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Synthesis of Sulfonated Chitosan as An Active Agent of Antibacterial Packaging for Fish Fillets

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Abstract

Modifying chitosan by increasing its hydrophilic properties through sulfonation reactions will increase its solubility in water and antibacterial activity and expand the application of chitosan. This study aims to determine the antibacterial activity of sulfonated chitosan film applied as fish fillet packaging using the total plate count method. Sulfonated chitosan in this study was successfully synthesized by reacting chitosan with 1,3-propane sultone. Sulfonated chitosan products were characterized using FTIR and UV-Vis spectrophotometers, while antibacterial activity was measured using the disc diffusion method. The optimum temperature for sulfonated chitosan synthesis was 60°C (SCS60). The resulting SCS 60 was a yellowish-white solid soluble in water with a yield of 61.46% and a degree of substitution of 33%. The diameters of the SCS 60 inhibition zone against *E. coli* and *S. aureus* isolates were 7 and 10 mm, respectively. SCS60-gelatin film is elastic (elongation 272%) and more hydrophobic with a contact angle of 90.12° compared to chitosan-gelatin film (62.8°). SCC60-gelatin film was able to suppress bacterial growth in fish fillets by up to 0.3 × 10^4 cfu/g compared to unpackaged fish (30 × 10^4 cfu/g). Sulfonated chitosan has the potential to be an antibacterial food packaging material.

1. Introduction

Food safety is a critical concern in relation to human health today. One of the key factors in ensuring food safety is the role of food packaging, which protects food from damage during processing, transportation, and storage. A major cause of food spoilage during these processes is contamination by microorganisms, such as bacteria, resulting from environmental, physical, and biochemical factors inherent to the food itself [1]. Bacterial contamination leads to spoilage, manifesting as alterations in taste, appearance, odor, nutritional value, and microbial composition, rendering the food unfit for human consumption and potentially causing illness [2]. Common bacteria responsible for food spoilage include *Escherichia coli*, *Staphylococcus aureus*, *Listeria monocytogenes*, and *Salmonella* [3]. Using antibacterial packaging materials offers a promising solution to inhibit or eliminate bacterial growth during transportation and storage, thereby preserving food quality and ensuring both health and safety for consumers [4].

Chitosan has long been recognized as a packaging material capable of forming antimicrobial and antifungal films, thereby extending the shelf life of various foods [5]. Its application in food preservation has been extensively studied, particularly in the extension of the shelf life of several fish species, including Solea solea and *Merluccius merluccius* [6], mackerel (*Rastrelliger* sp.) and catfish (*Clarias batrachus*) [7], fresh anchovies *[8], Sardinella longiceps* [9], and catfish [10]. Chitosan exhibits broad-spectrum antibacterial activity, effectively inhibiting the growth of both spoilage and pathogenic bacteria in food products [11, 12]. Specifically, chitosan has been shown to suppress the growth of spoilage bacteria, such as *Pseudomonas*, lactic acid bacteria, *Enterobacteriaceae*, and *Clostridium* spp. in poultry [13], as well as *L. monocytogenes* and *S. aureus* in beef and lamb [14]. Additionally, it has

demonstrated efficacy in inhibiting pathogenic bacteria, including *Aeromonas hydrophila*, *Edwardsiella ictaluri*, and *Flavobacterium columnare* in warm-water fish [15].

The studies mentioned above utilized dilute acid solvents, particularly acetic acid (1-2%), which is the most commonly used solvent for dissolving chitosan [16, 17]. However, the use of acetic acid in producing chitosan films presents several drawbacks regarding film characteristics. These include mechanical issues such as brittleness, ease of rolling, rapid drying, and poor barrier properties, specifically low water vapor permeability, and high water vapor absorption [18, 19, 20]. Additionally, the removal of acetic acid residue from the film is challenging, even after neutralization with a base like NaOH solution [19, 21]. The residual acetic acid in the film may migrate into the packaged food, potentially affecting its organoleptic properties, including odor, taste, and texture [19]. Casalini *et al.* [21] reported that the migration of acetic acid from chitosan films into cheese can adversely impact the quality and sensory characteristics of the product. The concern over acetic acid residue migration has led to regulatory measures limiting its presence in food to a maximum of 60 ppm [22].

Researchers have implemented several strategies to address these limitations, one of which involves modifying the structure of chitosan. Structural modification of chitosan has been widely reported as a straightforward and effective approach for altering its physical, chemical, and biological properties [23]. The amine group (C2) and primary hydroxyl group (C6) in chitosan are reactive sites that can be readily modified through chemical reactions such as sulfonation, alkylation, carboxylation, phosphorylation, and esterification [24, 25]. Transforming chitosan into water-soluble derivatives represents a promising solution to these challenges. Among these modifications, sulfonation is an economical and efficient method [26]. Sulfonated chitosan has attracted significant interest from researchers due to its water solubility, potent antibacterial activity, antioxidant properties, biocompatibility, and excellent metal chelation capacity [27, 28].

Sulfonated chitosan has been reported to exhibit potent antibacterial activity against both Gram-negative and Gram-positive bacteria. It has been shown to inhibit the growth of Gram-negative bacteria, including *Escherichia coli* [27, 28, 29, 30, 31], *Shigella dysenteriae*, *Aeromonas hydrophila*, *Salmonella typhimurium* [30], *Pseudomonas aeruginosa* [31], *Shewanella putrefaciens*, and *Pseudomonas fluorescens* [27]. Gram-positive bacteria inhibited by sulfonated chitosan include *Staphylococcus aureus* [27, 28, 29, 30, 31], *Bacillus cereus* [30], and *Propionibacterium acnes* [31]. Additionally, sulfonated chitosan demonstrates antifungal activity against several fungi, such as *Arthrinium sacchari* and *Botrytis cinerea* [29], as well as Malassezia furfur, *Malassezia pachydermatis*, *Trichophyton rubrum*, *Trichophyton mentagrophytes*, and *Candida albicans* [31].

Sulfonated chitosan has also been reported to exhibit strong antibiofilm activity against *E. coli* and *S. aureus* [28, 32]. Due to its potent antibacterial and antibiofilm properties, sulfonated chitosan holds promise for applications in the food processing industry. However, there remains limited information regarding the application and utilization of chitosan in food processing, particularly as a food packaging material. This study aims to determine the antibacterial activity of sulfonated chitosan films used as packaging for fish fillets. The sulfonated chitosan products were characterized using UV-Vis and FTIR spectroscopy, while their antibacterial activity against *E. coli* and *S. aureus* was assessed using the disc diffusion method. The antibacterial activity of the sulfonated chitosan films was further evaluated through the total plate count method.

