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Original Article

PRODUCTION AND CHARACTERIZATION OF CHITOOLIGOSACCHARIDE HYDROLYSATE PREPARED FROM *CHITOSANASE* ENZYME OF MARINE ISOLATES

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ABSTRACT

Objective: The present study was carried out to develop an enzymatic hydrolysate with unique biological properties targeting diabetic foot ulcers.

Methods: *Chitosanase*-producing organisms were isolated and used to create chitooligosaccharide hydrolysate. Various techniques, such as FTIR, NMR, and X-ray diffraction, were used. Antimicrobial activity was tested using disc diffusion and well diffusion methods. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) were determined through the Chitooligosaccharide-Broth Dilution Method.

Results: The study identified marine mud samples and isolated S9, S15, and SF12 as significant sources of *chitosanase* production. The partially purified chitosanolytic enzymes produced by these isolates were hydrolyzed in a 1% chitosan solution at 180 °C, revealing more prominent antimicrobial activity. The Chitooligosaccharide Hydrolysate (COS) preparation was fixed at 45 °C, pH 5.5, for 180 min. The chitosanase enzyme was soluble in four solvents and insoluble in ethanol, acetone, and diethyl ether. All COS hydrolysates prepared showed antimicrobial activity against foot ulcer pathogens, *Pseudomonas sp.,* and *Candida albicans*. S9 COS showed higher activity than SF12 hydrolysates against foot ulcer pathogens. The COS hydrolysate showed significantly stronger antimicrobial activities than chitosan and *chitosanase*.

Conclusion: The present study concludes that COS hydrolysate and its biological functions are applicable for diabetic foot ulcer treatment. Further investigation into the efficacy of COS against diverse infectious pathogens is needed.

Keywords: COS, Marine origin, Diabetic foot ulcer, Fourier transform infrared spectroscopy (FT-IR), Nuclear magnetic resonance (NMR), X-ray diffraction (XRD) analysis, Antimicrobial activity

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INTRODUCTION

The impact of the role of the biological activities of Chitooligosaccharide Hydrolysate (COS), chitooligomers, or chitosan oligomers is determined by the acetylated units, chain length, chemical modification, and their charge [1, 2]. COS has received increased attention in its pharmaceutical and medical applications due to its nontoxicity, high solubility properties, and biological and physiological effects. COS are prepared enzymatically using chitosanase and other non-specific enzymes. Enzymatic hydrolysis with various enzymes, including glycanases, proteases, and lipases derived from bacterial, fungal, mammalian, and plant sources [3]. After the enzymatic or chemical degradation of chitosan, the COS produced contains a mixture of oligomers. Several techniques for separating and purifying COS have been reported, such as gel filtration, ultrafiltration, ion exchange, and metal affinity chromatography [4, 5]. While the previously listed methods are necessary to achieve homogenous COS fractions, it usually takes a lot of effort and time to produce pure COS fractions. To reduce the application cost, further research has been conducted on enzymatic hydrolysate, a mixture of COS after enzymatic hydrolysis. This motivated carrying out the current study.

To overcome the drawbacks, low-molecular-weight chitosan and COS are preferred in several applications as antibacterial agents [6–8] and for antifungal effects [9]. It has been reported that chitooligomers of lower molecular weight exhibit better biological activities than chitosan [10]. COS and its derivatives exert antimicrobial effects against different groups of microorganisms, such as bacteria, fungi, and yeast [11]. The reactive functional groups of COS (an amino group as well as both primary and secondary alcoholic OH groups) at their C-2, C-3, and C-6 positions, respectively, during chemical derivatization result in increased antibacterial activity [12].

Chitosanase (EC 3.2.1.132) is an enzyme that hydrolyzes the β-(1-4) linkages in chitosan. They are metabolically active since they hydrolyze high-molecular-weight chitosan into shorter COS, which can be transported inside the cell and, in turn, used as a carbon and nitrogen source. Another function of *chitosanase* is the protection of microorganisms against the antimicrobial activity of chitosan. *Chitosanase* may act as a shield by hydrolyzing the high-molecular-weight chitosan into these short-chain chitosan forms, since neither low-molecular-weight chitosan nor COS exhibit inhibitory effects [13].

Based on their substrate specificity towards chitosan, *chitosanase* has been classified into subclasses I, II, and III [14]. Enzymes of subclass I hydrolyze A-D and D-D linkages; subclass II can hydrolyze D-D linkages only; whereas subclass III hydrolyzes D-A and D-D linkages. Subclass I enzymes were found in both families 46 and 75, and subclass III enzymes in family 46 [15].

