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► To cite this version:

Marie Renaudie, Christine Dumas, Stéphane Vuilleumier, Barbara Ernst. New way of valorization of raw coffee silverskin: biohydrogen and acetate production by dark fermentation without exogenous inoculum. *Bioresource Technology Reports*, 2022, 17, pp.100918. 10.1016/j.biteb.2021.100918 . hal-04733321

HAL Id: hal-04733321

<https://hal.science/hal-04733321v1>

Submitted on 13 Nov 2024

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New way of valorization of raw coffee silverskin: biohydrogen and acetate production by dark fermentation without exogenous inoculum

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Abstract

Coffee silverskin, a roasting by-product, was tested for hydrogen production by dark fermentation for the first time. The indigenous microflora converted organic matter into hydrogen without biomass pretreatment, reaching hydrogen yields of up to 24.1 mL H₂/g COD (Chemical Oxygen Demand). Hydrogen yield was maintained at high biomass load (minimal dilution) during semi-batch bioreactor operation. Acetate was the main metabolite produced, while butyrate, ethanol and propionate were produced in lower amounts. PCR amplicon sequencing of the 16S rRNA gene showed that hydrogen-producing bacteria affiliated to *Enterobacteriaceae* (27.5%) and *Clostridium* (5.1%) were selected from the indigenous microflora. In agreement with observed metabolites, acetate-producing *Enterococcus* (54.1%) was the main bacterial phylotype identified. This study opens a new approach for valorization of coffee silverskin through hydrogen production.

Keywords

Coffee silverskin, hydrogen, dark fermentation, indigenous microflora

1. Introduction

Hydrogen production by dark fermentation exploits the ability of acidogenic bacteria to convert organic matter into organic acids, hydrogen and carbon dioxide. In this fermentation process, hydrogen is the major product together with acetate and butyrate, with hydrogen yields of 4 and 2 mol of hydrogen per mole equivalent of hexose, respectively (Ghimire *et al.*, 2015). Most of the reported studies of hydrogen production by dark fermentation have focused on exogenous fermentation from organic waste using an external inoculum containing hydrogen-producing bacteria (Ghimire *et al.*, 2015). Recently, there has been growing interest in endogenous fermentation, *i.e.* from organic waste serving both as a substrate and as a source of hydrogen-producing bacteria (François *et al.*, 2021; Dauplain *et al.*, 2020; Marone *et al.*, 2014; Kim *et al.*, 2009). It was previously shown that development of a robust and substrate-specific community of indigenous hydrogen-producing bacteria depends on bioreactor operating conditions (Ernst *et al.*, 2014). Different types of biomasses were investigated for this purpose. Hexose-rich viticultural by-products (François *et al.*, 2021) afforded high hydrogen production yields (226 mL H₂/g VS). Lignocellulosic biomasses such as sorghum (60 mL H₂/g VS) (Dauplain *et al.*, 2020), or untreated food waste (0-4.4 to 24 mL-H₂/g VS) (Kim *et al.*, 2009, Marone *et al.*, 2014) were successful to different degrees. Valorization of lignocellulosic biomasses by dark fermentation is challenging, as they are not easily fermentable. Hydrolytic pretreatment of lignocellulosic biomass is also associated with release of phenolic compounds (furfurals, notably 5-

(Hydroxymethyl)furfural) (Kumar and Sharma, 2017), known to inhibit the metabolism of hydrogen-producing bacteria (Quemeneur *et al.*, 2012).

In the present study, we aimed to test the potential of a specific raw lignocellulosic biomass, coffee silverskin (CSS), for hydrogen production by dark fermentation. CSS is the thin outer-shell detached from the green coffee bean during the roasting stage, carried out at 180-200°C in our case (industrial source). CSS is rich in cellulose, hemicelluloses, lignin and proteins (23.8; 17.7; 26.6 and 18.7 g/100 g dry material, respectively) (Ballesteros *et al.*, 2014). Such waste is estimated to have no commercial value (dos Santos Polidoro *et al.*, 2018), and can be toxic as a fertilizer due to its content in phenolic compounds (Machado *et al.*, 2012). On average, 1 to 2 kg CSS waste is produced for 100 kg of roasted coffee (Iriondo-DeHond *et al.*, 2020).

