ENZYMATIC HYDROLYSIS AND FERMENTATION OF SOY FLOUR TO PRODUCE ETHANOL AND SOY PROTEIN CONCENTRATE WITH INCREASED POLYPHENOLS

BY

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THESIS

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ABSTRACT

Defatted soybean flour (DSF) is a protein-rich ingredient; however, it contains 30-35% carbohydrates, which is made up in parts by sucrose, flatulence-causing oligosaccharides, and water-insoluble cell-wall polysaccharides. A process was developed to produce soy protein concentrates (SPC) by substantially hydrolyzing these carbohydrates 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 a cellulase blend, pectinase blend, and α-galactosidase. The effect of process time on SPC protein concentration, overall protein recovery, carbohydrate hydrolysis, yeast growth, ethanol concentration, and total polyphenol concentration of SPC and hydrolysate were 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 in the production of SPC. After 12.25 hours 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. Protein recovery from DSF in the form of SPC was 84.4 ± 0.4%. Viable yeast count steadily declined from $1.6 \pm 0.1 \times 10^6$ CFU/mL slurry at the start of the process to $7.9 \pm 1.3 \times 10^5$ CFU/mL slurry after 12.25 hours. Flatulence causing raffinose-series-oligosaccharides were completely hydrolyzed into their monomeric sugars during this process. Total soluble carbohydrates in the EY treatment were consistently lower than control and EO treatment, suggesting that the yeasts were able to ferment sugars as they were becoming available. Total polyphenol concentration (TPC) of SPC increased more than twofold from 1.21 ± 0.04 mg gallic acid equivalent (GAE)/g dry SPC in control to 3.06 ± 0.03 mg GAE/g dry SPC in EY treatment. Similarly, hydrolysate TPC increased twofold from 179 ± 1 μg/mL in control to 371 ± 6 μg/mL in EY treatment. Thus, this novel process led to a protein and polyphenol-rich and reduced-carbohydrate SPC along with polyphenol and ethanol-containing hydrolysate.

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To Maa and Dadaji

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1. Introduction

Soy proteins are the largest source of low-cost plant-based dietary proteins. Whole soybean, which contains about 38-40% protein and 18-20% oil on dry matter basis, provides the most inexpensive source of high-quality protein and edible oil. China is purported to be the home of soybean and soybeans are supposed to be domesticated in the eastern half of northern China in the 11th century before the common era (Shurtleff et al., 2014). World's total soybean production increased by 75% from 220 million metric tons in 2005-2006 to 385 million metric tons in 2021-2022, with Brazil, United States, Argentina, China, and India contributing 37.4%, 31.2%, 13.5%, 4.9%, and 2.9% to total soybean production respectively. (SOPA, 2021). Soybean is a versatile crop, with products like edamame (dried immature soybeans), miso (fermented soybean paste), shoyu (soy sauce), soy milk (drink by soaking and grinding soybean), tofu (curdled soy milk), tempeh (fermented cooked soybeans) and kinako (powder of roasted whole soybeans) being conventional food products in eastern Asia.

In Western countries, non-traditional products of soybean, like soy flour, soy protein concentrates, isolates, and their textured products started attracting people's attention in the 1960s due to the advent of flash desolventizing, which reduced protein denaturation and facilitated the production of water-soluble protein ingredients (Phillips and Williams, 2011). The initial growth of soybean industry in the United States was driven by oil production rather than protein products (Liu, 2012). The non-traditional protein products were used as ingredients in formulated food products for their functional attributes like water solubility, water and fat absorption, emulsification, foaming, gelling, binding, etc. Phillips and Williams (2011) state that the consumption of soy products abruptly increased from 1997 due to the realization of various physiological properties of soybeans. On 26th October 1999, US FDA further confirmed the 'Soy Protein Health Claim' that soy protein included in a diet low in saturated fat and cholesterol may reduce the risk of coronary heart diseases by lowering blood cholesterol levels. The market was very receptive to this claim, and this allowed for higher proliferation in Western culture and diet. Proteins serve as a source of amino acids in human diets. Proteins from different sources have different amino acid profiles, thereby differing in levels of nutrition. The Joint FAO/WHO Expert Committee on Protein Quality Evaluation recommended the use of Protein Digestibility and Corrected Amino Acid Score (PDCAAS) as a metric of measuring protein quality on the basis of

protein content, amino acid profile, and protein digestibility in 1990 (World Health Organization, 1991), replacing Protein Efficiency Ratio (PER). Soy protein's PDCAAS score is 1.0, making it the highest in plant-based proteins. For reference, milk proteins and egg proteins have a score of 1.0, almond proteins' score ranges from 0.22-0.31, oats have a score of 0.66, and rice proteins have a score of 0.53-0.54 (Boye et al., 2012). Hence, this is also one of the reasons why soybeans are a major vegetable protein source: the protein is not only high in quantity but also high in quality.

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). Food-grade soy concentrates containing 70% proteins were first produced by Griffith Laboratories, Illinois (Liu, 2012). DSF contains 30 - 35% carbohydrates, which are divided into sucrose, water-soluble but flatulence-causing oligosaccharides like raffinose and stachyose, and water-insoluble polysaccharides like cellulose, hemicellulose, and pectic polysaccharides (Refstie et al., 2005).

The flatulence-causing oligosaccharides like raffinose and stachyose are called α-galactosides because a D-galactose molecule is linked to the D-glucose moiety of sucrose by an α(1→6) bond. Hence, chemically, α-galactosides are considered derivatives of sucrose, with raffinose, stachyose, and verbascose containing one, two, and three galactose molecules, respectively. They are non-reducing sugars and are soluble in water and aqueous alcohol solutions (Martínez-Villaluenga et al., 2008). α-galactosides cause flatulence upon consumption because the intestinal mucosa in human beings and many other non-ruminant animals does not possess the α-galactosidase enzyme, which hydrolyzes the α(1→6) bond between galactose and glucose, and the intact α-galactosides are too big to pass through the intestinal walls (Anderson et al., 1979). Hence, these sugars pass undigested through the small intestine into the large intestine, where they are subject to anaerobic microbial fermentation, which produces short-chain fatty acids, hydrogen, methane, and carbon dioxide (Cristofaro et al., 1972). Thus, the accumulation of this flatus causes discomfort, abdominal rumblings, and cramps, and hence is a well-studied reason for deterring people from eating pulses (Price et al., 1988). This also lowers the metabolizable energy of soy

flour since α-galactosides are not metabolized in the small intestine (Urbano et al., 2007). This makes soy foods less desirable and deters protein consumption and subsequent utilization.

Classically, structural cell wall polysaccharides have been grouped into cellulose, hemicellulose, and pectin (Scheller and Ulvskov, 2010). Cellulose is a linear homopolysaccharide of β-D-glucose units connected by β(1→4) glycosidic linkages and is crystalline in nature. Hemicelluloses are branched heteropolymers made up of xylose, arabinose, glucose, galactose, and small amounts of galacturonic and guluronic acids and are amorphous in nature (Karr-Lilienthal et al., 2005). In our study, we have classified xyloglucans (β(1→4) glucosyl backbone with C-6 partially substituted with α-linked xylose), xylans (β(1→4) linked xyloses), arabinoxylans β(1→4) linked xyloses with varying degrees of α-arabinose substitutions) and arabinogalactans β(1→4) linked D-galactose units with L-arabinose sidechains) as hemicelluloses (Aspinall and Cottrell, 1971; Cipriani et al., 2009; Huisman et al., 1996; Mendis and Simsek, 2014). Pectic polysaccharides include homogalacturonans, rhamnogalacturonans type I and II. Homogalacturonans are also called pectins and are homopolymers of α(1→4) D-galacturonic acid units. Rhamnogalacturonan type I is a polymer of alternating α(1→2) L-rhamnose and α(1→4) D-galacturonic acid units, with sidechains at C-4 positions. Rhamnogalacturonan type II has a backbone of α(1→4) D-galacturonic acid units, with oligosaccharide sidechains containing as many as 11 different monosaccharides at C-2 and C-3 positions (Navarro et al., 2019). Cellulose, hemicellulose, and pectic polysaccharides escape digestion in the small intestine and travel to the colon for their partial or complete hydrolysis via fermentation. Cellulose and hemicelluloses are usually insoluble, non-viscous fibers and are resistant to colonic fermentation but carry fermentable carbohydrates to the colon and contribute to fecal bulking. Pectic polysaccharides are partially soluble and are generally more completely fermented, contributing to the production of SCFA and lowering pH (Wong and Jenkins, 2007).

SPC is made by partial removal of water-soluble carbohydrates from DSF (Supplementary Fig. 7). 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). The 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 (Jankowski et al., 2009). Hence, a large ratio of wash solvent to DSF is used, producing a large waste stream called 'soy solubles' (Wang et al., 2004). The SPC from this process also has lower levels of total polyphenols than DSF since a significant portion of polyphenols in their native state are preferentially hydrophilic (Di Lorenzo et al., 2021), and are thus lost with the waste stream. The 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 (Pandjaitan et al., 2000).

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 (Supplementary Fig. 8). 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., 1998). SPCs and SPIs normally cost 2 – 2.5 times and 5 –7 times higher than DSF (Berk, 1992). Hence, from a mildly heat-treated DSF as raw material, which costs around 0.55 – 0.64 USD/kg (Berk, 1992; Markets Insider, 2021), SPC would cost 1.19 – 1.49 USD/kg and SPI would cost 2.98 – 4.16 USD/kg.

Cellulose and hemicellulose, comprising the bulk of insoluble fiber in DSF, can be hydrolyzed effectively by physical/chemical treatments (Walker and 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; De Almeida et al., 2014; Jacobsen et al., 2018; Yusoff

et al., 2015) and simultaneous yeast fermentation (Long and Gibbons, 2012) have been investigated. However, many of those processes did not study the production of SPC. The process that did produce SPC did not utilize simultaneous microbial fermentation to consume the monomerized sugars (Al Loman et al., 2016). 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 (Öhgren et al., 2007). 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.

Also, to the best of our knowledge, there hasn't been work done to investigate the effect of the hydrolytic enzymes on total polyphenol content of the end protein product. With the rising interest in plant-based meats, certain polyphenols play a major role in the stability of the end product (Beya et al., 2021). Hence, we decided to investigate the process of enzymatic hydrolysis and simultaneous yeast fermentation of DSF to produce SPC.

The objectives of this study were, thus, to test the hypotheses that this process

(1) produces an SPC with protein content greater than the SPC by 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 co-product ethanol, and

(4) causes the total polyphenol concentration of SPC and hydrolysate to change significantly compared to the SPC and supernatant produced by an equivalent acid-wash process.

1.1 Review of literature on previous work

Because soy proteins are used for their nutritional and functional properties, many studies on either enzymatic hydrolysis of protein or carbohydrates and on microbial fermentation of soybean meal, soy flour, or soybean white flakes have been done in the past.

Lamsal et al. (2006) used two endopeptidases and one exopeptidase to modify extruder expelled soy flour proteins to improve the protein's functionalities. The protein content of the resultant soy flour hydrolysate ranged from 46.6% to 53.9% on dry basis, depending upon jet

cooking or only spray drying treatments. The formation of smaller peptides enhanced the protein solubility, foam forming capacities, and foam and emulsion stabilities, with a reduction in emulsification capacities. The resultant proteins were indicated to be more bitter, astringent, or beany than the control samples. This study suggested that proteolysis of soy flour proteins may lead to functional and sensorial changes.

Jacobsen et al. (2018) conducted a study to examine the effect of crude enzymes produced by solid-state fermentation of defatted soybean meal (SBM) by *Aspergillus niger* on carbohydrates, proteins, inositol phosphate, and saponins in low moisture conditions. Crude protein content of soybean meal after enzymatic treatment increased slightly to 49.4% from 49.0% on dry basis in control soybean meal. The authors did not observe any significant effects on soluble, insoluble, and total dietary fiber, soluble protein, and certain saponin groups; this result was attributed to low moisture content (450 g/kg dry matter), lower enzyme to soy flour ratio (3 g enzyme/kg SBM), and shorter time of hydrolysis (70 minutes). Inositol-6-phosphate and Bb-DDMP saponin, two heat-stable antinutritional factors in soybean meal, were 84% and 58% hydrolyzed, respectively, and the hydrolysis was found to be highly dependent on time, moisture content, and enzyme loading for inositol-6-phosphate and mixing for Bb-DDMP saponin, respectively. This study thus elucidated the effect of reaction time, enzyme loading, moisture content, and mixing on effective hydrolysis of certain antinutritional factors of SBM.

Jung et al. (2006) investigated the potential of simultaneous and consecutive treatment of cellulase, hemicellulase, and pectinase enzymes on defatted soy flakes in the production of SPI at laboratory and pilot-plant scale. The objective of the study was to improve protein extraction yields from defatted soy flakes without causing protein degradation and to also compare the functionalities of a pectinase modified SPI with corresponding controls. Protein extraction yields due to simultaneous and consecutive addition of the commercial enzymes Puradax HA (cellulase) and IndiAge Super L (cellulase) were 63.2% and 63.3%, respectively, whereas the control protein yields for the same processes were 56.9% and 64.7%, respectively. The authors did not find any synergistic effect on protein extraction with either the combination of two of these cellulases or with a combination of a cellulase and a pectinase (IndiAge Super L and Multifect pectinase, respectively). The treatment with Multifect pectinase allowed the highest % increase in protein extraction yield in laboratory and pilot plant trials at 50% and 17%, respectively, over the controls. However, in the form of soy protein isolate, protein extraction yields were 28.0% and 40.7% for

pectinase and cellulase treatments, respectively, which suggested that a significant amount of protein remained in the whey fraction, thereby leading to similar protein yields corresponding to their controls. Finally, the authors also hypothesized increased isoflavone extractability since protein and isoflavone extraction were speculated to be influenced by similar parameters.

Hong et al. (2004) evaluated the effect of *Aspergillus oryzae* GB-107 fermentation on the nutritional quality of food soybeans and feed soybean meals. Protein content of fermented soybeans and soybean meal increased by 4.4% and 9.9% to 46.23 and 55.32%, respectively, due to, in part, decreased carbohydrate content. The essential amino acid profile also did not change significantly, owing to preferential utilization of certain amino acids by *A. oryzae*. There was an increase in the amount of small-sized peptides (<20 kDa), and a decrease in medium (20-60 kDa) and large (>60 kDa) sized peptides due to the protease secretion of *A. oryzae*, through which they speculated a change in functional properties of the resultant proteins. The authors also observed a 91.6% and 84.4% reduction in trypsin inhibitors, which is a major antinutritional factor. The authors finally speculated a reduced dry matter consumption by the fungi and its subsequent disappearance since the fermentation time was relatively lower (2 days) as opposed to 4 days by Sardjono et al. (1998). Thus, the authors were able to make fermented soybeans and soybean meal with eliminated trypsin inhibitor activities, increased protein content, and probiotics enriched product, which would make the respective soy foods more useful in human and livestock diets.

