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Advances in combined enzymatic extraction of ferulic acid from wheat bran

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ABSTRACT

Wheat bran could be utilised as feedstock for innovative and sustainable biorefinery processes. Here, an enzymatic hydrolysis process for ferulic acid (FA) extraction was optimised step by step for total wheat bran (Tritello) and then also applied to the outer bran layer (Bran 1). Proteins, reducing sugars, total phenols and FA were quantified. The highest FA yields (0.82–1.05 g/kg bran) were obtained either by rehydrating the bran by autoclaving (Tritello) or by steam explosion (Bran 1) using a bran/water ratio of 1:20, followed by enzymatic pretreatment with Alcalase and Termamyl, to remove protein and sugars, and a final enzymatic hydrolysis with Pentopan and feruloyl esterase to solubilise phenol. FA was recovered from the final digestate via solid phase extraction. A 40-fold scale-up was also performed and the release of compounds along all the process steps and at increasing incubation times was monitored. Results showed that FA was initially present at a minimum level while it was specifically released during the enzymatic treatment. In the final optimized process, the FA extraction yield was higher than that obtained with NaOH control hydrolysis while, in comparison with other FA enzymatic extraction methods, fewer process steps were required and no buffers, strong acid/alkali nor toxic compounds were used. Furthermore, the proposed process may be easily scaled-up, confirming the feasibility of wheat bran valorisation by biorefinery processes to obtain valuable compounds having several areas of potential industrial exploitation.

Introduction

Wheat is of great importance for the EU economy and wheat-derived by-products can be considered as feedstocks for biorefinery development. Current worldwide wheat production is more than 700 Mt/year (FAOSTAT, 2014 and 2016, www.faostat.org) with about one-fifth of the cultivated wheat total weight being converted into bran (90–150 Mt/year) [1]. Wheat bran is mainly used as a feed supplement, while its application in the food sector plays only a minor role [2]. However, there is great interest in innovative strategies for the valorisation of this residue through its transformation into added-value biomolecules [3]. Wheat bran is composed of approximately 8–12 % moisture, 13–18 % protein, 36–57 % carbohydrate (typically 40 % dietary fibres and 10 % starch), 5–6 % ash, 4–5.5 % fat and 1 % phenolic acids. Higher levels of phenolic compounds and dietary fibres have been reported in bran compared to the refined grain fraction [4–6]. Moreover, as bran itself has a multi-layer structure [1], sequential milling lead to different fractions, with increasing concentrations of protein and starch and a decreasing amount of dietary fibre from the outer to the inner layer. However, at an industrial scale, these milling fractions are usually collected together.

The chemically heterogeneous composition of wheat bran offers the potential to use it as a substrate in biorefinery processes. Possible valuable recovered compounds can be either building block chemicals to

Abbreviations: A, autoclaved; Alc, Alcalase; B, boiled; BSA, bovine serum albumin; Dris, Driselase; FA, ferulic acid; FAE, feruloyl esterase; GA, gallic acid; GLUC, glucose; Pent, Pentopan; SE, steam explosion; SPE, solid phase extraction; Term, Termamyl

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be used as precursors for higher-polymerized commodities or substances that are valuable *per se* but need to be separated and purified [4]. One of the most exploitable compounds is ferulic acid (FA). This hydroxycinnamic acid is mainly (80–90 %) present in bound insoluble form, linked to cell wall polysaccharides or to lignin via ester and ether bonds [6,7]. It has demonstrated beneficial health activities (such as antioxidant, anti-cancer, improved vascular function) exploitable in the food, health and cosmetic fields [1,2,4,6]. Another FA application is through its conversion into bio-vanillin [5], one of the most important flavouring agents in the food industry. Moreover, FA recovered from agricultural waste can be used as a building block for polymerization to obtain bio-plastics or as an additive in polymers to provide biological properties [8]. Over the last decade, research on FA exploitation in new material applications has increased rapidly. FA and several FA modified forms have been employed as monomers for the synthesis of homo- and co-polymers, such as polyferulic acid (PFA) and polydihydroferulic acid (PHFA, of particular interest because it mimics the thermal properties of polyethylene terephthalate, PET) [8,9], of nonisocyanate polyurethanes (NIPUs) [10] or of epoxy resins [11,12]. FA has also proved to be a good biocompatible antiradical additive for a sustainable approach to the stabilization of polymers in packaging [13,14]. Combining its health properties and capacity to be incorporated in polymers, several useful areas for exploitation can be found, such as FA-containing biodegradable polymers able to strategically release it at rates and concentrations appropriate for topical applications such as skin care products [15].

Protocols for FA recovery from crop by-products are often based on alkaline or acid hydrolysis aimed at the destruction of the lignin/phenolic-carbohydrate complex, but these treatments also release other phenolics, proteins, arabinoxylans and sugars and thus necessarily have to be followed by purification steps [4,16]. Nowadays, the extraction of FA is pursued by a microbiological or enzymatic approach through digestion with esterases [17,18]. In general, enzymatic treatments are considered more environmentally friendly than chemical methods as they are more energy efficient and selective. They may also produce a greater range of fractions with different chemical, functional and technological characteristics and, importantly, there is no need for solvent recovery at the end of the extraction process [2]. Moreover, the enzymatic treatment is more specific, releasing only certain compounds (*e.g.* FA) without damaging other valuable chemicals as happens during alkaline extraction [18]. Large efforts have been made in this direction on agricultural biomass, but few studies have been specifically performed on FA release from wheat bran via an enzymatic approach. Feruloyl esterase (FAE, EC 3.1.1.73) can catalyse the hydrolysis of ester linkages between FA and cell wall polysaccharides. Some FAEs have been studied for biotechnological production and specifically tested for FA release from wheat bran [7,17,18].

Here, total wheat bran (Tritello) and the outer bran layer (Bran 1) were subjected to enzymatic hydrolysis for FA extraction, for wheat bran valorisation through a biorefinery concept. In the final optimized process (both at laboratory and bioreactor scale), extraction steps were reduced to a minimum and the use of strong acid/alkali or toxic solvents was avoided, with the aim of setting up an exploitable process at an industrial level. Protein, reducing sugar and total phenol and FA yields were quantified at all processing steps.

