

Two-phase extraction, characterization, and biological evaluation of chitin and chitosan from *Rhizopus oryzae*

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ABSTRACT

Chitin is a polysaccharide based on the monomers of *N*-acetyl-glucosamine and chitosan is a deacetylated form of it. Our study is emphasized on the two-phase extraction of chitin and chitosan from fungus *Rhizopus oryzae* (NCIM 877) belonging to Zygomycota. Optimization studies such as utilization of carbon source, pH, and biomass were performed and maximum yield of chitosan (0.288 g/l) was obtained after 9 days of incubation. Characterization studies of chitin and chitosan were performed using Fourier transform infrared spectroscopy and X-ray diffraction methods. Studies on the degree of deacetylation, molecular weight, and viscosity for chitin and chitosan were found to be superior in comparison to standard. The anti-bacterial activity of chitosan was evaluated against the test organisms like *Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtilis*, and *Salmonella typhi*. The chitosan demonstrated higher anti-bacterial activity against gram-positive bacteria as compared with gram-negative ones. The extracted chitosan demonstrated anti-oxidant activity between 60% and 80% as evaluated by 2,2-diphenyl-1-picrylhydrazyl scavenging studies.

INTRODUCTION

Chitin is the second most abundant biopolymer in nature after cellulose (White *et al.*, 1979). It is a copolymer of *N*-acetyl glucosamine and *N*-glucosamine which are randomly distributed throughout the polymer chain. Chitosan, a versatile hydrophilic polysaccharide, is derived by deacetylation of chitin. It is a natural, modified, biodegradable, biocompatible, and non-toxic nitrogenous linear polysaccharide. Chitin and chitosan are the basic homopolymer polysaccharides found in the exoskeleton of shellfish and also in the cell wall of fungi of class Zygomycota (Zamani *et al.*, 2007). Chitosan is a linear, randomly distributed, heteropolysaccharide consisting of (1–4) linked 2-acetamido-2-deoxy-D-glucopyranose and 2-amino-2-deoxy-D-glucopyranose units. Commercially available chitosan has an average molecular weight (MW) ranging from 3,800 to 20,000 Da and degree of

deacetylation (DD) of 66%–95% (Alagesan *et al.*, 2016). The solubility of chitin and chitosan depends on DD, pH, and the protonation of free amino groups. Chitosan is readily soluble in most of the organic acids such as citric acid and tartaric acid while partially soluble in inorganic acids such as dilute sulphuric acid, acetic acid, hydrochloric acid, formic acid, and so on.

Industrially, the chitosan is derived from the chemical deacetylation of chitin, a waste by-product of the crustaceans obtained after industrial processing of seafood. The deacetylation is carried out using a strong alkali. The process is highly dependent on the seasonal availability and limited supply of the crustaceans. Moreover, the production of a large amount of waste of concentrated alkaline solution during the process contributes largely toward the pollution of the environment and is considered to be a major drawback. An alternative to solve these problems is chitosan production from fungi, whose main advantage is that it is environmental friendly. Therefore, physicochemical properties and yields of chitosan isolated directly from a fungus may be optimized by controlling fermentation and processing parameters. Fungal biomass can be produced by solid-state fermentation (SSF) and submerged fermentation (SmF). SmF has the specific advantage as fermentation method provides easier control of

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fermentation parameters such as pH and nutrient concentration in the fermentation medium (Amorim *et al.*, 2006).

Chitosan is no longer just a waste by-product from the seafood processing industry; it is effectively used as an anti-microbial agent, dietary supplement, and in weight loss management (Azuma *et al.*, 2015). The development of industrial applications of chitosan has expanded rapidly in the recent years. Chitosan was found to be polycationic, nontoxic, biodegradable, and possesses anti-microbial property. It has many applications, especially in agriculture, food, and pharmaceutical industries. Furthermore, it has been used in enzyme immobilization, wastewater treatment, as a food additive and anti-cholesterolemic, in wound healing, and in the pharmaceutical industry as drug delivery system (Kerch, 2015; Pandaya *et al.*, 2007). Chitosan is a versatile molecule which find wide applications in industries such as textile, food processing, paper, environmental, photography, chromatographic separations, solid-state batteries, light emitting devices (Du *et al.*, 2017; Pillai and Rari, 2001), biomedical (Azuma *et al.*, 2015; Xing *et al.*, 2018), agricultural (Malebra and Cerana, 2018), tissue engineering (Silva *et al.*, 2017), and so on.

