



Valorizing brewer's spent grain: A sequential pathway of supercritical extraction, hydrolysis, and fermentation

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ABSTRACT

The possibility of brewer's spent grain exploitation through a sequence of supercritical fluid extraction, acid hydrolysis, and fermentation was considered. All steps have been optimized within an interval of operative conditions, using specific designs of the experiments, and modeled through the Response Surface Methodology. The supercritical fluid extraction was optimized in the pressure range 20–40 MPa and temperature 40–100 °C. A fractional factorial design was applied considering the sulfuric (0.065–0.37 M) and nitric (0.01–0.5 M) acid concentration, and the liquid–solid ratio (8–12 w/w %) as independent factors for the hydrolysis step. The fermentation process of pre-treated BSG was also optimized using the Box-Behnken design with temperature (25–37 °C), inoculum volume (5–15 v/v %), and pH (4.5–6.5) as investigated factors. At optimal conditions, the overall process led to an ethanol yield of 82 % evaluated with respect to the theoretical one. Moreover, all the supercritical fluid extracts were richer in phenolic compounds than the ones obtained by the traditional Soxhlet method.

1. Introduction

The (bio)waste valorization, as principle of the biocascade approach, represents a possible route for food industries in reaching sustainability goals concurrently to the possibility of expanding their market and competitiveness (Madeddu et al., 2021; Goswami et al., 2022). This approach has the double impact of reducing the amount of wastes that would otherwise be landfilled or burned through their revalorization extracting valuable compounds and/or by conversion into energy and chemicals (Xiong et al., 2019; Kannah et al., 2020). Among all food industries, the brewing process was targeted for its relevant waste production and for its widespread diffusion all over the world. It has been reported that in 2021 the world beer production reached about 1.86 billion hectolitres, while in the EU the production was quantified at 33.1 billion litres (Statista, 2022; Eurostat, 2021). According to the estimate of 20 kg of brewer's spent grain (BSG) obtained for 100 L of beer produced (Mitri et al., 2022), the predicted amount of BSG annually available peaks at almost 40 million tons. Of this amount about 70 % is used as animal feed, 10 % for biogas production, and 20 % is landfilled

(Bianco et al., 2020). Even though its composition is strongly influenced by the type and quality of cereals used, malting and mashing conditions, and the harvest time, it generally consists of 15–27 % of lignin, 12–25 % cellulose, 19–42 % hemicellulose, 14–31 % protein on a dry weight basis. In addition, it also contains vitamins, minerals and phenolic compounds among which ferulic, coumaric, syringic, vanillic and p-hydroxybenzoic are noteworthy (Lisci et al., 2022). Recently, there has been growing interest in exploiting BSG potential as a component in the human diet, as a fermentation substrate for the production of bio-alcohols, as an extraction matrix for value-added compounds, and as a carrier for energy production through aerobic digestion and thermo-chemical conversion processes (Naibaho and Korzeniowska, 2021; Fernandez-Delgado et al., 2019; Connolly et al., 2021; Carlini et al., 2021).

The recovery of valuable compounds is traditionally performed through solid-liquid extraction. Meneses et al. (Meneses et al., 2013) tested different solvents like methanol, ethanol, acetone, hexane, ethyl acetate, water, and mixtures of methanol-water, ethanol-water, and acetone-water for extracting antioxidant phenolic compounds from BSG

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at a temperature close to the solvent boiling point. A similar approach was followed by Ikram et al. (Ikram et al., 2020) extracting antioxidant phenolic compounds using acetone solutions, while Junttila (Junttila, 2022) targeted the extraction of proteins using alkaline water in near subcritical conditions. Alternative extraction methods are also emerging as greener alternatives to traditional solid–liquid methods. Among all, supercritical fluid extraction (SFE) has shown promising results in the recovery of added-value compounds from different biowastes (Errico et al., 2023). For example, the method was used to recover a triacylglycerols-rich oil from spent coffee grounds (Coelho et al., 2020), as well as to valorize grape seeds (Passos et al., 2010), pomegranate peels (Kupnik et al., 2022), and avocado seeds (Páramos et al., 2020). Few works reported the application of BSG supercritical extraction as a stand-alone method for the recovery of bioactive compounds. Fernandez et al. (Fernández et al., 2008) optimized the extraction conditions based on the moisture content targeting tocopherols as a key class of components. The optimization of the extraction condition was also considered by Spinelli et al. (Spinelli et al., 2016) targeting the total polyphenolic compounds, the total flavonoids, and the antioxidant activity as selection criteria. A different approach was chosen by Alonso-Riaño et al. (Alonso-Riaño et al., 2022), while using the same criteria as Spinelli et al. (Spinelli et al., 2016), combined the SFE with a subsequent enzymatic hydrolysis step to examine the glucose concentration. The implementation of a sequential combination of unit operations opens the possibility of a further valorization of the BSG matrix by conversion of sugars to platform molecules or biofuels. As recently reported by Periyasami et al. (Periyasamy et al., 2023), the rising price of petroleum and the negative environmental impact of fossil fuels is a reason for the exploitation of lignocellulosic materials in the production of biofuels. This is also confirmed the expansion of the global biofuels request in 2022 driven by policies designed to reduce GHG emissions (Iea, 2022).

Bioethanol is still regarded as a valuable renewable energy source and the utilization of waste biomass is a way to reduce its price. The key to accomplish this task is to identify the best production conditions, especially for the pre-treatment stage which is necessary to break down the polymer's structure and increase the yield of fermentable sugars. A comprehensive review on the conversion of lignocellulosic biomass to bioethanol can be found in Periyasami et al. (Periyasamy et al., 2023), while specific studies on the impact of different pretreatments for BSG conversion have been reported by Ravindran et al. (Ravindran et al., 2018). To date, acid hydrolysis and enzymatic hydrolysis appear as the most applied methods. In particular, acid pre-treatment emerges as the most established technique used in the saccharification of lignocellulosic materials and, in addition, diluted sulfuric acid is widely used due to its low cost (Świątek et al., 2020; Arslan et al., 2012; Lenihan et al., 2010). Optimal pretreatment conditions aimed at achieving high carbohydrate recovery from the enzymatic saccharification of BSG were investigated by Rojas-Chamorro et al. (Rojas-Chamorro et al., 2018). Co-fermentation strategies for improving the yield of bioethanol by a simultaneous conversion of both hexose and pentose sugars have been studied by White et al. (White et al., 2008) and more recently by Rojas-Chamorro et al. (Rojas-Chamorro et al., 2020).

Despite all the contributions in studying possible ways of valorizing BSG, they are mainly based on single processes resulting in limited exploitation of this waste. The combination of different processes, and the diversification of the products obtainable could be the key to making BSG processing industrially more attractive. In this work, a three-step process was experimentally explored combining SFE for the recovery of a high-value fraction followed by bioethanol production through the fermentation of the acid hydrolysate obtained from the solid residue of the supercritical extraction. Supercritical carbon dioxide (scCO₂) was chosen as SFE solvent since it is environmentally friendly, largely available, non-flammable, and it avoids the problem of the elimination of traces of organic solvents for products aimed at the food, nutraceutical, or pharmaceutical market. Dilute acids are used to hydrolyse cellulose into monosaccharides and *Saccharomyces cerevisiae* was selected

for the fermentation step since it is the most established yeast already used on large scale. Design of experiment (DoE) approaches and surface methodology (RSM) have been used for identifying the optimal conditions for the SFE, the hydrolysis, and the fermentation. The SFE performance was evaluated based on the extraction yield and the total phenolic content while the glucose recovery and the ethanol yield were used as optimization variable for the hydrolysis, and fermentation, respectively. Different from most experimental works using the RSM, where the ability of the regression model to fit the experimental data is quantified by the coefficient of determination (R²) or the adjusted R² (Rojas-Chamorro et al., 2018; Iadecola et al., 2022; Gutierrez-Barrutia et al., 2022), in this work cross-validation was also used to assess the prediction ability of the models.

