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Research Article **Araştırma Makalesi**

Antibacterial Activity and Characterization of Water-Soluble Chitosan Compounds Produced from Enzymatic Deacetylation

Enzimatik Deasetilasyon ile Üretilen Su Çözülebilir Kitosan Bileşiklerinin Antibakteriyel Aktivitesi ve Karakterizasyonu

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Abstract: In this research, it was aimed to investigate the production of water-soluble chitosan from the enzymatic deacetylation of chitin by *Bacillus* (*B. cereus* (BC) and *B. thuringiensis* (BT)) strains. Characteristic properties involving molecular weight, degree of deacetylation, and antibacterial activity of chitosan samples were identified. The degree of deacetylation of BC and BT chitosan samples obtained at 393 and 213 ppm amounts was reported as 80.54% and 86.07% by the IR spectrum. As well as the degree of deacetylation, the molecular weights of samples showed a significant effect on antibacterial activity were 48.27 and 48.46 Da, respectively. Among the tested bacteria, the highest inhibitory effect was recorded in *Pseudomonas aeruginosa* and *Staphylococcus epidermis* for both chitosan samples. On the other hand, no antibacterial effect on *S. aureus,* Vancomycin-resistant Enterococci, *S. epidermidis, Klebsiella* sp., and *Salmonella* spp. were observed. Our results indicated a simple and cost-efficient method for the production of chitosan polymers showing antibacterial activity.

Keywords
- Antibacterial activity
- *Bacillus* sp.
- Chitosan
- Deacetylation
- Characterization

Özet: Bu araştırmada, *Bacillus* sp. (*B. cereus* (BC) ve *B. thuringiensis* (BT)) suşları ile kitinin enzimatik deasetilasyonundan suda çözülebilir kitosan üretiminin incelenmesi amaçlanmıştır. Kitosan örneklerinin moleküler ağırlıkları, deasetilasyon dereceleri ve antibakteriyel aktivitelerini içeren karakteristik özellikleri tanımlanmıştır. 393 (BC) ve 213 (BT) ppm miktarda elde edilen kitosan örneklerinin deasetilasyon derecesi IR spektrumu ile sırasıyla %80,54 ve %86,07 olarak belirlenmiştir. Deasetilasyon derecesinin yanı sıra antibakteriyel aktivitede önemli bir etki gösteren moleküler ağırlık değerleri ise 48,27 ve 48,46 Da'dır. Her iki kitosan bileşikleri için, test bakterileri arasında en yüksek inhibitör etki *P. aeruginosa* ve *S. epidermis*'e karşı kaydedilmiştir. Bunun yanı sıra *S. aureus*, Vankomisin dirençli Enterococci, *S. epidermidis, Klebsiella* sp. ve *Salmonella* spp'ye karşı hiçbir antibakteriyel etki gözlenmemiştir. Bizim sonuçlarımız, antibakteriyel aktivite gösteren kitosan polimerlerin üretimi için basit ve düşük maliyetli bir yönteme işaret etmektedir.

Anahtar kelimeler
- Antibakteriyel aktivite,
- *Bacillus* sp.
- Kitosan
- Deasetilasyon
- Karakterizasyon

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1. INTRODUCTION

Chitin, one of the most abundant biopolymers in nature, second only to cellulose, is a polysaccharide of animal origin. This biopolymer was identified as a natural poly-b-(1-4)-N-acetyl-D-glucosamine in 1884 and dissolves quickly in concentrated acid solvents (Brasselet et al., 2019; Junior et al., 2016). Chitosan, found by Rouget in 1859, is obtained from chitin by using chemical methods, including demineralization, deproteinization, and deacetylation processes (Junior et al., 2016; Santos et al., 2020). It comprises D-glucosamine and an N-acetyl D-glucosamine unit branched by b-(1-4) linkages and is a linear polycationic polysaccharide (Brasselet et al., 2019). It is a weak base and soluble in limited aqueous acidic solutions because of the presence of amine groups and the formation of cationic polyelectrolytes (Mohammadi et al., 2019).

