon source and regulated at 37°C (**Figure 4**). For each pH tested, the medium was buffered to limit early acidification. Microbial activity was detected over a pH range from 5 to 8. The maximum growth was observed at pH 6. From this point onwards, growth decreases as the pH value increases, until a total inhibition observed from pH 9. Metabolic activity does not follow the same pattern, as H<sub>2</sub> and butyric acid production increases progressively with increasing pH until a maximum is reached at pH 7. Thereafter production declines to no production from pH 9, concomitant with growth inhibition.

It has been shown several times that pH is an important parameter for H<sub>2</sub> production by dark fermentation, especially for its effect on hydrogenases activity and cell membrane charge (Cheawchanlertfa *et al.*, 2020; Liu *et al.*, 2020; Lan *et al.*, 2021). Our results were consistent with these observations as dark fermentation in buffered pH 7 enable the production of 645±93 mL<sub>H<sub>2</sub></sub>.L<sup>-1</sup> (**Figure 4**), twice as much as in unbuffered pH 7 condition (**Figure 3**). In addition, the working pH enables to control the purity of produced H<sub>2</sub> during dark fermentation, as the volume ratio H<sub>2</sub>/CO<sub>2</sub> increased from 1.3 to 2.6 between pH 5 and pH 8, respectively.

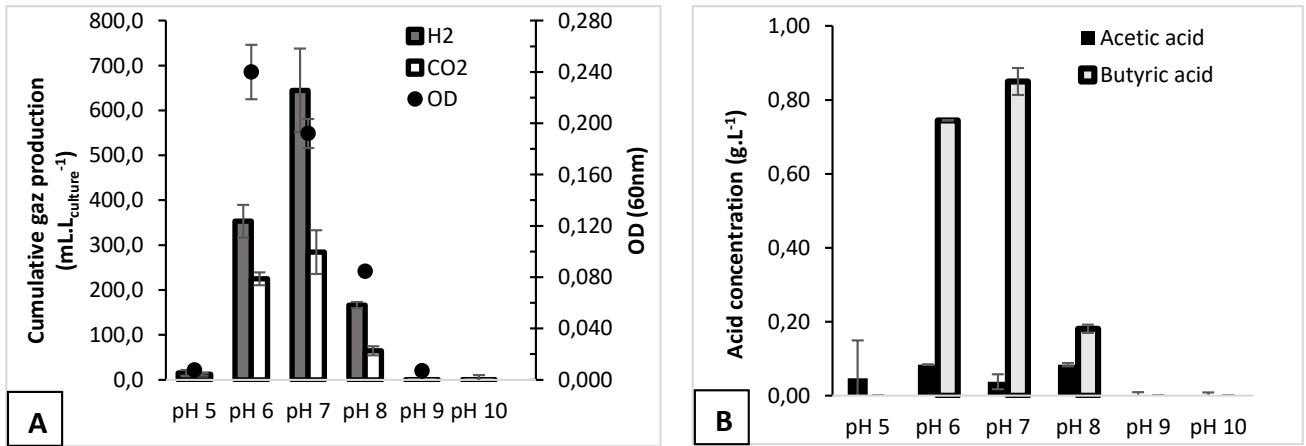


Figure 4: Effect of pH on *C. beijerinckii* C.sp.1.3. growth and on H<sub>2</sub> and CO<sub>2</sub> production (A), and on-acetic and butyric production (B), after 48h culture.

#### Acetic and butyric acid toxicity

Acetic and butyric acid produced during dark fermentation are known to inhibit cell growth (Khanal *et al.*, 2004; Van Ginkel and Logan, 2005; Zheng and Yu, 2005; Yin, Song and Wang, 2022). The toxicity of these organic acids was evaluated on 24 h cultures of *C. beijerinckii* C.sp.1.3 (Figure 5). Thus, the growth rate of the strain decreased linearly with the augmentation of the organic acids concentration.

The half maximal inhibitory concentration (IC<sub>50</sub>) is a quantitative measure that indicates how much of a particular “inhibitory” substance is needed to inhibit, a given biological process by 50%. According to this definition, butyric acid has a higher impact on the strain growth, with an estimated IC<sub>50</sub> of 8.7 g.L<sup>-1</sup>, while 9.2 g.L<sup>-1</sup> for acetic acid. As showed in the previous experiments (Figure 3 and Figure 4), the production of acetic and butyric acid are far from these concentrations. In addition, the fermentation of dairy waste that are presented in the next section showed a final concentration of 0.99 g.L<sup>-1</sup> and 2.46 g.L<sup>-1</sup> for respectively acetic and butyric acid. This represents an impact of 10% and 17% on the growth of the strain.

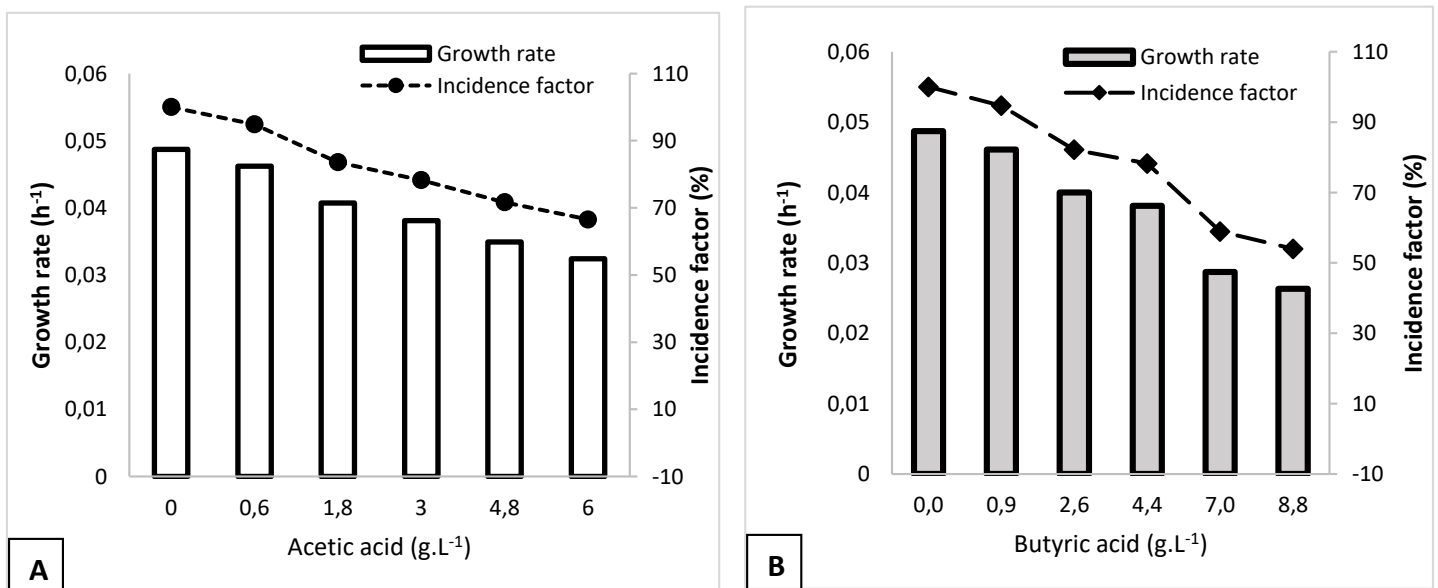


Figure 5: Inhibitory effect of acetic and butyric acid on the growth of *C. beijerinckii* 1.3.

