Physicochemical And Biological Properties of Chitosan Extracted from Fish Scales of Labeo Rohita

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Abstract

The current research was aimed to extract chitin and chitosan from the by-products (scales) of L.rohita by a sequence of chemical processes involving demineralization, deproteinization and deacetylation and studied its various physicochemical and biological properties. The most effective concentration for producing the best results was determined after considering several acid and alkali treatments. In the current research the features that make chitosan useful included Solubility (2.972), Fat Binding Capacity (111%), Water Binding capacity (106 %), and Ash Value (0.371%) have indicated the sample as good source of chitosan. The antibacterial property of chitosan against different pathogens like Pseudomonas fluorescens, Aeromonas hydrophila, Staphylococcus aureus, and Escherichia coli were evaluated. However Aeromonas hydrophila showed good antibacterial activity as compared to others. Antioxidant activity DPPH and Anti-inflammatory activity confirmed the antioxidant and anti inflamative properties of chitosan.

Keywords: Chitosan, Fat binding, Water binding, Ash, Moisture, Antibacterial, antioxidant, Anti-inflammatory activity.

1. INTRODUCTION

Aquaculture involves a number of species, rearing techniques and husbandry methods. Extensive aquaculture is the farming of fish in 'Natural' surroundings with no supplementary diet and with a low influence on the environment. On the other hand, intensive farming of fish commonly practiced in ponds and cages which require high-quality artificial feeds and medications with ensuing effects on the environment. Fish farm waste can change the environment beyond the immediate vicinity and the area directly impacted by the effluent wider coastal zone at different ecosystem levels, thus reducing the biomass, density and diversity of the benthos, plankton and nekton, and modifying natural food webs (Gowen, 1991; Pillay, 1991). The total fish produced worldwide in the year 2014 is 167.2 million tons, with a value of $217.5 billion and the fish industry's annual discard amount is approximately 25% of the total production. For centuries, India has had a traditional practice of fish culture in small ponds in Eastern India (FAO 2009) and today fishing is a major industry especially in coastal states of India, employing over fourteen million people. In India fish production has increased more than tenfold since its independence in 1947 (FAO 2013). According to the Food andAgriculture Organisation (FAO) of the United Nations, fish output in India is dialyzed between 1990 and 2010 (FAO 2013). Today India is the second largest producer of fish in

the world contributing about 5.43% of global fish production, which are third largest producer of fish and the second largest producer of fresh water fish in the world. Fish scale wastes are generally discarded in the environment through fish processing industries and local market vendors. The amount of fish wastes (by-products and by-catch) depends on many factors: fish species, fish size, and season and catch method, processing techniques, local regulations, market, etc. (SEAFISH 2001). In recent years it was dumped in one place and as an outcome, it contaminates the aquatic ecosystem. This plenteous waste may also pose severe environmental hazard due to easy deterioration. To avoid these problems, bioactive substances that are good for human health can be isolated from waste produced during the processing of fish.This procedure helps to reduce pollution while also raising the value of the byproduct of fish processing.A renewable source of useful enzymes and proteins are also offered by waste from fish processing that may be used to improve economics of seafood processing industry , while minimizing the environmental impact of waste disposal (Daboor et.al., 2012). Chitin and Chitosan are polysaccharides which are made up of N-acetylglucosamine, bound together in β-(1-4)-Nacetyl-d-glucosamine bonds. It's quite close to cellulose, but it has another amine group and a hydroxyl substituent on each monomer. According to Rudall and Kenchington (1973), it is the second most prevalent bio-polysaccharide after cellulose and is found in a variety of marine invertebrates, insects, fungi, and yeast (Muzzarelli et al 1981). Due to its compact structure, chitin is not soluble in the majority of solvents (Kurita 2001). This restricts its use, and numerous chemical changes have been made in an effort to create a more soluble and useful derivative, like chitosan. Thus, chitosan (β-(1-4)-linked D-glucosamine and N-acetyl-d-glucosamine) is the term used to refer to a collection of fully or partially deacetylated chitin .Due to its advantageous biological characteristics, such as biocompatibility, biodegradability, non-toxigenicity, non-antigenicity, adsorption, ability to form films, and ability to chelate metal ions, chitin and chitosan have recently attracted the attention of researchers (Rout 2001, Shahidi and Abuzaytoun2005). As a result, they have been used in several industrial domains (Shahidi et al. 1999; Ong et al. 2008). including poultry health and production in Malaysia (Kaikabo et al 2016a, 2016b).The features of chitin and chitosan may directly affect their use because of their natural origin and high level of chemical and physical diversity.The use of chitin and chitosan in a variety of industries, including food, medical, and agriculture, is closely linked to their physicochemical characteristics.Commercially, chitosan is made by chemically deacetylating chitin while subjecting it to a high alkali (Chatterjee et al 2005, Silva et al 2007).Chitin and chitosan are not identical because of variations in the raw materials, destructiveness of the isolation and conversion processes, caustic effects of the chemicals used in the extraction process, and variations in the levels of deacetylation and protein contamination (Nwe et al. 2002; Tajdinia et al. 2010) which restrict the use of these products. Due to the ongoing collection of these species without their replacement and their seasonal availability, commercial chitin and chitosan from shrimp, lobster, and crabs

