Uttar Pradesh Journal of Zoology

Volume 45, Issue 19, Page 434-445, 2024; Article no.UPJOZ.4217
ISSN: 0256-971X (P)

Production of Biopolymer & Preparation of Soap Using Chitin and Chitosan from *Brachyura* Shell

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

Article Information

DOI: https://doi.org/10.56557/upjoz/2024/v45i194549

Open Peer Review History:
This journal follows the Advanced Open Peer Review policy. Identity of the Reviewers, Editor(s) and additional Reviewers, peer review comments, different versions of the manuscript, comments of the editors, etc are available here: https://prh.mbimph.com/review-history/4217

Original Research Article

Received: 05/08/2024
Accepted: 09/10/2024
Published: 16/10/2024

ABSTRACT

Collagen, an extracellular protein, was extracted from eggshells using a combination of 5% EDTA, 0.45 M NaCl, 0.2% NaOH, 0.2% H_2SO_4, 0.7% citric acid, and 10% HCl, while chitosan, a naturally occurring polysaccharide, was extracted from crab shells (*Brachyura*) through demineralization with 1N Hydrochloric acid, deproteinization using 6% sodium hydroxide solution, and deacetylation with 40% NaOH, followed by decolorization using potassium permanganate and oxalic acid. The proximate analysis of chitosan revealed an ash content of 32% and a moisture content of 4.13%, while the protein content of the extracted collagen was found to be 70 µg/dL, with an alternative

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Cite as: *Indumathi Parameswaran, Rakul Kartikeyen J.R., and Nazareth Arockiamary. 2024. "Production of Biopolymer & Preparation of Soap Using Chitin and Chitosan from Brachyura Shell". UTTAR PRADESH JOURNAL OF ZOOLOGY 45 (19):434-45. https://doi.org/10.56557/upjoz/2024/v45i194549.*

method indicating 75 µg, equivalent to BSA. The molecular weights of collagen and chitosan were determined to be 300 kilodaltons and 190-310 kilodaltons, respectively, and their purity was confirmed using FTIR analysis. Hydrogels were prepared using a combination of collagen and chitosan and were tested for water absorption capacity and antimicrobial activity, demonstrating that both biomaterials possess significant antimicrobial and antioxidant properties. Additionally, chitosan's biocompatibility supports its use in creating edible films that can interact with other biopolymers and additives, indicating its potential for various industrial and biomedical applications.

Keywords: Collagen; chitosan; hydrogel; FTIR analysis; band–aid.

1. INTRODUCTION

Collagen is a naturally occurring protein that constitutes approximately 30% of the total protein in the human body. It is also derived from various animal sources such as bovine, porcine, and marine organisms, including fish, starfish, jellyfish, sponges, sea urchins, octopuses, squids, cuttlefish, sea anemones, and prawns [1]. Collagen is composed of amino acids, primarily proline, glycine, and hydroxyproline. To date, 28 different types of collagens have been identified, and they are generally classified into two major groups: fibrillar and non-fibrillar collagens. In humans, the main types of collagen include: Type I (present in skin, bone, teeth, tendons, ligaments, vascular ligature, and osteoid), Type II (found in eyes and cartilage), Type III (in reticular fibers, skin, muscle, and blood vessels), Type IV (in the epithelium-secreted layer of the basement membrane and the basal lamina), and Type V (in hair, cell surfaces, and placenta) [2]. Collagen is widely used as a humectant in moisturizing creams. Due to its high molecular weight, it cannot penetrate deep into the skin but can still promote skin hydration by retaining moisture on the surface [3].

Chitosan, a key component of the exoskeletons of many crustaceans such as crabs, shrimp, prawns, and lobsters, is also found in the cell walls of several fungi, including *Mucor* and *Aspergillus* species [4]. Chitosan is known for its affordability, biodegradability, and safety for consumption. It is widely utilized as a food additive, a moisturizing agent in cosmetics, a pharmaceutical ingredient, and an antibacterial agent. Chitosan has a weakly basic nature, is insoluble in both water and organic solvents, but it dissolves in weakly acidic aqueous media (pH ≤ 6.5). In an alkaline solution or in the presence of polyanions, chitosan precipitates, and at low pH, it forms a gel-like structure [5]. Despite its broad applications, the extraction of chitin and chitosan is relatively low compared to other sources due to limited availability. Insects, for instance, contain 30-45% protein, 25-40% fat, and 10-15% chitin [6]. Moreover, astaxanthin, a carotenoid extracted from crab shells, has potential as an eco-friendly alternative and is being explored as a supplement for fishmeal in aquaculture, although it has not yet been commercially produced on a large scale [7].

