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Isolation and Characterization of Chitin Nanowhiskers from Fermented Tiger Prawn Waste

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The objective of this study is to produce chitin nanowhiskers (CNW) from bacterial fermentation of tiger prawn waste. For this purpose, chitin was first extracted from tiger prawn waste using bacterial fermentation process followed by isolation of CNW using acid hydrolysis process. The isolated CNW from fermented tiger prawn waste (FCNW) were investigated using Fourier transform infrared (FTIR) spectroscopy, scanning electron microscopy (SEM), transmission electron microscopy (TEM), atomic force microscopy (AFM), x-ray diffractometer (X-RD) and thermogravimetric analysis (TGA). FTIR spectra analysis indicates that the acid hydrolysis of chitin did not altered the chemical structure of isolated FCNW. TEM analysis revealed that the produced FCNW displayed a nanoscale structure with an average length and width of 100 and 10 nm. AFM images of FCNW indicate the presence of spindle-like features. The X-RD analysis revealed that the acid hydrolysis process enhanced the crystallinity of FCNW from 20 to 46 % compared to chitin due to the removal of the amorphous region. The TGA results revealed that the FCNW is more thermally stable than fermented chitin (FC) and CNW from commercial chitin. The relatively good thermal stability of FCNW shows its suitability in a range of applications such as reinforcing fillers in green nanocomposites.

1. Introduction

For many years, chitin a natural polysaccharide having a linear polymer chain of N-acetyl-D-glucosamine linked by α (1, 4) glycosidic bond is popular among researchers due to its cheap and abundant availability worldwide. Chitin polymeric chains exists in α-crystalline form in exoskeleton of crustaceans such as crab, shrimp and lobster while β-chitin can be found in squid pens (Chaussard and Domard, 2004). Interestingly, in the last decade new interest in the use of chitin for nanocomposite materials has emerged because chitin is found to have desirable mechanical property due to their natural stacks of chitin nanocrystals embedded within the amorphous region. The amorphous region however can be removed through acid hydrolysis process. The remaining crystalline region normally exist in nanoscales can be further used as a reinforcing nanofillers for the development of green nanocomposites (Kelnar et al., 2015). Various methods have been employed in the production of chitin nanowhiskers including acid hydrolysis (Nair and Dufresne, 2003), TEMPO-mediated oxidation (Fan et al., 2012), and mechanical treatment (Fan et al., 2012).
Chitin is commercially obtained through harsh chemical treatments using acids and alkalis (Chandumpai et al., 2004) which discard large amount of chemical wastes that leads to environmental pollution. An alternative approach using biological processes (bacterial fermentation) to purify chitin from crustacean waste has also been reported which provides an environmentally cleaner approach and cheaper production cost (Zakaria et al., 1998). Besides chitin, this method also produces protein rich liquor which is suitable as protein source in aquaculture feed (Nor et al., 2011). Finding application for the by-product chitin would be an economical advantage to the frozen prawn processing industries which normally discard nearly 50 % of the whole

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processed prawn as waste. This study aims to produce CNW from biologically isolated chitin of tiger prawn waste origin to be used as polymeric reinforcement fillers in an attempt to replace the use of CNW from commercial chitin source which uses harsh chemical treatments.

2. Materials and methods

2.1 Isolation of chitin from fermented tiger prawn waste

Tiger prawn (Penaeus monodon) wastes was collected from a local prawn processing industry in Johor, Malaysia consisting of head, exoskeleton, and tail portion. Minced prawn waste (100 g) was fermented using 10 % v/w commercial starter EM and 10 wt% glucose in a lightly capped bottle and incubated at 37 °C for 72 h (Nor et al., 2011). The mixture was occasionally stirred especially during the first 24 h and the pH monitored. After 72 h, the fermented prawn waste silage is separated into solid fraction (chitin) and liquid fraction containing liquid protein. The chitin fraction was washed with distilled water and dried and is designated as fermented chitin (FC). For comparison purposes, commercial chitin obtained from Sigma-Aldrich (Malaysia) is designated as CC.

