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Enzymatic hydrolysis and fermentation of soy flour to produce ethanol and soy protein concentrate with increased polyphenols

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Abstract

There is a need to effectively concentrate soy protein from defatted soy flour (DSF) while simultaneously valorizing the carbohydrate-rich byproduct, which would otherwise be a waste. This study aims to evaluate a process developed to produce soy protein concentrates (SPC) by substantially hydrolyzing carbohydrates from DSF with the help of enzymes into water-soluble saccharides and monomeric sugars, which were simultaneously utilized by *Saccharomyces cerevisiae* for fermentation into ethanol. The enzyme mixture consisted of cellulase, pectinase, and α-galactosidase blend. The effect of process time on SPC, overall protein recovery, carbohydrate hydrolysis, yeast growth, ethanol concentration, and total polyphenol concentration (TPC) of SPC and hydrolysate was evaluated. Control and enzymes-only (EO) systems were maintained in conjunction with the enzymes + yeast (EY) system to individually assess the impact of isoelectric precipitation of soy proteins and enzymatic hydrolysis of carbohydrates without yeasts. After 12.25 h of EY process, 100 g of dry DSF produced 68.45 g dry SPC containing 72.23 ± 0.25% protein and 384 ml hydrolysate containing 9.76 ± 0.05 mg/ml ethanol. Flatulence-causing raffinose-series-oligosaccharides (RSOs) were completely hydrolyzed. Soluble carbohydrates in the EY treatment were consistently lower than in the control and EO treatment. TPC of SPC prepared by EY treatment increased by 2.5 times compared to the control. Thus, this novel process successfully produced a high-protein SPC with hydrolyzed RSOs, lower insoluble carbohydrates, high TPC, and a coproduct ethanol.

KEYWORDS
enzymatic hydrolysis, ethanol, fermentation, raffinose, soy protein concentrate

INTRODUCTION

Soy proteins are the largest source of low-cost plant-based dietary proteins. Soy protein products are classified into three categories based on protein content on dry matter basis: defatted soy flour (DSF) (50% protein), soy protein concentrate (SPC) (>65% protein), and soy protein isolate (SPI) (>90% protein) (Wang et al., 2004). Historically, SPC is a newer product, developed in 1959 from DSF, primarily to increase the protein content, decrease the beany off-flavor, and perform functional tasks which cannot be achieved by soy flour (Berk, 1992). DSF contains 30%–35% carbohydrates, which are divided into sucrose, water-soluble but flatulence-causing raffinose-series-oligosaccharides (RSOs) such as raffinose, stachyose, and verbascose, and water-insoluble polysaccharides such as cellulose, hemicellulose, and pectic polysaccharides (Refstie et al., 2005).

Abbreviations: ADF, acid detergent fiber; ADL, acid detergent lignin; ANOVA, analysis of variance; CFU, colony-forming unit; DRBC, dextrose rose bengal chloramphenicol; DSF, defatted soy flour; EO, enzymes only; EY, enzymes + yeast; FBGU, fungal β-glucanase unit; FC, Folin–Ciocalteu; FCR, Folin–Ciocalteu reagent; GAE, gallic acid equivalent; GalA, galacturonic acid; GalU, galactose unit; GE, glucose equivalent; HPLC, high-performance liquid chromatography; HSD, honest significant difference; NDF, neutral detergent fiber; RSO, raffinose series oligosaccharides; SPC, soy protein concentrate; SPI, soy protein isolate; SSF, simultaneous saccharification and fermentation; TPC, total polyphenol concentration.

SPC is made by partial removal of water-soluble carbohydrates from DSF. This process is commercially performed using three different methods: (1) aqueous alcohol wash process, (2) acid-wash process, and (3) heat denaturation process (Berk, 1992). The aqueous alcohol wash process works on the principle of extracting soluble carbohydrates without solubilizing globulin proteins from DSF using ethanol as a solvent. This process essentially rids the SPC matrix of polyphenols, which are micronutrients associated with potential health benefits, since polyphenols are preferentially more soluble in aqueous ethanol (Jokic et al., 2010). Acid-wash process exploits the principle of isoelectric precipitation, where the majority of soy proteins are the least soluble at pH 4.2–4.5, and, hence, are precipitated along with insoluble fiber, allowing water-soluble carbohydrates to be extracted from the matrix (Berk, 1992). However, complete removal of soluble carbohydrates is not achieved due to diffusional limitations (Al Loman et al., 2016). Hence, a large ratio of wash solvent to DSF is used, producing a large wastewater stream called "soy solubles" (Wang et al., 2004). The SPC from this process also has lower levels of total polyphenols than DSF because a significant portion of polyphenols in their native state are preferentially hydrophilic (Di Lorenzo et al., 2021) and thus lost with the waste stream. Heat denaturation or water wash process renders the soy protein insoluble by thermal denaturation and then allows its separation along with insoluble fiber, similar to the acid-wash process. This process follows similar drawbacks as the acid-wash process, and irreversibly denatures the soy proteins, making them functionally inactive (Berk, 1992). The polyphenol content of SPC is also significantly reduced (Wang et al., 2004).

The processes mentioned above do not remove any of the insoluble carbohydrates. SPI is produced by alkaline solubilization of proteins to remove insoluble fiber first, followed by isoelectric precipitation of proteins to remove soluble sugars. However, the protein recovery from this process is low (~60%) because one of the byproducts, "okara," takes away ~15% protein from the end product (Berk, 1992). The other byproduct, soy "whey," usually contains 1%–3% solids content and makes up for a costly effluent to discard (Alibhai et al., 2006). The polyphenol concentration is also significantly reduced per-unit amount of protein (Wang et al., 2004).

Cellulose and hemicellulose, comprising the bulk of insoluble fiber in DSF, can be hydrolyzed effectively by physical/chemical treatments (Walker & Wilson, 1991). However, the treatment is too harsh to be used on DSF with any intention of preserving the native structure of soy proteins. An enzymatic approach, thus, is a much milder way to hydrolyze the insoluble carbohydrates into smaller, soluble ones. Processes involving enzymatic hydrolysis of some or all of soy carbohydrates (Al Loman et al., 2016; Jacobsen et al., 2018) and simultaneous yeast fermentation (Long & Gibbons, 2012) have been investigated. However, many of those processes did not study the production of SPC. The process that did produce SPC (Al Loman et al., 2016) did not utilize simultaneous microbial fermentation to consume the monomerized sugars. Corn processing industries frequently utilize enzymatic hydrolysis with simultaneous yeast fermentation, also referred to as simultaneous saccharification and fermentation (SSF), for ethanol production from corn stover (Alfani et al., 2000). Alfani et al. (2000) have found that SSF has significant advantages of being time-efficient, cost-effective due to the use of fewer bioreactors, and preventing enzyme inhibition. This made an extrapolation of SSF on soy flour desirable with modified objectives. There is a continuous removal of hydrolysis end-products in SSF, which has the potential to reduce contamination risk (Li et al., 2018). However, that aspect was not explored in this study.

