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Research article

Effect of passively aerated biological pretreatment on different biomasses with diverse lignocellulosic fiber profiles

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ABSTRACT

Passively aerated biological pretreatment was applied to four different lignocellulosic biomasses with varying fiber content profiles: sugar beet pulp (SBP), brewery bagasse (BB), rice husk (RH), and orange peel (OP). In order to analyze the organic matter solubilization yield at 24 and 48 h, different percentages of activated sewage sludge (2.5–10%) were utilized as inoculum. The OP achieved the best organic matter solubilization yield in terms of soluble chemical oxygen demand (sCOD) and dissolved organic carbon (DOC) at 2.5% inoculation and 24 h: 58.6% and 20%, respectively, since some total reducing sugars (TRS) consumption was identified after 24 h. On the contrary, the worst organic matter solubilization yield was obtained with RH, the substrate with the highest lignin content among the tested, with percentages of 3.6% and 0.7% in terms of sCOD and DOC respectively. In fact, it could be considered that this pretreatment was not successful with RH. The optimum inoculation proportion was 7.5% (v/v) except for the OP (2.5% (v/v)). Finally, due to the counterproductive organic matter consumption at longer pretreatment durations, the optimal time for BB, SBP, and OP was 24 h.

Credit author statement

Amer Rouabhia: Writing-Original Draft, Investigation, Formal Analysis, Data Curation Visualization. **Carlos José Álvarez-Gallego:** Supervision, Methodology, Conceptualization, Writing – Review & Editing, Visualization. **Luis Alberto Fernández Güelfo:** Supervision, Methodology, Conceptualization, Writing – Review & Editing, Funding Acquisition, Visualization.

1. Introduction

Anaerobic digestion is -a biological process used in biorefineries-widely discussed in the scientific literature as a way to convert biomass to by-products with added value, such as biofuels (methane and/or hydrogen) and/or volatile fatty acids (VFAs).

Hydrolysis is the first stage of anaerobic digestion and, in the specific case of lignocellulosic waste, usually, it is the rate limiting-step of the process since vegetable fibers are hardly biodegradable. During the hydrolysis, carbohydrates, lipids and proteins are converted to monomers and then to VFAs as intermedial products (Bruni et al., 2021).

The VFAs are organic acids that contain six or less atoms of carbon (C2–C6), such as acetic, propionic, butyric, isobutyric, valeric, isovaleric, caproic and isocaproic acid. Although VFAs are widely used in industrial applications such as food, medicinal, petrochemical and cosmetics industries (Worwag and Kwarciak-Kozłowska, 2019), they are currently considered an excellent carbon source to produce polyhydroxyalkanoates (PHA) as bioplastic precursors (Anjum et al., 2016; Baumann and Westermann, 2016; Bhatia and Yang, 2017; Bravo-Porras et al., 2021; Esteban-Gutiérrez et al., 2018; Strazzera et al., 2018).

Prior to any biological process for lignocellulosic biomass valorization, the application of pretreatments (physical, chemical, biological or combinations) as process enhancer is very usual in order to promote the hydrolysis and solubilization of the organic matter and, hence, increase the production of added value by-products (Vu et al., 2021). Biological pretreatments are a recent approach to improve the digestibility of agricultural waste through the degradation of lignocellulosic structures and the hydrolysis of hemicellulose until fermentable sugars.

According to the literature, the main sources of microorganisms for biological pretreatments are fungal, microbial consortiums and specific enzymes (Safari Sinegani et al., 2005; Zabed et al., 2019). Certain fungi can biodegrade 40% of lignin from agricultural waste (Kainthola et al., 2019; Shi et al., 2019). In this sense, different groups of fungi (white rot,

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brown rot and soft rot) have been employed resulting the white rot group the most efficient for lignin degradation (del Cerro et al., 2021; Kamimura et al., 2019).

On the other hand, specific microorganisms such as pure cultures of *Cupriavidus basilensis* or sewage sludge (activated sludge) from wastewater treatment plants (WWTPs) have been also employed. In this case, the cellulose and hemicellulose of the biomass are also degraded in opposition to fungal pretreatment where lignin is the only one degraded (Li et al., 2020; Yan et al., 2017).

The use of enzymes also has the capacity to degrade cellulose and hemicellulose through specific ligninolytic enzymes such as pectinase, which have been applied with success on sugar beet pulp and orange peel (Kuo et al., 2019; Spagnuolo et al., 1997). In addition, these enzymes allow for reducing the pretreatment time with the simultaneous increase of the sugar conversion rate in comparison with the specific microorganisms as a source of biological agent but it has the handicap that the cost of enzymes is very high (Chan et al., 2020; Nguyen et al., 2019; Sharma et al., 2019; Wu et al., 2022; Zabed et al., 2018).

Taking into account the abovementioned issues, in this work a mixed culture of microorganisms, based on sewage sludge from WWTP, has been used as an inexpensive biological agent to pretreat in a passively aerated system four types of lignocellulosic biomass with different fiber composition profiles: sugar beet pulp (SBP), brewery bagasse (BB), orange peel (OP) and rice husk (RH). The main objective is to evaluate the organic matter solubilization yield and discuss how the fibers profile affects to the pretreatment performance in four different biomasses.

2. Methodology

Total solids (TS), volatile solids (VS) and soluble chemical oxygen demand (sCOD) have been determined using standard methods (2540B, 2540E and 5220C, respectively) (APHA, 2005). The pH was directly measured in the samples according to method 4500H$^+$ (APHA, 2005). The dissolved organic carbon (DOC) was analyzed using a TOC analyzer (Analytic Jena multi-N/C3100®) according to the 5310B standard method (APHA, 2005).

Individual VFAs were analyzed with a gas chromatograph (Shimadzu®, GC-2010), equipped with a flame ionization detector (FID) and a capillary column (Nukol® Merck KGaA, Darmstadt, Germany). The dimensions of the column were 0.25 mm × 25 μm x 30 m. The hydrogen was used as carrier gas at 50 mL/min and 75.5 kPa. According to Gómez-Quiroga et al. (2019), nitrogen and synthetic air gases were used as make up and oxidizer respectively. The gas flow was 30 mL/min at 75 kPa and 400 mL/min at 50 kPa for both

Before analyzing sCOD, DOC and VFAs, the samples were centrifuged at 4000 rpm for 15 min (Consul-21 Ortoalresa®) and then filtrated through 0.47 μm glass microfiber filter. In the case of VFAs determinations, 0.22 μm microfiber filters were used.

