

Extraction and Characterization of Chitin, Chitosan, and Protein Hydrolysate From the Invasive Pacific Blue Crab, *Portunus Segnis* (Forskål, 1775) Having Potential Biological Activities

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Abstract

The diversity of marine biomasses is a set of exploitable and renewable resources with application in several sectors. In this context, a co-culture based on three protease-producing bacterial isolates namely; *Aeribacillus pallidus* VP3, *Lysinibacillus fusiformis* C250R, and *Anoxybacillus kamchatkensis* M1V strains, was carried out in a medium based on the blue swimming crab *Portunus segnis* bio-waste. Proteases production was optimized using a central composite design (CCD). The highest level of proteases production obtained was 8,809 U/mL in a medium comprising 75 g/L of *Portunus segnis* by-product powder (P_{spp}). The biological value of P_{spp} and its obtained derivatives were evidenced via accredited protocols. The recovered protein hydrolysate (P_{Hyd}) was found to be active towards radical scavenging power, and against angiotensin I-converting enzyme (ACE). The blue crab chitin (BC) extraction efficiency was achieved with a yield of 32%. Afterward, chitosan was prepared through chitin *N*-deacetylation with a yield of 52%, leading to an acetylation degree (AD) of 19% and solubility of 90%. In addition, chitosan is found to be active against the growth of all pathogenic bacteria tested.

Highlights

- ▶ A co-culture of 3 protease-producing bacterial was carried on *Portunus segnis* by-products.
- ▶ The central composite design was used to optimize proteases production.
- ▶ The production of chitin and chitosan using eco-friendly methods was successful.
- ▶ The biological potentials for the recovered protein hydrolysate were very promising.
- ▶ The co-culture has proven its potential for biotechnological cleaner application.

1. Introduction

Crustaceans constitute the second most represented taxon of non-native species in the Mediterranean Sea (Zenetos et al., 2012). Indeed, among the 163 exotic marine species that have been reported in Tunisia, 24% were represented by the crustacean group (Dailianis et al., 2016). Such invasion occurred in 1869, since the creation of the Suez Canal where several Indo-Pacific marine species, denominated as Lessepsian, relocated from the Red Sea to the Mediterranean Sea (Galil and Zenetos, 2002), contributing to the modification of regional biodiversity.

The blue swimming crab, *Portunus segnis*, earlier known as *Portunus pelagicus* is one of the first Lessepsian invasive species that was registered in Egypt eventual the opening of the Suez Canal (Fox, 1924). The *Portunus segnis* frequents sandy-muddy and sandy territories till to 50 m deep, including areas next to reefs, mangroves, seagrass, and seaweed beds. In Tunisia, the first reported specimens were collected in coastal territories of the Gulf of Gabes during October 2014 (Bejaoui et al., 2017). Considering the problems generated by this newly introduced species to fisherman and the reluctance of local

consumer towards its consumption, the solution was to search for new markets for its export and/or to develop innovative processing including bio-wastes transformation (Tarhouni et al., 2019; Bouzgarrou et al., 2020; Ghedifa et al., 2021). In fact, the activity of valuing seafood sorting by-products is an activity to be developed in the coming years, given the ever-increasing demand for these products in several countries. Recently, the environmental concern has prompted manufacturers to consider the generated by-products by any transformation process (Besbes et al., 2017). Then, it has become crucial to find ways to promote these bio-wastes while integrating the concept of sustainable development (Gómez-Ríos et al., 2017; Hui et al., 2020; Uranga et al., 2020). Owing their high protein and polysaccharide contents, marine by-products offer numerous technological possibilities depending on the treatment to which they are subjected. Among modern techniques for upgrading bioactive proteins, peptides, chitin, and chitosan, the use of proteases has met a considerable success (Mechri et al., 2020a; Mechri et al., 2020b). This process allows the healthier solubilization of proteins in the form of protein and peptide hydrolysates. Indeed, marine by-products could be valued by transformation into homogeneous and fine flour which is mainly intended for biotechnological applications as proteases production (Jabeur et al., 2020; Mechri et al., 2019a; Mechri et al., 2019b). Again, the recovery of chitin requires recourse to strong acids and bases, which remains a double-edged sword because, although it allows the recovery of pure chitin, it can cause depolymerization of the chitin and seriously pollutes the environment.

The workflow of seafood processing is relatively easy to master. It results in the production of products having functional and nutritional properties that are particularly advantageous for the food, pharmaceutical, and cosmetic industries. Recent studies have demonstrated the anti-enzymatic and antioxidant properties of P_{Hyd} from crustacean's bio-wastes (Mechri et al., 2019b; Mechri et al., 2020b). Several research works focused on the identification and characterization of potentially active peptides, which could serve to develop functional foods for the prevention of several pathologies (Giordano et al., 2018; Jemil et al., 2014). In the same way, the proteolytic hydrolysis of crustacean's bio-wastes by proteases has been used to recover chitin. For example, Alcalase® from Novozymes Biopharma DK A/S (Bagsvaerd, Denmark) and serine alkaline protease (SAPN) from *Melghiribacillus thermohalophilus* Nari2A^T were proposed for the recovery of chitin from *Portunus segnis* and *Metapenaeus monoceros*, respectively (Mechri et al., 2019b; Mechri et al., 2020b). A comprehensive overview of the recent literature shows that several studies have been concentrated on an in-depth investigation of the bioactive compounds derived from *Portunus segnis* blue swimming crab products, using enzymatic and chemical biotechnological procedures (Hamdi et al., 2020; Hamdi et al., 2019). Previously, the production of bioactive compounds using biological tools was efficient with the co-culture of three bacterial strains on a medium based on peckled shrimp by-product *Metapenaeus monoceros* (Jabeur et al. 2020) in comparison to the use of just one bacterial stain in the same medium (Jabeur et al., 2020; Mechri et al., 2019a; Mechri et al., 2019b). In this context, this investigation was carried out to a clean valuing the invasive P_{spp} . A statistical approach using Box-Wilson CCD was considered to evaluate the effectiveness of a biological process using a cocktail of protease-producing strains to study some biological properties of the obtained P_{Hyd} and subsequently to recover chitin and chitosan. Besides, the P_{spp} and its obtained derivatives were well characterized by exhaustive accredited techniques, standards, and norms.

2. Materials And Methods

2.1. Materials

Commercial chitin and chitosan were from P-Biomedical, France. The commercial blue crab chitosan (BCC_{Chem}) was used again for comparison. The casein used to assess protease activity was from Merck (Darmstadt, Germany). Exotic blue crab specimens were purchased in fresh conditions from the local fishery souk in Sfax (Tunisia). Blue crab samples were washed, boiled, and shelled to peel the flesh from the crab shell. The latter was dried in the sun for 3 days and then milled as recently explained (Jabeur et al., 2020). The P_{spp} powder was used as an ingredient in the formulation of an economic culture media for proteases secretion. The entire other reagents and substrates were of the analytical mark.

2.2. Biochemical analyses

P_{spp}, BC, BCC_{Bio}, BCC_{Chem}, and P_{Hyd} characterizations were carried out in the Accredited Laboratory of Blue Biotechnology and Aquatic Bioproducts (B3Aqua) at the Institut National des Sciences et Technologies de la Mer (INSTM) following the requirements of the International standard ISO/IEC 17025 version 2017, and with the Tunisian Accreditation Council (TUNAC) applications rules with mutual recognition agreements with European Laboratory Accreditation Cooperation (ILAC) and European Cooperation for Accreditation (EA) for the accreditation of laboratories for analysis, testing, and calibration.

2.2.1. Protein content (Hartree method and MO/06)

The protein content was determined according to Hartree method (Hartree, 1972) using bovine serum albumin (BSA, Sigma-Aldrich, Steinheim, Germany) as a standard solution. This accredited method was adapted to microtitration where an amount of the product (0.45 g) was homogenized in 9 mL of distilled water using an Ultra-Turrax (IKA, Deutschland) and placing the tube in an ice bath. Following homogenization 250 μ L of the sample was taken for subsequent protein analysis as follows: 225 μ L of Solution A (2 g of KNaC₄H₄O \cdot 4H₂O + 100 g of Na₂CO₃ + 500 mL of 1 N NaOH) was added to 250 μ L of the sample, standard BSA and blank and placed in a water bath at 50°C for 10 min. Then 25 μ L of solution B (2 g of KNaC₄H₄O₆ \cdot 4H₂O + 1 g CuSO₄ \cdot 5H₂O + 90 mL H₂O + 10 mL 1N NaOH) are added, the mixture is then placed in the dark for 10 min at room temperature (23°C \pm 2°C). 750 μ L of solution C (1 mL of Folin reagent + 4 mL of ultrapure water) were added, the mixture was placed in a water bath for 10 min at 50°C. Finally, a measurement of the absorbance at 650 nm was carried out using a μ QuantTM microplate spectrophotometer plate well reader (Bio-Tek Instruments, Inc., USA) containing 500 μ L of final solutions.

2.2.2. Amino-acids (MO/09)

The determination of total amino acids is done after sample acid hydrolysis for 24 h at 110°C. Pre-column derivatisation is performed using O-Phtaldialdehyde (OPA) and 9-Fluorenylmethoxycarbonyl

chloride (Fmoc). Amino-acids contents were ascertained by chromatographic analysis using a HPLC system (Infinity 1260, Agilent, USA) equipped with a DAD detector ($\lambda = 338 \text{ nm}$; $\lambda = 208 \text{ nm}$). The amino acid separation was performed using a Zorbax eclipse AAA (4.6 mm ID and 250 mm bed length, Agilent, USA) at a temperature of 40°C. A Binary gradient mobile phase was used: valve (A): sodium phosphate monobasic NaH_2PO_4 solution (40 mM) adjusted to pH 7.8 with NaOH solution, valve (B): solution consisting of a mixture of acetonitrile/methanol/water (45/45/10) (v/v/v) according to ISO 13903: 2005 and ISO 17180-2013.

