Methane Production Using Brewery Spent Grain: Optimal Hydrothermolysis, Fermentation Of Waste And Role Of Microbial Populations

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Abstract

The hydrothermolysis variables temperature (150 - 210 °C) and time (10 - 20 minutes) were assessed to improve hydrolysis efficiency of brewery spent grain (BSG) for renewable energy generation. The intensification of the pretreatment was expressed by the severity variation (2.8 - 4.5) and the process was optimized with methane production of 411.6 ± 7.2 mL. g -1 STV (severity 4.2). The fermentation-methanogenesis of BSG and hydrolysate resulting from BSG hydrothermolysis process under severity of 4.2 (210 o C for 10 min.) was evaluated by central composite design (CCD) with the variables operation temperature (30 - 60 °C), BSG concentration (7.3 - 20.7 g.L -1) and hydrolysate (0 - 12.4 mL). The higher methane production observed was 305.8 mL.g -1 STV, with 14 g.L -1 of BSG, without hydrolysate at 45 °C. The main soluble metabolites were acetic acid (233.17 mg.L -1) and butyric acid (156.0 mg.L -1). On other hand, the lower methane production (108.5 ± 2.0 mL. g -1 STV) verified was 14 g.L -1 of BSG, 6.5 mL of hydrolysate at 60 °C, which revealed that in this condition propionic acid (947.4 mg.L -1) and acetic acid (599.2 mg.L -1) were expressive. In the optimal fermentation-methanogenic condition of pretreated BSG, Macellibacteroides and Sphaerochaeta (15.9 and 14.7%, respectively) were identified, as well as archaea similar to Methanosaeta (80.4%), favoring the acetoclastic methanogenic pathway.

1. Introduction

Due to population growth, energy consumption is expected to increase by approximately 50% from 2018 to 2050, reaching 1090 quadrillion British thermal units (Btu). The highest energy consumption is within the industrial sector, which is responsible for more than 50% of the total in relation to other sectors [1]. Therefore, national energy security, continuous use of renewable energy sources and energy recovery from waste are very relevant issues.

The conversion of residual lignocellulosic biomass, from various processes, into bioproducts and biofuels is environmentally suitable and in agreement with the concept of sustainability [2]. Whether biodiesel, bioethanol, hydrogen or methane, these biofuels can replace conventional fossil sources and potentially reduce carbon emissions [3]. Methane can be produced from diverse feedstock, including lignocellulosic materials, such as municipal, food and agroindustry wastes, considering availability, abundance and organic matter content.

Beer is one of the most appreciated and consumed drinks in several countries, regardless of culture or climate. Despite the technological advances in large-scale beverage production, some residues are process inherent, highlighting the brewery spent grain (BSG), which ranges from 14 to 20 kg for every 100 liters of beer produced [4]. Because of the wide, year-round availability of BSG, anaerobic digestion from this material for biofuel production is interesting and promising. The BSG use for bioproducts process like sugars extraction, proteins, enzymes and antioxidant acids have been evaluated, although the main destination is still animal feed [5]. BSG lignocellulosic structure, whose composition is 17-26% of cellulose, 19-42% of hemicellulose and 12-28% of lignin [6], can provide resistance to its degradation.

Hydrolysis of lignocellulosic material is a determining step of anaerobic digestion, since hydrolytic enzymes released by microorganisms are limited to completely disrupt the complex lignocellulosic structure. This step emerges in few days for proteins and lipids, and can occur within several days for lignocellulosic components [7]. Thus, the study of anaerobic digestion under different conditions and pretreatments applied in lignocellulosic biomass are necessary.

The pretreatment is employed to prepare the lignocellulosic substrate for microbial activity, in order to make it more accessible to the microbial consortium [8]. Therefore, some viability criteria must be considered, such as effective reduction of particle size, increased substrate porosity, degradability and solubility, production inhibitors and energy consumption [9]. Depending on the treatment applied, they can be classified as physical, chemical or biological.

Among the physical pretreatments, hydrothermolysis is related to the application of high temperatures, pressure and time, through which the rupture of the chemical bonds of the cell wall occurs and the release of cellular components in the liquid phase, making soluble and biodegradable portions that were previously insoluble [10]. The combination of the factors temperature and time of hydrothermolysis results in different pretreatment severities.

Camargo et al.[10] conducted the *citrus* peel hydrothermolysis under 10 bar, at 180 °C for 15 min. Rabelo et al.[11] pretreated the sugarcane bagasse by hydrothermolysis at 200 °C for 10 min. Ahmad et al. [12] evaluated the factors temperature (160, 180 and 200 °C) and time (5, 12 and 19 min) of hydrothermolysis from sugarcane bagasse pretreated by hydrogen peroxide. According to [12], 180 °C for 12 min were the optimized factors to increase the methane production up to 118.64%. Thus, it can be observed that optimal hydrothermolysis severity ranges according to the type of substrate.

Bochmann et al. [13] evaluated the methane production effects from BSG under 100 to 200 °C for 15 minutes, and verified optimal production at 140 °C (467.6 $NmL.g^{-1}$ VS), 14% more than the methane production obtained from the *in natura* substrate (409.8 $NmL.g^{-1}$ VS). Menardo et al. [14] also evaluated the temperature range (90 and 120 °C) of rice straw pretreatment for 30 minutes, and obtained increase of 24.5% on methane production with pretreated substrate (261 $NmL.g^{-1}$ VS) in relation to *in natura* (197 $NmL.g^{-1}$VS).

Conversely, [15] related greater methanogenic potential from the liquid fraction (376 ± 22 $mLCH_4.g^{-1}$ SV) compared to the solid fraction (75 ± 6 $mLCH_4.g^{-1}$ SV) resulting from the sugarcane bagasse hydrothermolysis process (150 °C for 40 min). Although the solid fraction was rich in sugars, the authors related the reduced methane production to hydroxymethylfurfural (HMF) and furfural (0.11 and 0.42 $g.L^{-1}$, respectively) verified in the assays with bagasse, while high organic matter content was observed in the assays with hydrolysate.

During the disruption of lignin-hemicellulose-cellulose structure under high temperatures, furanic and phenolic compounds are inevitable, which are toxic and can inhibit the growth of bacteria and archaea.

Phenolic compounds are released from the partial lignin rupture, while the 5-HMF and furfural are formed by the dehydration of pentoses and hexoses resulting from hemicellulose and cellulose degradation. In addition, formic acid is produced from 5-HMF and furfural, while levulinic acid is due to 5-HMF degradation [16]. However, contradictory, delignification is necessary to increase the biodegradability of waste [17].

Thus, to obtain reduced release of inhibitors concurrently with increasing solubilization of sugars, and consequently, the fermentation-methanogenesis efficiency, the lignocellulosic biomass pretreatment parameters can be statistically optimized [18]. In this study, BSG hydrothermolysis temperature (150 - 210 °C) and time (10 - 20 minutes) factors were evaluated regarding the maximum methane production. Then, the fermentation process was optimized through the central rotational composite design (CCD) and response surface methodology (RSM) in order to increase methane production. The effect of the hydrolysate from the hydrothermolysis process (0.6 - 12.4 mL), operation temperature (30 – 60 °C) and BSG concentration (7.3 – 20.7 $g.L^{-1}$) was evaluated. Subsequently, bacteria and archaea taxonomic characterization was carried out to map the BSG anaerobic digestion under optimal methane production conditions.

2. Materials And Methods

2.1 Substrate and inoculum

Brewery spent grain (BSG) used as substrate in the methane production assays was donated by Kirchen (São Carlos, SP, Brazil). The BSG was washed, dried at natural air temperature and milled with knife mill (kind Willey SL-31, trademark Solab) [19] and stored at 4 ^{o}C.

