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_{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 by proteases has been used for the recovery of 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 recent literature shows that, several studies have been concentrated on an in-depth investigation of the bioactive compounds derived from *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_{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 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_{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_{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_{spp}, 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 (Hatree 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 λ = 338 nm; λ = 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 *µ*mole 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_{spp} concentration

The experiments were realized in 500 mL Erlenmeyer flasks including 100 mL of liquid production medium containing various concentrations of P_{spp} (10-100 g/L). The flasks 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_{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).

2.5.2. 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:

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

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:

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

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:

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

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:

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

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:

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

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:

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

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

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

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 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 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 (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. 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:

$Y = -869003.28 + 39728.46 \times X1 + 707.23 \times X2 + 5097.37 \times X3 + 150.39 \times X4 - 474.64 \times X1 \times X1 - 3.97 \times X1 \times X2 - 14.72 \times X1 \times X3 + 13.88 \times X1 \times X4 - 3.99 \times X2 \times X2 + 3.96 \times X2 \times X3 + 0.69 \times X2 \times X4 - 286.45 \times X3 \times X3 - 26.02 \times X3 \times X4 - 9.98 \times X4 \times X4$ (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. The regression analysis possesses an *F*-value of 39.484 with a highly little probability value ($p < 001$) 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 α-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 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 (Table1). Such result highlights the efficiency of the deproteinization by this co-fermentation.

3.3. 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 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_{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} is 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} 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_{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} (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 $NH\text{-}COCH_3$ 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 CH_2 and CH_3 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 reachs 33.52% at 0.23 μg/mL (Fig. 6). However, toward the ABTS • +, the activity reached 84.87% at a concentration of 7.4 μg/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 μg/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 μg/mL, the ACEI inhibition of the P_{Hyd} was highly considerable with 68% of inhibition (Fig. 6), whereas the activity of captopril was at 93.29%. This result is analogeous 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 μg/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

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_{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_{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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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. and M.K. aided in interpreting the results and worked on the manuscript. All authors discussed the results and commented on the manuscript.

Availability of data and materials No data and materials were used to support this study.

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 Not applicable

Consent to publish Not applicable

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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	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_{spp}, *Portunus segnis* by-product powder. BC, Protein hydrolysate. BCC_{Bio}, Biological blue crab chitosan. BCC_{Chem}, Chemical blue crab chitosan. P_{Hyd}, Blue crab chitin.

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}
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
Aspargine	<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_{spp}, *Portunus segnis* by-product powder. BC, Protein hydrolysate. BCC_{Bio}, Biological blue crab chitosan. BCC_{Chem}, Chemical blue crab chitosan. P_{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	°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

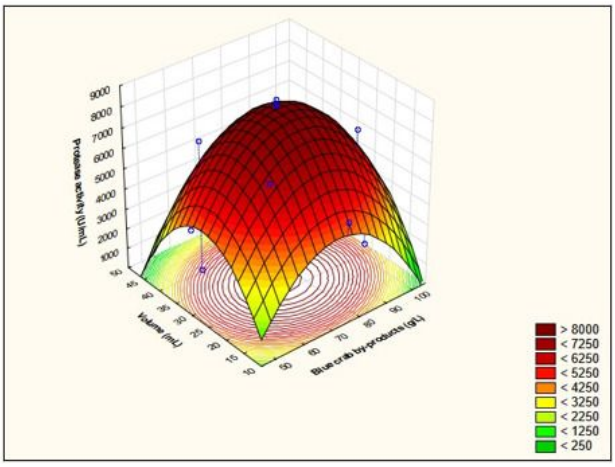
Table 4

Centered composite experiments plan with their experimental responses.

