



Biological chitosan production from shrimp waste and evaluation of anti-microbial activity of produced chitosan

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Abstract

Purpose: Chitosan is an important polysaccharide used in many fields. Today, it is produced with environmentally harmful chemicals and costly methods. In our study, the production of chitosan from waste shrimp shells by chitin deacetylase enzyme producing bacterial isolates from the Marmara Sea was demonstrated. Eco-friendly and low-cost chitosan production by five isolates and antimicrobial effect of produced chitosan was realized.

Method: Chitosan synthesis was induced in five marine bacterial isolates on chitin-rich medium containing waste shrimp shells, and the chitosan production was confirmed by P-nitroacetanilide, iodide inducers and FT-IR analysis. Subsequently, the bacterial isolates were identified by 16SrRNA analysis and the antimicrobial effect of chitosan from IK3-237 isolate was tested on conventional test bacteria.

Findings: The study provided a sustainable and cost-effective solution in which bacterial isolates from the Marmara Sea produced chitosan with a yield above 26% from chitin found in waste shrimp shells. In addition, the produced chitosan showed antibacterial effects similar to pure commercial chitosan.

Conclusion: We produced biologically produced chitosan from local isolates via enzymatic pathway in an environmentally friendly and cost-effective manner. We demonstrated that it has sufficient antimicrobial properties in comparison to pure commercial chitosan.

Keywords: chitin, chitosan, marine bacteria, antimicrobial activity

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Karides atıklarından biyolojik kitosan üretimi ve üretilen kitosanın anti-mikrobiyal aktivitesinin değerlendirilmesi

Özet

Amaç: Kitosan pekçok alanda kullanılan önemli bir polisakarittir. Günümüzde çevreye zararlı kimyasallar ve maliyeti yüksek yöntemlerle üretilmektedir. Çalışmamızda kitosanın, atık karides kabuklarından, beş Marmara denizi izolatu bakterinin ürettiği kitin deasetilaz enzimi ile eldesi gösterilmiştir. Doğa dostu ve düşük maliyetli üretim yapılmıştır ve üretilen kitosanın antimikrobiyal etkisi gösterilmiştir.

Yöntem: Kitin bakımından zengin atık karides kabukları içeren besiyerinde deniz izolatu beş bakterinin kitosan sentezlemesi indüklenmiş, kitosan üreten bakterilerin ürünü, P-nitroasetanilid, iyodür indükleyicileri ve FT-IR analizi ile doğrulanmıştır. Takiben bakterilerin tür tanısı 16SrRNA analizi ile yapılmış ve IK3-237 izolatu kitosanının antimikrobiyal etkisi konvensiyonel test bakterileri üzerinde denenmiştir.

Bulgular: Bu çalışma, Marmara Denizi'nden izole edilen bakterilerin atık karides kabuklarında bulunan kitinden %26'nın üzerinde bir verimle kitosan üretmesiyle sürdürülebilir ve uygun maliyetli bir çözüm sunmuştur. Buna ek olarak üretilen kitosan saf ticari kitosana benzer antibakteriyel etki göstermiştir.

Sonuç: Yerel izolatlardan enzimatik yolla biyolojik olarak üretilen kitosan, doğa dostu ve düşük maliyetle ürettik. Yeterli miktarda antimikrobiyal özelliği olduğunu saf ticari kitosanla kıyaslayarak gösterdik.

Anahtar kelimeler: kitin, kitosan, deniz bakterisi, antimikrobiyal aktivite

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1. Introduction

Chitosan derived from chitin is a linear and polycationic polymer consisting of 2-amino-2-deoxy-D-glucopyranose and 2-acetamido-2-deoxy-D-glucopyranose units linked by beta-(1-4)-glycosidic bonds. The most important advantage of chitosan is that it is soluble in common solvents and has high binding properties with free amino groups (-NH₃) (Figure 1) [1]. The functional groups of chitosan affect its solubility and mechanical properties, making it suitable for modifications.

When the acetylation of the β-D-glucosamine groups that make up chitin is less than 50%, the polymer is called chitosan. Like chitin, chitosan has biocompatibility, antioxidant, anticancer, biodegradability and antimicrobial properties [2, 3]. In addition, chitosan is highly advantageous over chitin as a robust, stable and processable biopolymer [4-6] and finds many applications. For example, it has been reported to have antimicrobial activity and is valuable in combating microbial infections [7-10]. In biomedicine and biotechnology, it is also used in drug transplantation [11], gene transplantation [12], wound and bone regeneration [13-14] and tissue engineering [15]. Similarly, in veterinary medicine, cosmetics and as accelerators in treatment protocols. It is used in pharmacology, agriculture, paper and food industry, wastewater treatment and textile industries [16, 17], with potential applications in biosensor technologies [18]. The versatility of chitosan and its various fields of application make it a valuable material in industries and research areas.

