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Evaluating the Efficacy of Chitosan Extracted from Crab Shells for the Preservation and Shelf-life Extension of Strawberries

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Authors' contributions

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

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ABSTRACT

A natural biopolymer derived from crab shells, was extracted and characterized by Fourier transform infrared spectroscopy (FTIR). The physicochemical properties and antimicrobial activity of chitosan (CH) in combination with cellulose derivatives like Carboxy Methyl Cellulose (CMC) and hydroxypropyl methylcellulose (HPMC) against various test organisms were evaluated in this study.

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The results showed that the combined treatment with CMC and HPMC significantly inhibited fungal decay and enhanced fruit quality attributes such as firmness, colour, and nutritional content. Moreover, the combination of HPMC and CH exhibited the highest zone of inhibition for *Pseudomonas* and *Rhizopus* at concentrations 2 & 3%. The combined treatment of CH and CMC with HPMC exhibited significant antibacterial activity against all tested organisms. In addition, the titratable acidity levels in strawberry fruits significantly decreased during cold storage, with uncoated fruits exhibiting lower values compared to those coated with chitosan. In conclusion, the efficacy of the chitosan was confirmed in enhancing the postharvest preservation of strawberries, particularly in mitigating ripening processes, minimizing microbial growth, and enhancing fruit firmness. The findings from this study could pave the way for the development of eco-friendly preservation strategies, promoting food security and extending the marketability of strawberries.

Keywords: CMC; HPMC; postharvest preservation; strawberry.

1. INTRODUCTION

Strawberries stand out as one of the most beloved fruits globally, cherished for their distinctive flavour profile and nutritional richness. However, their short postharvest shelf-life presents a significant challenge, leading to rapid deterioration and economic losses. Synthetic chemical fungicides have traditionally been the mainstay in postharvest disease control. However, consumer concerns about pesticide residues on foodstuffs, as well as pathogens' resistance to many current pesticides, have led to an increased need for alternatives to decay control [1]. In recent years, bio-active natural products have begun to replace synthetic fungicides as an effective decay control solution.

Chitin is a mucopolysaccharide that is found abundantly in nature [2]. This nitrogenous polysaccharide is white, hard, and has been built with stable molecules. Chitin links 2-acetamido-2-deoxy-b-D-glucose groups by (1→4) bond, which is called N-acetylglucosamine [3]. Following cellulose, chitin ranks as the second most prevalent biopolymer globally. It can be found in various sources including invertebrates, insects, marine diatoms, algae, fungi, and crustaceans such as crabs, shrimp, and lobsters [4]. Chitosan is a chitin derivative that can be obtained by N-deacetylation. Moreover, chitin is highly valued for its remarkable biological properties, including excellent biocompatibility, complete biodegradability, and inherent non-toxicity to living cells [5].

Notably, chitosan exhibits inherent antifungal properties and the ability to form protective films, making it an attractive candidate for extending fruit shelf-life. Numerous studies have delved into the application of chitosan coatings to mitigate postharvest decay, reduce physiological disorders, and maintain overall fruit quality during storage. Research findings indicate that chitosan coatings effectively retard ripening processes, minimize microbial growth, and enhance fruit firmness. However, further exploration is warranted to optimize chitosan extraction methods and fully understand its preservation mechanisms.

A critical aspect of chitosan research lies in characterizing its physicochemical properties, such as degree of deacetylation (DD) and molecular weight (MW), which significantly influence its efficacy as a coating material. By systematically examining these parameters, researchers can optimize chitosan formulations to maximize its preservative capabilities. Additionally, there is growing interest in exploring synergistic effects between chitosan and other additives, such as carboxymethyl cellulose (CMC) and hydroxypropyl methyl cellulose (HPMC), to enhance strawberry preservation further. The aim of this study is to add to the increasing body of knowledge on chitosan-based coatings for preserving strawberries. Ultimately, the findings from this research could pave the way for the development of eco-friendly preservation strategies, reducing postharvest losses and promoting food security.

2. MATERIALS AND METHODS

2.1 Materials

All chemicals used in this study were analytical grade purchased from Himedia suppliers, Chennai, Tamilnadu, India. The crab shells were collected from the Amjikarai fish market, Chennai, Tamilnadu, India. A local vendor verified that all the crab shells were from the same species. The exoskeletons underwent an initial wash with warm tap water to clear away soluble organics, adhesive proteins, and other contaminants. Subsequently, the shells were soaked for an hour to remove any residual tissues. Following this, they were oven-dried at

60°C for five hours to harden and alter the chitin crystal structure [6]. The final step involved grinding the dried shells into a fine powder using a regular mill.

2.2 Extraction of Chitin and Chitosan

Extraction was done with slight modifications in [7].

Deproteinization: 30g of exoskeletons were placed in a 250 ml beaker and the protein content was excluded by treating with 4% (w/v) sodium hydroxide solution at 45°C for 24 hours. The alkali phase was then separated by centrifuging for 15 mins at 4000 rpm, followed by washing with distilled water until the pH was neutral. To evaluate the rate of deproteinization, the supernatant was used to determine protein concentration by the Biuret method.

