

Full Length Research Paper

# Extraction and characterization of chitin and chitosan from crustacean by-products: Biological and physicochemical properties

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Chitin has been extracted from two Tunisian crustacean species. The obtained chitin was transformed into the more useful soluble chitosan. These products were characterized by their biological activity as antimicrobial and antifungal properties. The tested bacterial strains were *Escherichia coli* American Type Cell Culture (ATCC) 25922, *Pseudomonas aeruginosa* ATCC 27950 and *Staphylococcus aureus* ATCC 25923. Four fungi strains were also tested *Candida glabrata*, *Candida albicans*, *Candida parapsilensis* and *Candida kreusei*. Squilla chitosan showed a minimum inhibitory concentration (MIC) against the different fungi exceptionally for *C. kreusei*. Their antioxidant activity was investigated with 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity and inhibition of linoleic acid peroxidation. *Parapenaeus longirostris* Chitosan showed the highest radical scavenging properties. Chitin and chitosan produced were also characterized with Fourier Transform Infrared Spectroscopy (FTIR).

**Key words:** Antibacterial, antifungal, antioxidant, chitin, chitosan, crustacean.

## INTRODUCTION

Chitin, a homopolymer of N-acetyl-D-glucosamine (GlcNAc) residues linked by  $\beta$ -1-4 bonds, is the most widespread renewable natural resource following cellulose (Deshpande, 1986). The main source of chitin is crustacean waste, which is also the main cell wall material in most fungi (Nicol, 1991). Chitin and its derivatives have high economic value owing to their versatile biological activities and agrochemical applications (Hirano, 1996; Wang and Huang, 2001). The natural antibacterial and/or antifungal characteristics of chitosan

and its derivatives (Chung et al., 2003; El-Ghaouth et al., 1992; Kim et al., 1997; Papineau et al., 1991; Sudarshan et al., 1992) have resulted in their use in commercial disinfectants. Both chitin and chitosan have been shown to activate the defence system of a host and prevent the invasion of pathogens (Sudarshan et al., 1992).

Generally, chitosan has a higher antifungal activity than chitin, but it is less effective against fungi with a chitin or chitosan component in their cell walls (Allan and Hardwiger, 1979). Sudarshan et al. (1992) found that chitosan exhibited a differential antibacterial activity that manifested itself in order of decreasing effectiveness, as *Enterobacter aerogenes* > *Salmonella typhimurium* > *Staphylococcus aureus* > *Escherichia coli*. Many synthetic chemicals such as phenolic compounds are found to be strong radical scavengers; however, the use of synthetic antioxidants is under strict regulation due to their potential health hazards (Je et al., 2004). Therefore, the search for natural antioxidants as alternatives to synthetic product is of great importance. Recently, the

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**Abbreviation:** DPPH, 2,2-Diphenyl-1-picrylhydrazyl; ATCC, American type cell culture; MIC, minimum inhibitory concentration; ch PL, *Parapenaeus longirostris* chitin; CHS PL, *Parapenaeus longirostris* chitosan; ch SM, *Squilla mantis* chitin; chs SM, *Squilla mantis* chitosan; DMSO, dimethyl sulfoxide.

antioxidant activity of chitosan and its derivatives attracted an increased attention (Chiang et al., 2000).

From a technological point of view, it would be quite profitable to recover the by-products released from seafood processing because of its richness in compounds of high value added such as chitin products. Therefore, chitin and chitosan were extracted from *Parapenaeus longirostris* and *Squilla mantis* by-products, and characterized by biological activities such as antibacterial, antifungal and antioxidative activities. FTIR spectra were also established for chitin and its derivative products.

## MATERIALS AND METHODS

### Materials

Pink shrimp *P. longirostris* waste was provided from a Tunisian processing factory (Equimar Congelation), and the *S. mantis* was obtained from a local commercial trawler from la Goulette (Tunis). *S. mantis* inedible parts including heads, shells and tails were removed from whole body for chitin and chitosan extraction.