Chitosanase exhibits two types of cleavages, such as endo and exotype. The exotype *chitosanase* activity releases a single glucosamine residue and glucosamine oligomers. On the other hand, the endotype *chitosanase* has a high specific activity for producing functional chitooligosaccharides with a high degree of polymerization. The derivatives of chitosan and their enzymatic products have different structures and physicochemical properties, which result in novel bioactivities or findings of bioactive compounds [16]. It is impossible to establish an exact COS for the biological activities seen in various uses. There has been a surge in research on the biological properties of chitosan and its oligomers, with the idea that no single type of chitosan or oligomer possesses all of the properties mentioned above. Therefore, the main objective of our work was to develop an enzymatic hydrolysate with unique biological properties targeting diabetic foot ulcers.

MATERIALS AND METHODS

Chemicals and reagents

Nutrient Agar and Ethanol, Ethyl acetate, Diethyl ether, Methanol were purchased from Merkand Co. Antibiotics were obtained from Hi Media.

Isolation of chitosanase producing organisms and preparation of chitooligosaccharides hydrolysate

The muddy and dry soil samples (marine region) were collected from different places in Tamil Nadu and Pondicherry. To determine the activity of enzyme production, *chitosanase* detection agar plates were employed and observed for clear zone formation. Quantitative estimation of *chitosanase* activity was determined by the reduction of sugar, and the protein quantification of the crude extract was measured by the DNS and lowry methods, respectively. The biologically active COS hydrolysate mixture was prepared by incubating 1% chitosan with a partially purified *chitosanase* enzyme for 10 min to 48 h against the pathogens [17-19].

Characteristics of chitooligosaccharide hydrolysate

Solubility test

The solubility nature of the COS hydrolysate was tested at room temperature with multiple solvents with concentrations of 5 mg/ml, which include ethanol, water, ethyl acetate, glacial acetic acid, and diethyl ether [20].

Fourier Transform-InfraRed spectroscopy (FT-IR) analysis

An infra-red spectrophotometer was used to evaluate COS hydrolysate samples between 4000 and 400 cm^{-1} KBr pellets to identify their functional groups. The infrared spectra of chitosan oligosaccharide samples were analysed and compared with those of commercial chitosan [21].

X-ray diffraction (XRD) analysis

A PAN analytical X-ray diffractometer with Cu K (α) radiation of 1.54187 nm wavelength was employed to measure the X-ray diffraction patterns in the samples [22]. They were performed at 40 kV and 30 mA with a 2θ time constant, and the scanning was completed at a 2º anglebetween 20º and 80º at 0.02 deg min149^{-1}.

Nuclear magnetic resonance (NMR) spectroscopy

The samples were dissolved in D_2O, and then NMR spectra were recorded on a Varian Mercury Vx300 spectrometer at ambient temperature to determine the isotopic ratio of $^{13}C/^{12}C$ in the acetyl groups of each sugar residue [23].

Collection of test pathogens

The antimicrobial-resistant strains such as *Escherichia coli*, *Pseudomonas* sp., *Staphylococcus aureus*, and *Candida* sp. isolated from the diabetic foot ulcer samples were procured from KMCH (Coimbatore) and CMC (Vellore).

Determination of antimicrobial resistance pattern by disc diffusion assay

The antibiotic susceptibility pattern of the selected clinical pathogens was determined by the Kirby-Bauer disc diffusion method on Mueller Hinton (MHA) agar. The zone of inhibition defines sensitivity or resistance to different antimicrobial agents [24]. The antibiotics selected for the assessment were Penicillin-G (10 units), Ampicillin (10µg), Cloxacillin (30µg), Cephalexin (30µg), Cefuroxime (30µg), Ceftazidime (30µg), Cefepime, Cefeperazone/Sulbactum, Erythromycin (15µg), Gentamicin (10µg), Amikacin (30µg), Ciprofloxacin (5µg), Ofloxacin (5µg), Norfloxacin (10µg), Cotrimoxazole (25µg), Vancomycin (30µg), linezolid (30µg), Netromycin (10µg), Omnatax (5µg), Piperacillin/Tazobactum (10 µg), Amoxycilin/Clavulanic Acid (10 µg), and Imipenem (10 µg).

