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Integrating functional and techno-economic analyses to optimize black bean protein extraction: a holistic framework for process development

Jasmin S. Yang [a], Fernanda F.G. Dias [a], Karen A. McDonald [b,c], Juliana M.L.N. de Moura Bell [a,d,*]

[a] Department of Food Science and Technology, University of California, Davis, One Shields Avenue, Davis, CA, 95616, United States
[b] Department of Chemical Engineering, University of California, Davis, One Shields Avenue, Davis, CA, 95616, United States
[c] Global HealthShare® Initiative, University of California, Davis, One Shields Avenue, Davis, CA, 95616, United States
[d] Department of Biological and Agricultural Engineering, University of California, Davis, One Shields Avenue, Davis, CA, 95616, United States

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ABSTRACT

This study aimed to develop aqueous (AEP) and enzyme-assisted extraction processes (EAEP) for black bean proteins using an optimization framework that integrates functionality and industrial-scale profitability. A preliminary screening was performed to identify the best pH (AEP) and food-grade enzyme (EAEP) regarding total protein extractability (TPE), solubility, and *in vitro* protein digestibility. Techno-economic analyses revealed that the AEP at pH 7 and the EAEP with Alkaline Protease (AP) at pH 9 yielded the lowest overall cost of goods sold/kg of soluble and digestible protein. Experimental designs were performed to further guide the selection of solids-to-liquid ratio (SLR), extraction time, and enzyme concentration (EAEP only) to maximize discounted cash flow rate of return (DCFRR). The optimal conditions for the AEP (pH 7, 1:12 SLR, 15 min, 50 °C) and EAEP (pH 9, 1:12 SLR, 30 min, 0.5% AP, 50 °C) achieved TPEs of 66.2% and 80.8%, respectively, with DCFRRs (30-year project lifetime, $16.50/kg protein selling price) of 12.5% (AEP) and 18.2% (EAEP), demonstrating that despite the additional enzyme cost, the EAEP was more profitable. EAEP proteins exhibited significantly higher solubility (54%) in acidic conditions compared to AEP proteins (33%). However, higher enzyme loadings (0.5% AP) led to decreased emulsifying and foaming properties, especially in neutral conditions. This work offers valuable insights into the interconnected impacts of extraction conditions on protein yields, nutritional properties, and functionality, all while considering economic feasibility. Additionally, it underscores the effectiveness of holistic optimization strategies to develop protein extraction methods that are both efficient and commercially viable.

1. Introduction

The common bean (*Phaseolus vulgaris* L.) is one of the most important pulses in the world with respect to nutrition, economic value, and sustainability (Broughton et al., 2003; Hayat et al., 2014). Common beans are largely comprised of carbohydrates (36−40% starch and 23−32% total dietary fiber) (Tosh and Yada, 2010; Chung et al., 2008), but their high protein content (18−26%) makes them an important staple food in many parts of the world (Fordham et al., 1975). While beans are often consumed directly, common bean protein isolates and concentrates exhibit desirable techno-functional properties (e.g., solubility, emulsifying, foaming, gelation), making them highly promising fractions for use in a variety of plant-based food applications (Sathe and Salunkhe, 1981a; Wani et al., 2015; Shevkani et al., 2015; Hojilla-Evangelista et al., 2018; Yang et al., 2024a; de Paiva Gouvêa et al., 2023). The expansion of industrial common bean protein production would provide a valuable addition to the existing portfolio of commercially available plant protein ingredients produced from pea, chickpea, lentil, and mung bean (Good Food Institute, 2021).

The optimization of extraction strategies to maximize yields, produce high quality functional proteins, and achieve high profitability is critical in the development of any protein ingredient. Protein extraction from pulses is typically performed using aqueous extraction processes (AEP), in which pulse flours are dispersed in alkaline extraction media with or without mild heating, agitated for a given amount of time, then centrifuged to separate a protein-rich aqueous extract from the insoluble

* Corresponding author. Department of Food Science and Technology University of California, Davis One Shields Avenue, Davis, CA, 95616, United States.
E-mail addresses: jslyang@ucdavis.edu (J.S. Yang), ffgdias@ucdavis.edu (F.F.G. Dias), kamcdonald@ucdavis.edu (K.A. McDonald), jdemourabell@ucdavis.edu (J.M.L.N. de Moura Bell).

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starch/fiber-rich fraction (byproduct) (Boye et al., 2010). A scalable and easily implementable modification to aqueous extraction is the enzyme-assisted aqueous extraction process (EAEP), which involves the addition of commercial carbohydrases and/or proteases to the extraction slurry. The EAEP can improve extraction yields through cell-matrix disruption and protein solubilization (via proteolysis), while simultaneously inducing structural modifications that can affect the functional, thermal, and nutritional properties of the extracted proteins (Yang et al., 2024a; Dias et al., 2022; de Souza et al., 2020; Dias and de Moura Bell, 2022). To our knowledge, enzyme-assisted extraction for common beans has been limited to few studies using commercial carbohydrases (Bildstein et al., 2008), trypsin (Dias et al., 2010), and Alkaline Protease (Yang et al., 2024a, 2025). Other studies exploring post-extraction hydrolysis of pulse proteins have clearly demonstrated how proteases with unique specificities produce distinct patterns of hydrolysis, generating protein extracts with different physicochemical and functional properties (Xu et al., 2021; Zheng et al., 2019; do Evangelho et al., 2017; Vogelsang-O'Dwyer et al., 2022). This presented an opportunity to expand on previous work to gain a better understanding of the combined impact of extraction pH and the addition of commercial food-grade enzymes (carbohydrases and proteases alone or in sequence) during the extraction step, with a focus on both extraction yields and the resulting nutritional and functional properties of the extracted protein.

Importantly, enzyme usage also affects the economic feasibility of extraction processes due to the additional cost associated with food-grade enzymes. Furthermore, any potential changes in protein quality due to enzyme use could influence the selling price of the final product, thereby affecting overall process profitability. In this context, techno-economic analysis (TEA) is a valuable tool for assessing the economic feasibility of industrial processes *in silico* at scale, providing insights into capital investment/expenditures (CapEx), cost of goods sold (COGS), and discounted cash flow rates of return (DCFRR; profitability metric considering the capital investment as well as operating costs and time value of money) based on lab-scale experimental data. Petersen et al. (2022) used TEA to guide the selection of conditions (pH, temperature, time, solids-to-liquid ratio (SLR)) for the aqueous extraction of pea protein (using water or alkaline conditions) followed by recovery with ultrafiltration. The results of their study demonstrated that the technical optimum (i.e., highest protein recovery yields) did not align with the economic optimum (i.e., lowest minimum selling price) (Petersen et al., 2022). While the study provided a compelling illustration of the importance of considering economic indicators in process development, the optimization was limited to protein extraction yields and did not address the potential implications of extraction pH or time on protein functionality. With respect to enzymatic extraction, TEAs have been performed for the EAEP of soy and chickpea proteins (Cheng et al., 2019; Machida et al., 2022a), but to our knowledge, have only been used to compare final optimized processes, rather than being included in the selection of the extraction conditions per se.

Therefore, the major objectives of this work were to develop aqueous and enzyme-assisted extraction processes for black bean proteins by identifying the best pH (AEP) and commercial enzyme (EAEP) with a holistic selection criteria considering protein yield, nutritional (*in vitro* protein digestibility), functional (solubility at acidic and neutral pH), and biological (antioxidant, total phenolic content) properties, along with economic parameters including capital investments, unit production costs, and overall profitability over a 30-year project lifetime. Subsequently, the selected AEP and EAEP conditions were further optimized with respect to key extraction parameters (SLR, time, enzyme loading) based on economic metrics. Proteins from optimum extraction conditions for both AEP and EAEP were then characterized with respect to their physicochemical and functional properties of industrial relevance. This functionality-guided and economically driven approach is critical for the development of scalable protein extraction methods that produce protein ingredients of high quality and commercial applicability. We hypothesize that incorporating techno-economic modeling throughout every stage of process optimization can identify the extraction parameters with the greatest impact on economic indicators, allowing for a more informed selection of process conditions based on industrial feasibility. Overall, this work helps uncover relationships between extraction pH, proteolysis, protein extraction yields, functionality, and profitability, providing a comprehensive framework for the future development of extraction processes for other plant protein ingredients from a broader perspective.

2. Materials & methods

2.1. Materials

Black bean (*Phaseolus vulgaris*) flour was produced by milling whole black beans (Eclipse variety, 2020 crop, Inland Empire Foods, Riverside, CA, USA) with a WonderMill Electric Grain Mill (Pocatello, ID, USA). The proximate composition of the flour was 20% protein, 2% oil, 4% ash, 63% carbohydrates (by difference), and 11% moisture, as previously described (Yang et al., 2024a). The commercial enzymes used in this study were Bromelain (BIO-CAT, Troy, VA, USA), Flavourzyme® (Novozymes, Bagsværd, DK), Neutral Protease (BIO-CAT, Troy, VA, USA), Papain (BIO-CAT, Troy, VA, USA), FoodPro® Alkaline Protease (Danisco, Rochester, NY, USA), and FoodPro® CBL (Danisco, Rochester, NY, USA). Details regarding enzyme sources, activity, and optimal pH as reported by the manufacturers are detailed in Table S1. All reagents were of analytical grade and were purchased from Sigma Aldrich (St. Louis, MO, USA) unless otherwise stated.

2.2. Initial screening of black bean protein extraction strategies: effects of pH and enzyme on protein yields, solubility, and nutritional properties

2.2.1. Effects of extraction pH in the aqueous extraction process (AEP)

Aqueous extraction of black bean proteins was performed by dispersing black bean flour (50 g) in pre-heated deionized (DI) water (500 g, 50 °C) to achieve an SLR of 1:10 (Fig. 1A). The slurry was heated in a recirculating water bath (50 °C) and adjusted to the desired pH (7.0 or 9.0) with 1 M NaOH, followed by 1 h of extraction under constant stirring (120 rpm) and pH maintenance. For the pH shifting extractions (AEP-5/7 and AEP-5/9), the slurry was prepared as aforementioned and adjusted to pH 5.0 using 1 M HCl for an acidic pretreatment (30 min) followed by a pH shift to neutral (pH 7.0) or alkaline (pH 9.0) conditions for an additional 1 h of extraction (90 min total). To understand the effects of the acidic treatment alone (no pH shift), an acidic extraction (AEP-5) was performed for 30 min as a control. After all extraction processes, the slurries were centrifuged (3280 × *g*, 30 min, 4 °C) to obtain a protein extract (supernatant) and insoluble fraction (byproduct). Aliquots of the extracts were freeze-dried for further analysis (FreeZone, Labconco, Kansas City, MO, USA). All extractions were performed in triplicate.

2.2.2. Impact of commercial enzymes in the enzyme-assisted aqueous extraction process (EAEP)

Enzymatic extractions of black bean proteins were performed using five commercial proteases: Bromelain (B), Flavourzyme® (F), Neutral Protease (NP), Papain (P), and FoodPro® Alkaline Protease (AP) (Fig. 1B–Table S1). To accommodate the optimal pH range for each enzyme, the slurry pH of the EAEP with Bromelain, Flavourzyme®, Neutral Protease, and Papain were maintained at pH 7.0, while the EAEP with Alkaline Protease was maintained at pH 9.0. The EAEP was performed using the same conditions as the AEP (1:10 SLR, 50 °C, 1 h; section 2.2.1), with the addition of 0.5% enzyme (w/w, weight of enzyme/weight of bean flour) to the extraction slurry.

The impact of a carbohydrase pretreatment using FoodPro® CBL (C), a commercial cellulase complex with the ability to hydrolyze cellulose and arabinoxylans, was assessed prior to the AEP and EAEP (Fig. 1C). Carbohydrase pretreatments (pH 5.0, 30 min, 0.5% w/w carbohydrase)

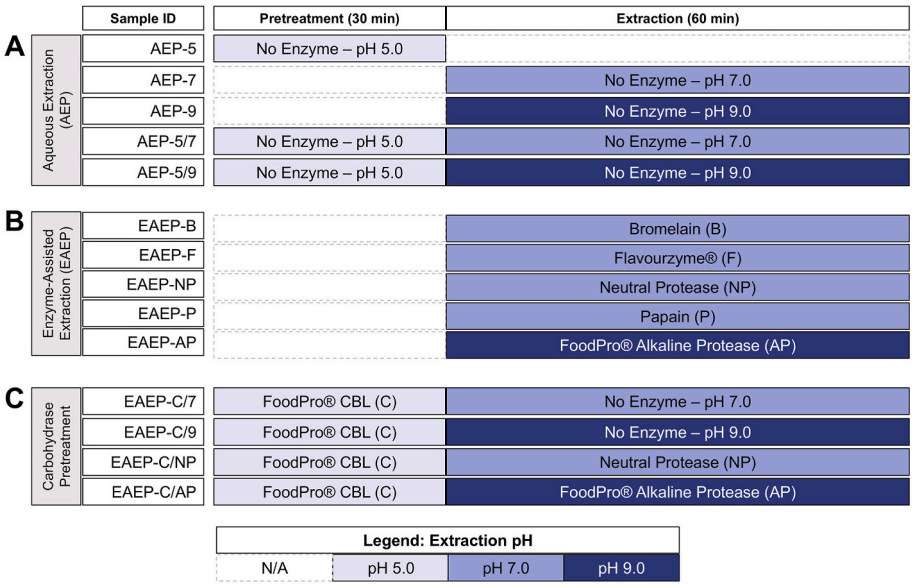

Fig. 1. Extraction conditions used in the pH and enzyme screening for the A) aqueous extraction process (AEP), B) enzyme-assisted aqueous extraction process (EAEP) with proteases, and C) carbohydrase pretreatment followed by AEP or EAEP.

followed by aqueous extraction at neutral (C/7) or alkaline (C/9) conditions were performed using the same general procedure as the pH shifting extractions in section 2.2.1. Preliminary data showed that Neutral Protease and Alkaline Protease were the most effective proteases in improving total protein extractability and solubility at pH 4.0. Therefore, carbohydrase pretreatments (pH 5.0, 30 min, 0.5% w/w carbohydrase) were followed by EAEP with Neutral Protease (C/NP: pH 7.0, 1 h, 0.5% w/w NP) or Alkaline Protease (C/AP: pH 9.0, 1 h, 0.5% w/w AP).

