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Unexpected high production of biohydrogen from the endogenous fermentation of grape must deposits

Emilie François[a], Christine Dumas[a], Régis D. Gougeon[b], Hervé Alexandre[b], Stéphane Vuilleumier[c], Barbara Ernst[a*]

[a] Université de Strasbourg, CNRS, IPHC UMR 7178, Laboratoire de Reconnaissance et Procédés de Séparation Moléculaire (RePSeM), ECPM 25 rue Becquerel F-67000 Strasbourg, France

[b] UMR PAM, Université de Bourgogne / Agrosup Dijon, Institut Universitaire de la Vigne et du Vin - Jules Guyot, 21000 Dijon, France

[c] Université de Strasbourg, CNRS, GMGM UMR 7156, 28 rue Goethe, 67000 Strasbourg, France

*Corresponding author. Email: Barbara.Ernst@unistra.fr

Abstract

The aim of this work was to assess the performances of wine byproduct biomass for hydrogen production by dark fermentation. Grape must deposits from two grape varieties (Pinot Gris and Chardonnay) were considered, either with external microbial *inoculum* or without. We show that grape must residues contain endogenous microflora, well adapted to their environment, which can degrade sugars (initially contained in the biomass) to hydrogen without any nutrient addition. Indeed, hydrogen production during endogenous fermentation is as efficient as with an external heat-treated *inoculum* (2.5 ± 0.4 $L_{H2}.L_{reactor}^{-1}$ and 1.61 ± 0.41 $mol_{H2}.mol^{-1}_{consumed\ hexose}$, respectively) with a lower energy cost. Hydrogen-producing bacteria were selected from the endogenous microflora during semi-batch bioreactor operation, as shown by T-RFLP profiles and 16S rRNA sequencing, with *Clostridium* spp. *(butyricum, beijerinckii, diolis, roseum)* identified as the major phylotype. Such hydrogen production efficiency opens new perspectives for innovating in the valorization of winery by-products.

Keywords

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Hydrogen, dark fermentation, endogenous fermentation, winery waste, valorization

1. Introduction

Today, around 89% of the world energy consumption relies on fossil fuels and this dependency has led to fossil fuel depletion and environmental issues. Transition towards renewable energies and their delocalized production is necessary. Biohydrogen as carbon-free energy, produced by dark fermentation is one of the most promising candidate technology (Kotay *et al.*, 2008). To produce hydrogen by dark fermentation at large scale, performances in terms of hydrogen productivity, yield and volume must be improved (Tapia-Venegas *et al.* 2015). One way to optimize the process is selection of the substrate. Simple substrates such as glucose or sucrose are the most commonly used carbon sources for hydrogen production by dark fermentation (Chen and Lin, 2003). However, the use of feedstocks for biofuel industrial-scale production increased the prices of such feedstocks (Ajanovic, 2011) such as cane sugar for the ethanol bioproduction, leading today to 2nd generation production approaches using by-products, waste or lignocellulosic biomass (Damay *et al.*, 2018). Consequently, many investigations are currently being conducted using organic agricultural (sugarcane waste, animal manure, etc.), industrial (dairy waste, starch-based waste, etc.) or municipal biomasses (organic fraction of solid waste, wastewater sludge, etc.) as interesting substrate for their biodegradable content (Chong *et al.*, 2009; Dong *et al.*, 2009; Guo *et al.* 2010, Lay *et al.*, 2012; Urbaniec and Bakker, 2015) and also for their non-competitive character with the food industry (Das and Veziroglu, 2008). Biomasses that are the best candidates to produce hydrogen from renewable resources must be rich in carbohydrates, abundant, available and cheap (Guo *et al.*,2010).

So far, dark fermentation studies have focused mainly on pretreatment (heat-, acid-, base-, chemical-treatments) of complex biomasses (Cheng *et al.*, 2011; Ntaikou *et al.*, 2010), and the on systematic addition of pretreated inocula (exogenous fermentation) (Ren *et al.*, 2008; Singh

and Wahid, 2015; Valdez-Vasquez *et al.*, 2006; Yin and Wang, 2016) and on the addition of macro- (N, P, S) and micro-nutrients (Fe, K, Mg, Ni, Ca, B, Mo, Zn, Co, Cu, Mn) to biomasses used as substrates (Hawkes *et al.*, 2007). Only few studies have used the endogenous microbial flora contained in the biomasses for hydrogen production by dark fermentation (endogenous fermentation) (Marone *et al.*, 2014; Noblecourt *et al.* 2018; Dauptain *et al.*, 2020; Asunis *et al.*, 2019).

Vine and wine by-products (with the exception of wine which is the fermentative product from vine) represent an annual world production of the order of several million tons and have already shown great potential for valorization strategies through the extraction of active compounds and/or the production of biogas (Martinez *et al.*, 2016). As a consequence of the numerous steps involved in winemaking, these by-products can be of various origins and natures, ranging from solid grape pomace after pressing, deposits from clarified musts, lees, to wastewater used for the different cleanings (Devesa-rey *et al.*, 2011). However, as far as energy is considered, most of the reported studies so far were focused on methane production at various scales (Fabbri *et al.*, 2015). Some studies already focused on grape pomace (Guo *et al.* 2014) and wastewater (Buitrón *et al.*, 2019) for hydrogen production by exogenous dark fermentation. In this study, we first investigated the feasibility of using grape must deposits (a winemaking biomass which corresponds to the deposits formed after must racking of grapes juice) as substrates for exogenous fermentation. Although not used so far for this purpose, such grape must deposits are rich in potentially easily metabolizable carbohydrates and may contain nutritional elements necessary for microbial consortium (Vidal *et al.*, 2000). We then studied the feasibility of using waste such biomass both as substrate and as microbial consortia to produce hydrogen by endogenous fermentation. The influence of heat-treatment was also investigated because of its beneficial effects (deactivation of hydrogenotrophic bacteria and selection and emergence of hydrogen producing bacteria). As regional wine characteristics and

grape variety exhibit specific wine grape microbiome (Bockulich *et al.*, 2013b), we further studied their influence on hydrogen production. These studies were complemented with biomolecular analyses to show the effect of different fermentation configurations on the composition of bacterial communities during dark fermentation.

2. Materials and Methods

2.1. Exogenous and endogenous fermentations

Two sets of experiments were carried out: exogenous and endogenous fermentations. Grape must deposits from two different grape varieties (Pinot Gris (PG) from Alsace and Chardonnay (Ch) from Burgundy) were used as biomass in both exogenous and endogenous fermentations. These deposits were collected from French wine producers after the static settlement of grape juice obtained after pressing freshly harvested grapes. They were stored at 4°C until use. Grape deposits are rich in carbohydrates (a mixture of glucose and fructose) with concentrations ranging between 90 and 230 g_{hexose}/L depending on the biomass. The grape deposits were diluted 1/14 in the bioreactor. Thus, the initial hexose concentration was between 6.4 and 16.4 g_{hexose}/L corresponding to 6.8 to 17.5 g eq. COD/L. Tartaric acid concentrations were 2.87 ± 0.05 g/L for Pinot Gris Grape deposits and 0.69 ± 0.23 g/L for Chardonnay grape deposits) and a smaller amount ethanol was also present in the initial medium. For Pinot Gris grape deposits, total solid (TS) content was 261 ± 1 $g_{TS}/kg_{biomass}$ with a volatile solid (VS) part of 172 ± 6 $g_{VS}/kg_{biomass}$. For Chardonnay grape deposits, total solid (TS) content was 328 ± 3 $g_{TS}/kg_{biomass}$ with a volatile solid (VS) part of 303 ± 3 $g_{VS}/kg_{biomass}$. In this study, exogenous fermentations consisted in adding external *inoculum* (0.65L) to the biomass (0.05L) used as substrate. Activated sludge (AS) from the anoxic zone of the local wastewater treatment plant was used as external *inoculum*. Activated sludge total solid content was 4.5 ± 0.5 g_{TS}/kg_{AS}. Endogenous fermentations involved using biomass (0.05L diluted in 0.65L tap

water) as the sole substrate, nutrients and *inoculum*. The fermentation experiments (exogenous and endogenous) were carried out for each biomass either with thermal pretreatment of the initial medium or without any thermal pretreatment.

