Production and Characterization of Chitosan extracted from crab shell as a clarifying agent for fruit juices

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Abstract:

Background and aim: The seafood industry generates enormous amounts of waste that negatively affect the environment. Seafood waste often contains chitosan, which can be converted to value-added and industrially significant products. The present study aimed to produce and characterize chitosan from crab shells as a clarifying agent in the food industry.

Methods: Different varieties of crab shells belonging to *Portunus pelagicus* (female)*, Portunus sanguinolentus, Portunus pelagicus* (male), and *Charybdis cruciata* were collected. Chitosan was extracted chemically, and its structural and physicochemical characterization was studied. Its use in fruit juice clarification was evaluated.

Results: Based on the yield and other physicochemical properties, *Portunus sanguinolentus* have been chosen for further studies. The antimicrobial activity of chitosan powder was checked against various food-borne microbes. It was observed to be effective against *Staphylococcus aureus*, *E. coli*, *Klebsiella* sp., *Shigella* sp., and *Salmonella* sp. Chitosan was used as a clarifying agent for muskmelon and watermelon juice and showed potent clarification ability.

Conclusion: It was proved that crab shell chitosan could be used as a natural alternative to a synthetic food additive as a clarifying agent in the food industries.

Keywords: Crab shell, Chitosan, *Portunus sanguinolentus*, Antimicrobial activity, Clarifying agent
Running title: Chitosan as fruit juice clarifier

1. Introduction:

Waste generated from a marine source and its processing industry has increased enormously, which might cause environmental degradation and severe pollution problems if not processed or recycled. The seafood industry generates about 106 tons of waste annually, most of which is destined for composting or converted as low-value-added products such as animal feed and fertilizers (1, 2). Marine waste is a potential resource with

high-added value as it contains proteins, lipids, and chitin. Chitin is one of the most significant polysaccharides in natural macromolecules and a typical component of crustaceans, fungal cell walls, molluscs, and insect exoskeletons. Chitin is insoluble in common solvents due to its high crystal structure, acetamido, and hydrogen bonding between hydroxyl and carbonyl groups. As they have poor solubility and biodegradability, the use of chitin for industrial applications has been limited (3, 4). Chitosan is a linear polysaccharide consisting of β-(1→4)-linked glucosamine and N-acetyl-d-glucosamine obtained on deacetylation of chitin. Since chitosan has better solubility, admirable biocompatibility, and degradability properties, it could be used for various industrial applications, including the food industries (5).

Clarification is an essential step in processing fruit juice to remove pectin and other carbohydrates present in the juice. Generally, clarifying procedures can be achieved by centrifugation, enzymatic treatment or applying clarifying agents such as gelatin bentonite, silica sol and polyvinyl pyrrolidone. However, these processes can be labour-intensive, time-consuming, and discontinuously operated. Chitosan is an effective coagulating agent in separating suspended particles from beverages (6). It is known to exhibit antimicrobial activity against some bacteria, yeasts, and moulds with low toxicity in mammalian cells and antioxidant activity. Hence, it could be used to extend the shelf-life of various fruit and fruit juices (7). The scope of the present work is to produce and characterize the chitosan extracted from crab shells and to evaluate the chitosan as a clarifying agent in fruit juice processing.

2. Materials and Methods:

2.1 Collection and Coarse purification of Crab shells

Different varieties of crab shells were collected from Ukkadam Fish Market, Coimbatore, Tamil Nadu, India and identified. The origin, distribution, scientific name, common name and vernacular name of the crab shells were noted. The collected crab shells were washed with distilled water to remove the impurities adhered to the shell and dried overnight at 110^{o}C in a hot air oven (8).

2.2 Extraction of Chitosan from Crab shells

The extraction of chitosan involved four steps, deproteinization, demineralization, decolourization and deacetylation. The collected crab shells were deproteinized with 3.5% (w/w) sodium hydroxide (NaOH) solution for 2 hours at 65^{o}C with constant stirring. Deproteinized shells were demineralized with 1N HCl for 30 minutes at ambient temperature with constant stirring. The above-treated crab shells were decolourized with acetone for 10 minutes and dried for 2 hours at ambient temperature. Following this, bleaching was done with 0.315% sodium hypochlorite solution for 5 minutes at ambient temperature. The shells were filtered, washed with distilled water, and oven-dried after each step. The deacetylation of chitin was performed with 50% concentrated NaOH solution at 100^{o}C for 5-6 hours, washed with distilled water and oven-dried at 60^{o}C for 24 hours. The processed crab shells were made into powder (9).