2.2.3. Crude fat (Folch method, MO/02)

The lipid content was performed following extraction of crude fat from 1 g of each sample according to the method characterized previously (Khemir et al., 2020) using a chloroform: methanol (2:1 v/v) extraction solution containing 0.01% butylated hydroxytoluene (BHT) as an antioxidant. After centrifugation (4,000×g, 4°C, 20 min), the lower phase (organic phase) containing the lipids was carefully collected with a Pasteur pipette and the solvent was evaporated to dryness. Fats were determined gravimetrically (Folch et al., 1957).

2.2.4. Fatty acids (ISO 12966-4 2015 and ISO 12966-2 2017, MO/03)

Lipid extracts were trans-esterified according to the standard ISO 12966-2:2017 procedure. Thus, the esterification of the crude fat extract was carried out in 2% sulfuric acid in absolute methanol (Carlo Erba, Val-de-Reuil, France) and the mixture was incubated for 12 h at 50°C. 1 mL of water was added and hexane (2 mL) (Sigma-Aldrich, Steinheim, Germany) was used for extraction to separate the layers. The resulting methyl esters were analysed using an Agilent Gas chromatograph system 6890N (Agilent Technologies, SC, USA), equipped with a flame ionization detector (FID), a splitless injector and a polar INNOWax fused silica capillary column (0.25 mm I.D × 30 m bed length × 0.25 μm film thickness). The temperature of the injector and the detector were 220°C and 275°C, respectively. Helium was used as a carrier gas with a 1.5 mL/min flow rate. Peaks were identified by comparing their retention times with those of a known mixture of standard fatty acids (PUFA N°3, Menhaden Oil, SUPELCO Sigma-Aldrich, Laramie, Wyoming USA). The results were expressed as percentages of the total fatty acids' methyl esters.

2.2.5. Carbohydrates (MO/11)

As described elsewhere, the carbohydrate quantity was determined (Brummer and Cui, 2005). For this, 0.5 g of the sample was weighed and added to 1 mL of ultra-pure water and homogenized using an ultra-Turrax (IKA, Deutschland), then centrifuged at 12,000 rpm/min for 15 min at 4°C. The supernatant is diluted 20 times with ultrapure. 2.5 mL of concentrated sulfuric acid were added after that, followed by 500 μL of 5% phenol. The absorbance was then measured at 490 nm using a LLG-UniSPEC 2 Spectrophotometer. The concentration of carbohydrates was determined using a calibration curve made by different known concentrations of glucose.

2.2.6. Determination of total volatile basic nitrogen (TVB-N) (MO/08)

TVB-N proportion was determined by flow injection analysis (FIA) as described formerly (Khemir et al., 2020). For this test, 1 g of each product was homogenised (DI-25, IKA, Germany) on ice in 2 mL ultrapure water for 1 min, 0.250 mL of 6% (v/v) perchloric acid was added and the extract was homogenized for a further 2 min. Homogenates were centrifuged at 12,000×*g* for 15 min and the supernatants was used to determine of total volatile basic nitrogen (TVB-N) using flow injection analysis (Ruiz-Capillas and Horner, 1999). A small volume (50 µL) of sample solution/standard is injected into a moving fluid (H₂O) and transported to the NaOH stream. The NaOH converts all ionic bases in the sample into volatile molecules (gas), which pass through the gas-permeable membrane and cause a change in the colour of the bromothymol blue (colour indicator). This colour change is detected by a spectrophotometric detector at a wavelength of 630 nm and recorded as a peak. The height of the peak is proportional to the concentration of TVB-N.

2.2.7. Moisture and crude ash (NFV04-401 MO/04 and NFV04-404 MO/05)

Correspondingly, the moisture and crude ash rates were ascertained in the guise of the NFV04-401 (MO/04) and NFV04-404 (MO/05) accredited internal standard methods. For both methods, the principle is based on weighing the test sample before and after drying. Moisture was determined by drying 1 g of each product in an oven (Memmert, Schwabach, Germany) at 104°C±3°C for 18 h. Crude ash content was determined by sample incineration for 6 h in a muffle furnace oven (Protherm, Ankara, Turkey) at 550°C.

2.2.8. Biogenic amines (ISO 19343:2017, MO/07)

The biogenic amines standards were purchased from Sigma Chemical Co. (St Louis, MO, USA). They were dissolved in deionized distilled water (DDW) with a 100 mg/100 mL concentration and used as the working solution. Biogenic amines determination was purchased by implying sample extraction with 100 mM HCl and derivatization with dansyl chloride (DCI) reagent. The samples were then filtered with a 0.45 µm PTFE-filter (Sartorius, Göttingen, Germany) and injected into the HPLC system (Knauer Smartline, Berlin, Germany). It consists of a quaternary pump (Knauer, model 1000) and a UV-Vis detector (Knauer, model 2000) set at 254 nm. The column was an Eurospher, 100-5 RP C18 (250 × 4.6 mm) and the mobile phase was composed by 350 mL of water and 650 mL of acetonitrile.

2.3. Growth conditions of proteases-producing bacterial strains

The pre-cultures were performed as described by Jabeur et al., (2020). Briefly, one colony of each bacterial strain agar culture was added to 100 mL of Luria-Bertani (LB) medium composed of (g/L): peptone 10, yeast extract 5, NaCl 5 at pH 7.4 in 500 mL Erlenmeyer flasks and incubated on a shaker incubator at 37°C for C250R strain and 45°C for VP3 and M1V strains for 24 h. Cultures in a liquid

medium containing only blue crab by-product powder as carbonaceous and nitrogenous substrate were established by simultaneously adding pre-cultures of strains VP3, C250R, and M1V. Experiments were carried out in 500 mL Erlenmeyer flasks to establish co-cultivation by an initial inoculum size (0.2) for each strain. In fact, VP3, C250R, and M1V pre-cultures were transferred simultaneously to 100 mL of medium and incubated at 45°C at 200 rpm for 24 h.

2.4. Protease activity assessment

Protease activity was measured as noticed elsewhere (Kembhavi et al., 1993). The culture medium's supernatant was used to measure protease activity after removing cellular debris by centrifugation at 10,000×*g* for 30 min. The reaction mixture consisted of 0.5 mL of diluted crude extract and 0.5 mL of 100 mM glycine-NaOH buffer (pH 10) containing casein at 10 g/L and incubated for 15 min at 70°C. To stop the hydrolysis reaction, 0.5 mL of (20%, *w/v*) trichloroacetic acid was added. Then, after standing for 15 min at room temperature (23°C±2°C), the mixture was centrifuged at 12,000×*g* for 15 min to eliminate the non-hydrolyzed casein. The acid-soluble material was assessed at 280 nm. One unit of protease activity was defined as the enzyme, yielding the equivalent of 1 μmole of tyrosine for every minute under the defined assay conditions.

2.5. Optimization of proteases production under co-culture

2.5.1. Influence of P_{spp} concentration

The experiments were realized in 500 mL Erlenmeyer flasks including 100 mL of liquid production medium containing various concentrations of P_{spp} (from 10 g/L to 100 g/L). The flasks were inoculated after sterilization and the proteases level was assayed.

2.5.2. Investigation of significant factors by CCD

A CCD of 36 experiments was used to ascertain the influence of four factors: temperature, the concentration of blue crab by-product powder, pH, and the medium's volume on proteases production at each of the five levels (Table 3). According to the preliminary tests, these factors were the most contributing ones on the activity of proteolytic bacteria.

2.6. Chitin and chitosan preparation

2.6.1. Blue crab chitin (BC) recovery

After CCD confirmation, the production of the protease was carried under the optimal medium. After centrifugation, the pellets were washed twice with distilled water, then filtered to eliminate the cell debris, and dried at 60°C for two days to excavate the BC. The later yield was estimated as BC derived according to the original wet amount of P_{spp} as reported elsewhere (Rao and Stevens, 2005). The infrared spectra of recovered BC was determined by FTIR then compared to the profile of commercial one as detailed previously (Zhu et al., 2018).

2.6.2. Blue crab chitosan (BCC_{Bio}) preparation

The switch of BC obtained through an ecofriendly biological procedure to BCC_{Bio} was done following the deacetylation procedure (Meramo-Hurtado et al., 2020). Briefly, the recovered BC was treated with 12.5 M caustic soda (NaOH) at a proportion of 1/10 (w/v) for 4 h at 140°C, to acquire chitosan entirely soluble in water under alkali conditions. Subsequent to filtration, the recovered residue was washed with distilled water till the neutral pH was attained, and the chitosan was kept in a dry heat incubator at 50°C for 12 h. The FTIR investigation of the extracted BCC was ascertained and compared to the commercial chitosan profile as detailed previously (Hamdi et al., 2018).

2.6.3. BCC physico-chemical characterization

2.6.3.1. Chromatographic conditions and analysis

Molecular weight of BCC_{Bio} was estimated by HPLC using Shodex sugar KS-802 polysaccharides (Showa Denko K.K., SDK, Kawasaki, Japan) analysis column (8 mm ID × 300 mm bed length) using H₂O as a mobile phase. Low molecular weight (1526.45 g/mol) of commercial chitosan BCC_{Chem}, with a 75% and 85% of DD, obtained from Sigma Aldrich (Hamburg, Germany) was used as standard. In fact, each sample was dissolved in a 1% acetic acid solution with continuous stirring to a final concentration of 50 mg/mL and sterilized by filtration through a 0.22 μm nylon membrane filter. The flow rate was 0.5 mL/min, the column temperature was 40°C and Shodex refractive index (RI) as a detector.