2.2.3. Total protein extractability (TPE)

The protein content of the protein extracts and insoluble fractions was measured using the Dumas combustion method (Vario MAX Cube, Elementar Analysensysteme GmbH, Langenselbold, DE) with a nitrogen conversion factor of 6.25. TPE was calculated using Eq. (1):

$$TPE\ (\%) = \left[1 - \left(\frac{Protein\ (g)\ in\ insoluble\ fraction}{Protein\ (g)\ in\ black\ bean\ flour}\right)\right] \times 100 \quad (1)$$

2.2.4. Protein solubility

Protein solubility was determined according to the method described by Rickert et al. (2004). Briefly, 1% w/w dispersions of the freeze-dried AEP or EAEP extracts were prepared in sodium acetate buffer (pH 4.0, 0.01 M) or sodium phosphate buffer (pH 7.0, 0.01 M) and were stirred for 1 h. The pH was monitored every 15 min and adjustments with 1 M NaOH or HCl were made if necessary. The dispersions were centrifuged (10,000 × *g*, 10 min, 20 °C; Sorvall RC-6 Centrifuge, Thermo Scientific, Waltham, MA, USA) and the protein content of the supernatants was analyzed using the Dumas combustion method (duplicate measurements of each extraction replicate (n = 3), totaling 6 measurements). Protein solubility was calculated with Eq. (2):

$$Solubility\ (\%) = \left(\frac{Protein\ content\ in\ supernatant\ (\%)}{Protein\ content\ in\ dispersion\ prior\ to\ centrifugation\ (\%)}\right) \times 100 \quad (2)$$

2.2.5. In-vitro protein digestibility

In-vitro protein digestibility (IVPD) was determined by performing the INFOGEST 2.0 simulated digestion protocol (Brodkorb et al., 2019), with a reduction in pancreatin concentration from 100 to 20 trypsin U/mL digest, as previously described (Yang et al., 2025). The reduction of pancreatin was performed to avoid very high enzyme blank values that may obscure the final calculation of IVPD. Following the modified INFOGEST digestion, undigested proteins were precipitated by adding 24% trichloroacetic acid (TCA, 20 mL) to each tube, incubating overnight at 4 °C, and centrifuging (3280 × *g*, 4 °C, 30 min). The pellets were then washed with acetone (10 mL), incubated for 1 h at −20 °C, then centrifuged once more (3280 × *g*, 4 °C, 30 min) to remove any residual TCA that may corrode the Dumas combustion elemental analyzer. Pellets were air-dried for >4 h to evaporate residual acetone, then were analyzed for total nitrogen content by the Dumas combustion method. IVPD (%) was calculated by performing a mass balance on nitrogen before and after digestion (Eq. (3)):

$$IVPD\ (\%) = \frac{N_{undigested\_extract} - \left(N_{digested\_pellet} - N_{enzyme\_blank}\right)}{N_{undigested\_extract}} \times 100 \quad (3)$$

where $N_{undigested\_extract}$ refers to the mass of nitrogen of the original undigested sample (i.e., 2.5 g AEP or EAEP extract), $N_{digested\_pellet}$ refers to the mass of nitrogen of the TCA-precipitated pellet after digestion, and N_{enzyme_blank} is the mass of nitrogen of the enzyme blank (2.5 g RO water instead of sample, n = 6) to account for the nitrogen contribution of the digestive enzymes and other components of the simulated digestive fluids. Single digestions were performed for each extraction replicate (n = 3).

2.2.6. Total phenolic content

Total phenolic content (TPC) was determined using the Folin-Ciocalteu method as previously described (Singleton et al., 1999) with modifications for a 96-well plate. Gallic acid (5–95 μg/mL) was used to build the standard curve. AEP and EAEP extracts were centrifuged (10,000×*g*, 20 min) to obtain a clear supernatant, which was further diluted 1:20 in DI water prior to analysis. Absorbance was read at 760 nm (SpectraMax iD5 Multi-Mode Microplate Reader, Molecular Devices, San Jose, CA, USA). TPC was reported as mg of gallic acid equivalents (GAE) per g of bean flour used in the extraction process. Each extraction replicate was measured in triplicate (n = 9).

2.2.7. Antioxidant capacity

Antioxidant capacity was determined using the ABTS radical cation decolorization assay, as described by Re et al. (1999), with

modifications for a 96-well plate (Al-Duais et al., 2009). Trolox (80−260 μM in ethanol) was used to construct a standard curve. AEP and EAEP extracts were centrifuged as described in section 2.2.6 and diluted 1:20 in ethanol prior to analysis. Absorbance was read at 730 nm (SpectraMax iD5 Multi-Mode Microplate Reader). Antioxidant capacity was reported as mmol Trolox equivalents per g of bean flour used in the extraction process. Each extraction replicate was measured in triplicate (n = 9).

2.2.8. Degree of hydrolysis

The degree of hydrolysis (DH) of the EAEP and AEP extracts was determined using the o-phthaldialdehyde (OPA) method reported by Nielsen et al. (2001). L-serine (0.9516 milliequivalents/L) was used as a standard. Protein extracts were diluted 1:50 in DI water prior to analysis and each extraction replicate (n = 3) was measured in duplicate (n = 6). Absorbance at 340 nm was measured using a Genesys™ 10 S UV–Vis spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The number of peptide bonds cleaved, h (milliequivalents/g protein), was determined as described by Nielsen et al. (2001), and the DH (%) was calculated with Eq. (4):

$$DH\ (\%) = 100 \times \frac{h}{h_{tot}} \tag{4}$$

where h_{tot} was 7.43 milliequivalents/g protein as previously determined by Tagliazucchi et al. (2015) for *Phaseolus vulgaris* (Pinto variety).

2.2.9. SDS-PAGE

The molecular weight distribution of the AEP and EAEP extracts was visualized using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in reducing conditions. Protein extracts were diluted 1:1 (v/v) with 2x Laemmli sample buffer (Bio-Rad, Hercules, CA, USA), containing 5% β-mercaptoethanol as the reducing agent, then heated at 85 °C for 10 min. Proteins were separated in the 10–250 kDa range using a 12% acrylamide Criterion™ TGX™ Precast Midi Protein Gel (Bio-Rad). Approximately 35 μg of protein was loaded into each well, and separation was performed at 200 V. Proteins were stained using Bio-Safe Coomassie Blue Premixed Staining Solution and imaged using a Bio-Rad Gel Doc™ EZ Imager (Bio-Rad).

2.3. Process simulation and techno-economic analysis (TEA) of the AEP and EAEP

Process models and economic analyses of the AEP and EAEP were performed using SuperPro Designer® Version 12, Build 3 (Intelligen, Inc., Scotch Plains, NJ, USA; http://www.intelligen.com) to evaluate the impacts of pH, enzyme use, and extraction time on key economic metrics. The process simulation modeled only the extraction process, which included four unit operations: pre-heating of the water via heat exchanger, batch extraction in a jacketed blending tank, cooling of the slurry via heat exchanger, and separation of the aqueous protein-rich extract using a decanter centrifuge (Fig. S1). Limiting the model to the extraction process allowed for a more streamlined screening of extraction conditions, as the other upstream/downstream unit operations (milling of bean flour, downstream protein isolation, spray-drying) would be similar in all cases. In addition, while there are inherent differences in the experimental and modeled unit operations (e.g., open beaker vs. closed tank, swinging bucket centrifuge vs. decanter centrifuge), the models capture the major differences in inputs and yields that provide sufficient information to compare conditions.

In all models, a greenfield scenario was assumed, with an annual production capacity fixed at 10,000 MT of bean protein (achieved by adjusting the number of batches per year, sizing of unit operations, and bean flour input), and 330 operating days/year. All monetary values are reported in U.S. dollars (USD, denoted as $). The costs of the main inputs are summarized in Table S2. The cost of the enzymes used in the EAEP were estimated to be $12.50/kg for the plant-derived proteases (Bromelain, Papain) and $20.00/kg for the microbially-derived proteases (Flavourzyme®, Neutral Protease, Alkaline Protease) (Guerrand et al., 2018). In the model, the component mass balance of bean protein was entered based on experimental results (TPE), whereas the mass balance of water following the centrifugation step was estimated by setting the solids concentration in the insoluble product stream to 28.5%. This value represents the approximate solids content of the insoluble fraction, which was determined experimentally for select conditions (data not shown). The slurry throughput and power requirements of the decanter centrifuge were estimated based on the particle size of the bean flour ($D_{4,3}$ = 185 μm (Yang et al., 2024a)). The power required for stirring was fixed at 2.32 kW/m^3, which was calculated based on the volumetric power input of the stirrer used for the lab-scale protein extractions. The specific heat of the bean flour, used to estimate the slurry heating and cooling times and utility requirements, was calculated as 1.744 kJ/kg•K based on the proximate composition of the flour, using the empirical model proposed by Heldman and Singh (1981).

Economic metrics (CapEx, COGS, and DCFRR) were obtained from the Economic Evaluation Report generated by SuperPro Designer®. The total CapEx included direct fixed capital (equipment purchase cost including 20% for unlisted equipment, and a Lang factor of 6.4 to account for equipment installation, piping, instrumentation, insulation, electrical facilities, buildings, yard improvements, auxiliary facilities, engineering, construction, contractor fees, and contingency), working capital, and start-up and validation costs (land costs, up front R&D, and up front royalties were not considered). COGS (without depreciation) was defined as the annual operating cost normalized by the annual production level, and considered the material costs, labor, lab quality control/assurance, utilities, waste disposal, equipment maintenance, factory expenses, local taxes, and insurance (McNulty et al., 2022). COGS was reported based on the amount of protein extracted (COGS/kg total extracted protein), and was also reported based on the amount of soluble or digestible protein in the extract (determined experimentally; sections 2.2.4 and 2.2.5) using a "functionality-based COGS" metric (Eq. (5)):

$$Functionality-Based\ COGS\left(\frac{\$}{kg\ soluble\ or\ digestible\ protein}\right) = \left(\frac{COGS\ (\$)}{kg\ total\ protein}\right) \times \left(\frac{kg\ total\ protein}{kg\ soluble\ or\ digesible\ protein}\right) \tag{5}$$

DCFRR, which provides a metric of overall profitability considering capital investment, corporate income taxes and the time-value of money, was calculated over a 30-year project lifetime, with a fixed selling price of $16.50/kg protein. Functionality-based DCFRR was similarly reported based on the total, soluble, and digestible protein (Eq. (6)):

$$Functionality-Based\ DCFRR\ (\%) = DCFRR\ (\%) \times \left(\frac{kg\ soluble\ or\ digestible\ protein}{kg\ total\ protein}\right) \tag{6}$$

While the functionality-based COGS and DCFRR are not

conventional metrics used in economic evaluations, they allow for the inclusion of functional and nutritional parameters in the assessment of process profitability, which is critical in comparing different extraction scenarios with a holistic perspective. In the case that realistic selling prices are available for products with different functional properties, COGS and DCFRR could be calculated based on these specific values; however, in early-stage process development and optimization, using simple functionality-based metrics can provide a preliminary assessment of extraction methods without extensive market research.

2.4. Optimization of extraction conditions for the AEP and EAEP

Following the selection of the most favorable pH (7.0) and enzyme (Alkaline Protease) based on the results of the enzyme screening, key extraction parameters were optimized using central composite rotatable designs (CCRD). For the AEP (pH 7.0, 50 °C), a 2^2 CCRD was performed with SLRs ranging from 1:8.6 to 1:20 and extraction times ranging from 15 to 75 min. These ranges were selected based on previous work showing that aqueous extraction of pulse proteins occurs very quickly in relatively dilute conditions (Yang et al., 2025; Machida et al., 2022a). To optimize the EAEP (pH 9.0, 50 °C, with Alkaline Protease), separate 2^2 CCRDs at discrete time points (30, 60, 90 min) were performed to optimize SLR (1:6.4 to 1:15) and the amount of enzyme (0.10–0.80% w/w). Our previous study demonstrated that the addition of Alkaline Protease was able to achieve high extractability in more concentrated extraction slurries (Yang et al., 2025); therefore, the SLR range for the EAEP was intentionally set at a more concentrated range than for the AEP. The enzyme range (maximum of 0.80% w/w) was also selected based on previous results showing that 1% w/w Alkaline Protease caused significant decreases in emulsifying capacity and foaming capacity/stability (Yang et al., 2025). The variable levels (coded and non-coded) for the CCRDs are shown in Tables S3 and S4. Extractions were performed as described in sections 2.2.1 (AEP) and 2.2.2 (EAEP with protease), with triplicates of the central point conditions (11 experiments in each CCRD). TPE was determined as described in section 2.2.3 (Eq. (1)). TEAs were performed for each experimental condition as described in section 2.3, with adjustment of the number of batches per year, sizing of unit operations, and bean flour input to achieve an annual production level of 10,000 MT of total bean protein in the aqueous extract stream.