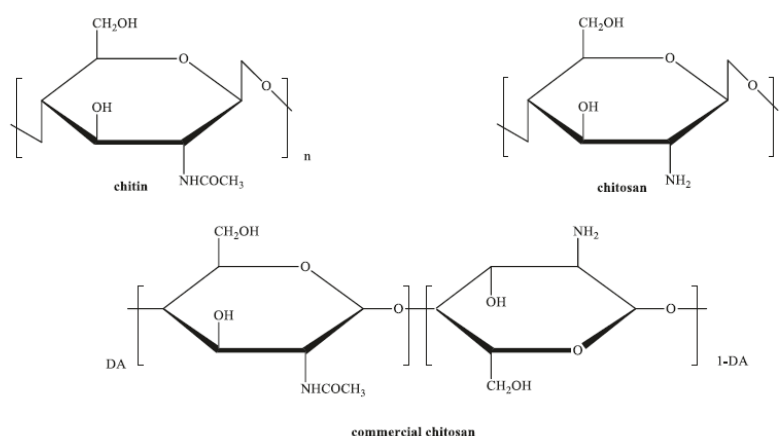


Figure 1. Schematic representation of acetylated chitin [poly(N-acetyl-β-d-glucosamine)], deacetylated chitosan [poly d-glucosamine] and commercial chitosan, a copolymer with an average degree of acetylation [1]

Chitin is commonly found in the exoskeleton of marine crustaceans and arthropods, insects, terrestrial crustaceans, fungi, and yeast cell walls [19]. According to the literature, more than 13 million tons of crustaceans are caught in marine habitats worldwide yearly [1, 17]. About half of the weight of this seafood consists of shells. The rate of chitin in these shells is about 12-46% [1]. Therefore, producing high-value-added products from the waste shells of seafood is an environmentally friendly approach where waste is recycled into the system in terms of the circular economy.

Chitosan can be produced from chitin generally by chemical, physical or biological methods [19]. Today's most widely used chemical method consists of demineralization, deproteinization and deacetylation steps, generally based on high-temperature treatment of strong acids and bases with chitin. This chemical method has important disadvantages, such as optimization to adjust the molecular weight of chitosan, removal of acid and base residues from the final product, and environmental damage of waste acids and bases. Therefore, it has high production costs. It has been reported that 1 kg of chitosan is obtained from approximately 35 kg of shells by chemical production [1, 19].

Physical chitosan production methods are based on ultrasonic wave, microwave and gamma radiation. This method allows the production of chitosan with a desired molecular weight [20]. However, the aforementioned physical forces alone cannot produce chitosan from chitin. Therefore, the method needs chemicals such as inorganic salts or organic solvents [19, 20]. Overall, chemical and special equipment use together with generating harmful waste streams makes the method expensive and not very efficient. [17].

The enzymatic method, which is a biological method, produces chitosan from chitin with the enzyme deacetylase [7]. Enzymatic production is a faster, relatively low-cost and environmentally friendly method compared to chitosan production by chemical and physical methods [1, 17, 20]. On the other hand, detection, isolation and reproduction of microorganisms that produce the enzymes required for the production of chitosan are of critical importance [7]. For this reason, there is increasing interest in studies on organisms that produce chitinase, chitosanase and glucanase, especially chitin deacetylase enzymes [1, 16].

Bacteria known as good chitin digesters are usually of the genus *Bacillus*. These include *Bacillus megaterium* [8], *B. cereus* [20], *B. Subtilis* [21], *B. licheniformis* [7], *B. amyloliquefaciens*, *Paenibacillus* sp., *Vibrio* sp., *Rhizobium* sp., *Acenobacter* sp., *Shewanella* sp., *Arthrobacter* sp., *Klebsiella* sp., *Enterobacter* sp., *Serratia* sp., *Citrobacter* sp. and *Corynebacterium kutscheri* have also been reported as producers of chitosan from chitin [20]. In addition to bacteria, *Drosophila melanogaster*, *Helicoverpa armigera* and *Mamestra configurata*, some fungal species, *Mucor* sp., *Aspergillus* sp. and *Saccharomyces* sp. can produce chitosan [1]. According to the literature, chitin deacetylases found in bacteria, insects, fungi, molds and yeasts show significant differences in their structures. These differences are primarily species dependent and are also influenced by factors such as the structure and size of the chitin substrate with which the enzyme interacts. Variations in chitin deacetylase structures highlight the diverse adaptations of these enzymes between different organisms [21]. Therefore, studies have focused on soil and water samples collected from different habitat in the literature [21, 22]. In particular, *Bacillus* species have been extensively studied as effective chitin deacetylase producers for enzymatic chitosan production in both soil and marine environment [8]. Among these species, *B. licheniformis* is especially preferred in many studies due to its high chitin deacetylase activity [7]. The antimicrobial effect of chitosan, which is also included in our study, has been reported [22].

