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ENZYMATIC EXTRACTION OF FERULIC ACID FROM BREWER'S SPENT GRAIN: EFFECT OF PHYSICAL PRETREATMENTS AND OPTIMIZATION USING DESIGN OF EXPERIMENTS

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Abstract

Brewers' grains (BSG), the main waste product of the beer industry, are widely available and rich in fiber and bioactive compounds. Ferulic acid (FA) is the main phenolic compound in BSG, it has antioxidant and antibacterial properties that make it a valuable product for cosmetic, food and pharmaceutical industries. Alkaline hydrolysis is the common technology for extracting FA from biomass, this technology needs to be substituted as it is non-selective and requires harsh conditions making purification more complicated and expensive. This research work presents enzymatic hydrolysis as a green alternative for FA extraction. Five commercial enzymes were screened and compared for their hydrolytic efficiency to release FA. Prior to the enzymatic hydrolysis, autoclave was selected as the most effective pretreatment. Depol 740L was selected as the desired enzyme based on its high feruloyl esterase activity (0.4 U/mL). The enzymatic extraction parameters (pH, temperature and enzyme dosage) were optimized using response surface methodology. The highest FA recovery, 1.06 ± 0.01 mg/g dry weight (43.13 % in comparison with the alkaline hydrolysis), could be achieved at pH 5.27; temperature 60 °C and 1.72% enzyme. The optimal extraction conditions were found to be sufficient within 22 hours, as determined by a kinetic study.

Keywords: Brewer's spent grain, enzymatic extraction, ferulic acid, optimization, phenolic compounds, biomass valorization

1. Introduction

The beer industry has seen significant growth in recent years. According to the BarthHaas Report 2020/2021 (Carlos Inaraja, 2021), beer production worldwide was 1.86 billion hectoliters in 2021, almost 71 million hectoliters (+4 percent) higher than it was in 2020. As a result, brewer's spent grains (BSG), the main waste product of the beer production process, are widely available and can potentially be utilized as a feedstock in biorefineries. BSG contain high levels of polysaccharides, proteins, and phenolic compounds, including ferulic acid (FA), which makes 0.18% up to 0.32% of its dry weight (Bartolomé et al., 1997; Bartolomé & Gómez-Cordovés, 1999). FA has a range of beneficial properties, including antioxidant, antimicrobial, and anti-inflammatory effects, making it useful in various industries such as food, health, cosmetics, and packaging (Kumar & Pruthi, 2014).

Ferulic acid is often esterified to polysaccharides or etherified to lignin in brewers' grains, and the breaking of these bonds is required for its release. There are several methods that have been reported for extracting FA from BSG, including alkaline hydrolysis (Sibhatu et al., 2021a), supercritical carbon dioxide (Sun et al., 2006), solvent extraction, and ultrasound-assisted extraction (Bonifácio-Lopes et al., 2020). Alkaline hydrolysis is the common method due to its high efficiency in releasing FA from BSG. However, it requires harsh conditions such as high temperature, high pH, and long reaction times, which can have negative impacts on the environment and may cause degradation of the FA and an increase in salt content of the extract. In addition, these harsh conditions can lead to the solubilization of other components, including proteins and lignin, which can complicate downstream processing and increase the time and cost demands. As a result, there is a need for the development of more environmentally friendly and selective methods for extracting FA from BSG.

Enzymatic hydrolysis has been investigated as a potential sustainable method for extracting ferulic acid (FA) from brewers' grains (Dupoirion et al., 2017; Valério et al., 2021). This method offers several advantages, including higher extraction yields, higher quality extracts, environmentally friendly extraction, scalability of the process, and high selectivity as it can specifically target bonds holding FA without damaging other valuable compounds. Feruloyl esterase (FAE) is the main enzyme involved in releasing FA from lignocellulosic biomass. However, due to the high price of FAE and the need for sugar degrading enzymes to solubilize polysaccharides, commercial enzyme cocktails are often used. These cocktails, which contain both FAE and sugar degrading enzymes, are considered as enzymatic pretreatment, and they are effective at releasing bound compounds and increasing overall yield (Guido & Moreira, 2017). However, enzymatic pretreatment alone is not sufficient to extract FA due to the presence of the lignin barrier and high cellulose crystallinity (Beldman et al., 1987). To overcome this issue, the application of physical pretreatments, which break down the plant matrix and increase

enzyme accessibility, can facilitate the transfer of compounds from the intracellular to extracellular medium.

There has been limited research conducted on the impact of combining physical pretreatments with commercial enzymatic cocktails for the recovery of FA from BSG (Guido & Moreira, 2017; Macias-Garbett et al., 2021). In a recent review published in 2021, Marcias-Garbett et al. emphasized the significance of pretreatment and extraction steps in determining the overall yield of phenolic compound extraction from BSG (Macias-Garbett et al., 2021). Mechanical procedures such as maceration, grinding, and homogenization are commonly employed (Niemi et al., 2012), along with chemical pretreatments such as autohydrolysis (Pinheiro et al., 2019), alkaline hydrolysis (Ideia et al., 2020), and enzymatic treatments (Xiros et al., 2009). Pulsed electric fields have also been proposed as a solvent-free and environmentally friendly pretreatment method (Kumari et al., 2019). These steps aim to enhance the recovery yields of phenolic and other bioactive compounds from BSG. In their 2017 review, Guido and Moreira emphasized the extraction of phenolic compounds from BSG using various methods (Guido & Moreira, 2017). Conventional solvent-based extraction techniques (SLE) involving organic solvents (Meneses et al., 2013), enzymatic reactions without physical pretreatment (Panagiotou et al., 2006), and alkaline reactions (Mussatto et al., 2007a) are commonly employed. Among these methods, SLE utilizing 60% (v/v) acetone under specific conditions has shown efficiency, but it is associated with disadvantages such as high solvent usage, time consumption, operating costs, and environmental concerns. To the best of our knowledge, no studies have investigated the effects of combining physical pretreatments, such as ultrasound (using water as the solvent), milling and autoclave, with a novel commercial enzymatic cocktail.

