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HETEROGENEOUS SULFATION REACTIONS OF LOW MOLECULAR WEIGHT CHITOSAN

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In this study, the heterogeneous sulfation of low molecular weight chitosan (LMWC, Mw = 12.2 kDa, DP = 73, PDI = 1.25) using sulfuric acid in isopropanol medium was systematically investigated. A series of sulfated chitosan derivatives were synthesized under varying reaction parameters, including temperature (-30 to +5 °C), time (1–24 h), and H_2SO_4 molar ratios (1.0–8.0 mmol/mmol GlcNU). The molecular characteristics of the obtained products varied within a wide range: DP = 58-73, DS = 0.026–1.289, sulfur content = 0.51–14.16%, SO_3Na = 1.64–45.58%, with yields ranging from 10.12% to 80.12%. FTIR spectroscopy confirmed successful sulfation through characteristic $-SO_3^-$ absorption bands at 1220, 1060, 987, and 814 cm^{-1}, indicating that sulfation predominantly occurred at the C-6 position of glucosamine residues via hydroxyl and amino groups. The optimal reaction conditions were determined to be 4.0 mmol/mmol H_2SO_4 per glucosamine unit at -20 °C for 12 hours, ensuring high DS values and minimal depolymerization. Elemental analysis supported FTIR findings and showed high correlation between reaction variables and functional group incorporation. These results demonstrate that controlled heterogeneous sulfation of LMWC in isopropanol is an effective method to produce water-soluble, highly substituted chitosan derivatives with preserved backbone integrity. The obtained derivatives are promising for further biomedical applications such as drug delivery, anticoagulant formulations, and bioactive coatings. This study provides a clear understanding of how reaction parameters influence structural and functional outcomes in chitosan sulfation chemistry.

Keywords: low molecular weight chitosan, heterogeneous sulfation, degree of polymerization, degree of substitution, sulfuric acid, isopropanol, rate of sulfation.

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Introduction

Chitosan is a widely distributed polysaccharide in nature, primarily obtained by the alkaline deacetylation of chitin [1]. It is a linear cationic polysaccharide composed of glucosamine and N-acetylglucosamine residues [2]. Chitosan is extensively used as an important polymer in various fields such as food, cosmetics, biotechnology, medicine, pharmaceuticals, and environmental protection. Its broad applicability is attributed to its biodegradability, biocompatibility, non-toxicity, and its specific binding ability to cell lectins, making it a promising biopolymer for enhancing the efficacy and delivery of various bioactive compounds [3–5]. These features make chitosan a versatile natural polymer of particular importance in the development of next-generation biomaterials [6]. However, the poor solubility of high molecular weight chitosan at neutral pH and its limited biological activity restrict its practical applications [7]. Therefore, in recent years, the development of LMWC and the introduction of various functional groups into its polysaccharide backbone have gained increasing attention. These modified derivatives are considered valuable for obtaining effective therapeutic agents with wide applicability. Such chitosan derivatives are characterized by improved water solubility, enhanced bioactivity, and stable physicochemical properties. They are considered promising in drug delivery systems, tissue engineering, and the development of biocompatible materials [8–11].

Among the various modification methods, sulfation reactions are regarded as one of the most promising approaches, as they enable the synthesis of biologically active compounds similar to natural glycosaminoglycans such as heparin and dermatan sulfate [12, 13]. Sulfated chitosan derivatives exhibit binding affinities to growth factors, cytokines, and cell receptors, making them suitable for targeted drug delivery and controlled release systems

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[14, 15]. Their biological activity mainly depends on the number, position, and distribution of sulfate groups, molecular weight, and macromolecular conformation [16].

Currently, various approaches have been developed for chitosan sulfation, including homogeneous methods using SO_3·pyridine or chlorosulfonic acid complexes in amide-type solvents (DMF, DMAc/LiCl, DMSO), as well as heterogeneous techniques in aqueous alcohol or biphasic systems. Homogeneous methods allow high degrees of substitution but often lead to chain degradation due to the harsh conditions, while heterogeneous systems are considered milder and better preserve the polysaccharide backbone. In addition, alternative strategies using sulfamic acid, carbodiimide-mediated activation, or enzymatic sulfation have also been explored in recent years [11, 17, 18].