2. Materials and methods

2.1. Chemicals and reagents

Sulfuric (98 %) and nitric (65 %) acids used for acid hydrolysis were purchased from Merck (Darmstadt, Germany). 1 M NaOH (Reagecon, Co. Clare, Ireland) was used for the neutralization of hydrolysates. For the preparation of yeast growth inoculum, yeast extract (VWR Chemicals LLC, USA), glucose (Merck, France), anti-foam (Sigma-Aldrich, China), and sodium chloride (VWR International, Belgium) were used. Standard solutions for HPLC analysis were prepared using 70 % ethanol (Merck, Darmstadt, Germany), L-(+)-Arabinose (≥99 %, Sigma-Aldrich) and D-(+)-Xylose (≥99 %, Sigma-Aldrich). Other compounds used are: CaCl₂·2H₂O (Sigma-Aldrich, Croatia); (NH₄)₂SO₄ (Sigma-Aldrich, USA); Na₂CO₃ (Sigma-Aldrich, Germany); MgSO₄ (Sigma-Aldrich, Germany); KH₂PO₄ (Sigma-Aldrich, USA); gallic acid monohydrate (Sigma-Aldrich, China); ethanol (Supelco Germany, absolute), ethyl acetate (VWR Poland, 99.8 %), n-hexane (VWR France, 97 %), acetone (VWR France, 100 %), Folin-Ciocalteu reagent (Reagecon, Ireland); carbon dioxide (AGA Denmark, 99.8 %).

2.2. BSG, yeast, and inoculum

The BSG was kindly supplied by Vestfyen brewery (Assens, Denmark). After the collection, it was stored at −4 °C. As needed, fresh BSG was oven dried at 60 °C for 24 h in a humidity-controlled oven (Memmert HCP 108). Dried samples were vacuum sealed in moisture barrier bags and refrigerated at 2 °C until use. The average particle size was estimated in duplicates using Tyler series sieves. BSG retained in each sieve was: mesh 8 (35 % ± 0.4 %), mesh 10 (26.3 % ± 0.5 %), mesh 12 (16.9 % ± 0.3 %), mesh 20 (16.6 % ± 0.1 %), mesh 28 (3.0 % ± 0.1 %), mesh 35 (0.8 % ± 0.1 %), and a leftover of 0.9 % ± 0.1 %.

A commercial strain of *Saccharomyces cerevisiae* (De Danske Gaerfabrikker A/S, Malteserkors) was grown overnight at 30 °C in an orbital shaker at 150 rpm using a growth medium containing the following compounds: 20 g L⁻¹ glucose; 6 g L⁻¹ yeast extract; 0.23 g L⁻¹ CaCl₂·2H₂O; 4 g L⁻¹ (NH₄)₂SO₄; 1 g L⁻¹ MgSO₄; 1.5 g L⁻¹ KH₂PO₄ (Sivakesava et al., 2001). The medium was stored at 4 °C until use.

2.3. Soxhlet extraction

BSG extractions were performed using distilled water, EtOH, ethyl acetate, n-hexane, and acetone according to the AOAC method (Latimer, 2023). Sequential extractions were performed in the order of water – EtOH and EtOH – water. For each extraction, 7 g (±0.7 g) of dried BSG and 180 mL of solvent were used. The extraction time was set to 6 h. The condenser temperature was set at 3 °C and the heating plate temperature ranged between 140 °C and 200 °C depending on the solvent used. Extracts were dried in a rotary vacuum evaporator (BUCHI, Rotovapor R-210) and placed in an air circulation oven (Memmert HCP 108) at 80 °C until constant weight. Extraction yields for all extraction methods were calculated as the ratio between the mass of the dried extract and the

Table 1
DoE for the SFE.

Run	Pressure [MPa]	Temperature [°C]	Density [kg m ⁻³]
1	20	40	834
2	20	70	659
3	20	100	481
4	30	40	910
5	30	70	788
6	30	100	662
7	40	40	956
8	40	70	857
9	40	100	757

mass of the dried sample.

2.4. Supercritical CO₂ extraction

Details on the supercritical extraction setup used were reported by Montesantos et al. (Montesantos et al., 2019). In brief, the supercritical extractions were performed in semicontinuous mode, consisting of a batch of BSG charged into the high-pressure extractor and extracted by a continuous flow of scCO₂. The extractor is cylindrical and equipped with a basket insert of 178 cm³ (i.d. 2.9 cm; height 27 cm). The basket was entirely filled with the BSG before each run. The BSG was pressed during the charge and about 35 g ended up being loaded into the basket in each run. Therefore, the extraction bed was characterized by a length-to-diameter ratio of 9.3 and the apparent density of the BSG was close to 200 g/L. After the charge of the BSG, the extractor is pressurized with scCO₂, and heated to the desired operating conditions. Then, the extractor is kept closed at the target pressure and temperature for 30 min. A drum-type gas-meter (Ritter TG 3/5, max flow: 6 L min⁻¹; min flow: 0.1 L min⁻¹; accuracy 0.5 % across full flow rate range), equipped with a gas thermometer, is used to measure the CO₂ flow rate, which was kept in the range 3.3 to 3.5 L min⁻¹ (at 20–21 °C and atmospheric pressure). All extractions were continued until a solvent-to-feed ratio (S/F) of 30 g_{CO₂}/g_{BSG} was reached. The extracts were collected in a dry trap, consisting of a vial inserted in a gas washing bottle. The extracts consisted of a low-volatility and viscous liquid with a dark yellow/amber color. The collection vial was changed as the S/F increased of 5 g_{CO₂}/g_{BSG}, thus giving six samples for each run. Each sample was weighed to track the extraction yield over time. All extracts of each run were mixed to guarantee an appropriate sample quantity for the analyses.

2.5. Dilute acid hydrolysis

The experiments were carried out by autoclaving (SHP Laboklav ECO 135 M, SHP Steriltechnik AG, Germany) the dried BSG obtained after supercritical fluid extraction with 200 mL of acid solutions in 250 mL flasks, at 120 °C for 20 min. The liquid phase was separated from the solid part by centrifugation at 4500 rpm and 25 °C for 20 min (Avanti J-HC, Beckman Coulter, United States), and then vacuum filtered and characterized in terms of its sugar composition.

2.6. Fermentation of BSG hydrolysate

In order to evaluate the time required by the yeast to consume the substrate, preliminary fermentation experiments were conducted in duplicate in 1 L fermentation flasks working in anaerobic conditions at 30 °C in an orbital shaker (IKA KS 4000 ic control, Germany) at 150 rpm for 24 h. The working volume of acid hydrolysate for both flasks was approximately 170 mL. Before being inoculated, the pH was brought to 5.5 using 0.1 M NaOH until a pH of 5.5 was reached, then 10 % (v/v) of inoculum was added. The run was conducted for 24 h, during which samples were taken at different times, filtered and stored for sugars and ethanol determination. Subsequent experiments for process

optimization were performed in 500 mL fermentation flasks using approximately 200 mL of hydrolysate. Each of them was carried out for 9 h under different experimental conditions in terms of temperature, inoculum volume, and pH.