The hydrothermal pretreatment (hydrothermolysis) was carried out by the addition of milled BSG and 210 mL of water in the hydrothermal system [20]. At the end of the reaction, the solid fraction (pretreated BSG) was separated from the liquid fraction (hydrolysate) with a 2 mm diameter pore sieve. In the optimization assays under hydrothermolysis conditions (B1-B7) only the solid fraction (10 $g.L^{-1}$) was used, while in the optimization assays under fermentative-methanogenic conditions (C1-C17), the solid and liquid fractions were used at different ratios (Table 2).

Granular sludge from upflow anaerobic sludge blanket reactor (UASB) from a poultry slaughterhouse (Ideal Poultry, Pereiras, SP, Brazil) was used as inoculum source. The inoculum was kept at 4 ^{o}C and, prior to batch reactors inoculation, the granules were ground were in a domestic blender, kept for 7 days at 37 ^{o}C for the recovery of microbial activity and removal of biodegradable organic matter, eliminating possible methane production from sludge. The control assays (C) were conducted under the same conditions as the others, without BSG, to verify the methanogenic potential from sludge.

According to analysis of the solid series [21], the sludge used as inoculum was composed of 60.7 $g.L^{-1}$ total solids (TS) and 56.0 $g.L^{-1}$ of total volatile solids (TVS).

2.2 Batch reactors

The assays for the optimization of hydrothermolysis conditions (B1-B7, Table S1, Supplementary Material) and the assays for the optimization of fermentative-methanogenic conditions (C1- C17) were performed in triplicates of batch reactors with 125 mL of reactional medium and 125 mL of headspace. 2 $g.kg^{-1}$ TVS of inoculum and 1 $g.kg^{-1}$ TVS of BSG (inoculum /substrate, ratio 2) were added according to [18]. The Zinder reaction medium [22], plus yeast extract (1$g.L^{-1}$) and sodium bicarbonate (10% p/v), was prepared according to [12]. The reaction medium pH was adjusted to 7.0, the reactors were subjected to N_2 atmosphere (100%) for 10 minutes, closed with a butyl cap and plastic screw and incubated.'

2.3 Optimized fermentative-methanogenic conditions from BSG

The optimization assays of fermentative-methanogenic conditions (C1 to C17) were performed with analysis of the variables operation temperature (x_1), pretreated BSG concentration (210 °C; 10 min, severity 4.2) (x_2) and volume of liquid fraction residual from hydrothermolysis (hydrolysate) (x_3). The matrix of combinations between the variables was assembled according to central composite design (CCD) 2^3 (Table1). The hydrolysate had pH 4.3, and organic matter concentrations, total soluble carbohydrates, phenols and acetic acid, respectively, of 17554 ± 21.6 $mg.L^{-1}$, 4240.0 ± 16.97 $mg.L^{-1}$, 767.0 ± 10.6 $mg.L^{-1}$ and 93.0 ± 14.1 $mg.L^{-1}$.

Table 1 DCC matrix for evaluate temperature (x1), BSG concentration (x2) and hydrolysate volume (x3) on methane production

	T (x_1)	BSG (x_2)	Hydrolizate (x_3)	T (°C)	BSG ($g.L^{-1}$)	Hydrolizate (mL)
C1	-1	-1	-1	36	10	3
C2	1	-1	-1	54	10	3
C3	-1	1	-1	36	18	3
C4	1	1	-1	54	18	3
C5	-1	-1	1	36	10	10
C6	1	-1	1	54	10	10
C7	-1	1	1	36	18	10
C8	1	1	1	54	18	10
C9	-1.68	0	0	30	14	6.5
C10	1.68	0	0	60	14	6.5
C11	0	-1.68	0	45	7.3	6.5
C12	0	1.68	0	45	20.7	6.5
C13	0	0	-1.68	45	14	0.6
C14	0	0	1.68	45	14	12.4
C15	0	0	0	45	14	6.5
C16	0	0	0	45	14	6.5
C17	0	0	0	45	14	6.5

Interactive effects between the variables maximum methane production response (*P*) were analyzed using the response surface methodology by Statistica 9.0 software package. The significance of experimental results (90% confidence level) was validated with F-test analysis of variance (ANOVA). For the statistical model validation, reactors were operated in triplicates for optimal conditions of hydrothermolysis and fermentative-methanogenesis.

2.4 Physicochemical, chromatographic and microscopy analysis

In natura and pretreated BSG (210 °C for 10 min) were analyzed relative to insoluble lignin content (Klason lignin) and total soluble carbohydrates according to National Renewable Energy Laboratory protocol (NREL) [23] and by the phenol-sulfuric acid colorimetric method [24], with glucose as standard.

Morphological structure was analyzed by scanning electron microscopy (SEM) and samples were prepared in accordance with [25], and the observation was performed using the Digital Scanning Microscope (DSM), Zeiss, model DSM-960.

Organic matter concentration was measured by Chemical Oxygen Demand (COD), phenols concentration by the 4-aminoantipyrine method and pH according to Standard Methods for the Examination of Water and Wastewater [21]. The total soluble carbohydrates analysis used the phenol-sulfuric acid colorimetric method [24], with glucose as standard.

Organic acids were quantified by a Shimadzu® (GC-2010) gas chromatograph according to [26] and biogas composition was determined using a Shimadzu® gas chromatograph (GC-2010) by [27]. The cumulative methane production data were adjusted using the Gompertz model (Equation 1) modified by [28] :

$$CH_4 = P.exp\{-exp[(Rm.e)/P.(\lambda - t) + 1]\} \quad \text{(Equation 1)}$$

Where, CH_4= cumulative methane production, *P* = maximum methane production (mL de $CH_4.g^{-1}$ TVS); R_m= maximum methane production rate (mL de $CH_4.g^{-1}$ TVS. h); λ= time to start methane production (h); *e* =Euler number (2.71828182). The adjusted experimental data was performed for the average of the values obtained by triplicate reactors using the software Origin Pro 9.0 software.

2.5 Microbial community analysis

The microbial characterization by massive sequencing was carried out with samples collected from the batch reactors at the end of the operational period. The samples were washed with PBS buffer (NaCl 8%, KCl 0.2%, Na_2HPO_4 1.44%, KH_2PO_4 0.24%) and total DNA was extracted using protocol according to [29]. Purity (260/280 nm ratio) and quantity ($ng.L^{-1}$) of the extracted DNA were measured in a Nanodrop spectrophotometer Thermo Fisher Scientific, USA).

Massive sequencing was accomplished by Genone Biotechnologies (Brazil-Rio de Janeiro, RJ), on a HiSeq PE250. The primers 341F/806R were used for the *Bacteria* domain (V3V4 RNAr 16S region) and U519F/806R (16S V4 region) for the *Archaea* domain. The sequences were sent to the National Center for Biotechnology Information (NCBI) database, under identification SAMN 13230687 (B2) and SAMN 13230692 (C18).

Operational taxonomic units (OTUs) were clustered considering 97% of similarity and the bacteria and archaea populations with relative abundance greater than 1% were considered in the results and discussion section.