Demineralization: The primary mineral in the shell is calcium carbonate. To prevent hydrolysis, hydrochloric acid was employed to dissolve and eliminate the calcium carbonate. The deproteinized shells was treated with a 4% (v/v) hydrochloric acid at 30°C for 12 hours to remove minerals. The decalcified membrane was separated by centrifugation at 4000 rpm for 15 minutes. In addition, the separated part was washed with distilled water to remove the acid and was dried at 40°C overnight to obtain chitin. The degree of demineralization was evaluated by determining the ash content of the solid.

Decolorization: The chitin found is pink in colour. The obtained chitin treated with 1% potassium permanganate for 30 minutes, followed by immersion in 1% oxalic acid for 30 minutes to 2 hours to attain decolourized product. It was then filtered, washed, and dried. Finally, the resulting product was determined to be pure crab shell chitin.

Deacetylation: Chitosan was prepared by deacetylation process by treatment with 65% (w/v) NaOH at 30°C for 3 days. The alkaline phase of chitosan was separated by centrifugation at 4000 rpm for 15 minutes. Most of the alkali was filtered off and the mixture was washed with distilled water to reduce the pH. The obtained chitosan (CH) phase was dried at 40 °C overnight and stored at room temperature for further analysis.

2.3 Characterization of Chitosan

Solubility: 10 mL of 1% acetic acid was added to 0.1 g of extracted chitosan in a centrifuge tube and centrifuged at 10,000 rpm for 30 minutes. After decanting, the chitosan was washed with 25 mL of deionized water, centrifuged at 6000 rpm, dried at 60°C for 24 hours, and weighed to calculate the dissolution percentage [8].

Water uptake capacity (WUC): In a centrifuge tube containing 0.5 g of extracted chitosan, 10 mL of deionized water was added. The chitosan was dispersed by rotating the tube for approximately 5 minutes. Every 10 minutes for a total of 30 minutes, the tube was shaken for 5 seconds. Subsequently, the sample underwent centrifugation at 3500 rpm for 30 minutes. After centrifugation, the supernatant was carefully removed, and the sample was weighed to determine the Water Uptake Capacity (WUC). [9] :

$$WUC(\%) = \frac{Bound\ water\ (ml)}{Initial\ chitosan\ weight\ (g)} \times 100$$

Oil uptake capacity (OUC): In a centrifuge tube, 0.5 g of chitosan was combined with 10 mL of sunflower oil. The mixture was shaken for 5 minutes to disperse the chitosan evenly. Throughout a 30-minute period, the sample was vortexed for 5 seconds every 10 minutes. Following this, the tube was rotated at 3500 rpm for another 30 minutes. After centrifugation, the supernatant was decanted, and the sample was weighed to determine the Oil Uptake Capacity (OUC) [9]:

$$OUC\ (\%) = \frac{Bound\ oil\ (ml)}{Initial\ chitosan\ weight\ (g)} \times 100$$

FT-IR Analysis: The chitosan was confirmed by FT-IR analysis using FT-IR spectrometer instruments in (Saveetha Medical College and Hospital, Chennai). Ranges from 4000-400 cm^{-1} with a resolution of 4.0 cm^{-1} [10]. The DD (%) was determined by using FTIR spectroscopy. The spectra of chitosan samples were obtained using an I.R instrument (SHIMADZU-8400, Japan) with a frequency range from 4,000 to 400 cm-1. The DD was calculated using [11] equation:

$$DD\ \% = 100 * (1 - \frac{\mathrm{A1655}}{A3450} \times \frac{1}{1.33})$$

A^{1655} and A^{3450} show absorbance at 1655 cm^{-1} for the amide I band as an indication of the content of the N-acetyl group and the hydroxyl group at 3450 cm^{-1} as a standard for the layer or gap of chitosan. The factor number “1.33” was

A^{1655}/A^{3450} and fully N-acetylated chitosan. This value was taken as zero for fully acetylated chitosan.

To determine the average viscosity MW, chitosan solution made by mixing up with 0.2 M acetic acid/0.3 M sodium acetate. The Ubbelohd viscometer is used to measure the intrinsic viscosity (η). The Mark-Houwink equation. $\eta = k.(M_w)^a$ was used to determine the MW in kDa [12]. The values of the empirical viscometer constants k and a are 0.078 cm^3/g and 0.76 [13].

2.4 Preparation of Edible Coating Solutions

Hydroxypropyl methylcellulose (HPMC), Carboxymethyl cellulose (CMC) and solvents used in the analysis were from Himedia suppliers, Chennai, Tamilnadu, India. CMC (1% w/v) was prepared by dissolving 1 g of CMC powder in a water-ethyl alcohol mixture (3:1 ratio) at 75°C for 15 minutes using a magnetic stirrer. Ethanol was added to expedite drying and enhance the glossiness of methylcellulose. Glycerol monostearate (0.75%) was incorporated into the mixture and stirred vigorously for 10 minutes [14].