Also, to the best of our knowledge, there has not been work done to investigate the effect of the hydrolytic enzymes on total polyphenol content of the protein end product. With the rising interest in plant-based meats, certain polyphenols play a major role in the stability of the end product (Carocho et al., 2014). Hence, we decided to investigate the process of enzymatic hydrolysis and simultaneous yeast fermentation of DSF to produce SPC. The proposed process is similar to the acid-wash process, but has the potential to significantly reduce the time required to concentrate the proteins and also not require multiple wash steps to enrich the protein content. The objective of this study was, thus, to test the hypotheses that this process (1) produces an SPC with protein content greater than that in SPC obtained from an equivalent acid-wash process, (2) hydrolyzes carbohydrates to a greater extent than an equivalent enzymatic process without simultaneous yeast fermentation, (3) allows the formation of a coproduct ethanol, and (4) causes the total polyphenol concentration (TPC) of SPC and hydrolysate to change significantly as compared to the SPC and supernatant produced by an equivalent acid-wash process.

MATERIALS AND METHODS

Chemicals and materials

DSF (7B Baker's Soy Flour) was provided by Archer Daniel Midlands (ADM, Decatur, IL, USA). The chemical reagents Folin–Ciocalteu reagent (FCR), cellulase blend (Novozyme Viscozyme L, 130 fungal β-glucanase unit [FBGU]/ml), pectinase blend (Novozyme Pectinex Ultra SPL, 4236 U/ml), and chloramphenicol were obtained from Sigma-Aldrich (St. Louis, MO, USA). Active dry yeast *Saccharomyces cerevisiae* (Red Star®) and α-galactosidase enzyme produced by *Aspergillus niger*

and sold as commercial dietary supplement Beano® (400 galacturonic acid unit [GalU]/tablet) were purchased from a local grocery store (Champaign, IL, USA). Vendor literature was used to estimate enzyme activities. Dextrose rose bengal chloramphenicol (DRBC) agar was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Liquid silicon antifoam (Fermfast, LD Carlson, Kent, OH, USA) was purchased from a local brewing supply store. All other reagents used were of analytical grade, and sugar standards, glycerol, and ethanol used were of high-performance liquid chromatography (HPLC) grade and purchased from Sigma-Aldrich (St. Louis, MO, USA).

Batch experiments in the centrifuge tubes

Batch experiments of enzymatic hydrolysis and yeast fermentation were done in 50 ml centrifuge tubes in biological triplicate (i.e., a separate batch of yeast inoculum was used for every replicate). Each tube contained 25 ml of slurry, which was a 15% (w/w) suspension of DSF in water along with enzyme mixture and yeast inoculum. Preliminary studies (not shown) with higher solid loadings (up to 25% w/w suspension) pointed out agitation limitations in the current experimental setup by requiring much longer times (up to 45 h) to hydrolyze carbohydrates effectively. This allowed microbial spoilage products (acetic and lactic acid) to increase in the product matrix as a consequence. Thus, higher solid loadings were not evaluated in this study.

The enzyme mixture was made such that, when added to the slurry, it would constitute 8.1 FBGU/g DSF of cellulase blend, 43.6 U/g DSF of pectinase blend, and 10.4 GalU/g DSF of α-galactosidase blend on dry matter basis.

Yeast inoculum was prepared by activating 1:5 (w/w) active dry yeast in sterile distilled water in an orbital shaker for 30 min at 39°C. Fermentation was started with 2% (v/w) inoculum in the slurry.

During our preliminary investigation, we realized that foam was formed while bringing the pH of slurry closer to the isoelectric point of soy proteins (pH 4.5). We also noted that this foam not only caused incorrect pH adjustment but also hampered enzyme dosing and yeast inoculation, resulting in under-catalyzed hydrolysis and subsequent fermentation. Hence, before adjusting the pH of the slurry, liquid antifoam at 0.8 mg/ml slurry was added to prevent foaming. Bulk 1-L slurry was adjusted to pH 4.5 with 12 M hydrochloric acid by stirring with a magnetic stir bar at 25°C in a 2 L beaker. Finally, 0.2 mg/ml chloramphenicol was added to avoid bacterial contamination.

After the simultaneous addition of enzyme mixture and yeast inoculum, the slurry was incubated at a 45° angle on a custom fabricated inclined-tube rack fixed on an orbital shaker at 150 rpm and 39°C. Samples were taken immediately after enzyme addition and yeast inoculation and at 4, 8, and 12 h, respectively, by withdrawing the entire tube from the incubator. The contents of the tubes were boiled on a heating block for 15 min to deactivate enzymes and yeasts. Subsequently, the slurry was centrifuged at 4696 × *g* at 4°C for 1 h to separate the solids and supernatant. The supernatant was decanted and termed hydrolysate. The solids were freeze-dried, ground, and were denoted as SPC. Figure 1 summarizes the entire process in the form of a flow chart with mass balance.

Two additional systems, namely an enzymes-only (EO) system (without yeasts) and a control system (without enzymes or yeasts), were maintained in each batch of experiments to investigate and distinguish the effect of isoelectric precipitation of soy proteins and the impact of enzymes without yeasts in this process of producing SPC. The control system consisted of an equal volume of sterile deionized water in place of enzyme mixture and yeast inoculum, whereas the EO system consisted of the enzyme mixture but an equal volume of sterile deionized water in place of yeast inoculum. All three systems were prepared in bulk first to ensure consistent DSF loading, enzyme dosing, and yeast inoculation, and were then distributed in triplicate in sterile centrifuge tubes corresponding to 0.25-, 4.25-, 8.25-, and 12.25-h process times, respectively.

Analytical procedures

Proximate composition

DSF, SPC, and active dry yeast were analyzed for nitrogen by Dumas combustion method 990.03 (AOAC, 2005) using a Leco FP628 analyzer (Leco Corp., St. Joseph, MI, USA). A conversion factor of 6.25 was used to estimate crude protein content, which is represented on dry basis. Moisture content was analyzed by drying at 105°C in a convection oven until a constant weight was achieved according to standard National Renewable Energy Laboratory method (Sluiter et al., 2008). Fat and ash content of DSF were measured by official method 945.39 (AOAC, 2005). Carbohydrate concentration was calculated by difference. Soluble and insoluble dietary fiber in DSF was measured based on method 991.43 (AOAC, 2005) using Ankom Fiber Analyzer (Ankom Technology, Macedon, NY, USA), and acid detergent fiber (ADF), neutral detergent fiber (NDF), and acid detergent lignin (ADL) by Ankom Technology methods 12, 13, and 9, respectively (Ankom A2000 Fiber Analyzer, Ankom Technology, Macedon, NY, USA). Cellulose was calculated as ADF-ADL and hemicellulose was calculated as NDF-ADF. Soluble dietary fiber was assumed to represent pectic polysaccharides. Proximate composition of DSF is given in Table 1.

