Article

Extraction and characterization of chitin from granary weevil, *Sitophilus granaries* L. (Coleoptera: Curculionidae)

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Abstract

Insect chitin has been extracted from granary weevil - *Sitophilus granarius* L, commonly called wheat weevil in laboratory by treating it with 1 M HCl and 1 M NaOH. This was followed by decolorization. The yield of chitin from this species is 20.2%. The chemical structure and physicochemical properties of α-chitin was characterized using Fourier transform infrared (FTIR), X-ray diffraction, scanning electron microscopy. Chitin exhibited similar chemical structures and physicochemical properties to earlier investigated chitin form different insect as well as commercial chitin. The Degree of Acetylation (DA) value for chitin was 97.48% and chitin crystalline index (CrI) was 78.77%. Thus it is a promising alternative for source of chitin.

Keywords *Sitophilus granaries*; chitin; characterization; FTIR; XRD; SEM.

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1 Introduction

Chitin is the earliest known polysaccharide and the second most common natural biopolymer existing after cellulose. It is a linear polysaccharide composed of β-1,4N-acetyl-glucosamine (GlcNAc) units occurring naturally in three polymorphic forms. The orientations of the microfibrils, α, β, and γ chitin causes polymeric forms (Sashiwa and Aiba, 2004; Pilai et al., 2009). It is a major constituent of the cell wall of many fungi, insect exoskeletons, and crustacean shells. Chitin isolates from crustaceans or other aquatic invertebrates and arthropods exclusively from those with a hard exterior usually belongs to α-form. The chains in α–forms are aligned in anti-parallel manner giving rise to strong hydrogen bonding which makes it more stable. In living world, the major source of commercial chitin is crustaceans. It is obtained mainly from the shells of crabs, shrimps, lobsters, crayfish, squids and krill shells etc (Martin, 1998; Muzzarelli, 1999).

Research on alternative sources of chitin is still lacking. Moreover, as chitin being chief component of insect cuticle;recently, the production of chitin and its derivatives from insect sources has drawn substantial attention. Chitin is becoming more popular because of its interesting properties, such as biodegradability, biocompatibility, non-antigenicity and non-toxicity (Shahidi and Abuzaytoun, 2005; Khor and Lim, 2003). Chitinalso depicts its prominent role in biomedical fieldswhich includes wound healing properties, enhancement of immune system, hemostatic and antimicrobial activity (Ong et al., 2009; Aranaz et al., 2009). Large infestation of *S. granarius*is commonly seen in harvested stored grains every year significantly decreasing the crop yields. To overcome this control measures such as drying, surface treatment and usage of fumigant is commonly practiced.

Studies on many potential sources of chitin and chitosan was outby (Kaya et al., 2015) and was noted *Leptinotarsa decemlineata,*commonly called Colorado beetle possess 20% chitin content. Interestingly, as the polymer is obtained from renewable resources, it was proposed to explore *Sitophilus granaries.* Thus, the main objective was to exploit *Sitophilus granaries* for obtaining the chitin since the raw material comes from renewable resource and is economically significant due to the pest status of organisms.

For the extraction of chitin, standard methods of acid and alkaline treatments, followed by decolorization with potassium permanganate was performed. The physicochemical properties of chitin were characterized by Fourier transform infrared (FTIR), X-ray diffraction (XRD), and Scanning Electron Microscopy (SEM) methods. These physicochemical properties were also compared with standard commercial shrimp chitin.

2 Materials and Methods

The study was conducted in PG Department and Research center, KRT Arts BH Commerce and AM Science College, Nashik during the academic year 2021-22. Following are the details of the requirements.

2.1 Standard shrimp chitin

Himedia shrimp chitin (CAS No.1398-61-4) was used as standard reference.

2.2 Organism for chitin extraction

The source for extract of chitin was *Sitophilus granarius*, reared in the PG Department and Research Centre in Zoology, KRT Arts B H Commerce and AM Science College(KTHM College), Nashik, Maharashtra.

2.2.1 Chitin extraction

The chitin was extracted from *Sitophilus granarius* using standard chemical method as they are simple and inexpensive techniques. The adult insects were separated from the culture, and were starved for 48 hours to remove the gut contents. Later they were washed with water and killed by freezing. They were thawed at room temperature and air dried for 3 days. Dried insects were crushed and grounded to powder and sieved through a 25-micron mesh and stored in air tight container at 4ºC. This powder was then subjected to demineralization and deproteinization for extraction of chitin.

