Trends in valorization of the invasive crab Portunus segnis for cleaner production of chitin, chitosan, and protein hydrolysate

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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 invasive blue crab Portunus segnis bio-waste. Optimization of proteases production was performed 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 (Pspp). The recovered protein hydrolysate (P Hyd) was found to be active towards radical scavenging power, and against angiotensin I-converting enzyme (ACE). The extraction efficiency of the blue crab chitin (BC) 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%. The spectrum of chitin and chitosan were depicted by Fourier-transform infrared spectroscopy (FTIR). The biological value of Pspp and its obtained derivates were evidenced via accredited protocols. These data constitute a roadmap towards a circular and sustainable bio-economic strategy for a clean transformation of a recalcitrant waste to bio-based products.

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 registred 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, counting areas next to reefs, mangroves, seagrassm, plus seaweed beds. In Tunisia, the first reported specimens were collected in coastal territories of the Gulf of Gabes during the month of October 2014 (Bejaoui et al. 2017). Taking into account the problems generated by this newly introduced species to fisherman and the reluctance of local consumer towards its consumption, the solution was therefore to search for new markets for its export and/or to develop innovative processing including bio-wastes transformation. 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 take into account 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 (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 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 pollute the environment.

The workflow of seafood processing is relatively easy to master. It results in the production of products having functional and nutritional properties which are particularly advantageous for the food, pharmaceutical, and cosmetic industries. Recent studies have demonstrated the anti-enzymatic and antioxidant properties of \( P_{\text{Hyd}} \) 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 for the development of 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 has been used for the recovery of chitin. For example, Alcalase\(^\circ\) from Novozymes Biopharma DK A/S (Bagsvaerd, Denmark) and serine alkaline protease (SAPN) from \textit{Melghribiacillus thermohalophilus} Nari2A\(^\dagger\) were proposed for the recovery of chitin from \textit{Portunus segnis} and \textit{Metapenaeus monoceros}, respectively (Mechri et al. 2019b; Mechri et al. 2020b). A comprehensive overview of recent literature shows that, several studies have been concentrated on an in-depth investigation of the bioactive compounds derived from \textit{Portunus segnis} swimming blue crab products, using enzymatic and chemical biotechnological procedures (Hamdi et al. 2020; Hamdi et al. 2019).

In this context, this investigation was carried out with the aim of a clean valuing of the invasive \( P_{\text{spp}} \). 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 \( P_{\text{Hyd}} \) and subsequently to recover chitin and chitosan. Besides, the \( P_{\text{spp}} \) and its obtained derivates 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 chemical blue crab chitosan (BCC\(_{\text{Chem}}\)) was used again for comparison. The casein used for assessment of protease activity was from Merck (Darmstadt, Germany). Exotic blue crab specimens were purchased in fresh conditions from the local fishery souk in Sfax (Tunisia). The sample 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 \( P_{\text{spp}} \) powder was used as ingredient in the formularization of an
economical culture media for proteases secretion. The entire of other reagents and substrates were of analytical mark.

2.2. Biochemical analyses

P$_{sp}$, BC, BCC$_{Bio}$, BCC$_{Chem}$, and P$_{Hyd}$ characterisations 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) in accordance with the requirements of the National standard NT 110-200 (version 2017), 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 v02)

The protein content was defined according to Hartree method with a slight adaptation of the Lowry method using BSA as a normal (Hartree 1972). Briefly, 0.45 g of each sample was meticulously homogenized in 9 mL of distilled water. Two dilutions were made with a final 200 dilution factor. After that, fractions of 250 μL from the BSA or diluted sample solutions were taken for succeeding protein analysis by adding consecutively the equivalent stoichiometric reactive solutions and quantification the absorbance at 650 nm using 96 wells microplates containing 500 μL of final solutions.

2.2.2. Amino-acids (MO/09 v01)

Amino-acids contents were ascertained by High Performance Liquid Chromatography (HPLC-DAD at $\lambda = 338$ nm; $\lambda = 208$ nm, Agilent 1260 Infinity), according to ISO 3903/d ISO 17180-2013 (MO/09 V02).

2.2.3. Crude fat (Folch method, MO/02 v01)

The lipid content was determined gravimetrically following an extraction of crude fat from 1 g of each sample according to the way characterized elsewhere (Folch et al. 1957; Khemir et al. 2020) using chloroform: methanol (2:1, v/v) solution containing 0.01% butylhydroxytoluene (BHT).