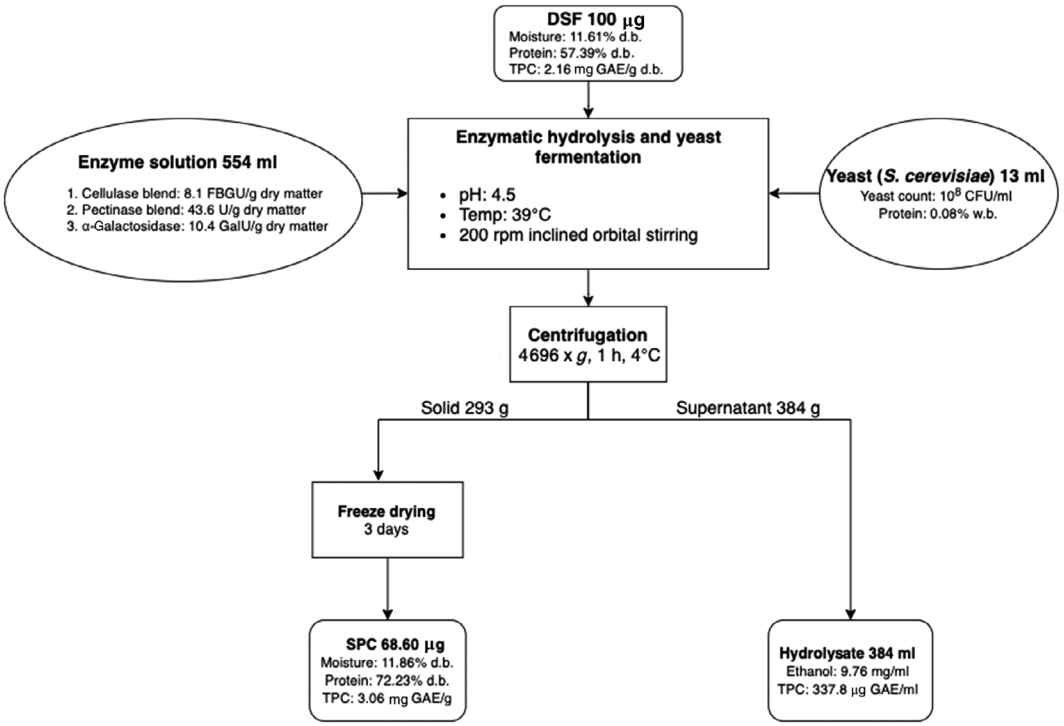

FIGURE 1 Flowchart with material balance for the production of SPC via enzymes and yeast treatment for 12.25 h

TABLE 1 Proximate composition of defatted soy flour

Component	Composition (% wet basis)
Moisture	7.2 ± 0.1
Protein	50.9 ± 0.1
Fat	1.2 ± 0.3
Ash	6.5 ± 0.1
Carbohydrate	34.2 ± 0.3
Dietary fiber	23.0 ± 3.7
Soluble dietary fiber	5.5 ± 0.7
Pectic polysaccharides	5.5 ± 0.7
Insoluble dietary fiber	17.5 ± 3.6
Cellulose	6.8 ± 2.2
Hemicellulose	10.2 ± 2.8
Lignin	0.6 ± 0.3
Sugars	11.4 ± 0.2
Stachyose	3.1 ± 0.1
Raffinose	1.9 ± 0.1
Sucrose	6.4 ± 0.2

Note: Sum of components: 100.2 ± 3.7%.

Carbohydrate and ethanol analysis

The hydrolysate was boiled on a heating block for 15 min to denature and precipitate water-soluble peptides. It was then centrifuged for 20 min at 4696 × *g*, 4°C, and aliquots of supernatant were taken for subsequent analyses. Stachyose, raffinose, sucrose, galacturonic acid, glucose, fructose, xylose, galactose, glycerol, and ethanol were quantified using HPLC (Agilent 1200 Series; Agilent Technologies, Santa Clara, CA) with a refractive index detector and the Rezex ROA-Organic acid H+ (8%) column (Phenomenex Inc., Torrance, CA). Isocratic elution with 5 mM H_2SO_4 mobile phase, 50°C column temperature, and 0.6 ml/min flowrate was used. All samples were centrifuged at 17,000 × *g* for 30 min, followed by filtration through a 0.45-μm syringe filter before chromatographic analysis.

Total soluble carbohydrate concentration was estimated by phenol sulfuric acid assay. Briefly, to 150 μl of sample's supernatant, 500 μl concentrated sulfuric acid and 150 μl freshly prepared 5% phenol were added in rapid succession. The mixture was vortexed, then heated in a boiling water bath for 5 min. After cooling for 5 min in an ice bath, the solution was further vortexed, then centrifuged at 2000 × *g* for 1 min, and its absorbance at 490 nm was measured in a spectrophotometer. The quantification was done with glucose as reference. A 1:100 dilution of the sample's supernatant was used to fit in the range of this assay (100–400 μg glucose equivalent [GE]/ml).

Yeast enumeration

Before the slurries were boiled at the end of each treatment's process, an aliquot was collected and cooled in a 25°C water bath and stored for 20 h at 4°C till enumeration. Serial dilutions of this sample were inoculated by

spot plating, and enumeration was carried out on DRBC agar plates, incubated at 30°C for 24 h. The results are reported as $\log_{10}$ colony-forming units (CFU)/ml slurry.

Total polyphenol content determination

Polyphenols were extracted from DSF, SPC, and hydrolysate by the method of Georgetti et al. (2009). One-half gram of solid sample or 0.5 ml of liquid sample was mixed with 80% methanol (1:10 w/v or v/v) and put under agitation in dark for 2 h at 25°C. A 1-ml aliquot was then centrifuged at 17,000 × *g* for 20 min. A 1:20 dilution of the resultant supernatant extract of DSF and SPC samples and undiluted supernatant extract of the hydrolysate were used for the subsequent analysis.

TPC was measured by the reduction of FCR with a slight modification in the method of (Mujić et al., 2011). Specifically, 500 μl of the final extract mentioned above was mixed with 63 μl of 2 N FCR and vortexed. After 4 min, 375 μl 10% sodium carbonate was added and vortexed again. The mixture was then incubated at 40°C for 30 min in a water bath, followed by 1 min in an ice bath. Finally, the mixture was centrifuged at 2000 × *g* for 1 min, and its absorbance was measured at 765 nm in a spectrophotometer. Gallic acid dilutions in 80% methanol were used for making the calibration curve (5–40 μg/ml), and the results were expressed in gallic acid equivalents (GAE).

Statistical analysis

All batch experiments were carried out in biological triplicates. A separate batch of yeast inoculum was used for each replicate to include the variations of commercial manufacture of instant dry yeast in the standard deviation. Every replicate's protein and moisture assays, HPLC analyses, and yeast enumeration plates were repeated twice; total soluble carbohydrate and TPC assays were repeated thrice. The data are expressed as mean ± standard deviation of six or nine values accordingly. One-way analysis of variance (ANOVA) was performed to examine the differences across treatments. For the datasets where the residuals were not normally distributed, Box-Cox optimized transformation was applied. Post hoc analyses were done using Tukey's honest significant difference (HSD) test to compare means across all treatments. All statistical analyses were done using *R*.