Antimicrobial activity of crude enzyme, chitosan and COS by well diffusion method

To determine the antimicrobial activity, the overnight broth cultures *of Escherichia coli, Pseudomonas sp., Staphylococcus aureus,* and *Candida sp*. were used [25, 26]. Wells were cut on the nutrient agar plates, and pathogens were streaked on these plates. The enzyme solution, chitosan, and COS hydrolysate were added in different concentrations (10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 µl), and then the plates were incubated at 30 °C for 24 h. After the incubation period, the zone of inhibition was measured and tabulated.

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of chitooligosaccharide-broth dilution method

The chitosan oligosaccharide hydrolysate samples were diluted to final concentrations of 0.025, 0.05, 0.25, 0.5, 1.0, 2.0, 4.0, 5.0, 10.0, 15.0, 25.0, 50.0, 75.0, and 100 mg/ml. About 100 µl of 105 CFU/ml of the test culture was inoculated in tubes with an equal volume of nutrient broth and COS hydrolysate samples. The tubes were incubated aerobically at 37 °C for 24 h. Two control tubes were maintained for each strain (media control and organism control). The dilutions that showed no turbidity were incubated further for 24 h at 37 °C. The lowest concentration that produces no visible turbidity after 48 h of incubation is regarded as the final MIC. The MBC value was determined by sub-culturing the test dilution (which had no visible turbidity) on freshly prepared nutrient agar media. The plates were incubated for 24–48 h at 37 °C. The highest dilution that yields no single bacterial colony is considered MBC [27, 28].

RESULTS AND DISCUSSION

Preparation of chitooligosaccharides hydrolysate

In this study, the marine mud sample was identified, and the isolates S9, S15, and SF12 were found to be the most significant source for *chitosanase* production isolation. The partially purified chitosanolytic enzymes produced by the isolates were hydrolyzed in a 1% chitosan solution at various incubation times at 180 °C. It possesses more prominent antimicrobial activity than the hydrolysate obtained at other time intervals. COS hydrolysate prepared by partial hydrolysis of chitosan provides an important biologically active derivative, as per [29], supporting our results. The enzymatic hydrolysate consists of mixtures of oligomers, and the increasing concentration of the monomer is inversely proportionate to the concentration of tetramers and pentamers with hydrolysis time and vice versa [30].

The antimicrobial activity of the monomer is lesser in comparison with the mixture, which was again matched with our results that the hydrolysate antimicrobial activity decreases with the hydrolysis time, and the reason might be due to the increase in the monomer concentration [31]. Hence, for the COS hydrolysate preparation in the present study, hydroxylation with the *chitosanase* enzyme of the isolates was fixed at 45 °C, pH 5.5, for 180 min.

Characterization of chitooligasaccharide hydrolysate by analytical and spectroscopic studies

Solubility test for Chitooligosaccharide hydrolysate samples

From the results (table 1), it was proven that COS was soluble in four solvents, such as distilled water, ethyl acetate, and glacial acetic acid, and was insoluble in ethanol, acetone, and diethyl ether. The COS dissolved in water immediately, but in ethyl acetate and glacial acetic acid only with constant stirring for an hour and 30 min, respectively [32]. From the result, it is evident that the COS water solubility proved that the chitosan has been hydrolyzed since it is insoluble in water [33].

Fourier transform-InfraRed spectroscopy (FT-IR) analysis

From the spectrum analysis, the peak values of S9 were analyzed by comparing the standard spectrum chart available in the NIST library (fig. 1). The peak signal recorded at 723.95 may be due to C-H deformation vibration (carbohydrates) [34], and the sharp peak observed at 723.95, 756.10, and 802.39 cm^{-1} indicates the presence of C-H stretch vibration [35]. The peaks at 979.84 cm^{-1}, 910.40 cm^{-1}, and 879.54 cm^{-1}, revealed the presence of carbon and hydrogen bonds (C-H deformation). Again, the vibration stretches up to 1604.77, 1404.18, and 1172.72 cm^{-1}, showing the presence of alcohol and ketone (C-H, C-O, and C=N stretch) [36]. A frequent band appears at 3008.95, 2823.79, and 2785.21 cm, indicating the presence of alkyl groups (CH_3 stretch). The SF12 peak signal at 733.34, 756, and 803 cm^{-1} and the S15 peak at 759.77 and 804 cm^{-1} were due to C-H stretch vibration. The peaks of SF12 and S15 at 912.34, 950.21 cm^{-1}, and 1031.65 cm^{-1}, respectively, confirm the presence of carbon and hydrogen bonds (C-H deformation). The

bands at 2875, 2940.54 cm1 of SF12 and 2781.25, 2945.13 cm^{-1}show the presence of alkyl groups (CH_3 stretch) [37].