2.2 Preparation of chitin nanowhiskers (CNW)

CNW were prepared through acid hydrolysis process (Nair and Dufresne, 2003) of FC (1.0 g) in boiling 3 N HCl for 1 h. After hydrolysis, chitin was diluted with distilled water (40 mL) and centrifuged (Hettich, Newport Pagnel, England) at 3,200 rpm for 15 min and were repeated thrice. The chitin sediment was dialysed in cellulose dialysis tubing against continuous water flow for 2 h. The dialysis process was continued by immersing the tubing in a beaker of distilled water until the dipping solution reaches pH 4 before the suspension was sonicated for 10 min. The extracted chitin is designated as FCNW and were sealed and kept at 4 °C until further use. Similar steps were repeated in producing CCNW from commercial chitin, CC.

2.3 Characterisation of CNW

Fourier transform infrared (FTIR) spectroscopy was performed using Perkin Elmer 1600 infrared spectrometer (USA). FC and CC were characterised using the KBr method in the ratio of 1 : 100 and made to a pellet while FCNW and CCNW were characterised using liquid method by suspension casting. All samples were scanned within the wave number range 370 to 4,000 cm^{-1}.

Morphological structure analysis was done using SEM (JEOL JSM-6390 LV) at an operating voltage of 15 kV and samples were sputter-coated with gold prior to observation. Dimensions of the samples were obtained from transmission electron microscopy (TEM) with a model LEOLIBRA at an accelerating voltage of 120 kV. Atomic force microscopy (AFM) was performed using SPA-300 HV atomic force microscope with an SPI 3800 controller. A dilute drop of chitin suspension was dispersed on the surface of an optical glass slide and allowed to dry at room temperature prior to analysis.

The crystallinity of the samples was studied using X-ray diffractometer (X-RD) (SIEMENS XRD D5000) and Ni-filtered Cu Kα radiation at an angular incidence of 10° to 50° (2θ angle range). The operating voltage and current were 40 kV and 50 mA. Since all of the chitin samples in this study had XRD patterns of the α-chitin type, the crystallinity index of each sample was calculated from the diffraction intensity data according to the reported method by Fan et al. (2010). The crystalline-to-amorphous ratio of materials was determined using Eq(1).

$$\text{Cr.I (\%)} = \frac{I_{110} - I_{am}}{I_{110}} \text{ x } 100\ \% \quad (1)$$

where, Cr.I is the crystallinity index, I_{110} is the maximum intensity of the diffraction from the plane at 2θ = 19.6°, and I_{am} is the intensity of the background scatter measured at 2θ = 16°. The crystal sizes of the [110] directions were measured from the widths at half heights of the diffractions peaks at 19.6°, using Scherrer's equation (Fan et al., 2008).

The thermal stability of samples was characterised using a thermogravimetric analyser (TGA), model 2050 (TA Instruments, New Castle, DE). The specimens were scanned from 30 °C to 600 °C at a rate of 10 °C min^{-1} under a nitrogen gas atmosphere.

3. Results and Discussion

3.1 FTIR spectroscopy analysis

The chemical structures of obtained FC, CC, FCNW and CCNW after the treatment were analysed through FTIR absorption spectroscopy. FTIR spectra of all samples are illustrated in Figure 1. Based on FTIR spectra, all samples showed similar absorption bands indicating that all samples have similar chemical compositions.

From Figures 1 (a) to (d), there are main absorbance region which can be observed around 3,420 cm^{-1}. This absorbance represents O-H groups stretching. The characteristic carbonyl (C=O) stretching around 1,640 cm-1 is attributed to the carbonyl vibrations of amide bond. The presence of amide group was further strengthened by appearance of C-N absorption band at around 1,370 cm^{-1} and N-H bending around 720 - 770 cm^{-1}. Meanwhile, the frequency at around 1,020 cm^{-1} is attributed to the bending vibration C-O inside the chitin ring and of hydroxyl group (Rumengan et al., 2014).