The present study focused on the extraction of chitosan from crab shells and collagen from eggshells. These two biomaterials were subsequently combined to form hydrogels, which were then tested for water absorption capacity and antibacterial activity. Both chitosan and collagen are recognized for their exceptional wound-healing properties, making them valuable in various industrial applications.

2. MATERIALS AND METHODS

2.1 Extraction of Collagen from Egg Shell

Collagen from Egg shells were extracted by several methods like chemical method, biological method, and physical methods. The chemical methods using modified procedure of [8] one from basic egg shell extraction method (Acetic acid method) and another from [9] fish scale collagen extraction method (Hydrochloric acid method). Egg shell was collected from local market at Annanagar. Washed thoroughly several times with distilled water and soaked with 5% EDTA solution for 24 hours. From egg shell, egg shell membrane was easily separated after 24 hours of an EDTA Treatment.

2.2 Pre-treatment

To remove non collagenous components such as lipid, calcium, and other proteins sources the egg shell membrane was blended and treated with 1:6(w/v) cold water for 4 hours and filtered. Then the filtered component was soaked with 0.45 M NaCl solution and kept in magnetic stirrer for 5 minutes, and then filtered and equal volume of distilled water was added and subjected for centrifugation at 10,000 rpm for 20 minutes. Then supernatant was discarded and remaining

precipitates were added with equal volume of 0.2%NaOH, 0.2% H_2SO_4 and 0.7% citric acid respectively and each kept in magnetic stirrer for 5 minutes. Finally discard the floating particles and washed with distilled water and pH was adjusted to 7. After adjusting discard distilled water and precipitates were soaked with 10% HCl for 24 hours. After 24 hours filter the solution and add equal volume of 1% alkaline hydrogen peroxide in 0.01 N NaOH solution and incubated for 24 hours. After 24 hours solutions were filtered and neutralized with distilled water.

2.3 Extraction of Collagen

After pre-treatment process egg shell membrane were treated with 1 M acetic acid solution and kept in cooling water bath for 2 hrs at 4°C. Mixtures were then removed by centrifugation process at 6000 rpm for 5 minutes. Then discard supernatant and precipitates were kept at cooling water bath for 24 hours at 4°C. The finally centrifuged at 10000 rpm for 20 minutes. Discard supernatant and collagen were obtained.

2.4 Extraction of Chitosan

Demineralization: The crab shells were grinded and soaked with 1N Hydrochloric acid solution for 24 hours at room temperature to remove the calcium salts with a solid/solvent ratio of 1:15(W/V) and filtered with the help of Whatman filter paper. Later they were washed with deionized water until it was neutralized and the samples were dried for about 2 hours at 60°C and weighed.

Deproteinization: Demineralised sample were treated with 6% sodium hydroxide solution in the ratio of 1:10 and kept in incubation for 48 hours. The contents were filtered with help of Whatman No.1 filter paper and washed thoroughly with deionized water to remove sodium hydroxide.

Decolourization: Chitin residues was further treated with potassium permanganate solution in the ratio of 1:10 for 1 hour and after the treatment chitin residues were further washed with oxalic acid at 1:15ratio for 3 hours until decolourization. The decolourized chitin sample was washed thoroughly with distilled water for about more than 7 to 10 times. The samples were dried and further deacetylated.

Deacetylation: The extracted crab chitin was treated with 40% NaOH in the ratio of 1:15 and kept in hot air oven at 105°C for 4 hours. Then the sample was washed thoroughly using distilled water. Finally, the sample was dried and weighed.

Confirmatory Test for Chitosan: 100 mg of chitosan powder was partially dissolved in distilled water in a test tube. Distilled water was placed in half of another test tube. Then mix the partially dissolved chitosan solution in the distilled water test tube. Then add 2-3 drops of Iodine/Potassium iodide. The mixture was then acidified with 3-6 drops of sulfuric acid. The solution was mixed using a stirring rod. After the addition of Iodine/Potassium iodide, the precipitate turned dark brown colour. This indicates presence of chitosan.

Estimation of protein: To 1 g of collagen sample, 5 ml of 0.1 M phosphate buffer was added and centrifuged at 5000 rpm for 20 minutes. Supernatant was collected and the pellet was discarded. To the supernatant added 1 ml of 10% TCA and incubated at room temperature for 30 minutes. The sample was centrifuged at 5000 rpm for 10 minutes, supernatant was discarded, the pellet was dissolved in 5 ml of 0.1N sodium hydroxide and the amount of protein present in collagen sample were analysed using Lowry's method. The molecular weight of the collagen and chitosan was determined to be 300 kilo dalton and 190 to 310 kilo dalton respectively.

