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EXTRACTION AND CHARACTERIZATION OF CHITOSAN FROM SHRIMP WASTE FOR APPLICATION IN THE FEED INDUSTRY

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Abstract: Chitosan is an amino polysaccharide prepared by processing shrimp waste (shell) which involves partial deacetyla-tion of chitin. Chitosan, a versatile natural polysaccharide, the second most abundant natural polymer. Many biochemists have found that chitosan as biocompatible, biodegradable and non toxic which made wide applicability in conventional pharmaceutics as a potential formulation excipient. In the present research mainly focused on the extraction of chitosan which is suitable for the feed industry. The crude chitin was collected from exoskeleton of Shrimp waste which are then processed by different methods to obtain chitosan and it was analyzed for its physiochemical parameters.
Keywords: Chitosan, extraction, characterisation.

INTRODUCTION

Shrimp waste has been used in poultry rations as a source of protein (Okoye *et al.,* 2005, Oduguwa *et al.,* 2004) or as a source of prebiotic (Khempaka *et al.,* 2011, Zhou *et al.,* 2009) with varying results. Most of the studies concluded that shrimp waste can be included between 5-15 % in poultry diets as a protein source and Chitosan as a prebioitc can be included upto 0.5 – 2.5% of diet. Chemicals i.e. acid and alkali have been used to extract chitosan from shrimp waste but the disadvantage is their corrosive nature and environmental pollution. Hence, in the present study chitosan was extracted form shrimp waste meal using organic acids (lactic, propionic and fumaric) and yeast culture for feed industry application. Extraction of chitosan from the natural sources involves demineralization, deproteination and deacytelation processes for which many methods such as use of acids and alkali, enzymes, ensiling, bioremediation using probioitics (Prameela *et al*, 2010, Jag Pal *et al*, 2014) etc have been tried.

MATERIALS AND METHODS

Fresh shrimp waste (SW) was sun dried for 3 days and subjected to grinding in a hammer mill. Representative samples of dried shrimp waste were taken, pooled, ground in a Wiley mill and analyzed for proximate composition (AOAC, 2005).

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Chitosan extraction

The process involved demineralization, deproteinization and deacetylation. In the conventional chemical method, inorganic acids like HCl are used which are highly corrosive. Hence, in the present study organic acids i.e. fumaric, lactic and propionic acids were used for demineralization. Deprotenation and deacetylation in the conventional method is brought about by using NaOH. In the present study baker yeast *(saccharomyces cerevisiae)* was used for deprotenation followed by deacetylation using NaOH.

Standardization of demineralization process

HCl and other organic acids were used in different strengths to arrive at the appropriate strength of acid that causes maximum demineralization of shrimp waste meal. HCl : 20 g of the shrimp waste meal was suspended in 1,4 and 8% HCl (1: W/V) for 72 h. Organic acids : 20 g shrimp waste meal was suspended in 5,10 and 20%of Fumaric, Propionic and Lactic acid (1:4 W/V) for 72 h. The pH of the solution was recorded at 24,48 and 72 h intervals using a digital pH meter. After 72 hours, the residue was washed over muslin cloth with distilled water until free of any traces of acid. The residue was dried in hot air oven at 60^0 c for 24h and the total ash content in it was estimated in muffle furnace at 550^{o}C for 3.0 h. The efficiency of demineralization was calculated (Monarul Islam *et al.,* 2011).

Demineralization (%) =

$$\frac{(\text{g of sample} \times \text{Ash content (\%) of sample}) - (\text{g of residue} \times \text{ash content (\% of residue)}}{\text{g of sample} \times \text{Ash content (\%) of sample}} \times 100$$

Standardization of deproteinization process

The demineralized shrimp waste residue obtained by using HCl in the previous step was deproteinized using boiling 4 % NaOH for 1 h (Monarul Islam *et al.,* 2011) whereas the residues obtained from the use of organic acids were deproteinized using yeast culture (Prameela *et al*.,2010) using 5% inoculum of baker yeast, 15% molasses and 0.02 % salt and fermented for 72 h. Later filtered through a muslin cloth and the residue left over was chitin. The efficiency of deprotenation was calculated:

$$\frac{(\text{g of sample} \times \text{CP content (\%) of sample}) - (\text{g of residue} \times \text{CP (\% of residue)}}{\text{g of sample} \times \text{CP content (\%) of sample}} \times 100$$

Deacetylation process:

Removal of acetyl groups from the chitin was achieved by using 70% NaOH solution with a solid to solvent ratio of 1:14 (w/v) at room temperature for 72 hours. The residue was

washed to neutrality in running tap water and the pH was checked by pH paper. The chitosan residue was dried overnight at 60^0 c and weighed (Monarul Islam *et al.,* 2011).