Song et al. (2008) evaluated the immunoreactivity reduction by natural and induced fermentation of soybean meal with *Lactobacillus plantarum*, *Bifidobacterium lactis*, and *Saccharomyces cerevisiae*. The authors found that during the natural fermentation, the surface microflora contributed to the growth of microbes and that lactic acid bacteria dominated the fermentation and inhibited the growth of other microbes by lowering the pH. The fermentation with *S. cerevisiae* showed the highest reduction in IgE immunoreactivity, up to 89% against soy-sensitive human pooled plasma. An increase in smaller-sized peptides (<10 kDa) was observed and was subsequently attributed to the reduction of immunoreactivity in the fermented soybean products. Crude protein content of SBM also increased from 47.08% in unfermented SBM to 58.08% in *S. cerevisiae* fermented SBM, and the increase was speculated due to the growth of microorganism load. The authors also found an unchanged essential amino acid profile in *S. cerevisiae* fermented SBM, with the exception of cysteine showing a sharp increase of 56%.

Al Loman et al. (2016) developed an enzymatic process to produce SPC and SPI without indigestible carbohydrates by using enzymes from *Aspergillus niger*. This process also produced soluble reducing sugar, which the authors describe as a potential fermentation feedstock. The enzyme system consisted of xylanase, pectinase, cellulase, α-galactosidase, and proteases. SPC was recovered by isoelectric precipitation after the enzymatic hydrolysis of DSF for 48 h at 50°C and was further ethanol washed to increase the protein content from 70% to 80% on dry basis. There was a considerable amount of protein loss in the hydrolysate after the separation of SPC solids, and hence the proteins from the hydrolysate were extracted by heat precipitation and further centrifugation to yield SPI with 89% protein on dry basis. The protein loss was attributed to the proteolytic activity of the *A. niger* enzyme system, which led to the formation and subsequent dissolution of smaller peptides into the hydrolysate that did not precipitate during isoelectric precipitation of SPC. The amino acid profile of DSF and SPC were found to be similar. Potentially undigestible oligosaccharides were substantially eliminated, and polysaccharides were greatly reduced in the resultant SPC, which was speculated to offer improved digestibility of these soy protein products. Overall, the enzyme system from *A. niger* gave one of the highest protein yields of 91.7%, with 82.2% protein recovered in the form of SPC with 70% protein content and 17.8% protein in the form of SPI with 89.3% protein content.

Long and Gibbons (2012) investigated the effect of commercial cellulase, β-glucosidase, and pectinase, and *Saccharomyces cerevisiae* NRRL Y-2034 and *Scherffersomyces stipitis* NRRL Y-7124 on ground soybeans, SBM, soybean hulls, and soybean white flakes. The enzymes were used to saccharify the carbohydrates in the respective matrices, followed by the yeasts in simultaneous fermentation of these reduced glucans to ethanol for a period of 96 h at 50°C. The protein content of SBM and white flakes after 96 h SSF by *S. cerevisiae* reduced from 52.60% to 48.32% and from 51.16% to 49.90% on dry basis respectively, with greater reductions by SSF from *S. stipitis*, attributing the loss to protein catabolism by yeasts. Ethanol titers for both SBM and white flakes increased as their respective solid loading in the SSF increased. *S. cerevisiae* produced more ethanol than *S. stipitis* in both substrates, with the maximum ethanol titer of 16.25 g/L and 12.75 g/L at 20% solid loading without enzymes in white flakes and with enzymes in SBM, respectively. The authors also reported a 78.8% and 72.3% reduction in crude fiber in white flakes and SBM after 96 h SSF with *S. cerevisiae*, respectively. The authors had anticipated that

the reduction of fibers would imply a conversion of some of the resultant sugars to biomass and, therefore, an increase in protein levels, which did not happen.

All these previous studies provided some guidance for the current study in different aspects of enzymatic hydrolysis and fungal fermentation of DSF. Accordingly, many clues about enzyme selection, enzyme dosage, yeast inoculation, solid loading, temperature, duration, and pH of enzymatic hydrolysis and fermentation, along with agitation levels, were used in the completion of this study. The difference between a conventional process of manufacturing SPC and the process investigated in the study is only at the protein precipitation and subsequent carbohydrate removal step. The downstream processing investigated in this study is a centrifugation step to separate solids and supernatant. Processes like membrane filtration and electro-acidification also exist to separate proteins from soluble carbohydrates (Alibhai et al., 2006). Thus, any variation in subsequent downstream processing in conventional manufacture is easily extrapolatable after the protein precipitation step in this process. Conventional protein precipitation in a batch manufacturing process of SPC may achieve equilibrium in 0.5 – 1 hour, depending on the agitation technique used. In this study, protein precipitation and enzymatic hydrolysis along with simultaneous fermentation for 4, 8, and 12 hours are explored to see when protein precipitation and carbohydrate diffusion in the continuous phase achieve equilibrium.

2. Materials and Methods

2.1 Chemicals and Materials

Defatted Soy Flour (7B Baker's Soy Flour) was provided by Archer Daniel Midlands (ADM, Decatur, IL, USA). The chemical reagents including 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 was 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). DRBC agar was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Liquid silicon antifoam (Fermfast) was purchased from a local brewing supply store (Champaign, IL, USA). All other reagents used were of analytical grade, and sugar standards, glycerol, and ethanol used were of HPLC grade and purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2 Batch experiments in the centrifuge tubes

Batch experiments of enzymatic hydrolysis and yeast fermentation were done in 50 mL centrifuge tubes. 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.

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 minutes 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. Slurry pH was then adjusted to 4.5 with 12 M hydrochloric acid. 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 (Supplementary Figs. 4 and 5) at 150 rpm and 39°C. Samples were taken immediately after enzyme addition and yeast inoculation and at 4, 8, and 12 hours respectively by withdrawing the entire tube from the incubator. The contents of the tubes were boiled on a heating block for 15 minutes to deactivate enzymes and yeasts. Subsequently, the slurry was centrifuged at 4,696×g at 4°C for 1 hour to separate the solids and supernatant. The supernatant was decanted and termed hydrolysate. The solids were freeze-dried, ground, and were denoted as SPC. Fig. 2 summarizes the entire process in the form of a flow chart with mass balance. Supplementary Figs. 9 and 10 summarize the process and mass balance for control and enzymes only treatments at 12.25 h, respectively.

Two additional systems, viz. an enzymes-only (EO) system (without yeasts) and a control system (without enzymes or yeast), 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 enzymes-only 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.

2.3 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 (Mariotti et al., 2008), which are represented on dry basis. Moisture content was analyzed by drying at 105°C in a convection oven till constant weight was reached according to standard NREL 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, hemicellulose was calculated as NDF−ADF. Soluble dietary fiber was assumed to represent pectic polysaccharides. Proximate composition of DSF is given in Table 1.

Carbohydrate and Ethanol Analysis

The hydrolysate was boiled on a heating block for 15 minutes to denature and precipitate water-soluble peptides. It was then centrifuged for 20 minutes at 4,696×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 high-performance liquid chromatography (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 minutes, followed by filtration through a 0.45 μm syringe filter before chromatographic analysis.

Total soluble carbohydrate concentration was estimated by phenol sulfuric acid assay (Masuko et al., 2005). 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 minutes. After cooling for 5 minutes in an ice bath, the solution was further vortexed, then centrifuged at 2000×g for 1 minute, 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). 0.5 gm 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 minutes. 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.

Total polyphenol concentration 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 2N FCR and vortexed. After 4 minutes, 375 µL 10% sodium carbonate was added and vortexed again. The mixture was then incubated at 40°C for 30 minutes in a water bath, followed by 1 minute in an ice bath. Finally, the mixture was centrifuged at 2000×g for 1 minute, 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).

2.4 Statistical Analysis

All experiments were carried out in biological triplicates. Every replicate's protein and moisture assays, HPLC analyses, and yeast enumeration plates were repeated twice; total soluble carbohydrate and total polyphenol concentration assays were repeated thrice. The data are

expressed as mean $\pm$ standard deviation of six or nine values. One-way analysis of variance (ANOVA) was performed to examine the differences across treatments. For the data sets 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* (R Core Team, 2021).

3. Results and Discussion

Table 1 Proximate composition of defatted soy flour (DSF)

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

Sum of components: 100.2 ± 3.7%

3.1 Protein enrichment in SPC

Globulins are storage proteins, which according to Osborne classification, are insoluble in water, but soluble in dilute saline. 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 Fig. 1. Water-soluble carbohydrates like 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 4.25 hours, suggesting that solubilization of carbohydrates had peaked. In the EO treatment, carbohydrate hydrolyzing enzymes were breaking down cellulose, hemicellulose, and pectic polysaccharides 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 and 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. 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 process (Fig. 1).

SPC prepared by EY method is already towards the upper end of protein concentration (70-75%) in commercial SPC manufacture (Joint FAO/WHO Codex Alimentarius Commission, 2019), which comprises refining of SPC by multistage washing. However, this SPC has been 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 h 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 (Supplementary Fig. 3). Hence, a lesser amount of higher protein SPC was produced. However, protein recovery decreased drastically ($p<0.05$) compared with control (Supplementary Fig. 2). 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.

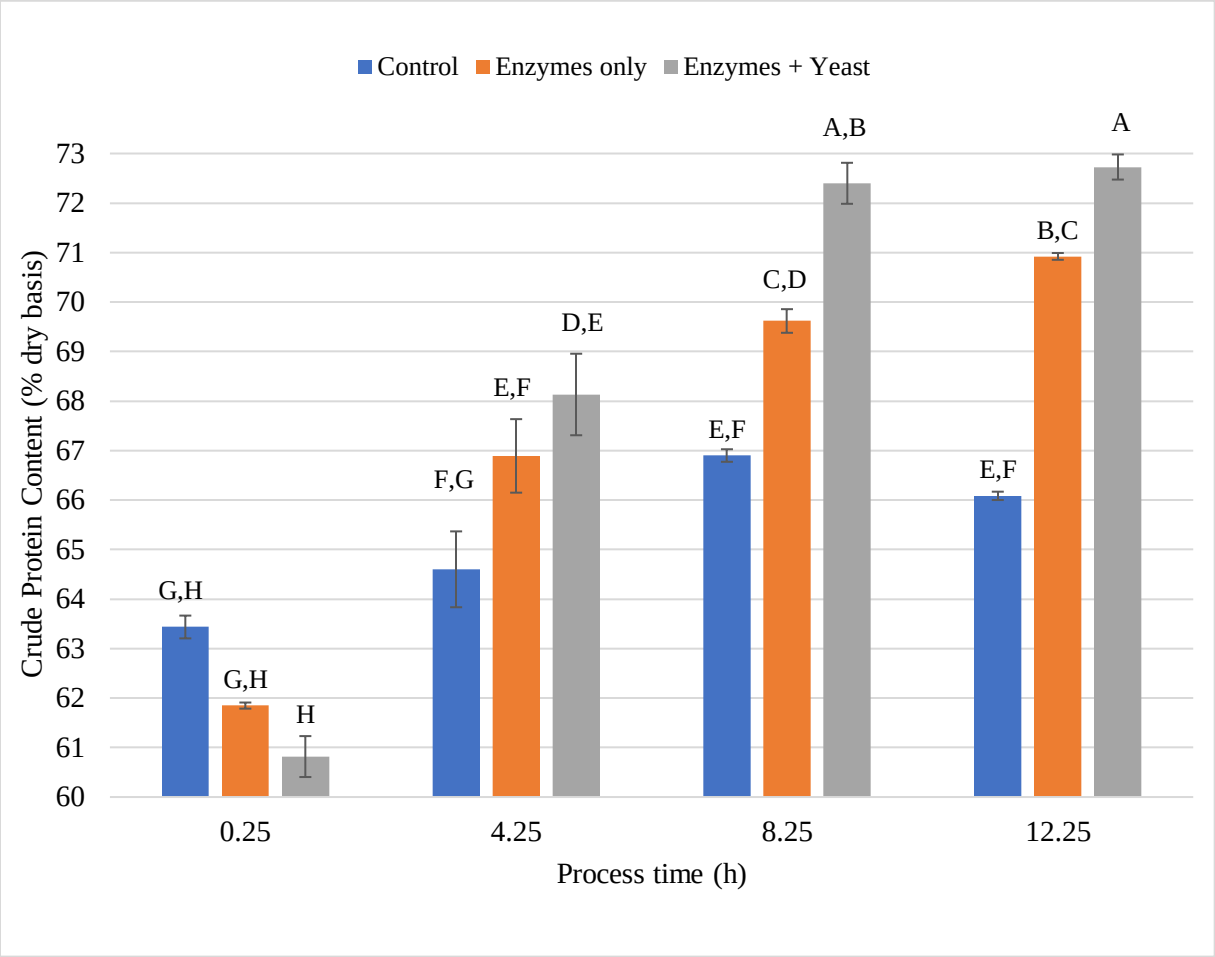

Fig. 1 Protein content of SPC prepared by Control, Enzymes only, and Enzymes + Yeast treatments as a function of process time. Error bars represent std. error (n=6). Treatments with the same letters are not significantly different, ($p<0.05$, Tukey's HSD test)

Table 2 Comparison of SPC protein concentration, 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}

Different letters across the rows in the same column indicate significant statistical differences, ($p<0.05$, Tukey's HSD test)

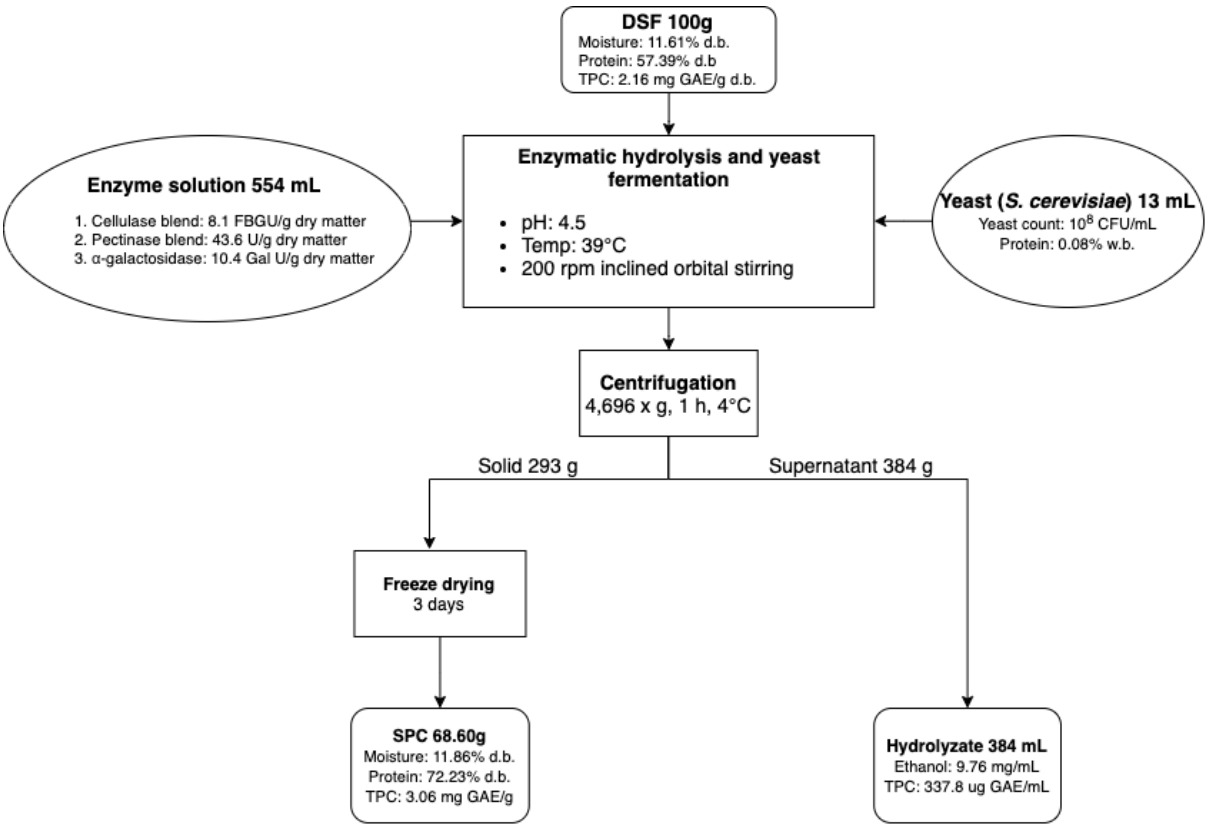

Fig. 2 Flowchart with material balance for the production of SPC via enzymes and yeast treatment for 12.25 h

3.2 Carbohydrate Hydrolysis and Fermentation Analysis

Fig. 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 this analysis was that xylose, fructose, and galactose co-eluted, and hence, were not quantified individually because their retention times were very close (9.134, 9.289, and 9.181 minutes respectively). Fig. 4 shows the individually quantified carbohydrates, glycerol, and ethanol as functions of process time and treatments. Supplementary Fig. 6 shows all the individual soluble components and their sums in the hydrolysates in a stacked format.