Estimation of glycine: To confirm the extracted collagen and chitosan from egg shell and crab shell respectively, Glycine by Sorenson formal titrations was performed.

2.5 Proximate Analysis

Ash test: An empty clean and dry crucible was weighed and known amount of 1g of chitosan and collagen sample was taken in the crucible. The crucible was weighed and placed in preheated oven for 400°C for 4 hours. It was kept in muffle furnace till the substance completely turned to ash. Then it was cooled and weighed. The value was then noted. The ash content of the sample was calculated using the following formula,

$$\% \text{ of Ash content} = \frac{(wt.of\ crucible\ with\ ash\ content) - (wt\ of\ empty\ crucible)}{Sample\ weight} \times 100$$

Moisture test: An empty clean and dry crucible was weighed and a known amount of 1g of finely powdered Chitosan and Collagen was taken in it. The crucible was placed in over 100°C for 3 hours. It was then cooled and weighed. This procedure was repeated for 2 to 3 times till the constant weight was obtained. The moisture content of sample was calculated using the formula.

2.6 FTIR Analysis

FTIR analysis was carried out for both collagen and chitosan the wavelength ranges from 600-3400 was measured. The frequency ranges from 600-800 is C-H Alkenes, 1500-1700 is C=C Alkenes and 3200-3600 is O-H Phenol.

Degree of deacetylation using FTIR: To calculate the deacetylation degree of chitosan absorption band from 1320-1420 cm^{-1} were taken. The first band is characteristics of the acetylated amine or amide function, where the second band was chosen as the reference band. Using the following equation the degree of deacetylation was determined.

DD%=100-(A1320/A1420-0.3822) X 1 / 0.03133

Whereas,
DD: degree of deacetylation.
A: absorption point

Hydrogel preparation: Alginate-based hydrogels were prepared using sodium alginate, glycerol, calcium chloride, agarose solution, chitosan and collagen were casted in a mold. Hydrogel was later used for preparing band aid.

Water Absorption test: Hydrogel were cut into two pieces and initial weight is noted. Later the gels were immersed in distilled water. The water absorption capacity was noted at regular interval of time such as after 1 to 5 hours and finally after 24 hours.

Antimicrobial property by agar well diffusion method: The plates were prepared by pouring 20 ml of media in a sterile Petri plate. The plates were allowed to solidify for 5 minutes and 100 µL of inoculum suspension was spread uniformly with L-rod and the inoculum was allowed to dry for 5 mins. And then the wells were prepared in the seeded agar plates with the help of a cork borer (8.5 mm). The chitosan - collagen based hydrogel were introduced into the well and the saturated sterile discs (5 mm in diameter) were impregnated in the solidified media and allowed to diffuse at room temperature for 2 hours. The plates were then incubated in the upright position at 37 °C for 18 hrs for bacteria. At the end of the incubation, inhibition zones formed around the well were measured with transparent ruler in millimetre. The experiments were performed in triplicate. Sterilized paper discs are used as a negative control. The ampilicin (10 µg disc) were used as positive controls for fungi and bacteria.

3. RESULTS

3.1 Extraction of Collagen

Method I: Samples treated with EDTA, lipids pigments were removed, and latter treated with 1 M acetic acid solution. The samples were centrifuged and collagens were obtained.

Method II: Samples were treated with EDTA, 30% Hydrochloric acid solution. Later acid solution was discarded and membranes were washed with distilled water and pH was adjusted to 7. Then centrifuged at 12000 rpm for 15 minutes and supernatant was discarded then add 2 N HCl solution and stored at refrigerator for 24 hours, and discard floating material. Obtained collagen was stored in the refrigerator for further usage (Fig. 1).

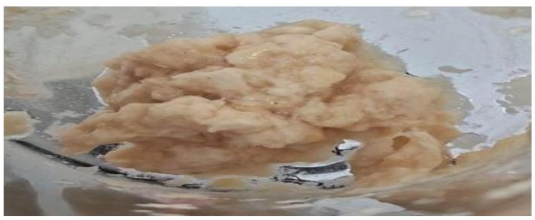
3.2 Sample Collection and Extraction of Chitosan

The Crab shell procured from local fish market of Ambattur, Chennai and washed thoroughly in tap water and then with distilled water. Then shells were dried and powdered finely, latter the samples were subjected to various processes such as demineralization, deproteinization and decolourisation and the extracted chitin was further deacetylated, resulting in the production of chitosan. The qualitative test by treating the sample with Iodine/Potassium iodide, the precipitate was turned into dark brown colour shows the presence of chitosan (Fig. 2).