2.3 Screening of Chitosan

A 0.1 g of chitosan powder was taken, added with 2-3 drops of

iodine/potassium iodide (KI) solution and mixed well. The mixture was then acidified with 2-3 drops of 1% H_2SO_4 (Sulphuric acid). A characteristic colour change to dark brown on the addition of iodine and a further change to purple on the addition of H_2SO_4 indicated the presence of Chitosan (10).

2.4 Estimation of Chitosan

A 50 mg of chitosan powder was accurately weighed, wetted with 0.2 ml of distilled water, and soaked for 15 minutes for swelling of the matrix in sintered glass funnels. About 0.2 mL of Bromocresol purple dye solution was added, and the excess dye was drained with 0.5 mL of deionized water. After that, 95% ethanol was added to remove the colours. The chitosan-bound dye was stripped off with 20 mL of 1N HCl solution and filtered through a 0.45 µ membrane filter. 5 mL aliquots were withdrawn from each sample, transferred to a 50 mL volumetric flask, and made to the volume of 50 mL with 1N NaOH solution. The development of blue colour was observed and measured spectrometrically at 589 nm (UV-Vis spectrophotometer 118, Systronics, Gujarat, India) (11).

2.5 Structural characterization of Chitosan

The chitosan has been characterized with FTIR (Fourier-Transform Infrared) spectroscopy (Shimadzu – IR affinity 1, Kyoto, Japan). The chitosan was mixed with 200 to 250 mg of KBr (Potassium bromide) and subjected to the IR spectrum, which measures the wavelength of 4000 cm^{-1} to 400 cm^{-1}. The functional group of chitosan was determined with the obtained spectra (12).

2.6 Physicochemical characterization of Chitosan

a) Yield

The yield of chitosan was determined by comparing the weight of raw material to the weight of chitosan obtained after treatment (13).

b) Solubility

The solubility of chitosan was measured according to the procedure of Kumari *et al*. (14). The chitosan was dissolved in 1% acetic acid and subjected to centrifugation. The supernatant was discarded, and the undissolved solid mass was taken, oven-dried and weighed. The solubility of chitosan was evaluated by using the formula,

Solubility (%) = [Initial weight of tube + Chitosan (g)] – [Final weight of tube + chitosan (g)] / [Initial weight of tube + Chitosan (g)] – [Initial weight of tube] x 100

c) Ash content

The ash content of chitosan was evaluated by the gravimetric method. Chitosan was taken in a crucible, heated with a spirit lamp until it burnt entirely and turned ash. Then the crucible was put in a muffle furnace at 600°C for about 3 to 5 hours. It was cooled in a desiccator, and the weight of the crucible was noted. The crucible was again heated in the muffle furnace for half an hour, cooled and weighed. This process was repeated until two consecutive weights were found to be the same. Finally, the ash content of chitosan was determined using the formula (15),

Ash content (%) = Weight of ash (g) / Initial weight of chitosan (g) x 100

d) Moisture content

The moisture content of chitosan was determined by the gravimetric method. The chitosan's dry weight and wet

weights were measured before and after drying. Then the moisture content was calculated by using the formula (14),

Moisture content (%) = Wet weight of chitosan (g) / Dry weight of chitosan (g) x 100

e) Water binding capacity (WBC)

The water binding capacity of chitosan was evaluated by weighing the tube containing 0.5 g of chitosan and added with 10 mL of water. It was then mixed in a vortex mixer for 1 minute and left at ambient temperature for 30 minutes with shaking of 5 seconds every 10 minutes. The contents were centrifuged at 3500 rpm for 25 minutes, and the supernatant was discarded. The tube was weighed again, and WBC was calculated by using the formula (16),

WBC (%) = Water bound chitosan (g) / Initial weight of chitosan (g) x 100

f) Fat binding capacity (FBC)

The FBC of chitosan was evaluated by weighing the tube containing 0.5 g of chitosan and added with 10 mL of soybean oil. It was then vortexed for 1 minute and left at ambient temperature for 30 minutes with shaking of 5 seconds every 10 minutes. The contents were centrifuged at 3500 rpm for 25 minutes, and the supernatant was discarded. The tube was weighed again, and FBC was calculated by using the formula (16),

FBC (%) = Fat bound chitosan (g) / Initial weight of chitosan (g) x 100

2.7 Antimicrobial activity of Chitosan against food-borne pathogens

The antimicrobial activity of chitosan against food-borne pathogens was evaluated using the agar-well diffusion method. Briefly, the overnight bacterial strains, including *Staphylococcus aureus*, *Bacillus* sp., *Escherichia coli*, *Klebsiella* sp., *Salmonella* sp., and *Shigella* sp., were prepared and adjusted with 0.5 McFarland standards. Mueller Hinton agar (MHA) plates were prepared, and a 6 mm well was made using a sterile cork borer. The bacterial strains were swabbed onto the surface of the MHA. Then the chitosan sample was dissolved in sterile distilled water and serially diluted. The chitosan sample with the concentration of 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} was loaded in the wells of MHA and incubated at 37ºC for 24 hours. Followed by incubation, the zone of inhibition was measured (17).

