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Optimization of Chitin Extraction, Physicochemical and Functional Properties of Chitosan Production from Shells of Karamote Shrimp *Peneaus* (*Melicertus*) *Kerathurus* in Western Greece

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Abstract

This research aims to optimize by chemical methods the extraction of chitin and chitosan from shells of *Karamote shrimp Peneaus (Melicertus) kerathurus*. Shrimp waste can be used as source of high value compounds. Chitin is a major component of the exoskeleton of invertebrates and chemically is a linear polysaccharide of β (1→4) linked N-acetylglucosamine monomers. Chitosan is a deacetylated form of chitin having d-glucosamine repeating units linked by β (1-4) glycosidic bond. The extraction method uses different concentrations of sodium or potassium hydroxide in the deproteinization (protein separation) and deacetylation (remove acetyl groups) step and hydrochloric acid for demineralization (separation of calcium carbonate and calcium phosphate) to yield optimum output. Among all experiments, results of 1.0 N solution of HCl for demineralization, 2 N for deproteination and 12.5 N NaOH solutions for deacetylation at solid to solvent ratio of 1: 15, clearly demonstrate a significant yield of chitin and chitosan. The results obtained from this study show also that the solubility of chitosan in 1% acetic acid solution achieved up to 90%.

Introduction

In recent years great interest has been expressed in isolating components using by-products and wastes. Fish wastes include byproducts or many fish species having no or low commercial value, undersized or damaged commercial species. Large amounts of culture wastes are associated with the environmental impact on aquatic ecosystems, since the release of organic wastes might significantly change the community structure and biodiversity of the benthic assemblages [1,2]. The objective of reducing fishery discards and to avoid environmental problems can be achieved by establishing alternative solutions, such as technologies to enhance and transform fish wastes as an economic resource, for example by developing techniques of extraction and concentration of the bioactive compounds they contain. Crustaceans, belonging to the Decapoda order, include prawns, shrimps, lobsters, crayfish and crabs. *Melicerthus kerathurus* known as karamote prawn is one of the above group. It is a demersal crustacean, widely distributed inhabiting the Mediterranean Sea and the eastern Atlantic from the south coast of England to Angola where it lives on soft bottoms of the continental shelf, less than 60 m depth [3]. The shell of crustaceans consists of 20-30% chitin, 30-40% protein and 30-50% calcium carbonate and calcium phosphate, and other minor constituents, such as lipids, astaxanthin and other minerals [4]. Chitin and chitosan are β (1-4) glycans whose chains are formed by 2-acetamide-2-deoxy-D-glucopyranose and 2-amino-2-deoxy-D-glucopyranose units, respectively. Chitin is the second most abundant polysaccharide on earth, following the cellulose. Chitin can be obtained from the cell wall of fungi, the exoskeleton of arthropods, the shells of mollusks and the beaks of cephalopods including cuttlefish, octopuses and squids. Chitin is presented mainly in three allomorphs: α-chitin, with antiparallel chains, is the most abundant and it is isolated from the exoskeleton of crustaceans, particularly from shrimps and crabs; β-chitin, with parallel chains, is presented in the cell walls of diatoms and in the skeletal structures of cephalopods, and commonly extracted from squid pens; γ-chitin is presented in fungi and yeast, which is a combination of the α and β allomorphs [5]. Chitosan is generally prepared by the deacetylation of chitin with alkali. Due to their useful biological properties (biocompatibility, biodegradability, antimicrobial activity) and chemical modification potentials because of their reactive functional groups (-OH, -NH_2, and -COOH) both have been used in a wide range of fields including biomedical, food production, and wastewater treatment fields [6-8]. The current research was performed to evaluate the suitable acid and alcali concentration for extraction of high-quality chitin and chitosan from shells of *Melicertus kerathurus*.

Materials and Methods

Description of the fishing area

The experimental fishery of the shrimps was carried out twice in October 2019 in Kalamos channel, along the Western Greek coast of the Ionian Sea, near the Kalamos Island (**Lat.**38.61581° N, **Log.**20.90102° E). Administratively, the island belongs to the Prefecture of Lefkada Island covering an area of 25 km^2.

