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Study on the production of chitin and chitosan from shrimp shell by using *Bacillus subtilis* fermentation

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Abstract—Fermentation of shrimp shell in jaggery broth using *Bacillus subtilis* for the production of chitin and chitosan was investigated. It was found that *B. subtilis* produced sufficient quantities of acid to remove the minerals from the shell and to prevent spoilage organisms. The protease enzyme in *Bacillus* species was responsible for the deprotenisation of the shell. The pH, proteolytic activity, extent of demineralization and deprotenisation were studied during fermentation. About 84% of the protein and 72% of the minerals were removed from the shrimp shell after fermentation. Mild acid and alkali treatments were given to produce characteristic chitin and their concentrations were standardized. Chitin was converted to chitosan by N-deacetylation and the properties of chitin and chitosan were studied. FTIR spectral analysis of chitin and chitosan prepared by the process was carried out and compared with spectra of commercially available samples.

Keywords: *Bacillus subtilis*; *Metapenaeopsis dobsoni*; Shrimp shell; Chitin; Chitosan

1. Introduction

Chitin, the second most abundant biopolymer next to cellulose and its derivatives like chitosan, carboxymethyl chitin, etc., are widely recognized to have immense applications in many fields.[1] They are widely used in the food industry, medicinal fields, chemical industries, textiles, water treatment plants, etc.[2,3] Glucosamine is another value-added product prepared from chitin by hydrolysis and it has versatile applications in pharmaceutics.[4] The prerequisites for the greater use of these biopolymers in various industries are cost of the manufacturing process and the technical grade with specific properties.[5] The commercial method of preparation of chitin from shrimp shell involves strong acid and alkali treatment to remove the minerals and proteins, respectively.[6] However, the use of these chemicals causes depolymerisation of the product and therefore affects properties such as molecular weight, viscosity and degree of deacetylation. These chemical treatment methods bring about hazardous environmental problems like disposal of wastewater. The cost of the chemicals is another drawback of this approach.

To overcome the problems of chemical treatments, different microorganisms[7–9] and proteolytic enzymes[10,11] were used to remove the proteins and mineral content. During fermentation with microbes, deprotenisation takes place by the activity of proteases in the microorganisms and demineralization by the acid produced by the microorganisms during fermentation.[12]

Commercial bacterial proteases are derived from *Bacillus* broth.[13] *B. subtilis*, the commercial starting material, contains a neutral protease, that is, characterized by its pH activity and neutral stability.[14] Zinc is essential for catalytic activity and calcium is required to maintain the structural rigidity of the enzyme. Many reports have demonstrated the application of proteases for the degradation of proteinaceous waste to useful biomass.[15–17] *B. subtilis* and *B. firmus* are the most common exploited species. Many authors[14,18] reported the application of *B. subtilis* for deprotenisation of crustacean waste to produce chitin.

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In the present work, an attempt was made to produce chitin by fermentation using *B. subtilis* and to study its various properties. Chitosan was also prepared from the chitin and its properties were analyzed.

2. Results and discussions

2.1. Changes in pH

During fermentation, the pH of the liquor samples first showed a decreasing trend up to the eighth day and thereafter started rising (Fig. 1). An initial decrease in pH was noticed in samples and that could be due to the ability of the strain *B. subtilis* to initially use sugar as substrates for their growth[19] and simultaneously they produce acid via pyruvate.[20] Low pH condition prevents the decay of shrimp shell, which are colonized by spoilage and pathogenic organisms.[21] By the eighth day, about 87% of the sugar in the jaggery broth was utilized by the organism. Therefore, when the sugar substrate was depleted, acid production decreased and pH started increasing.[22] The acid produced was responsible for the extent of demineralization noticed in the samples at the end of fermentation.[12] During fermentation, proteolysis occurs and ammonia is released due to utilization of amino acids for the growth of the bacteria.[19,23–26] This also might have caused the increase in pH.

2.2. Extent of deprotenisation and demineralisation

The deprotenisation of the shrimp shells was brought about by the activity of the neutral protease of *B. subtilis*.[13,18] Yang et al.[14] found that the optimum activity of the protease produced by *Bacillus subtilis* took place at 30 °C and pH of 6. In the present work also the protease showed maximum proteolytic activity of 10.6875 g tyrosine/g protein after 48 h of fermentation when the pH of the liquor was 5.9. The proteolytic activity remained fairly constant for 5–6 days during fermentation (Fig. 2). These changes are clearly supported by the reports of Allagheny et al.[26] and Sarkar et al.[19]

During fermentation, when the available carbohydrate source was utilized by the microorganisms for their growth, acid was released and this was responsible for calcium solubilization from the shrimp shell.[12] As the calcium ion concentration increased in the fermenting media, proteolytic activity showed a decreasing trend. Tsuru et al.[27] reported that calcium is recognized to prevent protease autolysis.