2.2.2 Demineralization

This was the first step of chemical method which involved treatment of powdered exoskeleton of *Sitophilus granaries* with 1 M HCl solution at room temperature (15:1 v/m, 0.5 hours). The resulted solid fraction was washed with distilled water until neutral pH was accomplished.

2.2.3 Deproteinization

The next step involved was deproteinization. Here solid fraction was subjected to alkaline treatment with 1 M sodium hydroxide (100ºC, 8 hours, under agitation).

2.2.4 Decolorization

The resulted product was then washed with distilled water till neutrality was reached. At last, it was washed with hot ethanol and later boiled in acetone to eliminate remaining impurities. Light brown chitin was obtained

which was washed with distilled water and dried at 50ºC in oven.

2.2.5 Percent yield of Chitin

The purified chitin was dried in a vacuum oven at 50ºC to constant weight (Abdou et al., 2008; Rodde et al., 2008; Majtan et al., 2007). The chitin content was determined from the weight variances of the raw materials and that of the chitin obtained after acid and alkaline treatments as discussed by (Kaya et al., 2016). This was presumed pure chitin and the proximate chitin content was calculated.

2.3 Structural characterization of extracted Chitin

Structural characterization of extracted chitin from *Sitophilus granarius*was determined by infrared spectroscopy, X-ray diffraction (XRD) and Scanning electron microscopy (SEM).

2.3.1 Infrared spectroscopy and FTIR

2 mg of chitin samples dried overnight at 50ºC and was homogenized with 100 mg KBr. Later the mixture was dried for 24 hours and subjected tocharacterization by infrared spectrophotometry (Spectrum Two FT-IR Spectrometer, Part Number L1600115, Hyphenated Technology, USA). The analysis was carried out at 4,000 to 400 cm^{-1} . KBr was used as reference.

The deacetylation (DA) of chitin samples was determined by comparing the absorbance of the measured peak to that of the reference peak. The DA was calculated from the absorbance (A) ratios according to the following equation (Majtan et al., 2007):

$$DA = (A1655/A3450) \times 100$$

2.3.2 X-ray diffraction of chitin powder

XRD analysis was performed to detect the crystalline nature of extracted chitins. The patterns were recorded using a Benchtop Powder X-Ray Diffraction (XRD) Instrument (Rigaku, Tokyo, Japan) with Cu radiation k-alpha, wavelength λ=1.54059 nm at the Department of Chemistry, Baburaoji Gholap College, Sangvi, Pune. Data were collected at a scan rate of 1°/min with the scan angle from 5°C to 40°C.

The crystalline index (CrI) was determined by the following equation:

$$CrI = (I_o - I_{am})/I_o$$

where $I_o$is height of the (hkl=110) peak and I_{am} is height of amorphous scattering at 2θ=16° (Yuan et al., 2011).

2.3.3 Scanning Electron Microscopy (SEM)

Surface of chitin was studied by using Field Emission Scanning Electron Microscope (FESEM), FEI (Nova NanoSEM 450) with Energy Dispersive Spectrometer (EDS), Bruker (XFlash 6I30) at Common Instrumentation Facility (CIF), Savitribai Phule Pune University, Pune.

3 Results

3.1 Chitin isolation and extraction

Chitin extraction is summarized in the Fig. 1.

3.2 Structural characterization of extracted Chitin

Infrared spectroscopy-Fourier transformed Infrared spectra (FTIR), X-ray diffraction (XRD) and Scanning electron microscopy (SEM) were carried out to study the structural characterization of extracted chitin from *Sitophilus granaries.*

3.2.1 Fourier Transformed Infrared Spectra (FTIR) Analysis

The chemical configuration of the chitin samples was characterized using Fourier Transformed Infra-red (FTIR) analysis (Fig. 2). There are three peaks were visible in both, i.e., commercial chitin from shrimp and chitin from *Sitophilus granaries.* The functional groups of the samples were compared with the peaks associated with functional groups of standardα-chitin from commercially available shrimp chitin. The α-chitin shows characteristic vibration bands near 1621.21, 1552.45 and 1376.04 cm^{-1} which correspond to the amide I

stretching of C = O, amide II stretching of N-H and amide III stretching of C-N respectively in FTIR spectra (Fig. 2). The corresponding chitin peak from *Sitophilus granaries* were seen at 1643, 1554 and 1373 cm^{-1}. The other significant peaks observed are: N–H stretching of amide groups (3264 cm^{-1}); O–H stretching vibration (2898 cm^{-1}); C–O–C symmetric stretching (1153 cm^{-1}) as seen in Fig. 2. Table 1 explains the assignments of the relevant bands from IR spectra of chitin of shrimp (standard/reference) and *Sitophilus granaries.*