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

To ascertained the fatty acid content, fatty acid methyl esters were recovered from the removed fat according to the standard ISO 12966-2:2017 procedure that comprises dissolution of glycerides in isooctane and trans-esterification via potassium hydroxide methanol solution, and then resolved using gas chromatography in the guise of the ISO 12966-2 (version 2012) and ISO 12966-4 (version 2015) (MO/03).

2.2.5. Carbohydrates

Carbohydrate quantity was determined as described elsewhere (Brummer and Cui 2005).
2.2.6. Determination of total volatile basic nitrogen (TVB-N) (MO/08 v01)

TVB-N proportion was determined by flow injection analysis (FIA) as described formerly (Khemir et al. 2020; Ruiz-Capillas and Horner 1999).

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

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, correspondingly.

2.3. Protease activity assessment

Protease activity was measured as noticed elsewhere (Kembhavi et al. 1993). The supernatant of the culture medium was used for measurement of 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±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 defines as the amount of the enzyme yielding the equivalent of 1 µmole of tyrosine for every minute under the defined assay conditions.

2.4. Optimization of proteases production under co-culture

2.4.1. Influence of P<sub>spp</sub> concentration

The experiments were realized in 500 mL Erlenmeyer asks including 100 mL of liquid production medium containing various concentrations of P<sub>spp</sub> (10-100 g/L). The asks were inoculated after sterilization and the proteases level was assayed.

2.4.2. Investigation of significant factors by CCD

A CCD of 36 experiments was used to ascertain the influence of 4 factors on proteases production at five levels each.

2.5. Chitin and chitosan preparation

2.5.1. Blue crab chitin (BC) recovery

After CCD confirmation, the proteases production 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. BC yield was estimated as BC derived according to the original wet amount of P<sub>spp</sub> 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).

2.5.2. Blue crab chitosan (BCC) preparation

The switch of BC to BCC 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).

2.5.3. BCC physico-chemical characterization

2.5.3.1. Determination of acetylation degree (AD)

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±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:

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

Where 161.16 refer to the mass (g/mol) of chitosan monomer; $(V_2 - V_1)$ the amount of base consumed (mL); $N$ to the normality of the base; and $W_1$ is the mass (g) of sample after elimination for moisture. After that, the DA is deduced as follow:

$$\text{AD} \, (\%) = 100 - \text{DD}$$

(Eq. 2)

2.5.3.2. Functional properties of BCC

2.5.3.3. Solubility

This propriety 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±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 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:

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

(Eq. 3)

Where $W_1$ is the mass (g) of the tube with initial chitosan; $W_2$ is the mass (g) of the tube with final chitosan and $W_0$: mass of the empty tube.
2.5.3.4. Water binding capacity (WBC)

The WBC was ascertained in accordance 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 during 30 min at 23±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:

\[ WBC = \left( \frac{m_f}{m_0} \right) \times 100 \]  \hspace{1cm} (Eq. 4)

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

2.5.3.5. 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, then incubated for 30 min at 23±2°C with short shaking every 10 min. The solution was centrifuged (3,500 rpm for 25 min), the supernatant was discarded, and the pellet was weighed. To determine the FBC the subsequent procedure was used:

\[ FBC = \left( \frac{m_f}{m_0} \right) \times 100 \]  \hspace{1cm} (Eq. 5)

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

2.6. Assessment of biological activities of the \( P_{Hyd} \)

2.6.1. Antioxidant activities

The DPPH radicals scavenging assay was performed in accordance 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 pursue:

\[ ArA (%) = \left[ \frac{A_{570\ nm\ of\ the\ control} - A_{570\ nm\ of\ test\ sample}}{A_{570\ nm\ of\ control}} \right] \times 100 \]  \hspace{1cm} (Eq. 6)

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

\[ SA (%) = \left[ \frac{A_{715\ nm\ of\ control} - A_{715\ nm\ of\ test\ sample}}{A_{715\ nm\ of\ control}} \right] \times 100 \]  \hspace{1cm} (Eq. 7)

2.6.2. 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 nm} of the sample - A_{228 nm} of the sample blank) / (A_{228 nm} of control - A_{228 nm} of control blank)]  

(Eq. 8)

2.7. 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). The regression model was built on the basis of the SPSS approach. The responses for each experiment represent the average 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 high amount of ash, carbohydrates, and appreciable quantity of protein, lipids, and TVB-N. The stated value of ash indicated 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 ($\omega$-3) were present in $P_{spp}$ with moderate amounts suggests that the $P_{spp}$ can be ranked as a prospective source of dietary supplement. 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.