This research aims to investigate the effect of combining physical and enzymatic pretreatments on the release of FA from BSG. Three physical pretreatments (milling, autoclave and ultrasonic) and five commercial enzymatic cocktails were studied. Furthermore, several physicochemical parameters such as temperature, pH, enzyme dosage and incubation duration were optimized using response surface methodology in order to maximize the efficiency of the enzymatic hydrolysis.

2. Material and method

2.1. Raw material

Brewer's spent grains were kindly provided by the company la Brasserie La Christelloise (Witry les Reims, France) and stored in the dark at -20 °C. The malt composition of the beer was 90% French Pilsen malt and 10% of caramel malt 50. Prior to usage, the wet BSG was dried at 60 °C for 24 h.

2.2. Extraction procedure of FA from BSG

In this study, our primary focus was on extracting FA as it is the predominant hydroxycinnamic acid identified in BSG. Although other hydroxycinnamic acids such as caffeic acid, p-coumaric acid, and cinnamic acid are present in trace amounts (UHPLC chromatogram in Figure S1, supplementary data file), we did not specifically investigate or analyze them in this research. Our main objective was to explore the extraction of FA from BSG. The process of extracting FA from BSG is shown in Figure 1.

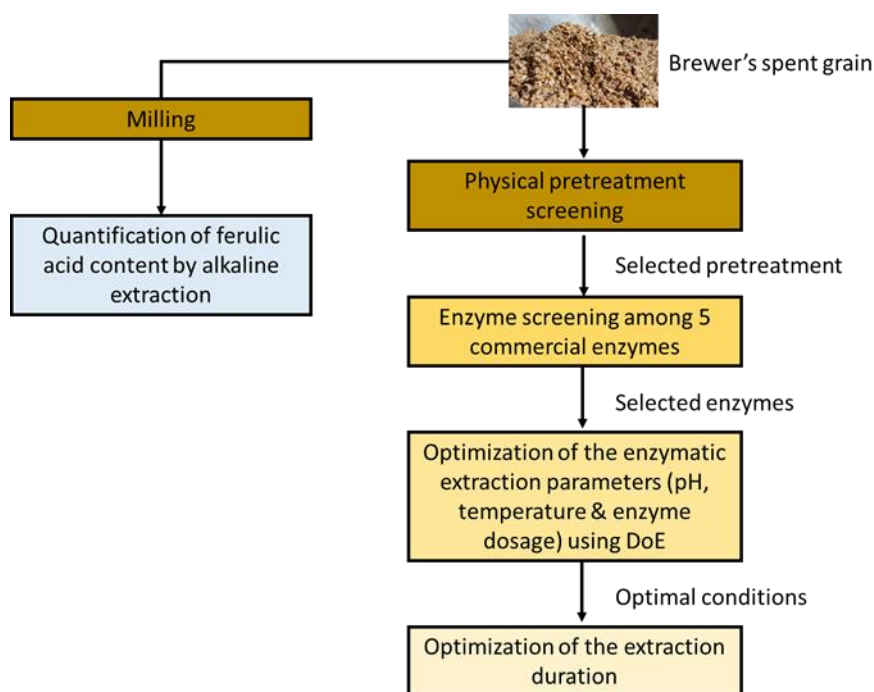


Figure 1. Extraction procedure of FA from BSG

The first step involves determining the amount of FA present through an exhaustive alkaline extraction. The second step involves testing different physical pretreatment methods, including milling, autoclaving, and ultrasound. These pretreatments will be carried out using pure feruloyl esterase (FAE). After identifying the most effective pretreatment method, we compared the performance of five commercial enzymes, focusing on their FAE activity. The best enzyme was selected and the conditions for enzymatic extraction (pH, temperature, enzyme dosage and time) were optimized.

2.3. Alkaline extraction

Alkaline extraction was carried out in order to determine the amount of FA present through an exhaustive alkaline extraction. The extraction was conducted by mixing 2.5 g of milled BSG with 50 mL of 2 M NaOH in an Erlenmeyer flask, which was heated at 50 °C and agitated at 400 rpm for 3 hours

using a heating plate. The mixture was then centrifuged at 4500 g for 10 minutes, and samples for HPLC analysis were taken from the resulting hydrolysate. The solid residue was subjected to two additional alkaline extractions (exhaustive extractions) to maximize the extraction of FA. The total amount of esterified ferulic acid in the BSG was calculated using equation 1

$$\text{Total Esterified FA (mg/g BSG)} = \frac{\text{FA alkaline process (mg)}}{\text{g of dry BSG}} \quad (\text{Eq 1})$$

2.4. Pretreatments screening

Autoclave (AC) and ultrasound (US) treatments were applied as pretreatment methods for milled and unmilled BSG, in addition to milled and unmilled BSG being tested without any pretreatment (Figure 2).

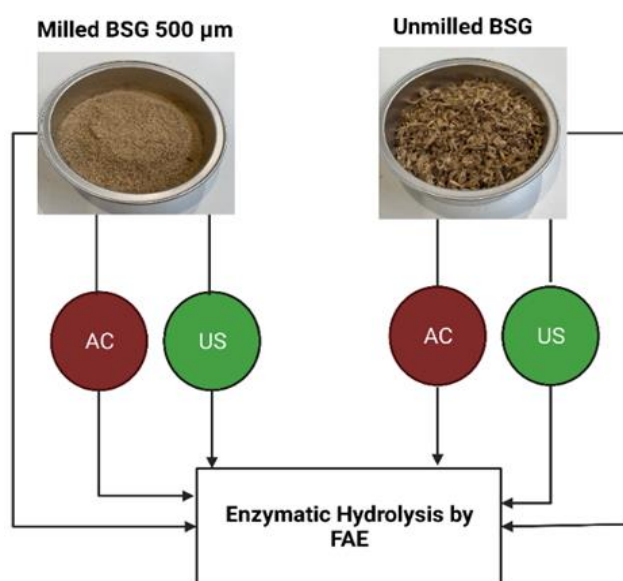


Figure 2. Schematic representation of the pretreatment screening procedure (AC: Autoclave, US: Ultrasound)

After the pretreatments, the extraction of FA will be carried out using pure FAE to eliminate the influence of cellulase and xylanase enzymes present in the commercial cocktails. The control is milled BSG without pretreatment and enzyme.

