agriloop

high-value products from agricultural residues through sustainable chains

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Deliverable D3.1

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Table of content

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1. Executive Summary

Agricultural waste and by-products such as brewery's spent grains, tomato pomace and potato peels are a sustainable source for the production of biobased chemicals. However, the carbohydrates are mainly present in the agricultural waste in the form of complex polymers like cellulose, hemicellulose, and lignin. Current pretreatments to release these fermentable carbohydrates require high temperatures (up to 200°C), the addition of strong acids, and subsequent cooling and neutralization prior to any bioprocess. The AgriLoop approach is developing highly-efficient and more cost-effective hydrolysis technologies than the current acid hydrolysis pre-treatment. Specifically, the AgriLoop is focused on the application of thermophilic fungi (UGENT), subcritical water treatment coupled with mild acid hydrolysis (NID), and supercritical fluids (UNIROMA).

UGENT successfully grew three selected thermophilic fungi: *M. thermophila*, *T. aurantiacus* and *T. lanuginosus* on brewery's spent grains. The crude extracts rich in fungi enzymes from these three thermophilic fungi were recovered and directly used for the hydrolysis and solubilization of the brewery's spent grains. After three days, up to 0.34 gCOD/gCOD-BSG was solubilized, likely in the form of complex carbohydrates polymers and/or proteins.

NID characterized the three selected agro-industrial residues and performed an acidic hydrolysis as a baseline for comparison between project partners. In parallel, NID developed a subcritical waste pre-treatment. This pretreatment showed better results for the brewery's spent grains and the potato peels residues. For both materials the temperature of 190 °C seems to be the most suitable, with low concentration of degradation products being produced, exception made for the lactic acid present on the PP SCW hydrolysate.

UNIROMA assessed acidogenic fermentation potential of three types of agro-industrial residues (tomato peels, brewery's spent grain and potato peels. Among these residues, potato peels yielded the highest fermentation values, up to 83.8 ± 1.0 % (COD basis). Propionate was the main fermentation product (over 50%, COD/COD), making the potato peels of particular interest as feedstock for the production of the poly(hydroxybutyrate/valerate) copolymer.

UNIROMA also evaluated the possibility of recovering value-added components (i.e., carotenoids, polyphenols, and flavonoids) by supercritical CO_2 extraction. The solid residues after the pre-treatment showed a decrease in the COD content up to 23% for the brewery's spent grains. In principle, the reduction of COD content in the solid residues should correspond to the amount of components of interest (i.e., carotenoids in particular lycopene for tomato peels, flavonoids for BSG, and polyphenols for potato peels) recovered in the extracted samples. Analyses for the characterisation and quantification of all extracted components are in progress.

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Abbreviations

TP – Tomato Pomace
BSG – Brewers´ Spent Grain
PP – Potato Peels
ASL – Acid soluble lignin
AIL – Acid insoluble lignin
5 -HMF – 5-Hydroxymethilfurfural
SCW – Subcritical water

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2. Introduction

Agricultural waste and by-products such as brewery's spent grains, tomato pomace and potato peels are highly rich in carbohydrates and are a sustainable source for the production of biobased chemicals like medium chain fatty acids, microbial proteins or biopolymers. However, these carbohydrates are mainly present in the agricultural waste in the form of cellulose, hemicellulose, and lignin. These compounds have a complex and heterogeneous structure, making it difficult to access their sugar fraction. Current pretreatments are based on physical, chemical, or physicochemical mechanisms and have been widely studied in the last decades. The amount of sugars released with these pretreatments is usually high, however they require high temperatures (up to 200°C), the addition of strong acids, and subsequent cooling and neutralization prior to any bioprocess. Additionally, these treatments may also produce inhibitory compounds for microbial growth, challenging the subsequent valorisation steps where microorganisms act as biocatalysts. Novel highly-efficient and more cost-effective hydrolysis technologies than the current acid hydrolysis pre-treatment are required. AgriLoop focuses on innovative pre-treatment methods: thermophilic fungi (UGENT), subcritical water treatment coupled with mild acid hydrolysis (NID), and supercritical fluids (UNIROMA) on a range of selected agri-residues feedstock (e.g., brewers 'spent grains, tomato peels, and potato peels) to unlock their full potential by solubilizing carbohydrates (and proteins).

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I. Feedstock characterization

1. Experimental

Tomato Pomace (TP) was supplied by TomaPaint Srl. (Italy) and received at NID facilities in February 2023. Brewers' Spent Grain (BSG) from a local Belgium brewery was sent by UGent on March 2023, while Potato peels (Agristo NV) were received at NID facilities in June 2023, also sent by UGent.

All the three agro-food wastes were characterized. TP and PP were dried through lyophilization and part of it was ground in a mill. BSG was already supplied dried, it has been only subjected to milling. All the wastes, raw and milled, were stored in sealed bags at room temperature until further use.

The moisture content of the raw materials was determined by drying a sample (10 g) through lyophilization. Dried samples were further subjected to pyrolysis at 550 °C, for 24 h, to evaluate their ash content. The particle size distribution of the wastes before and after milling was determined by submitting a sample (50 g) through a nest of five different sized sieves (250 – 2000 µm). The fraction collected in each sieve was weighed. The protein content of the three wastes was assessed by elemental analysis, using a Flash EA 1112 CHNS analyser (Thermo Scientific, Waltham, MA, USA), through a nitrogen-to-protein conversion factor of 6.25 (Mariotti et al. 2008). The lipids' content of each waste was determined using Soxhlet extraction with hexane according to the standard methods (APHA 1995). The characterization in terms of elements present in each waste (after acid hydrolysis) was conducted by inductively coupled plasma atomic emission spectroscopy (ICP-AES) through an Ultima ICP spectrophotometer (Horiba Jobin-Yvon, Bensheim, Germany) equipped with a RF generator (40.68 MHz), a monochromator (1.00 m Czerny-Turner), AS500 automatic sampler and Concomitant Metals Analyzer (CMA) device.

The hemicellulose, cellulose, and lignin contents of TP and BSG wastes was determined as described by Sluiter et al. (2008). The lignin and suberin complex content of the PP was assessed as described by Liang et al. (2014).

2. Results

The results of the characterization of the raw materials in terms of moisture, ash content, hemicellulose, cellulose, lignin (ASL and AIL), and protein are summarized in Table I.1. As can be seen, the materials' moisture content was highly variable, where BSG presented the lowest content (5.5 ±0.1 %), as expected, considering it was received dried. On the other hand, the TP and PP were found to have high moisture contents of 69.8 ± 0.4 % and 89.4 ± 0.2 %, respectively.

As for the dry weight of the three raw materials, a large percentage was represented by lignocellulosic polymers (cellulose, hemicellulose, lignin). Both TP and BSG had contents of lignocellulosic polymers over 60% (64.1% and 62.6%, respectively); however, potato peels showed a significantly lower content (26.9%), as expected, due to the

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absence of cellulose and hemicellulose. Lignin was found to be the lignocellulosic polymer with the highest content in all the raw materials, corresponding to 44.1%, 31.0%, and 26.9%, of the total dry weight of TP, BSG, and PP, respectively. It is worth noting that, in the case of the PP, this value represents the content of both lignin and suberin.

TABLE I.1. COMPOSITION OF THE RAW MATERIALS (TOMATO PASTE, BSG AND POTATO PEELS).

	Tomato Pomace	BSG	Potato Peels
Moisture (%)	69.8 ± 0.36	5.5 ± 0.11	89.4 ± 0.21
Ash content (%) [a]	3.3 ± 0.03	5.0 ± 0.04	7.8 ± 0.23
Protein (%) [a]	18.8	25.9	*
Lipids (%) [a]	11.5 ± 0.28	9.9 ± 0.18	*
Cellulose (%) [a]	13.7 ± 1.97	17.5 ± 2.98	N.A.
Hemicellulose (%) [a]	6.2 ± 0.52	14.1 ± 1.03	N.A.
Total Lignin (%) [a]	44.1 ± 5.27	31.0 ± 0.19	26.9 ± 6.67 [b]
ASL (%) [a]	3.9 ± 0.04	8.0 ± 0.11	1.5 ± 0.06 [b]
AIL (%) [a]	40.2 ± 5.31	23.0 ± 0.08	25.5 ± 6.58 [b]

[a] – on a dry weight basis
[b] – lignin and suberin
* – Waiting for analytical results
N.A. – not applicable

The content of hemicellulose in BSG (14.1%) was significantly higher than the content present in TP (6.2 %). In contrast, the cellulose content of these materials was comparable, corresponding to 17.5 % and 13.7 %, respectively. Aside from the lignocellulosic polymers, the dry raw materials were characterized in terms of protein, lipid, and ash content.

BSG had the highest estimated protein content (25.9%), while TP showed a considerably lower content (18.8%), and the content of the PP is still in the process of being determined. The lipid content of the PP has not yet been quantified. On the other hand, the remaining raw materials showed similar lipid contents of around 10 %. Additionally, all the raw materials were found to have low ash contents (<10%), with the potato peels having the highest content (7.8%) and TP the lowest (3.3%). This inorganic content was complemented by ICP analysis of the dry raw materials, where sodium, potassium, calcium, and phosphorus were found in the highest amount, respectively representing 1.2%, 0.6%, 0.2% and 0.2% of TP, 0.4%, 0.1%, 0.2% and 0.5% of BSG, and 0.9%, 1.7%, 0.1% and 0.1% of PP.

The particle size distribution of TP, BSG, and PP before and after milling is represented in Figure I.1. Before milling, all the dry raw materials were mainly composed of particles larger than 1.0 mm, representing 93.7%, 85.2%, and 72.5% of TP, BSG, and PP particles, respectively. As can be seen in Figure 1, the milling process was effective in reducing the particle size of the raw materials, and, in all case, most of the particles in the milled raw materials smaller than 0.71 mm. Furthermore, over half of the particles of milled BSG (70.3%) and potato peels (64.2%) were smaller than 0.5 mm and 0.25 mm,

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respectively. Particles with smaller sizes favours further material treatments (e.g., hydrolysis) by increasing the surface area to volume ratio.