In this study, it is aimed to screen bacterial isolates obtained from the Marmara Sea in terms of their chitin production yield and determine the best species to be utilized for biological chitosan production from waste shrimp shells. Thus the potential of marine microorganisms in chitosan production will be investigated and the quality of the products obtained from chitosan will be evaluated by comparing with the commercial ones. The study is a good example for the circular economy, where wastes are reintegrated into the system, used as raw materials, and environmental and economic balance is ensured without compromising the quality of the product.

2. Materials and methods

2.1 Selection of the chitosan producing bacterial isolates and methodology

The bacteria used in the study were selected from our Marmara Sea bacterial collection, which includes 698 pure bacterial species in total, isolated from the Marmara Sea through our projects and thesis studies in our laboratory in previous years, and whose morphological and biochemical tests have been largely completed [23]. For the present study pre-selection was made according to Gram (+), catalase (+) and oxidase (-) properties since chitosan production by Gram positive aerobic bacteria is well known in literature. The chitin deacetylase-producing isolates were identified and five bacterial isolates were used for chitosan synthesis in the present study. They were grown on medium containing waste shrimp shells and the conversion of chitin to chitosan by isolates was confirmed by P-nitroacetanilide, iodide inducers and FT-IR analysis. Afterwards, chitosan production yield of the isolates were determined and 16S rRNA analysis was performed to identify the selected bacteria at species level. In the second part of the study, the anti-bacterial effect of the chitosan produced was investigated and compared with the commercial chitosan.

2.2 Preparation of shrimp shells media

Waste shrimp shells were obtained from markets and fish restaurants. The shells were washed three times with distilled water, then kept at -20°C for 24 hours, then easily broken and transferred to a filter paper (SigmaAldrich) and kept in an oven (Binder ED 115, Germany) at 55-60°C (approximately 24 hours) until completely dry. It was then crushed into powder in a mortar. We used a modified medium given by Kaur et al. with Nutrient Broth (NB, Merck) and Nutrient Agar (NA, Merck) together with prepared shell powder to trigger chitin deacetylase enzyme production [20]. Selected isolates were grown in this media (500 ml NB and 0.5 g shrimp shells; 500 ml NA and 0.5 g shrimp shells)

2.3 Screening chitin deacetylase producers

Bacterial isolates were taken from stocks at -20°C, inoculated into 2 mL of Tryptic Soy Broth (TSB, Merck) medium and incubated at 30-37°C for 24-96 hours. Whatman filter paper (Cytiva, Whatman) strips impregnated with p-Nitroacetinalide (Alfa Aesar) were used to evaluate whether bacteria grown on shrimp shells medium produce chitin deacetylase. When chitin deacetylase removes the acetyl groups of p-Nitroacetinalide, which has a chitin-like structure, the colourless solution turns yellow. This indicator was tested using both modified liquid (NB) and solid medium (NA). The chitosan production study was continued with the isolates with suitable colour changes.

2.4 In situ chitosan production from shrimp shells

Experiments were carried out by incubating (Edmund-Bühler, Germany) selected isolates in 10ml NB containing 0.1g shrimp shell powder at 30°C for 24h. Then, the culture was centrifuged (Beckman Coulter, USA) at 12,000 rpm for 15 minutes, and the supernatant was discarded. Next, 0.1N NaOH (Merck) was added to the pellet,

mixed, and centrifuged. Collected pellets were dissolved in 2% acetic acid, agitated, and left overnight in the incubator at room temperature. Following the centrifugation step, the supernatant was collected and neutralized with 1N NaOH (Merck). For qualitative analysis to verify the presence of the chitosan, the samples were centrifuged (Gyrozen, South Korea) at 4000 rpm, washed with distilled water, placed in a petri dish and dried in an oven at 50-55°C for 2-4 hours.

2.5 Chitosan characterization

Chitosan has been verified by colour change with an iodide solution and the Fourier Transform Infrared Spectroscopy (FT-IR) analysis (Perkin Elmer Spectrum, USA). The dark purple colour of the precipitates, on which the iodine solution (Merck) was dripped, showed that chitosan production was achieved from the shells. Therefore, commercial chitosan (Sigma-Aldrich) was used as a positive control. FT-IR spectroscopy provides structural and compositional information resulting from the vibrations of macromolecules and their functional groups. FT-IR analysis of chitosan was performed at a resolution of 4 cm⁻¹ in the band range of 650-4000 cm⁻¹.