2.4.1. Ultrasound pretreatment

The extraction was carried out in a 100 mL Erlenmeyer flask using an ultrasound probe (UP400St, 400W, 24 kHz) and generator (Hielscher, Germany). 2.5 g of BSG was mixed with 50 mL of sodium acetate buffer (pH 7) and subjected to ultrasound pretreatment for 20 minutes. The final temperature of the liquor was 92°C. The samples were then cooled to room temperature and 10 µL of

pure feruloyl esterase (8 U/mL), produced by rumen microorganisms, was added to each sample. The samples were incubated at 40 °C and agitated at 130 rpm for 24 hours.

2.4.2. Autoclave pretreatment

2.5 g of BSG were placed in 250 mL Erlenmeyer flasks and 7 mL were used to moisten the biomass. Then, AC was run at 121 °C, 1 bar for 20 min. After the AC is over, and the mixture is cooled down, 43 mL was added to reach a total buffer of 50 mL. 10 µL of pure 8 U/mL feruloyl esterase was added to each sample and incubated at 40 °C, 130 rpm for 24 h.

The enzymatic reactions were stopped by heating at 90 °C for 10 min in a water bath. The samples were then centrifuged at 4500 g for 10 min, purified, and prepared for HPLC analysis.

2.4.3. Milling pretreatment

A laboratory blender (WARING COMMERCIAL, UK) was used for milling pretreatment prior to sieving the BSG. The fraction of BSG with particles smaller than 500 µm was then used for extractions.

2.5. Commercial enzymatic cocktail screening

Five commercial enzymes, described in Table 1, were tested for the extraction of FA. Depending on their FAE activity and sugar hydrolysis activities, one commercial enzyme was selected for further optimization.

Table 1. Commercial enzymes used in the screening stage

Enzyme	Microorganism	Supplier
Ultraflo® XL	<i>Humicola insolens</i>	Novozymes
Depol 740L	<i>Humicola insolens</i>	Biocatalysts
Depol 686L	<i>Trichoderma sp.</i>	Biocatalysts
Deltazyme VR AC-100	<i>Trichoderma longibrachiatum</i>	WeissBioTech
Viscozyme L	<i>Aspergillus sp</i>	Novozymes

2.6. Enzyme activity assays

2.6.1. Determination of ferulic acid esterase activity

FAE activity was measured based on the protocol defined by Novozymes (Novozymes A/S, Denmark) with some modifications. The activity of the enzyme was determined by measuring released ferulic acid in an assay using ethyl ferulate as substrate. 1.46 mg/mL of methyl ferulate was prepared as substrate with citrate phosphate buffer (pH 5.8) assisted by 2% (w/w) of polysorbate-80 to facilitate the solubilization since methyl ferulate is poorly soluble in water. Enzymes were diluted

with 1% (w/v) sodium chloride. 5 mL of substrate was incubated at 50 °C in a glass tube for 5 min. Then, 200 µL of diluted sample is added and well vortexed, incubated at 50 °C for 60 min. After 60 min, the reaction is stopped by adding 1 mL of oxalic acid, mixed well and samples are prepared for HPLC analysis. In the blank, 200 µL of 1% sodium chloride was added instead of enzyme samples. The FAE activity is calculated in U/mL using the equation 2:

$$FAE (U/mL) = \frac{(Area_{Sample} - Area_{blank}) \times 6.2 \times V_{total}}{\frac{\alpha \times 0.2 \times M_{enzyme}}{60}} \quad \text{Eq (2)}$$

Where, $Area_{Sample}$ area of ferulic acid peak in samples, $Area_{blank}$ area of ferulic acid peak in blank sample, α slope of calibration curve (area units/µmol), V_{total} total volume of diluted sample (mL), (6.2) total volume of assay (mL), (0.2) volume of sample in assay (mL), M_{enzyme} is the amount of enzyme (mL) and (60) is to convert from 1 h to 1 min.

2.6.2. Determination of cellulase and xylanase activities

Cellulase and xylanase activities of the five commercial enzymes were measured by K-CellG3 and XylX6 toolkits from Megazyme (Megzyme, Ireland). The results were expressed as unit (U) per mL of enzyme. One unit of cellulase activity is defined as the amount of enzyme needed to produce 1 µM of 2-chloro-4-nitrophenol from CellG3 in 1 min at pH 6.0 and 40 °C. One unit (U) of xylanase activity is defined as the amount of enzyme required to release 1 µM of *p*-nitrophenol from the XylX6 in 1 min at pH 6 and 40 °C.

2.7. Optimization of the enzymatic hydrolysis

After selecting the appropriate pretreatment and the best commercial enzymatic cocktail (Depol 740L) based on its enzyme activities, especially its FAE activity, response surface methodology (RSM) accompanied by Box-Behnken experimental design was employed to optimize the enzymatic hydrolysis and to evaluate the effects of the operating conditions of the enzymatic extraction on the release of FA. Box-Behnken design and the results were processed using MODDE software (Version 12.0.1, Sartorius, Sweden). The design comprises 15 trials from three independent variables at three levels of the system (Table 2).

Table 2. Variables and levels used in the Box-Behnken design

Variable code	Factor level		
	Temperature (°C)	pH	Enzyme dosage (w/w _{dry BSG})
-1	40	4	1
0	50	5	2
1	60	6	3

Maximum and minimum treatment levels were chosen according to the optimum condition ranges of the enzymes given by the manufacturer and to the literature reports (Bautista-Expósito et al., 2020). The response optimized is the FA recovery (%) which is calculated based on equation 3:

$$FA\ recovery\ (\%) = \frac{mg\ of\ FA\ from\ the\ enzymatic\ extraction}{mg\ FA\ from\ the\ alkaline\ extraction} \times 100 \quad Eq\ (3)$$

To assess the stability of the process and its intrinsic variability, three repetitions of the central point were performed in a randomized manner to minimize the impact of unpredictable variations in the observed outcomes resulting from external factors, and a second-degree polynomial equation (Equation 4) was used to fit the experimental data.

$$Y = \beta_0 + \sum_{i=1}^k \beta_i X_i + \sum_{i=1}^k \beta_{ii} X_{ii} + \sum_{i=1}^k \sum_{j=i+1}^k \beta_{ij} X_{ij} + \varepsilon \quad Eq\ (4)$$

Y represents the response; β_0 is the model intercept; β_i , β_{ii} and β_{ij} denote the coefficients of the linear, quadratic, and interactive effect, respectively; X_i , and X_j are the coded independent variables (dimensionless variables that range from -1 to 1).; k is the number of the tested factors ($k = 3$), and ε is the difference between the experimental and the predicted values.