No methane was detected (within the 0.01% (v/v) limit of detection) at the reactor outlet for both sets of experiments. This showed the effectiveness of our bioreactor device especially for extracting hydrogen produced by nitrogen gas sparging.

2.2. Bioreactor set-up

All experiments were based on the optimized working conditions of a 1 L semi-batch reactor with continuous gas extraction (Clion *et al.*, 2015); the substrate (grape must deposits) being added once at the beginning. The optimized operating parameters were: a mesophilic working temperature (37°C), a stirring speed set at 220 rpm, a nitrogen sweeping gas with a flow rate of 50 $mL.min^{-1}$, (controlled by a mass flow meter) to achieve anaerobic conditions and extract out produced gases. The working pH was kept over 5.7 and the initial pH adjusted at 7.0.

Dark fermentation experiments were realized with a 0.7 L working volume, with biomasses diluted to 1:14 with either external *inoculum* (exogenous fermentations) or tap water (endogenous fermentations). When heat-treatment (ht) was used (70°C for 1h), it was performed on the mix of substrate/microbial consortium without any gas flow and under static air.

During runs, samples were withdrawn and centrifuged; supernatants were filtrated over 0.2 µm filters. Then, supernatants and pellets were stored at -20°C prior to analysis.

2.3. Chemical analyses

Gas composition was measured online and analyzed with a micro-gas chromatograph (M200, Agilent) composed of two modules including two columns: the first is a 5Å molecular sieve

column and the second is a capillary column CP-PoraPLOT U using argon and helium as carrier gas, respectively. The micro-gas chromatograph is equipped with two thermal conductivity detectors.

The composition in alcohols and organic acids in the liquid phase, *i.e.*, ethanol, butanol, acetate, propionate, butyrate, isobutyrate, valerate and isovalerate was determined by injection of acidified supernatant into a gas chromatograph (7850A Agilent) equipped with a flame ionization detector and a DB-FFAP column.

Carbohydrates were measured using the anthrone-sulfuric acid method (Dreywood *et al.*, 1946). H_2 production performances were evaluated according to the H_2 production yield (Y_{H_2}) ($mol_{H_2}.mol^{-1}_{consumed\ hexose}$), H_2 cumulative production ($L_{H_2}.L^{-1}_{reactor}$) and H_2 productivity ($mmol.L^{-1}_{reactor}.h^{-1}$) which were determined using the modified Gompertz equation (Lay *et al.*, 1999).

$$H = H_m \cdot \exp \cdot \left\{ -\exp \cdot \left[\frac{R_m \cdot e}{H_m} (\lambda - t) + 1 \right] \right\}$$

where H is the cumulative H_2 production, H_m is the maximum cumulative H_2 production ($mmol.L^{-1}_{reactor}$), R_m is the H_2 productivity ($mmol.L^{-1}_{reactor}.h^{-1}$), λ is the lag time (h), t is the incubation time and e is exponential function of 1.

2.4. DNA extraction and PCR amplification

Microbial cells were collected after the centrifugation of ten milliliters of liquid. Genomic DNA was extracted using E.Z.N.A. Soil DNA kit and following the manufacturer's instructions (Omega). Quantification of genomic DNA was performed using Quant-iT™ PicoGreen® (Invitrogen).

Bacterial 16S rRNA gene fragments were PCR-amplified using 6-carboxyfluorescein (6-FAM) labelled 27f primer (5'- AGAGTTTGATCMT GGCTCAG – 3') (Weisburg *et al.*, 1991) and 927r primer 5'- CCGTCAATTCCTTTGAG TTT -3' (Schwieger *et al.*, 1998) were used for T-RFLP

analysis.

PCR mixtures contained 10 µL of iProof 1X buffer (Bio-Rad), 200 µM of each deoxynucleoside triphosphate (dNTP), 0,8 µM of each primer, 1U of iProof Taq polymerase (Bio-Rad) and 50 ng of DNA. Reaction tubes were placed in a thermal Master-cycler (Eppendorf) that was preheated at 98°C before amplification. PCR amplification involved an initial denaturation at 98°C for 2 min, followed by 20 cycles of denaturation at 98°C for 30 s, annealing at 52°C for 30 s, and extension at 72°C for 30 s and then a final 2min extension at 72°C. PCRs were carried out in triplicate for each DNA sample and primer pair, and replicates were pooled and stored at 4°C until they were used. Bacterial 16S rRNA gene fragments (0.9 kb) were then concentrated with a vacuum SpeedVac Concentrator (Savant) to obtain 30 µL final volume before being dropped off in a 1% agarose gel. Bands obtained were then purified with the Geneclean® Turbo kit (Mp) according to the manufacturer's recommendations.

2.5. T-RFLP analysis

T-RFLP analysis was realized as described by Penny *et al.* (2010). DNA samples were digested at 37°C for 16 h with 20 U AluI (AGCT) or HhaI (GCGC) (Fermentas), purified with a QIAquick gel extraction kit (Qiagen) used according to the manufacturer's recommendations. 6 µL of digested and purified samples were added to 12 µL of a mix containing Hi-Di™ formamide (Applied Biosystems), 1:20 (vol/vol) 6-FAM- or carboxy-X-rhodamine (ROX)-labeled MapMarker 1000 (Bioventures). The mix was then denatured at 95°C for 5 min and snap-cooled on ice for 5 min before being centrifuged at 12000 rpm for 15 min at 4°C. Denatured restriction fragments were size separated by capillary electrophoresis using an ABI Prism 3130 XL Genetic Analyzer (Applied Biosys-tems). T-RFLP electropherograms were analyzed with the PeakScanner V1.0 software (Applied Biosystems).

2.6. Sequencing

Genomic DNA sample of interest was sequenced. A portion of the 16S rRNA was amplified using the barcoded, universal primer set (515WF/918WR) (Wang and Quian, 2009). Pools were submitted for sequencing on Illumina MiSeq instrument at GeT-PlaGe (Auzeville, France). Sequences were processed using Mothur (version 1.36.1) according to MiSeq SOP pipeline (Schloss *et al.*, 2009).

Barcodes, primers, and sequences showing homopolymers of more than 8bp were discarded. Sequences showing 100% homology were grouped in unique sequences, then in OTUs (operational taxonomic unit, based on 97% homology). Sequences were then assigned to match to a sequence in SILVA (version 123) and PR2 databases for procaryotes and eucaryotes respectively to identify the genus level. A total of 23948 procaryotes sequences were obtained for the sample of interest.

3. Results and discussion

3.1. Endogenous bacteria from grape must deposits produce substantial hydrogen

Grape must residues from two grape varieties, Pinot Gris (PG) and Chardonnay (Ch), were firstly tested as substrate added to activated sludge (AS) used as external *inoculum* (exogenous fermentation) (Fig. 1a). The effect of performing heat-treatment on the mixture (biomass/*inoculum* in the case of exogenous fermentation or biomass/tap water in the case of endogenous fermentation) was also assessed.