2.7. Sugars and ethanol quantification

Liquid samples from acid hydrolysis and fermentation steps were centrifuged and filtered through 0.20 μm membranes (Sartorius, Germany), and then analyzed by HPLC. The HPLC system (Ultimate 3000, Thermo Fisher) was equipped with a refractive index detector (Dionex Softron GmbH, Germany), and a Phenomenex Rezex RHM-Monosaccharide H+ (8 %) analysis column working at 79 °C with ultrapure water as mobile phase (0.6 mL/min). Glucose, xylose, arabinose, and ethanol were identified using standard solutions and quantified through calibration curves. The limits of the curve were [0.1–50 g L⁻¹] for glucose, [1–10 g L⁻¹] for xylose, [1–10 g L⁻¹] for arabinose, and [0.15–81 g L⁻¹] for ethanol, with a R_{Glucose}² = 0.9993, R_{Xylose}² = 0.9917, R_{Arabinose}² = 0.9997, and R_{Ethanol}² = 0.9995.

2.8. Total phenolic compounds determination

Supercritical carbon dioxide extracts were diluted in 25 mL absolute ethanol and analyzed for the total phenolic content (TPC) according to the Folin-Ciocalteu method described by Singleton et al. (Singleton et al., 1999) adapted for a 96-well microplate reader. Briefly, 156 μL of distillate water were added to the sample wells, followed by 4 μL of Folin-Ciocalteu reagent, and 4 μL of sample. The wells were shaken for 1 min before adding 30 of 20 % weight solution of Na₂CO₃ and shaken for a further 60 min before reading the absorbance at 760 nm (Thermo Scientific, Varioskan LUX). Results were reported as mg of gallic acid equivalent per gram of extract (mg GA g_{ext}⁻¹) by using a GA calibration curve [20–400 g/L], R_{GA}² = 0.997. The analyses were performed in triplicates.

2.9. Data analysis

The process was optimized by means of the RSM using a polynomial equation to fit the experimental data. When possible, the RSM was complemented by a 5-fold cross-validation technique to assess the model's predictive performance (Anguita et al., 2012). Additionally, the optimal conditions predicted by the model conditions were experimentally validated. The response variables were the extraction yield for the supercritical fluid extraction at S/F = 30 g_{CO₂}/g_{BSG} and the TPC, the glucose recovery for acid hydrolysis (g_{glucose}/100 g_{BSG}), the ethanol yield relative to the theoretical value for the fermentation. The coefficient of determination R² was employed to assess the model's accuracy in calibration over all the experimental data. Stepwise backward elimination was used to find the significant parameters to fit the experimental data (Hocking, 1976). The significance level was set as 5 % for all the hypothesis tests carried out. The coefficient of determination in cross-validation R_{Cv}² was used to evaluate the model's performance in predicting the experimental response values for the data points excluded at each step of the 5-fold cross-validation (Refaeilzadeh et al., 2009). Minitab® 21 Statistical Software (Minitab Inc., State College, PA, 2021) was used for data analysis.

3. Design of experiments

The use of waste biomass for the recovery of valuable products is strongly dependent on the raw material. For this reason, it is crucial to properly design the experimental campaign so that the description and understanding of the variation in the data are simplified, and the impact of each considered variable is statistically evident. Different procedures for an optimal DoE have been used for the SFE, hydrolysis, and

Table 2
DoE for sulfuric and nitric acid pre-treatment.

run	H ₂ SO ₄				HNO ₃			
	C _{acid} [M]		L/S ratio [g _{liq} /g _{BSG} ⁻¹]		C _{acid} [M]		L/S ratio [g _{liq} /g _{BSG} ⁻¹]	
	real	coded	real	coded	real	coded	real	coded
1	0.065	-1	12	-1	0.01	-1	12	-1
2	0.37	1	12	-1	0.5	1	12	-1
3	0.37	1	8	1	0.5	1	8	1
4	0.18	0	10	0	0.1	0	10	0

Table 3
DoE for the BSG fermentation process.

run	Temperature (°C)		Inoculum volume (% v/v)		pH	
	real	coded	real	coded	real	coded
	1	25	-1	5	-1	5.5
2	25	-1	15	1	5.5	0
3	37	1	5	-1	5.5	0
4	37	1	15	1	5.5	0
5	25	-1	10	0	4.5	-1
6	25	-1	10	0	6.5	1
7	37	1	10	0	4.5	-1
8	37	1	10	0	6.5	1
9	30	0	5	-1	4.5	-1
10	30	0	5	-1	6.5	1
11	30	0	15	1	4.5	-1
12	30	0	15	1	6.5	1
13	30	0	10	0	5.5	0
14	30	0	10	0	5.5	0

Table 4
Extraction yield achieved for solvents with different polarity.

Solvent	Yield [(g _{extract} /100g _{BSG})]
H ₂ O + EtOH	9.47±0.12 + 9.75±0.02
EtOH + H ₂ O	10.21±0.21 + 9.90±0.08
Acetone	6.05±0.04
Ethyl acetate	6.31±0.01
n-hexane	5.36±0.20

fermentation of the BSG and they are described in the following.

3.1. Supercritical fluid extraction

The DoE for the SFE was defined based on the work of Alonso-Riaño et al. (Alonso-Riaño et al., 2022) where 20, 30, and 40 MPa were used. Differently from the studies of Spinelli et al. (Spinelli et al., 2016), and Alonso-Riaño et al. (Alonso-Riaño et al., 2022), the temperature range was extended up to 100 °C. It is widely reported that polyphenols thermal degradation is observed at 80 °C for conventional extraction methods. However, this value can be stretched to 150–200 °C for pressurized liquid extraction (Antony and Farid, 2022).

Table 1 summarizes the conditions explored in this study corresponding to a 3² full factorial design. For each pair pressure-temperature, the CO₂ density is also reported (Lemmon et al., 2019) since the solubility increases with it (Marcus, 2018). In particular, this effect is rather large for phenols, with association numbers in the Chrastil equation observed in the range 3 to 4 for several 1-ring phenols and for typical sCO₂ density ranges for industrial applications (Montesantos and Maschietti, 2020). Therefore, this value is expected to be a strong indicator of the sCO₂ power as a solvent for phenols. As noted previously, the extractions were performed until a value of S/F equal to 30 g_{CO₂}/g_{BSG} was reached. This value was selected by preliminary tests, which showed that at the investigated conditions most of the extract is obtained for S/F values up to 20 g_{CO₂}/g_{BSG} and no additional extract is

obtained for S/F >30 g_{CO₂}/g_{BSG}. Therefore, the extraction yield (g_{extract}/100 g_{BSG}) reported here can be assumed as the maximum yield attainable with scCO₂ at the conditions under investigation. Run 5 and 7 were performed in duplicate.

3.2. Acid hydrolysis

A half-fractional factorial design DoE was used to investigate the effects of the process conditions on the acid pre-treatment performance (Gunst and Mason, 2009). The acid concentration and liquid-solid (L/S) ratio were selected as design factors, while the temperature and process time were kept constant. The L/S ratio was defined according to Mussatto and Roberto (Mussatto and Roberto, 2005), while the concentrations of sulfuric and nitric acid were defined according to the works of Beldman et al. (Beldman et al., 1987) and Ascencio et al. (Ascencio et al., 2020) respectively. In particular, the following ranges were investigated: [8–12 g_{liq}/g_{BSG}⁻¹] for the L/S ratio, [0.065–0.37 M] and [0.01–0.5 M] as the concentration range for H₂SO₄ and HNO₃, respectively. Table 2 shows the design of experiments obtained as a function of the two independent variables investigated.