2.8. Ultra-High-Performance Liquid Chromatography

The FA content in the enzymatic hydrolysates were determined by ultra-high-performance liquid chromatography (UHPLC) (Thermo scientific, Ultimate 3000) equipped with a quadratic pump, an autosampler, a column furnace, a diode array detector and an accucore aQ C18 column (100 x 3 mm, Thermo scientific). The injection volume was 2.5 μ L, the oven temperature was 48 °C and the flow rate was 0.8 mL/min. The elution method was a 10 min gradient with acetonitrile (A) and formic acid 0.1% (B) as mobile phases: 0 min: 2% of A – 98% of B, 3 min: 10% of A – 90% of B, 8 min: 30% A – 70% of B and 9 min: 2% of A – 98% of B. Chromatograms were acquired at 320 nm and processed with Chromeleon software (version 6.8). FA calibration curve with 6 points was made using commercial FA from Carbosynth (Carbosynth Ltd, United Kingdom). Samples were acidified with HCl and diluted (1:1) in acetonitrile and filtered using regenerated cellulose 0.2 μ m filters.

2.9. Statistical Analysis

All experiments were performed at least twice to ensure accuracy. The error bars displayed in each figure represent the standard deviations. A Tukey test was applied to identify significant differences among groups. The statistical analysis was conducted using R software (R Core Team, 2013).

3. Results and discussion

3.1. Quantification of ferulic acid in BSG

The alkaline hydrolysis can disrupt the structure of lignin/phenolic-carbohydrate complexes, resulting in the production of phenolics, soluble sugars, insoluble lignin, and carbohydrates (Buranov & Mazza, 2009). In order to ensure thorough extraction (exhaustive extraction) and prevent the use of harsh conditions, the FA was extracted serially, a total of three times from the same biomass. The results are displayed in Figure 4.

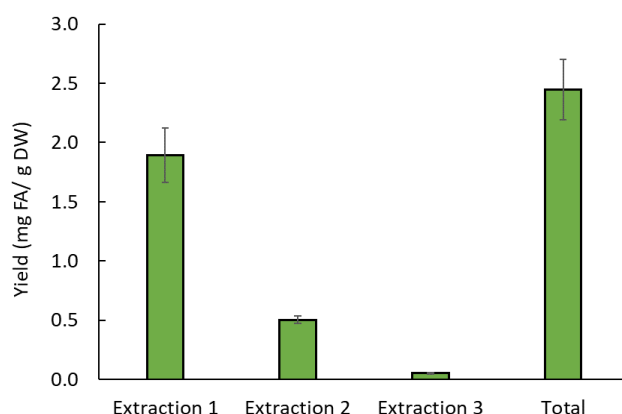


Figure 4. Total esterified ferulic acid by alkaline extraction from BSG.

Extraction conditions: milled BSG: 2.5 g; solvent: 50 mL of 2 M NaOH; temperature: 50 °C; agitation: 400 rpm; duration: 3 h.

As shown in Figure 4, the first extraction releases 1.89 ± 0.23 mg FA/g_{DW}, followed by 0.50 ± 0.03 mg FA/g_{DW} from the second extraction. The third round of alkaline hydrolysis released only 0.05 ± 0.01 mg FA/g_{DW} which indicates the complete extraction of FA from the solid residue. Thus, the total esterified FA from BSG was calculated to be 2.45 ± 0.25 mg FA/g_{DW} (0.245%w of dry weigh of BSG).

Recently, Sibhatu et al., 2021b achieved a yield of 46.17 mg of ferulic acid per 100 g of Ethiopian BSG using 2 (w/v) % NaOH concentration, at 120 °C. In another study by Codina-Torrella et al., 2021, the most favorable results were obtained using 1.45 (w/v)% NaOH at a temperature of 80 °C, with a yield of ferulic acid ranging from 156.53 to 290.89 mg per 100 g of material. In a previous study conducted by Mussatto et al., 2007, they found that 9.65 mg of ferulic acid and 9.22 mg of p-coumaric acid were obtained per gram of solubilized lignin during an alkaline extraction of BSG using a 2 (w/v)% NaOH concentration at 120 °C for 90 minutes.

3.2. Screening of physical pretreatments

Autoclave and ultrasound pretreatments with milled and non-milled BSG were tested and combined with enzymatic extraction using pure feruloyl esterase. The efficiency of the pretreatment technologies was evaluated based on the FA recovery from the total esterified FA. Figure 5 expresses the results from the different pretreatments conditions used in this study.

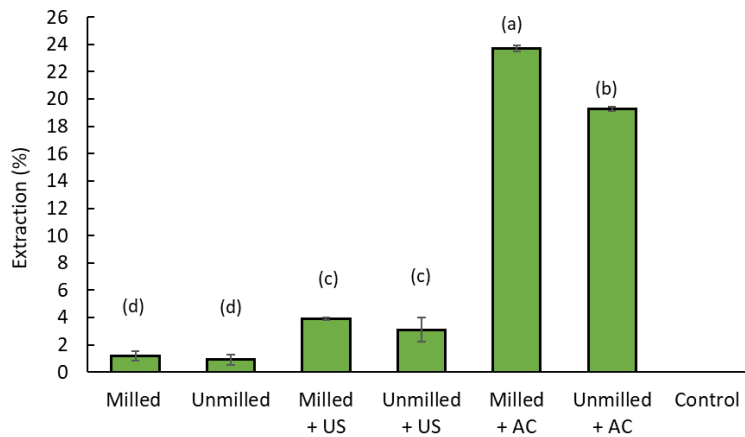


Figure 5. Ferulic acid extraction (%) by different pretreatments. Extraction conditions: Enzyme: 10 μ L of pure feruloyl esterase (8 U/ml); Incubation temperature: 40 °C; Agitation: 130 rpm; Incubation time: 24h. Control corresponds to Unmilled BSG without enzyme. Bars with distinct labels represent significant differences ($p < 0.05$) as determined by the Tukey test.

