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Efficient recovery of functional biomolecules from shrimp (*Litopenaeus vannamei*) processing waste for food and health applications via a successive co-culture fermentation approach

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ABSTRACT

This study developed a food-grade fermentation process that efficiently isolated proteins and minerals from shrimp-processing waste (SPW). The in vitro antioxidant and enzyme inhibitory effects of SPW hydrolysates obtained from the fermentation process were investigated. SPW broths were prepared from the head (SPW-SH) and body carapace (SPW-SS) of Pacific white shrimp (*Litopenaeus vannamei*) and fermented using a 5-day successive co-culture fermentation approach with *Bacillus amyloliquefaciens* TISTR-1880 and *Lactobacillus casei* TBRC-388. This bacterial combination demonstrated optimal efficiency in extracting proteins (up to 93% deproteinization) and minerals (up to 83% demineralization) from SPW samples compared with other studied co-culture combinations. The resulting SPW-SH and SPW-SS hydrolysates were rich in proteins (~70 and ~59 g/100 g dry weight, respectively). They exhibited significantly enhanced antioxidant potential compared to their corresponding non-fermented controls at up to 2.3 and 3.7-fold higher, respectively as determined by the ORAC, FRAP, and DPPH radical scavenging assays. The two SPW hydrolysates also had significantly higher inhibitory activities against angiotensin-converting enzyme, α-amylase, and lipase than the controls, indicating their improved anti-hypertension, anti-diabetes, and anti-obesity properties, respectively; however, both SPW-SH and SPW-SS hydrolysates did not inhibit α-glucosidase at the tested concentrations. The SPW hydrolysates produced in this study showed high potential for use as functional ingredients in food and nutraceutical products. Knowledge gained from this study can promote the prospective valorization of industrial SPW as an inexpensive source of functional biomolecules for food-related applications using a fermentation approach. This will increase the commercial value of SPW and reduce the environmental impact.

1. Introduction

The manufacture of crustacean products is increasing worldwide and plays a key role in the global economy. Industrial processing of crustaceans creates a large volume of non-edible waste, amounting to more than 8 million tons per year (Mao et al., 2017). The slow natural degradation rate of this waste has led to increased public environmental concern, especially in Thailand, China, Vietnam, and India as the world's leading shrimp exporters (Arbia et al., 2013). Shrimp-processing waste (SPW) including the head, shell, and tail represents 50–70% of the shrimp weight (De Holanda and Netto, 2006), and consists primarily of chitin, proteins, and mineral salts (mainly calcium carbonate), accounting for 17–18%, 42–47%, and 23–34% dry weight (DW), respectively. SPW also contains carotenoids, a group of natural antioxidant pigments (Venugopal, 2008). Attention regarding the utilization of SPW has mainly focused on the chemical extraction of chitin and its

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derivatives to serve as biopolymers for various purposes such as food preservation, water treatment, and biomedical applications (Venugopal, 2008). Chemical approaches involving strong acid and alkaline solutions generate hazardous by-products, and except for chitin, other valuable components of SPW are decomposed and cannot be recovered (Arbia et al., 2013; Mao et al., 2017). Over the past decade, fermentation has been promoted as a green strategy to produce chitin from SPW, with the concomitant recovery of proteins and minerals (Y.N. Tan et al., 2020). The fermentation process involves dissolution of proteins and minerals in SPW by protease-producing and acid-producing microorganisms, respectively. This approach is considered safe, eco-friendly, and cost-effective and is highly reproducible (Arbia et al., 2013; Mao et al., 2017).

Previous studies reported the effective use of bacterial fermentation to isolate chitin from SPW using a single strain of bacteria, e.g., *Pseudomonas aeruginosa*, *Bacillus cereus*, *Enterococcus faecalis* or *Lactobacillus paracasei* (Ghorbel-Bellaaj et al., 2018; Narayan et al., 2010; Sedaghat et al., 2017), as well as the combined use of two different strains. Compared with single-strain fermentation, co-culture fermentation of SPW using a proteolytic bacterium like *Bacillus* spp. and a lactic acid bacterium like *Lactobacillus* spp. resulted in high-quality chitin, and provided greater potential for recovering proteins and minerals from SPW (Chakravarty et al., 2018; Y.N. Tan et al., 2020; Zhang et al., 2022). The protein-rich liquid hydrolysate produced during SPW fermentation also exhibited biological functions as a result of releasing bioactive peptides from the parent protein by proteolysis (Ghorbel-Bellaaj et al., 2018). However, few studies have focused on the fermentation of SPW hydrolysates to produce SPW-derived chitin, and the development of a fermentation process to obtain food-grade functional molecules from the resulting SPW hydrolysates remains underexplored.

This study developed a fermentation process for efficient recovery of functional biomolecules, particularly proteins and minerals, from SPW for food and health applications. Generally Recognized as Safe (GRAS) strains of *Bacillus* spp. and *Lactobacillus* spp. were screened to serve as protease and acid producers in the co-culture fermentation of SPW, respectively. The in vitro bioactivities (antioxidant, anti-hypertension, anti-diabetes, and anti-obesity properties) and the nutritional compositions of the resulting SPW hydrolysates were also determined to evaluate their potential for use as functional ingredients in food and nutraceutical products. Knowledge gained from this study can be used to promote SPW as an inexpensive source of functional biomolecules for food-related applications in the food industry.

2. Materials and methods

2.1. Materials and chemicals

Fresh Pacific white shrimp (*Litopenaeus vannamei*) processing waste as head (SPW-SH) and body carapace (shell and tail, SPW-SS) was provided by the Thai Union Group PCL., Thailand and kept refrigerated during transport. Upon arrival at the research facility, the two SPW products were dried in a hot-air tray dryer (Model King 0.37 kw/0.5 HP, King Kluaynamthai, Bangkok, Thailand) at 65 °C overnight. The samples were then ground into powder (particle size <2.0 mm) using a grinder (Model JPS-500, Yongkang Horus Industry and Trade, Zhejiang, China) and stored in vacuum bags at −20 °C until used. Physical appearances and proximate compositions of the powdered samples are presented in Supplementary Fig. S1 and Table S1, respectively.

Five strains of *Bacillus* spp. (*B. amyloliquefaciens* TISTR-1880, *B. licheniformis* TISTR-013, *B. subtilis* TISTR-025, *B. subtilis* TISTR-1528, and *B. subtilis* MR-10) exhibiting high extracellular proteolytic activity and five strains of *Lactobacillus* spp. (*L. acidipiscis* BCC-42383, *L. brevis* BCC-47677, *L. casei* TBRC-388, *L. casei* CRL-431, and *L. plantarum* DSM-6595) possessing the ability to deal with aerobic growth were screened in our preliminary experiments. These bacteria, with the sources shown in Supplementary Tables S2 and S3, are not pathogenic to humans and have GRAS status. The bacteria were maintained in media supplemented with 20% (v/v) glycerol and stored at −40 °C.

Microbiological media and agar-agar were obtained from Merck (Darmstadt, Germany). All chemicals and enzymes were sourced from Sigma-Aldrich (St. Louis, MO, USA) and were of the highest quality unless otherwise specified.

