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Alessandra Cesaro, Anna Conte, H el ene Carr ere, Eric Trably, Florian Paillet, et al.. Formic acid pretreatment for enhanced production of bioenergy and biochemicals from organic solid waste. *Biomass and Bioenergy*, 2020, 133, 10.1016/j.biombioe.2019.105455 . hal-02947342

HAL Id: hal-02947342

<https://hal.inrae.fr/hal-02947342v1>

Submitted on 28 Jul 2023

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1 Formic acid pretreatment for the enhanced production of bioenergy and biochemicals from 2 organic solid waste

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24 25 **ABSTRACT**

26
27 Organic solid waste is one of the most promising feedstocks for the implementation of the circular
28 economy principles in waste management. Its anaerobic treatment can indeed promote organic
29 matter conversion into a number of value-added products as well as energy carriers. However, the
30 identification of sustainable strategies to handle organic solid waste in a biorefinery framework still
31 poses technological as well as economic challenges. The aim of this study was in enhancing the
32 potential of the organic fraction of municipal solid waste (OFMSW) to produce bioenergy and
33 biochemicals by combining dark fermentation with a formic acid pretreatment. Hydrogen yields up
34 to 31.6 ml/g_{VS} were obtained pre-treating the OFMSW with 5% formic acid, at 80 C for 70 minutes.
35 Concomitantly, a wide range of metabolites of market significance, including acetic acid, butyric
36 acid and ethanol, accumulated. The concentration of these metabolites further enhanced after the
37 dark fermentation of the pretreated substrates. Experimental tests highlighted the influence of the
38 different pretreatment operating conditions on the relative production of hydrogen and main
39 metabolites as well as the related pathways. It was found that the acid concentration plays a key
40 role in promoting the biological conversion of OFMSW and that the adjustment of the operating
41 temperature and treatment time can be targeted towards the production of either building blocks or
42 energy carriers, so as to ensure the viability of the process for its scale up.
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55 **Keywords:** biohydrogen, biomass, biorefinery, dark fermentation, building blocks, recovery.
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1. Introduction

The spread of the direct biomass utilization in the industrial sector is turning the organic solid waste into one of the most promising feedstocks for the implementation of the biorefinery concept, referred to the conversion of biomass into a wide spectrum of competitive bio-based products with market significance [1]. The potential of plant-based raw materials in replacing a large fraction of fossil resources for the production of energy carriers, chemicals as well as materials is well-established and in recent years research has mostly focused on agricultural crops, lignocellulosic biomasses and algae. However, the organic fraction of municipal solid waste (OFMSW) is raising as a widely available biomass which can be used in biorefinery facilities to produce multiple, value-added target products [2]. Such approach may also stand as a novel waste management strategy, perfectly fitting the circular economy principles via the organic waste to bio-chemical supply chain [3].

The lignocellulosic fraction (LF) and the organic fraction (OF) of municipal solid waste (MSW) are globally and continuously produced in large amounts and the availability of these substances at low cost makes them attractive to reduce the negative environmental impact of the use of fossil resources [4]. The organic waste is indeed a mixture of carbohydrates, proteins and fats, whose basic components, namely sugars, amino-acids and fatty acids, can be conveniently turned into a number of value added products, including ethanol, lactic acid, polylactic acid (PLA), polyhydroxyalkanoates (PHAs), succinic acid, 1,4-butanediol (BDO), farnesene, isobutene, acrylic acid, adipic acid, ethylene and polyethylene [3]. Nevertheless, such approach poses important challenges from the technological, economic and environmental perspectives [5], related to the need to identify highly efficient and sustainable conversion strategies of the organic waste.

This kind of waste has been traditionally handled via biological processes and the anaerobic digestion (AD) is currently one of the preferred treatments for the intensive biodegradation of the OFMSW. It is considered an up to date waste to energy technology, ending in the production of a biogas mainly composed of methane, which is obtained from a stand-alone AD process or by means of a two-stage process, with a dark fermentation (DF) stage for the additional production of hydrogen [6]. Hydrogen is a promising carbon-free clean fuel [7], that can be used either directly in combustion engines [8] or to produce electricity via fuel cell systems [9].

During dark fermentation the production of hydrogen comes along with the accumulation of intermediate products, namely soluble metabolites like volatile fatty acids (VFAs), carboxylic acids and alcohols, with growing market potential [10, 11]. The generation of VFAs is of particular interest due to the wide range of applications that these metabolic products hold in the cosmetic and pharmaceutical industry [12], in the production of solvent [13], in the generation of energy from microbial fuel cells [14], in the production of biodegradable polymer [15] as well as in the nutrient removal from wastewater [16].

1 In this context, the pretreatment of the substrate for the anaerobic process is a necessary step to
2 improve the bioconversion efficiency, so that recent advances in research are being directed
3 towards the identification of suitable techniques. Chemical, physical and biological methods, as
4 well as their adequate combination, can be used to treat the organic waste prior to anaerobic
5 digestion [17].

6 Among chemical pretreatments, acids have been applied to release the nutrients locked up in
7 organic waste and other lignocellulosic materials for subsequent fermentation [18, 19]. Extensive
8 research has been conducted on the use of chemicals to break down the lignin and hemicellulose
9 network and disrupt the crystalline cellulose structure, in order to promote the bioconversion of the
10 lignocellulosic materials into both biofuels and biochemicals [20]. Currently, both the dilute acid and
11 the alkaline pretreatments are the most mature technologies for commercialized application,
12 having shown high effectiveness on several agricultural residues [21, 22].

13 The use of organic solvents has been also studied for the production of high-quality intermediates:
14 it was found to be highly efficient for the biomass fractionation and easy to recover by means of
15 distillation processes [20]. Among the available organic solvents, the formic acid has shown a great
16 potential as active agent for the acceleration of biomass hydrolysis [23, 24]. It is the simplest
17 carboxylic acid and it is regarded as an effective pretreatment option, able to increase the surface
18 area of the substrate and to promote its solubilization into value-added pulps [25].

19 Most of the research works have been carried out to assess the efficiency of the formic acid
20 pretreatment on lignocellulosic substrates, whereas its potential for the processing of the OFMSW
21 destined to anaerobic processes has not been explored yet. The use of formic acid to promote an
22 extensive hydrolysis could result in the faster conversion of OFMSW into products of chemical
23 interest. Similarly, the formic acid could promote its greatest valorization when the OFMSW is
24 mixed with less rapidly biodegradable, lignocellulosic fractions. In full scale biological treatment
25 facilities, mixing OF and LF substrates is common to reduce the compaction of the more
26 putrescible waste within either anaerobic reactors or aerobic stabilization piles. Furthermore the
27 recent addresses towards the circular economy approach make the combination of different
28 organic substrates particularly attractive to gain their greatest valorization by implementing a sole
29 process.

30 The aim of this work was in assessing the effects of the formic acid pretreatment on the
31 bioconversion of the OFMSW into bioenergy and biomolecules under various operating conditions.
32 To this end, differently composed organic waste were considered and the recovery of carbon
33 sources was evaluated together with the biohydrogen yields, in order to discuss the feasibility of
34 this pretreatment for the OFMSW and address further studies for its scale up.

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2. Material and Methods

2.1 Raw materials

Experimental tests were carried out using artificial OFMSW samples, whose composition (Table 1) had been defined from previous studies [26 - 28], so as to reduce the influence of the composition variability of real samples.

The OFMSW was used as a single substrate as well as to prepare a mixture with a lignocellulosic fraction (LF): in the latter case, the samples contained 70% of OF and 30% of LF, expressed as mass percentage, on wet basis. The LF substrate contained tree leaves (50% wet weight) and wheat straw (50% wet weight), which represents the typical agricultural product used as alternative to energy crops in anaerobic processes. After being collected, the substrates were ground to reduce the particles size up to approximately 3 mm and stored at 4 °C until use.

Waste activated sludge was used as inoculum for the biological tests. It was sampled at a French municipal wastewater treatment plant and pretreated by heat/shock (90°C, 30 min) to inhibit methanogenic microbial activities consuming hydrogen (H₂).

The main characteristics of the substrates and the microbial inoculum are reported in Table 2.

2.2 Experimental setup

The pretreatment tests were conducted according to a 2ⁿ type factorial design, in which “n” is the number of the factors, namely the formic acid concentration, the operating temperature and the process time, and “2” is the number of levels chosen for the factors (Table 3). This factorial design established 8 different experimental test runs, as reported in Table 4.

The proposed pretreatment was carried out in 300 ml flask with specific solid-liquid ratio [29]. The 10% w/v substrate was immersed in a formic acid/water solution and pretreated in autoclave. Further samples were pretreated adding the formic acid without autoclave processing, in order to better understand the effects of the formic acid on the substrate degradation.

After the pretreatment, the samples were characterized by their main chemical-physical properties as well as by their biohydrogen potential.

2.3 Analytical methods

A detailed chemical-physical characterization of the substrates was carried out before and after the pretreatment.

In the untreated samples Total Solids (TS) and Volatile Solids (VS) were determined according to Standard Methods [30]. The C/N ratio was assessed as well: to this end, the Total Carbon (TC) was calculated as the sum of the Total Organic Carbon (TOC) and the Inorganic Carbon (IC) determined using a Shimadzu TOC-VCSN Total Organic Carbon Analyzer coupled to a Shimadzu

1 ASI-V tube rack; the Total Kjeldahl Nitrogen (TKN) was measured with Digest Automat K-438 and
2 an AutoKjeldahl Unit K-370, BUCHI.