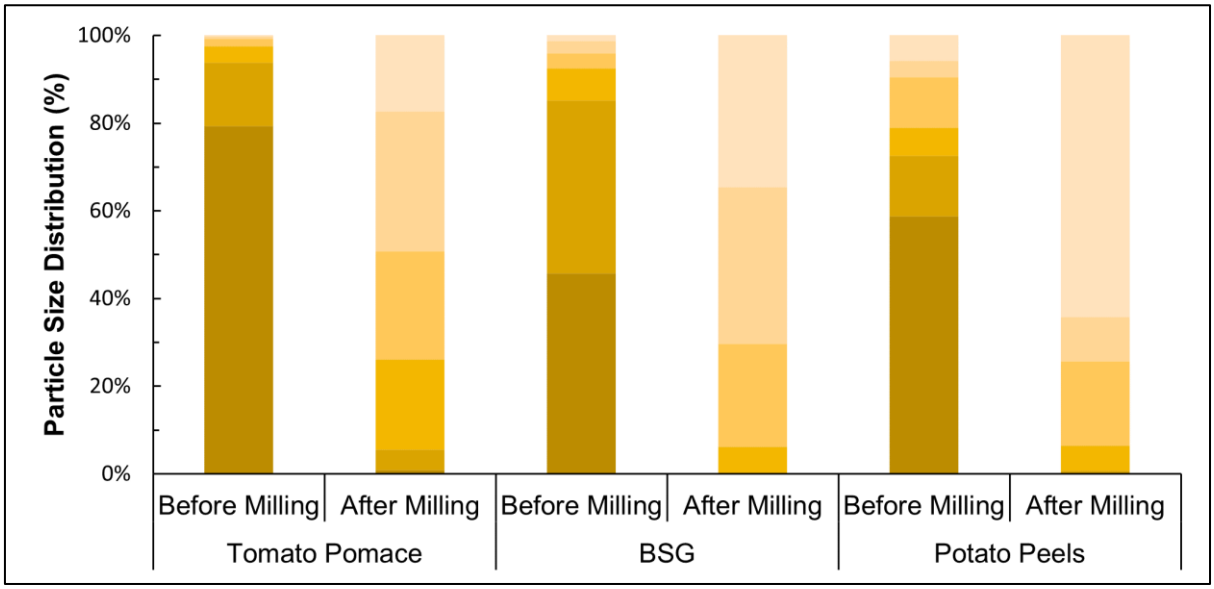

Figure I.1. Particle size distribution of dry TP, BSG, and PP, before and after milling. ■ > 2000 µm; ■ 1000 µm – 2000 µm; ■ 710 µm – 1000 µm; ■ 500 µm – 710 µm; ■ 250 µm – 500 µm; ■ < 250 µm

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II. Acidogenic fermentation (AF)

1. Introduction

In general, about one third of the total food annually produced is wasted throughout the whole value chain from primary producers to consumers, and this corresponds to around 1.3 billion tonnes of wasted food globally generated (Fritsch et al., 2017; Sharma et al., 2020). Although the reduction is certainly the preferred option in the management of food residues and by-products, approaches aimed at their recovery and valorisation are becoming routes of ever-increasing relevance, particularly in the context of the development of the circular bioeconomy concept. In this frame, in the AgriLoop project, the valorisation of these residues is performed, among the others, through the acidogenic fermentation (AF) process to obtain carboxylic acids, which have broad and relevant industrial applications as intermediate feedstock chemicals (Agler et al., 2011; Wainaina et al., 2019). Also, carboxylic acids are direct precursors for the biosynthesis of polyhydroxyalkanoates (PHAs), which are fully biodegradable polymers and represent a target product of the project (Medeiros Garcia Alcântara et al., 2020; Raza et al., 2018; Valentino et al., 2017).

During the first period of activity of task 3.1, AF batch experiments were performed with the three selected agro-industrial residues (i.e., TO, PP, and BSG) to assess their potential to be converted into carboxylic acids.

2. Materials and methods

2.1. Operation of acidogenic fermentation experiments

AF experiments were performed in a batch mode in serum bottles (250 mL total volume), filled with mineral medium and anaerobic fermentative sludge (as inoculum) for a total volume of the liquid phase equal to 150 mL. The pH of the medium was buffered at 5.5 to inhibit the methanogenic activity (Marchetti et al., 2023). Once prepared, all bottles were flushed for 15 minutes with a N_2/CO_2 (70/30, v/v) gas mixture to establish anaerobic conditions. As for the feedstocks, TP was provided by the TomaPaint Srl partner, while BSG and PP were provided to UNIROMA by Gent University. Prior to starting the AF tests, all residues were characterized in terms of COD and moisture content and were stored at -20°C before their usage (except for BSG, which was delivered in a dried form). AF experiments were all performed in duplicate, in the same operating conditions, consisting of ambient temperature, an initial concentration of agro-industrial residues of 2gCOD/L, and a corresponding ratio between the inoculum (as grams of volatile suspended solids, VSS) and the initial substrate concentration (I/S) of 0.2 gVSS/gCOD. Control tests in absence of by-products were also performed. Moreover, to assess the possibility of increasing their fermentation potential, tests using agricultural residues after undergoing freeze-drying (for TP and PP) and milling (for all three substrates) were also performed. Also, after 55 days of operation, due to the low conversion yield achieved in tests containing TP and BSG, these were augmented with glucose as reference synthetic substrate, to identify the possible presence of inhibitory or recalcitrant compounds contained in these

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residues on the microbial activity. Measurement of pressure inside the bottles to check their tightness was periodically taken. The liquid phase and the headspace of all bottles were periodically sampled to measure the production of fermented products and to verify the absence of methane production, respectively. A picture of some of the performed AF batch tests is reported in Figure II.1.

Figure II.1: Acidogenic fermentation batch tests with the three agro-industrial residues.

2.2. Analytical measurements

VSS were determined according to standard methods (APHA, 1995). For organic acids and ethanol analyses, liquid samples collected throughout the experimentation were filtered with cellulose acetate syringe filters (0.20 mm porosity) and stored at -20°C freezer until the analyses. For the latter, 1µL of sample (taken from a 1mL solution containing also 100 µL of oxalic acid 0.33M) was injected into a gas-chromatograph (GC 8860, Agilent Technologies, DB-FAPP column, USA). Standards with known concentrations were prepared and analysed with the same instrument to obtain a calibration curve that enables obtaining the concentration value from the peak area values. The concentration of organic acids and ethanol were converted into COD in accordance with the following oxidation stoichiometry: 1.07 $gCOD/g_{Acetate}$, 1.51 $gCOD/g_{Propionate}$, 1.82 $gCOD/g_{Butyrate}$, 2.04 $gCOD/g_{Valerate}$, 2.21 $gCOD/g_{Hexanoate}$, and 2.09 $gCOD/g_{Ethanol}$. The concentration of glucose was converted into COD by using the conversion factor of 1.07 $gCOD/g_{Glucose}$. The total COD (tCOD) of all residues, referred to the dry weight, was measured by using a commercial test (Tube test NANOCOLOR CSB 1500 Hg-frei, Macherey Nagel) consisting of glass cuvettes containing potassium dichromate, and concentrated H_2SO_4 (80-98%). After samples digestion at 160 °C for 30 minutes, the COD content was determined using a spectrophotometer (NANOCOLOR® Advance, Macherey Nagel) at a wavelength of 605 nm. The gas analysis was carried out by injecting 50 µL of the gaseous sample by a gas-tight Hamilton syringe into a “Dani Master” gas-chromatograph (glass column packed with

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Carbopack; He carrier gas 25 mL/min; oven temperature 50 °C; FID temperature 200 °C). Freeze-drying was carried out by means of Heto DRYWINNER (DW 1,0-60E Lab). The residues were subsequently milled by using a mortar.

2.3. Calculations

The fermentation yield was used as a main parameter to evaluate the conversion potential of the used residues into fermentation products. The yield was defined as the sum of each individual fermentation product (expressed as COD equivalents) at the maximum concentration data point (ΣP_{END}, typically corresponding to the last day of operation) minus its equivalent in the control test ($\Sigma P_{CONTROL}$) divided by the initial total COD added for each assay (tCODi). However, since in this study fermented products were typically not detected in the control tests performed in the absence of organic substrates, a simplified equation was used in which the second term of the equation was set to zero, as follows:

Fermentation Yield (%, COD/COD) = $((\Sigma P_{END})/(tCODi)) \cdot 100$

3. Results and discussion

Data regarding the characterization of the three residues, in terms of both COD and moisture content, are reported in Table II.1. A slight increase in the COD content was obtained by freeze-drying and milling the TP, and by milling the BSG. Probably, these types of pre-treatments on these two residues allowed the breaking of lignocellulosic bonds making available a higher content of organic matter. In the case of PP, a different trend was observed.

Table II.1. Moisture percentage and COD content in all agro-industrial residues.

Item	COD (gO_2/g)	w_{H2O} (%, wt/wt)
TP	1.64 ± 0.3	64.9 ± 0.5
TP_M	1.78 ± 0.3	-
BSG	1.10 ± 0.1	0.76 ± 0.05
BSG_M	1.37 ± 0.1	-
PP	1.40 ± 0.1	87.0 ± 0.1
PP_M	1.15 ± 0.5	-

The results of AF experiments, in terms of concentration of total fermentation products, are reported in Figure II.2. which highlights the occurrence of a very similar trend for the milled and not milled residues, especially for TP, TP_M, BSG and BSG_M, as well as the presence of not easily fermentable compounds in them. However, a rapid conversion into fermentation products was obtained in the presence of PP and PP_M,

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already starting from the first days of the tests operation. In addition, an easily biodegradable substrate (glucose) was added after 60 days of operation in the control tests, and in the tests with TP and BSG, to identify the possible presence of recalcitrant substances in these residues as well as to verify the effectiveness of the bacterial activity. In particular, glucose was supplied at a concentration of 2 gCOD/L in the control tests and at a concentration of 1 gCOD/L in the TP and BSG tests (in which, by also taking into account the amount of residues initially supplied, the total supplied COD accounted for 3 gCOD/L). As reported in Figure II.2., the addition of glucose resulted in a fast and almost complete conversion into fermentation products in the control test, equal to 1.83 ± 0.04 gCOD/L (accounting for a conversion of over 90%, COD/COD). Total glucose conversion was also achieved in the presence of TP and BSG, reaching a conversion yield higher than 100%. This indicates that, following the addition of glucose, bacteria were driven also to consume a portion of the agricultural residues along with the supplied synthetic substrate. This finding denotes that the substrate concentration chosen to perform the batch tests was not inhibitory for the microbial activity, thus the incomplete conversion of the supplied COD into fermentation products with TP and BSG was likely not achieved due to the presence of non-biodegradable and recalcitrant constituents in these residues. This also opens interesting perspectives on the possibility of adopting appropriate pre-treatment strategies (among those investigated in Task 3.1) to enhance the biological transformation of such by-products into valuable compounds.

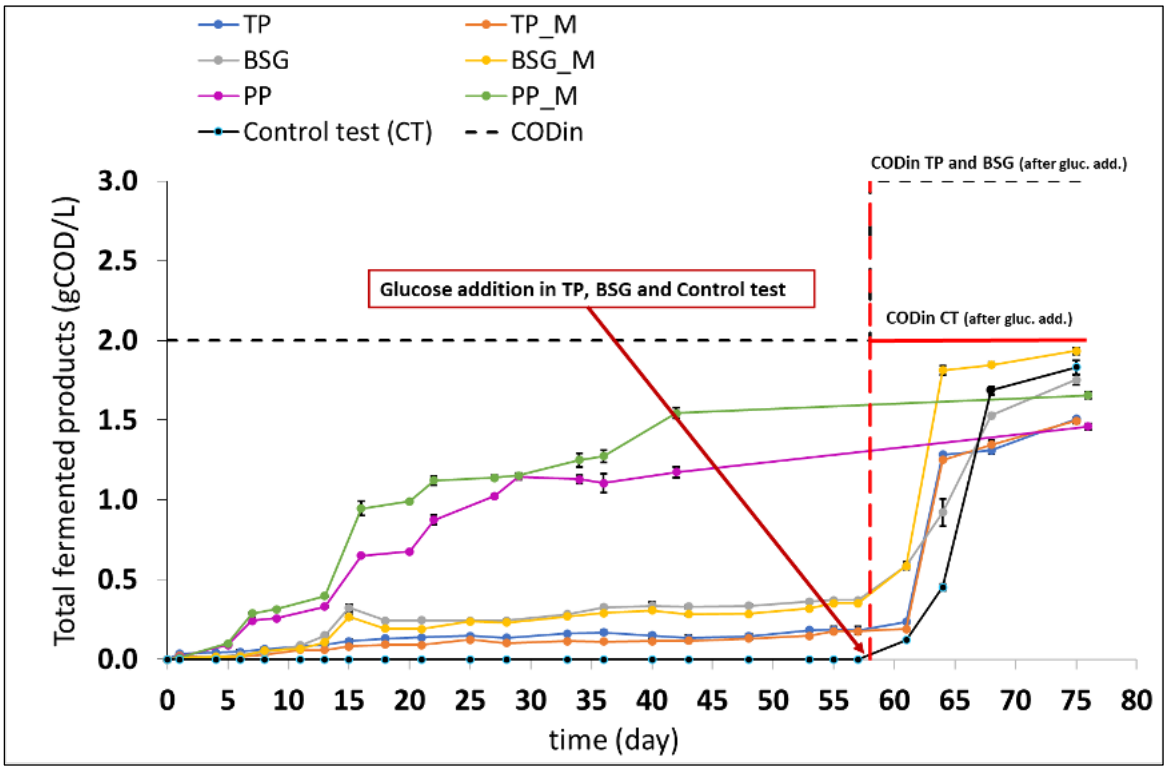

Figure II.2. Trend of the total concentration of fermentation products in AF tests performed with all agro-industrial residues.