2.2. Preparation of bacterial inocula and SPW broth

Bacillus strains were grown in Luria-Bertani (LB) broth at 37 °C, 150 rpm for 24 h, and *Lactobacillus* strains were cultivated in De Man, Rogosa and Sharpe (MRS) broth at 37 °C for 24 h. Bacterial cultures were centrifuged (4000×*g*, 15 min, 4 °C), resuspended in sterile peptone water and adjusted to an inoculum density of around 9 log CFU/mL.

The SPW-based broth was prepared following the method of Ghorbel-Bellaaj et al. (2012) with some modifications. The powdered sample, as either SPW-SH or SPW-SS, was mixed with 5% (w/v) glucose solution at a sample-to-solution ratio of 1:5 (w/v). The resulting broth was adjusted to pH 7.0 with 5 N NaOH, sterilized at 121 °C for 15 min, and used as a fermentation medium.

2.3. Investigation of bacterial activity in SPW broth

The primary selection of bacterial starters was conducted by investigating the protease activity and acid-producing capacity of *Bacillus* spp. and *Lactobacillus* spp. in SPW-SH broth, respectively. A 250-mL flask containing 100 mL of sterile SPW-SH broth was inoculated with a strain of *Bacillus* or *Lactobacillus* (~9 log CFU/mL, 2% v/v) and placed in a shaking incubator (Model ZWYR-2102C, Labwit Scientific, Victoria, Australia) at 37 °C, 150 rpm for 5 days. Changes in protease activity and pH of the broth were monitored daily. The strains of *Bacillus* and *Lactobacillus* that exhibited the highest protease activity and acid-producing capacity in the broth, respectively were paired and employed as starter co-cultures for further investigation.

2.4. Selection of starter co-culture and inoculum percentage

The co-culture fermentation of SPW-SH broth was performed in two successive steps using four starter co-cultures of the chosen *Bacillus* and *Lactobacillus* strains. To isolate proteins from the SPW-SH material, *Bacillus* cultures (2% v/v) were transferred into fermentation flasks (250 mL) containing sterile SPW-SH broth (100 mL) and cultivated at 37 °C, 150 rpm for 3 days using a shaking incubator. Sequentially, *Lactobacillus* cultures (2% v/v) were aseptically transferred into the *Bacillus* fermentation contents. The fermentation was then continued in the shaking incubator for another 2 days for mineral dissolution. The changes in protease activity and pH during fermentation were observed. After the 5-day fermentation, the SPW-SH residues were separated from the liquid hydrolysates by filtration through cheesecloth, washed repeatedly with deionized water, dried overnight at 65 °C, and analyzed to determine their protein and mineral contents. A competitive starter co-culture corresponding to the highest sample recovery of proteins and minerals was selected.

A successive co-culture fermentation process for SPW-SH using the aforementioned procedure was conducted, with the inoculum percentage of each selected bacterial culture varied from 2% to 4% and 6% (v/v) to evaluate the effect of inoculum percentage on fermentation efficiency. The residues obtained after fermentation were analyzed as described above. The optimal inoculum percentage of nutrient recovery from the sample was chosen, giving co-culture fermentation conditions that enabled efficient isolation of proteins and minerals from SPW. This fermentation process was further employed to ferment both SPW-SH and SPW-SS samples. The resulting SPW hydrolysates were collected by centrifugation (10,000×*g*, 15 min, 4 °C), freeze-dried and kept in aluminum foil bags at −20 °C for further bioactivity analyses.

2.5. Chemical and microbiological analyses

2.5.1. Protease activity, pH and degree of hydrolysis

The protease activity was determined following the method of Chakravarty et al. (2018), with casein used as a substrate. Enzymatic reactions were monitored at 660 nm using a 96-well microplate reader (Model Synergy HT, BioTek Instruments, Inc., Winooski, VT, USA) with BioTek Gen5 software. One unit of protease activity was defined as the amount of enzyme required to release 1 μmol of L-tyrosine per minute. Results were expressed as units (U) per mL of liquid hydrolysate produced during SPW fermentation. The pH of the hydrolysate was measured using a pH meter (Model Starter 2100, OHAUS Corporation, Parsippany, NJ, USA). The degree of hydrolysis (DH) was determined following the method of Benjakul and Morrissey (1997) based on the comparison of free amino groups in the hydrolysate to the total amino groups in the raw material after acid hydrolysis with 6 M HCl (100 °C, 24 h). The amount of free amino groups was spectrophotometrically analyzed at 420 nm using a microplate reader, with L-leucine employed as a standard.

2.5.2. Proximate composition

The nutrient analysis of SPW samples and their hydrolysates was conducted following the methods of the Association of Official Analytical Chemists (AOAC, 2019). Briefly, total fat content was determined after acid hydrolysis and petroleum ether extraction using a Soxtec system. The total nitrogen and chitin nitrogen were analyzed by the Dumas combustion method. Corrected protein content was obtained by subtracting chitin nitrogen from total nitrogen and multiplying by a conversion factor of 6.25 (Tshinyangu and Hennebert, 1996). The ash content was determined by burning samples in a muffle furnace at 550 °C. The moisture content was determined by drying samples at 105 °C. The total carbohydrate content was calculated by subtracting the contents of the aforementioned nutrients from 100, and the energy was calculated using multiplication factors of 9 for fat and 4 for carbohydrate and protein. The calcium content was determined using a flame atomic absorption spectrometer.

2.5.3. Deproteinization, demineralization and decalcification

The efficiencies of protein and mineral recovery from the SPW samples were reported as percentages of deproteinization (DP) and demineralization (DM) (also decalcification, DC), taking into account the masses of the nutrients before and after fermentation (Chakravarty et al., 2018; Ghorbel-Bellaaj et al., 2012). The calculations were performed using the following equations

$$\%DP = [(PO \times O - PR \times R)/(PO \times O)] \times 100,$$

where PO and PR are protein contents before and after fermentation, and O and R are masses (g DW) of the original sample and fermented residue, respectively

$$\%DM = [(AO \times O - AR \times R)/(AO \times O)] \times 100,$$

where AO and AR are ash contents before and after fermentation, and O and R are masses (g DW) of the original sample and fermented residue, respectively and

$$\%DC = [(CO \times O - CR \times R)/(CO \times O)] \times 100,$$

where CO and CR are calcium contents before and after fermentation, and O and R are masses (g DW) of the original sample and fermented residue, respectively.

2.5.4. Bacterial enumeration

The number of bacterial cells was determined by viable counts on culture media using the standard plate count method (BAM, 2001), and the total bacterial count was determined using plate count agar. The *Bacillus* count was determined on LB agar, and the *Lactobacillus* count was determined on MRS agar. For all microbial assays, culture incubation times were 24–48 h at 37 °C. Bacterial colonies that appeared on the agar media were counted and reported as CFU per mL of sample.

2.6. In vitro investigation of bioactivities

Dried SPW hydrolysates were resuspended in deionized water to the desired protein concentrations and analyzed promptly. Protein contents in the solutions were measured using a microplate reader at 660 nm using the Lowry method (Lowry et al., 1951).

