Valorization of Crab Shell Waste into Bacterial Enzyme Production and Chitin Characterization

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Abstract

Chitin, a valuable biopolymer with significant economic and industrial applications, is primarily found in aquatic crustaceans, particularly in crab shells, which serve as the main commercial sources. The study involves deproteinization and demineralization to remove proteins and minerals and extract 15.45% chitin from crab shell waste. Chitin was characterized by determining its physicochemical properties using Fourier transform infrared spectroscopy and Scanning electron microscope. The purified chitins from crab shell waste were comparable to those of commercially purchased chitin. Purified chitin further implicates in deacetylation process to convert the extracted chitin into chitosan. Chitosan-infused media were used to isolate 31 chitinolytic bacteria, with the highest enzyme activity at 82.45U/ml on 7X5ZA isolates. The culture filtrate of selected bacteria degrades 2% of crab shell powder resulting in 10.5% weight loss in the raw crab shell powder (substrate) and explores the production of N-acetyl glucosamine (97.53U/ml), post reaction with enzyme at incubation period of 48hrs at 47°C.

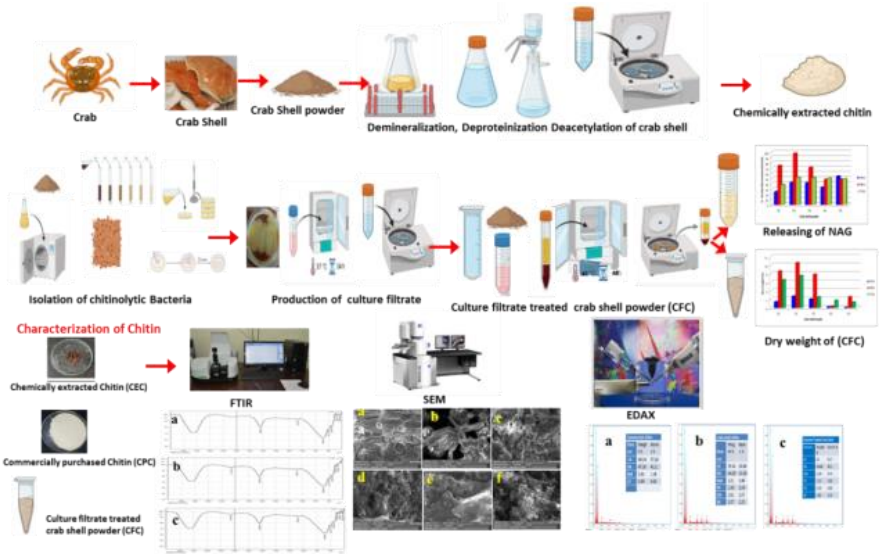
An alternative solution for the chemical process to extract chitin from the crab shell is an enzymatic process. Crab shell powder is treated with culture filtrate to obtain chitin which has similar chemical composition, surface morphology and elemental content compared with both commercially purchased chitin and chemically digested chitin of crab shell. This study suggests that crab shell waste can be used for effective chitin extraction and discovery of chitinase-producing bacteria to conserve a safe environment.

Keywords: Crab shell waste, Chitin, Deproteinization, Demineralization, FTIR, SEM, Chitinase.

Introduction

Chitin is a renewable and copious natural polymer that is assembled in marine invertebrates, insect exoskeletons and the cell walls of fungi and algae. Seafood processing industries have dumped chitinous matter, approximately 10% of the total global and approximately 10^{12} tons of chitin waste accumulates in the ocean every year.[7,13,34,39] The perpetual process of ecdysis (shedding of the cuticle) results in the constant accumulation of chitin at the ocean base, which is recognized as marine snow, while there is no quantitatively substantial deposit of chitin in the sediments of the ocean owing to its proficient degradation and metabolism by bacteria.[32,46] The main commercial sources of chitin in seafood are crustacean shells because of their high content and ready obtainability.[21,38]

Large amounts of crabs are being used in the seafood industry and pollution has increased due to the dumping of crab shell waste into the environment, which can be reduced by utilizing the crab shell for the manufacture of chitin and its derivatives and the production of chitinase for the degradation of crab chitin waste.