For HPMC (1% w/v) preparation, 1 g of HPMC powder was dissolved in 100 mL of distilled water at 80°C for 2 hours, then cooled to room temperature. Glyceryl monostearate (0.75%) was added as a plasticizer and stirred for 10 minutes under identical conditions [15]**.** To prepare CH (1% w/v), CH powder was dissolved in water containing 0.5% v/v glacial acetic acid at 40°C for 12 hours, with continued infusion under heat. Glycerol monostearate (0.75%, 0.1 ml) was added as a plasticizer to enhance the solution's strength and elasticity. The pH was adjusted to 5.6 using 1 N NaOH. The solution was heated to 45°C and stirred with a magnetic stirrer until completely dispersed.

2.5 Assessment of Antimicrobial Properties

The antimicrobial activity was determined by the agar well diffusion technique. Culture of *Pseudomonas aeruginosa* ATCC 9027, *Bacillus subtilis* ATCC 6051, *Rhizopus stonolifer* ATCC 42555, *Mucor mucedo* ATCC 48559 were obtained from culture centre, India. Test strains of bacteria and fungi were pre-cultured into respective sub-culture media. The former was sub-cultured in Tryptic Soy Agar (TSA) at 37℃ for 24 hours, while the latter in Potato Dextrose Agar (PDA) at 25℃ for 5 days. The cultures were kept refrigerated in respective agar slants at 4℃ during the experiment. Muller Hinton Plate (MHA) was prepared and labeled. A 24-hour test culture of $1\text{-}2\times10^8$ CFU/ml was obtained in a fresh culture with a McFarland ratio of 0.5. The bacterial suspension was evenly seeded and streaked onto MHA plates using sterile cotton swabs. Wells were punctured and 20µl of the sample was loaded in the wells of the agar plate using a micropipette. Following incubation at 37°C for 24 hours, the diameter of the inhibition zones was measured using a caliper and recorded in millimeters. All the tests were performed in triplicates [16].

2.6 Evaluation of Shelf Life with the Coating Solution

Strawberries (Fragaria ananassa) from the rosaceae family were purchased at the local market. Samples were selected for uniform size, absence of physical damage and fungal diseases, and a red area of >75%. The fruits are washed and dried for two hours at room temperature, and after two minutes they are surface-dipped with a 2% sodium hypochlorite solution. There were 15 fruits in each treatment, distributed to five treatment groups, and each treatment was repeated three times. The fifth group, control and untreated (T5), had untreated fruits were soaked in distilled water.

Four groups were assigned to one of three treatment options (T): T1-CMC (1%, 2%, 3%), T2-CMC + CH (1%, 2%, 3%), T3 - HPMC (1%, 2%, 3%), T4 - HPMC + CH (1%, 2%, 3%). After formulating the solutions, they were cooled to 20°C and the fruits were placed the formulation for 60 seconds to facilitate contact on the surface of the entire sample and create a uniform film. For each treatment, Samples were dried and stored in small plastic PET containers in a controlled environment at 4°C with 95% relative humidity, intermittently removed for analysis [17].

2.7 Physicochemical Evaluation of the Fruits During Storage

2.7.1 Determination of weight loss percentage

Strawberry samples (15 fruits per replicate) were weighed for every four days according to the standard method [18] Fruit weight loss was taken at the beginning of the experiment (i.e., day 0) and at the end of each storage period, and the

results were expressed as a percentage of the initial weight.

$$Weight\ loss\ (\%) = \frac{initial\ weight - final\ weight}{initial\ weight} \times 100$$

2.7.2 Determination of decay percentage

Fungal decay was visually monitored daily during storage. Strawberries that showed mycelial growth were considered rotten and their percentage was calculated every four days. A portion of 15 fruits (in triplicate) was attached to each coat.

$$Decay(\%) = \frac{No.of\ decayed\ fruits}{Total\ no.of.fruits} \times 100$$

2.7.3 Determination of pH

Strawberries were cut into small pieces and homogenized, and 1 g of grounded strawberries were suspended in 10 ml of distilled water and filtered. The pH of the sample is measured using a pH meter.

2.7.4 Determination of Titratable Acidity (TA)

Alike to that of pH assessment, the berries were processed. 5ml of the smashed strawberry with phenolphthalein as indicator was titrated against 0.1N NaOH. TA was determined using [19] method. The results were expressed as the percent of citric acid**.**

2.7.5 Sensory evaluation

The method described by [20] was slightly modified for the purposes of sensory analysis of coated and uncoated strawberries (control). Sensory ratings based on overall visual appeal, colour, and apparent structural integrity were performed using a 9-point hedonic scale. The rating is as follows: 1-4=dislike (extremely, very much. Moderately, slightly), 5=neither like nor dislike, 6-9=like (slightly, moderately, very much, extremely). Fruits with a score above 4 were considered acceptable. Sensory evaluation was performed by 10 untrained assessors. The effects of each treatment were assessed on the first and last day. Only fruits without signs of fungal rot were assessed.

3. RESULTS AND DISCUSSION

In this study, the biopolymer was extracted using seafood processing waste. This value-added product was designed to protect berries. The yields of extracted chitin and produced chitosan were measured. Chitin content from dried crab shell was 12%. The yield of chitosan obtained from extracted chitin was 76.8%, which is consistent with the results reported in the literature [21]. The results of this study were above average and our study shows that crabs can be one of the main sources of chitin and chitosan among other crustacean groups.