2.6.1. Antioxidant activity

Antioxidant activities were determined using the oxygen radical absorbance capacity (ORAC), ferric ion reducing antioxidant power (FRAP), and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assays, conducted following the protocols described by Sripum et al. (2017) with no modifications. In brief, the ORAC assay relies on the reaction between a sample and 2,2,-azobis(2-methylpropionamidine) dihydrochloride (AAPH) solution. Antioxidant activity was observed kinetically as a fluorescence decay curve of fluorescein at 37 °C using excitation and emission wavelengths of 485 nm and 528 nm, respectively. The FRAP assay was performed by incubating a sample with FRAP reagent at 25 °C for 8 min, and the absorbance was measured at 600 nm. The DPPH radical scavenging assay was performed by incubating a sample with DPPH reagent at 25 °C for 30 min, and the absorbance was measured at 520 nm. Results obtained from all the antioxidant activity assays were expressed as μmol Trolox equivalent (TE) per gram of protein.

2.6.2. Enzyme inhibitory activity

Disease suppressive effects were investigated in vitro by inhibition of the key enzymes that control the diseases. Inhibition of the hypertension-related angiotensin converting enzyme (ACE) was observed following the method of Schwager et al. (2006). The inhibition of type II diabetes and obesity-associated enzymes (α-amylase/α-glucosidase and lipase, respectively) were observed following the protocols described by Sirichai et al. (2022) with no modifications. Enzymatic reactions were monitored using a microplate reader at particular wavelengths (Supplementary Table S4). Enzyme inhibitory activities were reported as percentages of inhibition at a particular protein concentration and calculated as follows: % Inhibition $= [1-(B-b)/(A-a)] \times 100$, where A is the initial reaction rate of the control with enzyme but without sample, a is the initial reaction rate of the control without enzyme and sample, B is the initial rate of the enzymatic reaction with sample, and b is the initial rate of the reaction with sample but without enzyme.

2.7. Statistical analysis

All experiments were conducted at least in triplicate, with results expressed as mean ± standard deviation. One-way analysis of variance (ANOVA) and Duncan's multiple range test were used to detect significant differences between mean values. An unpaired *t*-test was employed to compare significant differences between the mean values of the two data sets. Values were considered significantly different at $p < 0.05$. All statistical analyses were performed using SPSS software Version 23.0 (SPSS Inc., Chicago, IL, USA).

3. Results and discussion

3.1. Proteolytic and acid-producing activities of bacteria in SPW broth

The strains of *Bacillus* and *Lactobacillus* tested were separately incubated in SPW-SH broth. During a 5-day fermentation period, proteolytic and acid-producing activities of *Bacillus* spp. and *Lactobacillus* spp. were examined, with the results presented in Fig. 1.

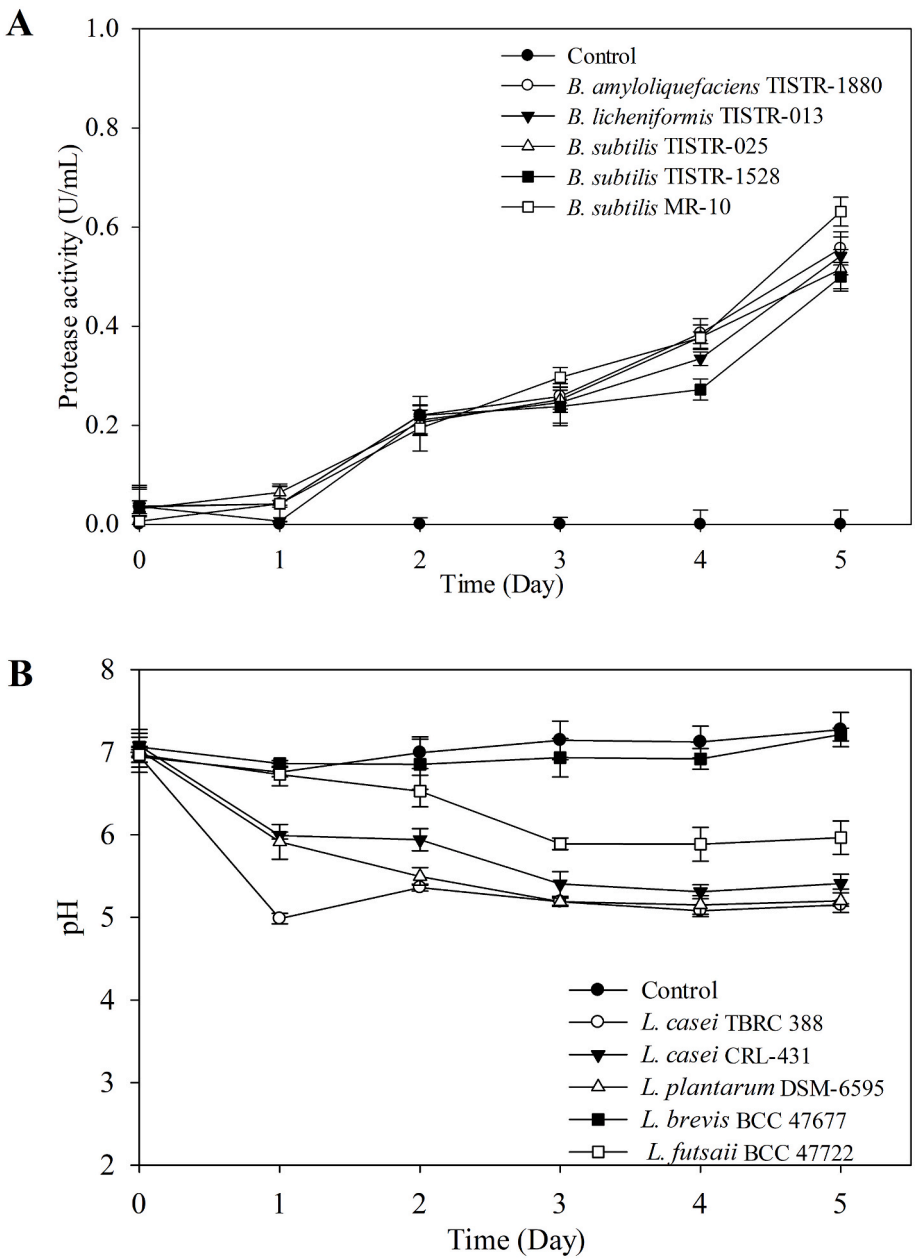

Fig. 1. Changes in (A) protease activity of SPW-SH broths fermented with *Bacillus* spp. and (B) pH of SPW-SH broths fermented with *Lactobacillus* spp. Data are shown as the mean ± SD of the experiments that were conducted in triplicate.

As shown in Fig. 1A, all SPW-SH broths inoculated with single strains of *Bacillus* spp. exhibited protease activities, which increased gradually with increasing fermentation time from ≤0.1 U/mL on day 1 to 0.5–0.6 U/mL on day 5. The control broth without bacterial cells did not exhibit protease activity throughout the fermentation period as expected. Results suggested that all the tested *Bacillus* strains were capable of producing proteolytic enzymes and excreting them into SPW-SH broth. During the 5 days of fermentation, *B. subtilis* MR-10 exhibited the highest protease-producing potential in SPW-SH broth followed by *B. amyloliquefaciens* TISTR-1880, with maximum protease activities of 0.63 and 0.56 U/mL detected on day 5, respectively. The cell density of these bacteria also increased by around 1.0 log CFU/mL at the end of fermentation, indicating their ability to grow in the broth. By contrast, a decrease in number of bacterial cells (<0.5 CFU/mL) after fermentation was observed in *B. licheniformis* TISTR-013, *B. subtilis* TISTR-025, and *B. subtilis* TISTR-1528 (data not shown). Our findings concurred with previous studies reporting that the proteolytic potential of *Bacillus* spp. varied significantly among different strains and were dependent on the proliferation and/or survival of the strains during fermentation (Hashmi et al., 2022; Wongputtisin et al., 2012). Protease production and enzyme stability in *Bacillus* spp. are influenced by various extrinsic environmental factors such as medium composition, temperature, pH, and aeration rate. These factors play crucial roles in shaping the metabolic activity, protein synthesis, and enzyme characteristics (Hashmi et al., 2022; Queiroga et al., 2013).