2. Experimental

2.1. Tools and Materials

The equipment used in this study included an Ubbelohde viscometer, standard laboratory glassware (Pyrex), a hot plate stirrer (Corning PC-420D), an incubator (Memmert IN 30), a caliper (Krisbow), a UV-Vis spectrophotometer (Genesys 10S, Thermo Scientific), an infrared spectrophotometer (Perkin Elmer UATR Spectrum Two), and pH indicator paper (Merck). The materials utilized were low molecular weight chitosan (CV. ChiMultiguna), 1,3-propane sultone (Chongqing Rongguangda Chemical Co., LTD), NaCl (Merck), NaOH (Merck), bovine gelatin (Rousselot), acetone (Merck), glacial acetic acid (Merck), distilled water (Bratachem), Mueller Hinton Agar (MHA) (HiMedia), Mueller Hinton Broth (MHB) (HiMedia), plate count agar (HiMedia), and bacterial isolates of *E. coli* and *S. aureus*.

2.2. Procedures

2.2.1. Determination of Molecular Weight of Chitosan

The average molecular weight of chitosan was determined by measuring its intrinsic viscosity using a Ubbelohde viscometer and calculating it using the Mark-Houwink equation (1).

$$\eta = KM^{\alpha} \quad (1)$$

Where, η represents the intrinsic viscosity, K and α are the viscometric constants for chitosan in 0.1 M CH_3COOH and 0.2 M NaCl solvents (1.81×10^{-3} and 0.93, respectively), and M denotes the average molecular weight of chitosan (Da) [33].

A chitosan stock solution was prepared at a concentration of 1 g/mL using a mixture of 0.1 M CH_3COOH and 0.2 M NaCl (3:1 v/v). From this stock solution, chitosan solutions of varying concentrations (0.001, 0.002, 0.003, 0.004, and 0.005 g/mL) were prepared. The flow time for each concentration was measured with five repetitions to ensure consistency in the results.

2.2.2. Determination of The Degree of Deacetylation of Chitosan (%DD)

The degree of deacetylation was determined using FTIR data, measured at wavenumbers ranging from 4000 to 400 cm^{-1}, employing the baseline method. The degree of deacetylation was calculated by comparing the

absorbance of the carbonyl/acetyl group at 1655 cm^{-1} with that of the hydroxyl group at 3450 cm^{-1}. The calculation of the degree of deacetylation can be expressed mathematically using Equation (2).

$$\%DD = 100 - \left[\left(\frac{A1655}{A3450}\right) \times \left(\frac{100}{1.33}\right) \right] \quad (2)$$

Where, A1655 represents the absorbance of the carbonyl functional group at a wavenumber of 1655 cm^{-1}, A3450 corresponds to the absorbance of the hydroxyl functional group at a wavenumber of 3450 cm^{-1}, and 1.33 is the ratio of the absorbance of the carbonyl to hydroxyl groups for fully deacetylated chitosan [34, 35].

2.2.3. Synthesis of Sulfonated Chitosan

The synthesis of sulfonated chitosan was conducted following the method developed by Sun *et al.* [29], with modifications. Five grams of chitosan (1.67×10^{-5} mol) were dissolved in 150 mL of 2% acetic acid. A total of 4.8 g (40 mmol) of 1,3-propane sultone was added dropwise, and the mixture was refluxed for 6 hours at varying temperatures of 25, 40, 60, and 80°C. After refluxing, the resulting mixture was combined with cold acetone and stirred vigorously until a white solid precipitate formed. The precipitate was filtered using a Buchner funnel and washed with acetone five times. The solid product was dried in an oven at 50°C for 8 hours, resulting in sulfonated chitosan as a powdered solid. The synthesis products were characterized using FTIR and UV-Vis spectrophotometers.

2.2.4. Film Preparation

Chitosan and sulfonated chitosan-gelatin films were prepared using the solution casting method. A total of 15 mL of chitosan and sulfonated chitosan, each at a concentration of 1% in acetic acid, were combined with 0.7 g of gelatin and 0.3 mL of glycerol. The film solution was stirred gently using a magnetic stirrer for 30 minutes at a temperature of 70°C, resulting in a final concentration of 0.75% for both chitosan and sulfonated chitosan in the film solution. The resulting film solution was then poured into a petri dish with a diameter of 10 cm and dried in a drying oven at 40°C for 8 hours to yield chitosan and sulfonated chitosan-gelatin films. The films were subsequently subjected to contact angle analysis [36], tensile strength and elongation tests [19], and biodegradability assessment [36].

2.2.5. Antibacterial Activity Testing

The antibacterial activities of chitosan and sulfonated chitosan film solutions were assessed using the disc diffusion method, following the guidelines established by the International Clinical Laboratory Standards [37]. This assessment was conducted against *E. coli* and *S. aureus* isolates. Additionally, the antibacterial activity of the chitosan and sulfonated chitosan films was evaluated using the total plate count method, specifically with fish fillets [38].

2.2.6. Antibacterial Activity of Disc Diffusion Method

The antibacterial activities of chitosan and sulfonated chitosan were evaluated using the disc diffusion method in accordance with the International Clinical Laboratory Standards [37]. This assessment targeted *E. coli* and *S. aureus* bacterial isolates, utilizing both chitosan film solutions and sulfonated chitosan samples. Ciprofloxacin at a concentration of 500 ppm served as the positive control, while a gelatin and glycerol solution in 1% acetic acid acted as the negative control.

A total of 100 µL of bacterial suspension, standardized to a concentration of 0.5 McFarland, was inoculated onto sterile MHA media in a 150 × 25 mm petri dish, serving as the test medium. The bacterial suspension was evenly distributed across the entire surface of the test medium using a sterile cell spreader. Sterile filter paper discs, 6 mm in diameter, were impregnated with 10 µL of each sample at a concentration of 0.75% and placed onto the surface of the test medium. The dishes were incubated for 24 hours at 37°C. The diameter of the resulting inhibition zones was measured using a caliper.

2.2.7. Antibacterial Activity of Total Plate Count Method

Antibacterial activity was assessed using the total plate count method on chitosan and sulfonated chitosan (SCS60) film samples, which exhibited the most significant antibacterial activity in the disc diffusion assay. Fresh fish fillet samples, weighing 25 g and purchased from a local market, were wrapped in chitosan-gelatin film and SCS60-gelatin film, while unwrapped fish fillets served as the control. The fish samples were stored at 4°C for 24 hours.

