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Use of carbohydrases to promote protein extraction from rice bran and soybean meal: A comparative study

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ABSTRACT

An in-depth study of commercial carbohydrases was performed to select those that are most performing to increase the protein content of two valuable agri-food waste, rice bran (RB) and soybean meal (SM). In particular, defatted RB (DRB) and SM were subjected to hydrolysis mediated by both one and the combination of two commercially available enzyme formulations, *i.e.* Ceremix® Plus MG, Celluclast® 1.5L, Ultraflo® L and Viscozyme® L. Sugar extraction yields, a useful parameter to evaluate the carbohydrase efficiency, were calculated as weight of the dried supernatants obtained after each enzymatic treatment, with respect to the starting weight. Instead, the percentage of proteins of the solid residues (DRBP and SMP) was evaluated according to the AOAC Dumas method, using elemental analysis. In case of RB, starting from a 19% protein content, a protein enrichment of 105 ± 4% was achieved using Ceremix® Plus MG and Celluclast® 1.5L. Regarding SM, an enrichment of 33 ± 4% was reached with Viscozyme® L. In both cases, the highest sugar extraction yields, specifically 64 ± 1% and 49 ± 2%, were obtained for DRB and SM, respectively. Results showed that the selection of specific carbohydrases tuned according to the polysaccharides composition is essential to increase the availability of proteins present in agri-food waste.

1. Introduction

The valorization of food waste (FW) for the production of high value-added products through integrated biorefineries is gaining a growing scientific attention (Girotto et al., 2015). Cereals and legumes, *e.g.* rice and soybean, are, among others, crops that generate large amounts of FW during their harvesting and manufacturing (about 25% of total FW) (Soybean Meal INFO Center, 2023).

Rice is currently one of the most abundant food crops worldwide and, according to Food and Agriculture Organization (FAO), its annual production in 2022 reached around 517 million tonnes (FAO Food and Agriculture Organization, 2022). Rice grain consists of three main parts: endosperm or white rice (~70%), hull/husk (~20%) and bran (~10%) (Phongthai et al., 2017), the former being the only edible fraction. Paddy rice (or rough rice) undergoes several processing steps to manufacture the rice for consumers, *i.e.*, husking, milling with rubber roll dehullers, abrasive milling (whitening), polishing, and glazing. Rice bran (RB) is the waste derived from the rice whitening and it makes up to 5–8% of rough rice (Orthoefer, 2005). Thus, taking into account the huge amount of worldwide production of rice, RB represents a massive FW.

Soybean is another primary staple food for more than half world population, as well as rice. In 2018, its production accounted for around 360 million tonnes and a further 28% growth is expected by 2030 (Prisacaru & Sevciuc, 2020). Soybean finds its way on the market both as grain and as many soybean-based food products, *i.e.* curds, milk, lecithin and oil (Nile et al., 2022). Regarding the latter, soybean oil is recognized among the most common vegetable oil, less expensive than corn and sunflower ones; moreover, it has many desirable characteristics, such as high linoleic acid content and low saturated fatty acid content (Asbridge, 1995). To produce oil, soybean seeds undergo several pre-treatments, affording soybean flakes, which are extracted with

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different methods (Dunford, 2012). Soybean meal (SM) is the cake obtained as residue from the extraction of oil and it accounts for 63% of soybean seeds (Soybean Meal INFO Center, 2023).

Such agri-food residues, *i.e.*, RB and SM, have been mostly unexploited so far and mainly used as animal feed or litters (Schramm et al., 2007). However, RB and SM are relatively rich in proteins with respect to other agri-food residues, thus they have high potential to produce value-added products, like, among others, protein hydrolysates. They have been reported to possess good nutritional value and functional properties, such as emulsifying and foaming capacity; moreover, they show several biological functions, *i.e.*, anticancer, antihypertensive, antioxidant activities, *etc*. (Fabian & Ju, 2011; Kim et al., 2021). Thus, they find application as functional food ingredients, nutraceutical supplements, flavour enhancers, ingredients in cosmetic formulations, or biostimulants in horticulture and many others (Tumma et al., 2022).