As shown in Fig. 1B, the pH of all SPW-SH broths inoculated with the tested *Lactobacillus* strains, except for the broth inoculated with *L. brevis* BCC-47677, decreased from pH 7.1–7.3 to pH 5.2–6.0 after 5 days of fermentation. This decrease in the pH occurred because the *Lactobacillus* strains metabolized glucose in the broth and produced lactic acid as a major product. However, diverse lactic acid bacterial strains have different lactic acid production yields and productivity depending mainly on the temperature (5–45 °C), pH (3.5–9.6), and availability of nutrients in their environment (Abedi and Hashemi, 2020), thereby

explaining the minimal pH change observed during the fermentation of SPW-SH broth with *L. brevis* BCC-47677. As anticipated, the pH of the cell-free broth used as a control remained relatively constant throughout the investigation, confirming that the reduction in pH level detected in *Lactobacillus*-fermented broths resulted from the bacterial acid-producing activities. The pH of the broths decreased during the first 2 days of fermentation, and generally remained constant until the end of the fermentation. This phenomenon was attributed to an increase in the buffering capacity of the broth due to the dissolution of minerals and the subsequent increase in free ions (Castro et al., 2018; Prameela et al., 2017). Among the *Lactobacillus* strains studied, the two optimal acid producers *L. casei* TBRC-388 and *L. plantarum* DSM-6595 (minimum pH values of 5.15 and 5.20 detected on day 5, respectively). Typically, *Lactobacillus* spp. exhibit high tolerance to acidic conditions and thrive in environments with low pH. The acid tolerance of bacteria is strain-specific and dependent on environmental and growth conditions (Barbosa et al., 2015). A significant decrease was observed in the number of *Lactobacillus* cells (up to 2 log CFU/mL) in SPW-SH broths after the 5-day fermentation period (data not shown), possibly due to the depletion of essential nutrients necessary for bacterial growth.

3.2. Changes in protease activity and pH during successive co-culture fermentation

Based on the aforementioned results, two *Bacillus* strains (*B. amyloliquefaciens* TISTR-1880 and *B. subtilis* MR-10) and two *Lactobacillus* strains (*L. casei* TBRC-388 and *L. plantarum* DSM-6595) were used for a 5-day successive co-culture fermentation process. The fermentation was performed using starter co-cultures as (1) *B. amyloliquefaciens* TISTR-1880 and *L. plantarum* DSM-6595, (2) *B. amyloliquefaciens* TISTR-1880 and *L. casei* TBRC-388, (3) *B. subtilis* MR-10 and *L. plantarum* DSM-6595, and (4) *B. subtilis* MR-10 and *L. casei* TBRC-388. Changes in protease activity and pH during the fermentation of the SPW-SH broth are presented in Fig. 2.

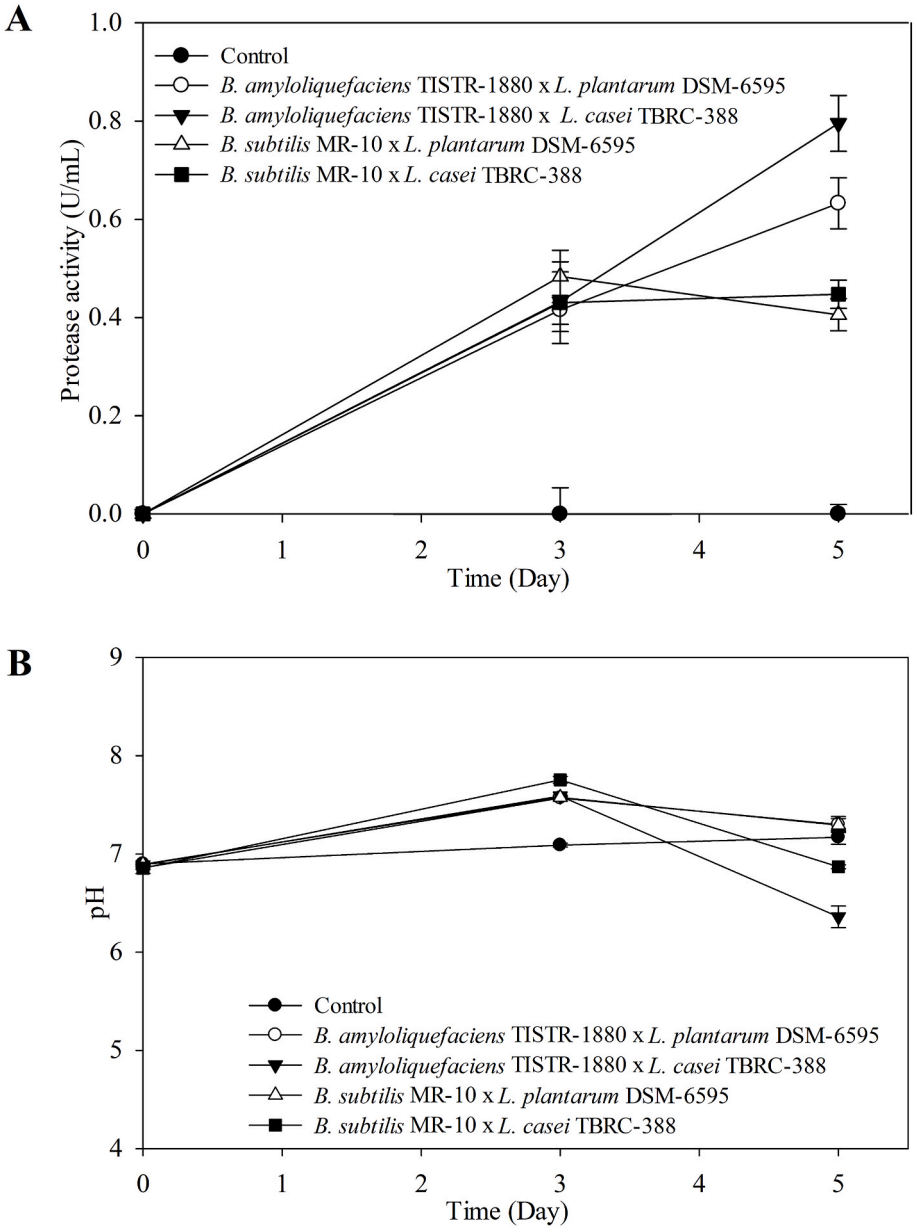

Fig. 2. Changes in (A) protease activity and (B) pH during the fermentation of SPW-SH using different combinations of *Bacillus* spp. and *Lactobacillus* spp. as starter co-cultures. The 5-day fermentation was initiated by *Bacillus* culture and sequentially co-performed with *Lactobacillus* culture starting on the 3rd day. Data are shown as the mean ± SD of the experiments that were conducted in triplicate.