Following the incubation period, the fish samples were homogenized with 225 mL of sterile 0.9% NaCl solution. The homogenate was subsequently subjected to serial dilutions, ranging from 10^{-1} to 10^{-6}, using sterile 0.9% NaCl as the diluent. A total of 1 mL from each dilution was transferred into a sterile petri dish, which was then filled with plate count agar (PCA) media maintained at approximately 45°C. The petri dishes were incubated at 37°C for 24 hours. The number of bacterial colonies in each dish was counted visually, adhering to the criteria of having 25 to 250 colonies per petri dish. The concentration of bacterial colonies was reported as the number of colonies per gram (cfu/g).

3. Results and Discussion

3.1. Characterization of Chitosan

The average molecular weight and degree of deacetylation of chitosan significantly influence its antibacterial properties and the modification process. Chitosan with a lower molecular weight exhibits higher solubility in water compared to its higher molecular weight counterparts, which in turn affects the modification process [27]. In this study, the results of intrinsic viscosity measurements conducted using an Ubbelohde viscometer, along with calculations utilizing the Mark-Houwink equation, indicated that the average molecular weight of the chitosan utilized was 299,992 Daltons. This corresponds to an estimated number of approximately 1,852 monomers. The chitosan employed in this study is categorized as having a medium molecular weight, ranging from 150 to 700 kDa [39].

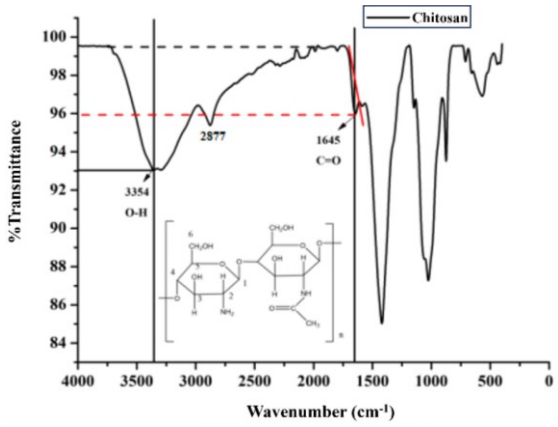

Figure 1. FTIR spectra of chitosan

The FTIR spectra of chitosan (Figure 1) reveal several significant absorption peaks. The peak at a wavenumber of 3354 cm^{-1} indicates the overlap of the hydroxyl (-OH) and amino ($-NH_2$) functional groups. The absorption observed at 2877 cm^{-1} corresponds to the stretching vibration of aliphatic C_3-H bonds. Additionally, the bending vibration of the carbonyl (C=O) acetyl group is identified at a wavenumber of 1645 cm^{-1}. In this study, the chitosan was determined to have a degree of deacetylation of 59%, calculated using the baseline method. The estimated number of monomers present in the chitosan is approximately 1,093. Chitosan, with a high degree of deacetylation, contains a more significant number of amino groups, which enhances its antibacterial properties. Furthermore, the modification of chitosan through a sulfonation reaction is more effectively achieved when using chitosan with a higher concentration of amino groups [27].

3.2. Synthesis and Characterization of Sulfonated Chitosan

The synthesis of chitosan with 1,3-propane sultone under weakly acidic conditions yields water-soluble sulfonated chitosan. This method is currently regarded as the most efficient due to its minimal chemical usage and environmentally friendly approach [40]. The synthesis mechanism involves a nucleophilic substitution reaction, wherein the sulfonate group preferentially attaches to the amino ($-NH_2$) group on the C2 carbon atom of chitosan rather than the hydroxyl (-OH) group on the C6 carbon atom [26, 27]. The mechanism of the chitosan sulfonation reaction is illustrated in Figure 2. The resulting sulfonated chitosan product appears as a yellowish-white solid and is soluble in water, except for the sulfonated chitosan synthesized at 80°C, which does not yield any solid sulfonated chitosan.

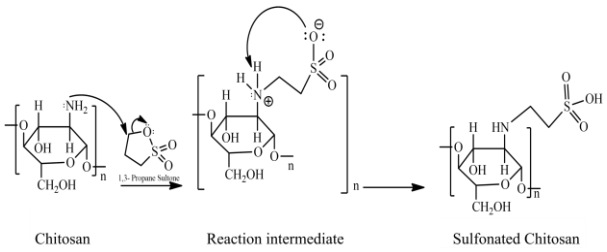

Figure 2. Reaction mechanism for the synthesis of sulfonated chitosan

The variation in reaction temperature during the synthesis of sulfonated chitosan results in products with differing yields and degrees of substitution, as presented in Table 1. The optimal condition identified in this study for sulfonated chitosan synthesis was a reaction temperature of 60°C. This finding aligns with the studies conducted by Tamer *et al.* [41] and Sun *et al.* [29], which reported high degrees of substitution at reaction temperatures of 65°C and 60°C, respectively. Notably, at a reaction temperature of 80°C, no sulfonated chitosan product was generated. This absence of product formation can be attributed to the inhibitory effect of elevated temperatures, which alters the equilibrium of the chemical reaction, driving it in the opposite direction for exothermic reactions, such as the sulfation process [42].

The FTIR characterization results for SCS 25, SCS 40, and SCS 60 (Figure 3) revealed strong absorption peaks at wavenumbers 1027, 1025, and 1024 cm^{-1}, along with a peak at 1378 cm^{-1}, respectively (Table 2). These absorptions correspond to the stretching of the S=O group, indicating the presence of sulfonate groups in the sulfonated chitosan. Additionally, strong absorptions around 1634 cm^{-1} and 1528 cm^{-1} are attributed to NH bending and C=O stretching, respectively. These findings are consistent with previous studies conducted by Sun *et al.* [29], Tamer *et al.* [41], and Rwei and Lien [43].

The appearance of new peaks at wavenumbers 1027, 1025, and 1024 cm^{-1} for SCS 25, SCS 40, and SCS 60, respectively, compared to chitosan, can be linked to the presence of sulfopropyl groups in the synthesized products. Moreover, the intensity of the peaks associated with the sulfopropyl groups at wavenumbers between 1027 and 1024 cm^{-1} increases with the degree of substitution. Notably, SCS 60 exhibits a higher attachment of sulfopropyl groups to the main chitosan chain compared to SCS 25 and SCS 40.