Graphical representation of chitin extraction and its characterization

Fig. 1: Chitin preparation from the crab shells (Chemically extracted chitin)

Chitinase can be designed for various valued implementations including biocontrol agents for pathogenic fungi[21,23] and detrimental insects[39], production of single-cell proteins, production of bioenergy, eradication of dengue and various applications in the medical, food, agriculture, pharmaceutical and chemical industries.[2,3,28]

Chitin is an amino polysaccharide with a simple unit of N-acetyl glucosamine bonded by a β (1-4) linkage and is insoluble in water. Chitin and its derived products have biological properties, such as biocompatibility, biodegradability and non-toxicity and have attracted significant attention in the fields of medicine, cosmetics, pharmacology, agriculture, biological control and wastewater treatment to produce highly value-added products. Research on chitin has kindled the interest of many investigators and made them curious about new promising sources of chitin.[3,18,28] Several techniques have been proposed and have been used to extract chitin from different sources, most of which rely on chemical treatments involving the removal of proteins and minerals.

The best practice for the removal of pigments from extracted chitin, which improves its color using chemical oxidation and solvent extraction methods,[4,46] and the large-scale industrial production of chitin from crustaceans, typically involves the removal of proteins at high temperatures with alkali usage (deproteinization) and mechanical crushing using tough acid (demineralization).[5,41] The goal of this study was to obtain crab chitin and analyze it along with purified chitin to stimulate the isolation of chitinolytic bacteria for the production of chitinase. Utilizing potent bacteria to break down crab shell waste is expected to resolve waste generation issues and address environmental concerns.

Material and Methods

Crab Shell collection: Crab shells were collected from a nearby fish market (Ukkadam), washed and the flesh was scraped from the shell. The shell was then cleaned with water, dried under sunlight until its weight was constant and finally ground into a powder.

Demineralization and deproteinization of crab shell: The demineralization process was performed at a ratio (1:10) and 20 g of the shell powder was treated with 12N concentrated HCl (200 ml) for 2 h at room temperature with shaking at 100rpm. For deproteinization, 0.3M sodium hydroxide (1000 ml) was added and incubated for 1h at 80^{o}C. To obtain a neutral pH and to remove excess HCl and NaOH, the extract was washed several times with cold water (Fig. 1). Chitin powder was dried, sterilized and stored in an airtight container for further use.

Processing of colloidal chitin: The procedure described in the study of Jabeen and Qazi[12] was followed to produce colloidal chitin: 20 g of extracted chitin from crab shells was blended with 400 ml of 6N concentrated HCl, agitated for two hours. Slowly add twice the amount of cold distilled for washing. Subsequently, the extracted chitin was strained using filter paper in a Buchner flask. Following this, the filtrate was mixed with 1000 mL of 50 % ethanol, continuously agitated, strained through filter paper, centrifuged at 6000 rpm and neutralized with distilled water. Subsequently, dried colloidal chitin was stored in a bottle at 4°C.

Isolation of chitinolytic bacteria: Colonies were isolated from the soil samples using a serial dilution technique on a basal medium containing 1 % colloidal chitin. The zones of hydrolysis-producing isolates were purely cultured and well-preserved in glycerol stocks for further use.

Estimation of chitinolytic activity: The selected colonies were used for enzyme production using 1% colloidal chitin in a basal medium (pH 7). Culture-inoculated flasks were incubated at 37^{o}C at 100 rpm for 48 hours. After incubation, the culture supernatant was collected and enzyme activity using the DNS method was measured at 540 nm. Chitin degradation was analyzed by reducing the sugars released from chitin.[35] N –N-acetyl glucosamine (NAG) was used as a standard. One unit of enzyme activity was designated as 1 µmol/ml of N – acetyl glucosamine concentration. Protein estimation was performed for each sample using Lowry's method.