Estimating weight loss during the roasting process at 20% due to water and CO₂ release (Illy and Viani, 1995), an annual world production of CSS of about 150 kt can be predicted. Valorization of CSS for energy production was investigated, as substrate for bio-oil production by pyrolysis (del Pozo *et al.*, 2020; dos Santos Polidoro *et al.*, 2018), or for production of biogas by anaerobic co-digestion (Battista *et al.*, 2016). More recently, enhancing fermentable sugar release by CSS pretreatment (Giacobbe *et al.*, 2018; Niglio *et al.* 2019a, Corrêa *et al.*, 2020), and fermentation of pretreated CSS for production of ethanol, butanol and succinic acid (Niglio *et al.* 2019b; Hijosa-Valsero *et al.*, 2018; Mussato *et al.*, 2012), was also investigated. For the applications mentioned above, CSS was systematically pretreated with at least one of the following approaches for valorization by fermentation: by enzymatic treatment, in acid or alkaline solution, in an autoclave, by sonication and/or by heat-treatment.

In the present work, we investigated the feasibility of using raw CSS without any pretreatment, and testing it both as substrate and potential source of hydrogen-producing bacteria for hydrogen production by dark fermentation, without exogenous inoculum.

2. Materials and Methods

2.1. CSS characterization and preparation

CSS from Les cafés SATI (Strasbourg, France) was collected and stored at -18°C . Before each fermentation test, CSS was thawed at 4°C . Total solids (TS), volatile solids (VS) and chemical oxygen demand (COD) were measured according to standard methods (APHA, 2005). Soluble total carbohydrates (including hexoses and more complex sugars as cellulose) were quantified using the anthrone-sulfuric acid method (Dreywood *et al.*, 1946).

CSS was composed of 0.82 ± 0.03 g TS/g raw CSS, with 94% of VS and 6% of ash. Initial COD was evaluated at 1.06 ± 0.04 g COD/g TS, in accordance with previous reports (Janissen and Huynh, 2018). Content in total soluble carbohydrates for raw CSS was 14.3 mg hexose/g raw CSS. Neither glucose nor fructose were detected by HPLC-RID in the soluble phase.

2.2. Dark fermentation tests

Tests were performed in a 1 L double jacket stirred tank bioreactor with a working volume of 0.7 L. CSS was diluted with tap water to two final concentrations: 31 g COD/L-reactor and 62 g COD/L-reactor. The pH was initially adjusted at 7.0 ± 0.2 , and kept above 5.7 during fermentation by automated addition of NaOH 1M. Temperature

was maintained at 37°C. Liquid medium was stirred at 220 rpm, and swept with nitrogen at a flow rate of 50 mL/min controlled with a mass flow meter, in order to maintain anaerobic conditions and to enhance extraction of produced gases (Clion *et al.*, 2015). Tests were stopped when hydrogen production dropped below 0.05 mL/min. Samples of liquid medium were collected through time and centrifuged. Supernatants and pellets were separated and stored at -18°C prior to analysis.

2.3. Analytical methods

Gas composition was measured online, and analyzed using a micro-gas chromatograph (M200, Agilent Technologies, USA) composed of two modules (two columns) equipped with two thermal conductivity detectors. The first column was a 5Å molecular sieve and the second was a capillary column CP-PoraPLOT U using argon and helium as carrier gas, respectively.

Content and composition of alcohols in the liquid phase was determined by injection in split mode of filtered (0.2µm, PES, VWR) supernatant diluted with an internal standard (isopropanol, 1000 mg/L), into a gas chromatograph (7890A GC, Agilent Technologies, USA) equipped with a flame ionization detector and HP-INNOWax column (30 m x 0.250 mm, 0.5 µm) (Agilent Technologies, USA). Helium (0.6 mL/min) was used as carrier gas. In each run, the column was heated at 45°C for 4 min, followed by temperature increase with a 10°C/min ramp up to 200°C.