3.3 Confirmatory Test for Chitosan

100 mg of chitosan powder was partially dissolved in distilled water in a test tube. Distilled water was placed in half of another test tube. Then mix the partially dissolved chitosan solution in the distilled water test tube. Then add 2-3 drops of Iodine/Potassium iodide. The mixture was then acidified with 3-6 drops of sulphuric acid. The solution was mixed using a stirring rod. After the addition of Iodine/Potassium iodide, the precipitate was turned into dark brown colour.

Egg shell



Collagen

Fig. 1. Extraction of Collagen from Egg shell

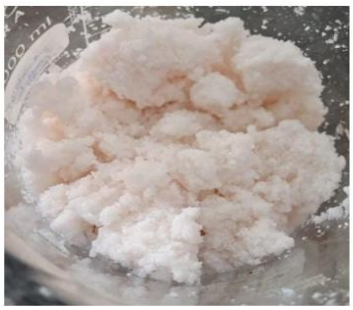
Crab Shell

Powdered Crab Shell

Demineralization

Deproteinization - Powdered Crab Shell

Decolourization- treated with $Kmno_4$



Decolourization- treated with Oxalic acid

Deacetylation

Extracted chitosan

Fig. 2. Collection of crab and extraction of Chitosan

3.4 Proximate Analysis

The moisture and ash content of Chitosan and collagen was kept in muffle furnace until the substance was completely turned to ash, cooled, and weighed (Table 1).

Table 1. Proximate analysis of Chitosan and Collagen extracted from Crab shell and egg shell

S.no	Substance	Ash	Moisture
1.	Collagen	35%	3.67%
2.	Chitosan	32%	4.13%

Estimation of glycine: The total amount of glycine present in the chitosan extracted from the crab shell and collagen extracted from the egg shell was found to be 2.33 mg/dl and 2.39 mg/dl respectively (Fig. 3).

Estimation of Protein: The Amount of protein was analysed using Lowry's method using standard Bovine serum albumin. The total amount of protein present in collagen extracted from method I is 70 µg/ dL and method II 75 µg equivalent to BSA.

SDS-PAGE: SDS-PAGE was performed to confirm the identity and Purity of the extracted collagen and chitosan along with the standard protein marker ranges between 29-207 KD. Collagen revealed the pattern ranges from 115 kDa to 130 kDa.

3.5 FTIR Analysis

FTIR analysis was carried out for both collagen and chitosan the wavelength ranges from 600-3400 was measured. The frequency ranges from 600-800 is C-H Alkenes, 1500-1700 is C=C Alkenes and 3200-3600 is O-H Phenol (Figs. 4, 5, 6 and Table 2).

Degree of deacetylation % of chitosan using FTIR: The degree of deacetylation for chitosan was determined using the standard formula and the results revealed 81.1% degree of deacetylation (Fig. 7).

Hydrogel preparation: Alginate-based hydrogels were prepared using sodium alginate, glycerol, calcium chloride, agarose solution, chitosan and collagen were casted in a mold. Hydrogel was later used for preparing band aid (Figs. 8, 9).

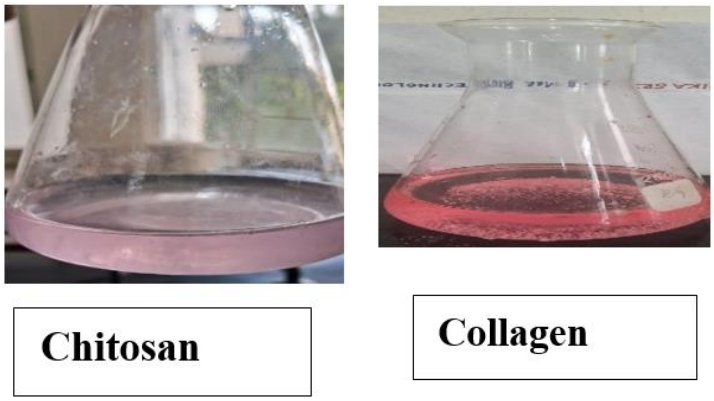


Fig. 3. Estimation of glycine from Chitosan and Collagen

Table 2. FTIR analysis of Chitosan, Collagen Method I and Method II

Bond	Type	Frequency range cm-1	Nature
O-H	Hydrogen bonded Alcohols, Phenol	3200-3600	Strong
C=C	Alkenes	1610-1680	Strong
C-H	Alkanes	1340-1470	Strong
C-O	Alcohols, ethers, carboxylic acids, ester	1050-1300	Strong
C-H	Alkenes	675-995	Stong
-C≡C-H	Alkynes	2100-2270	Weak
C-H	Alkanes	2850-3000	Stetching
O-H	Alcohol	3584-3700	Weak
C-O-C	Alkenes	871.82 – 671.233	Stretching