Analysis of crab shell degradation: The culture filtrate was added and incubated with crab shell powder in acetic acid buffer. Crab shell powder (0.1, 0.2, 0.3, 0.4 and 0.5 g) was mixed with acetate buffer (pH-5.4) and 10 ml of culture filtrate was added.

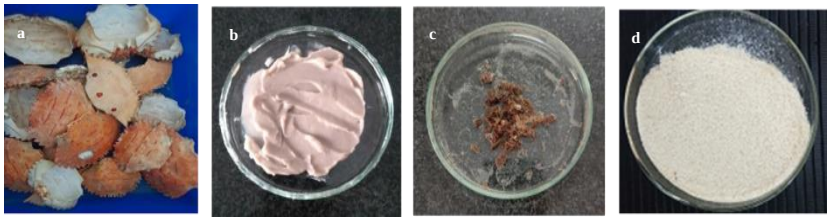


Fig. 2: Chitin from the crab shell wastes: a) crab shell b) After demineralization and deproteinization of crab shell c) dried extracted chitin d) commercially purchased chitin

The mixture was incubated in a shaking incubator at time intervals of 24, 48 and 72 hrs at 47 °C. After incubation, the reaction mixture at 6000rpm and the supernatant were removed separately for the DNS assay to determine the presence of GlcNAc (Fig. 2B). Crab shell powder (0.1, 0.2, 0.3, 0.4 and 0.5 g) was mixed with acetate buffer (pH-5.4) and 10 ml of culture filtrate was added. The mixture was incubated in a shaking incubator at time intervals of 24, 48 and 72 hrs at 47 °C. After incubation, the reaction mixture at 6000rpm and the supernatant were removed separately for the DNS assay to determine the presence of GlcNAc (Fig. 2B).

Pellets were collected and dried in an oven to measure the dry weight of chitin before and after degradation (Fig. 2A). The optimized conditions for the degradation of crab shell powder and its dried chitin pellet (culture-filtrate-treated crab shell powder (CFC)), were further characterized using FTIR, SEM and EDAX for comparison with chemically extracted chitin (CEC) and commercially purchased chitin (CPC).

Characterization of chitin: Ensuring the chitin properties, the samples were analyzed using Fourier Transmission Infra-Red Spectroscopy (FTIR), Scanning Electron Microscopy and EDAX. The results were compared with chemically extracted chitin (CEC) and commercially purchased chitin (CPC).

Fourier Transmission Infra-Red spectroscopy (FTIR): Chemically extracted chitin from crab shells (CEC), chitin from culture filtrate-treated crab shell powder (CFC) and commercially purchased chitin (CPC) were analyzed using an FTIR system. KBr pellets (2% KBr pellets were used to prepare the samples at 2:100 (w/w). Absorbance was measured at a resolution of 2 cm-1 and 4 scans.

Scanning electron microscopy (SEM): The samples were fixed on an SEM sample holder, dried using a critical point dryer (LADD 28000) and layered with a thin gold coat of 3 mm using a sputter coater (JBS E5150) for conductivity. TESCAN MIRA3 FESEM scanning electron microscope at Rao Research Center, Avinashilingam Institute, India was used to examine the microstructures of chemically extracted chitin from crab shells (CEC), chitin from culture-filtrate-treated crab shell powder (CFC) and commercially purchased chitins (CPC) under magnification range 5 - 20µm in 10kV. The elemental study was observed using EDAX APEX software.

Results and Discussion

Seafood waste is easily available in large quantities throughout the year[46] and tons of crab shells are collected from seafood industries. Annually, the seafood industry produces more than 10^6 million tons of seafood waste such as cuticles, scales and shells due to the high consumption of seafood such as crab, shrimp and prawn, which result in the dumping of waste into the environment, which requires proper waste management to resolve environmental issues.[36,39,40] The composition of crab shells is 30–50% chitin, with other contents such as proteins, minerals, lipids and pigments. The polysaccharide remains bound to proteins and minerals in the shell.[8,16] The major mineral found in the exoskeleton of shrimp is calcium carbonate ($CaCO_3$), followed by magnesium.[30] Chitin was isolated from shell waste using chemical[8], enzymatic[6,24] or microbiological[22] methods.