2.6.3.2. Determination of the degree of acetylation (DA)

The deacetylation degree (DD) was ascertained with the titration method as described elsewhere (Sarbon et al., 2015). In brief, 0.1 g of chitosan was assorted with 25 mL of 60 mM HCl and incubated at 23°C±2°C for 1 h. The solution was then diluted with 50 mL of distilled water and titrated with a 0.1 N NaOH solution until pH 8. The DD of the samples was calculated as given in the following formula:

$$DD (\%) = 161.16 \times (V_2 - V_1) \times N / W_1 \text{ (Eq. 1)}$$

Where 161.16 refer to the molar mass (g/mol) of chitosan monomer; (V₂-V₁) is the amount of base consumed (mL); N is the normality of the base; and W₁ is the mass (g) of sample after elimination of moisture. After that, the DA is deduced as follows:

$$DA (\%) = 100 - DD \text{ (Eq. 2)}$$

2.6.3.3. Solubility

This property was determined as previously mentioned (Fernandez-Kim, 2004). In fact, 0.1 g of chitosan is dissolved in 10 mL of 1% acetic acid, and incubated at 23°C±2°C for 30 min. After incubation, the solution is placed for 10 min at 100°C and centrifuged (8,000 rpm for 10 min). The recovered pellet is

dissolved in 25 mL of distilled water and re-centrifuged under the same conditions. Finally, the pellet is dehydrated in an oven at 50°C for 24 h. The solubility of BCC was determined according to this formula:

$$\text{Solubility (\%)} = (W_1 - W_2) / (W_2 - W_0) \times 100 \text{ (Eq. 3)}$$

Where W_1 is the mass (g) of the tube with the initial chitosan; W_2 is the mass (g) of the tube with the final chitosan and W_0 is the mass of the empty tube.

2.6.3.4. Water binding capacity (WBC)

The WBC was ascertained according to the earlier statement (Ocloo et al., 2011). Practically, 0.5 g of the prepared BCC was mixed for 1 min with 10 mL of distilled water and incubated for 30 min at 23°C±2°C. The solution was shaken for 5 s every 10 min. After that, the solution was centrifuged (3,500 rpm for 25 min), the supernatant was discarded and the pellet was weighed. The WBC is determined via the subsequent formula:

$$\text{WBC} = (m_f / m_0) \times 100 \text{ (Eq. 4)}$$

Where m_f is the water bound (g) and m_0 is the initial chitosan mass (g).

2.6.3.5. Fat binding capacity (FBC)

The FBC is determined as detailed previously (Knorr, 1982; Ocloo et al., 2011). Therefore, 0.5 g of chitosan was rigorously mixed with 10 mL of oil for 1 min, and then incubated for 30 min at 23°C±2°C with short shaking every 10 min. The solution was centrifuged (3,500 rpm for 25 min), the supernatant discarded, and the pellet weighed. To determine the FBC, the subsequent procedure was used:

$$\text{FBC} = (m_f / m_0) \times 100 \text{ (Eq. 5)}$$

Where m_f is the fat bound (g) and m_0 is the initial chitosan weight (g).

2.6.4. Antibacterial activity of BCC

2.6.4.1. Bacterial strains

The antibacterial activity of BCC has been evaluated against pathogenic bacterial strains: four Gram-positive: *Staphylococcus aureus* subsp. *aureus* ATCC® 6538P™; *Micrococcus luteus* LB 14110; *Bacillus cereus* ATCC® 14579™; *Micrococcus* sp. LB 14110 and four Gram-negative: *Listeria monocytogenes* ATCC® 19117™; *E. coli* ATCC® 10536™; *Pseudomonas aeruginosa* ATCC® 15442™; *Salmonella enterica* ATCC® 14028™ serotype Typhimurium.

2.6.4.2. Agar-well diffusion assay (AWDA)

The antibacterial activity of BCC_{BiO} against pathogenic bacterial strains was evaluated by the solid medium diffusion technique AWDA. Thus, the blue crab chitosan was dissolved in a 1% acetic acid

solution with continuous stirring at a final concentration of 50 mg/mL and sterilized by filtration through a 0.22 μm nylon membrane filter. Subsequently, a culture suspension of the indicator bacteria was plated on Mueller-Hinton agar wells (6 mm of diameter) perforated in the agar medium and inoculated with sterile Pasteur pipettes, then loaded with 100 μL of chitosan solution. The negative control consisted of 1% acetic acid, which was used to dissolve the chitosan sample. Ampicillin at a concentration of 25 $\mu\text{L}/\text{mL}$ was used as a positive control to determine the sensitivity of each bacterial strain. Petri dishes were stored for 2 h at 4°C, before being incubated for 24 h at 37°C. Antibacterial activity was assessed by measuring the inhibition zone (the clear area around the well), including the well diameter of 6 mm, relative to the bacterial strains tested. All tests were duplicated using the same method.

2.6.4.3. Minimum inhibitory concentration (MIC)

The MIC of chitosan was defined as the lowest sample concentrations that inhibited the visible growth of the microorganisms tested after overnight incubation. It was estimated in sterile 96-well microplates with a final volume of 100 μL in each microplate well. The BCC_{Bio} solution, previously dissolved in 1% acetic acid at a concentration of 50 mg/mL and sterilized by filtration through a 0.22 μm nylon membrane filter, has undergone a double serial dilution over the range of 0.012 mg/mL to 25 mg/mL, in Luria Bertani (LB) liquid medium. Each well of the microplate contained 50 μL of chitosan solution, 40 μL of medium and 10 μL of cell suspension. Bacteria incubated only in LB and 1% acetic acid was used as positive and negative controls, respectively. The plates were then incubated at 37°C for 24 h.

As an indicator of the viability of microorganisms, 25 μL of 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT) bromide (0.5 mg/mL), dissolved in sterile water were added to the wells and incubated at 37°C for 30 min. The solution in the well remained clear after incubation with MTT, if the bacterial growth were inhibited. The assessments of MIC values were duplicated.

2.7. Assessment of biological activities of the P_{Hyd}

A co-culture based on blue crab waste was carried out under the optimal conditions found by the methodology of the experimental designs and subjected in a centrifugation to remove the cellular debris. Subsequently, the supernatant was concentrated by rotavap followed by lyophilization to obtain a protein hydrolyzate, which will be subjected to a set of biological activity tests.

2.7.1. Antioxidant activities

The DPPH radicals scavenging assay was performed according to the formerly described method (Kirby and Schmidt, 1997), with minor modifications as previously explained (Mechri et al., 2020b). The percent of antiradical activity (ArA) was estimated as follows:

$$\text{ArA (\%)} = [(A_{570 \text{ nm}} \text{ of the control} - A_{570 \text{ nm}} \text{ of test sample}) / A_{570 \text{ nm}} \text{ of control}] \times 100 \text{ (Eq. 6)}$$

As previously described, the scavenging potential of the radical cation ABTS • + (SA) was assessed using ABTS as a substrate, (Re et al., 1999). The SA was presented through the formula:

$$SA (\%) = [(A_{715 \text{ nm}} \text{ of control} - A_{715 \text{ nm}} \text{ of test sample}) / A_{715 \text{ nm}} \text{ of control}] \times 100 \text{ (Eq. 7)}$$

2.7.2. Evaluation of angiotensin I-converting enzyme inhibitory activity (ACEI)

The ACEI was determined in the guise of Cushman & Cheung, (1971) by using hippuryl-L-histidyl-L-leucine at 6 g/L as substrate (Cushman and Cheung, 1971). The ACEI was calculated as following:

$$ACEI (\%) = 1 - [(A_{228 \text{ nm}} \text{ of the sample} - A_{228 \text{ nm}} \text{ of the sample blank}) / (A_{228 \text{ nm}} \text{ of control} - A_{228 \text{ nm}} \text{ of control blank})] \text{ (Eq. 8)}$$

2.8. Statistical analyses

The results obtained following the CCD were interpreted via SPSS statistical software (version 11.0.1. 2001, LEAD Technologies, Inc. United States) and the response surface was created under the Microsoft Excel program (version 2007, Microsoft Office, Inc., USA) using ANOVA analysis. Differences were considered significant at $p < 0.05$. The regression model was built based on the SPSS approach. The responses for each experiment represent the regular of the three independent tests.

3. Results And Discussion

3.1. Biochemical composition of P_{spp}

The study showed that P_{spp} is an interesting source of nutrients (Tables 1 and 2). It contained a high amount of ash, carbohydrates, and an appreciable quantity of protein, lipids, and TVB-N. The stated ash value indicates that the bio-waste is a good source of minerals, fairly as the quantity reported for some other crab shells (Cabrera-Barjas et al., 2020; Hamdi et al., 2020). In fact, the ash content represents the minerals preserved in P_{spp} , particularly the calcium carbonate, which is the main element of exoskeleton of crustacean's shells. A substantial body of literature highlights the involvement of carbohydrates, lipids and proteins compounds of crustaceans bio-wastes in the culture of microorganisms for enzymes production (Mechri et al., 2019b). In addition, available saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), amino-acids, plus Omega-3 (ω -3) were present in P_{spp} with moderate amounts suggests that the P_{spp} can be ranked as a prospective source of dietary supplement (Besbes et al., 2017). However it is worth noting that crustacean bio-waste undergoes significant seasonal change suggesting a deeper investigation on crab waste for an efficient utilization.

3.1.2. Proteases production under co-culture

An optimal protease activity (2,486 U/mL) was achieved with 90 g/L of P_{spp} . So, VP3, C250R, and M1V strains were able to acquire their needs for carbon, nitrogen, and energy sources directly from this bio-waste.