2.8 Clarification of fruit juice with chitosan

Watermelon and muskmelon juice was prepared, and it was clarified by adding 0.2% of chitosan obtained from crab shell and stirred. Then it was subjected to centrifugation at 5000 rpm for 10 minutes. The juice sample without chitosan served as a control (18).

3. Results and Discussion:

3.1 Collection and Processing of Crab shells and Extraction of Chitosan

Among the various types of crabs, four varieties of crab shells were collected from Ukkadam Fish Market, Coimbatore, Tamil Nadu, India and identified (Figure 1). The origin, distribution, scientific name, common name, and vernacular name of the collected crab shells were tabulated (Table 1). The chitosan was extracted from the crab shells by deproteinization, demineralization, decolourization, and deacetylation processes. The processed chitosan was made into powder and stored in an air-tight container for further use.

Figure 1. Collection of crab shells

(a)Blue swimming crab (Male)

(b)Blue swimming crab (Female)

(c)Three-spot swimming crab

(d)Christ shell crab

Table 1. Identification of collected crab shells

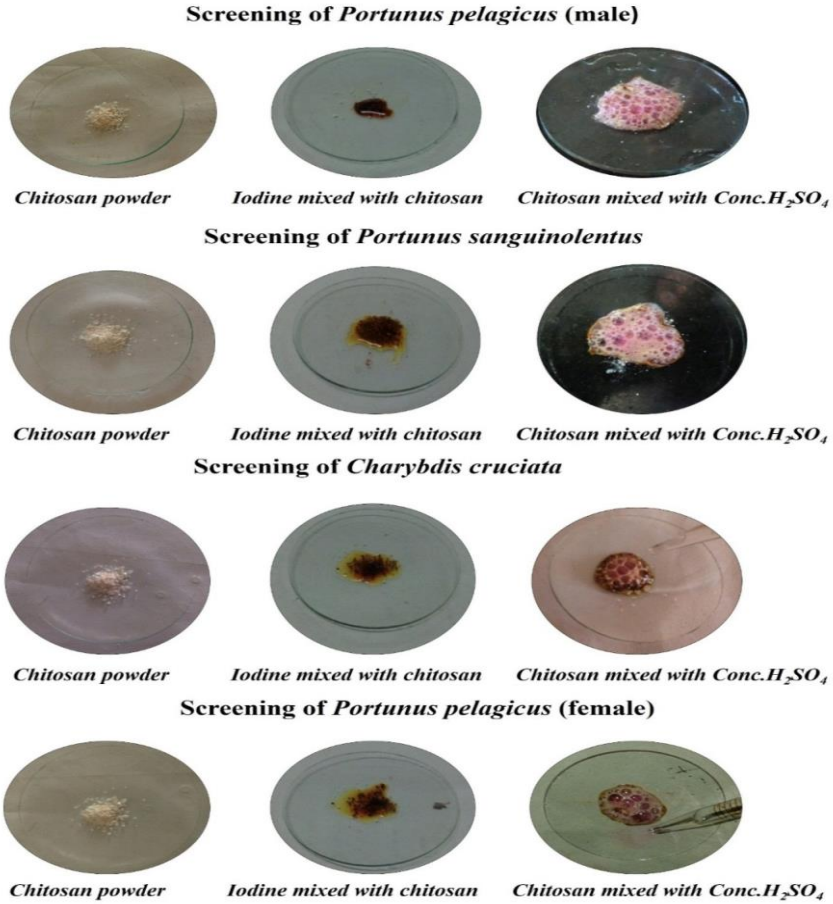
Blue swimming crab (Male)	
Origin	Intertidal estuaries of Indian and West Pacific Ocean
Distribution	Australia, Eastern Africa, Indonesia, New Zealand, Persian Gulf, Southeast Asia
Scientific name	*Portunus pelagicus*
Common name	Blue crab, Blue manna crab, Blue swimmer crab, Flower crab, Sand crab
Vernacular name	Olakkal nandu, Pulli nandu
Blue swimming crab (Female)	
Origin	Intertidal estuaries of Indian and West Pacific Ocean
Distribution	Australia, Eastern Africa, Indonesia, New Zealand, Perdian Gulf, Southeast Asia
Scientific name	*Portunus pelagicus*
Common name	Blue crab, Blue manna crab, Blue swimmer crab, Flower crab, Sand crab
Vernacular name	Olakkal nandu, Pulli nandu
Three-spot swimming crab	
Origin	Intertidal estuaries of Indian and West Pacific Ocean
Distribution	Read sea, Persian Gulf, Mozambique, South Africa, Madagascar, Mauritius, Pakistan, India, Maldives, Srilanka, Myanmar, Thailand, Japan, Korea, Taiwan, China, Singapore, Indonesia, Philippines,