Determination of the degree of deacetylation

The degree of deacetylation (DDA) was measured by the acid-base titration method (Domard & Rinaudo, 1983) with modifications. In brief, chitosan (0.1 g) was dissolved in 30 ml HCl aqueous solution (0.1 mol/l) at room temperature with 5–6 drops of methyl orange added. The red chitosan solution was titrated with 0.1 mol/ litre NaOH solution until it turned orange. The DDA was calculated by the formula:

$$\text{DDA}\ (\%) = [(C\,1V1 - C2V2)\,/M \times 0.0994]\ \times 0.016$$

where:

C 1 – concentration of standard HCl aqueous solution (mol/l)

*C*2 – standard NaOH solution (mol/l)

*V*1 – volume of the standard HCl aqueous solution used to dissolve chitosan (ml)

*V*2 – volume of standard NaOH solution consumed during titration (ml)

M – weight of chitosan (g)

The number 0.016 (g) is the equivalent weight of NH_2 group in 1 ml of standard 1 mol/l HCl aqueous solution, and 0.0994 is the proportion of NH_2 group by weight in chitosan. (Jun Zhang, *et al*., 2011)

Characterization of chitosan:

The chitosan so obtained was characterized using Fourier Transform-Infra Red Spectroscopy (FT-IR). A sample of 10µg was mixed with 100 µg of dried potassium bromide (KBr) and compressed to prepare a salt (10 mm diameter) and characterized by the FT-IR in the range of 500-4000 cm^{-1} (Arafat *et al.,*2015).

Solubility

The shrimp chitosan powder was placed into centrifuge tube of known weight and then dissolved with 10 ml of 1% acetic acid for 30 min using an incubator shaker operating at 240 rpm and 25^0 C. The solution was then immersed in a boiling water bath for 10 m, cooled to room temperature and centrifuged at 10,000 rpm for 10 min. The supernatant was then decanted and the undissolved particle were washed in distilled water (25 ml) and centrifuged at 10,000 rpm. The supernatant was removed and the undissolved pellet was dried at 60^0 c for 24 h and finally the particles were weighed and percentage solubility was determined (Huthman *et al.*,2013).

$$\text{Percentage of solubility} = \frac{(\text{initial weight of tube + chitosan}) - (\text{final wt of tube + chitosan})}{(\text{initial weight of tube + chitosan}) - \text{initial weight of tube}} \times 100$$

Extraction of Chitosan in large scale

After the initial laboratory evaluation, it was observed that 8 % HCl and organic acids (20% fumaric, lactic and propionic acids) at a ratio of 1:4 ratio (W/V) yielded better demineralized and deprotenized chitin. Hence, these levels of acids were used for large scale chitosan extraction.

Demineralisation

7 kg of sun dried shrimp waste meal was taken in plastic tubs of 100 L capacity and soaked for 72 h in 28 liters of 8% HCl or 20% Fumaric or lactic or propionic acid (1:4 w/v.) for demineralization process. The supernatant was discarded and the residue was washed thoroughly in tap water and filtered using a muslin cloth. The pH of the residue was checked using filter paper to ensure it was free of traces of acids. The residue was collected in a tray and dried for 48 h in hot air oven at 60^0C.

Deprotenization

The dried residue remaining from the 8% HCl treatment was then treated with boiling 4% NaOH for 1h and dried in a oven at 60^0C for 48 h whereas the demineralized residue of 20% fumaric, lactic or propionic acid was treated by using 5% baker yeast, 15% molasses, and salt 0.02% for 72 h and filtered through a muslin cloth. The liquid portion was collected and used for the enrichment of deoiled rice bran. The weight of residue of each treatment was recorded. The liquid from the HCl treatment was discarded.