A key issue for the utilization of proteins derived from plant by-products is their limited solubility. Moreover, some of them are linked to the lignocellulosic fraction, hampering their extractability (del Mar Contreras et al., 2019; Wang et al., 1999). In particular, the proteins of RB are hardly available because they possess a complex tertiary structure, tendency to aggregate and, moreover, they are difficult to separate from the other components of the vegetable matrix (Liu et al., 2021). Hydrolysis of proteins is a useful approach to improve their solubility, extractability and then their applicability. Among the several available methods, enzymatic hydrolysis mediated by proteases is the most promising one, because it requires mild conditions (low temperature and almost neutral pH), avoids side reactions and does not decrease the nutritional value of the protein source.

However, protease-mediated hydrolysis is often insufficient to efficiently extract most of the proteins from agri-food residues. Therefore, several methods have been reported to enhance the extraction efficiency of proteins. Among them, preliminary treatments with carbohydrases, *i.e.*, enzymes that catalyze the cleavage of glycosidic linkages of the naturally occurring polysaccharides of cell walls, such as pectinases, amylases, cellulases, xylanases, are often used, thus favouring the release of proteins from the lignocellulosic fraction (del Mar Contreras et al., 2019).

In this work, we report the study of hydrolytic protocols based on the use of four commercially available carbohydrase formulations (Viscozyme® L, Ultraflo® L, Celluclast® 1.5L and Ceremix® Plus MG) and their combinations, in order to provide information for optimized polysaccarides hydrolysis of RB and SM, thus increasing the protein content of these agri-food residues. Despite many research efforts have been made on the protease-mediated hydrolysis of RB and SM (Fabian & Ju, 2011), to the best of our knowledge, few or no studies report a comparative study of the capacity of different carbohydrases to improve proteins extraction efficiency during enzymatic pre-treatments.

2. Materials and methods

2.1. Materials

All solvents and reagents were purchased from Sigma-Aldrich, Merck and Fluorochem and were used without further purification. Rice bran and soybean meal (RB and SM) were kindly supplied by Riseria Fossati (Briona, Italy) and ILSA S.p.A. (Arzignano, Italy), respectively. The following carbohydrases, kindly provided by Novozymes® (Bagsværd, Denmark), were used: endo-1,3(4)-β-glucanases (side activities: xylanases, hemicellulases, pectinases) Viscozyme® L derived from *Aspergillus aculeatus* (enzyme activity: 100 FBG g^{-1} (Fungal Beta-Glucanase Units)); Ultraflo® L, another glucanase (side activities: cellulase, xylanase) derived from *Humicola insolens* (enzyme activity: 45 FBG g^{-1}); cellulase Celluclast® 1.5 L derived from *Trichoderma reesei* (enzyme activity: 700 EGU g^{-1} (Endo-Glucanase Units)) and Ceremix® Plus MG, which is a mixture of enzymes (endo-1,4-xylanases, α-amylases, endo-1,3(4)-β-glucanase, neutral proteases; enzymes activity: 130 FXU g^{-1} (Farvet Xylan Units), 115 KNU–B g^{-1} (Kilo Novo Units), 380 BGU g^{-1} (β-glucosidase Units), 0.3 AU-N g^{-1} (Anson Units), respectively) produced by submerged fermentation of several organisms: *Humicola insolens*, *Bacillus amyloliquefaciens*, *Bacillus licheniformis*. ^{1}H NMR experiments were performed in D_2O at 298 K on a 400 MHz Bruker AVANCE 400 spectrometer equipped with TOPSPIN software package (Bruker, Karlsruhe, Germany); chemical shifts (δ) are given in ppm and are referenced to the solvent (δ_H D_2O 4.71 ppm).

2.2. Treatment of defatted rice bran and soybean meal with carbohydrases

Defatted rice bran (DRB), prepared as previously reported (Bagnasco et al., 2013), was suspended in distilled H_2O (10% w/v) and the resulting mixture was treated with carbohydrase(s) (5% w/w_{DRB}) under magnetic stirring (700 rpm). For each enzyme, reaction conditions were set according to optimal temperature and pH using 0.1 M HCl, while regarding enzymes combination, an average of temperature and pH optimal values for single enzymes were used, see Table 1. After 4 h, the enzyme(s) were inactivated by heating at 100 °C for 15 min. Each reaction mixture was then centrifuged at 9000 rpm for 15 min, to separate the supernatant containing mostly the soluble carbohydrates from the DRB enriched in insoluble proteins (DRBP). DRBPs were dried at 60 °C overnight until constant weight. Supernatants were freeze-dried and weighted, and sugar extraction yields of each enzymatic treatment were calculated, see Fig. 1A and Table S1.