3. Results And Discussion

3.2 Methane production with BSG pretreated by hydrothermolysis, severity 4.2

The range of BSG hydrothermolysis temperature (150 to 210 °C) and time (10 to 20 minutes) according to the central composite design (CCD), showed maximum methane potential production (*P*) from 235.0 ± 10.1 to 411.6 ± 7.2 mL. g^{-1} STV under different pretreatment severities (from 2.4 to 4.5) (Figure S1 and Table S2, Supplementary Material). Optimal severity of 4.2 for *P* (411.6 ± 7.2 mL. g^{-1} STV) was confirmed in assay B2, with methane production rate (*Rm*) of 2.1 ± 0.16 $mL.g^{-1}$ STV .h, the condition in which total carbohydrates and organic matter removal was verified (90.4% and 81.1%, respectively) (Figure S3 and S4, Supplementary Material). In B1 and B3, with BSG under lower hydrothermolysis severities (2.5 and

2.8, respectively), lower values of *Rm* (0.9 ± 0.06 and 0.8 ± 0.14 mL.g^{-1} STV. h, respectively), as well as of *P* (250.0 ± 9.9 and 235.0 ± 10.1 mL.g^{-1}STV, respectively) were obtained, suggesting mild changes in the lignocellulosic matrix. Under severity of 4.5 (B4), *P* of 333.0 ± 11.3 mL. g^{-1} STV and *Rm* of 0.8 ± 0.03 mL.g^{-1} STV.h, were observed, highlighting that the increase in hydrothermolysis severity from 4.2 implied damage to BSG anaerobic digestion (Figure S4, Supplementary Material). Acetic acid consumption, verified in all assays and (54.2 to 92.0%) simultaneous to methane formation, evidences the predominance of acetoclastic methanogenesis in these conditions, where there was also consumption of butyric (43.0 to 96.6%) and propionic acids (3.8 to 69.6%) (Figure S2, Supplementary Material).

In light of this, it was decided to continue this study with pretreated BSG by hydrothermolysis under severity of 4.2 (210 °C for 10 min). When the hydrothermolysis effects on BSG structure was evaluated under the above-mentioned condition, there was no significant change on the lignin content of the material (29.1%) when compared to BSG *in natura* (26.5%). However, after the pretreatment, an increase of total carbohydrates solubilization from 33.5% (BSG *in natura*) to 53.5% (pretreated BSG) was confirmed, as well as morphological modification on the structure of BSG fibers, verified by Digital Scanning Microscope (DSM) (Figure S5, Supplementary Material).

Kinetic parameters *P*, *Rm* and λ were obtained (Table 2) under different conditions of operation temperature (30 to 60 °C), pretreated BSG concentration (7.3 to 20.7 g.L^{-1}) and hydrolysate volume (0.6 to 12.4 mL). In relation to *P*, the greater results were obtained for C13 and C3, with 305.8 ± 7.79 and 286.6 ± 3.47 mL.g^{-1}·STV, respectively. In these assays, the start time of methane production (λ) were similar, 16.5 ± 7.4 and 14.3 ± 7.83 hours, *Rm* of 1.7 ± 0.1 and 1.7 ± 0.2 mL. g^{-1} STV.h, respectively. In C13 and C3, lower hydrolysate volumes were added, 0.6 and 3.0 mL, respectively, supporting the negative effect of this variable.

The effect of hydrolysate addition on *P* can also be observed when comparing assays C13, C14, C15, C16 and C17, with 14 g BSG.L^{-1}, operated at 45 °C and with different hydrolysate volumes. In C13, with 0.6 mL of hydrolysate, 305.8 ± 7.79 mL CH_4.g^{-1}·STV was produced. Concurrently to the increase of hydrolysate volume to 6.5 mL (C15, C16 and C17) and 12.4 mL (C14), there was a decrease of 32.4 and 38.3% in maximum methane production, respectively. Hydrolysate produced after hydrothermolysis may contain inhibiting compounds, like furfural and 5-HMF, therefore the microbial community requires a longer adaptation period [30], which may have compromised the BSG anaerobic digestion, causing lower methane production in C14, C15, C16 and C17 compared to C13.

After performing assays with concentrations of 0.4, 0.8 and 2.0 g.$L^{-1}$of furfural and 5-HMF, [17] reported total inhibition of methanogenesis with 2.0 g.$L^{-1}$of both aldehydes, and partial inhibition with 0.8 g.$L^{-1}$of 5-HMF, under mesophilic (35 °C) and thermophilic (55 °C) conditions. According to the authors, the thermophilic microbial consortium was more sensitive to the increase of intermediate compound concentrations released from the hydrolysis of lignocellulosic biomass when compared to mesophilic consortium, characterized by reduced methane production. These results corroborate those verified in this

study, where assays with lower hydrolysate volumes (0.6 and 3.0 mL) and lower concentrations of compounds derived from BSG lignocellulosic disruption, were favorable conditions to anaerobic digestion, since there was greater methane production (305.8 ± 7.79 and 286.6 ± 3.47 $mL.g^{-1}$·STV).

Table 2 Methane production (P), methane production rate (Rm) and time to start methane production (λ) from BSG pretreated under various methanogenic conditions

Assays	Conditions			Methane			
	T (°C)	BSG ($g.L^{-1}$)	Hyd. (mL)	*P* ($mL.g^{-1}STV$)	*Rm* ($mL.g^{-1}STV.h$)	λ (h)	R^2
C1	36	10	3	189.9 ± 3.2	4.1 ± 0.4	1.7 ± 2.2	0.97
C2	54	10	3	155.0 ± 2.2	1.8 ± 0.1	7.0 ± 2.6	0.99
C3	36	18	3	286.6 ± 3.5	1.7 ± 0.2	14.3 ± 7.4	0.98
C4	54	18	3	182.9 ± 2.4	1.8 ± 0.1	7.7 ± 2.3	0.99
C5	36	10	10	165.0 ± 4.0	1.8 ± 0.2	6.2 ± 4.5	0.99
C6	54	10	10	187.0 ± 5.0	1.5 ± 0.1	1.2 ± 4.6	0.97
C7	36	18	10	186.9 ± 5.4	1.5 ± 0.1	11.6 ± 4.4	0.98
C8	54	18	10	145.1 ± 5.2	0.8 ± 0.1	22.5 ± 5.2	0.98
C9	30	14	6.5	182.6 ± 3.5	2.0 ± 0.2	40.8 ± 3.6	0.98
C10	60	14	6.5	108.5 ± 2.0	1.5 ± 0.1	51.8 ± 2.9	0.99
C11	45	7.3	6.5	203.2 ± 1.8	2.1 ± 0.1	37.4 ± 1.9	0.99
C12	45	20.7	6.5	267.9 ± 3.2	2.3 ± 0.1	8.8 ± 2.5	0.99
C13	45	14	0.6	305.8 ± 1.8	1.7 ± 0.1	16.5 ± 7.8	0.99
C14	45	14	12.4	188.8 ± 3.3	2.2 ± 0.2	48.8 ± 3.5	0.97
C15	45	14	6.5	206.8 ± 3.3	2.7 ± 0.2	4.7 ± 3.2	0.98
C16	45	14	6.5	203.8 ± 3.0	2.4 ± 0.1	6.7 ± 2.6	0.99
C17	45	14	6.5	210.2 ± 2.6	3.5 ± 0.2	8.2 ± 2.0	0.99

*T: Temperature, BSG: Brewery spend grain concentration, Hyd: hydrolisate volume

Lower *P* was observed in C10 (108.5 ± 2.0 mL. g^{-1} STV), where 14 g $BSG.L^{-1}$ and 6.5 mL of hydrolysate were added, operated at 60 °C, evidencing the negative effect of high temperature on microbial consortium. Additionally, higher λ was found in the assays, of 51.8± 2.9 hours and *Rm* of 1.50 ± 0.13 $mL.g^{-1}$ STV.h, demonstrating the difficult microbiota adaptation to 60 °C. Similarly, Kim et al. [31] related the temperature influence on methane production from food wastes in reactors inoculated with mesophilic sludge, showing greater stability and methanogenic potential at 35 °C (230 $mL.g^{-1}$ VS) when compared to 55 °C (170 $mL.g^{-1}$ VS). For the same substrate, Zamanzadeh et al. [32] also observed better methane production (480 $mL.g^{-1}SV$) at 37 °C when compared to reactors operated at 55 °C (448 $mL.g^{-1}SV$). Various authors have reported superior performance of fermentative-methanogenic reactors conducted at mesophilic temperature in relation to thermophilic temperature, mainly when inoculated

with a mesophilic microbial consortium, which may be related to the reduced adaptation ability of microbial groups to wide-ranging temperature variations [33, 34]. For Chen and Chang [35], the ideal temperature range for the methanogenesis stage is from 35 to 42 °C, and an increase to 55 °C can especially inhibit the activity of methanogenic archaea, even after the acclimatization of mesophilic inoculum at 55 °C for 13 days. Thus, in this study, the methanogenic potential of the inoculum from UASB operated at mesophilic temperature was affected in reactors conducted at thermophilic temperature, probably due to the greater abundance of protein denaturation of mesophilic bacteria in the inoculum.