The most influencing factors (temperature, pH, concentration of $P_{spp}$, and the volume of the culture) were optimized using a CCD (Table 3). 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 increasing 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 (Table 4).
The established model is expected by the subsequent equation:

\[ Y = -869003.28 + 39728.46 \times X_1 + 707.23 \times X_2 + 5097.37 \times X_3 + 150.39 \times X_4 - 474.64 \times X_1 \times X_1 - 3.97 \times X_1 \times X_2 - 14.72 \times X_1 \times X_3 + 13.88 \times X_1 \times X_4 - 3.99 \times X_2 \times X_2 + 3.96 \times X_2 \times X_3 + 0.69 \times X_2 \times X_4 - 286.45 \times X_3 \times X_3 - 26.02 \times X_3 \times X_4 - 9.98 \times X_4 \times X_4 \]  

(Eq. 9).

Where \( Y, X_1, X_2, X_3, \) and \( X_4 \) represent the protease activity, the temperature, \( P_{spp} \) concentration, the pH, and the culture volume, correspondingly. The regression analysis possesses an \( F \)-value of 39.484 with a highly little probability value (\( p < 0.001 \)) designating a elevated significance of the model. The adjacency of the experimental and the anticipated protease activity was justified as presented by the regression coefficient of \( R^2 = 0.97 \) whom 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 betwixt the diverse factors. According to this model, the activity reaches its maximum (8,803 U/mL) at a concentration of \( P_{spp} \) at 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 are 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 cuticle of crabs and shrimps. Its 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 dimeneralisation (Table 1).

Considering the obtained FTIR patterns, both chitins displayed archetypal \( \alpha \)-chitin structure, with absorbance bands roughly 3270.09, 2921.57, 1622.28, 1399.21, 1396.99, 627.51, 619.47, and 583.57 (Fig. 3A-C). Particularly, the spectrum of BC showed major bands at around 1622.28 cm\(^{-1}\), indicating an intra-molecular hydrogen bonding \( \text{CO-HOCH}_2 \) (Hamdi et al. 2017). The peaks having an absorbance at 3270 cm\(^{-1}\) marked the existence of the NH group which reflects the vibrational modes implicated in the intermolecular hydrogen bonding. The peaks which have an absorbance at 2921.57 cm\(^{-1}\) reflect symmetrical plus asymmetrical stretches in the C-H bond. However, no discernable band was found at
1540 cm$^{-1}$, suggesting the presence of trace protein in the recovered chitin, and confirming biochemical analysis (Table 1). Such result highlights the efficiency of the deproteinization by this co-fermentation.

### 3.3. BCC$_{\text{Bio}}$ 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 propriety 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$_{\text{Bio}}$ 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$_{\text{Bio}}$ is noted (0.51 g/100 g DW). However, the chemical blue crab chitosan (BCC$_{\text{Chem}}$) had a higher protein level (Table 1), suggesting a better deproteinization using biological process.

The DD of BCC$_{\text{Bio}}$ were found to be significantly high (81%) but within the range of results found in 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 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 to 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$_{\text{Bio}}$ 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$_{\text{Bio}}$. Indeed, several studies have proven that the BCC$_{\text{Bio}}$ 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$_{\text{Bio}}$ (Fig. 5A) has a typical commercial chitosan structure (Fig. 5B) with distinctive absorbance bands around 3245.25, 1626.64, 1404.31, 1152.39, 1022.74, 870.26, and 572.51 cm$^{-1}$ (Fig. 5C). In truth, for both spectra, the presence of a peak having a stretching wavelength at 1626.64 cm$^{-1}$ is attribute to the amide I band (C=O in the NH-COCH$_3$ group). This observation discloses an augment in the DD of the BCC (Erdogan and Kaya 2016). The peak at 1404.31 cm$^{-1}$ designated the C-H bending vibrations of CH$_2$ 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 870.26 cm⁻¹ has been ascribed to the absorption peaks of β-(1,4) glycosidic bond in BCC. Finally, the band at 3245.25 cm⁻¹ have been attributed to the stretching vibration of OH and NH (Ramasamy et al. 2014).