The concentration of the total reducing sugars (TRS) was analyzed by the dinitro-salicylic acid (DNS) method. After the centrifugation of the samples at 10,000 rpm for 10 min, 0.25 mL of the sample was mixed with 0.25 mL of DNS reagent and the resulting solution was heated at 105 °C for 10 min and subsequently cooled with ice for 5 min. Finally, the absorbance was measured at 540 nm using a UV–visible spectrophotometer (Gonçalves et al., 2010).

Fiber analysis was performed according to the Van Soest method (Van Soest et al., 1991) to determine proteins, cellulose, hemicellulose and lignin fractions.

2.1. Feedstocks and inoculum

Four types of biomasses were used as lignocelluloses wastes: orange peel (OP), sugar beet pulp (SBP), brewery bagasse (BB) and rice husk (RH). The inoculum was obtained from the activated sludge biological reactor of the urban wastewater treatment plant (WWTP) placed in Puerto Real (Cádiz, Spain). The OP was collected from the canteen of the Faculty of Science of the University of Cádiz (Cádiz, Spain). It was dried in an oven at 40 °C for 48 h. The SBP was supplied by an industrial sugar factory of the AB Sugar Group® placed in Jerez de la Frontera (Cádiz, Spain) and the RH was obtained from a rice processing factory placed in Seville (Spain). Finally, the BB was collected from a local craft brewery sited in Puerto Real (Cádiz, Spain) and it was dried at 60 °C in an oven for 24 h. All substrates were stored in a freezer at 4 °C till they were used.

2.2. Experimental procedure

Before the experiments, all of the substrates were processed as follows: firstly, they were ground and sieved until 1.7 mm of particle size and later, just before using, they were rehydrated for 24 h with deionized water to adjust a final TS content to 8% (w/w) (Aboudi et al., 2017).

100 mL was the total volume of the pretreatment tests. Each test includes 8% (w/w) of biomass and an inoculum percentage of 2.5%, 5%, 7.5% and 10% (v/v). All the assays were performed at standard conditions (298 K, 1atm) and the operation times were 24 h and 48 h. The experiments were run without agitation and the pH was corrected every 24 h. The initial pH was adjusted to 6 ± 0.5 with NaOH (0.5 M). All tests were performed by duplicate.

The organic matter solubilization performance of the pretreatment was calculated according to equation (1) in terms of concentration increment and (2) in terms of yield at weight basis:

$$\Delta_{concentration}(\%) = 100 \times (\mathrm{OM_F} - \mathrm{OM_0})/\mathrm{OM_0} \tag{1}$$

Where $\mathrm{OM_F}$ and $\mathrm{OM_0}$ are, respectively, the final and initial solubilized organic matter concentration of the samples expressed in terms of COD, DOC and/or TVFA.

$$\mathrm{Yield_{weight\ basis}}\ (\%) = (\text{mass of product} \ / \ \text{mass of feedstock}) \times 100 \tag{2}$$

2.3. Statistical analysis

A statistical study of the pH data was developed using the statistical software IBM® SPSS® Statistics version 29.0. Firstly, the Tukey test (significant difference) was applied to determine if the pH data have a normal distribution. Values of significance (S) higher than 0.05 imply that experimental data have a normal distribution. In the case of pH values, the Tukey test backs ANOVA analysis. Secondly, an analysis of variance (One-Way ANOVA) was carried out to estimate if each factor (pretreatment time, type of biomass and inoculation percentage) has a significant effect on the pH.

3. Results and discussion

3.1. Analytical characterization of the substrates

The physicochemical characterization of the four types of biomasses and the inoculum has been summarized in Table 1.

As it can be seen in the table, the VS percentage (related to TS content) was 88.6%, 93.2%, 94.7% and 84.4% in SBP, BB, OP y RH respectively. In general terms, the pH of the biomasses was slightly acid and the solubilized organic matter content, expressed as sCOD, DOC and VFAs, was low. It could be remarkable the highest VFA content was registered in the BB tests. This result could be due to the fact that this type of biomass comes from a biological transformation process in the brewery. About the TS percentage, RH and SBP showed a low water content while OP and BB were more wet substrates.

If the fiber content is considered, the four selected biomasses represent a wide range of different types of biomasses. RH has a high lignin and cellulose concentration, 14.0% and 32.8% respectively. SBP presents low lignin content biomass with a high NDF soluble fibers content (3.50% and 42.2% respectively). In the case of BB, its contents are 33.7%, 7.01%, 16.3% and 38.0% of hemicellulose, lignin, cellulose and NDF soluble fibers (mainly starch and pectin, respectively). Finally,

Table 1
Physicochemical characterization of inoculum and biomasses. All the results have been expressed in % (w/w) and referred to dry basis except TS (expressed in wet basis) and pH (expressed in pH units); n.d.: Non-detected.

Parameter	Inoculum	SBP	BB	RH	OP
VS (g/kg)	1.32 ± 0.3	739 ± 0.2	261 ± 0.2	772 ± 0.1	195 ± 0.1
TS (g/kg)	7.99 ± 1.2	833 ± 0.5	280 ± 0.4	915 ± 0.0	206 ± 0.8
sCOD (g/kg)	4.54 ± 0.2	10.6 ± 0.2	21.9 ± 0.7	1.29 ± 0.0	41.0 ± 0.4
DOC (g/kg)	0.37 ± 0.0	4.09 ± 0.1	7.93 ± 0.1	0.52 ± 0.1	10.1 ± 0.0
TVFA (g HAc/kg)	n.d.	0.87 ± 0.0	1.39 ± 0.0	0.03 ± 0.0	0.43 ± 0.0
pH (pH units)	7.15 ± 0.2	4.37 ± 0.1	5.44 ± 0.2	5.54 ± 0.5	4.17 ± 0.3
NDF-Soluble fibers[a] (%)	n.d.	42.2 ± 1.4	38.0 ± 1.1	16.5 ± 1.2	66.8 ± 1.2
Cellulose (%)	n.d.	21.1 ± 1.4	16.3 ± 0.4	32.8 ± 0.4	15.7 ± 2.2
Hemicellulose (%)	n.d.	22.5 ± 0.4	33.7 ± 0.5	22.2 ± 0.6	9.11 ± 0.8
Lignin (%)	n.d.	3.50 ± 0.0	7.01 ± 0.9	14.0 ± 1.0	1.26 ± 0.1
Rest (%)	n.d.	10.7 ± 1.4	4.99 ± 1.1	14.5 ± 0.3	7.13 ± 0.3

[a] Soluble fibers NDF are mainly composed of proteins, pectin, starch, mucilages.

the OP is a fresh substrate without any previous industrial processing and, hence, it maintains all its natural characteristics as a high pectin concentration (66.8%) and low lignin percentage (1.26%). These percentages are very similar to those reported by other authors for the same types of biomasses (Krishania et al., 2013).