Effect of hydrolysis on water-soluble carbohydrates

Fig. 3 shows the presence of water-soluble carbohydrates, which included oligosaccharides like 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 Fig. 4a, 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. 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. Fig. 4b also suggests that raffinose, whose extraction got similarly enhanced as stachyose in control, got around 83% and 87% hydrolyzed in EO and EY treatments, respectively. The hydrolysis products, i.e., sucrose, glucose, fructose, and galactose, are shown in Figs. 4c, e, and f, respectively (fructose and galactose are aggregated with xylose). 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 were 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 gotten 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 till 8.25 h due to the combined effect of invertase acting on sucrose as well as cellulase acting on cellulose. We hypothesized that the bulk of glucose came from the hydrolysis of sucrose, stachyose, and raffinose, and in some parts, 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 process and fermented by yeasts into ethanol (Fig. 4i). Fructose and galactose, quantified collectively with xylose (Fig. 4f), 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 (D'Amore et al., 1989; 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 since 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 (Millipore Sigma-Aldrich, n.d.). Accordingly, its primary rationale of inclusion was to convert insoluble polysaccharides like cellulose and hemicellulose into their smaller, soluble constituents. Since 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.

Fig. 4g shows the concentration of arabinose, a monomer primarily associated with hemicellulose, inexistent 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 to not 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 were 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.

Fig. 4f shows the concentration of xylose, another monosaccharide found in hemicellulose, as aggregated with fructose and galactose. We were unable to make any distinction in changes of xylose concentration as a function of process time, since 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 like xylose and arabinose, but it ferments hexoses like fructose and galactose (Fernandes and 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 (Fig. 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 like cellulose and hemicellulose, hence allowing subsequent enrichment in protein concentration in the respective treatments' SPC.

Pectinex Ultra SPL is a pectinase enzyme derived from *Aspergillus aculeatus* and contains polygalacturonase, pectin methylesterase, and pectin transeliminase, followed by small amounts of cellulase and hemicellulase activities (Millipore Sigma-Aldrich, n.d.). 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 (Fig. 4f). The release of galacturonic acid in the

hydrolysate (Fig. 4d) 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 corelates 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 h 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 (Protzko et al., 2018). 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

Fig. 4i suggests 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 as compared to EO treatment (Figs. 3d, g, and f, respectively) also suggests that yeast fermentation had a synergistic effect on enzymatic hydrolysis of water-insoluble carbohydrates. Fig. 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. Supplemetary Fig. 6 also shows the amount of soluble carbohydrates made available by the action of enzymes, the subsequent reduction of some soluble carbohydrates, and an overall reduction of soluble components as detected by HPLC. 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 co-product.

Yeast enumeration (Fig. 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 significant ($p<0.05$) decrease for the 12.25 h process. Fig. 4h 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 (Bettiga et al., 2009; Nijland and Driessen, 2019). The use of a pentose fermenting strain would not only help increase ethanol concentration but 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).

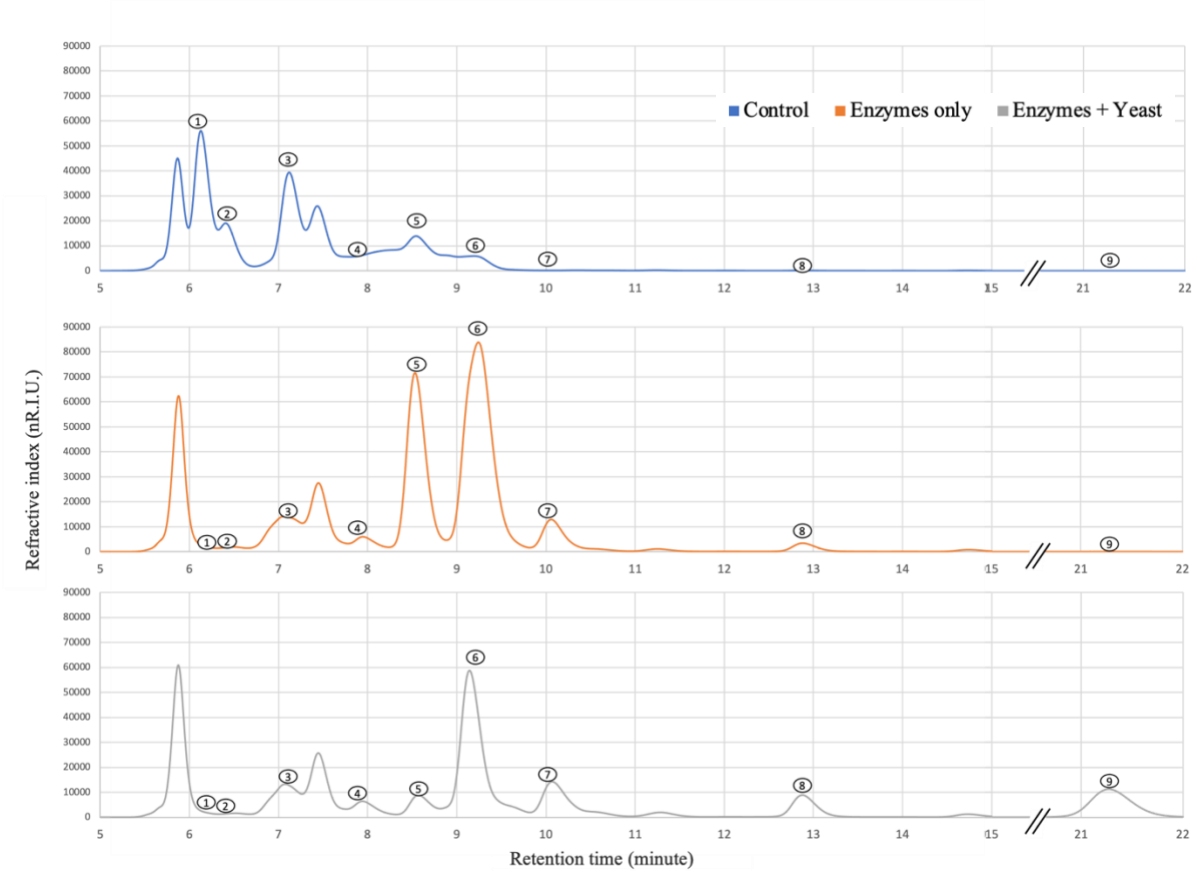

Fig. 3 HPLC chromatograms of hydrolysates after 12.25 h process, representing soluble saccharide profile, glycerol and ethanol. (1) Stachyose, (2) Raffinose, (3) Sucrose, (4) Galacturonic acid, (5) Glucose, (6) Xylose + Galactose + Fructose, (7) Arabinose, (8) Glycerol, (9) Ethanol

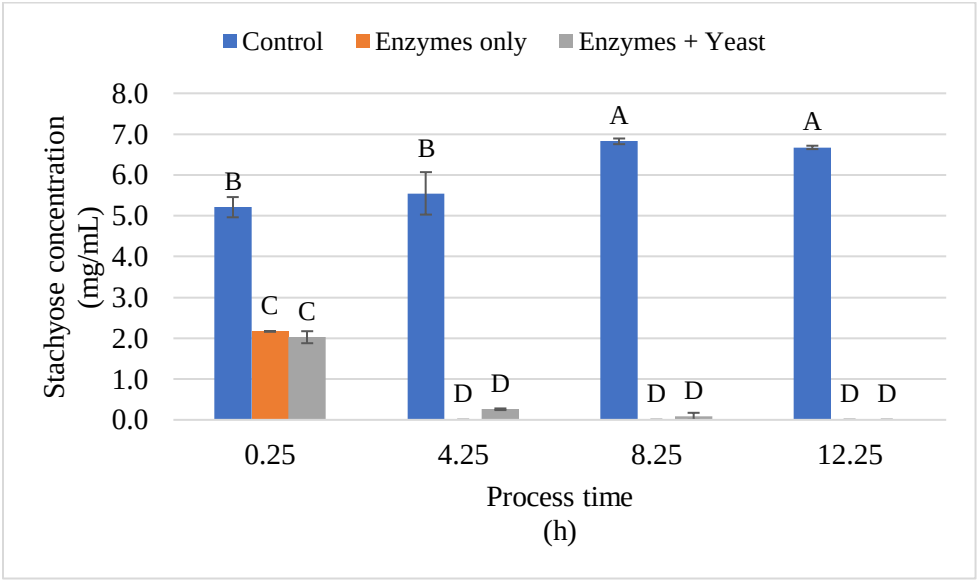

Fig. 4a

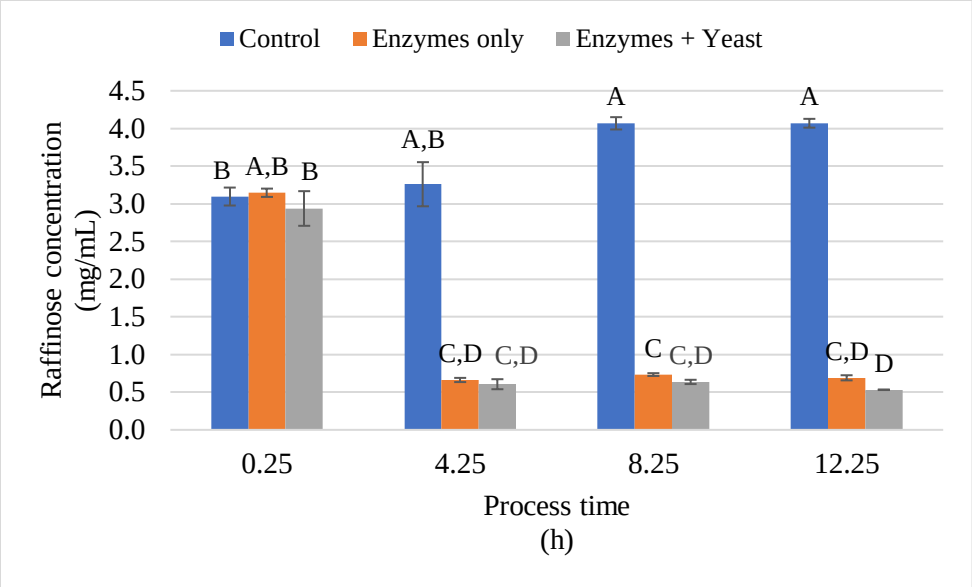
Control
Enzymes only
Enzymes + Yeast
Raffinose concentration (mg/mL)
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
B
A,B
B
A,B
C,D
C,D
A
C
C,D
A
C,D
D
0.25
4.25
8.25
12.25
Process time (h)

Fig. 4b

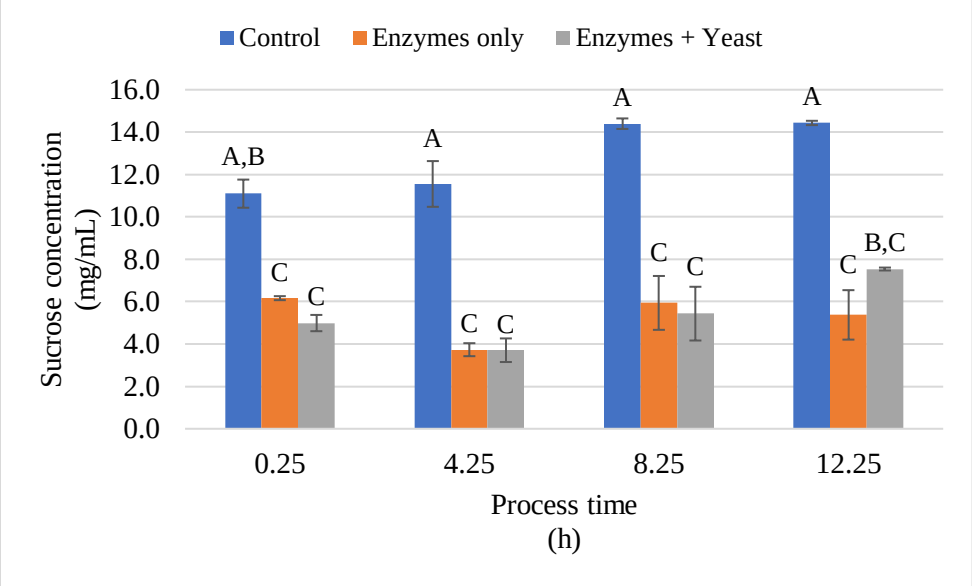
Control
Enzymes only
Enzymes + Yeast
Sucrose concentration (mg/mL)
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
A,B
C
C
A
C
C
A
C
C
A
C
B,C
0.25
4.25
8.25
12.25
Process time (h)

