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Original Research

The research determines appropriate parameters in the synthesis process of syringic acid grafted chitooligosaccharides

Bui Van Hoai[1,2], Dai-Nghiep Ngo[1,3,*]

ABSTRACT

Introduction: The aim of this study is to determine appropriate parameters in the synthesis of syringic acid onto chitooligosaccharides (COSs) with an ascorbic acid/hydrogen peroxide redox pair in order to obtain the derivative with the highest grafting degree. **Methods:** In this study, syringic acid grafted COSs, catalysed by an ascorbic acid/hydrogen peroxide redox pair were investigated. The synthesis conditions were investigated, including the mass ratio between syringic acid and COSs, pH, temperature and synthesis time. Characteristics of the derivative were evaluated by Thin Layer Chromatography (TLC), Ultraviolet-Visible (UV-vis) and FourierTransform Infrared (FT-IR) spectroscopy. The activities of COSs and derivative were evaluated by antimicrobial ability. **Results:** The results showed, that the best conditions for the synthesis were the mass ratio between syringic acid and COSs at 0.5:1, pH 5, temperature 27^{o}C, for 6 hours with grafting degree at 32%. The TLC assay showed, that free ascorbic acid and syringic acid are not present in the product. The UV-vis and FT-IR data confirmed, that syringic acid was successfully conjugated onto COSs. Furthermore, the antibacterial assay showed that syringic acid grafted onto COSs had minimum inhibitory concentration against foodborne pathogenic bacteria at 1%. **Conclusion:** The syringic acid onto chitooligosaccharides were successfully synthesized by free radical mediated grafting method with an ascorbic acid/hydrogen peroxide redox pair. The grafting degree of syringic acid onto COSs was greatly affected by many factors, including COSs, syringic acid, pH, as well as temperature and time of reaction. Moreover, the new derivative showed enhanced antibacterial capabilities, as compare to free COSs.

Key words: chitooligosaccharides, FTIR, antimicrobial, syringic acid, UV-vis

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INTRODUCTION

Chitin is the second most abundant polysaccharide, mainly extracted from the exoskeleton of sea creatures, such as crayfish, lobster, prawns, crab and shrimp. Besides, various microorganisms also produce chitin (for instance, cell walls of fungi and yeasts contain chitin). Chitin, in the form of films and fibers, is used in numerous applications (e.g. biosensor)[1]. Chitin shows various bioactivities, such as antioxidative[2], immunostimulanting[3], enzyme inhibitory, antimicrobial, anticancer and anticholesteremic activities[4]. Chitosan, a partially deacetylated polymer of N-acetyl glucosamine, is prepared by alkaline deacetylation from chitin, with the degree of deacetylation of chitin reaching about 50%[1]. Chitosan is also biologically active and possesses antioxidative[5], antimicrobial[6,7], immunity enhancing[8] and anticancer[9] features. Due to its non-toxic, non-antigenic, biocompatible and biodegradable properties, chitosan has wide applications in food, tissue engineering, pharmaceutical, textile, agriculture, water treatment and cosmetics industries[10]. Although chitin and chitosan have many advantages, they have poor solubility in water and thus have limited application in the living systems[11,12]. Chitooligomers (COSs), partially hydrolyzed by enzyme and chemistry methods products of chitosan, are of great interest in pharmaceutical and medicinal applications due to their non-cytotoxic and high water-soluble properties[13]. The COSs and its derivatives offer a broad spectrum of health-beneficial biological activities for humans, including anti-inflammatory, anti-oxidant, anti-cancer, anti-microbial activities[12,14,15]. In recent years, an interest to the synthesis of COSs derivatives to enhance its has increased.