3 In order to measure the pH, untreated samples were destined to a water extraction procedure [31].
4 They were mixed with water in a ratio 1:20 (wet weight) and continuously stirred for 1 hour at room
5 temperature. The mixture was then centrifuged (1500 rpm, 15 min) and the supernatant analyzed
6 using a pHmeter Eutech Instrument pH510. The supernatant was then filtered at 0.45 μm to obtain
7 the soluble fraction for the chemical oxygen demand (COD) analysis. This was performed using an
8 Aqualytic 420721 COD Vario Tube Test MR (0-1500 mg/l). Two mL of sample were pipetted into
9 each tube and then they were placed inside a ECO 25 Thermoreactors for COD (Velp Scientifica)
10 at 150 $^{\circ}\text{C}$ for 2 h. Soluble COD (sCOD) concentrations were determined using an Aqualytic
11 Multidirect spectrophotometer.

12 After acid hydrolysis of the substrate with sulfuric acid (solution 10% v/v H_2SO_4 98% with 1 g TS/
13 of substrate and agitation for 24 h), the carbohydrate content was determined by the method
14 proposed by Dubois et al. [32] and the protein concentration was performed following the modified
15 Lowry method [33].

16 The same analysis were performed also on the pretreated samples, following the same analytical
17 methods: in this case, the soluble fractions were obtained by a centrifugation at 5000 rpm for 10
18 min and filtration at 0.45 μm ; the sCOD concentrations were calculated by subtracting the sCOD of
19 the formic acid added in each sample.

20 The concentration of both organic acids and other metabolic end-products was assessed on the
21 samples after the pretreatment with formic acid as well as after the biological tests. To this end, 20
22 g of wet biomass was extracted by deionized water (1:2), mixed for 30 minutes, centrifuged at
23 5000 rpm for 10 min and filtrated at 0.45 μm [34].

24 Volatile fatty acids (VFA) composition, ie. acetic (C2), propionic (C3), butyric and iso-butyric (C4
25 and iC4), valeric and isovaleric (C5 and iC5) and caproic (C6) acids, was determined with a gas
26 chromatograph (GC-3900 Varian) equipped with a flame ionization detector. The formic acid
27 concentration was also determined with GC using a specific control sample.

28 High performance liquid chromatograph (HPLC) was used to measure the concentrations of non-
29 VFA metabolic products, such as ethanol and lactate: to this end, a further filtration step at 0.2 μm
30 was performed on the soluble fraction of the samples. The HPLC was coupled to refractometric
31 detection (Waters R410). Chemicals were separated by an Aminex HPX-87H column (300 mm on
32 7,8 mm, Biorad) equipped with a protective precolumn (Microguard cation H refill cartridges,
33 Biorad). The eluting solution corresponded to 2 mM H_2SO_4 under a flow rate of 0.4 ml min^{-1} . The
34 column temperature was set at 35 $^{\circ}\text{C}$ and the refractive index detector (Waters 2414) worked at 45
35 $^{\circ}\text{C}$.

36 The concentration of the metabolic products was expressed as $\text{COD}_{\text{eq}} \text{L}^{-1}$. The equivalent COD
37 values were calculated using their respective COD conversion factors, in $\text{g}_{\text{COD}}/\text{g}$ compound: lactic

1 acid 1.07, acetic acid 1.07, propionic acid 1.51, butyric acid 1.82, valeric acid 2.04, caproic acid
2 2.21 and ethanol 2.09 [35].

3 The initial concentrations of the formic acid used for the pretreatment were subtracted from the
4 concentrations of VFAs considered for discussion.
5

6 **2.4 Biohydrogen Potential tests**

7 Biochemical Hydrogen Potential (BHP) tests were carried out in 400 ml flasks on both raw and
8 pretreated samples.

9 The medium was composed of 220 ml of substrate, 5 - 130 ml of 2-(N-morpholino) ethanesulfonic
10 acid (MES) buffer (50 mM) and 1 - 10 ml of 3.2% NaOH (2 N). Heat treated inoculum was added to
11 reach a substrate/inoculum ratio of 10 g_{VS} substrate/g_{VS} inoculum. The pH was initially adjusted at
12 6.0 ± 0.2 using MES buffer and NaOH. After inoculation and pH adjustment, the head space of
13 reactors was purged with nitrogen gas to remove oxygen traces and keep the anaerobic
14 conditions; then, the bottles were sealed with a rubber stopper and locked with an aluminum
15 screw. Batch tests were incubated at mesophilic temperature (37 ± 1 °C) in a water bath and
16 carried out until the hydrogen production stabilized.

17 All batch reactors were connected to a multiplexed R3000 micro-gas chromatograph (μGC) with
18 two analytical capillary columns (SRA instrument, Marcy l'Etoile, France) to monitor gas production
19 on line. The first column was dedicated to carbon dioxide analysis and corresponded to a 5Å
20 molecular sieve (10 m length and 0.32 mm diameter) with argon as carrier gas at a pressure of 30
21 PSI. The second column dedicated to oxygen, hydrogen, nitrogen, and methane analysis was a
22 PLOT Q (8 m length and 0.32 mm diameter) with helium as carrier gas (20 PSI). The injector and
23 column temperatures were 90°C and 80°C, respectively. The detector was a microthermal
24 conductivity detector (μTCD). Multiplexing the channels allowed the simultaneous connection of 16
25 batch tests with a measure of the total gas production every 2 hours by pressure measurement. To
26 maintain a constant pressure in headspace, the gas composition was evaluated by sampling only
27 when the pressure was higher than 1 bar.

28 Each batch test run was carried out in quadruplicates and average values were considered for
29 discussion.
30

31 **2.5 Data Analysis**

32 **2.5.1 Hydrogen production estimation and kinetics**

33 The volume of hydrogen (V_{H2}) produced in the time interval between each measurement (t - t-1)
34 was calculated according to Equation 1 [36]:
35

$$36 V_{H_2} = V_{biogas} \cdot C_{H_2} \quad (\text{Eq. 1})$$

1
2 where: V_{biogas} is the volume of the biogas produced at time t ;
3 C_{H_2} is the concentration of hydrogen measured at time t .
4
5

6 The volume of biogas (V_{biogas}) was calculated in accordance with the ideal gas law, taking into
7 consideration the biogas moles (n_{biogas}), which, in turn, had been calculated as described in
8 Equation 2 [36].
9

$$n_{\text{biogas}} = V_{\text{HS}} \cdot (P_t - P_{t-1}) / R \cdot T_{\text{DF}} \quad (\text{Eq. 2})$$

10
11 where: V_{HS} is the head-space volume at time t ;
12 P_t is the pressure measured at time t before the analysis;
13 P_{t-1} is the pressure measured at time $t-1$ after the analysis;
14 R is the ideal gas constant;
15 T_{DF} is the temperature of the water bath during the dark fermentation tests.
16

17 The hydrogen production kinetic parameters were assessed by fitting the data to the modified
18 Gompertz equation [37], as described below.
19

$$H(t) = H_{\text{max}} \cdot \exp \cdot \left\{ -\exp \left[\frac{R_{\text{H}_2} \cdot e}{H_{\text{max}}} \cdot (\lambda - t) + 1 \right] \right\} \quad \text{Eq. (3)}$$

20
21 where: t is the time [d];
22 $H(t)$ is the cumulative hydrogen production at time t [ml H_2];
23 H_{max} is the maximum cumulative hydrogen production [ml H_2];
24 R_{H_2} denotes the maximum H_2 production rate [ml/d];
25 $e = 2.71828$;
26 λ is the lag-phase time [d] for hydrogen production.
27
28

29 30 3. Results and Discussion

31 32 3.1 Effects of the pretreatment on the substrate characteristics

33 The application of the formic acid resulted in significant solubilization enhancement of both kinds
34 substrates, as depicted by the variation in sCOD (Figure 1). The increase in sCOD was found to
35 range between 6.4-19.9% and 7.1-19.2% for OF and OF+LF, respectively. These values are
36 consistent with previous studies on the pretreatment of organic solid waste [38, 39] and, as
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1 expected, the different operating conditions promoted diverse solubilization pathways of the
2 organic matter.

3 The high acid concentration (15%) was observed to be more effective than the low one (5%) in
4 terms of OFMSW solubilization (Figure 1a); such effect was particularly evident when the high acid
5 concentration was applied under high temperature, as for the combination f (15% - 120°C - 35 min)
6 and h (15% - 120°C - 70 min). The sCOD variation was up to 100% higher than the one recorded
7 for the substrate pretreated at ambient temperature with 15% formic acid. These results indicate
8 that the organic substance of the samples were effectively solubilized by high concentrations of
9 formic acid. The solubilized fraction in the pretreated substrates was thus expected to result in
10 additional amounts of easily available substrate for the microbial growth, reportedly leading to an
11 enhanced organic waste digestibility [40, 41]. However, when the 15% formic acid was used at the
12 lower temperature (80°C) for 35 minutes, the sCOD variation was significantly lower than the one
13 achieved either at the higher temperature (combination f: 15% - 120°C - 35 min) or after a longer
14 treatment time (combination g: 15% - 80°C - 70 min).

15 At the same acid concentration and duration, the higher temperature improves the extent of
16 organic matter solubilization, as already reported by Alvarez-Gallego et al. [42]. These authors
17 found a better solubilization of the organic matter at temperatures up to 160°C and concluded that
18 losses by thermal decomposition, polymerization or caramelization of organic matter were not
19 induced. Similar consideration can be raised also for the OF+LF samples. Figure 1b shows that,
20 under the same pretreatment conditions, the improvement of sCOD for the mixed samples is
21 generally higher than the one obtained for the single OF substrates.

22 The degradation promoted via the application of the formic acid pretreatment can either solubilize
23 or mineralize the organic matter, basically depending on the complexity of the organic molecules
24 reacting with the acid under the defined operating conditions. Differently from the solubilization,
25 which is a desirable effect, the occurrence of mineralization phenomena should not be promoted
26 during the pretreatment of substrates destined to anaerobic conversion processes. The
27 transformation of organic materials into inorganic products would, indeed, result in the decrease of
28 substances available for the anaerobic bioconversion, with the consequent reduction of the overall
29 process yields. In this study, the mineralization effects were referred to the reduction in VS content,
30 plotted in Figure 2.