The peak was observed at 1320, 1443, and 1330.01. cm^{-1} of S9, SF12, and S15, respectively, which confirmed the acetyl group and, aligned with the results reported about these peaks, indicated an increase in the degree of acetylation.

The peaks were between 1420 and 1435 cm1, which confirmed the secondary structural changes in the polysaccharide were also similar to our report [38]. The analysis of the spectrum shows typical absorption bands corresponding to NH stretching of carbohydrates, proteins, and peptide bonds [39], which provides concrete evidence that the substance contained carbohydrates in its structure.

Table 1: Solubility pattern of the chitooligosaccharides hydrolysate

S. No.	Samples	Solvents	Soluble	Time taken for solubility
1	S9	Water	Yes	Immediate
		Ethanol	No	No
		Diethyl ether	No	No
		Ethyl acetate	Yes	60 min with constant stirring
		Glacial acetic acid	Yes	30 min with constant stirring
2	S15	Water	Yes	Immediate
		Ethanol	No	No
		Diethyl ether	No	No
		Ethyl acetate	Yes	60 min with constant stirring
		Glacial acetic acid	Yes	30 min with constant stirring
3	SF12	Water	Yes	Immediate
		Ethanol	No	No
		Diethyl ether	No	No
		Ethyl acetate	Yes	60 min with constant stirring
		Glacial acetic acid	No	30 min with constant stirring

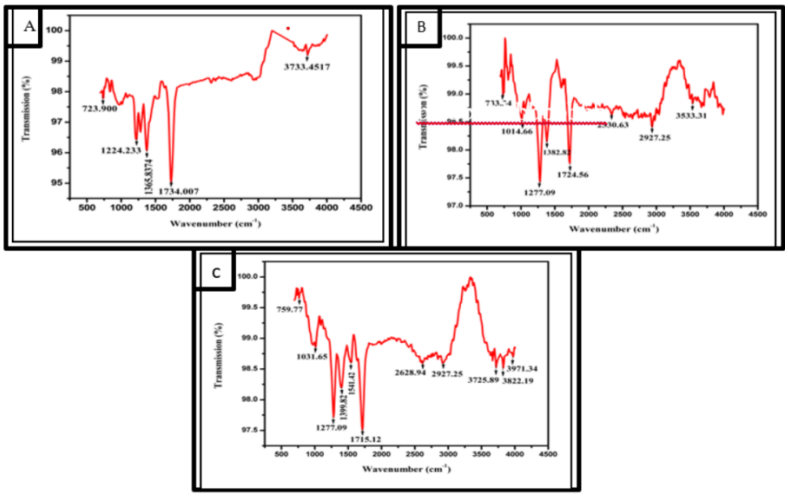

Fig. 1: FTIR pattern of chitooligosaccharides hydrolysate, a) S9 COS, b) S15 COS and c) SF12 COS

X-ray diffraction (XRD) analysis

The diffractogram of S9 showed a diffused peak, indicating its amorphous nature. The diffraction pattern of S9 represents less intense crystalline peaks, especially those situated between 20 and 30 (2θ). The S9 mainly indicated the ''annealed'' type pattern identified by the characteristic diffraction peaks at 21°, 33°, 45°, and 66°. S15 showed the characteristic peaks at 21°, 34°, and 45° (fig. 2). The peaks observed were similar to the results [40], where a new solid phase with a lower degree of crystallinity has been formed due to the breakdown of the original crystal lattice of the carbohydrate or polysaccharide.

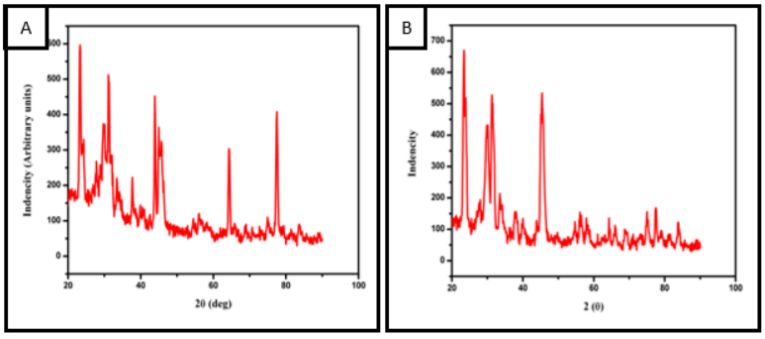

Fig. 2: X-ray diffraction pattern of the chitooligosaccharides hydrolysate, a) S9 b) S12