Natural phenolic compounds from the plant source s and food products have been showed to possess bioactivities such as anti-oxidant, anti-microbial, anti-inflammatory, anti-mutagenic, anti-allergenic and prevention of diabetes and cancer diseases effects[17]. Grafting of phenolic compounds onto COSs in order to enhance their activity is of particular interest to researchers. The syringic acid is among the m. Until now, most of COSs synthesis uses only chemical method with carbodiimide based

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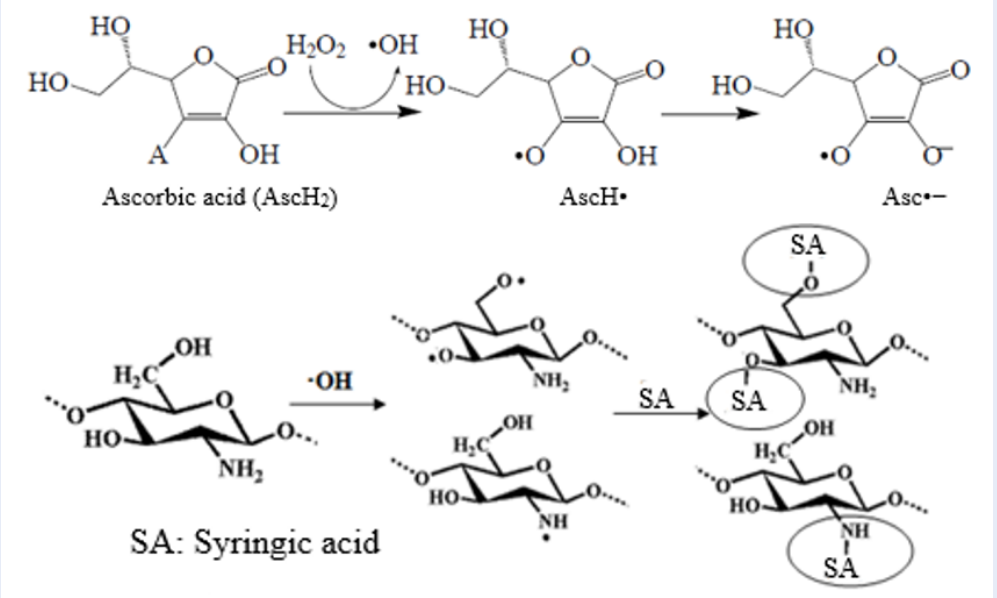

Figure 1: **Proposed mechanisms for the synthesis of syringic acid-g-COSs**[16].