31 The formic acid resulted in VS reduction ranging between 10 and 34%, with the most relevant
32 effects occurring at the high concentration (15%) for the OF samples. Relevant mineralization was
33 also observed when reducing the acid concentration and increasing the treatment time under mild
34 temperature, as depicted for the combination c (5% - 80°C - 70 min). In particular, comparing the
35 results obtained under this combination with those observed for the combination e (15% - 80°C -
36 35 min), the increase in both solubilization and mineralization occurred, pointing out an overall
37 enhancement of the pretreatment extent. For the OF+LF samples, higher VS decrease was

1 detected for the high acid concentration (Figure 2b): however, under the combination c (5% - 80°C
2 - 70 min), the mineralization effects were comparable with those obtained for the combination e
3 (15% - 80°C - 35 min), suggesting that under mild temperature conditions, mineralization can be
4 promoted by either high acid concentration or long treatment duration.

5 The influence of the pretreatment parameters was also investigated with reference to the variation
6 in the biochemical properties of the substrate. Due to the importance of the carbon to nitrogen
7 (C/N) ratio in anaerobic processes [43], this is an essential indicator to control the biochemical
8 conversion of the biomass. Usually, a C/N ratio between 35 and 45 is advised to pursue good
9 performances in fermentation processes [44]. In this study, the C/N ratio was 5.5 and 5.6 for the
10 OF and OF+LF, respectively, and it was found to enhance after the formic acid pretreatment
11 (Figure 2). The increase in C/N ratio is affected by the reduction in nitrogen content, which followed
12 the formic acid pretreatment despite the applied operating conditions. In this view, Wang et al. [45]
13 pointed out that significant decrease in nitrogen content may occur in sulfuric acid hydrothermal
14 treatment processes, due to the formation of ammonium sulfate, which may decompose and
15 release ammonia gas. A similar mechanism likely occurred for the OF+LF samples, as the
16 variation in C/N ratio shows the same trend of the VS reduction. Nevertheless, for the
17 combinations a (5% - 80°C - 35 min) and b (5% - 120 °C - 35 min), a low reduction in VS was
18 recorded together with relatively high values of the C/N ratio, because a decrease in carbon
19 content also occurred. Similar considerations can be drawn for the OF samples pretreated under
20 the combinations a (5% - 80°C - 35 min) and e (15% - 80°C - 35 min), suggesting that mild
21 temperatures and short treatment time result in the primarily mineralization of the carbon.

22 Although higher enhancement of the H₂ yields was expected for the samples characterized by C/N
23 ratio greater than 35, it is worth pointing out that Chen et al. [46] reported that the dark
24 fermentation of organic components characterized by a C/N ratio between 7 and 25, in the pH
25 range 5 - 8, can result in the simultaneous production of hydrogen and VFA, up to 100 mL/g_{COD}
26 and 311 g_{COD}/kg_{COD}, respectively.

3.2 Effects of the formic acid pretreatment on hydrogen production

27 Throughout the batch experiments, only H₂ and CO₂ were detected in the gas phase. Methane
28 (CH₄) was not observed among the gaseous products, pointing out that the heat-shock treatment
29 of the inoculum had efficiently suppressed the methanogenic activity.

30 In the control substrates (untreated samples), H₂ production attained yields of 13.3 and 8.8 ml/g_{SV}
31 for OF and OF+LF substrate, respectively (Figure 3). These H₂ yields were lower than the
32 previously reported ones, obtained from pretreated lignocellulosic and organic waste [44, 47].
33 Angeriz-Campoy et al. [48] reported that the specific H₂ production from the dark fermentation of
34 OFMSW was as low as 9 mL/g_{VS} at 1.2-days hydraulic retention time. The authors attributed this
35 outcome to the difficult colonization and hydrolyzation of the waste solid particles by the hydrolytic
36

1 microorganisms. The same condition likely occurred in this study, since the H₂ production from the
2 sample containing the LF, which is hard to biodegrade, was observed to be lower than that
3 obtained from the single OF substrate. Nonetheless, differences in the total H₂ productions were
4 observed, depending on the operating conditions (Figure 3).

5 The formic acid concentration remarkably affected the H₂ yield of the OF samples, with the best
6 performances being observed at the lower value (5%). The maximum H₂ yield was obtained under
7 the operating conditions of the combination c (5% - 80°C - 70 min), that resulted in 31.6 mL H₂/g_{VS}
8 for the OF substrates. It is evident that the process at low temperature and for long time enhanced
9 the fermentative degradation of the organic substrates, which was particularly evident under
10 weakly acidic conditions. Conversely, at higher formic acid concentration, the greatest increase in
11 H₂ production was observed for the substrates pretreated at the most extreme conditions
12 (combination h: 15% - 120°C - 70 min).

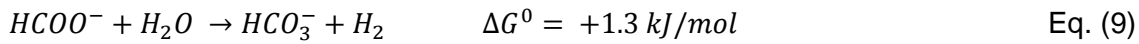
13 The low acid concentration promoted the H₂ generation from the dark fermentation of OF samples,
14 despite the operating temperature and time. According to Bolado-Rodríguez et al. [49], comparing
15 the effects of thermal, acid, alkaline and thermo-alkaline pretreatments on the methane production
16 of wheat straw and sugarcane bagasse, the use of 1.5% w/w HCl solution at 121°C for 60 minutes
17 generated 5-hydroxymetilfurfural (HMF) and furfural in the pretreatment liquids, accounting for the
18 inhibition of the subsequent bioconversion process. Similar results were also reported by Monlau et
19 al. [50].

20 The pretreatment of the OF+LF substrates under the high acid concentration and the same
21 operating temperature led to increasing total H₂ yield for decreasing pretreatment duration: this
22 aspect can be observed comparing the results of the test runs e (15% - 80°C - 35 min) and g (15%
23 - 80°C - 70 min). At low acid concentration, similar trends can be observed comparing the results
24 of the test runs a (5% - 80 °C - 35 min) and c (5% - 80 °C - 70 min) as well as those of the test
25 runs b (5% - 120 °C - 35 min) and d (5% - 120 °C - 70 min). Conversely, the most extreme acid
26 and temperature conditions resulted in higher H₂ production at the longer treatment duration.

27 Moreover, the decrease of the temperature (from h: 15% - 120°C - 70 min to g: 15% - 80°C - 70
28 min as well as from f: 15% - 120 °C - 35 min to e: 15% - 80°C - 35 min) promoted an increase in H₂
29 production. For the same kinds of substrates, less relevant difference was observed, at high
30 temperature and pretreatment duration, when the formic acid concentration was reduced (from h:
31 15% - 120°C - 70 min to d: 5% - 120°C - 70 min). These results confirmed that the degradation of
32 the LF in the mixed samples during the dark fermentation is affected by the temperature of the
33 pretreatment [51].

34 It is worth pointing out that the greatest H₂ yields were not obtained under the pretreatment
35 conditions that resulted in higher solubilization increase. This evidence could be a strong indication
36 of the release of inhibitory compounds, such as HMF and furfural, in the liquid phase.
37 Nevertheless, it should be taken into account that H₂ might be also produced from sources other

1 than the degradation of the substrate organic matter. Indeed, the pretreatment conditions could
2 have promoted the production of H₂ from formic acid decomposition. In biological systems the
3 formic acid is converted to H₂ due to the facultative anaerobic microbes [52], which can develop
4 under anaerobic conditions, according to the following equation:



7 This conversion pathway depends on the formate hydrogen-lyase (FHL) system, which facilitates
8 the H₂ production via the direct break down of formic acid [53]. The treatment of the substrates
9 under operating temperature of around 80°C [54], stimulates the oxidation of the formic acid
10 present into the medium. This likely happened when a low initial acid concentration was treated on
11 long term at a given temperature (combination a: 5% - 80 °C - 35 min). Nevertheless, whatever the
12 pretreatment combination applied, the presence of the formic acid in the samples which were not
13 processed in autoclave promoted a significant inhibition of H₂ production performances in terms of
14 lag phase, yield and maximum production. The sole addition of the acid to the samples, without
15 their processing in the autoclave, did not allow the occurrence of the fermentative mechanisms.

16 The application of the pretreatment before the dark fermentation also contributed to reduce the
17 period of acclimation of the microorganisms, thus accelerating bacterial growth and maximizing H₂
18 production. These trends were consistent with those reported in previous studies, pointing out that
19 the lag phase in the dark fermentation process of organic waste ranged from 1.5 to 8 h, depending
20 on the pH, inoculum and medium characteristics. The addition of the heat-shocked sludge and the
21 adjustment of the initial substrate to biomass ratio of the medium for the BHP tests resulted in
22 moderating the duration of the lag phase [55, 56] and also allowed the optimization of the process
23 kinetics [34].

24 Figure 4 plots the cumulative H₂ yields over time for the OF substrate, both untreated and
25 pretreated under the combination c (5% - 80°C - 70 min), which was observed to provide the
26 highest H₂ yield. In the control substrates, H₂ production started after more than 20 hours and went
27 on increasing for up to 5 days, whereas in the pretreated samples, the lag phase lasted only 2
28 hours and the generation of H₂ kept enhancing over time. The Gompertz curves showed that the
29 application of the pretreatment resulted in H₂ production trends evolving consistently with the
30 theoretical ones for both the control and the pretreated OF samples.

31 At the end of the fermentative process, the H₂ concentration decreased in all tests due to both the
32 depletion of readily available substrates and the biological H₂ consumption [57]. Since no CH₄
33 production was detected, H₂ consumption may have been caused by either the propionic
34 fermentation [58] or homoacetogenesis [59].