One noteworthy aspect is that the combination between the mildest treatment in terms of acid concentration and the highest L/S ratio was removed. The reason for this choice is related to an expected low glucose yield under such conditions as reported by Pinheiro et al. (Pinheiro et al., 2019) for enzymatic hydrolysis. The experimental domain was further simplified by removing the central level (denoted as 0) and extreme levels (i.e., -1 and +1, in coded notation) combinations. Hence, only combinations of extreme levels and the central point of the design were considered, resulting in 8 experimental conditions. The experimental glucose recovery was calculated as g of glucose per 100 g of dried BSG. Each condition was carried out in duplicate.

The efficiency of the pre-treatment performed with the two acids was assessed by determining the glucose recovery defined as g of glucose per 100 g of BSG present in the liquor.

3.3. Fermentation of the hydrolysate

The Box-Behnken experimental design was applied to identify the optimal conditions for the fermentation process. Temperature (25, 30, 37 °C), inoculum volume (5, 10, 15 % v/v) and pH (4.5, 5.5, 6.5) are the three key process variables chosen as factors for the experimental design. A total of 14 experimental combinations were obtained, including two points in the center of the experimental domain. The uncoded and coded values of these factors are shown in Table 3.

4. Results and discussion

This section presents the results obtained for the valorization of BSG through the extraction of added-value compounds and the conversion of residues to ethanol. Soxhlet extractions are discussed first, since the results in terms of yield, are commonly used as a basis for comparison for the SFE method.

4.1. Soxhlet extractions

The results of the Soxhlet extractions are presented in Table 4. Torres-Mayanga et al. (Torres-Mayanga et al., 2019) obtained extractives yields of 5.70±0.3 and 13.6±0.20 g_{extract}/100g_{BSG} in water and ethanol respectively. The authors referred to NREL (Sluiter et al., 2008) as the experimental procedure, however, the extraction time was not specified and a direct comparison with the values obtained in this study could be considered speculative. Paz et al. (Paz et al., 2019) observed an extraction yield in ethanol of 14.39±0.10 after 24 h. This value appears aligned with the value obtained in this work considering the difference in the extraction time. Extraction yields in acetone and n-hexane are in agreement with the results reported by Herbst et al. (Herbst et al., 2021)

Table 5
Extraction yield and TPC achieved through SFE.

Run	Pressure [MPa]	Temperature [°C]	Yield [$\text{g}_{\text{extract}}/100\text{g}_{\text{BSG}}$]	TPC [$\text{mgGA}_{\text{eq}}\text{g}_{\text{extract}}^{-1}$]
1	20	40	3.02	6.73 ± 0.47
2	20	70	2.58	5.73 ± 0.20
3	20	100	1.16	4.95 ± 0.30
4	30	40	3.32	7.19 ± 0.67
5	30	70	4.51 ± 0.06	4.45 ± 0.45
6	30	100	4.65	5.62 ± 0.57
7	40	40	3.37 ± 0.25	8.23 ± 0.12
8	40	70	4.94	5.21 ± 0.28
9	40	100	5.30	5.63 ± 0.45

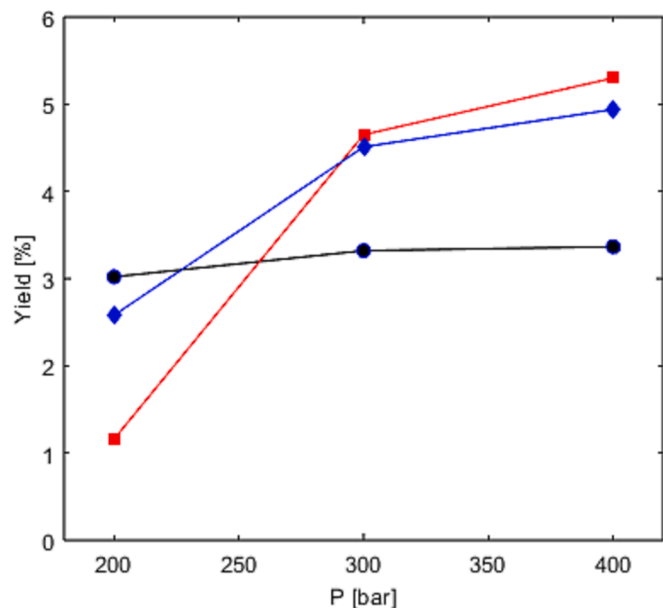


Fig. 1. Extraction yield as function of pressure. (●) 40 °C, (◆) 70 °C, (■) 100 °C.

and Qin et al. (Qin et al., 2018). Regarding the sequential extractions where water was followed by ethanol and vice versa, the two-sample *t*-test was employed to assess the significance of the difference in their extraction yield. The significance level was set equal to 5 %. Based on the hypothesis test, the extraction yields for water and ethanol were significantly different ($p = 0.026$ and $p = 0.046$, respectively). Similar

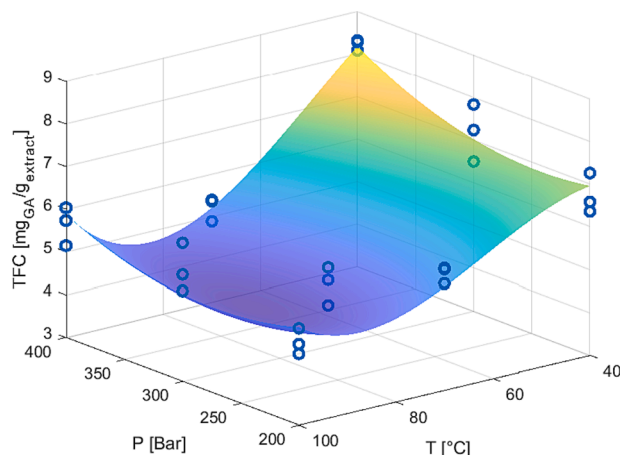
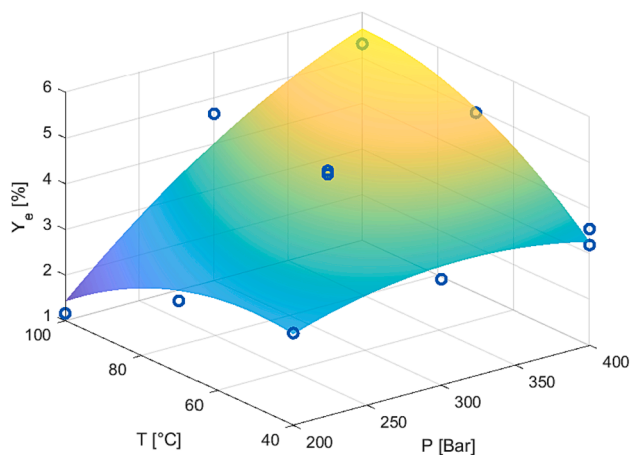


Fig. 2. Extraction yield (left) and TPC (right) as a function of temperature and pressure. Experimental points are marked in blue. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

results were observed considering the total yield ($p = 0.011$). Unlike the results reported by Herbst et al. (Herbst et al., 2021), the extraction sequence with the highest yield was the ethanol-water. A similar behavior was observed by Spigno et al. (Spigno et al., 2007) for the extraction of phenolic components from grape seeds. In fact, with respect to the extraction yield, mixtures of ethanol and water were more efficient than the corresponding single-solvent system.

4.2. Supercritical fluid extraction

The extraction yield and TPC for the P, T pairs considered in the DoE are reported in Table 5.