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As for potato residues, the achievement of a high concentration of fermentation products, , equal to 1.46 ± 0.02 gCOD/L (PP) and 1.66 ± 0.02 gCOD/L (PP_M), was in accordance with their high sugar content, around 63-80 % of total carbohydrates (Lima et al., 2021; Liu et al., 2003), mainly composed of starch that is easily fermentable.

The results of AF experiments in terms of products distribution and conversion yield (before the augmentation of glucose) are reported in Figure II.3. The assessment of the AF potential was mainly evaluated by considering the conversion yield, which represents the fraction of the initial total COD converted into fermentation products. As a main result, it was found a higher conversion yield with the potato peels compared with the two other residues, with values (on COD basis) accounting for 58.9 ± 1.8% (PP) and 78.1 ± 1.6% (PP_M), after 40 days of operation. A further increase was observed, reaching a maximum conversion yield equal to 73.2 ± 1.1% (PP) and 83.8 ± 1.0% (PP_M) by the end of the experiments (day 76), suggesting the presence of also slowly biodegradable COD in this residue. Moreover, the obtained results indicate that the reduction of the residue size increases the rate of conversion into fermentation products.

On the contrary, the milling pretreatment did not bring any benefit to the AF process for TP and BSG, achieving similar conversion yields, equal to 8.7 ± 1.1% (TP) and 8.4 ± 1.0% (TP_M), 18.1 ± 1.0% (BSG) and 17.2 ± 1.0% (BSG_M) on COD basis. Indeed, as previously discussed, the less biodegradable component contained in these residues corresponds to the fiber content, whose total percentage is typically much higher in TP and BSG compared to PP, around 12 to 22% (wt/ wt) for the lignin content in BSG, up to 41.9% (wt/wt) for hemicellulose (Lynch et al., 2016; Silva et al., 2004), and from 75 to 82 % (wt/wt) of dietetic fiber content in TP residue (from TomatoPaint Srl analyses), versus around 3% (wt/wt) in potatoes (Camire et al., 1997; Tolessa & Shunka Tolessa, 2018). This is fully in agreement with the lower observed acidogenic fermentation potential of TP and BSG compared with potato peels.

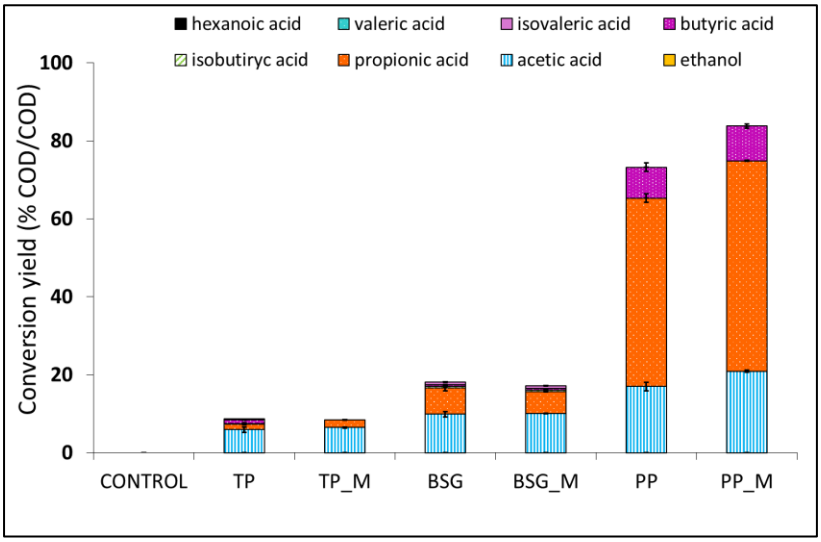

Figure II.3. AF tests with agro-industrial residues: conversion yield and distribution of fermentation products on the last day of operation for PP and PP_M, and before the glucose addition for control test, TP, TP_M, BSG, and BSG_M.

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For all residues, fermentation mainly resulted in the production of acetic, propionic, and butyric (at a low extent) acids. Also, a slight production in isobutyric acid was obtained with BSG and BSG_M (around 1% of the total COD). In addition, in the presence of BSG a slight alcoholic fermentation was triggered on the first days of operation, up to day 21th, with a maximum conversion of 1% (COD/COD) on day 15th. Regarding the TP residue, a slight production of hexanoic acid was also detected, up to day 60 th (before glucose addition), with a maximum conversion equal to 0.5% on day 36 th. However, being all AF tests performed at the same temperature and pH conditions, with the same inoculum, the slightly different profiles observed for the fermented products are more likely attributed to the different characteristics (in terms of carbohydrates, fibers, etc.) of the three agro-industrial residues.

Notably, with potato residues, propionic acid represents a very high fraction (over 50%, COD/COD) of all the fermentation products, and this aspect is particularly interesting from the perspective of exploiting this residue as feedstock for the production of the poly(hydroxybutyrate/valerate) copolymer. This behaviour is in agreement with what observed in a previous work (Marchetti et al., 2023) whereby, with the same operative conditions of pH and temperature, in presence of farinaceous by-products (e. g., reground pasta and bread crust), the concentration of propionic acid accounted for a high fraction of the overall fermentation products (with values between 35% and over 50% on a COD basis). This outcome could be likely attributed to the metabolic pathway of bacteria selected during the fermentation process. As an example, at genus level, Prevotella is associated with complex carbohydrate dietary patterns and previous studies have shown that Prevotella-dominated microbiota can produce up to 2–3 times more propionate than the other class of Bacteroidetes microbiota (Chen et al., 2017; https://doi.org/10.1038/s41598-017-02995-4). Indeed, potato peels (as the farinaceous by-products) are mainly composed of complex carbohydrate (i.e., starch), unlike tomato peels and BSG, justifying the different trend in the composition of propionic acid product. This hypothesis will require further investigations to be confirmed.

Finally, Figure II.4. reports the yields of conversion and the composition of fermentation products in control tests and in tests performed with TP and BSG after glucose supply. In these tests, the main fermentation products were acetic, propionic, and butyric acid, the latter especially in the control tests.

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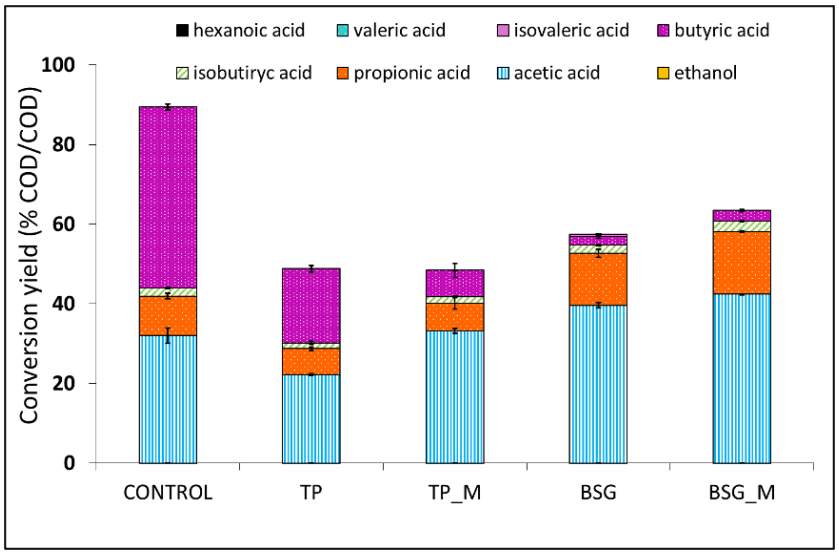

Figure II.4. Conversion yield and distribution of fermentation products in AF tests with TP, TP_M, BSG, and BSG_M, and in control tests after glucose addition.

As expected, under the applied working conditions no methane production was observed in all the AF tests.

4. Conclusions

The obtained results have shown the highest conversion yield of PP among all the tested residues into fermentation products, with values up to values of 73.2 ± 1.1% (PP) and 83.8 ± 1.0 % (PP_M) by the end of the experiments (day 76). Also, notably, a very high fraction (over 50%, COD/COD) of the fermentation products was represented by propionic acid, making PP particularly interesting as feedstock for the production of the poly(hydroxybutyrate/valerate) copolymer. The low conversion of TP and BSG into fermentation products was attributed to the presence in these two residues of compounds which are recalcitrant, but not inhibitory to the microbial activity. This also opens interesting perspectives on the possibility of adopting appropriate pre-treatment strategies (among those investigated in Task 3.1) to enhance the biological transformation of such by-products into valuable compounds.

Overall, further fermentation tests will be needed to assess the effective improvement in fermentation performance following at least one of the pre-treatment approaches tested in Task 3.1.

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III. Thermophilic fungi pre-treatment

1. Introduction

Fungi are considered key players in the degradation of lignocellulosic materials in natural environments (Tiquia-Arashiro SM. et al., 2019). They are well-known for their capacity to degrade lignin, cellulose, and hemicellulose due to the production of extracellular lignocellulosic enzymes. In the past decades, several studies have focused on the use of these extracellular lignocellulosic enzymes for the release of sugars from lignocellulosic materials for, among others, the production of ethanol (Garcia-Torreiro M. et al., 2016; Savachua D. et al., 2011). These extracellular lignocellulosic enzymes mainly consisted of a mix of cellulases, hemicellulases and ligninolytic peroxidases obtained from mesophilic fungi species (Dashtban M. et al., 2009). However, thermophilic fungi and the application of their lignocellulosic enzymes may provide new and more optimal lignocellulosic enzymes for the conversion of lignocellulosic materials into fermentable sugars (Maheshwari R. et al., 2000). Thermophilic temperatures (45-62°C) can result in increased conversion rates leading to shorter incubation times and lower enzyme loadings, as well as reducing the risk of microbial contaminations by competing microorganisms for fermentable sugars (McClendon SD et al., 2012).

Myceliophthora thermophila is a fast growing thermophilic fungus isolated from soil and from self-heating masses of composted vegetable matter (Singh B. et al., 2016). It is proficient in degrading wood and other cellulosic substances faster than other thermophilic and mesophilic fungi. *Myceliophthora thermophila* secrets a large number of hydrolytic enzymes enabling the fungus to grow on a variety of substrates (Singh B. et al., 2016). *Thermoascus aurantiacus* is another thermophilic fungus first isolated from self-heating and able to grow efficiently on different lignocellulosic biomass(McClendon SD et al., 2012). Similarly, *Thermomyces lanuginosus,* fungus normally present in compost heaps, can produce cellulase-free thermostable xylanases, with interesting applications also in the pulp and paper industry where materials with higher content of hemicellulose are used (Brar KK et al., 2023). Overall, *M. thermophila, T. aurantiacus* and *T. lanuginosus* are promising species and, yet to be fully explored, for the production of thermophilic enzymatic cocktails for the degradation of lignocellulosic materials.