3.3. Effects of the pretreatment on the generation of metabolites

In this study, the total production of metabolites varied depending on the substrate composition as well as on the process parameters. The pretreatment promoted the overall metabolite generation, which increased in both the OF and OF+LF substrates. The dark fermentation process further enhanced these concentrations up to 3.39 mg_{COD}/g_{VS} for the OF substrate pretreated under the combination f (15% - 120°C - 35 min) and 11.47 mg_{COD}/g_{VS} for the OF+LF substrate pretreated under the combination e (15% - 80°C - 35 min).

The organic solvent pretreatment promoted the production of metabolites as already obtained from the dark fermentation of the raw substrates, which accounted for 0.42 mg_{COD}/g_{VS} and 1.08 mg_{COD}/g_{VS} for OF substrate and OF+LF substrate, respectively. For the former one (OF substrate) the concentrations of acetic and butyric acids increased up to 0.11 mg/g_{VS} and 0.11 mg/g_{VS}, respectively; in the case of the mixed samples, such increase reached 0.30 mg/g_{VS} for the acetic acid and 0.28 mg/g_{VS} for the butyric one. Nissilä et al. [60] reported a total VFA production of 4.5 g/l from pure cellulose and starch biomass fermentation, while pretreated lignocellulosic biomass provided a VFA yield of 0.54 g/g_{VS} [61]. The dark fermentation of food waste and kitchen waste produced VFA concentrations up to 0.18 g/g_{VS} [55] and 0.76 g/g_{VS} [62]. As already observed for the generation of H₂, although diverse increases were observed for pretreated samples, the experimental results obtained in this study show lower values than the ones reported in scientific literature. Beyond the presence of solid waste particles, which accounts for the difficulties in degradation by microorganisms, the lack of mixing may have further limited the proper process development [63].

Nevertheless, experimental results highlight that different metabolite pathways occurred and some of the pretreatment combinations influenced those related to the generation of hydrogen.

For both the untreated OF and OF+LF samples, the increase in acetic and butyric acid concentration was observed, suggesting that the biological fermentation of these substrates mainly developed via acetic and butyric acid pathways.

Garcia-Aguirre et al. [64] and Oshoma et al. [65] showed that at slightly acidic conditions both the butyric and acetic acids are the main products of dark fermentation. Similarly, in our study, the highest increase in the content of butyric acid was achieved when the pretreatment of both OF and OF+LF samples was performed under low concentration of formic acid (combination b: 5% - 120°C - 35 min; combination c: 5% - 80°C - 70 min; combination d: 5% - 120°C - 70 min;). In this cases, the concentration of the acetic acid in the pretreated samples increased as well. The fermentation of the samples pretreated under the combination c (5% - 80°C - 70 min), which gave the highest H₂ production from OF samples, resulted in a butyric acid/acetic acid (Bu/Ac) ratio of 3.60, which did not fall within the optimal range of 0.25 - 1.5 suggested by Ghimire et al. [55] to enhance the H₂ production. Nevertheless, the Bu/Ac ratio might not always give a relevant indication for high H₂

1 generation. Guo et al. [37] reported that the homoacetogenic activity can influence the
2 concentration of end-metabolites due to the production of acetate from H₂.

3 In this view, relevant content of acetic acid was found in the effluents of the dark fermentation of
4 both OF and OF+LF samples pretreated with formic acid at high concentration, as in the
5 combination f (15% - 120°C - 35 min) and h (15% - 120 °C - 70 min). This evidence might indicate
6 the prevalence of a homoacetogenic activity responsible for the lower H₂ yields. Moreover, the high
7 formic acid concentration at high temperature applied during the pretreatment step promoted the
8 accumulation of the lactate, as showed in Figures 5 and 6, that further accounted for the low H₂
9 generation [66].

10 The lactate is an intermediate metabolic product, which at low concentrations can be converted to
11 other products, such as acetate, butyrate and propionate [67]. For both OF and OF+LF samples, it
12 was observed a reduction in lactate concentration and an increase in acetic acid content (Figures 5
13 and 6) after the dark fermentation of the substrates pretreated with 15% of acid concentration at
14 120°C for 35 minutes. This likely indicates that the lactic acid was efficiently converted into acetate,
15 which was probably further consumed within homoacetogenic process, leading to the reduction in
16 H₂ generation.

17 Therefore, the acidic conditions are crucial to pursue the valorization of the dark fermentation
18 pretreatment effluents. As the lactic acid production is not desired in dark fermentation due to its
19 inhibitory effect on H₂ production but it has a high market value, it should be first extracted from the
20 dark fermentation medium and destined to recovery [35].

21 Through acidogenic fermentation, VFA-rich organic waste could be valorized in order to obtain
22 several intermediates to be used for bio-plastic production. Some investigations have been done
23 but further efforts are needed for the scale up [68]. The identification of the most suitable
24 valorization scheme for the pretreated organic waste should take into account the VFA composition
25 that can be promoted during the pretreatment step. In this view, different operating conditions can
26 be set up to address the production of specific metabolites, so as to fulfill the market needs.

4 Conclusion

27 The effects of a thermo-chemical pretreatment with formic acid were investigated on differently
28 composed organic waste samples.

29 Experimental results pointed out that the higher acid concentration selected for the purposes of this
30 study (15%) promoted both the solubilization and a partial mineralization of the samples;
31 conversely, the lower value (5%) was found to enhance H₂ production from the dark fermentation
32 of pretreated substrates. This evidence is consistent with the composition of the soluble fraction of
33 the samples, since the most extreme pretreatment conditions (15% formic acid concentration and
34 120°C) were found to promote the generation of metabolic intermediates whose presence is not

1 desirable for H₂ production, such as ethanol and lactate in concentrations up to 2.41 mg/g_{VS} and
2 4.27 mg/g_{VS}, respectively, depending on the kind of substrate as well as on the pretreatment
3 duration.

4 In this study the highest H₂ generation from OF was obtained when the pretreatment was applied
5 at the low formic acid concentration (5%), under mild temperature (80°C) for 70 minutes: under
6 these conditions, the H₂ generation from the pretreated samples was 1.4-fold higher than the one
7 recorded for the untreated substrates. For the mixed OF+LF substrates the greatest H₂ generation
8 was achieved when the formic acid at low concentration (5%) was combined with high temperature
9 (120°C) for 35 minutes.

10 Experimental outcomes showed that the formic acid concentration plays a key role in the
11 conversion of the investigated substrates into a variety of reduced products such as H₂, ethanol,
12 lactate and organic acids: adjusting the other pretreatment parameters, namely operating
13 temperature and time, the subsequent biological process can be targeted towards the production
14 of either building blocks or energy carriers. Such approach can be particularly suitable for
15 lignocellulosic substrates, since the extent of the investigates thermo-acid pretreatment was found
16 to provide better results for the mixed OF+LF samples.

17 The combination of the organic solvent pretreatment and dark fermentation processes could thus
18 contribute to the bio-based society, reducing organic residues and increasing the efficiency of the
19 biomass-based production of energy and valuable commodities, while addressing the biorefinery
20 concept in waste management.

21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 **Acknowledgements**

38 The preparation of this manuscript was carried out in the framework of the project SUPER
39 (Sustainable Production of Energy from organic Residues), funded by the Università Italo
40 Francese/Université Franco Italienne (UIF/UFI).
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| Fraction | Percentage ^a [%] |
|--------------------------------|-----------------------------|
| Cooked meat-cooked fish-cheese | 16.2 |
| Fruit peeling | 32.2 |
| Vegetable peeling | 32.1 |
| Bread and cooked pasta | 19.5 |
| Total | 100 |

^a Determined by weight, wet basis

Table 1. Composition of the OFMSW

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| Parameter | (OF + LF) _{untreated} | OF _{untreated} | Aerobic sludge |
|--|--------------------------------|-------------------------|----------------|
| pH | 6.4 ± 0.3 | 6.7 ± 0.2 | 6.7 ± 0.2 |
| TS [% wet weight] | 78.5 ± 2.3 | 29.8 ± 2.8 | 10.1 ± 2.0 |
| VS [%TS] | 22.8 ± 0.7 | 49.2 ± 3.7 | 34.3 ± 0.9 |
| sCOD [g/l] | 33.1 ± 7.7 | 22.3 ± 10.7 | 2.7 ± 0.6 |
| TOC [mg/l] | 1.65 ± 0.04 | 0.70 ± 0.07 | 0.2 ± 0.2 |
| TKN [mg/kg _{DS}] | 3.0 ± 1.3 | 5.8 ± 1.9 | 1.9 ± 1.1 |
| Carbohydrates [% _{wet weight}] | 18.8 ± 1.9 | 27.1 ± 4.7 | - |
| Protein [% _{wet weight}] | 11.9 ± 3.1 | 15.3 ± 2.3 | - |

Table 2. Characteristics of substrates and inoculum used in this study

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| Factors | Low value | High value |
|-------------------------------|------------------|-------------------|
| Formic acid concentration [%] | 5 | 15 |
| Operating temperature [°C] | 80 | 120 |
| Operating time [min] | 35 | 70 |

Table 3. Selected values of the operating parameters for the pretreatment under investigation

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| Run | Formic acid concentration | Operating temperature | Operating time |
|-----|---------------------------|-----------------------|----------------|
| | [%] | [°C] | [min] |
| a | 5 | 80 | 35 |
| b | 5 | 120 | 35 |
| c | 5 | 80 | 70 |
| d | 5 | 120 | 70 |
| e | 15 | 80 | 35 |
| f | 15 | 120 | 35 |
| g | 15 | 80 | 70 |
| h | 15 | 120 | 70 |

Table 4. Combinations of the pretreatment operating conditions for the experimental test runs

1 **Figure captions**

2
3 **Figure 1.** Variation of sCOD for OF (a) and OF+LF (b) samples after the pretreatment in
4 comparison with sCOD of untreated substrates.

5
6 **Figure 2.** Variation of VS content and C/N ratio for OF (a) and OF+LF (b) samples after the
7 pretreatment, with respect to the untreated substrates.