The variation of the extraction yield with the pressure is reported in Fig. 1. For each value of the temperature considered it is possible to observe an increase in the extraction yield when the pressure is increased with a less pronounced effect at the lowest temperature. This can be justified by the increase in the scCO_2 density with the pressure and constant temperature. At the lowest pressure examined (20 MPa) a decrease of the yield by increasing the temperature is observed. This trend is reversed at the other pressures considered. As can be seen in Fig. 1 the cross-over pressure lies somewhere in the interval 200–300 bar. The existence of the cross-over pressure is due to contrasting effects. High temperatures increase the vapor pressure of the component extracted, and the mass transfer parameters, but the scCO_2 density is lowered. Using 200 bar and 40 °C as reference, the density of scCO_2 decreases by 21 % between 40 °C and 70 °C and by 42 % between 40 °C and 100 °C. At 300 bar, the decrease is 13 and 27 % respectively, while at 400 bar it is 10 % and 21 %. At 200 bar, the effect of the decrease of the solvent density on the efficiency of the extraction prevails over the effect of the increase of the vapor pressure of the solutes and of the mass transfer parameters, thus leading to the behavior on the yield reported in Table 5 and Fig. 1.

The maximum yield obtained in this study is equal to $5.30 \text{ g}_{\text{extract}}/100 \text{ g}_{\text{BSG}}$ at 40 MPa and 100 °C, comparable to the value of $5.7 \text{ g}_{\text{extract}}/100 \text{ g}_{\text{BSG}}$ obtained by Alonso-Riaño et al. (Alonso-Riaño et al., 2022) for 40 MPa and 80 °C and to the value of 5.49 reported by Kitryte et al. (Kitryte et al., 2015) at 35 MPa and 40 °C.

The highest values for the TPC were observed for the lowest temperature of the DoE independently of the pressure considered. This result differs from the TPC distribution obtained by Alonso-Riaño et al. (Alonso-Riaño et al., 2022) which reported a value of $16.6 \text{ mg GA/g}_{\text{extract}}$ at 400 bar and 80 °C which is double of the optimal value of $8.23 \text{ mg GA/g}_{\text{extract}}$ obtained at 400 bar and 40 °C. However, a decrease in the TPC with the extraction temperature was observed for different plant extracts as reported by Tyśkiewicz et al. (Tyśkiewicz et al., 2018).

Of particular interest is the comparison with the Soxhlet extracts

Table 6

Glucose recovery for sulfuric acid and nitric acid pre-treatment. Results reported as average value \pm standard deviation.

Run	L/S ratio [g _{liq} /g _{BSG}]	H ₂ SO ₄		HNO ₃	
		C _{acid} [M]	GR [g _{glucose} /100 g _{BSG}]	C _{acid} [M]	GR [g _{glucose} /100 g _{BSG}]
1	12	0.065	10.10 \pm 0.19	0.01	2.53 \pm 0.04
2	12	0.37	14.71 \pm 0.322	0.5	11.14 \pm 0.18
3	8	0.37	15.98 \pm 0.076	0.5	15.19 \pm 0.26
4	10	0.18	15.14 \pm 0.37	0.1	12.04 \pm 0.015

with the highest yield. For the water and ethanol extracts the TPC was 1.41 and 0.47 mg GA/g_{extract} respectively, far lower than all the extracts obtained by SFE. The TPC for the extract in n-hexane resulted below the lowest value of the calibration curve. If the comparison is performed in terms of total phenolic yield (mg GA/100 g_{BSG}), for all the scCO₂ extraction conditions, except 20 MPa 70 °C and 100 °C, the yield resulted higher than 20 mg GA/100 g_{BSG} with the maximum of 29.8 mg GA/100 g_{BSG} observed at 40 MPa and 100 °C. For the Soxhlet extraction

with water the total phenolic yield was 13.4 mg GA/100 g_{BSG} proving that a high extraction yield does not equal a high recovery of valuable compounds. Lastly it should be noted that differences in the TPC between different studies could be related not only to differences in the BSG composition but also to the method used for its determination.

The extraction yield and the TPC were modeled using the RSM. In particular for the estimated extraction yield (\hat{Y}_e) and the estimated TPC (TPC_e), pressure (P, in bar) and temperature (T in °C) are the factors employed to model the process. An empirical second-order polynomial model is assumed, with coefficient selected using a forward stepwise procedure. The significance level was set to 5 %. The estimated extraction yield was obtained using the two-degree polynomial equation reported in Eq. (1).

$$\hat{Y}_e = 0.0165 P - 0.000657 T^2 - 0.000047 P^2 + 0.000329 T^* P R^2 = 0.9428 \quad (1)$$

In the case of the TPC a second-order polynomial was not sufficient to properly fit the experimental data. Hence, the third-order polynomial equation reported in Eq. (2) was proposed.

$$TPC_e = 0.4890 T - 0.007229 T^2 + 0.000054 P^2 - 0.000902 T^* P + 0.000032 T^3 + 0.000006 T^2 * P R^2 = 0.8492 \quad (2)$$

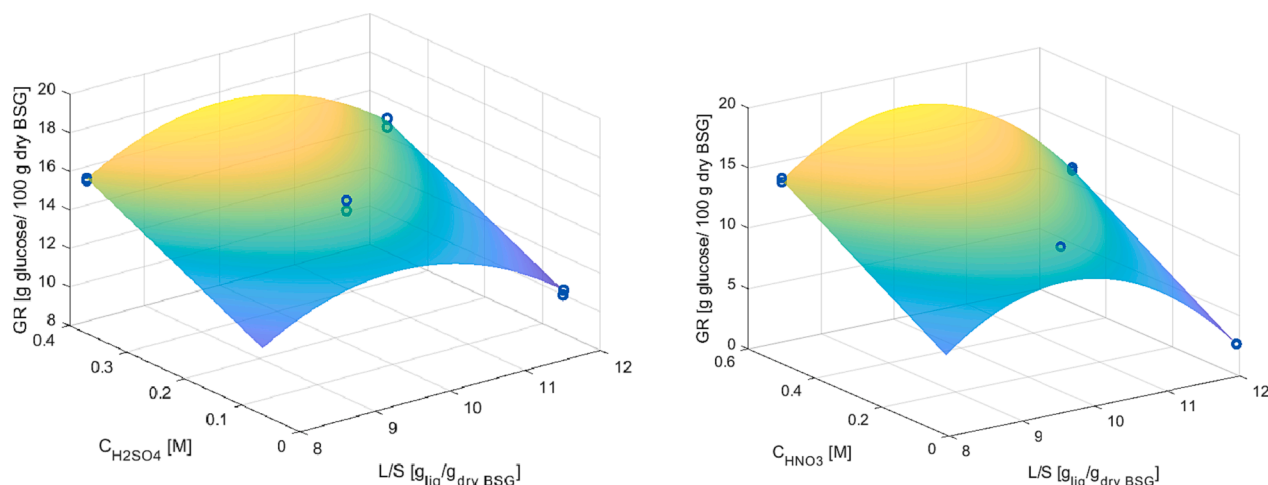


Fig. 3. Glucose recovery as a function of H₂SO₄ (left) and HNO₃ (right) concentration and L/S ratio. Experimental points are marked in blue. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