### Chitin and chitosan preparation

Chitin and chitosan were prepared from shrimp and squilla shell waste according to Gopalakannan et al. (2000). Dried shell waste was washed with tap water and deproteinised by boiling in 3% aqueous sodium hydroxide for 15 min. After draining the alkali, the process was repeated for the removal of residual protein from the shell and washed with tap water. The deproteinised shell was demineralised by HCl (1.25 N) at room temperature for 1 h. The acid was drained off and washed thoroughly with tap water followed with distilled water. The chitin was dried at ambient temperature ( $30 \pm 2^\circ\text{C}$ ). The dried chitin was pulverised into powder using a dry grinder. The chitosan was prepared by deacetylation of chitin by treating with aqueous sodium hydroxide (1:1; w/w) at  $90$  to  $95^\circ\text{C}$  for 2 h. After deacetylation the alkali was drained off and washed with tap water followed by distilled water. Finally, the chitosan was dried at ambient temperature ( $30 \pm 2^\circ\text{C}$ ).

### Antioxidant activity

The lipid peroxidation inhibition activity of the chitin and chitosan was measured in a linoleic acid emulsion system according to the methods of Osawa and Namiki (1985). Briefly, a sample (1.3 mg) of the chitin or chitosan was dissolved in 10 ml of 50 mM phosphate buffer (pH 7.0), and added to a solution of 0.13 ml of linoleic acid and 10 ml of 99.5% ethanol. The total volume was then adjusted to 25 ml with distilled water. The mixture was incubated in a conical flask with a screw cap at  $40 \pm 1^\circ\text{C}$  for 5 days in a dark room, and the degree of oxidation was evaluated by measuring the ferric thiocyanate level according to Mitsuda et al. (1996). A total of 100  $\mu\text{l}$  of the oxidised linoleic acid solution (described above) was mixed with 4.7 ml of 75% ethanol, 0.1 ml of 30% ammonium thiocyanate, and 0.1 ml of 0.02 M ferrous chloride solution in 3.5 % HCl. After stirring (3 min), the absorbance was measured at 500 nm.  $\alpha$ -tocopherol was used as a reference substance and distilled water as a control. The antioxidative capacity of inhibiting the peroxide formation in linoleic acid system was expressed as follows:

$$\text{Inhibition (\%)} = [1 - (\text{Absorbance of sample}/\text{Absorbance of control})] * 100$$

### 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

The ability to scavenge DPPH radical by chitin and chitosan was estimated by the method of Yamaguchi et al. (1998). 0.5 to 2 mg product in 1 ml 0.1 M Tris HCl buffer (pH 7.4) was mixed with 1 ml of DPPH (250  $\mu\text{M}$ ) with vigorous shaking. The reaction mixture was stored in the dark at room temperature for 20 min and the absorbance was measured against blank samples at 517 nm. The scavenging activity was calculated by the following equation:

$$\text{Scavenging activity (\%)} = (\text{Absorbance Blank} - \text{Absorbance sample}/\text{Absorbance Blank}) * 100$$

## Antibacterial and antifungal activities

### Micro organisms

Various chitin and chitosans were individually tested against a panel of microorganisms including different American Type Cell Culture (ATCC) reference bacteria and fungi.

**Bacteria strains:** *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27950.

**Fungi strains:** *Candida glabrata* ATCC 90030, *Candida albicans* ATCC 90028, *Candida parapsilensis* ATCC 22019 and *Candida kreusei* ATCC 6258.

The different American Type Cell Culture (ATCC) reference bacteria and fungi were used as well as clinical isolates.

### Determination of antibacterial and antifungal with micro-well dilution assay

The minimal inhibitory concentration (MIC) values were studied for the bacteria and fungi strains, being sensitive to the chitin or chitosan in the agar diffusion assay. The inocula of the bacterial strains were prepared from 12 h broth cultures and suspensions were adjusted to 0.5 McFarland standard turbidity. The chitin and chitosan were first dissolved in 10% dimethyl sulfoxide (DMSO) (this DMSO concentration does not offer inhibition to microorganism growth) and then diluted to the highest concentration (20 mg/ml) to be tested, and the serial twofold dilutions were made in 10 ml sterile test tubes containing nutrient broth. MIC values of the chitin or chitosan against bacterial strains were determined based on a micro-well dilution method as previously described (NCCLS, 2001).