The highest hydrogen volume produced by exogenous fermentation was obtained with Pinot Gris grape must deposits with heat-treatment: 2.4 $L_{H_2}.L_{reactor}^{-1}$ (Fig. 1a). This value was not significantly ($p > 0.05$; t-test) different from 2.1 $L_{H_2}.L_{reactor}^{-1}$ obtained from the other grape variety (Chardonnay). Hydrogen productions from the exogenous fermentation of grape must deposits increased from 0.5 to 2.4 $L_{H_2}.L_{reactor}^{-1}$ for Pinot Gris and from 0.7 to 2.1 $L_{H_2}.L_{reactor}^{-1}$ for

Chardonnay when heat-treatment was performed. It is also worthy to mention that using grape must deposits without heat-treatment produced significant volumes of hydrogen. Surprisingly, cumulative hydrogen productions obtained by endogenous fermentation (Fig. 1b) were higher (3.6 and 2.6 $L_{H_2}.L_{reactor}^{-1}$ for Pinot Gris and Chardonnay, respectively) than those obtained by exogenous fermentation (Fig. 1a). This shows the remarkable efficiency of microbial consortia indigenous to grape must media, which are selected by heat-treatment compared to an external *inoculum* (AS). Unexpected high hydrogen production was also obtained without heat-treated grape must deposits (2.8 and 2.3 $L_{H_2}.L_{reactor}^{-1}$ for Pinot Gris and Chardonnay, respectively), about 3-6 times higher than those obtained in unheated exogenous fermentations. Short lag times observed in unheated compared to heat-treated endogenous fermentations, as well as a quite complete sugar degradation to hydrogen (>97%), also suggest an adaptation of endogenous microbial consortia to dark fermentation conditions. It is also noteworthy that none of the fermentation tests were added with nutrients, which indicates that biomasses are not only rich in hydrogen producers but also rich in nutrients necessary for bacterial growth. Consequently, hydrogen can be produced from endogenous fermentation of grape must deposits without the need for an energetically expensive heat-treatment.

3.2. Hydrogen production performances

Highest hydrogen productivities were obtained from heat-treated exogenous fermentations: 8.91 and 8.18 $mmol.L^{-1}_{reactor}.h^{-1}$ for Chardonnay and Pinot Gris grape must deposits, respectively (Fig. 2a). Hydrogen productivities obtained from endogenous fermentations (2.97 and 3.80 $mmol.L^{-1}_{reactor}.h^{-1}$ for Chardonnay and Pinot Gris grape must deposits, respectively) and from heat-treated endogenous fermentations (1.78 and 5.05 $mmol.L^{-1}_{reactor}.h^{-1}$ for Chardonnay and Pinot Gris grape must deposits, respectively) were lower than those obtained by exogenous fermentations. This is consistent with the size of the microbial source which is

fourteen times smaller in endogenous fermentation (0.05L of grape must deposits diluted in 0.65L of tap water) than in exogenous fermentation (0.05L of grape must deposits diluted in 0.65L of activated sludge). The discrepancy between values obtained from the two different grape varieties (especially for exogenous fermentation without heat treatment and heat-treated endogenous fermentations) could be explained by (1) a difference of resistivity of indigenous bacterial consortia to heat-treatment and (2) possibly a different interaction (competition) between the exogenous *inoculum* and the indigenous bacterial populations of the biomass. Indeed, to support the first point, it seems that hydrogen producers from the Chardonnay grape variety are less heat-resistant than those from the Pinot Gris variety. This could explain the lower hydrogen productivity (1.78 $mmol.L^{-1}_{reactor}.h^{-1}$) obtained by heat-treated endogenous fermentation of Chardonnay grape must deposits compared to intermediate values obtained by heat-treated endogenous fermentation of Pinot Gris grape must deposits (5.05 $mmol.L^{-1}_{reactor}.h^{-1}$) whereas unheated endogenous fermentations of Pinot Gris and Chardonnay grape must deposits present quite similar hydrogen productivity (3.80 and 2.97 $mmol.L^{-1}_{reactor}.h^{-1}$, respectively). To support the second point, it seems that the use of the exogenous non-pretreated *inoculum* has a positive effect on the hydrogen productivity for Chardonnay grape must deposits, with likely a collaborative effect between the exogenous and indigenous bacterial communities (3.0 and 4.5 $mmol.L^{-1}_{reactor}.h^{-1}$ for exogenous and endogenous fermentations, respectively). Contrastingly, in the case of Pinot Gris grape must deposits, an inhibitory effect is observed with a hydrogen productivity divided by 2 between endogenous (3.8 $mmol.L^{-1}_{reactor}.h^{-1}$) and exogenous (1.4 $mmol.L^{-1}_{reactor}.h^{-1}$) fermentation experiments.

Highest hydrogen yields (in moles of hydrogen produced per mole of hexose consumed: $mol.mol^{-1}$) (Fig. 2b) were obtained from heat-treated exogenous and endogenous

fermentations with both Chardonnay and Pinot Gris varieties, and from Pinot Gris endogenous fermentation: 2.0 ± 0.12 $mol.mol^{-1}$ on average. The value obtained from Pinot Gris endogenous fermentation (1.30 $mol.mol^{-1}$) was significantly lower ($p < 0.02$; t-test) than the corresponding Chardonnay value (1.93 $mol.mol^{-1}$) but still higher than hydrogen yields obtained from exogenous fermentations (0.83 vs 0.48 $mol.mol^{-1}$ for Chardonnay and Pinot Gris varieties, respectively). Association of low hydrogen yields with low cumulative hydrogen volumes (such as exogenous fermentations of Pinot Gris and Chardonnay grape must deposits) indicates the presence of hydrogenotrophic bacteria with or without bacteria outcompeting for the substrate (to a lesser extent); while association of low hydrogen yields with high cumulative hydrogen volumes (such as endogenous fermentations of Pinot Gris grape must deposits) indicates the presence of bacteria outcompeting for the substrate with or without hydrogenotrophic bacteria (to a lesser extent).

Initial soluble sugars concentrations in the bioreactor (Fig. 2b), mainly composed of glucose and fructose, ranged between 6.8 - 17.5 $g_{eq.\ COD}.L^{-1}$. Differences of concentrations can be explained by the fact that biomasses were stored at 4°C after collections in wineries. This shows that the biomass storage at 4°C was not efficient enough to prevent maturation. Indeed, although all the tests were carried out over 36 days, a loss of half of the initial sugar content for the Pinot Gris and 30% for the Chardonnay was observed in 11 and 17 days of storage, respectively.

3.3. Metabolism

The mass balance of soluble chemical organic demand (COD) was calculated for each test using the final equivalent COD (from hydrogen and major metabolites produced) and the initial equivalent COD (from initial major metabolites and sugars) (Fig. 3). The mass balance was incomplete due to an analysis of only major metabolites produced and due to the bacterial

growth not evaluated in this study. Soluble metabolites and hydrogen produced obtained for each test are represented in figure 3. Acetate and butyrate were the major metabolites produced but in different quantities for all fermentations except for exogenous fermentations for which propionate was co-dominant with acetate. Figure 4 confirmed that heat-treatment applied on external *inoculum* inactivated propionate producing bacteria. Indeed, high amounts of propionate were produced during exogenous fermentation (0.35 ± 0.15 $mol_{propionate}.mol^{-1}_{initial\ hexose}$ on average) accompanied with a low production yield of hydrogen (0.7 ± 0.2 $mol_{H2}.mol^{-1}_{initial\ hexose}$ on average) compared to heat-treated exogenous fermentations (0.01 ± 0.01 $mol_{propionate}.mol^{-1}_{initial\ hexose}$ on average and 2.1 ± 0.1 $mol_{H2}.mol^{-1}_{initial\ hexose}$ on average). This is in accordance with equation (1) and explains low H_2/CO_2 ratios obtained for exogenous fermentations (0.33 ± 0.13 on average) compared to heat-treated exogenous fermentations (0.87 ± 0.11 on average). Besides, if we consider that hydrogen was produced via the acetate (2) and butyrate (3) pathways, it is possible to calculate the quantity of hydrogen produced from acetate and butyrate concentrations: 2*(butyrate + acetate); and to compare it to the measured production of hydrogen (H_2). In exogenous fermentations, calculated H_2/2*(butyrate + acetate) ratios were lower than 1 and were due to the consumption of a part of hydrogen for the production of propionate. Other hydrogenotrophic pathways such as homo-acetogenesis (4) or the sulfate-reducing pathway (5) could also be used. This was in accordance with the association of low hydrogen yields and low cumulative hydrogen volumes obtained from exogenous fermentations.