RESULTS AND DISCUSSION

Protein enrichment in SPC

Globulins are storage proteins, which according to Osborne classification, are insoluble in water, but soluble in dilute saline (Chéreau et al., 2016). They make up to 90% of soybean seed proteins (Chéreau et al., 2016). Globulins' isoelectric precipitation at pH 4.5 was leveraged in this study to produce SPC while carrying out enzymatic hydrolysis of carbohydrates within the range of enzymes' optimum pH conditions. Protein concentrations of SPC produced by control, EO, and enzymes + yeast (EY) treatments as a function of process time are given in Figure 2. Water-soluble carbohydrates such as stachyose, raffinose, and sucrose were partially separated in all treatments, allowing the protein concentration to increase. The protein concentration of control SPC did not increase after

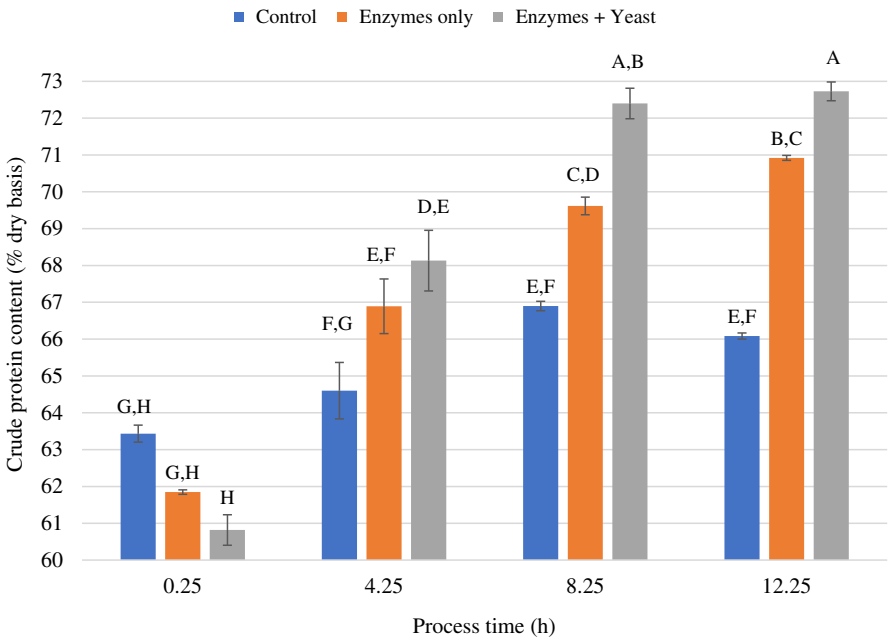

FIGURE 2 Protein content of SPC prepared by control, enzymes only, and enzymes + yeast treatments as a function of process time. Error bars represent SE ($n = 6$). Treatments with the same letters are not significantly different ($p < 0.05$, Tukey's honest significant difference test)

4.25 h, suggesting that solubilization of carbohydrates had peaked. In the EO treatment, carbohydrate hydrolyzing enzymes were breaking down cellulose, hemicellulose, and pectin into smaller polysaccharides, thereby making them water-soluble and subsequently allowing its separation from the protein matrix. This process allowed the protein to be enriched in EO SPC compared to control SPC, as can be seen for process times 8.25 and 12.25 h, respectively. Protein enrichment of SPC produced by EY treatment can be attributed, in part, to the inoculation of yeasts, as active dry yeast contained 47.68 ± 0.22% protein (N × 6.25) on dry basis. However, protein from *S. cerevisiae* has an isoelectric point of pH 3.2 (Yamada & Sgarbieri, 2005), which suggests that not all yeast protein precipitated at pH 4.5 along with soy proteins, and instead, may have escaped in the hydrolysate. Hence, the inoculation of yeasts alone is not enough to explain the increasing difference in protein concentration of this treatment's SPC as the processing time increased. We hypothesize that this increase is due to the synergy of enzymatic hydrolysis and yeast fermentation: as the carbohydrates were being hydrolyzed, yeasts were simultaneously able to ferment them into ethanol, thereby alleviating the product-inhibition effect on the enzymes (Andrić et al., 2010). The enzymes, in turn, were able to solubilize and separate more water-insoluble polysaccharides. Hence, the protein concentration of SPC prepared by EY treatment was significantly higher ($p < 0.05$) than the one produced by the EO treatment for 8.25- and 12.25-h processes (Figure 2).

SPC prepared by EY method is already toward the upper end of protein concentration (70%–75%) in commercial SPC manufacture (Berk, 1992), which comprises refining of SPC by multistage washing. However, this SPC was collected by centrifugation without washing, which caused it to retain 76.8 ±1.2% liquid. This liquid contained the same soluble solids present in the hydrolysate, thereby not enabling its separation from the protein matrix. The results from Al Loman et al. (2016) show that after washing with 60% ethanol with a solvent-to-solid ratio of 10, the protein concentration of centrifuged and dried SPC increased from 69.5% to 81.4%. This increase, however, came with a loss of SPC recovery from 58.8% to 48.5% and protein recovery from 71.5% to 69%. This suggests that a similar washing technique could be employed for EY SPC, if SPC higher than 72% protein concentration is desired for a specific application.

Because the protein concentration of SPC with EY treatment was not significantly different ($p < 0.05$) for 8.25- and 12.25-h processes, it was assumed that no further protein enrichment would be obtained under the present experimental conditions. Hence, SPC yield and protein recovery were compared and evaluated across treatments for a 12.25-h process. SPC yield was calculated as the amount of SPC obtained from 100 g of DSF on a moisture-free basis. Protein recovery was calculated as the amount of protein retained as SPC from total protein in DSF on a moisture-free basis (Table 2). The reduction of SPC yield from EY treatment as compared to control is rational since the control SPC contained a significant amount of carbohydrates, which were hydrolyzed in the EY treatment. Hence, a lesser amount of higher protein SPC was produced. However, protein recovery decreased significantly ($p < 0.05$) compared with control. We hypothesize that the amino acids from soy proteins were used to make yeast proteins, which did not necessarily precipitate at pH 4.5. Also, commercial enzymes, like the ones used in this study, contain some amount of proteases (Ribeiro et al., 2013), which could have caused the native soy proteins to break down into smaller peptides, which also did not precipitate at pH 4.5, and possibly escaped into the hydrolysate matrix, essentially becoming unrecoverable.

Carbohydrate hydrolysis and fermentation analysis

Figure 3 compares the HPLC chromatograms of water-soluble carbohydrates, glycerol, and ethanol from the hydrolysate of three treatments for a processing time of 12.25 h. One shortcoming of the HPLC column used in this study was that xylose, fructose, and galactose would co-elute, and hence, were not individually quantified because their retention times in the chromatograms were very close (9.134, 9.289, and 9.181 min, respectively). Therefore, xylose, fructose, and galactose are shown as an aggregate. Figure 4 shows the individually quantified carbohydrates, glycerol, and ethanol in stacked form as functions of process time and treatments.

TABLE 2 Comparison of soy protein concentrate (SPC), SPC yield, and protein recovery from different treatments after 12.25 h of process time

Treatment	SPC protein content (%)	SPC yield (g/100 g DSF)	Protein recovery (%)
Control	65.58 ± 0.08^{A}	81.66 ± 0.39^{A}	93.3 ± 0.01^{A}
Enzymes only	70.42 ± 0.07^{B}	69.00 ± 0.28^{B}	83.8 ± 0.01^{B}
Enzymes + yeast	72.23 ± 0.25^{C}	68.45 ± 0.71^{B}	84.4 ± 0.01^{B}

Note: Different letters across the rows in the same column indicate significant statistical differences ($p < 0.05$, Tukey's honest significant difference test).
Abbreviation: DSF, defatted soy flour.