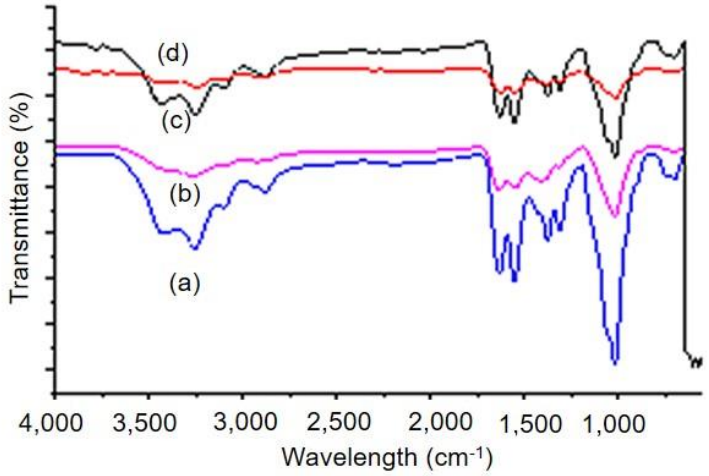

Figure 1: FTIR spectra of (a) CC, (b) FC, (c) CCNW and (d) FCNW

The IR spectra showed that there was no significant difference between the chitin and its respective nanowhiskers indicated that acid hydrolysis did not alter the chemical structures. It only involves the breaking of the glycosidic bond that linked chitin groups but does not involve breaking of carbonyl (amide) bonds although it is noted that the treatments of chitin in boiling HCl can dissolve the amorphous domain as well as ether and amide linkages (Zeng et al., 2012). By controlling the acid concentrations and reaction time, hydrolysis of amide bond can be avoided. This proves that the chemical groups of the resulting materials were stable and no strong chemical reaction occurred. Their surface morphologies however may be affected as discussed in the next section.

3.2 Morphological structure studies

The surface morphology of FC and CC were observed using SEM (Figure 2(a) and 2(b)). FC shows a rougher surface morphology compared to CC. The rough morphology of FC may be due to presence of protein and $CaCO_3$ which might still be attached on FC after the fermentation process. Zakaria et al. (1998) reported that although fermentation of prawn waste is an environmentally friendly biological method of isolating chitin from prawn waste, the chitin still contains minimal amount of calcium carbonate (< 1 %) and protein (5 %) within the chitin composite. CC that undergoes harsh acid and alkaline treatment in its industrial production exhibits smoother surface morphology. The differences are only very slight and that gives the milder fermentation method an added advantage since it is chemically less polluting to the environment.

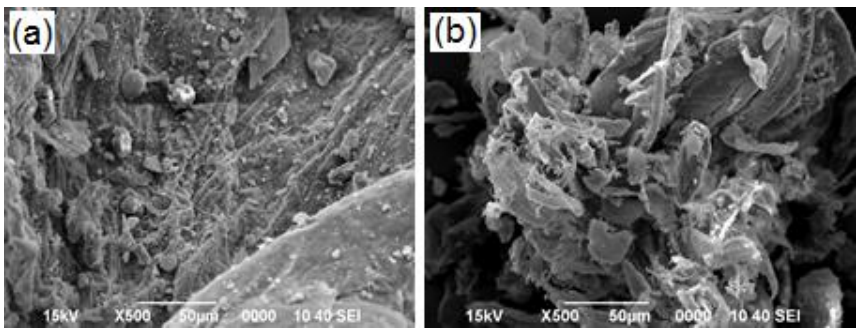

Figure 2: SEM images of (a) FC and (b) CC

To confirm the separation of individual crystallites or nanowhiskers, freeze dried nanowhiskers suspension of FCNW and CCNW were observed using TEM as shown in Figure 3(a) and 3(b). TEM is considered as a powerful technique in characterisation of nanowhiskers. The average size of FCNW and CCNW were estimated to be more than 100 nm in length and less than 10 nm in width. The dimension of chitin nanowhiskers prepare from various sources will have a small difference (Ma et al., 2014). It can be observed that chitin nanowhiskers particles aggregated to some extent more likely due to the evaporation of water.