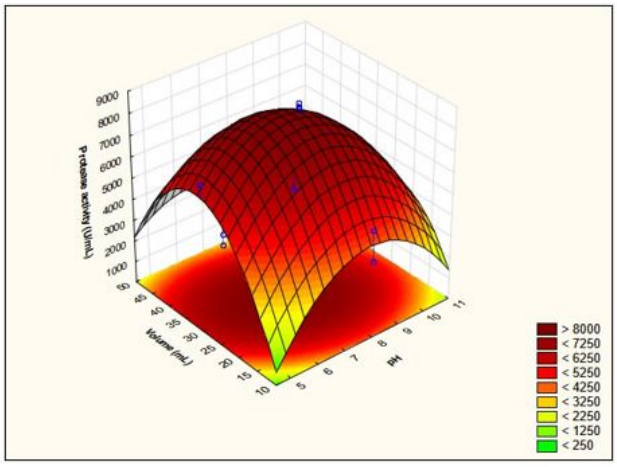
Run	X_1	X_2	X_3	X_4	Protease activity (U/mL)
1	-1	-1	-1	-1	3,939
2	-1	-1	-1	1	2,278
3	-1	-1	1	-1	2,692
4	-1	-1	1	1	89
5	-1	1	-1	-1	2,635
6	-1	1	-1	1	1,782
7	-1	1	1	-1	1,528
8	-1	1	1	1	26
9	1	-1	-1	-1	6,696
10	1	-1	-1	1	6,550
11	1	-1	1	-1	5,007
12	1	-1	1	1	4,937
13	1	1	-1	-1	3,191
14	1	1	-1	1	6,057
15	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

A



B



C

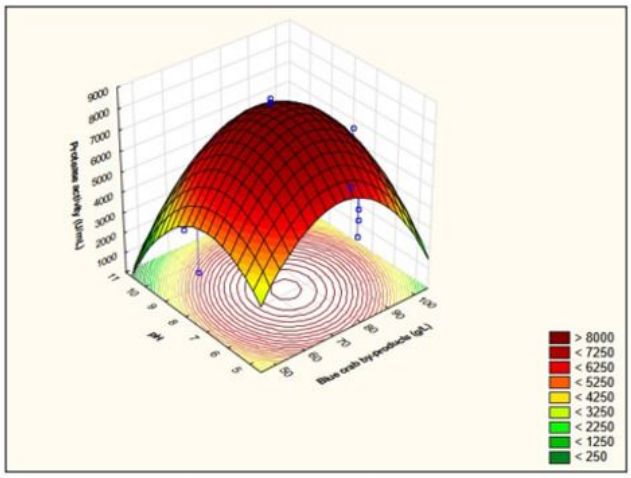


Figure 1

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).

