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ORIGINAL ARTICLE

Enhancing the fermentability of brewer's spent grains to short- and medium-chain carboxylates through dilute alkaline pretreatment

Grégoire B. L. Henry[1] · **Arnaud Isenborghs**[1] · **Emilie Walhain**[1] · **Thomas Nicolay**[1] · **Benoit A. Stenuit**[1] · **Patrick A. Gerin**[1]

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Abstract
The substitution of fossil resources with renewable carbon requires the development of cost-efficient treatments and conversion processes for biomass. Brewer's spent grains (BSG) are low-cost organic residues from the agroindustry. The valorization of BSG has been investigated to produce carboxylates through acidogenic fermentation. However, the high lignocellulose content of BSG limits their fermentability. This study examines moderate-temperature dilute alkaline pretreatments of BSG to improve their fermentability and their conversion to short- and medium-chain carboxylates (SCC and MCC) using mixed microbial fermentation. Room temperature and 80 °C pretreatments for 40 or 120 min with a KOH concentration of 0.25 M or 0.5 M were tested. The KOH added for pretreatment was then successfully neutralized by sparging CO_2 to adjust the pH to 6 prior the fermentation. The alkaline pretreatment increased the BSG to carboxylate conversion yield from 15 to 21%$_{\text{Total_COD}}$. It increased the final metabolite production by 29 ± 9% as compared to raw BSG. Carboxylates accounted for 89 to 93% of the identified metabolites; SCC represented 85 to 99.5% of the carboxylates produced; and the remaining 0.5 to 15% were MCC. Increasing the pretreatment temperature is detrimental to the production of MCC. The highest MCC proportion was obtained from BSG pretreated at room temperature under 0.25 M KOH. Alkaline pretreatment of BSG enabled a considerable reduction in fermentation time, reaching in 4 days the metabolite production obtained in 14 days with raw BSG.

Keywords Lignocellulosic biomass · Mixed microbial culture · Acidogenic fermentation · Volatile fatty acid · Medium chain carboxylic acids · Chain elongation

1 Introduction

The exploitation of fossil resources sparked off the industrial age, driven by technical and economic advances that transformed handmade agricultural to industrial production [1]. During the last two centuries, the fossil resources' consumption, the overharvesting of our lands, and the global poor waste management have led the society to the current environmental issues [2]. To address climate change, the depletion of fossil fuels, global population growth, rising energy demand, and waste production, society and industries need to replace fossil carbon sources with renewable ones and develop a sustainable and competitive circular economy [3]. Biorefinery processes using organic by-products coming from agriculture, forestry, or food industry as renewable carbon source to produce energy and/or chemicals do not compete with the food supply chain and are in line with the circular economy principles [4].

Brewer's spent grain (BSG) is an example of undervalorized organic by-products, currently mainly used as animal feed [5, 6]. BSG is roughly 85% of the solid by-products of a brewery, with a world production estimated at 36.4 Mt in 2020 [5, 7]. Although numerous studies have been reported in the literature in the past decade, current valorization approaches are not yet concrete [8, 9]. Bioconversion by methane-arrested anaerobic digestion, i.e., acidogenic fermentation, has recently been tested with BSG as organic substrate [10–13]. Methane-arrested anaerobic digestion is an established bioprocess catalyzed by microbial communities. It aims at transforming the fermentable macromolecules of organic residues to volatile fatty acids, H_2, ethanol and/or

✉ Patrick A. Gerin
patrick.gerin@uclouvain.be

[1] Laboratory of Bioengineering and Biorefining, Earth and Life Institute - Applied Microbiology, Université catholique de Louvain, Croix du Sud, 2, box L7.05.19, 1348 Louvain-la-Neuve, Belgium

lactate [14]. Owing to their versatile structure and functionality, mixed microbial cultures are able to convert complex organic substrates, thanks to a cascade of reactions catalyzed by different microorganisms [15]. The target molecules are volatile fatty acids, also called short chain carboxylates, made of 1 to 5 carbon aliphatic chain, linked to a terminal carboxylic acid. They are currently produced from fossil resources and are in demand by the conventional organic chemistry industry as they are precursors of esters, ketones, aldehydes, alcohols, or alkanes but could also be used in new bioprocesses for the production of biopolymers such as polyhydroxyalkanoates [16, 17]. Short-chain carboxylates can also be elongated to medium-chain carboxylates via reverse β-oxidation by microbial communities in the presence of electron donors such as ethanol, lactate, or H_2 [10, 18, 19]. Medium-chain carboxylates have the same structure as the short chain ones but with longer carbon chain from 6 to 12 C atoms. There are more easily extracted from aqueous media owing to their higher hydrophobicity and have a higher market value than the short-chain carboxylates [19].

To date, acidogenic fermentation of BSG has shown low conversion yields of biomass to carboxylates (from 20 to $25\%_{BSG_COD}$) and long fermentation time (several weeks) [10, 12, 20]. The BSG chemical composition varies according to several factors, e.g., type of barley used, the additives added during the beer production or BSG storage techniques [5]. However, BSG generally contains only a small proportion of fermentable components like proteins ($15–25\%_{Dry\ Matter}$), residuals starch ($2–9\%_{DM}$), lipids ($4–9\%_{DM}$), and soluble sugars ($\sim 1\%_{DM}$), compared to more than $50\%_{DM}$ of the poorly fermentable structural components hemicellulose, cellulose, and lignin [5–7, 10, 21, 22]. Hemicellulose is generally the most abundant component of lignocellulose in BSG ($19–40.2\%_{DM}$), followed by cellulose ($9–25.4\%_{DM}$) and lignin for ($7.7–20\%_{DM}$) [23]. The hydrolysis step of these macromolecules is expected to be the rate-limiting step of BSG fermentation, similarly to most lignocellulosic complex organic feedstocks [14, 24]. The low fermentability of lignocellulose can be explained by the limited accessibility of microbial enzymes to the structural polysaccharides due to the tight bonding between the hemicellulose, cellulose, and lignin constituents and the high crystallinity of cellulose [25–27].

To increase the fermentability of lignocellulosic biomass, various types of pretreatments can be used to degrade, solubilize, and make the lignocellulosic components more accessible to microorganisms and their enzymes [28, 29]. Among them, alkaline pretreatments would be particularly adapted to increase the conversion of BSG to carboxylates by acidogenic fermentation. Indeed, alkaline pretreatment increases the interfacial surface area of the substrate by swelling the lignocellulose structure, reducing the cellulose crystallinity as well as the polysaccharide degree of polymerization. They also solubilize some parts of the lignin by hydrolyzing ester linkages of the lignin-carbohydrate complexes. Alkaline pretreatments release acetyls and some uronic acids from hemicelluloses, thereby enhancing the accessibility of enzymes to polysaccharides and promoting the release of fermentable sugars [26–28, 30, 31]. To obtain the best reducing sugar yields, xylan and glucan recovery, dilute alkaline pretreatment should avoid temperature higher than 120 °C. Indeed, above these temperatures, alkaline solutions degrade carbohydrates leading to a reduction in fermentable sugars due to peeling and stopping reaction pathways. These processes result in the formation of isosaccharinic acids and metasaccharinic acids, respectively [27, 32].