According to preliminary tests, the most influencing factors of the protease activity are: temperature, pH, concentration of P_{spp} , and the volume of the culture. Thus, we have proposed a CCD for their optimization (Table 3) and co-cultures were carried out at 45°C and with stirring at 200 rpm with an initial absorbance at 600 nm of about 0.2 for each strain, in 500 mL Erlenmeyer flasks for 24 h.

The analysis of this matrix shows that the proteases production depends on the different parameters, leading to a cumulative effect of the tested factors with a notable variation in activity distinguished among the 36 experiments (Table 4). The best protein production was obtained in run 26 resulting in 8,703 U/mL protease activity with an increase of 3.54-times than the initial activity. The most influencing factors were a temperature of 42°C, a pH equal to 7.3, a culture volume of 30 mL, and a concentration of P_{spp} at 75 g/L.

The established model is expected by the subsequent equation:

$$Y = -869003.28 + 39728.46 \times X1 + 707.23 \times X2 + 5097.37 \times X3 + 150.39 \times X4 - 474.64 \times X1 \times X1 - 3.97 \times X1 \times X2 - 14.72 \times X1 \times X3 + 13.88 \times X1 \times X4 - 3.99 \times X2 \times X2 + 3.96 \times X2 \times X3 + 0.69 \times X2 \times X4 - 286.45 \times X3 \times X3 - 26.02 \times X3 \times X4 - 9.98 \times X4 \times X4 \text{ (Eq. 9)}$$

Where Y, X1, X2, X3, and X4 represent the protease activity, the temperature, P_{spp} concentration, the pH, and the culture volume, correspondingly. According to ANOVA analysis, an *F*-value of 39.484 with a highly little probability value ($p < 001$) designates an elevated significance of the model. The adjacency of the experimental and the anticipated protease activity was justified as presented by the regression coefficient of ($R^2 = 0.97$) which indicates that exclusively 0.03% of the total dissimilarity could not be elucidated through the created model. The adjusted R Square (predicted R^2) of 0.9 proved the fine agreement involving the experimental and the expected results. This model takes into account the secondary effect of all factors over and above the second order interactions between the diverse factors. According to this model, the activity reaches its maximum (8,803 U/mL) at a concentration of P_{spp} of 75 g/L, in a volume medium of 30 mL with a pH of 7.3 at 42°C with an agitation of 200 rpm.

As the surface plot explains merely two continuous variables at a time, any additional variables are held at a stable rank. Indeed, a surface plot can comprise just two continuous variables although other factors are fixed at the level 0 coded value. The response surface is curved since the model includes quadratic terms that are statistically momentous. In our case, the utmost values of protease production are in the superior right corner of the plot, which correlates with high values of the culture volume (mL) and the concentration of P_{spp} (g/L) (Fig. 1A). The nethermost values of protease production area in the lower-left corner of the plot, corresponding to low values of volume (mL) and pH (Fig. 1B). The third predictor pH and P_{spp} concentration are displayed in the third plot (Fig. 1C).

3.2. BC characterization

Chitin is the major constituent of the cuticle of crabs and shrimps. It is closely related to proteins, minerals, and fats. This is why the low protein and mineral contents is one of the factors determining its

superior feature. The recovered BC yield (Fig. 2) is estimated to 32%. BC shows very low quantities (%) of proteins, lipids, and TVB-N, which proves its purity. However, BC minerals content remains high, which necessitates further studies to promote its demineralisation (Table 1). This demineralization is a common process that can be achieved by several methods as reported in (Borić et al., 2020). In this study, the author resorted to the use of a hybrid process composed of nitrogen-based plasma to ensure the deproteinization of shrimp shell wastes and demineralization using lactic acid for the extraction of chitin. This allowed the elimination of 90% of the proteins and the complete elimination of minerals. In addition, the research of (Al Shaqsi et al., 2020) was carried out in the aim of optimizing the demineralization process for the removal of mineral contents from *P. segnis* bio-waste and has shown that an optimal concentration of 2 M HCl was effective and sufficient to remove the maximum of calcium and phosphorus compound compared to the other concentrations tested.

FTIR spectroscopy, a powerful tool for studying the obtained chitin physicochemical structure, showed that both chitins displayed archetypal α -chitin structure with absorbance bands roughly 3270 cm^{-1} , 2921 cm^{-1} , 1622 cm^{-1} , 1399 cm^{-1} , 1396 cm^{-1} , 627 cm^{-1} , 619 cm^{-1} , and 583 cm^{-1} (Fig. 3). Particularly, the spectrum of BC, gave a characteristic $-\text{NH}_2$ band of 3270 cm^{-1} and a carbonyl group band of 1622 cm^{-1} . However, no discernable band was found at 1540 cm^{-1} , suggesting the presence of protein trace in the recovered chitin, and confirming biochemical analysis (Table 1). Such a result highlights the efficiency of the deproteinization by this co-fermentation.

3.3. Assessment of biological activities

3.3.1. BCC_{Bio} characterization

Chitin and chitosan are mainly characterized by their DD, which represent the number of acetyl group compared to non-acetyl group. The deacetylation process excessively removes acetyl groups from chitin to obtain chitosan (Sharma et al., 2020). The property of chitosan depends on the source and the recovery procedure, as well as the type of analytical procedures adopted (Sarbon et al., 2015; Vázquez et al., 2018).

The yield of the recovered BCC_{Bio} from BC is estimated to be 52% (Fig. 4). Such value was higher than that reported in other studies including the chitin retrieved from *Callinectes sapidus* (12.1%) (Kaya et al., 2016) and from *Penaeus kerathurus* (22.23%) (Hamdi et al., 2017). In this study, a very small amount of protein in BCC_{Bio} was noted (0.51 g/100 g DW). However, the chemical blue crab chitosan (BCC_{Chem}) had a higher protein level (Table 1), suggesting a better deproteinization using biological process.

The DD of BCC_{Bio} was found to be significantly high (81%) but within the range of results found in the literature (Hajji et al., 2015). Despite its huge availability, the use of chitin has been restricted by its intractability and insolubility. Thus, manipulation with chitosan, representing the deacetylated derivative of chitin, was proposed as a good alternative (Erdogan et al., 2017). Yet, the solubility of chitosan is principally affected by the elimination of the acetyl group from chitin (Sarbon et al., 2015).

In this essay, the standard compound (commercial chitosan) and BCC_{Bio} were detected by HPLC (Fig. 5). The retention time of commercial chitosan was about 12.029 min while that of BCC_{Bio} was 12.329 min. This suggests that our biopolymer is of low molecular weight on the order of 1526 g/mol. It is well known that the degree of deacetylation is one of the most important chemical characteristics, which could influence the performance of chitosan in many applications like food packaging, which relies on the antibacterial power of the chitosane, and it is tied to its molecular weight. In fact, the number of amino groups binding to C-2 on the chitosan backbones is large since a large amount of amino groups is able to increase the process of inhibiting the proliferation of pathogenic bacteria. As a result, native chitosan with a higher DD and low molecular weight has been reported to show a stronger antibacterial effect than a molecule with a lower DD.

In this work, chitosan was found almost wholly soluble in 1% acetic acid with solubility up to 90%, which is comparable to the yield of 90.04% obtained from blue crab chitin (Hamdi et al., 2017). Nevertheless, chitosan extracted from mud crabs possess inferior solubility of ~53% (Sarbon et al., 2015). In addition, chitosan extracted from blue crab *portunus segnis* by-products possess WBC and FBC around 164% and 355%, respectively, showing that it could absorb or bind fat and water. Comparatively, high WBC of 582.40% and 180% were previously reported for the shrimp and mud crab chitosans, respectively (Ocloo et al., 2011; Sarbon et al., 2015). However, the BCC_{Bio} showed a lower WBC than shrimp shell chitosan (748%). This can be explained by the various sources as well as the preparation process of the BC and BCC_{Bio}. Indeed, several studies have proven that the BCC_{Bio} extraction process has a dramatic effect on its WBC and FBC abilities. In fact, these properties could be highly affected once the deproteinization stage was executed prior to the demineralization stage (Fernandez-Kim, 2004).

The FTIR pattern of the BCC_{Bio} has a typical commercial chitosan structure with distinctive absorbance bands around 3245 cm⁻¹, 1627 cm⁻¹, 1404 cm⁻¹, 1152 cm⁻¹, 1023 cm⁻¹, 870 cm⁻¹, and 573 cm⁻¹. In truth, for both spectra (Fig. 6), the presence of a peak having a stretching wavelength at 1627 cm⁻¹ is attribute to the amide I band (C=O in the NH-COCH₃ group). This observation discloses an increase in the DD of the BCC_{Bio} (Erdogan and Kaya, 2016). The peak at 1404 cm⁻¹ designated the C-H bending vibrations of CH₂ as shown previously (Kumari et al., 2015). The small peak at about 2810 cm⁻¹ has been attributed to the CH₂ and CH₃ groups (Zhang et al., 2012). The band at 870 cm⁻¹ has been ascribed to the absorption peaks of β-(1, 4) glycosidic bond in BCC_{Bio}. Finally, the band at 3245 cm⁻¹ has been attributed to the stretching vibration of OH and NH (Ramasamy et al., 2014).

3.3.2. Antibacterial abilities of BCC_{Bio}

Chitosan is a biopolymer derived from deacetylation of chitin in crustacean cuticles which has powerful antimicrobial properties against a broad spectrum of bacteria, yeasts and molds (Gumienna and Góna, 2021). Thus, looking for new alternatives in food and beverage packaging, antimicrobial, and active packaging are at the forefront of current development for food packaging. One of the rarest biopolymers on the market with antibacterial properties is chitosan which is characterized by its biodegradability and

biocompatibility (Priyadarshi and Rhim, 2020). In fact, in the current study, four Gram-negative and four Gram-positive bacteria were used to investigate the antibacterial effectiveness of BCC_{Bio}.