The early research describing the anti-microbial potential of chitin, chitosan, and their derivatives dated from the 1980–1990. Chitin and chitosan have been investigated as anti-microbial materials against a wide range of target organisms like algae, bacteria, yeast, and fungi in experiments involving *in vivo* (Chen and Tsaih, 1998) and *in vitro* (Young *et al.*, 1982) interactions with chitosan in different forms (solutions, films, and composites). Generally, in these studies, the chitosan is considered to be a bactericidal or bacteriostatic agent. The present study reports production of chitin and chitosan using *Rhizopus oryzae* under submerged growth conditions. The traditional two-phase extraction method employs acetic acid and hot sulphuric acid treatment (Zamani *et al.*, 2007). Our modified method of extraction for chitin and chitosan uses dilute hot sulphuric acid alone which is economical and eco-friendly. Both chitin and chitosan were characterized for material properties using Fourier transform infrared spectroscopy (FTIR) and X-ray diffraction (XRD) techniques. Also, their biological evaluation was done by demonstrating anti-bacterial and anti-oxidant activities.

MATERIALS AND METHODS

Microorganism and culture conditions

The fungus *R. oryzae* (NCIM 877) from Zygomycota class was selected for the study. The culture was procured from National Collection of Industrial Microorganisms (NCIM), NCL, Pune, India. The fungal culture was subcultured and maintained in Sabouraud's dextrose medium and incubated for 4–5 days at room temperature. The culture was preserved in the refrigerator at 4°C till further use. Standard chitosan (shrimp source) was procured from Sisco Research Laboratories (SRL) Pvt. Ltd. India. The fungal culture (3.2×10^6 spores/ml) was inoculated from preserved culture into 5 ml of Sabouraud's dextrose broth and incubated for 48 hours at room temperature. The inoculated broth was then transferred into 100 ml medium and incubated for 3, 6, and 9 days at 25°C at 150 rpm. During the batch growth of the organism, profiling studies for process parameters such as utilization of

carbon source (DNS method; Miller, 1959), pH change of the medium, and weight of biomass were performed.

Two-phase extraction studies

After growth, fungal mycelium was filtered through Whatmann filter paper No. 41 and the mycelium was dried at 60°C in a hot air oven for 8–10 hours. The extraction of chitin and chitosan was performed by the two-phase extraction process. In the first step, dealkylation of dried fungal biomass was done with 1 N sodium hydroxide (NaOH) solution followed by deacetylation using 1% hot sulphuric acid. A known quantity (1 g) of dried fungal biomass was treated with 50 ml of 1 N NaOH, the mixture was homogenized and autoclaved at 121°C, 15 lbs pressure for 20 minutes. Then, the mixture was centrifuged at 6,000 rpm for 20 minutes. The pellet was collected and washed repetitively with distilled water till the pH of the pellet reached neutral and dried at 40°C–50°C. The dried pellet now can be called as alkali insoluble material, which was treated with 1% hot sulphuric acid and autoclaved at 121°C for 20 minutes. The acid insoluble fractions were separated by centrifugation at 6,000 rpm for 15 minutes. The supernatant was collected and filtered through Whatmann filter paper no. 41 on an ice bath. The liquid was then centrifuged at 6,000 rpm for 15 minutes to recover the chitosan. The extraction of chitin and chitosan was performed according to the method of Zamani *et al.* (2007) with a slight modification in deacetylation step where hot dilute sulphuric acid was used instead of dilute acetic acid for the easy recovery of chitin and chitosan. The modified method avoids the longer digestion period at high temperature.