### Effect of different Carbon source

The diversity of wastes, feedstock and consequently of carbon source have an impact on the H<sub>2</sub> production. Therefore, several carbon sources susceptible to be found in agriwastes were examined for dark fermentation using *C. beijerinckii* C.sp.1.3. This includes monosaccharides (glucose, xylose), disaccharides (cellobiose, lactose, maltose, saccharose), polysaccharides (starch, cellulose), acid sugar (galacturonic acid) and polyols (inositol, glycerol). The fermentation potentials were determined on liquid minimal media supplemented with individual carbon source set at a concentration of 5 g.L<sup>-1</sup>.

*C. beijerinckii* C.sp.1.3 was able to produce H<sub>2</sub> on all tested mono- and di-saccharides (**Figure 6**) with, in decreasing order of effectiveness, cellobiose (904±13 mL<sub>H<sub>2</sub></sub>.L<sub>culture</sub><sup>-1</sup>) > sucrose (731±3 mL<sub>H<sub>2</sub></sub>.L<sub>culture</sub><sup>-1</sup>) and glucose (713±3 mL<sub>H<sub>2</sub></sub>.L<sub>culture</sub><sup>-1</sup>) > maltose (597±14 mL<sub>H<sub>2</sub></sub>.L<sub>culture</sub><sup>-1</sup>), xylose (529±0.05 mL<sub>H<sub>2</sub></sub>.L<sub>culture</sub><sup>-1</sup>) > lactose (399±25 mL<sub>H<sub>2</sub></sub>.L<sub>culture</sub><sup>-1</sup>). Production was also detected on galacturonic acid at a comparable level (561±19 mL<sub>H<sub>2</sub></sub>.L<sub>culture</sub><sup>-1</sup>). Low productions and growth are obtained on starch, cellulose and glycerol. No growth was observed on inositol.

This carbon phenotype was compared to two collection strains (**Figure 6**), *Clostridium beijerinckii* DSM 791 and *Clostridium acetobutylicum* DSM 792, one of the most representative and widely used *Clostridium* strains. The study showed differences in phenotypes between the strains. Surprisingly, the carbon phenotype of *C. beijerinckii* C.sp.1.3 was found to be closer to that of *C. acetobutylicum* DSM 792 than to *C. beijerinckii* DSM 791 strain. These results are very interesting considering that the *C. beijerinckii* C.sp.1.3 and *C. beijerinckii* CB 791 strains are phylogenomically closer than *C. acetobutylicum* DSM 792. The strongest differences in H<sub>2</sub> production between strains were observed for galacturonic acid, inositol and glycerol. A genome comparison was performed to determine whether the phenotypic difference was supported at the genomic level. It was found that only *C. beijerinckii* DSM 791 has a complete operon encoding the inositol metabolic pathway (supplementary data Figure 1), which is consistent with the results obtained. For the two other carbon sources, the genomic architecture does not explain the results (supplementary data Figure 2, 3).

*C. acetobutylicum* is known in the literature for its H<sub>2</sub> production from glycerol, (Jáuregui, Ladino and Malagón-Romero, 2018). Although, our results are inconsistent with this literature.

Our results are quite consistent with the literature for *C. beijerinckii*. Other strains of *C. beijerinckii* have been successfully used for dark fermentation (i) on lignocellulosic compound (Birgen *et al.*, 2021); (ii) on starchy compound (Ezeji, Qureshi and Blaschek, 2005; Ozmihci and Kargi, 2010; Masset *et al.*, 2012; Nasr *et al.*, 2017) ; (iii) showed weak growth on glycerol (Keis, Shaheen and Jones, 2001; Ujor *et al.*, 2014).

In conclusion, the isolated strain *C. sp.1.3*. was identified as a *Clostridium beijerinckii*, and phylogenetically closed to the *Clostridium beijerinckii* DSM 791. *C.sp.1.3*. was able to produce H<sub>2</sub> on monosaccharides, disaccharides and on real dairy wastewaters. Surprisingly, the ability of the isolated strain to produce H<sub>2</sub> on different carbon source showed that its phenotype is closer to *Clostridium acetobutylicum* DSM792 than to *Clostridium beijerinckii* DSM791.