Effect of hydrolysis on water-soluble carbohydrates

Figure 3 shows the presence of water-soluble carbohydrates, which included oligosaccharides such as stachyose, raffinose, and sucrose, along with a small amount of monosaccharide glucose in control hydrolysate. α-Galactosidase catalyzes the hydrolysis of stachyose and raffinose into their component sugars galactose and sucrose. From Figure 4, it can be seen that stachyose was completely hydrolyzed in both EO and EY treatments in the first 4.25 h itself due to the catalysis by α-galactosidase. It was also noticed that longer process times (8.25 and 12.25 h) significantly ($p < 0.05$) enhanced stachyose extraction in the control treatments, as can be seen by increasing concentration of stachyose in Figure 4. This suggested that more stachyose must have gotten extracted in EO and EY treatments as well and was completely hydrolyzed due to the action of α-galactosidase. Figure 4 also suggests that raffinose, whose extraction from control was similarly enhanced as stachyose, was 83% and 87% hydrolyzed in EO and EY treatments, respectively. The hydrolysis products, that is, sucrose, glucose, fructose, and galactose, are shown in Figure 4. Sucrose concentration was significantly lower than control throughout the entire process for both EO and EY treatments. Invertase catalyzes the hydrolysis of sucrose into glucose and fructose. Cao et al. (2016) have previously reported invertase as the second enzyme in Beano® tablets, which was used as a source of α-galactosidase enzyme in this study. Thus, the presence of invertase explains the hydrolysis of sucrose in EO treatment. *S. cerevisiae* is also a known producer of invertase (Carlson et al., 1983). Hence, we hypothesize that sucrose could have been hydrolyzed in EY treatment partially due to the effect of invertase produced by yeasts as well.

Glucose concentration was negligible in control throughout the process but was increasing significantly in the EO treatment until 8.25 h due to the combined effect of invertase acting on sucrose as well as cellulase acting on cellulose (Figure 4). We hypothesized that the bulk of glucose came from the hydrolysis of sucrose, stachyose, and raffinose, and in some parts,

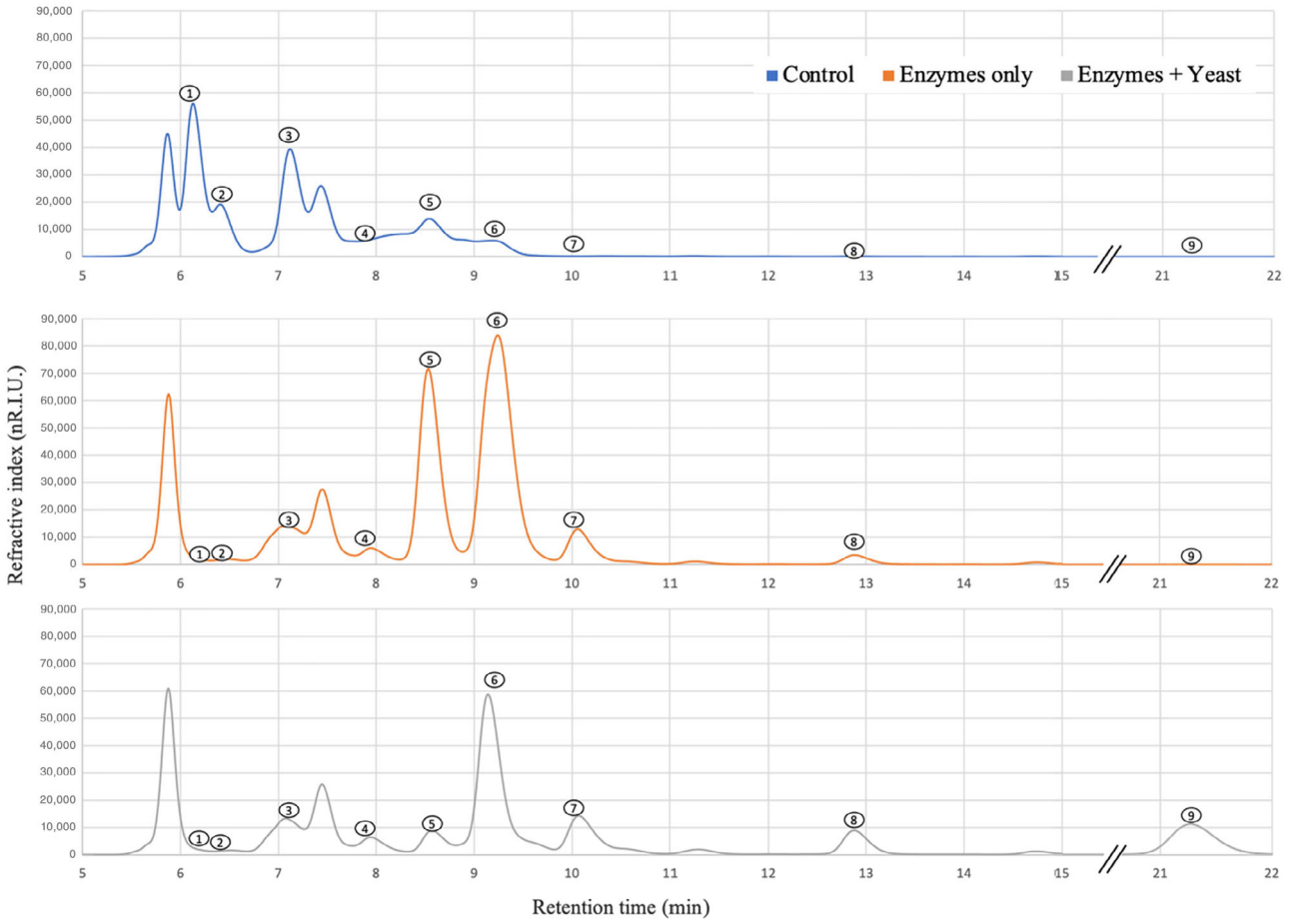

FIGURE 3 High-performance liquid chromatograms of hydrolysates after 12.25-h process, representing soluble saccharide profile, glycerol, and ethanol. (1) Stachyose, (2) raffinose, (3) sucrose, (4) galacturonic acid, (5) glucose, (6) xylose + galactose + fructose, (7) arabinose, (8) glycerol, (9) ethanol