3.2. pH control and evolution

In Table 2, the initial (0 h) and daily pH values (after 24 h and 48 h) before the daily adjusting procedure are shown. The pH correction consisted of the daily addition of an alkali agent (NaOH 0.5 M) until pH reached a value of 6.0 ± 0.5.

Table 2
pH initial values (0 h) and daily values (after 24 h and 48 h) before pH adjustment.

Time (h)	Inoculation percentage (v/v)	SBP	BB	RH	OP
0	**2.5**	5.31 ± 0.0	6.10 ± 0.0	6.22 ± 0.0	5.84 ± 0.0
	5	5.36 ± 0.0	6.14 ± 0.0	6.15 ± 0.0	5.84 ± 0.0
	7.5	5.43 ± 0.0	6.13 ± 0.0	6.32 ± 0.0	5.85 ± 0.0
	10	5.51 ± 0.0	6.18 ± 0.0	6.32 ± 0.0	5.87 ± 0.0
24	**2.5**	4.53 ± 0.0	4.38 ± 0.0	5.58 ± 0.0	4.81 ± 0.2
	5	4.37 ± 0.0	4.27 ± 0.0	5.61 ± 0.1	4.66 ± 0.1
	7.5	4.35 ± 0.0	4.27 ± 0.0	5.69 ± 0.0	4.68 ± 0.0
	10	4.29 ± 0.0	4.19 ± 0.0	5.78 ± 0.0	4.68 ± 0.0
48	**2.5**	4.69 ± 0.0	3.95 ± 0.0	6.08 ± 0.1	5.01 ± 0.0
	5	4.71 ± 0.0	4.00 ± 0.0	6.06 ± 0.0	5.04 ± 0.0
	7.5	4.83 ± 0.2	4.01 ± 0.0	6.07 ± 0.1	5.06 ± 0.0
	10	4.77 ± 0.2	3.93 ± 0.0	6.04 ± 0.0	5.10 ± 0.1

A preliminary statistical study of the pH data was developed. An analysis of variance (One-way ANOVA) was carried out in order to estimate if each factor (pretreatment time, type of biomass and inoculation percentage) has a significant effect on the pH. If the significance value is less than 0.05 indicates that there are significant differences among the data. As it can be seen in Table 3, pretreatment time, type of biomass and their interaction unequivocally show a significant effect on pH. On the contrary, statistical results for inoculation percentages and interactions 'Pretreatment time' x 'Inoculation percentage' and 'Inoculation percentage'·x 'Type of biomass' are not conclusive.

As it can be seen in Table 2, a decrease in the pH of all substrates after 24 h of pretreatment has been observed. OP and BB showed a higher pH decrease in 24 h versus what was observed for SBP and RH. In the specific case of RH, it only showed a very low decrease (0.54–0.63 units of pH) in the first 24 h. This diminishing does not seem to be related to the inoculation percentage as had been denoted by the statistical analysis.

On the contrary, the pH sharply decreases in BB and OP tests after 24 h. In these cases, the pH diminishing after 24 h was progressively higher as the increase in the inoculation percentage. The decrease in pH ranged between 1.72-1.99 and 1.03–1.19 units in BB and OP respectively.

However, in the following 24 h, for the OP the pH decrease was less important (0.77–0.83 units of pH) and, one more time, the inoculation percentage influence is not clear. Consequently, it can be concluded that for this biomass a higher solubilization of organic acids takes place in the first 24h. The same behavior has been observed for the SBP, showing a progressive diminishing of pH values (0.78–1.22 units of pH) in the first 24 h with the inoculation percentage and a less intensive pH decreasing (0.62–0.74 units of pH) in the following 24 h.

In the case of BB, the final 24 h were even more intense in terms of pH decline. The final pH values after 48 h from the beginning of the test were 2.15–2.25 units of pH lower than the initial pH values. Apparently, the biological pretreatment goes on with a high release of organic acids after 24 h. This fact may be probably related to the germination of the grain before brewing in the BB, which has allowed the access of the microorganisms to a wider portion of the organic matter during the pretreatment.

3.3. Organic matter solubilization

Even when the evolution of the pH has shown a clear profile that could be directly related to the solubilization processes, more specific analytical monitoring has been applied in order to clarify the fate of the organic matter in the biological pretreatment of the selected biomasses.

In order to evaluate the level of net solubilization of organic matter related to the applied biological pretreatment, data from sCOD and DOC are presented in Fig. 1.

The values of sCOD and DOC have been determined at 0 h, 24 h and 48 h. As the biological pretreatment includes a certain proportion of organic matter consumption due to microbial metabolism, the differences between initial and final values of sCOD and DOC represent the net solubilization process (Fdez.-Güelfo et al., 2011).

In general terms, biological pretreatment has been successful to increase the solubilized organic matter for SBP, BB and OP tests (see in Fig. 1). Nevertheless, the solubilization yield was extremely low in the

Table 3
Statistical Analysis from One-way ANOVA for pH data.