3.4. Assessment of biological activities

3.4.1. P_Hyd characterization (ISO 19343, 2017 (Fr))

The P_Hyd is an attractive source of nutrients including proteins, amino-acids, and 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. In fact, the biogenic amines are produced via whichever 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 besides be formed during agmatinase pathway, whom 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 fishery products is ascertained by 100 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 together 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, plus the management of this feature is besides a manner to promise and guarantee food security (Chaidoutis et al. 2019).

P_Hyd which contain great amounts of proteins, amino-acids, and biogenic amines are believed to 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).

3.4.2. 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 to 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 test.
Regarding the trapping activity of the free radical DPPH •+, the activity reaches 33.52% at 0.23 \( \mu \)g/mL (Fig. 6). However, toward the ABTS •+, the activity reached 84.87% at a concentration of 7.4 \( \mu \)g/mL, while the activity of BHT was of 26.91 and 40.88% for DPPH and ABTS respectively for the same concentration (Fig. 6). 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 concentration of 100 \( \mu \)g/mL resulting in 95% radicals neutralization (Mechri et al. 2020b).

### 3.4.3. 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 standard. For the same concentration of 0.23 \( \mu \)g/mL, the ACEI inhibition of the P\textsubscript{Hyd} was highly considerable with 68% of inhibition (Fig. 6), 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 and 71.52 \( \mu \)g/mL for the captopril and P\textsubscript{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

Bio-wastes generated from fishery offers outstanding opportunities for exploitation. This present scenario is part of the development of fishery by-products and especially of crustacean’s waste, with the aim of obtaining products with high added value. In this research, the chemical components of the P\textsubscript{spp} and its derivatives (protein, chitin, and chitosan) recovered by a biological way has been well analysed by the means of accredited approaches. Further works, a few of which are presently in progress in LMBEB, and B3Aqua laboratories, are still required to maximize the proteins removal from P\textsubscript{spp}. Complementary studies seems inevitable to evaluate these biological activities *in-vivo* and to purify the peptides which are responsible for them.

### Declarations

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References

Arena M, Manca de Nadra M (2001) Biogenic amine production by Lactobacillus. J Appl Microbiol 90:158-162

Bejaoui S, Ghribi F, Hatira S, Chetoui I, Rebah I (2017) First investigation in the biochemical analysis of the invasive crab Portunus segnis from Tunisian waters. J Am Oil Chem Soc 94:673-682

Besbes N, Joffraud J J, Khemis IB, Sadok S (2017) Bio-Preservation of refrigerated peeled Shrimp (Parapenaeus longirostris) using cactus fruit peels polyphenolic extract. J Biotechnol Biochem 3:36-47
Brummer Y, Cui SW (2005) Understanding carbohydrate analysis Food Carbohydrates: Chemistry, physical properties and applications:1-38

Cabrera-Barjas G et al. (2020) Utilization of industrial by-product fungal biomass from Aspergillus niger and Fusarium culmorum to obtain biosorbents for removal of pesticide and metal ions from aqueous solutions. J Environ Chem Eng 8:104355

Chaidoutis E, Migdanis A, Keramydas D, Papalexis P (2019) Biogenic amines in food as a public health concern an outline of histamine food poisoning. Arch Hell Med 36:419-423

Choi CW et al. (2002) Antioxidant activity and free radical scavenging capacity between Korean medicinal plants and flavonoids by assay-guided comparison. Plant Sci 163:1161-1168

Cushman D, Cheung H (1971) Spectrophotometric assay and properties of the angiotensin-converting enzyme of rabbit lung. Biochem Pharmacol. 20:1637-1648

Dailianis T et al. (2016) New mediterranean biodiversity records (July 2016). Mediterr Mar Sci 17:608-626

Erdogan S, Kaya M (2016) High similarity in physicochemical properties of chitin and chitosan from nymphs and adults of a grasshopper. Int J Biol Macromol 89:118-126

Erdogan S, Kaya M, Akata I Chitin extraction and chitosan production from cell wall of two mushroom species (Lactarius vellereus and Phyllophora ribis). In: AIP Conference Proceedings 1809, 2017. vol 1. AIP Publishing LLC, p 020012

Fernandez-Kim S-O (2004) Physicochemical and functional properties of crawfish chitosan as affected by different processing protocols, M. S. Dissertation, Seoul National University, Seoul

Folch J, Lees M, Sloane Stanley G (1957) A simple method for the isolation and purification of total lipides from animal tissues. J Biol Chem 226:497-509

Fox HM (1924) The migration of a red Sea crab through the Suez Canal Nature 113:714-715

Galil BS, Zenetos A (2002) A sea change-exotics in the Eastern Mediterranean Sea. In: Invasive aquatic species of Europe. Distribution, impacts and management. Springer, pp 325-336

Giordano D et al. (2018) Chapter Five - Biotechnological Applications of Bioactive Peptides From Marine Sources. In: Poole RK (ed) Advances in Microbial Physiology, vol 73. Academic Press, pp 171-220.