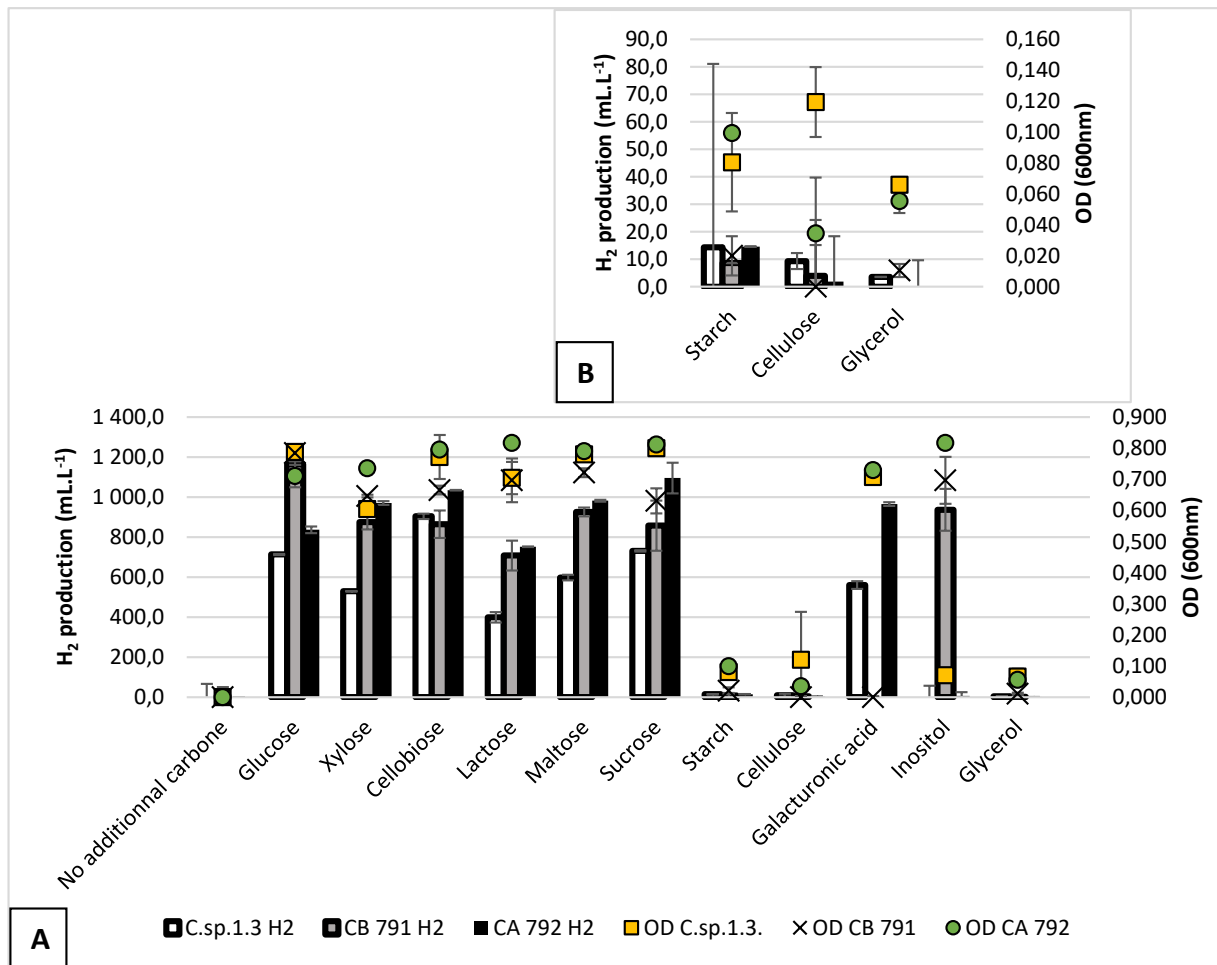


Figure 6: (A) Effect of different carbon sources growth and on H<sub>2</sub> production by the *C. beijerinckii* C.sp.1.3., and the collection strains CB791 and CA792 (DSMZ). (B) Zoom on the results for starch, cellulose and glycerol.

### Validation of the isolate *C. beijerinckii* C.sp.1.3. as a H<sub>2</sub> producer from dairy wastes

The strain *C. beijerinckii* C.sp.1.3 has been cultivated, in anaerobic conditions, into the mixture of CW and F1 at the optimal pH and temperature previously determined, i.e. pH 7 and 40°C, respectively (Figure 7). H<sub>2</sub> production was observed after a lag phase of 0.3 day, and entered an exponential phase until 1.5 days during which the maximal productivity reached 833.1 mL<sub>H<sub>2</sub></sub>·L<sub>culture</sub><sup>-1</sup>·d<sup>-1</sup>. Then, the production rate declined until 3 days for a final cumulative H<sub>2</sub> production of 880.8 mL<sub>H<sub>2</sub></sub>·L<sub>culture</sub><sup>-1</sup>. Thus, 95 % of the H<sub>2</sub> was produced in 1.7 days. On rich media, the production of H<sub>2</sub> of the isolated strain is lower than other strain, as for Zhao et al., (2011) who obtained a maximal production of 2000 mL<sub>H<sub>2</sub></sub>·L<sub>culture</sub><sup>-1</sup> at 35°C, pH 7 on MYP media with 5 g·L<sup>-1</sup> glucose. However, the production, productivity and yield obtained on real wastewaters were in the wide range of the literature data for the same type of substrate, in batch conditions, with cultures of *Clostridium*. (Ellis, Sims and Miller, 2014).

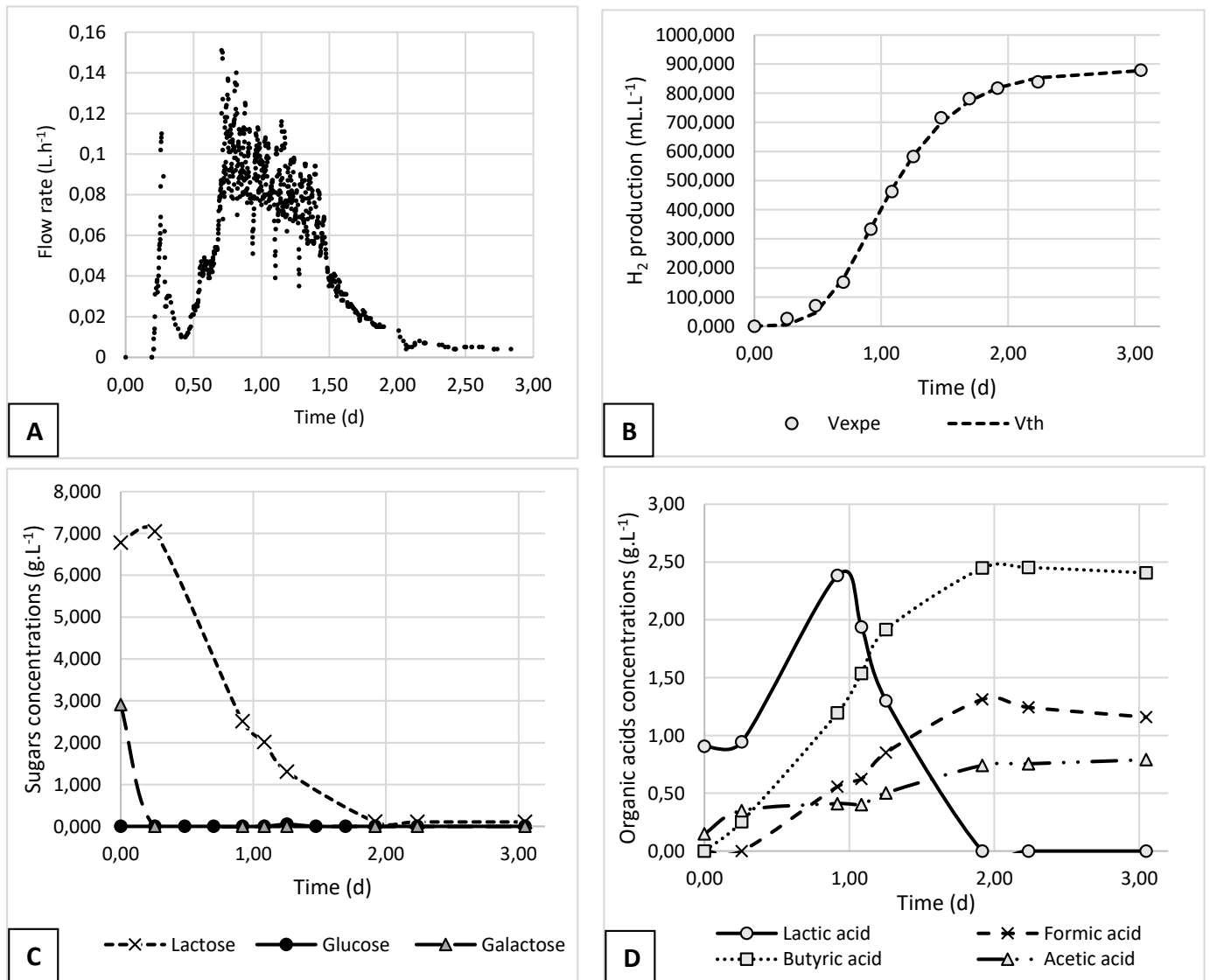


Figure 7: Experimental data of the dark fermentation from dairy wastes. (A) Biogas production flow rate (L). (B) Cumulative H<sub>2</sub> production (mL.L<sub>culture</sub><sup>-1</sup>), fitted with modified Gompertz modele (mL.L<sub>culture</sub><sup>-1</sup>). (C) Lactose, glucose and galactose consumption (g.L<sup>-1</sup>). (D) Lactic, formic, butyric and acetic acid concentrations (g.L<sup>-1</sup>) during dark fermentation of dairy wastewaters.