$$C_6H_{12}O_6 + 2H_2 \rightarrow 2CH_3CH_2COOH + 2H_2O \quad (\Delta G'_0 = -279.4\ kJ.mol^{-1}) \tag{1}$$

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2 \quad (\Delta G'_0 = -206.3\ kJ.mol^{-1}) \tag{2}$$

$$C_6H_{12}O_6 \rightarrow CH_3CH_2CH_2COOH + 2CO_2 + 2H_2 \quad (\Delta G'_0 = -254.8\ kJ.mol^{-1}) \tag{3}$$

$$2CO_2 + 4H_2 \rightarrow CH_3COOH + 2H_2O \quad (\Delta G'_0 = -104\ kJ.mol^{-1}) \tag{4}$$

$$SO_4^{2-} + 4H_2 + 2H^+ \rightarrow H_2S + 4H_2O \quad (\Delta G'_0 = -152.2\ kJ.mol^{-1}) \tag{5}$$

When endogenous bacterial consortia were used, heat-treatment had only little effect on produced metabolites, while grape variety had an impact (Fig. 3). Indeed, heat-treatment applied on Chardonnay grape must deposits reduced 3 times the butyrate production without altering the production of hydrogen. H_2/CO_2 ratio became higher than 1 with heat-treated endogenous fermentation (1.08) compared to 0.88 obtained with endogenous fermentation (without heat-treatment). Heat-treatment applied on Pinot Gris grape must deposits increased the concentrations of acetate and butyrate by a factor 8.9 and 1.3 respectively, accompanied with 1.4 times increase of the hydrogen production. Besides, as no propionate was produced without heat-treatment during endogenous fermentations, this means that propionate producing bacteria found in exogenous fermentations originated from the external *inoculum* (activated sludge) and not from the biomass (grape must deposits).

While there was little difference between metabolites and gases produced during heat-treated exogenous fermentations (typically used in the literature) and endogenous fermentation from Chardonnay grape must deposits; the Pinot Gris grape variety showed differences. The major one came from the amount of acetate produced which was 56 times lower in endogenous fermentation than in heat-treated exogenous fermentation. The high $H_2/2$*(butyrate + acetate) ratio calculated for endogenous fermentation from Pinot Gris grape must deposits (2.2) meant that acetate was consumed for the production of other metabolites. Moreover, low hydrogen yield obtained for this test without low cumulative volume of hydrogen. Thus, for equivalent hydrogen productions, distinct metabolites patterns could be identified according to the grape variety: butyrate pathway for Pinot Gris, and acetate and butyrate pathways for Chardonnay grape must deposits. More extensively, this suggests regional wine characteristics can have an influence on nutrients contents of the biomass leading to either different wine grape microbiomes and/or different metabolic pathways.

3.4. Evolution of bacterial communities during fermentation

Samples were withdrawn at different durations of fermentation (Fig. 5). The comparison of bacterial compositions assessed by T-RFLP before and during endogenous fermentation ~~(Fig. 5)~~ exhibited some selection and emergence of bacterial hydrogen producers during operating conditions of the bioreactor. Major terminal-restriction fragments (T-RF) were analyzed using our internal software and compared to the database from www.trflp.info (2013). T-RF couples clearly identified are indicated in Table 1. T-RFLP profiles revealed a moderate bacterial diversity and richness (Table 2) represented by one dominant population: 74 ± 2 bp for the AluI profile and 374 ± 3 bp for the HhaI profile with relative abundances of 67% and 61% respectively; and few minor populations with relative abundances below 15%. At 20h of hydrogen production, the bacterial composition changed with a decrease of the relative abundance of the initial major population and an emergence of the phylotype γ (firmicutes). This bacterial phylotype was then preserved until a time of fermentation ranging between 28h and 54h. T-RFLP profiles showed moderate richness and bacterial diversity which allow bacterial hydrogen producers to be selected during semi-batch bioreactor operation. At 54h, several new populations emerged, implying a change in richness and bacterial diversity. While in endogenous fermentation, moderate richness and bacterial diversity of the grape must deposits microbiome enabled the selection and emergence of hydrogen producers; in exogenous fermentation, T-RFLP profiles (~~Fig. 6)~~ revealed the need of using heat-treatment to reduce the larger bacterial diversity and richness (Table 2) related to the activated sludge (Zhang *et al.*, 2012). From 5h to 28h exogenous fermentation, two bacterial populations emerged, including the phylotype γ representative of Firmicutes phylum, and including the Clostridium genus.

3.5. Comparison of bacterial communities during endogenous and exogenous fermentations

T-RFLP profiles showed one or two major phylotypes (Table 1): α (*Clostridium* in Chardonnay grape must deposits) and γ (*Firmicutes* phylum including *Clostridium* genus, in Chardonnay and Pinot Gris grape must deposits) when heat-treatment was carried out in exogenous fermentations in contrast, for untreated fermentations where bacterial communities were complex with sub-dominant populations (relative abundance below 27%). Besides, *Clostridium butyricum* was also identified as a minor phylotype (β) in heat-treated exogenous fermentations of Chardonnay grape must deposits. This confirmed that heat-treatment inactivated hydrogenotrophic microbial populations (in exogenous fermentation) which explains low hydrogen yields and volumes obtained by untreated exogenous fermentations. Heat-treatment also favored *Clostridium* known to produce hydrogen but never identified so far in winemaking (Bokulich *et al.*, 2013a, 2013b; David *et al.*, 2014; Grangeteau *et al.*, 2016, 2017). An important outcome is therefore that our bioreactor conditions disable the growth of classical bacterial consortia for winemaking in favor of bacterial hydrogen producers.

Unlike exogenous fermentations, heat-treatment had only little or no effect on bacterial populations of endogenous fermentations. Indeed, with or without heat-treatment, T-RFLP profiles showed the phylotype γ as dominant with relative abundance above 50%, one sub-dominant population with relative abundance ranging between 17 to 38% and few minor populations with relative abundances below 13%. This can be explained by the fact that endogenous bacteria composing grape must deposits are heat-resistant.

Similar T-RFLP profiles obtained between heat-treated exogenous, endogenous and heat-treated endogenous fermentations for Chardonnay and Pinot Gris varieties indicated that grape must deposits contain moderate microbial diversity (table 2) which does not need to be

reduced by heat-treatment for the selection of hydrogen producers unlike the activated sludge.