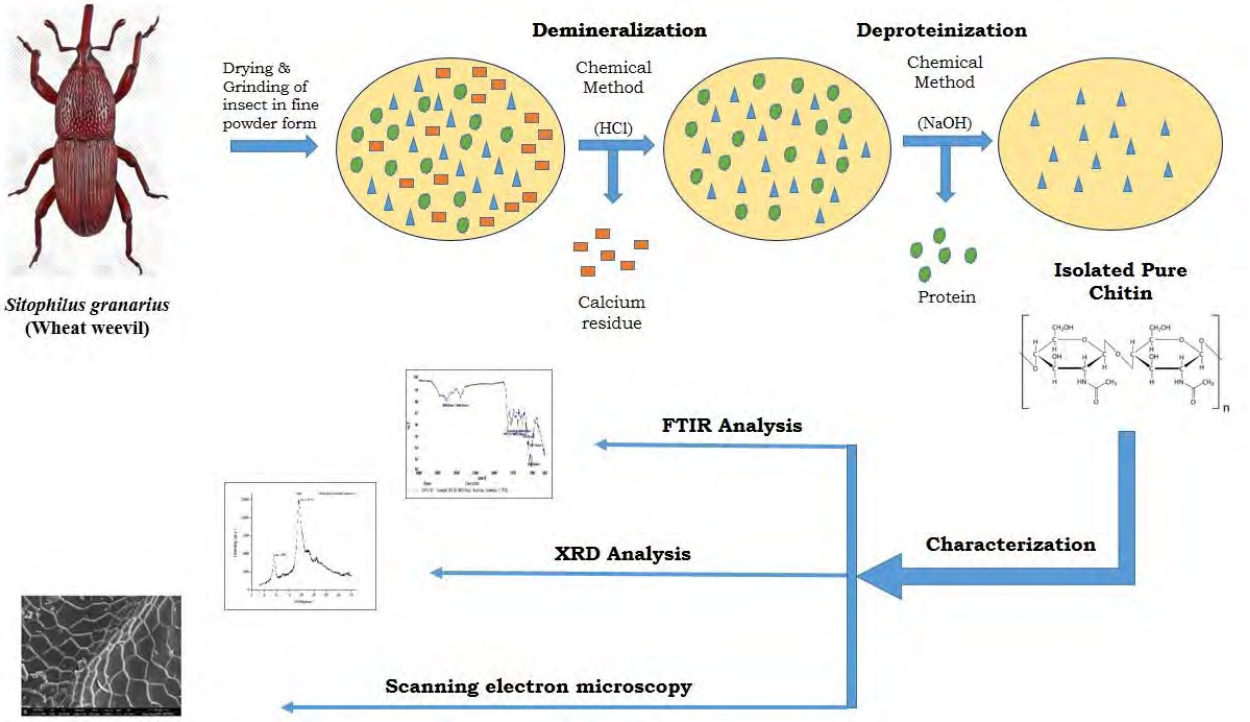

Fig. 1 Flow chart of chitin extraction.

Table 1 Major peaks and assignments in FTIR studies of chitin from *Sitophilus granaries*.

Peak No	Shrimp chitin		*Sitophilus granaries* chitin		Assignment
	%T	Wavenumber (cm^{-1})	%T	Wavenumber (cm^{-1})	
1	94.55	3261.84	98.11	3264.91	Presence of O-H/N-H
2	97.08	2876.87	98.32	2898.87	Presence of C-H, OH stretching
3	92.66	1621.21	95.64	1643.61	s, C=O stretching
4	91.73	1552.45	95.94	1554.91	δ, (N-H of N-acetyl group)
5	92.60	1376.04	95.94	1373.54	m, C-N, $-CH_3$
7	96.05	1203.83	96.06	1244.30	m, C-C skeletal vibration
8	93.21	1153.99	95.42	1153.20	s, C-O stretching vibration
11	86.04	1010.98	92.35	1008.05	s, C-H in plane deformation vibration
12	91.34	951.82	94.79	951.72	m, C-H wagging/rocking vibration