Nuclear magnetic resonance (NMR) spectroscopy

Fig. 3 shows the ^{13}C NMR spectra of COS in D2O. The six-strong signals of S9 were observed at 98.07, 56.26., 72.65, 77.6, 75.4, and 61.01 ppm attributed to carbon residues at C1, C2, C3, C4, C5, and C6 positions, respectively, whereas signals of S12 were observed at 100, 58.98, 72.6, 79.1, 77.4, and 62.18 ppm at respective C1, C2, C3, C4, C5, and C6 positions. The orientation of the residues of the acetyl group on C1, C2, C3, C4, C5, and C6 positions confirms the structure of oligosaccharides based on data found in the literature [41].

The increase in the peak height of the CH3 position and the C = O position of the S9 and S12 mixture with that of chitosan indicates an increase in the degree of acetylation [42].

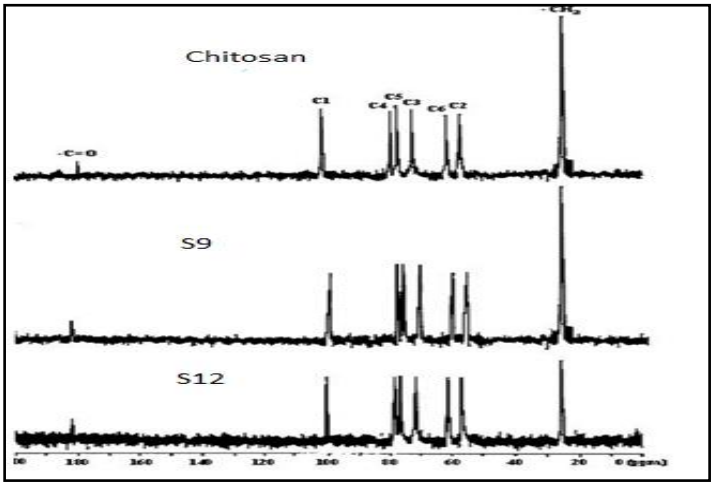

Fig. 3: NMR spectrum studies of S9, S12 and chitosan

Antimicrobial activity of the foot ulcer pathogens by Kirby-Bauer disc diffusion method

The antimicrobial activity of diabetic foot ulcer pathogens exhibited multiple drug resistances, which included Penicillin-G (10µg), Ampicillin (10µg), Cloxacillin (30µg), Cephalexin (30µg), Cefuroxime (30µg), Ceftazidime (30µg), Cefepime, Cefeperazone/Sulbactum, Erythromycin (15µg); Gentamicin (10µg); Amikacin (30µg); Ciprofloxacin (5µg); Ofloxacin (5µg); Norfloxacin (10µg); Cotrimoxazole (25µg); Vancomycin (30µg); linezolid (30µg); Netromycin (10µg) and Omnatax (5µg), Piperacillin/Tazobactum (10µg), Amoxycilin/Clavulanic acid (10µg) and Imipenem (10µg).

The COS hydrolysate prepared from the enzymatic hydrolysis with *chitosanase* was checked for its antimicrobial activity against *E. coli*, *S. aureus*, *Pseudomonas* sp., and *Candida albicans* (table 2 and fig. 4).

Table 2: Antimicrobial activity of the chitosan, chitosanase and chitooligosaccharides hydrolysate against diabetic foot ulcer pathogens by well diffusion method

Isolates	Inhibitory zone in diameter (mm)					
	Chitosan (100µl)		Chitosanase (100µl)		COS (100µl)	
	E. coli	*S. aureus*	*E. coli*	*S. aureus*	*E. coli*	*S. aureus*
SF12	11.04±0.10	17.88±0.02				
S 15			1.96±0.41	0.86+0.01	22.45±0.14	24.56±0.31
S 9			-	-	32.12±0.21	28.12±0.11
S 12			-	-	14.87±0.01	14.05±0.51

The values are means triplicates determinations (N=3,). Data are Presented mean ± standard deviation.