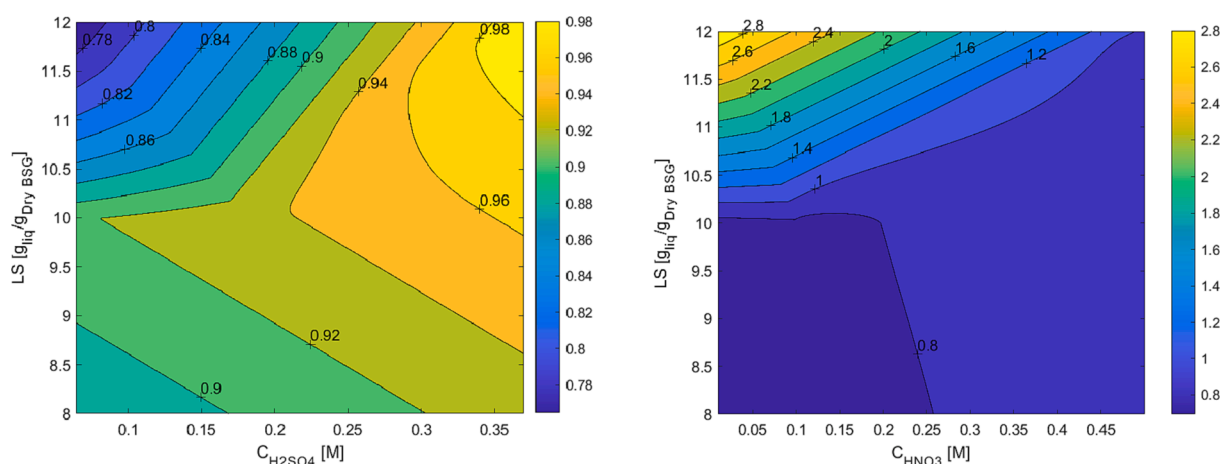


Fig. 4. Contour plots depicting the selectivity obtained through the acid hydrolysis step using sulfuric acid (left) and nitric acid (right).

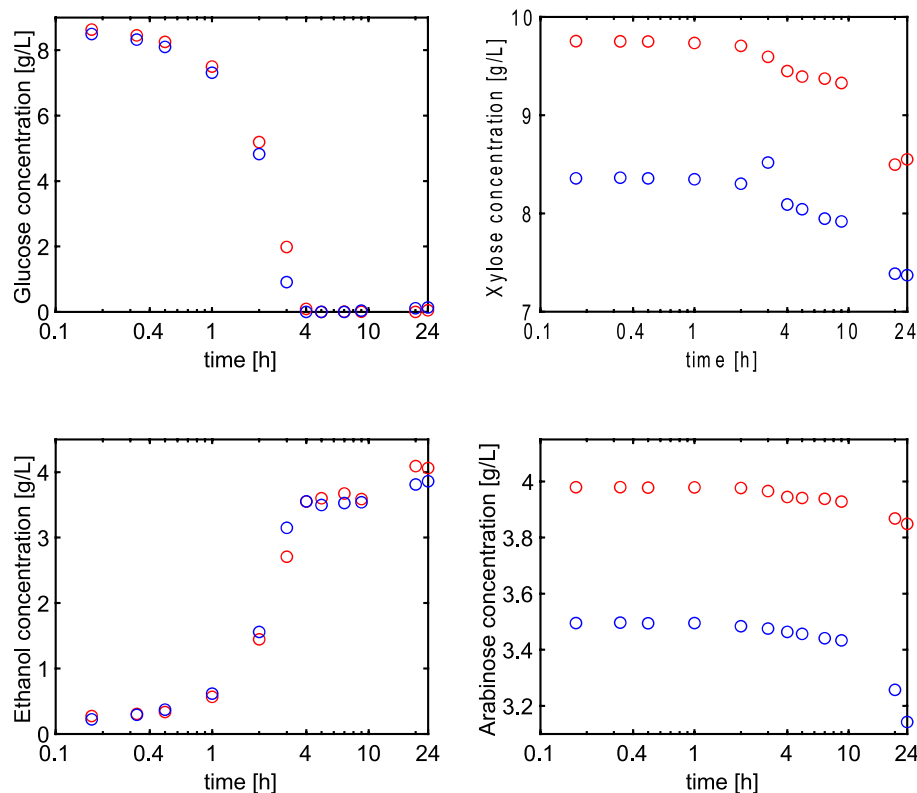


Fig. 5. Glucose (top left panel), xylose (top right panel) and arabinose (bottom right panel) consumption and ethanol (bottom left panel) production during the fermentation process for run 1 (red circle) and run 2 (blue circle). Time is in logarithmic scale. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 7
Results of DoE for the fermentation.

Run	T [°C]	iv [% v/v]	pH	Y_{EtOH}^* [%]
1	37	15	5.5	78
2	30	10	5.5	83
3	30	10	5.5	79
4	37	10	4.5	78
5	30	15	4.5	80
6	30	15	6.5	79
7	37	5	5.5	68
8	30	5	4.5	71
9	25	15	5.5	80
10	30	5	6.5	66
11	37	10	6.5	81
12	25	10	4.5	86
13	25	10	6.5	73
14	25	5	5.5	59

As it can be seen, the significant terms reported in Eqs. (1) and (2) indicate that both T and P have a significant impact on the extraction yield and TPC, individually and through their interaction.

The performance of the models performance was evaluated using a 5-fold-cross-validation approach, and the coefficient of determination in cross-validation resulted 0.7880 and 0.7500 for the extraction yield and TPC respectively.

The three-dimensional plot for extraction yield and TPC is shown in Fig. 2. For the extraction yield reported in the left panel, the complex shape of the plot highlights the strong interaction between T and P. The magnitude of the linear pressure term suggests its primary contribution to the extraction yield. Furthermore, the positive coefficient for the linear interaction term between T and P indicates that an increase in temperature amplifies the impact of P on \hat{Y}_e and vice-versa. The second-

degree term has a smaller effect on \hat{Y}_e but yet still present.

For the TPC reported in the right panel of Fig. 2, the interaction effect between T and P is predominant in describing the experimental data. Indeed, the linear interaction term resulted to be negative, indicating that an increase in temperature reduces the impact of pressure on the TPC, as it can be deduced from a visual inspection of the surface plot. A slight change of surface concavity along temperature is observed when the pressure increases due to the positive linear-quadratic interaction term.

Further analysis of the variation of the mass of extract with respect to the ratio solvent to feed and the variation of the extraction yield with the scCO₂ density and are reported in the Supplementary Material (Figs. S1-S10).

4.3. Acid hydrolysis

The residual BSG corresponding to the highest extraction and total phenolic yield obtained through SFE was further considered for the acid hydrolysis step. The efficiency of acid pre-treatment was evaluated by considering the glucose release via the hydrolysis of cellulose and hemicellulose for different acid concentrations and L/S ratio. The glucose recovery has been evaluated as grams of glucose on biomass basis ($g_{\text{glucose}}/100 g_{\text{BSG}}$). Glucose recovery mean values and standard deviations of each experimental run are reported in Table 6 for H₂SO₄ and HNO₃.

