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Ultrasonication mode for the expedition of extraction process of chitin from the maritime shrimp shell waste

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Worldwide, marine crustacean waste is a major problem for environmental pollution, and it is a severe risk to the coastline area. Shellfish wastes consist of some commercially valuable products, mainly the chitin. The extraction of chitin from the shellfish waste is very complicated and required a successive pretreatment process. Sonication can improve the process of extraction of chitin from the shrimp shell waste. In this study, the conventional and ultrasonication method of pretreatment was applied and compared for the extraction of chitin. By the conventional method, 12 h was required for the removal of calcium and proteins each. In contrast, only 6 h was required for the complete removal of calcium and proteins each, by the ultrasonication assisted method. After pretreatment, the results were analyzed and compared by the already purified commercial chitin using Fourier-transform infrared spectroscopy. Ultrasonication improves the rate of reaction of the pretreatment by the process of cavitation. By this work, the ultrasonication technique was proved to be much faster than the conventional method for the pretreatment process.

Keywords: Chitin, FTIR, Pretreatment, Shrimp shell

The crustacean shell waste production is around 6-8 million metric tons worldwide per annum[1]. Chitin is the chief element in crustacean shells and is the second most abundant biopolymer[2] on Earth that can be used as a potential supplement for chitinase production[3]. Chitinase can utilize to produce biomolecules of major importance from the degradation of chitin. Shrimp is an important seafood consumed worldwide. Shrimp processed in food industries for packaging, where only 25% of the total weight of the shrimp is used as an edible product, and the rest of the 75% of the total weight of the shrimp is waste (Fig. 1) that consists of the outer shell and head of the shrimp. Each year, the processing and consumption of seafood generate hundreds of tonnes of shellfish waste. The total estimated production of chitin is around 2.5 million tonnes[4]. The generation of waste in the food processing industries is not avoidable, and disposal of waste is harmful to the environment. The use of shellfish waste in various applications is important not only to rescue from environmental problems but also to make an alternative to disposal of shellfish waste for the waste treatment[5]. The by-products of marine wastes may often have a number of available substances of major importance that may have significant health benefits and might be an excellent source of proteins, pigments[6], chitin and minerals; especially the shell component is an enriched source of chitinous material. About 40% of the total waste is chitin wastes[7]. To date, the waste of marine food production, primarily of crustaceans shells are the chief source for the production of industrial chitin.

There is a severe concern for the good utilization of chitin, which is the most available biopolymer next to cellulose. It is mostly found in the exoskeleton of arthropods like insects, crustaceans, and molluscs (beaks) and endoskeleton of cephalopods. Some microorganisms like bacteria, fungi, and yeasts also contain chitin in their cell wall. In soil, fungi provide the most considerable amount, *i.e.*, 6-12 % of chitin[8]. Chitin is generally obtained from shrimp and crab shells, as it has the chitin content of 15-40%, which is

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Abbreviations: atm, Atmospheric pressure; C, Carbon; $CaCl_2$, Calcium chloride; cm, Centimetre; FTIR, Fourier-transform infrared spectroscopy; g/L, Gram per litre; h, Hours; H, Hydrogen; HCl, Hydrochloric acid; K, Kelvin; mL, Millilitre; N, Nitrogen; NaOH, Sodium hydroxide; O, Oxygen; rpm, Rotation per minute

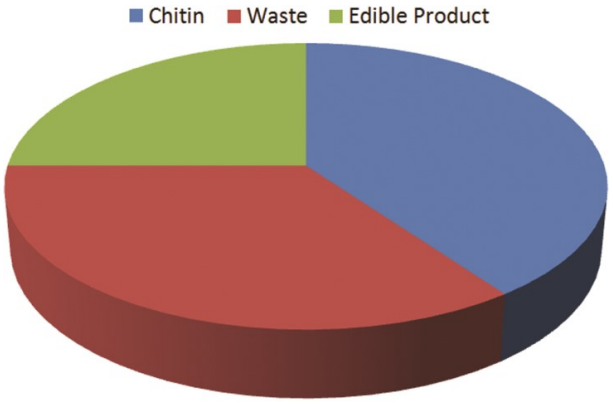

Fig. 1 — Pie chart showing the distribution of chitin, waste, and edible products in the shrimp. The complete pie is showing the shrimp that goes to the processing to make some edible products. The green colour is showing 25% of the total weight of the shrimp that used as edible products. The blue and red colour are combined for the 75% of the total weight of shrimp that is treated as waste, from that 75% the blue colour is showing 40% of chitin