The present study aims at increasing the yields and rates of carboxylate production in a one-pot process combining pretreatment, neutralization, and mixed culture acidogenic fermentation, starting from brewer's spent grain as a raw complex organic feedstock. The hypothesis of this study is based on the complementarity between the alkaline pretreatment effect increasing the interfacial surface area of the substrate and the hydrolytic activity of microbial communities. Since microbial communities have versatile hydrolytic activities, the strength of the alkaline pretreatment could be reduced. Decreasing the strength of the alkaline pretreatment brings benefits for microorganism by reducing the production of fermentation inhibitors as well as reducing the degradation of the soluble carbohydrates. From an industrial point of view, reducing the temperature, pressure or concentration reduces the cost of the process. In this way, low temperature, short duration, and dilute alkaline concentration were investigated on raw BSG. The impact of temperature (room temperature (RT) vs. 80 °C), the hydroxide concentration (water, 0.25 M KOH or 0.5 M KOH), and the holding time of the pretreatment (40 vs. 120 min) on the brewer's spent grain solubilization degree and total metabolite conversion yields were investigated. Similarly, to reduce the cost of the process, the study tested an initial neutralization of residual hydroxide by CO_2 sparging. This approach circumvents the need for expensive acids prior to starting acidogenic fermentation. The neutralization step is essential to avoid inhibition of the acidogenic microorganisms due to a too high pH ($pH > 13$) resulting from the alkaline pretreatment.

2 Material and method

2.1 Material

2.1.1 Substrate

Brewer's spent grain (BSG) was collected at the Bertinchamps craft brewery in Gembloux, Belgium. It was received fresh on the day of the brew and then directly

pressed under 3 bars on a sieve with a hydraulic press (Speidel 20 L) until the liquid has stopped flowing, finally divided in 1 kg samples, and stored in plastic bags in a freezer at − 20 °C. Table 1 summarizes the BSG composition.

2.1.2 Inoculum

Two fermentation experiments began a week apart with activated sludge as inoculum coming from the municipal wastewater treatment plant in Mont-Saint-Guibert, Belgium. Inocula were collected the morning of the day each fermentation experiment began. The sludges were concentrated by sedimentation for 4 h and removal of the supernatant. The settled sludges were used to inoculate the bioreactors. The composition of each concentrated inoculum (A and B) is presented in Table 1. We considered that the microbial composition of the activated sludges A and B was similar, as there was only 1 week between the two samplings. Other studies have shown that microbial composition of activated sludge was stable over longer periods of time [33–35].

2.1.3 Stock solutions

Alkaline solution for pretreatment A 0.5 M potassium hydroxide stock solution was prepared to carry out the dilute alkaline pretreatments. The 0.25 M KOH was obtained by diluting the stock solution with distilled water.

Mineral medium and pH adjustment solution for fermentation A mineral medium was supplemented to the broth from three concentrated stock solution as described in Henry et al. [10]. Solutions of 2 M KOH and 1 M H_3PO_4 were used to adjust the pH.

2.2 Alkaline pretreatments and neutralization

2.2.1 Pretreatment preparation

For each condition, pretreatment was performed in triplicates, each replicate in a 2-L Schott Duran® GL45 glass bottle, that served as bioreactor directly after the pretreatment. A quantity of 63.9 ± 0.2 g_{BSG_FM} (31.3 ± 0.1 g_{COD}) was added to each bioreactor bottle to reach a BSG concentration in the pretreated liquor (PL) of 116.6 ± 0.3 g_{COD_BSG} L_{PL}^{-1}. A total volume of 270 mL of solution of KOH and/or distilled water was added to the bioreactor to adjust the alkaline pretreatment conditions (Table 2). The bioreactors were weighed after every addition and the masses recorded. An initial pH measurement of the liquor (Fig. 1, number 1 as indicated on each graph) was carried out using a pH-meter (WTW pH-Electrode SenTix® 41, WTW Multiline P4 universal meter). A water bath (GFL 1083, with back-and-forth agitation) was preheated to reach a temperature between 85 and 90 °C, resulting in a temperature of 80 °C inside the bioreactors. Before the experiment, each bioreactor bottle was weighed empty and full of water, the difference between the values allowed to determine accurately the specific individual net volume of each bioreactor.

Table 1 Physicochemical characterization of the substrate and inoculum (mean + / − standard deviation of triplicate analyses)

	Brewer's spent grain	Inoculum	
		A	B
Total solid$_{105\,°C}$ (g_{TS}/g_{FM})	0.326 ± 0.004	0.0235 ± 0.0002	0.0167 ± 0.0004
Volatile solid$_{550\,°C}$ (g_{VS}/g_{TS})	0.9562 ± 0.0003	0.434 ± 0.005	0.466 ± 0.002
COD (g_{COD}/g_{FM})	0.49 ± 0.01	0.018 ± 0.001	0.012 ± 0.01

FM fresh matter, *TS* total solid, *VS* volatile solid, *COD* chemical oxygen demand

Table 2 Brewer's spent grain pretreatment conditions and associated starting fermentation inoculum

Conditions	BSG conc. (g_{COD} $L^{-1}{}_{PL}$)	KOH conc.(M)	Pretreatment temperature (°C)	Pretreatment duration (min.)	Number of CO_2 sparging steps (number)	Inoculum
A — Raw BSG	116.6 ± 0.3	0	22	0	0	B
B — 80 °C – H_2O – 120′	116.6 ± 0.1	0	80	120	1	A
C — RT – 0.25 M KOH – 120′	116.4 ± 0.1	0.25	22	120	1	B
D — RT – 0.5 M KOH – 120′	116.4 ± 0.1	0.5	22	120	2	A
E — 80 °C – 0.25 M KOH – 120′	116.7 ± 0.4	0.25	80	120	1	A
F — 80 °C – 0.5 M KOH – 120′	116.9 ± 0.4	0.5	80	120	2	B
G — 80 °C – 0.25 M KOH – 40′	116.8 ± 0.4	0.25	80	40	1	B
H — 80 °C – 0.5 M KOH – 40′	116.3 ± 0.2	0.5	80	40	2	B