Fig. 4c

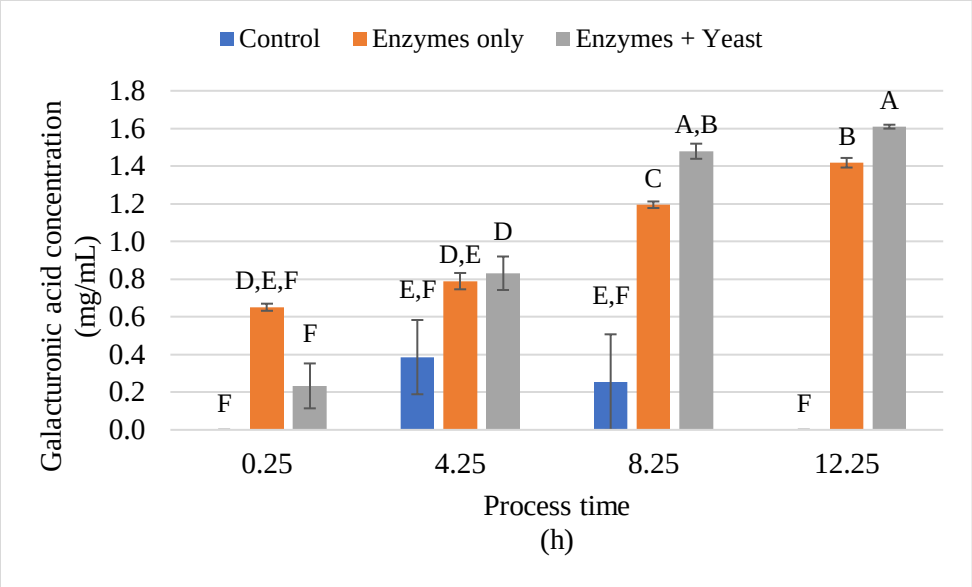
Control
Enzymes only
Enzymes + Yeast
Galacturonic acid concentration (mg/mL)
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
D,E,F
F
E,F
D,E
D
E,F
C
A,B
F
B
A
0.25
4.25
8.25
12.25
Process time (h)

Fig. 4d

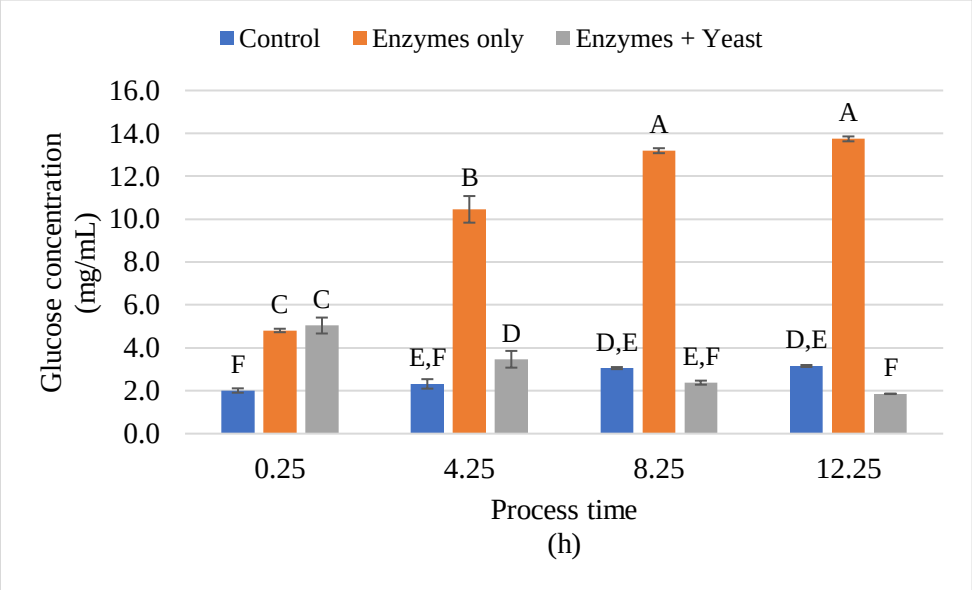
Control
Enzymes only
Enzymes + Yeast
Glucose concentration (mg/mL)
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
F
C
C
E,F
B
D
D,E
A
E,F
D,E
A
F
0.25
4.25
8.25
12.25
Process time (h)

Fig. 4e

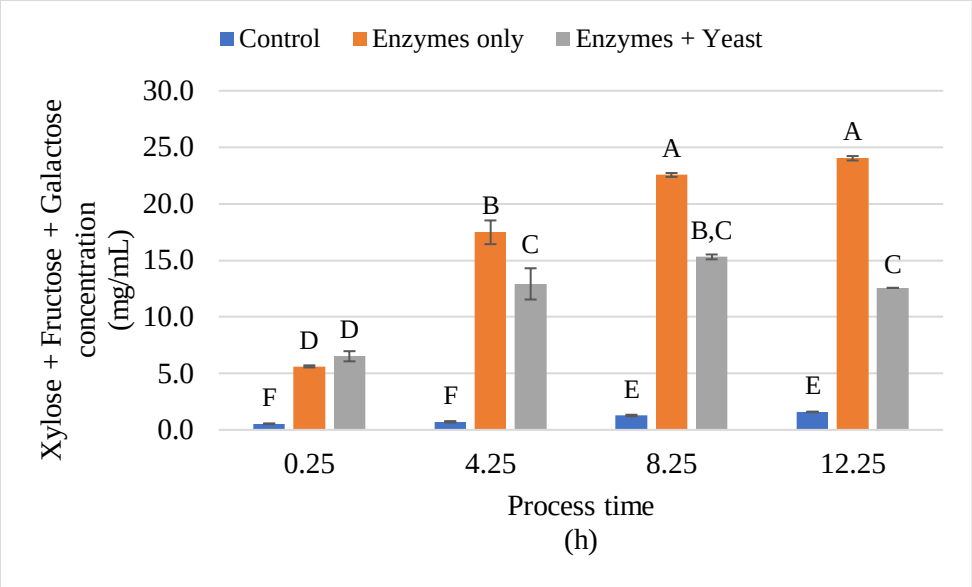
Control
Enzymes only
Enzymes + Yeast
Xylose + Fructose + Galactose concentration (mg/mL)
30.0
25.0
20.0
15.0
10.0
5.0
0.0
F
D
D
F
B
C
E
A
B,C
E
A
C
0.25
4.25
8.25
12.25
Process time (h)

Fig. 4f

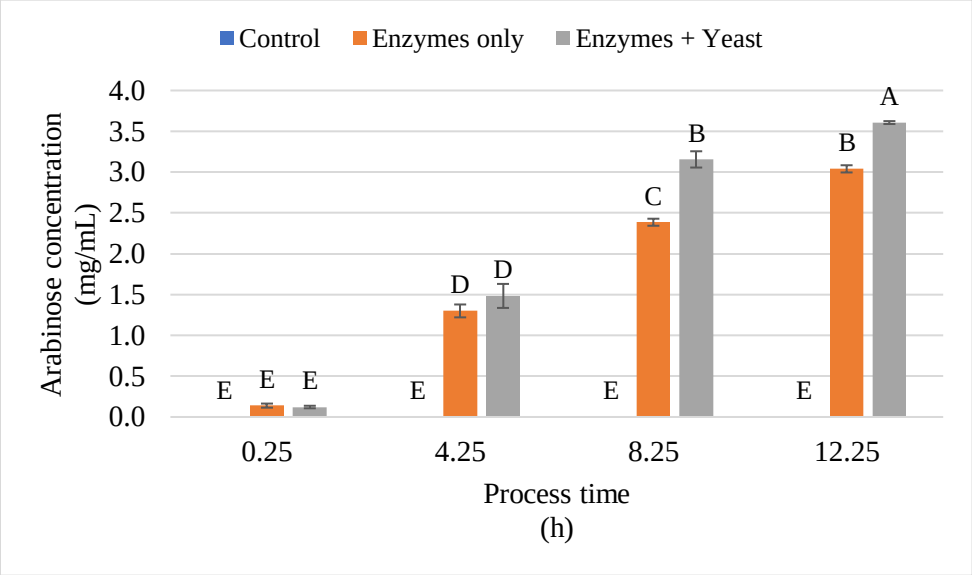
Control
Enzymes only
Enzymes + Yeast
Arabinose concentration (mg/mL)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
E
E
E
E
D
D
E
C
B
E
B
A
0.25
4.25
8.25
12.25
Process time (h)

Fig. 4g

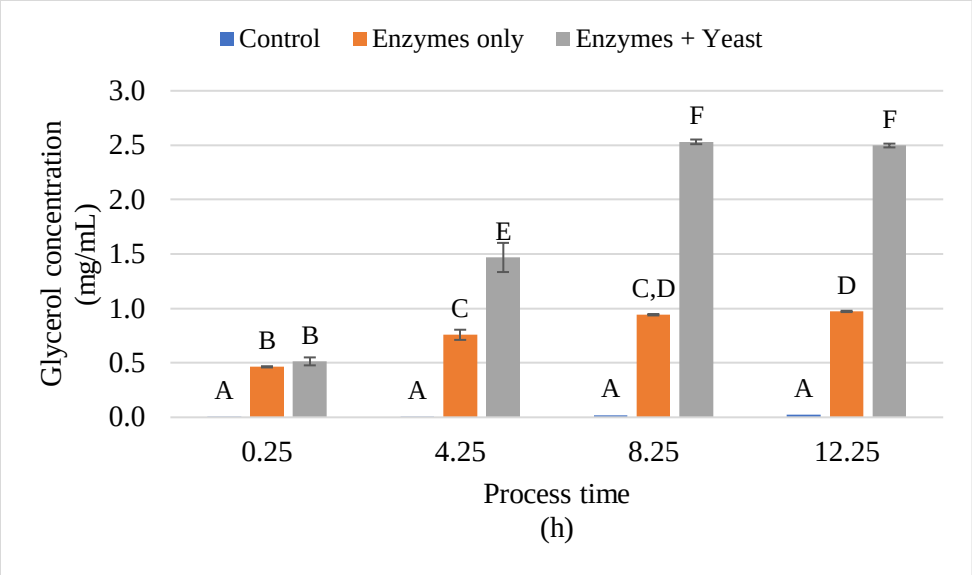

Fig. 4h

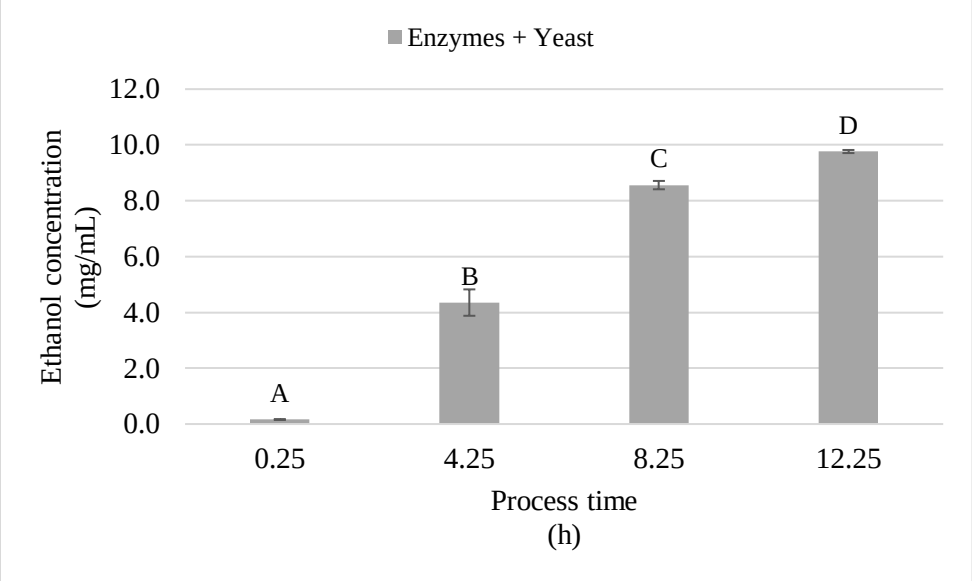

Fig. 4i Evaluation of the effect of control, enzymes only and enzymes + yeast treatments on carbohydrate hydrolysis, glycerol and ethanol fermentation as a function of process time. (a) Stachyose, (b) Raffinose, (c) Sucrose, (d) Galacturonic acid, (e) Glucose, (f) Xylose + Fructose + Galactose, (g) Arabinose, (h) Glycerol, (i) Ethanol. Error bars represent std. error (n=6). Treatments with the same letters are not significantly different, ($p<0.05$, Tukey's HSD test)

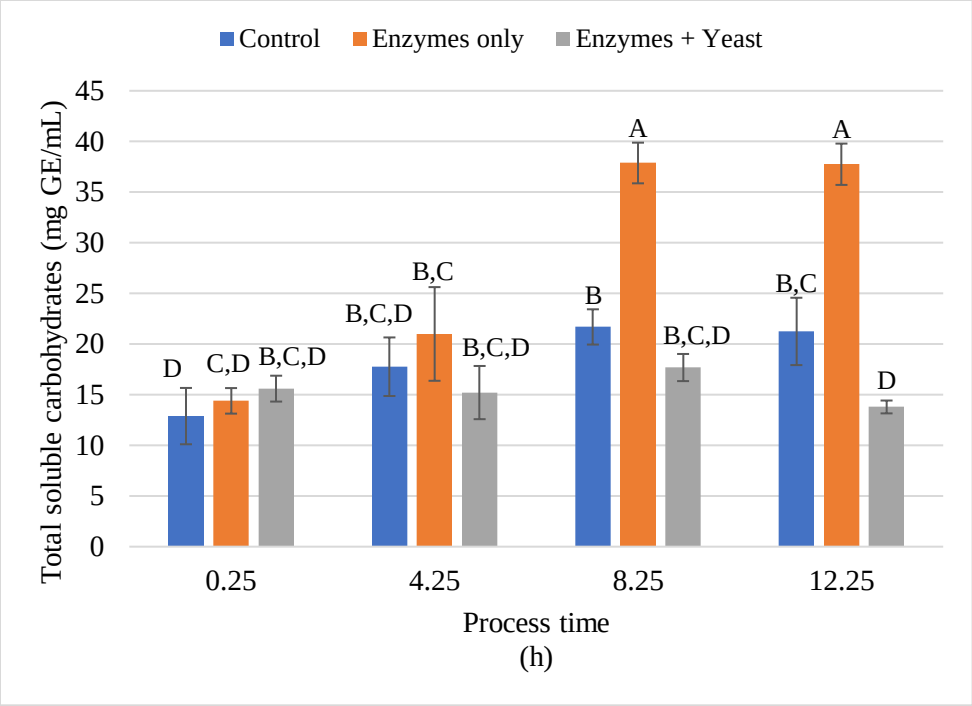

Fig. 5 Evaluation of the effect of control, enzymes only and enzymes + yeast treatments on total soluble carbohydrates as a function of process time. Error bars represent std. error (n=9). Treatments with the same letters are not significantly different, ($p<0.05$, Tukey's HSD test)

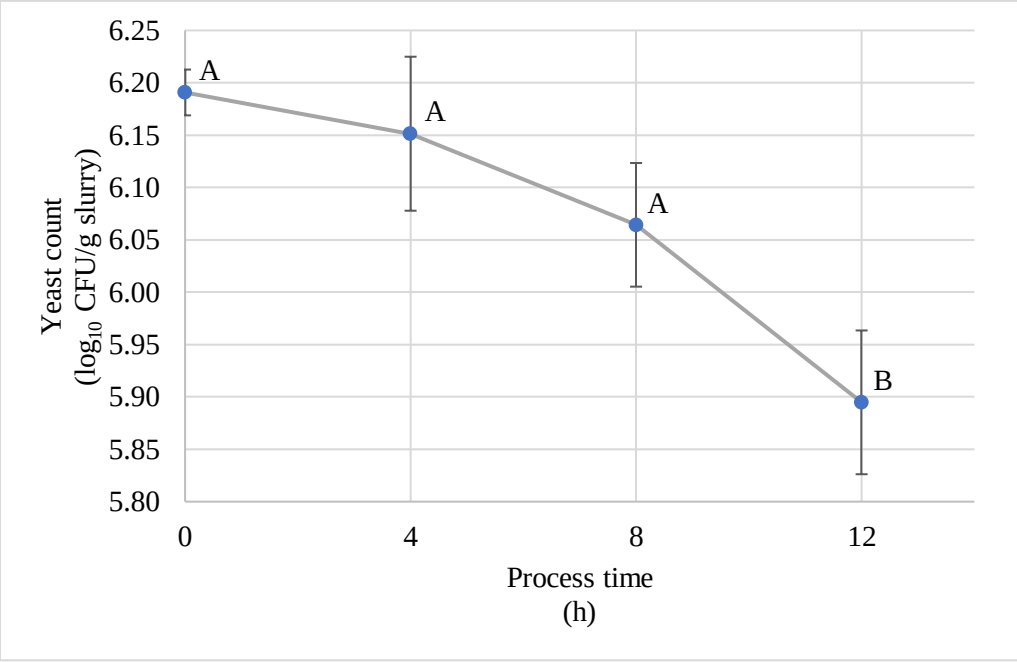

Fig. 6 Yeast concentration in slurry as a function of process time for the enzymes + yeast treatment. Error bars represent std. error (n=6). Counts with the same letters are not significantly different, ($p<0.05$, Tukey's HSD test)

3.3 Total Polyphenol Concentration changes

Polyphenols are phytochemicals that are associated with various health-promoting attributes (Pandey and Rizvi, 2009). Soy polyphenols include isoflavones, chlorogenic acid isomers, caffeic acid, and ferulic acid, which are antioxidants in nature (Pratt and Birac, 1979).