The maximum recovery was obtained from a pretreatment using AC with the following percentages $23.7 \pm 0.2\%$ for milled BSG and $19.3 \pm 0.1\%$ for non-milled BSG. On the other hand, US pretreatment resulted in a lower recovery ($3.9 \pm 0.1\%$ for milled BSG and $3.1 \pm 0.9\%$ for non-milled BSG). It should also be noted that a very low amount of FA ($1.0 \pm 0.1\%$) was detected after US pretreatment before the enzymatic extraction which could be associated with free FA. US has been reported as an effective method for the extraction of free phenolic acids (Medina-Torres et al., 2017). Untreated BSG showed very low recovery ($0.9 \pm 0.4\%$) in comparison to the pretreated ones, which shows the significance of pretreatments to increase the release of FA.

It can be noticed from Figure 5, that combining milling and AC can increase the FA extraction. Indeed, obtaining a smaller particle size leads to easier medium penetration and better enzyme attack. The high FA recovery after the AC pretreatment can be attributed to facilitated enzymatic hydrolysis in comparison with the US-pretreatment, due to a destruction of the fiber structure. This finding is in accordance with the outcomes reported by Debiagi et al. (2020), who investigated the efficiency of AC and US as pretreatment methods for lignocellulosic biomass. The combined action of high temperature (120 °C) and pressure (overpressure of 1 bar) weakens the biomass and assists further breakdowns

(Torget & Teh-An, 1994). The effect of steam and temperature results in hydrolyzing of hemicellulose and softening of cellulose, allowing a better enzymatic attack and greater substrate accessibility, which releases more FA. (Bautista-Expósito et al., 2020) treated wheat bran by AC at 121 °C for 30 min prior to enzymatic extraction by different commercial enzymes, showing that AC led to an increase of total soluble phenolic compounds and free FA by 2.4- and 41-fold, respectively.

In comparison with US, besides its high efficiency, AC is inexpensive and simple process that can be used for several biomasses at high volumes. Therefore, herein, AC was chosen for the further experiments as the main pre-treatment method.

3.3. Screening of the enzymatic commercial cocktails

3.3.1. Enzymatic activities

As the enzyme activity plays a major role in the release of FA into the medium, xylanase, cellulase and FAE activities of five commercial enzymes (Depol 740 L, Depol 686L, Ultraflo XL, Deltazyme VR-AC 100 and viscozyme L) were investigated. Results are presented in Figure 6.

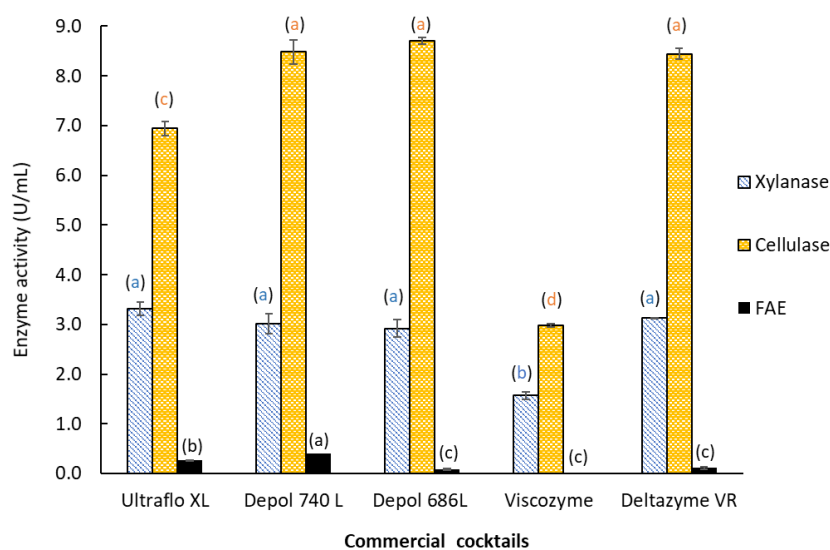


Figure 6. Xylanase, cellulase and FAE activity of the five selected commercial enzymes. Bars with distinct labels represent significant differences ($p < 0.05$) as determined by the Tukey test.

Cellulases cleave cellulose from the cell wall, while xylanases break down the xylan backbone and liberate xylose monomers. Afterwards, the FAE enzymes attack arabinose substituents, releasing FA (Dodd & Cann, 2009). The optimal enzymatic cocktail should possess a high activity of FAE as well as high cellulase and xylanase activities.

The highest cellulase activity was observed with Depol 686L and Depol 740L, 8.70 ± 0.07 U/mL and 8.48 ± 0.25 U/mL respectively, while the highest xylanase activity was seen with Ultraflo XL, Depol

740L, Depol 686L and Deltazyme, showing values of 3.31 ± 0.13 U/mL, 3.01 ± 0.21 , 2.92 ± 0.19 and 3.13 ± 0.01 U/mL respectively. Notably, Depol 740L showed the highest FAE activity, at 0.4 U/mL, followed by Ultraflo XL (0.26 U/mL), aligning with results previously reported in literature (Bautista-Expósito et al., 2020). While xylanase and cellulase play important role in breaking down saccharides to enhance FA release, it is apparent that FAE is the key enzyme in the process. Therefore, it can be inferred that Depol 740L is the most effective enzyme for FA extraction.