Lactose, galactose and lactic acid were the available substrates at the beginning of the fermentation, concentrated at 6.77, 2.9 and 0.59 g.L<sup>-1</sup> respectively. During the H<sub>2</sub> production lag phase (Figure 7B) galactose was totally consumed, in association with a peak of the biogas production flow rate (Figure 7A). Indeed, the flow rate rocketed from 0 at 0.2 day to 0.11 L.h<sup>-1</sup> at 0.3 days. A relative low production of acetic (0.20 g.L<sup>-1</sup>) and butyric acid (0.25 g.L<sup>-1</sup>) is also observed during this phase (Figure 7D). Since the galactose is fully consumed during this phase (Figure 7C), we emitted the hypothesis that this carbon source is rather associated with the biomass production, than with H<sub>2</sub> production. This hypothesis is conserved in our carbon balance and our H<sub>2</sub> production yield calculation. This phenomena of catabolic repression could have huge impact on a continuous fermentation.

The H<sub>2</sub> production is divided into two distinct metabolic activities. From 0.3 to 0.9 days, the biogas production increased to reach a flow rate up to 0.15 L.h<sup>-1</sup>. This production is strongly associated with a lactose consumption (6.85 g.L<sup>-1</sup>.d<sup>-1</sup>), and a production of lactic acid (2.18 g.L<sup>-1</sup>.d<sup>-1</sup>), butyric acid (1.42 g.L<sup>-1</sup>.d<sup>-1</sup>). A minor production of acetic acid (0.09 g.L<sup>-1</sup>.d<sup>-1</sup>) and formic acid (0.05 g.L<sup>-1</sup>.d<sup>-1</sup>) is also observed.

From 0.9 to 1.5 days, the flow rate decline slowly from 0.08 to 0.05 L.h<sup>-1</sup>. In opposition to the previous phase, the H<sub>2</sub> production is associated to a lower consumption of lactose (3.60 g.L<sup>-1</sup>.d<sup>-1</sup>), and consumption of lactic acid (3.20 g.L<sup>-1</sup>.d<sup>-1</sup>). A higher production of acetic acid (0.28 g.L<sup>-1</sup>.d<sup>-1</sup>), and butyric acid (2.15 g.L<sup>-1</sup>.d<sup>-1</sup>) is also concomitant to this production. The production of formic acid remained constant.

After 1.5 days of experiment , the H<sub>2</sub> production rate decline very strongly. The lactose and lactic acid are almost completely consumed and the production of metabolites reach a plateau at 1.9 days.

The theoretical ratio (R<sub>th</sub>) has also been calculated. This ratio defined by Equation 3 offers a quick analysis of the H<sub>2</sub> metabolism. For this batch, R<sub>th</sub> is equal to 0.81, implying that H<sub>2</sub> production is limited by other factors in competition. This conclusion is consistent with the high production of formic acid (1.16 g.L<sup>-1</sup>).

$$R_{th} = \frac{H_2}{2 * (Ac + Bu)} \quad (3)$$

Indeed, the carbon balance (Supplementary data Figure 4) is completed up to 91.9 %. Lactose and galactose represented 91.6 % of the carbon sources, and lactate 8.4 %. Regarding the products, CO<sub>2</sub>, acetic acid, butyric acid and biomass represented respectively 4.7, 6.0, 30.3 and 43.9% of the carbon. 8.1 % of the carbon at the end of the fermentation is unidentified. One hypothesis could be the production of solvents such as ethanol that were not analysed in this study. Solventogenesis is indeed well known in *Clostridium spp.* The accumulation of acetic and butyric acids is toxic to cells, and leads to a drop in pH. This favors solventogenesis to detoxify the environment and avoid inhibitory effects (Van Ginkel and Logan, 2005).

The H<sub>2</sub> production yield was determined of 2.03 mM<sub>H<sub>2</sub></sub>.mM<sub>eqlactose</sub><sup>-1</sup>. The literature shows a broad range of yields to compare with. For the same substrate type Ellis, Sims and Miller, (2014) obtained of 1.18 mM<sub>H<sub>2</sub></sub>.mM<sub>eqlactose</sub><sup>-1</sup> with a culture of *Clostridium spp.* in batch conditions. On the other hand, (Patel *et al.*, 2016) obtained with a pure culture of *Clostridium sp.* IODB-O3 and 7.5 g.L<sup>-1</sup> of lactose in their whey wastes obtained a yield of 6.34 mol<sub>H<sub>2</sub></sub>/mol<sub>lactose</sub>. Other studies for the same type of substrate in batch conditions but with sludge as an inoculum showed also interesting yields. For instance, Wong *et al.*, (2018) obtained 4.6 mM<sub>H<sub>2</sub></sub>.mM<sub>eqlactose</sub><sup>-1</sup> with 84.6 g.L<sup>-1</sup> of carbohydrates, with pre-treated landfill leachate sludge as an inoculum, and Romão *et al.*, (2014) obtained 4.08 mM<sub>H<sub>2</sub></sub>.mM<sub>eqlactose</sub><sup>-1</sup> with 20 g.L<sup>-1</sup> lactose, with a microbial consortium provided by the effluent treatment plant of a local dairy company as an inoculum. Comparison with these results suggests that the strain is within the range of values obtained in the literature for this type of strain.

These results highlight the question of the impact of substrate (lactose concentration) and inoculum on H<sub>2</sub> production. While complex microbial populations, such as sludge, appear to have higher yields than pure cultures, the control of pure cultures may be better suited to industrial applications. There is also the question of the robustness of the microbial community: while pure cultures are easier to control, more complex communities may offer greater adaptability and robustness to changes in operating conditions (temperature, etc.).



## Conclusion and perspectives

The isolate *Clostridium beijerinckii* C.sp.1.3 was able to produce H<sub>2</sub> from mono, di and polysaccharides, galacturonic acid and glycerol. Its ability to produce H<sub>2</sub> from a mix of dairy wastes is of special economic significance. Under optimum condition of temperature and pH (40°C, pH 7) the maximum productivity in batch for 5 g.L<sup>-1</sup> of Total Organic Carbon (TOC) was 833.1 mL H<sub>2</sub>.L<sup>-1</sup>.d<sup>-1</sup>, with a yield of 2.03 mM<sub>H<sub>2</sub></sub>.mM<sub>lactose</sub><sup>-1</sup>. If those results on real dairy wastes rich in lactose are decent, it is also promising for other type of wastes. Specifically, the carbon phenotype indicates that H<sub>2</sub> production could be stronger on waste rich in cellobiose. However, as the strain produce very few H<sub>2</sub> on cellulose, different strategies could be experimented: a co-culture of the isolated strain with other bacteria able to decompose cellulose wastes, or a physical, chemical, or enzymatic pre-treatment of cellulose wastes. This valuation of biomass and wastewaters is of special economic significance, and is candidate for an industrialisation of the process.