Deacetylation

The deproteinized and dried residue of each treatment was soaked (1;14 W/V) in 70% NaOH for 72 h, washed with tap water until free of alkali and filtered using a muslin cloth, dried in a hot air oven at 60^0C for 48 h and the weight of the residue was recorded to calculate the yield of Chitosan (Monarul Islam, *et al*., 2011).

RESULTS AND DISCUSSION

Demineralization and Deproteinization of shrimp waste meal results in chitin and the chitin is subjected to deacetylation to produce chitosan. Percot *et al*.(2003) reported that using HCl for the demineralization of chitin results in detrimental effects on the molecular weight and the degree of acetylation that negatively affects the intrinsic properties of the purified chitin. The authors elaborated on the importance of the optimization of the extraction process parameters (pH, time, temperature and solids to acid ratio) in order to minimize chitin

degradation and bring the impurity levels down to the satisfactory level for specific applications. Using organic acids such as lactic and/or acetic acids for the demineralization process is a promising idea since organic acids: (a) can be produced from low cost biomass such as cheese whey, (b) are less harmful to the environment, (c) can preserve the characteristics of the purified chitin and (d) the resulting organic salts from the demineralization process can be used as an environmentally friendly anti-icing agents and/or as preservatives (Subha Ganguly *et al*, 2013). Therefore, in the present study HCL at 1,4, and 8% strength and organic acids i.e. Fumaric, Propionic and Lactic acid at 5,10 and 15 % were used to treat the shrimp waste meal for 24 to 72 h so as to observe for pH changes. 8 % HCl and 20 % organic acids upto 72 h of treatment of shrimp waste meal resulted in lower pH than at 24 or 48 h. Based on these results 8 % HCl and 20 % organic acids upto 72 hrs of incubation was selected for demineralization of shrimp waste meal. The percentage demineralization was 29.5 for 1 % HCl and 78.3 for 8% HCl. The demineralization was 32.1, 67.1, 60.0 (for 5%), 60.2, 74.3, 68.7 (for 10%) and 89.9, 90.6, 85.6 (for 20%) strength of Fumaric, lactic and propionic acids, respectively.

Deproteinization can be done by using alkali or by the biological methods. This is an advanced and a new technique for chitin extraction. It can be done using proteolytic microorganisms or fungi or purified enzymes. It results in the production of oligomers with an optimum degree of polymerization for different applications and does not denature the chitin. Enzymatic extraction can be highly specific and yield chitin with higher molecular weights. Besides environmental advantages as compared to the chemical method, the use of enzymes also eliminates the hazards associated with the reactive reagents. Even the extracting cost of chitin by biological method can be optimized by reducing the cost of carbon sources (Jag Pal,*et al.,*2014). In the present study baker yeast (saccharomyces cereviciae) was used to bring out deproteinization of shrimp waste meal.Highest deproteinization (%) of 84.6 for 8 % HCl, 60.2, 53.9 and 71.0 % was observed for 20 % Fumaric, Lactic and Propionic acids, respectively.

The degree of deacetylation of the chitin was 65, 40, 44 and 47 % for 8% HCl and 20 % fumaric, lactic and propionic acids, respectively. It is indicated that the degree of deacetylation (DD) is influenced by NaOH concentration. Acetyl groups bounded in chitin are difficult to be removed. So, it needs high concentration of NaOH and temperature (Hargono *et al*., 2003). In this case, more NaOH will be disposed and more purified water has to be required to get chitosan product. The degree of deacetylation of shrimp chitosan

samples ranged from 45.5% to 81.24% based on different concentration of NaOH treatment (No and Meyers, 1995) and from 56% to 99% (Hossain and Iqbal, *et al*.,2014). It is a very important parameter that influences other parameters like solubility, chemical reactivity and biodegrability. DD of the commercially available chitosan ranges between 75 and 85%. The value of DD ranges from 30 to 95% and depends on various factors such as the type of analytical method employed, sample preparation, type of instrument used and it has been reported that the DD is one of the most important chemical characteristics which would influence the performace of chitosan in many of its applications (Huthman *et al*.,2013).