Validation experiments were performed for the AEP and EAEP using several conditions that achieved high overall profitability (DCFRR). To validate the other initial screening parameters, protein solubility, IVPD, TPC, antioxidant capacity, and DH were determined as described in sections 2.2.4−2.2.8.

2.5. Physicochemical and functional characterization of optimized AEP and EAEP extracts

2.5.1. Isoelectric point (pI) determination

Zeta potential of the extracted proteins was measured using a Zetasizer Advance–Pro (Malvern Instruments Ltd., UK) with DTS1070 capillary cells at 25 °C. AEP and EAEP extracts were diluted to 0.2 mg/mL protein in DI water, and the pH was adjusted to 3.0, 3.5, 4.0, 4.5, 5.0, 6.0, 7.0, and 9.0 with 0.5 M HCl or 0.5 M NaOH. Measurements of each extraction replicate (n = 3) were performed in triplicate (n = 9). The pI was calculated by linearly interpolating the pH at which the zeta potential was zero.

2.5.2. Surface hydrophobicity

Surface hydrophobicity was measured as described by Zhang et al. (2013) using a fluorescent probe (1-anilino-8-naphthalenesulfonate, ANS; VWR Inc., Chicago, IL, USA) with modifications for a 96-well plate as described by Dias and de Moura Bell (2022). Each extraction replicate (n = 3) was measured in duplicate (n = 6) and surface hydrophobicity was reported as the slope of the fluorescence intensity vs. concentration line.

2.5.3. Interfacial properties

To determine interfacial properties, protein dispersions (1% w/w protein) were prepared by resuspending freeze-dried powder (AEP and EAEP extract) in 0.01 M sodium acetate buffer (pH 4.0) or 0.01 M sodium phosphate buffer (pH 7.0), followed by hydration for at least 1 h at room temperature with stirring at 350 rpm.

Briefly, emulsifying capacity (EC) was determined using a visual titration method in which 5 mL of the 1% protein dispersion was continuously homogenized (10,000 rpm, Polytron PT 2500, Kinematica AG, Lucerne, CH) as soybean oil (containing 4 ppm Sudan Red 7 B) was added dropwise until emulsion breakage occurred. EC was reported as the g of oil emulsified (at the point of breakage) per g of protein (Bian et al., 2003).

Foaming properties were determined by whipping 5 mL (V_i) of the 1% protein dispersion at 20,000 rpm for 1 min (Polytron PT 2500) and measuring the volumes of the foam immediately (V_0) and 60 min after whipping (V_{60}). Foam overrun (Eq. (7)) and foaming stability (FS; Eq. (8)) were calculated as follows (Yang et al., 2022):

$$\textit{Foam Overrun}\ (\%) = \left(\frac{V_0\ (mL)}{V_i(mL)}\right) \times 100 \tag{7}$$

$$FS\ (\%) = \left(\frac{V_{60}\ (mL)}{V_0\ (mL)}\right) \times 100 \tag{8}$$

2.6. Statistical analyses

Statistical analyses (one-way and two-way analysis of variance (ANOVA) with Tukey's post hoc test, multivariate Pearson's correlation) were performed using JMP® 16.1 (Cary, NC, USA) with $p < 0.05$. The regression models and contour plots for the central composite rotatable designs were generated using the Protimiza Experimental Design Software (https://experimental-design.protimiza.com.br/, Campinas, BR) with $p < 0.05$.

3. Results & discussion

3.1. Initial screening of black bean protein extraction strategies: evaluating impacts on yields and functionality

3.1.1. Effect of extraction pH and enzyme on total protein extractability

Aqueous extractions of black bean proteins were performed in acidic (pH 5), neutral (pH 7), and alkaline (pH 9) conditions, as well as with pH shifting from acidic to neutral (pH 5/7) and acidic to alkaline (pH 5/9) conditions. Protein extractability increased significantly as the extraction pH increased, with AEP-5, AEP-7, and AEP-9 achieving 24, 66, and 75% TPE, respectively (Fig. 2A). Notably, acidic extractions were not expected to yield high TPE due to its proximity to the pI of common bean proteins (pH 4 to 5), but it was included in this study for purposes of comparison and to reveal potential impacts of acidic treatments on protein functionality and biological properties. Improved protein extractability at pH 9.0 compared to 7.0 has similarly been reported for lentil and pigeon pea (Jarpa-Parra et al., 2014; Mizubuti et al., 2000). Alkaline conditions increase the surface charge of proteins by promoting deprotonation of amino acid residues, which in turn reduces protein-protein and protein-matrix interactions. As a result, increased protein charge improves protein hydration and solubilization into the extraction medium, thus enhancing overall extractability (Sathe and Salunkhe, 1981b; Shen et al., 2008).

Protein extractability significantly decreased when an acidic pretreatment was applied (62% for AEP-5/7 vs. 66% TPE for AEP-7, and 65% for AEP-5/9 vs. 75% TPE for AEP-9) despite the same final extraction pH (Fig. 2A). A similar observation was made by Rosenthal et al. (2001) for the EAEP of soybean involving a cellulase pretreatment

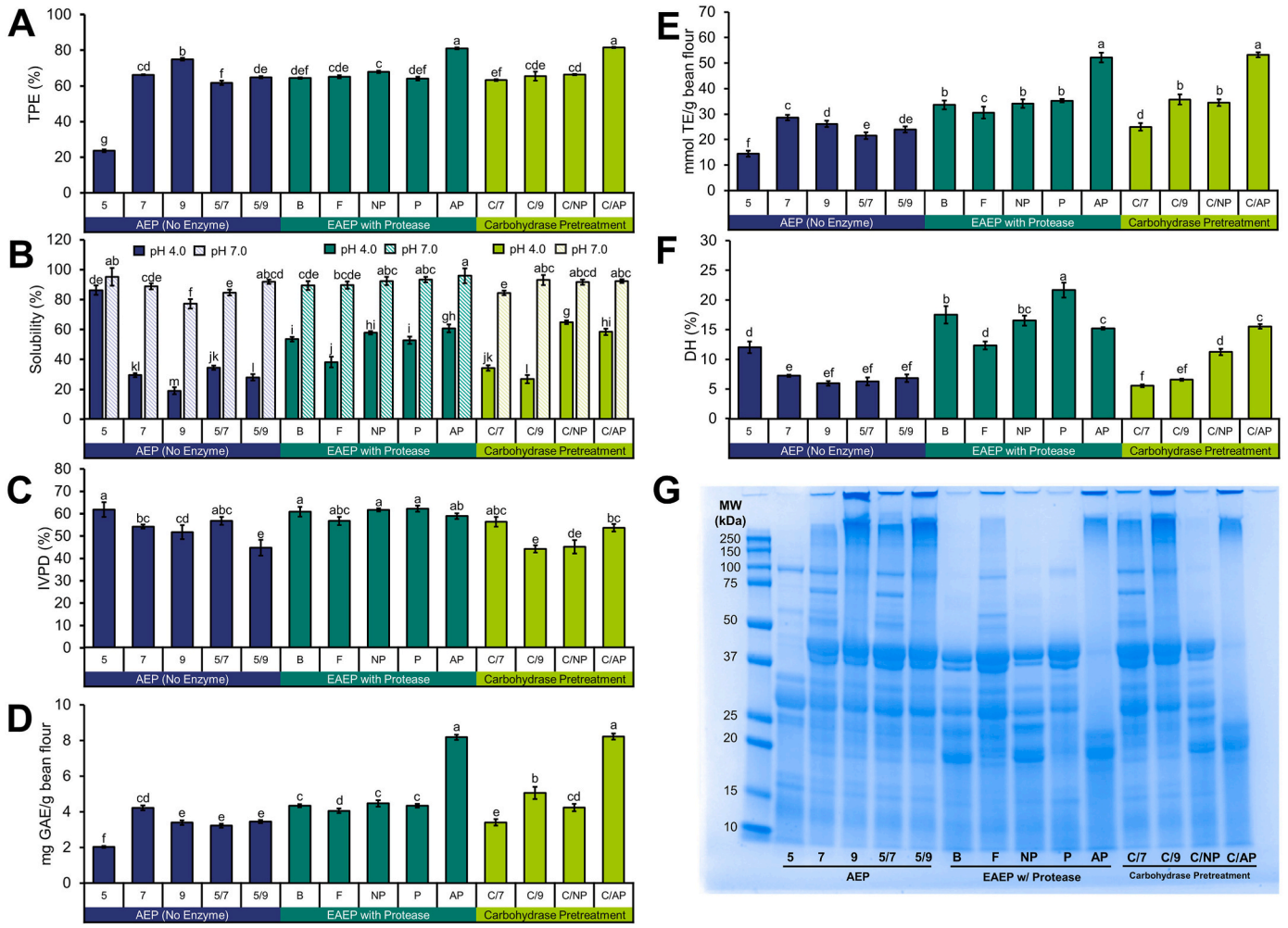

Fig. 2. A) Total protein extractability (TPE, %), B) protein solubility (%) at pH 4.0 and 7.0, C) *in-vitro* protein digestibility (IVPD, %), D) total phenolic content (mg gallic acid equivalents/g bean flour), E) antioxidant capacity (mmol Trolox equivalents/g bean flour), F) degree of hydrolysis (DH, %), and G) reducing SDS-PAGE gel of protein extracts produced using the aqueous (AEP) and enzyme-assisted aqueous extraction process (EAEP). B: Bromelain, F: Flavourzyme, NP: Neutral Protease, P: Papain, AP: FoodPro® Alkaline Protease, C: FoodPro® CBL.

at pH 5 followed by alkaline extraction at pH 9. The authors speculated that permanent structural changes occurred during the acidic pretreatment that prevented subsequent protein solubilization at more alkaline pH, as pH 5 was near the pI of soybean proteins. For beans specifically, previous structural studies of phaseolin (the major storage protein in beans) revealed that at pH 5, phaseolin aggregated into a tetrameric form (Blagrove et al., 1984). This suggests that the acidic pretreatment conditions may have induced irreversible denaturation in the proteins that prevented re-solubilization in alkaline conditions. Additionally, He et al. (2020) reported an increase in surface hydrophobicity and a decrease in solubility with low pH-shifting treatments (pH 1.0–3.0 to pH 7.2) for black bean protein isolates, which agree with the decreased extractability with pH-shifting observed in the present study.

For the EAEP with proteases, Alkaline Protease (AP, pH 9.0) yielded the highest extractability (81% TPE), and significantly improved protein extractability compared to AEP-9 (75% TPE) (Fig. 2A). Conversely, at pH 7.0, there was no increase in TPE for any of the EAEPs (Bromelain, Flavourzyme®, Neutral Protease, Papain; 64–68% TPE) compared to AEP-7 (66% TPE). Proteolysis typically improves protein extractability due to the breakdown of protein bodies and subsequent release of more soluble protein subunits and peptides (Rosenthal et al., 2001). Despite the similar TPEs, there was extensive hydrolysis for the extractions performed with Bromelain, Flavourzyme®, Neutral Protease, and Papain as evidenced by the degree of hydrolysis and SDS-PAGE (Fig. 2F and G). This could indicate that these proteases primarily hydrolyzed the already extracted protein and did not aid in the further extraction of proteins embedded in the protein matrix. In contrast, for the EAEP with Alkaline Protease, there was a significant increase in TPE, which could be explained by the effects of alkaline-induced cell matrix disruption as previously discussed, which simultaneously improved the accessibility of embedded proteins to the protease. This exposure likely facilitated proteolysis, which released more soluble protein fragments and enhanced overall protein extractability.

The carbohydrase pretreatments followed by neutral (C/7) and alkaline (C/9) extraction did not significantly affect TPE compared to the respective pH-shifting controls with no enzyme. Utilizing a combination of carbohydrases and proteases in extraction can sometimes have synergistic effects in increasing the porosity of the cell matrix, therefore facilitating mass transfer and accessibility to protease action (Rosenthal et al., 2001; Marquez and Lajolo, 1981). The results of the present study, however, indicated that the carbohydrase used may not have had the required specificity to degrade the bean cell wall matrix, or that the effects of cell wall degradation were not sufficient to have appreciable impacts on TPE. In several other studies, carbohydrases similarly did not affect protein extraction yields from rice bran, chickpea, or lentils (Miranda et al., 2022; Hanmoungjai et al., 2002; Machida et al., 2022b). Miranda et al. (2022) reported that for lentil protein extraction, carbohydrase pretreatments containing Viscozyme® L (carbohydrase

complex with arabinose, 1−4% w/w) did not change the soluble solids content (i.e., °Brix) of the aqueous extract. This suggests that the lentil cell wall was extremely resistant to hydrolysis. However, Perović et al. (2022) reported that chickpea protein extractability was significantly improved (68–93%) when an arabinofuranoside or cellulase/xylanase pretreatment was applied prior to alkaline extraction, demonstrating that perhaps other carbohydrases or carbohydrase cocktails may be effective depending on the plant matrix. In the present study, the combined use of carbohydrase and protease (66% TPE for Carbohydrase/Neutral Protease, 82% TPE for Carbohydrase/Alkaline Protease), did not increase TPE compared to extractions with protease alone (68% TPE for Neutral Protease, 81% TPE for Alkaline Protease) (Fig. 2A). However, when considering the observed decrease in extractability with the acidic pretreatment (AEP-7 > AEP-5/7 and AEP-9 > AEP-5/9), the results suggest that the proteases were able to hydrolyze some of the denatured proteins, therefore masking the effects of the irreversible denaturation that occurred in acidic conditions.