With regard to soluble metabolites in C1- C17, acetic acid concentrations were verified in the initial samples, from 35.0 to 301.1 $mg.L^{-1}$ (Figure 1). Disruption of the lignocellulosic structure by hydrothermolysis contributed to the acetic acid content in the initial samples, since this acid is a product from hemicellulose degradation. In fact, it was confirmed that there was 93.0 ± 14.1 $mg.L^{-1}$ of acetic acid in the hydrolysate from BSG hydrothermolysis, which surely contributed to the respective initial concentrations. Thus, the assays with higher acetic acid concentrations in the initial sample were C7 (279.0 $mg.L^{-1}$) and C14 (301.1 $mg.L^{-1}$) with 10 and 12.4 mL of hydrolysate, respectively.

During the exponential phase of methane production (Figure 1 II), there were expressive concentrations of propionic (165.4 to 847.3 $mg.L^{-1}$), butyric (175.8 to 966.62 $mg.L^{-1}$), valeric (43.0 to 255.8 $mg.L^{-1}$), isovaleric (12.3 to 105.4 $mg.L^{-1}$), isobutyric (13.2 to 53.3 $mg.L^{-1}$) acids and, mainly, of acetic acid (158.2 to 2.075.3 $mg.L^{-1}$).

The highest concentration of propionic acid (836.1 $mg.L^{-1}$) in the intermediate phase was found in C10, the assay with the lowest methane production (108.5 ± 2.0 $mL.g^{-1}$ STV). The accumulation of this acid indicates reactor disorder, since the overload is a consequence of the high partial pressure of hydrogen, which in turn suggests an unfavorable condition for acetogenesis, where a concentration between 900 and 2000 $mg.L^{-1}$ is considered toxic to the process [36]. Therefore, at the end of the assays (Figure 1 III), in C10 there was an increase of propionic acid (947.4 $mg.L^{-1}$) and decrease of acetic acid (599.2 $mg.L^{-1}$), demonstrating the unfavorable condition of acetogenesis and methanogenesis at 60°C.

The organic matter data (Figure S6, Supplementary material) corroborates the decrease in total organic acids concentration from the exponential phase (II) to the final methane production phase (III). The highest concentration of total organic acids at the end of operation corresponded to assay C8 (5613 $mg.L^{-1}$), where the increase of acetic acid (2909.8 $mg.L^{-1}$) was verified. Probably, the low methane production observed in C8 (145.1 ± 5.2 $mL.g^{-1}$ STV) occurred due to the inhibition of acetoclastic methanogenic activity, indicated by the acetic acid stock and caused due to the high temperature (54 °C) and hydrolysate volume (10 mL).

Depending on the condition, there was final pH ranging from 5.7 ± 0.07 to 7.2 ± 0.07. The lowest values were related to C8 (5.7 ± 0.07) and C10 (5.9 ± 0.10), where the high final concentrations of total organic

matter were measured (5613.3 and 1639.0 $g.L^{-1}$, respectively), which certainly contributed to the lower methane recovery (145.1 ± 5.2 and 108.5 ± 2.0 $mL.g^{-1}$ STV , respectively). The assays C3, C12 and C13, operated with the highest *P* values (286.6 ± 3.5, 267.9 ± 3.2 and 305.8 ± 1.8, respectively) ended with pH of 6.7 and 6.8. From the co-digestion of rice husk and *Salvinia molesta,* Syaichurrozi et al. [37] verified that in assays with initial pH 6.9 and final pH of 6.8 ± 0.05, the methane content was 65.45%, similar values found for assays conducted with initial pH 7.0 and final pH of 7.0 ± 0.05 (62.64%). As in this study, the pH close to 6.8 favored the anaerobic digestion of lignocellulosic substrates.

3.3 Statistical optimization of methane production with BSG pretreated by hydrothermolysis, severity 4.2

In the optimization of methane production from pretreated BSG by hydrothermolysis (severity 4.2), the effects of the variables operation temperature (x_1), BSG concentration (x_2) and hydrolysate volume (x_3) were statistically analyzed. The methane production profiles (*P*) resulted from x_1, x_2 and x_3 variations (shown in Table 3), which were delineated according to the central composite design (CCD) in Figure 2.

The statistical significance of the model was determined by ANOVA (Table 3) and the correlation coefficient calculated (R^2) was 89.9%, describing the quality of the obtained adjustment. The second order polynomial equation (Equation 2) represents methane production as a function of significant variables ($p > 0.10$):

$$Y = 227.70 - 20.79x_1 - 22.94x_1^2 + 15.57x_2 - 23.89x_3 - 16.46x_1x_2 + 14.76x_1x_3 - 18.19x_1x_3 \quad \text{(Equation 2)}$$

Where Y= predicted methane production ($mL.g^{-1}$ STV), x_1= temperature operation (°C), x_2= BSG concentration ($g.L^{-1}$) and x_3= hydrolysate volume (mL).

For temperature (x_1), a significant negative effect was verified (square and linear). BSG concentration variable (x_2) had significant positive linear effect, and the variable hydrolysate volume (x_3) presented significant negative linear effect. Significant effects were also observed for the interactions between factors ($x_1.x_2$, $x_2.x_3$ and $x_1.x_3$).

Table 3 ANOVA for the effects of temperature (x_1), BSG concentration (x_2) and hydrolysate volume (x_3)

Variable	Sum of Squares	Degrees of Freedom	Mean Square	F-value	P-value	Factors
x_1 (L)	5903.84	1	5903.84	14.33705	0.006835	S
x_1 (Q)	8013.53	1	8013.53	19.46027	0.003114	S
x_2 (L)	3308.05	1	3308.05	8.03337	0.025249	S
x_2 (Q)	301.74	1	301.74	0.73274	0.166878	
x_3 (L)	7794.01	1	7794.01	18.92719	0.003353	S
x_3 (Q)	979.67	1	979.67	2.37905	0.166878	
$x_1 . x_2$	2166.47	1	2166.47	5.26110	0.055503	S
$x_1 . x_3$	1741.98	1	1741.98	4.23026	0.078741	S
$x_2 . x_3$	2647.37	1	2647.37	6.42895	0.038917	S
Error	21.2	7	411.780			
Total	39178.2	16				

*S: Significant effect.

The operation temperature (negative square effect) represented the greatest magnitude effect on methane production, followed by hydrolysate volume (negative linear effect) and BSG concentration (linear effect). Based on the significant factors, the response surface for maximum methane production was developed, evidencing the optimal points resulting from the interaction of variables (Figure 3).

Throughout the statistical model, the optimal predicted operation temperature was at 36 °C, with reduced hydrolysate volumes (lower than 3.0 mL), and 18 $g.L^{-1}$ of BSG. In this conditions, optimal predicted methane production was 302.4 $mL.g^{-1}$STV.