A second-order polynomial was employed to model the relationship between the molar acid concentration and L/S ratio ($g_{\text{liq}}/g_{\text{BSG}}$) with the glucose recovery. From the experimental data reported in Table 6, regression Eqs. (3) and (4) have been derived.

$$GR_{H_2SO_4} = -51.910 + 12.980 LS + 15.103 C_{H_2SO_4} - 0.665 LS^2 R^2 = 0.9932 \quad (3)$$

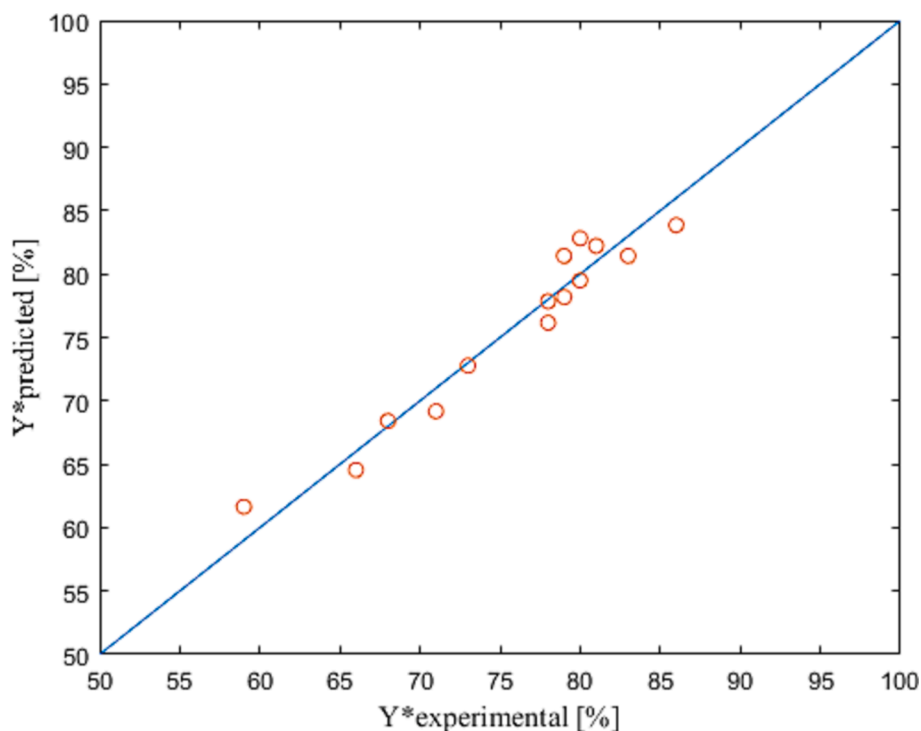


Fig. 6. Comparison between experimental values and values predicted from the model.

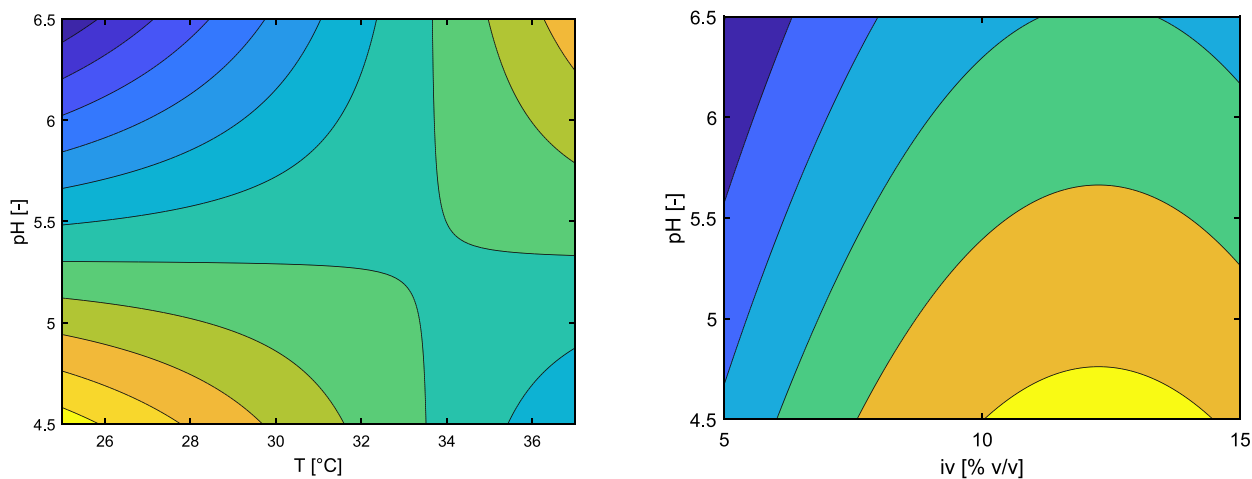


Fig. 7. Contour plot of the yield response. Isolevel curves with respect to T and pH, iv set to 12.25 (left panel), isolevel curves with respect to pH and iv, T is set to 25 °C (right panel).

$$GR_{HNO_3} = -127.13 + 28.497 LS + 17.562 C_{HNO_3} - 1.476 LS^2 R^2 = 0.9994 \quad (4)$$

The response surfaces to estimate the glucose recovery over acid concentration and L/S ratio are shown in Fig. 3.

It is possible to observe that both regression models well describe the experimental data obtained and, in both cases, the glucose recovery exhibits a positive linear trend with the acid concentration and a quadratic relationship with the L/S ratio.

The optimal value of glucose recovery predicted by the model corresponds to 18.12 g_{glucose}/100 g BSG and 19.24 g_{glucose}/100 g BSG for sulfuric acid and nitric acid, respectively. This condition corresponds to a L/S ratio of 10 g_{liq}/g_{BSG} and the maximum acid concentration investigated. Since the difference between the optimal glucose recovery between the two acids is small, the monomeric sugar selectivity was

analyzed. It is relevant to note that the yeast used to ferment the hydrolysates prefers glucose to other monomeric sugars in solution. The selectivity was analyzed by defining the ratio between glucose and xylose concentrations for each experimental run. The results, reported as contour plots, are shown in Fig. 4.

Based on the selectivity analysis sulfuric acid resulted as the optimal choice. Then, the optimal conditions obtained for the sulfuric acid were experimentally validated obtaining a glucose recovery equal to 18.3 ± 0.1 g_{glucose}/100 g BSG, which is quite close to the value of 18.12 g_{glucose}/100 g BSG calculated by Eq. (3).

4.4. Fermentation of optimized hydrolysate

Two different types of experiments were carried out for studying the fermentation of the hydrolysate. The first set of experiments was aimed

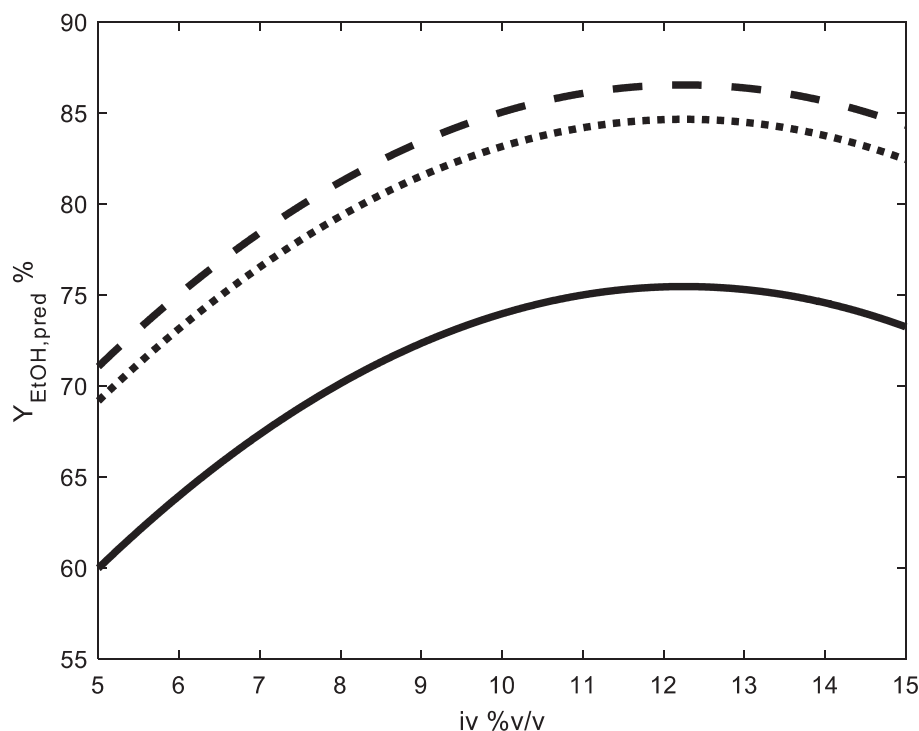


Fig. 8. Ethanol yield vs inoculum volume at $pH = 4.5$, $T = 25^{\circ}C$ (dashed), at $pH = 6.5$, $T = 37^{\circ}C$ (point), at $pH = 6.5$, $T = 25^{\circ}C$ (line).

at evaluating the time required for obtaining the maximum ethanol production from the fermentable sugars, which means that it corresponds to monitor the process until steady state is detected for ethanol concentration. The profile of sugars and ethanol concentration during the fermentation is reported in Fig. 5, using a logarithmic scale for the time coordinate in order to facilitate the visualization of the changes before and after glucose depletion.