The same protocols of carbohydrase(s)-mediated hydrolysis were carried out using soybean meal (SM) as starting material, thus achieving SM enriched in proteins (SMP). Again, sugar extraction yields of each enzymatic treatment were calculated, see Fig. 1B and Table S2. Two control extracts for both DRB and SM (CTR_{DRB} and CTR_{SM}, respectively) were also prepared by following the same procedure (T = 50 °C, pH = 5) without the addition of any enzyme. Sugar extraction yields were calculated (Tables S1 and S2) and both CTR_{DRB} and CTR_{SM} were

Table 1
Optimal pH and temperature values of the commercial carbohydrases used (individually or in combination[b]).

Enzymatic hydrolysis	Enzyme trade name	pH	Temperature (°C)	References
A	Viscozyme® L	5.0	50	Gama, Van Dyk, & Pletschke, 2015
B	Ultraflo® L	4.5	60	Heo et al. (2005)
C	Celluclast® 1.5L	4.5	50	Gama et al., 2015
D	Ceremix® Plus MG [a]	6.0	50	Peng et al., 2019; Tomasik & Horton, 2012; Ao et al., 2018
E	Viscozyme® L + Ultraflo® L	4.7	55	[b]
F	Viscozyme® L + Celluclast® 1.5L	5.0	50	[b]
G	Viscozyme® L + Ceremix® Plus MG [a]	5.5	50	[b]
H	Ultraflo® L + Celluclast® 1.5L	4.5	55	[b]
I	Ultraflo® L + Ceremix® Plus MG [a]	5.3	55	[b]
J	Celluclast® L + Ceremix® Plus MG [a]	5.3	50	[b]

[a] Being a mixture of different enzymes (endo-1,4-xylanases, *α*-amylases, endo-1,3(4)-*β*-glucanase, neutral proteases), optimal conditions were selected as the average of pH and temperature optimal values of the single enzymes.

[b] Reaction conditions for the combination of two carbohydrase formulations were set as the average of pH and temperature optimal values of the individual ones.