2.6 Chitosan yield

Following small-scale trials, the samples were produced on a larger scale using 250 mL flasks. 50 mL of NB (Merck) medium containing 0.5 g of shrimp powder was prepared, and the precipitate obtained following the above procedure was taken to the petri dish and left to dry in an oven at 55°C. The tare of the petri dishes used was taken. The yield was calculated by weighing the amount of chitosan formed. Based on the data that raw shrimp shell contains 12-46% chitin [1, 17], the average chitin content of raw shrimp shell is accepted as 30%. For this reason, chitosan production efficiency from chitin was calculated according to the following equation. Here, m_{chs} represents the amount of chitosan produced in g, m_{shs} represents the shrimp shells used in grams, and 0.3 represents the fraction of chitin content of the shells by mass.

$$\text{Yield} = \frac{m_{chs} \text{ (g)}}{0.3 \times m_{shs} \text{ (g)}} \times 100$$

2.7. Molecular Identification of Bacterial Isolates

2.7.1. DNA Isolation

Genomic DNA isolation from selected bacterial isolates was performed using the QIAamp DNA Mini Kit (Qiagen, Germany) according to the manufacturer's protocol. The isolates were incubated in Tryptic Soy Broth (TSB, Merck) at 30°C for 24 hours. 1.5 ml of the bacterial culture was taken and centrifuged at 12,000 rpm for 5 minutes. The pellet obtained was used for DNA isolation. The purity and concentration of the isolated DNA were measured using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA) and stored at -20°C.

2.7.2. PCR Amplification

Universal bacterial primers were used for the amplification of the 16S rRNA gene: 27F (5'-AGAGTTTGTGATCTGGCTCAG-3') and rP2 (5'-ACGGCTTTTGTACGACTT-3'). The PCR reaction mixture was prepared in a total volume of 25 µl: 5.5 µl 5X PCR Master Mix (containing 0.75 U Taq DNA polymerase, reaction buffer solution, 2 mM MgCl₂, and 250 µM dNTP), 1 µl forward primer (10 pmol/µl), 1 µl reverse primer (10 pmol/µl), 1 µl template DNA (50 ng), and 16.5 µl dH₂O. PCR conditions were applied on an Applied Biosystems Veriti 96-Well Thermal Cycler (Thermo Fisher Scientific, USA) as follows: Initial denaturation at 94°C for 1 minute; 40 cycles of denaturation at 94°C for 1 minute, annealing at 57°C for 1 minute, and extension at 72°C for 1 minute; final extension at 72°C for 10 minutes. PCR products were run on a 1% agarose gel at 100V for 30 minutes and visualized under UV light using the DNR-BIOIMAGING SYSTEMS gel imaging system.

2.7.3. DNA Sequencing and Analysis

PCR products were purified using the ExoSAP-IT purification kit. The sequencing reaction was performed using the BigDye Terminator v3.1 Cycle Sequencing Kit. Sequencing was carried out on an ABI 3500 Genetic Analyzer (Applied Biosystems). Raw sequence data were edited using BioEdit v7.2.5 software.

2.7.4. BLAST Analysis and Species Identification

The obtained 16S rRNA gene sequences were analysed using the Basic Local Alignment Search Tool (BLAST) in the National Centre for Biotechnology Information (NCBI) GenBank database. Sequence similarities and the closest species were determined. The obtained sequences were uploaded to GenBank and accession numbers were obtained and presented in Table 2.

2.7.5. Phylogenetic Analysis

The MEGA-X version 10.2.2 programme was used to determine phylogenetic relationships. The 16S rRNA gene sequences of reference species were downloaded from NCBI GenBank. Multiple sequence alignment was performed using the ClustalW algorithm. The phylogenetic tree was constructed using the Neighbour-Joining method. The reliability of the tree was tested using 1000 bootstrap analyses. Evolutionary distances were calculated using the Kimura 2-parameter model [24].

2.8. Antimicrobial activity

E. coli ATCC 25922, *P. aeruginosa* ATCC 15692, *S. aureus* ATCC 29213 and *Candida albicans* ATCC 10231 strains, representing Gram-negative and Gram-positive bacteria and yeast, were used for antimicrobial testing of chitosan since these are the mostly preferred species for antimicrobial activity tests. The optical densities of each test microorganism were spectrophotometrically equalized to 0.37 at OD 600 (Shimadzu, Japan). The produced chitosan from isolate IK3-237 and commercial chitosan were prepared and applied as 4% solutions in 2% acetic acid (Merck). The 4% solutions prepared with commercial chitosan was the positive control. Accordingly, 4% chitosan solutions prepared were absorbed into empty antibiogram discs (Bioanalyse, Türkiye) and placed in their petri dishes on which test microorganisms were spreaded on NA (Merck). Petri dishes were incubated (Edmund-Bühler, Germany) for 12 - 72 hours at 37°C for appropriate time periods according to the growth conditions of the experimental microorganisms and the effect zones around the discs were measured with a ruler.

3. Results

3.1 Selection of chitin deacetylase producers

The five bacteria selected for the study were cultured in TSB. After these revival cultures, they were grown on NB medium containing waste shrimp shells to induce the production of chitin deacetylase enzyme. In order to see the chitin deacetylase production of our selected isolates, Whatman paper strips impregnated with p-Nitroacetinalid indicator were placed in the tubes of these samples and a yellow color change was observed (Figure 2).