The separation process of chitin involves deproteinization, demineralization and decoloration steps. Chitin production is done annually by the utilization of chitin from crustacean waste for the production of chitinase by using microbes while solving ecosystem problems that will decrease pollution and will convert the waste into renewable resources. The bioconversion of chitin to monomers, glucan and chitooligosaccharide, was difficult due to the presence of hydrophobic properties between the two monomeric amino polysaccharides and the prompt conversion of chitin was achieved by enzymatic reactions.[15, 36]

Demineralization and deproteinization of crab shell: Chitin acts as a carbon source for chitinase production which is a copious product of the seafood industry. Pretreatment methods such as chemical and biological processes improve chitin degradation and chitinase production by microbes. In the present study, crab shells were purchased from a fish market, cleaned, dried, mashed, ground and subjected to demineralization and deproteinization processes for the removal of chitin minerals and proteins.[42] After processing, the chitin powder (Fig. 2) was washed with distilled water until it reached a neutral pH and was washed again with ethanol; a dehydrated 15.45 g chitin was obtained from 100g of the total crab shell waste. Similar results were reported[27,29] where chitin yields were 12.73% and 11.73 % respectively.

In this study, after treatment of acid and alkali, the processed 100g of crab shell powder was processed to obtain 15.45g of chitin and 4.25g of colloidal chitin. The extracted chitin was

subjected to further investigation to confirm the characteristics of chitin with commercial chitin. Surface morphological characteristics of extracted chitin showed the nonporous smooth arrangement and flaky chitin, similar to commercial chitin morphology. Related investigation of isolated chitin microparticles resulted as pasty, flaky, rough-surfaced and highly porous in SEM images. The morphology of the chitin microparticles is assembled as stacked layers like insect cuticles[9,26] and crustacean shells[44]. Deacetylation results in smoother surfaces and larger particle sizes, which may be caused by the microfibrillar structure of chitin[31].

Chitinolytic bacteria and Chitinase activity: Microbes are present under all environmental conditions, which help in the biogeochemical cycle in nature. These microbes have been chosen as better sources because of the rapid fermentation of complex compounds and the production of valuable products. Among various microbes, bacteria produce chitinase enzymes, isolation and identification of presumed chitinolytic bacteria are essential. In this study, the crab chitin content acted as the main carbon source. 31 isolates of bacteria from soil samples had the capacity to utilize crab chitin and produce chitinase at pH 7 and were incubated at 37ºC. The maximum zone of hydrolysis exhibited was 8 mm in ratio (zs/cs). Based on the limits of chitin hydrolysis, seven bacterial species were selected for further study.

The large zone of hydrolysis observed around the colonies indicates the potential for bacteria to produce chitinase. The ratio of the area to the size of the colony reflects the rate of diffusion of chitinolytic enzymes. This is a simple and inexpensive method for isolating chitinolytic bacteria from a source of bacterial diversity. Seven other isolates were selected based on their chitinolytic activity units (Table 1). The highest enzyme activity was observed on 7x5ZA (82.45 U/ml) with the lowest chitinase activity on 7x1RA (16.79 U/ml) (Table 1) after three days of fermentation at 37 °C. The complex structure of chitin from the treated crab shell became easily utilized by the microbes from the soil sample were investigated.

Around 31 colonies were isolated after the utilization of chitin on the chitin agar. Among 31 colonies, 7 isolates were selected for further studies based on zone of hydrolysis on the screening. Seki[37] discovered that 1010 bacterial cells per/cm^3 of soil could break down approximately 30 mg daily at a temperature of 25°C and calculated that chitin decomposition in the ocean takes around 140 days at 15 °C, 370 days at 5 °C and over 500 days below 5 °C. Similar results were revealed by Korany et al[17] among 34 isolates, only 4 isolates showed chitin hydrolysis in chitin agar medium.