In brief, the 96-well plates were prepared by dispensing into each well 95  $\mu\text{l}$  of nutrient broth and 5  $\mu\text{l}$  of the inocula. A 100  $\mu\text{l}$  of aliquot from the stock solutions of the extracts initially prepared at the concentrations of 20 mg/ml was added into the first wells. Then, 100  $\mu\text{l}$  from their serial dilution were transferred into six consecutive wells. The last well containing 195  $\mu\text{l}$  of nutrient broth without compound and 5  $\mu\text{l}$  of the inocula on each strip were used as negative control. The final volume in each well was 200  $\mu\text{l}$ . The plate was covered with a sterile plate sealer and then incubated for 18 h at  $37^\circ\text{C}$ . The MIC was defined as the lowest concentration of the compounds to inhibit the growth of micro-organisms, after incubation. The results were expressed in mg/ml (Smania et al., 1999).

### Infrared spectroscopy FTIR

The samples of chitin and chitosan produced were characterized in KBr pellets by infrared spectrophotometer in the range of 400 to 4000  $\text{cm}^{-1}$  (Brucker Equinox 55).

### Statistical analysis

The experiments were performed in triplicate. One way analysis of variance (ANOVA) was used, and mean comparison was performed by Duncan's test. Statistical analysis was carried out using the Statistical Package for the Social Sciences (SPSS) statistic programme (version 11.0) for windows. Means were accepted as significantly different at 95% level ( $P < 0.05$ ).

## RESULTS AND DISCUSSION

### Total antioxidant activity

We investigated the antioxidative activity of the various chitin and chitosan products, which were compared to  $\alpha$ -tocopherol, widely used as a natural antioxidant agent. The control (without antioxidant) had the highest absorbance value, indicating the highest degree of oxidation among samples, whereas the reference with  $\alpha$ -tocopherol had the lowest absorbance (Table 3). Various chitin and chitosans presented more than 60% inhibition of linoleic acid peroxidation, suggesting the presence of amino groups having antioxidant activity. Shrimp chitin presented the highest value of inhibition of linoleic acid (79.52 %) which indicated additional amino groups to enhance antioxidant properties. In linoleic acid system, oxidation of linoleic acid was effectively inhibited by *P. longirostris* chitin and chitosan extract ( $79.52\% \pm 0.068$  and  $78.07\% \pm 0.065$ ), followed by *S. mantis* chitosan ( $73.77\% \pm 0.02$ ) and chitin ( $60.56\% \pm 0.07$ ).

As shown in Table 3, chitin significantly inhibited lipid peroxidation in linoleic acid emulsion system and the activity was slightly lower than that of  $\alpha$ -tocopherol after 5 days. In this model system, peroxy (ROO $\cdot$ ) and alkoxy (RO $\cdot$ ) radicals, derived from the pre-existing lipid peroxide, were employed directly to initiate lipid peroxidation in the emulsified linoleic acid system (Cheng et al., 2003).

### DPPH radical scavenging activity

It is generally considered that the inhibition of lipid peroxidation by an antioxidant can be explained by various mechanisms. One is the free radical-scavenging activity where DPPH is a stable free radical with a maximum absorbance at 517 nm in ethanol. When DPPH encounters a proton-donating substance such as an antioxidant, the radical would be scavenged and the absorbance is reduced (Shimada et al., 1992). Based on this principle, the antioxidant activity of the substance can be expressed as its ability in scavenging the DPPH radical. Park et al. (2004) suggested that chitosan may eliminate various free radicals by the action of nitrogen on the C-2 position of the chitosan. The effect of chitin and chitosan on DPPH free radical scavenging is depicted in Figure 1. The chitosan extracted from *P. longirostris* had higher radical scavenging than the other products measured at the same concentration.

The scavenging activity of chitosan may be due to the reaction between the free radicals and the residual free amino group to form stable macromolecule radicals and/or the amino groups can form ammonium groups by absorbing hydrogen ions from the solution and then reacting with radicals through an additional reaction (Xie et al., 2001).