In recent years, heterogeneous sulfation techniques have garnered increasing attention in the field of polysaccharide modification due to several key advantages. These methods offer operational simplicity and are particularly favorable for thermally sensitive substrates, as they reduce the risk of polymer degradation during processing. Furthermore, heterogeneous systems often enable better regioselectivity, allowing for the targeted introduction of sulfate groups at specific hydroxyl positions within the polysaccharide backbone, which can significantly influence the material's biological and physicochemical properties [19, 20]. Despite these advantages, the sulfation outcome is highly dependent on various reaction parameters, including the type and concentration of sulfating agents, reaction temperature, time, and the nature of the solvent system employed. For instance, using different solvents such as dimethylformamide (DMF), dimethyl sulfoxide (DMSO), or alcohol-based systems like isopropanol can lead to substantial differences in product solubility, sulfation degree, and the positional distribution of sulfate groups [18, 21]. Consequently, a comprehensive and systematic investigation into these reaction conditions is essential to gain precise control over the sulfation pattern and the overall structure of the modified polysaccharide. Such studies not only facilitate the optimization of synthetic protocols but also provide a fundamental understanding necessary for tailoring sulfated polysaccharide derivatives with desired functionalities for biomedical, pharmaceutical, or industrial applications.

In the present study, the heterogeneous sulfation of LMWC was systematically investigated using a sulfuric acid/isopropanol solvent system. The main objective was to explore the effects of varying reaction parameters-including temperature, duration, and reagent concentration-on the structural and physicochemical characteristics of the resulting sulfated chitosan derivatives. The sulfation reactions were carried out under controlled low-temperature conditions to minimize thermal degradation and preserve the integrity of the polysaccharide backbone. To evaluate the outcome of the modification process, comprehensive characterization of the sulfated products was performed. Fourier-transform infrared (FTIR) spectroscopy was used to confirm the successful incorporation of sulfate groups and to identify key functional group transformations within the chitosan molecule. In addition, elemental analysis was employed to quantitatively determine the sulfur content, which was then used to calculate the degree of substitution (DS) and assess the extent of sulfation. The analytical results enabled the establishment of a clear relationship between reaction conditions and the structural-functional features of the sulfated chitosan products. Specifically, correlations were observed between temperature and DS, sulfation rate, and molecular integrity, allowing the identification of optimal parameters that yield high sulfation efficiency while maintaining favorable molecular characteristics. These findings provide a valuable foundation for designing sulfated chitosan-based materials with tailored biological properties, which could be leveraged in various biomedical applications such as drug delivery, wound healing, and regenerative therapies. Thus, the outcomes of this study contribute significantly to the ongoing development of next-generation bioactive and biocompatible chitosan derivatives.

Experimental part

The following reagents were used in the experiments: low molecular weight chitosan (LMWC, powder, DP = 73, Mw = 12.2 kDa; Sigma-Aldrich Chemie GmbH, China), isopropyl alcohol (reagent grade, TS6-09-402-87), sulfuric acid (96%, reagent grade, GOST 14262-78), and sodium hydroxide (analytical grade, GOST 4328-77).

Heterogeneous sulfation of LMWC with sulfuric acid in isopropanol medium. Isopropanol was placed into a three-neck flask equipped with a thermometer, dropping funnel, and stirrer, which was then immersed in an ice bath at -20 °C. Concentrated sulfuric acid was added dropwise to the isopropanol (volume ratio isopropanol: sulfuric acid = 1 : 0.05). After stirring for 10 minutes, LMWC (Mw = 12.2 kDa, DP = 73, PDI = 1.25) [22] was added to the reaction mixture (molar ratio chitosan: sulfuric acid = 1.0 : 1.0–8.0). The reaction temperature was gradually raised to between -30 and +5 °C, and sulfation was carried out for 1 to 24 hours. Upon completion, the reaction mixture was filtered under vacuum using a glass filter, and excess sulfuric acid was removed by washing with acetone. The obtained product was neutralized by dissolving in 1 M NaOH solution with stirring and left stirring for 1 hour to ensure complete neutralization. Subsequently, the sulfated chitosan derivatives were purified from impurities by dialysis using a dialysis membrane (MWCO 1000 Da, Spectrum Laboratories, USA) and then dried by lyophilization.

