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Eco-Friendly Extraction, Optimization and Modification of Chitosan from Crustaceans Waste for Sustainable Application in Pakistan

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ABSTRACT

Shell waste from the sea food operations is one of the most significant problems causing serious risks to the environment and human health. The most frequent method employed for its disposal is burning which becomes environmentally costly due to low burning capacity of shells. In this instant recycling of shrimp shell waste to chitosan would provide valuable product with many applications. Chitosan has obtained greater attention as a functional biopolymer. The anti-solvent precipitation method was used to modify chitosan and five carboxylic acids were used. The products were evaluated for changes in degree of deacetylation, solubility characterization. Chitosan derivative of chitin after the process of deacetylation has multiple of commercial and possible medical uses based on its degree of deacetylation. Therefore, this research was aimed to extract chitosan from shrimp shell waste and to study its characterization and modification. Results investigated that shrimp waste contained the highest moisture content (19%), fat (9.61%) and fiber (61%) while the chitosan contained negligible fat (0.02%), reduced protein (1.83%) and fiber (0.37%). Furthermore, bioactive properties of shrimp waste were also evaluated in which antioxidant activity observed in the range of 66.62- 74.52%, FRAP between 46.43-55.7mg/g and TPC from 1.79 to 2.72mgGAE/g extracted from different solvents. Additionally, modified chitosan observed increased degree of deacetylation with a decrease of viscosity. They also showed 70-80% solubilization at neutral pH because of the replacement of amino group with carboxylic group. Based on investigated results, chitosan could be used in edible films, food preservation and as an emulsifier in food industry.

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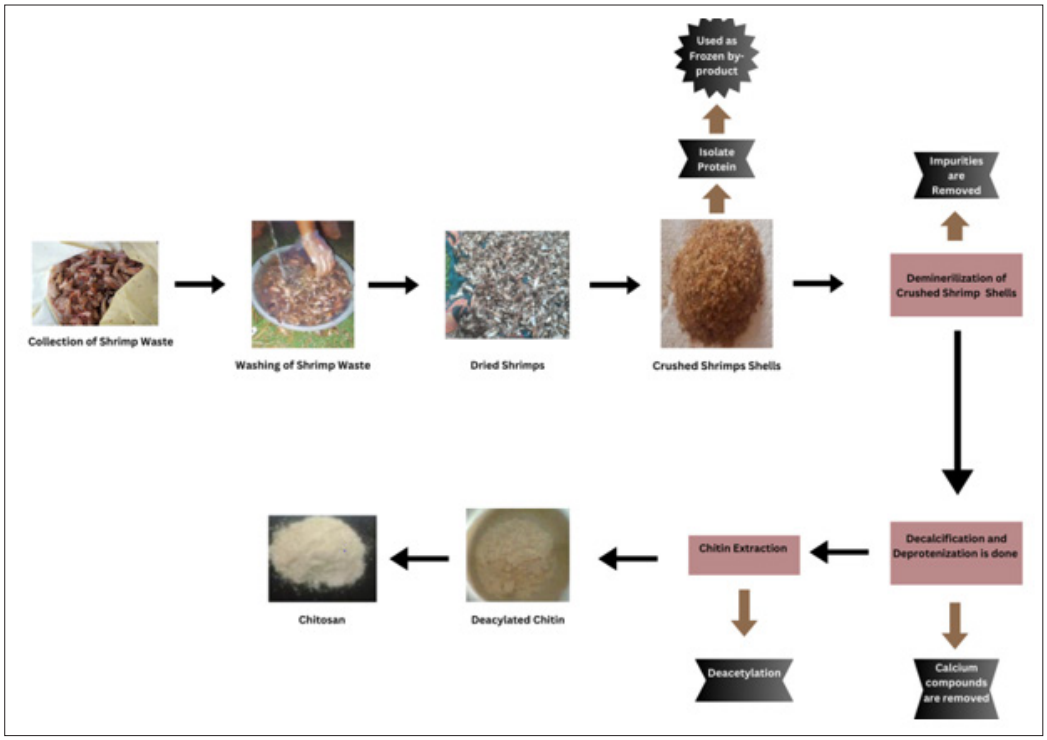
Abbreviation