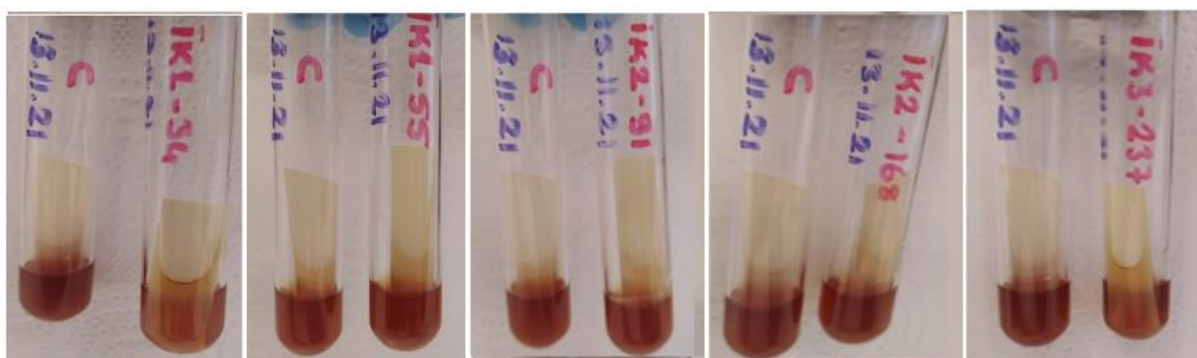


Figure 2. Color changes of bacteria grown on TSB medium with chitin when p-Nitroacetinalid impregnated filter papers were added

Another control method of chitin deacetylase production is the control method with iodine solution. Accordingly, the pellets turn purple if chitosan is present in the medium. In the next verification step, the chitosan produced by the isolates were subjected to FT-IR analysis.

3.2 Production of the chitosan

The five isolates selected for the experiment were incubated for 24 h at 30°C in 50 ml NB containing 0.5 g of shrimp shell powder on a larger scale. After centrifugation and washing, the pellets were dried at 50-55°C for 2-4 hours. In the yield calculation, Arabia et al. reported that 1 kilogram of chitosan was obtained from 35 kilograms of raw shrimp shells (0.029 g chitosan/g shrimp shell powder) by chemical production [1, 2]. Based on literature data [1, 17], approximately 30% of the shrimp shells is regarded to be chitin and the yield of the chemical chitosan production from shrimp chitin is calculated around 10%. When we look at the yield calculations of our isolates, the amount of chitosan produced per 1g of raw shrimp shell was calculated between 0.114 g - 0.0806 g. This showed that the isolates yielded between 26% and 38%. The chitosan yield of our isolates is found to be 3-4 times higher than chemical production (Table 1), the impurities which might be present in the chitosan produced in the present study might cause obtaining the higher yield value as compared to the commercial chitosan production from shrimp shells.

Table 1. The amount of chitosan produced by marine isolates of the Marmara Sea

Selected isolates for chitosan production	g chitosan / g shrimp shell powder	Yield (%)*
IK1-34	0.1046 g	34,86%
IK1-55	0.0806 g	26,86%
IK1-91	0.114 g	38%
IK1-168	0.0948 g	31,6%
IK3-237	0.0834 g	27,8%

*In yield calculation, the chitin content of raw shrimp shell was taken as 30% resulting in 0.30 g of chitin for 1 g raw material.

3.3 FT-IR validation of IK3-237 Chitosan to be used for Anti-Bacterial Analysis

Since strain IK3-237 was selected for the antibacterial study among the validated chitosans, only the FT-IR analysis of this strain is presented (Figure 3). Accordingly, the product of IK3-237 isolate was confirmed by FT-IR analysis using commercial chitosan as control. In commercial chitosan shown in blue, -NH₂ aberrations are observed at 1625 cm⁻¹, C-N stretches at 1525 cm⁻¹ and P=O signals at 1050 cm⁻¹. Similarly, in IK3-237 chitosan, -NH₂ deviations occurred at 1652 cm⁻¹, C-N stretches at 1500-1520 cm⁻¹ and P=O signals at 1029 cm⁻¹. The similarities between these graphs confirm that the substance produced by IK3-237 is chitosan. In contrast, the strong band around 1391 cm⁻¹ in chitosan from shrimp waste usually indicates an extra ionic/mineral component and is of course not seen in pure commercial chitosan. In other words, chitosan produced by bacteria in the laboratory, isolated in acetic acid and not completely neutralized, can lead to the formation of -NH₃⁺-OAc- (chitosan acetate). This can form a strong band in the region 1390-1370 cm⁻¹ and is usually assigned to amide III (C-N stretching) in chitin/chitosan systems.