3.3.2.1. Determination of inhibition zone diameter

Due to its biological properties, chitosan and its derivatives have been recognized as an antimicrobial biomaterial against a wide range of target organisms such as algae, bacteria, yeasts and fungi in several experiments involving *in vitro* and *in vivo* activities (Duran et al., 2016). The results of the agar diffusion method revealed that, as reported in several scientific studies concerning chitosans, BCC_{Bio} was effective against all bacterial strains tested with a variation of the inhibition zone diameter as shown in Table 5. In fact, diameters of 9 mm to 24 mm for Gram-positive bacteria and 10 mm to 17 mm for Gram-negative bacteria were reached at 50 mg/mL of BCC_{Bio}. Therefore, the inhibition values vary between 15 mm for *Salmonella enterica*, serotype Typhimurium and 20 mm for *Micrococcus luteus* and *Listeria monocytogenes* for the Gram-negative and Gram-positive strains, respectively, at 50 mg/mL of BCC_{Bio}.

3.3.2.2. MIC evaluation

The MIC of chitosan is defined as the lowest sample concentration that inhibits the visible growth of the microorganisms tested after overnight incubation. Indeed, blue crab chitosan was more effective against *M. luteus* with a MIC value of 1.565 mg/mL (Table 5). However, complete inactivation of all bacteria tested Gram-negative (*E. coli*, *Pseudomonas aeruginosa*, and *Salmonella enterica*, serotype Typhimurium) requires an amount of at least 3.12 mg/mL of BCC_{Bio}.

3.3.3. P_{Hyd} characterization (ISO 19343, 2017 (Fr))

The P_{Hyd} is an attractive source of nutrients including proteins, amino-acids, and a class of biogenic amines (Tables 1 and 2) of health benefits. Under this co-culture and due to the microbial breakdown of proteins, liberated amino-acids are formed rapidly. They can then be transformed into biogenic amines using appropriate enzymes. The biogenic amines are produced via enzymatic decarboxylation of amino-acid or transamination of aldehydes and ketones. Putrescine can be amassed with a unique-step decarboxylation pathway through ornithine decarboxylase. However, putrescine can be formed during agmatinase pathway, which without intermediary transforms agmatine to urea and putrescine, or by agmatine deiminase pathway which transforms arginine to agmatine through arginine decarboxylase (Arena and Manca de Nadra, 2001; Kalac and Krausová, 2005). Previously, histamine has been reported as a marker of the quality of histidine rich dark muscle fish (Prester, 2011). For that, the maximum acceptable level of histamine in fish products is ascertained by 100 mg/Kg and 50 mg/Kg in the EU and USA, respectively. In our case, the level of this amine (13.24 mg/Kg) is too much lower than this threshold. However, putrescine and cadaverine represent 67.6% of biogenic amines in P_{Hyd}. One of the benefits of these amines is the decrease of catabolism of histamine when it interacts by amine oxidases, accordingly promoting intestinal assimilation and preventing its detoxification. Additionally, they can play a vital function as quality and/or adequacy markers in certain foods. (Chaidoutis et al., 2019).

P_{Hyd} which contains great amounts of proteins, amino-acids, and biogenic amines exert health-beneficial effects. Indeed, previous examinations have been accomplished on the generation of biologically active amino-acids, peptides, proteins, and biogenic amines using microbial fermentation (Liu et al., 2020; Mechri et al., 2020a; Mechri et al., 2020b).

3.3.4. Ascertainment of antioxidant activities

Antioxidants have been frequently used in food industries to prevent spoilage and maintain nutritional value. They are also interesting to health professionals and biochemists since they can help the body protect itself from oxidative damage (Choi et al., 2002). DPPH and ABTS • + free radical scavenging activities are the most regularly used anti-radical activity tests (Liu et al., 2014). Actually, the anti-radical effect allows to prevent all diseases related with oxidative stress (Mohammadian et al., 2017). By this way, the antioxidant potential of the P_{spp} was appraised by the DPPH and ABTS • + free radical scavenging tests.

Regarding the free radical DPPH • + trapping activity, the activity reaches 33.52% at 0.23 $\mu\text{g}/\text{mL}$ (Fig. 7). However, toward the ABTS • +, the activity reached 84.87% at a concentration of 7.4 $\mu\text{g}/\text{mL}$, while the activity of BHT was of 26.91% and 40.88% for DPPH and ABTS respectively for the same concentration (Fig. 7). The obtained data are in agreement with preceding works where the hydrolysate resulting from the fermentation of *Anoxybacillus kamchatkensis*M1V in a medium containing only shrimp by-products showed a significant antiradical power, principally compared to ABTS cation radical whither the hydrolysate was as efficient as BHT at a concentration of 100 $\mu\text{g}/\text{mL}$ resulting in 95% radicals neutralization (Mechri et al., 2020b).

3.3.5. ACEI inhibiting potential

The ACEI has an essential role in blood pressure regulation via the kinin-kallikrein and renin-angiotensin systems. In the present study, the hydrolyzate displayed interesting activities compared to those obtained for captopril as a standard. For the same concentration of 0.23 $\mu\text{g}/\text{mL}$, the ACEI inhibition of the P_{Hyd} was highly considerable with 68% of inhibition (Fig. 7), whereas the activity of captopril was at 93.29%. This result is analogous to previous study where the shrimp by-products hydrolyzate obtained following the fermentation of *Anoxybacillus kamchatkensis* M1V recorded a stronger ACEI in comparison with the activity of captopril. The corresponding IC50s were 85.33 $\mu\text{g}/\text{mL}$ and 71.52 $\mu\text{g}/\text{mL}$ for the captopril and P_{Hyd} , in that order (Mechri et al., 2020b). Indeed, in vitro assessment of the inhibitory activity of ACE has shown that the hydrolyzate obtained from shrimp by-products can be used as crude in food complements or in pure form as latent pharmaceuticals for the control of blood pressure.

4. Conclusions

This present scenario is part of the development of fish by-products especially of crustacean's waste, to obtain products with high added value. In this research, the chemical components of the P_{spp} and its derivatives (protein, chitin, and chitosan) recovered in a biological way have been well analyzed by means

of accredited approaches. The highest level of proteases production obtained was 8,809 U/mL in a medium containing 75 g/L of P_{spp} as the unique carbon, nitrogen, and energy sources. The biological value of P_{spp} and its obtained derivatives were evidenced via accredited protocols approved by B3Aqua Lab. The extraction efficiency of the BC was achieved with a yield of 32%. Afterward, chitosan was prepared through chitin *N*-deacetylation with a yield of 52%, leading to an AD of 19% and solubility of 90%. Interestingly, BCC exhibited remarkable antibacterial activity. More interestingly, the recovered P_{Hyd} was found to be active towards radical scavenging power, and against ACE and containing interesting protein, polypeptide, soluble amino acids, and biogenic amines for health benefits. These data constitute a roadmap towards a circular and sustainable bio-economic strategy for clean transforming a recalcitrant waste to bio-based products. Further works, which are presently in progress in our laboratories, are still required to maximize the removal of proteins and minerals from P_{spp}. Complementary studies seem inevitable to evaluate these biological activities *in-vivo* and purify the peptides responsible for them.

Declarations

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Authors' Contributions

F.J. carried out the experiment. F.J. and S.M. wrote the manuscript with support from B.J., N.B. and S.S. Y.B.N., M.K., I.G., F.M., S.B., A.B., R.A, and M.J. aided in interpreting the results and worked on the manuscript. All authors discussed the results and commented on the manuscript.

Competing financial interests

The authors declare no competing financial interests.

Compliance with ethical standards

Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Consent to participate

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Tables

Table 1 Proximate composition of P_{spp}, BC, BCC_{Bio}, BCC_{Chem}, and P_{Hyd}.

Parameters		P _{spp}	BC	BCC _{Bio}	BCC _{Chem}	P _{Hyd}
Composition	Unit					
Moisture	%	9.57±0.40	10.79±0.45	11.31±0.51	4.77±0.21	ND
Ash	%	41.50±1.71	50.40±2.01	56.64±2.10	1.18±0.05	ND
Proteins	g/100 g	2.52±0.07	0.51±0.02	0.02±0.00	0.42±0.02	28.88±1.00
Carbohydrates	g/100 g	21.98±0.84	9.87±0.42	10.27±0.35	<LQ	<LQ
Lipids	g/100 g	0.57±0.04	0.30±0.01	0.21±0.01	1.26±0.03	<LQ
TVB-N	g/100 g	3.362±0.11	2.58±0.12	3.07±0.21	3.29±0.23	<LQ
SFA	g/100 g	0.23±0.01	0.13±0.00	0.12±0.00	0.88±0.06	<LQ
MUFA	g/100 g	0.23±0.00	0.12±0.00	0.07±0.00	0.19±0.00	<LQ
PUFA	g/100 g	0.04±0.00	0.01±0.00	<LQ	0.02±0.00	<LQ
ω-3	g/100 g	0.02±0.00	<LQ	<LQ	0.02±0.00	<LQ
ω-6	g/100 g	<LQ	0.06±0.00	<LQ	<LQ	<LQ
Amino-acids	g/100 g	11.02±0.41	6.4±0.26	11,07±0.45	25.69±1.03	33.18±1.32
Yield	%	-	32±1.18	52±1.94	-	-

LQ, Low quantity. ND, Not determined. P_{spp}, *Portunus segnis* by-product powder. BC, Blue crab chitin. BCC_{Bio}, Biological blue crab chitosan. BCC_{Chem}, Chemical blue crab chitosan. P_{Hyd}, Protein hydrolysate.

The values described in the table shows the average of three independent tests and the means of standard deviation (±SD) were reported.