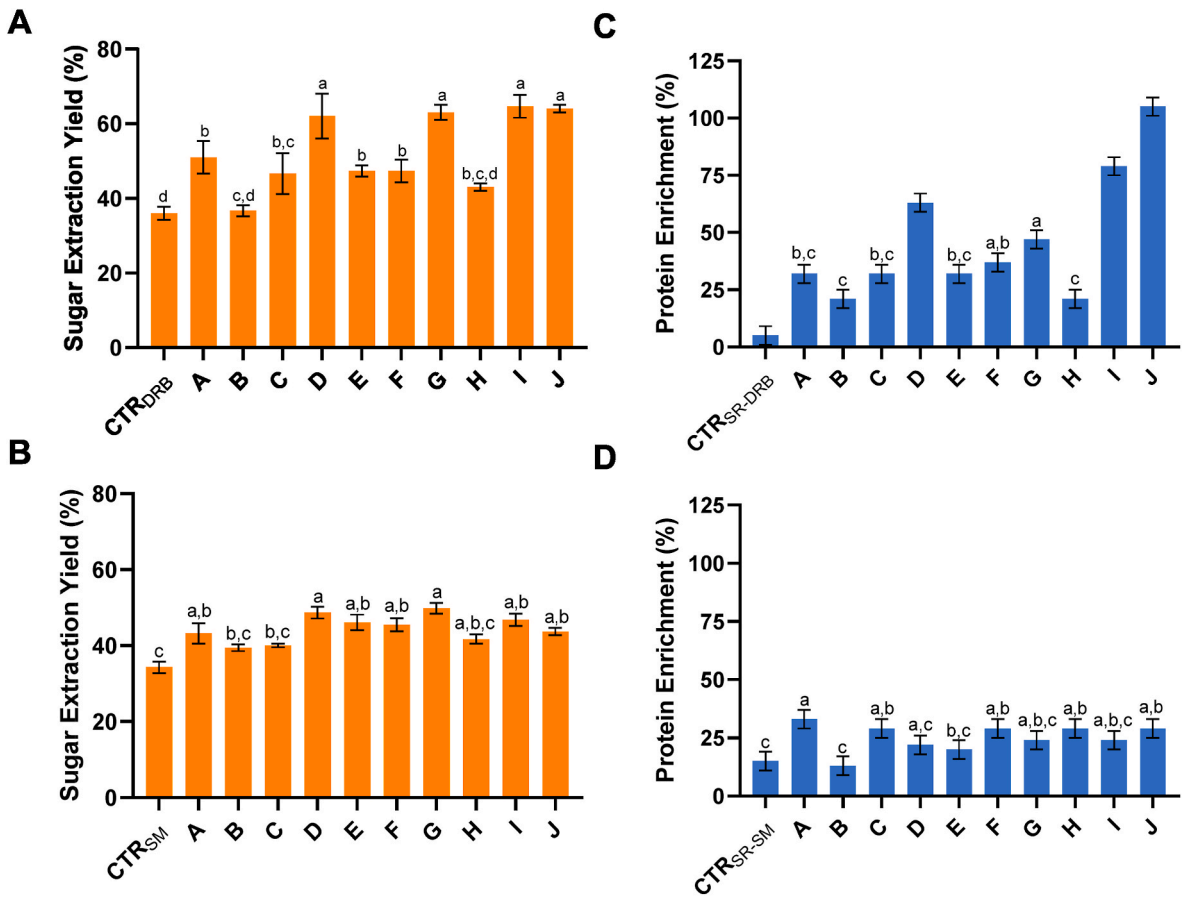

Fig. 1. Sugar extraction yields of (**A**) defatted rice bran (DRB) and (**B**) soybean meal (SM) and protein enrichment values[†] of (**C**) defatted rice bran enriched in protein (DRBP) and (**D**) soybean meal enriched in protein (SMP), as a function of the hydrolysis conditions used (see Table 1). Different letters mean statistically different values ($p < 0.05$).

[†] Calculated as the increase in %P with respect to the starting material ((($\%P_{DRBP}$ - $\%P_{DRB}$)/$\%P_{DRB}$)x100 and (($\%P_{SMP}$ - $\%P_{SM}$)/$\%P_{SM}$)x100, respectively).

submitted to ^{1}H NMR analysis carried out in D_2O, see Figs. S1 and S2.

2.3. Elemental analysis, protein content and enrichment

The protein content of DRBPs and SMPs obtained from each enzymatic hydrolysis was assessed using the AOAC (Association of Official Analytical Chemists) method (Thiex, 2023). A statistical factor of 6.25 was used to convert the results from elemental analysis (Perkin Elmer, Series II CHNS/O analyzer, Perkin Elmer, Massachusetts, USA) into the percentage of protein (%P, protein content). Data are reported in Tables S3 and S4. Moreover, the protein enrichment of DRBPs and SMPs was calculated as the increase in %P with respect to the starting material ((($\%P_{DRBP}$ - $\%P_{DRB}$)/$\%P_{DRB}$)x100 and (($\%P_{SMP}$ - $\%P_{SM}$)/$\%P_{SM}$)x100, respectively) (Fig. 1C and D). Elemental analyses were carried out on the solid residue ($CTR_{SR\text{-}DRB}$ and $CTR_{SR\text{-}SM}$) of control samples (Tables S3 and S4).

2.4. Statistical analysis

Statistical analyses were carried out on GraphPad Prism (8.0.1) software. Data were subjected to one-way ANOVA and the Sidak test was used to compare the means of different variables; different letters, if present, represent significant differences among treatments ($p < 0.05$).