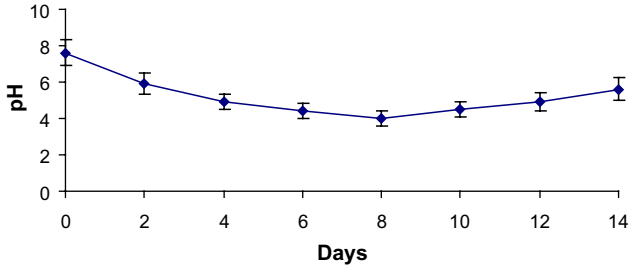

Figure 1. Changes in pH of the protein liquor.

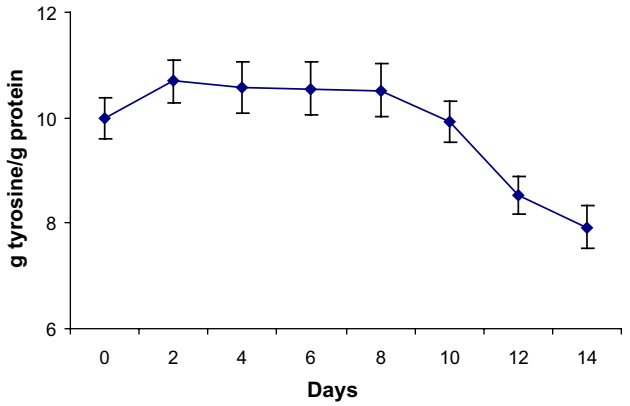

Figure 2. Changes in proteolytic activity.

2.3. Chemical treatment of fermented residue

Table 1 shows the protein and ash content of the fermented residue. About 84% of the protein and 72% of the minerals were removed from the shrimp shell at the end of fermentation. To produce chitin of standard quality, residual protein and ash content of the fermented residue have to be removed by mild acid and alkali treatments. Different concentrations of hydrochloric acid and sodium hydroxide were tried to remove ash and protein, respectively (Figs. 3 and 4). Considering the statistical significance ($P < 0.05$) in the extent of demineralization trials, we have used 0.8 N HCl for the demineralization study. Similarly, among different alkali concentrations tried, 0.6 M NaOH was used for the deprotenisation ($P < 0.05$).

Many previous papers reported the chemical treatment of the fermented residue to remove the residual protein and ash.[8,28,29] The optimum concentrations of the acid and alkali required depend on the residual protein and ash content. This was dependent on the type of the raw material, type of the starter culture, fermentation time, etc.

2.4. Characteristics of chitin prepared from shrimp shell

Table 2 presents the characteristics of the chitin prepared by this treatment. The chitin obtained is α-chitin

Table 1. Protein and ash content of the fermented residue

Particulars	% of protein content	% of ash content
Raw material	38.10 ± 2.15	18.538 ± 0.89
Fermented residue	6.096 ± 0.4	5.191 ± 0.3

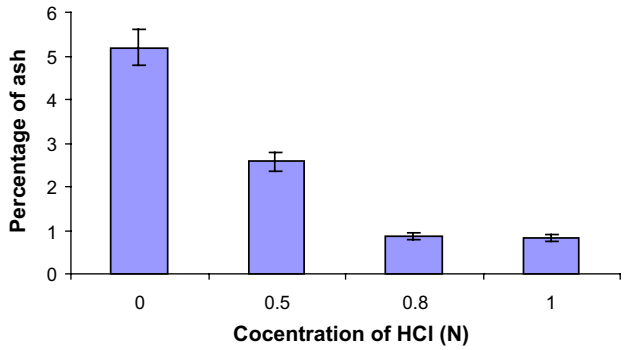

Figure 3. Percentage of ash content in the fermented residue treated with different concentrations of acid.