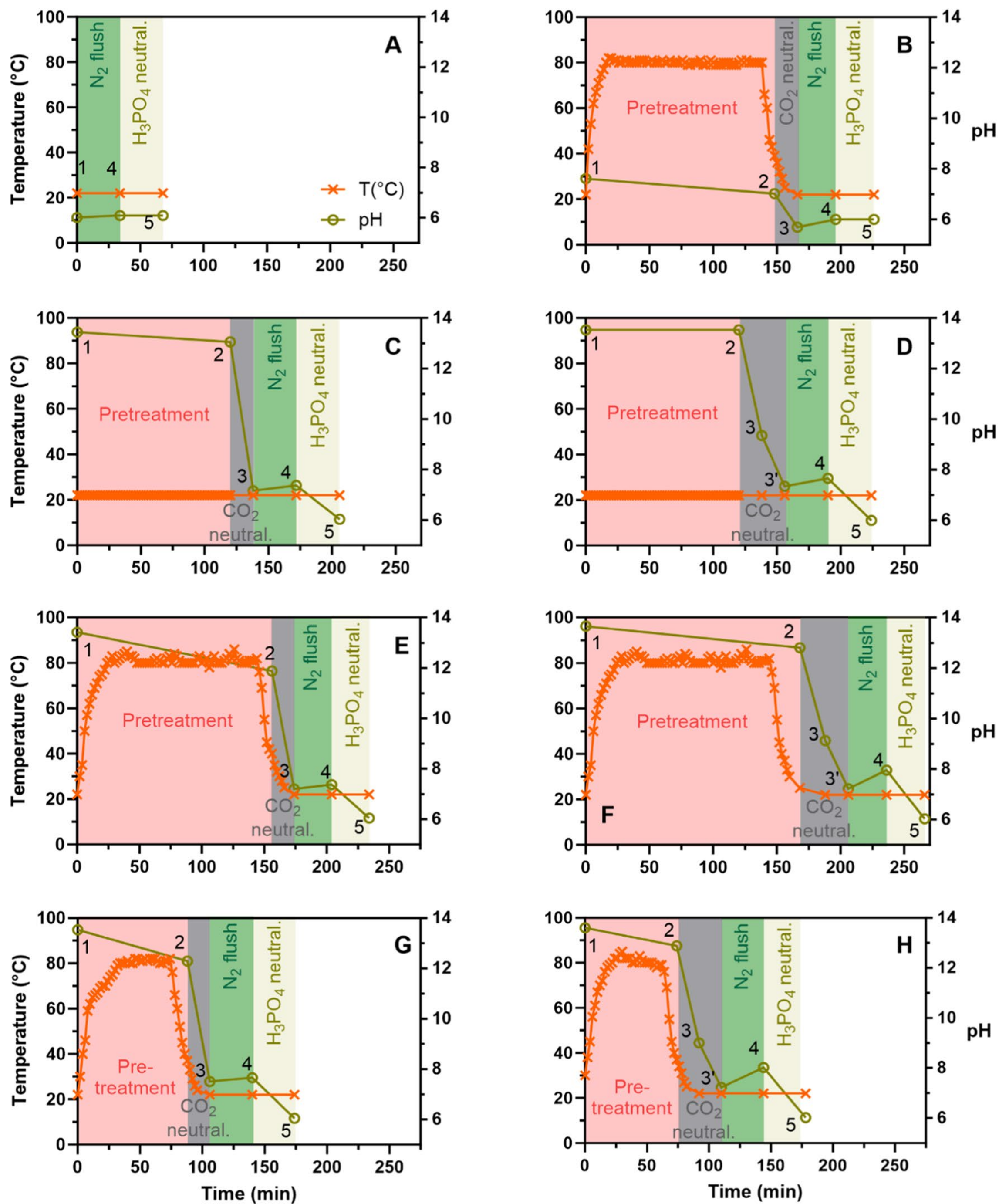

Fig. 1 Evolution of the temperature (line with cross symbol) and the pH (line with circle symbol) for the tested conditions (**A** raw BSG; **B** H_2O – 80 °C – 120 min; **C** 0.25 M KOH – RT – 120 min; **D** 0.5 M KOH – RT – 120 min; **E** 0.25 M KOH – 80 °C – 120 min; **F** 0.5 M KOH – 80 °C – 120 min; **G** 0.25 M KOH – 80 °C – 40 min, and **H** 0.5 M KOH – 80 °C – 40 min) during the pretreatment phase (from number 1 to number 2), CO_2 neutralization phase (from number 2 to number 3 after the first and number 3′ after the second CO_2 neutralization step), N_2 flush phase (from number 3 or 3′ to number 4) and the addition of H_3PO_4 phase as final pH adjustment before starting fermentation (from number 4 to number 5)

2.2.2 Pretreatment

Once BSG and the pretreatment solution were added to the bioreactors and the water bath was at the adequate temperature, two bioreactors per triplicate were sealed with GL45 screw cap and one bioreactor with a pierced GL45 screw cap in which a glass thermometer was placed for direct measurement of liquor temperature. For the

pretreatment conditions at 80 °C, the three replicates were plunged simultaneously in the pre-heated water bath with the one containing the thermometer positioned in the middle. Regarding the 80 °C pretreatment condition, the recording of the pretreatment duration and temperature began when the three replicates where closed and in the water bath. The 40- and 120-min pretreatment countdown began when the temperature of the liquor in the bioreactor reached 80 °C. After the desired pretreatment holding time, the bioreactors were removed from the hot water bath and immediately cooled under a stream of cold water for 10 min with a temperature measurement every 2 min. The bioreactors were weighed again to assess the amount of evaporated water. If needed, distilled water was added. Regarding the room temperature (RT) pretreatments, the pretreatment duration monitoring started directly after the addition of the pretreatment solution. Once the pretreatment time was elapsed, the bioreactors were weighed again to assess the amount of evaporated water. If needed, distilled water was added. The room temperature (RT) was stable at 22 °C, it was measured at the beginning and at the end of the pretreatments. After the pretreatment step, for all the conditions, the pH of the liquor was measured (Fig. 1, number 2) before being neutralized by sparging CO_2 if needed.

2.2.3 KOH neutralization by sparging CO_2

The three replicates were neutralized simultaneously by sparging CO_2 directly into the liquor for 2.5 min at a flow rate of 4 L min^{-1} previously adjusted with a gas rotameter (Masterflex™ 65-mm Correlated Flowmeter with Valve). The pressure in the bioreactors was adjusted with CO_2 to 1.4 bar with a UNIK 5000 manometer (GE, USA) with a three-way valve at its top and connected to the two-way valve of the bioreactor gas sampling port. The three bioreactors were agitated on an orbital shaker (Gyrotory Shake Model G10) at 60 rpm for 15 min. For the pretreatment conditions with 0.5 M KOH, the CO_2 sparging step and the 15 min stirring step were performed twice. Measurements of the pH were carried out after every CO_2 sparging and stirring step (Fig. 1, number 3 and 3′) to quantify the OH^- neutralized.

2.2.4 End of the pretreatment and neutralization steps

The bioreactors were weighed; a well-homogenized sample of 81 mL of the pretreated liquor was collected from each bioreactor with a 50-mL-polypropylene syringe (BD Plastipack, USA) to leave a final volume of 189 mL of pretreated liquor in the bioreactor, at a pretreated BSG concentration of 116 g_{COD_BSG} $L_{PL}{}^{-1}$. The bioreactors were weighed again. The samples were placed in two 50-mL-centrifuge plastic tubes (Falcon™ 50-mL Conical Centrifuge Tubes), weighed, and centrifuged at 4700 rpm for 10 min (ThermoScientific Heraeus Megafuge 40 centrifuge). After centrifugation, the supernatants were separated and collected in a new 50-mL Falcon™ tube. The masses of the solid and liquid fractions were weighed. The fractions were stored at − 20 °C for further analyses.

To prepare the fermentation steps, the remaining pretreated liquor was sparged with N_2 at a flow rate of 5 L min^{-1} for 2 min. This step was performed to avoid any inhibition of the fermentations due to the remaining soluble CO_2 [10]. The pH was measured after the first N_2 flush (Fig. 1, number 4).

2.3 Fermentation

2.3.1 Bioreactor preparation

Fermentations were performed in the same glass bottle used in the pretreatment and neutralization steps. The 189 mL of pretreated liquor contained in the bioreactor were supplemented with 20 mL of inoculum (0.36 g_{COD} and 0.24 g_{COD}, respectively, for inocula A and B) and 11 mL of mineral medium to reach a final fermentation working volume of 0.22 L. The bioreactors were weighed with a balance (OHAUS navigator NVT6201) after each supply. The mixed liquor (ML) contained an average final BSG concentration of 101.6 ± 3.8 g_{COD} $L^{-1}{}_{ML}$. The final pH was adjusted to pH 6 with H_3PO_4 prior the start of the fermentation (Fig. 1, number 5). The average pH at the beginning of the fermentation was 6.03 ± 0.04. Bioreactors were then sealed with GL45 caps; the ML as well as the headspace of the bioreactor sparged again for 2 min with N_2 gas at 5 L min^{-1} as described in detail in Henry et al. [10]. Twenty-milliliter-gas samples were taken to analyze the starting headspace gas composition (see section "Analytical methods") and ensure that there is no trace of remaining O_2. The N_2 pressure was measured with a hand manometer (UNIK 5000 manometer, GE, USA) and adjusted to 1.1 bar absolute pressure by N_2 addition. Bioreactors were incubated for 21 days on an orbital shaker (Kühner Shaker Lab-Shaker) at 120 rpm in a thermostatic chamber at 35 °C in the dark.