Chitosan has unique properties, such as chemical resistance, low toxicity, chelating with metal ions, biodegradability, wound healing, biocompatibility, antiviral, antimicrobial, antitumor, and antioxidant has been researched in a wide range of pharmaceuticals and medical applications (Prabu & Nataraja, 2012; Qin & Li, 2020). It and its derivates in different forms (solutions, films, and composites) are well-known as antimicrobial agents against target microorganisms like Gram-positive bacteria, Gram-negative bacteria, yeast, and fungi (Saito et al., 2019; Sharma et al., 2019).

This antimicrobial activity depends on its biological origin, molecular weight, and degree of acetylation. With its low molecular weight and degree of acetylation, Chitosan has been reported to inhibit the growth of several fungi, Gram-positive and Gram-negative bacteria, in many studies (Shariah & Masson, 2017; Goy et al., 2019).

Three antimicrobial mechanisms related to chitosan and its derivates have been revealed: 1) the interaction between the $NH3^+$ cationic groups in chitosan molecules and negatively charged microbial cell wall (Dragland et al., 2019), 2) the interference of mRNA synthesis due to penetration into cell nucleus (Abdeltwab et al., 2019) and 3) the preventing toxin production and microbial growth because of metal chelation characteristic of chitosan (Mohammadi et al., 2019).

This work aims to investigate the characterization of chitosan produced by the bioconversion of chitin and the antibacterial capacity of this biomaterial.

2. MATERIAL AND METHODS

2.1. Isolation of *Bacillus* sp. strains

Chitin deacetylase (CDA, EC 3.5.1.41), the bioconversion of chitin to chitosan by hydrolysis of acetamido groups of N-acetyl-D-glucosamine, belongs to the hydrolases enzyme family. CDA-producing *Bacillus* sp. strains were isolated from soil samples provided by Adana Karataş beaches (36°33′45″N35°22′49″E) in Türkiye. This isolation was performed by a method declared by Mercimek Takci et al. (2019). Bacterial strains were identified by applying microbiological methods (Gram staining, spore-forming, and cell morphology). According to these methods, VITEK 2 compact system (microbial identification system) was employed for bacterial identification.

2.2. Qualitative analysis and purification of chitosan obtained by bioconversion

Bacillus sp. strains that produced CDA were grown Luria-Bertani media overnight at 37°C. Subsequently, 5 mL of inoculum (Mc Farland 0.4) was transferred into a medium including 1% yeast extract, 0.4 g $(NH_4)_2SO_4$, 0.15 g KH_2PO_4, 2% sucrose, and 1% colloidal chitin with an initial pH of 8.0 at 37°C for 12 h with shaking (180 rpm). After incubation, the pellet was harvested using centrifugation at +4ºC, 5500 rpm for 30 min. The pellet containing bacterial cells, chitin, and chitosan was suspended in 10 mL of 0.1 N NaOH and autoclaved. After cooling, the suspension was again centrifuged at +4ºC, 5500 rpm, for 30 min. Pellet washed with 10 mL of 2% glacial acetic acid was left on a shaker at room temperature for 24 h for the dissolving of chitosan biopolymer. After centrifugation at 5500 rpm for 15 min., the discarded white precipitate was washed twice in distilled

water. Pellet suspended in 5 mL sterile water was evaluated as crude chitosan preparation and preserved at +4ºC (Kaur et al., 2012).

For qualitative analysis of chitosan, 1 mL of this chitosan solution was dried in a glass plate at +55ºC for 2-6 h. 2-3 drops of iodine (0.3% w/v)/potassium iodide (0.5% w/v) solution was then added to the dried precipitate and mixed. This mixture was acidified by adding 2-3 drops of 1% H_2SO_4, The formation of the white precipitate by neutralization with 1 N NaOH and the transformation of dark brown color to dark purple by adding H_2SO_4 indicates the presence of chitosan (Kaur et al., 2012).

2.3. Chitosan characaterization

Quantitative analysis of crude chitosan solutions was spectrophotometrically performed using 0.35% (w/v) ninhydrin reagent prepared in methanol. 1 mL ninhydrin reagent and 5 mL of chitosan solution were mixed. Moreover, tubes immediately capped were heated in the water bath for 30 min and diluted with 3 mL of 50% ethanol-distilled water (v/v) into a reaction mixture cooled below 30ºC. The absorbance of solutions stirred by a vortex was measured at 570 nm against a blank (Prochazkova et al., 1999). The concentration of chitosan is calculated depending on a calibration curve plotted using standard chitosan. All measurements were performed three times.