Material and methods

Wheat bran

Two types of wheat bran, Tritello and Bran 1, were provided by Barilla Spa (Parma, Italy). The wheat sequential milling process at an industrial level produces different fractions. Tritello is the complete industrial bran by-product, coming from the union of all milling fractions. Bran 1 consists of only the outer layer of wheat grains and was manually collected after the first industrial milling step.

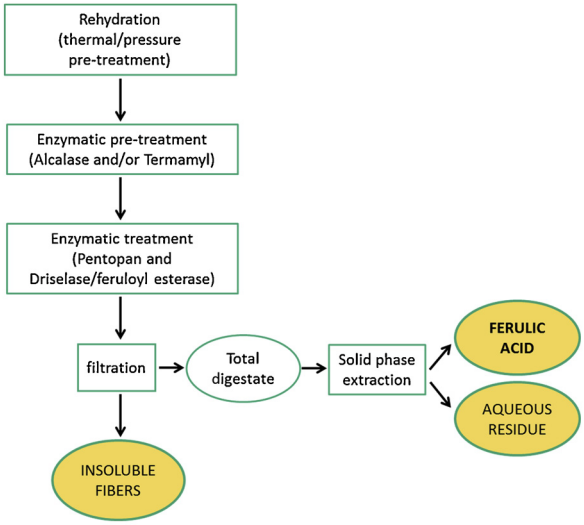

Fig. 1. Layout of extraction processes.

Ferulic acid extraction

Ferulic acid (FA) release from Tritello by enzymatic hydrolysis was optimised (Fig. 1) at the scale of 2 g of bran in 20 or 40 mL of water. For steam explosion samples, bran was wetter and the equivalent weight of the original 2 g was used. The choice of enzymes and their concentrations were based on previous tests performed within the frame of the NAMASTE FP7 project (European Union FP7 cooperation project, under grant agreement No 245267, [5]). The first step was bran rehydration, associated with heat and pressure. Three rehydration methods were tested: wheat bran boiled (B) for 20 min at 100 °C; autoclaved (A) for 20 min at 120 °C and 1 bar; or steam explosion (SE) under 5 different conditions (Table 1). SE was performed in a 25 L batch steam explosion device. The bran was dosed as such, without impregnation, and the vessel was closed, heated with direct steam and kept at the target temperature for a defined time; then the bottom valve was opened quickly so that the contents experienced a sudden pressure release and were shot into a product cyclone after which they were recovered for further treatment. The time temperature profile was characterized by a severity factor used during lignocellulosic pre-treatment. Aliquots of rehydrated samples (termed A t0, B t0, SE t0) were collected and stored at −20 °C until further analyses. The second step was an enzymatic pre-treatment with 2.6 U/g the protease Alcalase (Alc, Sigma-Aldrich, Milan, Italy) and/or 200 U/g α-amylase Termamyl (Term, Sigma-Aldrich). The pH of the rehydrated bran water suspensions was measured and, if needed, adjusted to 6.4 ± 0.1. Enzymes were added directly to the mixtures and samples were incubated at 60 °C for 1 h in a shaking water bath at 150 rpm. Processes were performed either with one enzyme, both enzymes simultaneously or none. Enzymes were inactivated by boiling for 10 min and, if needed, water was added up to a final volume of 40 mL. The third process step consisted of an enzymatic

Table 1
Steam explosion (SE) conditions.

	Pressure (bar)	Target temperature (°C)	Target time (min)	Severity factor
SE1	16	200	0.75	2.9
SE2	16	200	1.5	3.2
SE3	16	200	2.5	3.4
SE4	10	180	5.0	3.1
SE5	7	165	2.9	2.9

treatment. The pH was measured and, if needed, adjusted again to 6.4 ± 0.1. A combination of 2800 U/g xylanase Pentopan (Pent, Sigma-Aldrich) and 0.5 U/g mixture of cell wall degrading enzyme Driselase (Dris, Sigma-Aldrich) or 0.5 U/g pure feruloyl esterase (FAE, recombinant E-FAERU from rumen microorganism, from Astori Tecnica snc, Poncareale (BS), Italy) was added and the mixtures incubated at 40 °C for 3 h in a shaking water bath at 150 rpm before enzyme inactivation. Solid residues were removed by means of a nylon mesh filter (50 µm). The pH of the liquid phase (total digestate) was measured and aliquots were stored at −20 °C until further analyses. The fourth step was FA solid phase extraction (SPE). Aliquots of total digestates were loaded onto a Strata-X column (33 µm polymeric sorbent, 60 mg in 3 mL, Phenomenex Inc., Castel Maggiore (BO), Italy) according to the manufacturer's instructions. After elution, methanolic FA extracts were recovered and stored at −20 °C until further analyses.

An alkali-extractable hydrolysis was conducted as a control [16,17]. Aliquots of 1 g of bran were rehydrated in 20 mL water by boiling for 20 min. After cooling to room temperature, NaOH pellets were added to a 2 M final concentration and samples were incubated at 30 °C for 2 h in a shaking water bath at 150 rpm. The mixtures were neutralised with 12 N HCl and solid residues were removed by means of a nylon mesh filter. Aliquots of total digestates were subjected to FA SPE adsorption. Liquid extracts were stored at −20 °C until further analyses.

For SE samples, an extra control was performed to evaluate possible acid autohydrolysis. These control reaction mixtures (termed SE no enz) were incubated following the same steps as those of enzyme-treated samples, but without pH adjustment.