2.3.2 Bioreactor monitoring

The bioreactor monitoring routine at each day of measurement was carried out as detailed in Henry et al. [10]. Briefly, in the thermostatic chamber, for each bioreactor, the initial bioreactor mass was recorded; the initial pressure was measured; an initial gas sample was analyzed; and a ML sample was collected with a 50 mL syringe (BD, plastipack). The required quantity KOH or H_3PO_4 was reinjected by using the 50-mL syringe to adjust the pH to 6. To keep constant mixed liquor volumes in each bioreactor, distilled water was added to the bioreactor to counterbalance the volume sampled. One

syringe was dedicated to each bioreactor to avoid any transfer of microorganisms. Sampling and supply of liquid and gas took place without external air entry or release of gas from the headspace, thanks to a three-way Luer lock valve and Mohr pinchcock on the connecting tubes (detailed in the supplementary material of Henry et al. [10]).

At the end of the 14 days of fermentation, the remaining ML was determined by difference with the mass of the empty bioreactor. After ML centrifugation, the supernatant and pellet fractions were separated, weighed, and analyzed separately for total solids, volatile solids, and ash content. The liquid fraction was also analyzed for soluble COD content and fermentation metabolites.

2.4 Analytical methods

All the following analytical methods and equipment are described in detail in Henry et al. [10]. Total solids, volatile solids, ash content, and total COD (CODt) were measured according to the standard method (APHA, 2019). Soluble COD (CODs) was measured using the COD Cell Test method (Spectroquant® kits 1.14555.0001, Spectroquant Pharo 300, Merck KGaA, Germany) according to the provider's instructions. The headspace gas composition was analyzed by gas chromatography (CompactGC3.0, Global Analyser Solution) with a thermal conductivity detector (GC-TCD). This gas analyzer was calibrated to quantify the proportions of H_2, N_2, O_2, CO_2, CH_4, and H_2O present in the gas sample. Soluble metabolites were extracted from supernatant of the ML with liquid–liquid extraction in diethyl ether and analyzed by gas chromatography with flame ionization detector (Trace GC-TCD, Thermo Scientific). The detected and quantified components were ethanol (EtOH), propanol (PropOH), isopropanol (iPropOH), butanol (ButOH), isobutanol (iButOH), acetate (C2), propionate (C3), butyrate (C4), isobutyrate (iC4); pentanoate (C5), isopentanate (iC5), hexanoate (C6), heptanoate (C7), octanoate (C8), nonanoate (C9), decanoate (C10), phenylacetate (PhC2), and phenylpropionate (PhC3) as described in Henry et al. [10].

2.5 Calculations

2.5.1 Solubilization degree

The brewer's spent grain solubilization degree was used to characterize the impact of the pretreatment on BSG solubilization. The solubilization degree (SD) is calculated by Eq. (1) and expressed in $g_{COD_soluble}/g_{COD_added_BSG}$.

$$SD = \frac{\text{Soluble COD after pretreatment}}{\text{Total BSG initial COD added}} \left[\frac{g_{COD_soluble}}{g_{COD_added_BSG}} \right] \quad (1)$$

2.5.2 Severity factor

The severity factor (*SF*) is an established metric developed by Overend et al. [36] combining time and temperature to describe the non-catalyzed aqueous and steam pretreatment of hardwood. The severity concept has been further adapted in order to take into account the effects of acid and base catalysts on the lignocellulose degradation [25, 37]. The SF expressed in Eq. (2) allows to compare different pretreatment conditions by combining the effects of pretreatment temperature, holding time, and pH [25, 37].

$$SF = \log\left(\int_0^t \exp\left[\frac{T(t) - T_{ref}}{14.75}\right] dt\right) + |pH - 7| \quad (2)$$

where t is the pretreatment holding time (min); T is the recorded temperature; and T_{ref} is the reference temperature.

In this study, given that the pretreatment temperatures are maintained below 100 °C, we modified the T_{ref} value of 100 °C used by Overend et al. [36] and set it at 22 °C for adequate comparison with their reference conditions at room temperature. The severity factor was used only as a reference indicator to compare the different pretreatment conditions.

2.5.3 Normalized metabolite productions

Since the final volume of ML slightly varies (+ − 4.5%) between conditions, the metabolite productions were normalized by the final mixed liquor volume L_{ML} of the corresponding bioreactor to be perfectly comparable.

2.6 Statistics

2.6.1 Fermentations

The 8 pretreatment conditions were carried out in triplicate, resulting in 24 bioreactors containing pretreated BSG. Each bioreactor was used to start a fermentation, giving 3 biological replicates for each BSG pretreatment conditions. The results are based on the mean with standard deviations for the triplicates.

2.6.2 ANOVA Tukey's multiple comparison test

The initial BSG solubilization degree and the total metabolite productions were analyzed, respectively, with an ordinary one-way and 2-way ANOVA Tukey's multiple comparison test performed with Graphpad software grouped analysis function, with a p-value at a confidence level of 95%. The letters representing significantly different groups were generated manually by comparing the p-value of each comparison test in pairs.

3 Results

3.1 Evolution of temperature and pH during dilute alkaline pretreatment of brewer's spent grains and impact of pretreatment on COD solubilization

The temperature and the pH evolution are presented for all the conditions in Fig. 1. The negative control condition (raw BSG) showed stable temperature (22 °C) and pH (pH 6). For pretreatments at 80 °C, between 16 and 34 min were needed to reach 80 °C. Then, the temperature oscillated between a minimum temperature of 76 °C to and a maximum temperature of 86 °C with an average temperature of 80.5 ± 1.4 °C during the whole pretreatment. Cooling the bioreactors needed 10 to 18 min to reach a temperature below 40 °C.

The negative control condition required no pH adjustment (Fig. 1A). The 80 °C water condition led to a slight acidification during the pretreatment, decreasing from pH 7.61 ± 0.08 to pH 7.02 ± 0. 02. One step of CO_2 sparging was enough to decrease the pH to 5.69 ± 0.01 (Fig. 1B).

Under the alkaline pretreatment conditions (Fig. 1C-H), the pH decreased slightly but remained above pH 12. (Fig. 1, from number 1 to 2). The 0.25 M KOH conditions were neutralized to pH 7 in only one step of CO_2 sparging. Two steps of CO_2 sparging were needed to neutralize the 0.5 M KOH liquors (Fig. 1, number 3 or 3′). A higher increase in pH after the N_2 sparging step was observed for the 0.5 M KOH conditions, as compared to the 0.25 M KOH conditions (Fig. 1, from numbers 3 or 3′ to 4). Therefore, the 0.5 M KOH conditions required a higher amount of H_3PO_4 to decrease the pH to 6, as compared to the 0.25 M KOH conditions (Fig. 1, from numbers 4 to 5).

Figure 2 shows that the higher the severity factor, the greater the solubilization of BSG. The 80 °C-water pretreatment condition results in the weakest severity factor (*SF*) of all the pretreatments tested. The main contribution to the severity factor was the alkalinity of the pretreatment. Under alkaline condition, the pH component represents (6.4 to 6.6) out of the total *SF* of (6.4 to 10.5), while the temperature represents only (3.5–3.9) out of the total *SF*. Under alkaline conditions at 80 °C, KOH concentration and the increase in pretreatment duration barely affect the severity factor that remains in the range (10.1 to 10.5).

The solubilization degree of the raw initial BSG represents 1.2 ± 0.1% of the total COD added (Fig. 2). When the BSG is 80 °C-water pretreated, the BSG solubilization degree increases to 3.0 ± 0.1% and is the lowest of all the pretreatment conditions tested (Fig. 2).