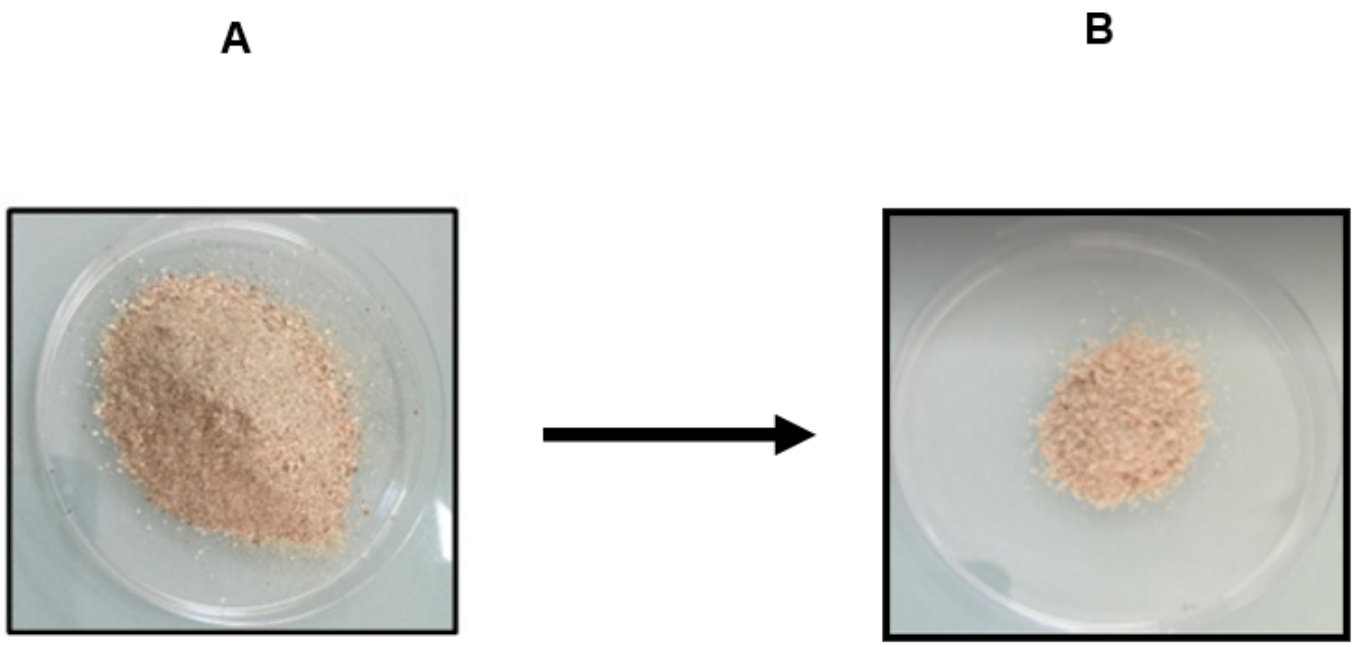


Figure 2

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