Similarly, Ajayi et al[1] selected 24 isolated, among the 36 chitinolytic species, based on high chitinolytic activity. Similarly, well diffusion methods aided in the identification and characterization of chitinolytic bacteria based on the observation in the hydrolysis on the chitin agar media.[11] Based on the limits of chitin hydrolysis, seven bacterial species were selected for the next step of research. The larger and clearer area of chitin hydrolysis reveals the stronger chitin degradation. This method was considered the first quantitative test for the stabilization of chitinolytic bacteria the ratio of the area size to the size of the colony reflecting the rate of release of chitinolytic enzymes. It is a simple and inexpensive technique to select chitinolytic bacteria from a source of bacterial diversity.

All seven isolates were mesophilic and thermostable, indicating their ability to function under environmental conditions. Seven colonies were selected for secondary screening in colloidal chitin broth medium and were analyzed for enzyme activity using the DNS assay method. Based on the maximum chitinase production after 72hrs of incubation, 7x5ZAisolate gave high enzymatic production and activity 82.45U/ml, the least enzyme activity shown in 7X1RA as 16.79 U/ml. The above result exposed that the highest enzyme activity gives a potential isolate as 7X5ZA, isolated from the soil sample of fish market area respectively and was selected for further studies which will be employed in chitin degradation process for resolution of chitin dumping waste to secure the environment.

The efficiency of chitin degradation using partially purified culture filtrate: Propitious strains among the isolates were used to utilize chitin in the medium. After incubation, the separated culture filtrate was analyzed by DNS assay, using N-acetyl glucosamine used as standard. Protein estimation was performed using Lowry's et al method. Table 1 depicted the result as efficient strain isolate (7X5ZA) among the seven. Further analysis of the degradation of chitin as an alternative method (enzyme treatment) to overcome chemical digestion of crab shell, the culture filtrate of 7x5ZA directly reacts with the crab shell powder in the presence of acetate buffer at different substrate concentrations and time intervals at 47°C.

Identification of the chitin degradation ability of culture filtrate through dry weight measurement of crab shell powder 2% substrate after 48 hours incubation shows 10.5 % weight loss on the crab shell powder while compared to the 3%, 4% and 5%. Chitin degraded into simple subunits analyzed by DNS assay method as shown in fig. 3B. The results showed that the high activities of culture filtrate on crab shell degradation enhanced the production of N-acetyl glucosamine 97.53U/ml with 2% crab shell powder as a substrate after 48 hrs of incubation at 47°C.

Next to that, figure 3A displays 1% and 3% of substrate concentration showing the enzyme activity 74.14U/ml and 70.74U/ml. The extracted chitin from the crab shell waste was applied to physiochemical investigations using FTIR and SEM to confirm the characteristics of commercial chitin. The processed chitin structure loses the complexity structure easily utilizing the carbon and nitrogen sources by microbes

to convert the chitin into a valuable product.[20,43] The isolates will aid in overcoming the treatment of harsh chemicals treatments which spoil the environment easily.

Characterization of chitin

Fourier Transmission Infra-Red spectroscopy (FTIR): FTIR evaluates the CPC samples with CEC and CFC, as exposed in figure 4 (Fig. 4 A, B and C) which depict small differences in the percent transmittance up to wave number 1072; thereafter, the detailed differences became prominent for a very short segment of the wave number. Approximately 1 mg of each crustacean chitin sample was analyzed using an FTIR spectrometer (IR Prestige 21, Shimadzu) with absorption spectra from 4000 to 400 cm^{-1} depicting IR bands.

The crude and purified chitin prepared during the course of this study had many comparable patterns of percent transmittances with more prominent vertical oscillation in the case of the purified polymer. The chitins extracted and purified in this study resemble the % transmittance of crab chitin more closely than that of commercial chitin. FTIR patterns showed that the bands corresponded to stretching and vibration of O-H, N-H, CO and saccharide bonds as shown in table 2.