Triplicates of reactors (assay C18) were carried out under these optimal predicted conditions for the respective variables, to validate the statistical model. 274.2 ± 5.6 $mL.g^{-1}$ STV of methane production, *Rm* of 1.91 ± 0.10 $mL.g^{-1}$ STV, λ of 18.6 ± 3.9 hours and R^2 of 0.99 were obtained. Therefore, there was 90.6% of accuracy in relation to the predicted values, thus, the data fit the model.

3.4 Taxonomy of microbial communities and potential BSG conversion pathways

The structure of microbial communities (Bacteria and Archaea domains) was performed based on two samples, one of them was from the hydrothermolysis optimization assay (B2), and the other from the optimization assay of fermentative-methanogenic activity (C18).

B2 assay was operated with 10 $g.L^{-1}$ of pretreated BSG by hydrothermolysis (severity of 4.2) at 37 °C, while C18 was operated with 18 $g.L^{-1}$of pretreated BSG by hydrothermolysis (severity of 4.2) plus 3 mL of hydrolysate, at 36 °C. Based on the taxonomic characterization of both samples, it was possible to infer

the effect of substrate concentration increase of 80% from B2 to C18 and the hydrolysate (3 mL) addition in C18.

The Bacteria domain populations had organisms belonging to the phylum *Bacteroidetes* (28.7 and 31.8%), *Spirochaetes* (22.1 and 17.4%), *Proteobacteria* (14.1 and 6.8%), *Firmicutes* (10.4 and 27.6%), *Cloacimonetes* (8.2 and 7.0%), *Chloroflexi* (7.6 and 4.3%) and *Synergistetes* (5.5 and 3.5%) for B2 and C18, respectively (Figure S8, Supplementary Material). For the Archaea domain, the prevalence of the phylum *Euryarchaeota* was found in both samples, with 99.5 and 99.4% of relative abundance in B12 and C18, respectively (Figure S9, Supplementary Material).

Considering the relative abundance of the bacterial phylum, changes in bacterial dominance can be verified because of the operational differences between B2 (10 $g.L^{-1}$ of BSG) and C18 (18 $g.L^{-1}$ of BSG plus 3 mL of hydrolysate in C18). In B2, *Bacteroidetes* (28.7%) and *Spirochaetes* (22.1%) were the prevalent phylum, and in C18, *Bacteroidetes* (31.8%) and *Firmicutes* (37.6%).

Bacteroidetes and *Firmicutes* are widely found in mesophilic reactors and are related to acidogenesis and the ability to metabolize organic molecules, such as carbohydrates and proteins, to organic acids [38]. Paranhos et al. [39] reported predominance of the phylum *Firmicutes* (43%) and *Bacteroidetes* (37%) when characterizing poultry manure sample applied as inoculum in anaerobic reactors for lignocellulosic biomass co-digestion. In the present study, bacteria belonging to the phylum *Bacteroidetes* and *Firmicutes* may also have resulted from poultry manure, since the inoculum used was anaerobic sludge from UASB reactor fed with poultry slaughterhouse wastewater. Thus, in this study, members of this phylum probably converted complex organic molecules from BSG to soluble carbohydrates and organic acids.

Bacteria belonging to *Spirochaetes* are often found in anaerobic reactors and also in natural aerobic environments. They are associated to acetate, ethanol and lactic fermentation from sugars [40]. Lee et al. [41] related *Spirochaetes* to acetate oxidation, a thermodynamically unfavorable reaction that results in hydrogen production, through a syntropic relationship with hydrogenotrophic methanogenic archaea. This metabolic pathway probably occurred in B2 and C18, since hydrogenotrophic methanogenic archaea (*Methanolinea*, *Methanoregula* and *Methanospirillum*) were identified.

Members of the phylum *Chloroflexi* (7.6 and 4.3% in B2 and C18 samples, respectively) are able to metabolize carbohydrates and amino acids as well as oxidize hydrogen via homoacetogenesis [42]. *Cloacimonetes* (8.2 and 7.0%, in B2 and C18, respectively) are proteolytic and amino acids consumers, commonly found in wastewater treatment plants [43]. Additionally, it is reported that the phylum *Firmicutes*, *Chloroflexi* and *Cloacimonetes* are resistant to toxic compounds resulting from corn straw pyrolysis, such as phenolic compounds 5-HMF and furfural [44], like in this study, this phylum was identified in the assays where the substrate was subjected to extreme temperature and pressure conditions, which may have released 5-HMF and furfural in the reaction medium [12, 45].

The difference between the bacterial genera identified in B2 and C18 was even more significant (Figure 4). In B2, the main bacteria identified were similar to *Treponema* (*Spirochaetes*), *Syntrophorhabdus* (*Proteobacteria*), *Macellibacteroides* (*Bacteroidetes*), unidentified_*Spirochaetaceae* (*Spirochaetes*) and *Sphaerochaeta* (*Spirochaetes*) with relative abundance of 11.8, 7.5, 7.4 6.0 and 3.8%, respectively. In C18, *Macellibacteroides* (*Bacteroidetes*) were also among the main bacteria (15.9 %), in addition to *Sphaerochaeta* (*Spirochaetes*). *Clostridium* (*Firmicutes*), *Hydrogenispora* (*Firmicutes*), *Pseudomonas* (*Proteobacteria*), *Bacteroides* (*Bacteroidetes*), *Petrimonas* (*Bacteroidetes*), with relative abundance, respectively, of 14.7, 8.1, 4.7, 4.5, 4.4 and 3.2%.

Treponema, dominant genus in B2 (11.8%), use a wide variety of carbohydrates and amino acids as carbon source and can metabolize short and long organic acids chains [46]. Furthermore, they accomplish homoacetogenesis, through which hydrogen and carbonic gas consumption occurs with acetate production [47]. This metabolic characteristic is probably associated with the low relative abundance of methanogenic hydrogenotrophic archaea, since hydrogen was converted to acetate and methane by homoacetogenesis - acetoclastic methanogenesis.

Syntrophorhabdus (7.5% in B2) allows the breakdown of compounds such as phenols, p-cresol, 4-hydroxybenzoate, isophthalate and benzoate in syntrophy with hydrogenotrophic methanogenic archaea [48]. Probably, this organism is involved in the conversion of phenolic compounds released from BSG lignocellulosic degradation, which favored the methanogenic activity in B2, the highest methane production (411.6 ± 7.2 mL. g^{-1} STV) in assays B1 to B7.

Macellibacteroides and *Sphaerochaeta*, belonging to the *Spirochaetaceae* family, are fermenters identified in both reactors. *Spirochaetaceae* (6.0% in B2 and 2.1% in C18) convert carbohydrates and amino acids into organic acids, mainly into acetic and butyric acids [49]. Usually found in mesophilic anaerobic reactors, bacteria similar to *Macellibacteroides* (7.4% in B2 and 15.9% in C18) have optimal growth at 35 - 40 °C and pH 5.0 - 8.5, which can convert sugar monomers primarily into lactate, acetate, butyrate and isobutyrate [50]. Similarly, *Sphaerochaeta* (3.8% in B12 and 14.7% in C18) can consume carbohydrates and produce, predominantly, acetate, formate and ethanol [51]. Regarding C18, it is likely that the sugar concentrations for BSG hydrolysis and hydrolysate favored *Macellibacteroides* and *Sphaerochaeta*, which were predominant in the reactor.