Degree of acetylation (DA) of chitin from shrimp and*Sitophilus granaries* was calculated using the formula mentioned in materials and method. This is a structural parameter which actually represents the proportion of N-acetyl-glucosamine units with respect to the total number of repeated units. DA is known to influence the physico-chemical parameters such as molecular weight, elongation at break, tensile strength and biological properties like wound healing, anti-microbial, biodegradable property etc. The calculated values for degree of acetylation (DA) of chitin from shrimp chitin and *Sitophilus granarius* was found to be 98.00% and 97.48% respectively.

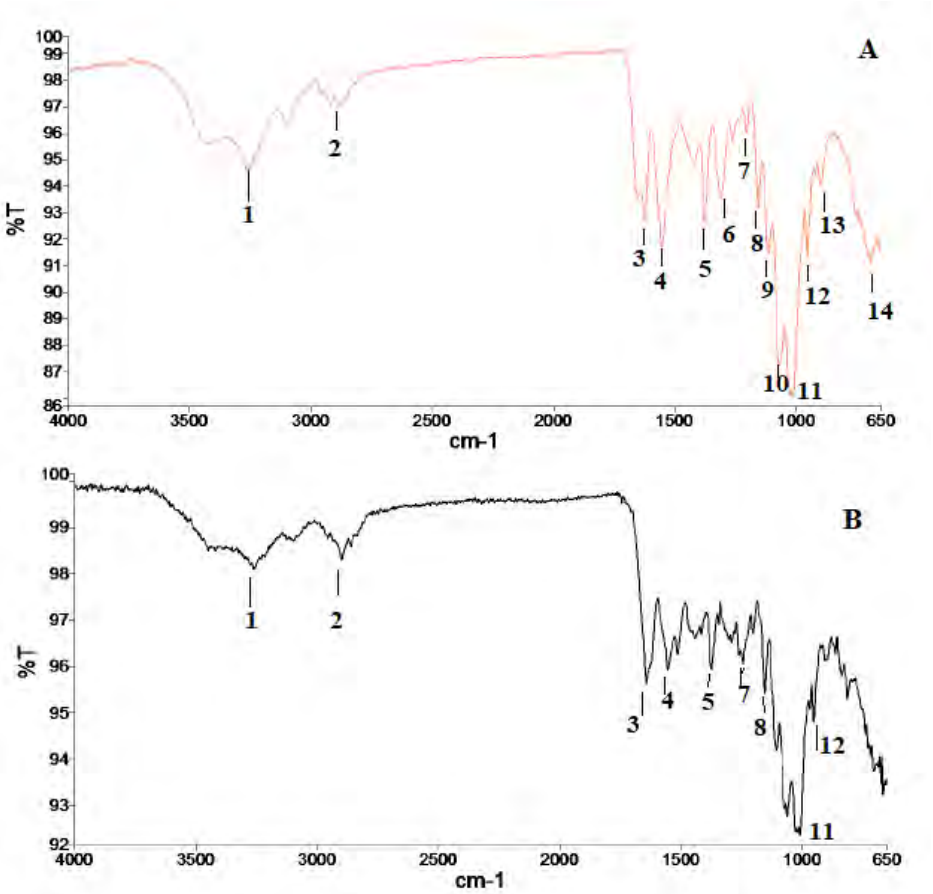

Fig. 2 Fourier transform infrared spectrum of chitin. A: Standard commercial chitin from shrimp; B: Chitin isolated from *Sitophilus granaries.*

3.2.2 X-ray chitin powder diffraction analysis

The XRD patterns of the chitin samples obtained from acid-base method presented in Fig. 3. This involves X-ray diffraction by a crystalline structure in the sample which allows the study of atomic and molecular structure of crystal. *Sitophilus granarius* chitin samples showed significant strong peak at 8.9°C, 9.2°C, 18.7°C, 25.6°C and minor peaks at 12.3°C, 22.8°C similar to the commercial shrimp chitin (8.9ºC, 9.2ºC, 12.3ºC, 16.3ºC, 18.7ºC, 22.8ºC, 25.6ºC).