Factors	Significance
Pretreatment time	0.000
Inoculation percentage	0.416
Type of biomass	0.000
Pretreatment time x Inoculation percentage	0.115
Pretreatment time x Type of biomass	0.000
Inoculation percentage x Type of biomass	0.741

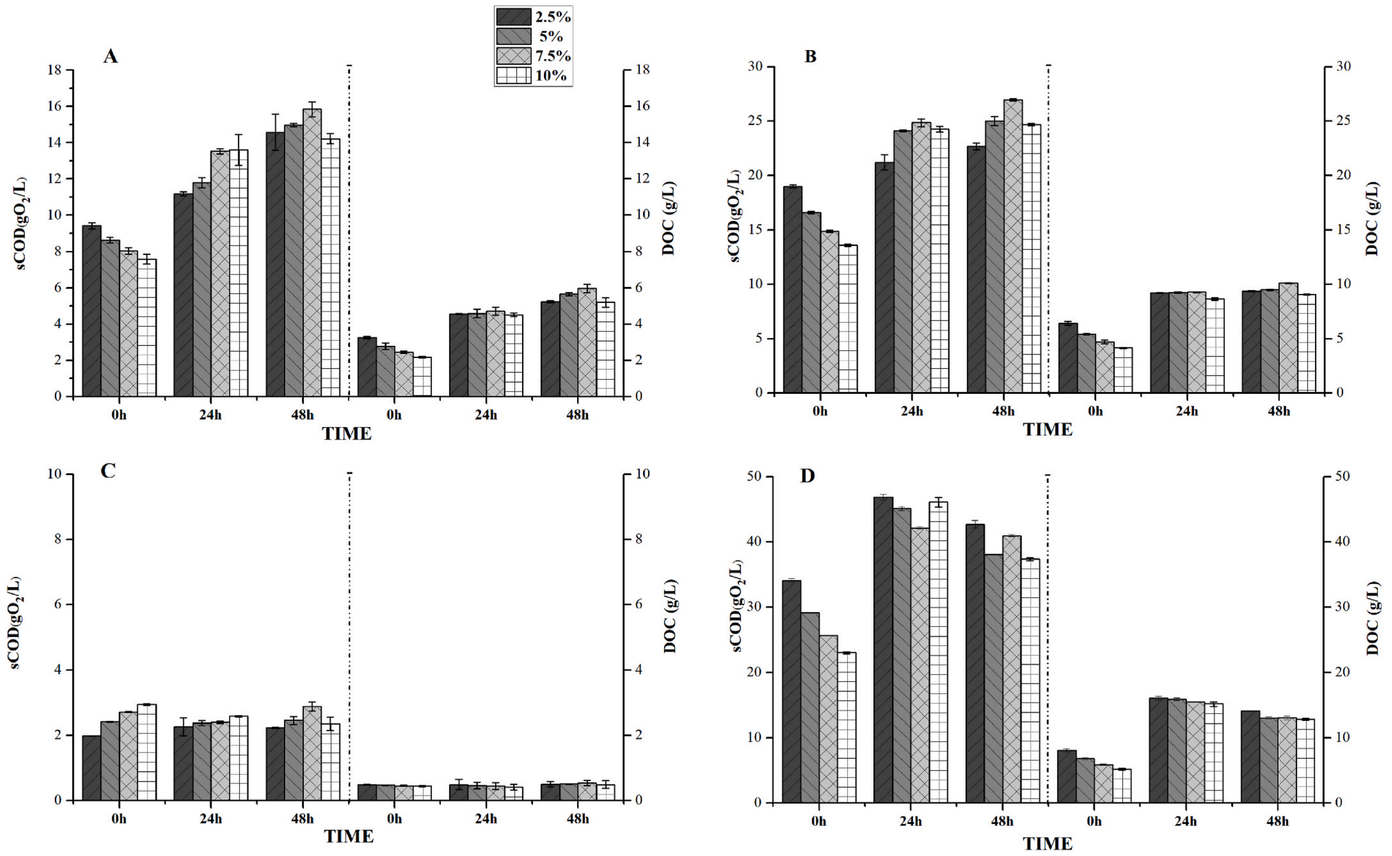

Fig. 1. Net solubilization estimated by sCOD and DOC data. A) SBP, B) BB, C) RH and D) OP.

case of RH. This result, it is according to the relatively low decrease observed in the pH evolution. Both of them, sCOD and DOC after 48 h of pretreatment are too close to initial values to consider any estimable extension of the solubilization process. The maximum values of sCOD and DOC in the tests with RH were 2.7 g O_2/L and 0.5 g C/L respectively. These values were more than one order of magnitude lower which have been observed for the BB or OP tests. This behavior could be related to the presence of high lignin and hemicellulose content in RH (14.0% and 22.2% respectively). Hemicellulose and lignin have a protective role against the microbial degradation in vegetal tissues. In fact, lignin (as macromolecule composed of heteropolyphenols) is difficult to be degraded by microorganisms by itself even in an anaerobic way (Matin and Hadiyanto, 2018). Yu et al. (2009) confirmed the hardest degradation of the rice hull (or rice husk) when a biological pretreatment with fungi was applied. However, the combined pretreatment with H_2O_2 (2%, 48 h) and the sequential pretreatment with *P. ostreatus* during 18 days was more effective than alone pretreatment with *P. ostreatus* for 60 days, where the intact structure of lignin and hemicellulose would have preserved the substrate from the microbial degradation.

On the other hand, the maximum concentration obtained from SBP (15.8 g O_2/L and 6.0 g C/L) and BB (26.9 g O_2/L and 10.1 g C/L) tests were observed at 48 h with an inoculum percentage of 7.5% (v/v). The maximum yields in terms of sCOD were 19.8% for SBP and 33.7% for BB. In the case of DOC values, the maximum yields were 7.47% for SBP and 12.66% for BB. Ozkan et al. (2011) applied an alkaline thermal pretreatment to SBP biomass (NaOH 2M and 121 °C at 1.5 atm for 30 min) obtaining a 58.0% of sCOD yield. In addition, Parchami et al. (2021) obtained 83% and 48% of starch and protein solubilization yield when a hydrothermal pretreatment was applied to bagasse spent grain from the brewing process. The pretreatment was applied at 1:4 (w/w) ratio and 140 °C (4 h) and 180 °C (30 min) respectively. However, those successful thermal and thermochemical pretreatments have the disadvantage of the high operation cost in comparison with the passively aerated biological pretreatment applied in this study.

For the OP tests, the maximum values (46.9 g O_2/L and 16.0 g C/L) were detected after 24 h with an inoculum percentage of 2.5% (v/v). In this case, the maximum solubilization yield was 58.6% for sCOD and 20.0% for DOC for 2.5% (v/v) tests. A really high level of delignification (86% and 92%) in OP was reached by Utekar et al. (2021) by applying sonication or alkali addition respectively during 4 h. NaOH 1N was added to OP at 2%(w/v) and the sonication pretreatment was performed with a 100 W of power and 70% of duty cycle at the room temperature.