8
9
10 **Figure 3.** Total H₂ production from both untreated and pretreated OF (a) and OF+LF (b) samples,
11 with the indication of the pH before and after BHP tests.

12
13 **Figure 4.** Cumulative H₂ production yields for OF, both untreated and pretreated under
14 combination c (5% - 80°C - 70 min). Lines indicate Gompertz curves.

15
16
17 **Figure 5.** Share of metabolites generated from both untreated and pretreated OF samples before
18 (a) and after (b) BHP tests.

19
20 **Figure 6.** Share of metabolites generated from both untreated and pretreated OF+LF samples
21 before (a) and after (b) BHP tests.

Figure 1a

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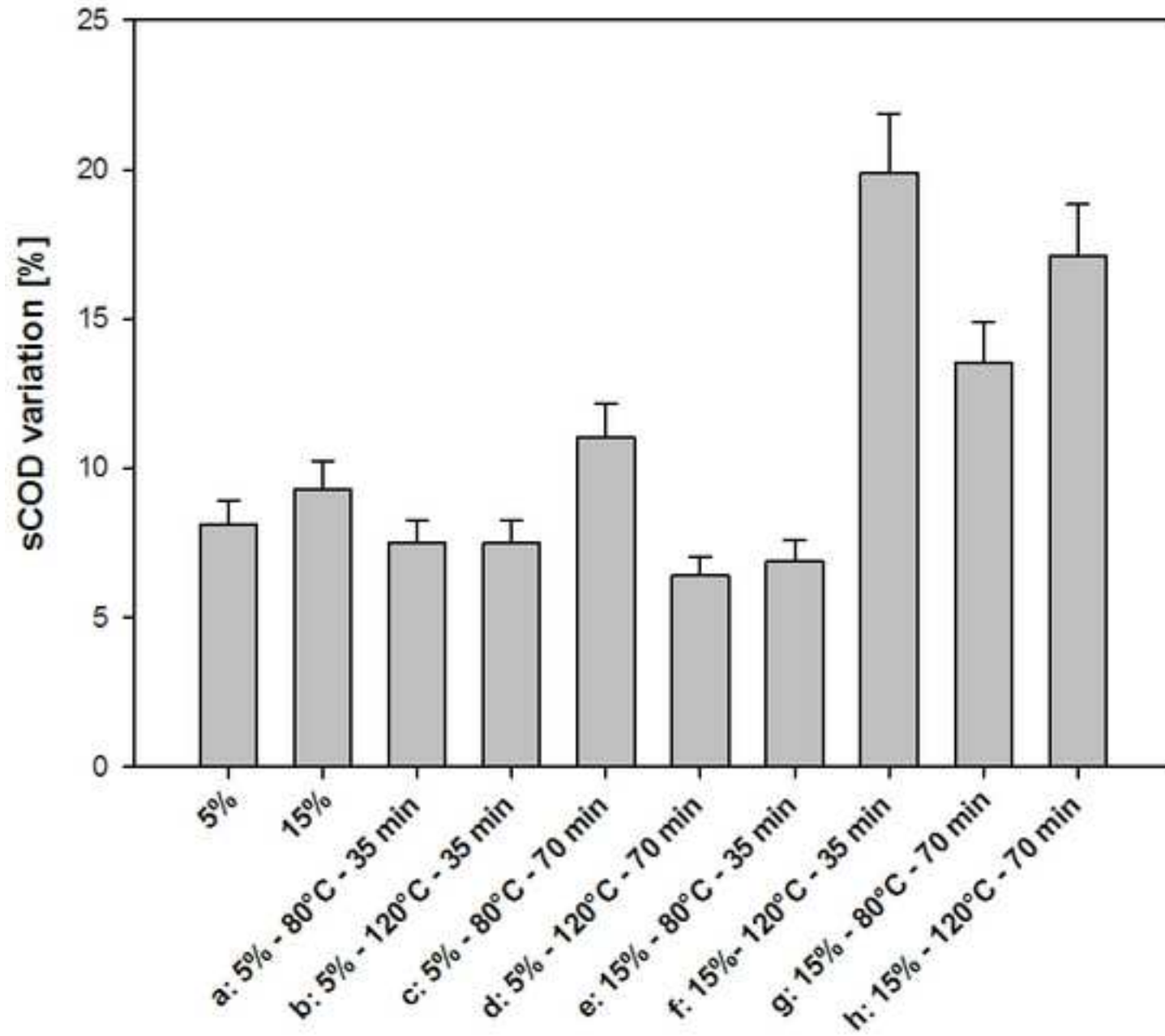


Figure 1b
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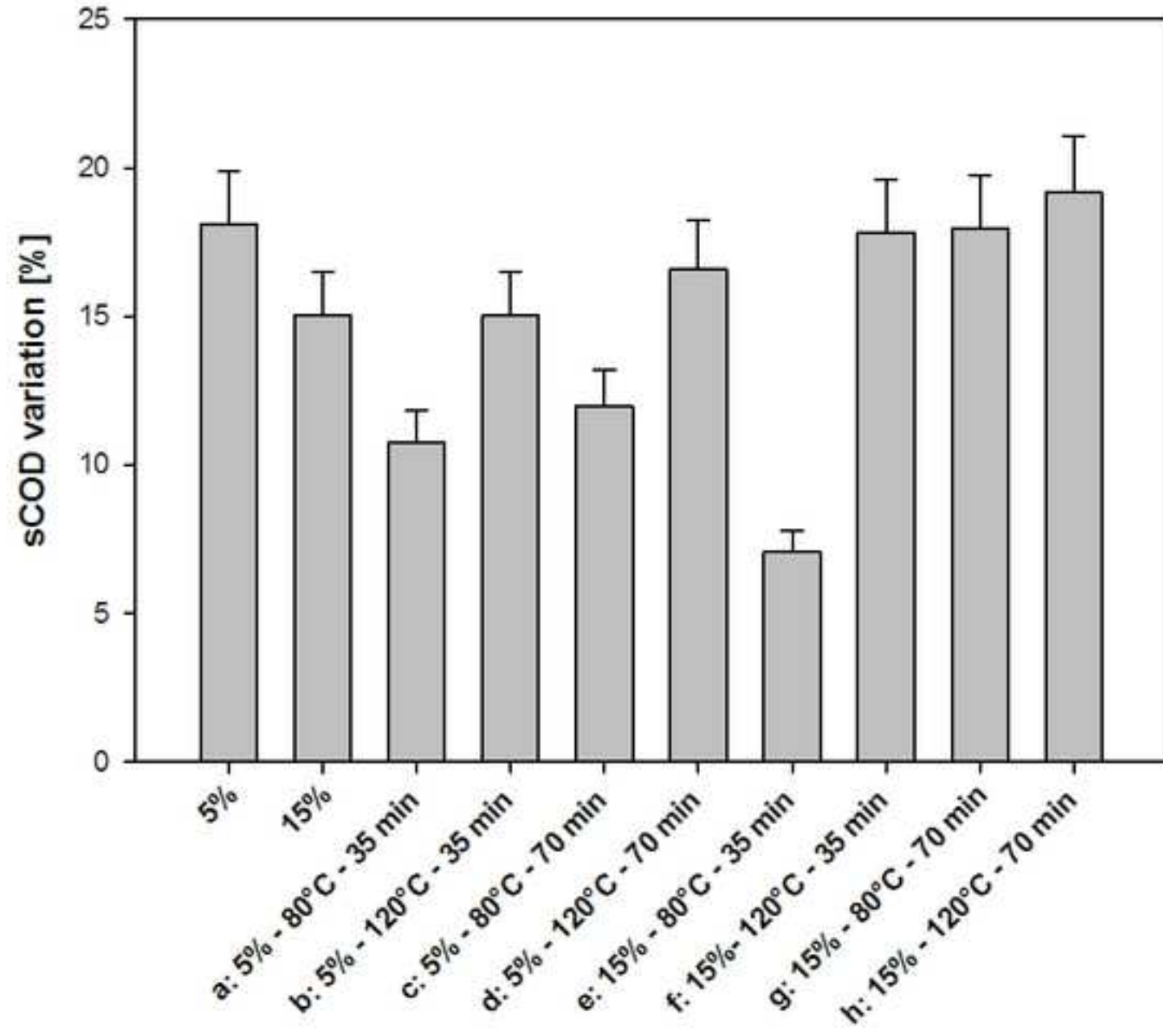