Results shown in Fig. 2A revealed that the protease activities of all inoculated SPW-SH broths increased steadily during the initial 3 days of fermentation when *Bacillus* spp. fermented the broths alone. After this period, *Lactobacillus* spp. coexisting with *Bacillus* spp. in the broths showed significant increases in protease activities until the end of the fermentation when using *B. amyloliquefaciens* TISTR-1880 as the *Bacillus* culture (co-cultures 1 and 2). Protease activities of the broths fermented with *B. subtilis* MR-10 were retarded in the presence of *Lactobacillus* spp. (co-cultures 3 and 4). This discrepancy related to the responses of *B. amyloliquefaciens* TISTR-1880 and *B. subtilis* MR-10 when co-cultivated with *Lactobacillus* spp. highlighted the complexity of microbial interactions between these two bacterial genera during co-fermentation and concurred with a previous report (Zhang et al., 2021, 2022). Compared to the results obtained from single-strain fermentation (Fig. 1A), successive co-culture fermentation of SPW-SH broth using *B. amyloliquefaciens* TISTR-1880 and *Lactobacillus* spp. demonstrated higher proteolytic potential. This effect was particularly notable in the co-fermentation with *B. amyloliquefaciens* TISTR-1880 and *L. casei* TBRC-388, which exhibited protease activity of 0.8 U/mL at the end of fermentation, 1.4-fold higher than fermentation with *B. amyloliquefaciens* TISTR-1880 alone. These results indicated that the *Lactobacillus* strains used in this study positively impacted the production and/or stability of proteases from *B. amyloliquefaciens* TISTR-1880. Strong protease activity is essential for the deproteinization of crustacean shells (Chakravarty et al., 2018) and the combined use of *B. amyloliquefaciens* TISTR-1880 and *L. casei* TBRC-388 as a starter co-culture was a competitive choice to achieve high protein isolation throughout the fermentation period.

As shown in Fig. 2B, the successive co-culture fermentation process involving *Bacillus* spp. and *Lactobacillus* spp. induced significant changes in the pH of SPW-SH broth. The pH values of all inoculated broths gradually increased from 6.8 to 7.6–7.8 during the first 3 days of fermentation when *Bacillus* spp. were present alone, probably associated with the formation of alkali products (e.g., ammonia) through protein hydrolysis (Kittibunchakul et al., 2023). The increase in pH promoted catalytic activity of the *Bacillus* proteases produced in the SPW-SH broth. This speculation was supported by a previous study suggesting that alkaline proteases from *B. amyloliquefaciens* and *B. subtilis* functioned effectively within a pH range of 6–11, exhibiting peak activity at pH 8–9 (Hashmi et al., 2022). As expected, a sharp drop in pH was observed after 3 days of fermentation when *Lactobacillus* spp. were co-fermenting the broth with *Bacillus* spp., thus reflecting lactic acid production by the *Lactobacillus* culture. Interestingly, the decrease in pH during this fermentation period was more pronounced in the presence of *L. casei* TBRC-388 (co-cultures 2 and 4) than in the presence of *L. plantarum* DSM-6595 (co-cultures 1 and 3). Reduction in pH during the lactic acid bacterial fermentation was related to enhanced demineralization of crustacean shells, primarily due to the chelation effect of lactic acid on the calcium molecules in the shells (Chakravarty et al., 2018). Comparing the starter co-cultures studied, using *B. amyloliquefaciens* TISTR-1880 and *L. casei* TBRC-388 in a successive manner resulted in the highest protease activity and also the lowest pH value (~pH 6.4) at the end of the fermentation. This bacterial co-culture showed high potential for recovery of proteins and minerals from SPW using a fermentation process.

3.3. Selection of starter co-culture and inoculum percentage for isolating biomolecules from SPW

Fig. 3 demonstrates the calculated percentages of deproteinization, demineralization, and decalcification obtained by comparing the protein, ash, and calcium contents remaining in SPW-SH after fermentation with their initial amounts in the raw material (Supplementary Table S1). Degrees of deproteinization, demineralization, and decalcification from SPW-SH obtained under all studied co-culture fermentation conditions were higher than without fermentation. Sequential fermentation of SPW-SH with *B. amyloliquefaciens* TISTR-1880 followed by *L. casei* TBRC-388 exhibited proteinization of 86.65%, significantly higher than fermentation using the other co-cultures tested, with efficiencies in demineralizing and decalcifying SPW-SH (~74% and ~66%, respectively) comparable to efficiencies of the co-culture of *B. subtilis* MR-10 and *L. plantarum* DSM-6595 and the co-culture of *B. subtilis* MR-10 and *L. casei* TBRC-388. The lowest degrees of deproteinization, demineralization, and decalcification (~82%, ~65%, and 62%, respectively) were obtained when fermenting with co-culture of *B. amyloliquefaciens* TISTR-1880 and *L. plantarum* DSM 6595. These results aligned with the changes

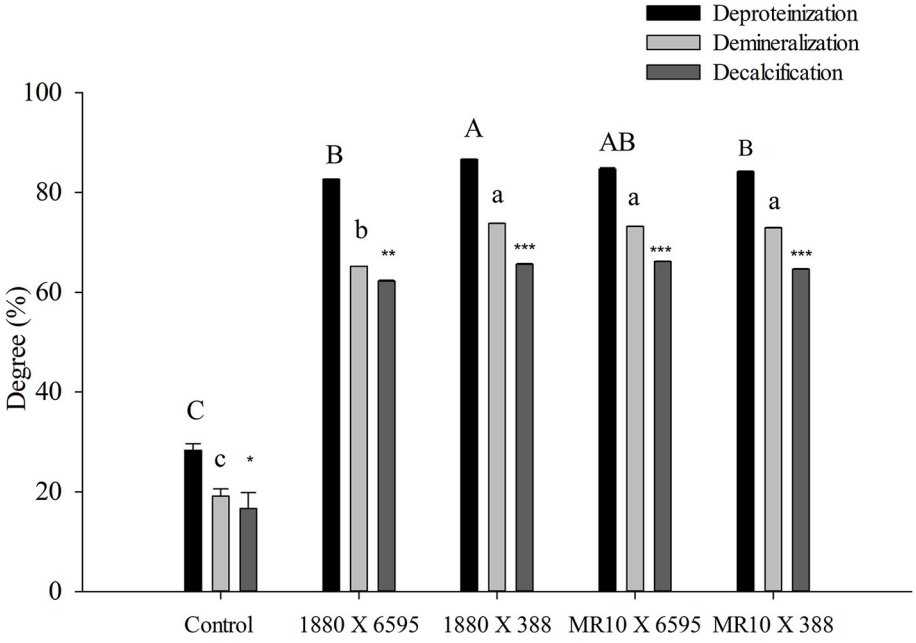

Fig. 3. Percentages of deproteinization, demineralization, and decalcification from SPW-SH obtained under co-culture fermentation conditions using different combinations of *Bacillus* spp. and *Lactobacillus* spp. as starter co-cultures. Data are shown as the mean ± SD of the experiments that were conducted in triplicate. For each measurement, different letters/symbols above the bars indicate significant differences at $p < 0.05$ analyzed using an ANOVA and Duncan's multiple range test.

in protease activity and pH observed during fermentation (Fig. 2) and verified the contributions of *Bacillus* spp. and *Lactobacillus* spp. to the isolation of biomolecules from SPW discussed in the previous section. The co-culture of *B. amyloliquefaciens* TISTR-1880 and *L. casei* TBRC-388 possessed the highest potency in isolating biomolecules from SPW under the studied successive fermentation conditions; hence, this was selected as the starter co-culture for further experiments. Earlier studies suggested that several factors influenced the efficiency of deproteinization and demineralization during fermentation of SPW including SPW concentration, species and quantity of inoculums, type of carbon source, initial medium pH, incubation time, temperature, and agitation rate (Andressa Caroline et al., 2017; J. S. Tan et al., 2020). Therefore, optimizing these fermentation parameters was a strategic approach to enhance the efficiency of protein and mineral recovery from SPW.