The 40-fold scaled up extraction process was also performed by means of a bioreactor (Applikon biotechnology, Delft, Netherlands) in a 2.5 L glass vessel containing 80 g autoclaved Tritello and 1.6 L water. Alc, Term, Pent and FAE were added, incubated and inactivated as described above. The mixture was continuously stirred at 350 rpm by two Rushton turbines and pH was kept constant at 6.4 ± 0.3. Sample aliquots (termed S) were collected during the process and stored at −20 °C until further analyses: S1, after autoclaving; S2-S3, after 30 and 60 min enzymatic pre-treatment; S4, after enzyme inactivation; S5-S8, after 30, 60, 120 and 180 min enzymatic treatment; S9, after enzyme inactivation. The best extraction conditions optimised for Tritello were repeated with the Bran 1 wheat fraction. At least two replicates were performed for all extraction conditions.

Protein, reducing sugar and total phenol spectrophotometric quantification

Total digestates were analysed for protein [19], reducing sugar [20] and phenol contents [21,22]. The results were expressed, respectively, as g bovine serum albumin (BSA), glucose (GLUC) and gallic acid (GA) equivalents per kg initial bran by means of calibration curves.

Ferulic acid and phenolic compound quantification by HPLC-DAD and UPLC-PDA-MS/MS

The profile of phenolic compounds was determined in all SPE methanolic FA extracts by HPLC-DAD (High Performance Liquid Chromatography-Diode Array Detector) as described previously [23,24]. Sample aliquots (equivalent to 1 mL total digestate) were completely dried in a speed vacuum at 45 °C and resuspended in 200 µL of 1:9 acetonitrile/0.2 % (v/v) acetic acid before being directly injected into the HPLC system (Gemini C18 column, 5 µm particles, 110 Å, 250 × 4.6 mm; pre-column SecurityGuard Ea; Phenomenex, Castel Maggiore (BO), Italy) equipped with an on-line diode array detector (MD-2010, Plus, Jasco Europe, Cremella (LC), Italy). Eluents were acetonitrile and 0.2 % acetic acid mixed by means of a dynamic gradient in which the acetonitrile % changed as follows: 0 min 9 %, 3 min 9 %, 8 min 12 %, 10 min 14 %, 14 min 15 %, 15 min 16 %, 17 min 24 %, 21 min 32 %, 23 min 35 %, 32 min 37 %, 35 min 100 %, 37 min 100 %, 41 min 9 %, 45 min 9 %. The flow was constant at 1 mL/min. The adopted HPLC-DAD separation procedure allowed the simultaneous analysis of FA and a further 20 different compounds, including hydroxycinnamic and phenolic acids, flavanols and flavonoids: ferulic, caffeic, chlorogenic, *p*-coumaric, sinapic and *trans*-cinnamic acids; gallic, protocatechuic, syringic and vanillic acids; catechin, epicatechin, epigallocatechin gallate, epicatechin gallate, epigallocatechin; vanillin, naringenin, quercetin, rutin, myricetin, and kaempferol. FA was quantified by means of a calibration curve using a FA standard (between 5 and 300 µM), by analysing chromatograms at 323 nm.

Two extracts were diluted 1:100 (v/v) in LC–MS grade water, filtered through Minisart RC4 filters (Sartorius, Göttingen, Germany) and 1 µL was injected. 1 mg of FA acid commercial standard (Sigma Aldrich, Milan, Italy) was dissolved in 1 mL 100 % (v/v) methanol and diluted with LC–MS water grade up to the final concentration of 100 pg/µL and 1 µL was injected. UPLC-PDA-MS/MS (Ultra-Performance Liquid Chromatography-Photo Diode Array-tandem Mass Spectrometry) analyses were performed as previously described [25] with the only modification that the analyses were performed with a MS^e method using a ramp energy from 20 to 60 V in function 2.

Statistical analyses

All extractions, including the bioreactor scale-up processes, were performed at least twice. The analyses on extraction fractions were performed at least twice in two technical replicates. The results were expressed as the mean (n = 2) ± SD. Statistically significant differences between data sets were analysed by one-way ANOVA tests followed by post-hoc corrected two tail student-*t* tests assuming equal variance.

Results and discussion

Enzyme selection

Each step involving enzymatic FA release from Tritello wheat bran was optimised by successive improvements. First, the study focused on the required enzymes. Proteins, reducing sugars and total phenols were quantified in all the total digestates and the FA amount was determined in the final extracts obtained after solid phase extraction (SPE) (Table 2). Alkaline hydrolysis was conducted as a control. The use of Alc led to up to a 2.7-fold increase in protein solubilisation compared to the process without enzymatic pre-treatment, while the amount of reducing sugars was up to 1.6-fold higher after Term treatment. Maximum solubilisation of proteins and sugars was obtained when Alc and Term were used in combination, also leading to an increased yield of total phenols (up to 1.3-fold) and FA (up to 19.9-fold). For such processes, proteins and residual starch are usually considered interferents and previous studies dealing with FA recovery from wheat bran have included a preliminary purification step with ethyl acetate and/or diethyl ether [16,26], or treated the bran with potassium acetate followed by extensive washing with water to remove starch [17,27]. One report [18] utilized two enzymes, an amylase and papain, for starch and protein removal from wheat bran before FA release by fungal extracts, in a process that included a large number of steps and the use of buffers and the toxic sodium azide. In contrast, the proposed enzymatic pre-treatment includes few steps and avoids the use of toxic chemicals.

For specific release of FA from plant cell walls, a commercial xylanase (Pent) was tested together with two different commercial enzymes, Dris or a pure FAE. Both combinations were assayed for FA release from Tritello wheat bran (Table 2). FAE was able to increase the FA content in the extract by up to 0.528 g/kg, about 12.8-fold more than the maximum level obtained with Dris. The amount of FA obtained after FAE hydrolysis was largely increased by the enzymatic pre-treatment, in particular the use of Alc alone (14.1-fold compared to the process without pre-treatment) or in combination with Term (19.8-fold). On the other hand, Dris was able to increase the amount of solubilised protein, reducing sugars and total phenols more than FAE.