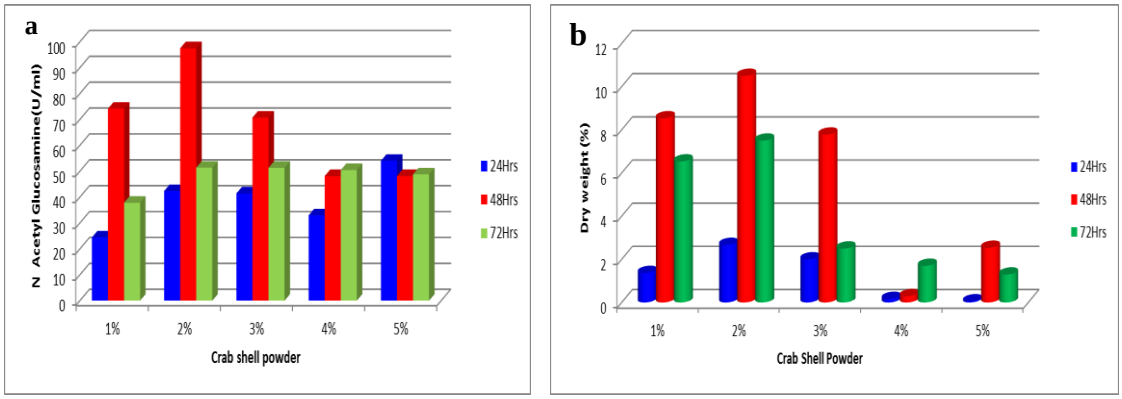


Fig. 3: Treatment of Crab shell powder using culture filtrate of 7X5ZA: a) Release of N acetyl glucosamine. b) Dry weight of degraded crab shell powder

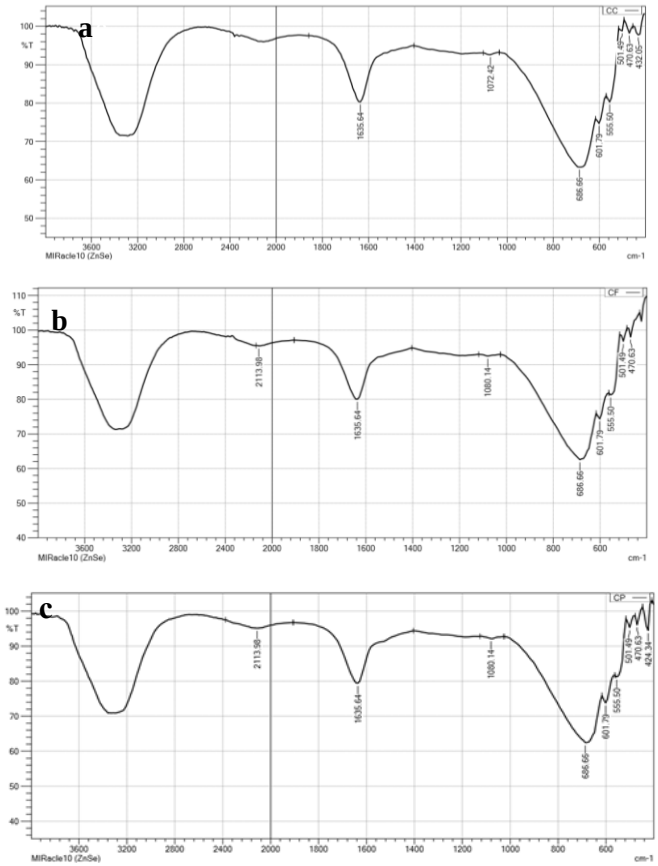


Fig. 4: FTIR analysis of Chitin: a) Commercial purchased chitin, b) Chemically treated chitin and c) Enzyme treated chitin

Table 1
Chitin hydrolysis isolates and chitinase activity on chitin medium

Location at Coimbatore	Isolates	Colony color and appearance	Ratio of zone size to colony size (ZS/CS)	Chitinase Activity (U/ml)
Periyakulam lake	7x1(WA)	Brown	3	40.04
	7x1(RA)	Red	2.6	16.79
Perur lake	7x3(PA)	Pale white	5	41.55
	7x4(MC)	Lemon yellow	6	54.89
Fish market Ukkadam	7x5(ZA)	Mango yellow	8	82.45
	7x6(XA)	Transparent	6.3	60.27
Vellalore land fills	7x8(BA)	White	5.5	76.42