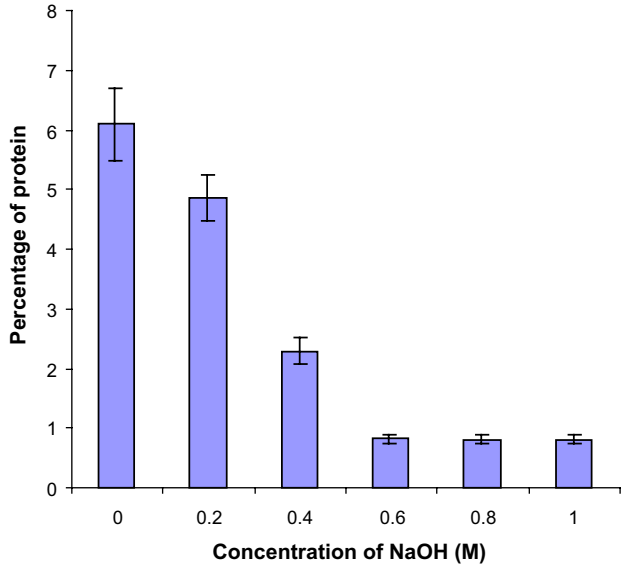

Figure 4. Percentage of protein content in fermented residue treated with different concentrations of alkali.

Table 2. Properties of chitin prepared

Moisture	5.1 ± 0.4%
Ash	0.854 ± 0.04%
Protein	0.815 ± 0.5%
Chitin	93.2 ± 0.6%
Appearance	White flakes
% of N-acetylation	84.4 ± 5.3
pH	7.8 ± 0.2

and the chitin content of the product was found to be 93.2%. Mineral content (calcium, sodium, potassium and magnesium) in the chitin prepared from the shrimp shell is shown in Figure 5. From the figure, it is clear that calcium content is more than other minerals in chitin. This is due to the fact that the main constituent of minerals in shrimp shell is calcium carbonate.[22] Mild acid treatment after fermentation, reduced the mineral content to permissible limits in the chitin. One of the factors determining the good quality of chitin is the low mineral content.[30]

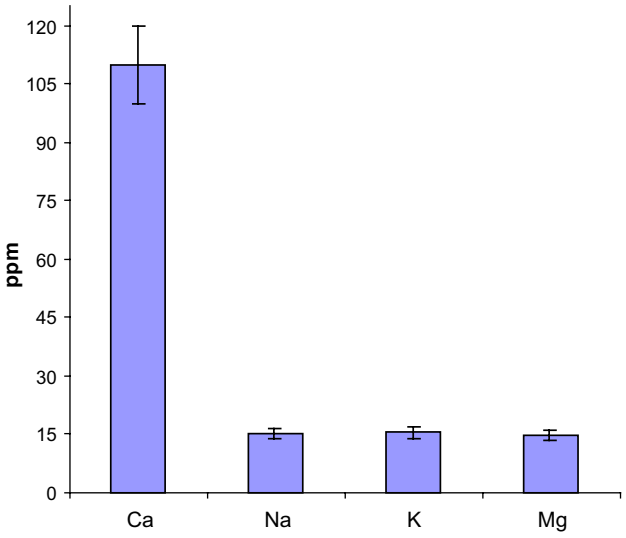

Figure 5. Mineral content of the chitin.

The FTIR spectrum of the chitin prepared by this method is shown in Figure 6. The spectrum was found to be similar to that of the commercial chitin (Fig. 7). There are no impurities found. Because, it has a better percentage of individual functional groups, the chitin prepared by the *Bacillus* fermentation compares favorably with the commercial chitin, with regard to purity. The percentage of N-acetylation, calculated from the FTIR spectrum, was remarkable, 84.4%. Other characteristics of the chitosan prepared are given in Table 3. The FTIR spectrum of the prepared chitosan is presented in Figure 8 and is similar to that of commercial chitosan (Fig. 9).

3. Conclusion

From this study it was found that *B. subtilis* was found to be an efficient starter culture for fermentation of shrimp shells. About 84% of the protein and 72% of the minerals were removed from the fermented residue at the end of fermentation. Chitin and chitosan were prepared from the fermented residue and the physiochemical properties of these products were found to be similar to commercial grades of these materials.