Raw material

A total number of 234 specimens were collected. Firstly, the shells were removed from the animal and secondly the specimens were packed in polyethylene bags, placed on ice, transported to the laboratory and were stored in a freezer at -20 °C until further use.

Reagents

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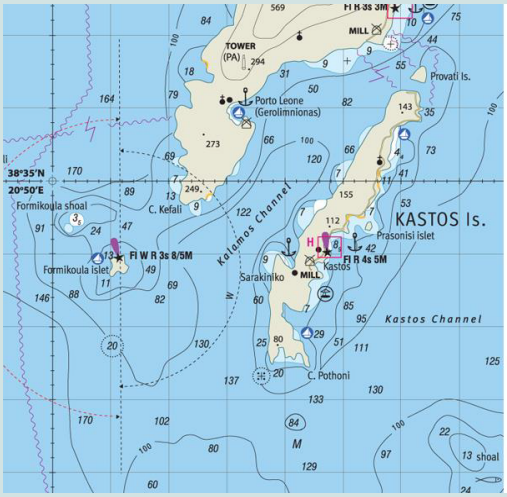

Figure 1A: Map of the study area in Kalamos channel along the Western Greek coast.

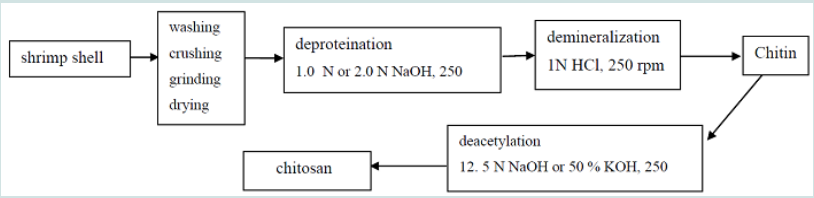

Figure 1B: Flow diagram of extraction of chitosan from shells of shrims.

All the chemicals and solvents used were purchased from Sigma-Aldrich at the analytical grade or highest level of purity available and used as received. A commercial chitosan with a deacetylation degree of 75% was chosen. All solutions were freshly prepared in distilled water.

Methods

Laboratory sample preparation was needful to convert the shell sample into a homogeneous material suitable for analysis. Before grinding, the biggest parts of shell samples were crushed and divided in smaller. Drying of samples was obtained by heating in a drying oven (model R. Espinar, S.L.) at 100-103 °C until constant weight was obtained between two sequential measurements [9,10]. Drying samples grinded in a mill (System POLYMIX® PX-MFC 90 D) into smaller particles using sieve with 2 mm wide openings. pH measurements were made using a digital laboratory pH meter (model WTW pH 525) which was calibrated using certified pH= 4.0 and pH= 7.0 buffer solutions, according to the official method [9]. The Ether Extract (EE) was determined using method of Soxhlet. Approximately 2000 mg of solid sample were mixed with anhydrous sodium sulfate, placed in an extraction thimble and were extracted using an appropriate solvent in the Soxhlet extractor. The distilled solvent was condensed and in final drying step the remaining traces of solvent was evaporated from the boiling flask. The mass of the extract (total fat) was measured after subtracting initial from final weight of the boiling flask. Ash contents were determined using dry ashing method. The samples (2000 mg) were ashed for about 8 hr. until a white or grey ash residue had been obtained using a furnace (model P. Selecta, 3000 W) where temperature had been gradually increased from room temperature to 450 °C in 1 h [9,10]. The *solubility of chitosan* was carried in dilute solution of acetic acid. 1000 mg of chitosan obtained from the deacetylation process was dissolved in 100 mL of 1% acetic acid solution and stirred by magnetic stirrer until a homogeneous solution was obtained. The chitosan acidic solution was then filtered using a vacuum pump. The procedure was repeated three times. The insoluble content was calculated from the weight of insoluble particles obtained on the filter and the weight of chitosan dissolved. The *water binding capacity (wbc)* was calculated as follows; 10 ml of distilled water with 1000 mg of chitosan was mixed on a vortex for 15 min and centrifuged at 3500 rpm for 30 min. After centrifugation, supernatant water was poured off and the sample was weighed. WBC (%) = [Bound water (g)/Initial chitosan weight (g)] * 100. The *oil binding capacity (obc)* was calculated as follows; 10 ml of sunflower with 1000 mg of chitosan was mixed on a vortex for 15 min and centrifuged at 3500 rpm for 30 min. After centrifugation, supernatant oil was poured off and the sample was weighed. OBC (%) = [Bound oil (g) / Initial chitosan weight (g)] * 100 [10].