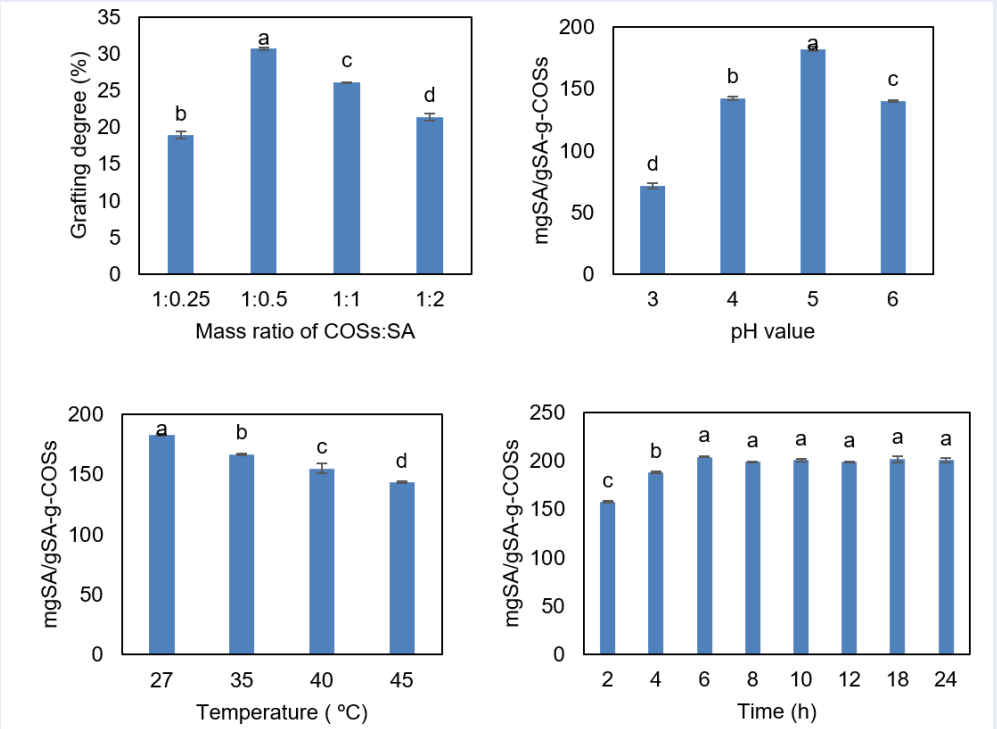

Figure 2: **The results of optimization of grafting conditions**: (**A**) Influence of COSs:SA ratio (COSs:SA from 1:0.25 to 1:2, pH 5, at 27^{o}C, for 24 hours); (**B**) Influence of pH (pH ranged from 3 to 6, COSs:SA ratio was 1:0.5, at 27^{o}C, for 24 hours); (**C**) Influence of temperature (temperature from 27^{o}C to 45^{o}C, COSs:SA ratio was 1:0.5, pH 5, for 24 hours); (**D**) Influence of time (time from 2 hours to 24 hours, COSs:SA ratio was 1:0.5, pH 5, at 27^{o}C). All statistical analyses were performed on the data, derived from three independent experiments and the data are shown as means ± SD. Means with different letters (a,b,c,d) are significantly different at $p < 0.05$ level.

chemical coupling reagents, such as 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS) reaction, or EDC and Dicyclohexylcarbodiimide (DCC) [12,14]. EDC and NHS, or EDC and DCC reagents are usually required in large amounts, considered environmentally disadvantageous and difficult to remove completely from the reaction media. As compared to these approaches, free radical mediated grafting of COSs is considered to be quicker and eco-friendlier. The redox pair system does not generate toxic reaction products and it is possible to perform the reaction at room temperature, thus avoid ing degradation of antioxidants [18]. Efficiency of synthesis is affected by many factors, such as the mass ratio of the grafting substrate to COSs, pH, temperature and time. Until now, the method of free radical mediated reagent, in order to graft syringic acid onto COSs is not reported yet. Thus, the aim of this work is to determine optimal condition in the synthesis process and to evaluate, whether syringic acid can be successfully grafted onto COSs, using ascorbic acid/hydrogen peroxide redox pair in order to improve COSs activity.

MATERIALS AND METHODS

Materials

Ascorbic acid (VWR, Leuven, Belgium), lactic acid (HiMedia, Mumbai, India), sodium bicarbonate (HiMedia, Mumbai, India), ethanol 99.99% (HiMedia, Mumbai, India), sodium hydroxyl (Merck, Darmstadt, Germany). Syringic acid (HiMedia, Mumbai, India), hydrogen peroxide (HiMedia, Mumbai, India), nutrient broth (HiMedia, Mumbai, India), nutrient agar (HiMedia, Mumbai, India), folin (Merck, Darmstadt, Germany). TLC silica gel 60 F254 (Merck, Darmstadt, Germany). All other chemicals were of analytical grade, or of the highest grade, available commercially.

We used the COSs powder, left from our previous research. Briefly, 0.8% chitosan solution was prepared in 0.8% lactic acid solution. The chitosan solution was hydrolyzed by cellulase with optimal parameters. After hydrolysis, the COSs were filtered through an ultrafiltration membrane, with membranes ranging in molecular weight cut-offs (MWCO). COSs fractionation was carried out by spray drying (SD-06AG, LabPlant, UK), in order to created COSs powder. The COSs was packed in vacuum for storage [19].