4. Materials and methods

4.1. Shrimp shell

The shrimp (*Metapenaeopsis dobsoni*) shells used for the experiment were procured in fresh condition from the shrimp processing plant located at Cochin, Kerala,

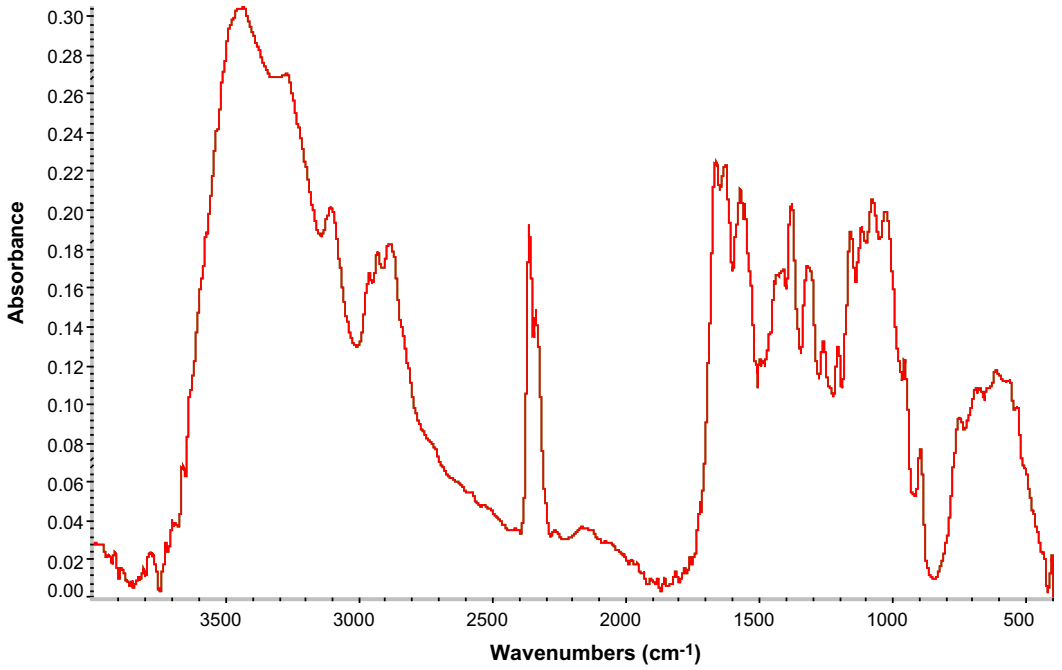

Figure 6. FTIR spectrum of chitin prepared by the *Bacillus* fermentation.

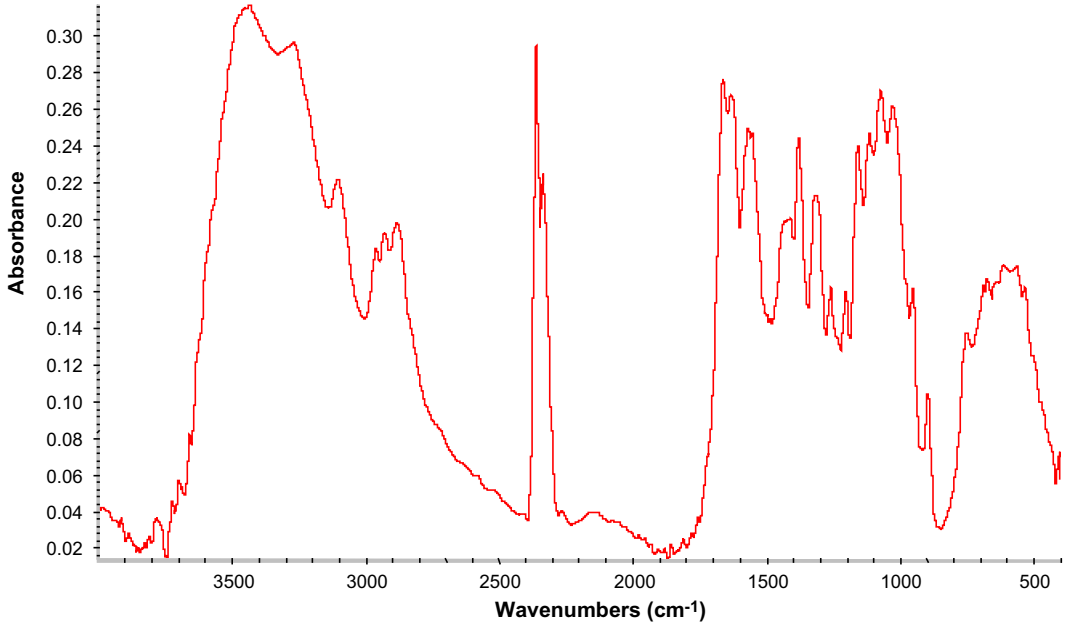

Figure 7. FTIR spectrum of commercially available chitin.