If the inoculum proportion is considered, the sCOD and DOC values in the tests with SBP and BB, increase as the inoculation proportion increases till 7.5% (v/v) in the first 24 h. In this case, a slight decline in sCOD and DOC values was observed especially at 48 h in the tests with a 10% (v/v). It could be hypothesized that the microbial consumption of previously solubilized organic matter was more important with time advance and the higher presence of microbial agents. So, it is crucial to determine the optimum time and the inoculum proportion to guaranty an extensive net solubilization without a strong and subsequent organic matter consumption associated to microbial nutritional requirements

In this study, the sCOD and DOC concentrations for SBP and BB tests approximately increased two times between 0 h and 48 h at 7.5% (v/v). COD increased from 8.0 to 15.8 g O_2/L for SBP and 14.9–27.0 g O_2/L for BB while DOC increased from 2.5 to 6.0 g C/L for SBP and 4.7–10.1 g C/L for BB. Zhang & Zang (Zhang and Zang, 2016) obtained a similar increase in the sCOD (1.21–3.03 times) by applying a 48 h alkaline pretreatment of the BB. The BB was mixed with a solid waste known as calcinated red mud (CRM) rich in CaO proceeding from a bauxite refining process in a proportion of 0.4 g CRM/g BB.

The OP tests showed a different profile. Organic matter solubilization was faster and the consumption was more important at shorter times and lower inoculum proportions. In fact, the 5% (v/v) tests showed a clearly lower value of sCOD and DOC in comparison to 2.5% (v/v) in the OP tests. The maximum values for both parameters were the maximum for the study proving that the organic matter in OP is easily solubilized but rapidly metabolized by the microorganisms.

3.4. *Volatile fatty acids (VFAs) production*

In Fig. 2, the initial and daily total volatile fatty acid (TVFA) concentrations are shown. VFA are not expected to be high after a very short biological pretreatment. In fact, a high level of VFA could be an indicator of microbial degradation of the previously solubilized organic matter. The final value at 48 h of TVFA in the tests with SBP and BB was maximum with an inoculation percentage of 7.5% (v/v). This information is coherent with the observed trend in the organic matter net solubilization measured as sCOD and DOC. However, the maximum daily increase in sCOD and DOC was related to the first 24 h. In this sense, the 68.5% and 67.1% of the sCOD increase in SBP and BB tests at 7.5% (v/v) respectively occurred in the first 24 h. A similar assertion could be done if the daily DOC increase is examined. Nevertheless, the TVFA increase seems to be delayed. Only the 6.9% and the 28.5% of the TVFA increase takes place in the first 24 h in SBP and BB tests at 7.5% (v/v) respectively. However, in the second 24 h, TVFA sharply decreased by around 250% in OP tests as a consequence of the remarkable final organic matter consumption.

Hence, as VFAs are a very remarkable biorefinery brick, the suitable condition for obtaining the best concentration of VFAs is 48 h, because at 24 h most of the solubilized organic matter is not as VFAs with the exception of OP. In fact, the higher yield (weight basis) for TVFAs was registered in the BB tests (5.48%) at 48 h.

Similarly, the 10% (v/v) inoculation tests were less effective in terms of TVFA accumulation as it was observed for sCOD and DOC evolutions. The high presence of microorganisms in all these tests could promote the consumption of solubilized organic acids. The loss of TVFA is roughly important in the case of the OP, where almost all the released VFAs had been lost at 48h in the 10% (v/v) test.

The TVFA concentrations in the RH tests were always extremely according to its previously commented organic matter levels. The increase in TVFA concentration was almost negligible in absolute terms but the evolution is similar to the previously commented for BB and SBP tests where the maximum values were registered at 48 h and 7.5% (v/v).

In general, the main individual VFA was acetic acid. The acetic acid percentage in the TVFA accumulation for all substrates was always above 80% in SBP, BB, OP and RH tests at 7.5% (v/v) at 48 h (97%, 90%, 91% and 84% respectively). In all the cases, the second most abundant VFA was butyric acid.

The higher values of TVFA concentration were approximately 1.6–6 times higher than the initial ones: 91.3–599 mg HAc/L in the SBP, 57.8–358 mg HAc/L in the BB, 91.3–332 mg HAc/L in the OP and 24–40 mg HAc/L in the RH tests. So the applied biological pretreatment implies net solubilization of the organic matter and a following releasing of some quantity of VFAs. As final consideration must be highlighted that the final values of the TVFA in the pretreated biomasses have been