Determination of molecular weight. The molecular weight values and molecular weight characteristics of the samples were determined by gel chromatography. Analyses were performed using an Agilent 1260 Infinity II chromatographic system equipped with the following components: Agilent 1260 Infinity II Iso Pump (G7110B) (Agilent Technologies, GmbH, Germany), Suprema Lux 1000 Å (8 × 300 mm, 5 µm) and Suprema Linear S (8 × 300 mm, 5 µm) columns (PSS Polymer Standards Service GmbH, Germany), G7800A 1260 GPS/SEC MDS detector, UV-VIS detector (G7115A 1260 DAD WR), refractive index detector (1260 Infinity II RI), light scattering detector (1260 Infinity II Dual Angle LS), and viscometer detector (1260 Infinity II Viscometer). Chromatographic analyses were conducted at 40 °C. The eluent consisted of an aqueous solution of 0.1 M $NaNO_3$ and 8.0 mol/L NaN_3. Column calibration was performed using pullulan standards (Sigma-Aldrich Chemie GmbH, Germany).

The degree of polymerization (DP) of the samples was calculated based on their molecular weight and the molecular weight of the polysaccharide monomer (Mr (monomer)) using the following formula:

$$DP = \frac{M\omega}{Mr(monomer)}$$

Determination of elemental composition. The degree of substitution (DS) of carbon atoms by sulfate groups in the samples, as well as the percentage content of sulfur, nitrogen, and carbon atoms (S%, N%, C%) in the samples, were determined using a EuroEA3000 CHNS-O elemental analyzer (EuroVector Srl, Italy).

The degree of substitution (DS) was calculated using the following formula:

$$DS = \frac{Ms \cdot (100 - S\%)}{Mm \cdot S\%}$$

where Ms is the molecular weight of the sulfate group, Mm is the molecular weight of the monomer unit, and S% is the sulfur content determined by elemental analysis.

Product Yield Formula:

$$\text{Yield (\%)} = \frac{\text{Actual amount of product obtained (mg)}}{\text{Theoretical amount of product expected (mg)}} \cdot 100\%$$

Explanation: Actual amount of product obtained (mg): The mass of the product actually obtained from the experiment.

Theoretical amount of product expected (mg): The maximum possible mass of the product calculated based on stoichiometry or reaction equations.

Results and Discussion

In this study, the sulfation reactions of LMWC using isopropanol/sulfuric acid were investigated. The reaction proceeds via an electrophilic substitution mechanism between the sulfating reagent and the -OH groups of chitosan. The sulfation reaction of chitosan with isopropanol/sulfuric acid is illustrated in Figure 1.

Sulfation reactions of LMWC using H_2SO_4 were carried out in an isopropanol medium. The study was performed using a LMWC sample with a degree of polymerization (DP) of 73 (molecular weight = 12.2 kDa) at temperatures ranging from -20 to +5 °C, over 1–24 hours, with H_2SO_4 amounts varying from 1.0 to 8.0 mmol per mmol of D-glukozamin (GlcN) unit (GlcNU). Sulfated products with different molecular weights (MM = 9.38–12.0 kDa, DP = 58–73, degree of substitution (DS) = 0.026–1.289, yield 10.12–80.12%) were obtained. The effects of temperature, reaction duration, amount of sulfating reagent, and reaction medium on the sulfation of LMWC were investigated. Changes in DS and DP values, sulfation rate, and product yield were studied during the reaction. Reaction conditions and molecular sizes of the resulting samples are presented in Table.