Composition in organic acids (mainly succinate, lactate, formate, acetate, propionate, butyrate) was determined by high pressure liquid chromatography equipped with a variable wavelength detector. Filtered (0.2 µm, PES, VWR) supernatants were injected

onto a HiPlex-H column (7.7 m × 300 mm, 8 µm) (Agilent Technologies, USA) heated at 65°C, and eluted isocratically with 5 mM aqueous H₂SO₄ at 0.4 mL/min.

2.4. Microbiological analysis

Desoxyribonucleic acid (DNA) was extracted from centrifugation pellets stored at -18°C using the Fast DNA Spin Kit for Soil (MP Biomedicals, USA), according to the protocol described by the manufacturer, and then stored at -18°C. Concentration of extracted DNA was determined fluorometrically with the Qubit® dsDNA HS assay kit (Invitrogen, USA) following the manufacturer's instructions.

Variable regions V4-V5 of the 16S ribosomal ribonucleic acid (rRNA) gene were amplified by polymerase chain reaction as described previously (Renaudie *et al.*, 2021), and sequenced on a MiSeq sequencer (Illumina) by a commercial provider (INRAE Narbonne). Sequences of over 97% identity were grouped in Operational Taxonomic Units (OTU). Identification of bacterial taxa was performed online using NCBI Nucleotide Blast and the 16S ribosomal RNA sequences database. Bacterial diversity was estimated using Simpson's index.

3. Results and discussion

3.1. Gas production

Endogenous fermentation tests were performed using CSS at 31 g COD/L reactor (in triplicates) and at 62 g COD/L reactor as both substrate and source of hydrogen producing bacteria (Figure 1). With CSS at 31 g COD/L reactor, hydrogen production profiles were very similar, highlighting the good reproducibility of the performed tests. Hydrogen production started after 6 h of lag time. For each replicate, hydrogen production was maximal (0.4-0.5 mL/min) at 23 h, and then decreased until the end of

the test (55 h). CSS concentration was doubled (62 g COD/L reactor) to the operational upper limit of the process for maintaining fermentation in the liquid phase (CSS absorbed added water and blown up significantly). The spongy state of the biomass explained variations in the hydrogen production flow rate observed in Figure 1 because produced gases were kept and released by the matrix and analyzed intermittently. Hydrogen production started after 8 h of lag time, peaked at 22 h (0.55 mL/min), decreased slightly and increased to a maximum of 0.61 mL/min at 36 h, and then decreased slowly until the end of the test (120 h).

Except for the maximum hydrogen production rate ($H_2 R_{max}$) with values ranging from 18.6 to 28.6 mL/L reactor/h, fermentation tests without exogenous inoculum at 31 g COD/L reactor gave similar results with a mean H_2/CO_2 molar ratio (0.71 ± 0.05), and reached a mean hydrogen production of 20.8 ± 1.2 mL H_2/g VS, and up to 27.2 mL H_2/g VS for the test with a higher loading in CSS (Table 1), corresponding to yields of 18.4 ± 1.0 mL H_2/g COD and 24.1 mL H_2/g COD, respectively. The value of hydrogen production per VS added is in the same order of magnitude as in the study of Marone *et al.* (2014) for endogenous fermentation of untreated vegetable waste (24 mL H_2/g VS), and higher than those obtained of Kim *et al.* (2009) for endogenous fermentation of untreated food waste (0-4.4 mL H_2/g VS).

To the best of our knowledge, the use of CSS as a substrate for dark fermentation has not yet been reported. Moreover, production of hydrogen from coffee production by-products so far always involved the use of an external microbial *inoculum* (Figure 2). For example, Miñón-Fuentes and Aguilar-Juárez (2019) reported a hydrogen yield of 49.2 mL H_2/g COD from fermentation of coffee pulp with a heat-treated external inoculum (anaerobic sludge), Petrosyan *et al.* (2020) obtained 2.75 mL H_2/g from spent

coffee grounds (SCG) with *E. coli*. The previous trial of endogenous hydrogen production from coffee mucilage Cárdenas *et al.* (2019) remained unsuccessful, despite a similar organic matter content (40 g COD/L reactor) to that of the present study. In contrast and as shown in Table 1, our experiments with CSS, solely relying on its associated microflora for fermentation, yielded an average of 16.0 ± 0.7 mL H₂/g raw CSS, equivalent to up to 24.1 mL H₂/g COD.