Hajji S, Younes I, Rinaudo M, Jellouli K, Nasri M (2015) Characterization and in vitro evaluation of cytotoxicity, antimicrobial and antioxidant activities of chitosans extracted from three different marine sources. Appl. Biochem. Biotechnol 177:18-35

Hamdi M, Hajji S, Affes S, Taktak W, Maâlej H, Nasri M, Nasri R (2018) Development of a controlled bioconversion process for the recovery of chitosan from blue crab (Portunus segnis) exoskeleton. Food
Hamdi M, Hammami A, Hajji S, Jridi M, Nasri M, Nasri R (2017) Chitin extraction from blue crab (*Portunus segnis*) and shrimp (*Penaeus kerathurus*) shells using digestive alkaline proteases from *P. segnis* viscera. Int J Biol Macromol 101:455-463

Hamdi M, Nasri R, Dridi N, Li S, Nasri M (2020) Development of novel high-selective extraction approach of carotenoproteins from blue crab (*Portunus segnis*) shells, contribution to the qualitative analysis of bioactive compounds by HR-ESI-MS. Food Chem 302:125334

Hamdi M, Nasri R, Li S, Nasri M (2019) Bioactive composite films with chitosan and carotenoproteins extract from blue crab shells: Biological potential and structural, thermal, and mechanical characterization. Food Hydrocol 89:802-812

Hatree E (1972) Determination of protein: a modification of the Lowry method that gives a linear photometric response. Anal Biochem 48:422-427

Hui C et al. (2020) Chitin degradation and the temporary response of bacterial chitinolytic communities to chitin amendment in soil under different fertilization regimes. Sci Total Environ 705:136003

Jabeur F, Mechri S, Kriaa M, Gharbi I, Bejaoui N, Sadok S, Jaouadi B (2020) Statistical Experimental design optimization of microbial proteases production under co-culture conditions for chitin recovery from speckled shrimp *Metapenaeus monoceros* by-product. BioMed Res Int 2020

Jemil I et al. (2014) Functional, antioxidant and antibacterial properties of protein hydrolysates prepared from fish meat fermented by *Bacillus subtilis* A26. Process Biochem 49:963-972

Kalac P, Krausová P (2005) A review of dietary polyamines: Formation, implications for growth and health and occurrence in foods. Food Chem 90:219-230

Kaya M, Dudakli F, Asan-Ozusaglam M, Cakmak YS, Baran T, Mentes A, Erdogan S (2016) Porous and nanofiber α-chitosan obtained from blue crab (*Callinectes sapidus*) tested for antimicrobial and antioxidant activities. LWT-Food Sci Technol 65:1109-1117

Kembhavi AA, Buttle DJ, Knight CG, Barrett AJ (1993) The two cysteine endopeptidases of legume seeds: Purification and characterization by use of specific fluorometric assays. Arch Biochem biophys 303:208-213

Khemir M, Besbes N, Khemis IB, Di Bella C, Lo Monaco D, Sadok S (2020) Determination of shelf-life of vacuum-packed sea bream (*Sparus aurata*) fillets using chitosan-microparticles-coating CyTA. J Food 18:51-60

Kirby AJ, Schmidt RJ (1997) The antioxidant activity of Chinese herbs for eczema and of placebo herb-I. J Ethnopharmacol 56:103-108
Knorr D (1982) Functional properties of chitin and chitosan. J Food Sci 47:593-595

Kumari S, Rath P, Kumar ASH, Tiwari T (2015) Extraction and characterization of chitin and chitosan from fishery waste by chemical method. Environ Technol Innov 3:77-85

Liu B, Cao Z, Qin L, Li J, Lian R, Wang C (2020) Investigation of the synthesis of biogenic amines and quality during high-salt liquid-state soy sauce fermentation. LWT-Food Sci Technol 133:109835

Liu Y-R et al. (2014) ABTS+ scavenging potency of selected flavonols from *Hypericum perforatum* L. by HPLC-ESI/MS: Reaction observation, adduct characterization and scavenging activity determination. Food Res Int 58:47-58