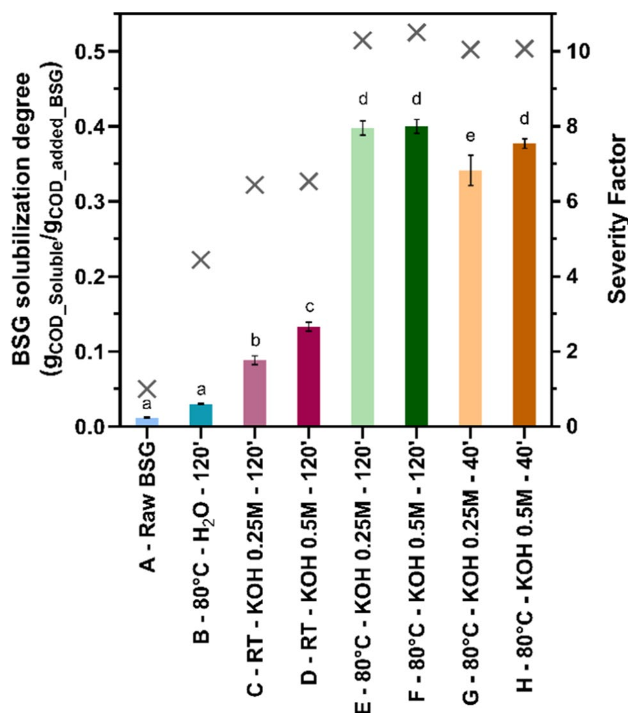

Fig. 2 BSG solubilization degree after pretreatment (left axis, bar plot) and the severity factor (right axis, cross dots) associated to the pretreatment conditions. Different letters represent significantly different groups (Tukey multiple comparison test, $p < 0.05$). RT represents 22 °C (reference temperature)

The difference in BSG solubilization degree between the raw BSG reference condition and the 80 °C-water pretreated is not-significant according to the Tukey's multiple comparison test (at *p*-value 0.6046). The BSG solubilization degree of the alkaline pretreatment at room temperature (RT) was multiplied by a factor of nearly 3 and more than 4 for the 0.25 M KOH and the 0.5 M KOH conditions, respectively, as compared to the 80 °C-water condition. These improvements are significant (*p*-value < 0.05) (Fig. 2).

The most severe pretreatments (*SF* = 10.5 and 10.3) were observed when the temperature effect was combined with the alkaline effects for 120 min. The initial solubilization degree reached the highest values of 39.7 ± 1.0 and 40.0 ± 0.9% of the total COD added, respectively, for the 120 min 0.25 M KOH and 0.5 M KOH at 80 °C pretreatments (Fig. 2). According to the Tukey multiple comparison test, the shorter pretreatment duration, decreasing from 120 to 40 min, significantly decreased the solubilization degree only for the 0.25 M KOH at 80 °C for 40-min condition compared to the three other 80 °C alkaline pretreatment conditions (*p*-value < 0.01) (Fig. 2).

3.2 Kinetics of fermentation of pretreated BSG and conversion to metabolites

Figure 3A shows that raw BSG and the least severe pretreatments (80 °C-water and room temperature under both alkaline concentrations) did initially solubilize only a small fraction of the organic solubilizable matter of the BSG; the fermentation released additional soluble COD components with time, mostly as fermentation metabolites (Fig. 3B). The most severe pretreatments combining temperature and alkalinity effect had solubilized initially between 35 to 40% of the total COD_{BSG} added before the fermentation. The fermentation did not solubilize more COD and even degraded/removed part of the soluble COD (Fig. 3A). The organic matter solubilized by the pretreatment was not more fermentable, as Fig. 3 B shows no increase of fermentation metabolites, as compared to the room temperature alkaline pretreatments. The soluble COD concentration (Fig. 3A) higher than the total identified metabolite COD concentration (Fig. 3B), especially for the most intensive pretreatment, suggests that part of the solubilized components were not fermentable. Figure 3 B shows that all alkaline pretreatments increased the fermentation kinetics, gaining 2 to 4 days of lag phase, and reaching similar total metabolite concentrations as the raw BSG but about 10 days earlier

Figure 4 shows that the production of the total metabolites, H_2 and CO_2, began sharply during the first two days of fermentation for all the alkaline pretreatment conditions and slowly for the 80 °C-water pretreated condition, while a lag phase of almost 4 days was observed for the reference raw BSG. After day 4, the alkaline pretreatment conditions have produced in average 74 ± 4% of the final total metabolites, 96 ± 2.2% of the final total H_2, and 87 ± 4.3% of the final total CO_2. Over the same time, the raw BSG reference condition produced only 4.7 ± 0.2% of the final total metabolites, 15.1 ± 0.1% of the final total H_2, and 9.8 ± 0.1% of the final total CO_2. The BSG 80 °C-water pretreated presented an intermediate fermentation activity with the production of 49.4 ± 0.1% the final total metabolites, 79.4 ± 0.3% of the final total H_2, and 52.1 ± 0.1% of the final total CO_2 (Fig. 4A). At the end of the fermentation, the average increase in total metabolite production obtained after alkaline pretreatment compared to the reference raw BSG condition was $29 \pm 7\%_{Tot_metabo}$. The highest H_2 production was obtained when BSG was pretreated under alkaline condition at room temperature (Fig. 4B). The three highest final CO_2 productions corresponded to BSG pretreated with 0.5 M KOH. The three intermediate CO_2 productions corresponded to BSG pretreated with 0.25 M KOH (Fig. 4C). Within both series, the CO_2 production increased as the severity factor decreased.

At day 4, the metabolite production did not start for the raw BSG reference condition, in contrast to the BSG pretreated with water at 80 °C that had already reached half of its final production (Fig. 5). Starting from BSG pretreated under alkaline conditions, metabolite production has already reached values ranging from $13.6 \pm 0.2\ g_{COD}\ L^{-1}{}_{ML}$ to $18.0 \pm 0.8\ g_{COD}\ L^{-1}{}_{ML}$.

The dotted line on Fig. 5 shows the total metabolite production in the raw BSG reference condition on day 14. According to the Tukey's multiple comparison test, the productions achieved for all alkaline conditions are already similar to this value after 4 days of fermentation (no significant difference at *p*-value 0.005, except for the BSG pretreated under 0.25 M KOH at RT for 120 min showing a *p*-value of 0.0078) (Fig. 5). The highest production is obtained when BSG is pretreated at 80 °C with 0.25 M KOH for 120 min and reached $18.0 \pm 0.8\ g_{COD}\ L^{-1}{}_{ML}$. This represents a significant increase of $33\%_{Total_metabolite_COD}$ compared to its corresponding condition under 0.25 M KOH at RT (Fig. 5).

At day 14, the final production of metabolites from the raw reference BSG condition reached $16.7 \pm 1.9\ g_{COD}\ L^{-1}{}_{ML}$. All the BSG pretreated under alkaline conditions produced

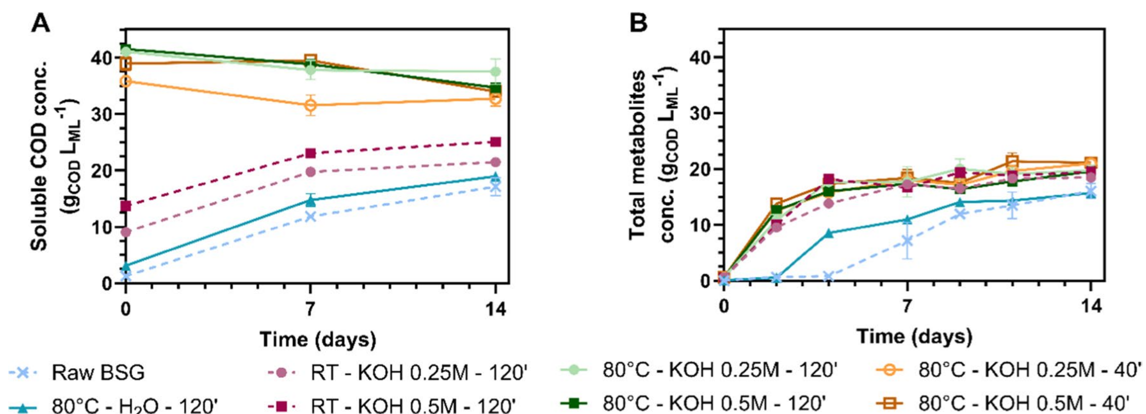

Fig. 3 Evolution of the soluble COD concentration (**A**) and the total identified fermentation metabolites (**B**) during the 14 days of fermentation after all tested pretreatment conditions. When error bars are not visible, they are present but smaller than the dot symbol