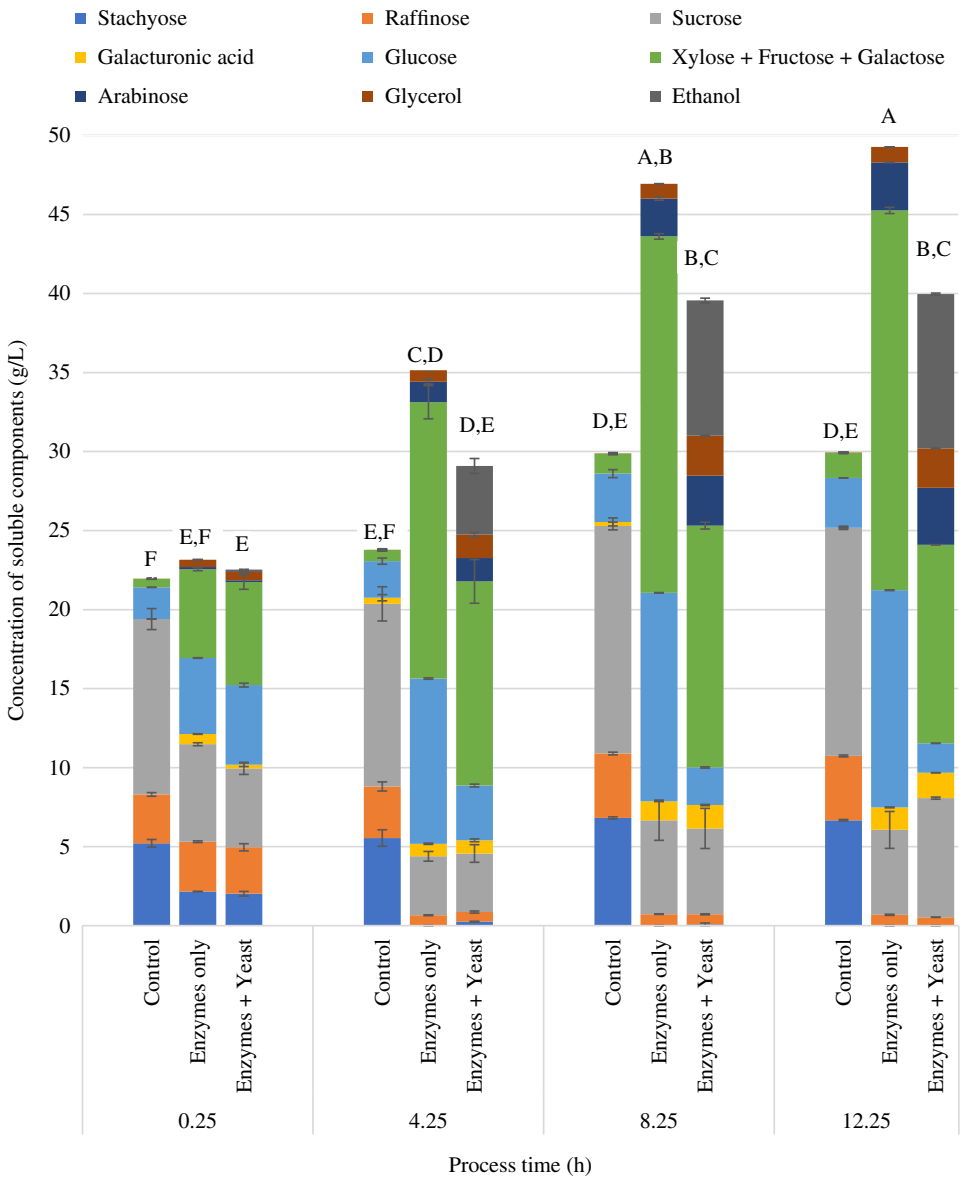

FIGURE 4 Soluble components as identified by high-performance liquid chromatography in hydrolysate of control, enzymes only, and enzymes + yeast treatments as functions of process time. Error bars represent SE ($n = 6$) of individual components. Sum of components with the same letters are not significantly different ($p < 0.05$, Tukey's honest significant difference test)

from the hydrolysis of cellulose. Ouhida et al. (2002) have reported that various commercial carbohydrase enzymes, including Viscozyme L, solubilize soybean hull cellulose and hemicellulose but do not completely monomerize them. Hence, we hypothesized that the cellulose in DSF matrix was also not completely monomerized due to the action of Viscozyme L's cellulase. Glucose was consumed significantly throughout the EY process and fermented by yeasts into ethanol (Figure 4). Fructose and galactose, quantified collectively with xylose (Figure 4), showed an increase in concentration in EO treatment compared to control throughout the process. At the same time, a decrease in their aggregate concentration in EY treatment was observed, suggesting that yeasts were able to metabolize some fructose and galactose to ethanol. It is well known that yeasts preferentially metabolize glucose, fructose, and then galactose (Huisjes et al., 2012).

Effect of hydrolysis on water-insoluble carbohydrates

Water-insoluble carbohydrates in DSF included cellulose, hemicellulose, and pectic polysaccharides (Table 1). Lignin, which is not a carbohydrate, is associated with these cell-wall polysaccharides (Knudsen, 1997). Pectic polysaccharides include homogalacturonans (commonly referred to as pectin) and rhamnogalacturonans type I and II, which are polymers made by alternating rhamnose and galacturonic acid backbones, with sidechains containing varying amounts of arabinose, galactose, fucose, and xylose (Navarro et al., 2019). Rhamnose and fucose were not analyzed in this study. Pectin, which by itself is a soluble fiber, is unextractable by water in the DSF matrix because it is linked to other cell-wall polysaccharides (Ouhida et al., 2002). Viscozyme L is a multicomponent carbohydrase derived from *Aspergillus aculeatus*, which

contains arabanase, cellulase, β-glucanase, hemicellulase, and xylanase (vendor literature). Accordingly, its primary rationale of inclusion was to convert insoluble polysaccharides such as cellulose and hemicellulose into their smaller, soluble constituents. Because we hypothesized that cellulose was not completely monomerized, we were unable to quantitatively determine the hydrolysis of cellulose on the basis of glucose released from the DSF matrix.

Figure 4 also shows that the concentration of arabinose, a monomer primarily associated with hemicellulose, is nonexistent in control's hydrolysate throughout the process but steadily increasing in EO and EY treatment's hydrolysate. This suggested that hemicellulose was getting hydrolyzed into its smaller, water-soluble saccharides. However, like cellulose, hemicellulose was also assumed not to be completely monomerized. Comparison between EO and EY treatments for 8.25- and 12.25-h processes suggests that there is slightly, but statistically significant ($p < 0.05$), more arabinose in the latter's hydrolysate. It was not clearly distinguished if there was more hydrolysis of hemicellulose, more monomerization of subunits of hemicellulose to arabinose, or both, but we were able to conclude that the synergism of enzyme hydrolysis and yeast fermentation did increase the amount of reducing sugar and potential feedstock for bioethanol production.

Figure 4 also shows the concentration of xylose, another monosaccharide found in hemicellulose, as aggregated with fructose and galactose. We were unable to distinguish changes in xylose concentration as a function of process time because xylose concentration is shown as an aggregate with fructose and galactose. However, baker's yeast, *S. cerevisiae*, does not naturally contain the pathways to metabolize pentose sugars such as xylose and arabinose, but it ferments hexoses like fructose and galactose (Fernandes & Murray, 2010). There was also a visible difference in the retention time of the aggregate of xylose, fructose, and galactose in EO and EY treatments' hydrolysate (Figure 3); the aggregate's peak in the chromatogram of EY treatment's hydrolysate had visibly shifted closer to the elution time of xylose. Thus, these two facts led us to hypothesize that the majority of the aggregate concentration of xylose + fructose + galactose in EY treatment's hydrolysate at 12.25 h represented xylose, followed by small amounts of unfermented galactose and fructose. Although the extent of hydrolysis of cellulose and hemicellulose was not clearly distinguishable, it is clear that the cellulase blend was instrumental in hydrolyzing insoluble carbohydrates such as cellulose and hemicellulose, hence allowing subsequent enrichment in protein concentration in the respective treatments' SPC.