Figs. 7a and 7b show the total polyphenol concentration (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 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 (Gama et al., 2015). 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 is has been well discussed that polyphenols are bound to other polymers like 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 as the other reason why the TPC count increased in SPC and hydrolysate matrices. FC assay has previously been reported (Alessandri et al., 2014; Huang et al., 2005; Magalhaes et al., 2006) to be sensitive to various reducing sugars and subsequently be non-specific to polyphenol estimation. We have various reducing sugars in SPC and hydrolysate in the enzymes only and enzymes + yeast 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.

Soy protein concentrate has been a de-facto ingredient in the manufacture of formulated meat products, high moisture meat-analogues, and texturized protein products (Zhang et al., 2021). Most of these end products suffer from lipid and protein oxidation during cooking, handling, and storage due to high moisture, protein, and lipid contents (Nikmaram et al., 2018; 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 (Trujillo-Mayol et al., 2021), plum juice concentrate in hams (Nunez de Gonzalez et al., 2008), pomegranate rind powder in cooked chicken patties (Naveena et al., 2008) and so on. The SPC made from our process naturally contains a significantly high level of polyphenols, and therefore may allow for exclusion of any extraneously added antioxidant, thereby having one less ingredient to label on the package.

Soy polyphenols are the richest source of isoflavones (Fukuda et al., 2017). We speculate most polyphenols in the hydrolysate to 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 serves as a rich source for extracting polyphenols for nutraceutical, food, and cosmetic purposes.

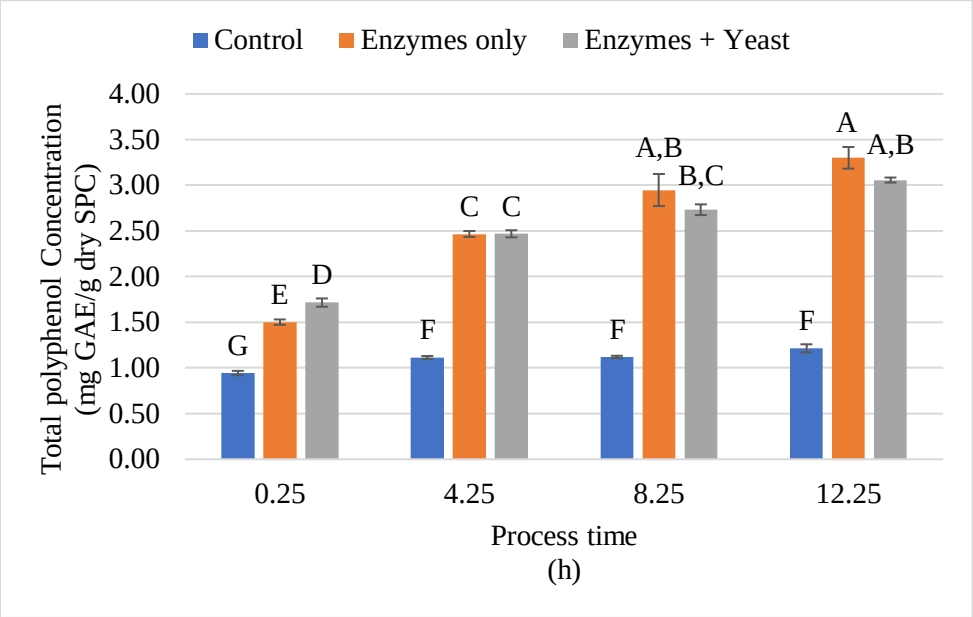

Fig. 7a

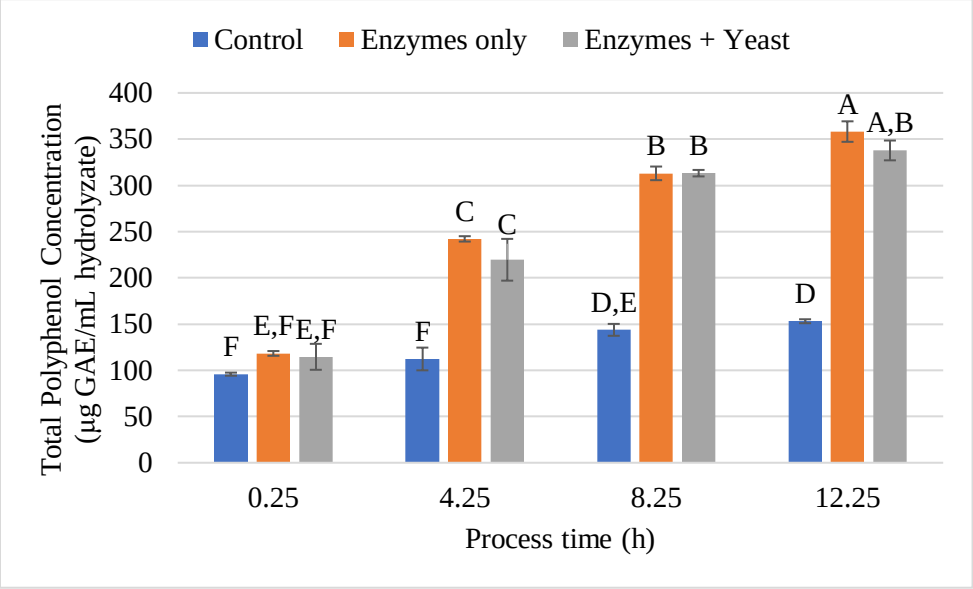

Fig. 7b Evaluation of TPC in (a) SPC, and (b) hydrolysate due to control, enzymes only and enzymes + yeast treatments as a function of process time. Error bars represent std. error (n=9). Treatments with the same letters are not significantly different, ($p<0.05$, Tukey's HSD test)

4. 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 co-product 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 co-product 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 $\pm$ 0.25% protein and 384 mL hydrolysate containing 9.76 $\pm$ 0.05 mg/mL ethanol, with 84.4 $\pm$ 0.01% protein recovery. SPC and the hydrolysate both contained significantly increased amounts of polyphenols. Enzymes-only treatment also produced high-quality SPC at 63.25 g dry matter with 70.42 $\pm$ 0.07% protein and 374 mL hydrolysate containing hexose-rich feedstock, with 83.8 $\pm$ 0.01% protein recovery. The enzymes-only treatment, thus, validates a similar work done by Al Loman et al. (2016), where SPC was made by enzymatic hydrolysis of carbohydrates of DSF for 48 h at 50°C using enzymes extracted from a fermentate of soy hulls by *Aspergillus niger*. Enzymatic hydrolysis coupled with simultaneous fermentation by yeasts, as explored in our study, enables further enzymatic hydrolysis of carbohydrates by synergism, thereby accelerating the protein concentration, allowing the process to yield a desirable product by 8 - 12 h, as opposed to 48 h. The process also produces a valuable co-product ethanol, along with a polyphenol-rich hydrolysate. A techno-economic analysis is further warranted to determine if the developed process would be cost-effective for SPC production at an industrial scale. Thus, the availability of such an SPC would make it a much more desirable ingredient in comminuted meat products or plant-based meat analogues.

4.1 Scopes For Future Work

The complex structure of hemicelluloses and pectic polysaccharides coupled with their varying solubility in the slurry proved to limit the catalytic activity of hydrolytic enzymes. Insoluble cellulose, along with its crystalline structure, was also hypothesized to be not easily accessible to enzymatic hydrolysis, as similarly reported by Jung et al. (2006) and Al Loman and

Ju (2016). We speculated that the simultaneous utilization of monomerized carbohydrates helped reduce the product inhibition of enzymes, which subsequently allowed those enzymes further to hydrolyze more carbohydrates (Fig. 4d, g). The exact mechanisms of such a dynamic relationship between the enzymes (cellulases, hemicellulases, pectinases) and their respective substrates have not been completely understood (Bansal et al., 2009; McCann and Carpita, 2008). The enzymatic hydrolysis of cellulose in itself is a sequence of processes that include either adsorption of cellulase to a cellulose microfibril or binding to a single molecule of cellulose by the active site of the catalytic domain, the disruption and penetration of cellulose microfibrils by certain other proteins, followed by cellulose breakdown only at the accessible glycosidic bonds at the cellulose/fluid interface (Jeoh et al., 2017). The degree of compactness of cellulose microfibrils also affects the available hydrophobic and hydrophilic surface areas of cellulose and the subsequent accessibility to glycosidic bonds by cellulases, as found in molecular dynamics calculations by Sinko and Keten (2015). Thus, it is crucial to have a mechanistic understanding of the action of such carbohydrase enzymes on their substrates with varying solubility at a molecular level. The elucidation of rate-limiting steps in the enzyme-substrate reactions and sturdy kinetic models can help decide the enzyme dosage for these kinds of simultaneous saccharification and fermentation processes, which would help rational reaction engineering for optimizing reactor operations and process designs (Jeoh et al., 2017).

The rationale to use chloramphenicol as an antibiotic in this process was to suppress the growth of competing microbes like lactic acid bacteria. Chloramphenicol is prohibited in food systems for many years for its nephrotoxic, aplastic anemic, and mutagenic effects (Doğan, 2020). Nisin is an approved food preservative (E234) and is similarly effective against gram-positive bacteria like lactobacillales (Gharsallaoui et al., 2016). Thus, a study with nisin under the conditions of the enzymatic hydrolysis and fermentation process elucidated in this study would allow the production of SPC that would fit human consumption. Optimization of enzyme dosing and yeast inoculation ratio also needs to be investigated with an objective of maintaining a carbon-limited environment such that the sugar is fermented as soon as it is made available by the action of hydrolytic enzymes so that the competing microbes do not have sufficient nutrients to grow and compete with yeasts, ultimately, eliminating the need to use antimicrobials.

The inclusion of yeast proteins into the SPC matrix has the potential to alter the amino acid, aroma, and taste profile of the resultant ingredient. Yeast extract is known to have a meaty aroma

and umami taste (Alim et al., 2020). The umami taste is often attributed due to the presence of free glutamic acid (Alim et al., 2020). As an ingredient widely used to make texturized protein products that mimic meat texture and flavor (Zhang et al., 2021), SPC with a savory flavor profile may make the end-product more palatable than an SPC which is bland or moderately beany. Thus, this hypothesis needs further investigation, which would enable the resultant SPC to be well suited to make plant-based meats based on the flavor profile.

The enzymatic hydrolysis and fungal fermentation process can be extrapolated to other plant based proteins sources to prepare their protein concentrates without using alcoholic solvents or harsh heat denaturing treatments. The isoelectric point of certain plant based proteins like oats, wheat germ, yellow pea, chickpeas, and lupins fall within pH 4.0 – 7.0 (Ge et al., 2000; Lan et al., 2018; Ma and Harwalkar, 1984; Ruiz Jr and Hove, 1976; Sánchez-Vioque et al., 1999), which also happens to be the range of activity of the carbohydrase enzymes and yeast fermentations used in this study. Thus, by making some substrate dependent enzyme dosage changes, proteins from these sources can be similarly concentrated while valorizing the carbohydrate residue by simultaneously fermenting it into ethanol. Thus, this process has a potential to be a versatile platform for making protein rich products.