Table 1. Synthesis products of sulfonated chitosan at different temperatures

No.	Sample	Yield (%)	Degree of substitution (%)*
1	SCS 25	71.73	26
2	SCS 40	61.24	31
3	SCS 60	71.82	33
4	SCS 80	-	-

SCS 25 = sulfonated chitosan product at 25°C; SCS 40 = sulfonated chitosan product at 40°C; SCS 60 = sulfonated chitosan product at 60°C; SCS 80 = sulfonated chitosan product at 80°C.
*Determined by FTIR

Table 2. Interpretation data of IR spectra of sulfonated chitosan

Wavenumber (cm^{-1})				Functional group vibration
Chitosan	SCS 25	SCS 40	SCS 60	
3354	3367	3364	3270	O-H stretching
1645	1633	1632	1634	>C=O stretching
1589	1528	1528	1528	N-H bending
-	1378	1378	1378	S=O stretching
1024	1025	1025	1024	C-O stretching

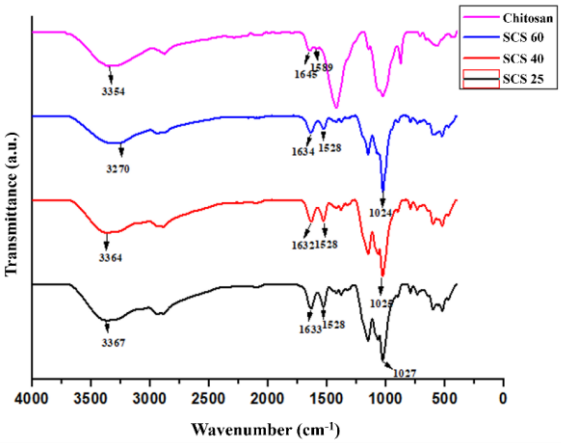

Figure 3. FTIR spectra of sulfonated chitosan

The comparison of FTIR spectra between chitosan and sulfonated chitosan at various temperatures indicates a shift in wavenumbers, suggesting that the sulfonation reaction primarily occurs in the amino group. Utilizing the ratio of C=O stretching absorption, NH bending, and S=O stretching relative to OH stretching reveals that sulfonation predominantly occurs at the amino group ($-NH_2$) of chitosan. NH and OH absorption ratios are relatively consistent, measuring 0.63 for SCS 25, 0.63 for SCS 40, and 0.60 for SCS 60. This consistency illustrates that sulfonation is more likely to occur in the amine group (C2) compared to the hydroxyl group (C6) of chitosan, as amines exhibit greater nucleophilicity than hydroxyl groups [44].

UV-Vis analysis of chitosan and sulfonated chitosan demonstrates a shift in the maximum wavelength (Figure 4). The success of the chitosan sulfonation reaction is evident from the absorption band of SCS 25, observed at a wavelength of 221 nm, which indicates the $\pi \rightarrow \pi^*$ electronic transition of the S=O chromophore group. Similarly, the successful sulfonation of chitosan at 40°C is indicated by an absorption band at 223 nm, also associated with the $\pi \rightarrow \pi^*$ of the S=O chromophore group. The peak at 295 nm reflects the $n \rightarrow \pi^*$ electronic transition of the S=O chromophore group in conjunction with the OH group. The synthesis of SCS 60 is confirmed by the absorption band at 223 nm, indicative of the $\pi \rightarrow \pi^*$ of the S=O chromophore group, along with an additional peak at 300 nm representing another $n \rightarrow \pi^*$ electronic transition. An interpretation of the maximum wavelengths for chitosan and sulfonated chitosan is presented in Table 3.

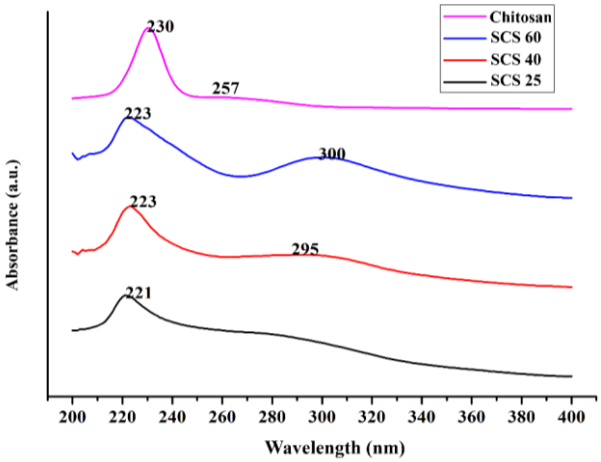

Figure 4. UV-Vis spectra of chitosan and sulfonated chitosan

3.3. Antibacterial Activity

The antibacterial activity of chitosan and sulfonated chitosan was evaluated using the disc diffusion method to determine the inhibition zones against *E. coli* and *S. aureus*. Ciprofloxacin, dissolved in a glycerol-gelatin solution in 1% acetic acid, served as the positive control, while a film solution was utilized as the negative control. The diameters of the inhibition zones for both chitosan and sulfonated chitosan are presented in Table 4.

Chitosan exhibits inhibition zones of 6 mm and 8 mm against *E. coli* and *S. aureus*, respectively. The antibacterial activity of chitosan is attributed to the presence of ammonium ions in its acetic acid solution. Generally, the antibacterial activity of sulfonated chitosan increases with the degree of substitution. Among the various formulations, SCS 60 demonstrates the largest inhibition zone compared to chitosan and other sulfonated chitosan products. The alkyl sulfonate groups in the main chain of chitosan increase its antibacterial properties [29]. Furthermore, SCS 60 exhibits greater effectiveness against gram-positive bacteria than gram-negative ones. This finding aligns with research conducted by Wang *et al.* [45], which indicates that the minimal inhibitory concentration of N-sulfopropyl chitosan against *S. aureus* is lower than that against *E. coli*. Conversely, other studies suggest that the antibacterial activity of sulfonated chitosan is stronger against *E. coli* than *S. aureus* [29, 32]. These discrepancies in antibacterial activity can be attributed to variations in the degree of substitution of sulfonated chitosan, as well as differences in the degree of deacetylation and molecular weight of chitosan employed in the synthesis of sulfonated chitosan [44, 45].