As seen from the table, when the sulfating reagent amount and reaction time remained constant (H_2SO_4 1.0 mmol/mmol GlcNU, 1 hour), changing the temperature significantly affected the molecular sizes of the obtained products. A decrease in temperature led to an increase in the DS values of the products. When the temperature was lowered to -20 °C, the DS value increased sharply up to 0.377; further lowering the temperature to -30 °C resulted in only a slight increase to 0.383. In this case, the increase in DS with decreasing temperature is related to the higher sulfation rate at lower temperatures. This correlation is supported by the observation that the sulfation rate at -20 °C was 0.0063 mmol/min, while at -30 °C it was slightly higher at 0.0064 mmol/min (Fig. 2a).

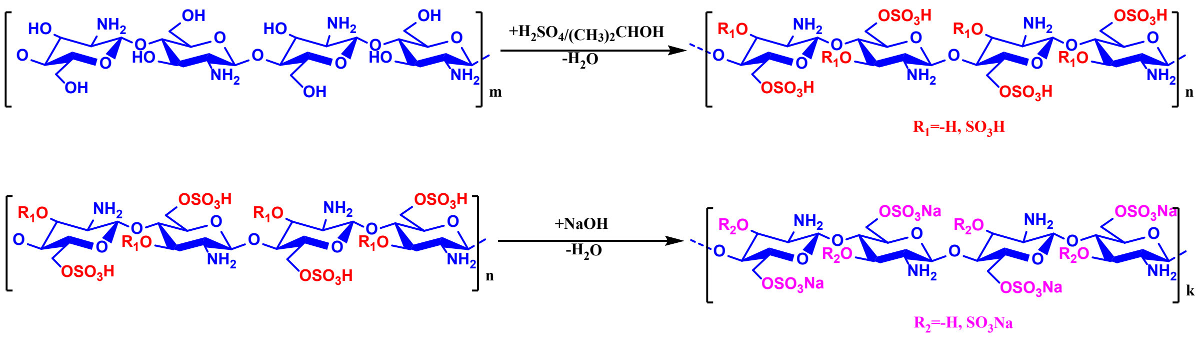

Fig. 1. Heterogeneous sulfation reaction of LMWC in isopropanol/sulfuric acid medium

Sulfation conditions of LMWC in isopropanol/sulfuric acid medium and molecular sizes of the obtained products

Sample	Reaction conditions			Results				
	H_2SO_4, mmol/mmol GlcNU	Temperature, °C	Time, hours	DP	S, %	SO_3Na, %	DS	Yield, %
LMWC	–	–	–	73	–	–	–	–
LMWC-SO_3Na1	1	+5	1	61	0.51	1.64	0.026	10.12
LMWC-SO_3Na2	1	0	1	65	1.32	4.33	0.069	20.25
LMWC-SO_3Na3	1	–5	1	68	2.82	9.08	0.156	29.41
LMWC-SO_3Na4	1	–10	1	71	4.43	14.26	0.260	34.37
LMWC-SO_3Na5	1	–20	1	72	6.05	19.47	0.377	37.21
LMWC-SO_3Na6	1	–30	1	73	6.13	19.73	0.383	39.90
LMWC-SO_3Na7	1	–20	4	72	7.50	24.14	0.496	48.86
LMWC-SO_3Na8	1	–20	8	71	8.85	28.45	0.620	57.36
LMWC-SO_3Na9	1	–20	12	69	10.06	32.33	0.745	63.86
LMWC-SO_3Na10	1	–20	16	67	10.57	34.02	0.802	65.81
LMWC-SO_3Na11	1	–20	20	65	10.70	34.44	0.817	66.52
LMWC-SO_3Na12	1	–20	24	63	10.77	34.66	0.825	66.87
LMWC-SO_3Na13	2	–20	12	68	11.85	38.14	0.958	69.17
LMWC-SO_3Na14	3	–20	12	64	12.67	40.78	1.169	74.05
LMWC-SO_3Na15	4	–20	12	62	13.83	44.51	1.244	78.13
LMWC-SO_3Na16	6	–20	12	59	14.01	45.10	1.274	79.71
LMWC-SO_3Na17	8	–20	12	58	14.16	45.58	1.289	80.12