3.4. Optimization of the enzymatic hydrolysis process

Box-Behnken design was used to maximize the FA recovery. The design is composed of 15 points (Table S1). The FA recovery ranges from $6.92 \pm 0.27\%$ to $42.74 \pm 1.84\%$. MODDE software generated a data presented in supplementary data file (Figure S3). The replicate plot (Figure S3-A) shows the variation in results for all experiments for a quick inspection of the raw data. Repeated experiments appear in blue (points 13, 14 and 15). The variability of the replicates is low. This will likely result in a very useful model. The summary of fit (Figure S3-B) shows the basic model statistics in four parameters, R^2 shows the model fit; 0.5 is a model with rather low significance, in our case R^2 is 0.97 which mean that our model fits very well. Q^2 shows an estimate for the future prediction precision. Q^2 should be greater than 0.1 for a significant model and greater than 0.5 for a good model, in our study Q is 0.90. Model validity is a test of diverse model problems. The model in this study has been found to be valid, with a score of 0.60, which means that there are no major issues present such as outliers, an incorrect model, or a transformation problem. Additionally, the reproducibility of the model, or the degree to which the model's results can be replicated, has been determined to be 0.99, which is well within acceptable limits. This suggests that the model's results are both valid and reliable. The coefficient plot, shown in figure (S3-C), indicates a positive or negative effect of the variables on the FA recovery. The effect is considered non-significant if the confidence interval crosses the origin ($p > 0.05$). Figure S3-D shows the plot of the residuals of the response and the normal probability of the distribution. All the points are on a straight line on the diagonal, which confirms that the residuals are normally distributed noise. Point number 2 was outside the red lines, which is considered as an outlier, thus this point was excluded.

In our study, the temperature and the quadratic terms of the pH and the quantity of enzyme is significant. Non-significant coefficients were removed from the model, the linear effect of pH and enzyme dosage were kept in the model since their quadratic effects are significant. The resulting in the regression model equation is presented in equation 5, the variables are expressed as coded values [-1,1].

$$FA (\%) = 28.59 + 16.66 \times Temperature - 1.08 \times pH - 0.72 \times Enzyme - 4.26 \times pH^2 - 4.18 \times Enzyme^2 \quad Eq (5)$$

The derived regression model equation describes a multipart process with linear and quadratic terms for all parameters with the FA recovery. The most significant factor that showed a linear effect on the yield is temperature (Figure 7A). A quadratic term was observed regarding the pH and enzyme dosage, meaning that their influences were not linear (Figures 7B and 7C respectively).

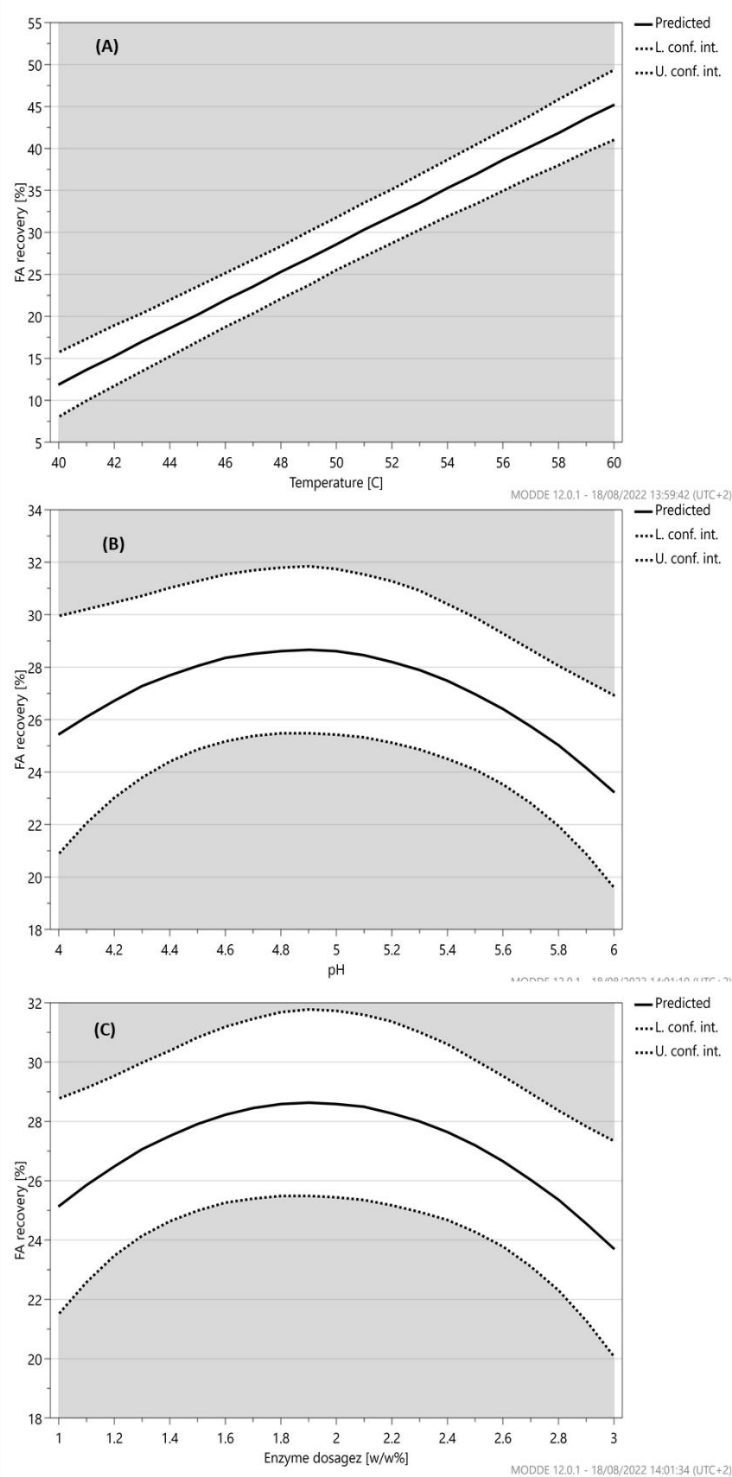


Figure 7. Main effect plot of factors: Temperature (A), pH (B), Enzyme dosage (C).