OACS: Oxalic Acid-Chitosan
CACS: Citric Acid-Chitosan
AACS: Ascorbic Acid-Chitosan
GACS: Glutaric Acid-Chitosan
SACS: Succinic Acid-Chitosan

Introduction

Over the past few decades, the sustainable shrimp revenues have grown substantially its value on a worldwide level, produced significant development, and observed an increase in aquaculture production [1]. Due to increasing demand for global shrimp markets and their cost-effectiveness, that plays an essential economic role in enhancing the strategy for food security by producing a high-value protein that has become more interest worldwide in sustainable shrimp farming [2]. The most important variables influencing the increasing number of organic shrimp aquaculture are the development of sustainable manufacturing methods and the green economy. The key concepts behind good aquaculture practices (GAP) are sustainable development includes the use of practices to improve biosecurity, lower environmental damage, and promote cost effectiveness. Globally, a significant amount of the total harvest from fisheries and aquaculture is discarded, resulting in approximately 35 percent of total production. Due to accumulation in the ecosystem and their slow up of degradation over time, which can have major effects on the environment, they are one of the most critical environmental issues and expand the environmental impact that the shrimp aquaculture sector produces [3,4]. Burning and dumping shrimp shells are the most prominent non-green disposal techniques [5]. Shrimp shells have a comparatively low commercial worth as a result they are used as 5% animal feed for the production of chitin and chitosan, a derivative of it with economical production costs and great commercial value [6]. Shrimp shells might be converted from worthless waste into economic wealth by extracting chitosan, a commercially significant substance with a variety of uses. This is one of the most economically advantageous conditions. Chitosan, a natural biopolymer with biocompatibility, biodegradability, and free toxicity, can be used as an environmentally friendly shell remediation solution in a variety of sustainable economic activities, including food, pharmaceuticals, medicine, cosmetics, agriculture, textiles, pulp, paper, biotechnology, environmental chemistry, and wastewater treatment [7,8]. Research investigations on the useful material found in shrimp shells have been conducted recently in an effort to reduce the ecological impact that the aquaculture sector has on the environment [9]. After cellulose, the most prevalent organic substance detected is chitin in nature. This biopolymer is insoluble in water and has a very linear structure. It has a low reactivity but dissolves rapidly in strong acids and certain fluoroalcohols. This biopolymer's large molecular weight and porous structure, which promote high water absorption, are further significant characteristics [10]. The fact that chitosan is a polymer that can be derived from renewable resources like fisheries, is nontoxic, nonallergic, biodegradable, and has antibacterial

properties is a major factor in the development of novel uses for this material [11]. However, this biopolymer yields chitosan, which has a structural function. These associations serve as a matrix that interacts with various components, including minerals in crab carapaces and phenolic tannins in insects [12, 13]. According to recent research, chitosan is a very versatile and potentially active biopolymer [14, 15]. Since it contains a free amino group, the most beneficial chitin derivative with a wide range of biological uses, it can be chemically modified to produce derivatives while it is biodegradable [16]. These derivatives are widely manufactured and traded commercially Chitin and chitosan are used in many different sectors, including as the pharmaceutical industry, tissue engineering, water treatment agriculture, cosmetics, and anti-tumor and anti-microbial chemicals [17,18].



Chitosan Production from Shrimp Waste

Material and Methods

Sample Preparation

Shrimp shells were obtained from a (Karachi market), Sindh, Pakistan. The shells were thoroughly disinfected with water to get rid of debris and other undesirable substances. They spent 48 hours being sun-dried. A blender machine was used to grind the dry materials into a coarse powder with a size range of 0.30 to 0.35 mm. For the purpose of producing chitin and analyzing its bioactive qualities, the ground coarse powder was stored in a container. The analytical-grade chemicals, including HCl and NaOH, were acquired from Sigma Aldrich.