The solubility of chitosan in 1% acetic acid solution was 99.29% ± 0.001. The high solubility of chitosan can be explained by the functional properties that occur during the deacetylation step. Temperature 150°C, deacetylation time 2 hours, alkali concentration 50% NaOH. The high solubility of chitosan in acetic acid shows a deacetylation rate of at least 85% [22]. The water absorption capacity of chitosan was 602.48% ± 49.25. The oil absorption capacity of chitosan was found to be 463.32% ± 8.46. Similar results were observed in [23].

The FTIR study of the extracted chitosan was performed to elucidate the functional groups present in them. A band at 3342.62 cm^{-1} corresponds to the stretching vibration of hydroxyl(-OH) and amino(-NH2) groups of chitosan (Fig. 1). Due to the overlapping vibrations of above-mentioned groups, the peak is broad often. The spectrum at 1585.79 cm^{-1} corresponds to the amide I band, which is associated with the stretching vibration of the c=o bond and spectrum at 1399.82 cm^{-1} corresponds to amide II band which involves N-H bending and C-N stretching vibrations. Peak at 1026.93 cm^{-1} indicate the C-O-C stretching found in the glycosidic linkages of molecules. The degree of deacetylation was found to be 82% and the molecular weight of the extracted chitosan was 12 K.da.

The antimicrobial activity of chitosan (CH) in combination with Carboxy Methyl Cellulose (CMC) and hydroxypropyl methylcellulose (HPMC) against various test organisms was evaluated in this study. The results depicted in Table 1 indicate a notable enhancement in the zone of inhibition (ZOI) with increasing concentrations of chitosan.

At a chitosan concentration of 1%, the combination of CMC and CH exhibited improved antimicrobial activity compared to CMC alone for all tested organisms. Particularly, the ZOI against *Pseudomonas* and *Bacillus* increased by 1 mm and 5 mm, respectively, when chitosan was added to CMC. Similarly, the combination of HPMC and CH showed enhanced activity against *Pseudomonas* and *Rhizopus* compared to HPMC alone.

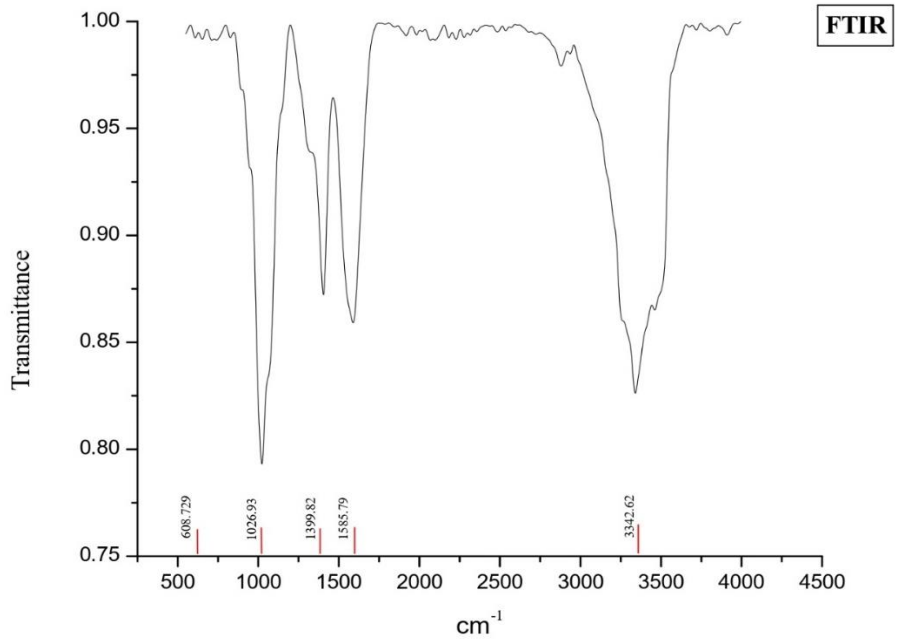

Fig. 1. FTIR analysis of extracted chitosan

Table 1. Antimicrobial activity of chitosan on test organism

1A. Zone of inhibition (mm) **– 1% formulations**				
Organism	**CMC**	**HPMC**	**CMC + CH**	**HPMC + CH**
Pseudomonas	6 ±0.5	5 ± 0.3	7 ±0.4	9±0.5
Bacillus	5±0.2	6±0.3	10±0.2	9±0.4
Rhizopus	5±0.4	7±0.2	8±0.3	12±0.3
Mucor	5±0.1	6±0.4	8±0.3	10±0.5
Data expressed as mean ± standard deviation of triplicate samples				
1B. Zone of inhibition (mm) **– 2%**				
Organism	**CMC**	**HPMC**	**CMC + CH**	**HPMC + CH**
Pseudomonas	7±0.5	6±0.2	9±0.4	13±0.1
Bacillus	6 ±0.5	5±0.3	7±0.3	8±0.5
Rhizopus	6±0.1	5±0.4	8±0.2	10±0.5
Mucor	6±0.3	8±0.2	8±0.2	10±0.5
Data expressed as mean ± standard deviation of triplicate samples				
1C. Zone of inhibition (mm) **– 3%**				
Organism	**CMC**	**HPMC**	**CMC + CH**	**HPMC + CH**
Pseudomonas	6±0.3	7±0.3	9±0.2	10±0.4
Bacillus	7±0.4	6±0.3	8±0.3	11±0.2
Rhizopus	6±0.5	5±0.4	10±0.2	11±0.3
Mucor	7±0.1	6±0.4	9±0.4	11±0.3
Data expressed as mean ± standard deviation of triplicate samples				