3.2. Metabolite production

Co-produced volatile metabolites were analyzed (Figure 3), except for replicate c for which no intermediate samples were available). At the lower organic load investigated (31 g COD/L), only ethanol was detected at the start of the fermentation (0 h).

Production of acetate, as well as of succinate, formate, and to a lesser degree lactate and propionate, began simultaneously with hydrogen production (9 h). Lactate, succinate and formate were subsequently consumed. After 24 h, acetate was the main detected metabolite, with butyrate present in smaller quantities. A similar distribution of major metabolites was observed at the higher organic load (62 g COD/L), but with butyrate, formate, propionate and ethanol present in markedly smaller quantities.

Hydrogen-producing bacteria yield 2 moles of hydrogen per mole of acetate released (Ghimire *et al.*, 2015). However, hydrogen production amounted to 23.8 ± 1.3 mmol/L and 45.5 mmol/L in 31 and 62 g COD/L reactors, respectively, compared to 41.9 ± 3.1 mmol/L and 86.6 mmol/L for acetate, respectively. This clearly suggests that a large part of the acetate produced resulted from homoacetogenesis (without hydrogen co-production) (Ghimire *et al.*, 2015). Moreover, production of propionate, as observed in the second phase of fermentation, is hydrogenotrophic (i.e. hydrogen-consuming) (Ghimire *et al.*, 2015). Similarly, ethanol production could compete with hydrogen

production by consuming the substrate with no benefit for hydrogen production. Thus, even if the fermentation process in our experiments is clearly not yet optimized with respect to hydrogen production, the observed hydrogen yield is still very significant compared to that observed from fermentation of other lignocellulosic coffee byproducts. Another beneficial aspect of the use of CSS as a substrate is that the dark fermentation bioreactor can be operated at a minimal dilution rate, thereby minimizing water requirements. The higher COD load experiment was thus investigated in more detail in terms of its microbial composition.

3.3. Microbiological analysis

The composition of bacterial communities of raw CSS in the 62 g COD/L-reactor was analyzed by sequencing of amplicons of the 16S rRNA gene at the start (0 h) and after 24 h of fermentation. Bacterial diversity decreased drastically during fermentation, as indicated by the observed evolution of the Simpson index, from 0.04 in the initial raw CSS substrate to 0.38 after 24 h of fermentation. The high bacterial diversity in raw CSS was also evidenced by the fact that almost half (41.5%) of the detected phylotypes represented less than 2% of total bacterial abundance (Figure 4). The most abundant phylotypes were *Corynebacterium* (11.9 %), *Lactobacillus* (7.9%), *Enterococcus* (6.4%), *Acinetobacter* (6.3%), and two unidentified genera from *Enterobacteriaceae* (5.4%) and *Streptophyta* (5.3%) (analyzed from two OTUs). The bacterial community of raw CSS was reminiscent of that of coffee processing wastewater and waste coffee tray leach in previous reports. In Pires *et al.*, 2017, the dominant genus was *Enterobacter*, while in Vilanova *et al.*, 2015, *Enterococcus* and *Pseudomonas* sp. were the main taxa, with *Stenotrophomonas*, *Sphingobacterium*, *Acinetobacter* also among the most abundant, in the bacteriome of waste coffee tray leach.