	Australia, Hawali
Scientific name	*Portunus sanguinolentus*
Common name	Blood-spotted swimming crab, Red-spotted swimming crab
Vernacular name	Mukkannu nandu
Christ shell crab	
Origin	Mediterranean Sea
Distribution	Indo-Pacific region, Persian Gulf to Japan, Indonesia and Australia, East Africa,
Scientific name	*Charybdis feriata / Charybdis cruciata*
Common name	Crucifix crab
Vernacular name	Siluvai nandu

3.2 Screening of Chitosan

A characteristic colour change to dark brown with the addition of iodine was observed. It was further changed to purple with the addition of sulphuric acid, which revealed the presence of chitosan obtained from the crab shells (Figure 2). This was well in accordance with the results of Panchani & Pandya (19), who qualitatively determined the chitosan using Lugol's iodine solution.

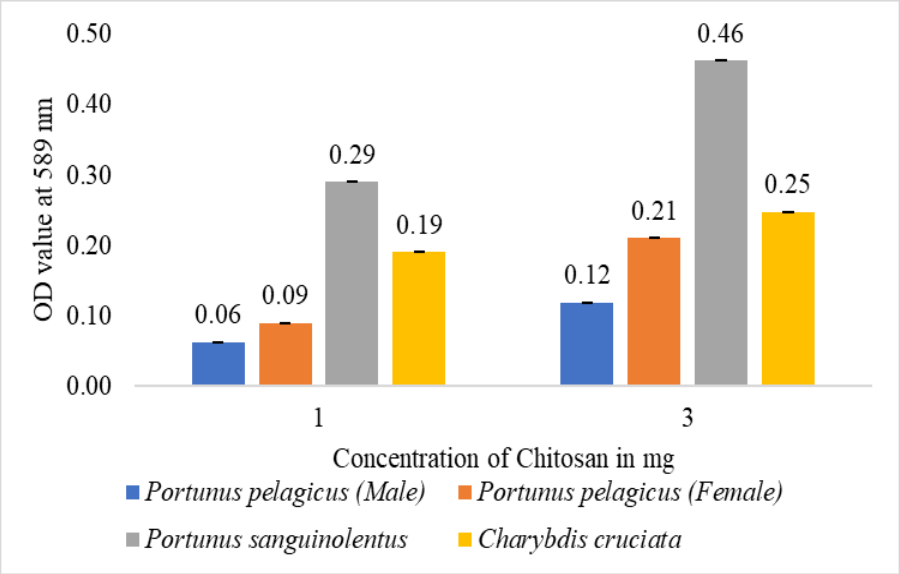
Figure 2. Screening of Chitosan



3.3 Estimation of Chitosan

The chitosan was quantitatively estimated, and it was observed that *Portunus saguinolentus* have higher amounts of chitosan than other chitosan extracted from crab shells (Figure 7). The estimation of chitosan was directly proportional to the degree of deacetylation process performed during the chitosan extraction, thereby it reflects the quantity of free amino group present in the chitosan relative to the fully acylated chitosan (chitin) as stated by Abou-Shoer, (11).

Figure 3. Estimation of chitosan



3.4 Structural characterization of Chitosan

The functional groups of chitosan were determined. The FTIR characterization of chitosan of *Portunus pelagicus* (Male) revealed that it has the presence of hydroxyl group (3641.6 & 956.69), alkanes (2885.51), imine / azo groups (1658.78), and silicon groups (1033.85) (Figure 4). The FTIR analysis of chitosan of *Portunus pelagicus* (Female) showed the presence of hydroxyl group (3441.01), alkanes (2924.09 & 2893.22), tertiary amine (2376.3), silicon hydroxide (2137.13), nitro group (1265.3), and carbonates (871.82) (Figure 5). The functional groups of chitosan of *Portunus sanguinolentus* were observed as hydroxyl group (3641.6), alkanes (2924.09), NH groups (1651.07), nitro group (1627.92), and aliphatic N-oxide (956.69 & 871.82) (Figure 6). The chitosan of *Charybdis cruciata* revealed a hydroxyl group (3633.89), alkanes (2924.09), NH group (2376.3), C-N & N-N groups (2144.84), sulphonic acid (1257.59), and silicon group (1072.42) as their active functional compounds (Figure 7). All these resultant peaks were well in accordance with the results of Ahyat *et al*. (20), Babatunde *et al*. (21), Varma & Vasudevan (22).