The degree of deacetylation of crude chitosan solutions was detected using The Thermo Scientific™ Nicolet™ iS™10 FT-IR Spectrometer. Powder samples acquired by drying at room temperature were analyzed in the IR at the wavenumber range of 400-4000 cm^{-1}. According to the equation stated below, the degree of deacetylation of samples was calculated (Kasaai, 2008).

%DD = 100 - [(A1655/A3450) x 100/1.33]

The absorbances of 1655 cm^{-1} of the amide-I band as a measure of the N-acetyl group content and 3450 cm^{-1} of the hydroxyl band as an internal standard are employed. 1.33 is the ratio of A1655/A3450.

The average molecular weight (Mv) of chitosan solutions was identified by viscometric measurements using an Anton Paar Modular Compact Rheometer 302. This value was calculated following the Mark-Houwink-Sakurada equation (MHS), in which [η] is the intrinsic viscosity (Yomota et al., 1993).

$[\eta] = KMv\alpha$

$\log Mv = (\log[\eta]-\log K)/\alpha$

where K=0.119 cm3 g-1 and α= 0.59 determined.

2.4. Antibacterial activity

Antibacterial activity of crude chitosan solutions were tested against the following bacterial isolates: (*Staphylococcus aureus, Bacillus subtilis, Pseudomonas aeruginosa, Klebsiella* sp., *Salmonella* spp., vancomycin resistance *Enterococci* (VRE), *Staphylococcus epidermidis*, *Listeria monocytogenes*). The strains cultured in nutrient broth were incubated at 37°C for 24h. Cultures were then centrifuged at 5500 rpm for 15 min to harvest cells. The optical density of cells suspended in saline solution was adjusted to 3.0 turbidity. This bacterial suspension was used as the inoculum. 0.1 mL of each test bacterium was inoculated in nutrient broth (pH 7.0), including chitosan, at a final concentration of 250 mg/L and incubated at 37°C and 120 rpm for 6h. 0.1 mL of bacterial samples were spread on Petri dishes, including plate count agar (PCA). After 12 h incubation, colonies on plates were counted, and antibacterial activity was calculated as a percentage according to the formula: [(C-T)/C]x100. C and T symbols are expressed as the colony numbers counted on the control and sample plates, respectively (Chung et al., 2011).

Control plates involved colonies formed by bacterial cultures grown without chitosan on PCA. The antibacterial capacity of crude chitosan solutions was compared with standard chitosan at the medium molecular weight (~400.000) (2 mg/mL dissolved in 0.1% glacial acetic acid solution). All counting was repeated three times.

3. RESULTS and DISCUSSION

Ten *Bacillus* spp. strains were isolated from the Karatas beach samples. The CDA production ability of these strains was screened based on the conversion of p-nitro acetanilide by the enzyme. Out of 10 isolates, only two *Bacillus* strains were seen to produce the enzyme chitin deacetylase (Figure 1).