Mechri S, Bouacem K, Amziane M, Dab A, Nateche F, Jaouadi B (2019a) Identification of a new serine alkaline peptidase from the moderately halophilic *Virgibacillus Natechei* sp. nov., strain FarD^T_ and its application as bioadditive for peptide synthesis and laundry detergent formulations. BioMed Res Int 2019 Article ID 6470897

Mechri S et al. (2019b) Purification and biochemical characterization of a novel thermostable and halotolerant subtilisin SAPN, a serine protease from *Melghiribacillus thermohalophilus* Nari2AT for chitin extraction from crab and shrimp shell by-products. Extremophiles 23:529-547

Mechri S et al. (2020a) Antioxidant and enzyme inhibitory activities of *Metapenaeus monoceros* by-product hydrolysates elaborated by purified alkaline proteases. Waste Biomass Valori:1-15

Mechri S et al. (2020b) A biological clean processing approach for the valorization of speckled shrimp *Metapenaeus monoceros* by-product as a source of bioactive compounds. Environ Sci and Poll Res:1-14

Meramo-Hurtado S, Alarcón-Suesca C, González-Delgado ÁD (2020) Exergetic sensibility analysis and environmental evaluation of chitosan production from shrimp exoskeleton in Colombia. J Clean Prod 248:119285

Mohammadian A, Ahmadvand H, Karamian R, Siahmansour R, Sepahvand A, Omidvari S (2017) Survey and comparison of the antioxidant activity, total phenolic, and flavonoid compounds of saffron petals sowing in different regions of the Lorestan province. Saffron Agron Technol 5

Ocloo F, Quayson E, Adu-Gyamfi A, Quarcoo E, Asare D, Serfor-Armah Y, Woode B (2011) Physicochemical and functional characteristics of radiation-processed shrimp chitosan. Rad Phys Chem 80:837-841

Prester L (2011) Biogenic amines in fish, fish products and shellfish: A review. Food Addit Contam: Part A 28:1547-1560

Ramasamy P, Subhapradha N, Shanmugam V, Shanmugam A (2014) Extraction, characterization and antioxidant property of chitosan from cuttlebone *Sepia kobiensis* (Hoyle 1885). Int J Biol Macromol 64:202-212
Rao MS, Stevens WF (2005) Chitin production by *Lactobacillus fermentation* of shrimp biowaste in a drum reactor and its chemical conversion to chitosan. J Chem Technol Biotechnol Int Res in Process, Environ Clean Technol 80:1080-1087

Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C (1999) Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radical Bio Med 26:1231-1237

Ruiz-Capillas C, Horner WFA (1999) Determination of trimethylamine nitrogen and total volatile basic nitrogen in fresh fish by flow injection analysis. J Sci Food and Agri 79:1982-1986

Sarbon N, Sandanamsamy S, Kamaruzaman S, Ahmad F (2015) Chitosan extracted from mud crab (*Scylla olivacea*) shells: Physicochemical and antioxidant properties. J Food Sci Technol 52:4266-4275

Sharma C, Bhardwaj NK, Pathak P (2020) Static intermittent fed-batch production of bacterial nanocellulose from black tea and its modification using chitosan to develop antibacterial green packaging material. J Clean Prod:123608

Uranga J, Etxabide A, Cabezudo S, de la Caba K, Guerrero P (2020) Valorization of marine-derived biowaste to develop chitin/fish gelatin products as bioactive carriers and moisture scavengers. Sci Total Environ 706:135747

Vázquez JA, Ramos P, Valcarcel J, Antelo LT, Novoa-Carballal R, Reis RL, Pérez-Martín RI (2018) An integral and sustainable valorisation strategy of squid pen by-products. J Clean Prod 201:207-218

Zenetos A et al. (2012) Alien species in the Mediterranean Sea by 2012. A contribution to the application of European Union's Marine strategy framework directive (MSFD). Part 2. Introduction trends and pathways. Mediterr Mar Sci 13:328-352

Zhang X, Geng X, Jiang H, Li J, Huang J (2012) Synthesis and characteristics of chitin and chitosan with the (2-hydroxy-3-trimethylammonium) propyl functionality, and evaluation of their antioxidant activity in vitro. Carbohydr Polym 89:486-491

Zhu W, Chen X, Struble LJ, Yang E-H (2018) Characterization of calcium-containing phases in alkali-activated municipal solid waste incineration bottom ash binder through chemical extraction and deconvoluted Fourier transform infrared spectra. J Clean Prod 192:782-789