3. Results and discussion

3.1. Composition, sugar extraction yields and protein enrichment of rice bran (RB)

The chemical composition of rice bran (RB) and soybean meal (SM) is significantly different and it is well known: carbohydrates (RB 37–60%; SM 30–35%), proteins (RB 13–19%; SM 44–49%), ash (RB 9–14%; SM 6–8%) and lipids (RB 10–23%; SM 2–3%), see Fig. 2 (Colletti et al., 2020; Fabian & Ju, 2011; Grieshop et al., 2003; Kim et al., 2021; Liu et al., 2021). It is important to underline that RB, being generated during the rice whitening process, still contains a noticeable lipid fraction, unlike SM, which is the residue deriving from the soybean oil production. To avoid RB rancidity problems caused by the presence of lipases (which tend to hydrolyze triglycerides to fatty acids) (Bhardwaj et al., 2001), RB was de-oiled with *n*-hexane, following the procedure reported elsewhere (Bagnasco et al., 2013), thus achieving defatted rice bran (DRB).

DRB and SM were submitted to enzymatic pre-treatments with a series of commercially available carbohydrase formulations, used individually or in combination, under the experimental conditions reported in Table 1. Then, sugar extraction yields (%, w/w) of DRB and SM were evaluated after freeze-drying of the supernatants obtained from each carbohydrase-mediated hydrolysis, in comparison with control treatments (see Tables S1 and S2). Experiments were performed in triplicate at optimal pH and temperature for each enzymatic formulation, while no enzyme was added for control samples. Only soluble sugars were extracted in the supernatants, as confirmed by ^{1}H NMR spectra of