Figure 4: FTIR analysis of chitosan of *Portunus pelagicus* (Male)

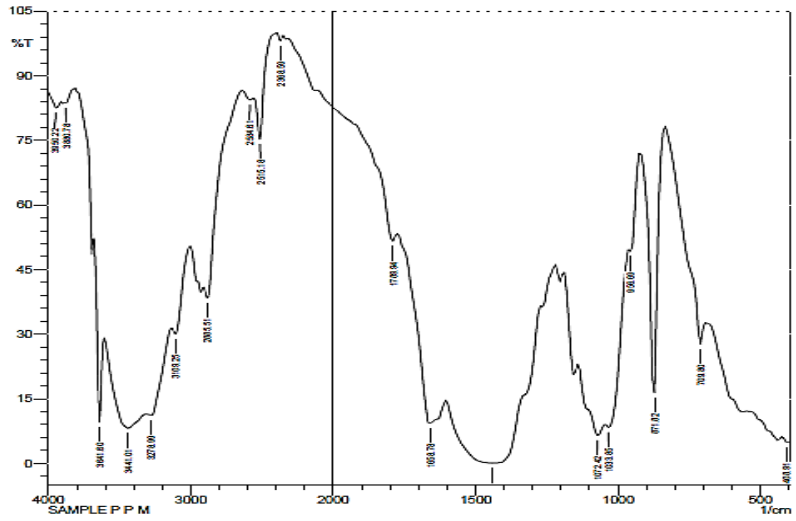


Figure 5. FTIR analysis of chitosan of *Portunus pelagicus* (Female)

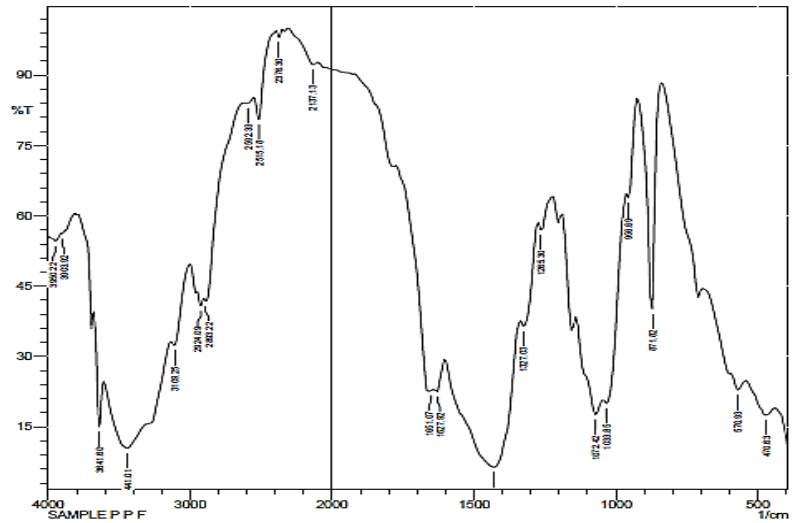


Figure 6. FTIR analysis of chitosan of *Portunus sanguinolentus*

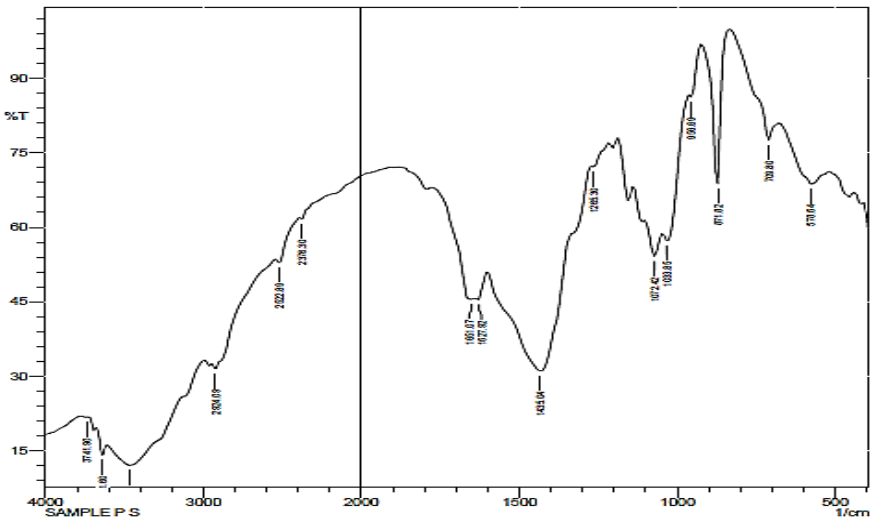
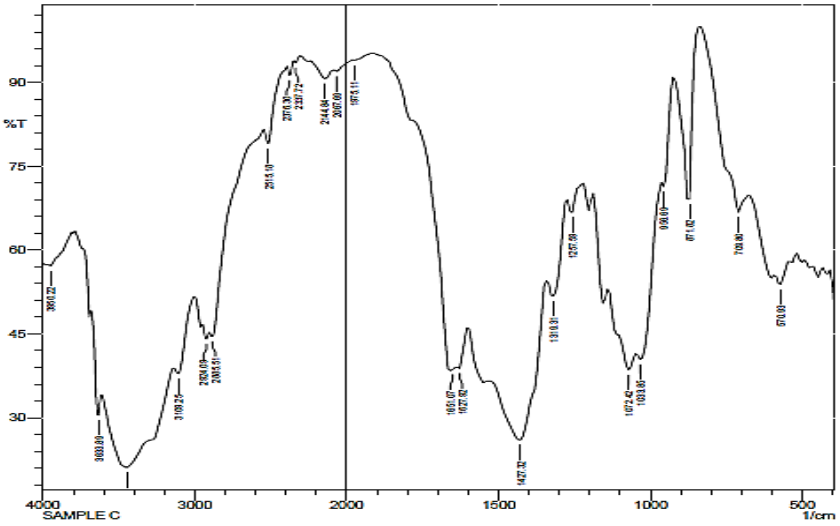