The crystallinity index (CrI) of chitin was calculated from the XRD data using equation mentioned in materials and methods. It was found to be 69.65% in commercialchitin and 78.77% in *Sitophilus granaries*.

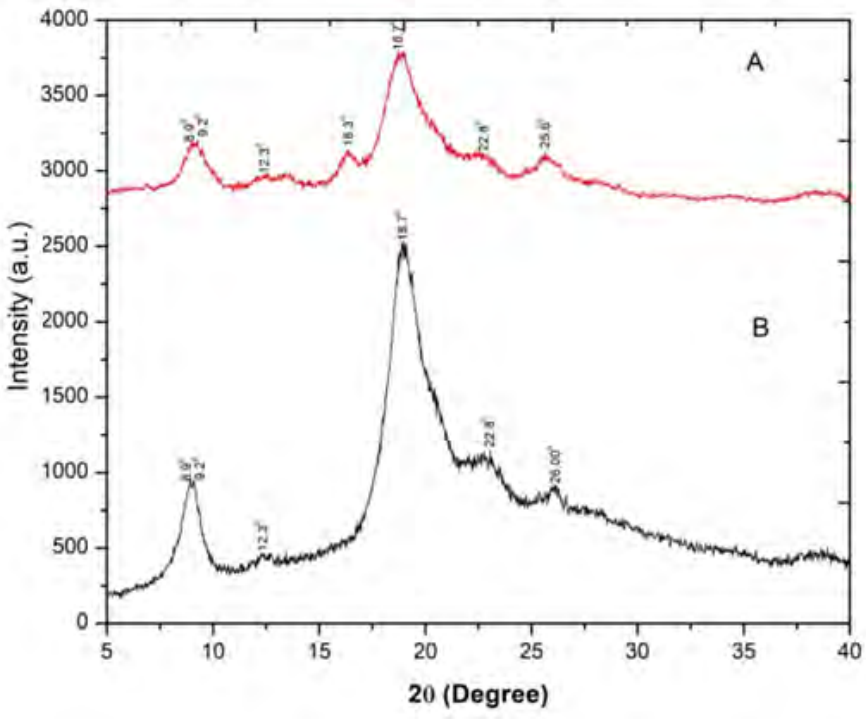

Fig. 3 XRD of chitin. (A) Standard commercial shrimp chitin; (B) *Sitophilus granarius* Chitin.

3.2.3 Scanning Electron Microscopy (SEM)

The surface morphology of extracted chitin from *Sitophilus granaries* was compared with shrimp chitin and the results are shown in Fig. 4a-f. From the SEM images it is observed that the chitin had smooth morphology. Moreover, the surface morphology showed that there are significant differences in the surface characteristics of the chitin obtained from wheat weevil.