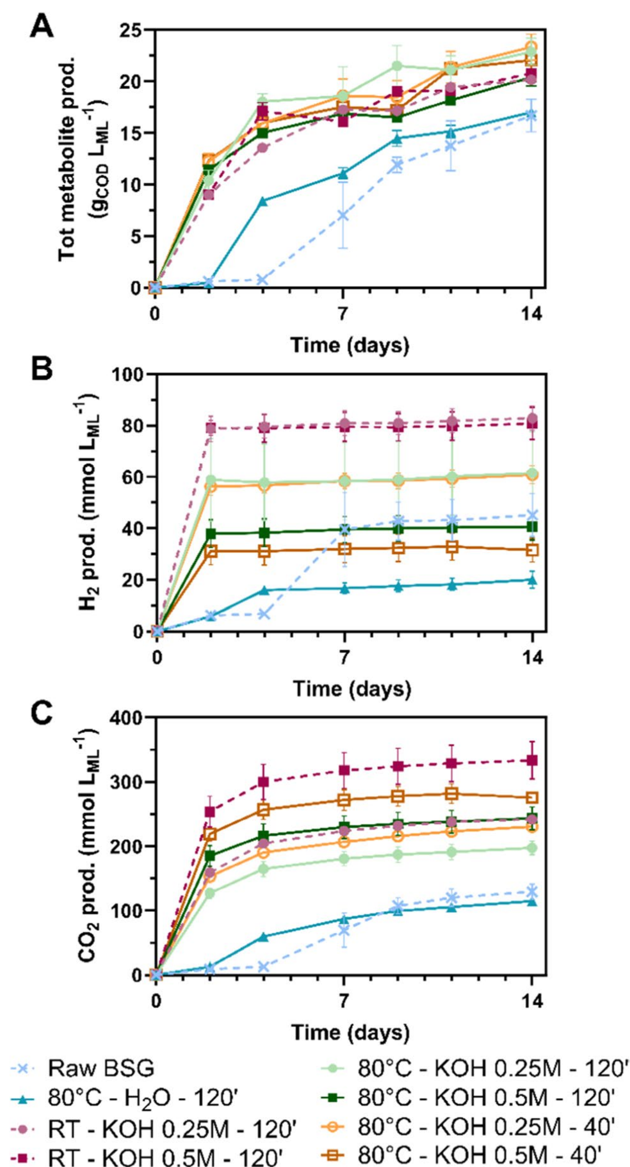

Fig. 4 Evolution of the cumulated production of total metabolite (**A**), H_2 (**B**), and CO_2 (**C**) during the 14 days of the fermentation for all the pretreated BSG conditions

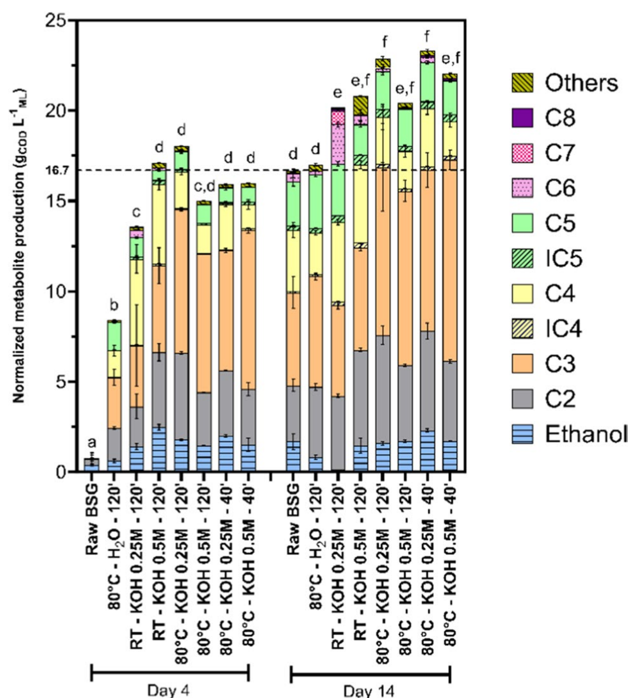

Fig. 5 Total and individual metabolite production normalized by unit volume of mixed liquor (L_{ML}), after 4 (left) and 14 days (right) of fermentation for all the pretreated BSG conditions. Others include all the metabolites analyzed that are not specified in the graph but in the "Material and method" section. The dotted line indicates the total metabolite production after the 14 days of fermentation of raw BSG. Different letters represent significantly different groups (Tukey multiple comparison test, $p < 0.05$)

a significantly enhanced total metabolite compared with the raw BSG. The percent increases ranged from a minimum of 21% to a maximum of 39% for BSG pretreated under 0.25 M KOH at RT for 120 min and 0.25 M KOH at 80 °C for 120-min condition, respectively (Fig. 5). The highest final productions of metabolites are obtained with BSG pretreated at 80 °C with 0.25 M KOH for 120- and 40-min, reaching, respectively, 22.9 ± 1.3 g_{COD} $L^{-1}{}_{ML}$ and 23.3 ± 1.3 g_{COD} $L^{-1}{}_{ML}$. The latter are significantly different from metabolite production detected under their reference condition (RT, KOH 0.25 M for 120 min), with a percent increase of 13.4 and 15.6$\%_{Total_metabolite_COD}$ respectively (Fig. 5).

Short chain carboxylates, particularly acetate, propionate, and butyrate were the most produced metabolites for all the conditions (Fig. 5). The alkaline room temperature pretreatment conditions and the raw BSG reference condition are the only three conditions producing more than 2$\%_{total_metabolite_COD}$ of medium-chain carboxylates (MCC). The BSG pretreated at RT for 120 min, with 0.25 M KOH produced 2.2 g_{COD} $L^{-1}{}_{ML}$ of caproate and 0.8 g_{COD} $L^{-1}{}_{ML}$ of heptanoate (representing 10.9 and 3.8$\%_{total_metabolite_COD}$, respectively). This is the only condition with almost no ethanol left in the mixed liquor at the end of the fermentation (0.1 g_{COD} $L^{-1}{}_{ML}$) (Fig. 5).

At the end of the fermentation, the conversion yield of BSG to metabolites reached similar values around 15$\%_{COD_BSG+Inoc}$ for raw BSG and 80 °C-water pretreated BSG, while the conversion yields are slightly higher, comprised between 18.6 ± 1.1 and $21 \pm 1.3\%_{COD_BSG+Inoc}$ for BSG pretreated under alkaline conditions (Fig. 6). The higher the severity of the pretreatment, the higher the non-identified soluble COD proportion.