3.1.2. Effect of extraction pH and enzyme on protein solubility

The solubility of food proteins serves as an indicator for overall protein functionality, as it can affect hydration, interfacial, and rheological properties (Kinsella, 1976). The solubility of the protein extracts was determined at pH 4.0 (close to the pI of bean proteins and the pH at which the effects of proteolysis on solubility are most apparent) and pH 7.0 to represent the conditions of acidic and neutral food products, respectively.

The results shown in Fig. 2B demonstrate that extraction pH had a significant effect on protein solubility at both pH 4.0 and 7.0. The AEP-5 proteins had the highest overall solubility at pH 4.0 (86%) and pH 7.0 (95%), which can be attributed to the selective extraction of acid-soluble proteins (largely albumins) in the extraction at pH 5.0. For the neutral and alkaline extractions, however, solubility decreased at pH 4.0 (AEP-7: 29%; AEP-9: 19%) and pH 7.0 (AEP-7: 89%; AEP-9: 77%) as the extraction pH increased from 7.0 to 9.0. Despite the higher protein extractability at pH 9.0 compared with pH 7.0, the alkaline pH may have induced structural modifications (e.g., post-extraction aggregation) that decreased protein solubility. Evidence of protein aggregation can be seen in the SDS-PAGE (Fig. 2G) with high molecular weight bands (>250 kDa) in the AEP-9 lane that were not present in the AEP-5 and AEP-7 lanes, and the substantial amount of protein that remained in the loading well for the AEP-9. Because these aggregates remained intact under reducing conditions (β-mercaptoethanol), they were likely stabilized by non-disulfide interactions (e.g., hydrogen bonds, covalent bonds) that may have caused low solubility (Rui et al., 2011; Sari et al., 2013). Decreased protein solubility with increasing extraction pH was similarly observed for pea and quinoa protein (Gao et al., 2020; Ruiz et al., 2016). In addition, a significantly lower thermal denaturation temperature of the AEP-9 proteins has been documented in our previous work comparing alkaline extracted bean proteins to albumin- and globulin-rich fractions (near-neutral conditions), demonstrating some degree of alkaline-induced denaturation (Yang et al., 2024a). While detailed characterization of the protein aggregates is beyond the scope of the present study, an important conclusion is although alkaline extraction conditions improved TPE, subsequent structural modifications and aggregation may decrease its solubility and be deleterious towards the overall functionality of the extracted protein.

When shifting the extraction pH from acidic to neutral in the AEP (AEP-5/7), solubility at both pH 4.0 (34%) and 7.0 (85%) was not significantly different compared to that of the AEP 7 (29% at pH 4.0; 89% at pH 7.0). However, the AEP-5/9 pH shift increased solubility from 19% to 28% at pH 4.0 and 77% to 92% at pH 7.0, compared to alkaline extraction alone (AEP-9), demonstrating that the acidic pretreatment followed by alkaline extraction improved the overall protein solubility. Typically, pH-shifting is performed by adjusting the protein solution to an extreme acidic or alkaline pH (e.g., pH 2 or 12) to induce a “molten globule” state, followed by a shift to neutral conditions (pH 7.0) to allow for protein refolding (Jiang et al., 2009; Li et al., 2020). This refolding may result in structural modifications that favor solubility such as the orientation of hydrophilic groups to the surface of the proteins. While the acidic conditions used in the present study (pH 5.0) were much milder, the significant improvements in solubility with the pH 5/9 shift suggest that it was still sufficient to achieve the benefits associated with pH shifting.

For the EAEP with proteases, proteins exhibited higher solubility at pH 4.0 in comparison to the AEP proteins, regardless of extraction pH. However, at pH 7.0, the solubilities were largely similar (89−96%). Improved solubility of EAEP proteins at acidic pH has similarly been reported for black bean (Yang et al., 2024a), lentil (Dias et al., 2024), and almond proteins (Dias and de Moura Bell, 2022), and can be attributed to the exposure of ionizable carboxyl and amino groups upon proteolysis (Wouters et al., 2016). Of the EAEP, the endoproteases (Bromelain, Neutral Protease, Papain, and Alkaline Protease) achieved higher solubility at pH 4.0 (53−61%) compared to Flavourzyme® (38%) and AEP 7 (29%). Clemente et al. (1999) reported that Flavourzyme® (a mixture of endoproteases, exoproteases, and aminopeptidases) was less effective in improving the solubility of chickpea protein isolates compared to Alcalase® (an alkaline endoprotease). This agrees with the results in our study and suggests that endoprotease action (cleavage of peptide bonds within the protein) may be more appropriate than exoprotease action (cleavage of amino acids from the C- or N-termini of the protein) with respect to improving black bean protein solubility. The observed differences in protein solubility between the EAEP extracts highlight the impact of enzyme specificity on protein functionality.

The carbohydrase pretreatments followed by neutral and alkaline extraction (C/7 and C/9) had no effect on protein solubility at pH 4.0 and 7.0 compared to the pH shifting controls, and the carbohydrase/protease combined treatments achieved similar solubility as the EAEP with the respective proteases. This is largely expected, as the carbohydrases would not impact protein structure and based on the similar TPE, would not generate extracts with distinct protein profiles.

3.1.3. Effect of extraction pH and enzyme on IVPD

Protein digestibility is an important factor in assessing the nutritional value and quality of proteins. The common bean is known to have limited protein digestibility due to the inaccessibility of native phaseolin to tryptic enzymes (Deshpande and Damodaran, 1989). The presence of antinutritional factors including trypsin inhibitors, phytate, and tannins, as well as the steric hindrance of bound sugar moieties (e.g., glycosylated phaseolin) can further inhibit protein digestibility (Deshpande and Damodaran, 1989; Sathe, 2002).

In the present study, IVPD ranged from 44 to 62%, demonstrating that processing conditions (extraction pH, type of enzyme) highly influenced IVPD (Fig. 2C). Previous reports on the IVPD of uncooked common bean protein isolates varied from 64 to 82% (Tang et al., 2009; Tan et al., 2014; Carbonaro et al., 1997; Higa et al., 2024). Importantly, the samples in the present study were only extracted, but not fractionated (i.e., isolation by isoelectric precipitation or membrane filtration); therefore, the lower IVPD range observed among all samples could be attributed to the presence of phenolics (see section 3.1.4), which has been demonstrated to impair digestion (Higa et al., 2024; De Toledo et al., 2013).

When assessing the impact of pH alone (AEP) on IVPD, the results show that AEP-5 had the highest IVPD (61.8%). For AEP-5, the high digestibility could be related to high solubility of the proteins (Fig. 2B) with the same rationale that the acidic extraction conditions selectively extracted highly soluble and digestible albumins. Prior studies on the IVPD of bean protein classes suggest that bean albumins are more digestible than globulins (Marquez and Lajolo, 1981), which supports this reasoning. The AEP-5/9 had the lowest IVPD despite exhibiting high solubility. This suggests that the shift from acidic to alkaline conditions may have altered the structure of the proteins, subsequently decreasing access to digestion sites by the digestive enzymes. While previous

studies on black bean and amaranth proteins have reported increased IVPD following pH-shifting, these pH shifts were performed in harsher acidic conditions (e.g., pH 1−3), which may have contributed to more severe partial unfolding/refolding of the proteins, and subsequent exposure of new cleavable sites by digestive enzymes (He et al., 2020; Figueroa-González et al., 2022).

Of the EAEPs with protease, there was no significant difference in IVPD (57−62%). However, compared to the AEP at the same pH condition (AEP-7 for Bromelain, Flavourzyme®, Neutral Protease, and Papain; AEP-9 for Alkaline Protease), all the EAEP extracts except EAEP with Flavourzyme® exhibited improved IVPD. This can be corroborated by the lower DH of that sample (EAEP-F; Fig. 2F), which suggests a lower extent of "pre-digestion" before simulated gastrointestinal digestion (Aryee and Boye, 2016). We showed in our previous work that the addition of 1% Alkaline Protease improved the IVPD of black bean proteins from 34 to 61% (Yang et al., 2025). In addition, enzymatic extraction was shown to reduce trypsin inhibitor activity (key anti-nutritional factor) in black bean proteins (Yang et al., 2025). Overall, these results suggest that EAEP with endoproteases is a useful strategy to improve IVPD. Similar results showing improved IVPD were also reported for almond and soy/zein mixtures hydrolyzed with neutral protease and papain, respectively (Wu et al., 2024; Dias et al., 2023).

Interestingly, IVPD was positively correlated with solubility at pH 4.0 ($p = 0.0022$; Table S5). This suggests that proteins with higher solubility at acidic pHs (generally reflective of acidic gastric conditions) may have higher overall digestibility. Future work is necessary to unveil the mechanistic rationale behind this correlation, but these preliminary results suggest that solubility in acidic pH has the potential to be used as a predictor of digestibility for black bean proteins.

3.1.4. Effect of extraction pH and enzyme on total phenolic content and antioxidant capacity

Black beans are rich in phenolic compounds (primarily concentrated in the seed coat) and have been demonstrated to confer health benefits, largely related to antioxidant capacity (Ganesan and Xu, 2017; Singh et al., 2017). In addition to polyphenols, certain peptides (potentially released in the EAEP) can also possess antioxidant properties (Garcia-Mora et al., 2015; de Castro et al., 2017). Therefore, to assess the impacts of extraction conditions on potential health-promoting properties, TPC (mg of gallic acid equivalents (GAE)/g bean flour) and antioxidant capacity (by ABTS radical scavenging) of the AEP and EAEP extracts were determined (Fig. 2D and E). Notably, proteins, peptides, and reducing sugars are known to interfere with the TPC assay, and therefore all results must be interpreted considering the different protein extractabilities (Fig. 2A). Overall, the trends observed for TPC and antioxidant capacity were very similar among all samples, and are therefore discussed together.

For the AEP, TPC ranged from 2.0 mg GAE/g bean flour (AEP-5) to 4.2 mg GAE/g bean flour (AEP-7). This range is slightly lower than previously reported for Eclipse black beans (1.28−6.89 mg GAE/g) using solvent extraction (methanol, ethanol, acetone) (Xu and Chang, 2007), but is similar to another study reporting a TPC of 1.6–3.7 mg GAE/g for black beans using ultrasound-assisted acidic ethanolic extraction (Madrera et al., 2021). Of the AEP conditions tested, AEP-7 yielded the highest TPC and antioxidant capacity. Typically, alkaline conditions (i.e., AEP-9) are known to free phenolics from plant cell matrices (Pinton et al., 2022); however, alkaline conditions may have degraded some of the pH-sensitive phenolics or induced the formation of insoluble protein/phenolic complexes (Shi et al., 2024; Friedman and Jürgens, 2000; Neta and de Castro, 2019). With respect to the acidic pretreatment, while the TPC of the AEP-9 and AEP-5/9 were not statistically different, the TPC and antioxidant capacity of AEP-5/7 was significantly lower than for neutral extraction alone. A possible explanation is the potential irreversible complexation of phenolics to proteins in acidic conditions. Upon shifting to neutral conditions, these complexes may have remained insoluble but were able to be released upon shifting to stronger alkaline conditions (pH 9.0).

For the EAEP, extraction with Alkaline Protease had significantly higher TPC and antioxidant capacity compared to the other commercial enzymes tested and exhibited a two-fold increase compared to the values for alkaline extraction alone (AEP-9). This suggests that the unique hydrolytic activity of Alkaline Protease could have released cell-matrix bound phenolics and may have also generated some antioxidant peptides. While the hydrolysis of pulse protein isolates to generate antioxidant peptides has been widely explored in existing literature (Chen et al., 2022; Ariza-Ortega et al., 2014; Aguilar et al., 2019), the effects of EAEP on the enzymatic co-extraction of phenolics in aqueous protein extraction processes have scarcely been reported, with only one study demonstrating the increase in antioxidant capacity of lentils extracts using a mixture of Flavourzyme® and Celluclast® (carbohydrase) (Neta and de Castro, 2019). Importantly, the TPE of the EAEP with Alkaline Protease was higher, which may have influenced the TPC values due to interfering absorbances of aromatic amino acids (Bastola et al., 2017).

Overall, the carbohydrase pretreatments did not affect TPC or antioxidant capacity with the exception of C/9, in which TPC and antioxidant capacity slightly increased compared to AEP-5/9. This suggests that the disruption of the cell wall matrix through the degradation of cellulose may have allowed for enhanced polyphenol extraction with the alkaline extraction. This is plausible due to the fact that most of the polyphenols present in black beans are concentrated in the fibrous seed coat. However, reducing sugars are also known to interfere with the Folin-Ciocalteu assay, and therefore these results should also be interpreted with caution as carbohydrase activity could generate more reducing sugars (Sánchez-Rangel et al., 2013). Correlation analysis between TPC and antioxidant activity demonstrated a strong positive association between these two parameters ($p < 0.0001$; Table S5), which aligns with previous findings (Gan et al., 2017; Yang et al., 2020).