Material characterization

The characterization of extracted chitin and chitosan was performed by using Fourier transform infrared spectroscopy (FTIR; NICOLET FTIR 6700) recorded in KBr pellet with a resolution of 4 cm⁻¹ and the wavelength ranging from 400 to 4,000 cm⁻¹. Using the spectra, material properties such as DD%, MW, and cP were calculated for chitin and chitosan. Commercial chitin and chitosan were used for the comparison study. The DD was determined by FTIR spectroscopic method (Pochanavanich and Suntornsuk, 2002) and measurements were taken in the absorbance mode with absorbance ratio of A_{1655} and A_{3450} by the following equation:

$$\%DD = 100 - 75.19 \left(\frac{A_{1655}}{A_{3450}} \right) \quad (1)$$

where A_{1655} is the absorbance of amide I band and A_{3450} corresponds to –OH groups.

The MWs of chitin and chitosan were calculated by Mark–Houwink's equation.

$$[\eta] = KM^a \quad (2)$$

where, η = intrinsic viscosity (cP), “K” and “a” are constant depends on the DD% of chitin and chitosan and M = molecular weight.

The viscosity of chitin and chitosan was determined by the Ostwald's viscometric method (Nadarajah, 2009). Chitin and chitosan (1% w/v) were prepared in 1% (v/v) acetic acid and

then stirred for 4 hours. The solution viscosity was measured by Ostwald's viscometer at 25°C.

The viscosity of chitin and chitosan was obtained by the following equation:

$$[\eta] = \left(\frac{\eta_{sp}}{C} \right) \quad (3)$$

where η is intrinsic viscosity, η_{sp} is specific viscosity, and C is the concentration of the material.

XRD patterns of chitin and chitosan were characterized by XRD (Bruker D8 Advance X-ray powder diffractometer) technique equipped with Cu α radiation ($\lambda = 1.54 \text{ \AA}$) and Ni filter. The relative intensities were recorded in the range of 10°–90° with a scanning rate of 5° minute⁻¹ (Nadarajah, 2009).

Anti-bacterial activity

The anti-bacterial activity of chitosan was determined by well diffusion method (Raafat *et al.*, 2008). One percentage of chitosan solution in dilute acetic acid (w/v) was inoculated against 2 g positive bacteria such as *Staphylococcus aureus* NCIM 2071 and *Bacillus subtilis* NCIM 2724 and 2 g negative bacteria such as *Escherichia coli* NCIM 2687 and *Salmonella typhimurium* NCIM 2501. The anti-bacterial activity of chitosan was observed by the development of zone of inhibition (ZoI) after incubation for 24 hours. Dilute acetic acid (1%) was kept as a control and acetic acid used to dissolve the chitosan exhibits inherent anti-bacterial property.

Anti-oxidant activity

The anti-oxidant property of chitosan was evaluated by 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay. Standard chitosan was used as a reference. The DPPH radical-scavenging activity of the chitosan was determined by the method as described by Trung and Duy Bao (2015). Reaction mixtures containing 1 ml of a chitosan sample (0.1–1.0 mg/ml) in 0.5% acetic acid solution, 1 ml ethanol, and 1 ml of 0.1 mM

DPPH ethanol solution were raised to a final volume of 4 ml by 0.5% acetic acid solution in test tubes. The mixtures were mixed thoroughly and then kept at 25°C for 30 minutes in the dark. The absorbance of the mixtures was measured at 517 nm against a blank (without DPPH) using a UV-Vis Spectrophotometer (Make: Lab India). Butylated hydroxytoluene (BHT) was used for comparison. The DPPH radical scavenging activity was calculated using the following equation:

$$\text{DPPH radical scavenging activity (\%)} = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100 \quad (4)$$

where, A_{control} and A_{sample} are the absorbance values for control mixture and sample mixture, respectively.