This study improved the successive co-culture fermentation process by exploring how the bacterial inoculum percentage impacted the recovery of proteins and minerals (especially calcium) from the SPW samples. As shown in Fig. 4, increasing the inoculum percentage of both *B. amyloliquefaciens* TISTR-1880 and *L. casei* TBRC-388 from 2% to 4% and 6% (v/v) enhanced the deproteinization, demineralization, and decalcification of SPW-SH. The degree of deproteinization observed after fermentation using 6% inoculum reached 93.13%, which was nearly 10% higher than achieved with 2% inoculum and 3.3-fold greater than the control without bacterial inoculation. The degrees of deproteinization obtained with 6% and 4% inoculum were similar but significantly higher degrees of demineralization and decalcification were achieved with 6% inoculum (~83% and ~80%, respectively) compared to 4% and 2% inoculum. The degrees of demineralization and decalcification after fermentation with 6% inoculum were 4.3 and 4.8-fold higher than the control, respectively. Thus, an inoculum percentage of 6% (v/v) was selected as the optimal inoculation condition to seed the fermentation of SPW in this study. The enhanced efficiencies in protein and mineral isolation observed with the increase in inoculum percentage were attributed to increasing protease activity and lactic acid production, resulting from a higher bacterial proliferation during fermentation (J. S. Tan et al., 2020).

The selected successive fermentation conditions employing a starter co-culture of *B. amyloliquefaciens* TISTR-1880 and *L. casei* TBRC-388, both with an inoculum density of around 9 log CFU/mL and inoculum of 6% (v/v), demonstrated greater potential in concomitantly extracting proteins and minerals from SPW compared to previously reported single-strain fermentation processes using either *Bacillus* spp. or *Lactobacillus* spp. as a starter. The maximal deproteinization and demineralization degrees of SPW obtained with a *Bacillus* fermentation approach were 90% and 72%, respectively (Ghorbel-Bellaaj et al., 2012). Fermentation with *Lactobacillus* spp. alone often results in less efficient deproteinization and renders a highly acidic product. Ximenes et al. (2019) fermented SPW with a mixture of *L. futsaii* and *L. plantarum* strains and achieved deproteinization and demineralization percentages of 85% and 89%, respectively with the pH of the fermented extract at around 4.5 while Chakravarty and Edwards (2022) also conducted a successive co-culture fermentation of SPW with *Serratia marcescens* and *L. plantarum* over 6 days. The 5-day fermentation process developed in this study resulted in higher deproteinization efficiency (~93% vs ~87%) and lower demineralization efficiency (~83% vs ~90%), related to the shorter period of acidification by the *Lactobacillus* culture. Nevertheless, our highly efficient food-grade fermentation approach represents a cost-effective and environmentally friendly green innovation. The use of microbial strains that are recognized as safe and commercially available enhances the feasibility of scaling up this research for industrial food-related applications.

3.4. Recoveries of biomolecules from SPW by successive co-culture fermentation

The efficiencies of the selected fermentation conditions for isolating residual proteins and minerals from SPW-SH and SPW-SS were investigated and compared. The recoveries of biomolecules from our SPW sample hydrolysates were also explored, with results shown in Table 1. The analysis results of SPW residues collected after fermentation revealed that the fermentation process developed in this study effectively isolated proteins, minerals, and calcium from SPW compared with

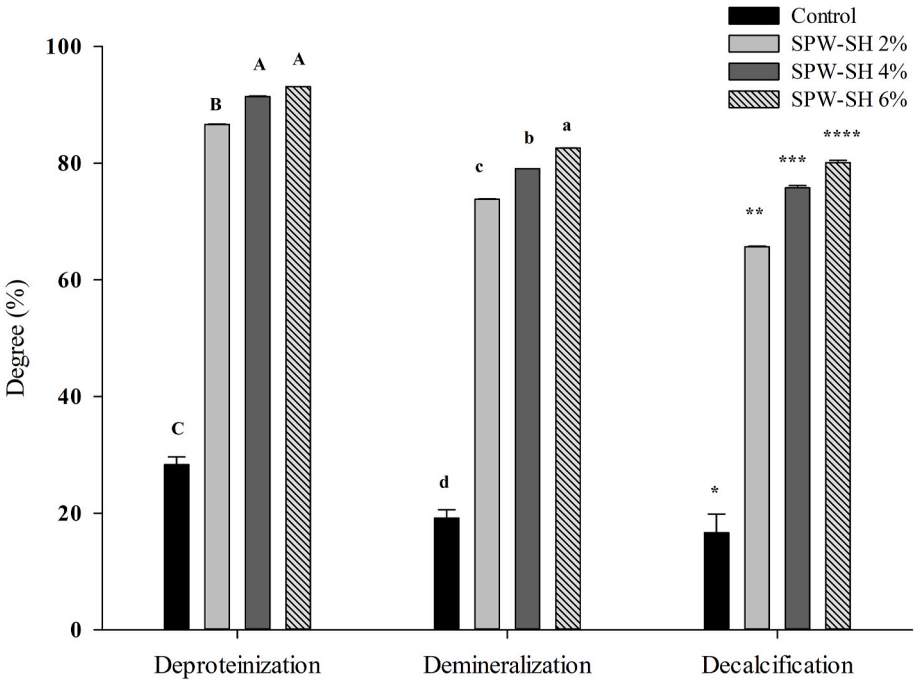

Fig. 4. Effects of inoculum size on the percentages of deproteinization, demineralization, and decalcification from SPW-SH obtained under co-culture fermentation conditions using *B. amyloliquefaciens* TISTR-1880 and *L. casei* TBRC-388 as a starter co-culture. For each measurement, different letters/symbols above the bars indicate significant differences at $p < 0.05$ analyzed using an ANOVA and Duncan's multiple range test.

Table 1
Percentages of deproteinization, demineralization, and decalcification from SPW samples achieved under selected co-culture fermentation conditions.