The scavenging activities of chitins and chitosans increased with increasing concentration from 1 to 2% (w/v). The results indicated that the radical-scavenging activity of *Squilla* chitin was not affected by the different concentrations. Additionally, this parameter varies within species.

### Antibacterial activities

The natural antibacterial and/or antifungal characteristics of chitosan and its derivatives (Chung et al., 2003; El-Ghaouth et al., 1992; Kim et al., 1997; Papineau et al., 1991; Sudarshan et al., 1992) resulted in their use in commercial disinfectants. According to literature (Jeon et al., 2001; Ueno et al., 1997), chitosan possesses antimicrobial activity against a number of Gram-negative and Gram-positive bacteria.

This study has been conducted to assess inhibitory effects of chitosan in terms of MIC. The effectiveness of chitosan bacteriostatic properties were tested against bacterial strains and fungi. Solution of chitin and chitosan from both species of shrimp and squilla inhibited all strains of bacteria (MIC, 0.156 to 5mg/ml) except for *P. aeruginosa* which was the most resistant bacteria strain studied (Table 1). *P. aeruginosa* is problematic as it has intrinsic resistance to several antibiotics and a capability to acquire resistance during antibiotic therapy (Beck et al., 1988).

Chitin extracted from *P. longirostris* exhibited important antibacterial activity against *Escherichia coli*; it was the most effective extract with the lowest MIC (0.156 mg/ml). Antibacterial activity of chitosan is influenced by its molecular weight, degree of deacetylation, concentration in solution, and pH of the medium (Lim and Hudson, 2003).

The protection of the host against bacterial infection is stimulated by chitosan (Iida et al., 1987). The mechanism underlying the inhibition of bacterial growth is thought to be that the cationically charged amino-group may combine with anionic components such as N-acetylmuramic acid, sialic acid and neuraminic acid on the cell surface, and may suppress bacterial growth by impairing the exchanges with the medium, chelating transition metal ions and inhibiting enzymes. Due to the positive charge on the C-2 of the glucosamine monomer below pH 6, chitosan is more soluble and has a better antimicrobial activity than chitin. The exact mechanism of the antimicrobial action of chitin, chitosan, and their derivatives is still unknown, but different mechanisms have been proposed. Interaction between positively charged

**Table 1.** MIC (mg/ml) Minimal inhibitory concentration of chitin and chitosan products against various microorganisms.

Chitin and chitosan products	MIC values (mg /ml)		
	<i>Staphylococcus aureus</i>	<i>Escherichia coli</i>	<i>Pseudomonas aeruginosa</i>
Ch PL	2.5	0.156	na
Ch SM	2.5	0.325	na
Chs PL	0.625	5	na
Chs SM	5	2.5	na

ch PL, *Parapenaeus longirostris* chitin; chs PL, *Parapenaeus longirostris* chitosan ; ch SM, *Squilla mantis* chitin; chs SM, *Squilla mantis* chitosan; na, not active.

**Table 2.** MIC (mg/ml) Minimal inhibitory concentration of chitin and chitosan products against various fungi.

Chitin and chitosan products	MIC values (mg /ml)			
	<i>Candida glabrata</i>	<i>Candida albicans</i>	<i>Candida parapsilensis</i>	<i>Candida kreusei</i>
Ch PL	5	0.156	0.156	0.156
Ch SM	0.325	0.156	0.325	1.25
Chs PL	5	1.25	0.325	0.325
Chs SM	0.156	0.156	0.156	1.25

ch PL, *Parapenaeus longirostris* chitin; chs PL, *Parapenaeus longirostris* chitosan; ch SM, *Squilla mantis* chitin; chs SM, *Squilla mantis* chitosan.

**Table 3.** Inhibition ratio of the linoleic acid oxidation by chitin and chitosan products measured by the ferric thiocyanate method.