RESULT AND DISCUSSION

The SmF process was employed for chitin and chitosan production from fungus. Figure 1 shows the growth profile of fungus with respect to its dry cell biomass, pH, carbon source, and chitosan yield. The growth pattern of fungus followed its late exponential phase that is 9 days at which maximum dry cell biomass was recovered. Thus, the optimum growth period of the fungus to recover maximum yield of extractable chitin and chitosan was determined during profiling studies. Figure 1 also depicts the effect of carbon source on the fungal growth. Initial carbon source (2% dextrose) was provided in the growth medium and as the growth of the fungus proceeds the utilization of carbon source is also enhanced. Hence, there is a depletion curve in the carbon content with respect to time. The pH of the growth medium eventually decreased from 5.5 to acidic value, thus affecting the vegetative growth of the fungus. The quantity of chitosan reported to be increasing with respect to time and reached the highest value of 0.288 g/l after 9 days of incubation (the yield of chitin and chitosan obtained was 132.71 and 37.99 mg/g of biomass, respectively).

The growth of fungus was maximum at its late exponential phase, which was depicted in terms of dry cell biomass. In this phase, fungal growth and yield of chitosan were

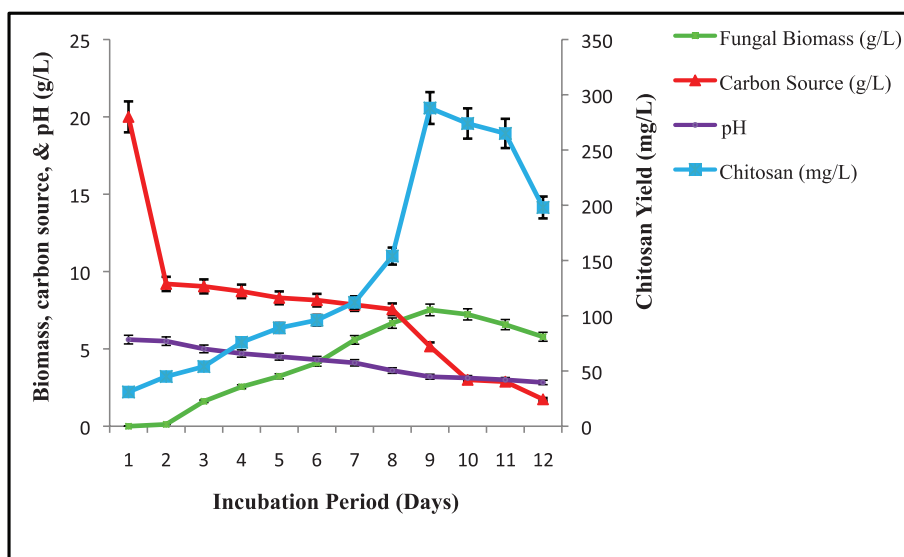


Figure 1. Growth profiling and yield of chitosan from *Rhizopus oryzae*.

higher, similar observation was made by Rhoades and Roller (2000). With respect to carbon source and pH, the depletion curve in both the cases was observed indicating effective utilization of carbon source and synthesis of acid metabolites. From the result, it is observed that the carbon source was utilized by the fungus for its growth as well as for the production of chitin and chitosan. At higher carbon source, the growth and yield of chitin and chitosan were higher indicating carbon source plays a vital role in the defining yield. Chitin and chitosan are the biopolymers which differ only in their positioning of carbon atom at its C-2 position. Chitin is a deacetylated form of chitosan. The two-step method of dealkylation and deacetylation is an easy and non-hazardous process of extraction for chitin and chitosan, where most of the chitin is deacetylated to form chitosan. The method of extraction is the highlight of our study which yielded higher chitosan with lesser impurities. The traditional methods use acetic acid or HCl for extraction and result into relatively lower yield with phosphate impurity but we used dilute hot sulphuric acid. According to earlier studies, chitosan yield from *R. oryzae* was 10.8 mg/g biomass in SSF (Kleekayai *et al.*, 2010) with conventional method of extraction using acetic acid. The production of chitosan from agro-industrial waste yielded 21.3 mg/g biomass from *Rhizopus arrhizus* (Cardoso *et al.*, 2012). Hence, the results show that chitosan yield from *R. oryzae* (NCIM 877) using the modified two-phase extraction is maximum compared to earlier studies.