Pectinex Ultra SPL is a pectinase enzyme derived from *A. aculeatus* and contains polygalacturonase, pectin methylesterase, and pectin transeliminase, followed by small amounts of cellulase and hemicellulase activities (vendor literature). Partial hydrolysis of hemicellulose and pectic polysaccharides contributed to galactose concentration of the hydrolysate in EO and EY treatments along with hydrolysis of stachyose and raffinose; however, its exact contribution was unable to be determined (Figure 4). The release of galacturonic acid in the hydrolysate (Figure 4) represents hydrolysis of galacturonan polymers of pectic polysaccharides by the pectinolytic enzymes blend. However, low concentration of galacturonic acid in EO and EY treatments' hydrolysate suggests that complete hydrolysis of pectic polysaccharides did not occur, as Knudsen (1997) has reported 4.8 ± 0.5% uronic acids in soybean meal, which correlates to 10.3 ± 1.1 mg GalA/ml hydrolysate in our study. Galacturonic acid release was significantly ($p < 0.05$) enhanced by the presence of yeast in 8.25- and 12.25-h EY processes. Partial hydrolysis of pectin and incomplete monomerization has also been reported by Rommi et al. (2014) during Pectinex enzyme aided cell wall disintegration on protein extractability from intact and dehulled rapeseed press cakes. Wild-type *S. cerevisiae* is known to be unable to metabolize galacturonic acid (Huisjes et al., 2012). However, it is also reported that a relatively low (2.5 g/L) concentration of galacturonic acid in acidic conditions inhibits galactose fermentation in wild-type *S. cerevisiae* and xylose and arabinose fermentation in *S. cerevisiae* engineered for pentose fermentation (Huisjes et al., 2012). This suggests that incomplete monomerization of pectin into galacturonic acid is a desirable aspect as long as pectin is hydrolyzed into smaller fragments, allowing easy separation from protein fraction by water washing.

Effect of yeast fermentation on hydrolyzed carbohydrates

Figure 4 shows the reduction in glucose, fructose, and galactose concentrations and increase in ethanol concentrations in the EY hydrolysate, suggesting that wild-type *S. cerevisiae* was able to ferment significant amounts of glucose, fructose, and galactose in the DSF slurry into ethanol. Meanwhile, the significant ($p < 0.05$) increase in arabinose, galacturonic acid, and possibly xylose concentrations in the hydrolysate of EY treatment compared to EO treatment also suggests that yeast fermentation had a synergistic effect on enzymatic hydrolysis of water-insoluble carbohydrates. Figure 5, which shows total soluble carbohydrates in the hydrolysate as a function of process time, suggests that yeasts were able to metabolize the fermentable feedstock as it was being made available by the hydrolyzing action of enzymes. Thus, it can be concluded that the simultaneous fermentation due to yeasts is able to reduce the product inhibition of at least those enzymes whose hydrolysis products were fermented into ethanol. The ethanol produced here can possibly be concentrated and feasibly extracted by a hydrolysate recycle process, allowing to valorize this coproduct. A techno-economic analysis is further warranted to determine if the developed method

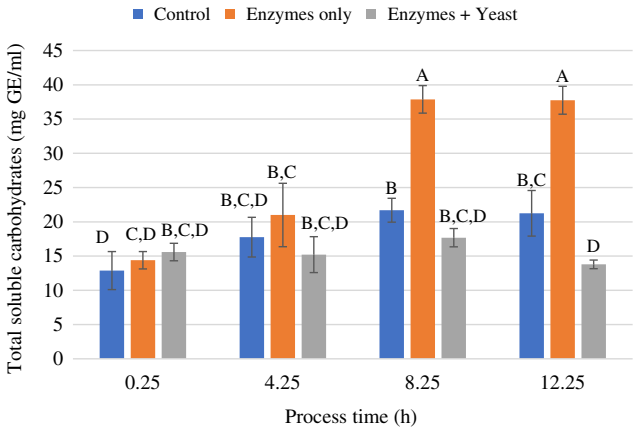

FIGURE 5 Evaluation of the effect of control, enzymes only, and enzymes + yeast treatments on total soluble carbohydrates as a function of process time. Error bars represent SE ($n = 9$). Treatments with the same letters are not significantly different ($p < 0.05$, Tukey's honest significant difference test)

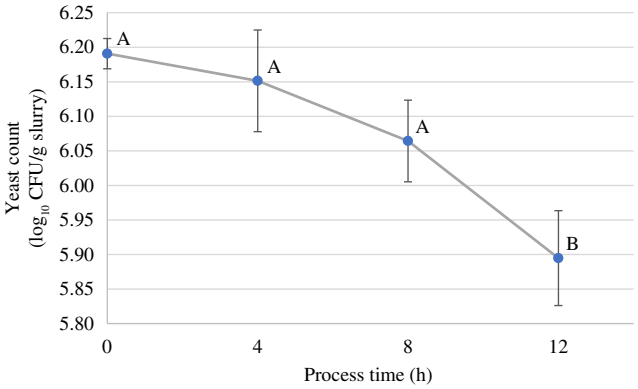

FIGURE 6 Yeast concentration in slurry as a function of process time for the enzymes + yeast treatment. Error bars represent SE ($n = 6$). Counts with the same letters are not significantly different ($p < 0.05$, Tukey's honest significant difference test)

would be cost-effective for SPC production at an industrial scale.

Yeast enumeration (Figure 6) was done to test the hypothesis that the soy flour slurry at pH 4.5 and 39°C is able to support yeast growth. It was observed that the viable yeast count kept decreasing as the processing time increased with a significant ($p < 0.05$) decrease for the 12.25-h process. Figure 4 shows glycerol concentration, and the trend of an increase in glycerol concentration indicates the thermal and osmotic stress the medium is exerting on the yeast (Ivit et al., 2020). The decline in viable yeast count, coupled with an increase in glycerol production, suggests that the wild type *S. cerevisiae* needs further adaptation at low pH and high-temperature conditions in a soy flour-rich medium so that the yeast can then spend all the metabolizable feedstock in making ethanol instead of glycerol.

The yeasts used in this study metabolized the hexose feedstock (glucose, fructose, and galactose) to produce ethanol, leaving the pentose (xylose and arabinose) and uronic acid feedstock unutilized. The existence of unutilized pentoses xylose and arabinose warrants a future investigation with pentose-fermenting yeasts since such yeasts exist via genetic engineering (Nijland & Driessen, 2019). The use of a pentose-fermenting strain would not only help increase ethanol concentration but also substantially reduce the effluent treatment costs associated with the hydrolysate. The enzymatic hydrolysis in this study took place at 39°C because the specific growth rate and ethanol productivity of yeast *S. cerevisiae* are known to reduce significantly, followed by an increase in glycerol productivity at higher temperatures (Aldiguier et al., 2004). A thermotolerant strain of *S. cerevisiae* would allow the enzymatic hydrolysis to operate at higher temperatures, thereby increasing the hydrolysis rate or permitting lower enzyme dosage. A subsequently shortened fermentation time, followed by high temperature and low pH conditions, would certainly help reduce the risk of microbial contamination (Aldiguier et al., 2004).

Total polyphenol concentration changes

Polyphenols are phytochemicals that are associated with various health-promoting attributes. Soy polyphenols include isoflavones, chlorogenic acid isomers, caffeic acid, and ferulic acid, which are antioxidants in nature (Seo & Morr, 1984).

Figure 7a,b shows the TPC in SPC and its respective hydrolysate from different treatments as a function of process time. There was no significant difference in TPC from EO and EY treatments in both matrices, except for the 0.25-h process in SPC. This suggests that the change in TPC is brought about by the enzymes used and is not significantly affected by the presence of yeast or the fermentation caused by it. In SPC and in its hydrolysate, TPC increased as processing time increased. This suggests that TPC did not just migrate from one phase to the other, but their overall concentration increased. For the 12-h process, TPC in SPC of EO and EY treatment was 2.72 and 2.53 times more than that of control, respectively. At the same time, TPC in the hydrolysate of EO and EY treatment was 2.34 and 2.21 times more than that of control.