3.6. Sequencing

Unlike T-RFLP fingerprinting which enables to follow the time evolution of a bacterial community, the sequencing assumes a classification of taxonomic families. It was realized from the 16S rRNA sequences obtained from genomic DNA extracted from samples withdrawn at 24h during Pinot Gris endogenous fermentation. Within the *Firmicutes* phylum (94.0%), 64.7% belonged to *Clostridiaceae,* 15.8% belonged to *Enterococcaceae* and 13.3% belonged to *Sporolactobacillaceae;* only 5.6% belonged to the *Gammaproteobacteria* phylum and more precisely to the *Enterobacteriaceae* family. Affiliation to sequences of main OTUs (Table 3) confirmed that *Clostridium* spp. *(butyricum, beijerinckii, diolis, roseum)* was the dominant phylotype (33.7% relative abundance) during Pinot Gris endogenous fermentation. Other subdominant phylotypes were: *Clostridium* sp., *Enterococcus villorum* and *Sporolactobacillus laevolacticus* with 19.9%, 15.8% and 13.2% relative abundances respectively. Main minor phylotypes were *Clostridium pasteurianum* (9.2%), *Klebsiella/Enterobacter/Proteus* (5.6%) and *Clostridium intestinale* (1.5%). The main dominant phylotype, *Clostridium* spp. *(butyricum, beijerinckii, diolis, roseum)* was previously reported to produce hydrogen (Davila-Vazquez *et al.*, 2007; Koskinen *et al.*, 2007). It is also consistent with the butyrate production during Pinot Gris grape must deposits endogenous fermentation.

Considering the metabolites analyses along with the association of a low hydrogen yield and a high cumulative hydrogen volume obtained for this test revealed the presence of bacteria outcompeting for sugar. Since uncomplete mass balance of COD was calculated and lactic phylotypes such as *Enterococcus villorum* and *Sporolactobacillus laevolacticus* were identified in this test, the lactate pathway is presumed to outcompete with *Clostridium* spp. for glucose

to produce lactate. It must be noted that lactic and enteric phylotypes could also play a role in the hydrogen production (Rafrafi *et al.*, 2013).

Thus, even if the heat treatment generally leads to a selection of heat resistant bacteria such as *Clostridium* known for their spore-forming capacity as well as their hydrogen production potential (Kan, 2013), this study shows that using grape must residues as both substrate and *inoculum* for hydrogen production by dark fermentation results in the emergence of *Clostridium* (probably initially present in the biomass at very low concentrations) even if no heat treatment was performed before fermentation.

3.7. Comparison of hydrogen production performances

Comparison with the literature clearly shows the potential for the use of grape must deposits in heat-treated exogenous fermentation. Indeed, the hydrogen yield (2.26 ± 0.07 $mol_{H2}.mol_{consumed\ hexose}^{-1}$), the hydrogen production (2.2 ± 0.2 $L_{H2}.L_{reactor}^{-1}$) and the hydrogen production rate (205 ± 12 $mL_{H2}.L_{reactor}^{-1}.h^{-1}$) were respectively seven, twenty-two and ten times those obtained by Hwang *et al.* (2011) when they used ripened grape slurry in heat-treated exogenous fermentation (0.1 $mol_{H2}.mol_{consumed\ hexose}^{-1}$; 0.31 $L_{H2}.L_{reactor}^{-1}$ and 19 $mL_{H2}.L_{reactor}^{-1}.h^{-1}$, respectively) and slightly over results obtained by Montoya-Rosales *et al.* (2019) for the fermentation of a hydrolyzed agave bagasse (0.92 $mol_{H2}.mol_{hexose}^{-1}$; 1.57 $L_{H2}.L_{reactor}^{-1}$ and 139 $mL_{H2}.L_{reactor}^{-1}.h^{-1}$, respectively). However, our results were consistent with these authors when they used ripened apple slurry (2.1 $mol_{H2}.mol_{consumed\ hexose}^{-1}$; 4.24 $L_{H2}.L_{reactor}^{-1}$ and 115.4 $mL_{H2}.L_{reactor}^{-1}.h^{-1}$, respectively) or with Sivagurunathan *et al.* (2014) when they used industrial beverage wastewater (1.92 $mol_{H2}.mol_{consumed\ hexose}^{-1}$; 2.4 $L_{H2}.L_{reactor}^{-1}$, respectively for hydrogen yield and hydrogen production). In endogenous fermentation, our results were six, ten and four times higher respectively in hydrogen production (2.5 ± 0.4 $L_{H2}.L_{reactor}^{-1}$), hydrogen yield (1.61 ± 0.44 $mol_{H2}.mol_{consumed\ hexose}^{-1}$ or 226 $mL_{H2}.g_{VS}^{-1}$) and hydrogen production rate (81 ± 14

$mL_{H2}.L_{reactor}^{-1}.h^{-1}$) in comparison with Marone *et al.* (2014) who used self-fermentation of vegetable wastes (0.4 $L_{H2}.L_{reactor}^{-1}$; 24 $mL_{H2}.g_{VS}^{-1}$ and 19.6 $mL_{H2}.L_{reactor}^{-1}.h^{-1}$, respectively).

4. Conclusion

The feasibility of using grape must deposits as substrate for an exogenous *inoculum*, selected after heat-treatment was demonstrated. For similar hydrogen productions, both the regional characteristics and the grape variety can impact hydrogen production pathways. Beneficial effects (emergence of bacterial hydrogen producers) obtained by performing heat-treatment were demonstrated through the decrease of bacterial diversity and increase of hydrogen production.

It was clearly demonstrated that without heat-treatment, grape must residues, used as both substrate and microbial consortium in endogenous fermentation, contain an adapted endogenous microflora composed of *Clostridiaceae* able to degrade the biomass to hydrogen, as efficiently as an external heat-treated *inoculum*.

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Appendix: supplementary data

Supplementary data for this work can be found in e-version of this paper online

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Author contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

CRediT authorship contribution statement

Emilie François: Investigation, Methodology, Writing - original draft, Christine Dumas: Methodology, Validation, Writing-Review, Régis Gougeon: Conceptualization, Resources, Writing-Review, Hervé Alexandre: Resources, Writing-Review, Stéphane Vuilleumier: Resources, Writing-Review, Barbara Ernst: Conceptualization, Supervision, Writing-Review & Editing.

Figure captions

Fig. 1 | Evolution of cumulative volume of hydrogen produced from a Exogenous fermentation, in presence of external activated sludge (AS) or b Endogenous fermentation of Pinot Gris (PG) and Chardonnay (Ch) grape must deposits with heat-treatment (ht) or without.

Fig. 2 | Hydrogen productivity (a) and hydrogen yield (b) obtained from Chardonnay (in full pattern) and Pinot Gris grape must deposits (in checked pattern); the initial sugars concentration is represented by circles (blue for Chardonnay; orange for Pinot Gris).

Fig. 3 | Mass balance of soluble chemical organic demand for the fermentation of Chardonnay (a) and Pinot Gris (b) grape must deposits fermentations.

Fig. 4 | Metabolites and gas produced during fermentations from a Chardonnay and b Pinot Gris grape residues.