FTIR spectra were used for the characterization of chitin and chitosan to know the possible functional groups present in the samples. The transmittance mode was used for the identification

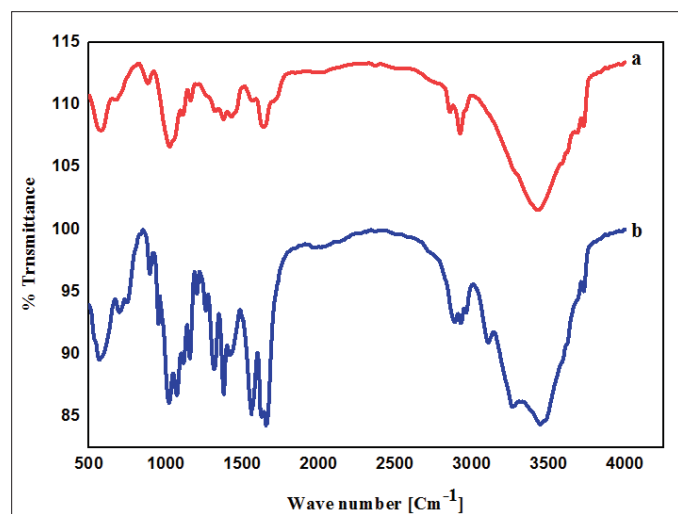


Figure 2. FTIR Spectrum of (a) chitin from *Rhizopus oryzae* and (b) standard chitin.

of the extracted material. Figures 2 and 3 show the transmittance spectra of chitin and chitosan recorded with frequency ranging from 400 to 4,000 cm^{-1} . The spectral bands of standard chitin and fungal chitin show the OH-axial stretching between 2,920.77 and 3,460.15 cm^{-1} , superimposed on NH stretching band; with the axial deformation of amide C=O at about 2,369.20 cm^{-1} . Angular deformation of -NH approximately at 1,639.00 cm^{-1} ; axial deformation of amide -CN around 1,425.54 cm^{-1} . Symmetrical angular deformation in CH_3 is 1,373 cm^{-1} . -CN axial deformation of amino groups between 1,160.65 and 1,119.29 cm^{-1} . Polysaccharide structure bands in the region between 579.75 and 1,021.92 cm^{-1} . The result confirms the material as chitin supported by the earlier studies (Pochanavanich and Suntornsuk, 2002). In the infrared transmittance spectrum, bands were observed related to OH-axial stretching between 3,430 and 3,726.68 cm^{-1} , superimposed on NH stretching band (Fig. 3). With the axial deformation of amide C=O at about 1,634.03 cm^{-1} , angular deformation of NH at approximately 1,553.11 cm^{-1} . Axial deformation of amide -CN at around 1,466.90 cm^{-1} , symmetrical angular deformation in CH_3 was 1,373 cm^{-1} . -CN axial deformation of amino groups was between 1,120 and 1,260 cm^{-1} . Polysaccharide structures exhibit bands in the region between 880 and 1,130 cm^{-1} . The literature for spectral analyses supports the infrared transmission spectral profile of extracted chitosan. Thus, the product is confirmed to be chitosan.

The absorbance mode of FTIR spectra was used to calculate DD. Table 1 demonstrates the DD, MW, and cP of chitin and chitosan obtained. The DD less than 60% indicated the presence of chitin and more than 60% indicated the presence of chitosan.

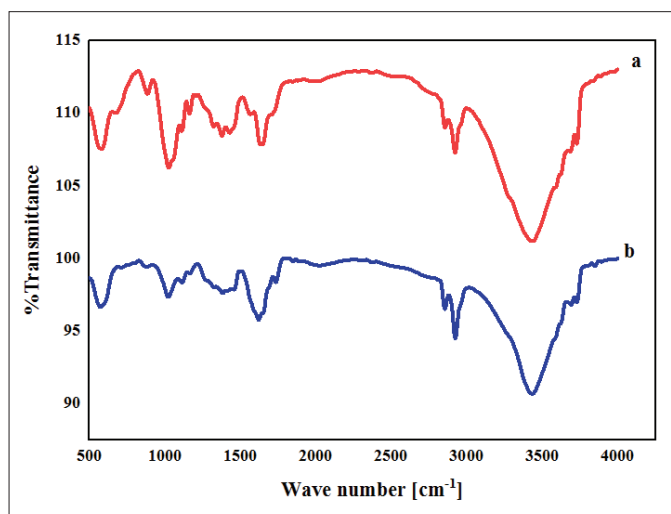


Figure 3. FTIR spectrum of (a) chitosan from *Rhizopus oryzae* and (b) standard chitosan.