Bioactive Analysis of Shrimp Waste

Extract Preparation for Analysis of Bioactive Compounds

Preparation of shrimp waste extract. Ten gram of shrimp waste powder put in different four solvents i.e. Water, Ethanol, Acetone and Methanol in 250 ml volumetric flask. The mixture was homogenized using orbital shaker for 8hrs subsequently transmitted through Whatman No. 1 filter paper filter. To extract the chitosan, the filtrate was put through a low-pressure evaporation process using a rotary evaporator (Heidolph, Germany) to remove any remaining solvent.

Total Phenolic Content (TPC) Analysis

Accurately measured 1ml of each extract was poured into a test tube containing 1ml of Folin-Ciocalteu reagent and 1ml of 7.5% Na2CO3 followed by incubation for 30 min. UV-VIS spectrophotometer Darmstadt (UV-VIS,3000, Germany) was used to check the absorbance at 765nm and total phenolic content was as expressed as mg GAE/g [19].

DPPH Free Radical Scavenging Activity

Accurately measured 2.9ml of Chitosan Extract was pipette in a test tube containing 0.1ml (0.02mM) of DPPH solution and incubated in a dark place for 30min at room temperature. After incubation the absorbance of the sample was measured at 517nm using spectrophotometer (UV-VIS, 3000, Darmstadt, Germany). Controlled solution was contained 2.9ml DPPH solution and 0.1ml methanol [20].

$$\text{DPPH\%} = \frac{absorbance\ of\ control - absorbnce\ of\ sample}{absorbance\ of\ control} \times 100$$

Determination of Ferric Reducing Antioxidant Potential (FRAP)

The protocol of was used to determine the capacity of samples for ferric-ion reduction. Accurately measured 200µl of Chitosan from shrimp waste extract was mixed with FRAP reagent and mixture was incubated for 30min at 37°C using water bath. Absorbance of the sample, standard and reagent blanks was measured at 593nm using UV-VIS spectrophotometer (UV-VIS, 3000, Darmstadt, Germany) [21]. The antioxidant potential of extract in terms of reducing ferric ions was expressed as µmol TE/g of dry chitosan from shrimp waste where TE is Trolox equivalent.

Production of Chitin

Deproteinization Process

Three grams of powdered shrimp shells were heated with 4% 2 N NaOH at 70˚C for four hours in order to deproteinize them. Washing with running water neutralized the substance. After the solid was gathered, distilled water was used to wash it. After

being vacuum-dried, the solid product was weighed using an analytical balance.

Demineralization Process

At room temperature, the deproteinized samples were treated with 10% HCl (3.25 N) at a ratio of 14 ml: 1 g (w/v) and left for four hours. After gathering the solid product, distilled water was used to wash it.

Deacetylation Processing

The demineralized sample was stirred for 75 hours at room temperature while being treated with 35% NaOH (8.75 N) at a ratio of 14 ml:1 g (w/v). Distilled water was used to filter and wash the deacetylated sample. After draining the alkali, distilled water was used repeatedly to reduce the pH. Chitosan was kept after being further dried at room temperature [22].

Analysis of Proximate Composition of Shrimp Waste and Chitosan

Moisture, ash, lipid, fiber and protein contents of shrimp waste and chitosan samples were determined [23].

pH Analysis

The pH measurement of chitosan was carried out using pH meter.

Modification of Chitosan

Following a little modification to the procedure described by, the antisolvent precipitation approach was used to perform the physicochemical modification of chitosan [24]. A solution of 2% glacial acetic acid was used to completely dissolve 500 mg of chitosan. Under constant stirring at 500 rpm, the resultant polymer solution was added to a 5% NaOH solution containing 500 mg of dicarboxylic acid (selected from ascorbic, citric, glutaric, oxalic, and succinic acids). After mixing, particle precipitation occurred, resulting in the formation of a milky-looking suspension. The obtained product was then oven-dried at 50 °C for a whole night after being filtered using Whatman filter paper.