After 24 h of fermentation, *Enterococcus* and the OTU of the *Enterobacteriaceae* family represented 54.1% and 27.5% of the total phylotypes analyzed, respectively. *Lactococcus* (10.3%) and *Clostridium* (5.1%) were other major enriched phylotypes. Strains of the *Enterococcus* genus are facultative anaerobes that grow under mesophilic conditions and are often able to survive on dry surfaces. They are known to metabolize complex carbohydrates as cellulose, and to produce acetate (without hydrogen co-production) as well as lactate and formate (Ramsey *et al.*, 2014). Thus, the biomass of CSS, rich in complex carbohydrates, appeared to favor growth of *Enterococcus* strains without hydrogen production. It is also possible that growth of *Enterococcus* strains inhibited growth of *Clostridium* strains, which are well-known hydrogen producers (Abo-Hashesh and Hallenbeck, 2012). Indeed, secretion of bacteriocins (enterocins) by *Enterococcus faecalis* causes inhibition of *Clostridium butyricum* (Liu *et al.*, 2011). As to the detected *Enterobacteriaceae*, they are often encountered in dark fermentation processes and produce hydrogen together with acetate coproduction, as well as ethanol (Abo-Hashesh and Hallenbeck, 2012). They may therefore also have contributed to hydrogen production in our experiments, and explain the ethanol detected. In contrast, the strong enrichment of lactate-producing (Nordkvist *et al.*, 2003) *Lactococcus* strains in the bioreactor requires further investigation, especially considering that only very low levels of lactate were observed in the medium. In the future, hydrogen production from CSS may potentially be improved by careful selection of operation conditions that limit the development of homoacetogenic strains that likely limit hydrogen production. For instance, the hydrogen potential of CSS could be improved by a hydrolysis step to increase the content of easily fermentable matter available in the reactive medium. Alternatively, a reaction medium enriched in acetate may be coupled with subsequent

photofermentation or anaerobic digestion, thereby potentially affording additional hydrogen production.

3.4 Estimated energy production for CSS valorization

For a given biomass, the dark fermentation process can constitute an additional brick to the production of methane by anaerobic digestion. Indeed, the effluent of dark fermentation is in our case concentrated in acetate, a compound which constitutes a substrate of choice for anaerobic digestion. Thus, the CSS energy valorization can be evaluated by the combined production of hydrogen and methane. Over a period of 48 h, hydrogen production corresponding to 16.0 L H₂/kg CSS (24.0 L H₂/kg COD) results in 173 kJ/kg CSS. The estimation of methane production from the amount of acetate produced considering that the conversion of acetate is total is 26.4 L CH₄/kg CSS equivalent to 764 kJ/kg CSS. Thus, the total amount of energy is 937 kJ/kg CSS without including the energy from the solid matter not metabolized by the dark fermentation. This value can be compared to the work of Battista *et al.* on the production of methane from coffee waste (mixture of coffee seed skin (CSS), coffee seed refuse (SR) and coffee product refuse (CPR)) of 15.9 L CH₄/kg (1.49 L biogas/L with a mixture of 60% methane for a loading rate of 0.0675 kg CSS/d, 0.0225 kg SR/d and 0.0225 kg CPR/d), representing an energy of 570 kJ/kg coffee waste, highlighting that the addition of the dark fermentation step seems to benefit the energy balance.

4. Conclusions

For the first time, hydrogen production from CSS were performed successfully, without any need of pretreatment or exogenous inoculum. Despite the high temperature of the

roasting process, the remaining active fraction of the indigenous microflora of coffee silverskin successfully supported hydrogen production with yields up to 24.1 mL H₂/g COD. Moreover, hydrogen production performance was maintained even at minimal dilution of biomass in the bioreactor. Simultaneously to hydrogen production, a high acetate production occurs (1.38 mmol/g COD_i). This study on the use of CSS as sole substrate and inoculum in dark fermentation opens the doors through a new way of valorization of this byproduct for hydrogen and acetate coproduction.

Acknowledgements

We gratefully acknowledge Les Cafés SATI (Strasbourg) for providing samples of coffee silverskin. The authors thank O. Théobald (ADEME) for his support of this work, Christelle Gruffaz (GMGM, Université de Strasbourg CNRS) for her assistance in DNA extraction and Anne-Clémence Aun (RePSeM-IPHC) for assistance with HPLC/GC analysis.

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Funding

This work was supported by a joint PhD grant to M.R. by the French Environment and Energy Management Agency (ADEME) and the Université de Strasbourg.

Author contributions

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

CRedit authorship contribution statement

Marie Renaudie: Investigation, Methodology, Writing - Review, Christine Dumas: Methodology, Validation, Writing original draft, Stéphane Vuilleumier: Resources, Writing Review, Barbara Ernst: Conceptualization, Supervision, Validation, Writing Review & Editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this paper.