Table 2 The amino-acids and biogenic amines compositions of P_{spp}, BC, BCC_{Bio}, BCC_{Chem}, and P_{Hyd}.

Parameters	P_{spp}	BC	BCC_{Bio}	BCC_{Chem}	P_{Hyd}
<i>Amino-acids (g/100g)</i>					
Aspartate	1.14±0.1	0.33±0.02	0.05±0.00	0.03±0.00	2.95±0.2
Glutamate	0.92±0.1	0.32±0.02	0.19±0.00	<LQ	5.08±0.4
Serine	0.46±0.02	0.14±0.00	0.06±0.00	<LQ	1.68±0.1
Asparagine	<LQ	<LQ	<LQ	<LQ	<LQ
Glutamine	<LQ	<LQ	<LQ	<LQ	<LQ
Histidine*	0.37±0.02	0.15±0.00	0.15±0.00	0.89±	0.89±0.06
Glycine	0.54±0.03	0.24±0.01	0.05±0.00	<LQ	2.93±0.1
Threonine*	0.30±0.02	0.09±0.00	0.03±0.00	0.16±0.00	1.31±0.1
Arginine*	3.51±0.2	4.09±0.2	8.51±0.4	16.47±0.8	3.90±0.2
Alanine	0.50±0.03	0.15±0.00	1.27±0.1	2.39±0.1	1.78±0.1
Tyrosine	0.18±0.00	0.06±0.00	<LQ	0.09±0.00	0.90±0.06
Valine*	0.42±0.02	0.10±0.00	0.05±0.00	<LQ	1.26±0.1
Methionine*	0.06±0.00	<LQ	0.07±0.00	0.14±0.00	0.75±
Tryptophane*	<LQ	<LQ	<LQ	<LQ	<LQ
Phenylalanine*	0.33±0.02	0.14±0.00	0.06±0.00	<LQ	1.16±0.1
Isoleucine*	0.21±0.01	0.07±0.00	0.07±0.00	<LQ	1.23±0.1
Leucine*	0.38±0.02	0.10±0.00	0.08±0.00	0.15±0.00	2.40±0.2
Lysine*	0.34±0.02	0.14±0.00	0.05±0.00	0.37±0.02	3.13±0.3
Hydroxyproline	0.65±0.05	0.65±0.04	0.80±0.04	5.55±0.4	0.70±0.05
Proline	0.71±0.05	0.44±0.02	0.81±0.04	0.30±0.02	1.13±0.1
<i>Biogenic amines (mg/Kg)</i>					
Histamine	ND	ND	ND	ND	13.24±0.08
2-phenylethylamine	ND	ND	ND	ND	55.65±0.24
Putrescine	ND	ND	ND	ND	330.94±2.1
Tyramine	ND	ND	ND	ND	14.24±0.09
Spermidine	ND	ND	ND	ND	31.66±0.2
Agmatine	ND	ND	ND	ND	35.52±0.21

Cadaverine	ND	ND	ND	ND	13.54±0.08
Spermine	ND	ND	ND	ND	<LQ

LQ, Low quantity. *, Essential amino-acids. ND, Not determined. P_{spp}, *Portunus segnis* by-product powder. BC, Blue crab chitin. BCC_{Bio}, Biological blue crab chitosan. BCC_{Chem}, Chemical blue crab chitosan. P_{Hyd}, Protein hydrolysate

The values described in the table shows the average of three independent tests and the means of standard deviation (±SD) were reported.

Table 3 Levels of the variables tested in the centered composite L36 design.

Code	Variables	Unit	Level -2	Level -1	Level 0	Level +1	Level +2
X1	Temperature	°C	37	39	41	43	45
X2	Blue crab by-products	g/L	45	60	75	95	105
X3	pH	-	4,5	6,5	8	9,5	11
X4	Volume	mL	10	20	30	40	50

Table 4 Antibacterial potential of blue crab chitosan (BCC_{Bio}).

Microorganisms	Positive control (mm) ^a	Diameter of inhibition zone (mm)	MIC (mg/mL)
Gram-negative			
<i>E. coli</i> ATCC® 10536™	20±0.7	10±0.4	3.12
<i>Lysteria monocytogenes</i> ATCC® 19117™	40±1.5	20±0.7	1.56
<i>Pseudomonas aeruginosa</i> ATCC® 15442™	18±0.7	14±0.5	3.12
<i>Salmonella enterica</i> , serotype Typhimurium ATCC® 14028™	25±0.9	15±0.5	3.12
Gram-positive			
<i>Staphylococcus aureus</i> ATCC® 6538P™	22±0.8	12±0.6	1.56
<i>Micrococcus luteus</i> LB 14110	14±0.5	15±0.5	1.56
<i>Bacillus cereus</i> ATCC® 14579™	30±1.1	20±0.7	6.25
<i>Micrococcus</i> sp. LB 14110	21±0.8	9±0.4	3.12

Well diameter: 6 mm; Acetic acid 0.1% was used as negative control; pH 5.5; Ampicillin was used as positive control.

The values described in the table shows the average of three independent tests and the means of standard deviation (\pm SD) were reported.

Table 5 Centered composite experiments plan with their experimental and predicted responses.

Run	X ₁	X ₂	X ₃	X ₄	Experimental activity (U/mL)	Predicted activity (U/mL)
1	-1	-1	-1	-1	3,939	3,371
2	-1	-1	-1	1	2,278	2,671
3	-1	-1	1	-1	2,692	2,342
4	-1	-1	1	1	89	81
5	-1	1	-1	-1	2,635	2,431
6	-1	1	-1	1	1,782	2,215
7	-1	1	1	-1	1,528	1,819
8	-1	1	1	1	26	42
9	1	-1	-1	-1	6,696	6,375
10	1	-1	-1	1	6,550	6,786
11	1	-1	1	-1	5,007	5,170
12	1	-1	1	1	4,937	4,020
13	1	1	-1	-1	3,191	4,879
14	1	1	-1	1	6,057	5,774
15	1	1	1	-1	4,907	4,090
16	1	1	1	1	3,567	3,424
17	-2	0	0	0	2,266	2,565
18	2	0	0	0	3,791	3,901
19	0	-2	0	0	4,282	4,729
20	0	2	0	0	5,585	4,611
21	0	0	-2	0	6,873	6,758
22	0	0	2	0	3,764	3,965
23	0	0	0	-2	5,059	4,985
24	0	0	0	2	2,555	3,550
25	0	0	0	0	8,445	8,262
26	0	0	0	0	8,703	8,262
27	0	0	0	0	8,300	8,262
28	0	0	0	0	8,200	8,262

29	0	0	0	0	8,237	8,262
30	0	0	0	0	8,037	8,262
31	0	0	0	0	8,500	8,262
32	0	0	0	0	8,202	8,262
33	0	0	0	0	8,114	8,262
34	0	0	0	0	8,318	8,262
35	0	0	0	0	8,475	8,262

Figures

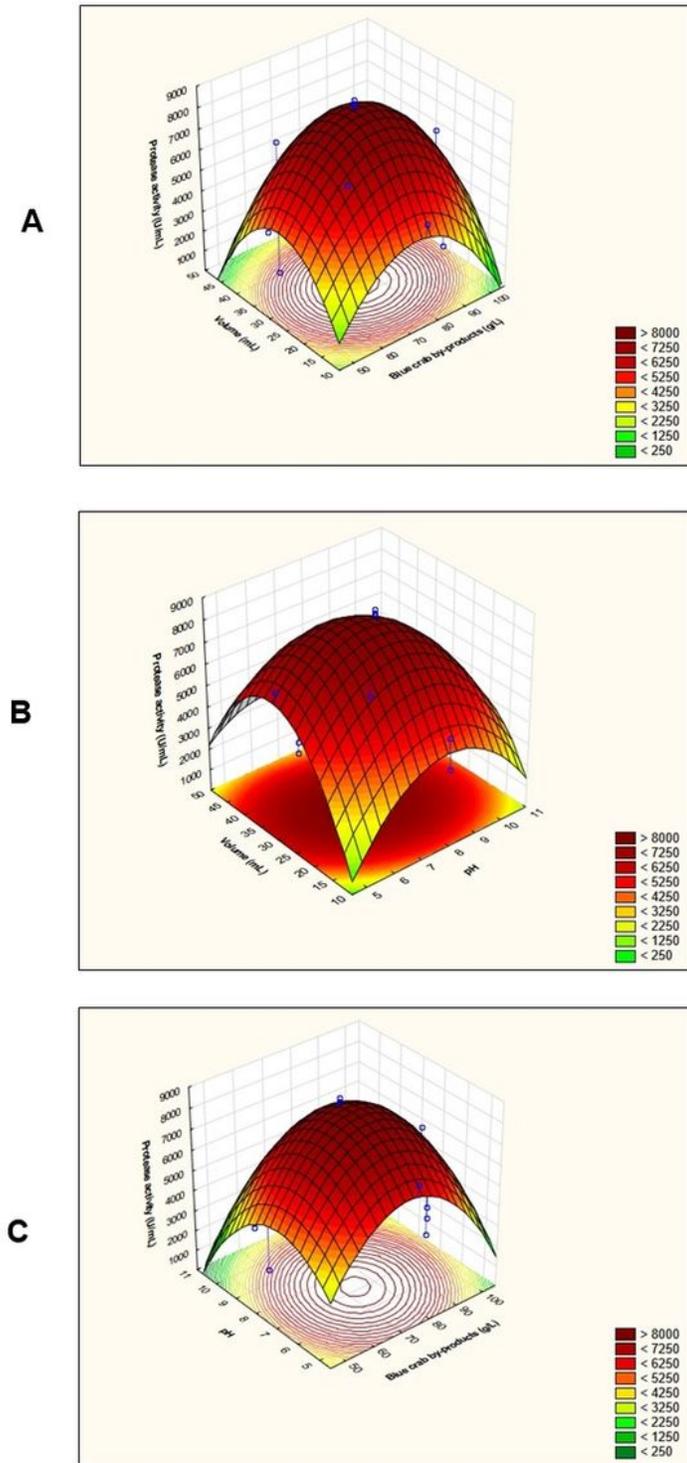


Figure 1

Response surface plot of proteases production showing the interactive effects of P_{spp} (g/L) and culture volume (mL) (A); pH and P_{spp} (g/L) (B); and pH and culture volume (mL) (C).