Table 2
Quantitation of proteins, reducing sugars, total phenols and ferulic acid amounts after different enzymatic treatments of boiled wheat bran and the NaOH control (2g wheat bran/40 mL water). In parentheses, the fold increase is shown for the process with the same feruloyl esterase but without enzymatic pre-treatment (raw 1 or 2). Different letters in superscript indicate a statistically significant difference (one-way ANOVA followed by post hoc two tail Student's *t*-test, $p < 0.05$) between the same type of data. Data are the mean ± SD (n = 2). Alc, Alcalase; Term, Termamyl; Pent, Pentopan; Dris, Driselase; FAE, feruloyl esterase; BSA, bovine serum albumin; GLUC, glucose; GA, gallic acid; FA, ferulic acid.

Enzymes					Proteins	Reducing sugars	Total phenols	Ferulic acid
Alc	Term	Pent	Dris	FAE	gBSAeq/kg	gGLUCeq/kg	gGAeq/kg	gFA/kg
		X	X		122.01 ± 3.14^{a}	503.86 ± 6.28^{a}	7.24 ± 0.01^{a}	0.041 ± 0.004^{a}
		X		X	45.05 ± 1.51^{b}	222.06 ± 15.12^{b}	5.64 ± 0.01^{b}	0.027 ± 0.003^{b}
X		X	X		158.31 ± 8.34^{c} (1.30)	491.71 ± 23.21^{a} (0.98)	9.23 ± 0.14^{c} (1.27)	0.039 ± 0.004^{a} (0.96)
X		X		X	120.99 ± 1.49^{a} (2.69)	287.22 ± 19.10^{c} (1.29)	7.45 ± 0.06^{d} (1.32)	0.376 ± 0.038^{c} (14.13)
	X	X	X		127.58 ± 7.87^{a} (1.05)	553.24 ± 37.15^{d} (1.10)	6.85 ± 0.01^{e} (0.95)	0.014 ± 0.001^{d} (0.34)
	X	X		X	95.38 ± 11.47^{d} (2.12)	355.50 ± 36.27^{e} (1.60)	4.74 ± 0.08^{f} (0.84)	0.009 ± 0.001^{e} (0.33)
X	X	X	X		144.39 ± 3.52^{c} (1.18)	521.42 ± 43.27^{d} (1.03)	8.42 ± 0.10^{g} (1.16)	0.034 ± 0.003^{a} (0.83)
X	X	X		X	117.08 ± 10.28^{a} (2.60)	387.20 ± 16.70^{e} (1.74)	7.15 ± 0.11^{a} (1.27)	0.528 ± 0.053^{f} (19.83)
NaOH control					180.45 ± 17.41^{e}	369.62 ± 17.51^{e}	18.10 ± 2.76^{h}	0.413 ± 0.041^{g}

This could be due to the fact that, according to the manufacturer's description, Dris is a cell wall degrading enzyme mixture which facilitates extraction processes by digesting cell wall carbohydrates with different feruloylated side-chains.

Regarding enzymatic treatment with FAE, previous studies suggested that the enzyme acts in synergy with main chain-degrading enzymes, such as xylanase, pectinase or α-L-arabinofuranosidase [5,7,17,18], leading to an increase in their activity and thereby giving FAE the possibility to reach its specific substrate when the cell wall matrix becomes partially degraded. Probably due to the high specificity of FAE, the FA extraction yield was slightly higher than that obtained in the NaOH control hydrolysis, which instead produced the highest content of total phenols (Table 2).

HPLC-DAD chromatograms of all the final extracts showed a large peak, with the same retention time and spectrum as the FA standard (Fig. 2). An aliquot of the purified extract was submitted to UPLC-PDA-MS/MS analysis in order to identify the main chromatographic peak, which was observed to absorb at the same wavelengths as FA standard. Photo Diode Array (PDA) analysis further confirmed the results and the high resolution MS identified the main molecule, with a retention time of 6.36 min and *m/z* feature of 193.0504 [M−H]- (calculated mass of 193.0501 [M−H]- with an error of 1.6 ppm) and a molecular formula of $C_{10}H_{10}O_4$. The *m/z* feature, fragmentation pattern and retention time of this molecule matched those of a pure FA commercial standard, thus confirming the identity of the molecule as FA (Fig. 2D–E). No other hydroxycinnamic acids or phenols were identified.

From the first set of experiments (Table 2), it was observed that pre-treatment with Alc and Term followed by treatment with Pent and FAE provided a double advantage: high degradation of interfering substances and high yields of target compounds (phenols and FA), hence this procedure was selected for further optimisation.