Table 1: Gas production performances from endogenous fermentation of CSS at 62 g COD/L reactor and 31 g COD/L reactor.

COD (g COD/L reactor)		31a	31b	31c	Average	62
H₂ production	(mL/g raw CSS)	17.0	15.6	15.4	16.0 ± 0.9	16.1
	(mL/g VS)	22.1	20.2	20.0	20.8 ± 1.2	27.2
H₂ Rmax (mL/L-reactor/h)		28.6	21.4	18.6	22.9 ± 5.2	29.3
H₂/CO₂ molar ratio		0.71	0.76	0.66	0.71 ± 0.05	0.67

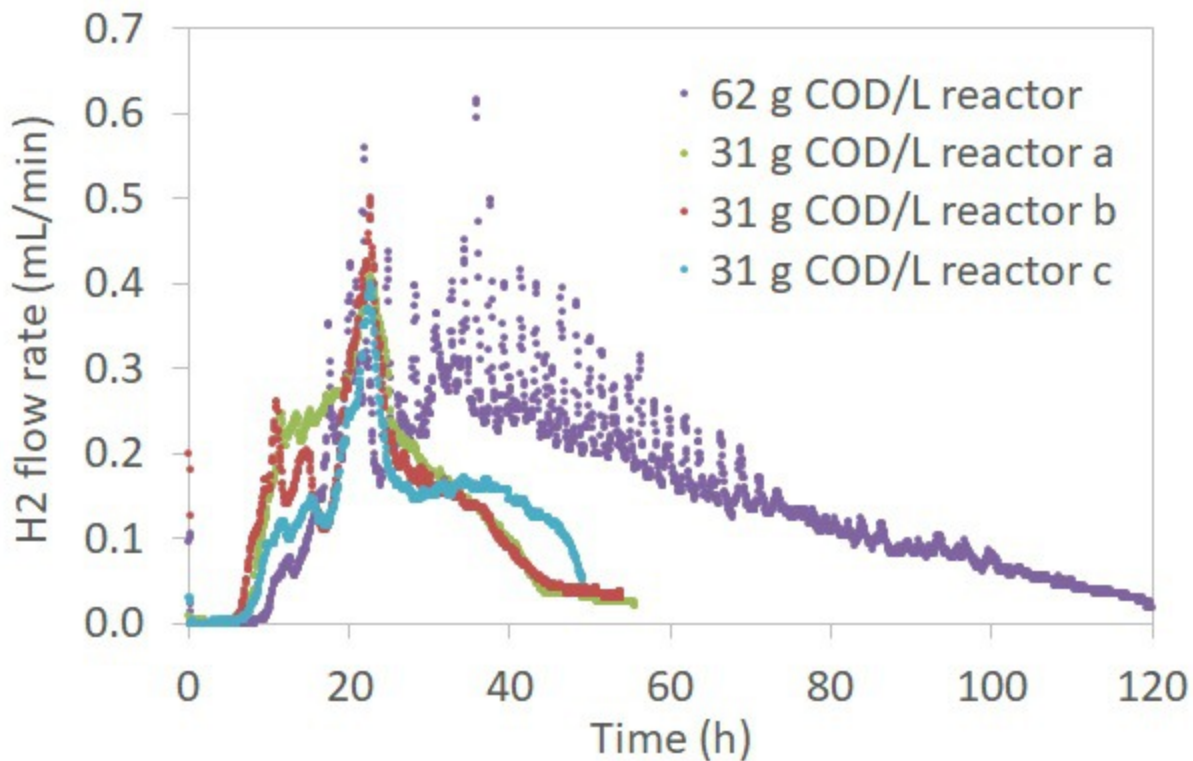
Figure Captions

Figure 1: Hydrogen flow rate as a function of time from coffee silverskin (CSS) endogenous fermentation

Figure 2: Reported studies of valorization of coffee production waste by hydrogen production.