Under C18 conditions, *Pelotomaculum* were verified (2.3%), a acidogenic bacteria which are strictly anaerobic and metabolize a limited number of compounds, including propionate, primary alcohols, low molecular weight aromatics and lactate [52]. Both *Hydrogenispora* and *Lutispora* are endospore-forming bacteria, found in C18 (4.7 and 1.1%, respectively). *Lutispora* consume, primarily, amino acids and release amino acids into iso-butyrate, propionate and iso-valerate [53]. *Hydrogenispora* grows on medium rich in sugars, starch and yeast extract, forming acetic acid, ethanol and hydrogen, and are found in both anaerobic sludge and vegetables [54, 55]. Thus, in this study, *Hydrogenispora* may have resulted from both BSG or anaerobic sludge.

Clostridium, identified in C18 (8.1%) and in B2 (1%) has been used as inoculum in reactors fed with lignocellulosic biomass due to its cellulolytic-fermentative ability, resulting in an increase of substrate degradability and, consequently, also due to the intermediates availability for methanogenesis [56]. Rabelo et al. [57] identified great quantities of *Clostridium* in reactors fed with *in natura* sugarcane bagasse (19%), as well as in reactors with the bagasse after hydrothermolysis (12.2%), which can metabolize organic compounds, such as carbohydrates and peptones, into diverse organic acids, acetones and alcohols. Moreover, *Clostridium* species are known for their resistance to high furan concentrations, as well as their ability to degrade them [30, 58]. In this study, the bacteria of the genus *Clostridium* probably degraded these compounds, whose presence is undeniable because of the hydrothermolysis severity applied to the BSG (4.2).

Other groups characterized by cellulolytic-fermentative activity were also identified, such as *Pseudomonas*, *Bacteroides*, *Gracilibacter*, *Ruminofilibacter*, *Ruminoclostridium. Pseudomonas* were identified in C18 (4.5%) and also in B2 (1.1%), a bacterial group characterized by cellulolytic activity to degrade cellulose, hemicellulose and lignin, in addition to consuming biphenols [59]. In addition to BSG polysaccharide degradation, *Pseudomonas* were probably involved in the conversion of long chain organic acids in the reactor, making substrates available for fermentative bacteria [60]. *Synergistaceae* (2.0% in B2 and 1.7% in C18), although belonging to the phylum with lower relative abundance in both reactors (*Synergistes)*, probably acted on amino acids metabolization producing short chain organic acids, such as acetic and butyric acids, releasing substrate for methanogenesis. *Bacteroides* (4.4% in C18) are found in the intestinal gut of animals [38] and, therefore, their probable presence in the sludge used as inoculum and collected from a poultry slaughterhouse wastewater reactor. Members of *Bacteroides* are related to carbohydrate hydrolysis and proteins and lipid degradation, becoming the available acetic, isovaleric, isobutyric and succinic acids [38, 61].

Gracilibacter, Ruminifilibacter, *Ruminoclostridium* and *Petrimonas* were observed in lower relative abundance (lower than 3.2%). *Gracilibacter* are saccharolytic and produces acetate, lactate and ethanol [62]. *Ruminofilibacter* and *Ruminoclostridium* are cellulolytic and available hydrogen, carbonic gas, ethanol, acetate as fermentation products, readily consumed in the following anaerobic digestion steps [63]. *Ruminoclostridium* are generally related to enriched microbial culture with potential for cellulose degradation and hydrogen production [63]. *Petrimonas* (3.2% in C18 and 1.4% in B2) ferments a large number of monomers, with acetate and propionate as the main products, in addition to low levels of succinate [64].

Other bacterial groups with fermentative and acidogenic function were found with relative abundance lower than 2%, such as *Gelria*, *Aminivibrio*, *Comamonas*, *Syntrophomonas* and *Syntrophus*. *Gelria* (1.2% in B2) are strictly anaerobic, chemo-organotrophic, saccharolytic and hydrogen-producing bacteria [65]. *Aminivibrio* (1.7% in B2) are related to amino acids fermentation and organic acids oxidation [66]. *Comamonas* (2% in B2) can degrade acetic, butyric and propionic acids [67]. *Syntrophomonas* (1.6% in C18 and 1.7% in B2) and *Syntrophus* (1% in B2) convert propionic and butyric acids into acetic acid and hydrogen, and *Syntrophus* can also oxidize aromatic compounds and benzoate [48].

Concerning the Archaea domain, the predominance of acetoclastic methanogenesis pathway is reiterated by the high relative abundance of the genus *Methanosaeta* (with 87.2 and 80.4%, respectively, in B2 and C18) (Figure S9, Supplementary Material). These archaea are consumers of electrons derived from propionate and butyrate oxidized into acetate [68], which may have been favored to more expressive concentrations of certain metabolites, such as acetic acid.

This configuration with a predominant acetoclastic pathway and less significant hydrogenotrophic methanogenic activity was previously described by Leclerc et al. [69] as the minimum microbiota necessary for the stability of anaerobic reactor. Stability was verified for both conditions, with methane production of 411 ± 7.2 and 274.2 ± 5.6 $mL.g^{-1}$ STV in B2 and C18, respectively. Archaea similar to *Methanolinea* (6.2 and 5.5% in B2 and C18, respectively), *Methanoregula* (5.4 and 8.3% in B2 and C18, respectively) and *Methanospirilum* (2.4% in C18) were identified at lower relative abundance. These are hydrogenotrophic methanogenic archaea, which reduce carbonic gas into methane using hydrogen molecules as electrons donors.

The degradation of recalcitrant compounds, such as lignocellulosic materials, occurs through various biochemical process and microbial interactions, and based on metabolic characteristics reported in the literature and on the results presented in this study, it was possible to correlate the metabolic pathways that appeared in the BSG anaerobic digestion (Figure 5).

Briefly, the BSG hydrothermolysis (severity 4.2) caused the rupture of BSG fibers, which favored substrate hydrolysis by *Clostridium* and *Pseudomonas*. BSG fermentation was also favored due to the higher release of soluble sugars which were converted into organic acids, mainly acetic acid, by *Macellibacteroides, Bacteroides, Sphaerochaeta* and *Clostridium.* Phenolic compounds resulting from hydrolysate and pretreated BSG did not compromise the fermentative-methanogenic process, since they were degraded by *Syntrophorhabdus*. Finally, acetic acid resulting from hydrolysate, released from the fermentation of soluble sugars, and formed from homoacetogenesis by *Treponema*, was converted into methane by *Methanosaeta*.

4. Conclusion

The application of hydrothermolysis to disrupt the lignocellulosic fibers of brewery spent grain (BSG) was crucial to increase methane production. Both temperature and time of BSG hydrothermolysis were statistically significant to increase the methane production to 75.1% with severity of 4.2.

Concerning the variables that can impact the pretreated BSG fermentation-methanogenesis, greater statistical significance was observed for the operational temperature, followed by hydrolysate volume and BSG concentration. According to response surface methodology, the optimal conditions predicted for methane production (302.4 $mL.g^{-1}$ STV) was at 36 °C, with 3 mL of hydrolysate and 18 g $BSG.L^{-1}$.

Changes were observed in the predominant bacterial phylum due to operational differences between B2 (10 $g.L^{-1}$ of BSG) and C18 (18 $g.L^{-1}$ of BSG and 3 mL of hydrolysate). In B2, *Bacteroidetes* (28.7%) was

predominant, and in C18, *Firmicutes* endospore forming bacteria were dominant (37.6%). With regard to methanogenesis, acetoclastic methanogenesis was the main pathway, with *Methanoseta* prevailing in both conditions (87.2 and 80.4% in B2 and C18, respectively).