Sample	DO 500 nm	Antioxidant activity (%)
Control	3.85 ± 0.25	0
α- tocopherol	0.632 ± 0.012	83. 58 ± 0.12
Chitin PL	0.788 ± 0.06	79. 52 ± 0.068
Chitin SM	1.519 ± 0.07	60. 56 ± 0.07
Chitosan PL	0.844 ± 0.06	78. 07 ± 0.065
Chitosan SM	1.01 ± 0.02	73. 77 ± 0.02

Values represents means ± se (n = 3). Control, Without antioxidant; antioxidant activity (%) = [1- (sample absorbance/control absorbance)]\*100; ch PL, *Parapenaeus longirostris* chitin; chs PL, *Parapenaeus longirostris* chitosan; ch SM, *Squilla mantis* chitin; chs SM, *Squilla mantis* chitosan.

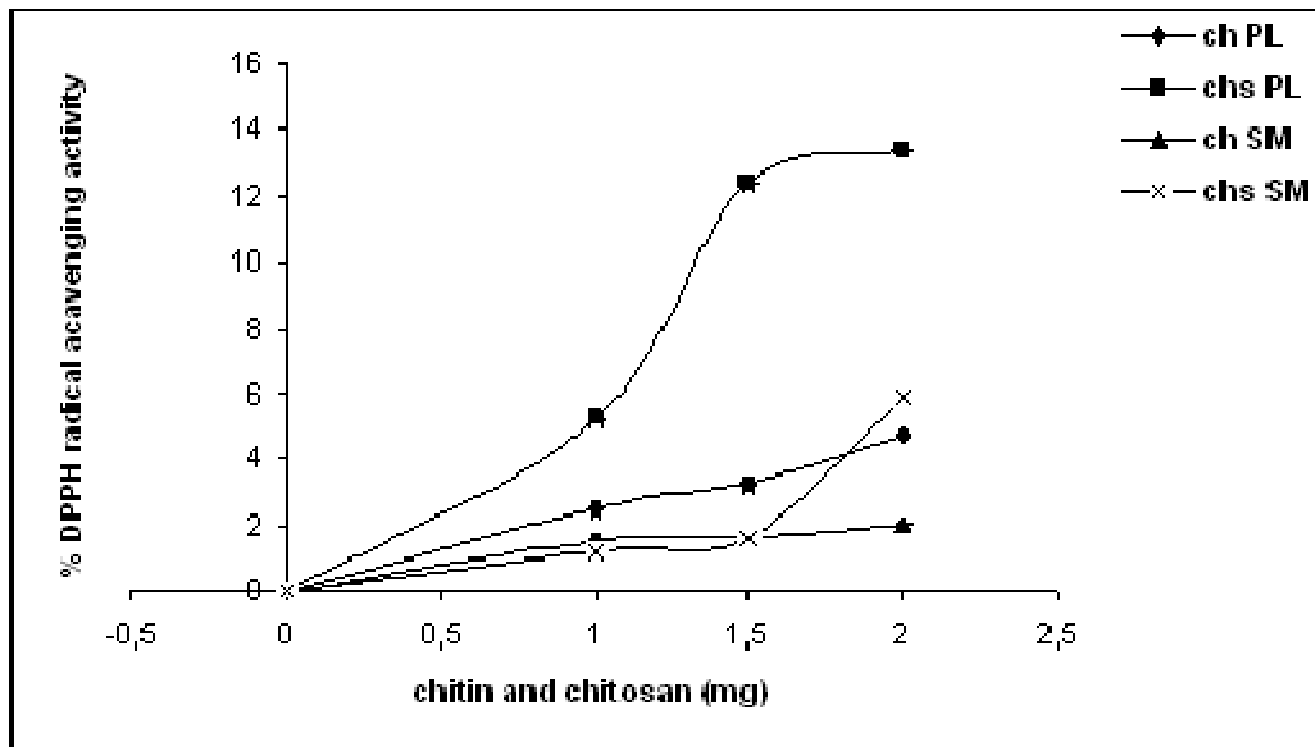
chitosan molecules and negatively charged microbial cell membranes leads to the leakage of proteinaceous and other intracellular constituents (Chen et al., 1998; Fang et al., 1994; Jung et al., 1999; Seo et al., 1992). Chitosan acted mainly on the outer surface of the bacteria. At a lower concentration (<0.2 mg/ml), the polycationic chitosan does probably bind to the negatively charged bacterial surface to cause agglutination, while at higher concentrations, the larger number of positive charges may have imparted a net positive charge to the bacterial surfaces to keep them in suspension (Papineau et al., 1991; Sudarshan et al., 1992).