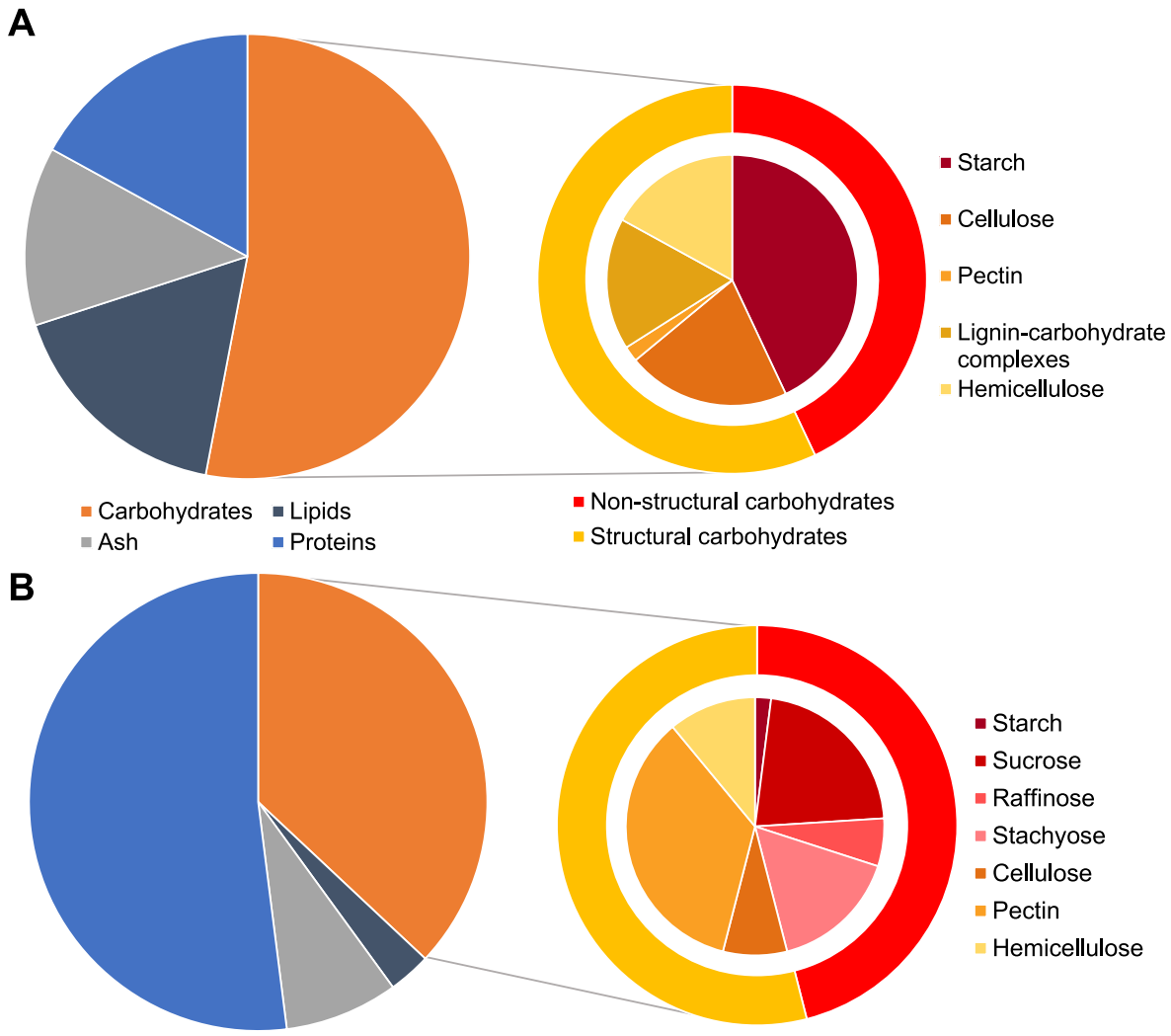

Fig. 2. Rough composition and distribution of types of carbohydrates of (**A**) rice bran (RB) and (**B**) soybean meal (SM).

control samples (CTR_{DRB} and CTR_{SM}), see Figs. S1 and S2. Minor traces of soluble proteins/short peptides and secondary metabolites were also detected. Specifically, glucose signals can be recognized in CTR_{DRB}, related to the high starch content in RB sugar fraction (Fabian & Ju, 2011). On the contrary, ^{1}H NMR spectrum of control SM supernatant (CTR_{SM}) reveals the presence of sucrose, raffinose and stachyose, as main components, according to literature data (Grieshop et al., 2003).

The protein content (%P) of the resulting solid residues from both enzymatic and control treatments, *i.e.*, the protein enriched fractions (DRBPs and SMPs) and control solid residues ($CTR_{SR\text{-}DRB}$ and $CTR_{SR\text{-}SM}$), was evaluated according to the AOAC Dumas method. The protein enrichment (%) was calculated as the increase in %P with respect to the starting material, *i.e.*, 19% and 45% for DRB and SM, respectively (Tables S1 and S2).

Regarding DRB, good sugar extraction yields were obtained with Viscozyme® L and Celluclast® 1.5L (51 ± 3% and 47 ± 3%, enzymatic treatments A and C, respectively). On the other hand, Ultraflo® L (B) was not able to properly hydrolyze sugar-based components of DRB to the same extent, resulting in a sugar extraction yield of only 37 ± 1%. In a recent study (Kim & Lim, 2016), similar sugar extraction yields were obtained using the same enzymes but longer incubation time (12 h *vs* 4 h of the present study), with Viscozyme® L resulting in the best performance (59 ± 2%). However, we found that even higher sugar extraction yields can be obtained using Ceremix® Plus MG (D) and its combination with Viscozyme® L (G), Ultraflo® L (I) and Celluclast® 1.5L (J) (62 ± 4%, 63 ± 1%, 65 ± 2% and 64 ± 1%, respectively), see Fig. 1A and Table S1. These sugar extraction yields are approximately two-time higher than that of control (no enzyme, 36 ± 1%). To the best of our knowledge, no data are reported regarding the use of Ceremix® Plus MG to hydrolyze DRB polysaccharides and only in few studies (Ansharullah et al., 1997; Fabian & Ju, 2011; Kim & Lim, 2016) two commercial carbohydrase formulations in combination are used during enzymatic pre-treatments.

Indeed, focusing on the protein content, Ceremix® Plus MG in combination with Celluclast® 1.5L (J) at T = 50 °C and pH = 5.3 leads to the greatest protein enrichment (+105 ± 4%, see Fig. 1C, starting from the initial value of 19 ± 2% of DRB and of 20 ± 2% of $CTR_{SR\text{-}DRB}$). Despite most of structural carbohydrates in RB are cellulose (21%) and hemicellulose (17%), along with lignin-carbohydrate complexes (17%), see Fig. 2A (Liu et al., 2021; Sapwarobol et al., 2021; Shih et al., 1999), the use of Celluclast® 1.5L, mainly constituted of cellulases, gave insufficient DRB protein enrichment, in agreement with the study of Ansharullah et al. (Ansharullah et al., 1997). On the other hand, its use in combination with a multi-component formulation (Ceremix® Plus MG) is advantageous in degrading the polysaccharide matrix of DRB. Indeed, Ceremix® Plus MG, along with β-glucanase and xylanases, contains α-amylases, which are able to properly hydrolyze starch, a non-structural carbohydrate, that represents roughly the 43% of total RB carbohydrates and hampers the protein extraction (Fig. 2A) (Fabian & Ju, 2011).