Figure 3: Major metabolites detected in the reactive medium during dark fermentation. For comparison of experiments at 31 g COD/L (average of tests a and b; error bar: standard deviation) and 62 g COD/L, measured concentrations (in mM) were expressed in relation to the initial COD (COD_i, in g/L) in the reactor.

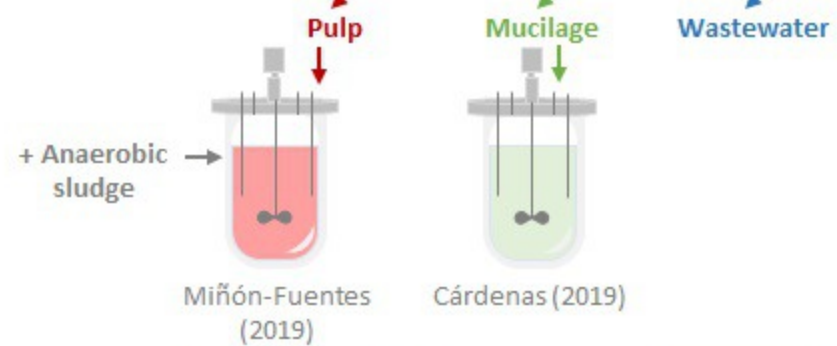
Figure 4: Relative abundance of dominant phylotypes (> 2%) in the reactive medium at 0 h (raw CSS, outer circle) and at 24 h (inner circle) for the test at 62 g COD/L.