### Antifungal activities

The antifungal activity of chitin and chitosan has been

reported by many investigators. This study has demonstrated that chitin and chitosan from both crustacean sources exhibited antifungal activity against a large number of human pathogenic fungi. The tested chitin compound has a significant effect against pathogenic *Candida* species (Table 2). However, like other studies chitosan has been observed to act more quickly on fungi than on bacteria (Cuero, 1999).

Our data demonstrated that both squilla and shrimp chitosan abolished germination of candida. All products tested are fungistatic. Furthermore, the results demonstrated that the antifungal activity of them was affected by their molecular weight obviously. Higher molecular weight resulted in better antifungal ability. These results agreed with the previous work (Jeon et al., 2001).



**Figure 1.** 1-1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity of chitin and chitosan. Results shown are mean values ( $n = 3$ ). ch PL, *Parapenaeus longirostris* chitin; chs PL, *Parapenaeus longirostris* chitosan; ch SM, *Squilla mantis* chitin; chs SM, *Squilla mantis* chitosan.

### FTIR spectroscopy

The shrimp chitin showed an intense peak at  $1552\text{ cm}^{-1}$  which corresponded to the N-H deformation of amide II (Duarte et al., 2001; Ravindra et al., 1998). The bands at  $1618\text{ cm}^{-1}$  and another at  $1651\text{ cm}^{-1}$  are attributed to the vibrations of the amide I band, and the band at  $1651\text{ cm}^{-1}$  corresponds to the amide I stretching of C = O. The band at  $1618\text{ cm}^{-1}$  could be attributed to the stretching of C–N vibration of the superimposed C = O group, linked to OH group by H bonding. These bands can be clearly observed in all samples.

The sharp band at  $1374\text{ cm}^{-1}$  corresponds to a symmetrical deformation of the  $\text{CH}_3$  group, and at  $1552\text{ cm}^{-1}$  corresponds to the N–H deformation of amide II (Duarte et al., 2001; Ravindra et al., 1998). The results of FTIR spectra of chitin are shown in Figure 3.

The spectra of Figure 2 correspond to the deacetylated sample with NaOH for 2 h. Note that for chitosan, the band at  $1552\text{ cm}^{-1}$  has a larger intensity than at  $1652\text{ cm}^{-1}$ , which suggests effective deacetylation for the two species. When chitin deacetylation occurs, the band observed at  $1652\text{ cm}^{-1}$  decreases, while a growth at  $1552\text{ cm}^{-1}$  occurs, indicating the prevalence of  $\text{NH}_2$  groups (Bordi et al., 1991). Figure 2 shows the spectrum of chitosan obtained from shrimp and squilla species.

### Conclusion

Chitin and chitosan have been extracted from two different sources of by-products which form cheap and abundant functional materials in Tunisia. This study had equally showed that we can generate various products of chitin and chitosan with high antibacterial and fungicidal activities.

There is no report on biological activities of chitin and chitosan prepared from *S. mantis*. Besides, antioxidative properties of the various chitin and chitosan extracts are of great interest in food industry, since their possible use as natural additives emerged from a growing tendency to replace synthetic antioxidants by natural ones. Owing to its excellent protective features exhibited in antioxidant activity tests, the chitin and chitosan extracts from the crustacean species could be concluded as a natural source that can be freely used in the food industry. This study identifies opportunities to develop value added products from crustacean-processing by-products with different biological activity such as antioxidant, antibacterial and antifungal properties. Chito-san is characterized by high antibacterial and fungicidal activities. The present results also indicate the possibility of exploiting the chitosan as an effective inhibitor of bacteria and fungi.