reasonably high than other sources. Other than chitin; they also constitute of 20-40% protein and 20-50% calcium carbonate[9-14]. The shells of crustacean possessed thick fibres of chitin, interlock by proteins *via* a covalent bond. Chitin-protein matrix then strengthened by the accumulation of minerals primarily by calcium carbonate. Further, the association of the protein with polysaccharides increased the complexity of the structure[15]. Hence, to extract the chitin, shrimp shell has to be treated to remove the adhered calcium deposition and protein. The depositions of calcium and proteins were removed by treating the chitin with concentrated acid (HCl) and alkaline (NaOH) solution, respectively[16-20].

Usually, the conventional method was used for the removal of calcium carbonate and proteins. However, the deproteinization step is difficult due to the presence of chemical bonds between chitin and protein. It is known to take much time to remove the proteins from the shell by using the conventional method. Ultrasonication assisted chemical treatment can be used to enhance the deproteinization as well as demineralization[21,22]**.** It was found that the sonication help to improve the rate of reaction by actions of cavitation. The process of sonication generates and breakdown lots of microbubbles that increase the temperature up to 5000 K and pressure up to 1000 atm of the solution. These circumstances create a cavitation zone with large cut, mechanical pressure waves, and instability, which are supposed to simplify heat and mass transfer in the chemical reactions[23]. The effect of cavitation might enhance the penetration of acid or alkaline solution, and this could be an apparent sense for the high extraction in a short period. It was also reported that the ultrasonication helps to increase reaction speed with the increase in the effective area of reaction[24]. The extracted chitin and its derivatives are having numerous applications in various industries[19,25-30]. Chitosan and chito-oligomers have received significant consideration in pharmaceutical industries because of their biocompatibility and nontoxicity[31-35].

Furthermore, Chitooligomers are used for numerous purposes, comprising drug delivery[36,37], antioxidant effect[38,39], and antimicrobial activity[40-43]. In this study, the extraction of chitin was performed using chemical conventional and ultrasonication methods[21,22]. It aims to estimate the time point required for complete removal of calcium carbonate and protein by both of the methods, *i.e.*, conventional method, and ultrasonication assisted method. The concentration of calcium carbonate and proteins was estimated by flame photometer and biuret test, respectively. The raw shrimp shell, demineralized shell, deproteinized shell, and commercial chitin samples were analyzed by FTIR[44], and the results were compared to obtain a more efficient method for chitin extraction from the shrimp shell.

Materials and Methods

Materials

Raw shrimp shell was procured from the local fish market. HCl, NaOH, $CaCl_2$, $CuSO_4.5H_2O$, and potassium sodium tartrate were procured from Sigma Aldrich (St. Louis, MO, USA). Probe ultrasonicator was purchased from Oscar Ultrasonics Pvt. Ltd. (Mumbai, Maharashtra, India). The magnetic stirrer was procured from Remi Sales & Engineering Ltd. (Mumbai, Maharashtra, India). Hot air over was purchased from Biotechnics India (Mumbai, Maharashtra, India). Digital weighing balance was procured from Contech Instruments Ltd. (Mumbai, Maharashtra, India), Flame photometer of ESICO International (Himachal Pradesh, India) was used from the Department of Chemical Engineering, National Institute of Technology, Raipur C.G. Bruker FTIR from Opus Bruker Optics was used from the Department of physics National Institute of Technology, Raipur C.G.

Pretreatment of shrimp shell

In order to obtain the purified chitin, two steps are involved in the pretreatment process of the shrimp shell,

i.e., demineralization followed by deproteinization for the complete removal of minerals and proteins. Conventional, as well as the ultrasonication method (Fig. 2), was used for the pretreatment. The complete procedure is as follows.