In the AgriLoop project, a two-step process is proposed for the enzymatic pre-treatment of agricultural residues at thermophilic conditions. First, 10-20% of the agricultural residues is used for the growth of selected thermophilic fungi species and consequent enzyme production. Second, the crude enzyme mix is separated from the remaining solids via centrifugation and filtration for further use. Third, the crude enzyme mix is used as enzymatic cocktail for the hydrolysis of the selected agricultural residues.

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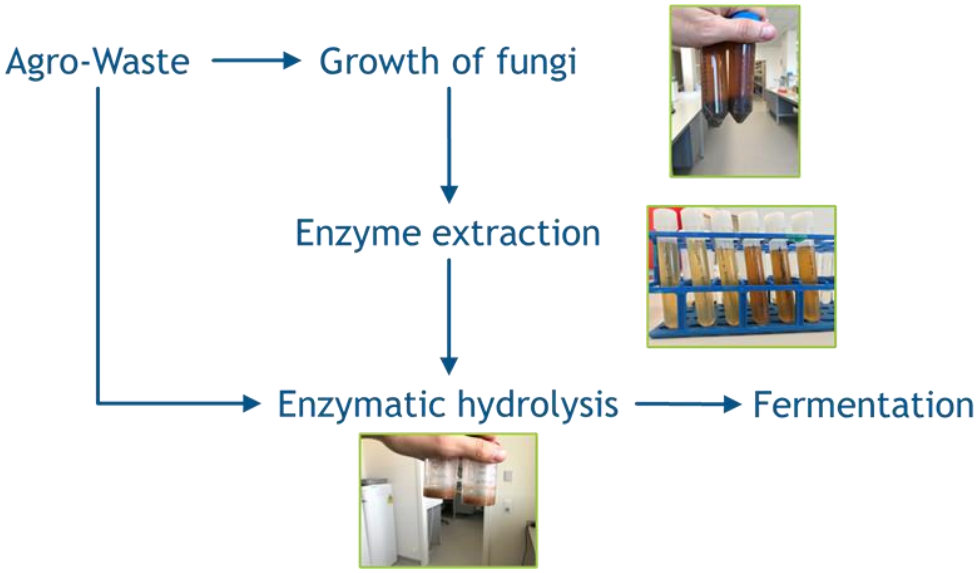

Figure II.1. Description of the thermophilic enzymatic pre-treatment of agricultural residues proposed in the AgriLoop project. The agricultural waste is not only hydrolyzed, but also used for the production of lignocellulosic enzymes by thermophilic fungi.

2. Materials and methods

2.1. Fungi growth

Freeze dried cultures of *Thermoascus aurantiacus*, *Thermomyces lanuginosus* and *Mycelyophtora thermophila* were obtained from the German DSMZ collection. The strains were first cultivated in both agar plates and liquid medium. After 5 days, the mycelium was collected, and kept at -80°C in glycerol/water (50% v/v) stocks before use. For the fungi experiments, an aliquot of these cultures was used to inoculate potato dextrose agar plates. Subsequently, the fungi cultures were incubated for 4-5 days at 50°C without shaking. After incubation, the agar plates were stored at 4°C pending experiments. Experiments took place no later than one month after incubation in the potato dextrose agar plates.

Five pieces of the agar plates (≈0.5 cm^2) were used to inoculate 250 mL Erlenmeyer flasks containing 50 mL of nutrients media and 0.5 or 1 g of dried brewery's spent grains. Nutrients media composition (g/L) consisted of 1.0 $(NH_4)_2SO_4$, 0.7 KH_2PO_4, 0.2 K_2HPO_4, 0.05 $MgSO_4 \cdot 7H_2O$ and 0.06 of yeast extract. In sterile tests, nutrients media and brewery's spent grains were previously autoclaved together for 15 min at 121°C. Inoculated Erlenmeyer flasks were incubated at 50°C for 5 days at 120 rpm in an orbital shaker.

2.2. Enzyme collection

After five days, the broth in the Erlenmeyer flasks was collected and centrifuged (7160 RCF for 10 min) to separate the solids from the extracellular enzyme solution. Then, the supernatant was filtered (vacuum filtration, 0.45µ pore size) to remove fungi spores. The crude filtrate obtained was stored at 4°C pending the hydrolysis tests.

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2.3. Hydrolysis tests

Enzymatic hydrolysis tests were performed with dried, autoclaved and non-autoclaved brewery's spent grains. Tests with autoclaved brewery's spent grains were performed in 250 mL Erlenmeyer flasks containing 35-40 mL of recovered crude filtrate and 500 mg of brewery's spent grains. Tests with non-autoclaved brewery's spent grains were performed in 50 mL plastic tubes. For this, 500 mg of brewery's spent grains were mixed with 20 mL of the recovered crude filtrate from the three selected fungi species. Subsequently, both the plastic tubes and the 250 mL Erlenmeyer flasks were incubated at 50°C and 100 rpm for 3 days.

2.4. Analytical methods and data analysis

Fungi growth on the potato dextrose agar and liquid medium was monitored by visual inspection. Fungi growth is obvious due to mycelium formation. The hydrolysis tests were monitored by liquid samples taken twice per day. Grab samples were stored at −20°C pending liquid analyses and were later used for soluble chemical oxygen demand (sCOD), sugars and carboxylic acids determination. Suspended solids were separated from the mixed liquor by centrifugation (19090 rcf for 2 min) and membrane filtration (0.2 µm pore size filters). Sugars (glucose and xylose) and carboxylic acids (lactic, acetic, propionic, (iso)butyric, (iso)valeric and caproic acid) were quantify by ultra-high pressure liquid chromatography using a Shimadzu HPLC LC-203 system equipped with a Biorad Aminex HPX-87H column (300x7.8 mm), a Shimadzu SPD-40/40V UV detector and a Shimadzu RID-20A RI detector with 5 mM sulfuric acid and 1% acetonitrile as mobile phase at 0.45 mL/min and 30°C. sCOD was determined with Nanocolor COD 1500 tube tests.

Solubilization yields are reported on a COD-basis and calculated from the amount of sCOD produced and brewery's spent grains (1.10 gCOD/gBSG) added, after three days of hydrolysis tests. Sugars and carboxylic acids yields are calculated from the amount of each component produced and brewery's spent grains added, after three days of hydrolysis tests.

3. Results and discussion

3.1. Fungi growth and hydrolysis tests with autoclaved brewery's spent grains

Three selected thermophilic fungi strains - *Thermoascus aurantiacus*, *Thermomyces lanuginosus* and *Mycelyophtora thermophila* - were first cultivated in potato dextrose agar plates (Figure II.2, left) and subsequently transferred to 250 mL Erlenmeyers flasks for cultivation at 50°C for 5 days with autoclaved brewery's spent grains. After 5 days, fungal mycelium was observed in all samples, but not in the controls with autoclaved brewery's spent grains, but no fungi inoculum. Due to shaking, the fungal mycelium was observed in suspension, and not as a homogeneous layer at the top of the liquid (Figure II.2, right). This characteristic mycelium can be better observed in the potato dextrose agar plates, as observed in Figure II.2. A different morphology was also

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Figure II.2. (Left) Characteristic morphology of the fungal mycelium of (from left to right): *T. aurantiacus, M. thermophila* and *T. lanuginosus* grown in potato dextrose agar at 50 °C for 4 days. (Right) Mycelium from *T. lanuginosus* in suspension observed after 4 days at 50 °C.

observed between the control flasks and those with autoclaved brewery's spent grains, showing the latter a more dense and opaque liquid.

Hydrolysis tests were performed with crude extracts from the three selected fungi grown for 5 days in autoclaved brewery's spent grains. After three days, 0.09 to 0.34 gCOD/gCOD-BSG of the brewery's spent grains was solubilized in tests with crude filtrate from the three selected fungi compared to only 0.02-0.04 gCOD/gCOD-BSG when no crude filtrate was added (Table II.1). Crude filtrate from *M. thermophila*, *T. aurantiacus* and *T. lanuginosus* showed similar solubilization yields. This is somehow counterintuitive, as the crude filtrate from *T. lanuginosus* lacked cellulase activity and therefore cellulose (that accounts for 17.5% of the brewery's spent grains) could not be hydrolysed. The sterilization step partially explain this outcome. The sterilization process consists of 15 min at 121°C, which likely disrupted the complex structure of the brewery's spent grains, making it more accessible for the other lignocellulosic enzymes.

Table II.1. Overview of yields (Y) obtained after three days in hydrolysis tests with crude extract from three different thermophilic fungi and autoclaved brewery spent grains.

Crude extract	**Replicate**	**Y_Solubilization**	**Y_Glucose+Xylose**	**Y_VFAs**
-	-	gCOD/gCOD-BSG	g/gCOD	g/gCOD
M. thermophila	A	0.21	0.01	0.00
	B	0.34	0.05	0.02
T. aurantiacus	A	0.23	0.03	0.01
	B	0.09	0.00	0.00
T. lanuginosus	A	0.27	0.03	0.03
	B	0.19	0.03	0.02
Control	A	0.04	0	0
	B	0.02	0	0

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In the final broths, mainly sugars were detected, but only represented up to 15% of the solubilized material (Table II.1). The fraction that was solubilized, but could not be identified by HPLC is likely rich in other complex molecules such as other carbohydrates that are not glucose and xylose and proteins. This fraction is likely readily biodegradable and can be used in subsequent steps for the production of carboxylic acids, microbial protein and polyhydroxyalkanoate. Compared to the fermentation tests performed by UniRoma, where only 15.9% (COD basis) of the brewery's spent grains were fermented to carboxylic acids after 18 days, the use of thermophilic enzymes as pretreatment for the solubilization and hydrolysis of the brewery's spent grains seems to be a promising alternative.

3.2. Fungi growth and hydrolysis tests with non-autoclaved brewery's spent grains

The same three selected thermophilic fungi strains - *T. aurantiacus*, *M. thermophila and T. lanuginosus* - were cultivated in Erlenmeyers flasks at 50°C for 5 days with non-autoclaved brewery's spent grains. After 5 days, visual inspection of the inoculated flasks and their controls (only dried brewery's spent grains and media, no fungi inoculum) clearly showed that microbial growth took place, likely due to the native microbial community present in the brewery's spent grains. No fungal mycelium was visually observed in any of the samples. A more dense and opaque liquid due to unwanted microbial growth was observed as compared with tests with autoclaved brewery's spent grains.

Unwanted microbial growth from the native microbial community present in the brewery's spent grains took place was not observed in tests with autoclaved brewery's spent grains. This may be a potential challenge for further scaling-up of the technology, as sterilization of waste materials can have a significant cost on the overall process. If needed, alternatives to sterilization are worth considering. This alternatives can aim to find selective conditions like lower pH or even higher temperatures than 50°C, where only a limited amount of microbial species can grow.

Even though unwanted microbial growth took place, a set of hydrolysis tests was still performed with the crude extracts in case the microorganisms present in the brewery's spent grains could also produce lignocellulosic enzymes or readily ferment the hydrolysed sugars. After three days, 13-18% of the brewery's spent grains was solubilized in tests with the crude filtrate from the three selected fungi compared to 10-13% when no crude filtrate was added (Table II.2). Crude filtrate from *M. thermophila* and *T. aurantiacus* showed higher hydrolysis yields than *T. lanuginosus*, likely cause by the fact that *T. lanuginosus* can only produce hemicellulose degrading enzymes and not cellulose and lignin degrading enzymes as *T. aurantiacus* and *M. thermophila*.

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Table II.2. Overview of yields (Y) obtained after three days in hydrolysis tests with crude extract from three different thermophilic fungi and non-autoclaved brewery spent grains.