Table 2
FTIR spectral peaks of commercial chitin and crab shell chitin

Functional Group and Vibration mode	Classification	Crab shell Chitin		
		Commercial	Chemical treated	Enzymatic treated
N-H stretching / O-H group	-	3286.70	3286.70	3286.70
CH 3 sym. stretching	Aliphatic compounds	-	2113.98	2113.98
C = O secondary amide stretch	Amide I	1635.64	1635.64	1635.64
C-O-C asym. stretch in phase ring	Saccharide ring	1072.42	1080.14	1080.14
CH ring stretching	Saccharide ring	686.66	686.66	686.66

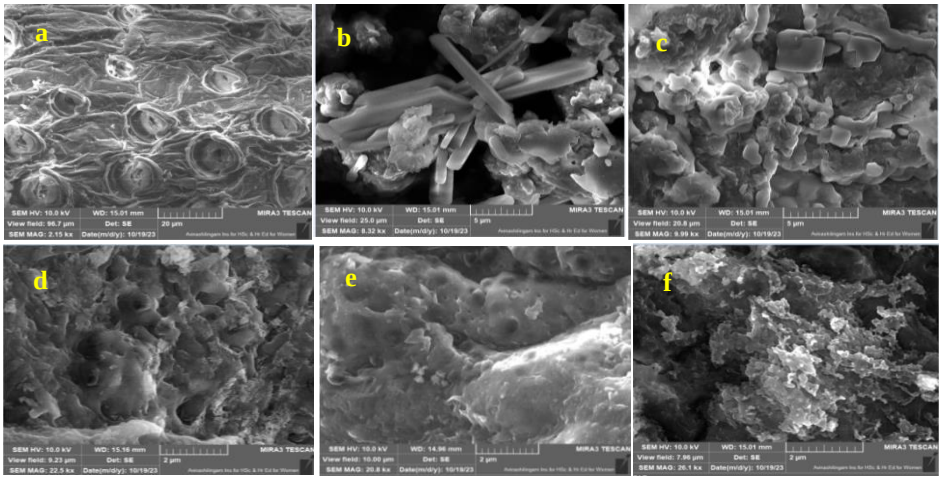


Fig. 5: SEM analysis of Chitin: a) Crab Shell Powder; b) Demineralized crab shell powder; c) Deproteinization of crab shell powder; d) After deacetylation of chitin; e) commercial chitin f) Enzyme treated Crab shell

Comparison of CEC and CFC with CPC showed bands at 3286 cm^{-1} (NH amide bond stretching/OH group). The infrared spectra of crab shell chitin are related to the literature, with absorption bands at 1635 cm^{-1} showing the presence of an amide group. The FT-IR spectra of crab shell chitin and commercially purchased chitin samples obtained by deacetylation showed progressive weakening of the band at 1635 cm^{-1} and disappearance at 1550 cm^{-1}.[24] The peak determined the saccharide ring stretch at 1072 cm^{-1} and 1080 cm^{-1} in all commercial chitin and chitin extracted from the crab shell.

Scanning Electron Microscope (SEM): Commercially purchased chitin (CPC) with chemically digested (CEC) and enzymatic digestion (CFC) crab chitin was analyzed using a Scanning electron microscope (TESCAN, MIRA3 XMU). Approximately 5 mg of each dried chitin sample was sputtered with gold using a Gatan Precision Etching Coating System and kept in a gold glaze system for 10–20 min before SEM analysis.

The surface morphology of chemically and enzymatically extracted chitin was examined and compared with that of commercial chitin using FESEM analysis. Commercial chitin and extracted chitin showed stacked layers with widely distributed fibers. The demineralized crab shell powder contained small shell fibers that were clustered together. Deproteinization causes the small fibrils to attach to each other, forming a nonporous smooth membranous plate-like crystalline chitin.