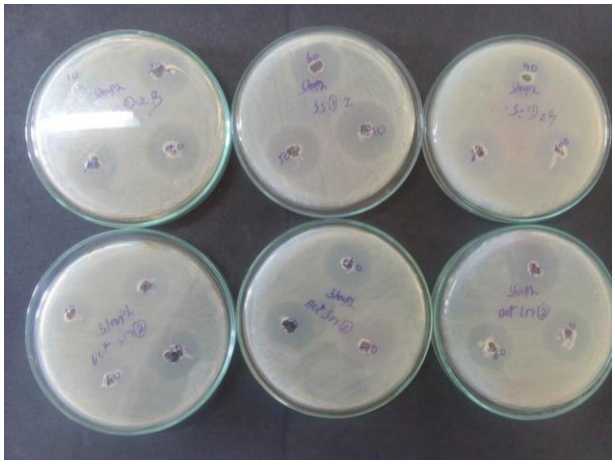

Fig. 4: Antimicrobial activity of *Staphylococcus aureus* at various concentrations

All the COS hydrolysates prepared at 180 °C showed antimicrobial activity and were then subjected to antibacterial activity at a concentration of 100 µl of 1 mg/ml against foot ulcer pathogens as well as *Pseudomonas* sp. and *Candida albicans*. Among the entire prepared COS, S9 COS showed higher activity than SF12 hydrolysates against the foot ulcer pathogens.

Table 4: Minimum Inhibition concentration (*Staphylococcus aureus)*

S. No.	Samples	Concentration (µl)											
		0.04	0.09	0.19	0.39	0.78	1.56	3.12	6.25	12.5	25	50	100
1	S15	-	-	-	-	+	+	+	+	+	+	+	+
2	S12	-	-	+	+	+	+	+	+	+	+	+	+
3	Chitosan	-	-	-	+	+	+	+	+	+	+	+	+
4	S9	-	+	+	+	+	+	+	+	+	+	+	+

(-)Absent, (+) Present

Table 5: Minimum Inhibitory concentration against *E. coli*

S. No.	samples	Concentration (µl)											
		0.04	0.09	0.19	0.39	0.78	1.56	3.12	6.25	12.5	25	50	100
1	Chitosan	-	-	-	+	+	+	+	+	+	+	+	+
2	S15	-	-	+	+	+	+	+	+	+	+	+	+
3	S12	-	-	-	+	+	+	+	+	+	+	+	+
4	S9	+	+	+	+	+	+	+	+	+	+	+	+

(-)Absent, (+) Present

The two concentrations (0.5 mg/ml and 1 mg/ml) of the COS were taken for the good diffusion method, and MIC range from 10µl to 100µl. The higher antimicrobial activity was observed with 1 mg/ml against foot ulcer pathogens. S15 and SF12 COS showed similar activity on both E. coli and S. aureus (Tables 5), whereas S9 COS showed higher activity over *E. coli* than *S. aureus.* The COS hydrolysate showed significantly stronger antimicrobial activities than chitosan and chitosanase. The inhibitory zones of COS hydrolysate were significantly higher than those of chitosan and chitosanase.

The present results exactly match those of the COS obtained by the partial enzymatic hydrolysis of chitosan with *chitosanase* [43], proven to have more effective antibacterial activity than the highly hydrolyzed chitosan materials. The water-soluble chitosan exhibited antibacterial activity against both Gram-positive and Gram-negative bacteria [44-46], which was similar in the present investigation except for S9. Our present investigation has controversial results [47] on the antibacterial effect of the COS hydrolysate that was greater than the chitosan and also disproved the results of multiple studies [48-51].

CONCLUSION

Thus, our findings signify considerable progress in the investigation of COS hydrolysates and their biological functions. The chemical nature of the substance has been determined by spectral analysis. Additionally, the findings of this study may shed light on the optimal concentration range required to produce COS hydrolysate. By evaluating its MIC and MBC, the current study demonstrated that the slightly hydrolyzed chitosan hydrolysate was more active than the chitosan and highly hydrolyzed chitooligomers. This information might serve as an outline for further investigation into the efficaciousness of COS against diverse infectious pathogens.

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ABBREVIATION

COS-Chitooligosaccharide, NMR-Nuclear Magnetic Resonance, FTIR-Fourier Transform Infrared Spectroscopy, XRD-X ray Diffraction, *S. aureus-Staphylococcus aureus, E-coli-Escherichia coli.*

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AUTHORS CONTRIBUTIONS

PV completed the research work plan, review of the literature and Manuscript writing. She did the Manuscript corrections and agree to the manuscript's published version.

CONFLICTS OF INTERESTS

The authors declared no conflicts of interest.

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