Fig. 2. Evaluation of shelf life with various treatment

As the chitosan concentration was increased to 2% and 3%, further augmentation in antimicrobial efficacy was observed. In most cases, the ZOI increased proportionally with the chitosan concentration, indicating a dose-dependent effect. Notably, the combination of HPMC and CH demonstrated the highest ZOI for *Pseudomonas* and *Rhizopus* at 2% and 3% chitosan concentrations, suggesting a synergistic effect between HPMC and CH.

With $p<0.05$, the values are found to be significant and these findings suggest that chitosan, particularly when combined with cellulose derivatives like CMC and HPMC, especially HPMC possesses significant antimicrobial properties against a range of test organisms.

The analysis of the shelf life of the strawberries yielded promising results. In comparison to the control group, which exhibited decay, the strawberries treated with the solutions displayed enhanced preservation. In accordance with microbial activity, the polymers on combination with chitosan showed remarkable results, especially HPMC. This suggests that the solutions contain preservative agents effectively inhibited microbial growth and extended the shelf life of the strawberries.

Fruit weight loss is mainly due to transpiration and evaporation of moisture through the skin [24]. Strawberries lose water quickly due to their thin skin, causing them to shrink and fall apart. The water loss rate is influenced by the water pressure gradient between the fruit tissue and the environment, as well as the storage temperature. Edible coatings function as protective barriers, shielding the fruit from physical damage, sealing minor wounds, and reducing moisture loss. Fig. 3 illustrates the gradual weight loss of strawberries stored at cool temperature (4°C). Uncoated strawberries experienced a 25% weight loss by the end of storage, whereas HPMC chitosan-coated strawberries had the lowest weight loss at 1.11%. These results indicate that chitosan coatings effectively limit moisture loss and prevent shape degradation by being selective barriers for O_2 and CO_2.

The amount of chitosan in the peel influences fruit damage caused by fungi. Control fruit showed signs of fungal damage after 3 days of storage at 4°C (Fig. 4). After 6 days of storage, fruit coated with 2% & 3% chitosan + HPMC/CMC and found no signs of fungal attack. Fruits coated with 1% chitosan were found to be less susceptible Chitosan coatings have demonstrated efficacy in inhibiting fungal growth on various crops. Increasing viscosity enhances the uniform deposition of chitosan on fruit surfaces, thereby optimizing coating effectiveness. The concentration of the coating agent notably impacts the accumulation of dry matter on the fruit surface. Application of chitosan solution with CMC or HPMC prevents fungal damage to fruit during storage.