Table 3. Interpretation of UV-Vis spectra analysis for chitosan and sulfonated chitosan

Sample	λ (nm)	A	ε ($cm^{-1}M^{-1}$)	Information
Chitosan	230	2.594	1.553×10^5	π→π* transition from carbonyl group (>C=O) to amide system
	257	0.386	2.310×10^4	n→π* transition from the carbonyl group (>C=O) and the N-H group
SCS 25	221	0.290	2.638×10^5	π→π* transition of carbonyl group (>S=O) of sulfonated chitosan at 25°C
SCS 40	223	0.218	1.482×10^4	π→π* transition of carbonyl group (>S=O) of sulfonated chitosan at 40°C
SCS 40	295	0.097	6.625×10^3	n→π* transition of the carbonyl group (>S=O) and the O-H group of sulfonated chitosan at 40°C
SCS 60	223	0.318	2.893×10^4	π→π* transition of carbonyl group (>S=O) of sulfonated chitosan at 60°C
SCS 60	300	0.182	1.656×10^4	n→π* transition of the carbonyl group (>S=O) and the OH group of sulfonated chitosan at 60°C

Table 4. Diameter of the antibacterial inhibition zone

No.	Compound	Concentration	Inhibition zone diameter (mm)	
			E. coli	*S. aureus*
1	Chitosan	0.75%	6	8
2	SCS 25	0.75%	7	6
3	SCS 40	0.75%	6	7
4	SCS 60	0.75%	7	10
5	Positive control	5 µg	20	20
6	Negative control	-	-	-

*Negative control: gelatin-glycerol solution in 1% acetic acid

The polycationic nature of chitosan and its modified forms is recognized as a crucial factor influencing their antibacterial properties [30, 44, 46]. SCS 60, which contains more sulfonate groups, exhibits increased acidity compared to SCS 25, SCS 40, and chitosan. The presence of a stronger acidic group facilitates the protonation of the amino group (-NH), leading to a more rapid formation of quaternary ammonium ions. Consequently, SCS 60 possesses a stronger positive partial charge than SCS 25, SCS 40, and chitosan. This enhanced positive charge enables more effective interactions with the negatively charged components of bacterial cell membranes, such as proteins and phospholipids, ultimately resulting in bacterial cell lysis due to the release of intracellular fluid [46, 47].

3.4. Characterization and Antibacterial Properties of Films

The characteristics of films, particularly their mechanical properties, hydrophobicity, and biodegradability, are critical factors for applications in food packaging. These properties are vital in protecting food, maintaining its quality, and extending its shelf life. The mechanical properties of the film can encompass tensile strength and elongation, while hydrophobicity can be quantified through the measurement of the contact angle.

Table 5 presents the mechanical properties (tensile strength, elongation, and contact angle) of chitosan-gelatin and SCS60-gelatin films alongside their antibacterial activity during the storage of fish fillets for 24 hours. Incorporating alkyl sulfonate groups into sulfonated chitosan did not significantly impact the tensile strength of the gelatin-based films. Abd-elnaeem *et al.* [48] reported a relatively low tensile strength of 4.95 MPa for sulfonated chitosan films. However, the presence of alkyl sulfonate groups in sulfonated chitosan enhanced the elongation properties of the SCS60-gelatin film compared to the chitosan-gelatin film, likely due to the elastic nature of sulfonated chitosan. The mechanical properties of the film are crucial for protecting food from external forces, including friction during handling and transportation processes [49].

The SCS60-gelatin film demonstrated greater hydrophobicity than the chitosan-gelatin film, as evidenced by a larger contact angle measurement. The contact angle for the SCS60-gelatin film was recorded at 90.12°, in contrast to 62.8° for the chitosan-gelatin film (Table 5). Farhadian *et al.* [50] reported a contact angle of 95.3° for a film containing 3000 ppm sulfonated chitosan applied to steel, indicating its hydrophobic nature. This

increased hydrophobicity can be attributed to alkyl groups ((-CH_2)$_3$-SO_3H) within the main chain of sulfonated chitosan. Wang *et al.* [49] noted that films exhibiting contact angles greater than 65° are classified as hydrophobic. Hydrophobic films play a crucial role in food preservation by preventing freezing, inhibiting spoilage (due to their antibacterial properties), and maintaining overall food quality [49, 51].

The antibacterial activity of the films was assessed using the total plate count method to determine the ability of chitosan-gelatin and SCS60-gelatin films to inhibit bacterial growth during the storage of fish fillets. In this study, the SCS60-gelatin film effectively inhibited the growth of bacteria in fish fillets stored at 4°C for 24 hours, as compared to unpackaged fish fillets (Table 5). The bacterial growth in fish fillets was reduced to 0.3 × 10^4 cfu/g when packaged with SCS60-gelatin film, in contrast to 30 × 10^4 cfu/g observed in the absence of film packaging. The antibacterial performance of the SCS60-gelatin film was found to be superior to that of the chitosan-gelatin film. The hydrophobic nature of the SCS60 film surface contributes to its enhanced antibacterial capability compared to the chitosan-gelatin film. Gelatin is widely utilized in food packaging materials, particularly for meat and fish products, due to its biodegradable properties, antioxidant capacity, antibacterial effects, and cost-effectiveness [52, 53]. Furthermore, gelatin can be combined with chitosan and its derivatives to enhance the mechanical properties, hydrophobicity, and antibacterial properties of the films, thereby preserving nutritional value and extending the shelf life of food [44].

The SCS60-gelatin film completely degraded after 10 days, whereas the chitosan-gelatin film degraded after 8 days (Figure 5). The chitosan-gelatin film decomposes more easily in soil than the SCS60-gelatin film due to its hydrophilic nature. This hydrophilicity arises from the active groups in chitosan, specifically the -OH and NH_2 groups. These groups are polar compounds that readily bind to water [54]. Film biodegradation begins with hydration, leading to increased water absorption, which causes the film to swell. This swelling allows enzymes and microorganisms responsible for film/polymer degradation to diffuse into the film [55]. The introduction of propyl sulfonate groups to sulfonated chitosan, an alkyl group, reduces the hydrophilic properties of chitosan. This result aligns with the contact angle analysis, where the SCS60-gelatin film exhibited greater hydrophobicity compared to the chitosan-gelatin film.

Table 5. Characteristics and antibacterial properties of chitosan-gelatin and SCS60-gelatin films

Sample	Tensile strength (MPa)	Elongation (%)	Contact angle (°)	Antibacterial activity (10^4 cfu/g)
Without film	-	-	-	30.00
Chitosan-gelatin film	1.21	222.5	62.80	0.36
SCS60-gelatin film	1.18	278.0	90.12	0.30

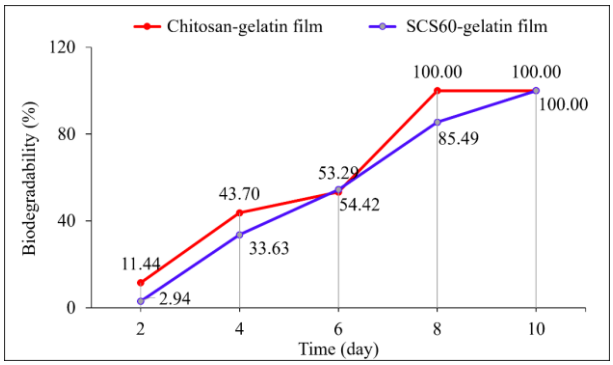

Figure 5. Biodegradability of chitosan-gelatin and SCS60-gelatin films

4. Conclusion

Based on the results of this study, the optimal temperature for synthesizing sulfonated chitosan using 1,3-propane sultone is 60°C. The resulting sulfonated chitosan product (SCS60) is a yellowish-white solid, water-soluble, with a yield of 61.46% and a degree of substitution (DS) of 33%. The antibacterial activity of SCS60 is better than that of chitosan, SCS25, and SCS40, showing inhibition zone diameters of 7 mm against *E. coli* and 10 mm against *S. aureus*. The SCS60-gelatin film effectively suppresses bacterial growth in fish to 0.3 × 10^4 cfu/g, compared to 30 × 10^4 cfu/g in unpackaged fish. Additionally, the SCS60-gelatin film exhibits better mechanical properties and hydrophobicity than the chitosan-gelatin film. Thus, sulfonated chitosan shows great potential as an antibacterial food packaging material.