Raw material

Shrimp's outer shells were collected from the local fish market. It was dried in sunlight for 3-4 days until complete moisture content was removed. The dried shrimp shells were crushed with the help of grinder and sieved with 150 mm sieve size. The sieved shrimp shells were stored in a dry place.

Demineralization

Sieved shrimp shells (50 g) were added with 500 mL of 0.6 M HCl, Sigma Aldrich (St. Louis, MO, USA) in the ratio of 1:10 and allowed to react on the magnetic stirrer for 12 h at 40°C and 400 rpm. Sample (5 mL) was collected at a regular interval of 1 h. The concentration of calcium carbonate was analyzed using a Flame photometer for the liquid sample, and the solid sample was used for the dry weight and ash content analysis. For the estimation of calcium with a flame photometer, the standard of $CaCl_2$ of concentration 20 ppm to 100 ppm of 10 mL each was prepared along with a blank, *i.e.*, without $CaCl_2$. The flame photometer was set with the air pressure between 0.4 to 0.6 kg/cm^2. All the known and unknown sample's reading was taken in the ppm unit. The unknown sample's concentration was calculated using the standard graph of $CaCl_2$. The solid samples were kept in a hot air oven at 70°C for 24 h, and the weight was measured to obtain the dry weight of the samples. The solid sample was kept in the muffle furnace at 800°C for 2 h, and the weight was taken using a weighing balance to obtain the ash content. The same procedure was followed for the pretreatment with ultrasonication assisted method. The reaction mixture was kept for ultrasonication for 6 h. The probe ultrasonicator was used with 20 kHz of frequency. After the demineralization process, the shells were washed with distilled water to reach the neutral pH. The demineralized shells were then used for the deproteinization.

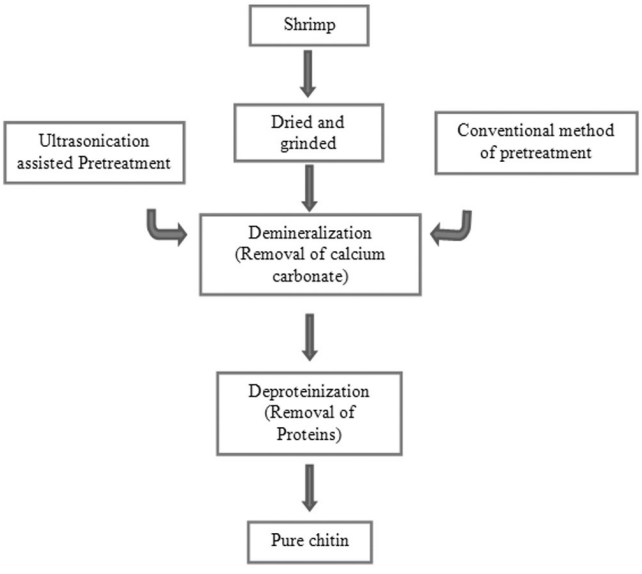

Fig. 2 — Steps of Pretreatment of shrimp shell. This flow chart showing the steps for the extraction of chitin from the shrimp shell by ultrasonication as well as the conventional method of pretreatment

Deproteinization

The demineralized shell was treated with 0.6 M NaOH, Sigma Aldrich (St. Louis, MO, USA) and allowed to react on the magnetic stirrer for 12 h at 40°C and 400 rpm and the other set of an experiment for deproteinization were carried out with ultrasonication for 6 h with the same conditions. At the regular interval of 1 h, solid and liquid sample was collected from the mixture. The liquid sample was analyzed for the concentration of protein by using the Biuret test, and the solid sample was used for dry weight, ash content analysis, and FTIR analysis. For the protein estimation, biuret reagent was prepared by adding 3 g of copper sulfate, pentahydrate ($CuSO_4.5H_2O$), and 1.2 g of potassium sodium tartrate in 50 mL of distilled water, after adding 10 g of NaOH, the solution was diluted to 100 mL of solution. BSA standard was prepared for the estimation of unknown proteins in the collected samples. 1 mL of prepared biuret reagent was added to each tube containing 2 mL of the known and unknown samples along with a blank solution. All the tubes were incubated for 30 min at room temperature, and absorbance was taken at 540 nm. The concentration of protein in unknown samples was calculated using the standard graph of BSA. For the FTIR analysis, the solid sample was coated with KBr, and absorbance spectra were collected for the wavelength range of 4500-400[45]. A diagrammatic representation of demineralization and deproteinization is given in (Fig. 3).