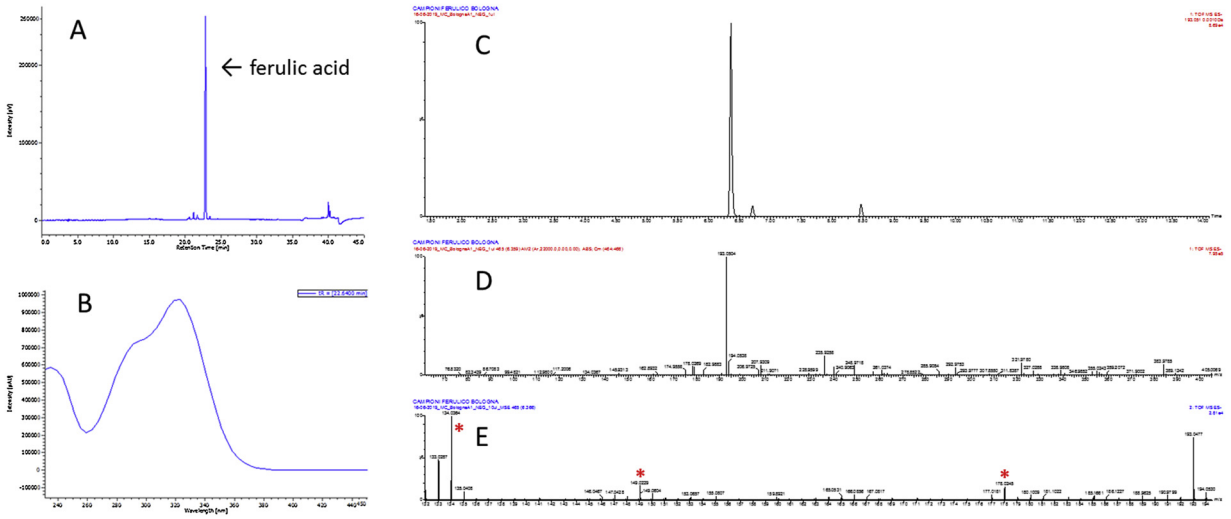

Fig. 2. HPLC-DAD (A, B) and UPLC-PDA-MS/MS (C, D, E) ferulic acid separation and identification in wheat bran extracts. HPLC-DAD chromatogram (wavelength 323 nm) (A) and acquired spectrum corresponding to the main peak (B). UPLC-qTOF extracted ion chromatogram (EIC) chromatogram of *m/z* 193.0501 (C), mass spectrum (D) and mass/mass spectrum (MS^{e}) (E) of the main peak. Asterisks indicate the ferulic acid fragments.

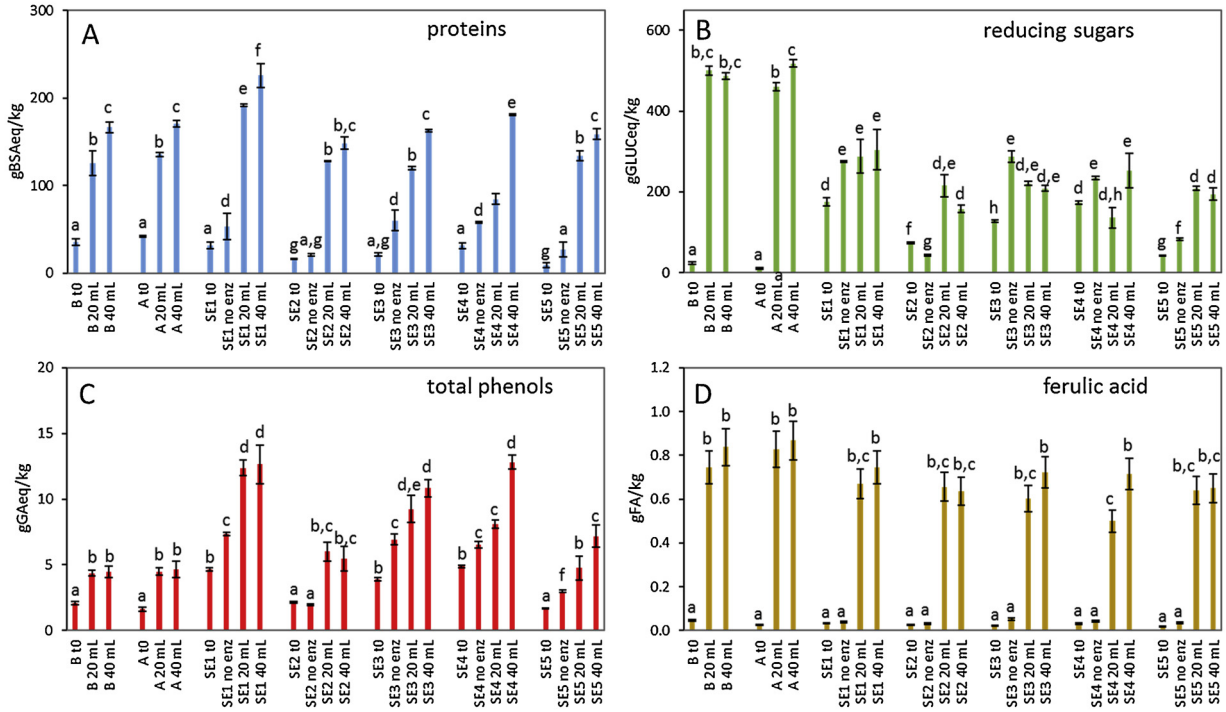

Fig. 3. Quantification of proteins (A), reducing sugars (B), total phenols (C) and ferulic acid (D) in different extracts of Tritello wheat bran. *t0* samples were collected after bran rehydration; *no enz* samples were collected at the end of the process performed with all the steps but without enzymes; *20 mL* samples and *40 mL* samples were collected after enzymatic treatment in 20 and 40 mL final volume, respectively. Different letters in superscript indicate a statistically significant difference (oneway ANOVA followed by post hoc two tail Student's *t*-test, $p < 0.05$) between the same type of data. Data are the mean ± SD (n = 2). A, autoclaved samples; B, boiled; SE, steam explosion; BSA, bovine serum albumin; GLUC, glucose; GA, gallic acid; FA, ferulic acid.

Bran rehydration method selection and solid/liquid ratio optimisation

The enzymatic process for FA release from Tritello wheat bran was further optimised by assaying different bran rehydration methods and solid/liquid ratios (see Methods). Proteins, reducing sugars and total phenols were quantified in all total digestates and the FA amount was determined in the final extracts after SPE (Fig. 3). No difference in protein and FA release was found between the rehydration methods themselves (Fig. 3, t0 samples), while three different SE conditions (samples SE1 t0, SE3 t0, SE4 t0) were able to release reducing sugars and total phenols more efficiently than the B or A methods (e.g. up to 15.8-fold and 3.0-fold, respectively, in SE4 t0 compared to A t0).

The different rehydration methods influenced the levels of degradation of interferents (starch and proteins) and of valuable compounds released during the following enzymatic extraction process (Fig. 3, 20 mL and 40 mL samples). Protein levels seemed not directly affected by the rehydration method (Fig. 3A), while more effects were observed for reducing sugars (Fig. 3B) and total phenols (Fig. 3C). The B and A methods led to similar results, while different effects were exerted by the five SE conditions. This is particularly evident in the case of the level of total phenols (Fig. 3C), which was e.g. 2.2-fold higher in SE1 total digestate compared to SE2. On average, phenols were 2-fold more solubilised during the SE process compared to B and A, probably as a result of bran matrix destruction during SE treatment, exposing more cell wall components to enzymatic digestion. Conversely, reducing sugars levels were on average 2.3-fold lower after SE compared to B and A (Fig. 3B). A change of colour was observed in SE-treated samples. Both the final brownish tint and the lower reducing sugar amounts were probably due to the Maillard reaction, which might occur between some amino acids and sugars present in the sample. This reaction typically produces brownish molecules, usually responsible for distinct food flavours (e.g. bakery products). FA levels in the final extracts were only slightly affected by rehydration method: the maximum average amounts were found in B and A samples (0.848 and 0.792 gFA/kg respectively), while SE rehydration led to an average of 0.653 gFA/kg with minor differences among the 5 SE conditions (Fig. 3D).