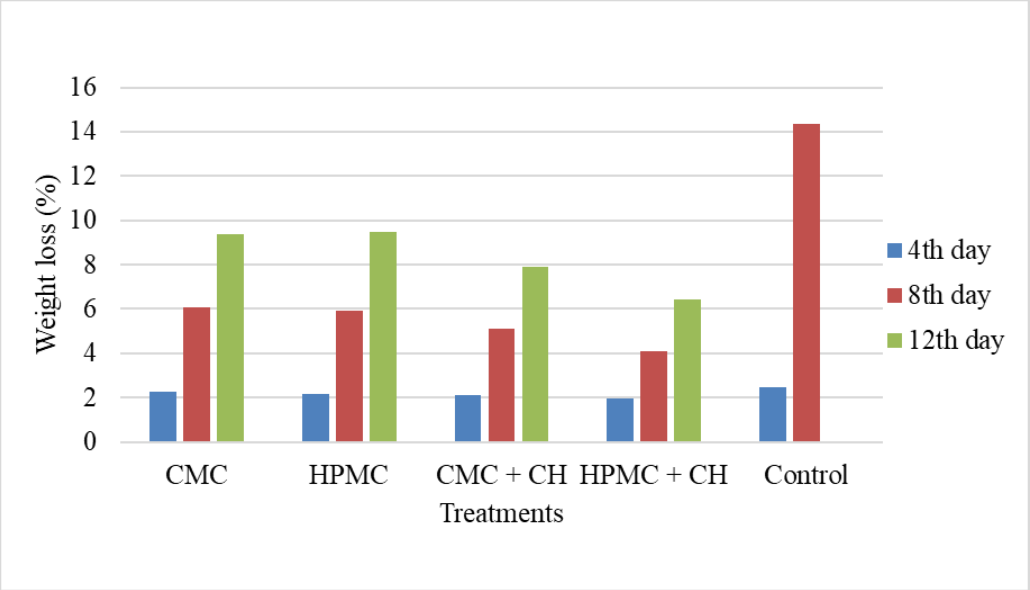

Fig. 3a. Weight loss evaluation of 1% formulations

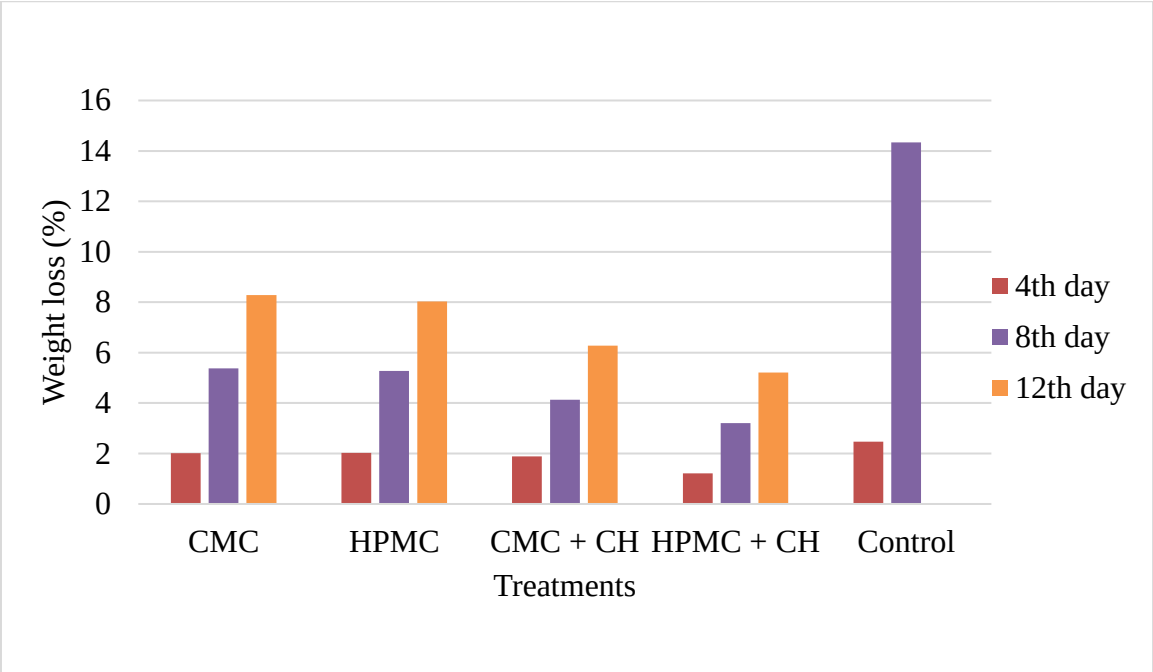

Fig. 3b. Weight loss evaluation of 2% formulations

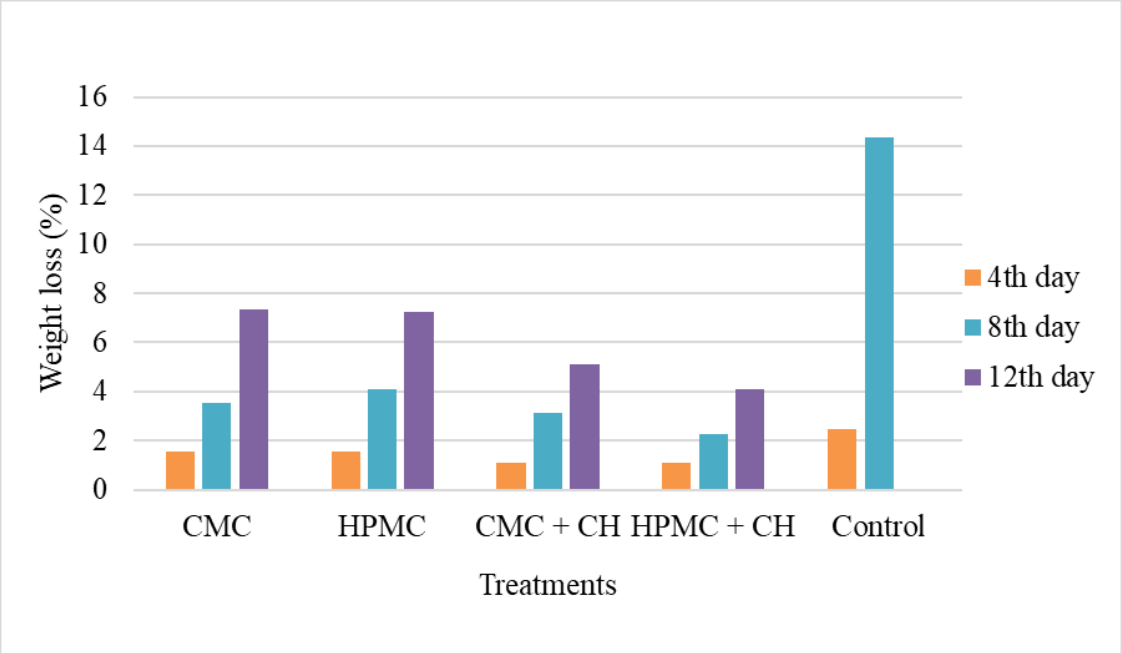

Fig. 3c. Weight loss evaluation of 3% formulations

The pH of strawberries increased after 4 days of storage and was significantly different between coated and uncoated samples. The parameters that determine crop quality and are commercially acceptable are pH = 3.27 to 3.86 [25]. Quality criteria for strawberries stored at 4°C include: uncoated samples showed a slight pH increase during storage, with no significant difference noted in various studies [26] - pH in field-ripened strawberries increases significantly during fruiting, while immature fruits exhibit stable pH levels. The decrease in respiration rate of fruit due to polymer coating may explain the delay of natural chemicals in respiratory enzyme activities.