may become impractical (Tajdinia et al 2010). Many insect species have been partially investigated as an alternate source of chitin and chitosan in an effort to diversify the chitin-chitosan base source and get chitin and chitosan of a better and more reliable grade (Zhang et al 2000, Ai et al 2008, Liu et al 2012).The majority of these research solely contrast insect chitin with synthetic chitin; little is known about how chitosan stacks up .Fish scales are available in large quantity in our society as bio-waste component and thrown away during food processing for which it easy to collect them. Therefore, the aim of the present study was to isolate chitosan and to analyze its various physicochemical and biological properties.

2. Materials and methods

2.1. Collection of samples

The scales of Labeo rohita were obtained in fresh conditions from a nearby fish market of Bhubaneswar, India. The scales were thoroughly washed with tap water and then distilled water to remove other pollutants including dust, leaves, fins, and gills.The scales were cleaned and then dried for 48 hours in a hot air oven at 500C. Then the fish scales were preserved in an air-tight container for further use.

2.2. Extraction of Chitin and Chitosan

For extraction of chitin, the conventional chemical method i.e., demineralization, deproteinization, and deacetylation was followed.

Demineralization

Demineralization is the process in which calcium carbonate and other calcium compounds are eliminated from the scales. Calcium Carbonate is removed to restrict the hydrolysis of chitin. The demineralization was done using the method (Sunita et al. 2016) with little modification. Hydrochloric acid in a 0.1Mol/ (0.1N solution) solution was used to demineralize the dried scales.The scales were entirely submerged in the 0.1Mol /L (0.1 normality) HCl solution and heated on a hot plate for 15 minutes at 900C. The solution is decanted once the scales have been heated, leaving the solute behind. This sample underwent many deionized water washes.

Deproteinization

Deproteinization is the technique used to remove the protein content from the produced chitosan . Deproteinization of chitosan is a necessary step for chitosan extraction because there are small molecules in chitosan samples which can be randomly distributed by various proteins. To this demineralised scales 1% NaOH (W/V) was added in a conical flask at 100 0C for 15 minutes in an incubator shaker.

Deacetylation

In deacetylation process an acetyl group is removed from the chitin so that the chitin turns into chitosan (Kumari et al. 2016). For deacetylation, chitin was treated with alkali, i.e., 1 g of chitin was added to 1% NaOH for 15 min at 100°C, followed by washing till it reaches neutral pH. After drying, the final product recovered was chitosan.

3. Physico-chemical properties of Chitosan

3.1. Moisture content

Chitosan samples were dried using a gravimetric technique in a hot air oven at

05^0C to determine their moisture content (Black et al).

3.2. Solubility

Chitosan is taken 100mg in duplicate and placed inside a centrifuge tube whose weight was previously recorded, then dissolved in 10ml of 1% acetic acid for 30 minutes in a shaker at 240 rpm at 25^0C.The centrifuge tube with the mixture is heated for 10 minutes in a water bath and cooled down. It is then centrifuged for 10 minutes with 5000 rpm at 25^0C. The supernatant is decanted leaving the pellet behind. The pellet is washed with 25ml of distilled water and this water is dried at 60^0C for 24 hours. Solubility of chitosan was determined according to the equation:

$$\text{Solubility (\%)} = \frac{[\text{initial weigh of tube + chitosan}] - [\text{final weight of tube + chitosan}]}{[\text{Initial weigh of tube + chitosan}] - [\text{weight of initial test tube}]} \times 100$$

3.3. Water binding capacity, (WBC)

The chitosan sample extracted from L rohita was taken as A1(lab made chitosan) and A2(commercial chitosan) and was measured using a method of Wang and Kinsella (1976). 0.5g M of each chitosan sample is taken in two centrifuge tubes, within it 10ml of water was added. After that, it is kept in an ambient temperature with intermittent shaking of 5 seconds in every 10 minutes and centrifuge at 3500 rpm for 25 minutes. After discarding the supernatant, the tube was weighed.