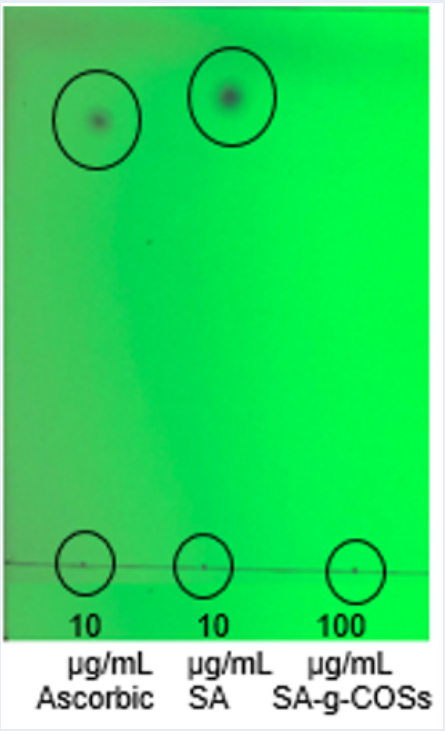

Figure 3: The TLC plate diagram of Ascorbic acid, syringic acid (SA) and SA-g-COSs. Syringic acid, ascorbic acid, and SA-g-COSs were developed on a silica gelplate () with chloroform - ethyl acetate - acetic acid (50:50:1) asmobile phase, heating at 100°C for 5 min. The developed TLC plate was observed under UV light. The experiment repeated three times.

Methods

Synthesis of syringic acid grafted chitooligosaccharides (SA-g-COSs)

The synthesis of SA-g-COSs was performed according to the method of Curcio *et al*., (2009) with slight modifications [18]. Briefly, the COSs (0.5 g) was dissolved in 25mL of water and then 1 mL of 1.0 M H_2O_2, containing 0.054 g of ascorbic acid was added. After 30 min, the syringic acid was added to the mixture at different amounts, with the mass ratios of SA, conjugated onto COSs chain being 1:0.25, 1:0.5, 1:1 and 1:2. The pH value of mixture changed from 3 to 6 and the reaction was carried out at the temperature ranging from 27°C (room temperature) to 45°C, for the periods of time, ranging from 2 to 24 hours. After the reaction, the mixture was allowed to rest at room temperature under atmospheric air, and then centrifuged with filter membrane 1 kDa in 50 mL centrifuge tube, in order to remove unreacted syringic acid and the catalyst. Finally, the resulting solution was lyophilized, using

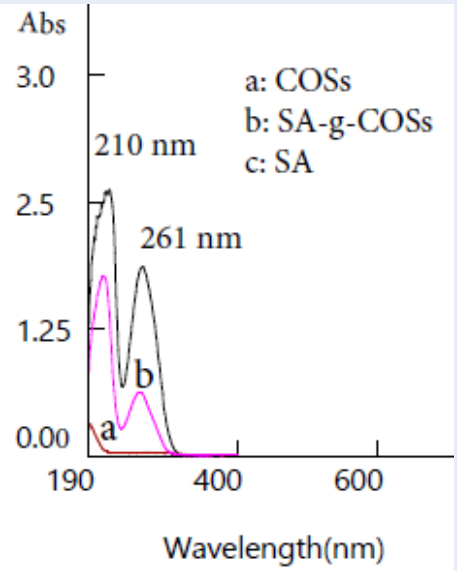

Figure 4: **The UV−vis spectra of COSs (a), SA-g-COSs (b) and Syringic acid (c).** The maximum absorbance of derivatives was determined by recording UV–vis absorption spectra (T60U UV-vis spectrophotometer, PG Instruments Ltd., US). The spectra of samples recorded at concentration of 50 ppm. The experiment repeated three times.

freeze dry system (FDU 2110, Eyela, Japan) to obtain SA-g-COSs solid samples (the moisture content of the SA-g-COSs, COSs and SA is the same).

Characterization of SA-g-COSs

To verify whether syringic acid was successfully grafted onto COSs backbones, TLC analysis was performed. Syringic acid, ascorbic acid, and SA-g-COSs were developed on a silica gel plate (TLC silica gel 60 F254, Merck, Darmstadt, Germany) with chloroform - ethyl acetate - acetic acid (50:50:1) as mobile phase, heating at 100°C for 5 min. The developed TLC plate was observed under UV light; The SA-g-COSs was characterized by UV – vis, FT-IR. The maximum absorbance of derivatives was determined by recording UV–vis absorption spectra (T60U UV-vis spectrophotometer, PG Instruments Ltd., US). Original and conjugated COSs samples (25 to 100 ppm) were dissolved in deionized water and then UV–vis absorption spectra were recorded in full scan mode from 190 to 400 nm. The FTIR spectra were recorded via the Alpha II FTIR spectrometer (Bruker, Germany). The dried sample was ground with potassium bromide (KBr) powder and pressed into a pellet for spectrometric measurement in the frequency range of 4000 – 400 cm−1 [20].