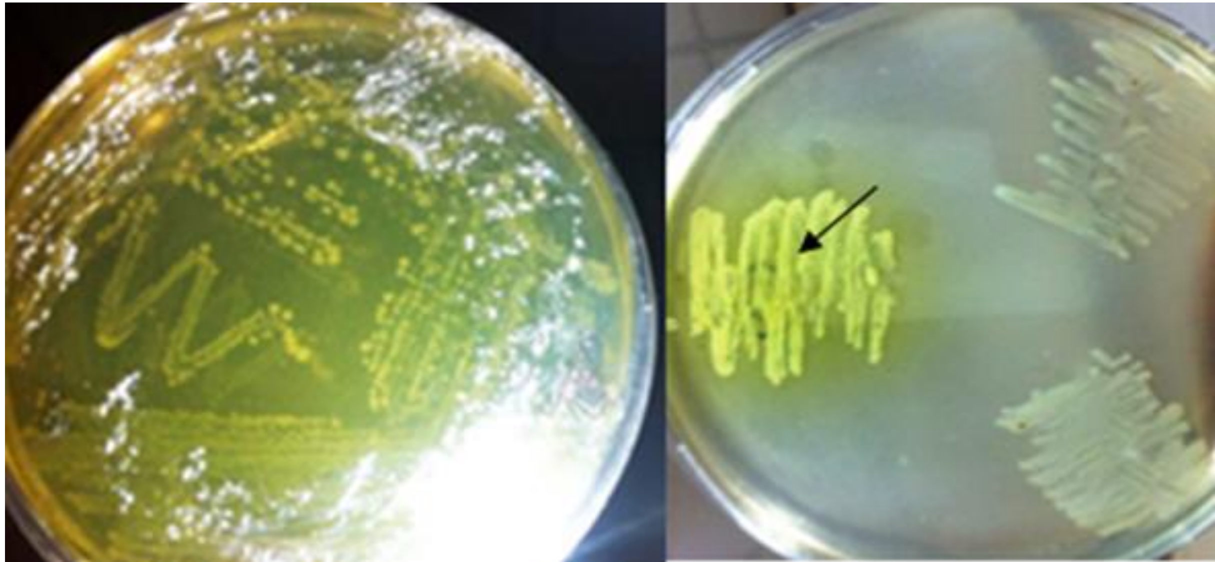

Figure. 1 Screening of chitin deacetylase (CDA) producing *Bacillus* isolates on a plate.

Two CDA-producing *Bacillus* isolates were identified as *B. cereus* (BC) and *B. thuringiensis* (BT) by using VITEK 2 microbial identification system, with an upper probability of 90%. Two *Bacillus* sp. strains were cultivated in broth medium, including 1% colloidal chitin. Following the incubation period, aqueous suspensions obtained from the fermented broth were qualitatively analyzed for the presence of chitosan biopolymer. Figure 2 is revealed that the dark purple coloration indicated bioconversion of the commercial chitin sample to chitosan for both samples. For *in vitro* rapid determination of chitosan concentration in an aqueous solution, colorimetric protocols may be used. A colored product is formed by acting between the free amino groups of chitosan and cationic sites of anionic dyes. This reaction is a reliable method for the quantification of chitosan polymers. The sensitivity of the ninhydrin essay is dependent on the type and properties (degrees of deacetylation and molecular weight) of chitosan performed (Leane et al., 2004).