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References

[1] M. S. Alamri, Akram A. A. Qasem, Abdellatif A. Mohamed, Shahzad Hussain, Mohamed A. Ibraheem, Ghalia Shamlan, Hesham A. Alqah, Ali S. Qasha, Food packaging's materials: A food safety perspective, *Saudi Journal of Biological Sciences*, 28, 8, (2021), 4490-4499 https://doi.org/10.1016/j.sjbs.2021.04.047

[2] Shraddha Karanth, Shuyi Feng, Debasmita Patra, Abani K. Pradhan, Linking microbial contamination to food spoilage and food waste: the role of smart packaging, spoilage risk assessments, and date labeling, *Frontiers in Microbiology*, 14, (2023), 1198124 https://doi.org/10.3389/fmicb.2023.1198124

[3] Jiaqi Lan, Shuo Yang, Yu Wang, Nan Guo, Xu Liu, Ketong Zhu, Pingsheng Gong, Shaowu Lv, Evaluation of Microbial Contamination in Cold Dishes and Prevalence of Foodborne Pathogens in Jilin Province, *Journal of Food Protection*, 85, 5, (2022), 728-734 https://doi.org/10.4315/JFP-21-328

[4] Tobi Fadiji, Mahdi Rashvand, Michael O. Daramola, Samuel A. Iwarere, A Review on Antimicrobial Packaging for Extending the Shelf Life of Food, *Processes*, 11, 2, (2023), 590 https://doi.org/10.3390/pr11020590

[5] Omar M. Khubiev, Anton R. Egorov, Anatoly A. Kirichuk, Victor N. Khrustalev, Alexander G. Tskhovrebov, Andreii S. Kritchenkov, Chitosan-Based Antibacterial Films for Biomedical and Food Applications, *International Journal of Molecular Sciences*, 24, 13, (2023), 10738 https://doi.org/10.3390/ijms241310738

[6] P. Fernández-Saiz, G. Sánchez, C. Soler, J. M. Lagaron, M. J. Ocio, Chitosan films for the microbiological preservation of refrigerated sole and hake fillets, *Food Control*, 34, 1, (2013), 61-68 https://doi.org/10.1016/j.foodcont.2013.03.047

[7] Rika Silvia, Sari Wahyu Waryani, Farida Hanum, Pemanfaatan kitosan dari cangkang rajungan (*Portonus sanginolentus* L.) sebagai pengawet ikan kembung (*Rastrelliger* sp) dan ikan lele (*Clarias Batrachus*), *Jurnal Teknik Kimia USU*, 3, 4, (2014), 18-24 https://doi.org/10.32734/jtk.v3i4.1651

[8] Mamiek Mardyaningsih, Aloysius Leki, Oktovianus D. Rerung, Pembuatan Kitosan dari Kulit dan Kepala Udang Laut Perairan Kupang Sebagai Pengawet Ikan Teri Segar, *Jurnal Rekayasa Proses*, 8, 2, (2016), 69-75

[9] C. O. Mohan, C. N. Ravishankar, K. V. Lalitha, T. K. Srinivasa Gopal, Effect of chitosan edible coating on the quality of double filleted Indian oil sardine (*Sardinella longiceps*) during chilled storage, *Food Hydrocolloids*, 26, 1, (2012), 167-174 https://doi.org/10.1016/j.foodhyd.2011.05.005

[10] Franklin Bonilla, Alexander Chouljenko, Vondel Reyes, Peter J. Bechtel, Joan M. King, Subramaniam Sathivel, Impact of chitosan application technique on refrigerated catfish fillet quality, *LWT*, 90, (2018), 277-282 https://doi.org/10.1016/j.lwt.2017.12.010

[11] Cai-Ling Ke, Fu-Sheng Deng, Chih-Yu Chuang, Ching-Hsuan Lin, Antimicrobial Actions and Applications of Chitosan, *Polymers*, 13, 6, (2021), 904 https://doi.org/10.3390/polym13060904

[12] Khaled M. El-Khawas, Bassam H. Mashat, Osama A. Attala, Gehan M. A. Kassem, Control of *Salmonella* and *Escherichia coli* in chilled chicken fillets using chitosan and lactic acid, *CyTA - Journal of Food*, 18, 1, (2020), 445-450 https://doi.org/10.1080/19476337.2020.1772887

[13] E. Latou, S. F. Mexis, A. V. Badeka, S. Kontakos, M. G. Kontominas, Combined effect of chitosan and modified atmosphere packaging for shelf life extension of chicken breast fillets, *LWT - Food Science and Technology*, 55, 1, (2014), 263-268 https://doi.org/10.1016/j.lwt.2013.09.010

[14] Vangelis Economou, Anestis Tsitsos, Alexandros Theodoridis, Ioannis Ambrosiadis, Georgios Arsenos, Effects of Chitosan Coatings on Controlling *Listeria* monocytogenes and Methicillin-Resistant *Staphylococcus aureus* in Beef and Mutton Cuts, *Applied Sciences*, 12, 22, (2022), 11345 https://doi.org/10.3390/app122211345

[15] M. Yildirim - Aksoy, B.H. Beck, Antimicrobial activity of chitosan and a chitosan oligomer against bacterial pathogens of warmwater fish, *Journal of Applied Microbiology*, 122, 6, (2017), 1570-1578 https://doi.org/10.1111/jam.13460

[16] Juan D. Giraldo, Bernabé L. Rivas, Direct ionization and solubility of chitosan in aqueous solutions with acetic acid, *Polymer Bulletin*, 78, 3, (2021), 1465-1488 https://doi.org/10.1007/s00289-020-03172-w