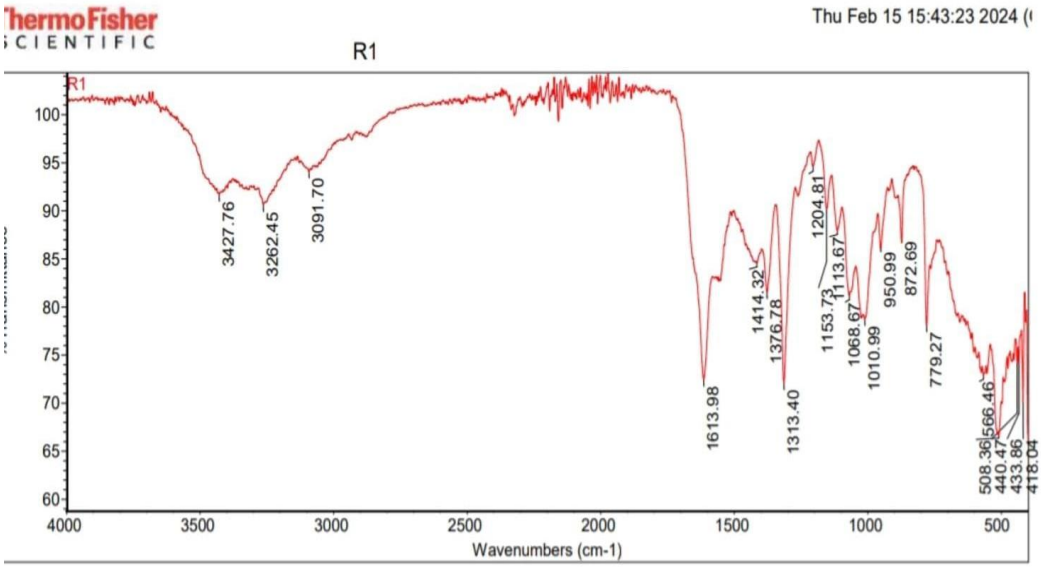


Fig. 4. FTIR analysis of Chitosan

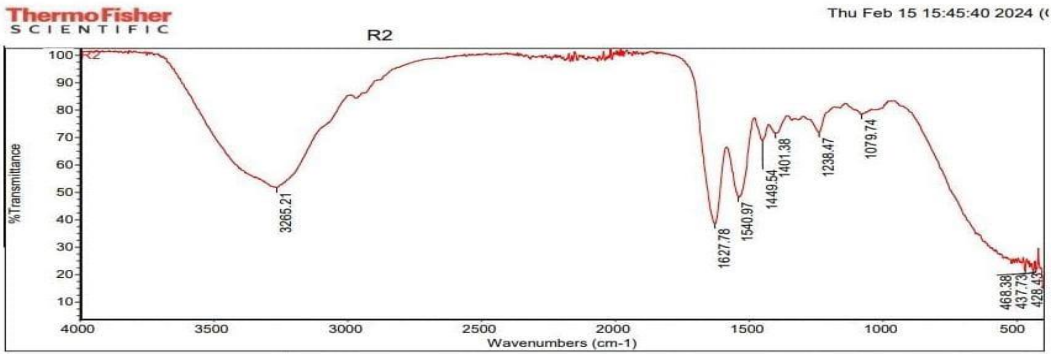


Fig. 5. FTIR analysis of Collagen Method I

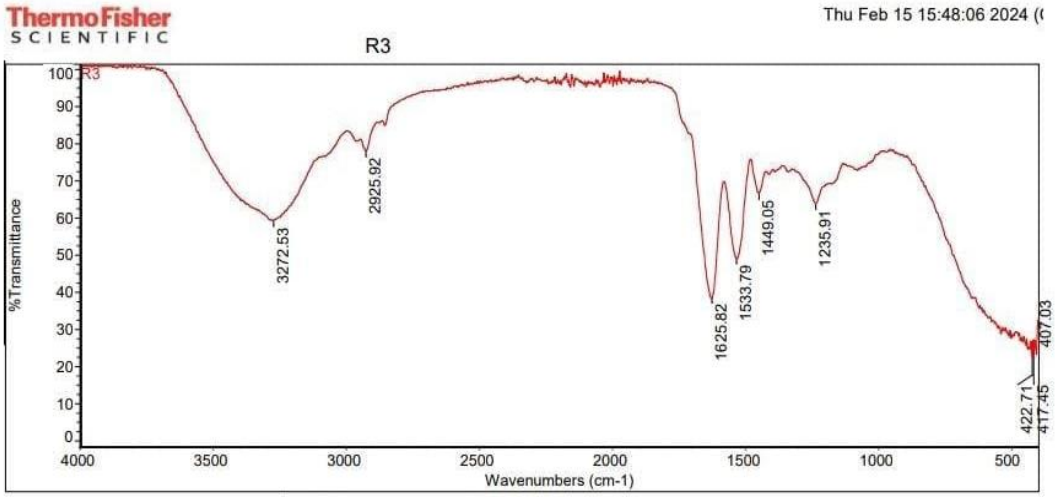


Fig. 6. FTIR analysis of Collagen Method II

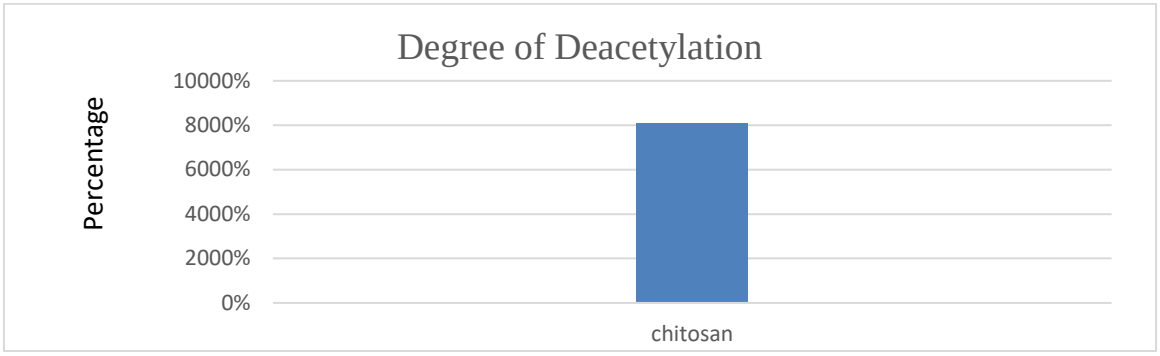


Fig. 7. Degree of Deacteylation

Fig. 8. Preparation of Hydrogel using chitosan extracted from crab shell and collagen from egg shell

Fig. 9. Preparation of band aid using Chitosan and Collagen- Hydrogel

Table 3. Water absorption capacity of hydrogel

s.no	Initial weight	After 1st hour	After 2nd hour	After3rd hour	After 4th hour	After 5th hour	After 24 hours
1	0.935	0.975	0.980	0.999	1.000	1.235	1.250
2	1.498	1.580	1.700	1.900	2.023	2.050	2.100

Water absorption Test: The biofilm was tested for water absorption that the film weight was increased when the time increase as 1hours, 2hours and 24hours. The film weight was observed and noted (Fig. 10, Table 3).

Antimicrobial property: The antibacterial effects of extracted chitosan - collagen based hydrogel have investigated against *staphylococcus aureus, Escherichia coli, Klebsiella pneumoniae* and *pseudomonas aeruginosa*. The test was performed with hydrogel as disc. It was observed that the clear zone of inhibition was against in S*taphylococcus aureus, Escherichia coli, klebsiella pneumoniae* and *pseudomonas aeruginosa* confirms the antibacterial property of extracted collagen chitosan-based hydrogel (Fig. 11, Table 4).