Determination of SA content in SA-g-COSs

The total syringic acid content of SA-g-COSs derivative was measured by the Folin – Ciocalteu method, describ ed by Liu, *et al*., (2013) with slight modifications [16]. Briefly, 1 mL of the sample solution was mixed with 1 mL of Folin–Ciocalteu reagent (10-fold dilution) and allowed to react at room temperature (27±2°C) for 5 min in the dark. Then, 3 mL of 2% Na_2CO_3 was added and the mixture was allowed to stand for 30 min before measuring at A760 nm. The syringic acid was used as the standard and the results were expressed as mg of syringic acid per gram of SA-g-COSs dry weight (mg SA/g SA-g-COSs). The grafting degree was calculated from the SA content in the SA-g-COSs and the utilized amount of SA in the preparation of the SA-g-COSs.

Antibacterial activity assay

The antibacterial activity of derivatives was performed using agar disk diffusion test, then minimum inhibitory concentration (MIC) values were determined [21]. Briefly, Indicator bacteria activated on nutrient broth medium (NB) (10 mL), The growth density was adjusted to match a MacFarland 0.5 standard (10^8 CFU/mL). A 1:100 diluted solution was prepared in a fresh NB and used as the inoculum (10^6 CFU/mL). Each bacterial suspension (100 µL) was uniformly spread on a nutrient agar medium disk. The five wells were formed on the surface of the agar disk [22]. 100 µL of the sample solution was poured to each well and plates were incubated for 18 h ours at 37^o C.

Statistical Analysis

Data were expressed as the mean ± standard deviation (SD) of triplicates. The least significant difference (LSD), Duncan ' s multiple range test and one-way analysis of variance (ANOVA) were used for multiple comparisons by stratigraphic centurion. The difference was statistically significant if $p < 0.05$.

RESULTS

Synthesis of syringic acid grafted chitooligosaccharides (SA-g-COSs)

The principle of synthesis of SA-g-COSs states, that ascorbic acid solution is present as a di-acid and can further react with H_2O_2 to generate hydroxyl radical (HO •), as well as resonance, stabilized tricarbonyl ascorbate free radical (AscH •). As AscH • has a pK = −0.86, it is not protonated and is present in the form of semi dehydroascorbate radical (Asc • −). Then the previously formed HO• attracts hydrogen

from amino and hydroxyl group of COSs, resulting in COSs macro radicals. Finally, the corresponding syringic acid monomers, which are in the close vicinity of reaction sites become acceptors of COSs macro radicals. Thus, forming COSs-syringic acid conjugates (**Figure 1**) [16].

The synthesis of derivative is influenced by factors, such as mass ratio of SA and COSs, pH, temperature and time. Therefore, these factors were studied by single factor experiments. As shown in **Figure 2**, the SA contents in the SA-g-COSs increased with increasing mass ratio of SA to COSs chain as 1:0.25, 1:0.5, 1:1, 1:2 were 84.00, 142.38, 228.24, 354.91 mg SA/g SA-g-COSs, respectively. The grafting degrees of SA onto COSs were 18,91%, 30.75%, 26.42%, and 21.42% with regard to 1:0.25, 1:0.5, 1:1, 1:2, respectively. According to our results, the optimal condition for the synthesis of SA-g-COSs was 1:0.5 as a mass ratio of SA to COSs chain. Because of different pH values in the synthesis process, the SA content in derivative is also different. The SA content in derivative showed an increase in pH values, ranged from 3 to 5. The SA contents at pH of 3, 4, 5, 6 were 71.47, 142.38, 182.18, 40.46 mg SA/g SA-g-COSs, respectively. These results show, that the optimal condition for the synthesis of SA-g-COSs are when pH value is 5. Our data are consistent with ascorbic acid stability in aqueous solution at pH levels from 4 to 6 [23]. The research results show that when temperature increases, the SA content tends to reduce as 183.19, 167.03, 155.11, 143.60 mg SA/g SA-g-COSs. This result suggests the most effective temperature of synthesis process be 27^o C. Regarding the time of synthesis, it was increased when in the time ranged from 2 h to 6 h and then not changed until 24 hours. Therefore, the synthesis process stopped at 6 h with the SA content of 204.40 mg SA/g SA-g-COSs, corresponding to grafting degree at 32 %.