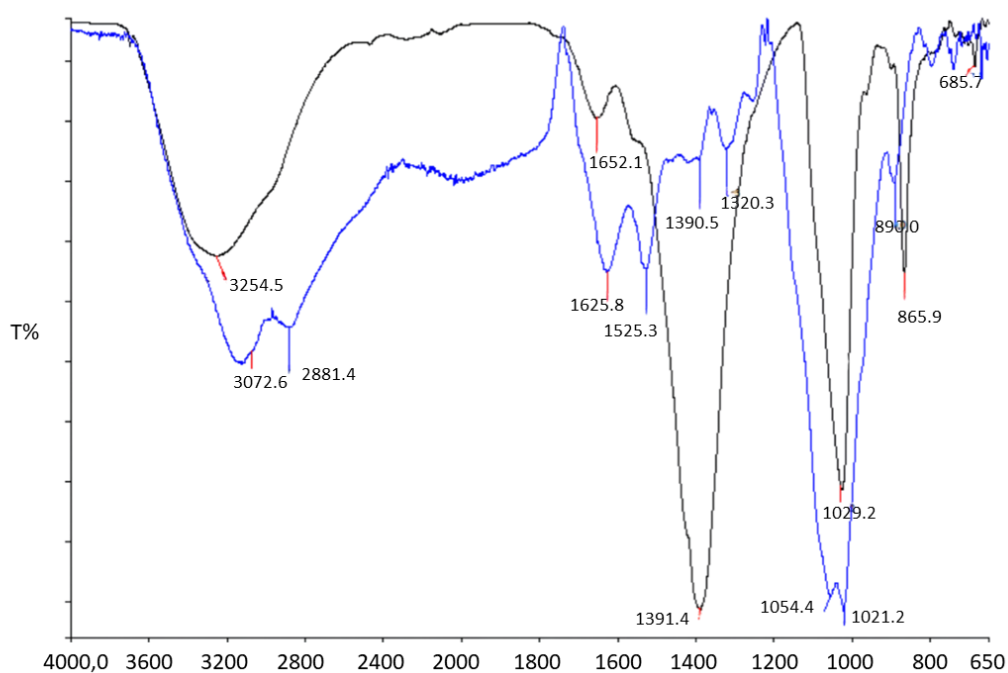


Figure 3. The samples selected in the spectrum between 650-4000, are shown. The fluctuations are quite similar when the FT-IR graphs of IK3-237 (Black) and commercial chitosan (CS, Blue)

3.4. Molecular Identification of Selected Isolates

PCR reactions produced approximately 1,500 bp of product for all five isolates (Figure 4). Agarose gel electrophoresis showed distinct bands of the predicted size for all isolates tested, confirming successful amplification of the 16S rDNA gene region.

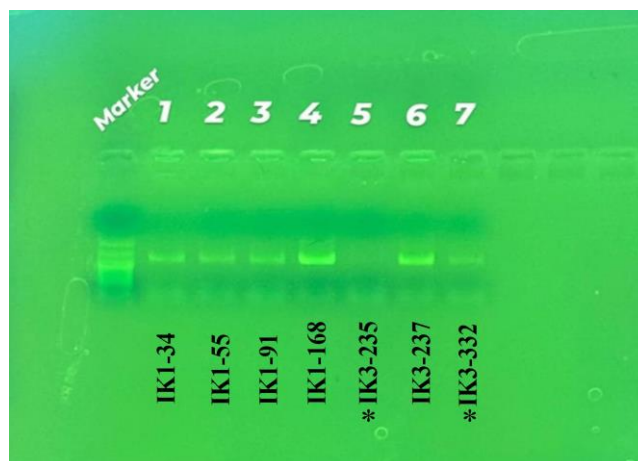


Figure 4. PCR amplification of 16S rRNA gene from chitin deacetylase-producing bacterial isolates. Marker: 1 kb DNA, 1-7: Isolates IK1-34, IK1-55, IK1-91, IK1-168, IK3-235, IK3-237, and IK3-332 expected product size: ~1500 bp. * low PCR yielded strains were eliminated

3.5. Molecular Characterisation of Selected Isolates

The 16S rRNA gene region was successfully amplified from the five isolates exhibiting chitin deacetylase activity as shown in Figure 4. The DNA products were extracted and DNA sequences were determined as described in materials and methods section. The sequences of their 16SrRNA were submitted to the GeneBank and accession numbers for the isolates and also for the nucleotide sequences were assigned. GenBank nucleotide accession numbers are written in paranthesis as follows: IK1-34 (KY419150.1), IK1-168 (MK824319.1), IK3-237 (cp-h37 EU584556.1), IK1-55 (MG470715.1) and IK1-91 (KX832708.1).

According to BLAST analysis, all isolates showed high similarity of over 99% with species belonging to the genus *Bacillus*. The isolates showed very high similarity (97.13-99.93%) with members of the *B. cereus* group (*B. anthracis*, *B. thuringiensis*).

Table 2. 16S rRNA gene sequence analysis results of all isolates showing chitin deacetylase activity

Isolate Code	Sequence Length (bp)	NCBI BLAST Result	Similarity (%)	GenBank Accession No.
IK1-34	~1400	<i>Bacillus</i> sp.	>99.0	PX138964
IK1-55	~1400	<i>Bacillus thuringiensis</i>	>99.0	PX139018
IK1-91	~1400	<i>Bacillus thuringiensis</i>	>99.0	PX139028
IK1-168	~1400	<i>Bacillus</i> sp.	>99.0	PX139029
IK3-237	1483	<i>Bacillus</i> sp.	99.93	PX139051

Notes: *bp*: base pair; *sp.*: species; NCBI: National Center for Biotechnology Information.