Solubility Studies of Modified Chitosan

At room temperature of 25 °C, the solubility of chitosan and its modified chitosan was assessed at several pH values: neutral pH = 7.0, acid pH = 5.0, CH3COOH solution, water and alkaline medium pH = 11.0, and 0.1 M NaOH solution. 100 mL of the aforementioned media were combined with 1.0 g of chitosan and its derivatives, and the mixture was swirled for three hours to determine solubility. After 1, 5, and 10 hours, qualitative observations of the samples that were exposed to the solubilization tests were recorded [25].

Viscosity

Viscosity of chitosan was determined at room temperature using a Brookfield digital viscometer.

Degree of Deacetylation

A homogeneous solution of chitosan was made with 0.01 mol/L of HCl and titrated against 0.1M NaOH. The pH value inflections were used to identify the termination point. There were primarily two inflections seen. The neutralization of HCl is represented by the first one, and the neutralization of chitosan's ammonium ions by the second. The number of amino groups in the chitosan chain, also known as the degree of deacetylation, is shown by the difference between two points [26].

DD % = 100- DA% [10]

Result and Discussion

Chitin Extraction Experimental Design Scheme at Various Temperatures and Times

Experimental design for chitin extraction at different temperatures and times were focused on two critical factors that influenced the efficiency and quality of the extracted chitin. It helped to optimize the extraction process and gained insights into the role of temperature and time in the reaction kinetics. Temperature 25°C, 40°C, 50°C, and 60°C were selected. According to table 1, temperature 25°C and 40°C showed the conditions allowed for the assessment of extraction efficiency under low heat treatment reduced the risk of degradation. Whereas, temperatures 50°C, and 60°C observed the increased the rate of reactions particularly denaturation of protein and removal of mineral. Moreover, 1-2 hours of duration evaluated the initial stage of extraction where rapid reaction took place. Between 3 to 4 hours, the extent of reactions over time were determined leading to improve extraction and undesirable degradation. Thus, this design identified the conditions that maximize chitin yield and purity.

Table 1: Scheme of experimental design for chitin extraction at different temperatures and time

Temperature (◦C)	Time(h)	Time(h)	Time(h)	Time(h)
25	1	2	3	4
40	1	2	3	4
50	1	2	3	4
60	1	2	3	4

Experimental Design Scheme for Deacetylation after Chitin Deproteinization at Different Temperatures and Times

Table 2 showed the experimental design for deacetylation of chitin (prepared at different temperatures and times) carried out over different incubation times. Chitin was deproteinized at temperatures 20°C, 40°C, 50°C, and 60°C with time intervals of 1,2,3, and 4 hours. According to our Table, it is observed that deproteinization achieved in longer time at low temperature i.e. 20°C but rate of reaction increased with the increase in temperature. Also, shorter duration might conserved energy but not achieved the desired deacetylation. As the time increased, chitosan was yielded with a higher degree of deacetylation. This whole system of temperature, time and deacetylation identified the optimal combination of deproteinization and deacetylation parameters for maximum yield and the quality of chitosan.

Table 2: Scheme of Experimental Design for Deacetylation of Chitin (Prepared at Different Temperatures and Times) Carried Out Over Different Incubation Times.