Table 1. Degree of deacetylation, molecular weight, and viscosity of chitin and chitosan from *Rhizopus oryzae* (NCIM 877).

Sl. No	Sample	Degree of deacetylation (%)	Molecular weight (Da)	Viscosity (cP)
1	Standard chitin	23.84	4.32×10^5	4.90
2	Standard chitosan	88.08	2.12×10^5	1.68
3	Chitin from <i>Rhizopus oryzae</i>	10.24	2.7×10^6	5.63
4	Chitosan from <i>Rhizopus oryzae</i>	72.51	3.5×10^5	3.08

The DD of standard chitin and chitin from *R. oryzae* was found to be 23.84% and 10.24%, respectively. Similarly, DD% of standard chitosan and chitosan from *R. oryzae* was found to be 88.08% and 75.21%, respectively. The result shows that effective deacetylation of chitin to chitosan has taken place with the modified method of extraction. The MWs for standard chitin and chitin from *R. oryzae* were reported to be 4.32×10^5 Da and 2.7×10^6 Da and that of standard chitosan and chitosan from *R. oryzae* were 2.12×10^5 Da and 3.5×10^5 Da, respectively. Thus, we obtained chitin and chitosan of high MW from a fungal source. Viscosity of standard chitin and chitin from *R. oryzae* is 4.90 and 5.63 cP, while for standard chitosan and chitosan from *R. oryzae* is 1.68 and 3.08 cP, respectively. The result shows that the corresponding viscosity with the increase in the MW has been achieved.

Previous studies (Nadarajah, 2009) have showed that the percentage of *N*-acetyl glucosamine is higher in chitin and is lower in chitosan. The DD% is an important material and functional property of chitosan (Pochanavanich and Suntornasuk, 2002). Higher the DD% in chitosan higher will be positive charge density, which makes chitosan a material with many industrial applications such as a coagulating agent in wastewater treatment and as an antimicrobial agent (He *et al.*, 2016). We have also observed that higher the MW, higher is the viscosity.

XRD method was used to study the crystalline nature of chitin and chitosan. The powder XRD pattern chitin is shown in Figure 4(a) and that of chitosan in Figure 4(b). The prominent diffraction peak exhibited at $2\theta = 13$ and 20° are corresponding to the (020) and (110) plane of the semi-crystalline chitin and chitosan. This is a clear indication for the formation of chitin and chitosan produced from *R. oryzae*. There are no significant changes observed in XRD pattern between commercial and extracted chitin and chitosan. However, as compared to the standard, extracted chitin and chitosan from the fungus were found to be broad diffraction peaks that symbolize the amorphous nature of the materials. The crystalline size of these extracted chitin and chitosan was calculated to be 20–50 nm using Scherer's formula: $d = K\lambda/\beta \cos\theta$, where K is the shape factor between 0.9 and 1.1, λ is the incident X-Ray wavelength (Cu $K\alpha = 1.542 \text{ \AA}$), β is the full width half maximum in radians of the prominent line, and θ is the position of line in the pattern.

Earlier studies showed that the chitin showed more prominent peaks as compared to chitosan at the range of $2\theta = 10$ – 20° intensity (Kaya *et al.*, 2015). The analysis also showed the same results which indicate that chitosan has lower crystallinity than its corresponding chitin, which makes the chitosan less heat stable than chitin. XRD is used to determine the polymorphic forms of a compound which has different crystalline structures for which distinct powdered XRD patterns are obtained. From the results, it is clear that the standard and the fungal chitin and chitosan resemble the earlier works on XRD (Kaya *et al.*, 2015). Hence, it is concluded that the extracted chitin and chitosan from *R. oryzae* (NCIM877) confirms the material identification and nature.