Results and Discussion

Dry Weight Analysis

The dry weight was measured for the samples collected during the demineralization and deproteinization process. It was observed that 22.4% of weight loss was

found after the demineralization process because of the removal of the calcium carbonate from the shells, and 36.4% of weight loss was observed after deproteinization due to the removal of proteins from the shell (Table 1).

Ash Content Analysis

The samples were kept in the muffle furnace at 800°C for 2 h, and the weight was measured. The ash content means the additional composition except for chitin in the shells, so the less the ash content, the more purified chitin will be obtained. It was observed that the ash content was decreased with the removal of minerals and proteins. The ash content of the raw shell and the commercial chitin was 39.56% and 6.28%, respectively. The ash content of chitin obtained by pretreatment of the raw shell using conventional and ultrasonication methods was 8.24% and 6.42%, respectively. It proves that the ultrasonication method is more efficient in removing non-chitinous material as compared to conventional material (Table 2).

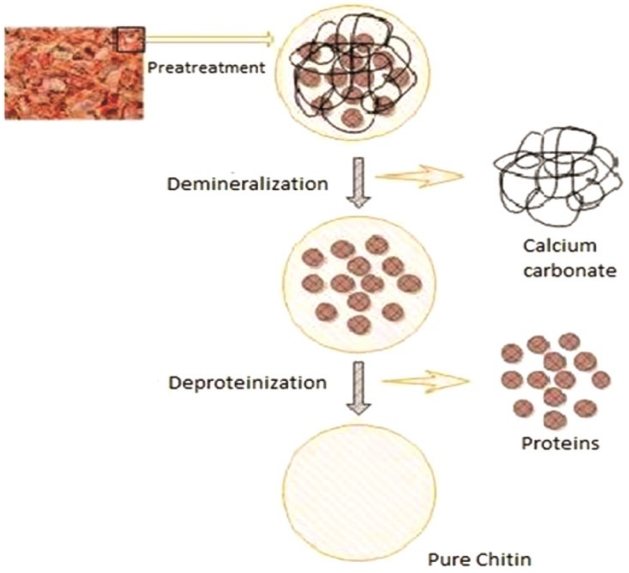

Fig. 3 — Diagrammatic representation for extraction of chitin from shrimp shell

Estimation of calcium carbonate

The flame photometer was used for the analysis of calcium concentration[46,47]. The liquid sample was collected during the pretreatment process. It was found that the calcium concentration was increased with respect to the time. In the sample isolated during the conventional and ultrasonication method of the pretreatment process, the calcium concentration was increased till 12 h and 6 h, respectively. Therefore it was observed that by the conventional method 12 h and by the ultrasonication method, 6 h was taken for the complete removal of calcium carbonate (Fig. 4).

Estimation of protein

The concentration of protein was evaluated using biuret test[48]. It was found that the concentration of

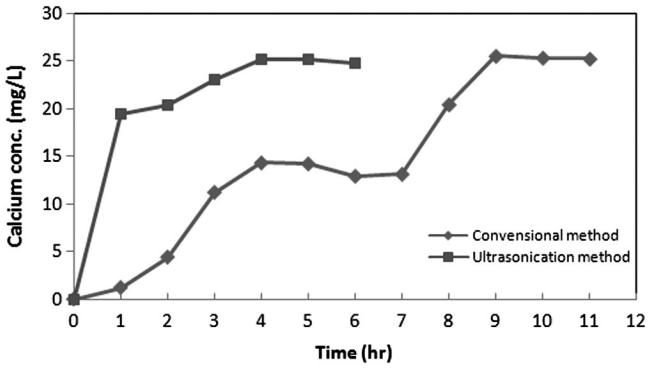

Fig. 4 — Analysis of removal of Calcium carbonate during pretreatment (Demineralization), Graph showing the gradual increase in the concentration of calcium carbonate during the process of demineralization by ultrasonication and conventional method, The concentration of calcium carbonate became constant after 4th and 9th day in case of ultrasonication method and conventional method respectively