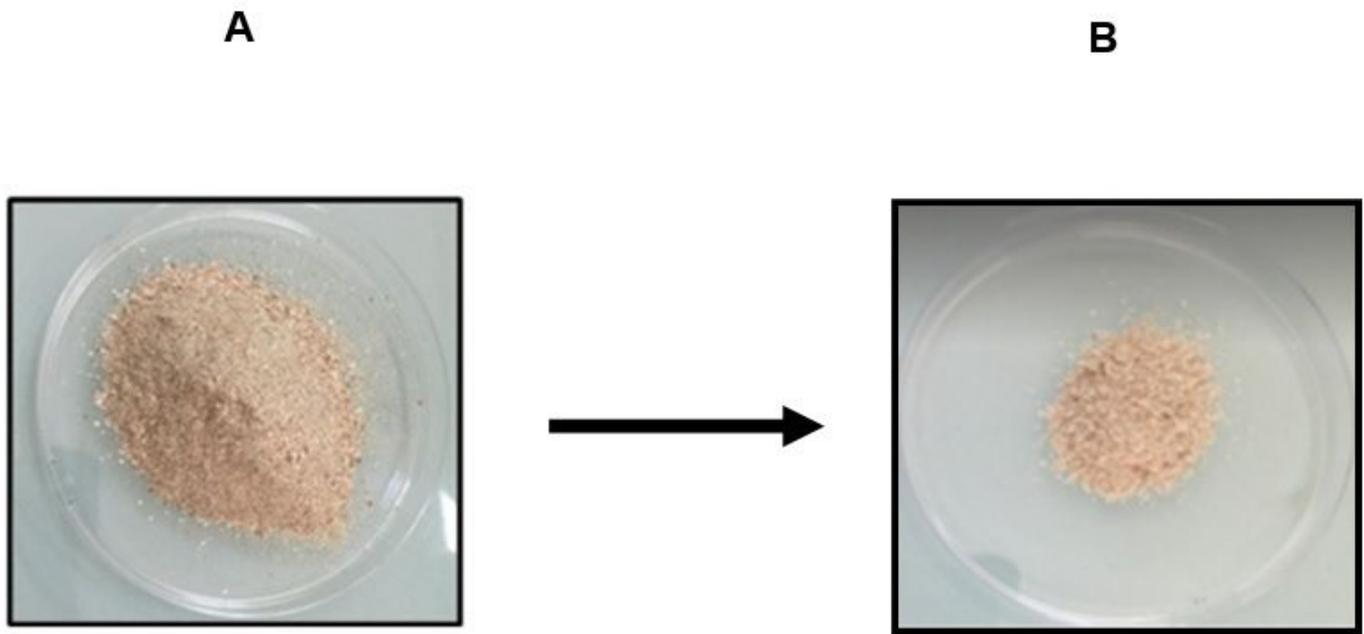


Figure 2

(A) P_{spp} ; (B) BC obtained by co-culture.

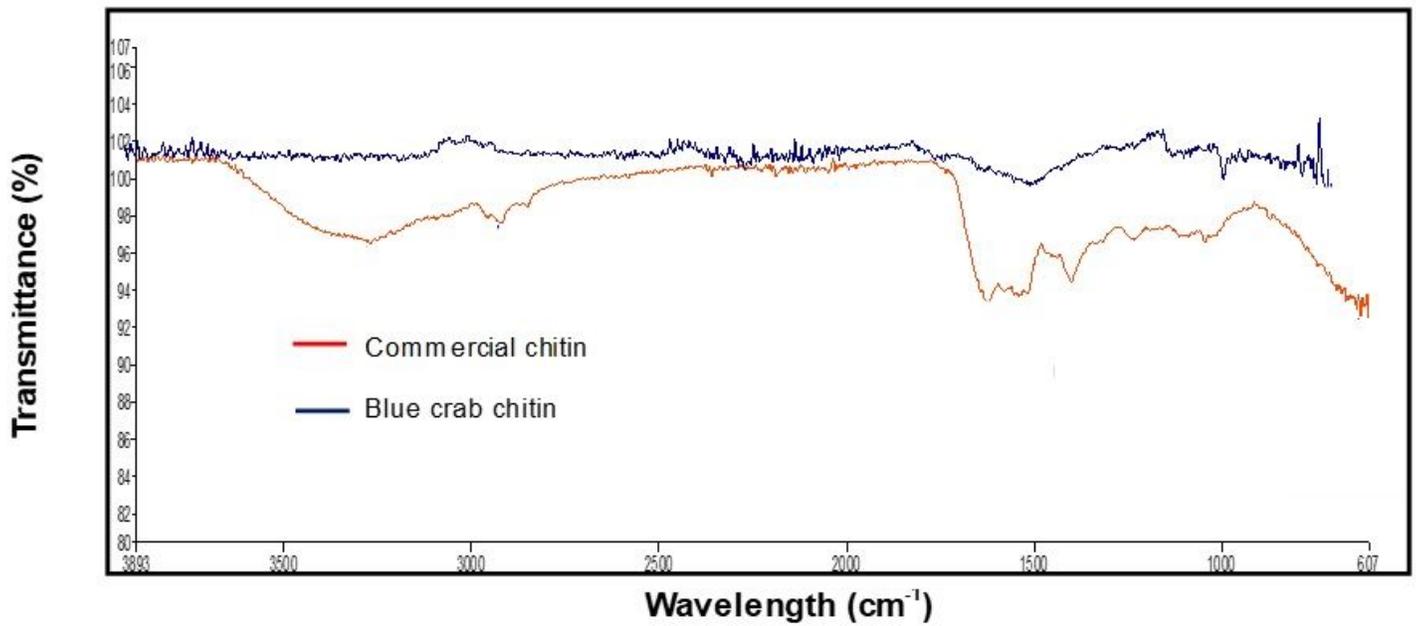


Figure 3

FTIR spectra of BC recovered compared to commercial chitin.

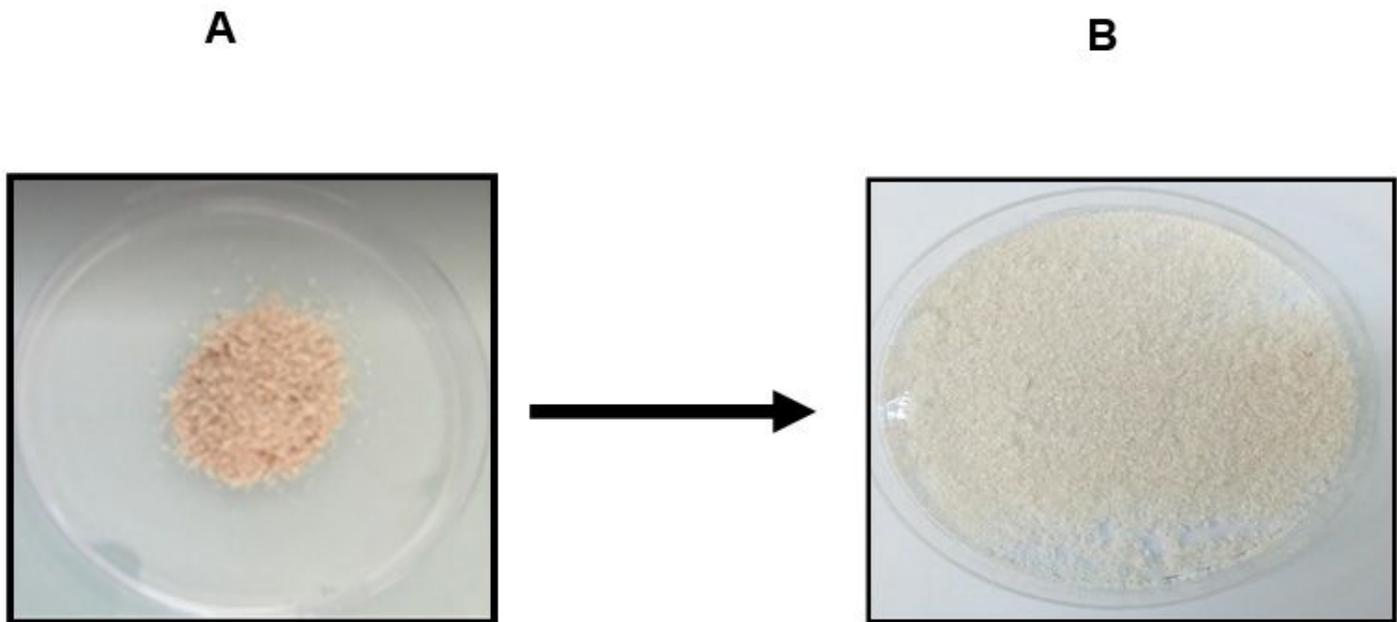


Figure 4

(A) BC obtained by co-culture; (B) *N*-deacetylated chitosan.