Figure 5 shows the anti-bacterial property of chitosan against test organisms. The inhibition has been demonstrated by the formation of ZoI. It is clear that the anti-bacterial activity of extracted chitosan is higher as compared to standard chitosan which is shown in Table 2. An observation was made from the results that,

chitosan demonstrates higher anti-bacterial activity against gram-positive bacteria as that of gram-negative bacteria. Recent data in the literature show that the chitosan acts as a bacteriostatic agent rather than bactericidal (Wei *et al.*, 2017). The exact mechanism is not fully understood and several other factors may contribute to the anti-bacterial action (Raafat *et al.*, 2008). Three models have been proposed for anti-bacterial activity, the most acceptable being the interaction between positively charged chitin/chitosan molecules and negatively charged microbial cell membranes. In this model, the interaction is mediated by the electrostatic force between the protonated NH^+ groups and the negative residues (Helander *et al.*, 2001). The electrostatic interactions result in two-fold interference. First, by promoting the changes in the properties of membrane permeability, thus provoking internal osmotic imbalances and consequently inhibiting the growth of microorganisms (Park *et al.*, 2004). Second, by the hydrolysis of the peptidoglycans in the cell wall leading to the leakage of intracellular electrolytes such as potassium ions and other low MW proteinaceous constituents (Didenko *et al.*, 2005; Young *et al.*, 1982).

Table 3 provides the % values for the anti-oxidant activity of chitosan in terms of DPPH scavenging activity. At a concentration range (0.1–1.0 mg/ml), the extracted chitosan showed maximum scavenging activity from 60% to 80%. Whereas, at the same concentration range, standard chitosan from shrimp source

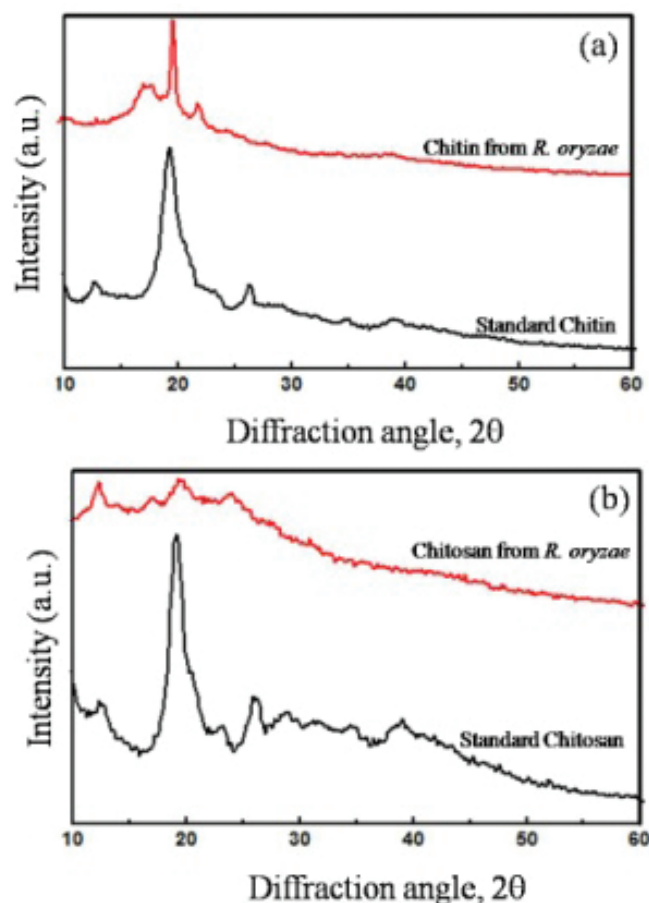


Figure 4. XRD Pattern of (a) standard chitin & chitin from *Rhizopus oryzae* and (b) standard chitosan and chitosan from *Rhizopus oryzae*.