**Tables**

**Table 1**

Proximate composition of \( P_{spp} \), BC, BCC\(_{Bio} \), BCC\(_{Chem} \), and \( P_{Hyd} \).
| Parameters | Composition | Unit | P\textsubscript{spp} | BC | BCC\textsubscript{Bio} | BCC\textsubscript{Chem} | P\textsubscript{Hyd} |
|------------|-------------|------|-----------------|----|-----------------|------------------|---------|
| Moisture   | %           |      | 9.57           | 10.79 | 11.31          | 4.77             | ND      |
| Ash        | %           |      | 41.50          | 50.40 | 56.64          | 1.18             | ND      |
| Proteins   | g/100 g     |      | 2.52           | 0.51  | 0.02           | 0.42             | 28.88   |
| Carbohydrates | g/100 g |      | 21.98          | 9.87  | 10.27          | <LQ              | <LQ     |
| Lipids     | g/100 g     |      | 0.57           | 0.30  | 0.21           | 1.26             | <LQ     |
| TVB-N      | g/100 g     |      | 3.362          | 2.58  | 3.07           | 3.29             | <LQ     |
| SFA        | g/100 g     |      | 0.23           | 0.13  | 0.12           | 0.88             | <LQ     |
| MUFA       | g/100 g     |      | 0.23           | 0.12  | 0.07           | 0.19             | <LQ     |
| PUFA       | g/100 g     |      | 0.04           | 0.01  | <LQ            | 0.02             | <LQ     |
| ω-3        | g/100 g     |      | 0.02           | <LQ   | <LQ            | 0.02             | <LQ     |
| ω-6        | g/100 g     |      | <LQ            | 0.06  | <LQ            | <LQ              | <LQ     |
| Amino-acids| g/100 g     |      | 11.02          | 6.4   | 11.07          | 25.69            | 33.18   |
| Yield      | %           |      | -              | 32    | 52             | -                | -       |

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

**Table 2**

The amino-acids and biogenic amines compositions of P\textsubscript{spp}, BC, BCC\textsubscript{Bio}, BCC\textsubscript{Chem}, and P\textsubscript{Hyd}.
| Parameters | \(P_{\text{spp}}\) | BC | BCC\(_{\text{Bio}}\) | BCC\(_{\text{Chem}}\) | \(P_{\text{Hyd}}\) |
|------------|-----------------|----|----------------|----------------|-----------------|
| **Amino-acids (g/100g)** | | | | | |
| Aspartate  | 1.14            | 0.33 | 0.05 | 0.03 | 2.95 |
| Glutamate  | 0.92            | 0.32 | 0.19 | <LQ  | 5.08 |
| Serine     | 0.46            | 0.14 | 0.06 | <LQ  | 1.68 |
| Asparagine | <LQ             | <LQ | <LQ  | <LQ  | <LQ  |
| Glutamine  | <LQ             | <LQ | <LQ  | <LQ  | <LQ  |
| Histidine* | 0.37            | 0.15 | 0.15 | 0.89 | 0.89 |
| Glycine    | 0.54            | 0.24 | 0.05 | <LQ  | 2.93 |
| Threonine* | 0.30            | 0.09 | 0.03 | 0.16 | 1.31 |
| Arginine*  | 3.51            | 4.09 | 8.51 | 16.47 | 3.90 |
| Alanine    | 0.50            | 0.15 | 1.27 | 2.39  | 1.78 |
| Tyrosine   | 0.18            | 0.06 | <LQ  | 0.09 | 0.90 |
| Valine*    | 0.42            | 0.10 | 0.05 | <LQ  | 1.26 |
| Methionine*| 0.06            | <LQ | 0.07 | 0.14 | 0.75 |
| Tryptophane*| <LQ           | <LQ | <LQ  | <LQ  | <LQ  |
| Phenylalanine*| 0.33         | 0.14 | 0.06 | <LQ  | 1.16 |
| Isoleucine*| 0.21            | 0.07 | 0.07 | <LQ  | 1.23 |
| Leucine*   | 0.38            | 0.10 | 0.08 | 0.15  | 2.40 |
| Lysine*    | 0.34            | 0.14 | 0.05 | 0.37  | 3.13 |
| Hydroxyproline| 0.65         | 0.65 | 0.80 | 5.55  | 0.70 |
| Proline    | 0.71            | 0.44 | 0.81 | 0.30  | 1.13 |
| **Biogenic amines (mg/Kg)** | | | | | |
| Histamine  | ND              | ND  | ND   | ND   | 13.24 |
| 2-phenylethylamine| ND        | ND  | ND   | ND   | 55.65 |
| Putrescine | ND              | ND  | ND   | ND   | 330.94 |
| Tyramine   | ND              | ND  | ND   | ND   | 14.24 |
| Spermidine | ND              | ND  | ND   | ND   | 31.66 |
| Agmatine   | ND              | ND  | ND   | ND   | 35.52 |
| Cadaverine | ND              | ND  | ND   | ND   | 13.54 |
| Spermine   | ND              | ND  | ND   | ND   | <LQ  |