Figure 2a

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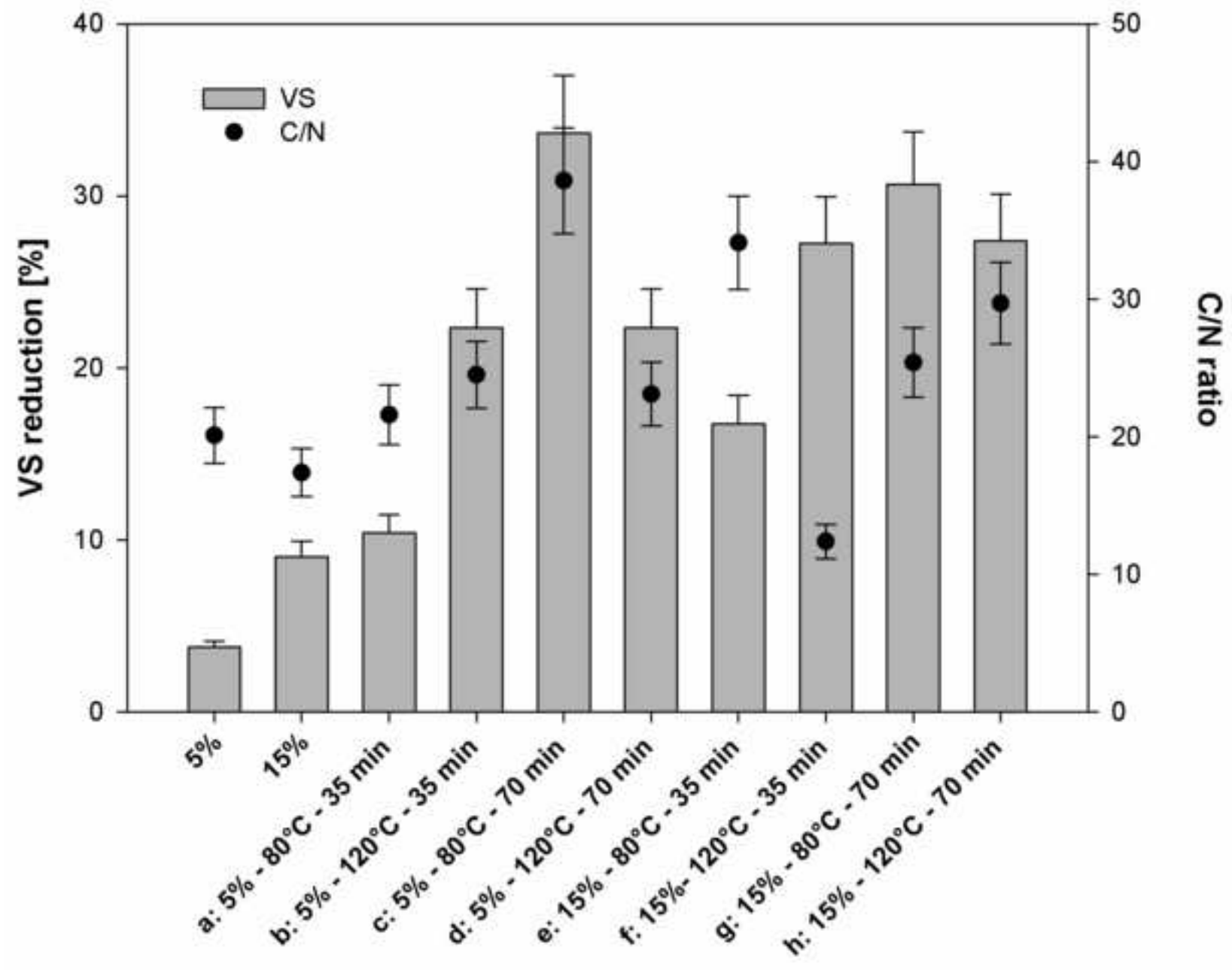


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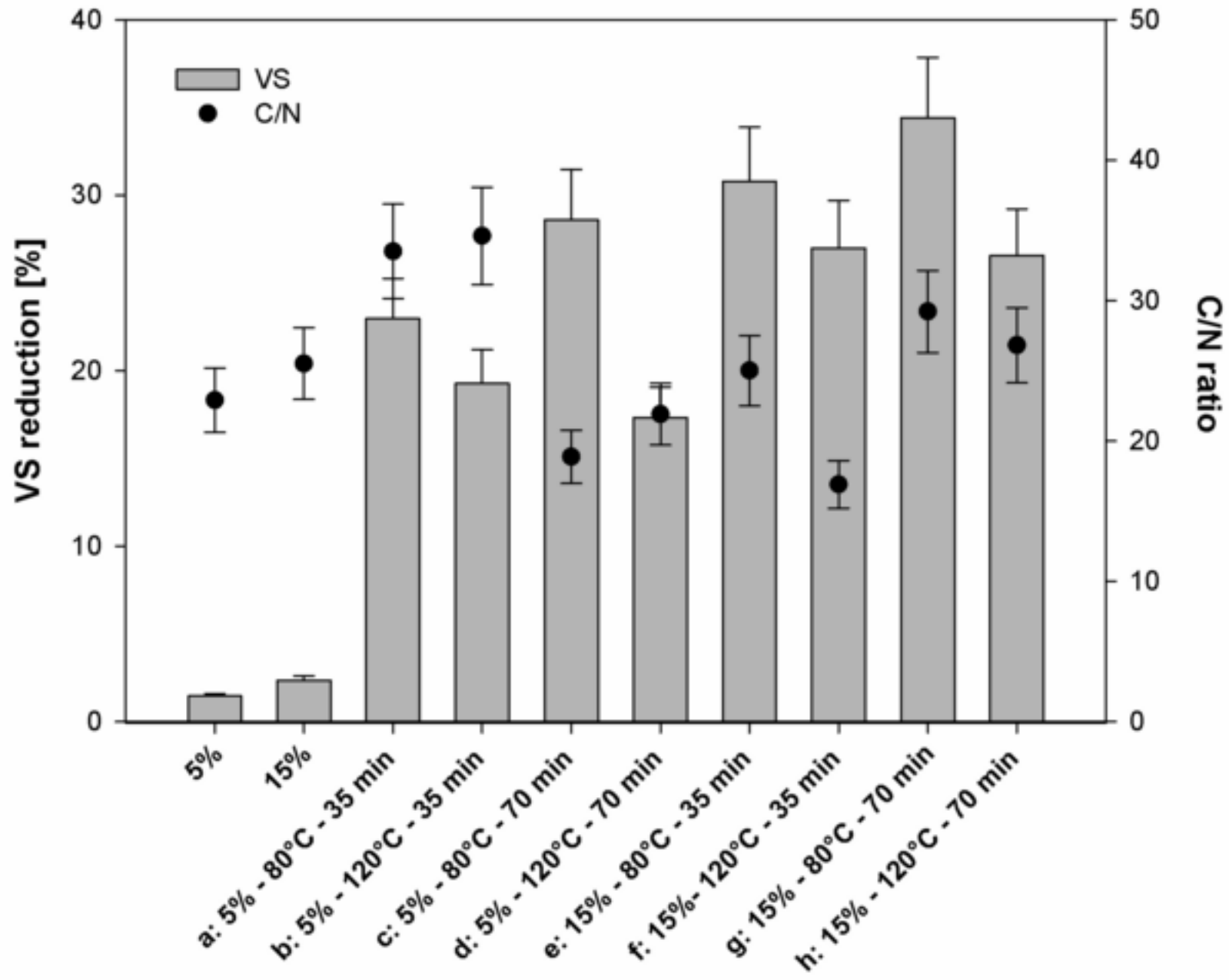


Figure 3a
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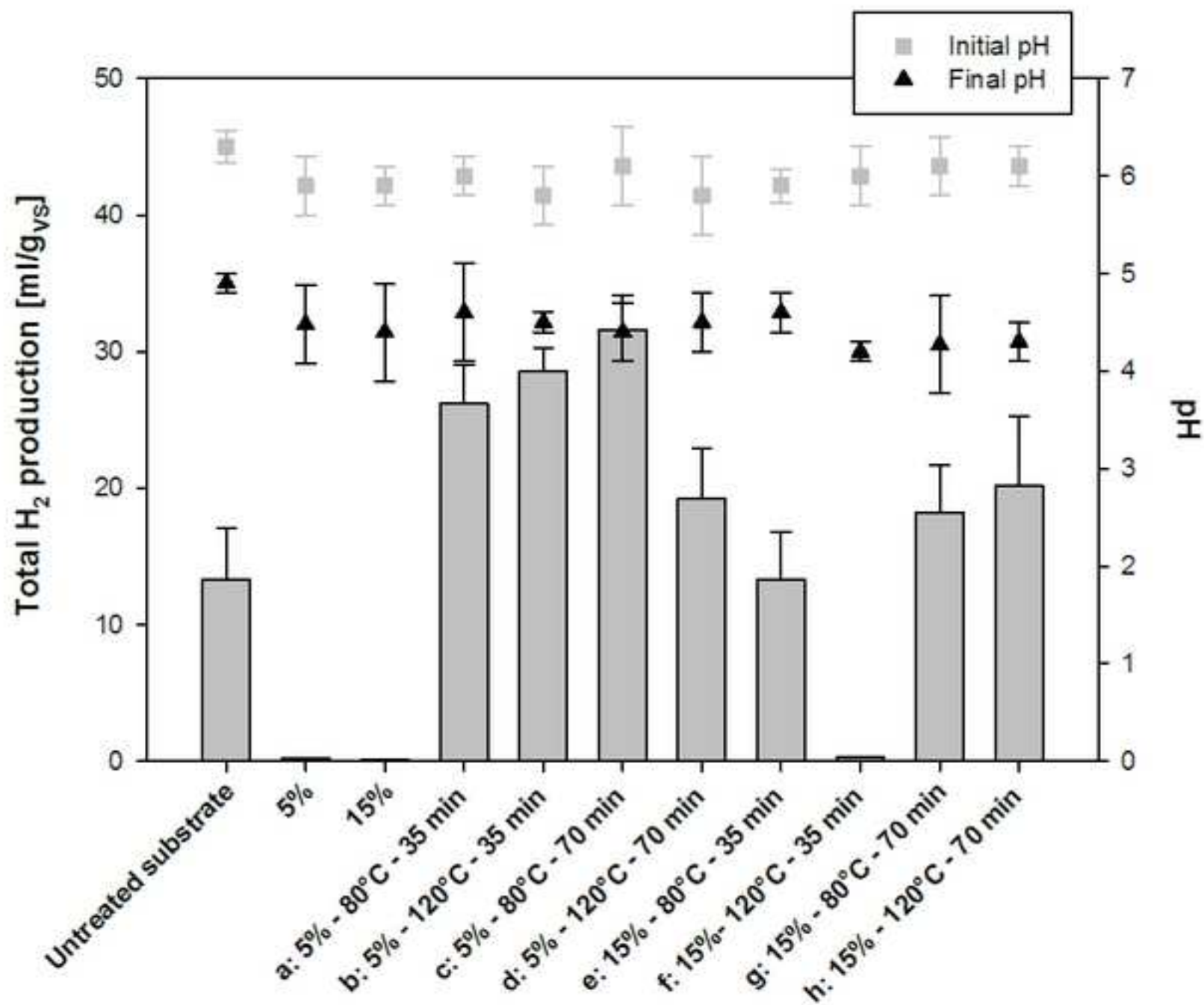