## Bibliographie

Abreu, A.A. *et al.* (2010) 'Engineered heat treated methanogenic granules: A promising biotechnological approach for extreme thermophilic biohydrogen production', *Bioresource Technology*, 101(24), pp. 9577–9586. Available at: <https://doi.org/10.1016/j.biortech.2010.07.070>.

Aziz, R.K. *et al.* (2008) 'The RAST Server: Rapid Annotations using Subsystems Technology', *BMC Genomics*, 9(1), p. 75. Available at: <https://doi.org/10.1186/1471-2164-9-75>.

Birgen, C. *et al.* (2021) 'Butanol production from lignocellulosic sugars by *Clostridium beijerinckii* in microbioreactors', *Biotechnology for Biofuels*, 14(1). Available at: <https://doi.org/10.1186/s13068-021-01886-1>.

Cheawchanlertfa, P. *et al.* (2020) 'Clostridium manihotivorum sp. nov., a novel mesophilic anaerobic bacterium that produces cassava pulp-degrading enzymes', *PeerJ*, 8, pp. 1–28. Available at: <https://doi.org/10.7717/peerj.10343>.

Chin, H.L., Chen, Z.S. and Chou, C.P. (2003) 'Fedbatch operation using *Clostridium acetobutylicum* suspension culture as biocatalyst for enhancing hydrogen production', *Biotechnology Progress*, 19(2), pp. 383–388. Available at: <https://doi.org/10.1021/bp0200604>.

Cripa, F.B. *et al.* (2020) 'Poultry slaughterhouse anaerobic ponds as a source of inoculum for biohydrogen production', *Journal of Bioscience and Bioengineering*, 129(1), pp. 77–85. Available at: <https://doi.org/10.1016/j.jbiosc.2019.07.006>.

Das, S.R. and Basak, N. (2021) 'Molecular biohydrogen production by dark and photo fermentation from wastes containing starch: recent advancement and future perspective', *Bioprocess and Biosystems Engineering*, 44(1). Available at: <https://doi.org/10.1007/s00449-020-02422-5>.

Davila-Vazquez, G. *et al.* (2009) 'Continuous biohydrogen production using cheese whey: Improving the hydrogen production rate', *International Journal of Hydrogen Energy*, 34(10), pp. 4296–4304. Available at: <https://doi.org/10.1016/j.ijhydene.2009.02.063>.

Dincer, I. (2012) 'Green methods for hydrogen production', *International Journal of Hydrogen Energy*, 37(2), pp. 1954–1971. Available at: <https://doi.org/10.1016/j.ijhydene.2011.03.173>.

Dinesh, G.H. *et al.* (2020) 'Simultaneous biohydrogen (H<sub>2</sub>) and bioplastic (poly-β-hydroxybutyrate-PHB) productions under dark, photo, and subsequent dark and photo fermentation utilizing various wastes', *International Journal of Hydrogen Energy*, 45(10), pp. 5840–5853. Available at: <https://doi.org/10.1016/j.ijhydene.2019.09.036>.

Ellis, J.T., Sims, R.C. and Miller, C.D. (2014) 'Microbial bioproducts from cheese whey through fermentation with wastewater sludge *Clostridium* isolates', *Canadian Journal of Microbiology*, 60(7), pp. 431–435. Available at: <https://doi.org/10.1139/cjm-2013-0803>.

Elsharnouby, O. *et al.* (2013) 'A critical literature review on biohydrogen production by pure cultures', *International Journal of Hydrogen Energy*, 38(12), pp. 4945–4966. Available at: <https://doi.org/10.1016/j.ijhydene.2013.02.032>.

Ezeji, T.C., Qureshi, N. and Blaschek, H.P. (2005) 'Continuous butanol fermentation and feed starch retrogradation: Butanol fermentation sustainability using *Clostridium beijerinckii* BA101', *Journal of Biotechnology*, 115(2), pp. 179–187. Available at: <https://doi.org/10.1016/j.jbiotec.2004.08.010>.

Farizoglu, B. *et al.* (2004) 'Cheese whey treatment performance of an aerobic jet loop membrane bioreactor', *Process Biochemistry*, 39(12), pp. 2283–2291. Available at: <https://doi.org/10.1016/j.procbio.2003.11.028>.

François, E. *et al.* (2021) 'Unexpected high production of biohydrogen from the endogenous fermentation of grape must deposits', *Bioresource Technology*, 320(August 2020). Available at: <https://doi.org/10.1016/j.biortech.2020.124334>.

Francois-Lopez, E. (2016) 'Production de biohydrogène par fermentation obscure : potentiel de diverses biomasses', *Manuscript de thèse*, pp. 1–8.

Van Ginkel, S. and Logan, B.E. (2005) 'Inhibition of biohydrogen production by undissociated acetic and butyric acids', *Environmental Science and Technology*, 39(23), pp. 9351–9356. Available at: <https://doi.org/10.1021/es0510515>.

Van Ginkel, S., Sung, S. and Lay, J.J. (2001) 'Biohydrogen production as a function of pH and substrate concentration', *Environmental Science and Technology*, 35(24), pp. 4726–4730. Available at: <https://doi.org/10.1021/es001979r>.

De Gioannis, G. *et al.* (2013) 'A review of dark fermentative hydrogen production from biodegradable municipal waste fractions', *Waste Management*, 33(6), pp. 1345–1361. Available at: <https://doi.org/10.1016/j.wasman.2013.02.019>.

Hallenbeck, P.C. and Benemann, J.R. (2002) 'Biological hydrogen production; Fundamentals and limiting processes', *International Journal of Hydrogen Energy*, 27(11–12), pp. 1185–1193. Available at: [https://doi.org/10.1016/S0360-3199\(02\)00131-3](https://doi.org/10.1016/S0360-3199(02)00131-3).

International Energy Agency, I. (2022) *Global Hydrogen Review 2022*. Available at: [www.iea.org/t&c/](http://www.iea.org/t&c/).

Jáuregui, M.A., Ladino, A. and Malagón-Romero, D. (2018) 'The effect of the initial concentration of glycerol on the hydrogen produced by strains of the genus *Clostridium* spp.', *International Journal of Sustainable Engineering*, 11(3), pp. 205–210. Available at: <https://doi.org/10.1080/19397038.2017.1387826>.

Jung, K.W. *et al.* (2011) 'Bioreactor design for continuous dark fermentative hydrogen production', *Bioresource Technology*, 102(18), pp. 8612–8620. Available at: <https://doi.org/10.1016/j.biortech.2011.03.056>.

Karadag, D. *et al.* (2014) 'A review on fermentative hydrogen production from dairy industry wastewater', *Journal of Chemical Technology and Biotechnology*, 89(11), pp. 1627–1636. Available at: <https://doi.org/10.1002/jctb.4490>.