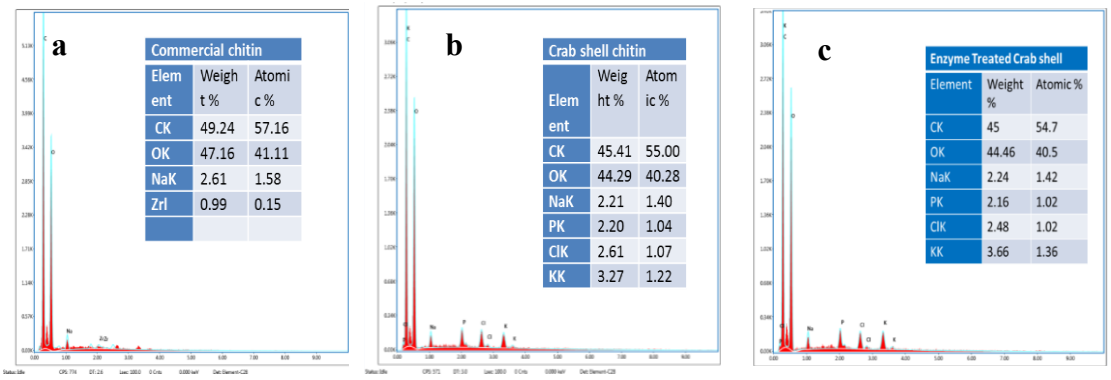


Fig. 6: EDAX analysis of Chitin: a) commercial chitin, b) Chemically treated crab shell chitin c) Enzyme treated crab shell

The enzymatically treated chitin exhibited a stacking layer with a cluster of small fibers. The SEM images of commercially purchased chitin indicated a nonporous smooth arrangement and the flaky chitin is depicted in fig. 5.

Energy Dispersive X – ray Analysis (EDAX): Elemental investigations of commercially purchased chitin (CPC) with chemically digested (CEC) and enzymatically digested crab chitin (CPC) were performed. The EDAX spectra of chemically and enzymatically raw crab shell chitin and purchased chitin exhibited the presence of carbon, oxygen, chlorine, sodium, potassium, zirconium and phosphate. The intensity of the peak for the existing elements was maximum for commercial chitin, whereas crab chitin showed nearly equal peak intensities (Fig.6).

The presence of elements on extracted chitin and commercial chitin indexed through EDAX showed a similar ratio of carbon and oxygen content with traces of other minerals. Similar EDAX spectrum investigation of different crustaceans such as crab, shrimp, fish and crayfish chitin and chitosan exhibited the highest carbon and oxygen content and a small amount of minerals (calcium, sodium, iron and zirconium).[35,37] Based on the previous studies, it was observed that the chitin extracted in this study is of high purity. EDAX spectra of crab shell chitosan and Safaco chitosan revealed different elemental compositions. Safaco chitosan contained metal minerals (Ca, Mg, Al and Si), while crab shell chitosan showed only two main peaks of C and O and no inorganic mineral content.

Conclusion

Next to cellulose, the most ample carbohydrate on the globe is chitin which provides a rich environment, necessary for the survival and growth of chitinolytic microorganisms. Therefore, the high diversity of organisms that contain exoskeleton structure as chitin in their structure, such as fungi, insects, marine arthropods, plants and animals produced chitinase for growth and development while microbes produce chitinase by decaying the organic matter like carbon and nitrogen from the chitin polymer. Soil bacteria act as an excellent source of chitinolytic enzymes and are the best rummage-sale for the renewable of chitin waste into useful products for various applications in biotechnology, medicine and agriculture. Chitin preparation was done from crab shell waste treated with culture filtrate of 7X5ZA. Characterizations of chitin samples were investigated with FT-IR and SEM analysis. The results of enzymatic degradation of crab shell wastes were relative to the chemical treatments.

The manufacture of chitin from crab shell waste insists not only on manufacturing purified chitin but also on solving environmental problems by converting the chitin to useful purposes for the isolation of chitinolytic bacteria. The potent chitinolytic bacteria were identified using simple and inexpensive techniques. The chitinase enzyme synthesis helps in the production of various useful byproducts that can be exploited for industrial purposes.

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