Declarations

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Figures

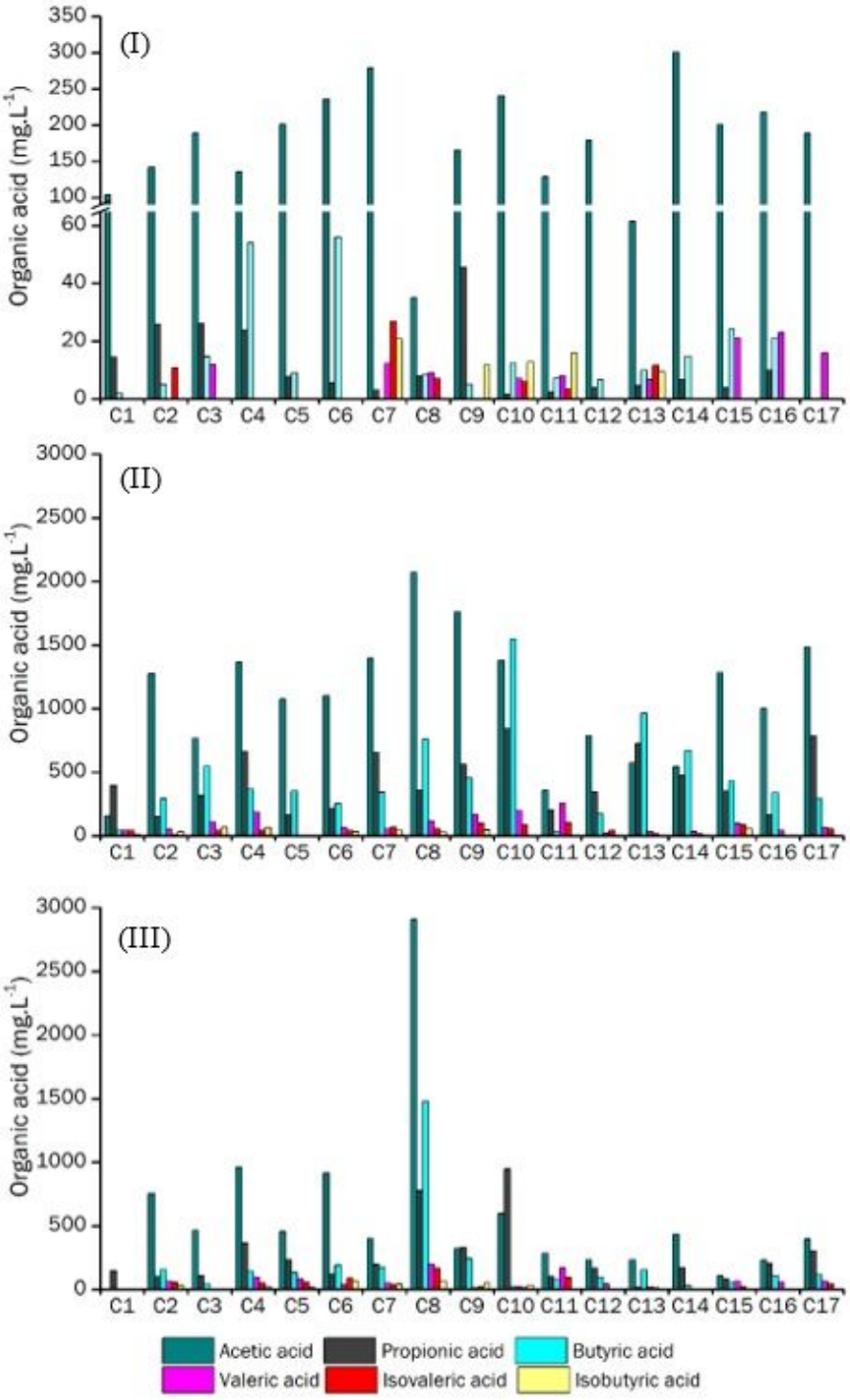

Figure 1

Organic acids content in fermentative-methanogenic assays with BSG pretreated, during initial (I), exponential (II) and final phases of methane production (III)

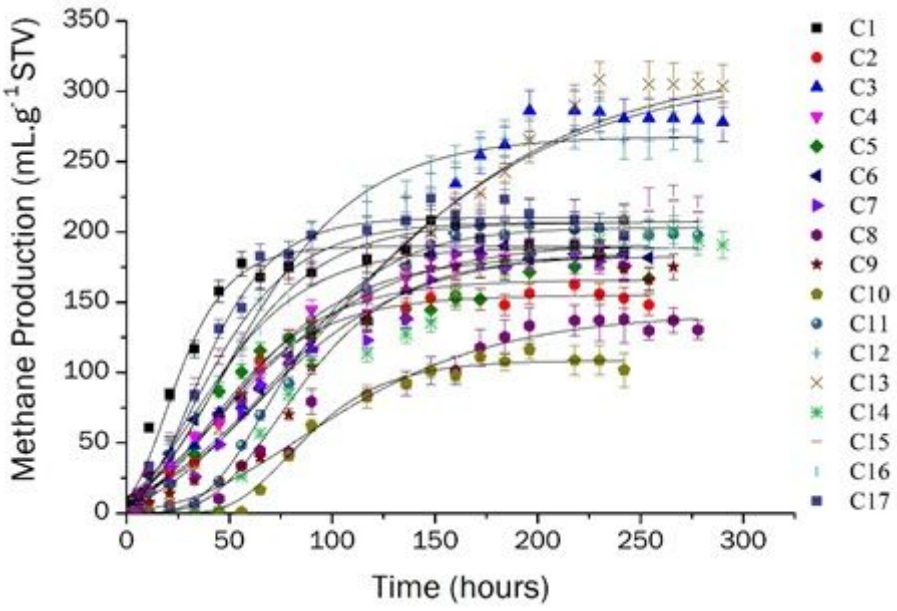

Figure 2

Methane production from BSG pretreated by hydrothermolysis according to DCC conditions

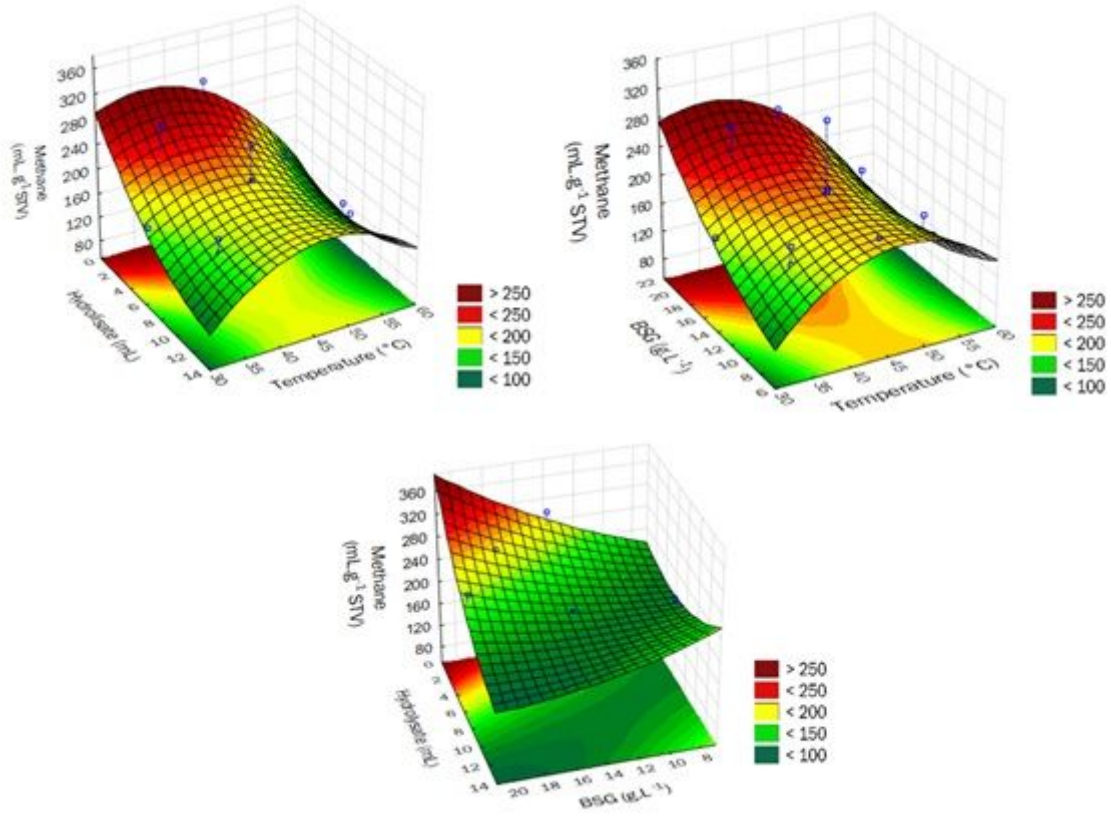

Figure 3

Response surfaces for the effect of the variables temperature, BSG concentration and hydrolysate volume on methane production