The increase in temperature leads to a significant improvement in the FA recovery. From 40 to 60 °C, the predicted yield enhanced by 275%. The effect of pH (Figure 7B) shows a characteristic curved quadratic shape. This means that the pH influence is not linear. The increase of the FA recovery observed is obtained for pH between 4.6-5.2. Above this pH, a significant drop is noticed. This fact is also noticeable in the response 4D contour plots presented in Figures 8. At pH 4, a low amount of FA was obtained indicating that Depol 740L does not perform well at this pH. These results are in agreement with those reported in the literature. Barberousse et al. in 2009 optimized the enzymatic extraction of ferulic acid from wheat bran by Depol 740L and xylanase. They found that the pH has the most significant effect in the release of FA. The optimal pH was reported to be between 5.3 and 5.9 giving 20% FA (Barberousse et al., 2009). Recently, Lau et al in 2020 found that the enzymatic extraction of FA from sweet corn cob increases with pH values between 4 and 6 and temperatures between 20 and 50 °C, values above which the FA released starts to decrease (Lau et al., 2020). In fact, the use of higher temperature (> 65 °C) does not improve the extraction yield, due to a loss of enzymatic activity at this temperature (Barberousse et al., 2009). The same trend is observed for the enzyme dosage as shown in the effect plot (Figure 7C). Increasing the enzyme dosage from 1% to 1.9% increases the recovery of FA by 14%. Adding more enzyme did not improve FA extraction.

The effect of the pH, temperature and enzyme dosage on the FA recovery is presented in the response 4D contour plots (Figure 8) in order to illustrate the effects of the interaction of two factors while keeping the third one in a fixed level.

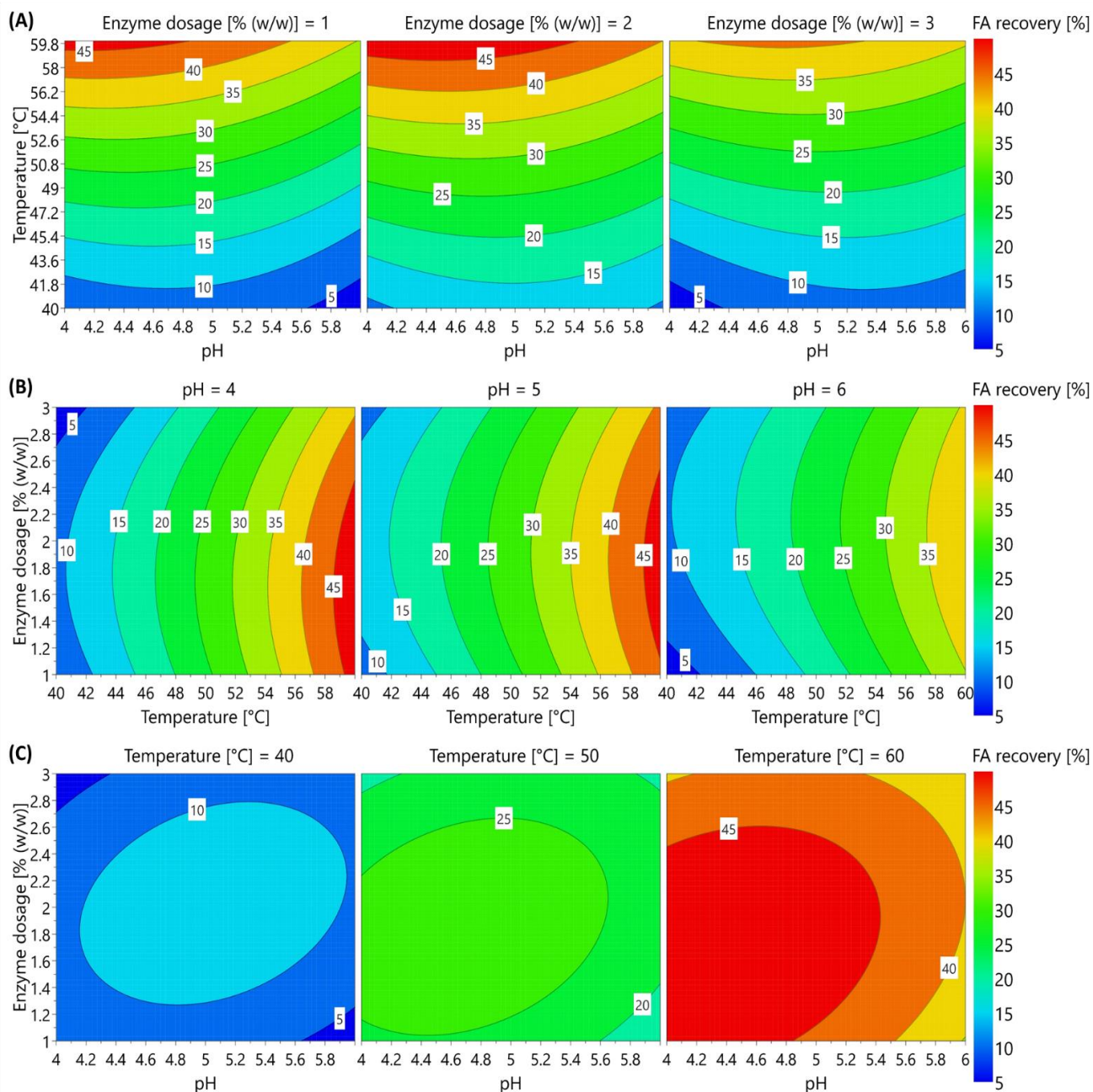


Figure 8. Response 4D Contour Plot describing the yield of extraction of FA according to extraction temperature, pH and enzyme dosage: (A) at fixed enzyme dosage, (B) at fixed pH and (C) at fixed temperature

Figure 8A shows the contour plot at varying temperature over a range of pH at fixed enzyme dosage. It can be seen that the increase in FA recovery is correlated with the increase in temperature whatever the pH and the enzyme dosages. However, the increase is more rapid at an enzyme dosage of 2% and at a pH between 4 and 5; the maximum yields around 40% were reached at 57 °C instead of 60 °C.