Extraction of chitin by chemical method

Deproteination (Dp): A total of 5-20 g dry samples of raw shrimp shell waste were treated with 1.0 N and 2.0 N NaOH at solid to solvent ratio 1:5, 1:15, 1:18 and 1:20 (w/v), with constant stirring at 200 rpm for 24 hours at room temperature, with pH ranged from 11-13. After that, the solution was filtered and the samples were washed with distilled water to neutrality in running tap water. Water from the samples was removed before performing the demineralization

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Table 1: Experimental details.

	Experiment				
Parameters	I	II	III	IV	V
Molarity of solution for deproteination	1.0 N	2.0 N	1.0 N	2.0 N	1.0 N
Solid to solvent ratio	1:15	1:15	1:18	1:5	1:20
Yield % after deproteination	47.7	62.73	60.71	58.14	60.24
Molarity of solution for demineralization	1.0 N	1.0 N	1.0 N	1.0 N	1.0 N
Solid to solvent ratio	1:10	1:10	1:10	1:10	1:10
Yield % after demineralization	34.8	31.18	29.31	34.1	28.89
Molarity of solution for deacetylation	12.5 N	12.5N	12.5 N	12.5 N	50 % KOH
Solid to solvent ratio	1:10	1:10	1:10	1:10	1:10
Yield % after deacetylation	56.45	79	38.13	49.07	63.43
Stirring period for Dp, Dm, Da **Rpm**	24 h 250	24 h 250	24 h 250	24 h 250	24 h 250
Centrifugation (wbc, obc) **Time**	3500 rpm 30 min	3500 rpm 30 min	3500 rpm 30 min	3500 rpm 30 min	3500 rpm 30 min

Table 2: Yield % of chitin and chitosan.

Experiment	Shrimp shells (g)	Chitin (g)	Chitosan (g)	Chitin yield (%)	Chitosan yield (%)	
					Yield after deacetylation (as a ratio to chitin)	Final yield (as a ratio to initial dry shell)
I	7	1.16	0.65	16.6	56.45	9.3
II	7	1.37	1.08	19.56	79	15.46
III	5.5	0.97	0.37	17.8	38.13	6.7
IV	20	3.96	1.94	19.82	49.07	9.7
V	5	0.87	0.55	17.41	63.43	11

Table 3: Physicochemical and functional properties of chitosan (values are expressed as Mean± S.D (n=5).

Parameters	Value
pH	8.3±0.1
ether extract	0.41±0.2
Ash	0.22±0.08
solubility in 1 % acetic acid	93±0.27
Wbc	565±50.43
Obc	384±26.92
Color	Whitish slightly brow

process.

Demineralization (Dm): Samples from deproteination process were treated with 1.0 N HCl in the ratio 1:10 (w/v), with constant stirring at 200 rpm for 24 hours with pH value ranged pH 1.0-2.5 at room temperature. After that, the solution was filtered and the samples were washed with distilled water to remove acid and calcium chloride. The samples were then dried for 3 hours using an oven at 80 °C until constant weight was obtained. The dried sample is now known as chitin.

Chitosan production

Deacetylation (Da): The deacetylation process was conducted by soaking dried chitin prepared from demineralization in a 12.5 N solution NaOH and 50% (w/v) solution KOH with constant stirring at 200 rpm for 24 hours at room temperature. After that, the product is known as chitosan. Chitosan was washed with tap water until neutral (pH 6.5-8.0) and dried as described in deproteination and demineralization.

Chitin and chitosan yield

The percentage of the *yield* of *chitin* was calculated by dividing the weight of extracted chitin to initial dry shrimp shell weight.