3.1.5. Effect of extraction pH and enzyme on degree of hydrolysis and molecular weight profile

The degree of hydrolysis (DH) is a key parameter for monitoring proteolysis (Nielsen et al., 2001). Although DH is not a direct measurement of extractability or functionality per se, it can often be related to different physicochemical and functional characteristics of proteins. The DH of the EAEP proteins with protease (12−22%) was significantly higher than that of the AEP proteins (6−7%) extracted at the corresponding pH (Fig. 2F). The DH of the AEP-5 extract was the highest of the AEP samples (12%), which could signify that of the protein species in the sample, there were more free amino ends (corresponding to a higher measured DH), or there may have been higher ε-amino groups from lysine in the extract that could have similarly reacted with the OPA reagent in the DH assay (Nielsen et al., 2001). This is corroborated by the SDS-PAGE (Fig. 2G) which shows that all the AEP-5 proteins were below 100 kDa in size. Of the proteases used in the enzyme screening, Papain and Flavourzyme® generated protein extracts with the highest (22%) and lowest (12%) DH, respectively. Interestingly, the hydrolysis of flaxseed protein using the same enzymes showed the opposite trend, with Papain and Flavourzyme® yielding the lowest and highest DH, respectively (Karamać et al., 2016), highlighting that the efficacy of commercial enzymes is matrix specific. Interestingly, the DH was positively correlated with all other parameters assessed in the screening, except TPE (Table S5). This suggests that DH could be a useful predictor of the functional/nutritional properties tested.

From the SDS-PAGE (Fig. 2G), it is evident that Alkaline Protease, whether applied alone or following a carbohydrase pretreatment at pH 5.0, was highly effective in hydrolyzing phaseolin (~45 kDa band) into peptides approximately half its original size (~26.5 kDa), as previously reported (Yang et al., 2024a). This is supported by previous findings that the central region of phaseolin is the most susceptible to hydrolysis (Deshpande and Nielsen, 1987; Zhang and Romero, 2020). The other proteases seemed to also hydrolyze phaseolin (thinner bands at ~45 kDa, particularly for Bromelain and Neutral Protease compared to

AEP-7), but to a lesser extent. Interestingly, the DH values seem to contradict what is observed in the SDS-PAGE, as Alkaline Protease exhibited lower DH compared to some of the proteases at pH 7.0. This discrepancy was similarly observed by Zhang & Romero (Zhang and Romero, 2020) for Great Northern and navy beans, in which Alcalase® achieved a higher DH than papain, but the phaseolin band was still visible in the SDS-PAGE. All proteases, except for Flavourzyme®, were able to hydrolyze the high molecular weight proteins around 70 and 100 kDa. There was no marked effect of the carbohydrase pretreatments prior to the use of proteases on the molecular weight profile of the protein extracts (Neutral Protease vs. Carbohydrase/Neutral Protease and Alkaline Protease vs. Carbohydrase/Alkaline Protease).

3.1.6. Effect of extraction pH and enzyme on large-scale economic feasibility

TEAs of the AEPs and EAEPs in the enzyme screening were performed to understand the impacts of extraction pH and enzyme use on key economic parameters (CapEx, COGS, and DCFRR). Because the pH-shifting (AEP-5/7, AEP-5/9) and carbohydrase pretreatments (C/7, C/9, C/NP, C/AP) did not yield significant improvements in extractability or functionality despite increased inputs and total extraction time, they were not considered in the TEAs or subsequent optimizations. To acknowledge the impacts of the different extraction conditions on key functional and nutritional value of the proteins obtained, functionality-based COGS and DCFRR were also reported, which scaled the metrics based on the amount of soluble protein (at pH 4 and 7) and digestible protein of the total protein extracted (Table 1). Notably, the TEA analysis presented allows for a relative comparison between extraction conditions, but the absolute values of the economic metrics may not be truly representative of industrial-scale costs. This limitation arises from fluctuations in materials, labor, and energy pricing over time, extrapolation of materials costs at scale, differences in process operations between lab/pilot data and industrial scale operations, and the assumption of a greenfield scenario, whereas existing processing facilities are often retrofitted to accommodate new processes.

Regarding the AEP (no enzyme), extraction at pH 9 exhibited a lower CapEx and COGS/kg total protein, and consequently, achieved a higher overall profitability (DCFRR) compared to AEP-7. These results can be attributed to the higher protein extractability achieved in alkaline extraction conditions (75% for AEP-9 vs. 66% for AEP-7; section 3.1.1). However, when considering the functionality of the proteins extracted, the AEP-7 had a lower COGS/kg for soluble and digestible protein, and extraction at pH 7 and 9 achieved nearly identical functionality-based DCFRRs based on acid-soluble protein (3.46% vs. 3.85%, respectively). This can be explained by the reduced solubility of AEP-9 proteins in acidic conditions (19%) compared to the AEP-7 (29%) (Fig. 2B). Given that solubility is important for protein functionality (e.g., foaming, emulsification, gelation, etc.), these findings reveal that, from an economic perspective, AEP-7 can produce proteins with higher and broader solubility across acidic to neutral conditions. This makes AEP-7 proteins particularly suitable for food products such as sports drinks, sodas, and other beverage applications requiring high protein solubility. More generally, these results also suggest a potential paradigm shift in the industry of sacrificing higher capital investments and unit production costs (per total extracted protein) to achieve lower production costs per unit of functional and/or digestible protein.

For the EAEP, across all economic metrics, EAEP with Alkaline Protease achieved the most favorable values. Remarkably, EAEP with Alkaline Protease required lower CapEx compared to both AEPs despite the additional cost of the enzyme, which was likely related to its high protein extractability (81% vs. 66−75%). In light of the results previously discussed for the AEP, these findings highlight that the proteolytic specificity of Alkaline Protease and resulting improvements in extractability and functionality successfully counteracted the negative effects of alkaline extraction alone (i.e., reduced solubility of AEP-9 proteins).

Overall, the results from the TEA emphasize the importance of considering not only the independent impacts of extraction conditions on protein extractability, functionality, and profitability, but also recognizing their interactive effects that can influence process desirability. Based on the results of the comprehensive extractability, functionality, and profitability screening that was performed, the AEP at pH 7 (AEP-7) and the EAEP with Alkaline Protease (EAEP-AP) were selected

Table 2
Impact of SLR and extraction time on protein extractability and economic feasibility of the AEP at pH 7.

Exp.	SLR	Time (min)	Batches /Year	TPE (%)	CapEx ($M)	COGS ($/kg protein)	DCFRR (%)
1	1:16.7	23.8	1070	70.1	96.28	14.33	11.35
2	1:9.4	23.8	1070	63.6	67.18	15.07	10.74
3	1:16.7	66.2	977	70.5	104.27	14.36	10.16
4	1:9.4	66.2	977	64.8	70.96	14.86	11.78
5	1:20	45	1021	70.6	115.57	14.58	7.69
6	1:8.6	45	1021	64.3	65.49	14.91	12.51
7	1:12	15	1092	67.3	74.81	14.41	14.47
8	1:12	75	960	69.3	80.9	14.18	14.83
9	1:12	45	1021	67.9	79.18	14.41	13.58
10	1:12	45	1021	67.7	79.37	14.47	13.12
11	1:12	45	1021	68.1	78.96	14.37	13.89

AEP: Aqueous extraction process.
TPE: total protein extractability.
SLR: solids-to-liquid ratio.
CapEx: capital expenditures.
COGS: cost of goods sold, without depreciation.
DCFRR: discounted cash flow rate of return, with selling price of $16.50/kg protein.

Table 1
Economic metrics for the aqueous (AEP) and enzyme-assisted aqueous extraction processes (EAEP) in the initial pH and enzyme screening. **Bolded** values show the most favorable economic metric for each extraction method.

Screening Condition	CapEx ($M)	COGS ($/kg protein)	Functionality-Based COGS ($/kg)[a]			DCFRR (%)	Functionality-Based DCFRR (%)[a]		
			Soluble protein at pH 4	Soluble protein at pH 7	Digestible protein		Soluble protein at pH 4	Soluble protein at pH 7	Digestible protein
AEP-7	72.04	14.84	**50.33**	**16.70**	**24.01**	11.75	3.46	10.44	6.37
AEP-9	**65.72**	**13.94**	73.07	18.06	25.70	**20.20**	**3.85**	**15.59**	**10.45**
EAEP-B	75.67	16.01	29.90	17.91	26.30	0.40	0.21	0.36	0.24
EAEP-F	74.78	15.78	41.27	17.61	27.82	2.93	1.12	2.63	1.66
EAEP-NP	71.64	15.39	26.63	16.66	24.95	7.97	4.61	7.36	4.92
EAEP-P	75.85	16.00	30.30	17.14	25.70	0.47	0.25	0.44	0.29
EAEP-AP	**64.23**	**14.68**	**24.15**	**15.31**	**24.91**	**15.63**	**9.50**	**14.99**	**9.21**

B: Bromelain, F: Flavourzyme®, NP: Neutral Protease, P: Papain, AP: FoodPro® Alkaline Protease; CapEx: capital expenditures; COGS: cost of goods sold without depreciation; DCFRR: discounted cash flow rate of return with a selling price of $16.50/kg.
[a] Functionality-based COGS only considering soluble or digestible protein fraction; calculated using Eq. 5.
[a] Functionality-based DCFRR only considering soluble or digestible protein fraction; calculated using Eq. 6.

for subsequent optimization through experimental designs.

3.2. Fine-tuning key extraction parameters for the AEP

To inform the selection of specific extraction conditions for the AEP and EAEP, CCRDs (2^2) were used to model the impacts of SLR, time, and amount of enzyme used (EAEP only) on TPE and economic feasibility. For the AEP, SLRs ranged from 1:8.6 to 1:20 and time ranged from 15 to 75 min (Table S3). For the EAEP, SLRs ranged from 1:6.4 to 1:15, while the enzyme loading ranged from 0.10 to 0.80% w/w (Table S4).

Experimental conditions and responses (TPE, CapEx, COGS, DCFRR) for the AEP-7 CCRD are shown in Table 2. Among all experiments, TPE values ranged from 63.6 to 70.6%, which aligned with the 66% TPE achieved for the AEP-7 (1:10 SLR, 60 min) in the initial screening. The regression model for TPE demonstrated that lower SLR (more dilute slurries) and longer extraction times corresponded to higher protein yields, although SLR had a higher impact compared to extraction time (Fig. 3A). This was expected due to the decreased slurry viscosity and increased concentration gradient between the proteins in the flour and bulk liquid, which facilitate protein dissolution and mass transfer (Machida et al., 2022a). However, from a practical perspective, low SLR may not be desirable due to the high water inputs, increased energy usage for heating the water, larger tank size requirements, and decreased number of batches that can be run per day. Low SLR systems would also generate larger volumes of effluent, thereby requiring more water to be evaporated in downstream processing to obtain a final powder (Van Der Goot et al., 2016). Therefore, considering economic metrics at this stage is critical for determining a suitable SLR/time combination that is both effective and economically feasible for large scale production.

The contour plot for CapEx generally matched the pattern for TPE, with lower SLR and longer times associated with higher CapEx (Fig. 3B). However, CapEx values were more sensitive to changes in extraction conditions compared to TPE. Specifically, CapEx nearly doubled when SLR decreased from 1:8.6 ($65.5 M, Exp. 6; most concentrated) to 1:20 ($115.6 M, Exp. 5; most dilute) (Table 2). This is likely related to the high investment required to accommodate larger slurry volumes for more dilute extraction conditions. Regarding COGS, SLR was the only significant variable, and the regression model exhibited a local minimum at an SLR of 1:14.3 (0.07) (Fig. 3C). Similarly, Petersen et al. (2022) found that for water extractions of pea protein, the estimated minimum selling price was the lowest at a solid loading of 6.7% (1:14.9); the authors attributed the increase in minimum selling price in more dilute conditions (e.g., 6.5% solid loading) to higher downstream energy costs and CapEx, resulting from the larger equipment sizes required (Petersen et al., 2022). With respect to overall profitability, experiments 7–11 (all performed at 1:12 SLR) had the highest DCFRR (14.4–16.1%) (Table 2). These results agree with the regression model (Fig. 3D), which