Seo and Morr (1984) showed that defatted soybean flakes, which are similar to DSF, contain 4 mg total phenolics/g of sample, distributed as about 72% isoflavonoids and 28% phenolic acids. The DSF used in this study had 2.16 ± 0.02 mg GAE/g dry matter TPC. Almost all isoflavones in DSF are in their glycoside form, and less than 1% exist as aglycones (Naim et al., 1974). We hypothesize that the increase in TPC

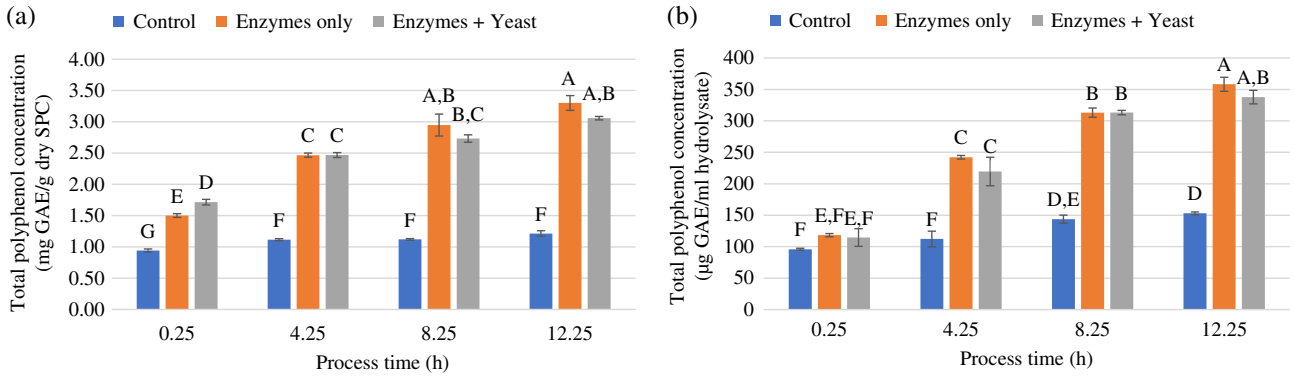

FIGURE 7 Evaluation of total polyphenol concentration in (a) soy protein concentrate (SPC) and (b) hydrolysate due to control, enzymes only, and enzymes + yeast treatments as a function of process time. Error bars represent SE ($n = 9$). Treatments with the same letters are not significantly different ($p < 0.05$, Tukey's honest significant difference test)

in SPC and its hydrolysate is primarily due to the formation of aglycones from their respective glycosides. Cellulose hydrolysis requires a β-glucosidase enzyme to catalyze the cleavage of glucose from cellobiose. This enzyme is included in the large array of cellulolytic enzymes in Viscozyme L. β-Glucosidases catalyze the hydrolysis of isoflavone glycoside into their aglycone form as well (Hu et al., 2018). Glycosides are not detectable by the Folin–Ciocalteu (FC) assay because their phenolic group is bound to either glucose, 6″-*O*-acetylglucoside, or 6″-*O*-malonylglucoside (Kaya et al., 2008). *S. cerevisiae* does not naturally possess the ability to metabolize cellobiose, and hence, does not possess any *endo* or *exo*-β-glucosidase activity (Tang et al., 2013). This further corroborates why there were no significant differences in the TPC counts between EO and EY treatments throughout the process.

It has been well discussed that polyphenols are bound to other polymers such as fibrous carbohydrates and proteins in soybean matrix (Rodriguez-Roque et al., 2013). Our study focused on the hydrolysis of such fibrous carbohydrates and some proteins. Hence, we hypothesize this is another reason the TPC count increased in SPC and hydrolysate matrices. FC assay has previously been reported (Alessandri et al., 2014) to be sensitive to various reducing sugars and subsequently be nonspecific to polyphenol estimation. We have various reducing sugars in SPC and hydrolysate in the enzymes only and EY treatment. However, exhaustive studies by Everette et al. (2010) indicate that almost all carbohydrates in the matrix did not interfere with FC assay. The lack of difference in TPC between EO and EY treatment, despite having significantly ($p < 0.05$) different amounts of reducing sugar due to its fermentation into ethanol, also further proves the specificity of FC assay to phenols in our study.

SPC has been a de facto ingredient in the manufacture of formulated meat products, high-moisture meat-analogs, and texturized protein products. Most of these end products suffer from lipid and protein oxidation during cooking, handling, and storage due to high moisture, protein, and lipid contents (Tarrega et al., 2020). Synthetic antioxidants have long been used, but recently a shift to antioxidants of natural origins has been found to be an acceptable strategy to alleviate this problem (Carocho et al., 2014) due to the rising demand for a clean label on processed food products. There have been numerous reports of uses of phenolic extracts from natural sources such as avocado peel extract in beef and soy-burgers, plum juice concentrate in hams, pomegranate rind powder in cooked chicken patties, and so on (Trujillo-Mayol et al., 2021). The SPC made from our process naturally contains a significantly high level of polyphenols and therefore may allow for an exclusion of any extraneously added antioxidant, thereby having one less ingredient to label on the package.

Soy polyphenols are the richest source of isoflavones (Di Lorenzo et al., 2021). We speculate most polyphenols in the hydrolysate represent isoflavones in their aglycone forms. Isoflavone aglycones are known to be more bioavailable and biologically active due to their enhanced liposolubility as compared to their glycoside forms (Di Lorenzo et al., 2021). Thus, the hydrolysate from this process may serve as a rich source for extracting polyphenols for nutraceutical, food, and cosmetic purposes.

CONCLUSION

The work presented here serves as a proof of concept of producing a high-quality SPC with high protein content, almost inexistent flatulence-causing oligosaccharides, low non-nutritional insoluble carbohydrates, and high polyphenols with the presence of yeast cells, along with a coproduct ethanol. The simultaneous operation of yeast fermentation along with enzymatic hydrolysis allowed for more carbohydrate hydrolysis, higher protein enrichment of SPC, and the formation of a coproduct ethanol. A 12.25-h process of enzymatic

hydrolysis and *S. cerevisiae* led fermentation of 100 g dry DSF led to 68.45 g SPC dry matter containing 72.23 ± 0.25% protein and 384 ml hydrolysate containing 9.76 ± 0.05 mg/ml ethanol, with 84.4 ± 0.01% protein recovery (Figure 1). SPC and the hydrolysate both contained significantly increased amounts of polyphenols. Thus, the availability of such an SPC would make it a much more desirable ingredient in comminuted meat products or plant-based meat analogs.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

AUTHOR CONTRIBUTIONS

Ruchir M. Agrawal: conceptualization, methodology, investigation, formal analysis, writing-original draft. **Michael J. Miller**: methodology, supervision, visualization, writing-review and editing, resources. **Vijay Singh**: conceptualization, supervision, visualization, writing-review and editing. **Hans H. Stein**: visualization, writing-review and editing, resources. **Pawan S. Takhar**: conceptualization, supervision, project administration, funding acquisition, visualization, writing-review and editing, resources.

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