The decrease in reaction temperature from +5 to -30 °C caused the degree of polymerization (DP) of the obtained product to change from 61 to 72, and the product yield ranged from 10.12 to 39.90%. This indicates a low degree of depolymerization of the polysaccharide chain during the reaction. The degree of substitution (DS), sulfation rate, and yield of the obtained product were inversely proportional to temperature; an increase in temperature led to a decrease in these parameters. The growth rates of these values were highest at -20 °C and changed only slightly at -30 °C. Therefore, -20 °C was selected as the optimal temperature for this sulfation reaction (Fig. 2b).

Under conditions where sulfuric acid amount and temperature remained constant (H_2SO_4 1.0 mmol/mmol GlcNU, -20 °C), the small molecular weight chitosan sample was sulfated for 12 hours. During the initial period of the reaction, a sharp increase in the DS of the obtained samples was observed. For example, within the first 12 hours, the DS increased sharply to 0.745, while further prolongation of the reaction time resulted in a slight increase, reaching 0.825 after 24 hours.

Moreover, during the study of the effect of reaction time on sulfation rate, it was found that the sulfation rate was high during the initial 12 hours of the reaction and reached a maximum value (0.00827 mmol/min) during the first 4 hours. In later stages, the sulfation rate decreased, although it remained relatively high between 8 and 12 hours (Fig. 3a). This indicates that the sulfation rate of the small molecular weight chitosan solution was highest during the initial 12 hours after adding sulfuric acid. This can be explained by the fact that at the early stages of the reaction, the medium contained a high amount of sulfuric acid and available chitosan -OH groups, resulting in a higher sulfation rate. Over time, as the amount of reactive -OH groups and sulfuric acid decreased, the sulfation rate decreased correspondingly.

Analysis of DS values of the obtained products showed that during the initial 1–12 hours of the reaction, 18.53–31.39% of the added H_2SO_4 was consumed. Partial reduction in the polysaccharide chain DP was also observed during the reaction. Additionally, the product yield increased during the first 12 hours, reaching 63.86%, and changed slightly afterwards, reaching 66.87% at 24 hours (Fig. 3b).

The obtained results showed that the increase in the product's degree of substitution (DS), sulfation rate, and yield was high during the first 12 hours and changed little afterward. Therefore, 12 hours is sufficient for the sulfation of small molecular weight chitosan, which helps prevent excessive depolymerization of the polysaccharide chain.

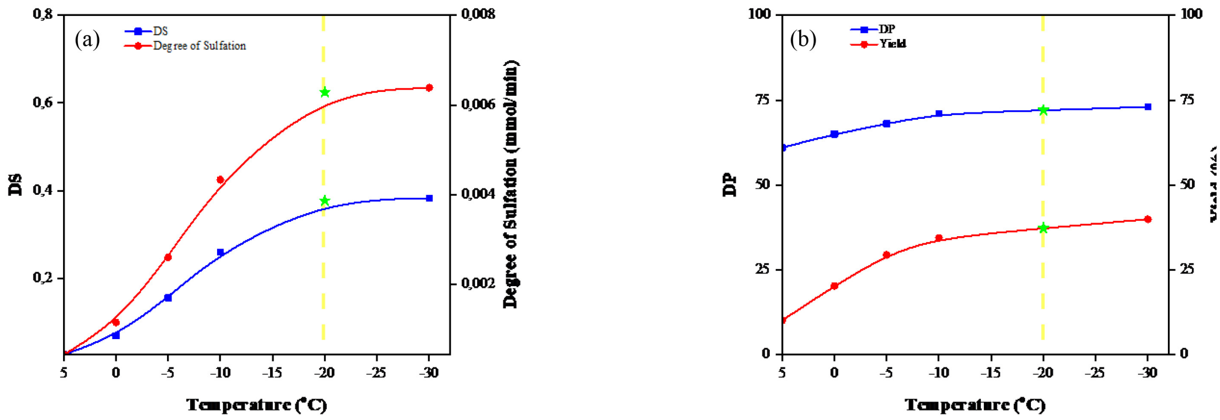

Fig. 2. Changes in the degree of substitution (DS) and sulfation rate (a), as well as degree of polymerization (DP) and product yield (b) of LMWC sulfated with sulfuric acid in isopropanol medium under the effect of temperature (H_2SO_4 1.0 mmol/mmol GlcNU, reaction time 1 hour)