Keis, S., Shaheen, R. and Jones, D.T. (2001) 'Emended descriptions of *Clostridium acetobutylicum* and *Clostridium beijerinckii*, and descriptions of *Clostridium saccharoperbutylacetonicum* sp. nov. and *Clostridium saccharobutylicum* sp. nov.', *International Journal of Systematic and Evolutionary Microbiology*, 51(6), pp. 2095–2103. Available at: <https://doi.org/10.1099/00207713-51-6-2095>.

Khanal, S.K. *et al.* (2004) 'Biological hydrogen production: Effects of pH and intermediate products', *International Journal of Hydrogen Energy*, 29(11), pp. 1123–1131. Available at: <https://doi.org/10.1016/j.ijhydene.2003.11.002>.

Kim, H.J. *et al.* (2008) 'Defined spatial structure stabilizes a synthetic multispecies bacterial community', *PNAS*, 105(47), pp. 2–7.

Kim, J.K. *et al.* (2008) 'Hydrogen production conditions from food waste by dark fermentation with *Clostridium beijerinckii* KCTC 1785', *Biotechnology and Bioprocess Engineering*, 13(4), pp. 499–504. Available at: <https://doi.org/10.1007/s12257-008-0142-0>.

Kim, T.H. *et al.* (2012) 'Effects of initial lactic acid concentration, HRTs, and OLRs on bio-hydrogen production from lactate-type fermentation', *Bioresource Technology*, 103(1), pp. 136–141. Available at: <https://doi.org/10.1016/j.biortech.2011.09.093>.

Kumar, G. *et al.* (2018) 'Biomass based hydrogen production by dark fermentation — recent trends and opportunities for greener processes', *Current Opinion in Biotechnology*, 50, pp. 136–145. Available at: <https://doi.org/10.1016/j.copbio.2017.12.024>.

Lan, Y. *et al.* (2021) 'Isolation and characterization of a newly identified *Clostridium butyricum* strain SCUT343-4 for 1,3-propanediol production', *Bioprocess and Biosystems Engineering* [Preprint], (0123456789). Available at: <https://doi.org/10.1007/s00449-021-02610-x>.

Liu, L. *et al.* (2020) 'Isolation of *Clostridium* from Yunnan-Tibet hot springs and description of *Clostridium thermarum* sp. nov. with lignocellulosic ethanol production', *Systematic and Applied Microbiology*, 43(5), p. 126104. Available at: <https://doi.org/10.1016/j.syapm.2020.126104>.

Masset, J. *et al.* (2012) 'Fermentative hydrogen production from glucose and starch using pure strains and artificial co-cultures of *Clostridium* spp.', *Biotechnology for Biofuels*, 5. Available at: <https://doi.org/10.1186/1754-6834-5-35>.

Mechichi, T. *et al.* (1997) 'New Aromatic O-Demethylating Homoacetogen From an Olive Mill Wastewater Treatment Digester', *International Journal of Systematic Bacteriology*, (1 999), pp. 1201–1209.

Meena, R.A.A. *et al.* (2020) 'Biohythane production from food processing wastes – Challenges and perspectives', *Bioresource Technology*. Elsevier Ltd. Available at: <https://doi.org/10.1016/j.biortech.2019.122449>.

Nasr, N. *et al.* (2017) 'Mono- and co-substrate utilization kinetics using mono- and co-culture of *Clostridium beijerinckii* and *Clostridium saccharoperbutylacetonicum*', *Bioresource Technology*, 241, pp. 152–160. Available at: <https://doi.org/10.1016/j.biortech.2017.05.086>.

Ozmihci, S. and Kargi, F. (2010) 'Comparison of different mixed cultures for bio-hydrogen production from ground wheat starch by combined dark and light fermentation', *Journal of Industrial Microbiology and Biotechnology*, 37(4), pp. 341–347. Available at: <https://doi.org/10.1007/s10295-009-0679-8>.

Patel, A.K. *et al.* (2016) 'Whey waste as potential feedstock for biohydrogen production', *Renewable Energy*, 98, pp. 221–225. Available at: <https://doi.org/10.1016/j.renene.2016.02.039>.

Rabelo, C.A.B.S. *et al.* (2018) 'Optimization of hydrogen and organic acids productions with autochthonous and allochthonous bacteria from sugarcane bagasse in batch reactors', *Journal of Environmental Management*, 223(July), pp. 952–963. Available at: <https://doi.org/10.1016/j.jenvman.2018.07.015>.

Romão, B.B. *et al.* (2014) 'Biohydrogen production through dark fermentation by a microbial consortium using whey permeate as substrate', *Applied Biochemistry and Biotechnology*, 172(7), pp. 3670–3685. Available at: <https://doi.org/10.1007/s12010-014-0778-5>.

Rosa, P.R.F. *et al.* (2014) 'Hydrogen production from cheese whey with ethanol-type fermentation: Effect of hydraulic retention time on the microbial community composition', *Bioresource Technology*, 161, pp. 10–19. Available at: <https://doi.org/10.1016/j.biortech.2014.03.020>.

Silva, J.S. *et al.* (2018) 'Cashew apple bagasse as new feedstock for the hydrogen production using dark fermentation process', *Journal of Biotechnology*, 286(April), pp. 71–78. Available at: <https://doi.org/10.1016/j.jbiotec.2018.09.004>.

Suresh, G., Kumari, P. and Venkata Mohan, S. (2023) 'Light-dependent biohydrogen production: Progress and perspectives', *Bioresource Technology*. Elsevier Ltd. Available at: <https://doi.org/10.1016/j.biortech.2023.129007>.

Ujor, V. *et al.* (2014) 'Glycerol supplementation of the growth medium enhances in situ detoxification of furfural by *Clostridium beijerinckii* during butanol fermentation', *Applied Microbiology and Biotechnology*, 98(14), pp. 6511–6521. Available at: <https://doi.org/10.1007/s00253-014-5802-8>.

Vallenet, D. *et al.* (2019) 'MicroScope: an integrated platform for the annotation and exploration of microbial gene functions through genomic, pangenomic and metabolic comparative analysis', *Nucleic Acids Research* [Preprint]. Available at: <https://doi.org/10.1093/nar/gkz926>.

Wang, J. *et al.* (2018) 'Biohydrogen production from space crew's waste simulants using thermophilic consolidated bioprocessing', *Bioresource Technology*, 255(January), pp. 349–353. Available at: <https://doi.org/10.1016/j.biortech.2018.01.109>.

Wong, Y.M. *et al.* (2018) 'Production of bio-hydrogen from dairy wastewater using pretreated landfill leachate sludge as an inoculum', *Journal of Bioscience and Bioengineering*, 127(2), pp. 150–159. Available at: <https://doi.org/10.1016/j.jbiosc.2018.07.012>.

Xiao, Y. *et al.* (2013) 'Effect of the culture media optimization, pH and temperature on the biohydrogen production and the hydrogenase activities by *Klebsiella pneumoniae* ECU-15', *Bioresource Technology*, 137, pp. 9–17. Available at: <https://doi.org/10.1016/j.biortech.2013.03.109>.