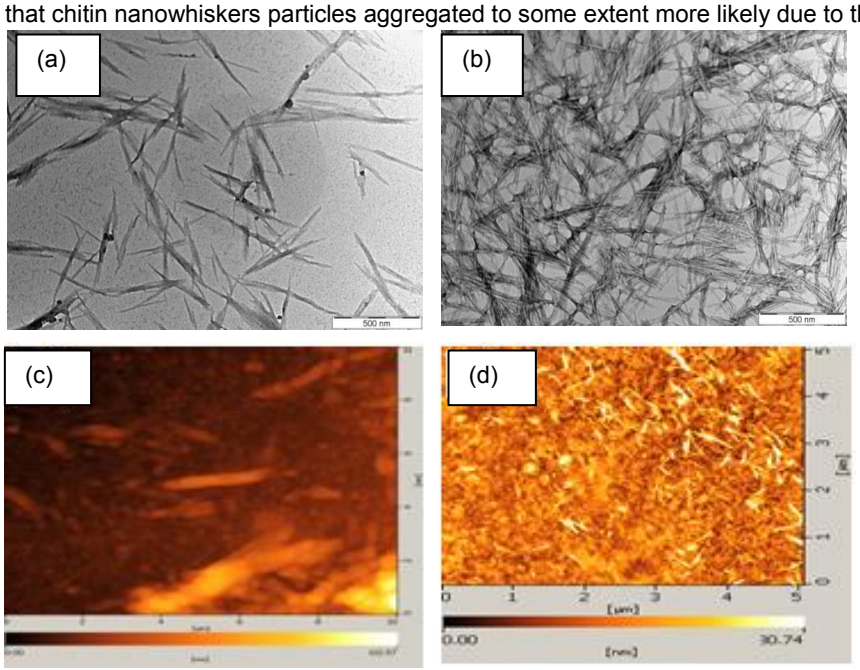

Figure 3: TEM and AFM images of (a) and (c) FCNW; (b) and (d) CCNW

AFM images of FCNW and CCNW are shown in Figures 3 (c) and (d). Even though TEM images have higher resolutions than AFM images, both images for the same sample corresponded well to each other. FCNW had spindle-like morphologies and some of them formed aggregation. CCNW consisted of mostly individualised rod-like whiskers with uniform widths and lengths. Similar observation was also previously reported by other researchers (Fan et al., 2010).

3.3 X-ray diffraction

The crystallinity of FC, CC, FCNW and CCNW were studied using XRD technique and their XRD spectra are shown in Figure 4. The crystallinity index of all samples and crystal size for plane (110) are summarised in Table 1.

The crystallinity index of chitin reported is similar to previous study done by Rumengan et al. (2014). The increase in crystallinity of chitin nanowhiskers compared to their respective chitin is mainly attributed to isolation of chitin nanocrystal through acid hydrolysis. Disorganised amorphous regions of chitin are highly hydrolysed and dissolved in the acid solution while highly crystalline regions of chitin are preferentially resistance to acid hydrolysis. Upon removal of disordered crystalline defects during acid hydrolysis, chitin rod-like nanowhiskers is produced.

The crystal size of the (110) plane determined from XRD pattern of FC and CC are both 0.46 nm. Similar findings were previously reported in study of crystallinity and hydrophilicity of chitin by Ioelovich (2014). There are no changes in the crystallite size of FCNW and CCNW which are also 0.46 nm.

3.4 Thermogravimetric analysis

Thermal stability of FC, CC, FCNW and CCNW were investigated through thermogravimetric analysis (TGA). The thermal degradation data (T_{10}), the peak degradation temperature (T_{max}) and the residual weight at 600 °C are tabulated in Table 2.