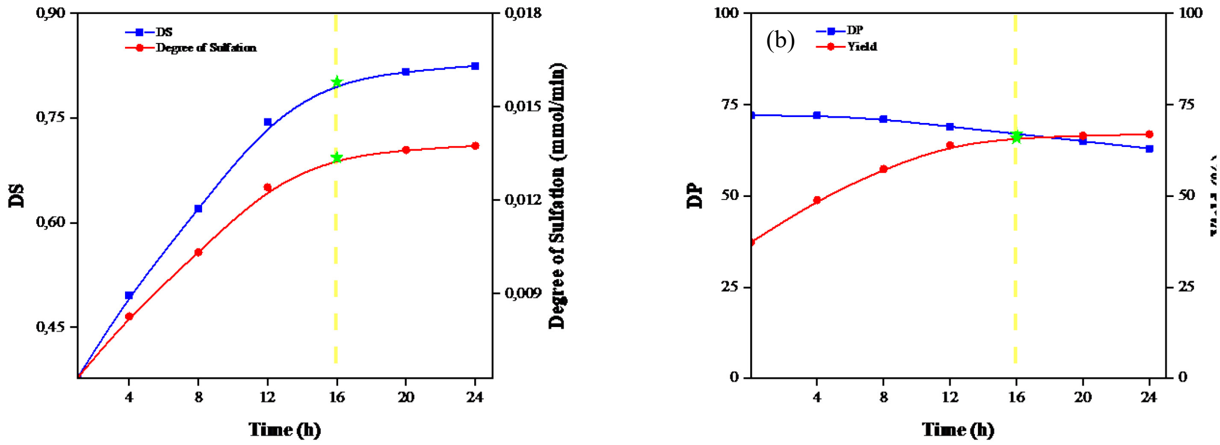

Fig. 3. Changes in the degree of substitution (DS) and sulfation rate (a), as well as degree of polymerization (DP) and yield (b) of small molecular weight chitosan during sulfation with sulfuric acid in isopropanol medium over reaction time (H_2SO_4 1.0 mmol/mmol GlcNU, temperature -20 °C)

Furthermore, in the next phase of the study, sulfation reactions were carried out at the optimal temperature and time (-20 °C, 12 hours) with varying amounts of H_2SO_4 (1.0–8.0 mmol/mmol GlcNU). The effect of sulfuric acid concentration on the product's DS, degree of polymerization (DP), and yield was investigated. Under constant temperature and reaction time (-20 °C, 12 hours), increasing the sulfuric acid concentration led to an increase in DS values. When the amount of H_2SO_4 was increased up to 4.0 mmol/mmol GlcNU, a sharp increase in DS up to 1.244 was observed. Further increases in sulfuric acid concentration resulted in minor changes in DS, with a maximum DS of 1.289 obtained at 8.0 mmol/mmol GlcNU (Fig. 4a).

from 0.958 to 1.244 were obtained (Table 1). Additionally, the influence of sulfuric acid concentration on the DP values of the products was studied. It was found that increasing H_2SO_4 concentration led to a decrease in DP values, but no sharp decline was observed (Fig. 4b). These results demonstrate that sulfation reactions carried out in isopropanol medium yield products with high DS values and relatively stable DP values.

The structures of the sulfated chitosan products were studied using FTIR spectroscopy, and the FTIR spectra of the obtained samples are shown in Figure 5. The results demonstrated that the FTIR spectra of chitosan and its sulfated derivatives differ from each other.