Yang, G. and Wang, J. (2019) 'Changes in microbial community structure during dark fermentative hydrogen production', *International Journal of Hydrogen Energy*, 44(47), pp. 25542–25550. Available at: <https://doi.org/10.1016/j.ijhydene.2019.08.039>.

Yin, Y., Song, W. and Wang, J. (2022) 'Inhibitory effect of acetic acid on dark-fermentative hydrogen production', *Bioresource Technology*, 364. Available at: <https://doi.org/10.1016/j.biortech.2022.128074>.

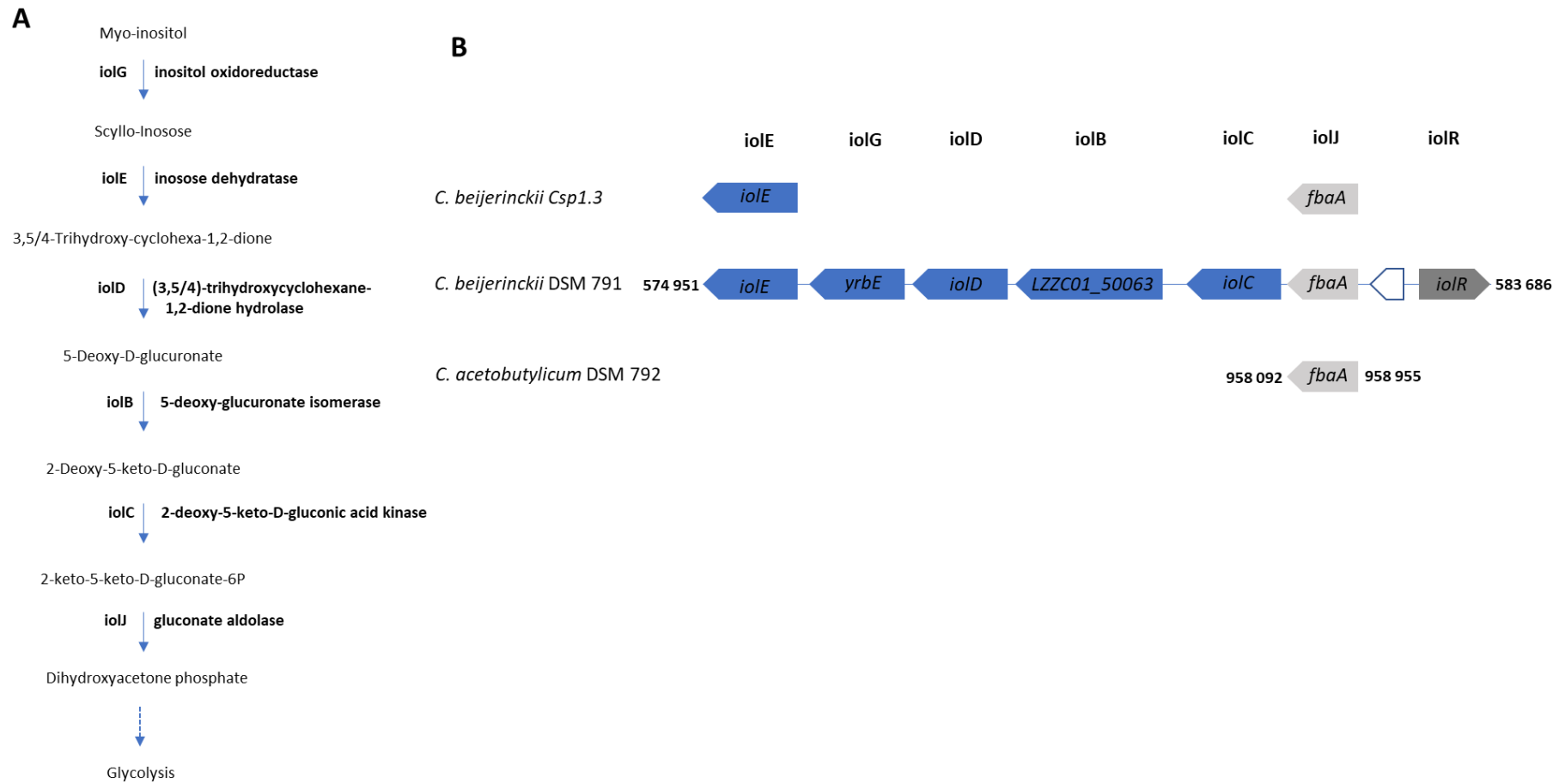
Zhao, X. *et al.* (2011) 'Hydrogen production by the newly isolated *Clostridium beijerinckii* RZF-1108', *Bioresource Technology*, 102(18), pp. 8432–8436. Available at: <https://doi.org/10.1016/j.biortech.2011.02.086>.

Zheng, X.J. and Yu, H.Q. (2005) 'Inhibitory effects of butyrate on biological hydrogen production with mixed anaerobic cultures', *Journal of Environmental Management*, 74(1), pp. 65–70. Available at: <https://doi.org/10.1016/j.jenvman.2004.08.015>.

## Supplementary data

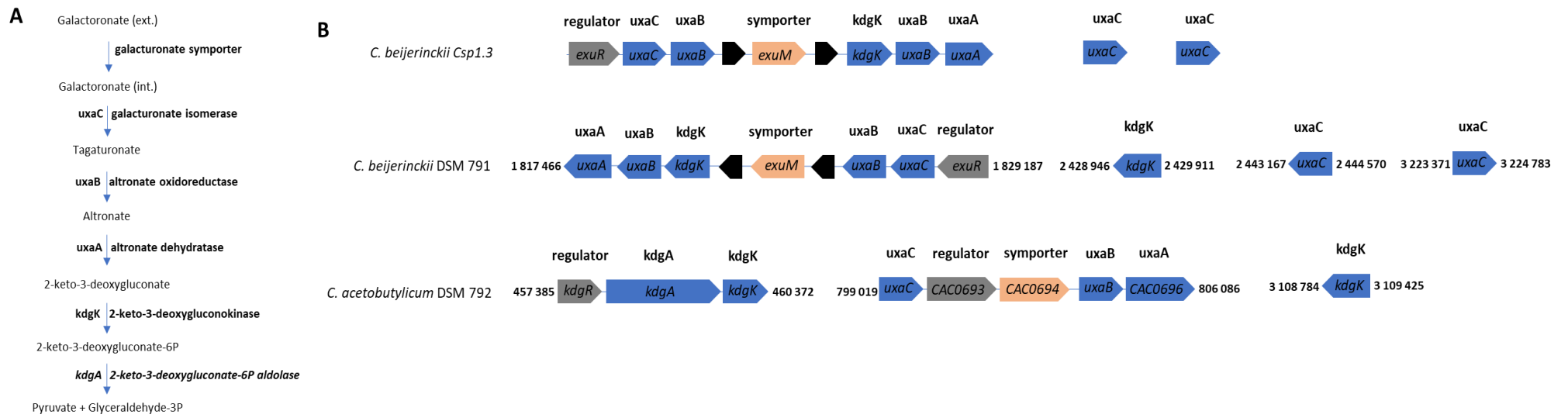
**Table 1: Complete genomes f *C. beijerinckii* strains used for species relationship analysis.**