Nomenclature

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
GalU	Galactose Unit
GE	Glucose Equivalent
HPLC	High Performance Liquid Chromatography
HSD	Honest Significant Difference
NDF	Neutral Detergent Fiber
SBM	Soybean Meal
SPC	Soy Protein Concentrate
SPI	Soy Protein Isolate
SSF	Simultaneous Saccharification and Fermentation
TPC	Total Polyphenol Concentration

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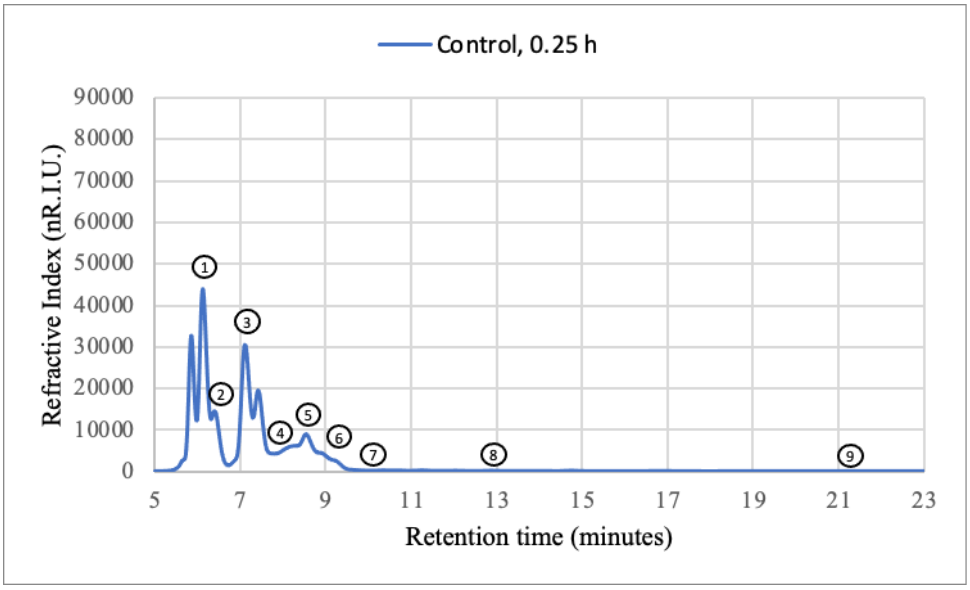
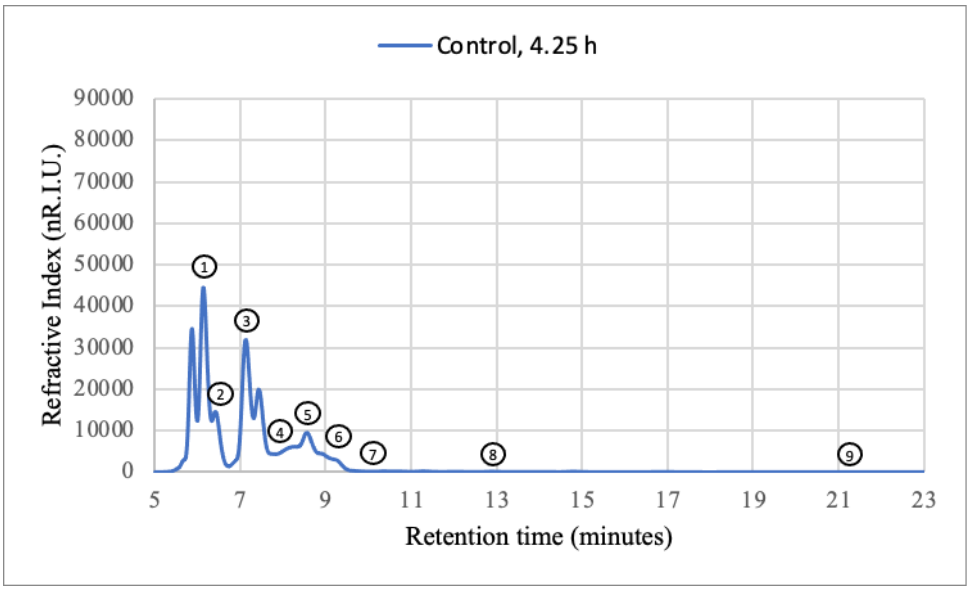
Appendix: Supplementary Figures



Supplementary Fig. 1a



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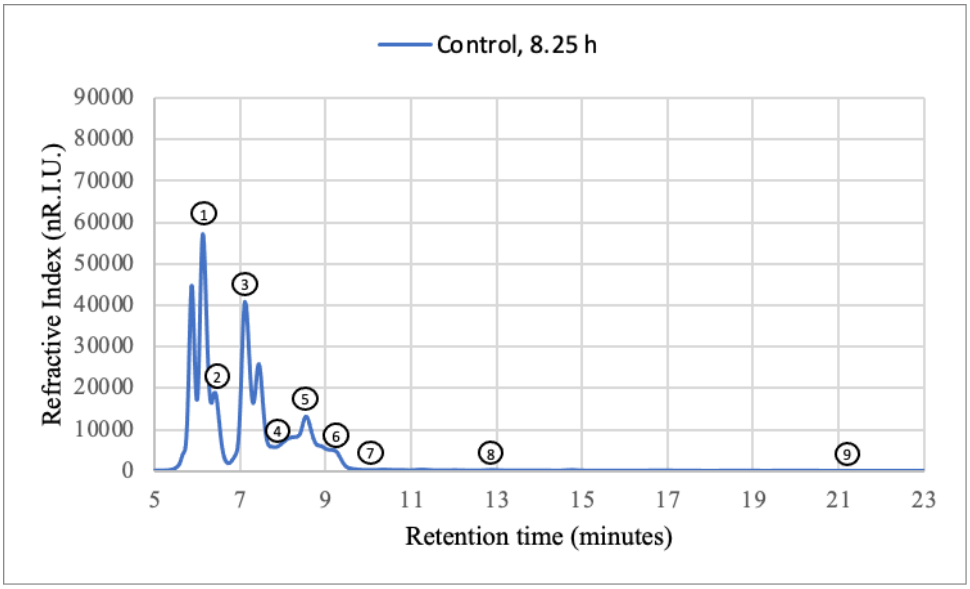

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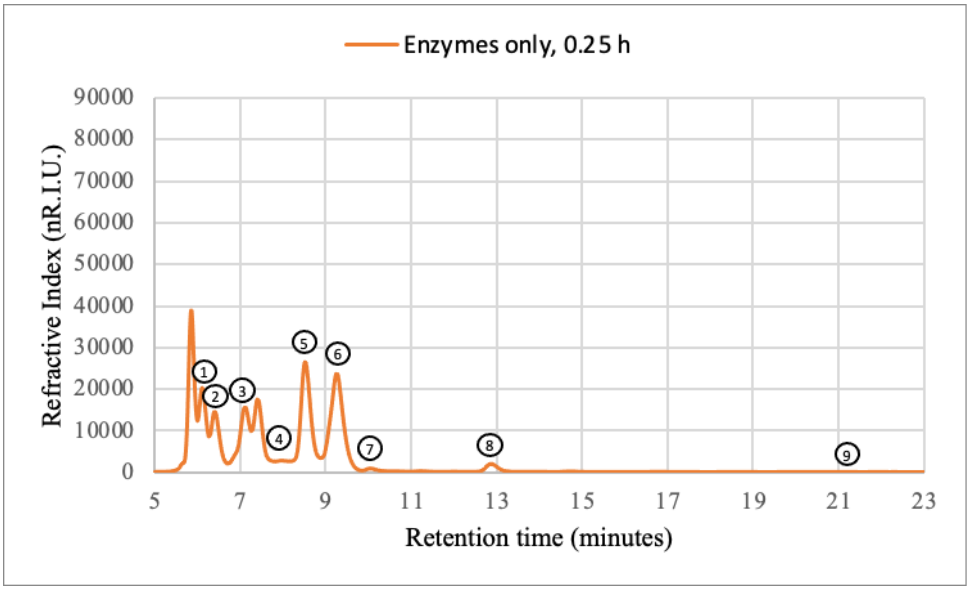

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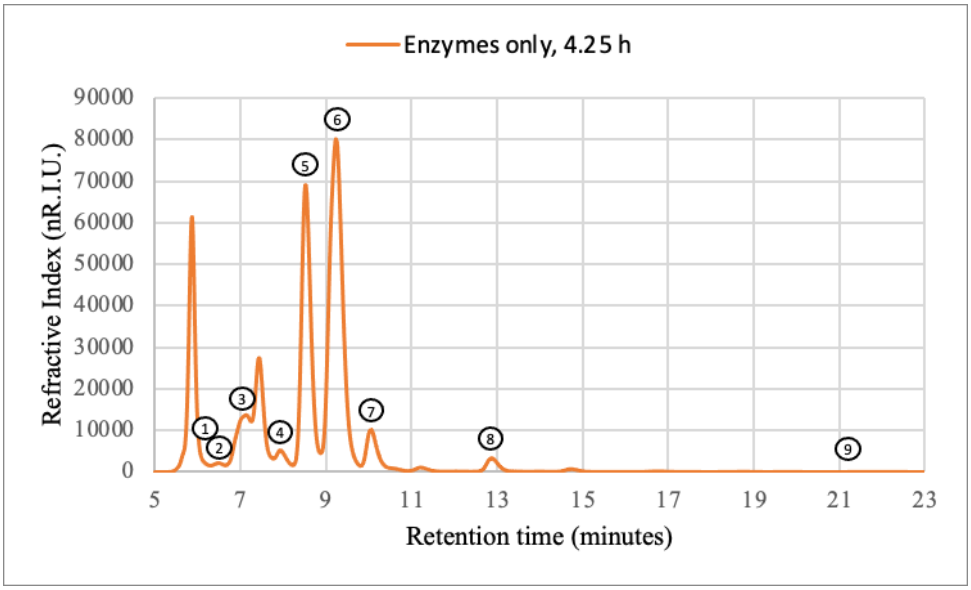

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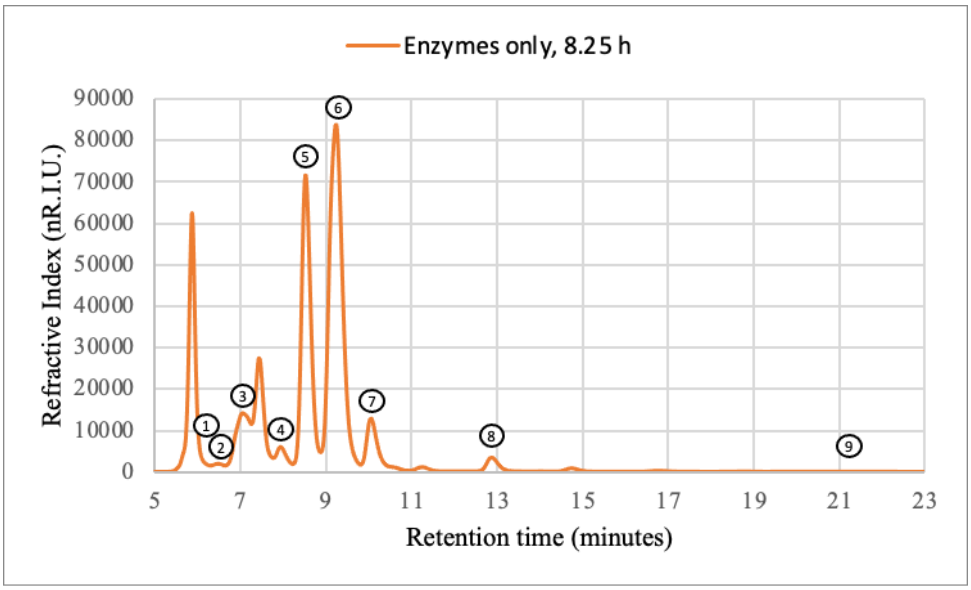

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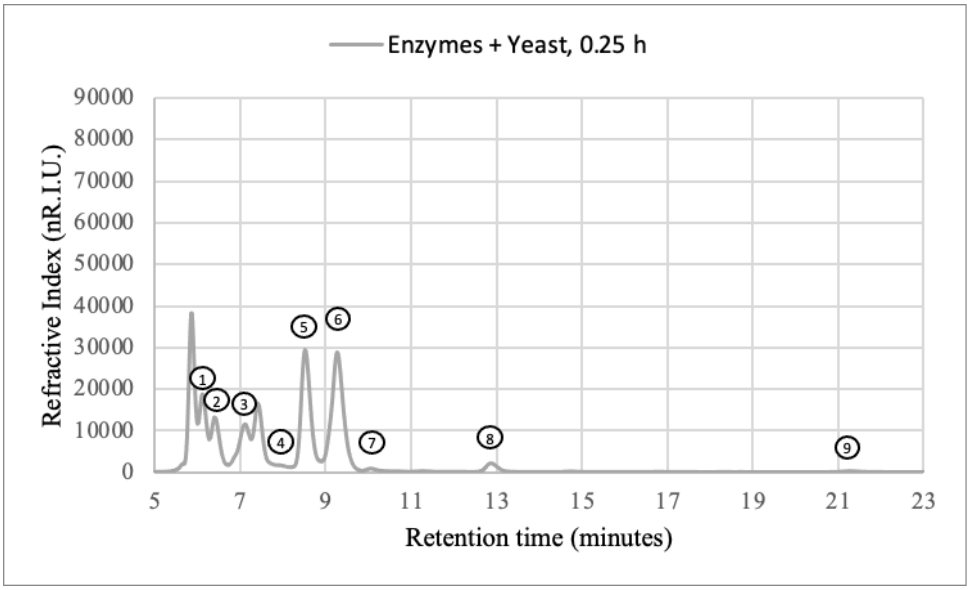

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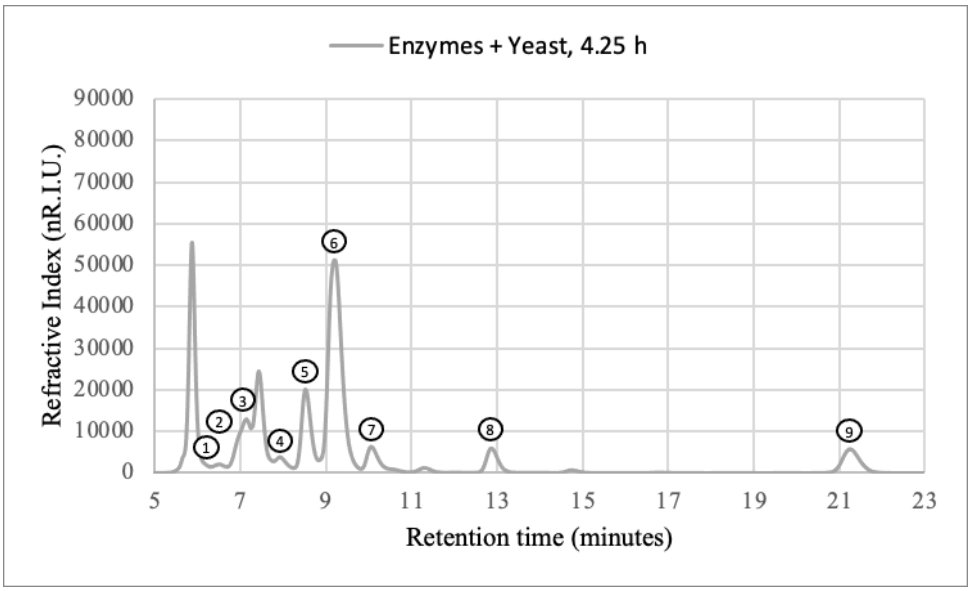
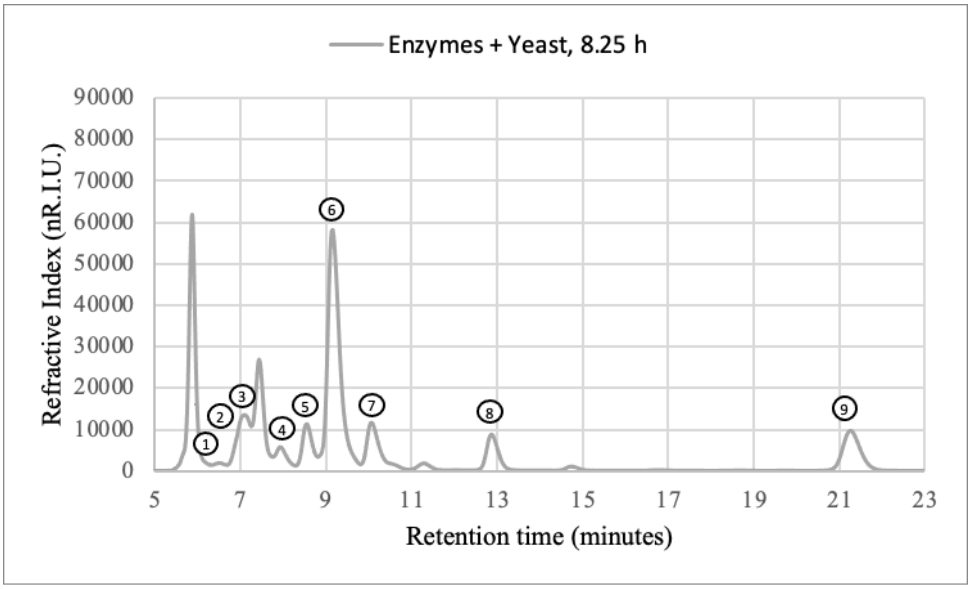
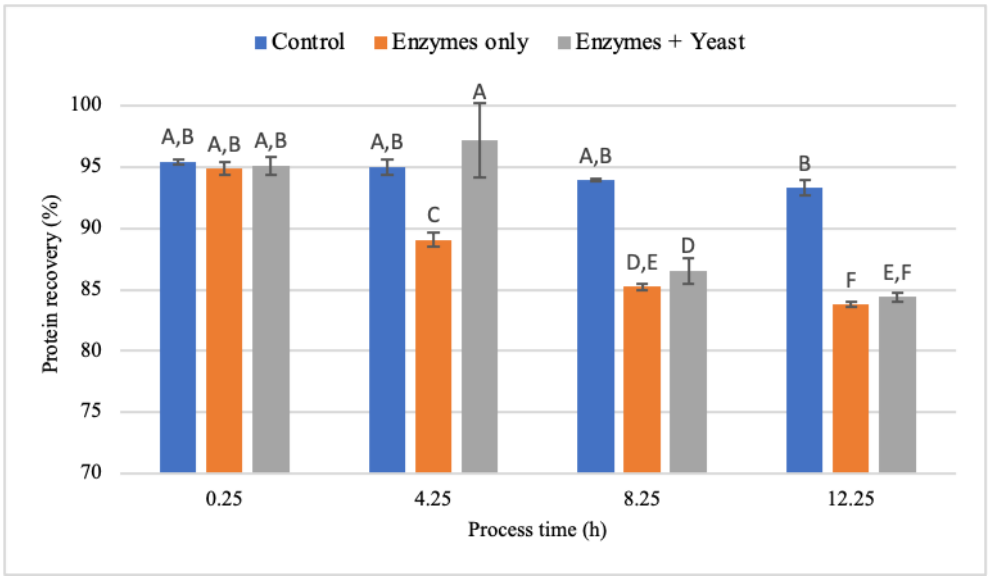