Sample Deproteinization Temperature, Time (Hour)	Deacetylation	Sample Deproteinization Temperature, Time (Hour)	Deacetylation	Sample Deproteinization Temperature, Time (Hour)
20 (1)	2	3	4	5
20 (2)	2	3	4	5
20 (3)	2	3	4	5

20 (4)	2	3	4	5
40 (1)	2	3	4	5
40 (2)	2	3	4	5
40 (3)	2	3	4	5
40 (4)	2	3	4	5
50 (1)	2	3	4	5
50 (2)	2	3	4	5
50 (3)	2	3	4	5
50 (4)	2	3	4	5
60 (1)	2	3	4	5
60 (2)	2	3	4	5
60 (3)	2	3	4	5
60 (4)	2	3	4	5

Bioactive properties of Shrimp Waste

Ferric Reducing Antioxidant Power (FRAP)

Ferric Reducing Antioxidant Power (FRAP) values of the extract were obtained using four different types of solvents i.e. water, methanol, acetone, and ethanol as shown in Figure 1. The highest value was observed by ethanol 55.77mg/g indicating its potential in extracting antioxidant compounds. Better extraction capacity was also shown by acetone and methanol having values of 49.67mg/g and 47.82mg/g respectively. The least value of FRAP was shown by water having a value of 46.43mg/g. so, the compounds having the high reducing power are less extracted by the water as compared to organic solvents like methanol, ethanol, and acetone.

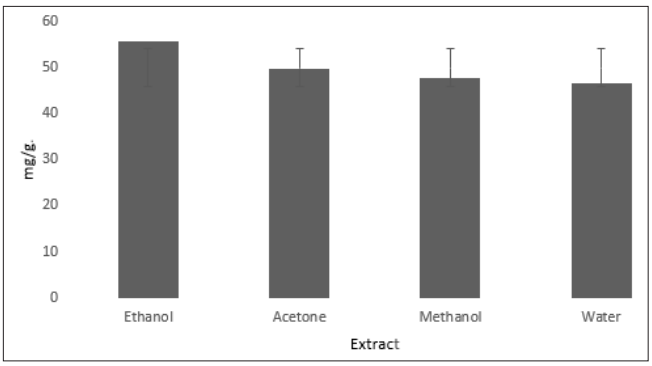


Figure 1: Effect of Different Extracts for the Determination of FRAP from Shrimp Waste All Values are Means of Triplicate Determinations. Means within a Column with Different Superscripts are Significantly Different at $P < 0.05$.

DPPH (Free Radical Scavenging Activity)

Figure 2 represented the Antioxidant Activity of shrimp waste extracted from four distinct solvents: water, methanol, ethanol, and acetone. According to the results, ethanol extract has the highest DPPH value, which is 74.54% that it was the most effective solvent in extracting antioxidant compounds from the sample, resulting in a higher reducing power. Whereas, acetone and methanol extracts showed intermediate DPPH values, with acetone at 70.35% and methanol at 67.44%. Both solvents demonstrated good extraction capability, but slightly less than ethanol. The small difference between these values indicated similar efficiency in extracting antioxidant compounds. Water extract recorded the least DPPH value of 66.42%. Although water is commonly used as a solvent due to its polarity, it appears to be less effective than the organic solvents (ethanol, acetone, methanol) in extracting compounds with Free Radical Scavenging activity.

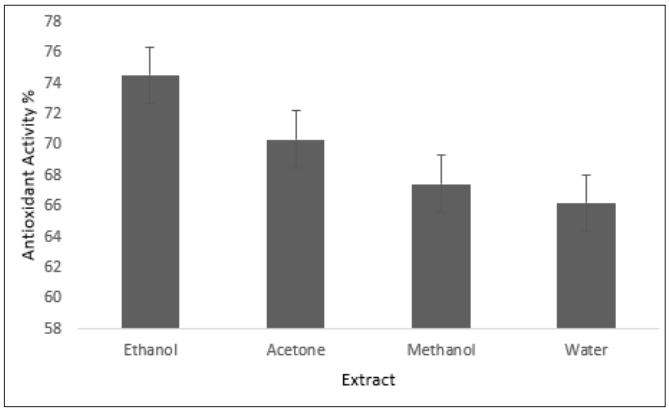


Figure 2: Effect of Different Extracts for the Determination of Antioxidant Activity from Shrimp Waste All Values are Means of Triplicate Determinations. Means within a Column with different Superscripts are Significantly different at $p < 0.05$.