[17] Nurul Liyana Lukman Hekiem, Aliza Aini Md Ralib, Maziati Akmal Mohd Hatta, Farah B. Ahmad, Anis Nurashikin Nordin, Rosminazuin Ab Rahim, Nor Farahidah Za'bah, Effect of chitosan dissolved in different acetic acid concentration towards VOC sensing performance of quartz crystal microbalance overlay with chitosan, *Materials Letters*, 291, (2021), 129524 https://doi.org/10.1016/j.matlet.2021.129524

[18] Julia Menegotto Frick Pavoni, Cláudia Leites Luchese, Isabel Cristina Tessaro, Impact of acid type for chitosan dissolution on the characteristics and biodegradability of cornstarch/chitosan based films, *International Journal of Biological Macromolecules*, 138, (2019), 693-703 https://doi.org/10.1016/j.ijbiomac.2019.07.089

[19] Jie Xu, Kun Liu, Wei Chang, Bor-Sen Chiou, Maoshen Chen, Fei Liu, Regulating the Physicochemical Properties of Chitosan Films through Concentration and Neutralization, *Foods*, 11, 11, (2022), 1657 https://doi.org/10.3390/foods11111657

[20] Luqing Cui, Shanshan Gao, Xiaoming Song, Lianqing Huang, Hehe Dong, Jinling Liu, Fushan Chen, Shitao Yu, Preparation and characterization of chitosan membranes, *RSC Advances*, 8, 50, (2018), 28433-28439 https://doi.org/10.1039/C8RA05526B

[21] Roberto Casalini, Filippo Ghisoni, Lorenzo Bonetti, Andrea Fiorati, Luigi De Nardo, Development of acid-free chitosan films in food coating applications: Provolone cheese as a case study, *Carbohydrate Polymers*, 331, (2024), 121842 https://doi.org/10.1016/j.carbpol.2024.121842

[22] Riccardo Toscani, Chitosan Film Formulations for Food Packaging: A Comparative Study of Acidic and Acid-Free Films, School of Industrial and Information Engineering, Politecnico di Milano, 2023

[23] Jianlong Wang, Shuting Zhuang, Chitosan-based materials: Preparation, modification and application, *Journal of Cleaner Production*, 355, (2022), 131825 https://doi.org/10.1016/j.jclepro.2022.131825

[24] Meenakshi Yadav, Babita Kaushik, Gyaneshwar K. Rao, Chandra Mohan Srivastava, Dipti Vaya, Advances and challenges in the use of chitosan and its derivatives in biomedical fields: A review, *Carbohydrate Polymer Technologies and Applications*, 5, (2023), 100323 https://doi.org/10.1016/j.carpta.2023.100323

[25] Suryani Suryani, Anis Yohana Chaerunisaa, I Made Joni, Ruslin Ruslin, Vica Aspadiah, Anton Anton, Ari Sartinah, La Ode Ahmad Nur Ramadhan, The Chemical Modification to Improve Solubility of Chitosan and Its Derivatives Application, Preparation Method, Toxicity as a Nanoparticles, *Nanotechnology, Science Applications*, 17, (2024), 41-57 https://doi.org/10.2147/NSA.S450026

[26] Weixiang Liu, Yukun Qin, Pengcheng Li, Design of Chitosan Sterilization Agents by a Structure Combination Strategy and Their Potential Application in Crop Protection, *Molecules*, 26, 11, (2021), 3250 https://doi.org/10.3390/molecules26113250

[27] Xiaoxiang Han, Zhehao Zheng, Chen Yu, Yunli Deng, Qing Ye, Fuge Niu, Qing Chen, Weichun Pan, Yanbo Wang, Preparation, characterization and antibacterial activity of new ionized chitosan, *Carbohydrate Polymers*, 290, (2022), 119490 https://doi.org/10.1016/j.carbpol.2022.119490

[28] Lin Wang, Yu Pang, Zhongwen Su, Meihua Xin, Mingchun Li, Yangfan Mao, Synthesis of N-isonicotinic sulfonate chitosan and its antibiofilm activity against *E. coli* and *S. aureus*, *Carbohydrate Research*, 542, (2024), 109194 https://doi.org/10.1016/j.carres.2024.109194

[29] Zhimin Sun, Changgu Shi, Xiangyang Wang, Qiang Fang, Jianying Huang, Synthesis, characterization, and antimicrobial activities of sulfonated chitosan, *Carbohydrate Polymers*, 155, (2017), 321-328 https://doi.org/10.1016/j.carbpol.2016.08.069

[30] Chee-Shan Chen, Wan-Yu Liau, Guo-Jane Tsai, Antibacterial Effects of N-Sulfonated and N-Sulfobenzoyl Chitosan and Application to Oyster Preservation, *Journal of Food Protection*, 61, 9, (1998), 1124-1128 https://doi.org/10.4315/0362-028X-61.9.1124

[31] Han-Fen Huang, Chien-Fang Peng, Antibacterial and antifungal activity of alkylsulfonated chitosan, *Biomarkers and Genomic Medicine*, 7, 2, (2015), 83-86 https://doi.org/10.1016/j.bgm.2014.09.001

[32] Jianying Huang, Yuhong Liu, Lijun Yang, Fengyan Zhou, Synthesis of sulfonated chitosan and its antibiofilm formation activity against *E. coli* and *S. aureus*, *International Journal of Biological Macromolecules*, 129, (2019), 980-988 https://doi.org/10.1016/j.ijbiomac.2019.02.079

[33] George A. F. Roberts, Julian G. Domszy, Determination of the viscometric constants for chitosan, *International Journal of Biological Macromolecules*, 4, 6, (1982), 374-377 https://doi.org/10.1016/0141-8130(82)90074-5

[34] Shobhan Sabnis, Lawrence H. Block, Improved infrared spectroscopic method for the analysis of degree of *N*-deacetylation of chitosan, *Polymer Bulletin*, 39, (1997), 67-71 https://doi.org/10.1007/s002890050121

[35] S. Bagheri-Khoulenjani, S. M. Taghizadeh, H. Mirzadeh, An investigation on the short-term biodegradability of chitosan with various molecular weights and degrees of deacetylation, *Carbohydrate Polymers*, 78, 4, (2009), 773-778 https://doi.org/10.1016/j.carbpol.2009.06.020

[36] Eli Rohaeti, Anna Rakhmawati, Application of *Terminalia catappa* in Preparation of Silver Nanoparticles to Develop Antibacterial Nylon, *Oriental Journal of Chemistry*, 33, 6, (2017), 2905-2912 http://dx.doi.org/10.13005/ojc/330625