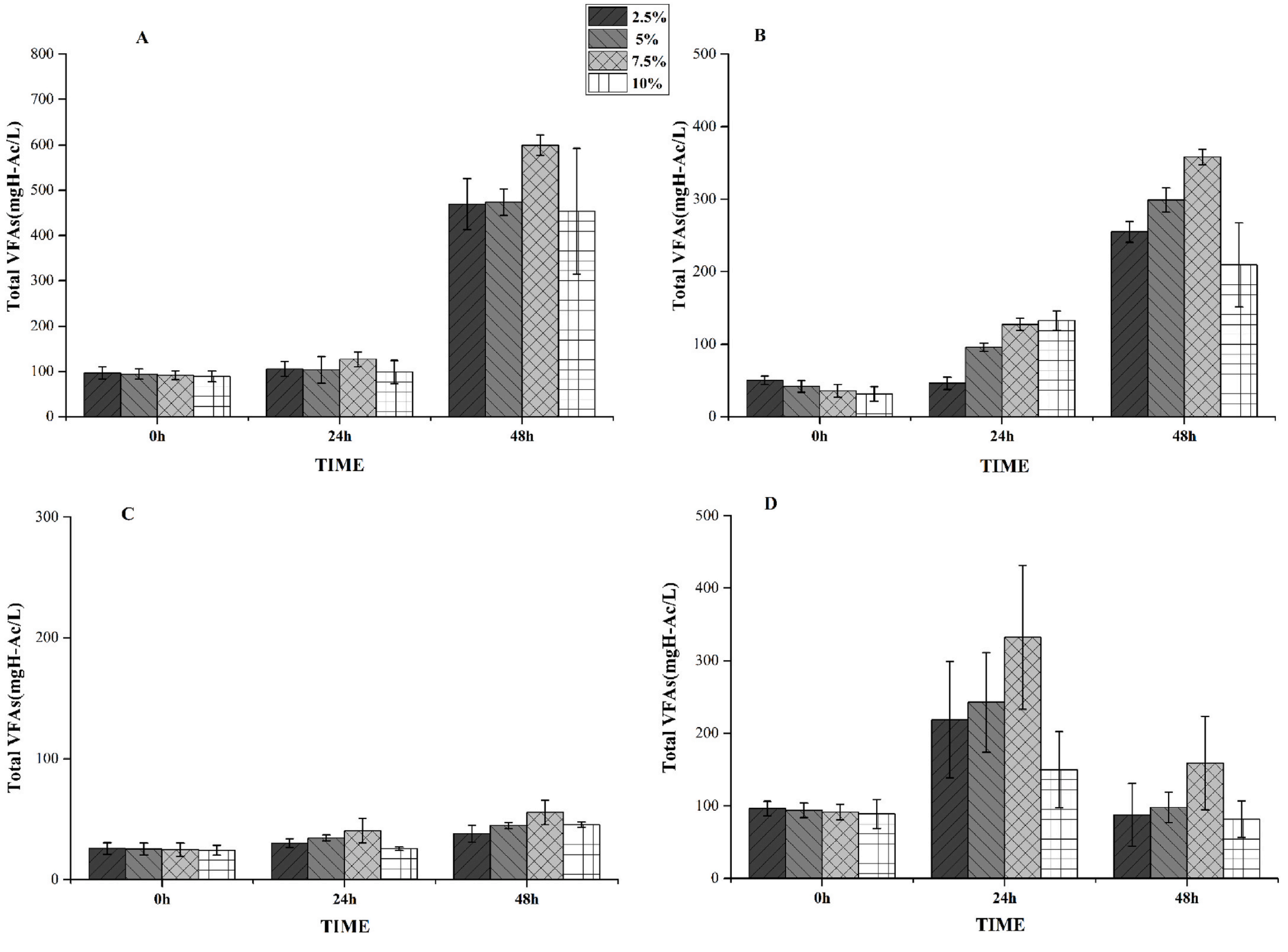

Fig. 2. Total volatile fatty acids (TVFA) accumulation (mg HAc/L). A) SBP, B) BB, C) RH and D) OP.

relatively low. As it will be discussed in section 3.6., even the higher values of TVFA (<600 mg HAc/L) are considerably lower than the net solubilization of organic matter. In fact, the higher proportion of VFAs in the solubilized organic matter was under 4%. So, acidogenesis has not been promoted by biological pretreatment.

3.5. Total reducing sugars

A reducing sugar is a substance that could act as a reducing agent in redox reactions because of its available aldehyde or ketone functional groups. All monosaccharides are reducing sugars and some disaccharides (maltose or lactose) are reducing sugars too. On the other hand, only some oligosaccharides (glycoprotein and glycolipids) and polysaccharides (such as starch, cellulose, hemicellulose or glycogen) could be oxidized in the reducing end (just one end for linear polymers). Because of this, even when high molecular carbohydrates could get a positive reaction for TRS analysis, the detection limit is high and the sensitivity of the analysis is low. In general terms, a hydrolyzed carbohydrate in monomeric/dimeric/oligomer form would give more response to TRS analysis than its polymeric original structure (BeMiller, 2019).

The obtained values for TRS analysis are shown in Fig. 3. TRS values at the initial time were 0.30, 4.15, 5.15 and 7.20 g/L for RH, SBP, BB and OP respectively. Obviously, the fiber structure of RH with a high-lignin content has a protective effect over the availability of reducing end for a DNS positive reaction. In fact, the scarce success of the pretreatment is confirmed by the constant and low level of TRS concentrations at any time and inoculation proportion for RH samples.

The mechanism of the pretreatment of the biomass starts with the enzymatic hydrolysis and depolymerization of carbohydrates fibers. Sweep electronic microscopy (SEM) observations indicate that a successful pretreatment includes physical changes in lignocellulosic structure and destruction and removal of lignin and hemicellulose which are placed in most external protective layers (Lara-Serrano et al., 2019). So, the cellulose fibers become more accessible to enzymes for following microbial activities. In the case of RH, this pretreatment has not been effective.

On the other hand, if the TRS concentrations for SBP, BB and OP tests are observed, the response to DNS reaction has been clearly higher than in RH. In general, no clear increase in TRS level has been detected in any pretreated biomass after 24 h or 48 h. In general, no discernible increase in TRS level was observed in any pretreated biomass after 24 or 48 h. In fact, the initial value of TRS has been maintained or even decreased. As a result, the available fibers have not been depolymerized till monomeric or dimeric sugars where the DNS reaction is more sensitive.

This behavior is related to the different profiles of fiber's content in each biomass. Herein, pectin it is the major abundant polymer in the NDF soluble fibers (40% in OP and 15–30% in SBP according to the literature) (Rivas et al., 2008; Spagnuolo et al., 1997) which has the ability to solubilize and dissolve in water (Bagde et al., 2017; Mathias et al., 2019). Also, in addition to the pectin content, there is a high fraction of sugar soluble in the OP (16.9%) and BB (Lynch et al., 2016; Westendorf and Wohlt, 2002). However, soluble monosaccharides and disaccharides are readily biodegradable substrates that could be rapidly metabolized for the microorganisms such as it has been observed for OP at 48 h. As it could be seen in section 3.6., TRS are a minor fraction of the total solubilized organic matter, revealing that most of the carbohydrates are in oligomer and polymer form after the pretreatment.