Fig. 10. Water absorption capacity of hydrogel using Chitosan and Collagen

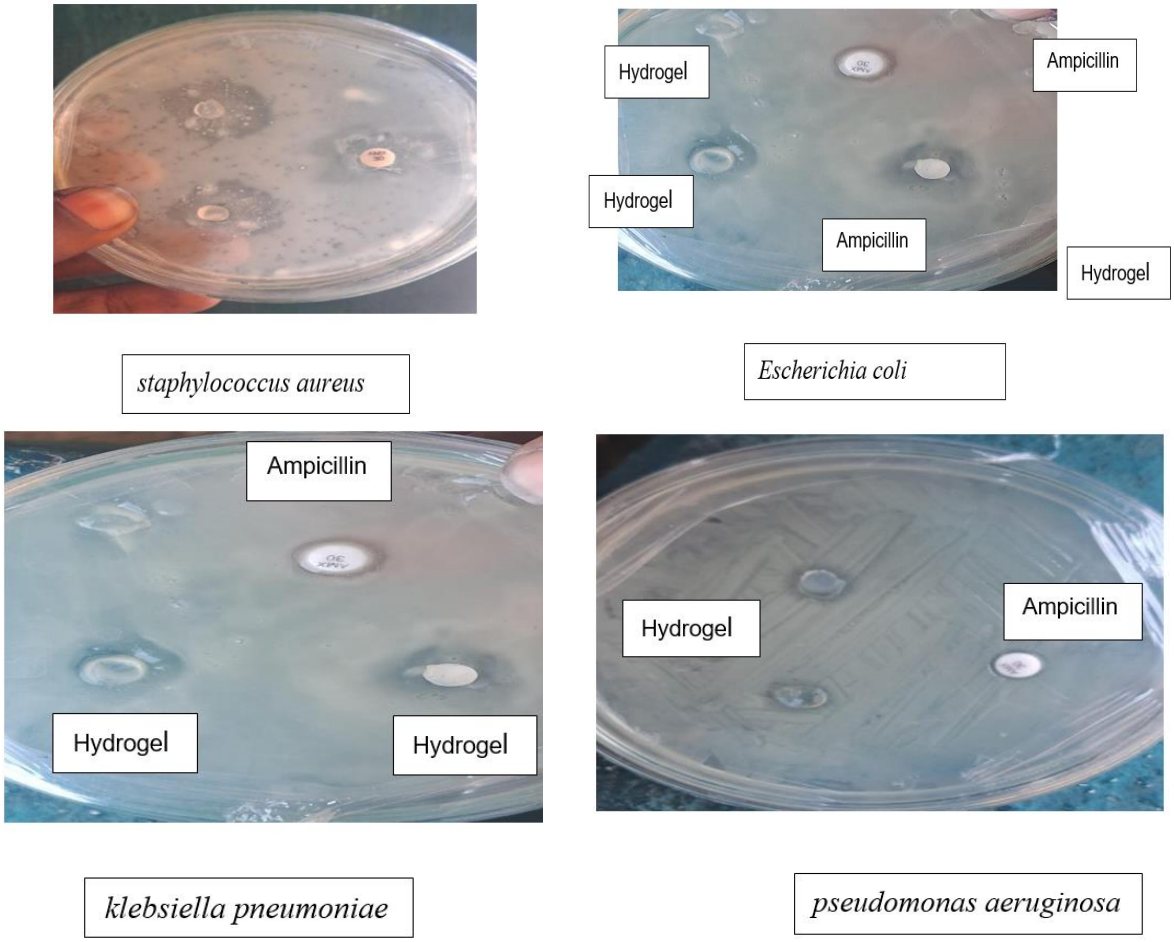


Fig. 11. Antimicrobial activity of hydrogel using Chitosan and Collagen

Table 4. Antibacterial effects of Chitosan - Collagen based hydrogel

S.no	Name of microorganism	Zone of inhibition (mm in radius)	
		Hydrogel	Antibiotic (Ampilcillin Disc)
1	*Staphylococcus aureus*	1.5	1.5
2	*Escherichia coli*	0.9	0.3
3	*klebsiella pneumoniae*	0.2	0.2
4	*pseudomonas aeruginosa*.	0.2	0.2

Whereas, Mn = 2; N = 2

4. DISCUSSION

Chitosan extracted from waste fungal biomass from Biotech industries, fungi that contain high concentrations of these polymers, or the fermentation of a fungus to produce chitin and chitosan [10]. The proximate analysis of *Actinidia deliciosa* has higher fat and protein content than *Persea americana* fruit, whereas fiber content of both the fruits are equivalent [11]. Chitosan extracted by deproteinization, demineralization and deacetylation processes should have a moisture content of less than 10%, because the higher water content in the chitosan structure, the faster damage of the polymer through hydrolysis reactions. The moisture content of chitosan obtained was 2.94%, which indicates that the moisture content was low; hence it is of good quality and suitable for scientific and industrial applications. Due to the hygroscopic nature of chitosan, it may yet absorb more moisture during storage. Ash content is slightly higher than 5% of ash content recommended for high-quality chitosan [12]. The degree of deacetylation of D-Glucosamine units was 12 hours and 42 hours (92%) hours were found to be 82 and 92%, respectively. The amount of free amino groups ($–NH_2$) in chitosan is an important functional group that acts as active sites in the reactions and bonding for removal of pollutants from wastewater [13]. Eggshell membrane extracted using organic acid contains 88.2% protein, 10.5% ash, 1.04% carbohydrate and 0.35% fat. The microspheres were prepared by the suspension cross-linking method using glutaraldehyde and loaded with hydroquinone of size range of 20–100 mm. It was found that release rate of hydroquinone was mainly controlled by the polymer cross-linking density and, thus, by the degree of swelling of the hydrogel matrix's microencapsulation [14]. Collagen chitosan-based hydrogels showed strong bacterial activity against Methycilin resistant *Staphylococcus aureus* (MRSA) with the minimum inhibitory concentration (MIC) 0.053 U/mL. Clear-cut inhibitory zones of the CCHL and lysostaphin solution were consistent and indicate the diffusion of lysostaphin from CCHL leading to inhibition of *S.aureus* and it is depends on the duration of contact time and doses up to 10 U/mL [15]. Bovine-derived collagen membrane for hemostasis and wound healing therapy increases the scores of hemostasis, granulation tissue development and epithelization with lower pain scores in a variety of intra-oral lesions [16]. **The collagen type I is the main component of bone matrix that play a role in the construction of ideal synthetic bone substitute materials.** Demineralised bone mixed with collagen matrix for bone transplant and replacement therapies demonstrates successful osseointegration and the appearance of new bone with vascular connective tissue surrounding periodontal osseous defect [17].