[37] Jan Hudzicki, Kirby-Bauer Disk Diffusion Susceptibility Test Protocol, *American Society for Microbiology*, 15, 1, (2009), 1-23

[38] Shun-Hsien Chang, Ying-Ju Chen, Hsiang-Jung Tseng, Hsin-I Hsiao, Huey-Jine Chai, Kuo-Chung Shang, Chorng-Liang Pan, Guo-Jane Tsai, Antibacterial Activity of Chitosan–Polylactate Fabricated Plastic Film and Its Application on the Preservation of Fish Fillet, *Polymers*, 13, 5, (2021), 696 https://doi.org/10.3390/polym13050696

[39] Ramón Román-Doval, Sandra P. Torres-Arellanes, Aldo Y. Tenorio-Barajas, Alejandro Gómez-Sánchez, Anai A. Valencia-Lazcano, Chitosan: Properties and Its Application in Agriculture in Context of Molecular Weight, *Polymers*, 15, 13, (2023), 2867 https://doi.org/10.3390/polym15132867

[40] Casper Van Poucke, Aurèle Vandeputte, Sven Mangelinckx, Christian V. Stevens, Green mechanochemical synthesis of water-soluble *N*-sulfonated chitosan, *Green Chemistry*, 25, 11, (2023), 4271-4281 https://doi.org/10.1039/D3GC00549F

[41] T. M. Tamer, A. M. Omer, M. M. Sabet, M. Elsayed Youssef, A. I. Hashem, M. S. Mohy Eldin, Development of polyelectrolyte sulfonated chitosan-alginate as an alternative methanol fuel cell membrane, *Desalination and Water Treatment*, 227, (2021), 132-148 https://doi.org/10.5004/dwt.2021.27290

[42] Tan Wang, Yue Zhou, Weiguo Xie, Lingyun Chen, Hua Zheng, Lihong Fan, Preparation and anticoagulant activity of N-succinyl chitosan sulfates, *International Journal of Biological Macromolecules*, 51, 5, (2012), 808-814 https://doi.org/10.1016/j.ijbiomac.2012.07.029

[43] Syang-Peng Rwei, Chia-Chun Lien, Synthesis and viscoelastic characterization of sulfonated chitosan solutions, *Colloid and Polymer Science*, 292, 4, (2014), 785-795 https://doi.org/10.1007/s00396-013-3115-6

[44] Anton R. Egorov, Anatoly A. Kirichuk, Vasili V. Rubanik, Vasili V. Rubanik, Alexander G. Tskhovrebov, Andreii S. Kritchenkov, Chitosan and Its Derivatives: Preparation and Antibacterial Properties, *Materials*, 16, 18, (2023), 6076 https://doi.org/10.3390/ma16186076

[45] Zhaodong Wang, Liuchun Zheng, Chuncheng Li, Shaohua Wu, Yaonan Xiao, Preparation and antimicrobial activity of sulfopropyl chitosan in an ionic liquid aqueous solution, *Journal of Applied Polymer Science*, 134, 26, (2017), 44989 https://doi.org/10.1002/app.44989

[46] Anna Guarnieri, Micaela Triunfo, Carmen Scieuzo, Dolores Ianniciello, Elena Tafi, Thomas Hahn, Susanne Zibek, Rosanna Salvia, Angela De Bonis, Patrizia Falabella, Antimicrobial properties of chitosan from different developmental stages of the bioconverter insect *Hermetia illucens*, *Scientific Reports*, 12, (2022), 8084 https://doi.org/10.1038/s41598-022-12150-3

[47] Dazhong Yan, Yanzhen Li, Yinling Liu, Na Li, Xue Zhang, Chen Yan, Antimicrobial Properties of Chitosan and Chitosan Derivatives in the Treatment of Enteric Infections, *Molecules*, 26, 23, (2021), 7136 https://doi.org/10.3390/molecules26237136

[48] Sara gamil Abd-elnaeem, Azza Ibrahim Hafez, kamel M. El-Khatib, Heba Abdallah Mohamed, Mai Mohamed Kamal El Din Fouad, Ehab Abadir, Parameters Affecting Synthesis of Sulfonated Chitosan Membrane for Proton Exchange Membrane in Fuel Cells, *Egyptian Journal of Chemistry*, 67, 5, (2024), 191-204 https://doi.org/10.21608/ejchem.2023.230907.8651

[49] Hongxia Wang, Fuyuan Ding, Liang Ma, Yuhao Zhang, Edible films from chitosan-gelatin: Physical properties and food packaging application, *Food*

Bioscience, 40, (2021), 100871 https://doi.org/10.1016/j.fbio.2020.100871

[50] Abdolreza Farhadian, Mikhail A. Varfolomeev, Alireza Shaabani, Saeed Nasiri, Iskander Vakhitov, Yulia F. Zaripova, Vladimir V. Yarkovoi, Aleksander V. Sukhov, Sulfonated chitosan as green and high cloud point kinetic methane hydrate and corrosion inhibitor: Experimental and theoretical studies, *Carbohydrate Polymers*, 236, (2020), 116035 https://doi.org/10.1016/j.carbpol.2020.116035

[51] Xin Li, Fenghong Li, Xuan Zhang, Weiyuan Tang, Mingzheng Huang, Qun Huang, Zongcai Tu, Interaction mechanisms of edible film ingredients and their effects on food quality, *Current Research in Food Science*, 8, (2024), 100696 https://doi.org/10.1016/j.crfs.2024.100696

[52] Marina Ramos, Arantzazu Valdés, Ana Beltrán, María Carmen Garrigós, Gelatin-Based Films and Coatings for Food Packaging Applications, *Coatings*, 6, 4, (2016), 41 https://doi.org/10.3390/coatings6040041

[53] Yanan Lu, Qijun Luo, Yuchan Chu, Ningping Tao, Shanggui Deng, Li Wang, Li Li, Application of Gelatin in Food Packaging: A Review, *Polymers*, 14, 3, (2022), 436 https://doi.org/10.3390/polym14030436

[54] Weiping Ban, Jianguo Song, Dimitris S. Argyropoulos, Lucian A. Lucia, Influence of Natural Biomaterials on the Elastic Properties of Starch-Derived Films: An Optimization Study, *Industrial & Engineering Chemistry Research*, 45, 2, (2006), 627-633 https://doi.org/10.1021/ie050219s

[55] Natalia Wrońska, Nadia Katir, Marta Nowak-Lange, Abdelkrim El Kadib, Katarzyna Lisowska, Biodegradable Chitosan-Based Films as an Alternative to Plastic Packaging, *Foods*, 12, 18, (2023), 3519 https://doi.org/10.3390/foods12183519