Table 3. Characteristics of chitosan prepared

Moisture (%)	7.6 ± 0.5
Ash (%)	0.832 ± 0.06
Appearance	Off white flakes
Acid insoluble ash	Nil
Solubility (1% of chitosan solution) in 1% acetic acid	95%
Viscosity (m Pa S)	1680 ± 138
Molecular weight ($\times 10^5$) (Da)	2.56 ± 0.15
Degree of deacetylation (%)	81 ± 7

India. The shell waste brought to the laboratory was minced in a meat mincer and used for the study.

4.2. Jaggery

Jaggery was used for the preparation of media for *Bacillus* growth and was obtained from the local market at Cochin. Its sucrose content was estimated and about

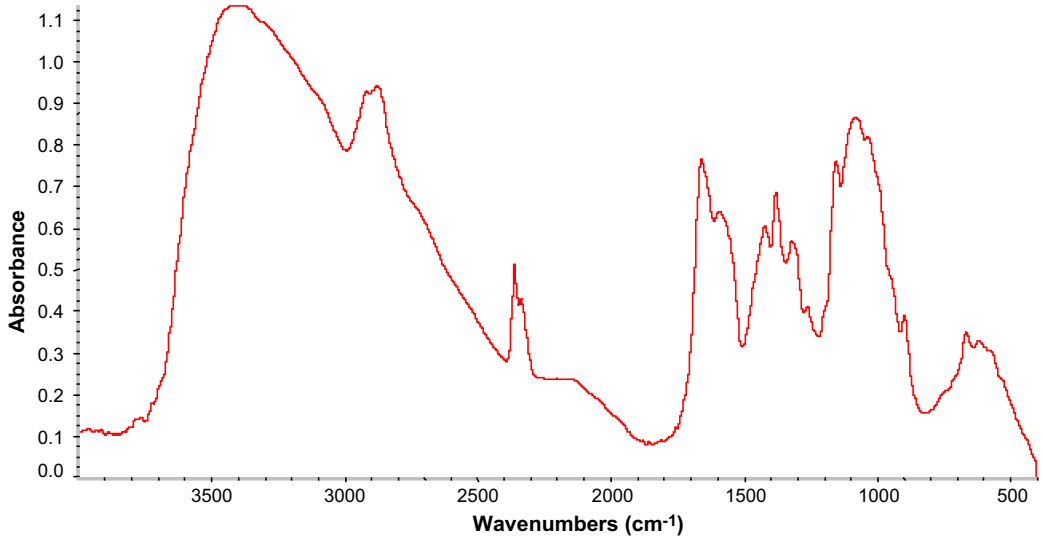

Figure 8. FTIR spectrum of chitosan.

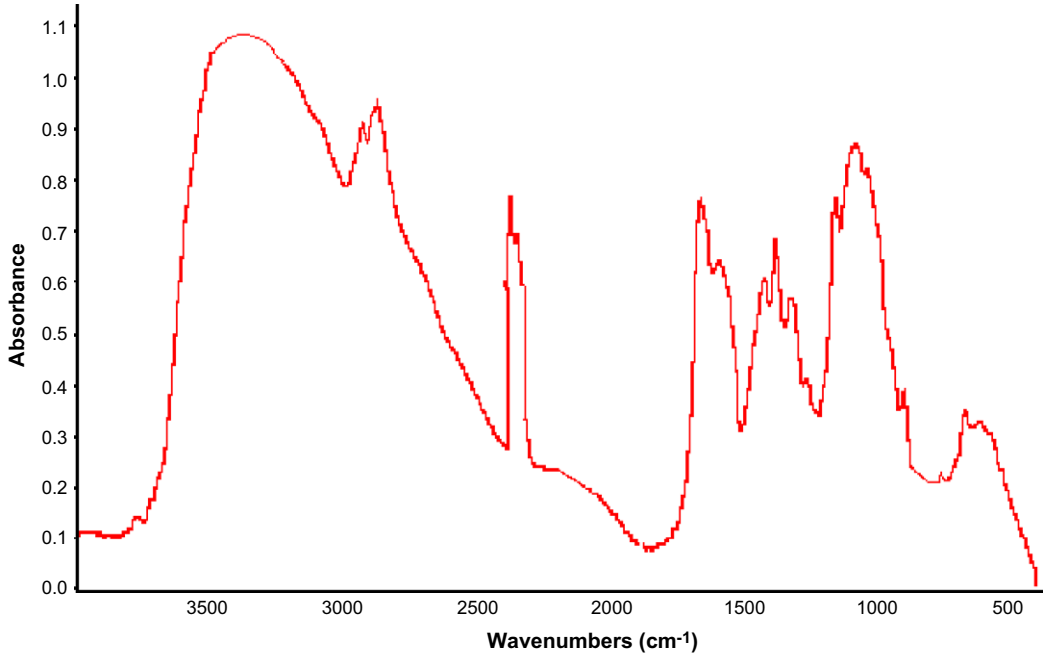

Figure 9. FTIR spectrum of commercial chitosan.