WBC (%) = [water bound in gram / initial sample weight in gram] x 100

3.4. Fat binding capacity, (FBC)

The chitosan samples were taken as A1 (lab made chitosan) and A2 (commercial chitosan) and was measured using a method of Wang and Kinsella (1976). 0.5gM of each chitosan sample is taken in two centrifuge tubes within it add 10ml of soyabean oil. Now it is kept in an ambient temperature with intermittent shaking of 5 seconds in every 10 minutes and centrifuge at 3500 rp for 25 minutes. After discarding the supernatant, the tube was weighed.

FBC (%) = [Fat bound in gram/initial sample weight in gram] x 100

3.5. Ash content

According to the AOAC (1990) method, the Ash content was assessed using a muffle furnace at the Central Institute for Women Agriculture. In a pre-weighed crucible with a lid, 2gm of each sample was taken. The sample has been burned. The sample and crucible were each weighed separately. After that, it spends 6 hours at 600 'C inside a muffle furnace that has been preheated. The crucibles are removed from the furnace and placed in a desiccator to cool afterward. The ash content is then determined by weighing the cooled ash.

ASH (%) = [Weight of Ash/Weight of sample] x 100

3.6. Percentage of Yield

By weighing, measuring, and keeping track of the sample's physical, chemical, and microbiological activity, yield can be estimated. To determine the yield percentage, the sample's size and quality are closely scrutinised.

Yield% = (Dry weight after extraction) / (weight of raw material) X 100

4. Biological Activity

4.1. Anti- bacterial Activity:

By using the agar disc diffusion method, the antibacterial activity of each chitosan sample was evaluated against four bacterial species.The Gram negative strains tested included E. coli, pseudomonas, Aeromonas and the Gram positivestrains used were Staphylococcus. The nutrient broth (NB) media was selected as growth medium for revival of all microbial cultures.The pH of medium was adjusted to 6.5−7.0pH and it was sterilized by autoclaving it for 20 min at 121 °C temperature and 15 psi pressure. Bacterial cultures 1% (v/v) were inoculated aseptically under laminar air flow cabinet in nutrient broth (NB) media (Walke et al). The culture suspension was seeded in nutrient agar plates by the pour plate technique. Four sterile filter paper discs (5 mmdiameter) were made at equal distance out of which three were filled with the laboratory prepared chitosan solution of concentrations 10 µl, 20µl and 30 µl respectively and one with the commercial chitosan solution of conc 10 µl and then incubated at room temperature for 24 hrs. The formation of a clear zone (restricted microbial growth) around the cavity is an indication of antimicrobial activity. (Vanitha et al).

4.2. Antioxidant Activity:

Since that the radical molecule is stable and need not be generated, DPPH is regarded as a legitimate accurate, simple, and cost-effective approach to assess the radical scavenging activity of antioxidants. By using an in vitro DPPH antioxidant scavenging assay, it can also be referred to as a free radical scavenging assay. DPPH(1,1-diphenyl-2picrylhydrazyl) radical scavenging ability of chitosan was measured by DPPH. According to Shimadak et al., a sample of chitosan (1.5 mg/ml) was taken in 50% methanolic solution containing 1ml of 1mM 2-2diphenyl-1-picrylhydrazyl (DPPH) in 1% acetic acid. This allowed for the measurement of the chitosan's 1,1-diphenyl-2picrylhydrazyl (DPPH) radical scavenging ability. After mixing, the mixture was left in a dim room for 30 minutes. Using a spectrophotometer, the absorbance was measured at 517 nm while the blank was 3 ml of 1% acetic acid.

4.3. Anti-Inflammatory Activity:

It can also be referred as the free radical inflammation assay by the involvement of in vitro anti-inflammatory assay. Chitosan's in vitro anti-inflammatory activity was assessed using the protein denaturation method, which was modified slightly from Murugan and Parimelazhagan's (2014) version. The reaction mixture Bovine serum albumin (5% aqueous solution) in 0.45 ml and distilled water in 0.05 ml made up the reaction mixture (0.5 ml, pH 6.3), which had its pH set at 6.3 using a little amount of 0.1N HCl. After being heated at 72°C for 15 minutes after being heated at 37°C for 20 minutes, samples were added to the reaction mixture at various concentrations (50, 100, and 150 g/ml of extracts) of stock solution at 10 mg/ml. 2.5 ml of phosphate buffer solution was then added. Using a spectrophotometer, absorbance at 600 nm was measured, and IC50 values were calculated. 0.45ml of bovine serum albumin and 0.05ml of distilled water were used as the negative control.

$$\%\ of inhibition = \frac{Abs\ of\ control - Abs\ of\ sample}{Abs\ of\ control} \times 100$$