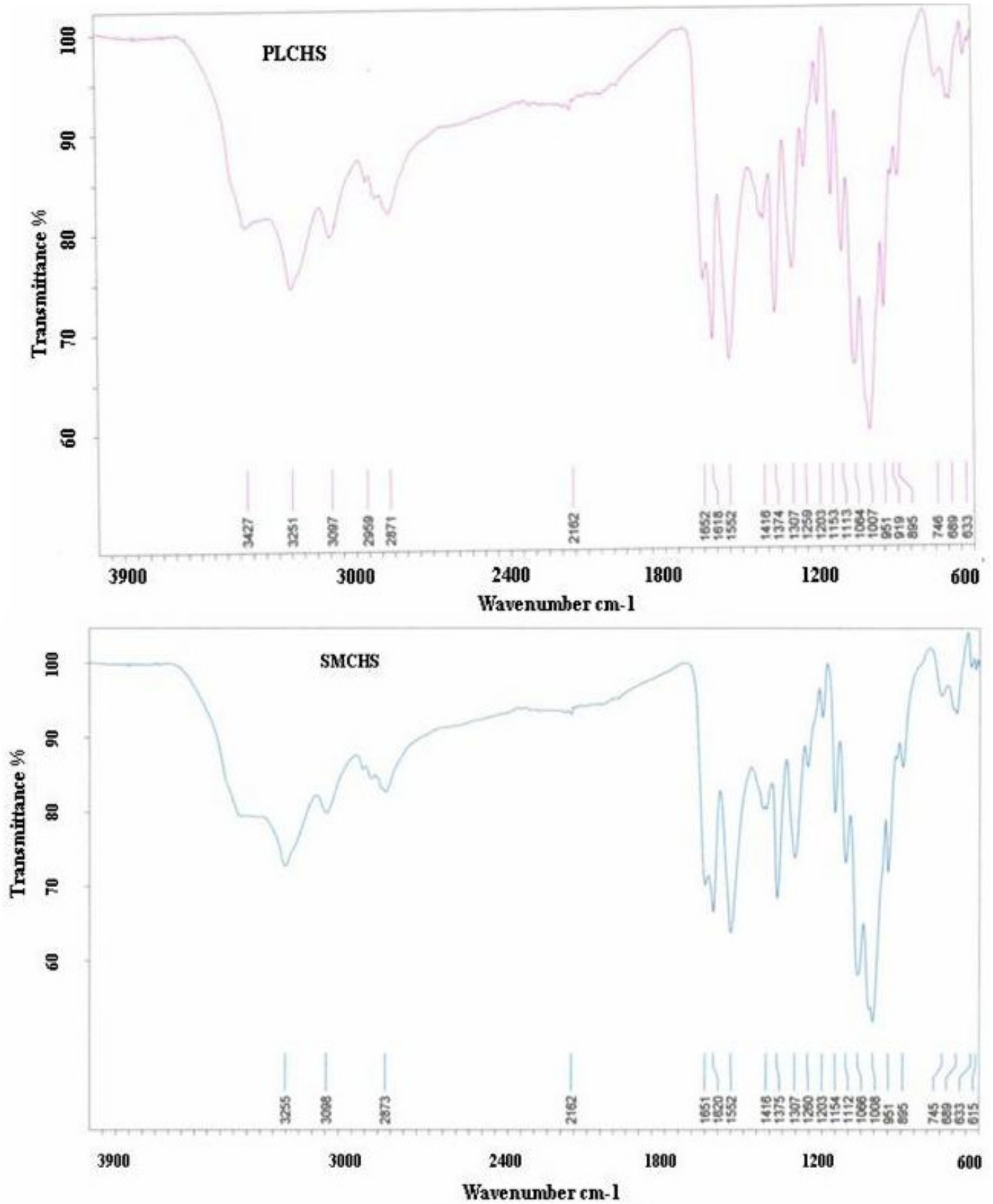


Figure 2. FTIR spectra of chitosan. PLCHS, *Parapeneus longirostris* chitosan; SMCHS, *Squilla mantis* chitosan.