The yield of Chitosan was 7.0, 8.0, 11.0 and 8.0 % from 8% HCl and 20 % fumaric, lactic and propionic acids, respectively. The yield obtained was found to be lower than 13.12% to 17.36% (Lertsutthiwong *et al*., 2002), 14% (Brzeski *et al*.,1982), 18.6% (Alimuniar and Zainuddin *et al.,* 1992) and 23% (Meyers *et al*.,1989). Variable yield of chitosan might be due to depolymerization of the chitosan polymer, loss of sample mass/weight from excessive removal of acetyl groups from the polymer during deacetylation and loss of chitosan particles during washing (Hossain, *et al*., 2014).

Characterization of extracted chitosan by FT-IR spectra (Table 1) showed the band spectrums of different bonds i.e. at 3267 cm^{-1} of OH group, 2924 for N-H stretching, 1629, 1540 and 1317 for amide I, amide II and amide III bands respectively. These observations are in close agreement with the reported values of (Puvvada *et al*., 2012).

The FT-IR of chitosan extracted using 8 % HCl or 20 % fumaric, lactic and propionic acids showed peak at 3846, 3839, 3844 and 3842 cm^{-1} , respectively representing the presence of OH group and compared well with the value of 3858 cm^{-1} for standard Chitosan (Puvvada *et al.,* 2012). The absorbance bands for NH stretching (Table 8), CH stretching, Amide 1 band, Amide 2 band, CH_2 bending, CO- stretching, CH3 wagging alone chain and NH-out of plane bending for the Chitosan extracted in the present with 8 % HCl or 20% organic acids compared well with the pure Chitosan spectra (Puvvada *et al.,* 2012). Kamala *et al*., (2010) reported that FT-IR spectrum of chitin extracted from shrimp waste using the chemical method showed a peak at 3293 cm^{-1} indicating presence of OH stretching and 2961 cm-1 indicating presence of N-H stretching, while 1214, 1138 and 933cm^{-1} for amide I, amide II and amide III bands, respectively. Liu *et al*., (2012) reported FT-IR spectra of chitin characterized by three significant amide bands at 1654, 1560, 1310 cm^{-1} corresponding to amide I stretching of C=O, the amide II of N-H and amide III of C-N, respectively.

The solubility of chitosan is one of important parameters for quality of chitosan, where higher solubility will produce a better chitosan. There are several critical factors affecting chitosan solubility including temperature and time of deacetylation, alkali concentration, prior treatments applied to chitin isolation, ratio of chitin to alkali solution, and particle size. The solubility, however, is controlled by the degree of deacetylation and it is estimated that deacetylation must be at least 85% complete in order to achieve the desired solubility (No *et al*., 1995). The solubility of chitosan obtained in this study was ranged from 90% for 8%HCl and 70.5%, 74.5%, 79.5% for 20% Fumaric, Lactic and Propionic acid respectively. Brine and Austin *et al.,* (1981) noted that lower solubility values suggested incomplete removal of protein and acetyl group. Since solubility of chitosan depends on the removal of acetyl group from chitin, therefore the lower DD value could adversely interfere with the results (Hossain and Iqbal, *et al*.,2014).

Conclusions

It was concluded that chitosan extracted by using organic acids i.e. 20% Fumaric, Lactic and Propionic acids along with yeast exhibited the characteristics of pure Chitosan.

Table 1: Main bonds observed in the FT-IR spectra of standard chitosan and extracted chitosan of the present study

	Std. Chitosan (cm^{-1}) (Puvvada *et al*., 2012)	**8 % HCl**	**20 % Fumaric Acid**	**20% Lactic Acid**	**20% Propionic Acid**
OH stretching	3858	3846	3839	3844	3842
NH stretching	3609	3295	3262	3253	3250
CH stretching	2862	2878	2845	2876	2877
Amide 1 band	1643	1646	1645	1643	1649
Amide 2 band	1552	1537	1529	1537	1532
CH2 bending	1421	1403	1403	1380	1400
CO- stretching	1022	1018	1007	1007	1010
CH3 wagging alone chain	752	-	662	687	684
NH-out of plane bending	564	-	598	600	-

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