4.4. Anti-Fungal Activity:

The chitosan sample was tested for its anti-fungal effectiveness against Pythium debarynum and Fusarium oxysporum. Two separate test tubes with potato dextrose broth were used to cultivate the organism. Fresh fungal cultures at 37 degrees Celsius after 24 hours of incubation have seen. Two Petri plates with identical amounts of potato dextrose agar were prepared and equally in two petri plates in the laminar air flow, and allowed to solidify. The plates were then swabbed with organisms. 5 wells were made at equal distance and lab-made chitosan sample with a variable concentration [25 µl, 50µl, 75µl, and 100µl] and clotrimazole was added respectively to each well. At 37°C temp plated were incubated in an incubator for 72 hours. The percentage of inhibition can be used to analyse antifugal activity.

5. Results and Discussion

5.1. Solubility

Chitosan is a deacetyl semi-crystalline biopolymer due to its rigid crystalline structure is insoluble in the majority of solvents, including water, alkali, and aqueous solution (pH -7). (Kumari et al 2016). But it is soluble in sodium acetate and acetic acid. The present study showed that solubility values of fish (L. rohita) chitosan and commercial chitosan values were found to be 2.972 and 3.370, respectively.

Table 1: Solubility test table of prepared (Fish) and Commercial Chitosan

Sl no.	Samples Taken	Solubility %
1	Fish Chitosan	2.972
2	Commercial Chitosan	3.370

5.2. Fat binding capacity (FBC)

Fat binding capacity is calculated by using solubility soya bean oil and Fish (L.rohita) Chitosan and commercial chitosan (Rout 2001). The average fat binding capacity of chitosan was 155% in commercial chitosan and 111% in fish (L.rohita) .

Table 2: FBC table of Fish (L.rohita) prepared and Commercial Chitosan

Sl no.	Samples Taken	FBC (%)
1.	Fish Chitosan	111
2.	Commercial Chitosan	155

5.3. Water binding capacity (WBC)

Water binding capacity is stated with respect to the molecular weight DD and degree of crystalline of chitosan. The binding of -OH groups, -NH groups with the surface area of chitosan. The increased DD gives many -NH2 groups which bind with water and increase the penetration of water molecules while crystallinity decreases. (Rout 2001). The

BC% of commercial chitosan is found to be 163% and 106% in L.rohita.

Table 3: WBC test table of fish(L.rohita)prepared and Commercial Chitosan

Sl no.	Samples Taken	WBC (%)
1	Fish chitosan	106
2	Commercial Chitosan	163

5.4. Ash value 1

By using two crucibles, the ash content of chitosan is investigated. The two samples were Fish (L.rohita)Chitosan and Commercial Chitosan. The muffle furnace was preheated and samples were placed inside the furnace for 6 hours. Following a six-hour cooling period, the crucibles are removed from the furnace using long tongs.

Table 4: Ash Content test table of prepared (Fish) and Commercial Chitosan

Samples	Time	Weight after charring	Final weight	% Ash
Fish Chitosan	6 hours	41.919	41.548	0.371
Commercial Chitosan	6 hours	42.498	42.040	0.458

5.5. Moisture content

The moisture content refers the total moisture percentage while chitosan is derived by the extraction process. The moisture content is calculated by weighing moist chitosan and then drying in the hot air oven. Chitosan is weighed after an additional drying period. This process has been done in triplicate whose values are as follows

Table 5: Moisture content table of chitosan prepared from L.rohita scales and Commercial Chitosan

Sample	Initial weight of chitosan	Final weight of chitosan	Moisture content
Commercial Chitosan	2.070gm	1.379 gm	0.691
Fish Chitosan	2.013 gm	1.337 gm	0.676

5.6. Yield Percentage

To determine the yield percentage, the sample's quality and quantity are carefully observed.

Yield% = (Dry weight after extraction) / (weight of raw material) X 100

= (40gm/100) X 100 =40%

6. Biological and TherapeuticActivity:

6.1. Anti- bacterial activity

Aeromonas hydrophila, Pseudomonas fluorescens, Escherichia coli, and Staphylococcus aureus were used as test organisms to determine the anti-bacterial activity of the chitosan sample. The bacterium was cultured in three separate test tubes containing nutritional broth. Fresh bacterial cultures seen after incubating the samples at 37°C for 24 hours. Nutrient agar was prepared, and poured equally in three petri plates in the laminar air flow, and left for solidification. After that plates were swabbed with organisms. The discs were placed on the plates. 10µl, 20µl and 30µl on three different discs and an ampicillindisc was placed in the middle of plate. The plates were incubated in an incubator at 37'Cfor 24 hours. Antibacterial activity can be studied by calculating the percentage of inhibition.