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

**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                | ºC   | 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

Centered composite experiments plan with their experimental responses.

| Run | $X_1$ | $X_2$ | $X_3$ | $X_4$ | Protease activity (U/mL) |
|-----|-------|-------|-------|-------|--------------------------|
| 1   | -1    | -1    | -1    | -1    | 3,939                    |
| 2   | -1    | -1    | -1    | 1     | 2,278                    |
| 3   | -1    | -1    | 1     | -1    | 2,692                    |
| 4   | -1    | -1    | 1     | 1     | 89                       |
| 5   | -1    | 1     | -1    | -1    | 2,635                    |
| 6   | -1    | 1     | -1    | 1     | 1,782                    |
| 7   | -1    | 1     | 1     | -1    | 1,528                    |
| 8   | -1    | 1     | 1     | 1     | 26                       |
| 9   | 1     | -1    | -1    | -1    | 6,696                    |
| 10  | 1     | -1    | -1    | 1     | 6,550                    |
| 11  | 1     | -1    | 1     | -1    | 5,007                    |
| 12  | 1     | -1    | 1     | 1     | 4,937                    |
| 13  | 1     | 1     | -1    | -1    | 3,191                    |
| 14  | 1     | 1     | -1    | 1     | 6,057                    |
| 15  | 1     | 1     | 1     | -1    | 4,907                    |
| 16  | 1     | 1     | 1     | 1     | 3,567                    |
| 17  | -2    | 0     | 0     | 0     | 2,266                    |
| 18  | 2     | 0     | 0     | 0     | 3,791                    |
| 19  | 0     | -2    | 0     | 0     | 4,282                    |
| 20  | 0     | 2     | 0     | 0     | 5,585                    |
| 21  | 0     | 0     | -2    | 0     | 6,873                    |
| 22  | 0     | 0     | 2     | 0     | 3,764                    |
| 23  | 0     | 0     | 0     | -2    | 5,059                    |
| 24  | 0     | 0     | 0     | 2     | 2,555                    |
| 25  | 0     | 0     | 0     | 0     | 8,445                    |
| 26  | 0     | 0     | 0     | 0     | 8,703                    |
| 27  | 0     | 0     | 0     | 0     | 8,300                    |
| 28  | 0     | 0     | 0     | 0     | 8,200                    |
| 29  | 0     | 0     | 0     | 0     | 8,237                    |
| 30  | 0     | 0     | 0     | 0     | 8,037                    |
| 31  | 0     | 0     | 0     | 0     | 8,500                    |
| 32  | 0     | 0     | 0     | 0     | 8,202                    |
| 33  | 0     | 0     | 0     | 0     | 8,114                    |
| 34  | 0     | 0     | 0     | 0     | 8,318                    |
| 35  | 0     | 0     | 0     | 0     | 8,475                    |

Figures
Figure 1

Response surface plot of proteases production showing the interactive effects of Pspp (g/L) and culture volume (mL) (A); pH and Pspp (g/L) (B), and pH and culture volume (mL) (C).
Figure 2

(A) Pspp. (B) BC obtained by co-culture.
Figure 3

(A) FTIR spectra of commercial chitin. (B) FTIR spectra of BC recovered from Pspp treated with the co-culture. (C) FTIR spectra of BC recovered compared to commercial chitin.
Figure 4

(A) BC obtained by co-culture. (B) N-deacetylated chitosan.
Figure 5

(A) FTIR spectra of commercial chitosan. (B) FTIR spectra of recovered BCCBio. (C) FTIR spectra of recovered BCCBio compared to the commercial one.
Figure 6

Anti-ACEI, anti-ABTS, and anti-DPPH of PHyd towards their standards at 7.4 µg/mL.

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