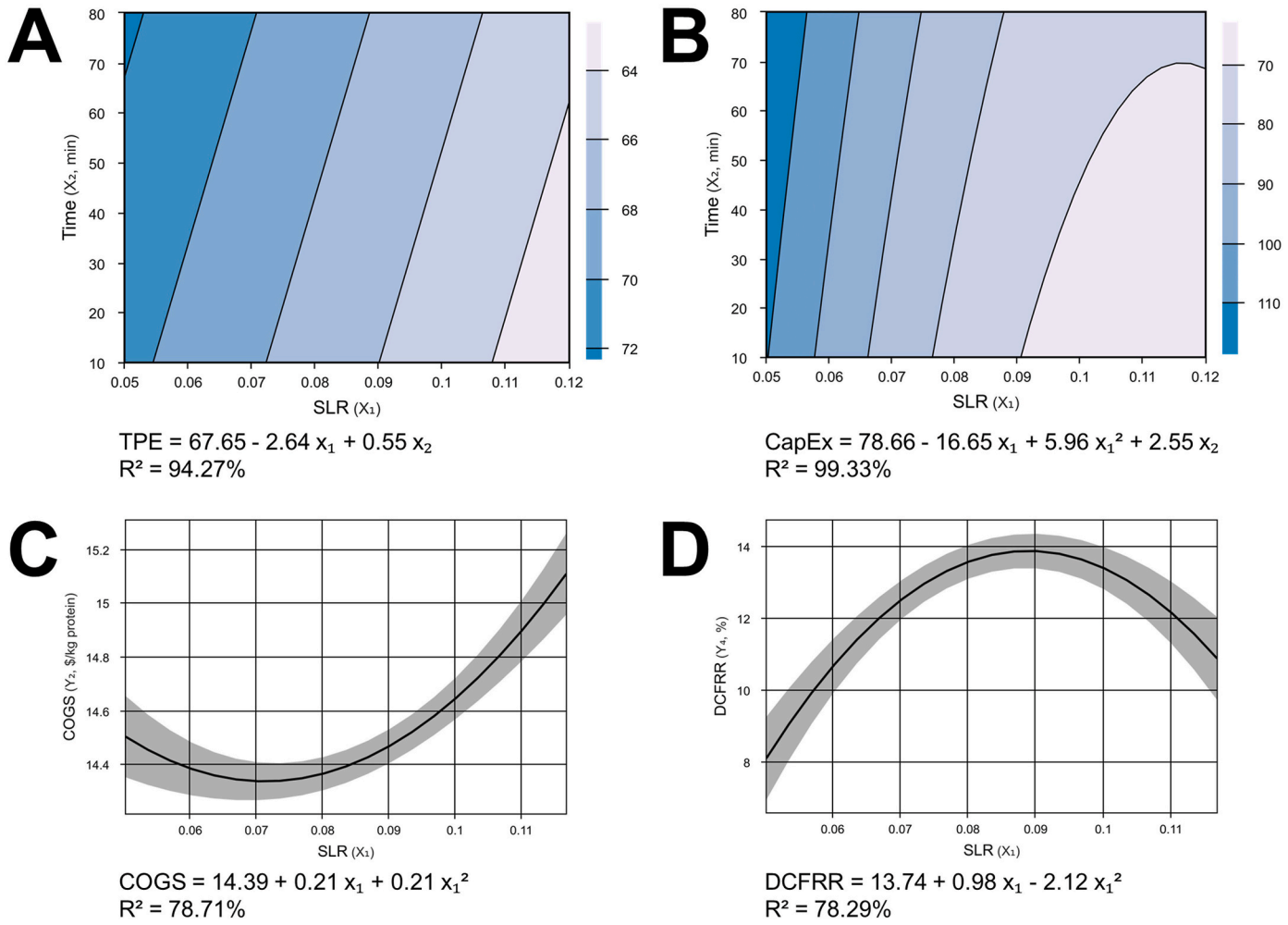

Fig. 3. Contour plots of the 2^2 central composite rotatable design for the AEP at pH 7, showing the effects of solids-to-liquid ratio (SLR) and/or time on (A) total protein extractability (TPE, %), (B) capital expenditures (CapEx, $M USD), (C) cost of goods sold without depreciation (COGS, $/kg protein), and (D) discounted cash flow rate of return (DCFRR, %), assuming a selling price of $16.50/kg protein. Variables and interactions with a significance level of $p < 0.05$ were included in the model.

revealed that there was a critical SLR (~0.088, equivalent to 1:11.4 SLR) in which increased dilution (lower SLR) became economically unfavorable despite achieving higher protein yields. Therefore, an SLR of 1:12 was selected (close to maximum), and extractions were performed at 15, 45, and 75 min to validate the accuracy of the models and guide the selection of the best extraction time for the AEP-7.

3.3. Fine-tuning key extraction parameters for the EAEP

For the EAEP-AP, separate experimental designs for discrete extraction times were performed, as the non-first order kinetics of enzymatic extractions could complicate the inclusion of time as a variable in the model (Yang et al., 2025). The variables and TPE for the 2^2 CCRDs at 30, 60, and 90 min of extraction time are shown in Table S6. The effect of extraction time on TPE was very minimal (<3.2% change when comparing the same Experiment # in the experimental design; i.e., same SLR and amount of enzyme but different extraction time). Therefore, TEAs were only performed for the EAEPs with 30 and 60 min of extraction (Table 3).

When comparing the economic metrics, the CapEx for the 60 min extractions was higher in every case. This can be ascribed to the lower number of batches/year due to the longer extraction time (1056 batches/year for 30 min vs. 990 batches/year for 60 min), which requires larger tank sizes to achieve the same annual production level of 10,000 MT of bean protein. The COGS was very similar for the EAEP at 30 and 60 min, which echo the results for the AEP in which extraction time was not a significant factor with respect to COGS. This implies that the additional energy required for the longer extraction did not greatly affect the operating costs. With respect to DCFRR, there were minor differences between 30 and 60 min of extraction, especially for the more concentrated slurries (e.g., 1:7 or 1:6.4 SLRs in Exp. 2, 4, and 6). Interestingly, DCFRR was higher for the 30 min extractions among all experiments except Exp 8 (1:9 SLR, 0.80% AP), in which the DCFRR was 5.88% for 30 min, and 11.81% for the 60 min extraction. This could be related to the 3% increase in TPE for the 60 min extraction (73.1% for 30 min; 76.4% for 60 min, Table 3). These results emphasize the sensitivity of the DCFRR metric to protein yields, as seemingly minimal differences in TPE had large impacts in the estimated profitability. Overall, the general trends in CapEx and DCFRR showed that 30 min of extraction was sufficient for most of the experimental conditions tested. Contour plots and regression equations for the EAEP with 60 min of extraction are shown in Fig. S2.

The contour plots and regression models for the EAEP at 30 min are shown in Fig. 4. With respect to protein yields, TPE was solely driven by SLR, with the highest extractability observed for the lowest SLR (Fig. 4A). CapEx increased for low SLR and high enzyme combinations, but the effects of SLR were much more prominent, as evidenced by the nearly vertical contour lines and higher coefficient for SLR (X_1) in the regression equation (Fig. 4B). This can be explained by the larger equipment sizes required in low SLR conditions, which aligns with the results for the AEP as previously discussed (Fig. 3B). Higher enzyme loadings marginally increased CapEx due to increases in equipment sizing, resulting from slightly lower TPE for higher enzyme loadings (although not significant in the regression model; Fig. 4A). For example, experiment 7 (1:9 SLR, 0.10% enzyme) had 2.6% higher TPE compared to experiment 8 (1:9 SLR, 0.80% enzyme), as shown in Table 3. The COGS increased as enzyme load and SLR increased (Fig. 4C), signifying that the addition of enzyme significantly increased the materials cost, as did extraction in more concentrated slurries. Similarly, He et al. (2021) found that the cost of Alcalase® in the extraction of proteins from brewers spent grain was an influential factor in determining MSP. The slightly lower TPEs with higher enzyme loadings also contributes to higher materials costs, as more bean flour was required to achieve the same annual production level. Among all experiments in the CCRD (Table 3), the enzyme cost ranged from 1.0 to 7.4% of the total cost of raw materials. In contrast, Cheng et al. (2019) demonstrated that for the EAEP of soy, the enzyme used (Protex 6 L) represented over 30% of the material cost. In the present study, the bean flour dominated the total material costs (~80−90%); the relatively cheaper feedstock of soybean used in the other study (whole, before dehulling or milling) could explain this difference. Interestingly, the DCFRR was only impacted by the enzyme loading (Fig. 4D). As DCFRR considers both capital investments and unit production costs, the results imply that for low SLR extractions, the high CapEx was offset by the lower COGS, therefore rendering SLR insignificant in determining the overall profitability. From a practical perspective, an SLR should be selected that minimizes waste (reducing the chemical and/or biological oxygen demand of the effluent that would require further treatment before disposal) and has a modest required capital investment to make it more practical for commercial adoption. Based on this rationale, a 1:12 SLR (0.08) was selected to achieve higher yields (i.e., less protein remaining in the insoluble byproduct) and to keep the CapEx under $70 M. While the model suggests that the lowest amount of enzyme yields the greatest profit, varying levels of enzyme use can significantly impact the physicochemical and functional properties of the extracted proteins. Therefore, EAEPs with a 1:12 SLR and 30 min of extraction time were performed for subsequent model validation and a more comprehensive sample characterization, with three concentrations of AP: 0.1, 0.3, and 0.5% w/w (denoted EAEP-0.1, EAEP-0.3, and EAEP-0.5).

Table 3
Impact of SLR and enzyme concentration at different extraction times on protein extractability and economic feasibility for the EAEP.

#	SLR	Enzyme (%)	Batches/Year		TPE (%)		CapEx ($M)		COGS ($/kg protein)		DCFRR (%)	
			30 min	60 min	30 min	60 min	30 min	60 min	30 min	60 min	30 min	60 min
1	1:12.5	0.20	1056	990	81.87	82.73	67.85	70.05	12.99	12.89	26.19	26.06
2	1:7	0.20	1056	990	75.60	73.22	49.80	52.82	13.60	14.04	29.42	24.11
3	1:12.5	0.70	1056	990	80.57	81.96	69.87	71.96	14.22	14.23	16.97	16.42
4	1:7	0.70	1056	990	73.30	72.29	52.41	56.15	15.21	15.59	12.76	7.75
5	1:15	0.45	1056	990	83.50	84.13	75.88	80.33	13.40	13.49	21.06	19.38
6	1:6.4	0.45	1056	990	70.74	69.67	51.09	53.70	15.10	15.52	14.34	9.06
7	1:9	0.10	1056	990	75.73	76.16	57.25	60.18	13.58	13.52	26.06	25.33
8	1:9	0.80	1056	990	73.14	76.37	61.85	62.00	15.67	15.07	5.88	11.81
9	1:9	0.45	1056	990	75.54	73.52	58.21	63.07	14.35	14.99	19.32	12.36
10	1:9	0.45	1056	990	74.61	74.60	62.07	62.29	14.56	14.72	16.39	14.92
11	1:9	0.45	1056	990	75.62	74.05	58.05	62.74	14.32	14.88	19.59	13.37

EAEP: enzyme-assisted aqueous extraction process.
TPE: total protein extractability.
SLR: solids-to-liquid ratio.
CapEx: capital expenditures.
COGS: cost of goods sold, without depreciation.
DCFRR: discounted cash flow rate of return, with selling price of $16.50/kg protein.

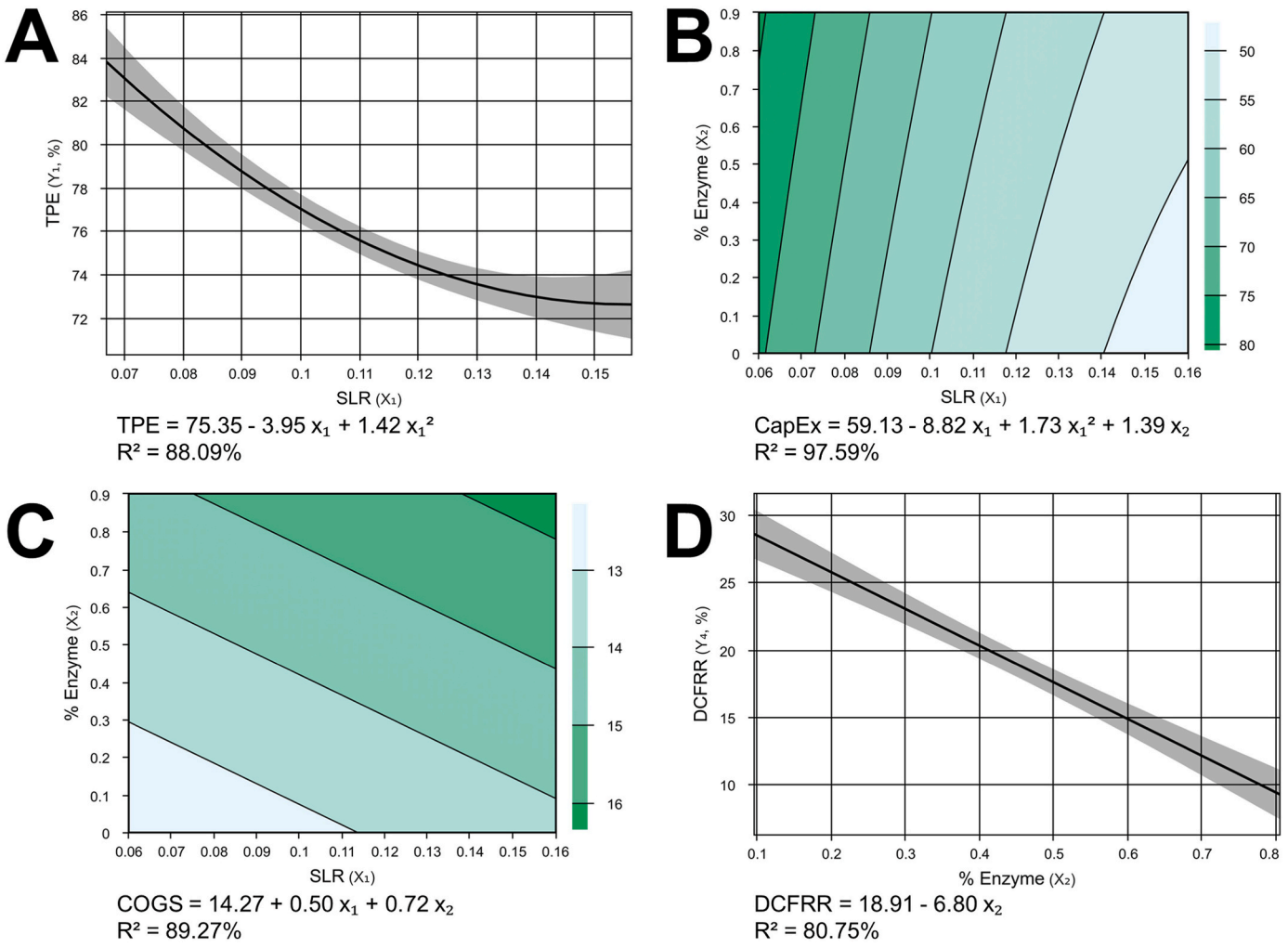

Fig. 4. Contour plots of the 2^2 central composite rotatable design for the EAEP with Alkaline Protease at 30 min, showing the effects of solids-to-liquid ratio (SLR) and/or enzyme loading on (A) total protein extractability (TPE, %), (B) capital expenditures (CapEx, $M USD), (C) cost of goods sold without depreciation (COGS, $/kg protein), and (D) discounted cash flow rate of return (DCFRR, %), assuming a selling price of $16.50/kg protein. Variables and interactions with a significance level of $p < 0.05$ were included in the model.