Crude extract	Replicate	Yield_Sol	Y_glucose and xylose	Y-VFAs
	-	gCOD/gCOD-BSG	g/gCOD	g/gCOD
M. thermophila	A	0.16	0.00	0.13
T. aurantiacus	A	0.18	0.00	0.11
T. lanuginosus	A	0.13	0.00	0.04
Control	A	0.13	0.00	0.04
	B	0.10	0.00	0.06

In the final broths, a mix of sugars and carboxylic acids were detected, representing 30 to 81% of the solubilized material (Table II.2). The presence of volatile fatty acids indicates that the hydrolysed sugars were directly fermented to a mix of volatile fatty acids by anaerobic microorganisms. These anaerobic microorganisms are likely already present in the raw brewery's spent grains. The solubilized fraction that could not be identified with the HPLC is likely rich in more complex molecules such as carbohydrates and proteins and it may require more than three days to be fermented. Potentially, most of the solubilized fraction can be further fermented. From a process perspective, the possibility to perform these tests with non-sterile conditions feedstocks broaden the potential application of these crude filtrates as a technology to valorise complex agricultural residues.

3.3. Discussion, bottlenecks and recommendations for future research

Crude extracts from three different thermophilic fungi were used for the hydrolysis and solubilization of brewery's spent grains. Higher solubilization yields were observed when the brewery's spent grains were autoclaved and the tests were performed in sterile conditions. For instance, up to 0.34 gCOD/gCOD-BSG of the brewery's spent grains were solubilized with autoclaved brewery's spent grains compared to only up to 0.16 or 0.18 gCOD/gCOD-BSG when the brewery's spent grains were not autoclaved or directly used for fermentation. This is likely due to the sterilization step. The sterilization step does not only inactivate the microorganisms present in the raw substrate, but it also likely has an effect on the internal structure of the brewery's spent grains. The higher temperature and pressure (121°C for 15 min) likely disrupts the lignin sheath, making it more accessible for the lignocellulosic enzymes. An alternative solution that may be worth considering would be to use autoclaved brewery's spent grains for the growth of the three selected fungi and non-autoclaved brewery's spent grains in the hydrolysis tests. In this way, enzyme production can be maximized and the hydrolysis step can be directly link with the fermentation step under non-sterile conditions.

Even though the crude extracts could be directly applied for the hydrolysis of the brewery's spent grains, up to 0.34 gCOD/gCOD-BSG of the brewery's spent grains was solubilized. These solubilization yields are usually found in literature when crude extracts (and not concentrated enzymatic cocktails) are directly applied to agricultural

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residues. They can be explained by the structural complexity of the brewery's spent grains. Brewery's spent grains are composed of 17.5% cellulose, 14.1% hemicellulose and 31% lignin, complex polymers that hinders the action of the produced enzymes. A higher lignin content is usually associated with a more recalcitrant structure, as the lignin shell provides compressive strength and stiffness to the plant cell wall. An alternative solution to improve the solubilization yields can be the coupling of the enzymatic pretreatment with the another of the pretreatments developed in the AgriLoop project. Another option worth exploring are physical pre-treatments to disrupt the structure of the brewery's spent grains

4. Conclusions

Agricultural waste such as brewery's spent grains are highly rich in carbohydrates and are a sustainable source for the production of biobased chemicals. Brewery's spent grains were successfully used for the growth of the thermophilic fungi: *M. thermophila*, *T. aurantiacus* and *T. lanuginosus*. The crude extracts from these three thermophilic fungi were directly used for the hydrolysis and solubilization of brewery's spent grains. After three days, up to 0.34 gCOD/gCOD-BSG was solubilized, likely in the form of complex carbohydrates polymers and/or proteins. Further research should focus on strategies to obtain higher solubilization yields, either by improving the enzymatic treatment or the coupling of the enzymatic treatment with another pretreatment like those developed in the frame of the AgriLoop project.

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IV. Acidic hydrolysis tests

3. Experimental

3.1. Preparation of hydrolysates

Milled BSG and PP were hydrolysed as described by Guarda et al. (2023). Briefly, the hydrolysis was carried out by adding a sulfuric acid solution (3% w/w), at a liquid to solid ratio of 10 g g^{-1} at 121 °C (1 bar) for 20 min. The hydrolysate liquors obtained after separation of the remaining solids through centrifugation (13000×g, 15 min), were filtered and then neutralized to pH 7.0. The supernatants were collected and stored at 4 °C for further analysis.

For TP another hydrolysis method was tested. Milled TP (40 g) was mixed with deionized water (600 mL) and was subjected to homogenization using an ultraturrax (19000xg, 4 min). Then, sulfuric acid solution was added to a ratio of 3% v/v and the hydrolysis was carried out at 121 °C (1 bar) for 45 min. The TP hydrolysate liquor obtained after separation of the remaining solids through centrifugation (13000×g, 30 min) was neutralized (pH 6.8-7.2) and stored at 4 °C for further analysis.

3.2. Characterization of hydrolysates

The hydrolysates were characterized in terms of sugars, organic acids, furfural and 5-hydroxymethylfurfural (5HMF), total phenolic compounds, phosphate and ammonium contents.

The sugars were quantified by high performance liquid chromatography (HPLC) using a CarboPac PA10 and AminoTrap columns (Dionex, Thermo Scientific, Sunnyvale, CA, USA), equipped with a pulsed amperometric detector (PAD) as to Araújo et al. (2021). The organic acids, furfural and 5HMF were also determined by HPLC as described by Araújo et al. (2021). NH_4^+ and PO_4^{3-} contents were determined by colorimetry, as implemented in a flow segmented analyser (Skalar 5100, Skalar Analytical, Breda, The Netherlands) (Carvalho et al. 2007).

4. Results

The results of the acidic hydrolysis of the different agro-food wastes in terms of total sugars and degradation products are represented in Table IV.1.

After acidic hydrolysis, TP, BSG and PP hydrolysates were characterized in terms of simple sugar concentration. PP hydrolysate was the one with the highest simple sugar concentration (42.5 g/L), being the major sugar monosaccharide glucose (82.66% w/w). BSG hydrolysate simple sugars was 28.2 g/L being mainly composed of xylose (49. 66% w/w), arabinose (25.06 w/w) and glucose (19.20% w/w). The hydrolysate with lower sugar content was that of TP (24.4 g/L), composed of xylose (46.94% w/w), arabinose (24.88% w/w) and glucose (21.04% w/w).

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TABLE IV.1. CHARACTERIZATION OF THE TP, BSG AND PP ACIDIC HYDROLYSATES.

	Tomato Pomace	BSG	Potato Peels
Simple Sugars	24.4 g/L	28.2 g/L	42.5 g/L
5HMF	38.3 mg/L	61.25 mg/L	*
Furfural	0	401.2 mg/L	*
Phenolic compounds	1.59 g/L	*	*
Acetic acid	1.49 ± 0.05 g/L	1.15 ± 0.152 g/L	0.98 ± 0.003 g/L
Lactic acid	1.09 ± 0.026 g/L	0.46 ± 0.037 g/L	10.80 ± 0.353 g/L
Phosphate	192.4 ± 1.42 mg/L	5.3 mg/L	200.3 ± 15.9 mg/L
Ammonium	353.5 ± 2.41 mg/L	758.7 ± 6.6 mg/L	456.1 ± 4.8 mg/L

* – Waiting for analytical results

Regarding the degradation products, the 5HMF and furfural contents were low both for TP and BSG. However, higher concentrations of furfural and 5HMF in the BSG hydrolysate, probably due to its higher glucose and xylose contents since they result from the degradation of these sugars, respectively.

Phenolic compounds are resultant from the hydrolysis of lignin. TP hydrolysate had 1.59 g/L phenolics content, resulting from the high fraction of lignin (44.1 ± 5.27%) of the waste.

Acetic acid was present in all the raw hydrolysates at concentrations ranging from 0.98 ± 0.003 g/L to 1.49 ± 0.05 g/L. On the other hand, lactic acid was also present in the three hydrolysates with concentrations between 0.46 ± 0.037 g/L and 10.80 ± 0.353 g/L. The high content of lactic acid on raw PP hydrolysate can be inhibitory for some bacterial strains.

Phosphate and ammonium ions were present in very low concentration in all the hydrolysates.

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V. Subcritical water experimental conditions

1. Materials and methods

Prior to the subcritical water treatment (SCW), the lipids were extracted from TP and from BSG using Soxhlet extraction with hexane according to Cruz et al., 2014. Then, each extract (TP and BSG after Soxhlet extraction and milled PP) was subjected to an extraction with water at a liquid to solid ratio of 10 g g^{-1} at 121 °C, 1 bar, 20 min. The extracts liquor obtained after separation of the remaining solids through centrifugation (13000×g, 15 min) were stored at 4 °C and used for the quantification of sugars. The remaining solid were subjected to subcritical water treatment. Batch reactions were performed in a Parr high pressure reactor (model 4547) (Figure V.1.).

Figure V.1. SCW apparatus used for SCW treatment.

Briefly, the apparatus consists in a 1.2 L stainless-steel vessel equipped with a magnetic stirrer. The temperature inside the reactor was maintained by a heating blanket placed around the reactor and a cooling coil inside the reactor both connected to a temperature controlled that monitors the temperature through a thermocouple inside the reactor. The initial pressure in the system is applied by pressurizing the system to 30 bar using industrial nitrogen. The final pressure of each experiment was dependent on the temperature applied. For these experiments the solid to liquid ratio (S:L) of 1:10 was maintained and only the target temperature was varied. For each experiment the reactor was filled with 40 g of dried material and 400 mL of distilled water. The reactor was heated under moderate stirring up to 140-280 °C and left 15 min in the target temperature. The total reaction time varied from 45 to 60 min. When the reaction time was completed, the reactor was cooled down and depressurized. The mixture was then separated by filtration, the liquors were stored for analysis and the remaining solid was freeze-dried and weighed before analysis. The selected end temperatures for TP were 190, 220, 250 and 280 °C, for BSG were 140, 190 and 220 °C and for PP were 190, 220 and 250 °C. In the case of TP, the extract subjected to the temperature of 190 °C was then subjected to 250 °C and the one subjected to 220 °C was then subjected to 280 °C. For BSG and PP the assays were performed individually at each selected temperature.

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2. Results

After lipids extraction, milled TP and BSG were subjected to hydrolysis with water at 121 °C, 1 bar, to extract the simple soluble sugars. PP milled raw material was subjected to the same hydrolysis. Table V.1. shows that the simple soluble sugars content was very low in all the raw hydrolysates. The higher content was for PP hydrolysate (0.31 g/L).

Regarding the degradation products, lactic acid was present in a high content (10.95 ± 0.179 g/L) in PP raw hydrolysate that is a similar concentration to the value obtained with the acidic hydrolysis. For TP and BSG, the lactic acid concentration was 1.49 ± 0.005 g/L and 0.83 ± 0.044 g/L, respectively. These values are also similar to the ones achieved with acidic hydrolysis. Acetic acid is present in the water raw hydrolysate in concentrations within 0.57 ± 0.030 g/L and 1.46 ± 0.023 g/L, while a low concentration of formic acid (0.16 ± 0.012 g/L) was only detected in BSG water hydrolysate. The soluble solids resulted from the drying process of liquid fraction. Its content was much higher for BSG (60.41 g/L) and PP (61.52 g/L) than for TP (16.95 g/L)

After the water extraction, the remaining biomass of each agri-food waste was subjected to a treatment with subcritical water. Subcritical water is an eco-friendly method for pre-treated lignocellulosic material, avoiding the use of solvents. During the SCW pre-treatment the hemicellulose fraction is mostly hydrolysed or solubilized, releasing the sugar present in the form of oligomers or monomers. Furthermore, during the pre-treatment other compounds could be released, namely proteins and phenolic compounds (Alonso-Riaño et al., 2022).