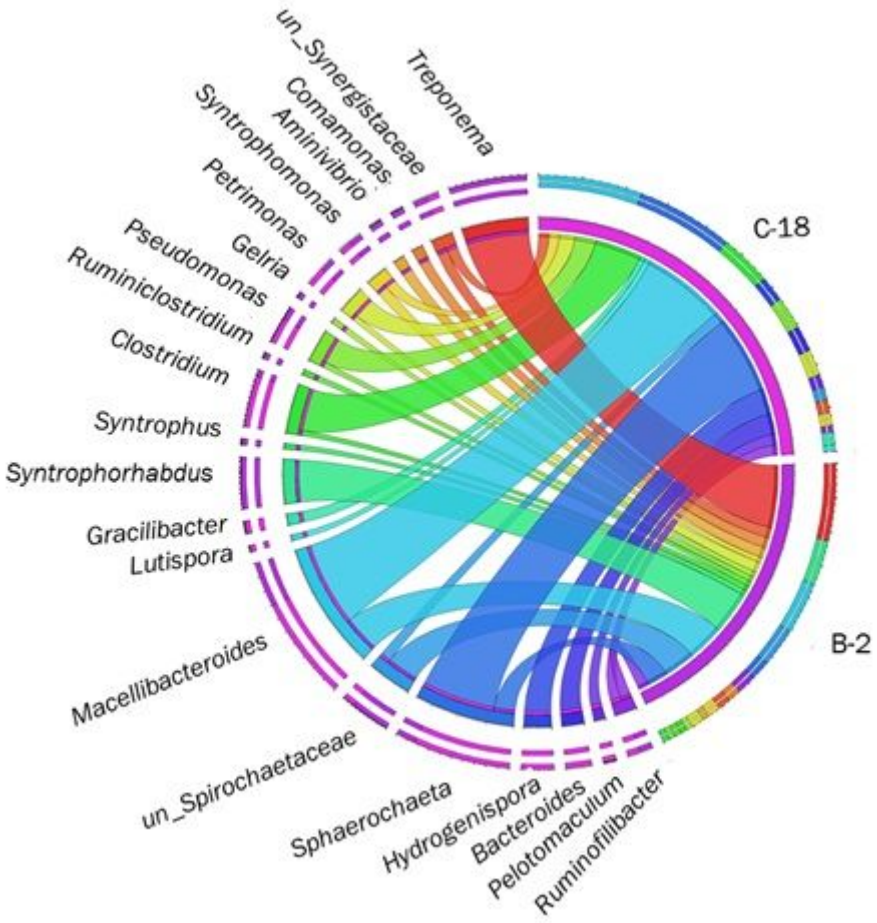

Figure 4

Circular ideograph “Circos” for bacterial genera identified in B2 and C18

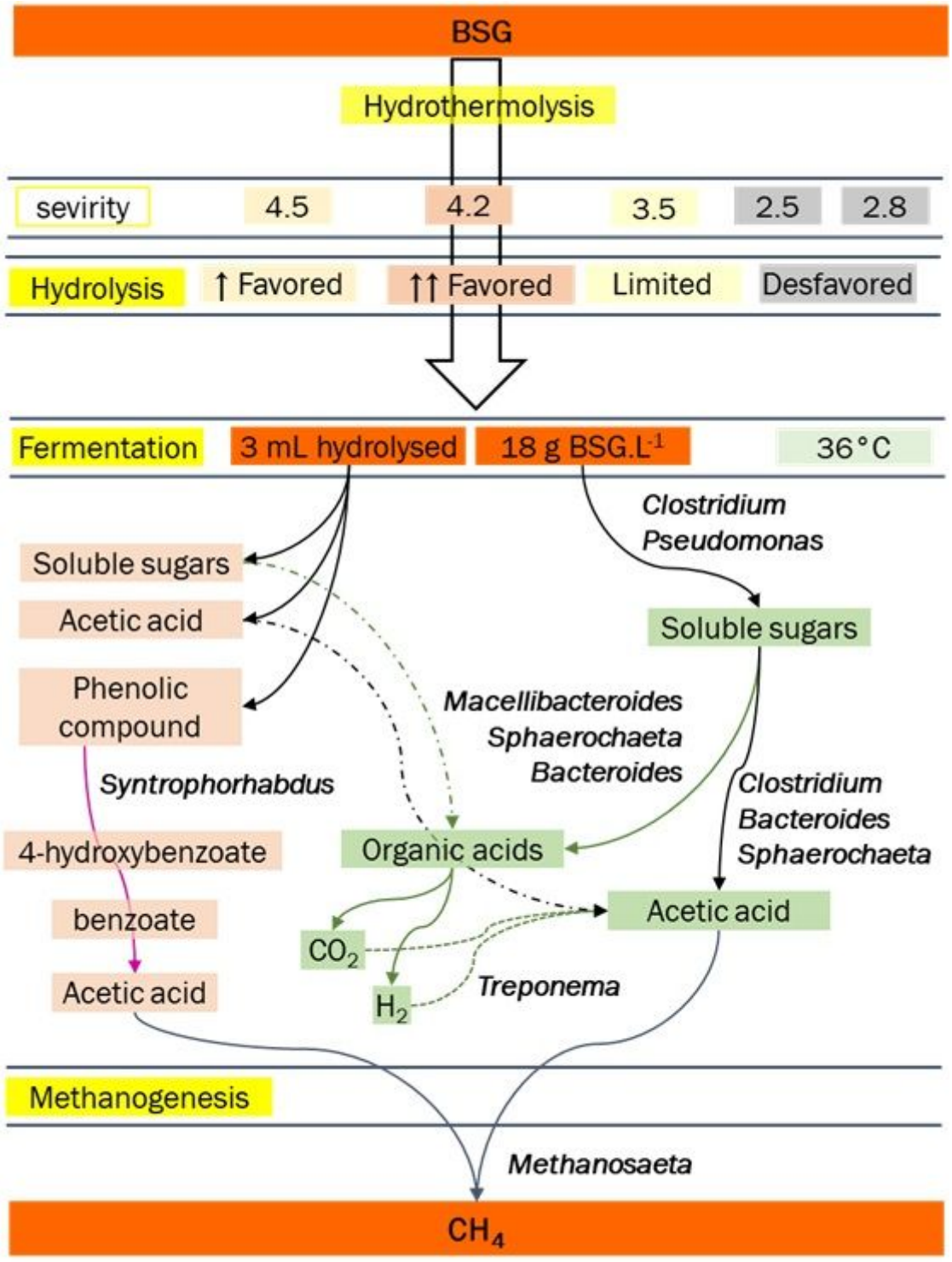

Figure 5

BSG conversion pathways into sugars, organic acids and methane

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