SPW residue	Degree (%)		
	Deproteinization	Demineralization	Decalcification
SPW-SH (control)	28.32 ± 1.34 [c]	19.13 ± 1.47 [d]	16.67 ± 3.18 [d]
Fermented SPW-SH	93.13 ± 0.04 [a]	82.58 ± 0.03 [a]	80.09 ± 0.39 [a]
SPW-SS (control)	31.17 ± 0.70 [c]	25.94 ± 0.12 [c]	35.8 ± 0.05 [c]
Fermented SPW-SS	84.36 ± 0.12 [b]	69.04 ± 0.48 [b]	73.12 ± 1.08 [b]

Data are shown as the mean ± SD of the experiments that were conducted in triplicate. Different superscripts within the same column indicate significant differences at $p < 0.05$ analyzed using an ANOVA and Duncan's multiple range test.

the corresponding non-fermented controls regardless of the specific part of the shrimp studied. The recoveries of proteins, minerals, and calcium from SPW-SH were 1.1–1.2-fold higher than the recoveries of these biomolecules from the SPW-SS sample, suggesting that the efficacy of the fermentation process varied depending on the type of raw material used. This finding concurred with previous studies demonstrating that fermentation of shrimp and crab shells under the same conditions resulted in different percentages of deproteinization and demineralization (Ghorbel-Bellaaj et al., 2012; Hajji et al., 2015). The DH values of proteins from SPW-SH and SPW-SS samples were 53.68% and 43.80%, respectively demonstrating that a higher extent of protein cleavage occurred in SPW-SH compared to SPW-SS. The DH measured in the fermented hydrolysates of our SPW samples was 2.4-fold higher than the DH measured in the raw materials (DH of fresh SPW-SH and SPW-SS were 18.22% and 11.44%, respectively). The increase in DH of protein substances observed after fermentation is associated with the proteolytic activity of the microorganisms involved, resulting in increased soluble peptide and free amino acid contents (Kittibunchakul et al., 2023). A key factor influencing the fermentation processes is the chemical composition of the raw materials, particularly proteins and carbohydrates that serve as sources of nitrogen and carbon for the microorganisms. These nutrients support microbial growth and also influence the production of metabolites such as organic acids and microbial enzymes (Neves et al., 2017). The SPW-SH contained higher protein than SPW-SS (22.5 and 18.9 g/100 g DW, respectively). Therefore, SPW-SH promoted higher growth and activity of the inoculated microorganisms. Ploydee and Chaiyanan (2014) suggested that shrimp heads had a suitable nutritional value for microbial fermentation, whereas the lower nutritional value of shrimp shells was more appropriate as a source of chitin. The yields of crude chitin obtained after fermenting SPW-SH and SPW-SS were 44% and 70% (w/w), respectively. However, the purification and characterization of the extracted chitin were beyond the scope of this study.

The chemical compositions of the hydrolysates derived from the fermentation of SPW-SH and SPW-SS were also different, with nutritive value per 100 mL of SPW-SH hydrolysate having a higher energy level compared to SPW-SS hydrolysate (13.16 kcal and 7.80 kcal, respectively). This finding concurred with the efficiency of the fermentation process in isolating proteins and minerals from the SPW samples as more effective for SPW-SH than for SPW-SS. For DW, protein content in the SPW-SH hydrolysate (70.03 g/100 g) was significantly higher than in the SPW-SS hydrolysate (59.07 g/100 g) while carbohydrate and ash contents were significantly higher in the SPW-SS hydrolysate (23.21 and 17.72 g/100 g, respectively) compared to the SPW-SH hydrolysate (14.19 and 14.99 g/100 g, respectively). The carbohydrates detected in the hydrolysates were attributed to the glucose added to the fermentation medium. Fat was not detected in the two hydrolysates, indicating that most of the energy in these hydrolysates emanated from their protein and carbohydrate contents. Our results concurred with other studies reporting that liquid hydrolysates from crustacean waste fermentation were mainly composed of protein and ash, with trace amounts of lipid (Castro et al., 2018; Hajji et al., 2015).

3.5. Antioxidant and enzyme inhibitory bioactivities of SPW hydrolysates

Antioxidant, anti-hypertension, anti-diabetes, and anti-obesity properties of the hydrolysates resulting from the fermentation of SPW-SH and SPW-SS broths were investigated in vitro, with results presented in Table 2.

The determination of ORAC, FRAP, and DPPH radical scavenging activities showed that non-fermented SPW-SH and SPW-SS broths that served as control samples exhibited antioxidant activities due to the presence of the carotenoid antioxidants, astaxanthin and β-carotene in shrimp (Prameela et al., 2017). The fermentation process significantly enhanced the antioxidant activities of SPW-SH and SPW-SS broths compared to their non-fermented counterparts (1.6–2.3-fold and 1.2–3.7-fold, respectively). This finding agreed with previous studies that reported increases in the antioxidant potential of crab shell (Hajji et al., 2015) and Jinga shrimp shell (Ghorbel-Bellaaj et al., 2012) extracts after fermentation. Earlier studies reported that the antioxidant activities of SPW-derived hydrolysates were attributed to the breakdown of proteins in the raw materials into short-chain biologically active peptides having 2–20 amino acids (Kim et al., 2016; Messina et al., 2021). The antioxidant power of hydrolyzed proteins increases with the reduction of peptide size, and protein hydrolysate fractions smaller than 3 kDa have higher antioxidant potential than fractions at higher molecular weight (Messina et al., 2021). The enhanced antioxidant activities observed in fermented crustacean products also resulted from the partial degradation of chitin into chito-oligosaccharides, which are effective electron donors and can stabilize free radicals (Ghorbel-Bellaaj et al., 2012; Hajji et al., 2015). The SPW-SH and SPW-SS hydrolysates produced in this study possessed superior antioxidant potential to a commercial protein hydrolysate prepared from sprats (ORAC and FRAP values of ~694 and ~13 μmol TE/$g_{protein}$) (Shekoohi et al., 2023). Comparing the antioxidant activities of our hydrolysate products, the SPW-SH hydrolysate exhibited 1.3-fold higher ORAC value and 1.8-fold higher FRAP value than the SPW-SS hydrolysate. By contrast, the SPW-SS hydrolysate exhibited 1.2-fold higher DPPH value than the SPW-SH hydrolysate. Our results concurred with Dayakar et al. (2022) who compared the antioxidant activities of carotenoprotein powders derived from shrimp heads and shrimp shells. They detected higher FRAP values in the powder from shrimp head and higher DPPH values in the powder from shrimp shells. Discrepancies in the antioxidant activity results were explained by differences in the reaction mechanism, sensitivity, and specificity of the assays (Apak et al., 2007)

An in vitro investigation of enzyme inhibitory activities revealed that the fermentation of SPW-SH and SPW-SS broths enhanced anti-hypertension, anti-diabetes, and anti-obesity properties compared to their non-fermented counterparts. Considering the inhibition of ACE, an enzyme associated with hypertension, SPW-SH and SPW-SS hydrolysates obtained after fermentation had 2.0 and 1.7-fold higher ACE inhibitory activities than the corresponding non-fermented control samples, respectively. This finding suggested that proteolysis could be used to extract anti-hypertensive substances such as certain types of bioactive peptides from SPW, concurring with previous research (Kim et al., 2016). ACE inhibitory activities of SPW-SH and SPW-SS hydrolysates determined at a protein concentration of 0.05 mg/mL were 75% and 71%, respectively. The superior ACE inhibition observed in the hydrolysate from SPW-SH indicated a greater quantity and/or type of ACE inhibitors produced during fermentation using this substrate. Due to the high ACE inhibitory activities, the half-maximal inhibitory concentration (IC_{50}) of our SPW hydrolysates on this enzyme was determined at <0.05 $mg_{protein}$/mL, which was better than the optimal values earlier reported for enzymatically-produced hydrolysates from marine-processing wastes such as red snow crab shell (IC_{50} ~0.4 $mg_{protein}$/mL) (Yoon et al., 2013) and inedible parts of northern shrimp (IC_{50} ~0.06 $mg_{protein}$/mL) (Kim et al., 2016).

Table 2
Antioxidant and enzyme inhibitory activities of SPW hydrolysates obtained under selected co-culture fermentation conditions.