3.6. Soluble organic matter distribution

Fig. 4 represent the distribution of the solubilized organic matter in

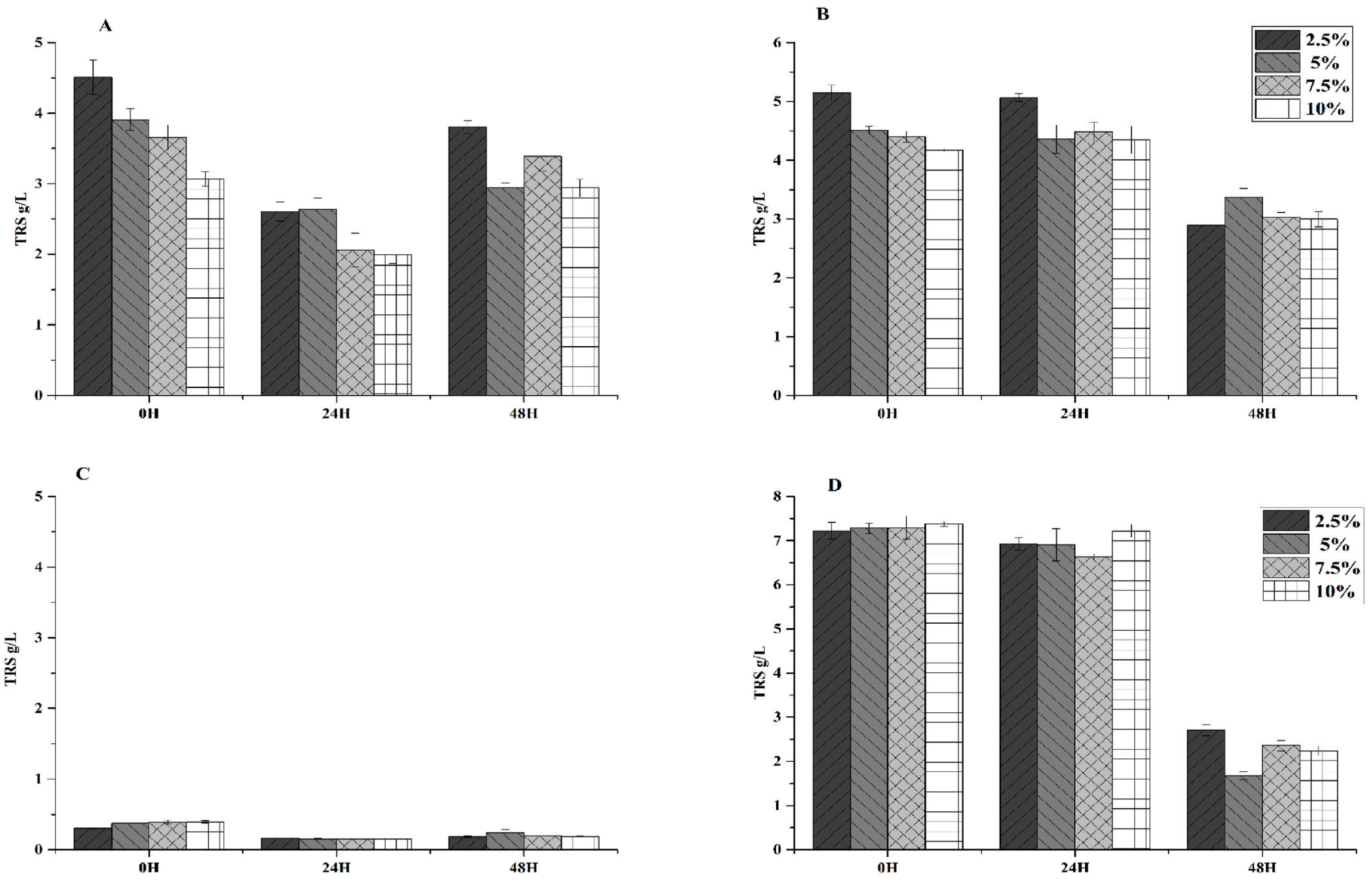

Fig. 3. The total reduced sugars (TRS) expressed in terms of g/L. A) SBP, B) BB, C) RH and D) OP.

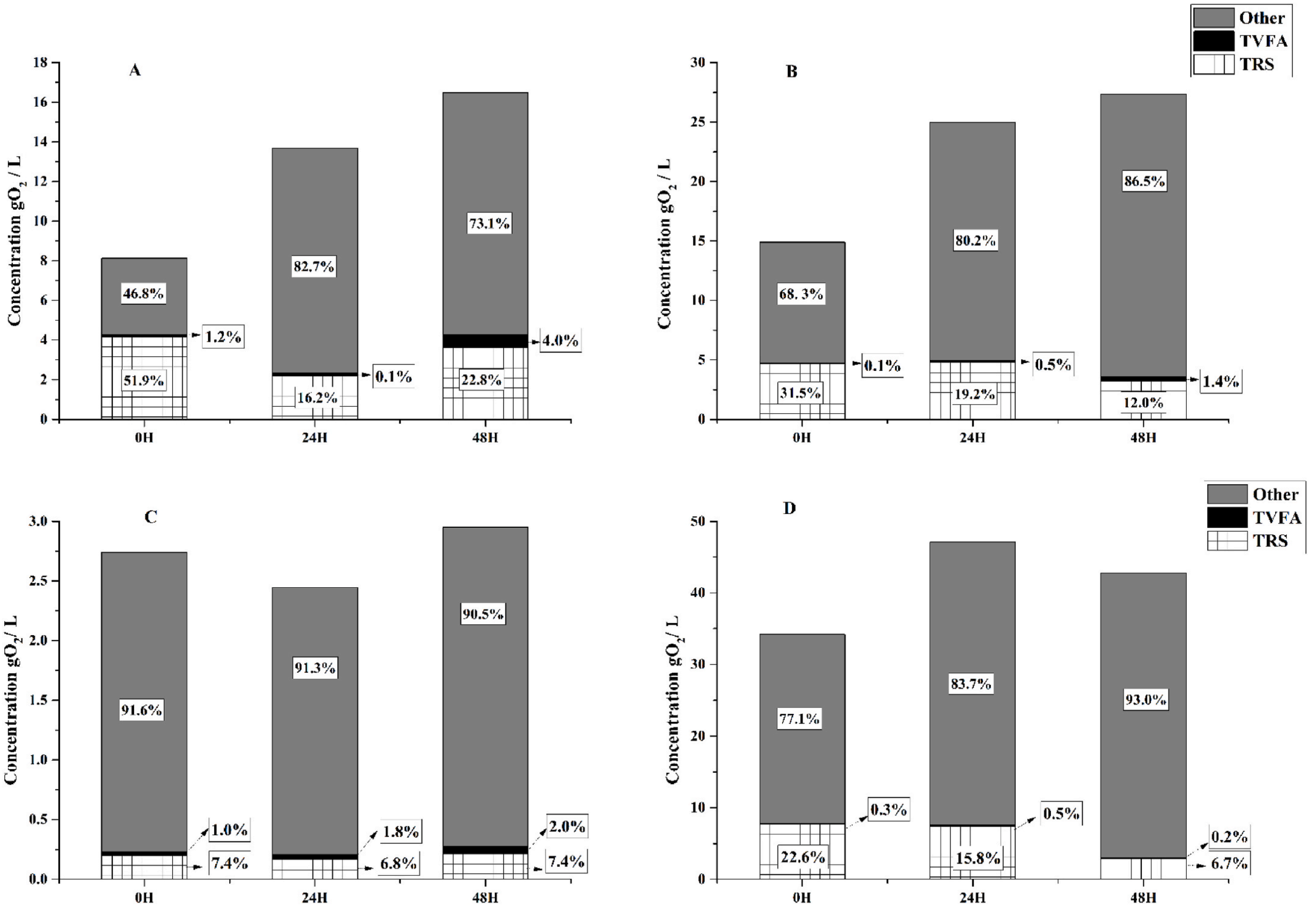

Fig. 4. Organic matter distribution (TVFA, TRS and others) expressed in terms of g O_2/L. The inoculation percentage was 7.5% (v/v) for SBP, BB and RH and 2.5% (v/v) for OP. A) SBP, B) BB, C) RH and D) OP.

equivalent units of COD (mg O_2/L). The showed data are referred to the tests with a 7.5% (v/v) for SBP, BB, RH and 2.5% (v/v) for OP tests which have been previously selected as the best one for each type of biomass.