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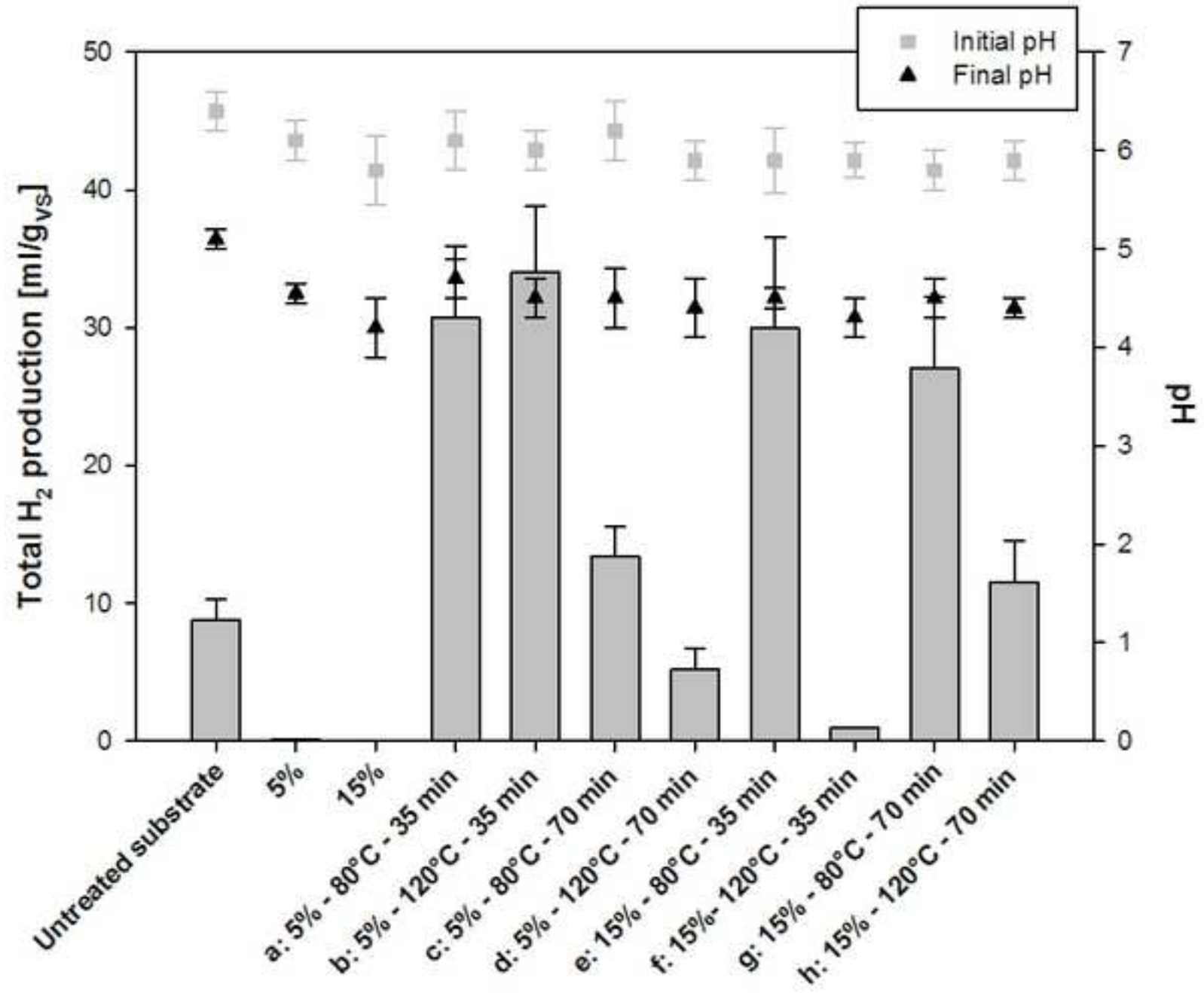


Figure 4
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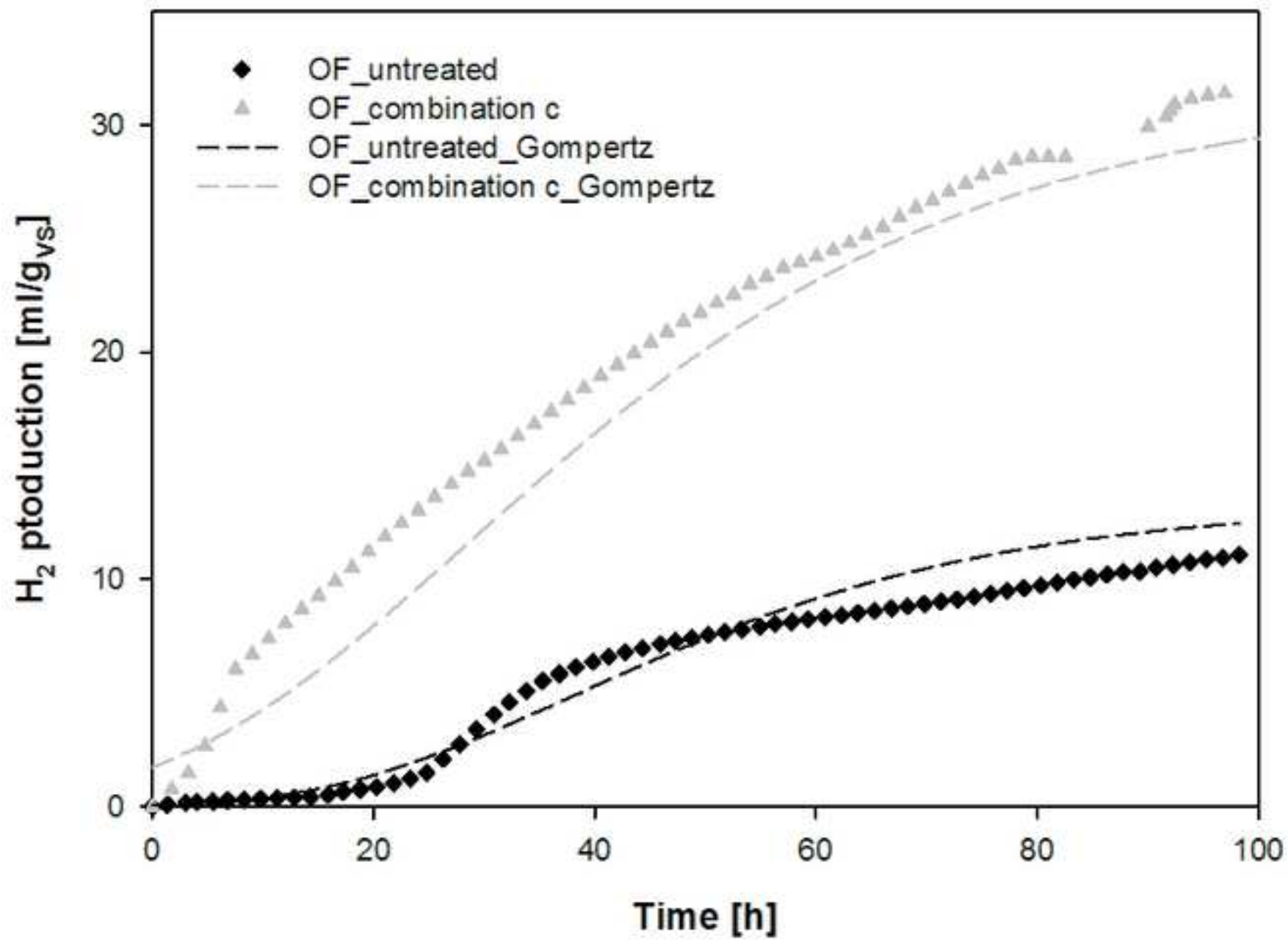