20% w/w broth prepared for the study was sterilized at 10 psi for 15 min.

4.3. Inoculum preparation

A freeze-dried culture of *B. subtilis* (ACC No. *121) was obtained from the Institute of Microbial Technology (IMTECH), Chandigarh, India. The *B. subtilis* strain was maintained in nutrient agar slopes at 4 °C. The inoculum was prepared by adding a loop full of cells to 10 mL nutrient broth and incubated at 30 °C for 24 h. Then, 0.5 mL from this inoculum was transferred to 100 mL of sterilized 20% w/v jaggery broth and incubated at 30 °C for 24 h. The prepared inoculum yielded a cell concentration of approximately 10^8 CFU/mL.

4.4. Fermentation

Thoroughly minced shrimp waste (200 g) was mixed with 24 h incubated 20% w/w jaggery broth (200 mL) containing 10^8 CFU/mL of *B. subtilis*. The flask was tightly closed and kept for fermentation for 15 days. Liquor samples were taken at 48 h intervals and analyzed for pH, proteolytic activity and sugar concentration.

After the completion of fermentation, sediments were removed, washed and estimated for its ash, chitin and protein contents.

4.5. Preparation of chitin

Solid fractions obtained after fermentation were treated with different concentrations of HCl (0.2, 0.5, 0.8 and 1 N) in 1:15 (solid:acid) ratio for 2 h at room temperature (30 ± 2 °C). The samples were then washed well and treated with different concentrations of NaOH (0.2, 0.4, 0.6, 0.8, 1 and 1.2 M) in 1:15 (solid:alkali) ratio for deprotenisation. Protein, ash and chitin contents were estimated. The FTIR spectra of the chitin samples were also measured and compared with that of the commercial chitin.

4.6. Preparation of chitosan

The purified chitin was boiled with 40% (w/w) NaOH until it gets deacetylated to chitosan. Chitosan formation was tested by its solubility in 1% acetic acid solution. Moisture, ash, acid insoluble ash, viscosity, molecular weight and degree of deacetylation were measured. The FTIR spectra of the chitosan samples were also measured and they are compared with the spectrum of commercially available chitosan. Commercial chitin and chitosan were obtained from M/s India Sea foods Ltd., Cochin, Kerala, India.

4.7. Analytical methods

pH was measured using Cyberscan pH meter.[31] Sugar content was estimated by the method of Hodge and Hofreiter.[32] The amount of protein was measured by the method of Lowry et al.[33] Proteolytic activity was analyzed with tyrosine as the standard compound.[7] Viscosity was measured using Brookfield DV-E Viscometer with ULA spindle (SMC—0.64) at 100 rpm. Molecular weight was determined by the method of Rutherford and Austin[34] and degree of deacetylation as per the procedure of Muzzarelli and Rocchetty.[35] The FTIR spectral analysis was carried out with a Nicolet AVATAR 360ESP FTIR Spectrometer. The average number of scans taken per sample was 16 in the spectral region between 400 and 4000 cm^{-1}. The degree of N-acetylation of the samples was calculated using the following equation:

$$\%\text{N-acetylation} = (A1655/A3450) \times 115.^{36}$$

Calcium, sodium, potassium and magnesium were analyzed for chitin using Varian SpectrAA 220 Atomic Absorption Spectrometer. Moisture, ash and acid insoluble ash contents were also estimated for chitin and chitosan samples as per AOAC.[37] Total nitrogen and chitin nitrogen were estimated by the Kjeldahl method. Corrected protein was obtained by subtracting CN from TN and multiplying by 6.25, the Kjeldahl conversion factor for meat protein, assuming that protein has 16% nitrogen.[38]

4.8. Statistical analysis

Results were expressed as mean ± S.D. Univariate analysis of variance was carried out and the statistical comparisons were done with Duncan's test using a statistical package program (SPSS 10.0 for Windows).

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