Table 1 — Dry weight analysis during pretreatment of chitin

S. No	Sample (g)	Conventional method (g)	% loss	Ultrasonication method (g)	% Loss
1.	Raw shells	5.0	-	5.0	-
2.	Demineralized shells	3.88	22.4	3.84	23.2
3.	Deproteinized shells	2.66	32.2	2.44	36.4

Table 2 — Ash content analysis during pretreatment of chitin

S. No	Sample (g)	Conventional method (g)	% Ash content	Ultrasonication method (g)	% Ash content
1.	Raw shells	0.3956	39.56	0.3956	39.56
2.	Demineralized shell	0.1552	15.52	0.175	17.5
3.	Deproteinized shell	0.0824	8.24	0.0642	6.42
4.	Commercial chitin	0.0628	6.28	0.0628	6.28

protein was increased in the sample collected during the reaction from the conventional and ultrasonication process of pretreatment. By the ultrasonication method, 2.5g/L of proteins was removed in 6 h. In contrast, the conventional method was removed only 0.5 g/L of proteins even after 12 h. To remove proteins from the shrimp shell; the ultrasonication method was much efficient as compared to the conventional method (Fig. 5).

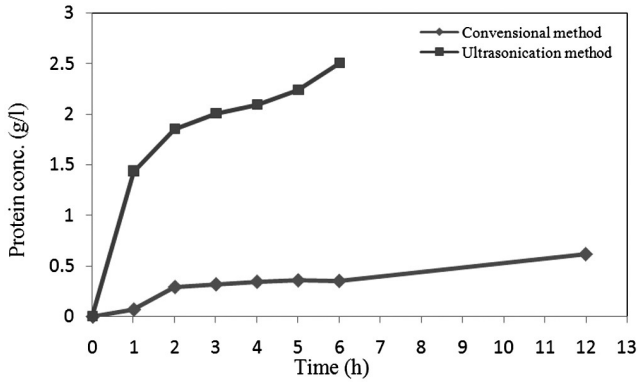

Fig. 5 — Analysis of removal of Proteins from the shrimp shell during pretreatment (Deproteinization), Graph showing the gradual increase in removed protein concentration by the ultrasonication method where the protein was utterly removed from the shell within 6 h, the protein removal by the conventional method is prolonged as compared to ultrasonication method

FTIR Analysis

FTIR analysis was done for the raw shrimp shell, commercial chitin, demineralized, and deproteinized shells obtained by conventional and ultrasonication assisted methods. All the results of FTIR were compared and found that the chitin purified by both of the methods is showing similarity with the commercial chitin. FTIR spectra of raw shrimp shell and the commercialized shell were compared with the demineralized shell and deproteinized shell obtained by the ultrasonication process (Fig. 6A) and by the conventional method (Fig. 6B) was compared. FTIR spectra of deproteinized shells obtained by both the methods revealed similarities with the commercial chitin. FTIR spectra (Fig. 7A-F) revealed similarities between the samples. All the common spectra were the vibrations due to the carbohydrate backbone. Absorbance between 800-900 cm^{-1} is assigned to glycosidic bond and C.H. stretching. Absorbance between 1020-1090 cm^{-1} is due to the absorbance of the