Characterization of SA-g-COSs

The TLC result (**Figure 3**) showed that no free syringic acid in derivative was observed. Yet free syringic acid and ascorbic acid appeared on the developed plate. This demonstrated, that syringic acid successful grafted onto the COSs and this was not a mixture condition between syringic acid and COSs. The UV-vis spectra of SA, COSs, and SA-COSs are presented in **Figure 4**. The absorbance peak of SA-COSs showed, that COSs were well conjugated with SA by the ascorbic acid/hydrogen peroxide catalyst. The COSs did not yield an absorbance peak in the range from 200 to 400 nm, whereas SA-COSs had two absorbance peaks at 210 and 261 nm. This pattern is identical to the characteristic absorbance peak o f SA, which should be assigned to the π-system of the benzene ring [24], The results indicate, that SA was successfully grafted onto COSs. In the FT-IR spectra, SA-COS shows absorbance significant peaks in 1732 and 1632 cm^{-1}, implying both ester and amide stretching, respectively [25]. Thus, our FTIR spectra in **Figure 5**indicate, that syringic acid was successfully conjugated onto COSs.

Antibacterial activity

The MIC values of COSs and SA-COSs derivative were determined as an evaluation of their antimicrobial activity against selected foodborne pathogenic bacteria. The result presented in **Table 1** shows the minimum inhibitory concentration of COSs derivative against the growth of *Escherichia coli* and *Bacillus subtilis*. The COSs did not inhibit growth for both bacteria species at concentrations, ranged from 0.5 to 4%. Yet, the derivative had a positive result for both bacteria species at 0.5%, but negative result for both bacteria species at higher concentrations, ranged from 1 to 4%. Therefore, the MIC of the derivative was determined at 1% concentration. This result shows that SA grafted on COSs chain increased antibacterial activity of COSs.

DISCUSSION

The results of this study show, that with the appropriate parameters, such as certain mass ratio between syringic acid and COSs (0.5:1), pH 5, temperature (27^o C), time (6 hours), the obtained derivative has syringic content, equal to 204 mg/g, corresponding to the level of grafting equal to 32%. The results of this study are similar to previous study, if mass ratio of gallic acid to chitosan is 0.5:1 [24]. The enhancement of grafting degree was possibly due to the increasing of SA mass ratio within the critical range. However, beyond that range, the excess of free SA molecules may inhibit grafting process, causing a reduction of grafting degree [16]. Our results offer the better grafting degree than the previous study of Eom *et al*., (2012) when N,N'-dicyclohexylcarbodiimide catalyst was used to conjugate phenolic acid onto COSs with the grafting degree at 10.26%. However, in that work, the factors which affected the synthesis process were not presented [14]. Liu *et al*., (2013) reported the synthesis of chitosan-gallic acid conjugate. The result of which shows gallic acid content at 128.3 mg/g chitosan-gallic acid, however, the study did not investigate the optimal conditions, such as pH, temperature and time of synthesis [16]. Cho *et al*., (2011) showed, that the synthesis of gallic acid grafted chitosan had the highest grafting degree at 53.88% [20].