When using bio-resorbable type I bovine collagen for hemostasis and wound healing therapies, gingival recession defects are significantly reduced and clinical assessment values (such as gingival tissue thickness, root coverage percentage, probing depth, and recession depth) are higher [18]. Collagens are used as a bone grafting material for bone transplant and replacement therapies stimulates the growth of new bone to rebuild the missing alveolar ridge around the dental implant [19]. Porcine-derived collagen used for bone regeneration therapies produces restoration of the bone defect in both the horizontal and vertical dimensions with no negative impacts for dental implants [20]. Collagen membranes are used in periodontal, implant treatments, as scaffolds and matrices for cell regeneration therapies, wound dressing products for healing and regeneration, and bone grafting materials for bone substitution and GBR**.** Due to its high biocompatibility, type I collagen is regarded as the benchmark in this area. It serves as the fundamental matrix in systems for cell culture. Collagen-based biomaterials, such as injectable matrices and scaffolds for bone regeneration, are frequently utilized in tissue engineering. These biomaterials are mostly made of collagen that forms fibrils, such as type I, II, III, V, and XI [21]. Collagen plugs/sponges are used as hemostatic materials Resorbable collagens are applied for industrial purpose. Type I collagen highly utilized to stop oral bleeding, speed up endogenous wound healing, and protect against infection [22]. Collagen-based products come in a variety of types and forms, such as sponge-like structures, plug-like structures, microfibrillar forms, matrices, scaffolds in oral wound dressing, accelerate wound healing, and close for graft and extraction site, and drug delivery carriers. Red blood cells play a supportive role in fibrin clot formation. Chitosan forms a coagulum with RBC. Polymorphonuclear neutrophils (PMN) clean wound site of foreign particles and cell debris. Chitin and chitosan attract PMNs to wound site. Macrophages consume dead cells, attract fibroblasts, support skin and blood vessel replacement and synthesis of the extracellular matrix. Chitin and chitosan attract macrophages.

Chitosan stimulates cytokine production (Transforming growth factor-β1, Platelet-derived growth factor, Interleukin-1). Fibroblasts helps in renewal of the dermis and synthesis of extracellular matrix. Chitosan indirect effect through macrophage cytokines and stimulates IL-8 production. Keratinocytes helps in renewal of epidermis. Chitosan indirect effect through macrophage cytokines [23]. Due to its ability to speed up the healing process of wounds, fungal chitosan can also be employed as a membrane in numerous medical applications, including wound dressings. A distinct biological pathway connected to the overall happening of growth and tissue regeneration is wound healing. As it advances, a range of cellular and matrix elements work in concert to replace lost tissue and rebuild the integrity of injured tissue through several interconnected and matching stages. Haemostasis, inflammation, proliferation, and remodelling are the four stages of the complicated and dynamic regenerative process known as wound healing [24].

5. CONCLUSION

Chitosan extracted from fungi are used in the food sector and human health care. Insects are the most prevalent eukaryotes on earth and are another promising source of chitin. Chitosan can create edible films due to its polycationic character, which enables it to collaborate with other biopolymers and additives. Bio-packaging composed of biodegradable materials is a popular trend in the field of food product preservation, it still possess a significant issue for food technicians because the materials must adhere to strict specifications to safeguard food goods. Hence the chitosan and collagen can be used as biodegradable materials and further work needed for its consistency.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc.) and text-to-image generators have been used during the writing or editing of this manuscript.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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