Total Phenolic Content (TPC)

Total Phenolic Content values from various extracts of shrimp waste were shown in Figure 3. The Acetone extract has the highest TPC value at 2.72 mgGAE/g. This indicated that Ethanol was the most effective solvent in extracting antioxidant compounds from the sample, resulting in a higher Total Phenolic Content. Ethanol and Methanol extracts showed intermediate TPC values, with Ethanol at 2.36 mgGAE/g and Methanol at 1.82 mgGAE/g. Both solvents demonstrated better extraction capability, but slightly less than Acetone. The small difference between these values indicates similar efficiency in extracting antioxidant compounds. The Water extract recorded the lowest TPC value of 1.79 mgGAE/g. Although water is commonly used as a solvent due to its polarity, it appears to be less effective than the organic solvents (ethanol, acetone, methanol) in extracting compounds with Total Phenolic Content.

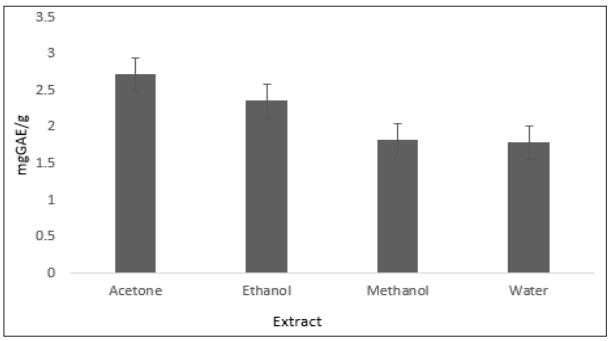


Figure 3: Effect of Different Extracts for the Determination of Total Phenolic Content from Shrimp Waste All Values are Means of Triplicate Determinations. Means within a Column with Different Superscripts are Significantly Different at $P < 0.05$.

Characterization of Shrimp Waste and Chitosan

The proximate composition and pH of the samples are represented in Table 3. Results indicated that the shrimp waste exhibited the highest moisture content (19.66%) than the chitosan (7.86%), while the ash content of both the samples were insignificantly different. It suggested that there was minimal loss of minerals during process because chitosan retained inorganic compounds. Reduction of fat and protein was observed significantly from 9.61% and 30.72% in shrimp waste to 0.02% and 1.83% chitosan respectively. This would be due to the effectiveness of the deacetylation and deproteinization process that lead to the purification of chitosan. Also, crude fiber was reduced from 61.80% in shrimp waste to 0.37% in chitosan. However, carbohydrate increased significantly from 31.6% in shrimp to 88.37% in chitosan. It showed that the successful isolation of polysaccharides was achieved. The pH of shrimp waste was 6.94 whereas chitosan is more alkaline i.e. 7.98. It is attributed to the alkaline treatment during extraction process that neutralizes acidic components and contributed to the basic nature of chitosan.

Table 3: Characteristics of Shrimp Waste and Chitosan

Parameters	Shrimp Waste	Chitosan
Moisture Content (%)	19.66±0.11	7.86±0.02^{c}
Ash (%)	1.56±0.11	1.53±0.05^{c}
Fat (%)	9.61±0.15	0.02±0.00^{a}
Protein (%)	30.72±.068	1.83±0.10^{b}
Crude Fibre (%)	61.80±0.15	0.37±0.35^{a}
Carbohydrate (%)	31.63±0.29	88.37±0.10^{b}
pH	6.94±.050	7.98±0.02^{a}

^{a}All values are means of triplicate determinations. Means within a column with different superscripts are significantly different at $P < 0.05$.