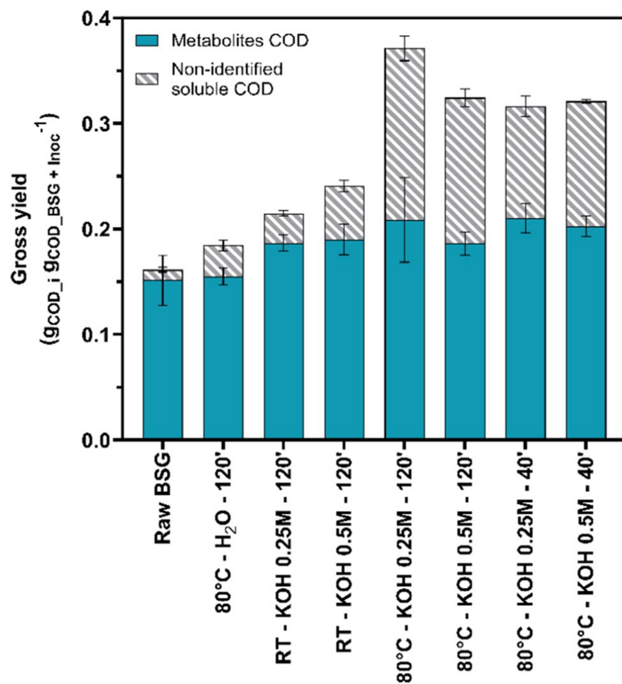

Fig. 6 Gross COD conversion yields of initial organic matter to identified soluble metabolites and non-identified soluble COD after 14 days of fermentation, for all the pretreated BSG conditions. Non-identified soluble COD corresponds to the difference between soluble COD and the total concentration of identified and quantified metabolites, normalized by the total amount of input COD. *i* corresponds to individual metabolites and soluble COD

4 Discussion

4.1 Benefits of alkaline pretreatment on brewer's spent grain fermentability

4.1.1 Alkaline pretreatments improve fermentation kinetics and yields

The main interest of the alkaline pretreatment is the improvement of the fermentation kinetics. Whatever the pretreatment time, temperature and hydroxide concentration, alkaline pretreatment achieved in 4 days a similar production of total soluble metabolites compared to that obtained with raw BSG after the 14 days of fermentation (Figs. 3 and 5). This gain of 10 days enabled by the alkaline pretreatment represents an increase in productivity, allowing at least 3.5 times more BSG to be processed in the same period of time and the same bioreactor volume.

At the end of 14 days of fermentation, the total metabolite production increased on average by 30 ± 9% under alkaline conditions compared with the raw BSG reference condition (Figs. 4 and 5). An equivalent improvement is also reflected in terms of productivity (Table 3). However, the best metabolite productivities are achieved after 4 days of fermentation if the BSG is pretreated under alkaline conditions (Table 3). Indeed, under alkaline conditions, 4 days of fermentation is sufficient to produce 74 ± 4% of the total final metabolites (Fig. 4). If these fermentations continue until the end of the 14 days, metabolite productivity drops considerably (Table 3). This suggests that pretreatment alkalinity is the factor influencing the most the rate of biomass conversion to metabolites. Nevertheless, the productivity at day 4 under alkaline condition oscillates between 3.4 and 4.5 $g_{COD_metabolites}$ $l^{-1}·d^{-1}$, respectively, for the 0.25 M KOH at RT, and the 0.25 M KOH at 80 °C conditions, indicating that temperature, hydroxide concentration, and pretreatment holding time, also have an influence on the biomass conversion but at a lower level (see Sect. 4.2).

Table 3 Metabolite productivity comparison between raw BSG and alkaline pretreated BSG after 4 and 14 days of fermentation

Conditions	Productivity [$g_{COD_metabolites}$ $L^{-1}·d^{-1}$]	
	After 4 days	After 14 days
Raw BSG	0.2	1.2
Alkaline pretreated BSG	3.4–4.5	1.4–1.7

4.1.2 Solubilized components are not all fermentable

Regarding all the alkaline pretreatment conditions, the BSG initial solubilization degree is higher compared to raw BSG reference condition or the 80 °C water pretreatment and increases with the severity of the pretreatment (Fig. 2). This leads to an increase in soluble COD directly accessible to microorganisms at the start of the fermentation (Fig. 3). However, only part of the initial solubilized COD leads to additional metabolite production, indicating that only part of the extra soluble COD is well fermentable by microorganisms. Additionally, the more severe the pretreatment, the higher the release of non-identified-non-fermentable soluble COD (Figs. 3 and 6). This means that for mixed microbial acidogenic fermentation, it is not necessary to use more aggressive alkaline pretreatments, the extra solubilized COD remains unfermentable.

According to the literature, alkaline pretreatment makes the lignocellulose to swell, extract carbohydrates, solubilized lignin and/or lignin-carbohydrate complexes, acetyls groups and uronic acid, de-crystalizes cellulose and removes part of the lignin [21, 26–28, 30, 38, 39]. In this case, the carbohydrate and acetyl group solubilizations are probably directly linked to the increase in total metabolite production, since carbohydrates can be metabolized by acidogens, while the lignin and lignin-carbohydrate complexes are probably part of the unconverted COD solubilization (Figs. 2 and 6).

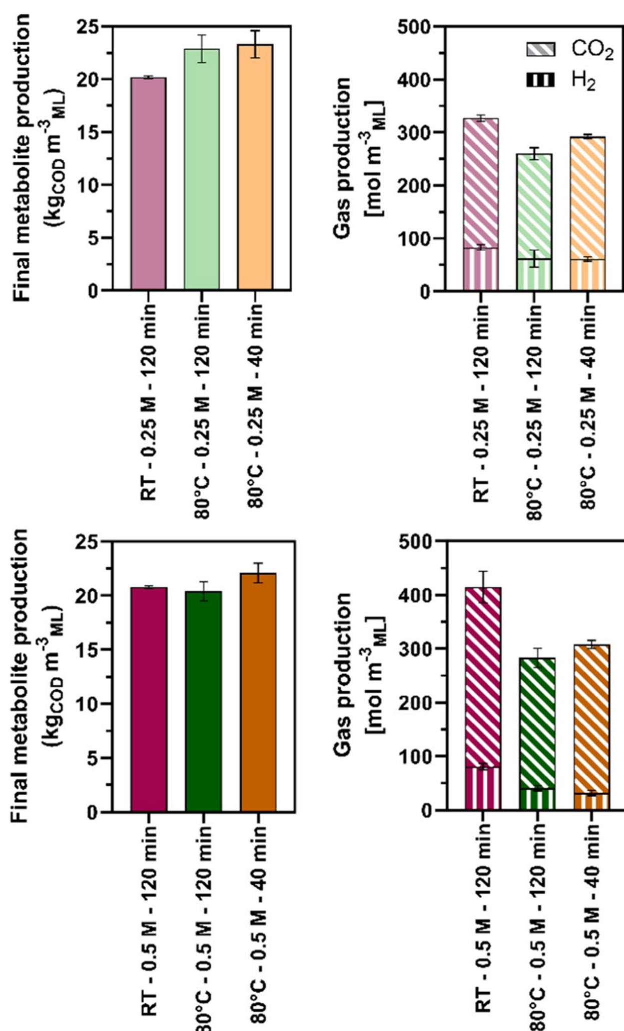

Fig. 7 Final production of metabolites (left), H_2 and CO_2 (right) at the end of the fermentation of BSG pretreated under hydroxide concentration of 0.25 M (top) and 0.5 M (bottom)

4.2 Moderate benefits from increased temperature, hydroxide concentration and pretreatment time

4.2.1 Increasing the pretreatment temperature

The only condition that exhibited a significant increase in total metabolite production at days 4 and 14 when the pretreatment temperature was increased from RT to 80 °C occurs under 0.25 M KOH (Figs. 4, 5, and 7). This explains the highest metabolite productivity of 4.5 $g_{COD_metabolites}$ $l^{-1}\cdot d^{-1}$ obtained with BSG pretreated with 0.25 M KOH at 80 °C for 120 min compared to the 3.5 $g_{COD_metabolites}$ $l^{-1}\cdot d^{-1}$ obtained under the same conditions at RT (Fig. 5 and Table 3). This suggests that, at an industrial scale, if temperature of 80 °C is available, it could be valorized to maximize the metabolite productivity.