Figure 7. FTIR analysis of chitosan of *Charybdis cruciata*



3.5 Physicochemical characterization of Chitosan

a) Yield

The percentage yield of chitosan was determined and has been tabulated (Table 2). It was found that *Portunus sanguniolentus* has a high yield of chitosan of 43.86%. Similarly, Abirami *et al*. (23) yielded chitosan of 37.5% from the crab shell. The maximum yield of chitosan was dependent on the chemical extraction of chitosan (24).

Table 2. Yield of Chitosan

Crab shell variety	Percentage yield of chitosan
Portunus pelagicus (Male)	37.40%
Portunus pelagicus (Male)	43.73%
Portunus sanguinolentus	43.86%
Charybdis cruciata	38.93%

b) Solubility

The solubility of chitosan was measured and tabulated (Table 3). It was observed that chitosan of *Portunus sanguniolentus* has high solubility of 87%. These results were in well accordance with the result of Metin *et al*. (25), who reported that the solubility of blue crab shells was 94.15%. Due to the presence of primary amino groups, chitosan was greatly soluble in dilute acidic solutions having a pH below 6. At low pH, these amines get protonated and become positively charged and which causes chitosan to become a water-soluble cationic polyelectrolyte (26).

Table 3. Solubility of Chitosan

Crab shell variety	Percentage solubility of chitosan
Portunus pelagicus (Male)	70.00%
Portunus pelagicus (Male)	72.72%
Portunus sanguinolentus	87.00%
Charybdis cruciata	54.54%

c) Ash content

The ash content of chitosan was evaluated and tabulated (Table 4). It was noted that *Charybdis cruciata* and *Portunus pelagicus* (Male) exhibited high ash content compared to *Portunus pelagicus* (Female) and *Portunus sanguinolentus*. Similarly, Putri *et al*. (27), obtained high ash content of 0.45% for chitosan obtained from Haruan Fish-Scales. However, Marei *et al*. (28) stated that a high-quality grade of chitosan must have a percentage of less than 1. Other than *Charybdis cruciata*, all other chitosan has an ash content percentage of less than 1, which in turn depicts the high quality of chitosan.

Table 4. Ash content of Chitosan

Crab shell variety	Percentage ash content of chitosan
Portunus pelagicus (Male)	0.73%
Portunus pelagicus (Male)	0.94%
Portunus sanguinolentus	0.82%
Charybdis cruciata	1.17%

d) Moisture content

The moisture content of chitosan was determined and tabulated (Table 5). Youn *et al*. (29) stated that based on Korea food and drug administration (KFDA), the moisture content of chitosan must be below 10%. The moisture content of all chitosan samples was observed to be less than 3%. Similarly, Gokilavani *et al*. (30) reported that the moisture content of chitosan extracted from the crab shell of *Paratelphusa hydrodromous* was 0.48±0.18%.

Table 5. Moisture content of Chitosan

Crab shell variety	Percentage moisture content of chitosan
Portunus pelagicus (Male)	0.13%
Portunus pelagicus (Male)	0.16%
Portunus sanguinolentus	0.22%
Charybdis cruciata	0.11%

e) Water binding capacity (WBC)

The water-binding capacity of chitosan was evaluated and tabulated (Table 6). The highest WBC was observed for *Portunus sanguinolentus* was 230%. The results were similar to the WBC of chitosan obtained from *Penaeus monodon* was 118%, as reported by Yusof *et al*. (31). It was known that the water absorption of chitosan depended on differences existed in the crystallinity of the products, particle size, number of salt-forming groups, and protein content, as stated by Tamzi *et al*. (32). It was also having a high negative correlation with physiological characteristics, such as viscosity and molecular weight, degree of deacetylation, and moisture content (32).