TABLE V.1. TP, BSG PP SUBCRITICAL WATER LIQUID FRACTION CHARACTERIZATION.

Assays		Liquid Fraction					
		Simple Sugars (g/L)	**Lactic acid (g/L)**	**Formic acid (g/L)**	**Acetic acid (g/L)**	**Soluble solids (g/L)**	**Yield (g/g)**
Tomato Pomace	Soluble Sugars after autoclave	0.17 ± 0.07	1.49 ± 0.005	ND	1.46 ± 0.023	16.95	0.13
	SCW190 °C	< 0.1	0.48 ± 0.029	0.57 ±0.032	0.54 ± 0.033	11.49	0.12
	SCW250 °C	< 0.1	0.49 ± 0.200	0.27 ± 0.107	0.39 ± 0.122	9.86	0.08
	SCW220 °C	< 0.1	0.27 ± 0.009	0.39 ±0.052	0.67± 0.055	12.25	0.17
	SCW280 °C	< 0.1	2.35 ± 0.013	N.D.	0.424±0.072	8.09	0.07
BSG	Soluble Sugars after autoclave	0.27	0.83 ± 0.044	0.16 ± 0.012	0.57 ± 0.030	60.41	0.30
	SCW140 °C	0.29	0.26 ± 0.013	0.05 ±0.001	0.23 ± 0.012	19.59	0.11

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	SCW 190 °C	1.46	0.49 ± 0.025	0.29 ±0.025	0.90 ± 0.001	33.91	0.30
	SCW220 °C	< 0.1	1.86 ± 0.062	0.82 ±0.083	1.38 ± 0.007	21.23	0.19
Potato Peels	Soluble Sugars after autoclave	0.31	10.95 ± 0.179	N.D.	0.76 ± 0.007	61.52	0.48
	SCW190 °C	0.66	2.07 ± 0.073	N.D.	0.22 ± 0.051	24.29	0.43
	SCW220 °C	1.41	3.05 ± 0.037	0.75 ±0.029	0.55 ± 0.09	16.92	0.30
	SCW250 °C	< 0.1	3.67 ± 0.094	0.72 ±0.032	0.75 ± 0.011	12.91	0.22

ND – not detected.

TP biomass was subjected to two different initial temperatures 190 °C and 220 °C (Table V.1.), then the solid fraction collected at the end of each run was then subjected to higher temperatures, namely 250 °C and 280 °C, respectively. This was performed to increase the efficiency of hemicellulose hydrolysis and in this way increase the extraction of fermentable carbohydrates. The analysis of the liquid fraction showed a very low concentration of simple sugars (<0.1 g/L) for any of the temperatures tested. The soluble solids achieved after drying were between 12.25 g/L and 8.09 g/L. resulting in very low yields (0.07 – 0.17 $g_{soluble\ solids}/g_{initial\ biomass}$). The hydrolysis/ solubilization decreased with the increase of temperature.

For BSG biomass after water hydrolysis, three different temperatures were tested on SCW runs, 140, 190 and 220 °C (Table V.1.). The higher simple soluble sugar concentration (1.46 g/L) attained was at 190 °C, despite still being a low concentration. This liquid fraction was then freeze dried resulting in 33.91 g/l of soluble solids, corresponding to the highest yield 0.30 $g_{soluble\ solids}/g_{initial\ biomass}$. The other soluble solid fractions were 19.59 g/L and 21.23 g/L, at 140 °C and 220 °C, respectively.

Regarding PP biomass the SCW pre-treatments were performed at the following temperatures 190 °C, 220 °C and 250 °C (Table V.1.). The highest simple sugar concentration in the liquid fraction was 1.41 g/L, achieved for the treatment at 220 °C, resulting in a soluble fraction of 16.92 g/l (yield of 0.30 $g_{soluble\ solids}/g_{initial}$). The highest yield and soluble fraction were achieved for the biomass treated at 190 °C, 0.43 $g_{soluble\ solids}/g_{initial}$ and 24.29 g/L, respectively.

The soluble fraction is constituted by oligosaccharides and could also have proteins. The fractions from the different wastes and temperatures are under analysis.

The degradation products were also evaluated for all the assays performed. The lactic acid higher concentrations were achieved for the highest temperatures tested for each biomass (Table V.1.). However, the highest concentrations were reached for PP runs, within 2.07 ± 0.073 g/L and 3.67 ± 0.094 g/L. Formic acid concentrations achieved were

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below 1 g/L, and in some runs were not detected. At least, acetic acid was detected in all the runs, being only above 1 g/l for the BSG SCW 220 °C pre-treatment.

3. Conclusions

The SCW pre-treatment showed better results for BSG and PP biomass. For both materials the temperature of 190 °C seems to be the most suitable, with low concentration of degradation products being produced, exception made for the lactic acid present on the PP SCW hydrolysate.

TP and BSG biomass have a high lipid (oil) content which is interesting to be used as substrate for biopolymers production.

Further assays will be performed accoupling enzymatic treatment to the pre-treated SCW biomass to increase the carbohydrate sugars concentration.

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VI. Supercritical fluid (s.c.) extraction (SFE)

1. Introduction

An ideal extraction method should be swift, yield quantitative recovery without degradation, and the extracts should be easily separated from the solvent. The development and application of alternative green technology to replace conventional extraction methods with improved extraction efficiency and low environmental impact for the determination of natural bioactive compounds is therefore, highly important. SFE is currently considered an appealing green alternative to traditional extraction methods. The properties of supercritical fluids (SFs) can be considered as intermediate between the ones of liquids and gases. Similarly to gases, SFs are highly compressible, but have high densities comparable to those of liquids. The combination of some of the properties of liquids with those of gases provides supercritical fluids with some very interesting features. For example, supercritical fluids can effuse through solid materials like a gas but can also act like a liquid and dissolve substances. In SFE, the organic phase used in typical solid–liquid extractions (SLE) is substituted by a supercritical fluid. The manipulation of both the temperature and pressure of the fluid can solubilize the substance of interest in a complex matrix and selectively extract it. Compared to SLE, SFE is indeed simpler, faster, and more efficient but without consuming large quantities of organic solvents, which are both expensive and potentially harmful (Marić et al., 2020). Other immediate advantages of SFE compared to traditional extraction techniques are process flexibility due to the continuous modulation of the solvent power/selectivity of the supercritical fluid and the elimination of polluting organic solvents, which also prevents expensive post-processing of the extracts for solvent removal. CO_2 is the most commonly used supercritical fluid thanks to its non-toxicity, chemical inertia, low cost, and most importantly, low critical values. Its low critical temperature (below 32 °C) makes CO_2 ideal for the extraction of thermolabile compounds. For these reasons, the use of CO_2 as an extraction solvent has been successfully reported in the literature for the isolation of many compounds from various sources, such as valuable compounds from food residues (Chronopoulou et al., 2019).

Here, the objective of the SFE treatment is to allow the disruption of lignocellulosic bonds in the matrices of interest, trying to increase the fermentative capacity of the solid residue as well as to recover high value-added components in the liquid extract.

2. Materials and methods

2.1. Operation of SFE experiments

Supercritical fluid extractions (SFE) were performed on a SFE 300 analytical extractor manufactured by Carlo Erba Instruments. The extractions took place in a metal tubular reactor of 1 cm^3 where the solid samples were introduced. Cooled CO_2 and in liquid state were fed into the high-pressure tubular reactor, pressurized at the desired target pressure by a syringe pump, and heated to the desired temperature with a system of recirculating air in the thermostatic chamber where the tubular reactor was located. In all the experiments, a static extraction was followed by a 5-min dynamic extraction

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performed through the depressurization of s.c.CO_2 in 2 mL of ethanol. For each extraction experiments, a fixed amount of milled agro-industrial residues was placed in the extraction cell without adding modifiers. Prior being used, all residues were characterized in terms of COD (representing the Chemical Oxygen Demand) and moisture content. Extraction experiments were performed on residues samples after freeze-drying (for TP and PP) and milling (for all three substrates), and residues are referred to as TP_M, PP_M, and BSG_M. On the basis of literature data, the operating parameters were varied in the pressure range of 20-30MPa for tomato peels, potato peels, and BSG; while temperature ranged between 40 to 80°C. The ethanol solutions recovered after CO_2 depressurization will be used for the further analytical evaluation of, among the others, lycopene (from tomato peels), flavonoids and polyphenols (from potato peels and BSG, respectively), through aluminium trichloride and Folin–Ciocalteu methods (Fernández et al., 2008; Lima et al., 2021), respectively. As for the solid residues after the SFE treatment, the total COD was measured in order to assess the variation in the intrinsic content of oxidizable organic matter of these matrices. A scheme of the extraction apparatus is shown in Figure VI.1.

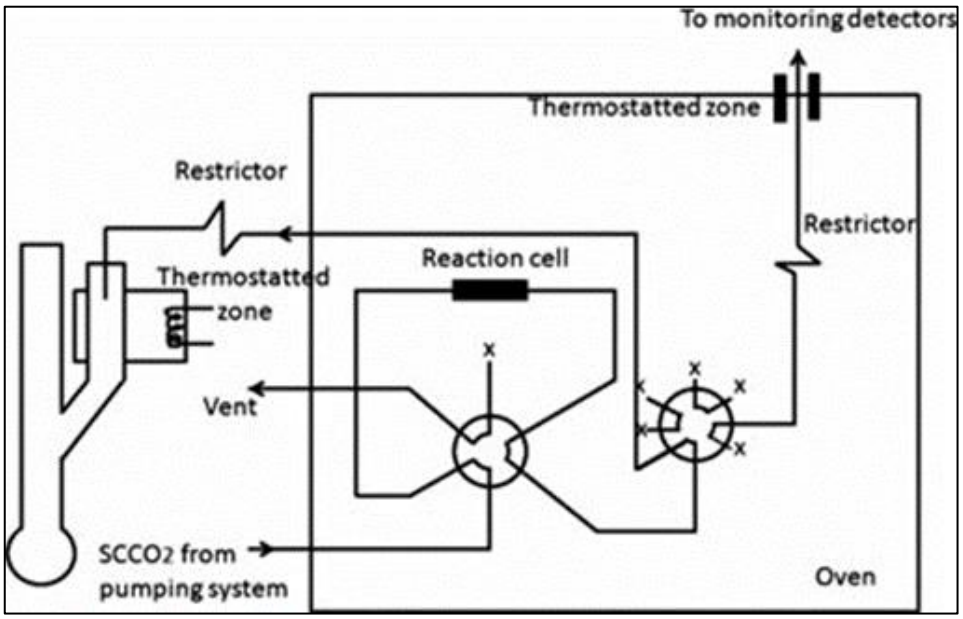

Figure VI.1. Schematic representation of the laboratory-scale SFE apparatus.

2.2. COD measurements and calculations

The total COD (tCOD) of all residues, referred to as the dry weight, was measured by using a commercial test (Tube test NANOCOLOR CSB 1500 Hg-frei, Macherey Nagel) consisting of glass cuvettes containing potassium dichromate and concentrated H_2SO_4 (80-98%). After samples digestion at 160 °C for 30 minutes, the COD content was determined using a spectrophotometer (NANOCOLOR® Advance, Macherey Nagel) at a wavelength of 605 nm. The COD of the agro-industrial residues was determined before and after the SFE with CO_2.

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The efficiency of the extraction procedure was estimated in terms of reduction of the COD in residues after the treatment with s.c CO_2 (*tCODf*) with respect to initial COD, measured before the treatment (*tCODi*); as follows:

Reduction tCOD (%, COD/COD) = ((tCODi – tCODf)/tCODi) · 100

In particular, the reduction in the COD content of the residues is attributed to the recovery of high value added substances in the extract samples. This needs to be confirmed by further analysis.