Yield was calculated as follows: Yield of chitin (%) = (extracted chitin, g)/shrimp shells, g) * 100

The percentage of the *yield* of *chitosan* in relation to chitin was calculated by dividing the weight of produced chitosan to dry chitin before deacetylation.

Yield was calculated as follows: Yield of chitosan (%) = (produced chitosan, g)/chitin, g) * 100

The percentage of the *final yield of chitosan* was calculated by dividing the weight of produced chitosan to initial dry shrimp shell weight.

Figure 1A and 1B presents the extraction of chitosan from shells of shrimp.

Results

Parameters and details of experiments are demonstrated in Table 1. Some differences can be observed attributed to the changes of molarity of solutions (NaOH, KOH, HCl) for deproteination, demineralization and deacetylation and to the ratio of solid to

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solvent. As a result, the percentage of yield differs in all experiments. The higher values of yield after deproteination were observed in exp II (62.73) and III (60.71) while the lowest value was observed in exp I. Also, the percentage of yield after demineralization (as a ratio to previous step of deproteination), varied from 29% to 35%. The higher values of yield after demineralization were observed in exp I (34.8), IV (34.1) and II (31.18) while the lowest value was observed in exp V (28.89). The percentage of yield after deacetylation (as a ratio to previous step of demineralization), varied from 38% to 79%. The higher values of yield after deacetylation were observed in exp II (79) and V (63.43) while the lowest value was observed in exp III (38.13).

The percentage of yield of chitin and chitosan was presented in Table 2. Differences can be observed in the experiments. The percentage of yield of chitin varied from 16.6 to 19.82, the higher values were observed in exp II (19.56) and IV (19.82) while the lower value in exp I (16.6). Also, the percentage of yield of chitosan varied from 9.3 to 15.46, the higher value was observed in exp II (15.46) and V (11) while the lower value in exp III (6.7).

Functional and physicochemical properties of chitosan that have been studied in this work have shown a variety of characteristics as it is demonstrated in Table 3. The color of chitosan is whitish slightly brown while the percentages of water binding capacity and oil binding capacity are 565 and 384, respectively. Chitosan shows high solubility in 1% acetic acid (93%) while values of ether extract and ash are below of 0.5%.

Discussion

Results from this work clearly demonstrate a variety of the percentage yield of chitin and chitosan. These values note the importance of the treatments of deproteination, demineralization and deacetylation and can be attributed to the differences of molarity of solutions (NaOH, KOH, HCl) and to the ratio of solid to solvent. Concerning the parameters of the experiments and the percentage yield it is believed that the extraction process can be improved to gain higher yields of chitin and chitosan [11-15]. In our experiment results of 1.0 N solution of HCl for demineralization, 2 N for deproteination and 12.5 N NaOH solutions for deacetylation at a solid to solvent ratio of 1:15, clearly demonstrate a significant yield of chitin and chitosan. Recovery of chitosan in the present study is similar to chitosan yield (15%) from shrimp shell waste reported by and slightly higher than yield of chitosan (12%) in study of [16,17].

Functional and physicochemical properties of chitosan indicate a good quality product with valuable properties [18]. Solubility is an important property to determine the quality of chitosan; high solubility refers to a good quality chitosan. Chitosan is soluble in dilute organic acids, like acetic acid or formic acid and insoluble in water and in basic pH solutions. Its solubility depends on distribution of N-acetyl and free amino groups. Chitosan is protonated because of the presence of amino group in the aqueous acid solution which leads to its solubility [19]. Also, higher values of solubility are combined with increasing degree of deacetylation due to removal of acetyl group from chitin [20]. In our experiments, chitosan showed high solubility in 1% acetic acid (93%) and its whitish slightly brown color is similar to color of chitosan of other studies [21]. The higher values of water and oil binding capacity of chitosan compared with reported studies found to be within the range. Finally, the lower values of the ash and ether extract content prove the purity of the sample indicating the completion of demineralization and deproteinization and confirm the quality of chitosan [22].

Conclusion

In the procedure developed in the present study, chitosan was obtained as a white slightly brown powder with sufficient functional physicochemical properties. Improving extraction processes by changing experiment parameters the yield percentage of chitin and chitosan could be increased giving an alternative solution in seafood industries to obtain valuable products.

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