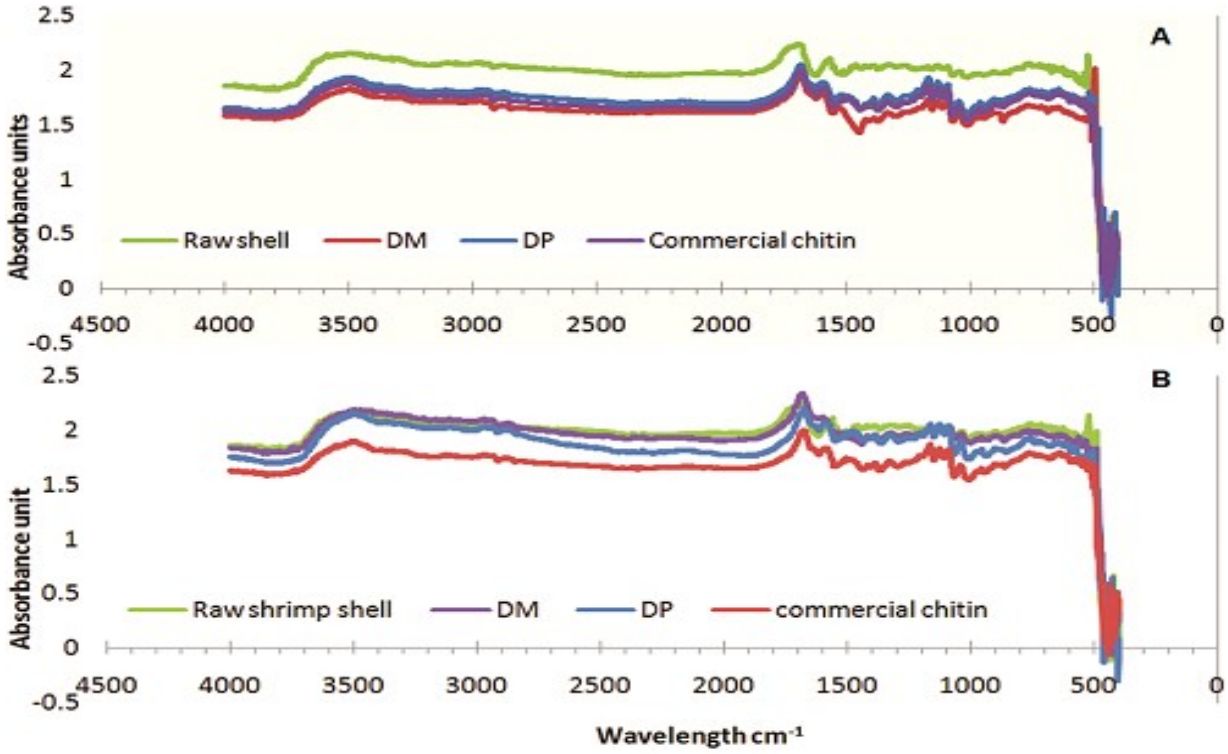

Fig. 6 — Comparison of FTIR data of raw shrimp shell and commercial chitin with the shell obtained after demineralization and deproteinization by (A) Ultrasonication assisted method and (B) Conventional method