Fig. 5 | Evolution of gas flow rate as a function of time ~~(a) and bacterial communities (b)~~ obtained from endogenous fermentation of Pinot Gris grape must deposits (a) and from exogenous fermentation of Pinot Gris grape must deposits with heat-treatment (b). Arrows correspond to samples withdrawn after different duration of fermentation. ~~Electrophoregrams correspond to samples withdrawn initially and after different duration of fermentation 20h, 24h, 28h and 54h. Colored triangles referred to the terminal-restriction fragment (T-RF) length in base pair units.~~

~~Fig. 6 | Evolution of gas flow rate (a) and bacterial communities (b) obtained from exogenous fermentation of Pinot Gris grape must deposits with heat-treatment. Electrophoregrams correspond to samples withdrawn initially, after heat-treatment and and after different duration of fermentation 5h, 20h, 28h and 43h. Colored triangles referred to the terminal-restriction fragment (T-RF) length in base pair units.~~

Figures

Fig. 1

a b

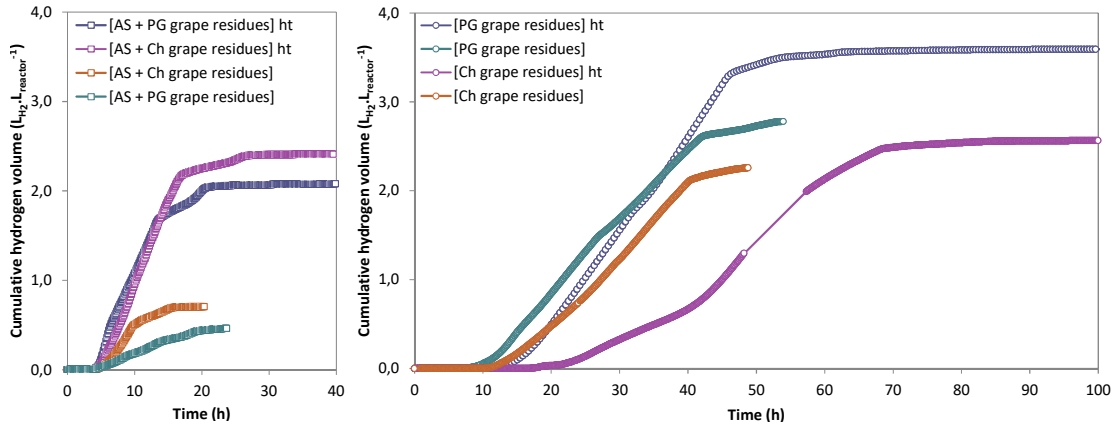
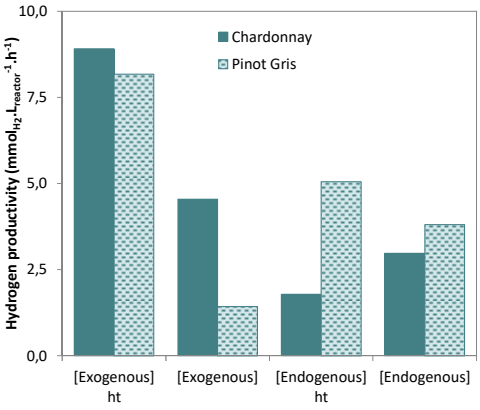


Fig. 2

a


Hydrogen productivity (mmol$_{H_2}$.L$_{reactor}^{-1}$.h^{-1})
10,0
7,5
5,0
2,5
0,0
Chardonnay
Pinot Gris
[Exogenous] ht
[Exogenous]
[Endogenous] ht
[Endogenous]

b

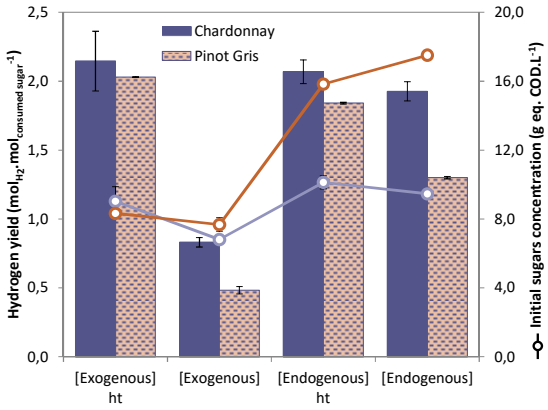

Hydrogen yield (mol$_{H_2}$.mol$_{consumed\ sugar}^{-1}$)
2,5
2,0
1,5
1,0
0,5
0,0
Initial sugars concentration (g eq. COD.L^{-1})
20,0
16,0
12,0
8,0
4,0
0,0
Chardonnay
Pinot Gris
[Exogenous] ht
[Exogenous]
[Endogenous] ht
[Endogenous]

Fig. 3

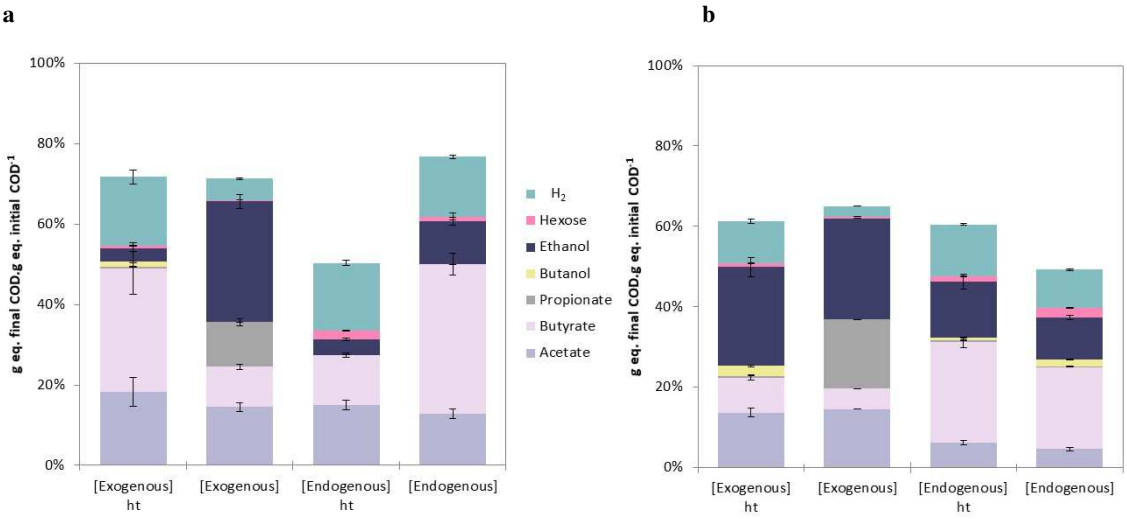

a
b
100%
80%
60%
40%
20%
0%
g eq. final COD.g eq. initial COD-1
[Exogenous] ht
[Exogenous]
[Endogenous] ht
[Endogenous]
H2
Hexose
Ethanol
Butanol
Propionate
Butyrate
Acetate