Figure 8B shows the contour plot at different enzyme dosages and temperatures at fixed pH. It can be observed that increasing the enzyme dosage above 1.8% does not improve the FA recovery. No significant interactions were found between enzyme dosage, pH and temperature.

Figure 8C indicates that the maximum FA recovery (45%) could be achieved when the pH is between 4.6 and 5.4, the enzyme dosage between 1.6 and 2.1% and the temperature at 60 °C. Indeed, at 60 °C, whatever the pH or the enzyme dosage, the minimum FA recovery is 40%. These findings suggest that it may be possible to optimize the enzymatic process by using water instead of buffer solutions, and by minimizing the amount of enzymes used. This could potentially reduce contamination of the hydrolysate by salts and additives from enzyme cocktails, make the purification step easier, increase the purity of the final product and lower the cost of the process.

3.5. Validation of the predictive model and kinetic of the enzymatic extraction

The optimizer tool of the software was used to predict the conditions that allows to maximize the response. The suggested conditions are: pH 5.27; temperature of 60 °C and 1.72% of enzymes. The FA recovery predicted by the maximizer tool is 45.88%. An average value of $43.13 \pm 4.43\%$ was obtained experimentally (Table 3). The obtained yield is 94% of the predicted yield by the maximizer tool, this validates the model equation, proving that the model is appropriate for optimizing the FA recovery from BSG.

Table 3. Validation of the model at optimum conditions

	pH	Temperature (°C)	Enzyme dosage (%)	FA recovery (%)
Predicted	5.27	60	1.72	45.88
Experimental*	5.27	60	1.72	43.13 ± 4.43
*Means \pm standard deviation (n=2)				

Enzymatic hydrolysis of FA under optimal conditions was monitored by taking samples at different times to find the best incubation time. The results are shown in Figure 9.

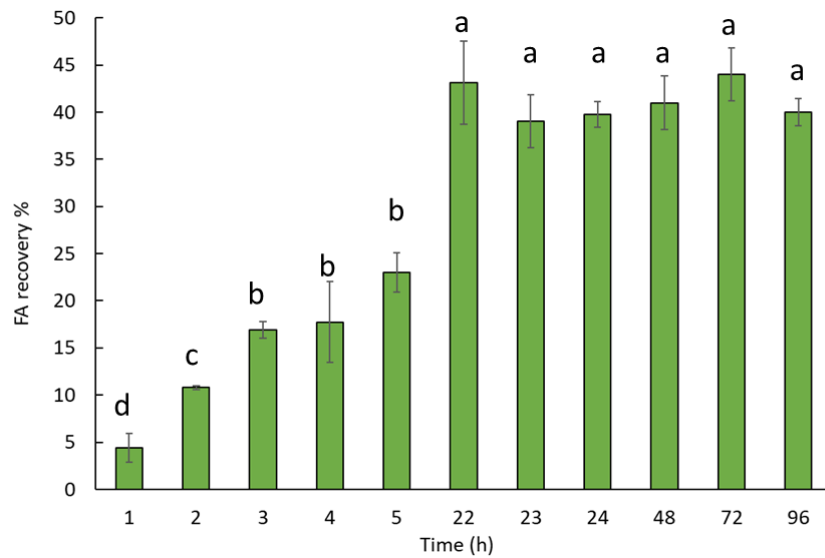


Figure 9. Kinetic of enzymatic hydrolysis of FA from BSG at 60 °C, pH 5.27 and 1.72% enzyme dosage. Bars with distinct labels represent significant differences ($p < 0.05$) as determined by the Tukey test.

The extraction of FA increases during the initial hours of hydrolysis, but extending the incubation time beyond 22 hours did not result in further release of FA. No significant difference was observed between extraction times of 22 hours and 29 hours. Due to its high sugar content, the hydrolysis of BSG at elevated temperatures can result in a rapid deterioration caused by microbial activity. Hence, it is not recommended to prolong the treatment duration under such circumstances. Therefore, it is recommended to minimize the duration of hydrolysis for optimal FA yield.

The stagnation of the release of FA after 22 h can be caused by the accumulation of products inhibiting the enzymatic activity (Frieden & Walter, 1963) or by the depletion of accessible substrates due to the complex macromolecular structure of the BSG matrix and its high lignin content as well as by the high crystallinity of cellulose (Beldman et al., 1987). The physical and steric factors caused by branching of the arabinoxylan backbone can restrict the enzyme action (Yu et al., 2002). In fact, Forssell et al in 2008 studied the hydrolysis of brewer's grains by carbohydrate degrading enzymes, including two preparations Depol 740 and 686. They found that the non-hydrolyzed fraction still contained more than 40% carbohydrates after enzymatic treatment despite high enzyme dosages used. The protein fraction was not affected by cellulases and xylanase. In addition to the recalcitrant carbohydrates, the residue was enriched with lignin and lipids (Forssell et al., 2008).

4. Conclusion

Brewer's spent grain is a low-cost and largely accessible agro-industrial waste that can be considered as a sustainable source of ferulic acid. This work revealed the interest of combining milling and autoclaving as physical pretreatments and commercial enzyme cocktails for the green extraction of ferulic acid from brewer's spent grain. The physical pretreatments made it possible to increase the extraction yield of ferulic acid by 23.7%. Depol 740L was selected as a potential enzyme cocktail due to its high feruloyl esterase activity, and also for its high carbohydrate degrading enzyme activities (cellulase and xylanase) which are considered as a pretreatment enzyme acting synergistically with feruloyl esterase for the release of ferulic acid from brewer's spent grain. Optimization of pH, temperature, enzyme dosage and incubation time of enzymatic hydrolysis significantly improved the recovery of ferulic acid. The maximum recovery of ferulic acid is 45%, meaning that more than 55% of the ferulic acid is not accessible by enzymes due to the complex macromolecular structure of the brewer's spent grain matrix and its lignin, lipid and protein content. The extraction and valorization of these compounds, upstream of the extraction of ferulic acid, would increase the efficiency of the biorefinery process of brewer's spent grain, to reduce the cost of the extraction process and to increase the yield of ferulic acid recovery by facilitating access to enzyme access.

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