3. Results and discussion

Experimental operating conditions to perform the SFE treatment with CO_2, were chosen based on literature studies. Depending on the type of agro-industrial residues, pressure and temperature values were set to improve the extraction of their main components to be extracted, as carotenoids (e.g., lycopene) for tomato peels (Hatami & Ciftci, 2023; Kehili et al., 2017; Machmudah et al., 2012), flavonoids for BSG (Alonso-Riaño et al., 2022; Fernández et al., 2008; Spinelli et al., 2016), and polyphenols for potato peels (Jimenez-Champi et al., 2023; Lima et al., 2021). The three residues were introduced into the extraction cell after freeze-drying and milling to ensure the absence of moisture in the sample and to allow selective and more efficient extraction of the components listed above.

Table VI.1. Operative conditions for each agro-industrial residues during SFE treatment with CO_2

Item	Pressure (MPa)	Temperature (°C)	Contact time (h)
TP	30	80	2– 3 – 5
BSG	20; 30	40; 60; 80	2 – 3 – 4 – 5
PP	20	80	2 – 3 – 5

Table VI.1. shows the temperature and pressure values adopted for the SFE with the three residues, which underwent different contact times with s.c. CO_2. At the end of each extraction procedure, the CO_2 present in the extraction cell was flowed inside ethanol, placed in a test tube, recovering all the (mostly apolar) substances extracted from the samples. Whereas, the solid residues were recovered in eppendorf tubes and then subjected to analysis of the COD content (as previously detailed). Regarding the characterisation and quantification of all extracted components, analyses are in progress. The obtained results in term of COD content of the solid residues, before and after the treatment, are reported In Figure VI.2. In particular, data in Figure VI.2A are referred to the COD trend obtained in the first set of the experiments, performed at 30 MPa and 80°C for TP_M and PP_M and 40 °C for BSG_M. A linear decrease of the COD content was obtained by increasing the contact time, with a maximum equal to about 16 %, 13 %, and 5 % (COD/COD) for TP_M, PP_M and BSG_M after five hours of treatment, respectively. Being the COD decrement significantly lower with BSG than with the other residues, further experiments were performed with BSG by changing the applied temperature and pressure (Figure VI.2B). The lowest decrease of the initial COD (about 5%) was obtained by applying a temperature of 40°C and a pressure of 20 MPa, while at 80°C and 30 MPa the highest decrease of 23% (COD/COD) was reached. In principle,

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the reduction of COD content in substrates should correspond to the recovery of components of interest in the extracted samples. In addition, the decompression of CO_2 at the end of the treatment should have led to the breaking of lignocellulosic bonds in the matrices, likely increasing the availability of COD for microorganisms during a further possible acidogenic fermentation step. These hypotheses will be confirmed by further AF batch tests using the post-treatment solid residues.

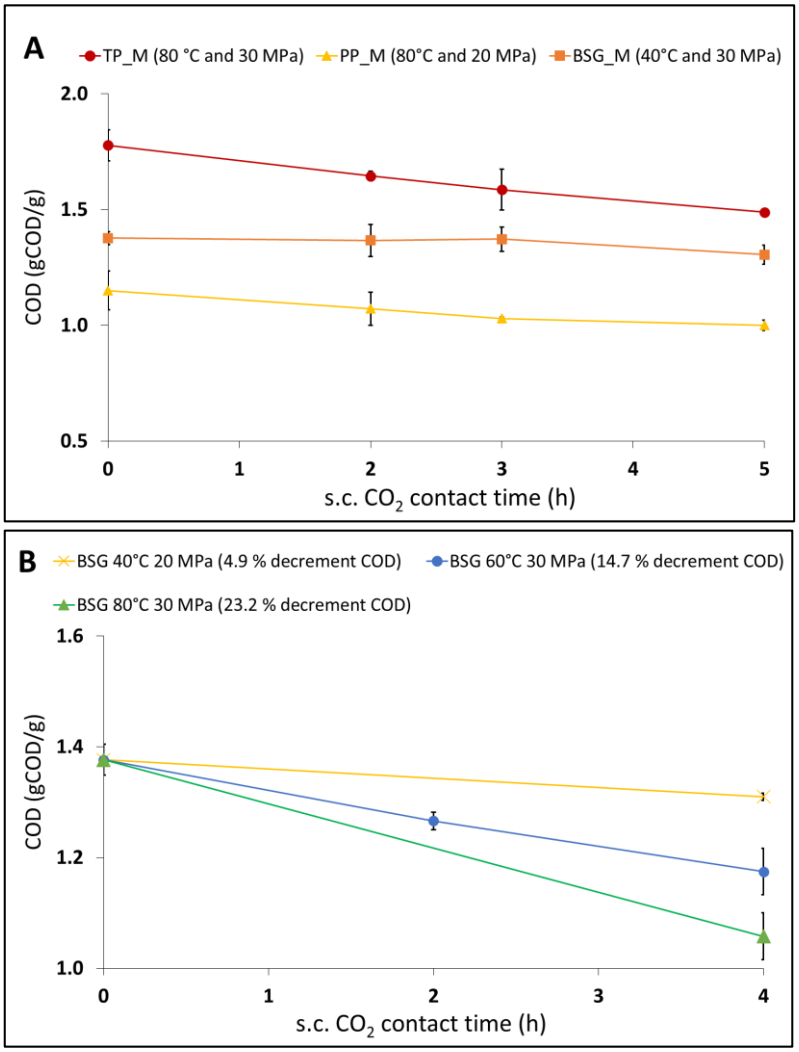

Figure VI.2. SFE tests with agro-industrial residues: trends of COD content per each substrate at different operative conditions(A); trends of COD content of BSG_M by changing the operative conditions (B).

4. Conclusion

Among the possible pre-treatments to be used to recover high value-added components form TP, PP, and BSG, UNIROMA has adopted SFE treatment with s.c. CO_2. Different operating conditions (in terms of temperature, pressure, and contact time) have been tested with the three residues. Analyses performed on solid residues after the pre-treatment, showed a decrease in the COD content up to 23% for BSG as temperature increased up to 80 °C (for pressure equal to 30 MPa) at a contact time of 4 hours. At the same temperature but at a contact time of 5 hours, the s.c. CO_2 pre-

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treatment caused a COD reduction of 16% and 13% for TP and PP, respectively. In principle, the reduction of COD content in the solid residues should correspond to the amount of components of interest (i.e., carotenoids in particular lycopene for tomato peels, flavonoids for BSG, and polyphenols for potato peels) recovered in the extracted samples. To confirm this, analyses for the characterisation and quantification of all extracted components are in progress.

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General conclusions

As part of Task 3.1. AgriLoop partners focused on the application of thermophilic fungi (UGENT), subcritical water treatment coupled with mild acid hydrolysis (NID), and supercritical fluids (UNIROMA) as biomass pretreatment for the release of fermentable sugars.

UGENT successfully grew three selected thermophilic fungi: *M. thermophila*, *T. aurantiacus* and *T. lanuginosus* on brewery's spent grains. The crude extracts rich in fungi enzymes from these three thermophilic fungi were recovered and directly used for the hydrolysis and solubilization of the brewery's spent grains. After three days, up to 0.34 gCOD/gCOD-BSG was solubilized, likely in the form of complex carbohydrates polymers and/or proteins. The sterilization of the BSG was identified as the main limitation for this process and further research is needed to determine whether the enzymatic pre-treatment can be performed under non-sterile conditions.

NID developed a subcritical water pre-treatment and performed an acidic hydrolysis. The subcritical water pre-treatment pretreatment showed better results for the brewery's spent grains and the potato peels residues. For both materials the temperature of 190 °C seems to be the most suitable, with low concentration of degradation products being produced, exception made for the lactic acid present on the PP SCW hydrolysate. After acidic hydrolysis, PP hydrolysate was the one with the highest simple sugar concentration (42.5 g/L), being the major sugar monosaccharide glucose (82.66% w/w). BSG hydrolysate simple sugars was 28.2 g/L being mainly composed of xylose (49. 66% w/w), arabinose (25.06 w/w) and glucose (19.20% w/w). The hydrolysate with lower sugar content was that of TP (24.4 g/L), composed of xylose (46.94% w/w), arabinose (24.88% w/w) and glucose (21.04% w/w).

As for the possible pre-treatments to be used to recover high value-added components form TP, PP, and BSG, UNIROMA adopted the SFE treatment with s.c. CO2. The latter was investigated at different pressure and temperature operative conditions, with the aim to also enhance the biological transformation of such by-products after the pre-treatment into organic acids. This treatment caused a COD content reduction in the solid residues of 23%, 16% and 13% for BSG, TP and PP, respectively. This reduction should correspond to the amount of components of interest (i.e., carotenoids in particular lycopene for tomato peels, flavonoids and polyphenols for potato peels and BSG) recovered in the extracted samples. To confirm this, analyses for the characterisation and quantification of all extracted components are in progress.

With reference to the assessment of the acidogenic fermentation (AF) potential of the investigated agro-industrial residues (performed by UNIROMA), the highest yield in terms of conversion into fermented products (up to 83.8 ± 1.0 %, COD/COD) was obtained with PP. Also, notably, a very high fraction (over 50%, COD/COD) of the fermentation products was represented by propionic acid, making PP particularly interesting as feedstock to produce the poly(hydroxybutyrate/valerate) copolymer. As for TP and BSG the low conversion yield obtained (around 8% and 18% on COD basis, for TP and BSG, respectively) could be attributed to the presence in these two residues of compounds which are recalcitrant, but not inhibitory to the microbial activity.

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Data Management Plan follow-up

N°	Dataset name	Open Data	Closed Data	Means of dissemination	Maximum delay before access	Data set access
8	WP3-Task 3.1 UNIROMA (Partner #12)	Data have been elaborated in excel files, available on request in the AgriLoop Dataverse.	Data reported in the deliverable are available on request	Scientific publications, International and national conferences and dissemination events	Once published and at the latest 2 years after the end of the project	https://entrepot.recherche.data.gouv.fr/dataverse/agriloop_wp3
22	WP3 -Task 3.1 NID (Partner #10)	Data have been elaborated in excel files, available on request in the AgriLoop Dataverse.	Data reported in the deliverable are available on request	Scientific publications, International and national conferences and dissemination events	Once published and at the latest 2 years after the end of the project	https://entrepot.recherche.data.gouv.fr/dataverse/agriloop_wp3
21	WP3 -Task 3.1 UGENT (Partner #15)	Data have been elaborated in excel files, available on request in the AgriLoop Dataverse.	Data reported in the deliverable are available on request	Scientific publications, International and national conferences and dissemination events	Once published and at the latest 2 years after the end of the project	https://entrepot.recherche.data.gouv.fr/dataverse/agriloop_wp3

This table above sums up the main information regarding the data produced for this deliverable, where is it stored and are the specific rules to respect concerning access, publication and FAIR principles.

N°	Dataset name	Owner	Name of the current contact	IPR issues	Use of third-party	Restrictions on data sharing (Y/N)
8	WP3-Task 3.1 UNIROMA (Partner #12)	UNIROMA	Angela Marchetti Marianna Villano	To be defined according to the Institution regulation	no	Yes, compliance with GDPR
22	WP3 -Task 3.1 NID (Partner #10)	NID	Cristiana Torres Maria Reis	To be defined according to the Institution regulation	no	Yes, compliance with GDPR
21	WP3 -Task 3.1 UGENT (Partner #15)	UGENT	Ángel Estévez Ramon Ganigué	To be defined according to the Institution regulation	no	Yes, compliance with GDPR

This table above sums up the main information regarding potential Intellectual property protection or GDPR issues.