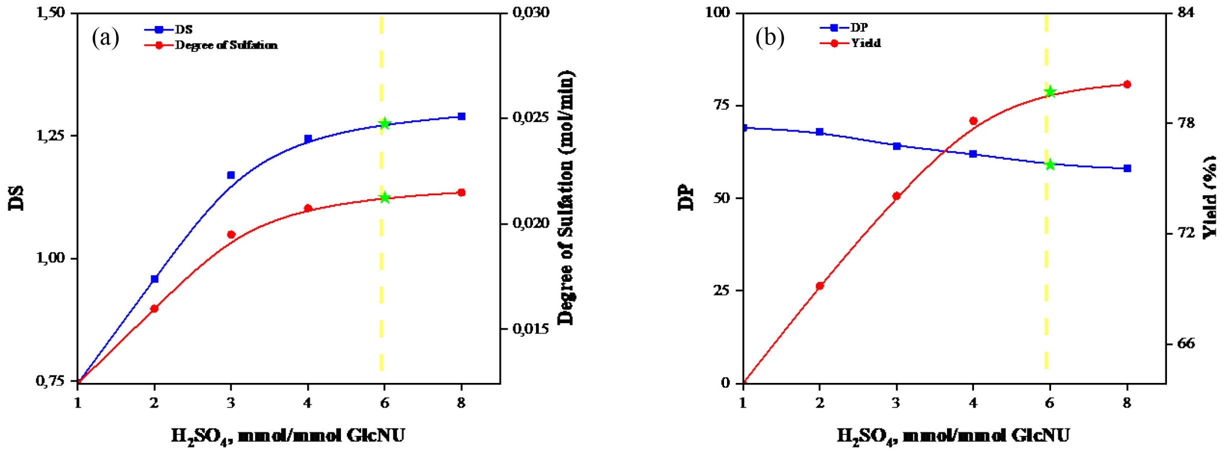

Fig. 4. Changes in the degree of substitution (DS) and sulfation rate (a), as well as polydispersity index (PDI) and product yield (b) of LMWC sulfated with sulfuric acid in isopropanol medium with increasing H_2SO_4 amount (temperature -20 °C, time 12 hours)

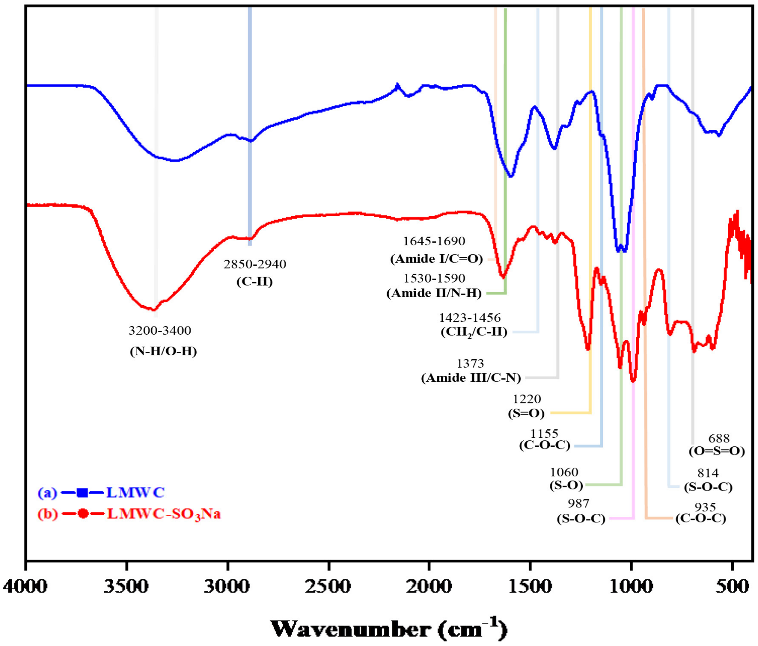

Fig. 5. FTIR spectra of chitosan and its sulfated derivatives (Spectra: (a) LMWC, Mw = 12.2 kDa, PD = 73; (b) LMWC-SO_3Na15