SE and other hydrothermal/pressure treatments are often employed in biomass pre-treatments before valorisation processes, because of their relatively simple technological requirements and the absence of corrosive and hard-to-recycle chemicals. The effects of these treatments are degradation and depolymerisation of hemicellulosic arabinoxylans together with some breakdown of cellulosic glucose, which is associated with a significant reduction in the amount of cross-linked phenolic acids and a release of FA and diferulic acids [3,28]. SE is more applicable in industry than other hydrothermal processes: it was found to be effective in phenolic acid extraction from wheat bran [16] and is often used as a pre-treatment to optimise yields in the enzymatic hydrolysis of sugars from cellulose, which has similarities with the process to release the FA from bran [28].

In case of SE-treated samples, a gradual acidification of the extraction mixture was observed during the process, in particular during the two enzyme incubation steps (down to pH 5.3 in sample SE4 at the end of the pre-treatment), and pH was therefore adjusted before each enzyme addition. In B and A treated bran, pH remained almost constant and adjustments were not required. To investigate pH modification due to a possible autohydrolysis effect, an extra SE control (Fig. 3, SE no enz samples) was performed by processing the reaction mixtures following all the steps but without enzyme addition or pH adjustment. Samples showed a progressive pH decrease, which may be due to acetic acid release as a result of the SE pre-treatment [28]. The final amounts of protein (Fig. 3A) and reducing sugars (Fig. 3B) were increased in SE no enz samples compared to SE t0 samples, while a minor effect was

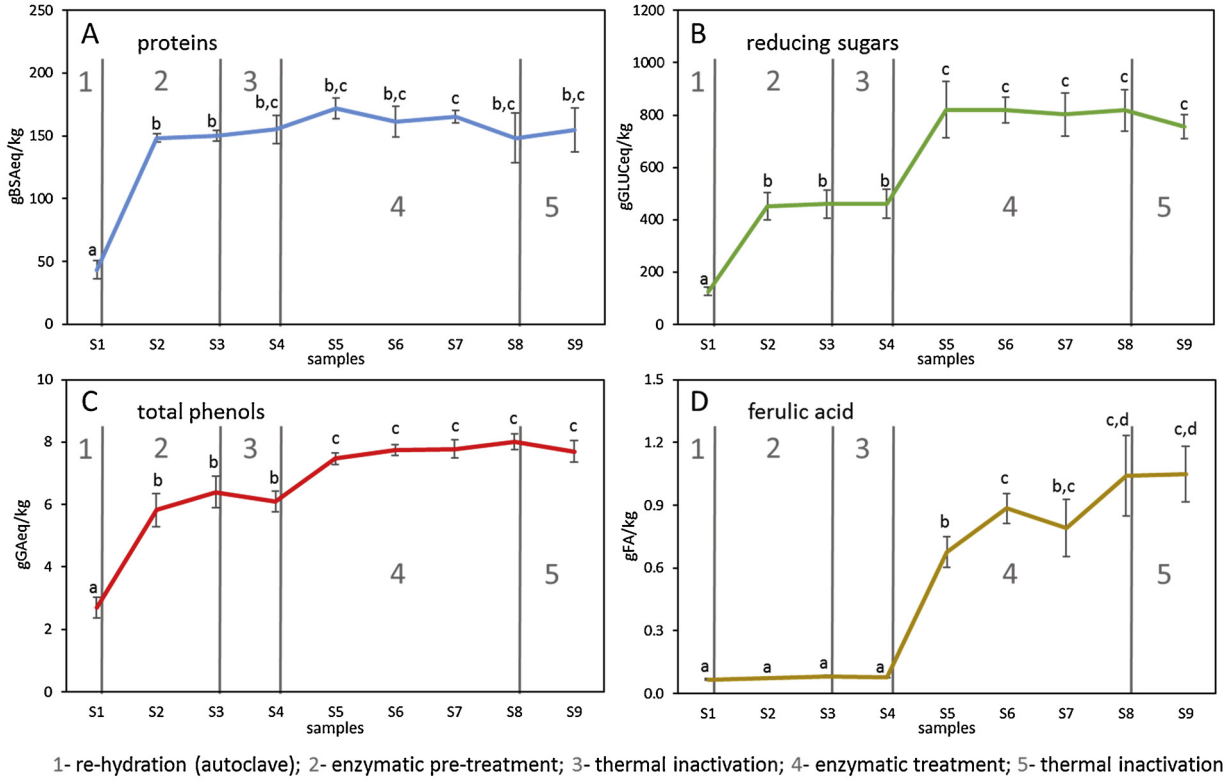

Fig. 4. Quantification of proteins (A), reducing sugars (B), total phenols (C) and ferulic acid (D) after the bioreactor 40-fold scale-up of enzymatic hydrolysis of autoclaved Tritello bran. Sample aliquots were collected at the following times: S1, after autoclaving; S2-S3, after 30 and 60 min of enzymatic pre-treatment; S4, after enzyme inactivation; S5-S8, after 30, 60, 120, 180 min of enzymatic treatment; S9, after enzyme inactivation. Different letters in superscript indicate a statistically significant difference (one-way ANOVA followed by post hoc two tail Student's *t*-test, $p < 0.05$) between the same type of data. Data are the mean ± SD (n = 2). BSA, bovine serum albumin; GLUC, glucose; GA, gallic acid; FA, ferulic acid.

observed for total phenols (Fig. 3C) and FA content (Fig. 3D). It has been reported that the release of acetic acid during the SE process can inhibit subsequent enzymatic bioconversions [28].