TA studies of fruits revealed that the main compounds found are citric acid and malic acids [27]. This characteristic is an important part of the organic quality of the fruit and varies from one variety to another. During freezing, fruits showed a significant decrease in TA, with uncoated fruits having lower values than chitosan-coated ones. Previous studies indicate that higher acidity loss in raw fruits is due to organic compound use during storage [28], chitosan application delays cold ripening and reduces damage in coated fruits.

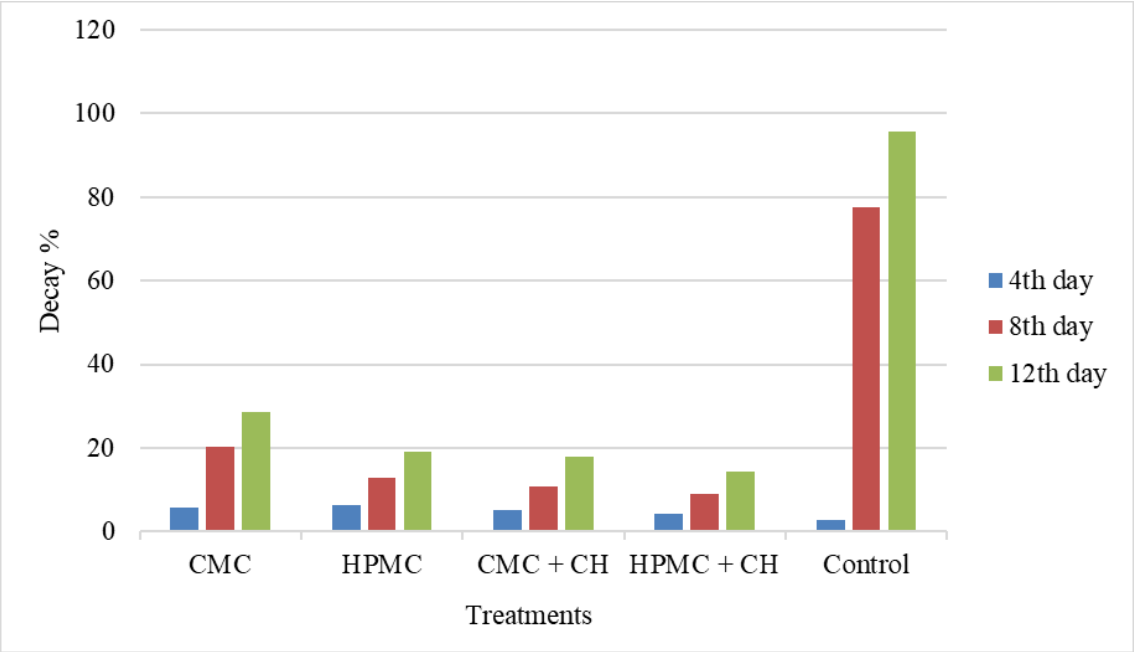

Fig. 4a. Decay percentage with 1% formulations

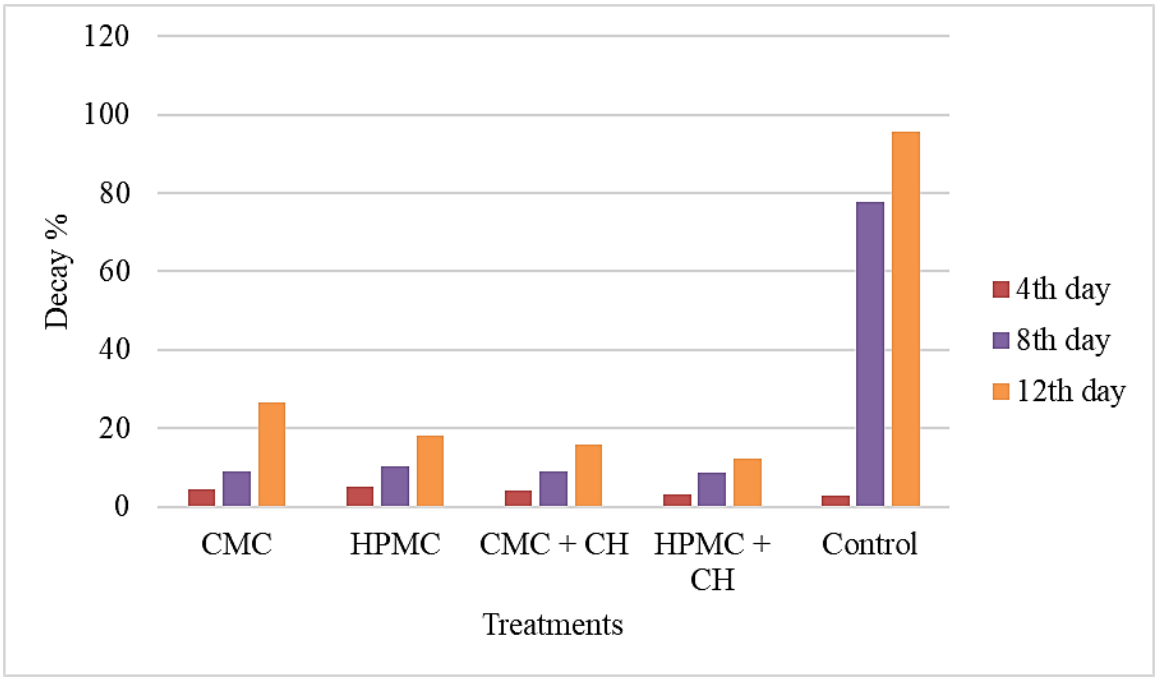

Fig. 4b. Decay percentage with 2% formulations

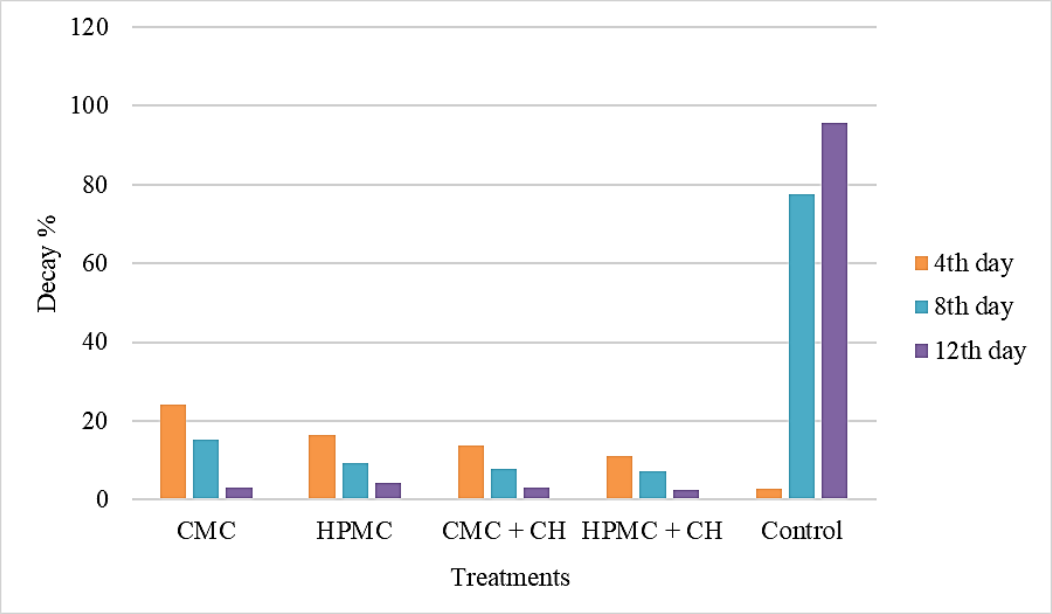

Fig. 4c. Decay percentage with 3% formulations

Sensory data were analysed for strawberries stored at 4°C and 95% humidity for one week. At baseline (day 0), participants expressed a preference for sweet fruit, as indicated by the mean acceptability score. The treatment did not cause discoloration of the strawberries and covered fruits are acceptable due to the shape of the skin. The content of the chitosan coating did not significantly change the appearance of the results, and addition other polymers did not affect the gloss or transparency of the coating. Not all results are acceptable to the eye during the storage period. After the fourth and eighth days, however, consumers showed a greater preference for the coated fruits, resulting in acceptable mean scores between the middle of the hedonic scale and like or dislike. On the 12th day of storage, the covered fruits were below acceptable limits. The high perception of consumers of unopened strawberries is related to the amount of drying and harvesting during storage.

Firmness is a critical physical parameter for assessing strawberry quality during ripening, storage, and distribution, influenced by initial differences in lignin content. The 3% chitosan coating showed better preservation effects compared to the 1% coating, although differences were not significant across all samples until day 6. Uncoated fruits exhibited visible firmness loss after four days of storage. Previous studies have explored the impacts of various nutrients and plant extracts, such as cactus extract, chitosan-oleic acid, calcium water, and volatile compounds from chitosan [17].

4. CONCLUSION

The results of the present study underscore the efficacy of chitosan coatings in enhancing the postharvest preservation of strawberries, particularly in mitigating fungal decay and maintaining fruit quality attributes such as firmness, colour, and nutritional content. The findings consistently demonstrate the potential of chitosan, especially at concentrations above 3%, to significantly extend shelf life and improve overall storability, even under varied storage conditions and in combination with additives like CMC and HPMC. Moreover, chitosan coatings exhibit promising outcomes in delaying senescence, and regulating activities critical for fruit quality maintenance. These results highlight chitosan as a viable and environmentally friendly solution for extending the marketability and enhancing the sensory appeal of strawberries, offering practical implications for the preservation of fruit quality in both temperate and tropical regions.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

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

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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