Absorption bands characteristic of symmetric and asymmetric stretching vibrations of O-H and N-H bonds in the monosaccharide residues of the samples were observed in the FTIR spectrum region of 3200–3400 cm^{-1}, while absorption bands corresponding to the stretching vibrations of H-C-H bonds were found in the 2850–2940 cm^{-1} region [23]. Signals characteristic of the stretching vibrations of -N-C=O bonds in N-acetyl groups were observed in the 1645–1690 cm^{-1} regions. Absorptions in the 1530–1590 cm^{-1} regions corresponded to the stretching vibrations of amid II -C-N-H bonds [24]. The C-H bonds of $-CH_2$ groups in the monosaccharide residue rings of the chitosan chain appeared in the spectra within the 1423–1456 cm^{-1} region. The absorption band near 1373 cm^{-1} corresponds to symmetric deformation vibrations of methyl ($-CH_3$) groups in sulfated chitosan samples [25]. These vibrations mainly relate to the remaining N-acetyl ($-NHCOCH_3$, amid III) groups in the deacetylated parts of the chitosan molecule. The presence of this absorption band indicates that acetyl residues still remain in the modified chitosan chain, i.e., the degree of deacetylation is not complete. Additionally, the absorption at 1373 cm^{-1} confirms that the main modification during sulfation occurred through amino or hydroxyl groups, while methyl groups did not undergo chemical changes, serving as an important spectral marker.

A strong absorption band around 1220 cm^{-1} in sulfated chitosan samples is characteristic of vibrations of the sulfate group ($-SO_3^-$), specifically the asymmetric stretching vibrations of the S=O bond, confirming the presence of sulfate groups in the modified chitosan chain. Absorptions corresponding to the stretching vibrations of C-O-C bonds in the monosaccharide residue rings of the chitosan chain were observed around 1155 cm^{-1} [26]. The absorption band near 1060 cm^{-1} in the FTIR spectra of sulfated chitosan corresponds to asymmetric stretching vibrations of the S-O bond in the sulfate group ($-SO_3^-$). The absorption around 987 cm^{-1} corresponds to the stretching and, in some cases, deformation vibrations of the S-O-C bonds in sulfated chitosan samples. This signal is especially pronounced when sulfation occurs at the C-6 position of chitosan chains or secondary hydroxyl groups. The absorption at 935 cm^{-1} is characteristic of deformation vibrations of β(1,4)-glycosidic bonds in the chitosan chain [27]. The absorption band observed at 814 cm^{-1} in sulfated chitosan samples corresponds to deformation vibrations specific to the $-SO_3^-$ group.

This signal is typically detected in sulfated polysaccharide derivatives, particularly when sulfation occurs at the C-6 position. The absorption at 688 cm^{-1} reflects deformation vibrations related to the O=S=O bonds of the sulfate group ($-SO_3^-$) in the sulfated chitosan sample [28].

Conclusion

Various samples of LMWC with different molecular sizes (DP = 58–73, S% = 0.51–14.16%, SO_3Na% = 1.64–45.58%, DS = 0.026–1.289, product yield 10.12–80.12%) were obtained through heterogeneous sulfation reactions. The study confirmed the feasibility of conducting heterogeneous sulfation of LMWC with H_2SO_4 in isopropanol media. The effects of reaction conditions on the molecular sizes of the resulting products were investigated. Furthermore, the optimal reaction conditions for sulfating LMWC were identified as using 4.0 mmol/mmol H_2SO_4 per glucosamine unit at -20 °C for 12 hours in an isopropanol medium. FTIR analyses confirmed the presence of sulfate groups ($-SO_3^-$) in the sulfated chitosan samples, their attachment at the C-6 position, and that modification primarily occurred via hydroxyl groups. Additionally, the spectra showed the presence of residual acetyl groups and preservation of the structural integrity of the chitosan chain.

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Conflict of Interest

The authors of this work declare that they have no conflicts of interest.

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