The enzymatic process was performed in two different solid/liquid ratios after each bran rehydration method: 1:10 (2 g bran/20 mL water) and 1:20 (2 g bran/40 mL water). To allow a comparison, data were reported per kg of bran (Fig. 3). A larger volume of water allowed a higher release of proteins (Fig. 3A) and in some cases of total phenols (Fig. 3C), while the level of reducing sugars (Fig. 3B) appeared to be affected differently by the solid/liquid ratio depending on the rehydration method applied. FA final yield (Fig. 3D) was, on average, 11.5 % higher in 1:20 solid/liquid conditions compared to the 1:10, with maximum yields obtained in samples A 40 mL and B 40 mL (Fig. 3D). Moreover, the increased amount of water reduced the mixture density allowing better stirring (a key point for scale-up and future potential industrial exploitation) and facilitating the interaction between enzyme and substrate. HPLC-DAD confirmed that FA was the major phenolic component in all extracts (Fig. 2). The best enzymatic process led to a final FA yield of 0.867 gFA/kg (Fig. 3D, A 40 mL) which is 2.1-fold higher than the NaOH control (Table 2). Thus the optimised enzymatic extraction proved to be a process as productive as alkaline hydrolysis previously indicated [16,17]. The process starting with autoclaved Tritello bran in a 1:20 solid/liquid ratio, followed by Alc and Term pre-treatment and Pent and FAE treatment, was selected for further experiments.

Process scale-up

A 40-fold scale-up of the selected process was performed in a bioreactor using autoclaved Tritello bran, a 1:20 solid/liquid ratio (80 g bran/1600 mL water), Alc and Term enzymatic pre-treatment and Pent and FAE hydrolysis. The release of interfering and target molecules was followed by sampling at all steps and times of incubation. The rehydration step did not produce a large release of protein and sugars or of phenols and FA (Fig. 4, S1 samples), as was observed at the smaller scale (Fig. 3). As expected, enzymatic pre-treatment increased protein, sugar and phenol levels (Fig. 4, samples S2-S3) and enzymatic treatment released further amounts of sugars and phenols (S5-S8), while the two enzyme thermal inactivations had no significant effects (S4 and S9). FA was initially present at a minimum level (on average 0.075 gFA/kg for S1-S4) while it was specifically released by the enzymatic treatment up to 1.049 gFA/kg in the final extract (S9). The data demonstrated that FA solubilisation was specifically caused by the combined action of Pent and FAE, with a rapid release in the first 30 min (S5, 0.676 gFA/kg) followed by slow increase up to the end of the process (S6-S9).

Comparing the total release of different compounds between the two process scales (Figs. 3 and 4), protein levels were similar (154.6 and 170.8 gBSA eq/kg, respectively, for bioreactor and 40 mL) with higher yields at bioreactor scale for reducing sugars (755.5 and 518.3 gGLUC eq/kg, respectively; 1.5-fold increase), total phenols (7.7 and 4.7 gGA eq/kg, respectively; 1.7-fold increase) and FA (1.049 and 0.867 gFA/kg, respectively; 1.2-fold increase). The improved hydrolysis performance in the bioreactor can be ascribed to more efficient stirring and to the continuous pH control, which allowed a homogenous reaction system and constant optimal enzyme activity conditions.

The final yield of FA obtained under the optimised process conditions at bioreactor scale (Fig. 4D; 1.05 gFA/kg) was comparable with