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References

Agler, M. T., Wrenn, B. A., Zinder, S. H., & Angenent, L. T. (2011). Waste to bioproduct conversion with undefined mixed cultures: The carboxylate platform. *Trends in Biotechnology*, *29*(2), 70–78. https://doi.org/10.1016/J.TIBTECH.2010.11.006

Alonso-Riaño, P., Melgosa, R., Trigueros, E., Illera, A. E., Beltrán, S., & Sanz, M. T. (2022). Valorization of brewer's spent grain by consecutive supercritical carbon dioxide extraction and enzymatic hydrolysis. *Food Chemistry*, *396*, 133493. https://doi.org/10.1016/j.foodchem.2022.133493

APHA. (1995). Standard methods for the examination of water and wastewater. 19th. *American Journal of Public Health*, *85*(8_Pt_2), 164–203. https://doi.org/10.2105/AJPH.85.8_Pt_2.P.164

Araújo, D., Costa, T., & Freitas, F. (2021). Biovalorization of Lignocellulosic Materials for Xylitol Production by the Yeast Komagataella pastoris. Applied Sciences, 11(12),

Brar KK, Raheja Y, Di Falco M, Tsang A, Chadha BS. Novel β-glucanases along with xylanase identified in Thermomyces lanuginosus secretome for enhanced saccharification of different lignocellulosics. *Biomass Conv Bioref*. 2023;13(1):273-286.

Carvalho, G., Lemos, P. C., Oehmen, A., & Reis, M. A. M. (2007). Denitrifying phosphorus removal: Linking the process performance with the microbial community structure. Water Research, 41(19), 4383–4396.

Cruz, M. V., Paiva, A., Lisboa, P., Freitas, F., Alves, V. D., Simões, P., Barreiros, S., & Reis, M. A. M. (2014). Production of polyhydroxyalkanoates from spent coffee grounds oil obtained by supercritical fluid extraction technology. Bioresource Technology, 157, 360–363

Camire, M. E., Violette, D., Dougherty, M. P., & Mclaughlin, M. A. (1997). *Potato Peel Dietary Fiber Composition: Effects of Peeling and Extrusion Cooking Processes*. https://pubs.acs.org/sharingguidelines

Chronopoulou, L., Dal Bosco, C., Di Caprio, F., Prosini, L., Gentili, A., Pagnanelli, F., & Palocci, C. (2019). Extraction of carotenoids and fat-soluble vitamins from Tetradesmus obliquus microalgae: An optimized approach by using supercritical CO2. *Molecules*, *24*(14). https://doi.org/10.3390/molecules24142581

Dashtban M, Schraft H, Qin W. Fungal Bioconversion of Lignocellulosic Residues; Opportunities & Perspectives. *International Journal of Biological Sciences*. 2009;5(6):578-595.

Fernández, M. P., Rodriguez, J. F., García, M. T., De Lucas, A., & Gracia, I. (2008). Application of supercritical fluid extraction to brewer's spent grain management. *Industrial and Engineering Chemistry Research*, *47*(5), 1614–1619. https://doi.org/10.1021/IE0708529

This project has received funding from the European Union's Horizon Europe research and innovation programme and the UK Research and Innovation fund under the UK government's Horizon Europe funding guarantee, grant agreement No. 101081776. Views and opinions expressed are however those of the author(s) only and do not necessarily reflect those of the European Union. Neither the European Union nor the granting authority can be held responsible for them.

Fritsch, C., Staebler, A., Happel, A., Cubero Márquez, M., Aguiló-Aguayo, I., Abadias, M., Gallur, M., Cigognini, I., Montanari, A., López, M., Suárez-Estrella, F., Brunton, N., Luengo, E., Sisti, L., Ferri, M., & Belotti, G. (2017). Processing, Valorization and Application of Bio-Waste Derived Compounds from Potato, Tomato, Olive and Cereals: A Review. *Sustainability*, *9*(8), 1492. https://doi.org/10.3390/su9081492

García-Torreiro M, López-Abelairas M, Lu-Chau TA, Lema JM. Fungal pretreatment of agricultural residues for bioethanol production. *Industrial Crops and Products*. 2016;89:486-492.

Guarda, E. C., Costa, E., Gil, C., Amorim, C. L., Galinha, C. F., Duque, A. F., Castro, P. M. L., & Reis, M. A. M. (2023). Acidogenic Fermentation of Brewers' Spent Grain Monitored through Two-Dimensional Fluorescence Spectroscopy. ACS Sustainable Chemistry & Engineering, 11(19), 7398–7406.

Hatami, T., & Ciftci, O. N. (2023). A step-by-step technoeconomic analysis of supercritical carbon dioxide extraction of lycopene from tomato processing waste. *Journal of Food Engineering*, *357*, 111639. https://doi.org/10.1016/j.jfoodeng.2023.111639

Jimenez-Champi, D., Romero-Orejon, F. L., Moran-Reyes, A., Muñoz, A. M., & Ramos-Escudero, F. (2023). Bioactive compounds in potato peels, extraction methods, and their applications in the food industry: a review. *CyTA - Journal of Food*, *21*(1), 418–432. https://doi.org/10.1080/19476337.2023.2213746

Kehili, M., Kammlott, M., Choura, S., Zammel, A., Zetzl, C., Smirnova, I., Allouche, N., & Sayadi, S. (2017). Supercritical CO2 extraction and antioxidant activity of lycopene and β-carotene-enriched oleoresin from tomato (Lycopersicum esculentum L.) peels by-product of a Tunisian industry. *Food and Bioproducts Processing*, *102*, 340–349. https://doi.org/10.1016/j.fbp.2017.02.002

Liang, S., & McDonald, A. G. (2014). Chemical and Thermal Characterization of Potato Peel Waste and Its Fermentation Residue as Potential Resources for Biofuel and Bioproducts Production. Journal of Agricultural and Food Chemistry, 62(33), 8421–8429.

Lima, M. de A., Andreou, R., Charalampopoulos, D., & Chatzifragkou, A. (2021). Supercritical carbon dioxide extraction of phenolic compounds from potato (Solanum tuberosum) peels. *Applied Sciences (Switzerland)*, *11*(8). https://doi.org/10.3390/app11083410

Liu, Q., Weber, E., Currie, V., & Yada, R. (2003). *Physicochemical properties of starches during potato growth*. www.elsevier.com/locate/carbpol

Lynch, K. M., Steffen, E. J., & Arendt, E. K. (2016). Brewers' spent grain: a review with an emphasis on food and health. *Journal of the Institute of Brewing*, *122*(4), 553–568. https://doi.org/10.1002/jib.363

Machmudah, S., Zakaria, Winardi, S., Sasaki, M., Goto, M., Kusumoto, N., & Hayakawa, K. (2012). Lycopene extraction from tomato peel by-product containing tomato seed

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using supercritical carbon dioxide. *Journal of Food Engineering*, *108*(2), 290–296. https://doi.org/10.1016/j.jfoodeng.2011.08.012

Maheshwari R, Bharadwaj G, Bhat MK. Thermophilic Fungi: Their Physiology and Enzymes. *Microbiol Mol Biol Rev*. 2000;64(3):461-488.

Marchetti, A., Salvatori, G., Astolfi, M. L., Fabiani, M., Fradinho, J., Reis, M. A. M., Gianico, A., Bolzonella, D., & Villano, M. (2023). Evaluation of the acidogenic fermentation potential of food industry by-products. *Biochemical Engineering Journal*, *199*, 109029. https://doi.org/10.1016/j.bej.2023.109029

Marić, B., Pavlić, B., Čolović, D., Abramović, B., Zeković, Z., Bodroža-Solarov, M., Ilić, N., & Teslić, N. (2020). Recovery of high-content ω–3 fatty acid oil from raspberry (Rubus idaeus L.) seeds: Chemical composition and functional quality. *LWT*, *130*, 109627. https://doi.org/10.1016/j.lwt.2020.109627

Mariotti, F., Tomé, D., & Mirand, P. P. (2008). Converting Nitrogen into Protein—Beyond 6.25 and Jones' Factors. Critical Reviews in Food Science and Nutrition, 48(2), 177–184

McClendon SD, Batth T, Petzold CJ, Adams PD, Simmons BA, Singer SW. Thermoascus aurantiacus is a promising source of enzymes for biomass deconstruction under thermophilic conditions. *Biotechnol Biofuels*. 2012;5(1):54.

Medeiros Garcia Alcântara, J., Distante, F., Storti, G., Moscatelli, D., Morbidelli, M., & Sponchioni, M. (2020). Current trends in the production of biodegradable bioplastics: The case of polyhydroxyalkanoates. *Biotechnology Advances*, *42*, 107582. https://doi.org/10.1016/j.biotechadv.2020.107582

Raza, Z. A., Abid, S., & Banat, I. M. (2018). Polyhydroxyalkanoates: Characteristics, production, recent developments and applications. *International Biodeterioration & Biodegradation*, *126*, 45–56. https://doi.org/10.1016/j.ibiod.2017.10.001

Salvachúa D, Prieto A, López-Abelairas M, Lu-Chau T, Martínez ÁT, Martínez MJ. Fungal pretreatment: An alternative in second-generation ethanol from wheat straw. *Bioresource Technology*. 2011;102(16):7500-7506.

Sharma, P., Gaur, V. K., Kim, S.-H., & Pandey, A. (2020). Microbial strategies for bio-transforming food waste into resources. *Bioresource Technology*, *299*, 122580. https://doi.org/10.1016/j.biortech.2019.122580

Silva, J. P., Sousa, S., Rodrigues, J., Antunes, H., Porter, J. J., Gonçalves, I., & Ferreira-Dias, S. (2004). Adsorption of acid orange 7 dye in aqueous solutions by spent brewery grains. *Separation and Purification Technology*, *40*, 309–315. https://doi.org/10.1016/j.seppur.2004.03.010

Singh B. Myceliophthora thermophila syn. Sporotrichum thermophile: a thermophilic mould of biotechnological potential. *Critical Reviews in Biotechnology*. 2016;36(1):59-69.

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Sluiter, A. (2008). Determination of Total Solids in Biomass and Total Dissolved Solids in Liquid Process Samples: Laboratory Analytical Procedure (LAP). Technical Report.

Spinelli, S., Conte, A., Lecce, L., Padalino, L., Alessandro, M., & Nobile, D. (2016). The Journal of Supercritical Fluids Supercritical carbon dioxide extraction of brewer's spent grain. *J. of Supercritical Fluids*, *107*, 69–74. https://doi.org/10.1016/j.supflu.2015.08.017

Tiquia-Arashiro SM, Grube M, eds. *Fungi in Extreme Environments: Ecological Role and Biotechnological Significance*. Springer International Publishing; 2019.

Tolessa, E., & Shunka Tolessa, E. (2018). Importance, Nutrient content and Factors Affecting Nutrient Content of Potato. In *American Journal of Food* (Vol. 3, Issue 3). http://www.aascit.org/journal/ajfnh

Valentino, F., Morgan-Sagastume, F., Campanari, S., Villano, M., Werker, A., & Majone, M. (2017). Carbon recovery from wastewater through bioconversion into biodegradable polymers. *New Biotechnology*, *37*, 9–23. https://doi.org/10.1016/j.nbt.2016.05.007

Wainaina, S., Lukitawesa, Kumar Awasthi, M., & Taherzadeh, M. J. (2019). Bioengineering of anaerobic digestion for volatile fatty acids, hydrogen or methane production: A critical review. *Bioengineered*, *10*(1), 437–458. https://doi.org/10.1080/21655979.2019.1673937

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