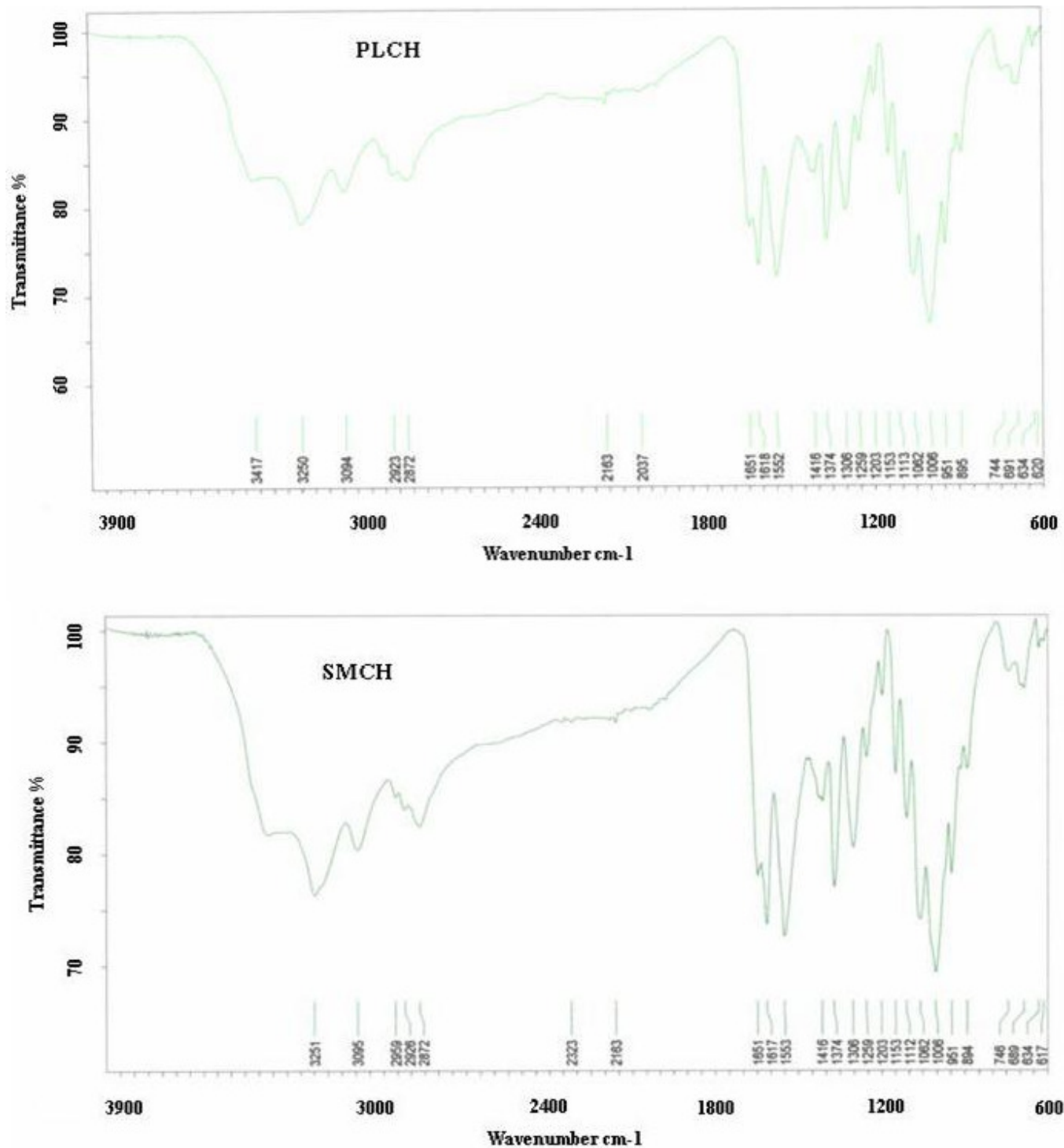


Figure 3. FTIR spectra of Chitin. PLCH, *Parapenaeus longirostris* chitin; SMCH, *Squilla mantis* chitin.

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