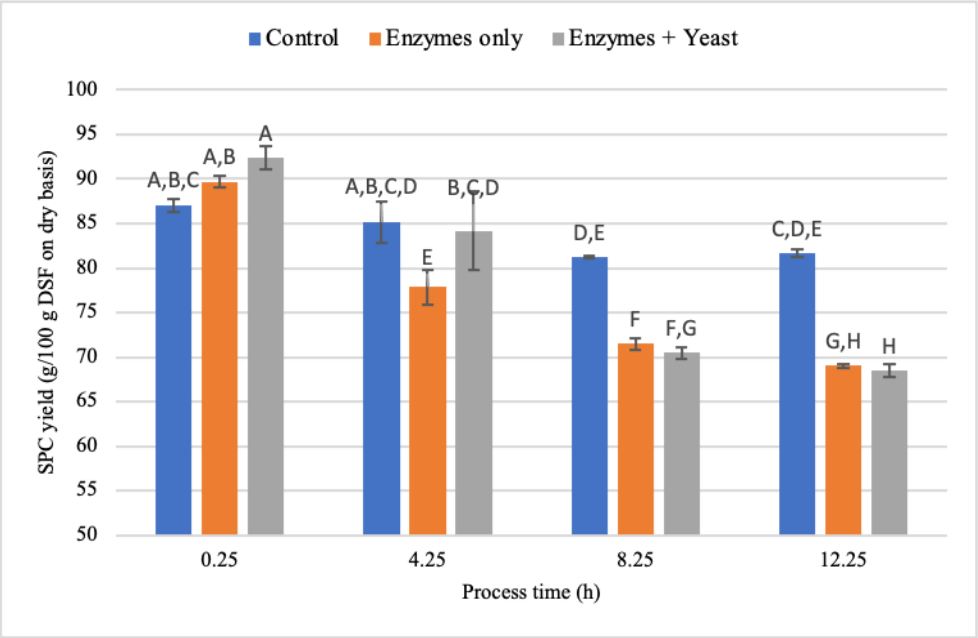
Supplementary Fig. 1h



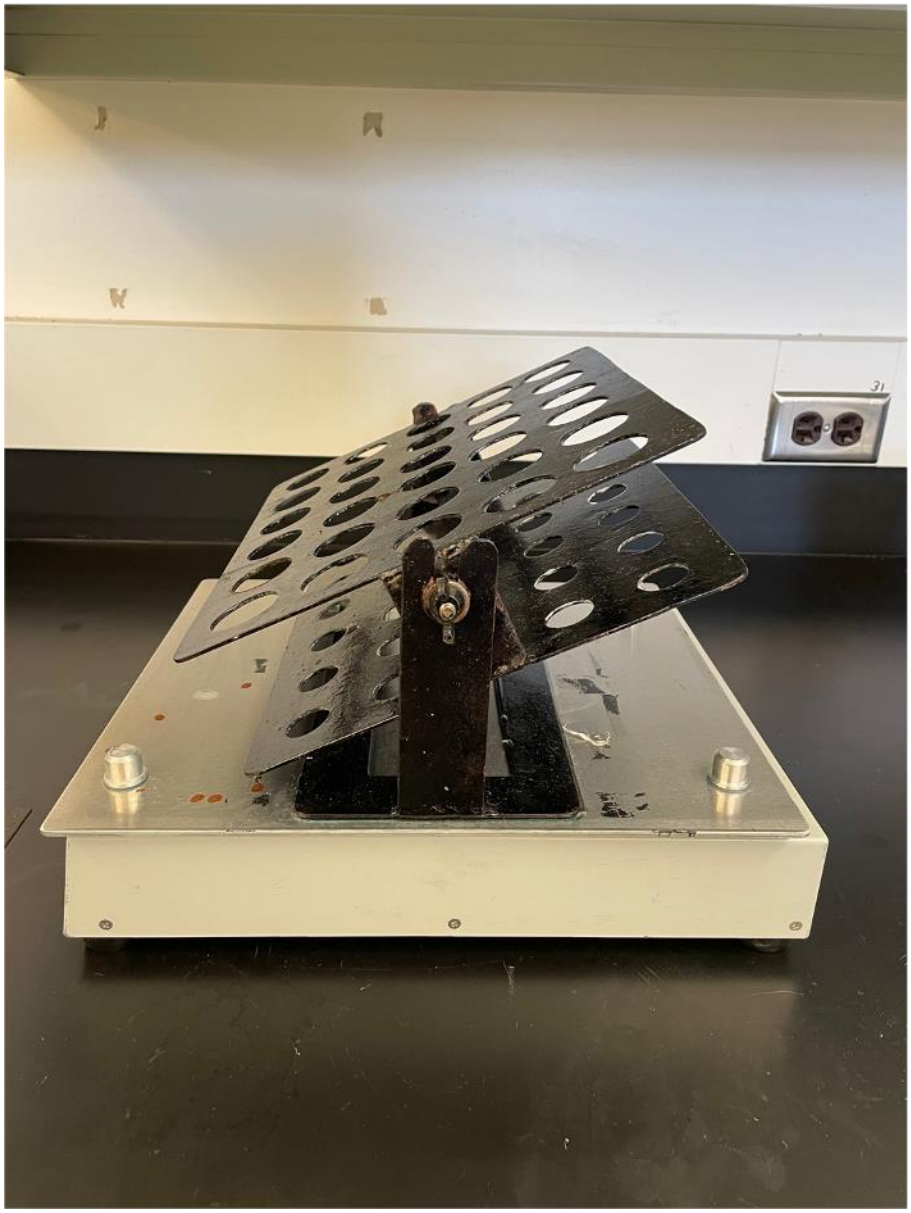
Supplementary Fig. 1i: HPLC chromatograms of control, enzymes only and enzymes + yeasts' hydrolysates after 0.25, 4.25, and 8.25 h respectively, representing soluble saccharides, glycerol and ethanol. (1) Stachyose, (2) Raffinose, (3) Sucrose, (4) Galacturonic acid, (5) Glucose, (6) Xylose + Galactose + Fructose, (7) Arabinose, (8) Glycerol, (9) Ethanol



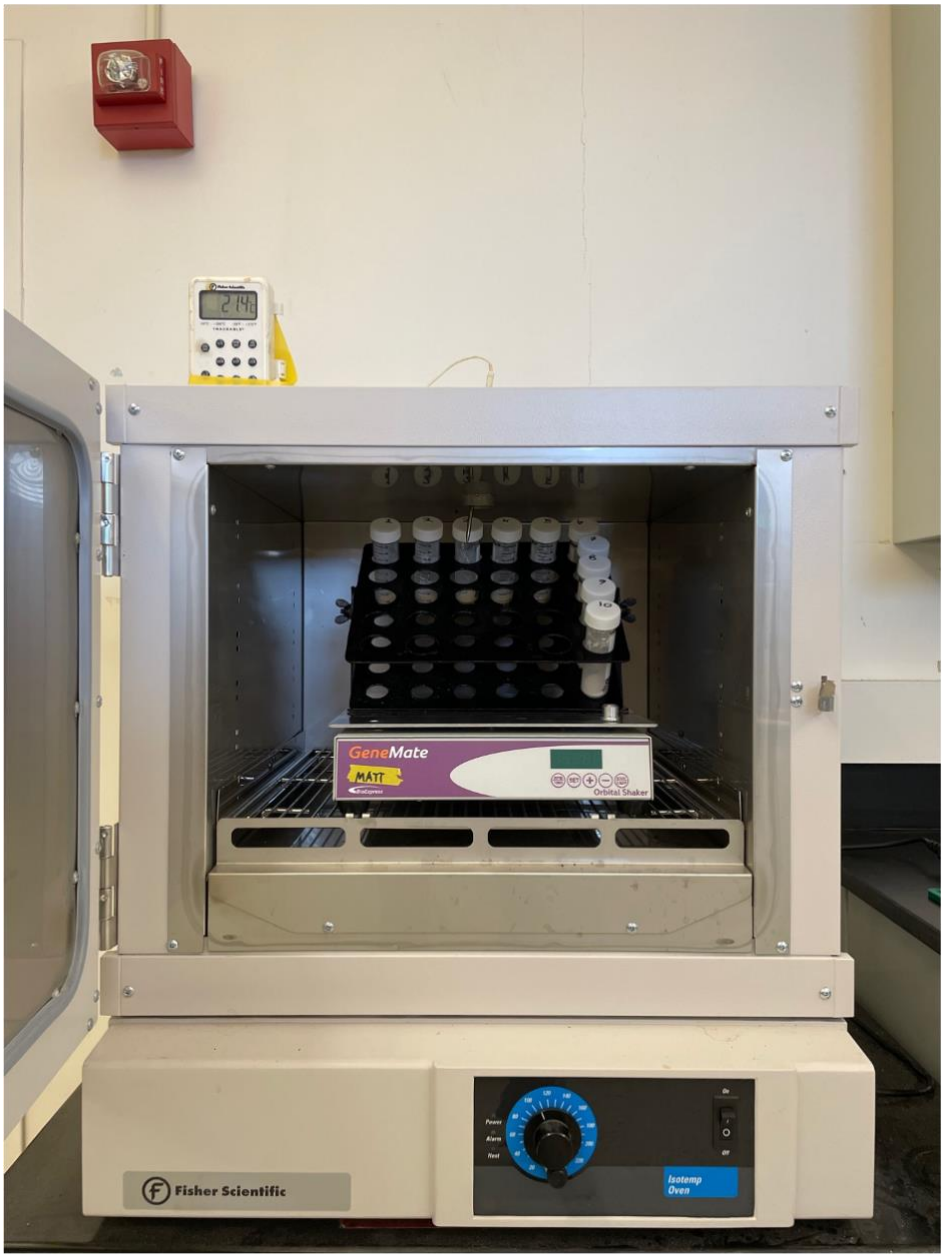
Supplementary Fig. 2: Protein recoveries from control, enzymes only, and enzymes + yeast treatments as a function of process time



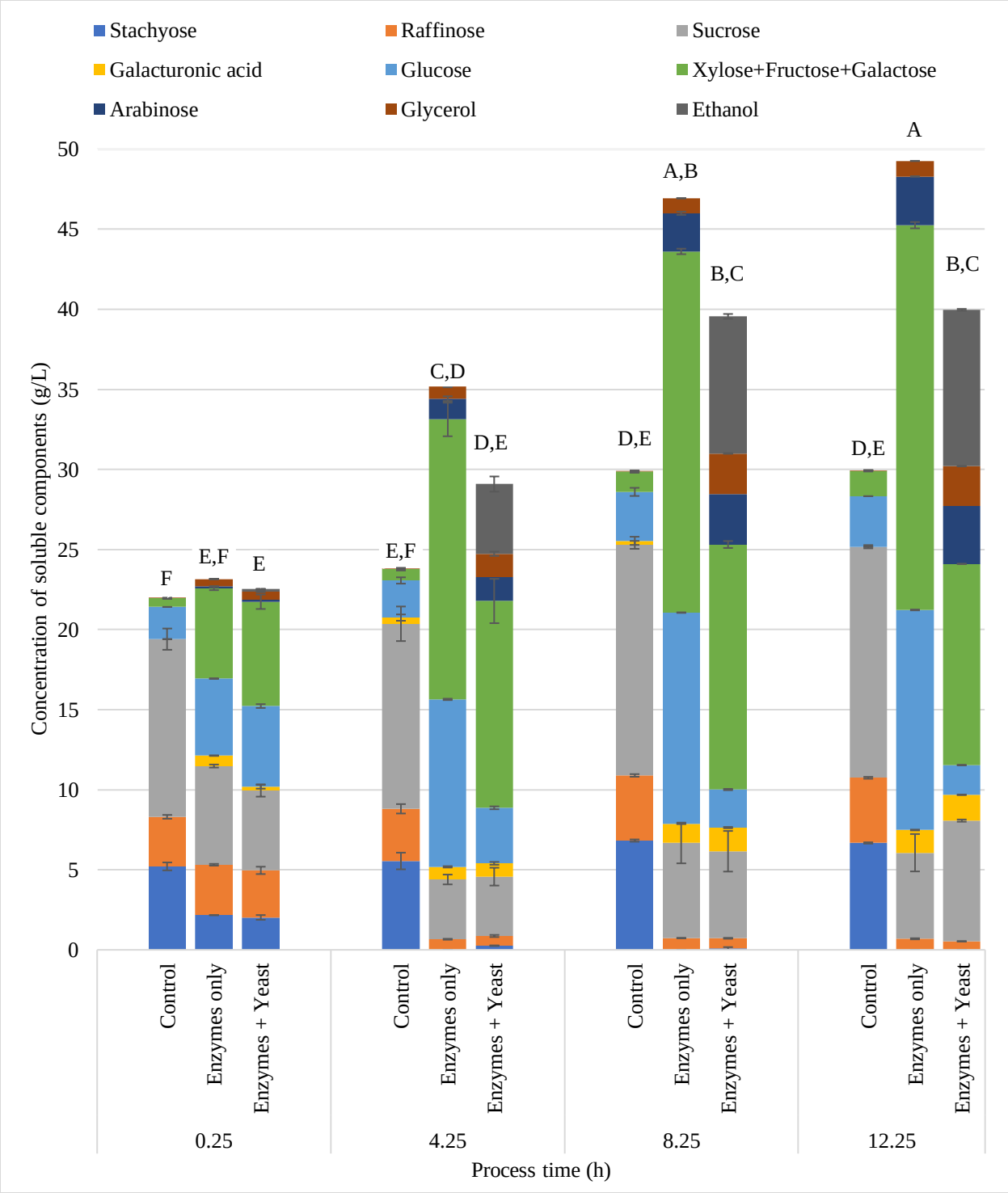
Supplementary Fig. 3: SPC yield from DSF on dry basis in control, enzymes only, and enzymes + yeast treatments as a function of process time