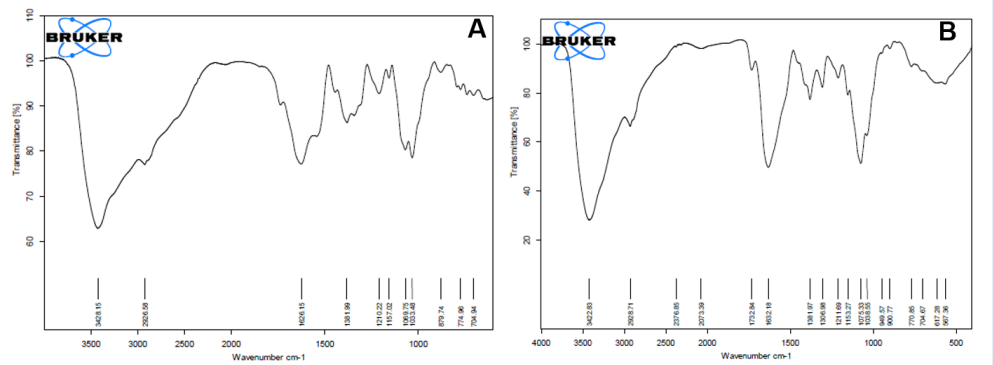

Figure 5: **FT-IR spectra of COSs (A) and SA-g-COSs (B)**. The FT-IR spectra were recorded in powder form in KBr discs in the range of 4000–400 cm^{-1}(Brucker, Germany). The experiment repeated three times.

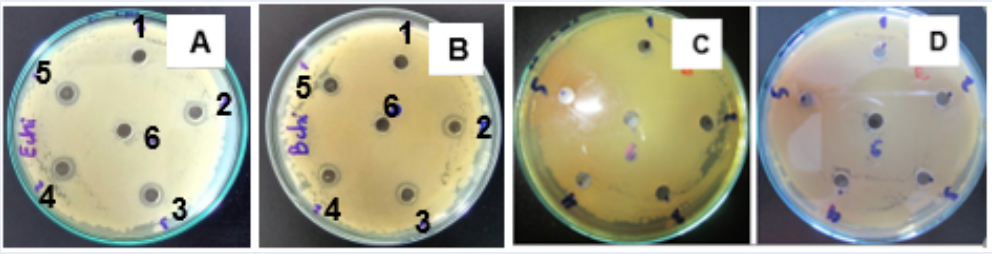

Figure 6: **Antibacterial activity of COSs and derivatives by agar disk diffusion test.** Concentrations ranged from 0.5 to 4%, (corresponding number changed from 1 to 5, control sample at centre) for each plate. A and B figures show result of derivatives against growth of *Escherichia coli* and *Bacillus subtilis*, respectively. C and D figures show result of COSs not against growth of *Escherichia coli* and *Bacillus subtilis*, respectively.

Table 1: The MIC results of COSs and SA-g-COSs

Bacteria	***Escherichia coli* (G-)**						***Bacillus subtilis* (G+)**					
Concentration (%)	0.5	1.0	2.0	3.0	4.0	Control	0.5	1.0	2.0	3.0	4.0	Control
COSs	+	+	+	+	+	+	+	+	+	+	+	+
SA-g-COSs	+	-	-	-	-	+	+	-	-	-	-	+

(+) not appearance of *inhibition zone, (-) appearance of inhibition zone. The experiment repeated three times (n=3).*

CONCLUSION

The catalyst of an ascorbic acid/hydrogen peroxide redox pair, which was used to graft SA onto COSs, was successfully achieved in inert atmosphere. The best conditions for the synthesis had the mass ratio between syringic acid and COSs at 0.5:1, pH 5, temperature 27^o C, time - 6 hours. Antibacterial assays showed that SA, grafted onto COSs improved COSs activity, comparing to free COSs.

ABBREVIATIONS

CFU: Colony forming unit

COSs: Chitooligosaccharides

FT-IR: Fourier Transform Infrared

MIC: Minimum inhibitory concentration

SA: Syringic acid

TLC: Thin Layer Chromatography

UV-vis: Ultraviolet-Visible

COMPETING INTERESTS

The authors declare that they have no competing interest.

AUTHORS' CONTRIBUTIONS

All authors of this manuscript have contributed to the work and approved contents of the final version.

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