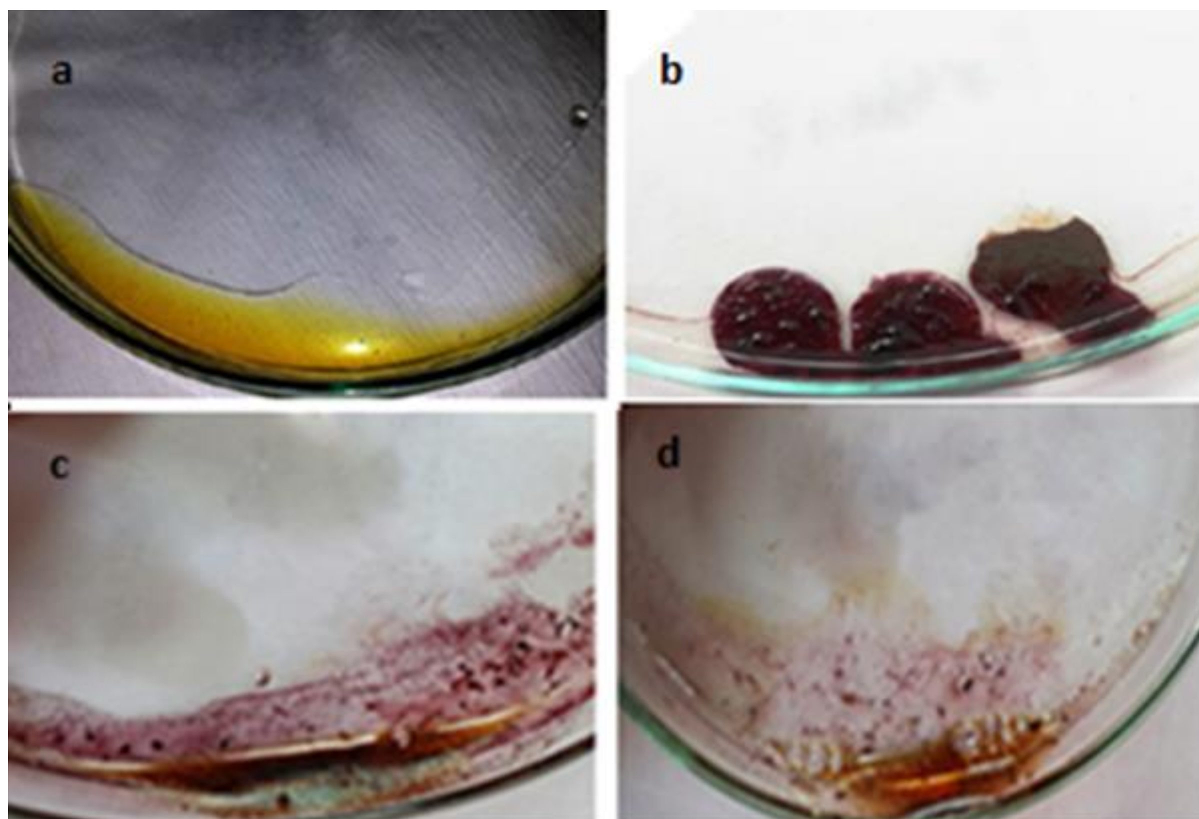

Figure 2. Dark purple coloring related to the production of chitosan by *Bacillus* sp.:
a, colloidal chitin; b, standard chitosan; c, BC chitosan samples; d, BT chitosan sample

In BC and BT solutions, chitosan concentrations revealed by ninhydrin binding were 393 and 213 ppm, respectively. MWs of the BC and BT chitosans obtained from the bioconversion was detected as 48.27 and 48.46 Da. Our results based on enzymatic deacetylation of various chitin samples can be discussed with other studies: ElMekawy et al. (2013) noted the increase in the degree of deacetylation of different chitinous substrates after treatment with CDA purified from *Alcaligenes* sp. ATCC 55938. Similar results were achieved by other researchers, such as Kaur et al. (2012), who declared the yield of chitosan 160 and 100 ppm using bioconversion of chitin by bacterial CDA (*Bacillus* spp. and *Serratia* spp.), values lower than our results. Similar results were described to convert various chitin substrates to partially deacetylated chitosan oligomers by fermentation the soil bacterium *Arthrobacte*r sp. as CDA producers (Tuveng et al., 2017).

The reaction of deacetylation is the release of chitosan, including a high degree of the chemical reactive amino group ($-NH_2$), by the process of the removal of acetyl groups from chitin. The quality of chitosan is expressed in its intrinsic properties, such as purity, molecular weight, and degree of deacetylation (DD). The degree of deacetylation is one of the main characteristics that affected the performance of chitosan in many of its applications. The degree of deacetylation of the final chitosan product may result in differences in the variation of the manufacturing process and origin (Ak Kalut, 2008). The degree of deacetylation (DD) of chitosans commercially prepared is in the range of 70-95% (Kaczmarek et al., 2019). FTIR results of purified (BC and BT) and commercial chitosans are depicted in Figure 3. In the FTIR spectrum of standard chitosan, internal reference bands were determined as the OH stretching band at 3289.08 cm^{-1}, $-CH_2$ stretching band in pyranose circle at 2870.71 cm^{-1}, -C=O (amide I) stretching band at 1649.74 cm^{-1}, NH (amid II) stretching band at 1567.25 cm^{-1}, C-O-C stretching band at 1149.43 cm^{-1} and C-O stretching band at 1023.48 cm^{-1}. The 1655 cm^{-1} and 3450 cm^{-1} wavenumbers of the characteristic peaks in the FTIR spectrum of standard

and purified chitosan samples were observed to shift to higher/lower wavelengths. This clarified some impurities that originated from the preparation process. So, the degree of deacetylation was calculated by taking reference to the characteristic stretching vibrations at 1647.57 cm^{-1} and 3281.96 cm^{-1} for the BC sample and 1624.27 cm^{-1} and 3264.85 cm^{-1} for BT. DD% acquired from IR of chitosan samples were 80.54 and 86.07%, respectively.