3.6. Phylogenetic Analysis

All five isolates form a monophyletic group, well supported by 16S rDNA sequences with bootstrap values >99%. All five bacteria were identified as belonging to the genus *Bacillus*. According to these data, isolates IK1-34 (KY419150.1), IK1-168 (MK824319.1), IK3-237 (cp-h37 EU584556.1) were identified as *Bacillus* sp. and IK1-55 (MG470715.1) and IK1-91 (KX832708.1) as *Bacillus thuringiensis* (Figure 5). When the phylogenetic tree showing these strains together was examined; tight clustering and >99% sequence similarity suggest that they share a recent common ancestor. The tree topology clearly places our chitosan-producing bacteria in a complex that includes *B. cereus*, *B. thuringiensis* and *B. anthracis*. The short branch lengths in this clade also indicate the established genetic affinity between members of the *B. thuringiensis* group, as species distinctions within this group are mainly based on plasmid-encoded traits rather than chromosomal variations.

Phylogenetic analyses confirm that these marine isolates belong to the *B. cereus* and *B. thuringiensis* group, as they exhibit chitin deacetylase activity. Based on 16S rRNA gene sequencing results, three of the five isolates were identified as *Bacillus* sp and two as *B. thuringiensis*. The ability of *B. cereus* and *B. thuringiensis* group bacteria to produce chitin deacetylase enzyme and chitinase is well documented in the literature [7, 8, 25] and supports the suitability of isolated strains IK1-34, IK1-55, IK1-99, IK1-168 and IK3-237 as candidates for biological chitinase production.

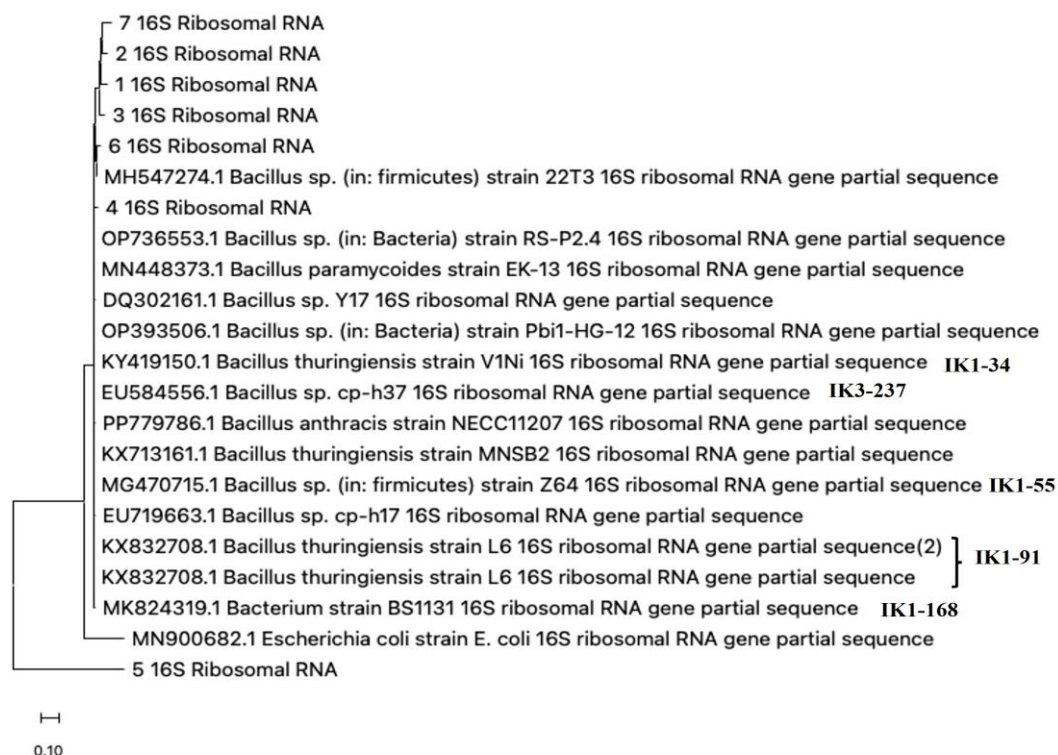


Figure 5. Neighbor-Joining phylogenetic tree of 16S rRNA gene sequences showing the relationship of isolates IK1-34, IK1-55, IK1-99, IK1-168 and IK3-237 with reference *Bacillus* species. Bootstrap values (1000 replicates) are indicated at nodes. Scale bar represents evolutionary distance

3.7 Antimicrobial activity of produced chitosan from isolate IK3-237

Chitosan of IK3-237 isolate and commercial chitosan selected for antimicrobial activity assay were compared in the presence of classical test microorganisms. For this purpose, 4% solutions of the two chitosans were impregnated on blank disks and placed in petri dishes containing the test microorganisms. The antimicrobial effect of both chitosan on the test organisms was evaluated by measuring the diameters of the antimicrobial zones. The antimicrobial zone diameters were not very wide in both samples. However, it is noteworthy that 98% purity commercial chitosan and the chitosan used as obtained from IK3-237 strain without extra purification step showed close results. In some test organisms, IK3-237 chitosan was even slightly better than commercial chitosan. This result confirms that IK3-237 chitosan has as much antimicrobial effect as pure commercial chitosan (Table 3).