Antibacterial activity was determined by calculating the percentage of inhibition of lab made chitosan against ***Staphylococcus aureus, Pseudomonas fluorescens, Escherichia coli and Aeromonas hydrophila***

Table: 6

Sl. No	**Concentration Of chitosan sample**		**Gram positive**	**Gram negative**		
			Staphylococcus aureus	***Pseudomonas fluorescens***	***Aeromonas hydrophila***	***Escherichia coli***
1	**Fish (L.rohita) chitosan**	**10 µl**	20mm	22 mm	23 mm	19 mm
		20 µl	23mm	24 mm	18 mm	20 mm
		30µl	23.5mm	26 mm	22 mm	21 mm
2	**Commercial chitosan**	**10 µl**	23mm	23.5 mm	24 mm	21.5 mm

6.2. DPPH Antioxidant Scavenging Assay:

Anti-oxidantactivity or DPPH assay was calculated by taking DPPH (1,1-diphenyl-2 picrylhydrazyl)radical activity. The chitosan sample was prepared at 1.5 mg/ml in a 50% methanolic solution that contained 1ml of 2,2-diphenyl-1-picrylhydrazyl (DPPH) in 1% acetic acid solution.

DPPH radical scavenging assay

Murugan and Parimelazhagan (2014) provided a description of the stable radical DPPH that was utilised to assess the activity of free radical scavengers. Chitosan oxidants lower the level of the free radical DPPH. The DPPH radical loses its purple colour when an antioxidant donates an electron or a hydrogen atom. Samples of stock 10 mg/ml at various

concentrations (50, 100, and 150 g/ml of extracts) were taken, and the volume was then adjusted with methanol to 1 ml. 2 ml of 0.1 mM methanolic solution of DPPH was added to chitosan samples and standard BHT. 1ml of methanol in 1.5ml of DPPH solution was used as a negative control. The entire reaction mixture was incubated for 30 min at room temperature in the dark, and then the absorbance at 517 nm was measured using a spectrophotometer against the blank to calculate the IC_{50} values.

$$\%\ of inhibition = \frac{Abs\ of control - Abs\ of sample}{Abs\ of control} \times 100$$

Table: 7 DPPH scavenging activity of Fish Chitosan

Volume (in ml)	% of DPPH Scavenging
0.5	-
1.0	-
1.5	21.42

6.3. Anti-Infllamatory Assay:

Protein denaturation method: The protein denaturation method was used to investigate the anti-inflammatory properties. The extracts showed highest inflammation activityat 200 µg/ml concentration.

Table 8: Protein denaturation assay of Chitosan

Volume (in ml)	% of Inhibition
0.5	-
1.0	-
1.5	93.64%

6.4. Antifungal Assay:

The chitosan sample was tested for its anti-fungal effectiveness against Pythium debarynum and Fusarium oxysporum. Two separate test tubes with potato dextrose broth were used to cultivate the bacterium. We acquire fresh fungal cultures after 24 hours at 37 degrees Celsius of incubation. Two Petri plates with identical amounts of potato dextrose agar were prepared, poured into the laminar air flow, and allowed to solidify. After that, organisms were swabbed onto the plates. Each plate was given one disc, which had a 50 l L. rohita sample poured upon it. During 72 hours, the plates were incubated at 37°C in an incubator. The percentage of inhibition can be used to study antifungal activity.

Table: 9 Antifungal tables of Fish(L.rohita) prepared Chitosan on ***Fusarium oxysporum*** **and** ***Pythium dibarium***

Concentration Of chitosan sample		**Name of the Organism**	
		Fusarium oxysporum	***Pythium debarynum***
	50µl	2 mm	5 mm

This study showed that fish (L. rohita) scale waste may be successfully used for chitosan extraction. Chitosan has been shown in an in vitro investigation to have antibacterial activity against a variety of gut pathogens, which may improve gut health. As a result, chitooligomers as a feed additive could take the role of antibiotics in animal feed, assisting in the problem of antibiotic residue in animal products.

7. Conclusion

Chitin derived from fish scales (L. rohita) can be converted into the more beneficial compound chitosan, which can be employed in a range of applications. According to this study, L. rohita chitosan exhibits good antibacterial action. Moreover, it has a wide range of antioxidant properties, including scavenging activities for superoxide anion radicals, hydroxylradicals, reducing power and ferrous-ion chelating activity. Fish (L. rohita) chitosan may be employed as a source of natural antioxidants, as a potential food supplement, or as an ingredient in the pharmaceutical sectors based on the findings.

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