Table 6. Water binding capacity of Chitosan

Crab shell variety	Percentage WBC of chitosan
Portunus pelagicus (Male)	122%
Portunus pelagicus (Male)	226%
Portunus sanguinolentus	230%
Charybdis cruciata	202%

f) Fat binding capacity (FBC)

The FBC of chitosan was determined and tabulated (Table 7). The rate of FBC ranged between 300-430% for the extracted chitosan. The result obtained in our study was higher compared with the commercially available chitosan (33). The FBC of chitosan was proportional to the steps involved in chitosan extraction. During the extraction process, if demineralization is performed as the first step, the rate of fat binding capacity will be elevated. However, if deproteinization is performed as the first step, then FBC content will be decreased (34). However, in contrast to this statement, deproteinization was the first step that proceeded in the study, high FBC of chitosan was obtained.

Table 7. Fat binding capacity of Chitosan

Crab shell variety	Percentage FBC of chitosan
Portunus pelagicus (Male)	378
Portunus pelagicus (Male)	346
Portunus sanguinolentus	430
Charybdis cruciata	304

Based on the higher yield and other physiochemical properties, chitosan extracted from *Portunus sanguinolentus* was chosen for further studies.

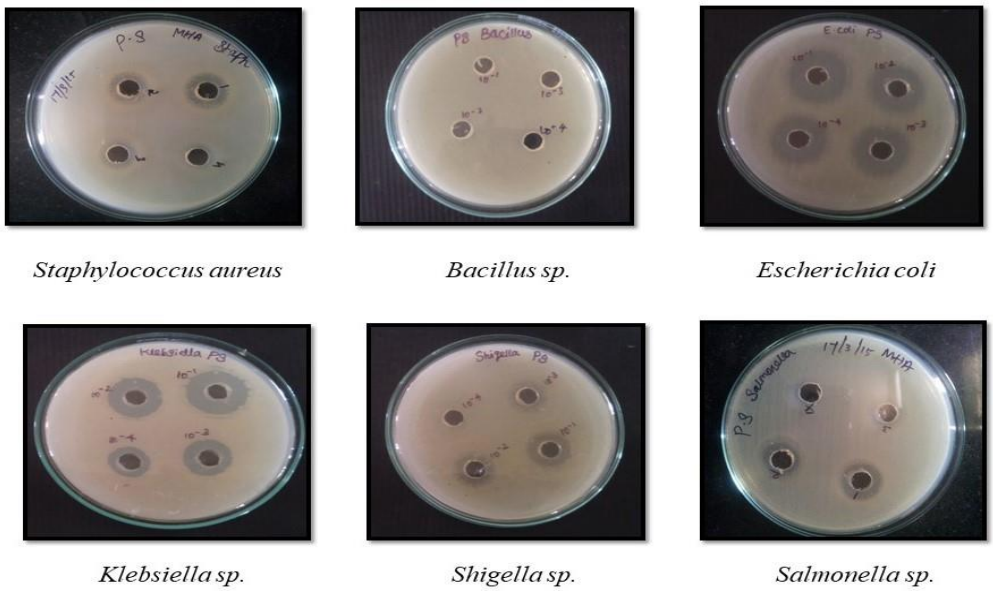
2.8 Antimicrobial activity of chitosan against food-borne pathogens

The antimicrobial activity of chitosan was evaluated, and it was observed that chitosan exhibited predominant antimicrobial activity towards *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella* sp., *Salmonella* sp., *and Shigella* sp. (Table 8; Figure 8). Similarly, Chien *et al*. (35) reported that chitosan has exhibited maximum activity of 12 mm against gram-positive and gram-negative bacterial pathogens. The exact mode of action of chiton on microbial pathogens was not completely understood. But it could be attributed that the antimicrobial activity of chitosan is due to the interaction between the positively charged amino group of chitosan and the negatively charged microbial cell wall. This disrupts the proteinaceous and other intracellular constituents of the microorganisms, or it promotes altering the properties of cell wall permeability, thus provoking internal osmotic imbalances, and consequently inhibiting the growth of microorganisms (36).