It is worth noting that both fermentation runs had been conducted under the same conditions, but the amount of pentoses at the beginning of the experiments was slightly different. This depends on the previous hydrolysis step which did not lead to the same production of arabinose and xylose. This aspect was not further investigated because the yeast used in this work is highly selective toward glucose. Indeed, the yeast completely consumed the glucose after almost 4 h (Fig. 5, top left panel), whereas arabinose and xylose were slightly consumed, and the consumption rate increased only after the total depletion of glucose. The evidence that ethanol is also obtained from pentoses is given by the increase in ethanol concentration after 9 h, which is almost 13 % in run 1 and circa 10 % in run 2. Furthermore, the final ethanol concentration is higher when starting from a reactant mixture containing a higher amount of pentoses. It is worth noting that arabinose and xylose had not been completely transformed to ethanol, because the amount theoretically obtainable from the consumption of pentoses is higher than the quantity produced in the time interval 9–24 h. Anyway, the production of ethanol from xylose and arabinose is quite scarce in presence of *Saccharomyces Cerevisiae*'s strain, confirming previous results (White et al., 2008). For this reason, experiments aimed at finding the best condition for glucose fermentation were chosen equal to 9 h.

4.4.1. Optimal condition for ethanol production

Table 7 reports the results of the experimental campaign for the fermentation in terms of the calculated ethanol yield (Y_{EtOH}^*) with respect to the theoretical one ($Y_{EtOH,theoretical} = 51\%$) obtained varying the independent factors: temperature (in $^{\circ}C$), inoculum volume (in % v/v), and pH .

The second-order regression model is represented by Eq. (5) and exhibits a determination coefficient $R^2 = 0.883$ and a R_{Cv}^2 equal to

0.684. Model adequacy is confirmed in Fig. 6, which shows that experimental measurements and computational predictions are in good agreement.

$$Y_{EtOH}^* = 152.7 - 3.42T + 7.21iv - 21.64pH - 0.2942iv^2 + 0.644T \cdot pH \quad (5)$$

As can be deduced from the coefficients of Eq. (5), temperature and pH exerts a negative influence on ethanol yield even though these influences are slightly attenuated by the positive interaction between both factors. The influence of inoculum volume is positive for low values, but it adversely affects ethanol production when it is greater than 24.51 % v/v. The conditions required for the obtainment of the maximum yield can be predicted by the model Eq. (5) and they are $pH = 4.5$, $T = 25^{\circ}C$ and $iv = 12.25\%v/v$, that is at the lowest extreme of the investigate pH and temperature range. The effect of the investigated parameters on the ethanol yield can be better appreciated by observing contour plots evaluated at the iv equal to 12.25 % (Fig. 7, left panel) and T equal to $25^{\circ}C$ (Fig. 7, right panel). It is interesting to note that the production of ethanol is favored by low pH and T or high pH and T . Considering the ethanol yield versus the inoculum volume (Fig. 8) at different values of temperature and pH , it evident that there is a small difference in ethanol production when considering one extreme of the range ($pH = 4.5$, $T = 25^{\circ}C$) and the other ($pH = 6.5$, $T = 37^{\circ}C$). On the other hand, there is a significant decrease of the yield if at high pH , the temperature is decreased, obtaining a minimum when $T = 25^{\circ}C$. Such results are extremely important from the operative point of view, because they evidenced that the interaction between temperature and pH is not trivial and that the effects of an eventually not controlled pH could be partially compensated by a proper temperature variation.

The validation of the regression model has been also carried out by performing a further experiment at intermediate conditions i.e., $T = 33.4^{\circ}C$, $pH = 6$ and $iv = 12\%v/v$. The measured yield is $Y_{EtOH,exp} = 81.03\%$ that is quite close to the model prediction $Y_{EtOH,pred} = 81.84\%$, confirming the model effectiveness. It is worth noting that the obtained results are in agreement with previous investigation on valorization of BSG (Rojas-Chamorro et al., 2020), where a theoretical yield equal to 78 % had been obtained when using a simultaneous saccharification and

fermentation in presence of microorganism able to convert xylose to ethanol.

5. Conclusions

Brewer's spent grain represents a valuable raw material with several areas of application. In this work, its possible use in a biorefinery context is considered through a 3-step process encompassing supercritical fluid extraction, diluted acid hydrolysis, and fermentation for bioethanol production. For each step, the operative conditions have been optimized and the models obtained through the response methodology method were experimentally verified.

The optimal extraction yield for the SFE was 5.3 % w/w at 40 MPa and 100 °C for an extraction time of about 3 h. This extract has a TPC of 5.63 mg GA/g_{extract} corresponding to a total phenolic yield of 29.9 mg GA/100 g_{BSG}. However, the highest TPC was measured at 40 MPa and 40 °C when the extraction yield is 36.5 % lower than the optimal value and the total phenolic yield 7.4 % lower than the value associated with the maximum yield.

Supercritical CO₂ BSG extracts are known to be rich in linoleic acid with relevant amounts of biocompounds providing antioxidant properties (Alonso-Riaño et al., 2022) however the choice to maximize the extraction yield or the TPC requires a deeper chemical characterization and an economic analysis. The hydrolysis step was optimized to maximise the amount of glucose released and the selectivity of the process toward the production of this sugar. The optimal conditions for the hydrolysis resulted in an H₂SO₄ concentration of 0.37 M with a L/S ratio of 10 g_{liq}/g_{BSG} corresponding to a glucose recovery of 18.3 g/100 g_{BSG}. Lastly, the fermentation step was performed at 25 °C, 4.5 as pH, and an inoculum volume of 12.25 % v/v. The ethanol yield resulted equal to 81.03 % evaluated with respect to the theoretical one.

The approach proposed in this work contributes to define a sequence of operations for biomass valorization leaving open the research and definition of different process sequences for a multiproduct portfolio. The experimental data provided in this study aim to contribute developing techno-economic evaluations of different possible scenarios for BSG valorization and their scale-up at pilot/industrial scale.

CRedit authorship contribution statement

Silvia Lisci: Writing – original draft, Methodology, Formal analysis, Data curation, Investigation, Validation. **Stefania Tronci:** Writing – original draft, Writing – review & editing, Methodology, Data curation, Conceptualization. **Massimiliano Grosso:** Writing – original draft, Writing – review & editing, Methodology, Data curation, Conceptualization. **Ron Hajrizaj:** Investigation, Data curation, Methodology. **Leonardo Sibono:** Writing – review & editing, Methodology, Formal analysis, Data curation, Investigation, Validation. **Henrik Karring:** Methodology, Conceptualization. **Alexandr Gerganov:** Investigation, Data curation. **Marco Maschietti:** Writing – review & editing, Methodology, Data curation. **Massimiliano Errico:** Writing – original draft, Writing – review & editing, Methodology, Formal analysis, Conceptualization, Project administration, Funding acquisition.

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

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ces.2023.119620>.

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