Table 4
Experimental (Exp.) and predicted (Pred.) protein extractability and economic metrics for selected AEP and EAEP conditions.

Extraction Condition	TPE (%)		Batches/ Year	CapEx ($M)		COGS ($/kg protein)		Functionality-based COGS ($/kg soluble protein at pH 4)[a]	DCFRR (%)		Functionality-based DCFRR (%) considering solubility at pH 4[a]
	Exp.	Pred.		Exp.	Pred.	Exp.	Pred.		Exp.	Pred.	
Aqueous Extraction Process (AEP)											
1:12 SLR, 15 min	66.2 ± 0.4^{b}	66.9	1092	75.8 ± 0.3^{c}	75.1	14.7 ± 0.1^{a}	14.4	44.5 ± 0.3^{c}	12.5 ± 0.7^{c}	13.7	4.1 ± 0.2^{c}
1:12 SLR, 45 min	65.2 ± 0.4^{b}	67.7	1021	81.5 ± 0.4^{b}	78.7	14.9 ± 0.1^{a}	14.4	n.d.	9.6 ± 0.8^{d}	13.7	n.d.
1:12 SLR, 75 min	65.6 ± 0.4^{b}	68.4	960	85.4 ± 0.5^{a}	82.3	14.9 ± 0.1^{a}	14.4	n.d.	9.0 ± 0.8^{d}	13.7	n.d.
Enzyme-Assisted Aqueous Extraction Process (EAEP)											
1:12 SLR, 0.1% AP	81.5 ± 0.4^{a}	79.9	1056	65.6 ± 0.3^{e}	66.3	13.1 ± 0.1^{d}	12.8	58.8 ± 0.3^{a}	26.6 ± 0.6^{a}	28.5	5.9 ± 0.1^{b}
1:12 SLR, 0.3% AP	81.6 ± 0.4^{a}	79.9	1056	65.9 ± 0.3^{e}	67.4	13.4 ± 0.1^{c}	13.4	48.1 ± 0.2^{b}	24.7 ± 0.5^{a}	23.0	6.9 ± 0.2^{b}
1:12 SLR, 0.5% AP	80.8 ± 0.7^{a}	79.9	1056	67.6 ± 1.1^{d}	68.5	14.1 ± 0.1^{b}	14.0	26.0 ± 0.3^{d}	18.2 ± 1.3^{b}	17.5	9.9 ± 0.7^{a}

AEP: aqueous extraction process; EAEP: enzyme-assisted aqueous extraction process; SLR: solids-to-liquid ratio; AP: Alkaline Protease; TPE: total protein extractability; CapEx: capital expenditures; COGS: cost of goods sold without depreciation; DCFRR: discounted cash flow rate of return, with selling price of $16.50/kg protein; n.d.: not determined.

Different superscript letters signify statistically significant differences between samples (same column) using one-way ANOVA with Tukey's post-hoc test ($p < 0.05$).

[a] Functionality-based COGS only considering acid-soluble protein fraction; calculated using Eq. 5.

[a] Functionality-based DCFRR only considering acid-soluble protein fraction; calculated using Eq. 6.

3.4. Model validation and economic evaluation of selected conditions

Lab-scale AEP and EAEP were performed under the selected extraction conditions, and TEAs were performed to validate the regression models and the economic metrics of the best conditions (Table 4). Overall, the experimental and predicted values were very similar, confirming the validity of the models. Slight variations between the actual and predicted DCFRR were observed for the AEP at 45 and 75 min, which could be due to the slightly lower actual TPE values of those extraction conditions (67.7 vs. 65.2% TPE for AEP at 45 min; 68.4 vs. 65.6% TPE for AEP at 75 min). This observation reiterates the extreme sensitivity of DCFRR to protein yields.

The TPEs and COGS for the AEP at 15, 45, and 75 min were not statistically different, but CapEx significantly increased with longer extraction times. Of the three AEP conditions tested, the shortest extraction time (15 min) yielded the highest DCFRR, so the AEP with only 15 min of extraction (1:12 SLR, pH 7.0; abbreviated AEP-15) was selected as the optimal condition for further physicochemical and functional characterization.

Among the EAEP, the TPE was similar regardless of enzyme loading, while the highest enzyme loading (EAEP-0.5) required significantly higher CapEx. COGS significantly increased with higher enzyme concentrations due to the higher material costs associated with the enzyme and NaOH use. The lowest enzyme loading (EAEP-0.1) yielded the best economic metrics overall (highest DCFRR), but considering the potential differences in functionality, all conditions were further characterized for solubility and digestibility as performed in the initial screening. While solubility at pH 7 (95−100%) and IVPD (44−49%) were not statistically different among any of the EAEP conditions, solubility at pH 4 significantly increased as more enzyme was used: 22.2%, 27.8%, and 54.4% for EAEP-0.1, EAEP-0.3, and EAEP-0.5, respectively (further discussed in section 3.5.2). Therefore, the same functionality-based COGS and DCFRR metrics, as used in the initial screening, were determined for the three EAEPs considering solubility at pH 4.0. This holistic approach revealed that EAEP-0.5 outperformed the extracts with lower enzyme usage when simultaneously considering functionality and profitability (9.92% functionality-based DCFRR considering acid-soluble proteins).

3.5. Characterization of optimized AEP and EAEP proteins

3.5.1. DH, zeta potential, and surface hydrophobicity

The optimized black bean extracts (AEP-15, EAEP-0.1, EAEP-0.3, and EAEP-0.5) were characterized for DH, zeta potential, and surface hydrophobicity to investigate the impacts of extraction conditions on protein physicochemical properties (Fig. 5). The DH of the AEP-15 proteins was 6.3%, which was significantly lower than the DH of the AEP-7 (1:10 SLR, 60 min) in the initial screening (7.2%). This could suggest that longer extraction times (even in neutral conditions) could contribute to partial unfolding of the protein structure (Feyzi et al., 2018), and that shorter extraction times might help retain the native structure of the proteins. Interestingly, the DH values of the EAEP-0.1 (5.1%) and EAEP-0.3 (5.5%) were slightly lower than that of the AEP-15 proteins (Fig. 5A). This could be attributed to the fundamental difference in extraction pH, as the alkaline conditions of the EAEP could induce partial denaturation and protein aggregation, effectively hiding free amino groups and decreasing measured DH using the OPA method (Mulcahy et al., 2017; Li et al., 2024). The low DH also implies that at lower enzyme concentrations, 30 min of extraction was insufficient to attain appreciable levels of proteolysis. While other reports of plant protein hydrolysis have demonstrated clear dose-dependent effects of enzyme concentration on DH (Rezvankhah et al., 2021; Yin et al., 2021), an important distinction of the present study is that hydrolysis occurred in a complex extraction slurry containing other bean flour components (starch, fiber, soluble carbohydrates) rather than purified protein. These other components could introduce molecular crowding or increases in slurry viscosity that could slow down reaction rates. In contrast, the EAEP-0.5 had a significantly higher DH (13.2%) compared to all the optimized samples, suggesting that a sufficient concentration of enzyme

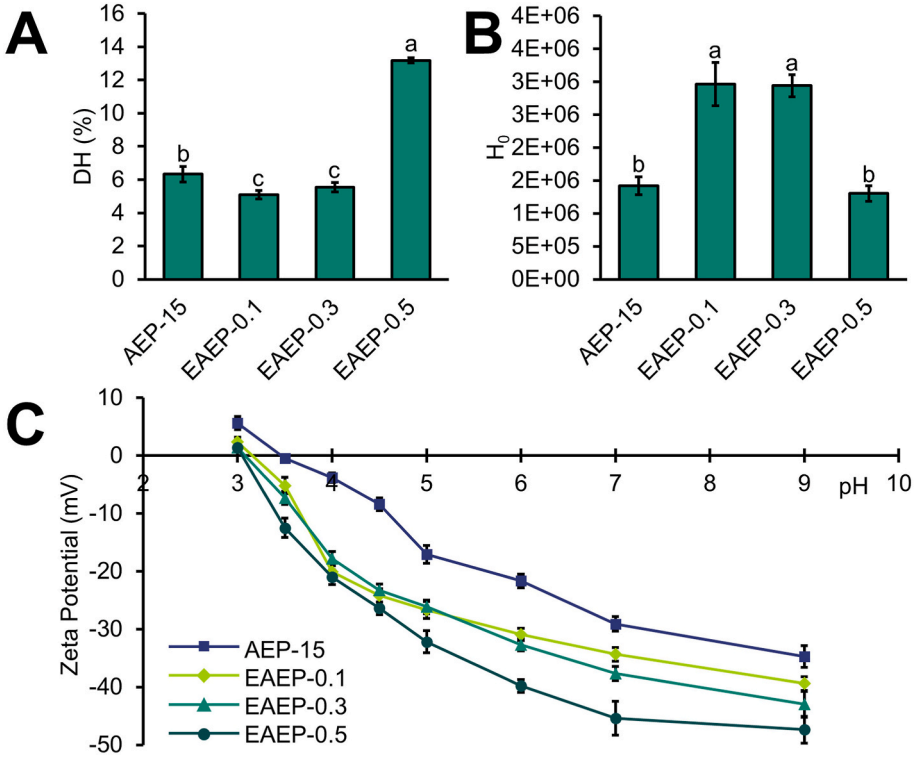

Fig. 5. A) Degree of hydrolysis (DH, %), B) surface hydrophobicity (H_0), and C) zeta potential (mV), of the optimized AEP-15 (pH 7.0, 1:12 SLR, 15 min), EAEP-0.1, EAEP-0.3, and EAEP-0.5 extracts (pH 9.0, 30 min, 1:12 SLR, with 0.1, 0.3, or 0.5% w/w Alkaline Protease, respectively). Different letters indicate statistically significant differences by one-way ANOVA followed by Tukey's test ($p < 0.05$).

was present to overcome the hurdles to proteolysis (i.e., short extraction time, complex matrix). However, the DH of the EAEP-0.5 was slightly lower than the EAEP with Alkaline Protease in the screening (15.2%), which could be attributed to the shorter extraction time (30 min vs. 60 min).

Surface hydrophobicity seemed to be inversely associated with DH, as the AEP-15 and EAEP-0.5 proteins, which exhibited the higher DH, exhibited relatively low surface hydrophobicity compared to the EAEP-0.1 and EAEP-0.3 proteins (Fig. 5B). This again alludes to potential partial denaturation and/or aggregation that occurred in alkaline conditions for the EAEPs with lower enzyme concentrations, which could have exposed hydrophobic regions of the proteins. The surface hydrophobicity of the EAEP-0.5 proteins was significantly lower than the other EAEP samples, demonstrating that more extensive hydrolysis may have led to the reburying of these newly exposed hydrophobic groups into the center of the protein fragment (Jung et al., 2005), or that the hydrophobic clusters were sufficiently disrupted so that the fluorescent probe was not able to sufficiently bind (Kundu and Guptasarma, 2002). A decrease in surface hydrophobicity upon proteolysis was similarly reported for black beans, fava beans, and lentils (Yang et al., 2024a; do Evangelho et al., 2017; Dias et al., 2024; Nawaz et al., 2022).