Under aqueous conditions without pH alkalinization, increasing temperature from RT to 80 °C alone is not enough to improve the BSG fermentability. This can be explained by BSG being already exposed to the same range of temperature in water, around 78 °C, at the end of the mashing step of the brewing process to inactivate amylases [40]. Hot-water soluble components are already extracted at this step and not present anymore in our raw BSG and 80 °C water pretreatments. This could explain that the 80 °C-water pretreatment has no significant impact neither on the initial BSG solubilization nor on the final total metabolite production, as compared to the raw BSG (Figs. 2 and 5).

4.2.2 Increasing the hydroxide concentration

Increasing the hydroxide concentration from 0.25 M to 0.5 M at 80 °C, whatever the pretreatment duration, led to a decrease in the final production of metabolite and H_2, combined with an increase in CO_2 production (Fig. 7). Although an increase in CO_2 production could mean a better conversion of biomass to reduced metabolites, in this case it is more likely to result from a higher release of the CO_2 absorbed during the neutralization step in the form of potassium carbonate. In summary, increasing hydroxide concentration during pretreatment should be avoided, since it increases reagent consumption (KOH) and decreases the yield of desired target metabolites (Fig. 7).

4.2.3 Increasing the pretreatment holding time

After 14 days of fermentation, the longer duration of pretreatment at 80 °C under both alkaline conditions slightly decreased the final metabolite production as well as the CO_2 production and had no impact on the H_2 production (Figs. 4 and 7). However, increasing the duration did not result in a significant increase in total metabolite production at day 4 (Fig. 5). This means that 40 min of alkaline pretreatment sufficiently affects the BSG lignocellulosic structure to allow a quicker microbial digestion.

4.3 Medium-chain carboxylate production

A normalized production of medium-chain carboxylate (MCC) higher than 0.5 kg_{COD} L^{-1} (representing at least 2.5$\%_{total_metabolite_COD}$) was only obtained from raw BSG or BSG pretreated at RT. MCC production was reduced with harsher pretreatment conditions (alkalinity, temperature, and duration) (Fig. 5). The BSG pretreated at RT under 0.25 M KOH produced the highest combined production of medium chain carboxylates (hexanoate, heptanoate, and octanoate) among all the conditions tested. Under this condition only, no ethanol remained at the end of the fermentation, suggesting a complete consumption of ethanol for the chain

elongation metabolism. Ethanol was probably used as electron donor for the reverse β-oxidation pathway leading to the formation of medium chain carboxylates [18, 19].

The production of MCC starting from BSG pretreated with KOH is in line with the study of Ji et al. [41], showing that a production of caproate from acetate and ethanol in mixed culture fermentation is possible at K^+ concentration of 12.5 g L^{-1}. Nevertheless, with this K^+ concentration, they showed a slight inhibitory effect increasing the lag phase of the caproate production and decreasing the caproate production yield [41]. In the present study, the K^+ concentration reached 9.75 and 19.5 g L^{-1}, respectively, for the 0.25 M and 0.5 M KOH pretreated BSG and allowed MCC production under both conditions. However, the MCC production strongly decreased for the three 0.5 M KOH pretreated BSG conditions compared to the 0.25 M KOH conditions, highlighting a possible inhibition effect of the K^+ concentration of 19.5 g L^{-1}.

It can be hypothesized that the microorganisms responsible for the chain elongation originate from the BSG and do not come from the activated sludge used as inoculum. Indeed, if the chain elongator would come from the activated sludge, then the pretreatment temperature and duration would not have any effect on the MCC production. However, both temperature and duration decrease the MCC production. Considering that the elongator comes from the BSG, the temperature of 80 °C would inactivate a large part of the elongating microbial community, the alkaline pH (pH > 12) and the K^+ concentration of 19.5 g L^{-1} would inhibit the remaining chain elongators, resulting in the poorest production of MCC when both conditions are cumulated, and whatever the pretreatment duration (Fig. 5). The lower production of MCC after fermentation of raw BSG compared to the BSG pretreated with 0.25 M KOH at RT would hypothetically be explained by the low availability of ethanol and acetate during the beginning of the fermentation, resulting in a microbial elongator population decline and a lower final MCC production compared to the BSG pretreated with 0.25 M KOH at RT. To confirm these hypotheses, evolution of microbial populations during fermentation after pretreatment should be the subject of further research.

4.4 CO_2 neutralization as a relevant pH adjustment technique for microbial fermentation

Before starting the fermentation, neutralizing the alkaline pH by sparging CO_2 seems to be an adequate technique. For all the conditions, a pH value close to 7 was reached, after one or two steps of CO_2 sparging. An acidic, near neutral or neutral pH are usually used to start acidogenic fermentations targeting chain elongation [15]. The use of phosphoric acid to decrease the pH to 6 was only used to mimic the raw BSG reference condition starting at pH 6. Nevertheless, as the fermentation naturaly acidify the broth, the initial artificial acidification with a strong acid (H_3PO_4 in the present case) can possibly be avoided in the future.

4.5 Severity factor as a reference indicator for standardizing pretreatments but not as an indicator of BSG fermentability

Since the temperature evolution during the heating and cooling phases of pretreatment varied among the experiments, the severity factor was used to standardize all the results (Fig. 1). The low variation of the SF between conditions indicates that the slight variation of pretreatment duration and the temperature oscillation are negligible comparatively to the change in the experimental conditions.

The higher the severity of the pretreatment, the higher the solubilization of the BSG at the end of the pretreatment phase (Fig. 2). Alkalinity and temperature seem to have a synergetic effect on BSG solubilization, since when these two effects are combined, the resulting degree of solubilization is more than twice the sum of the degrees of solubilization of each pretreatment effect taken separately. However, this synergistic positive impact is not recovered when looking at total final metabolite production (Figs. 2 and 6). Consequently, neither the severity factor nor the initial BSG solubilization should be used as a unique predictor of carboxylate fermentation efficiency from a complex organic substrate such as BSG.

5 Conclusion

Alkaline pretreatments of brewer's spent grains considerably boosted its fermentation kinetics and conversion yields. Fermentation of alkaline-pretreated BSG produced in 4 days a quantity of metabolites similar to that obtained after 14 days of fermentation of raw BSG. This substantial gain in productivity was mainly influenced by the alkalinity of the pretreatment. For the same fermentation time, alkaline pretreatments would allow to convert 3.5 times more BSG than the reference condition. The pretreatment temperature was a decisive factor and should be considered regarding the type of carboxylate targeted. Alkaline 0.25 M KOH at RT pretreatment should be preferred if production of medium-chain carboxylates is targeted, while 0.25 M KOH at 80 °C should be used to maximize the productivity of short-chain carboxylates. Increasing hydroxide concentration from 0.25 M to 0.5 M KOH should be avoided since it did significantly increase neither the targeted metabolites nor the H_2 production but increased the CO_2 production and the reagent consumption. Sparging CO_2 in the pretreated alkaline liquor appeared to be a suitable method to decrease the pH to 7, suitable for fermentation. The pretreatment severity factor and the

initial solubilization degree of the organic substrate did not appear to be effective indicators of potential gains in either carboxylate production or productivity. Better understanding of the mechanisms involved in the evolution of such a complex system would benefit from further investigation of the evolution of the BSG chemical composition during the alkaline pretreatment and of the microorganisms involved in the acidogenic fermentation.

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Author contribution GH: conceptualization and methodology, investigation, experimentation, data treatments and analysis, data visualization, resources, and writing original draft. AI, EW, and TN: experimentation and data treatments. BS and PG: supervision and draft review. All the authors read and approved the final manuscript.

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Data availability The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

Declarations

Competing interests The authors declare no competing interests.

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