3.2. Composition, sugar extraction yields and protein enrichment of soybean meal (SM)

Soybean meal (SM) was submitted to the same carbohydrase-mediated pre-treatments carried out on DRB. However, resulting sugar extraction yields of SM showed no significant differences among each other, according to the Sidak test (Fig. 1B). Furthermore, their values (all around 40–50%) resulted around 1.5 times higher than that of the control (CTR_{SM}, 34 ± 1%).

Despite both SM and RB are composed roughly by equal quantities of structural and non-structural carbohydrates (Fig. 2), their polysaccharide composition is extremely different. Specifically, non-structural carbohydrates of SM are predominantly sucrose (22%) and galactooligosaccharides, *i.e.*, raffinose and stachyose (6% and 16%, respectively) (Islam et al., 2018). Moreover, it is important to highlight that, unlike RB, SM contains only little amounts of starch (about 2%) and relatively lower amounts of cellulose (8%) and hemicellulose (11%), as structural carbohydrates. In SM, this fraction is mainly composed of pectin (roughly 35%), which is a heteropolysaccharide, whose principal chemical component is galacturonic acid (Li et al., 2020). It is known that pectin acts as a protective layer that covers cellulosic microfibrils, partially hindering cellulase activity (Islam et al., 2018). Indeed, the high percentage of cellulases in the carbohydrase formulations does not guarantee the complete hydrolysis of cellulosic fraction in SM, which strictly depends also on pectin hydrolysis (Islam & Ju, 2021).

The protein enrichment values (Fig. 1D) are consistent with sugar extraction yields: no significant differences were obtained among the investigated enzymatic hydrolysis. Indeed, all formulations, except for Ultraflo® L (B), led to a protein enrichment in the range +20–33%, starting from 45% of SM and 52 ± 2% of $CTR_{SR\text{-}SM}$. These results can be due to the specific composition of the used commercially available formulations, containing mainly cellulases, but lacking pectinases. Viscozyme® L (A) represented the only exception: its use led to the highest protein enrichment of +33 ± 4%, which can be related to its xylanases, hemicellulases and pectinases content. The simultaneous use of pectinases and cellulases, both present in Viscozyme® L (A), favours the breakdown of the pectin structure, thus making cellulose more accessible to the attack by cellulase, in agreement with the results reported by Rosset, Acquaro & Beléia using the same enzyme (Rosset et al., 2014).

4. Conclusions

In this study, the protein recovery from two relevant agri-food waste, *e.g.* RB and SM, was evaluated depending on the type of commercially available carbohydrase used in the enzymatic pre-treatment during protein extraction process. According to the rough chemical composition and distribution of types of carbohydrates in RB and SM, we observed that specific carbohydrases were able to selectively hydrolyze polysaccharides contained in these complex agri-food residues rich in protein, thus promoting protein recovery. Although in both cases an increase in the protein extraction was observed, carbohydrase-mediated hydrolysis using these enzyme formulations resulted to be a key step in the case of RB. Specifically, a noticeable protein enrichment was obtained using Ceremix® Plus MG and Celluclast® 1.5L, that are able to hydrolyze cellulose and starch, the main components of RB lignocellulose fraction. On the other hand, the differences between the enzymes employed for SM hydrolysis resulted statistically irrelevant as all carbohydrases were able to partially hydrolyze carbohydrates contained in SM.

CRediT authorship contribution statement

Letizia Scarabattoli: Investigation, Formal analysis, Writing – Original Draft; Sara Sangiorgio: Investigation, Writing – Original Draft; Fabio Romagnuolo: Investigation; Leonardo Gelati: Validation, Formal analysis; Denise Cavuoto: Investigation; Marco Rabuffetti: Writing – Original Draft, Writing – Review & Editing; Carlo Morelli: Validation, Writing – Review & Editing; Stefania Lupinelli: Resources, Project administration; Giovanna Speranza: Conceptualization, Project administration, Supervision.

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.lwt.2023.115060.

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