Zeta potential curves were determined for the optimized extracts to determine the impact of extraction conditions on the surface charge of the proteins at different pHs, and to estimate the pI of the proteins. The pI is a critical parameter for food proteins as charge and aggregation state can significantly impact the solubility and interfacial properties of proteins (Karaca et al., 2011; Yang et al., 2024b). The zeta potential curves show that generally, the EAEP proteins were more charged compared to the AEP-15 proteins, which could be attributed to the different protein species extracted in alkaline conditions that may inherently be more charged. In addition, the increased surface charge could be caused by the co-extraction of charged species (e.g., polysaccharides, polyphenols) in the alkaline conditions (Feyzi et al., 2018). Soluble pea polysaccharides have been previously demonstrated to increase the charge (more negative) when combined with pea proteins (Tran et al., 2024). In addition to co-extraction of charged components, the EAEP-0.5 extract exhibited the highest surface charge, likely due to the liberation of ionizable groups upon proteolysis (Wouters et al.,

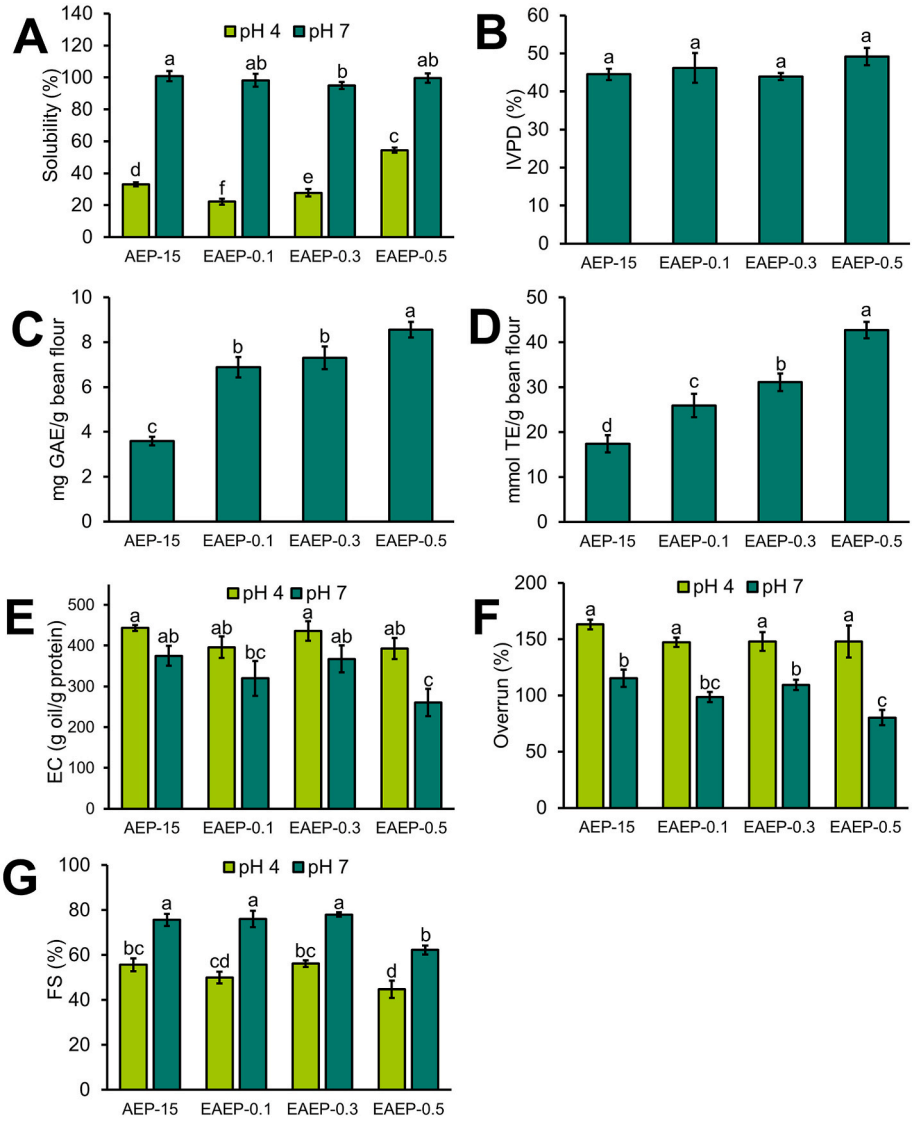

Fig. 6. Functional properties of the optimized AEP-15 (pH 7.0, 1:12 SLR, 15 min) and EAEP-0.1, 0.3, and 0.5 (pH 9.0, 1:12 SLR, 30 min with 0.1, 0.3, or 0.5% Alkaline Protease, respectively): (A) protein solubility at pH 4 and 7 (%), (B) *in vitro* protein digestibility (IVPD, %), (C), total phenolic content (mg gallic acid equivalents/g bean flour), (D) antioxidant capacity (mmol Trolox equivalents/g bean flour), (E) emulsifying capacity (EC; g oil/g protein), (F) foam overrun (%), and (G) foam stability (FS, %). Different letters denote statistically significant differences by one-way (B, C, D) or two-way (A, E, F, G) ANOVA followed by Tukey's test ($p < 0.05$).

2016). The pI of the AEP proteins was pH 3.46, while the pIs of the EAEP proteins were significantly lower (EAEP-0.1: pH 3.16; EAEP-0.3: 3.08; EAEP-0.5: 3.05). This change in pI with increased enzymatic hydrolysis has been similarly reported for other pulse proteins (Vogelsang-O'Dwyer et al., 2022; Klost and Drusch, 2019). Specifically, these results are in accordance with our previous work showing that the pI of AEP black bean proteins (pH 9.0, 1:15 SLR, 30 min, 50 °C) was pH 3.4, while the pI of EAEP proteins with a very high enzyme loading (1% Alkaline Protease), was < pH 2 (DH of 21%) (Yang et al., 2025). The findings in the present work underscore the significance of enzyme dosage in dictating the pI of the resulting protein extracts and suggest that the amount of enzyme in the EAEP could be tailored for the controlled modification of pIs.

3.5.2. Solubility, IVPD, TPC, and antioxidant capacity

The properties assessed in the original screening were also determined for the optimized AEP-15 and EAEP-0.1, 0.3, and 0.5 (Fig. 6A–D). All samples were highly soluble at pH 7 (95–100%), suggesting high commercial applicability in neutral foods or beverage applications. At acidic pH, solubility for the AEP-15 proteins was slightly higher (33%) compared to the AEP-7 (60 min, 30%) in the initial screening, which suggests that shorter extraction times can help maintain the native structure of the proteins and improve solubility. Notably, in acidic conditions, the solubilities of the EAEP-0.1 and EAEP-0.3 proteins (22 and 28%, respectively) were considerably lower than the solubility of the EAEP with Alkaline Protease in the initial screening (0.5% AP, 60 min; 61% solubility). This can be explained by the lower enzyme dosage compounded with the shorter extraction time and aligns with the low DH observed for these samples. However, the EAEP-0.5 achieved similar solubility as the screening condition (54%) and could be useful in potential acidic beverage applications. Most commercially available proteins have very low solubilities in acidic pH conditions (~5–10%) (de Paiva Gouvêa et al., 2023; Burger et al., 2022), which showcases both the AEP and EAEP bean proteins as potential novel protein ingredients.

The IVPD of the optimized samples (44–49%) were slightly lower than the IVPD values determined in the initial screening (54% for AEP-7, 59% for EAEP-AP) (Fig. 6B). For the AEP, this could be related to the shorter extraction times (75% reduction in time), that may have allowed the proteins to retain their undenatured state, as discussed in section 3.5.1. Previous studies have demonstrated that a certain level of denaturation (via thermal treatments) can be beneficial for digestion, as partial unfolding of proteins can facilitate the accessibility of digestion sites to gastric and intestinal proteases (Park et al., 2010). For the EAEP, the lower DH of the optimized samples compared to the EAEP with Alkaline Protease in the screening could explain the lower observed IVPD, as the proteins experienced a lower degree of "pre-digestion" by the commercial protease prior to simulated digestion (Wu et al., 2024). The small increment in protein solubility observed, combined with the slight reduction in protein digestibility, underscores the trade-off between these two properties. While solubility is important for food applications, a balance between solubility and digestibility must be carefully managed when optimizing protein extraction conditions. The TPC and antioxidant capacity were significantly higher for the EAEP extracts compared to the AEP-15 extracts (Fig. 6C and D), which matches the findings from the initial screening. Compared to the AEP-7 (1:10 SLR, 60 min) in the screening, the AEP-15 had slightly lower TPC and antioxidant capacities, likely due to the shorter extraction time. For the optimized EAEPs, TPC and antioxidant capacity increased as enzyme loading increased, suggesting that higher levels of proteolysis helped release more antioxidative compounds (phenolics and/or peptides).

3.5.3. Emulsifying capacity (EC)

Emulsifying capacity (EC) quantifies the amount of oil that can be emulsified by a given amount of protein under specific conditions before the emulsion breaks. Overall, high EC values were observed for all samples at pH 4 (393–443 g oil/g protein), with a slight downward trend (statistically insignificant, except for EAEP-0.5) in EC at pH 7 (261–375 g oil/g protein) (Fig. 6E). While EC is often enhanced at pHs further from the pI due to increased protein solubility and ability to migrate to the oil/water interface, higher EC near the protein pI can be attributed to the higher surface hydrophobicity of the proteins at pHs closer to the pI (Ngui et al., 2021). The exposure of hydrophobic patches may allow the proteins to interact more strongly with the oil phase of the emulsion, therefore "anchoring" themselves to the oil-water interface (Damodaran, 2005). Importantly, in the determination of EC, various visual cues signal the point of emulsion breakage, including the pooling of free oil on the top of the emulsion, viscosity changes, as well as complete emulsion inversion. For the EAEP-0.5, an emulsion formed, but the texture of the emulsion was markedly weaker than for the other samples at both pH 4 and 7, which could be due to the increased level of proteolysis that impaired the ability of the sample to form a strong lamella (film of continuous phase that surrounds oil droplets) at the interface (Damodaran, 2005). Importantly, this observation demonstrates the limitations of using EC alone to comprehensively assess the emulsifying properties of a protein ingredients. Future characterization of droplet size and emulsion stability could provide additional insight regarding the effects of proteolysis on emulsifying properties. Weakened emulsifying activity for hydrolyzed pulse proteins has also been reported for Alcalase-hydrolyzed *Phaseolus lunatus* (Betancur-Ancona et al., 2009) and chickpea (Yust et al., 2010).

3.5.4. Foaming properties

Foaming is an interfacial property that is especially important for plant-based dairy and egg analogues. Overall, all AEP and EAEP extracts displayed high foam overrun at pH 4 (147–163%) (Fig. 6F). At pH 7, however, the foam overruns were all lower, with the most hydrolyzed sample (EAEP-0.5) exhibiting the lowest value (80%). This decrease mirrors what was observed for EC with the EAEP-0.5. While solubility has been considered a prerequisite for foamability (Damodaran, 2005), several studies have found that pH conditions near the pI enhanced foaming capacity, as reviewed by Narsimhan and Xiang (2018). Similar observations have been reported for pea protein isolate, in which solubility was low at pH 3, but foaming capacity (defined by the foam volume per volume of gas) was relatively high (Othmeni et al., 2024). A potential explanation could be that in acidic conditions (close to the pI of the proteins), proteins were less charged and exhibited more protein-protein interactions, therefore decreasing the distance between molecules, resulting in the formation of a thicker protein film around air bubbles to support foam formation (Wani et al., 2015; Kinsella, 1981).

With respect to foam stability (FS), the opposite trend as foam overrun was observed, with overall higher stability in neutral conditions. Similar findings were reported for pea protein isolates that exhibited the highest foam expansion, but the lowest FS at pH 5 (close to the pI); the authors suggest that while the hydrophobic interactions and low net charge facilitated foam formation, the foam was less stable due to protein aggregation that led to coalescence of air bubbles (Othmeni et al., 2024). Importantly, in the present study, the volumes of the foams produced at pH 4 (9.3–10.3 mL) were significantly higher than those produced at pH 7 (5.1–7.5 mL); although FS is represented as a percentage of foam remaining after 60 min, the initial height of the foam could have an impact on the results due to increased drainage rates for foams that had a higher "holdup volume" (i.e., amount of liquid in the foam structure) (Germick et al., 1994). For the EAEP-0.5, there was a decrease in FS compared to the other samples, likely because the extensive molecular weight reduction (high DH) prevented the formation of a thick, cohesive film around air bubbles (Wouters et al., 2016). Similar losses in FS upon hydrolysis have also been reported for other pulse crops (Al-Ruwaih et al., 2019; Barac et al., 2012; Ahmed et al., 2019).

4. Conclusion

AEP and EAEP for black bean proteins were optimized using a holistic strategy considering protein yields, functional characteristics, and economic feasibility as estimated through industrial-scale techno-economic modeling. An initial screening of pHs and commercial enzymes, followed by subsequent optimization of SLR, time, and enzyme concentration to maximize functionality-based economic metrics (COGS and DCFRR considering the soluble or digestible protein fraction) resulted in the selection of one AEP (1:12 SLR, pH 7, 15 min, 50 °C), and three EAEPs with different enzyme concentrations (1:12 SLR, pH 9, 30 min, with 0.1%, 0.3% or 0.5% Alkaline Protease) that achieved high DCFRR (12.5−26.6% with selling price of $16.50/kg protein; production level of 10,000 MT protein/year, 30 year project lifetime). Shorter extraction times for the AEP appeared to better retain the native protein structure, which resulted in higher solubility, but lower digestibility. Lower enzyme levels in the EAEP were not effective in increasing the degree of hydrolysis, but an enzyme loading of 0.5% was sufficient to hydrolyze the proteins and improve protein solubility in acidic conditions. Interfacial properties were largely similar among the conditions except for the most hydrolyzed sample (EAEP with 0.5% enzyme), which had slightly lower performance due to the extensive proteolysis. This suggests that the AEP at pH 7 or EAEP with milder levels of hydrolysis (0.1 or 0.3% w/w, with ~6% DH) may be more suitable if foaming or emulsifying applications are desired. Overall, the findings of the present study confirm the need for functionality- and profitability-driven development of extraction processes, as processing parameters have a direct impact on the commercial viability and potential applications of the final protein product.

CRediT authorship contribution statement

Jasmin S. Yang: Conceptualization, Methodology, Investigation, Formal analysis, Writing – original draft, preparation, Writing – review & editing. **Fernanda F.G. Dias:** Conceptualization, Methodology, Investigation, Formal analysis, Writing – review & editing. **Karen A. McDonald:** Conceptualization, Methodology, Investigation, Formal analysis, Writing – review & editing. **Juliana M.L.N. de Moura Bell:** Conceptualization, Supervision, Project administration, Resources, Funding acquisition, Writing – review & editing.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crfs.2025.101078.

Data availability

Data will be made available on request.

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