(Wet) Process in producing countries



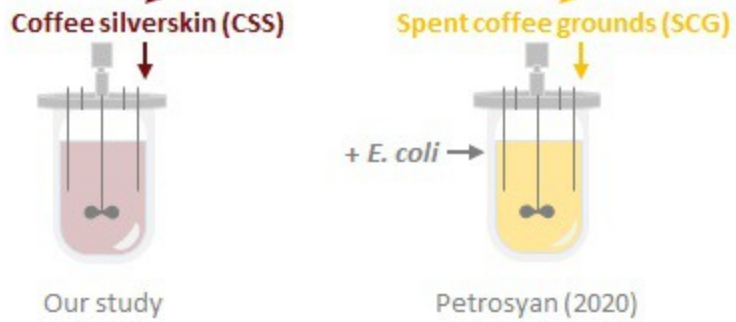
Waste in producing countries



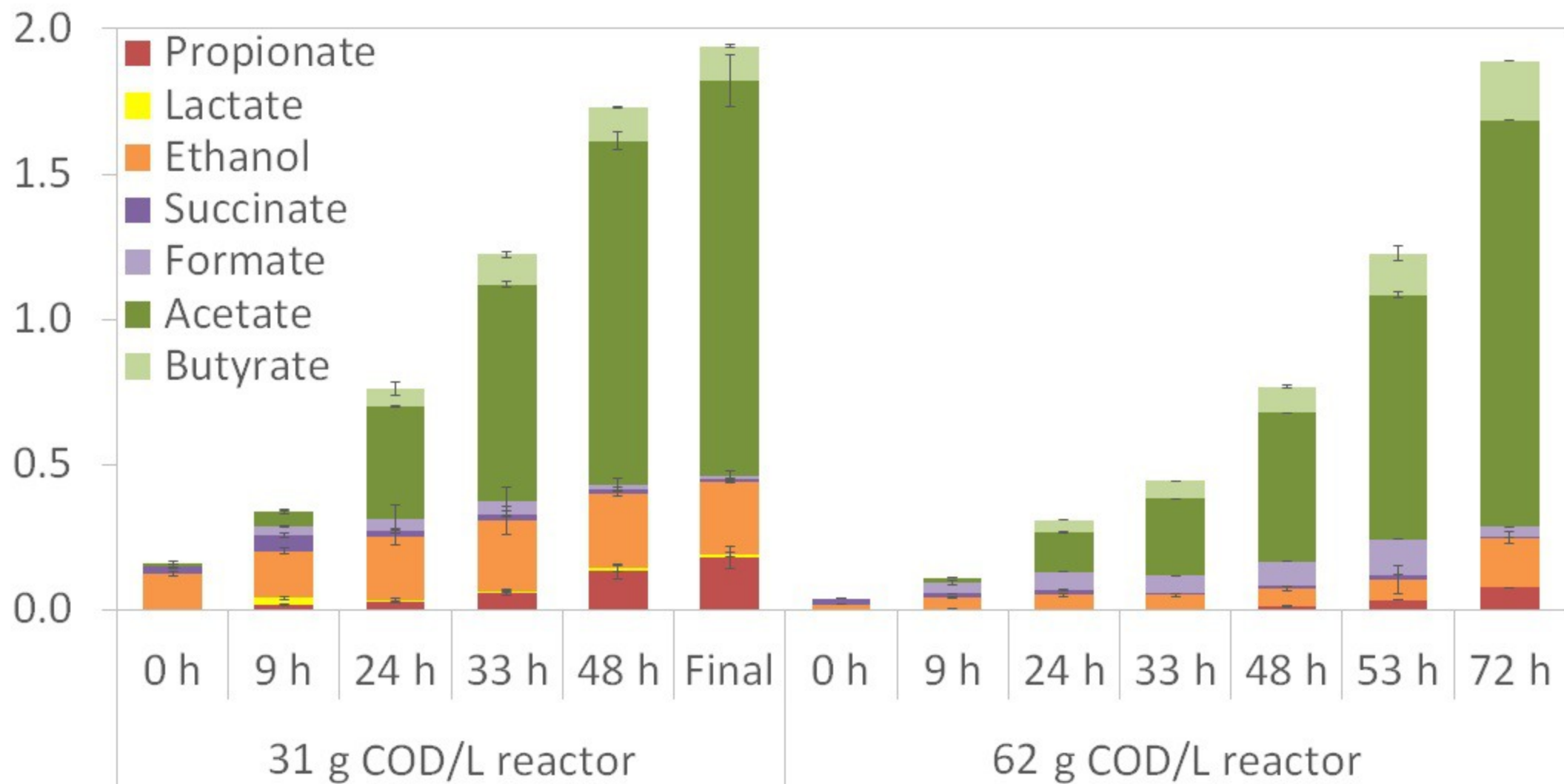
Process in consuming countries

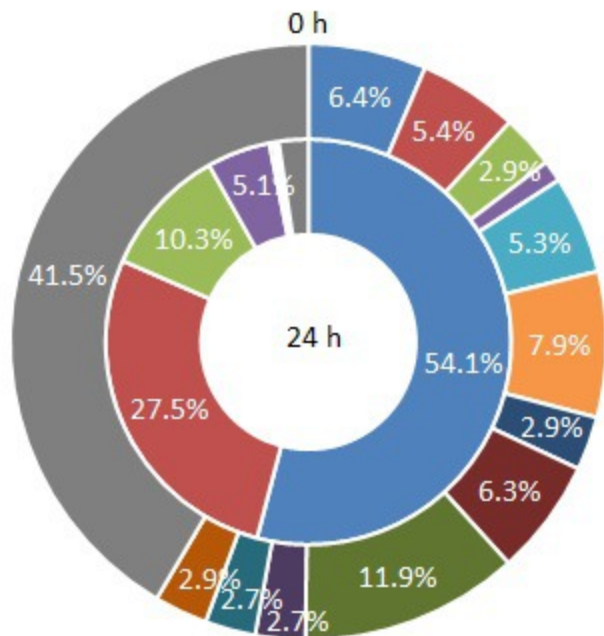


Waste in consuming countries



Concentration in metabolites
(mmol/g COD i)

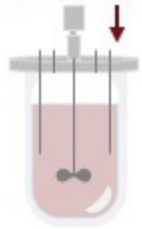




- *Enterococcus*
- *Enterobacteriaceae*
- *Lactococcus*
- *Clostridium*
- *Streptophyta*
- *Lactobacillus*
- *Weissella*
- *Acinetobacter*
- *Corynebacterium*
- *Comamonas*
- *Sphingobacterium*
- *Dysgonomonas*
- Others



Coffee silverskin (CSS)



Dark fermentation

- ✓ No added inoculum
- ✓ No pretreatment
- ✓ Up to 24.1 L H₂/kg COD