Fig. 4

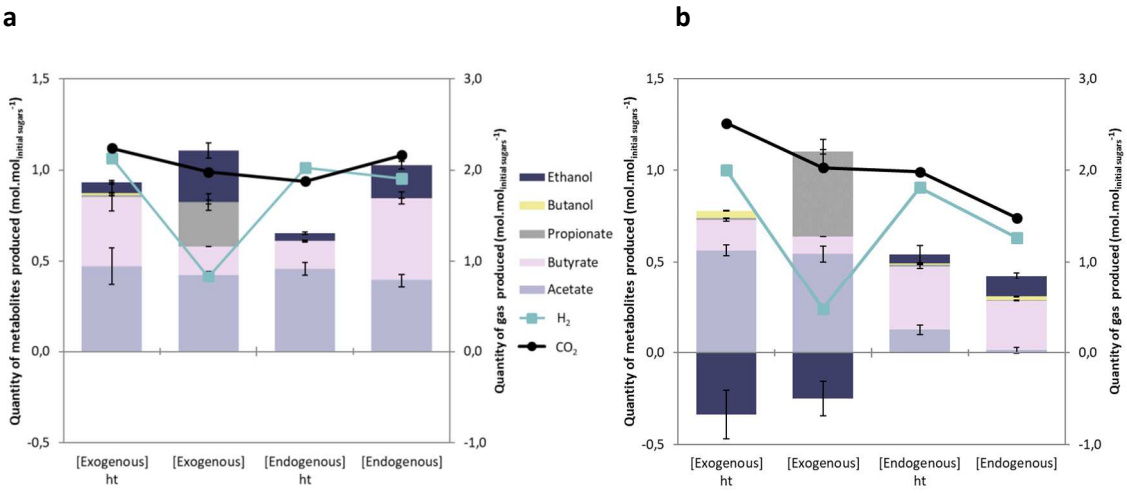
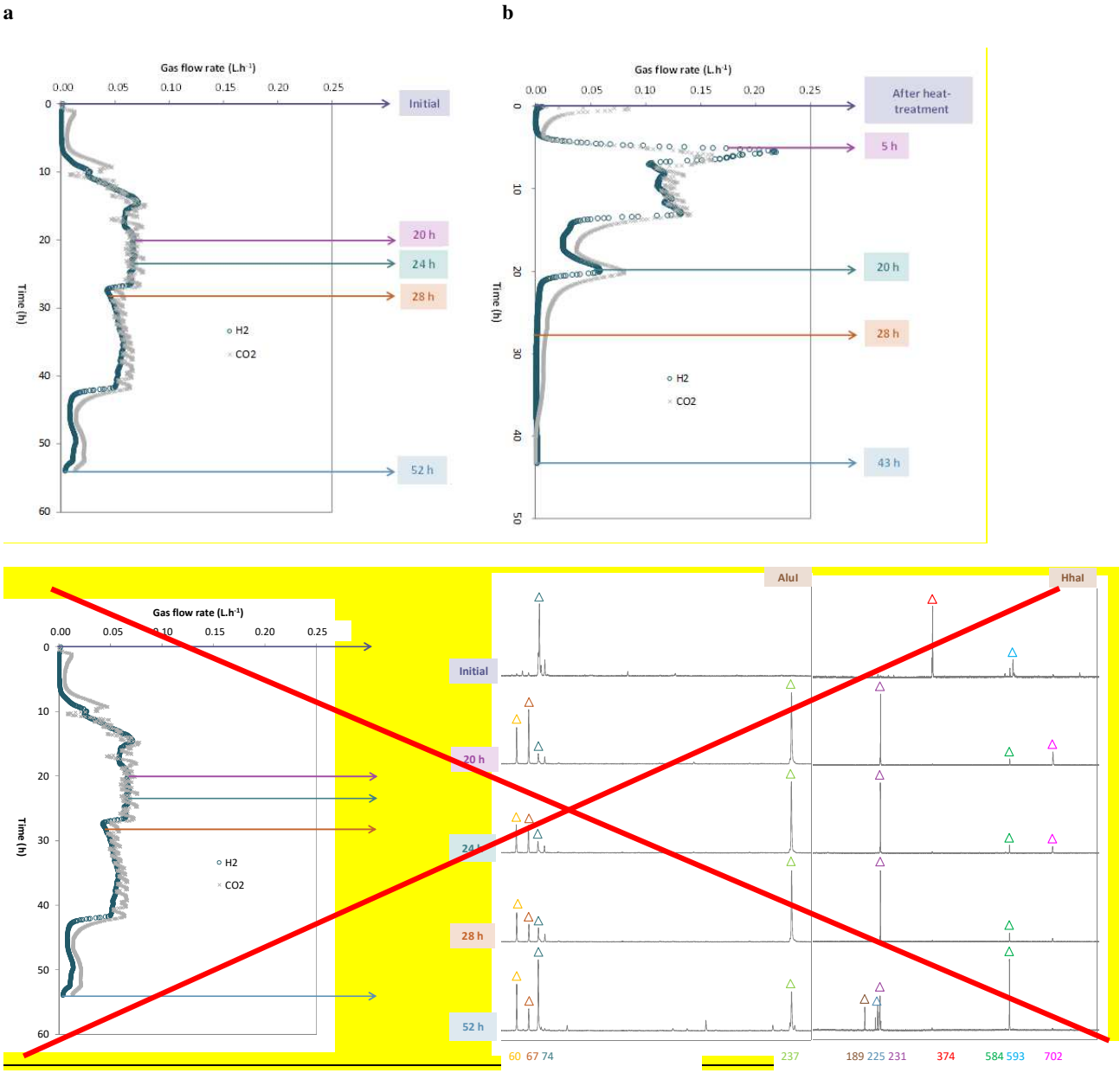
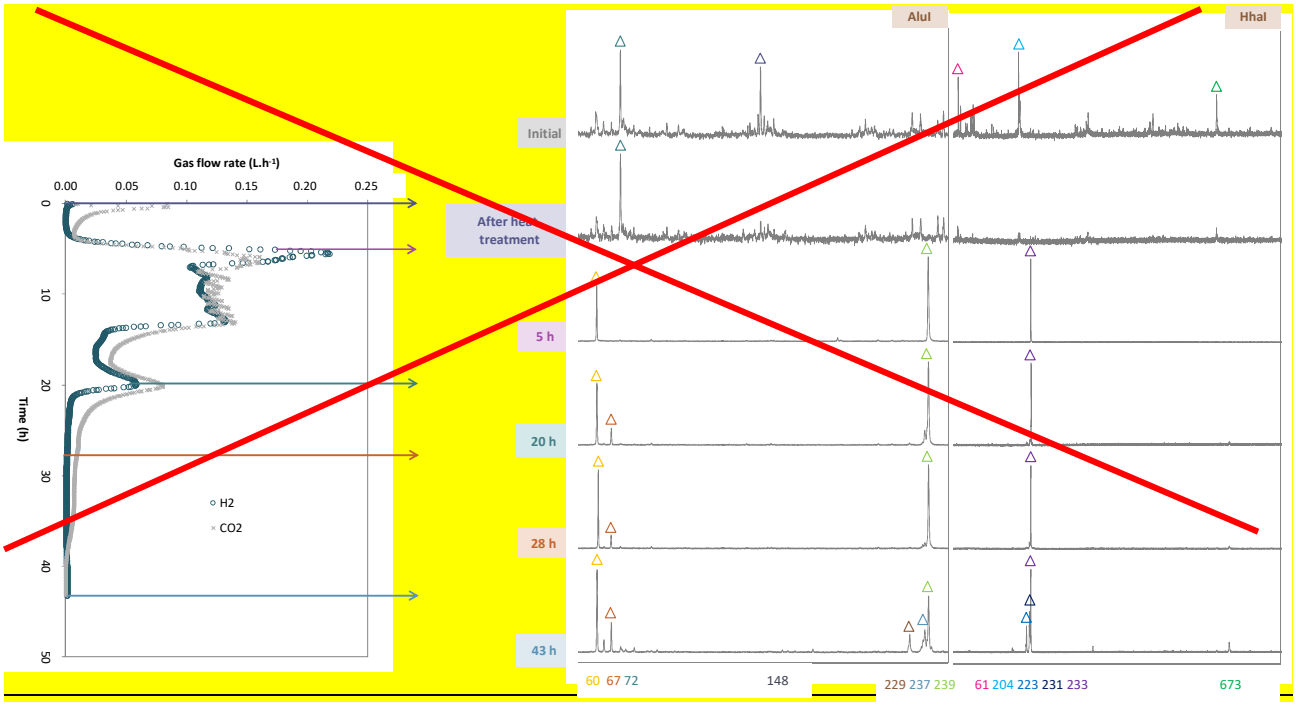

a
b
1,5
1,0
0,5
0,0
-0,5
3,0
2,0
1,0
0,0
-1,0
Quantity of metabolites produced (mol.mol$_{initial\ sugars}^{-1}$)
Quantity of gas produced (mol.mol$_{initial\ sugars}^{-1}$)
Ethanol
Butanol
Propionate
Butyrate
Acetate
H_2
CO_2
[Exogenous] ht
[Exogenous]
[Endogenous] ht
[Endogenous]