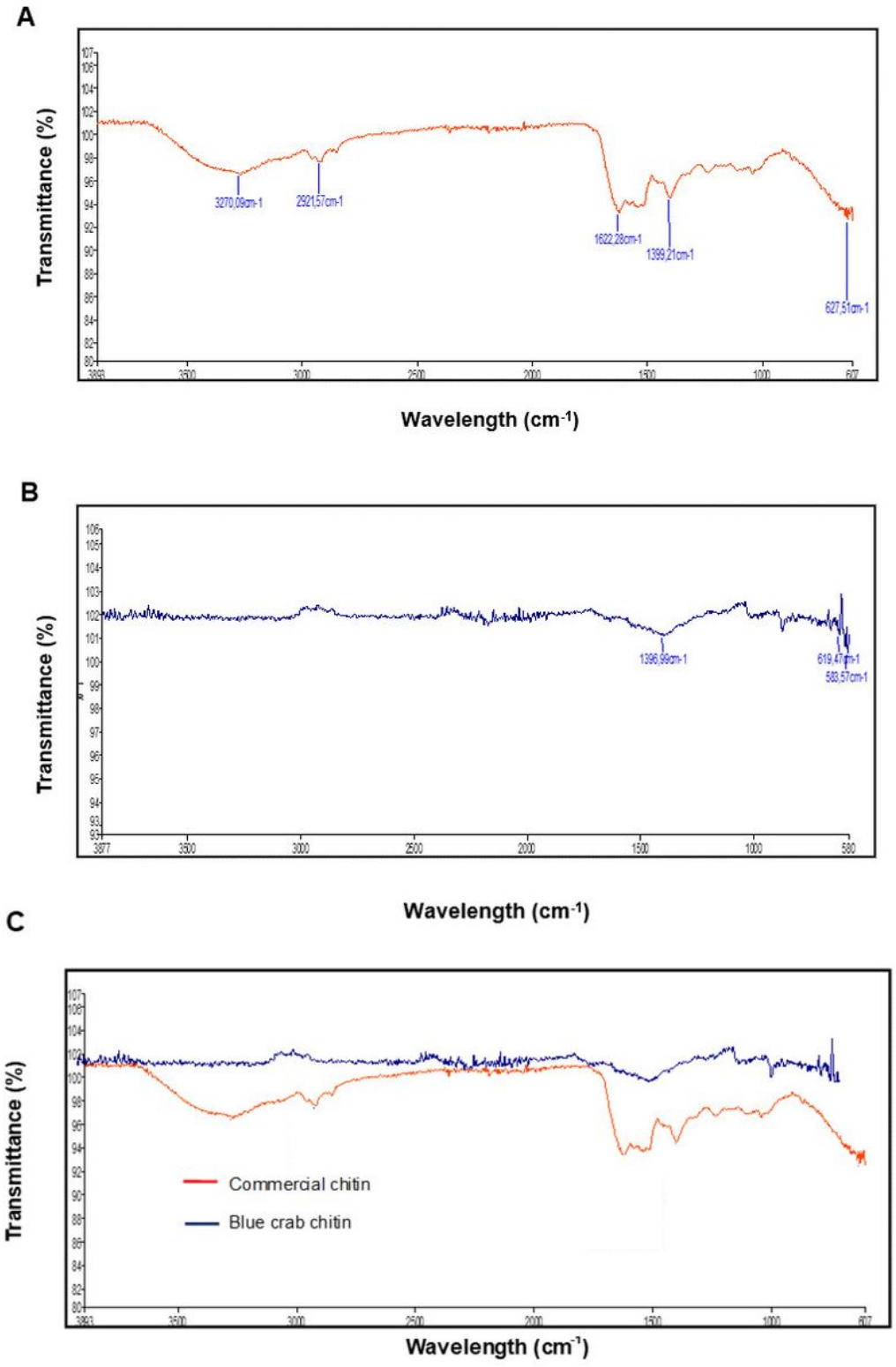


Figure 3

(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.