The major solubilized compounds are not TRS and VFAs. As final products of saccharification and acidogenesis, high levels of TRS and VFAs are not desired at the end of the pretreatment because are readily biodegradable substrates for the inoculated microorganisms. In addition, its presence in great quantities could be considered an indication of metabolic degradation of the biomass.

VFAs are the minor fraction of the non-pretreated and pretreated biomasses. Its concentrations were always under 4% in all the substrates and pretreatment times. Nevertheless, the proportion of TRS in pretreated biomasses has reached even a 22.8% over the total solubilized organic matter in the case of the SBP tests at 48 h. The global trend is a decrease in TRS proportion with the time of pretreatment except for data for 48 h with SBP. The probable reason for this diminishing is due to a double effect: net organic matter solubilization is greater than carbohydrate depolymerization and the solubilized monosaccharides and disaccharides could have been partially metabolized. From the point of view of a complete biorefinery process, the biological pretreatment should be fast and efficient in the solubilization of the organic matter but the presence of biorefinery bricks in this preliminary step is counterproductive since they could be subsequently consumed by the own microorganisms which carry out the pretreatment. In this sense, the results show that the pretreatment has been limited in terms of acidogenesis and saccharification of the solubilized organic matter.

3.7. *Comparative analysis*

In Fig. 5, it has been shown the results of the tests for the four biomasses, comparing the sCOD, DOC, TVFA and TRS yields (weight basis) at the inoculation percentage of 7.5% (v/v) for the BB, SBP and RH tests

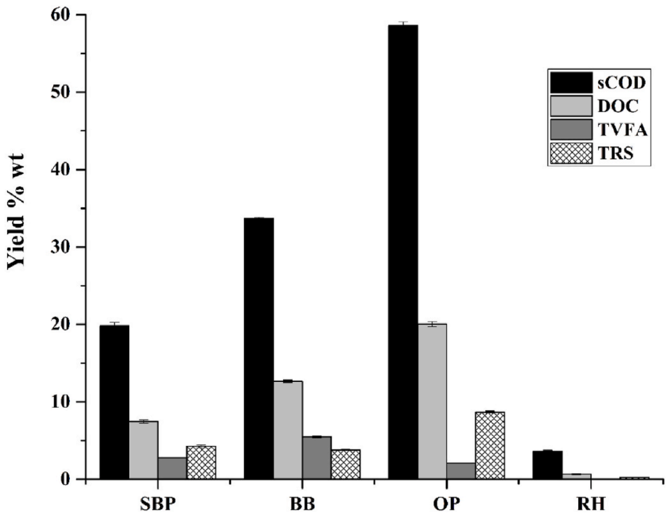

Fig. 5. Comparison of the solubilization yields % wt (weight basis) of sCOD, DOC, TVFAs and TRS at the best % (v/v) of inoculation ratio for each biomass.

(referred to 48 h). The OP values are referred to 24 h of pretreatment at 2.5% (v/v).

The best yields (weight basis) of the organic matter solubilization in terms of the sCOD were 58.59, 33.68, 19.80 and 3.6% for OP, BB, SBP and RH respectively. The TVFA yield was minor and the higher value was registered in the BB test (5.48%). Finally, the TRS yield was 8.6%, 4.2% and 3.72% obtained with the OP, SBP, BB respectively.

Subsequently, the different proximal compositions of the feedstock expressed as NDF soluble fraction, cellulose, hemicellulose and lignin (Table 1) have played a main role to produce effective solubilization through the biological pretreatment. The highest content in pectin (66.8%) of OP has contributed to smoothing the pH decrease which could be due to its sharp solubilization in 24 h and the presumable ammonium release associated with its high pectin content. In addition, the hemicellulose fraction –which has a protective role of cellulose against microbial attack-was the lowest in the study. These characteristics have triggered that OP has been the most solubilized substrate in the study and the extension of the VFAs and TRS production has been limited in low yields, at less, at the first 24 h.

On the other hand, the low lignin content in SBP and BB has permitted a wide solubilization of the organic matter but below the observed level for the OP tests. The sum of starch, pectin and cellulose fractions is according to the observed order in DOC and sCOD solubilization. However, if the TRS release is considered, the OP biomass shows a major solubilization yield in comparison with SBP and BB.

4. Conclusions

The current study investigates the effect of passively aerated biological pretreatment on four biomasses with various fiber content profiles: SBP, BB, OP and RH using varying inoculation amounts and pretreatment periods. The employed inoculum (activated sewage sludges from a WWTP) was able to hydrolyze and solubilize the organic matter at mild temperature and pH conditions (25 °C and pH 6). The fiber profile composition of the various types of biomasses affected the achieved solubility. The high-lignin content of RH induced a scarce solubilization level with an sCOD yield under 4%. In contrast, the other three biomasses were successfully processed, with a maximum sCOD yield of 19.8% for SBP with a 7.5% inoculation and 33.7% for BB with the same inoculation rate, and 58.6% for OP with a 2.5% inoculation proportion.

The substrates, in this case, were NDF-soluble sugars enriched biomasses with low to medium lignin concentration. Furthermore, the detectable synthesis of VFAs and TRS was restricted due to the short length of the pretreatment to avoid an early consumption of the solubilized organic matter during the pretreatment, particularly at 48 h. As a result, the optimal pretreatment time in terms of total solubilized organic matter for all biomasses was 24 h. Lastly, the increased inoculum ratio of 10% (v/v) resulted counterproductive due to solubilized organic matter consumption.

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Declaration of competing interest

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

Data availability

The authors do not have permission to share data.

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