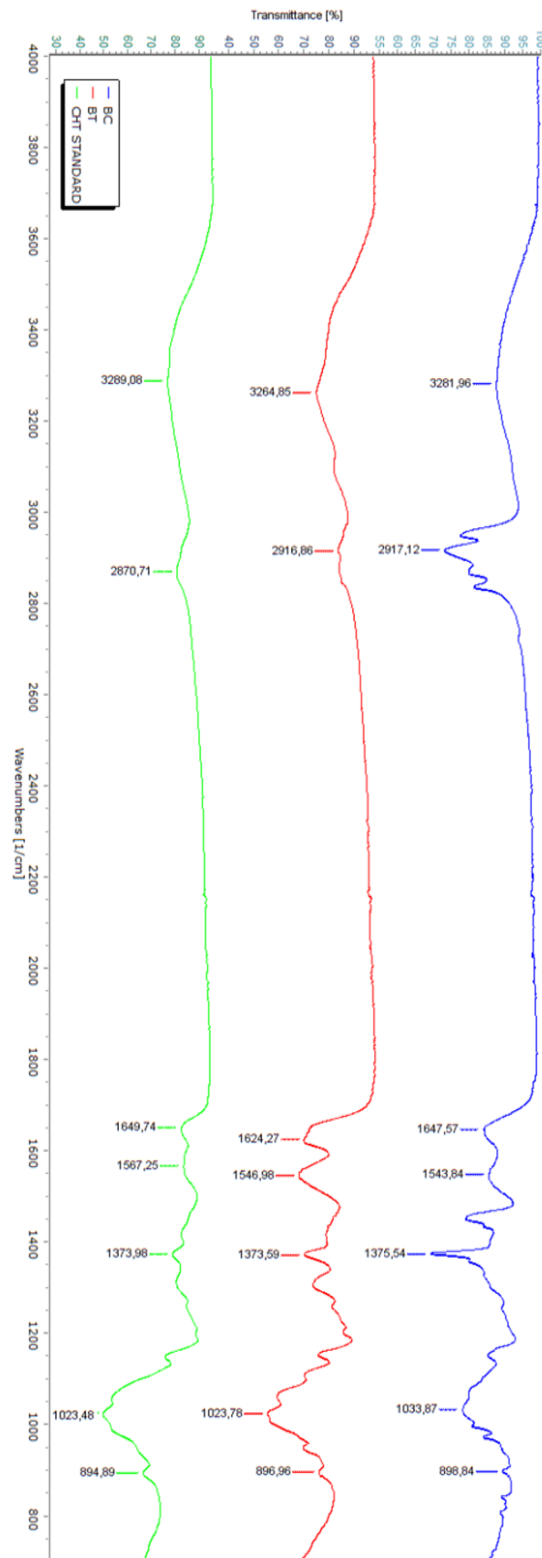

Figure 3. FTIR spectrum of the obtained water-soluble chitosan samples

In a study, Venugopal (2021) noted to obtain chitosan via the hydrolysis of Nacetylamido linkage of chitin by fungal chitin deacetylase produced from *Mucor rouxii*, *M. mechei*, and *Aspergillus niger*. A similar deacetylation pattern (79.52%) was observed after the fermentation of superfine chitin by using CDA obtained from *Penicillium oxalicum* (Pareek et al., 2019). In the other study, the degree of deacetylation of resultant chitosan in solid-state fermentation of *Aspergillus flavus* CDA was revealed as 83.35% at a 2.1 mg/g chitosan amount (Yonis et al., 2019). Compared to our chitosan solutions, the chitosan produced in this work was quite low. On the other hand, the degree of deacetylation of 83.35% was a little higher than the BC sample.

Table 1. Antimicrobial activities of standard and water-soluble chitosans prepared by enzymatic deacetylation (%).