Table 2 shows that based on T_{10} and T_{max} values, CC is more thermally stable than FC. The CNW shows different behaviour compared to the chitin whereby FCNW is observed to be more thermally stable than CCNW based on the same criteria. Interesting to note that T_{max} of FCNW is 4 °C higher than FC while CCNW is significantly lower than the original chitin (CC). The residual weight at 600 °C for both type of chitin

nanowhiskers were lower than the original chitin. The residual weight of FC is much higher than CC which reaffirmed the presence of excess protein and $CaCO_3$ that might still be attached in FC as previously observed in SEM images. Based on T_{10} and T_{max}, it can be concluded that the thermal stability of FC increased after it underwent acid hydrolysis treatment. The increase in thermal stability of FCNW compared to FC is attributed to the higher crystallinity index as previously reported. For CCNW, the T_{max} decreased due to the shorter molecule chain of CCNW, although the crystallinity index is high.

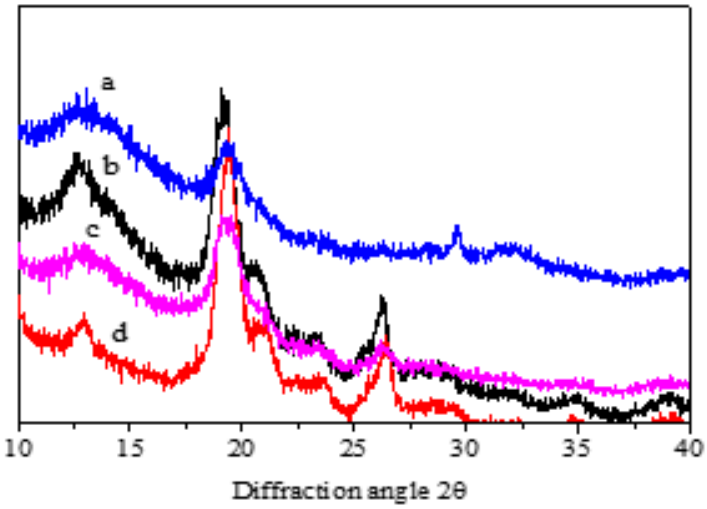

Figure 4: X-Ray diffractograms *of (a) FC, (b) CC, (c) FCNW and (d) CCNW*

Table 1: Crystallinity index and crystal size of FC, CC, FCNW and CCNW

Sample	Crystallinity Index (%)	Crystallite size [110] plane (nm)
FC	20.0	0.46
CC	38.0	0.46
FCNW	45.6	0.46
CCNW	66.7	0.46

Table 2: Thermal properties of FC, CC, FCNW and CCNW

Samples	Degradation Temperature, T_{10} (°C)	DTG peak temperature, T_{max} (°C)	Residual weight at 600 °C (%)
FC	271	322	5.6
CC	303	336	1.9
FCNW	282	326	0.6
CCNW	273	322	0.2

4. Conclusions

Chitin nanowhiskers was successfully produced through acid hydrolysis process from fermented prawn waste chitin. TEM results clearly indicated the nanosize dimension of fibrillated chitin nanowhiskers with length more than 100 nm and 10 nm in width. The XRD results showed that the crystallinity index of chitin nanowhiskers from both commercial and fermented chitins are higher than its respective chitin. This indicates that acid hydrolysis treatment performed is an effective approach in isolating the crystalline chitin molecules. Interesting to note that no significant changes between the chitin and its respective chitin nanowhiskers were observed through FTIR spectroscopy analysis which indicates that no changes of chemical structure is involved. The TGA results revealed that the FCNW is thermally more stable than FC and CCNW based on T_{max}. Based on the characterisation performed this material showed a great potential to be used as reinforcement agent for the development of green nanocomposites which can be applied in the biomedical, automotive as well as food industry applications.

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