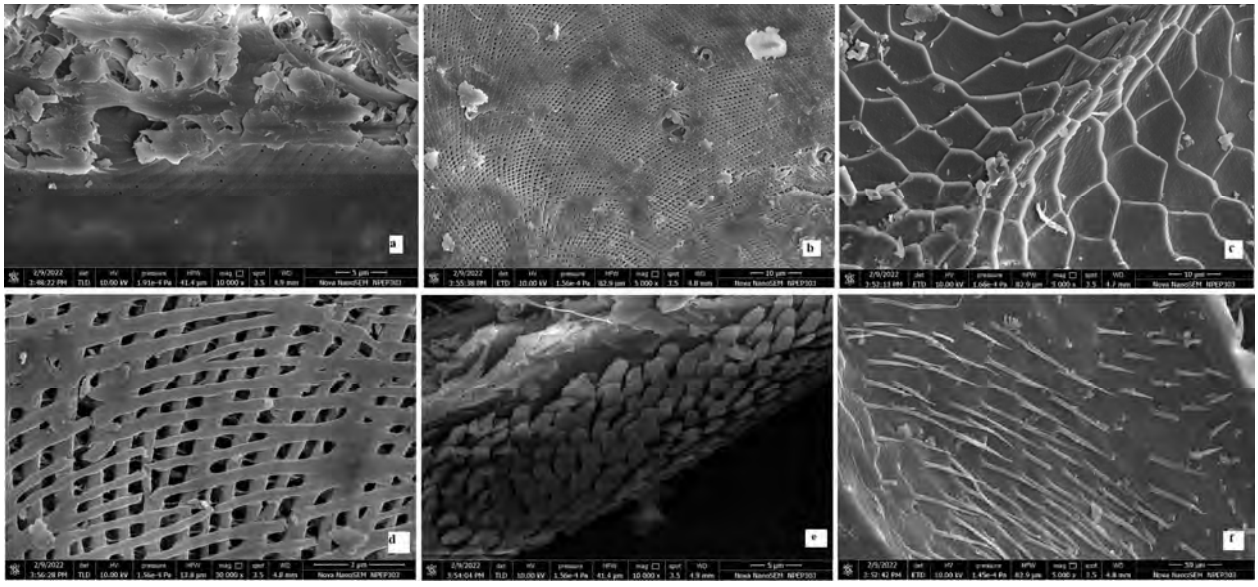

Fig. 4 Scanning Electron Microscopy (SEM) of chitin from *Sitophilus granaries*.

The chitin from wheat weevil showed smooth porous structure (Fig. 4a), parallel microfibrils (Fig. 4b, d), and well-organized repeating units of quadrilateral to hexagonal boxes (Fig. 4c, e). In addition to this we have also observed the spiny structure of chitin from isolated sample (Fig. 4f). Such spiny structure is never been reported before in chitin studies.

4 Discussion

Chitin is the major component of insect exoskeleton. As per earlier studies carried out by Majtan et al (2007), chitin is seen covalently bounded to catechol and sclerotin-like proteins.This biopolymer is insoluble in waterand prevents insects from desiccation which is an important adaptation for terrestrial living. Also, it protects the insects from microbial attack.

From 1998 to 2022 there is increased trend in insect chitin and chitosan studies (Kaya et al., 2017). The most common method for chitin extraction involves removal of catechol by acidictreatment followed by deproteinization. In this study, extraction and characterization of chitin from the adult wheat weevil - *Sitophilus granaries* is carried out by the method discussed by material and methods section. The acidic condition used for extraction of insect chitin is moderate as compared to crustacean shell. This is because insect chitin have low levels of inorganic material which is found to be less than 10% when compared to crustaceans shell (20-40%).

The water holding property is very promising feature in food industry. It was studied in chrysalis of silkworms, mealworms and grasshopper species and it was found to be very high as compared to shrimp chitin (Zhao et al., 2010). Chitin isolated from *Pterophylla beltrani* showed antifungal activity against the *M. anisoplia* (Torres-Castillo et al., 2015). Studies carried out by Ma et al (2015) have demonstrated better mechanical properties, tensile strengthof 62mPa and elongation at break as 10.4% for production of biodegradable film which is similar to commercial medical grape shrimp chitosan (Ma et al., 2015).

Scientists have proved the benefits of insect-based chitin in biomedical and food application. Conventional ethnobiological information has mentioned use if insects as an important source of nourishment and its vital usage in treatments of various diseases. Certain catastrophic insects are important vectors and pest causing huge damage to mankind, his domesticates and crops. Thus, it is important that they can be valorized. Therefore, here a humble effort is made to isolate chitin bio polymer from renewable resource- adult *Sitophilus granarius*.

For understanding the physico-chemical properties of chitin, the Fourier Transformed Infra-red (FTIR) analysis was carried out. The FTIR bands from isolates of wheat weevil chitin indicates presence of α-crystal form of chitin, like the commercial chitin extracted from shrimp. It was found that there is no band at 1540 cm^{-1} indicating absence of protein residues in the chitins.This is an indication of the successful deproteinization process (Tolaimate et al., 2003). Interior of insect cuticle which is formed of hypodermis contains protein that provide firmness, flexibility and elasticity to body. Tajiri et al (2017) have shown significance of cuticular proteins in mechanical control of whole body shape. These proteins are probably removed during deproteinization process whereas chitin – major component of cuticle attribute to the light but mechanically strong scaffold material in insects.

For understanding the Degree of acetylation (DA) of chitin from shrimp and *Sitophilus granaries* A1655 - the absorbance at 1655 cm^{-1} of the amide I band as a measure of the N-acetyl group content and A3450 - the absorbance at 3450 cm^{-1} due to hydroxyl group as an internal standard was used. The calculated values for degree of acetylation (DA) of chitin from shrimp chitin and *Sitophilus granarius* was found to be 98.00% and 97.48% respectively.