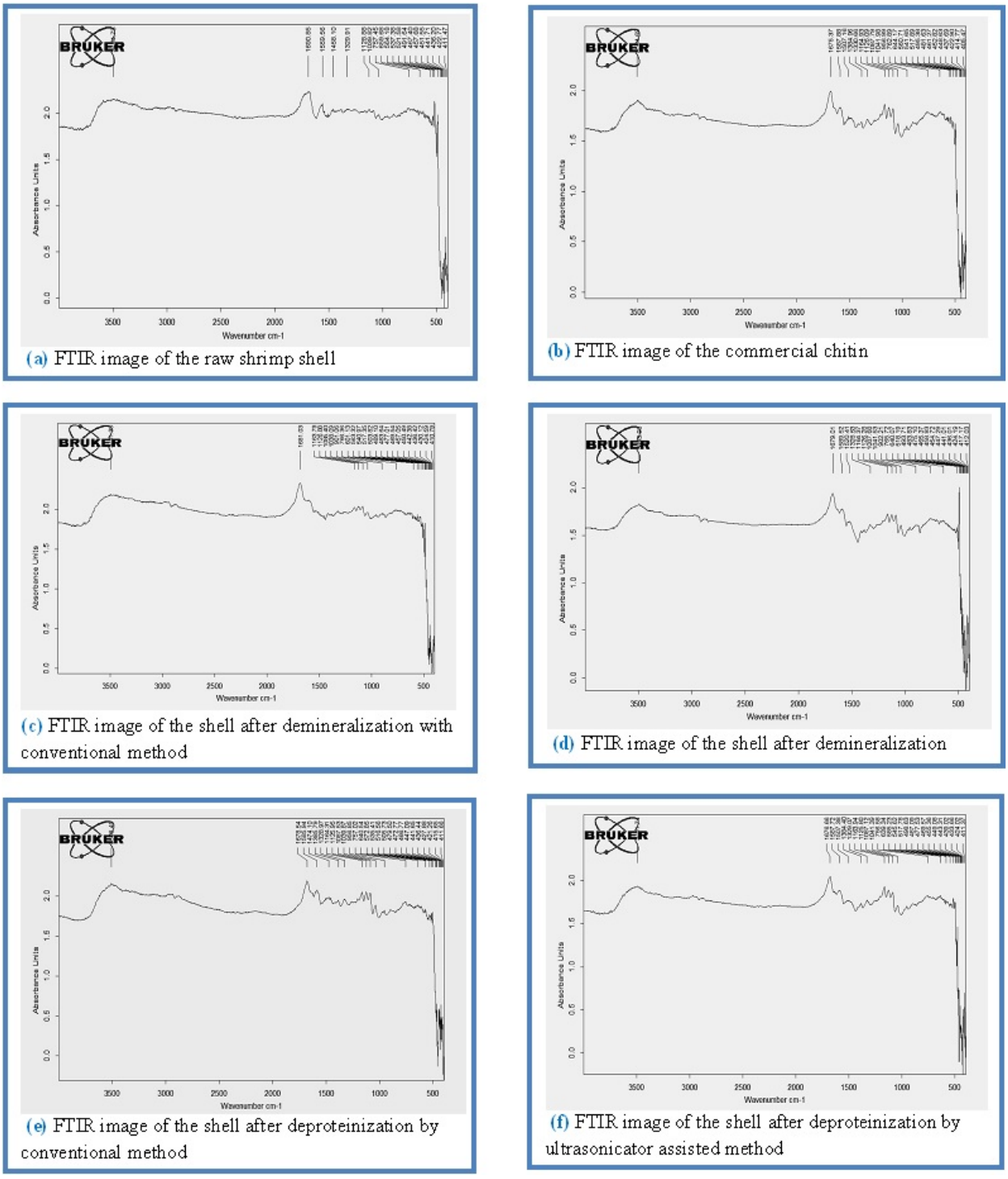

Fig. 7 — (A-F) FTIR images of shrimp shell during pretreatment

C-N bond. The absorbance between 1100 and 1200 cm^{-1} is assigned to C-C, and C-O modes, including a shoulder due to the vas C-O-C of the glycosidic linkage (1126 cm^{-1}) while the primary absorption is due to C-O weakly coupled to C-O-H. Absorbance between 1370-1430 cm^{-1} is due to the presence of C-H bending. Absorbance between 1550-1650 cm^{-1} is due to N-H. The presence of amino sugars shows the C=O of the amide I and N-H…C=O combination of the amide II absorbance bands between 1660-1740 cm^{-1}. The

observed spectra for the O-H bending modes and sugar O-H stretching vibrations are found in the region of 3200-3400 cm^{-1}[1,49,50] (Fig. 7A-F).

Conclusion

Shrimp shell is a constituent of chitin, proteins, and calcium carbonate. For the extraction of chitin from the shrimp shell, a conventional and ultrasonication technique was applied. Ultrasonication technique was found to remove calcium carbonate and proteins each in 6 h. In contrast, the conventional technique takes 12 h to remove both of the non-chitinous material. Dry weight analysis, ash content analysis, and estimation of calcium and protein concentration were done. From the dry weight analysis, it was concluded that the shrimp shells contain 20-40% of calcium and 20-50% of protein[9-14]. FTIR spectra of deproteinized shells obtained by both the methods revealed similarities with the commercial chitin. The Ultrasonication method was found as a much effective and time-saving technique for the efficient and complete removal of minerals and proteins as compared to the conventional method. Ultrasonication improves the rate of reaction of the pretreatment by the process of cavitation. This process produces lots of microbubbles, and their collides increases the temperature and pressure of the solution. These circumstances create a cavitation zone containing extreme shear, motorized waves, and instability; those are supposed to enable temperature and mass transfer in the biochemical reactions[23]. The cavities result in enhancing the dispersion of basic or acidic solutions, and it might be a reasonable goal for the maximum yield in the shortest time. Nevertheless, the frequency and energy of the ultrasonication need to be standardized to obtain an improved quality of chitin, which can be a new opportunity for further study.

Conflict of interest

All authors declare no conflict of interest.

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