Fig. 5


a
b
Gas flow rate (L.h-1)
0.00 0.05 0.10 0.15 0.20 0.25
Time (h)
Initial
20 h
24 h
28 h
52 h
H2
CO2
After heat-treatment
5 h
43 h
AluI
HhaI
60 67 74
237
189 225 231
374
584 593
702

~~Fig. 6~~

~~a~~ ~~b~~


Gas flow rate (L.h-1)
0.00
0.05
0.10
0.15
0.20
0.25
0
10
20
30
40
50
Time (h)
H2
CO2
Initial
After heat treatment
5 h
20 h
28 h
43 h
AluI
HhaI
60 67 72
148
229 237 239
61 204 223 231 233
673

Tables

Table 1 | Identification of major T-RF to bacterial phylotypes

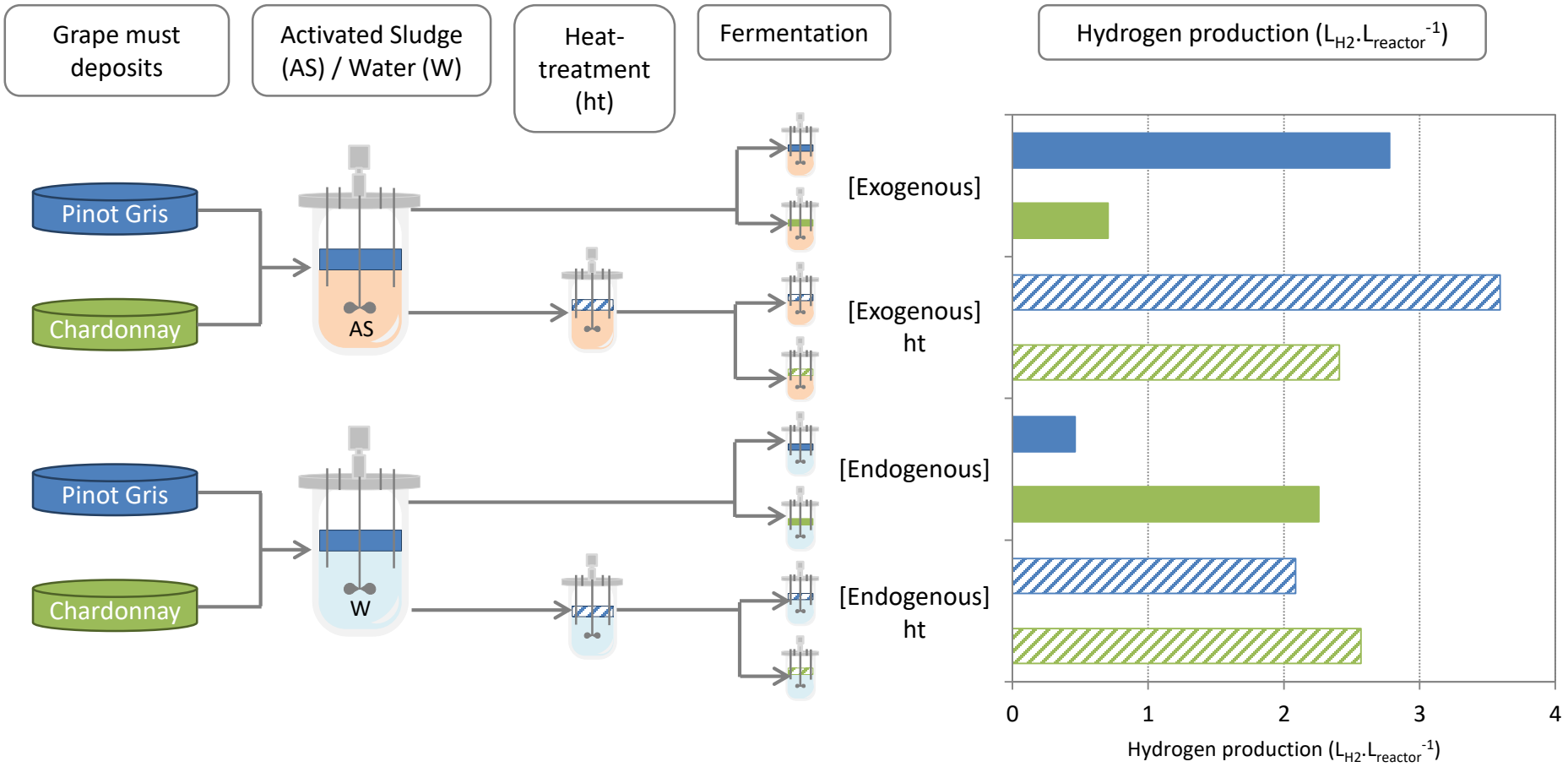
T-RF	AluI	HhaI	Phylotype
α	67 ± 2 bp	231 ± 3 bp	*Clostridium*
β	67 ± 2 bp	702 ± 3 bp	*Clostridium butyricum*
γ	237 ± 3 bp	231 ± 3 bp	*Firmicutes* including *Anaerobacter, Bacillus, Brevibacillus, Clostridium, Eubacterium, Lactobacillus* and *Pasteuria* gena

Table 2 | Bacterial diversities according to electrophoregrams

		AluI		HhaI	
		Shannon	Richness	Shannon	Richness
Chardonnay	heat-treated exogenous fermentation	1.05	3	1.23	4
	exogenous fermentation	2.27	13	0.00	1
	heat-treated endogenous fermentation	0.98	3	0.81	3
	endogenous fermentation	1.19	6	0.73	6
Pinot Gris	heat-treated exogenous fermentation	1.03	10	2.55	17
	exogenous fermentation	1.33	8	2.02	10
	endogenous fermentation	1.52	9	0.90	5
Pinot Gris heat-treated exogenous fermentation	initial	1.24	4	2.47	13
	after heat-treatment	0.54	2	0.00	0
	5h	1.03	10	2.55	17
	20h	1.22	6	0.00	1
	28h	0.99	4	0.54	6
	43h	1.87	11	1.85	10
Pinot Gris endogenous fermentation	initial	1.05	5	1.16	5
	20h	1.52	9	0.90	5
	24h	1.44	7	0.81	5
	28h	1.43	7	0.84	7
	54h	2.02	15	1.80	9

Table 3 | Phylogenetic affiliation of bacterial 16S rRNA sequences obtained by sequencing from sample withdrawn at 24h from endogenous fermentation of Pinot Gris grape residues

Closely relative to	Sequence identity (%)	Relative abundance (%)
Clostridium spp. *(butyricum, beijerinckii, diolis, roseum)*	99.5 %	33.7 %
Clostridium sp.	98.7 %	19.9 %
Enterococcus villorum	98.7 %	15.8 %
Sporolactobacillus laevolacticus	100.0 %	13.2 %
Clostridium pasteurianum	100.0 %	9.2%
Klebsiella spp. *(oxytoca, michiganensis, pneumoniae), Enterobacter cancerogenus, Proteus vulgaris*	99.7%	5.6%
Clostridium intestinale	99.7%	1.5%


Grape must deposits
Activated Sludge (AS) / Water (W)
Heat-treatment (ht)
Fermentation
Hydrogen production ($L_{H2}.L_{reactor}^{-1}$)
Pinot Gris
Chardonnay
AS
W
[Exogenous]
[Exogenous] ht
[Endogenous]
[Endogenous] ht
0
1
2
3
4
Hydrogen production ($L_{H2}.L_{reactor}^{-1}$)