XRD studies helps in the identification type of chitin and in *Sitophilus granaries* peaks between 4 and 26ºC were noted. Chitin from cicada sloughs (beetle)was reported to show a similar result, with reflections at 9.20°C, 12.60°C, 19.18°C, 20.68°C, 23.30°C and 26.48°C (Sajomsang and Gonil, 2010). There are slight differences seen between the reflections of *Sitophilus granarius* and that of shrimp chitin. Peak 16.3ºC, and 25.6ºC is missing in *Sitophilus granarius* chitin samples but 4 sharp peak at 8.9°C, 9.2°C, 18.7°C, 25.6°C are present. Presence of strong sharp peak at 8.9°C and 9.2°C indicates presence of α-chitin (Kaya et al., 2017; Soetemans et al., 2020).

Followed by XRD studies, CrI values were calculated. It helps in understanding the nature of the chitin with reference to crystallinity. From the current results of higher CrI-values of *Sitophilus granaries* (69.65%), it can be concluded that this chitin is more crystalline in nature. The results of CrI studies are in accordance with the studies carried out by Kurita (2001) and Kaya et al. (2015b) who have reported CrI of α-chitin of crustacea and grasshoppers between 63% to 73%. The CrI values of Adult BSF-chitin, pupae shells-chitin, and commercial chitin were reported 49.6%, 50.3%, and 60.1% respectively and is less crystalline (Zimri, 2018).

In another study, CrI values of insect species with ranges from 40% to 80% depending on the species, growth phase, sex and isolation method (Wasko et al., 2016). For example, chitin from beetle *Holotrichia parallela* had a similar high crystallinity (CrI values ~ 89%) to the tested commercial chitin from shrimp (Liu et al., 2012). As *Sitophilus granaries* shows 69.65% CrI value it is presumed that low molecular weight catechols may be present in chitin. This is in accordance with the studies carried out by Zhang et al (2000) which supports the low crystallinity. However, a much lower crystallinity was found in chitins from larva cuticles and silkworm pupa exuviae (*Bombyx mori*), about 54 and 58% respectively (Zhang et al., 2000).

Finally SEM studies of chitin from wheat weevil showed smooth porous structure, parallel microfibrils, and well-organized repeating units of quadrilateral to hexagonal boxes.

In addition to this some spiny structure of chitin from isolated sample were also seen. Such spiny structure is never been reported before in chitin studies. This kind of surface morphology probably due to usage of whole insect (including antennae, wings, legs, etc.) in isolation and extraction of chitin from *Sitophilus*. Kaya et al (2015) reported occurrence of such surface morphologies in isolates of chitin from orthopteran insects.

Chitin yield varies significantly which was evident from previous investigation on different beetles. The lowest value of chitin yield is reported in *Calosom rugosa* (5%) while the highest chitin yield in *Leptinotarsa decemlineata* (20.2%). In the present study yield of chitin from adult *Sitophilus granaries* was found to be 20.2%.

The yields of chitin from different insect varies within species and their developmental stages. The chitin from larval and pupalexcuvia of *Bombyx mori* were reported as 15 and 20% respectively (Zhang et al., 2000). In cicada sloughs, higher chitin yield of 36% was recorded (Sajomsang and Gonil, 2010). The yield of chitin from crustacean shells is reported between 7–40%, depending on the type of shell and species (Tolaimate et al., 2003). Though the amount of chitin yield in insect is low compare to crustacean, it was found that the chitin from insects was nontoxic and possessed antimicrobial traits. Therefore, they are suitable as alternative material for massive production and application for commercialization (Morin and Dufresne, 2002).

5 Conclusion

The isolation of chitin from *Sitophilus granarius* was successfully performed using standard acid-base methods. The recovery of chitin from *Sitophilus granarius* was higher than that of the crustacean shells due to low inorganic materials such as calcium carbonate. The characteristicsof chitin andits derivatives from *Sitophilus granaries* studied with FTIR, XRD and SEM analysisare similar to those of commercial chitin obtained from crustaceans (shrimp chitin). It was found that the DA of chitin derived from adult insect is found

to be 97.48%, which represent significant isolation of chitin.The X-ray powder diffraction (XRD) revealed that the chitin isolated from sample is having higher crystallinity compared to commercial shrimp chitin. SEM analysis indicates dense nanofibers surface structure of the chitin. The large number of *Sitophilus granaries* adults' pests captured from stored grains may provide a renewable source for the production of chitin and it does not disturb the food chain and ecosystem.

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