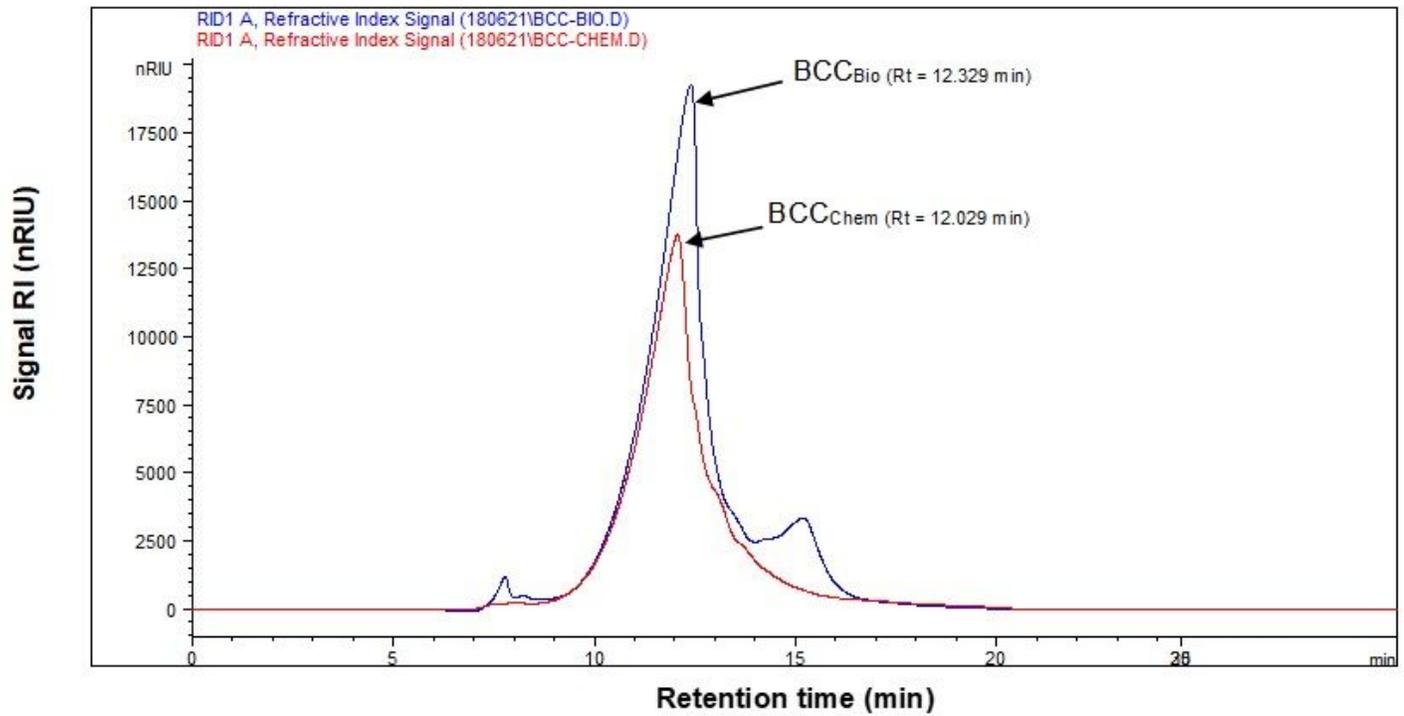


Figure 5

The HPLC chromatograms superposition of blue crab chitosan (BCC_{Bio}) and the commercial chitosan (BCC_{Chem}).

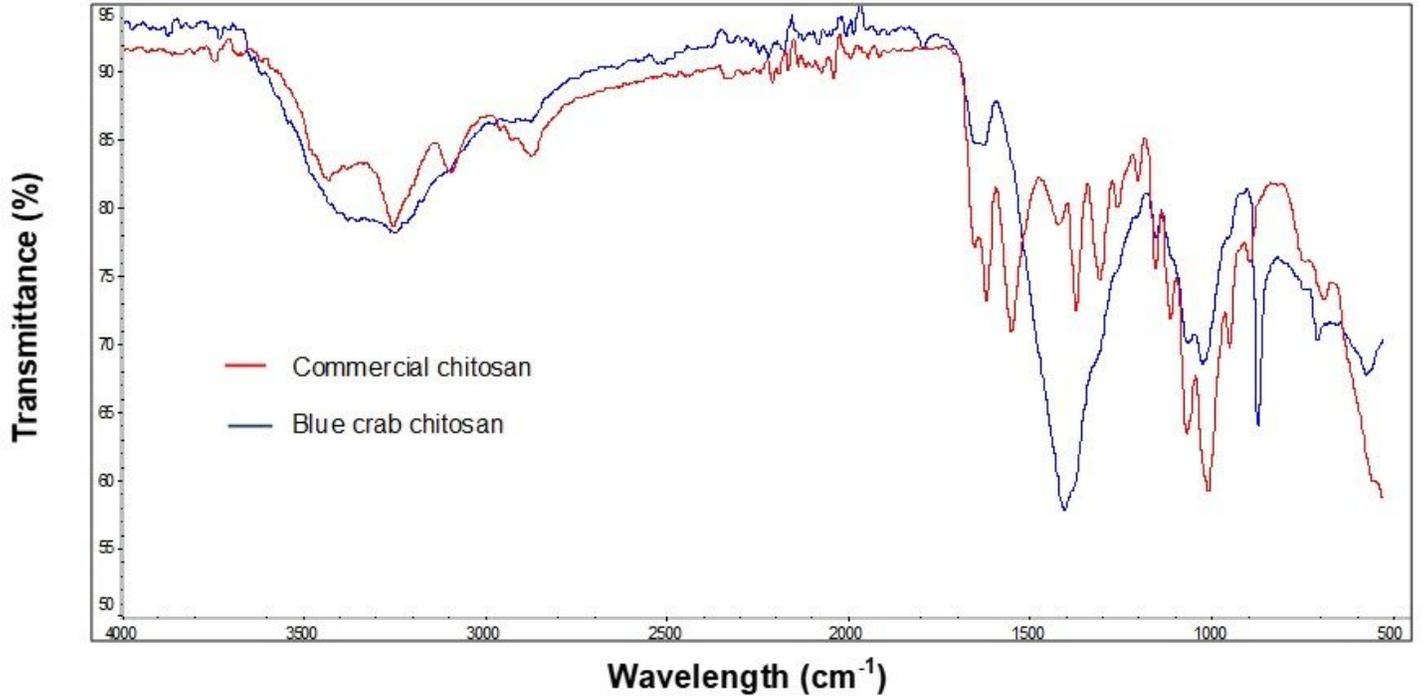


Figure 6

FTIR spectra of recovered BCC_{Bio} compared to the commercial one.

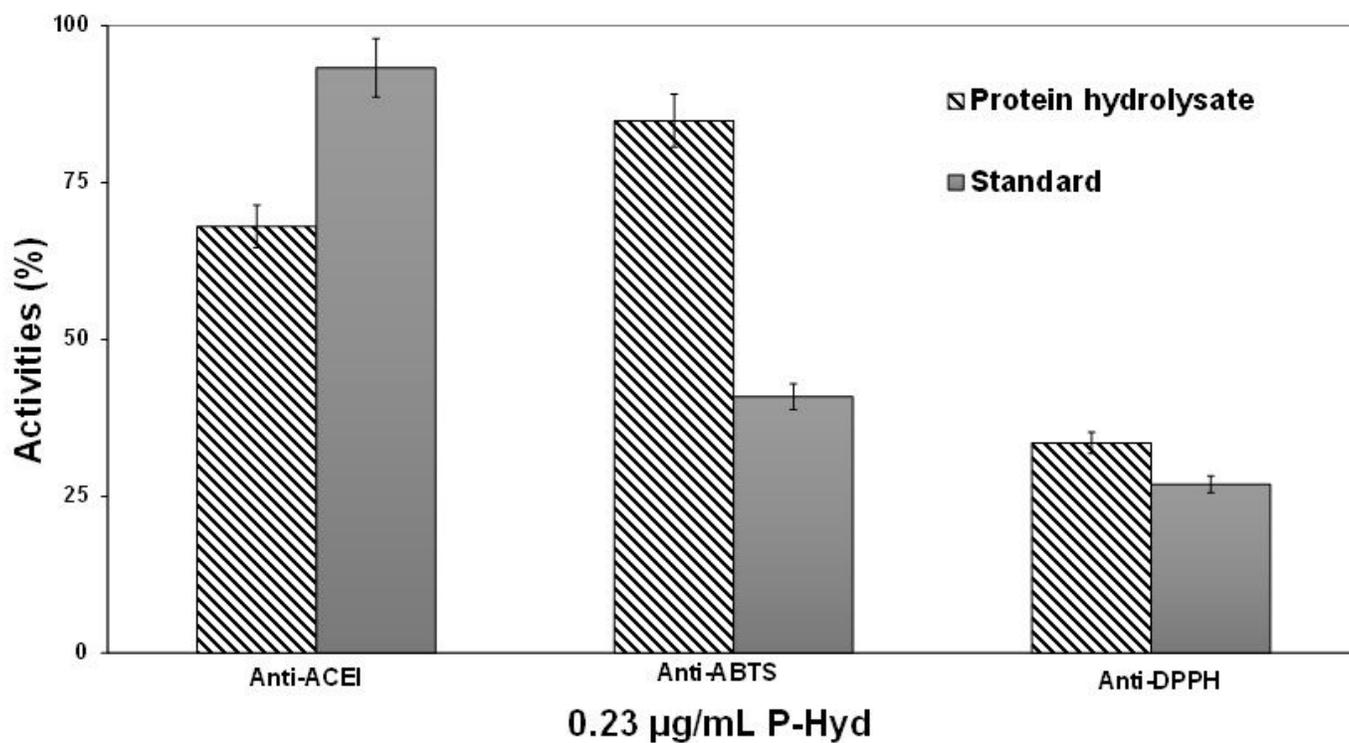


Figure 7

Anti-ACEI, anti-ABTS, and anti-DPPH of P_{Hyd} towards their standards at 7.4 µg/mL.

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