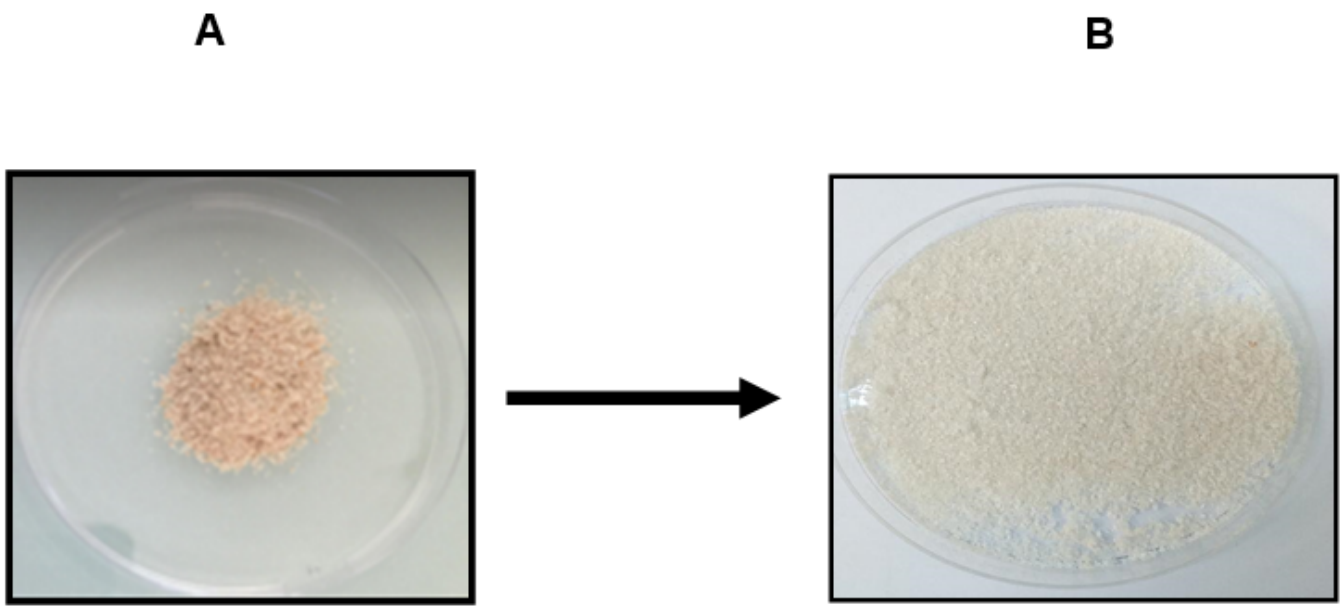


Figure 4

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

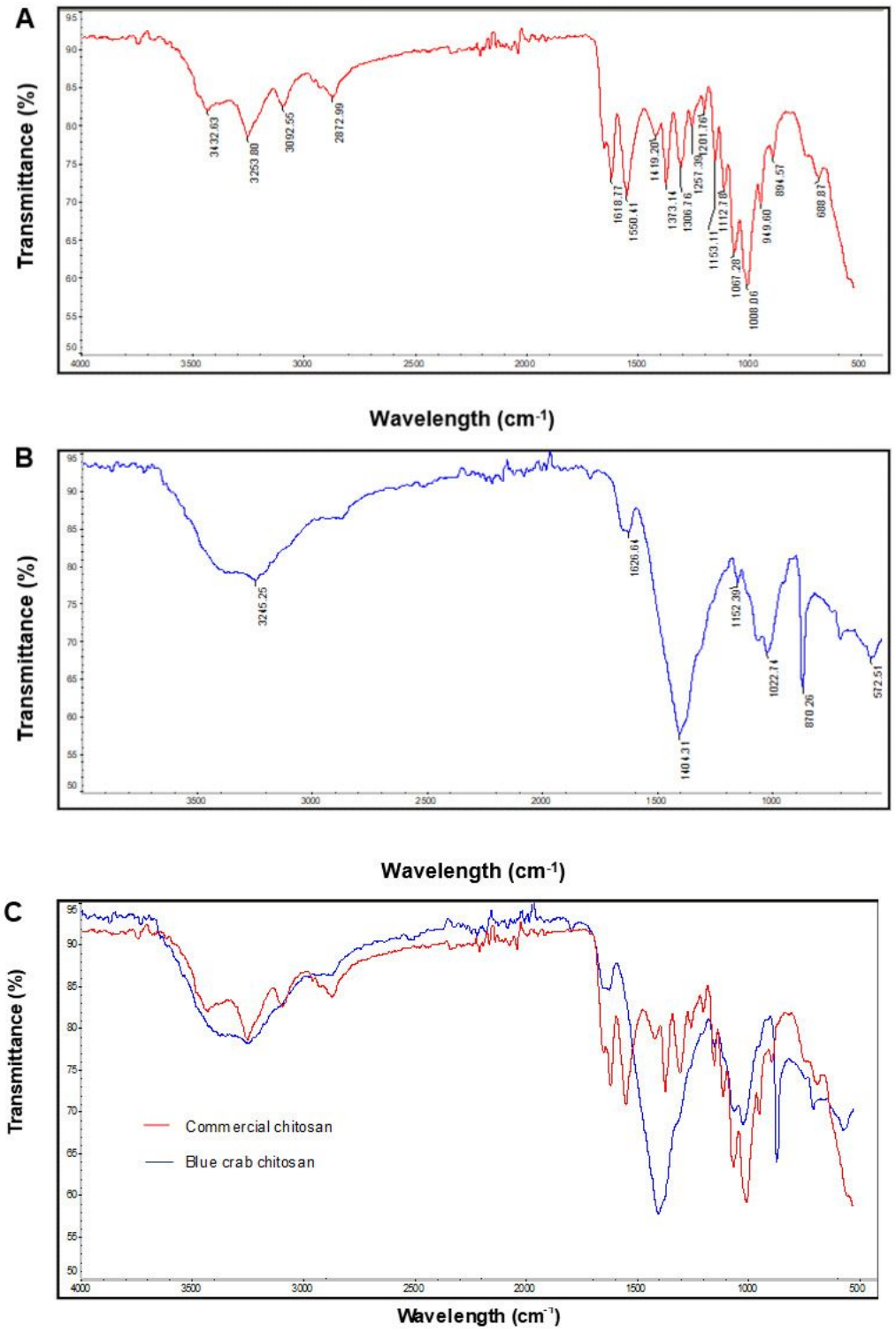


Figure 5

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