Table 3. Measurement of antimicrobial action zones formed by 4% solutions prepared with biological chitosan and commercial chitosan

Bacteria	IK3-237-4%	Commercial-4%
<i>E. coli</i>	3±1 mm	No-zone
<i>S. aureus</i>	4±1 mm	2±1 mm
<i>P. aeruginosa</i>	3±0.5 mm	2±0.5 mm
<i>C. albicans</i>	1±0.5 mm	1.5 ±0.5mm

4. Conclusions and discussion

In this study, chitin production from waste shrimp shells by five marine bacteria isolated from the Marmara Sea was evaluated. The yield of bacteria producing chitosan with the help of chitin deacetylase was calculated as 0.114 g - 0.0806 g of chitosan per 1 g of raw shrimp shell. This result indicates that the chitosan production efficiency of the isolates was 3-4 times higher than the chemical production (Table 1). The chitosan of IK3-237 isolate from five

bacteria, all of which were identified as *Bacillus* members, was compared with commercial chitosan in terms of its antimicrobial effect and it was determined that it was close to pure commercial chitosan.

The conversion of a valuable polysaccharide such as chitin, which is found in biological wastes such as shrimp shells at a rate of 30-47%, into valuable biopolymers such as chitosan through enzymatic processes by microorganisms is a desirable, sustainable, green-technology recycling product. In the chemical production of commercial chitosan, 1 kg of chitosan is obtained from 35 kg of shrimp shells, resulting in a yield of about 10.5% and many harmful chemicals released into the environment. In order to minimize environmental damage, in recent years, especially in biological chitosan production studies with soil bacteria, the yield has been determined between 10-16% [20]. The yield of our isolates from the Marmara Sea, which we used in this study, varies between 26-35%. Accordingly, chitosan production yields from our isolates are 3-4 times higher than chemical production and 2-3 times higher than biological production from soil bacteria. as is observed. At this point, with the advantage of being located in a geography surrounded by seas on three sides, attention has been drawn to the production of useful and valuable biopolymers that will support the national economy from our local marine isolate creatures and its usability has been shown. At the same time, a bioremediation model has been created for more efficient use of marine organisms and wastes.

In the second part of the study, the antimicrobial effect of 4% chitosan produced by IK3-237 bacteria without any extra purification step and isolated directly from the medium was compared with 98% pure commercial chitosan prepared in the same ratio. The test microorganisms chosen here were generally selected from those used in the literature. Both IK3-237 chitosan and commercial chitosan showed similar antimicrobial effect. Again, according to scientific studies, the antimicrobial effect of chitosan varies according to the structure of chitosan, bond properties and microorganisms [1, 6, 10]. For example, it has been reported in the literature that low molecular weight chitosan is more effective against *B. cereus*, *E. coli*, *S. aureus*, *P. aeruginosa*, *Salmonella enterica*, *B. subtilis*, *Listeria monocytogenes* and *Klebsiella pneumoniae* [7, 8, 21, 22]. Similarly, amino groups are a key element determining the physicochemical properties of chitosan and distinguishing it from chitin [1]. Chitosan's unique film-forming property and permeability barrier are also reported to support its antimicrobial effect [6, 9, 10]. When the detailed chemical characterization of the chitin deacetylase enzyme producing IK3-237 strain and the chitosans of other strains in our study is completed, their bond structures and chemical contents will be clarified.

In our study, five bacterial strains isolated from the Marmara Sea were evaluated for chitosan production efficiency using waste shrimp shells and the chitin deacetylase enzyme. Strains with remarkable efficiency were identified at the species level using BLAST analysis. Two of these five bacteria were identified as *Bacillus thrungiensis*, and three as *Bacillus* sp. The *Bacillus* sp. isolates, being from the Marmara Sea, an inland sea, suggest the possibility of being a local endemic species, which would be a valuable contribution to our country's microbiota. Additionally, the antimicrobial effect of chitosan produced by IK3-237 was found to be similar to that of pure commercial chitosan. This supports the idea that the chitosan quality of IK3-237 bacteria is close to that of commercial chitosan. In other words, the chitosan produced by at least one of our isolates appears to be a valuable biopolymer of a quality that can be processed and used for various purposes.

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