	Standard chitosan	**BC chitosan sample**	**BT chitosan sample**	**0.1% glacial acetic acid**
Staphylococcus aureus	0	0	0	0
Bacillus subtilis	96	14	16	0
Pseudomonas aeruginosa	99	41	49	0
Klebsiella sp.	99	0	0	0
Salmonella spp.	99	0	0	0
VRE	0	0	0	0
Staphylococcus epidermidis	0	34	33	0
Listeria monocytogenes	44	19	11	0

As represented in Table I, standard chitosan possessed a higher degree of inhibition than chitosan produced by bioconversion. Standard chitosan solution demonstrated antibacterial activity against 63% of all tested bacterial strains. This solution showed maximum inhibition in the range of 96-99% to *B. subtilis, P. aeruginosa, Klebsiella* sp., and *Salmonella* spp. The lowest activity, with 44% inhibition, was obtained against *L. monocytogenes*. However, no inhibitory action of standard chitosan on *S. aureus*, VRE, and *S. epidermidis* was noted. 0.1% glacial acetic acid was performed as a negative control for antibacterial capacity studies of standard chitosan and exhibited that negative control had no inhibitory effect on strains. For chitosan solutions, it was indicated that comparable antibacterial activity with the standard chitosan tested at a 2 mg/mL dose level was not recorded. BC and BT chitosan solutions were found to have no antibacterial effect on *S. aureus*, VRE and *S. epidermidis*, *Klebsiella* sp., and *Salmonella* spp. Among tested bacterial strains, the inhibitory effect at below 50% against only four strains (*B. subtilis, P. aeruginosa, L. monocytogenes,* and *S. epidermis*) was observed. The lowest inhibitory effect of both BC and BT solutions was noticed on *B. subtilis* and *L. monocytogenes* compared with the other microorganisms. The percentage of the maximum antibacterial activity of BC which is similar to the BT solution, was determined to be *P. aeruginosa*. Among all test bacteria, the most susceptible strain was *P. aeruginosa* against BC and BT chitosan solutions. As Chung et al. (2011) reported, the antibacterial efficiency of water-soluble chitosan obtained from shrimp shells with 90% N-deacetylation on *L. monocytogenes* was 11±5.0%. The inhibitory effect of bioconversion chitosan solutions in our study was higher than that of this value. Similarly, inhibition (22%) of the standard chitosan samples (at 2000 ppm) on *P. aeruginosa* observed by Ortega-Ortiz et al. (2010) was substantially lower than our results. Ali and Aldujaili (2022) reported that different concentrations of biogenic chitosan NPs synthesized by *B. subtilis* had an inhibitory effect on all tested. The highest inhibition zone with 26 mm showed against *S. aureus*.

The authors indicated that the antibacterial activity of chitosan polymers might be explained by the interaction of positively charged amino groups with the bacterial cell surface. The efficiency of this interaction depends upon the molecular weight and the degree of deacetylation of chitosan.

4. CONCLUSION

Enzymatic deacetylation of colloidal chitin by *Bacillus* sp. yielded water-soluble chitosans showing antibacterial activity. Especially the highest inhibition was reported against *P. aeruginosa* and *S. epidermis*. The degree of deacetylation and MW may be affected by the antimicrobial activity of chitosan as well as its concentration. The inhibitory effects of BC and BT chitosans at obtained amounts on tested bacteria are valued as compared with that of standard chitosans prepared by previous studies. However, the degrees of deacetylation of our chitosan samples are in the range of those used commercially. So, we here described that chitosan samples produced via enzymatic biotransformation of chitin by *Bacillus* sp. strains might be a promising candidate for industrial application.

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CONFLICT OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

AUTHOR CONTRIBUTIONS

Fiction: HAMT; Literature: HAMT, FM, FY, GG; Methodology: HAMT; Performing the experiment: HAMT, HIC; Data analysis: HAMT; Manuscript writing: HAMT, FM. Supervision: HAMT. All authors approved the final draft.

ETHICAL STATEMENTS

Local Ethics Committee Approval was not obtained because experimental animals were not used in this study.

DATA AVAILABILITY STATEMENT

Data supporting the findings of the present study are available from the corresponding author upon reasonable request.

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