SPW hydrolysate	Antioxidant activities (μmol TE/$g_{protein}$)			Enzyme inhibition (%)			
	ORAC	FRAP	DPPH	ACE [a]	α-Amylase [b]	α-Glucosidase [b]	Lipase [c]
SPW-SH (control)	940.95 ± 145.33 [b]	90.07 ± 0.90 [b]	0.03 ± 0.00 [d]	38.06 ± 2.11 [c]	ND	24.61 ± 1.95 [a]	7.50 ± 0.57 [c]
Fermented SPW-SH	1471.00 ± 227.28 [a]	145.91 ± 1.72 [a]	0.08 ± 0.00 [b]	74.73 ± 0.83 [a]	75.88 ± 1.79 [a]	ND	58.31 ± 1.41 [b]
SPW-SS (control)	312.34 ± 41.40 [c]	23.58 ± 0.25 [d]	0.06 ± 0.01 [c]	40.66 ± 3.19 [c]	ND	16.50 ± 1.71 [b]	5.46 ± 0.77 [c]
Fermented SPW-SS	1158.66 ± 107.24 [b]	80.28 ± 1.19 [c]	0.09 ± 0.02 [a]	70.80 ± 1.05 [b]	57.27 ± 2.23 [b]	ND	74.48 ± 4.13 [a]

Data are shown as the mean ± SD of the experiments that were conducted at least in triplicate. Different superscripts within the same column indicate significant differences at $p < 0.05$ analyzed using an ANOVA and Duncan's multiple range test.
[a] Protein concentration of all tested samples was 0.05 mg/mL.
[b] Protein concentration of all tested samples was 0.50 mg/mL.
[c] Protein concentration of all tested samples was 2.00 mg/mL; ND: not detected.

Both SPW-SH and SPW-SS hydrolysates showed promise to reduce the risk of diabetes by retarding the activity of α-amylase, a carbohydrate-hydrolyzing enzyme. This inhibitory effect was observed despite the absence of α-amylase inhibition in the control samples at the protein concentration examined. A similar finding on enhanced α-amylase inhibition after enzymatic treatment of shrimp shell was noted by Yuan et al. (2018). They suggested that this enhancement was linked to the liberation of small peptides (<4 kDa) with α-amylase inhibitory activity through the process of protein hydrolysis. At a protein concentration of 0.50 mg/mL, α-amylase inhibitory activities of SPW-SH and SPW-SS hydrolysates were 76% and 57%, respectively. The significantly higher α-amylase inhibition detected in SPW-SH hydrolysate was attributed to the smaller size and amino acid composition of their peptides (Henriques et al., 2021). The IC_{50} of the SPW hydrolysates obtained in this study on α-amylase was <0.50 $mg_{protein}$/mL, and better than some protein hydrolysates previously prepared using commercial proteases such as hydrolysates from blue whiting head (IC_{50} ~4.0 $mg_{protein}$/mL) (Henriques et al., 2021) and yellow field pea (IC_{50} < 2.9 $mg_{protein}$/mL) (Awosika and Aluko, 2019). Notably, no α-glucosidase inhibitory activities were observed in the SPW-SH and SPW-SS hydrolysates at the tested protein concentration (0.50 mg/mL) although mild α-glucosidase inhibition was detected in the control samples. This finding was likely attributed to the reduction in the effective peptide fragments that possessed α-glucosidase inhibitory activity following prolonged proteolysis during fermentation and consistent with the results of Zhang et al. (2023). Previous studies reported a dose-dependent effect of α-glucosidase inhibitory peptides present in protein hydrolysates (Awosika and Aluko, 2019; Henriques et al., 2021); thus, increasing the concentration of the tested hydrolysates might enable the detection of α-glucosidase inhibition.

For inhibitory activity against the lipid-hydrolyzing enzyme lipase, the SPW-SH and SPW-SS hydrolysates resulting from fermentation exhibited 7.8 and 13.6-fold better lipase inhibitions than their corresponding control samples, respectively. The considerable increase in lipase inhibitory activity observed in the SPW hydrolysates was related to the release and recovery of bioactive peptides and carotenoid pigments through the hydrolysis of SPW (Dayakar et al., 2022; Du et al., 2018). Lipase inhibitory activities of SPW-SH and SPW-SS hydrolysates measured at a protein concentration of 2.0 $mg_{protein}$/mL were 74% and 58%, respectively. Considering the inhibitory activities, the IC_{50} of these SPW hydrolysates on lipase was determined to be < 2.0 $mg_{protein}$/mL. Information on lipase inhibition by marine protein hydrolysates is limited but a recent study reported the IC_{50} values of protein hydrolysates derived from some fish species ranging from >15.0 $mg_{protein}$/mL to 3.3 $mg_{protein}$/mL (Tian et al., 2022). Our study opens the possibility of using SPW as a potent source of lipase inhibitors to alleviate the condition of obesity. The higher inhibitory activity of our SPW-SS hydrolysate compared to SPW-SH hydrolysate was attributed to astaxanthin, which is a potent lipase inhibitor and is more concentrated in shrimp shells than in shrimp heads (Du et al., 2018; Hu et al., 2019).

4. Conclusions

This study investigated the proteolytic and acid-producing activities of various food-grade *Bacillus* and *Lactobacillus* strains during SPW fermentation. Compared with the other bacterial combinations studied, *B. amyloliquefaciens* TISTR-1880 and *L. casei* TBRC-388 were identified as the most effective starter co-culture for efficient recovery of proteins and minerals from SPW samples using an optimized successive co-culture fermentation process. The resulting hydrolysates obtained after SPW fermentation were rich in proteins and exhibited antioxidant activities. They also possessed anti-hypertension, anti-diabetes, and anti-obesity properties through inhibition of disease-associated enzymes. The SPW hydrolysates produced in this study showed high potential for use as functional ingredients in food and nutraceutical products, following intensive safety assessments. However, a key limitation of the study is the absence of detailed peptide characterization. Future research should focus on identifying and characterizing the specific peptides responsible for the observed bioactivities, which will enhance scientific understanding and broaden the potential applications of SPW hydrolysates. Furthermore, additional studies are required to determine the IC_{50} values of SPW hydrolysates against disease-associated enzymes and to validate the in vitro findings through experiments involving cell lines or animal models. The knowledge gained from this study can be used to encourage prospective valorization of industrial SPW as an inexpensive source of functional biomolecules for food-related applications using a fermentation approach. This will increase the commercial value of SPW and reduce the environmental impact. This approach aligns with global efforts toward sustainable development and circular economy principles. The developed successive co-culture fermentation process could also be adapted for use with other crustacean-processing wastes.

CRediT authorship contribution statement

Varongsiri Kemsawasd: Methodology, Investigation, Data curation. **Weeraya Karnpanit:** Data curation, Formal analysis, Writing – original draft. **Sirinapa Thangsiri:** Investigation. **Pairote Wongputtisin:** Methodology. **Apinun Kanpiengjai:** Methodology. **Chartchai Khanongnuch:** Methodology. **Uthaiwan Suttisansanee:** Methodology. **Chalat Santivarangkna:** Methodology. **Suwapat Kittibunchakul:** Conceptualization, Methodology, Investigation, Data curation, Project administration, Writing – original draft, Writing – review & editing.

Declaration of competing interest

There are no conflict of interest among all authors.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crfs.2024.100850.

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