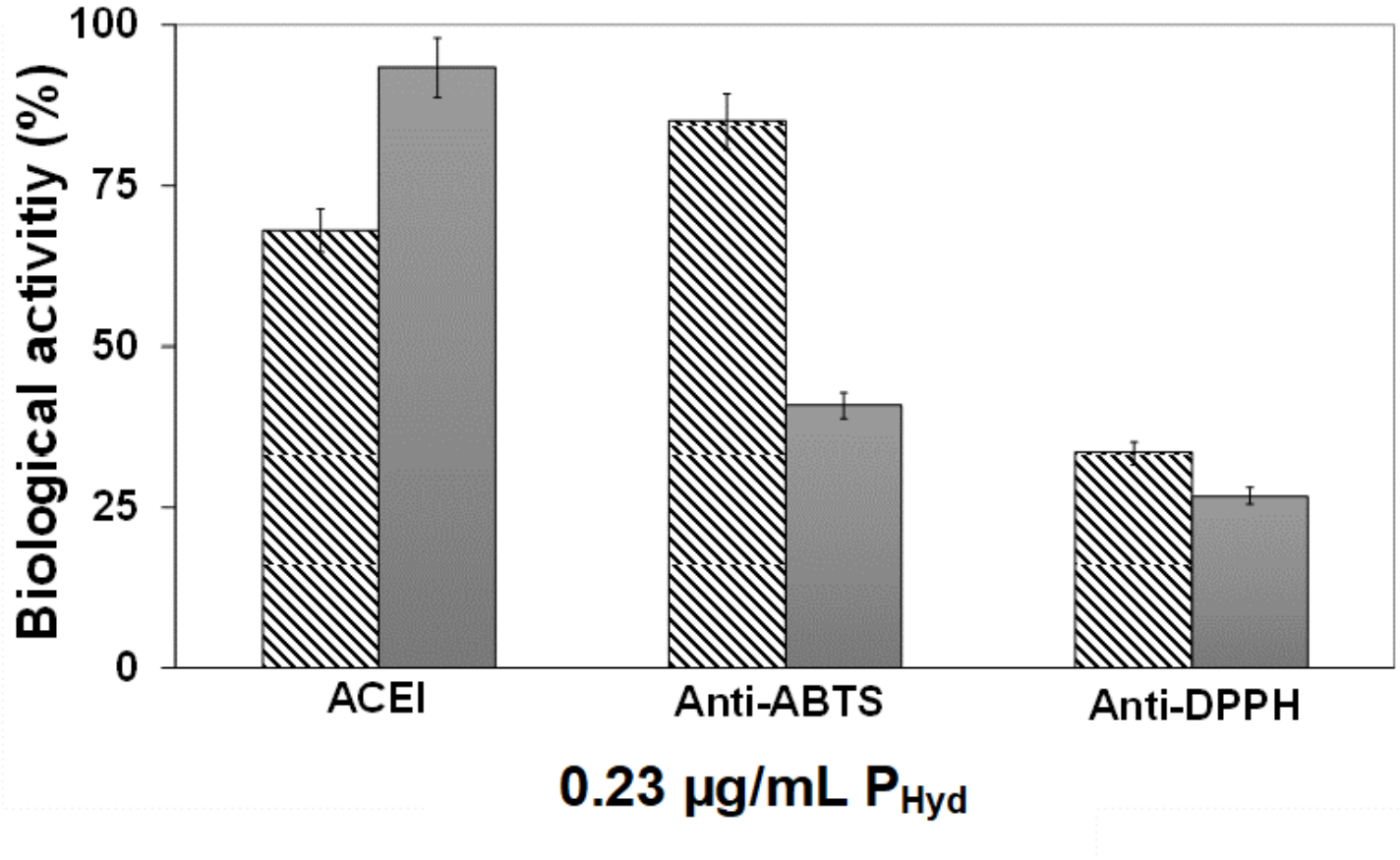


Figure 6

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

Supplementary Files

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