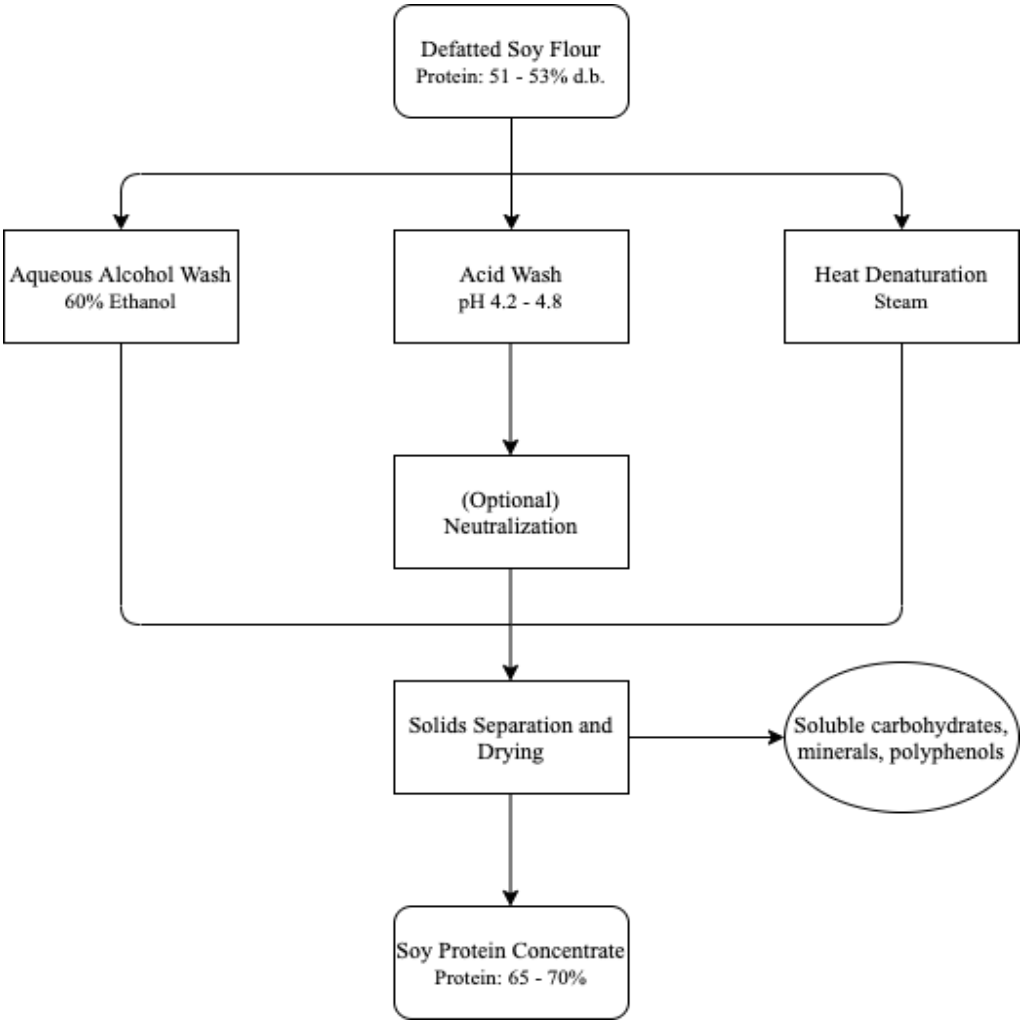
Supplementary Fig. 4: Custom fabricated tiltable centrifuge tube rack (30 tube capacity) fixed on an orbital shaker



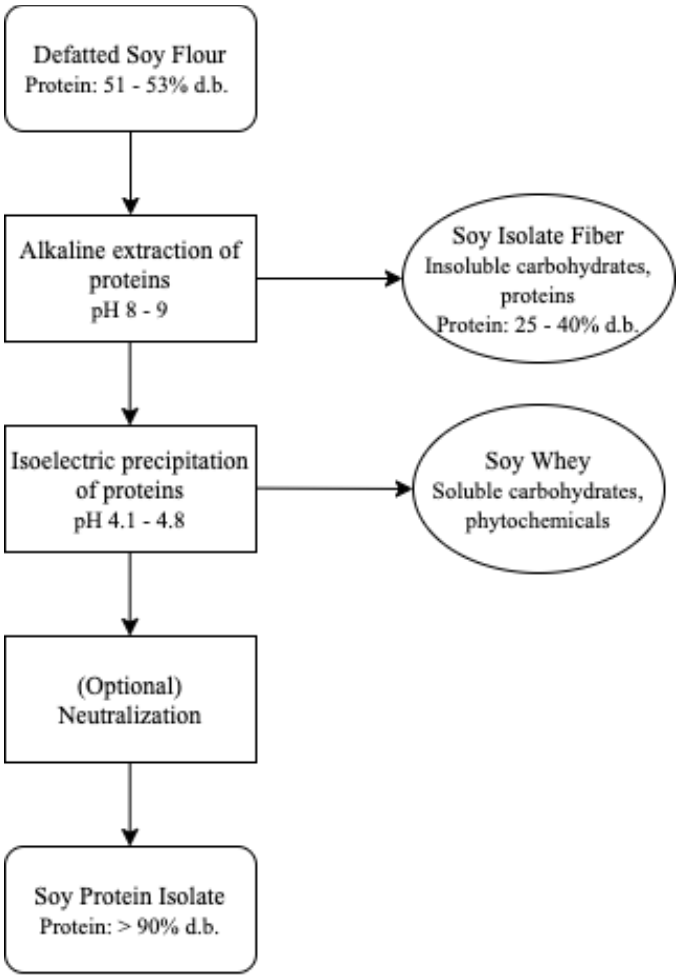
Supplementary Fig. 5: Custom tiltable tube rack fixed on an orbital shaker inside an isotemperature oven with a digital temperature readout via a thermocouple



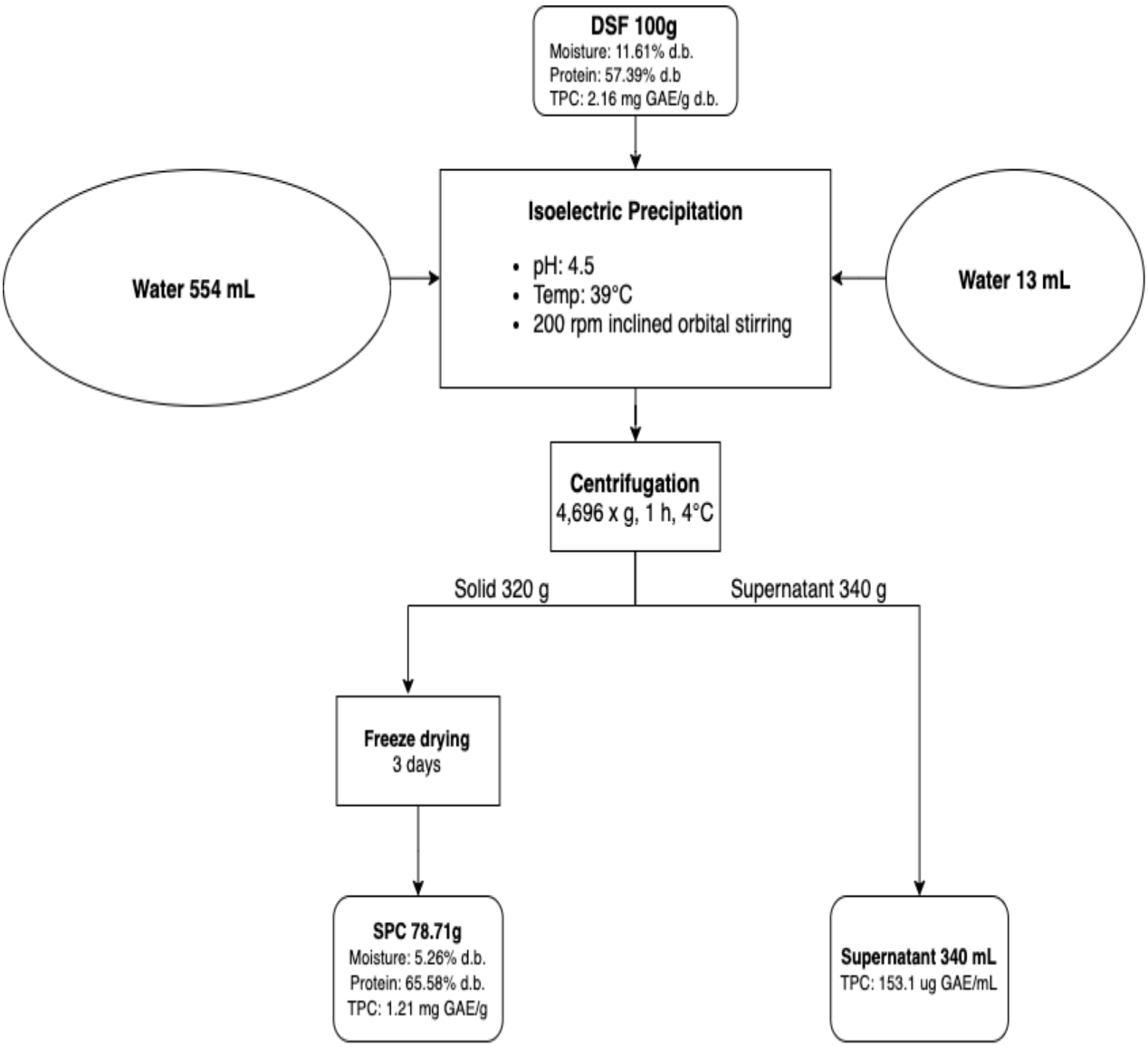
Supplementary Fig. 6: Soluble components as identified by HPLC in hydrolysate of control, enzymes only and enzymes + yeast treatments as functions of process time. Error bars represent std. error (n=6) of individual components. Sum of components with the same letters are not significantly different, ($p<0.05$, Tukey's HSD test)



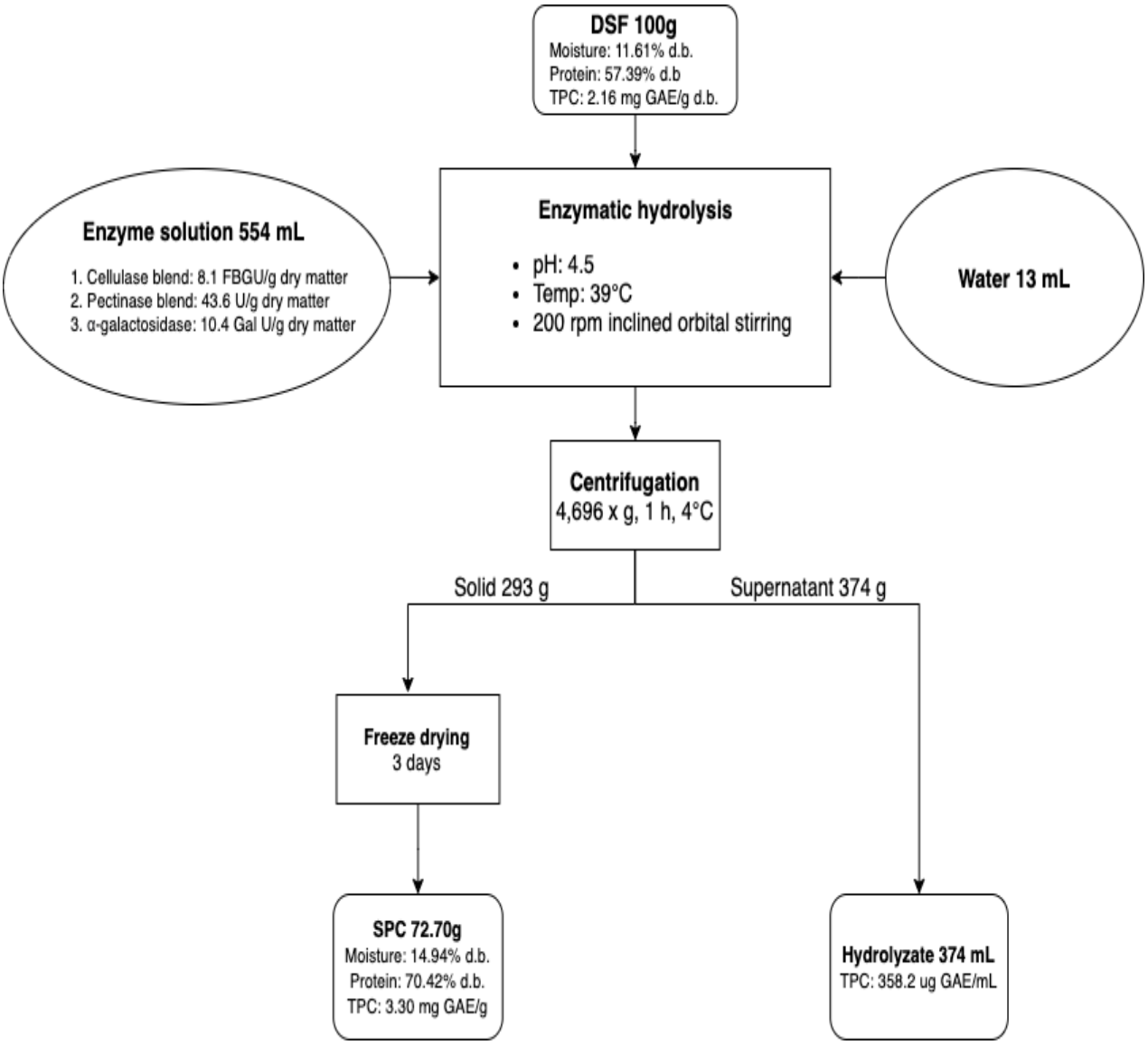
Supplementary Fig. 7: Conventional methods of soy protein concentrate manufacture



Supplementary Fig. 8: Conventional method of soy protein isolate manufacture



Supplementary Fig. 9: Flowchart with material balance for the production of SPC via control treatment for 12.25 h



Supplementary Fig. 10: Flowchart with material balance for the production of SPC via enzymes only treatment for 12.25 h