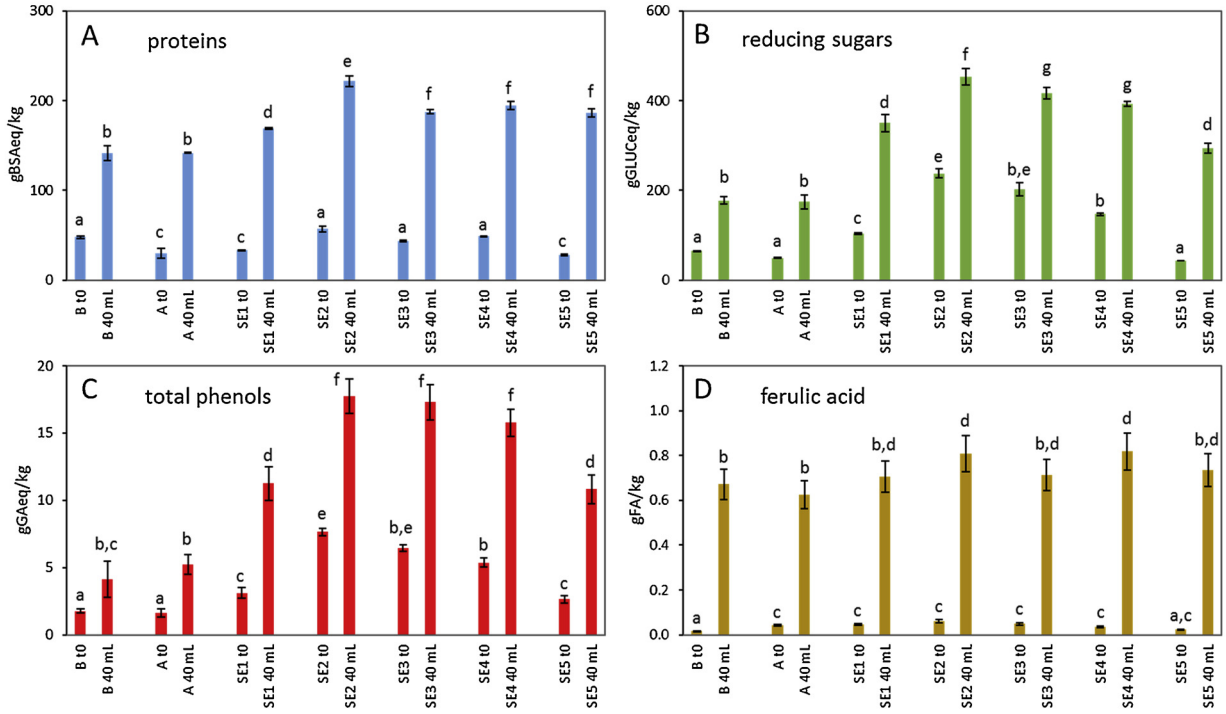

Fig. 5. Quantification of proteins (A), reducing sugars (B), total phenols (C) and ferulic acid (D) in different extracts of Bran 1 wheat bran. *t0* samples were collected after bran rehydration, *40 mL* samples were collected after enzymatic treatment in 40 mL volume. Different letters in superscript indicate a statistically significant difference (one-way ANOVA followed by post hoc two tail Student's *t*-test, $p < 0.05$) between the same type of data. Data are the mean ± SD (n = 2). A, autoclaved samples; B, boiled; SE, steam explosion; BSA, bovine serum albumin; GLUC, glucose; GA, gallic acid; FA, ferulic acid.

the few published data obtained for enzymatic digestion of wheat bran. Notably, it was 17 % higher than the FA content obtained with a process using the same enzymes but including several additional steps and greater water consumption ([5], 0.9 gFA/kg). Conversely, slightly higher FA levels were obtained by others [7,17], (respectively 1.3 and 1.9 gFA/kg), using different enzymatic treatments. Compared with other FA enzymatic extraction methods, the processes optimised here requires fewer steps and does not involve the use of buffers, solvents, strong acid/alkali nor toxic compounds.

FA extraction from Bran 1

The enzymatic extraction process optimised for Tritello wheat bran was repeated at a 40 mL scale with Bran 1 type of initial by-product (Fig. 5). All rehydration methods were tested, followed by enzymatic pre-treatment (Alc and Term) and treatment (Pent and FAE).

Data on solubilisation of interferents confirmed that, as observed with Tritello (Fig. 3), rehydration methods themselves (t0 samples) were able to release low amounts of protein (Fig. 5A), on average 41.1 gBSA eq/kg Bran 1, or 1.5-fold higher than with Tritello, and that different conditions hydrolysed variable amounts of reducing sugars (Fig. 5B), with a maximum in SE2 (221.8 gGLUC eq/kg). SE also led to an increased release of total phenols (Fig. 5C, SE t0 samples), at times even higher than that obtained after the entire process involving B or A rehydration (Fig. 5C, samples B 40 mL and A 40 mL). FA levels (Fig. 5D, t0 samples) confirmed that after the rehydration step, it was only minimally released, on average 0.039 gFA/kg, about 1.3-fold higher than in Tritello (Fig. 3D).

Final total digestates were in general richer in protein, reducing sugars and total phenols when Bran 1 was rehydrated by SE (Fig. 5A–C, 40 mL samples), compared to methods B and A. The process involving SE2 rehydration conditions seemed to be the most effective for protein, sugar and phenol solubilisation, 221.8 gBSA eq/kg, 453.4 gGLUC eq/kg, 17.8 gGA eq/kg, respectively, followed by SE3 and SE4 conditions (Fig. 5A–C), with generally higher measured levels of different compounds than those obtained with Tritello type bran (Fig. 3A–C). Maximum FA content was obtained with SE4 and SE2 Bran 1 processes, 0.818 and 0.810 gFA/kg, respectively, but these levels were about 6 % lower than the maximum levels extracted from Tritello (Fig. 3D).

With Bran 1 starting material, processes including SE seemed to be the most effective (Fig. 5), while B and A were found to be the most efficient for Tritello (Fig. 3). The different results obtained by applying the same processes to Tritello and Bran 1 can be ascribed to their specific structures and chemical composition. Bran 1 consists of the outer wheat bran layer, which contains more dietary fibre and less protein and starch compared to Tritello. These differences can lead to varied efficiencies of both rehydration methods (in particular SE) and enzyme reactions. These findings are in agreement with a previous published research in which the yield of solubilized material varied among wheat bran milling fractions, suggesting that differences in plant cell wall structure and in susceptibility to enzyme attack are important determinants of bran matrix solubilisation [5].

Conclusions

In the present study, total wheat bran (Tritello) was subjected to enzymatic hydrolysis before ferulic acid (FA) extraction, aiming at the valorisation of wheat bran by following a biorefinery concept. The process was optimised step by step at a 40 mL scale mainly including bran rehydration, enzymatic pre-treatment with Alc and Term for protein and residual starch degradation, and enzymatic treatment with Pent and pure FAE for specific FA release, followed by a SPE for FA recovery. A 40-fold bioreactor scale-up was performed, and the release of different compounds was monitored at all steps and at different

incubation times. The data showed that FA solubilisation was specifically obtained during Pent and FAE treatment. The FA extraction conditions were optimised for Tritello and repeated with the Bran 1 type by-product. In the case of Tritello starting material, the rehydration autoclaving step was the most efficient, while steam explosion worked best for Bran 1. Overall, the maximum FA yield (1.05 gFA/kg) was obtained with Tritello at a bioreactor scale.

In comparison with other published enzymatic protocols, the optimised process required fewer steps and did not involve the use of buffers, strong acid/alkali or toxic compounds. These aspects, together with the use of equipment commonly available in industrial plants, suggest that the scale-up of the present optimised process to an industrial level may be feasible. In addition to the final FA extract, the process generated two residues: a solid feedstock, mostly containing insoluble fibres, after enzymatic treatment and, after SPE, an aqueous solution containing sugars, peptides and amino acids. In view of a zero-waste closed-loop biorefinery process, these residues could be both further exploited for biogas or compost production or for more valuable applications, such as the production of ingredients for food and feed (given that the proposed processes does not involve the use of solvents or toxic reagents), nanofibers or biocomposites. The work confirms that wheat bran can be regarded as a versatile by-product, suitable for biorefinery challenges and valorisation.

Declaration of Competing Interest

None.

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