Solubility Data for Chitosan and Modified Chitosan

Different trends can be seen in the solubility profiling data for chitosan and its derivatives (OACS, CACS, AACS, GACS, and SACS) under various pH levels. All samples exhibited great solubility (90%) at acidic pH (4.0), indicating that the protonation of amino groups in the polymer structure makes chitosan and its derivatives soluble in acidic environments. All samples solubility dropped to 65% at neutral pH (7.0), most likely as a result of decreased protonation and chitosan's innate propensity to clump in these circumstances. None of the samples show solubility in alkaline conditions (pH 11.0), since the lack of charge stabilization causes the polymer to become deprotonated and insoluble. Different behaviors are revealed by solubility measurements under different pH values, as seen in Table 4. Whereas chitosan precipitates in neutral and alkaline conditions, it is completely soluble in acetic acid solutions. Given the protonation of amino groups along the polymer chain at low pH levels, this behavior is consistent with expectations. Because carboxylic groups gradually replace amino groups, chitosan derivatives exhibit a 65% solubilization (mass balance) at neutral pH. However, after pH 6.0, the resulting negatively charged structure does not completely dissolve, and there are still some undissolved parts visible. The derivatives stay insoluble at pH 11.0, which is consistent with chitosan's behavior [27].

Table 4: Data on chitosan and its derivatives' solubility profiles using mass balance analysis.

Samples	pH-4.0	pH-7.0	pH-11.0
Chitosan	90	65	00
OACS	90	65	00
CACS	90	65	00
AACS	90	65	00
GACS	90	65	00
SACS	90	65	00

^{a}All values are means of triplicate determinations. Means within a column with different superscripts are significantly different at $P < 0.05$.

Intrinsic Viscosity and Degree of Deacetylation in Modified Chitosan

The viscosity of a polymer solution gives a direct reflection of the hydrodynamic volume at a molecular level. It is influenced by the molecular size, chain length and the molecular weight of the polymer. Whereas the degree of deacetylation determines the number of free amino groups in the chitosan macromolecule which is directly related to its functionality, polarity and water solubility. Table 5 represented viscosity and degree of deacetylation of modified Chitosan. A comprehensive comparative analysis of various chitosan samples (CS) along with their modified counterparts grounded in two principal parameters i.e. viscosity and degree of deacetylation (DDA). The unmodified chitosan demonstrated the peak viscosity measurement of 10.65 accompanied with a comparatively low DDA of 67.78% which is typically the characteristic of chitosan. As the samples undergone modifications, a discernible trend emerged, characterized by the reduction in viscosity and an augmentation in DDA. For instance, viscosity experienced a marked declined in modified samples with the most diminished value recorded in SACS at 2.65. Simultaneously, the DDA escalated significantly with GACS and SACS exhibited values that surpass 100%.

Table 5: Chitosan and its Derivatives' Intrinsic Viscosity and Level of Deacetylation

Samples	Viscosity	Degree of Deacetylation
Chitosan	10.65	67.78
OACS	05.65	98.67
CACS	04.54	94.91
AACS	03.37	96.39
GACS	02.75	105.67
SACS	02.65	108.54

All values are means of triplicate determinations. Means within a column with different superscripts are significantly different at $P < 0.05$.

Conclusion

The study demonstrated the prospect of recycling shrimp shell waste into chitosan providing a sustainable solution for the risks that seafood sector waste causes to human health and the environment. Shrimp shell waste was collected from Pakistan, was dried, deproteinized, demineralized, and deacetylated to produce high-quality chitosan. Chitosan observed a high solubility of 90%, a low moisture content of 19.6%, and significant antioxidant characteristics (FRAP 55.77–47.82 mg/g, TPC 2.72–1.82 mgGAE/g, and 74.54–66.42% DPPH). These characteristics demonstrate that Pakistani shrimp shell waste is a desirable and feasible resource to produce industrial-grade chitosan, participating in waste removal, and encouraging environmentally friendly industrial processes.

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Declaration of Interest Statement

It is declared that we authors have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Funding Declaration

The authors did not receive support from any organization for the submitted work.

Disclosure Statement

The authors report there are no competing interests to declare

Data availability Statement

It includes both original data generated in my research and could be shared on your command.

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