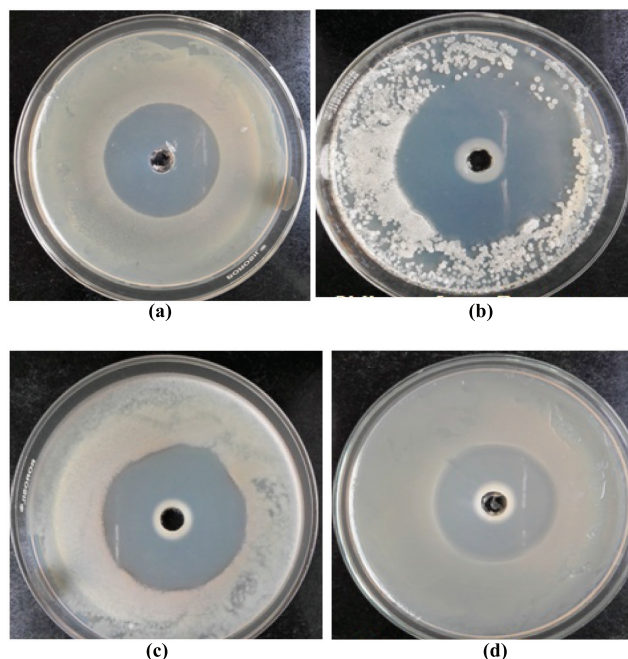


Figure 5. Anti-bacterial activity of chitosan from *Rhizopus oryzae* against various pathogens: (a) *Escherichia coli*, (b) *Staphylococcus aureus*, (c) *Bacillus subtilis*, and (d) *Salmonella typhi*.

demonstrated activity between 5% and 20%. The control BHT showed DPPH scavenging activity of 82%–90%. Hence, the results obtained for DPPH activity using extracted chitosan were similar to the earlier studies (Trung and Duy Bao, 2015; Yen *et al.*, 2007).

CONCLUSIONS

The present study emphasized the two-phase extraction of chitin and chitosan using a modified method of deacetylation step using hot sulphuric acid alone. We obtained an improvement in the yield of chitin and chitosan with this modified method (from 177 to 367 mg/g). Profiling studies were carried out for carbon source, pH, and chitosan yield using SmF process. Profiling studies showed that carbon source and pH have an influence on the weight of biomass and chitosan yield. FTIR results depicted functional groups and confirm the material nature. XRD data provided information on the crystallinity of the polymer. The XRD spectrum confirms that extracted chitin is more crystalline than chitosan. The extracted chitosan was of high MW and demonstrated effective anti-bacterial and anti-oxidant properties. Chitosan demonstrated maximum anti-bacterial activity against gram-positive bacteria as compared with gram-negative ones. Extracted chitosan demonstrated good anti-oxidant activity as compared to shrimp chitosan due to its high molecular weight.

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Table 2. Anti-bacterial activity of extracted chitosan against test organisms.

Sl. No	Test organism	Zone of inhibition (mm)	
		Chitosan from <i>Rhizopus oryzae</i>	Standard chitosan
1	<i>Escherichia coli</i>	3.0	2.1
2	<i>Salmonella typhi</i>	4.6	2.2
3	<i>Staphylococcus aureus</i>	3.8	2.0
4	<i>Bacillus subtilis</i>	3.0	2.0

Table 3. Anti-oxidant activity of standard chitosan and chitosan from *Rhizopus oryzae*.

Sl. no	Sample	DPPH scavenging activity (%) (0.1–1.0 mg/ml)
1	Butylated hydroxy toluene (control)	82–90
2	Standard chitosan	5–20
3	Chitosan from <i>Rhizopus oryzae</i>	60–80

CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

ABBREVIATIONS

NCIM	National Collection of Industrial Microorganisms
NCL	National Chemical Laboratory
SSF	Solid State Fermentation
SmF	Submerged Fermentation
DNS	Di-Nitro Salicylic Acid
AIM	Alkali Insoluble Material
AIF	Acid Insoluble Fractions
DD	Degree of Deacetylation
MW	Molecular Weight
cP	Viscosity
FTIR	Fourier Transform Infrared Spectroscopy
XRD	X-Ray Diffraction
ZoI	Zone of Inhibition
DPPH	2,2-diphenyl-1-picrylhydrazyl
BHT	Butylated Hydroxy toluene

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