Figure 5a

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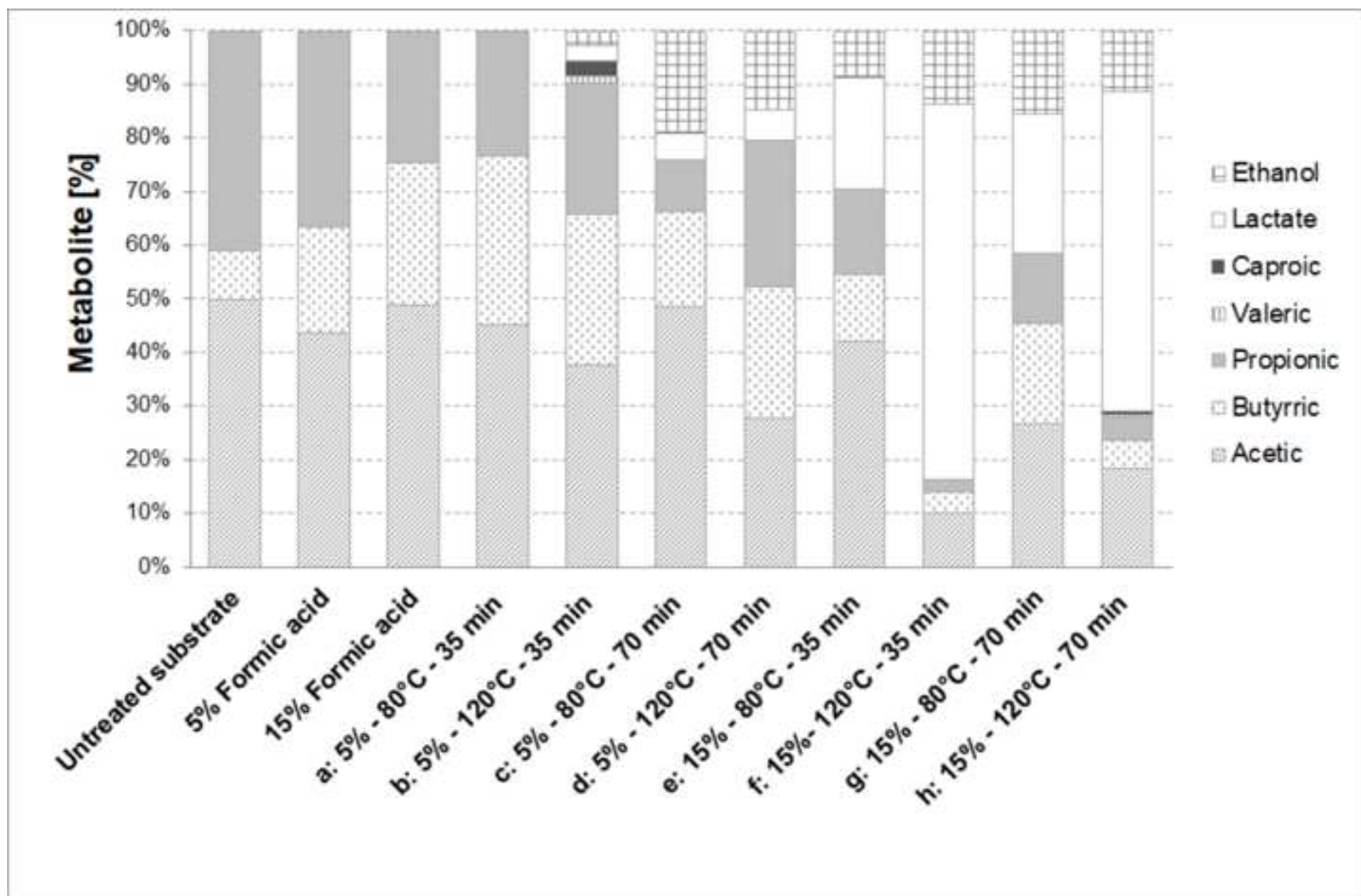


Figure 5b
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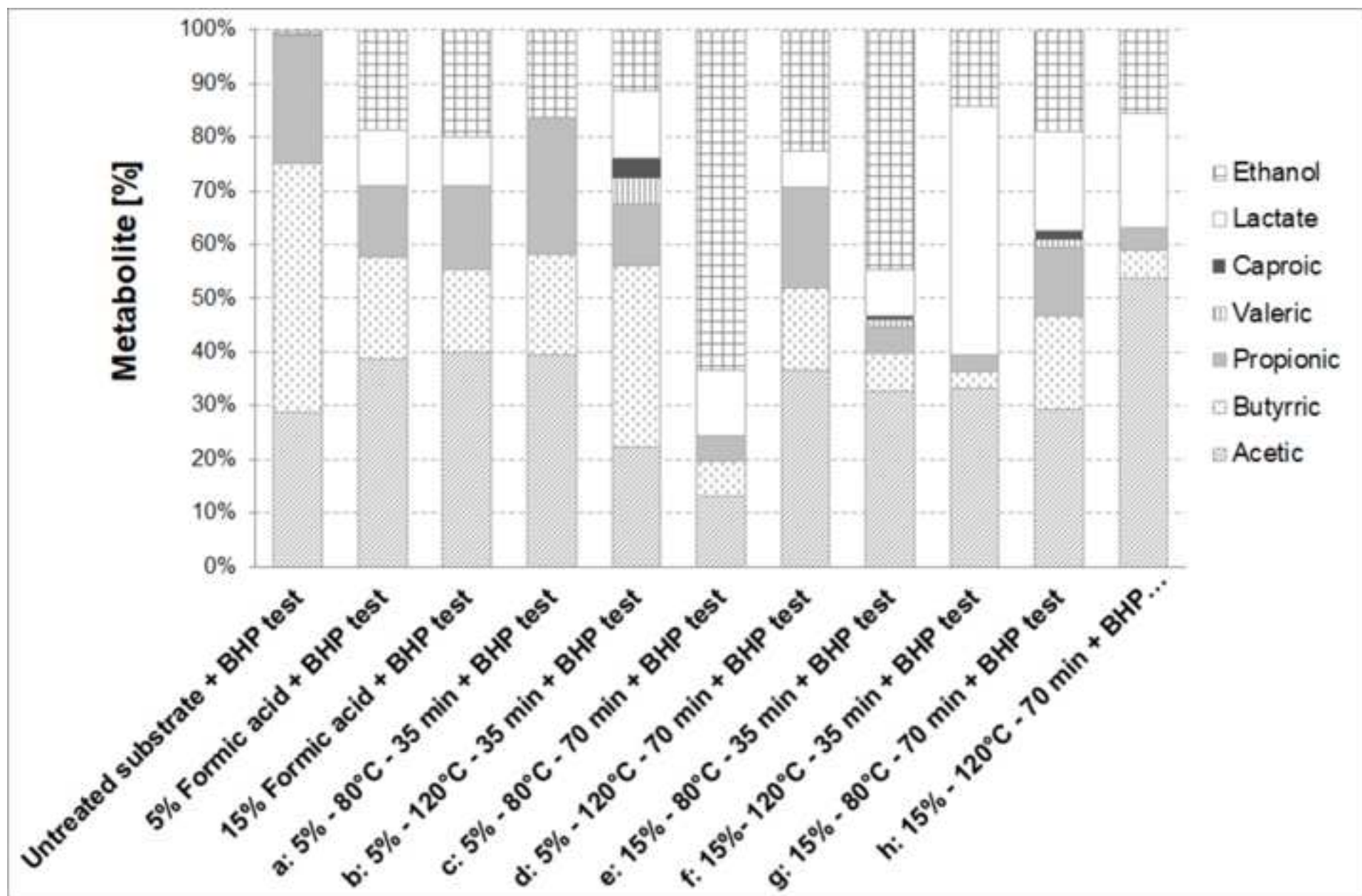


Figure 6a

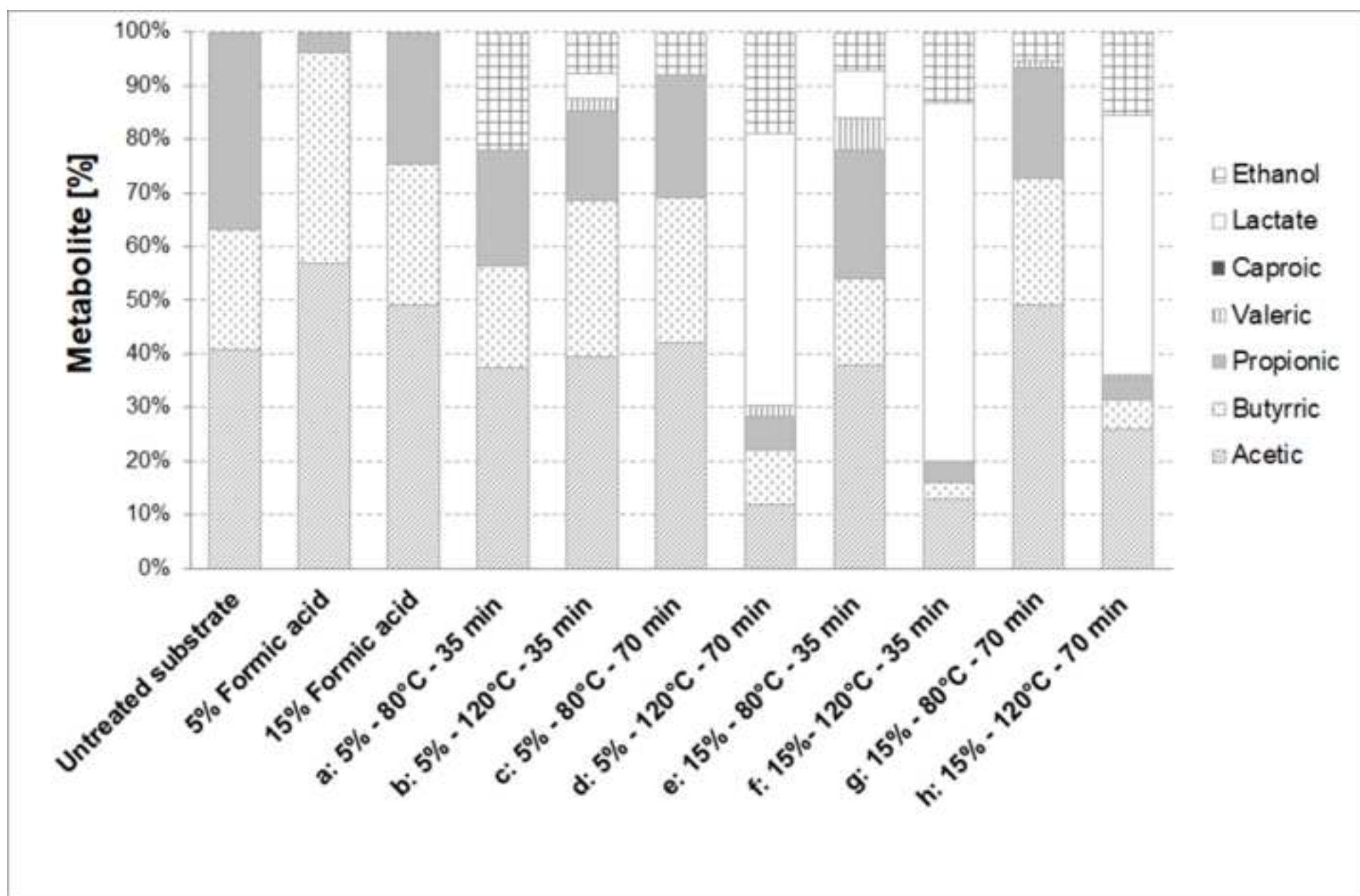
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Figure 6b

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