Table 8. Antimicrobial activity of Chitosan

Pathogens	10^{-1}	10^{-2}	10^{-3}	10^{-4}
Staphylococcus aureus	17	15	13	10
Bacillus sp.	-	-	-	-
Escherichia coli	24	22	21	20
Klebsiella sp.	22	20	18	15
Salmonella sp.	17	15	13	10
Shigella sp.	17	15	13	10

Figure 8. Antimicrobial activity of chitosan



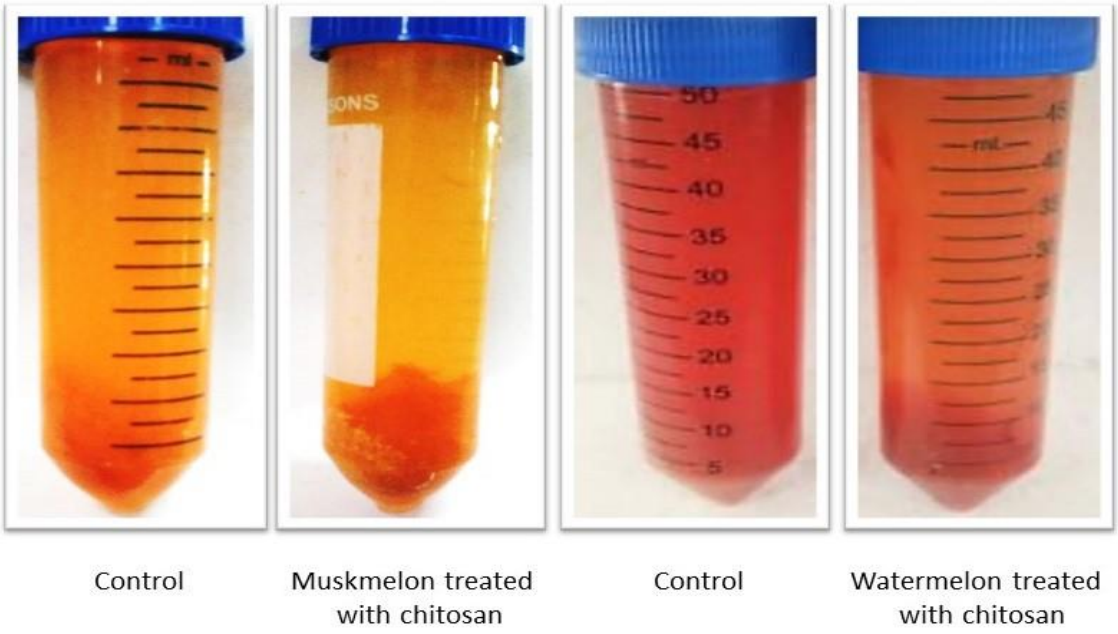
Staphylococcus aureus *Bacillus sp.* *Escherichia coli*

Klebsiella sp. *Shigella sp.* *Salmonella sp.*

2.9 Clarification of fruit juice with chitosan

Both watermelon and muskmelon juice were clarified with the chitosan of *Portunus saguinolentus*, and it was found that chitosan could be an excellent clarifying agent (Figure 9). It could also help in the preservation of fruit juices as they exhibit antimicrobial activity against food-borne pathogens. Similar to the results, Rungsardthong *et al*. (37) also performed the apple juice clarification with chitosan and observed with a gradual decrease in turbidity and reached maximum clarity. As chitosan is a cationic flocculant, it can be combined with the negatively charged pectin, soluble starch, protein and microparticles through positive and negative charge attraction to form floc precipitation. In addition, chitosan transfers small particles into large aggregates (bridge formation) and adsorbs dissolved organic substances onto the aggregates by an adsorption mechanism that can then be removed easily by filtration and sedimentation as stated by Ghorbel-Bellaaj *et al*. (38).

Figure 9. Fruit juice clarification using chitosan



Conclusion:

Clarification is a major step in the fruit juice processing industry. This work explored the use of chitosan, which was obtained from the crab shell (*Portunus sanguinolentus*), as a fruit juice clarifier proved to be highly effective. In this way, the clarification process will be carried out without enzymes and will be performed in a short time in accordance with the fruit juice industry. To further reduce the cost of chitosan production, the improvement of cheaper production methods with the development of greener technology would be opted. Furthermore, chitosan could propose to use as an alternative aid for fruit juice clarification with high performance. Chitosan which is obtained from crab shells would be economically significant in the fruit industry.

Conflict of Interest:

Each author declares that he or she has no commercial associations (e.g. consultancies, stock ownership, equity interest, patent/licensing arrangement etc.) that might pose a conflict of interest in connection with the submitted article

Authors contribution:

DDR, PS, SPR, KN, MA: Conceptualization, Methodology, Investigation, Data Curation; DDR, PS, SPR: Original draft preparation; DDR, KN, MA: Review and Editing

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