Strain	Origin (country)	Total sequence length (bp)	GC (%)	nb of plasmid	number of CDS	GenBank accession	assembly
Clostridium beijerinckii NCIMB 14988	Garden soil (England)	6,485,394	30	0	6046	GCA_000833105.2	
Clostridium BAS/B3/I/124	Unknown (Germany)	6,123,550	29.87	0	5798	GCA_002003345.1	
Clostridium beijerinckii NCIMB 8052	Unknown (United States of America)	6,000,632	29.86	0	5596	GCA_000016965.1	
Clostridium beijerinckii ATCC 35702	Unknown (United States of America)	5,999,050	29.85	0	5675	GCA_000767745.1	
Clostridium beijerinckii WB53	Woodchip bioreactor (United States of America)	4,287,191	29.31	1	4015	GCA_003129525.1	
Clostridium beijerinckii DSM791	Unknown (Germany)	5,781,472	29.66	0	5428	GCA_002006445.1	
Clostridium beijerinckii BIOML-A1	Human feces (United States of America)	6,182,260	29.62	0	5941	GCA_009876665.1	
Clostridium WB01_NA02	Pig feces (Germany)	5,627,834	29.61	0	5310	GCA_012843965.1	
Clostridium beijerinckii WB	Mangrove/sediment (China)	5,781,106	29.69	0	5369	GCA_002954125.1	
Clostridium beijerinckii G117	Soil collected from tropical lawn (Singapore)	5,803,412	29.7	0	5483	GCA_000280535.1	
Clostridium beijerinckii BGS1	Green land Soil (China)	5,880,896	29.71	0	5455	GCA_001685175.1	

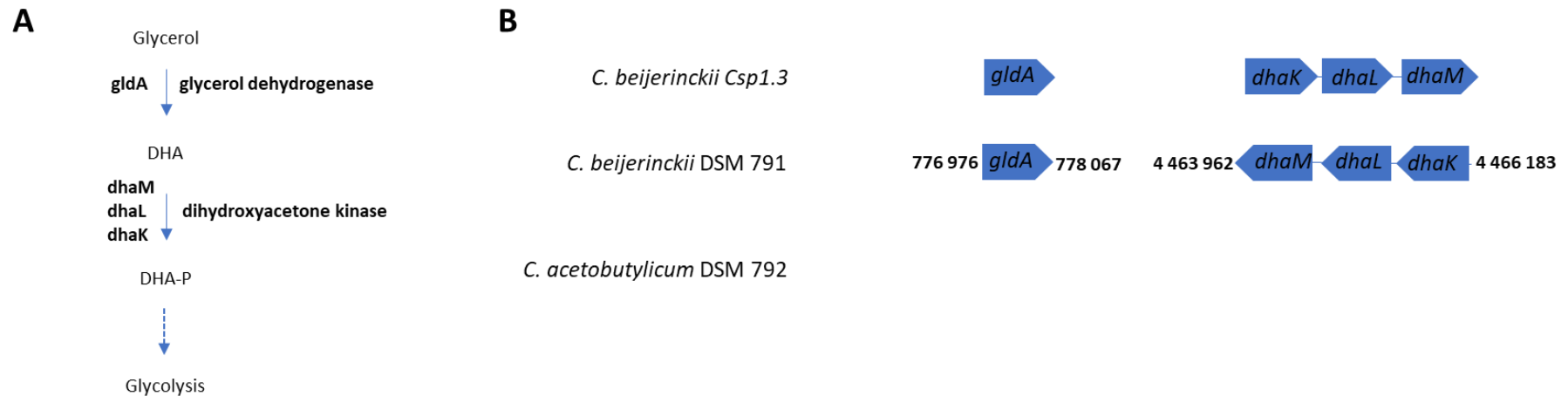


**Figure 1: Inositol metabolism. A) Inositol pathway and related enzymes. B) Conservation of the inositol operon between *Clostridium* species.**

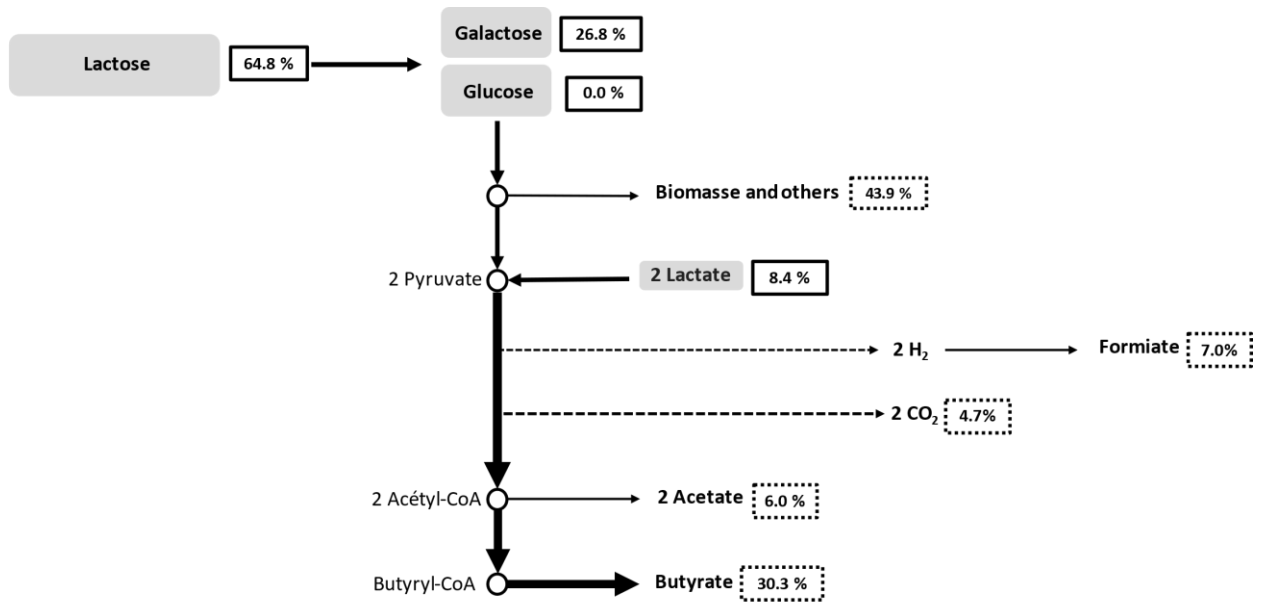




**Figure 2: Galacturonate assimilation pathway (A) and comparison of the genome organization of related genes between the *Clostridium* species studied (B).**



**Figure 3: Glycerol assimilation pathway (A) and comparison of the genome organisation of related genes between the Clostridium species studied (B).**



XXX : Carbone sources  
 → : Metabolic flow     
 XXX % : Molecular fraction of the metabolic flow of products     
 XXX % : Molecular fraction of the metabolic flow of substrates

**Figure 4: Metabolic flow distribution (carbone equivalent)**